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Arch Microbiol. Author manuscript; available in PMC 2013 March 01.

Published in final edited form as:
Arch Microbiol. 2012 July ; 194(7): 575–587. doi:10.1007/s00203-012-0793-2.

Isolation and identification of a bacteriocin with antibacterial and antibiofilm activity from *Citrobacter freundii*

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Abstract

Multi- and pan-antibiotic-resistant bacteria are a major health challenge in hospital settings. Furthermore, when susceptible bacteria establish surface-attached bio-film populations, they become recalcitrant to antimicrobial therapy. Therefore, there is a need for novel antimicrobials that are effective against multi-drug-resistant and surface-attached bacteria. A screen to identify prokaryote-derived antimicrobials from a panel of over 100 bacterial strains was performed. One compound isolated from *Citrobacter freundii* exhibited antimicrobial activity against a wide range of Gram-negative bacteria and was effective against biofilms. Random transposon mutagenesis was performed to find mutants unable to produce the antimicrobial compound. Transposons mapped to a bacteriocin gene located on a small plasmid capable of replication in *Escherichia coli*. The plasmid was sequenced and found to be highly similar to a previously described colicinogenic plasmid. Expression of the predicted bacteriocin immunity gene conferred bacteriocin immunity to *E. coli*. The predicted bacteriocin gene, *colA-43864*, expressed in *E. coli* was sufficient to generate anti-microbial activity, and purified recombinant ColA-43864 was highly effective in killing *E. coli*, *Citrobacter* species, and *Klebsiella pneumoniae* cells in a planktonic and biofilm state. This study suggests that bacteriocins can be an effective way to control surface-attached pathogenic bacteria.

Keywords

Citrobacter freundii; Bacteriocin; Antimicrobial; Biofilm

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Introduction

Members of the Enterobacteriaciae family of Gram-negative bacteria are among the most common food-borne and hospital-acquired pathogens. These organisms have had an immense impact upon human history through devastating diseases, such as the black plague and typhoid fever as well as more common urinary tract infections, dysentery, and hospital-acquired infections (Janda and Abbott 2006). Many members of the Enterobacteriaciae are well known for acquiring antibiotic resistance, including pan-resistant strains of *Klebsiella pneumoniae*, and carbapenem-resistant *Escherichia coli* and *Serratia marcescens* (Nordmann 1998; Bush 2010). The recent discovery of the *ndm-1*-coded carbapenemase has underscored the need to identify novel antibiotics (Bush 2010; Pillai et al. 2011).

Whereas bacteria can gain resistance through acquisition of additional DNA from a plasmid or other source, mutation of an antibiotic target site or a transporter protein, or other genetic mechanism, most bacteria can also gain antibiotic tolerance though formation of a biofilm (Mah and O'Toole 2001; Anderson and O'Toole 2008; Stewart and Costerton 2001). The mechanisms by which bacteria in biofilms resist killing by antibiotics are not completely elucidated. Proposed biofilm antibiotic resistance mechanisms include the presence of persister cells, microenvironments within the biofilm that prevent the antibiotic efficacy, and reduced antibiotic access to bacteria within a biofilm (Stewart and Costerton 2001; Hoiby et al. 2010; Lewis 2010). Regardless of the mechanism of resistance, the biofilm-associated infections are difficult to treat with antibiotics and tend to be persistent (Donlan 2001; Donlan and Costerton 2002). An antimicrobial capable of efficiently killing bacteria in biofilms would be of considerable use in treating chronic, biofilm-associated infections.

Bacteria have been competing for niches for hundreds of millions of years and have developed elaborate systems to inhibit competitors. The enormous genetic potential of microorganisms can be mined for useful compounds. Because of the pressing need for new antimicrobials that are effective against biofilms, we performed a screen for bacteria-produced antimicrobials. Here, we describe the identification of a bacteriocin from *Citrobacter freundii* and its characterization as an antimicrobial effective against planktonic and surface-attached bacteria. This is, to our knowledge, the first demonstration of established biofilm control by a bacteriocin.

Materials and methods

Bacterial strains, media, and culture conditions

The bacteria used in this study are listed in Table 1. Bacteria were grown routinely in lysogeny broth (LB) medium at 37°C. *E. coli* strain WM3064 was grown in medium supplemented with 0.3 mM diaminopimelic acid (DAP). Cells were enumerated as colony-forming units (CFU) on LB agar plates, when appropriate gentamicin was used at 10 μg ml^{-1} and kanamycin at 50 μg ml^{-1}.

Microbial inhibition assay

To examine the ability of the tested bacteria to produce antimicrobial compounds, bacteria were grown for 18 h in liquid broth. Thereafter, 20 μl of the overnight culture (~10^8 CFU ml^{-1}) was spotted on a lawn of microbial cells. Microbial lawns were prepared by spreading 100 μl of an overnight culture on an LB agar plate and incubated at 37°C. Positive production of a diffusible antimicrobial compound was visualized by the inhibition of the susceptible microbial lawn and a clear zone surrounding the examined bacteria colony.

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Crude extraction and biochemical analysis of antimicrobial compound from *C. freundii*

Citrobacter freundii was grown for 24 h in broth at 37°C. One milliliter of the overnight culture was centrifuged for 3 min at 12,000×*g*, and the supernatant was passed through a 0.2-μm pore-size filter to remove bacteria. Ten microliters of the filter-sterilized supernatant was spotted on a lawn of sensitive microbial cells and incubated for 24 h at 37°C. Positive antimicrobial activity was visualized by the development of a zone of inhibition where the filter-sterilized solution was spotted.

To gain insight into the nature of the antimicrobial compound, the following treatments were used: storage at −20°C for 16 weeks, heating for 15 min at 80°C, DNase-I treatment for 3 h (120 μg ml^{-1}), proteinase-K or trypsin treatment for 3 h (100 μg ml^{-1}), and filtration through several size-exclusion Microcon Centrifugal Filter Devices (Millipore, Billerica, MA).

Construction *C. freundii* transposon mutant library

Transposon mutagenesis and mapping were performed as previously described (Medina et al. 2008), except that *C. freundii* ATCC 43864 was used as the recipient strain. The mariner-based transposon delivery plasmid pBT20 (Kulasekara et al. 2005) was used to create a library of ~4,000 mutants.

Screening for genes involved in the production of *C. freundii* antimicrobial compound

To screen for mutants that are impaired in their ability to produce the antimicrobial compound, the *C. freundii* ATCC 43864 transposon mutant library was grown in LB medium for 24 h. A 96-prong multi-well transfer device (Dan-Kar MC96) was used to transfer aliquots of mutant libraries onto plates containing lawns of sensitive *C. freundii* NCTC 9750. The plates were incubated at 37°C for 24 h. Positive or negative production of the antimicrobial compound was assessed by the formation of a zone of inhibition surrounding each mutant colony. *C. freundii* ATCC 43864 wild-type and phosphate-buffered saline (PBS) was used as positive and negative controls.

Molecular techniques

The DNA sequence flanking transposon mutants were determined using arbitrary PCR, as described previously (Medina et al. 2008). The PCR products were sequenced using the TnM Int primer at the Molecular Resource Facility, New Jersey Medical School and compared with the GenBank DNA sequence database using the BLASTX program.

The bacteriocin and immunity gene from plasmid pCfc1, to be described later in the text, was cloned using a recombineering technique using *Saccharomyces cerevisiae* (Shanks et al. 2006). All plasmids used in this study are listed in Table 2. The bacteriocin gene was amplified using primers 2450, accgcttctgcgttctgatttaatctgtatcaTTAGTGATGGTGGTGATGGTGGTGATGTGCAGGTCG GATTAT TTC, and 2451, ctctctactgtttctccatacccgtaggaggaaaaagaATGCCTGGATTTAATTATGGTG that include an in-frame C-terminal poly-histidine tag (underlined), sequence to target recombination with expression vector pMQ124 (Shanks et al. 2009) (lower-case), and sequence to amplify the bacteriocin gene (upper-case). The bacteriocin immunity gene was amplified using primers 2446 (cgttgtaaaacgacggccagtgccaagcttgcatgcctgcGTTTGATTAAAAGGCAGTGT) and 2447 (gaattgtgagcggataacaatttcacacaggaaacatATGAATGAACACTCAATAGATAC), and primers sequences annotated as above. DNA was amplified with a high-fidelity polymerase (Phusion, New England Biolabs), using the manufacturers directions. The recombination reactions place the amino-terminus tagged histidine-tagged under transcriptional control of the *E. coli* P_{BAD} promoter on the ColE1-based pMQ124 vector and place the immunity gene

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under transcriptional control of the *E. coli* P_{lac} promoter on the pBBR1-based pMQ131 vector (Shanks et al. 2009). Plasmid constructs were verified by sequencing (University of Pittsburgh Genomics and Proteomics Core).

Purification of polyhistidine-tagged bacteriocin from *E. coli*

Escherichia coli S17-1 harboring pMQ348 was grown for 18 h in LB supplemented with 10 μg ml^{-1} gentamicin to reach a final concentration of OD_{600} = 0.2. One milliliter of the overnight culture was subcultured in 5 ml of fresh LB and left to grow for 2 h, arabinose, which induces expression of P_{BAD} promoter in pMQ348, was added to the culture (0.2% w/v final concentration) and the tubes were incubated for an additional 3 h. To obtain crude cell proteins, the bacteria were pelleted by centrifugation, washed twice in PBS and resuspended in fresh 500 μl of PBS. Cells were lysed by sonication on ice for 50 s using a VC505 sonicator set on 80% strength (Sonics and Materials Inc., Newtown, CT, USA). Cell debris was pelleted by centrifugation, and the supernatant was removed and passed through a 0.2-μm pore-size filter (hereafter referred to as crude cell extract).

Immobilized metal ion affinity chromatography (IMAC) was used to further purify the His_8-tagged bacteriocin, and 1 ml of the crude cell extract was mixed with 200 μl of Talon™ Metal Affinity Resin (Clontech Laboratories, Inc. Mountain View, CA) suspended washing-buffer (50 mM sodium phosphate, 300 mM NaCl and 10 mM imidazole pH-7). The mixture was stirred for 30 min and then centrifuged for 2 min at 1,000×*g*. The pelleted resin was collected in a 2-ml tube and the unbound supernatant discarded. The resin was washed twice with washing-buffer containing 60 mM imidazole. Elution of the tagged protein was performed by the addition of 1 ml of washing-buffer supplemented with 200 mM imidazole (elution buffer). The eluent was incubated for 2 min, centrifuged for 2 min, and the supernatant containing the His_8-tagged protein was collected in a new tube. The sample was then filtered through a 0.2-μm pore-size filter (hereafter referred to as IMAC-purified bacteriocin). Protein concentration was determined using Bio-Rad Quick start™ Bradford protein assay.

PAGE and Western blot analysis were performed on each purification fraction using standard techniques. The PAGE gel (4–20% gradient) was a precast mini-format gel (Precise Protein Gel, Pierce), and a Bio-Rad Protean 3 device was used for electrophoresis and transfer to an Immobilon transfer membrane (Millipore). The blot was probed with a mouse-anti-polyhistine antibody (Covance product number MMS-156P), and the secondary antibody was a goat-anti-mouse HRP-conjugated antibody (Pierce, product number 32430).

Bacteriocin anti-microbial activity

To assess the antimicrobial activity of bacteriocin on planktonically grown bacteria, tested bacteria were grown for 18 h at 37°C. Thereafter, 1 ml of the cells was pelleted by centrifugation, washed, and resuspended in 1 ml PBS. One hundred-microliter cell aliquots were placed in a 2-ml microfuge tube, and an equal volume of crude or IMAC-purified bacteriocin was added to the tube. Alternatively, as a control, 100 μl of sterile PBS was added to each tube. The tube was incubated at 37°C for the duration of the experiment. Quantification of viable bacteria before and following treatment was performed by CFU enumeration. Each experiment was carried out at least three times.

Bacteriocin (ColA-43864-His_8) anti-biofilm activity

Biofilms were formed in a non-tissue culture treated, 96-well polyvinyl chloride microtiter dishes (Becton–Dickinson, Franklin Lakes, NJ, USA) as previously described (O'Toole and Kolter 1998; Merritt et al. 2005). Briefly, microtiter wells were inoculated (100 μl per well) with bacteria that had been grown cell culture to stationary phase in LB medium and diluted

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1:100 in fresh LB media. *K. pneumoniae* biofilms were developed in M63 minimal salts supplemented with 1 mM $MgSO_4 \cdot 7H_2O$, 14 mM sodium citrate, and 34 mM L-proline (Kadouri et al. 2007). The plates were incubated for 18 h at 30°C to generate biofilms. To assess the antimicrobial activity of bacteriocin, the preformed biofilms were washed twice with PBS to remove planktonic cells, and 100 μl of tested bacteriocin sample was added to each well. Alternatively, as a control, 100 μl of sterile PBS was added to the wells. The microtiter dishes were incubated at 30°C for the duration of the experiment. Quantification of biofilm bacteria before and following treatment was performed by washing the microtiter plates with PBS, to remove non-adhering cells, 100 μl of fresh PBS was added to each well, and the samples were sonicated for 8 s using a VC505 sonicator, set on 40% strength, followed by dilution plating and CFU enumeration (Kadouri and O'Toole 2005; Kadouri et al. 2007). Each experiment was carried out at least three times.

Microscopy of biofilms was performed by first establishing biofilms on PVC cover slips in LB medium using the air liquid interface (ALI) method, described by Merritt et al. (2005). Biofilms of *C. freundii* ATCC 8090 were formed for 20 h, non-adherent bacteria were removed by washing with PBS and then incubated in PBS with either crude lysates from S17-1 + pMQ124 (empty vector) or S17-1 + pMQ348 (colicin expressing plasmid) to a final concentration of 11.6 μg ml^{-1} total protein. After 60 min, biofilms were again washed with PBS and stained with a commercial live-dead stain (Bac-Light, Invitrogen) according to the manufacturers specifications and visualized with an Olympus Fluoview 1000 confocal laser scanning microscope (CLSM) and Fluoview 2.1 software.

Results

Identification of an antimicrobial compound produced by *C. freundii* ATCC 43864

In a screen aimed at isolating new antimicrobial compounds, 105 bacteria, representing 42 species and 26 different genera, were cross-examined in a microbial inhibition assay [for a full list of bacteria tested, see Dashiff et al. (2011)].

One bacterial strain, *C. freundii* ATCC 43864, was found to produce a diffusible antimicrobial compound that inhibited the growth of other *C. freundii* strains (Fig. 1a, I), and other bacteria described below. Filter-sterilized supernatants of *C. freundii* ATCC 43864 grown in liquid broth (crude extraction method) also yielded the antimicrobial compound, suggesting that the compound was extracellular and did not require a competing organism to induce its production (Fig. 1a, II).

Microbial inhibition assays using both colonies and filter-sterilized supernatants were used to investigate whether additional members of the *Citrobacter* genus produce a similar antimicrobial compound and to evaluate their sensitivity to the antimicrobial compound produced by *C. freundii* ATCC 43864. As described in Table 3, an antimicrobial compound was produced only by *C. freundii* ATCC 43864. The compound was observed to inhibit the growth of *C. braakii* ATCC 43162, *C. freundii* NCTC 9750, and *C. freundii* ATCC 8090, but was not active in inhibiting the producing strain *C. freundii* ATCC 43864.

To further characterize the antimicrobial compound, crude extracts were isolated from *C. freundii* ATCC 43864 and subjected to a number of challenges. Treated extracts were spotted on a lawn of sensitive *C. freundii* NCTC 9750 to evaluate their antimicrobial activity. The antimicrobial compound was found to be resistant to DNase-I and freezing and sensitive to protease activity and heat. The compound was also found to have a molecular mass between 50 and 100 kDa based upon fractionation with size-exclusion filtration columns (data not shown). From this analysis, it was concluded that the antimicrobial compound was likely to be a protein.

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Construction of *C. freundii* ATCC 43864 transposon mutant library, and isolation of mutants defective in the synthesis of the antimicrobial compound

To isolate mutants defective in the synthesis of the antimicrobial compound, a mariner-based transposon was used to mutagenize *C. freundii* ATCC 43864. Mutants were transferred onto lawn of *C. freundii* NCTC 9750, which was found to be sensitive to the compound. The plates were incubated for 24 h until a zone of inhibition was seen surrounding each mutant (Fig. 1b). Using this approach from ~4,000 mutant colonies, a mutant unable to produce a zone of inhibition was isolated (Fig. 1c). This mutant was designated Cf-8A. No difference in growth rate was observed between Cf-8A mutants and the *C. freundii* recipient when grown in LB medium (data not shown).

Mapping of mutation Cf-8A to a predicted bacteriocin gene, *colA-43864*

The Cf-8A transposon mutation was mapped using arbitrary PCR to base pair 456 of a predicted bacteriocin gene, that is ~99% identical at the DNA level to the *colA* gene of *C. freundii* strain CA31, also known as *colA*-CA31. The resulting predicted protein has one amino acid different from the previously identified ColA-CA31, specifically a leucine to serine change at amino acid 328. Because the newly isolated bacteriocin gene is not identical to *colA-CA31* and is derived from a different strain, we named the gene *colA-43864*. Bacteriocins are bacterial-produced antimicrobial proteins, of which colicins are a well-characterized subgroup (Cascales et al. 2007).

Since the *colA-CA31* gene was found on a small plasmid that could replicate in *E. coli* (Morlon et al. 1982), we tested whether the Cf-8A mutation was also on a plasmid. To this end, total DNA was isolated from mutant strain Cf-8A and used to electroporate *E. coli*. If the Cf-8A mutation was on a plasmid capable of replication in *E. coli*, then we predicted that the plasmid could be selected for using the gentamicin resistance marker on the transposon. We were able to isolate gentamicin-resistant *E. coli* colonies harboring a plasmid isolated from the Cf-8A (data not shown), supporting that the Cf-8A was on a plasmid.

A small plasmid, named pCfc1, was isolated from the WT ATCC 43864 strain and sequenced (Genbank JF795024). Sequence analysis reveals that the plasmid is 6.72 kb in length, is ~99% identical to the pColA-CA31 plasmid of *C. freundii* strain CA31 (Morlon et al. 1988b), and has high similarity to bacteriocin-bearing plasmids from other *Citrobacter* species. The gene organization of pColA-CA31 (Morlon et al. 1988b) and the pCfc1 is identical and includes predicted genes for a bacteriocin, a bacteriocin immunity protein, a lysis protein, entry exclusion proteins, and plasmid mobility genes.

Antimicrobial activity of *E. coli* harboring *C. freundii* ATCC 43864 bacteriocin plasmid (pCfc1)

In order to establish that the genes involved in the synthesis of the antimicrobial compound are plasmid-borne and that no additional genes are required for the production of the bacteriocin, the pCfc1 was mobilized into *E. coli.* To accomplish this, we moved a selectable marker onto the plasmid, by mutagenesis with the pBT20-derived mariner transposon. *C. freundii* ATCC 43864 cells containing pCfc1 were mutagenized with the mariner transposon. The prediction was that, in a small subset of the mutant colonies, the transposon would localize to the pCfc1 plasmid rather than the chromosome, thereby adding a selectable marker to the pCfc1 plasmid. Mutant colonies were pooled, and plasmid DNA was isolated. The harvested plasmids were mobilized by electroporation into *E. coli* S17-1 cells, and the S17-1 cells were plated on LB agar supplemented with gentamicin. One Gm^r isolate (*E. coli*-21pA) was selected for further analysis. DNA sequencing of purified plasmid from *E. coli*-21pA confirmed that the cell harbors pCfc1 containing a transposon insertion at position 441 relative to the arbitrarily determined origin in an intergenic region between two

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predicted entry exclusion genes. This mutation is not predicted to interfere with transcription of the bacteriocin gene. The marked plasmid was designated as pCfc1-21A.

Wild-type *E. coli* S17-1, *E. coli* S17-21pA (*E. coli* S17-1-bearing pCfc1-21A in which the pCfc1 plasmid has transposon inserted in an intergenic region), and *E. coli* S17-8A (*E. coli* S17-1-bearing pCfc1-8A in which the pCfc1 plasmid has a transposon mutation in the *colA-43864* gene) were all spotted on a lawn of *C. freundii* NCTC 9750. As seen in Fig. 2, a clear zone of inhibition was seen around *E. coli* S17-21pA, supporting that the genes necessary for antimicrobial production are all present on pCfc1. As anticipated, no antimicrobial activity was seen on lawns that were inoculated with S17-1 wild-type or bearing the pCfc1-8A plasmid (Fig. 2. *E. coli* S17-1 wild-type and *E. coli* S17-8A, respectively).

Production and purification of bacteriocin (ColA-43864) in *E. coli*

To determine whether the *colA-43864* gene was sufficient for the observed antimicrobial phenotype, we cloned a poly-histidine tagged version of the gene and placed it under control of an arabinose-inducible promoter. The His_8-tag was placed separately on the C- and N-termini of the predicted ColA-43864 protein, and while both conferred antimicrobial activity, the C-terminal version exhibited higher relative antimicrobial activity (data not shown), and thus, subsequent work was performed with the carboxy-terminal tagged version. To express *colA-43864* in *E. coli,* pMQ348 (pMQ124-*colA-43864*-His_8) was mobilized by electroporation into *E. coli* S17-1 cells, to make *E. coli* S17-pMQ348. As a control, *E. coli* S17-1 was also transformed with empty vector control pMQ124, to generate *E. coli* S17-pMQ124.

When grown in the presence of 0.2% (w/v) glucose, no difference in growth was seen between *E. coli* S17-pMQ348 and *E. coli* S17-pMQ124 (Fig. 3). However, under promoter-inducing conditions, when 0.2% (w/v) L-arabinose was added to the media, a clear inhibition of *E. coli* S17-pMQ348 growth was measured compared to the empty vector control (Fig. 3). These data suggest that expressing the *colA-43864* gene in *E. coli* has a toxic effect.

To isolate crude extracts and pure recombinant ColA-43864 from *E. coli,* overnight cultures of *E. coli* S17-pMQ348 and empty vector control *E. coli* S17-pMQ124 were grown in the presence of arabinose and crude cell extracts were prepared, as described in materials and methods. ColA-43864 was isolated by IMAC, as described in materials and methods. PAGE analysis shows the purification of a single protein of the predicted mass in the elution fraction of *E. coli* S17-pMQ348 lysates, whereas no band was observed in the elution fraction of the empty vector control (Fig. 4a). To further confirm that the single band was the ColA-43864-His_8 construct, Western blotting was performed. We observed that the single eluted band seen on the PAGE gel was detected by an anti-polyhistidine antibody, whereas that band was absent in the empty vector control (Fig. 4b).

Antimicrobial activity of crude extract and purified ColA-43864-His_8

To characterize the bacteriocin's antimicrobial specificity, crude cell extracts from *E. coli* S17-pMQ348 and control *E. coli* S17-pMQ124 were prepared. Samples of the crude extracts (20 μl, containing 15 and 35 μg total protein from *E. coli* S17-pMQ348 and *E. coli* S17-pMQ124, respectively) were spotted on lawns of examined bacteria. As seen in Table 4, the crude ColA-43864-containing extract was able to inhibit the growth of several bacteria from the family *Enterobacteriaceae*, including *C. braakii*, *C. freundii, Enterobacter gergoviae*, *E. coli*, *Klebsiella pneumoniae*, and *Yersini pseudotuberculosis*. Several other tested species were immune to the bacteriocin-containing extracts (Table 4). No inhibition was seen when

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crude protein extract from empty vector control *E. coli* S17-pMQ124 was spotted (Table 4). Similar inhibition patterns were seen when *C. freundii* ATCC 43864 colonies were spotted on the lawns (data not shown).

To assess the antimicrobial activity of bacteriocin on planktonic bacteria grown in broth, crude extracts, isolated from *E. coli* S17-pMQ348 (37 μg total protein) and control *E. coli* S17-pMQ124 (87 μg total protein), were added to overnight cultures. Incubating *C. freundii* and *E. coli* for 30 min with crude cell bacteriocin extracts had resulted in a 8 and 6 log reduction in cell counts. A more moderate 2–3 log reduction was measured for *K. pneumoniae* and *Y. pseudotuberculosis*. No reduction was seen after incubation with PBS or crude cell extracts isolated from *E. coli* S17-pMQ124 empty vector control (Fig. 5a).

Whereas many antimicrobial agents are effective against planktonic cells, fewer are active against biofilms. Furthermore, while the results of one study suggest that bacteriocins can inhibit the development of biofilms (Hancock et al. 2010), it is not known whether bacteriocins have any effect upon cells in a biofilm. When placed on biofilms, crude cell extracts, isolated from *E. coli* S17-pMQ348 (75 μg total protein), were able to significantly reduce biofilm cell viability by up to 7 logs within 2 h of incubation (Fig. 5b).

To further assess the antimicrobial effect of ColA-43864 on pre-formed biofilms, CLSM was used to analyze bacteriocin-treated biofilms treated with live-dead fluorescent stains. Extracts from bacteria expressing recombinant ColA-43864-His_8 had a clear bacteriocidal effect on *C. freundii* biofilm cells (note red staining Fig. 5c, pMQ348 Dead), whereas most the *C. freundii* biofilm exposed to extracts without the recombinant ColA-43864-His_8 was largely composed of live cells (green staining Fig. 5c, pMQ124 Live). Furthermore, the colicin-exposed biofilms were stained red throughout the biofilms (data not shown), suggesting that ColA-43864 is able to penetrate the biofilms.

A range of purified recombinant ColA-43864-His_8 (0.02–2.00 μg) was used to test the impact of this protein on both planktonically grown bacteria and biofilms. Two micrograms of ColA-43864-His_8 caused a 6–7 log reduction in planktonically grown and biofilm cell viability (Fig. 6a, b). The reduction in cell viability was dose dependent (Fig. 6a, b). In an additional experiment, *K. pneumoniae* biofilms (composed of 2.5×10^6 CFU ml^{-1}) were incubated with 2 μg of ColA-43864-His_8. A 2 log reduction (1×10^4 CFU ml^{-1}) was seen within a 30-min incubation period. No reduction was measured for *K. pneumoniae* biofilms incubated with control PBS or mock IMAC-purified protein isolated from *E. coli* S17-pMQ124 (2.4×10^6 CFU ml^{-1} and 2.5×10^6 CFU ml^{-1}, respectively). Treating the bacteria with the elution buffer, used to elute the IMAC-purified protein, did not cause any reduction in bacterial CFU (data not shown).

Expressing C. freundii immunity gene provides resistance from ColA-43864 antimicrobial activity

In order to investigate whether the predicted immunity gene, from *C. freundii* ATCC 43864 plasmid pCfc1, is capable of providing resistance to the antimicrobial activity of ColA-43864, bacteriocin-sensitive wild-type *E. coli* S17-1, *C. freundii* NCTC 9750, and *C. freundii* ATCC 8090 were transformed by electroporation with pCfc-8A (pCfc1 with a mutated *colA-43864* gene). The transformants were spread on an LB agar plate, and 20-μl aliquots of crude ColA-43864-containing extracts were spotted on top of each lawn (15 and 35 μg total protein from *E. coli* S17-pMQ348 and *E. coli* S17-pMQ124, respectively). As seen in Fig. 7a, cells harboring the bacteriocin-defective plasmid, with an intact immune gene, were protected from ColA-43864-containing extracts. In another experiment, *E. coli* S17-1 was transformed with the immune gene, cloned under control of the *E. coli* P_{lac} promoter on a multicopy plasmid, to yield strain *E. coli* S17-pMQ345. Indicating that the

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immune gene was sufficient to produce ColA-43864-resistant *E. coli* (Fig. 7a, *E. coli* S17-pMQ345). CFU enumeration of planktonically grown bacteria also confirmed the resistance of *E. coli* S17-pMQ345 to the antimicrobial effect of crude cell extracts containing *ColA-43864* (Fig. 7b).

Discussion

In this study, we have determined the identity of an antimicrobial protein from a *C. freundii* strain ATCC 43864 using a genetic approach. The gene that codes for the antimicrobial, *colA-43864*, is on a small plasmid almost identical in sequence to the well-characterized colicin A-producing plasmid from *C. freundii* strain CA31 (Morlon et al. 1988a). The *colA-CA31* gene from *C. freundii* strain CA31 was previously described as a type-A bacteriocin (Davies and Reeves 1975) and used for several studies to characterize the role of "A" type bacteriocins (Varenne et al. 1981; Crozel et al. 1983). Based on the ~99% sequence identity, we predict that the biofilm-controlling ColA-43864 described in this study is also an "A" type bacteriocin. Bacteriocins are released through the action of lysis proteins rather than through traditional secretion systems (Cascales et al. 2007). The "A" type bacteriocins gain access to bacterial cells through binding to a complex of outer membrane proteins (OmpF and BtuB) and lipopolysaccharides (Chai et al. 1982; Cascales et al. 2007), then move through the periplasmic space with the aid of TolA and TolB (Hecht et al. 2010), and kill bacteria by disrupting their membrane and uncoupling the proton gradient (Schein et al. 1978; Cascales et al. 2007).

The plasmid pCfc1-21A was able to replicate in *E. coli* and was sufficient to confer antimicrobial activity to *E. coli*; however, when the predicted bacteriocin gene was mutated with a transposon, no antimicrobial activity was observed, suggesting that the antibacterial activity requires the *colA-43864* gene. To further confirm that the bacteriocin gene is necessary and sufficient for the antimicrobial activity, the *colA-43864* gene was cloned and placed expressed in *E. coli* under control of an arabinose-inducible promoter. Induction of the *colA-43864* gene resulted in a severe growth defect, supporting that expression of the *colA-43864* gene alone is toxic to the host cell. Crude fractions from the *E. coli* strain bearing pMQ348, but not the empty vector control, exhibited antimicrobial activity against species from several genera of Gram-negative bacteria including important pathogens *K. pneumoniae* and *E. coli*. Interestingly, only 8 out of 13 *K. pneumoniae* isolates exhibited sensitivity to the colicin. The source of bacteriocin resistance by the five bacteriocin-resistant *K. pneumoniae* isolates may be due to an altered or absent bacteriocin receptor, or they may harbor bacteriocin immunity genes. However, we were not able to detect immunity genes by PCR while using primers based on conserved sequences from multiple immunity genes (data not shown).

Plasmid pCfc1-8A or the predicted immunity protein gene alone, expressed on a multicopy plasmid, was able to provide protection from ColA-43864. This result further supports that the antimicrobial effect was specific to the ColA-43864, rather than some unrelated compound induced by production of ColA-43864.

A previous study showed that bacteriocin-producing *E. coli* strains could inhibit biofilm formation by non-bacteriocin-producing *E. coli* strains on catheter material (Hancock et al. 2010). While the study provided strong genetic evidence that bacteriocins could inhibit biofilm formation (Hancock et al. 2010), it was not determined whether established biofilms can be treated with bacteriocins or whether bacteriocins separated from other bacterial components could be used. Other studies have explored the influence of bacteriocins on biofilm formation by competing organisms (Kreth et al. 2005; Tait and Sutherland 2002). Tait and Sutherland showed that bacteriocin-producing strains have a selective advantage

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within a multi-species biofilm and their data suggest that bacteriocins may have evolved to function largely in the biofilms rather than among planktonic cells. Here, we show for the first time that a purified bacteriocin can effectively and rapidly kill cells in biofilms in a dose-dependent manner. The ability of the ColA-43864 to eradicate a large portion of the cells within a biofilm supports the model that diffusion of the ColA-43864 into the biofilm is not a limiting factor; however, it is possible that there are microenvironments within the biofilm where ColA-43864 would not function due to suboptimal pH or other conditions.

This study supports that bacteriocins could be used to effectively treat some bacterial biofilms. Future analysis will focus on determining the effect of bacteriocins in tandem with other antimicrobials and biocontrol agents such as phage and predatory bacteria to optimize methods for destroying established biofilms.

Acknowledgments

The authors would like to thank Nicholas Stella for technical assistance and Dr. Yohei Doi, Division of Infectious Diseases, University of Pittsburgh School of Medicine for kindly providing bacterial strains. This work was supported by funding from the Foundation of UMDNJ faculty research grant to D.E.K., and NIH AI085570 and a Research to Prevent Blindness Career Development Award to R.M.Q.S. Additional support was provided by NIH grant EY08098 and the Eye and Ear Foundation of Pittsburgh.

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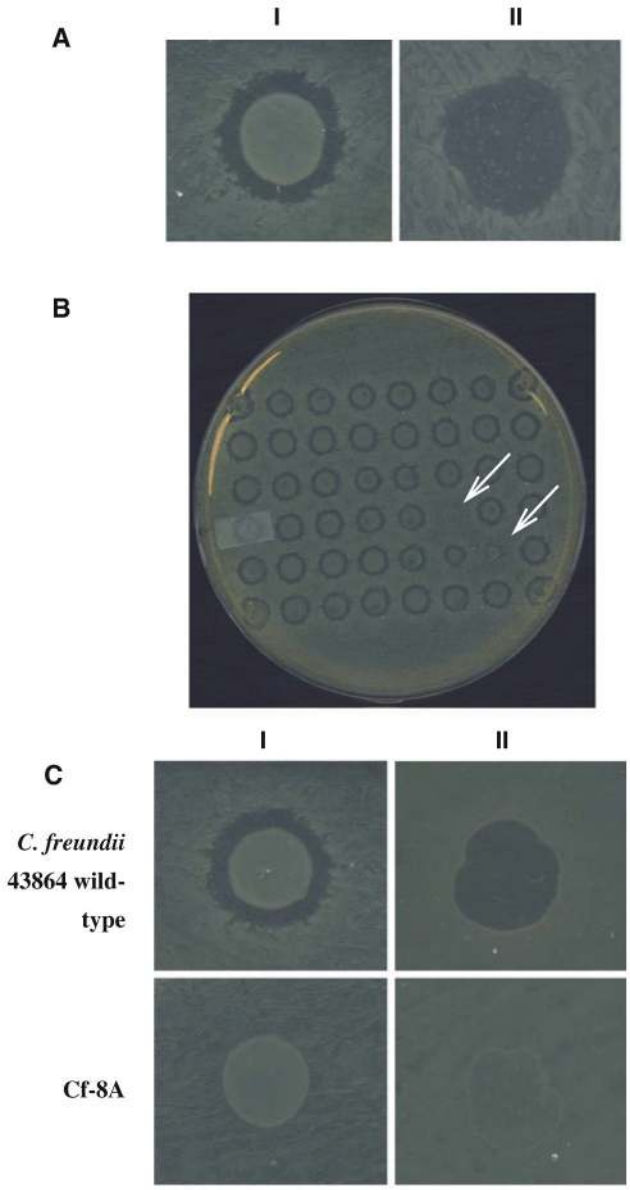

Fig. 1.

Identification of an antimicrobial compound from *C. freundii* ATCC 43864. **a** Microbial inhibition assay. *C. freundii* ATCC 43864 was grown in liquid broth for 24 h, and cells (I) or filter-sterilized supernatant (II) was spotted on a lawn of *C. freundii* NCTC 9750. Antimicrobial activity is seen by the formation of a zone of inhibition around or at the point of inoculation. **b** Screening for *C. freundii* ATCC 43864 mutants defective in the production of the antimicrobial compound. *C. freundii* ATCC 43864 transposon mutants were grown in a 96-well microtiter dish. Aliquots were transferred onto a lawn of sensitive *C. freundii* NCTC 9750. The plates were then incubated at 37°C and examined for the formation of a zone of clearing where the mutants were spotted. The *arrows* indicate the location of mutants impaired in their ability to produce antimicrobial compound. **c** Microbial inhibition assay of *C. freundii* ATCC 43864 antimicrobial-defective mutant (Cf-8A). *C. freundii* ATCC 43864 wild-type and Cf-8A were grown in liquid broth for 24 h, and cells (I) or filter-sterilized supernatant (II) was spotted on a lawn of *C. freundii* NCTC 9750.

Antimicrobial activity is seen by the formation of a zone of inhibition around or at the point of inoculation

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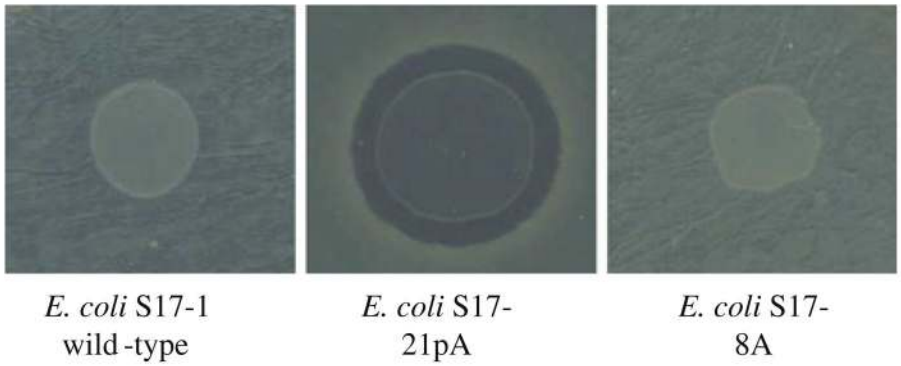

Fig. 2.

Transformation of *E. coli* with pCfc1. Overnight cultures of wild-type *E. coli* S17-1, *E. coli* S17-21pA, and *E. coli*-8A were spotted on a lawn of sensitive *C. freundii* NCTC 9750. Antimicrobial activity is seen by the formation of a zone of inhibition around the point of inoculation

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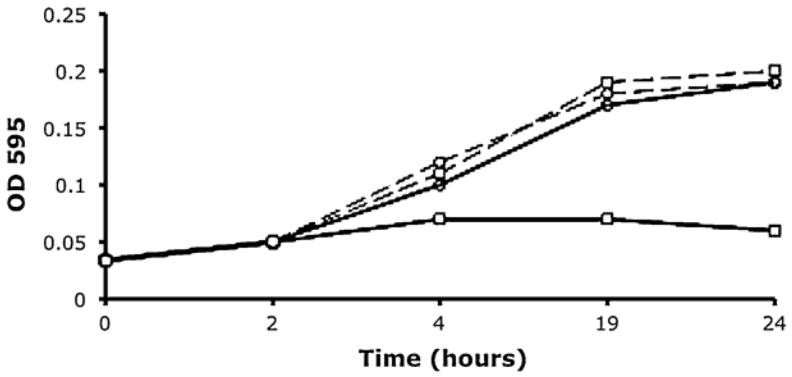

Fig. 3.

Growth of *E. coli* S17-1 expressing ColA-43864-His8. Cultures of *E. coli* S17-pMQ348 (*solid line*) and empty vector control *E. coli* S17-pMQ124 (*broken line*) were grown at 37°C in LB supplemented with 0.2% glucose (*colA-43864*-inhibiting condition, *circle*) or L-arabinose (*colA-43864*-inducing condition, *square*). Culture turbidity was measured by absorbance at A_{595} nm to determine growth

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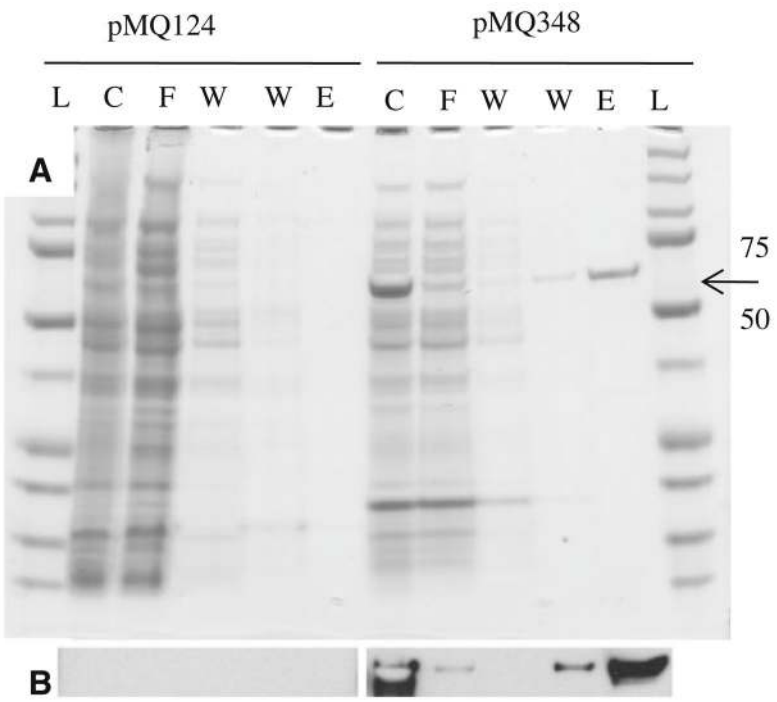

Fig. 4.

Purification of ColA-43864-His_8. **a** ColA-43864-His_8 purification fractions analyzed by PAGE. Crude lysates with empty vector negative control (pMQ124) or pMQ124 + His_8-*colA-43864-His8* (pMQ348) were generated identically and purified by IMAC. Fractions for crude fraction (C), column flow through (F), wash (W), and elution (E), and the size standard (L) are shown. The 50- and 75-kD size standards are numbered. **b** Immunoblot analysis of each fraction probed with an anti-poly-histidine antibody. Immunoblot lanes are aligned those of the PAGE in (**a**)

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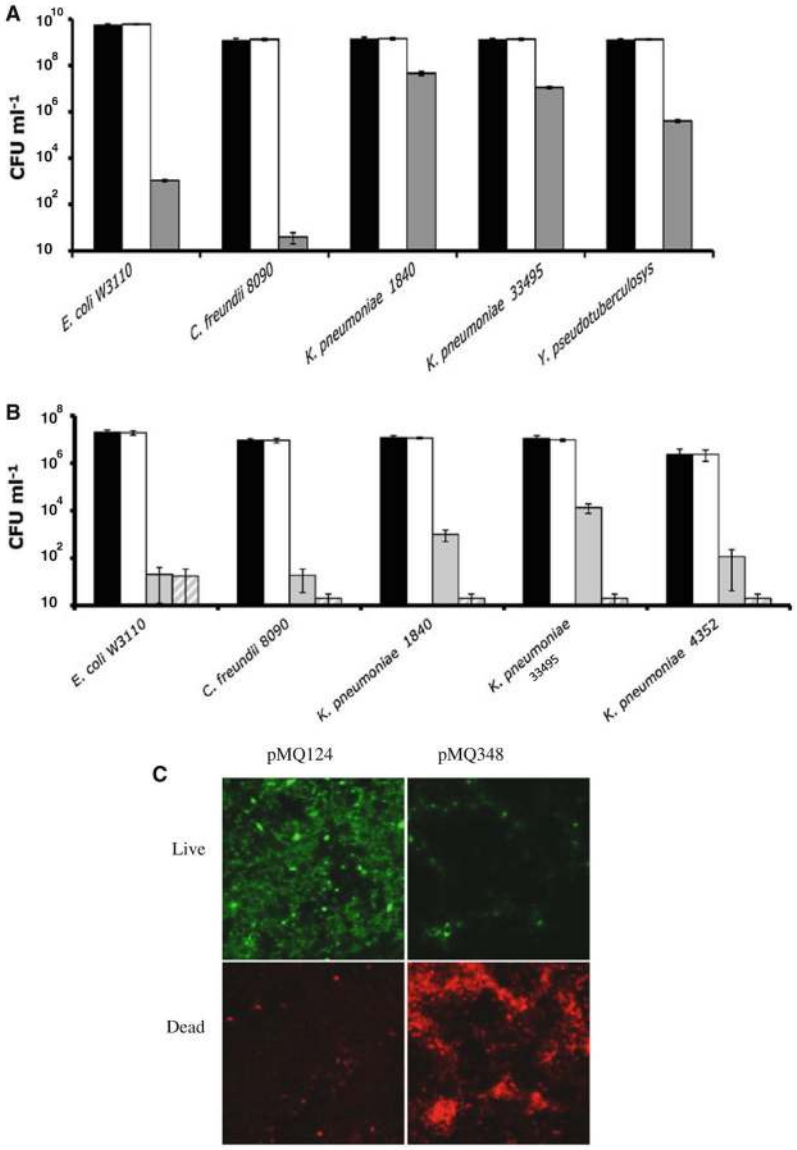

Fig. 5.

Antimicrobial activity of crude ColA-43864 extract. **a** Effect of ColA-43864 on planktonic bacteria. Tested bacteria (~10^9 CFU ml^{-1}) were incubated for 30 min with PBS (*black bars*), 87 μg protein extracted from empty vector control *E. coli* S17-pMQ124 (*white bars*), and 37 μg protein isolated from *E. coli* S17-pMQ348 (*gray bars*). Cell viability was measured at time 0 and following incubation. Each value represents the mean of 3 experiments. *Error bars* are shown as one-standard deviation. **b** Effect of ColA-43864 on biofilms. Overnight biofilms (composed of ~10^6–10^7 CFU ml^{-1}) were incubated for 120 min with PBS (*black bars*) and 175 μg protein extracted from empty vector control *E. coli* S17-pMQ124 (*white bars*). Biofilms were also incubated, for 30 min (*gray bars*) and 120 min (*striped gray bars*), with 75 μg protein isolated from *E. coli* S17-pMQ348. Cell viability was measured at time 0 and following incubation. Each value represents the mean of 3 experiments. *Error bars* are shown as one-standard deviation. **c** CLSM micrographs demonstrating the effect of ColA-43864 on biofilms. Overnight biofilms of *C. freundii* ATCC 8090 were incubated for 60 min with protein (11.6 μg ml^{-1} final concentration) extracted from empty vector control

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E. coli S17-pMQ124 or the colicin bearing plasmid *E. coli* S17-pMQ348. Thereafter, the biofilms were stained with Syto-9 (live) and propidium iodide (dead). A representative image is shown. Images were taken at the same exposures with a 40× magnification objective

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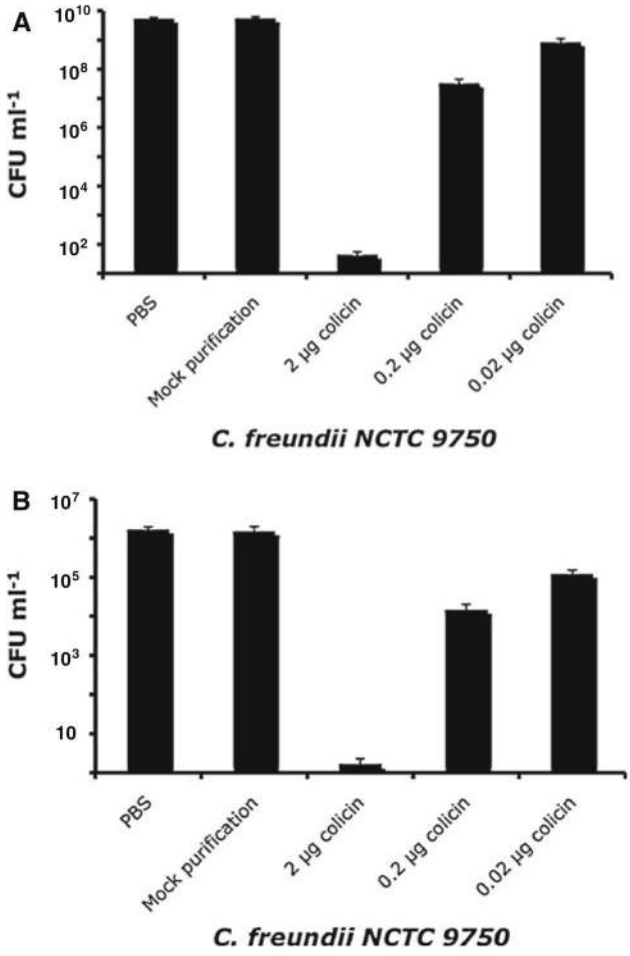

Fig. 6.

Antimicrobial activity of ColA-43864-His8. Effect of IMAC-purified ColA-43864 on planktonic (**a**) or biofilm (**b**) planktonic bacteria. *C. freundii* NCTC 9750 (6×10^9 and 2×10^6 CFU ml^{-1} for planktonic and biofilm, respectively) were incubated, for 30 min, in the presence of 2–0.02 μg IMAC-purified ColA-43864, PBS control and mock IMAC-purified sample from *E. coli* S17-pMQ124. Cell viability was measured at time 0 and following incubation. *Each value* represents the mean of 3 experiments. *Error bars* are shown as one-standard deviation

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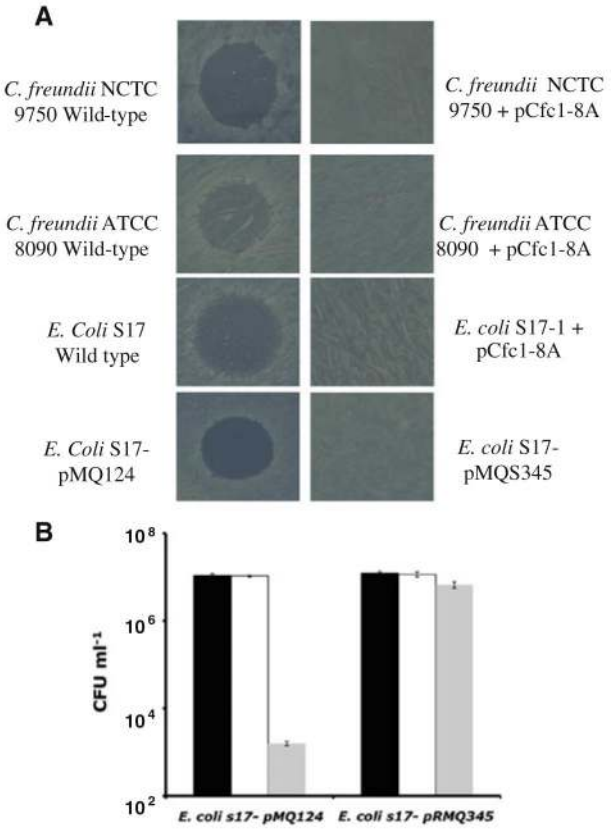

Fig. 7.

Expression of a predicted bacteriocin immunity gene in ColA-43864-sensitive bacteria. **a** Bacteriocin antimicrobial activity assay. Crude ColA-43864-containing extracts were spotted on microbial lawns of *C. freundii* NCTC 9750, *C. freundii* ATCC 8090, and *E. coli* S17-1, as well as transformants harboring plasmids: pMQ124 (empty vector), pCfc1-8A (*colA-43864* defective, intact immunity gene), or pMQ345 (vector + predicted immune gene from pCfc1). Antimicrobial activity is seen by the formation of a zone of inhibition, and the bacteriocin was spotted. **b** Effect of bacteriocin on *E. coli* harboring the immune gene. Planktonically grown *E. coli* S17-pMQ345 and an empty vector control *E. coli* S17-pMQ124 (~10^7 CFU ml^{-1} of each strain) were incubated for 30 min with PBS (*black bars*), 87 μg protein extracted from empty vector control *E. coli* S17-pMQ124 (*white bars*), and 37 μg protein derived from *E. coli* S17-pMQ348 (*gray bars*). Cell viability was measured at time 0 and following incubation. *Each value* represents the mean of 3 experiments. *Error bars* are shown as one-standard deviation

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Table 1

Strains used in the study

Name	Description	Source
Citrobacter		
C. braakii ATCC 43162	Wild-type	ATCC
C. braakii ATCC 51113	Wild-type	ATCC
C. freundii NCTC 9750	Wild-type	ATCC
C. freundii ATCC 43864	Wild-type	ATCC
C. freundii ATCC 8090	Wild-type	ATCC
C. koseri ATCC 27156	Wild-type	ATCC
Cf-8A	*colA-43864* mutant *C. freundii* ATCC 43864, bacteriocin defective	This study
C. freundii NCTC 9750-8A	*C. freundii* NCTC 9750-bearing plasmid pCfc1-8A	This study
C. freundii ATCC 8090-8A	*C. freundii* ATCC 8090-bearing plasmid pCfc1-8A	This study
Escherichia coli		
E. coli S17-1	ATCC 47055	ATCC
E. coli DH5α	Laboratory strain	Invitrogen
Twelve *E. coli* clinical isolates	Clinical isolates	University of Pittsburgh School of Medicine
E. coli strain WM3064	A diaminopimelic acid (DAP) auxotroph, derivative of strain B2155	(Croal et al. 2007)
E. coli S17-21pA	*E. coli* S17-1-bearing plasmid pCfc1-21pA (functional bacteriocin gene)	This Study
E. coli S17-8A	*E. coli* S17-1 bearing plasmid pCfc1-8A (mutated bacteriocin gene)	This Study
E. coli S17 pMQ348	pMQ124 + colA-43864-His8 C-tag	This study
E. coli S17 pMQ124	pMQ124 empty vector control	This study
E. coli S17-pMQ345	*E. coli* S17-1 + immune gene on pMQ131	This study

ATCC American Type Culture Collection

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Table 2

Plasmids used in the study

Name		Source
pCfc1	Bacteriocin-bearing plasmid from *C. freundii* ATCC 43864	This study
pCfc1-8A	pCfc1 with transposon mutation in *colA-43864*	This study
pCfc1-21A	pCfc1 with transposon mutation intergenic region	This study
pBT20	Mariner transposon delivery vector, *ori*R6K, *bla*, *aacC1*	Kulasekara et al. (2005)
pMQ124	Expression vector, *ori*ColE1, oripRO1600, *aacC1*	Shanks et al. (2009)
pMQ131	Broad host-range vector, *ori*pBBR1, *aphA-3*	Shanks et al. (2009)
pMQ345	pMQ131 + bacteriocin immunity gene from pCfc1	This study
pMQ348	pMQ124 + *colA-43864*-His_8	This study

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Table 3

Production of antimicrobial compound by *Citrobacter* spp

***Citrobacter* extracts tested**	***C. braakii* ATCC 43162**	***C. braakii* ATCC 51113**	***C. freundii* NCTC 9750**	***C. freundii* ATCC 43864**	***C. freundii* ATCC 8090**	***C. koseri* ATCC 27156**	**PBS control**
Citrobacter tested							
C. braakii ATCC 43162	–	–	–	+	–	–	–
C. braakii ATCC 51113	–	–	–	–	–	–	–
C. freundii NCTC 9750	–	–	–	+	–	–	–
C. freundii ATCC 43864	–	–	–	–	–	–	–
C. freundii ATCC 8090	–	–	–	+	–	–	–
C. koseri ATCC 27156	–	–	–	–	–	–	–

Citrobacter spp were grown in liquid broth for 24 h. Cultures and filter-sterilized supernatants were cross-examined in a microbial inhibition assay for their ability to produce an antimicrobial compound as well as their sensitivity to *C. freundii* ATCC 43864. Antimicrobial activity was measured by the formation of a zone of inhibition around or at the point of inoculation. (+) Positive zone of inhibition. (–) No zone of inhibition

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Table 4

Antimicrobial activity of bacteriocin

	***E. coli* S17-pMQ348**	***E. coli* S17-pMQ124**
Bacteria tested		
Citrobacter		
C. braakii ATCC 43162	+	–
C. braakii ATCC 51113	+	–
C. freundii NCTC 9750	+	–
C. freundii ATCC 43864	–	–
C. freundii ATCC 8090	+	–
C. koseri ATCC 27156	–	–
Enterobacter		
E. aerogenes ATCC 13048	–	–
E. aerogenes ATCC 51697	–	–
E. amnigenus ATCC 51816	–	–
E. cloacae ATCC 700323	–	–
E. cloacae ATCC 49141	–	–
E. gergoviae ATCC 35030	+	–
E. gergoviae ATCC 33028	–	–
Escherichia		
E. coli ZK2686/W3110	+	–
E. coli S17-1	+	–
E. coli DH5α	+	–
E. coli clinical isolate 1013	+	–
E. coli clinical isolate 1014	+	–
E. coli clinical isolate 1017	+	–
E. coli clinical isolate 1019	+	–
E. coli clinical isolate 1054	+	–
E. coli clinical isolate 1055	–	–
E. coli clinical isolate 1056	–	–
E. coli clinical isolate 1058	–	–
E. coli clinical isolate 1061	+	–
E. coli clinical isolate 1062	+	–
E. coli clinical isolate 1066	+	–
E. coli clinical isolate 1067	+	–
Klebsiella		
K. pneumoniae ATCC 13883	+	–
K. pneumoniae ATCC 4352	+	–
K. pneumoniae ATCC 33495	+	–
K. pneumoniae PIC 344	+	–
K. pneumoniae clinical isolate LAB 1840	+	–
K. pneumoniae clinical isolate LAB 1841	–	–

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	***E. coli* S17-pMQ348**	***E. coli* S17-pMQ124**
K. pneumoniae clinical isolate LAB 1842	–	–
K. pneumoniae clinical isolate LAB 1844	+	–
K. pneumoniae clinical isolate LAB 1963	+	–
K. pneumoniae clinical isolate LAB 1964	–	–
K. pneumoniae clinical isolate LAB 1965	–	–
K. pneumoniae clinical isolate LAB 1966	–	–
K. pneumoniae clinical isolate LAB 1967	+	–
Morganella		
M. morganii ATCC 25829	–	–
M. morganii ATCC 25830	–	–
Proteus		
P. mirabilis ATCC 35659	–	–
P. mirablis ATCC 43071	–	–
P. mirabilis ATCC 25933	–	–
P. mirabilis NCIMB 13283	–	–
P. mirabilis ATCC 7002	–	–
P. mirabilis PIC 366	–	–
P. morganii PIC 3661	–	–
P. rettgeri ATCC 9250	–	–
P. vulgaris ATCC 33420	–	–
P. vulgaris ATCC 49132	–	–
P. vulgaris ATCC 8427	–	–
P. vulgaris NCTC 4636	–	–
P. vulgaris PIC 365	–	–
Salmonella typhimurium PIC 3712	–	–
Serratia marcescens PIC 361	–	–
Shigella flexneri PIC 387	–	–
Yersinia		
Y. enterocolitica PIC 330	–	–
Y. pseudotuberculosis PIC 399	+	–

Filter-sterilized crude cell extracts from arabinose-induced *E. coli* S17-pMQ348 and *E. coli* S17-pMQ124 (empty vector control) were prepared. Twenty microliters of the crude extracts was spotted on lawns of examined bacteria (15 and 35 μg total protein from *E. coli* S17-pMQ348 and *E. coli* S17-pMQ124, respectively). Antimicrobial activity was measured by the formation of a zone of inhibition around or at the point of inoculation. (+) Positive zone of inhibition. (−) No zone of inhibition

Bacteria source: *PIC* Presque Isle Culture Collection, *ATCC* American Type Culture Collection, *LAB* lab collection