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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                                      |
| EtForm            | Ethyl formate                                      |
| 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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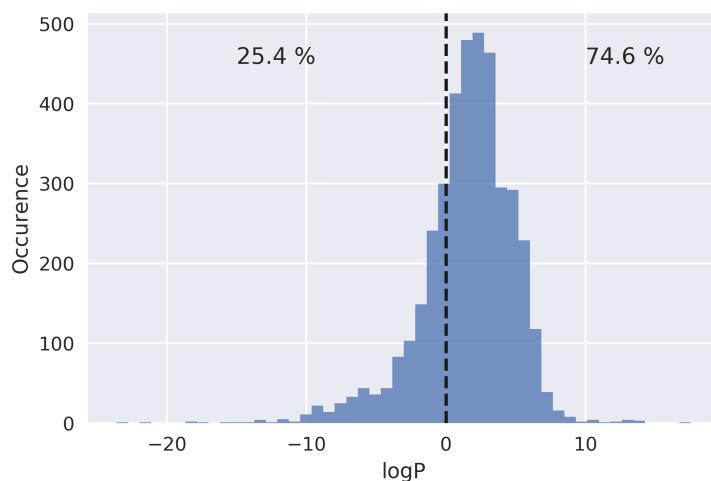
# 1. Introduction

## 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 (~ 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<br>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

|                       | <b>Component</b> | <b>Description</b>   |
|-----------------------|------------------|----------------------|
| 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

| <b>Manufacturer</b> | <b>Line</b>   | <b>Type</b>                | <b>Dimensions</b> |
|---------------------|---------------|----------------------------|-------------------|
| 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

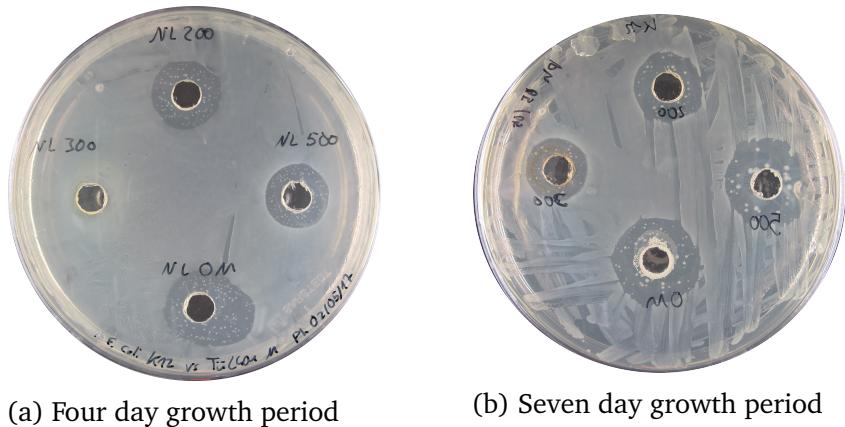
The strain Tü2401 was grown in seven different complex media for either four or seven days during preliminary experiments. Culture broth supernatants of NL 200, NL 300, NL 500 and OM media displayed antibiotic activity against *E. coli* K12on plate assays. A comparison between the viable production media 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 screening gradients and could not be extracted from the culture broth or biomass via ethyl acetate. The apparent high water solubility of the active compound 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 NL 200, NL 300, NL 500 and OM complex media for either four or seven days. 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 culture supernatant 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.



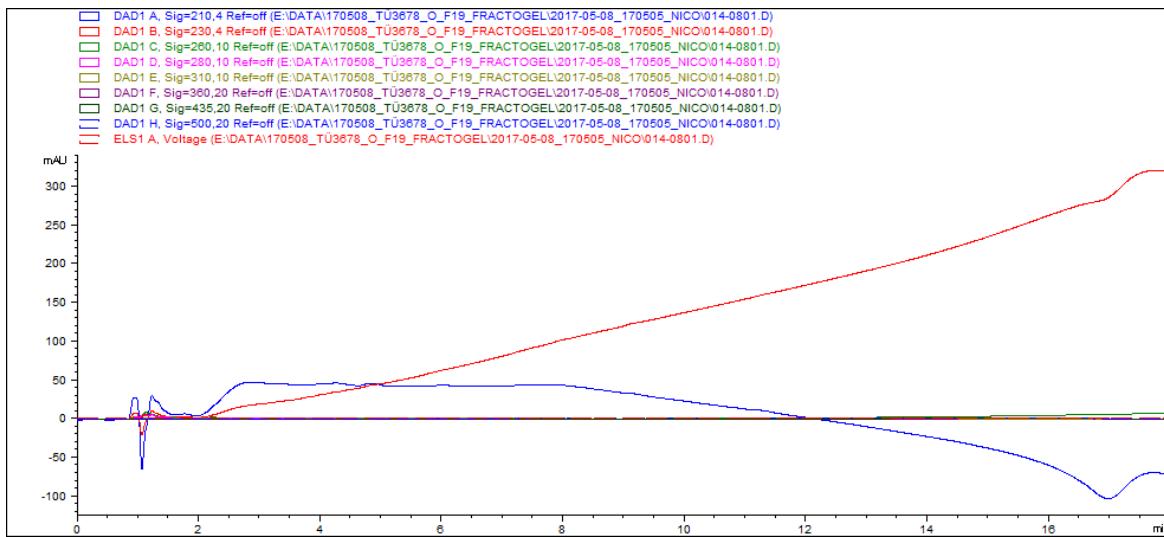
**Figure 3.1.: K12 Bioassay results with media supernatants** *Streptomyces* sp. Tü2401 was grown for either four (a) or seven days (b) in four different complex media. The filtrated culture broth supernatant was tested against *Escherichia coli* K12. Media: (Top) NL 200 (Left) NL 300 (Right) NL 500 (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 was antibiotically active, whereas the biomass and extracts thereof are not. The compound



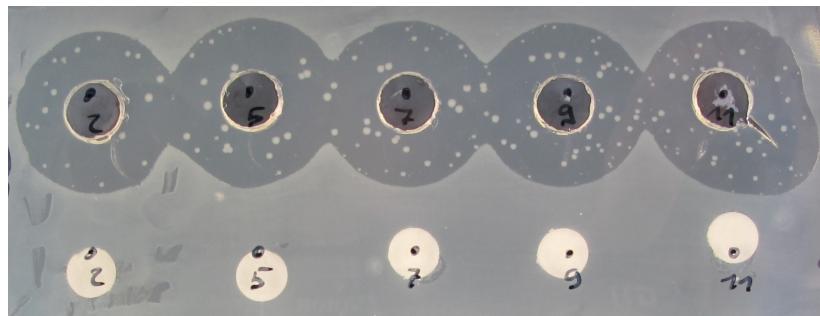
**Figure 3.2.: Chromatogram of OM medium supernatant screening.** 5  $\mu$ L of culture broth supernatant were injected and separated on a C18 column using a screening gradient of 4.5 to 100 % acetonitril. UV absorption was measured at 210, 230, 260, 280, 310, 360, 435 and 500 nm. ELSD voltage was captured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

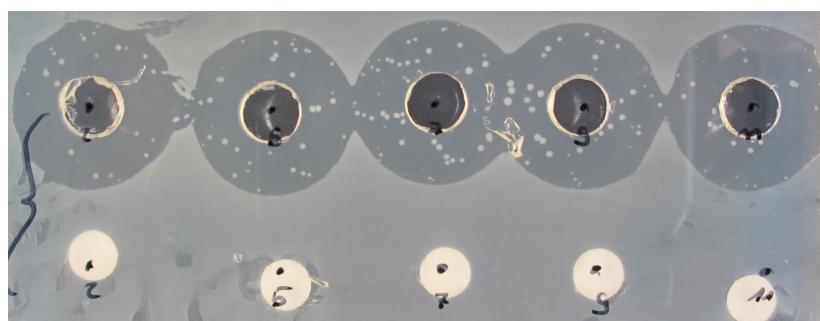
A hydrophilic molecule is likely to contain functional groups like amines or carboxylic acids, which are charged at certain pH-ranges. Adjusting the medium pH to reach the compounds point of zero charge could lower its water-solubility and, thus, enable extraction with an organic solvent. Additionally, 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 (EtAc), Methyl acetate (MeAc) and Ethyl formate (EtForm). 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



(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.

not be extracted with either ethyl acetate, methyl acetate or ethyl formate regardless of 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. Promotor induction in the *yorB*-assay (see Section 2.3.2) is indicated by a blue halo at the edge of the inhibition zone, where a sublethal concentration of antibiotic are present.

Both liquid culture extracts and agar plaques of Tü2401 were subject to the *yorB*-assay to determine the presence of the putative *yorB* inducer in extracts of liquid culture. Figure 3.4 depicts the assay results of ISP2 agar plaques after four days of growth as well as EtAc and MeAc extracts of OM culture broth supernatant.

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

**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 were taken from the Pubchem database and *Streptomyces* species names 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      |

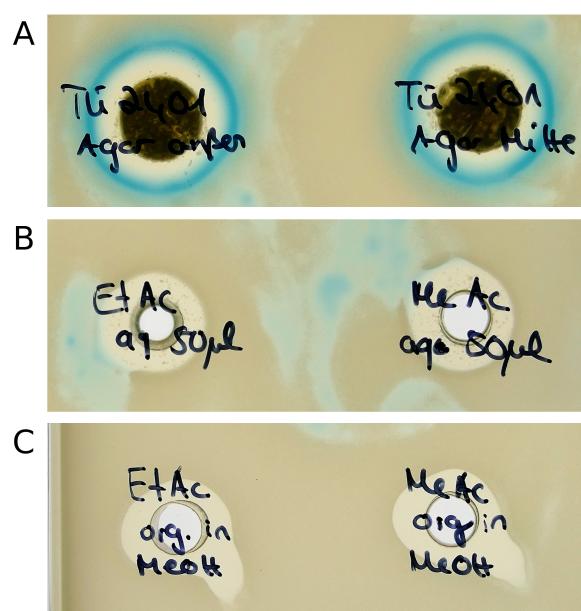


Figure 3.4.: Agar and Culture Extract Samples of Strain Tü2401 subjected to the *yorB* reporter gene assay

Samples were subject to the *yorB* reporter gene assay, incubated for 24 h and photographed. A: Agar plaques of Tü2401 taken after four days of growth on ISP2 agar. B: Aqueous phases of EtAc (left) and MeAc (right) extraction of OM culture broth supernatant. C: EtAc (left) and MeAc (right) extracts of OM culture broth supernatant dried and redissolved in MeOH. Blue halos indicate induction of the *yorB* reporter gene.

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 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 was 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-evaluable, 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-evaluable 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.

The lack of activity against *E. coli* K12 for all but one sample indicates, that the putative hydrophilic compound present in liquid cultures is not produced in the same amount in standing liquid or agar cultures of ISP2 medium. Testing of NL410 agar plate and standing cultures extracts has to be performed to determine whether the lack of activity is due to changes in medium or cultivation procedure.

The only samples able to induce the *yorB* reporter system were the ISP2 agar plaque and the aqueous phase of its EtAc extract. The plaque confirms the ability of strain Tü2401 to induce the reporter when grown on this particular solid medium, whereas aqueous extract sample indicates the active component(s) to be soluble in water, but not EtAc. The lack of activity in the aqueous BuOH extraction sample could be due to an inactivating reaction with BuOH during the extraction procedure. However, the activity of the aqueous EtAc extract was distinctly smaller than that of the agar plaque despite it being generated from multiple agar plates. The loss of activity could therefore also stem from low solubility or degradation during processing regardless of solvent. Variations in biomass used for

**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<br><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   |                |                    | -           | -                        |

the BuOH extracts could have resulted in an active-compound concentration below the detection threshold. LMPA extracts and samples were entirely inactive in the *yorB*-assay, indicating that the type of agarose also plays a role in determining production of the putative *yorB*-inducer.

The combined *yorB*- and antibacterial bioassays for different cultivation techniques largely did not lead to extraction of the *yorB*-inducing compound. Only the aqueous EtAc extract of ISP2 agar plates displayed minor activity. The inactivity of standing culture and LMPA extracts and samples shows that the *yorB*-inducing compound might only synthesized under specific growth conditions, indicating high regulation of the underlying cluster. As of now, the compound is only produced when grown on ISP2 agar plates and is not readily extracted from ground-up plates with organic solvents. Since the conditions which determine production are not yet determined, a wider array of media and culture techniques could be employed to narrow down the necessary stimuli. Especially minimal media or media with single limited nutrients could be used to explore if production is only dependent on sporulation or also on nutrient availability. An optimal production medium designed with this knowledge might be able to circumvent agar plate extraction, which in this assay proved to be insufficient in achieving significant concentrations of active compound. However, the assay highlighted the potential of a third compound, which is soluble in EtAc and BuOH and active against *B. subtilis* but not *E. coli*. Since this compound seems to be produced in liquid culture and be readily extracted, isolation and structure elucidation could be achieved far more swiftly. Overall, *Streptomyces* sp. Tü2401 displays a remarkable diversity in biologically active secondary metabolites, which all require different environmental stimuli.

### 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* 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

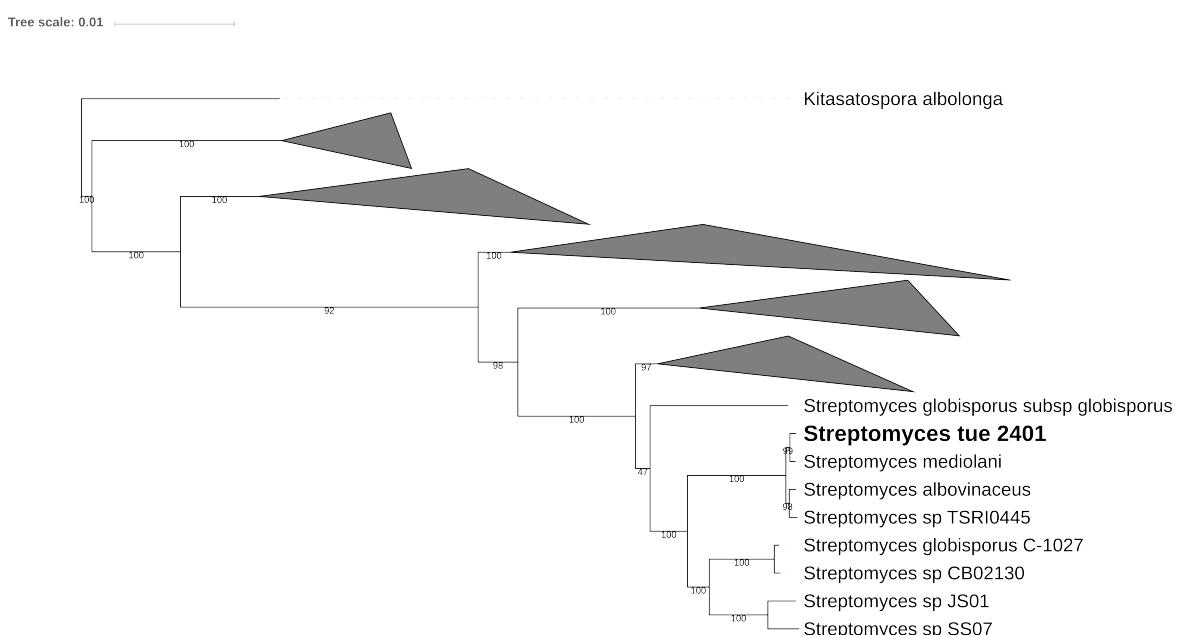


Figure 3.5.: Maximum likelihood tree of *Streptomyces* sp. Tü2401. The tree was constructed using a multiple-sequence alignment of 50 single-copy genes across 50 *Streptomyces* reference genomes. The node belonging to *Streptomyces* sp. Tü2401 is highlighted with bold text. Only the nine most closely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

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 (HSAF) 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.

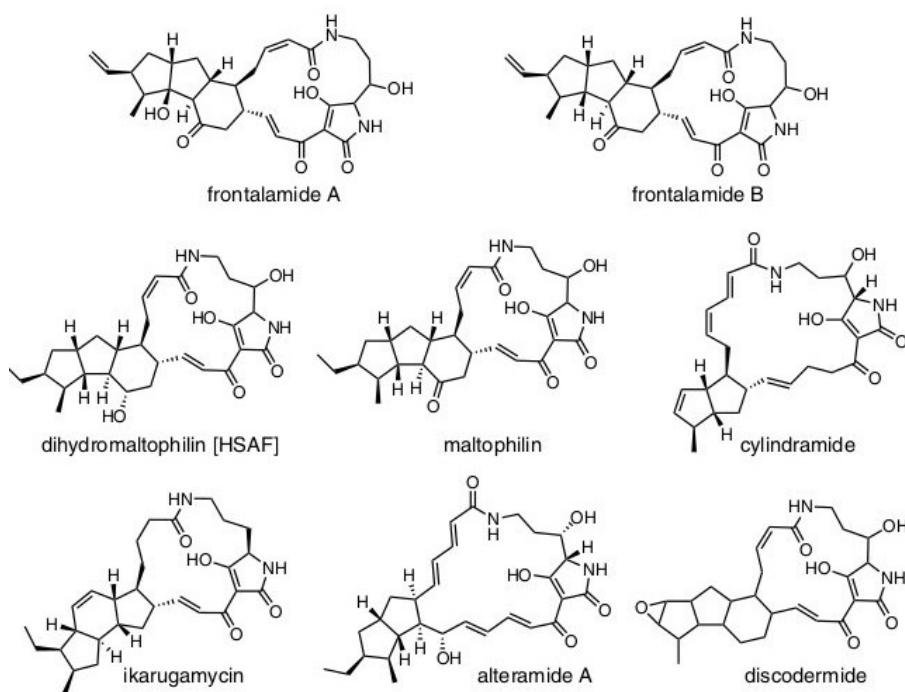


Figure 3.6.: **Structures of bacterial polycyclic tetramate macrolactams.** Adapted from Blodgett *et al.* (2010). Copyright lies with the respective authors.

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 HSAF 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 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> Electonic 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 hydrophic 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

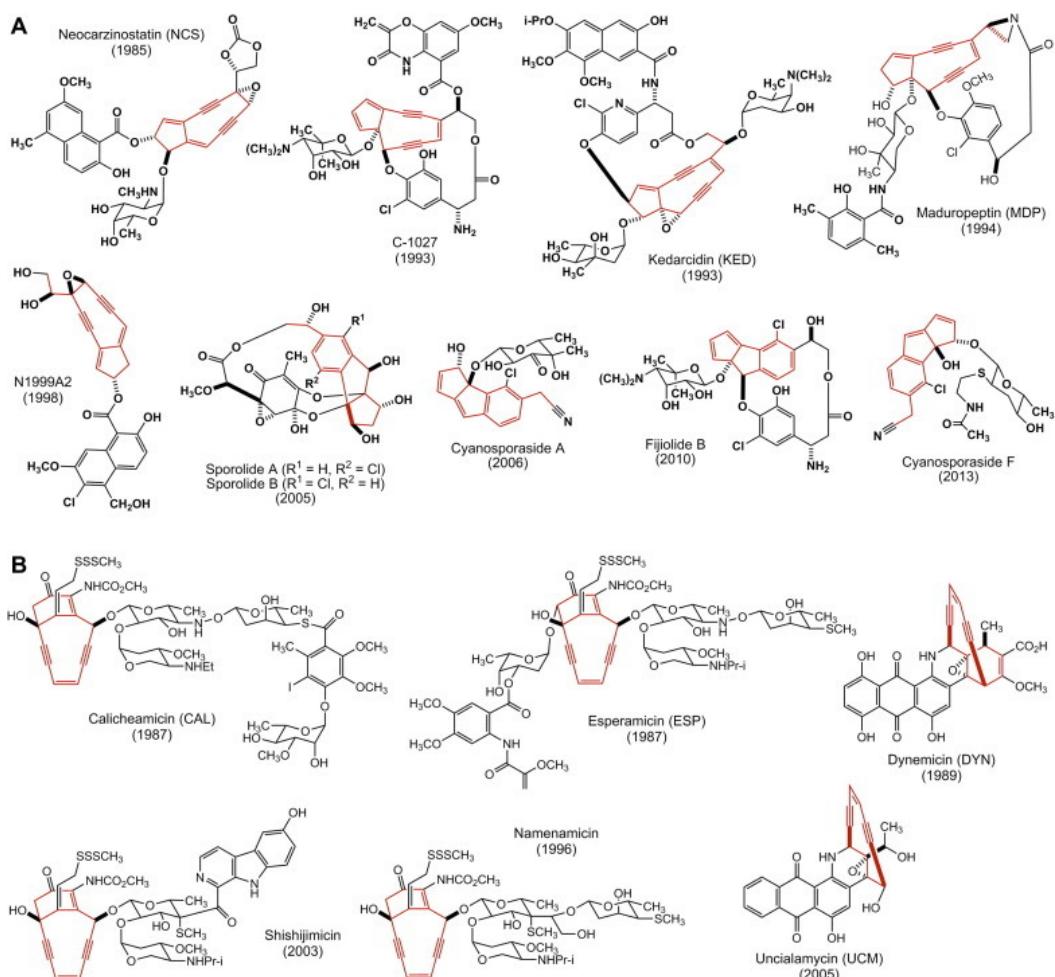


Figure 3.7.: **Structures of known enediyne natural products.** Enediyne cores are highlighted in red. (A) Compounds with nine-membered rings. (B) Compounds with ten-membered rings. The year of structure confirmation is displayed in parentheses. The sporolides, cyanosporolids and fijiolides do not contain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

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 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 10 L 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.

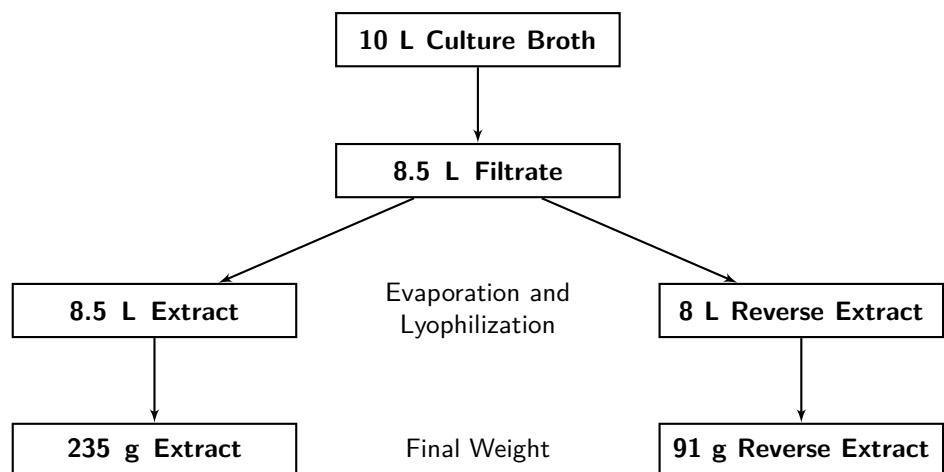


Figure 3.8.: Yield Diagram of *Streptomyces* sp. Tü2401 Fermentation.

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<br>B: Acetonitrile + 0.1 % Formic acid |
| Method           | Gradient 5 - 100 % B for 15 min<br>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<br>B: Acetonitrile + 0.1 % Formic acid |
| Method           | Isocratic 80 % B for 20 min<br>+ 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<br>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<br>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<br>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<br>B: Acetonitrile + 0.06 % Formic acid |
| Method               | Gradient 0 - 100 % B for 15 min<br>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<br>B: Acetonitrile + 0.1 % Formic acid |
| Method           | Gradient 5 - 100 % B for 20 min<br>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<br>B: Acetonitrile + 0.1 % Formic acid |
| Method           | Gradient 100 - 5 % B for 20 min<br>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

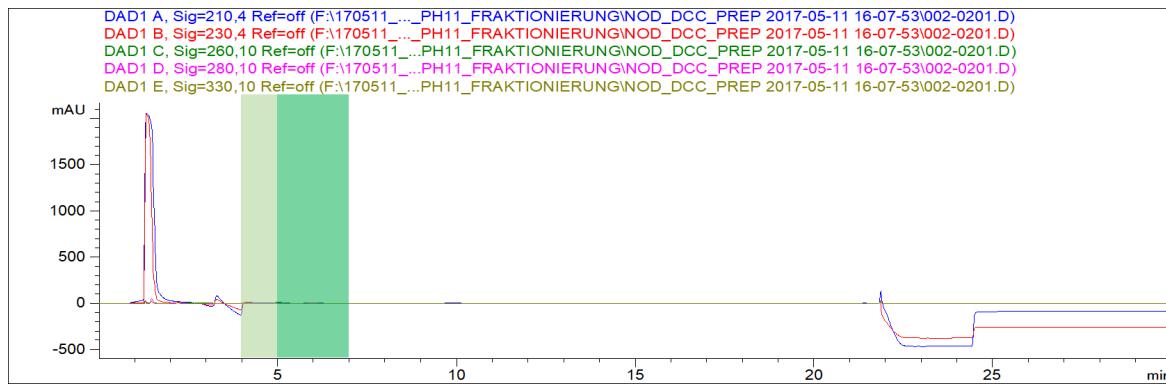


Figure A.1.: UV-chromatogram of reverse extract fractionation with the Luna<sup>®</sup> NH<sub>2</sub> column.testi

## 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. floridiae</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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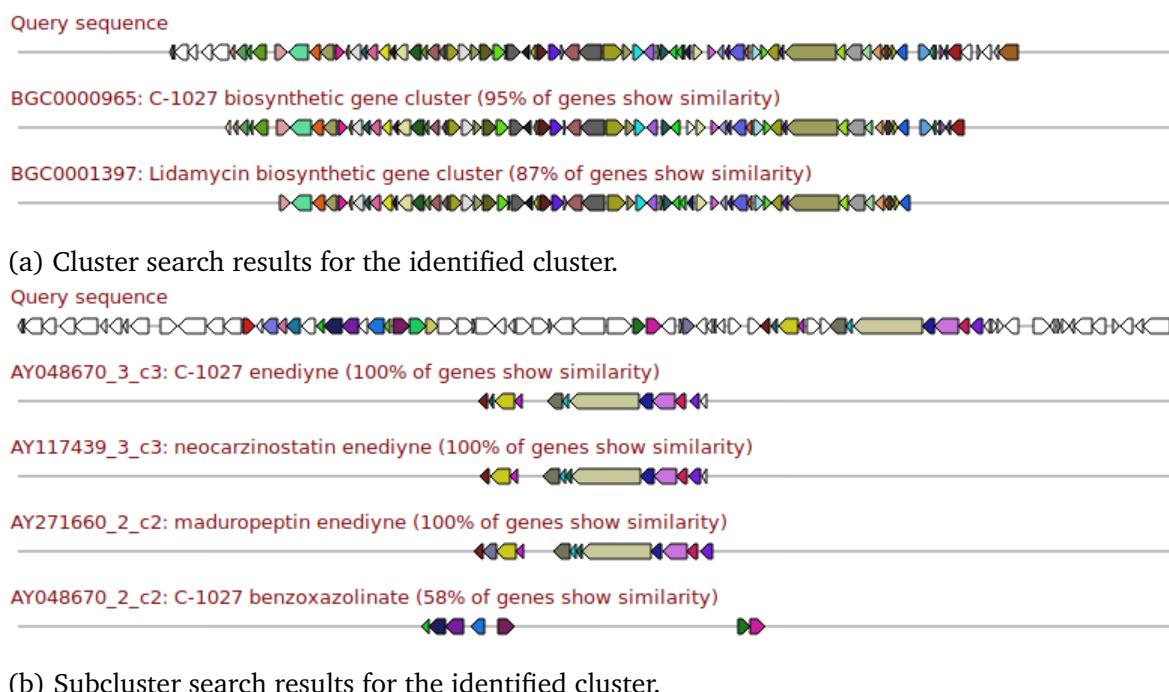
| TIGR ID | Description  |
|---------|--|
| 00008   | translation initiation factor IF-1   |
| 00033   | chorismate synthase  |
| 00060   | ribosomal protein uL18   |
| 00062   | ribosomal protein bL27   |
| 00118   | acetolactate synthase, large subunit, biosynthetic type                      |
| 00151   | 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.