Journal of Microbiological Methods xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Attenuated colicin-based screening to discover and create novel resistance genes

Kazuki Futai *

Life Science Center of Tsukuba Advanced Research Alliance, University of Tsukuba, Japan

ARTICLE INFO

Article history:
Received 25 December 2013
Received in revised form 6 March 2014
Accepted 10 March 2014
Available online xxxx

Keywords:
Resistance-associated gene screening
Attenuated colicin mutant
rstA
Fused gene
Directed evolution

ABSTRACT

I formulated a systematic approach to screen for colicin resistance-associated genes by using an attenuated colicin mutant and demonstrated its utility in a screen of genes related to colicin E5 resistance. Screening of an *Escherichia coli* genome library revealed *rstA* as a partial resistance gene to colicin E5. Transcript expression of BtuB and OmpF, proteins responsible for translocation of nuclease E colicins, was clearly inhibited in an *rstA*-overexpressing strain. In addition, the *tatA*::*recN* fusion gene provides resistance to an attenuated colicin E5 mutant. Improving *tatA*::*recN* by directed coevolution, I created a novel gene with enhanced resistance to colicin E5 and concluded that attenuated colicin-based screening is useful for the discovery and creation of novel colicin resistance-associated genes.

1. Introduction

Colicins are toxic proteins encoded on the colicinogenic plasmid pCol and are produced by *Escherichia coli* harboring pCol under stresses such as nutrient depletion and overcrowding (Cascales et al., 2007; Sharma et al., 2009). Colicins, through various modes of action, induce cell death in *E. coli* that are competent for colicin translocation, but do not harbor the corresponding immunity protein (Cascales et al., 2007). Colicinogenic *E. coli* co-express colicin and the corresponding immunity protein and thus escape colicin-induced suicide (Cascales et al., 2007). The first step of colicin invasion involves colicin binding to a cell surface receptor. The group E colicins, which bind to the vitamin B_{12} transporter BtuB, include 10 kinds of colicins (colicin A, E1 to E9) (Cascales et al., 2007; Di Masi et al., 1973). Colicin A and E1 produce a pore in the inner membrane that permits ion leakage. Colicins E2, E7, E8, and E9 randomly degrade DNA. Colicins E3, E4, and E6 cleave the 3′-end loop of 16S rRNA. Colicin E5 cleaves the anticodon loops of 4 tRNAs ($tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$) (Cascales et al., 2007; Ogawa et al., 1999). After binding to the BtuB receptor, unstructured N-terminal domain of these nuclease type E colicins (E2 to E9) threads through the outer membrane via OmpF or OmpC and capture TolB on the other side of the outer membrane for triggering colicin import (Housden et al., 2013). Colicin binding to TolB promotes the interaction of TolB with TolA, giving the colicin access to the inner membrane potential to drive entry of the C-terminal nuclease domain into the cytoplasm (Housden et al., 2013). After cytoplasmic entry, the C-terminal nuclease domain degrades its target nucleic acid (Cascales et al., 2007; Kleanthous, 2010).

Natural colicin-resistant *E. coli* strains have been identified (Cascales et al., 2007). Colicin resistance is genetically encoded; the immunity gene, which encodes an immunity protein, is the most remarkable example (Cascales et al., 2007). Genes encoding colicin receptor proteins (e.g., *btuB*) were identified by their phenotypes: suppression of these genes produces colicin resistance (Cascales et al., 2007; Di Masi et al., 1973). Other genes provide partial resistance (e.g., *gadX* and *gadY*) (Lei et al., 2011). In some cases, study of resistance-associated genes has revealed general cellular mechanisms; for example, translocation of the nuclease type E colicins is considered a model of protein translocation through the cell membrane (Sharma et al., 2009). The study of *btuB* and transporter BtuB has revealed the mechanism of nuclease type E colicin translocation (Cascales et al., 2007; Di Masi et al., 1973). The study of *gadX* and *gadY* revealed that GadX is a transcriptional inhibitor of *btuB* and a novel gene network in *E. coli* (Lei et al., 2011). An *E. coli*-based gene library, e.g., single-gene knockout or overexpression strains can be screened for colicin resistance to identify some, but not all, resistance-associated genes. For example, deletion of *lon*, which encodes an ATP-dependent protease, confers partial resistance to colicin E7 (Lee et al., 2006); however, a comprehensive genetic screen for colicin resistance in a single-gene knockout library of the entire *E. coli* genome did not yield *lon* (Sharma et al., 2009). Identification of genes that confer partial resistance is limited by screening conditions. Thus, to capture a greater diversity of colicin resistance genes, screening systems must be sufficiently broad to identify partial resistance genes.

* Center for Life Science Technologies, Yokohama Institute, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. Tel.: +81 45 503 9644.
E-mail address: futaikazuki@gmail.com.

http://dx.doi.org/10.1016/j.mimet.2014.03.003
0167-7012/

The lethality of colicin is so strong [a single molecule is thought to be sufficient to induce cell death (Shannon and Hedges, 1967)], current screening systems cannot identify all resistance-associated genes. To screen a greater diversity of colicin resistance-associated genes, I proposed to use an attenuated colicin derivative (Inoue-Ito et al., 2012). Genes that confer resistance to this attenuated colicin could then be screened for partial resistance to wild-type colicin and subsequently be used to elucidate novel cellular mechanisms. Furthermore, some genes could be improved by directed evolution to derive novel genes with enhanced resistance to wild-type colicin (Arnold, 1998).

To determine the utility of attenuated colicin for comprehensive screening of genes related to colicin resistance, I chose colicin E5 as a representative colicin and an attenuated E5 mutant (K60Q), which bears an amino acid mutation at the active site residue of colicin E5 C-terminal ribonuclease domain (E5-CRD) and thus invades to *E. coli* cell in the same manner as wild-type E5 (Inoue-Ito et al., 2012; Yajima et al., 2006). Transformants bearing plasmids containing *E. coli* genome fragments were incubated with the attenuated colicin E5 derivative. By sequencing the plasmids from surviving cells, genes that confer resistance to the attenuated E5 derivative were identified. Further study revealed that one of these genes is associated with resistance to wild-type E5, and this resistance is caused by a novel genetic network between the identified gene and genes encoding proteins used for invasion of colicin E5. By using the screened gene as material for directed evolution, I created a novel gene with enhanced resistance to wild-type E5.

2. Materials and methods

2.1. Purification of colicin/immunity protein complexes

Plasmids for expressing colicin E5 derivatives (colicin E5K60Q and E5I94M) were constructed from the wild-type ColE5 plasmid pKF601, which carries the colicin promoter, gene, and corresponding immunity protein (Im5)-encoding gene (Inoue-Ito et al., 2012; Yajima et al., 2006). Colicin E5K60Q bears a Lys-60 of E5-CRD to Gln mutation and its cytotoxicity is about 1/16–1/64 the cytotoxicity of colicin E5 (Inoue-Ito et al., 2012; Yajima et al., 2006). Colicin E5I94M bears an Ile-94 of E5-CRD to Met mutation and its cytotoxicity is about 1/4–1/16 the cytotoxicity of colicin E5 (Fig. 1). These plasmids were constructed using the QuickChange mutagenesis kit (Stratagene) and the mutation-generating primer pairs (Table 1). *E. coli* RR1 cells were transformed with the mutant or wild-type ColE5 plasmid. Overnight transformant cultures were diluted 100-fold in L-broth consisting of 10 g tryptone (Becton, Dickinson and Company), 5 g yeast extract (Becton, Dickinson and Company), and 5 g NaCl per liter, adjusted to pH 7. When the culture density (OD_{660}) reached 0.7, expression of colicin/immunity protein complexes was induced by mitomycin C 0.4 mg/L. The cultures were incubated for an additional 3 h at 37 °C. Each transformant culture was harvested by centrifugation and resuspended in 40 mL 20 mM Tris–HCl (pH 8.0) followed by sonication and centrifugation at 15,000 rpm for 30 min. The supernatant was applied to a DEAE-TOYOPEARL 650S (Tosoh) column and eluted with a KCl gradient from 0 to 500 mM in 20 mM Tris–HCl (pH 8.0). The colicin/immunity protein complex fraction was applied to a Mono S column (Amersham-Pharmacia Biotech) and eluted with a KCl gradient from 0 to 500 mM in 20 mM sodium acetate buffer (pH 6.0). Purified proteins were analyzed by SDS-PAGE, and concentrated to 2.5 μg/mL. Copurified immunity proteins are removed from colicin during the infectious event and do not inhibit colicin activity (Cascales et al., 2007). The cytotoxicity of colicin E5K60Q, E5I94M, and E5 was determined by the spot test (Masaki and Ohta, 1985). Colicins were serially diluted (1:4) from a starting concentration of 2.5 μg/mL with L-broth; 5 μL of each dilution

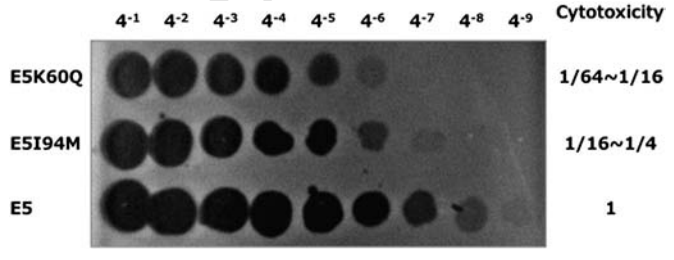

Fig. 1. Cytotoxicity of wild-type colicin E5 and attenuated mutants E5K60Q and E5194M, Each colicin was serially diluted (1:4) from a starting concentration of 2.5 μg/mL with L-broth an 5 μL of each dilution was applied to a soft agar surface of sensitive indicator strain (DH5a).

Table 1
Oligonucleotide sequences[a].

Oligonucleotide	Sequence (5′–3′)
Colicin E5 mutation–generation	
K60Q(+)	GTAGTCCGAAG**CAA**ACACCTCCAGAC
K60Q(−)	GTCTGGAGGTGT**TTG**CTTCGGACTAC
I94(+)	GGTGAGGTTACTCAG**NNS**AGTGATAAGACA GATCC
I94(−)	GGATCTGTCTTATCACT**SNN**CTGAGTAACCTC ACC
Drug resistance genes amplification	
Kanamycin F	CCTGCAGGCCCTGGGGGGAAAGCCACGTTGTG
Kanamycin R	TGCAGGGCCTGGGGGGCGCTGAGGTCTG
Chloramphenicol-1F	GCAGAAGGACCAAATCCTGGTGTCCCTGTTGA TAC
Chloramphenicol-2R	CATTGCCATACGAAACTCCGGATGAGC
Chloramphenicol-2F	GCTCATCCGGAGTTTCGTATGGCAATG
Chloramphenicol-3R	TGCGTATAATATTTGCCCATAGTGAAAACGGG
Chloramphenicol-3F	CCCGTTTTCACTATGGGCAAATATTATACGCA
Chloramphenicol-4R	AACGGGTCCACCGTTTTTATCAGGCTCTGG
rstA and *glpM* mutation–generation	
Δ*rstA*	TTTATATCTACCGTGT**AATAATAA**AACACTATC GTATTTG
Δ*glpM*	TTAAGGTAAAAAGATG**TAATAA**GTAATCAAAGC CGC
Screened genes amplification	
*rstA*F	TCTACCATGGATGTTATGAACACTATCGTATT TGTG
*rstA*R	TTTGGATCCTTATTCCCATGCATGAGGCG
*argR*F	GGGTGACCCATGGGAAGCTCGGCTAAG
*argR*R	GACGGGGGGATCCTTAAAGCTCCTGGTCG
*yhcN*F	TGTGCCATGGGTCACGAAACAAAGGC
*yhcN*R	CGATGGATCCTTATTTGTACAGTTCAGCCGTAGC
tatA::*recN*F	ACATCCATGGGTGGTATCAGTATTTGGCAG
tatA::*recN*R	GCAGGAAGGATCCTTACGCTGCAAGCAG
Real-time PCR	
16S rRNA-F	CCACGGAAGTTTTCAGAGATGAG
16S rRNA-R	ACCGCTGGCAACAAAGGA
btuB-F	TTCCCTATTGCGCTTGTCC
btuB-R	TCCCCACCCTGCTGAAA
ompF-F	TCGCCAACAAAACGCAAG
ompF-R	CACCAGATCAACATCACCGATAC
ompC-F	AGGTTTCGGTATCGGTGGTG
ompC-R	GAGCAGCCAGGTAGATGTTGTTAG
ompR-F	GCTGACGACTACATTCCAAAACC
ompR-R	AAAGCAATTACCGCCTCTTCC
tolA-F	GATCTGGAGTTCGTTCGATGAG
tolA-R	CGCTTGATTCCTGGCTTTG
tolB-F	AAACTGACCGGCATTAAAGGTG
tolB-R	GGTGAACGGTGAACGACAAA
tolQ-F	TCGGGGCGGTAAAACAAG
tolQ-R	TGGTTGAGGCGGTTGTAGG
tolR-F	GCCAGTGATTGTTGAAGTGTCTG
tolR-R	TCGGGTTGGCCTTGAAAC
tatA and *recN* amplification	
tatA-F	ACATCCATGGGTGGTATCAGTATTTGGCAG
tatA-R	AAAGGATCCTTACACCTGCTCTTTATCGTGGCG
recN-F	CGATCATGATGGCACAACTGACCATCA
recN-R	GCAGGAAGGATCCTTACGCTGCAAGCAG
tatA::*recN* ΔC-terminal amplification	
tatA N-F	ACATCCATGGGTGGTATCAGTATTTGGCAG
tatA N-R	GTTGGATCCTTAATCGGAACCGATGGAG
recN C-F	ATCGGTTCCATGGTCGATCCCAACCG
recN C-R	GCAGGAAGGATCCTTACGCTGCAAGCAG

[a] Bold letters show the nucleotides used to introduce the mutations.