Promicromonospora kermanensis sp. nov., a new *actinobacterium* isolated from soil

Fatemeh Mohammadipanah[1*], Maria del Carmen Montero-Calasanz[2,3*], Peter Schumann[2], Cathrin Spröer[2], Manfred Rohde[4] and Hans-Peter Klenk[2,3]

[1] Microbial Biotechnology Department, School of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, 14155-6455, Tehran, Iran

[2]Leibniz-Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Inhoffenstrasse 7b, 38124 Braunschweig, Germany

[3]School of Biology, Newcastle University, Ridley Building, Newcastle upon Tyne, NE1 7RU, United Kingdom

[4] Helmholtz Centre for Infection Research, Central Facility for Microscopy, Inhoffenstrasse 7, 38124 Braunschweig, Germany

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***Corresponding authors:**

Fatemeh Mohammadipanah, Tel.: +98-21-61113556; Fax: +98-21-66415081, e-mail: fmohammadipanah@ut.ac.ir, María del Carmen Montero-Calasanz, Tel.: +44 (0)191 20 84 700, e-mail: maria.montero-calasanz@ncl.ac.uk

The INSDC accession number for the 16S rRNA gene sequence of strain HM 533^T = DSM 45485^T = UTMC 00533^T = CECT 8709^T is KJ780745.

28 **Abstract**

29 A novel *Promicromonospora* strain, designated HM 533^{T}, was isolated from soil in Kerman
30 Province, Iran. It produced long and branched hyphae on ISP 2 medium that developed into
31 a large number of irregular shaped spores. It showed optimal growth at 25-30 °C and pH
32 5.0-8.0 with 0-4% (w/v) NaCl. The peptidoglycan type of strain HM 533^{T} is A4α L-Lys –
33 L-Ala – D-Glu. Whole cell hydrolyzates of strain HM 533^{T} contained the sugars ribose,
34 glucose and galactose. The main phospholipids were phosphatidylglycerol,
35 diphosphatidylglycerol, phosphatidylinositol, two unknown glycolipids and an unknown
36 phospholipid. MK-9(H_4) and MK-9(H_2) were the predominant menaquinones. The fatty-
37 acid pattern was mainly composed of the saturated branched-chain acids anteiso-$C_{15:0}$ and
38 iso-$C_{15:0}$. The 16S rRNA gene sequence analysis showed the highest pairwise sequence
39 identity (99.5-97.1 %) with the members of the genus *Promicromonospora*. Based on
40 phenotypic and genotypic features, strain HM 533^{T} was considered to represent a novel
41 species of the genus *Promicromonospora*, for which the name *Promicromonospora*
42 *kermanensis* is proposed with strain HM 533^{T} (DSM 45485^{T} = UTMC 00533^{T} = CECT
43 8709^{T}) as the type strain.

44

45 The family *Promicromonosporaceae* (Stackebrandt *et al.,* 1997, emend. Zhi *et al*., 2009)
46 comprises seven genera including *Cellulosimicrobium* (Schumann *et al*., 2001, emend
47 Brown *et al.,* 2006, emend. Yoon *et al*., 2007), *Isoptericola* (Stackebrandt *et al*., 2004),
48 *Myceligenerans* (Cui *et al.,* 2004, emend. Wang *et al*., 2011), *Xiangella* (Wang *et al.,* 2013),
49 *Xylanibacterium* (Rivas *et al.,* 2004), *Xylanimicrobium* (Stackebrandt & Schumann, 2004),
50 *Xylanimonas* (Rivas *et al.,* 2003) and *Promicromonospora* (Krasil'nikov *et al.,* 1961) as the
51 type genus. At current, the genus *Promicromonospora* includes 13 species (Euzeby, 2016)

52 with validly published names isolated from soil, sea sediment, air and insect cuticle
53 samples, and *Promicromonospora citrea* (Krasil'nikov *et al.*, 1961) as the type species.
54 Other two species *P. enterophila* and *P. pachnodae* were reclassified as *Oerskovia*
55 *enterophila* (Jáger *et al*., 1983; Stackebrandt *et al*., 2002, comb. nov.) and *Xylanimicrobium*
56 *pachnodae* (Cazemier *et al.,* 2003; Stackebrandt & Schumann, 2004, comb. nov.),
57 respectively. The members of the genus are characterized by the production of substrate
58 mycelium that fragments into rod or coccoid elements, iso- and anteiso-branched cellular
59 fatty acids, MK-9(H_4) as the major menaquinone, and a DNA G+C content of 70–75 mol %
60 (Schumann & Stackebrandt, 2012).
61 In our screening program on finding non-*Streptomyces* actinobacterial members, strain HM
62 533^T was recognized as a potentially new species in the genus *Promicromonospora*. In this
63 study, we determined the taxonomic position of this genomically distinct novel lineage
64 based on a polyphasic approach.
65
66 Rhizospheric soil samples were collected at ~10 cm depth from Kerman Province (Iran)
67 and then firstly air dried at room temperature (Nolan & Cross, 1988), followed by UV
68 irradiation at 254 nm for 10 min (Galatenko & Trekhova,1990). Strain HM 533^T was
69 isolated using the dilution plating method on Soil Extract agar (Hamaki *et al,*. 2005) after
70 14 days incubation at 28 °C and maintained on ISP (International Streptomyces Project) 2
71 agar slants at 4 °C and 20 % (v/v) glycerol suspensions at -70 °C. Cultural properties of
72 strain HM 533^T were evaluated according to the guidelines of the ISP as described by
73 Shirling & Gottlieb (1966), determining the culture colour by comparing with chips from
74 the ISCC-NBS colour charts (Kelly, 1964). The substrate mycelium characteristics were

75 observed on yeast extract-malt extract agar (ISP 2 medium) after 14 days at 28 ºC using the
76 cover-slip technique (Kawato & Shinobu, 1959). The morphology, ornamentation and
77 surface of the spores were observed using scanning electron microscopy. Samples were
78 fixed with 5% formaldehyde and 2% glutaraldehyde in HEPES buffer, dehydrated with a
79 graded series of acetone, critical-point dried and sputter-coated with gold/palladium.
80 Samples were examined in a Zeiss Merlin at an acceleration voltage of 5 kV with the inlens
81 SE-detector and HE-SE2 detector with 75:25 ratio.
82 Growth rates were determined for temperatures from 10-50 °C at 5 °C increments and for
83 pH values 4-12 (in increments of 1.0 pH units) on modified Bennett medium as described
84 by Williams *et al*. (1989) using Na-sesquicarbonate buffer system for maintaining the
85 alkaline condition. The utilization of carbon sources and acid production were determined
86 using GEN III Microplates in an Omnilog device (BIOLOG Inc., Hayward, CA, USA). The
87 GEN III Microplates were inoculated with a cell suspension made in a "gelling" inoculating
88 fluid (IF) A at a cell density of 98 % transmittance, yielding a running time of 4 days in
89 Phenotype Microarray mode at 28 °C. The exported data were further analyzed using the
90 opm package for R (Vaas *et al.,* 2012, 2013), using its functionality for merging subsequent
91 measurements of the same plate, statistically estimating parameters from the respiration
92 curves such as the maximum height, and automatically "discretizing" these values into
93 negative and positive reactions. Strain HM 533^{T} in comparison with reference strains *P.*
94 *umidemergens* DSM 22081^{T}, *P. alba* DSM 100490^{T}, *P. iranensis* DSM 45554^{T}, *P.*
95 *vindobonensis* DSM 15942^{T} and *P. sukumoe* DSM 44121^{T}, were studied in the GEN III
96 Microplates in two independent determinations. Reactions that gave contradictory results
97 between the two repetitions were regarded as ambiguous.

98 Strain HM 533^T developed a branched substrate mycelium with a wrinkly and twisty
99 surface, whose septate hyphae break, at a later stage, into fragments of various sizes and
100 rod-shape spore elements (Supplementary Fig. S1), a feature shared by all members of the
101 genus *Promicromonospora* (Krasil'nikov *et al.,* 1961; Schumann & Stackebrandt, 2012).
102 Substrate mycelium pigmentations varied slightly depending on the tested media. Strain
103 HM 533^T displayed good growth and a colour between cream and light yellow on ISP 2
104 medium, inorganic salts/starch agar (ISP 4 medium), glycerol/asparagine agar (ISP 5
105 medium) and tyrosine agar (ISP 7 medium). Relatively poor growth without spore
106 production and creamy coloured substrate mycelium was observed on oatmeal agar (ISP 3
107 medium) and peptone-yeast extract iron agar (ISP 6 medium). No diffusible pigments are
108 produced on any medium tested. HM 533^T grew well from 25 ºC to 30 ºC with no growth at
109 45 ºC and a weak growth at 10 ºC – a temperature at which *P. iranensis* could not grow.
110 Strain HM 533^T showed growth in the presence of 0-4 % NaCl and between pH 5-10 but
111 not at pH 4 or pH 11. In comparison to strain HM 533^T the type strain of *P. sukumoe* grew
112 at pH 12 while those of *P. iranensis* and *P. alba* could not grow at pH 5. In contrast to *P.*
113 *iranensis*, strain HM 533^T is not able to tolerate the 8% NaCl. According to the Biolog
114 system, strain HM 533^T oxidises dextrin, D-maltose, D-trehalose, D-cellobiose, β-
115 gentiobiose, sucrose, turanose, α-D-lactose, D-salicin, D-glucose, D-mannose, D-fructose,
116 D-galactose, L-fucose, inosine, D-mannitol, D-arabitol, glycerol, gelatin, L-aspartic acid,
117 L-glutamic acid, D-gluconic acid, D-glucuronic acid, D-saccharic acid, methyl pyruvate, L-
118 lactic acid, L-malic acid, bromo-succinic acid, β-hydroxy-butyric acid, acetoacetic acid,
119 propionic acid, acetic acid, and butyric acid, but not stachyose, D-raffinose, D-melibiose,
120 N-acetyl-D-galactosamine, N-acetyl-neuraminic acid, 3-O-methyl-D-glucose, D-fucose,

121 fusidic acid, D-serine, D-glucose-6-phosphate, D-fructose-6-phosphate, D-aspartic acid,
122 glycine-proline, L-arginine, L-serine, guanidine hydrochloride, L-galactonic acid- γ
123 -lactone, glucuronamide, mucic acid, quinic acid, D-lactic acid methyl ester, citric acid, α-
124 keto-glutaric acid, D-malic acid, potassium tellurite, γ -amino-n-butyric acid and sodium
125 formate. The full phenotype microarrays obtained using OmniLog device in comparison to
126 the type strains of other species of the genus *Promicromonospora* showed the biochemical
127 distinction of strain HM 533^T from related species ((Supplementary Fig. S2). A summary of
128 selected differential phenotypic characteristics is presented in Table 1.
129 For analysis of cell-wall amino acids and whole-cell sugars, polar lipids and respiratory
130 lipoquinones, strain HM 533^T was cultivated in trypticase soy broth (TSB) for up to 6 days
131 in shaking cultures at 200 rpm, pH 7.2 and 28 °C. Cell biomass was harvested by
132 centrifugation and washed twice with distilled water. Whole-cell sugars were prepared
133 according to Lechevalier & Lechevalier (1970), followed by thin layer chromatography
134 (TLC) analysis (Staneck & Roberts, 1974). Polar lipids were extracted, separated by two-
135 dimensional TLC and identified according to procedures outlined by Minnikin *et al.* (1984)
136 with modifications proposed by Kroppenstedt & Goodfellow (2006). Respiratory
137 lipoquinones were extracted from freeze-dried cell material using methanol as described by
138 Collins *et al.* (1977) and analysed by high-performance liquid chromatography (HPLC)
139 (*Kroppenstedt*, 1982). The isolation of the peptidoglycan and elucidation of its structure
140 were carried out according to published protocols (Schumann, 2011). A purified
141 peptidoglycan preparation was obtained after disruption of cells by shaking with glass
142 beads and subsequent trypsin digestion. The amino acids and peptides in cell wall
143 hydrolysates were analyzed by two-dimensional ascending thin-layer chromatography on

144 cellulose plates. The molar ratios of amino acids were determined by gas chromatography
145 and gas chromatography-mass spectrometry of *N*-heptafluorobutyryl amino acid isobutyl
146 esters. The amino-terminal amino acid of the interpeptide bridge was detected by
147 dinitrophenylation. The extraction of cellular fatty acids was carried out in duplicate from
148 biomass grown on TSA tubes held at 28 °C for 4 days. Analysis was conducted using the
149 Microbial Identification System (MIDI) Sherlock Version 6.1 (results evaluated against the
150 TSBA40 peak naming table database) as described by Sasser (1990).

151 The peptidoglycan of strain HM 533^T contains muramic acid and the amino acids lysine
152 (Lys), glutamic acid (Glu) and alanine (Ala) in the molar ratios 0.7:1.0:2.0:2.0, respectively.
153 The partial hydrolysate (4N HCl, 0.75 h, 100 °C) contained in addition to these amino acids
154 the peptides L-Ala – D- Glu, L-Ala – L-Lys, L-Lys – D-Ala and L-Ala – L-Lys – D-Ala.
155 Dinitrophenylation revealed that Glu represents the *N*-terminus of the interpeptide bridge.
156 Dinitrophenylated Ala was detected in lower amounts. From these data it was concluded
157 that strain HM 533^T shows the peptidoglycan type A4α (Schleifer & Kandler, 1972) L-Lys
158 – L-Ala – D-Glu (type A11.59 according to Schumann, 2011). Whole-cell sugar analysis
159 revealed ribose, glucose and galactose. HM 533^T contained primarily menaquinones MK-
160 9(H_4) (53 %) and MK-9(H_2) (33.5 %). The polar lipids profile consisted of
161 phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, an unknown
162 phospholipid and two unknown glycolipids (Supplementary Fig. S3). Major fatty acids
163 were the saturated branched-chain acids anteiso-$C_{15:0}$ (40.7 ± 0.5 %), iso-$C_{15:0}$ (27.0 ± 0.3
164 %), in agreement with the predominant fatty acids found in other members of the genus
165 *Promicromonospora* (Qin *et al*., 2012), complemented by anteiso-$C_{15:1}$ A (11.9 ± 0.3 %),
166 anteiso-$C_{17:0}$ (6.6 ± 0.1 %), and iso-$C_{16:0}$ (5.2 ± 0.1 %). The qualitative and quantitative

167 combination of fatty acids is diagnostic for species of the genus *Promicromonospora*, fatty
168 acid pattern 2c *sensu* (Kroppenstedt, 1985).

169

170 Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and
171 purification of the PCR product was carried out as described by Rainey *et al.* (1996).
172 Phylogenetic analyses and the rooting of the resulting trees were conducted as previously
173 described (Montero-Calasanz *et al.,* 2013) using the DSMZ phylogenomics pipeline
174 (Meier-Kolthoff *et al*., 2014) adapted to single genes integrated in the GGDC web server
175 (Meier-Kolthoff *et al*., 2013a) available at http://ggdc.dsmz.de/. Pairwise similarities were
176 calculated as recommended by Meier-Kolthoff *et al.* (2013b) for the 16S rRNA gene
177 available via the GGDC web server. For DNA-DNA hybridization tests cells were disrupted
178 by using a Constant Systems TS 0.75 KW (IUL Instruments, Germany). DNA in the crude
179 lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al*.
180 (1977). DNA-DNA hybridization was carried out as described by De Ley *et al*. (1970)
181 under consideration of the modifications described by Huss *et al*. (1983) using a model
182 Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6
183 multicell changer and a temperature controller with *in-situ* temperature probe (Varian, Inc).
184 The almost complete (1507 bp) 16S rRNA gene sequence of strain HM 533^{T} was
185 determined. The 16S rRNA gene sequence showed the highest similarity with the members
186 of the genus *Promicromonospora,* especially with the type strains of *P. alba* (99.5 %), *P.*
187 *umidemergens* (99.0 %), *P. iranensis* (98.7 %), *P. vindobonensis* (98.6 %), *P. sukumoe* (98.4
188 %), *P. aerolata* (98.3 %), *P. kroppenstedtii* (98.2 %) and *P. xylanilytica* (98.2 %). In
189 addition, the type strains of all members of *Promicromonospora* were placed within the

190 same phylogenetic group showing the maximum support by both maximum likelihood and
191 maximum-parsimony estimations (Fig. 1). The 16S rRNA gene sequences analysis strongly
192 supported the assignment of strain HM 533^{T} to the genus *Promicromonospora*. However,
193 16S rRNA gene sequence similarity between HM 533^{T} and closely related type strains
194 indicated the need to prove the genomic distinctness of the type strain representing the
195 novel species by DNA-DNA hybridization. Strain HM 533^{T} displayed a DNA-DNA
196 relatedness of 46.3 ± 0.9 % with the type strain of *P. alba*, 46.0 ± 5.6 % with the type strain
197 of *P. umidemergens* and 49.0 ± 0.8 % with *P. iranensis.* DNA-DNA hybridizations of strain
198 HM 533^{T} with the type strains of *P. sukumoe* and *P. kroppenstedtii* were not conducted
199 based on observations reported by Meier-Kolthoff *et al.* (2013b) that statistically confirmed
200 strains of the phylum *Actinobacteria* showing similarities 97.0-99.0 % generally result in
201 DNA-DNA hybridization values below the 70 % threshold recommended by Wayne *et al.*
202 (1987) to confirm the species status of novel strains, .
203 Several phenotypic characteristics apart from the phylogenetic analysis based on 16S rRNA
204 gene sequences support the distinctiveness of strain HM 533^{T} from all other
205 *Promicromonospora* species. Based on the phenotypic and genotypic data presented above,
206 we propose that strain HM 533^{T} represents a novel species within the genus
207 *Promicromonospora* with the name *Promicromonospora kermanensis* sp. nov.
208
209 **Description of *Promicromonospora kermanensis* sp. nov.**
210 *Promicromonospora kermanensis* [*ker.man.en'sis.* N.L. fem. adj. *kermanensis* referring to
211 Kerman, the province from where the type strain was isolated].

212 Aerobic, Gram-staining-positive actinobacterium which forms a well-developed branched
213 mycelium that break up into fragments of various size and rod-shaped spore elements (with
214 average size of 0.3 × 1 μm) (Supplementary Fig. S1) which turn to elipsoid shape when
215 culture gets old. Colonies on the ISP-media are wrinkled varying between creamy and light
216 yellow in colour. Optimal growth occurs at 28 ºC and at pH 5-8. Grows well in the presence
217 of 0-4 % NaCl. The utilization of carbon compounds and resistance to inhibitory chemicals
218 are shown in Table 1 and Fig. S2. The peptidoglycan type of strain HM 533^T is A4α L-Lys –
219 L-Ala – D-Glu. Whole cell hydrolyzates contain the sugars ribose, glucose and galatose.
220 The predominant menaquinones are MK-9(H_4) and MK-9(H_2). Phospholipids pattern
221 consists of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol and an
222 unknown phospholipid.
223 The type strain HM 533^T (= DSM 45485^T = UTMC 00533^T= CECT 8709^T) was isolated
224 from rhizosphere area collected at 10 cm depth in Kerman Province, Iran.
225

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230

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391

392 **Fig. 1.** Maximum likelihood phylogenetic tree inferred from 16S rRNA gene sequences,
393 showing the phylogenetic position of strain HM533^T and the type strains of related taxa.
394 The branches are scaled in terms of the expected number of substitutions per site (see size
395 bar). Support values from maximum-likelihood (left) and maximum-parsimony (right)
396 bootstrapping are shown above the branches if equal to or larger than 60 %.

397 **Table 1.** Differential characteristics of strain HM533^T and the type strains of other
398 *Promicromonospora* species

399 Strains: 1, *Promicromonospora* sp. nov. HM 533^T; 2, *P. alba* DSM 100490 ^{T} ; 3, *P.*
400 *iranensis* DSM 45554^T; 4, *P. umidemergens* DSM 22081^T; 5, *P. vindobonensis* DSM 15942^T;
401 6, *P. sukumoe* DSM 44121^T. All data are from this study, except chemotaxonomic data of
402 the reference strains.
403

Characteristics	*1*	*2*	*3*	*4*	*5*	*6*
Utilization of:						
Dextrin	+	+	+	-	+	+
D-Raffinose	-	+	-	+	-	-
D-Melibiose	-	+	+	+	-	-
Stachyose	-	+	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	+	-	-
L-Fucose	+	+	+/-	+	+	+
Inosine	+	+/-	+/-	-	-	-
Glycerol	+	+	+	-	+	+/-
D-Glucose-6-Phosphate	-	-	-	+/-	-	-
Acetoacetic Acid	+	+	+	-	+	+/-
Propionic Acid	+	-	+/-	+/-	-	-
Glucuronamide	-	-	-	+	-	-
D-Saccharic Acid	+	+	-	+	+	+
Methyl Pyruvate	+	-	+	+/-	-	+
D-Lactic Acid	-	-	-	+	-	-

Methyl Ester						
Citric Acid	-	-	-	-	-	+
α-Keto-Glutaric Acid	-	-	+	-	-	-
Bromo-Succinic Acid	+	-	+	+	-	+
L-Serine	-	-	-	-	+	-
L-Aspartic Acid	+	-	+	-	-	+
L-Glutamic Acid	+	+/-	+	-	+	+
Cell wall sugars*	rib, glu, gal	gal, glu, rha, rib [ȸ]	glu, rib [£]	rha, gal, glu [†]	rha, gal, glu [¥]	rha, glu, rib [ɋ]
Predominant menaquinones[a]	MK-9(H_4) and MK-9(H_2)	MK-9(H_4) and MK-9(H_2) [ȸ]	MK-9(H_4), MK-9(H_6), MK-8(H_4), MK-9(H_2) [£]	MK-9(H_6), MK-9(H_4), MK-8(H_4) [†]	MK-9(H_4), MK-9(H_2), MK-9(H_6) [¥]	MK-9(H_4), MK-9(H_0), MK-9(H_2), MK-9(H_6) [ɋ]
Phospholipids*	PG, DPG,PI, PL, 2GL	DPG, PG,PI, PIM, PGL, PL, GL [ȸ]	DPG, 2PL 2GL, 2PGL, PI, PG [£]	PG, DPG, PI, PL, 3GL [†]	DPG, PG, 3PGL, PL [¥]	PI, DPG [ɋ]

404

405 +, positive reaction; –, negative reaction; +/-, ambiguous; glu, glucose; rib, ribose; gal, galactose; rha,
406 rhamnose; ND, not determined; MK, menaquinones; ; i-, iso-branched, ai-, anteiso-branched; DPG,
407 diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PGL, unknow-n
408 phosphoglycolipid; PL, unknow-n phospholipid; GL, unknown glycolipid; PIM, phosphatidylinositol
409 mannoside.

410 a, only components making up ≥ 5 % peak area ratio are shown.

411 *, the components are listed in decreasing order of quantity.

412 ȸ, Data taken from Guo *et al.* (2016).

413 †, Data taken from Martin *et al.* (2010).

£, Data taken from Mohammadipanah *et al*. (2014).

¥, Data taken from Busse *et al.* (2003).

¶, Data taken from Takahashi *et al*. (1987).

Supplementary Fig. S1. Scanning electron micrographs showing the morphology of *Promicromonospora kermanensis* HM 533^{T} grown on ISP 2 medium for 10 days at 28 °C (at stationary phases); Bar 2µm.

Supplementary Fig. S2. The parameter "Maximum Height" estimated from the respiration curves as measured with the OmniLog phenotyping device and discretized and visualized as heatmap using the opm package. The Plates and substrates are rearranged according to their overall similarity (as depicted using the row and column dendrograms). Orange colour indicates positive reaction; purple colour indicates negative reaction; green colour indicates ambiguous reaction. Letters (A/B) indicate each replicate of experiment.

Supplementary Fig. S3. Total lipids profile of *Promicromonospora kermanensis* HM 533^{T}, after separation by two-dimensional TLC. Plate was sprayed with molydatophosphoric acid for detection of total polar lipid. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; GL (1-2), unknown glycolipid; PL, unknown phospholipid; L (1-4), unknown lipid.

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