Cultivating the uncultured

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Outline

• The human oral microbiome
• Uncultured oral bacteria
• Culturing uncultured bacteria
The oral microbiome

Fungi

Viruses

Protozoa

Bacteria and Archaea
- Saliva - $10^8 / \text{ml}$
- All oral surfaces colonised by biofilm
- Important role in tooth decay and gum disease
Cultural analysis of oral bacteria

• Oral bacteria typically fastidious and slow-growing – require complex media and long incubation times
• Many are strict anaerobes requiring care in sample collection, transport and incubation
• Comprehensive cultural analysis of samples difficult – only possible to analyse small number
• Around half of oral bacteria uncultivable
16S rRNA community profiling

DNA extraction →

- Transformation
- Ligation
- Plasmid vector
- 16S rRNA gene
- PCR

E. coli library → Sequence cloned genes and compare sequence to DNA databases to identify source organism in original sample
Next generation sequencing

• High throughput – e.g. Illumina MiSeq generates c 10M sequences per run
• Direct sequencing of amplicons – no cloning step
• For oral bacteria, V1-V2 is most informative
• Easy and quick to do with standard pipelines for analysis – explosion in publications
The goal of creating the Human Oral Microbiome Database (HOMD) is to provide the scientific community with comprehensive information on the approximately 700 prokaryote species that are present in the human oral cavity. Approximately 54% are officially named, 14% unnamed (but cultivated) and 32% are known only as uncultivated phylotypes. The HOMD presents a provisional naming scheme for the currently unnamed species so that strain, clone, and probe data from any laboratory can be directly linked to a stably named reference scheme. The HOMD links sequence data with phenotypic, phylogenetic, clinical, and bibliographic information. Genome sequences for oral bacteria determined as part of this project, the Human Microbiome Project, and other sequencing projects are being added to the HOMD as they become available. Genomes for 400 oral taxa (58% of taxa on HOMD) are currently available on HOMD. The HOMD site offers easy to use tools for viewing all publically available oral bacterial genomes. Welcome!

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The human oral microbiome
(Dewhirst et al., J. Bacteriol., 2010)

- 687 bacterial species-level taxa
- 6 phyla – *Firmicutes*, *Bacteroidetes*,
  *Proteobacteria*, *Actinobacteria*, *Spirochaetes*
  and *Fusobacteria* account for 96 % of species
- 116 cultured but un-named species
- 69 % of species cultured
Sequences vs cells

- DNA sequence information is valuable resource:
  - Can be used as biomarkers
  - Enables predictions of function
- But, for:
  - Experimental determination of function
  - Use in model systems
  - Potential use as probiotics
- Culture and description and naming of bacteria is required
Uncultivated taxa - *Bacteroidetes*

- Some belong to existing well-characterised genera
- Others constitute deep-branching lineages with no cultivable representatives
Reasons for lack of cultivation

• Undersampling – culture much more labour-intensive than culture-independent studies
  – High throughput culture reveals many “uncultured” bacteria; e.g. Browne et al. Nature 2016; 533: 543-6 - substantial proportion of intestinal bacteria cultured

• Dependence on other bacteria in the community
  – nutritional / signaling
  – epibionts and / or intracellular, e.g. TM7 (He et al. PNAS 2015;112:244-9)
“Microbioal Dark matter”

• Examination of environmental metagenomic sequence data revealed novel branch of phylogenetic tree largely unseen in 16S rRNA gene community profiling surveys - “Candidate Phyla Radiation”
• Mis-matches with currently used primers and inserts in 16S rRNA operons
• Some representatives of the CPR have small genomes and may be epibionts or parasitic on other bacteria
The phylum *Synergistetes*

*(Jumas-Bilak et al. 2009)*

- *Synergistes BH017*
- *Synergistes JV006*
- *Synergistes DO84*
- *Synergistes JV023*
- *Synergistes E2*
- *Synergistes D006*
- *Synergistes BH007*
- *Synergistes JV001*
- *Synergistes W028*
- *Synergistes E3*
- *Synergistes W090*
- *Jonquetella anthropi*
- *Synergistes RMA 14551*
- *Pyramidobacter piscolens*

- *Dethiosulfovibrio peptidovorans*
- *Dethiosulfovibrio russensis*
- *Dethiosulfovibrio acidaminovorans*
- *Dethiosulfovibrio marinus*

- *Aminobacterium mobile*
- *Aminobacterium colombiense*
- *Anaerobaculum mobile*
- *Anaerobaculum thermoderrenum*
- *Aminophilus restrictus*
- *Thermovirga lienii*
- *Thermanaerovibrio velox*
- *Thermanaerovibrio acidaminovorans*
- *Aminomonas paucivorans*
- *Synergistes jonesii*
- *Synergistes RMA 14605*
- *Synergistes RMA 16290*

**Cluster A** - uncultured

**Cluster B** - cultivable
Culturing an uncultivated Synergistetes

• Hypothesis: some uncultivated oral bacteria require presence of other bacteria and therefore could be grown in mixed culture \textit{in vitro}

• Aim of study: to cultivate representative of uncultivated \textit{Synergistetes} to obtain a pure culture
Plaque from periodontal pocket

(Vartoukian et al. Appl Env Microbiol 2009;75:3777-86)

Synergistetes Cluster A

Total bacteria (in red) with Synergistetes Cluster A
Method

Plaque sample from 8-mm periodontal pocket

\[\text{Cultured on Blood Agar incubated anaerobically for 10 d}\]

Subculture of *Synergistetes* “colonies” to fresh plate

\[\downarrow\]

? discrete *Synergistetes* colonies

\[\uparrow\]

Plates photographed, replica plated, and blotted on nylon membrane

\[\downarrow\]

Blot hybridised with *Synergistetes* probe
Synergistetes colony hybridisation of mixed primary culture

- Detection of scanty growth of Synergistetes
- Replica plate harvested in region of hybridisation and used to inoculate fresh plate
Composition of enriched community after 8 passages

• Culture:
  - *Parvimonas micra*
  - *Campylobacter rectus*
  - *Anaeroglobus geminatus*
  - *Tannerella forsythia*

• Clone library
  - As above plus:
    - *Synergistetes* phylotype W090
Passage 12, with *P. micra* streak

Clone library – *Synergistetes W090* only
Species naming and genome sequence

- Organism described and named *Fretibacterium fastidiosum* (Vartoukian et al. 2012)
- DNA extraction – small amount obtained
- Whole Genome Amplification (GenomiPhi)
- Sequenced at Sanger Institute
- Accession no. FP929056

- Hydroxyapatite-coated pegs
- Inoculated with saliva
- Media changed twice weekly
- Complex biofilm develops, with >250 OTUs / peg
In-vitro culture of previously uncultured oral bacterial phylotypes

Subgingival plaque samples

- 21-day culture on 4 complex media
  - One previously uncultivated taxon cultivated

- Community profiling
  - 93 uncultivated taxa detected

- Culture in biofilm model to 170 days
  - 26 uncultivated taxa seen in in-vitro biofilms
Culture of uncultivated oral taxa


- *Anaerolineae* HOT-439, the first oral taxon from the *Chloroflexi* phylum
- *Bacteroidetes* HOT-365
- *Peptostreptococcaceae* HOT-091

*Lachnospiraceae* HOT 500
*Veillonella dispar / parvula*
*Parvimonas micra*
Tannerella forsythia and Tannerella BU063

- *T. forsythia* strongly associated with periodontitis
- Uncultivated *Tannerella* phylotype BU063 (HOT-286) strongly associated with health
First Cultivation of Health-Associated *Tannerella* sp. HOT-286 (BU063)

- Cultivated from subgingival plaque
- Stimulated by *Propionibacterium acnes*
- Grew on membrane placed on *P. acnes* culture
Summary

• Culture is important!
• Oral bacteria have evolved as a community and many species are dependent on community lifestyle
• Microbial dark matter needs to be explored - extent of representation among human microbiome not yet clear
• New methods of co-culture are proving successful (but slow!) in cultivating previously uncultured oral bacteria
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