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Thermorudis pharmacophila WKT50.2T sp. nov., a novel isolate of class Thermomicrobia isolated from geothermal soil, and emended descriptions of Thermomicrobium roseum, Thermomicrobium carboxidum, Thermorudis peleae and Sphaerobacter thermophilus.

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Abstract:	<p>An aerobic, thermophilic and cellulolytic bacterium, designated strain WKT50.2T, was isolated from geothermal soil at Waikite, New Zealand. Strain WKT50.2T grew at 53-76 °C, and at pH 5.9-8.2. The DNA G+C content was 58.4 mol%. The major fatty acids were 12-methyl C18:0 and C18:0. Polar lipids were all linked to long chain 1,2-diols, and comprised of 2-acylalkyldiol-1-O-phosphoinositol (diolPI), 2 acylalkyldiol-1-O-phospho-acylmannoside (diolP-acylMan), 2-acylalkyldiol-1-O-phosphoinositol acylmannoside (diolPI-acylMan) and 2-acylalkyldiol-1-O-phosphoinositol mannoside (diolPI-Man). Strain WKT50.2T was utilised a range of cellulosic substrates, alcohols and organic acids for growth, but was unable to utilise monosaccharides. Robust growth of WKT50.2T was observed on protein derivatives. WKT50.2T was sensitive to ampicillin, chloramphenicol, kanamycin, neomycin, polymyxin B, streptomycin and vancomycin. Metronidazole, lasalocid A and trimethoprim stimulated growth. Phylogenetic analysis of 16S rRNA gene sequences showed that WKT50.2T belonged to class Thermomicrobia within the phylum Chloroflexi, and was most closely related to Thermorudis peleae KI4T (99.6 %). DNA-DNA hybridization of WKT50.2T and T. peleae KI4T was 18.0 %. Physiological and biochemical tests confirmed phenotypic and genotypic differentiation of strain WKT50.2T from T. peleae KI4T and other</p>

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Thermomicrobia. On the basis of its phylogenetic position and phenotypic characteristics, we propose that strain WKT50.2T represents a novel species for which the name *Thermorudis pharmacophila* sp. nov. is proposed, with the type strain WKT50.2T (=DSM 26011T =ICMP 20042T).

Emended descriptions of the strains *T. roseum*, *T. carboxidum*, *T. peleae* and *S. thermophilus* are also proposed and include the description of a novel respiratory quinone, MK-8 2,3-epoxide (23 %) in *T. roseum*.

1 ***Thermorudis pharmacophila* WKT50.2^T sp. nov., a novel isolate of class *Thermomicrobia* isolated**
2 **from geothermal soil, and emended descriptions of *Thermomicrobium roseum*, *Thermomicrobium***
3 ***carboxidum*, *Thermorudis peleae* and *Sphaerobacter thermophilus*.**

4

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16 Running title: *Thermorudis pharmacophila*

17 New Taxa: Other Bacteria

18

19 GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of *Thermorudis*
20 *pharmacophila* is HE794997.

21

22 Abbreviations: CMC, carboxymethylcellulose; TEM, transmission electron microscopy; TLC, thin layer
23 chromatography.

24

25

26 **ABSTRACT**

27 An aerobic, thermophilic and cellulolytic bacterium, designated strain WKT50.2^T, was isolated from
28 geothermal soil at Waikite, New Zealand. Strain WKT50.2^T grew at 53-76 °C, and at pH 5.9-8.2. The
29 DNA G+C content was 58.4 mol%. The major fatty acids were 12-methyl C_{18:0} and C_{18:0}. Polar lipids
30 were all linked to long chain 1,2-diols, and comprised of 2-acylalkyldiol-1-*O*-phosphoinositol (diolPI),
31 2-acylalkyldiol-1-*O*-phospho-acylmannoside (diolP-acylMan), 2-acylalkyldiol-1-*O*-phosphoinositol
32 acylmannoside (diolPI-acylMan) and 2-acylalkyldiol-1-*O*-phosphoinositol mannoside (diolPI-Man).
33 Strain WKT50.2^T was utilised a range of cellulosic substrates, alcohols and organic acids for growth,
34 but was unable to utilise monosaccharides. Robust growth of WKT50.2^T was observed on protein
35 derivatives. WKT50.2^T was sensitive to ampicillin, chloramphenicol, kanamycin, neomycin, polymyxin
36 B, streptomycin and vancomycin. Metronidazole, lasalocid A and trimethoprim stimulated growth.
37 Phylogenetic analysis of 16S rRNA gene sequences showed that WKT50.2^T belonged to class
38 *Thermomicrobia* within the phylum *Chloroflexi*, and was most closely related to *Thermorudis peleae*
39 KI4^T (99.6 %). DNA-DNA hybridization of WKT50.2^T and *T. peleae* KI4^T was 18.0 %. Physiological and
40 biochemical tests confirmed phenotypic and genotypic differentiation of strain WKT50.2^T from *T.*
41 *peleae* KI4^T and other *Thermomicrobia*. On the basis of its phylogenetic position and phenotypic
42 characteristics, we propose that strain WKT50.2^T represents a novel species for which the name
43 *Thermorudis pharmacophila* sp. nov. is proposed, with the type strain WKT50.2^T (=DSM 26011^T
44 =ICMP 20042^T).

45

46 Emended descriptions of the strains *T. roseum*, *T. carboxidum*, *T. peleae* and *S. thermophilus* are also
47 proposed and include the description of a novel respiratory quinone, MK-8 2,3-epoxide (23 %) in *T.*
48 *roseum*.

49 Members of the class *Thermomicrobia* are broadly distributed across a wide range of both aquatic
50 and terrestrial habitats including geothermally-heated soils, sediments and hot springs (Costa *et al.*,
51 2009; Engel *et al.*, 2013), mesophilic soils (Joynt *et al.*, 2006; Lesaulnier *et al.*, 2008) and rock (De la
52 Torre *et al.*, 2003). In addition, they have been detected in compost (Partanen *et al.*, 2010; Gladden
53 *et al.*, 2011), sewage sludge (Demharter *et al.*, 1989) and on human skin (Grice *et al.*, 2009).
54 *Thermomicrobia* is comprised of five characterised isolates with validly-published names:
55 *Thermomicrobium roseum* (Jackson *et al.*, 1973) isolated from a hot spring, *Sphaerobacter*
56 *thermophilus* (Hugenholtz & Stackebrandt, 2004) isolated from sewage sludge, *Nitrolancea*
57 *hollandicus* (Sorokin *et al.*, 2014) isolated from a nitrifying bioreactor, and *Thermorudis peleae* and
58 *Thermomicrobium carboxidum* (King & King, 2014b) isolated from a volcanic soil biofilm. Although
59 the genomes of both *T. roseum* and *S. thermophilus* are available (Wu *et al.*, 2009; Pati *et al.*, 2010),
60 they have only been partially characterised. It has further been suggested that *Candidatus*
61 “*Thermobaculum terrenum*” (Botero *et al.*, 2004) may also be incorporated into the
62 *Thermomicrobia*, although its phylogenetic position is uncertain and remains contentious (Kunisawa,
63 2011; Gupta *et al.*, 2013). All *Thermomicrobia* strains are neutrophilic, either thermotolerant or
64 thermophilic, and have a broad chemoorganotrophic substrate specificity.

65 Here we describe the phenotypic and phylogenetic characteristics of a novel *Thermomicrobia* strain,
66 WKT50.2^T, which was isolated from geothermally-heated soil in New Zealand. We propose that it
67 represents a novel species within the genus *Thermorudis*. In addition, we performed supplementary
68 characterisation of *T. roseum* (Jackson *et al.*, 1973), *T. carboxidum* and *T. peleae* (King & King,
69 2014b), and *S. thermophilus* (Demharter *et al.*, 1989) and propose the emendation of their formal
70 descriptions.

71 Strain WKT50.2^T was isolated from geothermally-heated soil at Waikite, New Zealand. Soil gas fluxes
72 at Waikite are low and are composed principally of CO₂ (>95 mol %) with minor concentrations of
73 NH₃ and CH₄ (Giggenbach *et al.*, 1994). The soil was sampled from a pink-coloured soil profile
74 directly above the hot spring. The soil sample had a pH of 4.5 (25 °C) and an *in situ* temperature of
75 64.8 °C. Soil crumbs were spread on AOM1 plates (Stott *et al.*, 2008) and incubated at 60 °C in an
76 aerobic atmosphere. Individual bacterial colonies were picked and purified with the streak-plate
77 method until an axenic culture was obtained (Stott *et al.*, 2008). Unless otherwise stated, all
78 physiological and metabolic characteristics were determined by growing WKT50.2^T at 65 °C in liquid
79 CPS medium (Supplementary Information), and all materials and methodologies for phenotypic
80 characterisation have been listed in the Supplementary Information.

81 Genomic DNA was isolated from strain WKT50.2^T using a NucleoSpin Tissue kit (Macherey Nagel,
82 Germany) according to the manufacturer’s instructions. The 16S rRNA gene was amplified using the

83 universal bacterial primers 9F and 1492R (Weisburg *et al.*, 1991). The near-complete 16S rRNA gene
84 sequence was 1357 bp (HE794997), and was manually checked for quality. Closely-related strains to
85 WKT50.2^T were determined by subjecting the 16S rRNA gene sequence to blastN discontinuous
86 megablast search (Altschul *et al.*, 1997). The 16S rRNA gene sequences from WKT50.2^T and closely-
87 related strains and phylotypes were aligned (all retrieved sequences were > 1212 bp in length), and
88 phylogenetic distances were calculated using the Jukes-Cantor correction within the ARB software
89 environment - SILVA SSU NR (non-redundant) Release 119 database (Jukes & Cantor, 1969; Ludwig
90 *et al.*, 2004). WKT50.2^T was most closely related to *T. peleae* K14^T (99.6 %), *T. carboxidum* K13^T (91.6
91 %) and *T. roseum* DSM 5159^T (91.2 %). A phylogenetic tree (Fig. 1) was constructed using MrBayes,
92 which uses a Bayesian inference model to calculate phylogeny (Ronquist *et al.*, 2012). The
93 phylogenetic tree in Figure 1 was calculated using a 0.25 burn, and Markov Chain Monte Carlo
94 (MCMC) estimation of 2,000,000 cycles, four chains (temperature parameter of 0.5) and a sampling
95 frequency of 500. The phylogenetic placement of strain WKT50.2^T shows that it groups within class
96 *Thermomicrobia* and is a member of the genus *Thermorudis* (Fig. 1).

97 Strain WKT50.2^T formed pale pink, convex and entire colonies of 2-3 mm diameter after two days of
98 incubation on CPS medium at 70 °C. Gram staining was negative. No cell lysis was observed when
99 cells were treated with a 3 % (v/v) solution of KOH (Halebian *et al.*, 1981). Neither motility nor spore
100 formation were observed under any growth conditions. WKT50.2^T cells had a characteristic dumb-
101 bell, or short rod morphology (Fig. 2). As the cultures aged, pairs or chains of cells were frequently
102 observed. Cells were between 2.1 and 2.8 µm long, and 0.9 to 1.1 µm wide (Fig. 2a). Cryo-TEM of
103 WKT50.2^T cells showed a classical Gram-negative structure of a cytoplasmic membrane, a
104 peptidoglycan layer, outer membrane, and putative thin proteinaceous and/or polysaccharide-rich S-
105 layer (Fig. 2b). The widths of the two cited lipid membranes are approximately 6 nm (data not
106 shown), which is consistent with a typical membrane thickness of 4-7 nm. The peptidoglycan
107 composition (Schumann, 2011) and cell wall sugars (Staneck & Roberts, 1974) of WKT50.2^T were
108 analysed by the Identification Service of the Deutsche Sammlung für Mikroorganismen und
109 Zellkulturen (DSMZ), Braunschweig, Germany. The analysis found substantial amounts of protein
110 present in the cell membrane, which could not be fully removed from the cell hydrolysate and
111 therefore prevented full analysis of the peptidoglycan content. No diaminopimelic acid (DAP) was
112 detected in WKT50.2^T cell walls, but ornithine, L-alanine and D-glutamic acid were identified and are
113 normally associated Gram-positive type cell membranes (Schleifer & Kandler, 1972). The same
114 amino acids were detected in the cell wall of *N. hollandicus* (Sorokin *et al.*, 2014). The cell envelopes
115 of *T. roseum* and *S. thermophilus* were also composed largely of protein, and only minor
116 concentrations of peptidoglycan could be detected (Merkel *et al.*, 1980) (Demharter *et al.*, 1989) The

117 cell wall sugars of WKT50.2^T were determined by TLC and contained xylose, mannose, glucose,
118 galactose, rhamnose and ribose. These sugars are not routinely reported in bacterial cell walls, but
119 with the exception of ribose, they reflect the composition of the large polysaccharide complex
120 bound to the peptidoglycan observed in the monoderm *Chloroflexus aurantiacus* (Jürgens *et al.*,
121 1987; Meissner *et al.*, 1988). These data suggest that the cell envelope of WKT50.2^T (Fig. 2b) includes
122 substantial polysaccharide and protein components.

123 Fatty acid methyl esters (FAMES) were prepared and analysed as previously described (Lagutin *et al.*,
124 2014). The fatty acid composition of WKT50.2^T is presented in Table S1. The cell membrane of
125 WKT50.2^T was primarily composed of the fatty acids 12-methyl C_{18:0} (54.0 %) and C_{18:0} (30.6 %). Polar
126 lipids were analysed using TLC and phospholipids were quantified by ³¹P-NMR (MacKenzie *et al.*,
127 2009) (Supplementary Information). TLC analysis of polar lipids indicated seven glycolipids and four
128 unknown phospholipids (Fig. S1). Phospholipids comprised 25.2 % of the total lipids and included
129 four unusual 1,2-diol-linked lipids (Lagutin *et al.*, 2014); 2-acylalkyldiol-1-*O*-phosphoinositol (diolPI)
130 (64.0 mol%), 2-acylalkyldiol-1-*O*-phospho-acylmannoside (diolP-acylMan) (17.9 mol%), 2-
131 acylalkyldiol-1-*O*-phosphoinositol acylmannoside (diolPI-acylMan) (11.5 mol%) and 2-acylalkyldiol-1-
132 *O*-phosphoinositol mannoside (diolPI-Man) (6.7 mol%) (Table S2, Figs. S2 and S3). Diol-linked lipids
133 were previously reported in *T. roseum* (Pond *et al.*, 1986) and *N. hollandicus* (Sorokin *et al.*, 2012)
134 but were not fully characterised.

135 DNA base composition and DNA-DNA hybridisation were determined by the Identification Service of
136 the DSMZ. The %G+C base content was determined by disrupting cells via French pressure cell and
137 purifying the DNA extract using a hydroxyapatite column (Cashion *et al.*, 1977). The DNA was
138 hydrolysed with P1 nuclease and dephosphorylated with bovine alkaline phosphatase and then
139 quantified using HPLC (Mesbah *et al.*, 1989). The DNA base composition was 58.4 mol% G+C. DNA-
140 DNA hybridisation between WKT50.2^T and *T. peleae* was performed as previously described (Ziemke
141 *et al.*, 1998). Strain WKT50.2^T and *T. peleae* K14^T exhibited a mean DNA-DNA similarity of 18.0 %,
142 which is below the recommended threshold value of 70 % for bacterial species delineation (Wayne
143 *et al.*, 1987). The extraction and determination of the respiratory quinones was undertaken using a
144 modified LCMS method (Supplementary Information) described by Nishijima and colleagues
145 (Nishijima *et al.*, 1997) and was determined as MK-8 (95.3 %) and MK-9 (4.7%).

146 Strain WKT50.2^T was unable to grow anaerobically using sulphur, sulphate or nitrate as terminal
147 electron acceptors (Supplementary Information). It had a growth range between 53–76 °C (optimum
148 68–73 °C), and the pH range for growth was pH 5.9–8.2 (optimum pH 6.8–7.0). No growth was
149 observed at or below pH 5.5, or at or above pH 8.9. Growth was observed with 0–1 % (w/v) NaCl, but

150 optimum growth occurred in the absence of NaCl. All substrate utilization experiments were
151 conducted in triplicate, with shaking incubation at 125 r.p.m. at 68 °C, using Castenholz salts solution
152 (Supplementary Information) with 50 % (v/v) air headspace. Table S3 contains a list of substrates
153 tested (0.2 % w/v unless otherwise stated). WKT50.2^T grew chemoorganotrophically using some di-
154 and trisaccharide substrates, but no growth was observed when using monosaccharides or sugar
155 derivatives as sole energy sources. WKT50.2^T was able to hydrolyse soluble starch (Sigma-Aldrich),
156 crystalline cellulose (Avicel™, Sigma-Aldrich) and sodium CMC (Hercules). Organic acids, with the
157 exception of sodium citrate, were used as sole carbon sources. Growth was also observed in media
158 containing peptone (Oxoid), Bacto™ tryptone (BD), Casamino acids (Difco) or BBL™ yeast extract
159 (BD), as well as in standard complex media such as R2A and nutrient broth. Ethanol and methanol
160 were utilised as sole carbon sources, but not 1-propanol or 2-propanol (all 0.05 % v/v). Oxidation of
161 carbon monoxide was tested as previously described (Hardy & King, 2001) but was not observed. No
162 homologs of the gene of the large subunit of carbon monoxide dehydrogenase (*coxL*) were found
163 using PCR primers (Table S4) based on the gene sequences from *T. roseum* and *S. thermophilus*, or
164 through analysis of the draft genome of WKT50.2^T. The preferred nitrogen source was determined
165 by providing different nitrogen sources in otherwise nitrogen-free Castenholz salts solution with
166 sucrose as a carbon and energy source (Supplementary Information). Nitrogen sources tested were
167 N₂, KNO₃, KNO₂, (NH₄)₂SO₄, yeast extract, urea, alanine, and serine. Strain WKT50.2^T was able to use
168 nitrate, ammonia and alanine as sole nitrogen sources.

169 Antibiotic sensitivity was assessed by growing strain WKT50.2^T on Castenholz salts solution with 5 g l⁻¹
170 peptone and 2.5 g l⁻¹ sucrose, with either 3 or 30 µg ml⁻¹ antibiotic. No growth was observed with
171 the addition of 3 µg ml⁻¹ ampicillin, which possibly supports a peptidoglycan assembly structure in
172 the cell, chloramphenicol, kanamycin, neomycin, polymyxin B, streptomycin or vancomycin. The
173 addition of 3 µg ml⁻¹ lasalocid A, or 3 or 30 µg ml⁻¹ metronidazole or trimethoprim resulted in a
174 higher OD₆₀₀ as well as a faster growth rate than the control with no antibiotic added (Table S5).
175 Further experiments showed a dosage-dependent increase in growth of WKT50.2^T following the
176 addition of up to 150 µg ml⁻¹ metronidazole or trimethoprim (data not shown). Neither
177 metronidazole, lasalocid A nor trimethoprim were able to support growth as sole carbon or nitrogen
178 sources.

179 Strain WKT50.2^T demonstrated negative reactions for both catalase and oxidase. Other enzyme
180 activities were assessed using the API ZYM kit (bioMérieux), prepared according to the
181 manufacturers' instructions. Esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase,
182 valine arylamidase, and trypsin activities were observed (Table S6).

183 In addition to the characterisation of WKT50.2^T we undertook further chemotaxonomic or
184 phenotypic analysis of the type strains of *T. roseum* (DSM 5159^T), *T. pelea* (DSM 27169^T), *T.*
185 *carboxidum* (DSM 27067^T) and *S. thermophilus* (DSM 20745^T). The materials and methods used for
186 this characterisation are described in full in the Supplementary Information, but where possible,
187 reflect the methodologies used for the WKT50.2^T characterisation. The temperature and pH ranges
188 and optima for growth, substrate utilisation (Table S3), preferred nitrogen sources and salinity
189 tolerance were determined for *T. roseum* and *S. thermophilus*. The growth responses to
190 metronidazole, lasalocid A and trimethoprim were also determined (Table S5). Full results are found
191 in the emended species descriptions for these type species. The respiratory quinone content of *T.*
192 *roseum* was determined by LC-MS (Supplementary Information) and was composed of primarily MK-
193 8 (74 %) along with low levels of MK-9 (3 %). A third peak (23 %) observed in the LC-MS
194 chromatograph (Fig. S4) corresponded to a novel quinone. We analysed this unknown peak
195 (Supplementary Information) and determined that it is an MK-8 epoxide with the epoxy structure at
196 the 2,3 position (see Supplementary Information and Figs. S5-S6). ³¹P-NMR analysis of the *S.*
197 *thermophilus* lipid extract identified the same four phospholipids as found in WKT50.2^T; diolPI (41.3
198 mol%), diolP-acylMan (17.0 mol%), diolPI-acylMan (8.4 mol%) and diolPI-Man (33.3 mol%) and
199 comprised 53.5 % of total lipid extract (Lagutin *et al.*, 2014). The FAME content of *S. thermophilus*
200 was dominated by 12-methyl C_{18:0} (63.2 %) and C_{18:0} (17.1 %) and reflects the FA profile of WKT50.2^T
201 (54.0 % and 30.6 % respectively). The unusual fatty acid 12-methyl C_{18:0} was previously determined
202 to be the major fatty acid in *T. roseum* (68 %) (Pond *et al.*, 1986) and *N. hollandicus* (Sorokin *et al.*,
203 2012), and was found at low levels (1.6 – 7.6 %) in *Thermogemmatispora* strain T81 (Vyssotski *et al.*,
204 2012). Outside the phylum *Chloroflexi*, 12-methyl C_{18:0} fatty acid has only been detected in low
205 concentrations (< 5 %) in *Rubrobacter xylanophilus* (Carretto *et al.*, 1996) and two strains of
206 *Rubrobacter taiwanensis* (Chen *et al.*, 2004). This fatty acid has not been found in the closely-related
207 species *R. radiotolerans* (Suzuki *et al.*, 1988), and thus could be considered as a useful taxonomic
208 marker for the class *Thermomicrobia*.

209 Despite the classical diderm-type cell envelope observed in Cryo-TEM imaging of WKT50.2^T (Fig. 2b)
210 and a negative Gram stain reaction, a number of chemotaxonomic observations reported here were
211 more consistent with the characteristic monoderm envelope structure normally associated with the
212 phylum *Chloroflexi*. The lack of detectable concentrations of DAP and presence of ornithine, L-alanine
213 and D-glutamic acid in the cell wall lysate, a negative KOH reaction and susceptibility to vancomycin
214 and ampicillin are all characteristic of Gram-positive, monoderm-type cell envelope structures.
215 Indeed, previous cell envelope classifications of *Chloroflexi* strains have been inconsistently reported
216 due to variable Gram-staining results for *Chloroflexus aurantiacus*, *Herpetosiphon* and *Roseflexi* spp.,

217 despite the prevailing *Chloroflexi* monoderm consensus (Sutcliffe, 2010 and references therein).
218 Recent publications reviewing the cell envelopes of *Chloroflexi* have argued that all *Chloroflexi*
219 genomes (including *T. roseum*, *T. terrenum* and *S. thermophilus*) lack crucial genes required for LPS
220 production and transport, and transporters specific to cell walls (Sutcliffe, 2010; Sutcliffe, 2011). We
221 have reviewed the draft genome of WKT50.2^T (not shown) and were also unable to find evidence of
222 crucial gene homologs required for the function and synthesis of diderm-type cells (Supplementary
223 information), nor secretory systems linked with the outer membrane. Based upon the lack of
224 genomic evidence for a diderm-type cell envelope and chemotaxonomic observations consistent
225 with monoderms, we conclude that WKT50.2^T has an atypical monoderm cell envelope rich in
226 proteinaceous and polysaccharide materials. Previous reports cite multi-layered outer membranes
227 structures for *Thermogemmatispora onikobensis* and *Thermogemmatispora foliorum* (Yabe *et al.*,
228 2011), *C. aurantiacus* (Hanada and Pearson, 2006) and other *Chloroflexi* spp. (Jürgens *et al.*, 1987),
229 although the composition and purpose of these structures have yet to be investigated. We therefore
230 caution that a focussed investigation of cell envelopes of *Chloroflexi* isolates that includes electron
231 microscopy, chemotaxonomic and genomic analyses is warranted and should be undertaken in the
232 future to provide clarity in cell envelope classification across this phylum.

233 Strain WKT50.2^T could be differentiated from other *Thermomicrobia* members on the basis of both
234 phenotypic and chemotaxonomic features, including differences in temperature and pH range,
235 substrate utilisation, oxidase and catalase reactions, Gram-staining and %G+C content of genomic
236 DNA (Table 1). It placed phylogenetically within the class *Thermomicrobia* and had the greatest 16S
237 rRNA gene sequence similarity with *T. peleae* (99.6 %), *T. carboxidum* (91.6 %) and *T. roseum* (91.2
238 %). DNA–DNA hybridization between *T. peleae* and WKT50.2^T was less than the 70 % DNA-DNA
239 similarity species-level threshold (Wayne *et al.*, 1987) confirming that WKT50.2^T is a novel species. In
240 addition, WKT50.2^T was able to utilise several substrates which *T. peleae* could not, including
241 sucrose, glycerol, ethanol, methanol and organic acids such as acetate, pyruvate and succinate.
242 WKT50.2^T was unable to utilise any monosaccharides tested (Table 1). In contrast, *S. thermophilus*
243 was able to utilize fructose as a monosaccharidic energy source, and *T. roseum* exhibited growth
244 using all monosaccharides tested (Table 1). The growth of WKT50.2^T was promoted by the
245 cultivation in the presence of lasalocid A, metronidazole and trimethoprim whereas *T. peleae* and *T.*
246 *carboxidum* only exhibited resistance to metronidazole and trimethoprim and not promoted growth.
247 In contrast to *T. roseum*, *S. thermophilus*, and *T. carboxidum*, WKT50.2^T was unable to use carbon
248 monoxide as an energy source or oxidise it constitutively. It is interesting to note that this inability is
249 also shared with *T. peleae* (King & King, 2014b), and it therefore is tempting to infer that this
250 observation could be used as a diagnostic tool to differentiate *Thermorudis* from *Thermomicrobium*

251 spp. However, we believe that this observation is likely coincidental particularly as the ability to
252 oxidise CO by other *Chloroflexi* such as by *Thermogemmatispora* spp. (King & King, 2014a) appears
253 to be independent of 16S rRNA gene phylogeny. These findings indicate that strain WKT50.2^T is
254 phylogenetically and physiologically distinctive from other species within the class *Thermomicrobia*
255 and we therefore propose that it represents a novel species within the genus *Thermorudis*, *T.*
256 *pharmacophila* WKT50.2^T. The descriptions of the species *T. roseum*, and *S. thermophilus* are
257 emended on the basis of new fatty acids, polar lipids, antibiotic resistance, quinones and substrate
258 utilisation data. Emended descriptions of *T. carboxidum* and *T. rudis* are also provided.

259 **Description of *Thermorudis pharmacophila* sp. nov.**

260 *Thermorudis pharmacophila* (phar.ma.co'phi.la. Gr. neut. n. *pharmakon* poison; N.L. adj. *philus* -a -
261 um (from Gr. adj. *philos* -ê-on) loving; N.L. fem. adj. *pharmacophila*, poison-loving).

262 Has the following characteristics in addition to those given in the genus description (King & King,
263 2014b). Cells are rods approx. 0.9 - 1.1 µm in diameter and 2.1 - 2.8 µm in length. Exhibits an
264 oxidative, heterotrophic metabolism. No growth observed on monosaccharides. Sucrose, trehalose
265 and raffinose act as sole carbon sources; lactose, maltose and cellobiose do not. Avicel™, CMC,
266 starch, and xylan are hydrolysed, but chitin, dextrin, gellan gum, galactomannan, glucomannan,
267 pectin and xanthan are not. Cells also grow on Whatman™ filter paper, glycerol, sorbitol and 0.05 %
268 (v/v) methanol or ethanol. Sodium acetate, formate, fumarate, lactate, pyruvate and succinate act
269 as sole carbon sources; sodium citrate, mannitol, galacturonic acid and 0.05% 1-propanol or 2-
270 propanol do not. Cells grow readily on yeast extract, peptone, tryptone and Casamino acids. Growth
271 inhibited by ampicillin, chloramphenicol, kanamycin, neomycin, polymyxin B, streptomycin and
272 vancomycin. Lasalocid A, metronidazole and trimethoprim stimulate growth. Ammonium ion, nitrate
273 ion or alanine can act as sole nitrogen sources. Optimal growth at 68-73 °C (range: 53-76 °C) and pH
274 6.8-7.0 (range: 5.9-8.2). NaCl tolerance up to 1 % (w/v). Growth on solid gellan-based medium
275 produces pink opaque colonies with circular and entire edges. G+C content of strain WKT50.2^T is
276 58.4 mol% and the principal quinone detected is MK-8 with minor concentrations of MK-9. The
277 major fatty acids are 12-methyl C_{18:0} and C_{18:0}, with minor amounts of C_{20:0}, C_{16:0}, 10-methyl C_{16:0} and
278 C_{14:0}. Polar lipids are diol-linked and include diolPI, diolP-acylMan, diolPI-acylMan and diolPI-Man.
279 The type strain, WKT50.2^T (=DSM 26011^T =ICMP 20042^T) was isolated from geothermally-heated
280 soils at Waikite, New Zealand.

281 **Emended description of *Thermomicrobium roseum* Jackson *et al.* 1973**

282 The description is as given by Jackson *et al.* (1973) with the following amendments.

283 Growth occurs on simple sugars; D-xylose, D-galactose, D-mannose, D-fructose, D-cellobiose, D-
284 maltose, sucrose, D-trehalose and D-raffinose. Dextrin, Phytigel™, starch, xylan and xanthan act as
285 sole carbon sources, but Avicel™ and CMC do not. Grows on short-chain alcohols including
286 methanol, ethanol, 1-propanol, 2-propanol and mannitol, but not sorbitol. Cells also grow on
287 glycerol and sodium acetate, formate, fumarate, lactate, pyruvate and succinate but not citrate.
288 Peptone, tryptone and Casamino acids act as sole carbon sources. Ammonium ion, nitrate ion or
289 yeast extract can act as sole nitrogen sources. Growth is stimulated by metronidazole and
290 trimethoprim. The predominant quinones are MK-8, MK-8 2,3-epoxide and MK-9, and the major
291 fatty acid is 12-methyl C_{18:0}. Fatty acids are linked to diols. Optimal growth at 65-70 °C (range: 52-77
292 °C) and pH 8.0-8.4 (range: 6.0-9.4). NaCl tolerance up to 1.5 % (w/v). Cells possess cell envelopes
293 composed largely of protein with only small amounts of peptidoglycan, and ornithine is present.
294 The type strain, *Thermomicrobium roseum*^T (= DSM 5159 = ATCC 27502) was isolated from a
295 geothermal hot spring in Yellowstone National Park, USA.

296 **Emended description of *Sphaerobacter thermophilus* Demharter *et al.* 1989**

297 The description is as given by Demharter *et al.* (1989) with the following amendments.

298 Growth occurs on D-fructose and D-cellobiose but not D-xylose, D-galactose, D-mannose, D-maltose,
299 sucrose, D-trehalose or D-raffinose. Avicel™, CMC, Phytigel™, starch and xylan act as sole carbon
300 sources but dextrin and xanthan do not. Grows on some short alcohols including ethanol, 1-
301 propanol, 2-propanol and mannitol but not sorbitol or methanol. Cells also grow on glycerol and
302 sodium acetate, citrate, formate, fumarate, lactate, pyruvate and succinate. Peptone, tryptone,
303 Casamino acids and nutrient broth act as sole carbon sources but yeast extract does not. Ammonium
304 ion, nitrate ion or yeast extract can act as sole nitrogen sources. Growth inhibited by
305 chloramphenicol, neomycin, kanamycin and trimethoprim, but growth is stimulated by
306 metronidazole. Optimal growth at 55-60 °C (range: 49-67 °C) and pH 8.0-8.4 (range: 6.1-9.0). NaCl
307 tolerance up to 1 % (w/v). The major fatty acids are 12-methyl C_{18:0} and C_{18:0}, with minor amounts of
308 C_{16:0}, 10-methyl C_{16:0} and C_{18:1} *n*-9. Polar lipids comprise diolPI, diolP-acylMan, diolPI-acylMan and
309 diolPI-Man.

310 The type strain, *Sphaerobacter thermophilus*^T (=DSM 20745^T = ATCC 49802^T) was isolated from a
311 sewage fermenter in Germany.

312 **Emended description of *Thermomicrobium carboxidum* King and King, 2014**

313 The description is as given by King and King (2014b) with the following amendments.

314 Growth inhibited by chloramphenicol, neomycin, streptomycin, and kanamycin but resistant to
315 metronidazole and trimethoprim.

316 The type strain, *Thermomicrobium carboxidum*^T (=DSM 27067^T = ATCC BAA-2535^T) was isolated from
317 geothermally-heated biofilm from Kilauea Iki crater (Hawai'i, USA).

318 **Emended description of *Thermorudis peleae* King and King, 2014**

319 The description is as given by King and King (2014b) with the following amendments.

320 Growth inhibited by chloramphenicol, neomycin, streptomycin, and kanamycin but resistant to
321 metronidazole and trimethoprim. Xanthan, dextrin and CMC do not support growth.

322 The type strain, *Thermorudis peleae*^T (=DSM 27169^T = ATCC BAA-2536^T) was isolated from
323 geothermally-heated biofilm from Kilauea Iki crater (Hawai'i, USA).

324

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329

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362 and the family *Chloroflexaceae* fam. nov., and the suborder *Roseiflexineae* subord. nov., containing
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456

457

458 **FIGURE CAPTIONS**

459

460 **Figure 1.** Phylogenetic tree based on 16S rRNA gene sequences of *Thermorudis pharmacophila*
461 WKT50.2^T (highlighted) and representative isolates and environmental clones of the phylum
462 *Chloroflexi* and class *Thermomicrobia*. The tree was constructed via a Bayesian inference model
463 (MrBayes), using Markov Chain Monte Carlo (MCMC) sampling methods to calculate posterior
464 distributions of trees in the ARB software environment. Posterior probability values $\geq 90\%$ are
465 indicated by open circles, $\geq 80\%$ by filled circles and $\geq 70\%$ by open diamonds. The scale bar
466 represents a 0.1 change per nucleotide position.

467

468 **Figure 2.** A) Negative stain TEM image of WKT50.2^T. Scale bar = 500 nm. B) Cryo-TEM image of
469 WKT50.2^T. The insert shows an enlarged view of the cell envelope structure, CM: cytoplasmic
470 membrane; OM*: outer membrane; P*: peptidoglycan; S*: S-layer; C: Formvar-carbon support
471 mesh. Scale bar = 200 nm.*; denotes that while the Cryo-TEM shows a classical Gram-
472 negative/diderm structure, chemotaxonomic and genomic data presented in the text suggest
473 WKT50.2^T has an atypical Gram-positive monoderm-type cell envelope.

474

475

476

477

Table 1. Differential characteristics of WKT50.2^T and selected *Thermomicrobia* strains. Strains: 1, WKT50.2^T; 2, *Thermorudis peleae* K14^T (King & King, 2014b); 3, *Thermomicrobium carboxidum* K13^T (King & King, 2014b); 4, *Thermomicrobium roseum* DSM5159^T (Jackson *et al*, 1973; Wu *et al.*, 2009); 5, *Sphaerobacter thermophilus* DSM20745^T (Demharter *et al.*, 1989); 6, *Nitrolancea hollandicus* (Sorokin *et al.*, 2012; Sorokin *et al.*, 2014). Data generated or repeated in this study is denoted by * and # respectively.

Characteristic	1*	2	3	4	5	6
Temperature (°C)						
range	50-78	55-70	50-70	52-77*	49-67*	25-46
optimum	68-71	65	70	65-70*	55-60	40
pH						
range	6.0-8.4	5.8-8.0	6.0-9.0	6.0-9.4	6.1-9.0*	6.2-8.3
optimum	6.5-7.0	6.7-7.1	7.0	8.0-8.4*	8.0-8.4*	6.8-7.5
Pigmentation	pink	pink	off-white	pink	opaque	greenish
Gram stain	-	+ #	+ #	-	+	+
Catalase reaction	-	-	+	+	+	NR
Oxidase reaction	-	+	+	-	+	NR
Utilisation of:						
D-xylose	-	-	-	+*	-*	NR
D-galactose	-	-	-	+*	-*	NR
D-glucose	-	-	-	+*	-*	-
D-mannose	-	-	-	+*	-*	NR
D-fructose	-	-	-	+*	+*	-
sucrose	+	-	-	+	-*	NR
glycerol	+	-	-	+	+*	NR
Avicel™	+	+	+	-*	+*	NR
CMC	+	-*	NR	-*	+*	NR
dextrin	-	-*	NR	+*	-*	NR
xanthan	-	-*	NR	+*	-*	NR

yeast extract	+	-	-	+	-*	-
CO	-	-	+	+	+	-
Antibiotic resistance	las [†] , met [†] , tri [†]	met*, tri*	met*, tri*	chl, met [†] *, str, tri [†] *	met [†] *	NR
Primary fatty acids	12-methyl C _{18:0} , C _{18:0}	12-methyl C _{18:0}	12-methyl C _{18:0}	12-methyl C _{18:0} , C _{18:0}	12-methyl C _{18:0} , C _{18:0} *	12-methyl C _{18:0}
Primary quinones	MK-8, MK-9	NR	NR	MK-8, MK-8 2,3-epoxide*	MK-8	MK-8
G+C content (mol%)	58.4	60.7	66.0	64.3	66.0	62.6

+, positive; -, negative; NR, not reported.

[†] stimulates growth; chl, chloramphenicol; las, lasalocid A; met, metronidazole; str, streptomycin; tri, trimethoprim

Figure 1

[Click here to download Figure: Figure_1_phylogenetic tree.jpg](#)

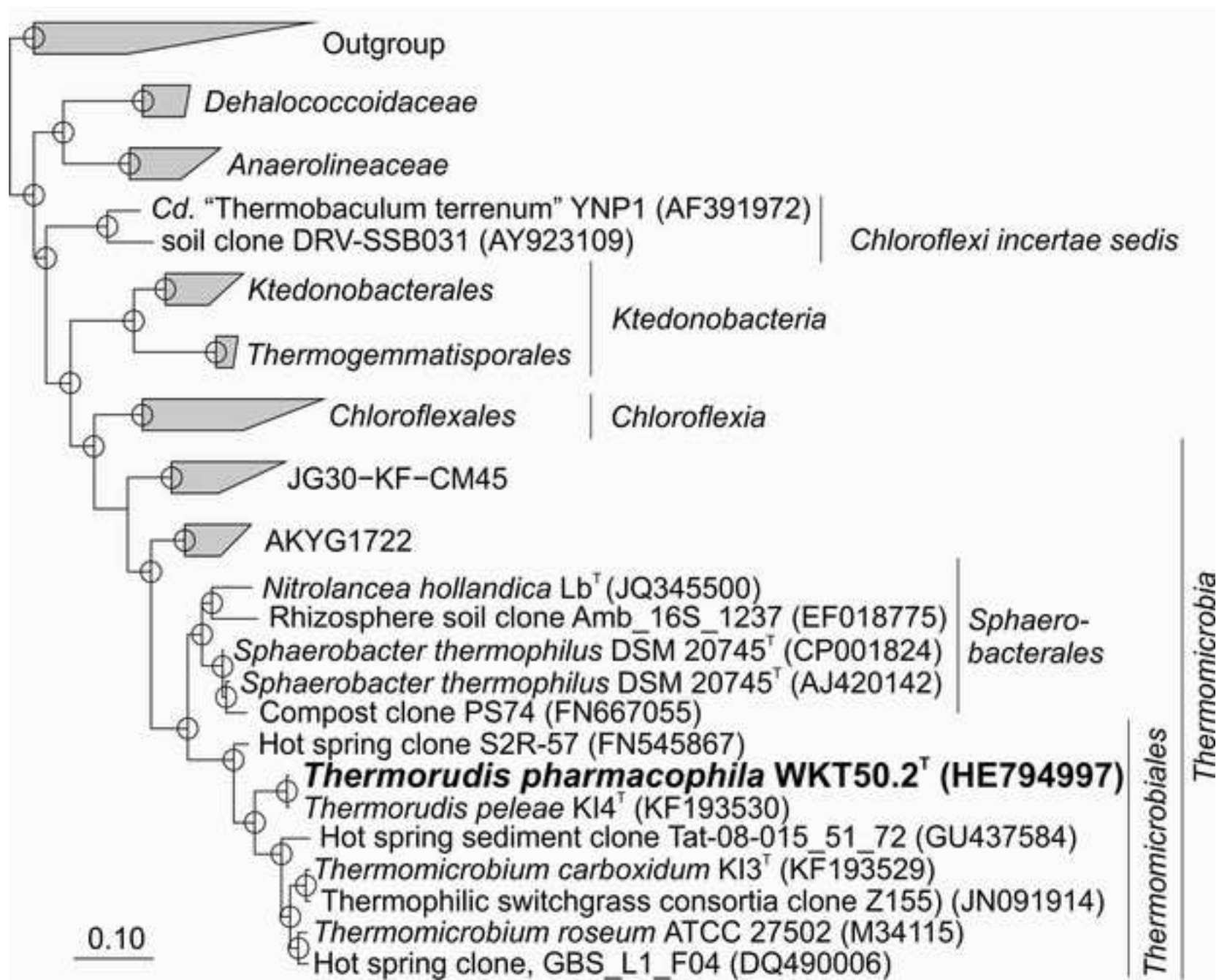
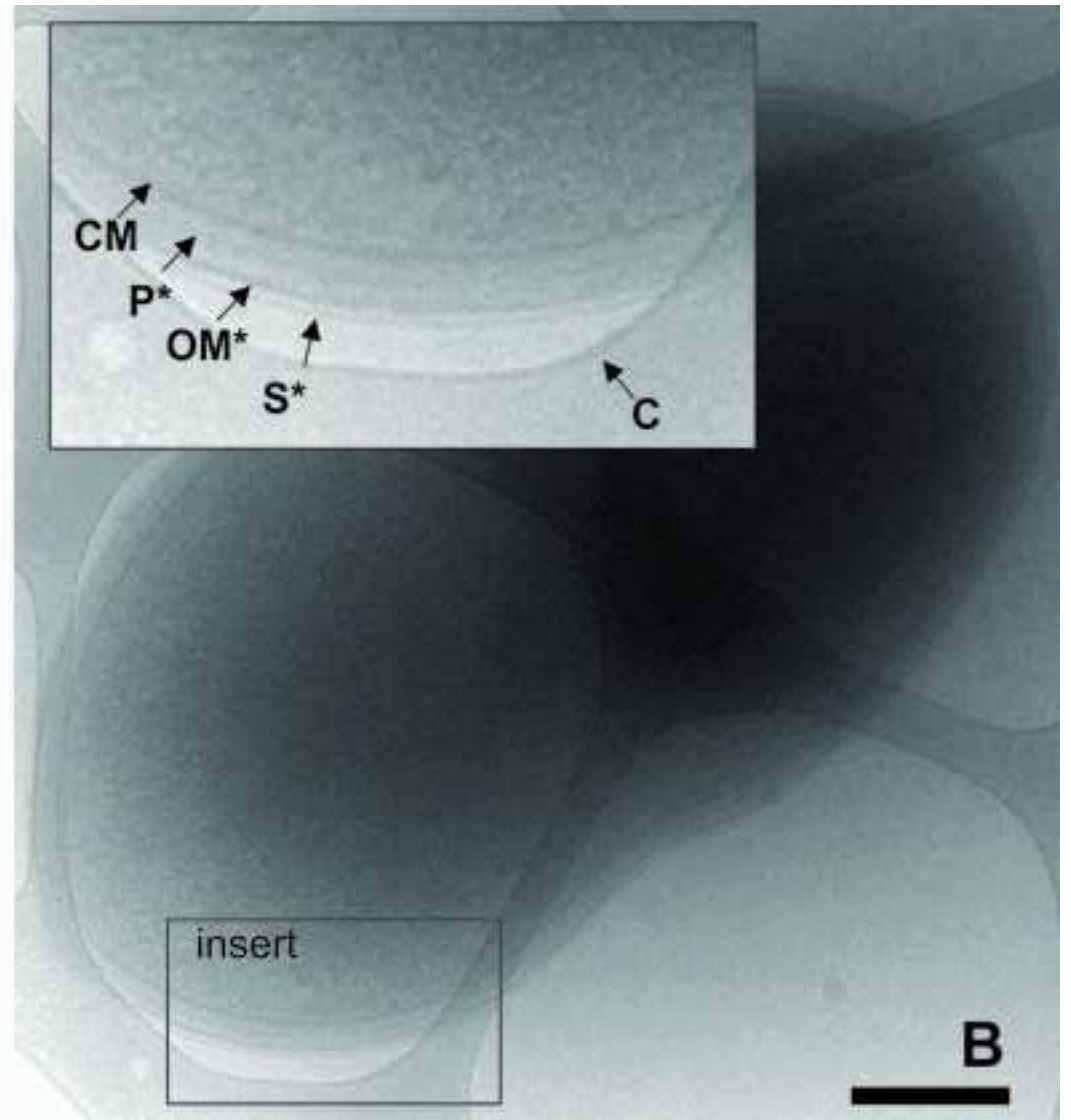
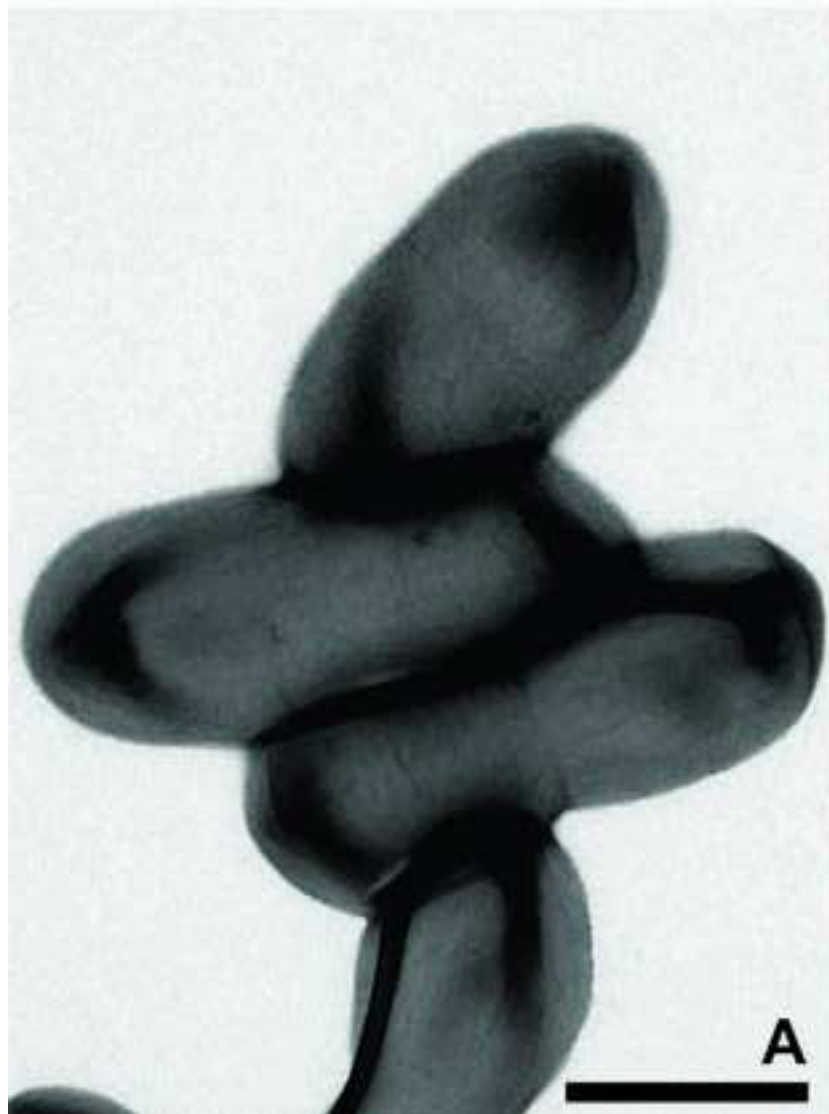


Figure 2

[Click here to download Figure: Figure 2_TEM.jpg](#)



1 **International Journal of Systematic and Applied Microbiology**

2

3 **SUPPLEMENTARY INFORMATION**

4

5 **Manuscript Title:**

6 ***Thermorudis pharmacophila* WKT50.2^T sp. nov., a novel isolate of class *Thermomicrobia***
7 **isolated from geothermal soil, and emended descriptions of *Thermomicrobium roseum*,**
8 ***Thermomicrobium carboxidum*, *Thermorudis peleae* and *Sphaerobacter thermophilus*.**

9

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22

23 **MATERIALS AND METHODS**

24

25 **General**

26 Unless otherwise stated, the following methodology was used for all media. The pH of all media was
27 adjusted prior to sterilisation with either 1 M NaOH or 1 M H₂SO₄. Medium pH was measured at
28 room temperature using a PHM250 Ion Analyser pH probe (Radiometer Analytical SAS, Lyon,
29 France). Crimp-top serum bottles or Schott™ bottles were filled such that the medium to headspace
30 ratio was approximately 1:1. The headspace was always aerobic unless otherwise stated, and the
31 bottles were sealed with butyl rubber septa prior to sterilisation. All media were autoclaved at 121 °C
32 and 15 lb/in² for 20 min using a P52014 Pressure Vessel (Mercer Stainless Limited, Henderson, New
33 Zealand) and were stored at 4 °C. Solid media were aseptically transferred immediately after
34 sterilisation at about 80 °C into sterile petri dishes and allowed to solidify. Growth was determined
35 via optical density at 600 nm (OD₆₀₀) using 1 ml polystyrene cuvettes with a pathlength of 1 cm

36 (Thermo Fisher Scientific Inc., Waltham, USA) or a 17 mm test tube holder (Perkin Elmer) on a
37 HALO VIS-10 Spectrometer (Dynamica, Australia).

38

39 **Phenotypic testing**

40 **General.** All substrate utilisation tests were performed using minimal medium (Castenholz salts
41 solution (Ramaley *et al.*, 1970): detailed below) without any energy source. All other metabolic testing
42 was conducted using Castenholz salts solution with the addition of 0.5 g l⁻¹ peptone and 0.25 g l⁻¹
43 sucrose (CPS), or 5 g l⁻¹ peptone and 2.5 g l⁻¹ sucrose (10x CPS). Inoculum for phenotypic
44 determination was generated by growing cells in 10x CPS for 2-3 days. Cells were cultured in
45 triplicate in 125 ml serum bottles sealed with rubber butyl septa, and containing an aerobic headspace
46 to medium ratio of 1:1. Cells were incubated in an orbital incubator at 68 °C (WKT50.2^T and *T.*
47 *roseum*) or 55 °C (*S. thermophilus*) and 130 r.p.m., and growth was determined based on the fastest
48 growth rate and highest OD₆₀₀ obtained. For all tests, a negative control with no carbon/energy source
49 and a positive control using the standard growth medium were applied in parallel.

50

51

52 **Castenholz salts solution** (Ramaley *et al.*, 1970)

Nitrilotriacetic acid	0.1 g l ⁻¹
MgSO ₄ .7H ₂ O	0.1 g l ⁻¹
CaCl ₂ .2H ₂ O	0.06 g l ⁻¹
NaCl	0.008 g l ⁻¹
NaNO ₃	0.689 g l ⁻¹
KNO ₃	0.103 g l ⁻¹
Na ₂ HPO ₄ .12H ₂ O	0.111 g l ⁻¹
FeCl ₂ solution	1 ml l ⁻¹
Nitsch element solution	1 ml l ⁻¹

53

54

55 **FeCl₂ Solution**

FeCl ₂ .4H ₂ O	0.44 g l ⁻¹
--------------------------------------	------------------------

56

57 **Nitsch Element Solution**

Concentrated H ₂ SO ₄	0.5 ml l ⁻¹
MnCl ₂ .4H ₂ O	2.8 g l ⁻¹
ZnSO ₄ .7H ₂ O	0.5 g l ⁻¹
H ₃ BO ₃	0.5 g l ⁻¹
CuSO ₄ .5H ₂ O	0.16 g l ⁻¹
Na ₂ MoO ₄ .2H ₂ O	0.03 g l ⁻¹
CoCl ₂ .6H ₂ O	0.046 g l ⁻¹

58

59

60 **TEM analysis.** Transmission Electron Microscopy (TEM) was performed at the Bio21 Institute
 61 Advanced Microscopy Facility, University of Melbourne, Australia. Two different methods were
 62 applied to visualise cells in exponential growth phase:

63 i) For negative staining, cells were absorbed on a glow-discharged formvar carbon-coated 400-
 64 mesh copper grid, and stained with 2 % (w/v) uranyl acetate. The resulting grids were observed
 65 on a FEI Tecnai F20 operating at 200 kV and digital micrographs were recorded with a Gatan
 66 Ultrascan 1000.

67 ii) For cryoTEM, cells were absorbed on glow discharge holey carbon grids and plunge frozen in
 68 liquid ethane. The resulting grids were observed on a FEI Tecnai F30 operating at 300 kV under
 69 low dose. Each micrograph was recorded on a Gatan Ultrascan 1000 with a total dose of ~2,000
 70 e⁻ nm⁻².

71

72 **Fatty acids analysis.** Cells were prepared for fatty acid analysis by combining cell biomass from 2 l
73 of 10x CPS enrichment grown aerobically at 68 °C (WKT50.2^T and *T. roseum*) or 55 °C (*S.*
74 *thermophilus*) at 130 r.p.m. Lipids were extracted from wet biomass (Bligh and Dyer, 1959) and
75 methylated (Carreau *et al.*, 1978). GC analysis of fatty acid methyl esters was performed on a
76 TraceGC Ultra instrument (ThermoFinnigan, USA), equipped with a FID and TG WaxMS (30 m ×
77 0.25 mm i.d., 0.25 µm) capillary column (ThermoFinnigan, USA). Helium was used as the carrier gas;
78 the split ratio was 1:100. The oven temperature was 190 °C. The injector and detector temperatures
79 were 280 °C. Samples were reanalysed on a GCMS-QP2010Ultra (Shimadzu) equipped MS detector
80 and TG WaxMS (30 m × 0.25 mm i.d., 0.25 µm) capillary column (ThermoFinnigan, USA) to confirm
81 fatty acid structural assignments. Helium was used as the carrier gas; the split ratio was 1:30.
82 Separation temperature was 200 °C. Injector temperature was 280 °C. Fatty acids were identified by
83 equivalent chain length (ECL) values (Stránský *et al.*, 1992), mass spectra, and comparison with fatty
84 acid mixtures of known composition derived from other bacteria.

85
86 **Polar lipid analysis.** Total cellular lipids were extracted from the biomass using as previously
87 described (Bligh *et al.*, 1959). Polar lipids were separated by two-dimensional thin layer
88 chromatography (silica gel, Macherey-Nagel Art. No. 818 033, cut to 10 x 10 cm). The first direction
89 was developed in chloroform: methanol: water (65:25:4, v/v/v), and the second in chloroform:
90 methanol: acetic acid: water (80:12:15:4, v/v/v/v) (Figure S1) (Tindall *et al.*, 2007). Primary amines
91 were detected with 0.2 % (w/v) ninhydrin in absolute ethanol. Phospholipids were detected with the
92 molybdate spray reagent (Vaskovsky *et al.*, 1975). Glycolipids were detected with 0.5 % (w/v)
93 anthrone in toluene. Another duplicate plate was charred by spraying with 10 % (v/v) sulfuric acid in
94 methanol and incubating at 120 °C for 30 min, in order to show total lipids. Phospholipid classes in
95 the extract were observed using ³¹P NMR (MacKenzie *et al.*, 2009).

96
97 **Anaerobic growth.** Cells were grown in a modified Castenholz salts solution with nitrate replaced by
98 0.58 g l⁻¹ NH₄Cl, and MgSO₄·7H₂O replaced with 0.08 g l⁻¹ MgCl₂·6H₂O. Sucrose (2.5 g l⁻¹) was used
99 as a carbon source for WKT50.2^T and *T. roseum*, and fructose (2.5 g l⁻¹) for *S. thermophilus*.
100 Anaerobic growth was tested using either elemental S⁰ (6.5 g l⁻¹), Na₂SO₄ (0.5 g l⁻¹) or NaNO₃ (0.5 g l⁻¹).
101 Resazurin (1 mg l⁻¹) and Na₂S·9H₂O (1 mM) were added to the medium, as an anaerobic indicator
102 and reducing agent respectively, prior to sterilisation. The headspace comprised (v/v): 85 % N₂, 10 %
103 H₂ and 5 % CO₂. Vials were incubated at 65 °C for WKT50.2^T and *T. roseum*, 55 °C for *S.*
104 *thermophilus*.

105

106

107 **Temperature range and optimum determination.** Cells were grown in 10 ml medium in 27 ml
108 anaerobic tubes sealed with butyl rubber stoppers. Headspaces were aerobic. The tubes were incubated
109 in duplicate in a temperature gradient incubator (Terratec Corporation, Hobart, Australia) set from 49
110 °C to 84 °C and agitated at 30 oscillations per min. There were 24 tubes over the temperature range
111 with an average of 1.5 °C between each subsequent tube.

112

113 **pH range and optimum determination.** The pH growth range was determined by growing cells in
114 buffered 10x CPS liquid medium between pH 4.5 and 9.2. The buffers used were acetate (buffer range
115 pH 4.5-6.5) or Tris (buffer range pH 6.9-9.2). pH was measured after sterilisation and inoculation.

116

117 **Salt tolerance.** Cells were grown in 10x CPS, with the addition of either 0.5, 1, 2, 3, 4 or 5 % (w/v)
118 NaCl. Growth was determined by the visible formation of biomass and measurement of OD₆₀₀ nm.

119

120 **Preferred N-source.** The preferred nitrogen source was determined using a modified version of
121 Castenholz salts solution without NaNO₃ or KNO₃, and sucrose or fructose (0.5 g l⁻¹) as carbon source.
122 Initially, dinitrogen gas as an N-source was tested by incubating strains in Castenholz salts medium
123 minus KNO₃. As no N₂-fixation was observed, no manipulation of the headspace was required to test
124 alternate N-sources. Nitrogen sources tested were either KNO₃ (0.689 g l⁻¹), (NH₄)₂SO₄ (1.48 g l⁻¹),
125 KNO₂ (1.7g l⁻¹), urea (0.23 g l⁻¹), alanine (0.70 g l⁻¹), serine (0.80 g l⁻¹), trimethoprim (30 µg ml⁻¹), or
126 metronidazole (30 µg ml⁻¹). All nitrogen sources were added after sterilisation by aseptically passing
127 them into the medium via a 0.22 µm syringe filter.

128

129 **Carbon monoxide utilisation.** The induced oxidation of carbon monoxide was determined using a
130 method previously described (Hardy *et al.*, 2001). WKT50.2^T cells were grown in Castenholz salts
131 solution with 0.05 % (w/v) yeast extract. The headspace contained 100 ppm of CO in air. For testing
132 constitutive oxidation, cells were grown in 10x CPS media initially with an aerobic headspace, then
133 100 ppm of CO was added once cells had grown to form visible biomass. For CO analysis, gas
134 samples were removed from vials and analysed immediately using a Peak Performer 1 Gas Analyser
135 (Peak Laboratories, Mountain View, C.A., USA) equipped with Unibeads 1S and Molecular Sieve
136 13X columns, and a Reducing Compound Photometer. The temperature was set at 105 °C, and
137 compressed air was used as a carrier. Detection limits were about 2 ppm. The instrument was
138 standardized using a certified CO standard (1044 ppm, Airgas Gulf States, Theodore, USA), and
139 laboratory dilutions of this standard.

140

141

142 PCR was performed using primers specific to the large subunit of carbon monoxide dehydrogenase
143 genes (King, 2003) to amplify putative *coxL* fragments. Further primer pairs (Table S5) were
144 designed on the basis of conserved motifs, including the active site, using the genomes of *T. roseum*
145 (http://www.ncbi.nlm.nih.gov/nuccore/NC_011959.1) and *S. thermophilus*
146 (http://www.ncbi.nlm.nih.gov/nuccore/NC_013524.1), using Geneious version 6.0.5 (Biomatters,
147 <http://www.geneious.com/>). PCR was carried out in 50 µl reaction volumes in 200 µl tubes containing
148 100 µM dNTPs, 0.5 µM primers and 1U Intron i-Taq™. The final concentration of MgCl₂ was 1.5
149 mM. DNA extracts from cultures were used at final concentrations of 10-50 ng. The PCR conditions
150 were as follows: 94 °C for 3 min; 10 cycles consisting of 94 °C for 45 sec, 62 °C for 60 sec, with a 1 °C
151 decrease every 2 cycles, and 72 °C for 90 sec; 30 cycles consisting of 94 °C for 45 sec, 58 °C for 60
152 sec and 72 °C for 90 sec; and a final elongation step at 72 °C for 20 min. PCR amplifications were
153 performed using a CGI-96 thermal cycler (Corbett Research, Sydney, Australia) with PalmCycler™
154 software, v2.1.10.

155
156 **Extraction of respiratory quinones.** Respiratory quinones were extracted using a modified version of
157 the method described by Nishijima and colleagues (Nishijima *et al.*, 1997). Briefly, approximately 5-
158 30 mg of freeze dried cell material was ground to a fine powder using a mortar and pestle and
159 extracted by addition of 2:1 chloroform/methanol. The resultant solution was ultrasonicated at room
160 temperature for 15 min then filtered through a 0.2 µm nylon syringe filter. The filtered solution was
161 dried under a stream of nitrogen and dissolved in acetone prior to analysis by LCMS.

162
163 **Analysis of respiratory quinones.** Analysis of extracts were carried out using a modified version of
164 the method described by Nishijima and colleagues (Nishijima *et al.*, 1997). Extracts dissolved in
165 acetone were analysed by LCMS on a system comprising of a Shimadzu 8040_LCMS system
166 equipped with photodiode array detector and triple quadrupole low resolution MS utilising an APCI
167 source for ionisation. High resolution MS by direct infusion was carried out on a Waters Premier QTof
168 instrument. LCMS chromatography was carried out on a Waters Acquity BEH C18 1.7 µm, 2x150
169 mm column at 30 °C. A linear gradient elution profile was employed. The initial elution condition
170 was 100 % methanol, which was held for 1 min. At 4 min, 2-propanol was injected to 5 % (v/v), then
171 the concentration increased at a linear rate to 100 % 2-propanol at 22 min, and held at this
172 concentration for 1 min before being reduced to initial conditions over 1 min. The column was
173 equilibrated for 7 min prior to a subsequent injection being made. A flow rate of 0.2 ml min⁻¹ was
174 employed. The class of respiratory quinones assigned to observed peaks in the UV-Vis and MS TIC
175 chromatograms were determined from a combination of their characteristic UV-Vis online absorption
176 spectra and their characteristic MS/MS fragmentation patterns. The number of isoprene units for each
177 quinone observed was determined from their retention times, with reference to quinone peaks

178 observed in chromatograms of microbial extracts of known composition, and their low resolution m/z
179 values indicating chain length.

180

181 The unique respiratory quinone peak observed in *T. roseum* extracts was tentatively identified as MK-
182 8 2,3-epoxide via a range of methodologies and observations: LCMS analyses of the *T. roseum* extract
183 (Fig. S4) showed the compound's peak eluted earlier than that of MK-8 (recorded at 270 nm). The
184 peak had a low resolution mass of 733 m/z [M+H]⁺ which suggested that the compound was 16 mass
185 units greater than MK-8. High resolution MS analysis of a quinone-rich extract of *T. roseum* by direct
186 infusion MS indicated a molecular formula of C₄₄H₇₇O₈ [M+H]⁺, consistent with presence of an
187 additional oxygen. The observation that the UV absorption spectrum of the unknown compound
188 (absorption maxima observed at 227, 265 sh, 303 nm - Fig. S5), was distinct from that of MK-8
189 (absorption maxima observed at 249, 263 sh, 270 nm - Fig. S6), suggested that the additional oxygen
190 substitution was to the main quinone ring rather than to the isoprene tail. An extract of *S.*
191 *thermophilus*, which almost exclusively produces MK-8, underwent epoxidation following the method
192 reported for epoxidation of MK-4 to MK-4 2,3-epoxide (Suhara, 2010). The resultant reaction
193 solution gave a single quinone peak on LCMS that had a low resolution mass of 733 m/z [M+H]⁺, and
194 a similar on-line UV absorption spectrum to that of the unknown peak. When the *T. roseum* extract
195 was mixed with the epoxidation reaction mixture and injected onto the LCMS it was found that both
196 peaks co-eluted as a single peak. These results support the hypothesis that the structure of the
197 unknown quinone is MK-8 2,3-epoxide.

198

199 As an analytical standard of MK-8 2,3-epoxide was not available for comparison, the relative levels of
200 quinones in *T. roseum* were determined by comparison of peak areas in the UV chromatograms
201 recorded on the LCMS. For relative levels of MK-8 and the putative MK-8 2,3 epoxide, peak areas at
202 248 nm (MK-8) and 266 nm (MK-8 2,3 epoxide) were compared with reference to the molar
203 extinction coefficients published for MK-4 and MK-4 2,3 epoxide at these wavelengths (Sherman
204 1981). For relative levels of MK-8 and MK-9, it was assumed that the molar extinction coefficients
205 for both compounds at 270 nm were the same (Nishijima *et al.*, 1997).

206

207 **Genome screening for enzymes involved in cell envelope synthesis and function.** The draft
208 WKT50.2^T genome was screened for genes encoding proteins involved in plasma membrane
209 translocation, and for genes involved in secretory systems and biosynthesis of the outer membrane.
210 The gene presence / absence analysis was undertaken via word search in the RAST server (Aziz *et al.*,
211 2008). Key protein family domains genes were referenced as per Sutcliffe (2011 – see below).

212

213 Representative Pfam domains for proteins associated with plasma membrane translocation pathways,
 214 and secretions systems and outer membranes. Adapted from Sutcliffe (2011). Presence of gene
 215 indicated by 'Y', absence 'N'.

System	Cellular location	Pfam domain	WKT50.2 ^T
Tat translocon	Plasma membrane	Pf00902	Y
Lipoprotein biogenesis	Plasma membrane	Pf01790	Y
	Plasma membrane	Pf01252	Y
Sec tranlocon	Plasma membrane	Pf00344	Y
Outer membrane assembly	Outer membrane	Pf07244 & Pf01103	N
	Periplasm	Pf00847	
Secretion systems *			
T1SS	Outer membrane & Periplasm	Pf02321	N
T2SS, T3SS	Outer membrane	Pf00263	N
	Outer membrane	Pf03958	N
	Outer membrane	Pf07660	
T4SS	Outer membrane	Pf03524	N
	Periplasm/plasma membrane	Pf04335	N
	Periplasm/plasma membrane	Pf03743	N
T5aSS	Outer membrane	Pf03797	N
T5bSS	Outer membrane	Pf03865	N
T5cSS	Outer membrane	Pf03895	N
T6SS	Tubule	Pf05638	N
T7SS	Outer membrane	Pf00577	N
T8SS	Outer membrane	Pf10614	N

216 * Secretion systems nomenclature as per Desvaux *et al.*, 2009.

217

218 **SUPPLEMENTARY TABLES**219 **Table S1.** Fatty acid composition of WKT50.2^T and *S. thermophilus* in weight % of total fatty acids.

220 Only components > 0.5 % are presented.

221

Saturated fatty acids	WKT50.2 ^T	<i>S. thermophilus</i>
C _{14:0}	1.2	1.5
C _{16:0}	5.4	9.5
C _{18:0}	30.6	17.1
C _{20:0}	7.3	-
Unsaturated fatty acid		
C _{18:1 n-9} *	-	2.3
Methyl-branched fatty acids		
10-methyl C _{16:0} *	1.5	5.5
12-methyl C _{17:0} *	-	0.9
12-methyl C _{18:0} *	54.0	63.2

222 * - positions of unsaturation and branching were identified by GCMS.

223

224 **Table S2.** Polar lipids of WKT50.2^T and *S. thermophilus*

	WKT50.2 ^T	<i>S. thermophilus</i>
Phospholipids	mol%	
diolP-acylMan	17.9	17.0
diolPI-Man	6.7	33.3
diolPI-acylMan	11.5	8.4
diolPI	64.0	41.3
1,2-diols	% 1,2-diols	
11-methyl C _{17:0}	-	0.8
13-methyl C _{18:0}	-	4.4
n19:0	15.2	20.2
13-methyl C _{19:0}	26.0	66.2
C _{21:0} <i>br</i>	-	3.3
n20:0	1.2	0.2
13-methyl C _{20:0}	0.3	0.7
n21:0	50.6	-
15-methyl C _{21:0}	1.4	-

225

226

227 **Table S3.** Substrate utilisation of WKT50.2^T, *S. thermophilus* and *T. roseum*. Substrates at 0.2 %
 228 unless otherwise stated.
 229

Group	Substrate	WKT50.2 ^T	<i>S. thermophilus</i>	<i>T. roseum</i>
Monosaccharides	DL-arabinose	-	ND	ND
	D-fructose	-	+	+
	L-fucose	-	ND	ND
	D-galactose	-	-	+
	D-glucose	-	-	+
	D-mannose	-	-	+
	rhamnose	-	ND	ND
	D-ribose	-	ND	ND
	D-xylose	-	-	+
Disaccharides	D-cellobiose	-	+	+
	lactose	-	ND	ND
	D-maltose	-	-	+
	sucrose	+	-	+
	D-trehalose	+	-	+
Trisaccharide	D-raffinose	+	-	+
Sugar derivatives	D-galacturonic acid	-	ND	ND
	D-glucuronic acid	-	ND	ND
	D-N-acetylglucosamine	-	ND	ND
Glucose-based polymers	Avicel TM	+	+	-
	CMC	+	+	-
	dextrin	-	-	+
	starch	+	+	+
	Whatman TM filter paper	±	ND	ND
Algal and plant-derived polymers	agarose	-	ND	ND
	sodium alginate	+	ND	ND
	galactomannan	-	ND	ND
	glucomannan	-	ND	ND
	lignin	-	ND	ND
	pectin	-	ND	ND
Bacterial exopolysaccharides	xylan	+	ND	ND
	Phytigel TM	-	+	+
Animal-derived polymers	xanthan	-	-	+
	chitin	-	ND	ND
Organic salts	sodium acetate	+	+	+
	trisodium citrate	-	+	+
	sodium formate	+	+	+
	sodium fumarate	+	+	+
	sodium lactate	+	+	+
	sodium pyruvate	+	+	+
	sodium succinate	+	+	+

++ very good growth; + growth; ± weak growth; - no growth; ND, not determined

Group	Substrate	WKT50.2 ^T	<i>S. thermophilus</i>	<i>T. roseum</i>
Alcohols (0.05 %)	methanol	+	-	+
	ethanol	+	+	+
	1-propanol	-	+	+
	2-propanol	-	+	+
Sugar alcohols	sorbitol	+	-	-
	mannitol	-	+	+
Protein derivatives / amino acids	yeast extract	+	-	+
	peptone	++	+	+
	tryptone	+	+	+
	Casamino acids	+	+	+
	alanine	-	ND	ND
	cysteine	-	ND	ND
	glutamic acid	-	ND	-
	glycine	-	ND	ND
	leucine	-	ND	ND
	lysine	-	ND	ND
	methionine	-	ND	ND
	serine	-	ND	ND
	tryptophan	-	ND	ND
	valine	-	ND	ND
Others	B-vitamins (Stott <i>et al.</i> , 2008)	-	ND	ND
	sodium benzoate	-	ND	ND
	ascorbic acid	-	ND	ND
	glycerol	++	+	+
	methylamine (0.05 %)	-	ND	ND
	trimethylamine (0.05 %)	-	ND	ND
	trimethoprim (30 µg ml ⁻¹)	-	ND	ND
	metronidazole (30 µg ml ⁻¹)	-	ND	ND
	Nutrient broth	+	+	ND
	R2A	+	ND	+

++ very good growth; + growth; ± weak growth; - no growth; ND, not determined;

230
231
232

Table S4. Primer sequences for coxL amplification

Name	Sequence 5' – 3'
OMPf *	GGC GGC TTY GGS AAS AAG GT
BMSf *	GGC GGC TTY GGS TCS AAG AT
O/Br*	YTC GAY GAT CAT CGG RTT GA
cox F1	TAC GTC GAC GAC GTC AAA CT
cox F2	GTC GAG TAC GAG CCG CTG
cox R1	CAR GGG CAG GGA CAC GAG AC
cox R2	TTY GCS GCC TAC ACG AAT TTC CCG C
Thermo cox F	TGC CGG TCT ACC CCG GCT AC
Thermo cox R	CTG TCG TAC TCC CAG CCG GT

*previously published (King, 2003).

233
234
235
236
237

238 **Table S5** – Growth response to metronidazole and trimethoprim of WKT50.2^T, *S. thermophilus* and *T.*
 239 *roseum*
 240

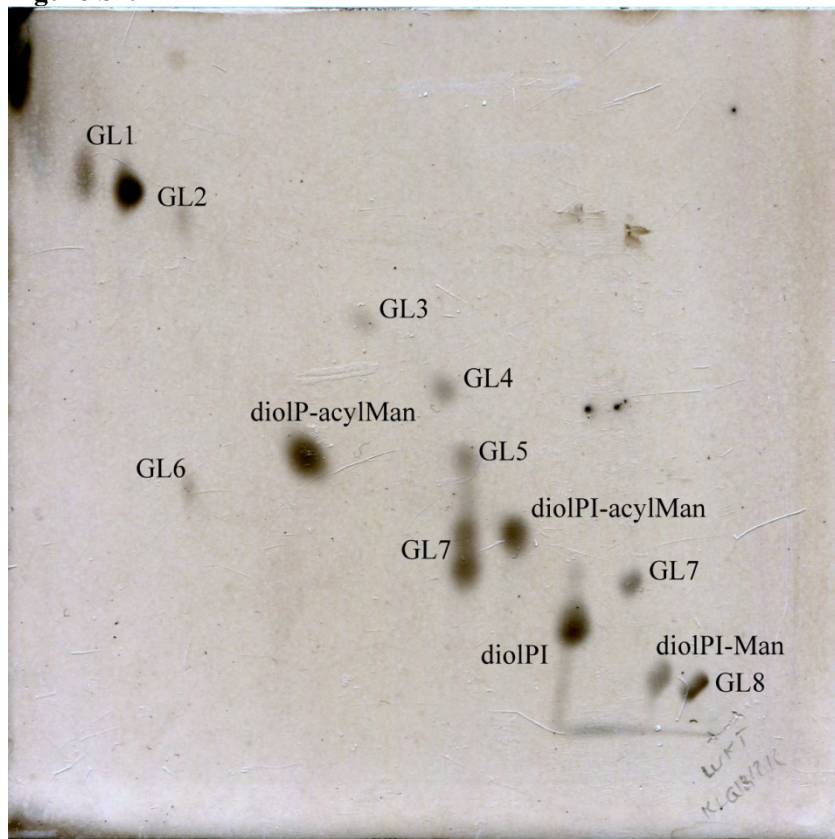
Antibiotic	% growth compared to positive control*		
	WKT50.2 ^T	<i>S. thermophilus</i>	<i>T. roseum</i>
metronidazole (3 µg ml ⁻¹)	113	39	196
metronidazole (30 µg ml ⁻¹)	343	105	202
trimethoprim (3 µg ml ⁻¹)	119	13	121
trimethoprim (30 µg ml ⁻¹)	135	2	134

241 *The mean OD₆₀₀ of three test cultures was compared to the mean of three controls, and the result is
 242 expressed as a percentage of growth compared to the control.
 243

244 **Table S6.** api[®]ZYM strips (bioMérieux) - selected enzyme activity for WKT50.2^T
 245

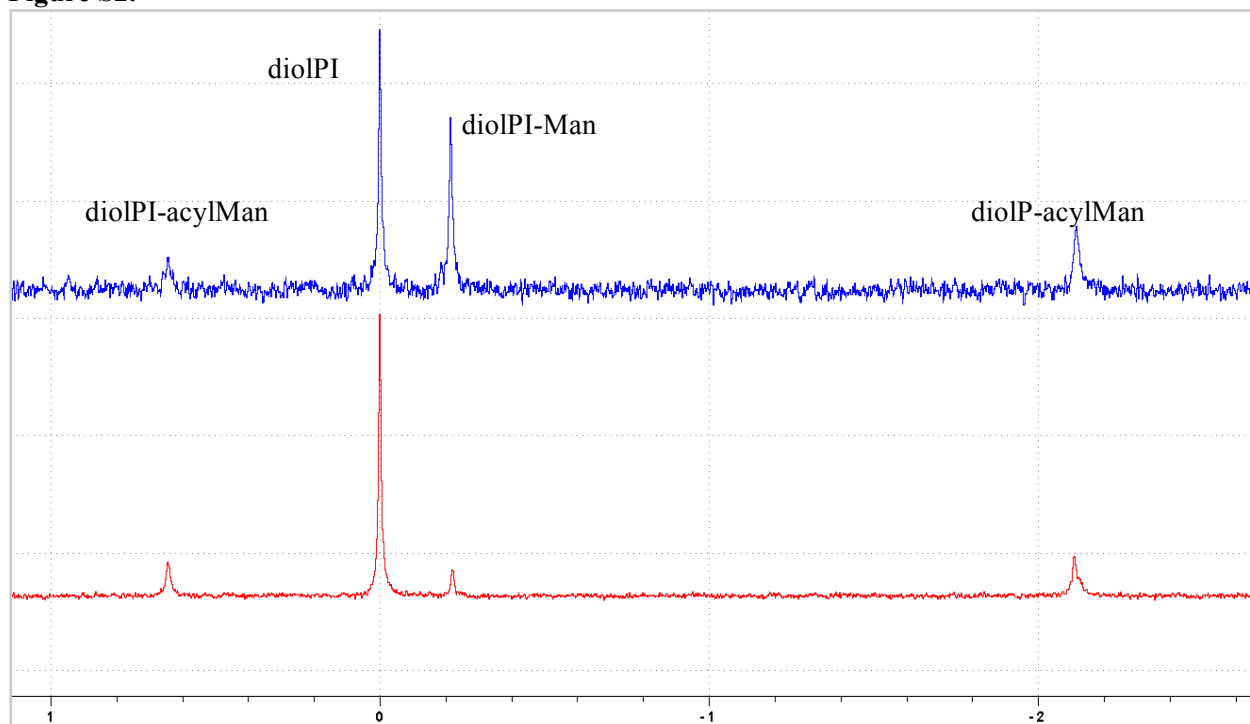
Positive activity	Weak or no activity
esterase (C4)	alkaline phosphatase
esterase lipase (C8)	cysteine arylamidase
lipase (C14)	α-chymotrypsin
leucine arylamidase	acid phosphatase
valine arylamidase	naphthol-AS-BI-phosphohydrolase
trypsin	α-galactosidase
	β-galactosidase
	β-glucuronidase
	α-glucosidase
	β-glucosidase
	N-acetyl-β glucosaminidase
	α-mannosidase
	α-fucosidase

246
 247
 248



253 **Figure S1.** 2D-TLC of strain WKT50.2^T, polar lipids visualised by charring after spraying with 10 %
254 v/v sulphuric acid in methanol. The first development is the vertical axis (bottom to top) and the
255 second development is the horizontal axis (right to left). GL – a glycolipid.

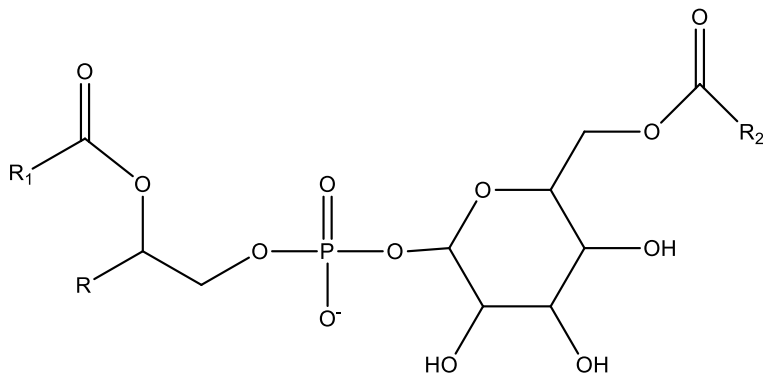
259 **Figure S2.**



260
261 **Figure S2.** ^{31}P NMR spectra of total lipid extracts from *S. thermophilus* (top) and WKT50.2^T
262 (bottom). The lipid extracts were dissolved in 10 % (w/w) sodium cholate, 1 % EDTA (w/w), aqueous
263 NMR detergent (80:20 v/v H₂O:D₂O, pH 7.2). Chemical shifts are relative to a 0.4 % (v/v) phosphoric
264 acid solution in a capillary insert.

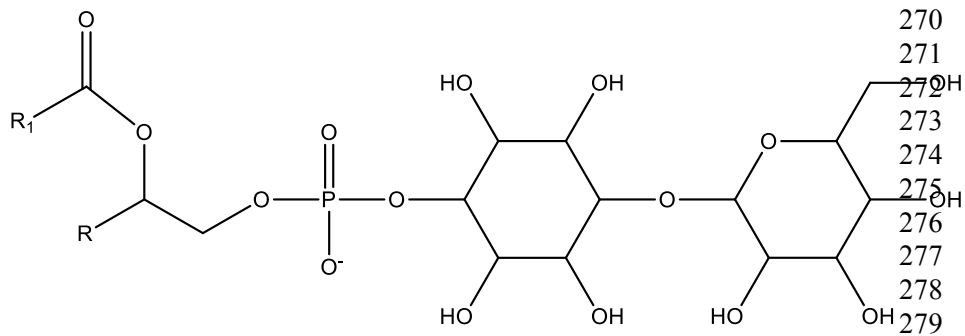
265
266

A

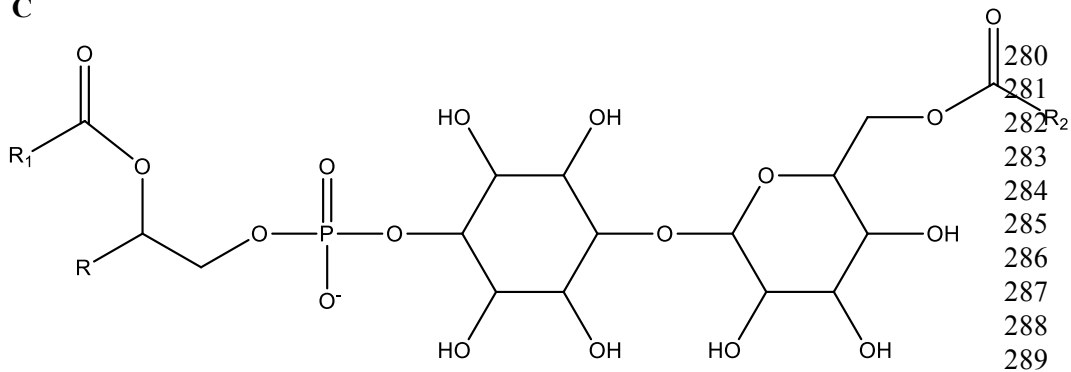


268

B

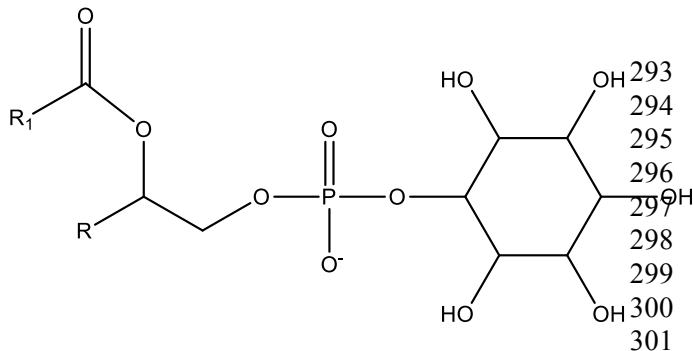


C



290

D

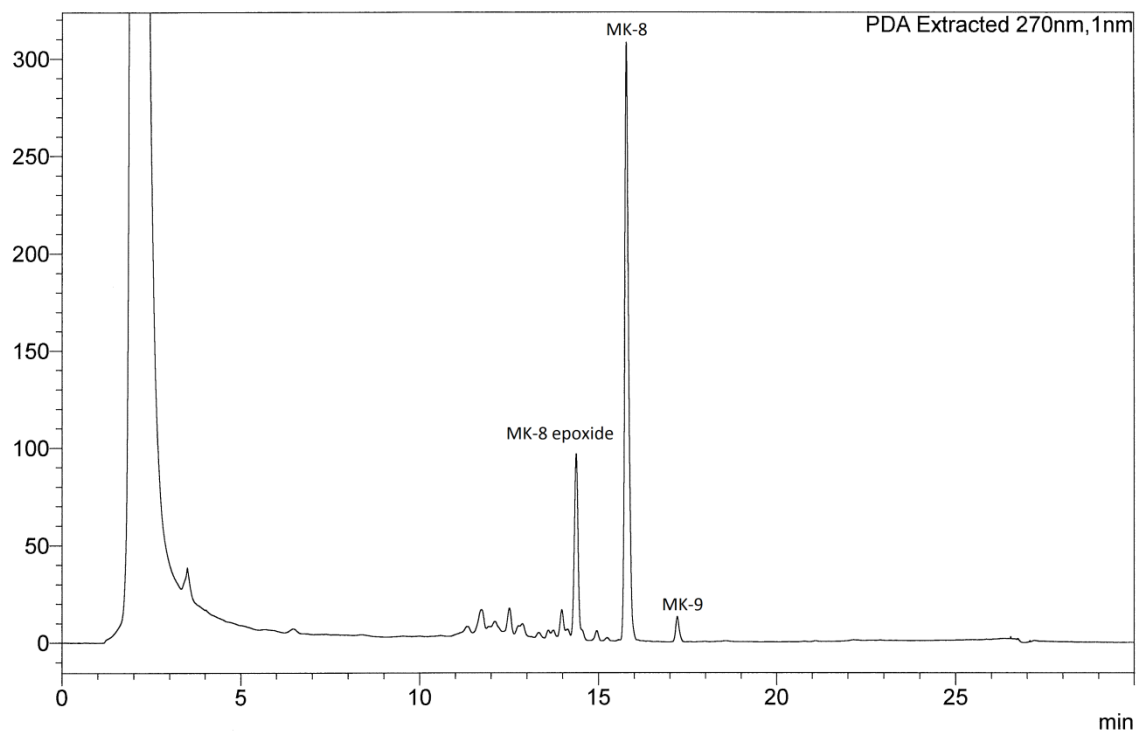


302 **Figure S3 A-D. Structures of the novel 1,2-diol based phospholipids found in WKT50.2^T and *S.***
 303 ***thermophilus*.** A) diol-phosphatidyl acylmannoside (diolP-acylMan), B) diol-phosphatidylinositol
 304 mannoside (diolPI-Man)*, C) diol-phosphatidylinositol acylmannoside (diolPI-acylMan)*, D) diol-
 305 phosphatidylinositol (diolPI). *The attachment position of the sugar to the inositol was not studied.

306

307 **Figure S4**

mAU

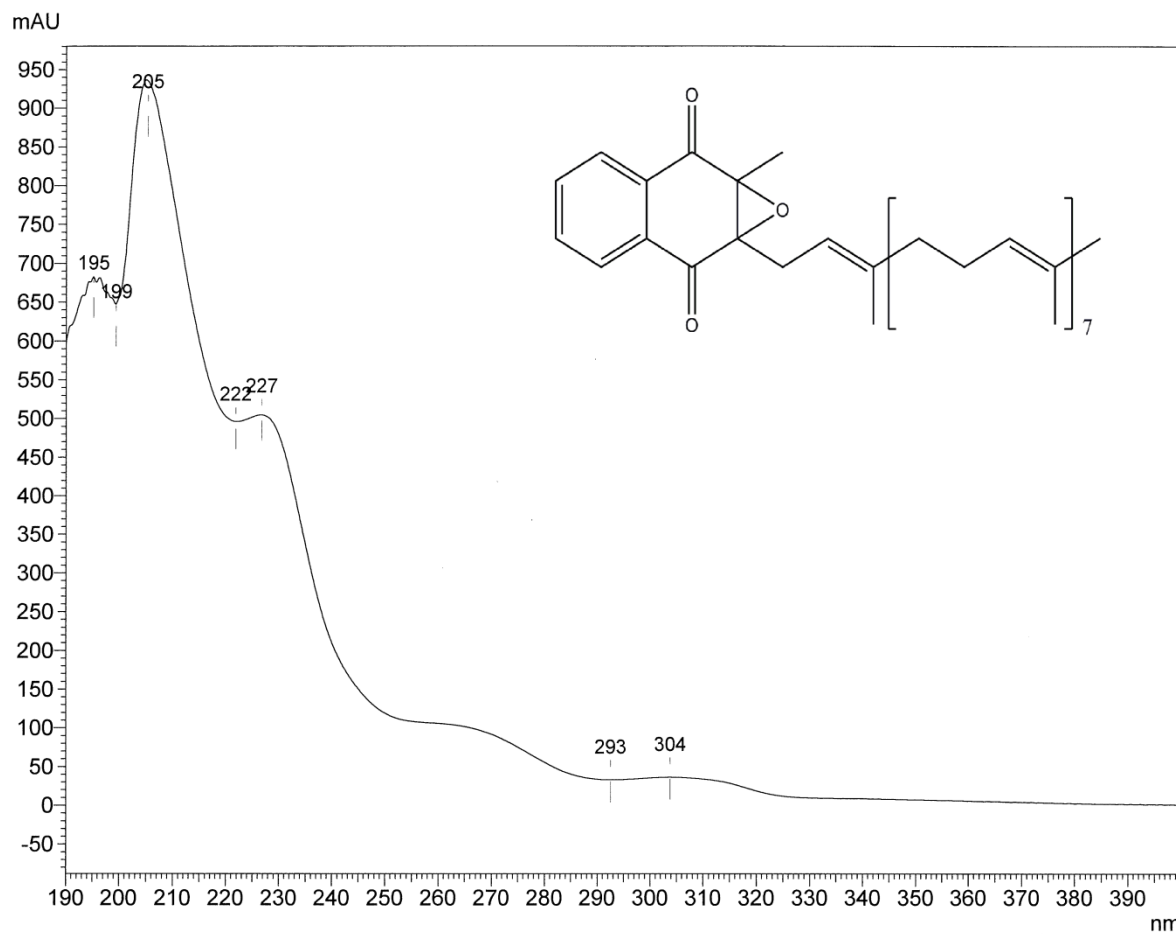


308

309 **Figure S4.** LCMS chromatogram of *T. roseum* extract recorded at 270 nm showing the three assigned
310 quinone peaks, MK-8 2,3-epoxide, MK-8, and MK-9.

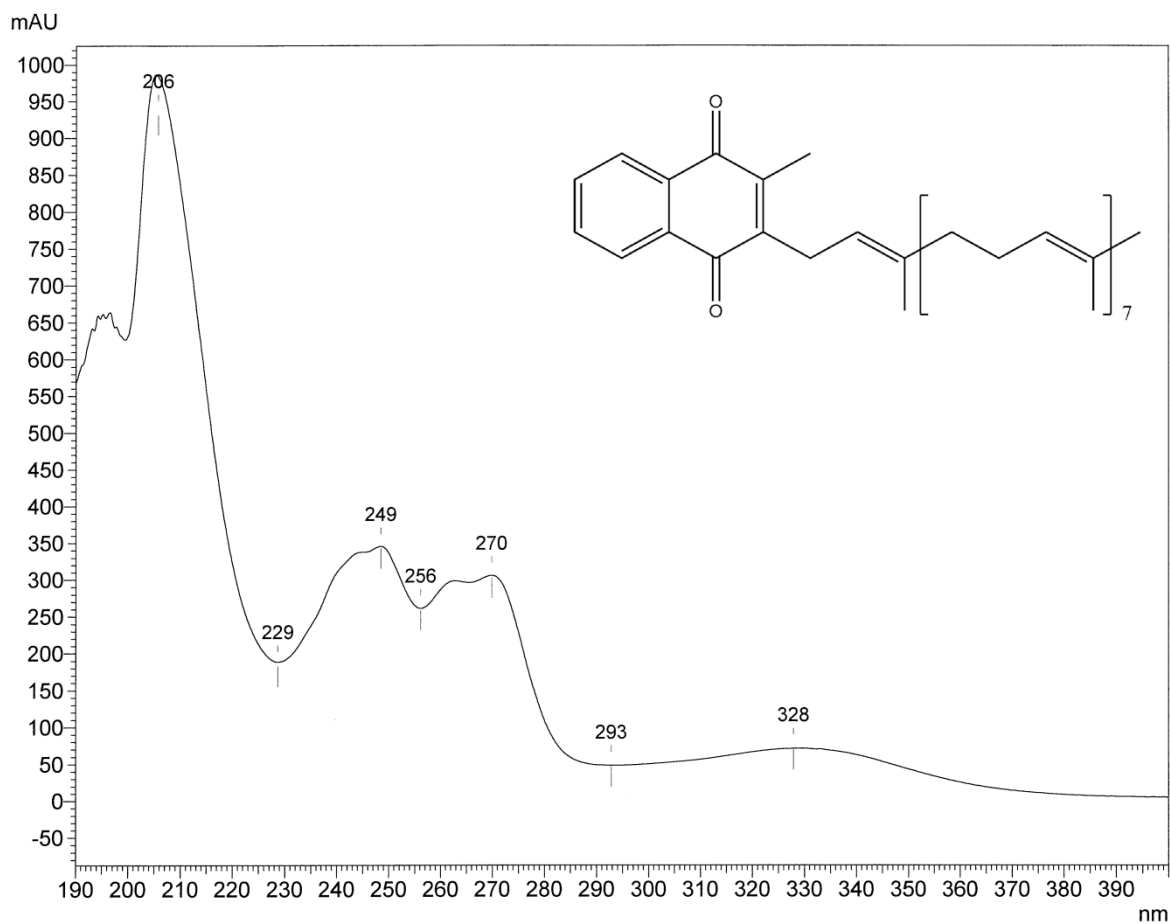
311

312 **Figure S5**



313
314 **Figure S5.** LCMS On-line UV-visible absorption spectrum of the peak proposed to be MK-8 epoxide
315 and the suggested structure of MK-8 2,3-epoxide.
316

317 **Figure S6**



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319 **Figure S6.** LCMS On-line UV-visible absorption spectrum of the peak representing MK-8 and the
320 structure of MK-8.

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