Resource

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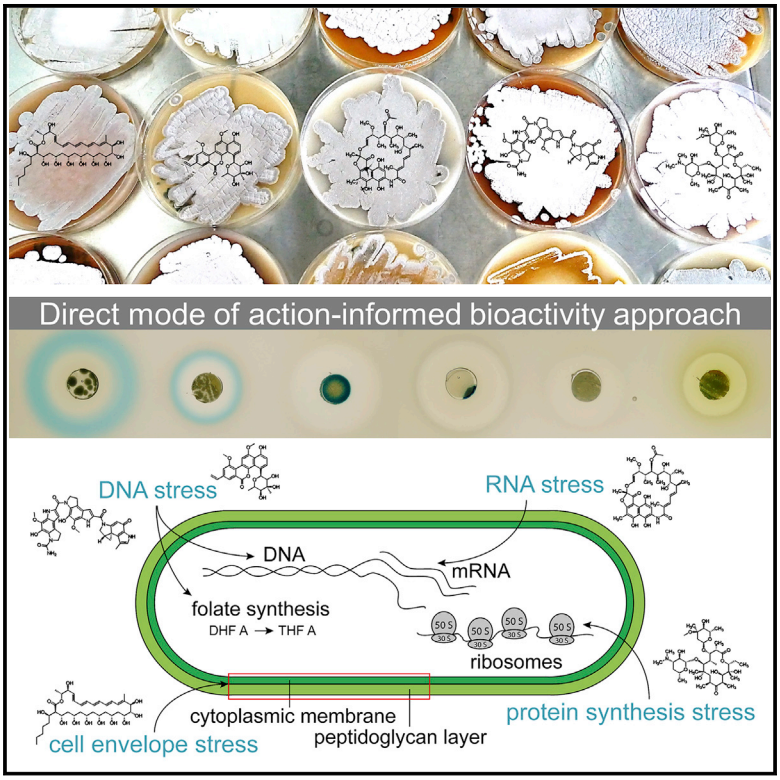
Bioreporters for direct mode of action-informed screening of antibiotic producer strains

Graphical abstract



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In brief

Wex et al. established a robust, sensitive, and rapid bioreporter-based approach for broad mode of action profiling of antibiotic producer strains, detecting also polypharmacology and synergism. Bioreporter specificity was validated with 90 known reference compounds and proof-of-concept was provided by screening 500 actinomycete producers of the Tübingen strain collection.

Highlights

- Direct mode of action-informed bioreporter screening of antibiotic producer strains
- Rapid mode of action profiling of unknown antimicrobials after overnight incubation
- Detection of polypharmacology and synergism
- Facilitated deconvolution process by an additional mode of action dimension

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Bioreporters for direct mode of action-informed screening of antibiotic producer strains

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SUMMARY

A big challenge in natural product research of today is rapid dereplication of already known substances, to free capacities for the exploration of new agents. Prompt information on bioactivities and mode of action (MOA) speeds up the lead discovery process and is required for rational compound optimization. Here, we present a bioreporter approach as a versatile strategy for combined bioactivity- and MOA-informed primary screening for antimicrobials. The approach is suitable for directly probing producer strains grown on agar, without need for initial compound enrichment or purification, and works along the entire purification pipeline with culture supernatants, extracts, fractions, and pure substances. The technology allows for MOA-informed purification to selectively prioritize activities of interest. In combination with high-resolution mass spectrometry, the biosensor panel is an efficient and sensitive tool for compound deconvolution. Concomitant information on the affected metabolic pathway enables the selection of appropriate follow-up assays to elucidate the molecular target.

INTRODUCTION

We are facing a post-antibiotic future, due to the rise of antibiotic resistance and the dried-out pipelines in antibiotic discovery and development (Aslam et al., 2018; Frieri et al., 2017; O'Neill, 2014). In recent years, the number of approved antibacterial drugs has been low, and the vast majority of these agents are derivatives of known antibiotic classes (Hutchings et al., 2019). The imprudent medical and economical handling of our available applicable antibiotics counteracts a sustainable use and further promotes rapid resistance development (Yang et al., 2019; WHO, 2015). The problem needs to be addressed from multiple sides to tackle this antibiotic crisis: we have to learn to handle our available precious drugs responsibly and stably finance antibiotic research and development. Furthermore, we urgently need to find new anti-infective agents, which are structurally different and either act on new targets or show novel binding modes on well-characterized targets, thereby avoiding cross-resistance (Bush et al., 2011). Resistance-breaking agents in combination with currently applied therapeutics are also of interest (Bush and Pucci, 2011; Laws et al., 2019; Melander and Melander, 2017). Although huge efforts have been made to overcome this problem by screening or rational design of optimized (semi-)synthetic molecules in target-based approaches, it became clear that the complexity of natural products is difficult to predict computationally or mimic by total synthesis (Brötz-Oesterhelt and Sass, 2010). A major issue in screening campaigns with synthetic libraries was the lack of bacterial cell entry or efflux problems of inhibitors identified against purified targets *in vitro* (Tommasi et al., 2015). The significant advantage of natural products is that they evolved for efficient target-binding and resistance-slowing polypharmacology. At the same time, their physicochemical properties facilitate entry and retainment within the bacterial cell. Therefore, natural products remain the most

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promising source in antibiotic discovery (Bérdy, 2012; Brötz-Oesterhelt and Sass, 2010).

After the golden age of antibiotic discovery (1940–1960), one of the main problems was the rediscovery of already known antibacterial agents, when following the classical phenotypic bioactivity-based screening approach mainly using actinomycetes producer strains isolated from soil (Genilloud, 2017; Tulp and Bohlin, 2005). To overcome this problem, current research is focusing on underexplored antibiotic producer species, e.g., those living in unique environmental niches, microbiomes, or those requiring specialized growth conditions (Niedermeyer, 2015; Zhang et al., 2017; Zipperer et al., 2016). Other approaches aim at harnessing the silent genomic capacity of known antibiotic producers (Wang et al., 2019). Producer strains often possess a large number of antibiotic gene clusters, and therefore have the genetic potential to produce a panel of different antimicrobial secondary metabolites when adapting to altered nutritional or environmental conditions, including microbe-microbe interactions (Culp et al., 2019; Handayani et al., 2018; Hug et al., 2018). Nevertheless, strains are usually discarded if they produce a known antibacterial agent, thereby potentially missing interesting compounds that are synthesized, e.g., in smaller amounts or under different growth conditions. The further development of effective screening tools, cultivation conditions, and extraction or dereplication methods form crucial steps in the discovery process of new bioactive molecules (Carrano and Marinelli, 2015; Wright, 2017). Cell-based screening methods that combine information on whole-cell bioactivity and mode of action (MOA) are promising tools to rapidly identify and characterize interesting bioactive molecules with the potential to cross the bacterial cell envelope and achieve sufficient target engagement in the whole-cell setting (Fischer et al., 2004; Wolf and Mascher, 2016; Nonejuie et al., 2016). Gathering MOA information at an early stage allows to prioritize activities targeting a specific metabolic pathway of interest. Identifying the targeted metabolic pathway also enables straightforward follow-up studies for tracking down the precise molecular target, which in turn is a prerequisite for rational lead optimization approaches.

The process of natural product discovery benefits from an MOA-informed screening and purification procedure in several ways. For known agents with known targets, the MOA information can confirm mass spectrometry-based compound identity by an independent method, facilitating compound dereplication. Besides, overlapping bioactivities in complex mixtures can be easily monitored and separated. Ideally, the setup of such a combined screening tool for antibacterial whole-cell activity and MOA should be easy to handle, suitable for high-throughput, quick to execute, and robust throughout the entire screening and purification process. Urban et al. (2007) reported on a panel of *Bacillus subtilis* whole-cell biosensors, derived from a large transcriptome study (Freiberg et al., 2006; Hutter et al., 2004), that are specifically induced upon antibiotic interference with the major biosynthetic bacterial pathways: DNA synthesis, RNA synthesis, protein synthesis, and cell envelope integrity. Their firefly luciferase-based reporter strain panel allowed for high-throughput screening of pure antimicrobial agents in solution. A substantial problem of most MOA analysis procedures is their dependency on preceding substance purification, which is time-consuming and requires separation processes with an inherent risk to miss out on interesting activities, especially in complex extract mixtures.

Here, we present an agar-based screening approach, which provides combined bioactivity and MOA information. Capitalizing on known and identifying a new biomarker, we developed a robust and versatile MOA profiling method that allows to directly screen antibiotic producer strains grown on agar without any purification, as well as culture supernatants, extracts, fractions, and pure substances. We verified the specificity and sensitivity of our bioreporters and assay conditions using an extensive library of reference antibiotics with known MOA and validated its suitability for direct screening with a set of characterized antibiotic producer strains. In a pilot screening campaign, we further tested 500 uncharacterized actinomycetes strains of the Tübingen strain collection. By following up on some activities from these strains, we could confirm that the metabolic stress sensed by the bioreporters matched the described MOA of the dereplicated substances in all cases.

RESULTS AND DISCUSSION

Selection of biomarker genes and optimization criteria for the setup of an agar-based bioreporter screening

As model organism we chose the best-investigated Gram-positive species *B. subtilis*, as there are extended datasets on the physiological stress response of this organism to antibiotic exposure, including transcriptome (Fischer et al., 2004; Freiberg et al., 2006), proteome (Bandow et al., 2003), or cytological profiling studies (Nonejuie et al., 2016). Furthermore, its high antibiotic susceptibility allows for a sensitive detection of most bioactive molecules, while the deduced MOA information can be often directly transferred to pathogenic species. To establish a bioreporter panel suitable for direct screening of producer strains, we selected biomarkers indicative of interference with the main bacterial metabolic pathways. We chose the promoters of the *B. subtilis* genes *yorB*, *bmrC* (*yhel*), *ypuA*, and *lial*, which had previously been shown to be specifically induced upon antibiotic stress (Urban et al., 2007; Freiberg et al., 2006; Mascher et al., 2004; Wenzel et al., 2014). The *yorB* gene encodes for a SPβ prophage protein of unknown function and is proposed to be part of the LexA regulon involved in the SOS response (Urban et al., 2007; Au et al., 2005). Its expression was described to be induced by compounds interfering with DNA synthesis and structure. Impairment of protein synthesis, or more specifically the occurrence of translation arrest, results in a specific induction of *bmrC.* In contrast, protein synthesis inhibitors which induce miscoding (e.g., most aminoglycosides) or result in truncated peptides (e.g., puromycin) do not increase *bmrC* expression (Urban et al., 2007). The BmrC protein is a subunit of a putative multidrug ABC transporter and it is still an enigma, why is it induced by a range of mechanistically similar yet structurally very diverse translation stalling agents. Besides, a deletion mutant of this transporter did not show increased sensitivity to compounds inducing its expression (Torres et al., 2009). Both the *ypuA* and *lial* promoters (P_{ypuA} and P_{lial}) serve as biomarkers for cell envelope stress. While P_{ypuA} reacts broadly to various kinds of stress affecting the cell wall or the cytoplasmic membrane, P_{lial} seems more selectively induced upon interference with the lipid-II cycle

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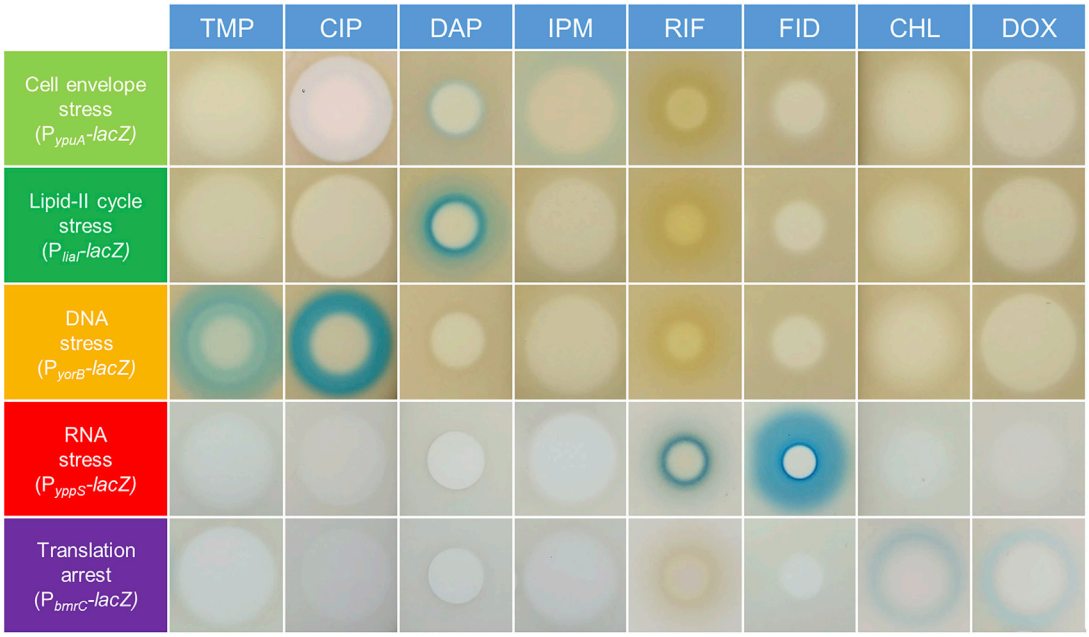

Figure 1. Induction pattern of exemplary reference antibiotics tested against the bioreporter strain panel

Pure compounds were spotted on agar, containing the respective *B. subtilis* reporter strain, in the following amounts: trimethoprim (TMP) 2 μg; ciprofloxacin (CIP) 5 μg; daptomycin (DAP) 2 μg; imipenem (IPM) 1 μg; rifampin (RIF) 1 μg; fidaxomicin (FID) 4 μg; chloramphenicol (CHL) 10 μg; and doxycycline (DOX) 10 μg. Promoter induction was detected as a blue halo following overnight incubation. The reporters for cell envelope stress (P_{ypuA}-*lacZ*, light green), lipid-II cycle stress (P_{liaI}-*lacZ*, green), and DNA stress (P_{yorB}-*lacZ*, yellow) were tested using lysogeny broth soft agar. RNA stress (P_{yppS}-*lacZ*, red) and translation arrest (P_{bmrC}-*lacZ*, violet) reporters were grown in Belitzky minimal soft agar.

as postulated by Mascher and colleagues (Urban et al., 2007; Jordan et al., 2006; Mascher et al., 2004), i.e., by compounds disturbing the cycling of the undecaprenyl phosphate carrier in the bacterial membrane. The gene of unknown function, *ypuA*, is regulated by SigM, an extracytoplasmic sigma factor involved in the cellular adaption to environmental stress (Jervis et al., 2007). The small membrane protein LiaI is encoded by the *liaIH-liaGFSR* operon, which is autoregulated via the two-component system LiaRS (Mascher et al., 2004). The promoter regions of the above-mentioned genes were individually fused to the β-galactosidase reporter gene *lacZ* and were chromosomally integrated into the *amyE* locus of *B. subtilis* 1S34, a sporulation-deficient derivative of *B. subtilis* 168 (Piggot, 1973). We also tried the *helD* (*yvgS*) promoter (P_{helD}) for our purpose, which was previously used to monitor stress within the RNA synthesis pathway (Urban et al., 2007), but P_{helD} only gave rise to a weak induction signal in the agar-based bioreporter setup, which did not meet the goal of robust detection. We re-analyzed previous microarray data (Freiberg et al., 2006) and identified an alternative, promising biomarker candidate, namely *yppS*, that had displayed strong upregulation upon treatment with the RNA polymerase inhibitors, rifampin and corallopyronin, in the transcriptome study. We constructed a reporter strain containing the chromosomal fusion of the *yppS* promoter (P_{yppS}) and the *lacZ* reporter gene, which reacted strongly to rifampin exposure and showed a more powerful induction signal than the P_{helD}-*lacZ* reporter strain (Figure S1). P_{yppS}-*lacZ* was selectively induced by direct inhibitors of the RNA polymerase and also allowed the detection of compounds interfering with RNA synthesis by intercalation into DNA, such as actinomycin D (compare Table S1). To yield sensitive detection in the agar-based assay setup, the handling of each bioreporter strain was optimized individually regarding growth phase, cell number, growth medium, supplements, substrate concentration, incubation time, and temperature, finally resulting in a robust protocol described in the STAR methods that can now be used for diverse applications without modification.

Induction specificity validated by pure antimicrobial substances with known MOA

Bacteria can show substantially different growth behavior and metabolism in liquid culture and agar. As previous studies employing some of our promoters were conducted in liquid media (Freiberg et al., 2006; Urban et al., 2007), we wanted to ensure that the bioreporters also generate reliable and selective signals in an agar-based setup. For a first round of validation, we tested 90 pure antibacterial agents with known MOA across the entire reporter panel. All of the five bioreporters showed selective induction by all tested antibiotics in accordance with their described MOA (Table S1). An exemplary panel of antibiotics is depicted in Figure 1. Reporter induction was detectable as a blue halo at the antibiotic diffusion borders, where the antibiotic concentration was not high enough to kill the *B. subtilis* reporter cells, but sufficient to cause a cellular stress response. Spotting of a concentration series of different antibiotics revealed a high sensitivity of the bioreporters, as already low antibiotic amounts gave rise to an induction signal (Figure S2). The most sensitive bioreporters even showed an induction at subinhibitory concentrations, i.e., a blue coloration of the agar was visible in the absence of a distinct zone of inhibition (ZOI).

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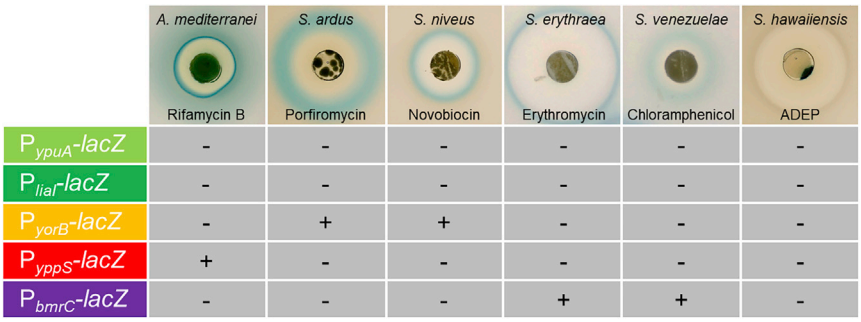

	Rifamycin B	Porfiromycin	Novobiocin	Erythromycin	Chloramphenicol	ADEP
P_{ypuA}-*lacZ*	-	-	-	-	-	-
P_{lial}-*lacZ*	-	-	-	-	-	-
P_{yorB}-*lacZ*	-	+	+	-	-	-
P_{yppS}-*lacZ*	+	-	-	-	-	-
P_{bmrC}-*lacZ*	-	-	-	+	+	-

Figure 2. Induction of the bioreporter strain panel by agar-grown producer strains of antibiotics from different mechanistic classes

Amycolatopsis mediterranei NBRC 14843, *Streptomyces ardus* NBRC 13490, *Streptomyces niveus* NBRC 12917, *Saccharopolyspora erythraea* NBRC 13426, *Streptomyces venezuelae* NBRC 13096, and *Streptomyces hawaiiensis* NRRL 15010 were precultured on agar. Agar plugs of well-grown cultures were sampled and embedded in fresh agar containing the different bioreporter strains. The induction status of the tested reporter strain is indicated by + (inducing) and – (non-inducing), with the pictures showing the respective induction zone arising around the actinomycetes agar plug. The names of the antibiotics known to be produced by the respective antibiotic producer strain are indicated in the corresponding panel. Porfiromycin is a mitomycin C derivative.

Each bioreporter specifically signaled stress inflicted onto a particular metabolic pathway or cellular structure, without elucidating the exact molecular target of the respective antibiotics. The DNA stress reporter (P_{yorB}-*lacZ*), as an example, showed induction after treatment with trimethoprim (a folate synthesis inhibitor limiting the thymidine precursor supply) (Gleckman et al., 1981), ciprofloxacin (a DNA gyrase inhibitor) (Lebel, 1988), or mitomycin C (a DNA intercalating and alkylating agent) (Verweij and Pinedo, 1990). For the detection of cell envelope stress, our two different biomarkers (*ypuA* and *lial*) allowed to more specifically classify the MOA of compounds acting in this pathway. While P_{ypuA}-*lacZ* was induced by all tested cell envelope-targeting compounds, except for the membrane-active ionophores (Table S1), an additional induction of P_{lial}-*lacZ* conveyed the information that those substances rather target membrane-associated steps of the lipid-II cycle (e.g., nisin or teicoplanin). Interestingly, all quinolones, mitomycin C, and phleomycin, which interfere with DNA synthesis, showed an additional weak induction of the cell envelope stress promoter P_{ypuA} besides their prominent induction of the DNA damage-sensing promoter P_{yorB}. This observation had not been made by Urban and colleagues in their screening in liquid media, and could be due to our agar-based setup (Urban et al., 2007). The overnight incubation might reveal secondary antibiotic effects that had not manifested in the liquid assay, e.g., due to shorter incubation times. One potential explanation for the induction of P_{ypuA} by this group of agents could be oxidative stress and the corresponding impairment of membrane function (Goswami et al., 2006; Dwyer et al., 2007; Hong et al., 2019). None of the five reporter strains showed an induction upon treatment with ionophores (e.g., gramicidin A), fatty acid synthesis inhibitors (e.g., cerulenin), protein synthesis inhibitors that act by miscoding (e.g., gentamicin), by inhibition of aminoacyl-tRNA synthesis (e.g., mupirocin), or with agents causing protein stress (e.g., diamide), which is in accordance with the anticipated reporter coverage (Table S1). Of note, the aminocyclitol antibiotics spectinomycin and hygromycin B, often described as unusual aminoglycosides, rather cause blocking of translation than miscoding (Borovinskaya et al., 2007, 2008) and accordingly led to an induction of the translation arrest reporter (P_{bmrC}-*lacZ*).

Validation of the agar-based bioreporter assay as a MOA profiling tool for direct screening of antibiotic producer strains

Having positively evaluated the sensitivity and specificity of the agar-based bioreporter system with known reference compounds, we set out to directly test antibiotic producer strains, without previous extract generation or further purification of metabolites. We cultivated six actinomycetes strains, known to produce specific, well-characterized antibiotics, on solid media for several days, mostly until sporulation became visible. Agar plugs of the grown producer strains were directly tested against our bioreporter panel. All strains showed antibacterial activity combined with the expected promoter induction reflecting the MOA of the produced antibiotic (Figure 2). For example, *Streptomyces niveus* NBRC 12917 is a known producer of novobiocin, which inhibits DNA gyrase (Sugino et al., 1978). When we tested an agar plug of the grown *S. niveus* strain against the reporter panel, we detected a selective induction of P_{yorB}-*lacZ*, indicative of interference with DNA synthesis (Figure 2). In accordance, the pure compound novobiocin showed the same induction pattern on the reporter panel (Table S1). As expected, the acyldepsipeptide (ADEP)-producing strain *Streptomyces hawaiiensis* NRRL 15010 showed no induction, as the ClpP protease activator ADEP causes cellular protein stress (Brötz-Oesterhelt et al., 2005; Sass et al., 2011), which is not covered by our reporter panel.

Direct combined bioactivity and MOA screening of uncharacterized antibiotic producer strains

We proceeded to directly screen uncharacterized actinomycetes strains, without subjecting them to any kind of extraction, enrichment, or fractionation procedure. A schematic overview of the direct agar-based bioreporter screening process is depicted in Figure 3. In total, we included ~500 actinomycetes strains from the Tübingen strain collection in the bioreporter screening. Of note, 290 of these strains had previously shown bioactivity against *Staphylococcus aureus*. All actinomycetes were grown on at least two different solid media and were first tested for growth inhibition of the *B. subtilis* 1S34 wild type in the agar-based setup. In addition, a subset of approximately 280 producer strains was tested against *E. coli* ATCC25922 to check

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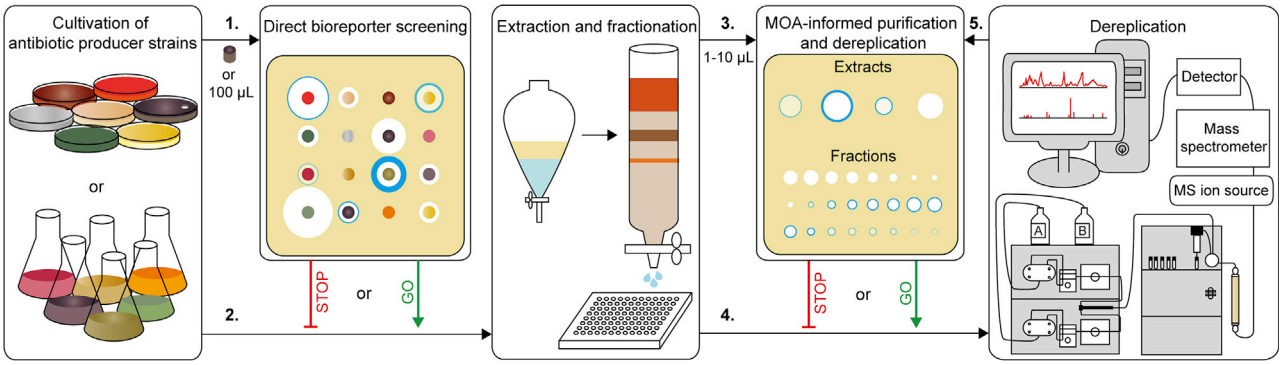

Figure 3. Schematic overview of the dereplication process using the agar-based bioreporter tool

(1) Well-grown samples of the antibiotic producer strains (agar plugs and/or supernatants) obtained under different growth conditions were directly tested against the reporter panel. (2) Samples with interesting bioactivities and reporter signals (blue halo) were selected for further investigation. Therefore, supernatants and/or agar plates were extracted and fractionated. (3) During this purification process, the previously shown activity and reporter signals were monitored in all samples. (4) This MOA-informed purification procedure allowed to selectively follow-up activities of interest, which could then be analyzed via high-resolution mass spectrometry. (5) Dereplicated, pure substances were again checked for the expected activity and reporter profile. The bioreporter setup allows for an evaluation at different steps (i.e., 2. and 4.) within the cultivation, purification, and dereplication processes, thereby enabling to specifically follow-up antibacterial activities of interest (indicated by "GO") or to deprioritize or set aside samples with less-interesting profiles (indicated by "STOP").

for anti-Gram-negative activity (Data S1). In this pilot study, none of the strains exhibited *E. coli*-only activity, which would have putatively hinted at a specific outer membrane target. All producer strains active against *E. coli* ATCC25922 also showed activity against *B. subtilis* 1S34, implicating conserved targets and demonstrating the capacity of the chosen bioreporter strain background for sensitive detection of a broad range of agents acting on a broad range of targets. Of course, antibacterial compounds selectively targeting the outer membrane without affecting the cytoplasmic membrane would be missed by our current bioreporter panel and would require the addition of an outer membrane stress bioreporter in a Gram-negative background. A total of 230 of the 500 tested producer strains showed no antibacterial activity against *B. subtilis* 1S34 and were excluded, while the remaining bioactive actinomycetes strains (~270) were tested against the full reporter panel. Figure S3 shows an exemplary screening plate containing the P_{yorB}-*lacZ* bioreporter. For some actinomycetes strains, we detected a blue coloration of the agar test plug. Generally, such agar plug coloration occurred with all five tested bioreporters and was considered unspecific, as it is most likely due to an inherent β-galactosidase production of the antibiotic producer strain. Promoter induction was only considered certain if a blue halo occurred at the borders of a defined ZOI. As 94 of the bioactive producer strains showed a reliable induction of at least one biomarker, we achieved a coverage of ~36%. An induction of more than one promoter (with the exception of the commonly observed P_{ypuA}-*lacZ* and P_{liaI}-*lacZ* combination) may either hint at the production of a single substance with a dual MOA or causing secondary antibiotic effects, or the production of multiple bioactive agents. Subsequent to the initial screening, we started to follow-up on several of the producer strains that had led to an induction of the bioreporter strains. Extracts were generated from the same agar plate or liquid culture previously used for the bioreporter assay, fractionated, and again checked for the previously detected reporter signal before analyzing them via high-performance liquid chromatography-mass spectrometry (HPLC-MS) and/or HPLC-tandem MS. The bioreporter signals not only allowed to monitor the purification procedure, but facilitated compound deconvolution by adding an additional MOA-informed dimension to the dereplication process. If a certain mass found in an extract suggested a known compound and the bioreporter signal matched the expected MOA, two independent technologies immediately confirmed each other in the occurrence of a particular agent.

For assay validation, we also considered certain compounds present in growth media of the producer strains (e.g., isoflavones, such as genistein and daidzein) or common metabolites from actinomycetes (e.g., N-acetyltyramine or aromatic compounds, such as indol-3-acetic acid and indole-3-propionic acid), which are often co-extracted and detected by HPLC-MS. To exclude unspecific induction signals, resulting from solvents or commonly purified media components or metabolites, we tested a subset of these substances on the bioreporter panel (Table S2). While some of these agents showed very weak antibacterial activity resulting in a turbid ZOI, none of them led to an induction of one of our five bioreporter strains.

In total, 28 compounds were dereplicated from different strains of the Tübingen strain collection, all of them matching the MOA proposed by the respective promoter induction (Table 1). Additional information on the antibiotic structures, detection methods, mass spectra, and a strain-specific assignment of the dereplicated substances can be found in Table S3 and Data S2.

One example for a correct MOA prediction was the detection of berninamycin C in the P_{bmrC}-inducing strain Tü2108 (Table 1). Berninamycin, a cyclic thiopeptide antibiotic, is known to inhibit protein synthesis by binding to the 50S subunit of the ribosome, thereby hindering the incorporation of amino acids (Reusser, 1969). The resulting stress response due to ribosomal stalling elicited the reporter signal indicating translation arrest. Strain Tü104 showed induction of both cell envelope stress promoters, P_{ypuA} and P_{liaI}. The combined induction suggested interference of a produced metabolite with biosynthesis, function, or integrity

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Table 1. Dereplicated antibiotics identified by direct bioreporter screening of antibiotic producers from the Tübingen strain collection

Reporter induction	Dereplicated antibiotics[a] (producer strain)
DNA stress (P_{yorB}-*lacZ*)	chartreusin (GöK9/13; GöK16/4); chrysomycin A (Tü2471); C-1027 (Tü2401); daunomycin (TüG111); rachelmycin (GöOber505)
RNA stress (P_{yppS}-*lacZ*)	actinomycin D, C_2 (GöWind756); chartreusin (GöK9/13; GöK16/4); cosmomycin B (Tü40/15; Tü2626); cosmomycin C (GöK8/12)
Translation arrest (P_{bmrC}-*lacZ*)	amicetin (KNN49.3e); berninamycin (Tü2108); chartreusin (GöK16/4); cycloheximide (A4/2); griseoviridin, viridogrisein (Tü3180); pactamycin (Tü6430); pristinamycin I, II (Tü2975)
Cell envelope and lipid-II cycle stress (P_{ypuA}-*lacZ* and P_{liaI}-*lacZ*)	aborycin (TüG349); FD-594 (Tü104); pentamycin (GöK12/7); telomycin (Tü2641)
No induction signal	albomycin δ_1 (Tü2401); boromycin (GöK12/9); griseorhodin A (TüG117); lobophorin A, E (GöSöt11); monactin (TüG102); omomycin (TüG343)

[a]The signals of the bioreporters concurred with the described MOA of the dereplicated antibiotics in all cases.

of the cell envelope, especially hinting at interference with the bacterial lipid-II cycle, also seen for antibiotics, such as teicoplanin (Table S1). HPLC-MS analysis revealed the production of antibiotic FD-594 (Data S2), belonging to the aromatic polyketides (Kudo et al., 2011). FD-594 is not well characterized but shares a related structural backbone with lysolipin I. It was proposed that lysolipin I would inhibit peptidoglycan synthesis by targeting bactoprenol-containing molecules (Drautz et al., 1975). The structural similarity and the shared reporter induction pattern (Table S1) hint at a similar MOA of antibiotic FD-594 and lysolipin I, a hypothesis that can now be tested by focused follow-up studies.

We were also interested to see what kinds of substances produced by the actinomycetes strains are not covered by our reporter panel. Therefore, we followed up some of the producer strains that showed antibacterial activity without inducing any of the five bioreporters. For these strains, seven different antimicrobial compounds could be identified. Among those were ionophores (monactin, boromycin, and omomycin), a siderophoric tRNA synthesis inhibitor (albomycin δ_1), and substances with unknown or poorly characterized MOA and target structures (lobophorins A and E, griseorhodin A) (Table 1). Mechanism-wise, these non-inducing compounds are in accordance with our expected coverage of the reporter panel that we had evaluated on the basis of 90 reference antibiotics with known MOA (Table S1). We further suspect some of the ZOI without reporter signal to be caused by aminoglycosides, which are known to be frequently produced by actinomycetes (Cox et al., 2017; Culp et al., 2019). Aminoglycosides would most likely be missed by our current screening strategy for two reasons, the *bmrC* bioreporter detects ribosome stalling but not miscoding and our applied purification procedure favors the extraction of hydrophobic substances. The expansion of the bioreporter panel is subject of current work. A bioreporter detecting interference with fatty acid synthesis as well as a bioreporter sensing protein stress, e.g., caused by the accumulation of mistranslated proteins (e.g., aminoglycosides), peptide fragments (e.g., ADEP), or prematurely terminated proteins (e.g., puromycin), are presently being developed. In addition, a Gram-negative bioreporter specifically responding to outer membrane stress is under investigation.

Exploiting the full potential of antibiotic producer strains

The advantages of our direct reporter screening approach are the large variety of compatible samples (agar plugs, supernatants, extracts, fractions, pure compounds) and the immediate detection of antibiotic activity combined with MOA information. This makes our technology a particularly useful tool to investigate changing antibiotic production patterns. When following up the hit strain Tü2401 that yielded an induction of the DNA stress reporter in the direct agar-based screening, we noted that we lost promoter induction, but not bioactivity, when the producer strain was grown in liquid media (Figure 4A). The ZOI were not distinguishable without the additional information of the divergent promoter signal. Substance extractions from agar and liquid media confirmed the cultivation-dependent production of two different natural products: albomycin δ_1 and C-1027 (lidamycin) (Figure 4B). When grown on agar, the strain produced C-1027 (Data S2), a very potent DNA intercalator that is able to introduce single- and double-strand breaks (Shao and Zhen, 2008), and our DNA stress reporter P_{yorB}-*lacZ* reacted accordingly (Figure 4A). In liquid culture, Tü2401 produced albomycin δ_1 (Data S2), a siderophore, which exerts intracellular activity against the seryl-tRNA synthetase (Zeng et al., 2012). Antibiotics interfering with tRNA synthesis are not covered by our reporter panel (compare mupirocin in Table S1), which concurred with the lack of promoter induction. This example highlights the potential of the direct MOA-informed bioreporter screening to quickly dissect divergent production of secondary metabolites that may occur under different growth conditions in a single producer strain.

The reporter strains were also useful to monitor the different steps of antibiotic purification. In cases where multiple antibacterial agents are produced, the additional information of the specific reporter signal helps to distinguish between these activities already at an early stage in the purification process. To demonstrate the potential to distinguish different substances hidden behind a single ZOI, we simulated the production of multiple bioactive metabolites by applying several combinations of two antibiotics, targeting different metabolic pathways, together in the same spot. Reporter induction sensitively detected the MOA of all agents in the different combinations (Figure S4). However, it should be kept in mind that the detection of reporter signals from both antibiotics is dependent on their concentration in the mixture and their diffusion coefficient in the agar. In particular, if one of the agents is quickly diffusing, very potent, or rapidly bactericidal, it might extinguish the signal of another

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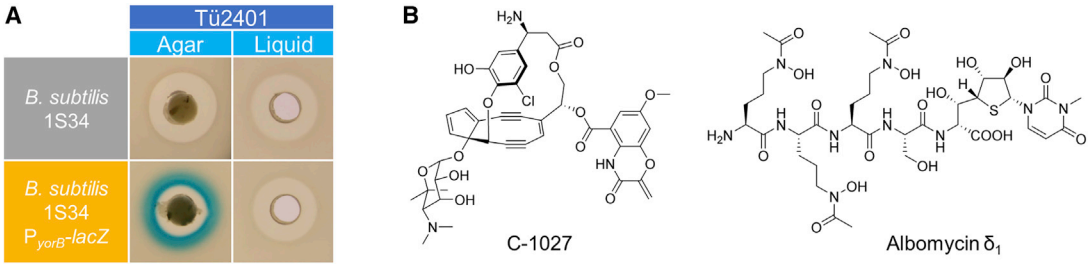

Figure 4. Rapid discrimination of distinct antibacterial metabolites synthesized by Tü2401 under different growth conditions

(A) Agar-based reporter screening of Tü2401 precultured either on OM agar or in OM liquid medium. An agar plug (left) or 100 μL of the liquid culture supernatant (right) were tested against *B. subtilis* 1S34 as well as our DNA stress reporter *B. subtilis* 1S34 P$_{yorB}$-*lacZ*. Both samples showed antibacterial activity against *B. subtilis* 1S34. Induction of the DNA stress reporter was only detected for the Tü2401 agar sample, allowing an immediate discrimination, and hinting at the production of two different substances.

(B) Structure of the isolated substances C-1027 (agar) and albomycin δ_1 (liquid).

less potent or less diffusible agent. Nonetheless, our reporter setup enables a bioactivity- and MOA-informed fractionation process, which will immediately solve the issues projected for simultaneous probing of two agents within the same ZOI. In an exemplary extract fractionation from Tü104, different active fractions separately eluted from the HPLC column could clearly be distinguished due to their divergent reporter signal (Figure S5). In combination with HPLC-MS data, the bioactivity- and MOA-based fractionation provides a valuable tool for selective compound identification and purification. The entire screening process is rapid and efficient and agar extraction has already worked well in many cases, omitting the prevalently used liquid cultivation for compound identification.

Promoter induction patterns provide information about dual and/or secondary MOA

For strain GöK16/4, we observed the induction of three different promoters, which corresponded to DNA, RNA, and protein synthesis stress, respectively (Table 1). This result initially suggested the production of several antibiotic agents with different MOA. However, dereplication revealed only a single bioactive substance: the antitumor glycoside antibiotic chartreusin (Data S2). For the pure compound chartreusin, we obtained the same promoter induction pattern (Figure 5A), thereby disproving the idea of several antibiotics produced by strain GöK16/4. At lower chartreusin concentrations, the signal on our translation arrest reporter was not detectable in the agar-based reporter setup (Figure 5A). Chartreusin-mediated P$_{bmrC}$-*lacZ* induction mainly appeared in the inner radius of the ZOI, indicating that higher concentrations might be required to induce the protein synthesis stress. In comparison, the DNA or RNA synthesis stress signals, induced by the intercalating MOA of chartreusin (Portugal, 2003), could be detected at all concentrations (Figure 5A). This observation might also explain why we could only detect P$_{yorB}$-*lacZ* and P$_{yppS}$-*lacZ* signals for the less bioactive strain GöK9/13, which was also shown to produce chartreusin but in lower amounts (Table 1). Our triple promoter induction pattern of chartreusin is supported by an observation of Li et al. (1978), who detected strong effects of chartreusin on RNA and DNA synthesis in an incorporation assay with radioactive precursors. Protein synthesis was also affected; however, to a lesser extent and requiring a higher concentration of chartreusin. The weak induction of the P$_{bmrC}$ promoter upon chartreusin treatment in our study hinted at an additional bacterial target structure or a secondary metabolic effect, which led to translation arrest. The interference with protein synthesis had also been observed in a study using reticulocyte ribosomes. Here, it was shown that chartreusin is attached to ribosomes and inhibits the binding of aminoacyl tRNAs, thereby hindering polypeptide chain elongation (Gregg and Heintz, 1972). This finding was confirmed by demonstrating the binding of chartreusin to human tonsil ribosomes and its inhibition of peptide translocation from the A to the P site (Vazquez et al., 1974).

The detection of polypharmacology by our bioreporter system was also verified for the pure substances 5′-fluorouracil and SCH-79797. 5′-Fluorouracil induced both the DNA stress and translation arrest biomarkers (Table S1). The detection of both signals concurs with its known dual MOA. 5′-Fluorouracil inhibits thymidylate synthase, which restricts and unbalances pyrimidine *de novo* synthesis leading to DNA stress. In addition, metabolites of 5′-fluorouracil are incorporated into RNA and DNA (Cohen et al., 1958; Longley et al., 2003; Noordhuis et al., 2004). Incorporation into RNA was described to disturb mRNA processing in eukaryotes and protein synthesis in general (Horowitz and Chargaff, 1959; Noordhuis et al., 2004; Will and Dolnick, 1989), the latter matching the observed ribosome stalling reporter signal. SCH-79797 is a recently reported antibacterial agent with broad-spectrum activity, very low induction of resistance, and a new, dual MOA (Martin et al., 2020). Motivated by the emerging pharmacological profile of the compound, the authors put substantial efforts into elucidating the molecular mechanism of the new compound and applied a range of different assays, including rather demanding methodology, such as cytological profiling, thermal proteome profiling, and metabolomics, to narrow down on the target (Martin et al., 2020). As a result, they found that SCH-79797 inhibits dihydrofolate reductase and disturbs membrane integrity. Interested in finding out what our easy-to-handle bioreporter strains would reveal about the compound, we also tested SCH-79797 and could immediately detect an interference with both cell envelope reporters, as well as the DNA stress reporter (Figure 5B). The combined induction of P$_{ypuA}$-*lacZ* and P$_{liaI}$-*lacZ* hinted at a membrane target (lipid-II cycle), while a P$_{yorB}$-*lacZ* signal indicated the induction of the SOS response due to DNA stress, which is induced e.g., by gyrase

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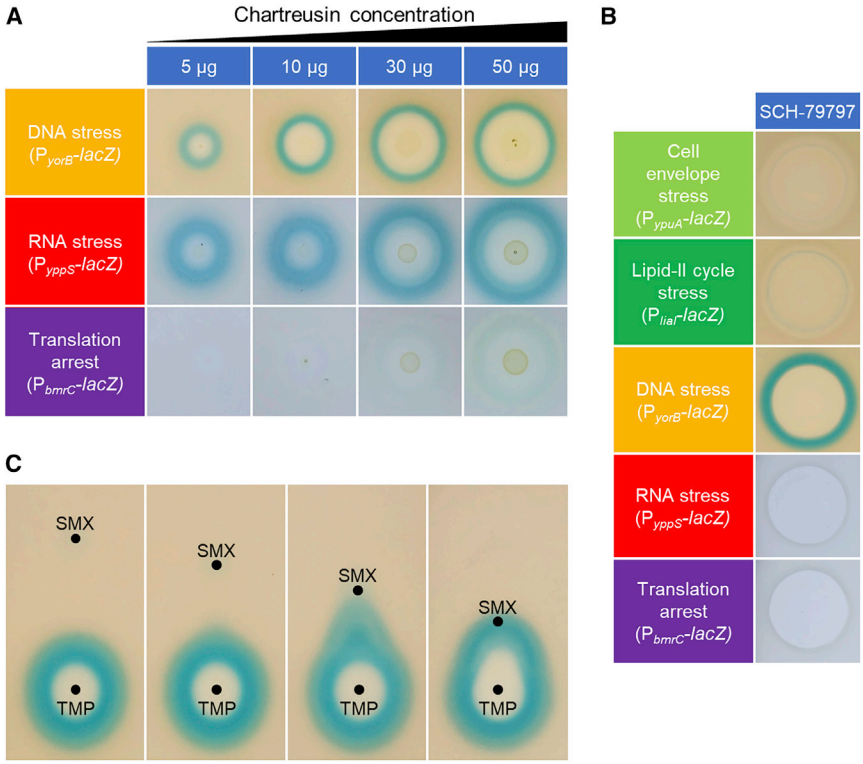

Figure 5. Reporter-dependent visualization of antibiotic polypharmacology and synergism

(A) Induction of the DNA, RNA, and protein synthesis stress reporters after treatment with a concentration range of chartreusin (5, 10, 30, or 50 µg). All tested concentrations induced the DNA and RNA stress reporters, while induction of P_{bmrC}-*lacZ* indicative of translation arrest was only detected at increased chartreusin concentrations.

(B) Diverse induction pattern after treatment with 40 µg SCH-79797. A strong induction for P_{yorB}-*lacZ* could be detected. SCH-79797 also showed weak signals for both cell envelope stress bioreporters P_{ypuA}-*lacZ* and P_{liaI}-*lacZ*.

(C) The synergistic effect of sulfamethoxazole (SMX) and trimethoprim (TMP) visualized by reporter induction. SMX at an amount of 10 µg showed no antibiotic activity. Trimethoprim at 1 µg showed a large ZOI and an induction of P_{yorB}-*lacZ*. After reducing the spotting distance, the synergistic effect of both substances was detectable by an additional induction zone between both antibiotic spots.

inhibitors, folate synthesis inhibitors, or some intercalators (Table S1). The latter example demonstrates that the reporter panel is well suited for rapid MOA profiling of new bioactive agents.

Detection of synergistic effects with the bioreporter tool

Having evaluated this reliable screening approach for sensitive MOA verification, we were interested to see if we could also detect synergistic antibiotic effects. The combination treatment and the synergism of trimethoprim and sulfamethoxazole, two folate synthesis inhibitors, is well established in antibiotic therapy (Minato et al., 2018). We spotted the two substances on agar, containing the DNA stress reporter, and gradually reduced the spotting distance. Sulfamethoxazole was used in a concentration showing no activity against *B. subtilis* 1S34 and neither did it elicit a reporter signal. As soon as the two compounds came into close vicinity, the induction signal of P_{yorB}-*lacZ* became visible where both antibiotic diffusions zones overlapped, clearly visualizing their synergistic effect on DNA synthesis (Figure 5C). This particular application could be of special interest not only for purified compounds, but also in co-cultivation studies with diverse producer species grown in close proximity.

Conclusions and perspectives

In summary, we developed and extensively evaluated a panel of bacterial bioreporters suitable for agar-based screening that respond specifically to distinct modes of antibiotic action, thereby signaling DNA stress, RNA stress, translation arrest, and interference with cell envelope integrity. Assay conditions were probed and optimized to enable reliable and sensitive signal detection after overnight incubation with the antibiotic. The assay proved to be fast, suitable for substantial throughput, and robustly worked with any material along the purification pipeline, from unprocessed producer strains, extracts, and partially purified fractions to pure compounds. To reliably validate the approach, in this study we focused on actinomycetes producer strains from the Tübingen strain collection grown under standard laboratory growth conditions. Knowing this to be a rather richly mined source and expecting to encounter many known compounds, we wanted to evaluate if the bioreporters manage to reliably signal the expected MOA of a broad range of diverse agents during the production and purification process, which indeed they did. In addition, they led us to a range of compounds that had not been isolated from the Tübingen strain collection before. However, importantly, our assay is not limited to this bacterial order of antibiotic producers, and there is also no preference for certain groups of bacteria.

The current proof-of-concept study aimed at demonstrating the broad applicability of the bioreporter-based technology. By using the different bioreporters in parallel, their MOA profiling capacity can be harnessed best. Screening campaigns benefit from the additional MOA information that adds a further dimension to the dereplication process. Known compounds with known MOA can be rapidly deconvoluted by correlating the HPLC-MS data with the bioreporter signal(s). Agar extraction of the single Petri dish of the cultivated producer strain, previously used for the bioreporter screening, is more than sufficient for dereplication of most bioactive agents. With an aim to expand the MOA coverage, to ideally assign a target area to most natural products tested, we are working toward the detection of further antibacterial mechanisms. Besides such global profiling, our bioreporter method offers potential as a direct selection tool in a screening process. For instance, if a certain target area is of particular interest, only a single bioreporter from the current set can be utilized.

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Notably, the potential of the technology extends beyond screening and can be applied to a variety of further research questions, such as unraveling challenging compound mixtures, detecting synergistic activities, or exploring multiple mechanisms inherent in a single compound. The bioreporter assay is also ideally suited as a rapid entry assay to guide the selection of appropriate follow-up experiments for elucidating the molecular target.

SIGNIFICANCE

With the dramatic increase in infections caused by antibiotic-resistant bacteria, there is an urgent need for new antibacterial agents to replenish dwindling antibiotic development pipelines. Although microbial secondary metabolites are acknowledged as the most promising source of antibiotics and efforts are directed toward finding new ones, rediscovery of known compounds binds valuable capacities. An efficient, sensitive, and rapid deconvolution strategy is mandatory to identify already known compounds quickly, reliably, and without the need for laborious compound purification. High-resolution mass spectrometry has made tremendous progress and serves as the mainstay of current dereplication procedures, while at the biological end of the natural product discovery process, classical agar-based screening for bacterial growth inhibition is still the most widely used procedure, not least due to its ease of handling. Our agar-based bioreporter toolbox links such classical activity-based screening with the simultaneous acquisition of reporter-based MOA information. When combined with high-resolution mass spectrometry, this MOA-informed bioactivity screening represents a powerful dereplication tool. Without much additional effort in time and resources, the information content generated in the screening process is greatly enhanced. A particular strength of the bioreporter assay is its ease of handling, speed, low cost, and the fact that it can be performed with basic microbiological equipment, which in principle allows its application in any natural product isolating laboratory worldwide. Another great strength is its versatility. Biosynthesis-related applications include, but are not limited to, the assessment of the biosynthetic potential of unprocessed producer strains, tracking of substances throughout the purification process, detection of bioactive compounds in mixtures, and evaluation of the synergistic potential. Mechanism-wise, MOA hypotheses emerge already during the initial screening process, and polypharmacology is detected. Any producer of secondary metabolites is suitable for testing, which makes the setup highly instrumental in the early steps of antibiotic discovery.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chembiol.2021.02.022.

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AUTHOR CONTRIBUTIONS

H.B.-O. and A.B. devised the study concept. K.W.W., A.B., H.B.-O., J.S.S., S.G., F.H., Y.M., N.O., and T.H.J.N. designed experiments. K.W.W. designed and generated the bioreporter panel, established the assay conditions, and conducted all reporter screening experiments. J.S.S., F.H., N.O., and V.M. cultivated the uncharacterized producer strains. J.S.S., F.H., N.O., V.M., and A.K. purified and dereplicated the antimicrobial compounds. A.B., H.B.-O., S.G., Y.M., and T.H.J.N. supervised experiments. All authors discussed and analyzed data. K.W.W., A.B., and H.B.-O. wrote the paper. H.B.-O., S.G., Y.M., and T.H.J.N. acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Bacillus subtilis 1S34	(Piggot, 1973)	N/A
B. subtilis 1S34 *amyE*::pHJS105-P_{yorB}-*lacZ*	This study	N/A
B. subtilis 1S34 *amyE*::pHJS105-P_{ypuA}-*lacZ*	This study	N/A
B. subtilis 1S34 *amyE*::pHJS105-P_{lial}-*lacZ*	This study	N/A
B. subtilis 1S34 *amyE*::pHJS105-P_{bmrC}-*lacZ*	This study	N/A
B. subtilis 1S34 *amyE*::pHJS105-P_{yppS}-*lacZ*	This study	N/A
B. subtilis 1S34 *amyE*::pHJS105-P_{helD}-*lacZ*	This study	N/A
Escherichia coli XL1 Blue	Agilent	Cat# 200249
Amycolatopsis mediterranei NBRC 14843	National Institute of Technology and Evaluation (NITE) Biological Resource Centre (NBRC)	Cat# 14843
Streptomyces ardus NBRC 13490	NBRC	Cat# 13490
Streptomyces niveus NBRC 12917	NBRC	Cat# 12917
Saccharopolyspora erythraea NBRC 13426	NBRC	Cat# 13426
Streptomyces venezuelae NBRC 13096	NBRC	Cat# 13096
Streptomyces hawaiiensis NRRL 15010	Agricultural Research Service (ARS) Culture Collection	Cat# 15010
Biological samples		
~500 cultivated actinomycetes producer strains	Tübingen strain collection	N/A
Chemicals, peptides, and recombinant proteins		
5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal)	Thermo Scientific	Cat# R0402
Vancomycin antibiotic discs (30 μg)	Thermo Scientific	Cat# CT0058B
Teicoplanin antibiotic discs (30 μg)	Thermo Scientific	Cat# CT0647B
Ciprofloxacin antibiotic discs (5 μg)	Thermo Scientific	Cat# CT0425B
Rifampin antibiotic discs (30 μg)	Thermo Scientific	Cat# CT0104B
Chloramphenicol antibiotic discs (10 μg)	Thermo Scientific	Cat# CT0012B
Oligonucleotides		
Primers used in this study are listed in Table S4.	Integrated DNA Technologies (IDT)	N/A
Recombinant DNA		
pHJS105	Leendert Hamoen, University of Amsterdam	N/A
Software and algorithms		
Bruker Daltonics DataAnalysis 4.2	Bruker Daltonics	N/A

RESOURCE AVAILABILITY

Lead contact

Heike Brötz-Oesterhelt (heike.broetz-oesterhelt@uni-tuebingen.de), Department of Microbial Bioactive Compounds; Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen; Tübingen, Baden-Württemberg, 72076; Germany.

Materials availability

Requests for plasmids and reagents generated in this study may be sent to the lead contact.

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Data and code availability

This study did not generate datasets to be deposited.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains

All reporter strains were developed on the basis of the sporulation deficient *B. subtilis* 1S34 strain (Piggot, 1973). The integrative plasmid pHJS105 was used for the transformation of the respective reporter construct. pHJS105 integrates into the *amyE* locus and contains the antibiotic resistance marker spectinomycin (SPT) (Jahn et al., 2015). All reporter strains were grown in lysogeny broth (LB) (1% tryptone, 1% NaCl, 0.5% yeast extract, pH7.2) containing the antibiotic selection marker SPT at a final concentration of 100 μg/mL. For sub-cloning, the *E. coli* strain XL1 Blue was used for the construction and propagation of all plasmids.

METHOD DETAILS

Recombinant plasmid construction

The plasmid pHJS105 contains a xylose-inducible promoter fused to an N-terminal *gfp* tag, which was replaced by the respective promoter reporter construct. To modify the vector, we used a standard restriction-ligation protocol. The restriction sites *Sph*I and *Kpn*I were used to exchange the xylose-inducible promoter for the promoter regions of the chosen biomarker genes (P_{ypuA}, P_{lial}, P_{yorB}, P_{yppS}, P_{bmrC}, and P_{helD}), which were amplified via PCR (phusion high-fidelity polymerase (NEB)) from *B. subtilis* 1S34 genomic DNA, gel-purified and then ligated using T4 DNA ligase (NEB). In a second step, the *gfp* reporter was replaced for a *B. subtilis*-optimized *lacZ* gene amplified from pMutin4 (Vagner et al., 1998), using the restriction sites *Kpn*I and *Hin*dIII. Primers used in this work are listed in Table S4. After transformation of chemically competent *E. coli* XL1 Blue, transformants were selected on LB agar containing 100 μg/mL ampicillin overnight. Single colonies were inoculated in 5 mL LB (37°C, 190 rpm). Plasmids were isolated (GeneJET Plasmid Miniprep Kit (Thermo Scientific)) and verified by Sanger sequencing (LGC genomics GmbH). The plasmids were transferred into competent *B. subtilis* 1S34 cells and selected on LB agar, containing 100 μg/mL of SPT. Integration into the *amyE* locus was confirmed by streaking single colonies on starch containing agar (0.3% meat extract, 1% starch, 1.2% agar). Successful transformants were unable to metabolize the starch, which was detectable after iodine staining (3.3% iodine, 6.7% potassium iodide). For confirmation of sequence integrity, the integrated sequence was amplified via PCR (DreamTaq polymerase (Thermo Scientific)). Purified PCR products (Monarch PCR & DNA Cleanup Kit (NEB)) were then confirmed by Sanger sequencing.

Agar-based bioactivity screening

Before the bioreporter assay was conducted, antibiotic producer strains were pre-tested for antimicrobial activity against the *B. subtilis* 1S34 wildtype. A subset of producer strains was additionally tested for activity against *E. coli* ATCC25922. An overnight culture of the respective strain was diluted to an OD_{600} of 0.05 and re-grown to an OD_{600} of 1 (37°C, 190 rpm). *B. subtilis* 1S34 cells were concentrated 1:10 (4700 rpm, 4°C) and used to inoculate the LB soft agar with an adjusted cell number of 3×10^7 colony forming units per mL (CFU/mL). For *E. coli* ATCC25922, we adjusted the cell number to 1.5×10^6 CFU/mL to inoculate the LB soft agar. Agar plugs of the antibiotic producer strains were arranged in square Petri dishes, and the soft agar containing either *B. subtilis* 1S34 or *E. coli* ATCC25922 was poured around. Agar plates were left to solidify for 15 min and subsequently incubated overnight (37°C).

Agar-based bioreporter screening

For reporter testing, overnight cultures were inoculated from glycerol stocks in LB, containing 100 μg/mL of SPT. Overnight cultures of the bioreporter strains were diluted to an OD_{600} of 0.05 and incubated for ~3.5 h (37°C, 190 rpm) until they reached an OD_{600} of 1. Cells were concentrated 1:10 (4700 rpm, 4°C) and used to inoculate the soft agar. For testing the P_{ypuA}-*lacZ*, P_{lial}-*lacZ*, P_{yorB}-*lacZ*, and P_{helD}-*lacZ* reporter strains LB soft agar was used (1% tryptone, 1% NaCl, 0.5% yeast extract, and 0.75% agar). The P_{yppS}-*lacZ* and P_{bmrC}-*lacZ* reporter strains were tested in Belitzky minimal soft agar (Stülke et al., 1993). Cell numbers were adjusted to 3×10^7 CFU/mL for the P_{lial}-*lacZ*, P_{yorB}-*lacZ*, P_{yppS}-*lacZ*, P_{helD}-*lacZ*, and P_{bmrC}-*lacZ* reporter strains and 6×10^7 CFU/mL for the P_{ypuA}-*lacZ* reporter strain. The agar was supplemented with 5 mM $MgCl_2$ and 150 μg/mL X-Gal (Thermo Scientific). As positive controls antibiotic discs of 30 μg vancomycin (P_{ypuA}-*lacZ*), 30 μg teicoplanin (P_{lial}-*lacZ*), 5 μg ciprofloxacin (P_{yorB}-*lacZ*), 30 μg rifampin (P_{yppS}-*lacZ*), and 10 μg chloramphenicol (P_{bmrC}-*lacZ*) were used for reliable induction (see key resources table). For the screening of pure substances, extracts, and supernatants, the agar containing the reporter strain was poured in square Petri dishes and let to solidify for approximately 15 min. Subsequently, the respective amount of 1–5 μL test substance was spotted on the agar, and the plates were incubated overnight at 30°C for LB and 37°C for Belitzky minimal soft agar, respectively. For testing larger volumes, e.g. low-concentrated supernatants or weakly active substances, the samples were filled into holes, which were punched into the dried agar plates with a cork borer. Direct screening of actinomycetes strains was performed by transferring pre-stamped agar plugs of the grown antibiotic producer strains to a square Petri dish, before embedding them in soft agar containing the respective reporter strains. Control experiments, with different solvents used in the extraction and purification process, as well as agar plugs, and extracts of cultivation media (without bacterial growth), were always performed in parallel to exclude unspecific inhibitory effects or induction signals.

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Dereplication procedures

a) Agar cultivation conditions and compound extraction procedure (strains GöK8/12, GöK9/13, GöK12/7, GöK12/9, GöK16/4, GöOber505, GöSöt11, GöWind756, Tü40/15, TüG102, TüG111, TüG117, TüG343, TüG349). Agar plates were inoculated with 100 μL of cryo stock. Cryo stocks were prepared from a three-day culture by 1:1 (v/v) dilution with glycerol/water (1:1, v/v) and stored at −80°C. Actinomycetes strains were cultivated for 7–10 days at 28°C on the respective agar plates (Table S5).

Grown agar plate(s) were cut in ~5 × 5 mm pieces and transferred to a 50 mL falcon tube. The content was poured over by a mixture of isopropyl acetate/chloroform/isopropyl alcohol (3:1:1 v/v/v, 40 mL) and extracted two times for 1.5 h on an overhead shaker. Residual solids were filtered off, and the combined organic extracts were dried *in vacuo* to complete dryness. The extracts were stored at −20°C.

Pre-fractionation of the extract by normal-phase solid-phase extraction (NP-SPE) (strains GöK8/12, GöK9/13, GöK12/7, GöK12/9, GöK16/4, GöOber505, GöSöt11, GöWind756, Tü40/15, Tü104, Tü2626, Tü2641, TüG102, TüG111, TüG117, TüG343, TüG349). Bioactive extracts were fractionated at room temperature (25°C) by normal-phase SPE (Macherey-Nagel Chromabond® SiOH, 200 mg, 3 mL). The SPE cartridge was conditioned with 4 mL dichloromethane (DCM), DCM/n-hexane (1:1, v/v, 4 mL), n-hexane (4 mL). The extract (20 mg/mL in methanol, 50 μL) was loaded onto the silica bed and dried extensively by pressurized air to remove residual methanol. A stepwise gradient was used to elute the NP-SPE cartridge into a 96-well microtiter plate (PP): n-hexane (100%, 2 mL), n-hexane/EtOAc/MeOH (8/1.8/0.2, v/v/v, 2 mL), n-hexane/EtOAc/MeOH (7.25/2.35/0.4, v/v/v, 2 mL), n-hexane/EtOAc/MeOH (6.5/2.9/0.6, v/v/v, 2 mL), n-hexane/EtOAc/MeOH (5.75/3.45/0.8, v/v/v, 2 mL), n-hexane/EtOAc/MeOH (5/4/1, v/v/v, 2 mL), n-hexane/EtOAc/MeOH (4.25/4.55/1.2, v/v/v, 2 mL), n-hexane/EtOAc/MeOH (3.5/5.1/1.4, v/v/v, 2 mL), MeOH (100%, 2 mL). The volume of the eluent was set to 200 μL/well. After complete evaporation of the solvents using pressurized air, the fractions were re-suspended in methanol (100 μL). Daughter plates for HPLC-UV-HR-MS analysis were prepared by transferring an aliquot (20 μL) from each well of the master plate. The master plate was used for antimicrobial biological testing after evaporation, whereas the daughter plate was stored at −20°C for HPLC-UV-HR-MS analysis of the bioactive wells.

HPLC-UV-HR-MS setup (strains GöK8/12, GöK9/13, GöK12/7, GöK12/9, GöK16/4, GöOber505, GöSöt11, GöWind756, Tü40/15, Tü104, Tü2626, Tü2641, TüG102, TüG111, TüG117, TüG343, TüG349). 3 μL of the extract (3 mg/mL in methanol) and the pooled bioactive wells of the micro-fractionation daughter plate (no defined concentration) were subjected to HPLC-UV-HR-MS analysis performed on a Bruker MaXis 4G ESI-QTOF mass spectrometer equipped with a Dionex Ultimate 3000 HPLC system (Thermo Scientific). HPLC instrumental setup: Column: Macherey-Nagel Nucleoshell EC RP-C18 (150/2 RP18, 2.7 μm), flow rate: 0.3 mL min^{-1}, eluting solvents: Methanol (system B, containing 0.06% formic acid) and H_2O (system A, containing 0.1% formic acid), gradient: 0 min (10% B), 20 min (100% B), 25 min (100% B), 26 min (10% B), 30 min (10% B). Mass spectrometer instrumental setup: Electrospray ionization mass spectra (positive and negative ions) were recorded in the range of 100–1250 Da. The elemental composition was derived from the averaged mass spectra with high mass accuracy below 3 ppm. Sodium formate was used as internal calibrant. Nebulizer pressure of the ESI source was set to 0.4 bar with a dry gas flow of 4.0 l min^{-1} and a dry gas temperature of 200°C. The endplate offset was 500 V and capillary voltage = 3,000 V. Bruker Daltonics DataAnalysis 4.2 was used as software for the analysis of the HPLC-UV-HR-MS data.

HPLC-UV-HR-MS dereplication strategy (strains GöK8/12, GöK9/13, GöK12/7, GöK12/9, GöK16/4, GöOber505, GöSöt11, GöWind756, Tü40/15, Tü104, Tü2626, Tü2641, TüG102, TüG111, TüG117, TüG343, TüG349). For the dereplication process, the UV-DAD spectra of the extracts were analyzed for relevant signals first. The UV_{max} of prominent UV-signals were extracted and a database search was performed (UV_{max} +/− 10 nm). The Dictionary of Natural Products served as underlying database (Taylor and Francis Group, 2019). Additionally, the search criteria were extended by the molecular masses as well as molecular formulas of relevant peaks. In this step, the high abundant masses in the peaks observed in the Base Peak Chromatogram (BPC, m/z +/− 1) as well as the adduct pattern was used to select masses for the database search. For the selected masses, the molecular formulas were estimated (+/− 3 ppm) and added to the search criteria. Finally, the MS/MS spectra of positive hits were matched with an *in-house* natural product database, Global Natural Products Social Molecular Networking (Wang et al., 2016) or spectra from the literature and medfrag (Ruttkies et al., 2016). The same procedure was carried out for the bioactive fractions of the micro-fractionation. A substance was classified as 'clearly identified', if especially the match with the in-house database confirmed even regioisomers or if all four criteria (UV_{max}, MS, molecular formula, MS/MS) were in agreement with the database entry.

b) Liquid cultivation conditions and compound extraction procedure (strains Tü104, Tü2626, Tü2641, Tü2108, Tü2975, Tü6430, Tü3180, A4/2, KNN49.3e). For antibiotic production, strains were cultivated in 50 mL preculture medium (R5 or NL410) for 3 days at 30°C in 500 mL Erlenmeyer flasks (with steel springs) on an orbital shaker (180 rpm) (Kieser and Foundation, 2000). 5 or 10 mL of preculture were inoculated in 50 or 100 mL of production medium (Table S5), respectively, and cultures were grown for 4–10 days at 30°C in 500 mL Erlenmeyer flasks (with steel springs) on an orbital shaker (180 rpm). 5 mL culture were extracted with 5 mL ethyl acetate or butanol for 30 min at room temperature. Ethyl acetate and butanol samples were dried *in vacuo,* and redissolved in 0.25 mL of methanol. The culture extracts were stored at 4°C or −20°C. Methanolic and butanolic extracts were used for bioassays and HPLC-MS analysis.

HPLC-MS analysis (strains Tü2108, Tü6430, Tü3180, Tü2975, A4/2, KNN49.3e-for strains Tü104, Tü2626, Tü2641 see section "a)"). HPLC analysis was performed as described previously with a HP1090M system with ChemStation 3D software rev. A.08.03 (Agilent Technologies, Waldbronn, Germany) on a Nucleosil C18 column (5 μm, 125 mm × 3 mm) fitted with a precolumn (20 × 3 mm) and with a flow rate of 850 mL min-1 (Krause et al., 2020). HPLC-MS was performed with an Agilent 1200 series chromatography system (binary pump, high performance autosampler, DAD-detector) coupled with an LC/MSD Ultra Trap System XCT 6330

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(Agilent Technologies, Waldbronn, Germany). The sample was injected on a Nucleosil 100 C18 column (3 μm, 100 × 2 mm) fitted with a precolumn (3 μm, 10 × 2 mm) at a flow rate of 400 μL/min and a linear gradient from 10% solvent A (0.1% formic acid in water) to 100% solvent B (0.06% formic acid in acetonitrile) over 15 min at 40°C.

c) Cultivation conditions and compound extraction procedure for Tü2401.

Liquid precultures of Tü2401 were inoculated either with spores from the respective agar plates, or with 50 μL of the respective suspension of cryo-preserved spores. Precultures were inoculated in NL410 medium and incubated for three days at 29°C (180 rpm). 500 mL of preculture were used to inoculate 9.5 L OM medium for fermentation. Liquid cultures were cultivated at 27°C in a 10 L bioreactor at a rotor speed of 200 rpm, and an airflow of 5 L/min. For the growth on solid medium, ISP2 agar plates were inoculated with 50 μL of the preculture and incubated at 29°C for four days until sporulation occurred. The respective media used for cultivation are noted in Table S5.

For the extraction from agar, Tü2401 was cultivated on ISP2 agar plates. Two plates (approx. 40 mL) were sliced, transferred into a 50 mL falcon tube, and subsequently centrifuged at 20,000 g for 30 min, resulting in 10 mL supernatant. After filtration through a folded filter, the pH was adjusted to pH 4.0 using 0.1 M HCl. After centrifugation, $(NH_4)_2SO_4$ was added to the supernatant up to the saturation point and incubated for 4 hr at 4°C. Afterward, the precipitate was separated and dissolved in 10 mL 0.1 M K_2HPO_4 at a pH of 8.0. Finally, this solution was extracted with 10 mL ethyl acetate. After drying under reduced pressure, the pellet was dissolved in 2.0 mL MeOH. Methanolic extracts were used for bioassays and HPLC-MS analysis.

For the extraction of liquid culture, the supernatant of Tü2401 was filtrated (0.2 μm) and extracted five times for 30 min with 2 L of ethyl acetate. The aqueous phase was collected, frozen at −80°C, and subsequently dried using a Lyovac GT2 (Heraeus Holding, Hanau; Germany). Further detailed information on the dereplication strategy has been described previously (Ortlieb, 2019).

QUANTIFICATION AND STATISTICAL ANALYSIS

Bruker Daltonics DataAnalysis 4.2 was used as software for the analysis of the HPLC-UV-HR-MS data.