Microbial Interactions and Communities

Volume 1

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A. T. Bull

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A. T. Bull

Biological Laboratory
University of Kent
Canterbury
England

J. H. Slater

Department of Environmental Sciences
University of Warwick
Coventry
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Contributors

Alan T. Bull, Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, England

Richard G. Burns, Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, England

John Cairns, Jr., Center for Environmental Studies, Virginia Polytechnic Institute, Blacksburg, Virginia 24061, USA

Jan W. Drozd, Shell Biosciences Laboratory, Shell Research Centre, Sittingbourne, Kent ME9 8AG, England

John C. Fry, Department of Applied Biology, University of Wales Institute of Science and Technology, King Edward VII Avenue, Cardiff CF1 3NU, Wales

Alvin K. Jones, Department of Botany and Microbiology, University College of Wales, Penglais, Aberystwyth SY23 3DA, Wales

Jan C. Gottschal, Department of Microbiology, Biology Centre, University of Groningen, Kerklaan 30, 9751 NN Haren (GR), Netherlands

Don P. Kelly, Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, England

William J. Kelly, Department of Biochemistry, Lincoln College, Canterbury, New Zealand

J. G. Kuenen, Department of Microbiology, Biology Centre, University of Groningen, Kerklaan 30, 9751 NN Haren (GR), Holland

John D. Linton, Shell Research Ltd., Sittingbourne Research Centre, Shell Biosciences Laboratory, Sittingbourne, Kent ME9 8AG, England

Paul R. Norris, Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, England

R. John Parkes, Scottish Marine Biological Association, Dunstaffnage Marine Research Laboratory, P.O. Box 3, Oban, Argyll PA34 4AD, Scotland

Darryl C. Reanney, Department of Microbiology, La Trobe University, Bundoora, Victoria, Australia 3083

William P. Roberts, Department of Microbiology, La Trobe University, Bundoora, Victoria, Australia 3083

J. Howard Slater, Department of Environmental Sciences, University of Warwick, Coventry CV4 7AL, England
Keith M. Steinkraus, Institute of Food Science, Cornell University, Geneva, New York 14456, USA
Meyer J. Wolin, Division of Laboratories and Research, Environmental Health Institute, Tower Building, Empire State Plaza, Albany 12201, USA

Preface

In 1881 Robert Koch published the account of the methods he had devised to obtain pure cultures of microbes. This single paper, now one hundred years old, laid the foundations for the rise of modern bacteriology and microbiology but at the same time distracted attention from one important fact: namely, that most microorganisms naturally inhabit environments containing more than one species of organism and, indeed, in most instances containing very many different types. Thus, the expression of the microbial genome, through the growth and physiological capabilities of a particular organism, is normally modified, attenuated or enhanced by the activities of associated populations of microbes occupying the same environmental niche. By comparison with our understanding of topics such as microbial genetics, molecular biology or biochemistry, our knowledge of the effects of mixed microbial existence and growth is rudimentary. However, it is quite apparent that in many cases the sum of the individual microbial population activities, capabilities and behaviour is often less than, and sometimes markedly different from, those expressed by mixed microbial systems.

In the light of this general perception, this treatise has been started to reflect the growing interest in researches on the behaviour and properties of mixtures of microorganisms. Not only is there considerable interest in the intrinsic properties and features of interacting microbial communities—at various levels, including ecological, physiological, biochemical and genetic—but there is also substantial value in studying mixed cultures from more applied standpoints including medical, nutritional, and biotechnological applications. The treatise will be an occasional one designed to highlight the various topics relevant to each area, containing reviews written to provide a comprehensive coverage. Necessarily, in this fast-growing field of microbiology, some of the study areas are comparatively under-developed and it is hoped that their discussion will stimulate and encourge future developments. Indeed some facets of mixed culture interaction studies are in their infancy and one aim of this treatise is to foster their progress through comparison with the approaches already adopted in the more established fields. In-

evitably the reviews will reflect the particular biases of the authors but it is hoped that interested students and researchers will be able to develop their own interests through the cited literature associated with each chapter. In this first volume a number of major topics have been covered and succeeding volumes will seek to treat those areas, such as competition, prey–predator interactions, and mixed culture biodegradation, not dealt with in the present volume.

It is with considerable pleasure that we acknowledge the initial contribution made by Anthony Watkinson of Academic Press who encouraged us throughout the gestation period of the project and whose enthusiasm was clearly evident from the discussions which we had with him and from which this treatise was evolved. We are grateful also to Peter Brown of Academic Press who has guided the project to maturity along with the assistance of June Nelson, Deborah Sanderson, and the other staff of Academic Press. We also appreciate the good-natured assistance of our secretaries, Ann Branch and Vicki Wade at Warwick and Marian Williams at UWIST, whose help in the preparation of the manuscripts was indispensable.

We hope that these collaborators, as well as the readers, will be interested in the final product for which we, of course, shoulder any responsibilities for its failings or shortcomings.

Alan T. Bull
Biological Laboratory
University of Kent
Canterbury CT2 7NJ
Kent, England

J. Howard Slater
Dept. of Environmental Sciences
University of Warwick
Coventry CV4 7AL
Warwick, England

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Historical Perspectives on Mixed Cultures and Microbial Communities

A. T. Bull and J. H. Slater

1. INTRODUCTION

The vast array and variety of activities that constitute contemporary microbiology are dominated by one quintessential practice, that of the study of pure, or monospecies, cultures. Indeed, as Slater (1981) has remarked, mixed species cultures are normally considered to be the antithesis of good experimental technique and students of microbiology are nurtured in the pure culture doctrine from their earliest acquaintance with the subject. Undeniably most of our knowledge of the properties and behaviour of microorganisms derives from the examination of isolated single species, and such practice will persist as a mainstay of experimental microbiology. The reasons for the pervasive position of the pure culture tradition in microbiology are not difficult to define and will be considered below. Less readily comprehended is the relative neglect and avoidance of mixed cultures by the majority of microbiologists, especially when one considers that the monospecies is strikingly atypical of nearly all habitats that are colonized by microorganisms. This introductory chapter will trace the development of the pure culture tradition and suggest how interest in mixed culture systems has been stimulated and quickened in recent years.

2. THE ROBERT KOCH TRADITION

The existence of mixtures of microorganisms in natural habitats was demonstrated conclusively by van Leeuwenhoek, and his skill as a lens-maker and microscopist enabled him to distinguish different morphological types of bacteria. Van Leeuwenhoek's much-cited letter to the Royal Society of London concerning "animals in the scurf of the Teeth" (van Leeuwenhoek, 1684) contains particularly clear observations of bacteria. However, it was almost two centuries later before pure cultures of bacteria were unequivocally obtained. Meantime, the quest for pure culture methods was taken up by mycologists, among whom Micheli and Brefeld can be counted the pioneers.

Micheli was born in 1679 and from humble beginnings became the botanical director of the public gardens in Florence. Mycology was a particular interest of Micheli's and of the nearly two thousand plants decribed in his *Nova Genera Plantarum* of 1729, approximately half were fungi. Some of the new genera of microfungi characterized by Micheli included *Mucor*, *Botrytis*, and *Aspergillus*, and he developed simple but effective means of culturing them on the cut surfaces of fruits. On the separate faces of pyramids cut from melon, quince and pear, Micheli sowed the "seeds" of *Mucor*, *Botrytis*, and *Aspergillus*, and observed their germination and subsequent development of characteristic colonies, or "plants" as he described them. Micheli made serial subcultures of these moulds and recorded that he "always observed the same mode of growth in them, not in one trial only, but however often and whenever I attempted it" (Buller, 1915). Brefeld was born 102 years after Micheli's death in 1737, and his researches on fungi were made in a number of German universities where he held chairs of botany. Brefeld took Micheli's culture method several stages further and the influence which he must have had on Koch is obvious. He turned his attention to many different groups of fungi and was particularly interested in unravelling life cycles and tracing patterns of growth and morphological differentiation: the success of such studies was due very largely to his development of liquid cultures in glass flasks, in which he could observe fungal growth. An elegant example of Brefeld's careful experimentation, the elucidation of rhizomorph development in the honey fungus, *Armillaria mellea*, is recounted in E. C. Large's history, *The Advance of the Fungi* (Large, 1940). To Brefeld, success in achieving pure cultures was dependent on:

(i) the use of an appropriate nutrient solution that was both transparent and sterile;

(ii) inoculation of the nutrient solution with a single spore; and
(iii) the necessity of preventing contamination of the solution by other organisms.

The use of gelatin to solidify solutions is claimed to originate from Davaine in 1858 (Large, 1940) and Brefeld adopted the practice to prepare solid culture media for fungi. Brefeld's contributions to experimental microbiology have often gone unnoticed by students of the subject but his protocol for pure cultivation was simple, reliable, and innovative. Its one drawback was the necessity of manipulating single fungal spores with which to initiate the culture, an operation that was a practical impossibility with bacteria and similar sized organisms and which was resolved only later by Lister and Koch. But before considering bacterial culture methods, mention of one further contribution from the study of fungi is appropriate.

In 1869, Raulin, one of Pasteur's devotees at the *École Normal* in Paris, published his *opus magnum* on fungal growth, *Chemical Studies on Growth*. The object of Raulin's work was the development of a completely chemically defined nutrient medium for the growth of *Aspergillus niger*, so that:

(i) optimum conditions for growth could be defined;
(ii) effects of physical and chemical culture variables on growth could be assessed; and
(iii) the efficiency of nutrient assimilation into fungal biomass could be quantified.

Raulin certainly appreciated that pure culture was obligatory for his investigations and is at pains to point out that those cultures of *Aspergillus niger* that become contaminated by "foreign vegetation" (usually *Penicillium* species) had to be disregarded.

Attempts to resolve two closely interrelated controversies provided nineteenth-century microbiologists with the greatest impetus to develop pure cultures of bacteria: one was the microbial causation of diseases, particularly animal diseases, the other was pleomorphism. It was Henle who, in 1840, first recognized and advocated that isolation of the organism from the host was a *sine qua non* requirement in proving that microorganisms could induce disease. Unfortunately, Henle's perspicacity could not be translated into practice because the technology for pure cultivation of bacteria was a long way from being developed. During the nineteenth century, wide acceptance of a notion that came to be known as pleomorphism posed a serious challenge to the scientific progress of microbiology. Briefly stated, the advocates of

pleomorphism believed that microorganisms were endowed with considerable capacities for morphological and functional variability. With the benefit of hindsight it is obvious that the confusion created by these claims of pleomorphism stemmed, unwittingly, from the study of mixed microbial populations and an inability to recognize succession in such populations. It took the development and exploitation of chemostat techniques a century later for the extent of true phenotypic variability in microbial species to be appreciated (Bull and Brown, 1979). Proponents of the alternative monomorphist thesis lead by Pasteur and Koch regarded study of pure cultures to be decisive in any analysis of microbial activity and morphology.

The epic investigations of Pasteur and Koch on such problems as alcoholic fermentation and anthrax were made without the advantage of pure cultures. In both instances the high reproducibility of their results almost certainly reflected the selectivity of the nutrient media being used to culture the organisms. During his work for the French wine industry, Pasteur analysed numerous different sources of "ferment" which he knew to contain a variety of microorganisms. Good ferments contained a preponderance of one particular organism, ineffective ferments were dominated by other organisms. Thus the practice developed of using only organisms from good ferments to ensure the production of good wine. Koch's successful investigation on anthrax also enjoyed a fair proportion of aleatory experimentation. He isolated the anthrax bacillus from the blood of infected mice and observed its growth in serum and aqueous humour, but his procedures were not designed to ensure the development of cultures from single organisms. Fortune favoured Koch because *Bacillus anthracis* grew rapidly in the bodies of experimental animals and outcompeted any contaminating bacteria. Not all microbiologists trying to follow Koch's lead were so fortunate. The method used by Pasteur for the cultivation of the anthrax bacillus was to inoculate sterile urine or wort with a drop of blood from a diseased sheep. Following serial cultivation of the growth in sterile urine, Pasteur assumed that a pure culture had been obtained of anthrax bacillus. However, neither Koch's nor Pasteur's procedures guaranteed pure cultures and it was Lister who reasoned that microscopic categorization of sterility or pure culture was apparent, not real, due to the very small size of the observed sample in comparison with the size of its source. Lister circumvented this difficulty by serial dilution of the source material to extinction, and argued that a drop from the dilution which produced growth contained but a single organism. Lister's paper "On the lactic fermentation and its bearing on pathology" was published in 1878 and contained the first reliable isolation method for

pure cultures of bacteria. This dilution isolation method, elegant and simple as it was, had limited application and numerically minor bacteria in a mixed population would be lost by early dilution. By 1880, Koch was fully convinced of the importance of pure cultures for bacteriological research and aware that the necessary techniques were not yet available. One year later and Koch fulfilled that urgent requirement and thereby enabled a spectacular blossoming of medical microbiology.

The history of Robert Koch's career following his removal in July 1880 from Wollstein to the Imperial Health Office in Berlin will be familiar to most microbiologists, but a few recollections of his exploits are appropriate here. In Berlin Koch found himself part of a talented and active bacteriology section that included Gaffky, Löffler, and Proskauer. So productive was the Office that in 1881 it was able to sustain its own periodical, *Mitteilungen aus dem kaiserlichen Gesundheitsant*, and launching the first issue was Koch's "*Zur Untersuchung von pathogenen Organismen*", a paper described by Brock as the most significant for the rise of microbiology (Brock, 1961). Initially Koch followed Micheli's strategem of using natural materials on which to isolate and grow bacteria. The sterile slices of potato used by Koch, while facilitating the isolation of certain bacteria, was a very selective substrate and failed to support the growth of many species. Moreover, the wet surface and opaque nature of the material made colony development and identity a difficult matter. Subsequently, Koch experimented with gelatin as a setting agent for nutrient solutions, such as meat infusion, and his preliminary experiments, in which organisms were trapped in the medium as the gelatin cooled and solidified, laid the basis of the pour plate technique. Koch also prepared slabs of gelatin medium and with the aid of platinum wire streaked mixed populations of bacteria on their surfaces: isolated bacteria grew where they were dispersed and gave rise to colonies that were easily differentiated on the transparent substrate. A significant modification of Koch's streak plate technique involved replacement of gelatin by the algal polysaccharide agar–agar which was stable over a much wider temperature range and much less susceptible to microbial degradation. This revolutionary technique was demonstrated to an august assembly, including Pasteur and Lister, at the International Medical Congress at London, in 1881. The following year Koch isolated the causal bacterium of tuberculosis and for which the significance of his newly introduced pure culture technique was amply revealed. Thus was heralded in what has come to be known as the golden age of medical bacteriology. The years to the end of the century were dominated by the exploits of Koch's colleagues and

others—among them Löffler, Fehleisen, Gaffky, Kitt, Fraenkel, Escherich, Weichselbaum, Kitasato, Yersin, Shiga—who were instrumental in isolating the bacteria responsible for most of the common infectious diseases. It was during this period, and in connection with his study of tuberculosis, that Koch finally established the practical test of Henle's much earlier ideas, a test which universally has come to be known as Koch's Postulates.

Robert Koch died in 1910. His postulates were the product of an eminently pragmatic approach to the understanding, cure, and prevention of infectious diseases, and of having the objective of achieving the unequivocal attribution of the causative organism to the disease. Koch has frequently been dubbed the ablest of technicians and his great achievements in medical microbiology accounted for by "his simplicity of outlook" (Stephenson, 1949). Marjory Stephenson wrote "No guesses as to the 'how' are hazarded by this great man; he possessed the empirical outlook and aimed at the perfect technique. He was the right man at the right time and medicine probably owes as much to his limitations as to his great gifts." And earlier Topley and Wilson (Wilson and Miles, 1955) had concluded that "the advances which he made in staining methods, in the use of the microscope for the observation of bacteriological preparations, and in the technique of cultivating bacteria, revolutionized this branch (bacteriology) of science". Whatever epithet history bestowes on Koch, there can be no question that the experimental tradition which he founded had the most profound influence on the course of all facets of microbiology during the following 100 years.

3. STIMULI FOR CHANGE

The use of nutrient gelatin as a culture medium for microorganisms was soon shown to have limitations. Soil fertility was a question addressed by several notable scientists in the late decades of the nineteenth century and nitrogen transformations, in particular, received much attention. But, although nitrification in soil was proven to be a biological phenomenon in the 1870s, it was not until 1891 that Winogradsky succeeded in demonstrating the process in soil-free media and isolating nitrifying bacteria. This he did by avoiding the use of complex organic materials like gelatin in his culture media: instead he used simple salts, later to be made into a solidified medium by the means of silica gel, on which to grow bacteria of this so-called autotrophic type. These early studies of autotrophic bacteria revealed some pitfalls associated with the isolation of microorganisms into pure culture. Stephenson (1949)

recounts one such episode surrounding the work of Burri and Stutzer in 1895, who described the isolation of a *Nitrobacter*-like organism from soil on silica gel medium. This organism also grew in nutrient broth but lost its capacity to reduce nitrite even when subcultured back on to nitrite silica gel. Winogradsky's suspicions were roused by this report and he examined the culture himself. He showed that the *Nitrobacter* species was contaminated by three heterotrophs that grew in the nutrient broth but not subsequently on nitrite medium. Such associations of autotrophs and heterotrophs were repeatedly encountered by soil microbiologists in the first decades of the twentieth century, and the accompanying growth of heterotrophic bacteria with nitrifying species following serial subculture in inorganic media was not uncommon. Thus Sack in 1925 (cited in Stephenson, 1949) described mixed colonies of *Hyphomicrobium* species and *Nitrosomonas* species on silica gel medium which, if subcultured into nutrient broth, produced ammonia (due to growth of the heterotroph) and, if the amino acid concentration was not inhibitory, nitrite (from weak growth of the autotroph).

The study of distinct physiological groups of microorganisms, like the nitrifiers, lead Winogradsky and Beijerinck to the development of enrichment culture methods for their isolation. This universal microbiological method seeks to encourage the predominance of one type of organism by imposing selective growth conditions on a mixed microbial population: the type to be enriched can be varied at will by manipulating the physio-chemical conditions under which the isolation is made. Armed with such experimental techniques it is not surprising, therefore, that microbiology quickly blossomed to the extent where now we have a very detailed understanding of the physiology, biochemistry, and genetics of many diverse species. But, as in medical microbiology, this understanding has come from the study of pure cultures of organisms. The complementary studies of mixed populations have taken very much longer to develop and to gain acceptance. Thus, the effect of the all-important biological factor—species interactions—on microbial behaviour suffered neglect from all but a few microbiologists and in consequence remains the poorest understood facet of microbiology. There are several reasons why this situation arose and was perpetuated, not least being the established Koch tradition of experimental microbiology. The complexity of microbial communities in nature made their study a daunting prospect, added to which ecology was considered by most microbiologists to be something of a Cinderella subject until quite recent times. The predicament has been nicely construed by Brock (1966) in his *Principles of Microbial Ecology*: "ecology is physiology carried into the actual habitat; ecology is physiology under the worst possible conditions".

The analysis of microbial communities and the interactions occurring within them has been constrained by a number of conceptual and experimental dilemmas. Despite the ingenious work of pioneers, such as Gause, experimental studies of interacting microorganisms have only become satisfactorily feasible since the development of continuous-flow cultures such as the chemostat and its many elaborations. Most microbiologists now would recognize the chemostat as the most appropriate system for examining population interactions but it is pertinent to recall that the chemostat was introduced only 30 years ago and that its exploitation by those interested in mixed populations has occurred even more recently (Veldkamp and Jannasch, 1972). Among the conceptual constraints was that relating to the assumed instability of interacting populations (particularly those of a prey–predator type) in simple laboratory models of the sort used by Gause in the 1930s. On the one hand microbiologists appeared hesitant of accepting the Lotka-Volterra prediction of neutral stability and, on the other, animal ecologists were pointing to the necessity of spatial heterogeneity for the stability of prey–predator populations. In addition, a body of opinion grew up that, intuitively, was sceptical of the possibility of establishing stable microbial communities in the laboratory, irrespective of the interactions involved. Within the last few years these views have been emphatically refuted and many illustrations of the experimental proof will be found in this book. The recent work of Lewin and his colleagues on bacteria–bacteriophage dynamics in a chemostat reveals the extent of stable community complexity that can be established (Chao *et al.*, 1977): three populations of primary consumers in association with two populations of predators maintained in a habitat supported by a single limiting nutrient, glucose. In nature, microbial communities are exposed to a range of environmental perturbations and stresses but homeostatic responses generally enable them to retain their stability in the face of all but the most extreme stresses. The mechanistic bases for the various biological interactions that contribute to community homeostasis is reviewed later by Bull and Slater (pp. 13–44). Nevertheless, it is worth emphasizing at this point that stability within complex communities is usually affected by the simultaneous occurrence of different categories of interactions.

Stimuli for encouraging the study of microbial communities and the interactions upon which they are based have come from diverse sources. Quite naturally, the desire to treat microbial ecology in a synecological frame of reference has been a potent force for the development of mixed culture studies and laboratory model ecosystems. Systems of the latter type have seen increasing application in the area of en-