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# Efflux-mediated bis-indole resistance in *Staphylococcus aureus* reveals differential substrate specificities for MepA and MepR

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ABSTRACT

The bis-indoles are a novel class of compounds with potent antibacterial activity against a broad spectrum of Gram-positive and Gram-negative pathogens. The mechanism of action of these compounds has not been clearly defined. To study the mechanism of action of bis-indoles, selections for mutants of *Staphylococcus aureus* NCTC 8325 with reduced susceptibility to several chemically related bis-indoles were carried out using serial passages in subinhibitory compound concentrations. Resistant mutants were only obtained for one of the four bis-indoles tested (MBX-1090), and these appeared at concentrations up to 16X MIC within 10–12 passages. MBX-1090 resistance mutations produced a truncated open reading frame of *mepR* (SAOUHSC_00314), a gene encoding a MarR-like repressor. MepR regulates expression of *mepA* (SAOUHSC_00315), which encodes a member of the *M*ultidrug *a*nd *T*oxic Compound *E*xtrusion (MATE) family of efflux pumps. MBX-1090 resistance was reverted when *mepR* (wild type) was provided *in trans*. Microarray experiments and RT-PCR experiments confirmed that over-expression of *mepA* is required for resistance. Interestingly, MBX-1090 resistant mutants and strains overexpressing *mepA* from an expression vector did not exhibit cross-resistance to closely related bis-indole compounds. MBX-1090 did not induce expression of *mepA*, suggesting that this compound does not directly interact with MepR. Conversely, the bis-indoles that were not substrates of MepA strongly induced *mepA* expression. The results of this study suggest that MepA and MepR exhibit remarkably distinct substrate specificity for closely related bis-indoles.



## 1. Introduction

The bis-indoles represent a novel class of antibacterial agents that exhibit potent in vitro activity against a broad spectrum of important Gram-positive and -negative pathogens, such as *Bacillus anthracis*, *Staphylococcus aureus* (including methicillin resistant), and *Enterococcus faecalis* (including vancomycin resistant), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Yersinia pestis, Burkholderia pseudomallei*, and *Burkholderia cepacia*, including multidrug resistant strains.[1] The bis-indoles are rapidly bactericidal against *B. anthracis* and *Y. pestis* at concentrations equivalent to 4× their MICs in in vitro killing curve assays, and produce 99.9% cell death in less than 3 h.[1] In addition, two of the bis-indoles provided protection of mice that were lethally infected with *B. anthracis*, *S. aureus*, or *Y. pestis*.[1]

The mechanism of action of the bis-indoles is not well defined. Panchal et al. demonstrated that two of the bis-indole compounds inhibit DNA, but not RNA, protein, or cell wall biosynthesis in macromolecular synthesis assays.[1] The finding that these compounds share some structural features with compounds that bind to the minor groove of double-stranded B form DNA,[2] such as DAPI, suggest that these compounds may inhibit DNA synthesis by binding to DNA. Preliminary experiments have demonstrated that the bis-indoles preferentially bind to A–T rich sites in DNA.[3] However, if the antibacterial activity of the bis-indoles was solely due to DNA binding, the bis-indoles would be expected to inhibit both DNA and RNA synthesis, which is observed when bacteria are treated with antibiotics derived from distamycin, a DNA-minor groove binding compound.[4] Therefore, it is possible that the antibacterial activity of the bis-indoles involves another molecular target.

To further characterize the mechanism of action of the bis-indoles, we selected mutants of *S. aureus* with reduced susceptibility to these compounds and mapped the resistance mutations to identify potential molecular targets. Here we report analyses of mutants with reduced susceptibility to a novel bis-indole antibiotic. Genetic analyses revealed that the resistance mutations did not map to a gene encoding a potential molecular target, but resulted in over-expression of MepA, a member of the *M*ultidrug *a*nd *T*oxic Compound *E*xtrusion (MATE) family of efflux pumps.

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However, further experiments demonstrated an interesting substrate specificity of MepA and its substrate-responsive repressor MepR for the closely related panel of bis-indoles analyzed in this study, and reveals fundamental chemical characteristics of the substrates of this important efflux system. This study represents the first analysis of this type that utilizes a panel of closely related compounds.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. Strains were propagated in cation adjusted Mueller Hinton Broth (MHBca) at 37 °C.

### 2.2. Bis-indoles and antibiotics

All antibiotics, with the following exceptions, were purchased from Sigma–Aldrich (St. Louis, MO): Carbenicillin (MediaTech, Inc., Manassas, VA), Ciprofloxacin and Enrofloxacin (ICN Biochemicals, Aurora, OH), and Mupirocin (SynGen Biotech Co., Ltd, Hsin-Ying Tainan, Taiwan). The synthesis of MBX-1162 and the previously described bis-indole antibiotics[1] used in this study will be reported in a future literature report (manuscript in preparation).

### 2.3. Antibacterial susceptibility assays

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method[5] with the following modifications. Antibacterial compounds were dissolved in dimethyl sulfoxide (DMSO) and serial twofold dilutions of each compound were made in DMSO at concentrations that were 50-fold higher than the final concentration. Aliquots (2 μl) of each dilution were transferred to 96-well assay plates and 100 μl of an inoculum containing $1 \times 10^4$ cells was added to each well (final concentration of DMSO = 2%). The assay plates were incubated at 37 °C for 16–20 h and cell growth determined by measuring the absorbance at 600 nm. Reserpine (20 μg/ml) or anhydrous tetracycline (a-tet; 100 ng/ml) were added to the media of selected assays to inhibit efflux pumps or induce the expression vector pG154 (see below), respectively.

**Table 1**
Bacterial strains, plasmids, and primers used in this study

| Strain or plasmid | Description | Source/reference |
|---|---|---|
| *S. aureus strains* | | |
| NCTC 8325 (NRS-77) | Parent strain (WT) | NARSA[a,26] |
| 1090Rd20A1 | Mutant with reduced susceptibility to MBX-1090 isolated from population A on day 20. | This study |
| 1090Rd20B1 | Mutant with reduced susceptibility to MBX-1090 isolated from population B on day 20. | This study |
| 1090Rd20C1 | Mutant with reduced susceptibility to MBX-1090 isolated from population C on day 20. | This study |
| *ΔmepR::erm* | NCTC 8325 *ΔmepR::erm* | This study |
| *ΔmepRA::erm* | NCTC 8325 *ΔmepRA::erm* | This study |
| *ΔmepRAB::erm* | NCTC 8325 *ΔmepRAB::erm* | This study |
| WT (pMC-SK) | NCTC 8325 carrying shuttle vector pMC-SK | This study |
| WT (pMC-SK-*mepR*) | NCTC 8325 carrying pMC-SK with *mepR* under the control of its own promoter | This study |
| 1090Rd20A1 (pMC-SK) | 1090Rd20A1carrying shuttle vector pMC-SK | This study |
| 1090Rd20A1 (pMC-SK-*mepR*) | 1090Rd20A1 carrying *mepR* under control of its own promoter | This study |
| WT (pG154) | NCTC 8325 carrying expression vector pG154 | This study |
| WT (pG154-*mepA*) | NCTC 8325 carrying expression vector pG154 with *mepA* under the control of the a-tet inducible promoter | This study |
| *ΔmepRA::erm* (pG154) | NCTC 8325 *ΔmepRA::erm* carrying expression vector pG154 | This study |
| *ΔmepRA::erm* (pG154-*mepA*) | NCTC 8325 *ΔmepRA::erm* carrying expression vector pG154 with *mepA* under the control of the a-tet inducible promoter | This study |
| *Plasmids* | | |
| pMC-SK-GW | GateWay-adapted shuttle vector | This study |
| pG154 | Anhydrous tetracycline (a-tet) inducible expression vector | This study |
| pMC-SK-*mepR* | pMC-SK carrying *mepR* under control of its own promoter | This study |
| pG154-*mepA* | pG154 carrying *mepA* under the control of the a-tet inducible promoter | This study |

| Primer | Primer sequence (5′→3′) |
|---|---|
| *mepRA amplification* | |
| 314-L1 (PCR) | CAAAATGTTTATGGCGCAAG |
| 315-R1 (PCR) | TCCCGATTTAGTCGGTGTTT |
| *mepRA sequencing* | |
| 314-L2 | TTGCTAAAGCATTACAACGAACA |
| 314-R1 | TTCAATACTCCTTGTCTTTTCCAA |
| 314-R2 | TCTTCTCGTATCTTGTGCATCG |
| 315-L1 | TGATTGGTATGTTAGCTAGTGTAGGC |
| 315-L2 | CGCGATTGCAAGTTATGGTA |
| 315-L3 | GGCCATTTTACAAGGTGCAA |
| 315-R2 | TCCGACCGAAATATACTCAGC |
| 315-R3 | CAACAAAGATAACGCCGATAGA |
| *RT-PCR* | |
| *mepA*-F | GCGAGAGGTGAAACGTTAGC |
| *mepA*-R | CTGATTGCAGTACCCAAAGC |
| *mepR*-F | GCTAAAGCATTACAACGAACAGG |
| *mepR*-R | GCGATACGAGTGTTTGTTCC |
| *rho*-F | TTCAACGTTTGACGAACCAC |
| *rho*-R | TCCACCCGCTTCAATATTTC |

[a] NRS-77 was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) Program, supported under NIAID/NIH Contract No. HHSN272200700055C.

### 2.4. Selection of mutants with reduced susceptibility to MBX-1090

Mutants of *S. aureus* NCTC 8325 were selected in a serial passage experiment as follows. Eight independent populations of *S. aureus* NCTC 8325 (populations A–H) were grown in 96-well assay plates in MHBca at 37 °C in the presence of several concentrations of MBX-1066, MBX-1090, MBX-1113, and MBX-1162 (0.125–128× MIC). On day 1 of the experiment, each well of the assay plate was inoculated with approximately $1 \times 10^4$ CFU of exponentially growing parent strain. After 16–18 h growth, 50 μl culture was recovered from the wells with highest compound concentration that exhibited robust growth (>50% of untreated control) for each population, were diluted 1:1000 in fresh MHB, and were used to inoculate the assay plate containing one of the bis-indoles (0.125–128× MIC) for the next culture. This process was repeated for 20 days. The assay plates from each serial passage were stored at −80 °C after the addition of glycerol to each well at a final concentration of 20%. Several colonies were isolated and purified from the frozen MBX-1090 resistant cultures. Resistant strains were named according to the following convention: compound, day, population, and clone number. For example, 1090Rd20A1 is resistant to MBX-1090, was isolated from the day 20 culture, from population A, and is clone number 1. Decreased susceptibility to MBX-1090 of the purified resistant mutants was confirmed using MIC assays.

### 2.5. Mapping resistance mutations

Mutations that were present in mutants with decreased susceptibility to MBX-1090 were mapped using Comparative Genome Sequencing (CGS), which is a DNA microarray based technique that is provided as a service by NimbleGen Systems, Inc.(Madison, WI) and has been used to successfully to map daptomycin and metronidazole resistance mutations in *S. aureus*[6] and *Helicobacter pylori*,[7] respectively. The genome sequence of *S. aureus* NCTC 8325 (Genbank Accession number CP000253) was used to construct the DNA microarray used for the CGS experiments. The microarrays were used to compare genomic DNAs from the reference strain and individual mutants from three populations (1090Rd20A1, 1090Rd20B1, and 1090Rd20C1) with reduced susceptibility to MBX-1090. Mutations identified by CGS were confirmed by sequencing PCR products amplified from genes containing putative mutations. The nucleotide primers used for PCR and sequencing reactions are listed in Table 1. Sequencing reactions were performed by Sequegen (Worcester, MA).

### 2.6. Strain construction

Deletion mutants were constructed in *S. aureus* using standard allelic exchange methods.[8] The primers used for constructing allelic exchange cassettes are listed in Table S1 (Supplementary data). Allelic exchange cassettes were constructed in a three-molecule splicing by overlap extension (SOE) PCR using the method described by Herring and Blattner.[9] The spliced products were cloned into pCR8/GW/TOPO (Invitrogen; Carlsbad, CA) according to manufacturer's instructions and transferred to a GateWay®-adapted allelic exchange plasmid (pG200-GW) using LR clonase (Invitrogen; Carlsbad, CA). Detailed descriptions of the construction of allelic exchange cassettes and of the allelic exchange vector pG200-GW are presented in Supplementary data.

### 2.7. Construction of expression plasmids

For complementation experiments, a fragment containing *mepR* and its promoter region was amplified by PCR using primers shown in Table S1 (Supplementary data) and cloned into the shuttle vector pMC-SK, resulting in pMC-SK-*mepR*. For over-expression experiments in *S. aureus*, the *mepA* open reading frame was amplified by PCR and cloned in a Gram-positive expression vector pG154 under the control of $P_{xyl\text{-}tetO}$, a promoter regulated by anhydrous-tetracycline.[10] A detailed description of the vectors (pMC-SK and pG154) and of the construction of these expression plasmids are presented in Supplementary data.

### 2.8. Transcription profile analyses

Overnight cultures of *S. aureus* NCTC8 8325 or 1090R d20A1 were diluted 1/200 into 200 ml of TSB in a 1 L flask and grown at 37 °C with shaking (250 rpm) until they reached an optical density at 600 nm ($OD_{600}$) of 0.3, at which time 5 ml aliquots were transferred to three replicate culture tubes containing an antibiotic or DMSO (2% final concentration) and incubated at 37 °C for 3 h. Antibiotics were added to cultures at the following concentrations that were chosen because they the highest concentration that did not significantly (⩽25%) affect the rate of growth in growth curve experiments: MBX-1066 (0.4 μg/ml), MBX-1090 (0.8 μg/ml), and distamycin (50 μg/ml). Aliquots (5 ml) of the cultures were transferred to three replicate culture tubes containing antibiotic and incubation was continued for 3 h. Treated cells were harvested and the cell pellets were resuspended in 1 ml RNA protect bacteria reagent (Qiagen, Germantown, MD) and incubated at room temperature for 15 min. RNA was purified using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to manufacturer's instructions followed by an on-column DNase treatment using Qiagen's RNase-Free DNase-Set (Qiagen, Germantown, MD). RNA quality and concentration were verified by gel electrophoresis and by the ratios of absorbance at 260 nm and 230 nm (260/230), and 260 nm and 280 nm (260/280). At this point, purified RNA from replicate cultures was combined. Microarray experiments using the purified RNA samples from two biological replicates of this experiment were performed as a fee for service by NimbleGen Systems, Inc. (Madison, WI) using a custom chip design based on the genome sequence of *S. aureus* NCTC 8325 (Genbank Accession number CP000253). Normalized hybridization data for both biological replicates provided by NimbleGen Systems was analyzed using ArrayStar 3 (DNASTAR Inc., Madison, WI) to identify genes that exhibit ⩾4-fold change (⩾90% confidence) in expression compared with the WT or untreated cultures.

### 2.9. RT-PCR

The culture conditions and antibiotic concentrations used in these experiments are as described above, except the following additional antibiotics were used: MBX-1113 (0.5 μg/ml), and MBX-1162 (0.125 μg/ml). RNA samples for RT-PCR were prepared as described above for DNA microarray experiments. Two micrograms of RNA were treated with RNase-free DNase (Promega, Madison, WI) for 1 h, and 1 μg of treated RNA was used for cDNA synthesis using the random hexamer protocol of the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). Reverse Transcriptase enzyme was omitted in one replicate to be used as a control for DNase treatment. RT-PCR experiments were performed using the LightCycler 480 SYBR Green Master Kit (Roche Applied Science, Indianapolis, IN) for PCR reactions and a LightCycler 480 Real Time PCR System (Roche) according to manufacturer's instructions. The primers used for RT-PCR are listed in Table 1 and were designed to have an annealing temperature of 60 °C and to produce an amplicon of 150–250 bp using Primer3.[11] A standard RT-PCR regimen consisted of a 95 °C hot start for 10 min, followed by 45 amplification cycles (95 °C, 10 s; 54 °C, 20 s; 72 °C, 30 s). Amplification specificity was demonstrated by a typical melting curve program. The prevalence of *mepA* transcripts were compared to *rho*, a reference or 'housekeeping' gene expressed

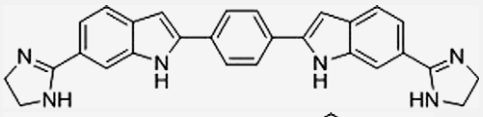
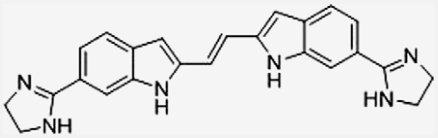
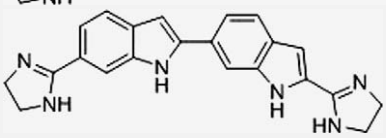
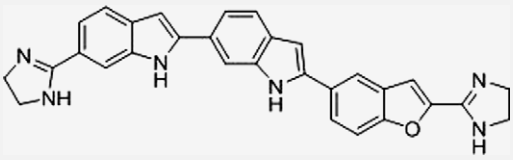
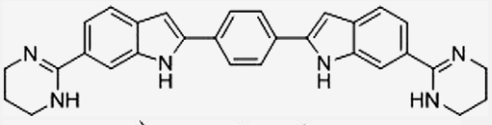
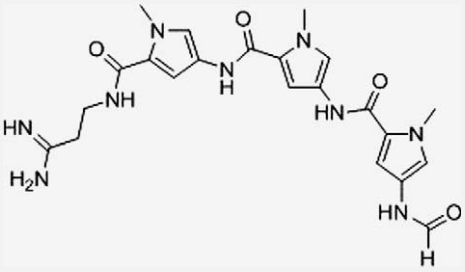
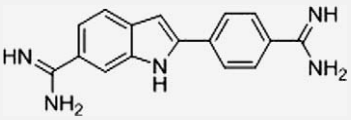
at generally constant levels.[12] Primer amplification efficiency for both *mepA* and *rho* samples was normalized with a primer-specific external standard curve made by amplifying varying concentrations of genomic DNA. A value was derived from the ratio of the average signal of several amplifications using *mepA* primers on a given cDNA to the average signal of several amplifications using *rho* primers on the same cDNA. The value for the cDNA from the untreated sample was set to 1, and values for the cDNA from treated samples were expressed as a comparison to the value of the untreated cDNA.

## 3. Results and discussion

### 3.1. Isolation of mutants with reduced susceptibility to bis-indoles

The bis-indoles shown in Table 2 represent a novel class of compounds with potent antibacterial activity against a broad spectrum of Gram-positive and Gram-negative pathogens.[1] To investigate the mechanism of action of the bis-indoles, we attempted to isolate and analyze mutants with decreased susceptibility to MBX-1066, MBX-1090, MBX-1113, and MBX-1162. Initial attempts to select directly for colonies of *S. aureus* NCTC 8325 with spontaneous mutations to resistance on agar plates containing these compounds were unsuccessful because the antibacterial activity of the bis-indoles decreases significantly in the presence of agar. When approximately $1 \times 10^8$ CFU of *S. aureus* were plated on MHB agar containing a bis-indole at concentrations up to 32-fold higher than the MIC a confluent lawn of bacteria was obtained (data not shown). Consequently, mutations with decreased susceptibility to the bis-indoles were selected by serial passage of 8 independent populations of susceptible NCTC 8325 (WT) in liquid media containing progressively increasing concentrations of one of the bis-indoles as previously reported.[1] Mutant populations with significantly reduced susceptibility ($\geqslant$4-fold) to MBX-1066,[1] MBX-1113,[1] and MBX-1162 (data not shown) did not appear after 20 serial passages. In contrast, all populations grown in the presence of MBX-1090 were able to grow in concentrations up to 16$\times$ MIC after 10–12 serial passages.[1] Moreover, reduced suscepti-

**Table 2**
Structures and physical properties of the compounds described in this study

| Compound | Structure | MW[a] | *c* Log *P*[b] | tPSA[c] (Å$^2$) | MSA[d] (Å$^2$) |
|---|---|---|---|---|---|
| MBX-1066 |  | 444.54 | 7.32 | 72.84 | 421.1 |
| MBX-1090 |  | 394.48 | 3.85 | 86.83 | 382.4 |
| MBX-1113 |  | 368.42 | 5.22 | 72.84 | 350.2 |
| MBX-1128 |  | 484.55 | 7.88 | 72.84 | 448.9 |
| MBX-1162 |  | 472.6 | 8.44 | 82.07 | 451.7 |
| Distamycin (DIST) |  | 495.55 | −0.39 | 175.99 | 449.5 |
| DAPI |  | 277.33 | 1.3 | 111.77 | 269.2 |

[a] MW, molecular weight.
[b] *c* Log *P*, predicted hydrophilicity using ChemDraw Ultra.
[c] tPSA, calculated topological polar surface area calculated using ChemDraw Ultra.
[d] MSA, calculated molecular surface area of fully energy minimized bis-indoles using default settings of Shrodinger software package.

bility to MBX-1090 appeared rapidly (within 1–2 serial passages) in each population, suggesting that acquisition of a single mutation is sufficient for the resistance phenotype.

To characterize the mutants further, individual clones from each of the resistant populations were isolated and MICs for MBX-1090 and MBX-1066 were determined to confirm decreased susceptibility to MBX-1090 and to test for cross-resistance. The MICs for three independent MBX-1090-resistant mutants (1090R d20A1, 1090R d20B1, 1090R d20C1) against MBX-1090 were 8–16-fold higher (8–16 μg/ml) than for the parent strain (WT), confirming reduced susceptibility of the mutants (data not shown). Interestingly, these mutants were not cross resistant to MBX-1066, indicating the resistance mutations are specific for MBX-1090 (data not shown). The susceptibility profile of one mutant (1090R d20A1) against additional bis-indoles and distamycin A was determined. The results, shown in Table 3, confirm that the highest increase in resistance of 1090R d20A1 was for MBX-1090, however, fourfold increases in the MIC for MBX-1113 and distamycin, an antibiotic that binds to the minor groove of DNA[13] were observed. In addition, the susceptibility profile of 1090R d20A1 against a panel of diverse antibiotics was determined (see Table S2, Supplementary data). The 1090R d20A1 strain exhibited modest fourfold and twofold increases in MICs for only two aminoglycoside antibiotics (streptomycin and kanamycin) and one fluoroquinolone (ciprofloxacin), respectively, but not against the other antibiotics tested including another fluoroquinolone (enrofloxacin), β-lactams, and tetracycline.

### 3.2. Identification of genetic changes in mutants with decreased susceptibility to MBX-1090

The genetic changes correlated with reduced susceptibility to MBX-1090 in three strains (1090R d20A1, B1, and C1) isolated from the serial passage experiment as compared to NCTC 8325 were identified using comparative genome sequencing (CGS).[7,14] The CGS method uses tiled DNA microarrays to generate high-resolution whole genome comparisons that can identify deletions, insertions, and single-nucleotide polymorphisms (SNPs). The CGS results for the three mutants are summarized in Table S3 (Supplementary data). All three mutants carried SNPs in the coding region of *mepR* (SAOUHSC_00314), a gene encoding a MarR-like repressor that regulates expression of the *mepRAB* locus.[15] MepR is comprised of a homodimer of 139 amino acid subunits that negatively regulates expression of *mepR* and *mepA* at the level of transcription by binding to operator sites upstream of the *mepR* and *mepA* genes that prevent RNA polymerase from binding to the promoters[15–17] (see Fig. 2). The *mepA* gene encodes an efflux pump that is a member of the *M*ultidrug *a*nd *T*oxic Compound *E*xtrusion (MATE) family of efflux pumps,[15] which mediates resistance to a wide range of cationic dyes, some fluoroquinolones, aminoglycosides and other structurally diverse antibiotics and drugs.[18,19] Transcription of the MepR regulon is induced when the DNA-bound repressor binds to a MepA substrate, which produces a conformational change in the repressor that decreases its affinity for DNA and allows RNA polymerase to initiate transcription. Inactivation of MepR has been shown to result in increased expression of the MepA efflux pump.[16,18] Interestingly, SNPs in *mepA* were present in two mutants (A1 and B1).

Because over-expression of MepA results in decreased susceptibility to diverse antibacterial agents,[15,16,18] it seemed likely that the SNPs in *mepR* were responsible for reduced susceptibility to MBX-1090 in the mutant strains. Consequently, the SNPs in *mepR* and *mepA* were confirmed by conventional sequencing. Each of the SNPs in *mepR* in the mutant strains is predicted to result in a truncated MepR protein. Interestingly, the *mepR* mutation in A1 (nt 153 C→T) produces a truncated protein of 117 amino acids that is missing a large portion of the dimer interface (alpha helices 5 and 6),which is predicted to result in an inactive repressor.[17] The *mepR* mutations in strains B1 and C1 are both insertions of a C after nt172, which results in a frameshift at codon 58 that encodes a truncated protein of 65 residues, which is predicted to be inactive as a repressor. The SNPs in *mepA* of mutants A1 and B1 were predicted to result in a semi-conservative (L379F) and a non-conservative (L337R) amino acid change, respectively. The role that these mutations play in reduced susceptibility to MBX-1090 is not clear. However, the fact that these mutations are not present in all three strains suggests that the *mepA* mutations are not required for resistance.

### 3.3. Confirmation of the role of MepR and MepA in resistance

The role of MepR in MBX-1090 resistance was confirmed by genetic complementation studies. A WT copy of *mepR* carried on a plasmid was introduced into 1090R d20A1 and the decreased susceptibility to MBX-1090 was reversed completely (see Table S4, Supplementary data). In addition, a defined genetic deletion of *mepR* in *S. aureus* NCTC 8325 (*ΔmepR::erm*) resulted in decreased susceptibility to MBX-1090 at levels similar to those of 1090R d20A1 (see Table 3). Transcription profiling experiments

**Table 3**
Genetic confirmation of the role of the *mepRAB* locus in reduced susceptibility to MBX-1090, bis-indole analogs, and distamycin

| Strain | Addition[a] | MIC (μg/ml) for MBX-: | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1066 | 1090 | 1113 | 1128 | 1162 | DIST |
| WT[b] | | 0.25 | 1 | 0.5 | 0.5 | 0.5 | 50 |
| WT | Reserpine | 0.125 | 0.5 | 0.25 | 0.25 | 0.125 | 50 |
| 1090R d20A1 | | 0.5 | 8 | 2 | 1 | 0.5 | 200 |
| *ΔmepR::erm* | | 0.25 | 4 | 1 | 0.5 | 0.25 | 100 |
| *ΔmepRA::erm* | | 0.5 | 1 | 0.5 | 1 | 0.5 | 100 |
| *ΔmepRAB::erm* | | 0.5 | 1 | 0.5 | 1 | 0.5 | 50 |
| WT (pG154) | | 0.5 | 1 | 0.5 | 1 | 0.5 | 50 |
| WT (pG154) | a-tet | 1 | 1 | 0.5 | 1 | 0.5 | 50 |
| WT (pG154-*mepA*) | | 0.5 | 1 | 0.5 | 1 | 0.5 | 50 |
| WT (pG154-*mepA*) | a-tet | 0.25 | 8 | 4 | 0.25 | 0.125 | 50 |
| *ΔmepRA::erm* (pG154) | | 0.5 | 1 | 0.5 | 1 | 0.5 | 50 |
| *ΔmepRA::erm* (pG154) | a-tet | 0.5 | 1 | 0.5 | 0.5 | 0.5 | 50 |
| *ΔmepRA::erm* (pG154-*mepA*) | | 0.25 | 1 | 0.5 | 0.5 | 0.5 | 50 |
| *ΔmepRA::erm* (pG154-*mepA*) | a-tet | 0.25 | 4 | 1 | 0.5 | 0.125 | 50 |

*Abbreviation*: DIST, distamycin.

[a] Reserpine was added to the indicated culture at a final concentration of 20 μg/ml to inhibit efflux pumps. Anhydrous tetracycline (a-tet) was added to the indicated cultures at a final concentration of 100 ng/ml to induce expression of *mepA* from the expression plasmid pG154-*mepA*.

[b] The wild type strain (WT) is *S. aureus* NCTC 8325.

comparing gene expression of 1090R d20A1 to the WT strain demonstrated increased expression of the *mepRAB* genes in the mutant (shown in Table 4), which is consistent with previous findings.[16,18] The decreased susceptibility to MBX-1090 of the *ΔmepR::erm* strain was reversed in a strain carrying deletions of both *mepR* and *mepA* (*ΔmepRA::erm*), which confirms the role of the MepA efflux pump in MBX-1090 resistance. The susceptibility of a strain carrying a deletion of the entire *mepRAB* locus (*ΔmepRAB::erm*) to MBX-1090 was the same as the *ΔmepRA::erm* mutant, suggesting that *mepB* does not play a role in the resistance phenotype. Taken together, the evidence supports the model shown in Figure 2 for the roles the MepR and MepA play in the MBX-1090 resistance phenotype.

To assess the cumulative role of all efflux systems on intrinsic resistance to the bis-indoles, susceptibility to these compounds was measured in the presence of reserpine, a general inhibitor of efflux pumps in Gram-positive bacteria,[20] including MepA.[16] As shown in Table 3, no significant difference in susceptibility to the bis-indoles tested was observed in the presence of reserpine, with the exception of MBX-1162 (fourfold decrease in MIC). This finding indicates that efflux does not play a major role in the intrinsic resistance of *S. aureus* to distamycin or most of the bis-indoles tested. Differences in susceptibility to some of these compounds are observed only when MepA is overexpressed, suggesting that the compounds tested are not MepA substrates, or do not bind to MepR, which would result in induction of MepA.

### 3.4. Substrate specificity of MepA

The substrate specificity of MepA was examined by measuring the susceptibility of WT and 1090R d20A1 strains to the panel of bis-indoles and distamycin. As shown in Table 3, 1090R d20A1 exhibited decreased susceptibility to MBX-1090, MBX-1113, and distamycin, suggesting that these compounds are MepA substrates. This finding was analyzed further by comparing susceptibility of WT and *ΔmepRA::erm* strains that over-expresses the MepA efflux pump from an expression vector (pG154) that is induced by addition of anhydrous tetracycline (a-tet). The results, shown in Table 3, demonstrate that MepA over-expression results in decreased susceptibility of the WT strain to MBX-1090 and MBX-1113 only. Interestingly, MepA over-expression in the *ΔmepRA::erm* strain resulted in decreased susceptibility to MBX-1090, but produced only a modest twofold decrease in susceptibility to MBX-1113. This finding suggests that a chromosomal copy of *mepRA* is required for resistance to MBX-1113 that results from over-expression of MepA. These results indicate MBX-1090 and MBX-1113 are the only substrates for the MepA efflux pump among the bis-indoles tested. In addition, the data suggests that the *mepA* mutation in 1090Rd20A1 plays a role in reducing susceptibility to distamycin.

**Table 4**
Transcription profiling of 1090R d20A1 and WT after exposure to MBX-1066, MBX-1090, or distamycin

| Sequence ID SAOUHSC_ | Description | Fold change in expression[a] | | | |
|---|---|---|---|---|---|
| | | 1090R d20A1 | 1066 | 1090 | DIST |
| *Up-regulated in 1090R d20A1* | | | | | |
| 00314 | *mepR* | +59.6 | +5.4 | +1.7 | +39.2 |
| 00315 | *mepA* | +109.0 | +8.7 | +2.0 | +72.6 |
| 00316 | *mepB* | +43.0 | +4.2 | +1.0 | +25.5 |
| 02290 | Conserved hypothetical protein | +2.1 | +6.8 | +15.3 | +14.2 |
| *Down-regulated in 1090R d20A1* | | | | | |
| 00069 | *spa* (protein A) | −6.8 | −3.1 | −3.1 | +2.0 |
| 01886 | *ribH* (riboflavin synthase, beta subunit) | −3.6 | +2.1 | −1.9 | −2.7 |
| 01887 | *ribA* (riboflavin biosynthesis protein) | −5.1 | +1.2 | −3.0 | −1.9 |
| 01888 | *ribE* (riboflavin synthase, alpha subunit) | −4.2 | +1.2 | −2.6 | −1.5 |
| 01889 | *ribD* (riboflavin biosynthesis protein) | −4.4 | +1.3 | −1.4 | −1.6 |

[a] Values represent ratio mutant or treated versus WT or untreated, respectively, with a 90% confidence as determined using ArrayStar (DNAStar).

### 3.5. Substrate specificity of MepR

Because substrates of the MepA efflux pump generally (but not always) also bind to MepR, we evaluated the ability of each of the bis-indoles and distamycin to induce *mepA* transcription. The binding of MepA substrates to MepR is thought to play an important role in gene regulation by inducing a conformation change in the repressor that reduces its affinity for specific DNA binding sites,[15] resulting in increased transcription of the *mepRAB* locus (see Fig. 2). To determine the effect of two bis-indoles (MBX-1090 and MBX-1060) and distamycin on *mepRAB* expression, transcription profiling experiments were performed. The results of these experiments, shown in Table 4, demonstrate that exposure to MBX-1066, which does not appear to be a MepA substrate, resulted in induction of *mepRAB* transcription. Distamycin, which is a weak substrate, strongly induced the *mepRAB* locus by 73-fold. Interestingly, MBX-1090 did not induce the *mepRAB* genes, even though it is a MepA substrate. To confirm and extend these findings, induction of *mepA* by exposure to various bis-indoles and distamycin was measured using RT-PCR. The results, shown in Figure 1, confirm that MBX-1090 does not induce *mepA* expression, whereas the other bis-indoles and distamycin induced transcription of mepA by varying degrees. Similar results were obtained when *mepR* transcription was measured (data not shown).

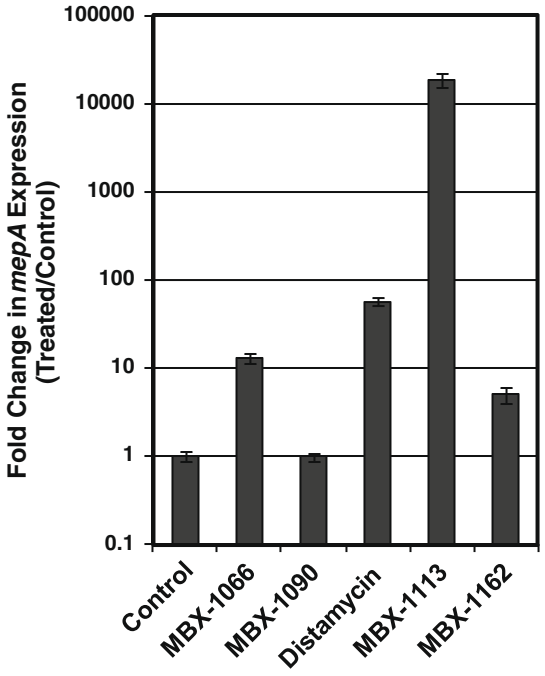



**Figure 1.** The relative number of *mepA* transcripts induced by exposure to bis-indoles and distamycin as compared to a housekeeping gene (*rho*) was measured using RT-PCR. *S. aureus* NCTC 8325 cultures were exposed to a subinhibitory concentration of a bis-indole or distamycin and the relative abundance of *mepA* RNAs normalized to a housekeeping gene (*rho*) were measured using RT-PCR. The fold-induction of *mepA* RNA as compared to an untreated control is shown for each compound. Each value represents the average of three experiments and the error bars represent the standard deviation for each average. The compounds and their concentrations (in parentheses) used in this experiment were as follows: MBX-1066 (0.4 μg/ml), MBX-1090 (0.8 μg/ml), MBX-1113 (0.5 μg/ml), and MBX-1162 (0.125 μg/ml), and distamycin (50 μg/ml).

The results of these experiments, summarized in Figure 2, demonstrate interesting differences in affinities of the bis-indoles for the MepA efflux pump and the MepR repressor that can be inferred from the assays described above. The decreased susceptibility of 1090Rd20A1, and WT and Δ*mepRA::erm* strains overexpressing MepA to MBX-1090, MBX-1113, and distamycin indicate that these compounds are substrates of the MepA efflux pump. Increased transcription of *mepA* and *mepR* in response to exposure to MBX-1066, MBX-1113, MBX-1162, and distamycin suggest that these compounds bind to MepR, resulting in a decreased affinity for its operator caused by a conformational change in the repressor.[15,17] Studies that measure directly the affinity of these bis-indoles for MepR would be required to confirm this finding. Interestingly, the results of this study indicate that the bis-indoles appear to fall into three classes with regards to their apparent affinities for MepA and MepR. First, MBX-1090 is a MepA substrate but does not bind to MepR. Second, MBX-1066 and MBX-1162 bind to MepR, but are not MepA substrates. Third, MBX-1113 and distamycin are MepA substrates and also bind MepR. Kaatz et al. have shown previously that two fluoroquinolones, ciprofloxacin and norfloxacin, appear to be MepA substrates,[16] but do not bind to MepR.[15] However, this study is the first analysis of several closely related compounds that vary in their ability to bind to MepA and/or MepR.

### 3.6. Bis-indoles and MepA or MepR binding specificity

To better understand the chemical features the bis-indoles required for recognition by the MepA efflux pump and/or MepR, the physical properties and chemical structures shown in Table 2 were examined. MepR binds to a variety of lipophilic monovalent and bivalent cationic biocides, drugs, dyes, antiseptics and disinfectants[15] that induce a conformation change that results in decreased affinity for its regulatory DNA binding sites.[17] All bis-indoles consist of planar conjugated ring systems and have terminal basic imadozoline residues, with the exception of MBX-1162, which has terminal basic tetrahydropyrimidine residues. The bis-indoles exhibit several similarities to 4′,6-diamidino-2-phenylindole (DAPI), including a terminal amidine with the potential to be positively charged, and an indole core (see Fig. 2). The estimated p$K_a$'s of the compounds are in the order of tetrahydropyrimidine (MBX-1162; p$K_a$ ~12.8[21]) > amidine (DAPI; p$K_a$ ~11.6[22]) > imidazoline (MBX-1066, MBX-1090; p$K_a$ ~9.6[23]). These p$K_a$'s are estimated based on literature values of the corresponding phenyl-substituted tetrahydropyrimidine, amidine, and imidazoline, respectively. The actual p$K_a$'s are expected to be similar, and maintain the same relative order. Kaatz et al. have shown that DAPI is a substrate of the MepA efflux pump[16] and that it binds to MepR using a direct binding assay.[17] The bis-indoles that bind to MepR exhibit an additional similarity with DAPI in that they have a phenyl group linked to the indole, which is absent in MBX-1090, the only compound that does not bind MepR. This suggests that the phenyl group is critical for MepR binding by the bis-indoles analyzed in this study. Kumaraswami et al. found that DAPI and R6G bind to MepR, but R6G binds with a significantly lower affinity.[17] The investigators hypothesized that the larger R6G (molecular surface area = 407.1 Å$^2$) does not fit the multidrug-binding pocket of MepR as well as the bivalent diamidine DAPI (MSA = 269.2 Å$^2$),[17] suggesting that size is an important factor for binding. Our results suggest that bis-indoles larger than R6G, such as MBX-1066 and MBX-1162, are able to bind to MepR. Therefore, other molecular properties must be critical for MepR binding. An analysis of the region of MepR that corresponds to the substrate recognition site of

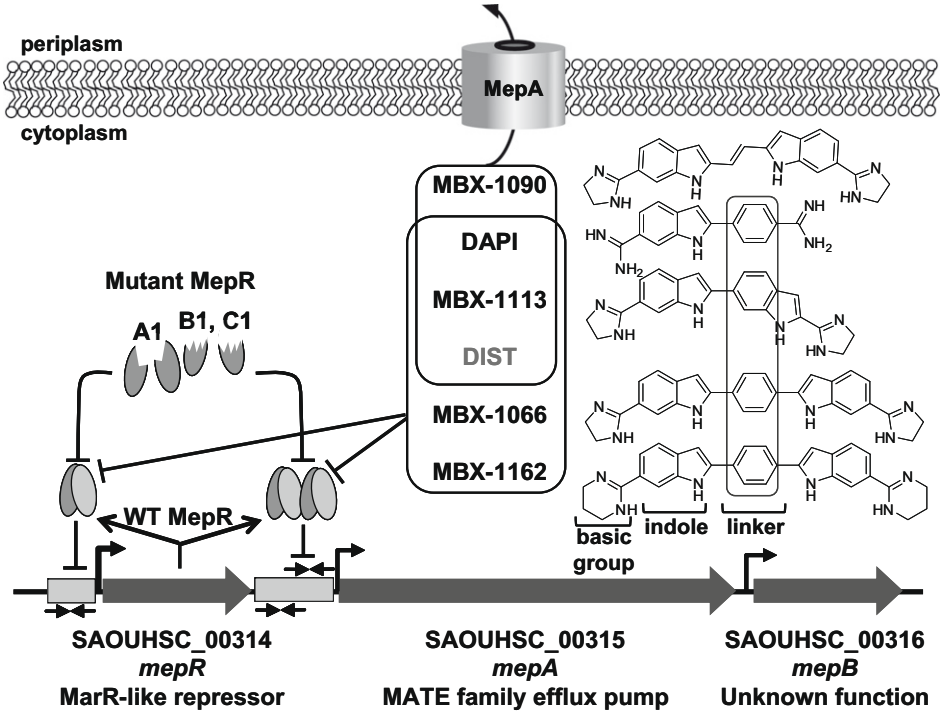



**Figure 2.** Model for the regulation of *mepRAB* by the MepR repressor, and a summary of the specificity of the bis-indoles for MepA and MepR. MepR is a MarR-like repressor that regulates expression of the *mepR* and *mepA* promoters by binding to upstream operator sites and blocking access to the promoter by RNA polymerase, which represses transcription.[17] Mutations resulting in truncated MepR are predicted to inactivate its repressor activity, resulting in increased expression of *mepR* and *mepA*. MepA is a member of the *M*ultidrug *a*nd *T*oxic Compound *E*xtrusion (MATE) family of efflux pumps, and MepB is a protein of unknown function. Several MepA substrates have been shown to bind to MepR, inducing a conformational change that decreases the affinity of MepR for its operator sites and results in increased expression of *mepRAB*. Gene expression studies suggest several bis-indoles (MBX-1113, MBX-1066, and MBX-1162), DAPI, and distamycin bind to MepR, which are indicated by the large box that is connected to MepR by a line that indicates a negative interaction. MBX-1090, MBX-1113, DAPI, and distamycin are MepA substrates as indicated by the smaller box connected to an arrow. Key: ⊥, negative interaction.

MexR that binds to the ArmR anti-repressor was determined in a repressor-anti-repressor co-crystal[24] reveals that it contains eight hydrophobic residues surrounded by four acidic or polar residues.[17] The nature of the putative multidrug-binding pocket of MepR suggests that basicity and hydrophobicity are important parameters that influence the binding of various bis-indoles to MepR. However, the data presented here do not exclude the possibility that MBX-1090 does bind to MepR but does not induce the conformational changes that result in loss of repressor activity. As stated above, direct binding studies will be required to confirm these hypotheses.

Substrates of bacterial multidrug resistance pumps, including MATE family pumps, include cationic compounds that are lipophilic.[25] However, there appears to be a clear negative correlation between *c* Log *P*, calculated hydrophilicity (see Table 2), and MepA substrates. Compounds with *c* Log *P* values $\leqslant$5.2 (e.g., MBX-1090, MBX-1113, distamycin, and DAPI) are substrates of MepA. In contrast, the bis-indoles with *c* Log *P* values $\geqslant$7.2 (e.g., MBX-1066, MBX-1162, and MBX-1128) are not MepA substrates, suggesting that there is an upper limit for lipophility of MepA substrates.

## 4. Concluding remarks

Our mechanism of action study has uncovered interesting trends in the substrate specificity of the MepA efflux pump and the substrate-responsive MepR repressor for a group of chemically related bis-indole antibacterial agents. Further studies of MepA and MepR substrate specificity using panels of chemically related compounds will increase our understanding of the structure activity relationships for binding.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.005.

## References and notes

1. Panchal, R. G.; Ulrich, R. L.; Lane, D.; Butler, M. M.; Houseweart, C.; Opperman, T.; Williams, J. D.; Peet, N. P.; Moir, D. T.; Nguyen, T.; Gussio, R.; Bowlin, T.; Bavari, S. *Antimicrob. Agents Chemother.* **2009**, *53*, 4283.
2. Brosh, R. M., Jr.; Karow, J. K.; White, E. J.; Shaw, N. D.; Hickson, I. D.; Bohr, V. A. *Nucleic Acids Res.* **2000**, *28*, 2420.
3. Opperman, T. J.; Li, J. B.; Lewis, M. A.; Houseweart, C.; Aiello, D.; Williams, J. D.; Peet, N. P.; Moir, D. T.; Long, E. C.; Bowlin, T. L. DNA Binding Activity of Novel Bis-indole Antibiotics. In *49th Interscience Conference on Antimicrobial Agents and Chemotherapy*; American Society of Microbiology: San Francisco, CA USA, 2009.
4. Ge, Y.; Difuntorum, S.; Touami, S.; Critchley, I.; Burli, R.; Jiang, V.; Drazan, K.; Moser, H. *Antimicrob. Agents Chemother.* **2002**, *46*, 3168.
5. CLSI, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*; *Approved Standard-Seventh ed.* Clinical and Laboratory Standards Institute: Wayne, PA USA, 2006; Vol. M7-A7 Vol. 26 (2).
6. Friedman, L.; Alder, J. D.; Silverman, J. A. *Antimicrob. Agents Chemother.* **2006**, *50*, 2137.
7. Albert, T. J.; Dailidiene, D.; Dailide, G.; Norton, J. E.; Kalia, A.; Richmond, T. A.; Molla, M.; Singh, J.; Green, R. D.; Berg, D. E. *Nat. Methods* **2005**, *2*, 951.
8. Bruckner, R. *FEMS Microbiol. Lett.* **1997**, *151*, 1.
9. Herring, C. D.; Blattner, F. R. *J. Bacteriol.* **2004**, *186*, 2673.
10. Geissendorfer, M.; Hillen, W. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 657.
11. Rozen, S.; Shaletsky, H. Primer3 on the WWW for General Users and for Biologist Programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology*; Krawetz, S., Misener, S., Eds.; Humana Press: Totowa, NJ, 2000; p 365.
12. Theis, T.; Skurray, R. A.; Brown, M. H. *J. Microbiol. Methods* **2007**, *70*, 355.
13. Chen, X.; Ramakrishnan, B.; Rao, S. T.; Sundaralingam, M. *Nat. Struct. Biol.* **1994**, *1*, 169.
14. Herring, C. D.; Palsson, B. O. *BMC Genom.* **2007**, *8*, 274.
15. Kaatz, G. W.; DeMarco, C. E.; Seo, S. M. *Antimicrob. Agents Chemother.* **2006**, *50*, 1276.
16. Kaatz, G. W.; McAleese, F.; Seo, S. M. *Antimicrob. Agents Chemother.* **2005**, *49*, 1857.
17. Kumaraswami, M.; Schuman, J. T.; Seo, S. M.; Kaatz, G. W.; Brennan, R. G. *Nucleic Acids Res.* **2009**, *37*, 1211.
18. McAleese, F.; Petersen, P.; Ruzin, A.; Dunman, P. M.; Murphy, E.; Projan, S. J.; Bradford, P. A. *Antimicrob. Agents Chemother.* **2005**, *49*, 1865.
19. Kuroda, T.; Tsuchiya, T. *Biochim. Biophys. Acta* **2009**, *1794*, 763.
20. Schmitz, F. J.; Fluit, A. C.; Luckefahr, M.; Engler, B.; Hofmann, B.; Verhoef, J.; Heinz, H. P.; Hadding, U.; Jones, M. E. *J. Antimicrob. Chemother.* **1998**, *42*, 807.
21. Brown, J. D.; Evans, R. F. *J. Chem. Soc.* **1962**, 4039.
22. Menger, F. M. *J. Am. Chem. Soc.* **1966**, *88*, 3081.
23. Fernandez, B.; Perillo, I.; Lamdan, S. *J. Chem. Soc., Perkins Trans. 2: Phys. Org. Chem.* **1973**, 1371.
24. Wilke, M. S.; Heller, M.; Creagh, A. L.; Haynes, C. A.; McIntosh, L. P.; Poole, K.; Strynadka, N. C. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 14832.
25. Huet, A. A.; Raygada, J. L.; Mendiratta, K.; Seo, S. M.; Kaatz, G. W. *Microbiology* **2008**, *154*, 3144.
26. Novick, R. *Virology* **1967**, *33*, 155.