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A simple and highly efficient method for gene silencing in *Escherichia coli*

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ABSTRACT

Here we present a simple and rapidly achievable protocol for gene silencing in *Escherichia coli* (*E. coli*). In this procedure, antisense RNA (asRNA) of 400-nucleotides (nt) length and absolute complementarity to the target is produced by an expression plasmid. The designed asRNA should ideally cover at least the −10 site of the promoter and the Shine-Dalgarno sequence, and additional 300-bp of the following open reading frame of the target gene. We show that the transcription process of the target is not affected at all, whereas the translation process is impaired. Based on high constitutive expression of asRNA we were able to extend the silencing effect to knock-out levels. By inducible expression, we show that also the modulation is possible. This technique should be widely useful to study gene function in *E. coli* and other bacteria.

1. Introduction

RNA interference (RNAi) via antisense RNA (asRNA) is a cornerstone for controlling the genetic flow in eukaryotic and prokaryotic cells (Khorkova et al., 2014; Saberi et al., 2016; Wagner et al., 2002). Since its discovery, RNAi has become a laboratory standard tool for the modulation of gene function in eukaryotic cell models (Bartel, 2004; Fire et al., 1998; Gregory et al., 2004; Sontheimer, 2005). The modes of action include the interference with transcription, translation and the induction of rapid degradation. Although the presence of asRNAs was firstly documented in bacteria in 1967, its importance remained unappreciated until recent years (Hindley, 1967). One of the first naturally occurring asRNAs was the *micF* RNA identified in *Escherichia coli* and other *Enterobacteriaceae*. The stress response gene *micF* encodes a 93 nt-long asRNA, which impairs expression of the outer membrane protein OmpF by binding *ompF* messenger RNA (mRNA) resulting in both inhibition of translation and degradation (Delihas and Forst, 2001; Green et al., 1986; Guillier et al., 2006). Interest in the field of antisense regulation in bacteria, however, has to be considered rather low in the following period, as by 2001 only twelve chromosomally encoded small RNAs were identified (Saberi et al., 2016). Emerging technologies like RNA-seq have attracted renewed interest and led to the identification of a plethora of regulatory mechanisms involving asRNAs in bacteria.

RNAi as a controlled instrument to modulate gene function as it is established in eukaryotic systems has long been considered impossible in bacteria due to the lack of a machinery similar to the RNA-induced silencing complex (RISC) as found in eukaryotic cells (Hannon and Rossi, 2004). Currently, most of the available protocols to study gene function in bacteria rely on various mutagenesis techniques. Since the discovery of the function of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), which codes for an adaptive immune system in bacteria and archaea (Deltcheva et al., 2011; Ishino et al., 1987; Jinek et al., 2012), it has been widely adopted as a novel tool for targeted genome editing and gene silencing in prokaryotic and eukaryotic cell systems as well (Cho et al., 2013; Cong et al., 2013; Hwang et al., 2013; Jiang et al., 2013; Mali et al., 2013; Qi et al., 2013). For example, by introducing *Cas9* derived from *Streptococcus pyogenes* and an engineered small guide RNA (sgRNA) into the cell system of interest, a versatile toolbox for genomic editing, transcriptional modulation, RNA targeting, and imaging is available (Jiang and Marraffini, 2015; Sternberg and Doudna, 2015). Nevertheless, deciphering the biological function of genes always requires the manipulation of the genetic background of the target organism, either by mutagenesis techniques or the acquisition of various elements that will interfere with cellular processes. These approaches always inherit the risk of off-target effects, genomic rearrangements or polar effects.

Here we describe a simple procedure for gene silencing in the model organism *E. coli*. The approach is based on the concept that the expression of asRNA of absolute complementarity to the target mRNA will specifically knock-down gene function. We determined the length of the asRNA and the critical genetic sites to be addressed for efficient gene silencing. We used either constitutive or inducible expression to evaluate the option to modulate the silencing effect.

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Table 1
Bacterial strains and plasmids.

	Relevant characteristics	Reference
Strains and plasmids		
NU14 wt	O18: K1: H7; cystitis isolate	(Hultgren et al., 1986)
NU14 Δ*ybtA*	*ybtA* deletion mutant	This study
NU14 Δ*ybtA* rec	Complemented mutant, pWKS30-P*ybtA*; Ap	This study
NU14 yRNAi	Wildtype, pHCE-RNAi for *ybtA*; Ap	This study
NU14 vc	Control strain; harbouring empty plasmids pHCE or pTrc99A	This study
NU14 Δ*galU*	*galU* deletion mutant	This study
NU14 Δ*galU* rec	Complemented mutant, pWKS30-P*galU*; Ap	This study
NU14 RNAi-*galU*	Wildtype, pHCE-RNAi for *galU*; Ap	This study
Plasmids		
pKD4	Kanamycin template plasmid	(Datsenko and Wanner, 2000)
pKD46	Lambda red recombinase helper plasmid	(Datsenko and Wanner, 2000)
pCP20	FLP recombinase helper plasmid	(Datsenko and Wanner, 2000)
pWKS30	Low-copy plasmid; Ap	(Wang and Kushner, 1991)
pTrc99A	Plasmid for inducible expression using IPTG; Ap	Pharmacia
pHCE	Plasmid for high constitutive expression; Ap	(Poo et al., 2002)
pWKS30-P*ybtA*	Expressing YbtA under the control of its native promoter; Ap	This study
pRNAi 1	pHCE expressing constituitively *ybtA* antisense RNA; Ap	This study
pRNAi 2	pTrc99A with inducible expression of *ybtA* antisense RNA; Ap	This study
pWKS30-P*galU*	Expressing galU under the control of its native promoter; Ap	This study
pRNAi-*galU*	pHCE expressing constituitively *ybtA* antisense RNA; Ap	This study

2. Material and methods

2.1. Bacterial strains and media

Bacterial strains and plasmids of this study are listed in Table 1. The uropathogenic *E. coli* (UPEC) strain NU14 was isolated from a patient with symptomatic cystitis (Hultgren et al., 1986). Bacteria were cultivated in lysogeny-broth (LB) medium and NBD medium [nutrient broth (NB) supplemented with 200 μM α,α'-dipyridyl (Sigma)]. NBD was used for experiments under iron deplete conditions. Use of antibiotics was provided as necessary [kanamycin 25 μg/ml (Km), ampicillin 100 μg/ml (Ap)].

2.2. Motility assays

Swimming motility was evaluated by using 0.3% LB soft agar plates. A late logarithmic phase culture was adjusted to an $OD_{600} = 1.0$ and standardised samples were stabbed into the middle of a soft agar plate and incubated at 37 °C. Motility was analysed after 8 h by measuring the diameter of motile bacteria. All experiments were performed in duplicates and repeated at least three times. For statistical analysis a paired *t*-test or the Mann-Whitney *U* test were performed and results were considered statistically significant if the *p*-value was lower than 0.05.

2.3. Construction of isogenic mutants

The isogenic deletion mutant NU14 Δ*ybtA* was generated according to the published protocol by Datsenko and Wanner (Datsenko and Wanner, 2000). Primers with 40-nucleotides (nt) homology extensions to the 5′- and 3′- sites of *ybtA* and 20-nt priming sequences for the template plasmids pKD4 carrying a resistance cassette flanked by FRT recognition target sites were designed (Table 2). The resulting PCR product was transformed into the wild-type strain harbouring the helper plasmid pKD46 with the lambda red recombinase under an arabinose-inducible promoter. In case of successful replacement, Km^R transformants were selected and the correct integration of the resistance cassette was confirmed by PCR.

2.4. Cloning and recombinant DNA techniques

Standard genetic techniques were used mainly as described by Sambrook and Russell (Sambrook, 2001). Enzymes were purchased from Fermentas Thermo Fisher Scientific (St. Leon-Rot, Germany) and used according to the manufacturer's protocol. Primers and plasmids used in this study are listed in Table 2. The plasmid pWKS30-P*ybtA* for complementation of the mutant strain was constructed by PCR amplification of the wild-type gene under the control of its own promoter. The PCR product was purified using a QIAquick PCR purification kit (Qiagen), digested with *Kpn*I and *Pst*I and cloned into low-copy plasmid pWKS30 (Wang and Kushner, 1991). For the construction of the plasmids pRNAi 1 and 2, both producing antisense RNA for gene silencing of *ybtA*, a 400-nt long fragment starting from the −10 region of the respective promoter was amplified and digested with *Bam*HI and *Sal*I (Fig. 1). The product was cloned into high constitutive expression plasmid pHCE (Poo et al., 2002), resulting in plasmid pRNAi 1. The vector pRNAi 2 is equipped with an IPTG (isopropyl-β-d-thiogalactopyranoside)-inducible *trc* promoter of plasmid pTrc99A. In both plasmids transcription of antisense RNA starts from the original 3'region of *ybtA*. This results in production of antisense RNA that is complementary to the region upstream of the gene of interest and the first 300-nt of the open reading frame of *ybtA*. Hybridization of the antisense RNA to the upstream region of *ybtA* assures covering of the Shine-Dalgarno sequence, which has been shown to be highly sensitive to antisense RNA (Dryselius et al., 2003), and it is supposed to inhibit translation of *ybtA* messenger RNA.

2.5. Western blotting

For detection of protein expression bacterial cultures grown in NBD were collected, corrected to an $OD_{600} = 1.0$ and finally analysed using rabbit polyclonal antiserum to YbtA (kind gift of A. Rakin) and FyuA (Feldmann et al., 2007). For loading control samples were loaded on a 10% SDS-PAGE and stained with Coomassie-Brilliant-Blue.

2.6. RNA extraction and quantitative real-time PCR (TaqMan)

RNA extraction was performed according to the Trizol (Invitrogen) method (Fu et al., 2013). Total RNA was first treated with DNase I (Fermentas) to remove contaminating genomic DNA. Then, first-strand cDNA was generated using random hexamers and RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas). PCR reactions were carried out in a final volume of 25 μl containing TaqMan Gene Expression Master Mix (Applied Biosystems), 30 ng of cDNA, primers, and probes.

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