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

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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, have been observed to affect the colonization of pathogenic *Enterobacteriaceae* species within hosts. Notably, to date, only five of these antimicrobials have been identified and all were derived from specific *Escherichia coli* and *Klebsiella pneumoniae* strains. We hypothesized that class IIb microcin production extends beyond these specific compounds and organisms. By employing an informatics-driven approach, screening bacterial genomes in publicly accessible databases, we have discovered a total of twelve previously unknown class IIb microcins. Our investigation unveiled that these microcins are harbored within a diverse array of *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 previously known ones. To validate their antimicrobial potential, we heterologously expressed these microcins, along with their immunity peptides, in *E. coli* and unequivocally demonstrated their efficacy against a variety of bacterial isolates, including plant pathogens like *Gibbsiella* species *and Rahnella victoriana*. Remarkably, two of these newly discovered class IIb microcins exhibit activity against gram-negative ESKAPE pathogens, such as *Acinetobacter baumannii* or *Pseudomonas aeruginosa* providing the first evidence that class IIb microcins can target bacteria outside of the *Enterobacteriaceae* family. Our findings hold significant promise for the development of innovative live biotherapeutic products tailored to combat these resilient bacteria and underscore the notion that class IIb microcins are more prevalent and more broad-spectrum in the natural microbial world than previously recognized.

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 microbiomes dynamics(11) and, specifically, several reports including ours have shown that a bacteriocins subclass, Class IIb microcins, mediates *Enterobacteriaceae* dynamics *in vivo*(12,13).

Class IIb microcins are ribosomally synthesized bacteriocins between 5 kDa to 10 kDa in size with activity against closely related strains or species(13–17). 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(18). Siderophores are iron-chelating molecules commonly employed by various bacteria to scavenge free iron to outgrow other bacteria, particularly in resource-scarce environments such as the gastrointestinal tract(15,16,19) and are often associated with increased pathogenicity or virulence(20–22). The iron chelating moiety of these siderophore antimicrobial peptides (sAMPs) 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(19,23–25). 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(12,13,16,26).

To date only five class IIb microcins have been described and only four have been characterized in terms of their antimicrobial activity. Specifically, the sAMPs MccE492 and MccG492 (uncharacterized) are solely present in *Klebsiella pneumoniae* (*Kp)*, whereas MccH47 is specific for *Escherichia coli (Ec)*(15). Additionally, truncated versions of *mciA* (MccI47) and *mcmM* (MccM) are present in *Kp* RYC492, whereas they are intact in the *Ec* CA46 genome(15). Interestingly, while the genes encoding for microcins 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 (Figure 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. In inhibition assays utilizing heterologous expression of these compounds, we demonstrate potent activity against a library of bacteria, including gram-negative ESKAPE and plant pathogens, .proving that class IIb microcins are more prevalent and more broad-spectrum in the natural microbial world than previously recognized.

Results

With the hypothesis that class IIb microcin production is a common trait among *Enterobacteriaceae*, we anticipated that the genes encoding for the antimicrobial and immunity would exhibit a high degree of dissimilarity to already known peptides as target specificity may result in accelerated adaptive coevolution(27). Therefore, in addition to the known microcin and immunity genes from MccE492, MccG492, MccH47, MccI47, and MccM, in our informatic approach we included the genes

that are necessary for mature class IIb microcin biosynthesis, extending our search to longer sequences for more reliable Basic Local Alignment Search Tool (BLAST) results(28). Moreover, we hypothesized that the amino acid sequences of genes responsible for posttranslational modification and microcin export would be less prone to evolutionary changes, thereby maintaining the functional integrity of the gene cluster(15,29). We then assessed their proximity in the respective genome location, because microcin genes are typically flanked by genes essential for toxin maturation(15). Further, we assessed small open reading frames upstream and downstream of the maturation genes, allowing us to identify novel class IIb microcins without significant sequence similarity to the known antimicrobials, enabling the discovery of compounds with new molecular targets or modes of action (see Methods).

Our informatics-driven analysis identified twelve promising class IIb microcin candidates from seven gene clusters with high similarity to *Ec* CA46 and *Kp* RYC492 in seven species across the *Enterobacteriaceae* family (**Figure 1A, Figure S2**): (i) *Brenneria goodwinii* (*Bg*; 2; GenBank: CP014137), (ii) *Gibbsiella quercinecans* (*Gq*; 1; CP014136), (iii) *Klebsiella oxytoca* (*Ko*; 1; CP033844), (iv) *Pantoea sp*. (*Ps*; 1; CP034363), (v) *Raoultella ornithinolytica* (*Ro*; 4; CP008886), (vi) *Salmonella enterica* (*Se*; 2; CP030220), (vii) *Serratia fonticola* (*Sf*; 1; CP033055). Remarkably, although it has traditionally been a defining characteristic of class IIb microcins that all required genes are encoded within the chromosome(12), the gene cluster we discovered for *Se* is situated on a 159 kbp plasmid. Phylogenetic sequence analysis of both the antimicrobial and immunity peptide genes revealed the presence of eight different clades represented in both trees, respectively (**Figure 1B,C**). Regarding the well-established class IIb microcins MccH47, MccI47, MccM, MccG492, and MccE492, we identified novel members for each group, supported by sequence similarity, amino acid identity, the closest BLAST match, and domain predictions (**Table 1, Figure S3**). In light of this discovery, we propose a new nomenclature for class IIb microcins that includes the species initials in which they were identified (*e.g.*, *Ec*, *Kp*), the closest relative already characterized class IIb microcin (G492, E492, H47, I47 or M), as well as the identifiers 'A' for antimicrobial or 'I' for immunity gene.

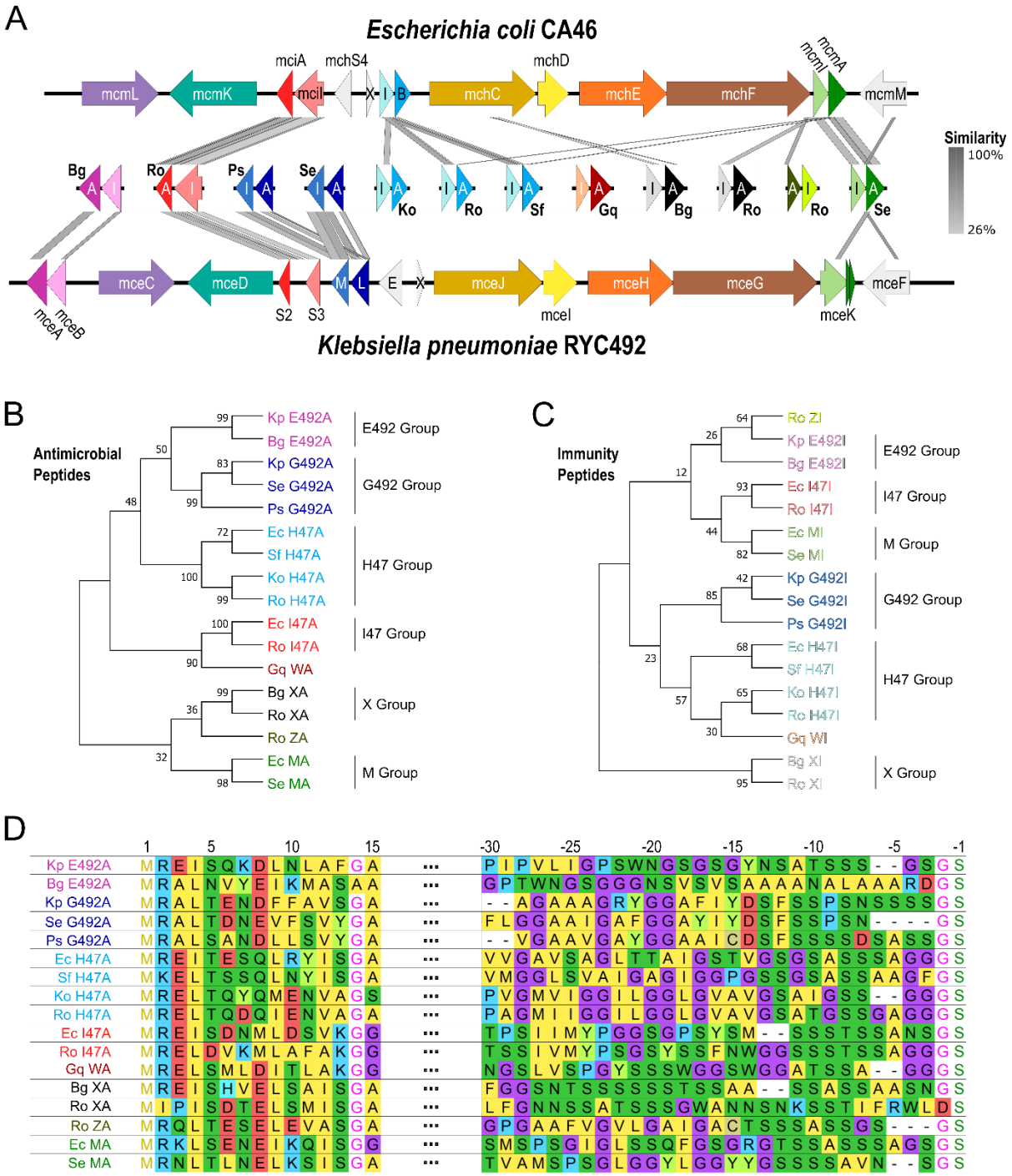

Figure 1: Novel class IIb microcins are found in numerous *Enterobacteriaceae* genomes. (A) Sequence alignments of the newly identified microcin and immunity genes with the gene clusters of *Ec* CA46 and *Kp* RYC492 using Easyfig(30). Antimicrobial (A) and immunity (I) genes in the center are represented 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(31) alignment of the amino acid sequence of the signal peptide sequence as well as the C-terminus of the antimicrobial peptides.

Thus, the novel G492 relative found in *Salmonella enterica* will be called *Se* G492 with the antimicrobial peptide identified as *Se* G492A and the immunity peptide identified as *Se* G492I. It is worth highlighting that in the case of the G492 group, all its members have the immunity gene located downstream of the antimicrobial gene, whereas for the other clades, this arrangement is reversed. In addition to uncovering eight novel variants of the five previously characterized microcins, we have identified four additional microcins that seem to belong to three entirely new clades. We name these microcins microcin W (MccW), microcin X (MccX), and microcin Z (MccZ). The two members of the microcin X group, found in *B. goodwinii* (*Bg* X) and *R. ornithinolytica* (Ro X), only show significant similarity between one another, but not to any of the other antimicrobial or immunity peptides (**Figure 1 B,C**, **Table 1, Table S1**). Similarly, MccW from *Gibbsiella quercinecans* (*Gq* W) does not show any sequence similarity to either the known or novel antimicrobial or immunity peptides in terms of sequence similarity, amino acid identity, the respective BLAST hits (**Table 1, Table S1**), or phylogenetic localization (**Figure 1 B,C**). Lastly, MccZ from *R. ornithinolytica* (Ro Z) shows insignificant similarity with *Ec* MA (*mcmA*) for the antimicrobial, whereas the immunity peptide does not have any match among the known or the novel microcins (**Table 1, Table S1**). Crucially, the identification of MccX, and MccZ within the same gene clusters as representatives of the E492 (*Bg* E492), H47 (*Ro* H47), and I47 (*Ro* I47) groups strongly implies that they are functional components of a gene cluster.

To test the newly identified microcins for antimicrobial activity in our previously established *Ec* overexpression system(13,16), all antimicrobial and immunity peptides were codon optimized, synthesized, and cloned into an inducible high copy vector (see Methods). Through static plate inhibition assays involving live-producing cells(13,16,26), we successfully validated the robust antimicrobial activity of eleven out of the twelve newly discovered microcins (Figure 2A). These microcins exhibit a range of specificities, with some inhibiting targets narrowly (*e.g., Ps* G492AI), while others exert a broader effect against multiple bacteria (*e.g., Se* G492AI). Notably, 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(15).

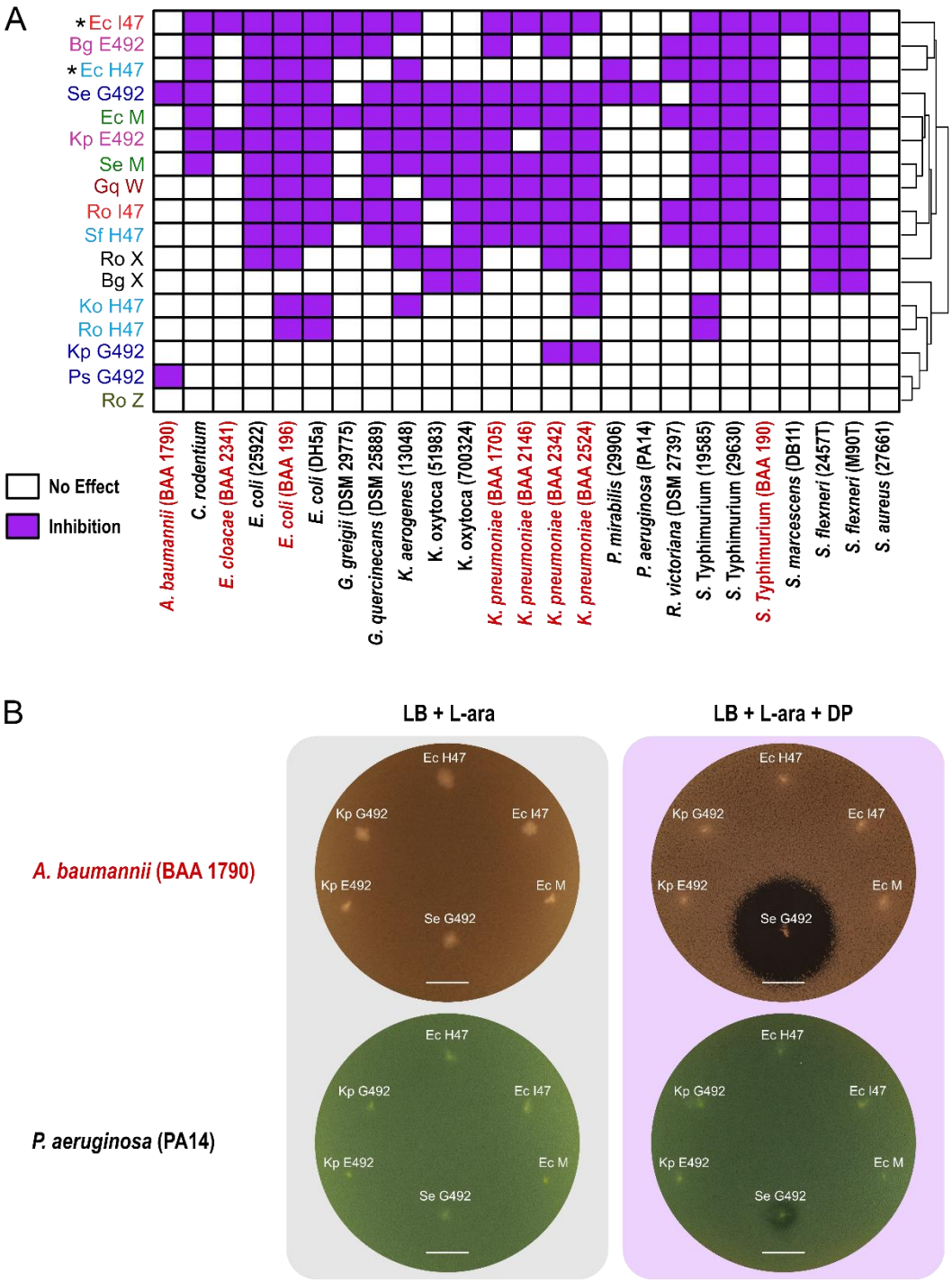

Figure 2: Novel sAMPs are effective inhibitors of *Enterobacteriaceae* and gram-negative ESKAPE pathogens. (A) Heatmap summarizing the inhibitory potential of known and novel class IIb microcins against a library of *Enterobacteriaceae*, *Pseudomonadales*, and gram-positive bacteria, including multidrug-resistant isolates (red) as determined by static inhibition assays with live producing bacteria. *=activity determined through microcin purification and minimum inhibitory concentration assays(13,16). (B) 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.

155 To this date class IIb microcins have been only shown to be very selective and only active
156 against different species within the *Enterobacteriaceae* family(13,15,16,26). While the activity for the
157 novel sAMPs varies, we here report, for the first time, antimicrobial activity outside of the
158 *Enterobacteriaceae* family. Specifically, we found that microcins *Ps* G492 and *Se* G492 have activity
159 against gram-negative multidrug-resistant ESKAPE pathogens with both being capable of inhibiting
160 *Acinetobacter baumannii* (BAA 1790), and with microcin *Se* G492 alone also showing activity against
161 *Pseudomonas aeruginosa* (PA14) (**Figure 2**).
162

163 **Discussion**

164 With a comprehensive analysis of publicly available bacterial genomes, we unraveled twelve
165 previously undiscovered class IIb microcins. Among these findings, we identified three novel
166 microcin clades, specifically MccW, MccX, and MccZ. Through heterologous expression, we showed
167 antimicrobial activity for all but one novel microcins and are the first to demonstrate activity for the
168 known class IIb microcin *Kp* G492. Hence, this research demonstrates that class IIb microcins exhibit
169 a higher prevalence in natural settings than previously anticipated. As a result, their impact on
170 ecological community dynamics in natural environments, including the growth of *Pseudomonadales*
171 species, is likely broader than previously thought. Antimicrobial and immunity genes were
172 overexpressed and tested in our *Ec*-derived expression system, which might have led to degrees of
173 incompatibility between the antimicrobial peptides and genes for microcin maturation.
174 Consequently, the process of posttranslational modification with the siderophore (*i.e.*,
175 monoglycosylated enterobactin) might be less efficient, and the export mediated by the native signal
176 peptide less targeted, potentially leading to reduced observed activity in the static plate assays.
177 Furthermore, it is important to note that these assays exhibit lower sensitivity compared to
178 purification approaches with minimum inhibitory concentration (MIC) assays. Thus, the activity
179 spectrums are likely to encompass a wider range than what has been described in this study.
180 However, historically the microcin literature proves that ideal approaches for purification and MIC
181 testing can vary between the antimicrobials(13,15,16,24).
182 Remarkably, we were able to expand the origins of class IIb microcins from the enteric
183 bacteria *Ec* and *Kp* to other members of the *Enterobacteriaceae* family, including well-known
184 phytopathogens(32–34). Specifically, *B. goodwinii* and *G. quercinecans* are associated with Acute Oak
185 Decline (AOD) and are frequently isolated together(35). Similarly, the two strains containing
186 microcin genes were isolated within the same research project. Notably, these bacteria grow
187 synergistically(36), while upregulating iron transporters during co-culture(37), hinting at class IIb

microcin-related competition. We were able to show activity of the overexpressed microcins against human-derived enteric isolates, however, their antimicrobial 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 tree pathogens *G. quercinecans, G. greigii* as well as *Rahnella victoriana*(36,38,39). 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(40,41). 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. We demonstrate that these newly identified class IIb microcins exert remarkable inhibitory effects on ESKAPE pathogen species not affected by any of the current ones. 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.

Materials and Methods

Bioinformatic class IIb microcin identification

We developed a pipeline that by leveraging BLAST(28) enabled us to mine publicly available genome databases for novel, previously undescribed sAMPs. 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*(28) against RefSeq(42), 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

221 gene clusters were guided by BLAST parameters sseqid (Genome ID) , pident (percentage of identical
222 positions) along with sstart (start of alignment in Genome) and send (end of alignment position in
223 Genome). Resulting hits were concatenated by genome ID and assessed for their proximity to one
224 another in the genome. These gene clusters should, at best, contain all the known genes required for
225 toxin maturation, including *mchCDEF* and *mcmL*(15,16). In addition to genomic hits to the known
226 microcins, small ORFs of 50 to 150 amino acids in size close to the biosynthesis genes were annotated
227 and their domains were predicted using SMART(43). The ORFs were meticulously examined and
228 assessed against established class IIb microcin criteria: (i) a serine-rich C-terminus culminating in a
229 final serine, (ii) the presence of fewer than one cysteine residue, (iii) a signal peptide within the initial
230 15 amino acids ending with GG or GA, and (iv) close proximity (≤200 bp) to an ORF featuring a
231 predicted transmembrane domain, typically encoding an immunity peptide. The identified genes
232 were included in the pipeline's input to expand the scope of gene detection. We repeated this process
233 iteratively through the pipeline until no additional genes were added to the output. Subsequently,
234 *blastp* was used to assess microcin similarity shown in Table 1 and Table S1.
235
236 *Phylogenetic analyses*
237 For the native full length coding sequence of the microcin and immunity genes a codon-based
238 sequence alignment was generated using the MUSCLE algorithm(31). Then, we determined the best
239 fit substitution models for maximum likelihood phylogenetic analyses, resulting in the General Time
240 Reversible model with discrete gamma distribution (GTR+G) and the Hasegawa-Kishino-Yano model
241 with discrete gamma distribution (HKY+G), respectively. A bootstrap test with 1000 replicates for
242 maximum likelihood and random seed was conducted for both trees. Alignment, model testing, and
243 tree building was performed in MEGA11(44).
244
245 *Plasmids and heterologous class IIb microcin expression*
246 ORFs of identified microcin and immunity genes were codon optimized for frequent *Ec* codon usage
247 without creating repetitive sequences and synthesized by Integrated DNA Technologies (Coralville,
248 IA) with 18 bp of native 5' upstream sequence and 20 bp of native 3' downstream sequence,
249 respectively. Using Gibson Assembly(45), the genes were cloned into our previously established *Ec*
250 class IIb microcin expression system that results in mature class IIb microcins posttranslationally
251 modified with a monoglycosylated enterobactin(13,16). Briefly, the antimicrobial and the immunity
252 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 at:

Static inhibition assays

Cultures of strains with confirmed plasmid assemblies were spread in LB agar plates containing 100 µg/ml ampicillin. Single colonies for each microcin were picked with a sterile pipet tip and inserted into solid LB agar 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 isolates. For the overlay, the microcin-producing bacteria in the stabs were inactivated using chloroform vapors and ten minutes under ultraviolet light. Then, target bacteria were diluted 1:2000 from overnight culture in LB media containing 100 µg/ml ampicillin and 0.2 mM 2,2-dipyridyl. *Ec* and *S. flexneri* strains were diluted 1:200 to acquire dense bacterial lawns. Finally, 0.5 ml of molten agar was added to the liquid media and the resulting soft agar was spread on the plate with the inactivated bacteria and incubated for 16 h at 37°C.

Availability of Data and Materials

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

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Competing Interests

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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.

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	2.0E-14	*Bg* E492I	*Kp* E492I (mceB)	14/94	6.0E-04
Bg X	*Brenneria goodwinii*	CP014137	*Bg* XA	*Kp* E492A (mceA)	21/98	6.9E-02	*Bg* XI	*Ec* MI (mcmI)	10/78	2.6E+00
Gq W	*Gibbsiella quercinecans*	CP014136	*Gq* WA	*Ec* I47A (mciA)	7/103	8.1E+00	*Gq* WI	*Ec* MI (mcmI)	8/74	3.3E+02
Ko H47	*Klebsiella oxytoxa*	CP033844	*Ko* H47A	*Ec* H47A (mchB)	32/77	1.0E-07	*Ko* H47I	*Ec* H47I (mchI)	19/69	2.0E-14
Ps G492	*Pantoea sp.*	CP034363	*Ps* G492A	*Kp* G492A (mceL)	43/89	6.0E-09	*Ps* G492I	*Kp* G492I (mceM)	37/85	2.0E-20
Ro Z	*Raoultella ornithinolytica*	CP008886	*Ro* ZA	*Ec* MA (mcmA)	21/64	1.0E-03	*Ro* ZI	*Ec* MI (mcmI)	5/74	2.0E+01
Ro H47	*Raoultella ornithinolytica*	CP008886	Ro H47A	*Ec* H47A (mchB)	31/79	2.0E-07	*Ro* H47I	*Ec* H47I (mchI)	21/69	4.0E-14
Ro I47	*Raoultella ornithinolytica*	CP008886	*Ro* I47A	*Ec* I47A (mciA)	37/79	4.0E-26	*Ro* I47I	*Ec* I47I (mciI)	64/135	1.0E-43
Ro X	*Raoultella ornithinolytica*	CP008886	*Ro* XA	*Ec* MA (mcmA)	18/99	4.4E-01	*Ro* XI	*Ec* MI (mcmI)	9/61	1.0E-02
Sf H47	*Serratia fonticola*	CP033055	*Sf* H47A	*Ec* H47A (mchB)	38/87	8.0E-11	*Sf* H47I	*Ec* H47I (mchI)	48/68	4.0E-22
Se G492	*Salmonella enterica*	CP030220	*Se* G492A	*Kp* G492A (mceL)	56/85	1.0E-11	*Se* G492I	*Kp* G492I (mceM)	38/84	1.0E-23
Se M	*Salmonella enterica*	CP030220	*Se* MA	*Ec* MA (mcmA)	34/90	1.0E-17	*Se* MI	*Ec* MI (mcmI)	38/71	6.0E-04

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