UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

The Isolation and Characterization of *Purgationimicrobium sucraloseivorans* gen. nov. sp. nov., and *Quisquiliimicrobium normanense* gen. nov. sp. nov.: Two Novel Wastewater Microorganisms Capable of the Anaerobic Metabolism of Two Anthropogenic Contaminants

A THESIS

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I dedicate this thesis to my family, a group of lovable eccentrics who taught me the first lesson: love is sharing knowledge.

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Abstract

As annual human production and intake of synthetic compounds surges alongside an ever-growing industrialized world population, anthropogenic contaminants become harder to ignore. Some, like sucralose and nitrilotriacetic acid (NTA), are thought to be of concern due to the possibility of health impacts including genotoxicity. This is exacerbated by a resistance to degradation in the environment, leading to proliferation into waterways used for drinking water among other things. This research is centered around the physiological response from microbial communities such as those found in wastewater treatment plants which consistently encounter these compounds and focuses on the isolation and characterization of three strains of two novel bacterial species, $9S^T$ and NTA5 of *Purgationimicrobium sucraloseivorans* gen. nov. sp. nov. and $NTA1^T$ of *Quisquiliimicrobium normanense* gen. nov. sp. nov., taken from wastewater enrichment media with sucralose or NTA as a sole carbon source. Comprehensive morphological, biochemical, and genetic analyses confirmed these organisms' classification within the *Azonexaceae* and *Actinomycetaceae* families, respectively. Identification of bacteria capable of metabolizing anthropogenic contaminants like these is a step towards understanding how microbial degradation takes place and if it might be a tool for bioremediation.

Chapter 1

Anthropogenic Contaminants in Our Waterways and the Search for a Microbiological Solution

Introduction to Xenobiotics and Bioremediation

The term “xenobiotic” has gained popularity to describe any exogenous chemical found within an ecosystem or organism. While these compounds are not solely anthropogenic, centuries of innovation in mass-production alongside a modern understanding of chemistry have thrust humanity’s role in the synthesis and proliferation of xenobiotic contaminants to the forefront. Decades ago, the first studies on the nascent effects of xenobiotics on microbiota began to reveal what would become a disturbing trend of negative effects [1], made more drastic by a recalcitrant nature for some of the most problematic [2]. As annual human production and intake of synthetic compounds surges alongside an ever-growing industrialized world population, anthropogenic contaminants become harder to ignore. A possible solution or tool is the application of microbial degradation of these chemicals as a means to remove or at least reduce the presence of these compounds [3]; however, a first step requires the identification of organisms with a suitable physiological toolset.

The Contaminants Targeted for Use as a Sole Substrate in this Study

Microbial degradation has been reported for some xenobiotic contaminants such as the chelator and detergent additive Nitrilotriacetic acid (NTA), for which environmental persistence and toxicology of this anthropogenic compound has been the subject of study [4-5]. Meanwhile, other contaminants have a relative dearth of information about how natural degradation may occur, such as in the case of sucralose. Being the most commonly used artificial sweetener across the globe, sales of sucralose make up nearly a third of the entire multibillion-dollar artificial sweetener market [6]. Problematic findings surround the sweetener including effects on body weight regulation hormones, potentially negative changes to gut microbiota, and a weakly mutagenic nature [7]. As recently as 2023 sucralose-6-acetate, a common impurity and intermediate in the manufacturing process with its own list of troubling properties including genotoxicity, was found to be present in commercial sucralose samples at levels up to 0.67% - enough that an individual ingesting one daily beverage sweetened with sucralose may exceed the threshold for toxicological concern [8]. In addition to known issues surrounding anthropogenic contaminants and their environmental persistence, it is likely that still worse problems will be uncovered over time with prolonged exposure.

After ingestion and passage through the human gastrointestinal tract, sucralose remains relatively unchanged with an estimated 85-95% being excreted via feces and urine [9]. Upon excretion it sequentially finds its way into wastewater treatment plants (WWTPs), where effluent wastewater is returned to surface waters still carrying the contaminant. These surface waters may in turn be used as source water for drinking water treatment plants (DWTPs) in

many places. Sucralose remains present before processing at DWTPs, in the effluent finished drinking water, and finally, in water making its way through distribution systems in amounts up to 2.4 ug/L (Fig. 1) [10-12]. While some studies show a decrease in concentration, such as when diluted in existing surface waters, sucralose is thought to make it back into homes through the recycling effect of water distribution systems - reappearing in domestic and commercial properties [11-12]. Because of this, even those individuals who do not personally use artificial sweeteners may still encounter the compound. Without the advent of remediation methods levels of sucralose and other recalcitrant xenobiotics will continue to rise, possibly alongside healthcare issues on a global scale.

To exacerbate matters, there is yet another set of possible issues concerning sucralose – thermal degradation into other polychlorinated compounds. Sucralose is purported to degrade entirely upon release of its final chlorine group at a temperature of up to 550°C [13], although such temps are unlikely to be experienced in waterways or the natural environment, some studies suggest degradation begins at a much lower temperatures, with hazardous byproducts forming as low as 98°C [14]. To be clear, this is a subject of ongoing debate, but of 28 publications discussing the matter from 1990-2020, 21 of them indicated an instability of sucralose at these lower temperatures [15]. For a product upon which a multibillion-dollar market has been built, this fact appears very telling [6].

Furthermore, among the products which are thought to occur from baking with sucralose or even heating in the presence of common cooking ingredients, polychlorinated aromatic hydrocarbons (PCAHs), chloropropanols, polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-p-dioxins (PCDDs) are thought to be of concern [14, 16-17]. It stands to reason that in places where sucralose is present in distribution system water, and this water is

subsequently used to cook, people may be unknowingly exposing themselves to these extremely toxic compounds. Although initial concentrations of sucralose suggest exposure to hazardous byproducts would be in small doses, the Maximum Contaminant Level for dioxin, for example, in drinking water set by the EPA under the Safe Drinking Water Act is 0.00003 parts per billion (ppb), and bioaccumulation is a known and problematic phenomenon for those undergoing repetitive exposure to PCDDs and PCDFs [18-19]. In the case of individuals who are unaware of their possibly repeated exposure, finding the cause of symptoms which could range from reproductive effects to carcinogenesis may be challenging [20].

Screening for Organisms Capable of Novel Metabolism

Increasing evidence associated with sucralose, and other anthropogenic contaminants like it, suggests a proactive approach towards bioremediation is prudent. It follows, that the microbial life which lives in wastewater undergoes consistent exposure to the recalcitrant chemicals which find their way down the sink in urban areas. In screening for organisms which are already mounting a response and evolving new mechanisms to degrade xenobiotics or adapting old ones, science can identify species which might shape the chemical landscape of tomorrow and appreciate the full range of tools with which environmental issues may be addressed.

The primary aim of this research was the pursuit of identifying organisms for future use in the bioremediation of sucralose and NTA. Few organisms have been identified which can metabolize these compounds, indeed on a review of the literature no organisms can utilize these compounds under anerobic conditions. An added complication is that sucralose has been shown

to have a bacteriostatic effect in some cases [21]. From the outset this work was high risk and proved to be so as days turned into weeks, turned into months with little signs of life as observed via microscopy, but eventually three isolates were cultivated and characterized as presented in Chapters 2 and 3.

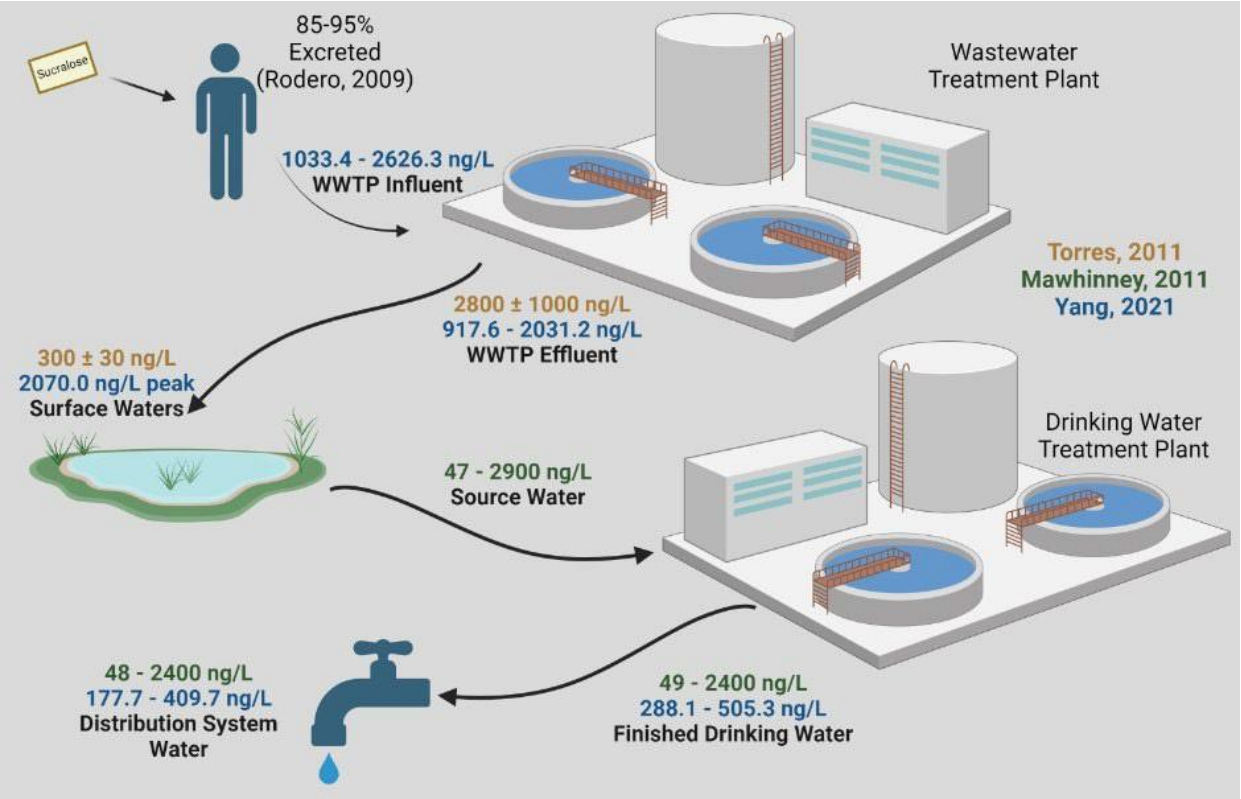

Fig 1. Diagram showing measurements of sucralose concentrations in three studies, taking place in the United States and China, showing the persistence of the compound as it cycles through managed water systems [9-12]. This diagram was created using BioRender. (BioRender.com)

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Chapter 2

Purgationimicrobium sucraloseivorans gen. nov. sp. nov., isolated from an anaerobic wastewater enrichment with an anthropogenic contaminant as the sole carbon source.

Abstract:

Two facultatively anaerobic, Gram-negative-staining, rod-shaped bacteria were isolated from enrichments conducted on a local wastewater sample, taken from Norman, Oklahoma, USA. The strains, designated NTA5 and 9S^T, were isolated from enrichment cultures containing nitrilotriacetic acid or sucralose as a sole substrate and have been shown to grow on either substrate in pure culture under anaerobic conditions. 16S rRNA gene sequences analysis revealed that the strains NTA5 and 9S^T were closely related with 98.9% sequence similarity. Furthermore, the ANI and dDDH values were 96.26% and 69.0% demonstrating that the two strains represented a single species. Strain NTA5 is most closely related to *Azonexus hydrophilus* (94.9%), *Azonexus caeni* (94.9%), and *Azonexus fungiphilus* (94.4%), while strain 9S^T shows the greatest similarity to *Azonexus caeni* (95.1%), *Azonexus fungiphilus* (94.9%), and *Azonexus hydrophilus* (94.8%). Strain NTA5 and 9S^T have a G+C mol% content of 63.16% and 63.20% respectively. Based on biochemical, phylogenetic, and genotypic criteria, these isolates constitute a novel species within a novel genus within the family *Azonexaceae*. The proposed name for this novel organism is *Purgationimicrobium sucraloseivorans* gen. nov., sp. nov., and the proposed type strain is 9S^T (=CCM 9401^T=NRRL B-65712^T). A second strain NTA5 (=CCM 9400^T=NRRL B-65710^T) was isolated from an enrichment from the same source but containing nitrilotriacetic acid (NTA) as the sole substrate.

1. Introduction:

The family *Azonexaceae*, located within the order *Rhodocyclales*, is currently composed of four genera of Gram-stain-negative, non-spore-forming bacteria. However, these genera, grouped on the basis of 16S rRNA similarity, show a diverse range of metabolic capabilities. They include obligate aerobes which are capable of fixing nitrogen, strict anaerobes which can respire ferric iron, and facultative anaerobes which make use of (per)chlorate for respiration [1]. Members of *Azonexaceae* have notably been isolated from samples taken from a wide variety of ecosystems under pressure from human activity. These range from soil used for the commercial production of rice, [2] freshwater lake sediment impacted by nearby mining, [3] industrial waste from paper production, [4] and activated sludge undergoing chlorination [5]. Both of the anthropogenic contaminants used as substrates in this study are substances consistently supplied to urban wastewater systems due to human use. In the case of sucralose, the overwhelming majority (as much as 96.7%) of the ingested compound is thought to be excreted by humans in an unchanged form [6]. This suggests that the communities of microorganisms which are already in use to treat wastewater come into frequent contact with the chemical, facing ecological and evolutionary pressure to adapt to its presence as humans consume more of it [7].

Utilizing a polyphasic taxonomic approach, two novel strains representing a novel species and genus with strain designations 9S^{T} and NTA5 were isolated from anaerobic enrichment of a wastewater sample taken from the City of Norman Water Reclamation Facility in Norman, OK, USA. The two closely-related strains represent a novel genus for which the name *Purgationimicrobium sucraloseivorans* gen. nov., sp. nov. is proposed.

2. Materials and Methods

2.1 Media:

The enrichment medium was prepared consisting of a basal medium, a stock solution of 20% (w/v) sucralose or 20% (w/v) nitrilotriacetic acid (NTA), and 1M $NaSO_4$ for half of the replicates. The basal medium contained (L^{-1}): 10 mL of mineral solution made without NaCl with the aim of promoting dechlorination of sucralose (an organochlorine sweetener), 8.75 mL vitamin solution, 5 mL Trace Metal solution, 5 g of TES buffer, 100 mg of yeast extract, and 2 g of sodium bicarbonate [8]. This basal medium was brought to a pH of 7 using H_2SO_4 before boiling for 1 minute to remove dissolved oxygen, and then transferred into chemically clean 160 mL serum bottles at a volume of 30 mL inside an anaerobic chamber before replacing the headspace with a 40 kPa atmosphere of 5% hydrogen, 10% carbon dioxide, and 85% nitrogen. The serum bottles this basal medium were then sterilized using an autoclave.

After cooling, half of the enrichment replicates received a 0.6 mL of a 20% (w/v) NTA stock solution and the other half received an equivalent volume of a 20% (w/v) sucralose stock solution, delivered through a butyl septum by way of needle and syringe with the addition of a 25 mm Puradisc 0.2 μm-pore filter for sterility. The solutions of these unbuffered substrates were adjusted to pH 7 before undergoing the process of boiling out dissolved oxygen. In the case of sucralose, for which evidence [9] suggests thermal degradation into harmful byproducts may occur at boiling water temperatures, the temperature was raised to 70°C before subjecting it to a vacuum of -25 psi for one minute to facilitate a low-temperature boil. In a similar fashion, 0.6 mL of a 1 M solution of $NaSO_4$ without dissolved oxygen and at a pH of 7 was added to half of both the replicates containing NTA and those with sucralose.

2.2 Isolation and Ecology:

Strains 9S^T and NTA5 were isolated from a wastewater sample taken on January 17th, 2023 from the City of Norman Wastewater Reclamation Facility at 3500 Jenkins Avenue, Norman, Oklahoma 73072 (35.176910, -97.441420). The sample was immediately transported on ice to the Lawson Lab (University of Oklahoma), where 40 mL aliquots were centrifuged at 5000 RCF for 20 minutes before decanting all but 2.5 mL of the supernatant. The remaining solution and resultant pellet were homogenized through the pulsed use of a vortex, before injecting 0.25 mL of this condensed inoculum into each bottle of the array of enrichments described above, and incubated at 30°C. Identical serum bottles for each condition were made and left uninoculated to serve as negative controls for assessing turbidity and the presence of microbial growth.

Successive enrichment ensued with transfers to new tubes containing a chemically identical medium taking place monthly, until signs of microbial activity were confirmed through phase contrast microscopy. Although somewhat expected due to the documented bacteriostatic nature of sucralose [10], the growth of microorganisms was extremely slow, with several initial transfers taking place monthly with only the slightest evidence of microbial activity observed. Solid media was made by incorporating noble agar into each of the sole-substrate broths +/- sulfate at an amount equal to 1.8% (w/v) noble agar. 0.1 mL of each enrichment culture was plated and incubated anaerobically at 30°C for 1 month using an Oxoid Anaerobic Jar with a headspace of 85% nitrogen, 10% carbon dioxide, and 5% hydrogen, with regular inspection for growth. Isolated colonies were streaked onto BHI agar medium, incubated at 30°C for 5-11 days, and stained to assess Gram reaction. Transfers took place biweekly due to the slow-growing nature of the strains on the given substrates, which would go

on to be a consistent theme even after isolation was achieved and the use of more nutritious media such as BHI and later TSA supplemented with 0.5% L-malic acid was implemented. All strains with a distinct morphology were preserved in 50% (v/v) glycerol and stored at -80°C. Upon 16S rRNA gene sequencing confirmation, novel organisms were then preserved using Microbank CryoBeads stored at -80°C for future use.

2.3 16S rRNA Gene Phylogeny

All strains with distinct morphology were processed and sent to GENEWIZ by Azenta (Genewiz.com, South Plainfield, NJ, USA) where nucleic acid extraction and Sanger sequencing of the 16S rRNA ribosomal subunit gene was performed. 16S rRNA gene similarities were compared to the most closely related taxa using the online database EZBioCloud [11]. The sequences of the nearest relatives were downloaded and aligned using MUSCLE [12] in MEGA 11 version 11.0.13 [13]. Phylogenetic relationships were inferred using both the maximum-likelihood method (Fig. 1) and the neighbor-joining method (Fig. 2) within MEGA 11, applying the Tamura–Nei substitution model. Bootstrap analysis with 1000 replicates was performed to assess the robustness of the phylogenetic trees.

2.4. Genome Features

Both potentially novel strains were then cultivated to a biomass exceeding 40mg. The biomass for each was transferred to a 1.5mL Eppendorf tube, washed in phosphate-buffered solution (1x PBS) before pelleting, decanting the supernatant of PBS, and resuspending each sample in 500uL of Zymo 1x DNA/RNA Shield and sent to Plasmidsaurus (Plasmidsaurus.com) for whole-genome Oxford-Nanopore sequencing.

2.5 Metabolic Feature Characterization

Gram stain reactions were determined by the use of the Advanced™ Gram Stain Kit with Stabilized Iodine (Hardy Diagnostics) and confirmed through the use of the KOH test [14]. Endospore-formation was determined through the process of an endospore stain [15]. Motility was determined using a needle stab inoculation of motility test medium [16]. The presence of catalase and oxidase were assessed for both strains by carrying out catalase and oxidase tests according to their respective protocols as written by the American Society for Microbiologists [17-18].

Metabolic function was assessed through the use of a BioLog Gen III MicroPlate™ (www.BioLog.com). Cultures of both strains were grown on fresh TSA + 5% (v/v) defibrinated sheep blood. Metabolically active cells of $9S^T$ were swabbed and introduced, at an amount calculated to 95% transmittance by a BioLog turbidimeter, to a tube containing Inoculating Fluid A (IF-A). The cell suspension was vortexed to ensure homogeneity, before being pipetted to a volume of 100uL into each well of a 96-well MicroPlate, covered, and incubated at 30°C for 36 hours. This process was repeated for strain NTA5.

Optimum temperature and temperature range for each strain were determined through cultivation in Tryptic Soy Broth (TSB) supplemented with 0.5% (w/v) malate at 4°C, 25°C, 30°C, 37°C, and 45°C, with growth being measured via optical density using a spectrophotometer (Spectronic 20D+, Milton Roy) set to 600 nm wavelength. Optimum and ranges for salinity and pH were also assessed by eye on a solid medium made from TSB supplemented with 0.5% malate and 1.5% agar, at increments of 1 pH in a range from 6 to 10 pH and increments of 1% NaCL (w/v) in a range from 1% NaCl to 10% NaCl in addition to the

previously used baseline 0.5% NaCl found in TSB. All characterization tests were carried out in triplicate.

2.6. In-silico Prediction of Polar Lipid Production

The glycerophospholipid metabolisms of strains $9S^T$ and NTA5 were assessed through the application *in-silico* methods, with particular attention paid to the polar lipid production predicted by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [19]. Amino acid profiles (.faa) were created through the use of the tool Prodigal [20] before submission to KEGG Mapper and analysis of gene prediction [19].

In this study, an *in-silico* approach was used to investigate glycerophospholipid metabolism, and the production of polar lipids was performed using the KEGG database [19]. An *in-silico* approach is now encouraged and routinely utilized for polar lipid profiling [21-25], and along with biochemical traits, these methods are now frequently used for species descriptions [26-27].

3. Results

3.1 16S rRNA Gene Phylogeny

Strains NTA5 and $9S^T$ were determined to be closely related with 98.9% sequence similarity, the nearest phylogenetic neighbors were *Azonexus hydrophilus* (94.9%), *Azonexus caeni* (94.9%), and *Azonexus fungiphilus* (94.4%). The cut off value routinely used to designate species into separate genera is approximately 6% sequence similarity [28]. Therefore, based on sequence comparisons, combined with the topology of the phylogenetic trees generated by the maximum-likelihood and neighbor-joining approaches, strongly support the classification of strains $9S^T$ and NTA5 as members of a novel species and genus within the family *Azonexaceae*.

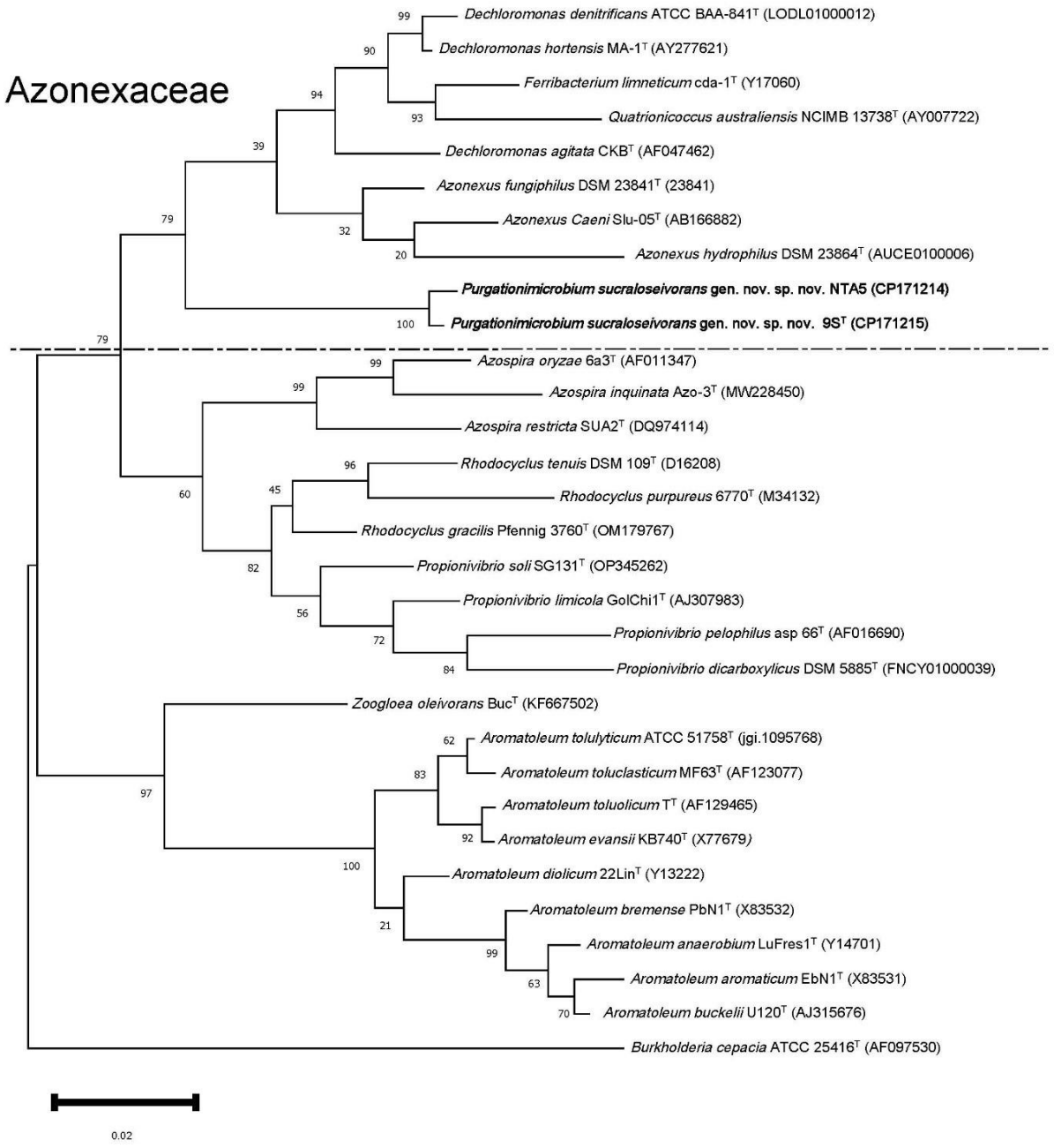

Figure 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequence similarity, demonstrating that *Purgationimicrobium* gen. nov. belongs to the family *Azonexaceae*. Bootstrap values at branch nodes were calculated from 1000 repetitions. *Burkholderia cepacia* serves as an outgroup.

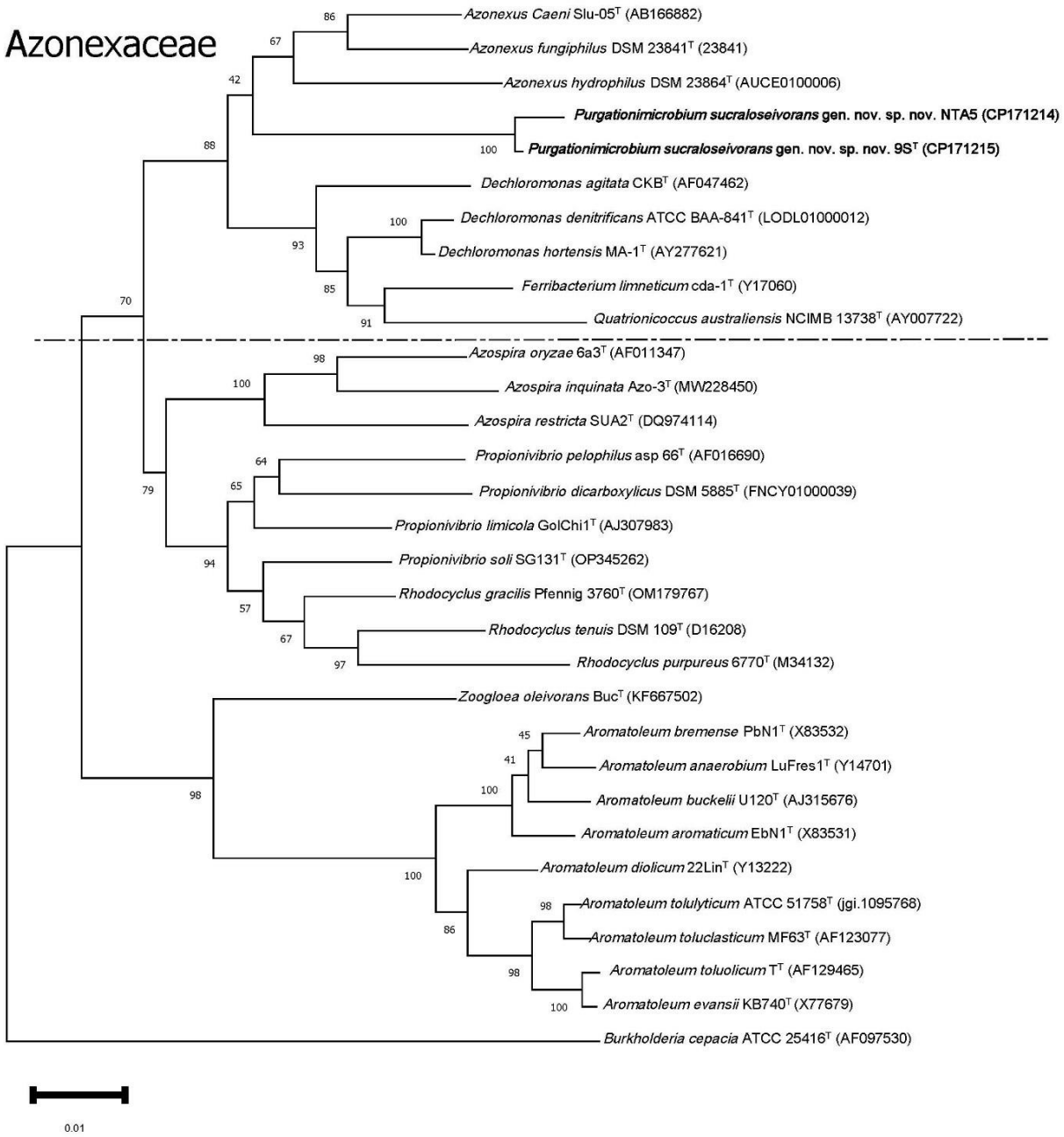

Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence similarity, demonstrating that *Purgationimicrobium* gen. nov. belongs to the family *Azonexaceae*. Bootstrap values at branch nodes were calculated from 1000 repetitions. *Burkholderia cepacia* serves as an outgroup.

3.2 Genome Features

The resultant genome sequences were each a single contig, suggesting no plasmids or contamination present. Both strains had an estimated genome size of 3.1 Mb with 2,888 genes annotated for strain $9S^T$ and 2,917 genes annotated for strain NTA5. To further investigate the relationship between the two strains, their genomes were submitted to EZBioCloud and the Type-Strain Genome Server (TYGS) [11][29]. The Average Nucleotide Identity (ANI) value between the two novel strains, as determined by EzBioCloud's OrthoANIu, was 96.26% [11]. G+C contents for $9S^T$ and NTA5 were determined to be 63.20% and 63.16% respectively, confirmed by both TYGS and EZBioCloud. Digital DNA-DNA Hybridization (dDDH, d_4 in %) between the strains was assessed by TYGS to be 69.0% [29]. A phylogram based on the genome similarity between strains $9S^T$ and NTA5 was made by TYGS analysis (Fig. 3).

These ANI and dDDH values demonstrated that strains $9S^T$ and NTA5 represent a novel species within *Azonexaceae* as ANI and dDDH values higher than 95%-96% and 70%, respectively, are established as the boundary for defining a prokaryotic species [30-31, 28]. The phylogenomic tree generated (Fig. 2) agreed with the phylogenetic placement of $9S^T$ and NTA5 from the 16S rRNA gene sequence tree (Fig 1). As seen below (Fig. 3) and with the data taken from these *in-silico* analyses, the two strains are predicted to fall within the taxonomic parameters used to describe a single species.

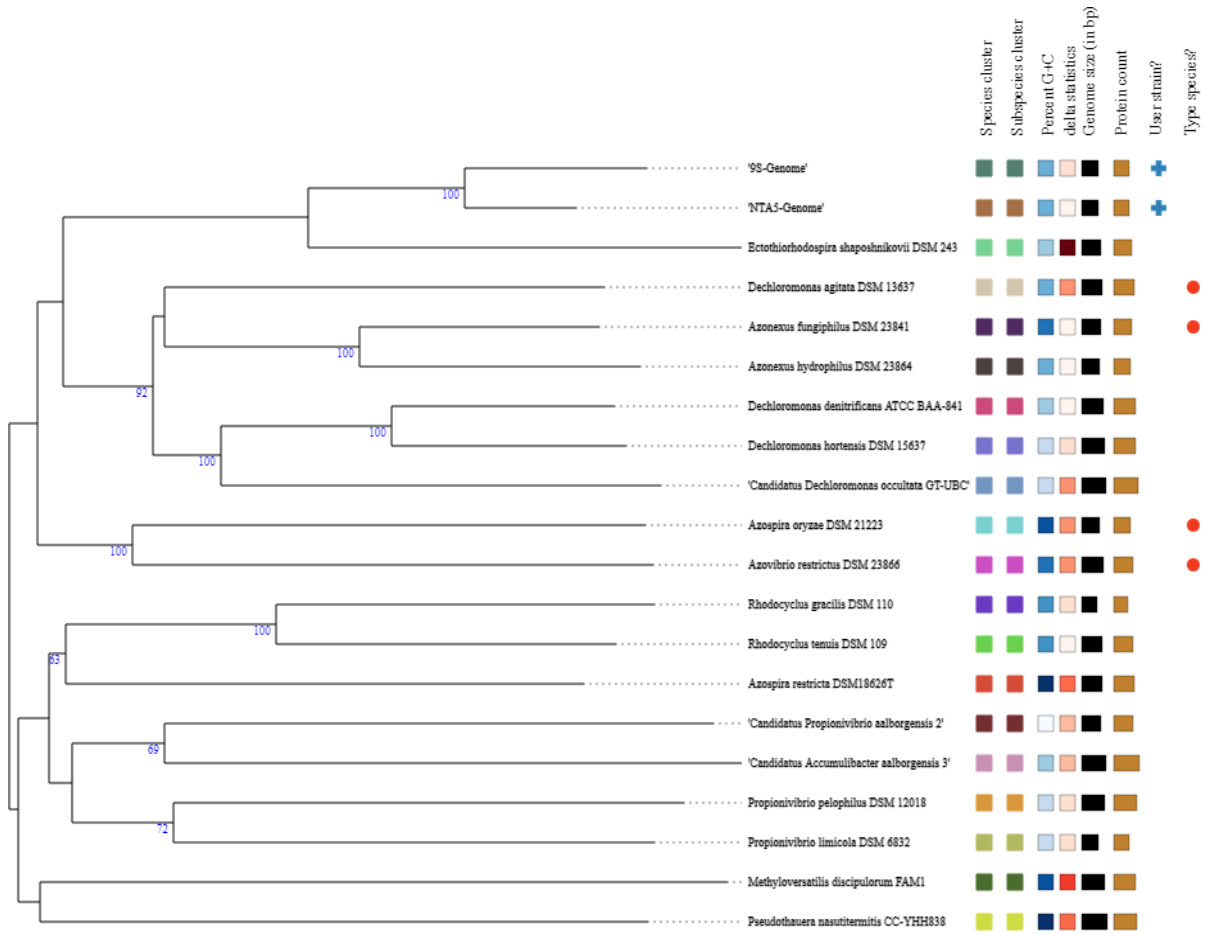

Figure 3. Phylogram computed by whole-genome sequence analysis of strains 9S^T, NTA5, and closely-related species by TYGS. Legend shows predicted species clusters, subspecies clusters, G+C%, delta statistics, relative genome size, relative protein count, user strains, and current type species [29].

3.3 Metabolic Feature Characterization

Both strains were found to be Gram-negative-staining rods through the use of an Advanced™ Gram Stain Kit with Stabilized Iodine (Hardy Diagnostics) and confirmed through the use of the KOH test [14]. 9S^T and NTA5 were assessed to be non-endospore-forming through the process of an endospore stain [15]. Both strains were subjected to a needle stab inoculation of motility test medium, the results of which suggested 9S^T and NTA5 were both nonmotile [16]. 9S^T and NTA5 were each determined to be catalase- and oxidase-positive through the

administration of the slide (drop) catalase test method and filter paper oxidase test method [17-18].

Metabolic function was characterized using a BioLog Gen III MicroPlate™ (www.BioLog.com). In both strains, L-malic acid was the only substrate of seventy-one tested to show metabolic activity after 36 hours of incubation at 30°C. For the chemical sensitivity portion of the assay, no wells showed activity for strain $9S^T$. Cells of strain NTA5 retained metabolic function in the positive control and in the presence of Rifamycin SV, Lincomycin, Vancomycin, and Aztreonam.

Optimum temperature and temperature range for each strain were determined through cultivation in Tryptic Soy Broth (TSB) supplemented with 0.5% (w/v) L-malic acid disodium salt at 4°C, 25°C, 30°C, 37°C, and 45°C, with growth being measured via optical density using a spectrophotometer (Spectronic 20D+, Milton Roy) set to 600 nm wavelength. Both strains showed an optimum of 30°C for their temperature, and growth in a range of temperatures from 25 - 37°C, with NTA5 showing a greater tolerance to 37°C with growth approaching that of its optimum temperature. Optimum and ranges for salinity and pH were also assessed by eye on a solid medium made from TSB supplemented with 0.5% malate and 1.5% agar, at increments of 1 pH in a range from 4 to 10 pH and increments of 1% NaCL (w/v) in a range from 1% NaCl to 10% NaCl in addition to the previously used baseline 0.5% NaCl found in TSB. Optimum pH for both strains was shown to be 7 pH with no growth present at any other level. Optimum salinity was shown to be 0.5% NaCl, with a range from 0.5 - 1% NaCl for both strains. All characterization tests were carried out in triplicate.

3.4. In-silico Prediction of Polar Lipid Production

As seen below (Fig. 4) both strains are predicted to possess identical glycerophospholipid metabolism. The results indicate that strains 9S^T and NTA5 synthesize phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG; cardiolipin), respectively. In addition, the strains also contain phosphatidylserine synthase (EC 2.7.8.8) used in the synthesis of phosphatidyl-L-serine (PS), an intermediate compound that the enzyme phosphatidylserine decarboxylase (PSD) (EC 4.1.1.65) further catalyzes in the production of phosphatidylethanolamine (PE) from phosphatidyl-L-serine (Fig. S2). Based on this analysis, the major polar lipids predicted to be produced by strains 9S^T and NTA5 are PE, PG, PS, and DPG consistent with other members in the family *Azonexaceae* [32].

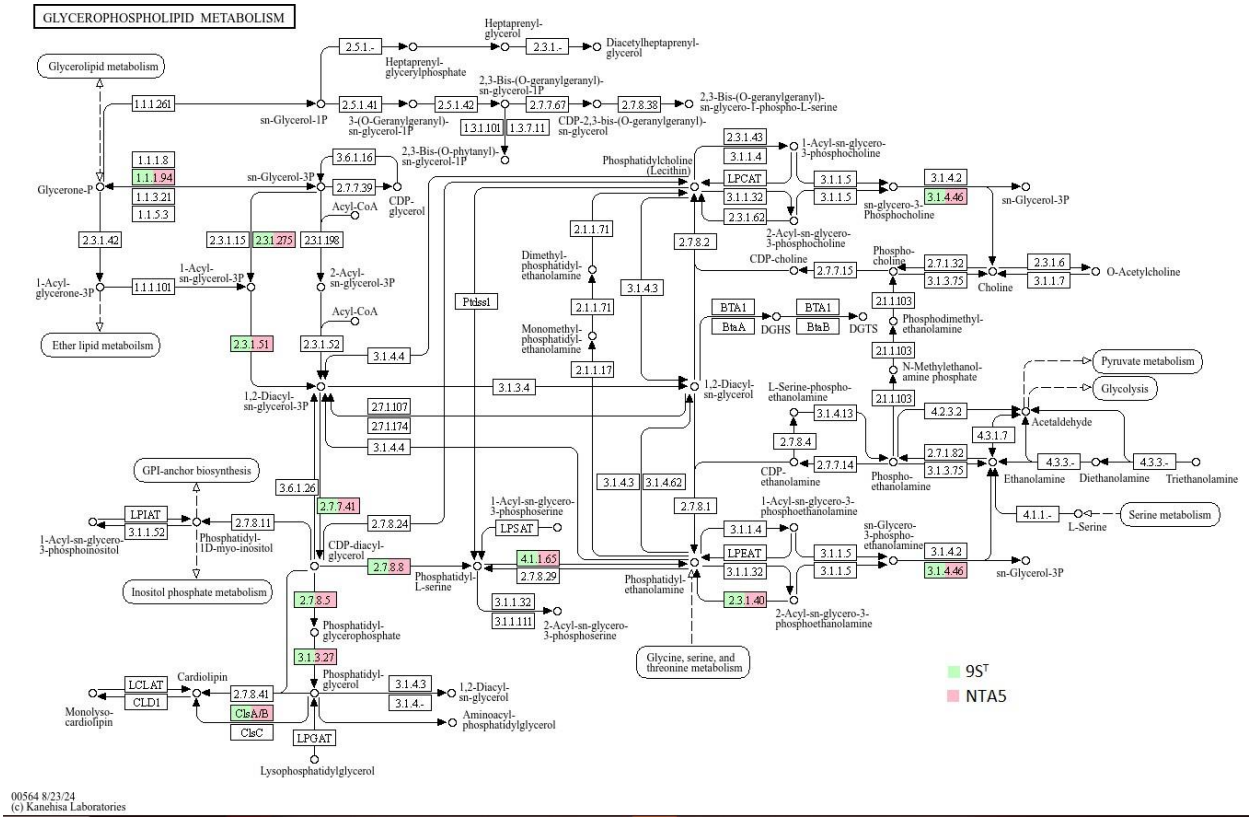

Figure 4. Glycerophospholipid metabolism map for both strains made with KEGG Mapper [19]. In the figure, genes for enzyme production found in the genome of 9S^T are denoted by green, and those of NTA5 by red.

Strains 9S^{T} and NTA5 were nonmotile Gram-stain-negative rods which exhibited facultatively anaerobic growth. Sporulation was not observed. Morphology, phenotypic, phylogenetic and genotypic analysis demonstrated that both strains represent a novel species and genus for which the name *Purgationimicrobium sucraloseivorans* gen. nov. sp. nov. is proposed.

Table 1. Table showing physiological properties of strains 9S^{T}, 1, and NTA5, 2, as well as seven close relatives of the proposed genus *Purgationimicrobium*. From left to right, the top row of numbers correlates to *Azonexus fungiphilus*, 3, *Dechloromonas* agitata, 4, *Ferribacterium limneticum*, 5, *Quatrionicoccus australiensis*, 6, *Aromatoleum aromaticum*, 7, *Propionivibrio limicola*, 8, and *Azospira oryzae*, 9. An oxygen tolerance value listed as +/- denotes an organism which exhibits facultatively anaerobic growth.

	1	2	3	4	5	6	7	8	9
Catalase	+	+	+	+	+	+	-	+	+
Oxidase	+	+	+	+	+	-	+	+	+
Gram-stain Reaction	-	-	-	-	-	-	-	-	-
Endospore Production	-	-	-	-	-	-	-	-	-
Motility	-	-	+	+	+	-	+	+	+
Oxygen Tolerance	+/-	+/-	+	+	-	+	+	-	+

Table 2. Table showing genomic data of strains $9S^T$, 1, and NTA5, 2, as well as seven close relatives of the proposed genus *Purgationimicrobium*. From left to right, the top row of numbers correlates to *Azonexus fungiphilus*, 3, *Dechloromonas* agitata, 4, *Ferribacterium limneticum*, 5, *Quatrionicoccus australiensis*, 6, *Aromatoleum aromaticum*, 7, *Propionivibrio limicola*, 8, and *Azospira oryzae*, 9. Asterisks denote data which were unavailable to the author. Where applicable, comparisons were made to strain $9S^T$. Digital DNA-DNA hybridization percentages (dDDH%) were calculated using the d_4 formula and accessed through the use of TYGS along with G+C%, genome size, and predicted proteins [29][21]. 16S rRNA gene similarity was assessed using EZBioCloud's online database [11].yy

	1	2	3	4	5	6	7	8	9
16S %Sim	100%	99.8%	94.88%	94.17%	93.96%	92.90%	92.91%	93.6%	92.90%
dDDH%	100%	69.0%	22.4 %	21.3%	*	*	19.5%	19.1%	19.9%
G+C%	63.2%	63.1%	65.58%	62.94%	66.40%	67.10%	64.70%	60.3%	66.33%
Culture Collection ID	CCM 9401	CCM 9400	DSM 23841	DSM 13637	ATCC 700589	CIP 107055T	DSM 19018	DSM 6832	DSM 21223
No. proteins	2,824	2,850	3,322	3,799	3,883	*	4,504	2,845	3,131
Base pairs (Mb)	3.10	3.14	3.55	3.96	4.15	*	4.73	3.10	3.45

Description of *Purgationimicrobium* gen. nov.

Purgationimicrobium (Pur.ga.ti.o.ni.mi.cro'bi.um. L. fem. n. *purgatio*, a cleaning out, cleansing; N.L. neut. n. *microbium*, a microbe; N.L. neut. n. *Purgationomicrobium*, a microbe from a (water) purification facility).

The cells are facultatively anaerobic, nonmotile, Gram-stain-negative rods in which sporulation is not observed. Optimal growth was observed at 30°C with 0.5% (w/v) NaCl at 7.0 pH. Growth was seen at temperatures ranging from 25°C to 37°C, at no other pH but 7, and at salinity ranging from 0-1% (w/v) NaCl. Isolate was taken from a wastewater enrichment with the sole substrate of sucralose. The cells can grow in anaerobic conditions on minimal medium containing noble agar and only either nitrilotriacetic acid or sucralose as a substrate. In-silico analysis predicts that PE, PG, PS, and DPG are produced.

The original wastewater sample was taken from the City of Norman Water Reclamation Facility in Norman, OK, USA. The G+C content is 63.2%. The proposed type species is *Purgationimicrobium sucraloseivorans*, for which the proposed type strain is $9S^T$ (=CCM 9401^T=NRRL B-65712^T).

Description of *Purgationimicrobium sucraloseivorans* gen. nov. sp. nov.

(su.cra.lo.si.vo’rans. N.L. neut. n. sucralose; L. pres. part. *vorans*, devouring; N.L. part. adj. *sucralosivorans*, sucralose-devouring).

The description is the same as for the genus. Strain $9S^T$ (=CCM 9401^T=NRRL B-65712^T) was isolated from a wastewater sample was taken from the City of Norman Water Reclamation Facility in Norman, OK, USA. A second strain NTA5 (=CCM 9400=NRRL B-65710) was isolated from an enrichment from the same source but containing Nitrilotriacetic acid (NTA) as

the sole substrate. Accession numbers for the strain 9S^{T} 16S rRNA gene and whole genome sequences are PP889578 and CP171215. Accession numbers for the strain NTA5 16S rRNA gene and whole genome sequences are PP935231 and CP171214.

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Chapter 3

Quisquiliimicrobium normanense gen. nov., sp. nov. isolated from an anaerobic wastewater enrichment with Nitrilotriacetic acid (NTA) an anthropogenic contaminant as the sole carbon source.

Abstract:

A facultatively anaerobic, Gram-positive-staining, non-acid-fast coccobacillus-shaped bacterial strain was isolated from an enrichment conducted on a local wastewater sample, taken from Norman, Oklahoma, USA. The strain, designated NTA1^T, was isolated from an enrichment culture containing nitrilotriacetic acid (NTA) as a sole substrate and has been shown to grow under anaerobic conditions. 16S rRNA gene sequence analysis revealed that NTA1^T shared a 16S rRNA gene similarity to its closest phylogenetic relative, *Scrofimicrobium canadense* of 94.15%, and a similarity ranging from 90.26% - 91.16% for the next ten closest relatives. Strain NTA1^T has a G+C mol% content of 64.0%. Based on biochemical, phylogenetic, and genotypic criteria, this isolate constitutes a novel genus and species within the family *Actinomycetaceae*. The proposed name for this novel organism is *Quisquiliimicrobium normanense* gen. nov., sp. nov., for which the type strain is NTA1^T (=CCM 9399^T=NRRL B-65709^T).

1. Introduction:

The clade *Actinobacteria* is a populous and heavily studied phylum of bacteria with a diverse set of physiological and morphological characteristics that make classification of its child taxa challenging [1]. However, this same extensive physiological toolset has garnered attention for some member species as a possible tool for use in bioremediation of contaminants, frequently anthropogenic in nature, such as pesticides, polycyclic aromatic hydrocarbons, or heavy metals [2-4]. The proliferation of these compounds accompanies increasing urbanization, industrialization, and agriculture [5], anthropocentric phenomena which are unlikely to stop or reverse in trend. Due to this trajectory, an emphasis must be placed on detection and reduction of the presence of contaminants in water systems in order to curb the impact on health which comes with community exposure [6-7]. With this goal in mind, identifying organisms capable of breaking down organic contaminants or sequestering heavy metals and understanding how to apply them to address contamination appears to be a natural step towards a solution [8-9].

In the case of the aminopolycarboxylic acid known as nitrilotriacetic acid (NTA), environmental exposure to the compound may entail exposure to trace amounts of heavy metals as well. NTA is used as a builder or bulking agent in detergents to replace phosphates in hopes of avoiding eutrophic effects of the latter on aquatic environments [10-11]. NTA works as a chelating agent which solubilizes Ca^{2+}, Co^{2+}, Cu^{2+}, and Fe^{3+} ions by forming metal-chelator complexes, effectively bringing metal ions into solution in waterways [12]. Unsurprisingly, both heavy metals and NTA have been linked to carcinogenicity [13-15].

In hopes of identifying bacteria capable of metabolizing this contaminant a polyphasic taxonomic approach was utilized, yielding a strain designated $NTA1^T$ which belongs to the

family *Actinobacteriaceae*. NTA1^T was isolated from an anaerobic enrichment medium featuring NTA as the sole carbon source, inoculated with a wastewater sample taken from the City of Norman Water Reclamation Facility in Norman, OK, USA. The strain represents a novel species and genus for which the name *Quisquiliimicrobium normanense* gen. nov., sp. nov. is proposed.

2. Materials and Methods

2.1 Media

The enrichment medium was prepared consisting of a basal medium, a stock solution of 20% (w/v) nitrilotriacetic acid (NTA), and 1M $NaSO_4$ for half of the replicates. The basal medium contained (L^{-1}): 10 mL of mineral solution, 8.75 mL vitamin solution, 5 mL Trace Metal solution, 5 g of TES buffer, 100 mg of yeast extract, and 2 g of sodium bicarbonate [16]. This basal medium was brought to a pH of 7 using H_2SO_4 before boiling for 1 minute to remove dissolved oxygen, then transferred into chemically clean 160 mL serum bottles at a volume of 30 mL inside an anaerobic chamber before replacing the headspace with a 40 kPa atmosphere of 5% hydrogen, 10% carbon dioxide, and 85% nitrogen. The serum bottles of basal medium were then sterilized using an autoclave.

After cooling, the enrichment replicates received a 0.6 mL of a 20% (w/v) NTA stock solution, delivered through a butyl septum via needle and syringe with the addition of a 25 mm Puradisc 0.2 μm-pore filter for sterility. The solution of this unbuffered substrate was adjusted to pH 7 before undergoing the process of boiling out dissolved oxygen. In a similar fashion, 0.6 mL of a 1 M solution of $NaSO_4$ without dissolved oxygen and at a pH of 7 was added to half of the replicates containing NTA.

2.2 Isolation and Ecology:

Strain NTA1^T was isolated from a wastewater sample taken on January 17th, 2023 from the City of Norman Wastewater Reclamation Facility at 3500 Jenkins Avenue, Norman, Oklahoma 73072 (35.176910, -97.441420). The sample was immediately transported on ice to the Lawson Lab (University of Oklahoma), where 40 mL aliquots were centrifuged at 5000 RCF for 20 minutes before decanting all but 2.5 mL of the supernatant. The remaining solution and resultant pellet were homogenized through the pulsed use of a vortex, before injecting 0.25 mL of this condensed inoculum into each bottle of the array of enrichments described above, and incubated at 30°C. Identical serum bottles for each condition were made and left uninoculated to serve as negative controls for assessment of turbidity and the presence of microbial growth.

Successive enrichment ensued with transfers to new tubes containing a chemically identical medium taking place every month, until signs of microbial activity were confirmed through phase contrast microscopy. The growth of microorganisms was extremely slow, with several initial transfers taking place with only the slightest evidence of microbial activity observed. Solid media was made by incorporating noble agar into media with NTA as the sole substrate +/- sulfate at an amount equal to 1.8% (w/v) noble agar. 0.1 mL of each enrichment culture was plated and incubated anaerobically at 30°C for 1 month using an Oxoid Anaerobic Jar with a headspace of 85% nitrogen, 10% carbon dioxide, and 5% hydrogen, with regular inspection for growth. Isolated colonies streaked onto BHI and BHI + 5% defibrinated sheep blood agar media, incubated at 30°C for 5-11 days, and stain to assess Gram reaction. Strain NTA1^T stood out for bucking the trend of a slow-growing nature that was seen with most other isolates, as well as for only growing on media supplemented with blood. All strains with a

distinct morphology were preserved in 50% (v/v) glycerol and stored at -80°C. Upon 16S rRNA gene sequencing confirmation, novel organisms were then preserved using Microbank CryoBeads stored at -80°C for future use.

2.3 16S rRNA Gene Phylogeny

All strains with distinct morphology were processed and sent to GENEWIZ by Azenta (Genewiz.com, South Plainfield, NJ, USA) where nucleic acid extraction and sanger sequencing of the 16S rRNA ribosomal subunit gene was performed. 16S rRNA gene similarities were compared to closest taxa using the online database EZBioCloud [17]. The sequences of the nearest relatives were downloaded and aligned using MUSCLE [18] in MEGA 11 version 11.0.13 [19]. Phylogenetic relationships were inferred using both the maximum-likelihood method (Fig. 1) and the neighbor-joining method (Fig. 2) within MEGA 11, applying the Tamura–Nei substitution model. Bootstrap analysis with 1000 replicates was performed to assess the robustness of the phylogenetic trees.

2.4. Genome Features

This potentially novel strain was then cultivated to a biomass exceeding 100mg. The biomass was transferred to a 1.5mL Eppendorf tube, washed in phosphate-buffered solution (1x PBS) before pelleting, decanting the supernatant of PBS, and resuspending the sample in 500uL of Zymo 1x DNA/RNA Shield and sent to Plasmidsaurus (Plasmidsaurus.com) for whole-genome Oxford-Nanopore sequencing.

2.5. Metabolic Feature Characterization

Gram stain reactions were determined by the use of the Advanced™ Gram Stain Kit with Stabilized Iodine (Hardy Diagnostics) and confirmed through the use of the KOH test [20]. Endospore-formation was determined through the process of an endospore stain [21]. Motility was determined using a needle stab inoculation of motility test medium [22]. Acid-fastness was assessed through the application of the Kinyoun method of acid-fast staining [23]. The presence of catalase and oxidase were tested according to according to their respective protocols as written by the American Society for Microbiologists [24-25]

Metabolic function was assessed through the use of a BioLog Gen III MicroPlate™ (www.BioLog.com). A culture of NTA1^{T} was grown on fresh TSA + 5% (v/v) defibrinated sheep's blood. Metabolically active cells were swabbed and introduced, at an amount calculated to 95% transmittance by a BioLog turbidimeter, to a tube containing Inoculating Fluid A (IF-A). The cell suspension was vortexed to ensure homogeneity, before being poured into a pipet reservoir. This cell suspension was then pipetted to a volume of 100uL into each well of a 96-well MicroPlate, covered with the lid, and incubated at 30°C for 36 hours.

Optimum temperature and temperature range for each strain were determined through cultivation on Tryptic Soy Agar (TSA) supplemented with 5% (v/v) defibrinated sheep's blood at 4°C, 25°C, 30°C, 37°C, and 45°C. Optimum and ranges for salinity and pH were also assessed on a solid medium made from TSB supplemented with 5% (v/v) defibrinated sheep's blood and 1.5% agar, at increments of 1 pH in a range from 6 to 10 pH and increments of 1% NaCL (w/v) in a range from 1% NaCl to 10% NaCl in addition to the previously used baseline 0.5% NaCl found in TSB. All characterization tests were carried out in triplicate.

2.6. In-silico Prediction of Polar Lipid Production

Glycerophospholipid metabolism of strain NTA1^T was assessed through the application *in-silico* methods, with particular attention paid to the polar lipid production predicted by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [26]. An amino acid profile (.faa) was created through the use of the tool Prodigal [27] before submission to KEGG Mapper and analysis of gene prediction [26].

In this study, an *in-silico* approach was used to investigate glycerophospholipid metabolism, and the production of polar lipids was performed using the KEGG database [26]. An in-silico approach is now encouraged and routinely utilized for polar lipid profiling [28-32], and along with biochemical traits, these methods are now routinely used for species descriptions [33-34].

3. Results

3.1 16S rRNA Gene Phylogeny

Phylogenetic analysis of the 16S rRNA gene sequences revealed that NTA1^T is most closely related to *Scrofimicrobium canadense* (94.15%), *Boudabousia marimammalium* (91.16%), and *Schaalia meyeri* (90.94%). The next ten phylogenetic relatives give similarity values ranging from 90.26% - 91.16%. The value of 6% sequence similarly is routinely used for the rank of genus. Based on sequence comparisons, combined with the phylogenetic trees generated by the maximum-likelihood and neighbor-joining approaches, the data strongly support the classification of strain NTA1^T as a member of a novel species and genus within the family *Actinomycetaceae*.

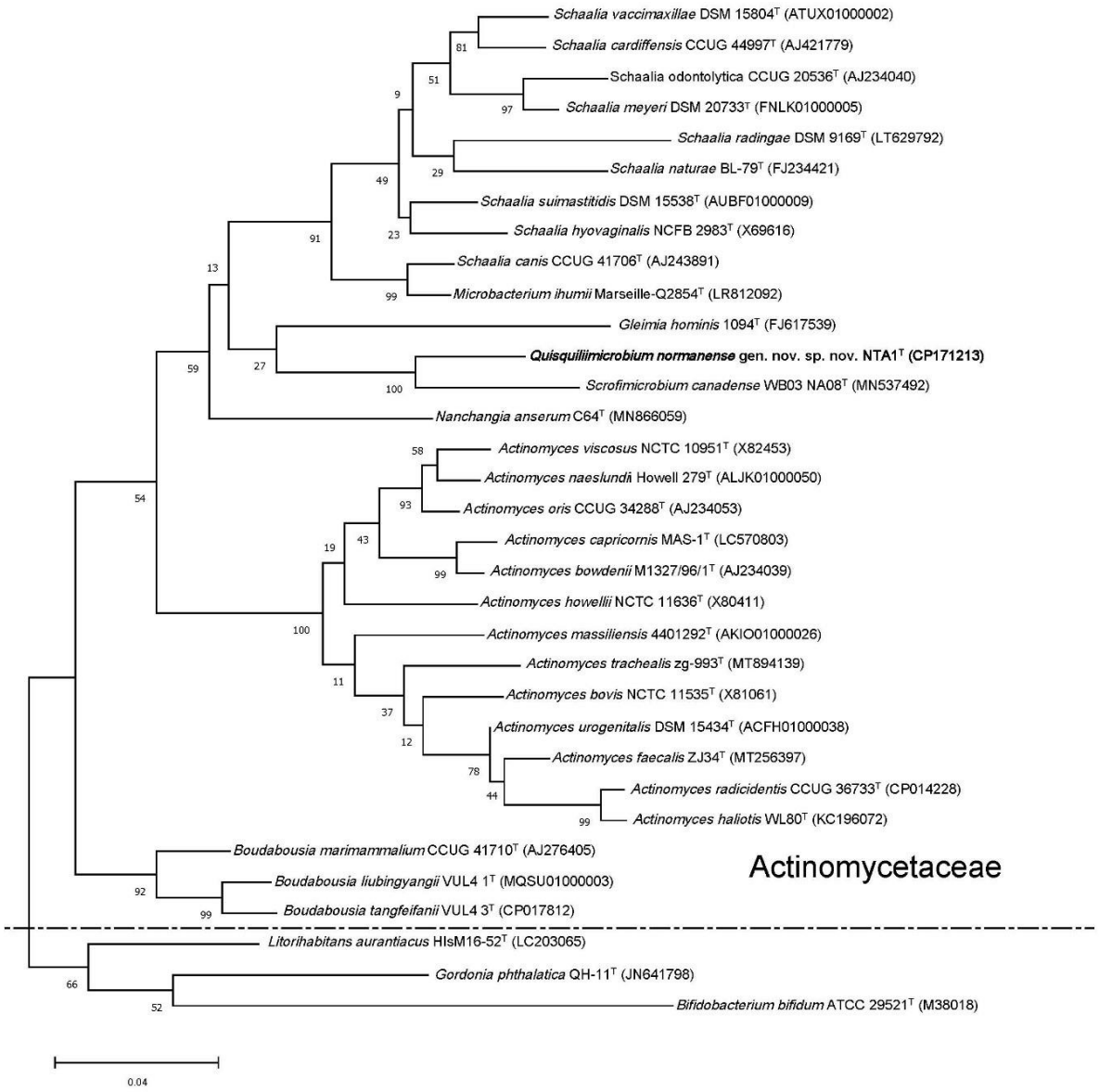

Figure 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequence similarity, demonstrating that *Quisquiliimicrobium* gen. nov. belongs to the family *Actinomycetaceae*. Bootstrap values at branch nodes were calculated from 1000 repetitions. *Gordonia phthalatica* serves as an outgroup.

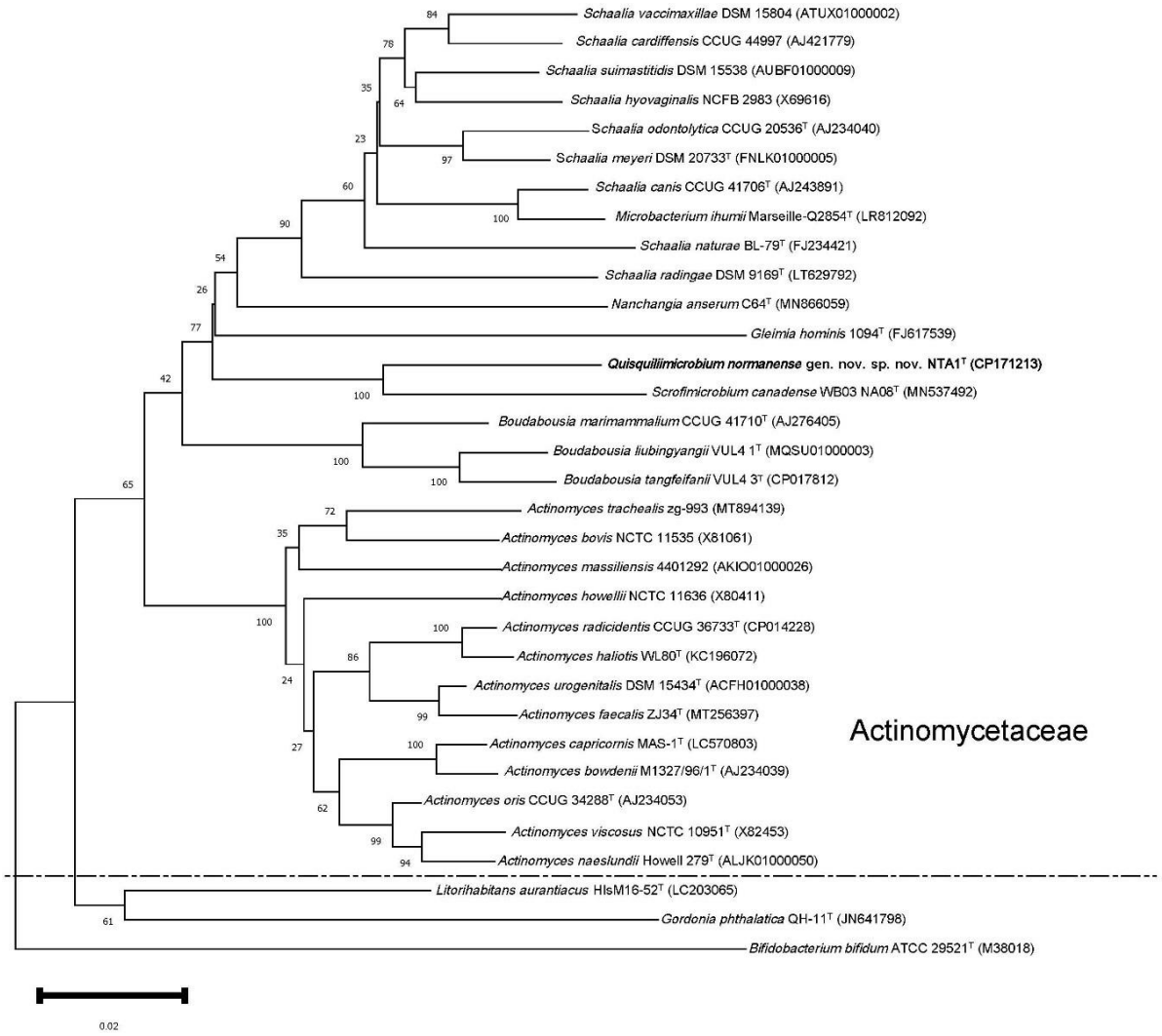

Figure 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence similarity, (Maximum Composite XXXX model)demonstrating that *Quisquiliimicrobium* gen. nov. belongs to the family *Actinomycetaceae*. Bootstrap values at branch nodes were calculated from 1000 repetitions.

3.2 Genome Features

The genome sequence was a single contig, suggesting no plasmids or contamination present. The isolate had a genome size of 2.3 Mb with 2,126 genes annotated at a coverage of 100x. To further investigate the relatedness between the NTA1^T and its closest relatives, its genome was submitted to the Type-Strain Genome Server (TYGS) [35]. Digital DNA-DNA Hybridization (dDDH, d_4 in %) between NTA1^T and its closest relative as determined by whole genome comparison, *Gordonia phthalatica* of the family *Mycobacteriaceae*, was shown to be 31.0% (Table 2) [36]. This prompted a submission of genomes belonging to NTA1^T, *G. phthalatica*, and the isolate's closest relative according to 16S rRNA gene similarity – *Scrofimicrobium canadense* - to EZBioCloud for OrthoANIu analysis [17]. This showed an ANI% value of 71.45% for NTA1^T and *G. phthalatica*, and one of 69.65% between NTA1^T and *S. canadense*. A phylogram based on the genome similarity between strain NTA1^T and other close relatives was made by TYGS analysis (Fig. 3). However, one must be careful of the interpretation, dDDH and ANI should only be used for closely related taxa, of the data. A 16S rRNA gene similarity value of 84.10% shared between NTA1^T and *G. phthalatica* demonstrates that these organisms are not closely related, subsequently dDDH and ANI are not suitable tools to use.

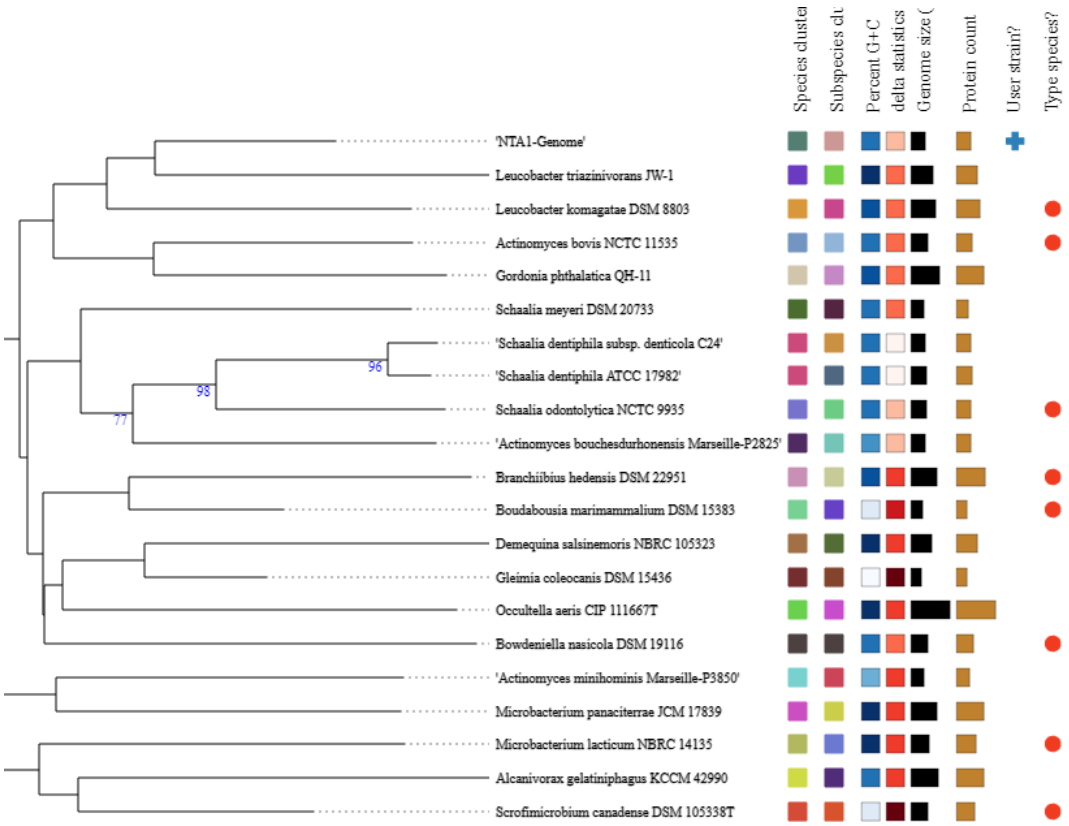

Figure 3. Phylogram computed by whole-genome sequence analysis of strain NTA1^{T} and its nearest relatives by TYGS. Legend shows predicted species clusters, subspecies clusters, G+C%, delta statistics, relative genome size, relative protein count, user strains, and current type species [35].

3.3 Metabolic Feature Characterization

NTA1^{T} was found to be a Gram-positive-staining coccobacillus through the use of an Advanced™ Gram Stain Kit with Stabilized Iodine (Hardy Diagnostics) and confirmed through the use of the KOH test [20]. The strain was determined to be non-endospore-forming through the process of an endospore stain [21]. Cells were subjected to a needle stab inoculation of motility test medium, the results of which suggested NTA1^{T} was nonmotile [22]. Due to the relatedness between the whole genome sequences of *Gordonia phalatica* and NTA1^{T} (Table 2),

an acid-fast stain was performed in triplicate, finding the strain to be non-acid-fast [23]. NTA1^T was assessed to be catalase- and oxidase-negative through the administration of the slide (drop) catalase test method and filter paper oxidase test method [24-25].

Metabolic function was characterized using a BioLog Gen III MicroPlate^{TM} (www.BioLog.com). Cells of strain NTA1^T showed metabolic function for 14 of the 71 tested substrates (Table 3). For the chemical sensitivity assay portion of the MicroPlate^{TM}, cells of strain NTA1^T showed metabolic activity in the positive control and in the presence of 10 of the other 23 chemical conditions (Table 4).

Optimum temperature and temperature range for the isolate was determined through cultivation on Tryptic Soy Broth (TSB) supplemented with 5% (v/v) defibrinated sheep blood at 4°C, 25°C, 30°C, 37°C, and 45°C, with growth being measured by eye. Strain NTA1^T showed an optimum temperature of 45°C, and growth in a range of temperatures from 25 - 45°C. Optimum and ranges for salinity and pH were also assessed by eye on a solid medium made from TSA supplemented with 5% (v/v) defibrinated sheep blood, at increments of 1 pH in a range from 4 to 10 pH and increments of 1% NaCL (w/v) in a range from 1% NaCl to 10% NaCl in addition to the previously used baseline 0.5% NaCl found in TSA. Optimum pH was shown to be 7 pH with growth at a pH range of 6-7. Optimum salinity was shown to be 0.5% NaCl, with a range from 0.5 - 1% NaCl. All characterization tests were carried out in triplicate.

3.4. In-silico Prediction of Polar Lipid Production

As seen below (Fig. 4) NTA1^T is predicted to possess a glycerophospholipid metabolism which differs from the strains discussed in Chapter 2. The results of a KEGG-based polar lipid analysis (Figure 3) indicate that strain NTA1^T contains the enzymes phosphatidylglycerophate synthetase

(E.C. 2.7.8.5) and but lacks phosphatidylglycerophate phosphatase (E.C. 3.1.3.27) required to synthesize phosphatidylglycerol (PG). However, it is likely that PG is present as this is an intermediate of DGP (Cardiolipin) and the enzyme cardiolipin synthase (E.C. 2.7.8.41) is present. Two explanations are possible (i) the lack of phosphatidylglycerophate phosphatase (E.C. 3.1.3.27) can be an annotation problem with KEGG or (ii) an otherwise unknown enzyme is taking the place of phosphatidylglycerophate phosphatase (E.C. 3.1.3.27). Strain NTA1^{T} also lacks phosphatidylserine synthase (EC 2.7.8.8) used in the synthesis of phosphatidyl-L-serine (PS), an intermediate compound that the enzyme phosphatidylserine decarboxylase (PSD) (EC 4.1.1.65) further catalyzes in the production of phosphatidylethanolamine (PE) from phosphatidyl-L-serine. Therefore, the predicted polar lipids produced are PG and DGP, consistent with other members in the family *Actinomycetaceae* [36].

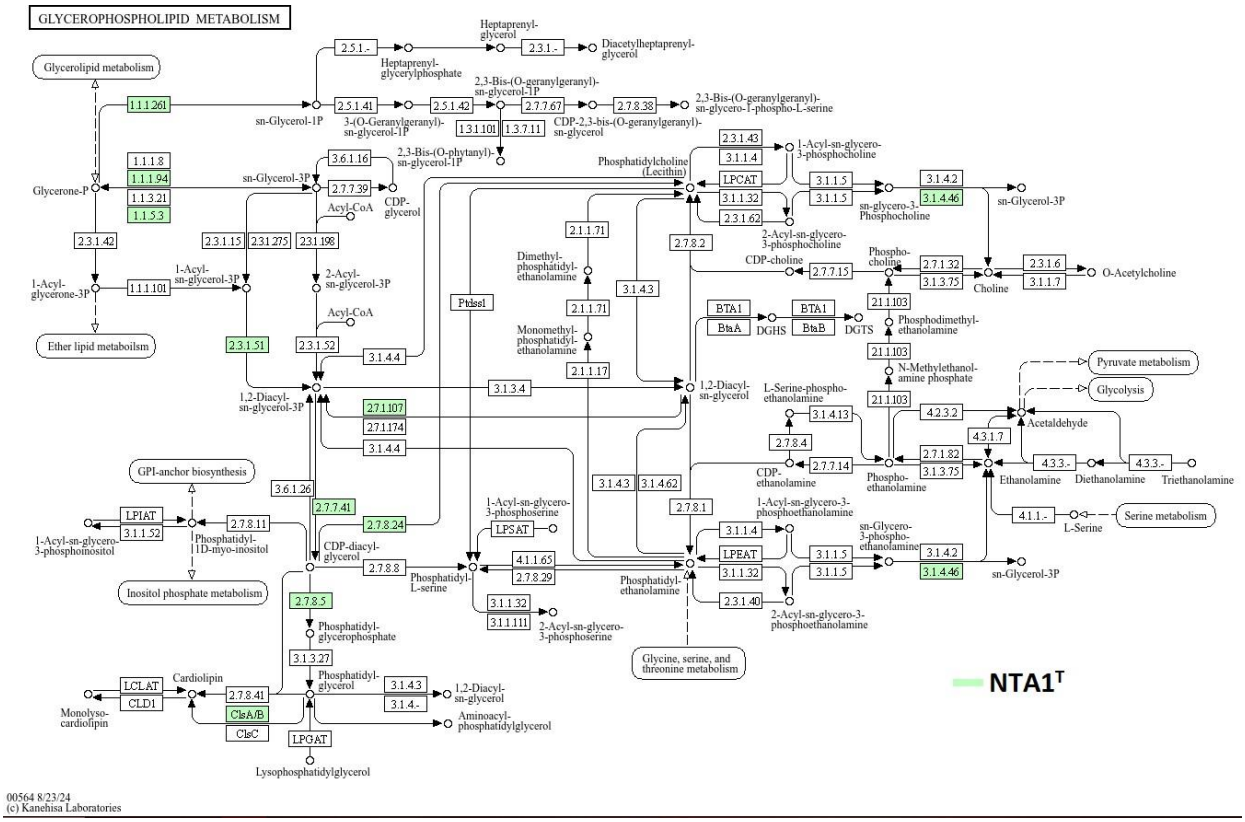

Figure 4. Glycerophospholipid metabolism map for three novel organisms made with KEGG Mapper [26]. In the figure, enzymes found in the genome of NTA1^{T} are shown in green.

Strain NTA1^{T} was a Gram-stain-positive coccobacillus that was facultatively anaerobic and nonmotile. Sporulation has not been observed. Morphology, phenotypic, phylogenetic and genotypic analysis demonstrated that the strain represents a novel species and genus for which the name *Quisquiliimicrobium normanense* gen. nov. sp. nov. is proposed.

Table 1. Table showing physiological properties of strain NTA1^T, 1, as well as seven close relatives of the proposed genus *Quisquiliimicrobium*. From left to right, the top row of numbers correlates to *Scrofimicrobium canadense*, 2, *Actinomyces hominis*, 3, *Boudabousia marimammalium*, 4, *Schaalia odontolytica*, 5, *Actinomyces bovis*, 6, *Microbacterium lacticum*, 7, and *Gordonia phthalatica*, 8. An oxygen tolerance value listed as +/- denotes an organism which exhibits facultatively anaerobic growth, and a value of u+ correlates to a microaerophile.

	1	2	3	4	5	6	7	8
Catalase	-	+	+	-	+	-	+	+
Oxidase	-	+	-	+	-	-	-	+
Gram-stain Reaction	+	+	+	+	+	+	+	+
Endospore Production	-	-	-	-	-	-	-	-
Acid-fastness	-	-	-	-	-	-	-	+
Motility	-	-	-	-	-	-	-	-
Oxygen Tolerance	+/-	+	u+	u+	-	+/-	-	+

Table 2. Table showing physiological properties of strain NTA1^T, 1, as well as seven close relatives of the proposed genus *Quisquiliimicrobium*. From left to right, the top row of numbers correlates to *Scrofimicrobium canadense*, 2, *Actinomyces hominis*, 3, *Boudabousia marimammalium*, 4, *Schaalia odontolytica*, 5, *Actinomyces bovis*, 6, *Microbacterium lacticum*, 7, and *Gordonia phthalatica*, 8. Asterisks denote data which were unavailable to the author. Digital DNA-DNA hybridization percentages (dDDH%) were calculated using the d4 formula and accessed through the use of TYGS along with G+C%, genome size, and predicted proteins [36-37].16S rRNA gene similarity was assessed using EZBioCloud's online database [17].

	1	2	3	4	5	6	7	8
16S %Sim	100%	94.58%	89.70%	91.36%	90.73%	89.67%	90.39%	84.10%
dDDH%	100%	26.3%	21.6%	23.2%	20.1%	20.3%	18.5%	31.0%
G+C%	64.0%	53.52%	49.60%	54.19%	64.88%	64.08%	70.53%	68.00%
Culture Collection ID	CCM 9399	DSM 105338T	DSM 22168	DSM 15383	NCTC 9935	DSM 43014	NBRC 14135	DSM 108671
No. proteins	2072	2497	1546	1518	2061	2167	2724	3902
Base pairs (Mb)	2.30	2.59	1.72	1.84	2.45	2.60	2.85	4.43

Table 3. Table showing metabolic activity of NTA1^T on assorted substrates.

Well	Substrate	Metabolic Activity (+/−)	Descriptor	Carbon Number
A1	Negative Control	−	Control	N/A
A2	Dextrin	+	Oligosaccharide	Variable
A3	D-Maltose	+	Disaccharide	C12
A4	D-Trehalose	−	Disaccharide	C12
A5	D-Cellobiose	+	Disaccharide	C12
A6	Gentiobiose	−	Disaccharide	C12
A7	Sucrose	−	Disaccharide	C12
A8	D-Turanose	−	Disaccharide	C12
A9	Stachyose	−	Oligosaccharide	C24
B1	D-Raffinose	−	Oligosaccharide	C18
B2	α-D-Lactose	−	Disaccharide	C12
B3	D-Melibiose	−	Disaccharide	C12
B4	β-Methyl-D-Glucoside	−	Glucoside	C7
B5	D-Salicin	−	Glucoside	C13
B6	N-Acetyl-D-Glucosamine	+	Amino sugar	C8
B7	N-Acetyl-β-D-Mannosamine	−	Amino sugar	C8
B8	N-Acetyl-D-Galactosamine	−	Amino sugar	C8
B9	N-Acetyl Neuraminic Acid	+	Sialic acid	C11
C1	α-D-Glucose	+	Monosaccharide (Hexose)	C6
C2	D-Mannose	+	Monosaccharide (Hexose)	C6
C3	D-Fructose	+	Monosaccharide (Hexose)	C6
C4	D-Galactose	+	Monosaccharide (Hexose)	C6
C5	3-Methyl Glucose	−	Methylated Monosaccharide	C6
C6	L-Fucose	+	Monosaccharide (Deoxyhexose)	C6
C7	L-Rhamnose	−	Monosaccharide (Deoxyhexose)	C6
C8	Inosine	+	Nucleoside	N/A
C9	1% Sodium Lactate	−	Control	N/A
D1	D-Sorbitol	−	Sugar alcohol	C6
D2	D-Mannitol	−	Sugar alcohol	C6
D3	D-Arabitol	−	Sugar alcohol	C5
D4	myo-Inositol	+	Sugar alcohol	C6
D5	Glycerol	+	Sugar alcohol	C3
D6	D-Glucose-6-PO4	−	Phosphorylated sugar	C6
D7	D-Fructose-6-PO4	−	Phosphorylated sugar	C6
D8	D-Aspartic Acid	−	Amino acid	C4
D9	D-Serine	−	Amino acid	C3
E1	Gelatin	−	Protein	Variable
E2	Glycyl-L-Proline	−	Dipeptide	Variable
E3	L-Alanine	−	Amino acid	C3
E4	L-Arginine	−	Amino acid	C6
E5	L-Aspartic Acid	−	Amino acid	C4
E6	L-Glutamic Acid	−	Amino acid	C5
E7	L-Histidine	−	Amino acid	C6
E8	L-Pyroglutamic Acid	−	Amino acid derivative	C5
E9	L-Serine	−	Amino acid	C3
F1	Pectin	−	Polysaccharide	Variable
F2	D-Galacturonic Acid	−	Sugar acid	C6
F3	D-Galactonic Acid Lactone	−	Sugar acid	C6
F4	D-Gluconic Acid	−	Sugar acid	C6
F5	D-Glucuronic Acid	−	Sugar acid	C6
F6	Glucuronamide	−	Amide	C6
F7	Mucic Acid	−	Sugar acid	C6
F8	Quinic Acid	−	Cyclic polyol	C7
F9	D-Saccharic Acid	−	Sugar acid	C6
G1	p-Hydroxy-Phenylacetic Acid	−	Aromatic acid	C8
G2	Methyl Pyruvate	−	Ketone	C4
G3	D-Lactic Acid Methyl Ester	−	Organic ester	C4
G4	L-Lactic Acid	−	Organic acid	C3
G5	Citric Acid	−	Organic acid	C6
G6	α-Keto-Glutaric Acid	−	Organic acid	C5
G7	D-Malic Acid	−	Organic acid	C4
G8	L-Malic Acid	−	Organic acid	C4
G9	Bromo-Succinic Acid	−	Organic acid	C4
H1	Tween 40	−	Surfactant	Variable
H2	γ-Amino-Butyric Acid	−	Organic acid	C4
H3	α-Hydroxy-Butyric Acid	−	Organic acid	C4
H4	β-Hydroxy-D,L-Butyric Acid	−	Organic acid	C4
H5	α-Keto-Butyric Acid	−	Organic acid	C4
H6	Acetoacetic Acid	+	Ketone body	C4
H7	Propionic Acid	−	Organic acid	C3
H8	Acetic Acid	−	Organic acid	C2
H9	Formic Acid	−	Organic acid	C1

Table 4. Table showing metabolic activity of NTA1^{T} in the presence of chemical conditions.

Well	Chemical Condition	Metabolic Activity (+/−)	Descriptor
A10	Positive Control	+	Control
A11	pH 6	+	pH condition
A12	pH 5	−	pH condition
B10	1% NaCl	+	Salt condition
B11	4% NaCl	−	Salt condition
B12	8% NaCl	−	Salt condition
C10	1% Sodium Lactate	−	Antimicrobial
C11	Fusidic Acid	−	Antibiotic
C12	D-Serine	−	Amino acid
D10	Troleandomycin	−	Antibiotic
D11	Rifamycin SV	−	Antibiotic
D12	Minocycline	−	Antibiotic
E10	Lincomycin	−	Antibiotic
E11	Guanidine HCl	+	Chaotropic agent
E12	Niaproof 4	−	Surfactant
F10	Vancomycin	+	Antibiotic
F11	Tetrazolium Violet	+	Redox indicator
F12	Tetrazolium Blue	−	Redox indicator
G10	Nalidixic Acid	+	Antibiotic
G11	Lithium Chloride	+	Salt condition
G12	Potassium Tellurite	+	Selective agent
H10	Aztreonam	+	Antibiotic
H11	Sodium Butyrate	+	HDAC inhibitor
H12	Sodium Bromate	−	Oxidizing agent

Description of *Quisquiliimicrobium* gen. nov.

Quisquiliimicrobium (Quis.qui.li.i.mi.cro'bi.um. L. pl. fem. n. *quisquiliae,* waste; N.L. neut. n. *microbium*, a microbe; N.L. neut. n. *Quisquiliimicrobium*, a microbe from waste).

The cells are facultatively anaerobic, nonmotile, non-acid-fast, Gram-stain-positive coccobacilli in which sporulation is not observed. Optimal growth was observed at 45°C with 0.5% (w/v) NaCl at 7.0 pH. Growth was seen at temperatures ranging from 25°C to 45°C, at a pH of 6-7, and at salinity ranging from 0-1% (w/v) NaCl. Isolate was taken from a wastewater enrichment with the sole substrate of NTA. Isolate can grow in anaerobic conditions on minimal medium containing noble agar and only nitrilotriacetic acid as a substrate. In-silico analysis predicts that the predicted polar lipids produced are PG and DGP, consistent with other members in the family *Actinomycetaceae.*

The proposed type species for *Quisquiliimicrobium* is *Quisquiliimicrobium normanense*, for which the proposed type strain is NTA1^{T} (=CCM 9399^{T}=NRRL B-65709^{T}). The original wastewater sample was taken from the City of Norman Water Reclamation Facility in Norman, OK, USA. The G+C content is 64.0%.

Description of *Quisquiliimicrobium normanense* gen. nov. sp. nov.

Quisquiliimicrobium normanense (nor.man.en'se. N.L. neut. adj. *normanense*, pertaining to the city of Norman, OK, USA, where the organism was first isolated.)

The description is the same as for the genus. The proposed type strain, NTA1^{T} (=CCM 9399^{T}=NRRL B-65709^{T}), was isolated from an enrichment containing nitrilotriacetic acid (NTA) as the sole substrate, inoculated with a wastewater sample taken from the City of Norman Water

Reclamation Facility in Norman, OK, USA. Accession numbers for the strain NTA1^{T} 16S rRNA gene and whole genome sequences are PP935230 and CP171213.

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Chapter 4

Discission: Regarding the Isolation of Fastidious and Otherwise Unique Microorganisms with Catabolic Effects on Anthropogenic Contaminants

The goal of this study was to isolate and identify organisms exhibiting interesting metabolic capabilities, specifically demonstrating the ability to degrade the anthropogenic contaminants sucralose and nitrilotriacetic acid (NTA) under anoxic conditions. Unravelling the causes for concern which have been and are still being documented by the scientific community added a sense of justification to these investigations. There is no misconception that a one-stop solution to all contamination is at hand, but a positive outlook is maintained on the incremental progress outlined by the work included in this thesis. From a personal perspective, witnessing bacteria capable of performing some unique metobolic activities is something truly wonderful.

Of these, three novel strains of two proposed genera and species were discovered. Strains $9S^T$ and NTA5 of *Purgationimicrobium sucraloseivorans* gen. nov. sp. nov. share the greatest 16SrRNA gene similarity with *Azonexus caeni* (95.1%), *Azonexus fungiphilus* (94.9%), and *Azonexus hydrophilus* (94.8%), while $NTA1^T$ of *Quisquiliimicrobium normanense* gen. nov. sp. nov. shares the greatest 16SrRNA gene similarity with *Scrofimicrobium canadense* (94.15%), *Boudabousia marimammalium* (91.16%), and *Schaalia meyerii* (90.94%).

Both strains of the *P. sucraloseivorans* grew anaerobically on media containing either sucralose or NTA as a sole substrate, while the single strain of *Q. normanense* only grew anaerobically on media containing NTA as the sole carbon source, but not that with sucralose. While it is assumed these are not the only bacteria capable of this metabolism, identifying

members of wastewater microbial populations which determine the fate of these compounds in the environment could be an important part of remediation efforts as fears about the accompanying effects of these chemicals' effects are confirmed by on-going investigations [1-8]. If the day comes when rising levels of NTA, sucralose, or other xenobiotic compounds which accompany industrialization, urbanization, or agricultural practices are to be addressed, manipulating an existing microbial response may be a component of strategies contributing to an effective solution with a minimal environmental impact.

Given the increasing prevalence of recalcitrant xenobiotic compounds in wastewater systems, the potential health risks associated with their persistence in drinking water sources warrant attention [9-12]. Microbial bioremediation offers a promising avenue for mitigating these risks and safeguarding public health [13-15]. It is hoped that policymakers prioritize research funding for bioremediation techniques and enforce regulations on the discharge of anthropogenic contaminants into wastewater systems. Establishing guidelines for acceptable levels of sucralose and NTA in treated effluents could help protect both natural ecosystems and human health.

Future research could focus on community-level interactions which might facilitate the growth of the organisms described here, the dechlorination of organochlorine carbohydrates, or the sequestration of heavy metals. Another focus of future research could be elucidating the specific metabolic pathways employed by which *P. sucraloseivorans* and *Q. normanense* conduct these catabolic functions. In addition to enriching our understanding of bacterial biochemistry, if individual genes for this metabolism could be identified and introduced into *E. coli*, or a similar species, the slow-growing nature of these organisms could be circumvented in the application of the genes which are responsible for the degradation of the anthropogenic contaminants in question.

As urbanization and industrialization continue to escalate, innovative solutions like bioremediation using microorganisms could be important in maintaining ecosystem integrity and promoting sustainable practices. The findings presented herein highlight the potential tools at our disposal, should harnessing the ongoing microbial response be chosen as a tool in the fight against chemical contamination such as this. The ability of *Purgationimicrobium* gen. nov., sp. nov. *sucraloseivorans* and *Quisquiliimicrobium normanense* gen. nov., sp. nov. to degrade persistent contaminants in wastewater may contribute towards global sustainability. The introduction of species such as these to wastewater treatment plants where they are undetected through the use of molecular methods may allow for incremental reduction in the contaminants they have been found to degrade before they become a cause for concern. By addressing the challenge of emerging pollutants, these organisms could contribute to more resilient and sustainable water management practices.

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Appendix A: Authored Publications During Graduate Studies

1. **Bacteroides vicugnae sp. nov., isolated from the fecal material of an alpaca**
    - Authors: Samuel Miller, Meredith Hendry, Jacobey King, Krithivasan Sankaranarayanan, Paul A. Lawson
    - Journal: *Anaerobe*, Vol. 88, 2024, Article ID 102862
    - Summary: This study reports on the isolation and characterization of *Bacteroides vicugnae* from the alpaca gut microbiome, detailing its phylogeny, genome characteristics, and potential functional roles within mammalian microbiomes.
2. **Clostridium tanneri sp. nov., isolated from the fecal material of an alpaca**
    - Authors: Samuel Miller, Meredith Hendry, Jacobey King, Krithivasan Sankaranarayanan, Paul A. Lawson
    - Journal: *International Journal of Systematic and Evolutionary Microbiology*, Vol. 74, 2024, Article ID 006372
    - Summary: This publication describes *Clostridium tanneri*, a novel strictly anaerobic species isolated from alpaca feces, including detailed taxonomic classification and genomic analysis.

Appendix B: Teaching Experience and Responsibilities

1. **Instructor of Record – Water Microbiology (University of Oklahoma)**

- Term: Fall 2024
- Responsibilities: Lectured on microbial aspects of water safety, and assessed students' laboratory notebooks for practical understanding of microbiological techniques in water quality testing, prepared and oversaw the implementation of class experiments, including methane-producing bioreactors.

2. **Instructor of Record – Microbiology Capstone Lab (University of Oklahoma)**

- Terms: Spring 2023 – Spring 2024
- Duties: Supervised student experiments including environmental isolation of targeted taxa, provided instruction in advanced microbiological techniques, including aseptic techniques and molecular biology methods, and offered mentorship on scientific reporting and data analysis.
- Awards: 2024 SBS Award for Excellence in Graduate Student Teaching

3. Teaching Assistant – Techniques in Microbiology

- Term: Fall 2022

4. Duties: Media preparation, assistance with course materials, proctoring exams and assisting in advanced microbiological techniques like transformation by electroporation.

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Anaerobes in the microbiome

Anaerobes in the microbiome

Bacteroides vicugnae sp. nov. isolated from the fecal material of an alpaca

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ABSTRACT

Two strictly anaerobic, Gram-stain-negative rod-shaped bacterial isolates, A2-P53^T and A1-P5, were isolated from an enrichment of fecal material from two alpacas (*Vicugna pacos*). Based on a comparative 16S rRNA gene sequence analysis, the isolates were assigned to the genus *Bacteroides* with the highest sequence similarities to *Bacteroides barnesiae* YS-aM39T (A2- P53T 97.7 % and A1-P5 97.9 %). Additionally, the average nucleotide identity and digital DNA-DNA hybridization values between these isolates and their closest relatives within *Bacteroides* were less than 92.1 % and 49.1 %, respectively. The average nucleotide identity between isolates A2-P53^T and A1-P5 was 99.9 %. The predominant cellular fatty acid for isolates A2-P53^T and A1-P5 was $C_{15:0}$ anteiso. The G+C % content of the isolates was 41.7 %. Based on biochemical, phylogenetic, genotypic, and chemotaxonomic criteria, these isolates A2-P53^T and A1-P5 represent two individual strains of a novel species within the genus *Bacteroides* for which the name *Bacteroides vicugnae* sp. nov. is proposed. The type strain of this species is strain A2-P53^T (CCUG 77273^T = CCM 9377^T = NRRL B-65693^T).

1. Introduction

There is an inherent bacterial cultivation bias towards humans as 400 % more bacteria have been isolated from humans than from the rest of the class *Mammalia* (https://bacdive.dsmz.de). Furthermore, there is a paucity of microbiome data regarding the alpaca (*Vicugna pacos*), and minimal microbiome studies have been exclusively focused on culture-independent methods [1–4]. These molecular inventories have provided tremendous insights into the microbial diversity and taxa richness within the gut of these modified ruminants. Similarly to the human gut, the alpaca gut presents itself as an environment for microbial discovery, as many phylotypes likely represent uncultured bacteria [5]. There is interest in pursuing culture-dependent approaches to recovering microorganisms and characterizing their physiological and metabolic properties that may be a source of bioactive compounds [6,7]. One such metabolic property of interest is the ability to degrade raw plant materials into products destined for human utilization. The consortium of mammalian rumen microbiota is uniquely poised to degrade this plant biomass [8–10] through the utilization of enzymes designated as carbohydrate-active enzymes (CAZymes), as described in the carbohydrate-active enzyme database (www.cazy.org) [11]. The prevalence of CAZymes within the genus *Bacteroides* is notably significant [12,13]. Genomic analyses of numerous *Bacteroides* species have revealed an impressive array of CAZymes [14]. While most of the studies focused on human gut-associated CAZymes, recently, other mammals, such as goats [15], cattle [16,17], yaks [18], sheep [19], and buffalo [16,20] have been studied for their CAZyme composition. CAZymes are classified into six broad categories: glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), glycosyl transferases (GTs), carbohydrate-binding modules (CBMs), and auxiliary activities (AAs).

In a continuing study of mammalian microbiomes within our laboratories, using a polyphasic taxonomic approach, two strains of a novel *Bacteroides* species, designated as strains A2-P53^T and A1-P5, were recovered from separate, anoxic pectin enrichments of fecal materials from two alpacas (*Vicugna pacos*), for which the name *Bacteroides vicugnae* sp. nov. is proposed.

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2. Materials and methods

2.1. Bacterial strains

Strains A2-P53^T and A1-P5 were isolated from freshly voided alpaca fecal samples obtained from two domesticated alpacas (a male, suri alpaca and a female, huacaya alpaca), herein denoted by A1 or A2, from the same privately owned alpaca ranch near Newcastle, Oklahoma, USA (35.24 N 97.59 W). The freshly voided fecal samples were collected and immediately placed into an anaerobic jar (oxoid) with an anaerobic gas pack (BD). The jar was placed on ice and transported to the Lawson Microbial Systematics Laboratory at the University of Oklahoma, Norman, for further processing. Multiple anoxic enrichments, using xylan, pectin, and cellulose as sole substrates, were prepared using 1.0 ml fecal slurry in a sealed serum bottle containing 100 ml modified Medium 2 (per 100 ml distilled water): Casitone (1.0 g), yeast extract (0.25 g), minerals solution (A) (15.0 ml), minerals solution (B) (15.0 ml), clarified rumen fluid (20 ml) [21], resazurin (0.0001 g), sodium lactate (70 % w/v) (1.0 g), sole substrate (0.2 g), cysteine HCl (0.05 g), sodium bicarbonate (0.4 g), distilled water (to 1000 ml). Minerals solution (A) contained (per 1000 ml); K_2HPO_4 (3.0 g). Minerals solution (B) contained (per 1000 ml) KH_2PO_4 (3.0 g), $(NH_4)_2SO_4$ (6.0 g), NaCl (6.0 g), $MgSO_4x7H_2O$ (0.6 g), $CaCl_2$ (0.6 g) as previously described [22]. The enrichment was incubated at 37 •C for 14 days with a headspace of 5 % hydrogen, 10 % carbon dioxide, and 85 % nitrogen to maintain anoxic conditions. After incubation, samples were inoculated onto Medium 2 agar, as previously described above, containing an additional 15.0 g of agarose per 1000 ml of media, containing the same sole carbon source as their original enrichment. Isolates were then sub-cultured onto BD Bacto™ Brain Heart Infusion (BHI) (Sparks, MD, USA) agar plates supplemented with 5 % defibrinated sheep's blood via multiple plate streaks until pure isolates were obtained as determined by Gram stain reaction and phase contrast microscopy (CX41, Olympus). Two of the resulting isolates were designated A2-P53^T and A1-P5. Strains A2-P53^T and A1-P5 were independently isolated from enrichments of different alpaca fecal material in modified Medium 2 supplemented with pectin. After primary isolation, A2-P53^T and A1-P5 were maintained under anoxic conditions on BHI plus 5 % sheep's blood medium at 37 •C and preserved in BHI plus 5 % clarified rumen fluid with 20 % (v/v) glycerol at −80 •C.

2.2. 16S rRNA gene sequence determination and phylogenetic analysis

Strains A2-P53^T and A1-P5 were sent to GENEWIZ (Genewiz.com), where near full-length 16S rRNA gene sequences were obtained using Sanger sequencing [23]. The nearest phylogenetic neighbors of strains A2-P53^T and A1-P5 were determined by a BLAST search against the GenBank database and EzBioCloud's Identify [24–26]. The 16S rRNA gene sequences for the strains nearest neighbors, in addition to the type strain for ***Bacteroides***, ***Bacteroides fragilis*** NCTC 9343^T (GenBank: KP326374), were downloaded from EzBioCloud, and these sequences, along with the newly derived sequences, were aligned using MUSCLE [27] and trimmed in MEGA 11 version 11.0.13 [28]. *Phocaeicola abscessus* CCUG 5592^T (GenBank: EU694176) was used as an out-group. A phylogenetic tree was reconstructed according to the maximum-likelihood method [29] (Fig. 1) and neighbor-joining method [30] (Fig. S1) using MEGA 11 software; the Tamura-Nei substitution model [31] and bootstrap analyses were performed using 1000 replications.

2.3. Determination of whole genome sequences and in-silico analysis

Cell biomass for A2-P53^T and A1-P5 was harvested from overnight cultures, and genomic DNA was extracted using the DNeasy DNA extraction kit (Qiagen) following the manufacturer's protocol. The DNA yields were quantified using a Qubit 3.0 fluorometer, and 100 ng of DNA was used as input for each genomic library construction. Briefly, the DNA was sheared using a QSonica 800R, followed by bead cleanup to

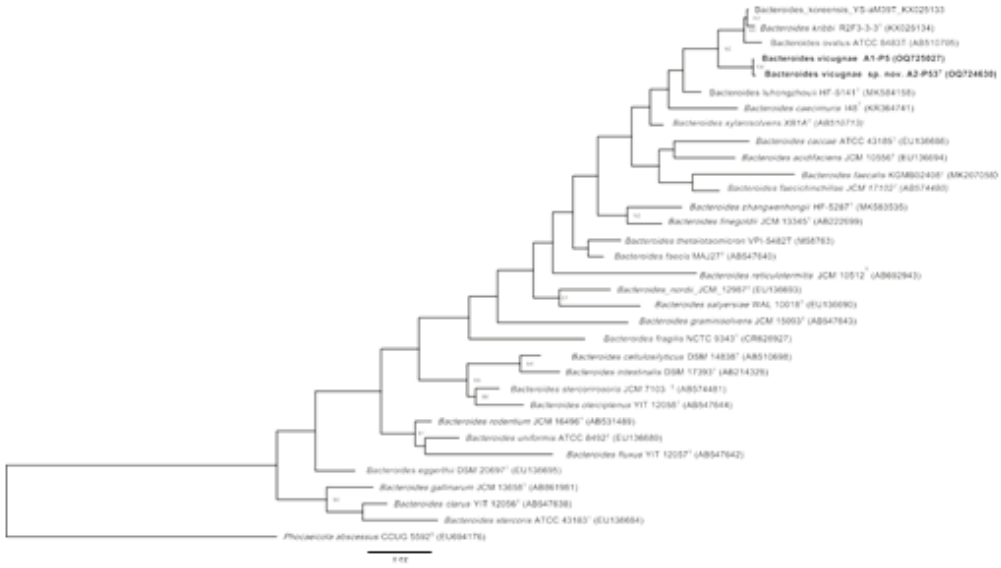

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationships between ***Bacteroides vicugnae*** sp. nov. A2-P53^T and A1-P5 within the ***Bacteroides*** genus. Bootstrap values (%) were obtained with 1000 replicates and are displayed at their branch nodes. *Phocaeicola abscessus* CCUG 5592^T (EU694176) was used as the out-group. Bar, 0.02 substitutions per site.

remove short fragments (<150 bp). Next, PCR sequencing libraries were generated using Illumina-specific barcoded primers and pooled from triplicate reactions. Finally, sequencing libraries were purified using bead cleanup (1.5X), quantified using a Tape Station, pooled in equimolar proportions, and sequenced on an Illumina MiSeq.

Using SPAdes v3.12.0 [32], the genomes for A2-P53^T and A1-P5 were assembled and annotated using the Bacterial and Viral Bioinformatics Resource Center [33] (BV-BRC) (https://www.bv-brc.org/). In addition, genomic sequences of close relatives for strains A2-P53^T and A1-P5, in addition to the type strain for ***Bacteroides***, ***Bacteroides fragilis*** NCTC 9343^T (GCA_000025985.1), were downloaded from BV-BRC.The evolutionary relationship between strains A2-P53^T and A1-P5 (GCA_034291865.1) and (GCA_034291865.1) and their nearest neighbors were evaluated with a phylogenetic approach. A whole genome-based phylogenetic tree was generated using the CodonTree method within BV-BRC [33]. The CodonTree method used the amino acid sequences from a defined number of BV-BRC's protein families (PATtyFams) [34] to build an alignment. A tree was generated based on the differences within those selected sequences. The protein sequences were used for each selected gene from the PATtyFams. The aligned proteins from single-copy genes were used for rapid RAxML analysis v8.2.11 [35,36]. The support values for the phylogenetic tree are generated using 100 rounds of the 'Rapid bootstrapping' option of RaxML, and visualization was accomplished with the Interactive Tree Of Life v3 [37]. ***Phocaeicola abscessus*** CCUG 55929^T (GenBank: GCA_000312445.1) was used as an out-group. The taxonomic status of strains A2-P53^T and A1-P5 was confirmed by comparing ANI and digital dDDH values between the strains A2-P53^T and A1-P5, in addition to their close relatives. Values for ANI between strains A2-P53^T and A1-P5 and close relatives were calculated using EzBioCloud's ANI Calculator, which uses the OrthoANIu algorithm [25,38]. The Genome-to-Genome Distance Calculator [39,40] v3.0 was used to determine dDDH. Prodigal [41] determined the DNA G+C (%) content. *In-silico* CAZyme analysis was performed using the dbCAN3 meta server [42,43] and CAZy (https://www.cazy.org/).

2.4. Phenotypic characterization

The Gram stain reaction was determined using the Advanced Gram Stain Kit (Hardy Diagnostics, CA) following the manufacturer's instructions. Aerotolerance was determined by placing cultures on media under aerobic conditions for one week at 37 •C. Cells were inspected for motility and endospore production by phase contrast microscopy (CX41, Olympus) at x1000 magnification. Temperature ranges for growth were determined in BH1 broth supplemented with 5 % clarified rumen fluid at 4 •C, 20 •C, 37 •C, 45 •C, 50 •C, and 55 •C salt tolerance was determined in BH1 supplemented with 5 % clarified rumen fluid at 0.5 % (w/v) and between 1.0 % (w/v) and 10.0 % (w/v), in increments of 1.0 % (w/v); pH ranges for growth were determined between 5.0 and 9.0 in increments of 1.0, and all tests were performed in duplicate. For the pH ranges, the following buffers were prepared at a final concentration of 10 mM: sodium acetate (pH 5.0), potassium phosphate (pH 6.0–8.0), and Tris-HCl (pH 9.0). Optimal growth conditions were determined by optical density (OD600) using a spectrophotometer (Spectronic 20D+, Milton Roy). An increase in OD600 greater than 0.1 after five days of incubation was considered growth. Additional biochemical traits were determined using the API-ZYM and API rapid ID32A test systems (bioM´erieux) and performed in duplicate using the manufacturer's instructions.

2.5. Cellular fatty acids analysis

Chemotaxonomic features were determined at the Center for Microbial Identification and Taxonomy (University of Oklahoma, Norman, Oklahoma). The cellular fatty acids of strains A2-P53^T and A1-P5 were harvested, extracted, and analyzed using the Microbial Identification System (MIDI) [44,45]. Analysis was performed with an Agilent Technologies 6890 N gas chromatograph equipped with a phenyl methyl silicone fused silica capillary column (HP-Ultra 2.25m × 0.2 mm × 0.33 μm film thickness) coupled with a flame ionization detector. Hydrogen was used as the carrier gas. The temperature program was preset at 170 •C and increased at 5 •C min−1 to a final temperature of 270 •C. Fatty acids were identified and expressed in percentages using the QBA1 peak naming database within the MIDI system.

3. Results and discussion

3.1. Phylogenetic analysis

Phylogenetic analyses based on 16S rRNA gene sequences demonstrated that strains A2-P53^T and A1-P5 were most closely related to ***Bacteroides koreensis*** Ys-aM39^T (GenBank: KX025133) (97.9/97.7 %), ***Bacteroides ovatus*** ATCC 8486^T (GenBank: DS264555) (97.4/97.6 %), and ***Bacteroides luhongzhouii*** HF-5141^T (GenBank: MK584158) (97.2/96.1 %). These values, along with the phylogenetic trees constructed by the Maximum Likelihood method [29] (Fig. 1) and the Neighbor-Joining method [30] (Fig. S1), support the proposal that strains A2-P53^T and A1-P5 were potentially novel and may represent a novel species based on the 16S rRNA gene percent similarity acceptance threshold of 98.8 % routinely used to demarcate this rank [46]. To determine if the isolates were indeed novel species, whole genome metrics such as ANI and dDDH were also performed.

3.2. Genomic analyses

Once assembled, the draft genome sequence for strains A2-P53^T and A1-P5 consisted of 49 and 46 contigs, with genome lengths of 6.2 Mbp, N50 lengths of approximately 389 Kbp, and an average G+C content of 41.7 %. Annotation of the A2-P53^T draft genome sequence in BV-BRC revealed 4741 protein-coding sequences, 63 tRNA genes, and 3 rRNA genes, whereas, following deposition in GenBank 4451 protein-coding genes, 63 tRNAs genes, and 2 rRNA genes were observed for A2-P53^T. The OrthoANIu values between strains A2-P53^T and A1-P5, and their close relatives were <92.1 % (Table 1). Values for dDDH between strains A2-P53^T, A1-P5, and their close neighbors were <49.1 % (Table 1). The OrthoANIu and dDDH between strains A2-P53^T and A1-P5 were ≥98 % (Table 1). These ANI and dDDH values indicated that strains A2-P53^T and A1-P5 represent a novel species within ***Bacteroides*** as ANI and dDDH values lower than 95%–96 % and 70 %, respectively, are established as the boundary for defining a prokaryotic species [39,47]. The phylogenetic tree generated (Fig. 2) agreed with the phylogenetic placement of A2-P53^T and A1-P5 from the 16S rRNA gene sequence tree (Fig. 1). The main genotypic characteristics of strains A2-P53^T and A1-P5 and close relatives are given in Table 1.

In this study, an *in-silico* approach was used to investigate glycerophospholipid metabolism, and the production of polar lipids was performed using the KEGG database [48]. An *in-silico* approach is now encouraged and routinely utilized for polar lipid profiling [49–53], and along with biochemical traits, these methods are now routinely used for species descriptions [54,55]. The results (Fig. S2) indicate that strains A2-P53^T and A1-P5 do not contain the enzymes phosphatidylglycerophosphate synthetase (EC 2.7.8.5) or cardiolipin synthase (EC 2.7.8.41) required to synthesize phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG; cardiolipin), respectively. Conversely, strains A2-P53^T and A1-P5 also contain phosphatidylserine synthase (EC 2.7.8.8) used in the synthesis of phosphatidyl-L-serine (PS), an intermediate compound that the enzyme phosphatidylserine decarboxylase (PSD) (EC 4.1.1.65) further catalyzes in the production of phosphatidylethanolamine (PE) from phosphatidyl-L-serine (Fig. S2). Based on this analysis, the major polar lipids predicted to be produced by strains A2-P53^T and A1-P5 is PE, consistent with other species in ***Bacteroides*** [56–58]. The identification of PS is interesting as this phospholipid is commonly absent from

Table 1
Genotypic differential characteristics of strains A2-P53^T, A1-P5, and their closest relatives.

Characteristic	1	2	3	4	5	6
GoC, %	41.8	41.7	41.9	42.1	42.1	41.5
16S rRNA gene Accession	OQ724630	OQ725027	DS264555	KX025134	KX025133	MK584158
16S rRNA gene % similarity	100.0	99.9	97.6	96.6	97.7	97.4
Genome Accession	GCA_03429187.5.1	GCA_03429186.5.1	GCA_00131499.5.1	GCA_00734139.5.1	GCA_00734137.5.1	GCA_00919329.5.2
% ANI	100.0	99.9	92.1	91.7	91.8	89.0
% dDDH	100.0	98.5	48.1	43.1	43.1	46.3

Strains: 1, A2-P53^T; 2, A1-P5; 3, *Bacteroides ovatus* JCM 5824^T; 4, *Bacteroides kribbi* JCM 31391^T; 5, *Bacteroides koreensis* JCM 31393^T; 6, *Bacteroides luhongzhouii* HF-5141^T.

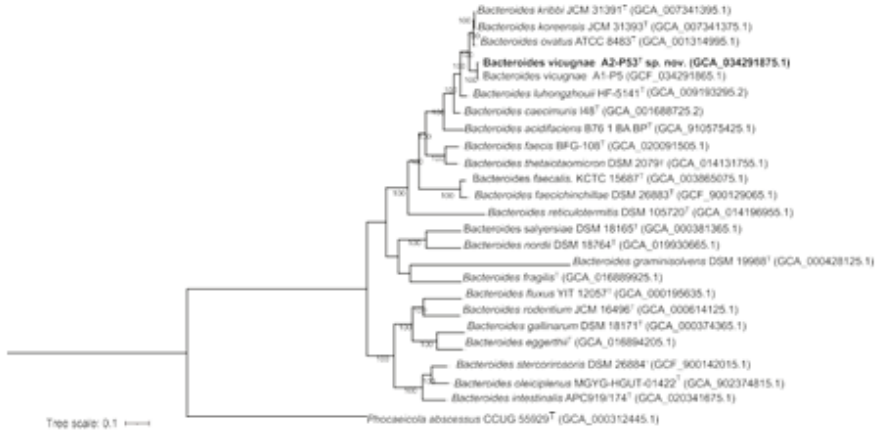

Fig. 2. Maximum-likelihood phylogenetic tree based on 445 single-copy genes showing the relationships between *Bacteroides vicugnae* sp. nov. A2-P53^T and A1-P5 within the *Bacteroides* genus. These genes were shared between all 31 genomes. A total of 447153 aligned nucleotides were used to build this tree. Bootstraps were generated with 100 rounds of the "Rapid" bootstrapping option within RaxML.

chromatography plates due to the conversion efficiency of PSD. A targeted LC-MS study recently described PS as part of a "stable core" of lipids within *Bacteroides* [59].

There were 486 CAZymes detected using at least two of three prescribed search algorithms for A2-P53^T and A1-P5, where 325, 69, 42, 28, and 25 functions were assigned to their respective GH, GT, PL, CBM, and CE CAZy families. These numbers are within the usually determined ranges of CAZyme families for other *Bacteroides* taxa as *in-silico* determined using similar pipelines [60].

3.3. Phenotypic characterization

The cells of A2-P53^T and A1-P5 are Gram-stain-negative rods that are strictly anaerobic. Cultures did not grow when the organisms were exposed to air. Colonies on BHI with 5 % sheep's blood medium were pink, circular, and mucoid on the agar surface after 48hrs at 37 •C. Cells were non-motile, and endospore production was not observed. A2-P53^T and A1-P5 could grow between 20 •C and 45 •C, with optimum growth at 37 •C. Additionally, both strains could grow at NaCl concentrations up to 3.0 % (w/v), with optimal growth occurring at 0.5 % (w/v) NaCl; strains grew at pH values between pH 6.0 to 9.0 with an optimum pH of 7.0. The API-ZYM test system produced positive reactions for Alkaline phosphatase, Esterase (C4), Esterase lipase (C8), Trypsin, Acid phosphatase, Naphthol-AS-BI-phosphohydrolase, α-Galactosidase, β-Galactosidase, β-Glucuronidase, β-Glucosidase, N-acetyl-β-Glucosaminidase, α-Mannosidase, and α-Fucosidase. Negative reactions were obtained for Lipase (C14), Leucine arylamidase, Valine arylamidase, Cystine arylamidase, α-Chymotrypsin, and α-Glucosidase. Additionally, the API rapid ID32A test system gave positive reactions for the α -Galactosidase, β-Galactosidase, α-Glucosidase, β-Glucosidase, N-acetyl-β-Glucosaminidase, Mannose, Raffinose, Indole, Alkaline phosphatase, Leucyl glycine arylamidase, Alanine arylamidase, Glutamic acid decarboxylase, α -Fucosidase, and Glutamyl glutamic acid arylamidase, whereas, negative reactions were obtained for Urease, Arginine dihydrolase, β-6-galphosphatase, α-Arabinosidase, β-Glucuronidase, Nitrate reduction, Arginine arylamidase, Proline arylamidase, Phenylalanine arylamidase, Leucine arylamidase, Pyroglutamic acid arylamidase, Tyrosine arylamidase, Glycine arylamidase, Histidine arylamidase, and Serine arylamidase. Strains A2-P53^T and A1-P5 exhibited identical physiological profiles in addition to identical profiles with the API-ZYME and API rapid 32A systems. Differences between these strains and their close relatives are denoted by positive reactions for α-Fucosidase (API-ZYME and API rapid 32A) and Glutamyl glutamic acid arylamidase (API rapid 32A) (Table 2).

3.4. Cellular fatty acids

Major fatty acids (≥10.0 %) for strains A2-P53^T and A1-P5 were anteiso-$C_{15:0}$ (42.7 % and 39.5 %), respectively (Table 3), which is consistent with the genus description of Bacteroides [63] and supports their placement within this genus.

Based on biochemical, phylogenetic, genotypic, and chemotaxonomic criteria, strains A2-P53^T and A1-P5 represent a novel species within *Bacteroides*. In addition to the phylogenetic analyses (Figs. 1 and 2 and Fig. S1), phenotypic and chemotaxonomic traits demonstrate the separateness of strains A2-P53^T and A1-P5 from their close relatives

Table 2
Morphological and physiological characteristics of strains A2-P53^T, A1-P5, and their closest relatives.

Characteristic	1	2	3	4	5	6
Temperature optimum	37 °C	37 °C	35–37 °C	35–37 °C	35–37 °C	35–37 °C
pH range	6.0–9.0	6.0–9.0	5.0–10.0	5.0–10.0	5.0–10.0	5.0–10.0
Salt range %	0.0–3.0	0.0–3.0	0.5–2.5	0.5–2.0	0.5–2.0	0.5–2.5
Isolation Source	Alpaca Feces	Alpaca feces	Human feces	Human feces	Human feces	Human feces
API-ZYME						
Alkaline phosphatase	+	+	–	NR	NR	+
Acid phosphatase	+	+	–	NR	NR	–
α-Fucosidase	+	+	–	–	–	–
α-Galactosidase	+	+	+	NR	NR	–
α-Glucosidase	–	–	–	+	+	–
α-Mannosidase	+	+	–	NR	NR	–
β-Galactosidase	+	+	–	NR	NR	–
β-Glucosidase	+	+	–	NR	NR	–
β-Glucuronidase	+	+	+	NR	NR	–
Esterase Lipase (C 8)	+	+	–	–	–	+
N-acetyl-β- Glucosaminidase	+	+	–	NR	NR	–
Trypsin	+	+	–	NR	NR	–
API rapid ID32A						
β-Glucosidase	+	+	+	–	–	+
α-arabinosidase	–	–	+	–	–	+
NAGA	+	+	+	–	–	–
Alkaline phosphatase	+	+	+	–	–	+
Leucyl glycine arylamidase	+	+	+	–	–	+
Alanine arylamidase	+	+	+	–	–	+
Glutamic acid decarboxylase	+	+	–	+	+	+
α-Fucosidase	+	+	–	–	–	–
Glutamyl glutamic acid arylamidase	+	+	–	–	–	–

Strains: 1, A2-P53^T (data from this study); 2, A1-P5 (data from this study); 3, *Bacteroides ovatus* JCM 5824^T [57,61]; 4, *Bacteroides fragilis* JCM 31391^T [57,62]; 5, *Bacteroides xylanisolvens* JCM 31393^T [57,62]; 6, *Bacteroides thetaiotaomicron* HF-5141^T [57]. The API rapid ID32A data for *Bacteroides ovatus* JCM 5824^T was taken from https://catalogue-crbip.pasteur.fr. The following characteristics were shared between all isolates, where data were reported: Gram Stain Reaction. (−), pH optimum (7), Salt optimum % (0.5), α-Chymotrypsin (−), Esterase (C 4) (+), Lipase (C 14) (+), Naphthol-AS-BI-phosphohydrolase (+), Valine arylamidase (−), Urease (−), Arginine dihydrolase (−), α-Galactosidase (+), β-Galactosidase (+), β-6-gal-phosphatase (−), α-Glucosidase. (+), β-Glucuronidase (+), N-acetyl-β-Glucosaminidase (+), Mannose (+), Raffinose (+), Nitrate reduction (−), Arginine arylamidase (−), Proline arylamidase (−), Phenylalanine arylamidase (−), Leucine arylamidase (−), Pyroglutamic acid arylamidase (−), Tyrosine arylamidase (−), Glycine arylamidase (−), Histidine arylamidase (−), and Serine arylamidase (−).

Table 3
Fatty acid profile of strains A2-P53^T and A1-P5 and close relatives.

Fatty acid (%)	1	2	3	4	5	6
$C_{14:0}$ iso	1.3	1.3	–	–	–	–
$C_{14:0}$	4.3	3.3	4.4	2.8	2.8	1.1
iso-$C_{16:0}$	–	–	9.6	**11.0**	**11.0**	8.9
anteiso-$C_{17:0}$	**39.5**	**42.7**	**40.0**	**39.0**	**40.0**	**32.0**
$C_{15:0}$ ω8c	1.6	1.3	–	–	–	–
$C_{15:0}$	–	–	3.0	tr	1.1	3.2
$C_{16:0}$	2.6	1.7	8.7	6.4	7.7	5.1
$C_{16:0}$ 3-OH	7.8	7.7	9.8	**11.0**	9.3	8.2
iso-$C_{16:0}$ 3-OH	1.0	1.1	–	–	–	–
$C_{18:1}$ ω9c	–	–	1.4	1.6	1.6	1.1
iso-$C_{17:0}$ 3-OH	–	–	9.2	**17.0**	**15.0**	**22.0**
$C_{17:0}$ 2-OH	1.6	1.9	2.4	3.2	2.9	6.6
Summed feature 4	4.5	2.8	–	–	–	–
Summed feature 8	5.9	4.4	–	–	–	–
Summed feature 12	**22.6**	**25.7**	–	–	–	–
Summed feature 15	8.6	9.8	–	–	–	–

Strains: 1, A2-P53^T (data from this study); 2, A1-P5 (data from this study); 3, *Bacteroides ovatus* JCM 5824^T [44]; 4, *Bacteroides fragilis* JCM 31391^T [44]; 5, *Bacteroides xylanisolvens* JCM 31393^T [44]; 6, *Bacteroides thetaiotaomicron* HF-5141^T [44]; NR, Not reported. The boldface indicates major fatty acids (>10 %); –, Not detected. *Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total.
Summed feature 4, $C_{15:0}$ and/or unknown $C_{15.849}$/$C_{15.8}$; summed feature 8, ($C_{18:1}$ ω9c and/or $C_{18:1}$ ω7c; summed feature 12, Unknown $C_{14.889}$/$C_{15:0}$ iso and or $C_{15:0}$ iso, summed feature 15, Unknown $C_{18.166/17.0}$ iso 3OH and/or $C1_{17.0}$ iso 3OH.

(Tables 1 and 2). It is proposed to name the novel strains A2-P53^T and A1-P5 as *Bacteroides vicugnae* sp. nov.

Description of *Bacteroides vicugnae* sp. nov. *Bacteroides vicugnae* (vi.cug'nae. N.L. gen. n. *vicugnae* pertaining to alpaca (*Vicugna pacos*) where the organism was first isolated).

The cells are Gram-stain-negative, strictly anaerobic rods. Non-motile and spores are not observed. Optimal growth was observed at 37 •C, with 0.5 % (w/v) NaCl, at pH 8. Positive for growth at 37, 40, and 45 •C. Growth with 5 %, but not 10 % (w/v) NaCl. Using the API-ZYM test system, positive reactions for alkaline phosphatase, esterase (C4), esterase lipase (C8), trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-Galactosidase, β-Galactosidase, β-Glucuronidase, β-Glucosidase, N-acetyl-β-Glucosaminidase, α-Mannosidase, and α-Fucosidase are obtained. Negative reactions are obtained for Lipase (C14), Leucine arylamidase, valine arylamidase, cystine arylamidase, α-Chymotrypsin, and α-Glucosidase. Additionally, using the API rapid ID32A test system, positive reactions are obtained for α-Galactosidase, β-Galactosidase, α-Glucosidase, β -Glucosidase, N-acetyl-β-Glucosaminidase, Mannose, Raffinose, Indole, Alkaline phosphatase, Leucyl glycine arylamidase, Alanine arylamidase, Glutamic acid decarboxylase, α-Fucosidase, and Glutamyl glutamic acid arylamidase. Negative reactions were observed for Urease, Arginine dihydrolase, β-6-gal-phosphatase, α-arabinosidase, β-Glucuronidase, Nitrate, Arginine arylamidase, Proline arylamidase, Phenylalanine arylamidase, Leucine arylamidase, Pyroglutamic acid arylamidase, Tyrosine arylamidase, Glycine arylamidase, Histidine arylamidase, and Serine arylamidase. The predominant cellular fatty acids were anteiso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The type strain is A2-P53^T (CCUG 77273^T = CCM 9377^T = NRRL B-65693^T), was isolated from the feces of a domesticated, male, suri alpaca, an additional strain A1-P5 (CCUG 77271 = CCM 9375 = NRRL B-65692), was isolated from the feces of a domesticated, female,

huacaya alpaca, which came from the same privately owned alpaca ranch near Newcastle, Oklahoma, USA (35.24 N 97.59 W). The G+C content of the isolates was 41.7 %. The 16S rRNA gene and genome sequences of strains A2-P53[T] and A1-P5 have been deposited in GenBank/EMBL/DDBJ under accession numbers OQ724630 and OQ725027 respectively (16S rRNA gene sequences) and GCA_034291875.1and GCA_034291865.1 (whole genome sequences).

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Ethical statement

This article does not contain any studies with animals performed by any of the authors.

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CRediT authorship contribution statement

Samuel Miller: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Meredith Hendry:** Investigation, Data curation. **Jacobey King:** Investigation, Data curation. **Krithivasan Sankaranarayanan:** Writing – review & editing, Visualization, Supervision, Software, Resources, Project administration, Formal analysis, Conceptualization. **Paul A. Lawson:** Writing – review & editing, Visualization, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no indirect or direct conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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MICROBIOLOGY SOCIETY

Clostridium tanneri sp. nov., isolated from the faecal material of an alpaca

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Abstract

A strictly anaerobic, Gram-stain-negative rod-shaped bacterium, designated A1-XYC3^T, was isolated from the faeces of an alpaca (*Lama pacos*). On the basis of the results of a comparative 16S rRNA gene sequence analysis, the isolate was assigned to the genus *Clostridium* with the highest sequence similarities to *Clostridium magnum* DSM 2767^T (96.8%), *Clostridium carboxidivorans* P7^T (96.3%) and *Clostridium acidtolerans* JW/YJL-B3^T (96.1%). The average nucleotide identity between A1-XYC3^T, *C. magnum*, *C. carboxidivorans* and *C. acidtolerans* was 77.4, 76.1 and 76.6%, respectively. The predominant components of the cellular fatty acids of A1-XYC3^T were $C_{14:0}$, $C_{16:0}$ and summed feature 10, containing $C_{18:0}$/$C_{17:0}$ cyclo. The DNA G+C content was 32.4 mol%. On the basis of biochemical, phylogenetic, genotypic and chemotaxonomic criteria, this isolate represents a novel species within *Clostridium sensu stricto* for which the name *Clostridium tanneri* sp. nov. is proposed. The type strain of this species is strain A1-XYC3^T (=CCM 9376^T=NRRL B-65691^T).

INTRODUCTION

The genus *Clostridium* within the family *Clostridiaceae* historically encompassed Gram-stain-positive, rod-shaped, anaerobic and spore-forming bacteria [1]. However, some species of the genus *Clostridium* display Gram-stain negative or Gram-stain-variable reactions as stated in the current genus description [2]. Currently, there are more than 150 species of the genus *Clostridium* with validly published names according to the List of Prokaryotic Names with Standing in Nomenclature (LSPN;https://lpsn.dsmz.de/genus/clostridium, April 2024). The genus *Clostridium* is a genus with historically well-documented taxonomic issues [1–3], culminating in a genus displaying a wide range of observable phenotypes and metabolic properties. With the application of molecular methods and whole-genome sequencing, the sheer depth of phylogenetic diversity within organisms designated as members of the genus *Clostridium* began to be uncovered [2–4]. In 2016, Lawson and Rainey proposed restricting the genus *Clostridium* to *Clostridium butyricum* and its close relatives [2], now recognised as *Clostridium sensu stricto*. Members of *Clostridium sensu stricto* can be found throughout the collective mammalian gastrointestinal environment [5–8], as well as modified ruminants (sheep and alpaca). Upon examining the literature, it was evident that the majority of non-human mammalian microbiomes are understudied, and recent studies have revealed that other understudied mammalian species, such as bats [9] and pandas [10], have the potential to increase our understanding of the diversity of the mammalian gut microbiome. In a continuing study of mammalian microbiomes, using a polyphasic taxonomic approach, a novel species of the genus *Clostridium* belonging to *Clostridium sensu stricto*, designated as strain A1-XYC3^T, was recovered during the enrichment of faecal material from an alpaca (*Llama pacos*). Strain A1-XYC3^T represents a novel species of the genus *Clostridium* for which the name *Clostridium tanneri* sp. nov. is proposed.

ISOLATION AND ECOLOGY

Strain A1-XYC3^T was isolated from a freshly voided alpaca faecal sample obtained from a domesticated alpaca on a privately owned alpaca ranch near Newcastle, Oklahoma, USA (35.24 N, 97.59 W). The freshly voided faecal sample was collected, processed and transported anoxically on ice to the Lawson Microbial Systematics Laboratory at the University of Oklahoma, Norman, for further processing.

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Keywords: 16S rRNA; *Clostridium tanneri*; genome; *in-silico*; novel species; phylogeny; taxonomy.
Abbreviations: ANI, average nucleotide identity; BV-BRC, Bacterial and Viral Bioinformatics Resource Center; dDDH, digital DNA-DNA hybridisation; orthoANIu, orthologous ANI using USEARCH.
The 16S rRNA gene and genome sequences of strain A1-XYC3^T have been deposited in GenBank under the accession numbers OQ724631 and GCA_033659955.1, respectively.
Three supplementary figures and one supplementary table are available with the online version of this article.

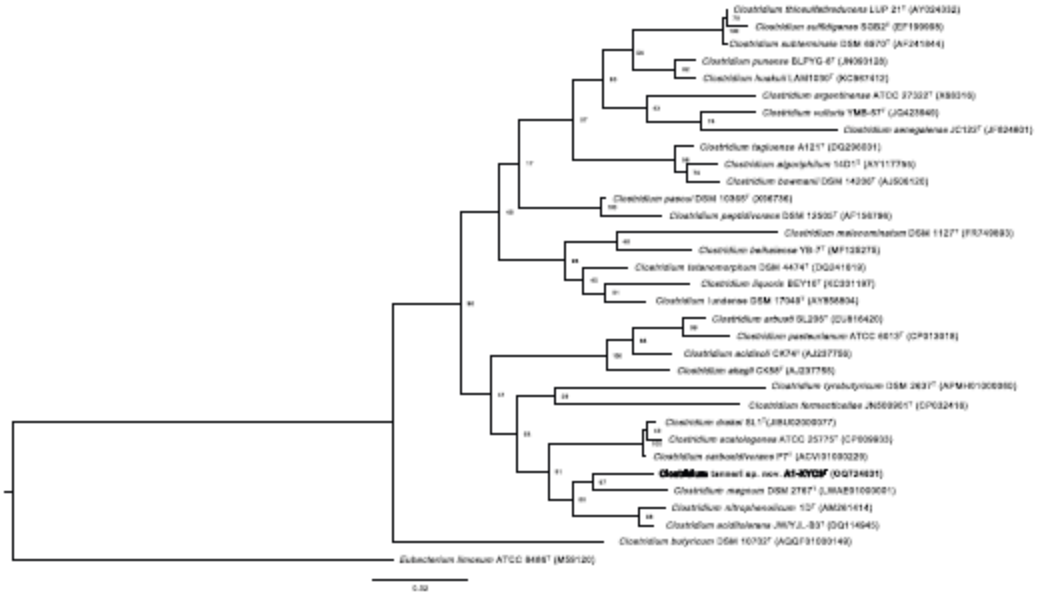

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationships between *Clostridium tanneri* sp. nov. A1-XYC3^T within *Clostridium sensu stricto*. Bootstrap values (percentages) were obtained with 1000 replicates and are displayed at their branch nodes. *Eubacterium limosum* ATCC 8486^T (M59120) was used as the outgroup. Bar, 0.02 substitutions per site.

Multiple anoxic enrichments, using xylan, pectin and cellulose as sole substrates [all added to 2% (w/v)], were prepared using 1.0 ml faecal slurry in a sealed serum bottle containing 100 ml modified Medium 2 (per 100 ml distilled water): casitone (1.0 g), yeast extract (0.25 g), minerals solution (A) (15.0 ml), minerals solution (B) (15.0 ml), clarified rumen fluid (20 ml), resazurin (0.0001 g), sodium lactate (70% w/v) (1.0 g), xylan (0.2 g), cysteine HCl (0.05 g), sodium bicarbonate (0.4 g) and distilled water (to 1000 ml). Minerals solution (A) contained (per 1000 ml); K_2HPO_4 (3.0 g). Minerals solution (B) contained (per 1000 ml); KH_2PO_4 (3.0 g), $(NH_4)_2SO_4$ (6.0 g), NaCl (6.0 g), $MgSO_4 \times 7H_2O$ (0.6 g) and $CaCl_2$ (0.6 g) as described previously [11]. The enrichment was incubated at 37°C for 14 days with a headspace of 5% hydrogen, 10% carbon dioxide and 85% nitrogen to maintain anoxic conditions. After incubation, samples were inoculated onto Medium 2 Agar containing the same sole carbon source as their original enrichment. Isolates were then sub-cultured onto BD Bacto Brain–Heart Infusion (BHI; BD) agar plates supplemented with 5% defibrinated sheep's blood via multiple plate streaks until pure isolates were obtained, as determined by Gram-stain-reaction and phase contrast microscopy (CX41, Olympus). One of the resulting isolates was designated A1-XYC3^T. A1-XYC3^T was isolated from an enrichment of modified Medium 2 supplemented with xylan. After primary isolation, A1-XYC3^T was maintained under anoxic conditions on BHI plus 5% sheep's blood medium at 37°C and preserved in BHI plus 5% clarified rumen fluid with 20% (v/v) glycerol at −80°C.

16s rRNA gene phylogeny

A1-XYC3^T was sent to GENEWIZ (Genewiz.com, South Plainfield, NJ, USA), where a near full-length 16S rRNA gene sequence (1399 bps) was obtained using Sanger sequencing. The nearest phylogenetic neighbours of A1-XYC3^T were determined by a BLAST search against the GenBank database and EzBioCloud's Identify [12–14]. The 16S rRNA gene sequences of A1-XYC3^T, its nearest neighbours, in addition to the type strain of the type species of the genus *Clostridium*, *Clostridium butyricum* DSM 10702^T (AQQF01000149), were downloaded from EzBioCloud, and these sequences were aligned using MUSCLE [15] and trimmed in MEGA 11 version 11.0.13 [16]. *Eubacterium limosum* ATCC 8486^T (M59120) was used as an outgroup. Phylogenetic trees were reconstructed according to the maximum-likelihood method [17] (Fig. 1) and neighbor-joining method [18] (Fig. S1, available in the online version of this article) using MEGA 11 software and the the Tamura–Net substitution model [19] and bootstrap analyses were performed using 1000 replications. The results of phylogenetic analyses based on 16S rRNA gene sequences indicated that A1-XYC3^T was most closely related to *Clostridium magnum* DSM 2767^T (96.8%), *Clostridium carboxidivorans* P7^T (96.3%) and *Clostridium aciditolerans* JW/YJL-B3^T (96.1%). These 16S rRNA gene similarity values, along with the phylogenetic trees reconstructed using the maximum-likelihood method [17] (Fig. 1) and the neighbor-joining

method [18] (Fig. S1) support the proposal that A1-XYC3^T represents a novel species. Additionally, the 16S rRNA gene sequence similarity between A1-XYC3^T and its close relatives is below the currently accepted threshold of 98.8%, which is now routinely used to demarcate this taxonomic rank [20].

GENOME FEATURES

Cell biomass for A1-XYC3^T was gathered from an overnight culture, and genomic DNA was extracted using the DNeasy DNA extraction kit (Qiagen) following the manufacturer's protocol. The DNA yield was quantified using a Qubit 3.0 fluorometer, and 100ng of DNA was used as input for genomic library construction. Briefly, the DNA was sheared using a sonicator (800R, QSonica), followed by bead cleanup to remove short fragments (< 150bp). Next, PCR sequencing libraries were generated using Illumina-specific barcoded primers and pooled from triplicate reactions. Finally, sequencing libraries were purified using bead cleanup (1.5×), quantified using a Tape Station, pooled in equimolar proportions and sequenced on a MiSeq (Illumina).

Using SPAdes v3.13.0 [21], the genome for A1-XYC3^T was assembled and annotated using the Bacterial and Viral Bioinformatics Resource Centre [22] (BV-BRC) (https://www.bv-brc.org/). In addition, genomic sequences of close relatives of A1-XYC3^T and the type strain of the type species of *Clostridium sensu stricto*, *Clostridium butyricum* DSM 10702^T (GCA_932751065.1), were downloaded from BV-BRC.

The evolutionary relationships between A1-XYC3^T (GCA_033659955.1) and its nearest neighbours were evaluated using a phylogenetic approach. A whole-genome-based phylogenetic tree was reconstructed using the CodonTree method within the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) [22] and rapid randomised accelerated maximum likelihood (RAxML) analysis v8.2.11 [23, 24]. The support values for the phylogenetic tree were generated using 100 rounds of the 'rapid bootstrapping' option of RaxML, and visualisation was accomplished with the Interactive Tree Of Life v3 [25]. *Eubacterium limosum* ATCC 8486^T (GCA_000807675.2) was used as an outgroup. The taxonomic status of A1-XYC3^T was confirmed by comparing average nucleotide identity (ANI) and digital DNA–DNA hybridisation (dDDH) values between A1-XYC3^T and its close relatives. Values for ANI between A1-XYC3^T and close relatives were calculated using EzBioCloud's ANI Calculator, which uses the orthologous ANI using USEARCH (OrthoANIu) algorithm [13, 26]. The Genome-to-Genome Distance Calculator [27, 28] v3.0 was used to determine digital DNA–DNA hybridisation. Prodigal [29]was used to determine the DNA G+C (mol %) content.

Once assembled, the draft genome sequence of A1-XYX3^T consisted of 91 contigs, with a genome length of 3.9 Mbp, an N50 length of 150735bp and an average DNA G+C content of 32.4 mol%. Annotation in BV-BRC revealed 3857 protein-coding sequences, 65 tRNA genes and 8 rRNA genes. The OrthoANIu values between A1-XYX3^T and close relatives were 75.6–77.4%. Values for dDDH between A1-XYX3^T and the six nearest neighbours were less than 23.5% (Table 1). These ANI and dDDH values indicated that A1-XYX3^T represents a novel species within the genus *Clostridium sensu stricto*, as ANI and dDDH values lower than 95–96% and 70%, respectively, are established as the boundary for defining a prokaryotic species [27, 30]. The phylogenetic tree (Fig. 2) was consistent with the phylogenetic placement of A1-XYX3^T from the 16S rRNA gene tree (Fig. 1). The main genotypic characteristics of A1-XYC3^T and close relatives are given in Table 1.

In this study, an *in-silico* approach was used to investigate glycerophospholipid metabolism, the production of polar lipids was performed using the Kyoto Encyclopaedia of Gene and Genomes (KEGG) database [31]. An *in-silico* approach is now encouraged

Table 1. Genotypic differential characteristics ofA1-XYC3^T and close relatives

Strains: 1, A1-XYC3^T; 2, *Clostridium drakei* SL1^T; 3, *Clostridium scatologenes* ATCC 25775^T; 4, *Clostridium carboxidivorans* P7^T; 5, *Clostridium magnum* DSM 2767^T; 6, *Clostridium nitrophenolicum* 1D^T; 7, *Clostridium acidotolerans* JM/YJL-B3^T, NR, Not reported.

Characteristic	1	2	3	4	5	6	7
DNA G+C content (mol%)	32.4	29.7	29.6	29.9	32.1	35.5	30.8
16S sequence accession number	OQ724631	JIBU02000077	CP009933	ACV101000229	LWAE01000001	AM261414	DQ114945
16S sequence similarity (%)	100.0	96.5	96.7	97.0	96.4	96.8	96.1
Genome accession number	GCA_033659955.1	GCA_003096175.1	GCA_000968375.1	GCA_001038625.1	GCA_900129955.1	NR	GCA_016316925.1
OrthoANIu (%)	100.0	75.8	75.6	76.1	77.4	NR	76.6
dDDH (%)	100.0	21.9	21.8	21.7	23.5	NR	23.1

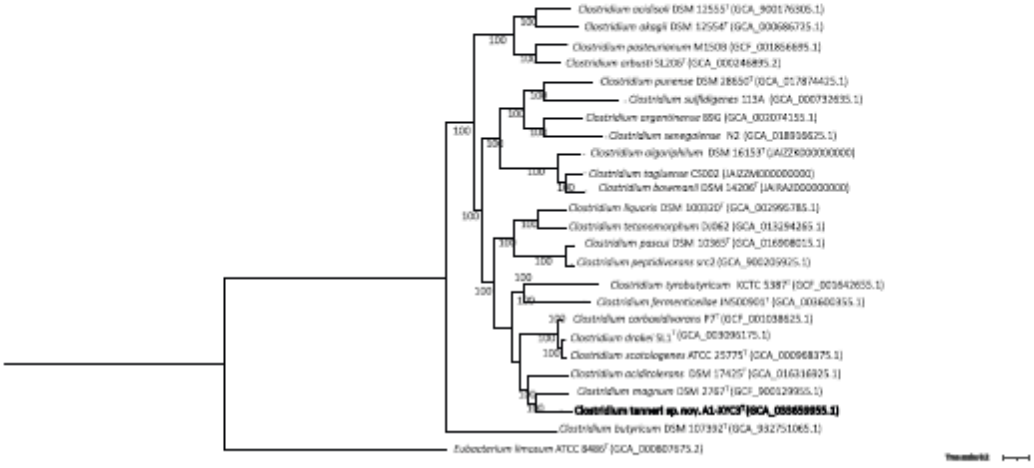

Fig. 2. Maximum-likelihood phylogenetic tree based on 605 single-copy genes, showing the relationships between *Clostridium tanneri* sp. nov. A1-XYC3^T and members of the genus *Clostridium sensu stricto*. A total of 610587 aligned nucleotides and 203529 amino acids to reconstruct this tree. Bootstraps were generated with 100 rounds of the 'Rapid' bootstrapping option within RaxML. *Eubacterium limosum* ATCC 8486^T (GCA_000807675.2) was used as the outgroup. Bar indicates the mean number of substitutions per site, 0.2.

and routinely utilised for polar lipid profiling [32–36], and along with biochemical traits, these methods are now routinely used for species descriptions [37–39]. The results (Fig. S2) indicate that A1-XYC3^T contains the enzymes phosphatidylglycerophate synthetase (E.C. 2.7.8.5) and phosphatidylglycerophate phosphatase (E.C. 3.1.3.27) required to synthesise phosphatidylglycerol (PG) from the essential building block cytidine diphosphate diacylglycerol. In addition, the enzyme cardiolipin synthase (E.C. 2.7.8.41) is present; this enzyme is required to produce diphosphatidylglycerol (DPG; cardiolipin). Furthermore, the genome of A1-XYC3^T also contains phosphatidylserine synthase (E.C. 2.7.8.8) used in the synthesis of phosphatidyl-L-serine (PS) an intermediate compound that the enzyme phosphatidylserine decarboxylase (PSD) (E.C. 4.1.1.65) further catalyses in the production of phosphatidylethanolamine (PE) from phosphatidyl-L-serine (Fig. S2). The identification of PS is interesting as this phospholipid is commonly absent from chromatography plates due to the conversion efficiency of PSD. Therefore, on the basis of the results of this analysis, the major polar lipids predicted to be produced by A1-XYC3^T are PE, PG, PS and DPG, consistent with those of other closely related species (Fig. S2) and other members of the genus *Clostridium sensu stricto* [40] determined using *in-silico* [36, 41] and wet lab methodologies [42]. The results of additional *in-silico* analysis of the cell wall of A1-XYC3^T indicated the presence of UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase (MurE) (E.C. 6.3.2.13), which is used to insert diaminopimelic acid (DAP) into the stem peptide of the peptidoglycan (Fig. S3) [43]. The presence of the diagnostic diamino acid DAP is consistent with the phenotypes of other members of the genus *Clostridium sensus stricto* [1, 2].

PHYSIOLOGY AND CHEMOTAXONOMY

The Gram stain reaction was determined using the Advanced Gram Stain Kit (Hardy Diagnostics) following the manufacturer's instructions. Aerotolerance was determined by placing cultures on media exposed to air. Cells were inspected for motility and endospore production by phase contrast microscopy (CX41, Olympus) at 1000× magnification.

Temperature ranges for growth were determined in BHI broth (supplemented with 5% (v/v) clarified rumen fluid) at 4, 20, 37, 45, 50 and 55°C. Salt tolerance was determined in BHI [supplemented with 5% (v/v) clarified rumen fluid] and tested at 0.5% (w/v) NaCl and between 1.0% (w/v) and 10.0% (w/v), in increments of 1.0% (w/v); pH ranges for growth were determined between 5.0 and 9.0, in increments of 1.0, and all tests were performed in duplicate.

For determination of the pH ranges, the following buffers were prepared at a final concentration of 10 mM; sodium acetate (pH 5.0), potassium phosphate (pH 6.0–8.0) and Tris-HCl (pH 9.0). Optimal growth conditions were determined from OD_{600} using a spectrophotometer (Spectronic 20D+, Milton Roy). An increase in OD_{600} greater than 0.1 after 5 days of incubation was considered growth. Additional biochemical traits were determined using the API ZYM test systems (bioMérieux) according to the manufacturer's instructions and performed in duplicate. Additionally, end products of metabolism for A1-XYC3^T were measured

Table 2. Morphological and physiological differential characteristics of A1-XYC3^T and close relatives

Strains: 1, A1-XYC3^T (data from this study); 2, *Clostridium drakei* SL1^T [47, 48]; 3, *Clostridium scatologenes* ATCC 25775^T [47, 48]; 4, *Clostridium carboxidivorans* P7^T [47]; 5, *Clostridium magnum* DSM 2767^T [49]; 6, *Clostridium nitrophenolicum* 1D^T [42]; 7, *Clostridium aciditolerans* JW/YJL-B3^T [50], NR, Not reported.

Characteristic	1	2	3	4	5	6	7
Gram-stain reaction	–	–	–	+	–	+	–
Endospore production	–	+	+	+	+	+	+
Motility	–	+	+	+	+	+	+
Temperature range	20–45°C	18–42°C	18–42°C	24–42°C	15–45°C	20–45°C	20–45°C
Temperature optimum	37°C	30–37°C	37–40°C	37–40°C	30–32°C	30°C	35.5°C
pH range	6.0–7.0	4.6–7.8	4.6–8.0	4.4–7.6	6.0–7.5	6.5–8.0	3.8–8.9
pH optimum	7.0	5.4–7.5	5.4–7.0	5.0–7.0	7.0	7.2	7.0–7.5
Salinity range (%)	0.0–2.0	NR	NR	NR	NR	0.0–1.0	0.0–1.5
Optimum salinity (%)	0.5	NR	NR	NR	NR	NR	0.0
Source	Alpaca faeces	Acidic mine sediment	Acidic mine pond	Settling lagoon	Enriched sediment	Subsurface soil	Wetland sediment

from cultures grown in basal medium with glucose at 37°C. Analysis was performed in duplicate from duplicate cultures using GC (Agilent 6890N GC, Agilent Technologies) as described by Liu *et al.* [44].

The cells of A1-XYC3^T are Gram-stain-negative rods (Table 2). A1-XYC3^T was strictly anaerobic. Cultures did not grow when the media was exposed to air. Colonies on BHI with 5% sheep's blood medium were opaque and circular on the agar surface after 24 h at 37°C. Cells were negative for motility and endospore production, visible with phase-contrast microscopy. A1-XYC3^T could grow at between 20°C and 45°C with optimum growth at 37°C. A1-XYC3^T could grow at NaCl concentrations between 0.5 and 2.0% (w/v) with optimal growth occurring at 0.5% (w/v) NaCl. A1-XYC3^T grew at pH values between pH 6.0 to 8.0 with an optimum pH of 7.0. A1-XYC3^T produced ethanol and acetate from glucose fermentation. The API ZYM test system detected positive reactions for esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Negative reactions were obtained for alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cysetine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Chemotaxonomic features were determined at the Centre for Microbial Identification and Taxonomy (University of Oklahoma, Norman, Oklahoma). The cellular fatty acids of A1-XYC3^T were harvested, extracted and analysed using the Microbial Identification System (MIDI) [45, 46]. Analysis was performed with a 6890N gas chromatograph (Agilent Technologies) equipped with a phenyl methyl silicone fused silica capillary column (HP-Ultra 2.25m×0.2 mm×0.33 µm film thickness) coupled with a flame ionisation detector. Hydrogen was used as the carrier gas. The temperature programme was preset at 170°C and increased at 5°C min^{-1} to a final temperature of 270°C. Fatty acids were identified and expressed in percentages using the QBA1 peak naming database within the MIDI system.

Major components of the fatty acids profile (≥10.0%) were $C_{14:0}$, $C_{16:0}$ and summed feature 10 (consisting of $C_{18:0}$/$C_{17:0}$ cyclo). Additionally, summed features 3, 4 and 12 represented the fatty acids present in moderate amounts (5.0%–9.9%) (Table S1), available in the online version of this article, which is consistent with the phenotypes of the members of *Clostridium sensu stricto*.

On the basis of biochemical, phylogenetic, genotypic and chemotaxonomic criteria, A1-XYC3^T represents a novel species within the genus *Clostridium sensu stricto*. In addition to the phylogenetic trees (Figs 1 and 2 and S1), phenotypic and chemotaxonomic traits indicate the separateness of strain A1-XYC3^T from its close relatives (Tables 1 and 2). It is proposed to name the novel strain A1-XYC3^T as *Clostridium tanneri* sp. nov.

DESCRIPTION OF CLOSTRIDIUM TANNERI SP. NOV.

Clostridium tanneri (tan'ne.ri. N.L. gen. n. tanneri, of Tanner, in honour of Ralph S. Tanner, a contemporary American microbiologist for his many contributions to the field of anaerobic microbial cultivation).

The cells are strictly anaerobic, motility-negative, Gram-stain-negative rods in which sporulation was not observed. Optimal growth was observed at 37°C, with 0.5% (w/v) NaCl, at pH 7. Positive for growth at 25, 30, 37 40 and 45°C and in 1 and 3%, but not 10% (w/v) NaCl. Using the API ZYM test system positive reactions are observed for esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Ethanol and acetate were produced as fermentative end products. Negative reactions were obtained for alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The major components of the fatty acids profile are $C_{14:0}$, $C_{16:0}$ and summed feature 10 (containing $C_{18:0}$/$C_{17:0}$ cyclo).

The type strain is A1-XYC3^T (=CCM 9376^T=NRRL B-65691^T), which was isolated from the faeces of a domesticated alpaca in Newcastle, Oklahoma. The DNA G+C content of the type strain is 32.4 mol%. The 16S rRNA gene and genome sequences of A1-XYC3^T have been deposited in GenBank under the accession numbers OQ724631 and GCA_033659955.1, respectively.

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Conflicts of interest
The authors declare that they have no indirect or direct conflicts of interest.

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