Comparative genomics and mutational analysis reveals a novel XoxF-utilising methylotroph in the Roseobacter group isolated from the marine environment

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Author contribution statement

JCM and YC conceived the project. AH conducted all lab work except sequencing, annotation and comparative genomics, which was conducted by JV and AK. CM, CG, MT, JT and JD provided guidance and insight during the project. AH and JV wrote the manuscript, with all authors providing constructive feedback and approval of the final manuscript.

Keywords

Methylotrophy, xoxF, marine environment, Roseobacter, Comparative genomics, Methanol, Methanol dehydrogenase

Abstract

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The Roseobacter group comprise a significant group of marine bacteria which are involved in global carbon and sulfur cycles. Some members are methylotrophs, using one-carbon compounds as a carbon and energy source. It has recently been shown that methylotrophs generally require a rare earth element when using the methanol dehydrogenase enzyme XoxF for growth on methanol. Addition of lanthanum to methanol enrichments of coastal seawater facilitated the isolation of a novel methylotroph in the Roseobacter group: Marinibacterium anthonyi strain La 6. Mutation of xoxF revealed the essential nature of this gene during growth on methanol and ethanol. Physiological characterisation demonstrated the metabolic versatility of this strain. Genome sequencing revealed that strain La 6 has the largest genome of all Roseobacter group members sequenced to date, at 7.18 Mbp. Multi-locus sequence (MLSA analysis showed that whilst it displays the highest core gene sequence similarity with subgroup 1 of the Roseobacter group, it shares very little of its pangenome, suggesting unique genetic adaptations. This research revealed that the addition of lanthanides to isolation procedures was key to cultivating novel XoxF-utilising methylotrophs from the marine environment, whilst genome sequencing and MLSA provided insights into their potential genetic adaptations and relationship to the wider community.

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1 Abstract

The Roseobacter group comprise a significant group of marine bacteria which are involved in global carbon and sulfur cycles. Some members are methylotrophs, using one-carbon compounds as a carbon and energy source. It has recently been shown that methylotrophs generally require a rare earth element when using the methanol dehydrogenase enzyme XoxF for growth on methanol. Addition of lanthanum to methanol enrichments of coastal seawater facilitated the isolation of a novel methylotroph in the Roseobacter group: *Marinibacterium anthonyi* strain La 6. Mutation of *xoxF5* revealed the essential nature of this gene during growth on methanol and ethanol. Physiological characterisation demonstrated the metabolic versatility of this strain. Genome sequencing revealed that strain La 6 has the largest genome of all Roseobacter group members sequenced to date, at 7.18 Mbp. Multi-locus sequence (MLSA analysis showed that whilst it displays the highest core gene sequence similarity with subgroup 1 of the Roseobacter group, it shares very little of its pangenome, suggesting unique genetic adaptations. This research revealed that the addition of lanthanides to isolation procedures was key to cultivating novel XoxF-utilising methylotrophs from the marine environment, whilst genome sequencing and MLSA provided insights into their potential genetic adaptations and relationship to the wider community.

2 Introduction

Previous research has shown that methanol in the oceans can reach concentrations of up to 420 nM (Williams *et al*., 2004; Beale et al. 2011; Dixon *et al*., 2011; Beale *et al*., 2013; Dixon *et al*., 2013; Read *et al*., 2012 and Kameyama *et al.*, 2010). There has long been a debate as to whether the ocean is a source or sink of methanol, however it has recently been revealed that various phytoplankton in laboratory cell cultures produce substantial concentrations of methanol (0.8–13.7 µM) (Mincer and Aicher 2016). Based on these data it was estimated that phytoplankton could be the largest global source of methanol, far exceeding terrestrial plant emissions. Given the availability of methanol in the oceans, it is not surprising that some marine bacteria are able to degrade it. Methylotrophic bacteria can use one-carbon compounds, such as methanol, as a carbon and energy source (reviewed in Anthony, 1982; Chistoserdova *et al*., 2009; Chistoserdova, 2011a). The first step in methanol oxidation is catalysed by methanol dehydrogenases (MDH). The best characterised MDH is the Ca^{2+} containing periplasmic pyrroloquinoline quinone (PQQ)-dependent MDH found in Gram negative methylotrophs, which is an $\alpha_2\beta_2$ protein encoded by *mxaF* and *mxaI* (Anthony 1986; Chistoserdova 2011). A second type of methanol dehydrogenase (XoxF) encoded by a homologue of *mxaF*, *xoxF*, has been discovered in many methylotrophs (Chistoserdova and Lidstrom 1997; Giovannoni et al. 2008; Chistoserdova 2011; Keltjens et al. 2014). This MDH is phylogenetically very diverse. With five clades (named *xoxF1-5*) and often multiple gene copies present, it is generally difficult to examine the exact role in methylotrophs of MDH enzymes encoded by *xoxF* (Chistoserdova 2011; Keltjens et al. 2014).

Knowledge of marine methylotrophs has arisen from their isolation and characterisation (Yamamoto et al. 1978; Strand and Lidstrom 1984; Janvier et al. 1985; Schaefer et al. 2002; Giovannoni et al. 2008) and through the use of functional gene probing (McDonald & Murrell 1997; Neufeld et al. 2007). For example, using *mxaF* primers, Dixon *et al*., (2013) identified methylotrophs such as *Methylophaga* sp., *Burkholderiales*, *Methylococcaceae* sp., *Paracoccus denitrificans*, *Methylophilus methylotrophus*, *Hyphomicrobium sp*. and *Methylosulfonomonas methylovora* in open Atlantic waters. Active marine methylotrophs have been found to be associated with phytoplankton blooms in the English Channel (Neufeld *et al.*, 2008), and uncultivated *Methylophaga* have been identified after enrichments with ^{13}C-labelled methanol or methylamine in DNA Stable Isotope Probing (DNA-SIP) experiments using seawater from the same location (Neufeld *et al*., 2007; Neufeld *et al*., 2008; Grob *et al*., 2015).

Marine bacteria of the Roseobacter group often comprise over 20% of the total bacterial community in coastal environments, and play key roles in the global carbon and sulfur cycles (Pradella, Päuker, and Petersen 2010; Wagner-Döbler and Biebl 2006; Buchan, González, and Moran 2005). Many strains are associated with phytoplankton (Jose M Gonzalez et al. 2000; Grossart et al. 2005; Amin, Parker, and Armbrust 2012; Amin et al. 2015) and some are known to utilise one-carbon compounds (J M Gonzalez et al. 1997; Schäfer et al. 2005; F. Sun et al. 2010). For example, the methylotroph *Marinovum algicola* was isolated from the dinoflagellate *Prorocentrum limais* (Lafay et al. 1995). Hence, it is possible that such close associations are due to the ability of some Roseobacter group members to use methanol and/or other one-carbon compounds excreted by phytoplankton as carbon and energy sources. Moreover, amplicon sequencing of *xoxF* genes from clade 5 (*xoxF5)* amplified from different coastal sites (Taubert *et al.,* 2015) revealed high relative abundances of sequences from the *Rhodobacteraceae* family such as *Sagittula* (a known marine methylotroph), but also of many

unclassified *Rhodobacteraceae* sequences, supporting the hypothesis that many members of the Roseobacter group are capable of methylotrophy *in situ*. It is therefore important that the methylotrophic abilities of the marine Roseobacter group is re-examined (Martens et al. 2006; Pradella, Päuker, and Petersen 2010).

Recent research has revealed the importance of rare earth elements (REEs) such as the lanthanides cerium and lanthanum during the growth of XoxF-utilising methylotrophs (Keltjens et al. 2014; Farhan Ul-Haque et al. 2015; Vu et al. 2016; Chistoserdova 2016). Not only have these lanthanides been shown to be present at the catalytic site of XoxF, but they are also involved in the up-regulation of the expression of *xoxF* and down-regulation of the expression of the *mxaFI* genes encoding the classic MDH (Nakagawa et al. 2012; Pol et al. 2014; Bogart, Lewis, and Schelter 2015; Wu et al. 2015; Keltjens et al. 2014; Farhan Ul-Haque et al. 2015).

REEs are highly insoluble and are rarely found in pure form (Hu et al. 2004) and due to the relative difficulty in quantifying REEs, they are not usually measured during environmental sampling. Studies have shown that concentrations can range from high nM in estuarine and coastal environments (Elderfield, Upstill-Goddard, and Sholkovitz 1990; Hatje, Bruland, and Flegal 2014) to pM concentrations in open oceans (Garcia-Solsona et al. 2014; Greaves, Rudnicki, and Elderfield 1991). However, very little is known about the bioavailability of REEs in the marine environment. The REE-specific *xoxF* gene is found in the genomes of a broad range of bacteria and is widely distributed throughout marine environments (Chistoserdova 2016; Taubert et al. 2015). It is clear, therefore, that the routine addition of REEs to enrichments is vital in capturing and isolating new methylotrophs. Here we report on the isolation of a novel methylotrophic Roseobacter (strain La 6) from lanthanum-supplemented enrichments containing methanol and seawater from the coast of Plymouth, UK. The methylotrophic nature of this strain was further characterised, and the genome sequenced and compared to other members of the Roseobacter group.

3 Methods

3.1 Strains, plasmids and culture conditions

Strains and plasmids used in this study are listed in Supplementary Table 1. Strain La 6 was maintained on Marine Broth 2216 (Difco, MB) (1.5% agar) or Marine Basal Medium (MBM) with 5 mM carbon source and grown at 25°C unless otherwise stated. *Escherichia coli* was grown at 37°C on Luria-Bertani (LB) (Sambrook and W Russell 2001). Antibiotics were used at the following concentrations (μg ml^{-1}): kanamycin (20), gentamicin (10) and rifampicin (20), unless otherwise stated. All carbon sources were added at 5 mM and lanthanides at 5 μM.

3.2 Lanthanide experiments and isolation of strain La 6

Seawater used for all experiments was collected from station L4 of the Western Channel Observatory, Plymouth, UK (50°15.0'N; 4°13.0'W). For lanthanide addition experiments, triplicate gas-tight 2 L bottles were filled with 0.75 L of surface seawater, with the addition of 0.1% marine ammonium mineral salts (MAMS) medium (Goodwin et al. 2001), 5 mM methanol and either 5 μM lanthanum, cerium, both or no metals (added as chloride heptahydrate salts). Enrichments were incubated at 25°C in a shaking incubator (50 rpm) and the methanol headspace concentration was monitored by gas chromatography as a proxy for methanol consumption in the liquid phase (methods described in supplementary information).

Strain La 6 was isolated in October 2014 using the same experimental set up as the lanthanide addition experiments, with only lanthanum as the added metal. Enrichments were incubated for 5 days, serial dilutions of this enrichment were then plated onto MBM medium containing lanthanum and incubated with methanol in the headspace of a gas tight chamber for 8 days. Colonies were re-streaked to purify and growth on methanol was confirmed by inoculation into liquid MBM containing methanol and lanthanum. Methods for physiological characterisation of the strain can be found in the supplementary information.

3.3 Genetic manipulations

A single allelic exchange method was used to generate an insertional mutation in the *xoxF* gene of *Marinibacterium* sp. La 6 (Todd et al. 2011). A 672bp internal fragment of the *xoxF* gene was amplified by PCR, ligated into the suicide vector pK19mob (Schäfer *et al.*, 1994) to form p672*xoxF* and transformed into *E. coli*. Plasmid p672*xoxF* was conjugated into strain La 6^{Rif}, a spontaneous rifampicin-resistant mutant, in triparental matings with helper plasmid pRK2013 (Figurski and Helinski 1979). Rif^R and Kan^R single cross over transformants were checked using colony PCR with primers that amplified a region spanning from within the disrupted genomic *xoxF* gene to inside the kanamycin cassette of the incorporated p672*xoxF* plasmid (Supplementary Table 1). The mutant strain was termed La 6 $XoxF^-$. To complement strain $XoxF^-$, the complete *xoxF* sequence was amplified by PCR, ligated into the broad host range vector pLMB509 (Tett et al. 2012) and transformed into *E. coli*. Transconjugants were screened using the primers that were used to originally amplify the *xoxF* gene and the insert was then sequenced. The confirmed plasmid was termed p509LA6. This plasmid was then conjugated into La 6^{Rif} using triparental matings, and the resulting complemented strain was termed La 6 $XoxF^-$ p509LA6.

3.4 Genome sequencing, assembly and annotation

Genomic DNA was extracted using the CTAB (cetyl-trimethylammonium bromide) method of Doyle & Doyle (1987). The genome of strain La 6 was sequenced as follows: standard and mate-pair sequencing libraries were produced using Illumina kits and run on a Miseq machine using V3 chemistry with a paired-end approach and 301 cycles per read. Reads were adapter-clipped and quality trimmed using Trimmomatic (Bolger, Lohse, and Usadel 2014). Mate-pair reads were additionally clipped, sorted and re-orientated using NxTrim (O'Connell et al. 2015). Potential PhiX and vector contamination were filtered out using fastq_screen (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/), while low complexity reads (consisting entirely of only one base type or direct short oligonucleotide repeats) were removed using prinseq (Schmieder and Edwards 2011). Potential overlapping paired-end reads were merged using FLASH (Magoč and Salzberg 2011). Assembly was done using Spades v.3.8. ORF-calling and annotation were done using the PROKKA pipeline v.1.12 (Seemann 2014). The draft genome sequence of strain La 6 is available in GenBank under accession number NSDV00000000; the strain deposit number is DSM 104755.

3.5 Comparative genomics

For MLSA, the unique core genome of 94 comparison genomes (including *Pavularcula bermudensis* HTCC2503 as the outgroup) consisting of 219 gene products with a combined length of 95,680 amino acid residues was determined using the bidirectional BLAST+ approach implemented in proteinortho5 (Lechner et al. 2011), excluding all genes with duplicates in any comparison genome. After alignment with muscle (Edgar 2004), the gene products were concatenated and un-alignable regions were filtered out using gblocks

(Castresana 2000), leaving 56,810 aligned amino acid residues for phylogenetic analysis. Clustering was performed using the Neighbour Joining algorithm with 1,000 bootstrap permutations.

For gene content analyses, a binary matrix was constructed, representing the presence or absence of orthologous groups identified by the bidirectional BLAST+ approach mentioned above. In order to prevent artefacts caused by fragmented or falsely predicted genes, all singletons were excluded from the analyses (requiring each considered orthologous group to be present in at least two different genomes). This resulting binary matrix was converted into a distance matrix and clustered using the neighbour joining algorithm and 1,000 bootstrap permutations.

4 Results and Dicussion

4.1 Isolation of a novel methylotroph using lanthanum

Traditional methylotroph enrichment and isolation experiments using water from station L4 of the Western Channel Observatory (Plymouth, UK; 50°15.0'N; 4°13.0'W) not supplemented with lanthanides frequently gave rise to the isolation of *Methylophaga* sp. (Howat 2017), whilst cultivation-independent research using DNA-SIP consistently showed that *Methylophaga* are also the dominant methylotrophs metabolising methanol in enrichment cultures (Neufeld et al. 2007; Neufeld, Chen, et al. 2008; Grob et al. 2015). *Methylophaga* spp. contain both *mxaF* and multiple copies of *xoxF*, and while there has been no direct evidence that *Methylophaga* spp. use MxaF rather than XoxF during growth on methanol, high levels of MxaF expression have been observed when methylotrophs are grown on methanol, suggesting the use of this calcium-containing methanol dehydrogenase enzyme (Choi et al. 2011; Kim et al. 2012). However, the model methylotroph *Methylobacterium extorquens* also contains both *xoxF* and *mxaF* genes, and work on this bacterium showed that it expressed XoxF instead of MxaF when lanthanide concentrations were higher than 100 nM (Vu et al. 2016). It may be possible that the seawater used in previous methanol enrichment experiments described above did not contain sufficient concentrations of REEs to support growth of XoxF-utilising methylotrophs. Therefore, the effect of the addition of lanthanides to seawater enrichments containing methanol was examined using surface seawater from station L4, Plymouth.

Methanol enrichments containing either 5 μM lanthanum, cerium or both showed a significant increase in methanol depletion ($p \leq 0.05$) compared to those without, suggesting that the bacterial oxidation of methanol was stimulated by the addition of the metals (Supplementary Figure 1). When lanthanum was then added to subsequent enrichments and isolation agar, a novel methylotroph (strain La 6) was isolated from station L4. This strain represented three out of 20 screened isolates selected for their ability to grow on methanol; all other strains being *Methylophaga* sp.). The corresponding 16S rRNA gene sequence of the isolate was 99% identical to *Marinibacterium profundimaris* strain 22II1-22F33T (Supplementary Figure 2) (Li et al. 2015) (Li et al. 2015). The relatively low colony counts of this isolated Roseobacter probably reflected the fact that they were a small proportion of the methylotrophs present in the seawater enrichment, however previous research using very similar enrichment procedures gave rise to no Roseobacters at all (Howat, 2017), suggesting that the addition of lanthanum aided methylotrophic growth of Roseobacters to support a population dense enough to be subsequently isolated.

PCR assays on genomic DNA from strain La 6 and subsequent Sanger sequencing indicated that the isolate contained only one copy of *xoxF* from clade 5 and no *mxaF* in its genome (later confirmed by genome sequencing, see below). When grown in MBM, strain La 6 exhibited lanthanum-stimulated growth on methanol, whilst there was an absolute requirement for lanthanum ions when grown on ethanol as carbon source **(Figure 1)**.

M. profundimaris was not previously tested for growth on methanol and its genome contained no predicted MDH. Therefore the physiology of strain La 6 was further characterised, the genome sequenced and its ability to grow methylotrophically was investigated to further understand the role of *xoxF5* in this marine strain.

4.2 Physiological characteristics

Strain La 6 utilised a wide range of carbon compounds including methanol, ethanol, propane and butane (for a full list of compounds see Supplementary Table 2). Tests for growth of the strain on methanol at concentrations higher than 5 mM yielded no increase in final cell density.

Strain La 6 is a Gram negative, ovoid rod, 0.8-2.2 µm long and 0.5-1.2 µm wide when grown on minimal medium. It is non-motile when tested on swimming, swarming or twitching motility plates and in liquid medium. Colonies are very pale cream and 0.5-1.0 mm in diameter, uniformly circular, convex and opaque after growth on MBM minimal media at 25°C for 6 days. Colonies are cream and 0.6-1.2 mm in diameter, uniformly circular, convex and opaque after growth on marine agar 2216 at 25°C for 4 days.

Temperature range for growth was 4-45°C, with the optimum at 37°C. The pH range for growth was pH 4.5-9 (optimum 7.5) and the NaCl concentrations for growth were 0-15% w/v (optimum 3%), with no growth at 20%. It did not grow under anaerobic conditions and did not reduce either nitrate or nitrite. It did not hydrolyse cellulose, gelatine or starch, nor did it ferment glucose or lactose aerobically or anaerobically. Strain La 6 was negative for thiosulfate oxidation. It produced indole-acetic acid when supplemented with tryptophan, but not without. Strain La 6 did not produce any acetone/methanol extractable pigments or bacteriochlorophyll *a* after growth in either a light/dark cycle or in the dark after 5 days at 22°C, therefore suggesting growth of the isolate is exclusively chemoheterotrophic and non-photosynthetic. Strain La 6 required vitamin B_{12} for growth, and was oxidase and catalase positive. Like many of the family of the *Rhodobacteraceae*, the principle fatty acid composition was 18:1ω7c (67.83%) and had a fairly similar profile to *M. profundimaris* 22II1-22F33, however it can be differentiated by the presence of summed feature 2 (14:0 3-OH/16:1) (7.31%), (Supplementary Table 3).

4.3 Genome sequencing and genome analysis of strain La 6

Sequencing of the genome of strain La 6 yielded 15 contigs covering a total length of 7.2 Mbp (mol % GC content 65.4). Based on sequence similarities, 73% of protein-coding genes could be assigned a putative function, whilst one quarter of them were classified as 'hypothetical', using the software tool PROKKA (Seemann 2014) (full genome statistics are summarised in **Table 1**). Assessment of the genome quality using CheckM (Parks et al. 2015) yielded a 'completeness' value of 99.41%, which is above the average value of 99.1% found in the currently published Roseobacter group genomes, indicating complete genome reconstruction (Supplementary Table 4). The genome suggested a complete tricarboxylic acid

cycle (TCA) pathway and genes for the pentose phosphate pathway, Entner-Doudoroff and Embden-Meyerhof pathways. It contained all genes required for ammonia assimilation (including glutamate dehydrogenase, glutamine synthetase, glutamine oxoglutarate amidotransferase and alanine dehydrogenase) and those encoding nitrogenase; it did not contain genes encoding ribulose-1,5-bisphosphate carboxylase/oxygenase.

4.4 Genome-inferred methylotrophic pathways in strain La 6

Genome sequencing confirmed that *xoxF* from clade 5 (*xoxF5*, one copy) was the only predicted MDH-encoding gene in the genome of strain La 6, and that it was adjacent to *xoxG* (encoding an associated cytochrome c used as an electron acceptor during methanol oxidation) and *xoxJ*, encoding a putative periplasmic binding protein (Chistoserdova 2011). Adjacent genes were similar to those found in the known methylotrophs *Rhodobacter sphaeroides* and *Paracoccus aminophilus* JCM7686, that employ the glutathione-dependent formaldehyde oxidation pathway (Wilson, Gleisten, and Donohue 2008; Dziewit et al. 2015) and only contain *xoxF5* (**Figure 2**).

In *R. sphaeroides*, the formaldehyde produced by XoxF is initially converted to S-hydroxymethyl-gluthathione (GS-CH_2OH) by a glutathione-formaldehyde activating enzyme (Gfa) or by a spontaneous reaction. This is then further oxidised by other enzymes to CO_2 to generate energy (Wilson, Gleisten, and Donohue 2008). However, unlike *R. sphaeroides*, the gene cluster around *xoxF5* of strain La 6 does not contain *gfa* (see Figure 2). BLAST searches of the genome using the Gfa from *R. sphaeroides* revealed some candidates, however none were more than 35% identical at the amino acid level. Searches for a formaldehyde activating enzyme gene, *fae*, which is used in other organisms revealed no candidates either (Vorholt et al. 2000). It is possible, therefore, that strain La 6 either does not contain a gene responsible for converting formaldehyde to GS-CH_2OH, relying solely on a spontaneous chemical reaction, or it has an as yet-unidentified mechanism (**Figure 3**).

La 6 contained *gmaS*, a key gene of the N-methylglutamate pathway for methylamine metabolism. It did not contain, *mauA*, the gene encoding for a subunit of an alternative methylamine degrading enzyme, methylamine dehydrogenase. However, the strain was unable to grow on methylamine as a carbon and energy source (**Supplementary information**). Lastly, strain La 6 also contains the gene encoding methyl-H4F reductase (MetF) which oxidises methyl-H4F originating from demethylation reactions such as in the metabolism of DMSP or chloromethane (Studer et al. 2001; Studer et al. 2002; Reisch et al. 2008; Curson et al. 2011). However, strain La 6 did not contain the *cmuAB* or *dmdA* genes that would suggest metabolism of chloromethane or DMSP (further discussed below).

For carbon assimilation, the genome of strain La 6 contains all the genes of the tetrahydrofolate-linked (H_4F) pathway. This pathway generates the key metabolite methylene-H_4F, which can either feed into the serine cycle for assimilation or serve as a further source of formate for generating energy (Chistoserdova, 2011). In strain La 6, this pathway may either rely on the spontaneous reaction between formaldehyde and H_4F or it may also be possible that FolD (bifunctional methylene-H_4F dehydrogenase–methenyl-H_4F cyclohydrolase) can function in the reductive direction and generate methylene-H_4F for assimilation (Chistoserdova, 2011). Formate generated through the glutathione-linked pathway could be fed *via* the reversible enzyme formyl-H_4F ligase (FtfL) and methenyl-H_4F cyclohydrolase (Fch) onto FolD. The genome of strain La 6 also contains genes encoding for three formate dehydrogenases (FDH); FDH1, 2, and 3.

Strain La 6 contained all the genes of the serine pathway. Methylotrophs utilizing the serine cycle require an additional pathway for regenerating glyoxylate; strain La 6 encodes all the genes for the ethylmalonyl-CoA pathway (EMCP) and does not contain isocitrate lyase, whilst it also had the potential to make PHB, containing the PHB synthase genes. A summary of predicted methylotrophic pathways based on the genome sequence and some physiological data is shown in **Figure 3**.

4.5 The role of XoxF during growth of strain La 6 on methanol and ethanol

XoxF5 is the sole MDH responsible for methanol oxidation in the two relatives of the Rosoebacter group, *R. sphaeroides* and *P. aminophilus*. However there are many Roseobacters that contain either a single *xoxF* from clade 5 but are unable to grow on methanol (or have not been tested) or the role of *xoxF5* of those that do grow on methanol was not previously examined (Shiba 1991; Lee et al. 2007; Li et al. 2015; Cho and Giovannoni 2006). Thus, we investigated the role of the *xoxF5* gene in strain La 6. Mutation of *xoxF5* in strain La 6 abolished the growth of the mutant strain La 6 XoxF$^-$ on both methanol and ethanol (**Figure 4**). Cell-free extracts of the wild-type strain grown on methanol contained substantial methanol dehydrogenase activity (262 nmol min^{-1} mg^{-1} protein; ± 6 s.e). SDS-PAGE and mass spectrometry analysis of the wild-type grown on various carbon sources (methanol, ethanol, succinate or benzoate) revealed the expression of XoxF in cells grown under all of these conditions, whilst the mutant did not express XoxF (**Supplementary Figure 3**). Complementation of the mutant with the wild-type *xoxF5* gene restored growth on both methanol and ethanol. SDS-PAGE analysis of cell free-extracts of this complemented *xoxF5* mutant confirmed restoration of expression of XoxF5 (**Supplementary Figures 3 and 4**). These data confirm that *xoxF5* is directly involved in the oxidation of methanol and ethanol in strain La 6 and that XoxF5 is essential for growth on these compounds.

4.6 Roseobacter-specific traits

Members of the Roseobacter group are known to grow on various aromatic and phenolic compounds (Buchan 2001; Buchan, Neidle, and Moran 2004; Alejandro-Marín, Bosch, and Nogales 2014). The ability of these organisms to degrade naturally occurring but potentially harmful compounds such as polycyclic aromatic hydrocarbons (PAHs) demonstrates the ecological importance of the Roseobacter group (Seo, Keum, and Li 2009). When tested, strain La 6 grew on a range of aromatics, including benzoate, 4-hydroxybenzoate, protocatechuate and catechol. Analysis of the genome revealed the presence of genes that could explain such capabilities, such as the *benABCD* cluster which encodes for benzoate dioxygenase, and the *pcaQDCHGB* cluster for protocatechuate metabolism (Buchan, Neidle, and Moran 2004; Alejandro-Marín, Bosch, and Nogales 2014). Strain La 6 was unable to grow on toluene, p-cresol, p-xylene, 3-hydroxybenzoate, benzene, naphthalene, vanillate or 4-chlorobenzoate.

Many Roseobacters are also able to metabolise the abundant sulfurous osmolyte dimethylsulfoniopropionate (DMSP), via demethylation and/or cleavage generating methanethiol or dimethylsulfide (DMS), respectively (Curson et al. 2011). DMS oxidation products in the atmosphere can act as cloud condensation nuclei, as chemo-attractants for many marine animals and are a major source of organic sulfur in the sulfur cycle (Schäfer et al. 2010; Curson et al. 2011; Moran et al. 2012). As with many Roseobacters, strain La 6 did not grow on DMSP as sole carbon source, but whole cells of strain La 6 did cleave DMSP,

generating DMS at a rate of 72 nmol min^{-1} mg^{-1} protein (4.8 s.e.). This DMSP-dependent DMS production is probably due to expression of the DMSP lyase gene *dddL* (which has 48% identy to DddL of *Sulfitobacter* sp. EE-36) that is present in the genome of strain La 6 (Curson et al. 2011). As mentioned previously, the genome of strain La 6 lacked a *dmdA* gene homologue, which encodes the DMSP demethylase enzyme (Moran et al. 2012), which is consistent with our finding that La 6 produced no MeSH above background levels (data not shown).

Recently Curson et al., 2017 discovered that some Roseobacters, such as *Labrenzia agreggata*, can produce DMSP and contain the *dsyB* gene, which encodes the key methylthiohydroxybutyrate methyltransferase enzyme of DMSP synthesis (Curson et al. 2017). The genome of strain La 6 contained a *dsyB* homologue (73 % amino acid identity to *L. agreggata* DsyB) and strain La 6 cell also synthesised DMSP at a rate of 2.3 nmol min^{-1} mg^{-1} protein (0.15 s.e.). It will be interesting to investigate why strain La 6 produces DMSP and what its intracellular function is in future studies. Some members of the Roseobacter group can also produce DMS independently of DMSP via methylation of methane-thiol, and contain the methanethiol methyltransferase enzyme termed MddA (Carrión et al. 2015). However, strain La 6 contains no MddA homologue and produced no DMS when grown in the absence of DMSP, irrespective of MeSH addition. The fact that strain La 6 produces DMSP but releases no detectable DMS in the absence of DMSP addition at high levels suggests that the DMSP lyase might only function when DMSP reaches high intracellular levels (J. Sun et al. 2016). Again, this aspect or organic sulfur metabolism in strain La 6 warrants further investigation in the future.

4.7 Comparative genomics

Members of the Roseobacter group are known for having large genomes, versatile metabolic capabilities and a relatively high GC contents (Luo and Moran 2014). Strain La 6 is no exception. Indeed, it has the largest genome of all sequenced members of the Roseobacter group to date, at 7.18 Mbp, compared to the next largest genome of *M. profundimaris* strain 22II1-22F33T at 6.15 Mbp (**Figure 5**). Although the high similarity of the 16S rRNA gene sequences suggests they are the same species, the estimated DNA-DNA-Hybridization (DDH) value between *M. profundimaris* 2II1-22F33 and strain La 6, determined using the GGDC online tool (Meier-Kolthoff, Klenk, and Göker 2014), is 35%. The probability for being the same species given by GGDC is <1%, therefore supporting the designation of strain La 6 as a new species within the genus *Marinibacterium.* Analyses of homologs shared between the two strains also reveal that whilst 74% of the protein coding genes of *M. profundimaris* have a homolog in strain La 6, only 64% of the protein coding genes in the genome of strain La 6 have a homolog in *M. profundimaris* (**Table 1**).

Multi-Locus Sequence Analysis (MLSA) was performed in order to examine the phylogenetic relationship based on sequence comparisons of the unique Roseobacter core genome, with a similar topology seen from previous analyses (Buchan, González, and Moran 2005; Newton et al. 2010; Luo and Moran 2014; Simon et al. 2017). Gene content analysis was performed and compared against the MLSA to investigate the similarities and differences in gene composition between genomes, thereby reflecting possible adaptations to individual niches and lifestyles (**Figure 6**). Overall, strain La 6 clusters deeply but coherently within subgroup 1 of the Roseobacter group, which currently consists of at least seven genera such as *Leisingera, Ruegeria, Sedimentitalea* and *Marinibacterium*. However, at a gene content level, strain La 6 (and *M. profundimaris*) clusters distinctly apart from subgroup 1 and far more closely with the *Oceanicola* and *Celeribacter* genera as well as *Ketogulonicigenium*

vulgare, indicating unique genetic adaptations. Bi-directional BLAST searches of all validly published Roseobacter genomes for *xoxF5* also showed that just under one fifth of all genomes harbour this gene (**Supplementary Table 5**).

5 Conclusions

By adding lanthanides to methanol seawater enrichments, we isolated a novel member of the Roseobacter clade that can use methanol as a carbon and energy source. This isolation arose due to the discovery that upon addition of either cerium or lanthanum to methanol seawater enrichments, there was a marked increase in methanol oxidation compared to enrichments without added lanthanides. Due to the difficulty in quantifying lanthanides in marine samples, at the time of sampling it was not possible to measure the standing concentrations of these in the coastal seawater samples. However, the results do suggest that concentrations were low enough such that the addition of 5 μM lanthanide was sufficient to stimulate an increase in biological methanol oxidation.

Whilst it is known that XoxF is a lanthanide dependent enzyme in some strains, our results from growth experiments with strain La 6 suggested that lanthanum was not strictly required for growth on methanol, only for ethanol, as there was only a slight stimulation upon addition of the metal. Contamination of lanthanides from glassware is sufficient to support the growth of some methylotrophs (Pol et al. 2014), however this does not explain why strain La 6 was completely unable to grow on ethanol in similar levels of lanthanide 'contaminants'. In order to understand the catalytic mechanism of this XoxF, further work should involve purification of the enzyme from cells grown with different metal compositions and the affinities of these enzymes for methanol, ethanol and other alcohols would need to be examined.

Elucidation of the role of XoxF in this strain is important since many members of the Roseobacter group contain *xoxF* genes. The role of *xoxF* in these marine bacteria warrants further investigation, especially in cultures that are supplemented with lanthanides. Our findings that just under 20% of the Roseobacter genomes examined in this study contain a *xoxF5* suggest that the potential for methylotrophy within this group is larger than previously thought. Since many Roseobacter strains harbour *xoxF5* sequences, this could have important implications for the capacity of the marine environment to act as a sink of methanol and needs to be investigated further, especially since many strains are associated with phytoplankton (Jose M Gonzalez et al. 2000; Grossart et al. 2005; Amin, Parker, and Armbrust 2012; Amin et al. 2015) which have recently been shown to produce high concentrations of methanol. Therefore further work will include investigating the distribution, diversity and activity of such methylotrophs in the marine environment using a variety of cultivation-independent techniques.

16S rRNA gene sequence comparisons place strain La 6 unambiguously within the genus *Marinibacterium*, while overall genome similarities to the type strain *M. profundimaris* 2II1-22F33, determined via digital DDH, were shown to be clearly below the common species cutoff of 70% (Goris et al. 1998; Meier-Kolthoff, Klenk, and Göker 2014). Furthermore, the vast differences seen between strain La 6 and its closest neighbours at the MLSA and gene content level clearly demonstrates the need for comparative genomics to be used as a tool to understand the ecological roles and metabolic plasticity of different members of the Roseobacter group. Based on this and the DDH values, we propose that the strain La 6 represents a novel species of the genus *Marinibacterium*. We propose the name

Marinibacterium anthonyi strain La 6 (in honour of the British microbiologist Professor Christopher Anthony).

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7 Author contributions

JCM and YC conceived the project. AH conducted all lab work except sequencing, annotation and comparative genomics, which was conducted by JV and AK. CM, CG, MT, JT and JD provided guidance and insight during the project. AH and JV wrote the manuscript, with all authors providing constructive feedback and approval of the final manuscript.

8 Conflict of Interest Statement

The Authors declare no conflict of interest with this manuscript.

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Figure Legends

Figure 1 Effect of the presence (black circles) or absence (white circles) of 5 μM lanthanum on the growth (solid lines) of strain La 6 on methanol (A, 5 mM initial concentration) and ethanol (B, 5 mM initial concentration). Dotted lines represent headspace methanol concentrations. Grey circles are no-inoculum controls containing lanthanum. Error bars are the standard error of three replicates.

Figure 2 Gene cluster surrounding the predicted methanol dehydrogenase gene *xoxF5* and comparison to the methylotroph *Rhodobacter sphaeroides* 241. Colours indicate predicted similar functions of genes between the two organisms. *adhI*, glutathione-dependent formaldehyde dehydrogenase; *soxH*, putative protein SoxH; *xoxF5*, methanol dehydrogenase; *xoxG*, cytochrome c-553i; *xoxJ*, hypothetical periplasmic binding protein; *gfa*, homologue of glutathione-formaldehyde activating enzyme; cytochrome c oxidase II.

Figure 3 Predicted metabolic pathway of methanol metabolism in strain La 6 based on genome sequence analysis. Enzymes are shown in red whilst compounds and names of pathways are in black. Solid arrows indicate enzymatic reactions, dashed arrows indicate reactions are non-enzymatic or unknown. Reactions within the blue box are part of the dissimilatory pathway, in green are the assimilatory pathway. XoxF, methanol dehydrogenase; GSH-FDH, glutathione-dependent formaldehyde dehydrogenase; FGH, *S*-formylglutathione hydrolase; FDH, formate dehydrogenase; PurU, 10-formyl-H_4F hydrolase; FtfL, formyl-H_4F ligase; FolD, bifunctional methylene-H_4F dehydrogenase– methenyl-H_4F

cyclohydrolase; Fch, methenyl-H_4F cyclohydrolase; MetF, methyl-H_4F reductase; EMC, Ethylmalonyl-CoA; PHB, polyhydroxybutyrate.

Figure 4 Growth of La 6 wild-type strain (black triangles), strain XoxF$^-$ (red triangles) or no inoculum controls (white circles) on 5 mM methanol initial concentration (A) and 5 mM ethanol initial concentration (B). Dashed lines in (A) represent methanol headspace concentrations. All conditions contained 5 μM lanthanum. Error bars show standard error of three replicate cultures.

Figure 5 Relationship between genome size and number of genes in the genome of strain La 6 compared to the genomes of 114 members of the Roseobacter group. The genome of strain La 6 is the represented by the black cross, the black triangle is the closest relative at the 16S rRNA gene sequence, *Marinibacterium profundimaris* strain 22II1-22F33T and grey circles depict all other members of the Roseobacter group.

Figure 6 Clustering of Roseobacter group genomes showing the relationships between sequenced strains based on Multi Locus Sequence Analyses (MLSA) as well as gene content. MLSA (left) is based on concatenated aligned core-genome gene product sequences and illustrates phylogenetic relationships with high resolution and confidence. Coherent clusters corresponding to the 5 subgroups originally described by Newton et al (2010) are marked in colour. Corresponding branches between the MLSA and gene content tree are indicated by identical numbering. For ease of viewing, genera and species consisting of multiple genomes which cluster coherently in the MLSA as well as the gene content tree are shown collapsed. Furthermore, the outgroup (*Parvularcula bermudensis* HTCC2503) is not shown. In contrast, gene content clustering (right) is based on the presence and absence of orthologs shared between the comparison genomes. This illustrates similarities and differences in gene composition between genomes, thereby reflecting adaptations to individual niches and lifestyles. Divergences between MLSA- and gene content-based clustering show that even closely related strains may possess strongly diverging gene compositions.

877 **Table 1** Genome statistics of strain La 6 compared to *M. profundimaris* strain 22II1-22F33T.

Genome data	**Strain La 6**	***M. profundimaris***
Genome size (bp)	7, 179, 825	6,152,202
GC content (%)	65.4	66.2
Number of contigs	15	60
Smallest contig (bp)	948	580
Largest contig (bp)	3,672,580	1,058,968
Average contig size (bp)	478,655	-
Median contig size (bp)	103, 981	-
N50	3,672,580	343,537
L50	1	5
Number of genes	6,844	5,628
Number of Coding Sequences (% of homologs with closest strain)	6,785 (64%*)	5,497 (74%**)
Number of hypothetical proteins (%)	1,835 (27)	-
tRNAs	52	44
rRNAs	6	4

878 *% of the protein coding genes in La 6 that have a homolog in *M. profundimaris*.
879 **% of the protein coding genes in *M. profundimaris* that have a homolog in strain La 6.

Figure 1.TIF

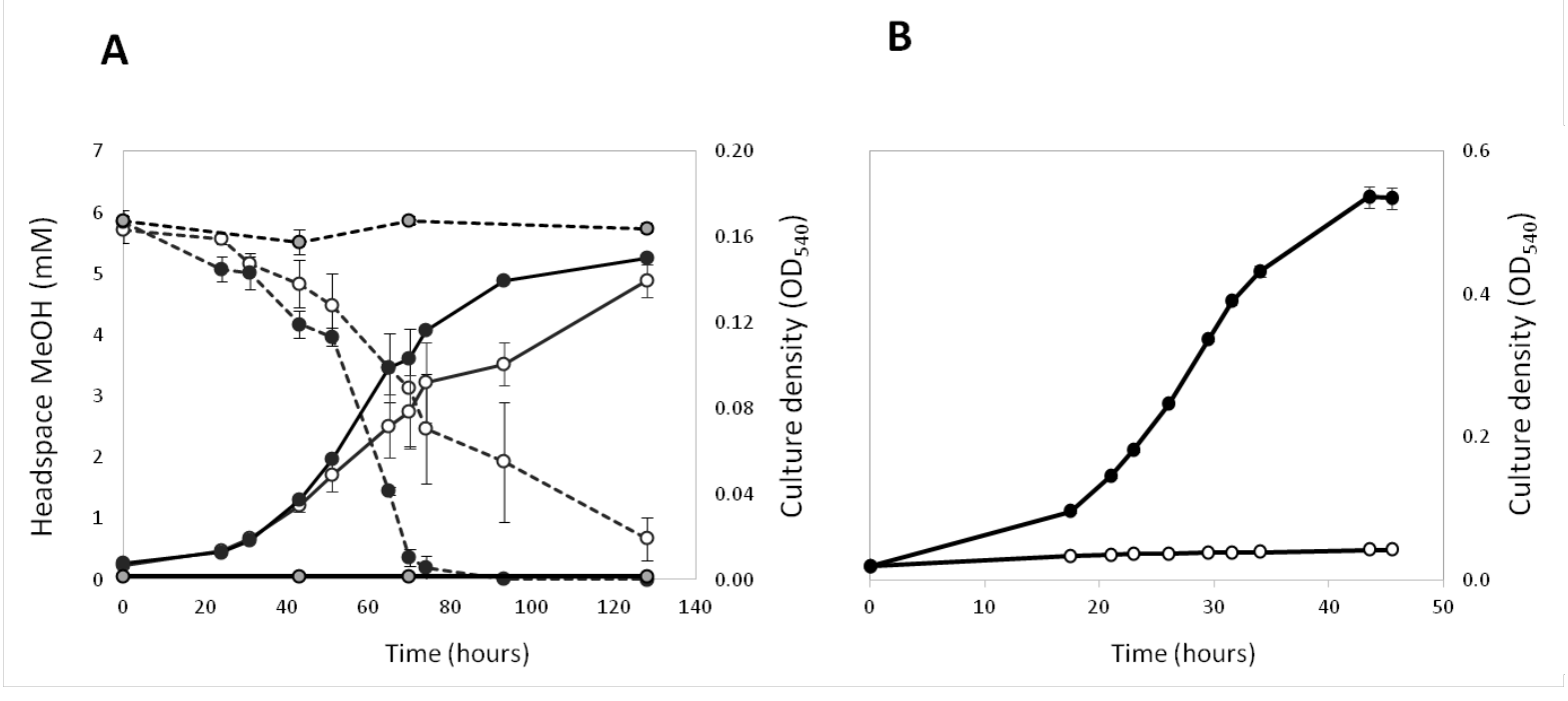
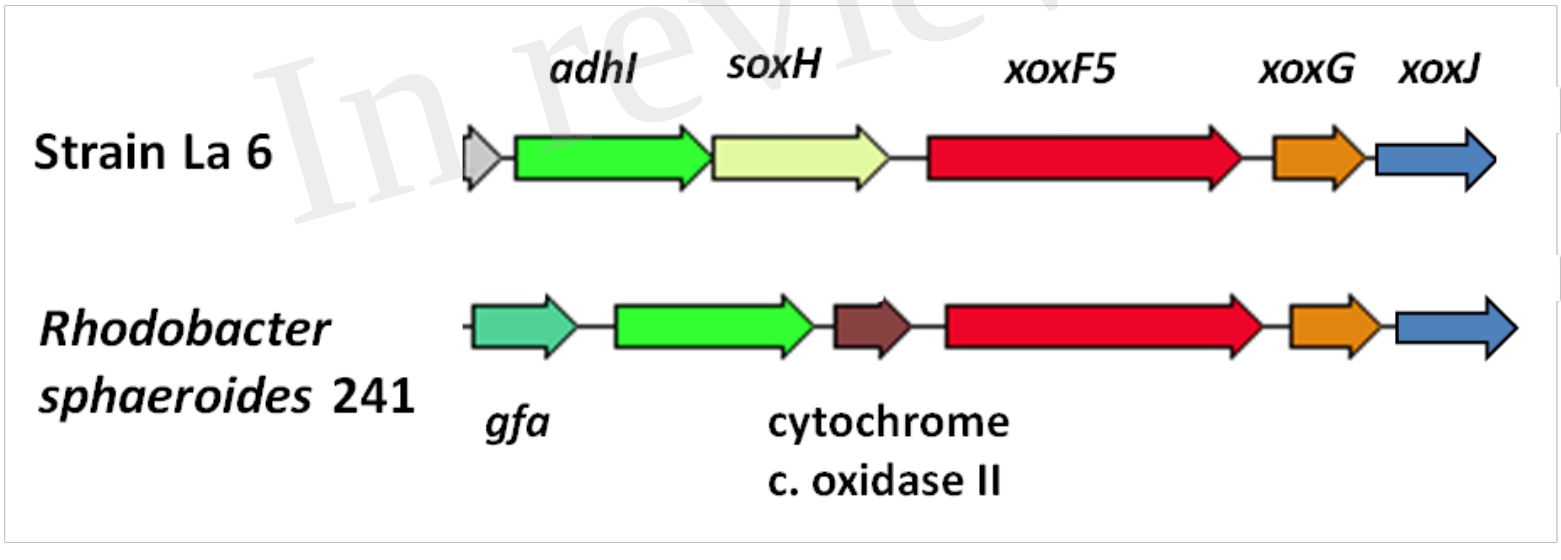


Figure 2.TIF



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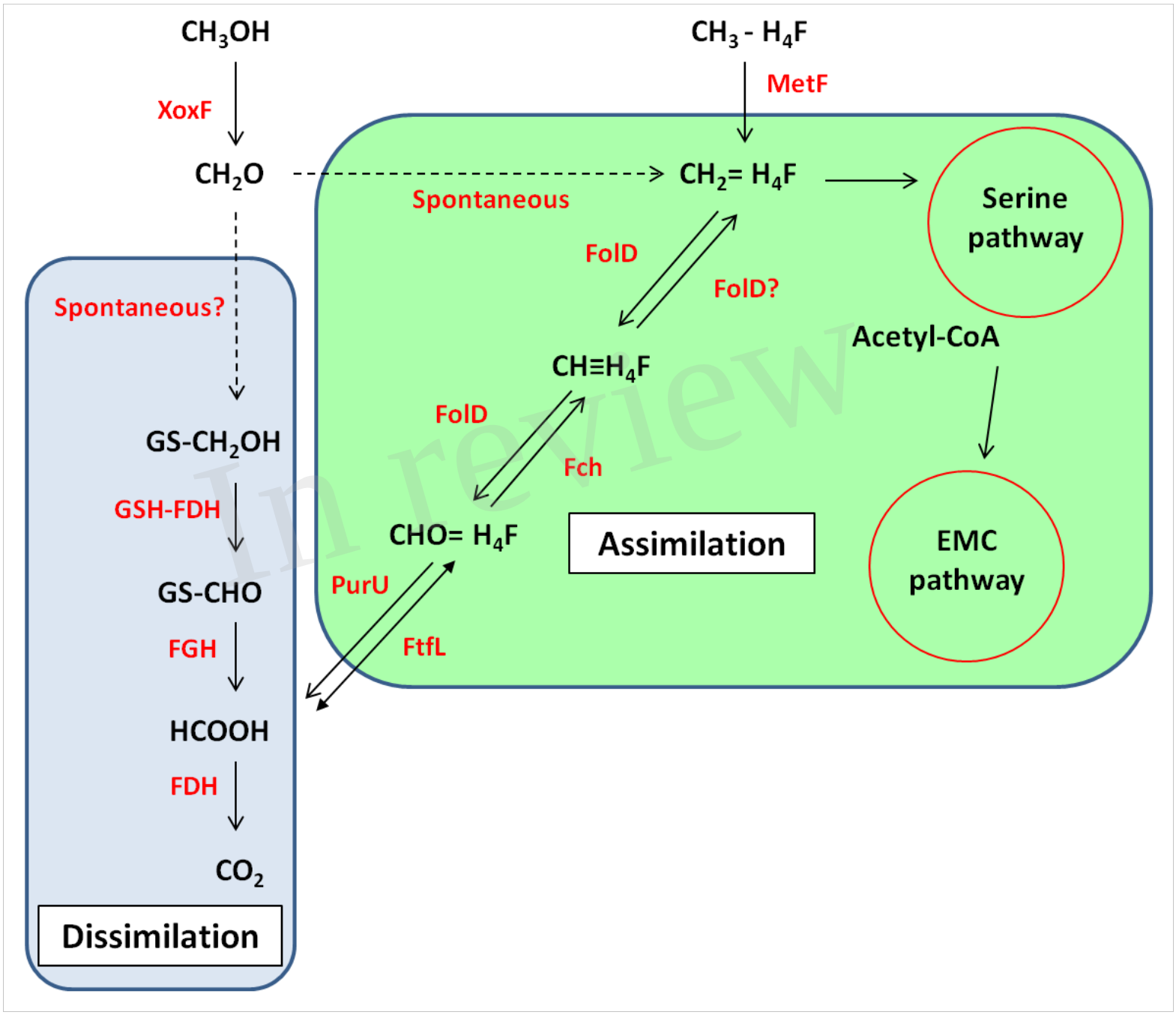

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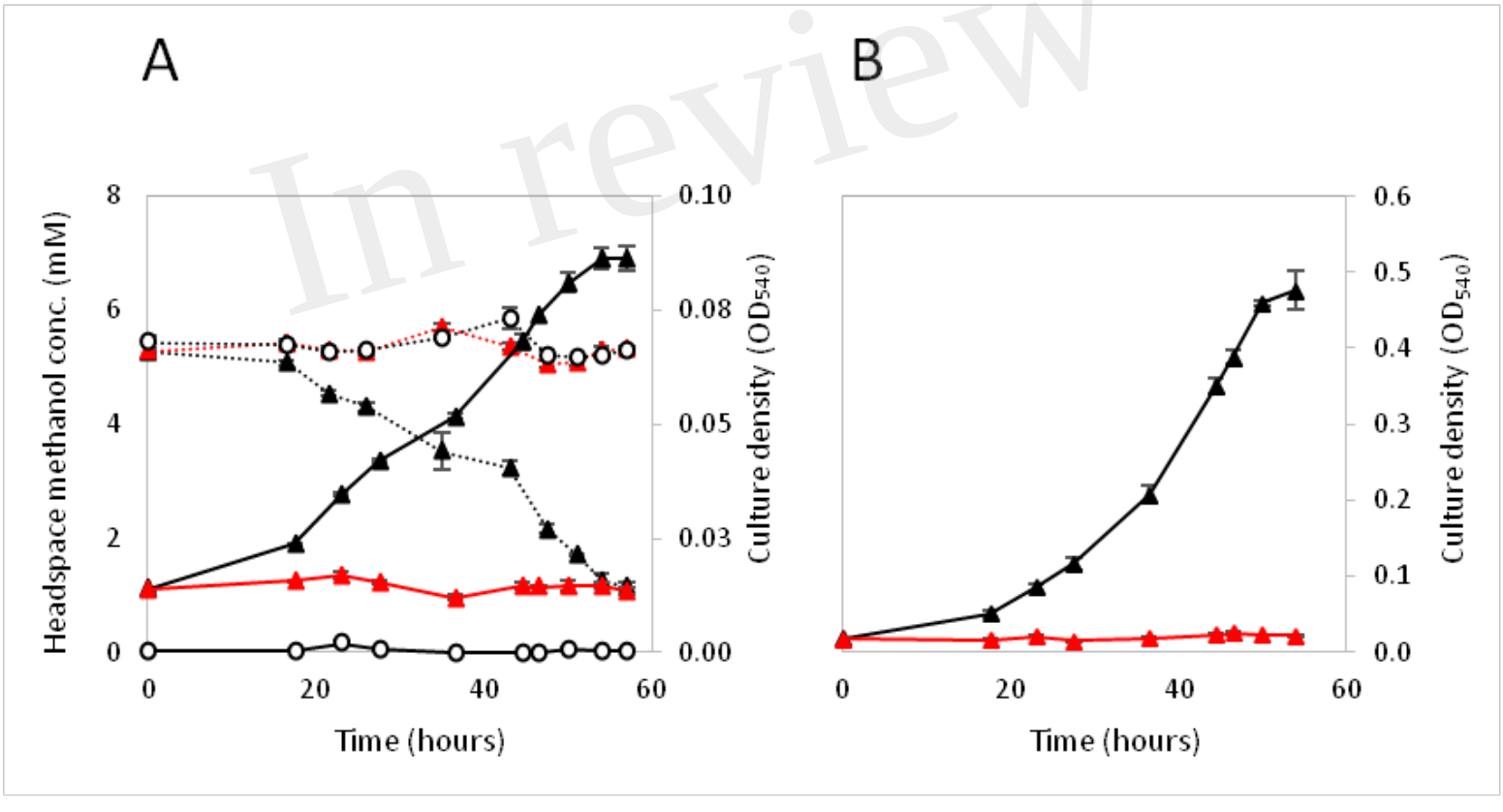

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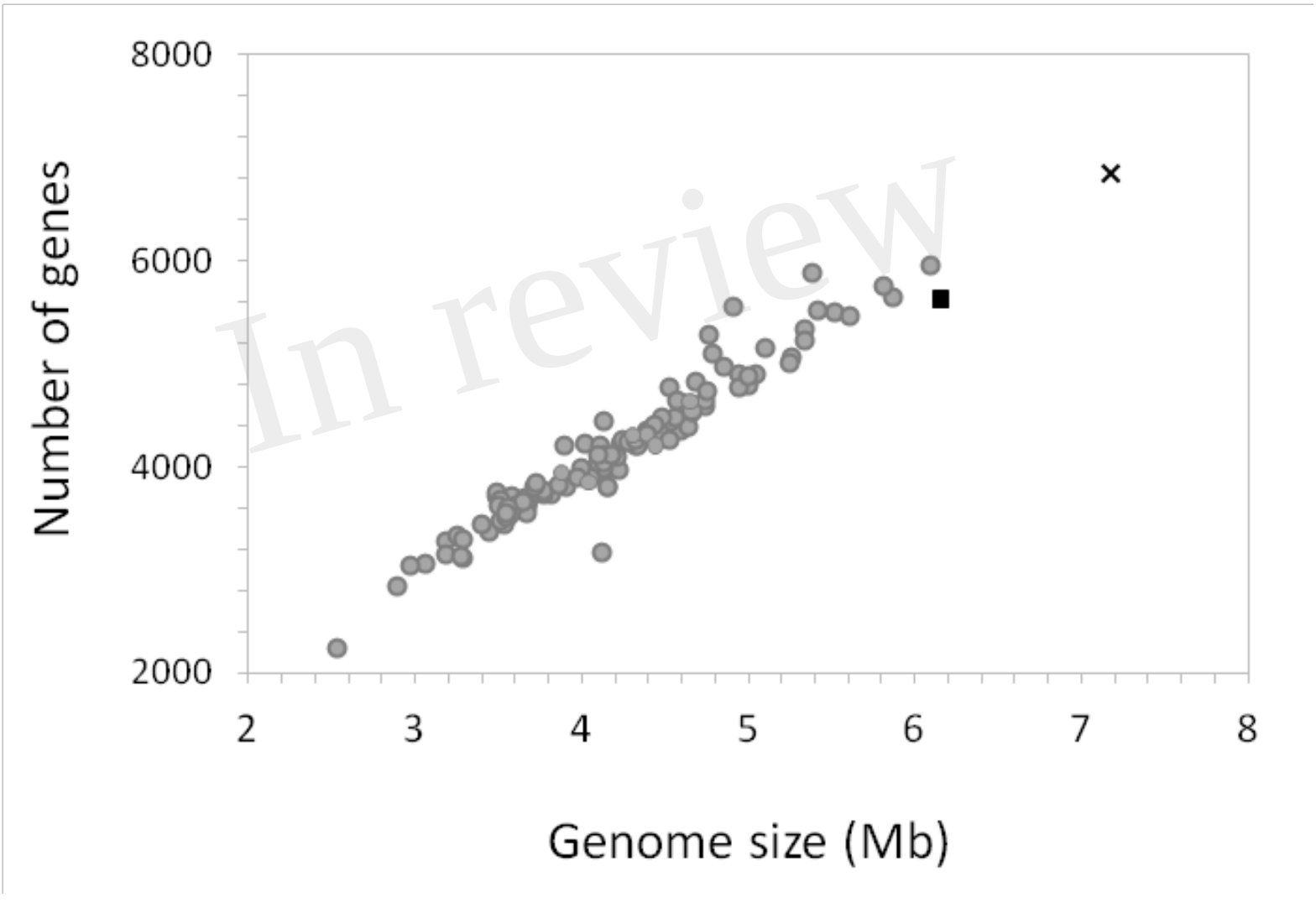

Figure 6.JPEG