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ORIGINAL RESEARCH PAPER

Gene-specific silencing induced by parallel complementary RNA in *Pseudomonas aeruginosa*

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Abstract To investigate whether parallel complementary RNA (pRNA) could induce gene-specific silencing in *Pseudomonas aeruginosa*, pRNA of the *mexA* gene was expressed in it. Compared to the control strains, the strain expressing pRNA of *mexA* showed a 50% decrease in minimum inhibitory concentrations (MICs) of several antimicrobial agents and a twofold increase in the initial accumulation rate of ethidium bromide, all of which are substrates of the MexAB-OprM efflux pump. These results suggest that gene-specific silencing was induced by pRNA. This is the first time that such a route for gene silencing has been reported in a bacterium other than *Escherichia coli*. Gene-specific silencing induced by pRNA may be useful as a novel biotechnology tool for gene regulation in prokaryotes.

Keywords Efflux pump · Gene-specific silencing · *mexA* · Parallel complementary RNA · *Pseudomonas aeruginosa*

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Introduction

Studies on gene-specific silencing by RNA have made great progress in elucidating both the mechanisms and potential applications of this technology (Win et al. 2007) since RNAi (gene silencing by double-stranded RNA) was discovered in *Caenorhabditis elegans* (Fire et al. 1998). Although this phenomenon has now been described for many different eukaryotes, the existence of RNAi in prokaryotes is still a hypothesis based on computational analysis (Makarova et al. 2006).

The discovery of gene-specific silencing, induced by parallel complementary RNA (pRNA) in *Escherichia coli*, revealed a novel route of gene regulation by RNA (Tchurikov et al. 2000). In *E. coli*, pRNA could form parallel RNA–RNA duplexes with mRNA of the *lon* gene to repress *lon* gene expression (Ponomarenko et al. 1992; Tchurikov et al. 1995, 2000).

If gene-specific silencing induced by pRNA could be proven to work in other prokaryotes besides *E. coli*, it would be a novel tool for regulating gene expression in prokaryotes, with many potential applications in biotechnology. We assayed a construct expressing pRNA of the *mexA* gene, which encodes the membrane fusion protein component of the MexAB-OprM multidrug efflux pump in the bacterium *Pseudomonas aeruginosa* (Akama et al. 2004; Nehme et al. 2004). We found that the efflux function of MexAB-OprM was inhibited in *P. aeruginosa* cells expressing pRNA of *mexA* (parmexA RNA).

Materials and methods

Bacterial strains and plasmids

Pseudomonas aeruginosa K854 is a MexR$^-$ and spontaneous streptomycin-resistant derivative of PAO1 wild type (Poole et al. 1996). The *mexR* gene, which encodes the repressor protein of the *mexAB-oprM* operon, is knocked out in K854 cells, leading to higher expression of the MexAB-OprM efflux pump (Poole et al. 1996).

Plasmid pAK1900-parmexA was constructed to express the pRNA of *mexA* (parmexA pRNA). Plasmid pAK1900-mirmexA, which produces an RNA transcript unable to form either an antiparallel or parallel duplex with the mRNA of *mexA* (see Fig. 1b), was constructed as a control. The parmexA DNA sequence used to express parmexA pRNA corresponded to the entire *mexA* gene and 278 bp upstream containing the *mexA* 5′-UTR. Restriction enzyme sites were added at the upstream end of the cloned sequence (*Hind*III, *Sal*I) and at the downstream end (*Sph*I, *Bam*HI). The *Hind*III-*Bam*HI fragment was cloned into pAK1900 to construct pAK1900-parmexA, and the *Sal*I-*Sph*I fragment was cloned in the opposite orientation of parmexA to construct pAK1900-mirmexA (mirmexA refers to "mirror symmetry sequence of *mexA*") (shown in Fig. 1a). The plasmids pAK1900-parmexA and pAK1900-mirmexA were screened in *E. coli* DH5α by β-galactosidase assays. All three plasmids, pAK1900-parmexA, pAK1900-mirmexA and pAK1900, were transformed into K854 by electroporation.

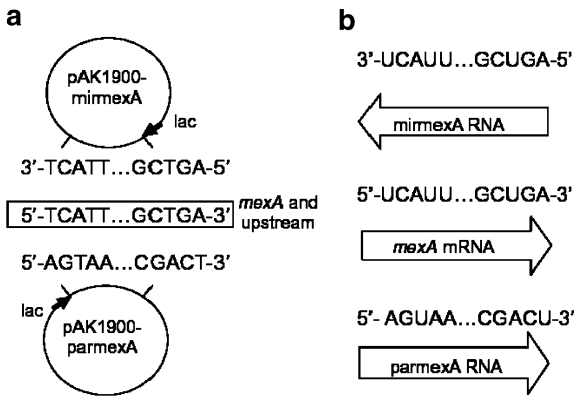

Fig. 1 Plasmids constructed and the relationship between *mexA* mRNA and RNAs expressed by the constructs. **a** The relationship between the *mexA* gene (with 278 bp upstream) and the synthesized DNA inserted in both orientations downstream of the *lac* promoter in pAK1900. The sense strands are shown with 5 bases at both ends. **b** The transcripts of *mexA* and the synthesized DNA in both orientations. In the *mexA* mRNA the corresponding region possesses the 278 bases upstream of the transcription start site plus the entire coding region. The parallel complementary relationship between *mexA* mRNA and parmexA RNA is shown, as well as the mirror symmetry relationship between *mexA* mRNA and mirmexA RNA

Antimicrobial susceptibility testing

The susceptibility of different *P. aeruginosa* transformants to specific antimicrobial agents (all substrates of the MexAB-OprM efflux pump) was tested by the spectrophotometric twofold dilution method as described by Devienne et al. (2002). Carbenicillin was added at 200 μg/ml to maintain the plasmids in the cells, since K854 lacking pAK1900-parmexA would be expected to grow faster in the presence of antimicrobial agents. Six parallel experiments were performed for each antimicrobial agent. Further details are shown in Table 1.

Calibration curve for intracellular concentration of ethidium bromide and fluorescence intensity

A calibration curve to determine the intracellular concentration of ethidium bromide (EB) from measured fluorescence intensity was generated as described by Narita et al. (2003). K854 cells at $OD_{650} = 0.3$ in MH broth were permeabilized with 1 mM EDTA at 80°C for 1 h, then mixed with EB at (up to 9 μM) and incubated at 24°C for 2 h. The fluorescence intensity of each concentration of EB was measured with a microplate reader at 30°C when excitation and emission were at 520 nm and 590 nm, respectively, and eight parallel experiments were performed for each concentration.

EB transport assay

EB transport was tested as described in previous studies (Narita et al. 2003). K854 cells were transformed with pAK1900-parmexA, pAK1900-mirmexA or pAK1900, and transformants were treated with 1 mM EDTA. EB was added to appropriate final concentrations (10 to 100 μM), and fluorescence intensity was measured consecutively. The assay was

Table 1 Susceptibility of *P. aeruginosa* transformants to various antimicrobial agents

Antimicrobial agent	MIC (μg/ml)*		
	pAK1900-parmexA	pAK1900-mirmexA	pAK1900
Ciprofloxacin	0.64	1.28	1.28
Sparfloxacin	0.8	1.6	1.6
Azithromycin	32	64	64
Tetracycline	40	80	80
Chloramphenicol	400	800	800

MIC minimum inhibitory concentration

The MIC was tested by inoculating 1 ml cultures of MH broth containing serial twofold dilutions of each antimicrobial agent with 5×10^6 organisms and 200 μg carbenicillin/ml. The OD_{650} of each culture was determined after incubation for 18 h at 37°C. OD was plotted versus concentration of each antimicrobial agent, and a curve was derived (see Supplementary Fig. 1). The MIC was defined as the inflection point to the terminal plateau. Six parallel experiments were performed for each antimicrobial agent

repeated eight times for each concentration of EB. Further details are shown in Fig. 2.

Results and discussion

Antimicrobial susceptibility of transformants of K854

The MexAB-OprM efflux pump functions to eliminate various antimicrobial agents from *P. aeruginosa* cells, including quinolones, macrolides, tetracyclines, β-lactams, and others, such as chloramphenicol (Masuda et al. 2000). Since the plasmid pAK1900 carries a β-lactamase gene, we tested the susceptibility of pAK1900-parmexA, pAK1900-mirmexA, and pAK1900 transformants of K854 to ciprofloxacin and sparfloxacin (quinolones), azithromycin (macrolides), tetracycline, and chloramphenicol. The minimum inhibitory concentrations (MICs) of antimicrobial agents are listed in Table 1 (further details are shown in Supplementary Fig. 1). For all five antimicrobial agents tested, pAK1900-parmexA transformants showed a 50% decrease in MICs compared with the two control transformant plasmid types, which suggested a decrease in the level of activity of the MexAB-OprM efflux pump. Results showed there was no significant difference between pAK1900-mirmexA transformants and the blank plasmid control pAK1900 transformants in the level of MICs, indicating that the higher load of plasmid insert DNA in a cell did not affect the results.

Measurement of EB accumulation rate in transformants of K854

The efflux activity of the MexAB-OprM efflux pump was monitored by determining the intracellular concentration of EB, which is a substrate of the MexAB-OprM efflux pump (Ocaktan et al. 1997). To measure the intracellular concentration of EB, K854 cells were permeabilized, and the fluorescence intensity of EB bound to nucleic acid was determined at equilibrium. A cubic equation, $y = 23.169x^3 - 473x^2 + 4293.5x + 1167.6$, was derived from the calibration curve for intracellular concentration of EB and fluorescence intensity, with an R^2 (coefficient of determination) of 0.9956 (the calibration curve is shown in Supplementary Fig. 2). The relative intensity value was shown as the fluorescence intensity. The intracellular concentration of EB corresponding to a given fluorescence intensity was calculated according to this equation.

Accumulation rates of EB in pAK1900-parmexA, pAK1900-mirmexA and pAK1900 transformants of K854 were measured by determining the fluorescence intensities of a series of concentrations of EB added to the reaction system. Since the curve of correlation equation for intracellular concentration of EB and time in each group appeared parabolic as measurement went on, especially when high concentrations of EB were added into the reaction system, quadratic equations were derived. The correlation equations for intracellular concentration of EB and time under different conditions (different transformants and

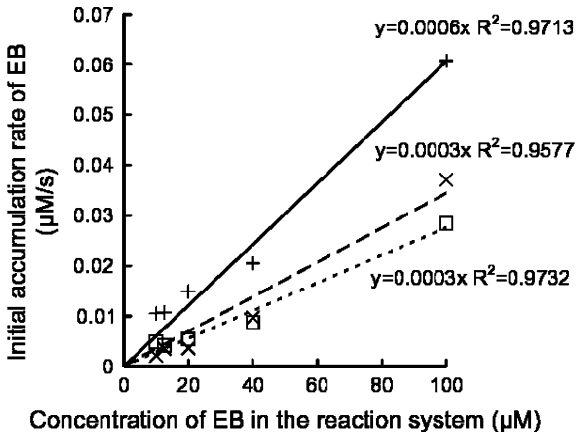

Fig. 2 Correlation curves for the initial accumulation rate of EB and the concentration of EB in the reaction system. Overnight cultures of pAK1900-parmexA, pAK1900-mirmexA and pAK1900 transformants of K854 were diluted 100-fold with MH broth containing 200 μg/ml carbenicillin and incubated at 37°C while rotating at 200 rpm. Cells were harvested by centrifugation when A_{650nm} was 1.0, diluted with MH broth to adjust the A_{650nm} to 0.3, and then permeabilized with 1 mM EDTA. After an incubation for 5 min at 30°C, EB was added to appropriate final concentrations (10, 12.5, 20, 40, 100 μM), and fluorescence intensity was measured at 30°C. Excitation and emission were at 520 nm and 590 nm, respectively. The first measurement was considered to be time 0, with fluorescence intensities subsequently determined every 4 s up to 56 s. All values were converted into intracellular concentrations of EB by the calibration curve equation. Correlation equations for intracellular concentration of EB and time under different conditions were then derived (further details are shown in Supplementary Table 1). The assay was repeated eight times for each concentration of EB. The derivative of a correlation equation for intracellular concentration of EB and time at time 0 ($x = 0$) was considered as the initial accumulation rate of EB under the corresponding condition. If no EB is added to the reaction system, the accumulation rate should be 0. The initial accumulation rates of EB in the pAK1900-parmexA (+), pAK1900-mirmexA (×), and pAK1900 (□) transformants of K854 were plotted as a function of the concentration of EB in the reaction system. The linear equations are shown for each curve. In the equations, y represents the initial accumulation rate of EB (μM/s); x represents the concentration of EB in the reaction system (μM); R^2 is the coefficient of determination

concentrations of EB in the reaction system) are listed in Supplementary Table 1.

To determine the initial accumulation rate of EB in different transformants when a series of concentrations of EB was added to the reaction system, the derivative of an equation in Table 1 at time 0 ($x = 0$) was considered as the initial accumulation rate of EB under the corresponding condition. If no EB is added to the reaction system, the accumulation rate should be 0. Correlation curves for the EB initial accumulation rate and EB concentration in the reaction system for the three transformants were derived. The curves were approximately linear with $R^2 > 0.95$ (see Fig. 2). A comparison of the slopes of the three curves showed that the initial accumulation rate of EB in the pAK1900-parmexA transformant of K854 (0.0006) was twofold higher than the initial EB accumulation rate of the two control transformants (0.0003). This result indicated that the expression of *mexAB-oprM* was repressed in the pAK1900-parmexA transformant of K854, which was consistent with the results showing increased susceptibility to antimicrobial agents in pAK1900-parmexA transformed K854 cells.

Conclusion

The experimental data presented in this study suggest that gene-specific silencing of *mexA* was induced by expressing *mexA* pRNA in *P. aeruginosa*. This is the first report of pRNA-mediated gene silencing in a prokaryotic organism other than *E. coli*. As previous analysis has shown, in different genomes numerous symmetrical sequences may be present, which could potentially be used to transcribe pRNAs (Tchurikov 1992). Thus gene silencing induced by pRNA might be a natural method for regulating gene expression in a variety of organisms. Gene-specific silencing induced by pRNA may represent a novel tool for gene regulation in prokaryotes, with numerous potential applications in biotechnology.

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