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MICROBIAL ECOLOGY AND THE DIRECT IDENTIFICATION OF MICROORGANISMS IN NATURAL HABITATS

A Historical Perspective

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I am convinced that microbiologists of my generation were quite fortunate indeed. The science of Microbiology as a well structured branch of biology, is not too old, just a few decades older than the science of Genetics which started in 1900 with the rediscovery of Mendel's laws. These two important sciences developed in a rather independent way, not paying too much attention to one another, particularly as far as Bacteriology was concerned, despite the fact that Genetics had adopted a few microorganisms as subjects for research in the first decades of this century. Some fifty years ago, the two sciences suddenly became inextricably linked to one another as a consequence of discoveries that mark what was perhaps the greatest revolution in biological knowledge of all times, when some of the most fundamental questions of genetics were answered by studies on bacteria and their viruses. In our young years, all the excitement was part of our lives; we could afford to have a "Renaissance" attitude toward the classical period of microbiology, were able to enjoy the wonders of the present, and attempt to imagine their projections to the future.

When I think of our priviledged position in the century, it comes to my mind the most familiar representation of the Roman god Janus, with one head and two faces, one looking to the classical period and the other to the future. In fact, this is not a good example. First, because the two faces of Janus were the symbols of departure and arrival, and, more important, because anyone over the age of forty is convinced that his best years are left behind and spends most of his time looking backwards and not forward. We should remember that Janus is also represented with a single face, as an old man, a symbol of balance.

I shall divide my presentation into two main subjects which are mentioned in the title. The first has to do with ecology, and the second with the direct observation of microorganisms in natural habitats.

During our life-time, it was interesting to follow the evolution of the science of ecology of plants and animals. This branch of biology is concerned with the analysis of interactions between living organisms and their natural habitats, and in part, with the relationships among organisms, since each member of the population contributes to modify the habitat shared with others. Natural populations thus achieve a certain balance because their members interact with one another and with the inanimate elements of the environment. At the beginning of the century, due to limitations in the knowledge of the elements involved, the ecology of plants and animals could only be formulated in general, descriptive terms. Today, thanks to significant advances in related sciences, the ecology of higher organisms has been elevated to a more central position than serving as a mere inventory of natural communities in various environmen-

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tal scenarios. This is mainly because we have realized that breaking the natural balance between the living and the inanimate worlds will be reflected in the long run on our own chances of survival in the planet. In other words, we are simply realizing that the quality of our lives strongly depends on the quality of the environment. But even today, progress in ecology is slow, because it is difficult to extrapolate to the real world the results of laboratory experiments, and there is a need for direct observations in the field, where formidable obstacles often conspire against rapid development.

While ecological studies of plants and animals were slow in developing, microbial ecology was placed from the beginning at the very core of the development of microbiology, and the roots of the impact caused by the work of sergei.

Winogradsky and Martinus Beijerinck's, are to be found in their ecological approaches.

The so-called modern approaches to the study of microbial communities in the wild were spelled half a century ago by Winogradsky himself, when, at the end of his career, he gave a bird's-eye view of the progress achieved in soil microbiology and summarized his own contributions to science. In the least chapter of his collected papers, Winogradsky made some remarks which may be pertinent to repeat here. He stated that pure culture methods had succeeded in the isolation and identification of important members of the soil microflora, such as the agents of nitrogen fixation or nitrification. However, studies limited to physiology and biochemistry of pure cultures of microorganisms in the laboratory, unless supplemented by ecological information, are of little help to clarify the role of microbes in nature, or to serve as a guide in the design of enrichment cultures for their isolation. Thus, members of the microflora capable of decomposing cellulose, such as *Cytophaga,* rapidly invade cellulose, but in the laboratory many of them are also capable of using glucose in pure culture. However, it would be impossible to achieve their isolation by using glucose in the enrichment culture, since many other organisms would rapidly invade the culture medium. A similar situation hods true in the nitrogen-fixing bacteria. *Azotobacter* readily uses carbohydrates to grow in nitrogen free media, and it can also be *grown* in the presence of combined nitrogen. However, ecological observations suggest that successful enrichment can only be achieved by the combination of two negative factors in the medium: absence of combined nitrogen and of "good" carbon sources. The combination lack of combined nitrogen + poor nutrients appears to be the right clue.

The recommendations that Winogradsky included in his last paper can be translated as follows:

1. resign to obligatory pure cultures;
2. avoid the use of cultures from culture collections;
3. for the purpose of experimentation, make use of organisms taken directly from natural environments;
4. try to avoid disturbing the organisms by the isolation procedure or by cultivation in media to which they are not adapted;
5. from the beginning of experimentation, search for conditions corresponding to the organisms' predilections, and stick to these conditions.

An important corollary of Winogradsky's experience is that the activity of the soil microflora cannot be conceived as the grand total of individual activities, but as the operation of an autoregulable community. In Winogradsky's words, 'the method of analysis of real activities of microorganisms in nature has to be based not on the behavior of species isolated from their natural milieu, but on the reactions of whole microbial communities in their own habitats'.

A quotation of this recommendation could very well be included in many of the papers that are published *today* in Applied and Environmental Microbiology and other journals devoted to environmental problems. Paradoxically, a good number of workers who have to deal with processes that are difficult or impossible to reproduce under laboratory conditions, have reached similar conclusions. This is a rather common situation now when one is dealing, for instance, with

the anaerobic microbial degradation of recalcitrant aromatic compounds. In many cases, it is impossible to experiment with pure cultures, either individually or in mixtures, and experimentation is reduced to the use of whole microbial 'communities' or 'consortia', two names who have become commonplace in the literature. There is a familiar ring in these expressions, already used by Winogradsky many years ago, although at present they do not emulate Winogradsky's grand vision, but are a reflection of our ignorance of the conditions required in the laboratory by the members of the natural microbial communities.

It must be admitted that, in spite of the spectacular success of the application of ecological principles to the discovery of agents of important microbial activities in natural cyclic processes, our knowledge of "microecology" is indeed very limited even today. A single crumb of soil has a large number of microhabitats, and individual bacteria actually interact with the components of a microenvironment limited to a few cubic micrometers. The limitations of our knowledge of bacterial ecology are most evident when it comes to defining the relationships of the various members of a microbial consortium operating at short distances from one another, particularly when they act on solid or poorly soluble substrates or when they collaborate at different stages in the degradation of certain compounds.

The second part of this presentation will refer to the observation of microorganisms in their natural habitats.

Figure 1 presents a very elementary scheme of analytical processing of field samples. Of course, this is a very simplified scheme in which each one of the steps could be expanded with many variations and recommendations that are found scattered in books or scientific papers, and often are part of laboratory folklore.

Samples are brought to the laboratory and processed following various experimental procedures. On the one hand, one can perform chemical analysis and estimate the biomass using various methods of different specificities. The most sophisticated form of chemical analysis includes the recognition of specific sequences of macromolecules (mainly nucleic acids) that can be extracted from the sample.

On the other hand, the analysis can be microbiological. The sample can be considered as an organ or as a whole organism, with which it is possible to do respirometry, microcalorimetry, etc., by traditional techniques, as ways of characterization of global biological activities. A second possibility is to isolate microorganisms by traditional methods. And a third option is to perform direct observation of microbes under some type of microscope (light, fluorescence, electron).

In the case of the fluorescence microscope, it is possible to recognize specific organisms

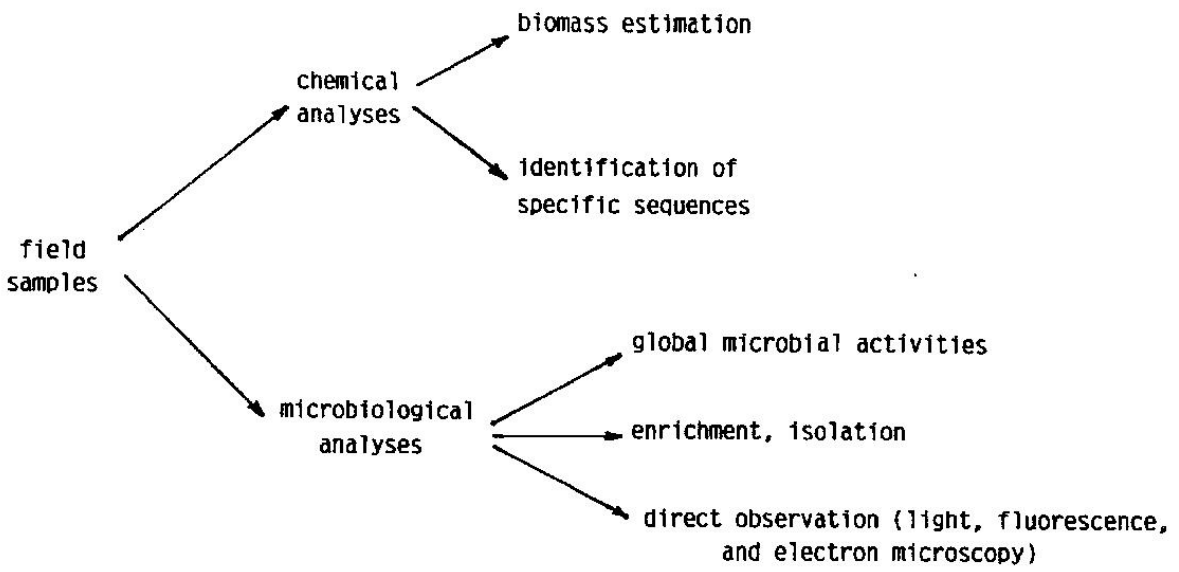

Fig. 1.-

through the use of probes conjugated with fluorescent reagents. The probes can be antibodies, or can be nucleotides complementary of sequences present in the organisms under investigation. Here, the methodology is common to that used for the recognition of specific sequences mentioned earlier under 'chemical analysis', but the macromolecule characteristic of a given organism is visualized in association with the whole cell, instead of being identified in the sample extract. In other words, molecular techniques allow the indirect identification of organisms by recognition of typical sequences extrated from the sample, or the identification of the sequences in the organisms themselves in microscopic preparations. A recent book edited by Levin, Seidler and Rogul (Microbial Ecology, McGraw-Hill, New York, 1992) analyzes the many aspects of the problems involved in the identification of organisms or their products under field conditions.

Of course, the quantitative ranges of these two approaches (the chemical and the microscopic) are vastly different. Recognition of a specific sequence in a field sample requires amplification, which can be achieved by the polymerase chain reaction (PCR), by means of which it is possible to increase the sensitivity of the identification reaction, to a point in which use of non-radioactive methods of detection become feasible. Thus, many methods of probing appear nowadays inextricably linked with amplification procedures, typically the PCR. Usually, the amplified target is fixed onto membranes, each of which can be hybridized with a specific probe. In addition, the "reverse-the-DNAs" approach has also been used, where the probe is immobilized on the membrane. A probe attached to long homomeric tail, is immobilized as individual spots onto the membrane. A real advantage of this approach is that only one hybridization is necessary, using the PCR-amplified target in solution. In the conventional method, membranes carrying the amplified product have to be subjected to individual hybridizations with the different probes.

Atlas and Bej (PCR Protocols. A Guide to Methods and Applications, Innis et al., editors, Academic Press Inc., San Diego, 1990, pp. 399) mention that the sensitivity of probes for direct detection of pathogenic bacteria in water samples is the order of 10^4 cells per ml, which is insufficient for sanitary control of the environment. Combination with PCR achieves the goal without sacrifice of specificity. The method consists in recovering all the cells (by centrifugation or filtration), release of DNA from all the cells, PCR amplification of required sequences, and detection of amplified genes by hybridization with specific probes. For total coliforms, for instance, the lacZ gene can be used; for fecal coliforms, *lamB*; for enteropathogenic bacteria, the heat labile toxin gene; for *Legionella,* a region of the 5S rRNA, etc. In this way, the sensitivity of detection reaches 1-10 fg of genomic DNA, which means from 1 to 10 cells per 1 to 100 ml of water.

The following example is taken from a paper by Steffan and Atlas (Annual Review of Microbiology 45, 137, 1991). In one gram of a soil sample, one cell of the organism to be identified may be present in the sample together with 10^{10} cells of other natural inhabitants. If the average genome size of all these organisms is about $5x10^6$ base pairs, and we have added a cell to the population of 10^{10} other organisms, a fragment of 500 base pairs characteristic of the introduced organism represents 1/10,000 of its cell genome, or one part in 10^{14} of the total DNA in the population, which means that if we continue our analysis with just one microgram of total DNA, in this aliquot we have 10^{-14} µg of fragment DNA. If at least 0.1 ng (that is, 10^{-4} µg) of DNA of 500 bp is required to see a band in an agarose gel, we need an amplification of the order of 10^{10} to reach this goal (this is equivalent to 2^{33}-2^{34}). If, instead, we transfer the band to a membrane and hybridize with a probe, the sensitivity increases about a thousand times, in other words, we only need an amplification of 10^7 to see the fragment. This amplification is equivalent to about 2^{24} times, which is easy to achieve by PCR.

Probes can use (a) total genomic DNA, (b) cloned fragments, or (c) synthetic oligonucleotides. Total genomic DNA has been used extensively in microbial taxonomy. Both possible directions can be used

in experiments, that is, either the known or the unknown DNA can be fixed onto the membrane. In these methods, proper discrimination is essential, since cross reactions are frequent, which often complicates considerably the interpretation of results.

b. Cloned fragments can be selected among those not reacting with other organisms (this has been used in sanitary bacteriology in the detection of *Salmonella*). Cloned probes using the ribosomal RNA (rRNA) determinants occupy a special place in the identification of organisms at various levels of relationships. The size of target per cell can be great, particularly with active cells, where the ribosome content is high.

c. Finally, synthetic DNA probes (usually of 15-20 bases) will hybridize with the target if perfectly matched. These probes can be very discriminatory, since minor mismatching can seriously affect hybrid stability.

The last point to be discussed here is the microscopic approach. This, for obvious reasons, can only be carried out in a much more limited scale than the recognition of specific sequences in sample extracts.

In devising microscopic methods for the observation of the soil microflora, we have to mention again Winogradsky, who was one of the first to perform direct observations of microorganisms in soils. A formidable obstacle that conspires against conclusions and generalizations is the fact that soils are the habitats for the most complex microbial populations and, therefore, drawing a background picture or a "ground level" for different soils, or even for the same soil represented by samples taken short distances apart, appears in principle as a truly formidable enterprise. This was fully appreciated by Winogradsky, who stated that it was essential to make observations on so-called "normal" soils with the idea of a) defining a stable or "type" population in places which have not been altered by human activities for a period of time, and b) obtaining an idea of the basic "biological state" of the soil. Once these parameters are defined, it would be possible to determine changes in the composition of the microflora provoked by experimental alterations.

In Winogradsky's experiments, the selection of a normal soil not affected by treatments was done by taking soil samples from places that had not received any fertilization for at least three years. A procedure of fractionation of soil suspensions was designed with inclusion of successive shakings and centrifugations. Smears of each supernatant and sediment obtained from each sample were made, and these were stained using acid dyes, because of the tendency of basic dyes to stain too deeply the inanimate components of soils.

Winogradsky's observations were rather unexpected. The microscopic appearance of prairie soils of diverse origins was remarkably uniform. The indigenous flora was found to be similar in all the samples, and it was called by Winogradsky autochthonous or humivorous. In contrast, the flora that develops after artificial treatments is much different and it is designated as zymogenic.

To my knowledge, these observations have not been repeated, but they left their imprint in the writings of modern general microbiologists. Thus, Professor H.-G. Schlegel (Allgemeine Mikrobiologie, 7th edition, Georg Thieme Verlag, Stuttgart, 1992) refers to autochthonous and allochthonous components of the microflora. The latter are the organisms that have survived and exist in a dormant stage, and can prosper under conditions of artificial treatments.

It would be impossible here to present a summary of the microscopical work that has been done on natural microbial habitats since the time of W., and the best one can do is to briefly mention a few striking modern pieces of research.

Interesting contributions have been reported for the identification of organisms by means of fluorescent antibodies. The methods have been usually adaptations from those used in medical bacteriology. The list of organisms identified by these procedures is very long and includes *Pseudomonas, E. coli, Rhizobium, Azotobacter, Chromobacterium,* nitrifiers, iron bacteria, sporeformers, anaerobes, fungi.

Particularly interesting work has been carried out in Professor Karl Schleifer's laboratory in Munich with nucleic acid probes in the identification of unculturable organisms (Amann et al., Nature 351, 161, 1991) which are parasites of *Paramecium*

caudatum. They belong to the genus *Holospora*. One species, *H. obtusa*, lives in the macronucleus of certain strains of *Paramecium,* while other two species (*H. elegans* and *H. undulosa*) attack other strains and live in the micronuclei. Schleifer's group constructed three types of probes. The first, conjugated with fluorescein, was complementary of a 16s rRNA sequence present in all bacteria. The second, conjugated with tetramethylrhodamine, was specific for H. *obtusa*, and was complementary to a fragment of the 23Ss rRNA, found only in this species. The third probe, also red because of rhodamine, was complementary of a less variable region of the 23s rRNA, which can be found in all *Holospora* species. These three types of probes are capable of clean differentiation of *Holospora* from the bacteria that were used as food for the growth of the host, and they also made possible the differentiation among the *Holospora* species. The results are strikingly illustrated in the original paper.

A variation of this approach that could be used in field samples was also tested in Schleifer's laboratory. The results were presented by Schleiferl at the third symposium on *Pseudomonas* which took place in Trieste in 1991, and published in the proceedings (*Schleifer et al., in Pseudomonas.* Molecular Biology and Biotechnology, Galli, Silver and Witholt, editors. American Society for Microbiology, Washington, D.C., 1992, p. 127). The workers used a) a mixture of *Escherichia coli* and *Comamonas testosteroni* cells; b) a mixture of *P. cepacia* and *P. fluorescens* cells, and c) a soil suspension with added *P. diminuta* cells.

In each of the cases, the organisms could be specifically distinguished in the smears by treatment with specific nucleic acid probes complementary of the rRNA gene sequences. In these, certain regions are very conservative and are present in all bacteria, while other segments show various degrees of variability, which allow specific recognition of bacterial genera, species and even different strains within a single species. The identification of the component members present in preparations *a* and *b* mentioned above could be achieved very precisely. In preparation *c*, the cells of *P. diminuta* and none of the other members of the microflora, could be clearly differentiated.

The methodology has undergone enormous progress since Winogradsky's time. We are now at the stage of developing many very sensitive tools able to fulfill the needs of specific situations. The tools will be of crucial help in attacking the numerous problems of modern microbial ecological research. It is to be hoped that the problems envisaged by the great pioneers of soil microbiology will soon be amenable to analysis, so that a more precise formulation of biological dynamics of natural media may be achieved in the near future.

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