The enigmatic Rid7C protein is an endoribonuclease involved in differentiation and A40926 production in *Nonomuraea gerenzanensis*

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Our Team/s

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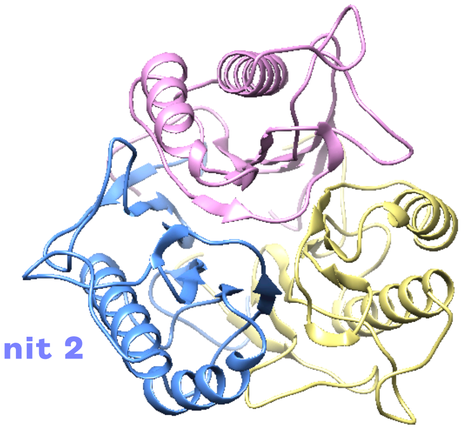
The protein family YjgF/YER057c/UK114 (Rid) is widespread in all domains of life and includes proteins involved in detoxification, RNA maturation, and control of mRNA translation.

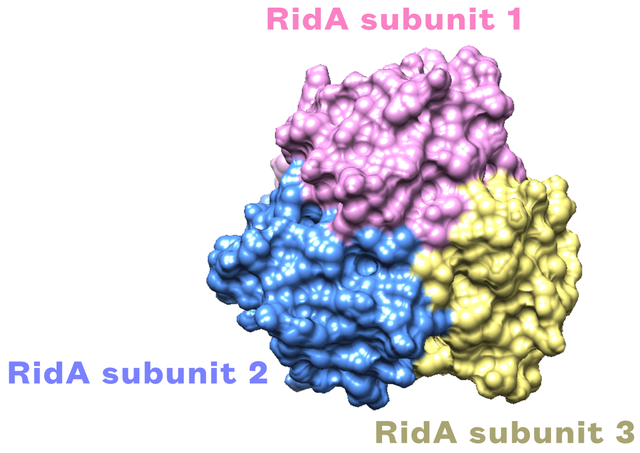
The only member of this superfamily biochemically well-characterized is the archetypal RidA.

3D model of human RidA (hRidA)

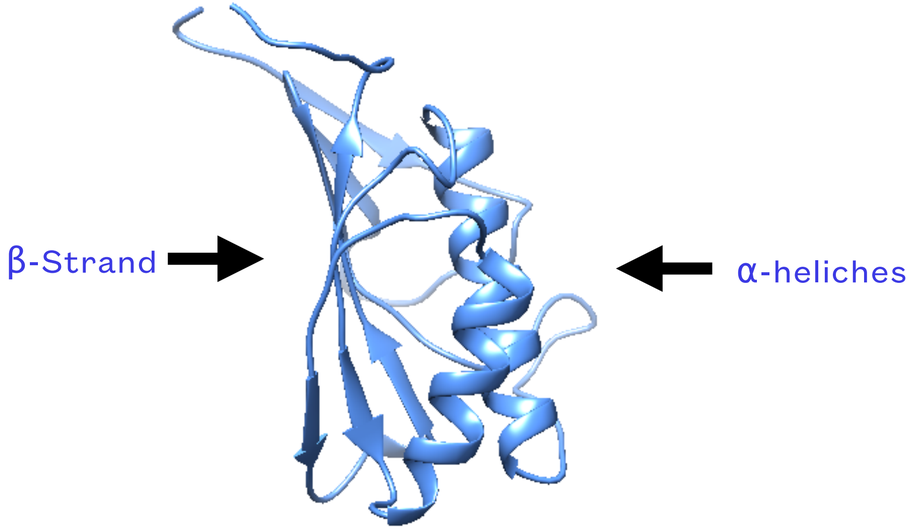
Trimeric model



Trimeric model



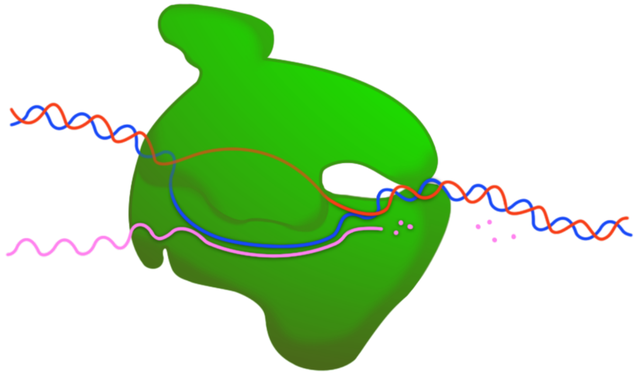
Monomer model



RidA hydrolyzes the reactive intermediates enamine/imine generated from the PLP-dependent serine/threonine dehydrates.

We studied *Nonomuraea gerenzanensis*, a rare actinomycete industrially used to produce A40926 (the precursor of **dalbavancin**, an FDA-approved antibiotic).

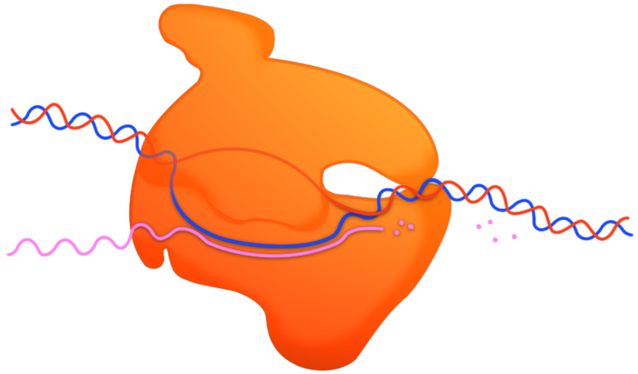
This actinomycete is characterized by the presence of a duplicated genes encoding β-subunit of RNA polymerase:



rpoB(S)

the wild-type gene

And



rpoB(R)

a specialist, mutant-type *rpoB* gene

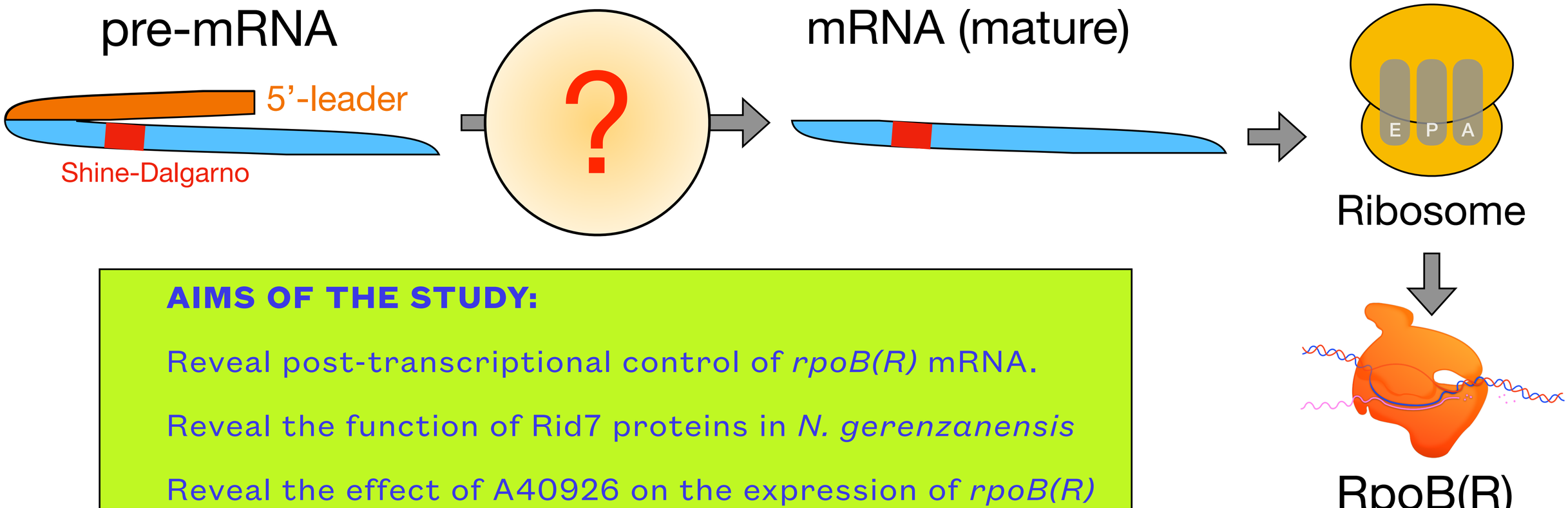
RpoB(R):

1. Controls the morphological differentiation;
2. Controls the activation of secondary metabolism (A40926 production);
3. Confers the natural resistance to rifamycins.

OUR HYPOTHESIS

Translation of the *rpoB(R)* mRNA is negatively modulated by a self-complementary hairpin loop in its 5'-UTR which hides the Shine & Dalgarno sequence.

Our hypothesis: Rid7 proteins may be involved in the 5'-UTR remotion



AIMS OF THE STUDY:

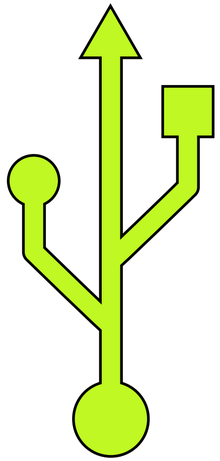
Reveal post-transcriptional control of *rpoB(R)* mRNA.

Reveal the function of Rid7 proteins in *N. gerenzanensis*

Reveal the effect of A40926 on the expression of *rpoB(R)*

Our approach

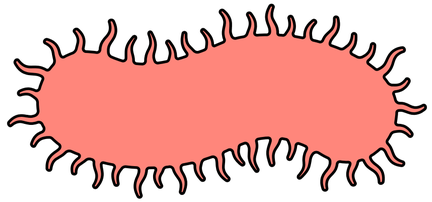
We use a combination of....



In silico methods

Protein modeling

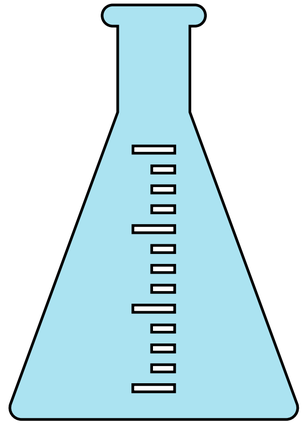
and more...



In vivo methods

molecular biology

and more...

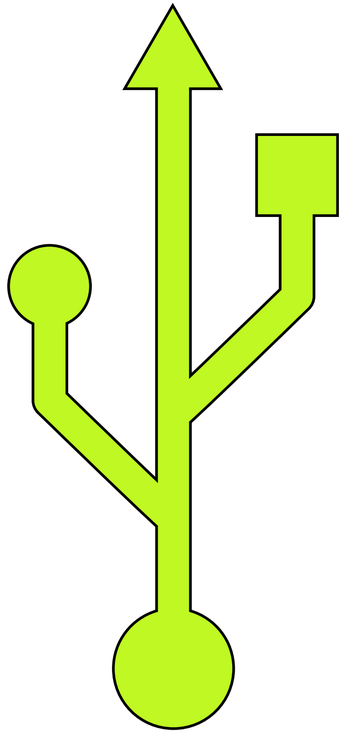


In vitro methods

Riboprobe (RNA) digestion

and more...

In silico analysis



Phylogenetic

Genome search revealed in *N. gerenzanensis* ATCC 39727 ten distinct YjgF/YER057c/UK114 (Rid) proteins.

Molecular phylogeny allowed us to assign tentatively these proteins to the following subfamilies:

Rid Family	Genes
RidA	SBO92579.1 SBO90862.1
Rid1	SBO91465.1
Rid3	SBO98760.1
Rid6	SBO94674.1
Rid7	SBO96935.1 (Rid7A) SBO95965.1 (Rid7B) SBP00267.1 (Rid7C) SBO92286.1

Rid7 protein

RidA and other seven families (Rid1 to Rid7) were identified in prokaryotes.

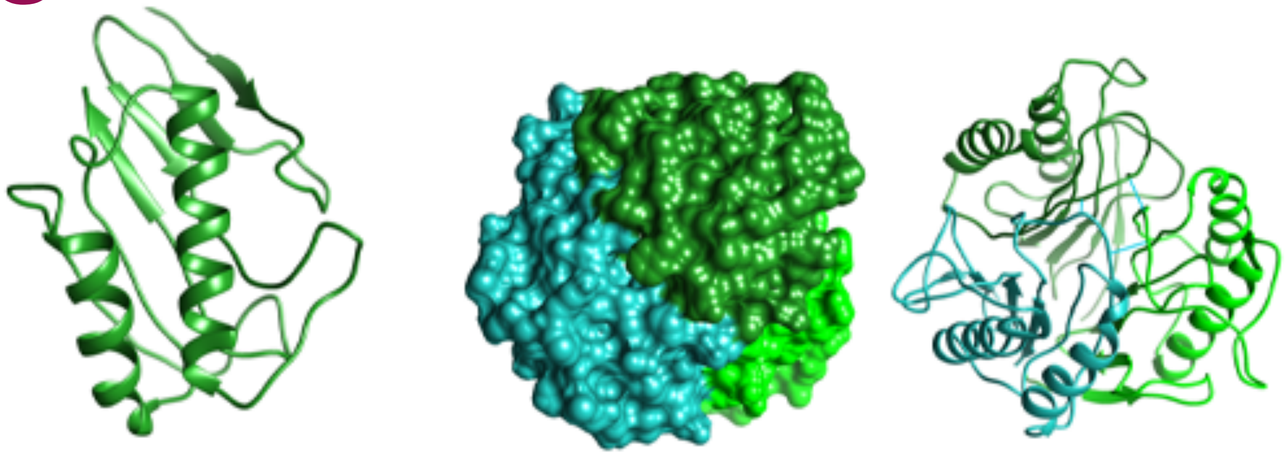
A conserved arginine (R) residue is shared by Rid members with the detoxifying activity.

Conversely, other proteins (e.g, Rid7) lack the R residue, and their role is mysterious.

Q94JQ4	YAKYFPAP----------SPA**R**STYQVAALPLN	***A. thaliana***	
P52758	YKQYFKSN----------FPA**R**AAYQVAALPKG	***H. Sapiens***	
P52759.3	YKTYFQGN----------LPA**R**AAYQVAALPKG	**RatL *(R. norvegicus)***	
SBO96935.1	LAARLG----------AAGAAPATTMLGVTRLAIP	**Rid7A**	
SBO95965.1	FNAGIEQA----------AREFDLATPPAALIGVEVLFEP	**Rid7B**	
SBP00267.1	YREYMG----------E--HDVPSTLLGVTVLGYT	**Rid7C**	
SBO92286.1	YRAYMG----------A--HDVPSTLLGVTVLGYN	**Rid7**	**Rid from *N. gerenzanensis***
SBO94674.1	IHEVFCKV----------RPACTGV**R**VAGLVDP	**Rid6**	
SBO91465.1	LAEVFGDQ----------GRHA**R**SAVGVAALPLD	**Rid1**	
SBO96592.1	YGEFFDES----------GPA**R**TTVAVHQLPHP	**Rid2**	
SBO90862.1	YAEFFDEE----------GPT**R**TTVAVHQLPHP	**Rid2**	
SBO98760.1	YRQFFANTDLASGQPVPVPLGTAPPAPPLEVNRARPA**R**VTIEIANLPVA	**Rid3**	
SBO92579.1	YKSYFNQP----------YPV**R**TTVGSDLMD--	**Rid7**	
Q7CP78	YEAFFTEH----------NATFPA**R**SCVEVARLPKD	***S. typhimurium* LT2**	

In silico modeling

Model of Rid7C



We used I-Tasser (Yang et al., 2015) to predict the structure of the Rid7A, Rid7B and Rid7C.

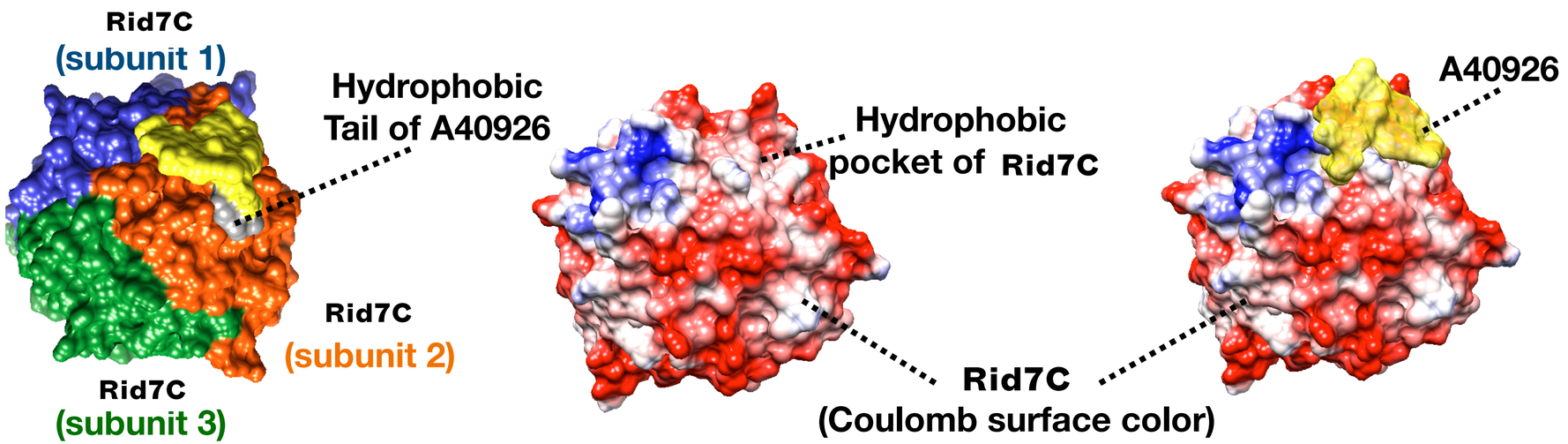
Various 3D crystal structures were used as templates to build I-Tasser models. The 4O4I (Tubulin-Laulimalide-Epothilone A complex) 3D crystal structure was used to predict the Rid7C ligand binding site.

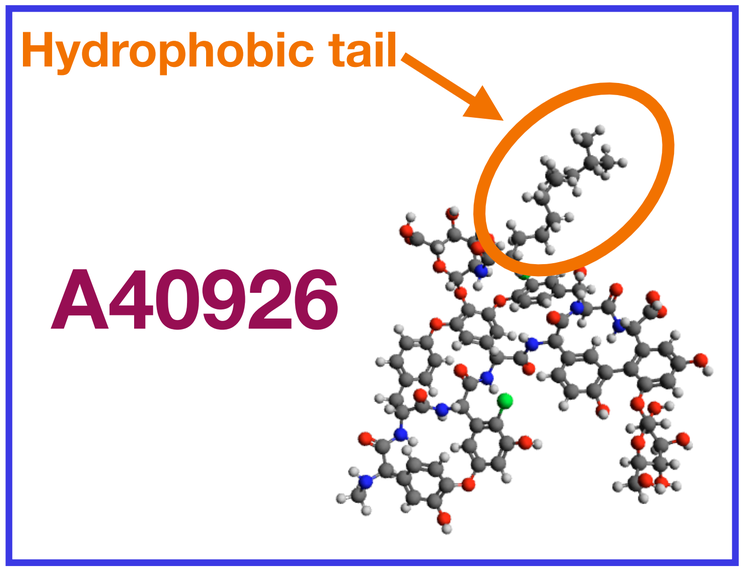
Laulimalide is an antibiotic....so we tested the binding with A40926

Yang, Jianyi, et al. "The I-TASSER Suite: protein structure and function prediction." *Nature methods* 12.1 (2015): 7-8.

In silico analysis: Binding Rid7C & A40926

Tool: Autodock (Morris et al., 2001)





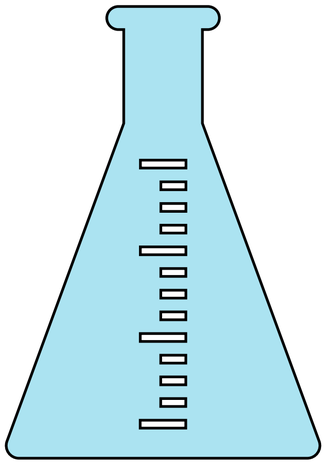
According to the docking results, the binding between Rid7C and A40926 is possible.

The binding could occur via the hydrophobic tail of the A40926.

So we decided to test this finding in vitro ...

Morris, Garrett M., et al. "AutoDock." *Automated docking of flexible ligands to receptor-User Guide* (2001).

In vitro analysis

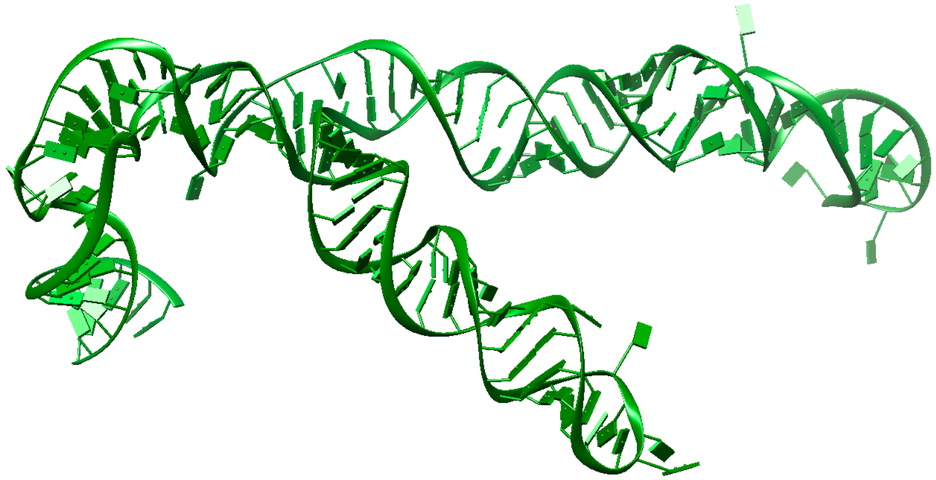


In vitro endoribonuclease activity

Sequence of the riboprobe

GGGGCGGGGGTTGTGGGATATTGATGGCGGCAGATGGAGTTGACACCATAAATAGGTGTGGACTTCCGTTTGCCTATTGCGTTAT

GCTGCCCTCCCTCGCCTTCGTATCCTCCGGAAGGACCTCTGTTGGCAGCCTCGCGCAACGCCTCTCCCGTACCCGCCGGTCCCCG



3D model of the riboprobe

RNA degradation experiments:

a 173 bp DNA fragment corresponding to the 5’-UTR leader sequence (129 bp) and the first coding (44 bp) regions of *rpoB(R)* was used as a template to carry out in vitro transcription.

The riboprobe was used in in vitro RNA digestion experiments

In vitro endoribonuclease activity

riboprobe

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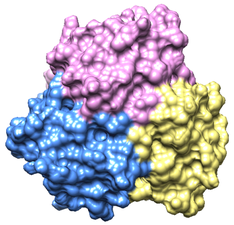
Rid7A

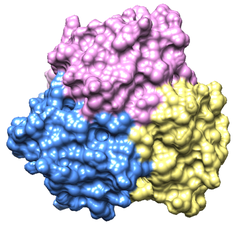


→ Riboprobe Digestion?

riboprobe

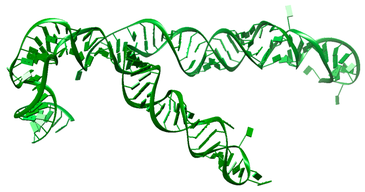
\+

Rid7B



→ Riboprobe Digestion?

riboprobe



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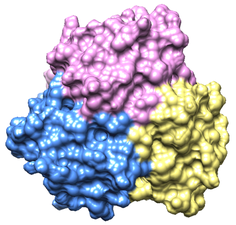
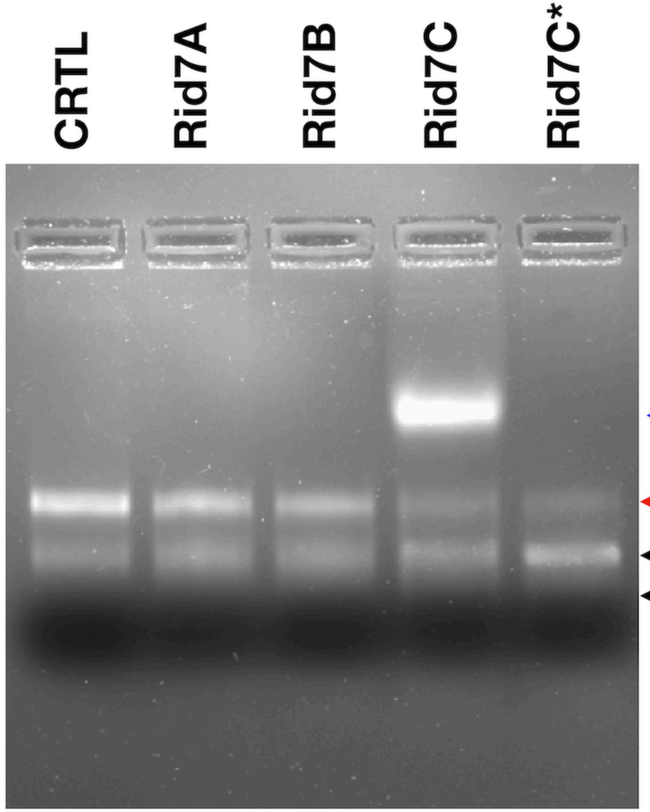
Rid7C



→ Riboprobe Digestion?

In vitro endoribonuclease activity



30°C
pH 7.8
$MgCl_2$ (1 mM)

The results of in vitro digestion were analyzed with an agarose gel. The Rid7C protein had an additional band, which was not the riboprobe but co-purified with the Rid7C protein.

band with molecular weight similar to RNA M1

In vitro synthesized Riboprobe [RpoB(R) 5'-UTR]

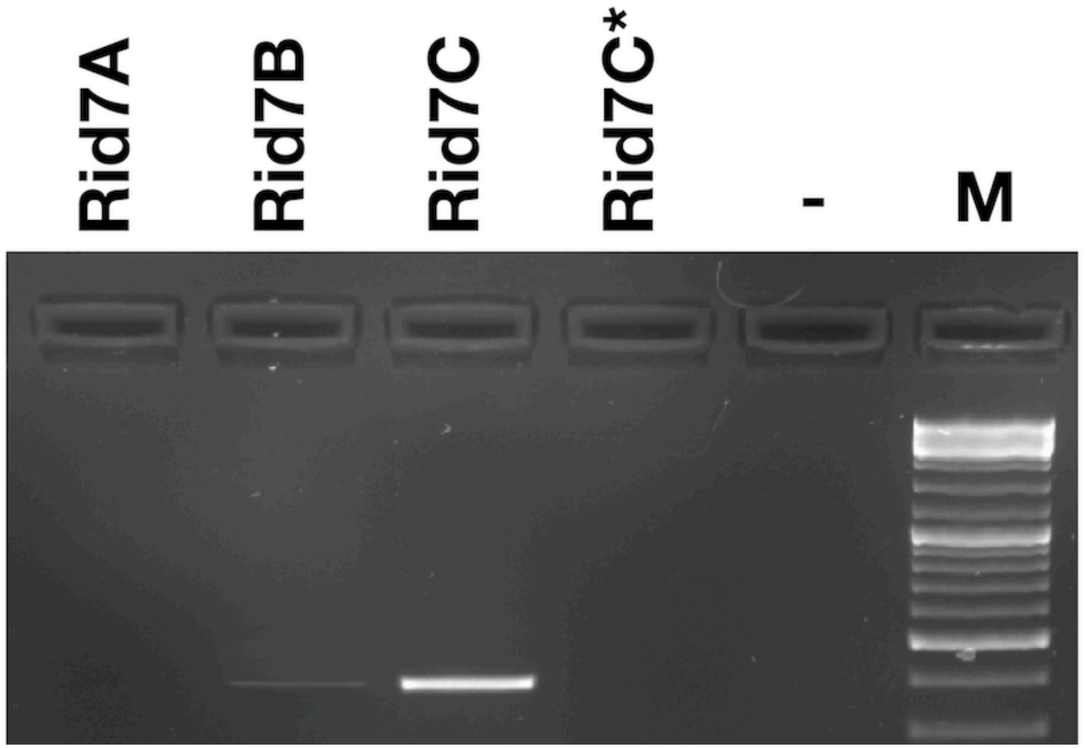
Degradation product of Riboprobe

Rid7C*=Rid7C purified with UREA (purified without RNA M1)

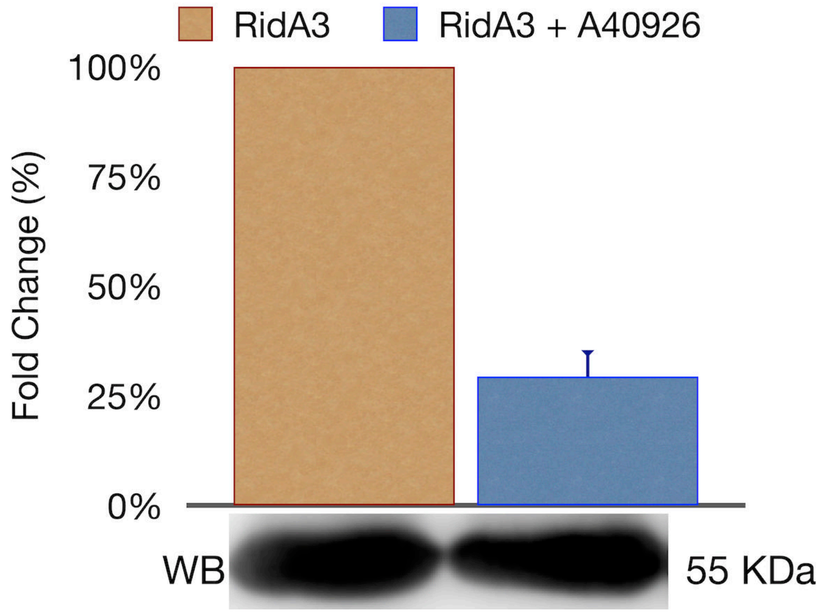
Rid7C* and Rid7C show better endoribonuclease activity

Binding between Rid7C and RNA M1

RNA M1 is the RNA component of RNaseP. This is a ribozyme involved in pre-tRNA maturation.



RT-PCR
Rid7C co-purification with RNA M1



RTq-PCR
Incubation with A40926 affects the binding and reduces the quantity of RNA M1 of 70%

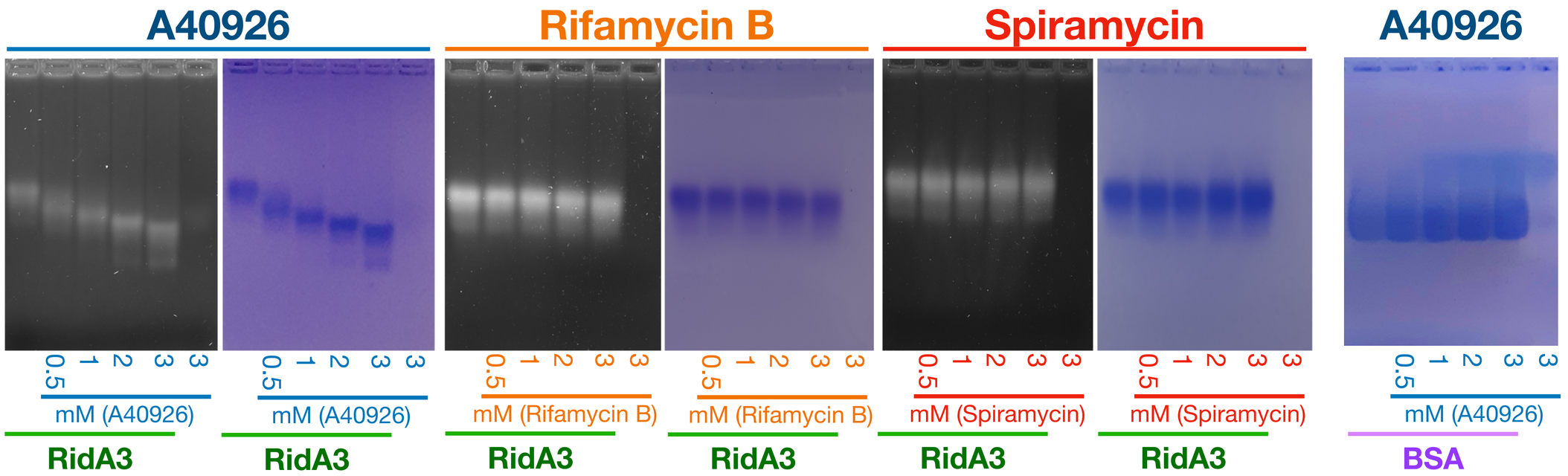
Binding between A40926 and Rid7C: effect on endoribonuclease activity

The A40926 binds Rid7C but does not bind BSA

Rifamycin B and Spiramycin does not bind Rid7C

→ Specificity of binding

Binding *in vitro*



Binding between A40926 and Rid7C: effect on endoribonuclease activity

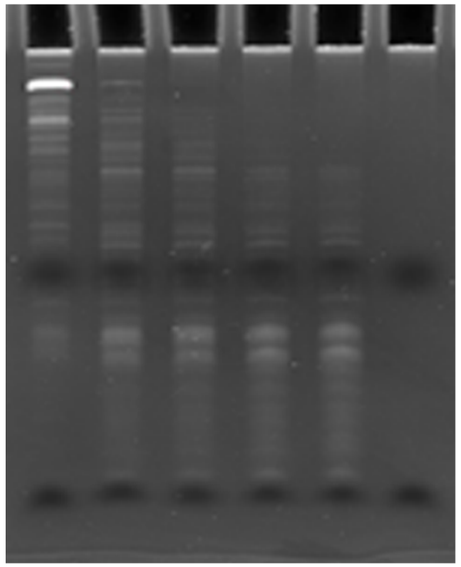
Time-course experiments

The product of nuclease activity was analyzed by 6% denaturing (8M urea) polyacrylamide gels.

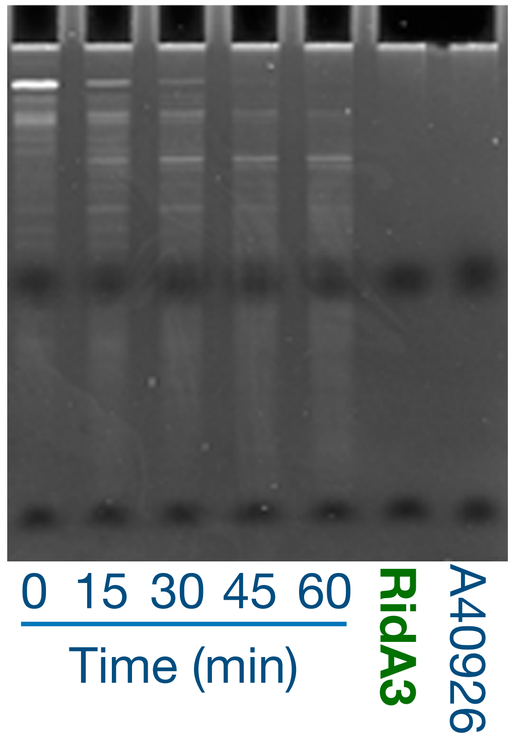
→

Only A40926 negatively modulates the Rid7C endoribonuclease activity

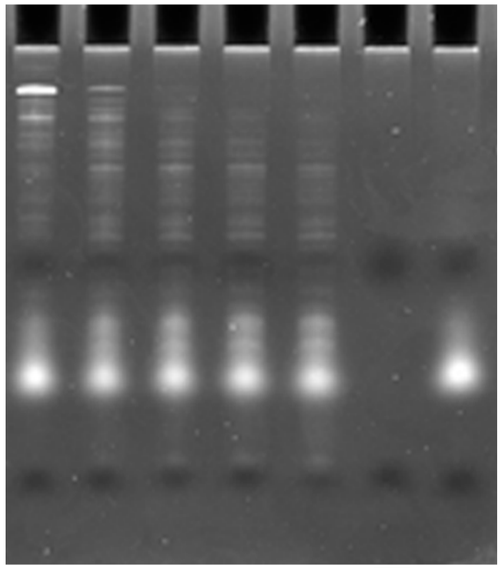
Control



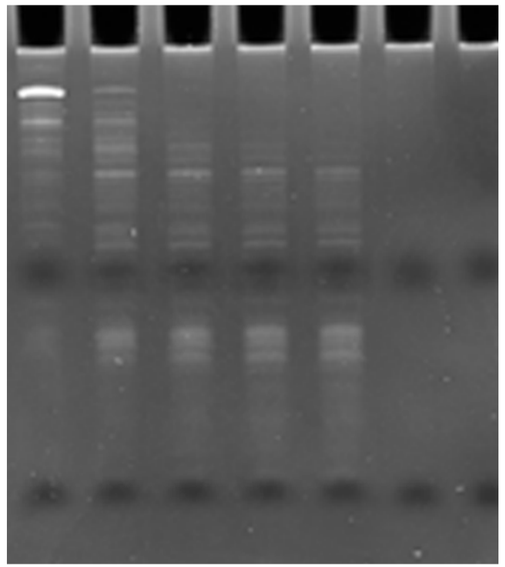
A40926



Rifamycin B



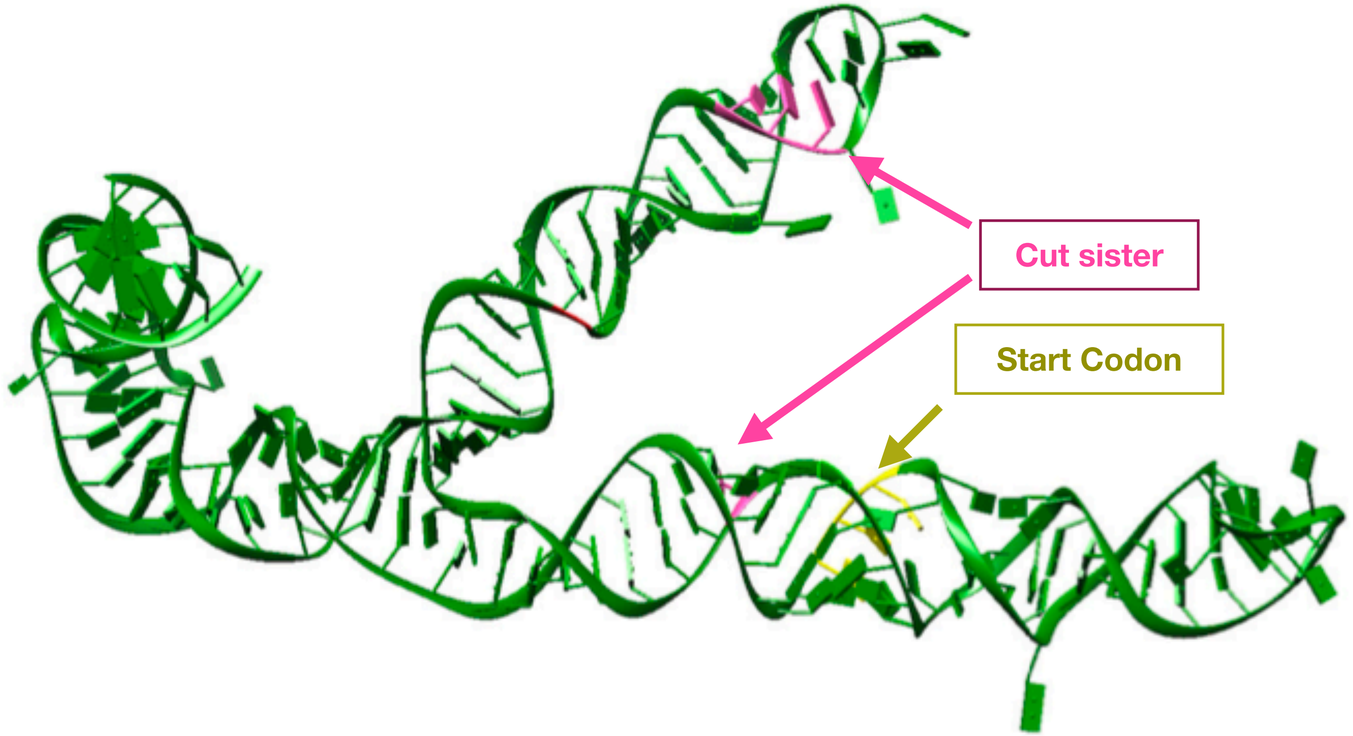
Spiramycin



RidA3: 5’-RACE (Rapid Amplification of cDNA Ends)

5’ rapid amplification of cDNA ends (5’-RACE) was performed with RNA fragments obtained from Rid7C ribonuclease activity

DNA sequencing of 5’-RACE products led to identify the 5’-ends of two degradation products (Site 1 and Site 2)



Cut Site 1 Cut Site 2

GGGGCGGGGGTTGTGGGATATTGATGGCGGCAGATGGAGTTGACACCATAAATAGGTGTGGACTTCCGTTTGCCTATTGCGTTAT

GCTGCCCTCCCTCGCCTTCGTATCCTCCGGAAGGACCTCTGTTGGCAGCCTCGCGCAACGCCTCTCCCGTACCCGCCGGTCCCCG

Coding sequence

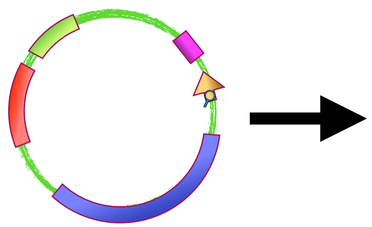
In vivo analysis

Streptomyces lividans: model organism

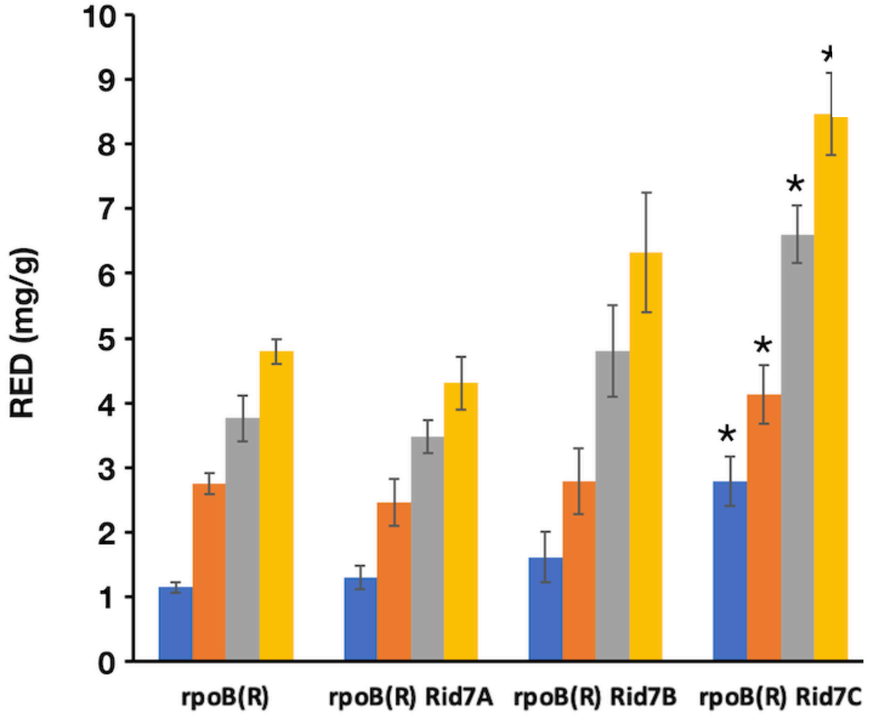
Producers of two antibiotics undecylprodigiosin (RED) and actinorhodin (ACT).

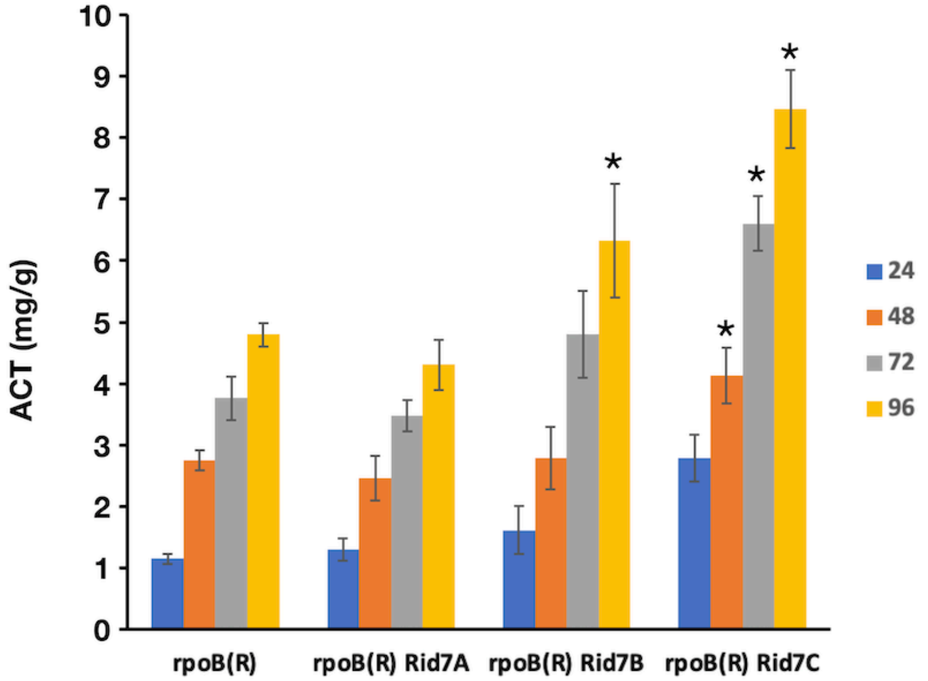
We used molecular biology to introduce Rid7 genes (A, B or C) and *rpoB(R)* into this microorganism.

Rid7A
or
Rid7B + RpoB(R)
or
Rid7C



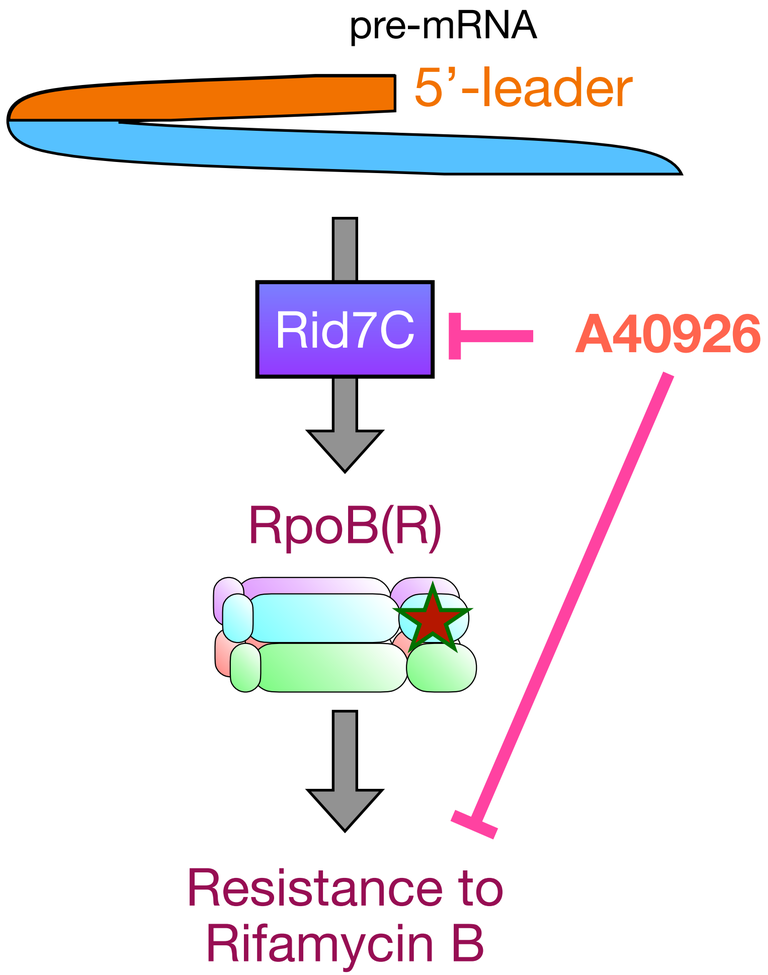
S. lividans

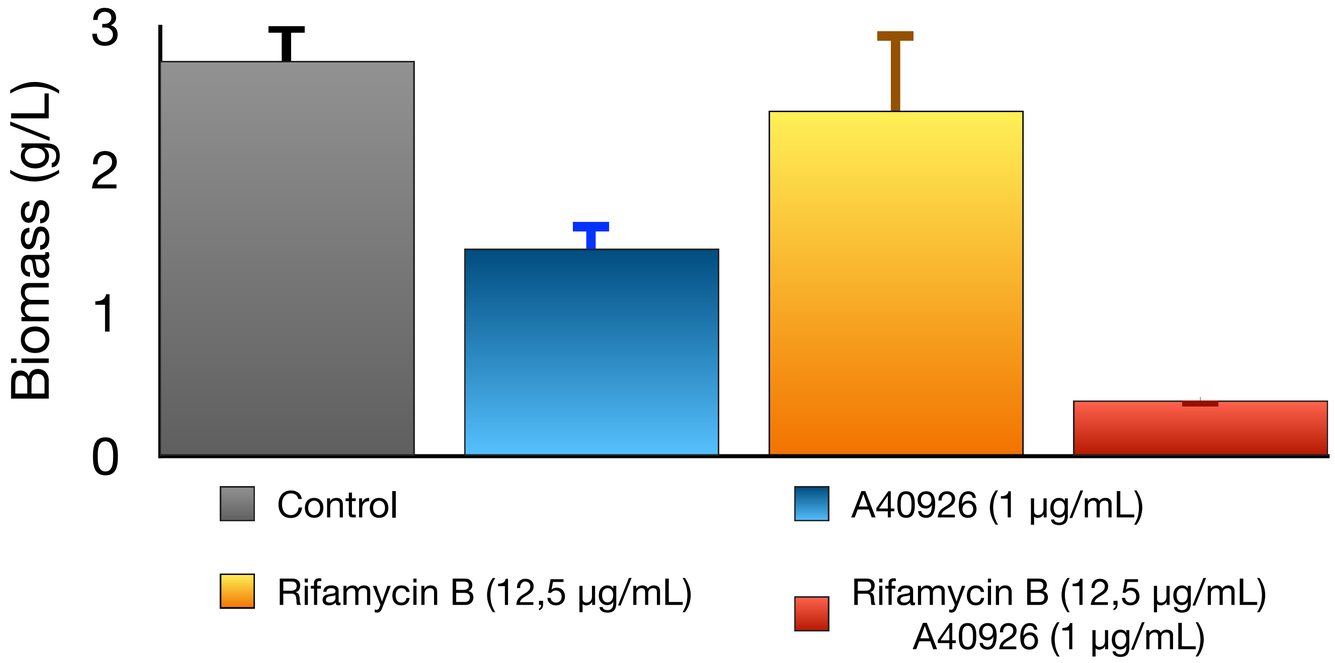




The Rid7C with the *rpoB(R)* induces a significant increase in antibiotic production.

In vivo inhibition of rifamycin resistance by A40926

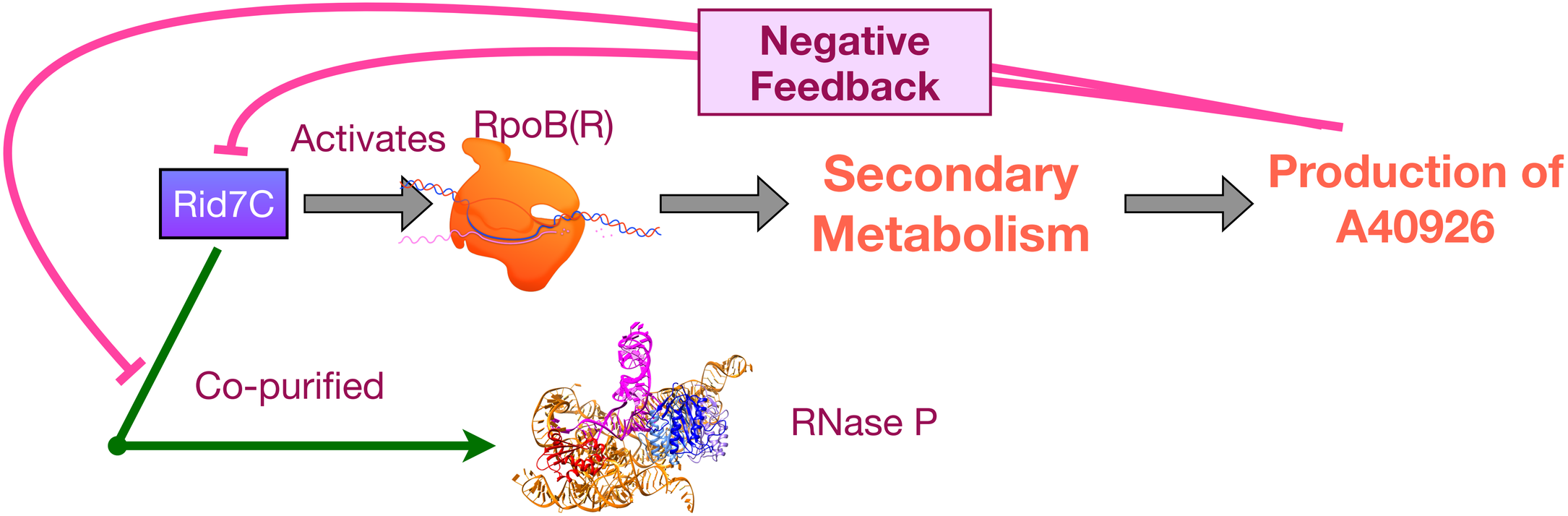




N. gerenzanensis is naturally resistant to rifamycin B. The bacterium shows sensitivity when A40926 and rifamycin B are administered simultaneously. This is because A40926 blocks the maturation of RpoB (R) mRNA, which gives resistance to rifamycin B.

Conclusions

- Rid7A, Rid7B e Rid7C are endoribonucleases
- Rid7C is involved in *rpoB(R)* mRNA maturation
- Rid7C co-purified with RNA M1
- The A40926 negatively regulates the endoribonuclease activity of di Rid7C



We have reported/discussed the full results in this paper

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RESEARCH ARTICLE

Rid7C, a Member of the YjgF/YER057c/UK114 (Rid) Protein Family, Is a Novel Endoribonuclease That Regulates the Expression of a Specialist RNA Polymerase Involved in Differentiation in *Nonomuraea gerenzanensis*

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Fabrizio Damiano and Matteo Calcagnile contributed equally to this work. Author order was determined in order of decreasing seniority.

Thanks!