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Identification of Lipstatin-Producing Ability in *Streptomyces virginiae* CBS 314.55 Using Dereplication Approach

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Summary

Streptomyces species are prolific producers of bioactive metabolites, such as β-lactone-containing lipstatin produced by *Streptomyces toxytricini*, an intermediate used in semi-synthetic process for production of anti-obesity drug orlistat. Understanding the distribution of identical or structurally similar molecules produced by a taxonomic group is of particular importance when trying to isolate novel biologically active compounds or strains producing known metabolites of medical importance with potentially improved properties. Until now, only two independent isolates of *S. toxytricini* species have been known to be producers of lipstatin. According to the current taxonomic criteria, *S. toxytricini* belongs to *Streptomyces lavendulae* phenotypic cluster. Taxonomy-based dereplication approach coupled with *in vitro* assay was applied to screen the *S. lavendulae* phenotypic cluster for production of lipstatin-like lipase inhibitors using synthetic *p*-nitrophenol derivatives of C_4 and C_{16} lipids. Screening the available strains from public collections belonging to *S. lavendulae* phenotypic cluster, high lipase inhibitory activity was identified in the *Streptomyces virginiae* CBS 314.55 culture supernatants. HPLC and LC-MS/MS confirmed lipstatin production by a new *Streptomyces* species for the first time. We have demonstrated that the new lipstatin-producing strain *S. virginiae* morphologically and physiologically differs from *S. toxytricini* substantially; however, the production capacity of the newly identified lipstatin-producing species *S. virginiae* is comparable to *S. toxytricini*. We have thus demonstrated the effectiveness of a simple and affordable dereplication approach for identification of potentially novel and useful industrial strains available in public culture collections.

Key words: lipstatin, *Streptomyces virginiae*, dereplication, lipase inhibitor

Introduction

While it is still important to isolate and screen novel species of *Streptomyces* for pharmaceutical and drug discovery programmes, and prevent costly rediscovery of known bioactive molecules (*1*), it is also important to fully characterise known clades to understand distribution of known bioactive metabolites and potentially find new

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structural or functional analogues. However, it is also of great importance to identify new strains producing known metabolites of industrial value with potentially improved properties, such as higher yield of the target product, improved purity profiles and other morphological or physiological properties, as it was the case in the past during the development of fermentation processes for production of biologically active compounds such as penicillin (*2*) and FK506 (*3*), where strains with advanced properties were introduced in order to improve the economy and quality of final product. Discriminating between microbial isolates which are morphologically similar and produce identical or similar metabolites is a particular issue in the identification of specific novel biosynthetic pathways in closely related strains, especially when constructing natural product libraries. Often referred to as the 'dereplication issue', it is of particular significance when dealing with Actinomycetes such as *Streptomyces*. The genus currently contains 568 species (*4*); however, this genus is still assumed underspeciated (*5*).

Lipstatin, and its medically important semisynthetic analogue orlistat (Fig. 1a), is a member of a small group of β-lactone-containing structures (Fig. 1a), which are exclusively produced by *Streptomyces toxytricini* species (*6–10*). The length of fatty acid moieties at the positions R1 and R3 (Fig. 1b) and the origin of the amino acid-derived residue attached to the hydroxyl group at the position R2 determine the structure of this family of compounds. Lipstatin irreversibly binds to pancreatic lipase with an IC_{50} value of 0.14 μM and exhibits a dose-dependent inhibition of triacylglycerol uptake in mice (*11*). In its chemically reduced form, tetrahydrolipstatin (orlistat) is used to prevent obesity through the interference with the degradation and absorption of lipid compounds in the intestine (*12*). Therefore, novel lipstatin-like analogues represent very interesting compounds with novel therapeutic potential. Interestingly, all these β-lactone-containing compounds reported in the literature are produced by the *Streptomyces* isolates belonging to the *S. lavendulae* phenotypic cluster, including panclicins (*13*), ebelactones A and B (*9*), valilactone (*6*), esterastin (*8*) and lipstatin (*14*).

The *S. lavendulae* phenotypic cluster was first proposed by Williams *et al.* (*15*), with many strains producing other commercially interesting metabolites such as cycloserine (*16*), saframycin (*17*), and virginiamycin (*18*). Attempts to resolve the complex taxonomy of this group have been inconclusive, with the most interesting work being that of Labeda (*19*), which evaluated and validated the specific status of 11 species as subjective synonyms of *S. lavendulae* based on DNA relatedness.

The aim of this study is to screen selected members of the *S. lavendulae* phenotypic cluster for alternative lipstatin-producing strains. A taxonomy-related approach was applied, where members of the *S. lavendulae* group were selected and further screened for their ability to produce metabolites capable of inhibiting porcine pancreatic lipase. Based on the dereplication approach, we succeeded in identifying a previously unknown lipstatin producer with novel morphological and physiological properties. This study indicates that bioactive metabolites still remain to be discovered in well-characterized strains, and the dereplication of natural products is a useful approach to finding novel strains with useful fermentation characteristics.

Materials and Methods

Bacterial strains and growth conditions

The *Streptomyces* strains used in this study are summarized in Table 1. All strains were grown on sporulation agar (*20*). Plates were incubated at 28 °C for 14 days. Spore stocks were prepared according to Kieser *et al.* (*21*).

Phylogenetic analysis

The 16S rRNA genes were downloaded from the NCBI database (*22*) and they were aligned using ClustalW with phylogenetic reconstruction performed using UPMGA and NJ methods in MEGA v. 4.0. The reliability of these trees was estimated by bootstrapping with 1000 replicates (*23*). 16S rRNA sequence not available from the database (See Table 1) was amplified by PCR, using the universal 16S rRNA primers according to Lane (*24*) and Muyzer *et al.* (*25*), followed by sequencing. The sequences were trimmed to identical lengths (1335 nt), aligned and the trees were computed. Partial 16S rRNA gene sequences were deposited in GenBank (accession numbers: JN201950-JN201955).

Physiological characterization

Morphological and physiological differences between *Streptomyces toxytricini* NRRL 15443 and *Streptomyces vir-*

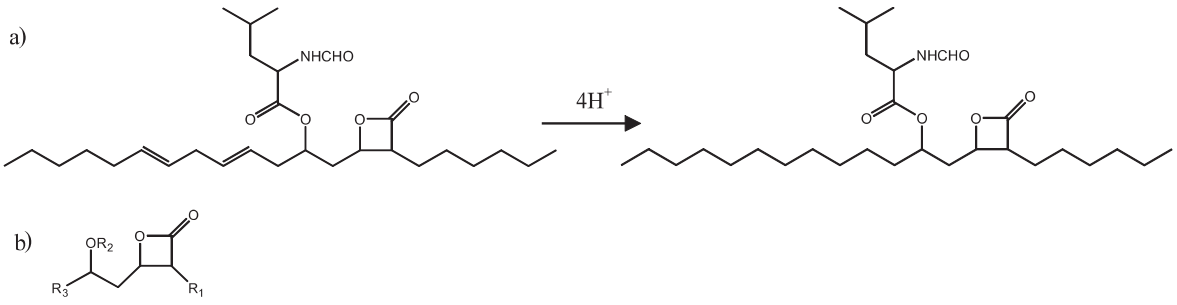

Fig. 1. Lipstatin and its medically important analogue orlistat (a). Basic structure of lipstatin-like metabolites containing typical β-lactone group (b) where R1 and R3 represent aliphatic chains of fatty acids of different lengths and R2 represents various amino acids or amino acid analogues attached through ester bonds to the hydroxyl group

Table 1. Strains belonging to the *Streptomyces lavendulae* phenotypic cluster used in this study with their public culture collection accession numbers

Strain	Culture collection accession number	16S rRNA accession number	Product
Streptomyces toxytricini	NRRL 15443	DQ442548	lipstatin
Streptomyces lavendulocolor	CBS 911.68 / NRRL B-3367	DQ442516	streptothricin complex
Streptomyces flavotricini	CBS 259.66	N201955	
Streptomyces goshikiensis	CBS 835.68 / NRRL B05428	EF178693	bandamycin
Streptomyces subrutilus	CBS 689.72 / NRRL B-12377	DQ442545	hydroxystreptomycin
Streptomyces racemochromogenes	CBS 937.68 / NRRL B-5430	DQ026656	racemomycin
Streptomyces polychromogenes	CBS 311.56 / NRRL 3032	AY999923	*O*-carbamyl-D-serine
Streptomyces katrae	CBS 748.72 / NRRL 3093	EF654092	
Streptomyces xanthophaeus	CBS 572.68 / NRRL B5414	DQ442560	geomycin
Streptomyces virginiae	CBS 314.55 / DSM 40094	JN201950	compound similar to streptogramin
Streptomyces lavendulae ssp. *grasserius*	DSM 40385	AY999841	grasseriomycin
Streptomyces lavendulae ssp. *lavendulae*	DSM 40558 / CBS 597.71	JN201953	vitamin B12
Streptomyces lavendulae ssp. *lavendulae*	DSM 40708	JN201952	megacidin
Streptomyces lavendulae ssp. *lavendulae*	DSM 40713	JN201951	ferrimycin (antibiotic A.9578)
Streptomyces lavendulae ssp. *lavendulae*	DSM 41088	JN201954	streptothricin

NRRL=collection of the National Center for Agricultural Utilization Research, Peoria, IL, USA; CBS=CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; DSM=German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

giniae CBS 314.55 in carbon utilization were determined using API ID 32 test (bioMérieux, Marcy l'Etoile, France). The API test strip was inoculated with spore suspension (10^6 spores/mL) and cultivated for three days.

Lipase inhibition assays

Inhibition of lipase activity by potential inhibitors produced by the different strains in this study was determined using two substrates, differing in aliphatic chain length: *p*-nitrophenyl butyrate (*p*NPB) and *p*-nitrophenyl palmitate (*p*NPP) (Sigma-Aldrich, St. Louis, MO, USA). To test the lipase inhibition, fermentation broth extracts were prepared by extracting with methanol in the ratio of 1:4 for 60 min. Samples were then centrifuged at 14 000 rpm for 10 min to remove cell debris and precipitates. Extracts were then serially diluted in methanol, used in lipase inhibition assay broth with the lipase from porcine pancreas (Sigma-Aldrich) and incubated with the *p*-nitrophenyl substrates. Assays using 10 % (by volume) *p*NPB in methanol were incubated in 3 mL of reaction mixtures containing 2.93 mL of 0.1 M phosphate buffer (pH=7.0), 25 μL of fermentation extract or 25 μL of H_2O (blank), and 30 μL of lipase (2.5 mg/mL prepared in 0.2 % Triton X-100). The reaction was started with 25 μL of *p*NPB solution. When using 0.125 % *p*NPB in the acetonitrile and ethanol ratio of 1:2, reactions were carried out in 2-mL volumes containing 1.75 mL of Tris-HCl (0.05 M, pH=8), 25 μL of fermentation extract or 25 μL of H_2O (blank), and 25 μL of lipase (2.5 mg/mL prepared in 0.2 % Triton X-100). The reaction was started with 200 μL of *p*NPP solution. The release of *p*-nitrophenol was measured spectrophotometrically at 400 nm for 10 min at 37 °C. The percentage of lipase inhibition was calculated from the decrease in specific lipase activity compared to the control sample (without the addition of culture extract) where molar absorption coefficient ($\varepsilon_{400\ nm}$) of *p*NPB was 14 800 M^{-1} cm^{-1} (*26*) and of *p*NPP it was 17 454 M^{-1} cm^{-1} (*27*).

HPLC analysis

Analysis of lipstatin production was carried out by reversed phase HPLC using a C-18 column (150 mm×4.0 mm, particle size 3 μm, Inertsil®, GL Sciences BV, Eindhoven, The Netherlands) using acetonitrile and 0.1 % H_3PO_4 (75:25 by volume) as the mobile phase at a flow rate of 1.5 mL/min at 45 °C. Lipstatin peaks were detected at 200 nm and identified using lipstatin external standard (obtained from Krka d.d., Novo Mesto, Slovenia).

LC-MS/MS analyses

LC-MS/MS analyses were performed on an Agilent 1100 with a reversed phase analytical C18 column (Gemini C18 column, 5 μm, 150 mm×2 mm i.d., Phenomenex, Torrance, CA, USA). The separation (10 μL injection volume) was performed at a flow rate of 0.250 mL/min and 45 °C using gradient elution with 0.5 % TFA as solvent A and acetonitrile as solvent B. The gradient program was as follows: 20 % A, 0–13 min; 20–2 % A, 13–13.1 min; 2 % A, 13.1–23 min; 2–20 % A, 23–23.2 min; 20 % A, 23.2–30 min.

The mass selective detector (Quattro Micro API, Waters, Milford, MA, USA) was equipped with an electrospray ionisation, and cone voltage of 20 V and capillary voltage of 3.0 kV were used for positive ionisation of the analytes. Dry nitrogen was heated to 350 °C and the drying gas flow was 8 L/min. Data were acquired in the multi-reaction monitoring mode. The collision energy was optimised for the following transitions: *m/z*=492.20>

315.18 and 492.20>333.19. Dwell time for each transition and inter-channel delay was optimized for high sensitivity.

Effect of nutrient source on lipstatin production in S. virginiae CBS 314.55

Seed medium (10 g of soya bean flour, 20 g of glycerol, 5 g of yeast extract per litre, pH=7) was aliquoted in 50-mL volumes in 500-mL Erlenmeyer flasks and inoculated with *S. virginiae* CBS 314.55 spores ($5 \cdot 10^7$ mL^{-1}) and incubated with shaking (28 °C, 210 rpm for 20–30 h). Aliquots of the above seed culture (10 % by volume) were used to inoculate 50 mL of fermentation medium in a 500-mL Erlenmeyer flask. Fermentation medium contained the following: 20 g of glycerol, 14.3 g of lecithin (Evonik Degussa, Hanau, Germany), 2 g of $CaCO_3$, 50 mL of soya bean oil, in 1 L of tap water (pH=7.2). The addition of different concentrations of complex nitrogen sources such as soya bean meal (Cargill, Conover, IA, USA), tryptone (Merck, Darmstadt, Germany), cottonseed meal (ADM, Decatur, IL, USA), yeast (Ardamine PH; Champlain Industries, Clifton, NJ, USA) and soya peptone (Sigma-Aldrich) were tested in order to determine the effect of nitrogen sources on lipstatin production. Lipstatin production process was carried out with shaking at 28 °C and 210 rpm for 6 days (*20*). Apart from the basic fermentation medium (with 4 % of soya bean meal and 5 % of soya bean oil) described above, supplementation with different mineral nitrogen sources such as sodium glutamate, urea, $(NH_4)_2SO_4$ and NH_4Cl (all from Sigma-Aldrich) were tested in the fermentation medium to assess their effect on lipstatin production. After optimization of nitrogen sources, different concentrations of soya bean oil were tested on lipstatin production using the basic fermentation medium (containing 4 % of soya bean meal) described above.

Results and Discussion

Bacteria from genus *Streptomyces* are an important source of industrially useful bioactive molecules, including β-lactone metabolites, which inhibit esterase and lipase activity (*6–8*). A number of lipstatin-like β-lactone-containing compounds, produced almost exclusively by *Streptomyces* species, have been reported in the literature (*6–10*). However, most of the β-lactone-producing strains have not been deposited in commercial culture collections and are thus not easily accessible. The focussed taxonomy-based and inexpensive approach to test a selected number of strains belonging to the *Streptomyces lavendulae* phenotypic cluster was therefore carried out as an approach to screen for novel structural and/or functional analogues of known metabolites based on phylogenetic relationships. Biosynthesis of lipstatin has previously been reported from *S. toxytricini* (*7,20*), and little is known about the production of β-lactone metabolites by other members of the *S. lavendulae* phenotypic cluster.

Screening the Streptomyces lavendulae phenotypic cluster for lipase activity

Three culture collections (CBS, NRRL and DSMZ) were examined to identify *Streptomyces* species, which were designated as belonging to the *Streptomyces lavendulae* phenotypic cluster (Table 1). A number of *Streptomyces* strains were deposited as independent isolates; however, based on the phenotypic properties (*e.g.* production of the identical secondary metabolite), a number of target strains was refined. Finally, 15 strains were selected, which were purchased from the corresponding culture collections (Table 1). These strains were cultivated in basic fermentation medium containing vegetable oil (Materials and Methods), which enhances the production of lipstatin-like β-lactones. The lipase inhibitory activity for each strain was evaluated using two esters of *p*-nitrophenyl (*p*NP): *p*NPB and *p*NPP. Extracts from *S. toxytricini* NRRL 15443 (the known lipstatin producer), *S. lavendulae* ssp. *lavendulae* DSM 40558, *S. racemochromogenes* NRRL 5430 and *S. virginiae* CBS 314.55 showed significant inhibitory action against C_4 esters of *p*NP across a range of dilutions (Table 2). However, screening of the same extracts against the C_{16} esters of *p*NP showed that fewer strains had a significant inhibitory activity; *i.e.* extracts from lipstatin producer *S. toxytricini* NRRL 15443 and extracts from *S. virginiae* CBS 314.55 (Table 2). The extracts from *S. lavendulae* ssp. *lavendulae* DSM 40558 and *S. racemochromogenes* NRRL 5430 did not inhibit the C_{16} activity of porcine pancreatic lipase in this assay.

Screening of a range of strains from the *S. lavendulae* phenotypic cluster revealed limited lipase-inhibitory activity against synthetic *p*NPP substrates (C_{16}); however, some activity was observed against synthetic *p*NPB substrates (C_4), suggesting that this group of organisms are a potential source for identification of novel esterase and lipase inhibitors. This disparity in activity between the two substrates may reflect differences in the solubility based on the aliphatic chains of the substrates. Weak lipase inhibitory activity in the extracts of *S. racemochromogenes* CBS 937.68 and *S. lavendulae* ssp. *lavendulae* DSM 40558 strains was observed. However, no inhibitory activity was measured when using *p*NPP as substrate, suggesting esterase activity. In the next stage, HPLC and LC-MS/MS analyses of the selected strains with lipase-inhibitory activity were used for screening of the metabolite corresponding to the mass of lipstatin.

Identification of a metabolite with lipase-inhibitory activity in the culture extract of Streptomyces virginiae CBS 314.55

Streptomyces toxytricini NRRL 15443 and *Streptomyces virginiae* CBS 314.55 both displayed similar lipase inhibition profiles when screened against *p*NP synthetic substrates (C_4 and C_{16}). HPLC analysis of the fermentation broth of *S. virginiae* CBS 314.55, when compared against authentic lipstatin standards and fermentation extracts of *S. toxytricini* NRRL 15443, indicated the presence of a metabolite with an identical retention time to lipstatin (Fig. 2) and the structure was finally confirmed by LC-MS/MS. Methanol extracts of *S. toxytricini* NRRL 15443 and *S. virginiae* CBS 314.55 were compared by applying two different chromatographic conditions using gradient starting with 80 and 50 % acetonitrile (ACN) in the mobile phase (Fig. 3), followed by MS/MS analysis that monitored the following transitions: *m/z*=492.20>315.18 and 492.20>333.19. Both strains produced an identical

Table 2. Lipase inhibitory activity of 16 strains belonging to the *Streptomyces lavendulae* phenotypic cluster determined using *p*-nitrophenyl butyrate (*p*NPB) and *p*-nitrophenyl palmitate (*p*NPP) as substrates. Activity is expressed as percentage of inhibition relative to the control (no inhibition). Only strains with *p*NPP activity are shown. *Streptomyces toxytricini* and *Streptomyces virginiae* (bold) showed highest inhibitory activity on both substrates

Strain	Accession number	Inhibitory activity/%			
		Dilution			
		1×	10×	100×	1000×
*p*NPB as substrate:					
Streptomyces toxytricini	**NRRL 15443**	**93.2**	92.6	88.8	76.4
Streptomyces lavendulocolor	CBS 911.68/NRRL B3367	30.2	10.8	0.0	5.5
Streptomyces flavotricini	CBS 259.66	42.8	15.2	8.8	0.0
Streptomyces goshikiensis	CBS 835.68/NRRL B5428	4.8	0.0	0.0	5.0
Streptomyces subrutilus	CBS 689.72/NRRL B12377	39.7	10.3	5.6	3.7
Streptomyces racemochromogenes	CBS 937.68/NRRL B5430	37.0	19.4	21.5	23.0
Streptomyces polychromogenes	CBS 311.56/NRRL 3032	16.7	6.4	2.9	9.3
Streptomyces katrae	CBS 748.72/NRRL 3093	26.7	7.3	1.3	14.1
Streptomyces xanthophaeus	CBS 572.68/NRRL B5414	23.6	0.0	0.0	0.0
Streptomyces virginiae	CBS 314.55/DSM 40094	**97.6**	96.5	78.3	19.1
S. lavendulae ssp. *lavendulae*	DSM 40385	21.8	11.8	19.3	14.0
S. lavendulae ssp. *lavendulae*	DSM 40558/CBS 597.71	44.8	13.5	16.5	22.9
S. lavendulae ssp. *lavendulae*	DSM 40708	31.5	5.0	0.0	8.1
S. lavendulae ssp. *lavendulae*	DSM 40713	38.4	10.7	11.1	12.3
S. lavendulae ssp. *lavendulae*	DSM 41088	23.1	19.2	0.0	0.0
*p*NPP as substrate:					
Streptomyces toxytricini	**NRRL 15443**	94.9	92.4	70.7	33.4
Streptomyces virginiae	**CBS 314.55/DSM 40094**	89.2	73.3	70.7	14.7

metabolite (*m/z*=492; Fig. 3c), and identical fragmentation pattern to the commercial lipstatin standard confirmed by MS/MS spectra (daughter scan of 492.20 was identical to 333 and 315; Fig. 3). Upon altering the chromatographic conditions from 80 to 50 % ACN, the retention time of lipstatin was altered identically in both strains and in lipstatin standard. The ion mass fragmentation spectra were also identical for the metabolite produced by both strains and the commercial standard confirming the production of lipstatin by *Streptomyces virginiae* CBS 314.55.

Comparative taxonomical characterisation of S. toxytricini and S. virginiae strains

Given the designated phylogenetic similarity between *Streptomyces toxytricini* NRRL 15443 and *Streptomyces virginiae* CBS 314.55, but substantially different physiological and morphological properties, further characterization of these two strains was undertaken to clarify the taxonomic relationship between them. The 16S rRNA sequences from the NCBI database for each of the strains in Table 1 and sequencing of a 16S rRNA gene fragment

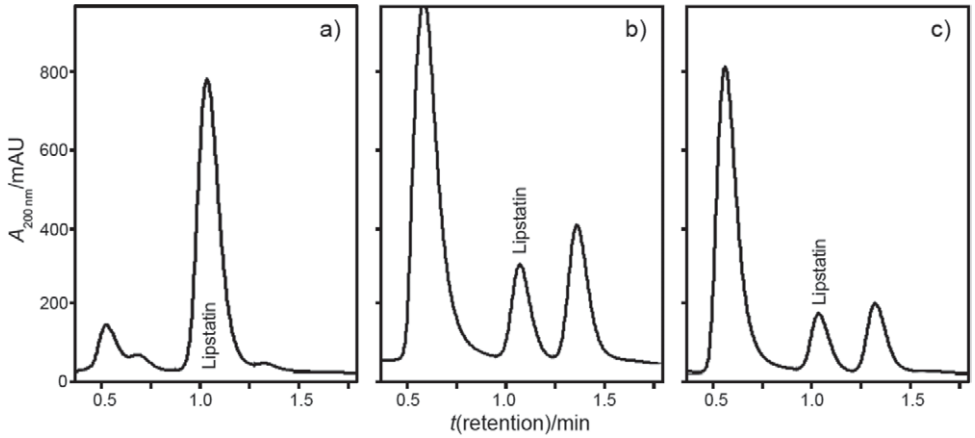

Fig. 2. HPLC analysis of: a) commercial lipstatin standard, and fermentation broth of: b) *Streptomyces toxytricini* NRRL 15443 and c) *Streptomyces virginiae* CBS 314.55 confirms the presence of lipstatin in the fermentation broth of *S. virginiae*
AU=arbitrary units

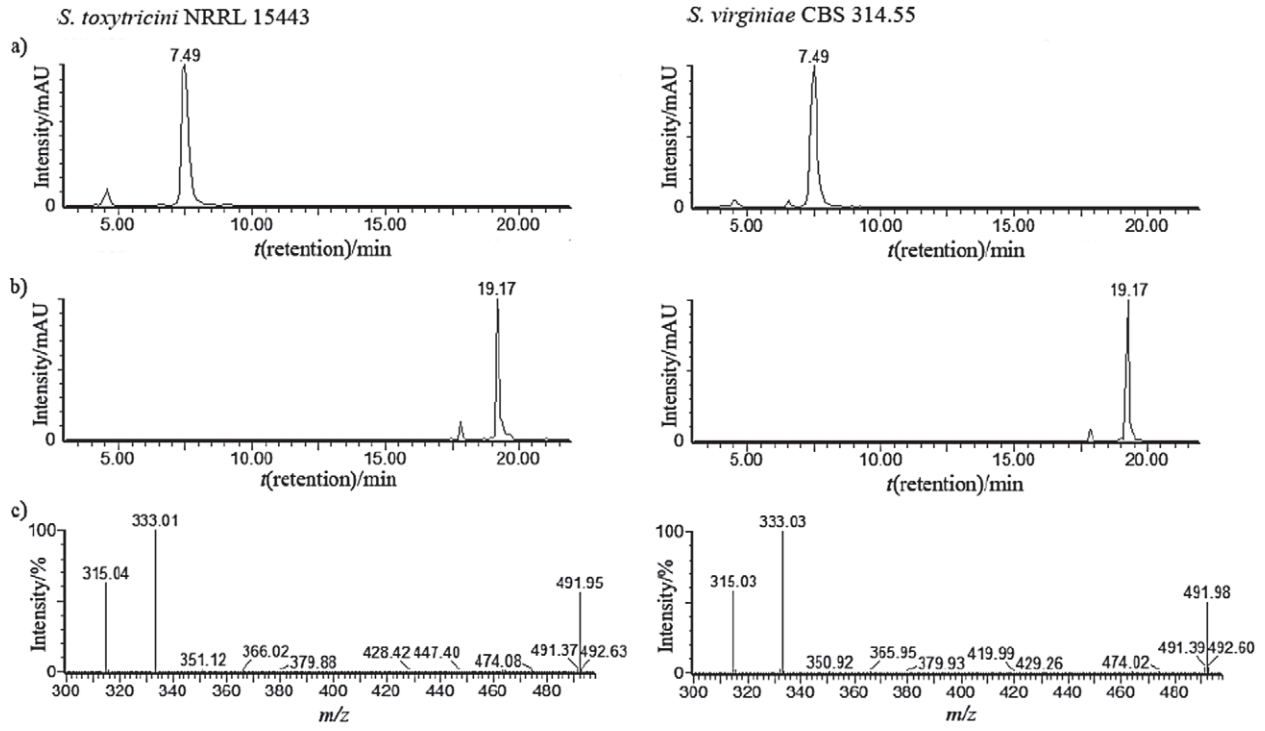

Fig. 3. LC-MS/MS analysis of *Streptomyces toxytricini* NRRL 15443 and *Streptomyces virginiae* CBS 314.55 culture broths compared under different chromatographic conditions: a) gradient starting with 80 % acetonitrile in the mobile phase, b) gradient starting with 50 % acetonitrile in the mobile phase, and c) MS fragment pattern of lipstatin peak at 7.49 min
AU=arbitrary units

for the remaining isolates allowed the construction of the phylogenetic tree (Fig. 4a), indicating a high degree of relatedness between the two lipstatin-producing strains, and the homogeneous nature of the *Streptomyces lavendulae* phenotypic cluster. Construction of the phylogenetic trees using either UMPGA or ML methods (*23*) showed highly similar topologies indicating confidence in the phylogeny. Our analysis has confirmed that, despite significant morphological and physiological differences, both strains belong to the same *S. lavendulae* phenotypic cluster group, which is confirmed by the initial designation in the culture collection.

Morphological and physiological characterisation of Streptomyces virginiae CBS 314.55

Examining colony morphology of the two lipstatin-producing strains on sporulation medium showed significantly different colony morphology (Fig. 4b). To examine the ability of the two strains to utilize a variety of carbon sources, API ID 32 (bioMérieux) tests were used. The differences between the two strains are summarised in Table 3; the results of assimilation were identical when using all other tested carbon sources. The newly identified *S. virginiae* strain displays entirely different colony

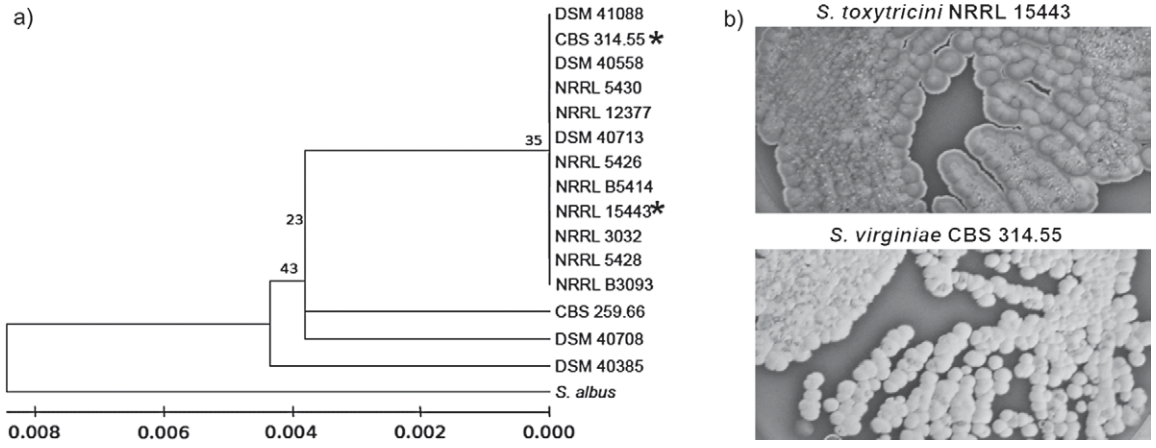

Fig. 4. Phylogenetic analysis of strains belonging to the *Streptomyces lavendulae* phenotypic cluster used in this work (a). An UPMGA tree (bootstrapped to 1000 replicates) based on the 16S rRNA indicates the close relationship of this cluster of species. Asterisks indicate lipstatin-producing strains. Colony morphology and colour differences in the two lipstatin-producing strains indicating the variation in the phenotypes of this cluster (b)

Table 3. Differences in the utilization of different carbon sources on API ID 32 test (bioMérieux) between *Streptomyces toxytricini* NRRL 15443 and *Streptomyces virginiae* CBS 314.55

Carbon source	*Streptomyces toxytricini* NRRL 15443	*Streptomyces virginiae* CBS 314.55
D-xylose	–	+
L-arabinose	–	+
D-cellobiose	–	+
D-lactose	+	–
Glucosamine	+	–

+ growth, – no growth

morphology on the agar plates, regarding the shape of the colonies as well as pigmentation (Fig. 4b). In addition, the two strains differ also in the capacity of assimilating five simple carbon sources, thus reflecting significant physiological properties.

The effect of nutrient source on the production of lipstatin by S. virginiae CBS 314.55

With the aim of examining the potential of *S. virginiae* CBS 314.55 as an industrial lipstatin producer, our experiments were based on industrial media previously shown to be suitable for the production of lipstatin by *S. toxytricini* strain (*20*). As demonstrated previously (*28–30*), fatty acids are the main source of precursors for lipstatin-like compounds and therefore significant yields of these compounds can only be achieved by using fatty acids (or vegetable oil) as a main source of carbon, as demonstrated in recent publication by Luthra and Dubey (*31*), where production medium was optimized by applying Plackett-Burman design, three factorial and one--variable-at-a-time approaches, where the effect of five medium ingredients on lipstatin production was evaluated, including glycerol, polypropylene glycol, soya bean oil, soya lecithin and soya bean meal. Results clearly demonstrate that soya bean oil has the most pronounced positive effect on the lipstatin production using *S. toxytricini* ATCC 19813 as a production strain (*31*). Therefore, the basic fermentation medium used in our experiments contained 5 % of soya bean oil and was used to assess the influence of nitrogen sources on the production of lipstatin. Altering the provision of nitrogen sources in the production medium (containing 5 % of soya bean oil as the main carbon source) and its effect on lipstatin production were examined using a range of complex nitrogen sources commonly used in industry. Additionally, supplementation of a range of simple mineral sources of nitrogen was also tested to examine their effect on lipstatin production. Maximum lipstatin production (122 mg/L) was obtained in the production medium containing 40 g/L of soya bean meal, although comparable lipstatin yields were also obtained using cottonseed meal and soya peptone. Yeast extract and tryptone showed a significantly reduced level of lipstatin production, suggesting the repression of biosynthesis by these nitrogen sources (Fig. 5a). Production medium containing 5 % of soya bean oil and 40 g/L of soya bean meal was used to test the effect of supplementation of mineral nitrogen sources on lipstatin production. Supplementation of the medium with 2.5 g/L of sodium glutamate slightly increased lipstatin production (139 mg/L); however, higher concentrations of sodium glutamate and the addition of urea or NH_4Cl significantly repressed lipstatin biosynthetic activity (Fig. 5b).

Following the testing of different nitrogen sources, effect of different fractions of soya bean oil was also examined. The provision of 1 to 10 % soya bean oil in production medium as a carbon source showed that lipstatin production was proportional to the soya bean oil increase, where the final yield of 123 mg/L of lipstatin was reached (Fig. 5c).

The highest yield of lipstatin achieved with *S. virginiae* CBS 314.55 was over 120 mg/L, thus showing good production capacity of the strain. In contrast, lipstatin yield between 45–70 mg/L, using the same medium, was achieved with *S. toxytricini* strain NRRL 15443.

Interesting difference between *S. virginiae* and *S. toxytricini* was observed in relation to the influence of increasing concentration of ammonium sulphate on lipsta-

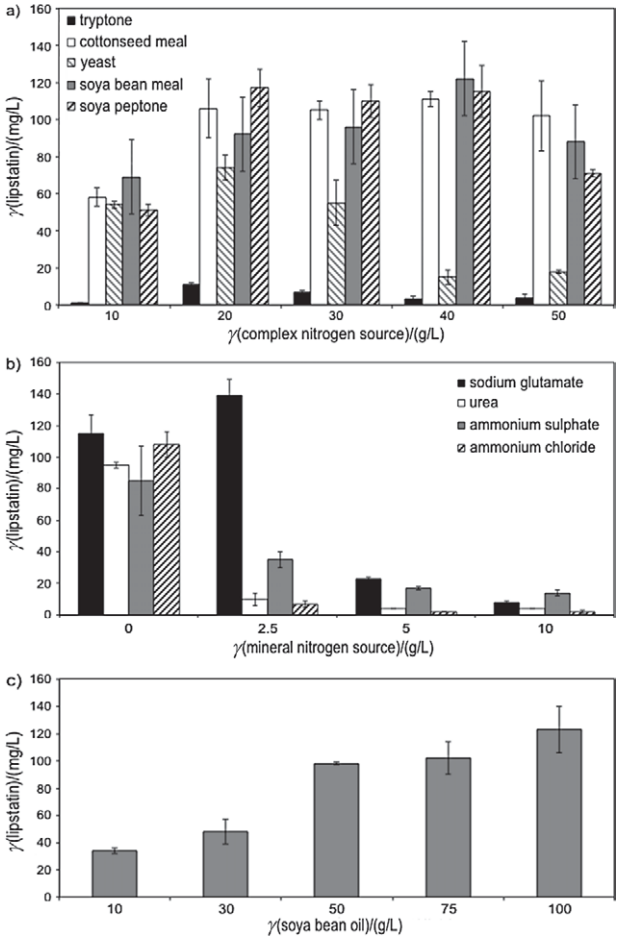

Fig. 5. The effect of carbon and nitrogen sources in the medium on lipstatin production by *Streptomyces virginiae*: a) complex nitrogen sources using fermentation medium with 5 % of soya bean oil, b) supplementation with simple/mineral nitrogen sources using fermentation medium containing 4 % of soya bean meal and 5 % of soya bean oil, and c) effect of the addition of different concentrations of soya bean oil. Error bars present standard deviation from two independent biological replicates

tin production. Supplementation of the basic production medium with different concentrations of $(NH_4)_2SO_4$ significantly repressed lipstatin production by *S. virginiae* strain, but not by *S. toxytricini.* When using *S. virginiae*, the addition of 2 g/L of $(NH_4)_2SO_4$ decreased lipstatin yield for more than 50 % and further increase in $(NH_4)_2SO_4$ concentrations reduced the yield of lipstatin for more than five times. However, when using *S. toxytricini* strain, initial increase of $(NH_4)_2SO_4$ displayed a positive effect on lipstatin production (Fig. 6). Addition of 2 g/L of $(NH_4)_2SO_4$ resulted in the highest lipstatin yield (157 mg/L) when using *S. toxytricini* strain, where lipstatin production was increased more than twice, compared to control. Concentrations higher than 5 g/L of ammonium sulphate showed negative effect on lipstatin production with both strains. Thus, *S. virginiae* displayed higher nitrogen repression phenotype with nitrogen as mineral source, compared to *S. toxytricini*.

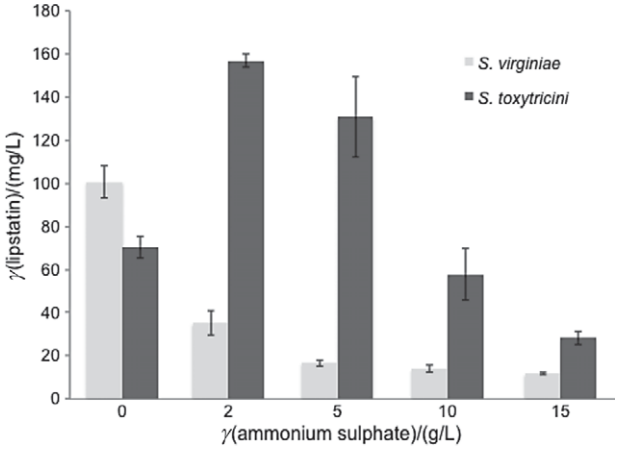

Fig. 6. The effect of ammonium sulphate supplementation in production medium, containing 4 % of soya bean meal and 5 % of soya bean oil, on lipstatin production by *Streptomyces virginiae* and *S. toxytricini* strains

The composition of the industrial production medium and how the bioprocess is carried out play a significant role in terms of product yield, purity profile and overall economy of the process (*32*). The use of vegetable oil in industrial fermentations is widespread and vegetable oil is often used as a carbon source or, as in our case, an important source of precursors for biosynthesis of lipstatin (*33–36*). Linoleic acid is major component of soya bean oil and therefore substrate for biosynthesis of main C_{14} precursor (tetradeca-5,8-dienoic acid), which is needed for lipstatin production. The provision of rich organic nitrogen sources, such as cottonseed meal, soya peptone and soya bean meal, exhibited significant positive effects on lipstatin production in *S. virginiae*. It has been shown that organic nitrogen sources, rich in amino acids and peptides, can significantly affect carbon source uptake in actinomycetes (*37*) or *N*-formyl-L-leucine, which is derived from the amino acid leucine and is also important biosynthetic precursor of lipstatin. Similar observations were obtained by Luthra and Dubey (*31*) using *S. toxytricini* ATCC 19813 strain, where the highest lipstatin production was obtained in the medium consisting of (in g/L): glycerol 22.5, soya bean meal 35, soya bean oil 15, soya lecithin 25 and polypropylene glycol 0.5. In this study, soya bean oil, soya lecithin and soya bean meal had significant effects on lipstatin production.

Conclusions

This work has shown for the first time the capability of *S. virginiae* CBS 314.55 to produce lipstatin. We have demonstrated that it is possible to identify target compound(s) produced by a new *Streptomyces* species after careful analysis of the taxonomic data available on strains from public culture collections using this affordable approach. Interestingly, the *S. lavendulae* group was difficult to resolve using 16S RNA methods alone, corroborating the work suggesting that secondary metabolic capability may have a role in strain designation (*19,38*). The identification of previously unknown lipstatin production in *S. virginiae* CBS 314.55 highlights the importance of phylogenetic dereplication in the construction of natural product libraries. The production of the same or similar metabolites by related taxa is an issue that can be potentially time and resource consuming in an industrial context when a novel activity is being screened for. However, dereplication can be useful when identifying novel strains with potentially advanced physiological or morphological properties.

Clearly, considering morphological and physiological properties, and lipstatin productivity of these two lipstatin-producing strains, *S. virginiae* CBS 314.55 strain displays at least comparable (if not superior) properties. Therefore, the newly identified lipstatin producer *S. virginiae* shows potential for its industrial application. Naturally, further work is needed to assess the properties and potential advantages of *S. virginiae* in the industrial settings and during process scale up.

It is most likely that many *Streptomyces* strains that were screened for antibacterial activity in the 1950s and 1960s may produce metabolites of interest in a wider medical context or are more tractable industrially. These data suggest that dereplication and re-examination of deposited strains can lead to affordable discoveries of potentially useful industrial strains.

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