Title
Comparison of proteomic responses as global approach to antibiotic mechanism of action elucidation

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Abstract

New antibiotics are urgently needed to address the mounting resistance challenge. In early drug discovery one of the bottlenecks is the elucidation of targets and mechanisms. To accelerate antibiotic research, we provide a proteomic approach for the rapid classification of compounds into those with preceded and unprecedented modes of action. We established a proteomic response library of *Bacillus subtilis* covering 91 antibiotics and comparator compounds, and a mathematical approach was developed to aid data analysis. The Comparison of Proteomic Responses (CoPR) allows the rapid identification of antibiotics with dual mechanisms of action as shown for atypical tetracyclines. It also aids in generating hypotheses on mechanisms of action as presented for salvarsan (arsphenamine) and the antirheumatic agent auranofin, which is under consideration for repurposing. Proteomic profiling also provides insights into the impact of antibiotics on bacterial physiology through analysis of marker proteins indicative of the impairment of cellular processes and structures. As demonstrated for trans-translation, a promising target not yet exploited clinically, proteomic profiling supports chemical biology approaches to investigating bacterial physiology.
Introduction

Antibiotics are essential medicines that reduce the mortality as well as the economic and societal impact of bacterial infections. In 1910, the first antibiotic introduced to the market was salvarsan (arsphenamine) for the treatment of syphilis. To this day, its mechanism of action has not been investigated. The use of salvarsan was discontinued due to toxicity when safer drugs like cell wall biosynthesis-inhibiting β-lactams or protein biosynthesis-blocking tetracyclines became available. Numerous antibiotic classes were discovered during the ‘Golden Age of Antibiotic Discovery’ (1940 to 1960). The most successful classes were brought to market and subsequently refined over several generations to reduce side effects, to broaden the spectrum of activity, and to overcome resistance. Antibiotic approvals peaked in the 1980s but declined since. In decades, no structurally new antibiotic class was discovered that entered the market. Today, multidrug-resistant bacteria like methicillin-resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa pose enormous challenges. With resistance spreading, infectious diseases returned as one of the leading causes of death worldwide and it is estimated that, by 2050, 10 million people will die annually due to antimicrobial resistance.

While antibiotic releases are still at a low, the investigation of bioactive molecules of natural and synthetic origin are experiencing a renaissance. Promising recently described compounds include teixobactin, a cyclic depsipeptide produced by Eleftheria terrae that inhibits cell wall biosynthesis, and murgocil, a synthetic inhibitor of cell wall biosynthesis that synergizes with β-lactams against MRSA. To find novel antibiotics, extensive compound libraries are screened for antibacterial activity (whole-cell activity screening) or for inhibition of a particular protein target or cellular process (target-based screening). Characterizing these initial hits is a bottleneck in antibiotic research, as it is laborious and resource intensive. Therefore, it is important to identify promising lead structures early on, to focus resources. One of the impediments in characterizing hits from whole cell activity screens (as opposed to target-based screens) is the time-consuming elucidation of the antibacterial target and mechanism of action. This is usually approached by identifying affected pathways by means of precursor incorporation, reporter gene assays, identification of targets based on mapping mutations in spontaneous resistant mutants, or by investigating the effects of treatment of bacteria on a system scale by transcriptome or proteome analysis. Systems-based approaches are particularly useful to investigate the effects of compounds with novel molecular targets (stemming from either screening approach) on the bacterial physiology as a whole, as was first shown by vanBogelen and Neidhardt.

Here we provide a major update of the B. subtilis proteomic response library, which had its origins in the late 1990s when large pharmaceutical companies investigated natural products as potential new antibacterial agents. It was shown that the acute proteomic response reflects the physiological impact of an antibiotic as well as cellular strategies to control and overcome the physiological challenge. Since compounds with a similar impact on physiology elicit similar responses, the proteomic profiles were used to identify inhibition of the peptidyl transferase reaction as mechanism of action of the natural product Bay 50-2369 based on the
similarities to chloramphenicol and tetracycline. Proteome analyses also aided in elucidating the mechanism of action of the natural product acyldepsipeptide, which targets ClpP. When industry largely moved to target-based antibiotic discovery, the library was expanded to include agents that inhibit experimental target areas, such as fatty acid biosynthesis or, inspired by the clinical success of daptomycin, the bacterial membrane. In fact, proteome analysis contributed to a better understanding of the mechanism of action of daptomycin itself. The mounting antibiotic resistance challenge increases the urgency to find new antibacterial agents with novel targets and mechanisms of action. Recent, largely academic efforts led to the emergence of compounds that need mechanism of action analysis and innovative drug discovery projects that require a better understanding of the physiological impact of inhibiting a novel target. Recognizing this demand, we offer the Comparison of Proteomic Responses in support of antibiotic research.

To propel future mechanism of action studies and to obtain insights into the physiology of antibiotic action, we gathered 55 of our previously published proteomic profiles of Bacillus subtilis 168 and investigated 36 further proteomic responses. This adds up to a library of response profiles for 91 antibacterial agents and comparator compounds (see supplementary information 2020_Senges_CoPR_Library.xlsx; Tab. S1-S71; Fig. S1-S35). The library covers clinically relevant and experimental drugs like topoisomerase inhibitors ciprofloxacin and nalidixic acid, aminoacyl-tRNA synthesis inhibitors mupirocin and AN3334, RNA polymerase inhibitor rifampicin, fatty acid biosynthesis inhibitors platensimycin and platencin, and substances used in research like the ionophores calcimycin and ionomycin, which disturb the ion homeostasis. Our data is made publicly available on the SubtiWiki platform (link to be provided upon acceptance of the article) and in the supplementary information (2020_Senges_CoPR_Library.xlsx; Tab. S1-S71; Fig. S1-S35).

To aid the analysis of new compounds, we introduce an approach we termed ‘Comparison of Proteomic Responses’ (CoPR) that combines 2D-PAGE with the mathematical comparison of the response profiles. A step-by-step protocol on how to use CoPR is available in the supplementary information. CoPR allows deduction of target areas for cytoplasmic, non-protein, and extra-cytoplasmic targets. Marker proteins indicative of the impairment of a specific cellular process or structure were delineated. As examples, we investigated the mechanisms of atypical tetracyclines (which can have dual mechanisms), salvarsan and auranofin, an antirheumatic drug considered for repurposing, and trans-translation inhibitors targeting a process not yet clinically exploited.

Results

Construction of the B. subtilis proteomic response library

To build the proteomic response library, we chose the gram-positive model organism B. subtilis 168, which is susceptible to most antibiotics, limiting constraints due to resistance, compound uptake, or efflux, which can impede this type of analysis for pathogens, and, in particular, multi-resistant pathogens. The proteome of
B. subtilis has been investigated in-depth and an extensive knowledgebase exists on its regulatory circuits and protein functions, which provide an optimal basis for a thorough interpretation of proteomic responses. While it is not possible to directly transfer knowledge of the specific proteins upregulated in response to an antibiotic challenge from B. subtilis to pathogens, the inferred target area is not species specific and hypotheses on target proteins can be tested in model organisms or pathogens.

The goal of the proteome analysis is to capture the acute antibiotic impact and bacterial response to antibiotic treatment. From a technical perspective, the construction of the proteomic response library can be divided into three steps: sample generation (Fig. 1a), data generation (Fig. 1b), and data evaluation (Fig. 1c).

To generate samples, exponentially growing cultures were treated with antibacterial agents in early to mid log-phase using ‘physiologically effective concentrations’ (PEC) that inhibit growth of the cultures by 50 to 80%. These concentrations were identified individually for each agent in growth experiments. Proteins produced during a 5 min pulse, starting 10 min after compound addition, were labeled with $^{35}$S-methionine and the cells harvested. Based on total protein quantitation and scintillation counting, global relative protein synthesis rates after antibiotic treatment were calculated in relation to untreated controls. Proteins were separated by 2D-PAGE and relative synthesis rates determined for individual protein spots based on the autoradiographs. To be designated ‘marker proteins’, the relative synthesis rates had to be at least 2 in each biological replicate. The exquisite sensitivity of pulse labeling allows monitoring of changes in the allocation of the cellular translation capacity, revealing adaptations of the proteome, which in gel-free mass spectrometry-based proteomic approaches remain hidden in a background of accumulated proteins.

Working with the growing library of proteomic profiles required the development of a data evaluation concept (Fig. 1c). The mathematical approach to the Comparison of Proteomic Responses (CoPR) is based on a matrix of pairwise comparisons of the similarity between two antibiotic responses. For each pairwise comparison a cosine similarity score (CoPR score) was generated based on the regulation factors of the marker proteins. The scores range from 1 (perfect similarity) to 0 (perfect dissimilarity), which allows the rapid identification of similar proteomic responses. Proteomic responses can be further interpreted individually based on the marker proteins, protein modifications, and knowledge on protein regulation and function. This is typically necessary to generate hypotheses on potential new molecular targets or to understand physiological consequences of antibacterial action.

**The proteomic response library**

The library of proteomic responses covers clinically used antibiotics, experimental antibiotics, comparator compounds, and toxic substances. For some of the compounds molecular targets and mechanisms have been described, while others have not been characterized yet (Fig. 2, Fig. 3, see Tab. 1; 2020_Senges_CoPR_Library.xlsx). On average, the proteomic responses of B. subtilis comprise 20 different marker proteins with the number of induced proteins ranging from 0 to 56. Overall 486 different proteins were identified as marker proteins for at least one condition, 130 of which are proteins of unknown function.
The CoPR-based similarity analysis facilitated the mechanism of action-dependent sorting of 86 response profiles. The dendrogram built based on the CoPR scores separates into two main branches: compounds, which mostly affect cytoplasmic components or processes (Fig. 2) and compounds with extra-cytoplasmic targets (Fig. 3). As discussed by vanBogelen et al.\textsuperscript{11}, proteins can be used as proteomic signatures to diagnose physiological states. In the context of antibiotics, they can specifically indicate which processes or structures are disturbed. The recurring marker proteins shown alongside the dendrogram (Fig. 2 and Fig. 3) allow a rapid matching of new compounds with target areas that are covered by compounds in the library. The homogeneity of proteomic responses varies for different cellular structures and pathways targeted (Fig. 4). Most compounds that impact the structural integrity of the cytoplasmic membrane, for instance, elicit the upregulation of certain marker proteins, most prominently LiaH, which is upregulated in response to 100% of these agents. The less well-defined target area of ‘redox and metal homeostasis and nucleic acid metabolism’ is represented by more diverse responses with varying combinations of marker proteins. The most consistent marker for this target area is YvyD with approximately 50% representation.

Of all target areas, fatty acid biosynthesis inhibitors, which group in the first branch of the dendrogram, gave the most homogeneous response (Fig. 2, 4). Irrespective of the protein target, the tested inhibitors (FabF inhibitors cerulenin and platensimycin, FabI inhibitor triclosan, FabF, FabHA, and FabHB inhibitor platencin) resulted in upregulation of FabHA and FabHB, FabF, and FabI\textsuperscript{13}. The induction of the fatty acid biosynthesis pathway is a direct countermeasure to impairment of the pathway by the inhibitors.

Two neighboring branches comprise antibiotics that both interfere with protein biosynthesis but affect physiology differently (Fig. 2). Antibiotics like tetracycline, erythromycin, or chloramphenicol interfere with chain elongation, thereby inhibiting protein biosynthesis. They share ribosomal proteins like RpsB, RpsF, RplJ, or the elongation factor Tu (TufA) as markers. Upregulation of ribosomal proteins and TufA counteracts the reduction in protein synthesis rates. A different response is observed for agents that compromise translation fidelity and protein quality. Puromycin, which leads to premature termination of translation, aminoglycosides, which interfere with ribosomal decoding and proof reading, and acyldepsipeptide, which causes uncontrolled proteolysis by ClpP\textsuperscript{12} elicit the upregulation of chaperone systems GroEL/GroES and DnaK/DnaJ as well as proteases ClpC and ClpE. These proteins prevent aggregation of misfolded proteins, facilitate refolding, or aid in the degradation of dysfunctional proteins.

Another branch harbors compounds that elicit oxidative stress responses, interfere with metal homeostasis, and/or cause nucleic acid stress (Fig. 2). Some of the agents, like nitrofurantoin and salvarsan, calcimycin and ionomycin, or nalidixic acid and rifampicin, elicit the upregulation of enzymes that detoxify reactive oxygen species (ROS), including superoxide dismutases SodA and SodF, catalase KatA, and/or alkyl hydroperoxide reductase AhpC/AhpF. Especially for comparator compounds, but also for the clinically used antibiotic nitrofurantoin, the upregulation of ROS detoxifying proteins can occur together with an upregulation of chaperones and proteases, indicating that protein homeostasis is critically impaired. Some of the agents, like nitrofurantoin, calcimycin, novobiocin, mupirocin, and trimethoprim elicit an upregulation...
of marker proteins involved in the protection of the cell from oxidative damage. Examples are MrgA and Dps, which mainly protect DNA, and/or OhrA/OhrB, the thioredoxin system (TrxA/TrxB), and BrxA/BrxB, which protect proteins. It was discussed previously that all antibiotics or all bactericidal antibiotics cause oxidative stress, which then leads to cell death.\textsuperscript{34,35} Although upregulation of oxidative stress-related marker proteins occurs in response to many compounds in this branch as well as the branch covering extracytoplasmic functions, the library shows that, at least during the acute proteomic response with sublethal doses of antibiotics, this is not the case for all antibiotics or all bactericidal antibiotics. No ROS-responsive proteins were upregulated in response to most fatty acid biosynthesis inhibitors, protein biosynthesis inhibitors, or cell wall biosynthesis inhibitors, including bactericidal aminoglycosides, vancomycin, and methicillin.

The second main branch of the dendrogram contains compounds acting on the cell envelope and its functions (Fig. 3). The most prominent marker proteins for this branch are LiaH, PspA, and YceC/H. The paralogous proteins LiaH and PspA stabilize the membrane from the inside. PspA is upregulated in response to loss of membrane integrity, LiaH in response to inhibition of membrane-based steps of cell wall biosynthesis. YceC and YceH are proteins of unknown function that were previously described as markers for cell envelope stress.\textsuperscript{32,33} Other marker proteins that are frequently observed include RacX, NadE, and YoxD.\textsuperscript{29,31} The amino acid racemase RacX is involved in cell envelope modification. The NAD\textsuperscript{+} synthase NadE has been described as a marker protein for membrane stress and indicates impairment of membrane-associated processes related to energy metabolism\textsuperscript{31}, and YoxD, a protein of unknown function, could be involved in the synthesis of alternative lipids, reorganizing the membrane to make it less susceptible to antimicrobial peptides.\textsuperscript{28,31,33} The ‘extra-cytoplasmic’ branch splits into three branches (Fig. 3). Compounds in the first branch are vancomycin, bacitracin, and the lantibiotics (NAI-107, gallidermin, mersacidin, and nisin), all of which interfere with the function of lipid II and/or the bactoprenol cycle. They share YtrB and YtrE as additional markers. Both proteins are cytoplasmic components of a postulated ABC transporter known to be upregulated in early stationary phase.\textsuperscript{36} Based on the proteome data, we speculated that the ABC transporter has a function related to the bactoprenol cycle. Indeed, in support of this hypothesis we found that a mutant, which constitutively expresses the \textit{ytrGABCDEF} operon is resistant to the acute effects of nisin (Fig. S36). The second branch includes compounds that interfere with membrane integrity but do not invoke sporulation-related marker proteins. Compounds in the third branch also target the membrane but share the sporulation and cell division-associated marker Spo0M and sporulation-associated transcriptional regulator SpoVG.

Not all antibiotics elicit an upregulation of marker proteins in \textit{B. subtilis}. Neither the inhibition of late steps of cell wall biosynthesis nor the inhibition of cell division resulted in changes of the cytosolic proteome (Tab. 1). Accordingly, no marker proteins were observed for inhibition of the transpeptidase reaction by methicillin\textsuperscript{10} or for inhibition of cell division proteins FtsZ or FtsI by PC190723\textsuperscript{38} and cephalexin,
respectively. No marker proteins were observed either for rotenone, an electron transport chain inhibitor to which *B. subtilis* is intrinsically resistant (Fig. S35). On the other end of the spectrum, some compounds have such a profound impact on the proteome that an interpretation of the 2D-PAGE-based proteomic response based on the CoPR approach is obstructed. When peptide deformylases (YkrB and Def) are inhibited by actinonin or down-regulated in a conditional mutant, global interference with cotranslational processing of protein N-termini leads to a pI-shift in most newly synthesized proteins. Using the approach described here it is not possible to unambiguously match pI-shifted protein spots with spots of control conditions on autoradiographs of the 2D gels. While proteins could still be identified using mass spectrometry, the calculation of 2D-PAGE-based regulation factors is impaired. However, the pI shifts are a direct consequence of the antibiotic action and can thus be used as a proteomic signature that provides insights into the mechanism of action.

**The CoPR similarity matrix provides insights into dual mechanisms of action**

The CoPR approach allows for a rapid detection of compounds with potential dual mechanisms of action since they elicit proteomic responses that show similarity to the profiles of compounds with two different target areas. Such compounds are strong candidates for clinical use because they might slow resistance development or lead to an overall bactericidal effect. In a recent proteome-based study we showed that the atypical tetracycline chelocardin has a dual mechanism of action with a concentration-dependent differential impact on physiology. Taking chelocardin as an example, the CoPR matrix reflects the concentration-dependent similarity with protein synthesis inhibitors as well as compounds in the extra- and cytoplasmic branch (Fig. 5a). At the physiologically effective concentration, chelocardin shares key marker proteins with both, as depicted exemplarily for tetracycline, daptomycin, and gramicidin S (Fig. 5b, c). The same is true for the atypical tetracycline anhydrotetracycline (Fig. 5d) - it also shares marker proteins at the physiologically effective concentration with protein synthesis inhibitors and with compounds affecting the cytoplasmic membrane. Interestingly, the proteomic response to 2-carboxamido-2-deacetyl-chelocardin (amidochelocardin), a recently described derivative of chelocardin that is active against multidrug-resistant pathogens, does not show indications of a dual mechanism (Fig. 5e). No marker proteins characteristic for protein synthesis inhibition were upregulated in response to amidochelocardin, even at low concentrations. In congruence with the proteomic response, the protein synthesis rates dropped to 50% at 12 µg/ml chelocardin, as measured by incorporation of 

**Metals and metalloids – opportunities for extending the elemental building set for antibiotics**

One approach to quickly introducing new antibiotics is to repurpose medicines approved for other indications. A promising candidate for drug repurposing is auranofin, a late stage antirheumatic drug. Auranofin, a gold-based organometallic, has been shown to inhibit antibiotic-resistant pathogenic gram-positive bacteria like MRSA. It is an inhibitor of thioredoxin and is thought to exert its antirheumatic...
effects through interactions with cysteines in transcription factors like AP-1 or NF-κB. While auranofin’s antibacterial mechanism is not fully elucidated, thioredoxin is indeed an essential protein in \( B. subtilis \)\(^{44} \). Another compound that contains an element rarely used in medicines is the arsenic-based organo-metalloid salvarsan. While its mechanism has not been proven experimentally, it is believed to be based on the reaction of arsenic with thiols. Salvarsan’s use for treatment of syphilis was discontinued when safer antibiotics became available. However, with resistance of \( Treponema pallidum \) to second line macrolide antibiotics on the rise and penicillin resistance impending\(^{42} \), salvarsan might be worthwhile revisiting as an antibiotic or an inspiration for the design of new drugs.

We investigated the mechanisms of action of auranofin and salvarsan by proteomic profiling using arsenic salts \( \text{As}_2\text{O}_3 \) and \( \text{As}_2\text{O}_5 \) as comparator compounds. The CoPR similarity matrix revealed that the responses to auranofin, salvarsan, and \( \text{As}_2\text{O}_3 \) were similar (Fig. 6a). The CoPR scores also reveal a high similarity to nitrofurantoin, an oral antibiotic used to treat lower urinary tract infections, the mechanism of which is not fully elucidated. Among the marker proteins of auranofin and salvarsan was arsenate reductase ArsC (Fig. 6b), which aids in the detoxification of arsenate. Both salvarsan and auranofin elicit a number of marker proteins that are involved in protecting the cell from oxidative protein damage (KatA, SodA, MrgA, chaperones, PepF) or replenishing the cysteine pool (MccB, CysC, CysK, YrhB, YxeK, and YxeP) (Fig. 6b). These marker proteins and the high similarity to the diamide and allicin proteomic responses (both compounds are known to react with cysteines\(^{46,47} \)) corroborate that the mechanisms of action of auranofin and salvarsan likely involve direct or indirect thiol-targeting.

**Proteomic profiling of trans-translation inhibitors**

Compound classes that exploit novel target areas are highly sought after to combat resistant pathogens. One promising novel target area is ribosome rescue, a process required to release ribosomes from stalled translation complexes. In bacteria the most important ribosome rescue process is *trans*-translation, which not only releases the ribosome but also targets the defective mRNA and the incomplete nascent peptide for degradation\(^{48} \). In some bacteria including \( E. coli \) and \( B. subtilis \), alternative factors have been found that can release ribosomes, but do not eliminate the mRNA or nascent peptide\(^{49,51} \). For \( E. coli \), a conservative estimate is that 2-4% of translation reactions require *trans*-translation to release ribosomes\(^{52} \). Stalled ribosomes can quickly lead to the depletion of actively translating ribosomes by trapping other ribosomes in polysomes. We investigated the *trans*-translation inhibitors KKL-35 and KKL-40 (oxadiazoles), derivatives of which have been shown to crosslink to the 23S rRNA\(^{53} \), and KKL-55\(^{54} \) (tetrazole), which likely has a different molecular target.

Decreased protein synthesis rates were observed after treatment with the oxadiazoles (KKL-35 36%, KKL-44 33%), but not to the same extent for the tetrazole (KKL-55 83%). According to the CoPR similarity matrix, the proteomic profiles show low to no similarity to the profiles of either of the two groups of protein biosynthesis inhibitors, represented by tetracycline (inhibition of elongation phase of translation) or kanamycin (causing proteotoxic stress). Instead, the inhibitors of *trans*-translation evoke a response similar
to that of ionophores that disturb metal homeostasis (calcimycin) and other agents causing oxidative stress responses (4-nitroquinoline oxide and allicin) (Fig. 7a). Several marker proteins of trans-translation inhibitors are indicative of oxidative stress and iron limitation (Fig. 7b), responses that are co-regulated by PerR and Fur in *B. subtilis* (reviewed by Moore and Helmann). As bacteria often upregulate compensatory measures, we hypothesize that trans-translation is of particular importance under oxidative stress, a condition shown to lead to elevated levels of defective mRNAs (reviewed by Wurtmann and Wolin).

**Discussion**

To support antibiotic research, we offer a system-based ‘Comparison of Proteomic Responses’ (CoPR) approach that provides insights into the impact of antibiotics on bacterial physiology and the bacterial strategies to cope with antibiotics. The CoPR library currently comprising responses of *B. subtilis* to 86 compounds as well as a step-by-step guide for comparative analysis are made publicly accessible (Link to be provided; see supplementary information Tab. S1-S7; Fig. S1-S35; 2020_Senges_CoPR_Library.xlsx). The CoPR approach replaces labor-intense one-on-one comparisons with an expedient and reliable process that identifies the most closely matching responses. The analysis of the response to tetracycline is a demonstration of the robustness of the method across laboratories and long timeframes: The response to tetracycline was analyzed twice using two different protocols and different equipment as described in the material and method section and the references provided almost 15 years apart. Both response profiles group in the same target area in the dendrogram (Fig. 2). Much like the microscopy-based mechanism of action investigation introduced by Nonejuie *et al.* CoPR provides a means of grouping compounds by target area and mechanism (Fig. 2, Fig. 3) without directly disclosing molecular targets or mechanisms of action. In addition, the proteome profiles provide complementary information by providing detailed insights into the effects of antibiotics and bacterial stress responses on the system level.

As demonstrated for the atypical tetracyclines, compounds with dual mechanisms can rapidly be identified using the CoPR approach (Fig. 5). Chelocardin, which was investigated in a phase II clinical trial in 1977, is active against tetracycline-resistant pathogens. The biosynthetic gene cluster of *Amycolatopsis sulphurea* was recently engineered for the synthesis of derivatives. The dual mode of action of chelocardin was first described based on proteome profiling. At low concentrations, the proteomic profile is more similar to that of protein biosynthesis inhibitor tetracycline indicating protein synthesis is inhibited, while with rising concentration the similarity to membrane-targeting compounds like daptomycin increases. In the original study, deriving this conclusion required elaborate one-on-one comparisons to each proteomic profile in the library. CoPR scoring now rapidly revealed that anhydrotetracycline also has a dual mechanism of action, while no marker proteins of protein synthesis are upregulated in response to amidochelocardin. Structurally, anhydrotetracycline is very similar to tetracycline while amidochelocardin is very similar to chelocardin, showing that CoPR can distinguish dual mechanisms even between closely related compounds.
Aside from assessing the novelty of antibiotic mechanisms, proteome profiling can also serve to inform structure-activity relationship studies, as was shown for PM47, a derivative of fatty acid biosynthesis inhibitor platensimycin, for which proteome analysis revealed that it no longer inhibits fatty acid biosynthesis\textsuperscript{13}. Proteome analysis also offers a starting point for mechanism of action elucidation of compounds with unprecedented targets. \textit{B. subtilis} often reacts to the stress exerted by an antibiotic by inducing proteins that counteract the disturbance of cellular homeostasis\textsuperscript{10,13}. The simplest reaction is the compensation of the loss of a particular function by upregulation of the target, as observed for example for translation inhibitors, tRNA-synthetase inhibitors, acyldepsipeptide (ClpP), and fatty acid biosynthesis inhibitors\textsuperscript{10,12,13}.

In past analyses, the in-depth interpretation of proteome profiles based on marker proteins has proven particularly useful for generating hypotheses on the mechanism of action of antibiotics with non-protein targets, like daptomycin or antimicrobial peptides, which act on the cytoplasmic membrane\textsuperscript{14,31}. Here, we used proteomic profiling to investigate metal and metalloid-containing antibiotics, revisiting the first antibiotic, salvarsan, and investigating auranofin, an antirheumatic drug with potential for repurposing. In 2005, 100 years after its discovery, the chemical structure of salvarsan was finally solved\textsuperscript{59}. Its antimicrobial mechanism is believed to be based on arsenic but has not been confirmed experimentally. Almost 40\% of the marker proteins for salvarsan are related to detoxification of arsenic, replenishing of the cysteine pool, and reaction to toxic protein modifications. Combining the information on marker proteins and the similarity of the salvarsan proteomic profile to that of As\textsubscript{2}O\textsubscript{3}, those of diamide and allicin (protein-thiol modifying agents\textsuperscript{46,47}), and that of auranofin (described to inhibit the bacterial thioredoxin reductase\textsuperscript{43}), we suggest that the antibiotic mechanism of salvarsan involves the targeting of protein thiols (Fig. 6). Interestingly, the physiologically effective concentrations of auranofin, salvarsan, and As\textsubscript{2}O\textsubscript{3} are vastly different. The physiologically effective concentration of auranofin is 0.06 µg/ml (88 nM), congruent with a single or a very small group of thiol-containing protein targets, such as thioredoxin reductase. The physiologically effective concentration of As\textsubscript{2}O\textsubscript{3} (64 µg/ml (647 µM)) is on the order of that of diamide (170 µg/ml (998 µM)), which reacts broadly with all thiols\textsuperscript{46}. The physiologically effective concentration of salvarsan is 2 µg/ml (10.8 µM), almost one order lower than that of allicin (14 µg/ml (86 µM)). It remains to be investigated if the comparably good antibacterial activity of salvarsan over As\textsubscript{2}O\textsubscript{3} and diamide is due to improved delivery of active compound to the target(s) in the cell or if salvarsan increases the selectivity for certain critical protein thiols.

Complementary to target-centric approaches, our system-based approach provides insights into the physiological impact of antibiotics. This can be exploited to study the physiological importance or the mechanism of a cellular process. Many aspects of ribosome function were elucidated using antibiotics. In such a chemical biology approach, antibiotics are used to cause a rapid loss of a critical cellular function to then observe the cellular response, and to deduce the impact of the loss-of-function on physiology. We employed \textit{trans}-translation inhibitors from a recent compound screening\textsuperscript{19}, to study the physiology of...
ribosome rescue in *B. subtilis*. Many of the marker proteins are indicative of oxidative stress and a disturbance of metal ion homeostasis (Fig. 6). Since compensatory measures are often induced in response to antibiotic stress, we hypothesize that ribosome rescue is of particular importance for the ability of *B. subtilis* to deal with mRNAs affected by oxidative stress. It has been shown that mRNAs are prime targets of oxidation by reactive oxygen species and that ribosomes stall on oxidized mRNAs when decoding is prevented\textsuperscript{56,60}.

As the study on *trans*-translation inhibitors shows, there is still much to learn about basic cellular processes. The presented library of proteomic response profiles reveals other gaps in our current understanding of bacterial physiology in general and the responses to antibiotics in particular. Although we based this study on the well-characterized model organism *B. subtilis*, across all proteome responses 27% of the marker proteins have uncharacterized or understudied functions. The compilation of profiles enabled the deduction of marker proteins indicative of an antibiotic’s main target area by association. Among those with high regulation factors are for example YtrB/YtrE and YceC as marker proteins for agents with extra-cytoplasmic targets. Antibiotics might contribute to elucidating their functions. YtrB/YtrE, for instance, are cytoplasmic components of an ABC transporter that we found upregulated in response to lantibiotics. We showed that the constitutive production of the ABC transporter protects cells from the inhibitory effect of nisin. As this example shows, in some instances, follow-up experiments might reveal strategies of *B. subtilis* and perhaps related pathogens like *B. anthracis* or *S. aureus* to cope with antibiotic stress and stress in general.

Proteomic profiling provides valuable insights into the effects of antibiotics on bacterial physiology and bacterial strategies to overcome antibiotic stress. The CoPR approach maximizes the utility of proteomic profiling by facilitating rapid comparisons of proteomic responses. We highlighted a variety of uses ranging from antibiotic mechanism of action studies, to investigation of bacterial physiology using antibiotics in a chemical biology approach, to analysis of gene function. Future applications may include the characterization of the bacterial response to non-antibiotic drugs, which have recently been shown to impact on human commensals\textsuperscript{61}, or investigations of the effects of secreted metabolites on microorganisms that share a habitat\textsuperscript{17}. By making available the data and tools, we expect to expand the use of the technique across the scientific community.
Materials and Methods

Antibacterial agents. If not stated otherwise substances were dissolved in dimethyl sulfoxide (DMSO). 2-carboxamido-2-deacetyl-chelocardin (amidochelocardin) was supplied by T. Lukezic and H. Petkovic. AN3334 was provided by Anacore Pharmaceuticals Inc, Palo Alto, USA. Anhydrotetracycline, GE2270A, linezolid, nalidixic acid, and nocathiacin I were purchased from Merck KgaA, Darmstadt, Germany. Nalidixic acid was dissolved in H$_2$O. As$_2$O$_3$ and As$_2$O$_5$ were purchased from Alfa Aesar, Haverhill, USA. As$_2$O$_3$ was dissolved in 1 M NaOH and As$_2$O$_5$ in A. dest.. ASD was synthesized/purified as described by Salmi et al. and dissolved in 0.9% NaCl. Auranofin was purchased from Fisher Scientific, Hampton, USA. BA234 was prepared according to previously described procedures, while using osmocene carboxylic acid instead of ruthenocene carboxylic acid. Cephalexin, CCCP, cinnamaldehyde, ciprofloxacin, monensin, mupirocin, rotenone, and tunicamycin were purchased from Sigma Aldrich, St. Louis, USA. Ciprofloxacin was dissolved in 0.1 M NaOH. Monensin was dissolved in ethanol. Closthioamide was synthesized as described by Kloss et al. cXRX was supplied by K. Scheinpflug. Kirromycin was supplied by S. Grond. KKL-35, KKL-40, and KKL-55 were supplied by K. C. Keller. MP66 and MP159 were prepared as described by Albada et al. NV503 was synthesized as described below. PC190723 was prepared as described previously. Salvarsan was prepared as described by Christiansen and Fargher, Squalamine was synthesized/purified as described by Zhang et al. and dissolved in H$_2$O. Telithromycin was provided by Anacor Pharmaceuticals. UC41 was synthesized as described by Czaplewski.

Synthesis of NV503. All solvents were purified according to reported procedures, and the reagents used were commercially available. Methanol, ethyl acetate, and dichloromethane were purchased from SDS and used without further purification. Column chromatography was performed on SDS silica gel (70-230 mesh). $^1$H NMR and $^{13}$C NMR spectra were recorded in MeOD on a Bruker AC 300 spectrometer working at 300 MHz and 75 MHz, respectively (abbreviations: s: singlet, d: doublet, t: triplet, q: quadruplet, m: multiplet). Tetramethylsilane was used as the internal standard. All chemical shifts are given in ppm. Mass spectroscopy analyses were performed by the Spectropole (Analytical Laboratory) of the University Paul Cézanne (Marseille). The purity of the compounds was checked by analytical HPLC (C$_{18}$ column, eluent CH$_3$CN-water-TFA (90:10:0.025, v/v/v), 0.5-1 ml/min) with a PDA detector spanning from 210 nm to 310 nm. All compounds possessed purity above 95%, as determined by analytical HPLC-PDA at 214 and 254 nm.

A mixture of progesterone (123 mg, 0.39 mmol), titanium(IV)isopropoxide (573 μl, 2.02 mmol) and spermine (2.02 mmol) in absolute methanol (5 ml) was stirred under argon at room temperature for 12 h. Sodium borohydride (114 mg, 3 mmol) was then added at -78 °C and the resulting mixture was stirred for an additional 2 h. The reaction was then quenched by adding water (1 ml) and stirring was maintained at room temperature for 20 min. The resulting inorganic precipitate was filtered off over a pad of Celite and washed with methanol and ethyl acetate. The combined organic extracts were dried over Na$_2$SO$_4$, filtered and concentrated in vacuo to afford the expected crude amino derivative, which was purified by flash
chromatography affording the expected amino derivative. Purification by column chromatography (silica gel; CH$_2$Cl$_2$/MeOH/NH$_2$OH(32%), 7:3:1) afforded a pale-yellow solid in 63% yield; $^1$H NMR (300 MHz, CD$_3$OD): $\delta = 5.28$ (s, 1H), 3.69-3.54 (m, 1H), 3.13 (m, 1H), 2.78-2.68 (m, 1H), 2.32-0.76 (m, 40H). $^{13}$C NMR (75 MHz, CD$_3$OD): $\delta = 148.60$, 128.04, 71.22, 67.32, 59.50, 57.61, 56.24, 50.90, 45.96, 43.79, 41.01, 38.53, 33.45, 30.80, 28.59, 27.21, 25.55, 24.27, 22.63, 19.99, 15.91, 13.21. C$_{31}$H$_{58}$N$_4$O MS (ESI$^+$) m/z 503.4673 (100%, [M+H]$^+$).

**Proteome analysis.** *Bacillus subtilis* 168 (trpC2) was cultured in Belitzky minimal medium$^{70}$ as described previously$^{10}$ and exposed at an OD$_{600}$ of 0.35 to antibacterial agents at the following concentrations:

- amidochelocardin 5, 10, and 20 μg/ml; AN3334 3 μg/ml; anhydrotetracycline 0.5, 2, and 4 μg/ml; As$_2$O$_3$ 64 μg/ml; As$_2$O$_5$ 40 μg/ml; ASD 2 μg/ml; auranoﬁn 0.06 μg/ml; BA234 6.25 μg/ml; CCCP 0.5 μg/ml; cephalaxin 0.0675 μg/ml; cinnamaldehyde 90 μg/ml; ciprofloxacin 25 μg/ml; closthoamide 0.7 μg/ml; cXRX 16 μg/ml; GE227A 8 μg/ml; kirromycin 50 μg/ml; kirrothricin C 100 μg/ml; KKL-35 1 μg/ml; KKL-40 0.25 μg/ml; KKL-55 2 μg/ml; KKL-896 1 μg/ml; linezolid 0.1 μg/ml; monensin 3 μg/ml; L-MP66 5.25 μg/ml; D-MP66 3.75 μg/ml; MP159 5 μg/ml; mupirocin 0.06 μg/ml; nalidixic acid 250 μg/ml; nocathiacin I 0.15 μg/ml; NV503 4.2 μg/ml; PC190723 64 μg/ml; rotenone 20 μg/ml; salvarsan 2 μg/ml; squalamine 1 μg/ml; telithromycin 5 μg/ml; tunicamycin 25 μg/ml; UC41 8 μg/ml.

In this study, pulse labeling with L-[35S] methionine and the 2D PAGE-based proteome analysis were performed as described by Wenzel et al.$^{13}$ with the exception of kirrothricin C, which was analysed as described by Bandow et al.$^{10}$. Briefly, for radioactive labeling with L-[35S] methionine, 5 ml of bacterial culture were treated with an antibiotic for 10 min before 1.8 MBq radioactive methionine (Hartmann Analytic, Braunschweig, Germany) were added. Incorporation was stopped after 5 min by addition of 1 mg/ml chloramphenicol, an excess of non-radioactive L-methionine, and cooling of the cells on ice. Cells were harvested and washed before cell disruption using a VialTweeter sonicator (Hielscher, Teltow, Germany). Cell debris was removed by centrifugation and protein concentrations were estimated using Roti NanoQuant (Roth, Karlsruhe, Germany). For radioactive gels, 50 μg of protein (300 μg for non-radioactive gels) were loaded onto 24 cm immobilized pH gradient strips pH 4 to 7 (GE Healthcare, Little Chalfont, United Kingdom) by passive rehydration for 18 h. Proteins were separated by isoelectric focusing in the first dimension using a Multiphore II electrophoresis system (GE Healthcare). In the second dimension proteins were separated according to molecular size by SDS-PAGE using the Ettan DaltTwelve system (GE Healthcare). For few antibiotic conditions (marked with an asterisk (*) in the figures) the Millipore 2-D Gel Electrophoresis System (Merck KgaA) was used for SDS-PAGE. Radioactive gels were dried on Whatman paper and exposed to storage phosphor screens (GE Healthcare). Screens were scanned using a Typhoon Trio+ (GE Healthcare) with 633 nm excitation wavelength and 390 nm emission filter. Non-radioactive gels were stained with 0.003% ruthenium(II)-tris(4,7)diphenyl-1,10-phenanthroline disulfonate and scanned on the Typhoon Trio+ with excitation at 532 nm and a 610 nm emission filter. Image analysis was performed as described by Bandow et al.$^{71}$ using Decodon Delta 2D 4.2.1 (Decodon, Greifswald, Germany). After...
background subtraction, signal intensities of protein spots were normalized to the total signal on the autoradiograph and set in relation to the synthesis rate in the respective control to obtain relative synthesis rates (regulation factors, see Eq. 1) for individual protein spots. To be designated ‘marker proteins’, the relative synthesis rates had to be at least 2 in each biological replicate, and the protein had to accumulate in sufficient amounts to allow protein identification from a preparative gel.

\[
RF = \frac{\text{relative signal intensity}_{\text{antibiotic treated}}}{\text{relative signal intensity}_{\text{untreated control}}} \quad \text{(Eq. 1)}
\]

RF: regulation factor of an individual protein spot

Proteins were identified from preparative 2D gels after tryptic in-gel digest either by MALDI ToF/ToF MS\textsuperscript{13} or by nUPLC-ESI-MS/MS\textsuperscript{24} as described previously and the data was uploaded to the PRIDE repository\textsuperscript{72}.

Data from literature was included in the downstream analysis only when experiments were performed according to the same protocols\textsuperscript{10,13} and satisfied the same quality standards. Thus, all data included was recorded for \textit{B. subtilis} 168 grown in Belitzky minimal medium, using radioactive pulse labeling to delineate relative synthesis rates, 2D-PAGE for protein separation, and mass spectrometry for protein identification.

For an overview on treatment times, concentrations used, and number of replicates of data gathered in this study and from literature, see supplementary information 2020_Senges_CoPR_Library.xlsx.

**Comparison of proteomic responses.** Regulation factors (RF) for marker proteins (see definition above) were determined based on relative signal intensity on autoradiographs compared to untreated controls (Eq. 1). To calculate CoPR scores, the data were prepared as follows: regulation factors of the independent replicates were averaged and logarithmized and values for non-marker proteins were set to 0. If literature data lacked regulation factors due to the low abundance of a protein in the control, the regulation factor was set to 20. If a protein was identified in several spots, the value in the library reflects the regulation factor of the spot with the highest volume-% representing the most intense spot with the highest protein synthesis rate. Unidentified marker proteins are omitted from the analysis.

Pairwise comparisons based on cosine similarity were performed using the regulation factors of an antibiotic a (RF\textsubscript{ABa}) and an antibiotic b (RF\textsubscript{ABb}) yielding a CoPR score for each pairwise comparison (Eq. 2). In this approach, proteomic profiles are treated as vectors, with proteins as dimensions and regulation factors as dilatation into these dimensions. A dot product is calculated, giving the sum of products of all pairwise multiplications of regulation factors of one protein, under both conditions. To give the CoPR score for the pairwise comparison, the dot product is divided by the product of the multiplication of the vector lengths. A value of 1 represents perfect similarity between response profiles while 0 represents perfect dissimilarity.

\[
\text{CoPR Score} = \frac{\|\log_{10}\text{RF}_{\text{ABa}}\| \cdot \|\log_{10}\text{RF}_{\text{ABb}}\|}{\|\log_{10}\text{RF}_{\text{ABa}}\| \cdot \|\log_{10}\text{RF}_{\text{ABb}}\|}
\]

\[
\text{(Eq. 2)}
\]

CoPR Score: Comparison of Proteomic Responses Score
RF_{AB_a}: regulation factors of antibiotic a

RF_{AB_b}: regulation factors of antibiotic b

Calculations were performed using Microsoft Excel and R 3.3.1 with the spatialEco package in version 1.1-0. For a step-by-step protocol see supplementary information.
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Author contributions

CHRS and JEB designed research and wrote the paper. JJS, MW, NR, ÜA, YM, PP, MVH, AY, BS, NBMJ, HLW, MK, JB, and JEB collected data. CHRS, JJS, MW, NR, and SS evaluated data. CHRS assembled the data. MB created mutants. BZ and JS integrated the data into SubtiWiki. JNA, BA, MP, TL, NAS, NL, and RM synthesized or purified compounds. LIL, KS, HBO, CH, JTS, HP, JMB, KCK, and NMN supplied substances.

Competing financial interests

Tadeja Lukežič and Hrvoje Petković worked at Acies Bio (Slovenia) at the time the experiments were performed.

Data Availability

Data for protein identification by nUPLC-ESI-MS was uploaded to the PRIDE repository (Project Name: Comparison of Proteomic Responses, Project accession: PXD011640). Protein regulation factors are supplied in the supplementary file 2020_Senges_CoPR_Library.xlsx and the SubtiWiki repository (http://subtiwiki.uni-goettingen.de/v4/downloads). Original proteomic profiles and a step-by-step protocol on how to perform the mathematical comparison are available as supplementary material.
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### Tab. 1 Antibiotics not amenable to CoPR analysis.

| compound     | compound class | target structure/process                  | comment                                                                                           | reference |
|--------------|----------------|-------------------------------------------|---------------------------------------------------------------------------------------------------|-----------|
| methicillin  | penicillin     | cell wall biosynthesis                    | no protein met 2-fold upregulation requirement of marker proteins                                   | (10)      |
| cephalaxin   | cephalosporin  | cell wall biosynthesis, cell division     | no proteins met 2-fold upregulation requirement                                                   | this work |
| PC190723     | benzamide      | cell division                             | no proteins met 2-fold upregulation requirement                                                   | this work |
| rotenone     | isoflavone     | respiratory chain                         | no proteins met 2-fold upregulation requirement                                                   | this work |
| actinonin    | nonribosomal peptide | protein synthesis, peptide deformylase | pl shift of protein spots after actinonin treatment prevents quantitative comparison of proteomic profile to control | (37)      |
**Fig. 1** Construction of the *Bacillus subtilis* proteomic response library. 

**a** Sample generation: Newly synthesized proteins of *B. subtilis* are pulse labeled for 5 min with $^{35}$S-L-methionine 10 min after addition of an antibiotic at a concentration that leads to a 50-80% growth inhibition. Subsequently, incorporation of $^{35}$S into protein of control and antibiotic-treated samples is determined by scintillation counting, to calculate relative protein synthesis rates. 

**b** Data generation: Autoradiographs of 2D-gels are used for software-based image analysis to identify marker proteins (regulation factor ≥2 in each of the biological replicates). Marker proteins are excised from non-radioactive gels and identified by mass spectrometry. 

**c** Data evaluation: Identified marker proteins and regulation factors are added to the CoPR library to calculate a similarity matrix. The similarity of a proteomic profile to responses in the library is utilized, in conjunction with knowledge on the proteins (function, localization, regulation, etc.) from the SubtiWiki database and literature, to generate a hypothesis on the mechanism of action of compounds.

**Fig. 2** Similarity of proteomic responses to antibiotics affecting cytoplasmic targets and processes. *B. subtilis* was treated for 10 min with physiologically effective concentrations (PEC) of the agents prior to radioactive labeling of newly synthesized proteins and 2D-PAGE-based proteomic profiling. To generate the dendrogram Ward’s method was applied on the CoPR scores. 

Abbreviations: AATS – amino acyl-tRNA supply; FAB – fatty acid biosynthesis; IH – ion homeostasis; OS – oxidative stress; PQ – protein quality; PS – protein synthesis. Colors underlying the dendrogram indicate groups of antibiotics with similar antibiotic effects and responses: purple – inhibition of FAB; dark blue – inhibition of PS; light blue – effects on PS resulting in proteotoxic stress; red – effects on redox homeostasis, metal homeostasis, and nucleic acids. 

Squares indicate marker proteins informative of cellular structures or processes according to the following color code: purple – FAB; dark blue – PS; light blue – proteotoxic stress; dark red – sulfur metabolism; red – detoxification of ROS; orange – prevention of oxidative damage; yellow – general stress; dark green – membrane (structural integrity); medium green – membrane (associated functions); gray – regulation of sporulation and cell division. References indicate the sources of proteomic data. # - Proteomic response recorded in this work. * - Proteome response was recorded on a different gel system.

**Fig. 3** Similarity of proteomic responses to antibiotics affecting extra-cytoplasmic targets. *B. subtilis* was treated for 10 min with physiologically effective concentrations (PEC) of the antibacterial agents prior to radioactive labeling of newly synthesized proteins and 2D-PAGE-based proteomic profiling. To generate the dendrogram Ward’s method was applied on the CoPR scores. 

Abbreviations: CWB – cell wall biosynthesis; IH – ion homeostasis; PS – protein synthesis. Colors underlying the dendrogram indicate groups of antibiotics with similar antibiotic effects and responses: light green – inhibition of CWB by interference with bactoprenol recycling; dark green – interference with membrane structure; medium green – interference with membrane structure and membrane-associated processes. Squares indicate marker proteins informative of cellular structures or processes according to the following color code: dark blue – PS; light
The most common marker proteins for each target area. For each branch of the dendrogram, the relative prevalence for the seven most frequently observed marker proteins is shown. Color coding: purple – inhibition of FAB; dark blue – inhibition of PS; light blue – effects on PS resulting in proteotoxic stress; red – effects on redox homeostasis, metal homeostasis and nucleic acids; light green – inhibition of CWB by interference with bactoprenol cycle; dark green – interference with membrane structure; medium green – interference with membrane structure and membrane-associated processes.

**Fig. 5** Concentration-dependent shifts in the mechanism of action of atypical tetracyclines. 

- **a** Similarity of the proteomic responses of *B. subtilis* to different concentrations of tetracyclines and reference antibiotics. High similarity is indicated by a score close to 1 and high intensity of blue color. Profiles originating from treatment with physiologically effective concentrations (PEC) are written in bold letters.
- **b** Up-regulation of marker proteins in response to reference antibiotics.
- **c-e** Induction of marker proteins by different concentrations of **c** chelocardin, **d** anhydrotetracycline, and **e** amidochelocardin. For **b-e** the scale represents the log<sub>10</sub> of the marker protein regulation factor.
- **f** The black line corresponds to treatment with the PEC. For proteomic responses to amidochelocardin and anhydrotetracycline see supplementary Fig. S4 and S5. Proteomic profiles for tetracycline and chelocardin were taken from Stepanek et al.²² and profiles for daptomycin and gramicidin S from Müller et al.¹⁴ and Wenzel et al.²⁸. All sections reflect averages of three biological replicates.

**Fig. 6** Comparing proteomic responses of *B. subtilis* 168 to auranofin and salvarsan.

- **a** Similarity of the proteomic response of *B. subtilis* against salvarsan, auranofin, arsenic salts, and reference antibiotics. High similarity is indicated by a score close to 1 and high intensity of blue color.
- **b** Comparison of marker proteins of salvarsan and auranofin. Shown are the marker proteins with highest regulation factors for both compounds. Averages of three biological replicates with standard deviations are given for the marker proteins of each antibiotic (regulation factors ≥2 in all replicates).

**Fig. 7** Proteomic response of *B. subtilis* 168 to inhibition of trans-translation.

- **a** Similarity matrix comparing proteomic responses of *B. subtilis* to the named compounds. Coloring indicates similarity, with high scores being close to 1 and indicated by high intensity of blue color.
- **b** Comparison of marker proteins of trans-translation inhibitors. Shown are regulation factors of marker proteins related to responses against oxidative...
stress and iron limitation. Averages of three biological replicates with standard deviations are given for the marker proteins of each antibiotic (regulation factors ≥2 in all replicates).
Fig. 1 Construction of the Bacillus subtilis proteomic response library. a Sample generation: Newly synthesized proteins of B. subtilis are pulse labeled for 5 min with $^{35}$S-L-methionine 10 min after addition of an antibiotic at a concentration that leads to a 50-80% growth inhibition. Subsequently, incorporation of $^{35}$S into protein of control and antibiotic-treated samples is determined by scintillation counting, to calculate relative protein synthesis rates. b Data generation: Autoradiographs of 2D-gels are used for software-based image analysis to identify marker proteins (regulation factor $\geq 2$ in each of the biological replicates). Marker proteins are excised from non-radioactive gels and identified by mass spectrometry. c Data evaluation: Identified marker proteins and regulation factors are added to the CoPR library to calculate a similarity matrix. The similarity of a proteomic profile to responses in the library is utilized, in conjunction with knowledge on the proteins (function, localization, regulation, etc.) from the SubtiWiki database and literature, to generate a hypothesis on the mechanism of action of compounds.
Fig. 2 Similarity of proteomic responses to antibiotics affecting cytoplasmic targets and processes. *B. subtilis* was treated for 10 min with physiologically effective concentrations (PEC) of the agents prior to radioactive labeling of newly synthesized proteins and 2D-PAGE-based proteomic profiling. To generate the dendrogram Ward’s method was applied on the CoPR scores. Abbreviations: AATS – amino acyl-tRNA supply; FAB – fatty acid biosynthesis; IH – ion homeostasis; OS – oxidative stress; PQ – protein quality; PS – protein synthesis. Colors underlying the dendrogram indicate groups of antibiotics with similar antibiotic effects and responses: purple – inhibition of FAB; dark blue – inhibition of PS; light blue – effects on PS resulting in proteotoxic stress; red – effects on redox homeostasis, metal homeostasis, and nucleic acids. Squares indicate marker proteins informative of cellular structures or processes according to the following color code: purple – FAB; dark blue – PS; light blue – proteotoxic stress; dark red – sulfur metabolism; red – detoxification of ROS; orange – prevention of oxidative damage; yellow – general stress; dark green – membrane (structural integrity); medium green – membrane (associated functions); gray – regulation of sporulation and cell division. References indicate the sources of proteomic data. # – Proteomic response recorded in this work. * – Proteome response was recorded on a different gel system.
Fig. 3 Similarity of proteomic responses to antibiotics affecting extra-cytoplasmic targets. *B. subtilis* was treated for 10 min with physiologically effective concentrations (PEC) of the antibacterial agents prior to radioactive labeling of newly synthesized proteins and 2D-PAGE-based proteomic profiling. To generate the dendrogram Ward’s method was applied on the CoPR scores\(^{26}\). Abbreviations: CWB – cell wall biosynthesis; IH – ion homeostasis; PS – protein synthesis. Colors underlying the dendrogram indicate groups of antibiotics with similar antibiotic effects and responses: light green – inhibition of CWB by interference with bactoprenol recycling; dark green – interference with membrane structure; medium green – interference with membrane structure and membrane-associated processes. Squares indicate marker proteins informative of cellular structures or processes according to the following color code: dark blue – PS; light blue – proteotoxic stress; dark red – sulfur metabolism; red – detoxification of ROS; orange – prevention of oxidative damage; yellow – general stress; dark green – membrane (structural integrity); medium green – membrane (associated functions), light green – inhibition of CWB by interference with bactoprenol recycling; gray – regulation of sporulation and cell division. References indicate the sources of proteomic data. # Proteomic response recorded in this work. * Proteome response was recorded on a different gel system.
Fig. 4 Relative prevalence of marker proteins for each target area. For each branch of the dendrogram, the relative prevalence for the seven most frequently observed marker proteins is shown. To this end the number of conditions within a target area for which a protein is a marker protein was divided by the total number of conditions in that category. Color coding: purple – inhibition of FAB; dark blue – inhibition of PS; light blue – effects on PS resulting in proteotoxic stress; red – effects on redox homeostasis, metal homeostasis and nucleic acids; light green – inhibition of CWB by interference with bactoprenol cycle; dark green – interference with membrane structure; medium green – interference with membrane structure and membrane-associated processes.
Fig. 5 Concentration-dependent shifts in the mechanism of action of atypical tetracyclines. **a** Similarity of the proteomic responses of *B. subtilis* to different concentrations of tetracyclines and reference antibiotics. High similarity is indicated by a score close to 1 and high intensity of blue color. Profiles originating from treatment with physiologically effective concentrations (PEC) are written in bold letters. **b** Up-regulation of marker proteins in response to reference antibiotics. **c–e** Induction of marker proteins by different concentrations of **c** chelocardin, **d** anhydrotetracycline, and **e** amidochelocardin. For **b–e** the scale represents the $\log_{10}$ of the marker protein regulation factor. For **c–e** the black line corresponds to treatment with the PEC. For proteomic responses to amidochelocardin and anhydrotetracycline see supplementary Fig. S4 and S5. Proteomic profiles for tetracycline and chelocardin were taken from Stepanek *et al.*\(^2\) and profiles for daptomycin and gramicidin S from Müller *et al.*\(^4\) and Wenzel *et al.*\(^2\). All sections reflect averages of three biological replicates.
Fig. 6 Comparing proteomic responses of *B. subtilis* 168 to auranofin and salvarsan. **a** Similarity of the proteomic response of *B. subtilis* against salvarsan, auranofin, arsenic salts, and reference antibiotics. High similarity is indicated by a score close to 1 and high intensity of blue color. **b** Comparison of marker proteins of salvarsan and auranofin. Shown are the marker proteins with highest regulation factors for both compounds. Averages of three biological replicates with standard deviations are given for the marker proteins of each antibiotic (regulation factors \( \geq 2 \) in all replicates).
Fig. 7 Proteomic response of *B. subtilis* 168 to inhibition of trans-translation. **a** Similarity matrix comparing proteomic responses of *B. subtilis* to the named compounds. Coloring indicates similarity, with high scores being close to 1 and indicated by high intensity of blue color. **b** Comparison of marker proteins of trans-translation inhibitors. Shown are regulation factors of marker proteins related to responses against oxidative stress and iron limitation. Averages of three biological replicates with standard deviations are given for the marker proteins of each antibiotic (regulation factors ≥2 in all replicates).
The diagram shows the relative prevalence of marker proteins in a biological system. The proteins are categorized into different functional groups:

- Inhibition of fatty acid biosynthesis
- Inhibition of protein biosynthesis
- Protein stress effects on redox status
- Membrane transporters
- Membrane integrity
- Associated functions

The proteins listed include:

- FabHA
- FabHB
- FabF
- FabI
- PanB
- PIsX
- Ald
- RplJ
- TuA
- RpsB
- RpsF
- Ads
- PyrAA
- PyrAB
- GroEL
- CipC
- CipE
- DnaK
- GroES
- CipP
- PtsH
- YvdD
- KatA
- AhpF
- AhpC
- MrgA
- YtxH
- YuaE
- LiaH
- YceC
- YtrB
- YtrE
- TrmB
- YceH
- NadE
- LiaH
- PspA
- YceC
- YvlB
- YceH
- Abh
- YfmS
- YceC
- NadE
- PspA
- LiaH
- RacX
- SpoFM
- YoxD

The graph visually represents the relative abundance of each protein, possibly indicating their importance or activity levels in the context of the study.
