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# Zusammenfassung

## Abstract

The strain *Streptomyces* sp. Tü2401 is capable of producing antimicrobial compounds which are active against diverse types of bacteria. Preliminary screening showed potent activity against several multiresistant *Escherichia coli* strains as well as the Gram-positive strains *Bacillus subtilis* and *Staphylococcus aureus*. Additionally, a mode-of-action *Bacillus* reporter strain indicated the presence of substances, which inhibit DNA synthesis. The broad activity spectrum and the unusual mode of action make this strain a valuable target of bioactivity-guided isolation of its natural products.

The

# Danksagung

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# Abbreviations

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AntiSMASH	Antibiotics & Secondary Metabolites Analysis Shell
EtAc	Ethyl acetate
GC	Gas Chromatography
MeAc	Methyl acetate
MS	Mass Spectrometry
HPLC	High Performance Liquid Chromatography
OD <sub>600</sub>	Optical Density at 600 nm
TLC	Thin Layer Chromatography
TMS	Trimethylsilane
Tü2401	<i>Streptomyces</i> sp. Tü2401

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# 1. Introduction

## 1.1. Natural products from *Streptomyces*

## 1.2. Hydrophilic Antibiotics

A common molecular parameter to determine hydrophilicity is  $\log P$ , the logarithm of the partition coefficient between 1-octanol and water.<sup>1</sup> Since accurate solubility prediction is of great importance in drug design, numerous methods have been implemented to calculate  $\log P$  from chemical structures.<sup>2-4</sup>

The StreptomeDB 2.0 database provides a unique, manually curated overview of natural products synthesized by *Streptomyces* species.<sup>5</sup> It contains over 4000 unique compounds produced by over 2500 host organisms and can be utilized to generate an overview of the hydrophilicity of published secondary metabolites. The distribution of  $\log P$  in the dataset (see Figure 1.1) reveals, that the vast majority ( $\sim 75\%$ ) of structures is on the hydrophobic side of the spectrum. In reality, the proportion is likely even higher as  $\log P$  only applies to uncharged structures, whereas many natural products are ionized under physiological conditions. The discovery process of *Streptomyces* secondary metabolites seems to favour hydrophobic molecules. Extraction with organic solvents followed by separation via High-Performance Liquid Chromatography (HPLC) is often used in isolation processes, while techniques more suitable to hydrophilic compounds still need to achieve the same level of prevalence.<sup>6</sup>

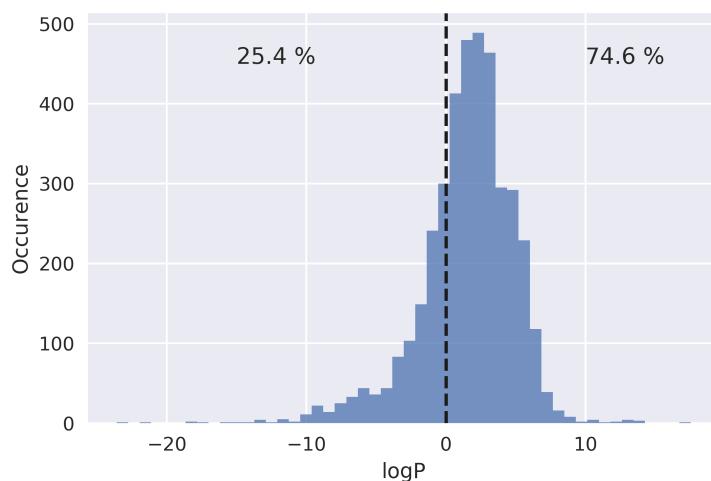


Figure 1.1.: **Distribution of  $\log P$  in the StreptomeDB Database** The molecular partition coefficient ( $\log P$ ) was calculated for 3985 entries in the StreptomeDB 2.0 secondary metabolite database<sup>5</sup> using the descriptor methods implemented in the RDKit cheminformatics suite.<sup>7</sup> 74.6 % of compounds are predicted to have  $\log P$  value  $> 0$  and 25.6 %  $\leq 0$ .

### 1.3. Antibiotics against Gram-negative bacteria

### 1.4. Isolation strategies for hydrophilic natural products

### 1.5. Aim of this thesis

## 2. Methods

### 2.1. Chemicals & Instruments

All chemicals and solvents were supplied by Merck, if not specified otherwise. Vendors for specific solvents are listed in Table 2.1. Water was purified prior to use by a MilliQ filtration system.

Table 2.1.: Used chemicals and solvents

Supplier	Chemical
J. T. Baker	Acetonitrile Chloroform
Alfa Aesar	Methyl acetate
Fisher Chemicals	Ethyl acetate

High performance liquid chromatography (HPLC) systems were manufactured by Agilent. The components of the HPLC systems are listed in Table 2.2. Detailed specifications of used HPLC-columns are listed in Table 2.3.

### 2.2. Strain Cultivation

#### 2.2.1. Media

All media were prepared by dissolving the components listed in Table 2.4 in MilliQ-H<sub>2</sub>O and adjusting the pH with NaOH and HCl. For solid media, 2 % (w/v) agar was added. Media were sterilized by autoclaving at 121 °C and 230 kPa for 15 min. Fluid media were stored at ambient temperature, solid media at 8 °C.

Table 2.2.: Components of HPLC systems

	Component	Description
Agilent 1100 Series	G1322A	Degasser
	G1311A	Quaternary Pump
	G1313A	Autosampler
	G1316A	Column Compartment
	G1315B	Diode Array Detector
Agilent 1200 Series	G1379B	Degasser
	G1312A	Binary Pump
	G1367B	Autosampler
	G1330B	Thermostat
	G1316A	Column Compartment
	G1315B	Diode Array Detector
Agilent 1260 Infinity	G4225A	Degasser
	G1312C	Binary Pump
	G1329B	Autosampler
	G1330B	Thermostat
	G1316A	Column Compartment
	G1315D	Diode Array Detector

Table 2.3.: Column Specifications

Manufacturer	Line	Type	Dimensions
Merck	SeQuant®	ZIC®-HILIC 3.5 µm 100 Å	150 × 4.6 mm
Phenomenex	Luna®	NH <sub>2</sub> 5 µm 100 Å	250 × 4.6 mm
	Kinetex®	Polar-C18 2.6 µm 100 Å	150 × 4.6 mm
Dr. Maisch	Nucleosil-100	C18 5 µm 100 Å	100 × 2.5 mm

Table 2.4.: **Media components for the cultivation of strain Tü2401.** Amounts are given for preparation with one liter of MilliQ-H<sub>2</sub>O. The pH was adjusted with NaOH and HCl.

Name	pH	Component	Amount	Vendor
LB		Yeast extract	5 g	Roth
		Tryptone	10 g	Roth
		NaCl	10 g	Roth
ISP2	7.3	Yeast extract	4 g	Oxoid
		Malt extract	10 g	Thermo Fisher
NL 200	7.5	D(-)Mannitol	20 g	Merck
		Cornsteep Powder	20 g	Sigma-Aldrich
NL 300	7.5	D(-)Mannitol	20 g	Merck
		Cotton Seed	20 g	Pharmamedia
NL 410	7.0	Glucose	10 g	Roth
		Glycerol	10 g	Acros Organics
		Oatmeal	5 g	Holo Bio Hafergold
		Soymeal	10 g	Hensel
		Yeast extract	5 g	Oxoid
		Bacto Casaminoacids	5 g	Difco
		CaCO <sub>3</sub>	1 g	Merck
NL 500	8.0	Starch	10 g	Roth
		Glucose	10 g	Roth
		Glycerol	10 g	Acros Organics
		Fish Meal	15 g	Sigma-Aldrich
		Sea Salts	10 g	Sigma-Aldrich
OM	7.3	Oatmeal	20 g	Holo Bio Hafergold
		Trace metal mix	5 mL	
Trace metal mix		CaCl <sub>2</sub> · 2 H <sub>2</sub> O	3 g	
		Fe <sup>3+</sup> citrate	1 g	
		MnSO <sub>4</sub> · H <sub>2</sub> O	200 mg	
		ZnCl <sub>2</sub>	100 mg	
		CuSO <sub>4</sub> · 5 H <sub>2</sub> O	25 mg	
		Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> · 10 H <sub>2</sub> O	20 mg	
		CoCl <sub>2</sub> · 6 H <sub>2</sub> O	4 mg	
		Na <sub>2</sub> MoO <sub>4</sub> · 2 H <sub>2</sub> O	10 mg	

### 2.2.2. *Escherichia coli* K12 and *Bacillus subtilis* 168

*Escherichia coli* K12 and *Bacillus subtilis* 168 were cultivated in LB medium (10 g peptone, 5 g yeast extract, 10 g NaCl per liter; pre-mixed by Roth) at either 37 °C (K12) or 30 °C (168). Liquid cultures were shaken at 200 rpm in flasks with baffles and spirals. Plate cultures were grown in an incubator. Pre-cultures were inoculated with cells from stored agar plates and incubated for 16 h. Main cultures were inoculated with 1 % (v/v) of pre-culture and incubated until the desired optical density at 600 nm ( $OD_{600}$ ) was reached.

### 2.2.3. General cultivation of *Streptomyces* sp. Tü2401

Agar-plate cultures of *Streptomyces* sp. Tü2401 were grown on ISP2 medium at 29 °C for four to seven days. 100 µL of spore solution or liquid culture were used for inoculation. Liquid cultures were incubated at 27 °C in shake flasks with aluminium caps. Pre-cultures were inoculated with spores from plate-grown mycelium or 50 µL of glycerol spore-stock. They were grown for three days in 20 mL of NL 410 in 100 mL flasks. Main cultures were inoculated with 5 mL of pre-culture. They were grown in 100 mL medium in 500 mL flasks for four to seven days.

Standing cultures of *Streptomyces* sp. Tü2401 were grown in 500 mL flasks fitted with either aluminium or foam caps. 5 mL of pre-culture grown in NL 410 were used to inoculate 100 mL of ISP2 liquid medium. The cultures were grown for seven days at 27 °C.

### 2.2.4. Batch Fermentation of *Streptomyces* sp. Tü2401

*Streptomyces* sp. Tü2401 was cultivated at a ten-liter scale in a continuous stirred tank bioreactor. 500 mL of pre-culture were grown in five 500 mL shake flasks containing 100 mL of NL 410 medium without CaCO<sub>3</sub>. The pre-cultures were inoculated from stored ISP-agar plates and grown for 72 h at 27 °C. The pre-cultures were pooled and used to inoculate 9.5 L of OM medium for fermentation. 2 mL of polyethylene glycol were added at the start to suppress foam generation. The temperature was kept at 27 °C with an airflow of 5 L min<sup>-1</sup> and a rotor speed of 200 rpm. Control samples of 15 mL were taken throughout the process at regular intervals. Fermentation was stopped after 125 h and the culture broth was harvested. Further processing is described in section 2.4.3.

## 2.3. Bioassays

### 2.3.1. Agar Diffusion Assays

Agar diffusion bioactivity assays against *E. coli* K12 and *B. subtilis* 168 were conducted on LB-agar in petri dishes. Round petri dishes ( $\varnothing = 92\text{ mm}$ ) were filled with 20 mL of liquid agar, square dishes ( $120 \times 120\text{ mm}$ ) were filled with 40 mL. Solidified agar plates were stored at  $8^\circ\text{C}$ .

400  $\mu\text{L}$  (200  $\mu\text{L}$  for round plates) of liquid culture at an  $\text{OD}_{600}$  of 0.3 to 0.6 and were spread on the solid agar plate with a drigalski spatula until dry. Round wells ( $\varnothing = 7\text{ mm}$ ) were punched out of the agar and filled with 50  $\mu\text{L}$  of sample. Processed plates were stored for 1 h at ambient temperature, before incubating them over night at either  $30^\circ\text{C}$  or  $37^\circ\text{C}$ .

### 2.3.2. *yorB* Reporter Gene Assay

The *yorB* reporter gene assays were performed by Katharina Wex of the group of Prof. Dr. Heike Brötz-Oesterhelt at the Interfaculty Institute of Microbiology and Infection Medicine Tübingen

Agar-based *yorB* reporter gene assays were performed with *Bacillus subtilis* 1S34 pHJS105-*yorB-lacZ2*.<sup>8</sup> 20 mL of LB-agar supplemented with 50  $\mu\text{g mL}^{-1}$  spectinomycin were inoculated with 500  $\mu\text{L}$  of overnight culture and grown until reaching the stationary phase. 50 mL LB-softagar (0.7 % agarose) with 150  $\mu\text{g mL}^{-1}$  5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) was prepared and mixed with the strain to a concentration of  $3 \times 10^7$  colony-forming units per milliliter. The agar was filled into a square petri dish and prepared with sample wells. 50  $\mu\text{L}$  of test samples were applied and the plate was incubated at  $30^\circ\text{C}$  over night.

## 2.4. Sample Preparation and Extraction

Extracts and reverse extracts of *Streptomyces* sp. Tü2401 were generally obtained through filtrated culture broth supernatant. After cultivating the strain for 4 to 7 days, the harvested biomass was centrifuged at 9000 rpm for 20 min. The supernatant was collected and filtered through a 0.2  $\mu\text{m}$  sterile filter. The filtrate was stored at  $4^\circ\text{C}$ .

#### **2.4.1. Preparation of Culture Broth Extracts**

Culture broth filtrate extracts were prepared by adding an equal amount of solvent to the filtrate and shaking the mixture for 30 min. The phases were separated by centrifugation at 4000 rpm for 10 min. Both phases were collected separately and stored at 4 °C. Samples were concentrated by drying under vacuum at either 40 °C (ethyl acetate, methyl acetate) or 60 °C (butanol, water) and resuspending them in a fifth of the initial volume. Organic phases were resuspended in methanol, aqueous phases in water.

#### **2.4.2. Determination of Extraction Conditions**

Three sets of five 15 mL falcon tubes were filled with 5 mL filtered Tü2401 culture broth. For each set, the pH of the samples was adjusted to 2, 5, 7, 9 or 11 with NaOH and HCl. Each set was extracted with either ethyl acetate, methyl acetate or ethyl formate and both phases were collected. Each phase was tested for bioactivity against *E. coli* K12.

#### **2.4.3. Processing of Fermentation Culture Broth**

The harvested fermentation broth was supplemented with diatomaceous earth and filtered through Pall T 1500 filter plates (relative retention range 10 - 30 µm). The remaining filter cake was discarded and the filtrate transferred to a stirring bucket. Two liters of ethyl acetate were added to the filtrate and stirred for 30 min. After phase-separation, the organic phase was collected and the aqueous phase extracted again. The process was repeated five times. Both phases were collected separately and concentrated in a rotary evaporator at 40 °C. The concentrated aqueous phase was frozen at –20 °C and lyophilized. The organic concentrate was stored at 8 °C.

#### **2.4.4. Agar Plate Extraction**

Standard ISP2 agar plates were ground with a blender and extracted with an equal volume of butanol or ethyl acetate for 1 h. The mixture was centrifuged at 4000 rpm for 1 h and the supernatant collected. The remaining slurry was resuspended in the same amount of water, centrifuged at 4000 rpm for 1 h and the supernatant collected.

Special ISP2 agar plates with low-melting-point agarose (LMPA) were prepared by substituting the 2 % (w/v) agar of regular plates with 4 % (w/v) LMPA. 75 mL of LMPA

agar plates were melted in Schott-flasks at 70 °C and extracted with either butanol or ethyl acetate for 30 min. The organic phase was collected and the remaining agar extracted again with 50 mL of water. All collected organic extracts were dried at 40 °C (ethyl acetate) or 60 °C (butanol) and resuspended in 1 mL methanol.

## 2.5. Bioactivity-guided Isolation

### 2.5.1. Thin Layer Chromatography

Thin layer Chromatography was performed with reverse extracts of Tü2401 on TLC Silica Gel 60 F<sub>254</sub> plates by Merck. Aqueous samples were applied by pipetting 1 µL at a time and letting the plate dry until the next application. The TLC chambers were filled up to 1 cm with solvent and incubated for 12 h. The plates were run until either 75 % of the plate had been soaked or 2 h had passed. The solvents used as mobile phases are listed in Table 2.5.

Table 2.5.: Mobile phase compositions used for Thin-Layer Chromatography

Solvent	Ratio (v/v)
Acetonitrile / Water	85:15
Butanol / Acetic acid / Water	14:3:2 and 42:10:7
Butanol / Ethanol / Water	3:2:1
Ethyl acetate / 2-Propanol / Water	6:3:1

The working orcin staining solution was prepared by mixing two storage solutions, solution A and B, at a ratio of 10:1 (v/v). Solution A contained 1 % (w/v) Fe(III)Cl<sub>3</sub> in 10 % sulfuric acid, solution B contained 6 % (w/v) Orcin in ethanol. The plates were sprayed with the working solution and treated with a heat gun for a few seconds.

Preparative samples were obtained by scraping the silica off the unstained plate and collecting it in reaction tubes. The samples were then extracted with 1 mL methanol, vortexed and sonicated for 30 min. After centrifugation at 14,000 rpm for 5 min, the supernatant was transferred to a new tube. The extraction was performed twice. The methanolic samples were dried at 30 °C and resuspended in an amount of water equal to the sample initially applied on the TLC plate.

### 2.5.2. Ion Exchange Chromatography

Ion exchange chromatography was performed with both a strong anion (Diaion SA11A, 20-50 mesh, Cl<sup>-</sup> form) and a strong cation exchange resin (Dowex 50WX4, 100-200 mesh, Na<sup>+</sup> form). Three solutions were used for all operations: An acidic solution (1 % (v/v) formic acid, pH 2), a neutral solution (MilliQ-H<sub>2</sub>O, pH 7) and a basic solution (2 % (v/v) ammonium hydroxide, pH 11). Prior to column preparation, both resins were swollen for 24 h. The anion exchange resin (AnX) was swollen in the basic buffer and the cation exchange resin (CatX) in the basic one. 12.5 mm diameter glass columns were filled with resin up to a bed height of 10 cm (AnX) or 9.5 cm (CatX). Both columns were operated at a constant flow of 2.5 mL min<sup>-1</sup>. All method steps are listed in Table 2.6. The pH of the applied sample was adjusted to pH 2 (CatX) or pH 11 (AnX) with NaOH and HCl. The flow-through of each step was collected and stored at 4 °C.

Table 2.6.: **Method for ion exchange chromatography.** pH values and relative volume of the solutions used for ion exchange chromatography with both strong anion exchange (AnX) and cation exchange (CatX) resins. Both resins were loaded with 1 mL of sample.

Step	AnX pH	CatX pH	Column Volumes
Equilibration	11	2	2
Wash 1	7	7	1
Sample application	11	2	*
Wash 2	11	2	1
Wash 3	7	7	1
Elution	2	11	5

### 2.5.3. Trimethylsilane Derivatization and Gas Chromatography

*The derivatization and gas chromatography (GC) measurements were performed by Dr. Dorothee Wistuba of the mass spectrometry department at the institute of organic chemistry in Tübingen.*

Dried HPLC fractions were suspended in a mixture of 460 µL N,O-Bis(trimethylsilyl)-trifluoroacetamide and 40 µL pyridine, before heating them to 110 °C. After derivatization, samples were dried with nitrogen gas and redissolved in dichloromethane. The derivatized samples were analyzed with a Hewlett Packard (HP) 6890 GC-system coupled

to a HP 5973 mass selective detector. The Agilent DB5 column measured 13 m × 0.25 mm with a film thickness of 0.1 µm. Helium was used as the carrier gas.

#### 2.5.4. Preparative HPLC

Preparative HPLC was performed on either the Agilent 1100 Series or Agilent 1260 Infinity instrument coupled to an Agilent G1346C fraction collector. Coupling of a Sedex Model 85 evaporative light scattering detector (ELSD) was achieved by mounting a 1:5-splitter after the UV-Detector. 4/5 of the flow was directed to the fraction collector, the remaining 1/5 to the ELSD. The ELSD was operated at a temperature of 40 °C and with nitrogen gas at a pressure of 3.2 bar. Detailed information about the used columns and methods can be found in Table 2.3 and the appendix. The obtained data was analyzed with the Agilent Chemstation (Version B.04.03). All samples were centrifuged at 14,000 rpm, before transferring the supernatant to HPLC-vials. All fractions were collected by timeslices of 1 min, stored at 4 °C to 8 °C, and subsequently dried at 40 °C. The samples were resuspended in an amount of water equal to the amount of injected sample and stored at 4 °C to 8 °C. Detailed method descriptions are found in the appendix at section A.1.

#### 2.5.5. Analytical HPLC and Mass Spectrometry

For mass spectrometry, an Agilent 1200 series HPLC system was coupled to an Agilent 6330 IonTrap LC-MS mass spectrometer. It features electrospray ionization with alternating positive and negative modes. The instrument was controlled with 6300 Series Trap Control (Version 6.1) and data was analyzed using DataAnalysis for 6300 Series Ion Trap LC-MS (Version 3.4). Measuring parameters are detailed for each method in section A.1.

### 2.6. Genome Analysis

*The taxonomic analysis was performed by Mohammad Alanjary of the group of Prof. Dr. Nadine Ziemert at the Interfaculty Institute of Microbiology and Infection Medicine in Tübingen*

The phylogenetic tree was constructed by using 50 sequenced *Streptomyces* genomes (Table A.9) as reference. From these genomes and the concatenated contigs of *Streptomyces* sp. Tü2401, 50 single copy genes were identified by The gene sequences were

aligned using the MAFFT tool (version 7) and refined using trimAl (version 1.2).<sup>9-11</sup> The maximum-likelihood tree was constructed with RAxML (version 8).<sup>12</sup> The average nucleotide identity was calculated with the JSpeciesWS web tool.<sup>13</sup> The final tree was visualized using Interactive tree of life (iTOL) v3.<sup>14</sup>

All five contigs were uploaded individually to the Antibiotics & Secondary Metabolite Analysis Shell (AntiSMASH) webserver.<sup>15-17</sup> Clusters were identified with standard settings and ClusterFinder enabled.

## 3. Results & Discussion

### 3.1. Determination of Extraction Conditions

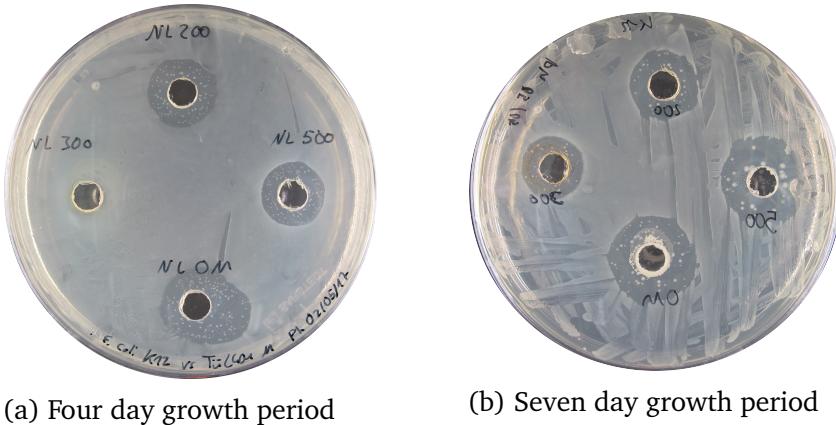
The strain Tü2401 was grown in seven different complex media for either four or seven days during preliminary experiments. Culture broth supernatants of NL 200, NL 300, NL 500 and OM media displayed antibiotic activity against *E. coli* K12on plate assays. A comparison between the viable production media and cultivation times is necessary to determine the optimal production medium for the following experiments.

Initial HPLC procedures failed to separate the active compound with standard reverse-phase screening methods. The compound displayed no retention on C-18 columns with acetonitrile/water gradients, making it likely to possess strong hydrophilic properties. Additionally, the compound was not able to be extracted from the culture broth or biomass via ethyl acetate. Its high solubility in water requires the development of an adapted extraction procedure to aid the purification process.

#### 3.1.1. Comparison of Production Media

To determine the optimal production medium, Tü2401 was grown in each of the media for either four or seven days, in which the strain previously proved to be active. The culture broth supernatants obtained were filtrated and subjected to the bioassay against *E. coli* K12. The assay results are displayed in Figure 3.1.

Most samples caused notable ( $\varnothing > 1$  cm) zones of inhibition in the agar diffusion assay. NL 500 after seven days and OM in both cases produced the largest inhibition zones ( $\varnothing > 1.5$  cm). Supernatant of NL 300 only produced inhibition zones after seven days of incubation. OM medium produced the largest inhibition zone after only four days of cultivation and, thus, seems best suited to production of the hydrophilic compound. NL 300 and 500 achieved maximum inhibition only after seven days, whereas NL 200 produced smaller inhibition zones in general.



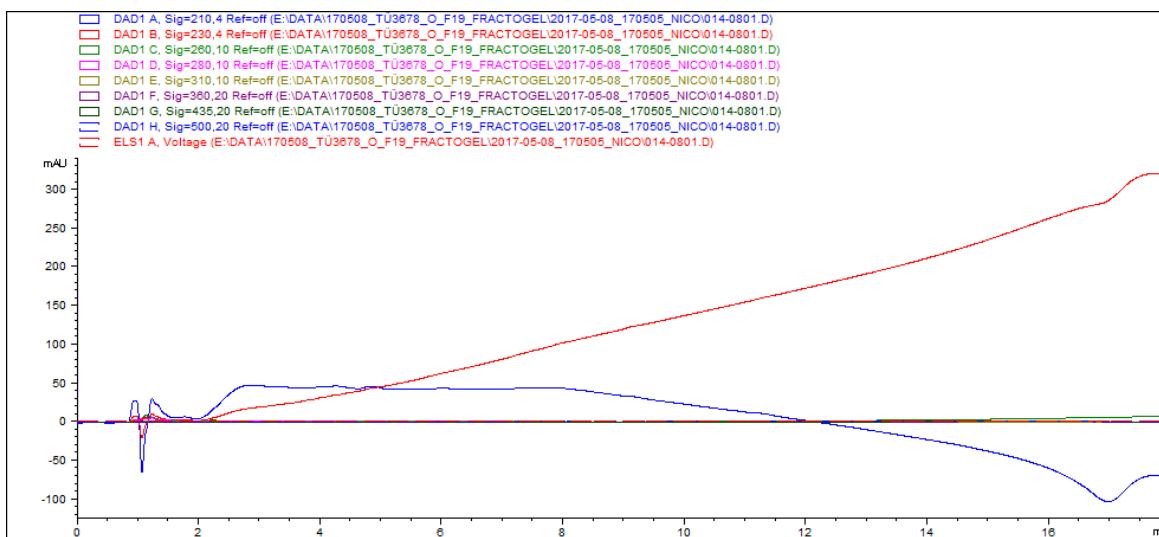
**Figure 3.1.: K12 Bioassay results with media supernatants** *Streptomyces* sp. Tü2401 was grown for either four (a) or seven days (b) in four different complex media. The filtrated culture broth supernatant was tested against *Escherichia coli* K12. Media: (Top) NL 200 (Left) NL 300 (Right) NL 500 (Bottom) OM.

NL 500 and OM supernatants were separated via an Agilent 1200 HPLC system equipped with a diode array detector (DAD) and evaporative light scattering detector (ELSD). A C18 column was used in combination with a 4.5 % to 100 % acetonitrile screening gradient (see Table A.1). The chromatograms are shown in Figure 3.2.

The screening chromatogram shows, that almost no UV- and ELSD-visible compounds in the OM broth supernatant were retained on the column. The OM supernatant seems to predominantly contain polar, water-soluble compounds with very low affinity to the C-18 solid phase. Since the antibiotic compound of interest also displayed no retention under similar conditions, the use of OM as a production medium would result in fewer impurities in the hydrophobic spectrum. The visible antibacterial activity of the supernatant against *E. coli* K12 after only four days and the low amount of hydrophobic impurities led to the choice of OM as the default production medium for further experiments.

### 3.1.2. Extraction experiments

One of the first steps in natural product isolation is usually the extraction via organic solvents to remove compounds of interest from a cellular matrix and enrich them in the solvent-phase.<sup>6,18</sup> In the case of strain Tü2401, the culture broth supernatant is antibiotically active, whereas the biomass and extracts thereof are not. The compound



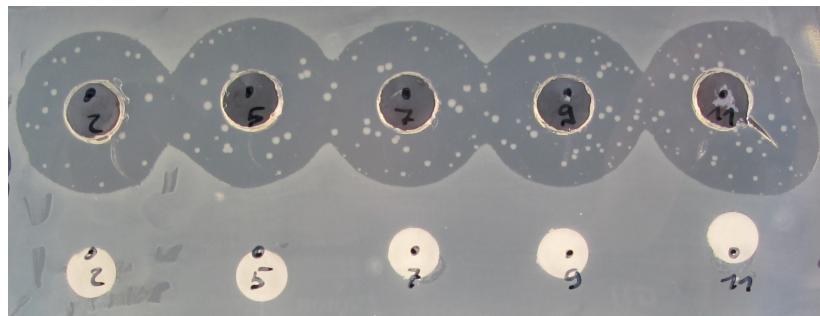
**Figure 3.2.: Chromatogram of OM medium supernatant screening.** 5 µL of culture broth supernatant were injected and separated on a C18 column using a screening gradient of 4.5 to 100 % acetonitril. UV absorption was measured at 210, 230, 260, 280, 310, 360, 435 and 500 nm. ELSD voltage was captured downstream of the UV detector.

therefore seems to be readily excreted to the medium, eliminating the need to separate it from cellular matter. However, an extraction process might still aid purification by removing hydrophilic contaminants such as salts easing further isolation steps such as HPLC.

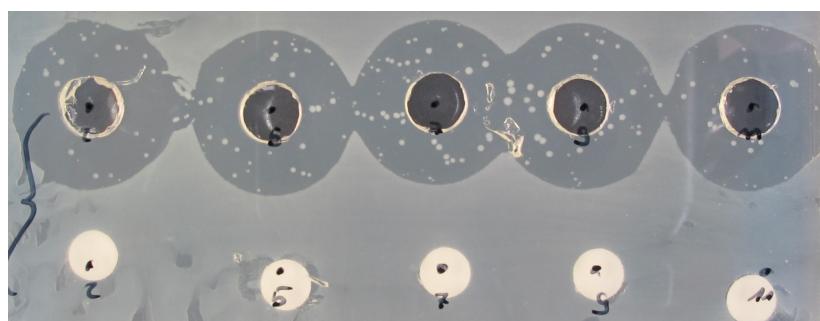
A hydrophilic molecule is likely to contain functional groups like amines or carboxylic acids, which are charged at certain pH-ranges. Adjusting the medium pH to reach the compounds point of zero charge could lower its water-solubility and, thus, enable extraction with an organic solvent. Additionally, solvents with higher polarity while still being immiscible with water could be applied.

OM medium supernatant was split into three sets of five aliquots each. Each aliquot was adjusted to either pH 2, 5, 7, 9 or 11. Each set was extracted with one following solvents (listed by descending polarity  $E_T(30)$ ):<sup>19</sup> Ethyl acetate, Methyl acetate and Ethyl formate. Organic and aqueous phases were separated and tested for bioactivity against *E. coli* K12 via agar-diffusion assay. The assay results are shown in Figure 3.3.

Neither of the organic phases produced any inhibition zone, whereas all aqueous phases produced inhibition zones of similar size ( $\varnothing > 1.5$  cm). The antibiotic compound could not be extracted with either ethyl acetate, methyl acetate or ethyl formate regardless of



(a) Ethyl acetate



(b) Methyl acetate



(c) Ethyl formate

**Figure 3.3.: Bioassay results of different OM culture broth extracts against *E. coli K12*.** Filtered supernatant of OM cultures of *Streptomyces* sp. Tü2401 were extracted with either (a) ethyl acetate, (b) methyl acetate or (c) ethyl formate. Sample-pH was adjusted prior to (from left to right) 2, 5, 7, 9 or 11. 50 µL of each phase subjected to agar-diffusion bioactivity assay against *E. coli K12*. Aqueous phases were pipetted directly into the wells in the upper row. Organic phases were applied to sterile filter discs and placed on the bottom row.

supernatant pH. This could be due to a high amount of polar but uncharged groups or the compound being zwitterionic. This would result in similar hydrophilicity over a wide pH range despite carrying no charge. However, the compound could possess a point of lowered water-solubility, but with a very narrow pH range or at an extreme point. The broad pH steps in the range of 2–14 covered in this experiment may have not hit this point. A repetition of the experiment with pH steps of 1 or 0.5 and a wider range of 1 to 14 could be applied to further explore the existence of a zero-charge point, that enables organic extraction.

The similar size of inhibition zones across all tested pH values also implies a resistance to pH-dependent degradation of the active compound over the range of 2–11. A notable trait of all inhibition zones caused by either pure broth supernatant or aqueous extraction phases is the presence of resistant colonies within. Since they grow seemingly random across the whole zone surface and not just the edges, concentration dependent resistance can be ruled out. It is more likely, that a resistant subpopulation of *E. coli* K12 is present in the glycerol stocks used to inoculate the test strain. The resistant subpopulation and the nonresistant strain must contain differences in antibiotic resistance genes. Genome sequencing of both strains with subsequent comparison of resistance genes could test this hypothesis and lead to valuable insights to the mode of action. From there on, classes of responsible compounds could be narrowed down and enable the development of more specialized isolation procedures.

Even though neither pH adjustment or change of solvent enabled extraction of the hydrophilic antibiotic, impurities in the hydrophobic spectrum are still removed by discarding the organic phase. Following samples of OM culture broth supernatant were preprocessed by performing a reverse extraction with Methyl acetate, thus keeping the aqueous phase.

## 3.2. Chromatographic Separation

### 3.2.1. Reverse-Phase HPLC

### 3.2.2. Hydrophilic Interaction Liquid Chromatography

- Short reiteration of HILIC

- Characteristics of NH<sub>2</sub> column
- Characteristics of ZIC-HILIC column

The Luna® NH<sub>2</sub> by Phenomenex features a silica matrix modified with 3-aminopropyl groups. The used model had a particle size of 3.5 µm and a pore size of 100 Å. The dimensions were 4.6 × 250 mm.

### Luna NH<sub>2</sub> Column

Separation via the Luna® NH<sub>2</sub> column was performed with an isocratic method (see A.2). The mobile phase consisted of 80 % acetonitrile and 20 % water at a flow of 2.0 mL min<sup>-1</sup>. Both solvents contained 0.1 % formic acid as a modifier. 50 µL of filtrated reverse extract at pH 11 were injected and fractions collected every minute. The fractions were subjected to the standard agar-diffusion bioassay against *E. coli* K12. The chromatogram is shown in

With this method, the water-soluble compound could be separated from the injection peak. Fractions 5, 6 and 7 produced noticeable zones of inhibition, correlating to elution between 4 and 7 min. With the injection peak eluting at 1.5 min, a relative retention of 2.5 to 5.5 min could be achieved. The majority seems to have eluted between 3.5 and 5.5 min, since the inhibition zones of fractions 6 and 7 were the largest with a diameter of 1.7 cm. Fraction 5 only produced a diameter of 1.2 cm. In the UV-chromatogram, two baseline-separated peaks with distinct spectra were detected in the timeframe correlated to bioactivity. The first was detected at 5 min, the second at 6 min. The UV-spectra indicate that two bioactive compounds with similar retention times have eluted right between the fraction collector timeframes. A single compound should have resulted in the detection of a rather broad peak with long fronting. However, if the compound does not possess a UV-chromophore, an additional method of detection is needed.

An evaporative light scattering detector (ELSD) can be used to detect analytes without chromophores, as long as they are less volatile than the solvent.<sup>20-23</sup> The detector can be coupled to a standard HPLC system and provides additional analytical data. To eliminate elution differences between different systems, both the ELSD and the fraction collector were attached to the same system behind the UV-detector. Since ELS-detection is inherently destructive, a splitter was used to divide the flow. One fifth was directed to the ELSD, the rest to the fraction collector. With both

### 3.2.3. Ion Exchange Chromatography

Water-soluble compounds contain polar functional groups, which in some cases can be protonated or deprotonated depending on the solvent pH. A comparison of acid dissociation constants ( $pK_a$ ) of natural products in the Antibase2008 database revealed, that the majority of entries are likely to carry a charge at pH 2–11.<sup>24</sup> 44 % of compounds contained an acidic functionality, 17 % a basic one, and 9 % both. The high prevalence of charged groups in natural products combined with the hydrophilic nature of the antibiotic compound make the presence of basic or acidic functionalities, such as amino- or carboxyl-groups, likely. This can be utilized for purification purposes via ion-exchange chromatography (IEC).

Ion exchange chromatography (IEC) utilizes charged functional groups bound to an insoluble resin matrix.<sup>18</sup> Charged analytes are separated by the equilibrium between themselves and the counterions associated with the matrix. Since the charge of natural products largely depends on pH and  $pK_a$ , the choice of buffers, resins and counterions has to be tailored to each experiment. Through optimised conditions for stationary and mobile phase, selective “catch and release” purification methods can be designed.

IEC has a wide range of applications. It is used in wastewater treatment and hydrometallurgy as well as to separate biological macromolecules like nucleic acids, carbohydrates and proteins.<sup>18,25–27</sup> It can also be used to separate small molecules and aid in their purification from complex sources.<sup>18,28,29</sup> As of now the molecular properties of the antibiotic compound remain unclear, yet they may be approximated by its behaviour in separation techniques. Exploration of the retention behaviour using IEC can be used to determine the presence of charged functional groups and approximate the  $pK_a$ .

An explorative IEC method similar to the approach of Måansson *et al.* was devised to test the applicability of IEC for separation.<sup>24</sup> Two ion-exchange resins with orthogonal selective properties were employed to determine if the compound contains acidic or basic functionalities. Both feature "strong" ion exchange groups, which retain their charge over a broad pH-range and are immobilized on divinylbenzene matrices. The strong cation exchanger (SCX), Dowex 50WX4, contains negatively charged sulfonic acids, while the strong anion exchanger (SAX), Diaion SA11A, contains positively charged quarternary ammonium groups. 1 mL aliquots of filtrated culture broth supernatant were adjusted to either pH 2, to protonate acidic groups or pH 11 to deprotonate basic groups. The sample at pH 2 was loaded onto the SCX whereas the pH 11 sample was loaded onto the

SAX. Washing steps at sample and neutral pH were performed to only ensure retention of compounds charged positively at pH 2 or negatively at pH 11. Bound analytes were eluted by reversing the buffer pH from 2 to 11 and vice versa. All loading, washing and elution fractions were collected, concentrated and subjected to the standard bioassay against *E. coli* K12.

The first three out of five SAX elution fractions displayed antibiotic activity in the bioassay, whereas the loading and washing fractions did not. The water-soluble compound was able to form an ionic bond with the sulfonic acid moieties of the SAX matrix at pH 11 and pH 7, but not at pH 2. This indicates the presence of one or more functional groups with a  $pK_a < 7$ , which are most likely carboxyl groups. As such, the compound might contain organic acid moieties.

None of the SCX fractions exhibited antibiotic activity in the bioassay. The compound was not eluted prematurely due to low affinity, since the loading and washing fractions were inactive. This could be due to an exceptionally strong bond of the compound to the cationic resin, which is still present at pH 11 and prevented elution with the used buffer. A strongly basic alkaloid or amine with a  $pK_a > 11$  for the protonated form would retain its charge and stay bound to the quaternary ammonium groups of the SCX. Another possibility would be a reaction with the SCX matrix, either deactivating the compound or binding it irreversibly. Since the presence of the compound was only inferred via bioactivity in the agar-diffusion assay, inactivation by prolonged exposure to high pH could have led to the negative assay results. However, samples stored at 8 °C and both high and low pH levels have retained antibiotic activity for several months without noticeable loss in produced inhibition zones. pH dependent degradation is therefore not an issue for the compound in question.

Chromatographic screening of the active SAX elution fractions on a C-18 column (see Table A.1) showed, that many impurities, especially in the hydrophobic spectrum, are still present. Ion Exchange Chromatography with a strong cation exchange resin is able to be applied for the separation of the hydrophilic compound from filtered culture broth supernatant, but it needs to be refined to achieve higher purities. Reverse extract samples can be used for separation instead of raw culture broth, to remove hydrophobic compounds beforehand. Variation of elution buffer pH might enable an elution profile more specific to the compound of interest and further narrow its acid strength.

### 3.2.4. Thin-Layer Chromatography

A classic method for separation of sugars is thin-layer chromatography (TLC).

### 3.2.5. Gel-filtration

## 3.3. Dereplication

### 3.3.1. HPLC Mass Spectrometry

### 3.3.2. Trimethylsilane Derivatization and Gas Chromatography

## 3.4. Activity Spectrum

### 3.4.1. Antibiotic Activity

*E. coli* K12 served as the primary test strain for the assessment of antibiotic activity of *Streptomyces* sp. Tü2401 cultures and extracts. Culture broth supernatant samples and extracts were perpetually tested positive in these assays. Additionally, culture broth supernatant also proved to be similarly active against multiresistant strains, namely *E. coli* 5309

Table 3.1.: Multiresistant *Escherichia coli* strains used in bioassays for antibiotic activity. Resistance against the given antibiotic is indicated by x. Legend: *amp* Ampicillin, *cam* Cloramphenicol, *nal* Nalidixic acid, *neo* Neomycin, *rif* Rifampicin, *spec* Spectinomycin, *strep* Streptomycin, *tet* Tetracyclin

Strain	amp	cam	nal	neo	rif	spec	strep	tet
JF438			x		x			
H5309	x			x			x	x
H5313	x					x	x	
H5434		x				x		x

**ToDo**

- How is resistance to the specified antibiotics conferred in these strains?
- Is there a common factor / mode of resistance in strains resistant to Tü2401 or those vulnerable to it?
- If there is, does it indicate a possible mode of action for Tü2401 antibiotic compound(s)?

### 3.4.2. Induction of the *yorB* Reporter System

Preliminary screening of *Streptomyces* strains on agar plates indicated strain Tü2401 to possess antibiotic activity against *Bacillus subtilis* and *Escherichia coli* strains. Mode-of-action screening with the specialized *Bacillus subtilis* 1S34 pHJS105-*yorB-lacZ2* strain showed, that disks of agar-cultures are also able to induce the *yorB* reporter system.

The *yorB* gene from *Bacillus subtilis* has been observed to be upregulated when cells were treated with quinolones.<sup>30</sup> Construction of a luciferase reporter system led to the hypothesis, that induction is due to DNA damage caused by inhibition of the Topoisomerase II alpha subunit.<sup>31</sup> Further characterization with greater natural product libraries strengthened this theory.<sup>8,32</sup> Table 3.2 gives an overview of tested *yorB*-inducing compounds, which either damage DNA directly or interfere with replication.

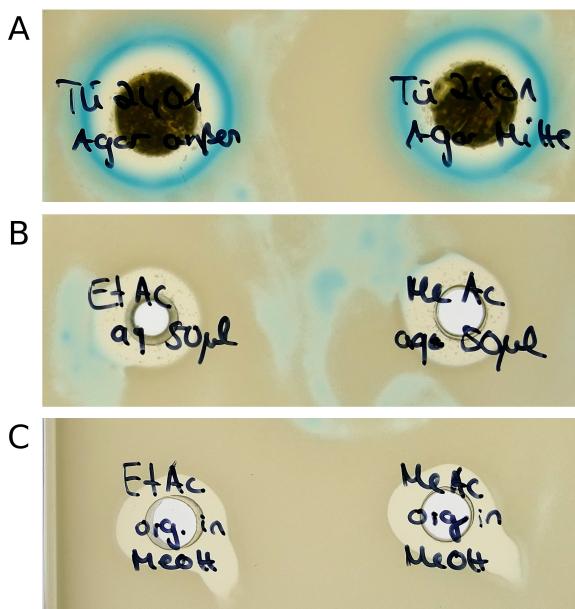
However, all samples and extracts stemming from liquid cultures were inactive in the *yorB* assay. The metabolite, which induced the *yorB* reporter system, is likely not produced in shake-flask cultures with OM-medium.

Loss of activity when transitioning from solid to liquid cultures is a known phenomenon in *Streptomyces* screening studies.<sup>33,34</sup> Secondary metabolite production is regulated by a plethora of interacting factors, which are dependent on the developmental stage of the cell, nutrient availability and other internal and external factors.<sup>35–38</sup> Cells grown in liquid cultures usually produce secondary metabolites during the stationary growth phase following nutrient depletion.<sup>38</sup> When grown on solid media, however, production coincides with the development of aerial hyphae. Antibiotic biosynthetic gene clusters have been observed to be influenced by the same regulators, which also govern morphological differentiation and spore development.<sup>39,40</sup>

The compound, which is able to induce the *yorB* reporter system of *Bacillus subtilis* was likely not produced during growth in OM liquid, but in solid NL 200 solid medium. Both

**Table 3.2.: Known inducers of the *yorB* reporter system.** Substances with known modes of action identified as inductors of the *yorB* reporter system in *Bacillus subtilis*.<sup>8,32</sup> Examples of origin are taken from the Pubchem database. *Streptomyces* species names were abbreviated.

Mode of Action	Name	Origin	PubChem ID
RNA polymerase inhibition	8-Hydroxyquinoline	Synthetic	1923
	Juglone	<i>Carya illinonensis</i>	3806
DNA gyrase inhibition	Novobiocin	<i>S. niveus</i>	54675769
DNA strand breaking	Alternariol	<i>Alternaria</i> sp.	5360741
	monomethylether		
	Bleomycin	<i>S. verticillus</i>	5360373
	Myxin	<i>Sorangium</i> sp.	72510
	Phleomycin	<i>S. verticillus</i>	72511
DNA intercalation	Streptonigrin	<i>S. flocculus</i>	5351165
	Chromomycin	<i>S. griseus</i>	5351560
	Daunorubicin	<i>S. peucetius</i>	30323
	Doxorubicin	<i>S. peucetius</i>	31703
DNA binding	Anthramycin	<i>S. refuineus</i>	5311005
	Desmethyllether-sibiromycin	<i>Streptomyces</i> spp.	6437361
DNA crosslinking	Porfiromycin	<i>S. arduus</i>	13116



**Figure 3.4.: Results of the *yorB* reporter system bioassay with strain Tü2401** Foto of the bioassay plate taken one day after incubation.

media are nutrient rich and complex, which points to the formation of aerial hyphae as a stimulus for production. Three cultivation strategies were developed to allow for both formation of air mycelium and extraction of the compound:

1. Standing liquid cultures could allow the formation of aerial mycelium at the medium surface. The compound could then be extracted from the fluid with standard procedures. Two 500 mL flasks were each filled with 100 mL of liquid ISP2 medium and inoculated with 1 mL of a one-week old NL 410 shake culture. One flask was sealed with an ordinary aluminium cap (AC), the other one with an air-permeable foam cap (FC). Both flasks were cultivated for four days, before the medium was centrifuged and the supernatant filtrated. 50 mL aliquots of each flask were extracted with either BuOH or EtAc, and both phases were collected separately. The organic phases were dried at 40 °C and solved in 1 mL MeOH. Both phases of each flask were subjected to the *yorB*-Assay.
2. Solid media cultivation on ISP2 agar proved to enable synthesis. Mincing of agar plates after cultivation could enable extraction with common solvents. Ten round ISP2 agar plates were inoculated with 100 µL of a one-week old NL 410 shake

culture and incubated for four days. One half of the plates was extracted with BuOH, the other half was extracted with EtAc.

3. ISP2 agar plates could also be prepared with low-melting-point agarose (LMPA). Melting the agar prior to liquid extraction might lead to higher yields due to greater surface area. Using LMPA reduces the temperature and, thus, risk of thermal decomposition during the process. Six ISP2 LMPA agar plates were inoculated with 100 µL of a week-old NL 410 shake culture and incubated for four days. The plates were then melted at 70 °C and extracted with BuOH and EtAc.

All generated extracts and their respective aqueous phases were tested for bioactivity in agar diffusion assays. The standard bioassay was used to determine the antibiotic activity against *E. coli* K12 and *B. subtilis* 168, while the *yorB* assay tested antibacterial activity as well as reporter induction. Additionally, agar stamps of ISP2 and ISP2 LMPA culture plates were tested in the *yorB*-Assay. A summary of the results is shown in Table 3.3.

The bioassay results indicate a high variety of activity for the strain *Streptomyces* sp. Tü2401 depending on cultivation procedure. Standing agar culture extracts were largely inactive in the antibiotic and *yorB*-assays. Ethyl acetate extracts of the foam and aluminium cap cultures mark the only exception. The foam cap extracts displayed high antibiotic activity against both *B. subtilis* strains, whereas the aluminium cap extracts were lightly active against *Bacillus subtilis* 1S34 pHJS105-*yorB-lacZ2*. Strong antibiotic activity against *Bacillus subtilis* 1S34 pHJS105-*yorB-lacZ2* was also present in BuOH extracts of both agar plate cultures and the EtAc extracts of LMPA. Antibiotic assay results of agar plate cultures against *B. subtilis* 168 were largely non-evaluatable, yet ISP2-agar EtAc extract and the aqueous phase of the BuOH extraction tested positive. Activity against *E. coli* was only lightly present in the aqueous phase of ISP2 EtAc extract.

Antibiotic activity against *B. subtilis* but not *E. coli* in a range of organic extract samples hints at the presence of lipophilic compounds active against Gram-positive bacteria, but not Gram-negative. Previous assays with organic extracts of liquid cultures further !!! The non-evaluatable results of the antibiotic assay against *B. subtilis* 168 impede a comprehensive comparison of activity against both *B. subtilis* strains and strengthen the need for further exploration of the activity spectrum of *Streptomyces* sp. Tü2401.

**Table 3.3.: Bioassay results from agar-plate and standing culture extraction.** Samples were subjected to the standard antibiotic assay against *E. coli* K12 and *B. subtilis* 168 as well as the *yorB*-Assay against *Bacillus subtilis* 1S34 pHJS105-*yorB*-lacZ2. Samples from standing cultures with foam cap (FC) or aluminium cap (AC), ISP2 agar plates (ISP2) and ISP2 agar plates with low-melting-point agarose (LMPA). Agar plaques were taken directly from the culture plate and subjected to the *yorB*-Assay without further processing. Samples were extracted with butanol (BuOH) or ethyl acetate (EtAc) and tested alongside their respective aqueous phases (aq.).

*Legend:* - No activity; + / ++ / +++ antibiotic activity with inhibition zone of 1 / 1.0-1.5 / >1.5 cm; n.e. result non-evaluable

Sample	Antibacterial			Induction <i>yorB</i>
	<i>E. coli</i>	<i>B. subtilis</i>	<i>yorB</i>	
FC BuOH	-	-	-	-
FC BuOH aq.	-	-	-	-
FC EtAc	-	+++	++	-
FC EtAc aq.	-	-	-	-
AC BuOH	-	-	-	-
AC BuOH aq.	-	-	-	-
AC EtAc	-	-	+	-
AC EtAc aq.	-	-	-	-
ISP2 BuOH	-	n.e.	+++	-
ISP2 BuOH aq.	-	+	-	-
ISP2 EtAc	-	++	-	-
ISP2 EtAc aq.	+	n.e.	-	+
ISP2 plaque			++	++
LMPA BuOH	-	n.e.	+++	-
LMPA BuOH aq.	-	n.e.	-	-
LMPA EtAc	-	n.e.	+++	-
LMPA EtAc aq.	-	n.e.	-	-
LMPA plaque			-	-

## 3.5. Bioinformatic Analysis

The genome of *Streptomyces* sp. Tü2401 was fully sequenced and obtained in five contigs. It encompasses 8,139,204 base pairs with a GC content of 71.4 %. 4627 open-reading frames were identified by automatic annotation. A brief summary of each contig is shown in Table 3.4. Analysis of the whole-genome sequence might lead to valuable insights into the phylogeny of the strain and its potential for secondary metabolite production.

Table 3.4.: Contig summary of the sequenced *Streptomyces* sp. Tü2401 genome.

Base pair count, automatically identified open-reading frames (ORFs) and GC-content is listed for each contig sorted by size. Total values were calculated from the summary of individual records.

Contig	Base pairs	ORFs	GC-content [%]
1	3 481 134	3221	71.6
2	2 271 151	2062	71.7
3	2 103 347	1953	71.2
4	159 117	135	69.1
5	124 275	145	70.6
Total	8 139 204	4627	71.4

### 3.5.1. Phylogeny of Strain Tü2401

Basic local-alignment search tool (BLAST) queries for the 16S-rRNA sequence of strain Tü2401 against the NCBI database revealed a variety of *Streptomyces* sequences with very high (>99 %) identity. 16S-sequence identity confirms the strain as a member of the genus *Streptomyces*, but is insufficient to define the species. To achieve this, 50 single-copy household genes were identified in the Tü2401- and 50 reference genomes of other fully sequenced *Streptomyces* strains. The multiple sequence alignments of these genes were used to construct the maximum-likelihood tree shown in Figure 3.5.

The closest relative to Tü2401 among the reference strains is *Streptomyces mediolani* NRRL WC-3934. The first members of this species were isolated 1969 in Milan and investigated for carotenoid production, yet publications regarding secondary metabolites are sparse.<sup>41,42</sup> (-)-8-O-methyltetrangomycin is mentioned to be synthesized by *S. mediolani* AC37, which is an angucycline with antibiotic activity against *Listeria monocytogenes*.

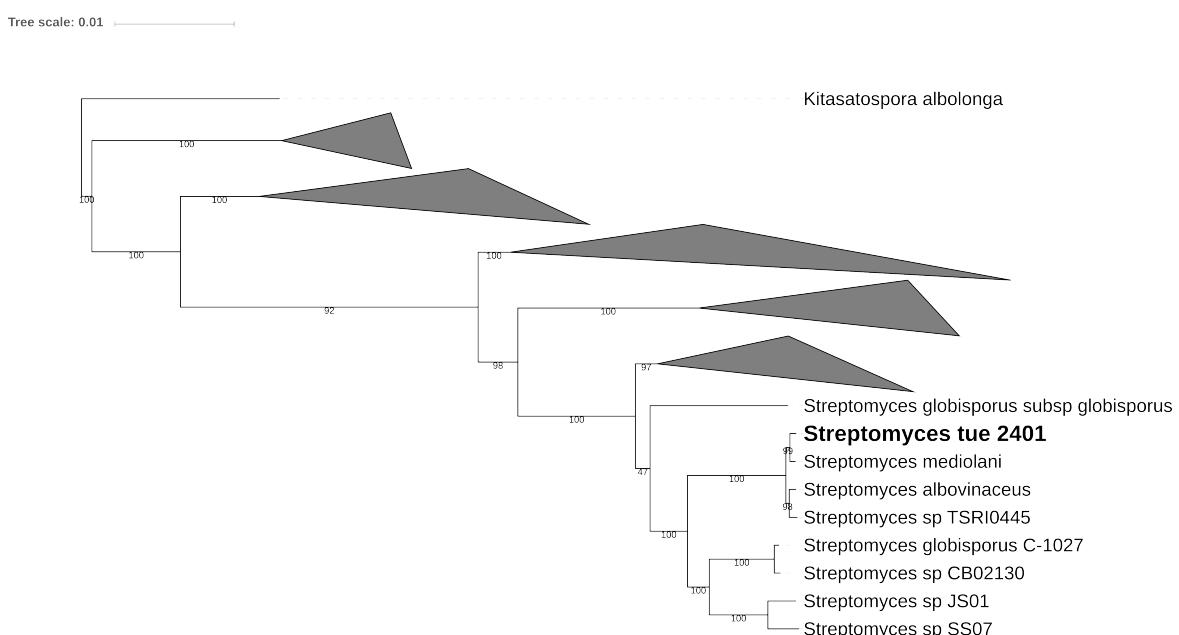


Figure 3.5.: Maximum likelihood tree of *Streptomyces* sp. **Tü2401**. The tree was constructed using a multiple-sequence alignment of 50 single-copy genes across 50 *Streptomyces* reference genomes. The node belonging to *Streptomyces* sp. **Tü2401** is highlighted with bold text. Only the nine most closely related nodes and the outgroup are shown. Dark triangles represented hidden, collapsed nodes.

and cytotoxic properties.<sup>43,44</sup> AntiSMASH analysis of strain Tü2401 (see Section 3.5.2) did not indicate the presence of angucycline biosynthetic gene clusters.

The multiple sequence alignment and tree construction establish the strain Tü2401 as a member of the genus *Streptomyces* and a close relative of *S. mediolani* NBRC15427.

### 3.5.2. AntiSMASH Cluster Identification

All five contigs were submitted to the antibiotics and Secondary Metabolite Analysis SHell (antiSMASH) to identify known biosynthetic gene clusters (BGCs).<sup>15–17</sup> 75 clusters were identified across the genome, seven out of which were  $\geq 95\%$  similar to characterized clusters.

#### Contig 1

Two clusters with 100 % gene identity to characterized BGCs were identified on this contig. The first is a Type1-PKS-NRPS hybrid and contains genes identical to a SGR PTMs BGC (BGC0001043), a Frontalamide BGC (BGC0000996) and a Heat-Stable Antifungal Factor BGC (BGC0000999).<sup>45–47</sup> The three clusters are associated with production of polycyclic tetramate macrolactams (PTMs), a diverse class of compounds with a wide range of biological activities.<sup>48</sup> Frontalamide A/B and the Heat-Stable Antifungal Factor (HSAT) displayed antifungal bioactivity; a mode of action commonly observed in bacterial PTMs.<sup>46–50</sup> PTM gene clusters are highly conserved and present in over 50 % of sequenced *Streptomyces* species but also appear across a wide range of phylogenetically diverse bacteria.<sup>46</sup> Figure 3.6 displays the structures of select bacterial PTMs.

The other cluster is predicted to be a lantipeptide BGC with genes identical to the AmfS BGC ( BGC0000496). The *amf* gene cluster was studied in *S. griseus* and is involved in the formation of aerial mycelium.<sup>51</sup> The AmfS peptide is postulated to be an extracellular morphogen and not directly associated with antibiotic activity.

The presence of a putative PTM biosynthetic gene cluster in the genome of *Streptomyces* sp. Tü2401 indicates a potential to synthesize antifungal compounds. Antifungal bioactivity assays can be employed to test culture supernatant and agar-disks. The associated Frontalamide A/B and HSAT were reported to be active against plant pathogens *Bipolaris sorokiniana*, *Fusarium graminearum*, *F. verticilloides* and *Ophiostoma minus*.<sup>46,47</sup> Isolation of a new variety or efficient production of PTM compounds could prove strain

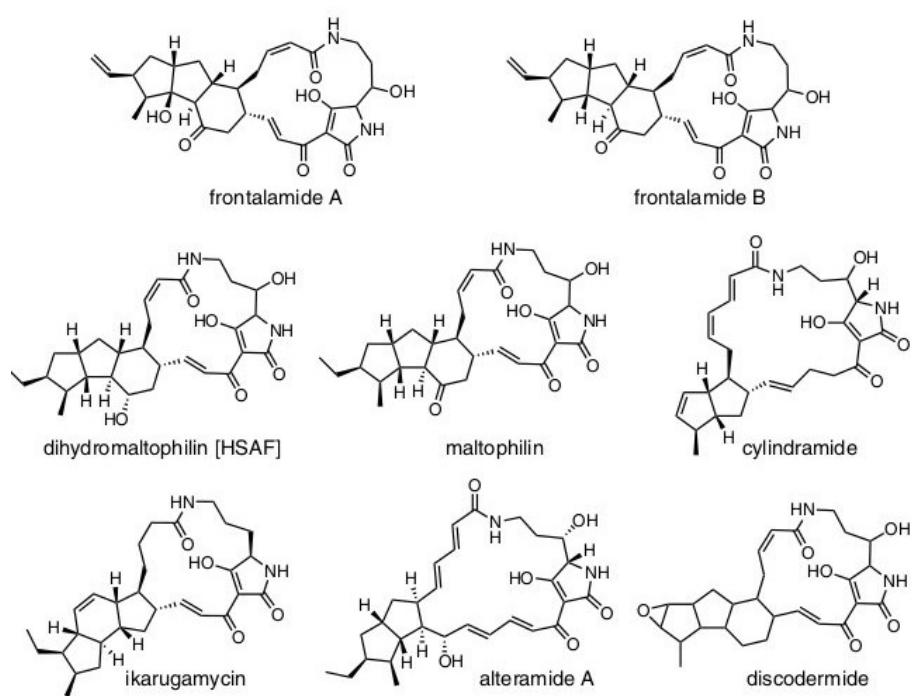


Figure 3.6.: **Structures of bacterial polycyclic tetramate macrolactams.** Adapted from Blodgett *et al.* (2010). Copyright lies with the respective authors.

Tü2401 as a promising source of antifungal compounds.

### Contig 2

Contig 2 contains three clusters with 100 % identity to characterized genes. The first one is highly similar to an isorenieratene biosynthetic gene cluster from *Streptomyces griseus* (MIBiG BGC-ID: BGC0000664). Isorenieratene is a rare carotene which is synthesized by green photosynthetic bacteria and few actinomycetes.<sup>52</sup> Carotenes mainly serve as protectants against oxidative stress and are unlikely to possess antibiotic activities.<sup>53</sup> Additionally, the orange coloration of carotenogenic strains has not been observed during growth of strain Tü2401. The gene cluster is likely not actively expressed under the used growth conditions. The two other clusters on this contig are highly similar to a griseobactin BGC (BGC0000368) from *Streptomyces* sp. ATCC 700974 and an ectoine BGC from *Halomonas elongata*. The former compound is an unusual siderophore from streptomycetes, the latter serves as an osmoprotectant.<sup>54,55</sup> Both are not associated with antibiotic activity.

### Contig 3

Only one cluster with high similarity to known BCG's was identified on Contig 3. Its genes are 100 % identical to the Desferrioxamine B BGC from *Streptomyces griseus* (BGC0000941).<sup>56</sup> Desferrioxamine B, like griseobactin, is a siderophore necessary to facilitate iron uptake.<sup>57</sup>

### Contig 4

One cluster was detected on contig four and identified as a Type1-PKS-NRPS hybrid. It shows a 95 % cluster identity to the C-1027 biosynthetic gene cluster from *Streptomyces globisporus* C-1027 (BGC0000965). Additionally, three homologous subclusters with 100 % identity were identified, which are associated with the synthesis of C-1027, Neocarzinostatin and Maduropeptin enediynes (see Figure A.2). The presence of this cluster could indicate, that the strain Tü2401 is capable of producing a compound similar to enediyne antibiotics.

Enediyne natural products are a class of cytotoxic bacterial compounds, which cause extensive DNA-damage.<sup>58–61</sup> 11 different enediyne natural products are known, all of

which feature either a bicyclo[7.3.0]dodecadienediyne core inside a nine-membered ring or a bicyclo[7.3.1]tridecadiyne core inside a ten-membered ring (Figure 3.7). The 9-membered family includes neocarzinostatin, C-1027 and maduropeptin. The 10-membered family includes calicheamicin  $\gamma_1^I$ , esperamicin A<sub>1</sub> and dynemicin A.<sup>58</sup> Enediynes are potent cytotoxic agents because of their ability to induce DNA double-strand breaks.<sup>62</sup> Electronic rearrangement of the carbocycle produces a benzenoid diradical, which abstracts hydrogen atoms from the DNA-backbone. These consulting radicals cause interstrand crosslinks or react with molecular oxygen. While the ten-membered enediyne compounds were isolated as free-standing chromophores, most of the compounds in the nine-membered family were isolated in conjunction with a protective apoprotein.<sup>58</sup> The nine-membered chromophore of C-1027, which was isolated from *Streptomyces globisporus* C-1027, is bound noncovalently to an 110 amino acid apoprotein.<sup>60,63-70</sup> The isolated chromophore has been shown to be very unstable, whereas the holo C-1027 did not lose activity under the same conditions.<sup>66-68</sup> The apoprotein binds specifically to the C-1027 chromophore, supposedly via hydrophobic pocket, which binds the benzoxazine side chain.<sup>67,71</sup> The enediyne antibiotics neocarzinostatin and maduropeptin, which were also isolated from actinomycetes, feature highly specific and protective apoproteins as well.<sup>60</sup>

The homologies of the identified cluster could be an indicator, that the strain Tü2401 is capable of synthesizing an enediyene antibiotic with a nine-membered core and a corresponding apoprotein. The potent DNA-strand-breaking capabilities of this compound-class could induce the *yorB* reporter system of *Bacillus subtilis* 1S34 pHJS105-*yorB-lacZ2* in the *yorB*-induction assay. A number of compounds, which cause DNA double strand breaks and crosslinks are reported to induce the system, though none of them belong to the family of endiyne antibiotics.<sup>8</sup> Whether this is due to inactivity or lack of representation in the assay is unclear, since the compound library is not publicly accessible. Nevertheless, the mode of action of enediyne compounds matches positive compounds closely.

Section 2.3.2 discussed, that the *yorB* inducing compound is produced when the strain is grown on an ISP2 agar plate, but not in liquid culture. Solvent extracts of plate cultures were also largely inactive with only minimal activity being present in the aqueous phase of ethyl acetate extraction. Maximum activity could only be observed in unprocessed plate culture samples. The apoprotein could have been detached during the extraction and concentration process, which also included high temperatures of 40 to 60 °C. Combined with incubation times of up to several days between extraction and assay, this could

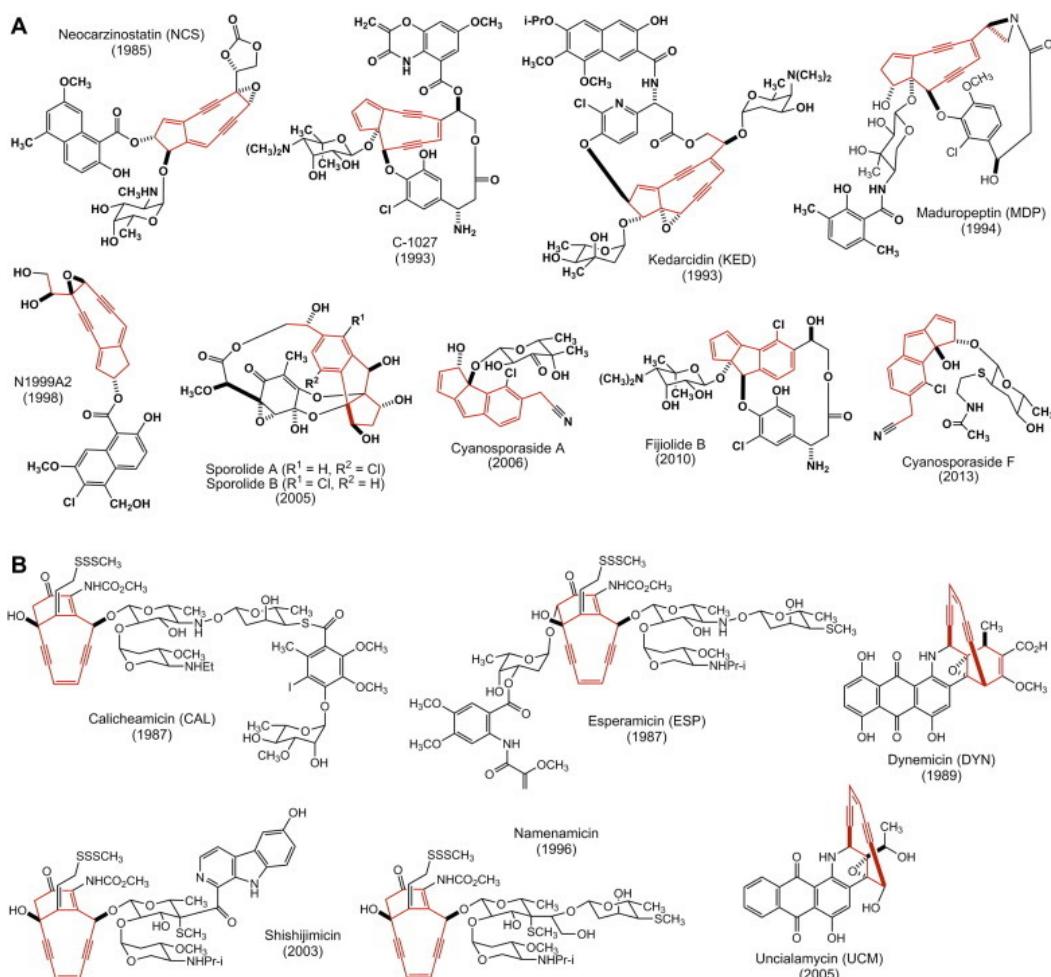


Figure 3.7.: **Structures of known enediyne natural products.** Enediyne cores are highlighted in red. (A) Compounds with nine-membered rings. (B) Compounds with ten-membered rings. The year of structure confirmation is displayed in parentheses. The sporolides, cyanosporolids and fijiolides do not contain an endiyene core, but are proposed to be derived from enediyne precursors. Adapted from Shen *et al.* (2015) Copyright 2014 Elsevier Ltd.

have led to the degradation of the chromophore below the sensitivity threshold. The high temperatures and long storage times also apply to the numerous HPLC-fractioning samples subjected to the *yorB*-induction assay.

To verify this assumption, the putative endiyne compound has to be extracted by an adapted protocol, dereplicated and subjected to the assay in pure form. The C-1027 antibiotic protein from *S. globisporus* was precipitated from the medium supernatant by the addition of ammonium sulfate and purified by dialysis and column chromatography.<sup>69</sup> The active chromophore could be extracted from the apoprotein with methanol at 0 °C.<sup>67</sup> However, as of now, untreated medium supernatant samples of the strain Tü2401 did not induce the *yorB*-reporter system. The cultivation in liquid OM medium is probably not sufficient for production of the putative endiyene compound and the protocol would have to be adapted for the extraction from ISP2 agar plates. To circumvent this, other cultivation media could be employed and tested for *yorB*-induction. The holo endiyene-apoprotein complex should be stable enough for routine testing of culture broth and its supernatant. The only alternative to optimizing production conditions would be heterologous expression of the biosynthetic cluster. For the endiyne compound family though, this has, as of now, only been partly achieved for the nine-membered endiyne neocarzinostatin.<sup>72</sup>

Only eleven compounds of the enediyne family are known to this date, yet several members are in use or development as anticancer drugs with promising results.<sup>58,73</sup> Isolation of a new endiyne compound could hold a strong promise for the discovery of a new anticancer drug lead structure, which makes strain Tü2401 a valuable target for further investigation.

### 3.6. Fermentation of *Streptomyces* sp. Tü2401

During the course of the thesis, *Streptomyces* sp. Tü2401 has been cultivated in 50–500 ml shake flasks without baffles. Culture volumes from 10 to 100 ml could be reached with this approach, leading to roughly 21 mg/ml biomass and 2 mg/ml reverse extract (see Section XXX). This yields of about 200 mg reverse extract per 500 ml flask, of which the content of antibiotic could be very small. Great amounts of extract, which are necessary for further purification and structure elucidation, are very labor intensive to achieve this way. This problem could be solved by cultivating of *Streptomyces* sp. Tü2401

at a greater scale, providing a larger amount of biomass in a labor-efficient manner. Additionally, fermentation would further investigate the growth conditions, under which the active antibiotic is produced. These can vary heavily between shake flasks and stirred bioreactors, especially in regard to oxygen levels and perturbation.<sup>74</sup>

The strain was fermented in OM medium at a 10 L scale in a continuous stirred tank bioreactor. The temperature was kept at 27 °C with 5 L min<sup>-1</sup> of airflow. Samples were drawn every 24 h and the culture broth supernatant subjected to the standard antibiotic assay against *E. coli* K12. The pH only decreased marginally throughout fermentation, decreasing from the initial 6.4 to the final 6.0. After 125 h the peak diameter of inhibition zone on agar plates had been observed and the culture was harvested. Cells and insoluble matter were removed by filtration, followed by extraction with ethyl acetate. Both phases were collected and concentrated by evaporation and lyophilization. The processing steps and final yields are shown in Figure 3.8. The filtrate and extraction phases were subjected to the antibiotic assay against *E. coli* K12 and *B. subtilis* 168. Both the filtrate and aqueous phase were active against *E. coli* and *B. subtilis* with inhibition zones comparable to samples from shake flasks. The organic phase was inactive.

The experiment demonstrated, that *Streptomyces* sp. Tü2401 is not only able to be cultivated in stirred bioreactors at a 10L scale, but is also able to synthesize the hydrophilic antibiotic under these conditions. This enables larger cultivation scales to obtain greater amounts of the active compound for purification and structure determination. When combined with sufficient quantization methods for the active compound, further optimization of culture conditions can be performed to maximize yields. The filtrate and extracts still need to be subjected to the *yorB*-assay to determine, whether the putative DNA-binding compound is synthesized in this setup.

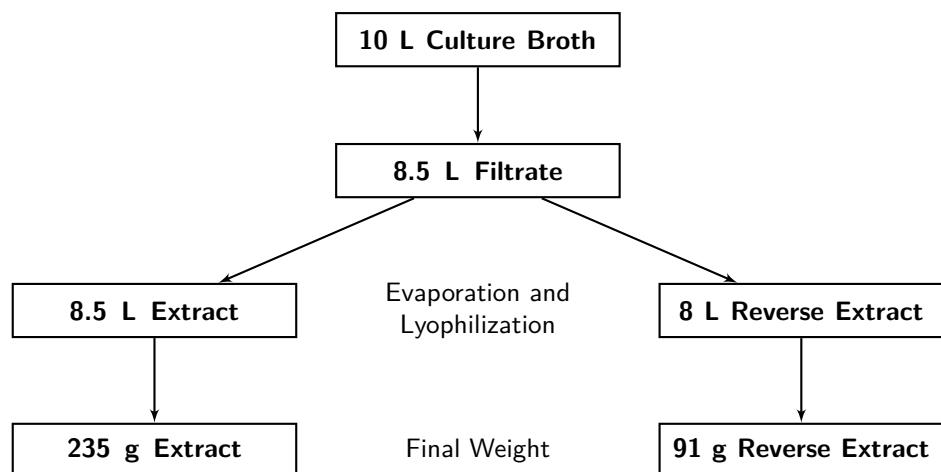


Figure 3.8.: Yield Diagram of *Streptomyces* sp. Tü2401 Fermentation.

Volumes of each processing step and final dry mass yields after fermentation of *Streptomyces* sp. Tü2401 in a 10 L stirred bioreactor.

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# A. Appendix

## A.1. HPLC Methods

Vielleicht wenn hier text steht

Table A.1.: Standard C18 screening method

Parameter	Value
Column	Nucleosil-100 C18 5 µm 150×3 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Gradient 5 - 100 % B for 15 min Plateau 100 % B for 3 min
Flow	0.85 mL min <sup>-1</sup>
Temperature	25 °C
Injection Volume	50 µL

Table A.2.: Standard aminocolumn method

Parameter	Value
Column	Luna NH2 5 µm 250×4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Isocratic 80 % B for 20 min + 100 % A for 10 min
Flow	2 mL min <sup>-1</sup>
Temperature	25 °C
Injection Volume	50 µL

**Table A.3.: Aminocolumn method adapted for MS coupling**

<b>Parameter</b>	<b>Value</b>
Column	Luna NH <sub>2</sub> 5 µm 250×4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Isocratic 80 % B for 60 min.
Flow	0.5 mL min <sup>-1</sup>
Temperature	40 °C
Injection Volume	50 µL
Capillary Voltage	3500 V
Injector Temperature	350 °C
Target mass	400 m/z

**Table A.4.: The standard ZIC-HILIC method**

<b>Parameter</b>	<b>Value</b>
Column	ZIC-HILIC 3.5 µm 150×4.6 mm
Solvents	A: 10 mM Ammonium acetate B: Acetonitrile
Method	Isocratic 80 % B for 45 min.
Flow	0.8 mL min <sup>-1</sup>
Temperature	25 °C
Injection Volume	50 µL

**Table A.5.: ZIC-HILIC method adapted for MS coupling**

<b>Parameter</b>	<b>Value</b>
Column	ZIC-HILIC 3.5 µm 150×4.6 mm
Solvents	A: 10 mM Ammonium acetate B: Acetonitrile
Method	Isocratic 80 % B for 60 min.
Flow	0.5 mL min <sup>-1</sup>
Temperature	40 °C
Injection Volume	50 µL
Capillary Voltage	3500 V
Injector Temperature	350 °C
Target mass	400 m/z

**Table A.6.: Screening method for HPLC-MS**

<b>Parameter</b>	<b>Value</b>
Column	Nucleosil-100 5 $\mu\text{m}$ 150 $\times$ 3 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.06 % Formic acid
Method	Gradient 0 - 100 % B for 15 min Plateau 100 % B for 2 min
Flow	0.4 mL min <sup>-1</sup>
Temperature	40 °C
Injection Volume	2.5 $\mu\text{L}$
Capillary Voltage	3500 V
Injector Temperature	350 °C
Target mass	400 m/z

**Table A.7.: Screening Method Polar-C18**

<b>Parameter</b>	<b>Value</b>
Column	Kinetex Polar-C18 2.6 $\mu\text{m}$ 150 $\times$ 4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Gradient 5 - 100 % B for 20 min Plateau 100 % B for 6 min
Flow	1.2 mL min <sup>-1</sup>
Temperature	50 °C
Injection Volume	50 $\mu\text{L}$

**Table A.8.: Reverse Screening Method Polar-C18**

<b>Parameter</b>	<b>Value</b>
Column	Kinetex Polar-C18 2.6 $\mu\text{m}$ 150 $\times$ 4.6 mm
Solvents	A: Water + 0.1 % Formic acid B: Acetonitrile + 0.1 % Formic acid
Method	Gradient 100 - 5 % B for 20 min Plateau 100 % B for 6 min
Flow	1.2 mL min <sup>-1</sup>
Temperature	50 °C
Injection Volume	50 $\mu\text{L}$

## A.2. Chromatographic Data

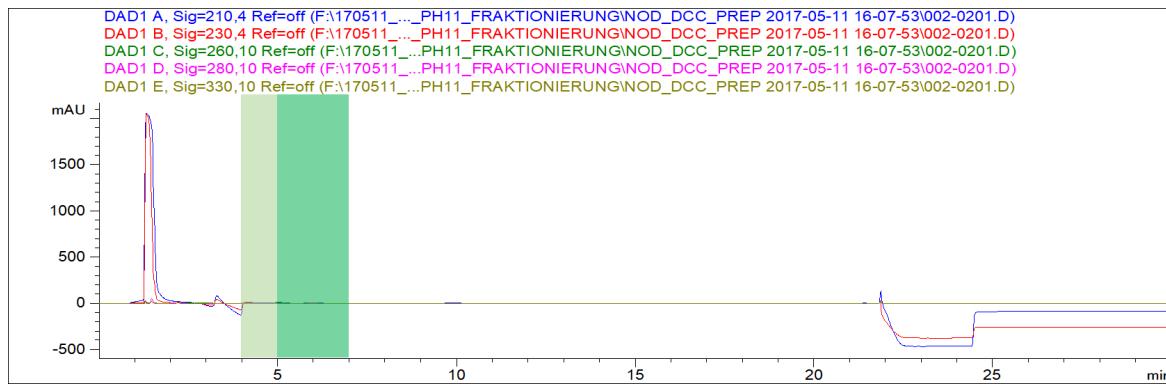


Figure A.1.: UV-chromatogram of reverse extract fractionation with the Luna<sup>®</sup> NH<sub>2</sub> column.testi

## A.3. Genomic Analysis

### A.3.1. Phylogenetic Data

Table including the reference genomes A.9

Table A.9.: Reference genomes for the construction of the phylogenetic tree

Assembly ID	Species name
GCF_000010605	<i>S. griseus</i> subsp. <i>griseus</i> NBRC 13350
GCF_000156455	<i>S. roseosporus</i> NRRL 15998
GCF_000156695	<i>S. roseosporus</i> NRRL 11379
GCF_000177175	<i>Streptomyces</i> sp. ACT-1
GCF_000239075	<i>Streptomyces</i> sp. W007
GCF_000261345	<i>S. globisporus</i> C-1027
GCF_000373305	<i>Streptomyces</i> sp. CcalMP-8W
GCF_000373405	<i>Streptomyces</i> sp. ScaeMP-e10
GCF_000377965	<i>Streptomyces</i> sp. CNB091
GCF_000385945	<i>S. fulvissimus</i> DSM 40593
GCF_000498935	<i>Streptomyces</i> sp. HCCB10043

GCF_000647875	<i>Streptomyces</i> sp. SolWspMP-sol2th
GCF_000702365	<i>Streptomyces</i> sp. CNS654
GCF_000716025	<i>S. baarnensis</i>
GCF_000716935	<i>S. alboviridis</i>
GCF_000716945	<i>S. albus</i> subsp. <i>albus</i>
GCF_000717015	<i>S. brasiliensis</i>
GCF_000717105	<i>S. anulatus</i>
GCF_000717215	<i>S. vinaceus</i>
GCF_000717645	<i>S. californicus</i>
GCF_000717665	<i>S. floridae</i>
GCF_000717795	<i>S. griseus</i> subsp. <i>griseus</i>
GCF_000717965	<i>S. purpeochromogenes</i>
GCF_000718135	<i>S. cyaneofuscatus</i>
GCF_000718205	<i>S. griseus</i> subsp. <i>rhodochrous</i>
GCF_000718235	<i>S. griseus</i> subsp. <i>rhodochrous</i>
GCF_000718245	<i>S. californicus</i>
GCF_000718455	<i>S. globisporus</i> subsp. <i>globisporus</i>
GCF_000718615	<i>S. purpeochromogenes</i>
GCF_000718695	<i>S. puniceus</i>
GCF_000718915	<i>Streptomyces</i> sp. NRRL WC-3540
GCF_000719035	<i>S. griseus</i> subsp. <i>rhodochrous</i>
GCF_000719195	<i>S. puniceus</i>
GCF_000719355	<i>S. griseus</i> subsp. <i>griseus</i>
GCF_000719585	<i>Streptomyces</i> sp. NRRL F-5681
GCF_000719655	<i>Streptomyces</i> sp. NRRL B-1381
GCF_000719925	<i>Streptomyces</i> sp. NRRL F-2202
GCF_000720055	<i>Streptomyces</i> sp. NRRL F-3218
GCF_000720065	<i>Streptomyces</i> sp. NRRL F-3273
GCF_000720915	<i>S. albus</i> subsp. <i>albus</i>
GCF_000721175	<i>S. anulatus</i>
GCF_000721205	<i>S. griseus</i> subsp. <i>rhodochrous</i>
GCF_000721415	<i>Streptomyces</i> sp. NRRL F-5702
GCF_000721575	<i>S. griseus</i> subsp. <i>griseus</i>
GCF_000721685	<i>S. mediolani</i>

GCF_000725705	<i>Streptomyces</i> sp. NRRL S-623
GCF_000743295	<i>Streptomyces</i> sp. JS01
GCF_000932225	<i>S. griseus</i>
GCF_000935135	<i>Streptomyces</i> sp. MNU77
GCF_001189025	<i>S. europaeiscabiei</i>
GCF_001270675	<i>S. griseus</i> subsp. <i>rhodochrous</i>
GCF_001278095	<i>Streptomyces</i> sp. CFMR 7
GCF_001279425	<i>Streptomyces</i> sp. NRRL F-2295
GCF_001279735	<i>Streptomyces</i> sp. MMG1522
GCF_001418625	<i>S. luridiscabiei</i>
GCF_001426325	<i>Streptomyces</i> sp. Root1295
GCF_001427565	<i>Streptomyces</i> sp. Root63
GCF_001434355	<i>S. anulatus</i>
GCF_001687325	<i>Streptomyces</i> sp. PTY087I2
GCF_001723115	<i>S. griseus</i>
GCF_001723125	<i>S. griseus</i> subsp. <i>griseus</i>
GCF_001746285	<i>Streptomyces</i> sp. EN16
GCF_001746305	<i>Streptomyces</i> sp. EN23
GCF_001746315	<i>Streptomyces</i> sp. EN27
GCF_001751305	<i>S. nanshensis</i>
GCF_001895105	<i>Streptomyces</i> sp. NBRC 110465
GCF_001905405	<i>Streptomyces</i> sp. TSRI0445
GCF_001905485	<i>Streptomyces</i> sp. TSRI0261
GCF_001905525	<i>Streptomyces</i> sp. TSRI0395
GCF_001905575	<i>Streptomyces</i> sp. CB00072
GCF_001905595	<i>Streptomyces</i> sp. CB00316
GCF_001905645	<i>Streptomyces</i> sp. CB02115
GCF_001905785	<i>Streptomyces</i> sp. CB02130
GCF_001905905	<i>Streptomyces</i> sp. CB02366
GCF_001931635	<i>Streptomyces</i> sp. Tue 6075
GCF_001983595	<i>Streptomyces</i> sp. IB2014 011-1
GCF_002082175	<i>S. violaceoruber</i>
GCF_002082585	<i>Kitasatospora albolonga</i>
GCF_002094995	<i>Streptomyces</i> sp. S8

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GCF_002154345	<i>S. fimicarius</i>
GCF_002154385	<i>S. albovinaceus</i>
GCF_002188345	<i>Streptomyces</i> sp. CS057
GCF_002217715	<i>Streptomyces</i> sp. SS07
GCF_002242735	<i>Streptomyces</i> sp. 2R
GCF_900090075	<i>Streptomyces</i> sp. MnatMP-M77
GCF_900090085	<i>Streptomyces</i> sp. Ncost-T6T-1
GCF_900090135	<i>Streptomyces</i> sp. LaPpAH-199
GCF_900091775	<i>Streptomyces</i> sp. ScaeMP-e83
GCF_900091865	<i>Streptomyces</i> sp. DvalAA-19
GCF_900091905	<i>Streptomyces</i> sp. OspMP-M43
GCF_900092005	<i>Streptomyces</i> sp. Cmuel-A718b
GCF_900105705	<i>S. griseus</i>
GCF_900187925	<i>Streptomyces</i> sp. PgraA7

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Table including the identified single-copy genes

Table A.10.: Single copy genomes for tree construction

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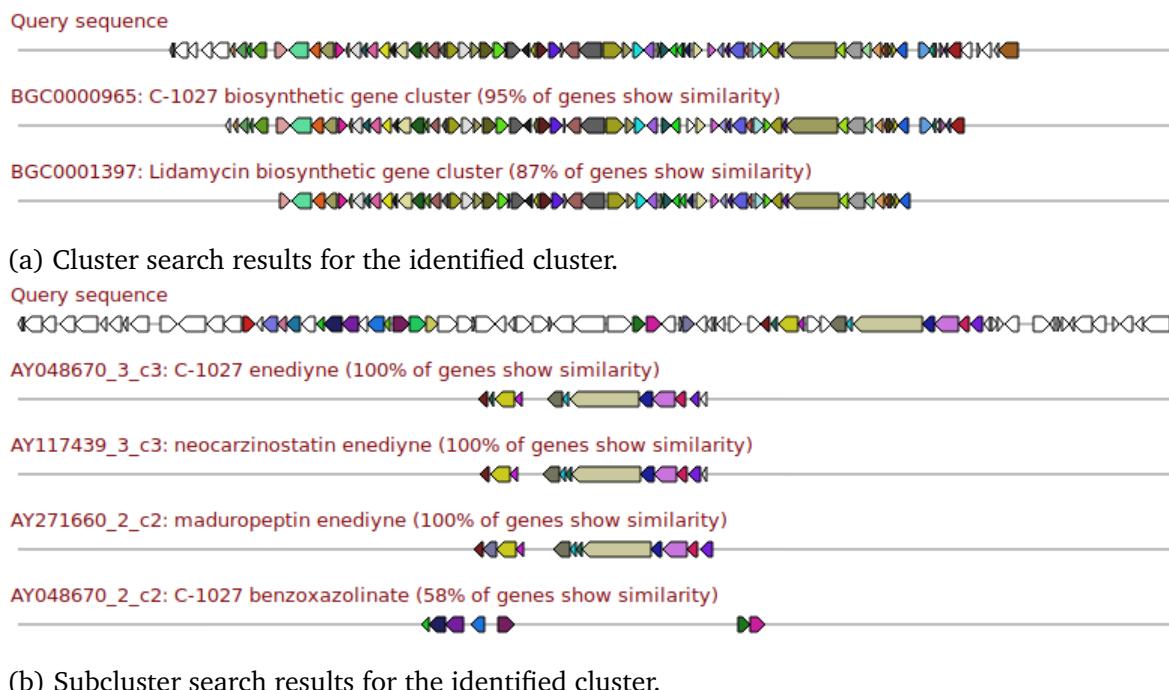
TIGR ID	Description
00008	translation initiation factor IF-1
00033	chorismate synthase
00060	ribosomal protein uL18
00062	ribosomal protein bL27
00118	acetolactate synthase, large subunit, biosynthetic type
00151	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
00171	3-isopropylmalate dehydratase, small subunit
00302	phosphoribosylformylglycinamide synthase, purS protein
00355	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
00382	ATP-dependent Clp protease, ATP-binding subunit ClpX
00431	tRNA pseudouridine(55) synthetase
00484	translation elongation factor G
00615	recombination protein RecR

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00631	exinuclease ABC subunit B
00708	cob(I)yrinic acid a,c-diamide adenosyltransferase
00962	ATP synthase F1, alpha subunit
00981	ribosomal protein uS12
01009	ribosomal protein uS3
01021	ribosomal protein uS5
01022	ribosomal protein bL36
01024	ribosomal protein bL19
01029	ribosomal protein uS7
01030	ribosomal protein bL34
01032	ribosomal protein bL20
01039	ATP synthase F1, beta subunit
01044	ribosomal protein uL22
01049	ribosomal protein uS10
01050	ribosomal protein uS19
01067	ribosomal protein uL14
01083	endonuclease III
01134	amidophosphoribosyltransferase
01162	phosphoribosylaminoimidazole carboxylase, catalytic sub-unit
01164	ribosomal protein uL16
01169	ribosomal protein uL1
01171	ribosomal protein uL2
01393	elongation factor 4
01980	FeS assembly protein SufB
02013	DNA-directed RNA polymerase, beta subunit
02027	DNA-directed RNA polymerase, alpha subunit
02156	phenylacetate-CoA oxygenase, PaaG subunit
02157	phenylacetate-CoA oxygenase, PaaH subunit
02952	RNA polymerase sigma-70 factor
03188	phosphoribosyl-ATP diphosphatase
03450	inositol 1-phosphate synthase
03631	ribosomal protein uS13
03632	ribosomal protein uS11

03687	ubiquitin-like protein Pup
03699	dehypoxanthine futilosine cyclase
03800	pyridoxal 5'-phosphate synthase, glutaminase subunit Pdx2

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**Figure A.2.: Cluster and subcluster search results for the cluster located on contig 4.** The 160 kb contig was submitted to AntiSMASH with the ClusterFinder option. Only the search results with the highest similarities are shown.