Unculturable bacteria—the uncharacterized organisms that cause oral infections

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The first bacterial culture media were broths made either by infusion or by enzymatic digestion of meat from various sources. Originally developed by Spallanzani in the 18th century and then refined by Pasteur in the 19th century, these allowed the recovery of bacteria from human disease sites[1,2]. However, it was quickly realized that such broths would be likely to contain mixtures of micro-organisms, and Robert Koch saw the need for development of solid culture media that would allow the physical separation of bacterial colonies. He first tried aseptically divided potatoes. Material taken from infected lesions was spread across the potato and then incubated at body temperature. Following incubation, bacterial colonies were seen which could be subcultured to further potatoes to give pure cultures. Although successful, Koch observed that only a limited number of the micro-organisms present in the sample grew on the potato. This was probably the first recognition of the phenomenon of unculturability *in vitro*. Nevertheless, the success of the technique led to the use of solidifying agents such as gelatin and agar to create solid media from the broths developed by Pasteur and others. This advance led to the golden age of medical microbiology, in the last quarter of the 19th century, when many of the bacteria causing serious infections in man were identified. This tremendous success, however, probably led micro-biologists to become complacent simply because so many important pathogenic bacteria could be cultured *in vitro* in this way.

UNCULTURABILITY

We are grossly ignorant of bacterial life on earth. Environmental microbiologists estimate that less than 2% of bacteria can be cultured in the laboratory. In the mouth we do rather better, with about 50% of the oral microflora being culturable[3]. For other body sites, the figure is unknown but is likely to be similar to that found in the mouth or higher. For example, the colonic microflora is suspected to be predominantly unculturable. It is therefore likely on numerical grounds alone that unculturable and therefore uncharacterized organisms are responsible for several oral and other human infections. A known instance is syphilis, caused by the spirochaete *Treponema pallidum*, which remains unculturable today.

MOLECULAR IDENTIFICATION OF BACTERIA

It is only with the advent of molecular biology that techniques have become available for studying mixed bacterial communities in their entirety, without the biases of culture. The theoretical basis for the development of these methods came first from Zuckerandl and Pauling[4], who suggested the use of biological macromolecules for the elucidation of evolutionary relationships among organisms. This idea, which developed into the branch of science known as molecular phylogeny, relies on the analysis of the DNA sequences of genes of common ancestry, or proteins themselves, in a range of organisms. Mathematical techniques are used to assess the similarity of these sequences and to construct phylogenetic trees which demonstrate the evolution of the whole organisms from which the DNA or proteins were isolated. In practice, 'housekeeping' genes are used for this purpose since they are widely distributed among different organisms and because their essential function has made them relatively conserved throughout evolution, allowing easy alignment of the sequences. The most widely used of these genes to date has been the small subunit (16*S*) ribosomal RNA gene[5]. At around 1500 base pairs in length this is both long enough to be informative and short enough to allow easy sequencing, particularly since the advent of automated DNA sequencers. Phylogenetic trees are constructed by first aligning sequences of the same gene from different organisms. Then the genetic distance between pairs of organisms in the dataset is calculated to give a matrix of similarities. This matrix is then further analysed by, for example, the neighbour-joining method in order to construct the phylogenetic tree or dendrogram[6].

Once such a tree has been constructed, the identity of an unknown organism can be determined by simply adding the sequence of its gene to the tree or by running a similarity search against other sequences of the same gene in a database. For 16*S* rRNA there are now several databases, containing around 12 000 sequences in total[7,8].

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CHARACTERIZATION OF MIXED BACTERIAL COMMUNITIES

Mixed bacterial communities can be characterized by adding polymerase chain reaction (PCR) and cloning steps to the procedure described above. DNA is first extracted directly from the biomass of the original sample without culture of the bacteria contained within it. Then, the gene encoding 16*S* rDNA is amplified by PCR with primers specific for conserved regions of the gene (i.e. regions present in >95% of bacterial species[9]). This will result in a mixture of all of the 16*S* rDNA molecules from the organisms in the original sample. These are then cloned into a plasmid vector, which is used to transform an *Escherichia coli* host, thereby establishing a library of 16*S* rDNA from the sample. The cloned genes can then be sequenced individually and submitted for identification to the databases mentioned above, typically via the World Wide Web. In this way, the composition of the microflora in the sample can be determined. If this procedure is complemented with traditional culture then the 16*S* rRNA genes sequenced from the culturable organisms can be 'subtracted' from the total microflora displayed by the molecular analysis, to reveal the unculturable portion. This technique, or a variation of it, was first used to describe the microflora of environmental sites such as hot springs and deep sea-water[10,11].

MOLECULAR ANALYSIS OF THE ORAL MICROFLORA

Molecular analysis of the oral microflora to date has focused on the bacteria associated with dento-alveolar abscesses and periodontitis. Dymock *et al.*[12] performed molecular analysis of pus aspirated with three dento-alveolar abscesses and found five groups of unculturable, and therefore previously uncharacterized, organisms that were predominant in the samples. Two of these represented totally novel eubacterial lineages. In addition, it was found that the numbers of two culturable species, *Fusobacterium nucleatum* and *Porphyromonas endodontalis*, were grossly underestimated in the samples. These observations suggest that some cultivable species include uncultivable biotypes.

A substantial number of novel taxa have also been identified in molecular studies of gingivitis[13] and periodontitis[14]. It is noteworthy that the majority of novel lineages isolated from oral infections are concentrated in two divisions of the phylogenetic tree—the Cytophagas and the low G+C Gram-positive bacteria.

The studies of the oral microflora described above have all made use of universal PCR primers for the initial 16*S* rDNA amplification, to maximize the recovery of organisms from the sample. The technique can also be adapted to use group-specific primers to specifically seek unculturable and uncharacterized organisms within specific regions of the phylogenetic tree. Spratt *et al.*[15] designed primers for the oral asaccharolytic Eubacterium branch of the low G+C Gram-positives and identified two groups of novel organisms within this branch from a single advanced periodontitis sample. Similar studies are now underway to investigate the microflora associated with endodontic infections, dentinal caries, and the early stages of plaque development.

WHY ARE SOME BACTERIA UNCULTURABLE?

As discussed above, it has been known for over a hundred years that *in-vitro* culture conditions may not allow the growth of all bacteria in a sample. Some of the possible reasons are that a required nutrient is not present in the culture medium, that the culture medium itself is toxic, or that other bacteria in the sample produce substances inhibitory to the target organism. In addition, we know that bacteria can depend on each other for growth. Oral bacteria, in particular, have evolved over millions of years in a mixed community in a biofilm. Over this time, some may have acquired mutations in essential synthetic pathways but are able to obtain the substances required from other bacteria in the biofilm. Of course, a bacterium dependent on another will not be able to grow independently *in vitro*. One example of this is *Bacteroides forsythus*, a Gram-negative anaerobe implicated in periodontitis, which has an absolute requirement for N-acetyl muramic acid[16], one of the essential components of peptidoglycan. This organism grows very poorly in pure culture but grows well either in co-culture with other organisms or in media supplemented with N-acetyl muramic acid.

Another reason for non-culturability *in vitro* may be the disruption of bacterial cytokine networks. Bacterial cytokines[17] are thought to be the mediators of bacterial–bacterial signalling and may be particularly important in coordinating the growth of component organisms in bacterial biofilms such as dental plaque. Mukamolova *et al.*[18] have reported the existence of a resuscitation-promoting factor (RpF) in *Micrococcus luteus* which stimulates the growth of other Gram-positive bacteria at picomolar concentrations. It is possible then that bacterial growth is controlled by networks of such cytokines, which may be responsible for the shifts in plaque composition in response to environmental factors. Obviously, the separation of bacteria on solid media would disrupt such networks and may explain why some organisms are unculturable.

CONCLUSIONS

The PCR-cloning-sequencing technique described in this review now allows the essentially complete description of complex bacterial communities. Originally developed for

the dissection of environmental ecosystems, these are now being applied to the human microflora, especially the flora associated with oral infections. These studies have confirmed that the estimates of bacterial unculturability made from combined microscopic/cultural studies are essentially correct and that 50% of the oral flora is unculturable. This group is highly likely to include novel pathogens, so we may well be entering a new golden era of microbiology when associations between specific organisms and infectious diseases can be assigned with greater certainty than ever before. We are also becoming increasingly aware of the extent to which pathogenic bacteria in mixed infections communicate with each other and their mammalian host. The normal flora organisms in man have evolved as a biofilm and may be dependent on each other for nutrition, working together in consortia to cause disease in susceptible hosts. *In-vitro* investigation of such organisms should take into account this interdependence.

REFERENCES

1 Foster WD. *A History of Medical Bacteriology and Immunology*. London: Heinemann, 1970

2 Collard P. *The Development of Microbiology*. Cambridge: Cambridge University Press, 1976

3 Wilson MJ, Weightman AJ, Wade WG. Applications of molecular ecology in the characterisation of uncultured microorganisms associated with human disease. *Rev Med Microbiol* 1997;**8**:91–101

4 Zuckerkandl E, Pauling L. Molecules as documents of evolutionary history. *J Theoret Biol* 1965;**8**:357–66

5 Woese CR. Bacterial evolution. *Microbiol Rev* 1987;**51**:221–71

6 Olsen GJ, Woese CR, Overbeek R. The winds of (evolutionary) change: breathing new life into microbiology. *J Bacteriol* 1994;**176**: 1–6

7 Neefs JM, Van de Peer Y, De Rijk P, Chapelle S, De Wachter R. Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res* 1993;**21**:3025–49

8 Maidak BL, Cole JR, Lilburn TG, *et al.* The RDP (Ribosomal Database Project). *Nucleic Acids Res* 1997;**25**:109–11

9 Lane DJ. 16*S*/23*S* rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. *Nucleic Acid Techniques In Bacterial Systematics*. Chichester: Wiley, 1991: 115–75

10 Giovannoni SJ, Britschgi TB, Mayer Ch, Field KG. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* 1990;**345**:60–3

11 Ward DM, Weller R, Bateson MM. 16*S* rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* 1990;**345**:63–5

12 Dymock D, Weightman AJ, Scully C, Wade WG. Molecular analysis of microflora associated with dentoalveolar abscesses. *J Clin Microbiol* 1996;**34**:537–42

13 Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci USA* 1999;**96**:14 547–52

14 Sakamoto M, Umeda M, Ishikawa I, Benno Y. Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16*S* rDNA libraries. *Microbiol Immunol* 2000;**44**:643–52

15 Spratt DA, Weightman AJ, Wade WG. Diversity of oral asaccharolytic Eubacterium species in periodontitis—identification of novel phylotypes representing uncultivated taxa. *Oral Microbiol Immunol* 1999;**14**:56–9

16 Wyss C. Dependence of proliferation of *Bacterioides forsythus* on exogenous N-acetylmuramic acid. *Infect Immun* 1989;**57**:1757–9

17 Kell DB, Young M. Bacterial dormancy and culturability: the role of autocrine growth factors. *Curr Opin Microbiol* 2000;**3**:238–43

18 Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB. A bacterial cytokine. *Proc Natl Acad Sci USA*, 1998;**95**:8916–21