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Letter

Microcin H47: A Class IIb Microcin with Potent Activity Against Multidrug Resistant *Enterobacteriaceae*

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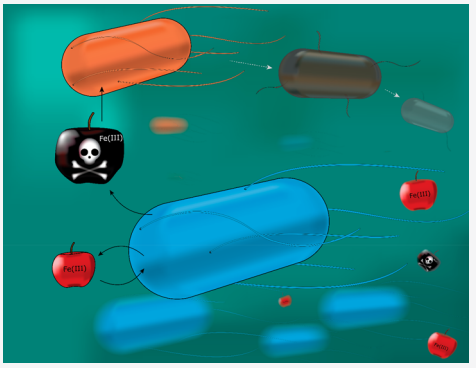
ABSTRACT: Microcin H47 (MccH47) is an antimicrobial peptide produced by some strains of *Escherichia coli* that has demonstrated inhibitory activity against enteric pathogens *in vivo* and has been heterologously overexpressed in proof-of-concept engineered probiotic applications. While most studies clearly demonstrate inhibitory activity against *E. coli* isolates, there are conflicting results on the qualitative capacity for MccH47 to inhibit strains of *Salmonella*. Here, we rectify these inconsistencies via the overexpression and purification of a form of MccH47, termed MccH47-monoglycosylated enterobactin (MccH47−MGE). We then use purified MccH47 to estimate minimum inhibitory concentrations (MICs) against a number of medically relevant *Enterobacteriaceae*, including *Salmonella* and numerous multidrug resistant (MDR) strains. While previous reports suggested that the spectrum of activity for MccH47 is quite narrow and restricted to activity against *E. coli*, our data demonstrate that MccH47 has broad and potent activity within the *Enterobacteriaceae* family, suggesting it as a candidate for further development toward treating MDR enteric infections.



KEYWORDS: *microcin, antimicrobial resistance, Salmonella, antimicrobial peptide, minimum inhibitory concentration, purification*

Prokaryotic antimicrobial peptides are abundant in nature and serve producing strains as a type of chemical warfare with neighboring cells.[1,2] While small molecule antimicrobials produced by bacteria have been exploited for decades as traditional antibiotics, antimicrobial peptides as therapeutic agents have just recently gained widespread interest as potential treatments for multidrug resistant (MDR) and extensively drug resistant (XDR) human pathogens.[3,4]

Medical complications related to MDR and XDR bacteria, including those from the *Enterobacteriaceae* (mainly *Klebsiella*, *Salmonella*, *Shigella*, and *Escherichia coli*), are a major issue in modern healthcare due to the increased morbidity, mortality, length of hospitalization, and related healthcare costs.[5] Every year, more than two million people acquire MDR infections, which result in over 23 000 directly related deaths and several more lethal outcomes from associated complications.[6] The gastrointestinal tract is both the locus of infection and the reservoir of resistance for several enteric pathogens, including XDR *Salmonella* Typhi,[7] and recent work has demonstrated microbiota-mediated colonization resistance to *Salmonella* infection.[8] Therefore, proteins and metabolites produced by gut commensal bacteria have become a major target for the discovery of new drugs to cure infections and prevent colonization by MDR/XDR pathogens.[9]

In order to expand the spectrum of viable therapies to treat MDR/XDR infections, we sought to further understand the capability of microcin H47 (MccH47), a class IIb microcin first identified by Laviña et al.,[10] produced and processed by the *mch* gene cluster in some *E. coli* strains.[11,12] Class IIb microcins are found in two forms, unmodified and post-translationally modified with a covalent linkage at the C-terminus to a glycosylated siderophore. Siderophores are small, ferric iron-binding molecules produced and consumed by many bacteria, including enterics.[13] During iron-depleted conditions characteristic of the mammalian gut, siderophore-microcins were shown to provide a competitive advantage to producing strains as they allow inhibition of other enterobacteria that occupy similar ecological niches.[12,14] The first characterized and arguably best studied class IIb microcin is microcin E492 (MccE492) from *K. pneumoniae* RYC49218,[15] which was shown to have inhibitory activity *in vitro* against a wide range of *Enterobacteriaceae* including *Klebsiella*, *Enterobacter*, *E. coli*, and *Salmonella*.[11] Similarly, microcin M (MccM) from *E. coli* was shown to inhibit *E. coli* and *Salmonella in vitro* and *in vivo*.[11,12] Both microcins were successfully purified via reversed-phase fractionation of culture supernatants.[11]

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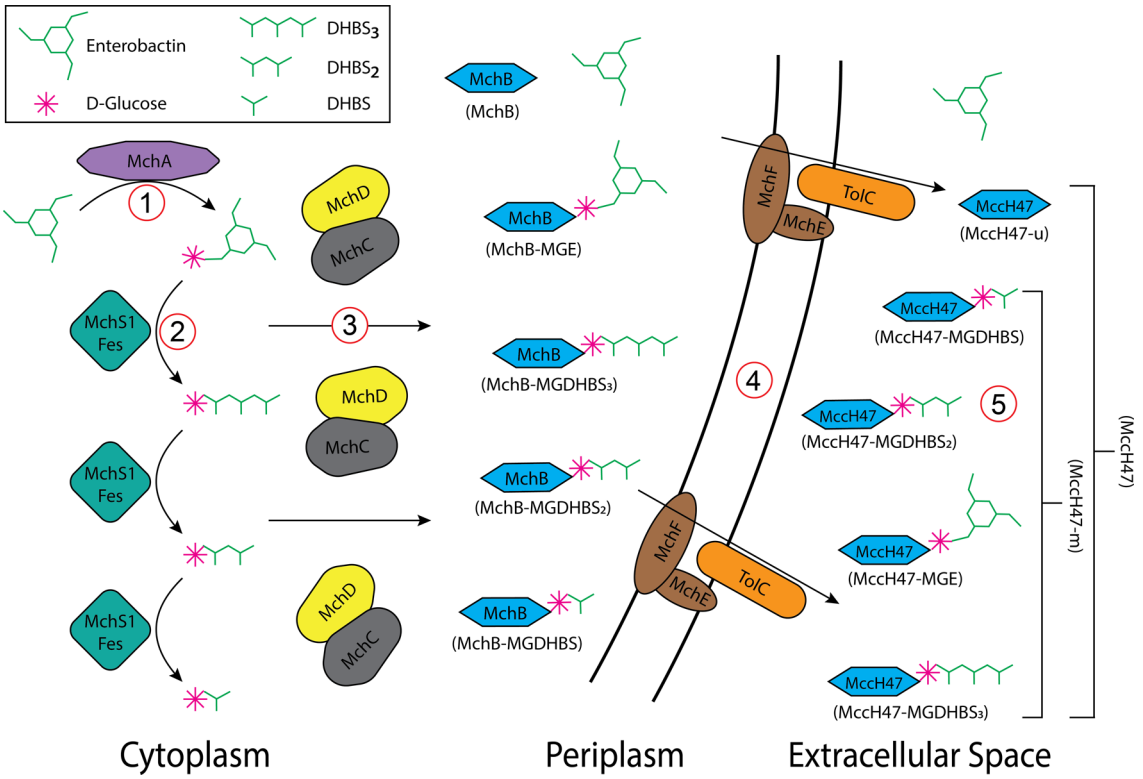

Figure 1. Overview of MccH47's biosynthetic pathway. (1) Enterobactin independently produced by the cell is glycosylated by MchA. (2) MchS1/Fes (and IroD, not pictured) linearize enterobactin and remove individual subunits of DHBS. (3) MGE/MGDHBS$_{3/2/1}$ is coupled via ester linkage to the C-terminal serine residue of MchB, a process catalyzed by MchCD. (4) MchB, with and without C-terminal post-translational modifications, is targeted for secretion by MchEF/TolC, creating an extracellular environment containing the mixture collectively referred to as "MccH47" (5).

MccH47 has gained significant interest recently, as it is produced by *E. coli* strain Nissle 1917 (*EcN*), a widely used engineered probiotic chassis strain, and has been heterologously expressed (from both *EcN* and other *E. coli* strains) in response to host-produced signals of dysbiotic conditions.[11,12,16] However, in contrast to MccE492 and MccM, MccH47 has been particularly difficult to purify, and most efforts to determine the inhibitory activity have utilized live-producing strains via a variety of methodologies.[14,16,17] This has resulted in conflicting reports of MccH47's efficacy in regards to the inhibitory activity of *Salmonella*, with some studies reporting inhibition[10,14,16] and others reporting no inhibition.[11,12] In 1990, Laviña et al. made the sole claim of MccH47 activity against additional members of the *Enterobacteriaceae* family,[10] yet no data were provided. Notably, MccH47 has never been purified to homogeneity,[1] and Vassiliadis et al. were unable to detect the inhibitory activity against targets other than *E. coli*.[11]

MccH47, like other class IIb microcins, is actually a mixture of compounds. The biosynthesis of MccH47 begins with the gene product of *mchB*, a 75-residue protein with a 15-residue N-terminal leader peptide and a serine-rich C-terminus (SASSSAGGGS). The C-terminal serine residue of MchB can be post-translationally modified (PTM) with a C-monoglycosylated (MG) linear enterobactin or enterobactin derivative, a process catalyzed by the activity of MchCD.[11,18,19] Enterobactin is a cyclic trimer of *N*-(2,3-dihydroxy-benzoyl)-serine (DHBS), and therefore, intracellular MchB with a C-terminal PTM of MG trimer, dimer, or monomer of DHBS will be denoted as MchB-MGDHBS$_3$, MchB-MGDHBS$_2$, and MchB-MGDHBS, respectively. The C-glycosylation of enterobactin is catalyzed by a glycosyltransferase, commonly MchA and/or IroB in microcinogenic strains, resulting in MG enterobactin (MGE).[20,21] MGE production is followed by conversion to MGDHBS$_{3/2/1}$ by the enterobactin esterases MchS1, IroD, and/or Fes, though conversion from enterobactin to DHBS$_{3/2/1}$ can occur prior to glycosylation.[20,22] Export of MchB and the PTM MchB forms is mediated by TolC and MchEF, which comprise an ABC-transporter and secretion (AMS) or peptidase-containing ATP-binding transport (PCAT) system with a high degree of similarity to CvaAB of the microcin V system.[23] The N-terminal, 15-residue leader peptide of MchB is cleaved during export, resulting in a 60-amino acid protein with or without C-terminal PTM, where the undefined mixture of such molecules will be collectively referred to as "MccH47". MccH47 lacking PTM (MccH47-u, where "-u" denotes the C-terminus is unmodified) is hypothesized to be unable to enter sensitive cells[20] yet is still readily secreted and detectable in the supernatant of a producing strain, as has been demonstrated in *EcN*.[11] Figure 1 provides an overview of the proposed MccH47 biosynthetic pathway and associated nomenclature. As we have defined "MccH47" as the mixture of secreted MccH47 forms with or without C-terminal PTM and "MccH47-u" as secreted MccH47 without C-terminal PTM, we also propose to refer to the subset of MccH47 with C-terminal PTM as "MccH47-m," where "-m" denotes that the C-terminus is modified. Other important gene products of the *mch* cluster include MchI, for immunity, MchS4, which increases enterobactin production, and MchX, which is believed to regulate its own production and the production of other downstream *mch* genes.[20,24,25]

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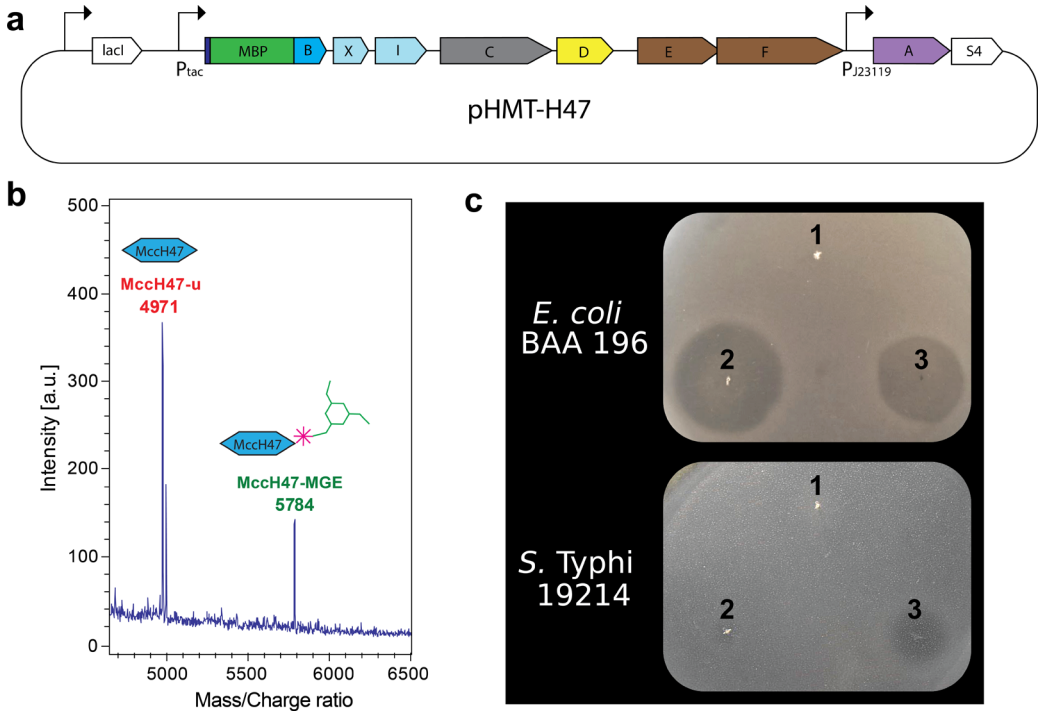

Figure 2. Construction of plasmid for overexpression and purification of MccH47. (a) Plasmid map of pHMT-H47, a pUC19-based plasmid that expresses a maltose binding protein (MBP)−MccH47-u fusion as well as the genes needed for post-translational modification. (b) MALDI-TOF analysis of purified MccH47 shows peaks for MccH47-u (red) and MccH47−MGE (green), with a monoisotopic mass difference of 813 Da. Monoisotopic masses for each species are 4948 Da (MccH47-u) and 5761 (MccH47−MGE), each observed here with a 23 m/z increase corresponding to a sodium adduct (see also Figure S2). An additional peak immediately adjacent to the primary labeled MccH47-u peak is a disodiated adduct. (c) Static inhibition assay comparing MccH47 overproduction in stabs and after purification against an ESBL-producing *E. coli* (top) or an MDR *S.* Typhi strain (bottom). (1) Stab of *E. coli* NEB10β harboring pUC19 (negative control), (2) stab of *E. coli* NEB10β harboring pS4BAD-H47, and (3) spot of ∼4 μg of MccH47 purified from *E. coli* NEB10β harboring pHMT-H47.

MccH47 is bactericidal, interacting with the F_o region of ATP synthase and allowing the unregulated influx of protons.[26] The uptake of MccH47-m is TonB dependent and mediated by the siderophore receptors Cir, Fiu, FepA, and IroN.[14,27] It is highly plausible, however, that any organism with siderophore receptors that can uptake enterobactin and/or its DHBS subunits is potentially susceptible to MccH47-m in iron limiting conditions. In fact, organisms as evolutionarily distant from *E. coli* as *Pseudomonas aeruginosa* have been demonstrated to import enterobactin conjugated with variable cargos (e.g., carboxylic acid) with relatively high promiscuity,[28] implying that variability in the susceptibility to MccH47-m among organisms capable of scavenging enterobactin/DHBS may be mostly dependent on F_o structure or some other feature not directly related to enterobactin/DHBS uptake.

Although MccH47 is a well-studied antimicrobial peptide with respect to its biosynthesis, secretion, mode of uptake, and mechanistic target, the variability in production methods has led to notable deficiencies regarding the spectrum of activity and potency. After attempting to purify MccH47 via reverse-phase chromatography and polyhistidine tags, which proved unsuccessful, we developed *E. coli* NEB10β pHMT-H47 (Figure 2a), expressing a maltose binding protein (MBP)−MccH47 fusion as well as a subset of eight genes from the *mch* cluster that were determined to be optimal for overexpression experiments (*mchACDXIEFS4*), on the basis of previously published literature.[17,20,24] The MBP−MccH47 fusion notably lacks the N-terminal leader peptide comprising the first 15 amino acids of MchB. The utilization of an MBP fusion for purification of an antimicrobial peptide (AMP) from *E. coli* is an established approach,[29,30] and we introduced a protease cleavage site for the Tobacco etch virus (TEV) protease between the MBP−MccH47 fusion to allow the release of MccH47 after MBP-mediated purification. Polyhistidine tags on MBP and TEV allowed for nickel agarose-based removal of these contaminants. Notably, the TEV recognition site utilized is 5′-ENLYFQS-3′, and TEV cleaves between the glutamine (Q) and serine (S), leaving an N-terminal serine as the first amino acid of the cleavage product, though this appeared to have a negligible impact regarding inhibitory activity.

MccH47 solutions were first analyzed and purified via polyacrylamide gel electrophoresis (PAGE) followed by mass spectrometry (MS) MALDI-TOF. PAGE analysis clearly reveals a band corresponding to MccH47, post digestion with TEV, that is absent in the undigested sample (Figure S1). MALDI-TOF analysis shows a monoisotopic peak at m/z = 4971, corresponding to the presence of the unmodified peptide MccH47-u (accounting for the additional serine residue; see above) as well as a second monoisotopic peak at m/z = 5784 (Figures 2b and S2), a difference in m/z of 813, which does not correspond to MccH47−MGDHBS$_{3/2/1}$ but instead corresponds to the monoglycosylated cyclic enterobactin form: MccH47−MGE. For both MccH47-u and MccH47−MGE, there is an observed increase of 23 m/z units caused by monosodiated adducts on the protein (Figure S2). Due to severe reduction in growth rate in strains overexpressing *mchS1*, we intentionally omitted the enterobactin esterase from the design of pHMT-H47. We hypothesized that Fes, a native enterobactin esterase, would linearize enterobactin and remove DHBS subunits for detectable levels of MccH47−

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$MGDHBS_{3/2/1}$. However, in pHMT-H47, *mchA*, the enterobactin glucosyltransferase, is constitutively expressed from a strong promoter, likely leading to elevated levels of MchA capable of rapidly glycosylating enterobactin. Cyclic, unglycosylated enterobactin is the preferred substrate of Fes,[22] and therefore, we hypothesize that the rapid glycosylation of cyclic enterobactin makes it immediately available for ester linkage to MccH47, making MccH47−MGE the only form of MccH47-m detectable in our experiments. Most interestingly, there appear to be no known instances of class IIb microcins which include a PTM of MGE, which serendipitously makes MccH47−MGE an entirely new form of MccH47. While our purified solution contains detectable levels of only MccH47-u and MccH47−MGE, because this remains a mixture of multiple MccH47 forms, we will continue to refer to this purified mixture as "MccH47."

Concurrent to PAGE and MS analysis, 7.5 μL (~4.0 μg) of MccH47 (see Figure 2b) were spotted onto LB agar plates, dried, and overlaid with the MDR *S.* Typhi strain 19214 (Figure 2c). A clear zone of inhibition is visible in the *S.* Typhi lawn corresponding to the MccH47 solution, demonstrating the first example of a purified form of MccH47 inhibiting *Salmonella*. Additionally, a second plasmid (pS4BAD-H47), containing all of the same genes as pHMT-H47, except lacking MBP, was developed for the overexpression and secretion of MccH47 in live-producing strains. It was stabbed into the agar medium, incubated for 36 h, and inactivated prior to the *S.* Typhi overlay, following previously established methods.[16,31] An additional static inhibitory assay was performed in the same manner utilizing an extended spectrum β-lactamase-producing *E. coli* (ESBL-*Ec*) strain BAA-196, which clearly demonstrates the inhibitory capability of *E. coli* NEB10β pS4BAD-H47. Comparing the effect of purified MccH47 with the active form produced from a strain growing in an agar stab, we clearly see a variable effect against the two targets. While ESBL-*Ec* is strongly inhibited by both and perhaps more so by the stabbed culture, *S.* Typhi appears to be more strongly inhibited by the purified form.

Utilizing the ability to purify MccH47 (see Figure 2b), we selected different representative members of *Enterobacteriaceae* for liquid minimum inhibitory concentration (MIC) assays and included several MDR strains of clinical relevance; the results are reported in Table 1. MccH47 activity demonstrated strong effects against all members of *E. coli*, *Shigella*, and *Salmonella* strains tested at concentrations lower than 75 μg/mL (13 μM) with no considerable difference between antibiotic-sensitive and MDR strains. Note that the reported MIC values are rather conservative due to the purification and the inclusion of MccH47-u, yet when comparing on a molar basis (Table 1), MccH47 potency is of the same magnitude as commonly used antibiotics.[32] No MIC was achieved against strains of *K. pneumoniae*, *K. oxytoca*, *Acinetobacter baumannii*, *P. aeruginosa*, *Staphylococcus aureus*, *Serratia marcescens*, or *Enterobacter cloacae*, even at concentrations as high as 650 μg/mL (113 μM) (Table S1). However, in liquid MIC assays, some minor growth retardation was observable for *K. oxytoca* and *K. pneumoniae* isolates, which led us to speculate that inhibitory assays on solid media may more clearly demonstrate the inhibitory activity. Spatially structured environments have been shown to impact bacterial toxin activity,[33] and indeed, halos of inhibition were observable against *K. pneumoniae* in aliquots containing as low as 1.75 μg of MccH47 (Figure S3). Interestingly, even though *A. baumanii* and *P. aeruginosa* are known siderophore scavengers, with the latter known to take up enterobactin linked to a wide variety of R-group cargos,[28,34] no alteration of growth pattern was observed in liquid MIC assays.

Table 1. Results of Minimum Inhibitory Concentration (MIC) Assays of Purified MccH47 against Multiple *Enterobacteriaceae* Species[a]

bacterial species	strain	MIC (μg/mL)	MIC (μM)
Escherichia coli	25922	30.8	5.3
Escherichia coli[b]	BAA-196	10.3	1.8
Escherichia coli	DH5α	6.3	1.1
Salmonella Typhimurium	19585	49.7	8.6
Salmonella Typhimurium	29630	36.6	6.3
Salmonella Typhimurium[b]	BAA-190	73.2	12.7
Salmonella Typhi[b]	19214	61.5	10.6
Salmonella Typhi	700931 (TY2)	52.3	9.0
Shigella flexneri	2457T	14.0	2.4
Shigella flexneri	M90T	25.2	4.4
Proteus mirabilis	29906	30.8	5.3

[a]Candidate members of *Klebsiella*, *Enterobacter*, *Staphylococcus*, *Acinetobacter*, and *Pseudomonas*, among others, were tested, but the MIC exceeded 650 μg/mL (113 μM) in each case (see Table S1). [b]Multidrug resistant, including carbapenemase, extended spectrum β-lactamase, and metallo-β-lactamase, producers.

To determine if inhibitory properties would depend exclusively on the interaction with different F_o subunits (A, B, C) of the ATP synthase of the target strains,[26] we built four phylogenetic trees (Figure S4) by aligning representative sequences of the target strains with respect to each F_o subunit and the marker gene 16S rRNA (see Methods). While subunit C does not show much variability between the different bacterial species (especially among *Enterobacteriaceae*), the subunits A and B roughly resemble the phylogenetic pattern of the 16S rRNA gene with one exception: While MccH47 susceptible *E. coli* and *Salmonella* are closely related with respect to F_o similarity, the other genus strongly inhibited by MccH47, *P. mirabilis*, is more distant than nonsusceptible strains including *E. cloacae*, *K. oxytoca*, and *S. marcescens*. This suggests that target affinity to ATP synthase is not the only factor determining MccH47−MGE susceptibility but other mechanisms, such as import through the siderophore receptors, may be involved.

It is difficult to determine the causative reason for the discrepancy across MccH47 literature and why MccH47 has been occasionally demonstrated to be ineffective against strains of *Salmonella* and even occasionally strains of *E. coli*. In Sassone-Corsi et al., the MccH47-producing strain is the wild-type *EcN*, whose chromosome contains *mchXIBCDEFS4* but lacks *mchAS1S2S3*.[12] Gaggero et al. demonstrated that MchA is essential for MccH47 production,[24] and while *EcN* lacks *mchA*, it does contain the *iroA* cluster, which is often found in uropathogenic *E. coli* and contains *iroB*, a glycosyltransferase homologous to *mchA* (69% aa identity).[24] While *EcN* theoretically contains all the necessary genes to make MccH47, it is likely that strictly defined environmental conditions play a significant role in determining MccH47 production. This is demonstrated by the apparently low-level MccH47 production by *EcN* as reported by Sassone-Corsi et al.,[12] compared to only MchB production and no detectable MccH47 from *EcN* as reported by Vassiliadis et al.[11] Additionally, MccH47 obtained from a recombinant *E. coli*

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strain with a plasmid containing *mchXIBACDEFS1S2S3S4* was able to inhibit strains of *E. coli* tested but not *Salmonella*, *Klebsiella*, or *Enterobacter*.[11] However, considering the MIC values reported here (Table 1), the lack of effect against *Salmonella* can most reasonably be explained as a concentration dependent effect. It is noteworthy that the MIC values reported here correspond to inhibition by the MccH47−MGE/MccH47-u mixture and that these values may vary if utilizing a different form or mixture of MccH47, as target organisms with variable siderophore receptor expression may import each variant at a different rate.

Regarding the capability of AMPs like MccH47 to be used as a new class of antibiotics, there are few points to consider. First, rather than systemic application of intravenous antibiotics or oral delivery of compounds intended for diffusion into the bloodstream, AMP's are proteins to be delivered to the point of infection: the mammalian gut. Sassone-Corsi et al. demonstrated the ability for *EcN* to release microcins within the physiological conditions of the murine gut, capable of reducing the growth of other *E. coli* and *Salmonella*.[12] This suggests the possibility of an *EcN* strain engineered to produce MccH47 in amounts comparable to those achieved in this work to decolonize undesirable enteric bacteria. Currently, engineered *EcN* is a pharmaceutical innovation on the leading edge of drug discovery, with engineered strains of *EcN* to treat phenylketonuria currently in human clinical trials.[35]

In this work, we clearly demonstrate the antimicrobial activity of MccH47 against multiple clinically relevant MDR *Enterobacteriaceae*. Additionally, we provide the first demonstration of a MBP−protein fusion to undergo post-translational covalent attachment to a glycosylated siderophore in the *E. coli* host, and we used this methodology to purify a novel form of MccH47 (MccH47−MGE). MccH47 has minimum inhibitory concentrations measuring <75 μg/mL (<13 μM) for all strains of *E. coli*, *Salmonella*, *Shigella*, and *Proteus* tested, with no measurable activity against any non-*Enterobacteriaceae* strains tested. We also show that MccH47 has an inhibitory effect on MDR *K. pneumoniae* in solid media assays, yet no measurable MIC was achieved in liquid assays, suggesting that structure-based environments may play a role in microcin susceptibility. Collectively, in this work, we establish MccH47−MGE as an inhibitory form of MccH47-m and have demonstrated a straightforward pipeline for the design, overproduction, and purification of other uncharacterized class IIb microcins. Moreover, we also demonstrate that MccH47 is a viable candidate for future *in vivo* challenge studies to determine its feasibility as a next generation antibiotic to achieve GI decolonization of MDR *Enterobacteriaceae*.

METHODS

Strains and Plasmids. Strains used in this study include *Escherichia coli* strain NEB10β (New England Biolabs, Ipswich, MA) and the strains listed in Table S1. All strains of Table S1 were purchased from ATCC (Manassas, VA). Plasmid constructs developed in this work were transformed by electroporation into *E. coli* NEB10β cells. All media and additional reagents listed in this study were purchased from Sigma-Aldrich, St. Louis, MO, unless otherwise indicated. Plasmids pHMT-H47 and pS4BAD-H47 were constructed using standard methods for Gibson Assembly[36] and the Gibson Assembly Master Mix (New England Biolabs. Ipswich, MA). To construct pHMT-H47, seven fragments were amplified by polymerase chain reaction (PCR)[37] and assembled in a single Gibson Assembly reaction, prior to transformation of *E. coli* NEB10β. Fragments include: (1) linearized pUC19, (2) chloramphenicol resistance cassette from pTARA (Addgene #39491[38]), (3) *lacI* and tac promoter from pMAL-c5X (New England Biolabs. Ipswich, MA), (4) MBP, amplified using primers to add a 6× Histidine N-terminal tag, from pMAL-c5X, (5) *mchB* from pEX2000,[24] (6) *mchXI* from pEX2000, and (7) *mchCDEFAS4* from pPP2000, an unpublished vector developed previously by combining *mchCDEF* from pEX2000, *mchA* from pJPMcH47,[16] and *mchS4* from pEX2000. To construct pS4BAD-H47, three fragments were amplified by PCR and assembled via Gibson Assembly. Fragments include: (1) linearized pUC19, (2) *araC* and P_{BAD} from pTARA (Addgene #39491[38]), and (3) *mchXIBCDEFAS4* from pPP2000. DNA files for the plasmids constructed in this work have been uploaded in Zenodo (DOI: 10.5281/zenodo.3483827) and can be opened with the free visualization software SnapGene Viewer (https://www.snapgene.com/snapgene-viewer/).

Inhibition Assays. Solid media inhibition assays were carried out in a manner similar to those described in previous work.[16,31] First, single colonies of MccH47-producing strains were selected by pipet tip and stabbed into iron-limited LB agar supplemented with 0.2 mM 2,2′-dipyridyl, an iron-chelating agent, and 0.4% L-arabinose to induce MccH47 production. Colonies were incubated for ∼36 h to allow for extended production of MccH47 and inactivated by chloroform. Then 7.5 μL (approximately 4 μg) of the purified MccH47 solution was spotted and allowed to dry, and the plate was placed under ultraviolet light for 10 min. Target strains were then diluted 1:500 from an overnight culture in 3 mL of LB with 0.2 mM 2,2′-dipyridyl; molten agar was added to a final concentration of 0.75%, and 3.5 mL of the inoculated soft agar medium was immediately overlaid and evenly spread on top of the MccH47 containing plate.

MccH47 Purification. The MBP−MccH47 was expressed and purified utilizing standard methods. Briefly, cultures of *E. coli* NEB10β pHMT-H47 were grown in 2 L of LB broth, under antibiotic selection (ampicillin and chloramphenicol), and in iron-limiting conditions to maximize enterobactin production, via the addition of 0.2 mM 2′2-dipyridyl, and induced with 0.5 mM IPTG when cultures reached an optical density at 600 nm (OD_{600}) of approximately 0.2. Cultures were grown for an additional 5−7 h postinduction, then pelleted, and frozen overnight at −20 °C. Cultures were then thawed in cold water and sonicated, and the crude lysate was passed through an amylose resin (New England Biolabs, Ipswich, MA) column to capture the MBP fusion proteins and then finally eluted with maltose. Elution was performed by adding the elution buffer (200 mM NaCl, 20 mM Tris-HCl, 10 mM maltose; pH 7.5), discarding the first 5 mL (∼8 mL of amylose resin was used), and then capturing the next 30 mL of eluent. The eluent was then concentrated using MilliporeSigma (Burlington, MA) MWCO 10 000 filters. The concentrated MBP−MccH47 was then digested by the addition of 10 μL of Tobacco etch virus nuclear-inclusion-a endopeptidase (TEV) (New England Biolabs, Ipswich, MA) and incubated overnight at 4 °C. The following day, the digestion was brought to room temperature; an additional 5 μL of TEV was added, and the digestion was allowed to incubate an additional 1−2 h, yielding a buffered solution of MccH47, TEV, and MBP. This solution was then further purified by subsequent rounds of resuspension with Ni-NTA agarose resin (Qiagen, Hilden,

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DE), as both TEV and MBP contained 6× Histidine tags. Specifically, Ni-NTA agarose was resuspended and washed in elution buffer and added to the concentrated MBP + MccH47 + TEV solution in a 1:2 volumetric ratio (i.e., 125 μL of slurry to 250 μL of digestion reaction). Ni-NTA slurry was pelleted by centrifugation, and the purified MccH47 in the supernatant was carefully removed by pipetting. This process was then repeated with fresh slurry, and the final MccH47 solution was quantified via Qubit fluorometric quantitation.

Minimum Inhibitory Concentration (MIC) Assays. MIC assays were performed by preparing two simple and robust media types: (i) 2× LB with 0.4 mM 2,2′-dipyridyl and (ii) 1× LB, 0.2 mM 2,2′-dipyridyl, and 0.5× amylose resin elution buffer (200 mM NaCl, 20 mM Tris-HCl, 10 mM maltose; pH ∼ 7.5). The first well of each MIC assay was loaded with 20 μL of 2× LB/0.4 mM 2,2′-dipyridyl and then with 20 μL of Ni-NTA purified amylose resin eluent, carrying the purified MccH47. This effectively rendered the first well with a media composition of 1× LB, 0.2 mM 2,2′-dipyridyl, 0.5× amylose resin elution buffer, containing the maximum MccH47 concentration for that particular MIC assay. The remaining wells were loaded with 20 μL of the second media solution, 1× LB, 0.2 mM 2,2′-dipyridyl, and 0.5× amylose resin elution buffer, and then 2-fold serial dilutions were conducted eight times. Cultures of target strains were grown overnight in LB with shaking at 37 °C to stationary phase and then diluted 10 000-fold for inoculation into each well individually for the MIC assay. MIC assay plates were incubated at room temperature with gentle agitation, and MICs were determined as the lowest concentration for which no observable growth could be seen after 24 h. All samples reported were done in at least triplicate, using at least three different MccH47 purifications. The median value of all assays was used as the reported MIC value.

Mass Spectrometry. For MccH47 to be analyzed via mass spectrometry, samples were purified, as described above, except that buffer replacement with deionized water (pH = 8.0) in MWCO 10 000 filters was immediately conducted after the initial MWCO 10 000 filter step. TEV digestion and Ni-NTA resin purification were performed in deionized water (pH = 8.0), and the resulting solution was processed at the Mass Spectrometry Core Facility at the University of Massachusetts Amherst. Mass spectra were acquired using a Bruker ultrafleXtreme MALDI-TOF mass spectrometer. A mixed matrix approach was found to yield the best overall signal. Matrices were prepared as follows: 15 mg/mL 2,5-dihydroxybenzoic acid in methanol, ∼10 mg/mL α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, and 0.1% trifluoroacetic acid in water. Five μL of 0.5 mg/mL protein of interest (POI) solution in water was mixed with 5 μL of each matrix solution, and after brief vortexing, 1 μL of the mixture was spotted on the MALDI target and allowed to dry at room temperature. Spectra were obtained using reflectron positive ion mode using sufficient laser fluency and shot number to obtain an acceptable signal/noise ratio.

Phylogenetic Analysis. For phylogenetic analysis, the nearly full length 16S rRNA sequence for the depicted strains was obtained from public databases; a sequence alignment was generated using MEGA X,[39] and all samples were reduced to the length of the shortest aligned sequence. For the ATP synthase F_o subunits A, B, and C, obtained amino acid sequences were aligned in MEGA X and utilized for tree building. A model test was used to estimate the best-fit substitution models for maximum likelihood phylogenetic analyses.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.9b00302.

Figure S1: polyacrylamide gel electrophoresis resulting from the overproduction of *E. coli* NEB10β pHMT-H47 and purification of MccH47−MGE; Figure S2: MALDI-TOF analysis of purified MccH47 with isotopic resolution; Figure S3: static inhibitory assay utilizing serial dilutions of MccH47−MGE against *K. pneumoniae*; Figure S4: phylogenetic analysis of the 16S rRNA and the ATP synthase F_o subunits of the tested bacterial species; Table S1: strains of bacteria tested for susceptibility against MccH47−MGE (PDF)

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Author Contributions

J.D.P., E.P., and V.B. discussed the initial project idea. J.D.P., B.M.M., and E.P. developed and performed the experimental work. J.D.P., B.M.M., and V.B. interpreted the data. J.D.P. and V.B. wrote the paper. M.W.S. provided microbiological insight and guidelines on strain engineering. B.A.M. helped with the inhibition experiments with *S.* Typhi strains. All the authors commented and approved the paper.

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Notes

The authors declare no competing financial interest.

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