Further Characterization of *Bacillus subtilis* Antibiotic Biosensors and Their Use for Antibacterial Mode-of-Action Studies[▽]

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We further examined the usefulness of previously reported *Bacillus subtilis* biosensors for antibacterial mode-of-action studies. The biosensors could not detect the tRNA synthetase inhibitors mupirocin, indolmycin, and borrelidin, some inhibitors of peptidoglycan synthesis, and most membrane-damaging agents. However, the biosensors confirmed the modes of action of several RNA polymerase inhibitors and DNA intercalators and provided new insights into the possible modes of action of ciprofloxacin, anhydrotetracycline, corrallopyronin, 8-hydroxyquinoline, and juglone.

Bacteria containing reporter genes that are fused to promoters that respond to antibiotic-induced stress are becoming increasingly popular for screening and characterization of inhibitors during the process of antibacterial drug discovery (26). A particularly useful set of strains has been developed using *Bacillus subtilis* 1S34 as the host. This set is based upon five promoter-luciferase reporter fusion strains (26) that signal the presence of inhibitors of fatty acid synthesis (*fabHB* promoter), DNA synthesis (*yorB* promoter), cell wall synthesis and cell envelope stress (*ypuA* promoter), RNA synthesis (*yvgS* promoter), and protein synthesis (*yheI* promoter).

Although these biosensors have already been validated with several classes of antibiotics with known modes of action (26), we sought to establish their utility for predicting or confirming the modes of action of additional inhibitors not previously examined. The inhibitors we tested comprised chemically distinct entities ranging from molecules with well-defined modes of action to those with poorly defined targets. In addition to further validation of the biosensors using agents with known modes of action, we have confirmed that the biosensors have the potential to reveal the biosynthetic pathways inhibited by poorly characterized agents, thus contributing to information on whether they might be suitable for development as new antibacterial agents.

The methodology used here followed published procedures. The MICs of inhibitors for *B. subtilis* 1S34 were obtained by microdilution in Mueller-Hinton broth according to the British Society for Antimicrobial Chemotherapy guidelines (22). The biosensors were used essentially as described previously (11, 26) by measuring the luminescence emitted by the strains in the presence of a range of concentrations of inhibitor that always included concentrations at, above, and just below the MIC of the inhibitor. Induction was expressed as a value relative to the value for drug-free controls, and maximal induction ratios were determined relative to the inducing concentration of the inhibitor. A minimum of three independent

experiments was conducted with each inhibitor-biosensor combination. Induction thresholds for detection of inhibitors by the biosensors have been experimentally defined as 2.5-fold for *yorB*, 2-fold for *yvgS*, *yheI*, and *fabHB*, and 1.7-fold for *ypuA* (26).

We analyzed the responses of the biosensors to a number of previously untested inhibitors (Table 1). Some of these agents have well-defined modes of action (category 1), whereas the targets of others are very poorly characterized (category 2) (Table 1). For agents in the first category, it was expected that telavancin, deoxyactagardine B, fosfomicin, fosmidomycin, D-cycloserine, daptomycin, XF70, XF73, clofazimine, cetyltrimethylammonium bromide (CTAB), valinomycin, Sepracor 155342, and anhydrotetracycline might induce *ypuA* (cell wall synthesis/cell envelope stress), corrallopyronin, myxopyronin B, and ripostatin (keto and hemiacetal isomers) might induce *yvgS* (RNA synthesis), mupirocin, indolmycin, and borrelidin might induce *yheI* (protein synthesis), and daunorubicin, doxorubicin, and chromomycin might induce *yorB* (DNA synthesis). Several of these agents, including telavancin, deoxyactagardine B, daptomycin, myxopyronin B, ripostatin (both isomers), daunorubicin, doxorubicin, and chromomycin did indeed induce the expected biosensor strain (Table 2). These results serve to further validate the usefulness of this set of biosensors for detection of bacterial inhibitors.

However, the biosensors failed to generate a signal with other agents that have well-defined modes of action, such as tRNA synthetase inhibitors (mupirocin, indolmycin, and borrelidin), some inhibitors of peptidoglycan synthesis (fosfomicin, fosmidomycin, and D-cycloserine), and most membrane-damaging agents (CTAB, clofazimine, XF-70, XF-73, Sepracor 155342, and valinomycin) in each case not demonstrating induction above the published thresholds (data not shown). These results indicate that the biosensors have limitations, since they are unable to detect certain classes of bacterial inhibitors. Similar conclusions were reached by Urban et al. (26) who noted that a number of established inhibitors, particularly those affecting protein synthesis, were not detected by this set of biosensor strains.

Intriguing data were obtained with anhydrotetracycline and corrallopyronin which both induced more than one biosensor (Table 2). Anhydrotetracycline, although capable of

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TABLE 1. Antimicrobial agents not previously examined with *B. subtilis* biosensors

Antimicrobial agent(s)	Known or suspected mode of action(s)	Source	Reference(s)
Category 1			
D-Cycloserine	Inhibition of peptidoglycan synthesis	Sigma Aldrich, Poole, UK	6
Deoxyactagardine B	Inhibition of peptidoglycan synthesis	Novacta Biosystems Ltd., Welwyn Garden City, UK	2
Fosfomycin	Inhibition of peptidoglycan synthesis	Sigma Aldrich, Poole, UK	6
Fosmidomycin	Inhibition of peptidoglycan synthesis	Invitrogen Ltd., Paisley, UK	13
Telavancin	Inhibition of peptidoglycan synthesis and membrane disruption	Theravance, San Francisco, CA, USA	7
Daptomycin	Membrane disruption	Cubist Pharmaceuticals, Lexington, MA, USA	8
CTAB	Membrane disruption	British Drug Houses Laboratory Supplies, Poole, UK	6
Clofazamine	Membrane disruption	Sigma Aldrich, Poole, UK	17
Anhydrotetracycline	Membrane disruption	Sigma Aldrich, Poole, UK	14
XF70 and XF73	Membrane disruption	Destiny Pharma, Brighton, UK	20, 21
Sepracor 155342	Membrane disruption	Sepracor Inc., Marlborough, MA, USA	16
Valinomycin	Potassium ionophore	Sigma Aldrich, Poole, UK	6
Ripostatin A (keto and hemiacetal forms)	Inhibition of RNA polymerase	G. Hofle, Helmholtz Centre for Infection Research, Braunschweig, Germany	12
Corallopyronin A	Inhibition of RNA polymerase	G. Hofle, Helmholtz Centre for Infection Research, Braunschweig, Germany	12
Myxopyronin B	Inhibition of RNA polymerase	T. Moy, Cubist Pharmaceuticals, Lexington, MA, USA	12
Chromomycin	DNA intercalator	Sigma Aldrich, Poole, UK	1
Daunorubicin	DNA intercalator	Sigma Aldrich, Poole, UK	6, 24
Doxorubicin	DNA intercalator	Sigma Aldrich, Poole, UK	24
Mupirocin	Isoleucyl tRNA synthetase inhibitor	Sigma Aldrich, Poole, UK	9
Indolmycin	Tryptophanyl tRNA synthetase inhibitor	Pfizer, Kalamazoo, MI, USA	9
Borrelidin	Threonyl tRNA synthetase inhibitor	Biotica Ltd., Cambridge, UK	9
Category 2			
Juglone	Inhibition of peptidyl-prolyl isomerase and RNA polymerase	Sigma Aldrich, Poole, UK	3
8-Hydroxyquinoline	Inhibition of RNA polymerase	Sigma Aldrich, Poole, UK	4, 5
Baicalein	Inhibition of DNA polymerase	Sigma Aldrich, Poole, UK	19
Holomycin and thiolutin	Inhibition of RNA synthesis	GlaxoSmithKline, Harlow, UK	15
Rose Bengal	Inhibition of RNA polymerase	Sigma Aldrich, Poole, UK	27

causing weak inhibition of bacterial protein synthesis in cell-free translation systems (23), is considered at the whole-cell level to act by promoting lethal membrane damage (14). The ability of anhydrotetracycline to induce both *yheI* (protein synthesis) and *ypuA* (cell wall synthesis/cell envelope stress) is consistent with the operation of both mechanisms of action and may offer new insights into the mode of action of this agent. Since a higher signal was generated with *yheI* than with *ypuA*, the more-dominant mode of action of anhydrotetracycline may actually be inhibition of protein synthesis, in contrast to the previous hypothesis that suggested membrane damage as the primary mechanism (14). Corallopyronin also induced two biosensors: in this case *yvgS* (RNA synthesis) and *fabHB* (fatty acid synthesis) (Table 2). Induction of *yvgS* is consistent with reports that corallopyronin inhibits bacterial RNA polymerase (12). However, the significance of the second apparent activity (inhibition of fatty acid synthesis) is currently unclear. Nevertheless, the primary mode of action of corallopyronin appears to be inhibition of RNA synthesis, since the induction signal for *yvgS* was greater than for *fabHB* (Table 2).

Since the *B. subtilis* biosensors appeared to be capable of detecting agents with additional modes of action, we considered whether it would be possible to detect both inhibition of DNA synthesis and membrane damage by ciprofloxacin, because for some time the fluoroquinolones have been suspected to have a secondary mode of action involving

membrane damage (10, 25). We were indeed able to detect induction of both the *yorB* promoter (DNA synthesis) (maximal induction ratio of 74.9 ± 5.6 at 1 $\mu\text{g/ml}$) and the *ypuA* promoter (cell wall synthesis/cell envelope stress) (maximal induction ratio of 1.8 ± 0.4 at 2 $\mu\text{g/ml}$) by ciprofloxacin (MIC of 0.125 $\mu\text{g/ml}$ against *B. subtilis* 1S34). The latter result supports a weak secondary mode of action involving membrane damage.

Regarding category 2 agents (i.e., those with poorly defined modes of action), the biosensors revealed that juglone and 8-hydroxyquinoline induced the *yorB* promoter (Table 2) consistent with inhibition of DNA synthesis by these agents. This contrasts with reported modes of action for these agents as inhibitors of peptidyl-prolyl isomerase and RNA polymerase (juglone) (3) and RNA polymerase (8-hydroxyquinoline) (4, 5). The biosensors were unable to reveal possible modes of action for the other poorly characterized inhibitors listed in Table 1 (baicalein, holomycin, thiolutin, and Rose Bengal), since no induction signals were obtained with any of these agents for the five biosensor strains examined here (data not shown).

In conclusion, we confirm and extend the usefulness of the *B. subtilis* biosensors first described by Urban et al. (26) for first-pass elucidation of the modes of action of bacterial inhibitors. Although the biosensors are not capable of detecting every class of antibacterial agent, they have been used, as

TABLE 2. Antimicrobial agents listed in Table 1 that induce one or more biosensors

Antimicrobial agent	<i>B. subtilis</i> 1S34 MIC (μg/ml)	Maximal induction ratio of the <i>B. subtilis</i> antibiotic biosensor ^a (inducing concn [μg/ml]) ^b				
		Cell wall synthesis and cell envelope stress (<i>ypuA</i>)	Protein synthesis (<i>yheI</i>)	RNA synthesis (<i>yvgS</i>)	DNA synthesis (<i>yorB</i>)	Fatty acid synthesis (<i>fabHB</i>)
Category 1						
Deoxyactagardine B	32 ^c	3.4 ± 0.7 (2)^c	1.0 ± 0.1 ^c	1.3 ± 0.4 ^c	0.9 ± 0.1 ^c	1.1 ± 0.1 ^c
Telavancin	0.031	3.1 ± 1.1 (2)	0.7 ± 0.2	1.3 ± 0.3	1.2 ± 0.4	1.3 ± 0.2
Daptomycin	2 ^c	2.6 ± 0.2 (1)^c	1.2 ± 0.1 ^c	1.2 ± 0.3 ^c	1.0 ± 0.2 ^c	1.2 ± 0.4 ^c
Anhydrotetracycline	0.5	1.9 ± 0.1 (1)	10.7 ± 1.7 (0.5)	1.5 ± 0.2	1.8 ± 0.1	1.6 ± 0.2
Ripostatin A						
Keto isomer	64	1.0 ± 0.1	1.3 ± 0.1	6.2 ± 0.5 (10)	1.1 ± 0.1	1.5 ± 0.1
Hemiacetal isomer	128	1.1 ± 0.4	1.3 ± 0.1	5.1 ± 0.5 (5)	1.2 ± 0.3	1.0 ± 0.1
Corralopyronin A	32	1.0 ± 0.1	1.3 ± 0.3	8.4 ± 1.0 (2)	1.7 ± 0.2	2.9 ± 0.4 (10)
Myxopyronin B	32	0.9 ± 0.1	1.5 ± 0.1	6.0 ± 0.4 (0.5)	1.4 ± 0.1	1.3 ± 0.3
Chromomycin	1	0.6 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	4.1 ± 0.8 (0.2)	1.4 ± 0.1
Daunorubicin	4	1.0 ± 0.1	1.2 ± 0.2	1.0 ± 0.2	66.6 ± 2.3 (10)	1.3 ± 0.1
Doxorubicin	4	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	38.2 ± 4.7 (5)	1.1 ± 0.1
Category 2						
Juglone	1	0.6 ± 0.1	1.1 ± 0.1	0.8 ± 0.2	5.9 ± 2.2 (0.2)	1.2 ± 0.1
8-Hydroxyquinoline	1	1.0 ± 0.2	1.2 ± 0.3	1.1 ± 0.2	4.7 ± 1.1 (1)	1.4 ± 0.2

^a The maximal induction ratio of the *B. subtilis* antibiotic biosensor is the maximum reporter signal $\pm$ standard deviation induced in the respective biosensor expressed as a ratio of the signal in noninduced control cultures. Signals above the published threshold (26) for induction of the respective biosensor are shown in boldface type.

^b The inducing concentration is the concentration of inhibitor demonstrating maximal induction (26) of the respective biosensor.

^c Determined in the presence of 50 μg of Ca^{2+}/ml .

described here, to provide new insights into the modes of action of several inhibitors.

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