Genome-Wide Screening Identifies Six Genes That Are Associated with Susceptibility to *Escherichia coli* Microcin PDI

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The microcin PDI inhibits a diverse group of pathogenic *Escherichia coli* strains. Coculture of a single-gene knockout library (BW25113; $n = 3,985$ mutants) against a microcin PDI-producing strain (*E. coli* 25) identified six mutants that were not susceptible ($\Delta atpA$, $\Delta atpF$, $\Delta dsbA$, $\Delta dsbB$, $\Delta ompF$, and $\Delta ompR$). Complementation of these genes restored susceptibility in all cases, and the loss of susceptibility was confirmed through independent gene knockouts in *E. coli* O157:H7 Sakai. Heterologous expression of *E. coli ompF* conferred susceptibility to *Salmonella enterica* and *Yersinia enterocolitica* strains that are normally unaffected by microcin PDI. The expression of chimeric *OmpF* and site-directed mutagenesis revealed that the K₄₇G₄₈N₄₉ region within the first extracellular loop of *E. coli OmpF* is a putative binding site for microcin PDI. *OmpR* is a transcriptional regulator for *ompF*, and consequently loss of susceptibility by the $\Delta ompR$ strain most likely is related to this function. Deletion of *AtpA* and *AtpF*, as well as *AtpE* and *AtpH* (missed in the original library screen), resulted in the loss of susceptibility to microcin PDI and the loss of ATP synthase function. Coculture of a susceptible strain in the presence of an ATP synthase inhibitor resulted in a loss of susceptibility, confirming that a functional ATP synthase complex is required for microcin PDI activity. In *trans* expression of *ompF* in the $\Delta dsbA$ and $\Delta dsbB$ strains did not restore a susceptible phenotype, indicating that these proteins are probably involved with the formation of disulfide bonds for *OmpF* or microcin PDI.

Escherichia coli strain 25 (*E. coli* 25; cattle origin) has an *in vitro* and *in vivo* competitive advantage against other *E. coli* strains that is linked to the production of the microcin PDI (MccPDI) (1–3). The inhibitory phenotype was first observed *in vitro* (4) and later called “proximity-dependent inhibition” (PDI), because inhibition occurred only when competing cells were in close proximity to sensitive cells (1). MccPDI appears to be most closely related to class IIa microcins, and the cluster of genes that encode MccPDI and associated immunity, activation, and export are located on a conjugative plasmid (2).

E. coli produces various antimicrobial bacteriocins that are classified as colicins or microcins. Microcins are distinguished by their lower molecular mass (<10 kDa) and require active transport across the membrane of producing cells. Microcins typically have a narrow spectrum of activity that is mediated through specific receptors expressed on the surface of susceptible bacteria. To date, 16 microcins have been described, including MccPDI. The receptors for seven of these microcins have been identified and include the outer membrane proteins Cir, FepA, Fiu, FhuA, and *OmpF*, all of which normally function in iron and other nutrient uptake (5–7).

While the gene encoding MccPDI has been identified and gene knockout and complementation studies have confirmed its inhibitory activity (2), little is known about how this microcin functions. The purpose of the current study was to identify any receptor and associated proteins required for MccPDI to recognize and inhibit susceptible *E. coli*. The *E. coli* Keio collection, a single-gene deletion library, was screened for mutants able to grow in the presence of the MccPDI-producing strain *E. coli* 25. The Keio collection is comprised of individual gene knockouts for all non-essential genes that are expressed by the PDI-susceptible *E. coli* strain BW25113. Screening of the full library followed by validation experiments demonstrated that strains lacking *atpA*, *atpF*,

dsbA, *dsbB*, *ompF*, or *ompR* were no longer sensitive to PDI, indicating that these genes are required for MccPDI to inhibit susceptible cells. Subsequent heterologous expression of *E. coli ompF* in *Salmonella enterica* and *Yersinia enterocolitica* conferred MccPDI susceptibility to these species. The mechanism by which MccPDI inhibits susceptible *E. coli* is not known, but here we demonstrate that MccPDI inhibition of susceptible cells requires a functional ATP synthase complex.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Bacterial strains used in this study (Table 1) were cultured in LB-Lennox medium (Difco, Franklin Lakes, NJ) or in M9 minimal medium (6 g liter⁻¹ Na₂HPO₄, 3 g liter⁻¹ KH₂PO₄, 0.5 g liter⁻¹ NaCl, 1 g liter⁻¹ NH₄Cl, 2 mg liter⁻¹ thiamine, 1 mM MgSO₄, 0.1 mM CaCl₂, and 0.2% glucose) at 37°C with shaking (200 rpm) unless otherwise indicated. Electroporation experiments were conducted using super optimal broth (SOB; 20 g liter⁻¹ Bacto tryptone, 5 g

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TABLE 1 Bacterial strains used in this study

Strain name	Designation	Relevant genotype/phenotype ^a	Reference or source
<i>E. coli</i> 25	E25	Wild type; SSuT ^r PDI ⁺	1
E25Δ <i>traM</i>	E25Δ <i>traM</i>	SSuT ^r Cm ^r PDI ⁺ , <i>traM</i> deletion prevents conjugation	3
E25Δ <i>mcpM</i>	E25Δ <i>mcpM</i>	SSuT ^r Cm ^r PDI ⁺ , <i>mcpM</i> deletion eliminates PDI inhibition phenotype	3
Keio collection			
BW25113	Kwt	Nal ^r , Keio collection wild-type K-12 strain	46
BW25113 (pMMB207 only)	Kwt(vector ctrl)	Nal ^r Cm ^r , BW25113 with empty pMMB207 vector	This study
51H12 Δ <i>atpA</i> (p207:: <i>atpA</i>)	KΔ <i>atpA</i> (<i>atpA</i>)	Kan ^r Cm ^r , <i>atpA</i> deletion complemented with <i>atpA</i>	This study
53B1 Δ <i>atpF</i> (p207:: <i>atpF</i>)	KΔ <i>atpF</i> (<i>atpF</i>)	Kan ^r Cm ^r , <i>atpF</i> deletion complemented with <i>atpF</i>	This study
65F11 Δ <i>dsbA</i> (p207:: <i>dsbA</i>)	KΔ <i>dsbA</i> (<i>dsbA</i>)	Kan ^r Cm ^r , <i>dsbA</i> deletion complemented with <i>dsbA</i>	This study
67G8 Δ <i>dsbB</i> (p207:: <i>dsbB</i>)	KΔ <i>dsbB</i> (<i>dsbB</i>)	Kan ^r Cm ^r , <i>dsbB</i> deletion complemented with <i>dsbB</i>	This study
7G4 Δ <i>ompF</i> (p207:: <i>ompF</i>)	KΔ <i>ompF</i> (<i>ompF</i>)	Kan ^r Cm ^r , <i>ompF</i> deletion complemented with <i>ompF</i>	This study
5F1 Δ <i>ompR</i> (p207:: <i>ompR</i>)	KΔ <i>ompR</i> (<i>ompR</i>)	Kan ^r Cm ^r , <i>ompR</i> deletion complemented with <i>ompR</i>	This study
7G4 Δ <i>ompF</i> (p207:: <i>dsbA</i>)	KΔ <i>ompF</i> (<i>dsbA</i>)	Kan ^r Cm ^r , <i>ompF</i> deletion expressing recombinant <i>dsbA</i>	This study
7G4 Δ <i>ompF</i> (p207 only)	KΔ <i>ompF</i> (vector ctrl)	Kan ^r Cm ^r , <i>ompF</i> deletion with empty pMMB207 vector	This study
51H12 Δ <i>atpA</i> (p207:: <i>ompF</i>)	KΔ <i>atpA</i> (<i>ompF</i>)	Kan ^r Cm ^r , <i>atpA</i> deletion expressing recombinant <i>ompF</i>	This study
53B1 Δ <i>atpF</i> (p207:: <i>ompF</i>)	KΔ <i>atpF</i> (<i>ompF</i>)	Kan ^r Cm ^r , <i>atpF</i> deletion expressing recombinant <i>ompF</i>	This study
65F11 Δ <i>dsbA</i> (p207:: <i>ompF</i>)	KΔ <i>dsbA</i> (<i>ompF</i>)	Kan ^r Cm ^r , <i>dsbA</i> deletion expressing recombinant <i>ompF</i>	This study
67G8 Δ <i>dsbB</i> (p207:: <i>ompF</i>)	KΔ <i>dsbB</i> (<i>ompF</i>)	Kan ^r Cm ^r , <i>dsbB</i> deletion expressing recombinant <i>ompF</i>	This study
5F1 Δ <i>ompR</i> (p207:: <i>ompF</i>)	KΔ <i>ompR</i> (<i>ompF</i>)	Kan ^r Cm ^r , <i>ompR</i> deletion expressing recombinant <i>ompF</i>	This study
O157:H7 Sakai	O157:H7	Wild type; Nal ^r	47
Δ <i>atpA</i>	O157:H7Δ <i>atpA</i>	Nal ^r Kan ^r , <i>atpA</i> deletion	This study
Δ <i>atpF</i>	O157:H7Δ <i>atpF</i>	Nal ^r Kan ^r , <i>atpF</i> deletion	This study
Δ <i>dsbA</i>	O157:H7Δ <i>dsbA</i>	Nal ^r Kan ^r , <i>dsbA</i> deletion	This study
Δ <i>dsbB</i>	O157:H7Δ <i>dsbB</i>	Nal ^r Kan ^r , <i>dsbB</i> deletion	This study
Δ <i>ompF</i>	O157:H7Δ <i>ompF</i>	Nal ^r Kan ^r , <i>ompF</i> deletion	This study
Δ <i>ompR</i>	O157:H7Δ <i>ompR</i>	Nal ^r Kan ^r , <i>ompR</i> deletion	This study
<i>ompF</i> heterologous expression, chimeric expression, and in <i>cis</i> His tagged			
7G4 Δ <i>ompF</i> (p207::Se. <i>ompF</i>)	KΔ <i>ompF</i> (Se. <i>ompF</i>)	Kan ^r Cm ^r , <i>ompF</i> deletion complemented with <i>Salmonella ompF</i>	This study
7G4 Δ <i>ompF</i> (p207:: <i>ompF</i> _{Ec1-72+Se68-363})	KΔ <i>ompF</i> (<i>ompF</i> _{Ec+Se})	Kan ^r Cm ^r , <i>ompF</i> deletion complemented with N-terminal sequence of <i>E. coli ompF</i> (bases 1–72) and C-terminal sequence (bases 68–363) of <i>Salmonella ompF</i>	This study
7G4 Δ <i>ompF</i> (p207:: <i>ompF</i> _{Se1-68+Ec73-362})	KΔ <i>ompF</i> (<i>ompF</i> _{Se+Ec})	Kan ^r Cm ^r , <i>ompF</i> deletion complemented with N-terminal sequence of <i>Salmonella ompF</i> (bases 1–68) and C-terminal sequence (bases 73–362) of <i>E. coli ompF</i>	This study
7G4 Δ <i>ompF</i> (p207::Ec. <i>ompF</i> ΔSYGG)	KΔ <i>ompF</i> (Ec.ΔSYGG)	Kan ^r Cm ^r , <i>ompF</i> deletion complemented with <i>E. coli ompF</i> sequence containing an SYGG deletion	This study
7G4 Δ <i>ompF</i> (p207::Ec. <i>ompF</i> ΔKGN)	KΔ <i>ompF</i> (Ec.ΔKGN)	Kan ^r Cm ^r , <i>ompF</i> deletion complemented with <i>E. coli ompF</i> sequence containing a KGN deletion	This study
<i>S. enteritidis</i> 22577(p207::Ec. <i>ompF</i>)	Se22577(Ec. <i>ompF</i>)	Nal ^r Cm ^r , Se22577 expressing recombinant <i>E. coli ompF</i>	This study
<i>Y. enterocolitica</i> JB580v(p207::Ec. <i>ompF</i>)	JB580v(Ec. <i>ompF</i>)	Nal ^r Cm ^r , JB580v expressing recombinant <i>E. coli ompF</i>	This study
BW25113:: <i>ompF</i> -His	Kwt:: <i>ompF</i> -His	Nal ^r Kan ^r , in <i>cis</i> His-tag insertion at C-terminus of <i>ompF</i>	This study
<i>S. enteritidis</i> 22577:: <i>ompF</i> -His	Se22577:: <i>ompF</i> -His	Nal ^r Kan ^r , <i>S. enteritidis</i> strain 22577 with in <i>cis</i> His tag insertion at C terminus of <i>ompF</i>	This study
Other			
BW25113Δ <i>tonB</i> Δ <i>tolA</i>	KΔ <i>tonB</i> Δ <i>tolA</i>	Kan ^r Cm ^r , <i>tonB</i> and <i>tolA</i> deletion strain, BW25113 background	This study
BW25113Δ <i>tonB</i> Δ <i>tolQ</i>	KΔ <i>tonB</i> Δ <i>tolQ</i>	Kan ^r Cm ^r , <i>tonB</i> and <i>tolQ</i> deletion strain, BW25113 background	This study
BW25113Δ <i>exbD</i> Δ <i>tolQ</i>	KΔ <i>exbD</i> Δ <i>tolQ</i>	Kan ^r Cm ^r , <i>exbD</i> and <i>tolQ</i> deletion strain, BW25113 background	This study
BW25113Δ <i>exbD</i> Δ <i>tolR</i>	KΔ <i>exbD</i> Δ <i>tolR</i>	Kan ^r Cm ^r , <i>exbD</i> and <i>tolR</i> deletion strain, BW25113 background	This study
<i>S. enteritidis</i> 22577	Se22577	Wild type, Nal ^r , <i>Salmonella enterica</i> serovar Enteritidis 22577	This study
<i>S. enteritidis</i> 21679	Se21679	Wild type, Nal ^r , <i>Salmonella enterica</i> serovar Enteritidis 21679	This study
<i>Y. enterocolitica</i> JB580v	JB580v	Nal ^r , <i>Yersinia enterocolitica</i> strain JB580v	48

^a SSuT^r, resistant to streptomycin, sulfonamide, and tetracycline antibiotics.

liter⁻¹ Bacto yeast extract, 0.5 g liter⁻¹ NaCl, 2.5 mM KCl, and 10 mM MgCl₂) and super optimal broth with catabolite repression (SOC; SOB with 20 mM glucose). The *E. coli* Keio knockout collection (termed the Keio collection), a set of single-gene deletion mutants for all nonessential

genes in *E. coli* K-12 strain BW25113, was purchased from Thermo Fisher Scientific Inc. To make it possible to isolate the wild-type BW25113 strain from a mixed culture, the strain was sequentially passaged five times in LB broth with increasing concentrations of antibiotic until it was capable of

growing in the presence of 30 $\mu\text{g ml}^{-1}$ nalidixic acid (1). Keio collection knockout strains originally were constructed by inserting a kanamycin resistance cassette into the gene of interest. For the current project, these strains were selected in culture and on agar plates with the addition of kanamycin. Depending on the experiment, antibiotics were added to media at the following concentrations: tetracycline, 50 $\mu\text{g ml}^{-1}$; chloramphenicol, 34 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; nalidixic acid, 30 $\mu\text{g ml}^{-1}$; and ampicillin, 100 $\mu\text{g ml}^{-1}$.

Screening for loss of MccPDI susceptibility. The Keio collection was employed to identify genes associated with susceptibility to MccPDI. Each mutant was grown overnight at 37°C without shaking in a 96-well plate containing 150 μl fresh LB (with kanamycin) per well. A 10-ml culture of *E. coli* 25 also was started at this time in LB (with tetracycline) and incubated at 37°C with shaking (200 rpm). The following day each mutant was individually placed into coculture with *E. coli* 25 in a sterile, U-bottom 96-well plate with 200 μl M9 medium per well. A 96-pin replicator (Boeckel Scientific, Feasterville, PA) was used to transfer overnight cultures ($\sim 1 \mu\text{l}$) of each strain for these competition experiments. The replicator was sterilized 3 $\times$ between each use by submerging pins into 70% ethanol and flaming. The *E. coli* 25 culture was poured into a sterile plastic trough and transferred in the same manner. Competition cultures were incubated overnight at 37°C, with shaking at 100 rpm. Approximately 24 h later cocultures ($\sim 1 \mu\text{l}$) were transferred onto LB agar containing kanamycin or tetracycline to select for the Keio strains or *E. coli* 25, respectively. The plates were incubated at 37°C for at least 6 h. Growth on tetracycline verified the presence of *E. coli* 25 in the culture. No growth on kanamycin indicated that the Keio knockout strain being tested was still susceptible to PDI. Growth of a Keio mutant strain on the kanamycin plate indicated the identification of gene knockout strains that potentially were no longer susceptible to PDI.

Direct competition assays. Bacterial strains were grown individually overnight in LB medium with the appropriate antibiotics. Equal volumes of each competing strain were inoculated into fresh M9 medium at a 1:200 dilution, and then the mixed cultures were incubated at 37°C for 24 h. When necessary, isopropyl- β -D-thiogalactopyranoside (IPTG; 100 μM unless specified otherwise) and chloramphenicol were added into M9 medium during the competition. Monocultures of each competing strain also were prepared as controls after inoculation into fresh M9 medium at the same dilution. The ATP synthase inhibitor resveratrol (Sigma-Aldrich) was added at a final concentration of 25 $\mu\text{g ml}^{-1}$ to determine if a functional ATP synthase complex is required for susceptibility to MccPDI (8). To quantify the CFU of each strain following competition, each culture was serially diluted in a 96-well plate containing sterile PBS and dropped (5 μl) onto LB agar supplemented with the appropriate antibiotics to select for each competing strain. Agar plates (3 per enumerated dilution) were incubated overnight at 37°C, and colonies were tallied to estimate total CFU.

Complementation of six Keio mutant strains. Genomic DNA from strain BW25113 was extracted by using a DNeasy blood and tissue kit by following the manufacturer's instructions (Qiagen, Valencia, CA). Primers (Table 2) were used to amplify the open reading frames of six genes from BW25113 genomic DNA with the addition of sequence to synthesize Flag tags at the C terminus of each recombinant protein. Each amplified fragment was digested with corresponding restriction enzymes and cloned into the vector pMMB207 (9), resulting in six recombinant plasmids: p207::atpA, p207::atpF, p207::dsbA, p207::dsbB, p207::ompF, and p207::ompR. The pMMB207 vector contains a chloramphenicol resistance gene. These plasmids were individually electroporated into *E. coli* S17-1 λ pir, after which these constructs were introduced by conjugation into the corresponding six different mutant strains from the Keio collection (strains 51H12, 53B1, 65F11, 67G8, 7G4, and 5F1) to generate the Δ atpA(atpA), Δ atpF(atpF), Δ dsbA(dsbA), Δ dsbB(dsbB), Δ ompF(ompF), and Δ ompR(ompR) complemented strains (Table 1). Δ ompF(dsbA) and Δ ompF(vector ctrl) strains also were constructed as controls to confirm that complementation was specific for a targeted trait.

For conjugation, donor S17-1 λ pir strains and recipient strains were inoculated separately into LB broth with appropriate antibiotics and incubated overnight at 37°C or 30°C. Overnight cultures then were mixed (500 μl of each) in a 1.5-ml tube, and the cells were pelleted by centrifugation (1,500 $\times$ g, 5 min, room temperature). The pellet was washed once with 1 ml of LB and then resuspended in 50 μl of LB. The conjugation mixture was spotted onto an LB agar plate and incubated overnight at 37°C or 30°C. Conjugants were isolated by directly streaking onto selective plates. All resulting complemented strains were competed against the *E. coli* 25 Δ traM strain in M9 medium with IPTG and chloramphenicol as described above. For competition experiments with the Δ ompF(ompF) strain, the concentration of IPTG was reduced to 20 μM . The *E. coli* 25 Δ traM strain was used for these experiments because it produces the PDI phenotype against susceptible strains but is nonconjugative due to the insertion of a chloramphenicol gene in traM (3).

PCR-mediated gene deletion. Gene-specific knockouts were generated using the homologous recombination system described by Datsenko and Wanner (10). Briefly, the gene of interest was replaced with a PCR-generated kanamycin or chloramphenicol resistance gene that originated from pKD4 or pKD3, respectively. Each primer incorporated a 36- to 50-nucleotide (nt) segment complementary to the region flanking the gene of interest as described in Table 2. PCR products were column purified using a QIAquick PCR purification kit (Qiagen), digested overnight at 37°C with DpnI (New England BioLabs, Ipswich, MA), purified again, and suspended in 30 μl 10 mM Tris, pH 8.0. The target strains carrying the λ Red plasmid pKD46 (Amp^r) were grown in SOB medium with 1 mM L-arabinose at 30°C to an optical density at 600 nm (OD₆₀₀) of ~ 0.6 . The cells then were made electrocompetent by washing twice with ice-cold water and once with 10% glycerol and concentrating the cells 100-fold in 10% glycerol. Electrocompetent cells (50 μl) were pulsed with $\sim 150 \text{ ng}$ of purified PCR product using a Gene Pulser Xcell (Bio-Rad, Hercules, CA). SOC medium was immediately added to the cells that were then incubated for 2 h at 30°C. Cells were plated on LB with kanamycin or chloramphenicol and incubated overnight at 30°C to select for transformants. PCR was used to verify the resistance gene cassette integrated at the desired location using primers within the antibiotic resistance gene combined with genomic primers adjacent to the sequence of interest (Table 2).

Heterologous ompF expression. MccPDI does not function against *Salmonella* or *Yersinia* (see Results). To determine if susceptibility can be conferred to these bacteria, the recombinant expression plasmid for *E. coli* ompF (p207::ompF) was introduced by conjugation from *E. coli* S17 λ pir into *S. enterica* serovar Enteritidis 22577 and *Yersinia enterocolitica* JB580v to generate Se22577(Ec.ompF) and JB580v(Ec.ompF) strains, respectively (Table 1). Both strains then were competed against the *E. coli* 25 Δ traM or *E. coli* 25 Δ mcpM strain in M9 medium with IPTG and chloramphenicol as described above. For these experiments, the *E. coli* 25 Δ mcpM strain, which does not synthesize a functional MccPDI (3), was used as a negative control. Heterologous expression of the recombinant OmpF was confirmed by Western blotting.

Western blotting. Strains were harvested from broth culture by centrifugation (13,800 $\times$ g, 2 min, 4°C), and the pellet was resuspended in Laemmli sample buffer and boiled for 5 min. The samples were electrophoresed by using Mini-Protean precast gels and subsequently transferred to a low-fluorescence polyvinylidene difluoride (LF-PVDF) membrane using the Trans-Blot Turbo transfer system (Bio-Rad). Membranes were blocked with 5% skim milk in Tris-buffered saline and probed with a His tag monoclonal antibody (1:2,500 dilution; Novagen), Flag-tagged monoclonal antibody (1:1,000; Thermo Scientific), or anti-DnaK monoclonal antibody (1:1,000; Enzo Life Sciences, Farmingdale, NY) for 1 h at room temperature. Blots then were incubated with anti-mouse DyLight 488 conjugate (1:5,000; Thermo Scientific) for 1 h at room temperature, followed by visualization using a ChemiDoc MP imaging system (Bio-Rad).

In cis expression of ompF fused with a C-terminal His tag. To generate an in-frame, 6-histidine tag on the C terminus of the endogenous ompF gene in *E. coli* strain BW25113 and *Salmonella* serovar Enteritidis

TABLE 2 PCR primers used in this study

Primer name	Sequence ^a (5'–3')	Purpose
atpA_p207_KpnI	CGGGGTACCATGCAACTGAATTCCACCGA	Used to construct recombinant plasmid for <i>atpA</i> complementation
atpA_p207_HindIII	CCCAAGCTTTTACTTGTGTCGTCATCGTCTTTGTAGTCCCAGGATTGGGT TGCTTTGA	
atpF_p207_EcoRI	CCGGAATTCATGGTGAATCTTAACGCAACAA	Used to construct recombinant plasmid for <i>atpF</i> complementation
atpF_p207_SalI	ACGCGTCGACTTACTTGTGTCGTCATCGTCTTTGTAGTCCAGTTCAGCGA CAAGTTTATC	
dsbA_p207_EcoRI	CCGGAATTCATGAAAAAGATTGGCTGG	Used to construct recombinant plasmid for <i>dsbA</i> complementation
dsbA_p207_SalI	ACGCGTCGACTTACTTGTGTCGTCATCGTCTTTGTAGTCTTTTTTCTCGGACAG ATATTTTC	
dsbB_p207_EcoRI	CCGGAATTCATGTTGCGATTTTGAACCA	Used to construct recombinant plasmid for <i>dsbB</i> complementation
dsbB_p207_SalI	ACGCGTCGACTTACTTGTGTCGTCATCGTCTT TGTAAGTCGCGACCGAACAGATCACGTT	
ompF_p207_EcoRI	CCGGAATTCATGATGAAGCGCAATATT	Used to construct recombinant plasmid for <i>ompF</i> complementation
ompF_p207_SalI	ACGCGTCGACTTACTTGTGTCGTCATCGTCTTTGTAGTCGAACTGGTAAACGATACC	
ompR_p207_EcoRI	CCGGAATTCATGCAAGAGAACTACAAGA	Used to construct recombinant plasmid for <i>ompR</i> complementation
ompR_p207_SalI	ACGCGTCGACTTACTTGTGTCGTCATCGTCTTTGTAGTCTGCTTTAGAGCCG TCCGGTA	
ΔatpA_H1_P1	CTTGACAGACGTCTTGACAGTCTTAAGGGGACTGGAGC	Used to generate <i>atpA</i> knockout strain
ΔatpA_H2_P2	GCCTTGCGGCCTGCCCTAAGGCAAGCCGCCAGACGT	
atpA_up	TGCTGCGATGGAAAAACGTC	
atpA_down	TTCTGGACGCTTGCGATCTT	
ΔatpF_H1_P1	TTTATTTAAAGAGCAATATCAGAACGTTAACTAAATAGAGGCATTGTGCT	Used to generate <i>atpF</i> knockout strain
ΔatpF_H2_P2	GGCGTAGGGCGAGCTACCGTAATAAAATTCAGACATCAGCCCTCCCTCC	
atpF_up	ATCGCTGTAGGTCTGGGTCT	
atpF_down	ATGTCCTGCCAGCGTTCTAC	
ΔdsbA_H1_P1	GTGAATATTCACGGGCTTTATGTAATTTACATTGAA	Used to generate <i>dsbA</i> knockout strain
ΔdsbA_H2_P2	AATTAACACCTATGTATTAATCGGAGAGAGTAGATC	
dsbA_up	AGCGGCAGGATGCATTATCA	
dsbA_down	GGGAAGATTACTGGCTGCGA	
ΔdsbB_H1_P1	AATAAATATAGCGGCAGGAAAAAAGCGCTCCCGCAGGAGCGCCGAATGGA	Used to generate <i>dsbB</i> knockout strain
ΔdsbB_H2_P2	AATGAATTGGTTTAACTGCGCACTCTATGCATATTGCAGGGAAATGATT	
dsbB_up	CAATGGCAGATGAAGCGAGC	
dsbB_down	TGCAAATGGGCTGGATAGCA	
ΔompF_H1_P1	GTTGTCAGAATCGATCTGGTTGATGATGTAGTCAAC	Used to generate <i>ompF</i> knockout strain
ΔompF_H2_P2	GTGATCGTCCCTGCTCTGTTAGTAGCAGGTACTGCA	
ompF_up	CGCTATCAGGGTAACGGGAG	
ompF_down	AGCACTTTCACGGTAGCGAA	
ΔompR_H1_P1	GGGCAAATGAACTTCGTGGCGAGAAGCGCAATCGCC	Used to generate <i>ompR</i> knockout strain
ΔompR_H2_P2	CTTACAAATTGTTGCGAACCTTTGGGAGTACAAACA	
ompR_up	TGTTGCGAACCTTTGGGAGT	
ompR_down	AGCAAGGTGACGATGAGCAA	
Ec.ompF_H1_His_P1	GACGACACGGTGTGCTGGGTATCGTTTACCAGTTCCATCATCACCATCA CCATTAA	In <i>cis</i> addition of His tag (C terminal) to <i>ompF</i> in <i>E. coli</i> strain BW25113
Ec.ompF_H2_P2	AAGTCCTGTTTTTTCGGCATTAAACAAAGAGGTGTG	
Se.ompF_H1_His_P1	GACGATCAGGCGGCTGTCGGTATTACTTACCAGTTCCATCATCACCAT CACCATTGA	In <i>cis</i> addition of His tag (C terminal) to <i>ompF</i> in <i>S. enteritidis</i> strain 22577
Se.ompF_H2_P2	AGTCCTGTTTTTGAGGCATAAAACAAAGGGGTCTG	
Ec1-72_EcoRI	CCGGAATTCATGATGAAGCGCAATATT	Used to construct KΔompF(<i>ompF</i> _{Ec+Se})
Ec1-72_R	CGGTGTTAATTTGAGTTTCCCTTTAAACCA	
Se69-363_F	GGAAACTCAAATTAACACCGATCTGACCGGT	
Se69-363_SalI	ACGCGTCGACTCAATGGTGATGGTGATGATGGAAGTGGTAAAGTAATACCGA	
Se1-68_EcoRI	CCGGAATTCATGATGAAGCGCAAAATCCT	Used to construct KΔompF(<i>ompF</i> _{Se+Ec})
Se1-68_R	CGGAATTGATCTGCGTTTCCCTTTAA	
Ec73-362_F	GGAAACGCAGATCAATTCCGATCTGACCG	
Ec73-362_SalI	ACGCGTCGACTTAATGGTGATGGTGATGATGGAAGTGGTAAACGATACC	

(Continued on following page)

TABLE 2 (Continued)

Primer name	Sequence ^a (5'–3')	Purpose
ΔSYGG-F	AATGGCGACATGACCTATG	Used to construct KΔompF(Ec.ΔSYGG)
ΔSYGG-R	GTTTTACCGTTACCTTG	
ΔKGN-F	GGTAAAAACAGTTACGGTG	
ΔKGN-R	GGAAAAATAATGCAGACCAAC	
ΔtolA_H1_P1	CTTGCTTGAAAGAGAGCGGGTAACAGGCGAACAGTTTTTGGAAACCGAGA	Used to knock out <i>tolA</i> for the Δ <i>tonB</i> knockout strain (Keio collection no. 67A9)
ΔtolA_H2_P2	CCGTGGCAACCGGTGCCTGATGTTGACCGTCCGAACAGTCAACATCGCGA	
tolA_up	TAATGACGCAGCCTATCTAA	
tolA_down	ATGCCTGCTTCATCATATCT	
ΔtolQ_H1_P1	AAAATGAAGCCTCGTGCCTTCCCAAGTCTATTGTGCGGGAGTTTAAGCA	Used to knock out <i>tolQ</i> for the Δ <i>tonB</i> knockout strain (Keio collection no. 67A9)
ΔtolQ_H2_P2	ATTCGGACTTGAGATCGCGACGACCTCGTCCACGCGCTCTGGCCATGGC	
tolQ_up	GTCAACGCCGAGAATACT	
tolQ_down	ACCAATACCAGACACTTCAA	
ΔtolR_H1_P1	TTCTGCACGCCAGGCGTTTACCGTTAGCGAGAGCAACAAGGGGTAAGCC	Used to knock out <i>tolR</i> for the Δ <i>exbD</i> knockout strain (Keio collection no. 57A12)
ΔtolR_H2_P2	AACTGTTTCGCTGTTACCCGCTCTCTTTCAAGCAAGGGAAACGCAGATGT	
tolR_up	TGGAAGTGAATTACGACAAC	
tolR_down	CAACTACCGCACCTGAAT	
P1	TGTGTAGGCTGGAGCTGCTTCG	Used to amplify the kanamycin or chloramphenicol resistance genes
P2	CATATGAATATCCTCCTTA	
C1	TTATACGCAAGGCGACAAGG	Used to verify the resistance gene cassette integration at the desired location combined with gene specific primers
C2	GATCTTCCGTCACAGGTAGG	
K1	CAGTCATAGCCGAATAGCCT	
K2	CGGTGCCCTGAATGAAGTGC	

^a For gene deletion mutants, the kanamycin or chloramphenicol primers P1 and P2 were added to the 3' end of homologous extension sequences (H1 and H2) of the *E. coli*-specific gene. Restriction enzyme sites are underlined. PCRs were performed in a volume of 50 μl containing 45 μl of Platinum PCR supermix (Invitrogen, Carlsbad, CA), 1 U *Taq* DNA polymerase, 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl₂, 220 μM each deoxynucleoside triphosphate, and stabilizers. PCR was carried out under the following conditions: 2 min at 94°C for 1 cycle; 30 s at 94°C, 30 s at 55°C, and 2 min at 68°C for 30 cycles; and 10 min at 68°C for 1 cycle.

strain 22557, the stop codon of *ompF* was replaced with a 6× histidine-stop sequence and a kanamycin resistance gene by using methods described by Datsenko and Wanner (10). Briefly, 36-nt sequences flanking the stop codon of *ompF* were chosen as homologous extension arms (H1 and H2) (Table 2). The His tag sequence was added to the C terminus of arm H1. These primers then were used to amplify the kanamycin resistance gene cassette from the pKD4 plasmid, and the remaining protocol was followed as described above.

OmpF chimera from *E. coli* and *S. enterica* serovar Enteritidis. Splice overlap extension PCR (11) was employed to construct two chimeric *ompF* sequences (*ompF*_{Ec1–72+Se68–363} [i.e., amino acids 1 to 72 of *E. coli ompF* and amino acids 68 to 363 of serovar Enteritidis *ompF*] and *ompF*_{Se1–68+Ec73–362}) based on templates from *E. coli* (strain BW25113) and *S. enterica* serovar Enteritidis (strain 22577). For the *ompF*_{Ec1–72+Se68–363} construct, primers Ec1-72_EcoRI, which incorporated an EcoRI site, and Ec1-72_R were paired, while primers Se69-363_F and Se69-363_Sall, which incorporated a Sall site, were paired to amplify the corresponding regions (Table 2). Both fragments contained a 10-bp complementary sequence and were used as the templates for the next PCR procedure, which used primers Ec1-72_EcoRI and Se69-363_Sall. The resulting fragment, containing amino acids 1 to 72 of *E. coli ompF* and amino acids 68 to 363 of serovar Enteritidis *ompF*, was inserted into the pMMB207 vector, generating recombinant plasmid p207::*ompF*_{Ec1–72+Se68–363}, to express the chimeric sequence of *E. coli ompF* and serovar Enteritidis *ompF* (*ompF*_{Ec+Se}). A sim-

ilar strategy was used to construct and express the *ompF*_{Se1–68+Ec73–362} chimeric sequence (*ompF*_{Se+Ec}) (Table 1).

Site-directed mutagenesis. Based on the chimeric OmpF data and amino acid variation between *E. coli*, *S. enterica*, and *Y. enterocolitica*, we identified the amino acid positions from OmpF that were most likely to play a functional role in binding to MccPDI. The corresponding DNA sequences in *E. coli* strain BW25113 were deleted by using mutagenesis primers (Table 2) and a Q5 site-directed mutagenesis kit (New England BioLabs). The manufacturer's protocol was followed to generate both the KΔompF(Ec.ΔSYGG) and KΔompF(Ec.ΔKGN) strains (Table 1).

Statistical analysis. One-way analysis of variance (ANOVA) was used to compare experimental results with pairwise comparisons made by using a Dunnett's one-way multiple-comparison test (SigmaPlot, version 12.5; Systat Software, Inc., San Jose, CA).

RESULTS

Six genes in *E. coli* are requisite for, or are correlated with susceptibility to, MccPDI. Mutants from the Keio collection ($n = 3,985$) were screened using a high-throughput 96-well plate method. Following two rounds of screening, six mutants (51H12, 53B1, 65F11, 67G8, 7G4, and 5F1) (Table 1) were consistently identified as resistant to inhibition by *E. coli* 25. These mutants then were placed into direct competition experiments (M9 medium) with *E. coli* 25, and all six strains were able to grow to a

TABLE 3 Gene knockout strains from the Keio collection that are no longer susceptible to killing by *E. coli* 25

Strain ID ^a	Gene knockout	Strain or gene function ^b	Log ₁₀ CFU/ml ± SEM recovered after coculture with ^c	
			<i>E. coli</i> 25	<i>E. coli</i> 25Δ <i>mcpM</i>
BW25113	None	Wild-type <i>E. coli</i> K-12 from Keio collection; positive control	3.48 ± 0.30	8.20 ± 0.04
51H12	<i>atpA</i>	ATP synthase, F ₁ complex, α subunit; also called <i>papA</i>	5.61 ± 0.20	6.57 ± 0.26
53B1	<i>atpF</i>	ATP synthase, F _o complex, β subunit; also called <i>papF</i>	6.81 ± 0.39	7.33 ± 0.17
65F11	<i>dsbA</i>	Periplasmic protein disulfide isomerase; involved with disulfide bond formation	7.37 ± 0.06	8.22 ± 0.11
67G8	<i>dsbB</i>	Disulfide oxidoreductase; involved with disulfide bond formation membrane protein; oxidizes periplasmic DsbA	7.60 ± 0.38	8.33 ± 0.17
7G4	<i>ompF</i>	Outer membrane protein, porin	6.98 ± 0.14	7.97 ± 0.08
5F1	<i>ompR</i>	Transcriptional regulatory protein OmpR; response regulator for osmoregulation	6.33 ± 0.33	8.27 ± 0.07

^a Strain identifier (ID) or Keio collection clone ID.
^b Putative gene function as defined in the Ecogene 3.0 database using the structural and functional annotation of the *E. coli* K-12 genome (<http://ecogene.org>).
^c Average CFU (±SEM) recovered after coculture with *E. coli* 25 (*n* = 3 independent replicates); recovery of >5 log₁₀ CFU/ml was considered resistant to MccPDI.

population density of 5.6 to 7.6 log CFU after 24 h, whereas the isogenic control (BW25113) only grew to a density of ~3.5-log CFU (Table 3). These knockout strains grew to a slightly higher density (6.5 to 8.3-log CFU) when competed against the *E. coli* 25Δ*mcpM* MccPDI knockout strain, which is defective in MccPDI synthesis and also harbors a constitutively expressed chloramphenicol resistance gene (Table 3).

To rule out the contribution of polar effects from insertion of a kanamycin resistance gene during construction of the Keio collection knockout strains, complementation experiments demonstrated that susceptibility to MccPDI could be restored in all cases, while the KΔ*ompF*(*dsbA*) and KΔ*ompF*(vector ctrl) control strains maintained resistance to MccPDI (Fig. 1). To validate these findings, independent deletion mutants for the six genes were generated in an *E. coli* O157:H7 Sakai background. Competition assays with *E. coli* 25 confirmed loss of sensitivity to MccPDI (see Fig. S1

in the supplemental material). Collectively, these data indicate that, at a minimum, these six genes each need to be expressed for *E. coli* to be susceptible to MccPDI.

OmpF is necessary for susceptibility to MccPDI. Because OmpF is a confirmed receptor for other microcins (12) and is the only known outer membrane protein from our list of six candidates, we hypothesized that heterologous expression of *E. coli ompF* in other bacterial species would make them susceptible to MccPDI. *S. enterica* and *Y. enterocolitica* strains synthesize OmpF homologues (see Fig. S2 in the supplemental material), but these species of bacteria are not susceptible to MccPDI (see Fig. S3). In *trans* expression of *E. coli ompF* (*Ec.ompF*) in *S. enterica* strain 22577 and *Y. enterocolitica* strain JB580v (Fig. 2A) conferred susceptibility to MccPDI (Fig. 2B).

These findings are consistent with OmpF being necessary for MccPDI to inhibit susceptible *E. coli*. Nevertheless, similar results might arise if susceptibility to MccPDI is a function of the abundance of OmpF in the outer membrane (*E. coli* or otherwise). Under this scenario, the expression of recombinant OmpF would exceed the threshold abundance needed for a bacterium to become susceptible to MccPDI. To test this alternative, the abundance of *in cis* His-tagged OmpF was qualitatively assessed for both the *E. coli* and *S. enterica* serovar Enteritidis strains. Western blotting showed that for the culture conditions employed in this study, the total protein level of native OmpF in *S. enterica* is substantially greater than that of *E. coli* (Se222577::ompF-His and Kwt::ompF-His, respectively) (Fig. 3A). Furthermore, when recombinant *S. enterica ompF* was expressed in the *E. coli* KΔ*ompF*(*Se.ompF*) strain, it did not recover the susceptible phenotype when competed against the E25Δ*traM* strain (Fig. 3B). These results are consistent with a specific MccPDI-*E. coli* OmpF interaction rather than a quantitative effect dependent on OmpF abundance.

The N terminus of *E. coli* OmpF drives the specificity of MccPDI. The extracellular regions (loops) of OmpF differ between *E. coli* and MccPDI-resistant species (*S. enterica*, *Y. enterocolitica*, and *Klebsiella pneumoniae*; see Fig. S2 in the supplemental material), suggesting that these regions serve as a specific ligand for MccPDI (13). To narrow the possibilities, two chimeric *ompF* sequences were constructed (*ompF*_{Ec1-72+Se68-363} and *ompF*_{Se1-68+Ec73-362}) (Table 1). After complementation into the Keio collection Δ*ompF* strain (7G4), competition against an MccPDI-producing strain (the E25Δ*traM* strain) demon-

FIG 1 Complementation restores susceptibility to MccPDI for the six Keio collection strains. The *ompF* mutant strain (KΔ*ompF*) with recombinant *dsbA* and empty pMMB207 vector were used as controls, indicating that complementation effects were specific. Results are expressed as the difference between mean log CFU (± standard errors of the means [SEM]; *n* = 3 independent experiments) during coculture and that during monoculture. *, significant ANOVA followed by a Dunnett's one-way multiple-comparison test versus control group (*P* < 0.001).

FIG 2 Heterologous expression of *E. coli ompF* converts resistant strains into MccPDI-susceptible strains. (A) Western blot detection of *E. coli* OmpF synthesis in *Salmonella enteritidis* (strain 22577) and *Yersinia enterocolitica* (strain JB580v). Lane 1, wild-type strains; lane 2, *Salmonella* 22577(*Ec.ompF*) and *Yersinia* JB580v(*Ec.ompF*) without IPTG induction; lane 3, *Salmonella* 22577(*Ec.ompF*) and *Yersinia* JB580v(*Ec.ompF*) after induction with IPTG. (B) Competition with *E. coli* 25 Δ *traM* (microcin-producing) and *E. coli* 25 Δ *mcpM* (no microcin production, negative control) after heterologous expression of *E. coli ompF* in *Salmonella* and *Yersinia*. Results are expressed as the difference between mean log CFU ($\pm$ SEM; $n = 3$ independent experiments) during coculture and monoculture. *, significant ANOVA ($P < 0.001$) for comparisons between paired competitions (*E. coli* 25 Δ *traM* strain versus *E. coli* 25 Δ *mcpM* strain).

trated that the *OmpF*_{Ec1-72+Se68-363} (*ompF*_{Ec+Se}) chimera restored susceptibility to MccPDI, while the second chimera, *OmpF*_{Se1-68+Ec73-362} (*ompF*_{Se+Ec}), did not (Fig. 3B). Consequently, the N-terminal sequence (amino acids 1 to 72) of the *E. coli* OmpF probably is required for interaction with MccPDI.

Based on these findings, the interspecific diversity of OmpF amino acid composition (see Fig. S2 in the supplemental material), and the crystal structure of OmpF (PDB accession number 2ZFG), two potential binding regions were identified (K₄₇G₄₈N₄₉ and S₅₃Y₅₄G₅₅G₅₆), both of which are located in the first extracellular loop of *E. coli* OmpF. These regions subsequently were deleted as part of a recombinant protein, and the modified sequences were expressed in a Δ *ompF* background [*KΔompF*(*Ec.ΔKGN*) and *KΔompF*(*Ec.ΔSYGG*), respectively]. Competition between these strains and an MccPDI-producing strain (the E25 Δ *traM* strain) indicated that deletion of K₄₇G₄₈N₄₉ blocked MccPDI susceptibility, whereas deletion of S₅₃Y₅₄G₅₅G₅₆ had no impact on

MccPDI susceptibility (Fig. 4). Together, these results indicate that K₄₇G₄₈N₄₉ within the first extracellular loop of *E. coli* OmpF is a binding site for MccPDI. Alternatively, it is possible that some of our OmpF chimeras and mutants, which lacked an MccPDI-susceptible phenotype, were not displayed in the membrane properly due to either improper folding or trafficking.

MccPDI activity requires a functional ATP synthase complex. Preliminary screening of the Keio collection demonstrated that Δ *atpA* and Δ *atpF* strains are no longer susceptible to MccPDI (Table 2). Both AtpA and AtpF are components of *E. coli* ATP synthase, which contributes to the production of ATP (14). Direct competition experiments between wild-type E25 and other knockout strains for the ATP synthase complex (*KΔatpB* [strain 54D1], *KΔatpC* [strain 45E6], *KΔatpD* [strain 45F6], *KΔatpG* [strain 89C11], and *KΔatpI* [strain 71G2]) did not confer resistance to MccPDI (see Fig. S4A in the supplemental material). In contrast, both *KΔatpE* (strain 54C1) and *KΔatpH* (strain 54A1) strains were resistant to MccPDI (see Fig. S4A).

Strains of *E. coli* with nonfunctional ATP synthase can grow on agar plates that are supplemented with glucose but not on plates that are supplemented with succinate or other C4-dicarboxylates (15). Accordingly, we cultured the Keio collection ATP synthase mutants on M9 agar plates supplemented with succinate or glucose to identify which mutations no longer have a functional ATP synthase complex. Only the mutant strains that were resistant to MccPDI (*KΔatpA*, *KΔatpE*, *KΔatpF*, and *KΔatpH* strains) were unable to grow on the succinate-supplemented plates, although they could grow on the glucose-supplemented plates (see Fig. S4B in the supplemental material). Consequently, MccPDI activity correlates with the presence of a functional ATP synthase complex. To determine if a functional ATP synthase is required for susceptibility, we conducted competition experiments in the presence of a previously described ATP synthase inhibitor, the polyphenol resveratrol (8). Inhibition of ATP synthase activity resulted in reduced susceptibility to MccPDI (see Fig. S4C). To ensure that this experimental design did not impact microcin production in the MccPDI-producing strain, both *atpA* and *atpF* were deleted from *E. coli* 25, resulting in strains that lack a functional ATP synthase complex. In both cases, the inhibitory phenotype from MccPDI was not affected. Consequently, MccPDI inhibition requires a functional ATP synthase within the susceptible strain. Interestingly, we found that in *trans* expression of *E. coli ompF* in two of the four *atp* gene knockouts (Δ *atpA* and Δ *atpH* mutants) restored susceptibility to MccPDI (Fig. 5). Similar in *trans* expression of *ompF* in the remaining two *atp* gene deletion strains (Δ *atpF* and Δ *atpE* mutants), however, did not restore susceptibility to MccPDI.

Potential roles of DsbA, DsbB, and OmpR in OmpF synthesis and maturation. The Δ *dsbA* and Δ *dsbB* strains from the Keio collection were no longer susceptible to MccPDI (Table 2). Deletion of *dsbA* is known to limit the abundance of OmpF in the *E. coli* cell membrane (16); consequently, finding *dsbA* and *dsbB* linkage to MccPDI susceptibility was not unexpected. Interestingly, in *trans* expression of recombinant *E. coli ompF* in these two strains did not recover the susceptible phenotype (Fig. 5). OmpR is known to regulate the expression of *ompF* and *ompC* via a two-component regulatory system in *E. coli* (17). This is consistent with our findings whereby the gene knockout for *ompR* lost susceptibility to MccPDI (Table 2) while complementation of *ompR*

FIG 3 N terminus of *E. coli* *ompF* drives specificity of MccPDI. (A) Western blot detection of OmpF synthesis from *E. coli* strain BW25113 (Bw) and *S. enteritidis* strain 22577 (Se). For both strains the endogenous *ompF* gene was fused with a C-terminal His tag to enable detection using an anti-His antibody. Endogenous DnaK served as a relative loading control. (B) Competition with *E. coli* 25ΔtraM (microcin producing) or *E. coli* 25ΔmcpM (no microcin production) with the KΔompF strain after complementation with *E. coli* *ompF* (Ec.ompF) or *Salmonella* *ompF* (Se.ompF) or one of two chimeric *ompF* constructs (*ompF*_{Ec+Se} and *ompF*_{Se+Ec}). Results are expressed as the difference between mean log CFU during coculture and monoculture ($n = 3$ independent replicates; error bars indicate SEM). *, significant ANOVA ($P < 0.001$) for comparisons between paired competitions (*E. coli* 25ΔtraM strain versus *E. coli* 25ΔmcpM strain).

restored susceptibility (Fig. 1), as did in *trans* expression of *E. coli* *ompF* in the KΔompR strain (Fig. 5).

The Ton and Tol systems are not involved in MccPDI activity. Many characterized microcins and colicins employ the Ton or Tol system in conjunction with siderophore receptors (FepA, FhuA, and CirA) to enter susceptible cells, whereas others use only a porin protein (OmpF, OmpA, and OmpW) to gain entry into susceptible cells (12, 18). Preliminary screening of the Keio collection did not identify any of the corresponding Ton and Tol knockout strains as being resistant to MccPDI (data not shown). This does not eliminate the possibility that redundant activity from these systems would mask effects from single-gene knockouts. Consequently, a series of double knockouts were constructed in the wild-type Keio collection strain (ΔtonB ΔtolA, ΔtonB ΔtolQ,

ΔexbD ΔtolQ and ΔexbD ΔtolR); however, competition against microcin-producing *E. coli* 25 showed no reduction in inhibition (see Fig. S5 in the supplemental material).

DISCUSSION

Microcins require outer membrane receptors on the target bacteria before they can recognize and inhibit these cells. The receptor-microcin interaction is highly specific, which explains the narrow spectrum of microcin activity (19). To identify proteins required for MccPDI activity, we screened a single-gene knockout library of the *E. coli* K-12 genome and identified six knockouts with reduced susceptibility to MccPDI (*atpA*, *atpF*, *dsbA*, *dsbB*, *ompF*, and *ompR*). OmpF is probably the only surface-exposed protein in this subset. Data from different experiments involving recombinant

FIG 4 KGN motif located on the first extracellular loop of *E. coli* OmpF is the putative binding site for MccPDI. KΔompF(Ec.ΔSYGG) and KΔompF(Ec.ΔKGN) are *ompF* mutant strains expressing *E. coli ompF* sequence containing deletions for the KGN or SYGG peptides, respectively (see the text). Competition experiments included either a microcin-producing strain (E25ΔtraM) or nonproducing strain (E25ΔmcpM). Results are expressed as the difference between mean log CFU during coculture and monoculture. Experiments were replicated independently ($n = 2$). Error bars indicate SEM. *, significant ANOVA ($P < 0.001$) for comparisons between paired competitions (*E. coli* 25ΔtraM strain versus *E. coli* 25ΔmcpM strain).

protein constructs, including heterologous expression in two different bacterial species, confirmed that OmpF serves as the receptor for MccPDI binding with a putative binding site being located in the first exterior loop of OmpF. OmpR is a response regulator involved in expression of *ompF* and *ompC* in *E. coli* (20). Consequently, it was not unexpected that the *ompR* knockout strain was resistant to MccPDI, and that subsequent *in trans* expression of *ompF* restored susceptibility for the Δ*ompR* strain.

Some colicins employ both OmpF and BtuB to translocate into susceptible cells (21), but the Δ*btuB* strain (55F12) from the Keio collection was fully susceptible to MccPDI (data not shown). Because others have demonstrated that the 55F12 strain harbors an intact copy of *btuB* in addition to the deleted locus (22), we generated an independent Δ*btuB* strain, but it also remained susceptible to MccPDI (data not shown). It should be noted, however, that there was no positive control for this experiment, and we cannot be certain that the parental background that we used (*E. coli* 186) does not also harbor a second copy of this gene. Nevertheless, this finding is consistent with the absence of any reports linking microcins to BtuB. Because the 96-well plate assay used in this study was principally a screening tool of undefined sensitivity, we also confirmed gene knockout strains for all known receptors or translocators used by other microcins and colicins (Δ*fhuA*, Δ*fepA*, Δ*cir*, Δ*fiu*, Δ*ompW*, Δ*ompA*, and Δ*tsx* strains) (5–7, 21, 23–25). All were fully susceptible to MccPDI in direct competition assays against the wild-type *E. coli* 25 strain (data not shown). Therefore, it appears that OmpF is the sole receptor for MccPDI, although we cannot rule out the possibility that more than one additional protein could serve as a redundant coreceptor that

FIG 5 *In trans* expression of *E. coli ompF* restores susceptibility to MccPDI in KΔatpA, KΔatpH, and KΔompR strains. Results are shown for competition with E25ΔtraM (microcin producing) or E25ΔmcpM (no microcin production) after complementation with *ompF* in different Keio collection strains (KΔatpA, KΔatpH, KΔdsbA, KΔdsbB, KΔompR, KΔatpH, and KΔatpE deletion strains). Results are expressed as the difference between mean log CFU counts during coculture and monoculture. Experiments were replicated independently ($n = 3$). Error bars indicate SEM. *, significant ANOVA ($P < 0.001$) for comparisons between paired competitions (*E. coli* 25ΔtraM strain versus *E. coli* 25ΔmcpM strain).

would not be detected by screening a library of single-gene knockout strains. If this was the case, these proteins would have to be highly conserved, because heterologous expression of *E. coli ompF* in *Salmonella* and *Yersinia* is sufficient to make these species susceptible to MccPDI.

All colicins and microcins described to date are thought to penetrate the outer membrane barrier to reach their site of action (periplasm or cytosol) in the target cells (26, 27). Molecules with a mass of approximately 600 Da are able to diffuse through the OmpF porin (28), but the predicted mass of MccPDI is approximately 12,000 Da (immature protein) and 10,000 Da after maturation (2). This mass difference suggests MccPDI cannot freely diffuse through the OmpF pore as a method for gaining entry into susceptible cells and may require a partner system for active transport. Microcins B17 and C are known to interact with OmpF (12), but there is no evidence that they require either the Ton or Tol system. Instead, these microcins rely on inner membrane systems, SbmA (29) and Yej (30), respectively, for active transport across the cell membrane. We individually tested these and other potential microcin transport-related genes (27) from the Keio collection (Δ*sbmA*, Δ*yejA*, Δ*yejV*, Δ*yejE*, Δ*yejF*, Δ*manY*, and Δ*manZ*) but found no loss of susceptibility to MccPDI (data not shown).

Given these results, it is possible that OmpF is the sole receptor for MccPDI. Under this scenario, and assuming that MccPDI crosses the susceptible cell membrane, MccPDI is probably secreted from the producing cell as a linear peptide. This mode of secretion has been predicted for colicin A and colicin N (31, 32). Colicin N is thought to bind the periphery of OmpF on the cell surface (33) and cross the outer membrane in an unfolded state, after which it forms a channel in the lipopolysaccharide (LPS) adjacent to OmpF (34). Future experiments will investigate the specific mechanism of MccPDI entry into susceptible *E. coli*.

Our screen of the Keio collection also identified $\Delta dsbA$ and $\Delta dsbB$ mutants as being required for MccPDI susceptibility. DsbA and DsbB are thiol-redox enzymes that catalyze disulfide bond formation between cysteine residues for proteins that are transported into the periplasm (35–38). In addition, Pugsley (16) reported that mutations in *dsbA* reduced the transcription of *ompF*, but when recombinant *ompF* was expressed in these knockout strains (this study), susceptibility to MccPDI was not restored. If deletion of *dsbA* or *dsbB* only impacted transcription of *ompF*, then complementation of *ompF* using an inducible promoter should have restored the MccPDI-susceptible phenotype. The lack of restoration indicates that these proteins play a role in post-translational modification of OmpF, either directly or indirectly (36), through trafficking or through folding, although OmpF lacks any cysteine residues.

It also is possible that these proteins play a role in posttranslational modification of the microcin protein, assuming that it enters the periplasm (consistent with entry as a linear peptide). Cysteine is the least abundant amino acid found in proteins (39) and is also one of the most reactive (40, 41). For these reasons, if a protein contains an even number of cysteines and is predicted to function outside the cytoplasm, it is likely that the cysteines are paired to form disulfide bonds (42). MccPDI has four cysteines in the C terminus, consistent with the prediction that disulfide bond formation occurs. Consequently, DsbA and DsbB may serve two functions in this system, an indirect impact on posttranslational modification of OmpF and a direct interaction with MccPDI.

E. coli ATP synthase is an important enzyme that provides energy for the cell by catalyzing the synthesis of ATP during oxidative phosphorylation (14, 43). A cluster of genes (*atpIBEFHAGDC*) is responsible for encoding the subunits of this large enzyme complex (44). Initial screening of the Keio collection knockout library demonstrated that the $\Delta atpA$ and $\Delta atpF$ strains were not susceptible to MccPDI activity; additional direct competition experiments indicated that the $\Delta atpE$ and $\Delta atpH$ strains also were not susceptible to MccPDI. Microcin H47, a class IIb microcin, targets the AtpB, AtpE, and AtpF proteins that form the membrane-bound F_o proton channel of ATP synthase (*a*, *c*, and *b* subunits) (25). In the case of MccPDI, deletion of the four genes that encode proteins for the *c*, *b*, δ , and α subunits of ATP synthase (*atpE*, *atpF*, *atpH*, and *atpA*) confers resistance to MccPDI activity while also causing the loss of ATP synthase activity. Deletion of the remaining five ATP synthase component genes did not affect susceptibility to MccPDI or ATP synthase activity. Using an ATP synthase inhibitor, we showed that a functional ATP synthase is necessary for MccPDI inhibition, and we confirmed that the loss of ATP synthase activity within the microcin-producing strain had no impact on the inhibition phenotype. Consequently, whereas microcin H47 exerts its activity by inhibiting the ATP synthase (45), MccPDI activity requires the presence of a functional ATP synthase in the susceptible cell.

In summary, a preliminary screen of the Keio collection identified six gene knockouts that were no longer susceptible to MccPDI activity. We show that *E. coli* OmpF is necessary to confer susceptibility to MccPDI through complementation experiments, independent gene knockout experiments (*E. coli* O157:H7), and heterologous expression of the recombinant OmpF in *Salmonella* and *Yersinia*. We speculate that DsbA and DsbB are required for proper folding and/or trafficking of OmpF for folding MccPDI upon entry into the susceptible cell. OmpR is a known transcrip-

tional regulator of *ompF*. Finally, a functional ATP synthase is required for a strain to be susceptible to MccPDI activity.

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We (L.J.E., L.H.O., T.E.B., and D.R.C.) have filed a U.S. patent application (US14/407,975) regarding the application of MccPDI in health and food safety.

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