



Molecular taxonomy of polymicrobial diseases – finding novel bacteria not previously considered to be associated with oral diseases

Introduction

Recombinant DNA technology and molecular biology have brought a revolution to the limitations imposed by traditional taxonomic methods of bacterial identification based solely on cultivation. Following on from the novel research of Carl Woese¹, molecular taxonomy is generally based on variations in the sequence of the 16S rRNA gene and has brought a new perspective to our understanding of the diversity of uncultured bacteria in a multitude of microbial habitats. Analysis of the polymicrobial oral diseases is no exception.

The two most prevalent oral infections are the polymicrobial diseases, periodontal disease and dental caries. In periodontal disease, progressive destruction of periodontal ligament and alveolar bone due to the presence of a polymicrobial population in the periodontal pocket can lead to the loss of a tooth (Figure 1). Cultured strains of *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* have been implicated as major pathogens involved in the progression of various forms of this disease. Dental caries, on the other hand, results from the acid fermentation products of oral bacteria that demineralise the tooth enamel. Species of streptococci, lactobacilli and actinomycetes have been implicated in the initiation of dental caries. However, the microbiology of the progression of the unarrested carious lesion once the outer enamel of the tooth has been breached and the infection spreads into the pulp and subsequently into the periapical region, has only recently been documented.

**Mangala Nadkarni, Roy Byun
and Kim-Ly Chhour**

Institute of Dental Research
Westmead Millennium Institute and
Westmead Centre for Oral Health
PO Box 533
Wentworthville, NSW 2145
Tel: (02) 9845-7826
Fax (02) 9845-7599
E-mail:
mnadkarni@dental.wsahs.nsw.gov.au

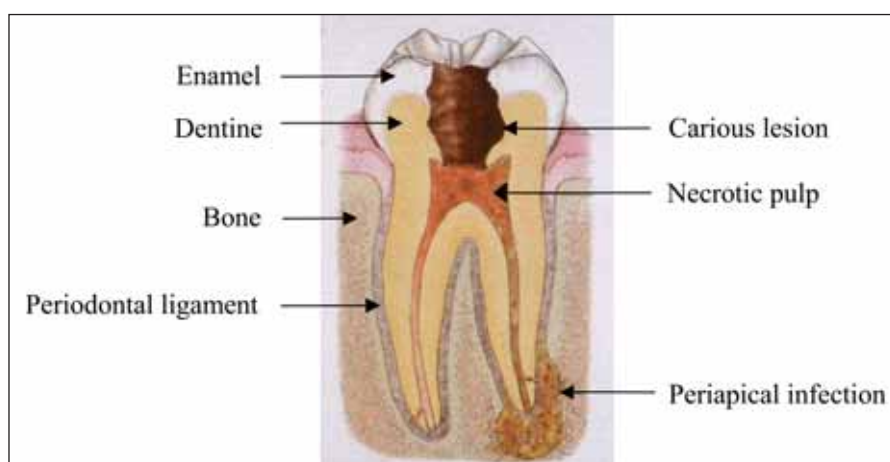
The application of a molecular taxonomic approach based on the 16S rRNA gene has emphasised the complex nature of these diseases and identified unique uncultured species of bacteria. These studies have shown that the composition of the oral microbiota comprises both aerobic and anaerobic Gram-positive and Gram-negative bacteria. Currently, more than 700 bacterial species have been identified as inhabiting the oral cavity². Most of these are strictly anaerobic bacteria with fastidious growth requirements. There is also increasing evidence that these polymicrobial infections exist due to

complex synergistic and/or inhibitory interactions between the different species and the host.

Novel bacteria associated with periodontal infections

Molecular analysis of bacteria associated with various forms of periodontal disease² has resulted in the identification of bacteria belonging to nine separate bacterial phyla. Several novel phylotypes have been identified in each of these nine phyla. In particular, members of three phyla, *Obsidian Pool* OP11, TM7 and *Deferribacteres*, have only previously been reported in extreme natural environments and no cultivable representatives have been identified. Most of these phyla are associated with periodontal disease. Using fluorescence *in situ* hybridisation, Brinig *et al.*³ found that members of the TM7 phylum isolated from human subgingival plaque exhibited a filamentous morphology that was composed of multiple cells. Quantification of members of this phylum

Figure 1. Schematic diagram of structure of tooth.





by real time PCR indicated that the subgroup IO25 was far higher in numbers at subgingival sites categorised as mild, moderate or severe and was rarely detected in healthy sites. These observations stressed the importance that these uncultured bacteria play in the progression of periodontal disease.

In a separate study, Kumar *et al.*⁴ compared the presence and absence of 39 bacterial species, including newly identified novel phylotypes using PCR amplification of the 16S rRNA gene. This study revealed that the uncultivated phylotypes, clone W090 from *Deferribacteres* phylum and clone BU063 from *Bacteroidetes* phylum, were more prevalent in people with healthy gingiva. In contrast, a strong association with disease status was shown by the presence of clones D084 and BH017 from the *Deferribacteres* phylum, clone AU126 from the *Bacteroidetes* phylum, IO25 clone from the TM7 phylum and clone X112 from the OP11 phylum.

Novel bacteria associated with advanced dental caries

Recently, our group at the Institute in Sydney has undertaken a comprehensive analysis of the bacteria associated with

advanced caries using the 16S rRNA gene for phylogenetic identification and quantification by real time PCR⁵. Multiple species of the two predominant families, *Lactobacillaceae* and *Prevotellaceae* (cultured and uncultured), were associated with carious dentine with as many as 31 different taxa in a given lesion. A total of 75 species or phylotypes was identified from a study of 10 teeth with a number of novel phylotypes present in numerically significant numbers. These included *Propionibacterium* sp strain FMA5, *Olsenella* sp. C3MLM018, *Prevotella* sp. IDR-CEC-0032, *Lachnospiraceae* sp. MCE9-31 and *Lachnospiraceae* sp. C4ALM087. A similar study by Munson *et al.*⁶ identified 31 previously unreported taxa in five teeth containing advanced carious lesions.

Analysis of the first of the two predominant families associated with carious dentine, the *Lactobacillaceae*, defined 18 different phylotypes of lactobacilli based on 16S rRNA gene sequences⁷. Several novel phylotypes that were present had only previously been isolated from unrelated habitats such as gastrointestinal tract of animals, sourdough, maize silage and semi-fermented wine. Of particular significance was *Lactobacillus ultunensis*

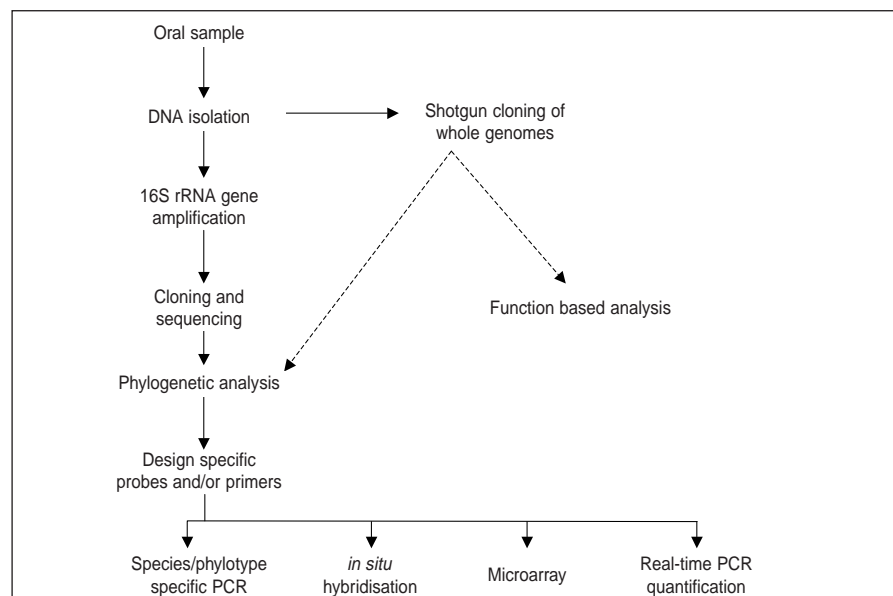
and a closely related *Lactobacillus* phylotype, together they comprised 19% of the sequenced clones. *Lactobacillus ultunensis* had previously only been associated with human stomach mucosa. Quantification by real time PCR indicated that *L. ultunensis* (and its related phylotype) along with *Lactobacillus gasseri* were present in higher numbers than other prevalent species. These species of lactobacilli had not previously been identified as being associated with oral disease.

The second predominant family comprised a complex array of novel prevotella phylotypes⁸. Phylogenetic analysis of prevotella specific 16S rRNA gene sequences grouped these prevotellae into six clusters. Clusters I to V were similar to previously reported but uncultured *Prevotella* phylotypes, whereas Cluster VI comprised a novel phylotype with 94% similarity to an uncharacterised *Prevotella*-like rumen bacterium. Real time PCR quantification of all six clusters showed that Cluster VI was the numerically dominant cluster. Recently, Sakamoto *et al.*⁹ isolated a non-pigmented, non-spore forming, non-motile, obligatory anaerobic Gram-negative rod from the oral cavity and showed that it possessed 99% 16S rRNA gene identity to our representative of cluster VI, oral phylotype IDR-CEC-0032. This bacterium has been given the species name, *Prevotella multisaccharivorax* sp. nov, based on its distinct sugar fermentation and cellular fatty acid composition⁹.

Conclusion

It is presently unclear why the oral cavity presents a favourable environment for the colonisation of such a diverse array of bacteria and how these complex groups of micro-organisms co-exist in the mouth. Dietary carbohydrates, degraded glycoproteins and the immunological status of the host all undoubtedly play a role, leading alternatively to the progression of dental caries or the onset of a periodontal infection.

Figure 2. Interactive approach for finding novel bacteria by molecular taxonomy.





Future directions

Currently a so called 'Human Oral Microbe Identification Microarray (HOMIM)' slide system is under development at the Forsyth Institute in Boston in the USA for use in the identification of about 600 known and novel bacterial phylotypes identified by molecular means from the oral cavity. Successful development of this technology could lead to simultaneous identification of cultured and uncultured bacteria in clinical samples at much lower costs than current DNA sequencing. Such arrays will help in diagnosing the onset of oral diseases and will ultimately have an impact on treatment protocols and outcomes.

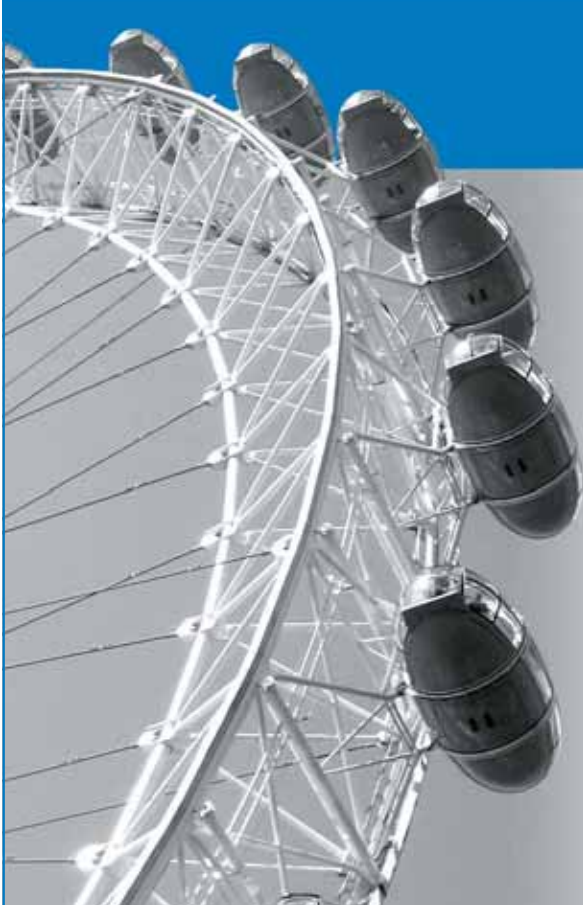
Culture-independent genomic analysis using whole genome shotgun cloning of collective microbial genomes, known as metagenomics, is also currently being pursued to study the physiology and

ecology of environmental micro-organisms¹⁰. In future, the application of similar analyses to polymicrobial oral infections might help explain how these complex microbial communities function in oral cavity and how they interact with their human host (Figure 2).


A recent NIDCR initiative to support a study employing metagenomics to re-build bacterial genomes forming oral biofilms associated with periodontal disease marks the beginning of this approach in biomedical research (<http://www.nih.gov/news/pr/dec2004/nidcr-07.htm>).

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