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Original Article

Pseudomonas laurentiana sp. nov., an Mn(III)-oxidizing Bacterium Isolated from the St. Lawrence Estuary

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ABSTRACT

Introduction: An aerobic, Mn(III)-oxidizing, Gram-negative, motile bacterium (strain GSL-010T) was isolated from deep waters of the St Lawrence Estuary (Canada) and subjected to a polyphasic taxonomic study. **Results:** Cells were mostly curved, motile rods (1.75 – 2.2 x 0.5 – 0.7 µm) with growth observed at 10 – 37 °C (optimum at 30 °C), pH 5 – 10 (optimum at 7.0 – 7.5) and with 0 – 3% (w/v) NaCl (optimum at 0.5%). Phylogenetic analysis based on 16S rRNA gene sequencing revealed strain GSL-010^T as a member of the genus *Pseudomonas*, most closely related to the type strains of *Pseudomonas plecoglossicida* (98.9%) and *Pseudomonas japonica* (98.6%). The major cellular fatty acids of strain GSL-010^T are a combination of $C_{16:1}$ ω7c and/or iso-$C_{15:1}$ 2-OH, $C_{16:0}$ and $C_{18:1}$ ω7c. The G+C mol content of the chromosomal DNA is 59.7%. The DNA–DNA hybridization values between strain GSL-010^T and *P. plecoglossicida* (32.0%) and *P. japonica* (34.4%) confirm the assignment of the bacterium to a new species. Conclusion: On the basis of phylogenetic analysis, DNA–DNA hybridization and physiological and biochemical characterization, strain GSL-010^T is clearly a unique bacterium and since it was isolated from the waters of the St. Lawrence estuary, the name *Pseudomonas laurentiana* sp. nov. is proposed. The type strain is GSL-010^T (=JCM 32154^T =NBRC 113027^T =KCTC 62392^T).

Key words: Manganese(III), Mn(III)-L, *Pseudomonas*, *Pseudomonas laurentiana*, *Pseudomonas* sp. nov., St. Lawrence Estuary.

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INTRODUCTION

Diverse both genotypically and phenotypically, well-known as causative agents of food spoilage[1] and as opportunistic pathogens,[2] the genus *Pseudomonas* comprises over 191 species that are found in a wide range of terrestrial and aquatic ecosystems. Many *Pseudomonas* are capable of specialized processes, including the enzymatic oxidation of soluble Mn(II) to insoluble Mn(III,IV) oxides. This process has been extensively characterized in *P. putida* strains GB-1 and MnB1[3-5] with very few studies focusing on Mn(III) oxidation. However, a recent re-evaluation of the previous Mn paradigm, which focused solely on the cycling between soluble Mn(II) and insoluble Mn(III,IV), has occurred. This was triggered by recent observation of significant concentrations of soluble ligand bound Mn(III) complexes (Mn(III)-L) in oxic, suboxic and some anoxic waters.[6-7] Thus, the isolation and investigation of Mn(III)-L oxidizing bacteria, particularly those from environmentally significant genera such as *Pseudomonas*, will provide a deeper insight into the role of bacteria in the overall cycling of Mn(III) in aquatic systems.

METHODS AND RESULTS

During a research cruise investigating the formation of Mn(III)-L complexes in the St. Lawrence Estuary, one bacterial strain, designated GSL-010^T, was isolated from deep (338m) waters sampled from Station 23 (48°42.032"N, 68°39.171"W). Isolation was achieved by selecting colonies positive for Mn(III)-citrate oxidation (as determined by the formation of dark brown oxides) on Minimal Media A (MMA) agar plates containing 100 µM Mn(III)-citrate at 30°C, pH 7.5 for 5-7 days in the dark (MMA: 0.238g $(NH_4)_2SO_4$ l^{-1}, 0.0602g $MgSO_4$ l^{-1}, 0.0488g $CaCl_2.2H_2O$ l^{-1}, 0.0204g KH_2PO_4 l^{-1} and 0.0284g Na_2HPO_4 l^{-1} before autoclaving, with sterile 4.5 mL 20% glucose, 200uL 5x Trace Elements Mix,[8] 1mL 3.7mM $FeCl_3.6H_2O$ and 20mL 1M HEPES (pH 7.8) added after autoclaving). The strain was purified by repeated streaking on Luria–Bertani agar (LB: 10 g tryptone l^{-1}, 5 g yeast extract l^{-1}, 10 g NaCl l^{-1}, 15 g agar l^{-1}, pH 7.5) and then routinely cultured in LB broth shaken at 120 rpm for 24 h. Strain GSL-010^T was stored at -80°C in LB supplemented with 20% (v/v) glycerol. As reference strains, *P. plecoglossicida* NBRC 103162^T and *P. japonica* IAM 15071^T were obtained from NITE Biological Resource Center (NBRC). The ability of the strains to oxidize Mn(II,III) were determined by observing Mn(IV) oxide formation during growth in MMA liquid media containing 100µM of Mn(II)-chloride, Mn(III)-citrate or Mn(III)-desferrioxamine B. The presence of Mn(IV) was detected using the Leucoberbelin blue assay as previously described.[9-10] The ability of strain GSL-010^T to form and remove of Mn(III)-L complexes has only recently been investigated and published by the authors.[10] The isolation of strain GSL-010^T highlights the first instance of a bacterium obtained through the observation of Mn(IV) oxide formation from Mn(III)-L oxidation. The results of phenotypical and physiological characterization are given in Table 1 and in the species description.

Purification of genomic DNA was achieved using the Wizard® Genomic DNA Purification Kit (Promega) as per the manufacturer's instructions and confirmed as previously described.[10] The 16S rRNA gene was amplified with the primer set 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- CGG TTA CCT TGT TAC GAC TT-3') using the GoTaq® Green Master Mix (Promega) as previously described.[12] The 16S rRNA PCR amplicon was sequenced using primers 27F, 1492R, 785F (5'-GGA TTA GAT ACC CTG GTA-3') and 518R (5'-GTA TTA CCG CGG CTG CTG G-3'). Sequencing was achieved by capillary electrophoresis DNA sequencing and was performed by the OHSU DNA Services Core (Oregon, USA) utilizing the 3730xl DNA Analyzer that was purchased with funding from NIH Shared Instrumentation Grant S10 OD010609. Individual sequences were analyzed using BioEdit 7.2.6,[13] corrected manually and assembled to form a contig that was 1424 bp in length. The 16S rRNA for strain GSL-010^T was uploaded to GenBank (KY471137) and sequence similarity assessed using EzTaxon-e,[14] with sequence similarities of 98.9% to *P. plecoglossicida* NBRC 103162^T and 98.6% to

Table 1: Differential physiological characteristics comparing strain GSL-010^T with the type strains of *P. plecoglossicida* and *P. japonica*. All data was generated in this study unless specified otherwise.

Characteristic	1	2	3
Habitat	Seawater (Canada)	*Plecoglossus altivelis* (Japan)[a]	Activated Sludge (Japan)[b]
Cell size (µm)	1.75–2.2 x 0.5–0.7	2.5–4.5 x 0.5–1.0*a*	2.0–3.5 x 1.3–1.7*b*
Temperature range (optima) (°C)	10–37 (30)	16–37 (30)	16–37 (30)
NaCl optima (%, w/v)	0.5	2.0	0.5
Maximum NaCl (%, w/v)	3.0	5.0	3.0
pH range	5–10	5–10	6–10
pH optimum	7–7.5	7.5	7
Utilization of:			
Sucrose	+	–	+
Tartaric acid	+	–	+
Xylose	+	–	+
Ascorbate	–	–	–
Xanthine	–	+	–
γ-hydroxybutyric acid	+	–	–
Succinic acid	–	+	+
L-Phenylalanine	–	+	+
G+C content of DNA (mol%)	59.7%	62.8%[a]	66.0%[b]
Oxidation of:			
Mn(II)-chloride	+	–	+
Mn(III)-citrate	+	–	+
Mn(III)-desferrioxamine B	+	–	+

Strains: 1, GSL-010^T; 2, *P. plecoglossicida* NBRC 103162^T; 3, *P. japonica* IAM 15071^T. All strains are positive for activities of catalase and assimilation of D-glucose, D-fructose, lactose, yeast extract, tryptone, formate, L-arabinose, peptone, casamino acids, succinamic acid and phenylacetic acid. Data taken from: *a,* Nishimori *et al.*[28] *b*, Pungrasmi *et al.*[29]

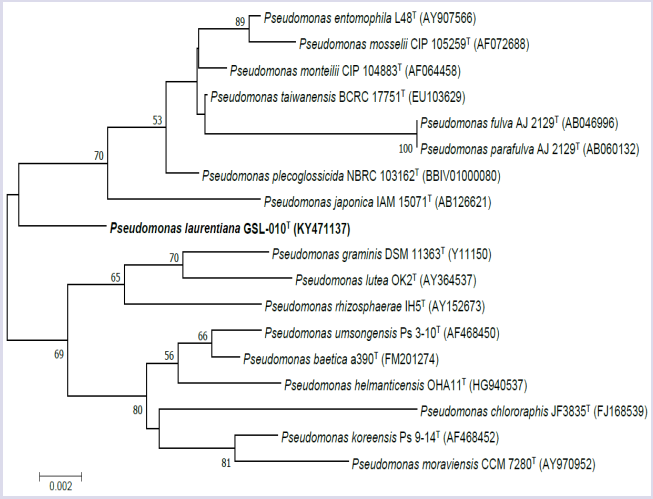

Figure 1: Neighbor-joining tree depicting the phylogenetic positions of strain GSL-010^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Numbers at nodes show the levels of bootstrap support based on data for 1000 replicates; only values that >50% are presented. Bar, 0.0020 nucleotide substitution rate (K_{nuc}) units.

P. japonica IAM 15071^T. Construction of the neighbor-joining[15] dendrogram was achieved using MEGA version 7.0.25[16] and is seen in Figure 1. Bootstrap analysis (1000 replications) was employed to determine the stability of the tree topology.[17]

Electron microscopy was performed at the Multiscale Microscopy Core (MMC) with technical support from the OHSU-FEI Living Lab and the OHSU Center for Spatial Systems Biomedicine (Oregon, USA). Cell size and morphology were examined after two days of growth in LB broth through negative staining, imaged at 120 kV on a FEI Tecnai™ Spirit TEM system (Figure 2). Visualization of strain GSL-010^T indicated that cells are curved-rods with lophotrichous flagella. Gram-staining was performed as per standard procedure[18] and determined that strain GSL-010^T is Gram-negative. Growth in MMA was tested at different temperatures (4, 10, 16, 23, 30, 37, 45 and 55°C) after 72 h at pH 7.5. The effect of pH (range 4.0–12.0, with intervals of 0.5 pH units) was assessed using MMA after 72 h at 30°C. NaCl (w/v) at increasing concentrations (0, 1, 2, 3, 5, 7.5, 10, 15 and 20%) was tested in MMA at 30°C, pH 7.5 and measured after 72 h. The following electron donors were tested for their ability to support growth: D-glucose, D-fructose, lactose, yeast extract, tryptone, formate, L-arabinose, peptone, casamino acids, succinamic acid, tartaric acid, xylose, ascorbate, xanthine, γ-hydroxybutyric acid, succinic acid, L-phenylalanine and phenylacetic acid. The ability of each strain to oxidize Mn(II), Mn(III)-L citrate or Mn(III)-desferrioxamine B was determined through the formation of dark brown oxides after 120 h, using MMA broth containing 100 µM of relevant Mn source at 30°C, pH 7.5. The ability for strain GSL-010^T

Fatty acid analysis, DNA G + C mol% content and DNA-DNA hybridization were performed by the identification service at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). Cellular fatty acid methyl esters were extracted and analyzed by saponification, methylation[19-20] and separated using GC-FID and a Sherlock Microbial Identification System (Microbial ID; Delaware, United States). Strains were grown under identical conditions (LB-medium with incubation for 48 h at 30°C) before submission to the DSMZ. Distinct differences were observed in the fatty acid composition of GSL-010^T compared to those of *P. plecoglossicida* and *P. japonica* (Table 2). The cellular fatty acid profile of strain GSL-010^T was characterized primarily by summed feature 3 (40.43%), $C_{16:0}$ (32.29%), $C_{18:1}$ ω7c (8.30%) and $C_{12:0}$ 2-OH (4.18%). The DNA mol% GC content was also determined through the disruption of cells, which were then hydrolyzed and the resulting deoxyribonucleosides measured using HPLC.[21-22] Published genome sequences representing a G+C range of 43-72 mol% were used as standards and G+C values were calculated as per the method of Mesbah *et al.*[23] The genomic DNA G+C content determined for strain GSL-010^T was 59.7%, which was lower than the values reported for *P. plecoglossicida* (62.8%) and *P. japonica* (66.0%). DNA-DNA hybridization was achieved initially through cell disruption using a Constant Systems TS 0.75 KW (IUL Instruments, Germany) and the DNA was purified by chromatography.[20] Hybridization was performed as previously described[24] as modified by Huss *et al.*[25] using a model Cary 100 Bio UV/VIS-spectrophotometer (Varian; California, United States). The DNA–DNA hybridization values between strain GSL-010^T and *P. plecoglossicida* NBRC 103162^T (32.0%) and *P. japonica* IAM 15071^T (34.4%) was significantly below the ≤70% threshold recommended for defining a new species.[26-27]

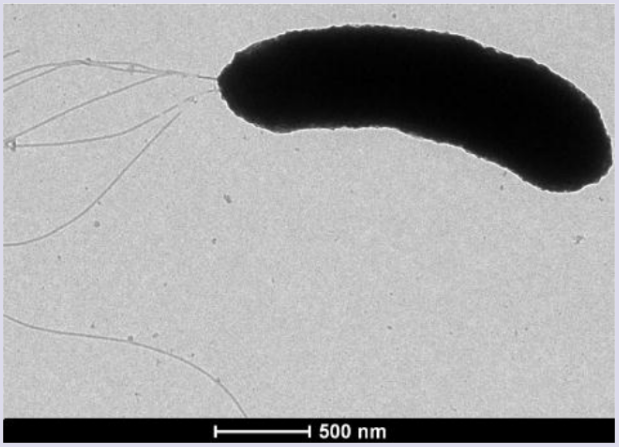

Figure 2: Negatively stained electron micrograph of *Pseudomonas laurentiana* GSL-010^T. Bar, 500 nm.

CONCLUSION

Based on the summary of genotypic and phenotypic results, we describe a novel species of the genus *Pseudomonas* represented by strain GSL-010^T and for which the name *Pseudomonas laurentiana* sp. nov. is proposed.

Description of *Pseudomonas laurentiana* sp. nov.

Pseudomonas laurentiana (lau.ren.ti.a'na. N.L. fem. adj. laurentiana [italic type] pertaining to Lawrence Estuary); the isolation source of the type strain.

Cells (curved-rods) are strictly aerobic and stain Gram-negative. Cells are 1.75 – 2.2 µm in length and 0.5 – 0.7 µm in width and are motile with lophotrichous flagella. The optimum temperature for growth is 30ºC; growth occurs at 10 – 30ºC but not at 4ºC or 37ºC. The optimal pH for growth is 7 – 7.5; growth occurs between pH 5.0 – 10.0. Growth is observed in the presence of 0 – 3 % NaCl (but not in ≥ 4 %) with an optimal concentration of 0.5%. Testing for catalase is positive. Assimilation of D-glucose, D-fructose, sucrose, lactose, yeast extract, tryptone, formate, L-arabinose, peptone, casamino acids, γ-hydroxybutyric acid, tartaric acid, succinamic acid and phenylacetic acid was observed; however, ascorbate, xanthine, succinic acid, maleic acid, L-asparagine, L-methionine or L-phenylalanine could not be utilized as sole carbon sources. Mn(IV) oxide formation was observed in cultures containing Mn(II), Mn(III)-citrate or Mn(III)-desferrioxamine B. The major cellular fatty acids are a combination of $C_{16:1}$ω7c and/or iso-$C_{15:1}$ 2-OH, $C_{16:0}$ and $C_{18:1}$ ω7c. The DNA–DNA hybridization values between strain GSL-010^T were: *P. plecoglossicida* (32.0%); and *P. japonica* (34.4%).

The type strain GSL-010^T (=JCM 32154^T =NBRC 113027^T =KCTC 62392^T) was isolated from deep waters sampled from the St. Lawrence Estuary. The genomic DNA G+C content of the type strain is 59.7%.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

Table 2: Differences in cellular fatty acid composition of *Pseudomonas laurentiana* GSL-010^T and phylogenetic neighbors.

Fatty Acid	1	2	3
Saturated fatty acid			
C10:0	0.11	–	–
C12:0	2.66	1.62	1.23
C14:0	1.76	0.41	0.23
C15:0	0.42	0.23	0.20
C16:0	32.29	25.73	25.48
C17:0	0.13	0.13	0.18
C18:0	0.18	0.24	0.39
Unsaturated fatty acid			
C16:1 ω5c	0.11	0.08	0.09
C17:1 ω8c	0.10	0.12	0.10
C18:1 ω7c	8.30	15.12	17.94
Branched fatty acid			
C17:0 cyclo	1.13	2.50	1.39
C18:1 11methyl ω7c	0.35	–	–
Hydroxy fatty acid			
C8:0 3-OH	–	0.08	0.15
C10:0 3-OH	3.83	6.49	8.04
C11:0 3-OH	–	0.04	–
C12:0 2-OH	4.18	5.83	1.23
C12:0 3-OH	3.66	4.22	4.32
C12:1 3-OH	0.07	0.78	0.61
Summed Features*			
2	–	0.09	0.24
3	40.43	35.92	33.65
7	0.17	–	–

Strains: 1, *Pseudomonas laurentiana* GSL-010; 2, *Pseudomonas plecoglossicida* NBRC 103162^T; 3, *Pseudomonas japonica* IAM 15071^T.

*Summed features are groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed feature 2 consists of $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$; Summed feature 3 consists of $C_{16:1}$ω7c and/or iso-$C_{15:1}$ 2-OH; Summed feature 7 consists of $C_{19:1}$ ω6c and/or $C_{19:1}$ cyclo ω10c.

ABBREVIATIONS

MMA: Minimal Media A.

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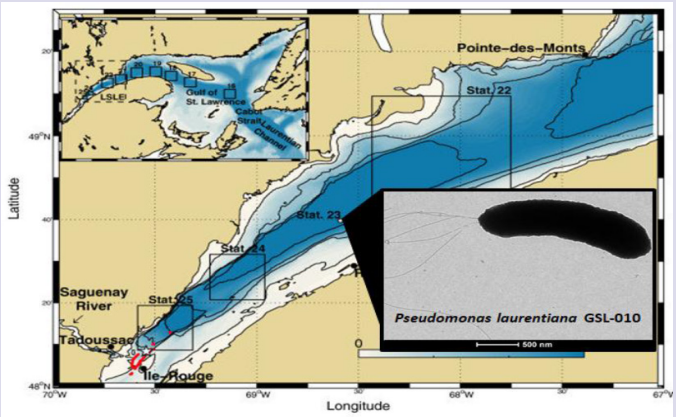
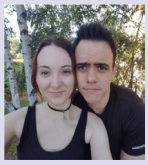
PICTORIAL ABSTRACT



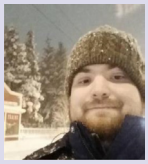
SUMMARY

- An aerobic, Mn(III)-oxidizing, Gram-negative, motile bacterium (strain GSL-010^T) was isolated from deep waters of the St Lawrence Estuary (Canada).
- Phylogenetic analysis based on 16S rRNA gene sequencing revealed strain GSL-010^T as a member of the genus *Pseudomonas*.
- DNA–DNA hybridization values between strain GSL-010T and *P. plecoglossicida* (32.0%) and *P. japonica* (34.4%) confirm the assignment of the bacterium to a new species.
- On the basis of phylogenetic analysis, DNA–DNA hybridization and physiological and biochemical characterization, strain GSL-010^T is clearly a unique bacterium.
- Since it was isolated from the waters of the St. Lawrence Estuary, the name *Pseudomonas laurentiana* sp. nov. is proposed.
- The type strain is GSL-010T (=JCM 32154^T =NBRC 113027^T =KCTC 62392^T).

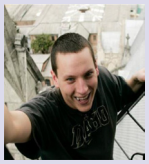
ABOUT THE AUTHORS



Dr. Mitchell H. Wright is a Geomicrobiologist who received his Ph.D. in 2014 for his work investigating the manganese reduction/oxidation characteristics of environmental bacteria. From 2016 to 2018 he undertook a postdoctoral researcher role under the mentorship of Prof. Bradley Tebo, where he explored the bacterial oxidative formation and removal of complexed Mn(III) and the implications of these processes on the global ocean. Upon returning to Australia, Dr. Mitchell H. Wright was recruited by First Choice College and to date, oversees their Department of Research and Development. Additionally, he has returned to his former lab (lead by Dr. Ian Cock) to continue his research into the antimicrobial potential of native plants.



Mr. John G. Hanna obtained his MSc (Biology/Biochemistry) at the Oregon Health & Science University under the supervision of Prof. Bradley M. Tebo and Dr. Mitchell H. Wright. He is most interested in extremophilic anaerobic respiration by thermophilic bacteria and intends to pursue his Doctorate in the future.



Mr. Derek A. Pica is a Research Associate with degrees from the University of Idaho and Oregon Health & Science University. He is currently involved in the identification and sequencing of genes associated with ophthalmic diseases in humans. Though his experience in science is largely lab-based, Derek has a passion for educating and has accepted a teaching position in Spain, which he begins in 2019.

Prof. Bradley M. Tebo is a distinguished Professor at the Oregon Health & Science University (OHSU) in Portland, Oregon (United States of America). He received his bachelor's degree in Biology at the University of California San Diego and his Ph.D. in Marine Biology at the Scripps Institution of Oceanography (UCSD), where his passion toward bacterial manganese biomineralization was first ignited. After successfully undertaking a postdoctoral role at the University of Washington, he found a more permanently role at Johns Hopkins University, Chesapeake Bay Institute in 1985. He returned to Scripps in 1987 where he rose through the academic ranks, eventually becoming a full Professor. He was recruited by OHSU in 2005 where he remains today and almost exclusively focuses on bacterial manganese oxidation. Prof. Bradley M. Tebo is a Fellow of the American Academy for Microbiology and the American Association for the Advancement of Science, a co-founding editor for Frontiers in Microbiology specialty section Microbiological Chemistry and Geomicrobiology, a member of the editorial board of the Geomicrobiology Journal and the author of over 65 publications.