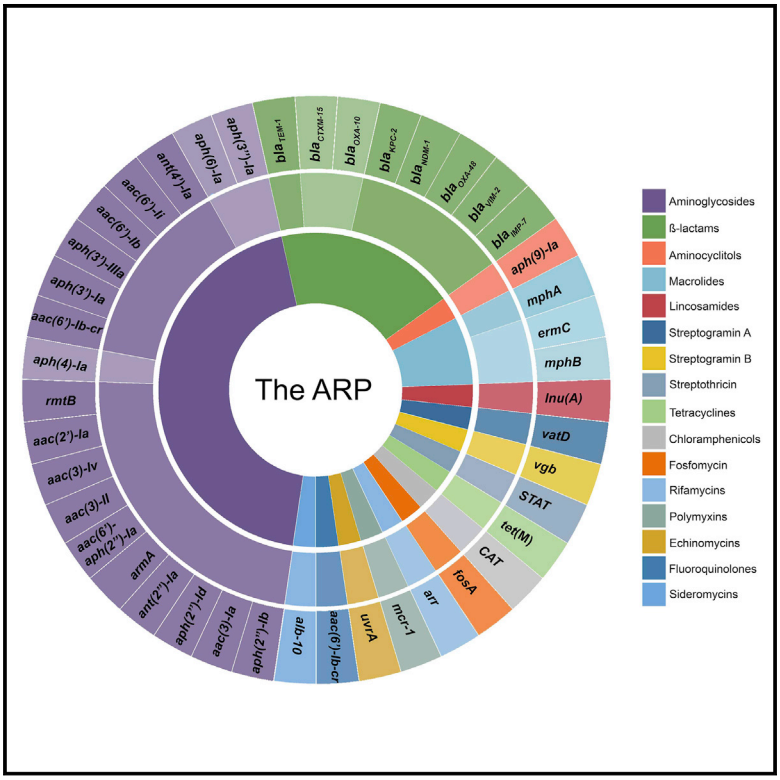
Resource

Cell Chemical Biology

A Common Platform for Antibiotic Dereplication and Adjuvant Discovery

Graphical Abstract



Highlights

- Development of a uniform antibiotic resistance platform (the ARP)
- The ARP is a versatile tool with dual functions
- The ARP allows for the discovery of new antibiotic adjuvants
- In dereplication mode, the ARP identifies common antibiotics

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In Brief

The antibiotic resistance platform (ARP) is a dual functioning system developed by Cox et al. to both identify new antibiotic adjuvants and to dereplicate known naturally occurring antibiotic scaffolds. The ARP will have broad application in confronting the antibiotic resistance crisis.

Cox et al., 2017, Cell Chemical Biology *24*, 98–109
January 19, 2017
http://dx.doi.org/10.1016/j.chembiol.2016.11.011

Please cite this article as: Cox et al., A Common Platform for Antibiotic Dereplication and Adjuvant Discovery, Cell Chemical Biology (2017), http://dx.doi.org/10.1016/j.chembiol.2016.11.011

A Common Platform for Antibiotic Dereplication and Adjuvant Discovery

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http://dx.doi.org/10.1016/j.chembiol.2016.11.011

SUMMARY

Solving the antibiotic resistance crisis requires the discovery of new antimicrobial drugs and the preservation of existing ones. The discovery of inhibitors of antibiotic resistance, antibiotic adjuvants, is a proven example of the latter. A major difficulty in identifying new antibiotics is the frequent rediscovery of known compounds, necessitating laborious "dereplication" to identify novel chemical entities. We have developed an antibiotic resistance platform (ARP) that can be used for both the identification of antibiotic adjuvants and for antibiotic dereplication. The ARP is a cell-based array of mechanistically distinct individual resistance elements in an identical genetic background. In dereplication mode, we demonstrate the rapid identification, and thus discrimination, of common antibiotics. In adjuvant discovery mode, we show that the ARP can be harnessed in screens to identify inhibitors of resistance. The ARP is therefore a powerful tool that has broad application in confronting the resistance crisis.

INTRODUCTION

The antibiotic crisis is one of the most pressing global health care challenges of the 21st century (Laxminarayan et al., 2013). The discovery and development of antibacterial drugs over the past 75 years has transformed not only infectious disease therapy, but also enabled much of modern medicine. With antibiotics in hand, clinicians undertook procedures unthinkable in the pre-antibiotic era. These life-saving and life-extending procedures are now at risk due to the evolution of resistance to our current drugs, coupled with a lack of new antibiotics coming to market. The reasons for the lack of new drugs are complex, involving changing priorities in the pharmaceutical industry in the face of a difficult regulatory environment and poor economic prospects. However, even if these issues could be managed easily, the scientific hurdles to new antibiotic discovery remain extremely challenging.

Chief among these challenges is the difficulty in identifying safe and effective antibiotic compounds. The major source of antibiotics during the "Golden Age" of discovery were natural products produced by bacteria and fungi (Brown and Wright, 2016). Of the principle antibiotic scaffolds in current clinical use only three, the fluoroquinolones, the sulfonamides, and trimethoprim are synthetic compounds not derived from microbial specialized metabolism. Efforts over the years to identify novel antibiotic scaffolds from synthetic chemical libraries have failed, and mining of soil bacteria or fungi generally identifies known scaffolds. The elimination of known antibiotics in natural product extracts, a process termed dereplication (Gaudencio and Pereira, 2015; Ito and Masubuchi, 2014; Nielsen et al., 2011; Tawfike et al., 2013), is laborious and resource intensive. Notwithstanding recent efforts to mine rare or previously unexplored microbial diversity for new antibiotics (Ling et al., 2015), faced with the challenge of dereplication and the poor track record of synthetic chemicals in this field, the pharmaceutical industry has pivoted toward more tractable disease areas.

With no new antibiotics on the horizon, efforts to preserve existing ones by blocking resistance with antibiotic adjuvants have emerged as a viable strategy. In this scenario, antibiotics are co-formulated with an inhibitor of resistance (Baym et al., 2016; Drawz and Bonomo, 2010; Wright, 2000). The inhibitor blocks the resistance element, freeing the antibiotic to target the bacterium. The first example of this approach was clavulanic acid, discovered in the 1970s (Reading and Cole, 1977). Clavulanic acid is a covalent inactivator of many serine β-lactamases that confer resistance to β-lactam antibiotics (Drawz and Bonomo, 2010). Since then, several co-formulations of β-lactam antibiotics and β-lactamase inhibitors have been brought to market or are in clinical trials (Bush, 2015; Drawz and Bonomo, 2010). This adjuvant strategy offers an orthogonal mechanism to new antibiotic discovery in preserving our existing drug arsenal.

Recognizing the challenges of antibiotic dereplication and the opportunity of adjuvants in drug discovery, we have developed a common antibiotic resistance platform (ARP) (Figure 1) that can be used for both adjuvant discovery and antibiotic dereplication. The use of antibiotic resistance in dereplication was proposed and applied >50 years ago (Stapley, 1958). However, previous anecdotes mostly relied on the use of poorly characterized mutant strains (Eisman et al., 1946; Stansly, 1946; Stapley, 1958). Harnessing the insight gained and the advances made in the field of antibiotic resistance over the last 50 years has allowed for curation of the ARP. The strength of the ARP lies within the mechanistic diversity and rigorously curated substrate

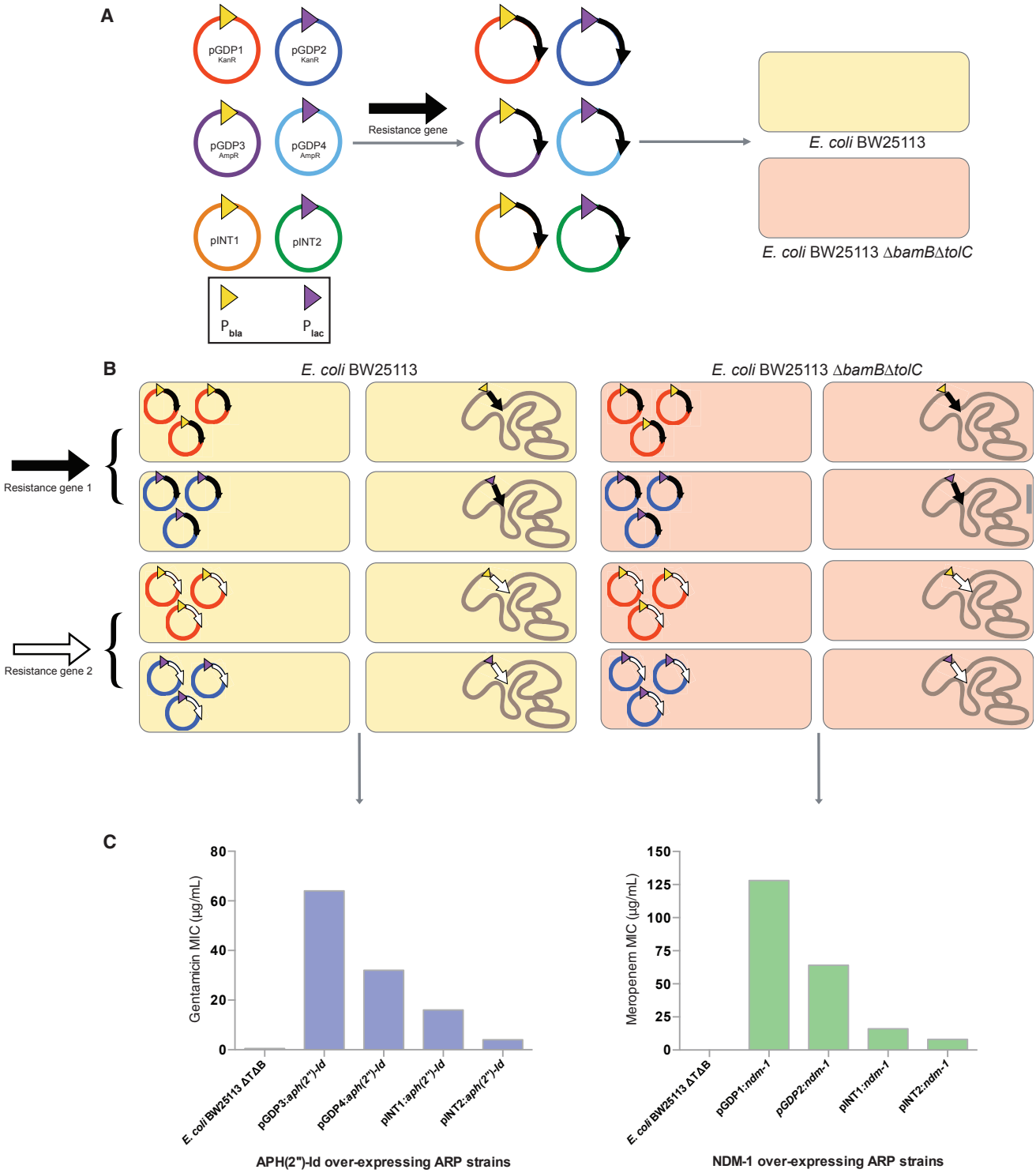

Figure 1. Overview of the Antibiotic Resistance Platform

For tight regulation of gene expression the ARP is composed of two series of plasmids; the pGDP series (low-copy-number plasmid-based system) and the pINT series (single-copy integration of genes into the chromosome). Gene expression is further regulated by the utilization of two different promoters (strong P_{bla} promoter and the weaker P_{lac} promoter). Antibiotic resistance genes can be ligated into the pGDP plasmids and/or integrated into the chromosome to achieve the desired level of gene expression. The constructs are then transformed into wild-type *E. coli* and/or the hyperpermeable, efflux-deficient mutant *E. coli* BW25113 Δ*bamB*Δ*tolC* (A). This process is iterative and repeated (as depicted by the arrows) for all antibiotic resistance genes (n = 42) in the array, creating the ARP (B). Regulation of minimum inhibitory concentrations (MICs) in the ARP (a reflection of gene expression levels) (C). See also Figure S1 and Table S1.

specificity of our resistance strains. The platform consists of a cell-based library of *Escherichia coli* expressing individual resistance genes. Using either a low copy plasmid or integration of the gene into the bacterial chromosome, we control resistance gene copy number. Expression is further regulated through the use of two promoter systems differing in strength. To our knowledge, no other descriptions of such a comprehensive tool in the public domain have been reported; indeed, any comparators remain shrouded in the confines of industry. We believe our platform will become the new gold standard for antibiotic dereplication and the identification of potentiators of existing antibiotics. Here we report development of the platform and demonstration of its versatility in both modes.

RESULTS

Platform Design: Construction of a Heterologous Expression System

Tight regulation of gene expression levels is crucial for a robust and sensitive screening platform. The ARP consists of a panel of resistance genes present in either a low copy plasmid-based system and/or in single copy via integration into the *E. coli* chromosome. For the plasmid-based expression system, four plasmids were constructed (pGDP series) (Figures 1 and S1). The origin of replication is derived from the widely used pBR322 plasmid (Bolivar et al., 1977) and the multiple cloning site (MCS) was modeled on a common pET vector to allow for facile movement of genes between systems (Figure S1). The pGDP series can be grouped based on selective marker (kanamycin; pGDP1 and pGDP2, or ampicillin; pGDP3 and pGDP4) and promoter strength (Figure 1). By utilization of two constitutive promoters of differing strength, the stronger β-lactamase P_{bla} promoter (pGDP1 and pGDP3) and the weaker P_{lac} promoter (pGDP2 and pGDP4), we are able to further tune expression levels (Figure 1C). Single-copy integration of genes into the chromosome was achieved by construction of the pINT plasmids (Figure 1), which enable integration into the arabinose operon in *E. coli* (Figure S1). These plasmids have the same MCS and promoters as the pGDP series (Figure S1).

Finally, to enable variations in the intracellular concentration of compounds, the platform was introduced into both wild-type *E. coli* BW25113 and a hyperpermeable, efflux-deficient mutant of *E. coli* BW25113 Δ*bamB*Δ*tolC* (King et al., 2014) (Figure 1). This strain has increased sensitivity to a number of antibiotics (Table S1).

Platform Development: the Antibiotic Resistance Gene Array

The ARP is comprised of >40 genes, which mediate resistance to >15 classes of antibiotics (Figure 2 and Table S1) and is designed to grow as new resistance genes are entered into the platform. We selected a wide-range of genes that are ideal candidates for the discovery of antibiotic adjuvants due to clinical prevalence. For utilization of the platform in antibiotic dereplication, we also selected genes that confer resistance to antibacterial natural products that have been categorized as being "high-frequency of discovery" (Baltz, 2005).

All genes were introduced into plasmids from the pGDP series and assessed for their ability to mediate antibiotic resistance (Table S1). In most cases we observed higher levels of resistance for genes under the control of the P_{bla} promoter, and lower levels of resistance when under the control of P_{lac} (Figure 1C and Table S1). Accordingly, for improved sensitivity during antibiotic adjuvant discovery screens, a selection of candidate genes were also integrated into the chromosome using the pINT series, which resulted in further reduction of gene expression (Figure 1C and Table S1).

Development of Antibiotic Dereplication Procedures Using the ARP

We designed the ARP for use in dereplication of antibiotics in natural product extracts derived from Actinobacteria; if an extract contains a known antibiotic, the antibacterial activity of this strain would be reduced in the presence of its cognate resistance gene. The substrate specificity of the resistance gene would reveal the identity of the antibiotic produced.

For increased robustness, we selected constructs that confer high levels of resistance (Table S1) and configured ARP genes into a 96-well plate format (Figure S2A). As a proof of principle, we selected two well-characterized producers of known antibiotics; *Amycolatopsis mediterranei* (rifamycin) (Verma et al., 2011; Zhao et al., 2010) and *Saccharopolyspora erythraea* (erythromycin) (Oliynyk et al., 2007). We successfully developed two ARP dereplication strategies (Figures 3A, 3B, S2, and S3). The first involves an agar overlay (Figures 3A and S2); the antibiotic-producing bacteria are spread over the surface of rectangular agar plates and cellulose ester paper placed on top. Following incubation, the paper is removed along with residual surface growth and the agar overlaid on the basal layer. The ARP is pinned onto the surface, followed by incubation (Figures 3A and S2). This strategy is readily amenable to high-throughput analysis of antibiotic-producing strains. Antibiotic production was detected in both control strains (Figure S2); for the *A. mediterranei* fermentation, only the ARP strain overexpressing the Arr ADP-ribosylating rifamycin resistance enzyme (pGDP3:*arr*) was able to grow on the conditioned agar (Figure S2C). Similarly, dereplication of *S. erythraea* was also successful, with only the macrolide methyltransferase ErmC (pGDP4:*ermC*), and the macrolide phosphotransferase MphA (pGDP3:*mphA*) ARP strains growing (Figure S2D).

The second dereplication method involves an agar plug procedure, where a section of fermented solid medium from the antibiotic producer is removed and the plug placed onto a lawn of an indicator ARP strain (Figure 3B). Antibiotic production is detected by measurement of the zone of inhibition surrounding the agar plug (Figures 3B and S3). ARP strains that show a decrease in the inhibitory zone area are able to resist the antibacterial agent, and therefore identify the antibiotic produced, as demonstrated with both control strains (Figure S3). This approach is also scalable for high-throughput implementation, especially to identify strains producing multiple antibiotics.

Rapid and Facile Dereplication of Antibiotic-Producing Actinobacteria

To investigate the robustness of dereplication, we selected ~60 strains from our in-house library of environmental actinomycetes that exhibit antibacterial activity against Gram-negative members of the ESKAPE pathogens (Boucher et al., 2009). Using

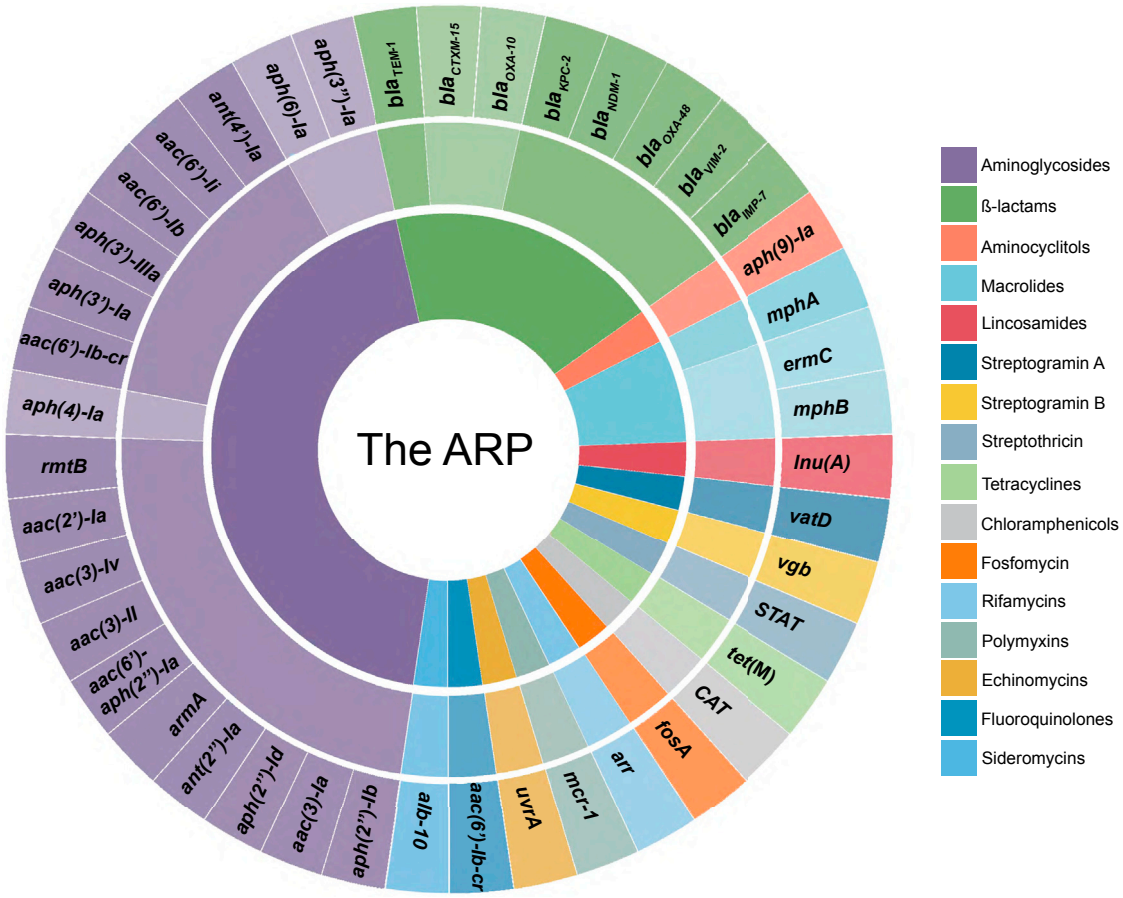

Figure 2. Antibiotic Resistance Spectrum of the ARP

The ARP consists of resistance elements to 15 classes of antibiotic, represented by the inner wheel. Each class is represented by a unique color (see key). The middle wheel divides the 15 classes into individual antibiotics found within each class (see Table S1). The outer wheel depicts the array of resistance genes within the ARP, grouped based on antibiotic substrate specificity.

the agar plug method described above, we successfully dereplicated this collection of unknown bioactive actinomycetes.

Overall, we observed that ~42% of these strains were producing streptothricin (Figure 3C), consistent with accounts that this antibiotic is a commonly found antibacterial natural product (Baltz, 2005). Analysis of these streptothricin-producing strains revealed them to be phenotypically and genotypically diverse (Figure S4). These strains were isolated from soil samples from >10 distinct locations. The second most commonly identified known antibiotic was streptomycin (~7% of strains). Liquid chromatography-mass spectrometry (LC/MS) confirmed streptothricin/streptomycin production. We also detected one strain producing a tetracycline antibiotic (Figures S2E and S3; WAC6725), which high-resolution mass spectrometry (HRMS) revealed to be oxytetracycline, with a mass of 460.1505 Da. Finally, a macrolide-producing organism was also identified (WAC9292) (Figure S2F), which HRMS (525.3282 Da) revealed to be pikromycin.

Dereplication Allows for the Enrichment of Rare Antibacterial Agents

Dereplication enabled us to rapidly weed out >50% of the antibiotic-producing environmental actinomycetes (Figure 3C), enriching for more unusual antibacterial agents. We found that three strains (WAC2567, 5858, and 6428) were producing derivatives of the bis-intercalating echinomycin peptides. Activity-guided purification of one of these strains (WAC2567) allowed for the identification of four known echinomycin antibiotics (Figure 4) (Socha et al., 2009); echinomycin, FD-991, depsiechinoserine, and echinoserine. The structure of purified echinomycin was confirmed by multidimensional nuclear magnetic resonance (NMR) spectroscopy (Table S2). We also detected the presence of a new molecule characterized as a sulfoxide derivative of echinoserine (Figure 4).

Since we identified three strains producing echino-peptides, we investigated known resistance mechanisms to this class of antibiotic. The excinuclease ATPase UvrA, identified within the echinomycin biosynthetic gene cluster (Watanabe et al., 2006), has been implicated in self-resistance during biosynthesis. We amplified the *uvrA* gene from the genome of WAC2567 and ligated the resulting amplicon into the pGDP series of plasmids (Table S1). Utilizing the agar plug dereplication method, *E. coli* overexpressing *uvrA* (pGDP1:*uvrA*) resulted in a reduction in the zone of inhibition of a fermented WAC2567 agar plug (Figure S5), demonstrating the flexibility of the ARP as a living platform that incorporates new mechanisms.

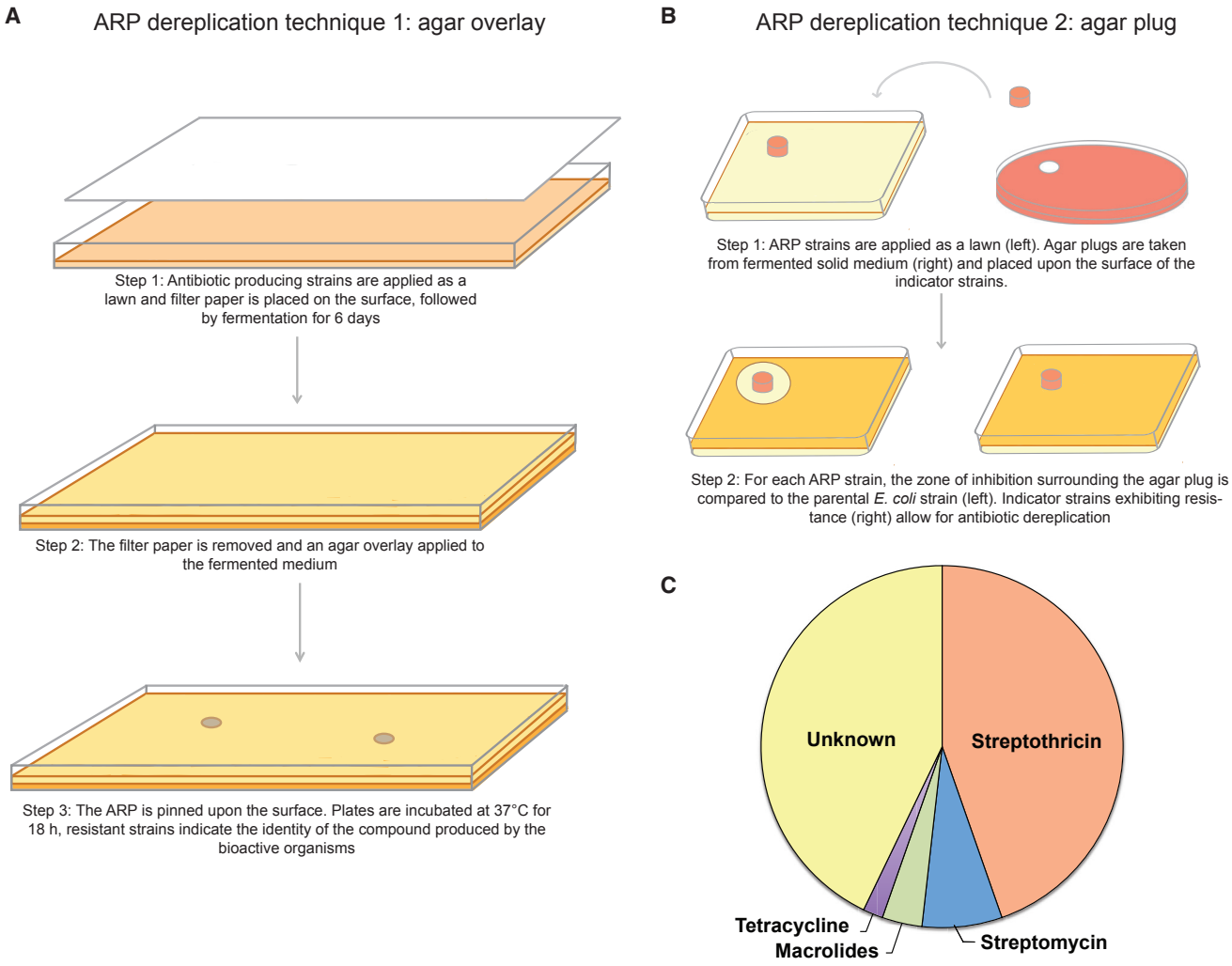

Figure 3. Antibiotic Dereplication Techniques Using the ARP

Agar overlay technique (A), agar plug technique (B), and dereplication of a collection of unknown antibiotic-producing actinomycetes (n = 60) using the ARP (C). In one step, it is possible to weed out >50% of known antibiotic-producing actinomycetes. See also Figures S2 and S3.

We next investigated two strains (WAC7452 and 7443) producing a dark brown bioactive pigment with UV/visible maxima of 233, 304, 326, and 442 nm. HRMS of the compound revealed a molecule with a mass of 463.1614 Da. A search of known compounds with an absorbance range of 440–450 nm, and our observed mass identified the unusual and poorly characterized angucycline, mayamycin (Schneemann et al., 2010) (Figure 4). The structure of the purified compound was confirmed by NMR spectroscopy (Table S3) and susceptibility testing determined that mayamycin has a minimum inhibitory concentration (MIC) of 16 μg/mL in *E. coli* Δ*bamB*Δ*tolC*.

Finally, six strains (WAC5484, 1512, 6076, 6620, 6428, and 6435) were found to be producing an orange/brown-colored water-soluble compound with UV/visible maxima of 280 and 420 nm, and HRMS revealed a mass of 1,045.2962 Da. Searching for known compounds with this mass did not reveal any suitable candidates. However, addition of a chelating ion-exchange resin reduced the mass to 992.3 Da, equivalent to the loss of an Fe^{3+} ion. Searching once more for known compounds with this mass drew our attention to the sideromycin, albomycin (Reynolds and Waksman, 1948; Stapley and Ormond, 1957). The identity of the compound was confirmed by mass fragmentation (Figure 4) and the previously identified albomycin biosynthetic cluster (Zeng et al., 2012) was located within the genome of WAC5484. During activity-guided purification, the compound exhibited potent activity against Gram-negative bacteria; however, spontaneous mutants readily emerged. Whole-genome sequencing of one of these mutants identified a nucleotide polymorphism (41× coverage) within the *fhuB* gene, the membrane subunit of the Fe^{3+} hydroxamate ABC transporter (Koster and Braun, 1986). Previous studies have shown that albomycin uptake relies on this ferric hydroxamate transport system (Pramanik and Braun, 2006). Indeed, these highly resistant uptake mutants can be utilized for dereplication of albomycin and other sideromycin antibiotics (Braun et al., 2009). However, to enable future dereplication efforts of albomycin specifically,

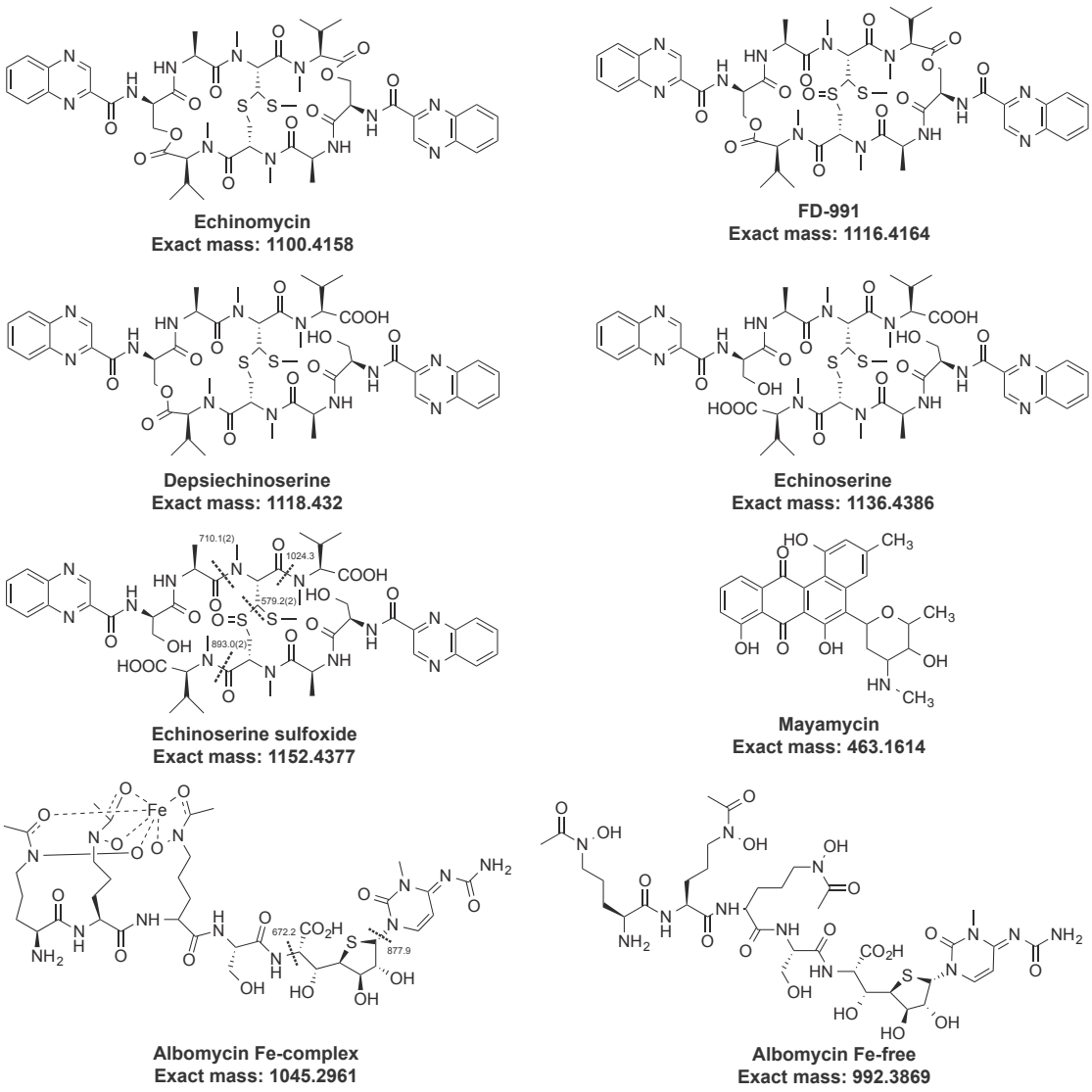

Figure 4. Bioactive Molecules Identified Following Dereplication Enrichment

All compounds are labeled and the high-resolution mass obtained during this study shown. For the new echino-peptide identified in this study (sulfoxide-echinoserine), the tandem mass spectrometry ion fragments are also shown $[M-H]^+$. Ions 710.1, 579.2, and 893.0 are fragments (MS^3) of the parent ion 1024.3 (MS^2). The tandem mass spectrometry ion fragments $[M-H]^+$ of the parent ion are also shown for albomycin. See also Tables S2 and S3.

the self-resistance seryl-tRNA synthetase gene (*alb-10*) (Zeng et al., 2009) from the albomycin biosynthetic cluster was included in the ARP. Overexpression of *Alb-10* (pGDP2:*alb-10*) resulted in a reduction in the zone of inhibition surrounding fermented agar plugs, and a 4-fold increase in *E. coli* BW25113 Δ*bamB*Δ*tolC* albomycin MIC levels (Table S1).

Utilizing the ARP for the Discovery of Antibiotic Adjuvants

The ARP can be utilized in cell-based screens of chemical libraries for the discovery of antibiotic adjuvants. Here the advantage of the varying MICs associated with the ARP is critical in selecting a strain with an appropriate screening window, where resistance is not overwhelming and thereby obscuring low abundance or weak inhibitors. Using a strain within the ARP we have recently discovered a natural product capable of restoring carbapenem activity against multidrug-resistant (MDR) strains overexpressing metallo-β-lactamases (King et al., 2014). To further demonstrate the utility of this platform in adjuvant discovery, we selected a clinically prevalent aminoglycoside-modifying enzyme; the aminoglycoside 2-*O* nucleotidyltransferase (ANT(2″)-Ia) (Cox et al., 2015). An appropriate screening window was achieved by cloning the gene into the pGDP system, with expression levels regulated by the weaker P_{lac} promoter (Table S1). This strain (*E. coli* BW25113 Δ*bamB*Δ*tolC* pGDP4:*ant(2″)-Ia*) was screened in the presence of sub-inhibitory concentrations of the substrate antibiotic gentamicin.

We evaluated a library of synthetic protein kinase inhibitors, the GlaxoSmithKline-Published Kinase Inhibitor Set (GSK-PKIS) (Drewry et al., 2014; Elkins et al., 2016), reasoning that

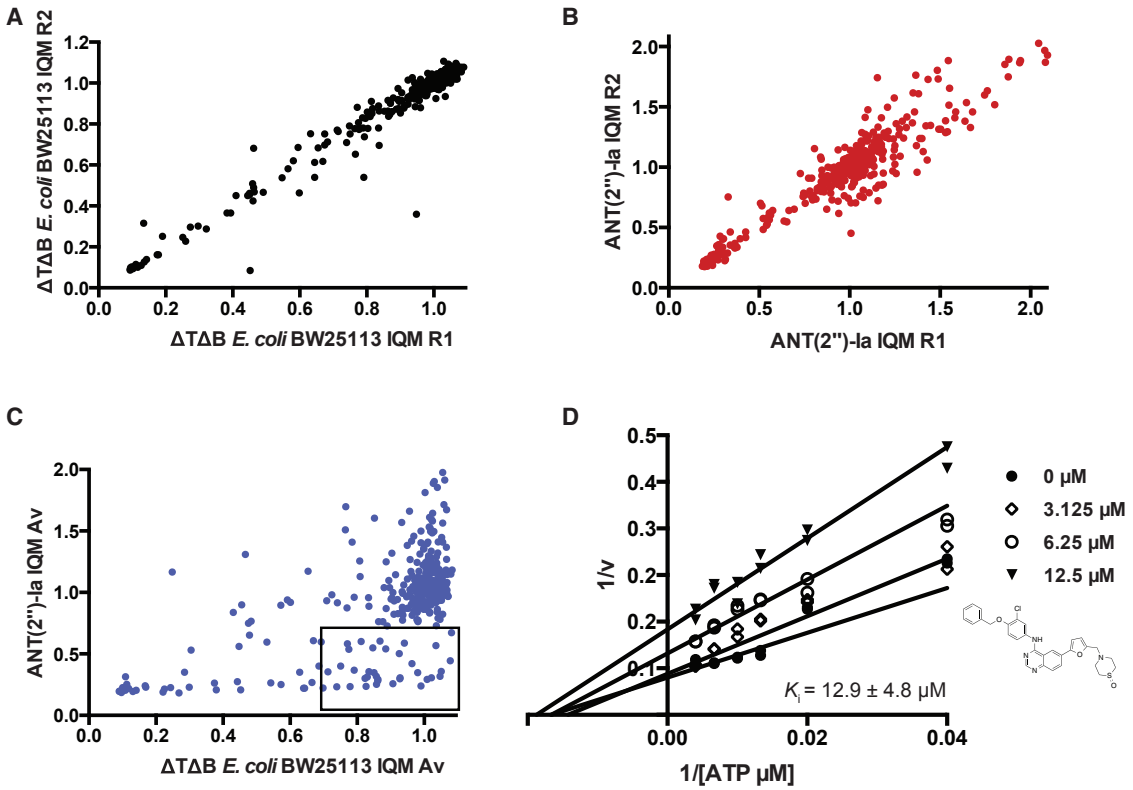

Figure 5. Screening the GSK-PKIS to Identify Inhibitors of the Aminoglycoside-Modifying Enzyme ANT(2″)-Ia

(A) Interquartile mean (IQM) normalized growth replica plot of *E. coli* BW25113 Δ*bamB*Δ*tolC* against the GSK-PKIS.

(B) Replica plot of *E. coli* BW25113 Δ*bamB*Δ*tolC* overexpressing ANT(2″)-Ia, in the presence of 4 μg/mL of gentamicin, against the GSK-PKIS.

(C) Comparative plot between the control *E. coli* BW25113 Δ*bamB*Δ*tolC* screen and *E. coli* BW25113 Δ*bamB*Δ*tolC* overexpressing ANT(2″)-Ia. Hits were compounds that inhibited normalized growth >0.68 in the *E. coli* BW25113 Δ*bamB*Δ*tolC*-harboring ANT(2″)-Ia strain and showed <0.7 normalized growth inhibition in the parent strain. Compounds falling within this criteria are highlighted with a rectangular box.

(D) In vitro determination of the inhibition constant (K_i) for GW583373A against ANT(2″)-Ia. The key depicts the different concentrations of inhibitor and the structure of the compound is shown.

since ANT(2″)-Ia utilizes ATP as a substrate for antibiotic inactivation, this PKIS may contain molecules that compete with ATP for ANT(2″)-Ia binding. Indeed, we identified 27 molecules that enhanced the activity of gentamicin (Figures 5 and 6 and Table S4); these molecules could be further grouped into four main chemotypes (Figure 6). We focused our attention on the 6-furanylquinazoline series of compounds. Of the 16 representatives of this chemotype, 8 blocked ANT(2″)-Ia-mediated resistance (Table S4). Two of these were selected for further study; GW574783B and GW583373A (Figure S6). Neither exhibited antibacterial activity alone (>100 μM), and both restored gentamicin activity against the ARP *E. coli* strain overexpressing ANT(2″)-Ia (Figure S6).

Both molecules exhibited dose-dependent inhibition of ANT(2″)-Ia activity in vitro. They exhibited a mixed inhibition pattern indicating that the compounds interfere with ATP binding to both the apoenzyme and to ANT(2″)-Ia-antibiotic complexes. This implies that the inhibitor binding site overlaps with both the ATP and kanamycin binding sites. This finding is not unexpected given the size of the compounds and the relative proximity of the nucleotide and aminoglycoside binding sites within the large active site cleft of ANT(2″)-Ia (Cox et al., 2015). GW583373A was found to be a more potent inhibitor of ANT(2″)-Ia (Figure 5D), with a K_i value of 12.9 ± 4.8 μM (GW574783B; K_i = 60 ± 18 μM).

DISCUSSION

Natural product antibiotic discovery efforts have dramatically declined over the last 50 years, driven by the wasteful rediscovery of known scaffolds. Furthermore, the effectiveness of existing antibiotics continues to be eroded by the emergence of resistance. To address these challenges, we have developed the ARP, which can be used to *both* systematically dereplicate known compounds in antibiotic discovery and identify inhibitors of resistance that can rescue existing drugs. In dereplication mode, the ARP provides a simple, low-cost, and effective method that does not rely on time-consuming and technologically intricate methods (Gaudencio and Pereira, 2015; Ito and Masubuchi, 2014; Nielsen et al., 2011; Tawfike et al., 2013). Indeed, the need for such a procedure is widely acknowledged since procuring antibiotics from natural sources is time consuming and costly. Even though the utility of exploiting resistance in antibiotic dereplication was recognized previously (Eisman et al., 1946; Stansly, 1946; Stapley, 1958), the development

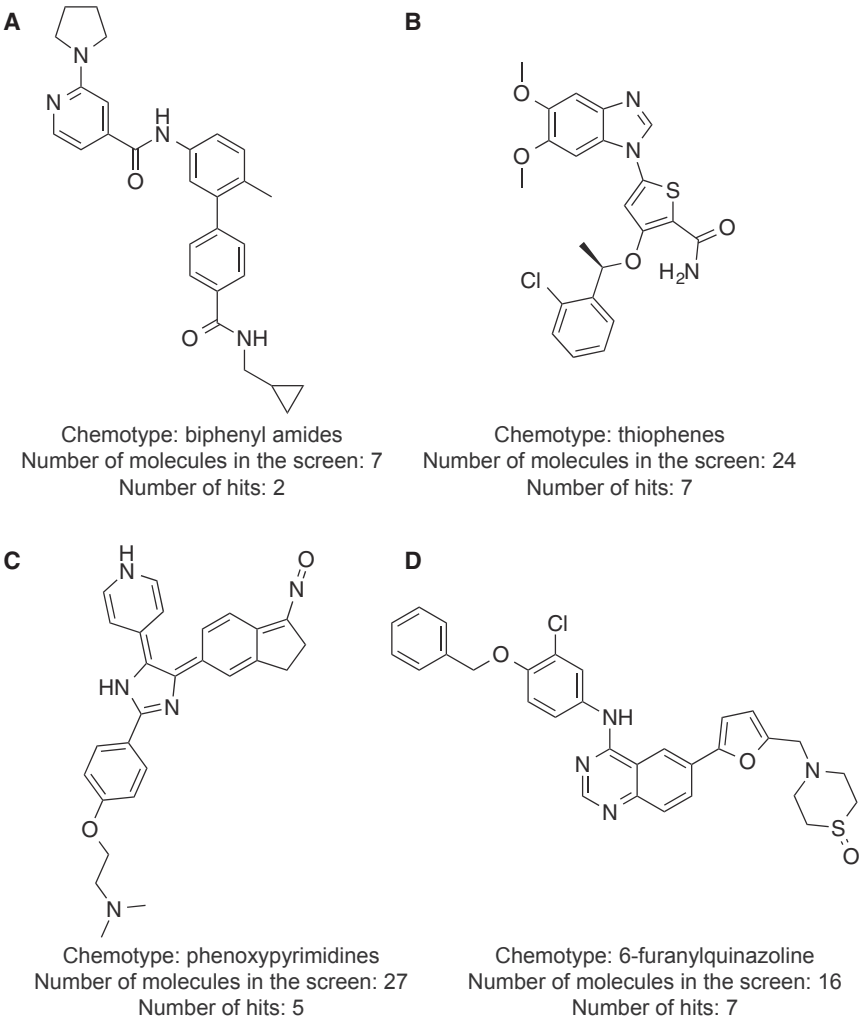

Figure 6. Representatives of the Four Main GSK-PKIS Inhibitor Chemotypes

(A) biophenyl amides; (B) thiophenes; (C) phenoxypyrimidines; (D) 6-furanylquinazolines. The 6-furanylquinazoline inhibitors (D) were selected for further characterization. Listed below each of the four chemotypes are the number of molecules in the GSK-PKIS with this scaffold and the number of hits. See also Table S4.

of a platform as comprehensive as the ARP is only now possible due to the increased insight we have gained into the molecular underpinnings of antibiotic resistance. One potential limitation of this approach is our ability to only dereplicate strains that are culturable in the laboratory setting. However, the ARP would be highly amenable in the dereplication of metagenomic libraries of so-called "unculturable" bacteria, whereby antibiotics are expressed in surrogate hosts. Furthermore, development of technologies such as the iChip, which allow for the isolation and growth of previously unculturable bacteria, circumvents this limitation. In addition to dereplication, the platform also enables the enrichment of specific scaffolds, a strategy that is especially useful in identifying biosynthetic programs for synthetic biology applications (Thaker et al., 2013).

Consistent with anecdotal reports from experience in the pharmaceutical industry (Baltz, 2005), we find that streptothricin is the most common antibacterial agent produced in a selection of our collection of antibiotic-producing soil-dwelling actinomycetes (Figure 3C). We also found that ~10% of the strains were producing the sideromycin, albomycin, revealing that this antibiotic is also highly prevalent. Since the molecule relies on iron for activity, it is possible that previous studies (Baltz, 2005) have not identified this antibiotic as having a high frequency of isolation due to a lack of this element in the fermentation media. Since streptothricin, albomycin, streptomycin, and echino-peptides are commonly produced antibiotics, in one simple step we can weed out ~70% of known antibiotics. This enriches for more unusual, low frequency of discovery molecules. Indeed, the isolation of mayamycin-producing actinomycetes highlights the strength of our enrichment.

With the rapid rate at which antibiotic resistance evolves, we intend to routinely update and enhance this platform to include newly discovered resistance determinants. Indeed, following the identification of strains producing the echinopeptides and albomycin, we were able to further improve the ARP by the addition of resistance determinants for future dereplication of these antibiotics.

The co-formulation of an antibiotic with an inhibitor of a respective resistance mechanism has thus far been limited to the β-lactams (Brown and Wright, 2016; Drawz and Bonomo, 2010; Pawlowski et al., 2016). This approach can be easily applied to other classes of antibiotic; the ARP has been constructed to include numerous candidate resistance determinants that will be utilized in future screening efforts. Our expression system fine-tunes gene expression levels (Figure 1C), allowing us to select the most sensitive screening conditions. Furthermore, the use of an identical genetic background allows for direct comparison between different targeted screens, which facilitates the identification of lead compounds. Indeed, the platform is purposefully designed to be a well-characterized, standard approach that enables screening in a robust and fully sequenced genetic background used by many other researchers, unlike most clinical strains.

We selected an aminoglycoside-modifying enzyme to exemplify the strength of our platform. The nucleotidylyltransferase ANT(2″)-Ia is a common cause of aminoglycoside treatment failure in North America due to infections caused by Gram-negative pathogens (Miller et al., 1997; Shaw et al., 1993; Shimizu et al., 1985). Only one class of inhibitor targeting this enzyme has been described (Hirsch et al., 2014). Screening a synthetic targeted library enabled the identification of a number of molecules that enhance the activity of aminoglycosides against the

resistance enzyme. These molecules are good chemical probe starting points to allow for structure-based drug design and more potent next generation adjuvants.

In this study we have demonstrated the versatility of the ARP; we believe this tool will be an invaluable resource in the fight against antibiotic resistance. The dereplication mode will enable prioritization of antibacterial natural product extracts while the adjuvant discovery mode will allow for the discovery of molecules that rejuvenate existing antibiotics. The modular nature of the ARP also makes it possible for the rapid addition of newly identified resistance determinants and combining several genes in a single construct, making it a valuable asset against the incessant and increasing emergence of MDR pathogens.

SIGNIFICANCE

Antibiotics are life-saving drugs. However, the clinical efficacy of these molecules is at risk due to the evolution of resistance, coupled with a lack of new antibiotics coming to market. Here, we describe how the antibiotic resistance platform (ARP) has been designed to tackle this problem. The ARP is a rigorously curated library of mechanistically distinct antibiotic resistance genes present in an identical genetic background. This platform can be used for both adjuvant discovery and antibiotic dereplication. In this study, we have demonstrated the versatility of the ARP in both applications; we believe this tool will be an invaluable resource in the fight against antibiotic resistance. Utilization of the ARP during dereplication will enable prioritization of antibacterial natural product extracts. On the other hand, the adjuvant discovery mode will allow for the identification of molecules that rejuvenate existing antibiotics. The modular nature of the ARP enables the rapid addition of newly identified resistance determinants, making it a valuable asset against the incessant emergence of multi-drug resistant pathogens.

EXPERIMENTAL PROCEDURES

Development of the pGDP Series

We constructed the ARP to enable easy movement of resistance genes between low-copy-number pGDP, integration pINT, and protein expression systems. The pGDP and pINT systems were therefore constructed with MCSs compatible with the commercially available pET systems (Novagen). The pGDP3 and pGDP1 plasmids differ by the presence of ampicillin- or kanamycin-resistance cassettes, respectively.

The P_{bla} promoter region for plasmids pGDP1 and pGDP3 was amplified using primers pBla-*BglII*-Fw (5′-CCT TTA GAT CTG GCC TAT TGG TTA AAA AAT G-3′) and pBla-*BglII*-Rv (5′-CCT TTT CTA GAG AAG CAT TTA TCA GGG TTA TTG-3′). The P_{Lac} promoter for plasmids pGDP2 and pGDP4 was extracted using the pLac-*BglII*-Fw (5′-CCT TTA GAT CTG CGG CGG CGG TGC TCA ACG G-3′) and pLac-*XbaI*-Rv (5′-CCT TTT CTA GAC CGG GCG CTA TCA TGC CAT ACC-3′) primers. The plasmid backbones from above and the promoter amplicons were digested with *XbaI* and *BglII* restriction enzymes and ligated, resulting in the four final pGDP plasmids.

Resistance genes were ligated into restriction endonuclease digested pGDP plasmids, all constructs were confirmed by Sanger sequencing.

Development of the pINT Series

The pINT series utilize the arabinose operon for integration in the chromosome. The vectors are based on the same backbone as the pGDP vectors, with the addition of the following; the PolB region was amplified from genomic *E. coli* BW25113 DNA using primers F1-Fw (5′-GGC ATT AAC GAT AGT GCC ATT G-3′) and F1-Rv (5′-CTT CGA AGC AGC TCC AGC CTA CAG CTG TAT AAA ACC ACA G-3′). The kanamycin-resistance cassette containing the flippase recognition target site was amplified from the Δ*tolC* Keio collection knockout strain using primers F2-Fw (5′-TGT AGG CTG GAG CTG CTT C-3′) and F2-Rv (5′-GCA GTT CGA AGT TCC TAT TCT C-3′). The two promoters P_{bla} and P_{Lac} were amplified from pGDP1 and pGDP2, respectively, using primers F3-Pbla-Fw (5′-CTA GAG AAT AGG AAC TTC GAA CTG C-3′), F3-Plac-Fw (5′-CTA GAG AAT AGG AAC TTC GAA CTG CGC GGC GGC GGT GCT AA-3′) and F3-Rv (5′-GCA TTT TTA TCC ATA AGA TTA GCG GCA TTT CCC CGA AAA GTG-3′). The AraC region was amplified from genomic *E. coli* BW25113 DNA using primers F4-Fw (5′-CCG CTA ATC TTA TGG ATA AAA ATG-3′) AND F4-Rv (5′-CCG CGA ATG GTG AGA TTG-3′).

Crossover PCR amplification was performed with all four amplicons, either P_{bla} (pINT1) and P_{Lac} (pINT2), using F1-Fw and F4-Rv primers, to yield one main amplicon. The pGDP4 plasmid was digested with *BlpI* and *BglII* restriction enzymes, followed by blunting using the Quick Blunting Kit (New England Biolabs). The crossover amplicons were ligated with the blunt-ended pGDP4 vector. Successful ligations were confirmed by sequencing. Finally, the *NcoI* site within the kanamycin cassette in pINT1 and pINT2 was removed using QuikChange mutagenesis (Agilent Technologies).

Construction of the Hyperpermeable and Efflux-Deficient *E. coli* Strain

The kanamycin cassette from the Keio collection deletion of the *bamB* gene (Baba et al., 2006) was removed using the pCP20 Flp recombinase system (Cherepanov and Wackernagel, 1995). For removal of the *tolC* gene from the resulting strain, the phage γ-red recombination system was utilized (Datsenko and Wanner, 2000). The Δ*bamB* strain was transformed with pKD46 using electroporation. For removal of the *tolC* gene, a region encompassing 500 bp upstream and downstream of the Δ*tolC* Keio collection knockout strain was amplified using primers NudF-Fw (5′-CGT TTG ACT ATC AGT CCC G-3′) and YgiB-Rv (5′-GCT GCG CCA TAG TTT TTA CC-3′). The pKD46-harboring Δ*bamB* strain was transformed with the resulting amplicon to remove the *tolC* gene, which was transformed with pCP20 to remove the kanamycin cassette from the *tolC* region. The *tolC* deletion was confirmed by PCR and sequencing (Central MOBIX Facility, McMaster University).

Chromosomal Integration of Antibiotic Resistance Genes

Following ligation of genes of interest into pINT1 and pINT2, the cassettes were amplified from these constructs using the F1-Fw and F4-Rv primers. The BW25113 Δ*bamB*Δ*tolC* strain was transformed with pKD46, which was then grown in the presence of arabinose to induce production of the integrase. The amplicon (200 ng) was then introduced into the pKD46-containing strain by electroporation and grown at 37°C in the presence of 25 μg/mL of kanamycin. Successful integrations were finally transformed with pCP20 to remove the kanamycin cassette.

Antibiotic Resistance Gene Array Susceptibility Testing

All strains were grown in Mueller Hinton II Broth (MHB II) (cation adjusted) (Becton Dickinson), at 37°C with aeration for 18 hr. Susceptibility testing was performed using the microdilution broth method, with inoculum prepared using either the growth or colony suspension method, according to CLSI guidelines (CLSI, 2012). For consistency, albomycin susceptibility testing was performed multiple times due to the emergence of spontaneous mutants.

Configuration of the ARP into a 96-Well Plate Format

In a sterile 96-well plate, a single colony of the various ARP strains was used to inoculate 100 μL of MHB II which was grown overnight at 37°C with aeration. Following incubation, these cultures were cryoprotected by the addition of 100 μL of sterile 50% glycerol, sealed with aluminum sealing foil (AlumaSeal; Sigma-Aldrich), and stored at −80°C.

Dereplication; Agar Overlay Procedure

For dereplication utilizing the agar overlay technique, Actinobacteria were fermented on 20 mL of Bennett's solid medium (Jones, 1949) in rectangular OmniTrays (Nunc). Strains were initially grown for 6 days at 30°C, in 3 mL of Streptomyces antibiotic medium (SAM). Following cultivation, 200 μL was applied as a lawn to the surface of the Bennett's medium. Bennett's medium

contained 1% potato starch, 0.2% casamino acids, 0.18% yeast extract, and 2 mL Czapek mineral mix per 1 L of medium (per 100 mL of mineral mix: 10 g of KCl, 10 g of $MgSO_4 \cdot 7H_2O$, 12 g of $NaNO_3$, 0.2 g of $FeSO_4 \cdot 7H_2O$, and 200 μL of concentrated HCl). Sterile mixed cellulose ester 0.45 μm filter paper (HAWP00010; EMD Millipore) was placed upon the surface and the plates were incubated for 6 days at 30°C. Following fermentation, the filter paper was removed and a 20 mL MH II solid medium overlay applied. Plates were incubated at 4°C overnight to allow for diffusion of bioactive molecules into the overlaid medium. Concurrently, the 96-well ARP glycerol library was used to inoculate 100 μL of MHB II in a sterile 96-well plate, using a sterilized manual 96-well pinning tool. Following incubation overnight at 37°C with aeration, the 96-well pinning tool was used to pin the cultured ARP strains upon the surface of the fermented and overlaid rectangular plates. Following incubation overnight at 37°C, these plates were analyzed for growth, which subsequently allowed for dereplication.

Dereplication; Agar Plug Procedure

To begin with, Actinobacteria were grown for 6 days at 30°C, in 3 mL of SAM; 100 μL of these cultures was applied as a lawn to the surface of Bennett's agar plates. These plates were then incubated for 6 days at 30°C and subsequently used to procure agar plugs. For dereplication, a single colony of *E. coli* ARP strains was used to inoculate 3 mL of MHB II, followed by incubation for 4 hr at 37°C with aeration. These cultures were adjusted to an optical density at 600 nm (OD_{600nm}) of 0.1–0.2 and 200 μL of this inoculum applied as a lawn to the surface of MH II solid medium square bioassay dishes (Nunc). Agar plugs (0.8 cm diameter) of the fermented Actinobacteria were then placed upon the surface of the *E. coli* ARP-inoculated bioassay dishes followed by incubation for 18 hr at 37°C. The bioassay dishes were imaged, and the radius of the zone of inhibition emanating from the agar plugs measured using the image-processing program ImageJ (Schneider et al., 2012).

Analysis of the Streptothricin- and Streptomycin-Producing Actinomycetes

Production of streptothricin and streptomycin was confirmed using LC/MS on an Agilent 1100 Series LC system (Agilent Technologies) with a C18 column (SunFire C18 5 μm, 4.6 × 50 mm, Waters) and an Agilent 1100 LC binary pump at a flow rate of 1 mL/min and the following parameters: isocratic for 1 min 5% solvent B (acetonitrile with 0.05% formic acid) and 95% solvent A (0.05% formic acid in water), followed by a linear gradient to 97% solvent B over 9 min. Commercially available streptothricin and streptomycin (Sigma-Aldrich) were used as a reference. The producing strains were fermented on 20 mL of Bennett's agar for 6 days at 30°C. The antibiotics were extracted with methanol and concentrated under reduced pressure. The resulting extracts were reconstituted in 1 mL of 100% (v/v) DMSO, 10 μL of which was used for analysis by LC/MS. The product ions from the reference antibiotics were used to extract in the producing crude extracts, product peaks were integrated using Analyst software (Agilent Technologies).

Genotypic analysis of the streptothricin-producing strains was achieved using BOX-PCR fingerprinting (Lanoot et al., 2004). Genomic DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories), 50 ng of DNA was used per reaction.

Mass Spectrometry and Reversed-Phase Purification of Bioactive Molecules

For purification and identification of the unknown bioactive molecules (mayamycin and echino-peptides), Actinobacteria were grown in 0.6 L of Bennett's medium for 6 days at 30°C with aeration. The cultures were filtered and the resulting cell biomass used for methanolic extraction at 4°C for 24 hr. The extract was concentrated under reduced pressure and purified using reversed-phase high-performance liquid chromatography (HPLC) with a C18 column (XSelect CSH 5 μm, 4.6 × 100 mm, Waters), at a flow rate of 1 mL/min and the above parameters. Resulting fractions were concentrated under reduced pressure and reconstituted in 200 μL 50% DMSO.

For purification of albomycin, the culture filtrate was applied on an HP-20 (50 g) column eluting with H_2O (200 mL), 25% MeOH (200 mL), 50% MeOH (200 mL), 75% MeOH (200 mL), and 100% MeOH (200 mL). The 50% elution fraction was concentrated under reduced pressure and applied to reversed-phase CombiFlash ISCO (RediSep Rf C18, Teledyne) with elution in a water-acetonitrile linear gradient system (0%–100% acetonitrile). The active subfractions were lyophilized and purified further using reversed-phase HPLC with a C18 column (Luna 5μ C18(2) 5 μm, 4.6 × 250 mm, Phenomenex), at a flow rate of 1 mL/min and the above parameters.

The activity of all fractions was assayed using the Kirby-Bauer disc diffusion assay; either *E. coli* BW25113 or *E. coli* BW25113 Δ*tolC*Δ*bamB* were applied as a lawn to square bioassay Mueller Hinton agar plates, as described above. Paper discs were placed on the surface and 20 μL of each extract was added to each disc. Plates were incubated for 18 hr at 37°C. Bioactive fractions were then assessed by reversed-phase LC/MS as described above. HRMS were obtained using an XL Orbitrap Hybrid mass spectrometer (Thermo-Fisher Scientific) equipped with an electrospray interface in positive ion mode. Structures were elucidated using 1D and 2D NMR experiments on a Bruker Avance III 700 MHz apparatus equipped with a cryoprobe in an appropriate deuterated solvent. Chemical shifts are reported in ppm relative to tetramethyl silane using the residual solvent as an internal signal.

ANT(2″)-Ia GSK-PKIS Adjuvant Screen

Inhibitor screening was performed at 1/4 the gentamicin MIC for *E. coli* BW25113 Δ*tolC*Δ*bamB* pGDP4:*ant(2″)-Ia* (4 μg/ml); 1/4 the MIC was used for selection based on our previous experience in compound combination screens. The parental strain *E. coli* BW25113 Δ*tolC*Δ*bamB* (no resistance enzyme) was screened as a control, to discount compounds that possess antibacterial activity alone. The robustness of the screen was determined by calculation of the Z′ factors (Zhang et al., 1999), it was calculated that both the control parental strain and the ANT(2″)-Ia-expressing strain had a Z′ factor of 0.5. The GSK-PKIS library (Drewry et al., 2014) was screened in duplicate, using a Beckman Biomek FX liquid handler (Beckman Coulter), 1.5 μL from a 1 mM stock of each compound, dissolved in 100% DMSO, was dispensed into the wells of a 384-well flat-bottom plate, followed by the addition of 28.5 μL of media inoculated with each strain and gentamicin (1/4 MIC), resulting in a final concentration of 50 μM. Diluted cell culture was prepared by dilution of an overnight culture 1:1,000 in MHB II. Plates were incubated at 37°C with aeration, the $OD_{600\ nm}$ of each plate was read following 18 hr incubation.

High-Throughput Screening Data Analysis

The data were normalized using the interquartile mean (Mangat et al., 2014) and analyzed using TIBCO Spotfire and Instant JChem version 15.4.27.0 (ChemAxon). Screen hits were compounds that inhibited normalized growth >0.68 in the *E. coli* BW25113 Δ*tolC* Δ*bamB*-harboring ANT(2″)-Ia strain and showed <0.70 inhibition of normalized growth in the parent strain. Both normalized growth inhibitions are above three times the SD of the mean. Data from the ANT(2″)-Ia screen has been deposited in PubChem (AID: 1159619).

Characterization of ANT(2″)-Ia GSK-PKIS Compounds

Compounds GW583373A and GW574783B were supplied by GSK for further studies, both compounds were reconstituted in 100% DMSO. The microdilution checkerboard method was used to determine if the compounds synergize with gentamicin in *E. coli* BW25113 Δ*tolC*Δ*bamB* pGDP4:*ant(2″)-Ia*. The assay was performed in MHB II at 37°C with aeration for 18 hr. Cell inoculum was prepared using the colony suspension method, according to CLSI guidelines (CLSI, 2012).

In vitro ANT(2″)-Ia pyrophosphatase assays were performed as described previously (Cox et al., 2015; Hirsch et al., 2014), with minor modifications. To improve compound solubility, Tween 20 was added to a final concentration of 0.01% (v/v) and compounds were added at a final concentration of 2% (v/v) DMSO. The enzyme (1 μg per 100 μL reaction volume) was incubated with compound for 10 min at 25°C, prior to initiation of the reaction with nucleotide. All inhibitory assays were performed with kanamycin B kept at K_m (Cox et al., 2015; Hirsch et al., 2014). Inhibitor K_i values against ATP were conducted in duplicate and data were fit to different models of enzyme inhibition, the most statistically favorable fit was assessed using software in GraFit version 5.0.13 (Erithacus Software) and GraphPad Prism version 6.00 (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.11.011.

AUTHOR CONTRIBUTIONS

G.C., G.D.P., A.M.K., and G.D.W. designed the study. G.D.P designed and developed the pGDP and pINT series. G.C. performed the majority of the experiments with contributions from A.S., A.M.K., A.C.P., and K.K. The manuscript was written by G.C. and G.D.W. with input from A.M.K., G.D.P., and K.K.

ACKNOWLEDGMENTS

We thank Linda Ejim, Laura Rossi, and Arlene Sutherland for assistance with initial cloning of resistance genes, Bill Zucker for access to the GSK protein kinase inhibitor library, and Tomasz Czarny for assistance with image design. This research was funded by a Canadian Institutes of Health Research grant (MT-13536), a Natural Sciences and Engineering Research Council grant (237480), the Ontario Research Fund (RE07–48), and by a Canada Research Chair in Antibiotic Biochemistry (to G.D.W.).

Received: September 20, 2016
Revised: November 7, 2016
Accepted: November 17, 2016
Published: December 22, 2016

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