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Culturing 'Uncultivable' Oral Bacteria

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Although the advent of next-generation sequencing has led to better appreciation of the true diversity of the human oral microbiome, it has not superseded the requirement for laboratory culture in the physiological and pathological characterisation of individual bacterial species. Yet over a third of oral bacteria are as-yet-uncultivated. Attempts to cultivate certain bacteria in isolation have been met with little success, possibly because species in biofilm communities may depend on each other for metabolic cooperation, such as, through sharing of iron-chelating siderophores.

With the aim of developing an in-vitro culture model for cultivation and isolation of previously-uncultivated oral bacteria, we have used a targeted approach focused on the uncultivable 'cluster A' of the recently-recognised phylum, *Synergistetes*, followed by an open-ended approach for isolation of non-specific novel targets. Microcolonies of cluster A *Synergistetes* in mixed culture on Blood Agar (BA) were targeted by rRNA-directed colony hybridisation at 14 days of anaerobic incubation. Repeated sub-culture of hybridisation-positive regions of cells, resulted in the enrichment and (after eight passages) eventual isolation of a novel taxon from this previously-uncultivated group, subsequently named *Fretibacterium fastidiosum*. Its growth was slow and significantly stimulated by other members of the original mixed bacterial community (*Parvimonas micra* and *Tannerella forsythia*) and helper strains (*Fusobacterium nucleatum* and *Staphylococcus aureus*).

On finding that a range of compounds with siderophore activity stimulated the growth of difficult-to-culture strains *F. fastidiosum* and *Prevotella* HOT 376, a culture model was developed, which incorporated 15µg siderophore (pyoverdines-Fe-complex or desferricoprogen) or 150µl undiluted subgingival plaque suspension into a central well on BA plates that were inoculated with heavily-diluted subgingival-plaque samples from deep periodontal pockets. After eight or more days of anaerobic incubation, microcolonies/colonies showing satellitism were passaged onto fresh plates cross-streaked with helper strains or onto cellulose-acetate membranes overlying lawn cultures of helper strains. Although several of the 77 colonies of interest did not survive passage or grew only on donor cross-streaks, four strains of previously-uncultivated taxa (1 *Bacteroidetes* HOT365 and 3 *Chloroflexi* HOT439 – the first oral taxon from this phylum to have been cultivated) were successfully isolated, as well as three strains of previously-cultivated uncharacterised taxa (2 *Bacteroidaceae* HOT272 and 1 *Capnocytophaga* HOT336). All except the latter were dependent on helper strains for growth.

In summary, colony-hybridisation-directed enrichment of cluster A *Synergistetes* resulted in the successful isolation of the first member of this previously-uncultivable group. An open-ended approach to the culture of previously-uncultivated bacteria led to the isolation of a further four novel strains. The resistance to culture of these novel taxa appears to be related to dependence on a biofilm lifestyle. Their characterisation will further our understanding of the human oral microbiome.

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