Written responses to open questions of the webinar 'Assay development for measuring antibiotic accumulation in Gram-negative bacteria' by Jessica Blair (JB), David A. Six (DAS) and Mark Brönstrup (MB) and moderated by Alice Erwin (AE). Originally broadcast on 19 May 2020. See webinar recording here: https://revive.gardp.org/assay-development-for-measuring-antibiotic-accumulation-in-gram-negative-bacteria/

Question asked	Response from the speakers
Jessica, what might be other methods of detection of compounds other than by measuring fluorescence?	JB - Various papers have used radioactive compounds. We talk about this in this review article https://mbio.asm.org/content/7/4/e00840-16.abstract Of course, the mass spec methods described by the other speakers is another alternative. DAS - We wrote a review that evaluated many methods of detection but focused on MS methods (DOI: 10.1016/j.cbpa.2018.05.005).
Do you think that the efflux pumps in Pseudomonas could play a role during subsistence phenotype already reported in several species of pseudomonas	AE - I had not heard the term subsistence phenotype, but a very quick search indicates it refers to the utilization of aminoglycosides (maybe other antibiotics?) as carbon source. My guess would be that regardless of whether an antibiotic has a detrimental effect on a bacterial cell process or if it is destined to be degraded, efflux would have essentially the same effect. Anyone else?
For Jessica, Ampicillin MIC (0.5) (if I am correct) for acrB knockout was lower than that for tolC knockout mutant (1.0). How is this explained?	JB - The MIC for WT was 1 and for each of the acrA, acrB orTolC knockouts was 0.25 (all data published). However, the inherent error in this method is known to be one doubling dilution so you can only conclude a real difference if the MIC difference is 2-fold or greater. MICs are relatively insensitive but other methods will show differences in the effect of loss of each component. TolC mutants tend to be affected the most because this is the outer membrane channel for multiple different pumps.
Is there any typical efflux-deficient strain known for Gram-positive bacteria (especially for S. aureus) in order to judge the impact of efflux processes after a comparison to a wild type?	AE - S aureus and other Gram-positive bacteria generally do not have the broad-spectrum multidrug pumps that are characteristic of Gram-negative bacteria. In Gram-positive bacteria, the known efflux pumps are more specific, such as for macrolides

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Will large peptide or oligonucleotides behave similarly to small molecules	AE - Small molecules cross the outer membrane mostly via porins, and peptides and oligonucleotides are too large to go through porins. The cation-mediated process by which aminoglycosides cross the outer membrane ("self-promoted uptake", look for papers by Robert Hancock) is probably the way that peptides and oligonucleotides enter the periplasm. Little is known about how these large molecules reach the cytoplasm (except when they lyse the inner membrane or create large holes in it.) DAS - Larger peptides and oligonucleotides cannot readily penetrate the outer membrane because they fold into structures that are too large to fit through porins and are too polar to cross the bilayers. Small linear peptides can certainly cross the outer membrane through porins. Many Gram-negative bacterial have di-, tri-, and/or oligo-peptide permeases in the inner membrane. These peptides are often the main carbon sources in growth media. Among many natural and synthetic antimicrobial peptides, many Cationic Antimicrobial Peptides (CAMPs), which often penetrate the outer membrane through "self-promoted uptake" or "self-facilitated uptake". The uncharged cyclic peptide natural product argyrin seems to penetrate directly across membranes with conformational changes likely from the bulk solution to lipid environments.
Does accumulation rate vary such that we may see no difference in a static situation like MIC, but may not reach equilibrium in a dynamic situation i.e. when concentrations change rapidly when PK is superimposed	AE - I do not know of any data on this. But it seems reasonable to assume that when bacteria are incubated with a small molecule for several hours in a microtiter plate, the system would reach equilibrium. In an infected animal, where the drug is circulating, exposure of bacteria to the drug may be transient and the end result is very different from the MIC conditions. JB - Accumulation rate or level can correlate poorly with MIC. The reason for this is that the time scales of these experiments are very different. Accumulation (or efflux rate) are measured on short time scales while MICs are measured over the course of ~18h. Accumulation level/efflux rate will be much more predictive over short time scales in a dynamic situation. DAS - MIC values are the clinically relevant gauge of antibacterial activity that when combined with PK help to determine the PK-PD drivers of efficacy for essentially all antibacterial drugs. In vivo, the half-life of most antibacterial drugs is usually longer than the doubling time of the bacteria. Recall that the doubling time for different bacteria under different growth conditions can vary from 20 min to hours.

Question asked	Response from the speakers
Is the flow cytometry technique useful in studying the antibiotics resistance pattern of Enterobacteriaceae?	JB - Yes. The assay works with many species of Enterobacteriaceae (and other bacterial families too). What the assay will allow you to do is to compare relatively how much compound accumulates between different strains.
Do you feel if the bacteria are resistant against one particular antibiotic, increasing the cut-off can solve the issues or shifting to next-generation antibiotic is the only solution?	AE - The cut off (breakpoint) for defining sensitivity vs resistance is intended to predict the outcome of treatment. (For an established drug, it is based on the clinical experience of which MICs are associated with clinical failure. For a new drug, the breakpoint is based on surveys of hundreds of recent clinical isolates.) Raising the MIC cut off would lead you to call an organism sensitive to a drug when in fact using that drug will not treat the patient's infection. When there are many patients with drug-resistant infections, we need new drugs. DAS - There is absolutely no question that we need new drugs, both next-generation drugs in current drug classes and first-generation drugs in new drug classes. IT is also true that in rare cases, clinicians can increase the dose of antibacterial drugs to cover bugs with higher MIC. This is sometimes reflected in the breakpoints for drugs with one breakpoint for Susceptible and a second higher breakpoint for Susceptible-Dose-Dependent. Despite these rare exceptions, for nearly all antibacterial drugs, higher doses are not the solution to resistance (usually because of safety/tolerability).
How selective are the efflux Inhibitors? Do they have any effect on the integrity of the bacterial wall?	JB - Depends on the inhibitor. There are some e.g. PABN which are relatively specific for RND systems. These are commonly thought to be competitive inhibitors that bind in the binding pocket of AcrB although the very latest evidence suggests they work by binding and changing the environment of the pocket rather than directly competing for binding sites. Others are less specific. For example, CCCP is an inhibitor used in many lab experiments and this dissipates the proton motive force so inhibits the function of al systems that rely on this energy source. Regarding the cell wall integrity - yes many of the existing compounds do interfere with membrane integrity at high concentrations but normally this is concentrations higher than used in lab assays. Ideally, new inhibitors should be sought that do not have this effect.

Question asked	Response from the speakers
Could Dr Brönstrup comment a bit more about lack of correlation between uptake/accumulation and MIC?	AE - In my experience, MIC does not correlate with uptake/accumulation, even if you control for potency vs the target (e.g. enzyme IC50). The studies I have done used just one concentration of drug and measured at a single time point. I think it is possible that correlation would be better if the uptake assay used a time course or dose-response or maybe even both - but I have not had the opportunity to pursue that approach. DAS - The correlation between uptake/accumulation and MIC is clear for many control compounds in isogenic pairs of strains with efflux/permeability differences, but as Dr Erwin points out, there are exceptions. It seems reasonable that the target potency-adjusted MIC would correlate best with subcellular accumulation in the target compartment (periplasm vs cytoplasm). New subcellular accumulation assays should be able to evaluate that better. MB - The lack of correlation is also due to different experimental conditions. In our case, uptake is determined from bacteria with a high start OD, low concentration, and short timespans (45 min). Whereas MIC determinations use bacteria at low start OD, have high concentrations and go over 18-24h. Using more similar conditions in experimental setups will probably improve the correlation.
For Mark. We are dealing with compounds which induce the distortion of a cell by causing spheroplastation in presence of partner compound. My question is whether we can evaluate the accumulation of both compounds in distorted periplasm?	MB - This should be possible when appropriate controls are used.
Can radioactivity be another option? I mean if we can selectively synthesize inhibitors with C14 and use the technique to selectively classify the different location.?	AE - Yes, it is certainly possible to measure uptake/accumulation using radioactivity instead of LC-MS or fluorescence. You still have the same issues of how long to incubate and how best to separate bacteria from the unbound compound at the end of incubation, with the same concerns about compound that might be stuck to the surface or might elute back out of the bacteria during washes.

Question asked	Response from the speakers
Out of interest, is there a correlation between efflux pump functionality and rescue systems for stalled ribosomes i.e. tmRNA in response to induction by antibiotics for accumulation profiles? And any speculation on growth at different phases of growth i.e. log and stationary phase.	JB - I am not sure a link has been shown (which certainly does not mean there is not one!). There is some evidence that efflux is differentially important depending on growth phase (Tom Silhavy) and we have a forthcoming paper addressing this!
What is the reason for the increased effect of erythromycin, if I got the graphs right, upon increase in time profile assay?	MB - I am not sure I got the question right. The changing ratio of uptake between WT and efflux strain might be a consequence of changing exporter expression profiles. But we have no experimental evidence to support this hypothesis.
For Dr Six - what method is used for calculating the dipole?	DAS - Venatorx used MOE to calculate dipoles using the 3D descriptors, based on minimized (or bound) conformations of the compounds after preparing them using “wash structures” at pH 7.
For measuring efflux in real-time, would one need to take into account the re-entry of a test compound into the cell; after the cells are energized?	JB - The assays are measured over a short time period and the concentration difference between inside and outside the cell is so great that the compound will initially only really move in one direction - out. We only use the initial drop in fluorescence to calculate the rate. However, the issue you mention is exactly the reason that steady state at the end of these assays must be interpreted with caution.
To Mark: Low Ery MIC in del TolC not correlated to accumulation? Is that kinetic question shorter than 12 h?	MB - The trend in the Ery experiment is right, but the difference in MIC's is much bigger than the differences in uptake, in spite of similar timespans. This tells us that cell proliferation is a complex process that is not just linear with compound concentration at the target. Target saturation above a (target-dependent) threshold over time is a crucial parameter.

Question asked	Response from the speakers
Do you use centrifugation thru a silicon layer instead of filtration and washing?	DAS - While I believe that centrifugation of cells through silicon oil is an excellent method of removing extracellular aqueous volume, it is a human resource and time resource-intensive process for more than a dozen or so samples. Because we routinely ran four 96-well plates in our assays, we chose the alternative filtration/wash method that we published (DOI: 10.1021/acsinfecdis.8b00299). Our filtration/centrifugation wash method to remove compounds not tightly cell-associated is more rigorous and likely removed both extracellular and cell-surface-associated compound. MB - We also apply 'normal' centrifugations in the fractionation assay, or rapid (15 second) filtration steps in the whole cell assay to separate compartments (see Prochnow et al., Anal Chem 2019).
Do you need a lot of bacterial cells to get enough sample to detect antibiotics by mass spec? Can you speak to the sensitivity of the mass spec and the bacterial numbers a little? I would imagine it is a lot of cells.	DAS - The number of bacterial cells needed to detect a specific compound that was incubated with those bacteria depends on 2 major factors: 1) the MS lower limit of detection (LLOD) of the compound in the chosen matrix; 2) the accumulation of that compound in the bacteria. The accumulation of compounds depends on how much compound is added to the cells, how long the compound is incubated, and how many cells are analysed. In practice, we used a cell density corresponding to OD600 of 10 in fresh medium. This large number of cells enabled us to detect compound accumulation even for compounds with high LLOD and/or low accumulation. Of course, with so many cells some compounds accumulate so well that their MS response is above the upper limit of quantification (ULOQ), so we also analysed 10x dilutions (DOI: 10.1021/acsinfecdis.8b00299). MB - David outlined all the important aspects. We use a starting OD between 1 and 5 (also depending on the chemical series and the sensitivity). However, this is still quite different from the start OD of 0.05 used for MIC determinations. LLOD's of our triple quadrupol mass spectrometer is typically in the range of 0.5-50 ng/ml.

Question asked	Response from the speakers
Have you ever tried a positive control (e.g. polymxin B) to show that you can increase accumulation by disrupting the membrane?	DAS - We strongly preferred to use isogenic pairs of bacterial strains with defects in efflux and/or permeability. (DOI: 10.1021/acsinfecdis.8b00299). There are many reasons to caution against using positive controls like polymyxin B/colistin. First, we found that the time course for positive controls to have an effect on accumulation was almost always longer than the standard compound incubation time (15 min). Longer pre-treatment with the positive controls created feasibility issues because our cells were grown exponentially until they reached OD600 of 0.6, whereupon they were concentrated to OD600 of 10 in fresh medium. If we incubated a positive control with the cells during growth the volume would be very large (using a large amount of compound), might affect the growth rate needed to reach the target OD and skewing the pre-incubation time, and might affect the cellular integrity to withstand the various centrifugation/filtration concentration & wash steps. Specifically, we found that polymyxin B/colistin caused a loss of cellular integrity (by flow cytometry) and CFU in our assay conditions. It is critical that the cells are intact at the end of the accumulation experiment. MB - Again, I agree with David. If you think of permeabilizing, the polymyxin B nonapeptide (PMBN) is the better agent, as it is devoid of antibacterial activity.
Methods for the accumulation of many antibiotics (radiolabelled or fluorescent) have been reported since the early 1990s, especially fluoroquinolones. Length of the assay was shown to be crucial and using low temperatures - without low temperature at the wash stage gives inaccurate low values are obtained even if kinetics are still observed. Also, values normalised to cell protein, not CFU due to cell death issues. Please can Dr Brönstrup say yes or no to whether centrifuging at 4oC?	DAS - We wrote a review that evaluated many methods of detection but focused on MS methods (DOI: 10.1016/j.cbpa.2018.05.005). There are several caveats to using low temperatures at any stage in accumulation assays. The top concern is that low temperatures affect membrane integrity in the short-term and temperature shifts exacerbate this problem when combined with centrifugation/filtration steps. Assuming cells are washed well to remove proteins released from both living and dead cells, cell protein can be used for normalization, but in practice, this is both human resource and time resource-intensive for more than a dozen or so samples. CFU normalization is also human and time resource-intensive for more than a dozen or so samples. We used four 96-well plates for our assay and found that OD600 was a reasonable normalization method (DOI: 10.1021/acsinfecdis.8b00299). MB - We usually incubate at 37°C to reflect the conditions of infection. However, all the following fractionation steps are performed at 4°C. In our plate assay, removal of media and wash steps are done at room temperature for technical reasons, but the steps are fast.

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Can cell dry weight also be used to normalise values?	AE - Yes, an assay could be normalized using dry weight, viable cell counts, or optical density DAS- the caveat with viable cell counts in colony-forming units or CFU is that quickly bactericidal compounds can affect CFU. We found this to be problematic for both polymyxins and fluoroquinolones. Polymyxins have the additional problem of inducing rapid lysis, while FQs do not.
Deletion mutants have 100s of gene expression changes so how do you know that the results are due to lack of tolC or another protein?	AE - For validating an uptake assay, we ask whether a mutation like tolC deletion has the same effect on both the MIC assay and the uptake assay. It is certainly true that deletion of tolC has many effects on bacterial physiology. JB - The impact of deletion of any of the efflux components is multifaceted and there many direct and many indirect effects. I agree you can correlate with MIC testing but we would also complement the deletion by putting tolC back in to make sure the phenotype would revert to ensure we are looking at the effect of TolC loss. DAS - To my knowledge, there have not been reports of efflux-independent effects of tolC deletion on MIC of antibacterial compounds. In contrast, there are multiple independent validations of the effect of tolC gene deletion on MIC and accumulation. 1) Dozens of papers have confirmed the relationship between TolC-mediated efflux, cellular accumulation, and MIC; 2) Efflux pump inhibitors recapitulate the deletion of efflux gene(s) on MIC. 3) The MIC shift upon tolC deletion can be recapitulated with the deletion of inner membrane efflux pump gene(s). More importantly, the reintroduction of a single efflux pump gene to the strain lacking all efflux pump genes could raise MIC.

Question asked	Response from the speakers
What type of overexpressors lose resistance? Not been widely published - usually accepted that such mutants are stable and no drug is required to maintain the phenotype	AE - Are you asking about screening expression libraries for clones that are less susceptible to antibiotics? Maybe Eric Brown at McMaster? JB - Not totally sure I understand the question but in clinical isolates, we often see overexpression of efflux systems caused by mutations in regulatory genes such as MarR or AcrR. This over-expression phenotype is maintained even with no drug present. DAS - Because any fitness cost of efflux overexpression is negligible, such mutants should be grown without selective pressure. In the absence of selective pressure, efflux pump overexpression could theoretically be lost due to suppressor mutations or reversion mutations. While these events are usually very rare, positive control compounds should always be run to confirm the expected phenotype of the efflux overexpression strain.
Efflux pumps are expressed more highly at early mid-log phase, so does Mark Brönstrup's assay better measure update?	MB - Also other assays are done with cells at the early mid-log phase. The most precise picture is probably obtained with long-term assays over 12-24h, that would correlate time-dependent uptake with efflux pump expression. Such studies are elaborate, though, and could only be performed for very few reference compounds.
Jessica Blair: Did I understand well that you measure the accumulation of fluorescently labelled antibiotics? What is the effect of the fluorophore on the internalization/efflux?	JB - No we have not done this but in theory, you could. We have used fluorescent dyes and drugs that are inherently fluorescent (e.g. fluoroquinolones). There are a few groups now able to produce fluorescently labelled drugs and these could be tested (e.g. Mark Blascovich).
Is there any typical efflux-deficient strain known for Gram-positive bacteria (especially for S. aureus) in order to judge the impact of efflux processes after a comparison to a wild type?	AE - S aureus and other Gram-positive bacteria generally do not have the broad-spectrum multidrug pumps that are characteristic of Gram-negative bacteria. In Gram-positive bacteria, the known efflux pumps are more specific, such as for macrolides

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Can anyone recommend the best broad-spectrum efflux pump inhibitor to use in whole-cell biochemical assays? This would be instead of using efflux pump knockouts.	JB - It depends on what you want to do. Happy to help if you email me directly. PABN targets all RNDs tested and as long as it is used as low enough concentrations the membrane effects can be minimised. Also seems to mimic a knockout reasonably well but if this is for screening purposes then a knockout would be much better because the way PABN works means it would affect some substrates more than others and you could get unfavourable interactions. CCCP will inhibit most efflux pumps as many rely on the PMF but this has a huge impact on cellular physiology.
Is there a requirement for the compounds with regard to physicochemical properties, e.g., solubility, lipophilicity for these assays to be useful?	AE - Solubility and lipophilicity certainly affect the total accumulation of compounds within bacteria. Also, they affect distribution of compounds across different parts of the bacterial cell (cytoplasm, periplasm, the two membranes) DAS - For any assay, practical considerations are critical and include: 1) solubility at concentrations and buffer/temperature conditions used in the assay; 2) compound stability in the assay conditions (buffer/temp/time); 3) recoverability in the assay conditions (taking into account binding to proteins/plastics); 4) dose-dependent detectability by the method being employed.
I would like to ask Jessica Blair about her slide showing the RND efflux pump (AcrAB-TolC pump). Recently, cryo-EM determined several types of efflux pumps including RND, ABC type pumps. In many recent data, membrane fusion proteins like AcrA, MacA directly interact with outer membrane protein and inner membrane transporter by bridging two proteins. I think the architecture model of the RND efflux pump in your slide is out of fashion. The architecture is hardly acceptable anymore.	JB - You are quite right. The simple schematic graphics I used are from an old slide deck that I have not updated. The cryo-EM structures and beautiful and updating these schematics are on the to-do list!

Question asked	Response from the speakers
Jessica, it is fine for dyes. In case you are working with your own compounds, the methods are useless. You may use fluorescence if applicable. The other issue is bacterial separation on FACS. It is very tricky due to the size of bacteria.	JB - In our paper regarding the method we show that you can get very good separation between background noise and bacterial cells. There are a few tricks here - filtering your buffer through a small filter to remove much of the background helps as does keeping the flow cytometer super clean with lots of washes makes a big difference. The other thing we did in the paper was use a dye (e.g. syto dyes) to stain the cells. These are DNA intercalating dyes and the accumulation of them is not affected by efflux. We use the fluorescence of the stain to gate for bacterial cells and then measure dye accumulation only in those events. I was clear I think in the webinar that this has currently only been used for fluorescent compounds. However, this could be adapted to look at the competition between substrates and the efflux assays would be particularly good for this. DAS - Flow cytometry analysis of bacteria can be tricky with older instrumentation and all samples need to have been pre-filtered to remove background particles. In addition to Dr Blair's work, I have published three recent papers that made use of bacterial fluorescent flow cytometry (DOI: 10.1074/jbc.M117.814962; DOI: 10.1111/mmi.14098; DOI: 10.1194/jlr.RA120000654)
Prof. Brönstrup: Are you planning to use it for *S. aureus*?	MB - We could develop a whole-cell assay for *S. aureus* that separates the cytoplasm from the membrane. But our focus is Gram-negative bacteria and mycobacteria for the moment.
What are the concentrations used? Over MIC? Below MIC? The same for all the strains?	AE - Assuming this refers to assays of accumulation: Most studies comparing several compounds have used just one concentration (such as 10 uM) that may be above the MIC for some compounds and below it for others. This might be justified by showing that in the conditions of the assay (usually incubating 15-20 minutes or less), bacteria remain viable. DAS - The ideal assay is done with multiple doses and multiple incubation times. For practical reasons, a single dose and time-point is often used but carries many caveats. Single-doses risk being too low for detection of all compounds tested and/or being so high that solubility/linear range is exceeded. It is good to test accumulation in matched

	(isogenic) strains with differential efflux/permeability [and MIC]. Comparing accumulation and MIC in these matched strains is often more valuable than for unrelated isolates.

Question asked	Response from the speakers
We have touched upon efflux, what assays can illustrate the importance of porins and uptake INTO the cell	AE - Both David Six and Mark Brönstrup talked about total accumulation, the net effect of influx and efflux. Measuring transit across porins is very tricky. Beta-lactams crossing through porins can be measured by liposome swelling assays, but this is a very difficult assay; hardly anyone except the Nikaido lab can get this to work. JB - The work of Helen Zgurskaya is worth a look as she has done some nice work looking at the importance of porins using strains with engineered porins with the larger size. It is important to note that this is also drug-specific, and the effect will be different for different drug classes. DAS - There are E. coli bacterial strains lacking up to 4 porins (see Dirk Linke paper DOI: 10.3389/fcimb.2017.00464). These bacterial show higher MIC to a large number of antibacterials and can serve as the host for exploring the pore specificity of various porins. There are many other species with single & double porin deletions that also show a shift in MIC to various antibacterials *(K. pneumoniae* and *A. baumannii* see www.KeMyth.com; DOI: 10.1093/jac/dkx285 and DOI: 10.1016/j.ijantimicag.2020.105918)
Are the Novartis P. aeruginosa efflux mutants different than those reported by Schweizer over several publications?	DAS - Novartis obtained many strains from Keith Poole and Herbert Schweizer. Charles Dean's group then made many further mutants and collections of isogenic mutants that were used in many publications to evaluate the effects on antibacterial activity for many classes of antibacterial. Many of these isolates were donated to NIAID and deposited at BEI Resources. I always recommend using published strains with well-defined genotypes and phenotypes - especially MIC assays done according to CLSI guidelines to facilitate inter-lab reproducibility.
Does compound accumulation/efflux change when bacteria are grown in more physiologically relevant conditions (ex. tissue culture media, serum, sputum etc..)?	JB - Almost certainly. I am not sure if anyone has directly this but we do know that expression of efflux systems changes in physiologically relevant media and also during infection so I would expect this would alter the amount of efflux/accumulation. DAS- Agreed. There is some data from Françoise van Bambeke and co-workers: DOI: DOI: 10.1093/cid/cis473; 10.3390/antibiotics9050218). Work by Victor Nizet and co-workers is also relevant: DOI: 10.1038/s41597-019-0051-4; DOI: 10.1093/jac/dkv487; DOI: 10.1016/j.ebiom.2015.05.021)

Question asked	Response from the speakers
How much useful is using a liposome reconstituted transport protein for measuring efflux?	AE - Two issues make liposomes more difficult than bacteria for measuring efflux: First, efflux is energy-driven. You would need to energize the liposomes. This can be done using membrane vesicles derived from bacteria (see Kaback in the 1970s or so) but would be more difficult in artificial liposomes. Second, the efflux pumps of greatest interest with regard to antibiotic discovery are the RND pumps, which span both outer and inner membrane. It is possible to reconstitute these in an artificial system (see Zgurskaya). JB - The beautiful work of Martin Picard has shown that tripartite systems can be reconstituted in two separate liposomes and active efflux can be measured by the transport of a fluorescent substrate. This system has the potential to be very powerful and could teach us a lot about the dynamics of the systems etc. DAS - The direct measurement of efflux in reconstituted systems is just one of several important facets to the clinical relevance of efflux. Three other critical variables are 1) the rate of entry of compounds into the periplasm; 2) the intrinsic potency of the antibacterial compound, and 3) the expression level of the relevant efflux pumps*. Together these factors strongly affect the relevance of efflux pumps for a given antibacterial compound against a given bacterial isolate. *The expression levels of efflux pumps are quite dynamic with constitutive and/or inducible expression in addition to high-frequency mutational upregulation.
How can external researchers gain access to tools, strains, and assays developed by GARDP and its partners?	AE - The research described by the speakers on May 19 was not supported by GARDP. Each of them came from a different lab with its own support. Some resources you might want to be aware of include the SPARK database and the bacterial strain collection in BEI Resources (https://www.beiresources.org/). DAS- In addition to BEI, many excellent E. coli tool strains are available from the Coli Genetic Stock Center at Yale (https://cgsc.biology.yale.edu/). Dr. Colin Manoil (University of Washington) also has transposon mutants of all non-essential genes in *P. aeruginosa*, *K. pneumoniae*, & *A. baumannii* (https://www.gs.washington.edu/labs/manoil/libraries.htm). JB - The mass spec assays described in my talk are part of platforms of the IMI programs 'ENABLE' (ending soon) and 'AMR Accelerator'. If one gets accepted to these programs, the platform is accessible. Otherwise, a direct collaboration with our or other mass spec labs (e.g. from Hergenrother) is possible.