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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**

Chemical	**Supplier**
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.

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_2 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

2.2 Strain Cultivation

2.2.1 Media

All media were prepared by dissolving the components listed in Table 2.4 in MilliQ-H_2O 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.

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 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. Tü2401were 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

Streptmyces 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

Table 2.4: **Media components for the cultivation of strain Tü2401.** All amounts are calculated for one liter of MilliQ-H_2O. 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
		$CaCO_3$	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		$CaCl_2 \cdot 2\,H_2O$	3 g	
		Fe^{3+} citrate	1 g	
		$MnSO_4 \cdot H_2O$	200 mg	
		$ZnCl_2$	100 mg	
		$CuSO_4 \cdot 5\,H_2O$	25 mg	
		$Na_2B_4O_7 \cdot 10\,H_2O$	20 mg	
		$CoCl_2 \cdot 6\,H_2O$	4 mg	
		$Na_2MoO_4 \cdot 2\,H_2O$	10 mg	

100 mL of NL 410 medium without $CaCO_3$. 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. The temperature was kept at 27 °C with an airflow of 5 L min^{-1} 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 ($\varnothing = 92$ mm) were filled with 20 mL of liquid agar, square dishes (120 × 120 mm) were filled with 40 mL. Solidified agar plates were stored at 8 °C.

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

2.3.2 *yorB* Reporter Gene Assay

Agar-based *yorB*-reporter gene assays were performed with *Bacillus subtilis* 1S34 pHJS105-yorB-lacZ2.[1] 20 mL of LB-agar supplemented with 50 µg mL^{-1} spectinomycin were inoculated with 500 µL of overnight culture and grown until reaching the stationary phase. 50 mL LB-softagar (0.7 % agarose) with 150 µg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was prepared and mixed with the strain to a concentration of 3×10^7 colony-forming units per milliliter. The agar was filled into a square petri dish and preparated with sample wells. 50 µL of test samples were applied and the plate was incubated at 30 °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 µm sterile filter. The filtrate was stored at 4 °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 seperately and concentrated in a rotary evaporator at 40 °C. The concentrated aquous 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 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_{254} 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) $Fe(III)Cl_3$ 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^- form) and a strong cation exchange resin (Dowex 50WX4, 100-200 mesh, Na^+ form). Three solutions were used for all operations: An acidic solution (1 % (v/v) formic acid, pH 2), a neutral solution (MilliQ-H_2O, 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 solution and the cation exchange resin (CatX) was swollen in the acidic solution. 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^{-1}. 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. Occasional coupling of an electric 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 was directed 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 **??**.

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).

2.6 Genome Analysis

The taxonomic analysis was performed by Mohammad Alanjary of the group of 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 **??**) 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).[2–4] The maximum-likelihood tree was constructed with RAxML (version 8).[5] The average nucleotide identity was calculated with the JSpeciesWS web tool.[6] The final tree was visualized using Interactive tree of life (iTOL) v3.[7]

All five contigs were uploaded individually to the Antibiotics & Secondary Metabolite Analysis Shell (AntiSMASH) webserver.[8–10] Clusters were identified with standard settings and ClusterFinder enabled.

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