Title:

Identification of cellular targets of a series of boron heterocycles using TIPA II – a sensitive target identification platform

Matthew S. Ward^a, Isba Silva^a, Walfre Martinez^a, Jameka Jefferson^a, Shakila Rahman^a, Jeanie M. Garcia^a, Divya Kanichar^b, Lance Roppiyakuda^b, Ewa Kosmowska^b, Michelle A. Faust^b, Kim P. Tran^b, Felicia Chow^b, Elena Buglo^b, Feimeng Zhou^c, Michael P. Groziak^b and H. Howard Xu^{a,*}

^aDepartment of Biological Sciences, California State University Los Angeles, Los Angeles, CA 90032, USA

^bDepartment of Chemistry and Biochemistry, California State University East Bay, Hayward, CA 94542, USA

^cDepartment of Chemistry and Biochemistry, California State University Los Angeles, Los Angeles, CA 90032, USA

* Corresponding author. Tel.: 323-343-2188; fax: 323-343-6451

e-mail: hxu3@calstatela.edu

Abstract

One of the hurdles in the discovery of antibiotics is the difficulty of linking antibacterial compounds to their cellular targets. Our laboratory has employed a genome-wide approach of over-expressing essential genes in order to identify cellular targets of antibacterial inhibitors. Our objective in this project was to develop and validate a more sensitive disk diffusion based platform of target identification (Target Identification Platform for Antibacterials version 2; TIPA II) using a collection of cell clones in an *Escherichia coli* mutant (AS19) host with increased outer membrane permeability. Five known antibiotics/inhibitors and 28 boron heterocycles were tested by TIPA II assay, in conjunction with the original assay TIPA. The TIPA II was more sensitive than TIPA because eight boron heterocycles previously found to be inactive to AG1 cells in TIPA assays exhibited activity to AS19 cells. For 15 boron heterocycles, resistant colonies were observed within the zones of inhibition only on the inducing plates in TIPA II assays. DNA sequencing confirmed that resistant clones harbor plasmids with *fabI* gene as insert, indicating that these boron heterocycles all target enoyl ACP reductase. Additionally, cell-based assays and dose response curves obtained indicated that for two boron heterocycle inhibitors, the *fabI* cell clone in AG1 (wild-type) host cells exhibited at least 11 fold more resistant under induced conditions than under non-induced conditions. Moreover, TIPA II also identified cellular targets of known antibacterial inhibitors triclosan, phosphomycin, trimethoprim, diazaborine and thiolactomycin, further validating the utility of the new system.

1. Introduction

Over the past twenty years, bacterial strains resistant to multiple classes of antibiotics have spread at an alarming rate, posing a serious threat to global public health.¹ Meanwhile, few antibiotics of novel structures have entered clinical use and the antibiotic drug development pipeline is scarce in the pharmaceutical industry.^{2, 3} These factors highlight an unmet medical need for discovery and development of novel antibiotics.² Bacterial functional genomics studies have identified roughly 200-300 essential proteins in a typical bacterium.⁴⁻⁸ However, only about 40 of these proteins have served as drug targets of currently available antibiotics,⁹ rendering the remainder as potential targets to be exploited for the discovery of novel antibiotics with perhaps new mechanisms of action.

High-throughput screening strategies have expanded our capacity to explore chemical diversity for the identification of novel inhibitors of bacterial cell growth.¹⁰⁻¹³ From the standpoint of antibiotic lead discovery process, there is a need for development of cost-effective, rapid, and comprehensive assay systems to identify targets of discovered antibacterial inhibitors. Recently, we reported the development and validation of a facile and inexpensive target identification platform for antibacterials (TIPA) which leverages inducible over-expression of essential genes in mixed pools of *Escherichia coli* clones harboring plasmid-borne essential genes.¹⁴ This approach is based on the concept that elevated concentrations of a target protein confer resistance because higher levels of a specific antibacterial inhibitor are needed to bind the excess target in order to inhibit cell growth.¹⁵ However, the host cells used in TIPA were “wild-type” laboratory strains of *E. coli*, which, like most Gram negative bacteria, possess an intact outer membrane that prevents penetration of many compounds,¹⁶⁻²⁰ making it difficult to identify novel chemical structures with limited potency. Conceptually, if the platform utilizes a host cell

which more readily permits passage of a diverse array of compounds into the cell, compounds with limited antibacterial activity along with their cellular targets may be easily identifiable. Identifying targets of new chemical entities albeit with weak activity provides a new path for advancing novel, high-value antibacterial inhibitor-target pairs through drug discovery and development process via medicinal chemistry manipulations. In this report, we describe the development of TIPA II system with improved sensitivity by using an outer-membrane mutant strain of *E. coli*, AS19, and validation of this new system by identifying cellular targets of a series of boron heterocycles with a varied activity against *E. coli* wild-type cells.

2. Results and discussion

2.1. Building a collection of 274 AS19 clones and configuration of six pools of mixed clones

To construct a collection of AS19 clones, individual plasmids, each containing an inducible essential gene in the pCA24N vector background, were isolated from 272 *Escherichia coli* AG1 cell clones (Table 1) and transformed into cells of AS19. Colonies of transformants were streaked to obtain pure cultures. The identity of each clone was confirmed by isolation of the plasmid from the cells and restriction enzyme digestion analysis of the plasmid (Fig. S1). All plasmids were confirmed to be successfully isolated and purified, and all plasmid clones contained the correct essential genes. The growth of AS19 clones was not adversely impacted by the presence of 0.25, 0.5 or 1.0 mM inducer (Isopropyl- β -D-1-thiogalactopyranoside). The AS19 clones were configured into six separate pools of mixed clones, with the number of clones between 39 and 48 per pool (Table 1). The compositions of the pools were rather random, primarily based on the numerical order of clone ID codes (JW numbers) described in the Pools #1 to #5 of TIPA,¹⁴ with additions of newly acquired clones in various pools to account for the entire essential gene landscape of *E. coli* (Profiling of *E. coli* Chromosome, PEC). For uniformity, the previous Pool #6 in TIPA which consists of 50 cell clones constructed in DH5 α host cells in our laboratory using pLEX5BA vector¹⁴ was replaced with pCA24N-based clones obtained from Nara Institute of Science and Technology,²¹ thus our entire collection of inducible essential gene cell clones can be maintained using chloramphenicol.

Table 1. List of TIPA II (AS19 host) cell clones and their assigned pool numbers

Clone ID/gene	Pool#	Clone ID/gene	Pool#	Clone ID/gene	Pool#	Clone ID/gene	Pool#	Clone ID/gene	Pool#	Clone ID/gene	Pool#
JW0023 ribF	5	JW0404 ribD	5	JW1629 tyrS	2	JW2893 pgk	5	JW3268 rpsH	3	JW3940 murB	4
JW0024 ileS	1	JW0405 ribE	5	JW1654 ribC	5	JW2909 metK	3	JW3269 rpsN	3	JW3941 birA	6
JW0025 lspA	1	JW0406 nusB	1	JW1703 pheT	2	JW2986 plsC	3	JW3270 rplE	3	JW3942 coaA	6
JW0027 ispH	5	JW0407 thiL	5	JW1706 rplT	2	JW2987 parC	3	JW3271 rplX	3	JW3944 secE	4
JW0029 dapB	5	JW0410 dxs	1	JW1709 thrS	2	JW2998 parE	3	JW3272 rplN	3	JW3945 nusG	4
JW0047 folA	5	JW0411 ispA	1	JW1729 nadE	2	JW3009 ribB	3	JW3273 rpsQ	3	JW3948 rplJ	4
JW0053 imp	1	JW0459 dnaX	1	JW1768 gapA	5	JW3028 cca	5	JW3274 rpmC	3	JW3949 rplL	4
JW0081 ftsL	1	JW0463 adk	1	JW1796 yaeZ	5	JW3030 folB	6	JW3275 rplP	3	JW3950 rpoB	4
JW0082 ftsI	1	JW0464 hemH	5	JW1855 aspS	2	JW3036 ygiD	3	JW3276 rpsC	4	JW3951 rpoC	4
JW0083 murE	1	JW0513 lpxH	5	JW1865 argS	2	JW3038 dnaG	3	JW3277 rplV	4	JW4000 ubiA	6
JW0084 murF	1	JW0515 cysS	1	JW1897 pgsA	2	JW3039 rpoD	3	JW3278 rpsS	4	JW4001 plsB	4
JW0085 mraY	1	JW0518 folD	5	JW2101 metG	6	JW3137 infB	3	JW3279 rplB	4	JW4003 lexA	6
JW0086 murD	1	JW0629 mrdB	1	JW2140 folE	5	JW3138 nusA	3	JW3280 rplW	4	JW4012 dnaB	4
JW0087 ftsW	1	JW0630 mrdA	1	JW2176 yejM	6	JW3143 glmM	6	JW3281 rplD	4	JW4020 ssb	4
JW0088 murG	1	JW0634 nadD	5	JW2225 gyrA	2	JW3145 ftsH	6	JW3282 rplC	4	JW4102 groS	4
JW0089 murC	1	JW0635 holoA	1	JW2228 nrdA	2	JW3150 obgE	6	JW3283 rpsJ	4	JW4103 groL	4
JW0091 ftsQ	1	JW0636 rlpB	5	JW2229 nrdB	2	JW3152 rpmA	3	JW3302 fusA	4	JW4107 efp	1
JW0092 ftsA	1	JW0637 leuS	1	JW2312 folC	5	JW3153 rplU	3	JW3303 rpsG	4	JW4121 psd	6
JW0093 ftsZ	1	JW0654 Int	1	JW2313 accD	2	JW3154 ispB	6	JW3304 rpsL	4	JW4122 rsgA	6
JW0094 lpxC	1	JW0666 glnS	1	JW2320 fabB	2	JW3156 murA	3	JW3347 trpS	4	JW4126 yjeE	1
JW0096 secA	1	JW0671 fldA	1	JW2395 gltX	2	JW3165 kdsC	6	JW3361 yrfF	6	JW4160 rpsR	1
JW0100 coaE	5	JW0897 mshA	2	JW2403 ligA	2	JW3166 yrbK	6	JW3396 asd	6	JW4185 ppa	1
JW0122 can	5	JW0898 lpxK	5	JW2404 zipA	2	JW3167 yhbN	6	JW3427 ftsX	4	JW4215 valS	1
JW0138 folK	5	JW0901 kdsB	2	JW2456 dapE	5	JW3168 yhbG	6	JW3428 ftsE	4	JW4218 yigP	6
JW0150 hemL	5	JW0905 mukF	5	JW2498 hisS	2	JW3199 rpsI	3	JW3429 ftsY	4	JW4325 dnaC	2
JW0152 yadR	5	JW0906 mukE	6	JW2499 ispG	5	JW3200 rplM	3	JW3530 glyS	4	JW4326 dnaT	2
JW0161 dapD	5	JW0907 mukB	5	JW2517 suhB	5	JW3204 degS	3	JW3531 glyQ	4	JW5007 secM	6
JW0163 map	1	JW0937 fabA	2	JW2547 acpS	2	JW3218 mreD	6	JW3583 gpsA	6	JW5154 yceQ	6
JW0164 rpsB	1	JW1056 mviN	5	JW2550 era	2	JW3219 mreC	6	JW3608 kdtA	4	JW5161 lolC	2
JW0165 tsf	5	JW1071 rne	2	JW2552 lepB	2	JW3220 mreB	6	JW3609 coaD	4	JW5162 lolD	2
JW0166 pyrH	1	JW1078 fabD	2	JW2569 pssA	2	JW3223 accB	3	JW3612 rpmB	4	JW5277 pheS	2
JW0167 frr	1	JW1079 fabG	2	JW2577 yfiO	5	JW3224 accC	3	JW3615 dut	4	JW5397 had	3
JW0168 dxr	1	JW1080 acpP	2	JW2587 rplS	2	JW3243 yrdC	6	JW3623 gmk	4	JW5403 der	6
JW0169 ispU	6	JW1084 tmk	2	JW2588 trmD	2	JW3248 def	3	JW3625 spoT	4	JW5414 ffh	4
JW0171 yaeL	1	JW1085 holB	2	JW2590 rpsP	2	JW3249 fmt	3	JW3678 dnaN	4	JW5550 murI	4
JW0172 yaeT	5	JW1104 lolE	2	JW2594 grpE	2	JW3256 rplQ	3	JW3679 dnaA	4	JW5625 gyrB	4
JW0174 lpxD	1	JW1195 pth	2	JW2596 uppS	3	JW3257 rpoA	3	JW3680 rpmH	4	JW5642 dfp	6
JW0175 fabZ	1	JW1198 prsA	2	JW2666 csrA	5	JW3258 rpsD	3	JW3681 rnpA	4	JW5740 orn	6
JW0176 lpxA	1	JW1199 ispE	2	JW2667 alaS	3	JW3259 rpsK	3	JW3683 yidC	4	JW5760 yigQ	6
JW0177 lpxB	1	JW1201 hema	5	JW2716 ispF	3	JW3260 rpsM	3	JW3707 glmS	6	JW5810 cdsA	6
JW0179 dnaE	1	JW1202 prfA	2	JW2717 ispD	3	JW3262 secY	3	JW3708 glmU	4	JW5829 infC	6
JW0180 accA	1	JW1203 prmC	5	JW2718 ftsB	3	JW3263 rplO	3	JW3756 rho	4	JW5847 prfB	6
JW0183 tilS	5	JW1206 kdsA	2	JW2750 eno	3	JW3264 rpmD	3	JW3776 hemD	6		
JW0361 hemB	5	JW1266 topA	2	JW2751 pyrG	5	JW3265 rpsE	3	JW3827 hemG	6		
JW0398 secD	5	JW1269 ribA	5	JW2796 lgt	3	JW3266 rplR	3	JW3835 polA	4		
JW0399 secF	5	JW1281 fabI	2	JW2892 fbaA	3	JW3267 rplF	3	JW3904 ftsN	4		

2.2. TIPA II validation - known antibiotics

The AS19 based TIPA II system was established in order to determine targets of antibacterial compounds, especially those with limited potency. First, TIPA II was validated using several known antibiotics and antibacterial inhibitors, including triclosan²² and phosphomycin²³ whose target protein over-expression conferred enhance cellular resistance to specific inhibitors.¹⁴ Just like in TIPA system, TIPA II disk diffusion assays generated resistant colonies within the zones of inhibitions surrounding triclosan- or phosphomycin-soaked disks only on induced assay trays containing Pool #2 mixed clones (Fig. 1). Subsequent colony purification, plasmid isolation and insert DNA sequencing indeed “identified” the targets of triclosan and phosphomycin by TIPA II (Fig. 1), with 100 percent of clones harboring plasmids containing *fabI* and *murA* genes, respectively. Besides triclosan and phosphomycin, we also tested trimethoprim and diazaborine, known to target dihydrofolate reductase (encoded by *folA* gene)²⁴ and enoyl [acyl carrier protein] reductase (enoyl ACP reductase) (encoded by *fabI* gene),²⁵ respectively. TIPA II assays showed that resistant cell colonies emerged within the zone of inhibition surrounding a filter disk impregnated with trimethoprim only on Pool #5 assay tray under induction (Fig. 2A) (Table 2). DNA sequence analysis of isolated plasmids demonstrated that the resistance colonies contained *folA* gene sequences, as expected. To determine quantitatively the increase in resistance, dose response curves were obtained from cell-based assays using AS19 clone (from AG1 JW0047 clone which contains plasmid pCA24N-*folA*) containing the *folA* gene on the plasmid (Fig. 2B). Under inducing conditions, the FolA clone became 133 fold more resistance as compared to non-inducing conditions (Fig. 2B). Similarly, the well known diazaborine, **1** (Table 2), was found to target enoyl ACP reductase via TIPA II assays, confirming the mechanism of action of this compound.²⁵

Figure 1. Images of zones of inhibition and resistance colonies (right panels) there within on TIPA II assay trays with filter disks containing either triclosan (top panels) or phosphomycin (bottom panels). Triclosan, 10 $\mu\text{g}/\text{disk}$; Phosphomycin, 200 $\mu\text{g}/\text{disk}$.

Figure 2. (A) Images of zones of inhibition and resistance growth occurred within the zone of inhibition (right) on TIPA II induced assay tray containing Pool #5 with filter disks applied with 1.1 mg of trimethoprim. (B) Dose responses and resistance testing for clone JW0047 containing plasmid-borne *folA* gene (AS19 host) against trimethoprim under induced (blue) or non-induced (red) conditions.

2.3. TIPA II validation – boron heterocycles

Recently, we and our collaborators reported synthesis, characterization and antibacterial activity of a series of 31 boron heterocycles.²⁶ Some of these compounds were active against *E. coli* cells, while the majority were not,²⁶ which are perfect examples that can be used to validate our TIPA II system for identification of cellular targets of compounds, in particular of those with poor activity. A total of 28 boron heterocycles were tested in both TIPA¹⁴ and TIPA II systems (Table 2). Due to a mutant outer membrane, AS19 cells are more permeable (thus more sensitive) to compounds as compared to AG1 host cells, as illustrated by disk diffusion assays conducted for both TIPA and TIPA II systems (Fig. 3). For example, **37** and **44** both failed to produce a zone of inhibition to cells of Pool #2 in TIPA (AG1 cells), either under induced or non-induced conditions (Fig. 3), while the same compounds created zones of inhibition to Pool #2 cells of TIPA II (AS19 cells) (Fig. 3). It was obvious that resistant colonies were present within the zones of inhibition for **37** and **44** (Fig. 3). Subsequent colony purification for **37** and **44** assays, plasmid isolation followed by insert DNA sequencing identified the targets of these two compounds to be enoyl ACP reductase.

Table 2. Compound structure, activities and summary results in TIPA and TIPA II.

Compound	Structure	Active in TIPA	Resistant colonies observed in TIPA/Target identified	Active in TIPA II	Resistant colonies observed in TIPA II/Target identified
Triclosan	[Sigma; CAS 3380-34-5]	Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase
Phosphomycin disodium	[Sigma; CAS 26016-99-9]	Yes	Yes/ UDP-N-acetylglucosamine-3-enolpyruvyltransferase	Yes	Yes/ UDP-N-acetylglucosamine-3-enolpyruvyltransferase
Trimethoprim	[Sigma; CAS 738-70-5]	ND*	ND*	Yes	dihydrofolate reductase
Thiolactomycin	[Sigma; CAS 82079-32-1]	ND*	ND*	Yes	Yes/ β -ketoacyl-ACP synthetase I and Acyl carrier protein
1 (diazaborine)		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase

25		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase
2b		No	Not applicable	No	Not applicable
15c		No	Not applicable	Yes	Yes/Enoyl ACP reductase
16b		No	Not applicable	No	Not applicable
17c		No	Not applicable	No	Not applicable
18c		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase

26		No	Not applicable	Yes	Yes/Enoyl ACP reductase
37		No	Not applicable	Yes	Yes/Enoyl ACP reductase
4c		No	Not applicable	No	Not applicable
22		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase
31		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase
24		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase

23		No	Not applicable	No	Not applicable
32		No	Not applicable	No	Not applicable
27		No	Not applicable	No	Not applicable
30		No	Not applicable	No	Not applicable
38		No	Not applicable	No	Not applicable

39		No	Not applicable	No	Not applicable
40		No	Not applicable	No	Not applicable
35b		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase
4a		No	Not applicable	Yes	Yes/Enoyl ACP reductase
5c		No	Not applicable	No	Not applicable

6c		No	Not applicable	No	Not applicable
3c		No	Not applicable	Yes	Yes/Enoyl ACP reductase
44		No	Not applicable	Yes	Yes/Enoyl ACP reductase
20a		No	Not applicable	Yes	Yes/Enoyl ACP reductase
41		No	Not applicable	Yes	Yes/Enoyl ACP reductase
33a		Yes	Yes/Enoyl ACP reductase	Yes	Yes/Enoyl ACP reductase

*ND, not determined.

Figure 3. Comparison of TIPA (AG1 as host cells) and TIPA II (AS19 as host cells). Images of zones of inhibition (or lack of) and resistance colonies within the zone of inhibition on TIPA II induced assay trays with filter disks containing either **37** or **44**. For TIPA assays, each disk was loaded with 750 μg . For TIPA II assays, 4 μg /disk for **37**, and 15 μg /disk for **44** were used.

Previous studies have demonstrated better cell envelope penetration of chemicals for AS19 as compared to wild-type strains.¹⁶⁻¹⁸ For example, while examining sequence-specific antisense peptide nucleic acids (PNAs) for their inhibition on *E. coli* cell growth, Good and colleagues observed that these PNAs had limited activity to wild-type laboratory strain of this species but were active to mutant strains with defective outer-membrane such as AS19.¹⁸ Similarly, Gabrielli and coworkers found that none of the D-arabinose 5-phosphate isomerase inhibitors inhibited the cell growth of wild-type strains of *E. coli* and *Pseudomonas aeruginosa* but exhibited slight activity against AS19.¹⁷

Among the 28 boron heterocycles tested by TIPA, seven were found to generate resistant colonies within zones of inhibition on TIPA induced assay trays containing Pool #2 cells (Table 2). Figure 4 shows representative TIPA disk diffusion assays for two of these compounds. Specifically, resistant colonies were observed (Fig. 4) and were collected within the zones of inhibition on the induced assay tray and the colony identities were determined by plasmid isolation and insert DNA sequencing. Sequencing results showed that 48 out of 50 from both **31** and **24** assays harbor plasmids containing the *fabI* gene, demonstrating that these antibacterial inhibitors target the enoyl ACP reductase enzyme in exerting their actions. The TIPA system has a separate pool (Pool #6) composed of DH5 α cell clones with pLEX5BA-borne essential genes, among which the *fabI* gene is represented. Resistant colonies were also observed on induced assay trays containing these four compounds. Similarly, nearly all of the 20 resistant colonies collected for **31** and **24** assays were found to contain the *fabI* gene as the plasmid inserts. To determine the degree to which over-expression of target protein confers cellular resistance, the cells of AG1 FabI clone (AG1/pCA24N-*fabI*, i.e., JW1281) were treated with a serial dilutions of **31** or **24**, under induced (1 mM IPTG) or non-induced (0 mM IPTG) conditions (Fig. 5). The

analyzed dose response curves indicated that the JW1281 cell clone became 18 fold more resistant to **31** under induction condition (Fig. 5A), while the cell clone was 11 fold more resistant to **24** when the inducer was present (Fig. 5B).

Figure 4. Images of zones of inhibition on TIPA assay trays containing Pool #2 with filter disks soaked with the following compounds: a **31**; b, **24**. Each disk was loaded with 75 μ g of compound.

Figure 5. Dose responses and resistance testing for clone JW1281 (AG1 host) against **31** (A) and **24** (B) under non-induced (0 mM IPTG) and induced (1 mM IPTG) conditions. The AG1 cell clone JW1281 harbors a plasmid containing the *fabI* gene whose expression is inducible by IPTG.

Among the 28 boron heterocycles tested by TIPA II, 15 were found to be active (producing zones of inhibition) and eight exhibited activity only in TIPA II but not in TIPA (Table 2). For all of these 15 active compounds, resistant colonies were observed and subsequent sequencing analyses determined that all or majority of the resistant colonies for each of the 15 compounds harbor plasmids with *fabI* gene as inserts (Table 2), indicating that their targets are enoyl ACP reductase. Representative disk diffusion assay results are shown in Figure S2. Specifically, four boron heterocycles (**25**, **2b**, **17c** and **37**) were tested in TIPA II, with images of zones of inhibitors (or absence thereof) shown for all six pools of mixed cell clones under non-induced and induced conditions (Fig. S2). Compounds **25** and **37** consistently produced zones of inhibition with similar diameters on all 12 assay trays (two trays per pool: one for non-induced and the second one for induced). On the induced assay tray of Pool #2, tiny resistant colonies emerged within zones of inhibition surrounding **25** and **37**, suggesting that clone(s) over-expressing the cellular target of these two inhibitors have gained resistance to **25** and **37**, respectively (Fig. S2). Subsequent genetic analyses via plasmid insert sequencing identified the targets of both **25** and **37** to be enoyl ACP reductase. Due to improved sensitivity, additional eight boron heterocycles were found to be active in TIPA II, all being found to target enoyl ACP reductase (Table 2).

It has been established that certain boron heterocycles in which the diaza-moiety and the boron atom form a six-member diazaborine ring inhibit enoyl ACP reductase.^{27, 28} In addition, structural activity relationship studies indicated that the boron atom and the diaza-moiety are essential for activity.^{25, 29} While it was anticipated that some of the active diazaborines that we synthesized may exert their actions via inhibiting FabI,²⁶ such predictions have to be experimentally verified. Here in this report, we not only demonstrated that seven of the 27 boron heterocycles target FabI enzyme using TIPA assays (Table 2), we have also validated the higher sensitivity of TIPA II assays in linking cellular targets of eight additional boron heterocycles with poor potency (Table 2). We and our collaborators recently reported that biochemical assays with four of the boron heterocycles showed that **14b**, **18c**, **35b** and **39** inhibited the *E. coli* FabI enzyme activity.³⁰ More importantly, through X-ray crystallographic studies using structural models of FabI with or without **14b** or **35b**, we found that the sulfonyl group can be replaced with an amide or thioamide without disruption of the mode of inhibition of the inhibitors.³⁰ Our TIPA and TIPA II assays confirmed the target-specific activity of two of the four compounds (**18c**, **35b**) examined.³⁰ While **39** was found active against the FabI enzyme,³⁰ we can only found a very small zone of inhibition in TIPA II assays and have failed to observe resistant colonies.

2.4. TIPA II – target identification of thiolactomycin

TIPA II was employed to test thiolactomycin to further validate the system. Thiolactomycin is a weak antibacterial inhibitor known to target β -ketoacyl-ACP synthase I encoded by *fabB* gene of *E. coli*. While it is inactive against AG1 cells (data not shown), thiolactomycin was able to generate zones of inhibition to AS19 cells of TIPA II (Figure 6). As shown in Figure 6, resistant colonies were present within the zone of inhibition only on the induced assay tray of

Pool #2. A total of 90 resistant colonies were analyzed genetically. Fifty-two out of 90 (58%) of the colonies harbor *fabB* containing plasmids, as predicted by published report.^{31, 32} Surprisingly, 38 out of 90 colonies (42%) turned out to harbor plasmids with *acpP* gene as insert. While such result is unexpected, this can be explained by the fact that acyl carrier protein (ACP, encoded by *acpP* gene) is a reaction co-factor of the enzymatic reaction catalyzed by β -ketoacyl-ACP synthase I and an excess amount of ACP may render higher specific activity of the FabB enzyme, resulting in enhanced resistant of the AcpP cell clone (JW1080).

While TIPa II has increased sensitivity and demonstrated its potential of revealing additional facets of the mechanism of action of an antibacterial inhibitor (such as in thiolactomycin), there some “caveats” that should be pointed out when this platform is applied to new antibacterial inhibitors whose targets are unknown. For example, over-producing an essential cofactor may confer resistance to the inhibitor but this cofactor may not be the primary target. Secondly, as discussed by us previously,¹⁴ such approach of over-expressing single essential protein per clone has the limitation that target(s) of an inhibitor that interacts with multiple subunits of a protein or an enzyme complex would be difficult to identify.

Figure 6. Images of zones of inhibition on assay trays with Pool #2 focusing on filter disks containing thiolactomycin ($300\text{ }\mu\text{g/disk}$) without induction (left) or with induction (right). Left panel shows complete clearing within zone of inhibition illustrating growth inhibition. Right panel shows emergence of resistant colonies within the zone of inhibition.

3. Conclusion

We have developed and validated a new version of TIPA, TIPA II, using *E. coli* AS19 as host cells and a collection of 272 individual cell clones configured into six pools of mixed cells. The TIPA II covers nearly the entire essential gene landscape of *E. coli* species and is simple and inexpensive to run. The TIPA II assays demonstrated higher sensitivity than TIPA assays in generating zones of inhibition (cell growth inhibition) for the antibacterial boron heterocycles. We employed both TIPA and TIPA II systems to test a series of 28 boron heterocycles in an attempt to identify their cellular targets. The former showed that the targets of seven boron heterocycles to be FabI, enoyl ACP reductase, while TIPA II was able to link the same enzyme as the target of 15 total boron heterocycles, eight more compounds whose targets were identified than what the TIPA system revealed. Moreover, the cellular targets of several known antibacterial inhibitors, triclosan, phosphomycin, trimethoprim, diazaborine and thiolactomycin, were identified. In particular, besides the known target (FabB, β -ketoacyl-ACP synthase I) of thiolactomycin identified, the TIPA II was able to reveal that another protein (AcpP, acyl carrier protein) may play an important role in the mechanism of action of this antibacterial inhibitor. In summary, the described TIPA II system will make contribution in discovery of novel antibacterial inhibitors with perhaps new mode of action.

4. Experimental

4.1. Bacterial strains, clones, culturing conditions and compounds

The collection of 272 *E. coli* AG1 clones (Table 1) containing the plasmid pCA24N with insert genes was procured from Nara Institute of Science and Technology (Nara, Japan)²¹. *E. coli* AS19 strain³³ was kindly provided by Dr. Q. Ye of Indiana University. All bacterial strains or cell clones were grown in Luria-Bertani (LB) broth or agar medium prepared from LB broth powder or agar granules purchased from Fisher Scientific (Tustin, CA). *E. coli* cell clones in AG1 host cells were grown in LB media supplemented with 30 µg/ml of chloramphenicol for maintenance of plasmids, while those in AS19 host cells were grown in LB media supplemented with 2 µg/ml of chloramphenicol. Bacterial strains or cell clones were preserved in frozen stocks containing 40% final glycerol, and stored at -80 °C. Isopropyl-β-D-1-thiogalactopyranoside was obtained from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide was purchased from Fisher Scientific. DNA ladders used in electrophoresis were purchased from Thermo Scientific (Carlsbad, CA). PCR was performed with Taq polymerase Master Mix from 5 PRIME (Hilden, Germany). Twenty-nine boron heterocycles were synthesized in the laboratory of Dr. Michael P. Groziak of California State University, East Bay. Known antibiotics were purchased from Sigma-Aldrich (St. Louis, MO).

4.2. Plasmid purification, confirmation and transformation into AS19

To isolate and purify plasmids from AG1 clones, frozen glycerol stocks of AG1 clones were streaked onto LB agar media plates containing 30 µg/ml chloramphenicol. The plates were incubated at 37 °C for 12-16 hours. Individual colonies were then picked from the agar plates and inoculated in 5 ml of LB broth with the same concentration of chloramphenicol. The cultures

were grown in a shaking incubator at 37° C for 12-16 hours. Plasmid DNA was purified using the Qiagen Miniprep Spin Kit, according to instructions of the vendor. The eluted plasmid DNA was stored at -20 °C. Each plasmid of the *E. coli* AG1 clones contains a unique essential gene as the insert in the pCA24N vector. The identities of plasmids were confirmed by routine restriction endonuclease digests, followed by agarose gel electrophoresis analysis of the patterns of digested DNA fragments. Comparison of experimental results with expected DNA digest patterns determined whether or not the identity of the plasmid is confirmed.

To transfer every plasmid into AS19 host cell, transformation experiments were performed using AS19 competent cells, which were prepared using the technique as described in Molecular Cloning: A Laboratory Manual.³⁴ Briefly, a single colony of freshly streaked AS19 cells was transferred into 100 ml of LB broth in a 1 L flask. Incubation of the culture lasted for three hours at 37 °C with vigorous agitation. The bacterial cells were then transferred to a chilled 50 ml polypropylene tube and the culture was cooled to 0 °C by storing the polypropylene tube on ice for 10 minutes. Once the cells had been cooled, the tube was centrifuged at 4,100 rpm for 10 minutes at 4 °C. Supernatant was decanted and the pellet is resuspended in 30 ml of ice cold magnesium chloride – calcium chloride solution (80 mM MgCl₂, 20 mM CaCl₂). Again the cells were centrifuged at 4,100 rpm for 10 minutes. Supernatant was decanted and the pellet was resuspended in 2 ml of ice cold calcium chloride (0.1 M CaCl₂) for every 50 ml of original culture. Frozen stocks of competent cells were prepared by adding 70 µl of dimethyl sulfoxide (DMSO) for every 2 ml of resuspended cells. This suspension was stored on ice for 15 minutes. An additional 70 µl of DMSO was then added to the suspension. The suspensions were mixed gently while in an ice bath. Aliquots of the suspension were dispensed into chilled, sterile

microfuge tubes. The competent cells were immediately snap frozen by immersing them in a dry ice/ethanol bath. The frozen tubes were stored at -80 °C.

To transform plasmids into AS19 cells, competent cell vials were thawed on ice. Fifty µl of competent cells were transferred to a sterile, chilled polypropylene tube using a chilled micropipette tip. One µl of the isolated plasmid DNA was added to the tube, with content of the tube being mixed gently using the pipet tip. After incubating the tube on ice for 30 minutes, the tube was moved to a preheated 42 °C water bath and was heat shocked for 90 seconds. Quickly, the tube was transported to an ice bath and allowed to cool for two minutes. Eight hundred µl of LB medium was added to the tube and the culture was incubated at 37° C for 45 minutes in a shaking incubator to allow time for the bacteria to recover and express the chloramphenicol resistance marker encoded in the plasmid. After the incubation, 100 µl of transformed competent cells were plated on LB agar plates containing 2 µg/ml chloramphenicol. The plates were stored at room temperature until the liquid had been absorbed and were then inverted and incubated at 37 °C for 12 – 16 hours.

4.3. Preparation of six pools of mixed clones

To test if cellular targets of antimicrobial compounds can be identified on solid media containing mixed clones, a disk-diffusion based procedure described previously¹⁴ was followed. The six pools from TIPA platform consists of five pools of AG1 clones harboring pCAN24N-based plasmids and one pool of DH5α clones harboring pLEX5BA-based plasmids, with significant overlap between the two types of clones in gene coverage.¹⁴ Because the two types of vectors have different antibiotic selection marker genes, TIPA pools were not convenient to manipulate. In TIPA II, in addition to the different host strain (AS19) with improved sensitivity, all 272 AS19 clones harbor plasmids based on the same vector (pCAN24N), making it more

efficient to perform the assays. The 272 transformed *E. coli* AS19 over-expression clones were grouped into six mixed pools. The six pools each contained between 39 and 48 clones (Table 1). To prepare the pools, one ml of LB containing 2 µg/ml chloramphenicol was added to each well of a 96-well deepwell block. Glycerol stocks of *E. coli* AS19 were inoculate into designated wells of 96-well deepwell blocks using sterile wooden applicators. The deepwell blocks were placed in a 35°C shaking incubator using a plate holder, and the cultures were allowed to grow for 16 hours. A Quadra 3 Automated Liquid Handler (Tomtec, Hamden, CT) was used to robotically transfer 100 µl of cell cultures from the 96-well deepwell block into a 96-well shallow microplate. The optical densities (OD₆₀₀) of the cell cultures were measured by a Tecan Infinite plate reader (Tecan, Durham, NC) and these values were used to calculate the volumes of the AS19 clones to be added to the designated mixed pools. Each clone sample was pipetted into a conical tube and, after all constituents of each pool were combined, the cells were vortexed to mix. One ml of the pooled sample was mixed with 99 ml of LB with 2 µg/ml chloramphenicol to obtain 100-fold dilutions of the pooled sample. Both the original pools and diluted pools were combined with 40% sterile glycerol solutions and their aliquots were stored at -80 °C.

4.4. TIPA and TIPA II assays

The TIPA assays were conducted as described previously.¹⁴ The TIPA II assays were performed similarly as TIPA procedures¹⁴ with the following modification. All six pools were grown in LB broth or agar media supplemented with 2 µg/ml of chloramphenicol.

Acknowledgements

This work was partially supported by grants from Army Research Office (grant W911NF-12-1-0059 to H.H.X.), National Institutes of Health (SC3GM83686 to H.H.X.; R01-GM105898 to F.Z.), CSUEB (Faculty Support Grants and Joan Sieber Interdisciplinary Research Award to M.P.G.), CSUPERB (Entrepreneurial Joint Venture to H.H.X.) and National Science Foundation (MRI grant 0420615, for the 500 MHz FT-NMR spectrometer). M.S.W., I.S. W.M. and J. J. acknowledge scholarship support from the NIH Minority Biomedical Research Support-Research Initiative for Scientific Enhancement (MBRS-RISE) Program (5R25GM061331). We also thank Nara Institute of Science and Technology (Nara, Japan) for sharing the *E. coli* gene clones.

References

1. Ho, J.; Tambyah, P. A.; Paterson, D. L. *Curr Opin Infect Dis* **2010**, *23*, 546.
2. Boucher, H. W.; Talbot, G. H.; Benjamin, D. K., Jr.; Bradley, J.; Guidos, R. J.; Jones, R. N.; Murray, B. E.; Bonomo, R. A.; Gilbert, D. *Clin Infect Dis* **2013**, *56*, 1685.
3. Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J. *Clin Infect Dis* **2009**, *48*, 1.
4. Forsyth, R. A.; Haselbeck, R. J.; Ohlsen, K. L.; Yamamoto, R. T.; Xu, H.; Trawick, J. D.; Wall, D.; Wang, L.; Brown-Driver, V.; Froelich, J. M.; C, K. G.; King, P.; McCarthy, M.; Malone, C.; Misiner, B.; Robbins, D.; Tan, Z.; Zhu Zy, Z. Y.; Carr, G.; Mosca, D. A.; Zamudio, C.; Foulkes, J. G.; Zyskind, J. W. *Mol Microbiol* **2002**, *43*, 1387.
5. French, C. T.; Lao, P.; Loraine, A. E.; Matthews, B. T.; Yu, H.; Dybvig, K. *Mol Microbiol* **2008**, *69*, 67.
6. Gerdes, S. Y.; Scholle, M. D.; Campbell, J. W.; Balazsi, G.; Ravasz, E.; Daugherty, M. D.; Somera, A. L.; Kypides, N. C.; Anderson, I.; Gelfand, M. S.; Bhattacharya, A.; Kapatral, V.; D'Souza, M.; Baev, M. V.; Grechkin, Y.; Mseeh, F.; Fonstein, M. Y.; Overbeek, R.; Barabasi, A. L.; Oltvai, Z. N.; Osterman, A. L. *J Bacteriol* **2003**, *185*, 5673.
7. Ji, Y.; Zhang, B.; Van, S. F.; Horn, Warren, P.; Woodnutt, G.; Burnham, M. K.; Rosenberg, M. *Science* **2001**, *293*, 2266.
8. Moule, M. G.; Hemsley, C. M.; Seet, Q.; Guerra-Assuncao, J. A.; Lim, J.; Sarkar-Tyson, M.; Clark, T. G.; Tan, P. B.; Titball, R. W.; Cuccui, J.; Wren, B. W. *MBio* **2014**, *5*, e00926.
9. Lange, R. P.; Locher, H. H.; Wyss, P. C.; Then, R. L. *Curr Pharm Des* **2007**, *13*, 3140.
10. Barrow, E. W.; Clinkenbeard, P. A.; Duncan-Decocq, R. A.; Perteet, R. F.; Hill, K. D.; Bourne, P. C.; Valderas, M. W.; Bourne, C. R.; Clarkson, N. L.; Clinkenbeard, K. D.; Barrow, W. W. *J Biomol Screen* **2012**, *17*, 946.
11. De La Fuente, R.; Sonawane, N. D.; Arumainayagam, D.; Verkman, A. S. *Br J Pharmacol* **2006**, *149*, 551.
12. Look, G. C.; Vacin, C.; Dias, T. M.; Ho, S.; Tran, T. H.; Lee, L. L.; Wiesner, C.; Fang, F.; Marra, A.; Westmacott, D.; Hromockyj, A. E.; Murphy, M. M.; Schullek, J. R. *Bioorg Med Chem Lett* **2004**, *14*, 1423.
13. Selin, C.; Stietz, M. S.; Blanchard, J. E.; Gehrke, S. S.; Bernard, S.; Hall, D. G.; Brown, E. D.; Cardona, S. T. *PLoS One* **2015**, *10*, e0128587.
14. Silva, I.; Real, L. J.; Ward, M. S.; Xu, H. H. *Appl Microbiol Biotechnol* **2014**, *98*, 5551.
15. Chopra, I. *J Antimicrob Chemother* **1998**, *41*, 584.
16. Bandekar, P. P.; Roopnarine, K. A.; Parekh, V. J.; Mitchell, T. R.; Novak, M. J.; Sinden, R. R. *Journal of medicinal chemistry* **2010**, *53*, 3558.
17. Gabrielli, L.; Merlo, S.; Airoidi, C.; Sperandeo, P.; Gianera, S.; Polissi, A.; Nicotra, F.; Holler, T. P.; Woodard, R. W.; Cipolla, L. *Bioorg Med Chem* **2014**, *22*, 2576.
18. Good, L.; Sandberg, R.; Larsson, O.; Nielsen, P. E.; Wahlestedt, C. *Microbiology* **2000**, *146* (Pt 10), 2665.
19. Liu, M.; Douthwaite, S. *Antimicrob Agents Chemother* **2002**, *46*, 1629.
20. Raneri, M.; Sciandrone, B.; Biani, F. *J Biomol Screen* **2015**, *20*, 627.
21. Kitagawa, M.; Ara, T.; Arifuzzaman, M.; Ioka-Nakamichi, T.; Inamoto, E.; Toyonaga, H.; Mori, H. *DNA Res* **2005**, *12*, 291.
22. McMurtry, L. M.; Oethinger, M.; Levy, S. B. *Nature* **1998**, *394*, 531.
23. Kim, D. H.; Lees, W. J.; Kempell, K. E.; Lane, W. S.; Duncan, K.; Walsh, C. T. *Biochemistry* **1996**, *35*, 4923.

24. Searle, M. S.; Forster, M. J.; Birdsall, B.; Roberts, G. C.; Feeney, J.; Cheung, H. T.; Kompis, I.; Geddes, A. J. *Proc Natl Acad Sci U S A* **1988**, *85*, 3787.
25. Baldock, C.; de Boer, G. J.; Rafferty, J. B.; Stuitje, A. R.; Rice, D. W. *Biochem Pharmacol* **1998**, *55*, 1541.
26. Kanichar, D.; Roppiyakuda, L.; Kosmowska, E.; Faust, M. A.; Tran, K. P.; Chow, F.; Buglo, E.; Groziak, M. P.; Sarina, E. A.; Olmstead, M. M.; Silva, I.; Xu, H. H. *Chem Biodivers* **2014**, *11*, 1381.
27. Baldock, C.; Rafferty, J. B.; Sedelnikova, S. E.; Baker, P. J.; Stuitje, A. R.; Slabas, A. R.; Hawkes, T. R.; Rice, D. W. *Science* **1996**, *274*, 2107.
28. Lu, H.; Tonge, P. J. *Acc Chem Res* **2008**, *41*, 11.
29. Grassberger, M. A.; Turnowsky, F.; Hildebrandt, J. *Journal of medicinal chemistry* **1984**, *27*, 947.
30. Jordan, C. A.; Sandoval, B. A.; Serobyan, M. V.; Gilling, D. H.; Groziak, M. P.; Xu, H. H.; Vey, J. L. *Acta Crystallogr F Struct Biol Commun* **2015**, *71*, 1521.
31. Jackowski, S.; Zhang, Y. M.; Price, A. C.; White, S. W.; Rock, C. O. *Antimicrob Agents Chemother* **2002**, *46*, 1246.
32. Tsay, J. T.; Rock, C. O.; Jackowski, S. *J Bacteriol* **1992**, *174*, 508.
33. Sekiguchi, M.; Iida, S. *Proc Natl Acad Sci U S A* **1967**, *58*, 2315.
34. Sambrook, J.; Russell, D. W. *Molecular cloning: a laboratory manual*; Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 2001.

Web reference

PEC, <http://shigen.nig.ac.jp/ecoli/pec/index.jsp> (ver. 4; accessed February, 2016).

Graphical Abstract

Non-induced

Induced