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Prevalence of ColE1-Like Plasmids and Colicin K Production among Uropathogenic *Escherichia coli* Strains and Quantification of Inhibitory Activity of Colicin K[▽]

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Colicin K exhibited pronounced inhibitory activity against uropathogenic *Escherichia coli* (UPEC) strains. Low prevalence of colicin K production and a relatively high prevalence of ColE1-like plasmids were determined among 215 UPEC strains from Slovenia. Sequencing of the colicin K-encoding pColK-K235 revealed a mosaic structure and the presence of the insertion sequence IS*2*.

An increasing health problem is the appearance and spread of antimicrobial resistance. Due to the global concern about antibiotic resistance, novel approaches, one being the use of bacteriocins, are being considered (10, 11). Colicins, bacteriocins produced by and active against *Escherichia coli* strains, are receiving renewed attention as an alternative means of preventing various *E. coli*-associated infections, for example, diarrhea, including serotype O157:H7 (15, 22, 28, 29, 30), and postweaning diarrhea and edema disease in swine (33). Colicins were recently shown to prevent colonization of urinary catheters (34). To date, no quantitative analysis of the inhibitory activity of colicins against uropathogenic *E. coli* (UPEC) has been performed.

Colicin K is a pore-forming colicin encoded on small ColE1-like plasmids (23). A cluster of three genes codes for the production and release of colicin: *cka* for colicin activity, *cki* for immunity, and *ckl* for lysis (23). The synthesis of colicin K is induced primarily by an increase in the alarmone ppGpp due to nutrient depletion (18, 19). Previous investigations found that approximately 30 to 50% of natural *E. coli* isolates produce colicins (27). Such a high prevalence of colicin production is in itself indicative of an ecological significance. Additionally, a recent study provided evidence that colicins E1 and E2 have an in vivo antagonistic role in promoting microbial diversity within *E. coli* populations in the mammalian colon (17).

In this study, we investigated the inhibitory activity of purified colicin K against 215 UPEC strains from Slovenia as well as the prevalence of colicin K production and ColE1-like plasmids among the studied strains. The nucleotide sequence of plasmid pColK-K235 encoding the investigated colicin K was determined and analyzed.

To gain insight into the ecological role of colicin K and to assess its antimicrobial efficacy, we initially determined the MIC of purified colicin K against 215 UPEC strains isolated from humans with urinary tract infections in 2001 and 2002 at the Institute of Microbiology and Immunology, Ljubljana, Slovenia (26).

Isolation of the strains was performed according to standard laboratory protocols, and UPEC isolates were from a bacterial urine monoculture of $>10^5$ CFU per ml. To elicit infections, extraintestinal pathogenic *E. coli* strains, including UPEC, possess virulence factors. Therefore, the investigated UPEC strains were PCR screened for virulence factor sequences as described previously (14, 20). As antimicrobial resistance is an increasing health problem, testing of susceptibility to antimicrobial agents was performed as described previously (26).

To isolate colicin K, the *cka* activity gene was amplified using PCR with primers ColK1 and ColK2 (Table 1). The PCR product was digested with restriction enzymes XhoI and MluI and cloned (ligated) into the expression vector pET8c (25), producing plasmid pMR1. Colicin K was expressed in *E. coli* strain BL21 (DE3) and large-scale expression was performed as previously described (2). The colicin K-containing fractions, as determined by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were dialyzed against 5 mM phosphate buffer and stored at −20°C. Protein purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein concentration was assayed using a bicinchoninic acid protein assay kit (Pierce).

An estimation of the lowest concentration of colicin K preventing the appearance of turbidity (MIC) was performed by spotting 5 μl of various concentrations (0.1, 1, 10, and 100 μg/ml) of Cka diluted in 10 mM Tris, pH 8, onto LB plates overlaid with soft agar harboring the individual investigated UPEC strains. Following an overnight incubation at 37°C, the plates were examined for colicin sensitivity (clear zones) of the overlaid strains.

The obtained results showed that, altogether, 68% of the tested UPEC strains were susceptible and 32% were resistant to colicin K (Fig. 1). However, among the sensitive strains, various levels of susceptibility were observed. Thus, 18% of the strains were inhibited by 0.1 μg/ml, 3% by 1 μg/ml, 16% by 10

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant property(ies)	Source or reference
Strains		
DH5α	*thi-1 recA hsdR17 lac*	BRL Life Technologies
MC4100	*araD139* Δ(*argF-lac*) *U169 rpsL150 relA flbB5301 ptsF25 deoC1*	R. Hengge-Aronis
BL21 (DE3)	High-level expression using T7 RNA polymerase-based expression systems	G. Anderluh
AB1133	Sensitive to all colicins	B. Bachman
UL98	*hlyA cnf1 usp iroN iucD fyuA fimH traT* Ap^r Tc^r Nal^r Cm^r Km^r St^r	26
UL114	K1 *iucD iroN fyuA usp traT* Ap^r Cip^r	26
UL31	*hly cnf1 papC papG sfa/foc iucD iroN usp* Cm^r Nal^r	26
UL173	K5 *iroN iucD fimH traT* Ap^r Sxt^r Cip^r	26
Plasmids		
pColK-K235	Wild-type plasmid	18
pUC19	Ap^r cloning vector	36
pBR322	Ap^r Tc^r cloning vector	3
pET8c	Ap^r expression vector	25

μg/ml, and 31% by 100 μg/ml of colicin K. The basis of such differences in susceptibility could be due to the different numbers of receptors per cell (32) or the shielding of colicin receptors by the O-antigenic chains of lipopolysaccharide (6, 35). Further, resistance to colicins is known to be due to the absence of functional receptors and resistant isolates appear spontaneously. Site-specific mutations in the FepA colicin receptor have been shown to affect susceptibility for colicins B and D (7).

From each susceptibility group (corresponding to concentrations of 0.1, 1, 10, and 100 μg/ml), two strains harboring virulence factor genes (Table 1) were chosen to follow the inhibitory activity of colicin K. The inhibitory activity of Cka was quantified in liquid medium essentially as described previously (33), and activity against one strain from each group is presented in Fig. 1. Briefly, overnight cultures of the tested UPEC strains were used to inoculate prewarmed LB to an approximate optical density at 600 nm (OD_{600}) of 0.05, followed by aliquoting 5 ml into tubes with various colicin concentrations. A total of 10 mM Tris, pH 8, was used to prepare constant volumes of added colicin (200 μl). Subsequently, the tubes harboring the tested strains and colicin were incubated with shaking at 37°C. To follow colicin activity against the tested strains, the OD_{600} was determined hourly. On the basis of growth inhibition as determined by OD_{600} values, it is evident that colicin K exhibited pronounced inhibitory activity against the tested UPEC strains.

An analysis of the nucleotide sequences of colicin-encoding plasmids is essential to elucidate their role in bacterial popu-

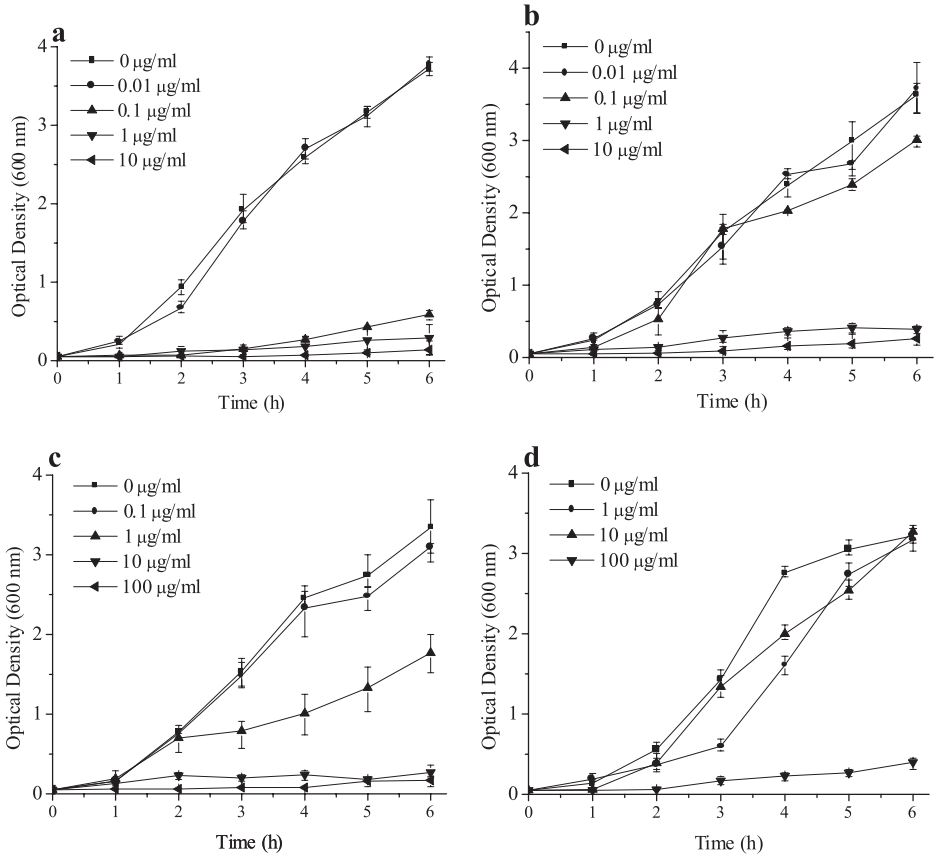

FIG. 1. Effect of colicin K on UPEC strains UL31 (a), UL173 (b), UL114 (c), and UL98 (d) (MIC as detected on plates containing 0.1, 1, 10, and 100 μg/ml, respectively). The experiments were repeated three times, and the means ± standard errors of the means (error bars) are shown. Only one of the two investigated strains from each sensitivity group is depicted; however, the effects of colicin K on both strains were comparable.

TABLE 2. Oligonucleotide primers used in this study

Oligonucleotide primer	Nucleotide sequence[a]	Position[b]
ColK1	TCA**CTCGAG**CATGGCTAAAGAAC TAAGTGGATATGGACC	2527–2555
ColK2	CC**ACGCGT**TAAATCCCTAACAA CTCATTAAGTTTGCTC	4172–4143
cka1	CAGAGGTCGCTGAACATGAAAC	3263–3284
cka2	CTCAGCTAAACGCTTCTCTGCTT	3336–3314
P1	TAACCGGGAAGGGGCGAGAGTTCT	6268–6291
P2	GAATGAACTGGACGCCGACG	5737–5718
P3	TTCTGAACTGACGGGGATCGC	4591–4611
P4	TGAAACACGCCAGTTCGCCC	1899–1918
P5	GCTTCCAGGGCCGCTCTTCCT	7154–7134
PK1	GGCGTATTTTCCCCGGTTTC	673–692
PK2	TGGTGCCAGCCAGTCTGCTC	594–575
PK3	GGGCGAACTGGCGTGTTTCA	1918–1899
PK4	ACACCCTTGTTGCTGCCGGA	2234–2215
PK5	TCCGGCAGCAACAAGGGTGT	2215–2234
PK6	AGCTGCGTGGCCTTTTCCGG	5216–5235
PK7	TCCGCCTTTCTCCCTTTGGG	5054–5035
PK8	TACAGCGCCCCTTTCAGCCT	6881–6862
PK9	AGGAAGAGCGGCCCGGAAGC	7134–7154
PK10	AGGCTGAAAGGGGCGCTGTA	6862–6881
PK11	CCCCTTCACGGATTTGCAGC	7531–7550
mob1	GGCAGTGGTCCGGTTGAT	6230–6248
mob2	TGCAGCCCGTAATGGTGA	6630–6612
rna1	AGGATCTTCTTGAGATCCTT	4632–4652
rm2	TATCCACAGAATCAGGGG	5291–5273
rom1	AAGCGGGCCATGTTAAGG	5774–5792
rom2	AATCAACCGGACCACTGC	6248–6230

[a] Nucleotides in boldface correspond to introduced XhoI and MluI restriction sites.
[b] Numbers indicate positions on plasmid pColK-K235.

lations and to gain a full understanding of their evolutionary histories. In spite of the high prevalence of colicin production among natural *E. coli* populations, to date, the complete nucleotide sequences of only a small number of colicin-encoding plasmids have been studied (8, 13, 21, 31). We therefore determined and analyzed the nucleotide sequence of the colicin K-encoding pColK-K235 (24). Nucleotide sequence data were obtained by a combination of subcloning of restriction fragments and primer walking with specific primers (Table 2). Sequencing reactions were performed using the Thermo Sequenase Cy5 dye terminator cycle sequencing kit and the ALFexpress II DNA sequencer (both from Amersham Biosciences). The nucleotide sequence was searched for potential open reading frames by GeneMark (4).

DNA sequencing revealed that the total length of plasmid pColK-K235 is 8,318 bp. The average GC content of the colicin K gene cluster was found to be 37.3%, while that of the remaining plasmid sequences was 52.9%, indicating that pColK-K235 has a mosaic structure.

Using BLAST (1), the positions of sequences identical to the genes *mob*, *ckr*, and *rom* of a number of other colicin-encoding plasmids were found. The positions of *oriV*, RNA I, and RNA II were determined by alignment with the sequences of plasmids pColE1 (5), pWQ799 (16), and pNTP1 (12). The position of *oriT* was likewise deduced by alignment with pColE1. The genes and functions of pColK-K235 are illustrated in Fig. 2.

The 1,331-bp-long insertion sequence IS*2* (9) was found upstream from the colicin K gene cluster. The latter has been extensively discussed elsewhere (23). The putative mobilization genes were designated *mbkA*, *mbkB*, *mbkC*, and *mbkD* according to the nomenclature (5).

Altogether, approximately 3 kbp of pColK-K235 (position numbers 5185 to 8162) is identical (97%) to pColD157 (13) in regions of the genes *rom* and *mbk* and the *ckr* determinant (Fig. 2). Shorter regions of similarity with other known colicin-encoding plasmids, namely, pColE1, pColA, and pScol7, are designated in Fig. 2. Our results demonstrate that pColK-K235 has a mosaic structure as different segments exhibit identity with different plasmids. Notable is the presence of the insertion sequence IS*2*, which can mediate recombination between homologous sequences on chromosomes or other plasmids, including conjugative plasmids.

Subsequently, the prevalence of related ColE1-like plasmids among the 89 colicinogenic UPEC isolates was examined by PCR probing for *mobA*-, *rom*-, and RNA II-specific sequences (Table 2). Our results demonstrated that 38 (43%) of the colicinogenic isolates and, thus, 18% of the altogether 215 UPEC isolates examined harbored sequences characteristic of ColE1-like plasmids.

To gain additional insight into the ecological role of colicin K, the prevalence of colicin K production among the studied UPEC strains was investigated. For this purpose, the 89 colicinogenic strains were PCR probed for *cka*-specific sequences (Table 2). A low prevalence of colicin K was determined as only two strains (1%) were positive by PCR for the tested sequences. Colicinogenic strains are immune to the produced colicin, and both strains were insensitive to colicin K as determined by bioassay, indicating that they indeed harbor and express the colicin K gene cluster. Our results thus demonstrate that, among the colicin K-insensitive strains, only approximately 6% exhibited immunity.

Our data show pronounced inhibitory activity of colicin K against UPEC strains. Nevertheless, for therapeutic purposes, it would be more effective to use a combination of colicins employing different receptors, translocations, and modes of action. Further, the here-described presence of IS*2* and the

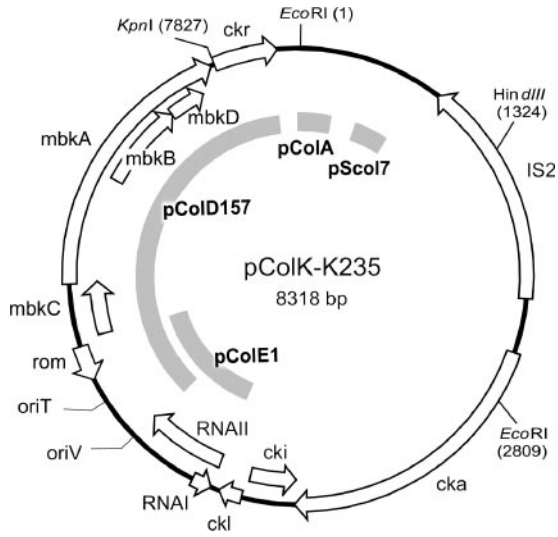

FIG. 2. Physical and genetic map of plasmid pColK-K235. Regions of similarity with other colicin-encoding plasmids are indicated. The arrows indicate the positions and orientations of functional genes on the basis of sequence comparisons. Restriction sites for several restriction endonucleases are indicated.

relatively high prevalence of ColE1-like plasmids indicate that these plasmids might play a significant role as vehicles of DNA rearrangements as well as gene mobilization.

Nucleotide sequence accession number. The obtained plasmid pColK-K235 sequence data have been deposited with the EMBL and GenBank data libraries under accession no. AY929248.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Anderluh, G., I. Gokce, and J. H. Lakey.** 2003. Expression of proteins using the third domain of the *Escherichia coli* periplasmic-protein TolA as a fusion protein. Protein Express. Purif. **28:**173–181.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
4. **Borodovsky, M., and J. McIninch.** 1993. Recognition of genes in DNA sequence with ambiguities. Biosystems **30:**161–171.
5. **Boyd, A. C., J. A. K. Karcher, and D. J. Sherratt.** 1989. Characterization of the ColE1 mobilization region and its protein products. Mol. Gen. Genet. **217:**488–498.
6. **Bradley, D. E., and S. P. Howard.** 1991. Colicinogeny of 0157:H7 enterohemorrhagic *Escherichia coli* and the shielding of colicin and phage receptors by their O-antigenic side chains. Can. J. Microbiol. **37:**97–104.
7. **Cao, Z., and P. E. Klebba.** 2002. Mechanisms of colicin binding and transport through outer membrane porins. Biochimie **84:**399–412.
8. **Chan, P. T., H. Ohmori, J. Tomizawa, and J. Lebowitz.** 1985. Nucleotide sequence and gene organization of ColE1 DNA. J. Biol. Chem. **260:**8925–8935.
9. **Ghosal, D., H. Sommer, and H. Saedler.** 1979. Nucleotide sequence of the transposable DNA-element IS*2*. Nucleic Acids Res. **6:**1111–1122.
10. **Gillor, O., B. C. Kirkup, and M. A. Riley.** 2004. Colicins and microcins: the next generation antimicrobials. Adv. Appl. Microbiol. **54:**129–146.
11. **Gillor, O., L. M. Nigro, and M. A. Riley.** 2005. Genetically engineered bacteriocins and their potential as the next generation of antimicrobials. Curr. Pharm. Dis. **11:**1067–1075.
12. **Grindley, J. N., and D. Nakada.** 1981. The nucleotide sequence of the replication origin of plasmid NTP1. Nucleic Acids Res. **9:**4355–4366.
13. **Hofinger, C., H. Karch, and H. Schmidt.** 1998. Structure and function of plasmid pColD157 of enterohemorrhagic *Escherichia coli* and its distribution among strains from patients with diarrhea and hemolytic-uremic syndrome. J. Clin. Microbiol. **36:**24–29.
14. **Johnson, J. R., and A. L. Stell.** 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. **181:**261–272.
15. **Jordi, B. J. A. M., K. Boutaga, C. M. E. van Heeswijk, F. van Knapen, and L. J. A. Lipman.** 2001. Sensitivity of Shiga toxin-producing *Escherichia coli* (STEC) strains for colicins under different experimental conditions. FEMS Microbiol. Lett. **204:**329–334.
16. **Keenleyside, W. J., and C. Whitfield.** 1995. Lateral transfer of *rfb* genes: a mobilizable ColE1-type plasmid carries the $rfb_{O:54}$ (O:54 antigen biosynthesis) gene cluster from *Salmonella enterica* serovar Borreze. J. Bacteriol. **177:**5247–5253.
17. **Kirkup, B. C., and M. A. Riley.** 2004. Antibiotic-mediated antagonism leads to a bacterial game of rock-paper-scissors in vivo. Nature **428:**412–414.
18. **Kuhar, I., and D. Žgur-Bertok.** 1999. Transcription regulation of the colicin K *cka* gene reveals induction of colicin synthesis by differential responses to environmental signals. J. Bacteriol. **181:**7373–7380.
19. **Kuhar, I., J. van Putten, D. Žgur-Bertok, W. Gaastra, and B. Jordi.** 2001. Codon-usage based regulation of colicin K synthesis by the stress alarmone ppGpp. Mol. Microbiol. **41:**207–216.
20. **Kurazono, H., M. Nakano, S. Yamamoto, O. Ogawa, K. Yuri, K. Nakata, M. Kimura, S. Makino, and G. B. Nair.** 2003. Distribution of the *usp* gene in uropathogenic *Escherichia coli* isolated from companion animals and correlation with serotypes and size-variations of the pathogenicity island. Microbiol. Immunol. **47:**797–802.
21. **Morlon, J., M. Chartier, M. Bidaud, and C. Lazdunski.** 1988. The complete nucleotide sequence of the colicinogenic plasmid ColA. High extent of homology with ColE1. Mol. Gen. Genet. **211:**231–243.
22. **Murinda, S. E., R. F. Roberts, and R. A. Wilson.** 1996. Evaluation of colicins for inhibitory activity against diarrheagenic *Escherichia coli* strains, including serotype O157:H7. Appl. Environ. Microbiol. **62:**3196–3202.
23. **Pilsl, H., and V. Braun.** 1995. Strong function-related homology between pore-forming colicins K and 5. J. Bacteriol. **177:**6973–6977.
24. **Pugsley, A. P.** 1985. *Escherichia coli* K12 strains for use in the identification and characteriziation of colicins. J. Gen. Microbiol. **131:**369–376.
25. **Raggett, E. M., G. Brainbridge, L. J. Evans, A. Cooper, and J. H. Lakey.** 1998. Discovery of critical TolA-binding residues in the bactericidal toxin colicin N: a biophysical approach. Mol. Microbiol. **28:**1335–1343.
26. **Rijavec, M., M. Starčič Erjavec, J. Ambrožič Avguštin, R. Reissbrodt, A. Fruth, V. Križan-Hergouth, and D. Žgur-Bertok.** 2006. High prevalence of multidrug resistance and random distribution of mobile genetic elements among uropathogenic *Escherichia coli* (UPEC) of the four major phylogenetic groups. Curr. Microbiol. **53:**158–162.
27. **Riley, M. A., and D. M. Gordon.** 1996. The ecology and evolution of bacteriocins. J. Ind. Microbiol. **17:**151–158.
28. **Schamberger, G. P., and F. Diez-Gonzalez.** 2002. Selection of recently isolated colicinogenic *Escherichia coli* strains inhibitory to *Escherichia coli* O157:H7. J. Food Prot. **65:**1381–1387.
29. **Schamberger, G. P., R. L. Phillips, J. L. Jacobs, and F. Diez-Gonzalez.** 2004. Reduction of *Escherichia coli* O157:H7 populations in cattle by addition of colicin E7-producing *E. coli* to feed. Appl. Environ. Microbiol. **70:**6053–6060.
30. **Schamberger, G. P., and F. Diez-Gonzalez.** 2004. Characterization of colicinogenic *Escherichia coli* strains inhibitory to enterohemorrhagic *Escherichia coli*. J. Food Prot. **67:**486–492.
31. **Šmajs, D., and G. M. Weinstock.** 2001. Genetic organization of plasmid ColJs, encoding colicin Js activity, immunity, and release genes. J. Bacteriol. **183:**3949–3957.
32. **Šmarda, J., and D. Šmajs.** 1998. Colicins—exocellular lethal proteins of *Escherichia coli*. Folia Microbiol. **43:**563–582.
33. **Stahl, C. H., T. R. Callaway, L. M. Lincoln, S. M. Lonergan, and K. J. Genovese.** 2004. Inhibitory activities of colicins against *Escherichia coli* strains responsible for postweaning diarrhea and edema disease in swine. Antimicrob. Agents Chemother. **48:**3119–3121.
34. **Trautner B. W., R. A. Hull, and R. O. D. Arouiche.** 2005. Colicins prevent colonization of urinary catheters. J. Antimicrob. Chemother. **56:**413–415.
35. **Walker, D., M. Rolfe, A. Thompson, G. R. Moore, R. James, J. C. D. Hinton, and C. Kleanthous.** 2004. Transcriptional profiling of colicin-induced cell death of *Escherichia coli* MG1655 identifies potential mechanisms by which bacteriocins promote bacterial diversity. J. Bacteriol. **186:**866–869.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–109.