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YojI of *Escherichia coli* Functions as a Microcin J25 Efflux Pump

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In the present study, we showed that *yojI*, an *Escherichia coli* open reading frame with an unknown function, mediates resistance to the peptide antibiotic microcin J25 when it is expressed from a multicopy vector. Disruption of the single chromosomal copy of *yojI* increased sensitivity of cells to microcin J25. The YojI protein was previously assumed to be an ATP-binding-cassette-type exporter on the basis of sequence similarities. We demonstrate that YojI is capable of pumping out microcin molecules. Thus, one obvious explanation for the protective effect against microcin J25 is that YojI action keeps the intracellular concentration of the peptide below a toxic level. The outer membrane protein TolC in addition to YojI is required for export of microcin J25 out of the cell. Microcin J25 is thus the first known substrate for YojI.

Microcin J25 (MccJ25) is a plasmid-encoded, 21-amino-acid antibacterial peptide produced by *Escherichia coli* (4, 21). It is active against *E. coli*, *Salmonella*, and *Shigella*. Four genes (*mcjA*, *mcjB*, *mcjC*, and *mcjD*) are required for MccJ25 synthesis, export, and immunity (26, 27). *mcjA* encodes the primary structure of MccJ25 as a 58-amino-acid precursor, from which a 37-amino-acid N-terminal leader is removed. The 21-residue mature peptide has a compact, extraordinary structure, which consists of an eight-residue lariat ring and a C-terminal tail which folds on itself and passes through the ring, where it is sterically trapped (2, 19, 30). The *mcjB* and *mcjC* gene products are involved in this assembly process (27). The *mcjD* product has a dual role. It works as a dedicated ATP-binding cassette (ABC) exporter of MccJ25 and, at the same time, by ensuring rapid secretion from the cytoplasm, protects cells from endogenous MccJ25 synthesized in producer cells, as well as from the exogenous microcin that gains entry (27).

MccJ25 intake is mediated by the outer membrane receptor FhuA and the inner membrane proteins TonB, ExbB, ExbD, and SbmA (22, 23). *E. coli* RNA polymerase is the target of antibiotic action (7, 31). The binding site for MccJ25 is located in the secondary channel of the enzyme (1, 16), which provides a route by which the nucleotide substrates reach the catalytic site. Thus, MccJ25 inhibits transcription by clogging the channel and blocking the access of substrates to the active center (1, 16).

In the course of experiments aimed at cloning an MccJ25-resistant mutation, we found a recombinant plasmid able to confer resistance to the antibiotic, but, unexpectedly, this plasmid did not contain the mutation. Analysis of this plasmid led to the identification of a previously described chromosomal locus, *yojI*, which, when present in multiple copies, protected cells from MccJ25. Gene disruption experiments showed that a protective effect against MccJ25 was exerted by the single-copy chromosomal gene *yojI*. On the basis of sequence similarities, *yojI* had been assumed to encode an ABC-type exporter (18). Here we provide evidence which strongly suggests that the resistance to MccJ25 mediated by YojI involves extrusion of the peptide and that YojI is assisted by the multifunctional outer membrane protein TolC.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strains and plasmids used in this work are described in Table 1. P1 *vir* transduction was used to introduce the *tolC*::Tn*10* allele into MC4100 and MG1655. The rich and minimal media used in this work were Luria broth (LB) and M9 minimal salts with 0.2% glucose (15), respectively. Solid media contained 1.5% agar. Antibiotics were added, when required, at the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; and tetracycline, 10 μg/ml. Incubations were done at 37°C except for strains bearing the mini-Mu plasmid pEG109 and its derivative pCLO4, which were grown at 30°C.

Sensitivity test and bioassay of microcin activity. Sensitivity to MccJ25 was tested by a spot-on-lawn assay, as follows. Doubling dilutions of a purified MccJ25 preparation were spotted (10 μl) onto LB plates and dried. Aliquots (50 μl) of cultures to be tested for sensitivity, in the stationary phase, were mixed with 3 ml of top agar (LB containing 0.7% agar) and overlaid onto the plates. After overnight incubation, the plates were examined for different degrees of inhibition.

To test a strain's ability to produce extracellular MccJ25, it was grown on solid LB medium, and a fresh colony was stabbed with a toothpick into the assay plate, which contained M9 medium. After 6 h or 24 h of growth at 37°C, cells were killed with chloroform. The plates were then overlaid with approximately 10^8 sensitive indicator cells in 3 ml of soft agar. The presence of excreted microcin was indicated by a zone of growth inhibition in the lawn of indicator cells surrounding the stabbed colony.

In vivo DNA cloning. In vivo cloning was done by introducing the mini-MudII4042-containing plasmid pEG109 (12) into strain SBG231 Mucts. Transformed chloramphenicol-resistant cells were grown and heated at 42°C to induce transposition during phage replication, and strain pop3001.6 was infected with the resulting lysate. Transductants were selected on LB containing chloramphenicol and MccJ25, purified on the same medium, and then rechecked for MccJ25 resistance.

Recombinant DNA methods. Plasmid DNA was isolated with the Wizard miniprep DNA purification system (Promega). Digestion with restriction endonucleases, ligation with T4 DNA ligase, transformation of competent cells by the $CaCl_2$ procedure, and agarose gel electrophoresis were done as described previously (24). Automated DNA sequencing was carried out by dideoxy termination (25). Database searches and sequence alignments were performed with the online BLAST Network Service at the National Center for Biotechnology Infor-

TABLE 1. *E. coli* strains and plasmids used in this work

Strain or plasmid	Genotype or description[a]	Reference or source[b]
E. coli strains		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacIZYA-argF*)*U169 deoR* [ϕ80*d*Δ*lacZ*M15]	11
MC4100	*araD139* Δ(*argF-lac*)*205* λ^- *flbB5301 ptsF25 relA1 rpsL150 deoCI*	CGSC
MG1655	λ^- *rph-1*	CGSC
pop3001.6	MC4100 Mucts	F. Moreno
SBG231	Spontaneous MccJ25^r mutant	7
Plasmids		
pEG109	MudII4042, *cat* (Cm^r), *rep*P15A	12
pCLO4	MudII4042::MccJ25^r-conferring 14-kb chromosomal fragment, Cm^r	This study
pUC18	High-copy-number cloning vector, *lacPOZ′* Ap^r	29
pCLO5	pUC18 with 4-kb HindIII fragment from pCLO4 containing *rcsB*, MccJ25^s Ap^r	This study
pCLO6	pUC18 with 10-kb SalI fragment from pCLO4 containing *yojI*, *alkB*, *ada′*, and 6.2 kb from left end of MudII4042, MccJ25^r Ap^r	This study
pUCyojIH	pUC119 *yojI yojH* under control of native promoter, Ap^r	17
pCLO7	pUC18 with 4.2-kb PstI-SmaI fragment from pCLO4 containing *yojI*, *alkB′*, and 1.7 kb from left end of MudII4042, Ap^r	This study
pUCyojIH	pUC119 *yojI yojH* under control of native promoter, Ap^r	17
pTrcHyojH	p*Trc*6His with *yojH* under control of *trc* promoter, expresses C-terminal His-tagged YojH, Ap^r	17
pTrcHyojI	p*Trc*6His with *yojI* under control of *trc* promoter, expresses C-terminal His-tagged YojI, Ap^r	17
pACYC184	Low-copy-number cloning vector, *rep*P15A, Cm^r Tc^r	5
pTUC203	pACYC184 with *mcjABCD*, MccJ25$^+$ ImmJ25$^+$ Cm^r	26
pTUC348	pACYC184 with MccJ25 synthesis genes (*mcjABC*) but MccJ25 export-immunity gene *mcjD* inactivated by 3′ end deletion, MccJ25$^+$ ImmJ25$^-$ Cm^r	This study
pJS300	pUC18 with *mcjD*, Ap^r	26

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; MccJ25$^+$, microcin J25 producer; ImmJ25$^+$, immune to MccJ25.
[b] CGSC, *E. coli* Genetic Stock Center.

mation, National Institutes of Health, Bethesda, Md. Restriction enzymes and DNA ligase were purchased from New England Biolabs.

Construction of a *yojI* chromosomal deletion. Deletion of the *yojI* gene was performed by use of the one-step chromosomal gene inactivation method described by Datsenko and Wanner (6). This technique uses the highly efficient phage λ Red recombination system encoded on the helper plasmid pKD46 and direct transformation of PCR products consisting of the *cat* marker gene with extensions identical to the sequences flanking the region targeted for deletion. *E. coli* strain DH5αΔ*yojI*::*cat* was constructed by transforming strain DH5α carrying the Red helper plasmid pKD46 (6) with a PCR product generated by using plasmid pKD3 (6) as the template with primers P1YojI (CTGCGGGCTGTAA TTCATTGTCCGGGTTTTCTGCTGTAGGCTGGAGCTGCTTC) and P2YojI (GACGGCAGCAGTCCGCCAGGTCAGCGTCGTAATACATATGAATAT CCTCCTTAG). These primers included 18 and 20 nucleotides (nt) of priming sites 1 and 2, respectively, of pKD3 and 35-nt homologous extensions flanking the 5′ and 3′ ends of the *yojI* gene. Transformants were selected at 37°C on LB agar containing chloramphenicol. Homologous recombination between the genomic DNA and the PCR product resulted in precise deletion of the entire coding region of *yojI* and replacement of this region with the *cat* gene. Representative gene disruption mutants gave a new fragment of the expected size (300 bp) in a PCR test using the locus-specific primer P3YojI (CCATCGACTGCCGCT ACAACC), located 129 nt upstream the start codon of *yojI*, and the *cat*-specific primer c1 (TTATACGCAAGGCGACAAGG) (6). The same primer combination gave negative results with parental strain DH5α.

RESULTS

Cloning of an *E. coli* K-12 chromosomal gene that confers resistance to MccJ25 when multiple copies of it are present. Most spontaneous MccJ25-resistant mutants affect outer and inner membrane components involved in MccJ25 intake (22, 23). In an attempt to identify the MccJ25 intracellular target, we isolated a novel spontaneous MccJ25-resistant mutant of *E. coli* K-12, designated SBG231. Genetic analysis located the mutation at 90 min on the *E. coli* genetic map. To clone the mutated gene, a mini-MudII4042 library was made by thermoinduction of strain SBG231 Mucts(pEG109), as described by Groisman et al. (12), and used to transduce strain pop3001.6. The rationale was that overexpression of the mutant gene would lead to microcin resistance. Transductants were selected on minimal medium supplemented with chloramphenicol and MccJ25. It was expected that only cells transformed with recombinant plasmids containing the mutation would grow. Plasmid DNA was extracted from one of four MccJ25-resistant clones and retransformed into pop3001.6 cells. Cells that received the plasmid, designated pCLO4, became resistant to a high MccJ25 concentration (1 mg/ml), while transformants with the control pEG109 plasmid were fully sensitive. A restriction map of pCLO4 showed that it included approximately 14 kb from the *E. coli* chromosome (Fig. 1). Unexpectedly, when we compared the physical map of the fragment cloned in pCLO4 with that of *E. coli* K-12 (20), no similarity was detected in the 90-min region. Instead, we found an overlap in the 49-min area. In fact, the fragment carried by pCLO4 was located at approximately 2,304 to 2,318 kb of the *E. coli* genome (3). This was interpreted to mean that a wild-type locus, and not the mutation we were seeking, was cloned from strain SBG231, which, when present in multiple copies, provided microcin resistance. (Cloning of the mutation at 90 min was achieved by a different approach and established that the RNA polymerase β′ subunit is the MccJ25 target [7].)

Subcloning of the MccJ25 resistance determinant. Cells containing pCLO4 formed extremely mucoid colonies. This was possibly due to overexpression of *rcsB*, a gene encoding a positive regulator of colanic acid synthesis (10, 13), which

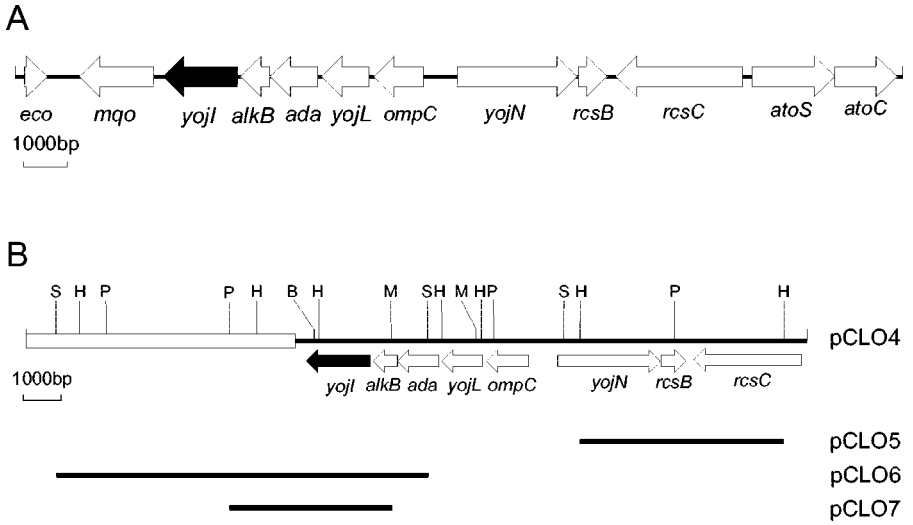

FIG. 1. (A) Overview of the *E. coli* genomic region containing *yojI*. (B) Restriction map and genetic organization of the *E. coli* chromosomal fragment cloned in pCLO4 (thick line). The relevant portion of the mini-Mu plasmid used for in vivo cloning is represented by the open box. Below the map, the horizontal lines indicate the pCLO4 segments carried by pUC18 derivatives used in this study. Relevant restriction sites are indicated (B, BamHI; H, HindIII; P, PstI; S, SalI; M, SmaI).

should be encompassed by the segment cloned in pCLO4 (Fig. 1). It was possible that the colanic acid capsule was responsible for the resistance to MccJ25 because it opposed a permeability barrier to antibiotic entry. This possibility was excluded, however, since a subclone of pCLO4 harboring *rcsB* (pCLO5 in Fig. 1) led to the mucoid phenotype but not to MccJ25 resistance. To identify the locus in pCLO4 responsible for MccJ25 resistance, additional subclones of this plasmid in the vector pUC18 were constructed. Two of them, pCLO6 and pCLO7 (Fig. 1), were able to confer MccJ25 resistance when they were transformed into DH5α cells. In particular, the 4.2-kb PstI-SmaI insert in pCLO7 should have encompassed only a single complete chromosomal open reading frame (ORF), *yojI*, flanked by a truncated *alkB* gene and 1.7 kb from the left end of the mini-Mu vector (Fig. 1). Sequencing of the ends of the insert and restriction analysis confirmed this organization. These results established that *yojI* is responsible for MccJ25 resistance.

Recently, Nishino and Yamaguchi (17) constructed a library of 37 *E. coli* ORFs assumed to be drug transporter genes on the basis of sequence similarities (18), including seven putative ABC-type ORFs. Among the latter were *yojI* and the adjacent ORF *yojH*. Although *yojH* was first assumed to be a drug resistance gene on the basis of its membrane topology, its product is actually a membrane-associated malate dehydrogenase and the gene has been renamed *mqo* (28) (Fig. 1A). Plasmids pUCyojIH, pTrcHyojI, and pTrcHyojH from Nishino and Yamaguchi's library were transformed into DH5α, and the transformants were tested for their resistance to MccJ25. We added 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to agar plates when we examined the susceptibility to MccJ25 of cells harboring the pTrc6His derivatives pTrcHyojI and pTrcHyojH. Cells carrying either pTrcHyojI or pUCyojIH, both of which express YojI, showed complete resistance to MccJ25 (no inhibition halos were detected in a spot-on-lawn test). As expected, synthesis of YojH alone from pTrcHyojH (used as a control) did not lead to resistance. These results confirmed that overexpression of *yojI* confers MccJ25 resistance.

YojI mediates export of MccJ25. One obvious explanation for the protective effect of YojI against MccJ25 is that it is capable of pumping out MccJ25 molecules, which would keep the intracellular concentration of the peptide below a toxic level. To prove this hypothesis, we tested whether YojI could substitute for McjD, the natural MccJ25 exporter, in secreting MccJ25. We subcloned a 4-kb HindIII-HincII fragment from plasmid pTUC203 (26) into pACYC184. This fragment carries the MccJ25 synthesis genes, *mcjABC*, but lacks the *mcjD* sequences encoding the C-terminal 117 amino acids of McjD, including the essential Walker B motif of the ABC exporter. The resulting plasmid, named pTUC348, was expected to be lethal to cells, since in the absence of a functional McjD the MccJ25 peptide would accumulate in host cells and kill them. Therefore, it was transformed into *E. coli* DH5α carrying pJS300 (26), a pUC18 derivative expressing McjD. When a mixture of plasmids pTUC348 (chloramphenicol resistant) and pJS300 (ampicillin resistant) was transformed into DH5α cells, selecting only for chloramphenicol resistance, all transformants which appeared were also ampicillin resistant, consistent with absolute coselection for the immunity plasmid pJS300. This expected result indicated that pTUC348 could not be propagated in the absence of an immunity gene. When the plasmid preparation with both pTUC348 and pJS300 was transformed into strain SBG231, containing an MccJ25-resistant RNA polymerase (7), clones harboring only pTUC348 were obtained. These transformants grew normally, indicating that the mutation overcame the inhibitory effect of accumulated internal MccJ25, but they were unable to give growth inhibition halos on an MccJ25-sensitive indicator strain (Fig. 2A). Introduction of a second plasmid containing either *mcjD* (pJS300) or *yojI* (pCLO7) rescued the MccJ25 secretion phenotype, as shown by the inhibition zones (Fig. 2B and C). This

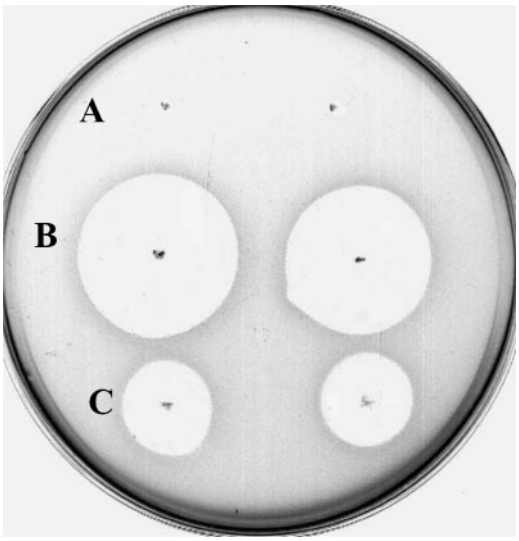

FIG. 2. Biossay for MccJ25 export by YojI. Microcin-resistant strain SBG231 transformed with pTUC348 (A), pTUC348 and pJS300 (*mcjD*$^+$) (B), and pTUC348 and pCLO7 (*yojI*$^+$) (C) was assayed for production of extracellular MccJ25. Plasmid pTUC348 directs MccJ25 synthesis but lacks the functional MccJ25 export-immunity gene *mcjD*. Two independent transformants were stabbed (black dots) in each row and pregrown for 6 h before they were overlaid with a lawn of sensitive cells and incubated overnight at 37°C.

result indicated that YojI was able to export MccJ25 out of the cells.

As noted above, under these assay conditions (6 h of pregrowth before overlaying with indicator cells) strain SBG231 (pTUC348) did not produce a detectable zone of inhibition. However, after the cells were pregrown overnight, a 0.5-cm halo was visible (results not shown). This relatively low-level secretion of MccJ25 may be explained by the presence in SBG231 of the single chromosomal copy of *yojI*.

TolC is required for microcin J25 secretion by YojI. ABC-type exporters usually function together with a membrane fusion protein (MFP), anchored to the inner membrane by either a single hydrophobic α-helix or a covalent lipid moiety, and an outer membrane channel, so that the pumped-out molecules do not accumulate in the periplasm (9). The *yojI* gene does not appear to be associated with any gene coding for an MFP, but it is possible that YojI may interact with a member of the MFP family and an outer membrane channel encoded elsewhere on the chromosome. It is noteworthy that McjD, the dedicated exporter of MccJ25, is believed to form a microcin export complex with TolC, but so far, no MFP constituent has been identified for this system (8). We presumed that TolC might also serve as the channel for the YojI efflux system. If so, the increased MccJ25 resistance seen in strains harboring pCLO7 (*yojI*$^+$) would be negated by a *tolC* mutation. To test this hypothesis, a *tolC*::Tn*10* insertion mutation was transduced into MC4100(pCLO7), and transductants were examined for resistance to MccJ25. The *tolC* mutation completely eliminated the MccJ25 resistance phenotype. In addition, when the *tolC* null allele was transduced into SBG231(pTUC348, pCLO7), microcin excretion was abolished. Again, it was necessary to use the microcin-resistant SBG231 background for this assay, since the accumulated microcin kills a TolC$^-$ MccJ25-producing cell. These results strongly suggest that TolC and YojI are components of an efflux complex that uses MccJ25 as a substrate.

Disruption of either *tolC* or *yojI* genes leads to increased sensitivity to MccJ25. We have long noticed that in twofold serial dilution assays on LB plates with several *E. coli* K-12 strains as indicators (including the widely used strains MC4100, MG1655, and DH5α), MccJ25 gives rise to turbid zones of growth inhibition, except for the first two or three dilutions, which produce clear halos with cloudy edges. As described previously (21), exposure of sensitive cells to MccJ25 results in filamentous growth. Microscopic observation during formation of a turbid zone of growth inhibition revealed that the cells gradually elongated over the first few hours of incubation. However, most of the filaments recovered and finally developed into colonies, which explains the final turbid appearance. These observations suggested that *E. coli* strains have intrinsic partial resistance to MccJ25. In light of the results described above, it was tempting to speculate that this phenotype results from removal of MccJ25 from the cells by the YojI-TolC complex. If so, one would predict that disruption of either *tolC* or *yojI* would make *E. coli* cells more sensitive to MccJ25. To test this hypothesis, a *tolC*::Tn*10* mutation was transduced into MC4100 and MG1655, and the strains were examined for sensitivity to MccJ25 in a spot-on-lawn test. The *tolC* derivatives were at least fourfold more sensitive to exogenous MccJ25 than their wild-type parental strains and gave completely clear spots. Microscopic examination of the halos revealed only isolated cells or short filaments dispersed all over the transparent zones. Apparently, the effect of MccJ25 on the *tolC* mutant cells was so disruptive that growth and division ceased without any gross changes in morphology. Introduction of plasmid pAX629, which carries a cloned copy of the wild-type *tolC* gene (14), into the *tolC* mutants restored the phenotype of the parent strains, showing that the effect of the *tolC* mutation on MccJ25 sensitivity is due to inactivation of the *tolC* gene itself rather than to the possible polarity of the insertion mutation on the expression of a downstream gene.

Next, we compared the resistance to MccJ25 of wild-type and *yojI* cells. To this end, the chromosomal *yojI* gene of strain DH5α was deleted by the method of Datsenko and Wanner (6), as described in Materials and Methods. A Δ*yojI* strain displayed increased susceptibility to MccJ25. In a spot-on-lawn test it was approximately eightfold more sensitive than the control strain DH5α and showed completely clear zones of growth inhibition (Fig. 3).

Taken together, the results described above support the concept that *yojI* is functional when a single copy is present and that, acting in concert with *tolC*, it is an important determinant of the baseline resistance of *E. coli* cells to MccJ25.

DISCUSSION

The main findings of the present study are as follows. (i) Overexpression of *yojI* confers increased resistance to MccJ25 and is correlated with an increased efflux of the antibiotic. Note that the plasmid-encoded immunity to MccJ25 is based on such a mechanism. (ii) Efflux of and resistance to microcin J25 mediated by YojI are dependent upon the outer mem-

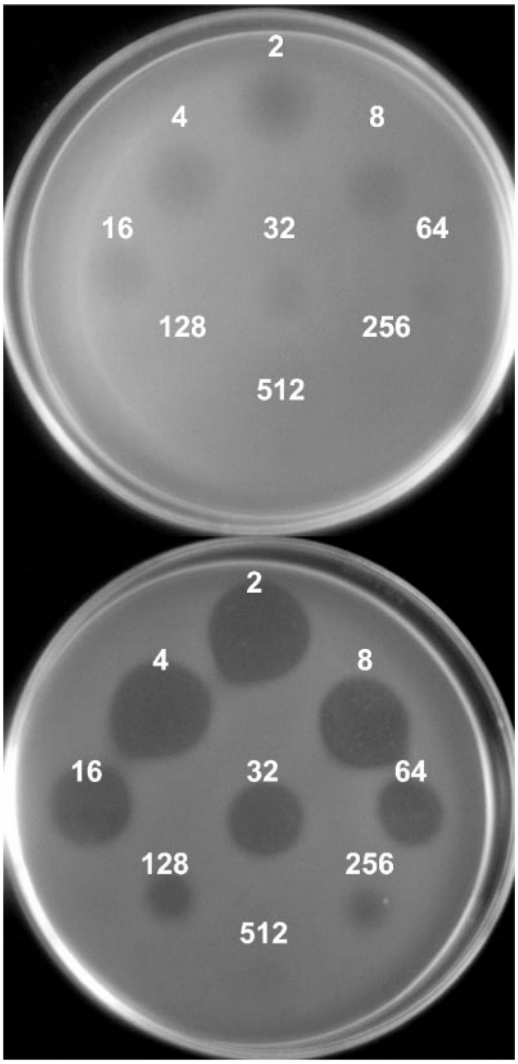

FIG. 3. Susceptibility of the Δ*yojI* mutant as quantitated by the critical dilution method. Twofold serial dilutions of MccJ25 were spotted on LB plates. After the spots dried, the plates were overlaid with a suspension of control DH5α cells (top) and DH5αΔ*yojI* cells (bottom). The numbers indicate the reciprocal of each dilution. The MccJ25 titer (i.e., the last dilution giving a halo) was 1:64 for the control (turbid spots) and 1:512 for the YojI-defective mutant (clear halos).

brane channel TolC. Therefore, it is likely that these proteins form a complex for MccJ25 excretion. (iii) We experimentally validated for the first time the previous inference (based on sequence homologies) that YojI is an exporter. (iv) Disruption of either the *tolC* or *yojI* gene renders *E. coli* K-12 cells more susceptible to MccJ25. This may be explained by assuming that either mutation results in loss of the efflux process, leading to an increase in the intracellular concentration of microcin. And (v) The Δ*yojI* mutant was viable, indicating that *yojI* is not an essential gene.

MccJ25 is the first substrate known for the YojI pump. Although the normal biological role of YojI is not yet understood, obviously it cannot be transport of MccJ25. The specificity of YojI is not known, but it was shown that overproduction of YojI did not alter the resistance to any of 26 representative antimicrobial agents and chemical compounds translocated by AcrAB or other major drug transporters (17). This suggests that it could be quite specific in its choice of substrate. Given the peptide nature of MccJ25, it is possible that YojI recognizes peptide compounds as substrates for transport.

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