2018

Isolation of Potential Photosynthetic N_2-Fixing Microbes from Topsoil of Native Grasslands in South Dakota

Nanfang Wang
South China Normal University

Shengni Tian
South China Normal University

Liping Gu
South Dakota State University

Lan Xu
South Dakota State University

Yeyan Qiu
South Dakota State University

See next page for additional authors

Authors
Nanfang Wang, Shengni Tian, Liping Gu, Lan Xu, Yeyan Qiu, Trevor Van Den Top, Jose L. Gonzalez-Hernandez, Michael B. Hildreth, Shaoshan Li, and Runbao Zhou

ISOLATION OF POTENTIAL PHOTOSYNTHETIC N_2-FIXING MICROBES FROM TOPSOIL OF NATIVE GRASSLANDS IN SOUTH DAKOTA

Nanfang Wang[1a, 1b, Ψ], Shengni Tian[1a,Ψ], Liping Gu[1a], Lan Xu[2], Yeyan Qiu[3], Trevor Van Den Top[1a], Jose L. Gonzalez-Hernandez[3], Michael Hildreth[1a], Shaoshan Li[1b]* and Ruanbao Zhou[1a]*

[1a]Department of Biology and Microbiology
[2]Department of Natural Resource Management
[3]Department of Agronomy, Horticulture and Plant Sciences
South Dakota State University
Brookings, SD; 57007

[1b] School of Life Science
Key Laboratory of Ecology and Environmental Science in Guangdong
Higher Education, South China Normal Normal University
Guangzhou 510631

*Corresponding authors, E-mail: ruanbao.zhou@sdstate.edu; lishsh@scnu.edu.cn
[Ψ]Shengni Tian and Nanfang Wang are co-first authors who made equal contributions.

ABSTRACT

Nitrogen fertilizer is one of the most limiting factors and costly inputs in agriculture production. Current fossil fuel-dependent ammonia production is both energy intensive and environmentally damaging. An economically practical and environmentally friendly solution for the production of ammonia is urgently needed. Solar-powered N_2-fixing cyanobacteria provide a unique opportunity and promise for applications in agriculture compared to all other N_2-fixing bacteria that cannot use solar energy. Isolation of nitrogen-fixing microbes from the topsoil of native grasslands may have the potential to use them in crop fields as living ammonia factories. This may be a mechanism to free farmers from heavy reliance on fossil fuels-dependent chemical nitrogen fertilizers and to improve soil health for sustainable agriculture. To screen for solar-powered N_2-fixing cyanobacteria in topsoil of native grasslands in South Dakota, we collected 144 topsoil samples from several native grasslands. Six photosynthetic microbial strains were isolated that are capable of growing well autotrophically in a nitrogen-free medium, suggesting that these six microbial strains have the ability to fix N_2. They were assigned the names: Xu15, Xu81, Xu86, Xu111, Xu141, and WW3. Based on cell morphology and its 18S rRNA gene sequence that we obtained, strain Xu15 was reassigned as *Chloroidium saccharophilum* Xu15, a common terrestrial coccoid green alga.

An acetylene reduction assay detected substantial ethylene production, suggesting nitrogenase activity occurrences in cultures Xu81 and Xu15. The other four are in the process of purification for testing their nitrogenase activity. Xu81, Xu111 and Xu141 are probably unicellular microalga, while WW3 and Xu86 are

likely filamentous cyanobacteria. Future research will focus on developing these validated N_2-fixing microbes as *in situ* living ammonia factories in crop fields.

Keywords

Sustainable agriculture, Nitrogen-fixation, Cyanobacteria, Microalgae, Soil health, Bio-solar ammonia factory

INTRODUCTION

Today's unsustainable supplies and volatile prices of fossil fuels have dramatically increased the price of ammonia fertilizer produced by the Haber-Bosch process, which is energy intensive and also contributes to greenhouse gas emissions. According to the report from the Food and Agriculture Organization of the United Nations (FAO), more than 188.31 million tons of ammonia will be needed annually to support the world's food production by 2020, and it is expected to grow annually on average by 1.5 percent (FAO 2017). The economic and environmental costs of the heavy use of fossil fuel-dependent, chemically synthesized ammonia fertilizers in modern agriculture have become pressing problems. Therefore, economical, environment-friendly sustainable production of ammonia must be urgently sought. Solar energy-driven nitrogen fixation by some cyanobacteria offers such a unique opportunity. In addition to reduced costs, cyanobacteria-based nitrogen fixation using solar energy and consuming CO_2 may help alleviate the two global problems: fossil fuel depletion and environment degradation due to elevated CO_2 emissions.

Biological nitrogen fixation refers to the process by which nitrogen-fixing microorganisms reduce atmospheric N_2 gas into ammonia through nitrogenase (Burris 1991; Rees et al. 2005; Mus et al. 2018). So far, biological nitrogen fixation is found exclusively in diverse prokaryotes including cyanobacteria. Through billions of years of evolution, some autotrophic cyanobacteria have gained the unique capability of using solar energy to aerobically reduce atmospheric N_2 into ammonia through nitrogenase (Fay 1992; Bergman et al. 1997; Berman-Frank et al. 2003; Zehr 2011).

The nitrogenase complex is encoded by a group of genes called *nif* genes, including the most important *nifH*. A minimum of 10 *nif* genes (*nif* F, J, H, D. G, K, U, S, V and B) enable anaerobic nitrogen fixation in an engineered *E. coli* (Yang et al. 2014; Vicente and Dean 2017). The global diversity of nitrogen-fixing microorganisms was assessed through analysis of 16989 *nifH* sequences from available databases (Gaby and Buckley 2011). The soil samples had the greatest diversity of *nifH* sequences among all the environments surveyed. Cyanobacterial *nifH* sequences were also found in topsoil (such as soil crusts) in certain arid ecosystems (Garcia-Pichel et al. 2001; Gaby and Buckley 2011; Hagemann et al. 2015; Zhang et al. 2016). The solar-powered N_2-fixing cyanobacteria in topsoil provides a definite advantage compared to heterotrophic nitrogen-fixing bacteria which fail to use solar energy, relying on other organisms for an energy source; and requiring anaerobic conditions. While this unique phenomenon of N_2-fixing cyanobacteria

has supported the nitrogen needs for native grasslands/forests for eons, mankind has largely ignored its potential application in crop production due to the wide availability of chemically synthesized ammonia driven by fossil fuel energy.

N_2-fixing microbes such as cyanobacteria have played a critical role in healthy soils that harmonize the soil biological, chemical, and physical properties to sustain huge annual biomass production (without applying nitrogen fertilizer) in native grasslands and native forests (Berman-Frank et al. 2003; Adam et al. 2016; Singh et al. 2016). Cyanobacteria can contribute at least 20-30 Kg of fixed N (≈55 Kg urea) ha^{-1} $season^{-1}$ as well as abundant organic matter to the soil (Pankratova 2006; Issa et al. 2014). The contribution of biomass by cyanobacteria only in arid land soil is estimated to reach 68 $x10^6$ tons of carbon, which could greatly improve both soil fertility and soil health (Garcia-Pichel et al. 2003). We hypothesize that the native grassland of South Dakota represents an unexplored territory where highly effective and stress-tolerant nitrogen-fixing cyanobacteria and other N_2-fixing microbes live. The objective of this research was to identify solar-powered, N_2-fixing cyanobacteria and other microbes in topsoil of native grassland in South Dakota. The long-term goal of this project is to engineer these N_2-fixing microbes with the highest capacity for nitrogen fixation and use them as a "solar-powered, living ammonia factory" in crop fields.

METHODS

Isolation and purification of potential N_2-fixing photosynthetic microorganisms. A total of 144 topsoil samples (1-3 cm depth) were collected from native grasslands at the Standing Rock Reservation near McLaughlin, Corson County, SD and native grasslands near Miller, Hand County, SD in May 2016, 2017. Approximately 3-5 grams of homogenized topsoil were added into a 35-ml small mouth bottle filled with 20-ml autoclaved mineralized water (AA/8N medium, a mineralized water without organic carbon sources) with combined nitrogen (Hu et al. 1981). These bottles were incubated under continuous light illumination (ca. 50 $\mu E/M^{-2}\cdot S^{-1}$) at 28-30 °C. After about 10 days, the "green" cultures that appeared were transferred into fresh AA/8N medium to isolate autotrophic microbes from the soil. The green cultures were then streaked onto $BG11_0$ (free of combined nitrogen) agar plates. After 12 times of re-streaking purification, we isolated six microbial strains that were able to reproducibly grow well on BG110 agar plates, suggesting that these six strains have N_2-fixing ability.

Morphology analysis by microscopy. Microscopy (Olympus, BX53) was used to examine the morphology of isolated cyanobacteria and microalgae.

Nitrogenase activity determined by acetylene reduction assay. A 7-day old culture growing on a $BG11_0$-agar plate (nitrogen-free medium) was cut and transferred into a 20-ml glass serum bottle (Wheaton). The bottle was sealed with a red rubber stopper (Wheaton) and injected with 1.0 ml of acetylene. The bottle was incubated under continuous light (50 $\mu E/m^2/s$, 120 rpm, 30 °C) for about 3 weeks. Five mL of headspace gas sample from the 20 mL culture bottle was

administered via a 1 mL GSV Loop to the GC-MS (Agilent 890A/5975C). To alleviate the vacuum in the serum bottle, we injected 4 mL of normal air and 1 mL CO_2 back into the 20 mL bottle. The volatile compounds were separated by CP7348 column (Agilent PoraBOND Q 25 m × 250 μm × 3 μm) with Pulsed Split mode at 20:1 ratio at a flow rate 0.8 mL/min using hydrogen as a carrier gas. The GC program was initiated at 32 ºC held for 4 min, and ramped at 110 °C to reach 232 °C. The scanning mass range of MSD was between 10 to 50 m/z.

RESULTS AND DISCUSSION

Isolation of four microalgae strains. Four photosynthetic micro-organisms were isolated from topsoil of native grassland in South Dakota. These four microbial strains reproducibly grew well autotrophically in the nitrogen-free medium plate (Figure 1, left panels) after more than 10 times re-streaking onto the nitrogen-free medium-BG110-agar plate (Figure 1). Xu15, Xu81 and Xu111 are ellipsoidal in shape (Figure 1, right panels), while Xu141 is spherical in shape (Figure 1). Based on their cell sizes (5.0-10 μm) and other visual microscopic morphology such as compartmentalized organelles, Xu15, Xu81, Xu111 and Xu141 are likely unicellular microalgae (Figure 1, right panels). Microalgae are unicellular microorganisms, having many compartmentalized organelles such as a nucleus, mitochondria and chloroplast within each cell; while cyanobacteria are usually multi-cellular microorganisms, although some cyanobacteria are unicellular organisms. Unlike microalgae, cyanobacteria lack a nucleus and mitochondria. Both microalgae and cyanobacteria can perform plant-type photosynthesis by using water as a source of electrons and releasing oxygen.

Isolation of two filamentous cyanobacterial strains. Two filamentous cyanobacterial strains were isolated from the topsoil of native grasslands in South Dakota. These two isolates reproducibly grew well autotrophically in the nitrogen-free medium-$BG11_0$-agar plate (data not shown), which suggests they are capable of nitrogen fixation. The cell size of strain Xu86 was in the range of 1.0-2.0 μm (Figure 2, Xu86 panel), while the cell size of strain WW3 was in the range of 2.0-3.0 μm (Figure 2, WW3 panel). Both Xu86 and WW3 strains had substantial motility on nitrogen-free medium-$BG11_0$-agar plates (data not shown).

Nitrogenase activity of culture Xu15 and culture Xu81. Since the culture of Xu15 and Xu81 appeared to be homogeneous at least microscopically, and given that they can grow well on the nitrogen-free medium, this strongly suggests that these two microalgae may have nitrogenase activity. The acetylene reduction assay was used to determine the nitrogenase activity for cultures Xu15 and Xu81. Both cultures showed a substantial ethylene production (Figure 3, C &D) after about 3 weeks of incubation with acetylene; while the controls with non-N_2-fixing cyanobacterium *Synechocystis* sp. PCC 6803 showed no ethylene production in conditions identical to that of Xu15 and Xu81(data not shown). The substantial ethylene production from the cultures of Xu15 and Xu81 suggests the presence of

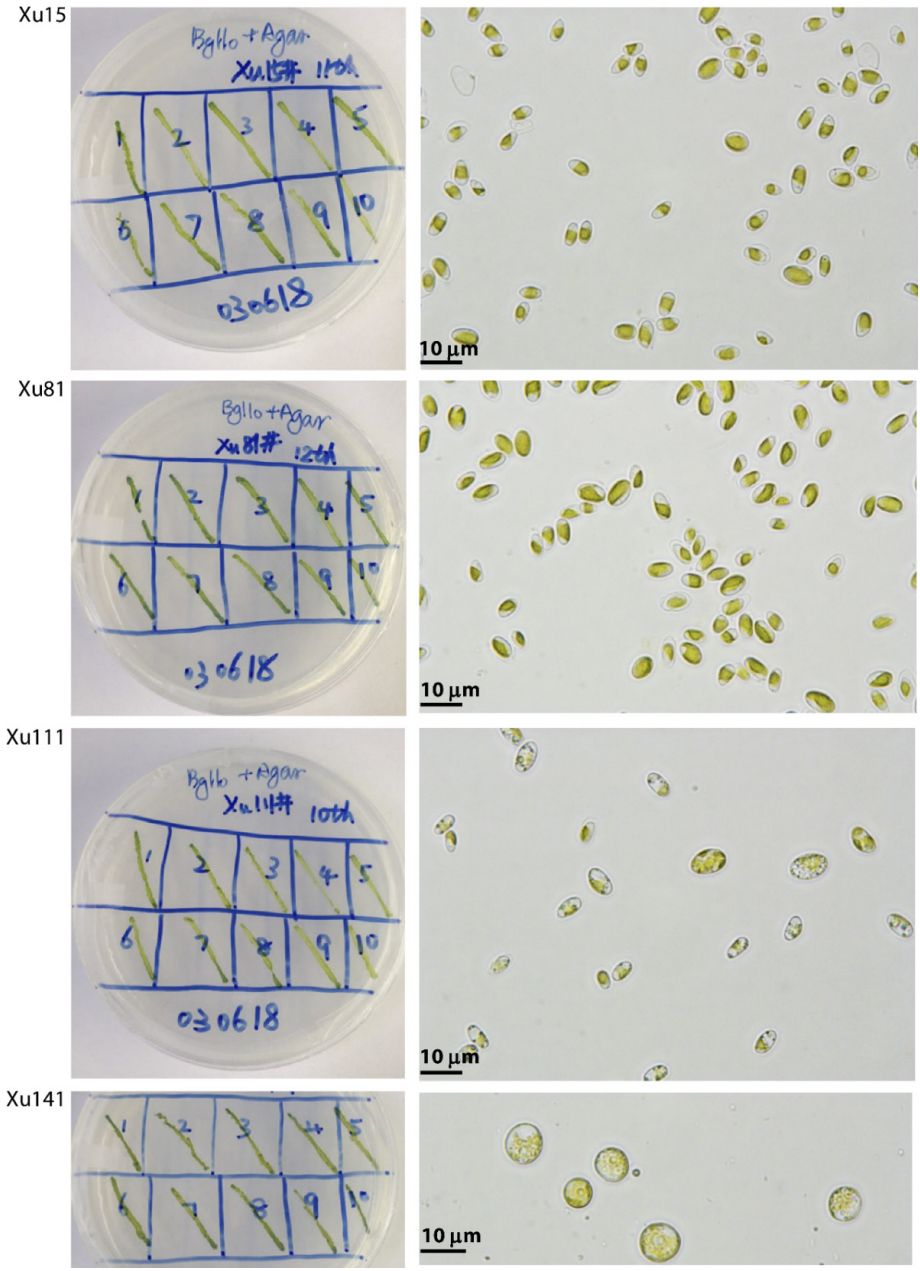

Figure 1. Four microalgae isolated from the topsoil of native grasslands in South Dakota. Left panels: four microalgae grown on BG11$_0$-agar plate (nitrogen-free medium). Right panels: bright-field images captured by an Olympus BX53 upright microscopy equipped with a digital camera.

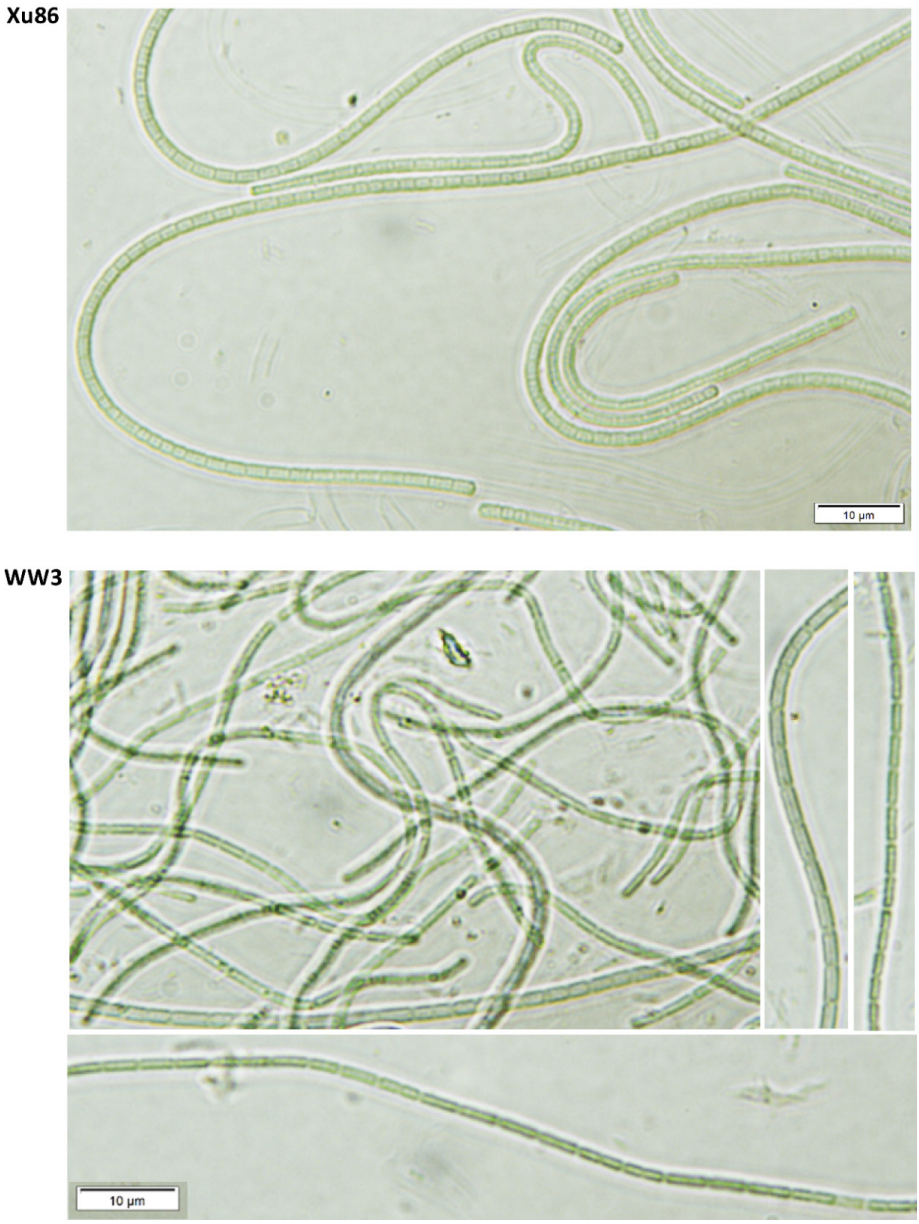

Figure 2. Two filamentous cyanobacterial strains isolated from the topsoil of native grasslands in South Dakota. These strains were able to grow well on free of combined nitrogen medium. Bright-field images for both Xu86 and WW3 grown in AA/8N (Hu et al. 1981) were captured by an Olympus BX53 upright microscopy equipped with a digital camera. The scale bars in all panels including 4 panels for WW3 were 10 µm.

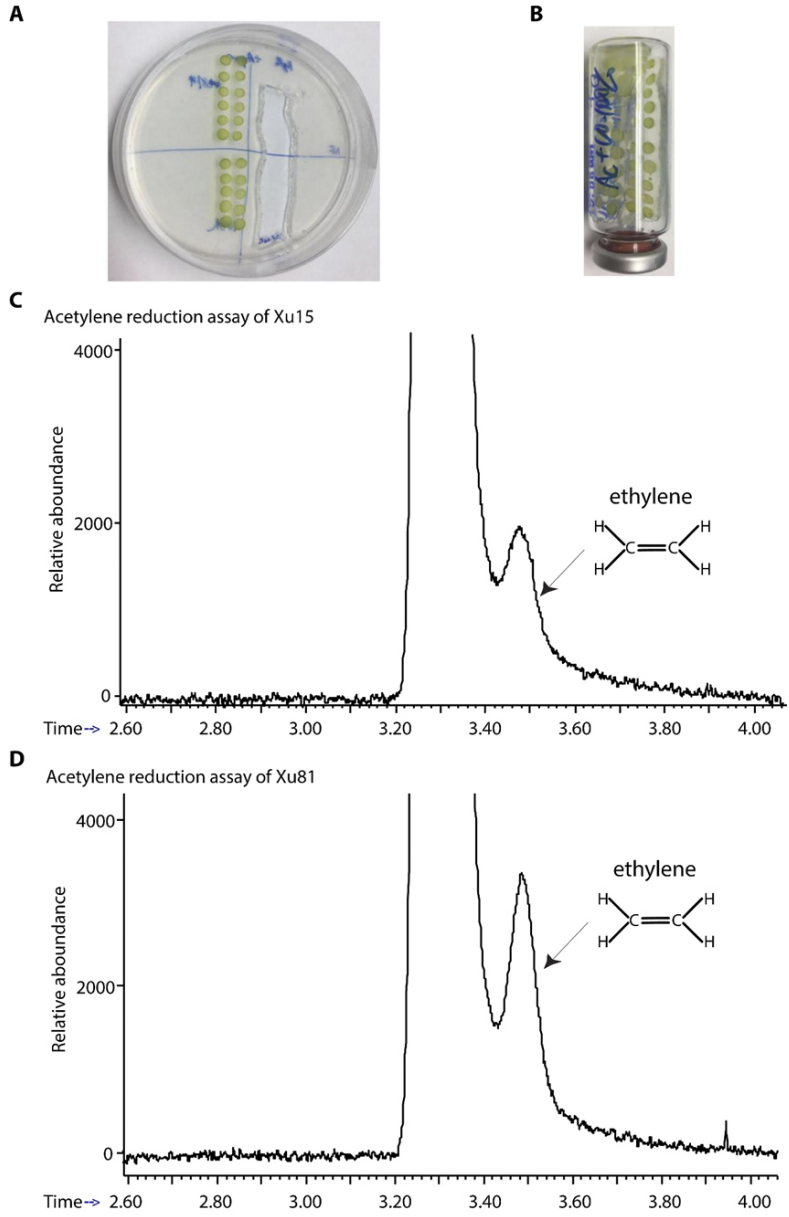

Figure 3. Acetylene reduction assay for cultures of Xu15 and Xu81 grown on BG11$_0$-agar plate (nitrogen-free medium). A) Xu15 or Xu81 culture incubated for about 7 days on BG11$_0$-agar plate under continuous light illumination (ca. 50 μE/M^{-2}·S^{-1}) at 28-30 °C. B) The culture along with the agar transferred into a 20 mL clear serum bottle and sealed with rubber stopper. C) Ethylene peak detected in the headspace gas sample of culture Xu15. D) Ethylene peak detected in the headspace gas sample of culture Xu81.

nitrogenase activity in these two cultures. However, the 18S rRNA sequences that we obtained identified Xu15 and Xu81 to be *Chloroidium saccharophilum*, a common terrestrial coccoid green alga (Darienko et al. 2010). Since there has been no report so far that *Chloroidium saccharophilum,* a eukaryotic microorganism, has nitrogenase activity, additional investigation and more evidences are required to further validate the nitrogen fixation ability of *Chloroidium saccharophilum* strains Xu15 and Xu81 that were isolated from South Dakota native grasslands.

Partial genome sequencing of Xu15 strain to obtain its 18S rRNA gene sequence. Very recently we started to sequence the genome for strain Xu15. The 18S rRNA gene containing contig_85 (bp 6718 to bp 10208) from Xu15 genomic DNA showed the highest identity (99.50% identical) to the partial 18S rRNA gene-containing sequences (GenBank access #: KC517116.1, KX024689.1) from both the *Chloroidium saccharophilum* strain CCM-UDEC-143 and the *Chloroidium saccharophilum* strain SHY184 (Song et al. 2016), a common terrestrial coccoid green algae (Darienko et al. 2010). The 18S rRNA gene sequence alignment among Xu15 and the two *Chloroidium saccharophilum* strains is shown in Figure 4. Based on the cell morphology and its 18S rRNA sequence data, we conclude that Xu15 belongs to *Chloroidium.* Therefore, strain Xu15 was reassigned *Chloroidium saccharophilum* Xu15. This newly isolated *Chloroidium saccharophilum* Xu15 strain will be stored at ATCC (the American Type Culture Collection). The 18S rRNA gene sequence will be deposited at GenBank.

Conclusion. Six photosynthetic microorganisms were isolated from native grasslands in South Dakota. These six isolates were all capable of growing autotrophically in nitrogen-free medium, which strongly suggests that these six isolates may have the ability to fix N_2 aerobically. Acetylene reduction assay detected a substantial production of ethylene, suggesting a nitrogenase activity for cultures Xu15 and Xu81. Four of the isolated strains (Xu15, Xu81, Xu111, Xu141) appeared to be unicellular microalgae. Based on its 18S rRNA gene sequence that we obtained, strain Xu15 was reassigned as *Chloroidium saccharophilum* Xu15 strain. Xu81 looks identical to Xu15. Both Xu86 and WW3 are likely filamentous cyanobacteria. To our best knowledge, this is the first successful isolation of photosynthetic micro-organisms from topsoil of South Dakota native grasslands. Future research will focus on validation and developing these validated N_2-fixing microbes as *in situ*, solar-powered living ammonia factories in crop fields.

ACKNOWLEDGEMENTS

We thank South China Normal University for funding the international joint training program for top graduate students (to N.W. & S. L.). This work was supported by South Dakota Nutrient Research and Education Council grant (to R. Z), and by the South Dakota Agricultural Experiment Station. We acknowledge use of the South Dakota State University Functional Genomics Core Facility supported in part by NSF/EPSCoR Grant No.0091948 and by the State of South Dakota.

LITERATURE CITED

Adam, B., I. Klawonn, J.B. Svedén, J. Bergkvist, N. Nahar, J. Walve, S. Littmann, M.J. Whitehouse, G. Lavik, M. M. Kuypers, and H. Ploug. 2016. N2-fixation, ammonium release and N-transfer to the microbial and classical food web within a plankton community. ISME Journal 10:450-459.

Bergman, B., J. Gallon, A. Rai, and L. Stal. 1997. N2 Fixation by non-heterocystous cyanobacteria. FEMS Microbiology Review 19:139-185.

Berman-Frank I., P. Lundgren, and P. Falkowski. 2003. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Research Microbiolgy 154:157-164.

Burris R.H. 1991. Nitrogenases. Joural of Biological Chemistry 266:9339-9342

Corpet F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Research 16:10881-10890.

Darienko T., L. Gustavs, O. Mudimu, C.R. Menendez, R. Schumann, U. Karsten, T. Friedl, and T. Proschold. 2010. Chloroidium, a common terrestrial coccoid green alga previously assigned to Chlorella (Trebouxiophyceae, Chlorophyta). European Journal of Phycology 45:79-95.

FAO. 2017. World fertilizer trends and outlook to 2020.

Fay P. 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiological Reviews 56:340-373.

Gaby J.C., and D.H. Buckley. 2011. A global census of nitrogenase diversity. Environmental Microbiology 13:1790-1799.

Garcia-Pichel F., J. Belnap, S. Neuer, and F. Schanz. 2003. Estimates of global cyanobacterial biomass and its distribution. Algological Studies 109:213-227.

Garcia-Pichel F., A. Lopez-Cortes, and U. Nubel. 2001. Phylogenetic and morphological diversity of cyanobacteria in soil desert crusts from the Colorado plateau. Applied and Environmental Microbiology 67:1902-1910.

Hagemann M., M. Henneberg, V.J. Felde, S.L. Drahorad, S.M. Berkowicz, P. Felix-Henningsen, and A. Kaplan. 2015. Cyanobacterial diversity in biological soil crusts along a precipitation gradient, Northwest Negev Desert, Israel. Microbiology Ecology 70:219-230.

Hu N.T., T. Thiel, T.H. Giddings Jr., and C.P. Wolk. 1981. New Anabaena and Nostoc cyanophages from sewage settling ponds. Virology 114:236-246.

Issa A.A., M.H. Abd-Alla, and T. Ohyama. 2014. Nitrogen fixing Cyanobacteria: future prospect. InTechOpen.

Mus F., A.B. Alleman, N. Pence, L.C. Seefeldt, and J.W. Peters. 2018. Exploring the alternatives of biological nitrogen fixation. Metallomics 10:523-538.

Pankratova E.M. 2006. Functioning of cyanobacteria in soil ecosystems. Eurasian Soil Science 39: S118–S127.

Rees D.C., F.A. Tezcan, C.A. Haynes, M.Y. Walton, S. Andrade, O. Einsle, and J.B. Howard. 2005. Structural basis of biological nitrogen fixation. Philosophical Transactions of the Royal Society of Mathematics, Physical and Engineering Sciences 363:971-984; 1035-1040.

Robert X., and P. Gouet. 2014. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Research 42: W320-324.

Singh J.S., A. Kumar, A.N. Rai, and D.P. Singh. 2016. Cyanobacteria: A precious bio-resource in agriculture, ecosystem, and environmental sustainability. Frontiers in Microbiology 7:529.

Song H., Y. Hu, H. Zhu, Q. Wang, G. Liu, and Z. Hu. 2016. Three novel species of coccoid green algae within the Watanabea clade (Trebouxiophyceae, Chlorophyta). International Journal of Systematic and Evolutionary Microbiology 66:5465-5477.

Vicente E.J., and D.R. Dean. 2017. Keeping the nitrogen-fixation dream alive. Proceedings of the National Academy of Science of the United States of America 114:3009-3011.

Yang J., X. Xie, X. Wang, R. Dixon, and Y.P. Wang. 2014. Reconstruction and minimal gene requirements for the alternative iron-only nitrogenase in Escherichia coli. Proceedings of the National Academy of Science of the United States of America 111:3718-3725.

Zehr J.P. 2011. Nitrogen fixation by marine cyanobacteria. Trends Microbiology 19:162-173.

Zhang B., R. Li, P. Xiao, Y. Su, and Y. Zhang. 2016. Cyanobacterial composition and spatial distribution based on pyrosequencing data in the Gurbantunggut Desert, Northwestern China. Journal Basic Microbiology 56:308-320.


Xu15_6718-10208
KC517116.1
KX024689.1
consensus>50

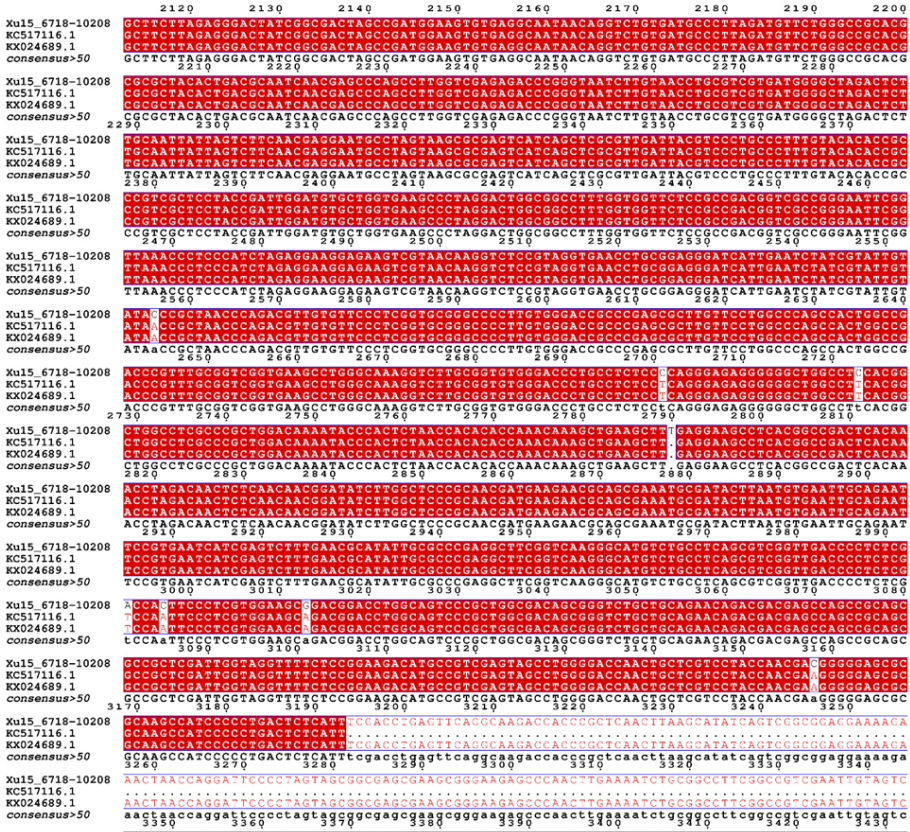

Figure 4. Sequence alignment of Xu15 strain 18S rRNA gene-containing genomic sequence with the partial 18S rRNA gene sequences from two **Chloroidium saccharophilum** ***strains.*** **Xu15_6718-10208:** ***Xu15 strain genomic sequence contig_85 that contains 18S rRNA gene sequence 3491bp (bp 6718 to bp 102208);*** **KC517116.1: Chloroidium saccharophilum** ***strain CCM-UDEC-143 partial 18S rRNA gene-containing genomic sequence 3192bp;*** **KX024689.1: Chloroidium saccharophilum** ***strain SHY184 partial 18S rRNA gene-containing sequence 3076bp. Sequence alignment was made using an online program MultAlin (Corpet 1988). The figure was generated by an online program ESPript 3.0 (Robert and Gouet 2014).***