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A Protein Important for Antimicrobial Peptide Resistance, YdeI/OmdA, Is in the Periplasm and Interacts with OmpD/NmpC▽

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Antimicrobial peptides (AMPs) kill or prevent the growth of microbes. AMPs are made by virtually all single and multicellular organisms and are encountered by bacteria in diverse environments, including within a host. Bacteria use sensor-kinase systems to respond to AMPs or damage caused by AMPs. *Salmonella enterica* deploys at least three different sensor-kinase systems to modify gene expression in the presence of AMPs: PhoP-PhoQ, PmrA-PmrB, and RcsB-RcsC-RcsD. The *ydeI* gene is regulated by the RcsB-RcsC-RcsD pathway and encodes a 14-kDa predicted oligosaccharide/oligonucleotide binding-fold (OB-fold) protein important for polymyxin B resistance in broth and also for virulence in mice. We report here that *ydeI* is additionally regulated by the PhoP-PhoQ and PmrA-PmrB sensor-kinase systems, which confer resistance to cationic AMPs by modifying lipopolysaccharide (LPS). *ydeI*, however, is not important for known LPS modifications. Two independent biochemical methods found that YdeI copurifies with OmpD/NmpC, a member of the trimeric β-barrel outer membrane general porin family. Genetic analysis indicates that *ompD* contributes to polymyxin B resistance, and both *ydeI* and *ompD* are important for resistance to cathelicidin antimicrobial peptide, a mouse AMP produced by multiple cell types and expressed in the gut. YdeI localizes to the periplasm, where it could interact with OmpD. A second predicted periplasmic OB-fold protein, YgiW, and OmpF, another general porin, also contribute to polymyxin B resistance. Collectively, the data suggest that periplasmic OB-fold proteins can interact with porins to increase bacterial resistance to AMPs.

Antimicrobial peptides (AMPs) are a major class of antibiotics naturally encountered by microbes in diverse environments. AMPs are produced by bacteria, archea, and eukarya and can lyse or prevent the replication of viruses, bacteria, and fungi (34, 72). Bacterial and eukaryotic cationic AMPs are amphiphilic and are represented by a wide range of sequences, lengths, and secondary structures. Linear alpha-helical peptides belong to the cathelicidin family and include LL-37 (38, 59). Bridged β-sheet peptides include bacterial lantibiotics and mammalian defensins. (36, 39, 57, 74). Lantibiotics are produced by gram-positive bacteria and limit interspecies competition (68). Defensins and cathelicidins are produced by epithelial cells and limit microbial growth on epithelial cell surfaces, such as in the gastrointestinal tract (37, 72). Polymyxin B is a cationic AMP derived from the soil bacterium *Bacillus polymyxa* (11). It consists of a peptide ring with a hydrophobic tail (51) and is commonly used in research laboratories as a model for AMPs because it is inexpensive, commercially available, and effective against a wide variety of bacterial species.

The increasing prevalence of antibiotic resistant pathogens has stimulated interest in the therapeutic use of AMPs, as well as in the understanding of bacterial mechanisms needed for responding to and resisting AMPs. *Salmonella enterica* utilize at least three different sensor-kinase systems to respond to cationic AMPs: PhoP-PhoQ (PhoPQ), PmrA-PmrB (PmrAB), and RcsB-RcsC-RcsD (RcsBCD) (20, 21, 30). All three of these signaling pathways are important for *S. enterica* virulence in mice (20, 22, 26, 29, 46, 48). The PhoPQ system directly regulates genes that confer AMP resistance, such as *pgtE*, which encodes an outer membrane protease that cleaves cathelicidins (27). PhoPQ also activates transcription of the gene encoding PagP, which transfers palmitate to lipid A, reducing outer membrane permeability and increasing bacterial viability in the presence of AMPs (31). The PmrAB sensor-kinase system is another target of PhoPQ. PmrA activates genes needed to modify lipopolysaccharide (LPS) with aminoarabinose or phosphoethanolamine, both of which decrease the local and overall negative charge of lipid A and thereby minimize electrostatic interactions with cationic peptides (28, 44, 73).

The RcsBCD (named for regulator of capsule synthesis) sensor kinase system also contributes to polymyxin B resistance (17, 20). RcsB and RcsC were initially identified based on their roles in capsule production (60), which requires the RcsA transcription factor in addition to the RcsB response regulator (61). RcsA, however, does not contribute to polymyxin B resistance (20). A whole-genome DNA microarray screen identified genes induced by polymyxin B in an RcsBCD-dependent, RcsA-independent pattern (20). One of the identified genes, *ydeI*, encodes a 14-kDa predicted periplasmic protein of unknown function that is limited to *S. enterica*, *Escherichia coli*, and *Shigella* spp. YdeI is important for resistance to polymyxin B in broth and for bacterial survival in mice upon oral, but not

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intraperitoneal inoculation, suggesting a role for YdeI in the gastrointestinal tract of mice (20). Bioinformatics analyses identified YdeI as an oligosaccharide/oligonucleotide binding-fold (OB-fold) protein (COG3111) (25). OB-fold family members have a structural fold of five antiparallel β-sheets that form a closed or partially opened barrel. The barrel of an OB-fold protein can function as a scaffold for the binding of small polymers, including nucleotides and sugars (62). However, bacterial OB-fold proteins lack residues appropriate for nucleotide binding (25) and are of unknown function.

To address how YdeI may function, we established whether the gene is regulated by known transcriptional activators of AMP resistance genes. In addition to RcsBCD (20), *ydeI* was regulated by PhoPQ and PmrAB, but *ydeI* mutant strains produced LPS similar to that of wild-type strains, indicating *ydeI* contributes to AMP resistance via an unknown mechanism. YdeI copurified with an outer membrane porin, OmpD/NmpC, and genetic data supported an interaction between YdeI and OmpD, since strains lacking either or both genes were similarly sensitive to AMPs. In cell fractionation experiments, YdeI localized to the periplasm. *S. enterica* serotype Typhimurium encodes a second predicted OB-fold periplasmic protein, YgiW (5, 25), which is also important for polymyxin B resistance. In addition, the porin OmpF, but not OmpE, for instance, contributed to polymyxin B resistance. These data suggest OB-fold proteins can protect bacteria from cationic AMPs, and that YdeI confers AMP protection in conjunction with the general porin OmpD. We suggest YdeI be renamed OmdA, for OmpD-Associated protein.

TABLE 1. *S. enterica* serovar Typhimurium strains

Strain	Genotype and/or description	Source or reference
SL1344	*hisG xyl rpsL* (wild-type serovar Typhimurium)	58
CSD221	14028s pKD46	Charlie Kim
CSD257	*galE*::Tn*10*	Bruce Stocker
FL416	SL1344 *phoP*::Cm	20
KDE445	SL1344 *ydeI*::Cm	20
KDE517	SL1344 *rcsB*::Cm	This study
KDE565	SL1344 *invA*::Cm	This study
KDE602	SL1344 *ydeI*Δ	This study
KDE612	SL1344 *ydeI*::HA::Kan	This study
KDE642	SL1344 *ydeI*::HA	This study
KDE662	SL1344 *phoP*::Cm, *rcsB*Δ	This study
KDE666	SL1344 *pmrA*::Cm	This study
KDE686	SL1344 with pYdeI-HA	This study
KDE735	SL1344 *ompD*::Cm	This study
KDE737	SL1344 *yddG*::Cm	This study
NMM748	SL1344 *ompD*::3xFLAG::Kan	This study
KDE755	SL1344 *ydeI*Δ *ompD*::Cm	This study
KDE759	SL1344 *ydeI*::HA *ompD*::3xFLAG::Kan	This study
NMM764	SL1344 *ompD*::3xFLAG	This study
KDE769	SL1344 *ydeI*::HA *ompD*::3xFLAG	This study
NMM800	SL1344 *ompE*/*phoE*::Cm	This study
NMM801	SL1344 *ompF*::Cm	This study
NMM802	SL1344 *ompN*::Cm	This study
NMM803	SL1344 STM1530::Cm	This study
NMM805	SL1344 *ompC*::Cm	This study
NMM806	SL1344 *ygiW*::Cm	This study
MCP847	SL1344 *ompD*::3xFLAG, with pGEX-3x	This study
MCP848	SL1344 *ompD*::3xFLAG, with pYdeI-HA	This study

MATERIALS AND METHODS

Bacterial strains and growth. All bacteria were grown and maintained at 37°C with agitation in Luria-Bertani (LB) broth or on LB agar plates. The following antibiotic concentrations were used: Streptomycin (Str) at 200 μg/ml, kanamycin (Kan) at 30 μg/ml, and chloramphenicol (Cm) at 10 μg/ml. Strains described in the present study (Table 1) were derived from wild-type *S.* Typhimurium strain SL1344 (58). The oligonucleotides used for generating deletion mutants are detailed in Table 2 (15). Mutations were made in the *S.* Typhimurium 14028 strain background (American Type Culture Collection, Manassas, VA), verified by PCR, and transduced into SL1344 using standard P22 phage transduction (63). Transductants were verified by growth on LB agar containing the appropriate antibiotic and by PCR.

Epitope-tagged strains KDE612 (*ydeI*::HA Kan^r) and NMM748 (*ompD*::3xFLAG Kan^r) were generated in SL1344 (65) by using oligonucleotides shown in Table 2. To generate double-tagged strains, the Kan resistance cassette was removed from KDE612 (*ydeI*::HA) by transformation with the plasmid pCP20 (15). The resulting strain, KDE642 (*yde*::HA KanΔ), was the recipient for transduction with a phage lysate from NMM748 (*ompD*::3xFLAG Kan^r), to generate strain KDE759 (*ydeI*::HA KanΔ *ompD*::3xFLAG Kan^r). The Kan resistance cassette was removed from KDE759 to yield KDE769 (*ydeI*::HA KanΔ *ompD*::3xFLAG KanΔ).

Real-time quantitative PCR. Overnight bacterial cultures were diluted 1:100 into 50 ml of selective LB medium and grown for 3 h at 37°C with shaking. Polymyxin B was added to a final concentration of 1 μg/ml, and incubation continued for 40 min. Bacteria were collected by centrifugation and processed for RNA as described previously (20). Total RNA was reverse transcribed into cDNA with the SuperScript III first strand synthesis system (Invitrogen, Carlsbad, CA). Gene expression was determined by using SYBR green (ABI, Carlsbad, CA) and primers specific to *ydeI* (forward primer, 5′-ATTGAGGATGGT TATCGCGGTA-3′; reverse primer, 5′-CCTGTTCGATGGTCATTTTTTCT-3′) or *gapA* (forward primer, 5′-TGTTTTCCGTGCTGCTCAGA-3′; reverse primer, 5′-TTGATTGCAACGATCTCGATGT-3′). Reactions were run on a 7900 HT real-time PCR system (ABI, Carlsbad, CA) and analyzed by using 7000 sodium dodecyl sulfate (SDS) 1.1 RQ software (ABI). The efficacy of each PCR primer set was determined with a standard curve of RNA isolated from SL1344 exposed to polymyxin B. *ydeI* mRNA levels were normalized to *gapA* for each strain, and the levels of *gapA* were similar across samples. Wild-type (SL1344) and *phoP* (FL416), *ydeI* (KDE455), *rcsB* (KDE517), *pmrA* (KDE666), and *phoP rcsB* (KDE662) mutant strains were examined.

LPS purification for SDS-polyacrylamide gel electrophoresis (PAGE). Bacterial strains (wild type [SL1344] and *phoP* [FL416], *galE* [CSD257], and *ydeI* [KDE602] mutants) were grown overnight in LB medium or MgM (100 mM Tris-chloride [pH 5.0], 5 mM potassium chloride, 7.5 mM ammonium sulfate, 0.5 mM potassium sulfate, 1 mM potassium phosphate monobasic, 8 mM magnesium chloride, 38 mM glycerol, and 0.1% Casamino Acids [10])–10 μM $MgCl_2$ as 25-ml cultures. Equivalent numbers of bacteria, as determined by optical density at 600 nm (OD_{600}), were harvested by centrifugation at 5,000 × *g* for 20 min and processed for LPS. Bacteria were resuspended in lysis buffer (2% SDS, 4% β-mercaptoethanol, 10% glycerol, 1 M Tris [pH 6.8]) and boiled for 15 min. Proteinase K (0.1%) was added to boiled lysates, followed by incubation at 60°C for 1 h. An equal volume of prewarmed 95% saturated phenol was added to lysates, followed by incubation at 70°C for 15 min with vortexing every 5 min. Samples were incubated on ice for 10 min, followed by centrifugation at 14,000 × *g*. The clear aqueous phase was extracted twice with an equal volume of ether. Samples were resolved on a SDS–12% polyacrylamide gel and processed for silver staining (64). Briefly, the gel was soaked overnight in 40% 2-propanol–5% acetic acid. Periodic acid was added to a final concentration of 0.7%, and the gel was incubated for 15 min longer and then rinsed in water for up to 2 h. The gel was stained with silver by using conventional techniques (49).

LPS purification and lipid A isolation for mass spectrometry. LPS was isolated from the indicated strains (wild type [SL1344] and *phoP* [FL416] and *ydeI* [KDE602] mutants) using a rapid small-scale isolation method for mass spectrometry analysis (71). Briefly, a 1.0-ml aliquot of Tri-Reagent (Molecular Research Center, Cincinnati, OH) was added to a cell culture pellet (1 to 10 ml of an overnight culture), resuspended, and incubated at room temperature for 15 min. Chloroform (200 μl) was added, and the samples were vortex mixed and incubated at room temperature for 15 min. Samples were centrifuged for 10 min at 13,400 × *g* and the aqueous layers were collected. Water (500 μl) was added to the lower layers and vortex mixed. After 15 to 30 min, the samples were centrifuged as described above, and the aqueous layers were again collected. Two more aliquots of water were added to each sample for a total of four extractions. The combined aqueous layers were frozen and lyophilized.

TABLE 2. PCR primers used in this study

Primer[a]	Sequence (5′→3′)[b]
invA-P1	TGAAAAGCTGTCTTAATTTAATATTAACAGGATACCTATAGTGTAGGCTGGAGCTGCTTC
invA-P4	ATATCCAAATGTTGCATAGATCTTTTCCTTAATTAAGCCCATGGGAATTAGCCATGGTCC
invA-5′PCR	GCAGAACAGCGTCGTACTAT
invA-3′PCR	CGGAACGAACTAATTCAGCG
ompC-P1	ATAAAAAAGCAATAAAGGCATATAACAGAGGGTTAATAACGTGTAGGCTGGAGCTGCTTC
ompC-P4	AAAAAGGGCCCGCAGGCCCTTTAGCAACATCTTTTGCTGAATGGGAATTAGCCATGGTCC
ompC-5′PCR	GTTAACCAGTAAGCAGTGGC
ompC-3′PCR	TACGCCGGAATAAGGCATGA
ompD-P1	GTTGAGGAAACACGCTAAGAAAATTATAAGGATTATTAAAGTGTAGGCTGGAGCTGCTTC
ompD-P2	GCCCTGAAAGGACTGGCTTTGTATTCAGACTACAACAAAAATGGGAATTAGCCATGGTCC
ompD-5′PCR	AAACGCCTCGTTTAACAATG
ompD-3′PCR	TACATCAAGAGAAAAAGCCA
ompD-3xFLAG-5′	ACCGACAACATCGTTGCTGTTGGTCTGAACTACCAGTTCGACTACAAAGACCATGACGGT
ompD-3xFLAG-3′	CCAGTGAACGTCTGCACGGCATACTCCTTATGACCGAGTCCATATGAATATCCTCCTTAG
ompF-P1	GCAGGTGTCATATAAAAAAACCAATGAGGGTAATAAATAGTGTAGGCTGGAGCTGCTTC
ompF-P4	AAGTCCTGTTTTTGAGGCATAAAACAAAGGGGTCTGCTGAATGGGAATTAGCCATGGTCC
ompF-5′PCR	CGGAATTTATTGACGGCAGT
ompF-3′*PCR*	GAGATAAAAAAACAGGACCG
ompN-P1	CAATCTTTTGCAAATAAGTTAAGTTTTTAAGGATAAAAAAGTGTAGGCTGGAGCTGCTTC
ompN-P4	GCCCGCCGAAACGGCGGGCTTGAGAAGAATTAATGAATAAATGGGAATTAGCCATGGTCC
ompN-5′PCR	TCAACGAATCTGTAGAAGTT
ompN-3′PCR	GTGGTGATGAAAAAAGAAAA
phoE-P1	TCCCGACAAATCATAGCGCGTAATTAAAACAGGAATGGAAGTGTAGGCTGGAGCTGCTTC
phoE-P4	ATGCCTGATGGCGCAGCGCCATCAGGCACAATGCGACTTAATGGGAATTAGCCATGGTCC
phoE-5′PCR	TTCCTGTTTTTTACCGGGTT
phoE-3′PCR	GTCGGCATAACCCTGCTGCC
pmrA-P1	CCGCAGATGATATTCTGCAACCGTGCAGGAGACTAAGCGAGTGTAGGCTGGAGCTGCTTC
pmrA-P4	GAAGGGTCATCGCTCTTCGCTGAAAACGCATCAGGCTCACATGGGAATTAGCCATGGTCC
pmrA-5′PCR	ACAAACGACGTATTACCAGG
pmrA-3′PCR	TGTCAGCATTAAACGCTGGC
STM1530-P1	TTGCGGAAAGTCCAAAAATAAGACAAATAAGGCATATAAAGTGTAGGCTGGAGCTGCTTC
STM1530-P4	AAGGAGGGTATCCCTAAACTGTCTTAATCAGCAAGTTTTAATGGGAATTAGCCATGGTCC
STM1530-5′PCR	GACAGTTCCGCGAGCAGGCT
STM1530-3′PCR	CAGTGACACAGTTTTGGGAA
yddG-P1	AACGCTAATAGGGCTTGTTGCCATCGTTCTGTGGAGCACGGTGTAGGCTGGAGCTGCTTC
yddG-P4	GCGGGTCGATTTAATATCAATATCGGCCCGCCCGTCGCTAATGGGAATTAGCCATGGTCC
yddG-5′PCR	AGCATGACATCACAAAAAGC
yddG-3′PCR	ATGAAGAGTGTCAAGGTACA
ydeI-HA-5′	GCGAAGGAACCGCTTGTTCGCGTGAACCGACTGCAAAAATATCCGTATGATGTTCCT
ydeI-HA-3′	CGCTTTTACAAGACCCATGGTATCAGGCCAGCCGCGTGCCCATATGAATATCCTCCTTAG
ygiW-P1	ATTAAATGGATCTGAAACGACATGAAAGGGAAAAGTAATCGTGTAGGCTGGAGCTGCTTC
ygiW-P4	GGGCGGCTGTGAATCCTGACCGATCTTGCGCAATGTGGGAATGGGAATTAGCCATGGTCC
ygiW-5′PCR	AAGTTGTTAAGGATTACCTT
ygiW-3′PCR	TGTGGCCTATTCTGCCCACC

[a] The primer name indicates the gene or operon deleted or the epitope tag, followed by the primer binding site or epitope tag. P1, P2, and P4 indicate priming sites in pKD3 or pKD4 plasmids from Datsenko and Wanner (15). 5′PCR and 3′PCR refer to PCR primers used to screen deletion mutants and correspond to serovar Typhimurium genomic sequences. Primer families are divided by lines of space.

[b] Underlined sequences denote priming sequences for P1, P2, or P4 sites (15) or epitope tag sites in pSU315 (HA) or pSUB11 (3xFLAG) for 5′ epitope tag primers (65).

Lipid A was isolated from LPS after hydrolysis in 1% SDS as described previously (13). Briefly, 500 μl of 1% SDS in 10 mM sodium acetate (pH 4.5) was added to a lyophilized sample. Samples were incubated at 100°C for 1 h, frozen, and lyophilized. To remove detergent, pellets were extracted three times by resuspension in 100 μl of water and 1 ml of acidified ethanol (100 μl of 4 N hydrochloric acid in 20 ml of 95% ethanol) and centrifugation at 4,000 × *g* for 5 min. Pellets were washed three times in 1 ml of 95% ethanol. This entire process was repeated a total of three times. Samples were resuspended in 500 μl of water, frozen on dry ice, and lyophilized.

Mass spectrometry for lipid A analysis. Negative ion matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry experiments were performed as described for the analysis of lipid A preparations with the following modifications (33). Lyophilized lipid A was dissolved with 10 μl of 5-chloro-2-mercaptobenzothiazole (Sigma-Aldrich, St. Louis, MO) MALDI ma-

trix in chloroform-methanol at1:1 (vol/vol) and applied (1 μl) to the sample plate. Experiments were performed using a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA). Each spectrum was an average of 200 shots. Calibration was performed with ES Tuning Mix (Agilent, Palo Alto, CA).

pTac-YdeI cloning. A pGEX-3X plasmid without the glutathione *S*-transferase (GST) open reading frame (pGEX/GSTΔ) was constructed by sequential restriction digestion with EcoNI and BamHI. The *ydeI* gene was amplified from an SL1344 strain in which the *ydeI* chromosomal gene was tagged with the hemagglutinin peptide (HA) epitope tag (KDE642, see above). PCR primers (KDE821 and KDE822) to amplify *ydeI*::HA included EcoNI (KDE821) and BamHI (KDE822) restriction sites (underlined) and were as follows: KDE821, 5′-GGC GGATATCTGGTTACCCGTCAAATAGACG-3′; and KDE822, 5′-GCGGGA TCCTCTCTAGACGCAACCAGACC-3′. Amplified *ydeI*-HA product was digested with EcoRV and BamHI restriction endonucleases, ligated into pGEX/GSTΔ digested with EcoNI and BamHI, and recovered in DH5α library efficiency *E. coli* (Invitrogen). pYdeI-HA plasmids were transformed into SL1344 and screened by immunoblotting for YdeI-HA expression.

Immunoaffinity chromatography. pYdeI-HA was purified from a wild-type SL1344 background (KDE686, MCP847, and MCP848) by immunoaffinity chromatography using a ProFound HA tag immunoprecipitation/coimmunoprecipitation kit and according to the manufacturer's instructions except where noted (Pierce Thermo Fisher Scientific, Rockford, IL). Briefly, 20-ml cultures at an OD_{600} of 0.5 to 0.6 were induced with 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 h. Cultures were centrifuged, and pellets were resuspended in B-PER lysis reagent (Pierce Thermo Fisher Scientific), which contains the detergent *n*-octyl-β-D-thioglucopyranoside (70), supplemented with lysozyme (100 μg/ml) and DNase I (100 U/ml). After 15 min of incubation at room temperature, lysates were centrifuged at 4,000 × *g* for 10 min, and the supernatant was harvested and loaded onto columns containing 20 μl of anti-HA agarose beads. YdeI-HA and GST-HA were eluted with either an HA dipeptide or at pH 2.8. The same procedure was carried out with the wild-type SL1344 strain. Samples were resolved by SDS-PAGE, which was transferred to polyvinylidene difluoride membrane for immunoblotting or stained either with silver or SYPRO-Ruby for mass spectrometric analysis, which was performed by the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry (http://mass-spec.stanford.edu). Five peptides unique to OmpD were identified, as described in the results, and the overall score for OmpD was 248. The peptides spanned amino acids 26 to 345, out of 362 total amino acids in OmpD. The protein with the next best score, 55, was ApbE, a 38-kDa thiamine biosynthesis lipoprotein precursor, for which a single 29-amino-acid fragment was identified. No peptides from porins other than OmpD were identified.

Coimmunprecipitation of chromosomally expressed proteins. Epitope-tagged strains (KDE769 and KDE642) were grown overnight in LB medium plus antibiotic. Equivalent numbers of bacteria were pelleted by centrifugation at 5,000 × *g* and washed with 10 mM Tris (pH 7.5). Pellets were frozen at −20°C and lysed by using B-PER lysis reagent (Pierce Thermo Fisher Scientific) with lysozyme (100 μg/ml). Insoluble material was removed by centrifugation at 14,000 × *g*. Supernatants were precleared by incubation with protein G-agarose beads at 4°C for 1 h, followed by centrifugation. Supernatants were incubated at 4°C for 4 h with protein G-agarose beads (Chemicon, Bedford, MA) coupled to either mouse anti-HA (clone F-2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or mouse anti-FLAG (clone M2; Sigma-Aldrich) at 2 μg of antibody per 25 μl of beads. Beads were pelleted by centrifugation, washed three times with Tris-buffered saline plus 0.05% Tween 20, and resuspended in an equal volume of 4× SDS sample buffer. Samples were analyzed by immunoblotting with rabbit anti-HA (a gift from T. Su) or rabbit anti-FLAG (Sigma-Aldrich) antibody.

Polymyxin b and cathelicidin antimicrobial peptide resistance. A modified microtiter plate assay was used to establish strain resistance to antimicrobial peptides (67). Briefly, overnight bacterial cultures were diluted 1:25 or 1:100 in MgM plus 10 μM magnesium chloride and grown to an OD_{600} of ~0.3 (~10^8 bacterial CFU/ml). Bacteria were harvested by centrifugation and resuspended in LB medium at 10^7 bacteria/ml. Then, 50-μl aliquots were incubated in 96-well polypropylene plates in triplicate with freshly prepared polymyxin B (Sigma-Aldrich) at 4 to 8 μg/ml or cathelicidin antimicrobial peptide at 50 μM (a gift from Robert Hancock). Plates were incubated at 37°C with agitation for 1 h and then diluted two- to fourfold in phosphate-buffered saline (PBS) on ice. To determine $CFU_{plus\ AMP}$, 100 μl of each culture was spread onto selective LB agar plates. CFU_{input} was determined by plating diluted untreated bacteria. The percent survival was determined by dividing the $CFU_{plus\ AMP}$ by CFU_{input}. The following strains were examined: wild type (SL1344) and *phoP* (FL416), *ydeI* (KDE602), *ompD* (KDE735), *ydeI ompD* (KDE755), *ygiW* (KDE806), *ompC* (KDE805), *ompF* (KDE801), *ompE/phoE* (KDE800), *ompN* (KDE802), STM1530 (KDE803), and *invA* (KDE565) mutants. Strains were tested in triplicate with a wild-type control, and *P* values within experiments were calculated from sample triplicates as indicated in the figure legends. *P* values were also calculated between experiments: *phoP* ($P = 0.000$), *ydeI* ($P = 0.002$), *ompD* ($P = 0.001$), *ygiW* ($P = 0.005$), *ompC* ($P = 0.025$), *ompE/phoE* ($P = 0.257$), *ompN* ($P = 0.061$), and STM1530 ($P = 0.238$).

Cell fractionation. Chloroform shock treatment was performed as previously described (3). Briefly, strain KDE642 (expressing chromosomal YdeI-HA) was grown aerobically at 37°C in LB/Str medium containing 0.2% maltose. Portions (2 ml) of overnight bacterial cultures were centrifuged at 4,000 × *g* for 10 min. Cell pellets were vortex mixed, resuspended in residual medium plus chloroform (20 μl), and incubated at room temperature for 15 min. Next, 200 μl of 0.01 M Tris (pH 8.0 at room temperature) was added, and samples were centrifuged at 6,000 × *g* for 20 min. Periplasmic and cytoplasmic proteins fractionate with the supernatant and pellet, respectively. Whole-cell lysates were prepared by pelleting 200 μl of overnight culture and resuspending the pellets in 200 μl of 0.01 M Tris.

Immunoblotting. Samples were mixed with 8× sample loading buffer, separated by SDS–15% PAGE, and transferred to polyvinylidene difluoride membrane by semidry blotting. Membranes were blocked with 5% nonfat powdered milk–PBS–0.05% Tween 20 for 1 h at room temperature. Primary antibodies were diluted in either PBS–0.05% Tween 20 or 5% milk–PBS–0.05% Tween 20 and incubated with blots either at 37°C for 45 min or at 4°C overnight. Primary antibodies were diluted as follows: anti-HA, 1:200 (clone F-2; Santa Cruz); anti-DnaK, 1:1,000 (clone 8E2/2; VWR, West Chester, PA); anti-maltose binding protein, 1:1,000 (NEB, Ipswich, MA), or anti-FLAG, 1:1,000 (Sigma-Aldrich). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) diluted 1:10,000 in PBS–0.05% Tween 20 for 1 h at 37°C. Proteins were visualized by enhanced chemiluminescence (GE Healthcare, Pittsburgh, PA).

Methyl viologen sensitivity disk assay. Wild-type (SL1344), *ompD* (KDE 735), *yddG* (KDE 737), and *ydeI* (KDE602) strains and 14028s, a wild-type strain used in previous methyl viologen assays (55), were grown overnight in LB medium with antibiotics at 37°C. Cultures were diluted 1:10 in PBS to an OD_{600} of 10^7 bacteria/ml and spread to LB- or M9-agar plates with or without antibiotics. A 0.5 M stock of methyl viologen was diluted in water to concentrations ranging from 0.1 to 100 mM. Methyl viologen was spotted onto sterile filter paper disks and placed in the center of the plates containing spread bacteria. Plates were incubated overnight at 37°C, and the diameter of the growth inhibition zones was determined as the average of the largest and the smallest diameters, which differed by <1 mm. Over the course of six experiments, differences in halo size between strains were not observed at any concentration of methyl viologen.

Statistics. *P* values were determined with two-tailed Student *t* tests. Error bars represent standard deviations.

RESULTS

***ydeI* expression is regulated by multiple sensor-kinase signaling systems.** The PhoPQ and PmrAB two-component regulatory systems confer bacterial resistance to polymyxin B by modifying the lipid A component of LPS (30). *pmrA* mutant strains are, like *ydeI* mutants strains, defective for virulence upon oral but not intraperitoneal inoculation, suggesting that both genes play roles in the gastrointestinal tract phase of infection (20, 29). However, *pmrA* is important for survival in 10 μM iron (69), whereas *ydeI* is not (data not shown). To establish whether *ydeI* induction in the presence of polymyxin B requires the PhoP or PmrA response regulators, *ydeI* mRNA accumulation was evaluated by real-time quantitative PCR in early exponential stage polymyxin B-treated wild-type and mutant strains (Fig. 1A). *ydeI* expression increased approximately threefold in wild-type *Salmonella* treated with polymyxin B, but not in strains lacking *rcsB*. This confirmed that *rcsB* is required for the induction of *ydeI* (20). *ydeI* mRNA levels were similarly low in strains lacking *phoP*, either in the presence or absence of polymyxin B. In *rcsB phoP* double mutant strains, *ydeI* transcript levels were similar to the background levels observed in strains lacking the *ydeI* gene. In the presence of polymyxin B,

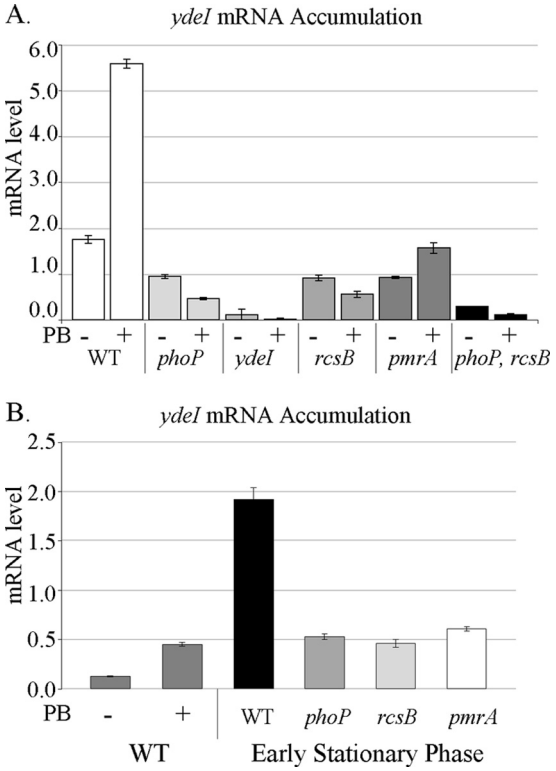

FIG. 1. *ydeI* mRNA accumulation requires PhoP and PmrA. Real-time quantitative PCR was used to evaluate *ydeI* mRNA levels and samples were normalized to a *gapA* control. (A) *ydeI* mRNA levels after exposure to polymyxin B for 40 min in LB medium using strains with the indicated genes deleted. (B) *ydeI* mRNA levels in early stationary phase cells in LB medium. WT, wild type. The data are representative of three independent experiments.

pmrA mutant strains had higher levels of *ydeI* mRNA than *rcsB* and *phoP* mutant strains but significantly lower levels than the wild type (Fig. 1A). These data show that *ydeI* induction via polymyxin B treatment is modulated by the response regulators of three sensor-kinase systems, RcsB, PhoP, and PmrA.

The RcsBCD system is activated in early stationary phase (35, 52), which may mimic the growth state of *S. enterica* in the gastrointestinal tract (40). To determine whether cells at early stationary phase require PhoP and PmrA for *ydeI* regulation, mRNA was examined. *ydeI* mRNA levels were approximately four times higher in wild-type strains at early stationary phase than in log phase after treatment with polymyxin B (Fig. 1B), a finding consistent with *ydeI* regulation by RpoS, the stationary phase sigma factor (20). *ydeI* accumulation was also clearly dependent on *rcsB*, *phoP*, and *pmrA*. Thus, the PhoPQ, PmrAB, and RcsBCD sensor-kinase systems play a role in inducing *ydeI* in early stationary phase, as well as in response to polymyxin B.

***ydeI* does not modify LPS based on SDS-PAGE and mass spectrometry analysis.** Molecular modifications of LPS lipid A, core, or O-antigen confer resistance to cationic peptides, including polymyxin B (2, 30). Genes that modulate O-antigen chain length in *S. enterica* are activated by the PmrAB and RcsBCD systems (16), and PhoPQ may play an indirect role in modulating O-antigen chain length (29, 41). We examined

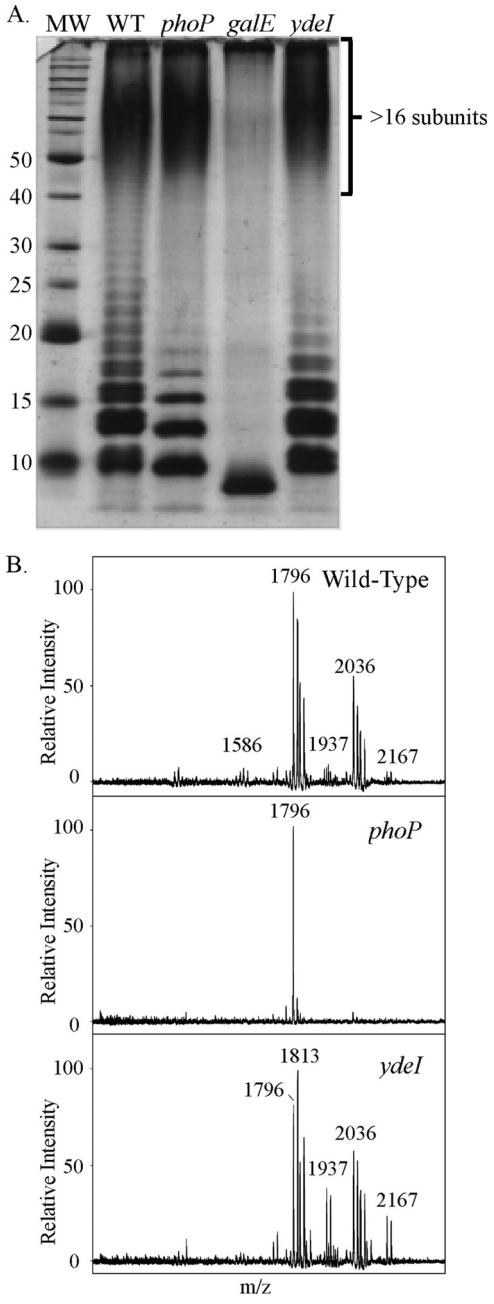

FIG. 2. LPS and lipid A from *ydeI* mutant strains and the wild type are indistinguishable. (A) LPS was isolated from wild-type (WT) and strains with deletions of the genes indicated, separated by SDS-PAGE, and silver stained. (B) MALDI-TOF mass spectra of lipid A from wild-type and *phoP* and *ydeI* mutant strains. The mass/charge (*m*/*z*) ratios and related structures are as follows: 1,796 unmodified hexa-acyl lipid A; 1,813, hexa-acyl lipid A with 2-OH myristate; 1,937, hexa-acyl lipid A with Ara4N; 2,036, unmodified hepta-acyl lipid A; and 2,167, hepta-acyl lipid A with Ara4N.

whether chain length is altered in *ydeI* mutants by SDS-PAGE analysis of LPS isolated from wild-type and *ydeI* mutant strains (Fig. 2A). A *galE* mutant control, a rough strain lacking O-antigen, had the lipid A and core region of LPS but lacked bands of higher molecular weight. *phoP* mutant-derived LPS had a banding pattern similar to that of the wild type, consis-

tent with previous observations (6, 23). In addition, *ydeI* mutants had LPS ladder profiles similar to that of the wild type. These results suggest the polymyxin B sensitivity of *ydeI* mutant strains is not due to a change in O-antigen length or status.

PhoPQ and the PmrAB sensor-kinase systems contribute to cationic AMP resistance by modifying the lipid A portion of LPS (28, 32, 73). Since PhoPQ and PmrAB regulate *ydeI*, the question of whether *ydeI* mutants can modify lipid A was examined. LPS was isolated from *Salmonella* wild-type and mutant strains, and the lipid A portion was analyzed by mass spectrometry. A *phoP* mutant strain was included as a negative control, since *phoP* is needed to modify lipid A by palmitoylation, phosphoethanolamine incorporation, or 4-amino-4-deoxy-L-arabinose addition (21). Negative-ion MALDI-TOF mass spectrometry revealed that the lipid A isolated from *ydeI* mutants contained modifications similar to those of the wild type (Fig. 2B). These results, along with the O-antigen chain length analysis, indicate that YdeI contributes to antimicrobial peptide resistance using a mechanism that does not involve LPS modifications.

OmpD specifically copurifies with YdeI. Since it does not appear that YdeI modifies LPS, a different approach was taken to understand how YdeI may contribute to AMP resistance. Immunoaffinity chromatography was performed to identify proteins that may interact with YdeI. YdeI was epitope tagged with HA and expressed from the pTac promoter (pYdeI-HA) on a plasmid in a wild-type strain. YdeI-HA was purified by using immunoaffinity chromatography and eluted with either an HA dipeptide or at low pH. Regardless of elution method, a discrete 40-kDa band of protein copurified with YdeI-HA (Fig. 3A). Control experiments with a wild-type strain yielded no bands, indicating that the 40-kDa band does not interact with the column. A 28-kDa protein copurified with YdeI-HA inconsistently between experiments and was not pursued further. The 40-kDa protein band was isolated and subjected to proteolytic digestion and tandem mass spectrometric analysis, which identified five peptides of 11 to 13 amino acids in length, each specific to OmpD, a major outer membrane porin.

To determine whether the interaction between OmpD, an abundant protein (4), and YdeI was specific, the experiment was repeated with two additional strains harboring chromosomal OmpD-3xFLAG, one with pGST-HA and one with pYdeI-HA. Silver staining shows coelution of a 40-kDa protein band with YdeI-HA whether or not OmpD was epitope tagged, but not with GST-HA. GST-HA comigrates with the 28-kDa nonspecific band, as expected (Fig. 3B). Immunoblotting of GST-HA flowthrough and eluate revealed OmpD-3xFLAG in the former but not in the latter, showing that OmpD-3xFLAG was produced but did not bind GST-HA (Fig. 3C). Thus, OmpD specifically interacts with YdeI.

OmpD coimmunoprecipitates with YdeI. To establish whether YdeI interacts with OmpD under conditions in which both proteins are regulated by their native promoters, coimmunoprecipitations were performed with chromosomally expressed YdeI (chrYdeI-HA) and OmpD (OmpD-3xFLAG). In these strains, an epitope tag sequence was inserted in-frame at the C terminus of the *ydeI* or *ompD* genes. When YdeI-HA was immunoprecipitated with anti-HA antibody, OmpD-3xFLAG coimmunoprecipitated, indicating that YdeI and OmpD interact (Fig. 4). However, when OmpD-3xFLAG was immunopre-

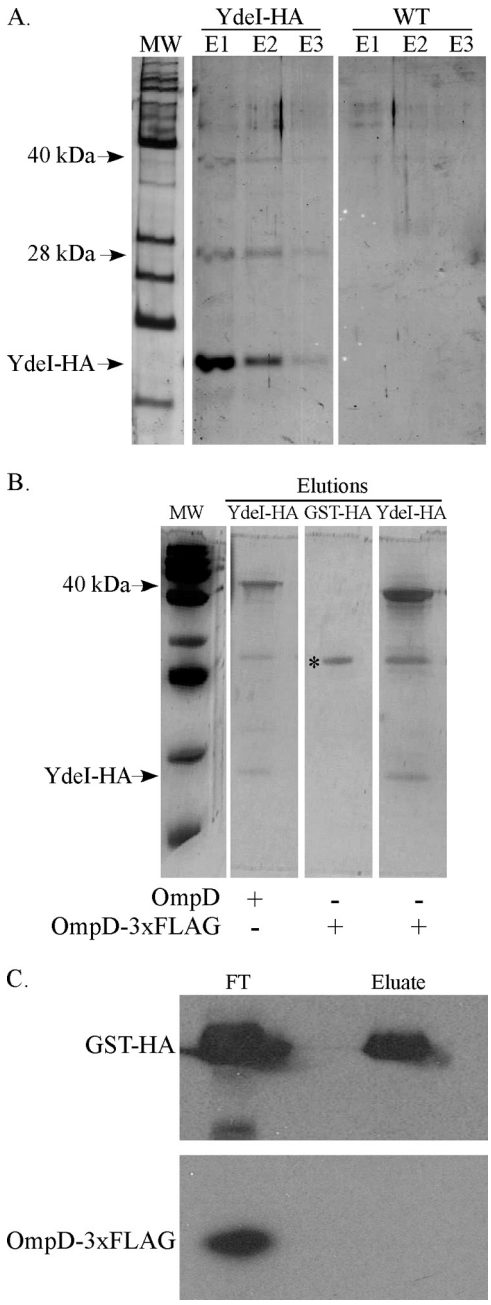

FIG. 3. OmpD specifically copurifies with YdeI. (A) Bound proteins were eluted from YdeI-HA using an HA dipeptide, resolved by SDS-PAGE, and analyzed by silver staining. Eluted fractions (E1 to E3) from a pYdeI-HA strain (left) and from a wild-type (WT) strain (right). The 40-kDa band was isolated and submitted for mass spectrometric analysis. Lanes shown are from the same gel, but the image was cropped to remove unloaded lanes. (B) Bound proteins were eluted from YdeI-HA or GST-HA with low pH, resolved by SDS-PAGE, and analyzed by silver staining. Each strain harbored endogenous OmpD or chromosomal OmpD-3xFLAG, as marked. GST-HA (28 kDa) is marked with an asterisk. Lanes shown are from the same gel, but the image was cropped to remove unloaded lanes. (C) The flowthrough (FT) and eluate from the GST-HA sample (B) were subject to immunoblotting with anti-FLAG and anti-HA antibodies.

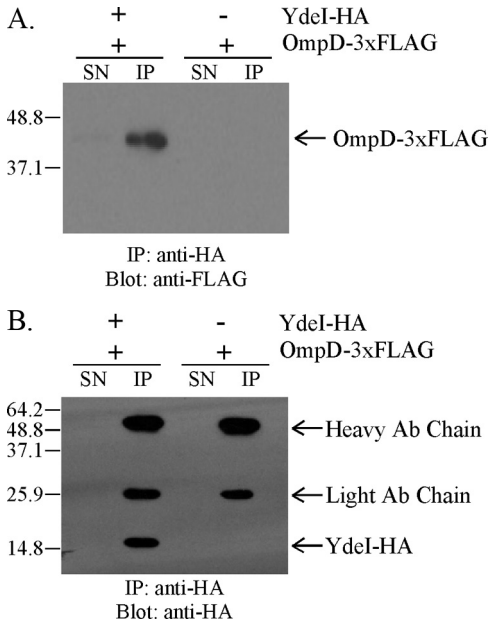

FIG. 4. OmpD-3xFLAG coimmunoprecipitates with chromosomally expressed YdeI-HA. Strains encoding chromosomally expressed, epitope-tagged YdeI-HA and OmpD-3xFLAG were grown overnight in LB medium, harvested, and lysed, and YdeI-HA was immunoprecipitated with mouse anti-HA antibody coupled to protein G-agarose beads. Supernatant (SN) or agarose bead immunoprecipitate (IP) from equivalent numbers of bacteria was resolved by SDS-PAGE and analyzed by immunoblotting with (A) an anti-FLAG antibody or (B) an anti-HA antibody. Molecular weight markers are indicated by bars to the left; the heavy and light chains of the immunoprecipitating antibody are indicated by arrows to the right of (B).

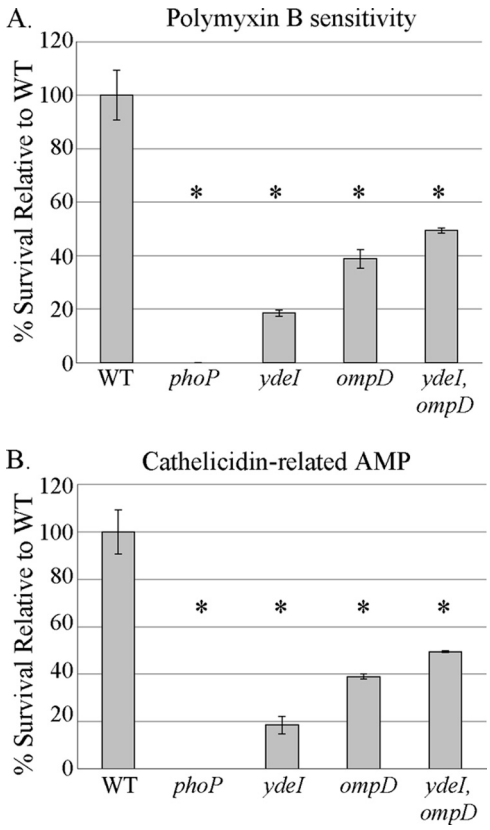

FIG. 5. *ompD* mutant strains are sensitive to polymyxin B and cathelicidin antimicrobial peptide. Strains harboring deletions of *ydeI*, *ompD*, or both genes were incubated in the presence of 4 μg of polymyxin B/ml (A) or 50 μM cathelicidin antimicrobial peptide (B) and plated for CFU. Controls included wild-type (WT) and a *phoP* insertion mutant. The data are reported as percent survival relative to the wild type. Asterisks represent *P* values on triplicate samples, as determined by a Student *t* test ($P < 0.001$).

cipitated with anti-FLAG antibody, YdeI-HA did not coimmunoprecipitate (data not shown), suggesting the anti-FLAG antibody disrupts the YdeI-OmpD interaction or that only a fraction of OmpD interacts with YdeI. Nevertheless, the data as a whole confirm the observation that YdeI interacts with the OmpD porin under native conditions.

OmpD is important for resistance to polymyxin B. Genetic analyses were performed based on the hypothesis that if YdeI and OmpD interact to confer antimicrobial peptide resistance, then strains lacking *ompD* would be sensitive to antimicrobial peptides. *ydeI* and *ompD* single mutant strains were both mildly sensitive to polymyxin B compared to the extremely sensitive *phoP* mutant control strain (Fig. 5A). This observation is consistent with the idea *ydeI* and *ompD* represent just one of the multiple mechanisms of AMP resistance activated by PhoP (21). In addition, *ydeI ompD* double mutant strains were only as sensitive to polymyxin B as the single mutant strains. If YdeI and OmpD functioned in independent pathways to confer AMP resistance, the double mutant strains would be expected to be more sensitive to AMP than either single mutant strain, particularly since the strains analyzed contain complete deletions of the corresponding genes. These data suggest YdeI and OmpD function in the same pathway to confer polymyxin B resistance.

YdeI and OmpD confer resistance to a mouse cathelicidin. *S.* Typhimurium strains lacking *ydeI* are attenuated in mice when delivered orally, the natural route of infection, but not when delivered into the peritoneal cavity (20). This pattern of attenuation suggests that YdeI may be important for resistance to gastrointestinal tract antimicrobial peptides, an idea consistent with the observation that the bacteria adhere to the mouse small intestine (7). Cathelicidin antimicrobial peptide is a murine cationic AMP produced by multiple cell types, including macrophages and epithelial cells (9, 14, 54), and is expressed in the small intestine (24). Single mutant *ydeI* and *ompD* strains were mildly sensitive to cathelicidin antimicrobial peptide exposure (Fig. 5B), showing that both genes are important for resistance to a physiologically relevant AMP. Double-mutant *ydeI* and *ompD* strains were only as sensitive to cathelicidin antimicrobial peptide as single mutant strains, suggesting that YdeI and OmpD function together to increase bacterial resistance to gastrointestinal tract AMPs.

YdeI is a periplasmic protein. To establish whether YdeI localizes to the periplasm and therefore could plausibly interact with OmpD, cell fractionation and immunoblotting of strains with chromosomal YdeI-HA were performed. YdeI-HA localized to both the cytoplasmic and the periplasmic samples, as determined by DnaK and maltose binding protein control proteins, respectively (Fig. 6). YdeI-HA in the cytoplasm may reflect ongoing protein translation or that YdeI has an un-

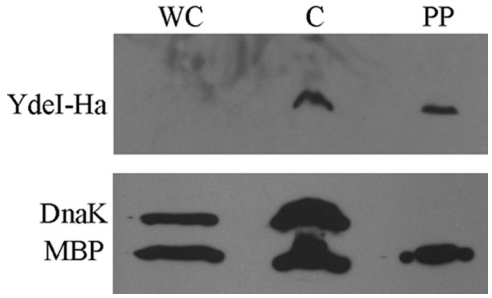

FIG. 6. YdeI is in the periplasm. Immunoblotting of whole-cell (WC) lysates and fractions from an YdeI-HA strain (KDE686). The upper blot was probed with an anti-HA antibody, and the lower with anti-DnaK and anti-maltose binding protein (MBP) antibodies to identify cytoplasmic (C) and periplasmic (PP) fractions.

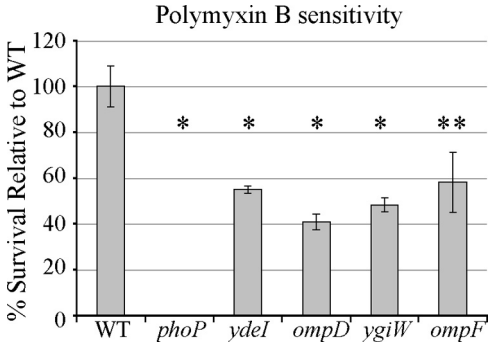

FIG. 7. *ygiW* and *ompF* mutants are sensitive to polymyxin B. Strains with the indicated gene deleted were incubated with 8 μg of polymyxin B/ml and plated for CFU. The data are reported as percent survival relative to the wild type (WT). Single and double asterisks represent *P* values on triplicate samples, as determined by a Student *t* test ($P < 0.01$ and $P < 0.001$, respectively).

known role in the cytoplasm. Nevertheless, the clear presence of YdeI-HA in the periplasm is consistent with a model in which YdeI interacts with the outer membrane protein OmpD in the periplasmic compartment.

The porin *ompF* and the OB-fold protein *ygiW* contribute to polymyxin B resistance. To establish whether other porins or OB-fold proteins may contribute to AMP resistance, strains with deletions of the following genes were constructed: *ompC*, *ompF*, *ompE*, *ompN*, and STM1530 (porins), and *ygiW* (OB-fold protein). *ompF* and *ygiW* mutants were moderately sensitive to polymyxin B (Fig. 7). The remaining porins and also an *invA* mutant strain, lacking an inner membrane protein, were not sensitive to polymyxin B ($P > 0.01$, data not shown). The results suggest that *S.* Typhimurium periplasmic OB-fold proteins, YdeI and YgiW, and the porins OmpD and OmpF may help protect the bacteria from AMPs.

DISCUSSION

To establish how YdeI contributes to AMP resistance, we first determined whether *ydeI* activation requires the PhoPQ or PmrAB sensor-kinase systems, which together regulate a large number of genes important for AMP resistance (30). *ydeI* induction required *phoP* and *pmrA* upon exposure to polymyxin B and in early stationary phase (Fig. 1). Whether transcription of *ydeI* is directly or indirectly regulated by PhoP, PmrA, and RcsB is unknown, but a putative PhoP-binding site exists 65 nucleotides upstream of the *ydeI* promoter (47; data not shown). PhoP and PmrA confer AMP resistance by activating genes needed to modify LPS (30), but *ydeI* does not appear to play a major role in altering O-antigen length or the charge or fatty acid composition of LPS (Fig. 2). To identify proteins that might interact with YdeI, mass spectrometry was performed on a 40-kDa band of protein that coeluted with YdeI (Fig. 3). The major protein identified was OmpD, an outer membrane protein, and immunoprecipitation indicated that chromosomal YdeI and OmpD interact (Fig. 4). OmpF is a 40.0-kDa protein that should migrate very similarly to OmpD (39.7-kDa). A band of ~40-kDa was sent for mass spectrometry and no peptides matching OmpF were found. Strains with a deletion in *ompD*, *ydeI*, or both genes are moderately sensitive to polymyxin B and to cathelicidin antimicrobial peptide (Fig. 5), an AMP expressed in the mouse intestine (24). Furthermore, YdeI localizes to the periplasm (Fig. 6). Collectively, the data suggest that periplasmic YdeI may interact with OmpD in the outer membrane to facilitate AMP resistance. We propose that YdeI be renamed OmdA, for OmpD-Associated protein.

OmpD is a general porin, defined as a protein that forms a trimeric outer membrane channel capable of transporting solutes based on size and charge (50). The channel of OmpD has a minimum pore size of 0.8 nm and preferentially transports cations (8). OmpD has been proposed to function in conjunction with a predicted inner membrane efflux pump, YddG, in resistance to methyl viologen (paraquat) and aromatic amino acids (19, 55, 56). We did not find that a deletion of *yddG* significantly altered polymyxin B sensitivity (data not shown), and strains lacking *ompD*, *ydeI*/*omdA*, or *yddG* were not more sensitive to methyl viologen than the wild-type parent strain (see Materials and Methods). In addition, several other general porins and also an inner membrane protein, InvA, did not contribute significantly to polymyxin B resistance. However, the porin OmpF and the OB-fold periplasmic protein YgiW did show evidence of conferring AMP resistance (Fig. 7).

In *Vibrio cholerae*, the porin, OmpU, has been implicated in bacterial resistance to multiple stressors, including polymyxin B, organic acids, and bactericidal/permeability-increasing protein (a 55- to 60-kDa protein on the surface of human gastrointestinal tract epithelial cells) (12, 43, 45, 66). In *Vibrio fischeri*, OmpU is important for resistance to anionic detergents such as bile salts and SDS (1, 53). OmpU may respond to multiple kinds of stress via the periplasmic protease DegS, which activates SigmaE, the stress-response RNA polymerase subunit (42). Whether the single OB-fold protein identified by bioinformatics in *Vibrio* species (25) plays a role in this pathway is unknown. In *S.* Typhimurium, YdeI/OmdA does not appear to be important for generalized stress responses, as *ydeI*/*omdA* mutants do not have increased sensitivity to the detergent deoxycholate (data not shown). These observations suggest the existence of multiple mechanisms by which porins contribute to resistance to small toxic molecules.

The *S.* Typhimurium porin OmpD and the OB-fold protein YdeI/OmdA may function together to protect bacteria from cationic AMPs. Both *ydeI*/*omdA* and *ompD* are important for virulence in mice, since *ydeI*/*omdA* mutants survive poorly in

mice upon oral, but not intraperitoneal, infection (18), and *ompD* insertion mutants have an oral 50% lethal dose that is 13-fold higher than that of wild-type strains (20). *ydeI*/*omdA* single mutants are slightly more sensitive to AMPs than either *ompD* single or *ydeI*/*omdA ompD* double mutants, indicating that YdeI/OmdA contributes to AMP resistance only when OmpD is present. One possibility is that cationic AMPs can enter the cell through OmpD, but YdeI either blocks AMP entry or increases export via OmpD. Since it is difficult to imagine how YdeI could block AMP import without blocking nutrient uptake through OmpD, it seems more likely that YdeI/OmdA passively increases cationic AMP export. For instance, YdeI/OmdA may capture cationic AMPs in the periplasm near the OmpD pore, creating an AMP concentration gradient and thereby increasing diffusion of AMPs out of OmpD. These observations collectively suggest a new mechanism by which gram-negative bacteria resist AMPs, involving interaction between a general porin and an OB-fold protein.

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