Revealing a world of biofilms – the pioneering research of Bill Costerton

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MICROBIOLOGY PIONEERS — ESSAY

Revealing a world of biofilms — the pioneering research of Bill Costerton

Hilary Lappin-Scott, Sara Burton and Paul Stoodley

Abstract | Bill Costerton is recognized as the founding father of the field of biofilms, which is the study of microorganisms attached to surfaces. He was a true pioneer and was passionate about directly observing living complex microbial communities to learn how they function in different ecosystems. His multidisciplinary approach to the study of biofilms forged a common way of thinking about the ways in which microorganisms survive and function in the environment as well as in medical, dental, industrial, agricultural, engineering and other contexts. In this Essay, we outline some of the achievements that Bill made during his scientific journey.

John William (Bill) Costerton (FIG. 1) (1934–2012) was an extraordinary scientific leader, an inspirational mentor and a generous collaborator. His influence will be felt for a long time to come by the hundreds of scientists whom he personally trained, mentored and collaborated with, as well as those who were inspired by his presentations at conferences. In this short Essay, we cannot do justice to all of Bill's scientific contributions (which include >700 publications in medical, veterinary, microbiology and engineering journals and books over 6 decades); rather, we focus on his development of the biofilm concept, his pioneering research on cell–cell signalling and his exquisite use of microscopy to unravel the complex processes that occur within biofilms. Other recent tributes to Bill's work have been published, which focused on his training and leadership in the field[1–3]. In this Essay, we consider the progression of Bill's scientific journey from his early days looking at bacterial cell coats, to the direct study of biofilms on various surfaces, to the elucidation of the processes and mechanisms underlying biofilm formation and persistence (FIG. 2).

Shaping the biofilm concept

Bill received his Ph.D. in bacteriology from the University of Western Ontario, Canada, in 1960. After taking part in missionary work in India, during which he established a pre-medical school, Bill undertook a postdoctoral fellowship at the University of Cambridge, UK, before moving to McGill University, Montreal, Canada, in 1968, where he began work on bacterial cell wall ultrastructure. Bill moved from McGill University to the University of Calgary, Canada, in 1970 and began to broaden his work to include bacteria in various different habitats, including the bovine rumen[4,5]. He noted that bacteria were attached to the gut or to cellulose fibres via a complex matrix[6], which was later termed the glycocalyx[7], and had little in common with the same species cultivated many times in the laboratory. Bill made the salient observation from transmission electron micrographs (TEMs) that clinical isolates of *Escherichia coli* had a thick glycocalyx (which he later found had an important role in attachment and biofilm formation) that was almost non-existent in high-passage reference strains. Before 1976, much of Bill's work involved using culture-grown bacteria to study their ultrastructure — the earliest report that we could find of electron microscopy imaging of bacteria directly on tissues is a 1976 study of beak necrosis in hens[8].

Bill took these observations and adapted the direct microscopic analysis of microorganisms that was used by ZoBell and Allen[9] and Henrici[10], by applying these techniques to rocks that were submerged in pristine mountain streams, exploiting the abundant

natural resources of the Marmot Basin in the Canadian Rockies[11]. With his colleagues (notably, Gill Geesey and Cam Wyndham, with whom he worked closely in the late 1970s), Bill confirmed that microorganisms attached readily to the surfaces of the rocks. Importantly, they also reported the numerical abundance of attached, sessile bacteria compared with the free-floating, planktonic bacteria in fast-flowing mountain streams. The paper[11] referred to 'slime' on submerged rocks and the authors were able to take the direct observations further using electron microscopy to observe the association of the attached microbial community, and importantly, noted that most of the bacterial activity occurred in the slime layers attached to the sediments and not in the fast-flowing waters above[12]. This observation had important implications in terms of estimating the bioremediation capacity of rivers that had been exposed to pollutants[12].

However, Bill's interests extended well beyond natural environments. He studied the cell envelopes and cellular ultrastructure of bacteria from a wide range of habitats and began to translate data from environmental systems into other, unrelated disciplines. His thinking was, 'how does a bacterium know whether it is in a urinary catheter or an alpine stream?', and he hypothesized that it would behave in the same manner growing as a biofilm on either of these surfaces[13]. This was summarized in a popular science article in 1978 (REF. 14), and this hypothesis formed the basis of his research for the next 35 years.

His 'big thinking' was characterized by the way in which he crossed the traditional disciplinary divides and moved away from pure culture planktonic laboratory studies towards studies of complex communities growing on surfaces[4,15,16]. The biofilm concept that Bill developed was that microorganisms are predominantly found in the attached 'sessile' state on wetted surfaces, rather than in the free-floating planktonic state, and that the cells that grow within the biofilm are phenotypically distinct from planktonic cells. These differences bring, in turn, varied responses to antimicrobial control strategies in infectious diseases, industrial processes and natural ecosystems. Thus, the study of well-stirred, liquid

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Figure 1 | **Bill Costerton relaxing in his office with a picture of his beloved mountains in the background.** Image provided by S. Hunt and the Center for Biofilm Engineering, Montana State University, USA, 2004.

monocultures would limit our ability to extrapolate and understand biofilms in the real world. Bill considered that biofilms had been overlooked as monoculture liquid growth was preferred and widely accepted as 'the norm' for the *in vitro* culture model for microbiology research. He championed the idea of a universal strategy of biofilm formation, regardless of the species and environments that are involved. As is common in science, such a proposed paradigm shift was not initially embraced by the scientific community. He used to recount the many times that this got him into trouble, most famously at a Gordon conference in the 1980s, where delegates robustly challenged his bold extrapolations from natural habitats to — for example — the human lung. Bill knew that he might have upset one of his scientific heroes — Professor Harry Smith from Birmingham University, UK — with this audacious leap, but they worked to resolve their differences. Today, of course, the cystic fibrosis (CF) lung is used as a well-studied example of the deadly consequences of the biofilm phenotype in infectious diseases.

Bill wished to follow up the observations that led to the evolving biofilm concept with quantitative data from time course experiments carried out in reproducible laboratory biofilm systems. The development of such systems involved the challenges of how to both observe and enumerate biofilm versus planktonic cells after various treatment regimes. As many of the biofilms that were studied were within tubular devices (such as catheters and industrial pipelines), the

approach focused on developing a device that had removable sections, whereby biofilms could be 'harvested' and studied. The resulting device was the Robbins device[17,18], which had removable sampling studs to enable the biofilm organisms to be visualized using scanning electron microscopy (SEM) and TEM. In addition, cell counts that were related to industrial, medical and environmental settings could be undertaken[19–22] to develop more effective cleaning regimes and enable research on surface materials. This greatly affected our understanding of microbial ecology within biofilms and the interactions of microorganisms with surfaces.

The development of the Robbins device as a reproducible laboratory system facilitated studies of the differing responses of biofilm and planktonic cells, particularly in response to antimicrobial agents. The first studies involved antibiotics, but this was later broadened to other antimicrobial agents. Results from these studies suggested that biofilms were only partially controlled by antimicrobial agents[23–26] and that concentrations that controlled planktonic cells were not effective when the same organisms were grown on a surface[27,28]. These important observations led to the emergence of a specific research area studying antimicrobial resistance in biofilms. In 1992, looking to overcome this resistance, although the mechanism was not well understood at that time, Bill explored the bioelectric effect, by which certain bacterial biofilms could be made susceptible to antimicrobial agents when exposed to an electric current[25]. Although the mode of action of the bioelectric effect is still not understood, there continues to be interest in its development for treating orthopaedic infections[29]. Although the early theories of biofilm resistance focused on the physical barrier that is provided by the thick biofilm matrix, Bill quickly developed microscopy imaging methods to help to address this question; others subsequently demonstrated the passage of antibiotics into biofilms[30]. This drove major international funding initiatives to elucidate the mechanisms of antibiotic resistance. Much of this thinking now centres on the diverse sociomicrobiology within biofilms, including recalcitrant subpopulations of the community, such as 'persister' cells.

Bill worked with collaborators in the growing field of implanted device-related infections, as these infections responded poorly to antimicrobial treatment and to host defence mechanisms. If biofilms were removed from indwelling devices (such as heart valves and the Jarvik artificial heart[31]),

and disrupted, they responded well to antibiotics, but these drugs were ineffective at controlling biofilm-grown microorganisms *in situ*.

Biofilms are increasingly considered to be vehicles for the increased transfer of antibiotic resistance plasmids. Recent studies that compared standard filter mating and biofilm conditions indicated that growth as a biofilm increases the transfer of antibiotic resistance plasmids and the emergence of resistance mutations in *Staphylococcus aureus*[32].

Biofilms: a microscopic approach

A constant theme of Bill's research was the use of microscopy to directly document bacteria on surfaces (FIG. 3). It is not surprising that Bill relied on direct microscopic observation, given that, as discussed above, his early research career used TEM-based examination of bacterial cells to reveal the presence of the outer glycocalyx[6]. Such images formed the basis of the direct visualization of the extracellular polysaccharide (EPS) matrix that is found in biofilms; the definition of EPS was subsequently altered by the biofilm research community to 'extracellular polymeric substance', when the chemical complexity of EPS was more fully appreciated.

This early use of microscopy to support Bill's development of the biofilm concept, was, in his own words, "Because of my ingrained tendency to place my trust in direct *in situ* observations of bacteria by light and electron microscopy." (REF. 33). Making direct observations remained Bill's preferred approach throughout his career. During the 1970s and 1990s, Bill rallied a network of clinical and academic collaborators. One of these early collaborations was with Niels Høiby of the University of Copenhagen, Denmark, which resulted in the development of the concept of CF infections as having a biofilm aetiology. This longstanding collaboration with Neils Høiby and later Mike Givskov, was recognized by the naming of the Costerton Biofilm Centre in Copenhagen in 2013. Armed with the fixation and electron microscopy techniques that are required to directly observe biofilms on biological and manufactured surfaces, and his increasing conviction that biofilm attachment to native tissue and medical devices could explain the recalcitrance of many chronic infections to antibiotic therapy, Bill used his boundless charm and enthusiasm for his research to begin to image an enormous variety of surfaces and samples for evidence of biofilms. The clinical samples that were analysed included the post-mortem CF lung[34], the

Clinical research

Chronic wounds | 2008–2012

Dental biofilms | 1996–2012

Biliary tract | 1988–1993

Osteomyelitis | 1985–1991

Orthopaedic implants | 1984–1988 | 2005–2012

Urogenital tract | 1984–1999

Cardiovascular | 1982–2002

Cystic fibrosis | 1980–1988

Basic research

Bioelectric effect | 1992–1996

Resistance to host immunity | 1990–2003

Biofilm vaccine development | 1987–1988 | 2009–2011

Cell signalling and development | 1986–2000

Glycocalyx ultrastructure | 1982–1993

Antibiotic resistance in biofilms | 1982–2006

Veterinary biofilms | 1976–1992

Cellulose degradation | 1975–1993

Cell wall and antibiotic resistance | 1969–1982

Cell envelope ultrastructure | 1967–1993

Industrial research

Groundwater biobarrier | 1997–1999

Wastewater | 1990–1995

Oil field ultramicrobacteria | 1986–1999

Industrial fouling and corrosion | 1981–1994

Petroleum microbiology | 1981–1994

Stream biofilms | 1977–1984

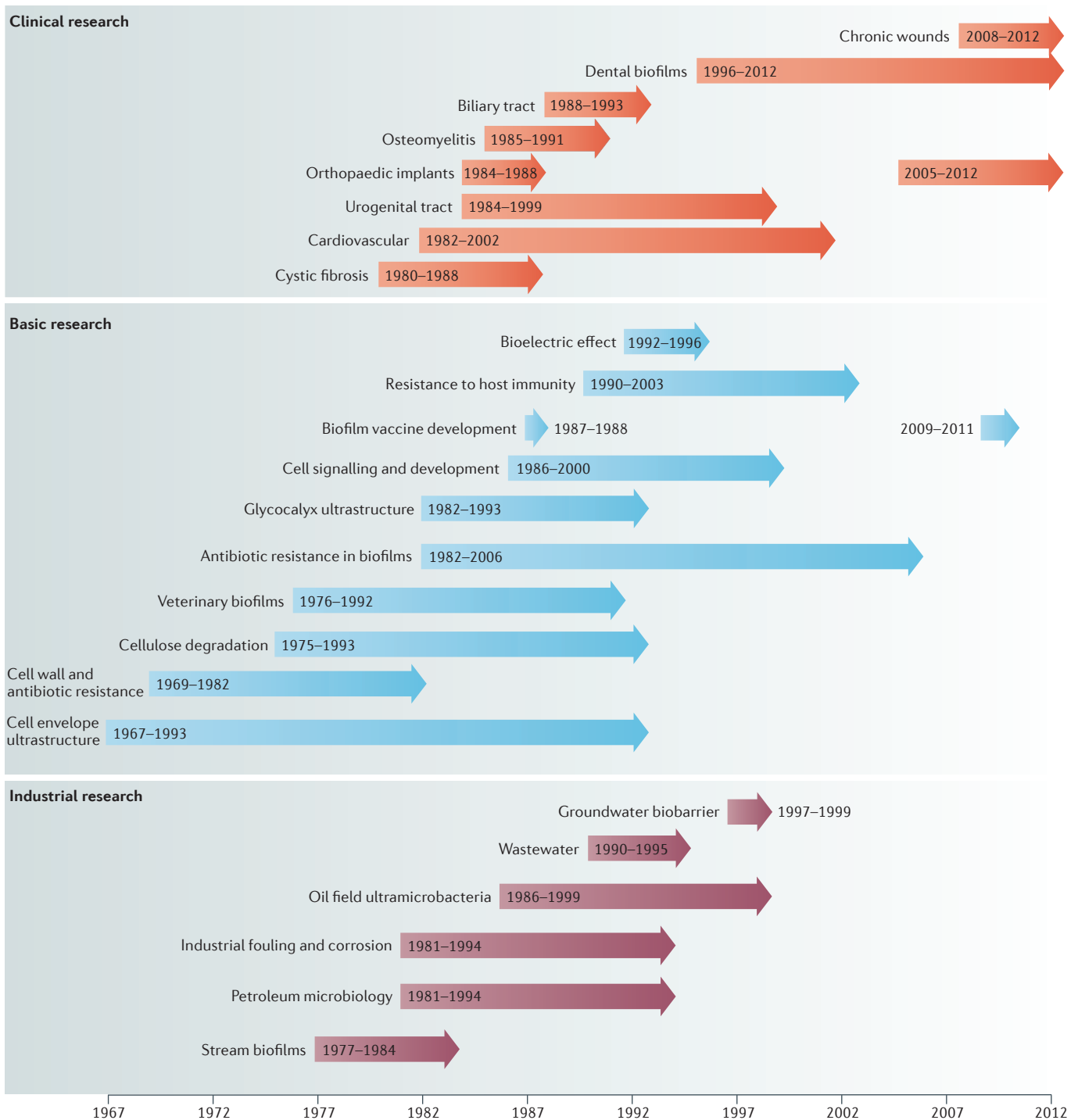1967 1972 1977 1982 1987 1992 1997 2002 2007 2012

Figure 2 | **A timeline of Bill's research interests and publications spanning his entire career.** This timeline illustrates Bill's diverse interests as the biofilm concept developed from basic cell ultrastructure in the early years, to agricultural, industrial, environmental, dental, medical , and many related, fields.

urogenital tract[35–37], orthopaedic samples[38,39], peritoneal dialysis catheters[40], intravenous catheters[41], ventricular shunts[42] and endotracheal tubes[43]. These images revealed details that have subsequently been recognized as hallmark features of biofilm infections, including the restricted penetration of antibodies and phagocytic cells into biofilms.

The images that were generated not only conveyed the extent of the complexity of biofilm communities to microbiologists but also provided Bill with materials with which to amaze his audience in visual presentations that were a perfect accompaniment to his narrative. Bill found that SEM images that showed an extensive staphylococcal biofilm

attached to a pacemaker lead, despite aggressive antibiotic therapy, were particularly illustrative of both the chronic nature and antimicrobial resistance of biofilm bacteria[44]. At the same time as Bill was studying clinical specimens, he was also using his visualization techniques to demonstrate the importance of biofilms in the industrial fouling of
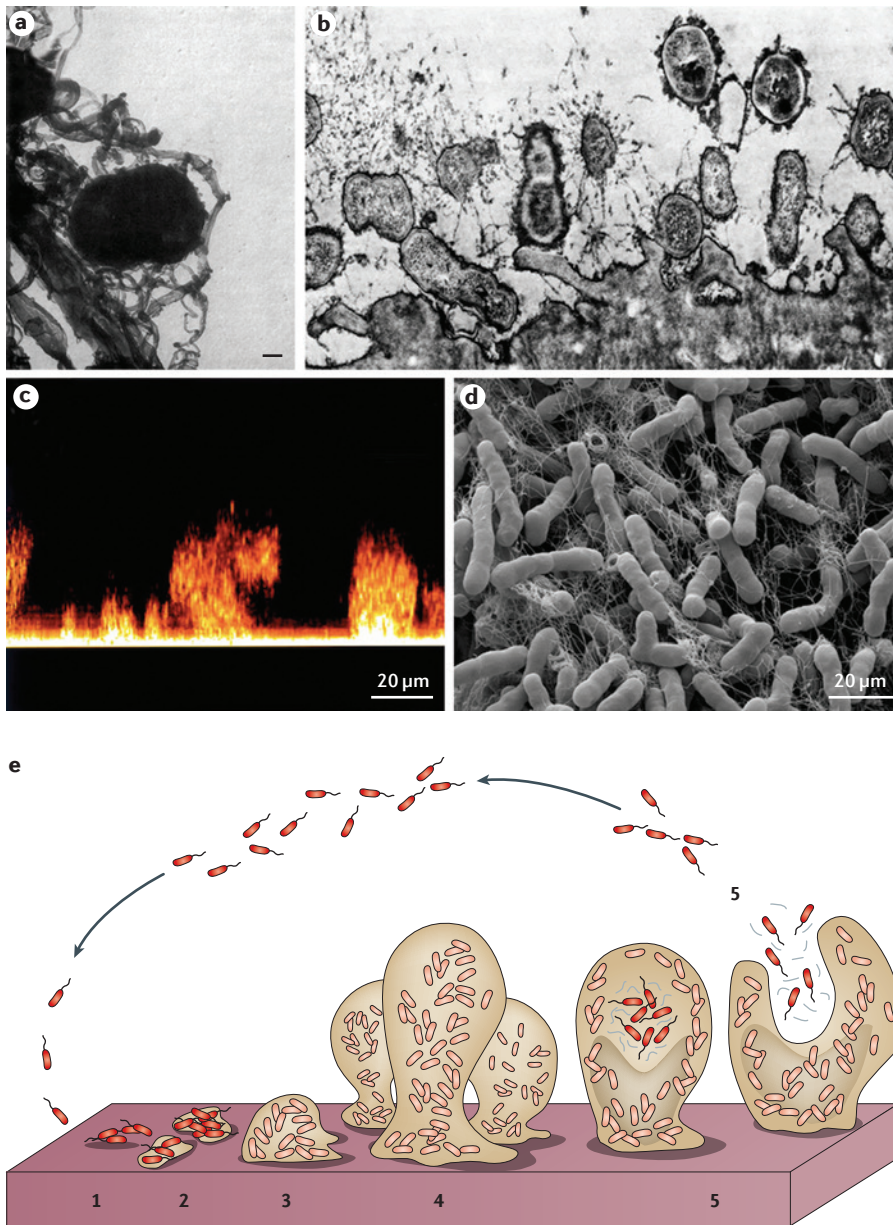
pipelines and oil field plugging and souring to diverse audiences from the petroleum industry, waste water engineers and physicians (particularly orthopaedic surgeons). Using images, Bill could tell the vivid story of biofilms and reveal for the first time a new world, in which bacteria could be physically interconnected, close enough for signal-based communication, and arranged in structures that were optimized for nutrient exchange and that protected the residents from antibiotics and host immunity.

While Bill was continuing his pioneering use of microscopy to directly examine surfaces, environmental microbiologists were discovering that culturing alone had other limitations. In 1977, Carl Woese[45] opened the door to the development of culture-independent 16S ribosomal RNA-based phylogenetic analysis, which began to reveal the inadequacies of culturing microorganisms to show the full complexities of environmental communities[46]. These new techniques enabled previously undiscovered bacteria to be identified by analysis of their 16S rRNA gene sequences and then microscopically imaged in their natural environment using fluorescent probes that were specifically targeted to rRNA. For Bill, this strengthened his conviction of the power of direct study, *in situ* or *ex situ* wherever possible, which prompted him to declare in an acceptance speech for his 2008 Sarton Chair medal, which was awarded by Ghent University, Belgium, "… and so we unlimbered our microscopes".

The next breakthrough in imaging came from the development of the confocal microscope. Bill immediately saw the power of confocal microscopy and, with Doug Caldwell and John Lawrence at the University of Calgary, he was part of the first team to apply this technology to the study of biofilms[47]. Although not as high resolution as electron microscopy, confocal microscopy enabled live imaging of fully hydrated specimens in three dimensions. Furthermore, fluorescent stains and probes were rapidly developed that could enable the investigation of pH, metabolic activity and viability on a local scale. The use of confocal microscopy to study biofilm development in flow cells spearheaded by the Center for Biofilm Engineering (CBE) in Bozeman, Montana, USA, the Calgary Group, and the group of Søren Molin (with whom Bill also maintained a close and long-standing relationship) at the Technical University of Denmark, Lyngby, revealed new phenomena at an almost breathtaking pace. Biofilms were now shown not to be uniform slime layers but to be complex

Figure 3 | The power of the image. The figure shows transmission electron microscopy (TEM), confocal microscopy and scanning electron microscopy (SEM) images that were taken between 1975 and 2011, which helped to shape Bill's thinking about biofilms and his incorporation of these concepts into conceptual schematic models. **a** | The TEM shows the rumen bacterium *Ruminococcus albus* (dark cocci in centre) attached to a fibre of cellulose by extracellular fibrils[5]. **b** | The TEM of a biofilm community attached to bovine rumen epithelium shows that the cells are held together and to the rumen by the extracellular bacterial glycocalyx[14], which was later understood to be extracellular polymeric substance (EPS). **c** | The confocal image of a laboratory-grown biofilm shows cell clusters (that is, bacterial cells held together and to the glass surface in EPS matrix) separated by water channels[50]. **d** | The SEM shows a biofilm on the bone of an infected jaw, clearly showing the bacteria connected by nanotubules, which were hypothesized to have conductive properties[57]. Later research confirmed that the biofilms were indeed conductive[56]. **e** | The schematic shows a conceptual model of biofilm formation as a developmental process, including structural components of discrete cell clusters separated by water channels[58]. Part **a** of the figure from *J. Bacteriol.*, 1975, **122**, 278–287, reproduced with permission from American Society for Microbiology. Part **b** of the figure reprinted from REF. 14, Nature Publishing Group. Part **c** of the figure from Evolving perspectives of biofilm structure., Stoodley, P., deBeer, D., Boyle, J.D., and Lappin-Scott, H.M., *Biofouling*, 1999, Taylor & Francis, reprinted by permission of the publisher (Taylor & Francis Ltd, http://www.tandfonline.com). Part **d** of the figure reprinted, with permission, from REF. 57, American Dental Association. Part **e** of the figure modified, with permission, from REF. 58, Annual Reviews.

arrangements of microcolonies that take on many forms, including 'streamers' and the famous 'mushroom' structures. By then, Bill was Director of the CBE, where the confocal microscope was also being used in laboratory flow cell studies in combination with microelectrodes and tracers for real-time studies of metabolic activity and mass transport, which were used to relate these newly discovered structures to function[48,49]. Collaborating with CBE resident science artist Peg Dircx, Bill was able to incorporate the newly acquired findings from confocal microscopy with his previous electron microscopy-based observations to develop a now-iconic schematic as a generalized working model of a biofilm[50,51], which is better known as the CBE model (FIG. 3). The ability to record time-lapse images of water flowing through the biofilms also stimulated Bill to think about biofilms not only as communities of casual acquaintances but actually as highly coordinated communities that have higher orders of organization, which is analogous to that of tissues in multicellular organisms. Confocal imaging was pivotal in establishing a role for cell signalling in shaping biofilm structure[52] and showing biofilm formation as a developmental process[53,54], as well as showing how biofilms could protect bacteria from phagocytic cells[55], as he had inferred from earlier electron microscopy studies. With the use of confocal microscopy as the imaging modality of choice for biofilms, SEM fell out of favour for biofilm research in the 1990s owing to suspicion that dehydration steps created artefacts in the biofilm structure. However, Bill later returned to using electron microscopy, as greatly improved preservation techniques enabled his group to show the presence of networks of nanotubes and electrically conductive nanowires that connect individual cells[56,57] (FIG. 3) at a resolution that is not possible with confocal microscopy.

As the imaging revealed ever-increasing complexity in the biofilm communities, Bill embraced this complexity and rapidly absorbed it into new, dynamic, conceptual models[58,59]. The images enabled an easy transition from the pages of narratives from an academic journal into schematics that could be used to illustrate key points and features that are easily understood by the non-specialist in talks and more interpretive articles.

Biofilm development and communication

Although imaging revealed some of the secrets of the physical complexity of biofilms, Bill was already speculating about some of the more intangible characteristics of bacteria in these organized communities, such as the possibility of highly adaptive physiology and programmed genetic expression[58]. Under Bill's leadership, a leap in our understanding of biofilms was driven by his advocacy of using the culture-independent nucleic acid-based methods that were being developed by environmental microbiologists, in growing recognition of the limitations of culture to probe natural communities.

One core characteristic of biological communities is communication. Intercellular communication is required to coordinate the behaviour of bacterial populations in processes such as, switching on the light organ of the bobtail squid (also known as *Eupryma scolopes*) by *Vibrio fischeri*. In addition to organized chemical signalling and developmental processes, bacteria may also exhibit many social activities and were considered to be a model for dissecting social behaviour at the genetic level, which led Parsek and Greenberg to introduce the term 'sociomicrobiology' (REF. 60). It was considered that this level of organization gave additional community characteristics to biofilms, which were no longer regarded as simple collections of cells. The use of molecular techniques enabled the determination of the gene regulatory mechanisms of Lux homologues in *V. fischeri* and the first report of density-dependent quorum sensing in bacteria[61]. The emerging exploratory studies[62,63] in bacterial cell–cell communication proved to be essential to the later development of our understanding of communication within biofilms.

Bill had begun to suspect that bacteria could communicate within biofilms and indeed formulated the hypothesis that biofilms increase both chemical and genetic communication between cells. The seminal work on cell signalling in biofilms was published in *Science* in 1998 by a multidisciplinary team that included Pearson, Iglewski, Parsek and Greenberg[53]. Moreover, social organization within higher organism communities was well documented. Bill led the biofilm research community in considering the roles of specialist cell 'types' in biofilms, which enables division of labour[64], and the recognition of persister cells as being fundamental in the recalcitrance of chronic infections to antimicrobial treatment[65]. There has been a recent resurgence in research within this field, which has led to our current understanding of biofilms as complex communities. It is now widely accepted that biofilms form persister cells, which are highly tolerant to many antibiotics. Indeed, these persister cells may themselves also prove to be useful targets to combat chronic infections by the use of synthetic compounds, such as brominated furanones: these compounds can reduce persistence during growth of *P. aeruginosa* and revert isolated persister cells to susceptible cells, although the precise mechanism of action remains undetermined[66]. However, the clinical challenges that are created by chronic biofilm infections, which were first recognized by Bill, remain recalcitrant to control. Costerton, Brady, Shirtliff *et al.,* developed novel hypotheses and methods to identify and test biofilm antigen vaccines against chronic osteomyelitis *S. aureus* infections[67]. However, although the initial vaccine had partial success, complete clearance of infection was not achieved. Thus, additional measures were designed to target and eradicate planktonic bacteria, and the use of a biofilm vaccine, concurrent with antibiotic therapy, is a promising approach[68]. These early studies, which investigated quorum sensing and sociomicrobiology were a precursor to our current understanding, which encompasses the connectivity of cell–cell signalling via bacterial quorum sensing and metabolic control within biofilms[69].

And finally …

Although Bill was based at Allegheny-Singer Research Institute, Pennsylvania, USA, during the latter part of his career, he had built a virtual network (that would have been the envy of any self-respecting biofilm) of collaborators all over the world. Bill had just agreed to serve on the scientific advisory board of the newly formed Singapore Centre on Environmental Life Sciences (SCELSI), directed by Stefan Kjelleberg, and revelled in the opportunity to fly half way around the world on a regular basis! Bill continued to be heavily involved in the application of imaging techniques to study clinical specimens, particularly dental[57] and periprosthetic orthopaedic infections[70], although he benefitted from being able to borrow techniques that were developed in the 1990s by environmental microbiologists to identify bacteria in an environment from their 16S rRNA and the use of fluorescence *in situ* hybridization (FISH) probes to confirm and microscopically map their presence[71]. Seeing the power of molecular techniques to identify unculturable biofilm bacteria in natural environments, Bill was sometimes frustrated by the recalcitrance of clinical microbiology in appreciating the value of these techniques for application in medical diagnostics. For

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Bill, medical microbiology was too beholden to Koch's postulates (which had functioned so well in diagnosing single causative pathogens in acute infections), noting that three of the four postulates were reliant on obtaining pure cultures, and as such, were of limited use in diagnosing chronic biofilm infections, which are difficult to culture and are often polymicrobial. Breaking this dogma is likely to be an important next step in medical microbiology education and the biofilm story.

In the latter part of his career, Bill returned to imaging EPS ultrastructure (for which he coined the phrase 'casernae' to convey the concept of EPS having a functional structural role), with the discovery of nanowires, nanotubes, membrane vesicles and extracellular DNA networks[57,58]. Elucidating the more subtle chemical and physical nature of EPS and its role in the function of the biofilm community, almost as an extension of the living cells within, is now an area of intense ongoing research, which illustrates just how visionary Bill was in his holistic way of thinking about biofilms and the way he drew upon his broad range of personal experiences to shape ideas in the field.

Bill possessed a rare gift of being able to think beyond the narrow confines of detail, as shown by his interests in both the fundamental cell processes and structures and the applied, more translational, aspects of his work in biofilms (FIG. 2). Thanks to his contributions spanning 6 decades and the tremendously large network of trainees and collaborators that he built, biofilms are now recognized as protected and privileged environments for microbial cell communication, survival and adaptation.

No doubt many of the paths that Bill trod during his career will be revisited with the benefit of higher-resolution fluorescence imaging and improved structural preservation[72] to reveal ever-increasing levels of complexity. Bill realized early on the power of direct imaging as a tool for discovery and saw the full possibility of the dynamic complexity of bacteria on surfaces in a still photographic plate, which demonstrates the insight, questioning and dogged perseverance that were needed to become the great scientist that he was. We leave the final words to Bill himself[13]: in a section within a book chapter that described the development of the biofilm concept, entitled 'Flights of fancy', Bill outlined his suggestions for important observations in microbiology. We encourage you to read and ponder on these words and would like to draw your attention to his premise that, although we are bound by our scientific honesty "…we are not prevented from using our full imaginations in experimental design … so let the wild ride begin".

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