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Colicins U and Y inhibit growth of *Escherichia coli* strains via recognition of conserved OmpA extracellular loop 1

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ABSTRACT

Interactions of colicins U and Y with the OmpA (Outer membrane protein A) receptor molecule were studied using site-directed mutagenesis and colicin binding assay. A systematic mutagenesis of the colicin-susceptible OmpA sequence from *Escherichia coli* ($OmpA_{EC}$) to the colicin-resistant OmpA sequence from *Serratia marcescens* ($OmpA_{SM}$) was performed in regions corresponding to extracellular OmpA loops 1–4. Susceptibility to colicins U and Y was significantly affected by the OmpA mutation in loop 1. As with functional analysis, a decrease in binding capacity of His-tagged colicin U was found for recombinant OmpA with a mutated segment in loop 1 compared to control $OmpA_{EC}$. To verify the importance of the identified amino acid residues in OmpA loop 1, we introduced loop 1 from $OmpA_{EC}$ into $OmpA_{SM}$, which resulted in the substantial increase of susceptibility to colicins U and Y. In addition, colicins U and Y were tested against a panel of 118 bacteriocin non-producing strains of four *Escherichia* species, including *E. coli* (39 strains), *E. fergusonii* (10 strains), *E. hermannii* (42 strains), and *E. vulneris* (27 strains). A majority (82%) of *E. coli* strains was susceptible to colicins U and Y. Interestingly, colicins U and Y also inhibited all of the 30 tested multidrug-resistant *E. coli* O25b-ST131 isolates. These findings, together with the fact that OmpA loop 1 is important for bacterial virulence and is evolutionary conserved, offer the potential of using colicins U and Y as specific anti-OmpA loop 1 directed antibacterial proteins.

1. Introduction

Colicins are exoproteins released by producer bacterial strains belonging to the *Enterobacteriaceae* family. They are likely involved in competition with closely related bacteria in microbial communities inhabiting gastrointestinal tracts (Gillor et al., 2009; Gordon and Riley, 1999; Kerr et al., 2002). Moreover, colicin synthesis appears to increase the ability of producer strains to colonize the intestines and increase bacterial virulence (Azpiroz et al., 2009; Budič et al., 2011; Micenková et al., 2014b; Petkovšek et al., 2012; Šmajs et al., 2010; Štaudová et al., 2015). In general, colicins show narrow antibacterial spectra as a consequence of their interaction with a specific outer membrane receptor or receptors (Cascales et al., 2007; Šmarda and Šmajs, 1998). Some of the colicin types, including colicin U, inhibit human and animal pathogens (Bosák et al., 2012; Patton et al., 2007; Šmajs et al., 1997; Šmajs and Weinstock, 2001a,b). Colicins U and Y are pore-forming colicins closely related in terms of protein sequence (Šmajs et al., 1997; Riley et al., 2000 Riley et al., 2000).

Outer membrane protein A (OmpA) is a major surface protein in *E. coli* that plays an important role in biofilm formation, bacterial conjugation, host cell adhesion and invasion, formation of transporting pores, and multidrug resistance (Confer and Ayalew, 2012; Ma and Wood, 2009; Martinez et al., 2014; Mohan Nair and Venkitanarayanan, 2007; Smani et al., 2014; Smith et al., 2007; Zakharian and Reusch, 2005). The crystal structure of OmpA (Ishida et al., 2014; Marcoux et al., 2014; Pautsch and Schulz, 2000) has revealed two domains: a transmembrane β-barrel and a globular periplasmic C-terminal domain. The transmembrane β-barrel is composed of eight membrane-spanning anti-parallel β-strands connected to four long extracellular loops and three short turns in the periplasmic place. In addition to important cellular functions, OmpA is highly immunogenic bacterial component due to its exposed epitopes on the cell surface (Guan et al., 2015) and it has also been shown that OmpA expression is essential for *E. coli* K1 virulence (Krishnan and Prasadarao, 2014). In addition, OmpA protein also serves as a receptor for antibacterial agents including several bacteriophages and colicins (Datta et al., 1977; Foulds and

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Chai, 1978; Morona et al., 1984; Porcek and Parent, 2015; Power et al., 2006; Pugsley, 1985). OmpA is a major receptor molecule for colicins U and Y; however, it also facilitates the lethal effects of colicins K and L on susceptible bacteria, where it serves as a secondary receptor (Cascales et al., 2007; Šmarda and Šmajs, 1998).

In this communication, we showed that colicins U and Y represent colicin types that are active on most strains of *Escherichia coli*, including multidrug-resistant O25b-ST131 isolates, and that this broad activity spectrum is due to recognition of the evolutionary conserved OmpA epitope.

2. Material and methods

2.1. Bacterial strains and growth conditions

Altogether, 118 bacteriocin non-producing strains from four *Escherichia* species were tested for susceptibility to 11 pore-forming colicins. A set of 39 *E. coli* strains were collected between 2007 and 2012 from male and female patients at the Faculty Hospital Bohunice, Brno, Czech Republic (Micenková et al., 2016; Šmajs et al., 2010). Strains of *E. fergusonii* (10 strains), *E. hermannii* (42 strains), and *E. vulneris* (27 strains) were originally collected in the České Budějovice region (Czech Republic) between 2001 and 2007 from human clinical material (mostly feces). These strains were supplied by the National Reference Laboratory for *E. coli* and *Shigella*, Center of Epidemiology and Microbiology, National Institute of Public Health, Prague, Czech Republic. None of these *Escherichia* strains produced any of the 30 known bacteriocin types (Micenková et al., 2016; Šmajs et al., 2010). In addition, 30 multidrug-resistant *E. coli* isolates belonging to O25b-ST131 group, which were characterized in study Micenková et al. (2014a), were also tested for colicin U/Y-susceptibility. The study was approved by the ethics committee of the Faculty of Medicine, Masaryk University, Czech Republic. Other bacterial strains and plasmids used in this study are shown in Table 1.

Bacterial strains were cultivated at 37 °C in TY medium containing 8 g tryptone (HiMedia, Mumbai, India), 5 g yeast extract (HiMedia), and 5 g NaCl per liter (pH 7), or on solid TY medium supplemented with 1.2% (w/v) agar. Ampicillin or kanamycin (0.05 g l^{-1} or 0.1 g l^{-1}; Sigma, St. Louis, USA) was used for the selection of transformants.

2.2. Crude colicin preparation and colicin activity assay

Altogether, eleven colicin producers were used for preparation of the crude colicins from lysates (Bosák et al., 2012). Briefly, overnight TY culture of a colicinogenic strain was diluted 20-fold and incubated (37 °C; 4 h). The producer bacterial culture was then induced by mitomycin C (0.0005 g l^{-1}, Sigma), incubated for an additional 4 h and centrifuged for 15 min at 4000 × *g*. The pellet was washed twice in distilled water, resuspended in 5 ml distilled water and sonicated. The resulting bacterial lysate was centrifuged for 15 min at 4000 × *g* and the supernatant was used as a crude colicin preparation.

Antibacterial colicin activity was tested by spotting 10-fold serial dilutions of crude colicins on agar plates inoculated with an indicator strain (i.e., with a strain from the set of 118 *Escherichia* strains used in this study). The indicator bacteria (≈10^8 cells) were added to 3 ml of 0.75% (w/v) TY agar and poured on a TY plate. The reciprocal highest 10-fold dilution of the crude colicin causing growth inhibition of susceptible bacteria was considered to be the colicin titer (in arbitrary units; A.U.). The data represent the average of at least three independent assays.

2.3. Heterologous expression of ompA variants

DNA regions containing *ompA* sequences of eleven different bacteria were amplified from a single bacterial colony resuspended in 100 μl of sterile water. Bacterial suspensions (1 μl) were used as a DNA template, which was amplified using *Taq* polymerase (New England BioLabs, Beverly, USA) and specific primers (Table S1). PCR started with denaturation at 94 °C for 5 min, which was followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and finished by extension at 72 °C for 10 min. PCR products were immediately cloned using a TOPO TA Cloning Kit (Life Technologies; Carlsbad, USA) according to the manufacturer's instructions. All constructs were verified by sequencing to avoid possible errors introduced by *Taq* polymerase. Several parallel clones were sequenced and tested for susceptibility to colicins.

2.4. Site-directed mutagenesis

Site-directed mutagenesis used PCR amplification with primers containing defined mutations (Table S1). The sequences of *ompA* genes from a susceptible strain of *E. coli* and a resistant strain of *Serratia marcescens* (*S. marcescens*) were analyzed using Lasergene software (DNASTAR, Inc., Madison, USA); pairs of complementary primers were designed for the heterologous parts. PCR products were amplified in low-stringency conditions using 1 μl of *Pfu* Turbo polymerase (Fermentas, Glen Burnie, USA). Plasmid encoding the OmpA from a susceptible *E. coli* (pDS1104) was used as the template DNA. PCR mutagenesis started with denaturation at 94 °C for 2 min, followed by 20 cycles at 94 °C for 30 s, 37 °C for 1 min, 68 °C for 12 min, and finished by extension at 68 °C for 20 min. After PCR amplifications, the template DNA was degraded with the *Dpn*I enzyme (New England Biolabs). Constructs were transformed using the bacterial strain *E. coli* BL21 *ompA*$^-$, which contained a frameshift mutation (deletion of 5 nucleotides CATGG) between coordinates 360 and 364 of the *ompA* gene. For protein analysis of recombinant OmpA molecules, nucleotide sequence encoding C-terminal 6 × His-Tag was added to the recombinant *ompA* sequences using an In-fusion® HD Cloning Kit (Clontech, Mountain View, USA). The prepared plasmid constructs are listed in Tables 1 and S1.

2.5. Quantitative real-time PCR

For analysis of expression at the mRNA level, total mRNA was isolated from bacterial cultures (containing ≈10^8 cells) using RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) and a RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Total RNA (1 μg) was used for reverse transcription with random primers (New England BioLabs) and SuperScript III Reverse Transcriptase (Life Technologies) in accordance with manufacturer's instructions. Samples of cDNA were prepared from independent biological duplicates. After transcription, real-time PCR with specific primers (Table S1) and FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland) was performed in technical triplicates. For the relative quantification of expression of *ompA*, the Pfaffl method (Pfaffl, 2001) and the housekeeping gene *idnT* (Zhou et al., 2011) were used.

2.6. Separation of membrane fractions and western blot analysis

For analysis of *ompA* expression at the protein level, membrane fractions from bacterial suspensions were separated, as was previously published (Laskowska et al., 2004; Marani et al., 2006). Briefly, overnight bacterial suspensions synthesizing His-tagged OmpAs (pDS1105–pDS1116) were diluted 20-times and cultivated (37 °C, 200 rpm, 6 h) to an OD_{600} of 0.5–0.6. Bacterial suspensions (100 ml) were centrifuged (30 min; 4000 × *g*), resus-