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**Screening of gastrointestinal lipase inhibitors produced by microorganisms isolated from soil
and lake sediments**

Maria Angeles Camacho-Ruiz ^{1,5}, Enrique Ordaz¹, Manuel R. Kirchmayr¹, Hugo Esquivel², Ali
Assaf⁴, Juan Carlos Mateos-Díaz¹, Frédéric Carrière³ and Jorge A. Rodriguez^{1*}

¹ Biotecnología Industrial, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Zapopan, Jalisco, Mexico.

² Biotecnología Médica y farmacéutica, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Guadalajara, Jalisco, Mexico.

³ CNRS, Aix Marseille Université, UMR 7282, Enzymologie Interfaciale et de Physiologie de la Lipolyse, Marseille, France.

⁴ Centro de Investigación en Alimentación y Desarrollo, A.C., Hermosillo, Sonora, 83340, México.

⁵ Departamento de Fundamentos del Conocimiento, Centro Universitario del Norte, Colotlán, Jalisco, Mexico.

* Correspondence to:

Jorge A. Rodriguez: Tel.: +52 (33) 33 45 52 00 ext. 2108; fax: ext. 1001. Email address:
jrodriguez@ciatej.mx

Running Head: Screening of lipase inhibitors

Abbreviations used:

β -CD, β -cyclodextrin; BCG, bromocresol green; BSA, bovine serum albumin; BtA, butyric acid; Gly-SM, Glycerol-Soy milk agar media; NaTDC, sodium taurodeoxycholate; PHIBLA, pH indicator-based lipase assay; RGL, rabbit gastric lipase; rHPL, recombinant human pancreatic lipase; TG(4:0), tributyrin; THL, Tetrahydrolipstatin; YMD, yeast extract-malt extract-dextrose agar media.

2 **Significance and impact of the study**

3 Only a few medications for obesity treatment are currently on the market. The inhibition of dietary
4 triglyceride absorption via inhibition of gastrointestinal lipases is the most successful pharmacological
5 treatment. Orlistat is the only FDA approved lipase inhibitor; however, it has recently been associated
6 with some cases of hepatotoxicity. Secondary metabolites of microbial origin represent an almost
7 unexplored potential source for obtaining gastrointestinal lipase inhibitors. In this work, we isolated and
8 identified new microorganisms producing gastrointestinal lipase inhibitors, from which even the crude
9 extracts obtained showed a 100% inhibitory effect on the activity of pancreatic and gastric lipases.

10

11 **Abstract**

12 Gastrointestinal lipase inhibitors are molecules of pharmaceutical interest due to their use as anti-
13 obesity drugs. In this study, forty strains isolated from soil and sediments were identified with the ability
14 to produce inhibition of gastrointestinal lipase activity. The biomass extract of these strains showed at
15 least 50% inhibition in the hydrolysis of tributyrin by recombinant Human Pancreatic Lipase (rHPL) or
16 Rabbit Gastric Lipase (RGL) by *in vitro* assays. Based on gene sequencing, the isolates were identified
17 mainly as Streptomyces. Moreover, none of the identified strains have been reported to be lipase
18 inhibitor producers, so they can be viewed as potential sources for obtaining new drugs. IC₅₀ values of the
19 three best inhibitor extracts showed that AC104-10 was the most promising strain for production of
20 gastrointestinal lipase inhibitors. AC104-10 shows 99% homology (16S rRNA gene fragment) to
21 *Streptomyces cinereoruber* strain NBRC 12756. An inhibitory study over trypsin activity revealed that
22 AC104-10 extract, as well as THL, had no significant effect on the activity of this protease, showing its
23 specificity for lipases.

24

25 **Keywords:** lipase inhibitor, *Streptomyces*, pancreatic lipase, gastric lipase.

26

27 **Introduction**

28 Obesity is defined as "abnormal or excessive fat accumulation that presents a risk to health". The
29 prevalence of obesity is increasing at an alarming rate and has become a worldwide public health
30 problem. In 2014, more than 1.9 billion adults (18 years and older) were overweight, of whom over 600
31 million were obese (WHO 2016). Obesity is a significant risk factor for increased morbidity and
32 mortality, especially for cardiovascular disease and diabetes. It also increases the risk of cancer and
33 chronic diseases, including osteoarthritis, liver and kidney disease, sleep apnea, and depression. Despite
34 the substantial adverse effects of obesity, weight loss can result in a significant risk reduction for the
35 majority of these comorbid conditions (Pi-Sunyer 2009).

36 Obesity is primarily regarded as a disorder of lipid metabolism and the enzymes involved in this
37 process could be selectively targeted to develop anti-obesity drugs. The lipolysis of triglycerides from
38 dietary lipid intake is due principally to gastrointestinal lipases. During whole lipid digestion, human
39 gastric lipase (HGL) and human pancreatic lipase (HPL) contribute to hydrolyze 17.5% and 48.5% of the
40 meal TG acyl chains, respectively (Carriere et al. 1993). When an optimal lipolysis occurs, triglycerides
41 are transformed into a 2-monoglyceride (2-MG) and two free fatty acids (FFA). These are absorbed by
42 the enterocytes to synthesize new triglyceride molecules, which are transported to different organs
43 through lipoproteins (chylomicrons) after a meal (Phan and Tso 2001).

44 For almost two decades, a successful pharmacological treatment of obesity has involved the
45 inhibition of dietary triglyceride absorption via inhibition of gastrointestinal lipases. A number of
46 inhibitors of pancreatic lipase have been described from natural sources. Alkaloids, carotenoids,
47 glycosides, polyphenols, polysaccharides, saponins and terpenoids are well studied, and lipophilic
48 compounds from microbial sources are the most active (Lunagariya et al. 2014).

49 However, of all reported inhibitors, the only clinically approved pancreatic lipase inhibitor is
50 Orlistat (XenicalTM, AlliTM). The active compound in Orlistat is tetrahydrolipstatin (THL), a hydrogenated
51 derivative of lipstatin, which is a β -lactone produced by *Streptomyces toxytricini* (Weibel et al. 1987).

52 THL inhibits pancreatic and gastric lipases from several species, including human, with comparable
53 efficiency, and has been shown to be an effective tool for the treatment of human obesity lipases (Carrière
54 et al. 2001). Nevertheless, Orlistat has been associated with cases of hepatotoxicity (Lau and Chan 2002;
55 Kose et al. 2015). So far, a cause and effect relationship of severe liver injury with Orlistat use has not
56 been established. Nevertheless, FDA has added information about reported cases of severe liver injury on
57 the label of XenicalTM and AlliTM (FDA 2009).

58 The success of Orlistat has led to the search and identification of new gastrointestinal lipase
59 inhibitors showing no such adverse reactions. To accomplish the search, a sensitive and reliable high
60 throughput screening (HTS) assay that takes into account the conditions in which gastrointestinal lipases
61 act should be devised. The use of a triglyceride (a natural substrate for these enzymes) is suitable for the
62 HTS lipase inhibition assay. HTS methods to detect pancreatic lipase inhibition using triglycerides have
63 been developed, including ultraviolet (UV) spectrophotometric assays to detect lipase activity using
64 triglycerides from *Aleurites fordii* seeds oil (tung oil) (Mendoza et al. 2012; Serveau-Avesque et al. 2013)
65 and *Punica granatum* seed oil (Pomegranate oil) (Ulker et al. 2016). However, these methods cannot be
66 usable at acid pH for detection of gastric lipase inhibition. A new spectrophotometric method called
67 PHIBLA (pH Indicator Based Lipase Assay) was reported to carry out assays in a broad pH range in the
68 presence of detergents such as bile salts (Camacho-Ruiz et al. 2015). This HTS method is suitable to
69 measure the inhibition of human pancreatic lipase (HPL) and human gastric lipase or homologue lipases
70 with many similarities such as porcine pancreatic lipase (PPL) (de Caro et al. 1981) and rabbit gastric
71 lipase (RGL) (Roussel et al. 1999), close to *in vivo* conditions in order to find specific inhibitors for these
72 kinds of lipases.

73 The aim of this work was to screen for potential new and specific inhibitors of gastrointestinal
74 lipases such as rHPL and RGL, from a collection of microorganisms isolated from unexplored Mexican
75 ecosystems: sediments of Chapala Lake, and soil samples from uncultivated land on the lakeshore and in
76 the Sonoran Desert.

77 **Results and discussion**

78 **Isolation of microorganisms and screening of gastrointestinal lipase inhibitors**

79 Microorganisms are found in all sorts of natural environments where their growth is affected both
80 by interactions with other populations (synergistic, antagonistic, etc.) and by the physical and chemical
81 characteristics of such environments. Because of these interactions, secondary metabolites are produced
82 with various biological activities which play an important role in their survival. For this reason, the
83 microorganisms explored in this work were isolated from both an extreme environment, such as the
84 Sonoran Desert, and a mesophilic environment, such as Chapala Lake (lakeshore soil and sediments).

85 One hundred sixty-six strains were isolated and incubated for a week on Petri dishes containing
86 yeast malt dextrose agar media (YMD) or Glycerol-Soy milk agar media (Gly-SM) for biomass and
87 subsequent secondary metabolite production. It is noteworthy that solid agar media is recommended for
88 exploration of chemical diversity because agar plug samples are easily analyzed for optimal
89 representation of the qualitative secondary metabolome (Frisvad 2012). In addition, when surface cultures
90 are made, the biomass can be easily separated from the culture medium by scraping with a spatula.

91 An acetone extraction was performed to the obtained biomass and subsequently fractionated with
92 hexane. Extracts were evaporated under vacuum and suspended in DMSO at a final concentration of 50
93 mg ml⁻¹ for inhibition test.

94 Extracts obtained from strains cultured in GLY-SM medium had a greater inhibitory effect on the
95 activity of gastrointestinal lipases than those obtained from cultures in YMD media (data not shown).
96 These results were expected since culture media formulated with soy products has been widely used for
97 the production of secondary metabolites with Streptomycetes, including pancreatic lipase inhibitors, with
98 satisfactory results (Luthra and Dubey 2012).

99 Extract samples from 40 strains showed an inhibitory effect of more than 50% for one or both
100 gastrointestinal lipases tested. Out of these 40 strains, 21 were isolated from lakeshore soil, 8 from lake

101 sediment and 11 from desert soil. It should be noted that the extracts that had the greatest inhibitory effect
102 in both lipases were obtained from cultures of strains isolated from lakeshore soil samples. These results
103 agree with those obtained by Kilaru (2005), who observed more strains producing lipase inhibitors
104 isolated from lakes than strains isolated from desert soils.

105 Residual lipase activity of rHPL and RGL on tributyrin (TG(4:0)) after addition of 1 μ L (50 μ g) of
106 extract samples are shown in **Fig. 1**. Complete inhibition of lipase activity was observed for AC104-10
107 and AC104-6 extract samples, both isolated from lakeshore soil samples taken at 10-20 cm depth.
108 Additionally, extract from AC3-4 strain caused a significant inhibitory effect for both lipases, obtaining a
109 residual activity of 20% for rHPL and 35% for RGL. This strain was isolated from a lakeshore soil
110 sample taken at <10 cm depth. Photographs of GLY-SM agar cultures of the three strains that showed the
111 higher inhibitory effect in both rHPL and RGL are shown in **Fig. 2**. It can be seen that the three strains
112 have different morphologies, suggesting that they are strains of different genus, species or variety.

113

114 **Identification of isolated strains producing lipase inhibitors**

115 Strains whose extracts showed an inhibitory effect greater than 50% on any tested lipase were
116 identified by sequencing the 16S ribosomal RNA gene or ITS region. The results showed that 27 of the
117 identified bacteria belong to the order of *Actinomycetales*, of which 24 belong to the genus *Streptomyces*.
118 Moreover, 4 filamentous fungi and 3 *Bacillales* were also identified (**Table 1**). These results were
119 expected because *Streptomyces* have been the major source of microbial lipase inhibitors for decades
120 (Lunagariya et al. 2014). However, none of the identified strains had been reported as lipase inhibitor
121 producers.

122 The strains that showed the greatest potential as producers of lipase inhibitors were: AC104-10
123 with a 99% homology with *Streptomyces cinereoruber* strain NBRC 12756; AC3-4 with a 99% homology
124 with *Nocardia yamanashiensis* strain IFM 0265; and AC104-6, which was not possible to identify.

125 The phylogenetic distribution of the isolates that produced lipase inhibitors, and reported lipase inhibitor
126 producing bacteria were analyzed by the neighbor-joining method (**Fig. 3**). Interestingly, AC104-10 strain
127 is in the same cluster as *Streptomyces albolongus*, a valilactone producing bacterium. Valilactone, a β -
128 lactone, was reported as a potent lipase and esterase inhibitor, with an IC_{50} of $0.00014 \mu\text{g}\cdot\text{ml}^{-1}$ over hog
129 pancreas lipase, and $0.029 \mu\text{g}\cdot\text{ml}^{-1}$ over hog liver esterase (Kitahara et al. 1987).

130

131 **Lipase inhibition effectiveness of the microbial extracts**

132 IC_{50} values of biomass extracts for pancreatic and gastric lipase inhibition were determined. The
133 results in **Fig. 4** show the dose-response curves of AC104-10, AC104-6 and AC3-4 extracts on rHPL and
134 RGL inhibition. All extracts markedly inhibited pancreatic and gastric lipase activity in dose-dependent
135 relationships. The findings showed that AC104-10 extract was the most effective lipase inhibitor with
136 IC_{50} values of $12.16 \text{ mg of extract}\cdot\text{mg}^{-1}$ ($0.0058 \text{ mg}\cdot\text{ml}^{-1}$) of rHPL and $7.63 \text{ mg}\cdot\text{mg}^{-1}$ ($0.03 \text{ mg}\cdot\text{ml}^{-1}$) of
137 RGL, followed by AC104-6 extract with IC_{50} values of $170.66 \text{ mg}\cdot\text{mg}^{-1}$ ($0.08 \text{ mg}\cdot\text{ml}^{-1}$) of rHPL and
138 $10.08 \text{ mg}\cdot\text{mg}^{-1}$ ($0.04 \text{ mg}\cdot\text{ml}^{-1}$) of RGL. AC3-4 extract was the least potent inhibitor among the extracts
139 tested with IC_{50} values of $421.28 \text{ mg}\cdot\text{mg}^{-1}$ ($0.2 \text{ mg}\cdot\text{ml}^{-1}$) of rHPL and $205.53 \text{ mg}\cdot\text{mg}^{-1}$ ($0.82 \text{ mg}\cdot\text{ml}^{-1}$) of
140 RGL. However, all extracts were less potent than THL (control) in inhibiting gastrointestinal lipases.
141 THL IC_{50} values obtained were $0.0071 \text{ mg}\cdot\text{mg}^{-1}$ ($3.41\times 10^{-6} \text{ mg}\cdot\text{ml}^{-1}$) of rHPL and $0.0013 \text{ mg}\cdot\text{mg}^{-1}$
142 ($5.2\times 10^{-6} \text{ mg}\cdot\text{ml}^{-1}$) of RGL. There is a remarkable difference between the extracts tested and THL, which
143 is attributed to THL being a pure molecule. We cannot ensure that the inhibitor molecules present in these
144 extracts will be more potent than THL once purified. However, a significant difference of rHPL/RGL IC_{50}
145 inhibition ratio was found for the best extracts tested in comparison with THL where values above 1 mean
146 greater inhibition of rHPL. For THL, an IC_{50} inhibition ratio rHPL/RGL of 5.46 was obtained, whereas
147 for the AC104-10, AC3-4 and AC104-6 extracts, IC_{50} inhibition ratios rHPL/RGL of 1.59, 2.04 and 16.93
148 were determinate, respectively. These results suggest that the extract AC104-6 shows a higher inhibition
149 for rHPL in comparison with RGL (as shown by THL), three times greater than THL, whereas the AC3-4

150 and AC104-10 extracts had a similar inhibition for both rHPL and RGL, with AC104-10 being the most
151 effective extract (IC₅₀) for lipase inhibition.

152 A separate study in which the effects of AC104-10 extract and THL were measured over trypsin
153 activity from porcine pancreas was carried out using BAPNA (N α -Benzoyl-L-arginine 4-nitroanilide
154 hydrochloride) as substrate. Neither AC104-10 extract nor THL had any effect over trypsin activity (data
155 not shown) suggesting that the lipase inhibitor produced by AC104-10 strain is as specific for lipases as
156 THL.

157

158 **Materials and methods**

159 **Chemicals**

160 Tributylin (TG(4:0)), butyric acid (BtA), BSA, NaCl, CaCl₂, sodium taurodeoxycholate (NaTDC),
161 β-cyclodextrin (β-CD), bromocresol green (BCG), *p*-nitrophenol, malic acid, MOPS, 2-Methyl-2-
162 propanol (*tert*-Butanol), DMSO, Tetrahydrolipstatin (THL), and Butylated hydroxytoluene (BHT) were
163 purchased from Sigma-Aldrich..

164

165 **Enzymes**

166 Recombinant human pancreatic lipase (rHPL) was expressed in *Pichia pastoris* and purified from
167 culture media as described by Belle et al. (2007). Porcine colipase was partly purified from lipid-free
168 pancreatic powder using the procedure described in Fernandez et al. (2007). Rabbit gastric lipase (RGL)
169 was purified according to Moreau et al. (1988).

170

171 **Isolation and identification of microorganisms**

172 Soil samples were collected from uncultivable land on the lakeshore (Las Tortugas, Jalisco,
173 Mexico; latitude 20°18'34"N, longitude 102°49'19"W, altitude 1562 m), from the Sonoran Desert
174 (Sonora, Mexico; latitude 28°48'10"N, longitude 111°12'13"W, altitude 115 m) and from sediment of
175 Chapala Lake (Jalisco and Michoacán, Mexico) where the following four sites were selected: San Juan
176 Cosala (latitude 20°16'51"N, longitude 103°20'20"W, altitude 1519 m), San Luis Soyatlan (latitude
177 20°12'8"N, longitude 103°18'11"W, altitude 1522 m), Tuxcueca (latitude 20°9'19"N, longitude
178 103°10'37"W, altitude 1521 m) and Cojumatlan (latitude 20°7'24"N, longitude 102°50'18"W, altitude
179 1522 m). At the lakeshore, two soil samples were collected at each spot, one on the surface layer, and the

180 other at a depth of about 10 to 20 cm. Isolation of strains was carried out using the procedure described in
181 Kuster and Williams (1964).

182 For molecular identification, pure liquid cultures were used for total DNA extraction with Dneasy® Plant
183 Mini Kit (Qiagen, Hilden, Germany). PCR reactions were carried on a Veriti™ 96-well thermal cycler
184 (Applied Biosystems, Foster City, USA) using OneTaq Hot Start DNA Polymerase (New England
185 Biolabs). Ribosomal 16S fragment of approximately 1,300 bp was amplified by PCR with primers 63F
186 and 1387R (Marchesi et al. 1998) for actinomycetes identification, and approximately 600 bp fragment
187 from the internal transcribed spacer (ITS) region in the nuclear ribosomal repeat unit was amplified by
188 PCR with primers ITS1 and ITS4 (Gardes and Bruns 1993) for filamentous fungi identification. PCR
189 products were sent to Macrogen USA Corp. for sequencing. Consensus sequences were obtained with
190 CLC Main Workbench 5.5 software package (CLCBio, Aarhus, Denmark) and identification was done
191 using the BLASTn algorithm from GenBank database comparing to recorded sequences.

192

193 **Biomass production and organic solvent extraction of inhibitors**

194 Isolated microorganisms were cultured on 60 × 15 mm Petri dishes on two different media. The
195 YMD media contained 4 g l⁻¹ yeast extract, 10 g l⁻¹ malt extract, 4 g l⁻¹ dextrose and 18 g l⁻¹
196 bacteriological agar (pH 7.0). Moreover, the soy milk-glycerol media was composed of 60% lipid free
197 natural soy milk (ADES®) and 2 % glycerol. Cultures were incubated at 30 °C for 7 days. After that,
198 biomass was carefully collected in a 2 ml micro tube from the agar surface by scraping with a spatula.
199 The biomass was stored at -20 °C until use.

200 Extractions from biomass were performed with acetone (containing 0.1 % of BHT) in a ratio of 2
201 ml of solvent per g of biomass for one hour at room temperature with sonication at 6 intervals of 10 min,
202 using an ultrasonic cell disruptor (SONICS® Vibro-Cell®). Biomass was then separated by centrifugation
203 at 9402 g. Supernatants were collected in new 2 ml micro tubes and one volume of hexane was added to
204 each one. Samples were mixed vigorously in a vortex mixer and centrifuged at 9402 g. The upper phase

205 was collected in new 1.5 ml micro tubes and dehydrated with anhydrous ammonium sulfate. After
206 filtration, extracts were evaporated in vacuum, dissolved in DMSO (at final concentration of 50 mg ml⁻¹)
207 and used to measure the lipase inhibitory effect.

208

209 **Lipase inhibition assay**

210 The enzyme-inhibitor pre-incubation method was used (Ransac et al. 1997), in aqueous medium
211 and the absence of substrate, to test for direct interactions that might occur between the lipase and the
212 inhibitor. For the lipase inhibition assay, 1 µl of extracts (50 mg ml⁻¹ in DMSO) was pre-incubated in a 96
213 well microplate with 20 µl of rHPL (0.48 ng µl⁻¹ in 2.5 mmol l⁻¹ MOPS buffer, pH 7.2) or RGL (4 ng µl⁻¹
214 in 1.5 mmol l⁻¹ malate buffer, pH 5.0) in the presence of 4 mmol l⁻¹ NaTDC. Residual enzyme activity
215 was measured after 1 h of continuous shaking (6000 g) pre-incubation at room temperature. THL was
216 used as positive control at an enzyme:inhibitor molar ratio of 1:100, and 1 µl of DMSO was used as a
217 negative control. Residual activity was expressed as the percentage of activity retained in the sample
218 relative to the negative control inhibition.

219

220 **Lipase activity assay**

221 Lipase activity was determined using the pH indicator based lipase assay (PHIBLA) (Camacho-
222 Ruiz et al. 2015) by indirect measurement of the release of butyric acid upon hydrolysis of tributyrin
223 (TG(4:0)). For rHPL activity assays, the substrate emulsion was prepared as follows: 0.5 ml of *p*-
224 nitrophenol stock solution (10 mmol l⁻¹ in *tert*-butanol) was dissolved in 9 ml of 2.5 mmol l⁻¹ MOPS (pH
225 7.2) buffer solution containing 150 mmol l⁻¹ NaCl, 1 mM CaCl₂, 3 mg·ml⁻¹ β-CD, 0.5 mmol l⁻¹ NaTDC
226 and colipase (in a five molar excess), then 0.5 ml of tributyrin stock solution (100 mmol l⁻¹ in *tert*-
227 butanol) was immediately added drop by drop in a continuous vigorous vortexing. For RGL activity
228 assays, the substrate emulsion was prepared similarly: 0.5 ml of bromocresol green stock solution (5

229 mmol l⁻¹ in *tert*-butanol) was dissolved in 9 ml of 1.5 mmol l⁻¹ malate (pH 5.0) buffer solution containing
230 150 mmol l⁻¹ NaCl, 1.5 μmol l⁻¹ BSA, 3 mg ml⁻¹ β-CD and 2 mmol l⁻¹ NaTDC, then 0.5 ml of tributyrin
231 stock solution (100 mmol l⁻¹ in *tert*-butanol) was immediately added drop by drop with continuous
232 vigorous vortexing. Substrate emulsions were prepared at the time of the test.

233 To each well of a microplate containing 20 μl of enzyme substrate, 100 μl of corresponding
234 substrate emulsion were added. Subsequently, the plate was positioned in a microtiter plate scanning
235 spectrophotometer (x-MarkTM, Bio-Rad) and shaken for 5 s before each reading. The decrease in
236 absorbance was recorded every 30 s at 410 nm for rHPL assays and 610 nm for RGL assays. Activity
237 assays were performed at 37 °C. Blanks without enzyme were carried out, and data were collected at least
238 in triplicate for 15 min.

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305

306 **Figure legends:**

307 **Figure 1.** Inhibitory effect of biomass extracts on gastrointestinal lipases activity. Biomass extracts of the
308 strains isolated from the 10-20 cm superficial lakeshore soil (A), <10 cm superficial lakeshore soil (B),
309 lake sediment (C), and desert soil (D). Only those extracts in which residual activity obtained was less
310 than 50 % for one or both enzymes are shown. *Biomass extracts that showed less than 35 % of residual
311 activity on both enzymes. Biomass was obtained from Gly-SM superficial cultures after 7 days of growth.
312 Residual activity was measured using PHIBLA with an emulsion of TG(4:0).

313

314 **Figure 2.** Photographs of strains selected as best producers of inhibitors in glycerol-soymilk agar culture
315 medium.

316

317 **Figure 3.** Phylogenetic tree for bacteria strains producing lipase inhibitors by neighbor joining analysis
318 based on their 16S rRNA gene sequences. Numbers at the nodes are bootstrap values (of 1,000
319 replicates).

320

321 **Figure 4.** Dose-response curves of the organic extracts of the strains AC104-10 (*Streptomyces*
322 *cinereoruber*), AC104-6 (not identified.), and AC3-4 (*Nocardia yamanashiensis*). Panel A, effect on
323 rHPL; Panel B, effect on RGL. The enzyme was pre-incubated with the inhibitor for 1 h in the presence
324 of 4 mmol l⁻¹ NaTDC. Residual activity was measured using PHIBLA with an emulsion of TG(4:0) as
325 substrate.

326

327

329 **Table 1.** Identification of isolated strains capable of producing gastrointestinal lipase inhibitors.

N°	Origin	Identification	Identity (%)	Accession number
AC104-3	Lakeshore, 10-20 cm superficial soil	<i>Amycolatopsis balhimycina</i>	99	NR 025564.1
AC104-5	Lakeshore, 10-20 cm superficial soil	<i>Streptomyces longwoodensis</i>	99	NR 041161.1
AC104-6	Lakeshore, 10-20 cm superficial soil	ND	ND	ND
AC104-7	Lakeshore, 10-20 cm superficial soil	<i>Streptomyces cylabdanicus</i>	99	NR 136867.1
AC104-10	Lakeshore, 10-20 cm superficial soil	<i>Streptomyces cinereoruber</i>	99	NR 112261.1
AC104-18	Lakeshore, 10-20 cm superficial soil	<i>Streptomyces gardneri</i>	99	NR 112575.1
AC104-20	Lakeshore, 10-20 cm superficial soil	ND	ND	ND
AC104-21	Lakeshore, 10-20 cm superficial soil	<i>Streptomyces resistomycificus</i>	99	NR 112287.1
ACS102-2	Lakeshore, 10-20 cm superficial soil	<i>Streptomyces polyantibioticus</i>	99	NR 043573.1
ACS102-12	Lakeshore, 10-20 cm superficial soil	<i>Streptomyces vinaceus</i>	100	NR 041131.1
AC3-1	Lakeshore, <10 cm superficial soil	<i>Streptomyces resistomycificus</i>	99	NR 112287.1
AC3-3	Lakeshore, <10 cm superficial soil	<i>Streptomyces resistomycificus</i>	99	NR 112287.1
AC3-4	Lakeshore, <10 cm superficial soil	<i>Nocardia yamanashiensis</i>	99	NR 024803.1
AC3-5	Lakeshore, <10 cm superficial soil	<i>Streptomyces chartreusis</i>	99	NR 114825.1
AC3-8	Lakeshore, <10 cm superficial soil	<i>Streptomyces longwoodensis</i>	98	NR 041161.1
AC3-9	Lakeshore, <10 cm superficial soil	<i>Streptomyces spororaveus</i>	99	NR 112469.1
AC3-11	Lakeshore, <10 cm superficial soil	<i>Streptomyces ederensis</i>	99	NR 112457.1
AC3-12	Lakeshore, <10 cm superficial soil	<i>Streptomyces rishiriensis</i>	99	NR 044141.1
AC5-2	Lakeshore, <10 cm superficial soil	<i>Streptomyces griseorubiginosus</i>	99	NR 112350.1
AC5-7	Lakeshore, <10 cm superficial soil	<i>Streptomyces chartreusis</i>	99	NR 114825.1
ACS1-1	Lakeshore, <10 cm superficial soil	<i>Streptomyces alboniger</i>	99	NR 043228.2
ACPLRF-1	Lake sediment	<i>Bacillus tequilensis</i>	99	NR 104919.1
ACPLRF-2	Lake sediment	ND	ND	ND
ACSLRF-7	Lake sediment	<i>Streptomyces albogriseolus</i>	99	NR 112487.1
ACSLRF-9	Lake sediment	<i>Streptomyces californicus</i>	99	NR 112257.1
ACSLRF-17	Lake sediment	<i>Streptomyces albogriseolus</i>	99	NR 112487.1
ACSLRF-25	Lake sediment	ND	ND	ND
ACSLRF-26	Lake sediment	<i>Streptomyces albogriseolus</i>	99	NR 112487.1
ACSLRF-27	Lake sediment	<i>Actinomadura geliboluensis</i>	100	NR 109059.1
A114	Desert soil	<i>Streptomyces regensis</i>	99	NR 112402.1
A127	Desert soil	<i>Streptomyces mexicanus</i>	99	NR 114647.1
A135	Desert soil	<i>Bacillus licheniformis</i>	100	NR 118996.1
A147	Desert soil	<i>Bacillus subtilis subsp. Spizizenii</i>	100	NR 118486.1
A62-A	Desert soil	<i>Aureobasidium pullulans</i>	99	JF439462.1
A95	Desert soil	ND	ND	ND
A99-B	Desert soil	<i>Streptomyces africanus</i>	99	NR 112600.1
B11	Desert soil	<i>Aspergillus nidulans</i>	100	KP278174.1

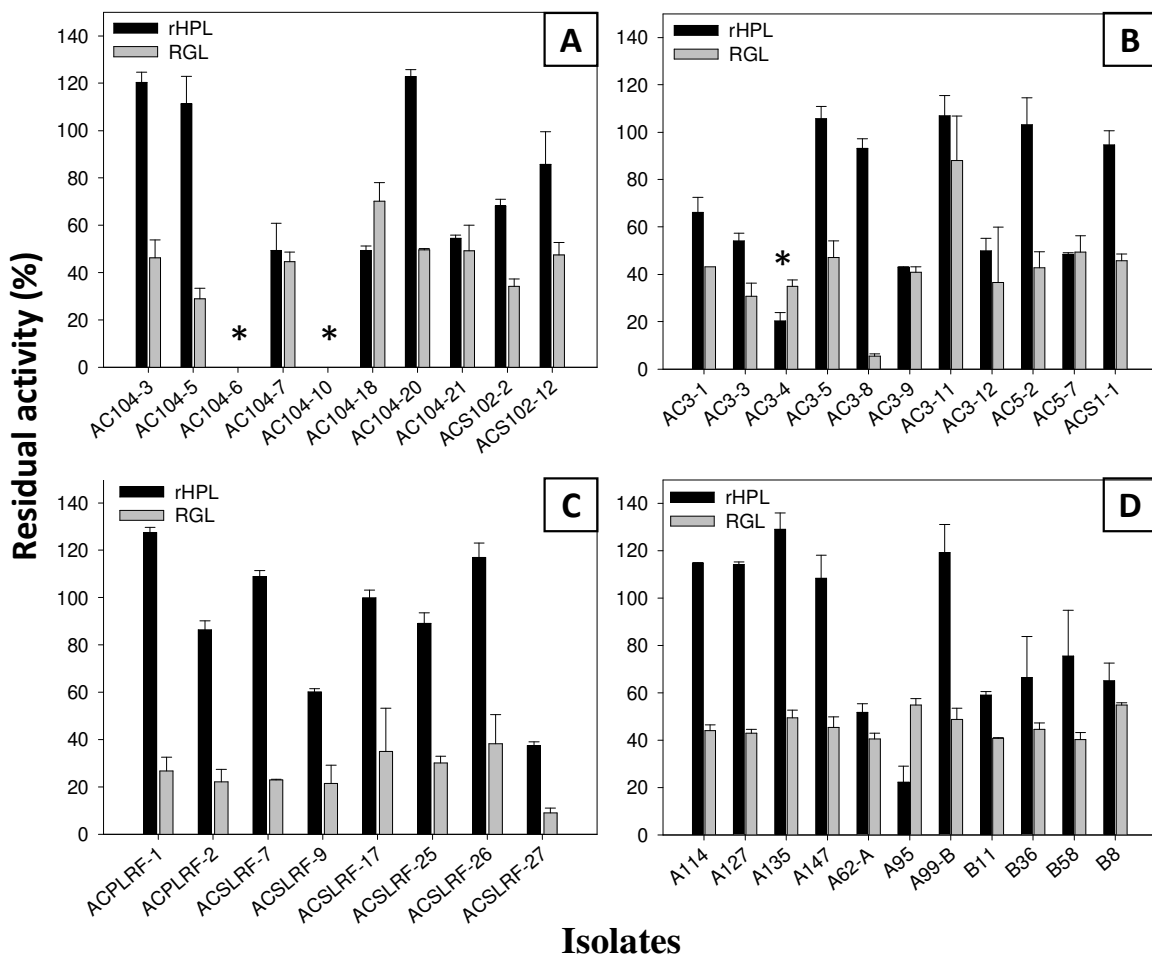
B36	Desert soil	<i>Ascomycota sp.</i>	96	JN561255.1
B58	Desert soil	<i>Aspergillus fruticosus</i>	99	AB248969.1
B8	Desert soil	ND	ND	ND

330 *ND: Not determined because there were no coincidences of alignment with reported sequences.*

331

332

333 **Figure 1.**



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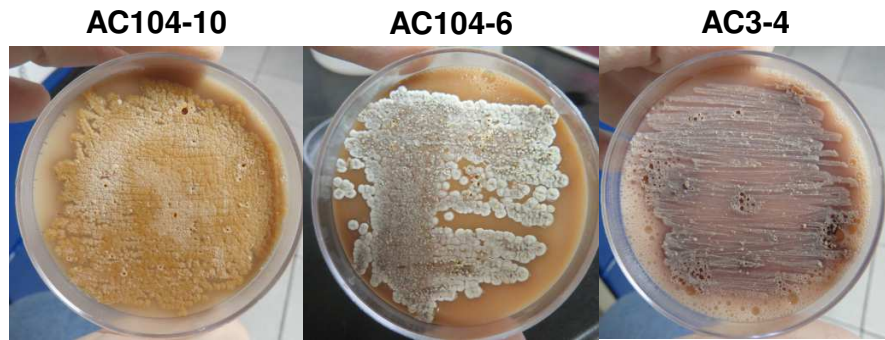
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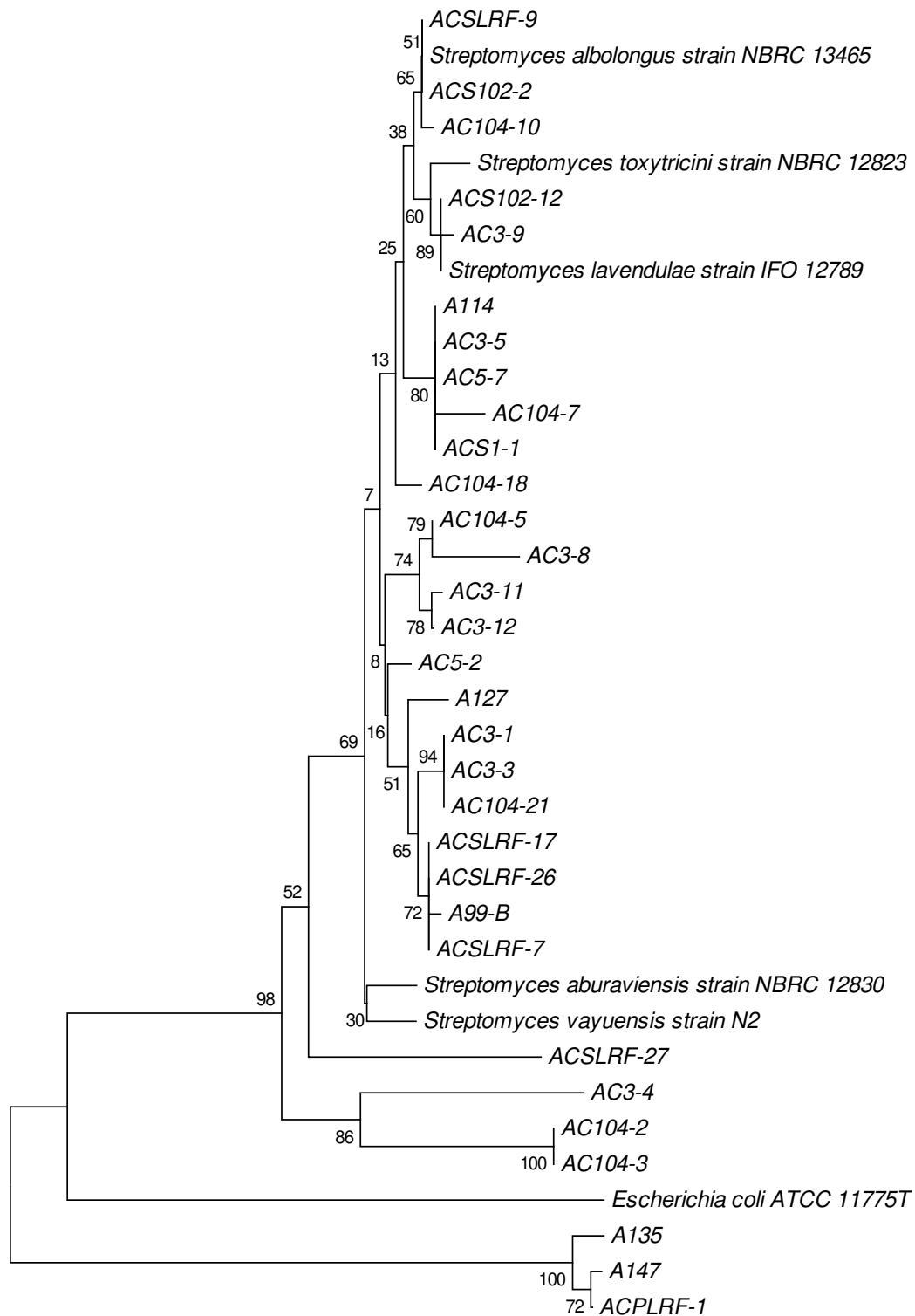
338 **Figure 2.**

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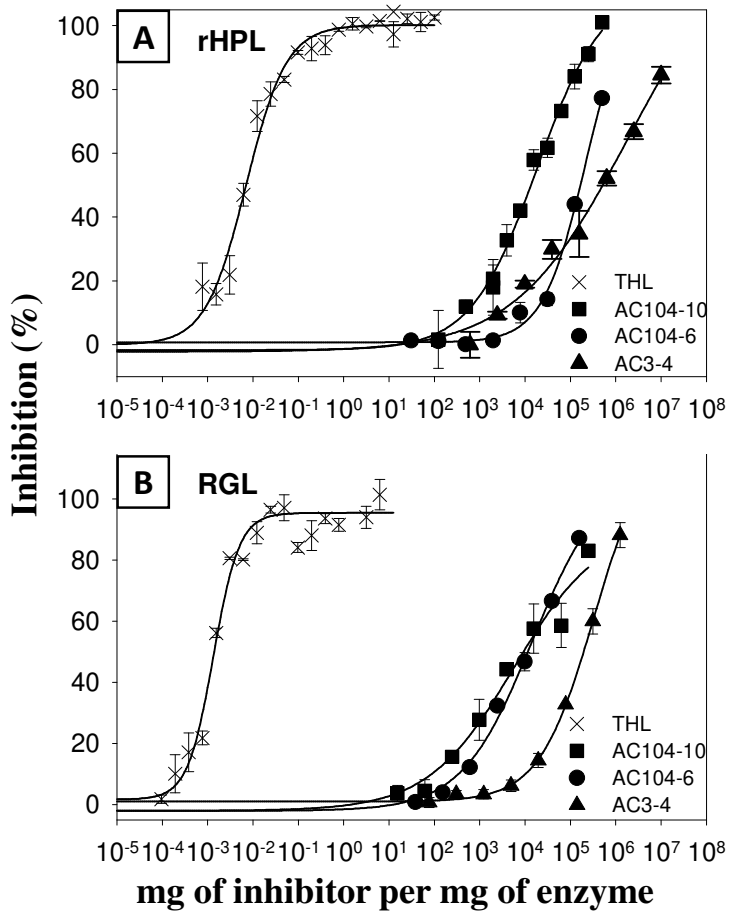
341 **Figure 3.**



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343 **Figure 4.**



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