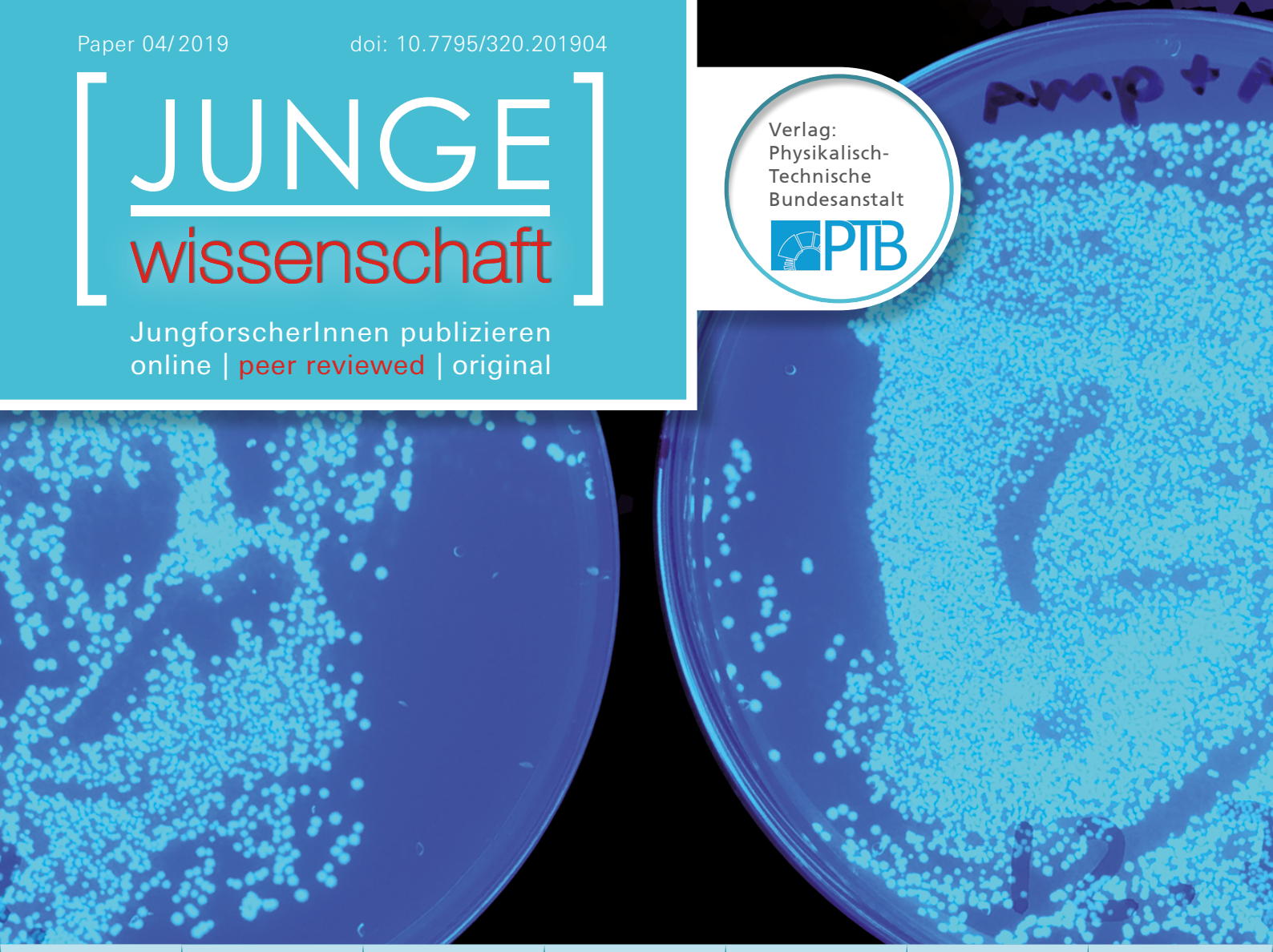
Paper 04/2019 doi: 10.7795/320.201904

JUNGE wissenschaft

JungforscherInnen publizieren
online | peer reviewed | original

Verlag:
Physikalisch-
Technische
Bundesanstalt
PTB



Biologie & Medizin

Counteracting Antibiotic Resistances

SMALL NON-CODING RNA INDUCED GENE SILENCING OF ANTIBIOTIC RESISTANCE GENES IN *E. COLI DH5α*

The aim was to determine whether small non-coding RNAs (sRNAs) are able to silence antibiotic resistance genes in bacteria. If so, the bacteria would lose their resistance. Therefore, such sRNAs were designed against GFP gene, tetracycline (Tc) and chloramphenicol (Cm) resistance gene. In presence of these sRNAs and the antibiotic, only few Tc resistant bacteria had grown, thus the Tc resistance gene was successfully silenced. In contrast, the number of Cm resistant bacteria remained unchanged and GFP-expressing bacteria continued to glow green, hence those genes were not silenced.

DIE JUNGFORSCHERIN

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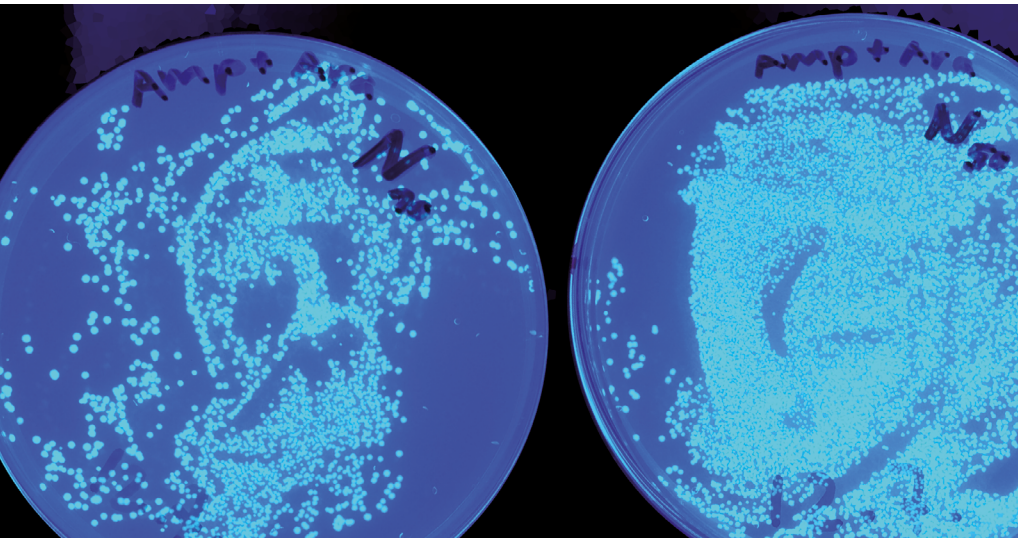
Eingang der Arbeit:
18.6.2017
Arbeit angenommen:
3.10.2017



Counteracting Antibiotic Resistances

SMALL NON-CODING RNA INDUCED GENE SILENCING OF ANTIBIOTIC RESISTANCE GENES IN *E. COLI DH5α*

1. Introduction

Before the early twentieth century, infectious diseases often were a death sentence. Only the discovery of antibiotic drugs by Sir Alexander Fleming in 1928 made it possible to treat and cure bacterial infections. Nowadays, however, bacteria have become resistant against most antibiotics, and some have even developed multiple resistances. This imposes a major threat to our health since bacterial infections can possibly not by treated with antibiotics anymore. Thus, it is but reasonable to search for solutions. One approach is to develop continuously new antibiotics. However, bacteria eventually become resistant against these new drugs too. Therefore, I was looking for an alternative to conventional antibiotics, and I came up with RNA interference.

1.1 Research Questions

I was asking myself whether the regulatory functions of small non-coding RNAs (sRNAs) on bacterial gene expression could be applied to antibiotic resistance genes. At least, GFP (green fluorescent protein) gene had been successfully silenced with sRNAs by MAN et al. in 2011 [1]. However, nothing such had been done before with antibiotic resistance genes. Therefore, I formulated the following research questions:

Is sRNA capable of silencing any gene in general and antibiotic resistance genes in particular? Do ineffective antibiotics regain an effect after sRNA-induced gene silencing of the respective resistance gene?

In this study, sRNAs against tetracycline resistance gene and chloramphenicol resistance gene were tested as I had access to bacteria which were resistant against those two antibiotics. Besides, sRNA against GFP gene was tested as well.

1.2 Theory [1, 2]

In order to allow adaptation to differing conditions and to avoid wasteful overproduction of proteins, bacterial cells use a variety of mechanism to regulate gene expression. These include inducible and repressible operons as well as small non-coding RNA (sRNA).

sRNA is composed of three different parts: an mRNA base pairing region (mRNA-BPR), an Hfq protein binding site and a Rho-independent terminator. The mRNA-BPR is 19–23 nucleotides (nt) long and shows partial complementarity to the 5′-untranslated region (5′-UTR) of the target mRNA. Through base pairing with the target mRNA, sRNAs prevent translation and promote degradation of the target mRNA. The latter is done by the enzyme RNase E, which cuts the 5′-end of the mRNA.

An AU-rich Hfq protein binding site of 12–19 nt length is required as the protein Hfq stabilises the sRNAs and stimulates the base pairing between sRNA and mRNA. The GC-rich Rho-independent terminator stops transcription of the sRNA.

In total, sRNAs are approx. 100 nt long. Additionally, they display two to four stem loops, one of which must lie within the mRNA-BRP and one within Rho-independent terminator region.

2. Materials and Methods

2.1 Bacteria Strains and Plasmids

For transformation experiments, the competent bacteria strain *Escherichia coli DH5α* (*E. coli DH5α*) was used. To provide the bacteria with antibiotic resistances, the plasmids pBR322 (with a tetracycline resistance gene) and pACYC184 (with a chloramphenicol resistance gene) were used. For GFP expression, the pGLO plasmid (BIO-RAD Laboratories, Inc.) was used. pGLO contains an ampicillin resistance

doi: 10.7795/320.201904

gene, and GFP gene is under control of a negative inducible arabinose operon. For vector cloning, the plasmids pACYC184 and pUC19 were used, as pACYC184 is compatible with pGLO and pBR322, and pUC19 is compatible with pACYC184.

2.2 Design of sRNAs

The mRNA-BPR was designed according to the 5′-UTR of the respective target mRNA. Sequences of 20–25 nt were chosen randomly in this area. Importantly, the Shine-Dalgarno consensus sequence (5′-AGGAGGU-3′) was included and remained unchanged. In the remaining part of the mRNA-BPR, up to 5 nt were changed arbitrarily. This procedure was repeated several times for each of the targeted genes. The sequence of the Hfq protein binding site and of Rho-independent terminator were taken from natural sRNAs [1]. All three components were assembled randomly into a series of possible sRNA candidates. An example is given in Figure 1.

2.3 Prediction of Secondary Structure and Selection of sRNAs

The secondary structures of all sRNA candidates were predicted using the *RNAFold WebServer* of the University of Vienna. Among these, those secondary structures were selected which formed two stem loops, one within the mRNA-BPR and one within the Rho-independent terminator region (Figure 2). Secondary structures containing the AUG start codon were sorted out.

Of the remaining secondary structures, those with high base pairing probabilities were favoured. Some additional nucleotide changes were made in order to either increase base pairing probability or to change the structure of the stem loop in the mRNA-BPR since a stem loop with 5–9 unpaired nucleotides in the loop is considered optimal.

The adjusted sequences of these secondary structures were run on the *RNAFold WebServer* once again to verify that the final secondary structure displays the desired features. Then, the mRNA-BPRs of these final candidates were aligned with the sequences of the target mRNAs to check the number of nucleotide matches. Those sRNAs were selected which showed a number of nucleotide matches between 60 and 90 percent. Finally, one of these sRNA sequences was chosen for each GFP gene, tetracycline resistance gene and chloramphenicol resistance gene. The selected sequences were named *antiGFP1*, *antiTc1* and *antiCm1*, respectively.

2.4 Vector Cloning

The sRNAs were rewritten from single-stranded RNA into double-stranded DNA. Then, *tac1* promoter sequence [3] was added at the 5′-end of the antisense strand and at the 3′-end of the sense strand of each sRNA-DNA-sequence. *BamH1* restriction site was inserted in front of the *tac1* promoter at the 5′-end of the antisense strand and the 3′-end of the sense strand. *HindIII* restriction site was inserted at the 3′-end of the antisense strand and the 5′-end of the sense strand.

These restriction enzymes were chosen as they are found only once within the plasmids pACYC184 and pUC19. Furthermore, they create different single-nucleotide overhangs (“sticky ends”), which ensures the correctly oriented insertion of the sRNA-DNA-sequences.

The sense and antisense DNA strands for each sRNA were ordered at *Sigma Aldrich Co. LLC*. Then, the corresponding sense and antisense DNA strands were

5′-CAGCGAAGGAAUUCUUCUCCUUUGCAAAUUAAUAAUAAAGGCACAGCCGCCAGUUCCGCUGGCGG CAUUUUU-3′

Fig. 1: Sequence of sRNA against GFP gene. Red: mRNA-BPR, Green: Hfq binding site, Blue: Rho-independent terminator.

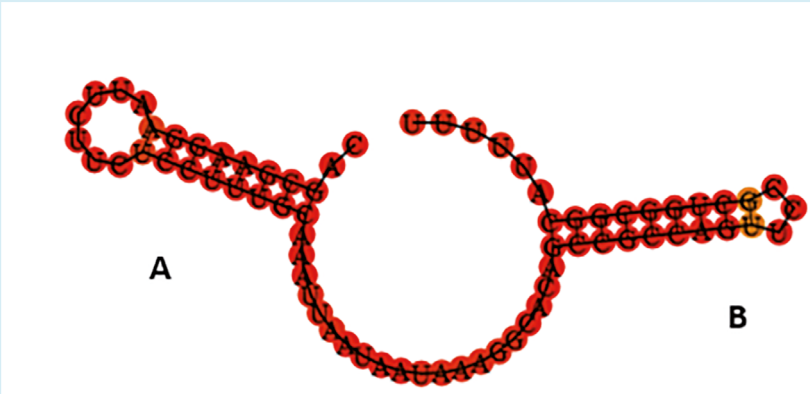


Fig. 2: Predicted Secondary Structure of sRNA against GFP. Those sRNAs were favoured which showed one stem loop within the mRNA-BPR (A) and one stem loop within the Rho-independent terminator region (B).

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annealed. The plasmids pACYC184 and pUC19 were both double digested with the restriction enzymes *BamH1* and *HindIII*. The annealed strands of antiGFP1 or antiTc1 were added to digested pACYC184 together with ligase. This resulted in the recombinant vectors pACYC184-tac1-antiGFP1 and pACYC184-tac1-antiTc1 (Figure 3), respectively. The annealed strands of antiCm1 were added to digested pUC19 together with ligase, which generated the recombinant pUC19-tac1-antiCm1 vector (Figure 3).

All three recombinant vectors were sequenced, and transformation experiments were only carried out after sequencing confirmed the correctness of the newly generated vectors.

2.5 Transformation and Growth of Bacteria

First, the bacteria were transformed with the recombinant vector. Therefore, frozen *E. coli DH5α* were thawed on ice. Then, 60 µl bacteria were mixed together with 2 µl (1ng/µl) of the recombinant vector. After 10 minutes of incubation on ice, the bacteria were added to preheated (37 °C) liquid growth medium and incubated overnight at 37 °C.

To transform *E. coli DH5α* with the second plasmid (i.e. pGLO, pACYC184 or pBR322), electroporation was used. 20 µl of transformed *E. coli DH5α* in liquid culture were incubated at 37 °C and 300 rpm (rotations per minute) for 2 hours in 1.0 ml YENB (Yeast Extract and Nutrient Broth) growth medium. Then, the bacteria were centrifuged twice for 3 minutes at 5000 g. The supernatant was discarded each time and the bacteria pellet was dissolved in 100 µl (1ng/µl) of the plasmid DNA which should be co-transformed in addition to the recombinant vector. The bacteria were electroporated using the *Gene Pulser Xcell™* (BIO-RAD Laboratories, Inc.) in a sterile 2 mm electroporation cuvette at 2500 V for 5.0 ms using the pre-installed program "Bacterial: E. coli cuvette 2 mm". After electroporation, the bacteria were transferred to 1.0 ml SOC (Super Optimal Broth with Catabolite Repression) medium and incubated for 1 hour at 37 °C and 180 rpm. After one hour, the bacteria were plated on different LB Agar plates (10 µl bacteria-SOC-solution per plate) and incubated again overnight at 37 °C. The list of LB Agar plates the co-transformed bacteria were plated onto is given in Table 1. Each co-transformation experiment was repeated twice. Hence, in total, three replicates were produced and examined.

Tab. 1: List of LB Agar plates the co-transformed bacteria were plated onto. Amp = Ampicillin, Tc = Tetracycline, Cm = Chloram-phenicol, Ara = Arabinose, Lac = Lactose.

Co-Transformed Vectors	pGLO + pACYC184-tac1-antiGPF1	pBR322 + pACYC184-tac1-antiTc1	pACYC184 + pUC19-tac1-antiCm1
LB Agar Plates	▪ Amp+Ara ▪ Amp+Ara+Lac ▪ Cm ▪ Amp+Cm ▪ Amp+Cm+Ara ▪ Amp+Cm+Ara+Lac	▪ Tc ▪ Tc+Lac ▪ Cm ▪ Cm+Lac ▪ Tc+Cm ▪ Tc+Cm+Lac	▪ Cm ▪ Cm+Lac ▪ Amp ▪ Amp+Lac ▪ Cm+Amp ▪ Cm+Amp+Lac

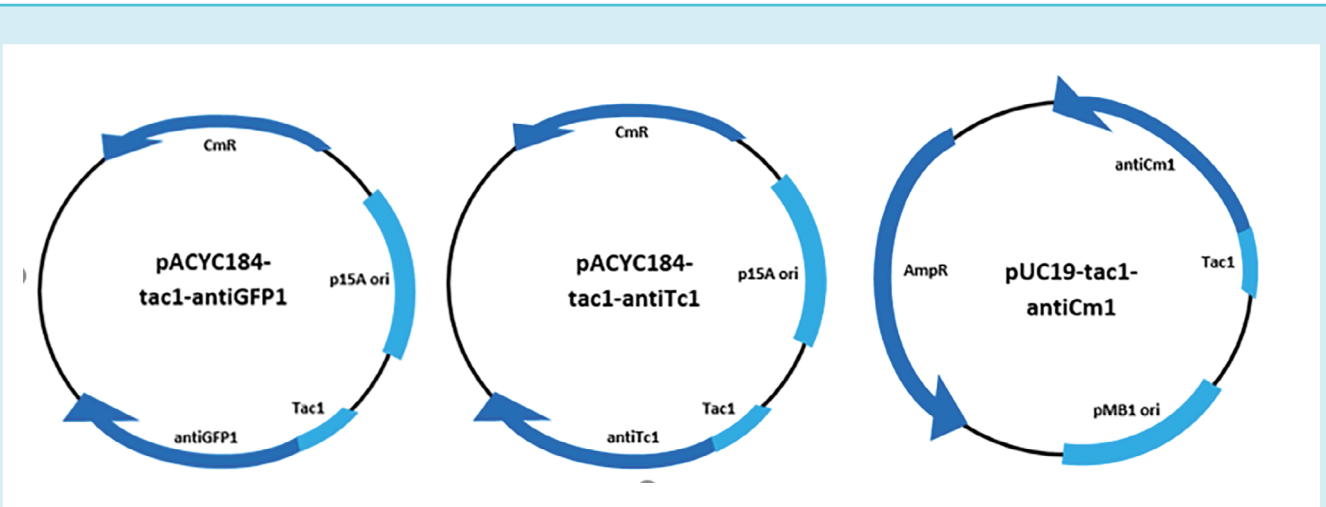

Fig. 3: Plasmid maps of the three newly created recombinant pACYC184 and pUC19 vectors

2.6 Measurement of Gene Expression and Silencing

To measure the effect of sRNA on the expression of its target genes, two methods were applied:

The silencing effect of sRNA against GFP was measured by counting the number of grown bacteria colonies on the different LB Agar plates and their ability to glow green when exposed to ultra violet light.

The silencing effect of sRNAs against tetracycline resistance gene and chloramphenicol resistance genes was measured by counting the number of bacteria colonies grown on the LB Agar plates in presence and absence of lactose (the inducer of *tac1* promoter and thus controlling the transcription of sRNA).

To draw any conclusions about the effectiveness of sRNA induced gene silencing, Chi-Squared-Test (χ^2-Test) was applied to the following four LB Agar plate combinations:

- Tetracycline (Tc) and tetracycline and lactose (Tc+Lac)
- Tetracycline and chloramphenicol (Tc+Cm) and tetracycline, chloramphenicol and lactose (Tc+Cm+Lac)
- Chloramphenicol (Cm) and chloramphenicol and lactose (Cm+Lac)
- Chloramphenicol and ampicillin (Cm+Amp) and chloramphenicol, ampicillin and lactose (Cm+Amp+Lac).

The null hypothesis (H0) and the alternative hypothesis (H1) were the same for all four χ^2-tests.

H0: There is no significant difference between the distributions of bacteria on the different plates.

H1: There is a significant difference between the distributions of bacteria on the different plates.

3. Results

3.1 Designing of sRNAs and Vector Cloning

sRNAs against GFP gene, tetracycline resistance gene and chloramphenicol resistance gene were designed according to the following principles (as described in Materials and Methods):

- sRNA is composed of three different parts: mRNA-BPR, Hfq protein binding site and Rho-independent terminator.
- In total, sRNA is of 70–80 nucleotides (nt) length.
- mRNA-BPR has a length of 19–23 nt and is partially complementary to the 5′-UTR of the target mRNA.
- Hfq binding site is an AU-rich region of 12–19 nt length.

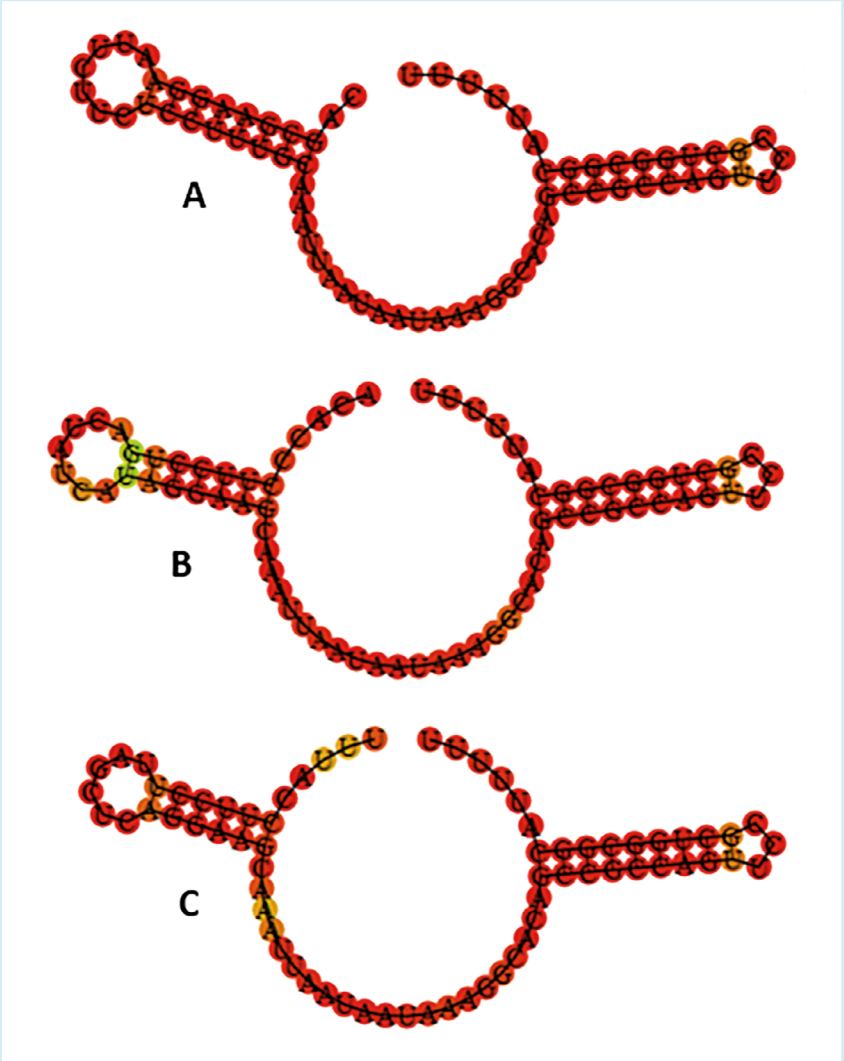

Fig. 4: The predicted secondary structures of all three selected sRNAs, namely antiGFP1 (A), antiTc1 (B) and antiCm1 (C). The different colours denote the base pairing probability, whereby red means high probability, yellow represents medium and green low probability

doi: 10.7795/320.201904

- Rho-independent terminator is a GC-rich sequence with an inverted repeat followed by a uridine-overhang.
- sRNA forms two stem loops. One stem loop is formed by mRNA-BPR and one by Rho-independent terminator. Hfq binding site is located between the two stem loops.
- The start codon AUG is to be avoided to prevent translation of sRNA.

This resulted in a few possible sRNA candidates for each gene. One sRNA was selected against each gene and was named accordingly. The secondary structures of these sRNAs are shown in Figure 4, and their properties are listed in Table 2.

These sRNAs were cloned into either pACYC184 or pUC19 vector. The newly generated recombinant vectors were sequenced. Although the sRNA sequences were correct, it appeared that both vectors have one mistake within the *tac1* promoter sequence. There was an additional guanine in recombinant pACYC184 and an additional adenine in recombinant pUC19.

Tab. 2: Some selected properties of the sRNAs antiGFP1, antiTc1 and antiCm1. Note that with "stem loop", the stem loop formed by the mRNA-BPR is meant.

	antiGFP1	antiTc1	antiCm1
Total Length of sRNA	72 nt	74 nt	71 nt
Length of mRNA-BPR	23 nt	25 nt	22 nt
Nucleotide Matches with target mRNA	15 nt (65.2 %)	17 nt (68.0 %)	19 nt (86.4 %)
Total Length of the Stem Loop	21 nt	19 nt	16 nt
Length of the paired part of the stem loop	14 nt	12 nt	10 nt
Length of the unpaired part of the stem loop	7 nt	7 nt	6 nt

3.2 Co-Transformation with pGLO and pACYC184-tac1-antiGFP1

Competent *E. coli DH5α* was transformed with pGLO and pACYC184-tac1-antiGFP1 by electroporation and plated onto different LB Agar plates. After overnight incubation at 37 °C, bacteria colonies had grown on each plate. The numbers of grown bacteria colonies (as an arithmetic mean of all three data sets) are given in Table 3. The standard deviation was calculated and did never exceed 13.

When assessed under UV light, only the bacteria on the Amp+Ara-plate, the Amp+Ara+Lac-plate, the Amp+Cm+Ara-plate and the Amp+Cm+Ara+Lac-plate glowed green. The bacteria on the other three plates did not glow green.

3.3 Co-Transformation with pBR322 and pACYC184-tac1-antiTc1

E. coli DH5α was co-transformed with pBR322 and pACYC184-tac1-antiTc1 and plated onto different LB Agar plates. After overnight incubation at 37 °C, bacteria colonies had grown on each plate.

On the Tc-plate, there was a dense bacterial layer of about 2000 bacteria colonies. In contrast, only 71 bacteria colonies grew on the Tc+Lac-plate (Figure 5). This equals a reduction of 96.5 percent. Comparison of these two plates with χ^2-test resulted in $\chi^2 = 3593.47$ for a critical value

Tab. 3: Arithmetic means of the three replicates of pGLO/pACYC184-tac1-antiGFP1 co-transformed bacteria on different LB Agar plates. Plates with glowing bacteria on it are marked with an asterisk (*).

LB Agar Plate	Amp	Amp+Ara*	Amp+Ara +Lac*	Cm	Amp+Cm	Amp+Cm +Ara*	Amp+Cm +Ara+Lac*
Number of Bacteria Colonies	1265	1284	1350	2000	1459	1476	1158

doi: 10.7795/320.201904

($\alpha = 0.05$, $v = 3$) of 7.815. On both the Cm-plate and the Cm+Lac-plate were dense bacterial layers with roughly 2000 colonies per bacterial layer. On the Tc+Cm-plate, 73 bacteria colonies were counted. However, there were only 48 bacteria colonies on the Tc+Cm+Lac-plate; this equals a reduction of 34.3 percent. Comparison of these two plates with the χ^2-test resulted in a value of $\chi^2 = 10.99$ for a critical value ($\alpha = 0.05$, $v = 3$) of 7.815. (The indicated numbers in the above paragraph are the arithmetic means of all three data sets, whereby standard deviation never exceeded 4 colonies.)

3.4 Co-Transformation with pACYC184 and pUC19-tac1-antiCm1

E. coli DH5α was co-transformed with pACYC184 and pUC19-tac1-antiCm1 and plated onto different LB Agar plates. After overnight incubation at 37 °C, bacteria colonies had grown on each plate. There were 44 bacteria colonies on the Cm-plate and 39 bacteria colonies on the Cm+Lac-plate. Addition of lactose lowered the number of bacteria colonies by 11.4 percent. χ^2-test analysis of these two plates resulted in a χ^2 value of 1.08 for a critical value ($\alpha = 0.05$, $v = 3$) of 7.815.

On both the Amp-plate and the Amp+Lac-plate were dense bacterial layers with approx. 2000 colonies in each bacterial layer.

With 38 bacteria colonies on the Cm+Amp-plate and 39 colonies on the Cm+Amp+Lac-plate, the number of bacteria colonies on these two plates was almost equal. In fact, it increased by 1 colony or 2.6 percent. Comparison of those two plates with χ^2-test resulted in a value of $\chi^2 = 0.44$ for a critical value ($\alpha = 0.05$, $v = 3$) of 7.815. (The indicated numbers in the above paragraph are again arithmetic means of all three data sets. Here too, standard deviation never exceeded 3 colonies.)

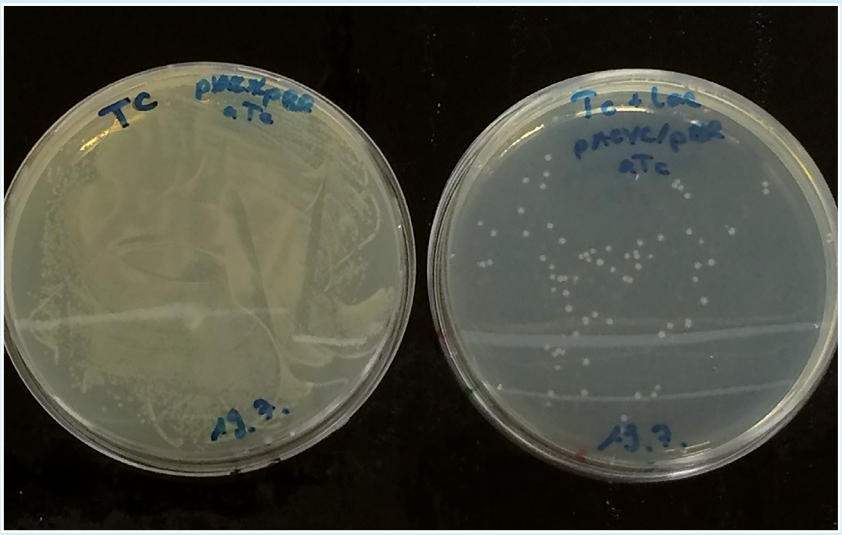

Fig. 5: pBR322/pACYC184-tac1-antiTc1 co-transformed bacteria on a Tc-plate (left) and a Tc+Lac-plate (right). A representative example from the three replicates is shown. Note that the horizontal white stripes on both plates are light reflections.

4. Discussion

4.1 Co-Transformation with pGLO and pACYC184-tac1-antiGFP1

E. coli DH5α was co-transformed with pGLO and pACYC184-tac1-antiGFP1. Bacteria grew on all plates, but only those on plates containing arabinose glowed green. This makes sense since arabinose activates the arabinose operon, which in turn initiates transcription of green fluorescent protein, GFP.

In total, 1265 bacteria colonies grew on the ampicillin-plate (Amp-plate). On the ampicillin-arabinose-plate (Amp+Ara-plate), however, 1284 bacteria colonies were counted. This increase in bacteria colony number of 19 colonies in absolute terms and 1.5 percent in relative terms clearly indicates that arabinose itself does not harm the bacteria or hinder their growth, respectively. A comparably high number of 1350 bacteria colonies was counted on the ampicillin-arabinose-lactose-plate (Amp+Ara+Lac-plate). Even though lactose was present on this plate, there were 66 colonies more than on the Amp+Ara-plate. This increase by 5.1 percent leads to the conclusion that lactose as well is not harmful to *E. coli DH5α* or its growth. Nevertheless, bacteria on both the Amp+Ara-plate and the Amp+Ara+Lac-plate glowed green. Inhibition of GFP expression on the Amp+Ara+Lac-plate might not appear due to several possible reasons:

First, the bacteria might not have been transformed with pACYC184-tac1-antiGFP1 as well. Hence, no sRNA against GFP was transcribed.

Second, *tac1* promoter might not be functioning, although this is very unlikely since the correctness of the *tac1* promoter sequence had been confirmed.

Third, concentration of lactose might have been too low to properly activate the *tac1* promoter.

Fourth, even though the sequence of antiGFP1 is correct and properly expressed, it might not be working due to some fundamental designing errors.

doi: 10.7795/320.201904

And last, it could be that GFP expression is much faster that sRNA transcription. Hence, a lot of GFP protein is already expressed and present when antiGFP1 starts to work. Since sRNA inhibits translation of mRNA and because GFP is an extremely stable protein, sRNA would have no visible effect even if designed, transcribed and working properly.

Nonetheless, as the bacteria grew on plates containing ampicillin, against which there is a resistance gene on pGLO, and glowed green under UV light exposure, these three negative control plates all confirmed that the bacteria have at least been successfully transformed with pGLO plasmid and that they express a functioning green fluorescent protein.

Next, the dense bacterial layer on the Cm-plate is sign of a successful pACYC184-tac1-antiGFP1 transformation since the bacteria must be chloramphenicol resistant to grow on this plate, and this is only possible if they express the chloramphenicol resistance gene on pACYC184.

However, only the growth of bacteria on the Amp+Cm-plate, the Amp+Cm+Ara-plate and the Amp+Cm+Ara+Lac-plate verified that *E. coli DH5α* was successfully co-transformed with both plasmids. There was only a small difference (1.2 percent) in bacteria colony number between the Amp+Cm-plate (1459 colonies) and the Amp+Cm+Ara-plate (1476 colonies). Nevertheless, only the bacteria on the Amp+Cm+Ara-plate glowed green under UV light. These results confirm once again that arabinose is not harmful to bacterial growth but induces the arabinose operon and thus initiates GFP expression. Still, only 1158 colonies were counted on the Amp+Cm+Ara+Lac-plate. Compared to the Amp+Cm+Ara-plate, these are 21.5 percent less colonies. This reduction could be due to chance or due to a harmful effect of lactose. Having said that, a harmful effect of lactose on bacterial growth was excluded by the negative controls. Also, a harmful effect of the lactose-arabinose-combination is very unlikely as there was no comparable effect in the negative controls. Consequently, chance seems to be responsible for this reduction in bacteria colony number. However, the bacteria on both the Amp+Cm+Ara-plate and the Amp+Cm+Ara+Lac-plate glowed green. Hence, there was no or too little inhibition of GFP expression. Since both plasmids, pGLO and pACYC184-tac1-antiGFP1, are present in the bacterial cells, the following explanations are possible:

Although there was only one mistake in the *tac1* promoter sequence, *tac1* promoter could be functioning improperly. By digestion of pACYC184-tac1-antiGFP1, insertion of another gene (GFP for instance) and repetition of the experiment, this hypothesis could be tested.

Concentration of lactose might have been insufficient to activate the *tac1* promoter. By alteration of the lactose concentration, the optimal lactose concentration for *tac1* activation could be found.

Even though the sequence of antiGFP1 is correct, it might not be working due to some fundamental designing errors.

And last, it could be that GFP expression is much faster that sRNA transcription. Hence, a lot of GFP protein is already expressed and present when antiGFP1 starts to work. Since sRNA inhibits translation of mRNA and because GFP is an extremely stable protein, sRNA would have no visible effect even if designed and transcribed properly.

Since the same concentration of lactose was used as [3] did, from whom the *tac1* promoter sequence was taken, and since the sRNAs were designed in accordance to the principles used by [1] the last explanation seems to be the most probable one. This hypothesis could be examined by plating the co-transformed bacteria on an Amp+Cm+Lac-plate first before transferring the grown bacteria colonies to an Amp+Cm+Ara+Lac-plate.

4.2 Co-Transformation with pBR322 and pACYC184-tac1-antiTc1

E. coli DH5α was co-transformed with pBR322 and pACYC184-tac1-antiTc1. Bacteria were plated and grew on every plate. However, huge differences persist among the plates.

A dense bacterial layer with an uncountable number of bacteria colonies was observed on the tetracycline-plate (Tc-plate). This negative control plate indicates that the bacteria have at least been transformed with pBR322, which carries a tetracycline resistance gene. One could argue that the vector plasmid pACYC184 contains both a chloramphenicol and a tetracycline resistance gene, and that the tetracycline resistance might originate from there as well. Since the tetracycline resistance gene on pACYC184 was destroyed by the insert of antiTc1 at this site, the bacteria's resistance against tetracycline must be due to successful pBR322 transformation.

As opposed to the Tc-plate, there were only 71 bacteria colonies on the tetracycline-lactose-plate (Tc+Lac-plate). Analysis with the χ^2-test resulted in a value of $\chi^2 = 3593.47$ for a critical value ($\alpha = 0.05$, $v = 3$) of 7.815. Since the χ^2 value is much higher that the critical value, the null hypothesis can be discarded. Consequently, the alternative hypothesis is accepted and states that the differences among the bacteria distributions are significant. Three reasons might explain why the number of bacteria colonies on the Tc+Lac-plate decreased drastically:

First, massive reduction of bacteria colonies could be due to a harmful effect of lactose on the bacteria.

Second, in the case of a successful co-transformation with pBR322 and

pACYC184-tac1-antiTc1, lactose could have activated the *tac1* promoter. Activation of *tac1* promoter would cause transcription initiation of the sRNA antiTc1 coded on the recombinant vector. Hence, sRNA induced gene silencing of tetracycline resistance gene could be the reason for a decreased number of bacteria colonies.

Third, it might also be the case that this time *E. coli DH5α* was not successfully transformed with pBR322. However, this last explanation is very unlikely since few colonies grew on the Tc+Lac-plate and therefore must contain the pBR322 plasmid.

Next, there were dense bacterial layers on both the chloramphenicol-plate (Cm-plate) and the chloramphenicol-lactose-plate (Cm+Lac-plate). These plates were negative controls too. Since pACYC184-tac1-antiTc1 contains a chloramphenicol resistance gene, bacteria growth on both of these plates indicates that *E. coli DH5α* has successfully been transformed with pACYC184-tac1-antiTc1 and expresses the chloramphenicol resistance gene. As there is no significant difference in bacteria growth between the Cm-plate and the Cm+Lac-plate, lactose does not have a harmful effect on the bacteria and their growth. Thus, it is likely that the massive reduction in bacteria on the Tc+Lac-plate was a result of sRNA induced silencing of tetracycline resistance gene.

The last negative control plate was the tetracycline-chloramphenicol-plate (Tc+Cm-plate). In total, 73 bacteria colonies were counted on this plate. Since this plate contains two antibiotics at the same time, bacteria must be resistant against both tetracycline and chloramphenicol to grow on the Tc+Cm-plate. Hence, bacteria growth on this plate indicates that the co-transformation with pBR322 and pACYC184-tac1-antiTc1 was successful. However, the number of bacteria colonies on the Tc+Cm-plate was much less when compared to the Tc-plate or the Cm-plate, on which there were dense bacterial layers. This could be a result of either increased selection pressure in presence of two antibiotics or low plasmid copy numbers per cell for both pBR322 (15–20 copies per cell) and pACYC184 (10 copies per cell), or both. It might also be that tetracycline and chloramphenicol interfere with each other.

Nevertheless, only 48 bacteria colonies were observed on the tetracycline-chloramphenicol-lactose-plate (Tc+Cm+Lac-plate), the actual test plate. This equals a reduction of 34.3 percent compared to the Tc+Cm-plate. Since the negative controls excluded a harmful effect of lactose on bacteria growth (namely the results provided by the Cm-plate and the Cm+Lac-plate), this decrease in bacteria number is very likely to result from sRNA induced gene silencing. This assumption is supported by the fact that the analysis with χ^2-test led to a value of $\chi^2 = 10.99$. As χ^2 is bigger than the critical value ($\alpha = 0.05$, $v = 3$) of 7.815, the null hypothesis can be discarded again and the differences in bacteria distribution on the different plates are statistically significant.

4.3 Co-Transformation with pACYC184 and pUC19-tac1-antiCm1

E. coli DH5α was co-transformed with pACYC184 and pUC19-tac1-antiCm1. Bacteria were plated and incubated. Although bacteria grew on each plate, differences persist among the individual plates.

In total, 44 bacteria colonies were counted on the chloramphenicol-plate (Cm-plate). Since bacteria need to be resistant against chloramphenicol to grow on the Cm-plate, bacteria growth on this plate shows that the bacteria have successfully been transformed with pACYC184.

On the chloramphenicol-lactose-plate (Cm+Lac-plate), only 39 bacteria colonies were counted. This equals a reduction of 11.4 percent compared to the Cm-plate. This decline in bacteria colonies may have several reasons:

In the case of a successful co-transformation with pACYC184 and pUC19-tac1-antiCm1, lactose could have activated the *tac1* promoter. Activation of *tac1* promoter would cause transcription initiation of the sRNA antiCm1 coded on the recombinant vector. Hence, sRNA induced gene silencing of chloramphenicol resistance gene could be the reason for a decreased number of bacteria colonies.

Another reason might be that lactose somehow hinders the bacteria in their growth. It might also be the case that this time transformation of pACYC184 was not as successful as on the previous plate. However, since the number of bacteria colonies is almost the same on both the Cm-plate and the Cm+Lac-plate, these two explanations do not seem to be very likely.

As a last possible explanation, differences between the Cm-plate and the Cm+Lac-plate might also be due to chance. At least, the difference in bacteria colony number is only 5 colonies in absolute terms and 11.4 percent in relative terms. Moreover, the χ^2 value of 1.08 for these two plates is much smaller than the critical value of 7.815. Hence, the null hypothesis cannot be rejected and the differences in the distribution of bacteria colonies are not significant.

Next, there were dense bacterial layers on both the ampicillin-plate (Amp-plate) and the ampicillin-lactose-plate (Amp+Lac-plate). Like the previous two plates, these plates were negative controls too. Since pUC19-tac1-antiCm1 contains an ampicillin resistance gene, bacteria growth on both of the plates indicates that *E. coli DH5α* has successfully been transformed with pUC19-tac1-antiCm1 and expresses the ampicillin resistance gene. As there is no significant difference in bacteria growth between the Amp-plate and the Amp+Lac-plate, lactose does not seem to have a harmful effect

on the bacteria and their growth. Thus, a harmful effect of lactose can be excluded as a reason for the reduction in bacteria colonies on the Cm+Lac-plate.

The last negative control plate was the chloramphenicol-ampicillin-plate (Cm+Amp-plate). In total, 38 bacteria colonies were counted on this plate. Since this plate contains two antibiotics at the same time, bacteria must be resistant against both chloramphenicol and ampicillin to grow on the Cm+Amp-plate. Hence, bacteria growth on this plate indicates that the co-transformation with pACYC184 and pUC19-tac1-antiCm1 was successful. However, the number of bacteria colonies on the Cm+Amp-plate was relatively low. Again, this could be a result of either increased selection pressure in presence of two antibiotics, or low plasmid copy numbers per cell for pACYC184 (10 copies per cell), or both.

Even so, 39 bacteria colonies were observed on the chloramphenicol-ampicillin-lactose-plate (Cm+Amp+Lac-plate). This equals an increase of 2.6 percent compared to the Cm+Amp-plate. Even though lactose was present on the test plate, there was no reduction in bacteria colony number. Hence, a silencing effect of sRNA can possibly be excluded. Supportingly, χ^2-test analysis resulted in a value of $\chi^2 = 0.44$ for a critical value ($\alpha = 0.05$, $v = 3$) of 7.815. The value of χ^2 is much smaller than the critical value, thus there is no reason to reject the null hypothesis. As a result, there are no significant differences in the bacteria distributions, and the relatively low increase in colony number is most likely to have originated from chance. Therefore, it is a fair assumption that the reduction in bacteria colonies on the Cm+Lac-plate compared to the Cm-plate was due to chance as well and not due to sRNA induced gene silencing.

5. Conclusion

sRNAs were designed against GFP gene, tetracycline resistance gene and chloramphenicol resistance gene. These sRNAs, named antiGFP1, antiTc1 and antiCm1, respectively, were cloned into either pACYC184 or pUC19 plasmid and put under control of lactose-activated *tac1* promoter. Thereby, the recombinant vectors pACYC184-tac1-antiGFP1, pACYC184-tac1-antiTc1 and pUC19-tac1-antiCm1 were generated. Sequencing confirmed the correctness of both the sequence and the insertion of the sRNA sequences. However, one single-nucleotide mismatch appeared within *tac1* promoter region. Whether or not this mistake had a negative influence on the outcome of this experiment must be tested in a subsequent study by inserting a reporter gene, such as GFP gene, behind the imperfect *tac1* promoter.

GFP-expressing or tetracycline and chloramphenicol resistant bacteria were transformed with the corresponding recombinant vector and plated onto selective LB Agar plates.

GFP-expressing bacteria continued to glow green even after transcription of antiGFP1 was induced. This result implies that sRNA did not successfully silence the GFP gene. A reason for that failure could be the stability of GFP and the fast expression of GFP gene. This hypothesis could be verified if co-transformed bacteria would first be plated onto selective plates with lactose only. This would allow sRNA transcription, but not GFP production. These bacteria, within which sRNA has accumulated, would be transferred to plates containing both lactose and arabinose to initiate GFP synthesis. As sRNA is already present within the cell, GFP expression should be prevented if the sRNAs work properly. Also, a constitutive sRNA-expression plasmid could be used to produce an observable sRNA-induced silencing of GFP gene.

In contrast, the number of tetracycline resistant bacteria was significantly reduced after the sRNA antiTc1 had been transcribed. In conclusion, tetracycline resistance gene was successfully silenced by sRNA. Consequently, tetracycline became effective again and was able to kill the bacteria.

However, the number of chloramphenicol resistant bacteria remained relatively unchanged, no matter whether sRNA against chloramphenicol resistance gene was present or not. This result indicates that chloramphenicol resistance gene was not silenced. Thus, chloramphenicol had no effect on the (still resistant) bacteria. This failure might originate from the secondary structure of antiCm1, which slightly differs from the structures of antiGFP1 and antiTc1. Adjusting the sequence and therefore the secondary structure of antiCm1 may lead to proper functioning of this sRNA. Also, it could be that although sRNA is transcribed, its amount is too little to repress the target mRNA and therefore doesn't evoke an effect on the phenotype. This hypothesis must be tested by applying Northern Blot and/or qRT-PCR.

In conclusion, sRNA might be in the position of silencing any gene in general, even though it was not demonstrated in this study. Nonetheless, sRNA was able to silence one of the two antibiotic resistance genes. Hence, in principle this method is suited to this propose.

However, more research must be carried out in order to understand the mechanism of sRNA-induced gene silencing even better, to determine essential sRNA properties and to increase effectiveness of (artificial) sRNA-induced gene silencing in bacterial cells.

Nevertheless, the results of this study may lead to a new antibiotic treatment one day. This new treatment could be put into practice in various ways. For example, sRNA could not only focus on silencing antibiotic resistance genes, but it might also attack other vital bacterial genes. Furthermore, sRNA could be introduced into pathogenic bacteria by bacteriophages (transduction).

doi: 10.7795/320.201904

This strategy would ensure that only pathogenic bacteria are attacked by the sRNAs; harmless bacteria and human cells would remain undisturbed. The great advantage of sRNAs as an adjuvant to antibiotics is the ability to response fast and adjust the sRNA in case the bacteria acquire a new resistance gene or change an old one.

Acknowledgements

I would like to thank everyone who helped me to realise this project. Special thanks go to my biology teacher Dr Michael Kappeler, who supported me from the start although my idea sounded unrealistic or even utopian. Then, I would like to thank Dr Oleg Georgiev for allowing me to work in his laboratory and use his equipment. He brought up much patience for my numerous questions, and his expertise in vector cloning was essential to my project. At last, I would like to thank Dr Hugo Stocker who drew my attention to errors and incorrectly used terminology.

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doi: 10.7795/320.201904

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doi: 10.7795/320.201904

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Junge Wissenschaft
c/o Physikalisch-Technische
Bundesanstalt (PTB)
www.junge-wissenschaft.ptb.de

Redaktion
Dr. Sabine Walter, Chefredaktion
Junge Wissenschaft
Paul-Ducros-Str. 7
30952 Ronnenberg
E-Mail: sabine.walter@verlag-jungewissenschaft.de
Tel.: 05109 / 561 508

Verlag
Dr. Dr. Jens Simon,
Pressesprecher der PTB
Bundesallee 100
38116 Braunschweig
E-Mail: jens.simon@ptb.de
Tel.: 0531 / 592 3006
(Sekretariat der PTB-Pressestelle)

Design & Satz
Sabine Siems
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