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Oscillospira and related bacteria – from metagenomics species to metabolic features

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Summary

Oscillospira is an under-studied anaerobic bacterial genus from Clostridial cluster IV that has resisted cultivation for over a decade since the first time it was observed. In recent years its 16S rRNA gene was identified in several human gut microbiota studies where it was often associated with interesting traits, especially leanness. However, very little is known about its metabolism or physiology. Here we use nearly complete genomes derived from shot-gun metagenomic data from the human gut to analyze *Oscillospira* and related bacteria. We used sequence similarity, gene neighborhood information and manual metabolic pathway curation to decipher key metabolic features of this intriguing bacterial genus. We infer that *Oscillospira* species are butyrate producers, and at least some of them have the ability to utilize glucuronate, a common animal-derived sugar that is both produced by the human host and consumed by that host in diets rich in animal products. These findings could help explain diet-related inter-individual variation in fecal *Oscillospira* levels as well as the observation that the presence of this genus is reduced in diseases that involve inflammation.

Keywords: Gut microbiome, butyrate, 16S rRNA. Oscillibacter, colon, microbiome, fecal microbiota,

Introduction

Quite often in molecular microbial ecology, putative genera or species that are quite prominent in cultivation-independent studies have few or no cultured isolates. *Oscillospira guilliermondii* represents a typical case: first observed in guinea pig caecal contents by Chatton and Pérard in 1913 (see (Tuffery, 1954)) it is at this time the only named species, albeit without a cultured type strain, in the genus *Oscillospira* (Parte, 2014). The cells of *O. guilliermondii* have been shown to be very large (up to 70 μm in length and about 5-7 μm in width), and subdivided into disk-like segments (Tuffery, 1954), which can be rod-shaped or oval (Mackie et al., 2003), and have internal structures that appear to be spores. Bacteria with *O. guilliermondii*-related 16S rRNA sequences were identified in the rumen of several herbivores, including cattle and sheep, and found to be more abundant when the diet was based on fresh forage (Mackie et al., 2003). The large and conspicuous morphology of

O. guilliermondii facilitated the isolation of its DNA and amplification and sequencing of the 16S rRNA genes of these bacteria (Yanagita et al., 2003), despite the inability to grow them in culture. Based on the sequence of its 16S rRNA gene, *O. guilliermondii* was placed within the clostridial cluster IV in the Clostridiales order in the Firmicutes phylum. The cultivated organism most closely related to *O. guilliermondii* (92.6–92.9 % sequence identity in the 16S rRNA gene) is *Oscillibacter valericigenes* isolated from the alimentary canal of a Japanese clam (Iino et al., 2007; Katano et al., 2012). *Oscillibacter* has been often wrongly assigned to the family Oscillospiraceae, a classification the persists in NCBI taxonomy. In contrast, *Oscillospira* is correctly placed in the family Ruminococcaceae in the NCBI taxonomy, which is consistent with its phylogenetic clustering, and that of *O. valericigenes* within this family based on their 16S rRNA sequence. *Oscillibacter ruminantium* isolated from the rumen of Korean native cattle (Lee et al., 2013), which has also been identified rarely in human blood infections (Sydenham et al., 2014), has slightly lower sequence similarity to *O. guilliermondii* (91.6 %). Since *O. guilliermondii* has never been grown in pure culture, and the first member of the *Oscillospira* genus has only recently been cultivated in a rumen fluid-containing medium (Cuiv et al., 2015), little is known about its ecology or physiology. Nevertheless, based on many 16S rRNA gene amplicon sequence studies of the human gut microbiome, it is recognized as a common and fairly abundant member of the fecal microbiota. It appears to be positively associated with leanness and age [(Makivuokko et al., 2010; Yatsunenko et al., 2012; Biagi et al., 2016), see also (Konikoff and Gophna, 2016)] and is less abundant in persons with inflammatory bowel disease (Walters et al., 2014) or steatohepatitis (Zhu et al., 2013). Recently, *Oscillospira* has also been shown to be negatively associated with looser stools (Tigchelaar et al., 2016). Since *Oscillospira* and/or its close relatives may be involved in human health, we reasoned that its putative metabolism is worth exploring in gut metagenomes. Since 16S rRNA gene sequences of *Oscillospira* were not part of the assembled nearly complete genomes derived from metagenomes (known as co-abundance gene groups (CAGs) or metagenomic species) that were reconstructed from human gut metagenomes (Nielsen et al., 2014), one could use BLASTN to identify directly which CAGs correspond to *Oscillospira*-related phylotypes.

However, since several *Oscillibacter* species have had their genomes sequenced, one could use a highly conserved protein, such as RpoB, the beta subunit of the bacterial RNA polymerase that is known to be an excellent phylogenetic marker (Case et al., 2007), as a BLASTP seed to detect their closest relatives, some of which could be expected to belong to the genus *Oscillospira*. We obtained the 10 best BLASTP matches to *O. valericigenes* RpoB to which additional homologs from several related species were added, aligned them by MAFFT (Supplementary file 1) and constructed a maximum likelihood-based phylogenetic tree (Fig. 1). This tree, representing a lineage within the family Ruminococcaceae, shows that along with the cluster that contains *Oscillibacter* species, including *O. valericigenes,* there is a sister group with two branches: one with the poorly documented *Oscillibacter sp.* ER4 (a genome obtained from a human gut isolate) and Firmicutes bacterium CAG:129 and the other branch that includes Firmicutes bacterium CAG:83 and *Oscillibacter* sp. CAG:241 (Firmicutes bacterium CAG:83 and Oscillibacter sp. CAG:241 (henceforth called CAG:83 and CAG:241 for short). We propose that either one or both of these branches consist of another genus rather than *Oscillibacter* that in all likelihood is *Oscillospira*. Notably, CAG:83 is the most common of these, found in about 60% of all individuals in several large metagenomic datasets of the fecal human microbiota (Table 1). Its genome is dominated by genes with either unknown functions or a general function prediction only, with relatively few genes inferred to be involved in carbohydrate transport and metabolism (Fig. 2), for a gut symbiont.

Oscillospira species are probably slow growers and associated with slow colonic transit times

It has been suggested that faster colonic transit times select for faster growing microbes, while slower transit/constipation allows for slower replicating organisms to be maintained in the lumen and avoid washout (Vandeputte et al., 2016). A recent study of 1126 adults Europeans used 16S rRNA gene amplicon sequencing to associate specific bacterial taxa with stool softness/hardness based on the Bristol stool scale, and observed that *Oscillospira* was positively associated with harder stools (Tigchelaar et al., 2016), with confidence for that association (i.e. statistical significance after correcting for multiple hypotheses testing) that was second only to *Methanobrevibacter*, a methanogen known to be linked to longer colonic transit times (Lurie-Weinberger and Gophna, 2015). Additionally, the only disease that *Oscillospira* has been linked to is gallstones (Keren et al., 2015), for which slow-transit / constipation is a well-established risk factor (Misciagna et al., 1996; Hofmann, 2005). Thus, we hypothesized that *Oscillospira* species are slow-growing bacteria. To infer approximate growth rates we used the number of tRNA genes in the genomes (obtained from *rrn***DB,** (Stoddard et al., 2015)), shown to be a strong predictor of microbial generation times (Vieira-Silva and Rocha, 2010). Generally, faster growing microorganisms have more tRNA gene copies in their genomes, and vice versa. Indeed, CAG:83 and CAG:241 had fewer than 40 tRNA genes (Table 2), which is very rare in bacteria and typical to very slow growing bacteria, such as the marine cyanobacterium *Prochlorococcus marinus* (35-40 tRNA genes, generation times of 17 h or longer). This is in contrast to fast growing gut microbes such as *Bacteroides fragilis* (72-72 tRNA genes, generation time 0.63 h), and *Peptoclostridium difficile* (82 tRNA genes, generation time of less than 70 minutes, (Carroll and Bartlett, 2011)), although some fast growing species have fewer tRNA gene copies (46 tRNA genes in the case of *Fusobacterium nucleatum* that has an optimal generation time of about 54 minutes). Very slow growth could help explain why *Oscillospira* relative abundance has been strongly positively associated with methane production (presumably by one or both of the human methanogens mentioned above) in women with chronic constipation (Parthasarathy et al., 2016), since even in the absence of any direct metabolic interaction between *Oscillospira* and methanogens, these microbes will benefit greatly from slow transit times in the gut. Such slow growth could also explain why *Oscillospira* species have remained uncultivated for over a century, and implies that investigators attempting their cultivation should wait for several weeks for visible growth.

Are *Oscillospira* species butyrate producers?

Multiple intestinal species of Firmicutes produce the short chain fatty acid butyrate known to play a key role in maintaining gut health by preventing cell proliferation, suppressing inflammation and providing energy to enterocytes. Butyrate is produced by intestinal bacteria from acetyl-CoA beginning with four enzymatic reactions that generate butyryl-CoA, and ending either by transferring the CoA to an acetate molecule by butyryl-CoA:acetate CoA-transferase or by a two-step process in which butyryl-CoA is first converted to butyryl-phospate by phosphate butyryltransferase, and is then dephosphorylated by butyrate kinase (Louis et al., 2004). Notably, butyrate was previously shown to be the major end product of sugar fermentation by *O. ruminantium* (Lee et al., 2013).

The enzyme catalyzing the first step in the pathway, thiolase, has homologs in all members of the *Oscillibacter* clade described in Figure 1, and this is also true for homologs for the enzyme responsible for step two, 3-hydroxybutyryl-CoA dehydrogenase (also known as beta-hydroxybutyryl-CoA dehydrogenase). The third enzyme, 3-hydroxybutyryl-CoA dehydratase, has homologs present only in CAG:83 and CAG:241, yet there are homologs that have over 70% identity to the CAG:83 and

CAG:241 enzymes that are annotated more generally as enoyl-CoA hydratases in *Oscillibacter sp.* ER4, as well as in *O. valericigenes* and *O. ruminantium* that could have dehydratase function. While no ORF in CAG:83 is annotated as a butyryl-CoA dehydrogenase, there is an acyl CoA dehydrogenase adjacent to the 3-hydroxybutyryl-CoA dehydrogenase mentioned above that has over 70% identity to those enzymes of known clostridial butyrate producers, such as *Faecalibacterium prausnitzii (Sokol et al., 2008)*. Close homologs to this enzyme > 80% identity) are found in all other members of the *Oscillibacter clade*.

CAG:83 appears to have no homolog of phosphate butyryltransferase, while CAG:241 has two paralogs of this enzyme, one of which is 46% identical at the amino acid level to a homolog in *Peptoclostridium difficile,* known to produce butyrate (Karlsson et al., 2000). Both CAG:83 and CAG:241, Unlike known *Oscillibacter* species, have ORFs annotated as butyrate kinase, that have over 60% sequence identity to homologs from *Eubacterium ventriosum* and other known butyrate producers (Barcenilla et al., 2000), while *Oscillibacter sp.* ER4 and CAG:129 and the top *Oscillibacter* clade in Figure 1 do not. As for the alternative single enzyme path from butyryl-CoA to butyrate, the four cultivated *Oscillibacter* species represented at the top of Figure 1, as well as CAG:129 and *Oscillibacter* sp. ER4 have homologs of butyryl-CoA:acetate CoA-transferase, which are over 60% identical to this enzyme from the known butyrate producer *Butyricicoccus pullicaecorum* (Eeckhaut et al., 2013). In contrast, both CAG:83 and CAG:241 only have homologs with lower similarity (<50% sequence identity) with more general functional annotations: CoA-transferase and acetyl-CoA hydrolase for CAG:83 and CAG:241, respectively. In conclusion, it appears likely that at least some members of the *Oscillibacter* clade can produce butyrate. The putative species that we associate with the genus *Oscillospira*, CAG:83 and CAG:241, especially the latter, unlike their relatives from the *Oscillibacter* clade, have the butyrate-kinase mediated pathway, which is complete in CAG:241.

Major substrates utilized by *Oscillospira* species

The only reliably-annotated sugar transporter in CAG:83 and CAG:241 is Cut1 family ABC transporter, two membrane subunits of which are present in both genomes (99% identity between CAG:83 and CAG:241), with homologs of over 70% identity in the other members of the *Oscillibacter* clade. Cut1 family transporters are known to be involved in uptake of oligosaccharides, glycerol-phosphate and polyols (Schneider, 2001), and although they have not been well studied in Clostridia, genetic evidence supports their role in plant fiber utilization by *Clostridium phytofermentans* (Mukherjee et al., 2014). Unfortunately, because of the broad substrate specificity of the Cut1 family it is currently impossible to predict the substrates of these transporters in *Oscillospira* and other *Oscillibacter* clade organisms.

CAG:83 has an additional protein annotated as "sugar ABC transporter", but this annotation is questionable, since nearly all its closest matches are annotated as heme ABC transporters. An additional protein annotated as a putative sugar ABC transporter ATP-binding protein in CAG:241 (the only putative sugar transporter in this CAG, other than the Cut1 family transporter described above), has a closely related homolog in CAG:83 (92% amino acid identity) that has been annotated more conservatively as an ABC transporter ATP-binding protein. These proteins have more distant homologs (less than 80% identity) in other *Oscillibacter* clade genomes, some of which are annotated as putative sugar transporters, while others are not. The paucity of sugar transporters in these organisms suggests that the major substrates of these strains are not plant-derived glycans.

In CAG:83, ORF CCX72803.1 is annotated as an uronate isomerase, while CAG:241 does not have a homolog for this enzyme. Additionally, CAG:83 ORF CCX72806.1 is annotated as a mannitol dehydrogenase domain protein, but most of its BLASTP homologs are annotated as altronate oxidoreductases, while CCX72805.1 is annotated as a sAF domain protein, but nearly all its homologs are annotated as altronate hydrolases (absent from CAG:241 and other members of the clade). These three ORFs, which in CAG:83 are adjacent to one another on contig 160, probably function together in the enzymatic pathway required to convert glucuronate to 2-dehydro-3-deoxy-D-gluconate, thus directing it into the pentose phosphate pathway.

Glucuronate is a common host-derived sugar in the human body, and a key component of heparin/heparan sulphate glycosaminoglycans that are found on the cell surface and in the extracellular matrix of most human tissues (Tripathi et al., 2012), and common to all vertebrates (Poulain and Yost, 2015). Glucuronate therefore represents a good substrate for gut symbionts, and its utilization by some *Oscillospira* strains could explain why the relative abundance of such strains increased in the cecum following prolonged fasting in a bird, a fish and a mouse (Kohl et al., 2014). Having a host sugar as a primary energy source may also explain why *Oscillospira* has been positively associated with leanness in multiple human studies (Tims et al., 2013; Goodrich et al., 2014; Konikoff and Gophna, 2016): on the one hand leaner individuals may consume lower amounts of starches than the over-weight or obese, while on the other, host glycan degradation by *Oscillospira* would cause energetic expenditure for the host that must regenerate at least some of these glycans. Obviously, mammalian-derived glycans will also be present in high amount in animal-protein rich diets, perhaps explaining why switching to such a diet rapidly increased the relative abundance of *Oscillospira* (David et al., 2014), and potentially why *Oscillibacter*-related taxa were increased in individuals that switched to a reduced carbohydrate weight loss diet (Walker et al., 2011). What are the primary energy and carbon sources for CAG:241, which lacks the glucuronate utilization module present in CAG:83, remains unclear - presumably it can either make do with whatever substrates the Cut1 transporter can take in, or it relies on fermentation products of neighboring bacteria.

Spore-formation associated genes in *Oscillospira*

In contrast to related organisms such as *Oscillibacter valericigenes* that are non-sporulating (Iino et al., 2007), *Oscillospira* were shown to contain spore like-structures and so are expected to contain sporulation-associated genes (Mackie et al., 2003). Indeed, homologs of the small acid-soluble spore protein, spore maturation proteins A and B, six stage III sporulation proteins, and the sporulation transcriptional regulators SpoIIID, and SpoVT, are present in the genome CAG:83. CAG:241 has all putative sporulation proteins present in CAG:83, with the exception if SpoVT and an additional 19 putative sporulation proteins. Thus, sporulation is likely to be a patchily distributed trait in the *Oscillibacter* clade. Interestingly, even in the non-sporulating *O. valericigenes* a few sporulation genes exist, in particular sporulation-related regulators (Katano et al., 2012), evidence that sporulation genes could have been co-opted for other roles, especially regulatory ones.

In conclusion, Some *Oscillospira* species are likely to be able to utilize host glycans and can probably secrete the important short chain fatty acid butyrate. Butyrate has been shown to be important for prevention of inflammation (Cushing et al., 2015), by inducing differentiation of regulatory T-cells (Furusawa et al., 2013) and down-regulating the expression of genes encoding pro-inflammatory cytokines (Chang et al., 2014). As a histone deacetylase inhibitor, butyrate also has a role in

preventing colonic tumors and promoting normal cell proliferation, differentiation and apoptosis, potentially modulating the wnt signaling that is critical in colorectal cancer (Malcomson et al., 2015). More recently, a reduction in butyrate producing bacteria has been observed in patients with type II diabetes (Qin et al., 2012; Karlsson et al., 2013), and vancomycin treatment that reduced butyrate producer levels also decreased peripheral insulin sensitivity (Vrieze et al., 2014), indicating that butyrate may also play an important role in metabolic diseases (Arora and Backhed, 2016). Thus, *Oscillospira* may have important contributions for human health, and its putative butyrate production may explain why it is reduced in several inflammatory diseases (Zhu et al., 2013; Walters et al., 2014).

Tables

Table 1. Relative frequencies of *Oscillospira*-related CAGs in large metagenomic datasets (from 139 American, 368 Chinese, 401 Danish, and 359 Spanish individuals)

	American	**Chinese**	**Danish**	**Spanish**
CAG:83	53%	47%	73%	62%
CAG:129	53%	23%	76%	44%
CAG:241	10%	4%	30%	9%

Table 2. Selected genome properties of *Oscillospira*-related CAGs

Taxonomy	**%GC**	**Genome size(Mb)**	**tRNA genes**
CAG:83 (TaxID 1262992)	60.7%	1.87	33
CAG:129 (TaxID 1263003)	61.1%	1.76	26
CAG:241 (TaxID 1262911)	60.6%	1.97	38
Oscillibacter sp. ER4	57.2%	2.73	49

Legends

Figure. 1. Maximum likelihood tree of RpoB proteins of *Oscillibacter* and related bacteria

The tree was inferred by using the Maximum Likelihood method based on the Le-Gascuel model (Le and Gascuel, 2008). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1245 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Fig. 2 Functional genome composition of CAG:83, based on COG (Galperin et al., 2015) categories

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