Synthetic oxepanoprolinamide iboxamycin is highly active against human pathogen
Listeria monocytogenes

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Abstract: Listeriosis is a dangerous food-borne bacterial disease caused by the Gram-positive Bacillota (Firmicute) bacterium Listeria monocytogenes. In this report, we show that the synthetic lincosamide iboxamycin is highly active against L. monocytogenes and can overcome the intrinsic lincosamide resistance mediated by VgaL/Lmo0919, a member of ABCF ATPase resistance determinants that act by directly removing the antibiotic from the ribosome. While iboxamycin is not bactericidal against L. monocytogenes, it displays a pronounced postantibiotic effect, which is a valuable pharmacokinetic feature. Experiments in L. monocytogenes infection models are necessary to further assess the potential of iboxamycin as a novel drug for treatment of listeriosis. We demonstrate that VmlR ARE ABCF of Bacillota bacterium Bacillus subtilis grants significant (33-fold increase in MIC) protection from iboxamycin, while LsaA ABCF of Enterococcus faecalis grants an 8-fold protective effect. Furthermore, the VmlR-mediated iboxamycin resistance is cooperative with that mediated by the Cfr 23S rRNA methyltransferase resistance determinant, resulting in up to a 512-fold increase in MIC. Therefore, emergence and spread of ABCF ARE variants capable of defeating next-generation lincosamides in the clinic is possible and should be closely monitored.
Introduction

Lincosamides constitute an important class of antibiotics used both in veterinary and human medicine [1]. These compounds inhibit protein synthesis by binding to and compromising the enzymatic activity of the peptidyl transferase centre (PTC) of the ribosome [2-5], resulting in bacteriostasis [6]. Representatives of this antibiotic class share a common architecture and are typically comprised of a 4'-substituted L-proline residue connected via an amide bond to a unique S-glycosidic aminosugar moiety (Figure 1A,B). The first lincosamide to be discovered, lincomycin (Figure 1A), is a natural product produced by *Streptomyces lincolnensis* ssp. *lincolnensis* and is active against streptococcal, pneumococcal and staphylococcal infections [7]. Its semi-synthetic derivative, clindamycin (Figure 1B), can be produced via a one-step stereoinvertive deoxychlorination of lincomycin [8]. Clindamycin is more potent than lincomycin and is currently the lincosamide of choice for human medicine [9]. Like lincomycin, clindamycin is mostly active against Gram-positive but not Gram-negative bacteria, which restricts the spectrum of its applications [10]. A cis-4-ethyl-L-pipeolic acid amide of clindamycin, pirlimycin, has a similar spectrum of antibacterial activity [11, 12] and is approved for veterinary applications in the United States and European Union. Finally, a recently developed semisynthetic derivative of lincomycin ('compound A') was shown to be able to overcome clindamycin resistance in *Staphylococcus aureus* mediated by ribosomal RNA (rRNA) methylation by ErmA and ErmB antibiotic resistance determinants [13].

Iboxamycin (Figure 1C) is a newly developed lincosamide with an exceptionally broad spectrum of antibacterial activity [14]. Featuring a fully synthetic, bicyclic oxepanoprolinamide aminocycl fragment, iboxamycin improves upon previous lincosamides in its activity against both Gram-positive and Gram-negative pathogens [14]. Iboxamycin was found to be more potent than clindamycin against Gram-positive pathogens and overcomes lincosamide resistance mediated by rRNA modification by Erm and Cfr 23S rRNA methyltransferases, both of which are highly clinically important and widespread antibiotic resistance determinants [15-18]. While Cfr grants strong protection against clindamycin in isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* (MIC >128 µg/mL), it confers only moderate resistance against iboxamycin (MIC of 2-8 µg/mL compared to 0.06 µg/mL for cfr- strains) [14]. Importantly, iboxamycin is also highly active against *Enterococcus faecalis* (MIC 0.06 µg/mL as compared to 16 µg/mL for clindamycin) – a species that is intrinsically resistant to ‘classical’ lincosamides as it encodes the LsaA antibiotic resistance (ARE) factor in its chromosomal genome [19], a member of the ABCF ATPase protein family that includes multiple resistance factors [20-22]. LsaA provides resistance against pleuromutilin, lincosamide and streptogramin A (PLS₂) antibiotics by displacing the drug from the ribosome [23], acting similarly to other ARE ABCFs [24-27]. As evident from the 96- to 256-fold higher sensitivity to clindamycin and lincomycin in a ΔlsaA *E. faecalis* strain as compared to *E. faecalis* ectopically expressing LsaA [23], LsaA is a potent lincosamide resistance determinant. The high sensitivity of *E. faecalis* to iboxamycin suggests that this compound has the potential to overcome resistance mediated by other ARE ABCFs as well.

Listeriosis is a dangerous food-borne bacterial disease caused by the Gram-positive Bacillota (formerly: Firmicute) bacterium *Listeria monocytogenes*, which infects people through contaminated meat, fish and dairy products [28, 29]. While it is a relatively rare infection that mainly affects people with weakened immune systems, or who are pregnant [30], the majority of listeriosis cases require hospitalisation and mortality rates can be as high as 20-30% even with antibiotic treatment [31, 32]. Antibiotic treatment options for *L. monocytogenes* infections include cell wall synthesis disruptors ampicillin and vancomycin, folic acid synthesis inhibitors sulfamethoxazole and trimethoprim, and protein synthesis inhibitors, such as gentamicin and azithromycin [33]. *L. monocytogenes* strains reported in recent years are often resistant to clindamycin, with the resistant fraction ranging from 29% to 76%, depending on the collection [34-37], thus excluding clindamycin as a viable option for treatment of *L. monocytogenes* infections. Importantly, just as *E. faecalis* encodes the ABCF ATPase LsaA, *L. monocytogenes* encodes the ARE ABCF PLS₄ resistance factor VgaL/Lmo0919 in its core genome [38]. As with LsaA, VgaL operates on the ribosome [23], and loss of VgaL results in increased sensitivity to lincosamides, with the Δlmo0919 *L. monocytogenes* strain being 8- to 16-fold more
sensitive to lincomycin as compared to the isogenic wild type [23]. Finally, a model Bacillota, *B. subtilis*, also encodes an ARE ABCF PLS$_A$ resistance factor – VmlR [27, 39].

In this report, using lincomycin and clindamycin as reference compounds, we i) characterised the efficacy of iboxamycin against *L. monocytogenes*, ii) probed its ability to specifically counter resistance mediated by ABCFs *L. monocytogenes* Lmo0919, *E. faecalis* LsaA and *B. subtilis* VmlR, iii) characterised its bactericidal/bacteriostatic mechanism of action and, finally, iv) assessed the strength of its post-antibiotic effect (PAE).

**Results**

*L. monocytogenes is highly sensitive to iboxamycin despite Vgal/Lmo0919 ABCF resistance factor*

To test the lincosamide sensitivity of *L. monocytogenes* we used two widely-used wild-type strains, both belonging to serovar 1/2a: EGD-e [40] and 10403S, a streptomycin-resistant variant of 10403 [41]. The two wild types are genomically distinct, e.g. the virulence master-regulator PrfA is overexpressed in EGD-e and the prophage content differs between the two strains [42]. In additional to the two wild types, we also tested a *L. monocytogenes* EDG-e derivative that was genomically modified to abrogate the expression of VgaL/Lmo0919 PLS$_A$ resistance factor (EDG-e Δlmo0919) [23].

Both wild-type *L. monocytogenes* strains are dramatically more sensitive to iboxamycin (MIC of 0.125-0.5 µg/mL) as compared to clindamycin (MIC of 1 µg/mL) and lincomycin (MIC of 2-8 µg/mL) (Table 1). In agreement with the higher sensitivity of Δlmo0919 EDG-e to lincomycin [23], this strain is 2-8-fold more sensitive to iboxamycin than the corresponding wild type. This indicates that while Vgal does confer some protection from iboxamycin, the high potency of the synthetic antibiotic would likely allow the drug to overcome resistance in clinical settings. A likely explanation is that increased affinity of the synthetic drug for the ribosome renders antibiotic displacement by ABCF ATPases inefficient.

Importantly, expression of Lmo0919 is not constitutive: it is elicited by antibiotic-induced ribosomal stalling on the regulatory short open reading frame upstream of the lmo0919 gene [38]. Therefore, the difference in iboxamycin sensitivity between wild-type and Δlmo0919 EDG-e strains reflects both the ability of Lmo0919 to protect the ribosome from the antibiotic as well as the efficiency of iboxamycin-mediated induction of Lmo0919. To deconvolute these two effects, we used engineered strains that allow for ectopic inducible expression of ABCF in the following experiments.

*E. faecalis ABCF LsaA grants a moderate protective effect against iboxamycin*

To test the ability of other ABCF PLS$_A$ resistance factors to confer resistance to iboxamycin, we compared a pair of *E. faecalis* strains: one lacking the chromosomally-encoded LsaA (ΔlsaA pCIE$_{spec}$) and the other allowing cCF10-peptide-inducible expression of LsaA (ΔlsaA pCIE$_{spec}$ LsaA) [23]. Using this experimental set up, we could specifically assess the ability of LsaA to protect the strain from lincosamides. While expression of LsaA dramatically increases resistance to clindamycin and lincomycin (96- to 256-fold, respectively), it results in a mere 8-fold protective effect against iboxamycin (MIC of 0.0625 and 0.5 µg/mL, respectively) (Table 1), demonstrating that iboxamycin can also largely overcome LsaA-mediated resistance.

*B. subtilis ABCF VmlR acts cooperatively with rRNA methyltransferase Cfr to grant significant protection against iboxamycin*

Next we tested a set of *B. subtilis* strains: wild-type 168 *B. subtilis*, ΔvmlR (VHB5) as well as a ΔvmlR strain in which VmlR is expressed under the control of IPTG-inducible P$_{hytspan}$ promoter (VHB44) [43] (Table 1). Disruption of vmlR results in a 33-fold increase in iboxamycin sensitivity (MIC of 2 and 0.06 µg/mL, respectively), and resistance is restored upon ectopic expression of VmlR (MIC of 4 µg/mL, 2-fold increase over the wild-type levels). The iboxamycin sensitivity of Δlmo0919 *L. monocytogenes* EDG-e and ΔvmlR *B. subtilis* is near-identical, indicating that the 16-/4-fold difference in iboxamycin sensitivity between wild-type *L. monocytogenes* and *B. subtilis* is due to the different efficiency of resistance granted by Lmo0919 and VmlR respectively.
Importantly, VmlR loss results in the same relative increase in sensitivity to all lincosamides tested – iboxamycin, clindamycin and lincomycin; 32-33-fold – regardless of the potency of the lincosamide (Table 1). This suggests that if the affinity of iboxamycin to the target were to be decreased by, for instance, rRNA modification, direct target protection by the ABCF could cooperatively lead to high levels of resistance. To probe this hypothesis, we have characterised the lincosamide sensitivity of \( B.\ subtilis \) strains that express Cfr 23S rRNA methyltransferase under the control of IPTG-inducible \( P_{\text{hy-spank}} \) promoter, either in the presence or absence of the chromosomally-encoded VmlR. Ectopic expression of Cfr in \( VmlR^+ B.\ subtilis \) effected a cooperative resistance to iboxamycin, resulting in MICs of 16-32 \( \mu \text{g/mL} \) as opposed to 2 \( \mu \text{g/mL} \) when either of these resistance determinants are expressed individually (Table 1). As expected, Cfr also granted high levels of lincomycin and clindamycin resistance when ectopically expressed in both wild-type and \( \Delta vmlR \) strains (MIC ranging from 320 to excess of 640 \( \mu \text{g/mL} \)).

**Iboxamycin is bacteriostatic against** \( L.\ monocytogenes \) **and displays a strong postantibiotic effect**

Macrolide antibiotics that tightly bind the ribosome and dissociate slowly are bactericidal, while macrolides that dissociate rapidly are bacteriostatic [44]. As with lincomycin and clindamycin, iboxamycin was shown to be bacteriostatic against a panel of bacterial species [14]. However, since effects on \( L.\ monocytogenes \) were not assessed in the original report – and the species is highly sensitive to iboxamycin – we tested for potential bactericidal effects of iboxamycin against this pathogen. The three \( L.\ monocytogenes \) strains that we used for the MIC measurements – wild-type 10403 and EGD-e as well as ABCF-deficient EGD-e \( \Delta m0919 \) – were treated with 4x MIC concentration of either iboxamycin, clindamycin and lincomycin for increasing periods of time (from 2 to 24 hours), washed, and then plated on BHI agar plates that contain no antibiotic. The bacterial growth expressed in Colony Forming Units, CFU, was scored after either 24- or 48-hour incubation of plates at 37°C. When the colony counting was performed after 24 h, we observed potentially bactericidal behaviour of iboxamycin, with almost a two \( \log_{10} \) drop in CFU after the 10-hour treatment with the antibiotic (Figure 2A-C). Importantly, no similar CFU decrease was observed for either clindamycin or lincomycin (Figure 2A-C). However, this apparent CFU drop effect of iboxamycin disappeared after 48 h of incubation (Figure 2D-F), suggesting slow regrowth rather than cidality and indicative of the so-called postantibiotic effect (PAE) [45, 46].

PAE is characterised by the time after antibiotic removal where no growth of the treated bacteria is observed. This prolonged action of iboxamycin has been previously noted for \( S.\ aureus \) and \( E.\ faecium \) [14]. Therefore, we next performed post-antibiotic effect experiments in \( L.\ monocytogenes \), demonstrating that, indeed, iboxamycin displays pronounced PAE, suppressing the growth of the wild-type 10403S and wild-type EGD-e for 6 and 8 hours, respectively (Figure 3B,C). Clindamycin demonstrates a weaker PAE against EGD-e (2 hours) and similar PAE against 10403S. No clear PAE is detectible for lincomycin. Compared with the isogenic wild-type, EGD-e \( \Delta m0919 \) displays similar PAE in the case of clindamycin, and, possibly, somewhat more pronounced PAE in the case of iboxamycin.

**Discussion**

In this report we have evaluated the efficiency of the oxepepanolaminamide iboxamycin against \( L.\ monocytogenes \). The antibiotic can largely overcome the intrinsic PLS\(_r\) resistance of this species that is mediated by the ribosome-associated ATPase VgaL/Lmo0919, and can similarly counteract the intrinsic resistance mediated by ARE ABCF LsaA in \( E.\ faecalis \). ARE ABCF PLS\(_r\) resistance factors are broadly distributed among bacterial pathogens [20, 22, 47, 48], and therefore the ability of iboxamycin to largely counteract the ABCF-mediated resistance is a valuable feature of the new antibiotic. However, given that \( B.\ subtilis \) VmlR does confer significant levels of iboxamycin resistance (33-fold increase in MIC) and is cooperative with the Cfr rRNA methyltransferase resistance determinant, emergence and spread of ABCF ARE variants capable of defeating next-generation lincosamides in the clinic is possible and should be closely monitored.

Furthermore, we demonstrate that iboxamycin displays a strong PAE against \( L.\ monocytogenes \), compromising bacterial re-growth for many hours post antibiotic removal. The PAE
is considerably stronger than that of clindamycin while lincomycin displays no PAE. It is possible that the strength of the PAE reflects how tightly the antibiotic binds to the target, the ribosome – and how slowly it dissociates from it. The pronounced PAE suggests that development of even more tight-binding lincosamides could produce effectively bactericidal drugs in the context of infection. Further biochemical studies are necessary to substantiate this hypothesis. Experiments in _L. monocytogenes_ infection models are necessary to further assess the potential of iboxamycin as a novel drug for the treatment of listeriosis.

**Materials and methods**

**Synthesis of Iboxamycin**

Iboxamycin was prepared according to the method reported by Mason et al. [49].

**Strains and media**

Wild-type _L. monocytogenes_ 10403S was provided by Daniel A. Portnoy, wild-type _L. monocytogenes_ EGD-e was provided by Jörgen Johansson, construction of _L. monocytogenes_ EDG-e Δmo0919 was described earlier [23], _E. faecalis_ ΔlsaA ( _lsaA_ :Kan) strain TX5332 [19] was provided by Barbara E. Murray, _E. faecalis_ ΔlsaA pCIEspec and _E. faecalis_ ΔlsaA pCIEspec LsaA were described earlier [23]. Wild-type 168 _B. subtilis_ ( _B. subtilis_ laboratory stock) was used. _B. subtilis_ strains trpCΔΔmIR (VHB5) and ΔmIR _thrC::P_{hyspank}-vmIR_ (VHB44) were described earlier [27]. To construct _B. subtilis_ _thrC::P_{hyspank-cfr}_ (VHB138) and ΔmIR _thrC::P_{hyspank-cfr}_ (VHB139), a PCR product encoding _Staphylococcus sciuri_ cfr gene optimized to _E. coli_ codon usage [50] was PCR-amplified from the pBRcfr plasmid using primers VHT25 (5′-CGGATAACACTGTTAAGCTTAGCAGGTGTTGCTCAGTGAACACACACAAACAAACG-3′) and VHT26 (5′-GTTCCTACCGAATCTGCGATGGTCTCAGTGAACACACACAAACG-3′). The second PCR fragment encoding a kanamycin-resistance marker, a polylinker downstream of the _P_{hyspank}_ promoter and the _lac_ repressor ORF – all inserted in the middle of the _thrC_ gene – was PCR-amplified from pHT009 plasmid using primers pHT002_F (5′-GTCGACTAAGCTTACCTGGACACCATCCAACCCATACCACATG-3′) and pHT002_R (5′-GATCAGCTAATCCGTCGAGCGCATGGC-3′). The two fragments were ligated using the NEBuilder HiFi DNA Assembly master mix (New England BioLabs, Ipswich, MA) yielding the pHT009-cfr plasmid (VHp439) which was used to transform either wild-type 168 _trpC2_ or ΔmIR (VHB5) strain. Selection for kanamycin resistance yielded the desired VHB138 and VHB139 strain.

Growth assays, MIC, cidality and post antibiotic effect assays with _L. monocytogenes_, were performed in MH-F broth, _E. faecalis_ MIC assays were performed in BHI broth and _B. subtilis_ MIC assays were performed in LB broth. The _L. monocytogenes_ _Δmo0919_ strain was used as per European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_man uals/Media_preparation_v_6.0_EUCAST_AST.pdf) and contained 95% Mueller-Hinton broth (MHB) media (Sigma, Lot# BCCB5572), 5% lysed horse blood (defibrinated 50% stock, Hatunala AB Cat. No. 139) and 20 mg/mL β-NAD (Sigma, Lot# SLCD5502). Prior to use the 50% horse blood stock was freeze thawed five times and clarified via centrifugation twice for 30 minutes at 18,000 rpm at 4 °C and then filtrated using 0.2 µm membrane filter, aliquoted and stored at -20°C. Solid agar plates were prepared from BHI broth media (VMR, Lot# G0113W) supplemented with 1% (final concentration) agar.

**Liquid growth assays**

_L. monocytogenes_ was pre-grown on BHI agar plates at 37 °C for 48 hours. Individual fresh colonies were used to inoculate 2 mL of MH-F broth in 15 mL round bottom tubes, which were then incubated overnight at 37 °C with shaking at 180 rpm. The overnight cultures were diluted then with MH-F broth to final OD600 of 0.005 and incubated for 8 hours in a water bath shaker (Eppendorf™ Inova™ 3100 High-Temperature) at 37 °C with shaking at 160 rpm. Bacterial growth was monitored by OD600 measurements every 30 minutes.
Antibiotic susceptibility testing

The Minimum Inhibitory Concentration (MIC) antibiotic sensitivity testing was performed according to EUCAST guidelines (http://www.eucast.org/ast_of_bacteria/mic_determination), as described earlier [23].

*L. monocytogenes* strains were grown in MH-F broth inoculated with $5 \times 10^5$ CFU/mL ($OD_{600}$ of approximately 0.0015) with increasing concentrations of antibiotics. After 24-48 hours of incubation at 37 °C without shaking, the presence or absence of bacterial growth was scored by eye.

*E. faecalis* strains were grown in BHI media supplemented with 2 mg/mL kanamycin (to prevent *lsa* revertants), 0.1 mg/mL spectinomycin (to maintain the *pCIE*<sub>spec</sub> plasmid), 100 ng/mL of cCF10 peptide (to induce expression of LsaA protein) as well as increasing concentrations of antibiotics, was inoculated with $5 \times 10^5$ CFU/mL ($OD_{600}$ of approximately 0.0005) of *E. faecalis* ΔlsaA (*lsa::Kan*) strain TX5332 transformed either with empty *pCIE*<sub>spec</sub> plasmid, or with *pCIE*<sub>spec</sub> encoding LsaA. After 16-20 hours at 37 °C without shaking, the presence or absence of bacterial growth was scored by eye.

*B. subtilis* strains were grown in LB medium supplemented with increasing concentrations of antibiotics was inoculated with $5 \times 10^5$ CFU/mL ($OD_{600}$ of approximately 0.0005), and after 16-20 hours at 37 °C without shaking the presence or absence of bacterial growth was scored by eye.

Time-kill kinetics assay

The protocol was based on that of [51] and Svetov [44]. Exponential *L. monocytogenes* cultures in MH-F broth ($OD_{600} \approx 0.3$) were diluted to $10^5$ CFU/mL ($OD_{600} = 0.001$) in 10 mL of MH-F broth either supplemented with appropriate antibiotic at four-fold MIC concentration or without antibiotics (positive growth control), and the resultant cultures were incubated at 37 °C without shaking. 1 mL aliquots were taken at incremental incubation times (0, 2, 4, 6, 8 and 10 h), spun down at 4000 rpm for 5 min at room temperature and cell pellets were gently washed twice with 900 µL of 1x PBS. Cell pellets were resuspended in 100 µL of 1x PBS, ten-fold serial dilutions were prepared in 96-well plates ($10^{-1}$-$10^8$), and 10 µL resultant ten-fold serial dilutions were spotted on BHI agar plates. Colony forming units were scored after 24- to 48-hour incubation at 37 °C.

Post Antibiotic Effect (PAE) assay

Exponential cultures of *L. monocytogenes* strains in MH-F blood broth media ($OD_{600} \approx 0.3$) were diluted to $10^5$ CFU/mL ($\approx OD_{600}$ of 0.001) in 5 mL of MH-F media either supplemented with appropriate antibiotic at four-fold MIC concentration or without antibiotics (positive growth control) and incubated at 37 °C for without shaking for 2 h. After the 2 h pre-treatment, antibiotics were removed by 1:100 dilution of 100 µL into 10 mL of fresh prewarmed MH-F blood broth media. At incremental time points (0, 2, 4, 6, 8 and 10 h), 1 mL of the 100x-diluted cell culture was harvested, centrifuged for 5 min at 4000 rpm, 900 µL of the medium was removed, and the pellets were resuspended in the remaining 100 µL. The volume was adjusted to 1 mL with 1x PBS. Control cultures without antibiotics were handled similarly. Cell solutions were then serially diluted ten-fold to $10^8$, and 10 µL were spotted on BHI agar plates. Plates for individual time points were incubated at room temperature until the last set of plates were spotted (10 h time point), and then incubated at 37 °C. The plates were scored after 24 and 48 h incubation at 37 °C and imaged using ImageQuant LAS 4000 (GE Healthcare). The last time point (24 h) was processed separately analogously to 0-10h time points (see above).

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Conflicts of Interest
AGM is an inventor in a provisional patent application submitted by the President and Fellows of Harvard College covering oxepanoprolinamide antibiotics described in this work. AGM has filed the following international patent applications: WO/2019/032936 ‘Lincosamide Antibiotics and Uses Thereof’ and WO/2019/032956 ‘Lincosamide Antibiotics and Uses Thereof’.
Table 1. Broth microdilution Minimum inhibitory concentration (MIC) testing of lincosamide antibiotics against \textit{L. monocytogenes}, \textit{E. faecalis} and \textit{B. subtilis} strains.

In the case of \textit{L. monocytogenes} strains, MIC testing was carried out in MH-F broth and growth inhibition was scored after 48 hours incubation at 37 °C. \textit{E. faecalis} MIC testing was carried out in BHI broth supplemented with 2 mg/mL kanamycin (to prevent \textit{lsa} revertants), 0.1 mg/mL spectinomycin (to maintain the pCIE\textit{spec} plasmid), 100 ng/mL of cCF10 peptide (to induce expression of LsaA protein). \textit{B. subtilis} MIC testing was carried out in either LB medium or LB supplemented with 1 mM IPTG to induce expression of either \textit{VmlR} or \textit{Cfr} protein, and growth inhibition was scored after 16–20 hours at 37 °C.

| Species / Strain                  | Antibiotic MIC, µg/mL |
|----------------------------------|------------------------|
|                                  | Lincomycin  | Cindamycin | Iboxamycin |
| \textit{L. monocytogenes} 10403S | 4-8         | 2          | 0.125-0.25 |
| \textit{L. monocytogenes} EDG-e | 8           | 1-2        | 0.125-0.5  |
| \textit{L. monocytogenes} EDG-e ∆\textit{lmo0919} | 0.25-1 | 0.125-0.5 | 0.0625     |
| \textit{E. faecalis} \textit{ΔlsaA} pCIE\textit{spec} | 0.125 | 0.15 | 0.0625 |
| \textit{E. faecalis} \textit{ΔlsaA} pCIE\textit{spec} LsaA | 16-32 | 16 | 0.5 |
| \textit{B. subtilis} wt 168      | 80          | 4          | 2          |
| \textit{B. subtilis} \textit{ΔvmlR} | 2.5         | 0.125      | 0.06       |
| \textit{B. subtilis} \textit{ΔvmlR} \textit{thrC::P\textit{hy-spank}−vmlR} (IPTG: 1 mM) | 160 | 8 | 4 |
| \textit{B. subtilis} \textit{thrC::P\textit{hy-spank}−cfr} (IPTG: 1 mM) | >640 | 640 | 16-32 |
| \textit{B. subtilis} \textit{ΔvmlR} \textit{thrC::P\textit{hy-spank}−cfr} (IPTG: 1 mM) | >640 | 320 | 2 |
Figure 1. Chemical structures of lincosamide antibiotics lincomycin (A), clindamycin (B) and iboxamycin (C).
Figure 2. Iboxamycin is bacteriostatic against *L. monocytogenes*.
Exponentially growing *L. monocytogenes* type strains; 10403S (A,D), EDG-e (B,E) or VgaA-deficient EDG-e Δ*lmo0919* (C,E) were treated with 4x MIC of either iboxamycin, clindamycin, lincomycin or no antibiotic as control. Cells were harvested at given time points and washed before plating. After 24 (A-C) or 48 hours (D-F) of incubation, colonies were counted to determine CFU/ml. All experiments were carried out in MH-F broth at 37 °C with shaking at 180 rpm, data points are from three biological replicates and standard deviation is indicated with error bars.
Figure 3. Iboxamycin displays strong postantibiotic effect against *L. monocytogenes*.

To determine the time taken for antibiotic treated *L. monocytogenes* strains to resume growth after a two-hour antibiotic treatment, exponentially growing type strains; 10403S (A), EDG-e (B) or VgaA-deficient EDG-e Δ*lmo0919* (C) were treated with 4x MIC of either iboxamycin, clindamycin, lincomycin, or no antibiotic as control, for two hours. Cells were then diluted by 100-fold to remove the antibiotic, and samples taken every two hours subsequently for viability counting. All experiments were carried out in MH-F broth at 37 °C with shaking at 180 rpm, data points are from three biological replicates and standard deviation is indicated with error bars.
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