

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.

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1 Methods

1.1 Chemicals & Instruments

Chemicals and solvents were supplied by Merck, if not specified otherwise. Differing vendors for solvents are listed in Table 1.1

Table 1.1: 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 1.2.

Table 1.2: 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 1.3: Column Parameters

Manufacturer	Line	Type	Size
Merck	SeQuant [®]	ZIC [®] -HILIC PEEK 3.5 μm	150 \times 4.6 mm
Phenomenex	Luna [®]	NH2 5 μ	250 \times 4.6 mm
	Kinetex [®]	Polar-C18 2.6 μm	150 \times 4.6 mm
Dr. Maisch	Nucleosil-100	C-18 5 μm	100 \times 2.5 mm

1.2 Strain Cultivation

1.2.1 Media

All media were prepared by solving the components listed in Table 1.4 in MilliQ-H₂O and adjusting the pH with NaOH and HCl. For solid media, 2 % (w/v) agar was added. Sterilization was performed by autoclaving at 121 °C and 230 kPa for 15 min. Fluid media were stored at ambient temperature, solid media at 8 °C.

1.2.2 *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 generated by adding 2 % (w/v) agar to the medium.

1.2.3 General cultivation of *Streptomyces* sp. Tü2401

Streptomyces sp. Tü2401 was cultivated primarily in liquid NL- or OM-medium. Agar-plate cultures were grown on ISP2 medium at 29 °C. 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 grown in 20 mL of NL 410 in 100 mL flasks and inoculated with plate-grown mycelium. Main cultures were grown in 100 mL medium in 500 mL flasks.

1.2.4 Batch Fermentation of *Streptomyces* sp. Tü2401

The strain 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 round flasks containing 100 mL of NL 410 medium without CaCO₃. 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 NL 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 1.3.4.

Table 1.4: **Media components for the cultivation of strain Tü2401.** All amounts are calculated for one liter of MilliQ-H₂O. 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	
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 · 2 H ₂ O	3 g	
		Fe ³⁺ citrate	1 g	
		MnSO ₄ · H ₂ O	200 mg	
		ZnCl ₂	100 mg	
		CuSO ₄ · 5 H ₂ O	25 mg	
		Na ₂ B ₄ O ₇ · 10 H ₂ O	20 mg	
		CoCl ₂ · 6 H ₂ O	4 mg	
		Na ₂ MoO ₄ · 2 H ₂ O	10 mg	

1.3 Chemical Screening of Reverse Extracts

Extracts and reverse extracts of *Streptomyces* sp. Tü2401 were generally obtained through filtrated medium supernatants. After cultivating the strain for 4 - 7 days, the harvested biomass was centrifuged at 9000 rpm for 20 min. The supernatant was collected and filtered before further processing.

1.3.1 General Sample Preparation

Concentrated reverse extracts of medium supernatant were used for most chromatographic experiments and bioassays. Filtered medium supernatant was concentrated by a factor of five by drying it under vacuum at 40 to 60°C and resuspending it in water.

1.3.2 Agar Diffusion Bioactivity 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 μ L (200 μ L for round plates) of liquid culture at an optical density of 0.3 to 0.6 at 600 nm 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 μ L of sample. Finished plates were stored for 1 h at ambient temperature, before incubating them at either 30°C or 37°C over night.

1.3.3 Determination of Extraction Conditions

5 mL filtered medium aliquots of Tü2401 were transferred to 15 mL falcon tubes. The pH of three of each tube was adjusted to either 2, 5, 7, 9 or 11 with NaOH and HCl. Each set of pH adjusted samples was then extracted with either ethyl acetate, methyl acetate or ethyl formate for 30 min. The phases were separated by centrifuging at 4000 rpm for 5 min. The organic phase was subtracted and stored in a new falcon tube. Samples of both phases were test for bioactivity against K12.

1.3.4 Processing of Fermentation 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 completed phase-separation, the organic phase was collected and the aqueous phase reused for further extraction. The process was repeated five times. Both phases were collected separately and concentrated in a rotary evaporator at 40 °C.

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1.3.5 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.

1.4 Bioactivity-guided Isolation

1.4.1 Thin Layer Chromatography

Thin layer Chromatography was performed with reverse extracts of Tü2401 on TLC Silica Gel 60 F₂₅₄ plates by Merck. Aqueous samples were applied by pipetting 1 μ L 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 1.5.

Table 1.5: 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
Chloroform / Methanol	8:3

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 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 and the extraction process repeated. 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.

1.4.2 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-H₂O, 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 1.6. 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 1.6: **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

1.4.3 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. Dried HPLC fractions were suspended in 500 µL *N,O*-Bis(trimethylsilyl)-trifluoroacetamide with added 40 µL pyridine and heated to 110 °C. Afterwards, the 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.

1.4.4 Preparative HPLC

Preparative HPLC was performed on either the Agilent 1100 Series or Agilent 1260 Infinity instrument coupled to an Agilent fraction collector. All samples were centrifuged at 14,000 rpm, before transferring the supernatant to HPLC-vials. Detailed information about the used columns and methods can be found in Table 1.3 and the appendix. The obtained data was analyzed with the Agilent Chemstation (Version B.04.03). All fractions were collected by timeslices of 1 min and stored at 4 °C to 8 °C, before drying them at 40 °C. Dried fractions were resuspended in an amount of water equal to the amount of injected sample and stored at 4 °C to 8 °C.

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1.4.5 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 control software was 6300 Series Trap Control (Version 6.1).

2 Results & Discussion

2.1 Determination of Extraction Conditions

2.2 Antibacterial Activity Spectrum

2.2.1 Extraction of yorB-inducing Compound

Tü2401 displayed positive results in the yorB-Assay when grown on ISP2 plates . However, all previously generated samples from liquid cultures only showed antibacterial activity. Three cultivation strategies were developed to induce production of the putative compound 2 (PC2):

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1. Standing cultures could allow the formation of aerial mycelium at the medium surface. Thus, enabling the synthesis of PC2 and allowing extraction of the liquid medium. 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 seperately. 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. ISP2 agar plates were previously known to enable synthesis of PC2 and could be extracted with prior breakup. 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). This would allow melting of the plates at lower temperatures. Thus, allowing for easier extraction with reduced risk of thermal decomposition during the process. Six ISP2 LMPA agar plates were inoculated with 100 μ L of a week-old NL 410 shake culture and incubated for four days. The plates were then melted at 70 $^{\circ}$ C and extracted with BuOH and EtAc.

All generated extracts and their respective aqueous phases were tested for bioactivity. The standard bioassay was used to determine the antibiotic activity against *E. coli* K12 and *B. subtilis* 168, and the yorB-Assay was used to test for the mode of action. Additionally, agar stamps of grown ISP2 and ISP2 LMPA plates were tested in the yorB-Assay. A summary of the results is shown in .

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Table 2.1: ASDASD

Cultivation	Solvent	Sample	<i>E. coli</i>	<i>B. subtilis</i>	yorB-Assay
***pt<=-	***pt<=-	FC	+	-	-
*pt>=	*pt>=	FC	-	-	-
Standing	FC aq.	-	-	-	-
Cultures	AC	++	+	-	-
	AC aq.	-	-	-	-
	***pt<=-	FC	-	+++	-
	*pt>=	FC	-	-	-
	FC aq.	-	-	-	-
	AC	-	-	-	-
	AC aq.	-	-	-	-

2.3 Chromatographic Separation