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Molecular methods for exploring the intestinal ecosystem

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Molecular methods have provided renewed impetus for the analysis of the composition of the intestinal microflora in health and disease. The polymerase chain reaction coupled with denaturing gradient gel electrophoresis provides a method whereby the bacterial communities in large numbers of samples can be compared efficiently and effectively. Altered bacterial populations associated with disease states can then be targeted for further investigation. In the long-term, an 'abnormal microflora' might be rectified by the use of probiotics or prebiotics.

Intestinal microflora: Molecular methods: Analysis

Common knowledge

The intestinal microflora of humans inhabits the distal regions of the bowel, especially the colon. Here, possibly hundreds of bacterial species form a bacterial community in which some members number about 10^{11} cells per gram (wet weight) of contents. The predominant species are obligately anaerobic and are extremely oxygen sensitive. Perhaps because of their fastidious requirement for anaerobiosis, but more likely due to their complex nutritional requirements, about 60 % of the bacterial community cannot be cultivated in the laboratory even when excellent bacteriological culture methods are used (Tannock *et al.* 2000). The 16S rRNA gene sequences of these viable, but non-cultivable bacteria, have been detected in phylogenetic studies of human microflora and it seems that the predominant bacteria comprising the faecal microflora of humans belong to the *Bacteroides-Prevotella* group, the *Clostridium coccoides* group, and the *Clostridium leptum* group (Franks *et al.* 1998; Sghir *et al.* 2000). Each of these phylogenetic groups, of course, contains many bacterial species belonging to several genera.

The bacterial community inhabiting the intestinal tract is strongly homeostatic and characteristic of the human host from which the sample was obtained (Tannock *et al.* 2000; Zoetendal *et al.* 1998). These phenomena have been observed when the gut microflora has been analysed using PCR coupled with denaturing gradient gel electrophoresis (DGGE). In this method of analysis, bacterial DNA is extracted from the faecal or intestinal sample and fragments of the 16S rRNA gene (no more than 400 bp in length) are amplified by PCR. One of the primers has a GC-rich 5′ end (GC clamp). 16S fragments are amplified from the bacterial community in the sample and denaturing gradient gel electrophoresis separates the 16S molecular species within the resulting mixture. The double-stranded 16S fragments migrate through a polyacrylamide gel containing a gradient of urea and formamide until they are partially denatured by the imposed chemical conditions. The fragments do not completely denature because of the GC clamp, and migration is radically slowed when partial denaturation occurs. Because of variation in the 16S sequences of different bacterial species, chemical stability is also different; therefore different 16S 'species' can be separated by this electrophoretic method. A profile of 16S sequences amplified from the sample is thus obtained (Muyzer & Smalla, 1998).

The uniqueness of individual microfloras extends even to the level of bacterial strains. Genetic fingerprinting methods such as ribotyping and pulsed field gel electrophoresis of DNA digests prepared from bacterial isolates has permitted analysis of the human faecal microflora at the strain level. Examination of bifidobacterial and *Lactobacillus* populations in monthly faecal samples collected over a 12-month period has shown that there can be marked variation in the complexity and stability of these bacterial populations between human subjects (Kimura *et al.* 1997; McCartney *et al.* 1996). It is clear from the results of these studies that individual humans harbour unique microfloras when considered at the level of bacterial strains, as well as at the level of 16S rDNA community profiles. Bifidobacterial populations were consistently large in all samples that were examined and strains that persisted in the faeces for at least 12 months could be recognised. *Lactobacillus* populations fluctuated in size in the case of most subjects but unique, persisting strains

Abbreviations: PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; bp, base pairs; rRNA, ribosomal DNA; rDNA, ribosomal DNA gene; SPF, specific pathogen free; IgE, immunoglobulin E; Th, T-helper; APS, adenosine-5′-phosphosulphate.

Note: For the definition of the terms inulin and oligofructose please refer to the introductory paper (p. S139) and its footnote.

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could often be detected (Kimura *et al.* 1997; McCartney *et al.* 1996).

Normal and abnormal

Practical application of knowledge about the composition of the intestinal microflora in medicine requires that the abnormal state can be differentiated from the normal. If this can be accomplished, remedial action might be able to be designed for humans suffering from diseases with which an abnormal microflora is associated. Candidate diseases in which an abnormal microflora might be implicated include:

- *Inflammatory bowel disease.* Members of the intestinal microflora provide a constant antigenic stimulus for the host's immune system. Normally, immunological tolerance towards the intestinal microflora prevents continuous intestinal inflammation. While the aetiologies of Crohn's disease and ulcerative colitis remain unknown, there is compelling evidence from animal models that this controlled, homeostatic response is lost in genetically susceptible hosts which therefore develop chronic immune-mediated colitis. This notion is supported by rodent models in which targeted deletion or over-expression of a variety of genes that regulate immune or mucosal barrier function lead to an overly aggressive cellular immune response confined to the intestine (Elson *et al.* 1995; Sartor, 1997).

  Results from experiments with germfree rodents, in which intestinal inflammation is absent or only very mildly expressed, indicate that bacteria are indispensable contributors to the pathogenesis of chronic, immune-mediated, intestinal inflammation. This concept is further supported by the rapid development of colitis when germfree HLA-B27 transgenic rats and IL-10 deficient mice are colonised with bacteria from healthy specific-pathogen-free (SPF) animals (Sartor, 2000; Sellon *et al.* 1998). Additionally, decreasing the bacterial load by the administration of broad-spectrum antibiotics reduces colitis (Rath *et al.* 1998). PCR coupled with DGGE provides a means of screening the composition of the gut microflora harboured by animals that differ in the absence or presence of colitis.
- *Atopic disorders*. Comparison of the immunological characteristics of germfree and conventional animals has demonstrated that the presence of the normal microflora stimulates the reticuloendothelial tissues of the animal, particularly those tissues associated with the intestinal tract. Peyer's patches and mesenteric lymph nodes are more developed, the lamina propria of the intestinal wall contains more neutrophils and lymphocytes, and the serum contains a higher concentration of immunoglobulins in conventional animals compared to the germfree situation (Gordon & Pesti, 1971). The influence of the intestinal microflora on the development of the immune system needs to be intensively studied. A practical application of such studies would be to understand the increasing incidence of atopic disorders such as asthma of children in affluent countries. Atopic disorders are characterised by dominant T-helper 2 (Th2) mechanisms and the production of immunoglobulin E (IgE) to common environmental antigens. Asthma is an atopic disorder characterised by activation and recruitment of eosinophils to the lung resulting in chronic swelling and inflammation of the airways. The reasons for the increase in atopic disorders in developed countries are unknown, but several factors in addition to hereditary predisposition that may be of significance, all concerning microbial exposure in childhood, have recently been identified in retrospective studies (Farooqi & Hopkin, 1998; Romagnani, 1997; Wickens *et al.* 1999). One of these factors concerns treatment of children with oral antibiotics during the first two years of life. Three categories of broad-spectrum antibiotics were identified in this role in a study conducted in the United Kingdom: penicillins, cephalosporins and macrolides (Farooqi & Hopkin, 1998). The mechanism by which antibiotics influence the programming and development of the immunological system most likely involves alterations to the collection of bacterial species inhabiting the intestinal tract. Treatment of young children with broad spectrum, oral antibiotics might produce perturbations in the composition of the intestinal microflora such that bacteria important in promoting Th1 mechanisms are depleted at a crucial age. This could result in Th2 dominance over Th1 immune responses to environmental antigens and an increased incidence of atopic disorders. The aetiology of atopic disorders is doubtless complex, but it is important that their molecular origins be determined in order to develop effective countermeasures. One such countermeasure may be the administration of appropriate bacteria to children if these bacterial types can be shown to programme the immune system towards a Th1 response as has been demonstrated with avirulent *Mycobacterium bovis* (Erb *et al.* 1998). But it is necessary, first, to determine the impact of antibiotic administration on the composition of the intestinal microflora of children. PCR coupled with denaturing gradient gel electrophoresis is a useful technique to employ in these investigations.

Tracking bacterial species and strains

Studies to date that have used PCR and denaturing gradient gel electrophoresis to analyse the intestinal microflora have utilised universal primers that amplify DNA from all of the bacteria in the sample. The aim in these studies has been to derive a community profile representing 90–99 % of the bacterial inhabitants (Zoetendal *et al.* 1998). PCR coupled with denaturing gradient gel electrophoresis can be developed, however, for the analysis of specific bacterial populations comprising the microflora. PCR primers that amplify DNA sequences characteristic of particular phylogenetic groups (for example *Bacteroides-Prevotella*,

Bernhard & Field, 2000) have been designed and these permit the differentiation of at least some of the species that comprise the group.

Analysis of bacterial communities using molecular techniques has so far targeted 16S rRNA gene sequences because, as revealed by Carl Woese, small ribosomal subunit RNA (16S rRNA in the case of bacteria) contains regions of nucleotide base sequence that are highly conserved and that these are interspersed with hypervariable regions (Woese, 1987). These hypervariable regions contained the signatures of phylogenetic groups and, sometimes, even species. An alternative approach is to analyse the community in terms of bacteria that carry out a particular metabolic activity. Sulphate-reducing bacteria, for example, may have an association with inflammatory conditions of the intestine. Bacteria that reduce sulphate are a heterogeneous group, but they have a common feature of activating sulphate to adenosine-5′-phosphosulphate (APS). Anaerobic bacteria then reduce this molecule to form bisulphite and then hydrogen sulphide. PCR primers that amplify APS reductase sequences from sulphate-reducing bacteria have been designed, and can be used to detect these bacteria in faecal or intestinal samples (Deplancke *et al.* 2000).

Recycling Robert Koch

Recognition of differences in the composition of microfloras begs the question of the biological significance of such observations. A decrease or increase in a bacterial population is really only the start of the investigation. What these changes mean to the host must then be determined. Doubtless, gnotobiotic research with experimental animals will have an important role in performing, as far as is feasible, experiments to satisfy Koch's postulates. A renaissance in gnotobiotic research can be envisaged after several decades of neglect of this field.

The derivation of remedies to rectify 'abnormal' microfloras will become realistic propositions when the biological significance of specific groups comprising the bacterial community is known. Specific probiotics and prebiotics may have roles as remedies. *Homo sapiens* is, however, a genetically diverse species, and universal remedies may be difficult to achieve given our current concept of the uniqueness of individual microfloras. However, it is surely worth trying.

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