Identification of the molecular target is a crucial step in evaluating novel antibiotics. To support target identification, a label-free method based on chromatographic co-elution (TICC) exploits the alteration of the elution profile of target-bound drug versus free drug in ion exchange (IEX) chromatography to identify potential target proteins from elution fractions. The applicability of TICC for antibiotic research is investigated by evaluating which proteins, that is, putative targets, can be monitored in Bacillus subtilis. Coelution of components of known protein complexes provides a read-out for how well the native state of proteins is conserved during chromatography. Rifampicin, which targets RNA polymerase, is used in a proof-of-concept study.

Beside antibiotic stewardship, the discovery and development of novel antibiotics is arguably the most crucial remedy to countering the rapid emergence of resistant bacterial pathogens. The development of analogues is an important strategy to stock the current antibiotic development pipeline. However, structurally novel compounds with unprecedented targets and modes of action (MoA) are needed to overcome the constant emergence of resistant pathogens.\(^1\) Omics approaches like proteomics or transcriptomics are routinely used to study the physiological impact of drugs and to delineate their MoAs.\(^2\) Yet, many omics approaches fail to reveal the molecular target directly. Knowledge of the target is essential for understanding the MoA on a molecular level, to estimate the risk of cross-resistance, and to facilitate optimization by medicinal chemistry. Among the global methods available for identifying drug targets on a molecular level, the most promising, like proteome-wide thermal profiling and target identification by chromatographic co-elution (TICC), do not require compound derivatization\(^3\) that may produce artefacts due to structure manipulation.

So far, TICC has been used mainly to study drug-target interaction in eukaryotic systems.\(^1\) Here, we assessed the performance of TICC regarding a) proteome coverage, b) the preservation of protein complex integrity, c) the coverage of essential proteins, d) identification of known antibiotic targets, and by e) performing a proof-of-concept study for antibiotic research using the clinically relevant antibiotic rifampicin. Bacillus subtilis served as model organism since it is susceptible to most antibiotics.\(^7\)

The general principle behind TICC is that the elution profile of a drug in native ion exchange (IEX) chromatography changes when the drug is bound to a protein target (as opposed to unbound drug). To allow target binding, the drug is incubated with cell lysate. This sample as well as a control sample containing only the drug in buffer are then subjected to native IEX chromatography. After time-controlled fractionation into microtiter plates, the antibiotic is quantified in all fractions using mass spectrometry (MS). Fractions with target-bound antibiotic can be identified by comparing elution profiles of the antibiotic in the buffer and lysate samples. Fractions into which the antibiotic is shifted by target binding are subjected to protein identification and quantitation by MS. Correlating the elution profiles of drug and proteins leads to a list of potential targets (for methods refer to Supporting Information).

First, we analyzed which proteins (i.e., potential targets) are amenable to TICC, which requires them to be soluble, separable, identifiable, and quantifiable. To this end we analyzed the proteins in all fractions in the absence of antibiotic in two independent replicates. As expected, some proteins do not interact with the IEX column material. These 222 proteins in the void volume (fractions 1–20) were not included in the tally. In the remaining fractions we identified a total of 920 unique proteins. As the B. subtilis genome encodes 4200 genes, 2515 of which are actively transcribed under the growth conditions used in this study,\(^12\) this technique covers 37% of transcribed genes. Of these, only few proteins were detected in >10 fractions (15% ± 2) with a maximum of 199 proteins per fraction (replicate 1:153, replicate 2:199; Figure 1a and Figure S1a, Supporting Information).
Figure 1. Efficacy of protein separation and protein complex integrity during TICC. a) The number of proteins per fraction demonstrates that the reduction of complexity of the sample using a relatively short gradient is reasonable to balance time and effort of target identification. b–d) For selected proteins, the relative amount eluting per fraction are shown. Elution profiles for known protein complexes demonstrate that complex integrity is preserved. For ATP synthase (b) and RNA polymerase (RNAP) (d) the subunit organization is shown for clarity. Data shown reflect the first replicate, data for the second replicate are shown in Figure S1, Supporting Information.

Information). On average, 60% of proteins eluted across less than three fractions, indicating that the chosen fractionation time of 96 min is an efficient trade-off between reduction of sample complexity and required instrument time (Figure S2, Supporting Information). IEX chromatography works best for cytosolic proteins while membrane proteins are not separated as efficiently by this method. The subcellular localization of proteins was predicted using TMHMM 2.0 Server, HMMTOP 2.0, SOSUI, Phobius, LipoP 1.0, and SignalP 3.0. As expected, 86.5% of the identified proteins were predicted to localize in the cytosol, 2.9% were lipoproteins, 3.4% secreted proteins, 6.7% integral membrane proteins, and 0.5% have unknown subcellular localization.

Target inhibition is a highly specific process which can require access to the catalytic core (i.e., platensimycin), certain amino acids near the active site (i.e., rifampicin) or interference with the tertiary structure (i.e., spectinomycin). Hence, it is critical to prevent protein denaturation and disintegration of protein complexes. We evaluated the structural integrity of known protein complexes based on co-elution in TICC. Selected elution profiles of representative protein complexes are shown (Figure 1 and Figure S1, Supporting Information). The ATP synthase, which is a clinically exploited antibiotic target, represents a structurally complex protein machinery (Figure 1b). Except for the membrane-bound α and ɛ subunits, all other components were identified and all components of the F1 complex co-eluted (Figure 1b and Figure S1b, Supporting Information). Additionally, co-elution is exemplarily shown for glycyllphenylalanyl tRNA synthetase (GlyQ/S, PheS/T), RNase J (RnjA/B), and glutamate synthase (GltA/B) (Figure 1c and Figure S1c, Supporting Information). PheS/T were found exclusively as co-eluting proteins, while the other proteins were also detected in fractions.
Table 1. Known antibiotic targets and their amenability to TICC. Limitations of TICC for target identification were determined experimentally (*) or derived at by reasoning. Proteins listed in bold were identified in at least one replicate.

| Process/structure          | Relevant target(s)                                                                 | Relevant proteins                                                                 | Excerpt of relevant antibiotics/inhibitors | (Potential) limitations of TICC |
|----------------------------|-----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------------------|--------------------------------|
| Membrane                   | Membrane                                                                          |                                                                                  | Lipopeptides, colistin antimicrobial peptides | Not a protein target |
| Cell wall biosynthesis     | Bactroprenol, lipid II                                                             |                                                                                  | Bacitracin, teicoplanin, lanthionines, vancomycin | Not a protein target |
| Penicillin binding proteins | DacA/B/C/F, PhpA(FtsI)/B/C/D/E/F/G/H/I/X, Pona, SpoVD                             |                                                                                  | β lactams                                     | Covalent binding |
| UDP-N-acetylgalcosamine-   | MurAA/A                                                                           |                                                                                  | Fosfomycin                                     |                                |
| enolpyruvyl-transferase    |                                                                                  |                                                                                  |                                            |                                |
| Cell division              | Divisome                                                                           |                                                                                  | PC190723, many structurally diverse molecules |                                |
| Folate biosynthesis        | Dihydrofolate reductase                                                            |                                                                                  | Trimethoprim                                    |                                |
| Fatty acid synthesis       | Acetyl-CoA carboxylase                                                             |                                                                                  | Sulfamethoxazole                                 | Resistance of B. subtilis |
| β-ketoacyl-ACP synthase II | FabF                                                                               |                                                                                  | Platensimycin, cerulenin, platencin            | Inhibition requires substrate binding |
| β-ketoacyl-ACP synthase III| FabHA/B                                                                            |                                                                                  | Platencin                                      |                                |
| Enol-ACP reductase         | Fabl                                                                               |                                                                                  | Triclosan                                      |                                |
| Replication                | DNA gyrase                                                                         |                                                                                  | (fluoro-) quinolone-novobiocin                 | Gyrase subunits do not co-elute due to DNA removal |
| Transcription              | RNA polymerase                                                                     |                                                                                  | Rifampicin                                      |                                |
| Translation                | Ribosome                                                                           |                                                                                  | Targeting rRNA: macrolides, chloramphenicol, tetracyclines, linezolide, KKL-35/40 |                                |
| Aminoacyl-tRNA synthetases | AlaS, ArgS, AsnS, AspS, CysS, GlnA/B/C, GlyQ/S, HisS/Z, IleS, LeuS, LysS, MetS, PheS/T, ProS, SerS, ThrS/T, TrpS, TyrS/Z, ValS, YtspR |                                                                                  | Mupirocin, indolmycin, AN3334                  |                                |
| Elongation factors         | FusA (EF-G), TuA (EF-Tu), Tfs (EF-Ts)                                              |                                                                                  | Fusidic acid, kirromycin, kirothricin C, pulvomycin, GE2770A |                                |
| Formyl-methionine deamylase| DefA, DefB (YkrB)                                                                  |                                                                                  | Actinonin                                       |                                |
| ATP synthesis              | ATP synthase                                                                       |                                                                                  | Bedaquiline                                     |                                |
| Protein homeostasis        | Proteases                                                                          |                                                                                  | Acyldepsipeptides, β-lactones, cyclomarin A, MJMS123, ecuminic, lassomycin |                                |
| Chaperones                 | DnaK, GroESL                                                                       |                                                                                  | Proline-rich peptides, hydroxyphenylamides, suramin, cloxacil, rafinoxide |                                |

not containing their respective interaction partner(s), where they could be uncomplexed or interacting with other proteins. RNaseJ, for instance, is known to form RnjA and RnjB homod and hetero-complexes with distinct functions.[21] Since we used rifampicin to assess the suitability of TICC for antibiotic research, Figure 1d and Figure S1d, Supporting Information, show the elution profile of the RNA polymerase (RNAP) complex. RNAP consists of four essential subunits (α2, β, β′), three accessory subunits (δ, ε, αo), and during transcription initiation a σ subunit for promoter recognition.[22] There are several σ factors present in the cell, each controlling transcription of a distinct regulon. Of these, the housekeeping sigma factor σA (SigA) is the most abundant. RNAP assembly is a recurring process between transcription cycles. Accordingly, we found fractions containing subunits of the core enzyme capable of transcription (fractions 47–48) with or without accessory subunits (fraction 48), partially assembled subunits (fractions 49–52), or free subunits (fractions 30, 35, 41, 42, 44, 45, 53, 56). Subunit ε (69 aa) was detected in fraction 46 but did not meet the quantitation requirement of three detected peptides.

To globally address protein complex stability, for all proteins found in both replicates elution profiles were analyzed using
correlation profiling. Perfect co-elution of two proteins is reflected by a correlation factor $r$ of 1 (e.g., PheS/T: $1 \pm 0.01$). For proteins that co-elute with multiple interaction partners or that elute in additional fractions, $r$ can be lower (e.g., $0.9 \pm 0.06$ for GlyQ/S, $0.89 \pm 0.02$ for RnjA/B, or $0.96 \pm 0.03$ for GltA/B). Lists of proteins co-eluting with significant correlation factors ($p$ value $\leq 0.01$) and elution profiles of each protein are available (Supporting Information). This data set by no means provides a comprehensive or validated list of *B. subtilis* protein complexes. The chromatographic conditions were not optimized for global protein complex studies but rather to suit the main purpose of antibiotic target identification. To our knowledge the only study globally probing for protein interactions in *B. subtilis* is based on a yeast two-hybrid approach and reports 2613 interactions, including homo-oligomerization and transient interactions. Excluding self-interactions and considering only protein pairs that were identified in both replicates, we found $\approx 16\%$ of these interactions in our untargeted approach.

Antibiotic targets are typically essential. For *B. subtilis* currently 257 essential genes are known, $169 \approx 66\%$ of which were detected in the analyzed TICC fractions. Table 1 gives an overview of current antibiotic targets. It is indicated in the table which of the targets were identified in this study and some rational and experimental evidence of limitations of TICC is provided. The prerequisites and limitations of the current TICC workflow are the following: A) the target has to be a protein, B) the protein of the organism under investigation has to bind the drug, C) antibiotics binding covalently to their targets are removed during protein precipitation and escape MS-based quantitation, D) antibiotics targeting proteins only when in complex with RNA or DNA (see Supporting Information for details) are not suitable since nucleic acids are digested before chromatography, (E) target identification is affected if antibiotic binding to the target requires the presence of a substrate, since these are diluted when the extract is concentrated using flow-through filters (see Figure S4, Supporting Information for platensimycin as example).

Rifampicin, which targets RNAP was used in a proof-of-concept analysis. Compared to the buffer control, in the presence of the protein extract rifampicin was shifted into the additional fractions 46–48 (replicate 1) and 41–49 (replicate 2). The shift fractions contained a total of 208 and 236 proteins in replicate 1 and 2, respectively. Using correlation profiling, the list of proteins co-eluting significantly in both replicates reduced the list of potential targets to two proteins ($p \leq 0.01$: RpoA, RpoB) or six proteins ($p \leq 0.05$: RpoA, RpoB, RpoC, LysS, PdhB, YxkC) depending on the cut-off chosen for $p$ values (Figure 2b).

For both replicates, rifampicin showed the best correlation with its known target RpoB ($r$ of $0.93$ and $0.94$, respectively). We demonstrate that TICC is suitable for antibiotic research. With reasonable instrument time and effort, a large set of potential antibiotic targets (clinically relevant antibiotic targets as well as essential proteins) is being covered and protein complexes are preserved intact. We conclude that this technique is a powerful tool ready to aid the development of critically needed antibiotics.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

Bacillus subtilis, mode of action, protein complexes, protein–protein interaction, rifampicin

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