

AEROBIC BACTERIAL METHANE SYNTHESIS IN THE HUMAN
GASTROINTESTINAL TRACT

by

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This thesis constitutes one the largest single bodies of work I have produced in my lifetime. Though one large document, it veils more than two years of triumph, discipline, frustration, and most frequently, learning in the form of many mistakes. To say that this was done alone would not only be disastrously wrong but fail to commend the many people without whom this work would not and could not exist. I would first and foremost like to acknowledge Dr. Seth Walk, whose oversight, planning, and guidance helped me to keep learning and keep innovating. I would also like to acknowledge the enormous contribution of Dr. Qian Wang. Not only was this project her intellectual progeny, but it was most directly her guidance, teaching, and seemingly endless patience that helped me grow from the hapless novice that I was when I first entered the Walk lab. I would also like to note the innumerable contributions made by the Walk lab personnel. Trenton, Karlin, Reece, Nick, and others all provided support and guidance in key times and areas, all of whom provided assistance of inestimable value and abatement of uncountable quantity. These mentioned are all mentors and coworkers deserving of emphatic praise, whom any scientist would be lucky to have in their employ. I would also like to extend my appreciation to the College of Agriculture at Montana State University. This college in part provided the funding for my tenure as a graduate student in the department of microbiology, which is nestled within the aforementioned College. Thank you all.

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ABSTRACT

Aerobic bacterial methane synthesis constitutes a paradigm-shifting novel metabolism recently described in aquatic environments. It challenges the traditional model of methanogenesis as being a strictly anaerobic process carried out by archaeal methanogens. To date, the presence of aerobic bacterial methane synthesis has not been studied within the context of the human gastrointestinal tract. The goal of this work was to investigate the possibility of the presence of such metabolisms in the human gut microbiome. To investigate this, fecal samples from six individuals were first screened for the ability to produce methane under aerobic conditions. Bacteria from two of those fecal samples were isolated and evaluated for their ability to utilize methylamine, a known substrate involved in aerobic bacterial methane synthesis, as a sole nitrogen source. The ability of those isolates to produce methane under aerobic conditions from methylamine was then evaluated. Additionally, a flask-independent culture-based assay was developed in order to screen larger numbers of future isolates for the ability to utilize methylamine as a sole nitrogen source. This work demonstrates the first evidence of aerobic bacterial methane synthesis from members of the human gastrointestinal tract, finding two isolates capable of producing methane under aerobic conditions. Such findings broaden the understanding of methane-generating pathways that may have implications for the development of dysbiosis and atherosclerosis in human hosts.

INTRODUCTION

The human gut microbiome is a diverse and complex community of microorganisms. With an estimated bacterial load on the same order of magnitude as the number of cells in the human body [2], these organisms have important impacts on host health and development. One area in which the impact of the human microbiome on host health has been studied is that of methane production. It is estimated that nearly all people (95.7%) harbor methane-producing microbes (methanogens) in their gastrointestinal tract [3] with the majority of studies being focused on either methane production itself, or some other aspect of methanogens.

Methane production in the gut has traditionally been attributed solely to anaerobic Archaea. In recent years, however, evidence has come to light that challenges the traditional paradigm of methanogenesis as being a strictly anoxic process [4-13]. For example, oxygenated portions of certain environments contain methane, the origin of which is poorly described by traditional methanogenesis [4, 10, 14-19]. Substrates for methanogenesis, including methylphosphonate [12, 13] and methylamine [10], have been shown to be precursors to non-methanogen methane production in aerobic environments. Additionally, a reactive oxygen species-dependent mechanism for methane production in cells of all domains of life, including human cells, has been demonstrated [20].

While much evidence of aerobic methane production by non-Archaeal microorganisms has been demonstrated in many environments, this metabolism has not been demonstrated in the human gut. Since the human metabolome includes potential substrates (methylated amines and methylphosphonate) for methane synthesis, we hypothesized that bacteria of the human microbiome generate methane using oxygen available along the gut mucosa. Our results

represent the first evidence that methane can be produced aerobically by bacterial members of the human gut microbiome.

BACKGROUND

Methanogenesis

Methanogenesis is a well-studied microbial metabolism. It is present in a wide range of environments, including in deep subsurface coal seams [21], hot springs in Yellowstone National Park [22], marshes and wetlands [23], and in the gastrointestinal tract of mammals [24].

Methane production has most typically been implicated for its role in global carbon cycling and as a potent greenhouse gas [23], with methane having a radiative effect 25 times that of carbon dioxide [25]. Additionally, methane is the second most abundant carbon compound in the atmosphere [26]. Biologically produced methane, or biogenic methane, is a product of microbial methanogenesis, wherein methane is produced as a by-product of respiration, using carbon dioxide as a terminal electron acceptor [23]. Carbon dioxide reduction can be coupled with the oxidation of several different compounds; hydrogen in hydrogenotrophic methanogenesis, acetate in acetoclastic methanogenesis, and various methylated compounds in methylotrophic methanogenesis. Electron donors in methylotrophic methanogenesis include methanol, formate, trimethylamine, and methylamine [23] (see fig. 1).

Historically speaking, methanogenesis has referred exclusively to the three dissimilatory processes just described. The steps in traditional models of methanogenesis were originally described in only a few archaeal phyla, with the majority being of the kingdom *Euryarchaeota* [27]. Methanogenesis also occurs in other Archaea, *Thermoplasmatota*, *Halobacterota*, and various *Candidata* [23, 27]. These methanogenic taxa generally occupy important ecological niches, metabolizing the by-products of heterotrophic metabolisms[21-23, 27]. It should be noted that a common feature across all archaeal methanogenic taxa is the enzyme methyl-

coenzyme M reductase (Mcr). Specifically, the McrA gene is commonly used in PCR-based assays as a proxy for the presence of methanogens, due to the nature of the gene being diagnostic for methanogenesis [10, 28]. This enzyme is responsible for producing methane in all methanogens, regardless of electron donor. It forms methane and a heterodisulfide from methyl-coenzyme M and HS-HTP [26]. It is the reduction of the formed heterodisulfide that generates ATP in methanogenic metabolisms.

Considering that methanogenesis has a historical and precise definition, this work describes a biologically mediated process of methane production that is utterly distinct from the historical and precise definition. Terms such as “biogenic methane production” will be used to describe any biological process that yields methane. Conversely, methanogenesis as it has been historically defined will commonly be referred to as “traditional” methanogenesis. While there is a need for a term to delineate traditional methanogenesis from other mechanisms of biologically produced methane, it is likely that methanogenesis itself should encompass any form of biologically produced methane to ameliorate confusion.

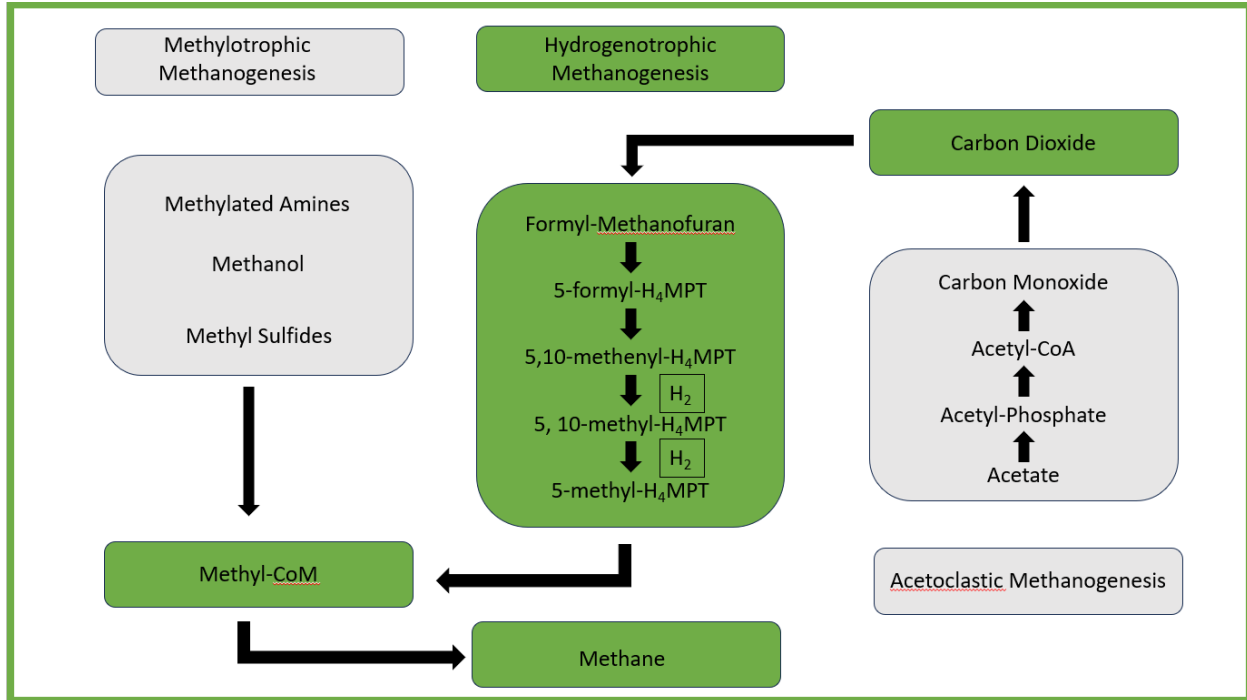


Figure 1: Diagram depicting the different pathways of methanogenesis. Hydrogenotrophic methanogenesis includes the steps in green, while acetoclastic methanogenesis and methylotrophic methanogenesis are shown in gray. Both acetoclastic methanogenesis and methylotrophic methanogenesis feed into the hydrogenotrophic methanogenesis pathway at different points.

It has been estimated that up to 90% of global methane emissions is microbial in origin [25]. This methane is produced largely in reduced, anoxic environments, as more oxidized environments are toxic to methanogens [23, 26]. These reduced environments, such as rice paddies, marshes, and portions of the mammalian gut, typically host a consortium of microbial metabolisms that are required for producing an environment conducive to methanogenesis. For example, such environments commonly contain glycolytic pathways that are limited to fermentation in the absence of oxygen. They produce diverse substrates including acetate, formate, carbon dioxide, and hydrogen [29-31], thereby enriching local environments with substrate for methanogens to produce methane. Thus, methanogens generally function in a given

environment as bottom-feeders, using the end products of glycolytic metabolisms as substrates for traditional methanogenesis.

There are several reasons why methanogenesis has traditionally been almost exclusively described as an anaerobic process. The first is due to the fact that several enzymes critical to methanogenesis are inhibited in environments with a high redox potential, rendering them ineffective in oxygenated environments [23]. The second is that methanogenesis is a low energy-yielding process and even oxygen-tolerant methanogens [7] are competitively excluded from oxic niches in most environments [23]. Therefore, traditional methanogenesis, with relatively few exceptions [7, 32], is an anaerobic process mediated by a phylogenetically narrow group of Archaea.

Biogenic Methane Production Under Aerobic Conditions

Archaeal methanogens are common inhabitants of lake sediments, which tend to become anoxic rapidly with depth [33, 34]. Hydrogenotrophic and acetoclastic methanogenesis are nearly ubiquitous among these methanogens in freshwater lake sediments [33]. Archaeal methanogens are therefore responsible for the majority of methane produced in the sediments of oceans and freshwater lakes. However, many of these lakes and oceans also contain oxic portions of the water column enriched in methane, called pelagic methane enriched zones (PMEZs) [5, 8, 9, 18, 35]. PMEZs have been identified in different lakes and appear to be a widespread phenomenon [5, 9, 10, 18]. If methanogenesis is strictly an anaerobic metabolism, the repeated observation of PMEZs is enigmatic. Multiple theories exist to explain the presence of PMEZs, including methane production from micro-anaerobic zones in organic particulate matter and benthic cycling from anoxic sediment-derived methane emissions [8, 17]. However,

these explanations are specific to a subset of water bodies [36], leaving the possibility of other, potentially biological [19], explanations for PMEZs.

Yellowstone Lake in Yellowstone National Park (USA) has a particularly well-studied PMEZ with a distinct methane peak in a well-oxygenated zone of the water column [19]. Studies also showed that *mrcA*, a ubiquitous gene among archaeal methanogens [23], was absent in the Yellowstone Lake PMEZ [10, 19]. Recently, bacterial species including *Acidovorax* sp., were isolated in culture from the Yellowstone Lake PMEZ and shown to produce methane when grown aerobically with methylamine (MeA) [10]. Transposon mutagenesis of the *Acidovorax* genome showed that a pyrodoxyl-5'-dependant aminotransferase was necessary for methane production [10], and therefore constitutes a fundamentally different way of producing biogenic methane than what is found in traditional archaeal methanogens. Similar aminotransferases are widespread in taxa that cannot produce methane, so the manner in which this enzyme helps generate methane or whether it might itself catalyze the key methane-producing reaction is not fully understood. Although strong evidence was put forth to demonstrate the necessity of the aminotransferase in the conversion of MeA to CH₄, it is not yet feasible to infer the presence of aerobic bacterial methane synthesis simply by screening for pyrodoxyl-5'-aminotransferases. Regardless, this methylotrophic, methanogenic metabolism does not appear to be dependent upon oxygen-sensitive enzymes [10, 19] and offers a new explanation for PMEZs in some freshwater lake systems.

Certain *Acidovorax* species are not the only organisms to have been implicated in aerobic methane synthesis. In fact, bacteria are not the only domain of life to which aerobic methane synthesis has been attributed. Phytoplankton such as *Emiliana huxleyi*, among others, have been

shown to produce methane in amounts that fluctuate based on environmental variables such as temperature and day/night cycles [37, 38]. These phytoplankton produce far less methane than is produced via classical methanogenesis. However, the collective potential for global methane emissions from this metabolism could be in large due to the immense amount of phytoplankton that collectively exist in global aquatic systems [38]. Other possibilities exist, such as cyanobacteria whose blooms in a Kenyan Lake correlate with methane levels [4]. There are additionally several reports of methane production from methylphosphonate under aerobic conditions [13, 15, 19] with potential contributions from both archaea [13] and bacteria [19].

In recent years, evidence of biogenic methane synthesis occurring in oxygenated environments has been mounting. Whether that evidence is of non-archaeal methanogens [10, 13, 15, 19, 37, 38], alternative methanogenesis pathways [16, 18], or oxygen-tolerant archaeal methanogens [7, 14], it is becoming clear that the traditional model of biogenic methane production being constrained to a phylogenetically narrow group of strictly anaerobic archaea needs revision. The finding that methane is produced in oxic environments has important implications for not only environmental microbiology, but also for the calculation of the global methane budget [17]. More research is needed to understand the molecular mechanisms underlying this metabolism in bacteria. Ultimately, the potential for methane to be produced despite the presence of oxygen constitutes an important and overlooked area of research that has been written off based upon the physiology of traditional methanogens. This view limits a complete understanding of how certain oxygenated lake environments function, as well as their potential contribution to larger ecosystems as a whole.

The Human Gut Microbiome

In addition to the deep subsurface [21], hot springs [22], and marine environments [27] methane is also produced in the mammalian gut. In fact, this environment constitutes one of the most intricate and complex environments harboring methane production [39]. People are estimated to be carrying an estimated 500-1000 different species of bacteria [40] that collectively make up ~100 trillion cells [41]. Not only is the human microbiome diverse overall, but it is also individual-specific, meaning that its taxonomic composition is highly variable between individuals [31, 40, 42]. Like most diverse microbial communities, DNA sequencing is often used to make inferences about its ecology, which limits current understanding about metabolisms and metabolites (metabolic byproducts). Despite this challenge, the human microbiome is clearly important to human health, interacting with a wide range of human cells and tissues. The human gut microbiome modulates host immunity [43], facilitates serotonin production [44], produces vitamins that support normal growth and physiology [45], and produces other important signaling molecules [41, 46]. The human gut microbiome additionally helps break down chemically complex compounds, making nutrients more available. Metabolic by-products like short-chain fatty acids produced from the degradation of indigestible polysaccharides by bacteria such as *Bacteroides thetaiotaomicron* provide the host with 10-15% of its daily caloric requirements [41, 46].

Despite wide variation in the interindividual microbiome, there are clear trends in taxonomic composition. The human gut microbiome is numerically dominated at the phylum level by *Firmicutes* and *Bacteroidetes*, with *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, and *Verrucomicrobia* present at lower abundances [47]. Evaluation of 39 individuals found that the

sequence-defined operational taxonomic units (OTUs) clustered into three groups, referred to as “enterotypes”. These enterotypes could be differentiated according to the abundance of *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and/or *Ruminococcus* (enterotype 3) [48]. Each enterotype displayed different functional gene profiles, with enterotype 1 enriched in carbohydrate degradation and enterotypes 2 and 3 enriched in mucin degradation. It was also noted that enterotypes 1 and 2 were more enriched in vitamin production compared to enterotype 3 [48]. Although certain phyla are consistently found in the human gut [49], there is a high level of diversity at the species level. Individual species typically make up <5% of the total microbiome community but are diverse with respect to genetic potential [48, 50]. For example, members of the Actinobacteria phylum are often found at a low abundance (<2%) but have a significant impact on microbe-microbe interactions [49] as well as degradation of complex starches [51].

Several factors, including diet [41, 52], exercise [53, 54], delivery method at birth and infant diet [55], influence gut microbiome diversity within individuals over time. This intra-individual variation can be seen clearly when comparing the microbiome of the small intestine to that of the large intestine. Although the colonic microbiome is dominated by phyla Bacteroidetes and Firmicutes, the microbiome of the ileum is dominated instead by Actinobacteria and Firmicutes [56, 57]. The small intestine in general contains more aerobic and facultatively anaerobic bacteria than the colon, and harbors far lower cell counts as well [58]. The variation between the small and large intestine is explained by differing nutrient transit times, oxygen availability, pH, and bile salt concentration between the small intestine and the large intestine [58] (see fig. 2), and points to a general trend in the gut microbiome that different areas have

different conditions which leads to differing community composition.. Age of the host should also be noted as a factor affecting the composition of the human gut microbiome, with *Akkermansia* being especially prevalent in children of roughly one year of age [47]. A given individual's microbiome also varies over time to some extent. The traditional view of the healthy gut microbiome is of it being stable over time [59], which makes sense if the composition of the human gut microbiome is shaped largely by deterministic processes. Although several studies have confirmed this stability [59, 60], they tend to show that there is only a subset of the human gut microbiome that is stable across time, while the rest of the gut microbiome is more temporally variable. The idea of a stable subset of microbes is also seen in a study that characterized *E. coli* clones across a 1- to 2-year period, finding that some of the *E. coli* clones were present for the whole study period, while others were much more transient in nature [1].

It is important to understand that the human gut microbiome is, like a subsurface fissure, pelagic hypersaline lake, or topsoil, an environment. The microbial community that exists within it is shaped by the same fundamental, underlying processes that shape microbial communities elsewhere. There are, however, many things that make the human gut microbiome a unique environment. The relatively large nutrient flux [61], myriad microbe-host interactions [44, 45, 62, 63], and varying chemical and environmental conditions throughout the length of the human gastrointestinal system [61] are some of the key features that shape microbial community structure within the gut. To an extent, effective and comprehensive study of the human gut microbiome is limited by the difficulty of procuring representative samples [64]. However, many aspects of the human gut microbiome can still be understood within the context of

ecological principles. This is exemplified by the human gut microbiome within the small intestine. The small intestine is composed, beginning with the stomach and ending at the colon, of the duodenum, jejunum, and ileum. Along its length, it becomes decreasingly aerobic [61]. The nutrient influx into the duodenum tends to be composed of simpler, more energy rich compounds compared with what is observed further down the small intestine [61]. Additionally, transit time of nutrients slows throughout the length of the small intestine, with nutrients from the stomach spending the least amount of time in the duodenum, and the most amount of time in the ileum [61]. In the relatively few studies characterizing the small intestinal microbiome, it has been shown that the community becomes more numerous and more diverse along its length [61, 65, 66]. These observations make sense. The quick transit time of nutrients would push microbes to metabolize the simple, high-energy sugars that are more prevalent in the duodenum. The metabolites produced from that primary degradation would not be available to microbes for long due to the quick transit time of nutrients in the duodenum. This leaves less niche space open to microbes, leading to the decreased diversity and cell count seen in the duodenum [61, 66].

Microbes in an energy-rich environment, in order to maximize growth rate, tend not to fully catabolize energy-rich compounds, even if they have the metabolic capacity to do so [67]. This would mean that primary degradation of the simple sugars entering the duodenum would produce various partially degraded metabolites, opening up niche space downstream in the jejunum and ileum. Accordingly, the increased niche space should allow for greater microbial diversity, which is what is seen along the small intestine [61]. Similar effects of a trophic cascade have also been documented in experiments simulating the colonization of particulate

organic matter in oceanic environments, with microbial diversity increasing alongside a greater array of available growth substrates produced from the catabolic breakdown of simpler compounds [68]. Although there are many more factors affecting community structure within the human gut microbiome, many of which are under-explored, it is clear that certain deterministic factors are at play. The human gut microbiome may be of critical importance to human health, but it still constitutes an environment, and an ecological perspective is therefore invaluable in understanding its functions and community dynamics.

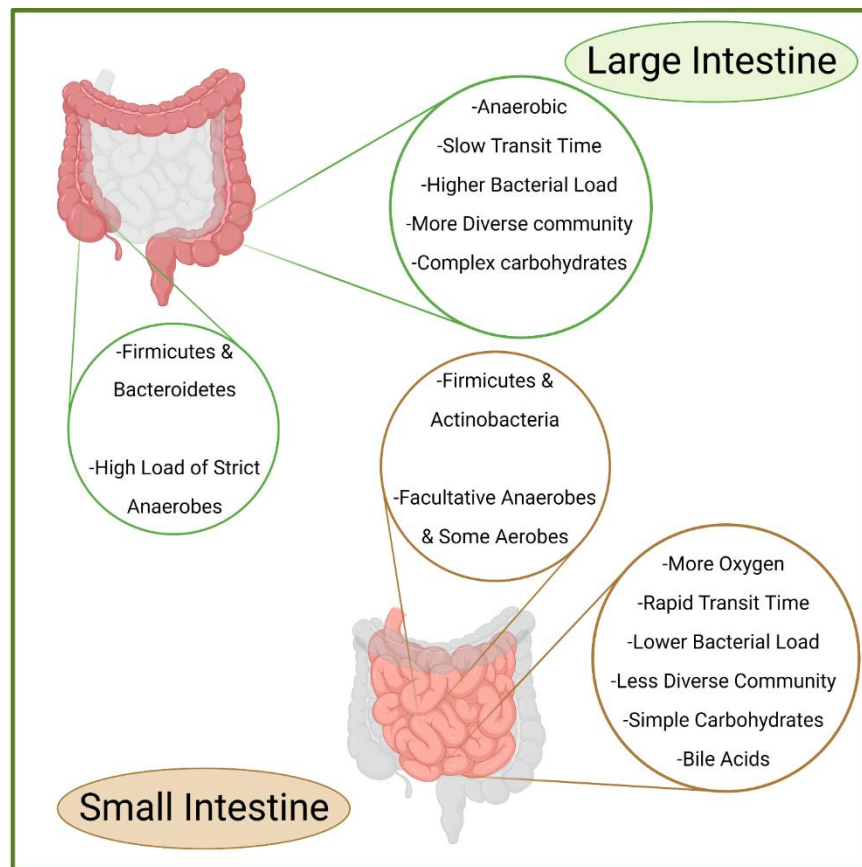


Figure 2: Diagram highlighting some of the differences between the small intestine and large intestine microenvironments. Differences concerning environment and community structure are shown.

It is abundantly clear that the human gastrointestinal tract is host to a wide array of bacteria that are beneficial, if not essential, to the health of the human host. However, not all bacteria present in the human gastrointestinal tract are beneficial. By extension, not all human gut microbiomes are beneficial, or at least not as beneficial as they should be. Besides the obvious cases of the overgrowth of pathogenic bacteria causing disease, there is much evidence to implicate certain abnormal compositions of the human gut microbiome in various disease states. Although an imprecise term, an imbalanced gut microbiome leading to a detrimental outcome has been termed a dysbiosis [69], with such dysbioses having been implicated in various diseases. For example, irritable bowel syndrome (IBS) has been shown to consistently feature a microbiome with lowered overall alpha diversity [69]. Additionally, small intestinal bacterial overgrowth has frequently been found in patients with diarrhea-dominated IBS [70], while microbially-mediated methane production has been implicated in constipation-dominated IBS [71]. It has also been shown that obesity rates correlate with an increased Firmicutes to Bacteroidetes ratio [69], as well as an increased abundance of microbes that produce short chain fatty acids [72]. One disease associated with dysbiosis is infection with *Clostridium difficile*, a nosocomial infection that occurs with surprising regularity [73]. A healthy, diverse microbiome is known to effect a barrier to colonization against any one given organism, a key function in the prevention of disease caused by enteric pathogens [74]. This provided resistance to colonization is key in preventing *C. difficile* infection, and pathogenic overgrowth of this bacteria typically only occurs when the resident microbiome is disturbed with antibiotics [75]. In fact, the presence of a healthy microbiome is so effectual in preventing or treating *C. difficile* overgrowth

that transplantation with a healthy microbiome via fecal microbiome transplantation has been implemented with good success to treat recurrent forms of *C. difficile* infection [75].

Bacteria and Archaea are not the only members of the human gut microbiome, with viruses also inhabiting the gastrointestinal tract of healthy and unhealthy individuals. It has been estimated that there are about the same number of viruses inhabiting a human host as there are bacteria, with around 10^9 virus-like particles (VLPs) per gram of feces [76]. The “virome” has been shown to display a large degree of inter-individual variability and within-host stability [76, 77], with most of the viral load in the human microbiome being composed of phages [78]. Besides viral pathogens, associations have been made between viruses and disease states. A good example is that of *Caudovirales* having been associated with ulcerative colitis across multiple studies [79, 80]. Viruses inhabiting the human gut microbiome are an important factor in the development and maintenance of the human gut microbiome, and may also be the source of novel treatments such as phage therapy [78].

As a result of the manifold ways in which the microbiome and host interact, they can therefore be thought of as a “superorganism,” sometimes referred to as a holobiont [81], wherein the human host and its associated microbiome exist in a state of super-charged symbiosis [82]. Host factors influence the microbiome as the microbiome is concurrently exerting influence upon the host [43, 62, 82]. The microbiome is critical to nutrient processing [45], immune system development [62], hormonal regulation [46], and myriad other aspects critical to the maintenance of human health. The study and understanding of the human microbiome, its constituents and consequences, and the potential for its change and manipulation, is of incredible importance and impact to human health.

Methane and Methanogens in the Human Gastrointestinal Tract

As mentioned previously, methane is also produced in the human gut [24]. This methane is detectable in some individuals via breathalyzer [83]. However, there are no known methane synthesis pathways in human cells. Therefore, the human microbiome is essentially the only other potential source of endogenous methane production. This is also true to ruminants, which are well-known sources of methane despite lacking any methane synthesis pathways in and of themselves [84]. The majority of the human microbiome is contained within the colon, which harbors a wide range of metabolites and a large array of bacterial, archaeal, and fungal diversity [72]. These organisms metabolize the milieu of carbohydrates, amino acids, fatty acids, and other compounds that are introduced into the colon through the diet and metabolism of the human host. The wide variety of microbial metabolisms present within the human gastrointestinal tract lead to a correspondingly vast array of metabolites produced by the human microbiome [72]. Accordingly, many of the end products of the trophic cascade in the human gut microbiome are relevant to methanogenesis. Carbon dioxide, produced by the human host and the gut microbiome, as well as copious amounts of hydrogen provide substrates for hydrogenotrophic methanogenesis [85]. Aceticlastic methanogenesis is also supported by the many fermentation pathways present in the colon [30]. Additionally, substrates for methylotrophic methanogenesis are abundant. Trimethylamine (TMA), Trimethylamine-N-Oxide (TMAO), methylamine (MeA), and methanol are all available to facilitate methylotrophic methanogenesis [30]. Despite this, few methylotrophic methanogens have been isolated from human gut samples, and those that have are estimated to exist in low abundance within the community [86, 87]. In contrast, hydrogenotrophic methanogens and aceticlastic methanogens

have been shown to comprise a larger portion of the microbial community within the human gastrointestinal tract [3].

The effects of methane on human health and disease constitute a developing area of research. Although it is clear that many individuals produce detectable levels of methane and that the overwhelming majority of people have methanogens in their gut, the effects of the methane that is produced on overall human health are understudied. Methane production at a level detectable by breathalyzer has been used in clinical diagnostics, having been shown to be useful in diagnosing small intestinal bacterial overgrowth [88]. Detectable levels of methane production have also been shown to decrease intestinal motility, and have accordingly been associated with constipation-related irritable bowel disease [83]. Methane production has otherwise been shown to decrease inflammation, and to attenuate the production of pro-inflammatory cytokines [88]. Importantly, the absence of methanogens can also be an indicator of dysbiosis-related disease, as has been shown in cases of severe malnutrition [89], where it was found that malnourished individuals also had a higher redox potential in their gastrointestinal systems. Endogenous methane production has also been implicated in colorectal cancer and obesity [90]. Although understudied, it is clear that methane production does have important impacts on host health and well-being and could be used as a diagnostic marker for the development of certain diseases.

The excretion pathways of methane from the body are twofold, with methane being expelled from the body through flatus, through exhalation, or both [24, 91]. Some sources claim that flatus accounts for most methane released from the body [24], while others claim it to be exhalation [90, 91]. Regardless of expulsion pathway, methane produced in the gastrointestinal

tract is relatively inert physiologically. It does not interact with host physiology in the ways that other gases such as nitrous oxide or hydrogen sulfide do [92]. Methane that is detectable via breathalyzer would have most likely been produced by the gut microbiome before entering the bloodstream and being subsequently exhaled [91].

The two most dominant methanogens in the human gut are *Methanobrevibacter smithii* (*M. smithii*) and *Methanosphaera stadmansii* (*M. stadmansii*), which produce methane hydrogenotrophically and methanotrophically, respectively [30]. Considering that *M. smithii* has been sequenced from stool samples of as many as 95.7% of participants in some studies [3], and *M. stadmansii* from 29.4% of those same participants [3], it is likely that the distribution of these archaeal methanogens is widespread and accounts for the majority of methane produced by the human gut microbiome. However, neither of these methanogens are able to utilize MeA or TMA to produce methane, relying instead upon hydrogen or methanol [30, 93]. Some studies have implicated certain *Methanomasssicoccus* as carrying the potential to metabolize methylated amines, but it is unlikely that these species are highly prevalent or abundant in the human gastrointestinal tract [30].

It should also be noted that the amount of methane produced by those individuals who do produce methane is significantly less than that produced in the ruminant gut. In fact, methane production in ruminants accounts for 96% of the methane emitted by gut fermentation globally [84]. Methane produced by humans is simply dwarfed by the amount that is produced by ruminants.

Production of Trimethylamine and Methylamine in the Human Gastrointestinal Tract

Methylated amines in the human gut are produced from dietary compounds metabolized first into L-carnitine or choline, then secondarily into TMA [94]. Although the substrates choline and L-carnitine are a product of host diet, conversion into trimethylamine from these substrates is exclusively a product of the microbiome and can happen both anaerobically and aerobically [94, 95].

Since the human host can produce TMAO from TMA, but not TMA itself, the microbiome plays key roles in the amount of TMAO present in a given individual. It is therefore important to understand the mechanisms by which methylated amines are produced, metabolized, and excreted by the human host and/or the human gut microbiome. There exist two primary metabolic pathways through which TMA, and subsequently TMAO or MeA, is produced by the human gut microbiome. The first, mediated by choline TMA-lyase (*cutC*), synthesizes TMA using choline as a metabolic precursor. The second, carnitine oxygenase (*cntA*), instead utilizes L-carnitine as a metabolic precursor to produce TMA [94, 96].

Additionally, a third potential pathway has been described, but was generally shown to possess a high degree of sequence similarity with *cntA* and has therefore been considered by some to belong to the same enzyme cluster as *cntA* [94]. Importantly, the metabolic potential to produce TMA (*cntA/cutC*) is widely distributed in the gut microbiomes of human hosts. One study that developed gene databases for the *cutC* and *cntA* enzyme clusters found fecal samples from each studied individual (N=50) contained the potential to produce TMA from choline, while 26% of those individuals also showed the potential to produce TMA from L-carnitine [94]. Each of these pathways was only represented in a minority of the microbial community. Yet, if this

sampling is indicative of TMA-producing potential, it can be inferred that the links between TMA and TMAO production and atherosclerosis [94-96] do not rely upon a large dosage of TMA or TMAO to be seen. These metabolic pathways are also putatively present in a wide range of taxa. Choline-dependent cutC has been shown to be present in *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, generally in anaerobic bacteria. Conversely, L-carnitine dependent cntA has been shown to be present in *Gammaproteobacteria*, *Betaproteobacteria*, and some *Firmicutes* [94, 96], being more commonly associated with obligate and facultatively aerobic metabolisms [94]. The ability of the host to produce TMAO from TMA is likely to be dependent on the gut microbiome. It has been shown using murine models that the absence of a microbiome leads to complete cessation of TMAO production, which was subsequently restored upon inoculation of those same mice with a “conventional” microbiome [97].

Importance of the Potential for Aerobic Methane Synthesis in the Human Gut

The concept of aerobic bacterial methane synthesis carries the potential to impact human health on a couple of different axes. Aerobic bacterial methane synthesis has the potential to affect the level of TMA, and therefore the level of circulating TMAO in the body. Additionally, it may also play a role in maintaining the anaerobicity of the colon, which is associated with dysbiosis if disrupted [98].

Once synthesized, TMA can either be further metabolized into MeA, or it can be absorbed by the intestinal epithelium and then be subsequently metabolized in the liver by flavin monooxygenase-3 [99] to trimethylamine-N-oxide (TMAO), a compound whose presence has been associated with atherosclerosis and cardiovascular disease [94, 95].

This association between TMAO and atherosclerosis is not insignificant. Levels of TMAO in the blood have been previously described as an independent risk factor for predicting the development of atherosclerosis [100], with the correlation having been shown to be dose-dependent [101]. The mechanisms by which TMAO can effect the development of atherosclerosis are numerous. The presence of TMAO has been shown to activate pro-inflammatory cytokines and activate inflammasomes [102, 103], as well as increasing oxidative stress at endothelial barriers [100]. Inflammation and oxidative stress play key mechanistic roles in the development of atherosclerosis [104, 105]. This activation of inflammatory pathways also leads TMAO to enhance the development of atherosclerotic plaques [100]. Additionally, TMAO has been shown to promote calcification of smooth vascular tissue. This was shown both in *in-vitro* and in rat models [106]. Calcification of vascular tissue, particularly in the coronary artery, is a known risk-factor for cardiovascular disease [107]. Given the effects of TMAO on the human host, it is unsurprising that high levels of TMAO, a molecule which enhances inflammation through multiple mechanisms at endothelial layers of vascular tissue, constitutes a risk factor for the development of atherosclerosis. Accordingly, microbial metabolisms capable of metabolizing TMA into methane would reduce the production of TMAO by host liver enzymes. The presence of MeA-based aerobic bacterial methane synthesis could therefore function to lessen the likelihood of TMAO-induced atherosclerotic development.

The human gut microbiome, as mentioned previously, is not a static entity. Nowhere is this more apparent than in the development of a nascent microbiome in neonates. The neonatal gut microbiome is influenced by far fewer factors, and is initially far simpler, than the adult microbiome [108, 109]. This neonatal microbiome is initially colonized by facultative anaerobes

and aerotolerant anaerobes [110], taking advantage of an uncolonized, oxygenated neonatal gastrointestinal tract [109]. Colonization by facultative anaerobes reduces the oxygen content of the neonatal gut, opening up niche space for *Bifidobacterium* and *Clostridium* [111, 112]. Importantly, this initial colonization of facultative anaerobes that subsequently create an anoxic environment is critical; never again throughout the life of an individual will their colon be a highly oxygenated environment [109, 112]. The anaerobic environment of the colon is therefore likely to be of incredible importance in maintaining a healthy microbiome. Irritable bowel disease (IBD), for example, has been shown to display a decrease in abundance of *Faecalibacterium prausnitzii*, an anaerobe, coupled with an according increase in facultative anaerobes of the family *Enterobacteriaceae* [98, 110, 113]. Although the causality between dysbiosis and IBD is unclear, nevertheless an association is evident between increased colonic aerobicity, dysbiosis, and IBD [114]. Coupled with the fact that the majority of a healthy colonic microbiome is composed of anaerobes [115], the maintenance of anaerobic conditions in the colon is essential to cultivating a healthy gut microbiome. The potential for consumption of oxygen in the metabolism of colon-associated bacteria capable of producing biogenic methane under aerobic conditions could therefore be important in the maintenance of colonic anaerobicity. Such a metabolism has the potential to play roles in maintaining a healthy colonic microbiome through the maintenance of its anaerobic environment.

HYPOTHESIS AND APPROACH

In view of the finding that there exist bacteria which can metabolize methylated amines such as TMA and MeA to produce methane under aerobic conditions [10], and given that those same methylated amines are present in the human gut, this study sought to investigate the possibility that similar metabolic processes as recently described in Yellowstone Lake could also be found in the human gut microbiome. Bacteria from the oral microbiome which are also common to the human gut microbiome, such as some *Bacillus* sp. and *Klebsiella* sp., have already been shown to grow on C1 compounds such as MeA [116]. This indicates further that the human gastrointestinal tract may harbor bacteria that can metabolize MeA, potentially to produce biogenic methane, a process which would act as a sink for TMA in the human metabolome. This is of interest to human health as TMA and associated TMAO production has been previously linked to the development of atherosclerosis, despite the microbial mechanisms underlying TMA production being only a minor part of the human gut microbiome [95]. Therefore, understanding a potential sink for TMA, that being the production of methane from TMA and its subsequent metabolites, especially MeA, may carry important implications for impeding the development of atherosclerosis. Additionally, with methane production having been associated with a number of conditions, understanding and characterizing sources of endogenous methane production carries important implications for human health. This study hypothesizes that bacteria in the human gastrointestinal tract metabolize methylamine to produce biogenic methane using oxygen available along the gut mucosa.

In order to evaluate this hypothesis, whole fecal slurries collected in Martinson, et al. [1] were first cultured in a defined, nitrogen-limited media within a sealed aerobic environment.

The extent to which these fecal slurries produced methane was evaluated using gas chromatography. Those fecal slurries showing increased methane production were then serially diluted and plated on the same enrichment media used to culture the whole fecal slurry. Colonies were picked and isolated, then screened for their potential ability to utilize methylamine as a growth substrate. Those isolates that displayed a potential ability to utilize MeA were then cultivated aerobically in sealed serum bottles, and the amount of methane produced was evaluated using gas chromatography. Growth curves were next performed on those isolates capable of producing methane from methylamine in an attempt to show MeA utilization of the isolates. A high-throughput, tetrazolium-based colorimetric assay was then developed to screen a large number of isolates for methylamine utilization. Isolates that were investigated in this way also had their genomes sequenced via sanger sequencing.

METHODS

Sample Selection

Six fecal samples used in a previously published study [1] were selected for this study. Although designated numerically in Martinson, Pinkham, et al., the six fecal samples were designated here P103, P121, P721, P420, P801, and P802. These samples were stored in cryogenic vials continuously at -80°C since the completion of Martinson, Pinkham, et al. Each sample was originally collected and processed according to the protocol described in Martinson, Pinkham, et al. Each whole fecal sample was stored after processing under anaerobic conditions and was re-opened for this study under anaerobic conditions. Although collected for use in Martinson, Pinkham, et al., the samples chosen for this study were not used, and were therefore simply collected and stored until the beginning of this study. The six samples used in this study were chosen randomly from a cohort of nine individuals comprising both males (6) and females (4). The individuals corresponding to the samples used in this study were between 25 and 45 years of age. Overall health characteristics were not surveyed [1].

Evaluation of Methane Production

Either whole fecal slurries or individual isolates were cultivated in 50ml. serum bottles stoppered with bromo-butyl rubber stoppers sealed with aluminum crimp caps to prevent gas exchange between the cultures and the environment. To evaluate the gas contents of the headspace of the serum bottles, 10ml of air from the surrounding room was first injected into the serum bottle through a filtered 23-gauge, 1 inch needle and 10ml syringe. The bottles were then shaken before 1ml of the headspace was transferred into a 10ml gas collection vial. The

collection vial was also stoppered with a bromo-butyl rubber stopper sealed with an aluminum crimp cap, and the transfer was performed with a sterile needle and syringe. These samples were then injected into an Agilent 7890 GC–MS gas chromatograph. The GC-MS had a Carboxen-1010 porouslayer open-tubular capillary GC column, set up in splitless mode. The 0.8mL of headspace sample was injected at 200°C, the ion source was set to 230°C. The carrier gas used was helium. Column flow rate was set to 1.5 mL/min. The oven was heated to 250°C at a rate of 25°C per minute after being set to 35°C for 1.5 minutes. That oven was held at 180°C for 1 minute and then at 250°C for 3.5 minutes. The mass spectrometer was run in scan/selected ion monitoring mode with a scan range of 10-100 m/z. This was used for scan/selected ion monitoring m/z 14, 15, 16, and 17.

Cultivation, Enrichment, and Isolation

Fecal samples and isolates were cultivated on a modified SAR11 media [117] containing 0.4% w/v glucose as a sole carbon source, 100 micromoles of potassium phosphate, and with the omission of sodium chloride, ammonium sulfate, and sodium biphosphate to form what was termed throughout this study as the “basal media.” Notably, this basal media lacks a nitrogen source. Accordingly, 10 millimoles of methylamine hydrochloride were added to the basal media (basal+MeA). In some cases, 10 millimoles of ammonium sulfate were added instead of 10 millimoles of methylamine hydrochloride. In other cases, both ammonium sulfate and methylamine hydrochloride were omitted. Additionally, 5% volume/volume lysogeny broth (LB) was added to the initial screening cultures, growth curves, and to cultures of individual isolates used to evaluate methane production. Throughout the development of the colorimetric assays, 0.5% v/v LB was added instead of 5% v/v LB. This was done in order to avoid masking

potential effects of MeA utilization in growth. The sensitivity of the colorimetric assay allowed for the change in LB concentration, despite overall growth metrics (optical density, fluorescence) being lessened as a result. Since SAR11 media was initially developed to cultivate oligotrophic isolates from marine environments [117], LB was added to account for the richer nutrient environment that isolates from fecal samples were presumably adapted to grow within. Enrichment cultures were grown under aerobic conditions at 37°C with vigorous shaking to maintain aerobicity.

Isolation of individual bacteria from whole fecal samples was accomplished by plating serial dilutions of the whole fecal samples initially grown in liquid basal+MeA (no LB) on plates made with basal+MeA and 1.5% w/v agar powder, also without LB. Individual colonies were differentiated based on their shape, size, opacity, and color. Each differing colony was then picked and individually plated. The process of identifying different colonies, picking, and then plating them was performed until all of the colonies on a single plate were identical, at which point the colonies on one plate containing only colonies with identical morphologies was considered as a single isolate.

Whole fecal slurries were also cultivated anaerobically in the basal media containing MeA. The basal media was made to be anaerobic by allowing the finished media to equilibrate in an anaerobe chamber for three days, thus allowing for the evacuation of any oxygen in the media. Whole fecal slurries, opened under anaerobic conditions, were cultured in a 50ml serum bottle containing basal media+MeA only after three days of equilibration of the media with an anaerobic environment. The serum bottles were stoppered with a bromo-butyl rubber stopper. The anaerobic environment was generated within a Coy Laboratories Airlock anaerobe chamber using a gas

environment containing 85% Nitrogen, 10% CO₂, and 5% Hydrogen. This gas environment was created using a commercially available pre-mixed gas mixture.

Initial Isolate Screening

Initial screening was performed using 14 ml. falcon-type tubes. Colonies from a plate containing a single isolate were picked and grown in basal media containing 5% v/v LB and MeA. Those cultures were grown overnight at 37°C under aerobic conditions and with vigorous shaking. From those overnight cultures, 50µl, was transferred to fresh tubes containing 5 ml basal media with 5% v/v LB, both with and without 10 mM MeA. These tubes were also incubated at 37°C with vigorous shaking to maintain aerobicity. After 24 hours, the cultures were removed from the incubator and shaken lightly by hand before reading the optical density of 1ml of each culture at 600 nM absorbance. An Amersham Biosciences Ultrospec 2100 pro spectrophotometer was blanked with water and used to take optical density readings. Those isolates that showed increased optical density when MeA was added to the basal media (with 5% LB) compared to when MeA was absent (also with 5% LB) were suspected of being able to utilize MeA to generate biomass and were therefore selected for further study. Isolates that failed to show increased optical density with the addition of MeA at this point were excluded from further study.

Optical Density Growth Curves

Growth curves were performed on those isolates that indicated from the initial screening process the potential for MeA utilization. As in the initial screening procedure, colonies were picked and grown in an overnight culture containing the basal media, 5% v/v LB, and MeA.

Overnight cultures were grown at 37°C under aerobic conditions and were subjected to vigorous shaking. From those overnight cultures, 150 µl was transferred to fresh 14ml falcon-type tubes containing 9 ml basal media with 5% v/v LB, both with and without 10 mM MeA. These cultures were also grown at 37°C under aerobic conditions with vigorous shaking. Growth curves were performed in triplicate. Growth was evaluated at every time point by measuring the optical density at 595 nanometers absorbance of 1ml. of culture using an Amersham Biosciences Ultrospec 2100 pro spectrophotometer which was blanked using water.

Characterization of the MTS Assay

In order to effectively screen a larger number of isolates concurrently, the Promega CellTiter 96® AQueous One colorimetric assay (MTS reagent) was chosen. It constituted a fluorescence-based approach wherein a tetrazolium-based reagent reacts with reducing agents produced by cells in culture.

The assay was first characterized with the fecal isolates under this study's growth conditions to ensure a strong relationship between fluorescence and growth, as well as to evaluate its overall accuracy and consistency. Initial work for the characterization of the colorimetric assay was performed by culturing an isolate with 5ml of media in a 50ml Erlenmeyer flask. The flasks were subjected to vigorous shaking under aerobic conditions in an incubator at 37°C for the duration of the culture period. At each time point that a culture was evaluated, the flasks were removed from the incubator and 15ul. of culture was transferred to a 96 well plate. The 96 well plate was then promptly placed into a 4°C refrigerator and the flasks were returned to the incubator. After the culture period was complete, 135ul of a mixture of 10% v/v MTS reagent and 90% v/v warmed (37°C) LB was added to each culture-containing well in

the 96 well plate. The 96 well plate was then transferred into a BioLog OmniLog plate reader that was programmed read fluorescence at 400nm in 30-minute intervals for four hours, recording the progress of the reaction between the MTS reagent and reducing agents produced by the cells in culture. The plate reader was set at 37°C and programmed to shake linearly in perpetuity for the duration of the reaction.

Cultures were prepared for a given MTS assay by first picking a colony of the isolate to be evaluated and growing it overnight in a 14ml falcon-type tube containing 2-3ml of basal media with 10mM MeA and 0.5% v/v LB. From this overnight culture, 2ml was transferred to a microcentrifuge tube and pelleted by centrifugation at 5.5xG for 5 minutes. It was then resuspended in 1X PBS and diluted to an optical density between 1.4 and 1.6. The optical density-adjusted suspension was then diluted by a dilution factor of ten or one hundred, depending on the particular experiment, by adding the appropriate amount of optical density-adjusted suspension into an appropriate amount of culture media.

Development of Isolate Screening Protocol

In order to gain the potential to screen a large number of human gut isolates, a high-throughput screening method was needed. Instead of screening cultures for MeA utilization via growth in falcon-type tubes measured by optical density, an approach similar to that used to characterize the MTS assay was chosen, with the main difference being that isolates were grown in a 24 well plate instead of in an Erlenmeyer flask. Growth was evaluated using a tetrazolium-based Promega CellTiter 96® AQueous One Solution (MTS reagent). Isolates were grown in 24-well plates containing 2ml of volume per well, termed the growth plates. Each well in the plate contained 750 µl of basal media +0.5% LB, basal media +10nMMeA and 0.5% LB, or only

LB as a positive control. The growth plate was kept within a BioLog OminLog, set to shake linearly at 37°C in order to facilitate the growth of each mutant being screened. As each culture was growing in the 96 well growth plate, at each time point that growth was to be evaluated, 15 microliters of culture were transferred to a new 96 well plate, termed the test plate, and stored in the refrigerator in order to arrest growth at that time point. After the 30-hour growth period, each culture in the test plate had 135 microliters of a 10:1 mixture of warmed (37°C) LB and Promega CellTiter 96® AQueous One Solution added to it before being placed into a BioLog OmniLog plate reader for processing. The BioLog OmniLog was set to shake linearly for four hours at 37°C, with fluorescent reads at 400nm absorbance occurring every 30 minutes. The resulting fluorescence data was then plotted using PRISM graphing software. The fluorescence data produced after three hours of reaction time in the test plate was used to evaluate the results.

DNA Extraction, Processing, and Analysis

Genomic DNA from a given fecal isolate was extracted from overnight cultures grown in LB under aerobic conditions using the Invitrogen EasyDNA extraction kit, with the following modifications. For isolate P121-2c, each step was followed according to the protocol, with the exception that 70% ethanol was used throughout the protocol where ethanol was needed as a reagent. For isolate P103-3c the protocol was modified to incubate samples at 65°C for twenty minutes, vortexing them on high for 10 seconds every two minutes. Additionally, P103-3c was allowed to incubate on ice in a 4°C refrigerator for 1 hour with 70% ethanol instead of the prescribed 30 minutes with 100% ethanol. In the extraction of both isolates, the final step of the protocol was changed to resuspend the DNA in Qiagen C9 buffer instead of the provided TE buffer, in order to facilitate PCR and sanger sequencing which would have otherwise been

inhibited by the use of the provided TE buffer. The genomic DNA was then amplified using an 8F/1492R primer pair [118] that targeted the 16s gene. Amplification of DNA was performed in an Eppendorf Mastercycler thermocycler. A 30 second denaturation step was performed at 95°C, a 30 second annealing step was performed at 49°C, and a 2-minute extension step was performed at 72°C, collectively constituting one amplification cycle. A total of 35 amplification cycles were performed for each PCR reaction. Amplification products were then kept at 4°C for further use. To sequence the DNA, the amplified PCR product was purified using a Qiagen QIAquick PCR purification kit, with each step being performed according to the provided protocol. The purified 16s DNA was sent to Azenta life sciences to be read via sanger sequencing. The forward and reverse reads were aligned using BLAST. Contigs generated from alignment were then blasted against the whole NCBI nucleotide database in order to establish the identity of a given fecal isolate.

Statistical Testing and Analysis

Paired T-tests were performed on the data presented in figure 10 with a 95% confidence interval. Unpaired T-tests were performed on the data in figures 3, 6, and 7, also with a 95% confidence interval. Since the variance in the tested data sets in figures 3, 6, and 7 was large (ratio of mean variance >2) Welch's correction was employed in the unpaired T-tests. A linear regression was performed upon data sets A and B presented in figure 13, and a Pearson correlation was used to evaluate the degree of interdependency between CFU/ml and MTS assay fluorescence in that figure, with the statistical significance of that interdependency being evaluated using a 95% confidence interval.

RESULTS

Aerobic and Anaerobic Methane Production in Whole Fecal Slurries

Whole fecal slurries from each participant were cultivated in sealed serum bottles, and the headspace of each culture was analyzed for CH₄ production after 24 hours via GC-mass spec. Fecal slurries from participants P103 and P121 both clearly produced excess methane when cultured in basal media containing MeA as the sole nitrogen source, as compared to the methane produced when those participants were cultured with ammonia as the only nitrogen source. The other four isolates, P420, P721, P802, and P801, did not show a meaningful increase in methane production when grown in media containing MeA (fig. 3). This experiment was reproduced with the P103 whole fecal slurry with the addition of 2-bromoethanesulfonate in order to inhibit McrA gene activity [119]. When the P103 fecal slurry was cultivated in this way, it again showed an increase in methane production when MeA was added to the media compared with when ammonia was the sole nitrogen source in the basal media (fig. 4). When the P121 fecal slurry was cultivated in this way, it only demonstrated an increase in methane production in the presence of MeA after 3 days of culturing. It should be noted that in the case of both P103 and P121 whole fecal slurries lacking 2-BES, the apparent increase in methane production was not calculated to be statistically significant.

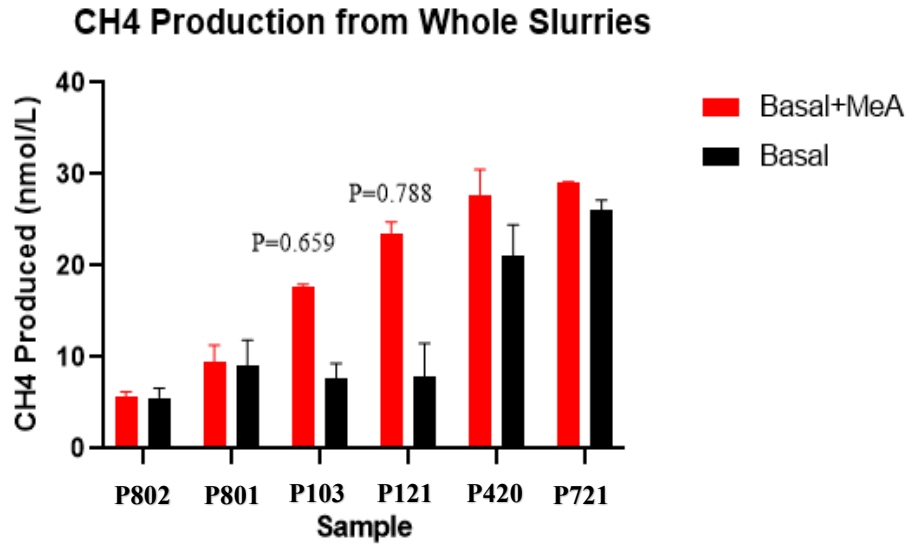


Figure 3: Methane measured in whole fecal slurries chosen from Martinson, et. Al. [1] and cultured aerobically in the basal media with and without MeA. Neither culture condition contained LB. Methane was measured from the headspace of a 50ml serum bottle via gas chromatography. Gas samples of the headspace were taken at T=0hr. and T=24hr. Data shown represents CH₄ measured at T=24hr. in excess of the CH₄ produced at T=0hr. The displayed P values reflect an unpaired T-test performed between a given slurry's two growth conditions using Welch's correction with a 95% confidence interval.

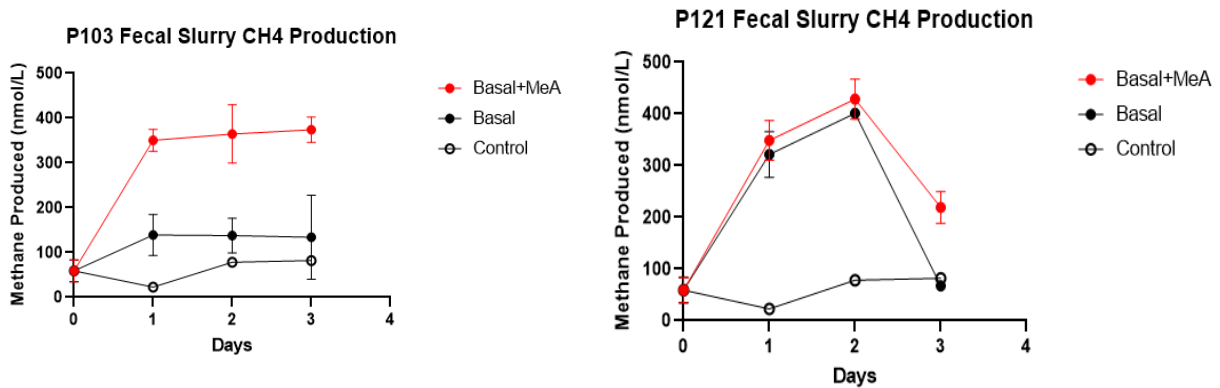


Figure 4: P103-3c and P121-2c cultured in basal media both with and without MeA. This culturing was performed for 72 hours within a sealed 50ml. serum bottle under aerobic conditions with the addition of 2-bromoethanesulfonate. Gas from the headspace of each culture was analyzed via gas chromatography every 24 hours for 72 hours.

Whole fecal slurries from each individual were also grown anaerobically in basal media containing MeA and no LB. Media was made to be anaerobic as described in the methods section. Methane production was evaluated after 24 hours using GC-mass spec as outlined in the methods section. Whole fecal slurries P801 (551 nmol/L), P802 (148 nmol/L), and P103 (179 nmol/L) did not produce large amounts of methane. However, slurries P121 (1837 nmol/L), P420 (3428 nmol/L), and P721 (1612 nmol/L) did (fig. 6). It should be noted that methane from the P103 fecal slurry grown in aerobic conditions was fully 9.5% of the methane produced when that fecal slurry was cultivated under anaerobic conditions. No other whole fecal slurry produced methane under aerobic conditions in amounts that were remotely comparable to the methane produced under anaerobic conditions.

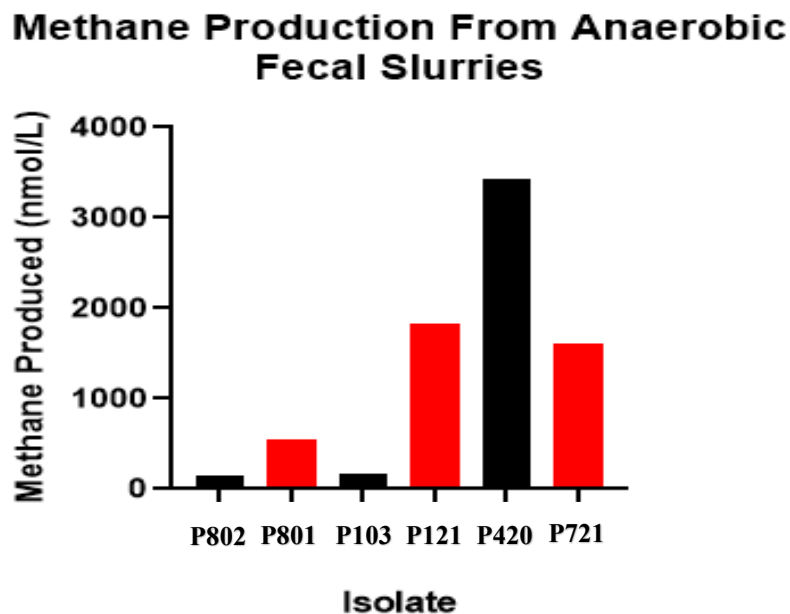


Figure 5: Methane production of whole fecal slurries cultivated in the basal media under anaerobic conditions from each individual in the study. Methane production was evaluated from the headspace of a sealed serum bottle after 24 hours culture using gas chromatography.

Differential Growth of Fecal Isolates in the Presence of MeA

Since two fecal slurries (P103 and P121) demonstrated increased methane production with MeA as a nitrogen source, those fecal slurries were then serially diluted and grown as enrichment cultures using basal media containing MeA. Each distinct colony from the enrichment cultures was then isolated and grown individually on the same enrichment media. The isolates from each enrichment culture were then screened for their ability to use methylamine as outlined in the methods section. In total, nine isolates from P103 and five isolates from P121 showed higher optical density (OD) after 24 hours of culture when grown in basal media containing MeA than in basal media lacking MeA. Notably, each P103 isolate (-1abc, -2abc, -3abc) showed clearly increased OD after culturing (fig. 6). Of those P103 isolates, P103-3C displayed the greatest difference in optical density when grown with MeA than without MeA. The increase in OD observed in each screened isolate was determined to be statistically significant within a 95% confidence interval. P121 isolates -2abc and -3bc demonstrated increased optical density after 24 hours of growth when grown with MeA than without MeA (fig. 7). Of those isolates, P121-2a, -2b, and -2c showed the greatest optical density difference when grown with MeA than without MeA and did so in comparable amounts. Additionally, the increase in optical density observed in P121 isolates -2abc and -3bc were each found to be statistically significant.

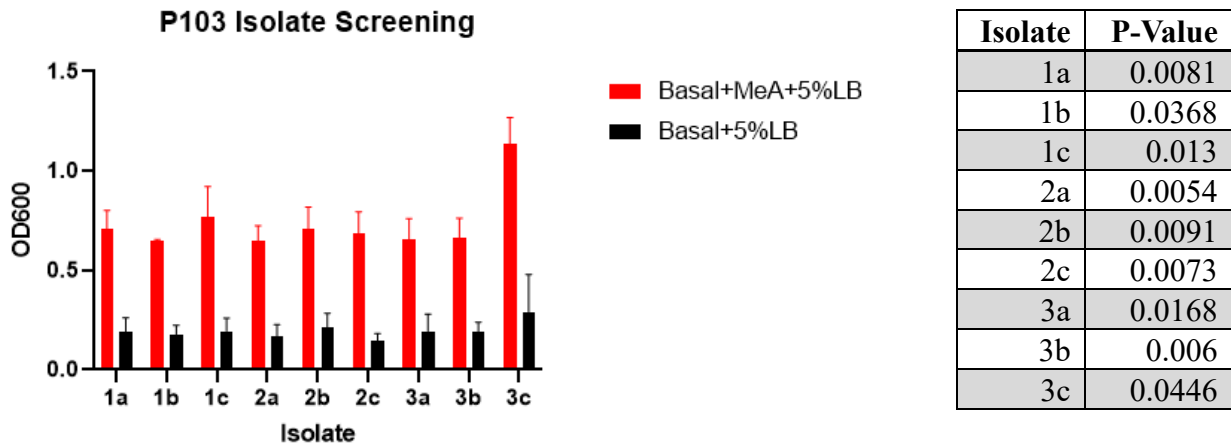


Figure 6: Optical density measurements from P103 isolates. Each culture was evaluated for growth using OD after 24 hours of growth in the basal media both with and without MeA. The displayed P values reflect an unpaired T-test performed between a given isolate's two growth conditions using Welch's correction with a 95% confidence interval.

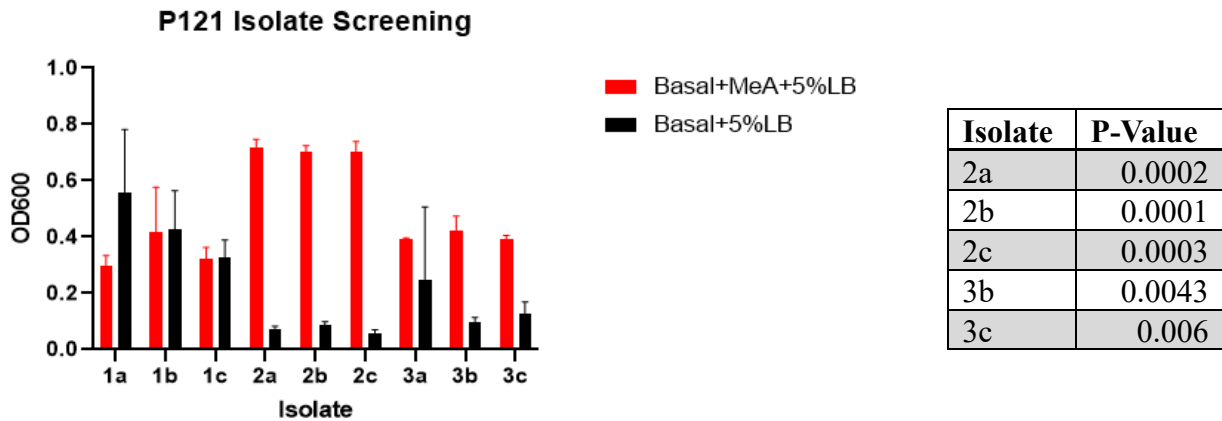


Figure 7: Optical density measurements from P121 isolates. Each culture was evaluated for growth using OD after 24 hours of growth in the basal media both with and without MeA. The displayed P values reflect an unpaired T-test performed between a given isolate's two growth conditions using Welch's correction with a 95% confidence interval.

Growth curves were then performed on five of the isolates, P103-3c, P121-2abc, and P121-3c, as they displayed the largest OD differences. Each test culture was set up in triplicate and growth curves were performed as described in the methods section. The optical density of

each test culture was evaluated at two-hour time intervals for a total growth time of 14 hours, except for P103-3c, which was evaluated over 18 hours of growth. Each of the cultures that showed the largest OD differences from screening produced more robust growth over the course of a growth curve, echoing the results from the screening cultures (fig. 8 & 9). The isolate P103-3c, consistent with the screening cultures, again showed the single largest OD difference out of all the isolates upon which growth curves were performed. The isolates P121-2a and -2b reached an optical density of between 0.6 and 0.8, matching what was seen in the screening cultures (fig. 7 & 8). The other P121 isolates, P121-2c and P121-3c, reached an optical density around 0.5 and 0.6, respectively. These results are different than the optical density results obtained from the screening cultures, displaying a higher (P121-3c) and lower (P121-2c) value (fig. 7 & 8).

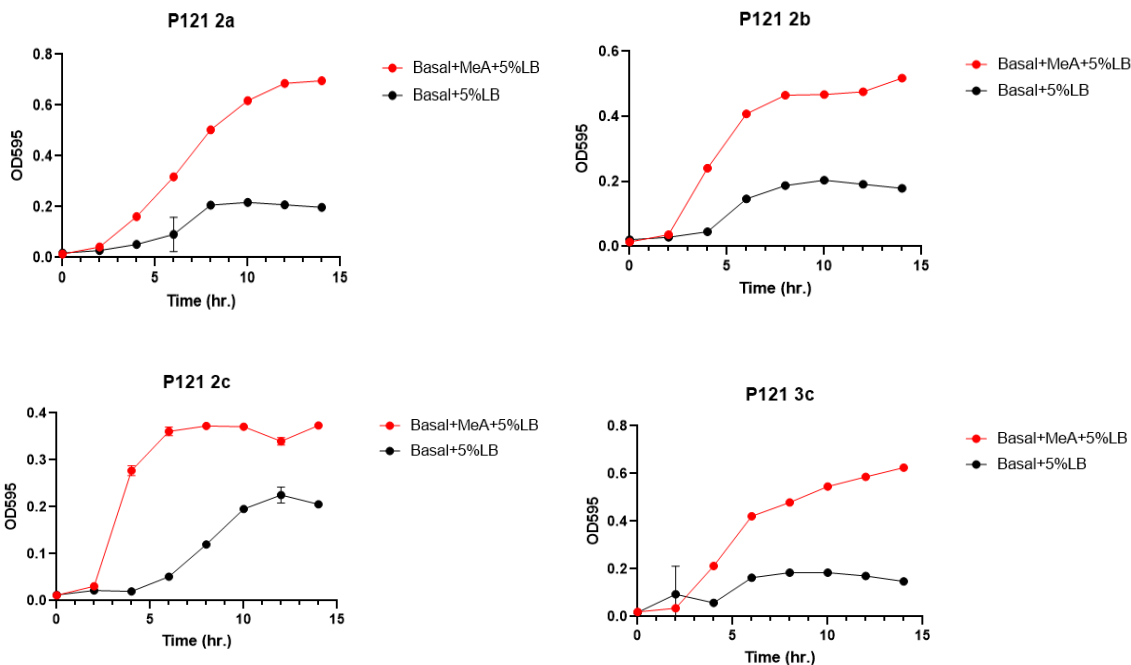


Figure 8: Growth curves performed on four isolates from the P121 fecal slurry. Isolates were selected for a growth curve based on their screening results. Growth was evaluated every 2 hours for fourteen total hours via optical density at 595nm absorbance.

The difference in optical density seen in the screening culture for P103-3c was also matched in a growth curve. This isolate also displayed the largest overall optical density, reaching an OD of 1.70 (fig. 9). This OD measurement eclipses that of the screening data for P103-3c, which yielded an OD of 1.16. It should also be noted that optical density numbers in the growth condition without MeA were higher than the growth condition with MeA until the tenth hour of growth, at which point the trend reversed for the remainder of the growth curve.

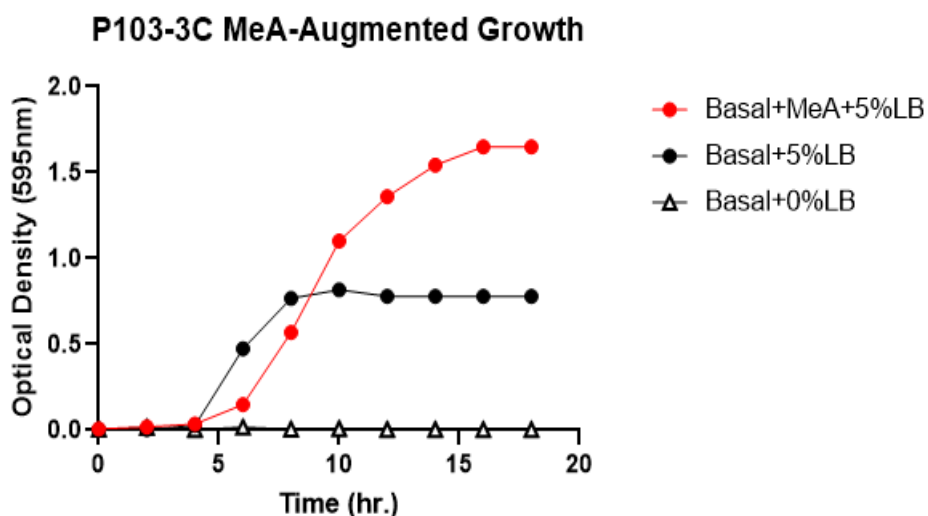


Figure 9: Growth curve performed on P103 isolate 3c. Growth was evaluated via optical density at 595nm absorbance every two hours for eighteen total hours. Error bars are depicted but are too small to be seen beneath the symbols.

Aerobic Methane Production by Selected Fecal Isolates

Mimicking the experiment done with whole fecal slurries, P103 isolates were cultured for 24 hours in a sealed serum bottle containing the basal media with MeA and 5% v/v LB. Gas in the headspace was then analyzed using gas chromatography. A control condition was also set up, omitting the MeA from the culture, leaving LB as the only nitrogen source in the media. It was

found that P103-3c, P103-3b, and P103-2c produced more methane after 24 hours of culture when MeA was added to the basal media, but not when it was omitted. In order to test the statistical significance of the observed increase in methane production from P103-2c, 3b, and 3c, separate paired T-tests were performed between the methane in the headspace of each isolate's serum bottles at T=0hr and T=24hr. (n=2). The p-values generated reflect a 95% confidence interval from a paired T test performed between the methane data at 0hr. and 24hr. It was found that the observed increase in methane production of isolates P103-2c and -3c were statistically significant. The observed increase in methane production in P103-3b was not found to be statistically significant.

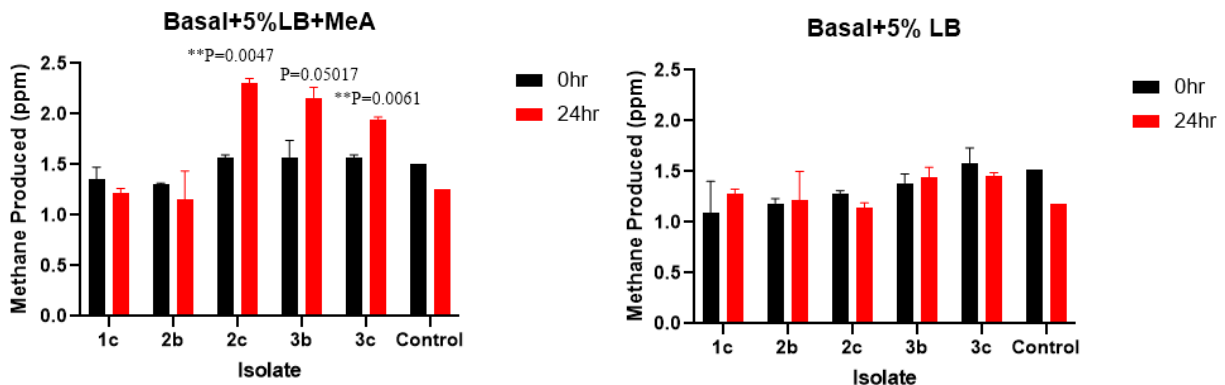


Figure 10: Methane measured in the headspace of sealed serum bottles at T=0hr. and T=24hr. Individual isolates from the P103 fecal slurry were cultivated in the basal media containing 5% v/v LB, both with and without MeA. Growth of these isolates was not evaluated in this experiment, but rather only the methane produced in the headspace of each isolate.

Characterization of the MTS Assay

Although it was clear that certain isolates were able to both utilize MeA and produce methane when it was present, it was also clear that screening cultures in flasks or falcon tubes was an inefficient method to test isolates for their potential to produce methane from MeA.

Although screening cultures in falcon tubes is effective on a small scale, it would not be a feasible way to screen large amounts of human gut isolates for future experiments. Therefore, colorimetric-based assay to screen for MeA utilization was developed, a process that would allow the screening of many isolates concurrently. Before using the colorimetric (MTS) assay however, it had to be characterized in order to evaluate its consistency and accuracy in measuring growth via fluorescence. Additionally, it had to be demonstrated that the same differences in growth with MeA compared to that without MeA could also be demonstrated by this approach.

To initially characterize the colorimetric assay, the isolate P103-3c, chosen because of its significant methane production and large growth advantage when grown with MEA, was cultured in both LB (n=4) and PBS (n=4). Inoculated cultures were run in triplicate. P103-3c was cultivated at an initial dilution of 10X. Samples were taken every hour for twelve hours in order to effectively demonstrate the relatively rapid growth of P103-3c in LB. As expected, the colorimetric assay showed a large amount of fluorescence from the samples cultured in LB, and relatively little from uninoculated controls or from samples cultured in PBS (fig. 11). Importantly, the fluorescence shown from the LB cultures appeared to contain a lag phase, log phase, and the beginning of a stationary phase, mimicking a classical growth curve pattern. Additionally, the assay demonstrated a large degree of consistency, suggested by diminutive error bars present in this initial round of culturing. Some background fluorescence was consistently seen from uninoculated cultures. This background fluorescence was observed consistently and at the same magnitude in every MTS assay performed.

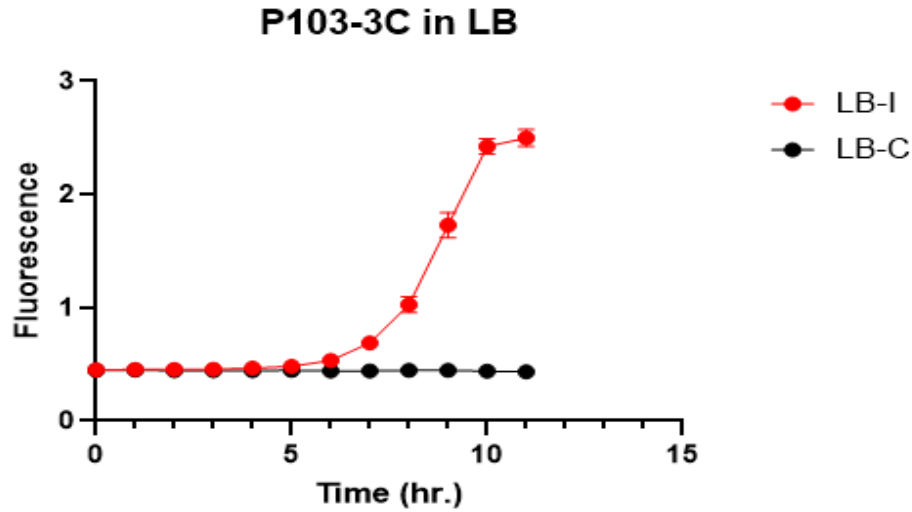


Figure 11: MTS assay using P103-3c grown in LB. Growth was evaluated every two hours for twelve total hours. The line labeled LB-I represents an inoculated test condition, while the line labeled LB-C represents an uninoculated control condition. Each culture condition was set up in triplicate.

Next, P103-3c was cultivated, at a 10X initial dilution, in basal media containing MeA and 0.5% v/v LB. Samples were run in triplicate and compared with uninoculated controls of the same media (fig. 12). The assay showed definitively increased fluorescence from inoculated cultures. Although the fluorescence pattern over time did not mimic a classical growth curve, fluorescence did steadily increase with culture time under the inoculated condition but did not in the uninoculated condition. Error bars remained small to diminutive, again suggesting a large degree of consistency present in the assay. It should also be noted that the magnitude of fluorescence of P103-3c grown in LB was much higher than when it was grown in basal media containing MeA and 0.5% v/v LB.

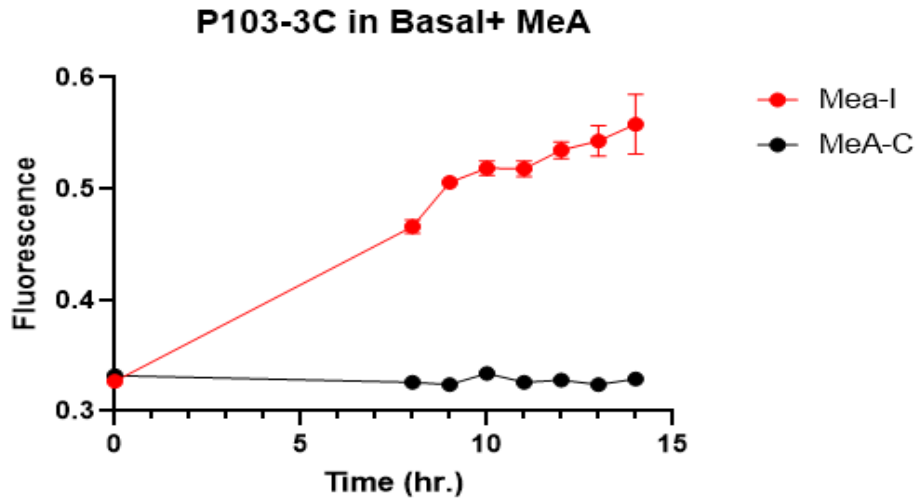


Figure 12: MTS assay using P103-3c grown in the basal media containing MeA and 0.5% v/v LB. Growth was evaluated at T=0hr. and from T=8hr. through T=14hr. The line labeled MeA-I represents an inoculated test condition, while the line labeled MeA-C represents an uninoculated control condition. Each culture condition was set up in triplicate.

Two experiments were then performed in order to determine the colorimetric assay's viability in demonstrating real growth from fluorescence data. P103-3c was grown under the same conditions as for the previous assays, but with a 100X initial dilution. The initial dilution of P103-3c was changed for these experiments from 10X to 100X, and the culture time changed from 14 hours to 30 hours in order to produce a clearer picture of how this isolate grew under the given culture conditions. Cultures were incubated under aerobic conditions at 37°C with vigorous shaking for 30 hours, with growth being evaluated every six hours. Additionally, colony-forming units (CFUs) were counted for each culture at each time point using serial dilutions and spread plating. Fluorescence was read the same way as in the previous MTS assays. Fluorescence was plotted against CFUs/ml in order to elucidate the relationship between real growth of P103-3c in culture and fluorescence values from the assay. A Pearson correlation was used to evaluate the degree of relationship between fluorescence and CFUs/ml. The Pearson

correlation from the first experiment was found to have an R^2 value of 0.8393, and the Pearson correlation from the second experiment an R^2 value of 0.7903. The R^2 value of both experiments had highly significant P-values of less than 0.001 with a 95% confidence interval (fig. 13). These data indicate that there is a reliably strong relationship between fluorescence from the MTS reagent and real growth of cells in culture.

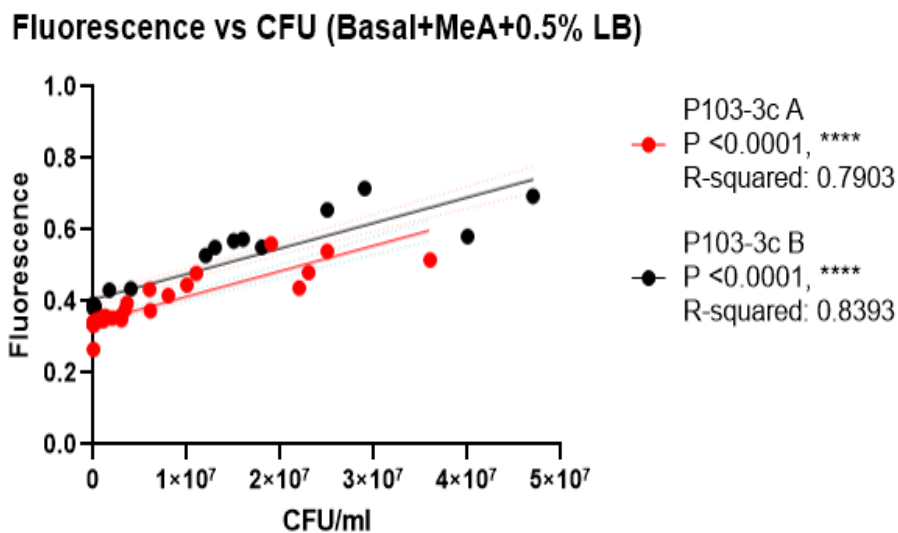


Figure 13: Two separate but comparable MTS assays, experiment A and experiment B, are represented in this graph. Both assays show P103-3c grown in the basal media containing MeA and 0.5% v/v LB. Both experiments differ only in the time points taken, with experiment A having had growth evaluated every two hours for twelve hours, and every 6 hours for the following 18 hours. Experiment B had growth evaluated every 6 hours for 30 hours. Fluorescence values of each culture at each time point in each experiment were plotted against their corresponding CFU/ml. A Pearson correlation was used to evaluate the degree of relationship between the CFU/ml and the corresponding fluorescence of each culture at each time point. A separate Pearson correlation was calculated for each experiment. Additionally, the data for each experiment was separately fit to a linear regression, demonstrated by the trendlines displayed in this figure.

Since the colorimetric assay had been shown to demonstrate real growth with P103-3c cultivated in basal media containing MeA and 0.5% v/v LB, a colorimetric assay was performed with P103-3c in order to determine the assay's ability to demonstrate a growth advantage similar

to what was shown in the screening cultures and initial growth curves. To do this, P103-3c was cultured in the same manner as previously described in the methods, with a 100X initial dilution into 5ml of media, contained within a 50ml Erlenmeyer flask. P103-3c was cultivated in basal media with 0.5% v/v LB, both with and without MeA. Each culture condition was performed in triplicate, with uninoculated cultures to serve as a negative control. Cultures were incubated aerobically with vigorous shaking for 30 hours, with growth being evaluated every six hours. Fluorescence was evaluated as previously described in the methods. It was shown that cultures of P103-3c showed greater fluorescence when cultured in the basal media containing MeA compared to the basal media without MeA. This increase in fluorescence was maintained throughout the course of the entire cultivation period. In both culture conditions, P103-3c cultures fluoresced more strongly than did their respective uninoculated controls (fig. 14). This data from the colorimetric assay is consistent with the data from previously mentioned optical density-based assays.

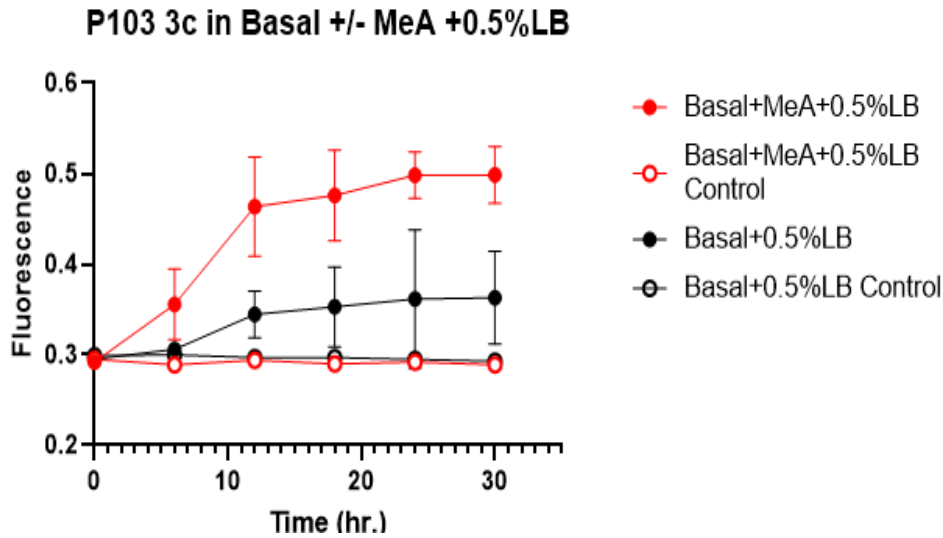


Figure 14: MTS assay using P103-3c grown in the basal media containing 0.5% v/v LB, both with and without MeA. Growth was evaluated every six hours for 30 hours. The line labeled “I” represents an inoculated test condition, while the line labeled “C” represents an uninoculated control condition. Inoculated test conditions were set up in triplicate.

A different fecal isolate, P121-2c, was cultured in the same manner that P103-3c was cultured. The isolate was added in a 100X dilution into 5ml. of basal media with 0.5% v/v LB, both with and without MeA. The cultures were grown at 37°C in 50ml. Erlenmeyer flasks, subjected to vigorous shaking, for a growth period of 30 hours. Growth was evaluated every six hours for the duration of the growth period. The isolate p121-2c demonstrated increasing fluorescence values for the first twelve hours of growth, followed by a decrease in fluorescence. This decrease was seen when MeA was both present and absent from the media, but that decrease was noticeably attenuated in the presence of MeA compared with its absence (fig. 15). The isolate grew at similar rates throughout the first twelve hours in both conditions, however, and reached a similar level of peak fluorescence. It is notable that this growth curve mimics the p121-2c growth curve produced in a falcon tube and measured with optical density (fig. 8). Additionally, it also mimics the pattern seen when methane production from P121 was measured

with BES (fig.4). The similarity of these patterns further cements the viability of the colorimetric assay as an effective tool to measure the growth of these isolates.

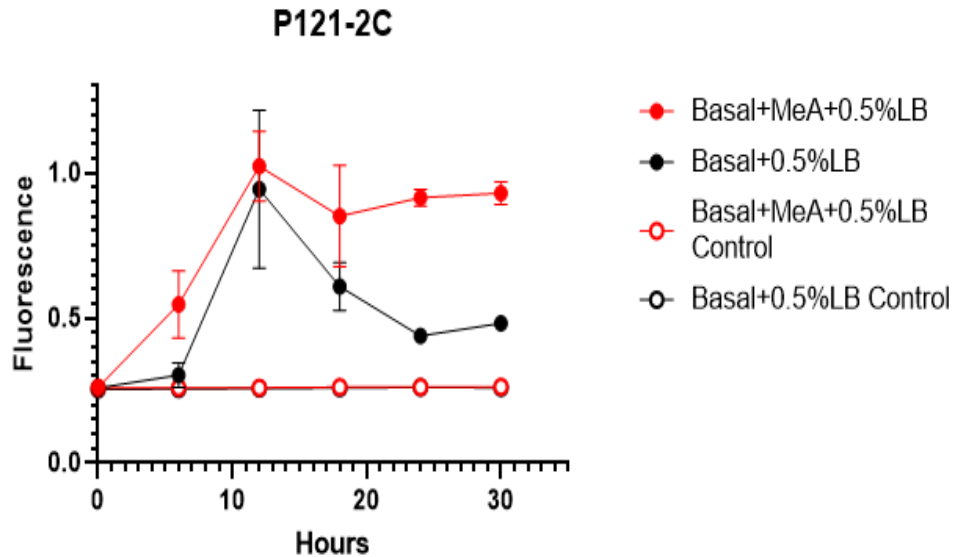


Figure 15: MTS assay using P121-2c grown in the basal media containing 0.5% v/v LB, both with and without MeA. Growth was evaluated every six hours for 30 hours. Lines labeled “control” represent an uninoculated test condition. Inoculated test conditions were set up in triplicate.

Culture of P103-3c in 24-Well Plates

Each isolate was then cultured in a 24-well culture plate, rather than in Erlenmeyer flasks, in order to facilitate the screening of multiple isolates concurrently. Each well in the plate held a maximum volume of 2ml. A 100X dilution of a P103-3c overnight culture adjusted to an optical density between 0.14 and 0.16 was added to 750 μ l of media in each tested well. After P103-3c was grown in the basal media containing 0.5% LB, with or without MeA, growth was evaluated in the same way as in figures 11-15. Growth was again demonstrated to be greater when P103-3c was grown in the presence of MeA, despite being grown in wells of a 24-well plate. It should

be noted that the fluorescence values of this MTS assay demonstrated a greater magnitude than that of previous MTS assays. CFUs per ml were not counted for this assay. Additionally, the overall trend of the growth from these isolates is unlike that demonstrated in fig. 14 in that fluorescence values decreased at hours 24 and 30 of growth.

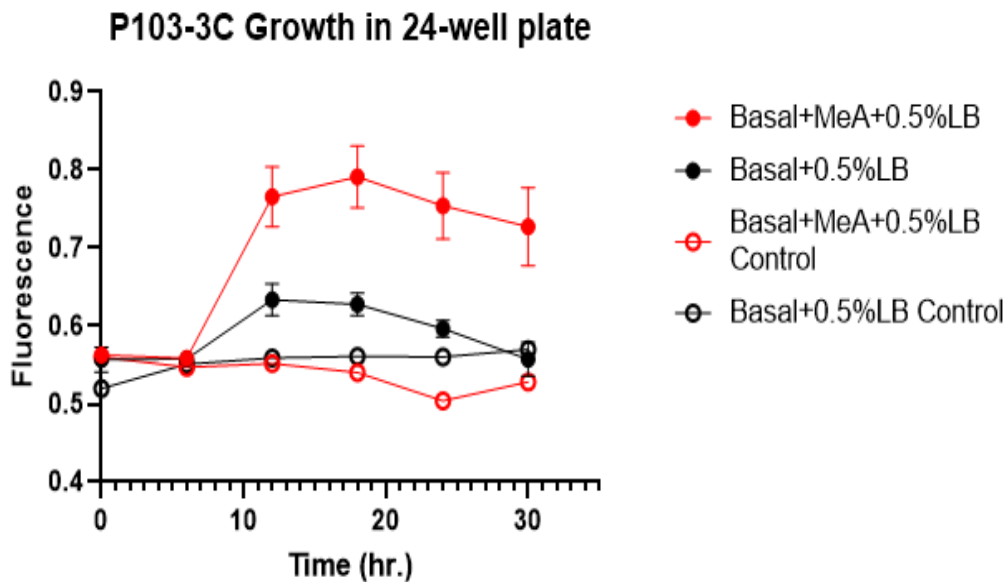


Figure 16: MTS assay with P103-3c grown in the basal media containing 0.5% v/v LB, both with and without MeA. Growth was evaluated every six hours for 30 hours. Lines labeled “control” represent an uninoculated test condition. Inoculated test conditions were set up in triplicate. Growth was performed in a 24-well plate at 37°C with vigorous shaking, and samples were transferred to a 96-well plate for evaluation. Plotted fluorescence values represent those measured after three hours of reaction time between samples and MTS reagent.

16s Sequencing of P103-3c and P121-2c

The identity of two of the fecal isolates, P103-3c and P121-2c, was identified via 16s sequencing. DNA was first extracted and purified as described in the methods section before being sent to Azenta life sciences for 16s sequencing. Reads from the forward and reverse primers were aligned using the NCBI blast tool. Contigs generated from read alignment were then blasted against the NCBI database, and the results of that query were used to identify both

isolates at the species level. The isolate P103-3c was identified at the species level with 100% Query coverage at a 99.64% ID as being *Micrococcus luteus*. The isolate P121-2c was also identified at the species level with 100% Query coverage at a 99.58% ID, as a *Bacillus subtilis* (Table 1).

Isolate	Closest Relative	Query Coverage	Percent ID
P103-3c	<i>Micrococcus luteus</i>	100%	99.64
P121-2c	<i>Bacillus subtilis</i>	100%	99.58

Table 1: BLAST results from the 16s gene sequences for isolates P103-3c and P121-2c. Genus and species, as well as query coverage and percent identity are shown.

DISCUSSION AND CONCLUSIONS

This work sought to investigate the possibility of aerobic bacterial methane synthesis occurring in the human gut. Contrary to the traditional model of biogenic methane being produced almost exclusively by archaeal methanogens [23], much evidence has surfaced in recent years describing bacterially-mediated biogenic methane production occurring under oxic conditions [8, 10, 11]. These bacteria were found to produce methane from methylated phosphonates and methylated amines through assimilatory pathways, using C-P lyases and aspartate aminotransferases to catalyze the formation of methane from such precursors. Methane producing bacterial metabolisms constitute a novel area of research, with most studies occurring in freshwater lake ecosystems.

Until now, little to no work has been done to understand the role, if any, that bacterially-mediated biogenic methane production plays in the human microbiome, one of the most impactful microbial environments on human health [63, 86, 93, 111]. The human gastrointestinal tract is known to produce TMA and MeA from dietary choline and L-carnitine [94]. Additionally, although the colon is an anaerobic environment, there is a steep oxygen gradient originating in the colonic epithelium [120]. In contrast, the small intestine harbors a higher oxygen content than does the colon [66]. Therefore, the human gut microbiome operates under conditions amenable to aerobic bacterial methane synthesis as it is currently understood, and subsequently constitutes another environment capable of harboring this novel metabolism.

Initial Culture Work: Isolation, and Evaluation of Isolates

In order to initially investigate whether the human gastrointestinal tract harbored bacteria capable of bacterial methane synthesis, whole fecal slurries from six individuals were evaluated for their ability to produce methane under aerobic and anaerobic conditions. Since methanogens are putatively present in a large proportion of the population [3], it was expected that the majority of fecal slurries would produce methane when grown under anaerobic conditions. In fact, only three (P121, P420, P721) of the six fecal slurries tested produced a relatively large amount of methane when grown anaerobically in the modified SAR11 medium. The fecal slurries of the other three individuals, P802, P801, and P103, produced a relatively small amount of methane. It should be noted that culture in the basal media was not intended to approximate the conditions found in the human gastrointestinal tract, but instead to promote the possible utilization of methylamine by a given fecal slurry to produce methane. Therefore, a lack of biogenic methane production under anaerobic conditions is not highly indicative of a lack of methane production within the host. However, it should be noted that the amount of methane produced when these fecal slurries were cultivated anaerobically is unequivocally higher than the methane produced when those same fecal slurries were cultivated aerobically. It seems most likely that this difference in methane production is in fact due to the activity of archaeal methanogens whose metabolisms were inhibited under aerobic conditions. Although some evidence has been shown to indicate the ability of some methanogens to tolerate oxygen [7], it still remains the case that the majority of archaeal methanogens are highly sensitive to oxygen [23]. Indeed, the two most common methanogens in the human gut, *Methanobrevibacter smithii*, and *Methanosphaera stadtmanae* [86], have not been shown to exhibit tolerance to oxygen.

Therefore, the lowered production of methane from whole fecal slurries when under aerobic conditions, compared to when they were cultivated under anaerobic conditions, is most likely due to the inhibition of archaeal methanogens by an oxygenated environment. That these fecal slurries could produce methane under aerobic conditions, however, was indicative of microbes able to biogenically produce methane aerobically. Despite the presumed inhibition of methanogenesis, whole fecal slurries P103 and P121 still produced more methane when cultivated with basal media containing MeA than when they were cultivated in basal media that omitted it. This indicated that fecal slurries P103 and P121 harbored members of their respective communities that could produce methane aerobically, and potentially so from MeA.

In order to confirm that the methane produced by whole fecal slurries under aerobic conditions was not due to the activity of aerotolerant archaeal methanogens, fecal slurries P103 and P121 were grown in the same manner as in figures 3 and 4, but with the addition of 2-bromoethanesulfonate (BES), which is a known inhibitor of archaeal methanogenesis [119]. Under these conditions, the whole fecal slurry P103 clearly produced more methane when MeA was present in the media than when it was omitted, indicating that methane production from members of the gut microbiome of that individual is influenced by the presence of MeA, even under aerobic conditions, and that the observed results are not due to aerotolerant classical methanogens. This pattern was not as clear with the P121 fecal slurry. It should be noted in figures 8 and 15 that a similar pattern was shown for the p121 whole fecal slurry and the isolate P121-2c, wherein a difference, growth or otherwise, between conditions containing or lacking MeA is not readily apparent until late in the testing period. Together, these data indicate that the

methane production of the p103 and p121 whole fecal slurries is in part dependent upon the presence of MeA under aerobic conditions.

To ascertain the members of the gut microbiome responsible for the results seen in figures 3 and 4, whole fecal slurries P103 and P121 were serially diluted and grown on plates containing the basal media with MeA. Methylamine was the only source of nitrogen in the media on those plates, enriching for organisms potentially capable of utilizing MeA as a sole nitrogen source. Nine isolates (1abc, 2abc, 3abc) each from the P103 and P121 fecal slurries were obtained. Isolates from other fecal slurries were not obtained since those fecal slurries did not show evidence of MeA-based methane production, based on the results from figures 3 and 5. Of the isolates, each of the P103 isolates and five of the P121 isolates (2abc, 3b, 3c) showed increased optical density when MeA was present in the media compared to when it was absent from the media. Of these isolates, P103-3c, P121-2a, -2b, -2c, and -3c all showed the greatest difference in optical density between the two growth conditions. Growth curves were next performed on those five isolates, which served to corroborate the results obtained in figures 6 and 7. These results together show that each of these human gut isolates (P103-3c, p121-2abc, p121-3c) are able to grow more effectively on methylamine as a nitrogen source. Consistent with previous work that showed bacteria capable of producing methane as a by-product of MeA assimilation [10], these results therefore point to the potential of these isolates to produce methane from methylamine.

The ability of an isolate to utilize MeA in an assimilatory manner does not mean that it produced methane, however. In order to positively link MeA utilization and methane production, P103-3c, P121-2abc, and P121-3c were cultivated in sealed serum bottles under aerobic

conditions for 24 hours, with and without MeA, before evaluating gases in the headspace of the bottles via gas chromatography. Two of the five isolates tested in this manner, P103-2c and P103-3c, produced methane over the 24-hour testing period in statistically significant amounts. Those two isolates, then, individually demonstrated an ability to utilize MeA in an assimilatory manner and use it to produce methane, constituting the first evidence of aerobic bacterial methane synthesis from isolates of the human gastrointestinal tract.

MTS Assay Development

A more effective tool than flask-based culture to evaluate aerobic methane production from future fecal isolates was deemed to be of import in order to establish a greater understanding of this metabolism. The MTS assay described in this study was chosen for its ability to be consistent and high-throughput and first needed to be validated as an effective tool in those regards. Initial experiments using the MTS assay showed it to be consistent and sensitive to bacterial cell counts. In support of the ability of the MTS assay to do this, the isolate P103-3c demonstrated increased fluorescence when grown in the presence of MeA. Additionally, the fluorescence pattern was reflective of the growth advantage seen in initial culturing work. In order to further confirm the validity of the MTS assay as an evaluating tool, the relationship between CFU/ml of culture growth and fluorescence in the assay was evaluated. The result of quantifying that relationship was that, to a large extent, fluorescence values shown by the MTS assay correspond to actual growth of P103-3c in culture. The confirmation of results obtained in figures 8 and 9 seen by figures 14 and 15 further serves to emphasize this point. However, each of these results were based on flask-mediated culture, which would diminish the effectiveness of the MTS assay to evaluate a large number of fecal isolates. As

shown in figure 16, this is not a limitation since initial growth of the isolates could be accomplished in a 24-well plate. Attempts were initially made to get around flask-based culture using a 96-well plate. These attempts were ultimately unsuccessful due to the diminutive volume available in the individual wells of 96-well plates, which prevented consistent results from being obtained (supplemental figure 1, Appendix A). Since culture using 750 μ l in a 24-well plate produced consistent results, it is therefore possible to screen 11 isolates per 24-well plate, representing a dramatic improvement over flask-based culture methods. Based on a highly significant correlative relationship between CFU/ml and fluorescence as well as a high level of repeatability, the MTS assay was shown to be an effective method to evaluate a larger number of fecal isolates for MeA utilization than could be achieved using flask-based culture methods.

The identities of the isolates P103-3c and P1212-2c were established by 16s rRNA gene sequencing. Each sequenced gene was analyzed against the NCBI BLAST database, resulting in the identification of P103-3c as a strain of *Micrococcus luteus* and the identification of P121-2c as a strain of *Bacillus subtilis*. Both of the BLAST queries expressed very high query coverage and 100% IDs, lending confidence to the identification of P103-3c and P121-2c at the species level. Since a minority of *M. luteus* are known to be gram variable [121], gram stains were performed on cultures of P103-3c at 6, 12, and 24 hours. The isolate P103-3c was found to be gram positive at all of these time points. *Bacillus subtilis* is known to be facultative anaerobe [122] while *Micrococcus luteus* is considered an obligate aerobe [123], which is consistent with the ability of these isolates to produce methane under aerobic conditions.

Limitations and Future Directions

These results, while valuable, certainly do not paint a complete picture of bacterially mediated aerobic methane production in the human gastrointestinal tract. This study only addresses the question of whether or not bacteria in the human gut are capable of producing methane from methylamine under aerobic conditions. It does not address whether this process happens in-vivo, which other bacteria can produce methane aerobically, how widespread these bacteria are in the population, or what amount of methane is produced by this process in-vivo. All of these questions are relevant to gaining a full understanding of the scope of bacterially mediated methane production as well as the potential impacts thereof on human health and disease.

Some of these questions may be quite difficult to answer. For instance, gaining an understanding of how much bacterially produced methane is generated within the human body is itself a challenge due to the difficulty of creating effective proxies of the GI microenvironment. A likely proxy to using human models for this would be monoculture of P103-3c or P121-2c within germ-free mouse models. Carefully controlling the gas environment in which subject mice live could theoretically allow for the measurement of any methane produced presumably as a result of the monoculture within the mouse models. Mice could also be fed a diet spiked with MeA and TMA, allowing researchers to observe any changes of the levels of those metabolites in the mice, if any. This could potentially allow for evidence that these fecal isolates impact the levels of methylated amines in-vivo, making a more compelling case that aerobic bacterial methane synthesis could play a role in host physiology.

The primary shortcoming of using fecal samples, namely that can only be a proxy for the gut microbiome [124], do not hamper the results of this study. Since this study primarily investigated the presence of methane-producing bacteria from the human gut, it was not reliant upon samples being a highly accurate representation of the colonic microenvironment. Other potential sampling methods, including biopsy, endoscopy, capsule-based methods, etc., confer expense and difficulty of access along with any advantages they may bring. Those advantages were not necessary to achieve the primary aims of this study. Therefore, the ease and inexpensiveness of using previously collected fecal samples in this study constituted a perfectly adequate sampling regime. However, attempting to deduce the extent to which this methane production is happening in-vivo may require samples that more closely approximate the colonic microenvironment.

Finally, the mechanisms by which P103-3c and P121-2c produce methane from methylamine should be elucidated. This can be accomplished using a transposon library, where mutants are screened using the MTS assay. Once mutants no longer capable of aerobic bacterial methane synthesis are identified, the locations of transposon insertions can be used to ascertain the enzymes involved in aerobic bacterial methane synthesis, which carries the potential to evaluate unculturable isolates for the ability to produce methane aerobically.

Conclusions

This work constitutes the first evidence of bacterially mediated aerobic methane synthesis in human gut isolates. Bacterially mediated aerobic methane synthesis is a newly discovered metabolism in bacteria that challenges the traditional model of methane synthesis as being carried out strictly in anaerobic conditions by archaeal methanogens. Previous work has been

focused primarily on lake environments. Therefore, evidence of aerobic bacterial methane synthesis in the human gut expands the breadth of environments and organisms capable of harboring this novel metabolism and raises important questions as to what roles such a metabolism might play in human health.

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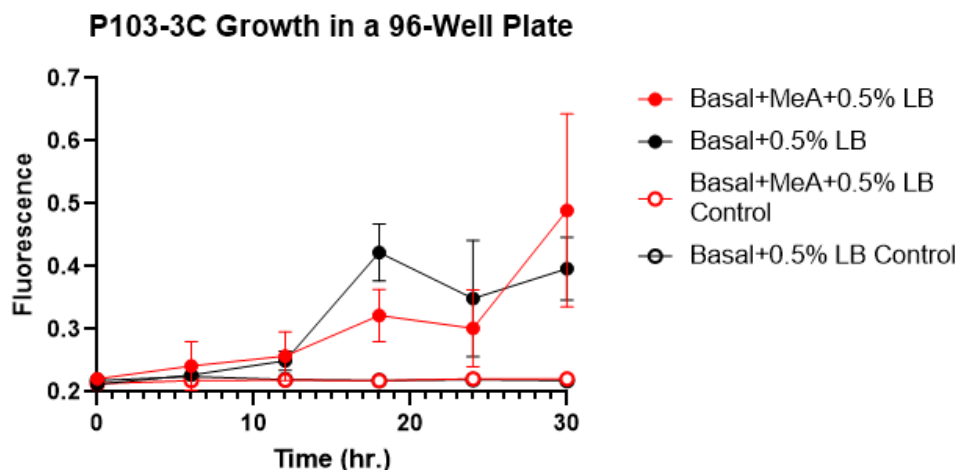
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APPENDIX

SUPPLEMENTARY DATA

MTS Assay When P103-3c is Grown in a 96 Well Plate

The isolate P103-3c was grown in the same way as in figure 13, except that instead of being grown in Erlenmeyer flasks, cultures were set up in a 96-well plate. All concentrations were kept the same as in previous experiments (fig. 11-14), but with a total culture volume of 200 μ l. As in those experiments, growth was evaluated every 6 hours for 30 hours, with 15 μ l. of culture being transferred at each time point to another 96-well “evaluation plate.” After 30 hours of culture in a plate reader at 37°C with vigorous shaking, the MTS reagent was added and the evaluation plate was evaluated in the same manner as described for the MTS assay in the methods section.



Supplemental Figure 1: MTS assay using P103-3c grown in the basal media containing 0.5% v/v LB, both with and without MeA. Growth was evaluated every six hours for 30 hours. Lines labeled “control” represent an uninoculated test condition. Inoculated test conditions were set up in triplicate. Growth was performed in a 96-well plate at 37°C with vigorous shaking, and samples were transferred to a 96-well plate for evaluation. Plotted fluorescence values represent those measured after three hours of reaction time between samples and MTS reagent.

Gram Stain Results of P103-3c

The isolate P103-3c was identified to be *Micrococcus luteus* (table 1). As some *M. luteus* are gram variable, a colony of P103-3c was picked from a plate and grown in LB at 37°C with vigorous shaking. The culture was gram stained at 6, 12, and 24 hours in order to establish the gram reaction of the isolate at multiple different time points.

Growth Time (hr.)	Gram Reaction
6	Positive
12	Positive
24	Positive

Supplemental Table 1: Gram stain results of the isolate P103-3c grown in LB. Gram stains were performed 6-, 12-, and 24-hours post-inoculation.