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Microcin PDI Inhibits Antibiotic-Resistant Strains of *Escherichia coli* and *Shigella* through a Mechanism of Membrane Disruption and Protection by Homotrimer Self-Immunity

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ABSTRACT Microcin PDI (MccPDI), a class IIa microcin that is produced by *Escherichia coli* strains 25 and 284, is known to inhibit foodborne pathogenic enterohemorrhagic *E. coli* serotypes O157:H7 and O26. Here we demonstrate that MccPDI can inhibit *Shigella* strains and *E. coli* isolates that are multidrug resistant, the latter including strains known to cause urinary tract infections in people and companion animals. Two exceptions out of 17 strains were identified. One of the two resistant *E. coli* isolates (AR0349) has a mutation in a critical amino acid residue that was identified in previous work as a requisite for the MccPDI precursor protein (McpM) to interact with outer membrane porin F (OmpF) on susceptible cells. The second resistant *E. coli* strain (MAD 96) had no mutations in *ompF*, but it was PCR positive for two antimicrobial peptides, of which colicin Ia/Ib likely inhibits the MccPDI-producing strain during coculture. Recombinant McpM was still effective against strain MAD 96. In an assessment of how MccPDI affects susceptible strains, results from both an extracellular ATP assay and a nucleic acid staining assay were consistent with membrane damage, while the addition of 200- to 600-Da polyethylene glycol (PEG) to cocultures protected against MccPDI (>600-Da PEG did not provide protection). Further studies using a paraformaldehyde cross-linking experiment and a bacterial two-hybrid assay demonstrated that MccPDI immunity protein (McpI) forms a multimeric complex with itself and presumably protects the producer strain from within the periplasm through an unknown mechanism.

IMPORTANCE Microcins represent potential alternatives to conventional antibiotics for human and veterinary medicine. For them to be applied in this manner, however, we need to better understand their spectrum of activity, how these proteins interact with susceptible cells, and how producer cells are protected against the antimicrobial properties of the microcins. For microcin PDI (MccPDI), we report that the spectrum of activity likely includes most *E. coli* strains due to a conserved binding motif found on an outer membrane protein. *Shigella* has this motif as well and is susceptible to MccPDI killing via damage to the bacterial membrane. Receptor specificity suggests that these proteins could be used without causing large-scale disruptions to a microbiota, but this also increases the likelihood that resistance can evolve via random mutations. As with conventional antibiotics, good stewardship will be needed to preserve the efficacy of microcins should they be deployed for clinical use.

KEYWORDS microcin PDI, multidrug resistant, UTI, urinary tract infection

Microcins are antimicrobial peptides (<10 kDa) that are produced by Gram-negative bacteria predominantly from the family *Enterobacteriaceae* (1–3). The recently described microcin PDI (MccPDI), a class IIa microcin (4) produced by *Esche-*

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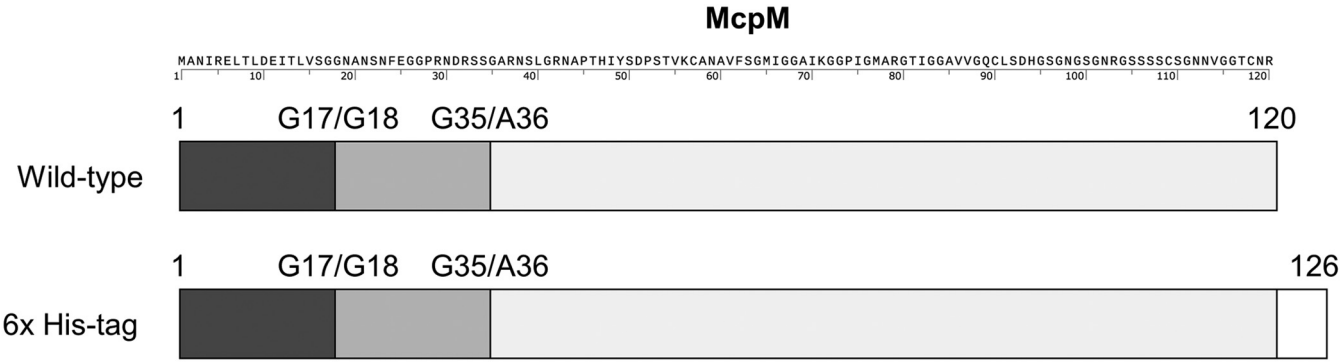

FIG 1 Full-length amino acid sequence of McpM (GenBank accession number JQ901381). The MccPDI precursor protein, McpM, consists of 120 amino acids. This graphical representation shows McpM and McpM with a 6×His tag (white) and the two signal peptides G17/G18 (dark gray) and G35/A36 (medium gray) where the precursor protein is cleaved during excretion to form the mature peptide (light gray) (7). The excreted protein has an approximate mass of 8.2 kDa and a theoretical pI of 9.58.

richia coli strains 25 and 284, inhibits growth of foodborne pathogenic *E. coli* strains, including enterohemorrhagic *E. coli* serotypes O157:H7 and O26 (5). The name microcin "PDI" was given due to the apparent need for the producing bacteria to be in close proximity to inhibit target bacteria (i.e., proximity-dependent inhibition) (6). The reason why proximity is required is unknown, although only minute quantities of the native protein appear to be secreted, and the proximity requirement may be a consequence of simple concentration dependence. The MccPDI system is plasmid encoded, consisting of five genes: *mcpM* (precursor protein), *mcpI* (self-immunity protein), *mcpA* (putative repressor protein with CaaX protease activity [5]), *mcpB* (export protein B), and *mcpD* (export protein D). Export proteins B and D comprise a type I secretion system (T1SS) that functions with TolC (an outer membrane protein) to secrete McpM (5). The excretion of McpM is accompanied by the cleavage of two signal sequences, leaving an 8-kDa mature peptide (Fig. 1), originally described by Zhao et al. (7). *E. coli* strains are susceptible to MccPDI when outer membrane porin F (OmpF) is present, which was demonstrated in part by heterologous-expression experiments (8). Previous site-directed mutagenesis experiments identified an amino acid motif in extracellular loop 1 of OmpF ($K_{47}G_{48}N_{49}$) that is required for inhibition of susceptible bacteria (8). This motif is present in multiple reported OmpF sequences from both *E. coli* and *Shigella* strains (see Fig. S1 in the supplemental material).

Microcins are being investigated as alternatives to medically important antibiotics (3, 9, 10). According to the U.S. Centers for Diseases Control and Prevention (CDC) and the World Health Organization (WHO), the increasing prevalence of multidrug-resistant (MDR) pathogens limits successful clinical outcomes for both people and animals (11–15). Antimicrobial resistance (AMR) is a significant global challenge for both community-acquired (16, 17) and hospital-acquired (18) infections. *E. coli* strains causing urinary tract infections (UTI) are a particularly challenging problem, with a pandemic of a multilocus sequence type 131 (ST131) strain afflicting people from both high- and lower-income countries (17, 19–21).

Microcins might be a versatile alternative antibiotic for these infections. These low-mass proteins (<10 kDa), of which fewer than 20 have been described (8, 22), appear to be stable and functional under a wide range of pH and ionic conditions (23). Microcins are typically highly specific for conspecific bacteria, making it possible to target specific pathogens causing UTI, pulmonary infections, and septicemia without harming "bystander" bacteria. The present work demonstrates that MccPDI can kill a diversity of bacteria, including multidrug-resistant *E. coli* strains from urinary infections and *Shigella* strains. We report two cases whereby UTI strains (Table 1) were resistant to MccPDI when cultured with *E. coli* strain 25 (Table 1), with the probable mechanisms of resistance involving mutation of a key amino acid residue in the OmpF protein and

TABLE 1 Bacterial strains used in this study

Strain	Relevant genotype or phenotype[a]	Reference and/or source[b]
Escherichia coli		
25	Wild type; SSuT^r PDI$^+$	5
25 Δ*mcpM* Δ*mcpI*	SSuT^r PDI$^-$; *mcpM* and *mcpI* deletion	7
25 Δ*mcpM* Δ*mcpI*/pMMB207	SSuT^r PDI$^-$; *mcpM* and *mcpI* deletion with pMMB207 vector (no-insert control)	This study
25 Δ*mcpM* Δ*mcpI*::pMMB207::*mcpM*	SSuT^r Chl^r PDI$^-$; *mcpM* and *mcpI* deletion complemented with *mcpM*	This study
25 Δ*mcpM*::pMMB207::*mcpM*	SSuT^r Chl^r PDI$^-$; *mcpM* deletion complemented with *mcpM*	7
25 Δ*mcpM* Δ*mcpI*/pFPV25.1::gfpmut3	SSuT^r Amp^r PDI$^-$; *mcpM* and *mcpI* deletion complemented with GFPmut3	This study
25 Δ*traM*	SSuT^r Kan^r PDI$^+$; *traM* deletion	5
25 Δ*traM*/pFPV25.1::gfpmut3	SSuT^r Kan^r Amp^r PDI$^+$; *traM* deletion with pFPV25.1 complemented with GFPmut3	This study
BW25113	Nal^r; Keio collection wild-type K-12 strain	65
BW25113 Δ*ompF*	Kan^r; Keio collection; *ompF* deletion	65
BW25113/pMMB207	Nal^r Chl^r; BW25113 with pMMB207 vector (no-insert control)	This study
BW25113/pMMB207::*mcpI*	Nal^r Chl^r; BW25113 with pMMB207 complemented with *mcpI*	This study
BW25113/pFPV25.1::tdTomato	Nal^r Amp^r; BW25113 with pFPV25.1 complemented with tdTomato	This study
PAP222	Wild type; Nal^r microcin V positive	66
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	Thermo Fisher
DH10B/pCR2.1::$p_{mic\text{-}210/0}$*mcpMIADB*	Amp^r; DH10B with pCR2.1 complemented with the native promoter −210 and PDI operon (4.9 kb)	This study
DHM1	Nal^r; F$^-$ *cya-854 recA1 endA1 gyrA96 thi1 hsdR17 spoT1 rfbD1 glnV44*(AS)	Euromedex
DHM1/pUT18/pKNT25	Nal^r Amp^r Kan^r; negative control with vectors pUT18 and pKNT25	This study
DHM1/pUT18::*mcpI*/pKNT25::*mcpI*	Nal^r Amp^r Kan^r; complemented with *mcpI*	This study
DHM1/pUT18::*mcpI*/pKNT25::*mcpM*Δ36	Nal^r Amp^r Kan^r; complemented with *mcpI* and *mcpM*Δ36	This study
DHM1/pUT18::*mcpI*/pKNT25::*ompF*	Nal^r Amp^r Kan^r; complemented with *mcpI* and *ompF*	This study
DHM1/pUT18C/pKT25	Nal^r Amp^r Kan^r; negative control with vectors pUT18C and pKT25	This study
DHM1/pUT18C::*mcpI*/pKT25::*mcpI*	Nal^r Amp^r Kan^r; complemented with *mcpI*	This study
DHM1/pUT18C::*mcpI*/pKT25::*mcpM*Δ36	Nal^r Amp^r Kan^r; complemented with *mcpI* and *mcpM*Δ36	This study
DHM1/pUT18C::*mcpI*/pKT25::*ompF*	Nal^r Amp^r Kan^r; complemented with *mcpI* and *ompF*	This study
DHM1/pUT18::*mcpM*Δ36/pKNT25::*mcpI*	Nal^r Amp^r Kan^r; complemented with *mcpM*Δ36 and *mcpI*	This study
DHM1/pUT18::*mcpM*Δ36/pKNT25::*mcpM*Δ36	Nal^r Amp^r Kan^r; complemented with *mcpM*Δ36	This study
DHM1/pUT18::*mcpM*Δ36/pKNT25::*ompF*	Nal^r Amp^r Kan^r; complemented with *mcpM*Δ36 and *ompF*	This study
DHM1/pUT18C::*mcpM*Δ36/pKT25::*mcpM*Δ36	Nal^r Amp^r Kan^r; complemented with *mcpM*Δ36 and *mcpI*	This study
DHM1/pUT18C::*mcpM*Δ36/pKT25::*mcpI*	Nal^r Amp^r Kan^r; complemented with *mcpM*Δ36 and *mcpI*	This study
DHM1/pUT18C::*mcpM*Δ36/pKT25::*ompF*	Nal^r Amp^r Kan^r; complemented with *mcpM*Δ36 and *ompF*	This study
DHM1/pUT18::*ompF*/pKNT25::*mcpI*	Nal^r Amp^r Kan^r; complemented with *ompF* and *mcpI*	This study
DHM1/pUT18::*ompF*/pKNT25::*mcpM*Δ36	Nal^r Amp^r Kan^r; complemented with *ompF* and *mcpM*Δ36	This study
DHM1/pUT18::*ompF*/pKNT25::*ompF*	Nal^r Amp^r Kan^r; complemented with *ompF*	This study
DHM1/pUT18C::*ompF*/pKT25::*mcpI*	Nal^r Amp^r Kan^r; complemented with *ompF* and *mcpI*	This study
DHM1/pUT18C::*ompF*/pKT25::*mcpM*Δ36	Nal^r Amp^r Kan^r; complemented with *ompF* and *mcpM*Δ36	This study
DHM1/pUT18C::*ompF*/pKT25::*ompF*	Nal^r Amp^r Kan^r; complemented with *ompF*	This study
DHM1/pUT18C::Zip/pKT25::Zip	Nal^r Amp^r Kan^r; positive control with vectors pUT18C::Zip and pKT25::Zip	Euromedex
K-12	Nal^r	6
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*(F′ *proAB lacI*^{q}ZΔM15 Tn*10* [Tet^r])	Agilent
XL1-Blue/pUT18::*mcpI*	Tet^r Amp^r; complemented with *mcpI* in vector pUT18	This study
XL1-Blue/pUT18::*mcpM*Δ36	Tet^r Amp^r; complemented with *mcpM*Δ36 in vector pUT18	This study
XL1-Blue/pUT18::*ompF*	Tet^r Amp^r; complemented with *ompF* in vector pUT18	This study
XL1-Blue/pUT18C	Tet^r Amp^r; with vector pUT18C	This study
XL1-Blue/pUT18C::*mcpI*	Tet^r Amp^r; complemented with *mcpI* in vector pUT18C	This study
XL1-Blue/pUT18C::*mcpM*Δ36	Tet^r Amp^r; complemented with *mcpM*Δ36 in vector pUT18C	This study
XL1-Blue/pUT18C::*ompF*	Tet^r Amp^r; complemented with *ompF* in vector pUT18C	This study
XL1-Blue/pUT18C::Zip	Tet^r Amp^r; positive-control vector pUT18C with leucine zipper of GCN4	This study
XL1-Blue/pKT25	Tet^r Kan^r; with vector pKT25	This study
XL1-Blue/pKT25::*mcpI*	Tet^r Kan^r; complemented with *mcpI* in vector pKT25	This study
XL1-Blue/pKT25::*mcpM*Δ36	Tet^r Kan^r; complemented with *mcpM*Δ36 in vector pKT25	This study
XL1-Blue/pKT25::*ompF*	Tet^r Kan^r; complemented with *ompF* in vector pKT25	This study
XL1-Blue/pKT25::Zip	Tet^r Kan^r; positive-control vector pKT25 with leucine zipper of GCN4	This study
XL1-Blue/pKNT25	Tet^r Kan^r; with vector pKNT25	This study
XL1-Blue/pKNT25::*mcpI*	Tet^r Kan^r; complemented with *mcpI* in vector pKNT25	This study
XL1-Blue/pKNT25::*mcpM*Δ36	Tet^r Kan^r; complemented with *mcpM*Δ36 in vector pKNT25	This study
XL1-Blue/pKNT25::*ompF*	Tet^r Kan^r; complemented with *ompF* in vector pKNT25	This study
Escherichia coli (urinary tract isolates)		
AR0346	Wild type	CDC
AR0349	Wild type	CDC
MAD 92	Wild type	This study, WADDL
MAD 95	Wild type	This study, WADDL
MAD 96	Wild type	This study, WADDL
MAD 98	Wild type	This study, WADDL
MAD 99	Wild type	This study, WADDL
MAD 101	Wild type	This study, WADDL
MAD 102	Wild type	This study, WADDL
MAD 107	Wild type	This study, WADDL
MAD 111	Wild type	This study, WADDL

(Continued on next page)

TABLE 1 (Continued)

Strain	Relevant genotype or phenotype[a]	Reference and/or source[b]
Shigella		
S. flexneri 24570	Wild type	BEI Resources
S. flexneri 2457T	Wild type	BEI Resources
S. sonnei WRAIR I virulent	Wild type	BEI Resources
S. dysenteriae Newcastle 1934	Wild type	BEI Resources
Shigella sp.	Wild type	BEI Resources

[a]Amp^r, ampicillin resistant; Chl^r, chloramphenicol resistant; Kan^r, kanamycin resistant; Nal^r, nalidixic acid resistant; SSuT^r, resistant to streptomycin, sulfonamide, and tetracycline antibiotics.

[b]*E. coli* isolates AR0346 and AR0349 were obtained from the CDC and FDA antibiotic resistance isolate bank. WADDL, Washington Animal Disease Diagnostic Lab; BEI Resources, Biodefense and Emerging Infections Research Resources Repository.

production of a colicin that likely kills the MccPDI-producing strain before it can affect the susceptible strain.

Due to technical challenges with obtaining sufficient quantities of recombinant McpM for our experiments, we employed three different methods to evaluate the activity of MccPDI. The inhibition activity of MccPDI was observed by coculturing a wild-type MccPDI producer strain (*E. coli* 25) *in vitro* with target bacteria and quantifying changes in the number of target cells over time. This methodology demonstrated that MccPDI affects a wide diversity of *E. coli* and *Shigella* strains, and it was used to determine that MccPDI kills susceptible bacteria via membrane damage. Using two different recombinant vectors, we were able to generate sufficient recombinant McpM to verify the bactericidal phenotype of MccPDI using spot assays on agar plates. Finally, we further characterized how the immunity protein (McpI) protects the MccPDI-producing strains. *E. coli* strain 25 is "immune" to the effects of McpM because of concurrent synthesis of a "self-immunity" protein (McpI) that forms a multimeric structure to protect the PDI producer strain via an unknown mechanism.

RESULTS

Microcin PDI inhibits *Shigella* strains and UTI *E. coli* strains. Previously validated coculture competition assays (5–8, 24) were employed to examine the sensitivity of the target bacteria to MccPDI. The coculture competition assays were conducted with *E. coli* strain 25 (MccPDI producer), six *Shigella* strains, and nine UTI *E. coli* strains that were resistant to between one and four classes of antibiotics (Table 2 and Fig. 2). Cell counts (CFU) were then determined by plating on agar with corresponding antibiotic selection. Coculture with strain BW25113 (positive control; MccPDI susceptible) resulted in a 5.2-log CFU reduction relative to monoculture, while coculture with the BW25113 Δ*ompF* strain (negative control; MccPDI nonsusceptible) resulted in a 0.64-log CFU reduction. Eight of the 10 tested UTI strains were inhibited between 3.5 and 8.5 log CFU relative to monoculture results, while *Shigella* strains were reduced between 5.0 and 8.8 log CFU (Fig. 2A and B; see also Fig. S2A and B in the supplemental material). Two strains, AR0349 and MAD 96, were not inhibited. A reversed coculture competition assay conducted to measure the survival of the MccPDI producer (*E. coli* 25) against all 12 tested *E. coli* strains showed that only MAD 96 was capable of inhibiting the producer (Fig. S3). Bacteriocin typing by PCR indicated that *E. coli* strain MAD 96 harbors genes for two bacteriocins (colicin Ia/Ib [Col Ia/Ib] and microcin V) (Fig. S4) that could inhibit *E. coli* strain 25, thereby blocking the MccPDI phenotype. We were able to obtain a strain of *E. coli* that produces only microcin V (strain PAP222) (Table 1) and demonstrated that this microcin does not inhibit *E. coli* strain 25 (Fig. S5). Presumably, colicin Ia/Ib is responsible for protecting MAD 96 by inhibiting growth of *E. coli* strain 25.

By spotting recombinant McpM (Fig. S6) onto agar plates that were prespread with target bacteria, we were able to confirm the bactericidal activity of McpM (Fig. S7). Interestingly, this was possible even when a key surface outer membrane protein, OmpF, was absent (Fig. S7). Spotted recombinant McpM also inhibited MAD 96 (Fig. S8). Spot assays with *E. coli* DH10B/pCR2.1::$p_{mic\text{-}210/0}$*mcpMIADB* (vector construct containing the native *mcpM* promoter and all five MccPDI genes) confirmed the competition assay

TABLE 2 Antibiotic profiles of bacteria used in this study[b]

Strain	Resistance profile[a]	Sample	Species
Escherichia coli			
MAD 92	AmcAmpXnl	Urine	Canine
MAD 95	AmcAmpFoxCpdCefEnoGenMarTimSxt	Urine	Canine
MAD 96	AmcAmpCefFoxCpdXnlPenTic	Urine	Canine
MAD 98	AmcAmpLexXnlEno	Urine	Canine
MAD 99	AmcAmpVecFoxCpdXnlCliEryOxaPenTic	Urine	Canine
MAD 102	AmpEno	Urine	Canine
MAD 107	AmcAmpCpdPen	Urine	Canine
MAD 111	AmcAmpVecCpdXnlChlDoxEnoGenMarPenTicTimSxt	Urine	Canine
AR0346	AmkAmpSamAtmCfzFepCtxFoxCipCstGenLvxTetTobSxt	NA	Human
AR0349	AmkAmpSamAtmCfzFepCtxFoxCipCstGenbLvxTetTobSxt	NA	Human
25	StrSulTet	Fecal	Bovine
BW25113	Nal	NA	NA
BW25113 Δ*ompF*	Kan	NA	NA
Shigella			
S. flexneri 2457T	Nal	NA	NA
S. flexneri 24570	Nal	NA	NA
S. sonnei WRAIR I virulent	Nal	NA	NA
S. dysenteriae Newcastle 1934	Nal	NA	NA
Shigella sp.	Nal	NA	NA

[a]Amc, amoxicillin-clavulanic acid; Amk, amikacin; Amp, ampicillin; Atm, aztreonam; Cef, cephalothin; Cfz, cefazolin; Chl, chloramphenicol; Cip, ciprofloxacin; Cli, clindamycin; Cpd, cefpodoxime; Cst, colistin; Ctx, cefotaxime; Dox, doxycycline; Eno, enrofloxacin; Ery, erythromycin; Fep, cefepime; Fox, cefoxitin; Gen, gentamicin; Kan, kanamycin; Lex, cephalexin; Lvx, levofloxacin; Mar, marbofloxacin; Nal, nalidixic acid; Oxa, oxacillin; Pen, penicillin; Sam, ampicillin-sulbactam; Str, streptomycin; Sul, sulfonamide; Sxt, trimethoprim-sulfamethoxazole; Tet, tetracycline; Tic, ticarcillin; Tim, ticarcillin-clavulanic acid; Tob, tobramycin; Vec, cefovecin; Xnl, ceftiofur. All resistance was determined by the MIC (Sensititre assays) (Washington Animal Disease Diagnostic Laboratory), with the exception of drug resistance of strains MAD 98, MAD 99, MAD 107, and MAD 111, which was determined by a disc diffusion assay. Resistance of *E. coli* strains AR0346 and AR0349 was determined by the MIC (Sensititre assays) (Centers for Disease Control and Prevention). Strains were considered intermediate at a typical concentration of 8 μg/ml.
[b]NA, information not available.

results, whereby zones of clearance were clearly evident where this strain was "spotted" onto the lawn of susceptible bacteria (Fig. S8). The zone of clearance in these assays indicates that even the MccPDI-producing strain succumbs to McpM, presumably due to the presence of an abundance of McpM that cannot be fully counteracted by the McpI immunity protein in this assay format. It is also possible that a bacteriocin-producing strain such as MAD 96 could produce a false-positive result with the spot assay by killing DH10B/pCR2.1::$p_{mic\text{-}210/0}$*mcpMIADB*, thereby leaving a zone of clearance. In this case, it appears that MAD 96 not only kills the MccPDI strain but also grows in the same location, which prevents a false-positive finding (Fig. S9).

OmpF mutations. Previous work demonstrated that *E. coli* OmpF is required for MccPDI to inhibit susceptible bacteria (8). An amino acid sequence alignment was used to compare OmpF from *E. coli* K-12 to OmpF sequences of different *Shigella* strains and *E. coli* AR0346, AR0349, and MAD 96 (Table 3). The *E. coli* AR0349 (GenBank accession no. MH665273) sequence differed from those of *E. coli* strains K-12 MG1655 and BW25113, *Shigella flexneri* 2a 2457T, *S. sonnei* 53G, *S. boydii* CDC3083-94, and *S. dysenteriae* Sd197 in multiple locations within extracellular loops 1, 2, 3, 4, 5, and 6 (loop identity is based on the OmpF crystal structure [25]) (Fig. S1), including mutations to a critical OmpF motif in extracellular loop 1 that is necessary for susceptibility to MccPDI (8). Mutations were not detected in loop 7 and loop 8. In contrast to the significant amino acid sequence differences in OmpF from strain AR0349, the amino acid sequences from several other strains, such as *Shigella flexneri* strain 2a 2457T and *S. sonnei* 53G, were identical to the conserved *E. coli* sequence within the extracellular loops.

Polyethylene glycol protects a susceptible *E. coli* strain from MccPDI. To determine if MccPDI functions by damaging the membrane of susceptible *E. coli* strains, we first conducted coculture assays in the presence of different mass fractions of polyethylene glycol (PEG) (200 to 8,000 Da). For these experiments, PEG is thought to act as an "osmoprotectant" (26) that blocks the MccPDI phenotype if the effector protein McpM permeabilizes the membrane. When susceptible *E. coli* strain BW25113 was cocultured with wild-type MccPDI producer *E. coli* strain 25, there was a typical 5.6-log CFU

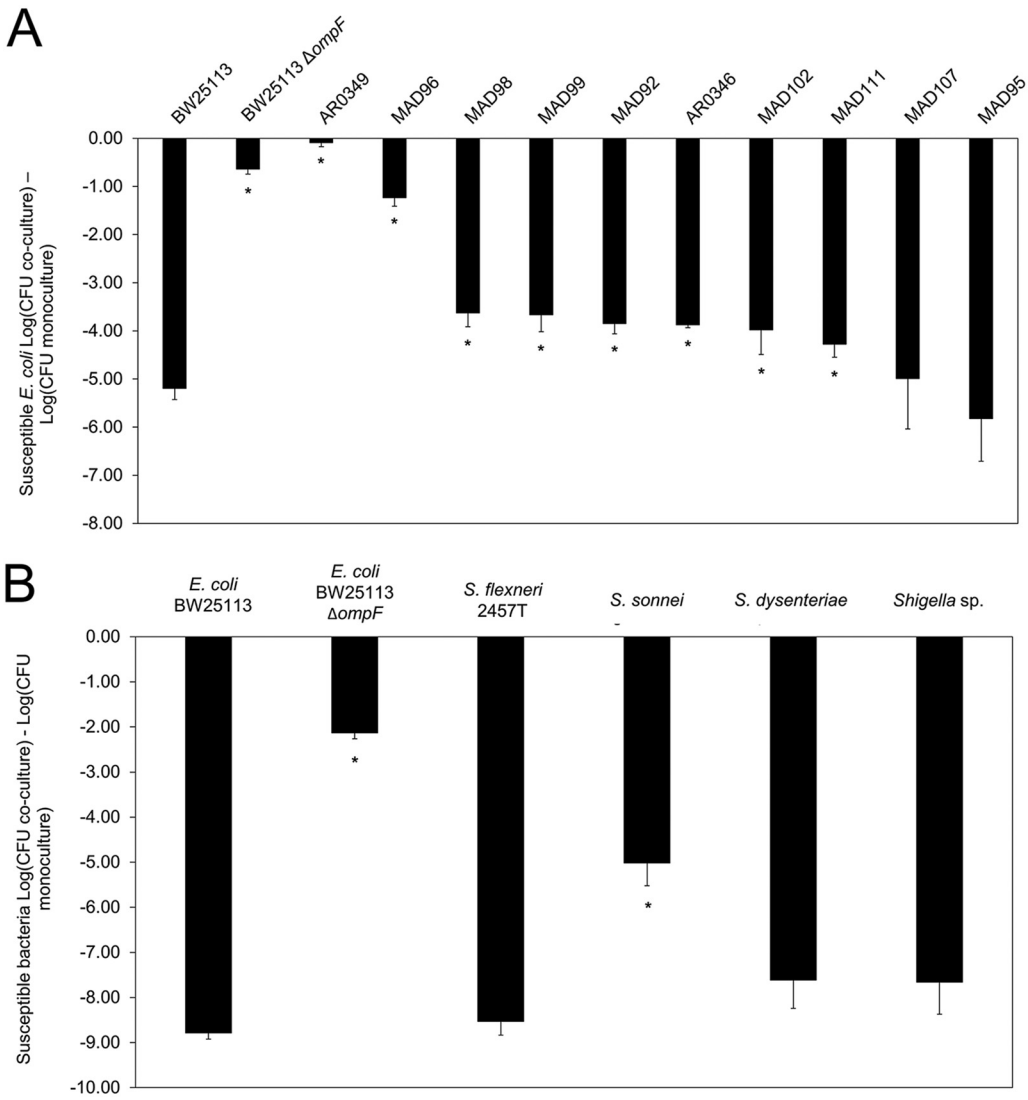

FIG 2 MccPDI is effective against UTI *E. coli* and *Shigella* strains. (A) Competition between MccPDI-producing strain *E. coli* 25 and positive and negative controls (BW25113 and BW25113 Δ*ompF*, respectively) as well as 10 other strains in M9 medium for 24 h. (B) Competition between the MccPDI producer *E. coli* 25 and target *Shigella* strains (*S. flexneri* 24570, *S. flexneri* 2457T, *S. sonnei* WRAIR I virulent, *S. dysenteriae* Newcastle 1934, and *Shigella* sp.) in M9 medium for 24 h. Results are expressed as the difference in mean log CFU during coculture and monoculture of the target strain ($n = 3$ independent replicates; error bars indicate standard errors of the means [SEM]). *, $P < 0.05$ compared to wild-type (BW25113) coculture based on one-way ANOVA.

reduction in the number of BW25113 cells after 24 h, compared with a 1.2-log CFU reduction when *E. coli* strain BW25113 expressed a recombinant immunity protein (with isopropyl-β-D-1-thiogalactopyranoside [IPTG] induction), McpI (Fig. 3) (5). The addition of 15% (wt/vol) PEG with a molecular mass of between 200 and 600 Da blocked apparent membrane damage, whereas higher mass fractions (1,450 Da and larger) did not affect the MccPDI phenotype.

Differential dye testing is consistent with membrane damage. To confirm that MccPDI damages susceptible bacterial membranes, we used a differential staining technique whereby 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) nucleic acid stain was added to cocultures containing an MccPDI producer strain (green fluorescence) and an MccPDI-susceptible strain (red fluorescence). The remaining green/red bacterial cells were quantified after MccPDI exposure. For the concentration of dye used in this experiment, only bacteria with membrane damage will take up the stain for detection by fluorescence (27). The MccPDI-producing strain (*E. coli* 25 Δ*traM*; conju-

TABLE 3 PCR primers used in this study

Primer	Sequence (5′–3′)[a]	Purpose	Reference
BACTH construct primers			
M1_36_pUT18_F	catgcatgAAGCTTgCGTAACTCACTGGGTCGAAA	Used to construct *mcpM*Δ36	This study
M1_36_pUT18_R	catgcatgGAATTCggTCGGTTACATGTTCCGC		
M1_36_pKNT25_F	catgcatgAAGCTTgCGTAACTCACTGGGTCGAAA	Used to construct *mcpM*Δ36	This study
M1_36_pKNT25_R	catgcatgGAATTCggTCGGTTACATGTTCCGC		
M1_36_pUT18C_F	catgcatgTCTAGAgCGTAACTCACTGGGTCGAAA	Used to construct *mcpM*Δ36	This study
M1_36_pUT18C_R	catgcatgGAATTCTTATCGGTTACATGTTCCGC		
M1_36_pKT25_F	catgcatgTCTAGAgCGTAACTCACTGGGTCGAAA	Used to construct *mcpM*Δ36	This study
M1_36_pKT25_R	catgcatgGAATTCTTATCGGTTACATGTTCCGC		
mcpI_pUT18_F	catgcatgAAGCTTgATGGAGGGCGCTACTATGTT	Used to construct *mcpI*	This study
mcpI_pUT18_R	catgcatgGAATTCggTTTCGCGGAGATTGTTCTT		
mcpI_pKNT25_F	catgcatgAAGCTTgATGGAGGGCGCTACTATGTT	Used to construct *mcpI*	This study
mcpI_pKNT25_R	catgcatgGAATTCggTTTCGCGGAGATTGTTCTT		
mcpI_pUT18C_F	catgcatgTCTAGAgATGGAGGGCGCTACTATGTT	Used to construct *mcpI*	This study
mcpI_pUT18C_R	catgcatgGAATTCCTATTTCGCGGAGATTGTTCTT		
mcpI_pKT25_F	catgcatgTCTAGAgATGGAGGGCGCTACTATGTT	Used to construct *mcpI*	This study
mcpI_pKT25_R	catgcatgGAATTCCTATTTCGCGGAGATTGTTCTT		
ompF_pUT18_F	catgcatgAAGCTTgATGATGAAGCGCAATATTCTGG	Used to construct *ompF*	This study
ompF_pUT18_R	catgcatgGAATTCggGAACTGGTAAACGATACCC		
ompF_pKNT25_F	catgcatgAAGCTTgATGATGAAGCGCAATATTCTGG	Used to construct *ompF*	This study
ompF_pKNT25_R	catgcatgGAATTCggGAACTGGTAAACGATACCC		
ompF_pUT18C_F	catgcatgTCTAGAgATGATGAAGCGCAATATTCTGG	Used to construct *ompF*	This study
ompF_pUT18C_R	catgcatgGAATTCTTAGAACTGGTAAACGATACCC		
ompF_pKT25_F	catgcatgTCTAGAgATGATGAAGCGCAATATTCTGG	Used to construct *ompF*	This study
ompF_pKT25_R	catgcatgGAATTCTTAGAACTGGTAAACGATACCC		
M13R49	GAGCGGATAACAATTTCACACAGG	Used to verify BACTH constructs	This study
pUT18C_R	GCATCAGAGCAGATTGTACTGAGAG		
pUT18_R	TTCGCGATCCAGGCCGC		
pKT25_R	CCAGGGTTTTCCCAGTCACG		
pKNT25_R	GCGATTGCTGCATGGTCA		
mcpI construct primers			
mcpI_flag_F	ccgGAATTCATGGAGGGCGCTACTAT	Used to construct pMMB207::*mcpI* with addition of a Flag tag in the C terminus	This study
mcpI_flag_R	acgcGTCGACCTACTTGTCGTCATCGTCTTTGTAGTCT TTCGCGGAGATTGTTCT		
Gene deletion mutant primers			
A1_mcpMI_KO_F	ggaAGATCTATGGCAATGCTGGAAGAG	Used to construct suicide plasmid pDM4Δ*mcpM*/Δ*mcpI*(A1+A2) to delete *mcpM* and *mcpI*	This study
A1_mcpMI_KO_R	AATCTTGCCGATCATATTTCCCCTATCGGT		
A2_mcpMI_KO_F	GAAATATGATCGGCAAGATTCATGGACTA		
A2_mcpMI_KO_R	acgcGTCGACCTTCATAATACGGAACTGTCAG		
mcpM_F	AGATGAGATAACGCTTGTCA	Used to confirm *mcpM* deletion and complementation	7
mcpM_R	ACTTCCTCTGTTACCACTTC		
mcpI_F	TATGTGGTTTGTTACTGGGAT	Used to confirm *mcpI* deletion and complementation	7
mcpI_R	CGCGGAGATTGTTCTTATTT		

(Continued on next page)

TABLE 3 (Continued)

Primer	Sequence (5′–3′)[a]	Purpose	Reference
SacI_pMIADB	ggtggtGAGCTCTGGAACGAGATGTACTGAACAGAGGCCGTG	Used to construct $p_{mic\text{-}210/0}$*mcpMIADB*	This study
HindIII_MIADB	ggtggtAAGCTTTCACCTCCTGTTGGGGTGATTATTTATATTATGAGTTCTGGC		
Construct tdTomato primers			
tdtomato_XbaI	tgcTCTAGATTTAAGAAGGAGATATACATATGGTGAGCAAGGGCGAGGA	Construct pFPV-tdTomato for labeling bacteria	This study
tdtomato_SphI	catGCATGCCTACTTGTACAGCTCGTCCATGC		
Bacteriocin primers			
mcmM_F	GCATTAGTTGGGGAGCCAGA	Used to detect the gene *mcmM* (microcin M)	This study
mcmM_R	CAACCCCACCAGGAACAGTT		
mchB_F	TGCGAGAAATAACAGAATCACAGT	Used to detect the gene *mchB* (microcin H47)	This study
mchB_R	CCAGCAGAAGAACTGGCACT		
mceA_F	CTTGGCCCGATGAGTACAGG	Used to detect the gene *mceA* (microcin E492)	This study
mceA_R	TTTTGGTGCAGGAGAGACCG		
cvaC_F	TTCAGGGCGTGATATTGCGA	Used to detect the gene *cvaC* (microcin V)	This study
cvaC_R	TCCCGCAGCATAGTTCCATG		
cia_F	TGTAAACCCTCCACGTGTCG	Used to detect the gene *cia* (colicin 1a)	This study
cia_R	GACAACCGGGTGTCCAGAAT		
cib_F	GGTGGTGACAGAGGATGTGG	Used to detect the gene *cib* (colicin 1b)	This study
cib_R	GTCTCCGCCATATGGACCTG		
mcbA_F	TGGAATTAAAAGCGAGTGAATTT	Used to detect the gene *mcbA* (microcin B17)	This study
mcbA_R	CACCGTTTCCACCACTACAA		
ompF sequence primers			
ompF_up	CGCTATCAGGGTAACGGGAG	Used to sequence *ompF* in *E. coli*	This study
ompF_down	AGCACTTTCACGGTAGCGAA		

[a]Restriction enzyme sites are underlined.

gation negative [5]) and the MccPDI-negative strain (*E. coli* 25 Δ*mcpM* Δ*mcpI* [7]) were transformed with pFPV25.1::gfpmut3 (green). Here, *E. coli* 25 Δ*traM* lacks the ability to be transferred to other cells by conjugation, which limits the likelihood of sharing the MccPDI plasmid and its immunity gene. *E. coli* 25 Δ*mcpM* Δ*mcpI* served as a negative control. MccPDI-susceptible *E. coli* BW25113 was transformed with pFPV25.1::tdTomato (red). After 2 h of coculture, there were similar proportions of green and red cells and no evidence of DAPI staining between the two groups (*E. coli* 25 Δ*traM* versus BW2511 and *E. coli* 25 Δ*mcpM* Δ*mcpI* versus BW25113) (Fig. 4). After 6 h, however, the MccPDI producer (*E. coli* 25 Δ*traM*) was numerically dominant over susceptible bacteria, and DAPI-stained cells were present (Fig. 4, top row), consistent with death of the susceptible bacteria as a result of membrane damage. In contrast, coculture with MccPDI-negative *E. coli* 25 (Δ*mcpM* Δ*mcpI*) did not result in a difference in the number of cells (green versus red) after 6 h.

ATP release assay confirms that MccPDI damages the bacterial cell membrane. We cultured *E. coli* 25 Δ*mcpM* Δ*mcpI* (microcin and immunity knockout) as a monoculture with or without induction with plasmid pMMB207 containing an IPTG-inducible *mcpM* sequence. In the presence of 0.5 mM IPTG (Fig. 5), MccPDI was produced, and extracellular levels of ATP increased from an unmeasurable background level to a peak at 8 h before rapidly dropping off. None of the other test conditions resulted in significant changes in the concentration of extracellular ATP. Results were confirmed by a reduction of approximately 4 log CFU at 8 h from the expected population size (Fig. S10). The 8-h peak in the extracellular ATP level and the reduction in CFU also correspond to the expected expression kinetics of *ompF* (28).

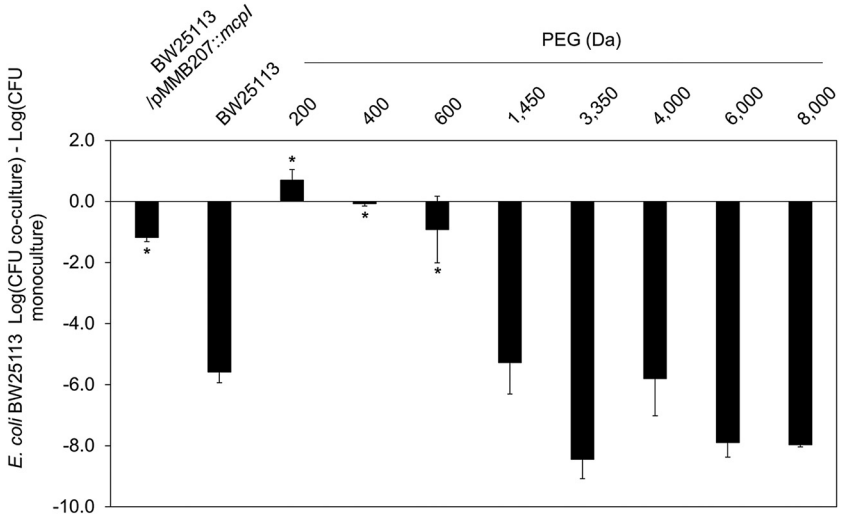

FIG 3 The osmoprotectant PEG protects PDI-susceptible bacteria. Results of competition between *E. coli* 25 and susceptible strain BW25113 with the addition of 15% (wt/vol) PEG of different mass fractions (200 to 8,000 Da). The MccPDI immunity gene (*mcpI*)-complemented strains of BW25113 served as negative controls for this assay. Results are expressed as the difference in mean log CFU during coculture and monoculture of the target strain ($n = 3$ independent replicates; error bars indicate SEM). *, $P < 0.05$ compared to susceptible *E. coli* strain BW25113 coculture based on one-way ANOVA.

McpI protein-protein interactions. To characterize how the MccPDI self-immunity protein (McpI) (8 kDa) may function to protect the MccPDI-producing strain, we conducted paraformaldehyde (PFA) cross-linking experiments with a Flag-tagged McpI protein (Fig. 6). Treatment with 2% PFA alone revealed the presence of three protein bands, consistent with protein-protein interactions (Fig. 6, lane 6). The approximate molecular mass of these protein bands was similar to what would be expected if McpI forms multimers. Only a combination of boiling (100°C for 10 min) and the presence of β-mercaptoethanol (βME) eliminated most of the putative dimer and trimer structures (lane 3).

Bacterial two-hybrid system confirms McpI-McpI interaction. We used a bacterial two-hybrid (BACTH) system (29) to determine if the immunity protein McpI forms a multimer with itself, or with McpM, and to investigate other potential protein-protein interactions with OmpF. This assay works by fusing two proteins of interest with the T18

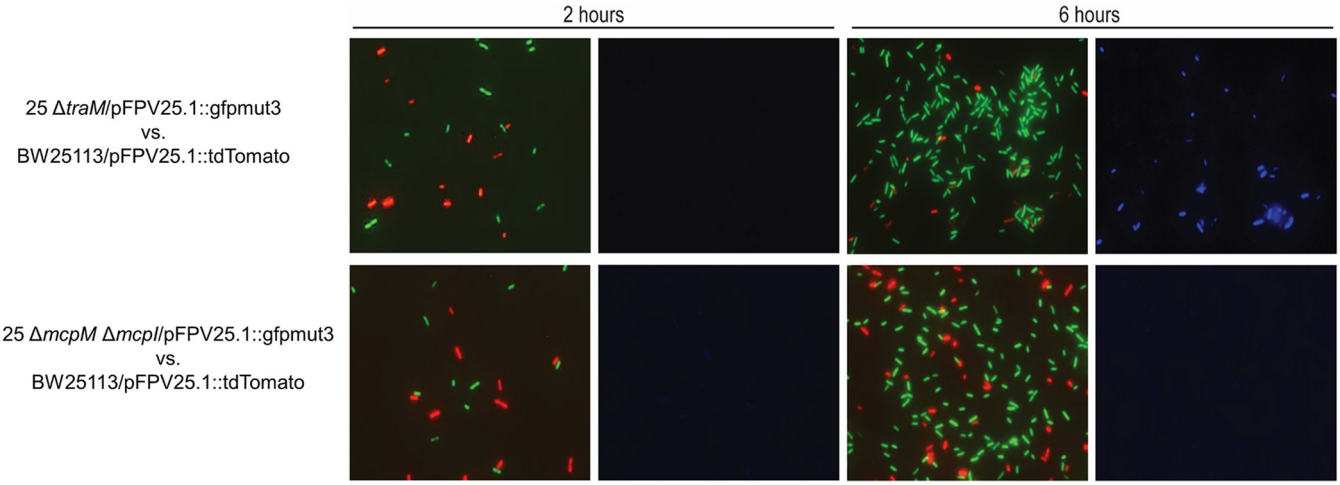

FIG 4 MccPDI induces membrane permeability in susceptible cells. Shown is DAPI staining of the fluorescence-labeled strains in monoculture and coculture for 2 h and 6 h. The green-labeled strains are *E. coli* 25 Δ*traM* and 25 Δ*mcpM* Δ*mcpI*, and the red-labeled MccPDI-susceptible strain is BW25113. Blue fluorescence indicates that the cells were stained by DAPI, consistent with increased membrane permeability given exposure to MccPDI.

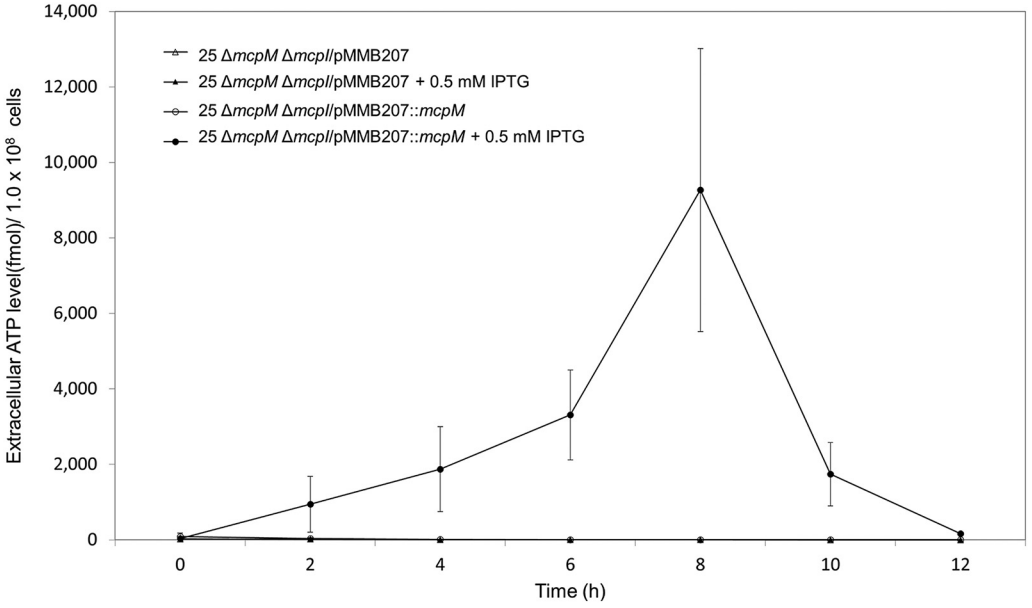

FIG 5 ATP detected with induced complementation for McpM. *E. coli* 25 Δ*mcpM* Δ*mcpI* with the vector (pMMB207) or with complemented *mcpM* (pMMB207::*mcpM*) without IPTG induction showed no detectable ATP in the extracellular environment. Upon induction (0.5 mM IPTG), *E. coli* 25 Δ*mcpM* Δ*mcpI*/pMMB207::*mcpM* generated elevated levels of ATP (2 to 8 h) compared to the vector-only strain. Results are mean luminescence values for three independent replicates (error bars indicate SEM).

and T25 subunits of adenylate cyclase from *Bordetella pertussis*, respectively. If these subunits are located proximally when two proteins interact, this allows adenylate cyclase activity, thereby releasing cAMP into the medium, which can be quantified by measuring β-galactosidase activity (30). McpM is synthesized as a 120-amino-acid (aa) peptide that undergoes two cleavage events (Fig. 1) and is secreted via a type I secretion system (5, 7). We constructed the McpM fusion protein with the putative mature McpM sequence (84 aa long; strain McpMΔ36). Out of nine tested combinations, only the McpI-McpI interaction was positive (McpI-T18/McpI-T25) (Fig. 7). Inter-

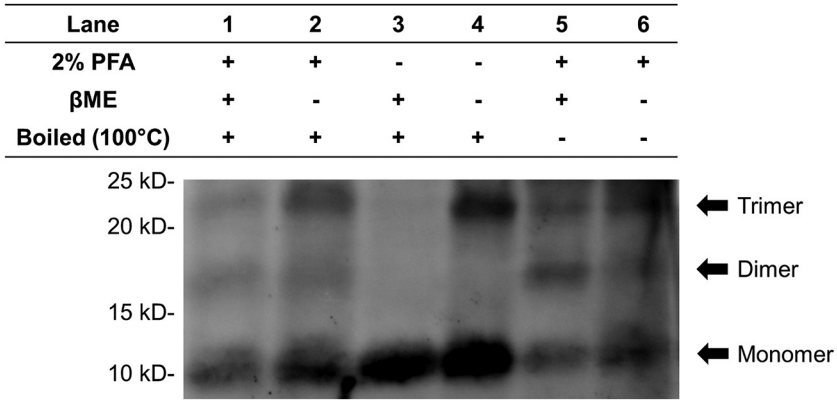

FIG 6 The McpI immunity protein forms multimers. Shown is detection of the microcin PDI immunity protein (McpI) (with a C-terminal Flag tag) forming a homotrimer structure. Samples consisted of the total bacterial lysate expressing McpI in the presence or absence of the 2% paraformaldehyde (PFA) cross-linking reagent β-mercaptoethanol (βME) and with or without boiling of the sample for 10 min to disrupt cross-linking. Black arrows point toward potential monomer, dimer, and trimer structures upon cross-linking. Crossed-linked and boiled samples show evidence for both dimer and monomer proteins with (lane 1) or without (lane 2) βME. Total reduction of the homotrimer structure into monomers (lane 3) occurred with a combination of βME and boiling. Boiled samples without βME lacked a detectable dimer (lane 4). Heated (60°C) cross-linked samples with (lane 5) or without (lane 6) βME showed evidence of both homotrimer and dimer proteins.

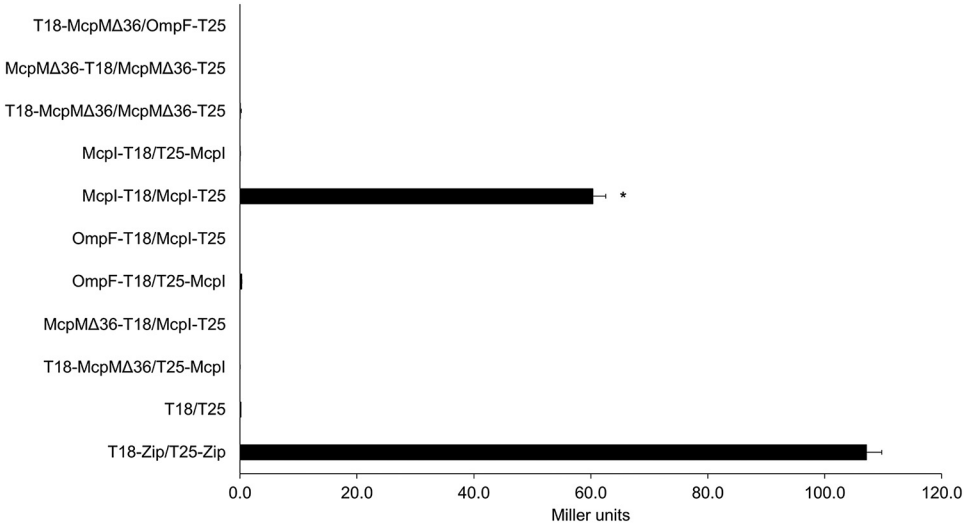

FIG 7 BACTH McpI-McpI interaction. A beta-galactosidase assay was used to confirm bacterial constructs to quantify the hybrid proteins' interaction. Compared to the positive control (T18-Zip/T25-Zip) and negative control (vectors only) (T18/T25), the McpI-T18/McpI-T25 interaction was clearly positive given an N-terminal T25 orientation (black bar), but no signal was detected with an McpI-T25 C-terminal orientation (McpI-T18/T25-McpI). No protein interaction was detected with McpMΔ36 or OmpF. All readings were performed in a 96-well plate at an OD_{420}, which are referred to as "Miller units" ($n = 3$ independent replicates; error bars indicate SEM). *, $P < 0.05$ in a comparison with the T18-Zip/T25-Zip positive control, based on one-way ANOVA.

estingly, the McpI-T18/T25-McpI interaction did not result in restoration of adenylate cyclase activity, consistent with a nonsymmetric assembly of multimers.

DISCUSSION

While MccPDI is demonstrably effective against multiple strains of *E. coli* (5, 6), this paper reiterates this efficacy in the context of antibiotic-resistant bacteria, including strains recovered from urinary tract infections and several strains of *Shigella*. Urinary tract infections are of particular interest given that, on a global scale, antibiotic-resistant strains afflict 150 million people each year (31–34). The recent discovery of *mcr-1*-encoded colistin resistance in combination with extended-spectrum beta-lactamase (ESBL) resistance (e.g., strains AR0346 and AR0349) (Table 2) is emblematic of this growing problem. Even in cases where these strains can still be controlled with traditional antibiotics, the use of these drugs can cause complications for the patient while also providing selective pressure on the normal gastrointestinal tract microflora (34–36). Shigellosis is a problem worldwide (13, 37), particularly in communities suffering from poor sanitation and hygiene. Consequently, there is considerable interest in novel antimicrobials, such as microcins, that have a high degree of specificity and efficacy against *E. coli* and *Shigella* infections.

Microcins are a class of antimicrobial peptides that interact with cell surface receptors before translocating into susceptible bacteria (2, 8). Microcin PDI is known to interact with *E. coli* OmpF, which must be present for MccPDI to kill the target bacteria during coculture (8). The effector protein McpM likely binds to a specific location in OmpF (8), where it probably translocates through the porin, as has been documented for other bacteriocins (e.g., colicin E3 [38]). Interestingly, purified recombinant McpM protein can still inhibit an *E. coli* strain that does not synthesize OmpF (Δ*ompF* mutant) (see Fig. S7 in the supplemental material). If McpM functions by entering the susceptible cell through OmpF, it is possible that recombinant McpM gains entry via mechanisms (e.g., other outer membrane porins) that may be concentration dependent, although coculture with *E. coli* 25 and *E. coli* BW25113 Δ*ompF* showed some evidence that BW25113 was susceptible to "natural concentrations" of MccPDI when OmpF was absent (Fig. 2A and B show reduced CFU for *E. coli* BW25113 Δ*ompF*, whereas the density of *E. coli* 25 was elevated when these strains were cocultured [Fig. S3]).

As with any antimicrobial compound, it is inevitable that resistance mechanisms will emerge. A likely "Achilles' heel" of MccPDI involves mutations in the OmpF protein that is located in the outer membrane of susceptible cells. Zhao et al. (8) determined that a $K_{47}G_{48}N_{49}$ amino acid motif found in the predicted outer loop 1 of OmpF is required for MccPDI to affect a susceptible cell. Strain AR0349 from the present study has a very divergent OmpF amino acid sequence that includes mutation in this $K_{47}G_{48}N_{49}$ motif (Fig. S1). We do not know what selection pressures led to the emergence of such mutations, but the $K_{47}G_{48}N_{49}$-to-$K_{47}D_{48}N_{49}$ mutation should confer resistance to MccPDI (8). While we surmise that this amino acid motif is required for McpM binding, the glycine-to-aspartic acid substitution exhibited by strain AR0349 would also change the charge characteristics of the OmpF outer vestibule (39), and this might prevent passage of McpM through the porin. Consequently, while the susceptible sequence of OmpF is largely conserved in *E. coli* strains, and this conservation extends to *Shigella* species (Fig. S1), potential therapeutic applications of McpM should be judicious to limit widespread selection for resistance mutations.

E. coli strain MAD 96 was also resistant to MccPDI during coculture, although MAD 96 is susceptible to recombinant McpM (Fig. S8), consistent with a susceptible *ompF* sequence (Fig. S1), or this is an artifact of the high concentration of McpM that was used with the spot assays. Observations in the laboratory (not shown) led us to speculate that MAD 96 produces one or more bacteriocins that might inhibit *E. coli* strain 25 before McpM can be upregulated sufficiently to impact MAD 96 (Fig. S3 and S4). This would not be unusual given that it is common for uropathogenic *E. coli* (UPEC) strains to produce bacteriocins (40, 41). MAD 96 was PCR positive for both colicin Ia/Ib and microcin V (Fig. S4). On its own, MccV does not appear to inhibit an MccPDI producer (*E. coli* 25) in medium (Fig. S5), and thus, it is likely that Col Ia/Ib is primarily responsible for inhibiting the MccPDI-producing strain (Fig. S3). According to Abraham et al. (41), the likelihood of UPEC plasmids carrying both MccV and Col Ia/Ib is as high as 84.5%. This form of "resistance" against MccPDI (and other microcins) would not be relevant if McpM is deployed as a recombinant protein (Fig. S7 and S8) or if it was produced by a non-*E. coli* heterologous expression host.

Data from multiple assays from the present study are consistent with the conclusion that the MccPDI precursor protein McpM kills susceptible strains via membrane disruption, and experiments here and elsewhere confirm that naturally susceptible strains are widespread (6–8, 24). OmpF is the key ligand for susceptibility (8, 42), but other genes have been identified that are requisite for susceptibility to MccPDI (*ompR*, *atpA*, *atpF*, *atpE*, *atpH*, *dsbA*, and *dsbB* [8]). OmpR regulates the expression of *ompF* as part of the EnvZ/OmpR two-component osmoregulatory system (42–44). The roles of ATP synthase (*atpA*, *atpF*, *atpE*, and *atpH*) and thiol-disulfide interchange protein (*dsbA* and *dsbB*) in MccPDI susceptibility remain unknown (8, 45–47). If McpM enters the periplasmic space to affect susceptible cells, we speculate that these other host proteins are needed for McpM maturation before membrane disruption occurs, or they may play an important role in the synthesis and/or function of OmpF. The former would not be unprecedented given that microcin V permeabilizes the bacterial inner membrane, and E492 is a channel-forming bacteriocin (1, 48–50).

Results from the osmoprotectant assay were consistent with low-mass PEG (200 to 600 Da) blocking pores that might be formed by McpM. It is worth noting, however, that PEG molecules of this mass fraction have hydrodynamic radii of 0.56 to 0.8 nm, while the next-highest mass fraction (1,450 Da) did not protect susceptible bacteria, and the expected size of these molecules is 1.2 nm (51). OmpF consists of a trimer complex that forms an "hourglass"-shaped porin, having an external vestibule approximately 1 nm in diameter that reduces in a cone shape to a constricted region of approximately 0.5 nm (39). It is possible that at sufficient concentrations, 200- to 600-Da PEG enters the vestibule and physically blocks McpM from entering the porin or physically blocks access to the binding site. All three of these mechanisms (osmoprotection, physical blockage, and binding blockage) would protect susceptible bacteria from MccPDI.

Like other microcin systems (10, 23), the MccPDI locus encodes a "self-immunity protein" (McpI) that protects the microcin-producing cell from its antibacterial activity (5). Less is known about how microcin immunity functions, although immunity to microcins B17, C, E, and J25 is conferred by concurrently expressed efflux pumps (1, 4, 23, 52). We found no evidence that McpI binds to either McpM or OmpF, but it instead forms a homotrimer structure based on the bacterial two-hybrid (BACTH) system and PFA cross-linking experiments. It is important to note that the BACTH system detects only protein-protein interactions that occur in the cytosolic space (30, 53). Consequently, the McpI-McpI protein-protein interaction revealed by the BACTH system is consistent with the assembly of McpI multimers in the cytosol, although this does not preclude assembly of multimers in the periplasm as well. The function of the multimeric McpI structures is unknown, but analysis of the amino acid sequence using tools such as InterPro and PfamScan suggests the McpI does not function as an enzyme (54, 55). Based on protein structure prediction, McpI consists of two transmembrane domains and was predicted to be a transmembrane protein (Fig. S11) (56–58). It is possible that the multimeric form of McpI enters the periplasm, where it serves as a simple molecular "plug" that blocks the entry of linear McpM peptides into the periplasmic space via OmpF or as a plug to block pores that are formed by McpM from outside the cell membrane. Alternatively, the McpI multimers could remain in the cytosol, where they would block McpM-mediated damage to the inner membrane. Further work is needed to determine how McpM damages the cell membrane and how McpI prevents McpM from killing the producing cell.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Unless otherwise stated, all strains described here (Table 1) were grown in LB Lennox (LB broth) medium (Difco Laboratories, Inc.) or in defined M9 minimal medium (6 g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 0.5 g/liter NaCl, 1 g/liter NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.2% glucose) supplemented with thiamine (1 mg/liter) and leucine (100 μg/ml). Bacteria were cultured at 37°C on a shaker table (200 rpm) (24). When used, the following antibiotics were added to media: ampicillin (Amp) at 100 μg/ml, tetracycline (Tet) at 50 μg/ml, kanamycin (Kan) at 50 μg/ml, nalidixic acid (Nal) at 30 μg/ml, and chloramphenicol (Cm) at 32 μg/ml. Unless otherwise noted, basic reagents and antibiotics were purchased from Sigma-Aldrich, Inc., and VWR International, LLC, respectively.

Plasmid/DNA extraction and construction of recombinant protein vectors. All plasmids were extracted from *E. coli* using the QIAprep spin miniprep kit (Qiagen), and *E. coli* genomic DNA was extracted using a DNeasy blood and tissue kit (Qiagen), according to the manufacturer's instructions. All primer pairs (Eurofins Genomics) and engineered restriction enzyme sequences (New England BioLabs) are listed in Table 3. Platinum PCR supermix (Invitrogen) was used with preparative PCR to engineer plasmids pMMB207, pCR2.1, pFPV25.1gfpmut3, pUT18, pUT18C, pKT25, and pKNT25. PCR was used to verify plasmid constructs (listed in Table 4), and reaction mixtures included DreamTag green PCR master mix (Thermo Scientific). PCR product identity was confirmed by Sanger sequencing (Eurofins Genomics).

Mutant construction. Methods described previously by Datsenko and Wanner were used to generate gene deletions (59). Briefly, primers (Table 3) were designed to incorporate a 36- to 50-nucleotide DNA sequence that complemented the region flanking the gene of interest. PCR with these primers generated a PCR product that joined the flanking sequences to a kanamycin resistance (Kan^r) gene, originally from plasmid pKD4 (Table 4). A QIAquick PCR column purification kit (Qiagen) was used to purify the PCR products, followed by DpnI restriction enzyme digestion (New England Biolabs, Inc.) for 4 h at 37°C to digest the DNA template (pKD4) before repeating the column purification step. The treated PCR product (150 ng) was then electroporated into *E. coli* strain 25 with a Gene Pulser Xcell instrument (Bio-Rad Laboratories, Inc.), using settings (1.8 kV, 25 μF, 200 Ω, and 1-mm-gap cuvette) described previously (24). In preparation for this procedure, *E. coli* strain 25 carrying the λ Red plasmid pKD46 (Amp^r) was grown to an optical density at 600 nm (OD_{600}) of ~0.6 in super optimal broth (SOB) medium (Fisher Scientific). Bacterial cells were then washed twice in ice-cold water and once in 10% glycerol before 50 μl of 10% glycerol was added to resuspend the cells for electroporation. Immediately after electroporation, 0.5 ml of SOB with catabolite repression (SOC) recovery medium (Fisher Scientific) was added to cells for recovery (2 h at 30°C with aeration) before plating on LB agar with kanamycin (LB-Kan) and incubation overnight at 30°C in a stationary incubator. PCR was used to verify *mcpM* and *mcpI* gene deletions (Table 3).

Coculture competition assay. Due to difficulties associated with purification of MccPDI, as an indirect method to examine the inhibitory effect of MccPDI, coculture competition assays (*in vitro* method) were performed using a modified protocol (7, 24, 60). Briefly, bacterial strains were grown individually overnight in LB broth. The following day, cultures grown overnight (50 μl) were transferred into three fresh tubes containing 5 ml M9 medium (*E. coli* 25 or *E. coli* BW25113 alone or 50 μl of both strains). M9 cultures were incubated for 24 h at 37°C with shaking. When appropriate, antibiotics and/or

TABLE 4 Plasmids used in this study

Plasmid	Relevant genotype and/or description[a]	Source or reference
pCR2.1-Topo	Amp^r; Topo cloning vector	Invitrogen
pCR2.1::$p_{mic\text{-}210/0}$*mcpMIADB*	Amp^r; containing *mcpM* promoter −210/0 and *mcpM*, *mcpI*, *mcpA*, *mcpD*, and *mcpB*	This study
pMMB207	Chl^r; RSF1010 derivative; IncQ *lacI*^{q} Tac *oriT*	67
pMMB207::*mcpM*	Chl^r; pMMB207 containing the *mcpM* gene with a 6×His tag at the C terminus	This study
pMMB207::*mcpI*	Chl^r; pMMB207 containing the *mcpI* gene with a Flag tag at the C terminus	This study
pFPV25.1::gfpmut3	Amp^r; vector containing GFPmut3	62
pFPV25.1::tdTomato	Amp^r; vector containing tdTomato	This study
pKD4	Kan^r; containing Kan^r cassette for PCR amplification	68
pKD46	Amp^r	59
pUT18	Amp^r; cloning vector	Euromedex
pUT18C	Amp^r; cloning vector	Euromedex
pUT18C-Zip	Amp^r; pUT18 containing the leucine zipper	Euromedex
pUT18::*mcpM*Δ36	Amp^r; pUT18 containing the *mcpM*Δ36 gene	This study
pUT18::*mcpI*	Amp^r; pUT18 containing the *mcpI* gene	This study
pUT18::*ompF*	Amp^r; pUT18 containing the *ompF* gene	This study
pUT18C::*mcpM*Δ36	Amp^r; pUT18C containing the *mcpM*Δ36 gene	This study
pUT18C::*mcpI*	Amp^r; pUT18C containing the *mcpI* gene	This study
pUT18C::*ompF*	Amp^r; pUT18C containing the *ompF* gene	This study
pKT25	Kan^r; cloning vector	Euromedex
pKT25-Zip	Kan^r; pKT25 containing the leucine zipper	This study
pKT25::*mcpM*Δ36	Kan^r; pKT25 containing the *mcpM*Δ36 gene	This study
pKT25::*mcpI*	Kan^r; pKT25 containing the *mcpI* gene	This study
pKT25::*ompF*	Kan^r; pKT25 containing the *ompF* gene	This study
pKNT25	Kan^r; cloning vector	Euromedex
pKNT25::*mcpM*Δ36	Kan^r; pKNT25 containing the *mcpM*Δ36 gene	This study
pKNT25::*mcpI*	Kan^r; pKNT25 containing the *mcpI* gene	This study
pKNT25::*ompF*	Kan^r; pKNT25 containing the *ompF* gene	This study

[a]Amp^r, ampicillin resistant; Chl^r, chloramphenicol resistant; Kan^r, kanamycin resistant.

0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; Fisher Scientific) was added. CFU were enumerated by first preparing serial dilutions (10-fold; 6 replicates each) in a 96-well plate containing sterile 1× phosphate-buffered saline (PBS). After mixing, 5 μl of each dilution was transferred onto an LB agar plate (with or without antibiotics). Plates were incubated at 37°C overnight before CFU were determined according to the methods of Chen et al. (60).

Bacteriocin typing for *E. coli* strain MAD 96. *E. coli* strains 25 and MAD 96 were picked from fresh colonies into 5 ml LB broth and inoculated at 37°C with shaking at 200 rpm. The culture grown overnight (1 μl) was used to PCR amplify different bacteriocin gene candidates using primer sets that were designed using SnapGene software (GSL Biotech) (Table 3).

McpM protein expression and purification. To generate recombinant McpM (with a C-terminal 6×His tag) for various experiments, 2 liters of a 0.5 mM IPTG (Fisher Scientific)-induced *E. coli* strain 25 Δ*mcpM*/pMMB207::*mcpM* culture grown overnight was pelleted by centrifugation at 4,000 × *g* at 4°C for 30 min. The pellet was resuspended in 15 ml of PBS (50 mM sodium phosphate, 500 mM NaCl [pH 8.0]). A protease inhibitor cocktail (cOmplete ultra tablets, mini, EDTA free; Roche) was added to the resuspended pellet, and the mixture was sonicated (S-450 Ultrasonics sonifier; Branson) for 6 min with 20-s on/off pulses. Ice was added to the water bath surrounding the tube to avoid sample overheating. After sonication, the tube was centrifuged at 30,000 × *g* (4°C for 30 min). The supernatant containing soluble McpM was then purified by using high-performance liquid chromatography (HPLC) with nickel-Sepharose Excel resin (GE Healthcare). HPLC conditions were as follows. Two milliliters of resin was packed into a tricorn 5/10 column (GE Healthcare) and attached to an Äkta Avant 25 chromatography system (GE Healthcare). The column was equilibrated at 2 ml/min for 10 column volumes (CV) with PBS (pH 7.6), followed by sample loading using a sample pump at 0.5 ml/min. Washout of the unbound sample was performed at 2 ml/min for 15 CV with PBS (pH 7.6) containing 20 mM imidazole. Sample elution was isocratic for 5 CV at 2 ml/min using PBS (pH 7.6) with 125 mM imidazole. McpM elution was monitored by reading the absorbance at 280 nm, and the peak containing McpM was collected in 500-μl fractions (8°C). Pooled McpM fractions were then desalted into 1× PBS (to remove imidazole) using a HiTrap desalting column (GE Healthcare), according to the manufacturer's instructions. Desalted McpM was collected by peak fractionation (UV at 280 nm) in 500-μl fractions at 8°C. All data were collected and analyzed using Unicorn chromatography management system version 7.0 (GE Healthcare) (see Fig. S6 in the supplemental material).

High-copy-number McpM expression vector. A second McpM expression vector was constructed to include the indigenous PDI promoter (−210/0, upstream of the *mcpM* gene) and the entire MccPDI operon (*mcpM*, *mcpI*, *mcpA*, *mcpB*, and *mcpD*), using the primers listed in Table 3. The vector construct (pCR2.1-Topo backbone; Invitrogen) is a high-copy-number plasmid that permits synthesis of more McpM than with pMMB207::*mcpM* (low-copy-number vector), without the need for induction. The vector construct methods were used as described above to produce pCR2.1::$_{pmic\text{-}210/0}$*mcpMIADB*, which was subsequently transferred into *E. coli* strain DH10B by electroporation (see "Spot plating assay," below).

McpI protein expression. To generate recombinant McpI, pMMB207::*mcpI* (with a C-terminal Flag tag) was constructed using the vector construct methods described above. The engineered plasmid was transformed into *E. coli* BW25113 (Table 4). A fresh BW25113/pMMB207::*mcpI* colony was inoculated overnight into 1 liter LB broth (0.5 mM IPTG and the appropriate antibiotic) and pelleted by centrifugation at 4,000 × *g* (4°C for 30 min). Cell pellets were subjected to a cross-linking experiment and Western blot analysis (see below).

Spot plating assay. Spot plating was developed as a secondary (and more rapid) protocol for detecting MccPDI-susceptible bacteria. Cultures (5 ml) of *E. coli* and *Shigella* strains grown overnight were diluted to an OD_{600} of ~0.8 with LB broth. Susceptible bacterial strains (e.g., *E. coli* BW25113) were spread onto an M9 agar or LB agar plate using a sterile cotton swab. Purified McpM protein (5 μl at 40 μg/ml) or a culture (OD_{600} of ~0.8) of DH10B/pCR2.1::$p_{mic\text{-}210/0}$*mcpMIADB* or the microcin PDI producer *E. coli* 25 (positive control) in PBS or M9 medium (negative control) was spotted onto the agar plate and air dried before being incubated at 37°C overnight (Table 1). Strains that exhibited zones of clearance associated with recombinant McpM or with the MccPDI-producing strain were considered positive for susceptibility to MccPDI.

Western blot analysis. To detect His-tagged and Flag-tagged recombinant proteins (McpM and McpI, respectively), total bacterial proteins were collected from a 10-ml culture grown overnight (with 0.5 mM IPTG) by centrifugation at 18,000 × *g* (4°C for 5 min). Cell pellets were resuspended in 1× Laemmli sample buffer (Bio-Rad Laboratories, Inc.) and boiled for 10 min. Any kD Tris-glycine precast gels (Bio-Rad) were used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein separation. A Trans-Blot turbo transfer starter system (Bio-Rad) was used for protein transfer onto a low-fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc.). Ponceau S staining was used to verify protein transfer prior to the addition of antibodies for specific protein detection. Anti-His tag or anti-Flag tag primary antibody (1:1,000) (Thermo Scientific) was used with secondary goat anti-mouse antibody (1:5,000) (DyLight 650, conjugate). A ChemiDoc MP imaging system (Bio-Rad) was used to detect fluorescent signals.

Osmoprotectant assay. To determine if pores were present in the bacterial membrane, we added 15% (wt/vol) polyethylene glycols (PEGs) (200 to 8,000 Da) to coculture competition assay mixtures for 24 h (26, 61). Competition assays and enumeration methods were used as described above.

DAPI staining of fluorescently labeled *E. coli*. To "tag" bacteria for imaging, we first constructed a reporter plasmid, pFPV25.1::tdTomato, by replacing gfpmut3 in pFPV25.1::gfpmut3 (62) with the tdTomato gene (62) using primers tdtomato_XbaI and tdtomato_SphI (Table 3). Standard cloning procedures were used, and sequencing was used to verify results. *E. coli* strain 25 Δ*traM* and *E. coli* strain 25 Δ*mcpM* Δ*mcpI* (Table 1) were transformed with pFPV25.1::gfpmut3 expressing green fluorescent protein (GFP), while target strain BW25113 was transformed with pFPV251::tdTomato expressing red fluorescent protein (tomato red). *E. coli* 25 Δ*traM* (conjugation negative) was chosen to prevent undesirable conjugation between MccPDI producer and target strains. Competition assays (described above) were conducted with the fluorescently labeled cells, and individual monocultures were run as controls. After 2-h and 6-h incubations, 1-ml samples were taken from each of the cultures, and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain (Thermo Scientific) was added at a final concentration of 0.25 μg/ml for 10 min at room temperature. The stained bacteria were pelleted by centrifugation for 1 min at 12,000 × *g* and resuspended in the same volume of PBS. Cells were then immobilized onto poly-L-lysine-coated glass slides (Sigma) for 20 min and covered with glass coverslips. Cells were observed and images were captured by using an inverted fluorescence microscope (Evos; Advanced Microscopy Group).

ATP depletion assay. Extracellular ATP was quantified by using a BacTiter-Glo microbial cell viability assay (Promega). Fresh bacterial colonies of *E. coli* Δ*mcpM* Δ*mcpI* with empty control vector pMMB207 or with complementation with *mcpM* (pMMB207::*mcpM*) were transferred to medium and grown at 37°C overnight with appropriate antibiotics (Table 1). Cultures grown overnight (10 μl) were pipetted into fresh M9 medium (90 μl) in a 96-well microplate with appropriate antibiotics and induced with 0.5 mM IPTG as needed (0, 2, 4, 6, 8, 10, and 12 h). According to the manufacturer's protocol, reagents (BacTiter-Glo buffer and substrate) provided in the kit were then combined and added to each well of inoculant (100 μl reagent to 100 μl culture), and samples were incubated for 5 min at room temperature before luminescence was recorded with an Infiniti M1000 Pro microplate reader (Tecan Systems). A luminescence signal was generated by recombinant luciferase through the detection of ATP. Each assay was repeated for three independent replicates with *E. coli* Δ*mcpM* Δ*mcpI*/pMMB207 and *E. coli* Δ*mcpM* Δ*mcpI*/pMMB207::*mcpM* (without 0.5 mM IPTG), which served as a negative control. CFU were enumerated at each time point using the method described above.

Paraformaldehyde cross-linking. We used a cross-linking experiment to determine if the MccPDI self-immunity protein, McpI, forms a multimeric structure. Formaldehyde is the smallest aldehyde (electrophilic molecule) that forms covalent bonds between two different macromolecules (63). Due to its small molecular size, formaldehyde cross-links macromolecules 2.3 to 2.7 Å apart (63, 64), which makes it particularly suitable to study the interactions between proteins in spatial proximity. Furthermore, the ability of PFA to permeate intact cells and cellular membranes easily makes it ideal for this experiment (64). A 5-ml culture containing *E. coli* BW25113/pMMB207::*mcpI* was induced overnight with 0.5 mM IPTG (Table 1). A modified protocol of a method previously described by Klockenbusch and Kast (64) was used to cross-link Flag-tagged McpI for analysis by Western blotting. Briefly, a 500-μl culture grown overnight was centrifuged at 18,000 × *g* for 1 min at room temperature. The resulting cell pellet was washed twice with 100 μl sterile PBS (pH 7.0), and prewarmed (37°C) 2% (wt/vol) PFA was then used to resuspend the pellet. Fixation with PFA proceeded for 30 min at room temperature. PFA-treated cells were recentri-

fuged at 18,000 × *g* for 1 min and washed once with 0.5 ml ice-cold 1.25 M glycine in PBS. After another recentrifugation step (18,000 × *g* for 1 min), the supernatant was discarded. Loading buffer (1× Laemmli buffer; Bio-Rad) was added to the cross-linked samples with or without βME. To reverse cross-linkages, samples were boiled for 20 min. To maintain partial cross-linkage, samples were heated for 10 min at 60°C before loading onto an SDS-PAGE gel for Western blot analysis.

Bacterial two-hybrid assays. A bacterial two-hybrid (BACTH) system (Euromedex) was used to identify potential protein-protein interactions between different MccPDI proteins (5) and the OmpF ligand from susceptible bacteria (8). Fusion proteins were first constructed for McpI, McpMΔ36 (mature McpM), or OmpF with either the T18 or T25 subunit of adenylate cyclase from *Bordetella pertussis* (29). According to standard molecular cloning techniques, primers (Table 3) and DNA templates (*mcpI*, *mcpM*Δ36, and *ompF*) were used to prepare PCR products for cloning. Restriction digestion and ligation (T4 ligate; New England Biolabs) were performed to insert PCR amplicons into plasmids pUT18 and pKNT25 (N-terminal T18/T25 adenylate cyclase subunits) or into pUT18C and pKT25 (C-terminal T18/T25). T18 and T25 vector constructs were individually transformed into *E. coli* XL1-Blue (Agilent) for plasmid propagation, and vector constructs were confirmed by Sanger sequencing (Eurofins Genomics). Plasmid vectors were cotransformed (e.g., a T18 and a T25 construct) into *E. coli* strain DHM1 (Table 1). The transformed bacteria were inoculated into LB broth with appropriate antibiotics at 30°C for 1 h with aeration before being plated onto LB agar that contained appropriate antibiotics, 50 mM 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) (Fisher Scientific), and 0.5 mM IPTG (Fisher Scientific) for blue and white screening. Plates were incubated for 1 to 4 days at 30°C in a stationary incubator.

Beta-galactosidase assays. We used a beta-galactosidase assay described previously by Karimova et al. (30) to verify potential protein-protein interactions that were identified from the bacterial two-hybrid assay. Briefly, a freshly grown colony was inoculated into 5 ml LB broth with 0.5 mM IPTG and incubated overnight on a shaker table at 37°C with appropriate antibiotics. The inoculant grown overnight (1 ml) was added to 5 ml M63 medium [15.1 mM $(NH_4)_2SO_4$, 100.0 mM KH_2PO_4, 1.8 μM $FeSO_4{\cdot}7H_2O$, 3.0 μM vitamin B_1 (pH 7.0) with KOH, and 0.4% (wt/vol) maltose], and the resulting OD_{600} was recorded. The diluted culture (1 ml) was transferred into a new glass 5-ml tube, to which toluene (30 μl) and 30 μl of 0.1% (wt/vol) SDS were added to permeabilize the bacteria, and this mixture was incubated for 30 min at 37°C with vigorous agitation. PM2 assay buffer (0.5 ml) (70 mM $Na_2HPO_4{\cdot}12H_2O$, 30 mM $NaH_2PO_4{\cdot}H_2O$, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$ at pH 7.0, and 100 mM βME) was added, and the solution was incubated at 28°C for 5 min before the addition of 0.25 ml *o*-nitrophenol-β-D-galactopyranoside (ONPG). An aliquot of this mixture (100 μl) was transferred to a single well of a 96-well microplate and incubated at 30°C until visible yellow color developed. The reaction was terminated with the addition of 50 μl stop solution (1 M Na_2CO_3). Data were recorded with a microplate reader at an OD_{420} (SpectraMax; Molecular Devices, LLC). The enzymatic activity, *A* (in units per milliliter), was calculated according to the following equation: $A = 200 \times [(OD_{420} - OD_{420}$ control tube)/total incubation time in minutes] × dilution factor. A level of β-galactosidase activity at least 5-fold higher than that measured for a negative control, DHM1(pKT25/pUT18C) cells, was considered positive for a protein-protein interaction (30).

Statistical analysis. Where appropriate, experimental results were compared by using one-way analysis of variance (ANOVA) with Dunnett's one-way multiple-pairwise-comparison test (SigmaPlot version 12.5; Systat Software, Inc., San Jose, CA).

Accession number(s). Newly determined sequence data for *ompF* (strain AR0349) were deposited in GenBank under accession number MH665273.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00371-19.

SUPPLEMENTAL FILE 1, PDF file, 6.6 MB.

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