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Kermanshah University Of Medical Sciences

عنوان مقاله ژورنال کلاب:

Description of Streptomyces naphthomycinicus sp. nov., an endophytic actinobacterium producing naphthomycin A and its genome insight for discovering bioactive compounds

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Description of *Streptomyces naphthomycinicus* sp. nov., an endophytic actinobacterium producing naphthomycin A and its genome insight for discovering bioactive compounds

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ACTIONS

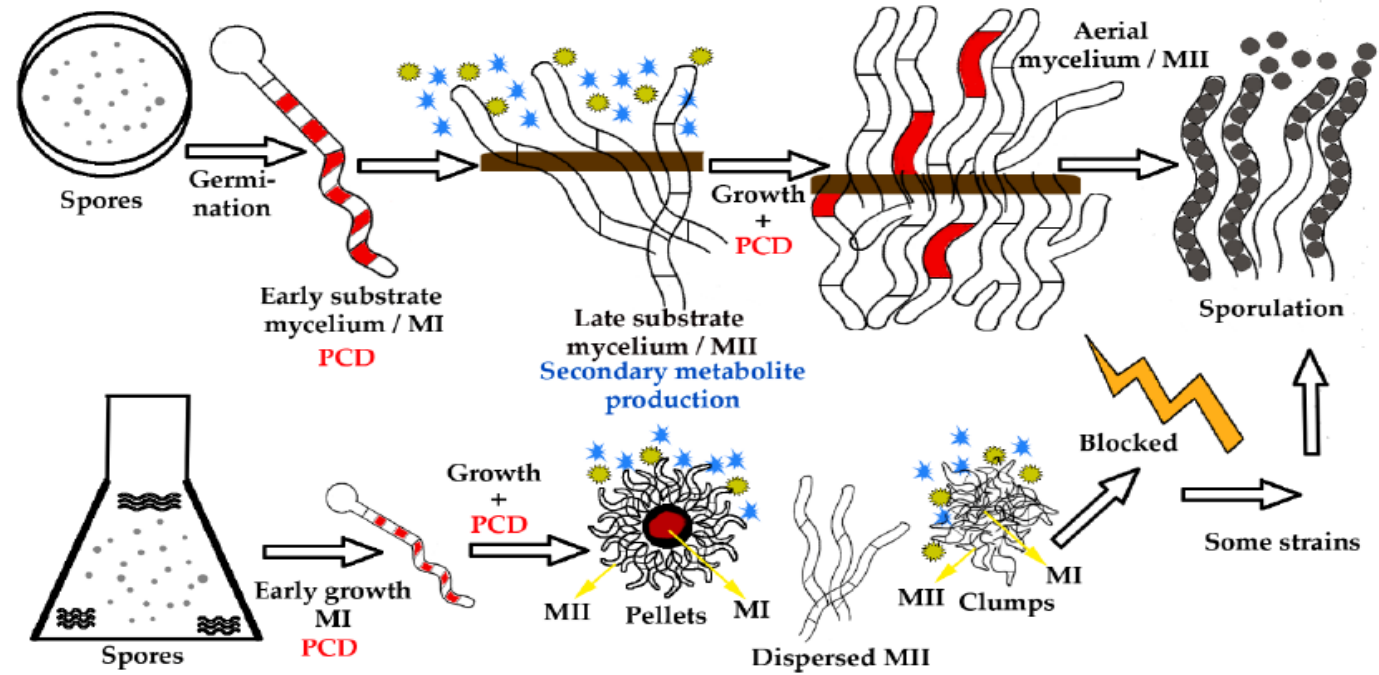


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introduction

- Endophytes
- Streptomyces
- biosynthetic gene clusters (BGCs)
- Regulatory mechanisms for Ab production
- liquid-state fermentation, negative side



<https://doi.org/10.3390/antibiotics7020041>

Aim of this survey:

- describe strain TML10T(Polyphasic taxonomic approach)
- investigate the production of antibiotics
- determine the structures of its bioactive compounds
- examine the biosynthesis gene clusters and genome data mining
- preliminary screening of arginine degradation, phylogenetic tree and sequence analysis of arginine deiminase

Materials and methods+ result

- Isolation of strain TML10T

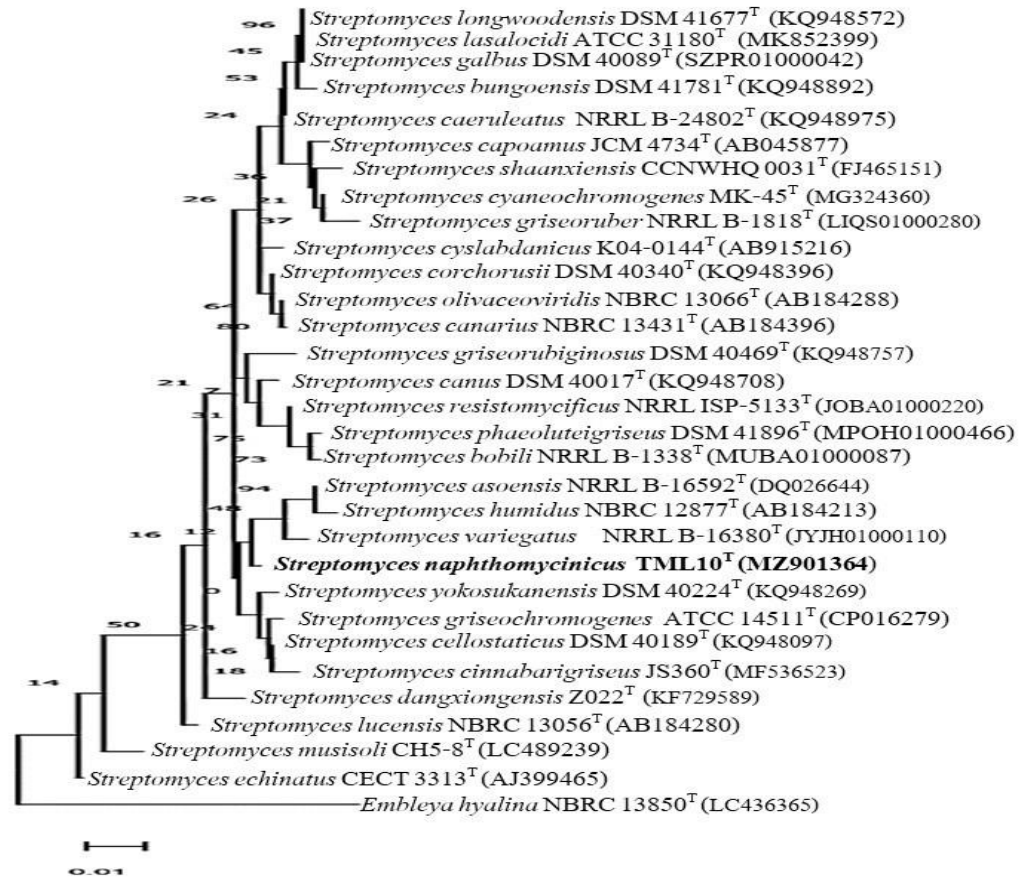
Humic acid vitamin B agar(HVA)/ VL70 gellan gum with the amino acid mixture /VL70 gellan gum with carboxymethyl cellulose

- 16s rRNA gene analysis and phylogenetic characteristic

- *S. cellostaticus* NBRC 12849T (99.2%), *Streptomyces yokosukanensis* NRRL B-3353T (99.0%), *Streptomyces bungoensis* DSM 41781T (99.0%), and *Streptomyces cinnabarigriseus* JS360T (99.0%).

- 1,453 bp/ maximum likelihood tree

16s rRNA gene analysis



Whole genome sequencing, and genome assembly and annotation of strain TML1

- short read platform(from Illumina)
- long-read technique(Oxford Nanopore Technologies (ONT))
- Quast (Version 5.2.0) genome assembly quality
- BUSCO (Version 5.4.6) was used to assess the completeness of a genome assembly quantitatively
- Prokka version 1.14.6 (Seemann, 2014) was applied to annotate the genome sequences.

- Longread technique
- 10.16 Mbp/ 8,570 genes/G + C content of 72.4
- BUSCO result=99.9%

Genome comparison study

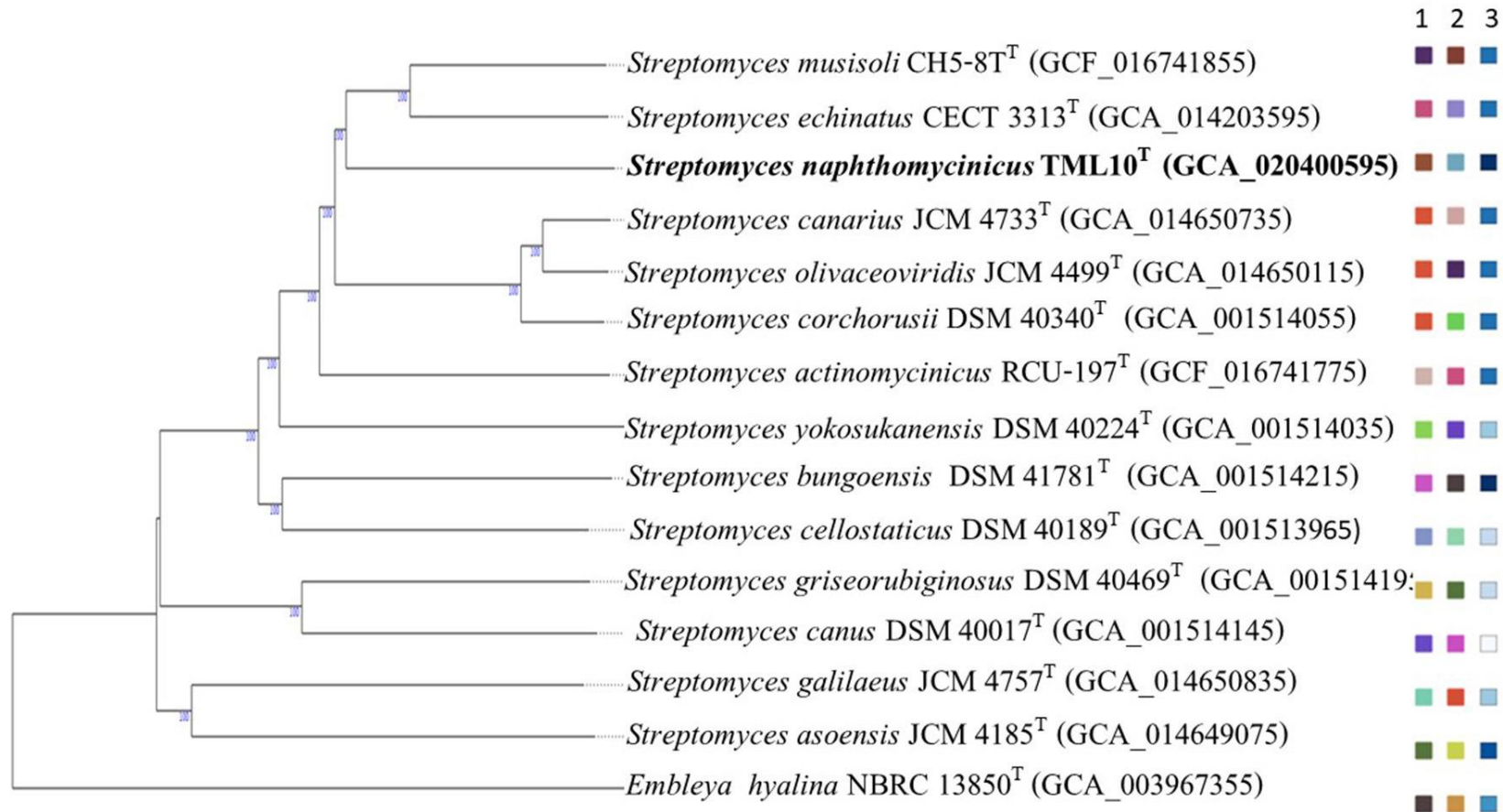
- Average nucleotide identity (ANI)
- JspeciesWS web service
- phylogenetic tree(Type (strain) Genome Server (TYGS))
- Digital DNA-DNA hybridization (dDDH)

TABLE 2 16S rRNA gene sequence similarity, average nucleotide identities, digital DNA:DNA hybridization values (%) between strain TML10^T and their related species.

Strain/comparison with 1.	2.	3.	4.	5.
16S rRNA gene similarity (%)	97.1	97.2	98.9	99.2
(ANiB) (%)	88.5	88.5	87.2	84.1
(ANIm) (%)	90.8	90.7	90.4	88.4
dDDH (%)	38.8 (C.I. model 36.4–41.4)	38.6 (C.I. model 36.1–41.3)	37.8 (C.I. model 35.3–40.3)	31.8 (C.I. model 29.4–34.3)
Genome completeness and contamination (%)	100, 1.9	99.86, 0.82	99.53, 1.33	99.47, 2.62

Strain: 1, *Streptomyces naphthomycinicus* TML10^T; 2, *Streptomyces musisoli* CH5-8^T; 3, *Streptomyces echinatus* CECT 3313^T; 4, *Streptomyces corchorusii* DSM 40340^T; 5, *Streptomyces cellostaticus* NBRC 12849^T.

Genome comparison study



Chemotaxonomic, cultural and morphological properties

- Whole-cell sugar
- diaminopimelic acid (DAP)
- Phospholipids
- Whole cell fatty acids

Medium/ Strain	Growth (Good /poor)	Aerial mycelium (color)	Substrate mycelium (color)
Strain TML10^T			
ISP 2	Good	Grayish green	Brown
ISP 3	Good	Greenish gray	Greenish yellow
ISP 4	Good	Grayish brown	Dark yellow
ISP 5	Good	Yellowish white	Yellow
ISP 7	Good	Grayish green	Dark brown with melanin pigment
Bennett's agar	Good	Grayish green	Olive green
1/2 Potato Dextrose agar	Good	Brownish gray	Dark brown
Nutrient agar	Moderate	white	Light yellow

Colonies were tough
spiny surfaces



- LL-diaminopimelic acid
- Whole-cell sugars : galactose, glucose, and mannose
- lipids: PE, PG, PI, an unknown lipid with amino group (LPA), and an unknown lipid with phosphate group (LPL)

Table S2. Whole-cell fatty acid composition (%) of *Streptomyces naphthomycinicus* TML10^T
 Only fatty acids detected at more than 0.1 % of the total are presented. -, not detected. Bold presents fatty acid more than 10%.

Fatty acids	Percent
<i>anteiso</i> -C _{13:0}	0.2
<i>iso</i> -C _{14:0}	1.9
C _{14:0}	0.6
<i>iso</i> - C _{15:1} G	0.3
<i>iso</i> -C _{15:0}	8.5
<i>anteiso</i> -C _{15:0}	23.8
C _{15:1} w8c	0.3
C _{15:1} w6c	1.4
<i>iso</i> -C _{16:1} H	1.1
<i>iso</i> -C _{16:0}	15.3
C _{16:0}	7.8
<i>anteiso</i> -C _{17:1} w9c	2.6
<i>iso</i> -C _{17:1} w10c	-
<i>iso</i> -C _{17:0}	5.7
<i>anteiso</i> -C _{17:0}	20.1
C _{17:1} w8c	0.4
C _{17:0} cyclo	0.4
C _{17:0}	0.7
C _{16:1} 2OH	0.4
<i>iso</i> -C _{18:1} H	0.4
<i>iso</i> -C _{18:0}	0.4
C _{18:1} w9c	1.2
C _{18:0}	1.2
<i>iso</i> -C _{20:0}	1.9
C _{16:1} w7c	1.8
C _{18:1} w7c	0.3
C _{16:0} 10-methyl	1.6

Phenotypic characterization

TABLE 3 Differential characteristics between *Streptomyces naphthomycinicus* TML10^T and related species of *Streptomyces*.

Characteristics/strains	1 [#] .	2 ^a .	3.	4.	5 [#] .
Spore chain	Loop	Spiral	Spiral ^c	Spiral ^c	Spiral ^c
Spore surface	Spiny	Rough	Spiny ^c	Smooth ^c	Spiny ^c
Aerial mass color on ISP 2	Grayish green	Medium gray	Gray ^c	Light grayish yellowish brown ^c	Pale yellowish green
Diffusion pigment on ISP2	-	+ (brilliant yellow)	- ^c	- ^c	-
Melanin pigment production on ISP 7	+	+	+ ^c	- ^c	+
Growth at pH 4	-	-	- ^a	+ ^b	-
Minimum pH for growth	5	6	6 ^a	nd	5
Maximum NaCl tolerance (% w/v)	5	7	3 ^a	2 (w) ^d	5
Maximum temperature for growth (°C)	37	45	42 ^a	nd	37
Hydrolyze of starch	+	-	w ^a	+ ^d	+

Strain: 1, *Streptomyces naphthomycinicus* TML10^T; 2, *Streptomyces musisoli* CH5-8^T; 3, *Streptomyces echinatus* JCM 4144^T; 4, *Streptomyces corchorusii* NBRC 13032^T, 5, *Streptomyces cellostaticus* NBRC 12849^T. +, positive or present; -, negative or absent; w, weak; nd, no report. Data was taken from ^aDuangupama et al. (2021); ^bTaké et al. (2015); ^cKämpfer (2012); ^dLaw et al. (2019); [#]all data from this study.

Data from the literatures used the same methods with this study except; ^aThe pH range for growth was tested in ISP 2 broth. ^dNaCl tolerance was tested in tryptic soy broth.

Based on the polyphasic taxonomy, strain TML10T was proposed as a novel species named *Streptomyces naphthomycinicus* sp. nov.

Preliminary screening for antimicrobial activity against bacteria and fungi

- The antimicrobial activity of strain TML10T was tested on HPDA using a cross-streak method described previously
- The antifungal assay by a dual culture method on HPDA

TABLE 4 Antimicrobial activity of strain TML10^T against tested microorganism by dual culture technique.

Strain	Inhibition against							
	<i>Bacillus cereus</i> ATCC 11778	MRSA DMST 20654	<i>Staphylococcus aureus</i> ATCC 25923	<i>Xanthomonas oryzae</i> PXO 71	<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Candida albicans</i> BCC 7390	<i>Curvularia lunata</i> BCC 15558	<i>Fusarium incarnatum</i> BCC 4829
TML10 ^T	++++	+++	+++	++	++	++++	++++	++

++++, strong inhibition; +++, good inhibition; ++, moderate inhibition; +, weak inhibition.

Liquid and solid-state fermentation of strain TML10

TABLE 1 Recipe of liquid and solid media for antibiotic production of strain TML10^T.

Media	Recipe (composition per 1 L RO water for broth media)
IM 22 broth	Glucose 15 g, soyatone 15 g, pharmamedia 5 g, CaCO ₃ 2 g, NaCl 5 g
ISP 2 broth	Malt extract 10 g, glucose 4 g, yeast extract 4 g
F26 broth	Glucose 20 g, soy bean flour 10 g, CaCO ₃ 4 g, CoCl ₂ .6H ₂ O 1 mg
F40 broth	Glucose 0.5 g, soluble starch 15 g, malt extract 5 g, profolo 3 g, corn steep liquor 2 g, CaCO ₃ 2 g, MgSO ₄ .7H ₂ O 1 g, NaCl 2 g, trace elements solution 1 mL Trace elements solution was (composition per liter); CuSO ₄ .5H ₂ O 1 mg, FeSO ₄ . 5H ₂ O 7 mg, MnCl ₂ .4H ₂ O 8 mg, ZnSO ₄ .7H ₂ O 2 mg
SI broth	Sucrose 20 g, CaCO ₃ 2.5 g, KNO ₃ 1 g, K ₂ HPO ₄ 0.5 g, MgSO ₄ .7H ₂ O 0.5 g, NaCl 0.5 g
Solid medium	Basmati Rice 20 g, 5 ml of LF 42 medium containing 1× HO-LE solution and 15 ml of RO water LF 42 medium; yeast extract 5 g, peptone 5 g, soya flour 5 g, glycerol 4 ml, soluble starch 2 g, CaCO ₃ 2 g, NaCl 2 g, K ₂ HPO ₄ 0.5 g and MgSO ₄ .7H ₂ O 0.5 g/L RO water HO-LE solution (1,000×) contained (g/L) H ₃ BO ₃ 2.85, MnCl ₂ .4H ₂ O 1.8, Sodium tartrate 1.77, FeSO ₄ .7H ₂ O 1.36, CoCl ₂ .6H ₂ O 0.04, CuCl ₂ .2H ₂ O 0.027, Na ₂ MoO ₄ .2H ₂ O 0.025 and ZnCl ₂ 0.020

Antimicrobial assay and antibiotics production of strain TML10

- Agar diffusion method
- *Pseudomonas aeruginosa*, *Escherichia coli*, *S. aureus*, methicillin-resistant *S. aureus*(MRSA), resistant *Candida albicans*
- Vancomycin (500µg/ml), colistin (500µg/ml), and amphotericin B (250µg/ml)
- Absolute methanol and sterilized liquid media were used as negative controls

TABLE 5 Antibiotics production of strain TML10^T in four liquid media and rice medium against test organisms by agar diffusion assay.

Medium	Inhibition zone (mm) [#]				
	<i>Staphylococcus aureus</i> ATCC 29213	MRSA 03120385	<i>Escherichia coli</i> JCM 109	<i>Pseudomonas aeruginosa</i> 06348315	<i>Candida albicans</i> ATCC 10231
ISP 2	-	-	-	-	-
Met+ISP2	-	-	-	-	-
F 26	14.3 ± 0.28ab	9.7 ± 0.28a	-	-	8.3 ± 0.28ab
Met+F26	-	-	-	-	-
F40	12.6 ± 0.12a	10.2 ± 0.28ab	-	-	8.2 ± 0.28a
Met+F40	-	-	-	-	-
S1	-	-	-	-	-
Met+S1	-	-	-	-	-
Rice medium	17.7 ± 0.28ab	18.3 ± 0.57ab	-	-	12.8 ± 0.28ab
Vancomycin 500 µg/ml	18.8 ± 0.28b	18.7 ± 0.57b	nd	nd	nd
Colistin 500 µg/ml	nd	nd	17 ± 0.28	11.5 ± 0.25	nd
Amphotericin B 250 µg/ml	nd	nd	nd	nd	14.5 ± 0.5b

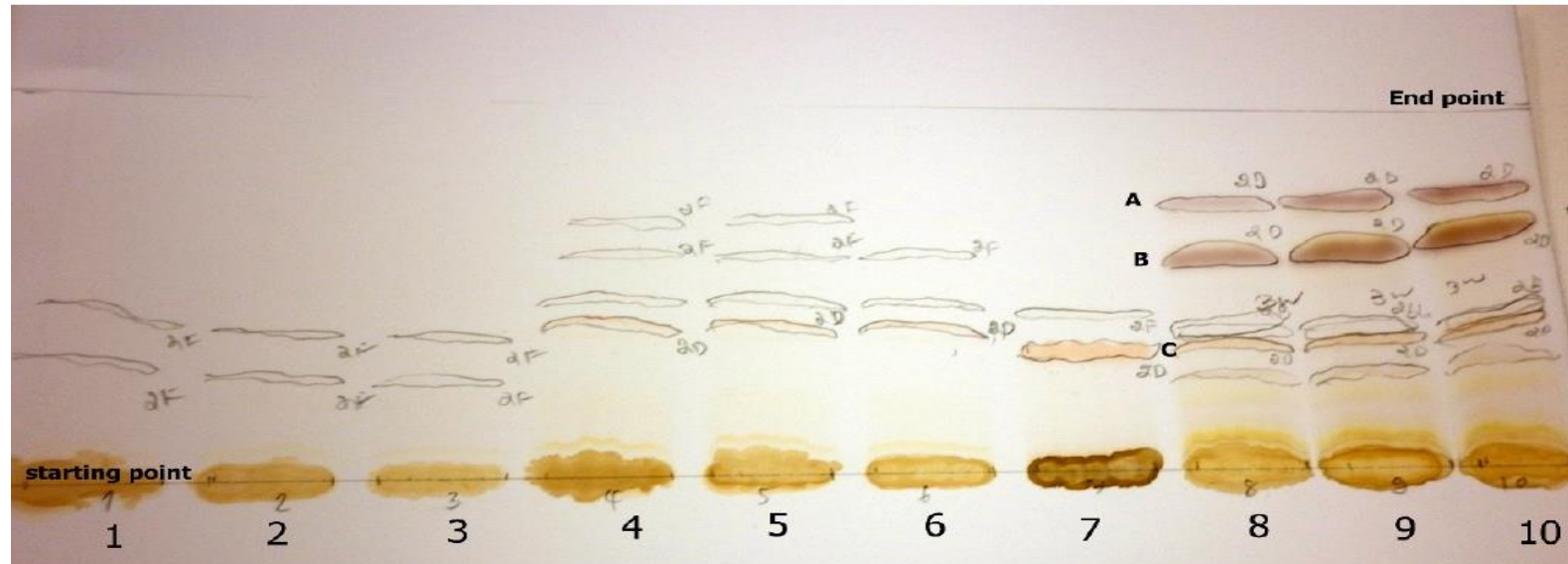
[#]Values are mean of triplicate determination ($n = 3$) ± standard deviation.

-, no zone of inhibition was found; Met+, methanol extracted from cells which grown on each medium; nd, not done. Lower case letters in the same column indicate significant differences ($p < 0.05$).

Antibiotic production of strain TML1

- Based on the bioactivity, F26 and rice medium were tested for a large scale antibiotic production
- against *S. aureus* ATCC 29213 and MRSA 03120385
- rice medium contained two compounds with antimicrobial activity: compound A (CA) (RF 0.69) and compound B (CB) (RF 0.53)
- concentrated compound from the F26 liquid medium collected on days 2 and 4 showed no activity

- rice medium was selected for large-scale antibiotic production to purify the antibiotic and its structural elucidation as the production of bioactive compounds was stable compared to the liquid medium F26.



Supplementary figure S1. TLC profile of antibiotics production of strain TML10 on different medium

(solvent system; chloroform: methanol (9:1)) A, compound A; B, compound B

Lane 1; Freeze dried broth of F26 harvest 2 d dissolved with water

Lane 2; Freeze dried broth of F26 harvest 2 d dissolved with 30% methanol

Lane 3; Freeze dried broth of F26 harvest 2 d dissolved with 50% methanol

Lane 4; Frozen dried broth of F26 harvest 4 d dissolved with water

Lane 5; Frozen dried broth of F26 harvest 4 d dissolved with 30% methanol

Lane 6; Frozen dried broth of F26 harvest 4 d dissolved with 50% methanol

Lane 7; Strain TML10 grown on ISP 2 agar and extracted with absolute methanol and dried compound dissolve with 50% methanol

Lane 8; Extracted rice medium dissolved with 30% methanol

Lane 9; Extracted rice medium dissolved with 50% methanol

Lane 10; Extracted rice medium dissolved with 70% methanol

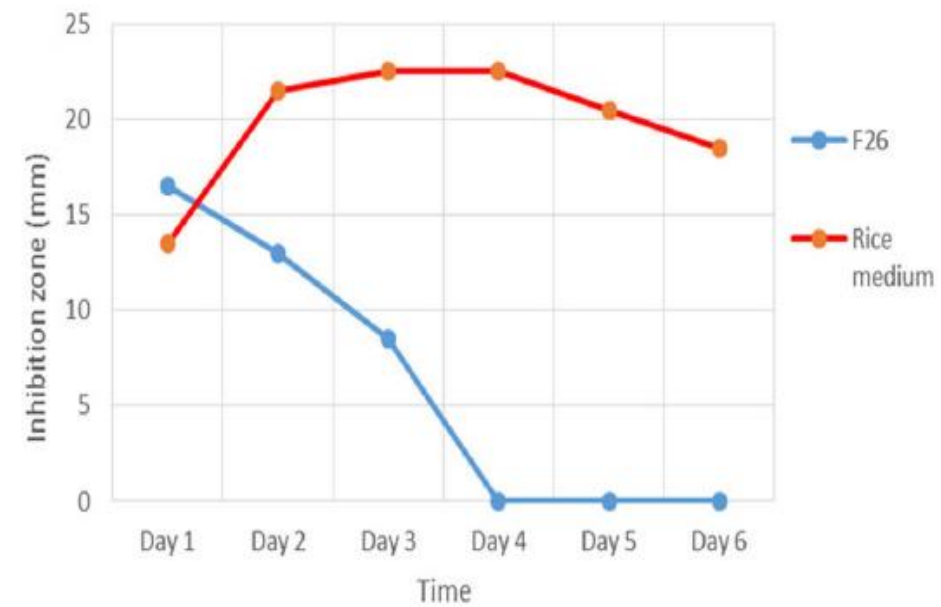
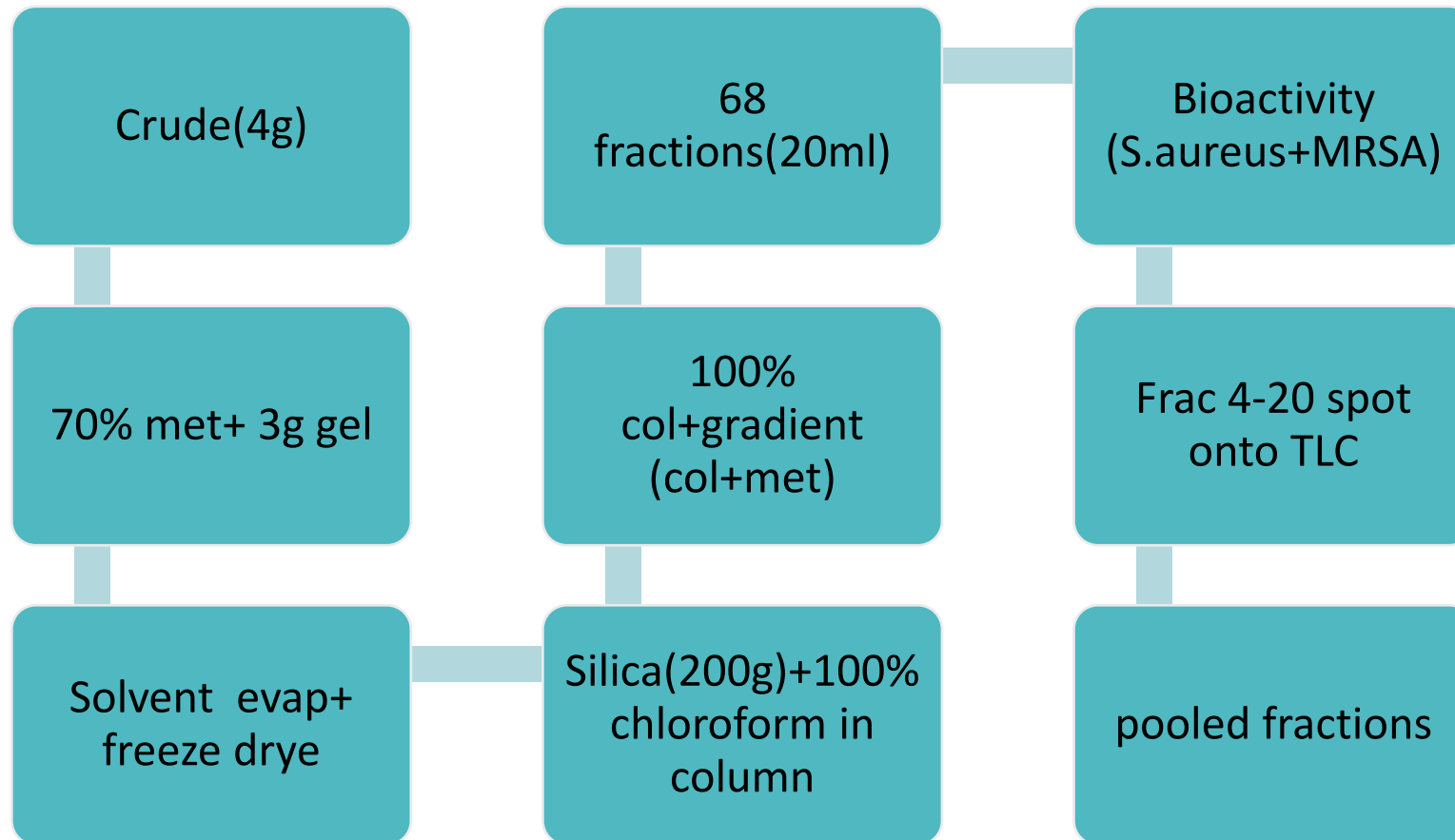


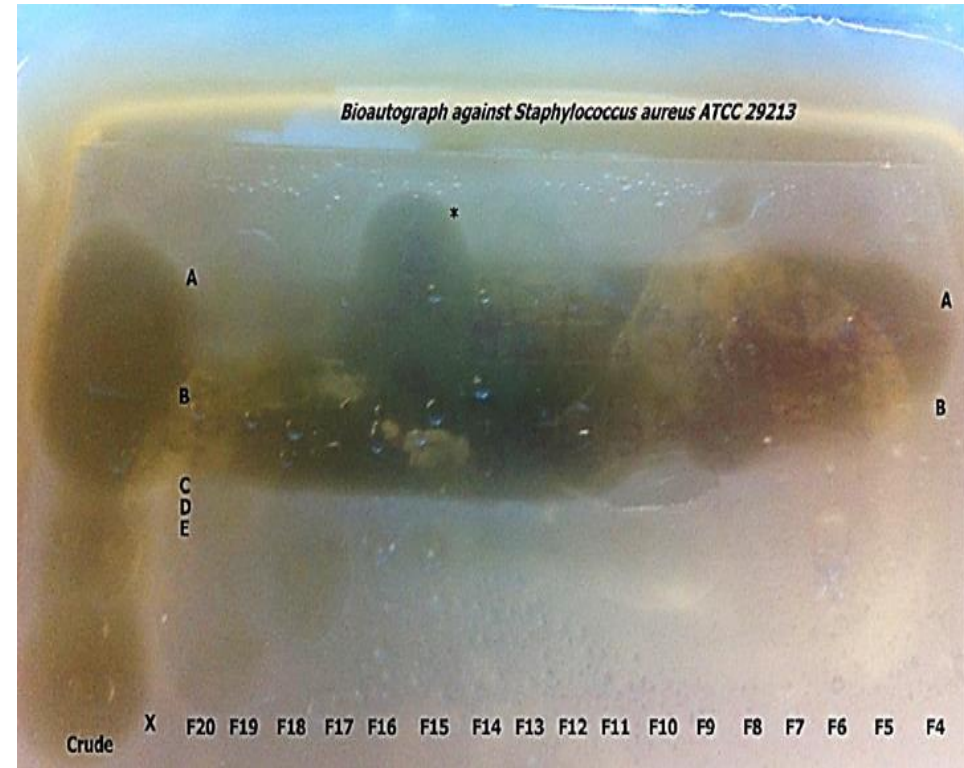
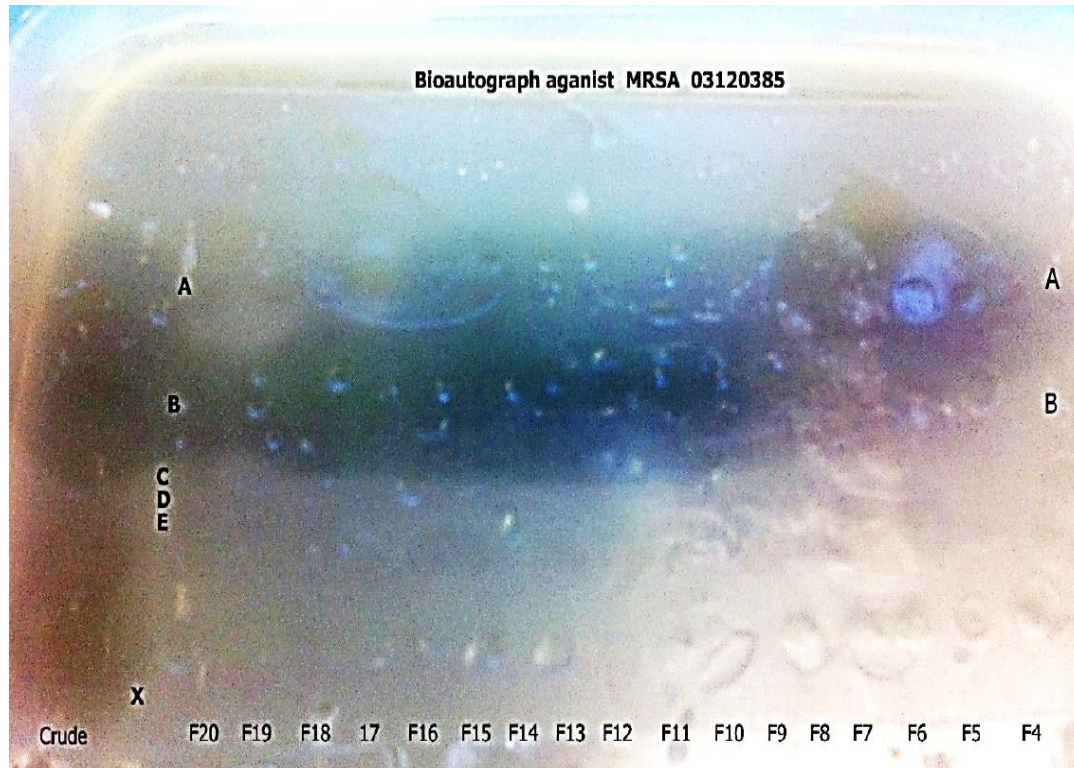
FIGURE 2
Antibiotic production of strain TML10^T in F26 liquid medium and solid state with rice grain inhibit *Staphylococcus aureus* ATCC 29213 for 6 days.

Using solid state fermentation

- would be beneficial in terms of consistency in production
- requiring less energy, and lowering cost by using a variety of agricultural wastes
- Based on this study, solid fermentation was better than liquid fermentation for a bioactive compound screening program

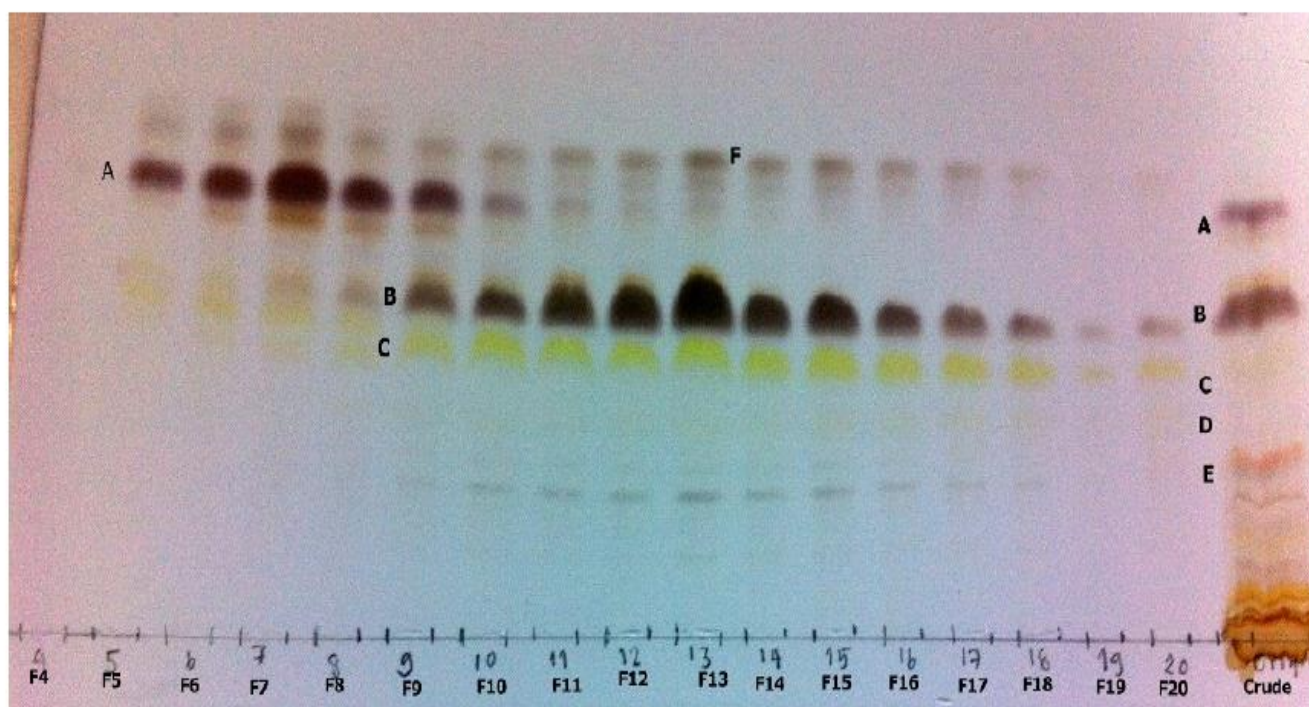
Purification of the antibiotic compounds





Fractions 4– 8 included the bioactive compound CA.

Fractions 9–11 contained a mixture of CA and CB, while fractions 12–16 showed major compound CB

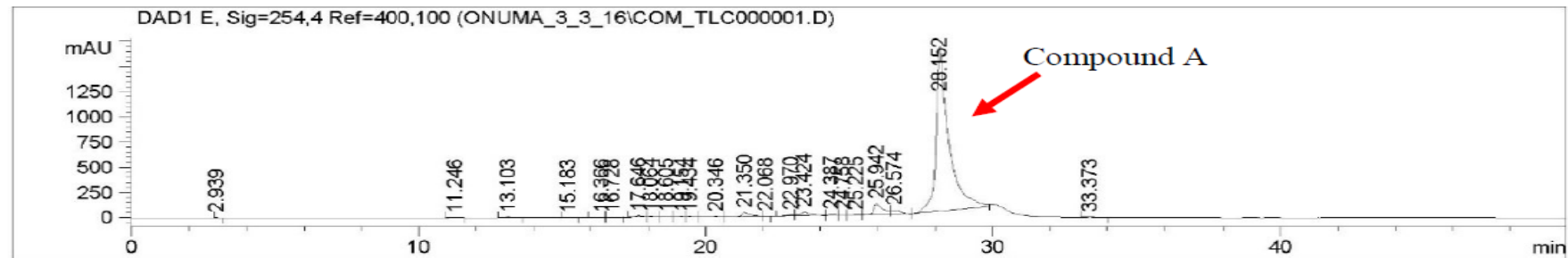


Supplementary figure S3 TLC on silicagel 60 F₂₅₄ of fraction 4-20 from silica gel column of bioactive compounds of strain TML10^T; F4 –F20, fraction 4 – 20; crude, crude methanol extract from rice. solvent system was chloroform: methanol (9:1). A, B, C, D, E and F; compound CA, CB, CC, CD CE and CF.

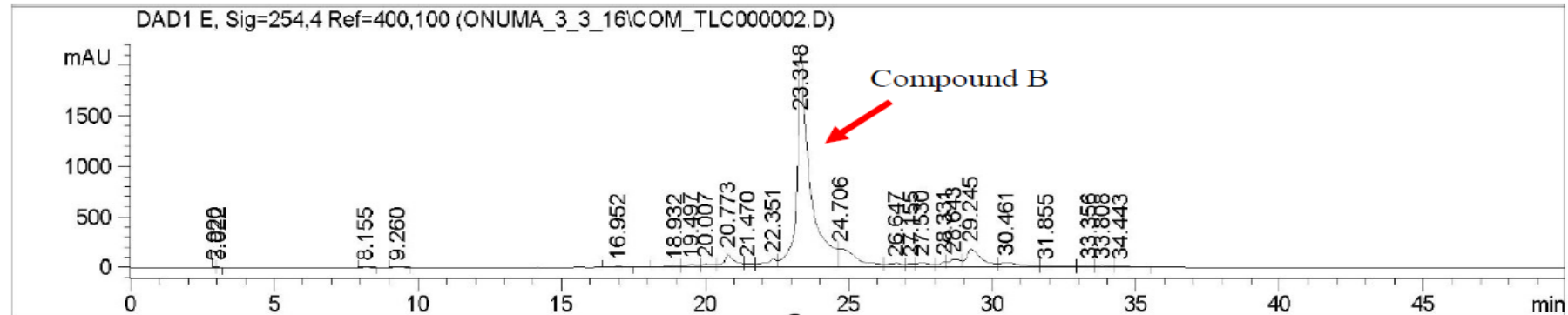
High performance liquid chromatography

solvent system was acetonitrile: water with 0.1% trifluoroacetic acid by increasing gradient of 10% acetonitrile to 90% acetonitrile within 40 mins at a flow rate of 1.0 ml/min at 27°C. All samples were repletely injected three times.

Structural elucidation by using LC-MS and NMR



A)



B)

Supplementary figure S4. HPLC analysis of bioactive compounds of strain TML10^T. A); compound A (retention time 28.15 min) and B); compound B (retention time 23.31 min) (red arrows) purified by silica gel 60 column and further prepared by preparative TLC.

- CA and CB corresponded to known compounds, naphthomycin A ($C_{40}H_{46}NO_9Cl$) and naphthomycin B ($C_{39}H_{44}NO_9Cl$), respectively
- Naphthomycin and its derivative are the antibiotics produced by many strains of *Streptomyces*. It belongs to ansamycin antibiotics

Secondary metabolite and biosynthesis gene cluster prediction

- Secondary metabolite analysis Shell (anti-SMASH) version 7.0
- There were six types of BGCs ($\geq 50\%$ similarity) detected from a draft genome of strain TML10T
- Although AntiSMASH is an effective tool for finding similar known BGCs, it is limited to annotating peptides and polyketides coded by modular assembly lines only.

TABLE 6 The distribution of BGCs of *Streptomyces naphthomycinicus* TML10^T based on “antiSMASH” prediction.

BGC type	Product	Span (nt)	BGC similarity (%)
Terpene	Albaflavenone	58,820–79,833	100
	Carotenoid	1,598,386–1,623,976	63
	Geosmin	928,798–950,957	100
	Hopene	309,016–334,735	92
Type 1 PKS (T1PKS)	Tripartilactam/niizalactam	1,408,322–1,541,131	100
	Naphthomycin A	1,387,838–1,498,759	71
Type 2 PKS (T2PKS)	Spore pigment	644,852–717,363	83
NRPS	Rimosamide	190,892–234,867	42
	ε-Poly-L-lysine	779,032–812,910	100
RiPP: Lanthipeptide	Informatipeptin	874,756–954,119	57
Others	Melanin	1,068,663–1,089,031	71
	Ectoine	549,991–559,379	100
	Desferrioxamin B/desferrioxamine E	840,565–852,334	83

- The in silico gene prediction results showed that strain TML10T contained various genes encoding antibiotic production: actinorhodin, gramicidin, tyrocidine, and an antitumor compound, chondramide
- arid area for 7 months in cold and summer seasons
- Strain TML10T contained a gene encoding arginine deiminase, an invaluable enzyme for anticancer

Preliminary screening of arginine deiminase production of strain TML1

- M9 medium (pH 6.8 at 27°C)
- point-inoculating their cells
- The pinky-purple zone around bacterial cells showed a positive result
- Blastp

Statistical analysis

- Antibiotic production of strain TML10T in different media against tested microorganisms was carried out in triplicates, and inhibition zones were expressed as mean \pm SD
- IBM SPSS Statistics version 29
- normality of the data was tested by the Shapiro–Wilk test
- (ANOVA) was adopted to determine a significant group

Description of *Streptomyces naphthomycinicus* sp. nov.

- Aerobic, catalase positive. Cells grow between 15 and 37°C with good growth at 27°C. Cells grow between pH 5 and pH 10

Conclusion

- The findings of this study provide valuable insights into selecting appropriate antibiotic production media, which is critical for a successful antibiotic screening program
- Genomic data mining revealed a correlation between the genotypic and phenotypic characteristics of this strain, which contains BGCs of naphthomycin A and the *arcA* gene encoding arginine deiminase.

- antiSMASH and Thin-Layer Chromatography profiling=+-Poly-L-lysine
- arginine deiminase production, a treatment for cancer worldwide

THANK YOU