

1 **QUORUM SENSING IN RHIZOBIA ISOLATED FROM THE SPORES OF THE MYCORRHIZAL**
2 **SYMBIONT *RHIZOPHAGUS INTRARADICES***

3
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11 RUNNING HEAD: Quorum sensing in AMF sporosphere rhizobia

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17 ABSTRACT

18 Most beneficial services provided by arbuscular mycorrhizal fungi (AMF), encompassing improved crop performance
19 and soil resource availability, are mediated by AMF-associated bacteria, showing key plant growth promoting (PGP)
20 traits, *i.e.* the production of indole acetic acid, siderophores and antibiotics, and activities increasing the availability of
21 plant nutrients by nitrogen fixation and phosphate mobilization. Such functions may be affected by the ability of AMF-
22 associated bacteria to communicate through the production and secretion of extracellular small diffusible chemical
23 signals, N-acyl homoserine lactone signal molecules (AHLs), that regulate bacterial behaviour at the community level
24 (quorum sensing, QS). This work investigated the occurrence and extent of QS among rhizobia isolated from AMF spores,
25 using two different QS reporter strains, *Agrobacterium tumefaciens* NTL4 pZRL4 and *Chromobacterium violaceum*
26 CV026. We also assessed the quorum quenching (QQ) activity among *Bacillus* isolated from the same AMF spores. Most
27 rhizobia were found to be quorum-signalling positive, including six isolates producing very high levels of AHLs. The
28 results were confirmed by microtiter plate assay, which detected 65% of the tested bacteria as medium/high AHL
29 producers. A 16S rDNA sequence analysis grouped the rhizobia into two clusters, consistent with the QS phenotype.
30 None of the tested bacteria showed QQ activity able to disrupt the QS signalling, suggesting the absence of antagonism
31 among bacteria living in AMF sporosphere. Our results provide the first evidence of the ability of AMF-associated

32 rhizobia to communicate through QS, suggesting further studies on the potential importance of such a behaviour in
33 association with key-plant growth-promoting functions.

34

35 **KEYWORDS:** arbuscular mycorrhizal fungi; mycorrhizospheric bacteria; *Sinorhizobium meliloti*; N-acyl homoserine
36 lactones production; quorum quenching; reporter strains.

37

38 INTRODUCTION

39 The agroecosystem services and beneficial activities provided by arbuscular mycorrhizal (AM) fungi (AMF) can be
40 mediated by a third component of the symbiosis, the microbiota living intimately associated with fungal structures, such
41 as spores, sporocarps and extraradical hyphae (Azcon-Aguilar and Barea 2015; Barea et al. 2002; Roupael et al. 2015).
42 Several molecular studies, utilizing PCR-denaturing gradient gel electrophoresis (DGGE), have revealed the complexity
43 and diversity of bacterial communities associated with the spores of different AM fungal species (Long et al. 2008; Roesti
44 et al. 2005). PCR-DGGE analyses have also revealed the occurrence of specific and diverse microbial communities tightly
45 associated with the spores of six different AMF isolates, consisting of bacteria belonging to Actinomycetales, Bacillales,
46 Rhizobiales, Pseudomonadales, Burkholderiales, and endobacteria related to the Mollicutes (Mre) (Agnolucci et al.
47 2015). Other studies, focused on the isolation and characterization of AMF-associated microorganisms, have reported the
48 occurrence of dense and active bacterial communities able to promote mycorrhizal establishment and biological control
49 of soilborne pathogens, fix nitrogen, and provide nutrients and growth factors (Alonso et al. 2008; Azcon-Aguilar and
50 Barea 1996, 2015; Barea et al. 2002). Recent studies have reported the isolation of bacteria associated with the spores of
51 the AMF species *Rhizophagus intraradices*, showing multiple functional Plant Growth Promoting (PGP) traits, such as
52 siderophore and indole acetic acid (IAA) production, phosphorus (P) solubilization from inorganic and organic sources
53 and nitrogen fixation (Battini et al. 2016b) and producing large increases in the uptake and translocation of P from the
54 soil to the host plant (Battini et al. 2017). Some of them were able to improve the biosynthesis of plant health-promoting
55 secondary metabolites and to affect the expression levels of transcripts encoding for key enzymes involved in their
56 biosynthetic pathways (Battini et al. 2016a).

57 So far, nothing is known about the ability of AMF-associated bacteria belonging to the same species to
58 communicate with one another through the production and secretion of extracellular small diffusible chemical signals,
59 called autoinducers, that regulate their behaviour at the community level, *i.e.* quorum sensing (QS) (Fuqua et al. 1994).
60 Such signalling molecules are represented by N-acyl homoserine lactones (AHLs) in Gram-negative bacteria, by a family
61 of small oligopeptides in Gram-positive bacteria and by other molecules (autoinducer-2) in both Gram-positive and
62 negative bacteria (Miller and Bassler 2001). QS can control the expression of many genes responsible for different

63 bacterial activities and functional traits, such as swarming and motility, biofilm formation, bioluminescence, plasmid
64 conjugal transfer and virulence, metabolite production, as well as the production of antibiotics, siderophores and
65 exoenzymes, and symbiotic interactions (Hartmann and Schikora 2012). As AMF-associated bacteria are able to carry
66 out many of the described activities, some of which are necessary for the optimal performance of mycorrhizal plants,
67 investigations able to reveal their QS phenotype are important and highly relevant.

68 Studies on the diversity of AHL-producing bacteria have focused on many diverse habitats, but only a few have
69 investigated the occurrence and diversity of AHL-producing bacteria in the soil environment and in particular in the
70 rhizosphere (Chan et al. 2011). So far, the details of QS processes taking place in the diverse bacterial species and strains
71 isolated from the mycorrhizosphere, and in particular from AMF spores, remain to be unravelled.

72 The aim of the present study was to assess the occurrence and extent of QS among bacteria living associated
73 with AMF spores. To this aim, we screened 28 Gram-negative bacteria previously isolated from the spores of *Rhizophagus*
74 *intraradices* IMA6, for AHL production, using two different QS reporter strains, *Agrobacterium tumefaciens* NTL4
75 pZRL4 (sensitive to medium, long chain AHLs) and *Chromobacterium violaceum* CV026 (sensitive to short chain AHLs).
76 In addition, using the latter reporter, we evaluated the ability of 9 Gram-positive bacteria isolated from the same ecological
77 niche, to interfere with QS, a process generally described as ‘quorum quenching’ (QQ), by assessing their ability to
78 degrade AHLs.

79

80 MATERIALS AND METHODS

81 *Bacteria*

82 The bacteria utilized in this work were previously isolated from the *Rhizophagus intraradices* IMA6 sporosphere (Battini
83 et al. 2016b) and maintained in the collection of the Microbiology Labs of the Department of Agricultural, Food and
84 Environment, University of Pisa, Italy (International Microbial Archives, IMA). Among the heterotrophic isolates
85 showing the mucoid morphotype and originating from TSA medium (tryptic soy agar) 28 Gram-negative bacteria were
86 screened for AHL production. For AHL degradation, 9 Gram-positive bacteria morphologically ascribed to Bacillaceae
87 were selected. All the bacterial isolates were molecularly analysed by 16S rRNA gene sequencing (Supplementary
88 material, SM, Tables 1 and 2), except four of them (TSA3, TSA26, TSA41, TSA50), which had been previously identified
89 (Battini et al. 2016b).

90

91 *Extraction of total DNA, PCR analysis and sequencing*

92 Genomic DNA was extracted from bacterial liquid cultures grown overnight at 28 °C using “MasterPure™ Yeast DNA
93 Purification Kit” (Epicentre®) following the manufacturer’s protocols. Bacterial isolates were identified based on 16S
94 rDNA sequencing, as reported by Battini et al. (2016b).

95

96 *Nucleotide sequence accession numbers*

97 The sequences of 16S rRNA genes were submitted to the European Nucleotide Archive (ENA) under the accession
98 numbers from LT984816 to LT984840 and from LT984844 to LT984851.

99

100 *Screening for AHL-producing bacteria*

101 The 28 Gram-negative bacteria were screened for AHL production using the AHL reporter strains *Agrobacterium*
102 *tumefaciens* NTL4 pZRL4 and *Chromobacterium violaceum* CV026 by microtiter plate assays (McClellan et al. 1997;
103 Shaw et al. 1997; Trovato et al. 2014). Bacteria were grown at 28 °C overnight with continuous shaking (120 rpm) on LB
104 broth until the exponential growth phase was reached and then centrifuged at 7,500 rpm for 10 min. A volume of 1 mL
105 of supernatant was transferred to a 1.5 mL Eppendorf tube and stored at -20 °C. Two mL of *A. tumefaciens* NTL4
106 preculture were inoculated in a 50 mL tube containing 18 mL of AB liquid medium (3 g/L K₂HPO₄, 1 g/L NaH₂PO₄, 1
107 g/L NH₄Cl, 0.3 g/L MgSO₄, 0.15 g/L KCl, 0.01 g/L CaCl₂, 2.5 mg/L FeSO₄, 0.5% glucose) supplemented with gentamycin
108 (30 µg/mL) and incubated at 28 °C with continuous shaking (120 rpm) for 24 h. A volume of 16.75 mL of bacterial
109 culture was mixed with AB agar (33.25 mL, 0.7% Agarose I; Euroclone) containing 5-Bromo-4-chloro-3-indolyl b-d-
110 galactopyranoside (X-Gal; 20 mg/mL) and gentamycin (30 µg/mL) previously melted and cooled at 43 °C. Aliquots of
111 200 µL of *A. tumefaciens*/AB agar mixture were poured in each of the wells of a sterile 96-well microtiter plate (Cellstar,
112 Greiner bio-one, Kremsmuenster, Austria). Upon solidification, 10 µL of overnight culture supernatant grown and
113 harvested as described above, were dispensed over the agar in the wells and incubated for 24 h at 30 °C. Negative control
114 wells contained 10 µL of sterile LB growth medium, while the medium amended with 10 µL of a 10 ng/µL solution of
115 N-octanoyl-L-homoserine lactone (OHL; Fluka Chemie GmbH Buchs, Switzerland) and subsequent fivefold stepwise
116 dilutions, was used as positive control. Bacteria able to produce AHL could be identified by the activation of the reporter
117 strains through blue coloration. Digital images of the results were acquired directly on an Epson Perfection 1240U flatbed
118 digital scanner. The bacterial isolates were further screened for the production of AHL by plate assay on TSA agar using
119 the bioreporter strain *C. violaceum* CV026. Briefly, the isolates were streaked against the reporter strain in a perpendicular
120 manner, incubated at 28 °C for 24 h and observed for violet colouration due to the induction of violacein pigment in the
121 reporter strain. *C. violaceum* CV026 streaked against itself was used as a negative control. Isolates testing positive to the

122 qualitative tests were subsequently analyzed for semi-quantitative AHL production by the same microtiter assay upon
123 performing serial 10:1 serial dilutions of the supernatants.

124

125 *AHL degradation assay*

126 The QQ activity was assessed in solid plate assays carried out with the AHL biosensor *C. violaceum* CV026 (McClean et
127 al. 1997; Romero et al. 2011). The 9 bacterial isolates were grown in 1 mL of TSA at 28 °C and 200 rpm for 24 h. A
128 volume of 40 µL of a stock solution (50 µg/mL) of N-octanoyl-L-homoserine lactone (OHL; Fluka Chemie GmbH Buchs,
129 Switzerland) were added to achieve a final concentration of 2 µg/mL, and incubated for further 24 h. In order to detect
130 the inhibition of OHL activity, 50 µL of the supernatants were spotted in wells made in TSA plates overlaid with 5 mL
131 of a 1:100 dilution of an overnight culture of *C. violaceum* CV026 in soft TSA (0.8% agar). Sterile water, sterile TSA
132 growth medium and OHL were used as negative controls. The formation of halo zones around wells indicated the capacity
133 of the bacteria to degrade OHLs after 24 h, eliminating the violacein production.

134

135 RESULTS

136 *Identification of bacteria*

137 Among the 37 bacteria analysed, 33 of them were 16S-sequenced and affiliated to genus and species using BLAST,
138 together with the four bacteria previously sequenced (Battini et al. 2016b). The 25 Gram-negative bacteria belonged to
139 *Sinorhizobium meliloti* (Table S1), while the 8 Gram-positive ones were affiliated to *Bacillus* and *Fictibacillus* spp. (Table
140 S2).

141

142 *Screening for AHL-producing bacteria (Quorum sensing)*

143 Most of the isolates were found to be quorum-sensing positive in one or both AHL assays, but they showed different
144 response patterns to the two AHL reporter strains. Specifically, *A. tumefaciens* NTL4 detected 23 out of 28 bacteria (82%)
145 as AHL producers, while *C. violaceum* CV026 detected 14 out of 28 bacteria (50%) as AHL producers. The bacteria
146 unable to produce AHL (*S. meliloti* TSA 22, 95, 99, 107, 137), as revealed by both reporter strains, were not further
147 investigated. According to the colour pattern observed in the semi-quantitative bioassay performed with *A. tumefaciens*
148 NTL4, 15 out of 23 (65%) and 8 out of 23 (35%) of the tested bacteria showed medium/high and low production of signal
149 molecules, respectively (Table 1). The semi-quantitative test did not reveal short signal molecules for 9 (39%) of the
150 rhizobia tested, consistently with the qualitative assay (Table 1). It is interesting to note that 16S rDNA sequence analysis
151 detected two clusters, corresponding with the level of AHL production (high versus low producers). Accordingly, the
152 non-short chain AHL producing strains, as assessed by *C. violaceum* CV026, grouped together in the second cluster (Fig.

153 1). The sequences of the tested bacteria were aligned, revealing two base changes, C→T at positions 1026 and 1146,
154 referring to the 16S rRNA gene of *Escherichia coli*.

155

156 *AHL degradation assay (Quorum quenching)*

157 The reporter strain AHL biosensor *C. violaceum* CV026, producing violacein in response to the presence of short-chain
158 AHLs, revealed that none of the tested Gram-positive bacteria was capable of interfering with AHL activity in the plate
159 bioassay.

160

161 DISCUSSION

162 This work provides the first evidence of the ability of rhizobia living associated with AMF spores to communicate through
163 the production of extracellular small diffusible signalling molecules, able to regulate their behaviour at the community
164 level, as most of the tested isolates showed the quorum sensing phenotype.

165 The 28 tested bacteria utilized in this work, previously isolated from AMF spores, belonged to *S. meliloti*. Their
166 occurrence in association with AMF may be ascribed to their ability to produce exopolysaccharides and to form biofilms,
167 thus allowing an efficient colonization of roots and mycorrhizal hyphae (Toljander et al. 2006). It is important to note
168 that, beyond biological nitrogen fixation, rhizobia produce phytohormones, improving plant nutritional status and
169 biocontrolling phytopathogens (Chandra et al. 2007). In particular, some of the *S. meliloti* isolates tested here - TSA3,
170 TSA26 and TSA41 - show multiple functional PGP traits, such as siderophore and IAA production, P solubilization and
171 phytate mineralization (Battini et al. 2016b).

172 A high percentage of isolates showed the QS phenotype (82%) and 6 produced very high levels of the signal
173 molecules AHLs, revealing the large extent of the phenomenon within the rhizobia isolated from AMF spores. Our data
174 on the abundance of AHL-producing bacteria are higher than those reported by previous studies performed on bacteria
175 isolated from the rhizosphere of wheat (8%), tomato (12%), tobacco (ca. 20%) and ginger (12%) (Chan et al. 2011;
176 D'Angelo-Picard et al. 2004; Pierson et al. 1998; Steidle et al. 2001). This can be explained taking into account that the
177 rhizobia tested here were isolated from a peculiar and specialised ecological niche - AMF spores - where the QS phenotype
178 may represent an important factor for their establishment and maintenance. Moreover, such isolates belong to a species,
179 *S. meliloti*, that has been reported to produce seven compounds with N-acyl-L-homoserine lactone signalling activity
180 (Cha et al. 1998).

181 Here, we report for the first time that the bacteria showing different QS activity differed in their 16S rDNA
182 sequences, a trait to be further investigated in order to develop a possible molecular marker for the rapid identification of
183 high AHL-producers.

184 The potential importance of the QS phenotype in the modulation of key PGP functions may be suggested by the
185 P solubilisation and phytate mineralization ability of some of the tested strains, one of which, *S. meliloti* TSA26, improved
186 root P content in maize plants (Battini et al. 2017). Indeed, diverse rhizobial strains have been reported to secrete organic
187 anions chelating cations bound to phosphate and to produce phytase/phosphatase enzymes (Owen et al. 2015).

188 Here, none of the tested Gram-positive bacteria was capable of interfering with AHL activity, i.e. of disrupting
189 the QS signalling, suggesting the absence of antagonism towards the rhizobia living in the same ecological niche, the
190 AMF sporosphere. This is an interesting finding, as the bacteria tested belonged to the species *Bacillus*, widely studied
191 for its efficient QQ activity (Grandclément et al. 2015); actually, the QQ trait is a strain characteristic and it is feasible
192 that the coexistence of bacteria showing QS and QQ activities in the same niche, entails the exclusion of competitive
193 strains, in favour of commensal/neutral ones.

194 In conclusion, our work provides the first evidence of AHL production in rhizobia living associated with AMF
195 spores. As the secretion of such compounds, able to regulate the QS behaviour, occurred in most of the bacteria analyzed,
196 we propose that it may represent an important mechanism allowing them to become established in the mycorrhizosphere,
197 where they may be functionally complementary to AMF, in the promotion of plant nutrition and health. Further
198 investigations will reveal whether the bacteria producing the highest AHL levels show also the best functional traits, in
199 order to select the appropriate AMF/bacteria consortia to be utilized in sustainable and innovative food production
200 systems, where soil biological fertility and natural biogeochemical cycles are protected and maintained.

201

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205

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282

283 TABLES

284 **Table 1** Semi-quantitative AHL production in rhizobia isolated from *Rhizopagus intraradices* spores, as assessed by
285 the microtiter assay upon performing 10:1 serial dilutions of the supernatants, using the reporter strains
286 *Agrobacterium tumefaciens* NTL4 and *C. violaceum* (CV026).

Isolates	Reporter strain	
	NTL4	CV026
<i>S. meliloti</i> TSA1	++++	++
<i>S. meliloti</i> TSA3	+++	++
<i>S. meliloti</i> TSA6	+++	++
<i>S. meliloti</i> TSA10	++++	+/-
<i>S. meliloti</i> TSA11	+++	+/-
<i>S. meliloti</i> TSA24	++++	++
<i>S. meliloti</i> TSA26	+++	++
<i>S. meliloti</i> TSA27	+++	++
<i>S. meliloti</i> TSA28	++++	++
<i>S. meliloti</i> TSA29	+++	++
<i>S. meliloti</i> TSA41	+++	+
<i>S. meliloti</i> TSA42	+++	+/-
<i>S. meliloti</i> TSA45	++++	++
<i>S. meliloti</i> TSA91	+	-
<i>S. meliloti</i> TSA94	+	-
<i>S. meliloti</i> TSA96	+	-
<i>S. meliloti</i> TSA98	+	-
<i>S. meliloti</i> TSA100	+	-
<i>S. meliloti</i> TSA101	+	-
<i>S. meliloti</i> TSA102	++	-
<i>S. meliloti</i> TSA105	+	-
<i>S. meliloti</i> TSA106	+	-
<i>S. meliloti</i> TSA139	++++	++

287

288 NTL4: *A. tumefaciens* NTL4 (pZLR4); CV026: *C. violaceum* CV026.

289 - no production; +/- = scarce production; + = low production; ++ = medium production; +++/++++ = high production.

290

291 FIGURE LEGEND

292 **Fig. 1** Affiliation of the sequences of the bacteria isolated from spores of *Rhizophagus intraradices* IMA6 with the existing
293 16S rRNA gene sequences, using Neighbor-Joining method based on the kimura 2-parameter method. Bootstrap
294 (1000 replicates) values below 50 are not shown. Evolutionary analyses were conducted in MEGA6. The DNA
295 sequences retrieved in this work are indicated by their isolate code and accession numbers.
296

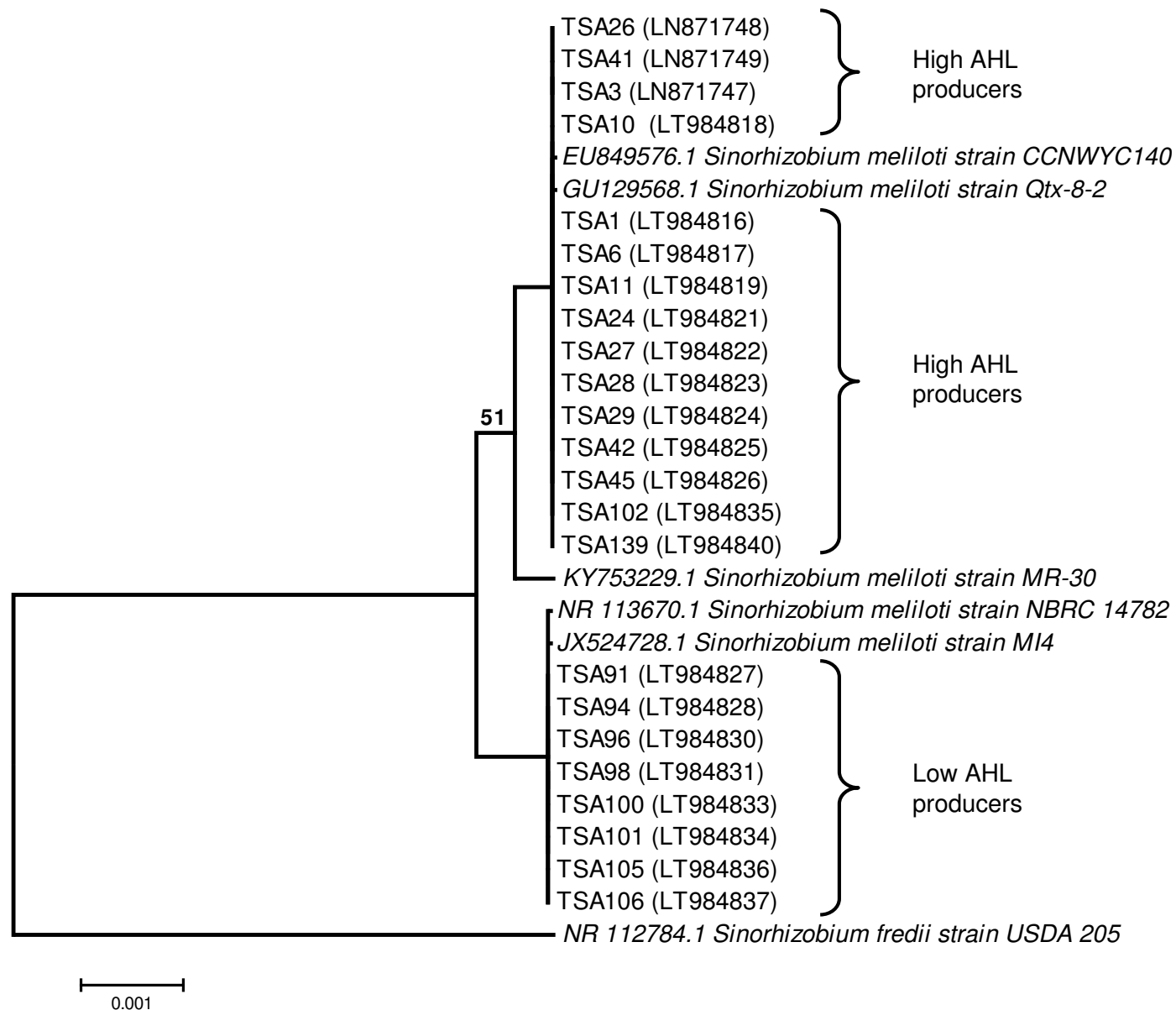


Fig. 1