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

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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

Abstract

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

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

Introduction

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

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

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

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

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

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

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

Results and discussion

Isolation of microorganisms and screening of gastrointestinal lipase inhibitors

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

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

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

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

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

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

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

Identification of isolated strains producing lipase inhibitors

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

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

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

Lipase inhibition effectiveness of the microbial extracts

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

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

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

Materials and methods

Chemicals

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

Enzymes

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

Isolation and identification of microorganisms

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

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

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

Biomass production and organic solvent extraction of inhibitors

Isolated microorganisms were cultured on 60 × 15 mm Petri dishes on two different media. The 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 bacteriological agar (pH 7.0). Moreover, the soy milk-glycerol media was composed of 60% lipid free natural soy milk (ADES®) and 2 % glycerol. Cultures were incubated at 30 °C for 7 days. After that, biomass was carefully collected in a 2 ml micro tube from the agar surface by scraping with a spatula. The biomass was stored at -20 °C until use.

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

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

Lipase inhibition assay

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

Lipase activity assay

Lipase activity was determined using the pH indicator based lipase assay (PHIBLA) (Camacho-Ruiz et al. 2015) by indirect measurement of the release of butyric acid upon hydrolysis of tributyrin (TG(4:0)). For rHPL activity assays, the substrate emulsion was prepared as follows: 0.5 ml of *p*-nitrophenol stock solution (10 mmol l⁻¹ in *tert*-butanol) was dissolved in 9 ml of 2.5 mmol l⁻¹ MOPS (pH 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 and colipase (in a five molar excess), then 0.5 ml of tributyrin stock solution (100 mmol l⁻¹ in *tert*-butanol) was immediately added drop by drop in a continuous vigorous vortexing. For RGL activity 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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Figure legends:

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

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

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

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

328 **Tables:**

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 fruticulosus</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

Figure 1.

338 **Figure 2.**

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341 **Figure 3.**

343 **Figure 4.**

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