Cultivating Previously Uncultured Bacteria from the Human Oral Cavity

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Dedication

This thesis is dedicated to my father, Pradeep Murugkar and my grandfather, Vasant Joshi. I wish you both were here to see your dream for me come true.
Acknowledgements
A journey of a thousand miles begins with a single step, and I would like to thank my advisor Kim Lewis for letting me take the first step and giving me a chance to be a part of his lab where I have had an exhilarating scientific journey. His patience, knowledge and his ability to inspire everyone to “think” and to “stop and smell the roses” is amazing. Words fall short when it comes to thanking Eric Stewart for all his help and support, excellent guidance, encouraging me to ask questions and for truly being the “fount of knowledge”. I would also like to thank my committee members, Slava Epstein, Katherine Lemon and Matthew Waldor for all the helpful suggestions and for being a committee that I looked forward to meeting and talking to. A big thank you to Ekaterina Gavrish for being such a wonderful friend and a great support throughout these five years. Thank you, to the “Unculturuble group” Kathrin Witt, Philip Strandwitz, Bijaya Sharma and Anna-Barbara Hachmann, for the fun discussions during the Tuesday “Unculturables” lunches and for being supportive when “nothing worked” and “it was a disaster”. I would also like to thank Sumayah Rahman and Srishti Prabha for all their help with my project. Big thanks to Lawrence Mulcahy and Iris Keren for their help with sorting, and Laura Fleck for helping with the deletion mutant library. These five years wouldn’t have been as memorable and fun if it hadn’t been for all the Lewis lab members. Thank you all for being such wonderful co-workers and awesome friends. I would like to thank Jon Clardy and Eric Dimise of Harvard Medical School, Floyd Dewhirst of the Forsyth Institute and George Weinstock of Washington University for all the help with my project. A special thanks to my friends Tejas, Sven, Ramya, Shilpa and Girish for being so wonderful and always having faith in me. Finally I would like to thank my mom and my brother for always believing in me, encouraging me and for being my support systems.
Abstract

Our knowledge of the bacteria that make up the human microbiome and the roles they play in health and disease is severely limited, and one of the greatest causes of that limitation is the inability to culture many of these organisms. It is estimated that 50% of the human oral flora is uncultured (Aas et al., 2005) and the essential challenge is to develop methods for cultivating these elusive organisms, in order to understand the role of the oral microbiome in human health. It was previously discovered that many natural bacterial isolates from environments outside of the human body were uncultured due to their dependence on growth factors that are normally provided by other organisms in the environment (D’Onofrio et al., 2010). The hypothesis is that similar interactions are responsible for the failure to culture many of the organisms that make up the human microbiome. The goal of this study was to cultivate previously uncultured organisms from the oral cavity using co-culture techniques, identify their limiting growth factors and to determine the ubiquity of these growth factor-requiring organisms in the oral cavity. Several dependent bacteria from the oral cavity were isolated using co-culture techniques. One previously uncultured bacterium, KLE1280, was chosen as the model organism to identify its growth factor requirement. KLE1280 is related to *Porphyromonas catoniae* by 16S rRNA sequencing, and KLE1280 was isolated in co-culture with an oral isolate closely related to *Staphylococcus hominis* which acts as a helper. We found that this isolate (KLE1280) would also grow in the presence of *E. coli*, which allowed for the screening of a library of deletion mutants in search of a growth factor. *E. coli* mutants lacking menaquinone biosynthesis genes were unable to induce growth of *Porphyromonas sp* KLE1280. Exogenously added menaquinone 4 (MK4)
induces growth of KLE1280. Along with MK4, heme (naturally occurring or synthetic) is also required for its growth. Based on these results, whole genome sequencing was done by our collaborator George Weinstock’s group at The Genome Institute at Washington University. It was confirmed that this isolate is indeed missing the menaquinone biosynthesis genes. It appears to be very specific in its requirement for MK4, as KLE1280 was not induced by any quinone except MK4 or an intermediate of the menaquinone pathway, 1,4-dihydroxy-2-naphthoquinoic acid (DHNA). Two other species of Porphyromonas were also dependent on MK4. We hypothesize that other uncultured bacteria might be deficient in the same or similar growth factors, and similar to the model organism studied here, could be very specific in their growth factor requirement. It is therefore necessary to identify more growth factors in order to cultivate more organisms from the human microbiome. Using this approach may allow us to isolate many more uncultured organisms.
# Table of Contents

Dedication ii  
Acknowledgements iii  
Abstract of Dissertation iv  
Table of Contents vi  
List of Figures vii  
List of Tables viii  
Chapter 1: Introduction 1  
Chapter 2: Experimental Procedures 11  
Chapter 3: Results 22  
Chapter 4: Discussion 44  
Chapter 5: Way forward 55  
Chapter 6: Conclusions 56  
Appendix 57  
References 62
<table>
<thead>
<tr>
<th>FIGURE NUMBER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1: Successive colonization of organisms within the dental plaque matrix.</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2: Screening for uncultured isolates using serial dilution and cell sorting approach</td>
<td>13</td>
</tr>
<tr>
<td>Figure 3: Genetic screen using <em>E. coli</em> deletion mutant library</td>
<td>16</td>
</tr>
<tr>
<td>Figure 4: Density dependent growth of dental plaque cells</td>
<td>23</td>
</tr>
<tr>
<td>Figure 5: Dependent isolates from cell sorting experiments</td>
<td>25</td>
</tr>
<tr>
<td>Figure 6: Potential uncultured isolates</td>
<td>26</td>
</tr>
<tr>
<td>Figure 7: Induction of KLE1280 by <em>Staphylococcus hominis</em> KLE1525 and by <em>E. coli</em></td>
<td>29</td>
</tr>
<tr>
<td>Figure 8: Identifying the biosynthetic genes for the growth factor</td>
<td>31</td>
</tr>
<tr>
<td>Figure 9: Testing deletion mutants in the menaquinone biosynthesis pathway in <em>E. coli</em></td>
<td>32</td>
</tr>
<tr>
<td>Figure 10: Genes involved in the menaquinone biosynthesis pathway identified bioinformatically in the genome sequence of KLE1280, <em>P. gingivalis</em> W83 and <em>E. coli</em></td>
<td>35</td>
</tr>
<tr>
<td>Figure 11: Heme biosynthetic pathway in <em>Porphyromonas gingivalis</em> W83 and KLE1280</td>
<td>36</td>
</tr>
<tr>
<td>Figure 12: Induction of <em>Porphyromonas sp.</em> KLE1280 by Ethyl acetate extracts</td>
<td>38</td>
</tr>
<tr>
<td>Figure 13: Heme requirement of KLE1280</td>
<td>42</td>
</tr>
<tr>
<td>Figure 14: Structures of menaquinone (general structure), menaquinone 4 and menadione</td>
<td>51</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th>TABLE NUMBER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1: Dependent isolates and their closest relatives</td>
<td>28</td>
</tr>
<tr>
<td>Table 2: Genes involved in the chorismate, menaquinone and heme biosynthesis pathway identified in the genome sequence of KLE1280.</td>
<td>34</td>
</tr>
<tr>
<td>Table 3: Quinone specificity of KLE1280</td>
<td>40</td>
</tr>
</tbody>
</table>
Introduction

Uncultivability - a puzzling phenomenon

Bacteria that have never been cultured in the laboratory are found in almost every environment (Rappe & Giovannoni, 2003); many of them are often referred to solely by 16s rRNA accession numbers (Dewhirst et al., 2010). The problem of uncultured bacteria is very old, stemming from observations made over a century ago (Winslow & Willcomb, 1905, Amann, 1911), where a stark difference between the large number of cells from water, soil or sewage samples and the few colonies produced on synthetic media was first observed. From environments such as soil and ocean, only 1% of microorganisms will grow in vitro, and this puzzling phenomenon became known as “the great plate count anomaly” (Staley & Konopka, 1985). Carl Woese described 11 bacterial phyla in 1987 based on 16S and 18S rRNA gene sequences (Woese, 1987). This number grew to 52 in 2003 (Rappe & Giovannoni, 2003) and has now grown to 85, the majority of which remain uncultivated (Keller & Zengler, 2004) (Stewart, 2012). Culture-independent methods have identified numerous bacteria that are members of entire phyla which have never been cultured (Rusch et al., 2007).

Uncultured bacteria are not confined to one particular habitat but are found in every environmental niche. Members of the uncultured division TM7 were detected in soil (Ferrari et al., 2005), activated sludge (Zhao et al., 2013) and also on apple flowers, along with members of the phyla Deinococcus-Thermus (Shade et al., 2013). Rare organisms belonging to the phyla Armatimonadetes, Bacteroidetes, Chlamydia, Chloroflexi, Cyanobacteria, Elusimicrobia, Fibrobacteres, Firmicutes,
Gemmatimonadetes, Spirochaetes, AD3, WS1, WS4, WS5, WYO, OD1, OP3, BRC1, TM6, WPS-2, and FCPU426 were detected in peatlands. These also included members from TM7 division (Serkebaeva et al., 2013). For a long time, the seawater organism SAR11 was very challenging to cultivate in the laboratory (Rappe et al., 2002).

The microbial communities associated with the human body are no exception to the ubiquity of uncultured bacteria (Dewhirst et al., 2010, Turroni et al., 2008, Alverdy & Chang, 2008).

Uncultured bacteria and the human microbiome

There are many communities of microorganisms associated with the human body, the number of bacteria in the human body are estimated to be 100 times the number of human cells (Wade, 2013). These microorganisms are capable of a broad range of nutrient utilization and metabolite synthesis that results from their complex metabolic interactions (Gill et al., 2006). These organisms were originally termed the “Human Microbiome” by Joshua Lederberg, meaning an ecological community of commensal, symbiotic and pathogenic microorganisms that share our body (Lederberg & McCray, 2001, Dewhirst et al., 2010). A major part of the gut microbiota still remains uncultured (Eckburg et al., 2005). Although the vaginal flora is not as diverse and extensively studied as the gut flora, previously uncultured bacteria have been reported to be present in abundance in the vaginal microbiome as well (Fettweis et al., 2012).

The organisms associated with the oral cavity are collectively referred to as the oral microbiome (Dewhirst et al., 2010). Of the 600 species present in the mouth
(Dewhirst et al., 2010), around 50% have never been cultivated before (Aas et al., 2005, Wade, 2004, Wade, 2013), though the oral cavity has been extensively studied due to the ease of accessibility and the correlation between change in the microbiota composition and chronic periodontal disease (Kolenbrander, 2011).

**Why grow these bacteria?**

Studies of oral microbiology provided a fairly good understanding of species that were pathogenic, and of species that are likely symbionts (Wade, 2013). This distinction is difficult to make for uncultured bacteria, however. Correlation studies show that some uncultured organisms may be pathogenic. For example, TM7 is often found in patients with periodontitis (Brinig et al., 2003). In a study of oral squamous cell carcinomas, 52 genera were identified, of which, 67% were sequences belonging to either uncultured bacteria or unclassified group (Pushalkar et al., 2011). Unculturable bacteria are also reported to be present at sites of infections in various diseases like dental caries and periodontitis (Sakamoto et al., 2002). However, correlation is not causality, and for this, the organism needs to be isolated and characterized to understand its biology, its interaction with other organisms and its role in health.

We hypothesized that previously uncultured bacteria require growth factors, small molecules from other bacteria and that identifying these growth factors will lead to increased recovery of rare and novel bacteria, eventually making it possible to close the gap of the great plate count anomaly.
Bacteria and growth factors

Bacteria that derive their growth factors from other bacteria are referred to as dependent bacteria and the organisms providing the growth factor are called helpers. Much of the work on helper and dependent organisms has been performed in the marine environment.

It has been shown that previously uncultivable marine bacteria will grow only in the presence of a mixed population of bacteria from the same environment (Rappe et al., 2002, Giovannoni et al., 2005). Growth of Prochlorococcus sp., an abundant planktonic bacterium in the ocean, requires the presence of heterotrophic helper bacteria to form isolated colonies on solid media (Morris et al., 2008). Studies done in our lab showed that previously uncultured organisms will grow on nutrient medium only in the presence of other species from the same environment (Kaeberlein et al., 2002, Nichols et al., 2008). Using co-culture as a bioassay, the first general class of compounds which act as growth promoters for uncultivable species from marine sediment was identified (D'Onofrio et al., 2010). These growth factors are siderophores, chelators of Fe(III). A diversity of uncultivable bacteria, some closely related to cultivable species, do not produce siderophores. By losing their ability to produce siderophores, uncultivable species apparently gain the ability to only grow in environments populated by favorable neighbors.

Oral microorganisms exist as a complex multispecies community (Kolenbrander et al., 2002), suggesting the presence of interactions between different species forming biofilms in the mouth. These biofilms are usually formed on the salivary pellicle, which
is a proteinaceous film derived from saliva covering the tooth enamel to protect it from acid demineralization (Lendenmann et al., 2000). Dental hygiene routines disrupt these biofilms regularly. However, the biofilms start re-forming immediately after disruption, in a defined order (Kolenbrander et al., 2002), with the primary colonizers attaching to the salivary pellicle within a matrix, followed by the late colonizers (Kolenbrander, 2011) (Figure 1).

These studies suggest that these organisms are dependent on the attachment and growth of other species for their colonization and possibly survival in the oral cavity. Their dependence could be on physical attachment or on the availability of small molecules and growth factors from other organisms.

Spent supernatant has been shown to facilitate growth of bacteria from the oral cavity (Gibbons & Macdonald, 1960, Bentley & Meganathan, 1982). Supernatant of whole saliva supported robust growth of Bacteroides melaninogenicus from the mouth (Gibbons & Macdonald, 1960). Supernatant of parotid saliva (saliva obtained directly from the parotid ducts that produce it), however, did not support growth of this bacterium, possibly because parotid saliva does not contain the growth factors and small molecules produced by other bacteria found in whole saliva (Gibbons & Macdonald, 1960).

These observations suggest that many uncultivable bacteria depend on growth factors produced by neighboring species for their growth.
**Figure 1:** Successive colonization of organisms within the dental plaque matrix. Salivary proteins on the tooth surface provide attachment for early colonizers. Once the early colonizers have attached, they in turn provide attachment via surface antigens, to late colonizers, thus forming the dental plaque matrix. Figure reproduced from (Kolenbrander et al., 2002).
Researchers have used a variety of media and additives such as blood, serum, saliva, hemin and menadione to name a few, in hopes of growing previously uncultivated microorganisms, but have not been able to grow all the organisms from the oral cavity (Dewhirst et al., 2010, Wade, 2013, Wyss, 1992).

**Quinones as growth factors**

Menaquinone 4 (MK4) is described in this study as being the growth factor for a dependent bacterium. Menadione, a synthetic quinone, has been added to media used to grow organisms from the human microbiome to satisfy the menaquinone (vitamin K) requirement of bacteria (Bentley & Meganathan, 1982). Vitamin K is a catch-all phrase describing all quinone-like compounds; the major quinones in bacteria are menaquinones and ubisquinones (Hiratsuka et al., 2008). Both ubisquinones and menaquinones are lipid soluble molecules that are considered to be a part of the electron transport chain (ETC) in bacteria, shuttling electrons between the components of the ETC (Newton et al., 1971). In humans and animals, vitamin K plays various roles in blood clotting, and also as anticancer agent (Cranenburg et al., 2007, Lamson & Plaza, 2003). Menaquinone biosynthesis is being studied as a target for novel antibiotics in *Mycobacterium tuberculosis* (Debnath et al., 2012).

Menaquinone is biosynthesized from chorismate, which in turn is derived from the shikimate pathway. There are eight genes involved in conversion of chorismate to menaquinone (Hiratsuka et al., 2008). The menB, menC, menD, menE, menF and menH gene products convert chorismate to 1,4-dihydroxy-2-naphthoate (DHNA). The first five
genes are clustered together on the \textit{E. coli} chromosome (Suvarna \textit{et al.}, 1998). This hydrophilic head is attached to a hydrophobic octaprenylphosphate tail to form menaquinone by the \textit{menA} and \textit{ubiE} gene products.

The menaquinones are based on the number of isoprenoid units attached to the quinone head. MK4 has four isoprenoid chain subunits whereas MK7 has seven, and so on. \textit{E. coli} produces MK8 as its major menaquinone, amongst others. The hydrophobicity of menaquinones increases with the increasing number of side chain subunits, and that is one reason why longer chain length menaquinones are not readily diffusible in aqueous media. This would restrict their spread and hence would prevent their easy uptake by organisms.

\textbf{Genus Porphyromonas}

The dependent isolate in this study is related to \textit{Porphyromonas catoniae} by 16S rRNA sequencing. Some of the members of the genus are \textit{Porphyromonas asaccharolytica}, \textit{P. gingivalis}, \textit{P. catoniae}, \textit{P. endodontalis}, \textit{P. cangingivalis}, \textit{P. canoris}, \textit{P. cansulci}, \textit{P. circumdentaria}, \textit{P. crevioricanis}, \textit{P. gingivicanis}, \textit{P. gulae}, \textit{P. levii}, \textit{P. macacae}, and \textit{P. salivosa}. Of these, the first four are associated with the human body while the others are usually found in cats, dogs and other animals (de Lillo \textit{et al.}, 2004). The genus \textit{Porphyromonas} is not a very well characterized genus, though a member of this genus \textit{Porphyromonas gingivalis} is extremely well studied owing to its pathogenecity in humans (Belanger \textit{et al.}, 2007, Genco \textit{et al.}, 1998) (http://www.pgingivalis.org/). \textit{P. catoniae}, previously known as \textit{Oribaculum catoniae}
(Willems & Collins, 1995), has been associated with healthy oral cavities of six month old to two year old children (Kononen et al., 1999, Crielaard et al., 2011).

*P. gingivalis* is usually isolated from sites of periodontal disease along with *Tannerella forsythia* (previously *Bacteroides forsythus*) and *Treponema denticola*. Together these are called the “red complex” and are suggested to have co-operative interactions (Rocas et al., 2001) (Suzuki et al., 2013). Growth of *P. gingivalis* was stimulated by the extract of *Tannerella forsythia* in an *in vitro* study to understand their interactions (Yoneda et al., 2005). *P. catoniae* was isolated from dental plaque growing on rich medium as satellite colonies around other bacteria (Kononen et al., 1999) suggesting that it derives growth factor/s from other organisms. These studies suggest that at least some, if not all, members of the *Porphyromonas* genus interact with other bacteria for growth and pathogenesis.
Owing to the complex nature of the dental plaque biofilms and the close interactions amongst individual members of the biofilm community, the general principle of reliance on growth factors from neighboring species thus seems to apply to the oral microbiota as well. The co-culture approach is therefore likely to succeed in isolation of previously uncultivated organisms from the oral cavity. Our aim is to grow all previously uncultured bacteria and close the gap in the great plate count anomaly. To this end, my goal is to isolate previously uncultivated organisms from the oral cavity and identify their growth factors using the co-culture approach.

The specific aims of this study are:

1. To isolate previously uncultivated organisms from the oral cavity using co-culture techniques
2. To identify the growth factor for one of these isolates
3. To determine the ubiquity of organisms requiring this growth factor in the oral cavity and determine their identity
Experimental Procedures

Sample collection

Dental plaque was collected from healthy individuals after explaining to them the nature of the study that was being conducted (IRB#08-11-15) and after obtaining their consent. Sterile toothpicks were provided to the healthy individuals, who provided their dental plaque samples by scraping their teeth to dislodge plaque. The scrapings were collected in sterile test tubes and were processed immediately. They were preserved and used separately.

Isolating previously uncultured organisms from the oral cavity

The goal of this experiment was to obtain isolates that were dependent on other bacteria for their growth. The hypothesis was that on relatively densely inoculated plates, dependent isolates will only grow near their helper organisms (the source of their growth factors). A corollary of this hypothesis is that dependent isolates will grow later than the helper organisms, once the helper organism has produced enough growth factor for the dependent isolate. Based on this hypothesis, dental plaque was plated with various densities of cells, the plates were incubated, and, of the resultant growth, small colonies (diameter <1 mm) growing close to large colonies (diameter >1 mm) on the agar medium were picked to test for dependence. All experiments were performed aerobically, but incubated anaerobically in an anaerobic chamber (Vinyl Anaerobic Chamber, Coy Lab Products, with a strict anaerobic atmosphere of 0-5 ppm oxygen using a palladium catalyst and gas mix comprised of 5% hydrogen, 5% CO2 and 80% nitrogen) (http://www.coylab.com/vinyl.htm).
Two different approaches were used to plate dental plaque, a serial dilution approach and a cell sorting approach.

1. **Serial dilution approach**

   Dental plaque samples were collected from healthy individuals and the number of cells counted microscopically (data not shown). 10 µl dental plaque was suspended in 1 ml each of Trypticase Soy Broth (TSB), Luria-Bertani broth (LB), Brain Heart Infusion broth (BHI) and Fastidious Anaerobe Broth (FAB). Samples were serially diluted in 10-fold dilutions, and 100 µl of each dilution was plated aerobically on R2A, Trypticase Soy Agar (TSA), Luria-Bertani Agar (LBA), Brain Heart Infusion Agar (BHIA), Blood Agar (BA), Chocolate Blood Agar (CBA), Fastidious Anaerobe Agar (FAA) and Fastidious Anaerobe Agar with 5% sheep blood and 5% pooled human saliva (FAABS) (See Appendix for recipes). Plates were incubated anaerobically at 37°C for 3-7 days. The plates were checked daily for presence of small colonies and late growers. Small colonies (diameter <1 mm) growing close to large colonies (diameter >1 mm) on plates that had 100 – 300 colonies were picked and spread on fresh media plates (Figure 2).
Figure 2: Screening for uncultured isolates. (a) Serial dilution spread plate of bacteria from dental plaque. (b, c, d) Small colonies growing in close proximity of larger colonies. These small colonies were picked and either spread or streaked onto fresh media plates for identifying dependent bacteria. (e) Cell sorting plate with 384 events from dental plaque sorted onto the plate.
2. **Cell-sorting approach**

Dental plaque samples from healthy individuals were sorted in arrays onto solid medium (Omni tray – a rectangular one well plate, dimensions – Length – 127 mm x Breadth – 85 mm) to allow for a defined distance between two cells on a plate. Briefly, 100 µl of dental plaque was diluted 1:1000 in phosphate buffered saline (PBS) and 384 cells were sorted with a BD Facs Aria II cell sorter into arrays of either 24, 96 or 384 cells onto Modified Fluid Medium (mFUM), FAABS and CBA. Single cells were also sorted into 96 well plates with liquid medium (mFUM) to allow for physical separation between cells. All plates were incubated anaerobically at 37°C for 3-7 days. These plates were also checked daily for the presence of small colonies and late growers. Similar to the serial-dilution approach, small colonies from the solid medium were picked and spread on fresh agar media (Figure 2).

**Helper-dependent pairs**

Colonies picked from both approaches were spread on the same medium from which they were picked. Either the large colony growing next to the small colony was spotted as a helper or a helper mix of many colonies growing around individual small colonies was spotted on these plates. Replated colonies that grew only in proximity to the helper/mix were selected for further validation. Colonies that only grew with a live helper were replated on all media and those which showed dependence on a helper on all the media were replated to verify their dependence.
Identification of Isolates by 16S rRNA Gene Sequencing

Potential dependent isolates that showed consistent growth dependence on other isolates to grow were stored in 15% glycerol stocks in the medium from which they were picked. Glycerol stocks were also made of the helper/s or helper mixes. All stocks were frozen and stored at -80°C. Those isolates that were selected for further analysis and their helpers were characterized by 16S rRNA gene sequencing. The isolates were plated from glycerol stocks and incubated to obtain isolated colonies. Colony PCR was performed on all selected isolates using the universal primers 27F and 1492R, which amplify a 1466 bp region of the 16S rRNA gene (Marchesi et al., 1998). The closest relative organism of these isolates was identified using the Ez-Taxon server (http://www.eztaxon.org/; Chun et al., 2007) and by comparison to the database at the Human Oral Microbiome Database (HOMD (Dewhirst et al., 2010); http://www.homd.org).

Identification of a growth factor for KLE1280 using *E. coli* as a genetic tool (Figure 3)

- Developing the screen

Libraries of deletion mutants of *E. coli* K-12 MG1655 with non-essential genes deleted (single and multiple) are available, and were ordered from [http://ecoli.aist-nara.ac.jp/](http://ecoli.aist-nara.ac.jp/) and [http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp](http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp) (Kato & Hashimoto, 2007, Baba et al., 2006). Some of the long, medium, short and single gene deletion mutants of *E. coli* were picked and reorganized into a smaller library consisting of mutants deleted in many of the non-essential genes of *E. coli* (Screen put together along with Laura Fleck and Kathrin Witt, fellow graduate students).
Figure 3: Genetic screen using *E. coli* deletion mutants. A library of some of the long, medium and short deletion mutants of *E. coli* covering many of the non-essential genes was assembled from available libraries. These strains were tested for growth induction of the dependent isolate. The mutant that did not show growth induction of the dependent isolate was subsequently analyzed further.
• **Testing the reorganized library to identify the growth factor for KLE1280**

35 µl of $10^7$ cells/ml of KLE1280 from a glycerol stock was spread onto FAABS plates and each plate was spotted with 5 µl of a deletion mutant. Plates were incubated anaerobically at 37°C for 3 days. Results were noted as presence and absence of growth induction around the helper.

• **Testing individual gene mutants of OCL67 genes**

Individual gene mutants corresponding to those deleted in OCL67 and the menaquinone biosynthesis pathway individual gene mutants were tested using the same procedure as the multiple gene deletion mutants.

**Whole genome sequencing of KLE1280**

Whole genome sequencing was performed by our collaborator George Weinstock and his team at The Genome Institute at Washington University, St. Louis, MO, using Illumina sequencing. The draft genome was annotated using the RAST (Rapid Annotations using Subsystems Technology) server (Aziz et al., 2008) and Tigra assembler. Presence of genes was determined by detection of open reading frames (ORF).

**Biochemical analysis**

Glycerol stocks of *E. coli* and *S. hominis* KLE1525 were inoculated in liquid medium and incubated anaerobically at 37°C for 7 days to allow for growth factor production in liquid medium. *E. coli* was grown in 1 L FAB supplemented with 5% defibrinated sheep blood and 5% pooled human saliva, while KLE1525 was grown in 1 L FAB. The resultant growth was centrifuged at 10000 g for 30 minutes and the supernatant
was filtered through a 0.22 µm filter. The cell pellets and supernatants were extracted by Eric Dimise, a postdoctoral scientist working with Jon Clardy at Harvard Medical School. The procedure is detailed below. Uninoculated broths of both media were treated the same way as controls. The dried extracts were resuspended in acetone at a concentration of 1 mg/ml. 100 µl of $10^7$ cells/ml of KLE1280 from glycerol stocks was spread onto FAABS/FAA plates and each plate was spotted with 5 µl and 10 µl of the extract. 5 µl and 10 µl acetone was spotted as diluent control. Plates were incubated anaerobically at 37°C for 3 days. Results were noted as presence or absence of growth induction around the extracts/fractions.

**Extraction and fractionation of supernatant**

- **Extraction using hexane and ethyl acetate:**

  1 L of supernatant was added to a 2 L separating funnel. 500 mL hexane was added to this and mixed by shaking. The two layers were allowed to separate and the hexane layer was siphoned off and set aside. This process was repeated three times in total and all the hexane phases were pooled. 500 mL ethyl acetate was then added to the funnel with hexane-extracted supernatant. The same process as with hexane was repeated thrice with ethyl acetate and the extractions were pooled. The hexane and ethyl acetate extracts were then dried with anhydrous sodium sulfate. They were then completely dried in a Rotovap. The dried material was stored at -20°C under Argon gas till further use.

- **Extraction using XAD4 resin:**
1 L of supernatant was slowly passed over Amberlite™ XAD4 resin (300 cc bed volume in H2O). The resin was then rinsed with two bed volumes ddH2O. The bound material was then eluted with increasing concentrations of methanol (25%, 50%, 75% and 100%). The elutions were dried completely in a rotovap. The dried material was stored at -20°C under Argon gas till further use.

**Extraction and purification of quinones**

Cells were re-suspended in 50 mL 3:2:1 ethanol, H2O, 25% sodium hydroxide. The cell suspension was then refluxed under inert atmosphere (argon) for 20 minutes at 100°C. The vessel was immediately cooled in an ice bath. The contents were then poured into a separating funnel and extracted three times with heptanes (~200 mL portions). The organic layers were collected, rinsed with brine and dried with anhydrous sodium sulfate. They were then completely dried in a rotovap. The dried material was stored at -20°C under Argon gas till further use.

**Testing addition of exogenous menaquinone and the specificity of quinones for growth induction of KLE1280**

Ubiquinones (Q) and menaquinones (MK) were mixed with warm FAA supplemented with 5% defibrinated sheep blood and 5% pooled human saliva at a concentration of 5 µg/ml, and 200 µl of the warm agar was added to 96-well plate wells. 10 µl of KLE1280 at various cell densities (diluted in FAB from glycerol stocks) was added on top of the solidified agar and the plates were incubated anaerobically at 37°C for 3 days. The commercially available (Sigma) quinones tested were MK4, Q1, Q2, Q4,
Q9 and Q10. Q8 and MK8 were extracted and purified from *E. coli*. MK4, MK5, MK6, MK7 and MK8 were obtained from *M. luteus*. Also tested was 1,4-dihydroxy-2-naphthoate (DHNA) (Sigma), an intermediate from the menaquinone biosynthesis pathway for induction of growth of KLE1280. This intermediate is the substrate for the *menA* gene product. Apart from these compounds, menadione (Sigma) was also tested.

**Heme requirement of KLE1280**

The heme requirement of KLE1280 was determined by plating 100 µl of glycerol stock of KLE1280 and then spotting 10 µl of various concentrations of MK4 (0.01 mg/ml, 0.1 mg/ml, 1 mg/ml, 10 mg/ml) on media with and without blood (5%) or hemin (10 µg/ml). The plates were incubated anaerobically at 37°C for 3 days. Along with blood and hemin, hemoglobin (100 µg/ml), a component of blood, was also tested for growth induction of KLE1280.

**Testing dependence of other Porphyromonas sp. on menaquinones and *E. coli* mutant OCL67**

Two strains of *Porphyromonas catoniae* Oral Taxon 283 (Forsyth Code – F0035 and F0037) were obtained from our collaborator Floyd Dewhirst’s lab at the Forsyth Institute, Cambridge, MA. 100 µl of glycerol stock of both these strains were plated on FAABS and tested for dependence by spotting a 5 µl spot of 1 mg/ml, 5 mg/ml, 100 mg/ml of MK4, DHNA and menadione. Dependence on a 5 µl spot of *E. coli* mutant OLC67 was also tested.
**Determination of quinone dependency in dental plaque**

10 µl dental plaque was serially diluted in FAB, plated on FAA supplemented with 5 µg/ml MK4 and incubated anaerobically at 37°C for 5 days. Colonies were replated on media with and without MK4. The isolates growing only on plates with MK4 were replated on fresh media with and without MK4 to confirm their growth dependence on MK4. Isolates were also tested for dependence on wild type *E. coli* and the deletion mutant OCL67.
Results

Isolation of uncultured bacteria

A sample of dental plaque from healthy volunteers was inoculated onto various commercial media and incubated anaerobically to obtain plates with moderately crowded growth (100-300 colonies). Two approaches were used to inoculate dental plaque on the media: spread plating serial dilutions of dental plaque and using a BD FACSARia II cell sorter to deposit dental plaque bacteria in an array on the media plates. The rationale behind using a cell sorter was to ensure separation of individual cells from each other. Plates were incubated anaerobically at 37°C.

Density dependent growth

We hypothesized that many uncultured bacteria depend on other bacteria for their growth. This would mean that the number of bacteria growing on a crowded plate would be more than those growing on a sparsely populated plate. To test this hypothesis, dental plaque from a healthy individual was vortexed and sonicated to disrupt cell clumps and sorted into arrays of 24, 96 (on plates and in single wells) and 384 cells onto mFUM agar, FAABS and CBA plates. A total of 1152 cells were sorted, of which 664 grew as colonies. Plates with 384 cell arrays gave the highest recovery of 35% although this did not reach statistical significance (Figure 4). It suggests a co-operative relationship between neighboring cells, perhaps giving a glimpse of interactions that might be going on in the natural environment between these cells. A comparison between single cells sorted onto Omni trays (96 cells) and in 96 wells showed that physically separating single cells from each other lowers the total recovery of bacteria (not statistically significant; p=0.06) (Figure 4).
Figure 4: Density dependent growth of dental plaque cells. Dental plaque was sorted into arrays of 24, 96 (on plates and in single wells) and 384 cells onto FAABS, mFUM agar, and CBA plates. Single cells sorted onto Omni trays (96 cells) gave a higher recovery than cells sorted in 96 well plates (Statistically not significant; \( p=0.06 \)). Plates with 384 cell arrays gave the highest recovery. Error bars represent standard deviation within replicates.
Of the 664 colonies, 150 colonies were picked and replated on the same media with a mix of surrounding colonies spotted as helpers. Of these replated colonies, 110 grew independently while 15 didn’t grow at all. The remaining 25 showed dependence on the helper mix. These dependent isolates were then plated on all three test media with helper mix spotted. 14 of these grew independently on at least one of the three media while seven failed to re-grow completely. Two isolates were not very consistent in their dependence pattern while two showed consistent dependence on the mix. Thus, out of 150 colonies that were picked, two were verified to be dependent isolates (Figure 5).

**Helper-dependent pairs**

Small colonies growing next to large ones (Figure 2) on plates from both approaches were chosen for evaluation of dependence, based on the hypothesis that the small colonies may be growing slower due to the necessity of utilizing growth factors produced by the neighboring colony. The small colonies were either streaked next to the potential helper strain (Figure 6a), or were suspended in liquid medium and spread onto fresh media plates. Potential helpers were then spotted onto these spread plates (Figure 6b-f). The potential helper was either a single colony growing next to the small colony or a mix of all the colonies growing around the small colony on the isolation plate. A dependent isolate was identified as one that grew only in the presence of a helper species, close to the helper and showed diminished/no growth away from the helper (Figure 6).
**Figure 5:** Dependent isolates from cell sorting experiments. (a) - Graph of the number of events sorted on three different media (FAABS, CBA and mFUM) and the number of colonies that grew. Of these, the colonies that were <1 mm in diameter were picked. (b) - The picked colonies were plated on three different media (including the one that they were picked from) and evaluated for independent growth on each of the three media. (c, d) - Two dependent isolates were obtained out of a total of 150 colonies that were picked originally.
Figure 6: Uncultured isolates. Potential uncultured isolates were tested for dependence by (a) streaking the dependent and helper mix close to each other and by (b-f) spreading each small colony suspension on fresh media plates and spotting with the helper/helper mix. The dependent isolates showed growth only in close proximity of the helper/mix. (a, b) *Eubacterium sp.* helped by a helper mix, (c) *Prevotella sp.* helped by a helper mix, (d) *Staphylococcus sp.* helped by *Deinococcus sp.*, (e) *Prevotella sp.* helped by a helper mix, and (f) *Porphyromonas sp.* KLE1280 induced by a helper mix.
Identification of Isolates by 16S rRNA Gene Sequencing

Through 16SrRNA gene sequencing, seven previously uncultured oral isolates were obtained, as determined by 16S rRNA gene sequence comparison to the database at the Human Oral Microbiome Database (HOMD (Dewhirst et al., 2010); http://www.homd.org). These isolates were consistently dependent on either a single helper or helper mix. They were closely related to *Eubacterium yurii* subsp. *yurii* ATCC 43713T (99.6%), *Prevotella tannerae* ATCC 51259T (99.0%), *Prevotella oulorum* ATCC 43324T (99.7%), *Lautropia mirabilis* AB2188T (99.0%), *Solobacterium moorei* JCM 10645T (99.2%) and *Candidatus Peptostreptococcus massiliae* 2002-69396 (99.2%) (Table 1). A single isolate, *Porphyromonas* sp. KLE1280 (Figure 4f) was chosen for further evaluation, due to its consistent dependence on an isolate obtained from a helper mix (KLE1525, which is closely related to *Staphylococcus hominis* subspecies *novobiosepticus* GTC 1228T (99.6%)), and since the dependent isolate belongs to a genus that contains the important pathogen *Porphyromonas gingivalis*, amongst others. KLE1280 is 96% similar by 16S rRNA sequencing to the closest type strain *Porphyromonas catoniae* 51270T.

Identification of a growth factor for KLE1280 using *E. coli* as a genetic tool

The ability of *E. coli* K-12 MG1655 to induce growth of KLE1280 was tested. *E. coli* induces growth of KLE1280 (Figure 7b) in addition to the natural helper, KLE1525 *S. hominis* (Figure 7a).
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Closest Relative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLE1280(^a)</td>
<td><em>Porphyromonas catoniae</em> ATCC 51270(^T) (96.4%)</td>
</tr>
<tr>
<td>KLE1500(^b)</td>
<td><em>Eubacterium yurii</em> subsp. <em>yurii</em> ATCC 43713(^T) (99.6%)</td>
</tr>
<tr>
<td>KLE1501(^b)</td>
<td><em>Prevotella tannerae</em> ATCC 51259(^T) (99.0%)</td>
</tr>
<tr>
<td>KLE1502(^b)</td>
<td><em>Prevotella oulorum</em> ATCC 43324(^T) (99.7%)</td>
</tr>
<tr>
<td>KLE1510(^b)</td>
<td><em>Lautropia mirabilis</em> AB2188(^T) (99.0%)</td>
</tr>
<tr>
<td>KLE1505(^b)</td>
<td><em>Solobacterium moorei</em> JCM 10645(^T) (99.2%)</td>
</tr>
<tr>
<td>KLE1524(^b)</td>
<td><em>Candidatus Peptostreptococcus massiliae</em> 2002-69396 (99.2%)</td>
</tr>
</tbody>
</table>

\(^a\) Helper – *Staphylococcus hominis* subsp. novobiosepticus GTC 1228\(^T\) – 99.6%

\(^b\) Helper – mix of clonies growing around dependent organism

**Table 1:** Dependent isolates and their closest relatives
Figure 7: Induction of *Porphyromonas* sp. KLE1280. KLE1280 was spread evenly onto Fastidious Anaerobe Agar supplemented with 5% defibrinated sheep blood and 5% pooled human saliva. 5 µl of the helper was spotted on top. (a) KLE1280 induced by *Staphylococcus hominis* KLE1525. (b) KLE1280 induced by *E. coli*.
There is a complete, ordered collection of 3985 *E. coli* knockout strains (Keio collection (Baba et al., 2006), and also sets of large deletions (Kato & Hashimoto, 2007, Hashimoto *et al.*, 2005). A smaller set of mutants was put together in a reorganized library. This reorganization was done by cherry picking mutants from the long, medium, short and single gene deletion libraries of *E. coli* so that together, these mutants covered many **non-essential** genes of *E. coli*. For the screen, KLE1280 was spread on plates of FAABS and a single *E. coli* mutant was spotted onto each plate (Figure 3). After incubation at 37°C under anaerobic conditions, the presence or absence of growth of KLE1280 around the spot of *E. coli* was noted.

One deletion mutant from this subset, *E. coli* OCL67, did not induce growth of KLE1280 (Figure 8b). This mutant has six menaquinone biosynthesis genes deleted along with ten other genes (Figure 8c). All 16 single deletion mutants of the *E. coli* genes within the OCL67 deletion were then tested individually for growth induction of KLE1280. Also tested were additional deletion mutants in the menaquinone biosynthesis pathway. *E. coli* deletion mutants ΔmenD, ΔmenC, ΔmenE and ΔmenB did not induce growth of KLE1280 (Figure 9). *E. coli* strains bearing deletions of other genes within the OCL67 deletion were still able to induce growth of this isolate, as were the menaquinone biosynthesis pathway gene deletion mutants ΔmenA and ΔubiE.
Figure 8: Identifying the biosynthetic genes for the growth factor. KLE1280 was spread on FAABS plates and individual mutants from the reorganized library were spotted onto these plates as helper spots. (a) KLE1280 induced by *E. coli*. (b) *E. coli* OCL67 showing no induction of KLE1280. (c) The shaded region depicts the deletion region of *E. coli* OCL67.
Figure 9: Menaquinone biosynthesis pathway in *E. coli*. Deletion mutants in each step were tested for induction of growth of KLE1280. *E. coli* mutants ΔmenD, ΔmenC, ΔmenE and ΔmenB did not induce growth of KLE1280.
Whole genome sequencing of KLE1280

Six deletion mutants of *E. coli* in the menaquinone biosynthesis pathway did not induce growth of KLE1280, or were impaired in their induction. This suggested the requirement by KLE1280 for menaquinone or an intermediate from the menaquinone biosynthesis pathway. These results also indicated that the genes responsible for menaquinone biosynthesis might be absent in KLE1280. In order to verify the presence or absence of menaquinone biosynthesis genes, the Washington University Genome Sequencing Center of our collaborator George Weinstock sequenced the genome of KLE1280. The draft genome was annotated using the RAST server (Aziz et al., 2008). Presence of genes was determined by detection of open reading frames (ORF). There were no hits for ORFs of *menD*, *menC*, *men H*, and *menB*. There were partial hits for *menF* and *menE* (Figure 10). ORFs were detected for *menA* and *ubiE* in the genome of *Porphyromonas sp.* but the protein alignment was shorter than the ORF on the stop codon end (Table 2). These genes are known to exist in the closest cultivable relative with a genome sequence, *Porphyromonas gingivalis* W83 (http://www.nmpdr.org). ORFs were detected for *menA* and *ubiE* in the genome of KLE1280.

Members of the genus *Porphyromonas* require heme for growth (Dashper et al., 2009) suggesting an incomplete heme biosynthesis pathway (http://www.nmpdr.org). Three of the five genes involved in the heme biosynthesis pathway are present in *Porphyromonas gingivalis*, while the other two are missing. ORFs for these three genes were detected in KLE1280 as well (Figure 11).
<table>
<thead>
<tr>
<th>Enzyme name</th>
<th>Gene</th>
<th>Gene ID (NCBI)</th>
<th>KLE1280</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinone biosynthesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-succinyl-5-enolpyruvl-6-hydroxy-3-cyclohexene-1-carboxylate synthase</td>
<td>menD</td>
<td>2553091</td>
<td>No hits</td>
</tr>
<tr>
<td>Naphthoate synthase</td>
<td>menB</td>
<td>2553097</td>
<td>No hits</td>
</tr>
<tr>
<td>Demethylmenaquinone methyltransferase</td>
<td>ubiE</td>
<td>2552085</td>
<td>ORF present</td>
</tr>
<tr>
<td>Isochorismate synthase, putative</td>
<td>PG_1525 (menF)</td>
<td>2553092</td>
<td>Partial hit in ORF region</td>
</tr>
<tr>
<td>Mandelate racemase/muconate lactonizing enzyme</td>
<td>PG_1522 (menC in P.g.)</td>
<td>2553098</td>
<td>No hit</td>
</tr>
<tr>
<td>O-succinylbenzoic acid--CoA ligase</td>
<td>PG_1521 (menE)</td>
<td>2553101</td>
<td>Partial hit in ORF ctg 460.4</td>
</tr>
<tr>
<td>Phospho-2-dehydro-3-deoxyxephtolate aldolase/chorismate mutase</td>
<td>PG_0885</td>
<td>2552917</td>
<td>ORF present</td>
</tr>
<tr>
<td>3-dehydroquinate synthase</td>
<td>aroB</td>
<td>2553182</td>
<td>ORF present</td>
</tr>
<tr>
<td>3-dehydroquinate dehydratase, type II</td>
<td>aroQ</td>
<td>2552429</td>
<td>ORF present</td>
</tr>
<tr>
<td>Shikimate 5-dehydrogenase</td>
<td>aroE</td>
<td>2552086</td>
<td>ORF present</td>
</tr>
<tr>
<td>Shikimate kinase</td>
<td>aroK</td>
<td>2552909</td>
<td>alignment goes beyond ORF region by few bases, possible start site within ORF region</td>
</tr>
<tr>
<td>3-phosphoshikimate 1-carboxyvinyltransferase</td>
<td>aroA</td>
<td>2552164</td>
<td>ORF covers alignment region, but no valid start site present in ORF</td>
</tr>
<tr>
<td>chorismate synthase</td>
<td>aroC</td>
<td>2551821</td>
<td>ORF present</td>
</tr>
<tr>
<td>mandelate racemase/muconate lactonizing enzyme family protein</td>
<td>PGTDC60_0781 (menC in P.g.)</td>
<td>10722510</td>
<td>No hit</td>
</tr>
<tr>
<td>Mandelate racemase/muconate lactonizing protein</td>
<td>Poras_0926 (menC in P.a.)</td>
<td>10575412</td>
<td>No hit</td>
</tr>
<tr>
<td>Heme biosynthesis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ferrochelatase; catalyzes the ferrous insertion into protoporphyrin IX</td>
<td>hemH</td>
<td>2552580</td>
<td>ORF present</td>
</tr>
<tr>
<td>Protoporphyrinogen oxidase</td>
<td>PG0375</td>
<td>2552298</td>
<td>ORF present</td>
</tr>
<tr>
<td>Mannosyltransferase</td>
<td>PG0129</td>
<td>2552197</td>
<td>ORF present</td>
</tr>
</tbody>
</table>

**Table 2**: Genes involved in the chorismate, menaquinone and heme biosynthesis pathway identified in the genome sequence of KLE1280. Whole genome sequencing for *Porphyromonas sp.* by performed by Illumina sequencing. The draft genome was annotated using the RAST server (Aziz et al., 2008) and Tigra assembler.
Figure 10: Genes involved in the menaquinone biosynthesis pathway identified bioinformatically in the genome sequence of KLE1280, *P. gingivalis* W83 and *E. coli*. Genes in filled green boxes have been identified bioinformatically in the genome sequence of the isolate (data from the National Microbial Pathogen Data Resource at [http://www.nmpdr.org](http://www.nmpdr.org) and [http://ecocyc.org/ECOLI/NEW-IMAGE?type=PATHWAY&object=PWY-5838&detail-level=2](http://ecocyc.org/ECOLI/NEW-IMAGE?type=PATHWAY&object=PWY-5838&detail-level=2)). *E. coli* has a complete pathway for menaquinone biosynthesis. With the exception of *menH*, whose gene has not been identified in *P. gingivalis*, this indicates a complete pathway for menaquinone biosynthesis in this organism. This is consistent with the known ability of *Porphyromonas gingivalis* to produce menaquinone (Shah & Williams, 1987). KLE1280 has 6 genes missing (or partial sequences) from the pathway (*menF*, *menD*, *menH*, *menC*, *menE* and *menB*).
Figure 11: Heme biosynthetic pathway in *Porphyromonas gingivalis* W83. Genes in green circles are present in KLE1280. This pathway is incomplete in *Porphyromonas gingivalis* W83 as well as KLE1280, thus limiting growth of these strains to media containing exogenous heme.
Extraction and fractionation of spent supernatant

*S. hominis* KLE1525 and *E. coli* both induce growth of KLE1280, likely providing one or more growth factors to the dependent isolate. To understand the interaction between the helper and the dependent and to identify the growth factor/s that the helper provides, biochemical extraction and fractionation of the supernatant of the helper was performed in parallel with whole genome sequencing. The hypothesis is that the helper produces one or more growth factors which can be extracted, purified and identified biochemically from the spent medium in which the helper has grown.

Both helpers of KLE1280, *E. coli* and KLE1525, were grown in liquid and solid media to allow for growth factor production. The resultant culture was centrifuged, filtered, concentrated, and extracted with hexane and then ethyl acetate. The cell pellets of both helpers were also extracted with ethyl acetate and hexane. All extracts were dried and resuspended in acetone at a concentration of 1mg/ml and tested for growth induction of KLE1280. The ethyl acetate extracts of supernatant of *E. coli* and the cell pellet extract of KLE1525 showed induction of KLE1280 (Figure 12a, b). These extracts were further fractionated by High Performance Liquid Chromatography. None of the resultant fractions induced growth of KLE1280.

An ethyl acetate extract of KLE1525 cells together with the solid medium it was grown on (FAA) also showed induction of KLE1280 (Figure 12c), but none of the further fractions showed induction of KLE1280.

Despite the extracts inducing growth of KLE1280, none of the fractions induced its growth, suggesting that either there was more than one fraction required for growth induction or activity loss of the fraction.
Figure 12: Induction of *Porphyromonas* sp. KLE1280 by Ethyl acetate extracts. (a) KLE1280 was spread evenly onto Fastidious Anaerobe Agar supplemented with 5% defibrinated sheep blood and 5% pooled human saliva. 10 µl of the ethyl acetate extract of the supernatant of *E. coli* grown in FAB supplemented with 5% defibrinated sheep blood and 5% pooled human saliva was spotted on top. (b) KLE1280 was spread evenly onto FAA. 10 µl of the ethyl acetate extract of the supernatant of KLE1525 grown in FAB was spotted. (c) KLE1280 was spread evenly onto Fastidious FAA. 10 µl of ethyl acetate extract of KLE1525 cells together with the solid medium it was grown on (FAA) was spotted.
Testing addition of exogenous menaquinone and the specificity of quinones for growth induction of KLE1280

Next, the ability of purified quinones to induce growth of KLE1280 was tested. Ubiquinones and menaquinones were mixed with Fastidious Anaerobe Agar supplemented with 5% defibrinated sheep blood and 5% pooled human saliva at a concentration of 5µg/ml, and KLE1280 was spread on the plates (Table 3). The commercially available (Sigma) quinones tested were MK4, Q1, Q2, Q4, Q9 and Q10. Q8 and MK8 were extracted and purified from E. coli. MK4, MK5, MK6, MK7 and MK8 were obtained from M. luteus in a previous project, and these were tested as well. 1,4-dihydroxy-2-naphthoate (DHNA), an intermediate in the menaquinone biosynthesis pathway was also tested for induction of growth of KLE1280. MK4 and DHNA induced growth of KLE1280. None of the other menaquinones or ubiquinones induced growth of KLE1280 (Table 3). Also, menadione did not induce growth.

Heme requirement of KLE1280

Exogenously added menaquinone 4 induces growth of KLE1280. The medium used to grow KLE1280, FAABS, contains saliva and blood. These were excluded from the medium one at a time to determine if they were required for growth of KLE1280. Saliva was not required for the growth of KLE1280.
Table 3: Quinone specificity of KLE1280. Various quinones were tested for induction of growth of KLE1280 at a concentration of 5 µg/ml added to FAABS. Menaquinone 4 (MK4) induced growth of KLE1280. One intermediate from the menaquinone biosynthesis pathway, 1,4-dihydroxy-2-naphthoate (DHNA) also induced growth of KLE1280. While it is expected that members of the Porphyromonas would utilize menaquinones (Grenier & Mayrand, 1986), ubiquinones were also tested, as an organism forced to scavenge for exogenous quinones may be more promiscuous than those that make their own.
In addition to MK4, blood was required, for growth of KLE1280. Blood could be excluded if hemin was present in the medium. Hemin is a synthetic source of heme added to various commercial media, such as FAA and BHI, used for growing bacteria associated with the human body. Different concentrations of MK4 were spotted on media with and without blood (5%) or hemin (10µg/ml). Along with blood and hemin, hemoglobin (100µg/ml), a component of blood, was also tested for growth induction of KLE1280. MK4 induced growth of KLE1280 in the presence of any of these three heme sources in the medium, but not in their absence (Figure 13). *E. coli* induced growth of KLE1280 on medium without any source of heme or menaquinone 4 (data not shown). This suggests that *E. coli* can provide both growth factors to KLE1280, and that KLE1280 may be deriving its growth factors from other bacteria in the oral cavity.

**Testing dependence of other *Porphyromonas* sp. on menaquinones and *E. coli* mutant OCL67**

The ability of MK4 to induce growth of other uncultured strains from the genus *Porphyromonas* was tested next to determine if a menaquinone requirement is specific to KLE1280 or if there are other members of the same genus that also require it to grow. Our collaborators at The Forsyth Institute had previously isolated two additional strains, *Porphyromonas* sp. (HOT-283 strain F0035) and *Porphyromonas* sp. (HOT-283 strain F0037) from the oral cavity using *Staphylococcus aureus* and *Propionibacterium acnes* as helpers. These strains were procured and tested for growth induction using MK4 and DHNA. Both MK4 and DHNA induced growth, while menadione did not.
Figure 13: Heme requirement of KLE1280. KLE1280 was spread evenly on FAA (without hemin) plates containing blood (a), hemoglobin (b), hemin (c), MK4 (d), hemoglobin and MK4 (e), blood and MK4 (f) and hemin and MK4 (g). KLE1280 grew only in presence of both MK4 and hemoglobin/blood/hemin (e-g)
**Determination of quinone dependency in dental plaque**

In order to understand whether the requirement for menaquinone is a one-genus occurrence or a generalized phenomenon in the oral cavity, ubiquity of quinone dependence was determined. Dental plaque was serially diluted in FAB, plated on FAA supplemented with 5 µg/ml of MK4 and incubated anaerobically at 37°C for 5 days. Colonies were replated on media with and without MK4. Those isolates growing only on plates with MK4 and not on the plates without MK4 were replated on media with and without the growth factor to confirm their dependence. The isolates were also plated with OCL67 as helper. Using this method, more isolates belonging to the genus *Porphyromonas* were isolated as being dependent on MK4. Another isolate, identified by 16S rRNA sequencing as Candidatus *Peptostreptococcus massiliae* 2002-69396 (99.32% similar) was induced by wild-type *E. coli* but not by the deletion mutant OCL67. This isolate was not induced by MK4 at 5 µg/ml concentration. These results suggest that this isolate either requires a different concentration of MK4 or a different quinone. Different concentrations of various quinones need to be used for this experiment to determine the exact requirement of quinones in the oral cavity. No other MK4 dependents were isolated at the concentration of MK4 tested. Thus it looks like quinone dependence is not restricted to *Porphyromonas sp.* There are other bacteria that dependent on quinones for growth.
Discussion

A considerable part of the oral microbiome remains uncultured, and this presents a significant barrier to understanding its role in health and disease. Indirect approaches such as metagenomics provide valuable information on the microbiome, but it is necessary to culture microorganisms in order to understand their physiologies, their growth requirements, their causalities in disease and their contribution to health in general. These organisms could also be sources of various secondary metabolites which could be accessed upon growth of these organisms. It was found that a common cause of “uncultivability” in the external environment such as soil or marine sediment is the dependence of uncultured bacteria on growth factors produced by cultivable neighboring species (D’Onofrio et al., 2010). The hypothesis is that a similar relationship exists between uncultured bacteria of the oral microbiome and their neighbors.

To obtain previously uncultured bacteria from the oral microbiome, a sample of dental plaque from healthy volunteers was selected as a source of material. Dental plaque is essentially a biofilm and the complex structure that has been proposed (Kolenbrander et al., 2002) allows for easy exchange of small molecules within the matrix of the dental plaque biofilm. Dental plaque therefore seemed to be an ideal candidate as a source of bacteria that require growth factors from their neighbors. Dental plaque was inoculated onto various commercial media and incubated anaerobically to obtain plates with moderately crowded growth (100-300 colonies). The hypothesis was that on such crowded plates some of the colonies were actually those of uncultured bacteria that grew because they were receiving growth factors from their neighbors. Two approaches were used to inoculate dental plaque on the media: spread plating serial dilutions of dental
plaque and using a BD FACSaria II cell sorter to deposit dental plaque bacteria in an array on the media plates, to ensure separation of individual cells from each other. Dental plaque was sorted into arrays of 24, 96 (on plates and in single wells) and 384 cells onto FAABS, mFUM agar, and CBA plates. Although statistically not quite significant, single cells sorted onto Omni trays (96 cells) gave a higher recovery than cells sorted in 96 well plates. Plates with 384 cell arrays gave the highest recovery, suggesting that the decreased cell-to-cell distance increased the chances of growth factor interaction, thus leading to higher recovery of dependent bacteria. These results agree with previous findings where growth of one bacterium from the oral cavity was dependent on others. *Fusobacterium* species was dependent on the presence of *Actinomyces* species for growth (Periasamy *et al.*, 2009). Another study showed cooperation between bacterial species within the oral biofilm network for survival (Kolenbrander *et al.*, 2010).

Small colonies growing next to large ones on plates from both approaches were chosen to be verified for dependence. These small colonies/slow growers could be deriving their growth factors from neighboring fast growers. These small colonies were therefore replated on fresh media with their potential helpers or a helper mix of all the colonies growing around the small colonies. A characteristic pattern was observed for dependent organisms. On a plate that had the dependent bacteria spread or streaked next to the helper, the dependent grew only close to the helper. There was an inverse proportion between colony size of the dependent and the distance between the helper and dependent; the colony size decreased as the distance between the dependent and the helper increased. This could be due to the decreasing amount of a growth factor produced by the helper that was unavailable away from the helper (D'Onofrio *et al.*, 2010).
Isolates verified to be dependent were identified by 16S rRNA gene sequencing. Of the seven isolates obtained, one isolate, KLE1280 was chosen for further evaluation, due to its consistent dependence on isolate KLE1525, which is closely related to Staphylococcus hominis subspecies novobiosepticus GTC 1228\(^T\) (99.6%). KLE1280 was consistently dependent on KLE1525 on various media tested. KLE1280 is 96% similar to the closest type strain Porphyromonas catoniae 51270\(^T\). P. catoniae is associated more with periodontal health rather than disease (de Lillo et al., 2004). It is interesting to note that P. catoniae was isolated from infants as satellite colonies, apparently dependent on other bacteria for growth (Kononen et al., 1999). Of the genus Porphyromonas, P. gingivalis is a principal pathogen causing gingivitis (Andres et al., 1998), though not all Porphyromonas are associated with pathogenicity. For example, in addition to P. gingivalis, P. endodontalis is associated with disease but not P. catoniae (de Lillo et al., 2004).

The ability of E. coli to induce growth of KLE1280 was tested, as this model organism readily lends itself to a genetic screen for growth factors. E. coli induces growth of KLE1280 in addition to the natural helper, S. hominis. Mutants of E. coli with non-essential genes (single and multiple) deleted, have been previously organized into libraries, which can be requested from http://ecoli.aist-nara.ac.jp/ and http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp (Kato & Hashimoto, 2007, Baba et al., 2006). The screen is based on the hypothesis that the missing gene(s) of a mutant that doesn’t induce growth would allow us to determine the identity of the growth factor. A genetic screen was thus developed using ordered libraries of long, medium, short and single deletion mutants of E. coli to identify the growth factors. The single gene deletion
ordered library (Keio collection) has 3985 single non-essential gene deletion mutants of *E. coli*. Testing all these individually would involve extensive amounts of resources and time. A less intensive *E. coli* deletion mutant screen was put together to use as a possible indicator towards the growth factors for previously uncultured organisms dependent on *E. coli*. This reorganization was done by cherry picking mutants from the long, medium, short and single gene deletion libraries of *E. coli*.

Mutants from the reorganized library were tested for induction of KLE1280. *E. coli* strain OCL67 showed no induction. This deletion mutant has six menaquinone biosynthesis genes deleted along with 10 other genes. The single deletion mutants of *E. coli* that were deleted in OCL67, as well as the additional *E. coli* single gene deletion strains present in the menaquinone biosynthesis pathway but not deleted in OCL67 were tested for induction of growth. Only *E. coli* mutants in *menD*, *menC*, *menE* and *menB* did not induce growth of KLE1280.

Other deletion mutants of *E. coli* that had either a menaquinone biosynthesis pathway gene deletion, or a chorismate pathway gene deletion, showed reduced abilities to induce growth of KLE1280 (data not shown). Chorismate is a precursor for menaquinone biosynthesis and a gene deletion mutant in chorismate biosynthesis would consequently result in absence of menaquinone production.

Gene deletion mutants of *E. coli* in the menaquinone biosynthesis pathway did not induce growth of KLE1280. This suggests that menaquinone is essential for growth and its biosynthesis machinery is missing in this isolate. In order to get a better understanding of the quinone biosynthesis of KLE1280, its genome was sequenced by our collaborator George Weinstock and his team at The Genome Institute at Washington University. The
draft genome was annotated using the RAST (Rapid Annotations using Subsystems Technology) server (Aziz et al., 2008). The sequence showed that the essential genes of the menaquinone pathway are either missing or partial (*menF, menD, menH, menC, menE* and *menB*). These genes are known to exist in the closest cultivable relative with a genome sequence, *Porphyromonas gingivalis* W83 ([http://www.nmpdr.org](http://www.nmpdr.org)). *menA* and *ubiE* were detected in the genome of KLE1280. These results are consistent with the observations from the previous experiment using the single deletion mutants of *E. coli* from the menaquinone biosynthesis pathway as helpers, where deletion mutants in *menA* and *ubiE* were able to induce growth of KLE1280. This suggests that DHNA could serve as a substrate for the *menA* gene product, and be converted to menaquinone in KLE1280. Exogenously added DHNA does indeed support the growth of KLE1280.

The heme biosynthetic pathway in KLE1280 is incomplete. The heme biosynthesis pathway is incomplete in *P. gingivalis* as well, which is consistent with its observed requirement for heme is a requirement for growth, which it is thought to acquire from the host (Dashper et al., 2009) or other bacteria.

The genetic evidence for menaquinone as a growth factor for KLE1280 was completely unexpected. The compound is an integral component of the respiratory chain, highly hydrophobic, and only small amounts should leak out of the cell (Bentley & Meganathan, 1982). In addition, the media contained menadione, a menaquinone analog specifically added to satisfy the quinone requirement in various organisms (Bentley & Meganathan, 1982).
In hindsight, it is fortunate that a genetic screen was done prior to a bioassay-driven purification. The bioassay-driven purification proved highly challenging. The spent supernatant of either *E. coli* or KLE1525 did not show consistent induction of KLE1280, even after concentration. The supernatants were nonetheless extracted with ethyl acetate, hexane and methanol. The ethyl acetate extract did show induction of KLE1280 but once it was fractionated further, none of the fractions showed any induction.

There are a few possible explanations for the inability of any of the fractions to induce growth. One, the growth factor could be degraded. A second possibility is that there is more than one growth factor and they get separated during fractionation. These would be particularly difficult to catch since the combinations of the number of fractions would be an exponential figure. A third possibility could be slight modifications in the structure of the growth factor, rendering it non-functional for the dependent.

KLE1280 seems to be very specific in its quinone requirement. Of the several ubiquinones and menaquinones tested for their ability to induce growth of KLE1280 at a concentration of 5 µg/ml (MK4, Q1, Q2, Q4, Q9 and Q10 (Sigma); Q8 and MK8 (*E. coli*); MK4, MK5, MK6, MK7 and MK8 (*M. luteus*)), only MK4 induced growth of KLE1280. As was hypothesized, DHNA, an intermediate in the menaquinone biosynthesis pathway and the substrate of MenA (Figure 8), induced of growth of KLE1280, strongly suggesting that the *menA* gene and gene product are active in this isolate.

The lack of a menaquinone biosynthetic pathway in KLE1280 agrees well with the requirement for an external quinone. According to the genome sequence, KLE1280
has the \textit{menA} and \textit{ubiE} genes, explaining why the precursor DHNA was able to induce growth. None of the other menaquinones or ubiquinones induced growth of KLE1280 (Table 3). Especially surprising was that menaquinone and ubiquinone from \textit{E. coli} did not help, even though \textit{E. coli} can induce growth of KLE1280. One reason could be that these quinones have long isoprenoid chains (8 subunits) which makes them hydrophobic and hence probably difficult to diffuse. It is possible that when released from \textit{E. coli}, these quinones are mobilized by adhering to outer membrane vesicles or some other form of a solubilizing agent.

Along with these quinones, menadione was tested. Menadione does not induce growth of KLE1280 at the standard (recommended) concentration tested. Menadione is a synthetic derivative of menaquinone without a side chain (Figure 14) that was originally present in our growth media, and is standardly used in media for the culture of oral microbes. While menadione is standardly added to growth media for oral microbes, KLE1280 does not grow at the recommended concentrations of menadione in the medium. These results indicate that KLE1280 is very specific in its quinone requirement and there could be more bacteria that are dependent on the same or similar growth factors that we are missing, that are not being added in the right concentration or is not specific.
**Figure 14:** Structures of menaquinone (MK) (general structure), menaquinone 4 and menadione. Menaquinone 4 has four isoprenoid side chains of which two are unsaturated. Menadione is a synthetic derivative of menaquinone without the isoprenoid side chain.
To determine whether higher concentrations of menadione induced growth of KLE1280, 1X, 2X, 5X, 10X and 100X recommended concentrations of menadione were tested. Menadione at 5 times the recommended concentration did not induce growth of single cells of KLE1280, but it did induce growth at 100 times the recommended concentration, however this was not the case for the other MK4 dependent *Porphyromonas* isolates, procured from The Forsyth Institute (data not shown). These results indicate that a specific quinone factor is essential for other uncultured strains in this genus as well. Menadione cannot support the growth of either of these strains at the recommended concentrations, and we will not be able to isolate other bacteria that might be dependent on quinones or other growth factors that are currently not being added in the right concentration.

Floyd Dewhirst and his team isolated a third *Porphyromonas* sp. (HOT-279 strain F0450) which required cross streaking with *Staphylococcus aureus* for growth, even in the presence of menadione. HOT-279 has a full genome sequence which was released September 4, 2012 (GenBank # ALKJ00000000). This isolate is missing the menaquinone biosynthetic operon as well, suggesting that this may be a general characteristic of some of the *Porphyromonas*.

Exogenously added menaquinone 4 allows the growth of KLE1280. The medium used to grow KLE1280, FAABS, contains saliva and blood. These were excluded from the medium one at a time to verify the dependence of KLE1280 on them. Saliva was not required for the growth of KLE1280. As expected, along with MK4, blood was required for growth of KLE1280, based on the incomplete heme biosynthesis pathway in its genome. Blood could also be excluded if hemin was present in the medium. Hemin is a
synthetic source of heme added to various media used to grow fastidious anaerobes and organisms associated with the human body. KLE1280 required a source of heme, either in the form of hemin, blood or hemoglobin along with MK4. In the oral cavity, blood could be the source of heme and MK4 could be obtained from other bacteria. *E. coli* induced growth of KLE1280 on medium without any source of heme or menaquinone 4 (data not shown). This indicates that *E. coli* can provide both growth factors to KLE1280, suggesting that KLE1280 may be deriving both of its growth factors from other bacteria in the oral cavity.

The ubiquity of menaquinone dependent bacteria was determined by plating dental plaque on media with MK4, picking colonies and plating them on media with and without MK4, and looking for those that only grow on media with MK4. Potential candidates were also screened for dependence on *E. coli* deletion mutant OCL67. Apart from *Porphyromonas sp*, there was one other isolate that appeared quinone dependent. This isolate was identified by 16S rRNA gene sequencing as Candidatus *Peptostreptococcus massiliae* 2002-69396 (99.32% similar). It was induced by wild type *E. coli* but not by its deletion mutant OCL67. This suggests a quinone requirement for this bacterium as well. Although it was not induced by MK4 at 5 µg/ml concentration, it might require a different concentration of MK4 or a different menaquinone. If specificity of KLE1280 for MK4 is any indication, there are many other bacteria that require specific quinones, the requirement for which will not be satisfied by menadione. This specificity then could be the reason why there was low recovery of quinone dependent bacteria from the oral cavity.
The previously uncultured *Porphyromonas sp.* KLE1280 depends on a growth factor produced by *S. hominis*. *E. coli* can also serve as a helper, and mutants deficient in menaquinone biosynthesis fail to induce growth of *Porphyromonas sp.* KLE1280. The genome of KLE1280 lacks key enzymes of the menaquinone biosynthetic pathway, and exogenous menaquinone supports its growth. It was a surprise to find an apparently essential component of the electron transport chain in the growth of an anaerobic bacterium, and that it was missing from the genome. While menadione is standardly added to growth media for oral microbes, KLE1280 does not grow on recommended growth media. In addition, it is puzzling why an obligate anaerobe would require an electron transport chain. Many obligate and facultative anaerobes will use alternative electron acceptors under anaerobic conditions, but we are unaware of a precedent for this process to be essential for growth on rich media. The above results suggest that KLE1280 lacks the ability to make its own menaquinones, yet apparently a functioning electron transport chain is essential for this organism. The closest sequenced species, *P. gingivalis*, makes menaquinone, and according to its genome has the biosynthetic pathway. This suggests that KLE1280 might have “lost” the ability to produce menaquinone as suggested in the “Black Queen Hypothesis” where an organism loses its ability to produce a certain growth factor that can be obtained from its neighboring bacteria (Morris *et al.*, 2012).
Way forward

Mechanism of uncultivability for KLE1280

KLE1280 has a requirement for hemin and MK4. These are known to be involved in electron transport. The hemin requirement of *Porphyromonas* has been reported before (Genco *et al.*, 1994, Smalley *et al.*, 1991). Hemin acts as a prosthetic group for proteins in respiratory chains (Vernon & Kamen, 1954); (Gibbons & Macdonald, 1960). MK4 has also been shown to be a part of the electron transport chain (Beyer, 1958, Brodie *et al.*, 1957). This is probably the role of heme and MK4 in KLE1280 where the MK4 could be to shuttling electrons between one complex and another. The other possibility is that either or both MK4 and heme could be acting as terminal electron acceptors. This can be investigated by using completely reduced and oxidized forms of hemin and hemoglobin, testing them to induce growth of KLE1280, the hypothesis being that if MK4 is involved in electron transport then completely reduced hemin or hemoglobin would not support the growth of KLE1280 (Conant & Tongberg, 1930, Conant, 1923). This experiment would be useful though, only if either of the compounds was the terminal electron acceptor in the electron transport chain. Another approach to determine the role of MK4 in electron transport would be to add either completely reduced or oxidized MK4 and then assay the resultant growth medium for presence of a mixture of both reduced and oxidized MK4. Detection of cytochromes in the medium could also indicate electron transport activity.
Conclusion
In conclusion, we have identified an intriguing dependency for growth in a previously uncultured bacterium. This organism, KLE1280, is closely related to *Porphyromonas catoniae* ATCC 51270\(^T\), and is incapable of growth in the absence of exogenous menaquinone 4 and heme. Menaquinone is an important part of the electron transport chain in many bacteria and this organism might be incapable of electron transport on its own. Furthermore, it might not be capable of alternative metabolic pathways that could be independent of the electron transport chain.

Current standard media cannot support the growth of this bacterium, and it is likely that many uncultured bacteria are deficient in the same or similar growth factors. Therefore, use of this growth factor may allow the isolation of significant number of additional uncultured organisms. This approach thus could be successful in isolating a number of previously uncultured bacteria and identification of novel growth factors.
Appendix

**Tryptic Soy Broth/Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto™ Tryptone (Pancreatic Digest of Casein)</td>
<td>17.0</td>
</tr>
<tr>
<td>Bacto Soytone (Peptic Digest of Soybean Meal)</td>
<td>3.0</td>
</tr>
<tr>
<td>Glucose (Dextrose)</td>
<td>2.5</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Dipotassium Hydrogen Phosphate</td>
<td>2.5</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C

**Blood Agar**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA</td>
<td>1L</td>
</tr>
<tr>
<td>Defibrinated sheep blood</td>
<td>50mL</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C

**Chocolate Blood Agar (CBA)/**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA</td>
<td>1L</td>
</tr>
<tr>
<td>Heated, lysed defibrinated sheep blood</td>
<td>50mL</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C
### R2Aagar/R2NP (broth)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Digest of Casein</td>
<td>0.25</td>
</tr>
<tr>
<td>Enzymatic Digest of Animal Tissue (Proteose Peptone)</td>
<td>0.25</td>
</tr>
<tr>
<td>Acid Hydrolysate of Casein</td>
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</tr>
<tr>
<td>Yeast Extract</td>
<td>0.5</td>
</tr>
<tr>
<td>Dextrose (Glucose)</td>
<td>0.5</td>
</tr>
<tr>
<td>Soluble Starch</td>
<td>0.5</td>
</tr>
<tr>
<td>Dipotassium Phosphate</td>
<td>0.3</td>
</tr>
<tr>
<td>Magnesium Sulfate Heptahydrate</td>
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<tr>
<td>Sodium Pyruvate</td>
<td>0.3</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C

### Luria-Bertani Broth/Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C
**Brain Heart Infusion broth/Agar**

<table>
<thead>
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<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef heart</td>
<td>5</td>
</tr>
<tr>
<td>Calf brains</td>
<td>12.5</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>2.5</td>
</tr>
<tr>
<td>D(+) glucose</td>
<td>2</td>
</tr>
<tr>
<td>Peptone</td>
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</tr>
<tr>
<td>Sodium chloride</td>
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</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C
**Fastidious Anaerobe Agar/Broth**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>23</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5</td>
</tr>
<tr>
<td>Soluble Starch</td>
<td>1</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>0.4</td>
</tr>
<tr>
<td>Glucose*</td>
<td>1</td>
</tr>
<tr>
<td>Sodium Pyruvate*</td>
<td>1</td>
</tr>
<tr>
<td>L-Cysteine HCl.H2O*</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium Pyrophosphate</td>
<td>0.25</td>
</tr>
<tr>
<td>L-Arginine*</td>
<td>1</td>
</tr>
<tr>
<td>Sodium Succinate</td>
<td>0.5</td>
</tr>
<tr>
<td>Hemin*</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin K*</td>
<td>0.001</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C

* - Added after autoclaving

**Fastidious Anaerobe Agar with 5% sheep blood and 5% pooled human saliva (FAABS)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAA</td>
<td>900 mL</td>
</tr>
<tr>
<td>Defibrinated sheep blood</td>
<td>50 mL</td>
</tr>
<tr>
<td>Pooled human saliva</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Final pH: 7.2 ± 0.2 at 25°C
**Modified Fluid Medium (mFUM) agar/broth**

- **Tryptone**: 10
- **Yeast extract**: 5
- **Glucose**: 3
- **Hemin**: 0.002
- **Menadione**: 0.001
- **Cysteine HCl**: 0.5
- **Dithiothreitol**: 0.1
- **Sodium chloride**: 2.9
- **Sodium carbonate**: 0.5
- **Potassium nitrate**: 1
- **Dipotassium phosphate**: 0.45
- **Mono potassium phosphate**: 0.45
- **Ammonium sulfate**: 0.9
- **Magnesium sulfate (heptahydrate)**: 0.188
- **Heat inactivated fetal bovine serum**: 20 mL
- **Pooled human saliva**: 500 mL

**Final pH**: 7.2 ± 0.2 at 25°C
References


