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The Critical Roles of Polyamines in Regulating ColE7 Production and Restricting ColE7 Uptake of the Colicin-producing *Escherichia coli**

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The ColE7 operon is an SOS response regulon, which encodes bacteriocin ColE7 to kill susceptible *Escherichia coli* and its related enterobacteria under conditions of stress. We have observed for the first time that polyamines confer limited resistance against ColE7 on *E. coli* cells. Thus, this study aims to investigate the role of polyamines in modulating the protective effect of the *E. coli* cells against colicin. In the experiments, we surprisingly found that endogenous polyamines are also essential for ColE7 production, and the rate of polyamine synthesis is directly related to the SOS response. Our experimental results further indicated that exogenous polyamines suppress the expression of TolA, BtuB, OmpF, and OmpC proteins that are responsible for ColE7 uptake. Moreover, two-dimensional gel electrophoresis revealed that the production of two periplasmic proteins, PotD and OppA, is increased in *E. coli* cells under ColE7 exposure. Based on these observations, we propose that endogenous polyamines may play a dual role in the ColE7 system. Polyamines may participate in initiating the expression of the SOS response of the ColE7 operon and simultaneously down-regulate proteins that are essential for colicin uptake, thus conferring a survival advantage on colicin-producing *E. coli* under stress conditions in the natural environment.

Polyamines (putrescine, spermidine, and spermine) are aliphatic cations ubiquitous to all living organisms (1). They have been shown to affect membrane permeability, gene expression, intracellular signaling, and apoptosis through noncovalent interactions or specific conjugation with proteins, nucleic acids, phospholipids, and other acidic substances (2–5). Because of their important regulatory functions, the biosynthesis, degradation, uptake, and excretion of polyamines are stringently regulated in order to maintain appropriate cellular levels (6, 7). Recently, polyamines have been shown to mediate the production of RecA, which together with LexA controls the SOS response regulon (8–10). Polyamines are also known to modulate the gating of ion channels such as *N*-methyl-D-aspartate receptors of neurons and the bacteria outer membrane porins OmpF, OmpC, and PhoE (11–13). In this modulation, putrescine and spermidine have been shown to bind to the aspartic acid residues located at positions 113 and 121 of OmpF and position 105 of OmpC, altering the charge and pore size of these porins and membrane permeability of *E. coli* cells (12, 14).

Bacteriocins are one of the most abundant and diverse classes of antimicrobial toxins produced by all major lineages of eubacteria and archaebacteria (15). The major function of bacteriocins appears to mediate population dynamics within species. One class of bacteriocins, the colicins produced by *Escherichia coli*, have served as the model for exploring the ecological role of these potent toxins (16).

All colicins found to date are plasmid-encoded. For example, the colicin E7 (ColE7) operon contains *cea*, *cei*, and *cel* genes located on a 6.2-kb native plasmid (pColE7-K317) (17). The ColE7 operon is transcribed as a polycistronic mRNA in the order *cea-cei-cel* by the ColE7 promoter that has two SOS boxes (ATCTGTACATAAAACCAGTG-GTTTTATGTACAGAT) located in an inverted repeat orientation and is regulated by the LexA repressor (17). The *cea* is a toxin structural gene, which encodes the DNase ColE7 of 576 amino acid residues, and the product of *cei* gene composed of 87 amino acids is the ColE7 inhibitor (Im7). Im7 binds to the C-terminal DNase (T_2A) domain of ColE7 forming a ColE7-Im7 complex and keeps ColE7 inactive inside the ColE7 producers (18, 19). In addition, the *cei* gene is also transcribed independently from its own constitutive promoter that is active at a low level (20). The *cel* gene encodes the lysis protein (Lys-7) for ColE7 secretion. Lys-7 is translated as a 47-amino acid precursor and then processed to a mature form of 28 amino acids (21).

The translocation mechanism of colicin across the membrane of *E. coli* and the mode of action of ColE7 have been well documented (22–24). Once ColE7 binds to the outer membrane receptor BtuB through its internal receptor binding (R) domain, it further translocates into the envelope of susceptible cells through interactions between the N-terminal membrane translocation (T) domain and the outer membrane porins OmpF or OmpC as well as the Tol/Pal translocation system, which consists of the TolA, TolB, TolQ, TolR, and Pal proteins (22, 24). As a result, the DNase T_2A domain extends into the periplasmic space. The T_2A domain of ColE7 is then cleaved from the rest of the colicin and transported into the cytoplasm to hydrolyze the chromosome of susceptible cells (25, 26). It has been claimed that proteins interacting with colicins during translocation are all indispensable for colicin uptake (22, 27).

In this study, we use ColE7 as a research model to investigate the roles of polyamines in the ColE7 production and the ColE7 transportation. We found for the first time that endogenous polyamines are essential for triggering ColE7 production under mitomycin C induction. We also found that both endogenous polyamines and exogenous spermidine render limited resistance to *E. coli* cells against ColE7, suggesting that polyamines play a role in restricting colicin translocation across the

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TABLE 1
The *E. coli* strains used in this study

Strains	Genotype	Source or Ref.
W3110	F$^-$, *mcrA mcrB* In(*rrnD-rrnE*)1, λ$^-$	ATCC
JM101	F′, [*tra*D36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *supE thi* Δ(*lac-pro*AB)	Ref. 63
JM109	F′, [*tra*D36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ (*lac-proAB*)	
M15		Qiagen
AB1111	F$^-$, *thr*-1 *proA*2 *thi*-1 *lacY*1 *galK*2 *mtl*-1 *xyl*-5 *ara*-14 *supE*44 *str*-25 *leu*-6 *Rac*-0 *hisC*3, λ$^-$	Coli Genetic Stock Center, Yale University
HT252 (EWH319)	F$^-$, Δ(*speA speB*) Δ(*speC glc*) Δ*speD thr*-1 *proA*2 *thi*-1 *lacY*1 *galK*2 *mt*l-1 *xyl*-5 *ara*-14 *supE*44 *str*-25, λ$^-$	ATCC (29)
HT306	F$^-$, Δ(*speB-speA*)97 *glc*-1 Δ*speD*98 *thr*-1 *proA*2 *thi*-1 *lacY*1 *galK*2 *mtl*-1 *xyl*-5 *ara*-14 *supE*44 *str*-25 *ampA*1 *cadA*2, λ$^-$	Coli Genetic Stock Center (30)
HT414	F$^-$, Δ(*speA speB*)97 *glc*-1 *thr*-1 *thi*-1 *lacY*1 *supE*44 *rplL*9 *fhuA*21 *hsdS*1, λ$^-$	Coli Genetic Stock Center
D18	*speD*3-1	ATCC (28)
HT375	F$^-$, Δ*speD98 lacZ43*(Fs) *str*	CGSC (28)

E. coli cell membrane. By using this ColE7 model, we have demonstrated in *E. coli* that the presence of endogenous polyamines induces ColE7 production but restricts ColE7 uptake. Thus, the ecological role of polyamines in conferring survival advantages for a colicin-producing cell is discussed.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Bacterial Cultures—*E. coli* strains used in this study are listed in Table 1. Strain W3110 was purchased from the ATCC (Manassas, VA). M15(pREP4), JM101, and JM109 were from our own collections and were used as hosts for expression of recombinant proteins. Polyamine-deficient mutants HT306, HT414, and HT375 were obtained from the Coli Genetic Stock Center, Yale University. HT252 and D18 were from the ATCC. HT375 and D18 were defective in spermidine biosynthesis and produced only putrescine (28). HT252, HT306, and HT414 were defective in both putrescine and spermidine biosynthesis (29–31). Strain AB1111, obtained from Coli Genetic Stock Center, Yale University, has many identical genomic markers of all the polyamine mutants used in this work, so that AB1111 and W3110 were used as polyamine wild type controls. The plasmid pColE7-K317 containing the ColE7 operon has been described previously (17). Vectors pQE30 and pQE70 (Qiagen, Valencia, CA) were used to generate recombinant proteins with the His_6 tag at the N or C termini, respectively. *E. coli* cultures were grown in LB or M9 medium. The M9 medium in this study was supplemented with 0.4 mM threonine, 0.4 mM proline, 0.2 mM histidine and methionine, 0.8 mM leucine, 1 mM serine, and 0.01% thiamine (32). Appropriate antibiotics were added to culture media as required.

Construction of Recombinant Plasmids pQE30-tolA, pQE30-tolB, pQE30-btuB, pQE30-pal, and pQE30-ceiE7—Portions of the *tolA*, *tolB*, *btuB*, *pal*, or *ceiE7* genes were amplified by PCR from *E. coli* K12 chromosome or from pColE7-K317 using primer pairs shown in Table 2. The amplified DNA fragments of the *tolA*, *tolB*, *btuB*, or *pal* genes were cloned into the BamHI, KpnI, HindIII, or SalI sites of pQE30, thus fusing the His_6 tag to the N terminus of each protein. Each recombinant plasmid was introduced into *E. coli* M15 containing pREP4, which encodes *lacI*^{q}. The expressed recombinant proteins, except for BtuB, were purified by nickel-nitrilotriacetic acid (Qiagen) affinity column chromatography as described previously (25). The BtuB protein was purified by elution from a 12.5% SDS-PAGE after electrophoresis.

Western Blotting—Rabbit polyclonal antibodies against the ColE7-Im7 complex, OmpF, TolA, TolB, BtuB, and Pal proteins were produced in this study. The antigen used to raise the anti-OmpF antibody was a synthetic peptide (KGNGENSYGGNGDMTY) corresponding to amino acid residues 47–62 of the OmpF protein. Other antigens used were the purified recombinant proteins from *E. coli* described above. Preparation of the anti-OmpC antibody has been described previously (33). Monoclonal antibody against the σ70 was purchased from NeoClone (Madison, WI). The procedure for Western blotting was the same as described previously (25). The σ70 was used as an internal control because its synthesis was independent of polyamines (34). The primary antibodies were used at a 1:50,000 dilution, and the secondary antibodies, anti-rabbit IgG-horseradish peroxidase and anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA), were diluted 5000- and 2500-fold, respectively. Images on x-ray films were scanned by a laser scanning densitometer (Amersham Biosciences), and band densities were quantified with the ImageQuant software (version 5.2; Amersham Biosciences).

TABLE 2
PCR primers for construction of recombinant plasmids pQE30-*tolA*, pQE30-*tolB*, pQE30-*btuB*, pQE30-*pal*, and pQE30-*ceiE7*

Genes	Primer pairs	PCR product
		bp
tolA	5′-CGCGGATCCGATGATATTTTCGGT	
	5′-ACGCGTCGACTTACGGTTTGAAGTCC	403
tolB	5′-CGGGGTACCGAAGTCCGCATTGTG	
	5′-CCCAAGCTTTCACAGATACGGCGA	1247
btuB	5′-CGGGGTACCGATACCAGCCCGGAT	
	5′-CCCAAGCTTTCAGAAGGTGTAGCT	1746
pal	5′-GCGGGATCCTGTTCTTCCAACAAG	
	5′-ACGCGTCGACTTAGTAAACCAGTACC	478
ceiE7	5′-TAATATGGGATCCAAAAATAGTATTAGTG	
	5′-CATAAGCTTTCCTTGTTGTGAAAGAAT	278

Assay of ColE7 Expression in Wild Type and Polyamine-defective Mutant Strains—Plasmid pColE7-K317 was introduced into AB1111, W3110, HT375, D18, HT252, HT306, and HT414 strains to investigate the effects of polyamines on ColE7 expression in various genetic backgrounds. The cells were grown overnight in M9 medium and diluted 100-fold into fresh M9 medium to grow overnight again for completely eliminating contaminations of exogenous polyamines. The overnight bacteria cultures were then diluted 100-fold into fresh M9 medium. When cell density reached A_{600} of ~0.3, the culture was divided into two portions, and mitomycin C (MMC)[2] was added directly to one of them to a final concentration of 0.5 μg/ml. ColE7 production in these cells with or without MMC induction was assessed by Western blotting at every 30-min interval for 2 h. For determining whether exogenous polyamines rescue ColE7 expression in polyamine-deficient mutants, cells were grown to A_{600} of 0.3 and then divided into several aliquots. Polyamines (putrescine or spermidine alone or mixture of putrescine and spermidine) were then added to each aliquot at concentrations ranging from 0 to 4 mM. MMC (0.5 μg/ml) was then added 30 min after addition of polyamines to induce ColE7 production. ColE7 expression was moni-

[2] The abbreviations used are: MMC, mitomycin C; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high pressure liquid chromatography.

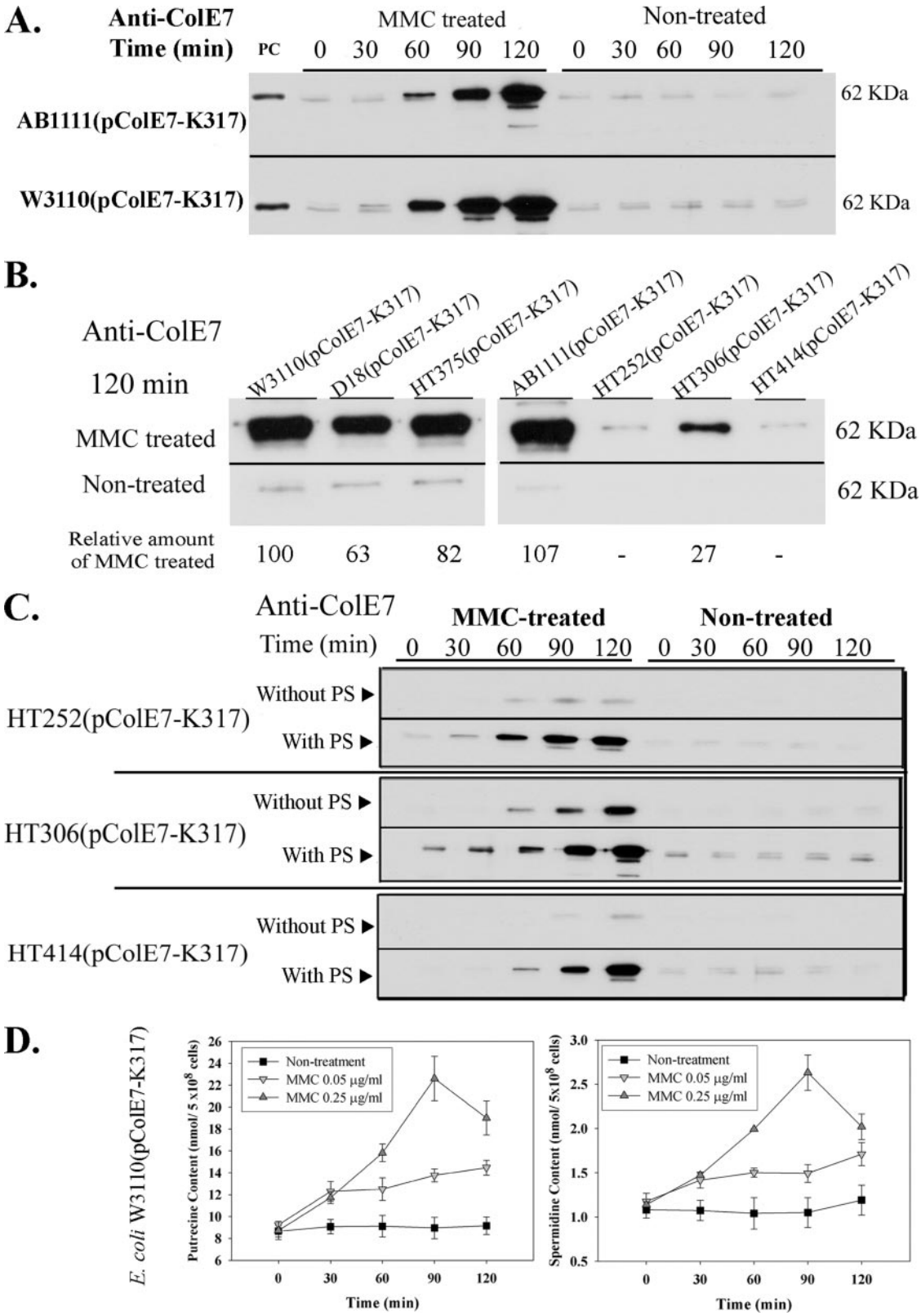

FIGURE 1. **Effect of polyamines on ColE7 production.** *A, E. coli* cells W3110(pColE7-K317) and AB1111(pColE7-K317) containing the ColE7 operon were grown in M9 medium to an A_{600} of 0.3, treated with or without 0.5 μg/ml MMC for 0, 30, 60, 90, and 120 min, and then assayed for intracellular ColE7 by Western blotting using anti-ColE7 antibody. Purified ColE7 protein was used as positive control (*PC*). *B,* ColE7 productions of *E. coli* strains W3110, D18, HT375, AB1111, HT252, HT306, and HT414 containing pColE7-K317 plasmid were examined. Cells were grown to an A_{600} of 0.3, treated with or without 0.5 μg/ml MMC for 0, 30, 60, 90, 120 min, and then assayed for intracellular ColE7 by Western blotting. For clarity, only the result of the 2-h time point is shown. *C,* strains HT252, HT306, and HT414 containing plasmid pColE7-K317 were cultured in M9 medium with or without 1 mM exogenous putrescine and spermidine (*1 mM PS*). ColE7 expressions were then detected after cell cultures were treated with or without 0.5 μg/ml MMC for 0, 30, 60, 90, and 120 min by Western blotting. Equal amounts of each cell fraction were loaded. *D,* W3110(pColE7-K317) cells were grown in M9 medium to an A_{600} of 0.3, treated with 0.05 and 0.25 μg/ml MMC for 0, 30, 60, 90, and 120 min, and then assayed for intracellular putrescine and spermidine levels by HPLC. *Solid triangles* (▲ or ▼) denoted polyamine levels of cultures that were treated with MMC, and *solid square* represent those of nontreated control cultures. Data are mean ± S.D. of triplicate experiments ($p < 0.005$).

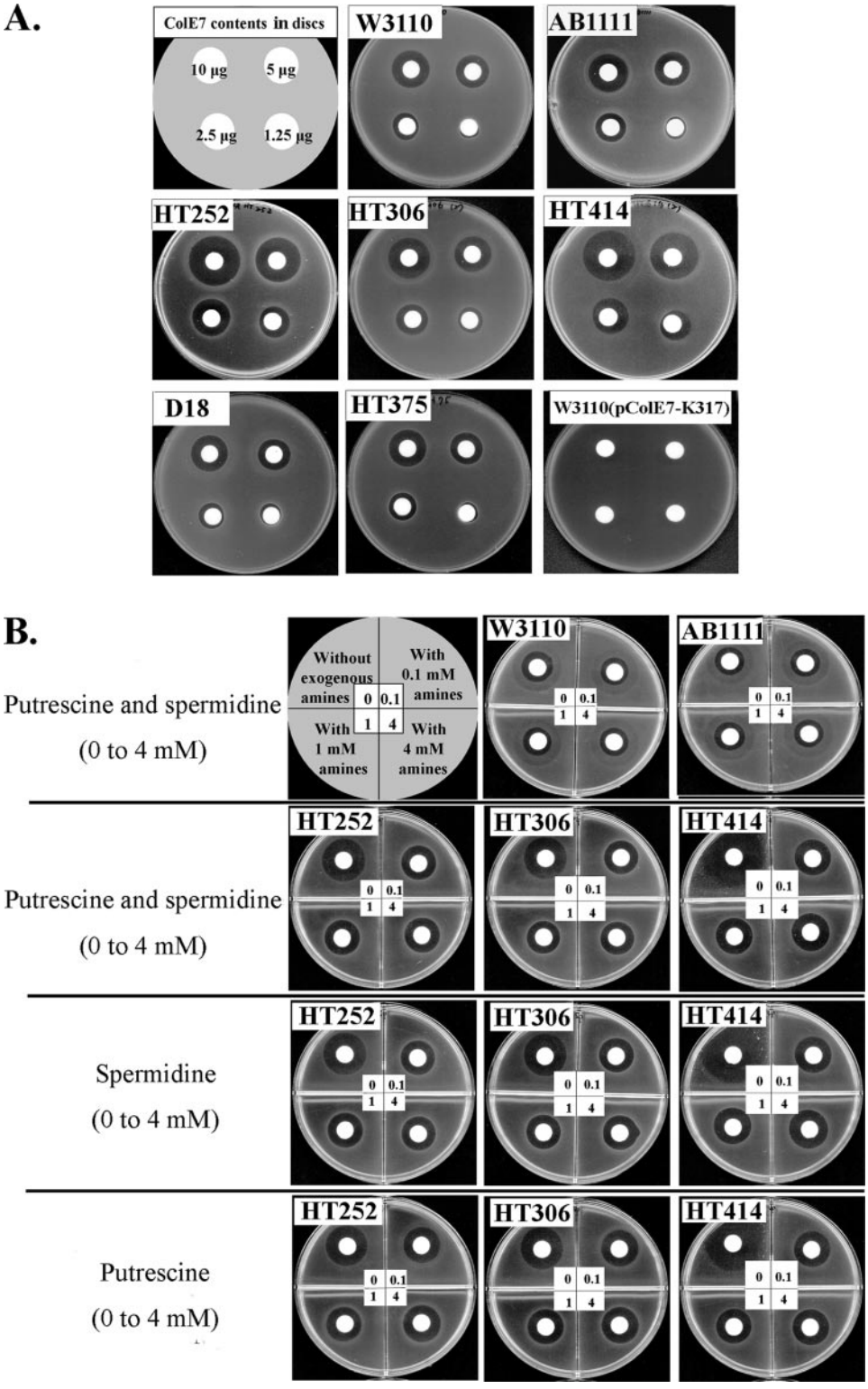

FIGURE 2. **Effect of polyamines on the susceptibility of *E. coli* to ColE7.** *A, E. coli* strains W3110, AB1111, D18, HT375, HT252, HT306, and HT414, with or without pColE7-K317, were separately plated on an M9 agar plate as a lawn. The filter paper disks impregnated with 10, 5, 2.5, or 1.25 μg of purified ColE7-Im7 complex were then placed on the lawn. The plates were incubated at 37 °C for 48 h. The clear zone surrounding the disk indicates the cells killed by ColE7. The W3110 and AB1111 were used as control groups. HT252, HT306, and HT414 were strains defective in both spermidine and putrescine synthesis, and D18 and HT375 were strains defective in spermidine synthesis. The strains containing pColE7-K317 were used as negative controls. *B, E. coli* W3110, AB1111, HT252, HT306, and HT414 were grown on M9 agar plates. Both top and bottom agars were made to contain 0 to 4 mM of putrescine, spermidine, or a mixture of putrescine and spermidine. Filter papers impregnated with 5 μg of purified ColE7-Im7 complex were placed on the lawn. The plates were incubated at 37 °C for 48 h. The wild type *E. coli* strains W3110 and AB1111 strains were also used as references.

tored by Western blotting as described above. It was noted that flasks used for cell cultures were pre-washed with diluted HCl to deplete any exogenous polyamine contamination.

Purification of Native ColE7-Im7 Complex—One liter of LB medium was inoculated with a 10-ml overnight culture of W3110(pColE7-K317). ColE7-Im7 complex was isolated from the culture as described by Liao *et al.* (25), and the purity of the isolated ColE7-Im7 complex was determined by SDS-PAGE as described by Chak *et al.* (35).

Susceptibility of Cells to the ColE7-Im7 Complex—Wild type *E. coli* strains and polyamine-deficient mutants were grown in M9 medium to an A_{600} of 0.4–0.6, and then 1×10^9 cells were mixed with 5 ml of soft agar in M9 medium and overlaid on a M9 agar plate containing 10 ml of bottom agar as a lawn. After a brief evaporation of the moisture on the lawn, filter paper disks impregnated with 10, 5, 2.5, or 1.25 μg of native ColE7-Im7 were placed on the lawn. The plates were incubated at 37 °C for 48 h, and the size of the clear zone surrounding the disks was meas-

ured. To investigate effects of exogenous polyamines on the susceptibility of these cells to ColE7-Im7, both top and bottom agars were made to contain 0–4 mM putrescine, spermidine, or a mixture of putrescine and spermidine.

Viable Counting of E. coli against ColE7-Im7—Wild type *E. coli* cells, W3110 and AB1111, and polyamine-defective mutants were grown in M9 medium to an A_{600} of 0.5. The cell cultures were then incubated with a final concentration of 3 ng/ml (sublethal dosage) of purified native ColE7-Im7 complex for 5 min. The cells were washed twice, and then 10-fold serial dilutions were made. The 10^{-5} to 10^{-7} dilutions were separately plated on LB agar plates. The plates were incubated at 37 °C for 48 h, and visible colonies were then counted.

Determination of Polyamines by HPLC—E. coli cells, with or without MMC (0.05–0.25 μg/ml) or ColE7-Im7 (0.2 mg/ml) treatment, were harvested and assayed for their intracellular spermidine and putrescine concentrations. 5×10^8 cells were pelleted, resuspended in 250 μl of pure water, and then processed for HPLC analysis as described previously (36, 37). The internal standard 1,7-diaminoheptane, standard spermidine, and putrescine (Sigma) were treated and analyzed in an identical manner as controls. Samples were analyzed on an HPLC system equipped with sample controller (Waters 600E) and automated sample injector (S5200 sample injection, SFD, Germany) using a Luna C18 column (00G-4252-E0, 4.6 × 250 mm Luna, Phenomenex, Torrance, CA) with a 1-ml/min flow rate. Analysis of polyamines was monitored by a fluorescence detector (FP-2020 Plus, Jasco, Tokyo, Japan) with an excitation wavelength of 340 nm and emission wavelength of 515 nm. All data were stored and analyzed using the SISC ChemStation (Scientific Information Service Corporation, Taipei, Taiwan), which quantifies each compound by calculating the peak area of an HPLC. Identification and quantification of spermidine or putrescine in a sample were achieved by comparing the peak retention time and peak volume of the sample to those of standard spermidine or putrescine that was analyzed in an identical manner.

Two-dimensional Gel Electrophoresis and Identification of Periplasmic Proteins of E. coli Cells—W3110(pColE7-K317) cells were grown in LB medium to an A_{600} of 1 and then treated with or without 0.2 mg/ml of native ColE7-Im7 for 2 h. Periplasmic proteins of these cells were then isolated after osmotic shock (38) for two-dimensional gel analysis. An immobilized pH gradient strip (13 cm, linear pH 4–7) was used in this study. Isoelectric focusing was performed on the IPGphor™ system (Amersham Biosciences) at 20 °C for a total of 93,890 Vhr including 30 V for 13 h, followed sequentially by 500 V for 1 h, 1000 V for 1 h, 4000 V for 2 h, 6000 V for 2 h, and 8000 V for 9 h. Preparative two-dimensional gels were stained overnight with 0.2% (w/v) Coomassie Brilliant Blue R-250 in 30% methanol and 5% acetic acid and then destained with 30% methanol. The analytical two-dimensional gels were stained with ammoniacal silver stain as described previously (39). Data acquisition for both preparative and analytical two-dimensional gels was achieved using the Image Master 2D Elite system (version 3.01, Lab San version 3.0, Amersham Biosciences). Spots representing differentially expressed proteins were excised manually and then transferred to an ultra-rigid skirted 96-well PCR plate (Thermofast® 96, Abgene, UK) for automatic in-gel digestion according to the procedures described in the MassPREP user's guide (Waters Associates, Milford, MA). The digested samples were subjected to MALDI-TOF mass spectrometry. Protein identification was performed as described previously (40).

RESULTS

Lack of ColE7 Production in Polyamine-deficient E. coli—Polyamines previously have been shown as an SOS-inducing mediator of *E. coli*

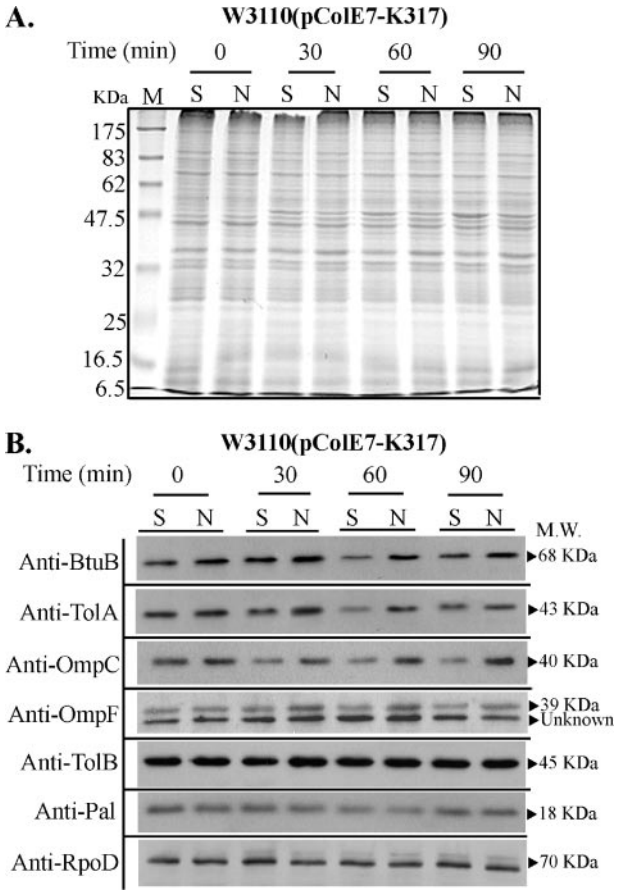

FIGURE 3. **Effect of polyamines on the production of proteins involved in ColE7-Im7 uptake.** *A*, W3110(pColE7-K317) cells were grown in M9 medium to an A_{600} of 0.3, incubated with (*S*) or without (*N*) 4 mM spermidine for 0, 30, 60, or 90 min. Equal amounts of each cell fraction were loaded and followed by SDS-PAGE analysis. *B*, changes in the levels of BtuB, TolA, OmpC, OmpF, TolB, and Pal of W3110(pColE7-K317) cells treated with polyamines were assayed by Western blotting. All cells were cultured in M9 medium to an A_{600} of 0.4, incubated with (*S*) or without (*N*) 4 mM spermidine for 0, 30, 60, or 90 min. The RpoD protein (σ70) was used as internal control.

(8–10), and the ColE7 operon is known to be regulated by an SOS-responsive promoter (17). To study to what extent polyamines are involved in colicin production, we examined the expression of ColE7 in wild type *E. coli* strains (W3110 and AB1111) and polyamine-deficient *E. coli* strains (HT375, D18, HT252, HT306, and HT414). The plasmid pColE7-K317 was introduced into these strains, and ColE7 production in cells with or without MMC treatment was determined by Western blotting every 30 min for 2 h. ColE7 production was significantly induced in W3110(pColE7-K317) and AB1111(pColE7-K317) at 60 min after MMC treatment (Fig. 1*A*). The spermidine-defective strains D18(pColE7-K317) and HT375(pColE7-K317) produced less ColE7 under MMC treatment at a 2-h time point (Fig. 1*B*). In contrast, the strains HT252, HT306, and HT414 containing pColE7-K317 produced none or very little ColE7 even at the 2-h time point (Fig. 1*B*). To ensure polyamines are essential for the expression of ColE7, we set up an experiment to test if exogenous polyamine can rescue ColE7 expression in polyamine-defective mutants. The rescue experiment showed that exogenous polyamines effectively rescue the ColE7 expression in strains HT252, HT306, and HT414 (Fig. 1*C*), suggesting that polyamines play a crucial role in regulating the expression of the ColE7 operon.

To find out whether the regulation of ColE7 production by polyamines is coupled with SOS response, we determined intracellular contents of putrescine and spermidine before and after MMC (0.05 or 0.25 μg/ml) treatment at various time points. We observed that putrescine

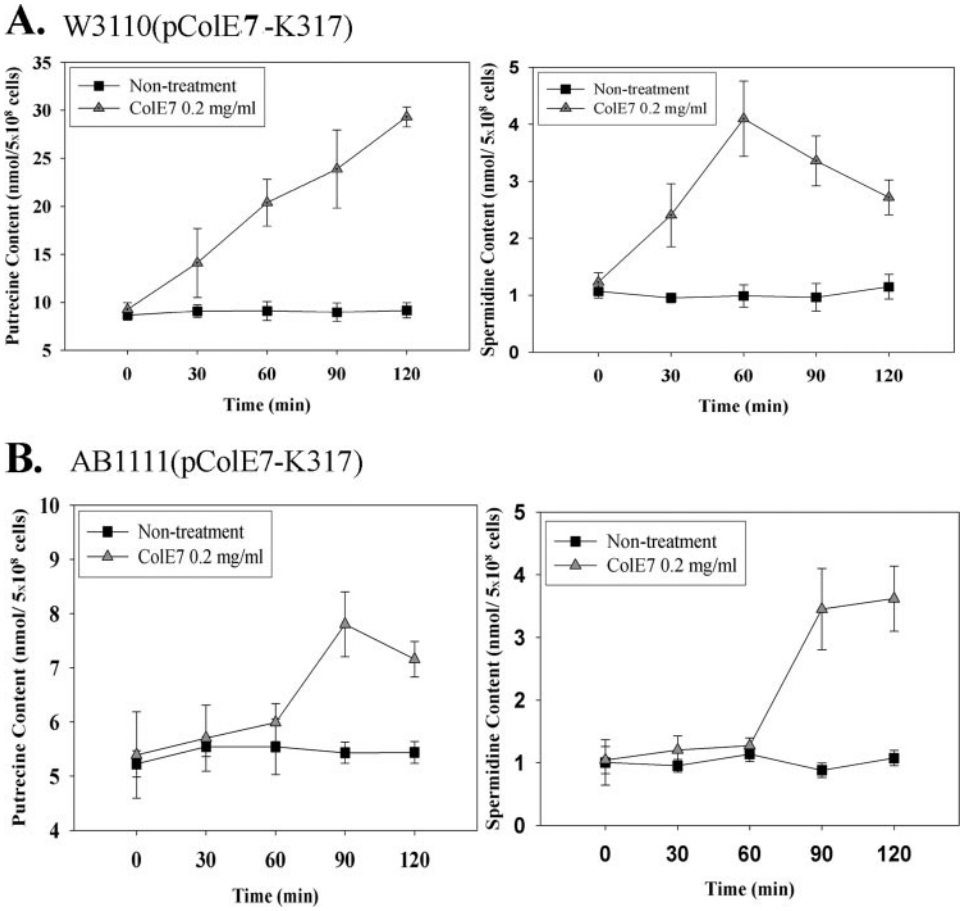

FIGURE 4. **Effect of ColE7 treatment on the synthesis of endogenous polyamines.** W3110-(pColE7-K317) cells (*A*) and AB1111(pColE7-K317) (*B*) were grown in M9 medium to an A_{600} of 0.3. One-ml aliquot containing 5×10^8 cells was treated with or without 0.2 mg/ml of native ColE7-Im7 for 0, 30, 60, 90, and 120 min. The treated and nontreated (the control) cells were harvested and then assayed by HPLC for intracellular putrescine (*left panel*) and spermidine (*right panel*) concentrations. Data are mean ± S.D. of triplicate duplicates ($p < 0.001$).

and spermidine levels were induced significantly in W3110(pColE7-K317) 30 min after MMC treatment (Fig. 1*D*). Similar results were obtained from *E. coli* strains AB1111(pColE7-K317) and W3110 (data not shown). These data indicated that biosynthesis of spermidine and putrescine in *E. coli* cells is indeed coupled with the SOS response.

Endogenous and Exogenous Polyamines Provide Limited Protection of E. coli against Colicin—It is already known that polyamines modulate the outer membrane permeability of *E. coli* cells (14). To investigate whether endogenous polyamines affect the susceptibility of *E. coli* to ColE7, wild type *E. coli* and *E. coli* defective in spermidine or in both putrescine and spermidine synthesis were assayed for their susceptibility to ColE7. Interestingly, larger clear zones surrounding the disks of HT252, HT306, and HT414 strains, defective in both spermidine and putrescine production, were observed in all four tested ColE7 concentrations compared with the controls (Fig. 2*A*). The strains D18 and HT37, defective in spermidine production, were also sensitive to ColE7 (Fig. 2*A*), and the viable counting assay indicated about 3-fold more sensitivity to ColE7 of stains HT375 and D18 compared with the controls (data not shown). These results might suggest that endogenous polyamine confers resistance on *E. coli* cells against ColE7.

To study the fine-tuning of polyamines in regulating the susceptibility of *E. coli* to ColE7, the agar plates containing 0–4 mM concentrations of exogenous polyamines were made to examine the sensitivity of polyamine-defective mutants to ColE7. Addition of putrescine and spermidine or only spermidine up to 0.1 mM significantly reduces the clear zone size of mutants HT252, HT306, and HT414 compared with the mutants without exogenous polyamine addition (Fig. 2*B*). It was noted that addition of polyamines beyond the 0.1 mM dose does not further increase the protective effect of the mutants against colicin. Similar protective effect of the wild type strains (W3110 and AB1111) has also been observed, indicating that probably 0.1 mM of exogenous polyamines is sufficient for exerting the limited protection of the cells against colicin.

Response of E. coli Proteins Involved in ColE7 Translocation to Polyamines—Proteins such as TolA, TolB, BtuB, OmpC, OmpF, and Pal are essential for the translocation of colicins across the *E. coli* cell membrane (41, 42). Because polyamines (putrescine or spermidine) have been proved to render *E. coli* cells the limited resistance to ColE7 (Fig. 2), experiments were then designed to resolve whether polyamine alters the production of proteins involved in the translocation process of colicin. The SDS-PAGE analysis of W3110(pColE7-K317) treated with or without 4 mM spermidine was shown in Fig. 3*A*, and Western blotting against BtuB, TolA, OmpC, OmpF, TolB, Pal, and RpoD was shown in Fig. 3*B*. Results of Western blotting demonstrated that production of BtuB, TolA, OmpC, and OmpF proteins was reduced to a certain extent in response to the spermidine treatment, and production of OmpC and OmpF was found to be reduced more rapidly than production of BtuB and TolA (Fig. 3*B*). No significant changes were observed in expression of TolB and Pal after spermidine treatment. The phenomenon of reduced production in TolA, BtuB, OmpF, and OmpC was observed in both ColE7 producing and nonproducing cells, including W3110 and AB1111 (data not shown). Thus, our results suggested that limited resistance of these cells against colicin conferred by spermidine treatment might be due to the suppressive effect of polyamines on the production of the proteins involved in colicin translocation.

Increase in Intracellular Polyamine Levels in Response to Colicin Treatment—We have confirmed that polyamine renders the cells resistance to ColE7 (Figs. 2 and 3), and it is interesting to know whether the

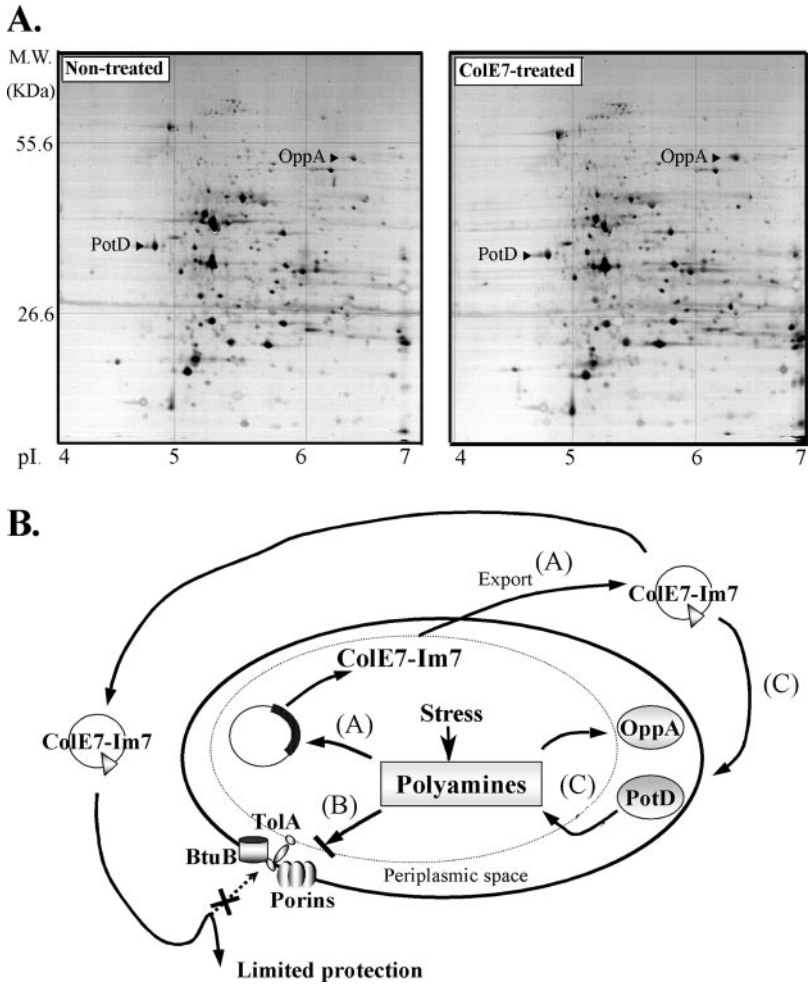

FIGURE 5. *A,* effect of ColE7 on the expression of periplasmic proteins PotD and OppA. W3110-(pColE7-K317) cells were grown in M9 medium to an A_{600} of 1.0 and then treated with 0.2 mg/ml native ColE7-Im7 for 2 h. Periplasmic proteins of ColE7-Im7-treated cells were isolated and analyzed by two-dimensional gel electrophoresis. Image Master 2D Elite system (version 3.01) was used to analyze two-dimensional gels and to quantitate the densities of protein spots. Spots (indicated by *arrowheads*) representing differentially expressed PotD and OppA proteins were isolated and identified by MALDI-TOF. *B,* the proposed model for the roles of polyamines in the induction of ColE7 production and the restriction of ColE7 uptake. Polyamines activate the ColE7 operon to produce ColE7-Im7, which is then excreted (*A*). To prevent the reentry of the excreted ColE7, production of BtuB, TolA, OmpF, and OmpC is suppressed by endogenous polyamines (*B*). The limited amount of reentry ColE7 stimulates the productions of periplasmic proteins PotD and OppA and polyamines. Therefore, higher polyamine concentration would further decrease the expression of essential proteins for ColE7 uptake to diminished ColE7 reentry (*C*). PotD is involved in the transport of polyamines from the periplasm into the cytoplasm. The increased level of polyamines induce the production of OppA, which scavenges oligopeptides; however, the significance of this function in relation to ColE7 production and transportation remains to be investigated.

increase of endogenous polyamines of the cells is coupled to colicin exposure. Experiments were set up to assay the contents of polyamines in the cells with colicin exposure, as shown in Fig. 4*A*. The content of polyamines, including putrescine and spermidine, in W3110(pColE7-K317) detected by HPLC increased rapidly with the time that the cells were treated with 0.2 mg/ml ColE7. It was noted that significant production of the amines in AB1111(pColE7-K317) was only starting at 60 min after the ColE7 treatment (Fig. 4*B*). The results clearly showed that *E. coli* W3110 responds better than *E. coli* AB1111 in polyamine production under ColE7 exposure (Fig. 4, *A* and *B*), indicating that production of polyamines is varied with different genetic backgrounds.

Increased Abundance of Periplasmic Proteins PotD and OppA in Response to Colicin Exposure—It has been documented that polyamines accumulate in the periplasmic space of *E. coli* cells during their synthesis and transportation (43, 44). In addition, we have confirmed in this work that production of intracellular polyamines of the cells is coupled with colicin stimulation (Fig. 4). Thus, it is very interesting to know what sort of periplasmic proteins will be induced in cells treated with ColE7.

Periplasmic proteins of *E. coli* strain W3110(pColE7-K317) treated with ColE7 were then prepared and analyzed by two-dimensional gel electrophoresis (see "Materials and Methods"), and protein spots showing differential expression after ColE7 treatment were picked and identified by MALDI-TOF (see "Materials and Methods"). Many proteins shown to be differentially expressed (Fig. 5*A*), among them the two periplasmic proteins PotD and OppA related to polyamine transportation and amine synthesis, respectively, were observed to be significantly increased after ColE7 treatment (Fig. 5*A*). The quantity of OppA and PotD extracted from cells with ColE7 treatment for 2 h were increased 2.2- and 1.5-fold, respectively, as calculated by Image Master 2D Elite system (version 3.01).

DISCUSSION

In *E. coli*, polyamines are required for the induction of several SOS-related genes, such as *recA*, *uvrA*, and *umu* genes, when the cells are exposed to DNA-damaging agents, including UV, MMC, γ-irradiation, and H_2O_2 (8–10). The native ColE7 operon is known to be driven by an SOS-responsive promoter and is regulated by the RecA and LexA proteins (17). However, it is not known whether polyamines play any role in triggering the expression of the ColE7 operon. The present study showed that the putrescine and spermidine double mutants HT252 and HT414 containing ColE7 plasmid are defective in ColE7 production with or without MMC induction (Fig. 1*B*), and ColE7 expression in the cells has been effectively rescued by addition of exogenous polyamines (Fig. 1*C*). In addition, HPLC analysis revealed that endogenous spermidine and putrescine are significantly increased in response to MMC induction in *E. coli* cells (Fig. 1*D*). These results clearly demonstrated that both endogenous putrescine and spermidine are necessary for ColE7 production, implying that endogenous polyamines may be involved in the promoter-specific activation of an SOS response operon. This notion is consistent with the finding that polyamines activate *recA* expression and probably mediate RecA polymerization (10, 45). The polyamine-defective mutant HT306(pColE7-K317) still produced a

basal level of colicin under MMC induction (Fig. 1*B*), implying that this mutant strain may still synthesize a small amount of polyamines (46).

Furthermore, we demonstrated that the presence of polyamines conferred limited protection on *E. coli* cells against colicin (Fig. 2). Several previous papers noted that polyamines were globally responsive to numerous environmental stresses, including oxidative stresses, cold shocks, and lower pH conditions (47–50). The limited protective effect of polyamine found in this paper is therefore not necessarily a specific phenomenon for ColE7 treatment. In contrast to the environmental stresses stated above, it is noteworthy that the mode of action of ColE7 is specific for the BtuB or BtuB/OmpF receptor for penetrating sensitive cells (27, 51). Thus, suppression of BtuB, TolA, OmpC, and OmpF production by exogenous polyamines determined previously suggests a specific mechanism for diminished colicin uptake (Fig. 3*B*).

Previous studies indicated that polyamines enhanced translational levels of several genes such as *oppA, cyaA,* and *rpoS* (σ^s), which were important for cell growth and the viability of the *E. coli* cells (34, 52, 53). The group of genes whose expression was enhanced by polyamines at the level of translation was further proposed as a "polyamine modulon" (4). However, reports as to how polyamines suppressed the expression of these genes are rare. It was reported that RpoS acted as a negative regulator of OmpF expression under nutrition limitation (54, 55). Probably, this information provides a clue to understand how polyamines mediate the suppression of the production of proteins involved in colicin translocation. Additionally, polyamines have been shown to bind to the aspartic acid residues located at positions 113 and 121 of OmpF and 105 of OmpC (12, 14, 56), thus altering the pore size of the porins. By modulating the gating of OmpF, exogenous spermine may affect the translocation of bacteriocins, colicin A and N, across the membrane of *E. coli* (57). Therefore, this binding may be another molecular basis by which colicin producers restrict the reentry of their own colicin.

Results of the present study also showed that the amounts of endogenous polyamines were increased when the ColE7 producers are under exposure to their own toxin (Fig. 4). Our proteomic study indicated that the levels of the two periplasmic proteins PotD and OppA are increased when the cells are exposed to ColE7 (Fig. 5). The PotD protein is a periplasmic substrate-binding protein of the ATP-binding cassette transporter that imports spermidine (K_m = 0.1 μM) and putrescine (K_m = 1.5 μM) from the periplasm into the cytoplasm of *E. coli* cells (6, 7). Similar to PotD, OppA is also located in the periplasm of Gram-negative bacteria. A major role of the Opp system is to recycle cell wall peptides as they are released from growing peptidoglycan (58). OppA captures peptides ranging in size from 2 to 5 amino acids from the periplasm and transports them into the cytoplasm (59–61). It is conceivable that the increase in OppA production is because of an increase in polyamines because polyamines have been shown to stimulate OppA synthesis (52, 53, 62). Thus, we would like to propose that accumulation of intracellular polyamines is a likely cellular response to the stress of ColE7 exposure, providing a protective mechanism against the reentry of colicin back to the colicin-producing cells.

We report for the first time that polyamines play an important role in conferring limited protection on the cells against colicin. Polyamines have been shown to regulate colicin production in this work. Therefore, we propose that polyamines also play a central role in maintaining the selective advantage that allows colicin producers to survive in the environment (Fig. 5*B*). Under environmental stress such as overpopulation or nutrient depletion, colicin producers synthesize more endogenous polyamines, which together with other inducers trigger the expression of the ColE7 operon to produce colicins in order to gain a survival advantage. Meanwhile, the increased levels of polyamines stimulated by reentry ColE7 suppress the production of TolA, BtuB, OmpF, and OmpC proteins, conferring a protective effect on the *E. coli* cell against reentry of the colicin it produced.

In conclusion, our present data suggest that polyamines play an important ecological role in regulating colicin production and translocation of the ColE7-producing cells, and this may render survival advantage to colicin-producing cells in natural environments. The mechanisms by which polyamines mediate ColE7 production and the expression of proteins for ColE7 uptake together with the significance of increased levels of OppA and PotD upon ColE7 exposure remain to be investigated.

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