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A High-Throughput Approach To Identify Compounds That Impair Envelope Integrity in *Escherichia coli*

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The envelope of Gram-negative bacteria constitutes an impenetrable barrier to numerous classes of antimicrobials. This intrinsic resistance, coupled with acquired multidrug resistance, has drastically limited the treatment options against Gram-negative pathogens. The aim of the present study was to develop and validate an assay for identifying compounds that increase envelope permeability, thereby conferring antimicrobial susceptibility by weakening of the cell envelope barrier in Gram-negative bacteria. A high-throughput whole-cell screening platform was developed to measure *Escherichia coli* envelope permeability to a β-galactosidase chromogenic substrate. The signal produced by cytoplasmic β-galactosidase-dependent cleavage of the chromogenic substrate was used to determine the degree of envelope permeabilization. The assay was optimized by using known envelope-permeabilizing compounds and *E. coli* gene deletion mutants with impaired envelope integrity. As a proof of concept, a compound library comprising 36 peptides and 45 peptidomimetics was screened, leading to identification of two peptides that substantially increased envelope permeability. Compound 79 reduced significantly (from 8- to 125-fold) the MICs of erythromycin, fusidic acid, novobiocin and rifampin and displayed synergy (fractional inhibitory concentration index, <0.2) with these antibiotics by checkerboard assays in two genetically distinct *E. coli* strains, including the high-risk multidrug-resistant, CTX-M-15-producing sequence type 131 clone. Notably, in the presence of 0.25 μM of this peptide, both strains were susceptible to rifampin according to the resistance breakpoints ($R > 0.5$ μg/ml) for Gram-positive bacterial pathogens. The high-throughput screening platform developed in this study can be applied to accelerate the discovery of antimicrobial helper drug candidates and targets that enhance the delivery of existing antibiotics by impairing envelope integrity in Gram-negative bacteria.

The emergence of multidrug-resistant (MDR) Gram-negative bacterial infections is a global health crisis demanding the urgent development of new therapeutic strategies. Treatment options are particularly limited due to the protective envelope of Gram-negative bacteria, which provides intrinsic resistance against multiple classes of antimicrobials (1). MDR strains acquire additional resistance by further fortifying their envelope barrier to exclude additional classes of antimicrobials through membrane modifications and overexpression of efflux pumps (1). Intrinsic resistance coupled with acquired resistance to cephalosporins and last-resort carbapenems by expression of AmpC-type extended-spectrum β-lactamases (ESBLs) and carbapenemases (NDM-1, KPC, VIM, and IMP), together with a frequent association with additional antimicrobial resistance genes, have virtually eliminated all viable treatment options against some high-risk MDR clones (2, 3).

The continuous development of novel antimicrobials requires investment in alternative drug discovery strategies, such as the establishment of high-throughput assays specifically designed for identification of compounds, here referred to as "helper" drugs, that restore susceptibility or allow alternative uses of current antimicrobials in clinical use. The cell envelope of Gram-negative bacteria is among the possible targets for helper drugs that may facilitate circumvention of bacterial resistance mechanisms by enhancing permeability and thus access of known antimicrobials to their intracellular targets. The envelope of Gram-negative bacteria is a multicomponent structure consisting of an inner membrane (IM), a peptidoglycan (PG) layer within the periplasmic space, an outer membrane (OM), and membrane-spanning efflux pumps (4). Intrinsic tolerance to antibiotics and environmental stress factors is mainly attributed to the asymmetrical OM, which blocks the passive diffusion of large (>600-Da) and hydrophobic agents, and to multidrug efflux pumps that expel compounds from the cell. Energy generation and envelope biogenesis machinery at the IM maintain envelope integrity by driving efflux pumps and enabling maintenance. Impairment of any of these envelope components, through gene inactivation or physical damage, can sensitize bacteria to antimicrobials and other environmental stressors (5–7). In particular, cationic compounds, such as polymyxins, can act as helper drugs, permeabilizing the OM to increase the access of other antimicrobials (7–11). This helper drug activity is especially effective with antimicrobials with activity against Gram-positive bacteria that are normally excluded by the envelope of Gram-negative bacteria. Similarly, cationic peptides and peptidomimetics have been reported to increase susceptibility to antimicrobials active against Gram-positive bacteria in Gram-negative bacterial species by compromising the integrity of the bacterial envelope (11–13).

High-throughput screening (HTS) may involve time-effective and robust assays for the identification of helper drugs that potentiate antimicrobial activity. Previous HTS approaches have fo-

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TABLE 1 Bacterial strains used in this study

Strain name	Relevant genotype or description	Reference or source
MG1655	*E. coli* F$^-$ λ$^-$ *ilvG rfb-50 rph-1*	Blattner et al., 1997 (19)
MG1655 Δ*lacI*	MG1655 *lacI*::Cm	This study
MG1655 Δ*lacI* Δ*lacY*	MG1655 *lacI lacY*::Cm	This study
MG1655 Δ*tolQRA*	MG1655 *tolQRA*::Cm	This study
MG1655 Δ*lacI* Δ*lacY* Δ*tolQRA*	MG1655 *lacI lacY*::Cm, *tolQRA*	This study
JW2496-3	BW25113 *bamB*::Kan	Keio Collection (20)
JW0052-1	BW25113 *surA*::Kan	Keio Collection (20)
JW5592-1	BW25113 *dapF*::Kan	Keio Collection (20)
JW0145-1	BW25113 *mrcB*::Kan	Keio Collection (20)
JW5503-1	BW25113 *tolC*::Kan	Keio Collection (20)
MG1655 Δ*lacI* Δ*lacY* Δ*bamB*	MG1655 *lacI lacY*::Cm *bamB*::Kan	This study
MG1655 Δ*lacI* Δ*lacY* Δ*surA*	MG1655 *lacI lacY*::Cm *surA*::Kan	This study
MG1655 Δ*lacI* Δ*lacY* Δ*dapF*	MG1655 *lacI lacY*::Cm *dapF*::Kan	This study
MG1655 Δ*lacI* Δ*lacY* Δ*mrcB*	MG1655 *lacI lacY*::Cm *mrcB*::Kan	This study
MG1655 Δ*lacI* Δ*lacY* Δ*tolC*	MG1655 *lacI lacY*::Cm *tolC*::Kan	This study
ATCC 25922	Quality control for susceptibility testing	EUCAST
UR40	ST131 clone containing $bla_{CTX\text{-}M\text{-}15}$	Cerquetti et al., 2010 (22)

cused on identification of compounds that enhance the activity of a particular antimicrobial in a resistant strain (14–17). The utility of this conventional HTS strategy is limited, as it can identify only compounds that synergistically inhibit growth in combination with a specific antimicrobial. Alternatively, antimicrobial-independent HTS assays that evaluate the perturbation of key aspects of bacterial drug resistance provide a wider scope of helper drug discovery.

Here we introduce an HTS platform designed to detect changes in envelope integrity based on conditional permeation of the β-galactosidase (LacZ) substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) across the envelope of the Gram-negative bacterium *Escherichia coli*. The aim of the study was to develop and validate the HTS platform for detection of envelope permeabilizers that restore antibiotic susceptibility by weakening the cell envelope barrier in Gram-negative bacteria. A 96-well format HTS assay was developed using polymyxin B and its nonlytic derivative, polymyxin B nonapeptide, as controls. The platform was further validated by measuring the rate of ONPG hydrolysis in gene deletion mutants with altered envelope integrity. As a proof of concept, a small library of selected peptides and peptidomimetics was screened, leading to the identification of two compounds that exert significant synergy with antibiotics to which *E. coli* is intrinsically resistant.

MATERIALS AND METHODS

Antimicrobials and media. Bacteria were routinely grown on Luria-Bertani agar (LA), in Luria-Bertani broth (LB), and in cation-adjusted Mueller-Hinton broth II (MHB II). All growth media were purchased from Becton Dickinson and prepared according to the manufacturer's instructions. The antibiotics and control compounds used in this study were purchased from Sigma-Aldrich, Denmark. Antimicrobial susceptibility testing by broth microdilution and checkerboard assays to determine antimicrobial interaction was performed in MHB II. A library consisting of a diverse set of 36 peptides and 45 peptidomimetics, comprising known antimicrobial compounds, cell-penetrating compounds, and immunomodulating compounds representing a diverse range of physicochemical properties, e.g., different degrees of cationicity and hydrophobicity as well as N-terminal modifications, such as acylation and lipidation, was assembled and provided by Henrik Franzyk. The library compounds were dissolved and diluted in deionized water or MHB II.

Bacterial strains and construction of gene deletion mutants. All *E. coli* strains and mutants used in this study are listed in Table 1. Gene deletion mutants were constructed by using the bacteriophage λ Red recombinase system (18). Briefly, an FLP recombination target-flanked chloramphenicol resistance cassette (Cm) was amplified from template plasmid pKD3 using primers containing a 39-base sequence homologous to the gene targeted for deletion. The resulting PCR product was purified by gel electrophoresis and extracted using a Thermo Fisher GeneJET gel extraction kit. Cells of electrocompetent strain MG1655 or its derivative cells containing expressed bacteriophage λ Red recombinase genes (by induction with arabinose) were electroporated with the purified PCR product and selected on 24 μg/ml chloramphenicol-LA plates (19). The chloramphenicol resistance cassette was removed from the deletion strains by transformation and subsequent curing of the temperature-sensitive pCP20 plasmid at 37°C as previously described (18). All gene deletions were confirmed by amplifying the 200-bp sequence up- and downstream from the locus of the deleted gene followed by DNA sequencing of the resulting PCR product. All primer sequences are available upon request. Sequencing was performed by Macrogen Europe, The Netherlands.

Transfer of gene deletion cassettes by P1 transduction. Gene deletion cassettes from the Keio Collection mutant strains were moved to the MG1655 Δ*lacI* Δ*lacY*::Cm strain by P1 transduction according to standard laboratory methods (20). Briefly, P1 phage lysates of donor deletion mutant strains were made in LB containing 0.2% glucose and 5 mM $CaCl_2$, treated with chloroform, and centrifuged to remove intact cells and cell debris. The supernatant was transferred to a new vial and stored at 4°C. An overnight culture of the MG1655 Δ*lacI* Δ*lacY*::Cm recipient strain was incubated at 37°C with the phage lysate for 30 min, followed by addition of LB containing 100 mM sodium citrate and an additional 1 h of incubation at 37°C with continuous aeration. Transductants were subsequently selected and purified on LA plates containing 25 μg/ml kanamycin and 10 mM sodium citrate. Gene deletions were confirmed by PCR.

HTS of a peptide and peptidomimetic library. An MHB II overnight culture of MG1655 Δ*lacI* Δ*lacY*::Cm or MG1655 Δ*lacI*::Cm was subcultured 1:100 in fresh MHB II and grown to an optical density at 600 nm (OD_{600}) of 0.2 at 37°C with continuous aeration. The culture was then transferred to 96-well plates, followed by addition of the compounds and a 20-min incubation at 37°C. After addition of 450 μg/ml ONPG, the absorbance at 420 nm and the optical density at 600 nm were recorded for 1 h in 3-min intervals by using a PowerWave XS microplate spectrophotometer (BioTek Inc.). The rate of ONPG hydrolysis (signal) relative to the cell density was determined as the A_{420} per OD_{600} per period of time (per minute). Data were analyzed using Gen5 data analysis software (BioTek Inc.) and background signal was re-

moved as follows: ($signal_{compound}$/$signal_{untreated\ control}$) − 1. The ONPG hydrolysis rate in the gene deletion mutant strains was measured as described above, except that the 20-min compound incubation step was omitted. Screening of the library was conducted with a 20-min incubation of 1 μM solutions of each library member as described above. Polymyxin B (0.625 μg/ml) and polymyxin B nonapeptide (10 and 5 μg/ml) were used as positive controls to estimate the degree of envelope permeabilization within a fixed range. Control concentrations were selected as the lowest concentrations that generated a statistically significant signal for HTS (Z′ score > 0.6) without having a significant impact on growth. Statistical significance of the signal generated by the controls or the library compounds was determined by Z′ score (21) or by two-tailed Student's *t* test from biological triplicates, respectively. Percent reduction of envelope integrity was calculated as follows: 100 × {1 − [($signal_{compound}$ − $signal_{0.625\ \mu g/ml\ polymyxin\ B}$)/($signal_{untreated\ control}$ − $signal_{0.625\ \mu g/ml\ polymyxin\ B}$)]}, where $signal_{compound}$ is the signal obtained with the test compound, $signal_{untreated\ control}$ is the signal obtained with the untreated control, and $signal_{0.625\ \mu g/ml\ polymyxin\ B}$ is the signal obtained with 0.625 μg/ml polymyxin B, which represented 100% reduction in the assay.

Antimicrobial susceptibility testing. The broth microdilution method was used to determine the MICs of the tested antibiotics and two library compounds for *E. coli* reference strain ATCC 25922 and for a CTX-M-producing clinical strain of sequence type 131 (ST131) (22) following CLSI guidelines (23). To rapidly assess the ability of the peptides and peptidomimetics to enhance antibiotic susceptibility, *E. coli* ATCC 25922 growth was assessed following exposure to fixed concentrations of a peptide or peptidomimetic (1 μM) and six antibiotics (benzylpenicillin, erythromycin, fusidic acid, novobiocin, rifampin, and vancomycin). The antibiotic concentrations were selected according to clinical resistance breakpoints. As no clinical breakpoints are available for the testing of antibiotics with activity against Gram-positive bacteria in *E. coli*, we used the EUCAST breakpoints for fusidic acid resistance in *Staphylococcus* spp. (resistance breakpoint [R] > 1 μg/ml), vancomycin resistance in *Enterococcus* spp. (R > 4 μg/ml), and erythromycin and rifampin resistance in *Streptococcus* spp. (R > 0.5 μg/ml) (www.eucast.org). For benzylpenicillin, we used a generic breakpoint (R > 2 μg/ml) designed for organisms that do not have specific breakpoints (www.eucast.org). In the absence of any established breakpoint, the concentration of novobiocin was defined using an arbitrary cutoff value (R > 1 μg/ml).

Checkerboard synergy assay. A two-dimensional checkerboard assay (24) was used to assess synergy between the most promising peptide (compound 79) and the antibiotics for which 1 μM peptide inhibited *E. coli* growth in the susceptibility testing experiment described above. Briefly, 2-fold dilutions of each compound-antibiotic combination were prepared in a 96-well plate and inoculated with the test strain according to the CLSI guidelines for broth microdilution (23). The fractional inhibitory concentration (FIC) index is {[MIC of $A_{(A+B)}$/MIC of A] + [MIC of $B_{(A+B)}$/MIC of B]}, where MIC $A_{(A+B)}$ represents the MIC of antibiotic A in the combination of antibiotic A and compound B and MIC $B_{(A+B)}$ represents the MIC of compound B in the combination of antibiotic A and compound B. The FIC index of each combination (antibiotic A and compound B) was calculated to determine whether the effect was synergistic (sum of FICs [∑FIC] ≤ 0.5), noninteracting (∑FIC > 0.5 to 4), or antagonistic (∑FIC ≥ 4) (25).

RESULTS

Development of HTS platform to measure *E. coli* envelope integrity. To establish an HTS platform that measures the integrity of the *E. coli* envelope, a classical β-galactosidase activity assay was miniaturized to a 96-well kinetic format in whole cells. The bacterial envelope imposes separation of the cytoplasmic β-galactosidase enzyme from its extracellular substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG), enabling quantification of envelope permeability by using ONPG as a probe. Under conditions of increased envelope permeability or cell lysis, the rate of ONPG hydrolysis increases either by ONPG entry into the cell or due to the extracellular release of β-galactosidase (Fig. 1). Hydrolysis of the chromogenic ONPG releases *ortho*-nitrophenolate (ONP) into the growth medium, producing a yellow color. Since ONP absorbs light at 420 nm, whereas the precursor molecule ONPG does not, β-galactosidase activity can be monitored by measuring the absorbance at 420 nm, and the rate of ONPG hydrolysis relative to the cell optical density (OD) over time (i.e., absorbance at 420 nm per OD_{600} per period of time) can be correlated to the degree of membrane permeability.

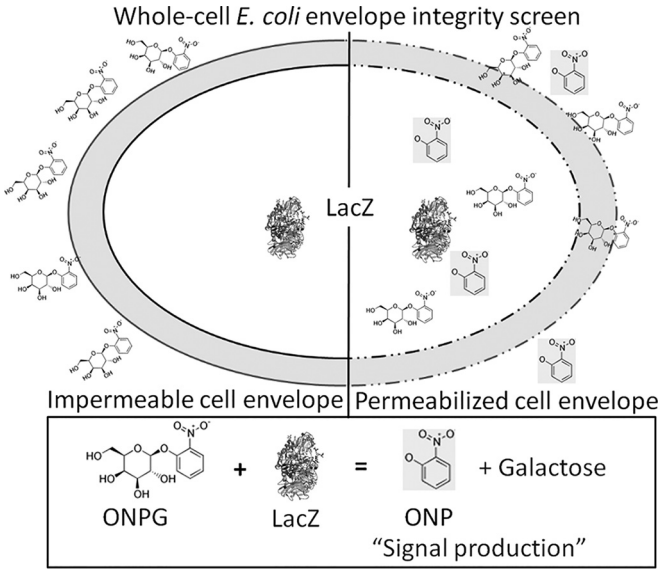

FIG 1 Illustration of the envelope integrity screening platform described in this study. The two concentric circles represent the inner and outer membranes of the envelope of Gram-negative bacteria. On the left half, the envelope is intact and the cytoplasmic β-galactosidase (LacZ, PDB accession number 1TG7 [45]) is compartmentally separated from its chromogenic substrate (ONPG). Permeabilization or lysis of the cell envelope (right) increases the rate of ONPG influx across the cell envelope or extracellular release of β-galactosidase and subsequent hydrolysis to produce ONP. The screening assay is based on the detection of hydrolyzed ONPG (ONP) at 420 nm as a signal indicating envelope impairment. This figure is not to scale.

To optimize screening sensitivity and consistency, the *lac* operon of MG1655 was modified. First, the transcriptional repressor, *lacI*, was deleted using the bacteriophage λ Red recombinase system to enable constitutive *lacZ* expression, permitting the flexibility to use various growth media and eliminating the need for inducers, which might be a potential source of variability in the assay (18). Next, since lactose permease is able to transport ONPG into the cytoplasm with a high efficiency, its corresponding gene (*lacY*) was deleted in the MG1655 Δ*lacI* strain (26), resulting in a significantly lower rate of ONPG hydrolysis (Fig. 2A). This reduction of the basal background signal enabled detection of the effect induced by lower concentrations of the envelope permeabilizer polymyxin B (Fig. 2B) (7). Since polymyxin B possess both lytic and envelope-permeabilizing properties, it was not clear whether the signal production was solely due to an increased rate of cell lysis or was also caused by increased envelope permeability (27). To differentiate between these two possibilities, a nonlipidated analogue of polymyxin B, polymyxin B nonapeptide, which lacks a lytic capability, was used for the detection of envelope permeabilization in the absence of cell lysis (27, 28). Interestingly, only the MG1655 Δ*lacI* Δ*lacY* strain was able to detect the envelope per-

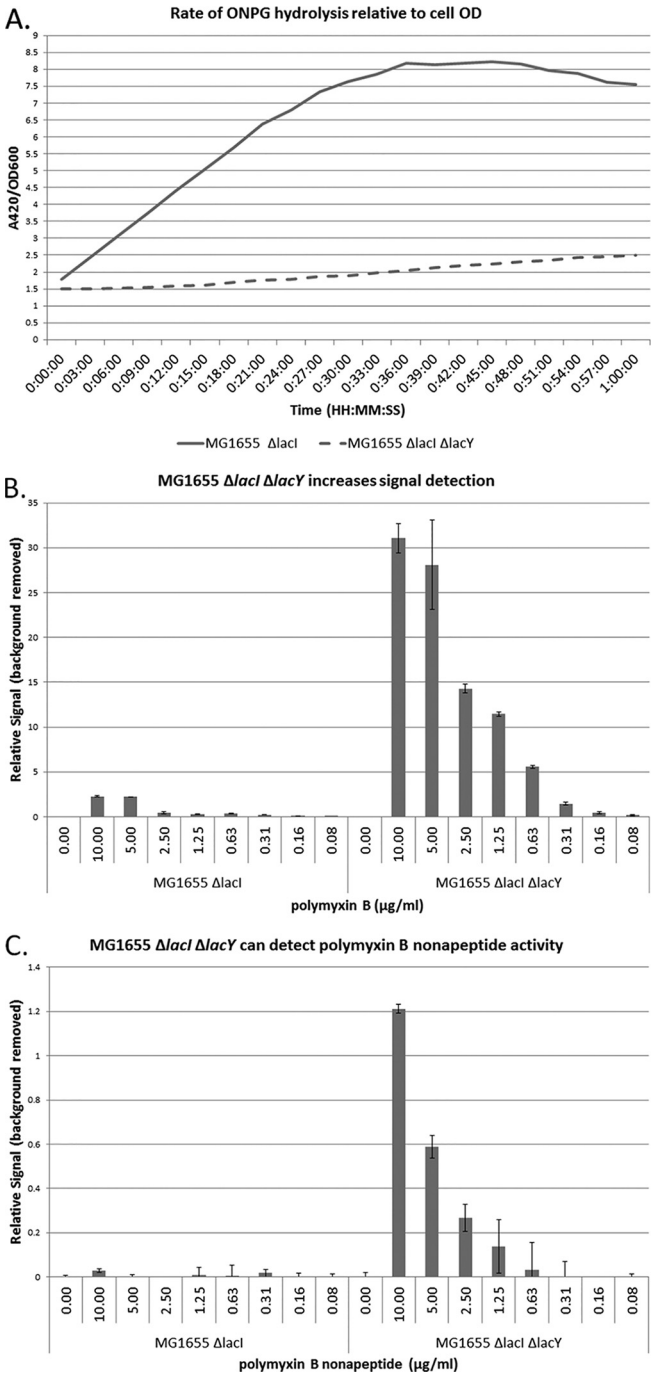

FIG 2 Rate of ONPG hydrolysis in the MG1655 Δ*lacI* and MG1655 Δ*lacI* Δ*lacY* strains. The MG1655 Δ*lacI* Δ*lacY* strain has a significantly lower rate of signal production than the MG1655 Δ*lacI* strain. (A) Signal production (A_{420}) relative to the cell OD (OD_{600}) is graphed over time. (B and C) Deletion of the lactose permease gene, *lacY*, increases the sensitivity of the assay and, consequently, the detection of the polymyxin B-mediated (B) and polymyxin B nonapeptide-mediated (C) envelope permeabilization signal. MG1655 Δ*lacI* and MG1655 Δ*lacI* Δ*lacY* strains grown to an OD_{600} of 0.2 were incubated for 20 min with various concentrations of polymyxin B or polymyxin B nonapeptide, followed by addition of 450 μg/ml ONPG. The amount of the ONPG hydrolysis signal (absorbance of ONP at 420 nm) divided by the cell OD (at 600 nm) was determined after 30 min of ONPG incubation. The amount of ONPG hydrolysis relative to the background signal was determined by division of the amount for the untreated control. Representative results from technical triplicates are shown.

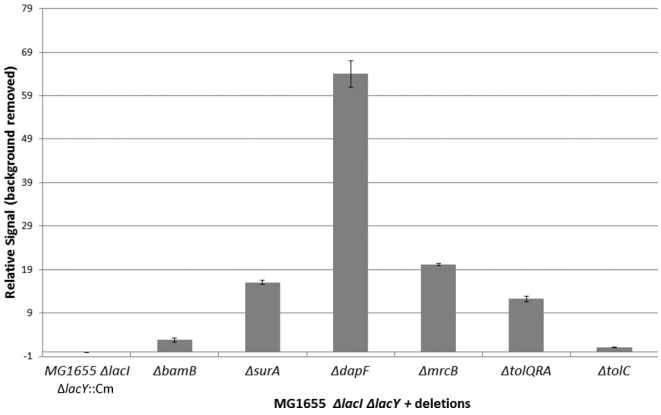

FIG 3 Gene deletions affecting envelope integrity. Envelope-impaired mutants and MG1655 Δ*lacI* Δ*lacY* were incubated with 450 μg/ml ONPG for 30 min, and the amount of the ONPG hydrolysis signal (absorbance at 420 nm) divided by the cell OD (at 600 nm) was determined. The amount of ONPG hydrolysis relative to the background signal was determined by division of the amount for the MG1655 Δ*lacI* Δ*lacY* control. Representative results from technical triplicates are shown.

meabilization activity of polymyxin B nonapeptide (Fig. 2C). The polymyxin B nonapeptide-induced permeabilization of the OM was confirmed by an increased rate of nitrocefin hydrolysis by MG1655 Δ*lacI* Δ*lacY* expressing the CMY-2 β-lactamase, indicating increased OM permeability (data not shown). Hence, the MG1655 Δ*lacI* Δ*lacY* strain was chosen as the model strain in the screening platform.

Screen validation using *E. coli* mutants with impaired envelope integrity. The screening platform was tested by including gene deletion mutants with impaired envelope integrity. Deletion cassettes of genes involved in envelope biogenesis (*bamB*, *surA*, *mrcB*), peptidoglycan precursor synthesis (*dapF*), efflux (*tolC*), and OM stability (*tolQRA*) were transduced by P1 transduction from previously constructed mutants into the MG1655 Δ*lacI* Δ*lacY* strain (20, 29–34). All gene deletion mutants, except the Δ*tolC* and Δ*bamB* mutants, were previously identified in an HTS assay as mutants with increased envelope permeability or an elevated rate of cell lysis (35). After incubation for 30 min with ONPG, the deletion mutants displayed increased signal production relative to the baseline signal of the MG1655 Δ*lacI* Δ*lacY* strain (Fig. 3). Notably, the Δ*tolC* mutant gave rise to the lowest level of signal production.

HTS of a peptide and peptidomimetic compound library to identify envelope permeabilizers acting synergistically with antibiotics. To establish a proof of concept, the HTS platform was used to screen for envelope permeabilizers in a peptide and peptidomimetic compound library selected to cover a broad range of structural diversity. Two peptides, compounds 33 and 79, produced the highest rate of ONPG hydrolysis (Fig. 4) and increased envelope permeability to a greater extent than the polymyxin B positive control with *P* values of less than 0.1 (Table 2). The two identified peptides were further studied for their ability to increase the susceptibility of the *E. coli* reference strain ATCC 25922 to six antibiotics. Both compounds potentiated the activities of novobiocin (1 μg/ml), fusidic acid (1 μg/ml), and rifampin (0.5 μg/ml) at therapeutically achievable levels, leading to inhibition of bacterial growth, whereas no interaction with benzylpenicillin or vancomycin was observed (Table 3). While the activity of erythromy-

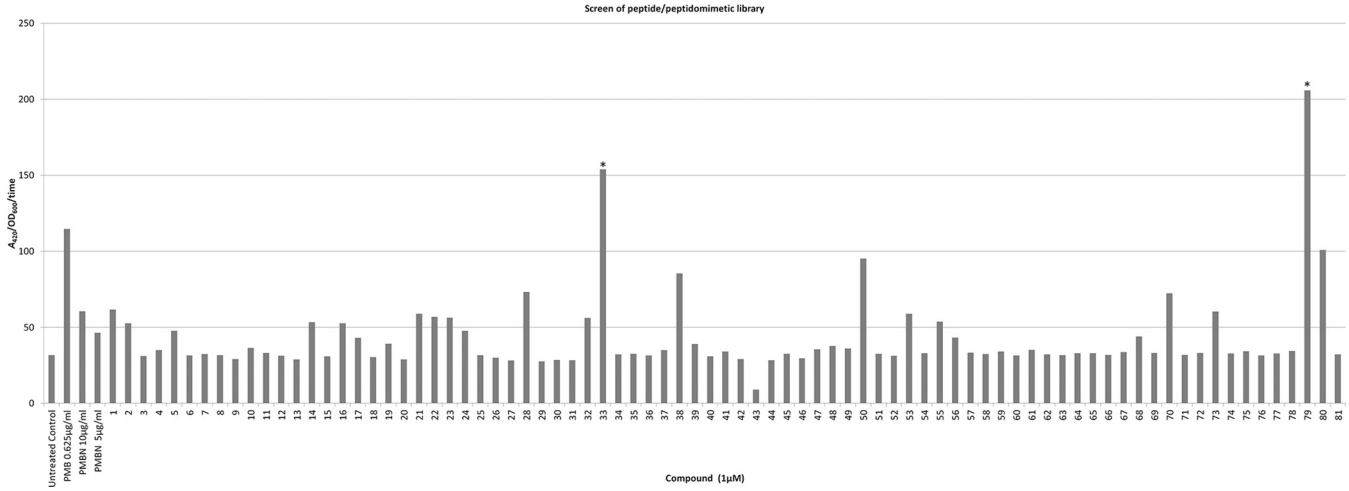

FIG 4 HTS of peptide and peptidomimetic library using the developed platform. The MG1655 Δ*lacI* Δ*lacY* strain was grown to an OD_{600} of 0.2 and incubated with 1 μM compounds for 20 min, followed by addition of 450 μg/ml ONPG and additional incubation for 1 h. The maximum rate of ONPG hydrolysis relative to cell density (A_{420}/OD_{600}) per minute is shown (the results represent the averages from triplicate assays). The two compounds producing the highest signal from ONPG hydrolysis are marked with asterisks. PMB, polymyxin B; PMBN, polymyxin B nonapeptide.

cin was potentiated to the *Streptococcus* breakpoint concentration of 0.5 μg/ml by compound 79, the less active compound 33 inhibited growth only in combination with 1 μg/ml erythromycin. To further assess the potential of the observed indication of synergy, the overall most active compound in the screen, compound 79, was tested in combination with the antibiotics in checkerboard assays. Compound 79 displayed efficient synergy (FIC index < 0.2) with all antibiotics tested (erythromycin, fusidic acid, novobiocin, and rifampin) (Table 4). Synergy was also apparent in the epidemic CTX-M-15-producing ST131 clone (Table 4). The synergistic combinations resulted in significant reductions of the MICs of erythromycin (16-fold for both ATCC 25922 and ST131), fusidic acid (31- and 125-fold, respectively), novobiocin (32- and 8-fold, respectively), and rifampin (66- and 16-fold, respectively), while the MIC of compound 79 was reduced by 8-fold (from 2 to 0.25 μM) in all cases. The synergistic effect of the compound reduced the MIC of rifampin below the resistance breakpoint for Gram-positive pathogens (R > 0.5 μg/ml) in both ATCC 25922 (from 2 to 0.03 μg/ml) and the ST131 clone (from 4 to 0.25 μg/ml). The MIC of erythromycin was reduced below the resistance breakpoint for *Staphylococcus* (R > 2 μg/ml) in ATCC 25922 (from 16 to 1 μg/ml) but not in the ST131 clone (from 256 to 16 μg/ml) due to the very high MIC for the latter strain, caused by the presence of a macrolide resistance gene, *mph*(A) (unpublished whole-genome sequencing data).

TABLE 2 Sequence, MIC, and average percent reduction of envelope integrity relative to that for polymyxin B for the two best peptide hits detected by the envelope integrity screen

Compound no.	Peptide sequence	MIC (μM)[a]	Avg ± SD % reduction[b]	*P* value
79	KKWRKWLKWLAKK-NH_2	2	236 ± 85	0.03
33	KWKLFKKVLKVLTTG-NH_2	2	166 ± 44	0.09

[a] MIC for *Escherichia coli* reference strain ATCC 25922.
[b] Average envelope integrity reduction based on three biological replicates.

DISCUSSION

In this study, an HTS platform for the discovery of compounds that impair envelope integrity in *E. coli* was developed and validated. This platform can be applied to characterize mutants with impaired envelope integrity, as demonstrated by the robust detection of *dapF*, *mrcB*, *surA*, and *tolQRA* gene deletion mutants. HTS of a peptide and peptidomimetic compound library led to identification of two peptides capable of increasing envelope permeability to a higher degree than the polymyxin B positive control. This finding indicates the potential of this HTS platform to identify antimicrobial helper drug candidates.

The screening platform developed here can detect deficiencies in different aspects of envelope integrity, including the overall

TABLE 3 Growth or no growth of *Escherichia coli* ATCC 25922 in the presence of peptide-antibiotic combinations

Antibiotic[a]	MIC[b] (μg/ml)	Antibiotic concn (μg/ml)	Growth[c]		
			Compound 79	Compound 33	No peptide
PEN	16	2[d]	+	+	+
ERY	16	0.5[e]	−	+	+
FUS	500	1[f]	−	−	+
NOV	16	1[g]	−	−	+
RIF	2	0.5[e]	−	−	+
VAN	250	4[h]	+	+	+
No antibiotic	NA[i]	NA	+	+	+

[a] PEN, benzylpenicillin; ERY, erythromycin; FUS, fusidic acid; NOV, novobiocin; RIF, rifampin; VAN, vancomycin.
[b] MICs of antibiotics individually.
[c] Compounds 79 and 33 were tested at a concentration of 1 μM each. +, growth; −, no growth.
[d] EUCAST generic breakpoint.
[e] EUCAST breakpoints for *Streptococcus* spp.
[f] EUCAST breakpoints for *Staphylococcus* spp.
[g] Arbitrary breakpoint defined in the absence of any clinical breakpoint.
[h] EUCAST breakpoint for *Enterococcus* spp.
[i] NA, not applicable.

TABLE 4 Checkerboard assay results for antibiotic-compound 79 synergy in *E. coli* ATCC 25922 and MDR CTX-M-15-producing ST131 strains

Antibiotic or compound	ATCC 25922			ST131		
	MIC alone[a]	MIC at FIC index[b]	FIC index[c]	MIC alone	MIC at FIC index	FIC index
Erythromycin	16	1	0.19	256	16	0.19
Fusidic acid	500	16	0.16	500	4	0.14
Novobiocin	16	0.5	0.16	16	2	0.25
Rifampin	2	0.03	0.14	4	0.25	0.19
Compound 79	2	0.25	NA[d]	2	0.25	NA

[a] MIC of each antibiotic (μg/ml) or compound (μM) individually.
[b] MIC in compound-antibiotic combination at the lowest fractional inhibitory concentration index.
[c] FIC index, fractional inhibitory concentration index.
[d] NA, not applicable.

permeability of the IM and OM and efflux pump activity, to various degrees. As the premise of the HTS platform relies on the limited permeation of ONPG across both the IM and OM, an increase in the permeability of either membrane can be detected. While the limited permeation of ONPG across the IM was established by an earlier assay for detection of cytoplasmic membrane permeabilization, data regarding ONPG permeation across the OM are lacking (36, 37). While ONPG is within the maximum size cutoff for porin diffusion, its low hydrophilicity (compared to that of lactose), nonionic state at physiological pH, and size (301 Da) would likely restrict its rate of diffusion through the OM porins (1). This assumption is supported by the ability of the platform to detect the activity of both polymyxin B, a permeabilizer of both the IM and OM, and polymyxin B nonapeptide, which is known to permeabilize the OM (7, 28). Preliminary work exploring the parameters of assay sensitivity also detected a similar degree of polymyxin B nonapeptide-induced OM permeabilization by using nitrocefin as a probe for OM permeability (38). However, as the usefulness of an HTS platform depends on its cost-effectiveness, the costly nitrocefin probe was replaced by ONPG, which displays a sensitivity for the detection of Gram-negative bacterial envelope permeabilizers similar to that of nitrocefin. Hence, the β-galactosidase activity-based HTS platform was optimized with ONPG, which is capable of robustly detecting deletion mutants affecting OM biogenesis. It is important to note that this assay does not distinguish between the enhanced permeation of small compounds across an intact membrane(s), leakage of cytoplasmic contents, or a low level of cell lysis. However, since defects in envelope integrity often result in an elevated rate of cell lysis or leakage of cytoplasmic contents, this is not detrimental for the identification of agents affecting envelope integrity (35, 39, 40).

Our HTS assay showed a limited ability to detect the Δ*tolC* gene deletion in the *acrAB-tolC* efflux pump system, suggesting a limitation in detecting the impairment of efflux systems. This limitation could be due to the poor affinity of ONP as a TolC substrate or to poor detection of the hydrolyzed LacZ substrate in the Δ*tolC* mutant. In a previous study evaluating multidrug efflux pump inhibitors in *E. coli* by using a fluorogenic LacZ substrate, it was found that while the Δ*tolC* mutation inhibited efflux activity, resulting in the hydrolysis of the fluorogenic substrate, the fluorophore was retained inside the cell with little fluorescence in the extracellular medium (41). Thus, the *tolC* deletion may also lead to retention of the ONP chromophore in the assay presented here, thereby obscuring accurate detection of envelope impairment due to the diminished transport of hydrolyzed substrate to the extracellular medium. Although it may not be possible to completely overcome this limitation in an HTS format, adaptation of the platform to measure the hydrolysis of different LacZ substrates, such as the fluorogenic substrate, may enable better detection of *tolC*-type envelope deficiencies. Importantly, this phenomenon was limited to the *tolC* deletion mutant, inferring that deletion of other efflux pump components, such as *acrA* or *acrB*, eliminated efflux activity and enabled both substrate hydrolysis and the release of the fluorophore into the medium (41).

The developed HTS platform proved useful for identification of envelope-permeabilizing compounds from a peptide and peptidomimetic library, and the subsequent finding of antibiotic-peptide 79 synergies confirmed the validity of this screen as an efficient tool to identify helper drugs capable of potentiating antibiotic activity. Although the growth of *E. coli* ATCC 25922 was not inhibited by breakpoint concentrations of benzylpenicillin and vancomycin in combination with 1 μM the peptides, the potential for synergy between these combinations cannot be ruled out by the susceptibility assay conducted here. The underlying mechanism(s) for the synergistic antibiotic-peptide combinations observed for erythromycin, fusidic acid, novobiocin, and rifampin was not directly investigated in this study. However, on the basis of the envelope-permeabilizing activity of the peptides, synergy likely involves increased permeation of the antibiotics, enabling them to reach their intracellular targets. In particular, the increased permeability of the OM may be the key factor. As the OM barrier is considered to be a primary cause for intrinsic antibiotic resistance in Gram-negative bacterial species, partial or transient disruption of this barrier would be expected to lower the MICs of these antibiotics close to the MICs observed in wild-type strains of Gram-positive bacterial species. Consistent with this reasoning, a low concentration (0.25 μM) of compound 79 reduced the MICs of rifampin and erythromycin in *E. coli* ATCC 25922 to be within the range of MICs for *Staphylococcus* strains (0.004 to 0.064 μg/ml and 0.032 to 1 μg/ml, respectively, according to EUCAST wild-type distributions), suggesting that this peptide permeabilizes the OM of *E. coli*. Since the combination of rifampin or erythromycin with compound 79 did not exhibit synergy in a susceptible *Staphylococcus* strain (data not shown), other factors, such as IM permeabilization, were unlikely to contribute significantly to the observed MIC reduction. Furthermore, the MIC reduction observed in this study was similar in magnitude to the reductions previously seen in mutants with hyperpermeable OMs (42) and by exposure to polymyxin B nonapeptide (7).

In the present study, we identified two Gram-negative bacterial envelope permeabilizers that have the potential for repurposing antibiotics with activity against Gram-positive bacterial species

for therapy in MDR Gram-negative bacterial infections by abolishing the intrinsic resistance to these antibiotics. Peptide 79 was shown to synergize with these antibiotics in two distinct *E. coli* genetic backgrounds, including the MDR CTX-M-15-producing ST131 strain. Further *in vitro* and *in vivo* studies are warranted to evaluate a possible clinical application of these findings. Compounds 33 and 79 are both short antimicrobial peptides (13 and 15 residues in length, respectively); compound 79 exhibited 20% hemolysis of washed human red blood cells at 200 μg/ml (~80 μM), while compound 33 was nonhemolytic to isolated sheep red blood cells on agar plates at a concentration of up to 500 μM. Both peptides are expected to act as antimicrobials via a membrane-associated mechanism of action (43, 44). The main difference between the two peptides is the tryptophan-rich face (assuming an α-helical active conformation) of compound 79, which increases its propensity to associate with the bacterial membrane, thereby disrupting its integrity. This structural difference may also explain the hemolytic activity of this peptide.

In conclusion, the HTS platform developed here offers a robust method for identifying envelope permeabilizers capable of potentiating the activity and spectrum of conventional antibiotics. This tool can be exploited to discover new antimicrobial helper drug candidates and targets as well as repurpose or resuscitate existing antimicrobials by circumventing both intrinsic and acquired drug resistance in high-risk MDR clones such as the *E. coli* ST131 clone.

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