Expanding the toolbox: Novel class IIb microcins show activity against Gram-negative ESKAPE and plant pathogens

Benedikt M. Mortzfeld[1,2,*], Shakti K. Bhattarai[1,2], Vanni Bucci[1,2,3]

[1]Department of Microbiology, University of Massachusetts Medical School, Worcester, MA, USA

[2]Program in Microbiome Dynamics, University of Massachusetts Medical School, Worcester, MA, USA,

[3]Program in Systems Biology, University of Massachusetts Medical School, Worcester, MA, USA

[*]Corresponding author

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Correspondence to:

Benedikt M. Mortzfeld benedikt.mortzfeld@umassmed.edu

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Abstract

Interspecies interactions involving direct competition via bacteriocin production play a vital role in shaping ecological dynamics within microbial ecosystems. For instance, the ribosomally-produced siderophore bacteriocins, known as class IIb microcins, affect the colonization of host-associated pathogenic *Enterobacteriaceae* species. Notably, to date, only five of these antimicrobials have been identified, all derived from specific *Escherichia coli* and *Klebsiella pneumoniae* strains. We hypothesized that class IIb microcin production extends beyond these specific compounds and organisms. With a customized informatics-driven approach, screening bacterial genomes in public databases with BLAST and manual curation, we have discovered twelve previously unknown class IIb microcins in seven additional *Enterobacteriaceae* species, encompassing phytopathogens and environmental isolates. We introduce three novel clades of microcins (MccW, MccX, and MccZ), while also identifying eight new variants of the five known class IIb microcins. To validate their antimicrobial potential, we heterologously expressed these microcins in *E. coli* and demonstrated efficacy against a variety of bacterial isolates, including plant pathogens from the genera *Brenneria*, *Gibbsiella,* and *Rahnella*. Two newly discovered microcins exhibit activity against Gram-negative ESKAPE pathogens, *i.e. Acinetobacter baumannii* or *Pseudomonas aeruginosa,* providing the first evidence that class IIb microcins can target bacteria outside of the *Enterobacteriaceae* family. This study underscores that class IIb microcin genes are more prevalent in the microbial world than previously recognized and that synthetic hybrid microcins can be a viable tool to target clinically relevant drug-resistant pathogens. Our findings hold significant promise for the development of innovative engineered live biotherapeutic products tailored to combat these resilient bacteria.

Introduction

A large body of theoretical and experimental work has shown that dynamics of microbiomes are shaped by the network of interbacterial interactions[1–4]. These cooperative and competitive interactions are often achieved *via* the secretion of cross-feeding metabolites[5], antimicrobial peptides[6] and bacterially produced small molecules[7, 8] and are crucial to ecological properties including stability and ability to respond to external perturbations[9, 10]. Among the competitive interactions, bacteriocin production is proposed to be a prominent mediator of microbiome dynamics[11] and, specifically, several reports including ours have shown that a bacteriocin subclass, Class IIb microcins, mediates *Enterobacteriaceae* dynamics *in vivo*[12–14].

Class IIb microcins are ribosomally synthesized bacteriocins between 5 kDa to 10 kDa in size with activity against closely related strains or species[13, 15–18]. Unlike all other microcins, they carry a serine-rich C-terminal motif for a posttranslational modification with a siderophore, here an enterobactin or an enterobactin derivative, before they are secreted into the extracellular space[19]. Siderophores are iron-chelating molecules commonly employed by various bacteria to scavenge free iron to compete with other bacteria, particularly in resource-scarce environments such as the gastrointestinal tract[16, 17, 20] and are often associated with increased pathogenicity or virulence[21–23]. The iron chelating moiety of these posttranslationally modified antimicrobial peptides is recognized by high-affinity receptors and functions as a Trojan Horse key to susceptible bacteria as it triggers import into the periplasmic space, where the peptide inhibits the molecular target of susceptible bacteria[20, 24–26]. Because of these features, delivery of class IIb microcins by wildtype and engineered probiotics has been recently proposed as a strategy to combat drug-resistant enteric bacteria[12, 13, 17, 27], which is in line with a growing body of work from the past decade that explores siderophore conjugation, including with enterobactin, to specifically deliver antibiotics and other small molecules to drug-resistant Gram-negative pathogens[28–30].

To date only five class IIb microcins have been described and only four have been characterized in terms of their antimicrobial activity. Specifically, the class IIb microcins MccE492 and MccG492 (uncharacterized) are solely present in *Klebsiella pneumoniae* (*Kp)*, whereas MccH47 is specific for *Escherichia coli (Ec)*[16]. Additionally, truncated versions of *mciA* (MccI47) and *mcmM* (MccM) are present in *Kp* RYC492, whereas they are intact in the *Ec* CA46 genome[16]. Interestingly, while the genes encoding for microcin posttranslational modifications are highly conserved between *Ec* and *Kp*, suggesting a conserved pathway for microcin maturation, the toxin and corresponding immunity genes are significantly more variable (**Fig S1**). We hypothesized that class IIb microcin production extends beyond these specific compounds and organisms and identified a total of twelve novel class IIb microcins in seven additional *Enterobacteriaceae* species. Utilizing heterologous expression of these compounds in our *E. coli* system optimized for enterobactin conjugation, we show potent antimicrobial activity by the encoded toxins against a library of bacteria, including Gram-negative ESKAPE and plant pathogens. This demonstrates that class IIb microcin genes are more prevalent in the microbial world than previously recognized and that synthetic hybrid microcins can be a viable tool to target clinically relevant drug-resistant pathogens.

Materials and Methods

Bioinformatic class IIb microcin identification

We developed a pipeline that by leveraging BLAST[31] enabled us to mine publicly available genome databases for novel, previously undescribed class IIb microcins. We included *mchCDEF* and *mcmL* for *Ec* as well as *mceCDGHIJ* for *Kp* for posttranslational modification and export, expecting more reliable hits for longer and functionally conserved proteins in close proximity to class IIb microcin and immunity genes. Thus, we first ran *tblastn*[31] against RefSeq[32], to screen for all genes related to biosynthesis pathways, known microcin genes, as well as immunity gene sequences exhibiting homology to the microcin gene clusters found in *Ec* CA46 and *Kp* RYC492. Homology to the microcin gene clusters were guided by BLAST parameters sseqid (Genome ID) , pident (percentage of identical positions) along with sstart (start of alignment in Genome) and send (end of alignment position in Genome). Resulting hits were concatenated by genome ID and assessed for their proximity to one another in the genome. These gene clusters should, at best, contain all the known genes required for toxin maturation, including *mchCDEF* and *mcmL*[16, 17]. In addition to genomic hits to the known microcins, small ORFs of 50 to 150 amino acids in size close to the biosynthesis genes were screened and annotated manually using the criteria described below as well as their domains were predicted using SMART[33]. The ORFs were meticulously examined and assessed against established class IIb microcin criteria known from *Ec* H47, *Ec* I47, *Ec* M, *Kp* E492, and *Kp* G492: (i) a serine-rich C-terminus culminating in a final serine, (ii) the presence of fewer than two cysteine residues, (iii) a signal peptide within the initial 15 amino acids ending with GG or GA, and (iv) close proximity (≤200 bp) to an ORF featuring a predicted transmembrane domain, typically encoding an immunity peptide. The identified genes were included in the pipeline's input to expand the scope of gene detection. We repeated this process iteratively through the pipeline until no additional genes were added to the output. Subsequently, *blastp* was used to assess microcin similarity shown in Table 1 and Table S1.

Phylogenetic analyses

For the native full length coding sequence of the microcin and immunity genes a codon-based sequence alignment was generated using the MUSCLE algorithm[34]. For phylogeny of all microcins, the nucleotide sequences without the respective signal peptides were codon-aligned. Subsequently, we determined the best fit substitution models for maximum likelihood phylogenetic analyses, resulting in the General Time Reversible model with discrete gamma distribution (GTR+G) and the Hasegawa-Kishino-Yano model with discrete gamma distribution (HKY+G),

respectively. A bootstrap test with 1000 replicates for maximum likelihood and random seed was conducted for all trees. Alignment, model testing, and tree building was performed in MEGA11[35].

antiSMASH analyses

To test if similar results of class IIb microcin identification could be obtained with automated bioinformatic tools, we ran antiSMASH 7.0[36], a widely used tool for microbial genome mining and biosynthetic gene cluster detection. As input, we utilized the seven genomes from the newly identified class IIb microcins: (i) *Bg* CP014137, (ii) *Gq* CP014136, (iii) *Ko* CP033844, (iv) *Ps* CP034363, (v) *Ro* CP008886, (vi) *Se* CP030220, (vii) *Sf* CP033055. As a positive control for the well-established microcins *Kp* E492 and *Kp* G492 as well as *Ec* H47 and *Ec* M we used the accession numbers CP127839 (*Kp* RYC492) and CP148105 (*Ec* Nissle 1917), respectively. Notably, using the "loose" setting, in none of the cases a class IIb microcin biosynthesis gene cluster was detected, nor were any microcin genes identified. This was the case for both, the novel microcins and the original, well-annotated, microcins.

Plasmids and heterologous class IIb microcin expression

ORFs of identified microcin and immunity genes were codon optimized for frequent *Ec* codon usage without creating repetitive sequences and synthesized by Integrated DNA Technologies (Coralville, IA) with 18 bp of native 5' upstream sequence and 20 bp of native 3' downstream sequence, respectively. Using Gibson Assembly[37], the genes were cloned into our previously established *Ec* class IIb microcin expression system that results in mature class IIb microcins posttranslationally modified with an MGE[13, 17]. Briefly, the antimicrobial and the immunity genes are co-expressed under the control of an arabinose-inducible pBad/araC promoter in a high copy plasmid with a pUC-derived origin of replication. All assemblies were verified using whole plasmid sequencing. DNA files for all used plasmids can be found as supplementary material.

Static inhibition assays

Cultures of strains with confirmed plasmid assemblies were spread in LB agar plates containing 100 µg/ml ampicillin. In addition to a pUC19 control without microcin expression, single colonies for each microcin were picked with a sterile pipet tip and all placed into the same solid LB agar plate containing 100 µg/ml ampicillin for plasmid retention, 0.2 mM 2,2-dipyridyl to create iron-limited conditions during the growth phase, and 0.4% L-arabinose for induction of gene expression. Plates were incubated at 37°C for up to 72 h, before they were overlaid with the target bacterial

152 isolates. Note that testing all microcin-expressing stains on the same plate allowed us to confidently
153 assess differential inhibitory activity between all 17 tested microcins. For the overlay, the microcin-
154 producing bacteria in the stabs were inactivated using chloroform vapors and ten minutes under
155 ultraviolet light. Then, target bacteria were diluted 1:2000 from overnight culture in LB media
156 containing 100 µg/ml ampicillin and 0.2 mM 2,2-dipyridyl. *Ec* and *S. flexneri* strains were diluted
157 1:200 to acquire dense bacterial lawns. Finally, 0.5 ml of molten agar was added to the liquid media
158 and the resulting soft agar was spread on the plate with the inactivated bacteria and incubated for
159 16 h at 37°C. The pUC19 control strain was unable to create any zone of inhibition against any of
160 the tested target bacteria.
161
162 *Relative MIC dilution factors*
163 For enrichment of microcin *Se* G492, an MBP-microcin fusion protein was expressed from pHMT-
164 SeG492 in *E. coli* BL21 cells as previously described[13, 17]. Harvested cells were resuspended in
165 column buffer (200 mM NaCl, 20 mM Tris-HCl, pH 7.5), lysed by sonication, and passed through a
166 high flow amylose resin (*New England Biolabs, Ipswich, MA*) as recommended by the manufacturer.
167 The protein was eluted with 10 mM maltose, cleaved with Tobacco etch virus (TEV) protease, and
168 further processed as previously reported[13, 17]. The relative MIC assays were conducted using
169 sterile 96-well round bottom microplates. The plates were prepared as follows: the first row
170 contained 20 µl of 2x LB with 0.4 mM 2,2'-dipyridyl and 20 µl of *Se* G492 containing solution in
171 amylose resin elution buffer (200 mM NaCl, 20 mM Tris-HCl, 10 mM maltose, pH 7.5). All other
172 wells were filled with 20 µl of 1x LB, 0.2 mM 2,2'-dipyridyl, and 0.5× amylose resin elution buffer,
173 and a two-fold serial dilution was performed across the plate. The target bacteria were grown
174 overnight in LB at 200 rpm and 37°C and were added to a final dilution of 10^{-4} into the wells. The
175 plates were then incubated in the dark at 37°C with gentle agitation. Relative MICs were
176 determined as the lowest concentration at which no growth was observed after 24 hours. All
177 reported values represent the median of at least three biological replicates.
178
179 **Results**
180 With the hypothesis that class IIb microcin production is a common trait among
181 *Enterobacteriaceae*, we anticipated that the genes encoding for the antimicrobial and immunity
182 would exhibit a high degree of dissimilarity to already known peptides as target specificity may
183 result in accelerated adaptive coevolution[38]. Therefore, in addition to the known microcin and
184 immunity genes from MccE492, MccG492, MccH47, MccI47, and MccM, in our informatic approach

185 we included the genes that are necessary for mature class IIb microcin biosynthesis, extending our
186 search to longer sequences for more reliable Basic Local Alignment Search Tool (BLAST)
187 results[31]. Moreover, we hypothesized that the amino acid sequences of genes responsible for
188 posttranslational modification and microcin export would be less prone to evolutionary changes,
189 thereby maintaining the functional integrity of the gene cluster[16, 39]. We then assessed their
190 proximity in the respective genome location, because microcin genes are typically flanked by genes
191 essential for toxin maturation[16]. Further, we manually assessed and annotated small open
192 reading frames (ORFs) upstream and downstream of the maturation genes, allowing us to also
193 identify novel class IIb microcins without significant sequence similarity to the known
194 antimicrobials, enabling the discovery of compounds with new molecular targets or modes of
195 action (see Methods).

196 Our informatics-driven analysis identified twelve promising class IIb microcin candidates
197 from seven gene clusters with high similarity to *Ec* CA46 and *Kp* RYC492 in seven species across the
198 *Enterobacteriaceae* family (**Fig 1A, Fig S2**): (i) *Brenneria goodwinii* (*Bg*; 2; GenBank: CP014137), (ii)
199 *Gibbsiella quercinecans* (*Gq*; 1; CP014136), (iii) *Klebsiella oxytoca* (*Ko*; 1; CP033844), (iv) *Pantoea*
200 *sp*. (*Ps*; 1; CP034363), (v) *Raoultella ornithinolytica* (*Ro*; 4; CP008886), (vi) *Salmonella enterica*
201 (*Se*; 2; CP030220), (vii) *Serratia fonticola* (*Sf*; 1; CP033055). Although it has traditionally been a
202 defining characteristic of class IIb microcins that all required genes are encoded within the
203 chromosome[12], the gene cluster we discovered for *Se* is situated on a 159 kbp plasmid.
204 Phylogenetic sequence analysis of both the antimicrobial and immunity peptide genes revealed the
205 presence of eight different clades represented in both trees, respectively (**Fig 1B,C**). Regarding the
206 well-established class IIb microcins MccH47, MccI47, MccM, MccG492, and MccE492, we identified
207 novel members for each group, supported by nucleotide sequence similarity, amino acid identity,
208 the closest *blastp* match, and domain predictions (**Table 1, Fig S3**). It is important to note that
209 application of established tools for secondary metabolite identification (*e.g.*, antiSMASH 7.0)[36] to
210 these genomes did not yield identification of any of the old or novel microcins providing support of
211 the relevance of our approach. In order to then ensure that these novel microcins are unique and
212 not part of any other microcin class, we performed phylogenetic analysis for all known microcin
213 genes from the classes I, IIa, and IIb and show distinct clustering for all newly described sequences
214 (**Fig S4**). In light of this discovery, we propose a new nomenclature for class IIb microcins that
215 includes the species initials in which they were identified (*e.g.*, *Ec*, *Kp*), the closest relative already
216 characterized class IIb microcin (G492, E492, H47, I47 or M), as well as the identifiers 'A' for
217 antimicrobial or 'I' for immunity gene.

218 Based on this, the novel G492 relative found in *Salmonella enterica* will be called *Se* G492
219 with the antimicrobial peptide identified as *Se* G492A and the immunity peptide identified as *Se*
220 G492I. It is worth highlighting that in the case of the G492 group, all its members have the
221 immunity gene located downstream of the antimicrobial gene, whereas for the other clades, this
222 arrangement is reversed. In addition to uncovering eight novel variants of the five previously
223 characterized microcins, we have identified four additional microcins through manual curation of
224 ORFs in proximity to the microcin maturation genes. These novel microcins, which we name
225 microcin W (MccW), microcin X (MccX), and microcin Z (MccZ), seem to belong to three entirely
226 new clades based on nucleotide similarity (**Fig 1 B,C**). The two members of the microcin X group,
227 found in *B. goodwinii* (*Bg* X) and *R. ornithinolytica* (*Ro* X), only show significant similarity between
228 one another, but not to any of the other antimicrobial or immunity peptides. This holds true for the
229 nucleotide similarity (**Fig 1 B,C**) as well as amino acid identity and the closest *blastp* hits (**Fig. 1D,**
230 **Table 1, Table S1**). Similarly, MccW from *Gibbsiella quercinecans* (*Gq* W) does not show any
231 sequence similarity to either the known or novel antimicrobial or immunity peptides in terms of
232 nucleotide similarity, amino acid identity, the respective *blastp* hits, or phylogenetic localization
233 (**Fig 1,Table 1, Table S1**). Lastly, MccZ from *R. ornithinolytica* (Ro Z) shows insignificant amino
234 acid similarity with *Ec* MA (*mcmA*) for the antimicrobial, whereas the immunity peptide does not
235 have any match among the known or the novel microcins (**Fig 1 C, Table 1, Table S1**). Crucially,
236 the identification of MccX, and MccZ within the same gene clusters as representatives of the E492
237 (*Bg* E492), H47 (*Ro* H47), and I47 (*Ro* I47) groups strongly implies that they are functional
238 components of a microcin gene cluster.
239 To test the newly identified microcins for antimicrobial activity we used our previously
240 established *Ec* overexpression system[13, 17]. All antimicrobial and immunity peptides were codon
241 optimized, synthesized, and cloned into an inducible high copy vector (see Methods). Thus, we
242 extracted the novel microcins out of their native genomic context of siderophore biosynthesis and
243 transferred them into a heterologous expression background optimized for microcin-
244 monoglycosylated enterobactin (MGE) linkage[13, 17]. This allowed us to create hybrid compounds
245 that could be efficiently tested for antimicrobial activity in an *E. coli* background. Through static
246 plate inhibition assays involving live-producing cells[13, 17, 27], we successfully validated robust
247 antimicrobial activity of eleven out of the twelve newly discovered microcins (**Fig 2A**). Notably,
248 antimicrobial activity was only observed in iron-depleted media (**Fig 2B,C**). The hybrid microcins
249 exhibit a range of specificities, with some inhibiting targets narrowly (*e.g., Ps* G492AI), while others
250 exert a broader effect against multiple bacteria (*e.g., Se* G492AI). Moreover, our study also provides

the first evidence of inhibitory activity by *Kp* G492, a microcin whose existence and function have only been proposed in the scientific literature based on genetic sequence[16].

To this date class IIb microcins have been only shown to be very selective and only active against different species within the *Enterobacteriaceae* family[13, 16, 17, 27]. While the activity for the novel microcins varies, we here report, for the first time, antimicrobial activity outside of the *Enterobacteriaceae* family utilizing hybrid antimicrobial peptides. We demonstrate that microcins *Ps* G492 and *Se* G492 have activity against Gram-negative multidrug-resistant ESKAPE pathogens with both being capable of inhibiting *Acinetobacter baumannii* (BAA 1790), and with microcin *Se* G492 alone also showing activity against *Pseudomonas aeruginosa* (PA14) (**Fig 2**). Specifically, compared to *K. pneumoniae* (BAA 1705) *Se* G492 is 256-times more effective against *A. baumannii* (BAA 1790), 128-times more effective against *E. coli* (BAA 196), and 8-times more effective against *P. aeruginosa* (PA14) (**Fig. 2B**).

Discussion

With a comprehensive analysis of publicly available bacterial genomes, we unraveled twelve previously undiscovered class IIb microcins. Among these findings, we identified three novel microcin clades, specifically MccW, MccX, and MccZ. Through heterologous expression, we showed antimicrobial activity for all but one novel microcins and are the first to demonstrate activity for the known class IIb microcin *Kp* G492. Hence, this research demonstrates that class IIb microcin genes exhibit a higher prevalence in *Enterobacteriaceae* genomes than previously reported. As a result, their impact on ecological community dynamics in natural environments, including the growth of *Pseudomonadales* species, might be broader than previously thought. For antimicrobial activity testing, microcin and immunity genes were overexpressed recombinantly in our *Ec*-derived expression system optimized for microcin-MGE production[13, 17]. The common process of posttranslational modification with the siderophore consolidated the import mechanism of the hybrid microcins towards enterobactin, the most characteristic siderophore of the *Enterobacteriaceae* family. This allowed us to test the target-specific antimicrobial activity of the microcins irrespective of siderophore production in the native genomic background. However, it is important to note that class IIb microcin activity is dependent on active import through siderophore receptors and consequently some of these microcins might display different activity spectrums when tested in their native genomic background of siderophore biosynthesis. Furthermore, static plate inhibition assays exhibit lower sensitivity compared to purification approaches with quantitative minimum inhibitory concentration (MIC) assays. Thus, the activity

spectrums of the hybrid microcins could encompass a wider range than what has been described in this study when tested as a purified product. However, historically the microcin literature proves that ideal approaches for purification and MIC testing can vary between the antimicrobials[13, 16, 17, 25].

We were able to expand the origins of class IIb microcins from the enteric bacteria *Ec* and *Kp* to other members of the *Enterobacteriaceae* family, including well-known phytopathogens[40–42]. Specifically, *B. goodwinii* and *G. quercinecans* are associated with Acute Oak Decline (AOD) and are frequently isolated together[43] and the two strains containing microcin genes were isolated within the same research project. Notably, these bacteria grow synergistically[44], while upregulating iron transporters during co-culture[45], hinting at class IIb microcin-related competition. We were able to show activity of the overexpressed hybrid microcins against human-derived enteric isolates, however, their native spectrum might have evolved to target more frequently encountered strains from the genus *Brenneria* or *Gibbsiella*. Further, we demonstrated activity of several class IIb microcins against the three tree pathogen genera *Brenneria*, *Gibbsiella*, as well as *Rahnella*[44, 46–49]. Thus, treatment with potent microcins, purified or produced in live bacteria, could present a viable option to target bacteria-caused plant diseases.

In health care settings the burden by Gram-negative ESKAPE pathogens and multidrug-resistant *Enterobacteriaceae* weighs heavily on modern medicine and novel antimicrobials are needed to develop new treatment options[50, 51]. In addition to enteric pathogens and pathobionts, bacteria outside of the *Enterobacteriaceae* family have also been shown to scavenge for and to import enterobactin, including *P. aeruginosa* and *A. baumannii* [52, 53]. Therefore, different siderophore conjugates could be a viable option to target these pathogens as well or to finetune the desired target range[28–30]. Antimicrobial peptides and particularly microcins are promising candidates for selective eradication of enteric pathogens and have been demonstrated to potently reduce pathogen colonization *in vivo*, when produced by a live probiotic[12, 13]. Here we present the most comprehensive library of class IIb microcins created so far, that is suited for heterologous expression and *in vivo* application for the development of novel live biotherapeutic products against drug-resistant enteric bacteria and Gram-negative ESKAPE pathogens.

In this study, we challenge the prevailing notion that class IIb microcin production is limited to *Ec* and *Kp*. Through comprehensive genomic analysis of publicly available bacterial genomes, coupled with heterologous overexpression, we unveiled a set undiscovered class IIb microcins across *Enterobacteriaceae* species. Our findings not only expand the known repertoire of class IIb microcins but also hold significant implications for synthetic hybrid compounds. We demonstrate

that these newly identified class IIb microcins exert remarkable inhibitory effects on ESKAPE pathogen species when expressed in a system for enterobactin-derived conjugation. This discovery underscores their potential as agents against a broader spectrum of pathogens, including those affecting humans and plants, thus opening new avenues for antimicrobial research and applications.

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Competing Interests

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Data Availability Statement

The genomes are accessible with the following GenBank numbers: *Brenneria goodwinii* (CP014137), *Gibbsiella quercinecans* (CP014136), *Klebsiella oxytoca* (CP033844), *Pantoea sp.* (CP034363), R*aoultella ornithinolytica* (CP008886), *Salmonella enterica* (CP030220), *Serratia fonticola* (CP033055). All information is included in the manuscript or supporting files. All plasmid sequences as well as annotation files to produce Figs 1A, S1, S2, and S3 are available as supplementary material.

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Author Contributions

B.M.M, S.K.B, and V.B conceptualized the study. S.K.B. performed bioinformatic data mining. B.M.M and V.B designed the experiments. B.M.M performed plasmid design, verification, and *in vitro* activity testing. B.M.M, S.K.B, and V.B wrote the manuscript.

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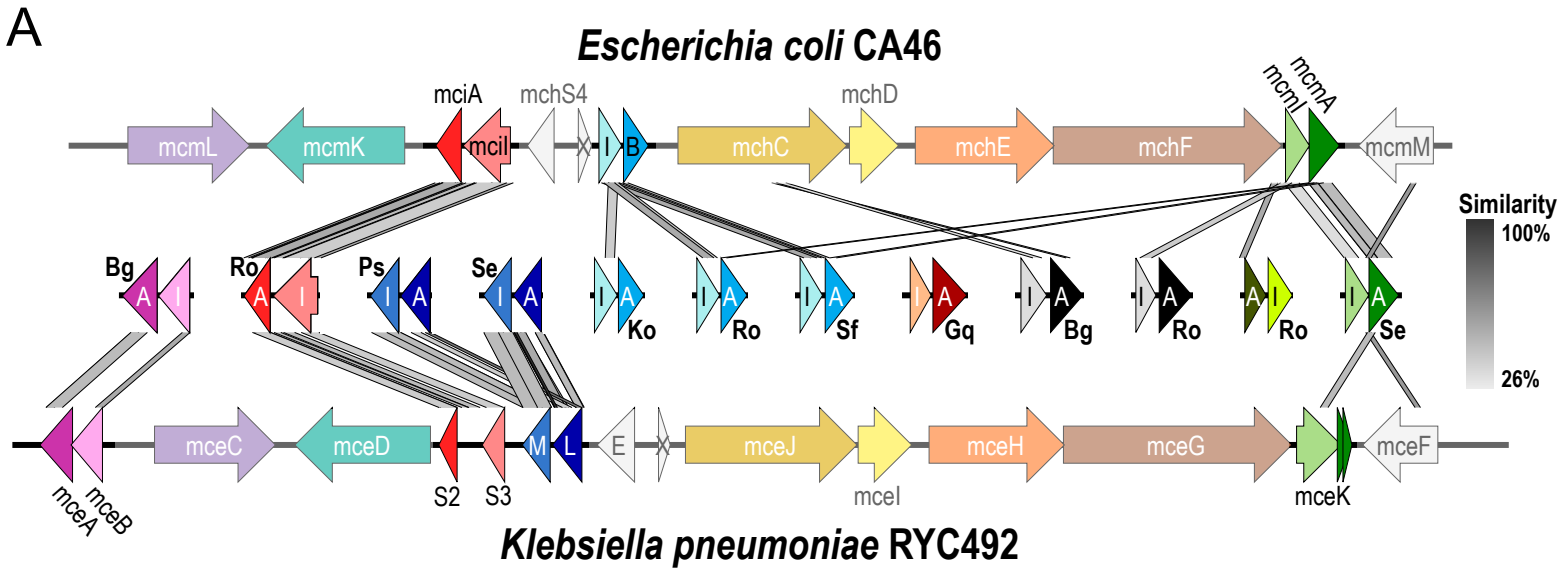
Figure Legends

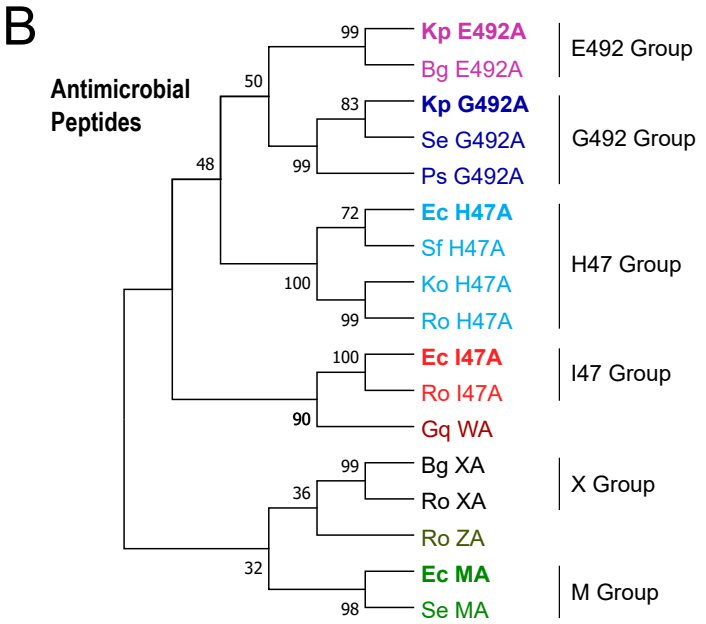
Fig 1: Novel class IIb microcins are found in numerous *Enterobacteriaceae* genomes. (A) Sequence alignments of the newly identified microcin and immunity genes with the gene clusters of *Ec* CA46 and *Kp* RYC492 using Easyfig[54]. Antimicrobial (A) and immunity (I) genes in the center are represented by darker and lighter shades, respectively. X=mchX, I=mchI, B=mchB, E=mceE, L=mceL, M=mceM. (B,C) Phylogenetic trees of antimicrobial and corresponding immunity genes using codon-aligned nucleotide sequences with General Time Reversible model with discrete gamma distribution (GTR+G) and the Hasegawa-Kishino-Yano model with discrete gamma distribution (HKY+G), respectively. (D) MUSCLE[34] alignment of the amino acid sequence of the signal peptide sequence as well as the C-terminus of the antimicrobial peptides.

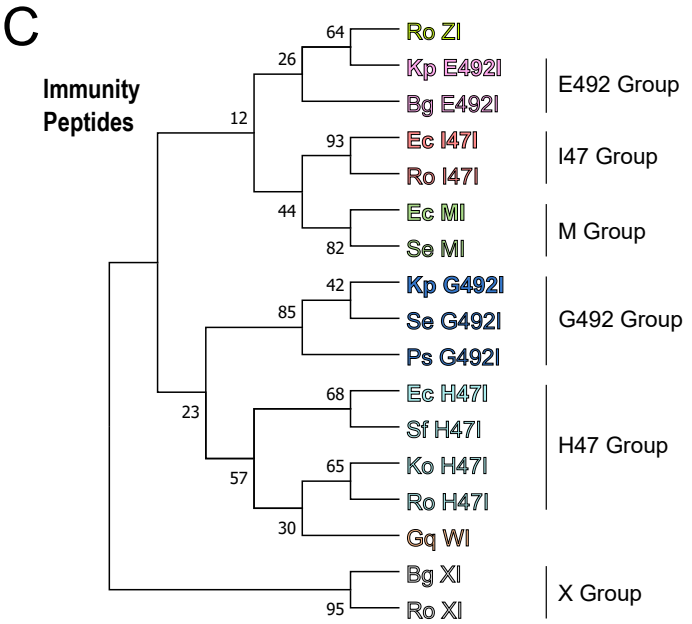
Fig 2: Novel class IIb microcins are effective inhibitors of *Enterobacteriaceae* and Gram-negative ESKAPE pathogens. (A) Heatmap summarizing the inhibitory potential of known and novel class IIb microcins against a library of *Enterobacteriaceae*, *Pseudomonadales*, and Gram-positive bacteria, including multidrug-resistant isolates (red) as determined by static inhibition assays with live producing bacteria. *=activity determined through microcin purification and minimum inhibitory concentration assays[13, 17]. (B) Relative minimum inhibitory concentrations for *Se* G492 against different bacterial species. Note that *Se* G492 is 256-times more potent against *A. baumannii* (BAA 1790) compared to *K. pneumoniae* (BAA 1705). (C) Static inhibition assays comparing *Kp* E492, *Kp* G492, *Ec* H47, *Ec* I47, *Ec* M, and *Se* G492 activity from single colony production against multidrug-resistant *A. baumannii* (BAA 1790) and *P. aeruginosa* (PA14). Note that iron-limited conditions (DP) are required for antimicrobial activity, confirming action of class IIb microcins. L-ara=L-arabinose, DP=2,2-dipyridyl, scale bars: 1 cm.

Table 1: *Blastp* results and closest matches to the known class IIb microcins MccE492, MccG492, MccH47, MccI47, or MccM. Red color indicates no significant match found.

Microcin Name	Species	Accession no.	Antimicrobial Gene Name	Antimicrobial closest match	Identical/Total Length	E-value	Immunity Gene Name	Immunity closest match	Identical/Total Length	E-value
Bg E492	*Brenneria goodwinii*	CP014137	*Bg* E492A	*Kp* E492A (mceA)	35/106	2.00E-14	*Bg* E492I	*Kp* E492I (mceB)	14/94	6.00E-04
Bg X	*Brenneria goodwinii*	CP014137	*Bg* XA	*Kp* E492A (mceA)	21/98	6.90E-02	*Bg* XI	*Ec* MI (mcmI)	10/78	2.60E+00
Gq W	*Gibbsiella quercinecans*	CP014136	*Gq* WA	*Ec* I47A (mciA)	7/103	8.10E+00	*Gq* WI	*Ec* MI (mcmI)	8/74	3.30E+02
Ko H47	*Klebsiella oxytoxa*	CP033844	*Ko* H47A	*Ec* H47A (mchB)	32/77	1.00E-07	*Ko* H47I	*Ec* H47I (mchI)	19/69	2.00E-14
Ps G492	*Pantoea sp.*	CP034363	*Ps* G492A	*Kp* G492A (mceL)	43/89	6.00E-09	*Ps* G492I	*Kp* G492I (mceM)	37/85	2.00E-20
Ro Z	*Raoultella ornithinolytica*	CP008886	*Ro* ZA	*Ec* MA (mcmA)	21/64	1.00E-03	*Ro* ZI	*Ec* MI (mcmI)	5/74	2.00E+01
Ro H47	*Raoultella ornithinolytica*	CP008886	Ro H47A	*Ec* H47A (mchB)	31/79	2.00E-07	*Ro* H47I	*Ec* H47I (mchI)	21/69	4.00E-14
Ro I47	*Raoultella ornithinolytica*	CP008886	*Ro* I47A	*Ec* I47A (mciA)	37/79	4.00E-26	*Ro* I47I	*Ec* I47I (mciI)	64/135	1.00E-43
Ro X	*Raoultella ornithinolytica*	CP008886	*Ro* XA	*Ec* MA (mcmA)	18/99	4.40E-01	*Ro* XI	*Ec* MI (mcmI)	9/61	1.00E-02
Sf H47	*Serratia fonticola*	CP033055	*Sf* H47A	*Ec* H47A (mchB)	38/87	8.00E-11	*Sf* H47I	*Ec* H47I (mchI)	48/68	4.00E-22
Se G492	*Salmonella enterica*	CP030220	*Se* G492A	*Kp* G492A (mceL)	56/85	1.00E-11	*Se* G492I	*Kp* G492I (mceM)	38/84	1.00E-23
Se M	*Salmonella enterica*	CP030220	*Se* MA	*Ec* MA (mcmA)	34/90	1.00E-17	*Se* MI	*Ec* MI (mcmI)	38/71	6.00E-04







D

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Bg E492A	M R A L N V Y E I K M A S A A				...	G P T W N G S G G G N S V S V S A A A A N A L A A A R D G S						
Kp G492A	M R A L T E N D F F A V S G A				...	- - A G A A A G R Y G G A F I Y D S F S S P S N S S S S G S						
Se G492A	M R A L T D N E V F S V Y G A				...	F L G G A A I G A F G G A Y I Y D S F S S P S N - - - - G S						
Ps G492A	M R A L S A N D L L S V Y G A				...	- - V G A A V G A Y G G A A I C D S F S S S S D S A S S G S						
Ec H47A	M R E I T E S Q L R Y I S G A				...	V V G A V S A G L T T A I G S T V G S G S A S S S A G G G S						
Sf H47A	M K E L T S S Q L N Y I S G A				...	V M G G L S V A I G A G I G G P G S S G S A S S A A G F G S						
Ko H47A	M R E L T Q Y Q M E N V A G S				...	P V G M V I G G I L G G L G V A V G S A I G S S - - G G G S						
Ro H47A	M R E L T Q D Q I E N V A G A				...	P A G M I I G G I L G G L G V A V G S A T G S S G A G G G S						
Ec I47A	M R E I S D N M L D S V K G G				...	T P S I I M Y P G G S G P S Y S M - - S S S T S S A N S G S						
Ro I47A	M R E L D V K M L A F A K G G				...	T S S I V M Y P S G S Y S S F N W G G S S S T S S A G G G S						
Gq WA	M R E L S M L D I T L A K G G				...	N G S L V S P G Y S S S W G G S W G G A T S S A - - G G G S						
Bg XA	M R E I S H V E L S A I S G A				...	F G G S N T S S S S S S T S S A A - - S S A S S A A S N G S						
Ro XA	M I P I S D T E L S M I S G A				...	L F G N N S S A T S S S G W A N N S N K S S T I F R W L D S						
Ro ZA	M R Q L T E S E L E V A S G A				...	G P G A A F V G V L G A I G A C T S S S A S S G S - - - G S						
Ec MA	M R K L S E N E I K Q I S G G				...	S M S P S G I G L S S Q F G S G R G T S S A S S S A G S G S						
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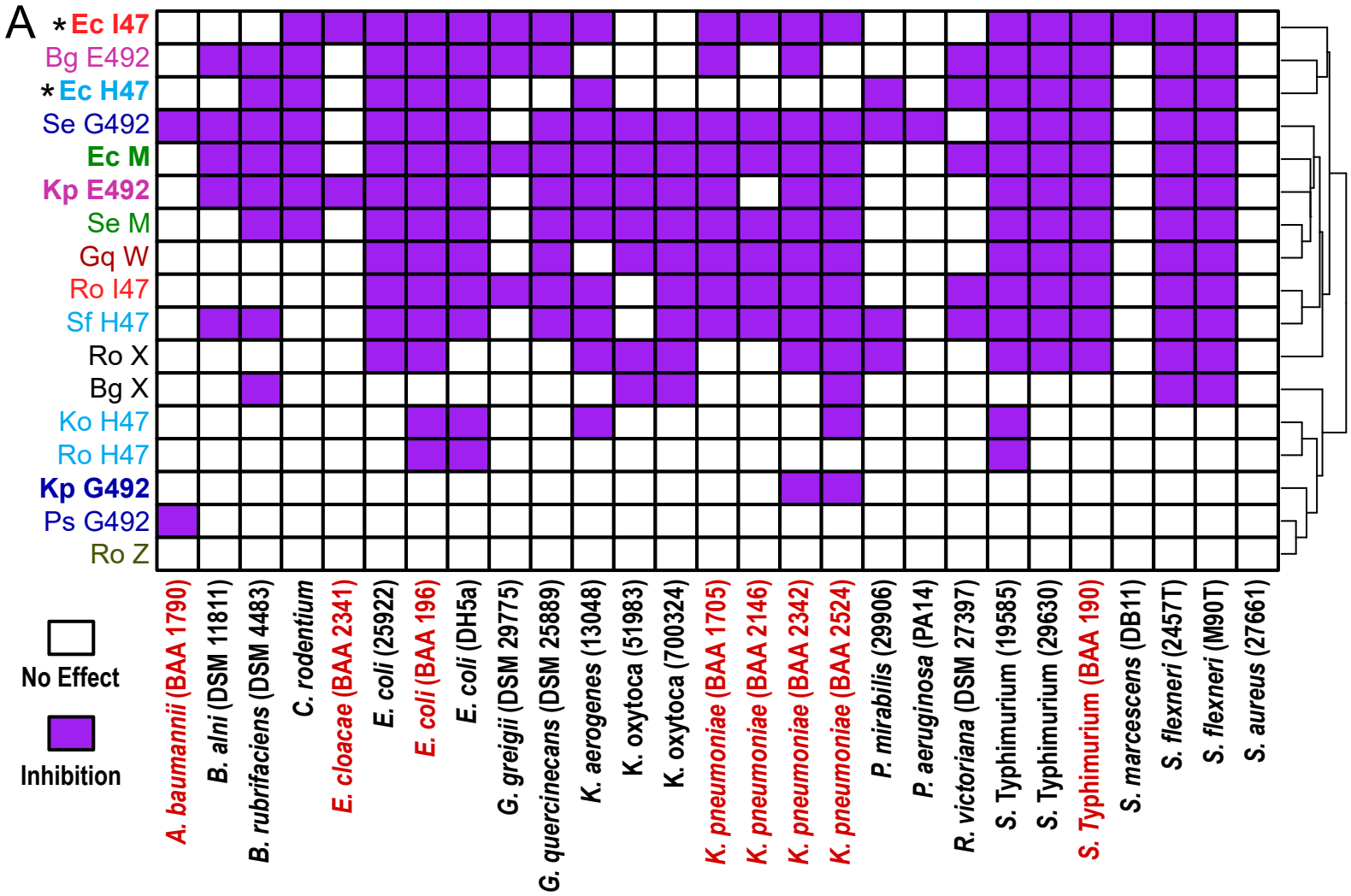
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Bg E492
*Ec H47
Se G492
Ec M
Kp E492
Se M
Gq W
Ro I47
Sf H47
Ro X
Bg X
Ko H47
Ro H47
Kp G492
Ps G492
Ro Z
No Effect
Inhibition
A. baumannii (BAA 1790)
B. alni (DSM 11811)
B. rubrifaciens (DSM 4483)
C. rodentium
E. cloacae (BAA 2341)
E. coli (25922)
E. coli (BAA 196)
E. coli (DH5a)
G. greigii (DSM 29775)
G. quercinecans (DSM 25889)
K. aerogenes (13048)
K. oxytoca (51983)
K. oxytoca (700324)
K. pneumoniae (BAA 1705)
K. pneumoniae (BAA 2146)
K. pneumoniae (BAA 2342)
K. pneumoniae (BAA 2524)
P. mirabilis (29906)
P. aeruginosa (PA14)
R. victoriana (DSM 27397)
S. Typhimurium (19585)
S. Typhimurium (29630)
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S. flexneri (2457T)
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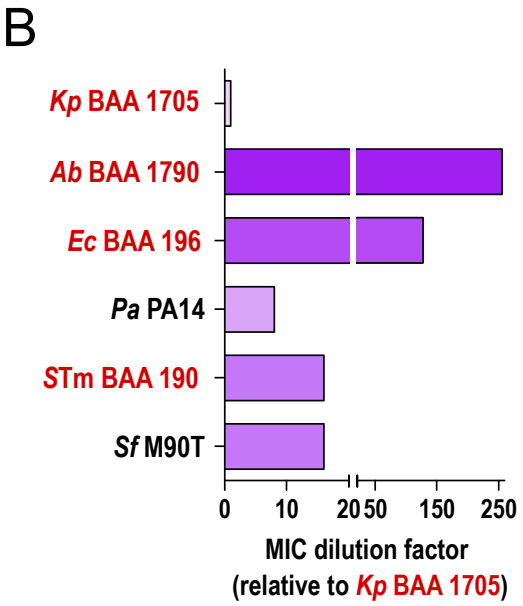
S. aureus (27661)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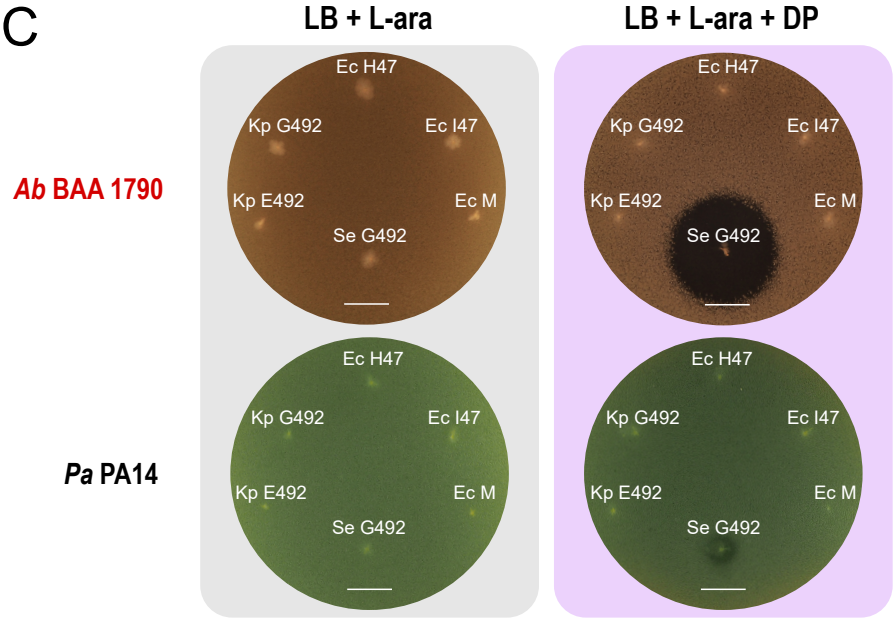
150
250
MIC dilution factor
(relative to Kp BAA 1705)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