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Advances and challenges in bacterial compound accumulation assays for drug discovery

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The identification of potent *in vitro* inhibitors of essential bacterial targets is relatively straightforward, however vanishingly few of these molecules have Gram-negative antibacterial potency and spectrum because of a failure to accumulate inside the bacteria. The Gram-negative bacterial cell envelope provides a formidable barrier to entry and couples with efflux pumps to prevent compound accumulation. Assays to measure the cellular permeation, efflux and accumulation of compounds in bacteria continue to be innovated and refined to guide drug discovery. Important advances in the label-free detection of compounds associated with or passing through bacteria rely on mass spectrometry This technique holds the promise of bacterial subcellular resolution and the throughput needed to test libraries of compounds to evaluate structure-accumulation relationships.

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Introduction

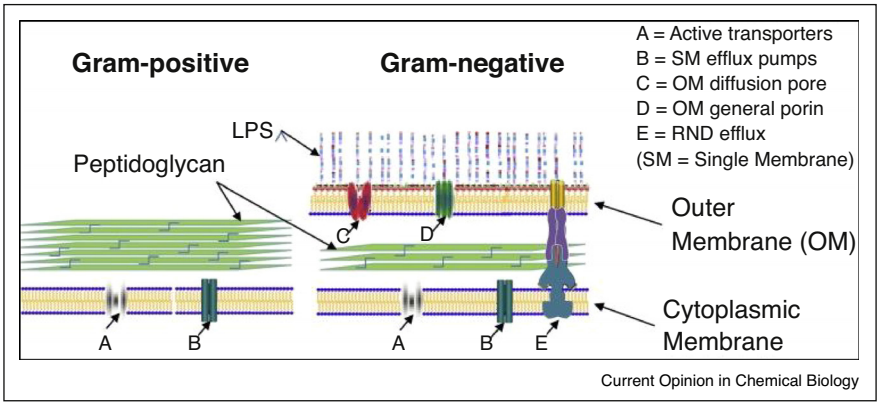
The identification of new safe and effective antibacterial drugs is one of the most important, yet challenging, areas of drug discovery and development [1]. Among the most pressing needs are new treatments for bacterial infections caused by the Gram-negative KAPE pathogens: *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacter species [1,2]. The discovery of new agents that target Gram-negative pathogens is particularly difficult because of the relatively impermeable Gram-negative cell envelope, coupled with efflux pumps that extrude diverse compounds [3]. The Gram-negative cell envelope (Figure 1) includes a unique outer membrane (OM) whose outer leaflet is composed of lipopolysaccharides (LPS or endotoxin). The LPS are cross-linked by divalent cations to provide a permeability barrier, especially to large, hydrophobic compounds [4]. Small, polar nutrients and metabolic substrates (and several classes of antibacterials) permeate across the outer membrane through integral outer membrane β-barrel proteins called porins (Figure 1) [3,5•]. Many classes of biochemically potent compounds have limited permeation across the OM into the periplasm. This, together with the impact of Gram-negative efflux pumps from the Resistance Nodulation Differentiation (RND) family (Figure 1), reduces the intracellular accumulation of compounds and therefore antibacterial activity [6,7].

There are two general approaches for small-molecule antibacterial drug discovery — target-based and phenotype-based. Often the failure to advance leads from both approaches can be attributed to the inability of the compound(s) to accumulate to sufficient intracellular concentrations to halt bacterial growth of clinically relevant strains [8,9]. Therefore, it is clear that the ability to measure the accumulation of compounds in bacteria would help guide SAR within scaffolds and comparisons among scaffolds [3,7,10,11]. Indeed, mammalian whole-cell permeation assays are widely and successfully used in drug discovery and development [12,13]. For example, permeation into and/or transport can be measured across monolayers of Caco-2 or Madin-Darby canine kidney (MDCK) cells (and specific uptake and efflux transporter mutants of these cells) [12,13]. In contrast to Caco-2 and MDCK cells, bacteria do not grow in monolayers with tight junctions, but are usually grown planktonically. This means that assays to measure compound accumulation in bacteria typically need wash steps. Although the mammalian drug permeability and transporter assays have demonstrated utility, the throughput is limited and the standard assays do not provide subcellular/organellar resolution [12,13]. For Gram-negative bacteria, there are essentially two non-contiguous subcellular compartments where antibacterial targets/pathways are located — the cytoplasm and the periplasm, located between the two membranes (Figure 1). Ideally, specific subcellular compound accumulation data would be obtainable, but this is quite difficult because of the small size of bacteria (approximately 2–5 μm in length, comparable to mitochondria) [14]. This review will examine and evaluate the various assay methodologies and technologies used to measure accumulation of compounds in bacteria, with and without subcellular localization.

Methods for dosing and washing bacteria

Assays to measure accumulation of compounds in bacteria have been described for well over seventy years [16].

Figure 1



Comparison of the cell envelopes of Gram-negative and Gram-positive bacteria. The Gram-negative outer membrane bilayer has lipopolysaccharide as the outer leaflet, which contributes to the formidable OM permeability barrier. Transit across the outer membrane occurs via porins (C and D) that permit passage of small, polar compounds and via outer membrane channels of RND efflux pumps (E) which actively expel compounds. The cytoplasmic membrane for both Gram-negative and Gram-positive bacteria also functions as a permeability barrier with transit facilitated by active transporters or permeases (A) and single membrane efflux pumps (B). Figure reproduced from Singh et al with permission [15].

Accumulation assays have two major components — the incubation and washing steps followed by the detection method for the analytes (Table 1). When compounds are incubated with bacteria, the remaining free compound not associated with the bacteria can interfere with many detection methods and usually must be washed away. Classical methods to wash cells include centrifugation [17,18], filtration [19,20], and centrifugation through oil [19–21,22••]. While these methods are useful, they have the common limitation that they have not been successfully adapted to automated high-throughput formats. In addition, centrifugation-based washing requires the cells to be spun and washed over many minutes, increasing the chance for cell lysis and efflux or diffusion of the analyte from the cells [19,23]. Filtration washing requires that the absolute cell numbers used do not completely clog the filter, preventing the supernatant from flowing through. These limited cell numbers translate to lower signal from the analytes. Filtration can also lead to cell lysis and loss of analyte [19]. Although oil centrifugation offers the most rapid wash method for each individual sample, it also requires careful manual aspiration of all layers to isolate the washed bacteria and to avoid contamination with the supernatant [20,21,22••,23]. The oil wash method has also been used in mammalian cell assays of compound accumulation with a similarly limited throughput [23]. Innovations in methodology to enable more efficient and automatable bacterial wash steps could thus provide significant impact towards assay throughput and sensitivity.

Specialized compound detection methods from intact or lysed bacteria

Among the many detection methods for bacterial compound accumulation assays, perhaps the gold standard is a radiometric assay, utilizing radiolabeled compounds [16,17,19–21,24]. Radioactivity offers excellent sensitivity, low background, and straightforward quantitation. However, radiolabeling is impractical for large numbers of compounds because it is resource intensive for production and waste handling. In addition, some compounds have reported instability due to self-radiolysis [25]. Radiometric assays also offer no subcellular resolution.

Other detection methods take advantage of a compound's intrinsic spectroscopic properties and have been used extensively with tetracyclines and quinolones [26–31]. However, few compounds of other classes have useful intrinsic absorbance or fluorescence properties. Furthermore compound analogs may have different signal intensities & detection wavelengths, and there is often background signal, making it challenging to quantitate exposure with this approach [32–34]. Intrinsic fluorescence can also be quenched or enhanced upon binding to bacterial proteins further contributing to these challenges. For example, fluorescence enhancement is seen for tetracycline with the TetR protein, but it remains unclear whether tetracycline analogs bind to TetR with the same affinities and have the same fluorescence enhancements [26,27,31].

Modification by bacterial enzymes inside intact cells is an intrinsic feature of some compounds like beta-lactams that has been employed for accumulation studies [5•,16,35]. Intracellular hydrolysis or modification of compounds can generate a colorimetric or fluorescence signal for exposure. This can be an excellent way to infer the presence of compounds inside the cell and has the added benefit of compartment-specific localization when the