***Curtobacterium glycinis* sp. nov. from *Glycine max*, *Curtobacterium gossypii* sp. nov. from *Gossypium hirsutum* and *Curtobacterium oryzae* sp. nov. from *Oryza sativa*, three new *Curtobacterium* species and endophytes from agricultural crops**

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ABSTRACT

Three new *Curtobacterium* species from healthy tissues of agricultural crop plants in the United States are reported. They are *Curtobacterium glycinis* sp. nov. from soybean in Missouri, *Curtobacterium gossypii* from cotton in Puerto Rico and *Curtobacterium oryzae* sp. nov. from rice in Texas.

Keywords: novel species, taxonomy, bacteria, prokaryotes, endophytes, agriculture, *Actinobacteria*, *Microbacteriaceae*

INTRODUCTION

Bacterial endophytes of plants have many beneficial interactions with their hosts and play a crucial role in promoting plant health (Finkel et al. 2017; Schenk et al. 2012). Microbiomes of the major agricultural crops including Cotton (*Gossypium hirsutum* L.), rice (*Oryza sativa* L.) and soybean (*Glycine max* (L.) Merr.) have been characterized (Edwards et al. 2015; Liu et al. 2019; Longley et al. 2020; Qiao et al. 2017; Roman-Reyna et al. 2020; Ullah et al. 2019), and beneficial endophytes have been detected (Bertani et al. 2016; de Almeida Lopes et al. 2018; Zhou et al. 2018). Plant-associated microorganisms are increasingly used for biotechnological applications, including biological control of

34 plant pathogens, plant growth promotion, or isolation of active compounds (Ryan et al.
35 2008; Glick 2012; Bouizgarne 2013; Dey et al. 2014).
36 *Curtobacterium* is a genus of *Actinobacteria* in the family *Microbacteriaceae*
37 comprising eight validly published species (Parte 2018). Several of the species have been
38 isolated from agricultural plants. These include *Curtobacterium albidum* shown to
39 counteract salt stress in rice (Vimal et al. 2019), *Curtobacterium flaccumfaciens* a
40 pathogen of bean, beat, tulip and poinsetta (Collins and Jones 1983), *Curtobacterium*
41 *herbarum* from grasses (Behrendt et al. 2002), and *Curtobacterium plantarum* from corn
42 and soybean seed (Dunleavy 1989).
43 In this study, three *Curtobacterium* strains were isolated from healthy plant tissues
44 in the United States. Strain OG107 from soybean in Missouri, strain VK105 from cotton in
45 Puerto Rico, and strain SS108 from rice in Texas. The strains were characterized using
46 molecular and phenotypic tests to determine their taxonomic placement. Our results
47 indicate that the strains represent three new species of *Curtobacterium*, and we propose the
48 names *Curtobacterium glycinis* sp. nov. (OG107), *Curtobacterium gossypii* sp. nov.
49 (VK105) and *Curtobacterium oryzae* sp. nov. (SS108). These strains are taxonomically
50 separated from the plant pathogens found within this genus.
51
52 **METHODS**
53 **Isolation.** Strains OG107 and SS108 were collected from the roots of healthy field-
54 grown *Glycine max* in Missouri and *Oryza sativa* seedlings in Texas, United States,
55 respectively. Plant tissue was washed with a mild detergent to remove particulates,
56 surface-sterilized with bleach (1% v/v sodium hypochlorite) and ethanol (70% v/v), and
57 homogenized. Serial dilutions of tissue homogenate were plated on a panel of media types
58 for endophyte cultivation. Strain OG107, a small (0.7 mm diameter), pale yellow colony,
59 arose on R2A agar after 5 days of incubation at 24°C, and strain SS108 arose as a non-
60 descript colony on Actinomycete Isolation Agar. Both strains were streaked to purity and
61 stored in glycerol (20% v/v) at -80°C until subjected to further testing.
62 Strain VK105 was isolated as described in Irizarry and White (2017). Briefly, seeds
63 of wild, non-cultivated *G. hirsutum* plants were collected roadside in Guayama, Puerto
64 Rico. Seeds were inoculated on Potato Dextrose Agar (PDA) and incubated at room
65 temperature (25°C). Strain VK105 arose as an irregular yellow-orange pigmented colony.
66 The colony was streaked to purity and stocked in 25% glycerol at −80°C until further
67 analysis.

68 **Motility.** Strains were tested for flagellar-dependent swimming and swarming
69 motility on R2A plates solidified with 0.3% and 0.6% agar, respectively. Three
70 independent colonies were inoculated onto R2A broth and grown for 36 hr at 24°C. Broth
71 cultures were normalized to an OD600 of 0.1, and 1.5 µl of culture was spotted directly
72 onto the surface of the motility agar. The diameter of colony expansion was measured for
73 5 days.
74 **Carbon source utilization.** Substrate utilization was assessed using Biolog GenIII
75 Microplates (Catalogue No. 1030) (Biolog Inc., Hayward, CA). Each bacterium was
76 inoculated in duplicate plates using Protocol A described by the manufacturer, with the
77 exception that plates were incubated at 30°C for 36 hr. Respiration leading to reduction of
78 the tetrazolium indicator was measured by absorbance at 590 nm.
79 **Biochemical analyses.** Catalase activity was evaluated by immediate effervescence
80 after the application of 3% (v/v) hydrogen peroxide solution via the tube method, a
81 positive reaction was indicated by the production of bubbles. *Staphylococcus aureus*
82 NCIMB 12702 and *Streptococcus pyogenes* ATCC 19615 were used as positive and
83 negative controls, respectively. Oxidase activity was evaluated via the oxidation of Kovács
84 oxidase reagent, 1% (w/v) tetra-methyl-p-phenylenediamine dihydrochloride in water, via
85 the filter-paper spot method. A positive reaction was indicated when the microorganism's
86 color changed to dark purple. *Pseudomonas aeruginosa* NCIMB 12469 and *Escherichia*
87 *coli* ATCC 25922 were used as positive and negative controls, respectively.
88 **Phylogenetic and genomic analyses.** DNA was extracted from pure cultures using
89 the Omega Mag-Bind Universal Pathogen Kit according to manufacturer's protocol with a
90 final elution volume of 60µl (Omega Biotek Inc., Norcross, GA). DNA samples were
91 quantified using Qubit fluorometer (ThermoFisher Scientific, Waltham, MA) and
92 normalized to 100 ng. DNA was prepped using Nextera DNA Flex Library Prep kit
93 according to manufacturer's instructions (Illumina Inc., San Diego, CA). DNA libraries
94 were quantified via qPCR using KAPA Library Quantification kit (Roche Sequencing and
95 Life Science, Wilmington, MA) and combined in equimolar concentrations into one 24-
96 sample pool. Libraries were sequenced on a MiSeq using pair-end reads (2x200bp). Reads
97 were trimmed of adapters and low-quality bases using Cutadapt (version 1.9.1) and
98 assembled into contigs using MEGAHIT (version 1.1.2) (Li et al. 2015). Reads were
99 mapped to contigs using Bowtie2 (version 2.3.4) (Langmead and Salzberg 2012), and
100 contigs were assembled into scaffolds using BESST (2.2.8) (Sahlin et al. 2014).

101 Average nucleotide identity analyses were performed using the pyani ANIm
102 algorithm (Richter and Rosselló-Móra 2009) implemented in the MUMmer package
103 (Kurtz et al. 2004) retrieved from https://github.com/widdowquinn/pyani.
104 16S rRNA gene sequences were extracted from genome assemblies using barrnap
105 (Seemann 2019) and submitted to GenBank. Phylogenetic analyses based on the 16S
106 rRNA gene were performed using FastTree (Price et al. 2010) with a General Time
107 Reversible substitution model. Taxon sampling for each species is described in the
108 respective phylogenetic tree figure legend.
109
110 **RESULTS**
111 **Phylogenetic and genomic analyses**
112 *Curtobacterium glycinis* sp. nov. strain OG107
113 Strain OG107 shared 98.9% 16S rRNA gene sequence identity with
114 *Curtobacterium citreum* DSM 20528^T and less with the remaining *Curtobacterium*
115 species. A phylogenetic tree using FastTree (Price et al. 2010) confirmed the affiliation of
116 strain OG107 with the genus *Curtobacterium*. OG107 was most closely related to *C.*
117 *herbarum* P 420/07^T with 84% bootstrap support (Figure 1). The top average nucleotide
118 identity (ANI) value of OG107 was 89.2% with *C. luteum* ATCC 15830^T. This value was
119 well below the threshold for species demarcation (Richter and Rosselló-Móra 2009; Chun
120 et al. 2018) providing further genomic support that strain OG107 represents a new
121 genomic species of *Curtobacterium.*
122
123 *Curtobacterium gossypii* sp. nov. strain VK105
124 Strain VK105 shared 99.8% 16S rRNA gene sequence identity with
125 *Curtobacterium citreum* DSM 20528^T and less with the remaining *Curtobacterium*
126 species. A phylogenetic tree using FastTree (Price et al. 2010) confirmed the affiliation of
127 strain VK105 with the genus *Curtobacterium*. VK105 formed a monophyletic group with
128 the species *C. albidum* and *C. citreum* supported by high bootstrap support (Figure 1) and
129 was equally related to either of the two species. Average nucleotide identity (ANI) values
130 of *C. albidum* DSM 20512^T and *C. citreum* DSM 20528^T to VK105 were both 87.7%.
131 These values are well below the threshold for species demarcation (Richter and Rosselló-
132 Móra 2009; Chun et al. 2018) providing further genomic support that strain VK105
133 represents a new genomic species of *Curtobacterium.*
134

135 *Curtobacterium oryzae* sp. nov. strain SS108

136 Strain SS108 shared 99.6% 16S rRNA gene sequence identity with *Curtobacterium*
137 *luteum* strain DSM 20542^T and less with the remaining *Curtobacterium* species. A
138 phylogenetic tree using FastTree (Price et al. 2010) confirmed the affiliation of strain
139 SS108 with the genus *Curtobacterium*. SS108 was most closely related to *C. luteum* with
140 79% bootstrap support (Figure 1). The top average nucleotide identity (ANI) value of
141 SS108 was 89.2% with *C. luteum* DSM 20542^T. This value was well below the threshold
142 for species demarcation (Richter and Rosselló-Móra 2009; Chun et al. 2018) providing
143 further genomic support that strain SS108 represents a new genomic species of
144 *Curtobacterium.*

145

146 **Morphology, physiology and biochemical characteristics**

147 *Curtobacterium glycinis* sp. nov. strain OG107

148 Strain OG107 stained Gram-positive. Cells had a slightly curved rod shape (0.2-0.4
149 µm in width and 1-3 µm in length); V and Y shaped forms were observed (Figure 2). They
150 were aerobic, non-sporulating and showed motility. On nutrient agar, colonies were cream,
151 entire, punctiform and convex. After 48 hr, good growth is observed on TSA, R2A and NA
152 at 22 and 30°C. Growth was poor to moderate on the same media and incubation times at
153 37°C. Growth is observed when media are supplemented with 4% NaCl (w/v). Strain OG
154 107 was positive for catalase, but not for oxidase or urease. Being aerobic, the strain has an
155 oxidative metabolism and according to the Biolog system, it is capable of using a range of
156 substrates as carbon sources that include sugars, alcohol sugars and organic acids.
157 Compared to its closest phylogenetic neighbor, *C. citreum* JCM 1345^T, OG17 uses N-
158 Acetyl-D-Galactosamine, D-arabitol, gentobiose, *myo*-inositol, mannitol and turanose as
159 carbon sources. These tests help to differentiate between the two strains. A detailed
160 description is given in Table 1 and the species description.

161 *Curtobacterium gossypii* sp. nov. strain VK105

162 The cells of strain VK105 stained Gram-positive, formed short irregular rods (0.4-0.6 µm
163 in width and 2-4 µm in length), with cells diving by bending (Figure 3). Y shaped-forms
164 were sometimes visible. Cells were non-motile and did not produce spores. Strain VK105
165 showed good aerobic growth on R2A and TSA agars. On both media, yellow to orange
166 colonies were observed after 2 days incubation at 22 and 30°C. At 7 days, colonies were
167 circular, smooth, glistening and slightly convex. The new strain produced catalase but not
168 oxidase. Strain VK105 has an oxidative metabolism and uses a range of substrates as

169 carbon sources that are also useful for to differentiate against its closest phylogenetic
170 neighbors, *Curtocabterium citreum* and *C. albidum*, namely D-arabitol, L-fucose, D-
171 glucuronic acid, and *myo*-inositol. These and other physiological characteristics are
172 presented in Table 1 and the species description.
173
174 *Curtobacterium oryzae* sp. nov. strain SS108
175 Cells stained-Gram positive, are non-motile, non-spore-forming and rod shaped or
176 slightly curved. In many cases, two cells remained together and formed V shapes (Figure
177 4). Average cell size ranged from 0.2-0.4 µm in width and 1.5 x 2 µm in length. Colonies
178 of strain SS108 were light yellow, entire, round and raised when grown on nutrient agar.
179 After 2 days, good growth was observed on TSA and R2A agars at 22, 30 and 37°C. On
180 nutrient agar, good growth was obtained at 30°C after 2 days and was moderate at 22 and
181 37°C. However, after 7 days, good growth was also seen at 22°C. Aerobic growth.
182 Tolerates 4% NaCl (w/v). Enzyme activity was detected for catalase, but not for oxidase or
183 urease. Several carbon sources are useful to differentiate between the new strain and *C.*
184 *luteum* JCM 1480^{T} and include the utilization of N-Acetyl-D-Galactosamine, D-arabitol,
185 gentobiose, *myo*-inositol and D-mannitol among others (Table 1). Other phenotypic
186 characteristics are given in the species description.
187
188
189 **DESCRIPTION OF CURTOBACTERIUM GLYCINIS SP. NOV.**
190
191 *Curtobacterium glycinis* (gly.ci'nis. N.L. fem. gen. n. *glycinis* of *Glycine max*, the
192 soybean, referring to the origin of the type strain)
193
194 Cells are Gram-stain positive, non-spore forming, motile and rod-shaped with V
195 and Y shapes produced. Aerobic and chemoorganotrophic. Colonies are circular, smooth,
196 glistening and slightly convex. On R2A and nutrient agar, colonies are cream color and
197 entire, punctiform and convex. Growth is abundant at 22 and 30°C but moderate at 37°C.
198 Grows in the presence of 4% NaCl. Growth is observed between pH 5-7. Positive for
199 catalase; negative for oxidase and urease. The following substrates are used as carbon
200 sources: N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-arabitol, D-cellobiose, L-
201 fucose, gentiobiose, *myo*-inositol, α-D-lactose, D-mannitol, D-melibiose, β-methyl-D-

202 glucoside, D-raffinose, turanose, D-gluconic acid, D-glucuronic acid, L-alanine, L-
203 glutamic acid (weak), L-rhamnose, D-Glucose-6-PO4.
204 The type strain is resistant to nalidixic acid and aztreonam.
205
206 The type strain OG 17^{T} was isolated from the roots of healthy field-grown *Glycine*
207 *max* in Missouri, USA.
208
209
210 **DESCRIPTION OF CURTOBACTERIUM GOSSYPII SP. NOV.**
211
212 *Curtobacterium gossypii (*gos.sy′pi.i. N.L. gen. n. *gossypii* of *Gossypium,* the
213 generic name of cotton, referring to the origin of the type strain)
214
215 Cells are Gram-stain positive, non-motile, non-spore-forming, coccoid or rod-
216 shaped. Aerobic and chemoorganotrophic. Yellow to orange colonies on R2A and TSA
217 agars. Growth on these media is observed after 48 h. Catalase is produced but not oxidase.
218 Grows in the presence of 1 - 4 % NaCl; weak at 8% and at pH 5-7. The following
219 substrates are used as sole carbon sources: dextrin, D-maltose, D-trehalose, D-cellobiose,
220 gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-
221 methylD-glucoside, D-salicin, N-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-
222 fructose, D-galactose, inosine, sodium lactate, D-mannitol, D-arabitol, *myo*-inositol,
223 glycerol, L-aspartic acid, L-glutamic acid, pectin, D-arabitol, D-gluconic acid, D-
224 glucuronic acid and glucuronamide.
225 The type strain is resistant to nalidixic acid and aztreonam.
226
227 The type strain, VK105^{T}, was isolated from seeds of wild cotton collected in Puerto
228 Rico, USA.
229
230 **DESCRIPTION OF CURTOBACTERIUM ORYZAE SP. NOV.**
231
232 *Curtobacterium oryzae* (o.ry′zae. N.L. gen. n. *oryzae* of rice, referring to the origin of the
233 type strain)
234

235 Cells are Gram-stain positive, non-motile, non-spore-forming and rod shaped or
236 slightly curved; V shapes are formed. Aerobic and chemoorganotrophic. Colonies are light
237 yellow, entire, round and raised on nutrient agar. Good growth is obtained at 22, 30 and
238 37°C on the media tested. Grows on media supplemented with 4% NaCl and at pH 5-7.
239 Positive for catalase, but negative for oxidase or urease. The following substrates are used
240 as carbon sources: N-Acetyl-D-Glucosamine, N-Acetyl-D-Galactosamine, N-Acetyl
241 Neuraminic Acid, D-Arabitol, D-Cellobiose, L-Fucose, Gentiobiose, myo-Inositol, α-D-
242 Lactose, D-Mannitol, D-Melibiose, β-Methyl-D-Glucoside, D-Raffinose, D-Sorbitol,
243 Turanose, D-Gluconic Acid, D-Glucuronic Acid, L-Alanine, L-Glutamic Acid, L-
244 Rhamnose, D-Glucose-6-PO4 and Acetic Acid.
245 The type strain is resistant to nalidixic acid and aztreonam.
246 The type strain, SS108^{T}, was isolated from *Oryza sativa* seedlings collected in
247 Texas, USA.
248

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253

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339
340

341 **TABLES**

342

343 **Table 1.** Physiological characteristics of newly described *Curtobacterium glycinis*, *C.*
344 *gossypii* and *C. oryzae* species and related *Curtobacterium* type strains. All strains were
345 positive for the assimilation of dextrin, D-fructose, D-galactose, α-D-glucose, D-mannose
346 and sucrose (data not shown).

	***C. gossypii* VK105**	***C. oryzae* OG107**	***C. glycinis* SS108**	***C. citreum* JCM 1345^T**	***C. pusillum* JCM 1350^T**	***C. luteum* JCM 1480^T**	***C. albidum* NBRC 15078^T**	***C. herbarum* DSM 14013^T**
General characteristics:								
Colony pigment		light yellow	ivory	yellow	yellow	yellow	ivory	orange
Motility	-	w	w	+	+	+	-	+
Oxidase	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Urease	-	-	-	-	-	-	-	nr
Growth with 4% NaCl	+	+	+	nr	nr	nr	nr	nr
Growth with 8% NaCl	+	-	-	nr	nr	nr	nr	nr
Utilization of:								
N-Acetyl-D-Glucosamine	+	+	+	+	-	+	w	nr
N-Acetyl-D-Galactosamine	-	+	+	-	-	-	-	nr
N-Acetyl Neuraminic Acid	-	+	-	nr	nr	nr	nr	nr
D-Arabitol	+	+	+	-	w	-	-	nr
D-Cellobiose	+	+	+	+	-	+	+	nr
L-Fucose	w	+	+	+	-	+	-	nr
Gentiobiose	+	+	+	-	+	-	+	nr
myo-Inositol	+	+	+	-	-	-	-	nr
α-D-Lactose	+	+	+	+	-	+	+	nr
D-Mannitol	+	+	+	-	+	-	w	+
D-Melibiose	+	+	+	+	+	+	w	+
β-Methyl-D-Glucoside	+	+	+	-	+	-	w	nr

D-Raffinose	+	+	+	-	-	-	+	nr
D-Sorbitol	w	+	-	-	+	-	+	+
Turanose	+	+	+	-	+	-	+	nr
D-Gluconic Acid	+	+	+	-	+	-	+	nr
D-Glucuronic Acid	+	+	+	-	-	-	-	nr
L-Alanine	+	+	+	-	-	-	w	nr
L-Glutamic Acid	+	+	w	+	-	-	-	nr
L-Rhamnose	w	+	+	-	-	-	-	+
D-Glucose-6-PO4	-	+	+	-	-	-	-	nr
Acetic Acid	w	+	-	-	-	-	-	nr

+, Positive; w, Weakly positive; -, Negative; nr, Not reported.

Data from this study, Aizawa et al. (2007) and Behrendt et al. (2002).

352

353 **FIGURES**

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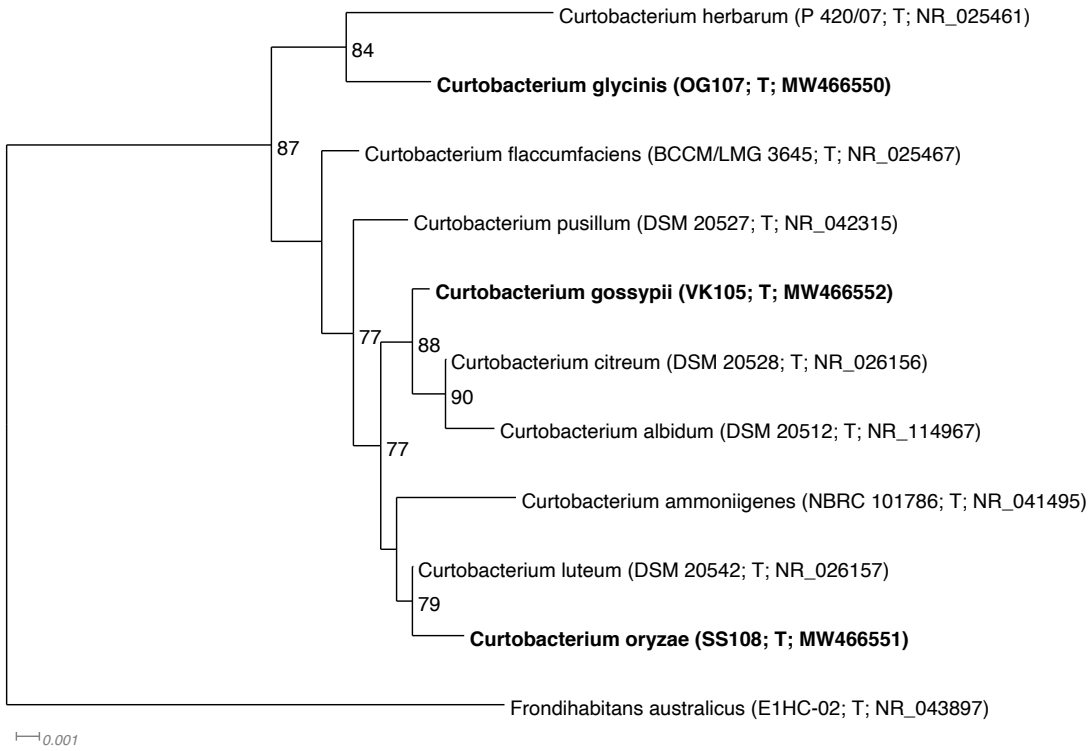

356

357

358 **Figure 1.** Phylogenetic 16S rRNA gene tree of *Curtobacterium glycinis* sp. nov.
359 strain OG107, *Curtobacterium gossypii* sp. nov. strain VK105, *Curtobacterium oryzae* sp.
360 nov. strain SS108 and relatives generated using FastTree (Price et al. 2010). All validly
361 published *Curtobacterium* species were included in the tree, the tree is rooted with
362 *Frondihabitans australicus*. New species are in bold. Strain identifiers and GenBank
363 accession numbers follow species names, T stands for 'type'. Support values above 70%
364 are given by the branches. *Curtobacterium glycinis* is most closely related to *C. herbarum*
365 with 84% support, *Curtobacterium gossypii* to *C. albidum* and *C. citreum* with 82%
366 support and *Curtobacterium oryzae* to *C. luteum* with 79% support. Branch lengths are
367 proportional to the changes along the branches, a scale bar is provided.

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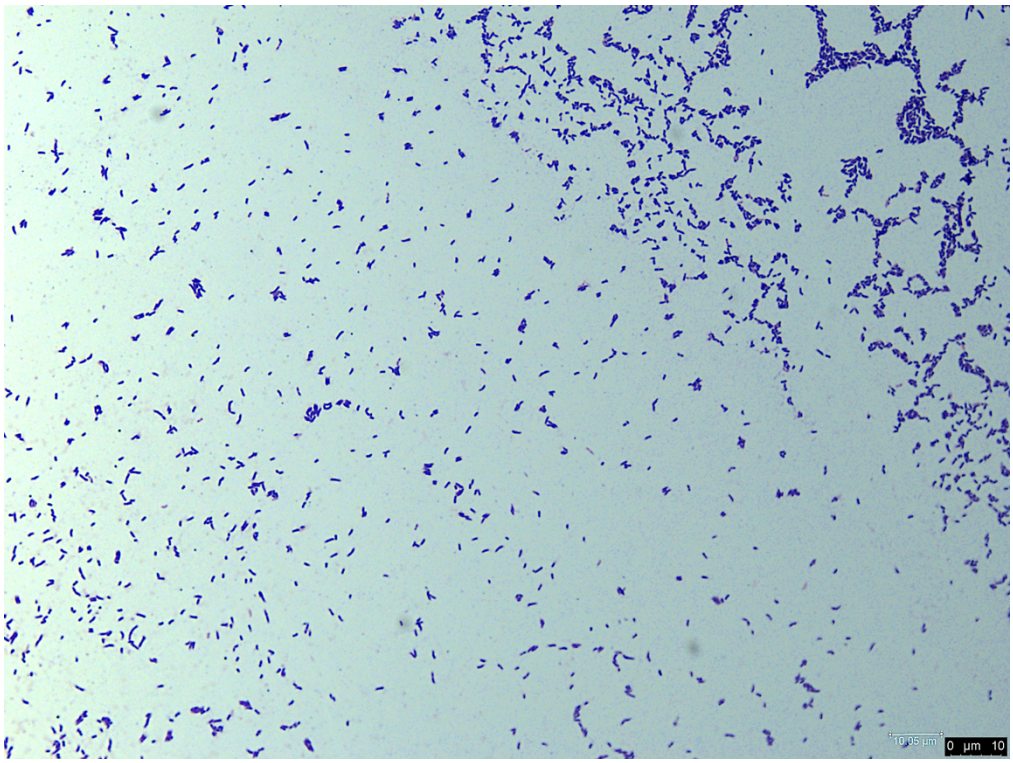


Figure 2. Morphology of *Curtobacterium glycinis* sp. nov. strain OG107 depicted following Gram stain using bright field microscopy. Bar = 10 µm.

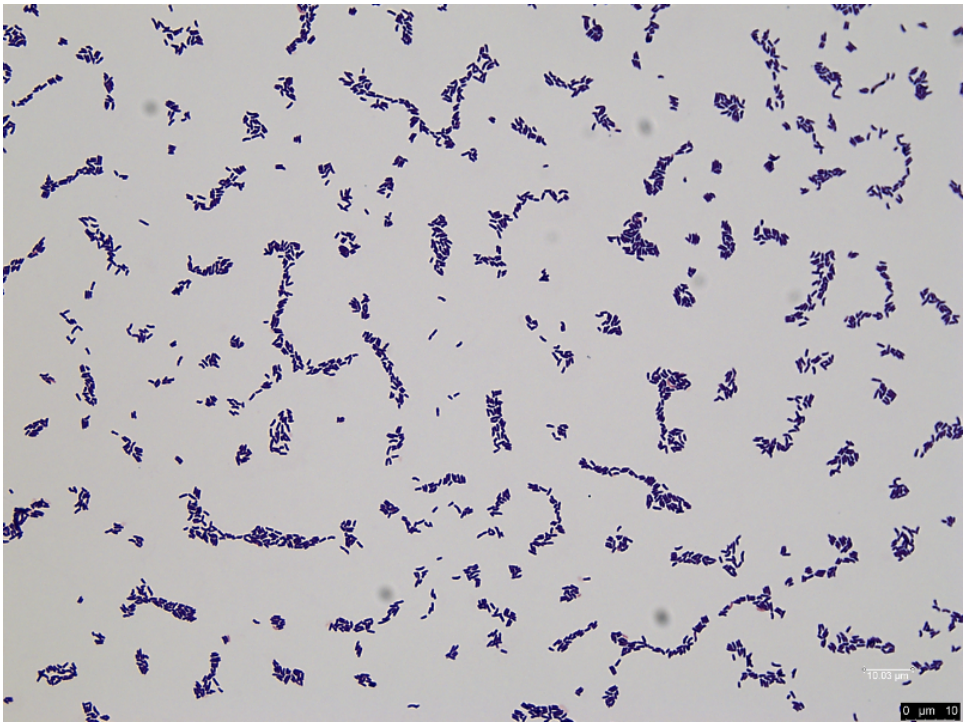
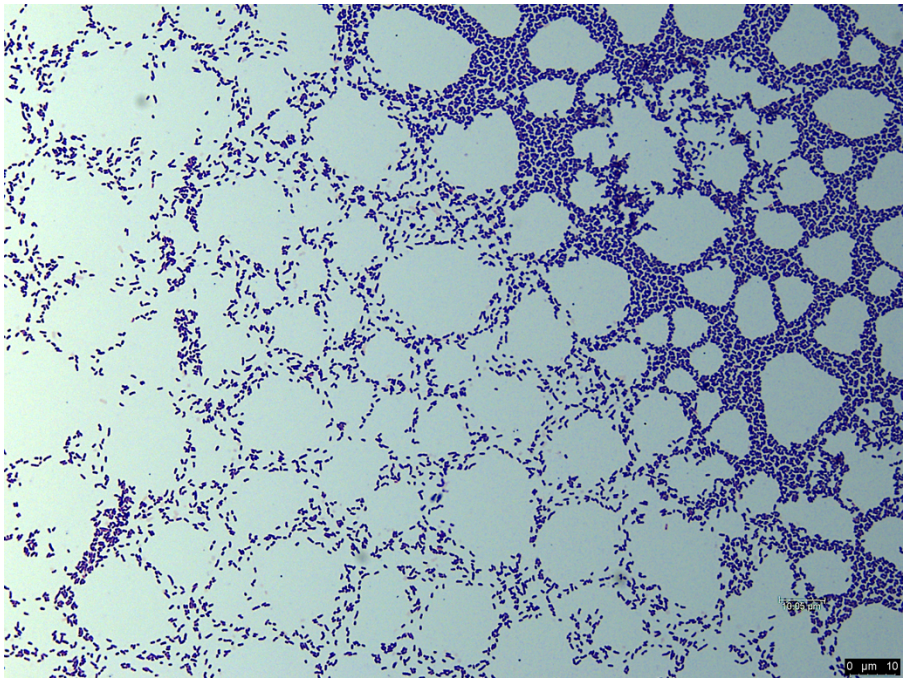


Figure 3. Morphology of *Curtobacterium gossypii* sp. nov. strain VK105 depicted following Gram stain using bright field microscopy. Bar = 10 µm.

392 

393

394 **Figure 4.** Morphology of *Curtobacterium oryzae* sp. nov. strain SS108 depicted following

395 Gram stain using bright field microscopy. Bar = 10 µm.