MaCuMBA

Transcriptomics and Metatranscriptomics

Wolfgang R. Hess

COURSE on "METAGENOMICS"

December 9-13, 2013

Campus de San Juan de la Universidad Miguel Hernández, Alicante, Spain

Marine microorganisms:

cultivation methods for improving their biotechnological applications

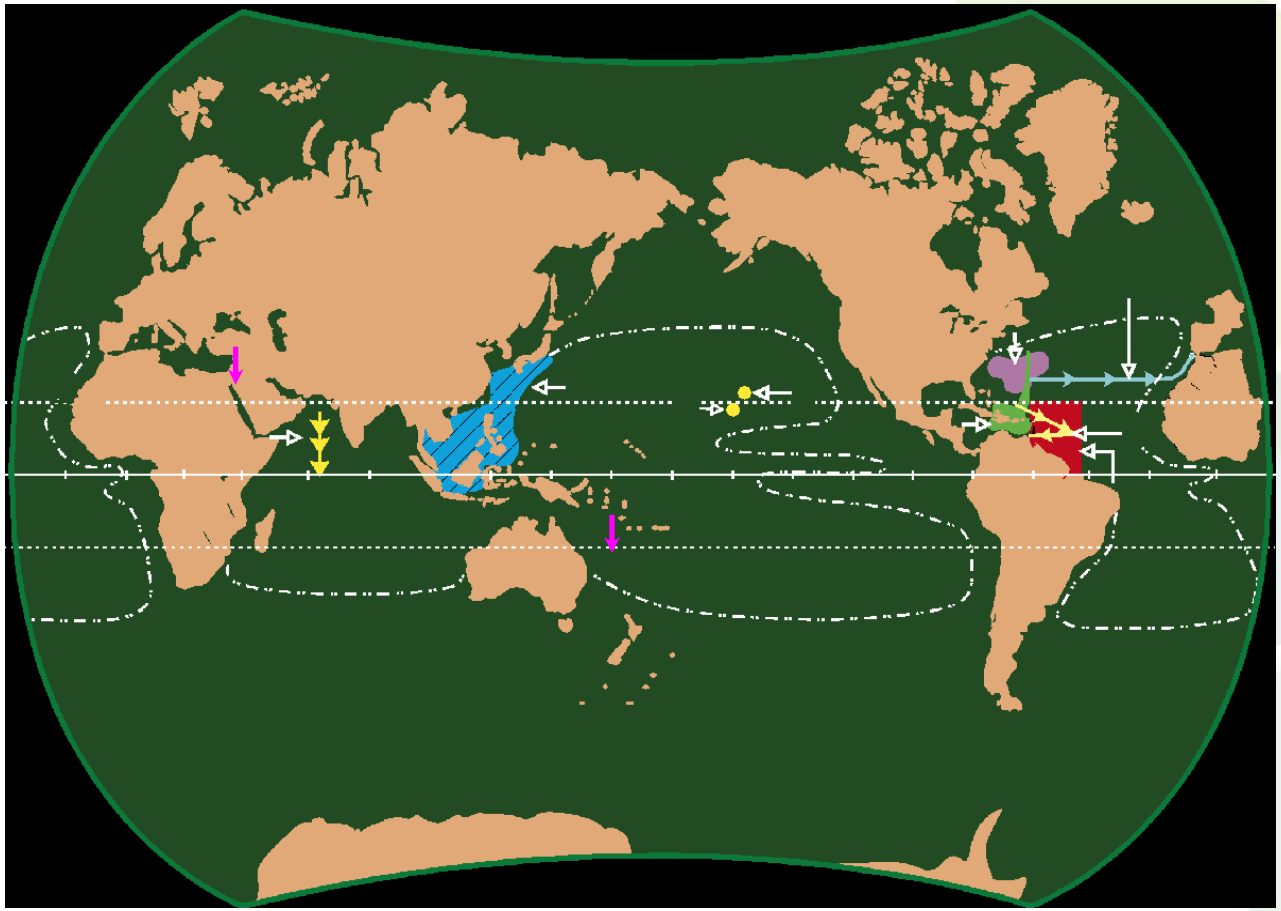
Topics covered

(1) Why doing Transcriptomics and Metatranscriptomics?

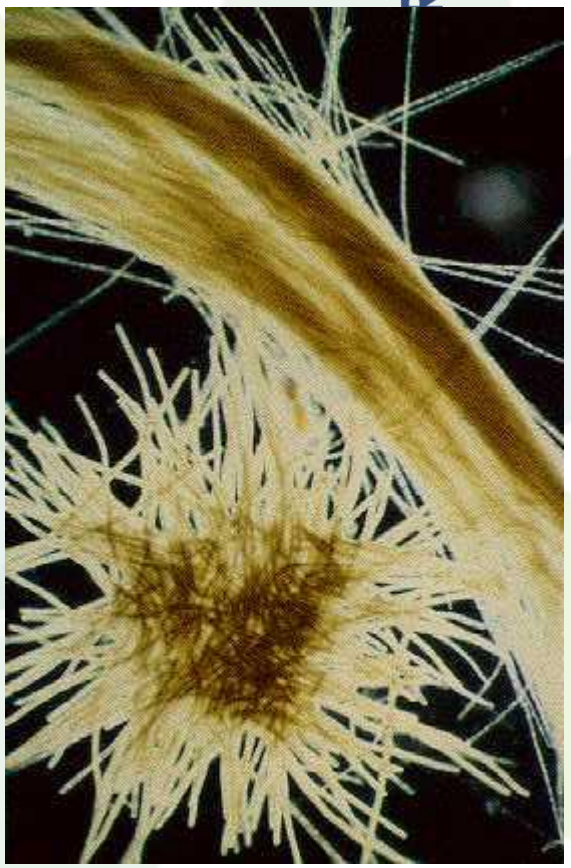
(2) How to do it?

(3) What kind of things can we find?

The organisms: *Trichodesmium* global distribution



Capone et al., 1997



picture: B. Bergman

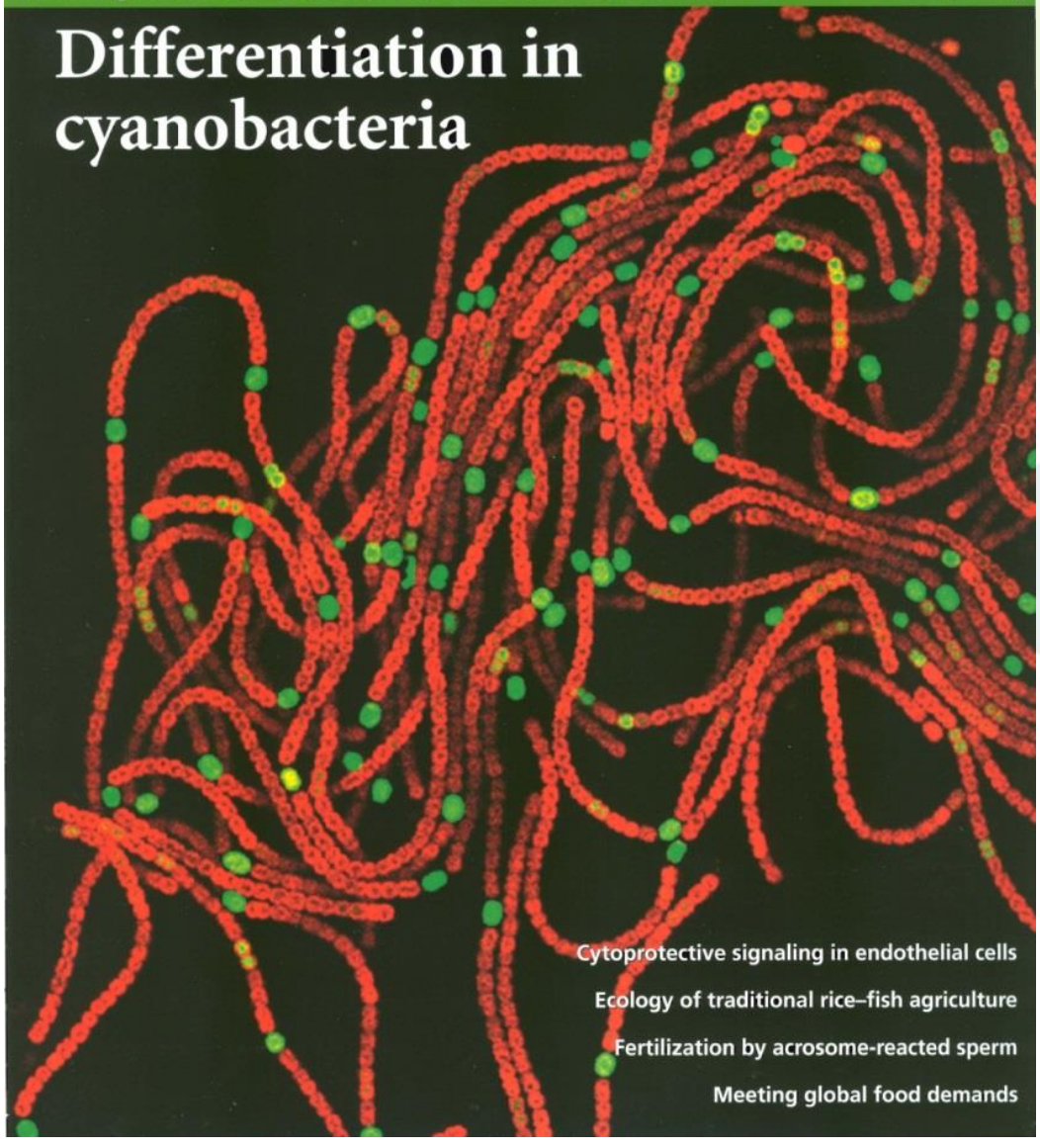
Trichodesmium is an enigmatic organism: a major contributor of ‚new' nitrogen, multicellular, forming large blooms, capabable of apoptosis and coordinated behavior [Rubin, Berman-Frank & Shaked „Dust- and mineral-iron utilization by the marine dinitrogen-fixer Trichodesmium" Nature Geoscience 4, 529–534]

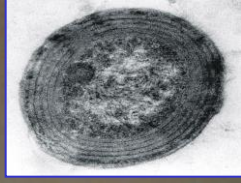
Anabaena PCC7120

December 13, 2011 | vol. 108 | no. 50 | pp. 19837–20272

PNAS

Proceedings of the National Academy of Sciences of the United States of America www.pnas.org





Variable pigmentation in cyanobacteria

Acaryochloris

(Chlorophyll *d*)

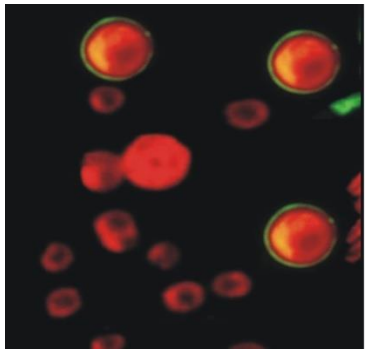
Synechococcus

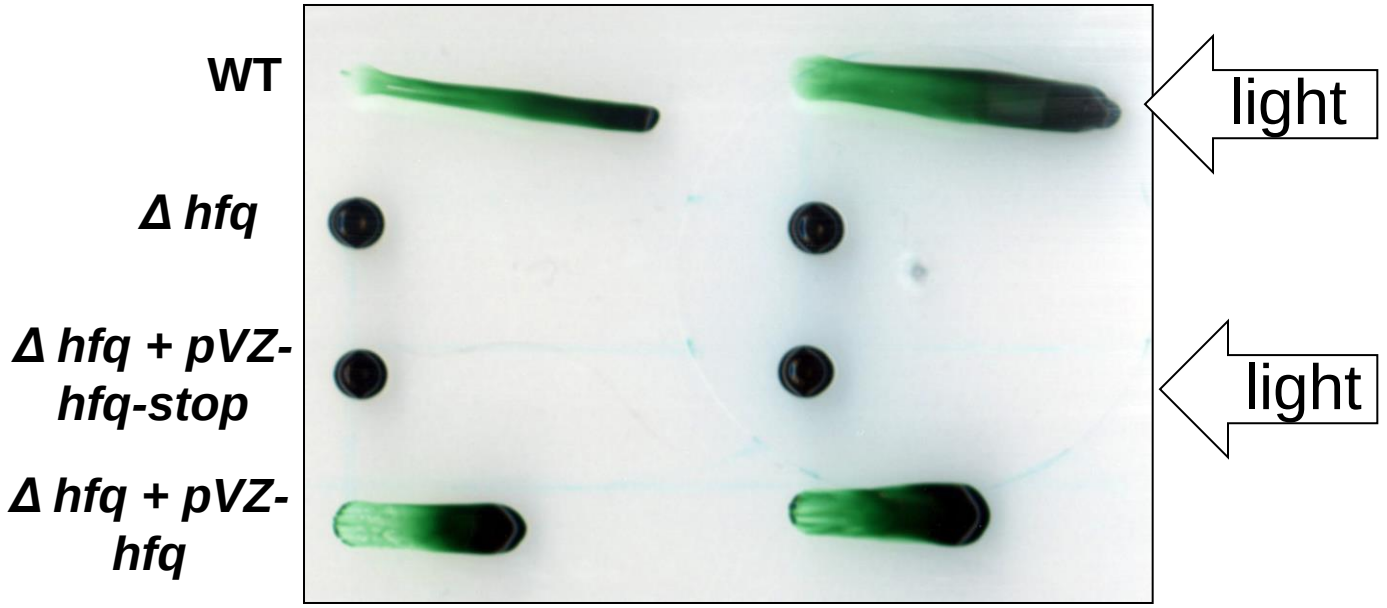
(Phycoerythrin)

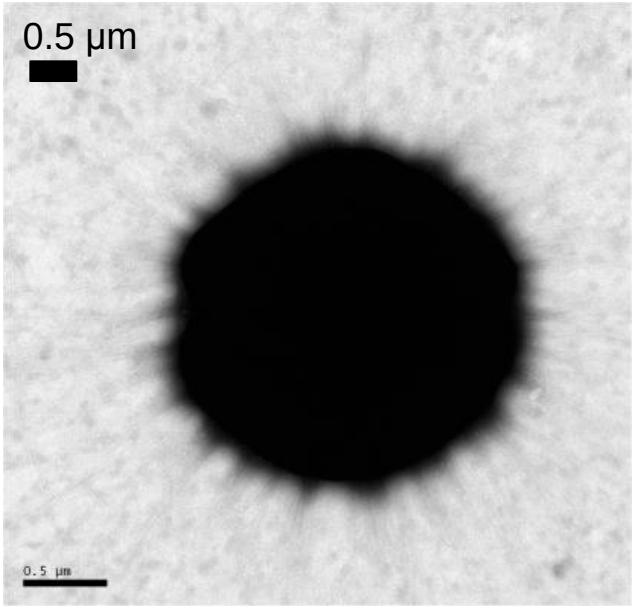
Prochlorococcus

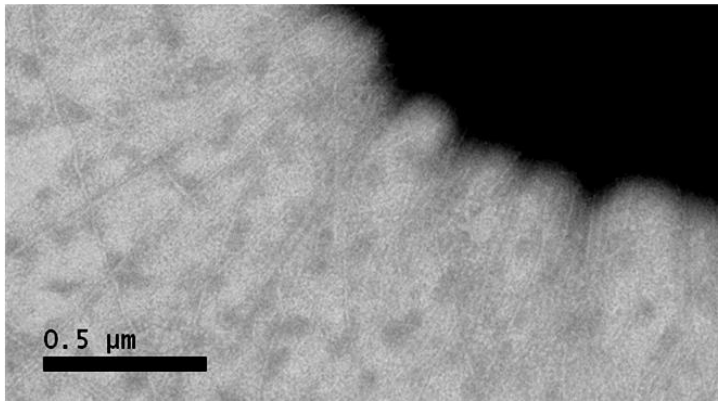
(DV-Chlorophylls *a*+*b*)

Synechocystis PCC6803





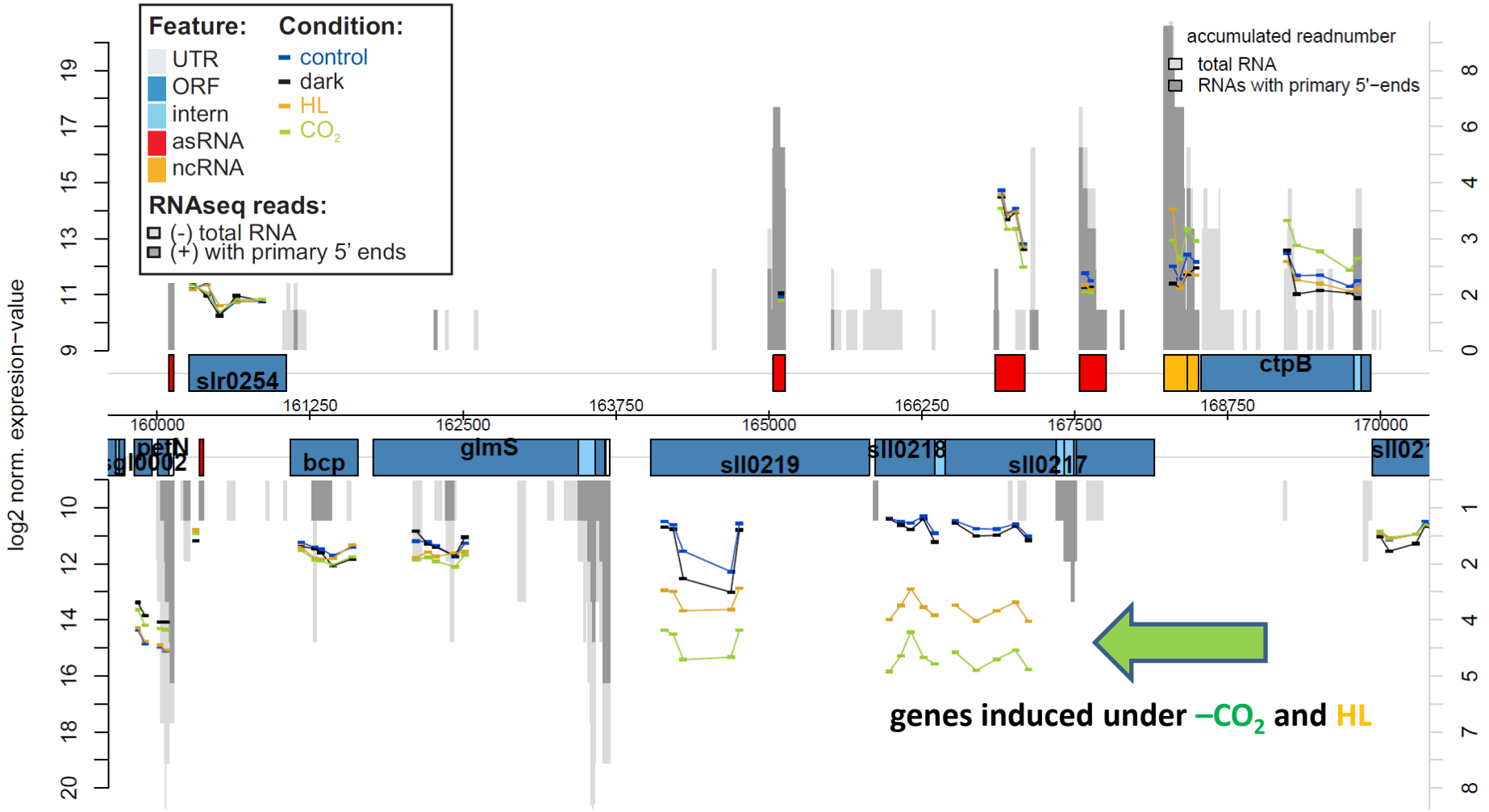




Dienst et al. (2008) *Microbiology* 154, 3134–3143

(1) Why doing Transcriptomics and Metatranscriptomics?

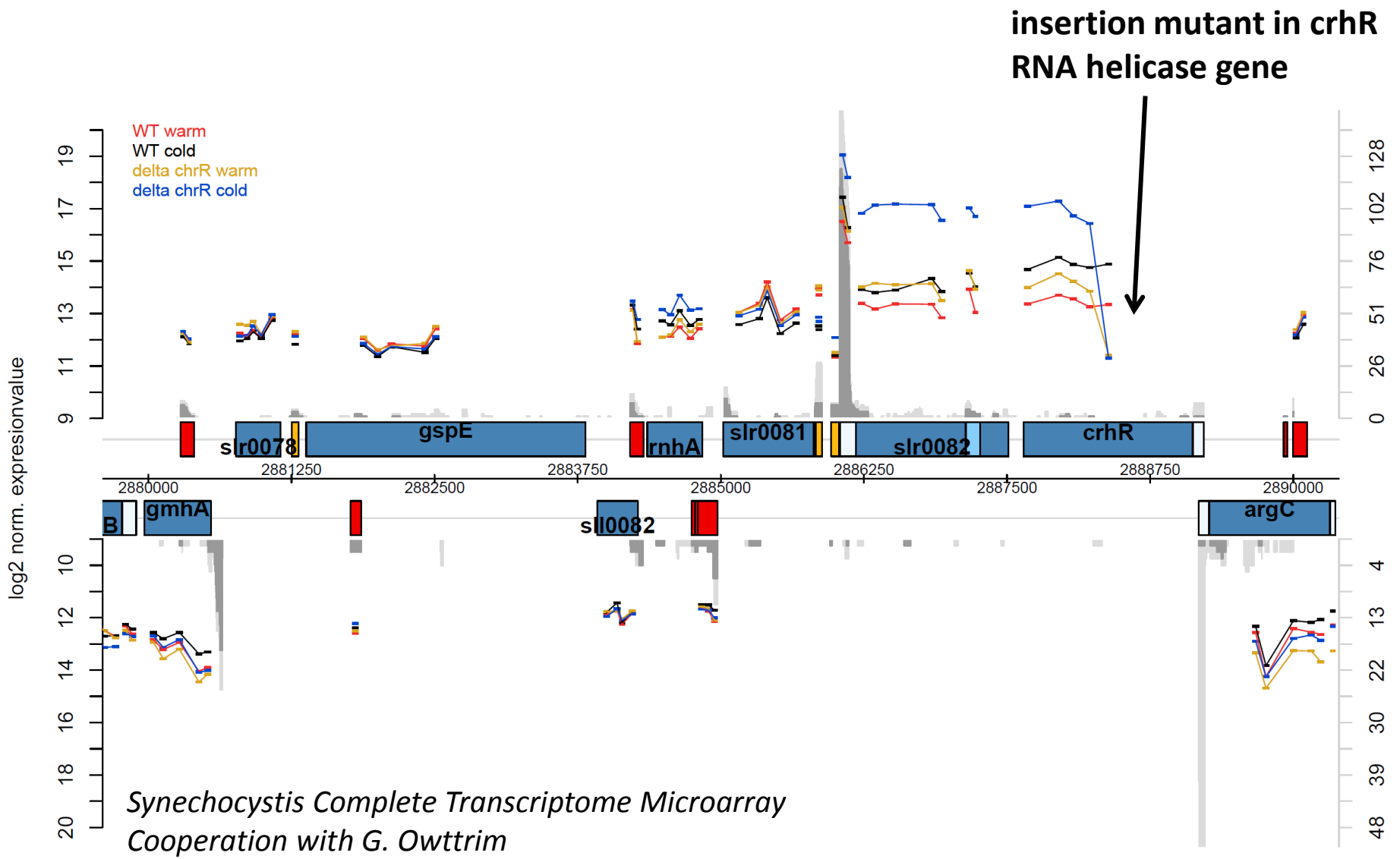
Methods that provide comprehensive information about the status of gene expression: under different conditions



Synechocystis Complete Transcriptome Microarray

sll0217-sll0219: flv4_flv2 operon, encoding an electron valve for photosystem II

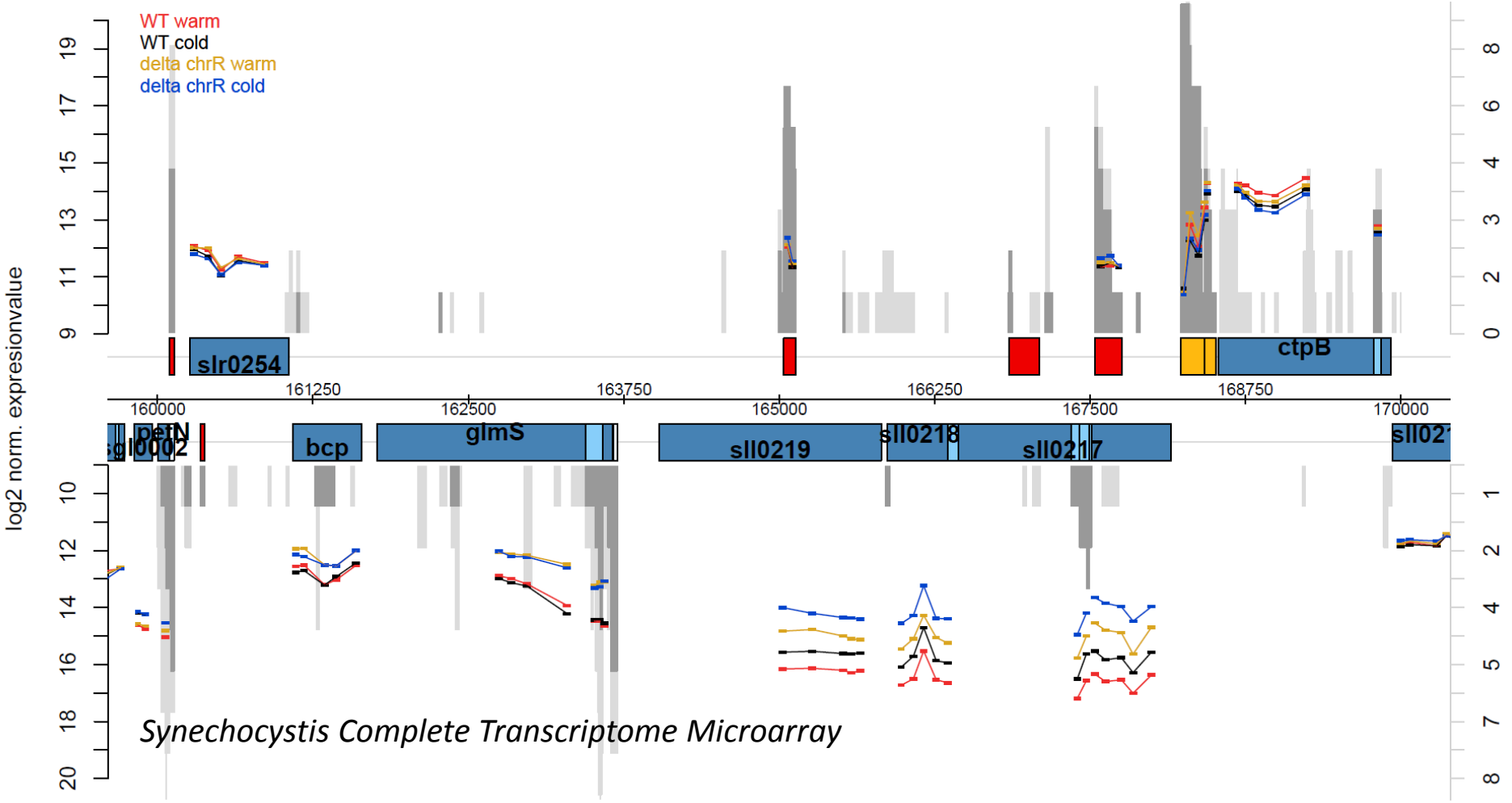
Methods that provide comprehensive information about the status of gene expression in mutant lines



Synechocystis Complete Transcriptome Microarray
Cooperation with G. Owttrim

Methods that provide comprehensive information about the status of gene expression in mutant lines

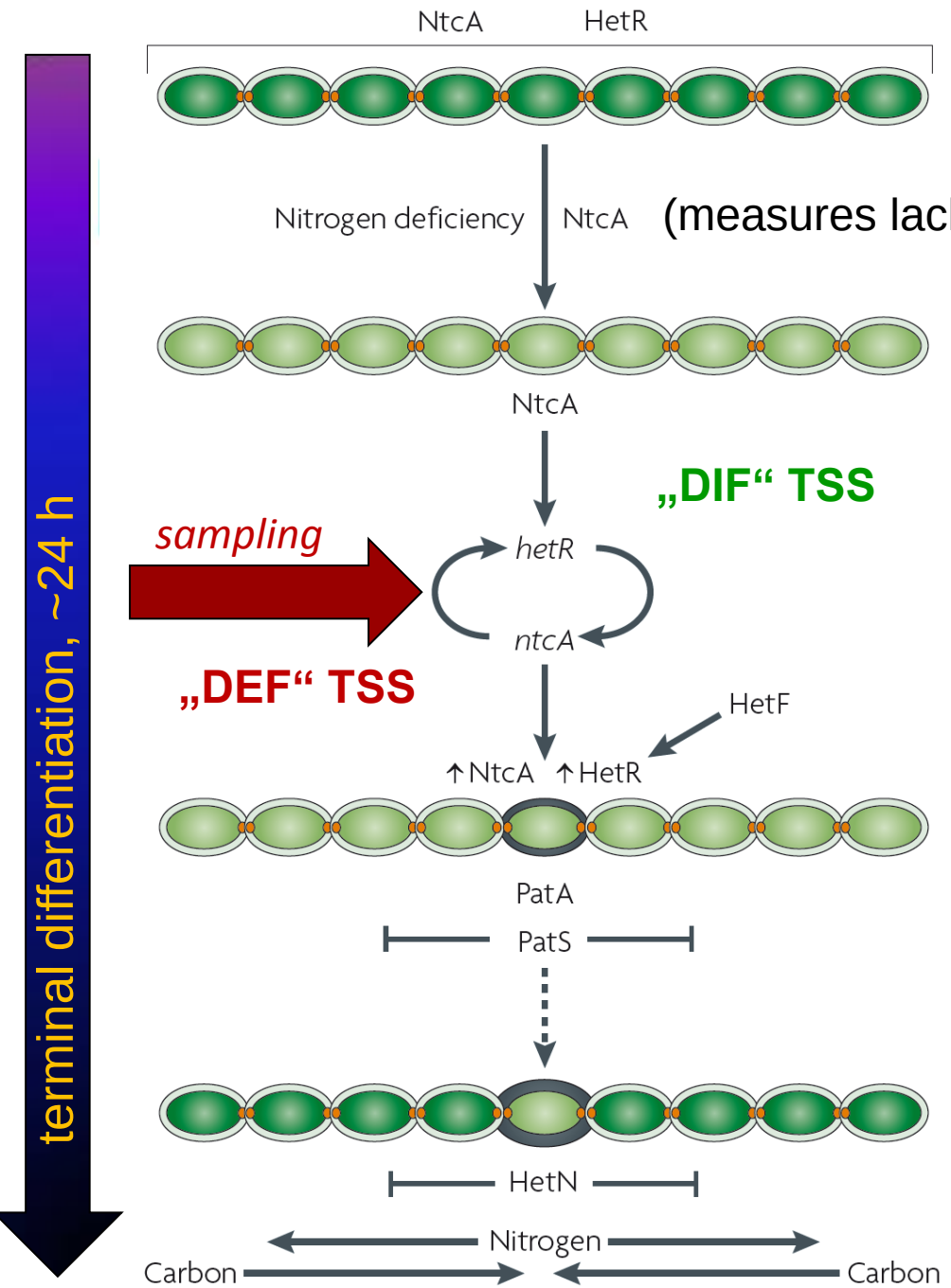
insertion mutant in crhR RNA helicase gene: effect on flv4_flv2 expression



Synechocystis Complete Transcriptome Microarray

Transcriptomic methods provide exact information about the status of gene expression under different conditions and in mutant lines and about the actually used TSS (promoters).

Anabaena PCC7120

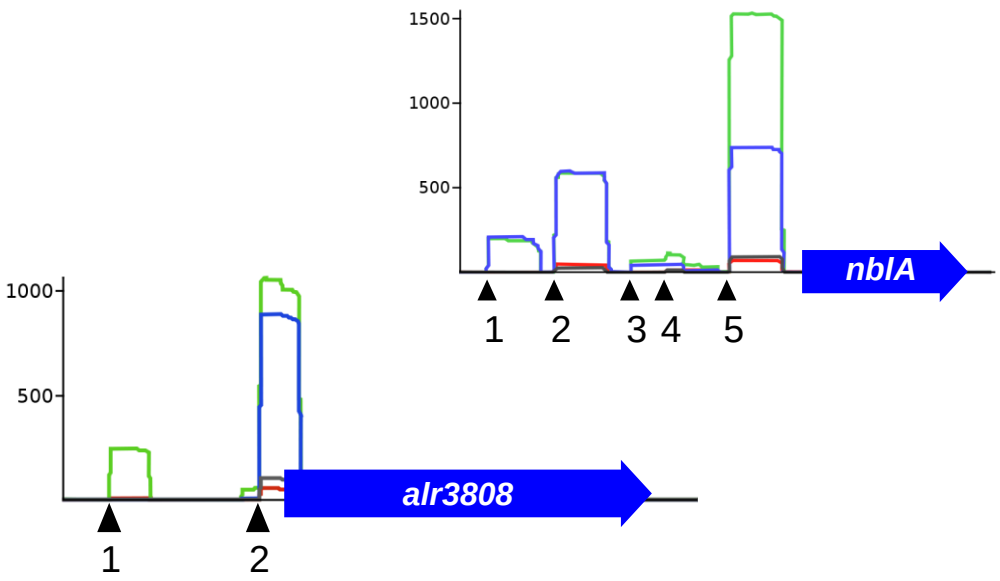


(measures lack of combined nitrogen in the cell)

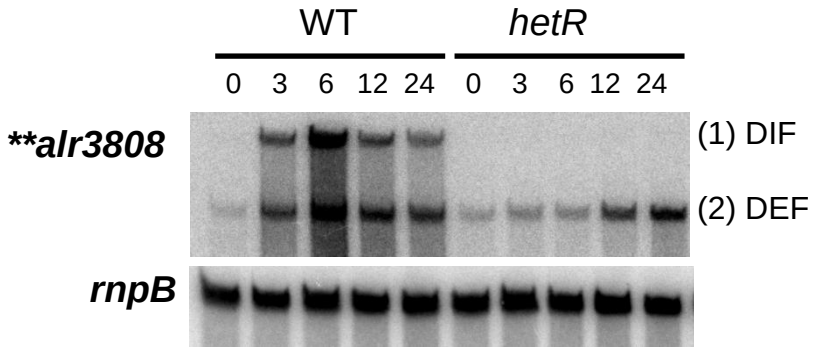
Flores & Herrero

NATURE REVIEWS | MICROBIOLOGY
VOLUME 8 | JANUARY 2010

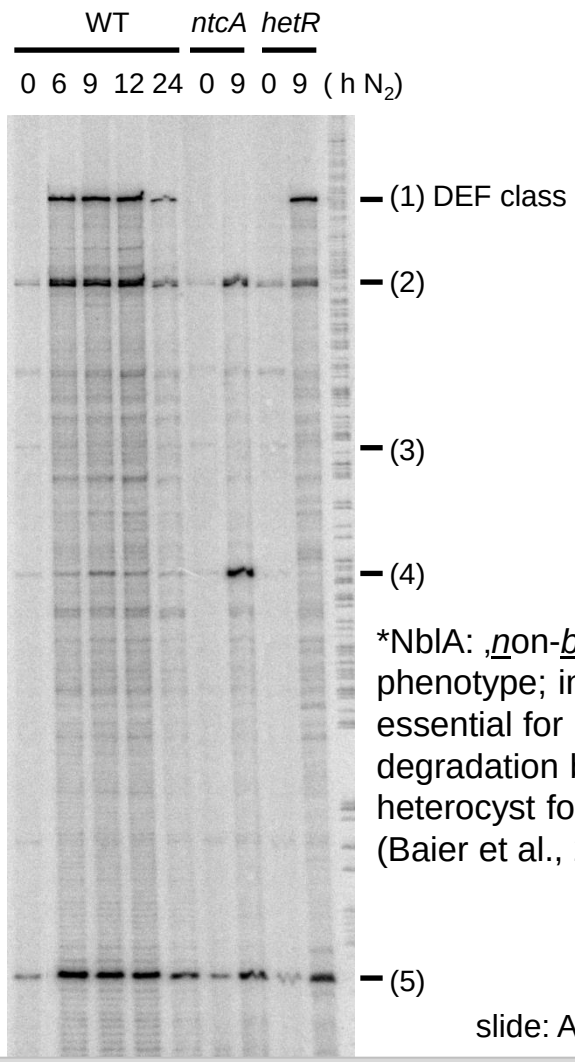
dRNA-seq of the cyanobacterium *Anabaena* 7120



***alr3808* encodes a DpsA homologue with known N-dependent regulation (Ow et al., 2008).



nblA*

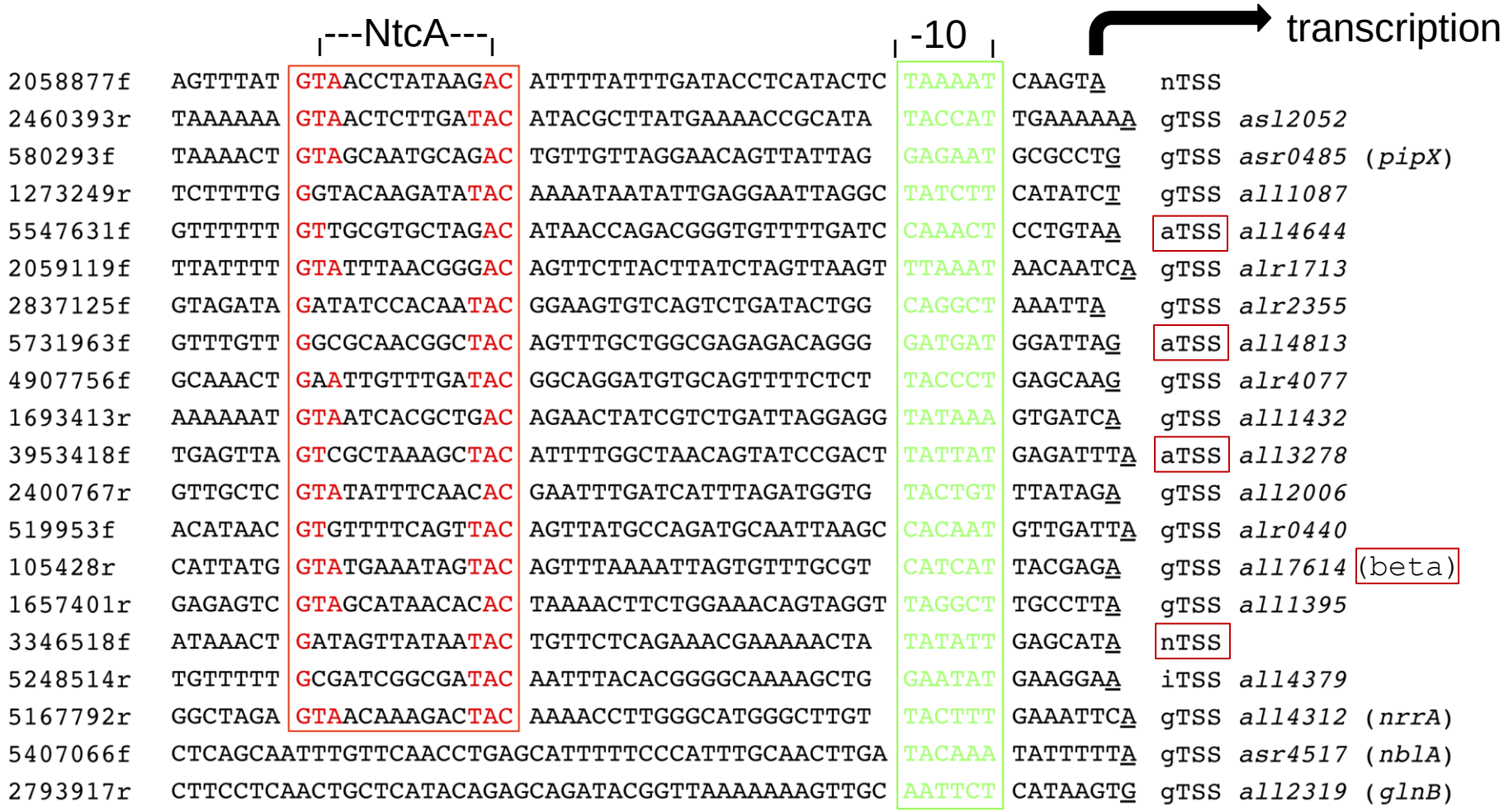


*NblA: ‚*non*-*bl*eaching' phenotype; in 7120 essential for PBS degradation but not for heterocyst formation (Baier et al., 2004).

slide: A. Muro-Pastor

WT NH_4^+ | WT 8 h N_2 | *ΔhetR* NH_4^+ | *ΔhetR* 8 h N_2

dRNA-seq provides exact information about the TSS (promoters) & allows the inference of binding sites for regulatory proteins



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2460393r	TAAAAAA	GTAACTCTTGATAC	ATACGCTTATGAAAACCGCATA	TACCAT	TGAAAAAA	gTSS	*as12052*	
580293f	TAAAACT	GTAGCAATGCAGAC	TGTTGTTAGGAACAGTTATTAG	GAGAAT	GCGCCTG	gTSS	*asr0485*	(*pipX*)
1273249r	TCTTTTG	GGTACAAGATATAC	AAAATAATATTGAGGAATTAGGC	TATCTT	CATATCT	gTSS	*all1087*	
5547631f	GTTTTTT	GTTGCGTGCTAGAC	ATAACCAGACGGGTGTTTTGATC	CAAACT	CCTGTAA	aTSS	*all4644*	
2059119f	TTATTTT	GTATTTAACGGGAC	AGTTCTTACTTATCTAGTTAAGT	TTAAAT	AACAATCA	gTSS	*alr1713*	
2837125f	GTAGATA	GATATCCACAATAC	GGAAGTGTCAGTCTGATACTGG	CAGGCT	AAATTA	gTSS	*alr2355*	
5731963f	GTTTGTT	GGCGCAACGGCTAC	AGTTTGCTGGCGAGAGACAGGG	GATGAT	GGATTAG	aTSS	*all4813*	
4907756f	GCAAACT	GAATTGTTTGATAC	GGCAGGATGTGCAGTTTTCTCT	TACCCT	GAGCAAG	gTSS	*alr4077*	
1693413r	AAAAAAT	GTAATCACGCTGAC	AGAACTATCGTCTGATTAGGAGG	TATAAA	GTGATCA	gTSS	*all1432*	
3953418f	TGAGTTA	GTCGCTAAAGCTAC	ATTTTGGCTAACAGTATCCGACT	TATTAT	GAGATTTA	aTSS	*all3278*	
2400767r	GTTGCTC	GTATATTTCAACAC	GAATTTGATCATTTAGATGGTG	TACTGT	TTATAGA	gTSS	*all2006*	
519953f	ACATAAC	GTGTTTTCAGTTAC	AGTTATGCCAGATGCAATTAAGC	CACAAT	GTTGATTA	gTSS	*alr0440*	
105428r	CATTATG	GTATGAAATAGTAC	AGTTTAAAATTAGTGTTTGCGT	CATCAT	TACGAGA	gTSS	*all7614*	(beta)
1657401r	GAGAGTC	GTAGCATAACACAC	TAAAACTTCTGGAAACAGTAGGT	TAGGCT	TGCCTTA	gTSS	*all1395*	
3346518f	ATAAACT	GATAGTTATAATAC	TGTTCTCAGAAACGAAAAACTA	TATATT	GAGCATA	nTSS		
5248514r	TGTTTTT	GCGATCGGCGATAC	AATTTACACGGGGCAAAAGCTG	GAATAT	GAAGGAA	iTSS	*all4379*	
5167792r	GGCTAGA	GTAACAAAGACTAC	AAAACCTTGGGCATGGGCTTGT	TACTTT	GAAATTCA	gTSS	*all4312*	(*nrrA*)
5407066f	CTCAGCAATTTGTTCAACCTGAGCATTTTTCCCATTTGCAACTTGA			TACAAA	TATTTTTA	gTSS	*asr4517*	(*nblA*)
2793917r	CTTCCTCAACTGCTCATACAGAGCAGATACGGTTAAAAAAAGTTGC			AATTCT	CATAAGTG	gTSS	*all2319*	(*glnB*)

dRNA-seq of the cyanobacterium *Anabaena* 7120

Mitschke et al. (2011b) Proc. Natl. Acad. Sci. USA, *108*, 20130-20135.

Metatranscriptomics & the typical question of microbial ecology:

Who is there and what are they doing?

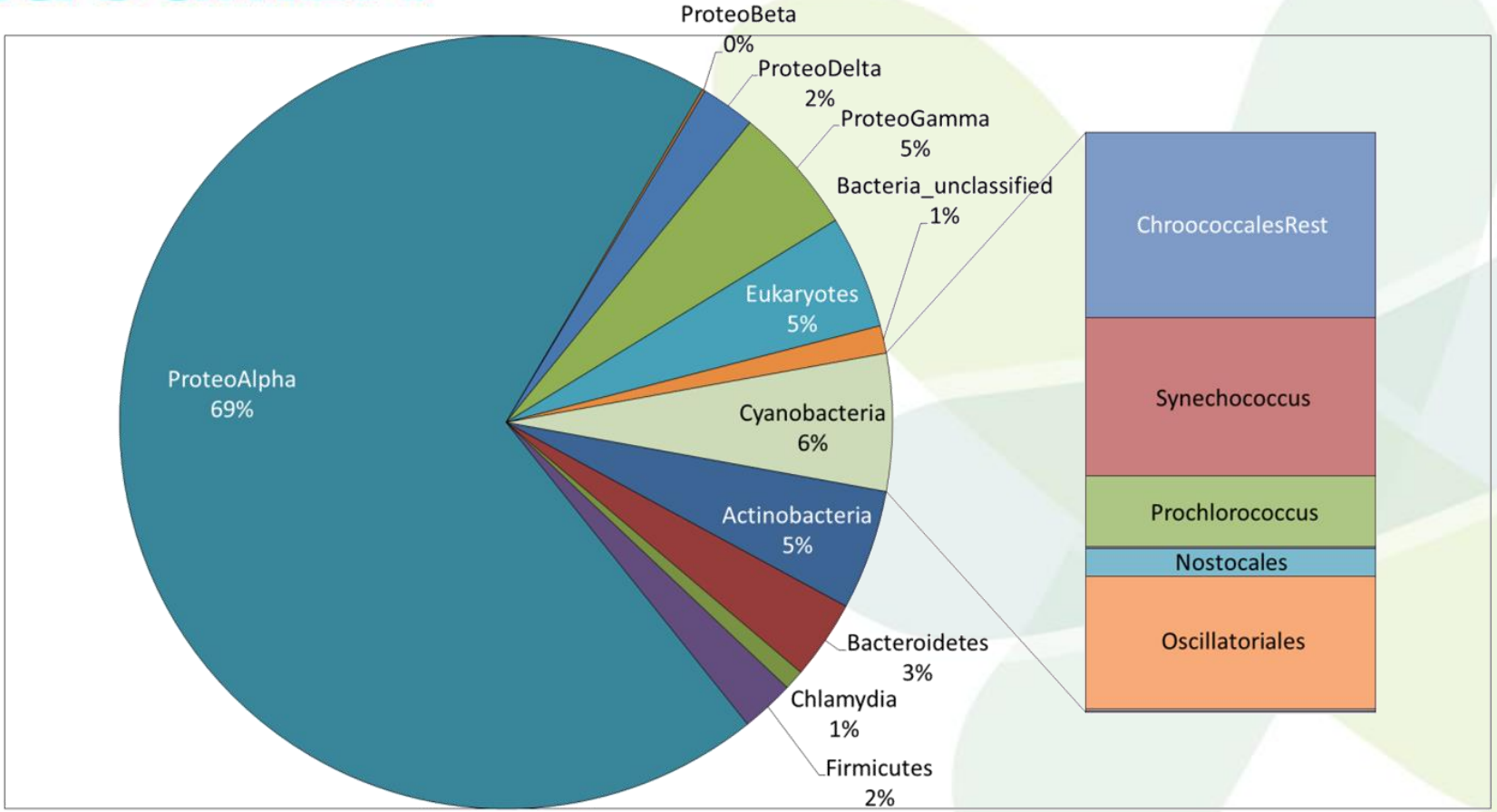
Methods that provide comprehensive information about the status of gene expression in populations

16S rDNA sequencing for bacteria and archaea RNA seq / dRNA seq **SW Pacific**

16S rDNA sequencing for bacteria dRNA seq / RNA seq **Red Sea**

Red Sea sampling 2012

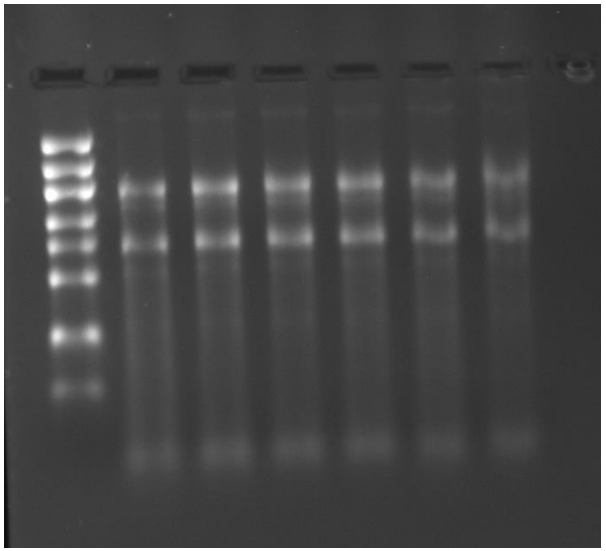
MaCuMBA



16S rRNA-based diversity

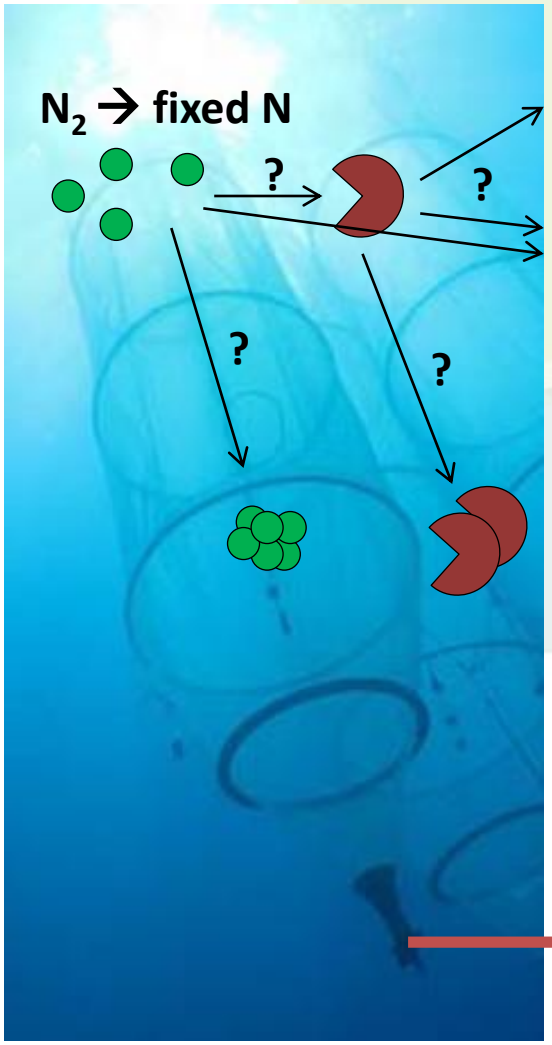
objective: nitrogen cycle

T12/13 24h sampling
M t0 t1 t2 t3 t4 t5

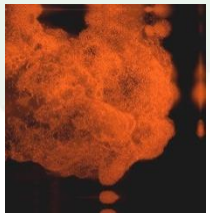


VAHINE total RNA

SW Pacific 2013



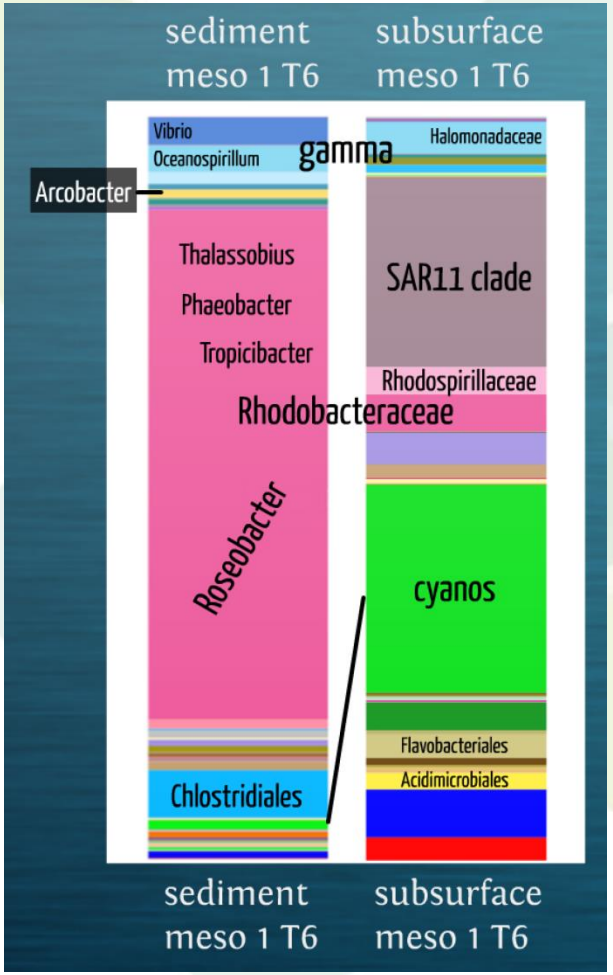
N_2 fixing cyanobacteria



collaboration with Sophie Bonnet

VAHINE sampling 2013

MaCuMBA



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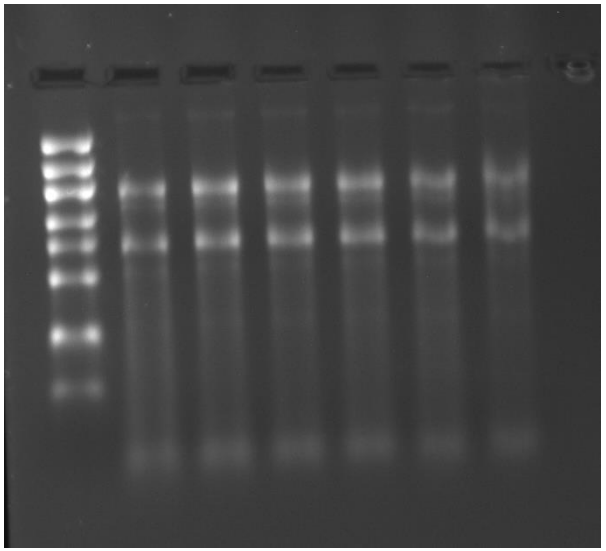

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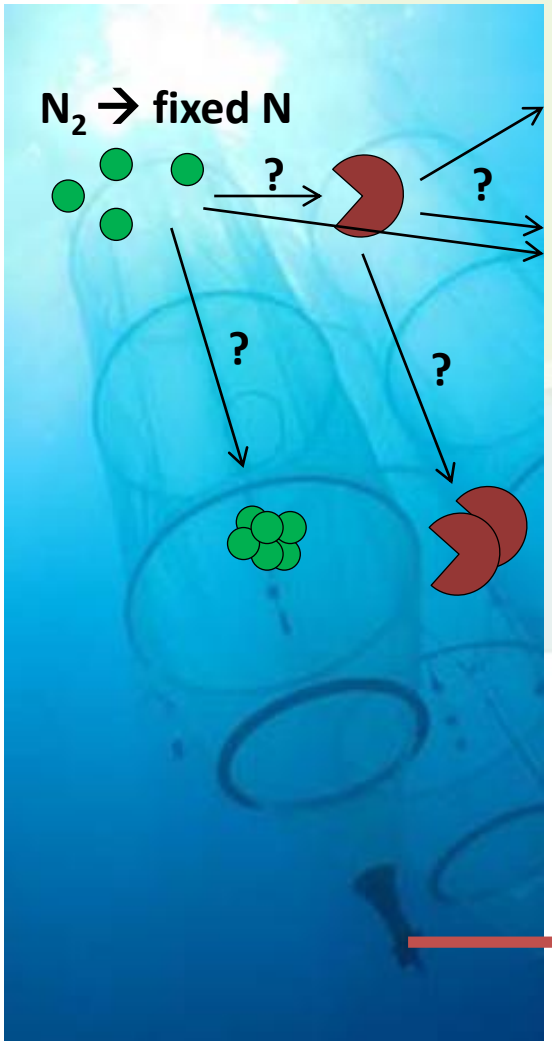

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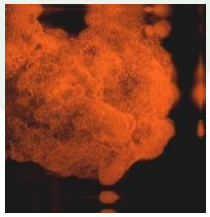
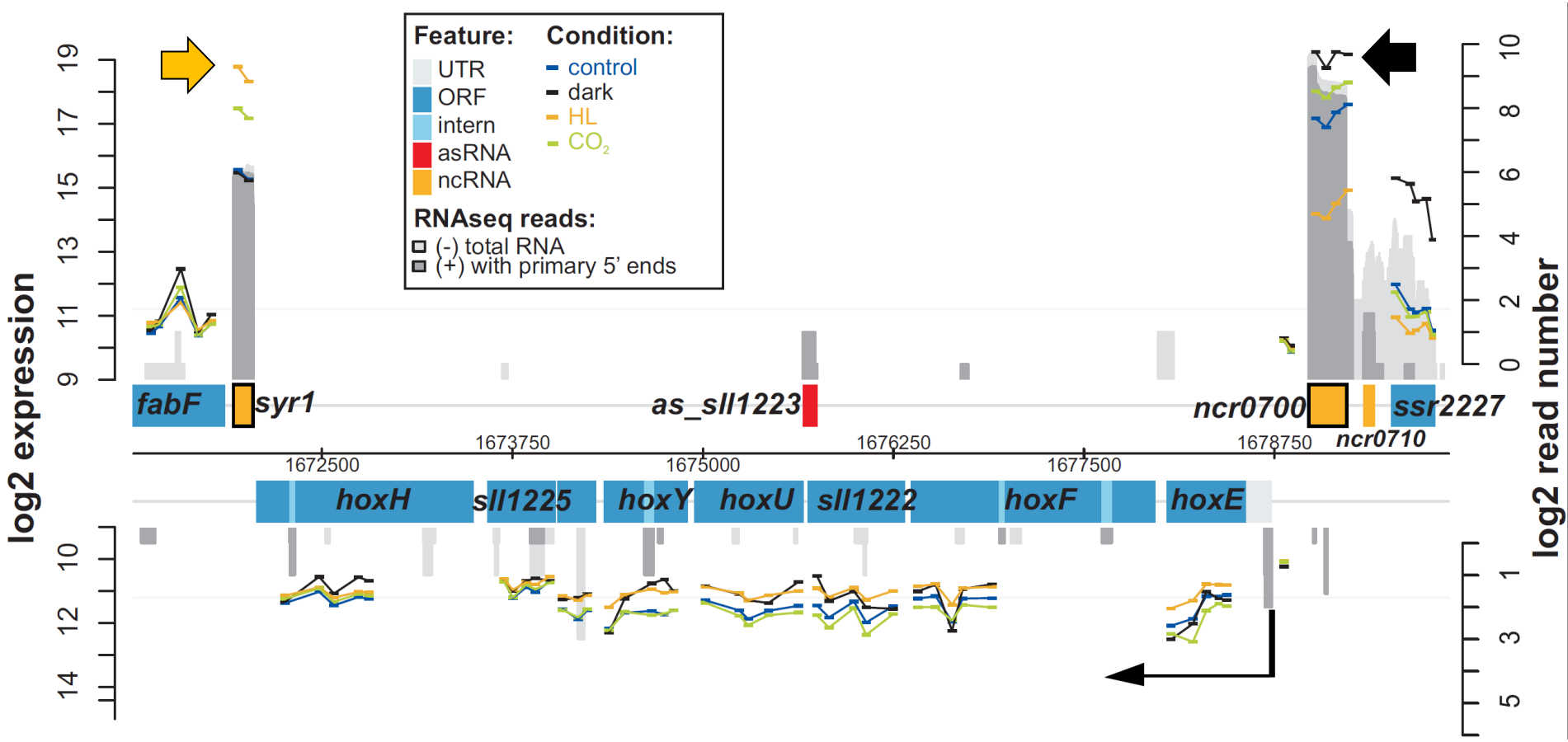


collaboration with Sophie Bonnet

Methods that provide comprehensive information about the status of gene expression: finding new players

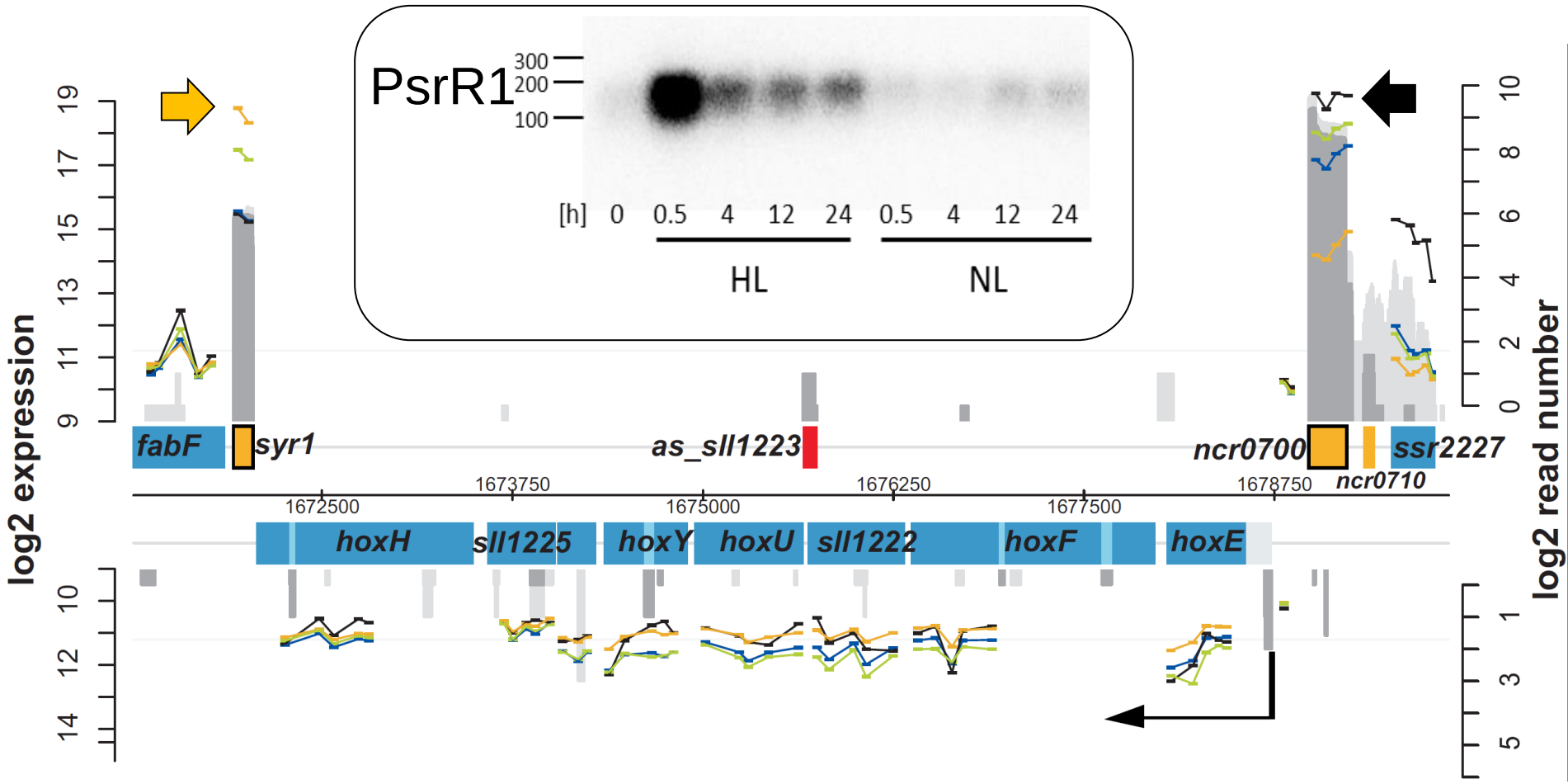


Synechocystis Complete Transcriptome Microarray

PsrR1 (SyR1), Photosynthesis regulatory RNA1

PNAS
An experimentally anchored map of transcriptional start sites in the model cyanobacterium *Synechocystis* sp. PCC6803

Methods that provide comprehensive information about the status of gene expression: finding new players



Synechocystis Complete Transcriptome Microarray

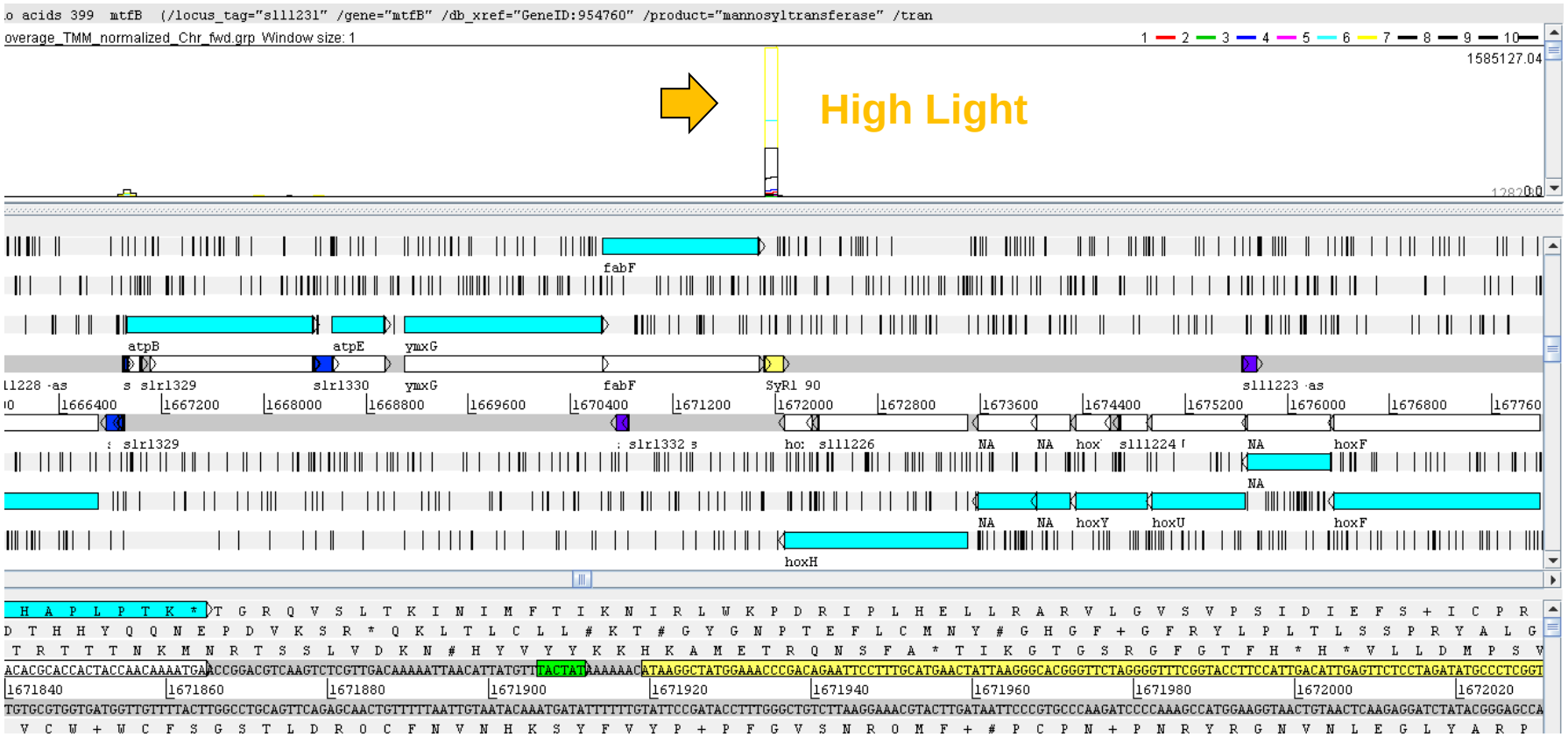
PsrR1 (SyR1), Photosynthesis regulatory RNA1

PNAS

An experimentally anchored map of transcriptional start sites in the model cyanobacterium *Synechocystis* sp. PCC6803

Jan Mitschke[a,1], Jens Georg[a,1], Ingeborg Scholz[a], Cynthia M. Sharma[b], Dennis Dienst[c], Jens Bantscheff[d], Björn Voß[a], Claudia Steglich[a], Annegret Wilde[a], Jörg Vogel[b], and Wolfgang R. Hess[a,e,f]

Methods that provide comprehensive information about the status of gene expression: finding new players by dRNA-seq

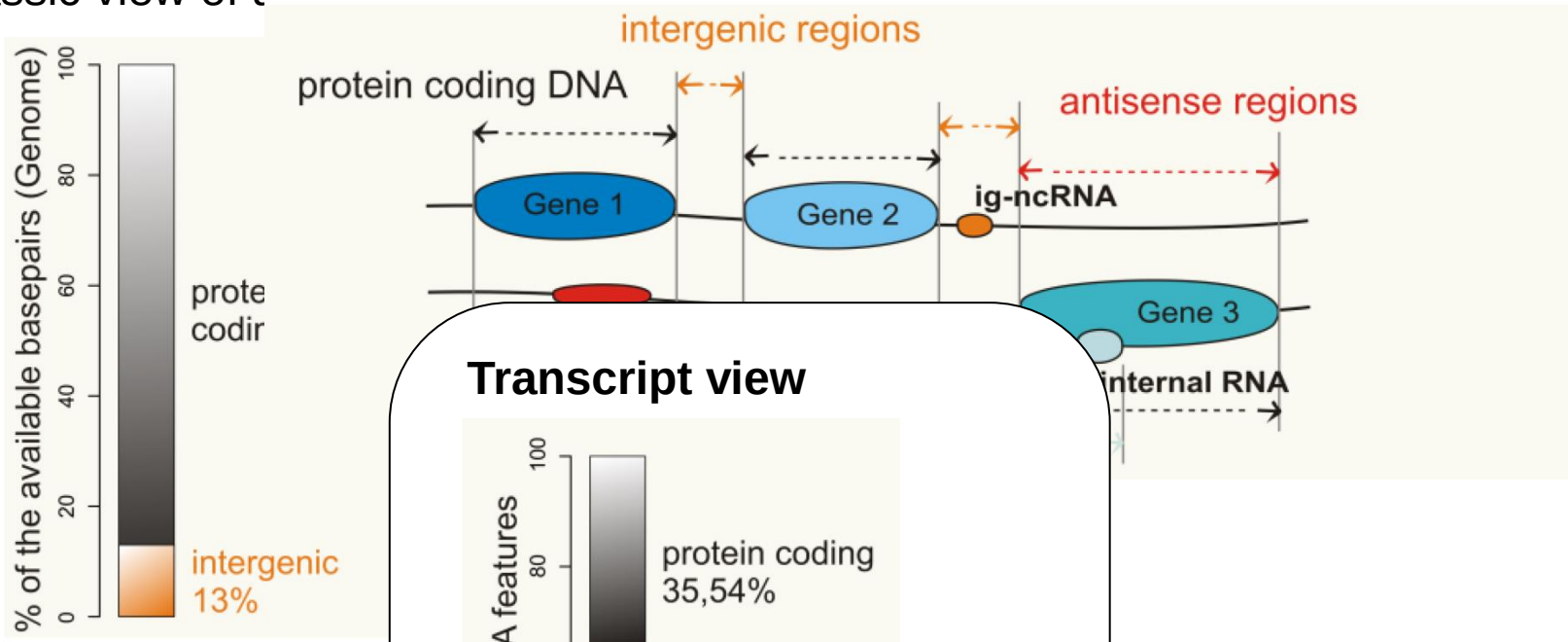


Synechocystis Double-comparative dRNA-seq Analysis of ten different conditions

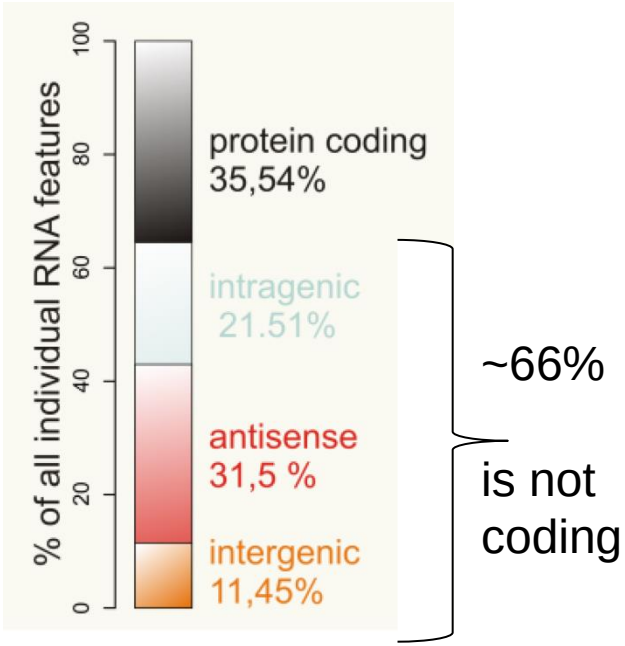
PsrR1 (SyR1), Photosynthesis regulatory RNA1

Are these new players relevant at all?

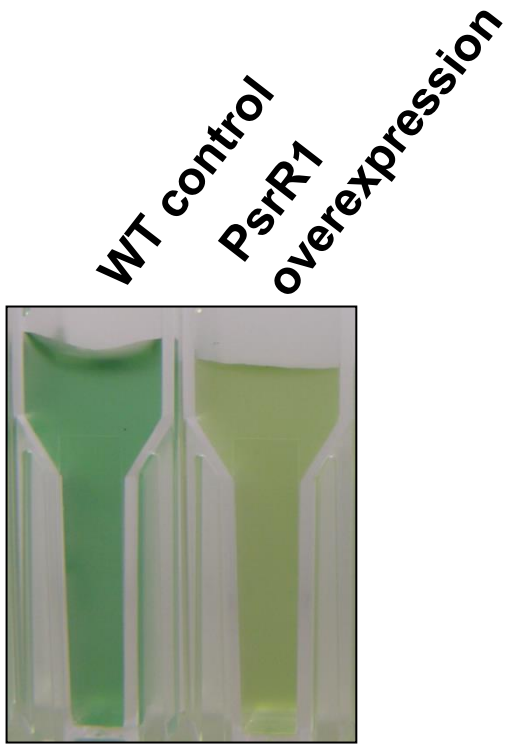
Classic view of a microbial genome

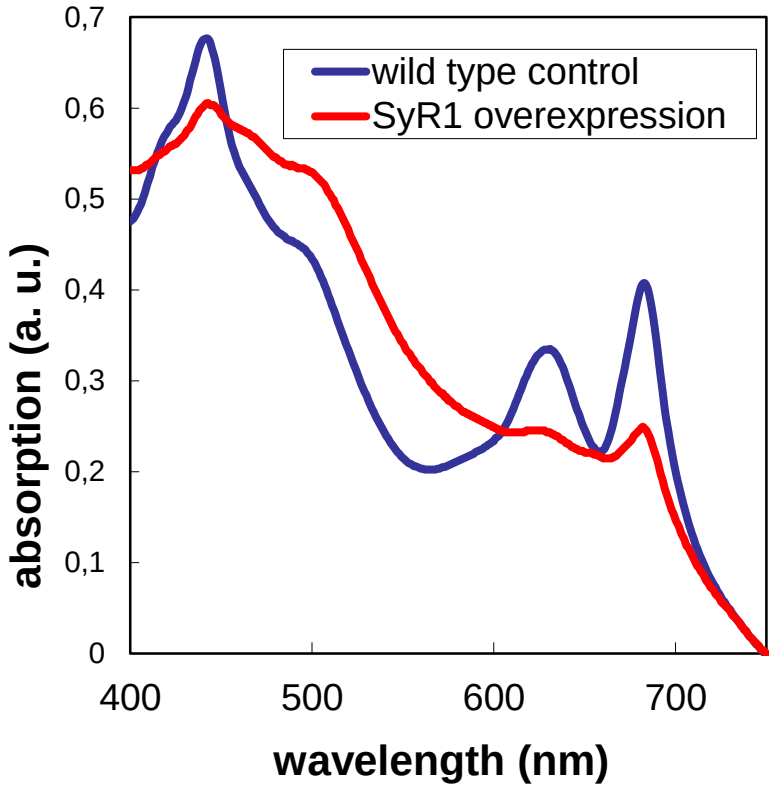


Transcript view



Pigmentation phenotype of PsrR1 overexpression in *Synechocystis*





PNAS

An experimentally anchored map of transcriptional start sites in the model cyanobacterium *Synechocystis* sp. PCC6803

Jan Mitschke[a,1], Jens Georg[a,1], Ingeborg Scholz[a], Cynthia M. Sharma[b], Dennis Dienst[c], Jens Bantscheff[a], Björn Voß[a], Claudia Steglich[a], Annegret Wilde[d], Jörg Vogel[b], and Wolfgang R. Hess[a,e,f]

Are these new players relevant at all?

Judged by

- their numbers (2/3 of all promoters give rise to non-mRNA transcripts),
- their regulation (exp., PsrR1 induction by high light),
- and the phenotypic effects when they are mutated or overexpressed (exp., PsrR1 overexpression causes disturbed pigmentation and photosynthesis).

YES!

(2) How to do it?

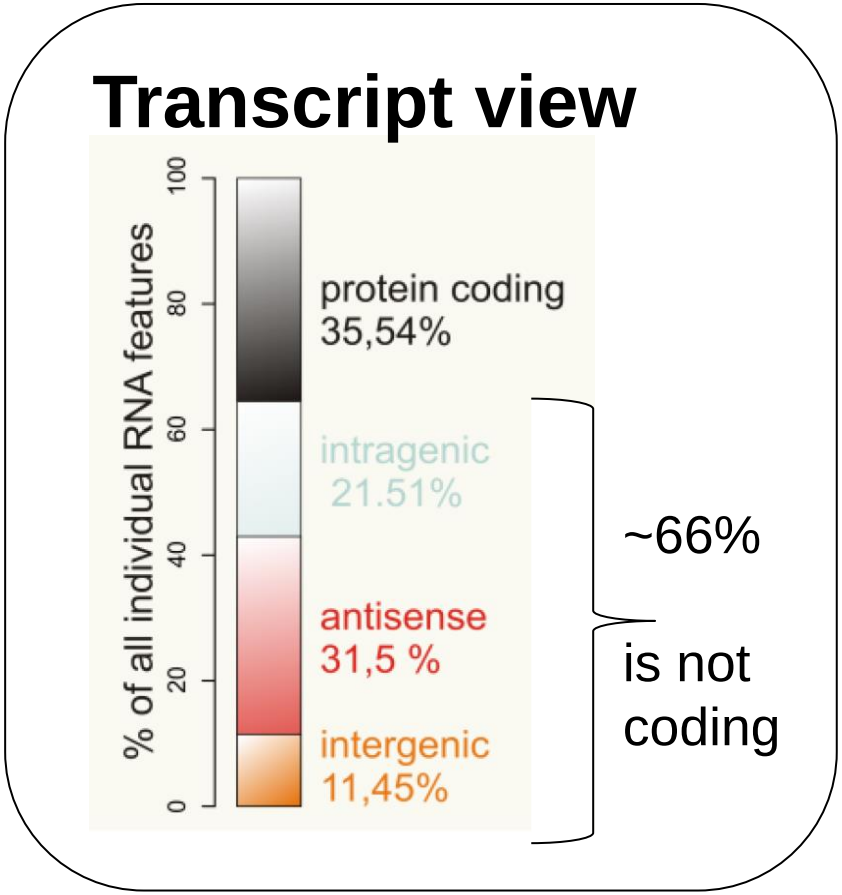
2.1 Computational predictions

2.2 Microarrays

2.3 RNA-seq

2.1 Computational predictions and validation in experiments

These RNA players are abundant.



Why are they not just annotated during normal genome annotation?

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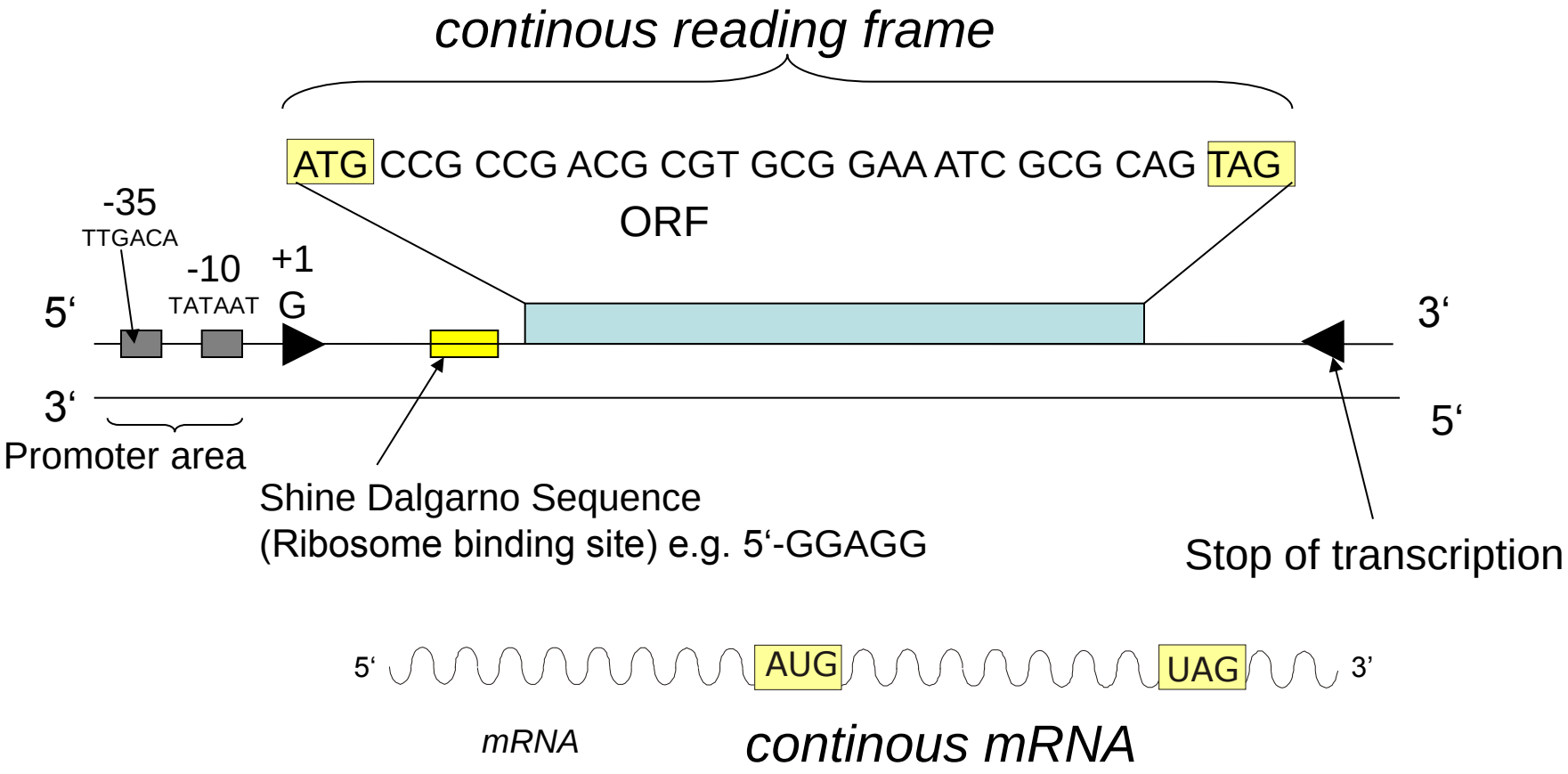
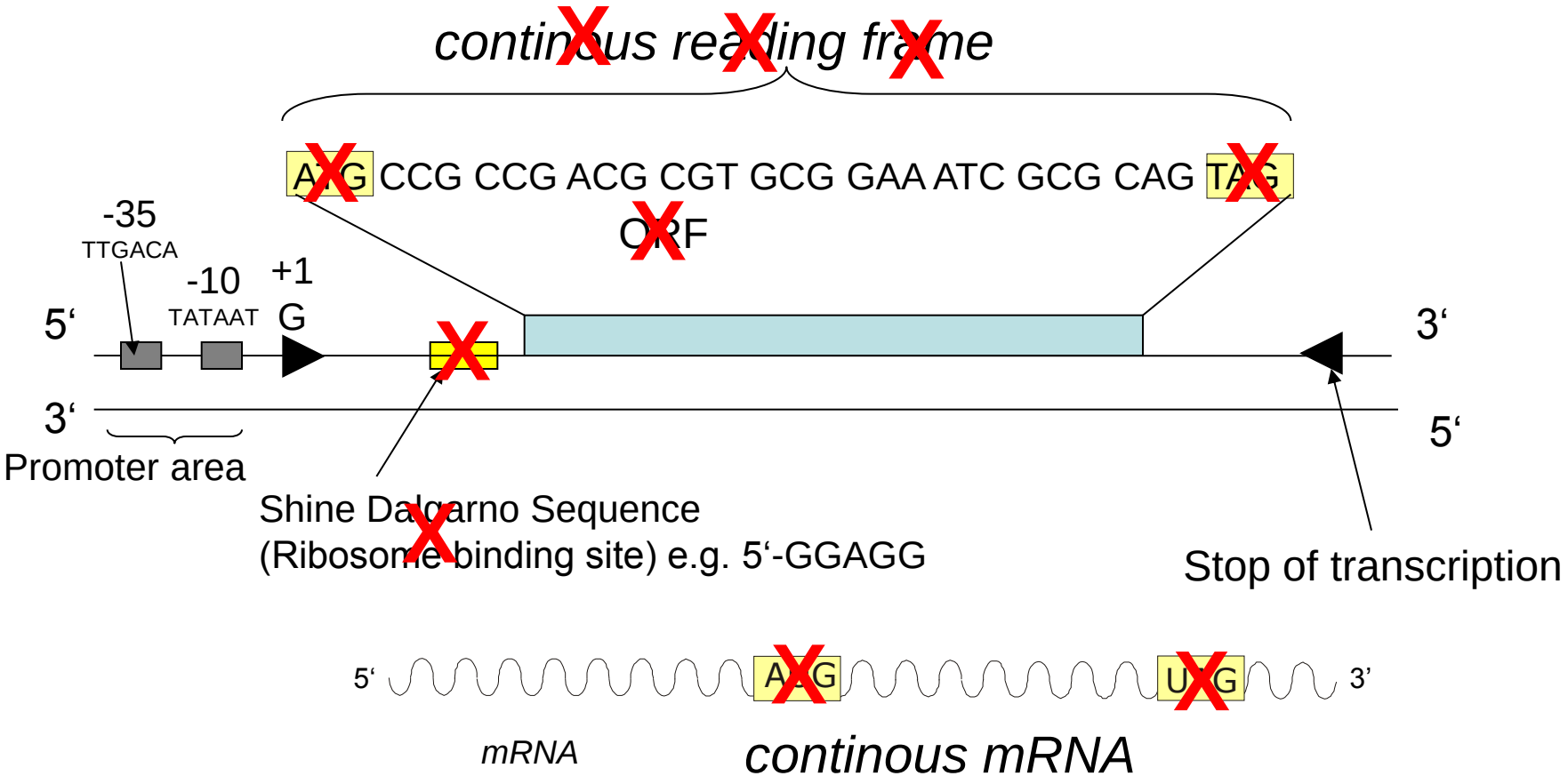


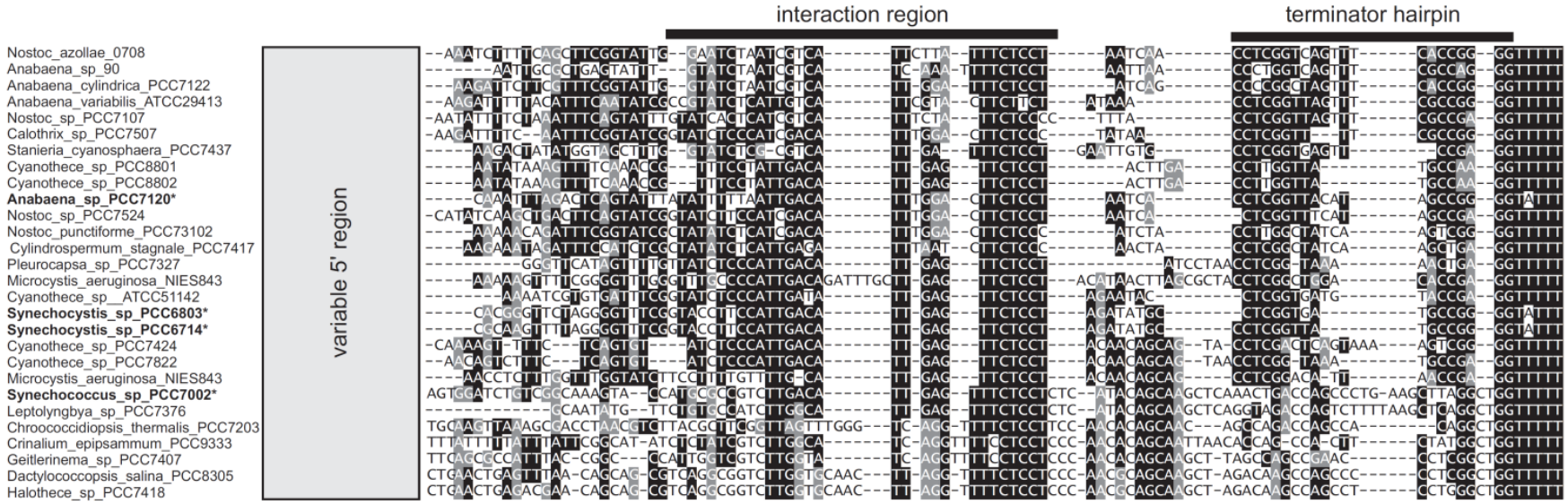
Scheme of a bacterial protein-coding gene

Why are they not just annotated during normal genome annotation?



Scheme of a bacterial non-protein-coding gene

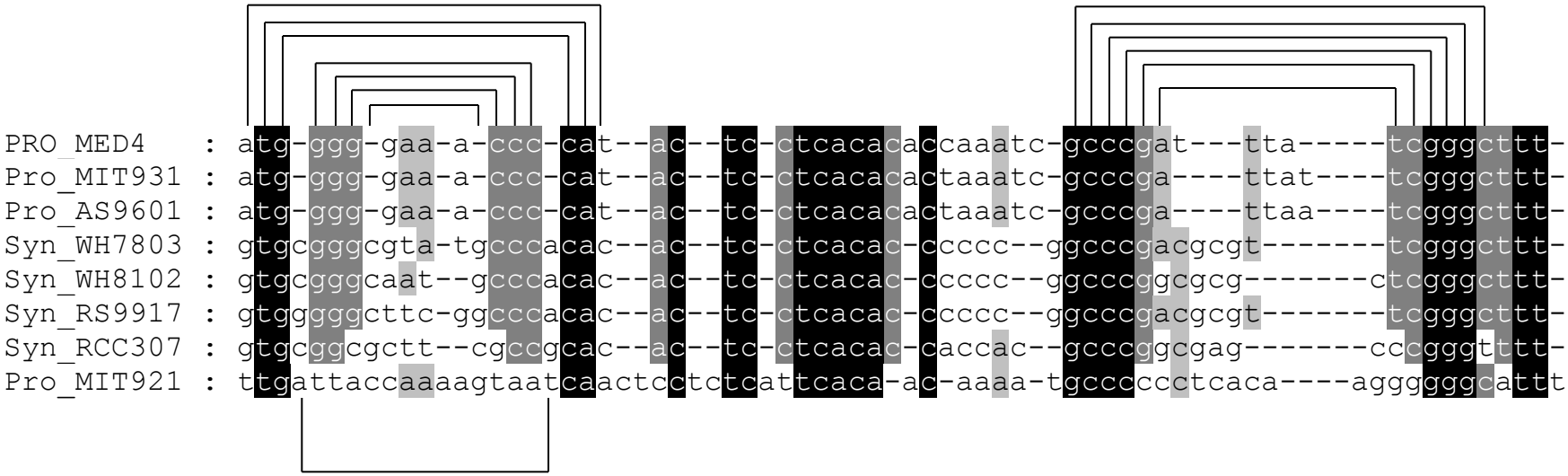
Alignment of PsrR1 from 28 different cyanobacteria



Usually, the sequence conservation of non-coding transcripts is not very good,

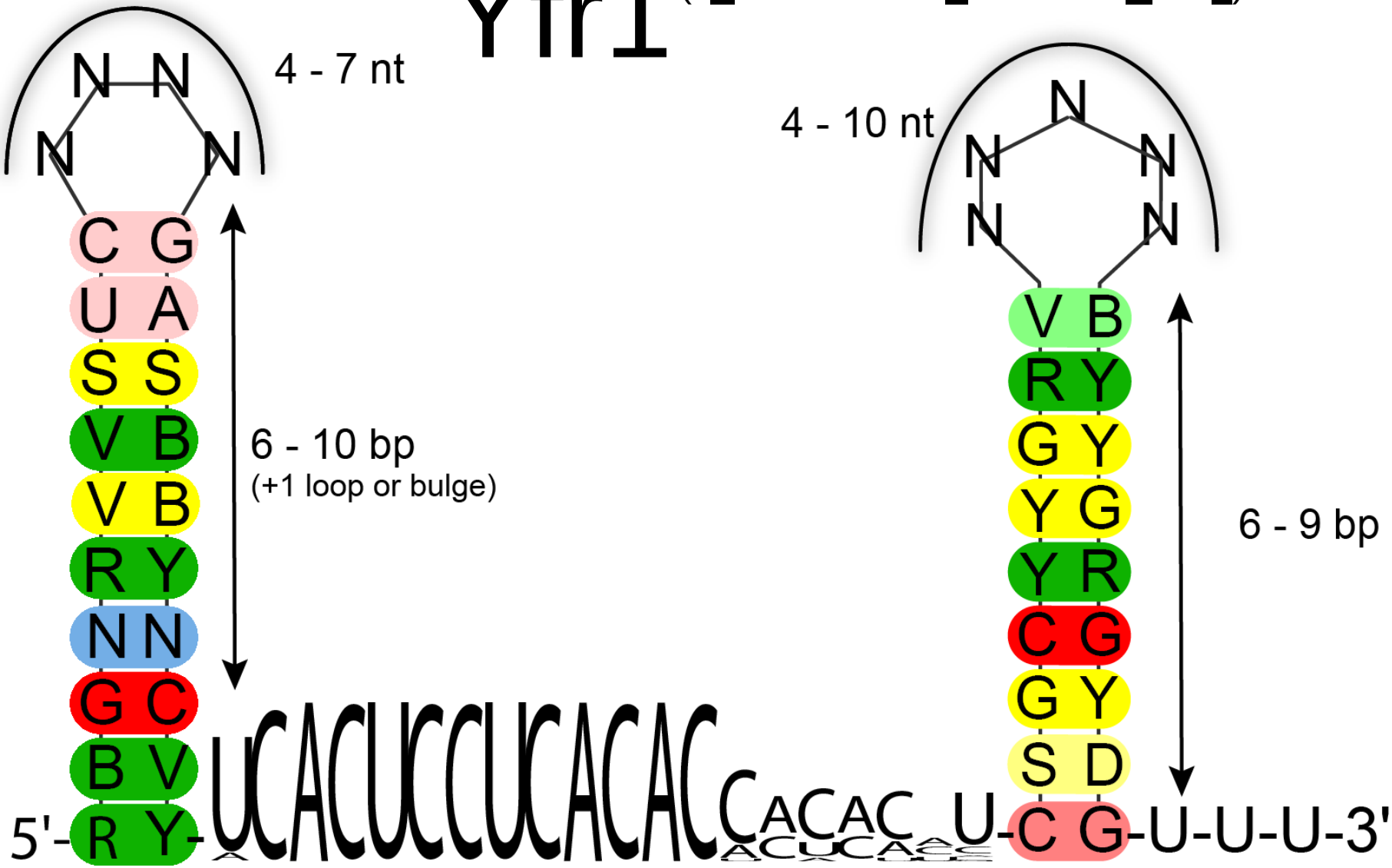
but the secondary structure conservation is better and might be utilized:

Complementary mutations indicate non-coding RNAs



Comparison of 4 *Prochlorococcus* and 4 *Synechococcus*

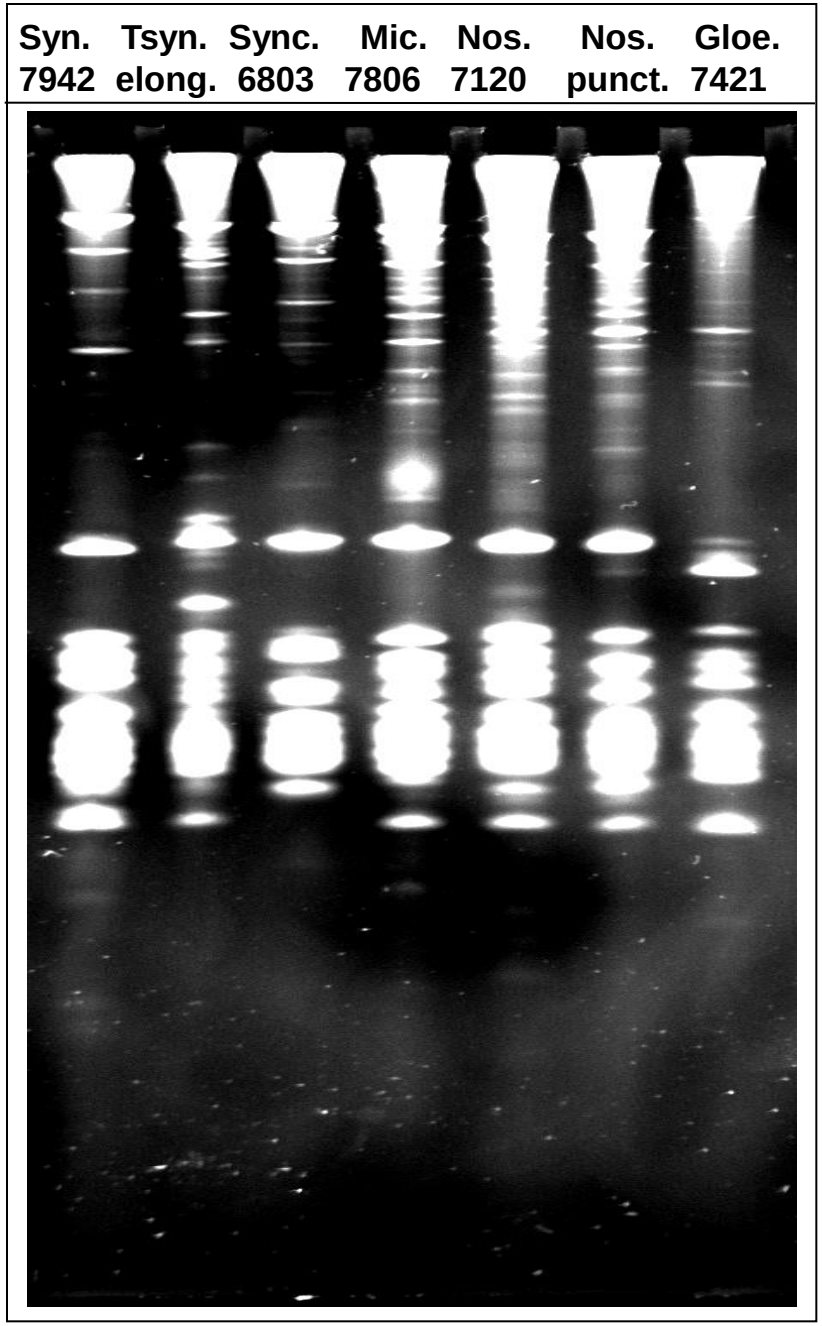
Yfr1 (c**Y**anobacterial **F**unctional **R**NA **1**)



Sequence/structure model for Yfr1 of 31 cyanobacteria.

Voss et al., (2007) *BMC Genomics* 8:375,

(R: A or G; Y: C or U; M: A or C; S: G or C; B: G, U or C; V: G, C or A; D: G, T or A).
Colors: number of different base pairs at this position (red = 1, yellow = 2, green = 3 and blue = 4 or more).
Shading: frequency of base pairing.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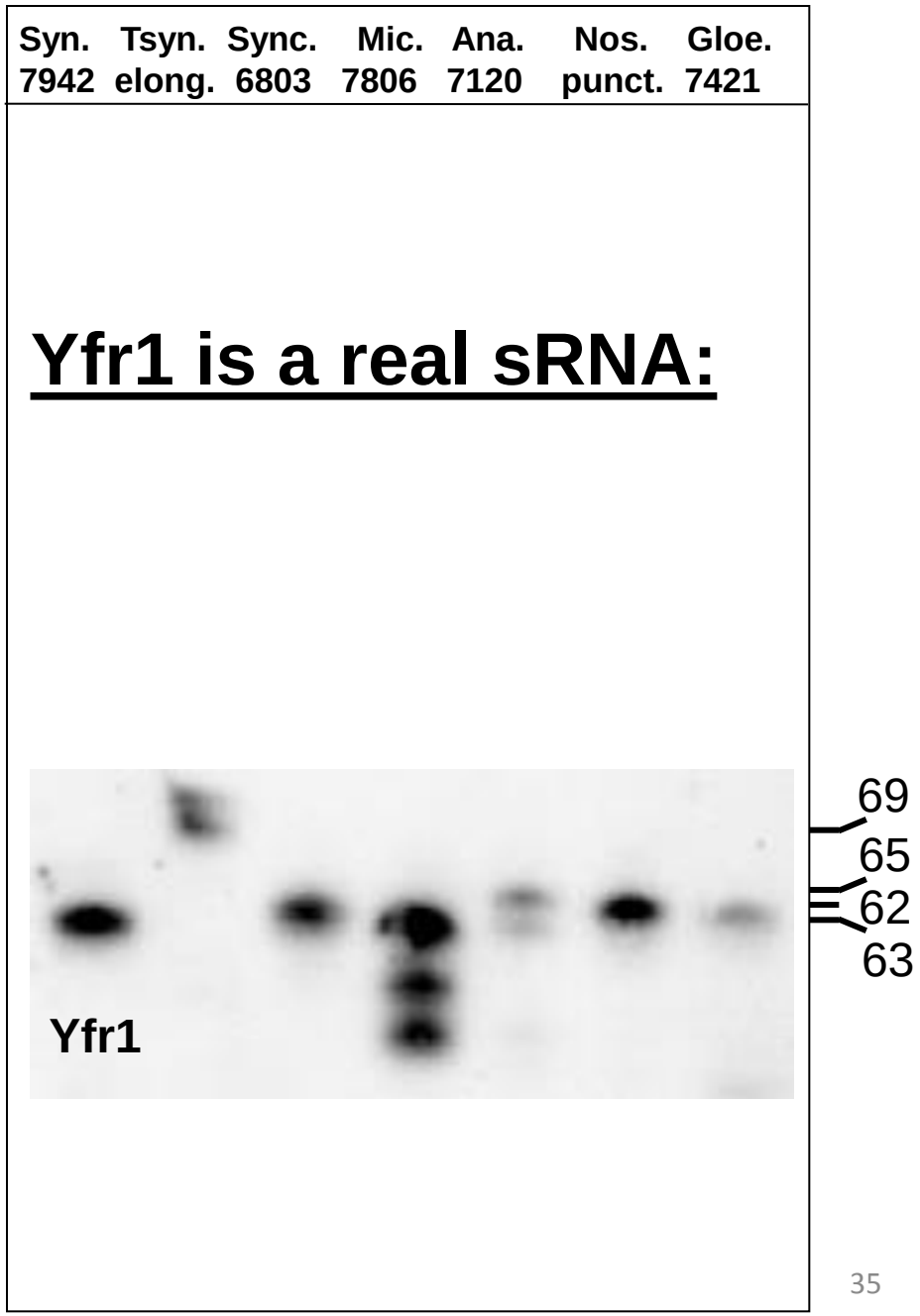
Tsyn. elong.
Sync. 6803
Mic. 7806
Nos. 7120
Nos. punct.
Gloe. 7421


Syn. 7942
Tsyn. elong.
Sync. 6803
Mic. 7806
Ana. 7120
Nos. punct.
Gloe. 7421
Yfr1 is a real sRNA:
Yfr1
69
65
62
63

But, how to find out the function of a bacterial sRNA in a marine microbe that can‘t even be manipulated ?

Functional analysis of Yfr1 – target prediction and experimental verification

***Synechococcus elongatus* PCC 6301**

→ homozygous mutants in *S. elongatus* PCC 6301 (Nakamura et al., 2007)

→ reduced growth under various stress conditions, e.g. oxidative stress and high salt stress conditions

→ about 18,000 copies/cell (vs. 146,400 ribosomes/cell)

→ putative target of Yfr1 in *Synechococcus* is *sbtA* (C transporter)

***Synechocystis* sp. PCC 6803** (work of Annegret Wilde Univ. Giessen)

→ Yfr1 is essential in *Synechocystis* sp. PCC 6803

→ mutants are not fully segregated

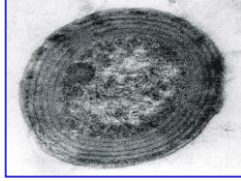
→ strong growth effects in the mutants under light stress

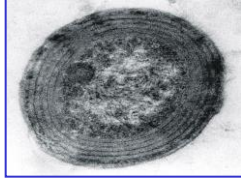
Prochlorococcus

→ ~ 100 molecules per cell (vs. ~ 2,000 ribosomes/cell)

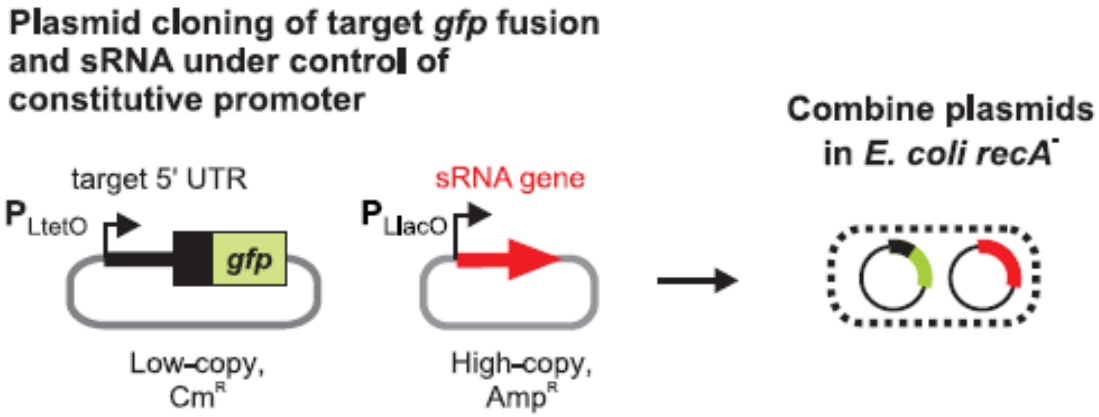
Target prediction with IntaRNA

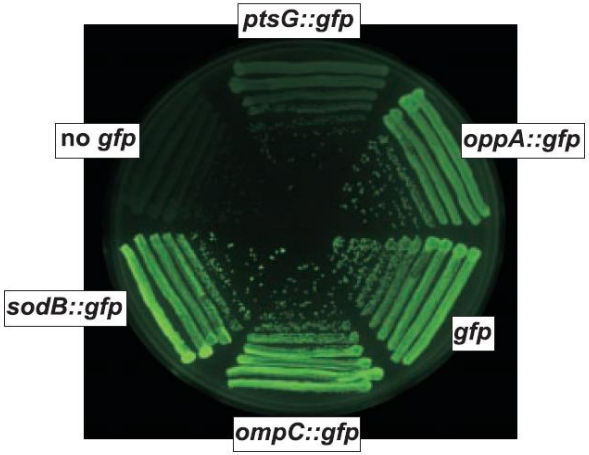
http://rna.informatik.uni-freiburg.de:8080/IntaRNA/Input.jsp



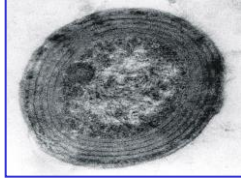


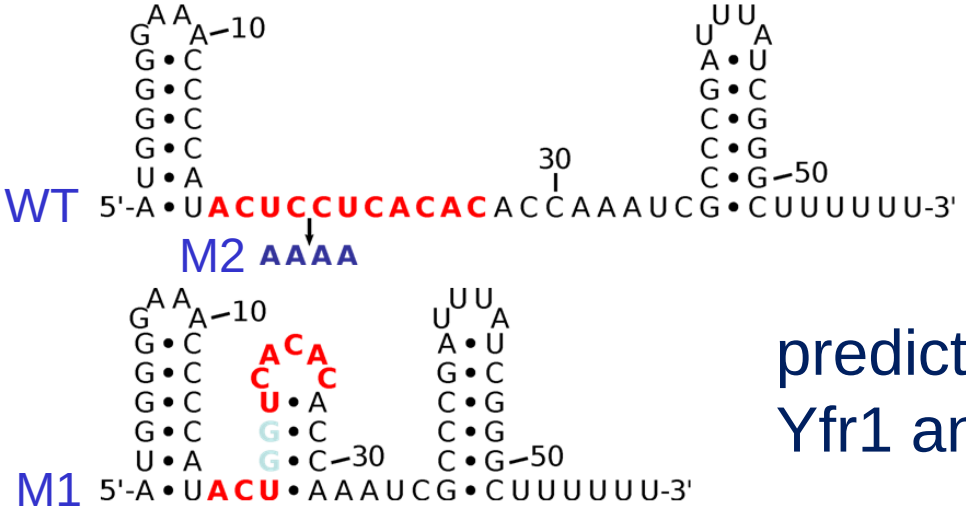
GFP reporter system



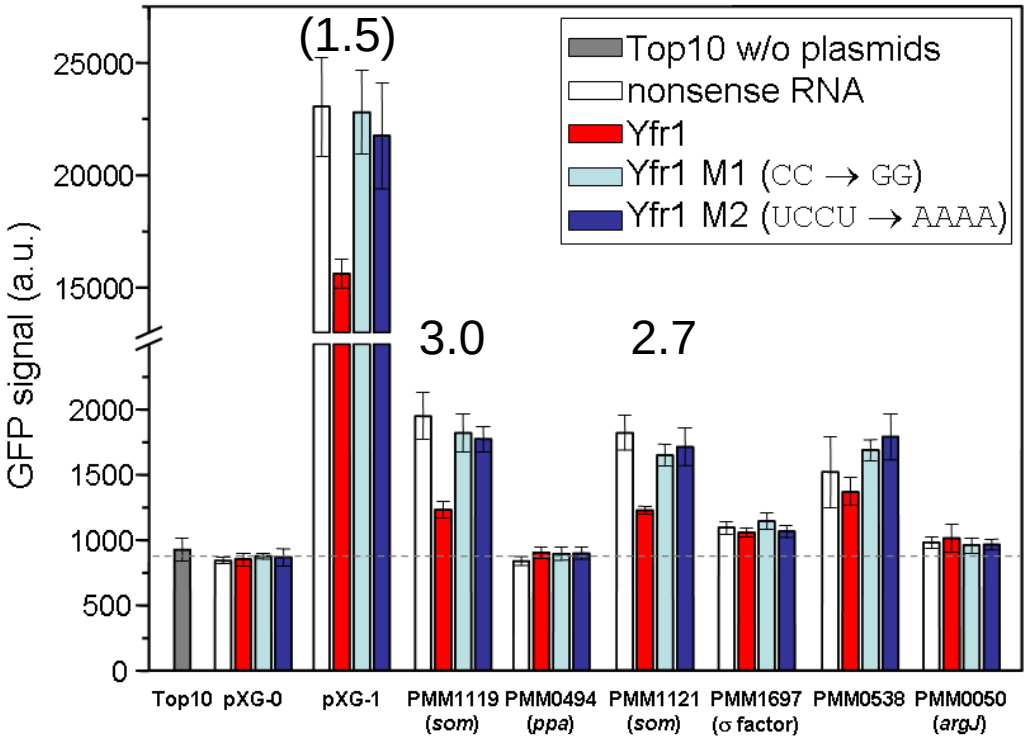


Johannnes Urban &
Jörg Vogel, NAR 2007



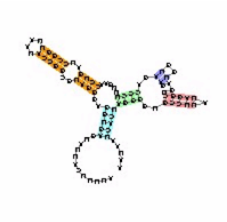


predicted structures of WT Yfr1 and mutated Yfr1



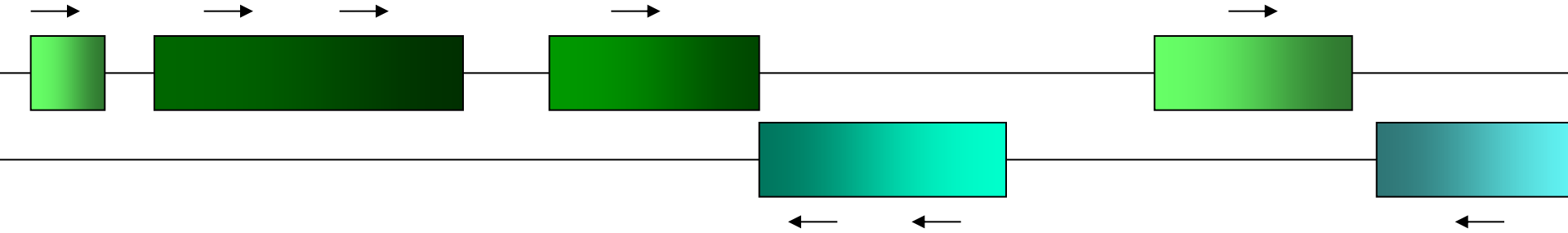
Flow cytometry measurements of GFP fluorescence of *E. coli* cells

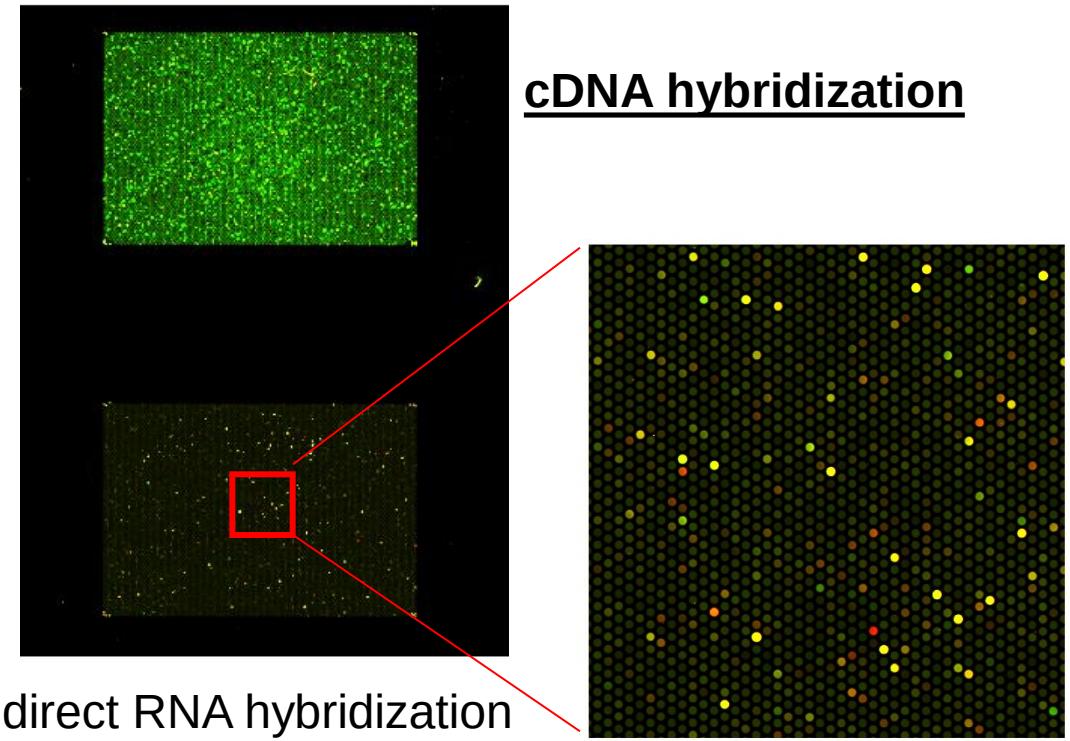
Yfr1 regulates the synthesis of porins, a class of outer membrane proteins



2.2 Microarrays

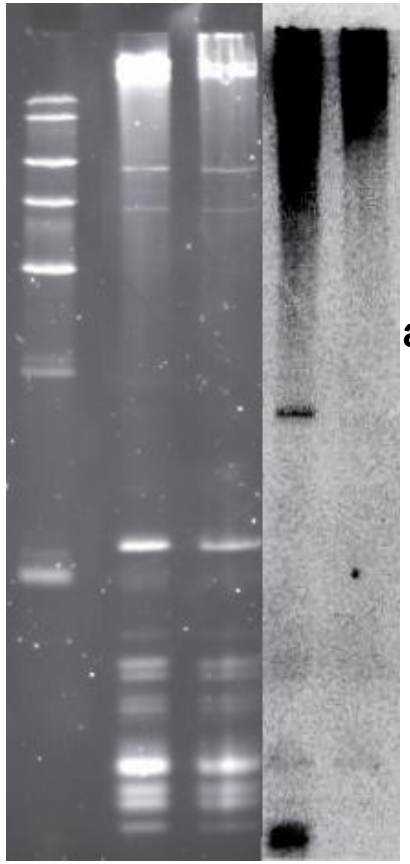
Synechocystis classical array (Eisenhut, et al. (2007) *Plant Physiol* 144:1946-59)





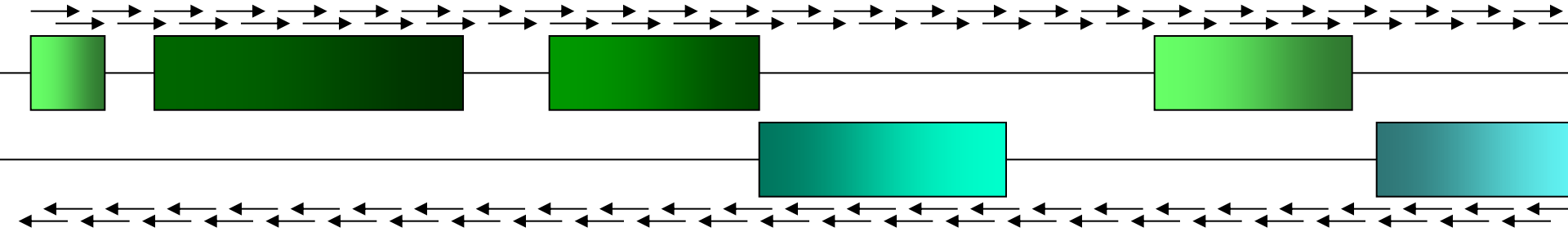
cDNA hybridization

direct RNA hybridization

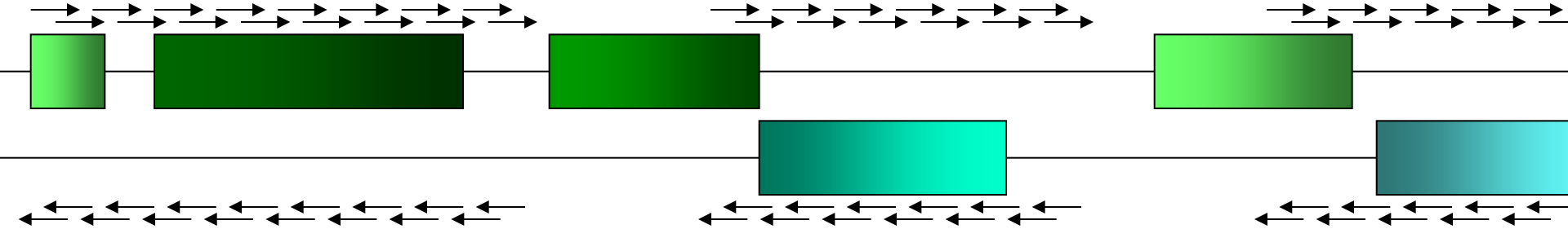


anti-slr1944 RNA

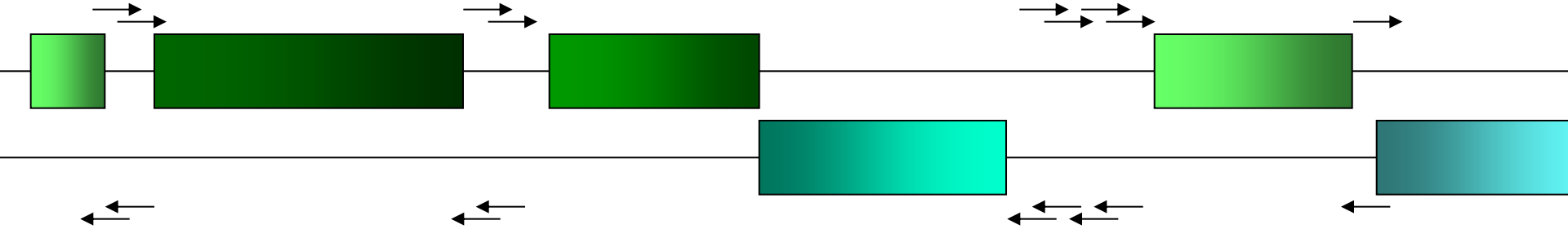
Types of microarrays



Full genome tiling array

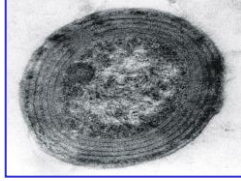


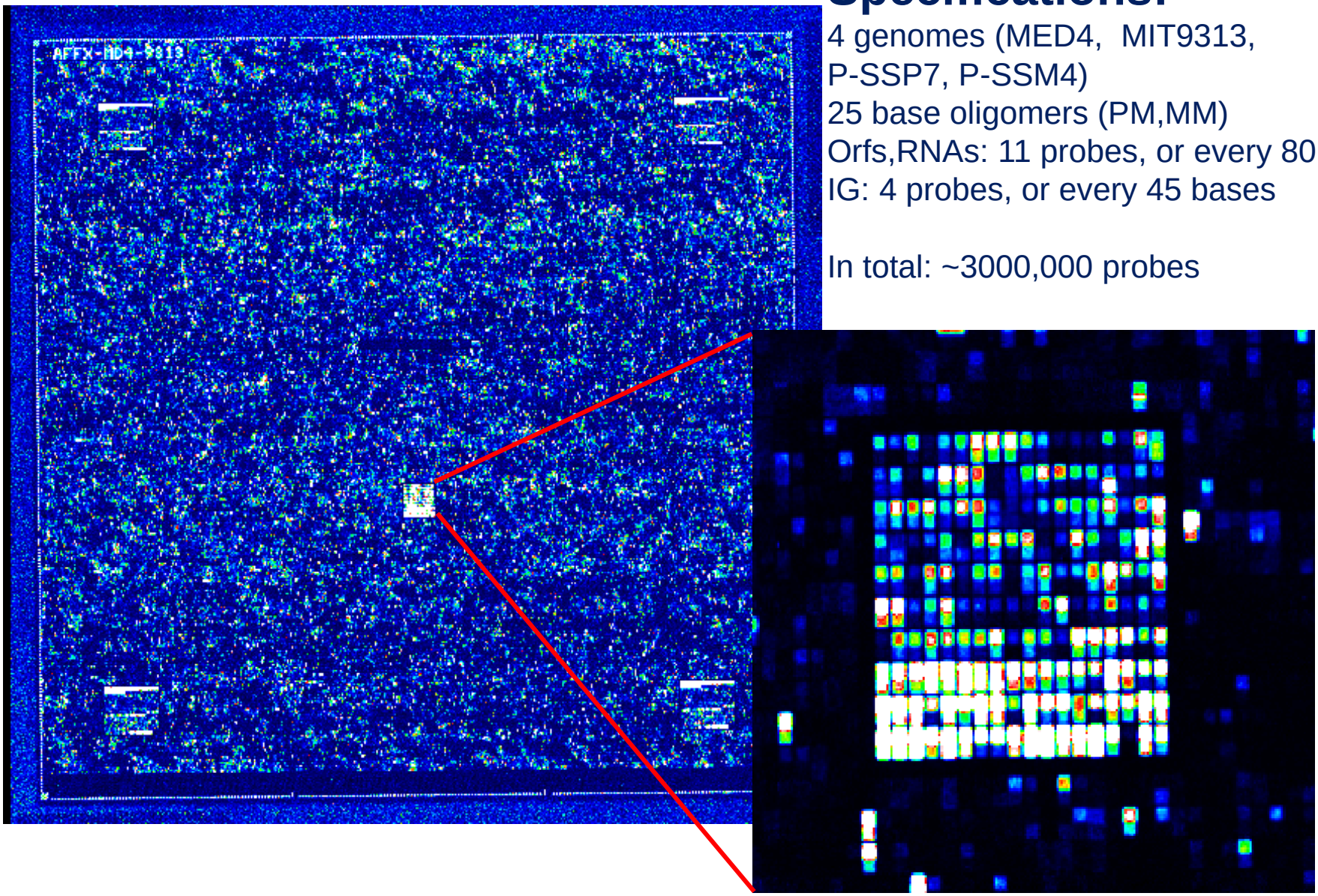
Synechocystis PCC6803: „1/3 genome tiling array“ (Georg et al., MSB 2009)



Synechococcus WH7803: „intergenic regions only“ tiling array (Gierga et al., ISME J. 2012)

Types of microarrays:
Prochlorococcus Affymetrix high density microarray



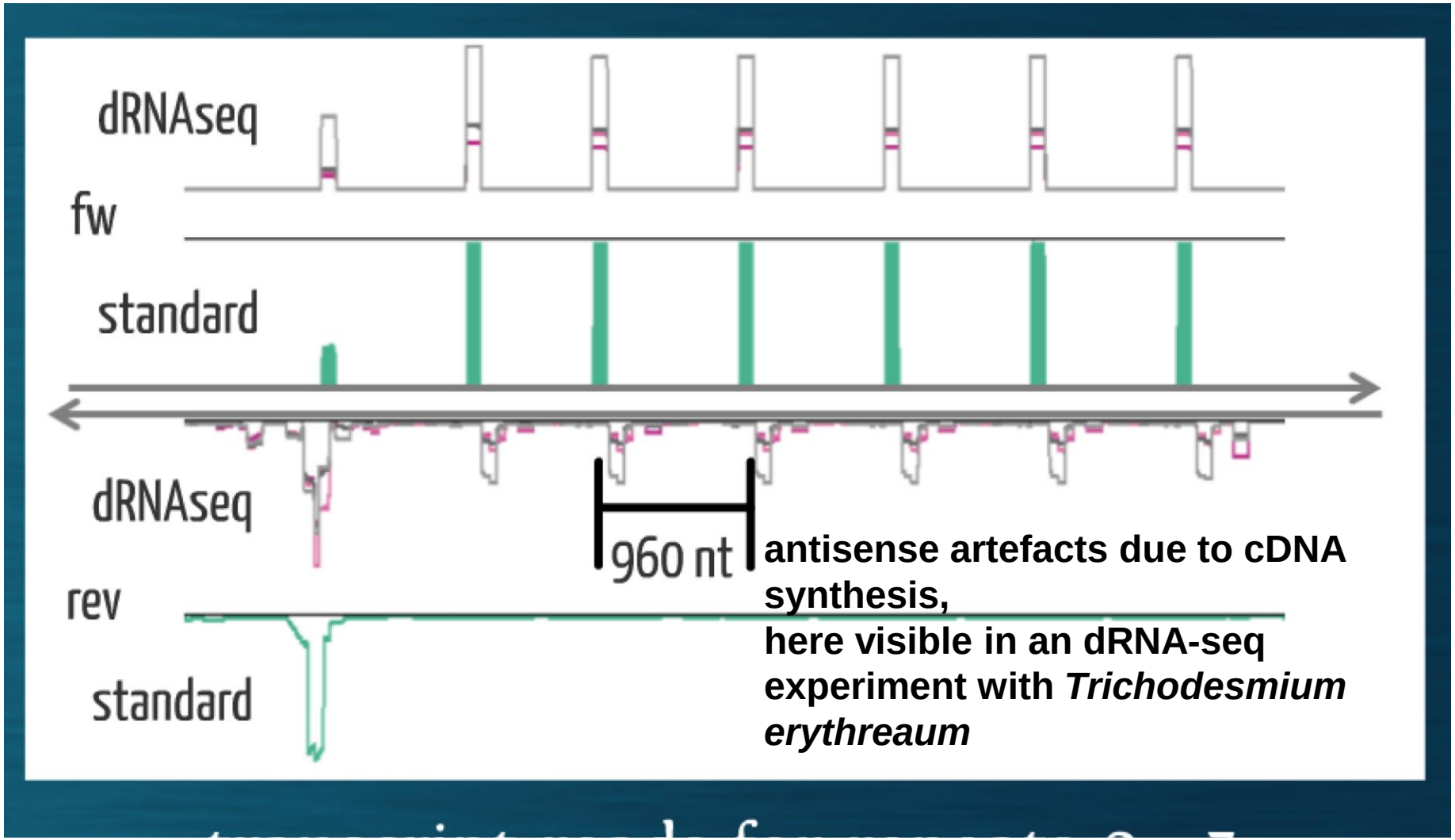


Specifications:

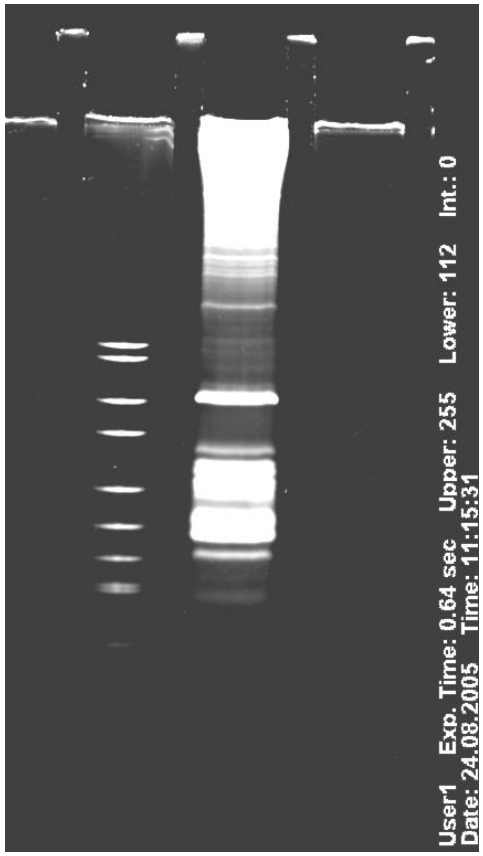
4 genomes (MED4, MIT9313, P-SSP7, P-SSM4)
25 base oligomers (PM,MM)
Orfs,RNAs: 11 probes, or every 80 bases
IG: 4 probes, or every 45 bases

In total: ~3000,000 probes

Most classical microarrays work with cDNA that may cause problems:



antisense artefacts due to cDNA synthesis,
here visible in an dRNA-seq experiment with *Trichodesmium erythreaum*



> 120 nt

5S RNA ~115 nt

90-110 nt

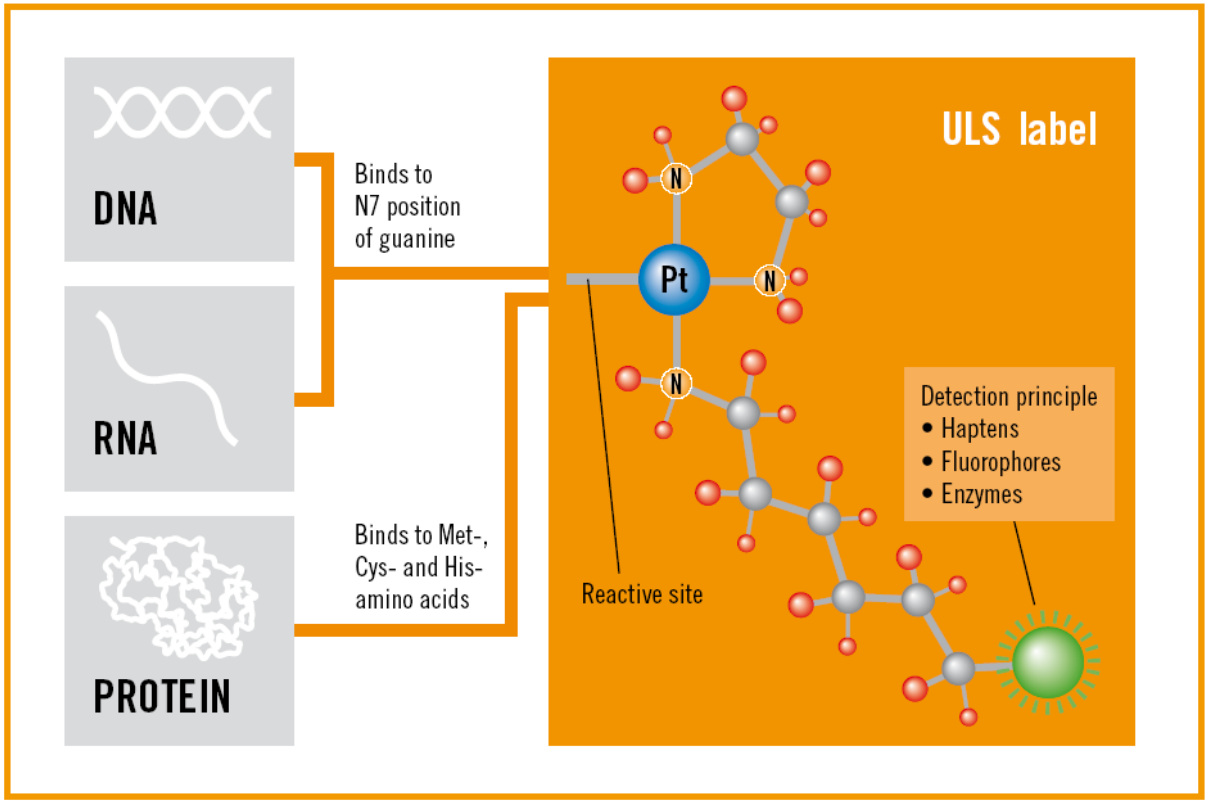
tRNAs 70 - 90 nt

< 70 nt

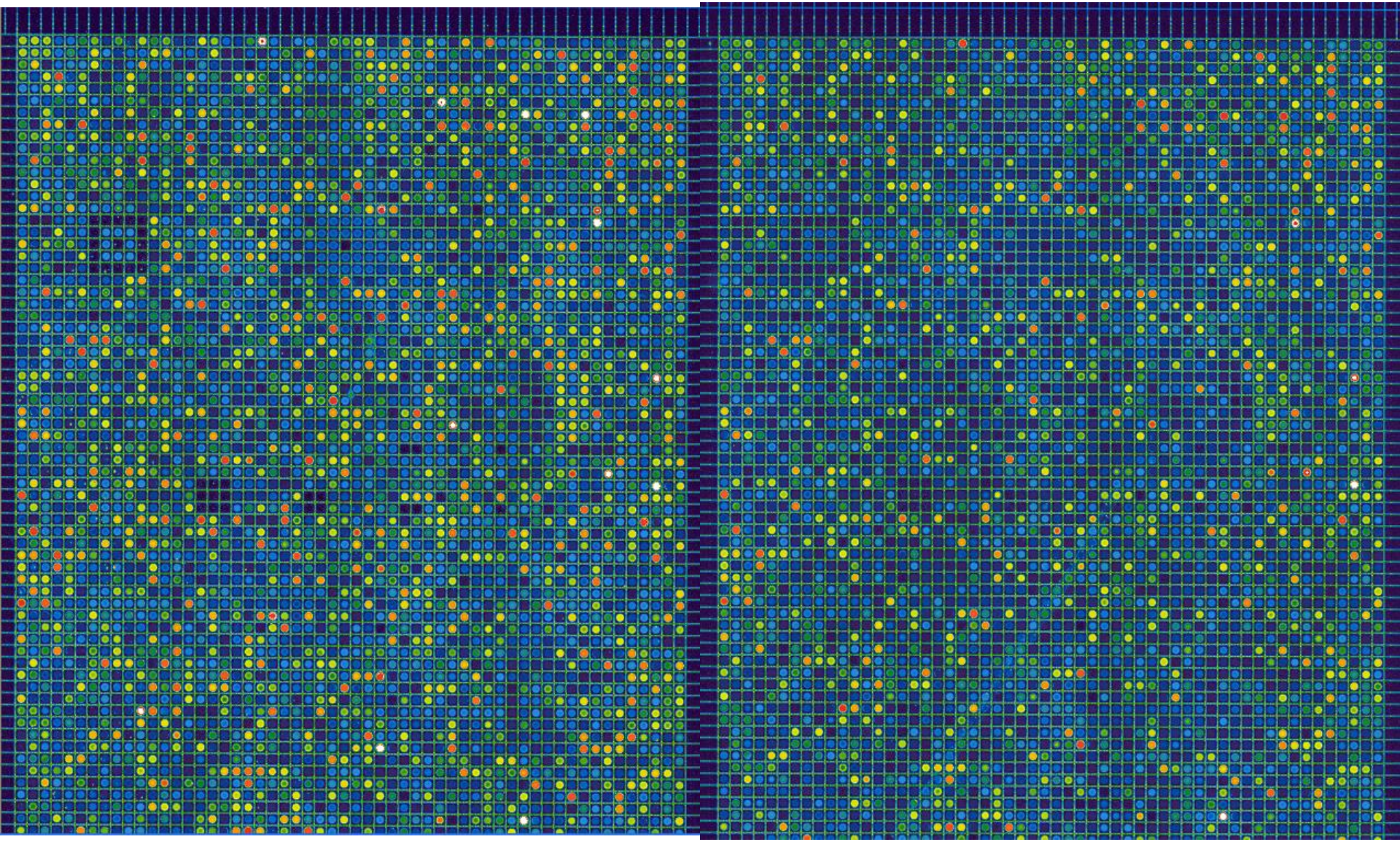
200 µg *Synechococcus* WH 7803 RNA

Therefore we introduced

Direct labelling of RNA via Platinum complexes



„Intergenic spacer only“ array *Synechococcus* WH 7803 and direct RNA labeling (tiling factor =11, sense + antisense); no protein-coding genes; Format: 12K



cold stress RNA

control RNA

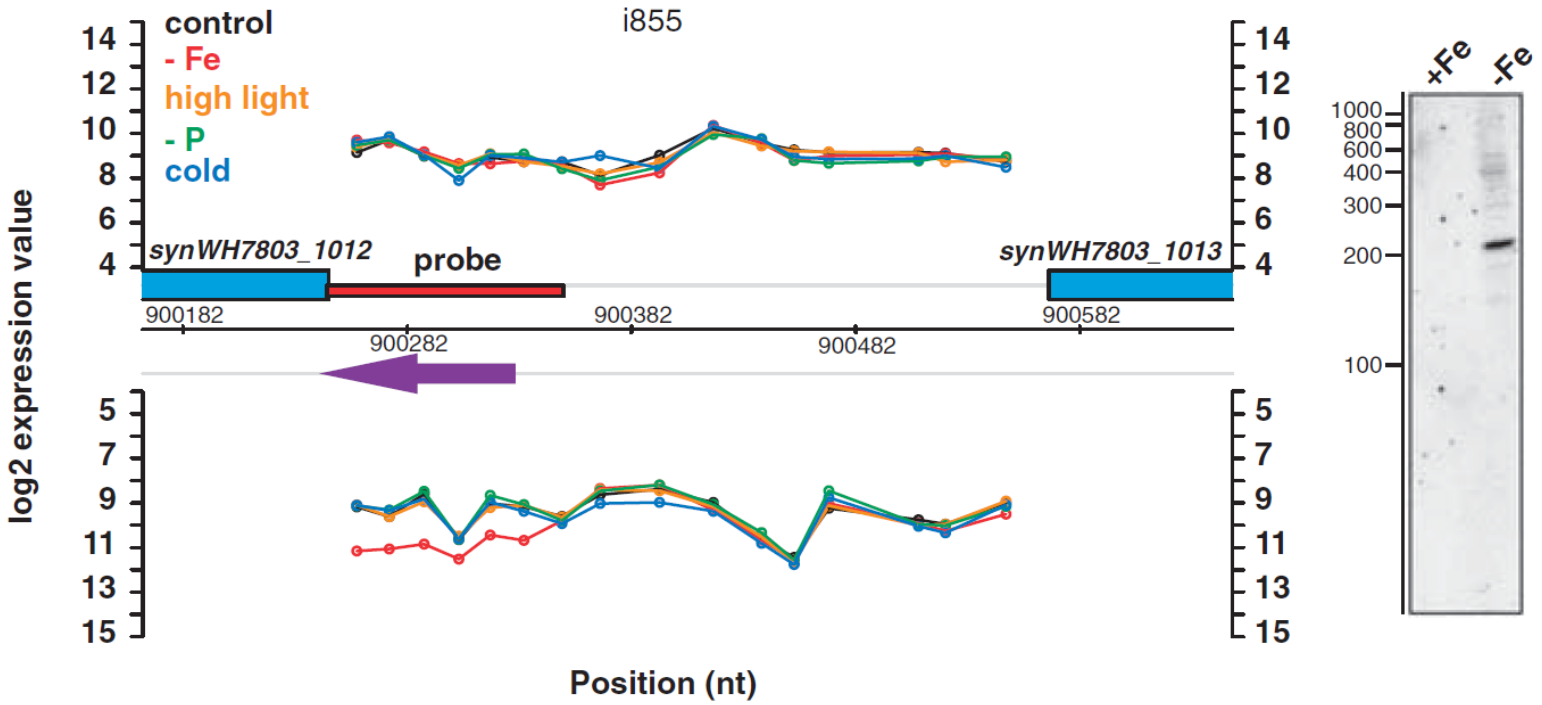
The ISME Journal (2012), 1–14

www.nature.com/ismej

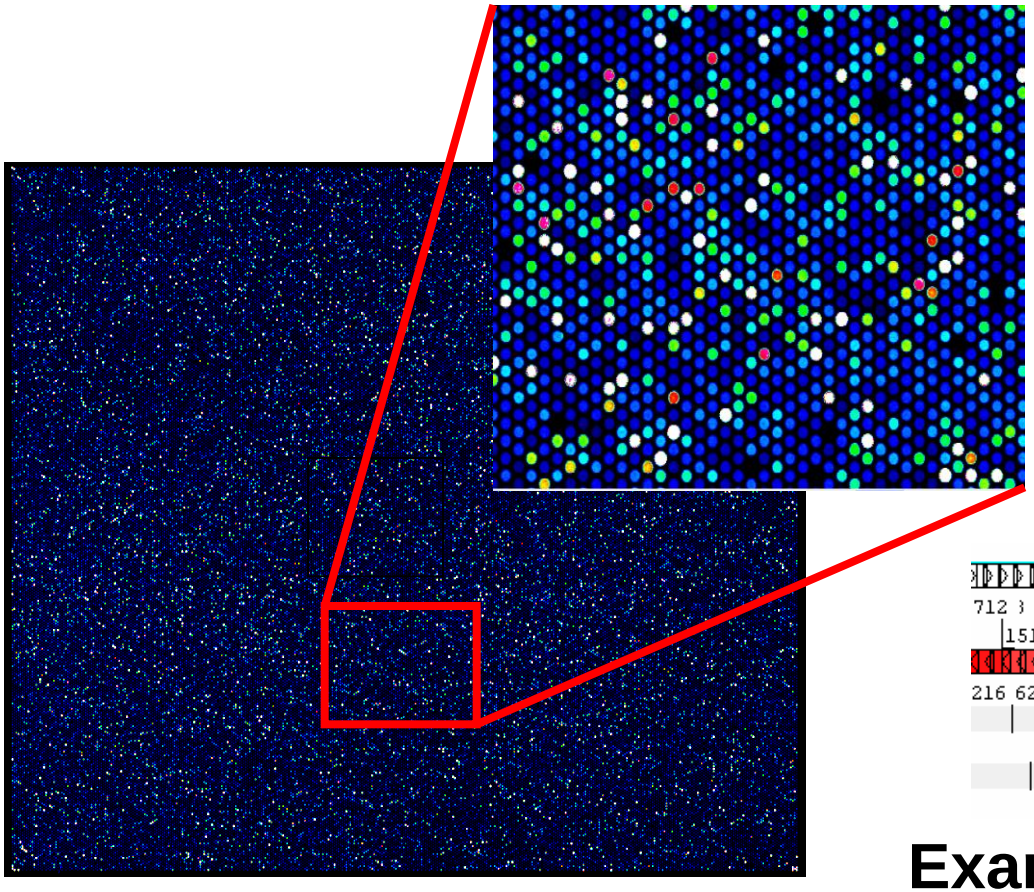
ORIGINAL ARTICLE

Non-coding RNAs in marine *Synechococcus* and their regulation under environmentally relevant stress conditions

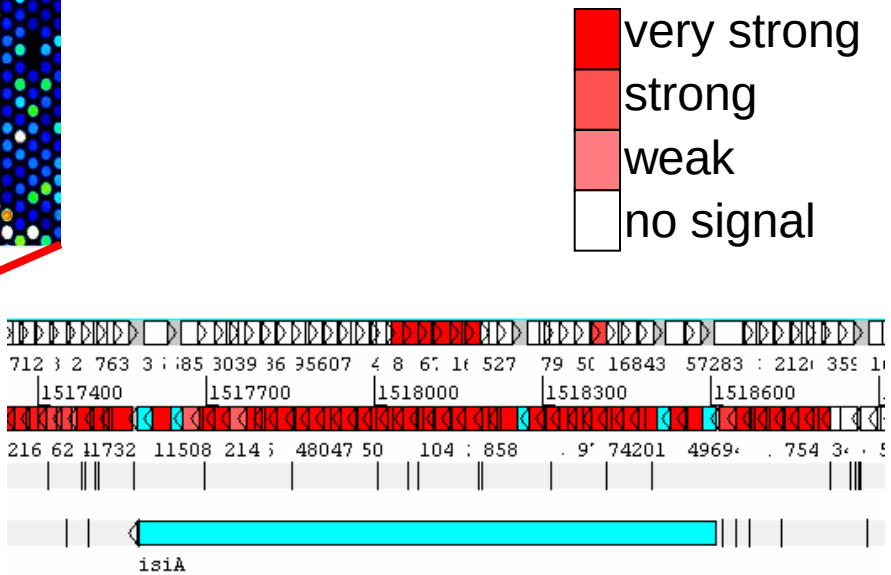
Gregor Gierga, Björn Voss and Wolfgang R Hess
Faculty of Biology, University of Freiburg, Freiburg, Germany



A 105K Agilent tiling array covering 1/3 of the *Synechocystis* genome and with probes on both strands was hybridized against directly labelled total RNA



***Synechocystis* 6803 array**



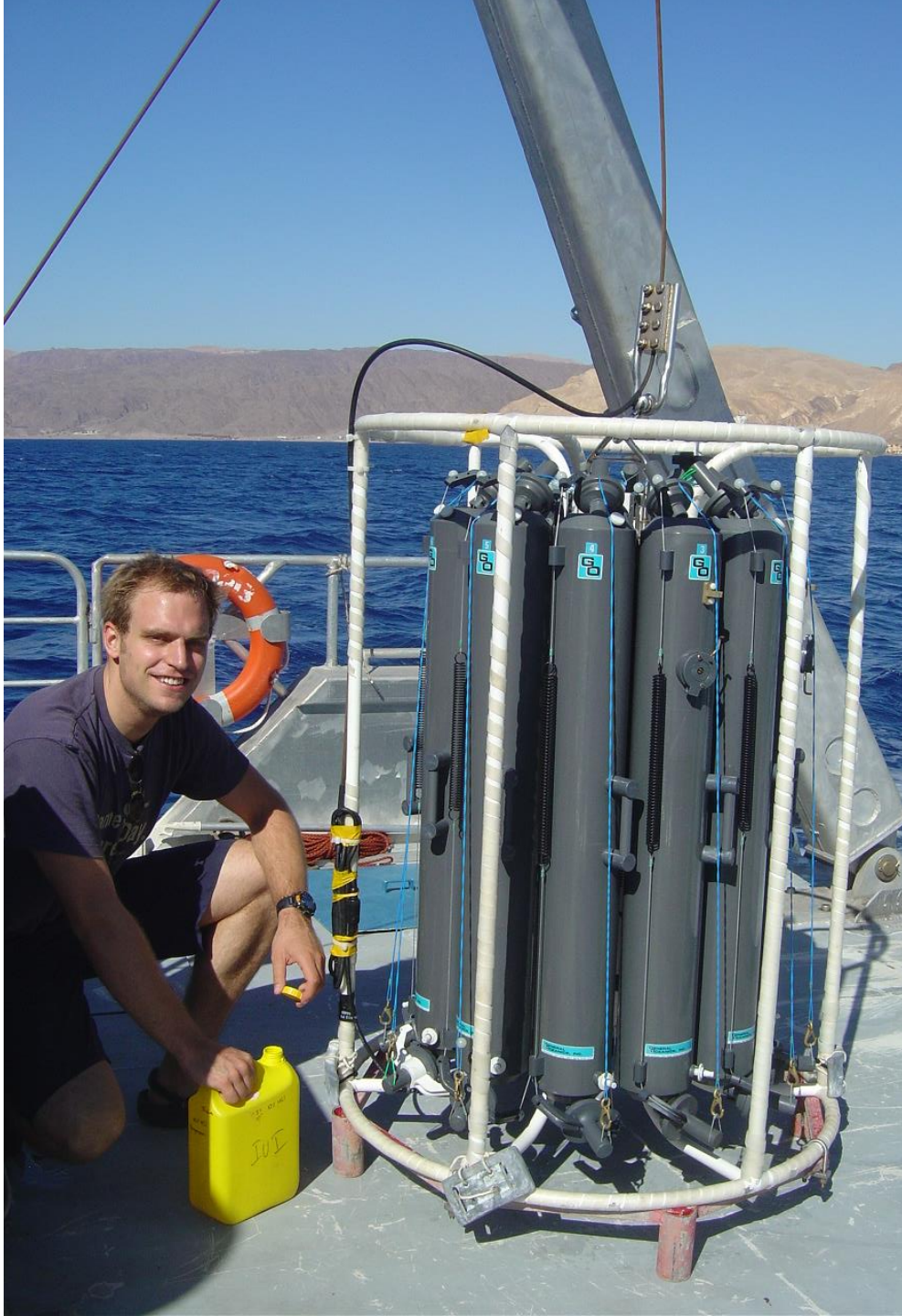
Example: *isiA* mRNA // IsrR antisense RNA

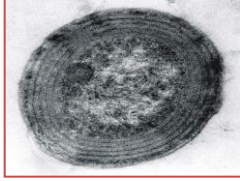
Other pitfalls:

<u>RNA half life</u>

RNA is not very stable as the gene as the gene expression profile is constantly changing in a cell.

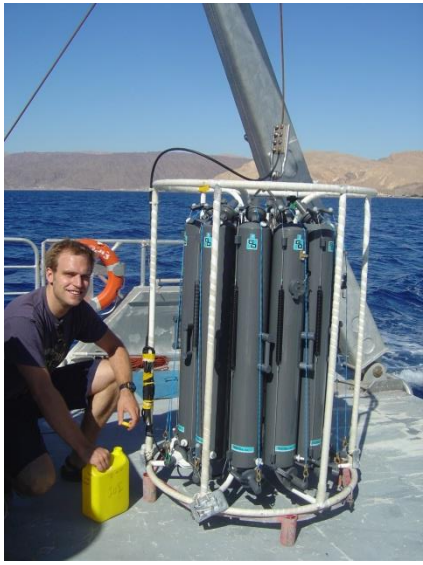
You don‘t want the gene epxression on deck of the ship to be measured (or in the dark centrifuge either)





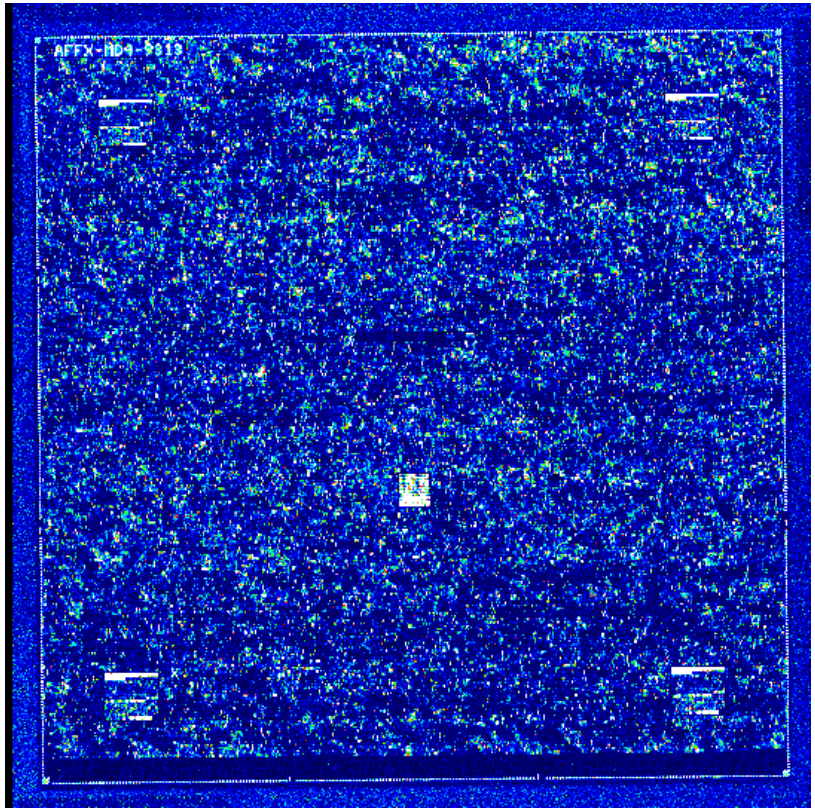
Other pitfalls:

RNA half life - What do you guess is the median half life of mRNA in *Prochlorococcus*?

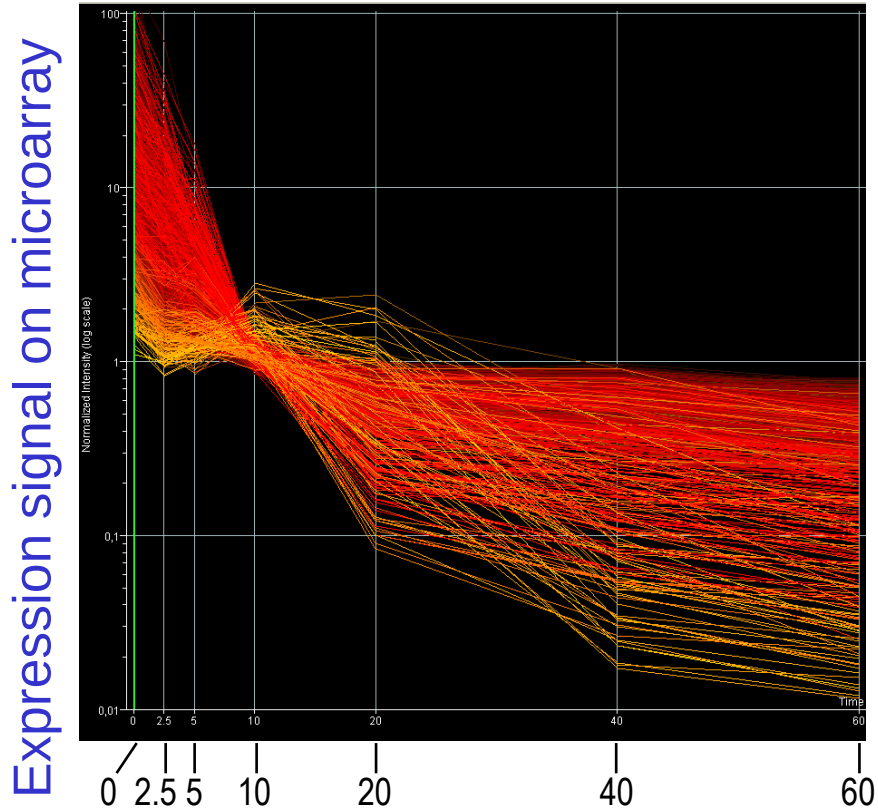


How to measure half-life time?

- rifampicin binds to beta subunit of RNA polymerase
- prevents initiation of new transcripts
- RNA stability can be measured



Affymetrix high density microarray



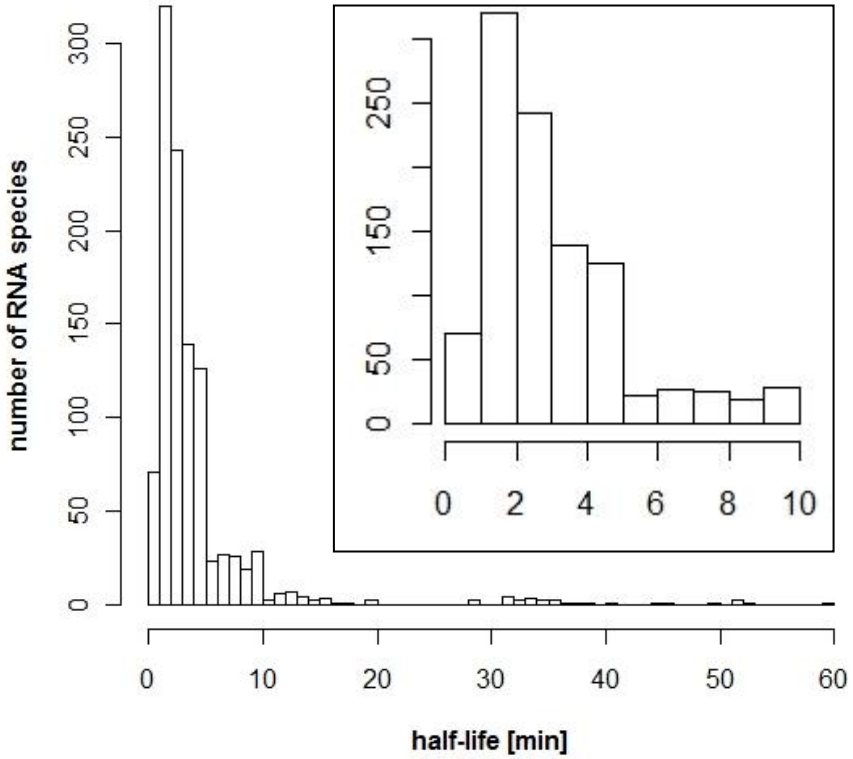
Time [min] after rifampicin addition

slide: C. Steglich

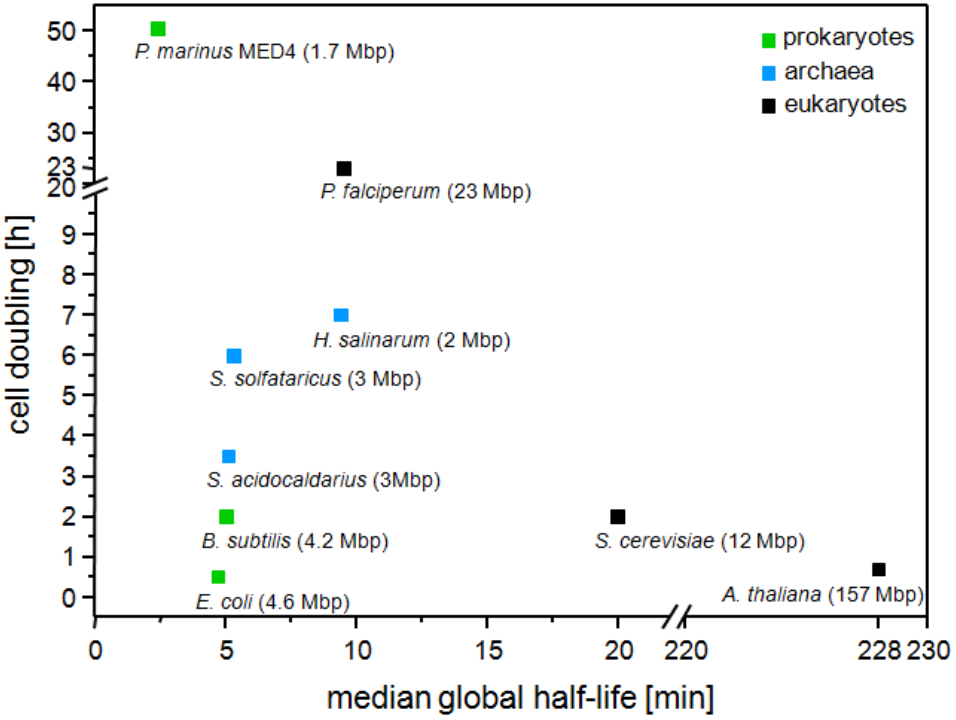
Steglich et al. Genome Biol 2010

Shortest global half-life ever measured for any organism

global half-life of 2.4 min

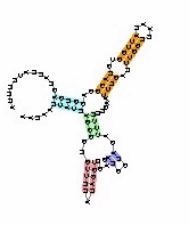


cell doubling vs. half-life



slide: C. Steglich

2.3 RNA-seq

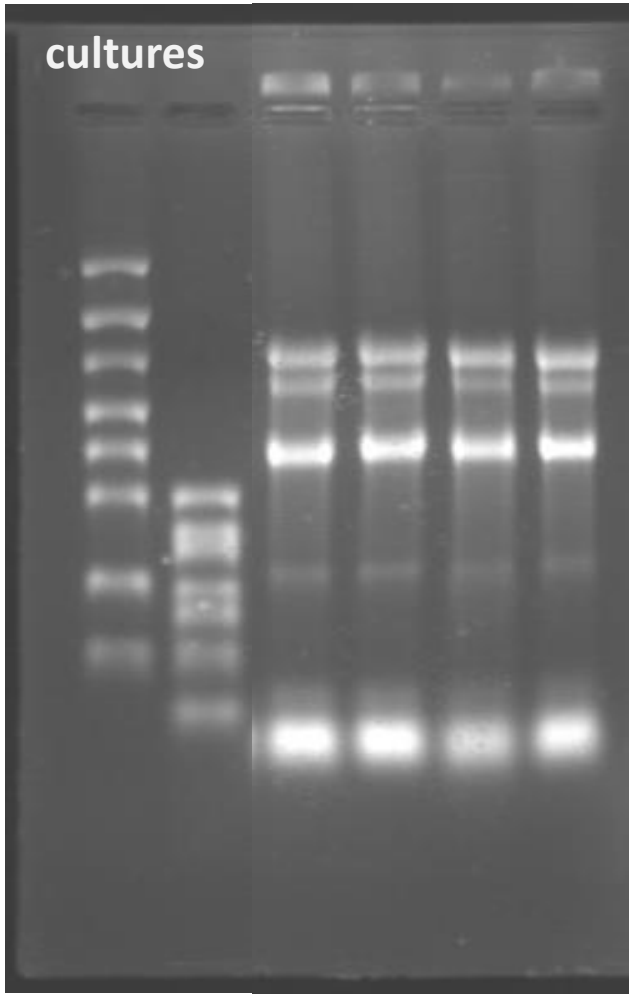


(Isolation and sequence analysis of all small RNAs; “dRNA-seq”, “RNomics“, 454, Solexa)

Transcriptome sequencing

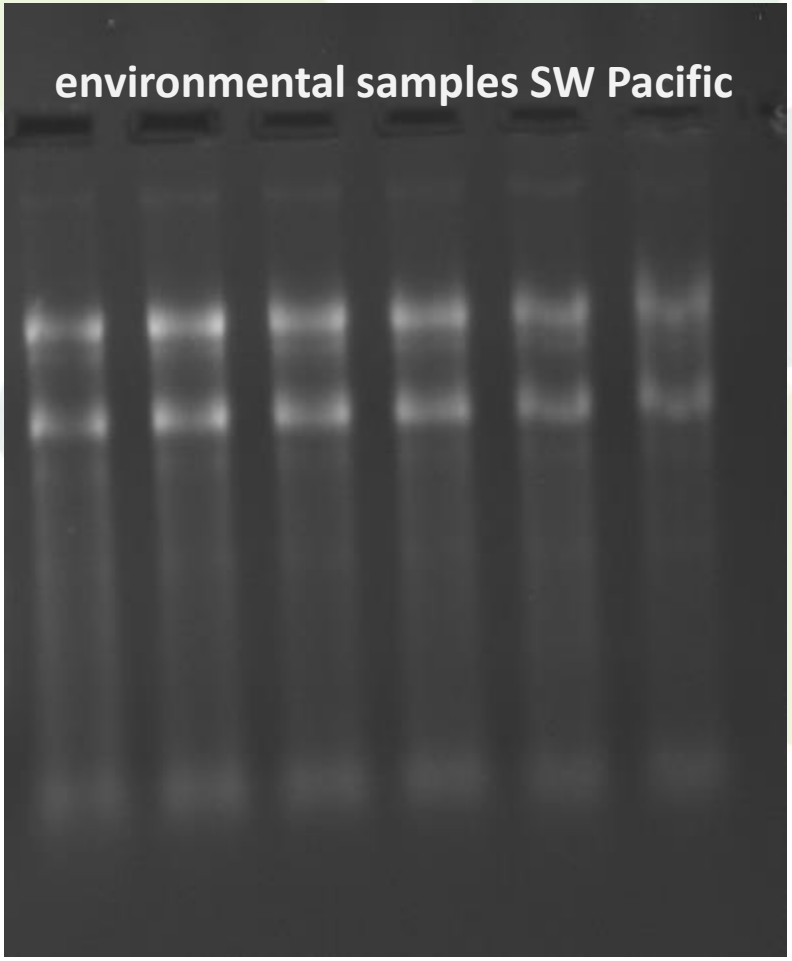
MaCuMBA

RNAseq mainly sequences cDNA of ribosomal RNA



rRNAs

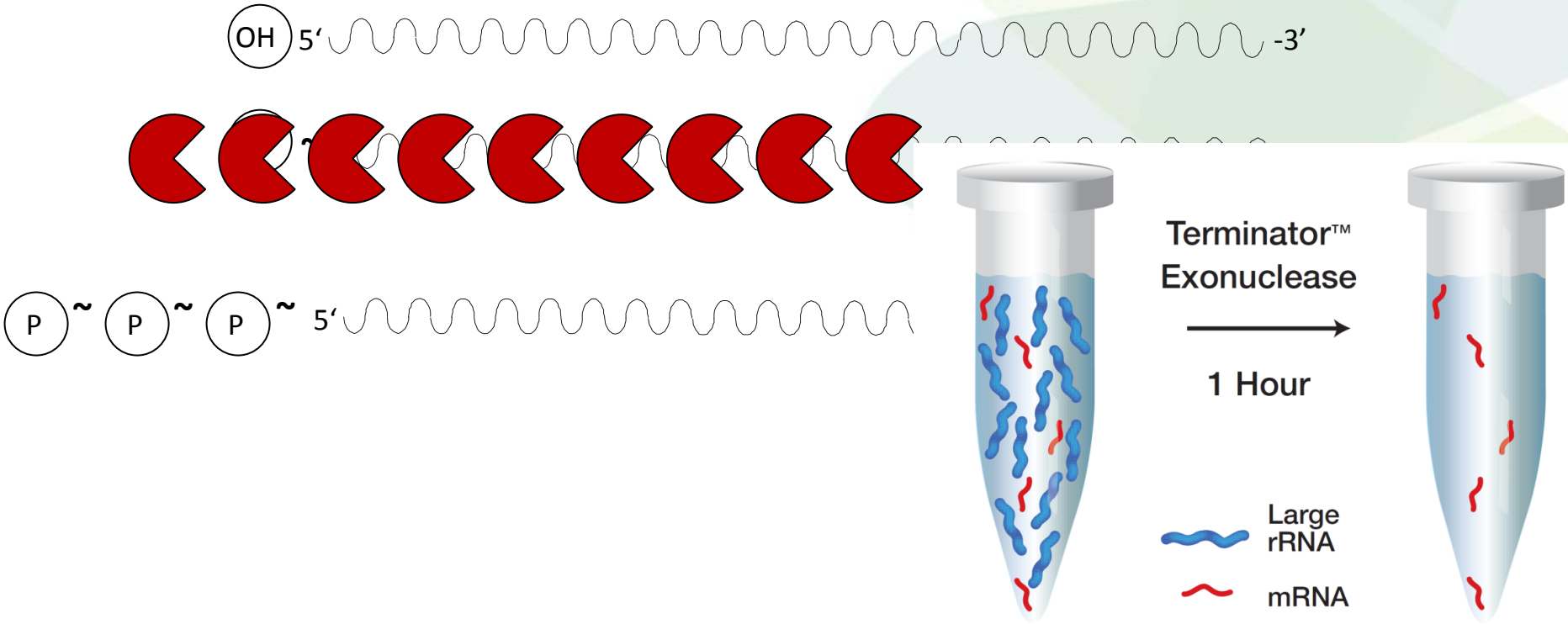
tRNA



dRNA-seq and rRNA depletion

Enzymatically - Terminator™ Exonuclease (TEX):

- In the cell rRNA is processed from a primary transcript → rRNA maturation
- Primary transcripts: 5‘-PPP
- Processed transcripts: 5‘-P, 5‘-OH (don’t ligate)



Differential RNAseq (dRNA-seq) according to Sharma et al. (2010)

TEX (5´PPase)-treatment removes not only rRNA, but all processed/degraded RNAs.

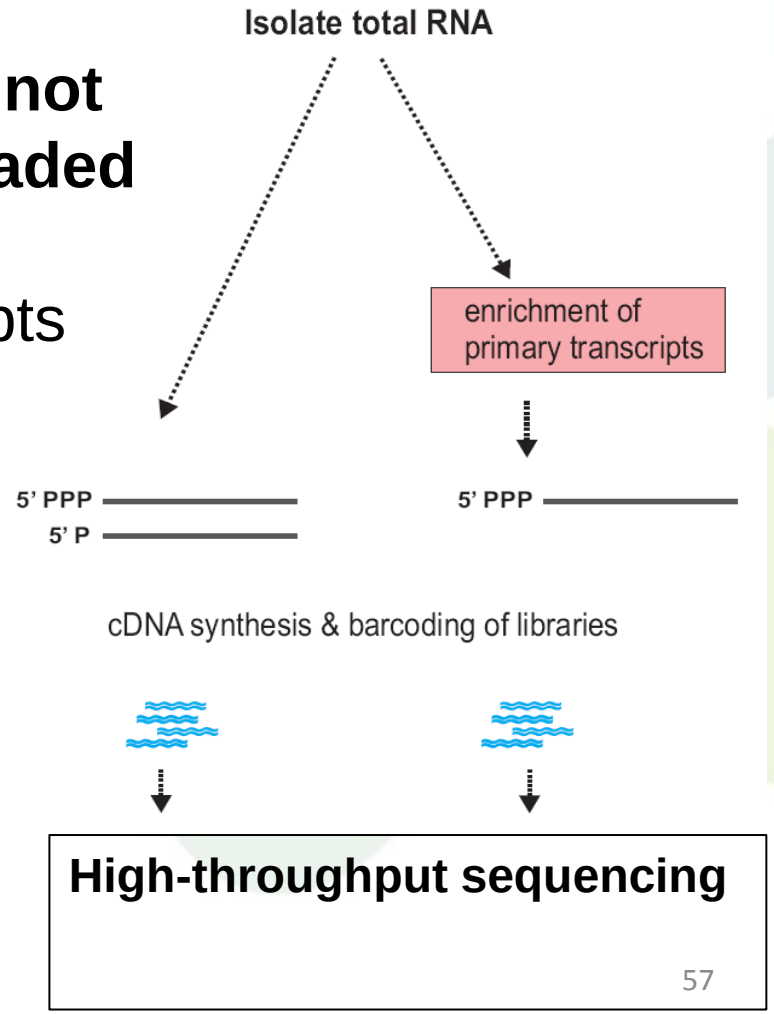
⇒ Enrichment for primary transcripts

→ mapping of TSS

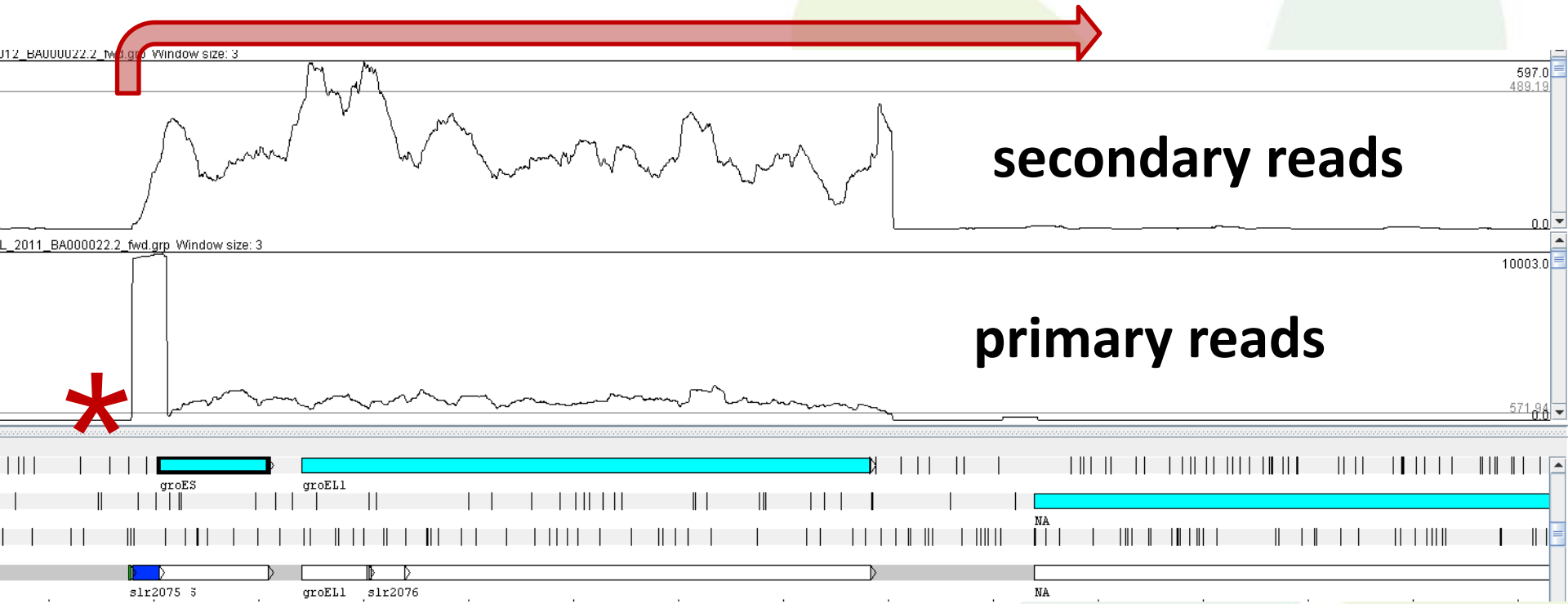
dRNA-seq:

• 2 libraries:

TEX-treated vs. untreated



Difference between TEX/TAP-treated and untreated RNA (dRNA-seq versus RNA-seq)

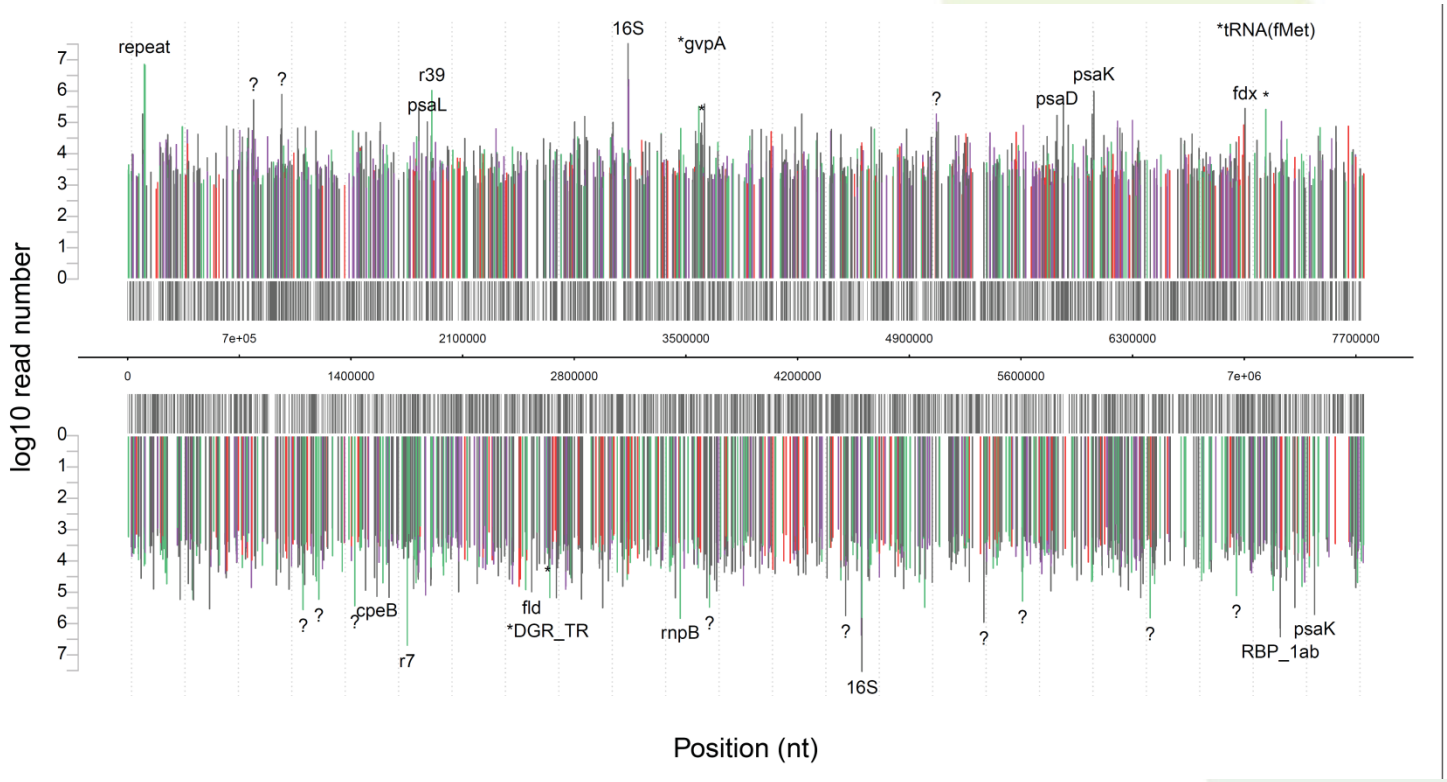


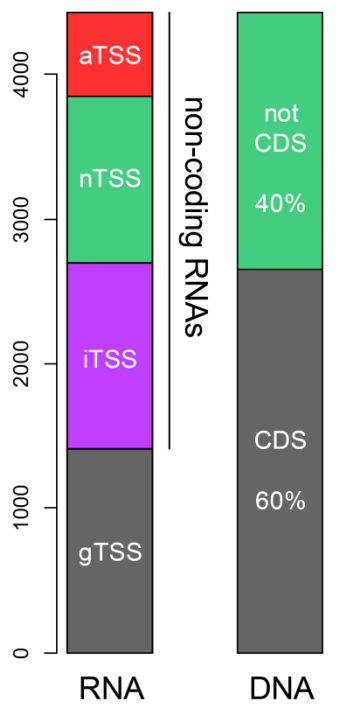
Example: Total RNA from *Synechocystis* 6803 (high light cultures)

*Transcriptional start site

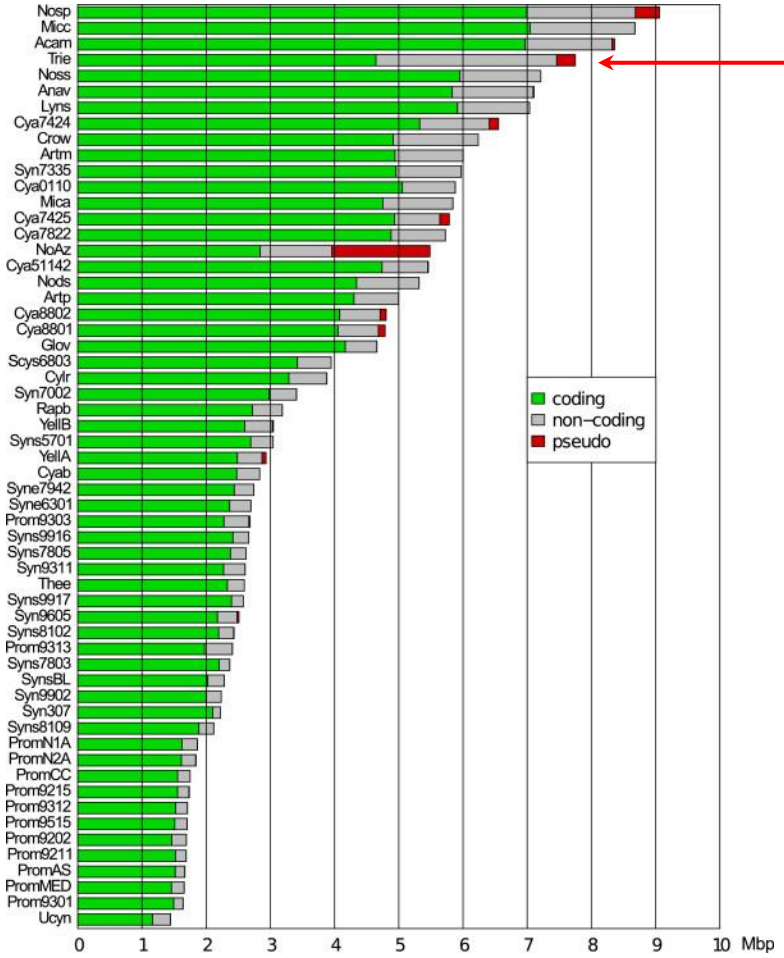
(3) What kind of things can we find?

The primary transcriptome of *Trichodesmium*





non-coding potential



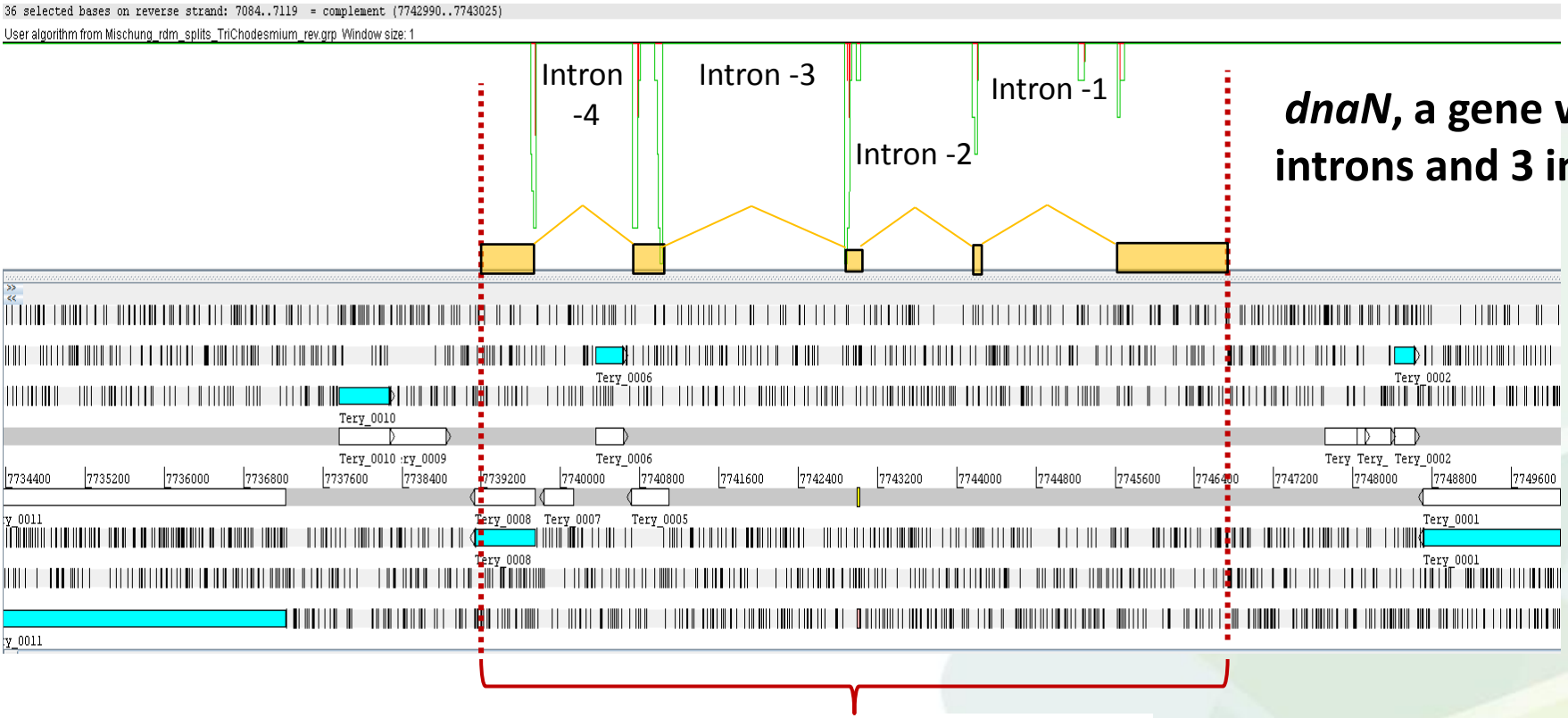
Larsson et al., 2011

Trichodesmium erythraeum

highest proportion of non-coding nucleotides amongst all sequenced cyanobacteria to date;

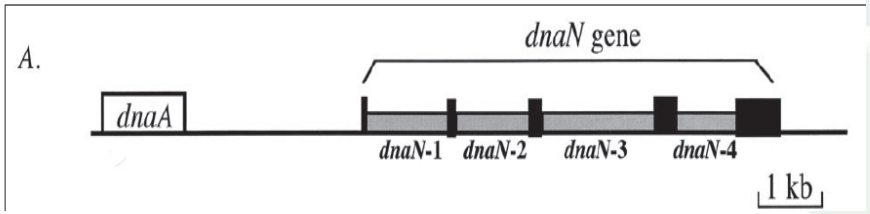
some analogy to eukaryotic genomes

mapping of split transcriptome reads in Trichodesmium



***dnaN*, a gene with 4 introns and 3 inteins**

ACCELERATED PUBLICATION: *Intron-encoded General Maturase*

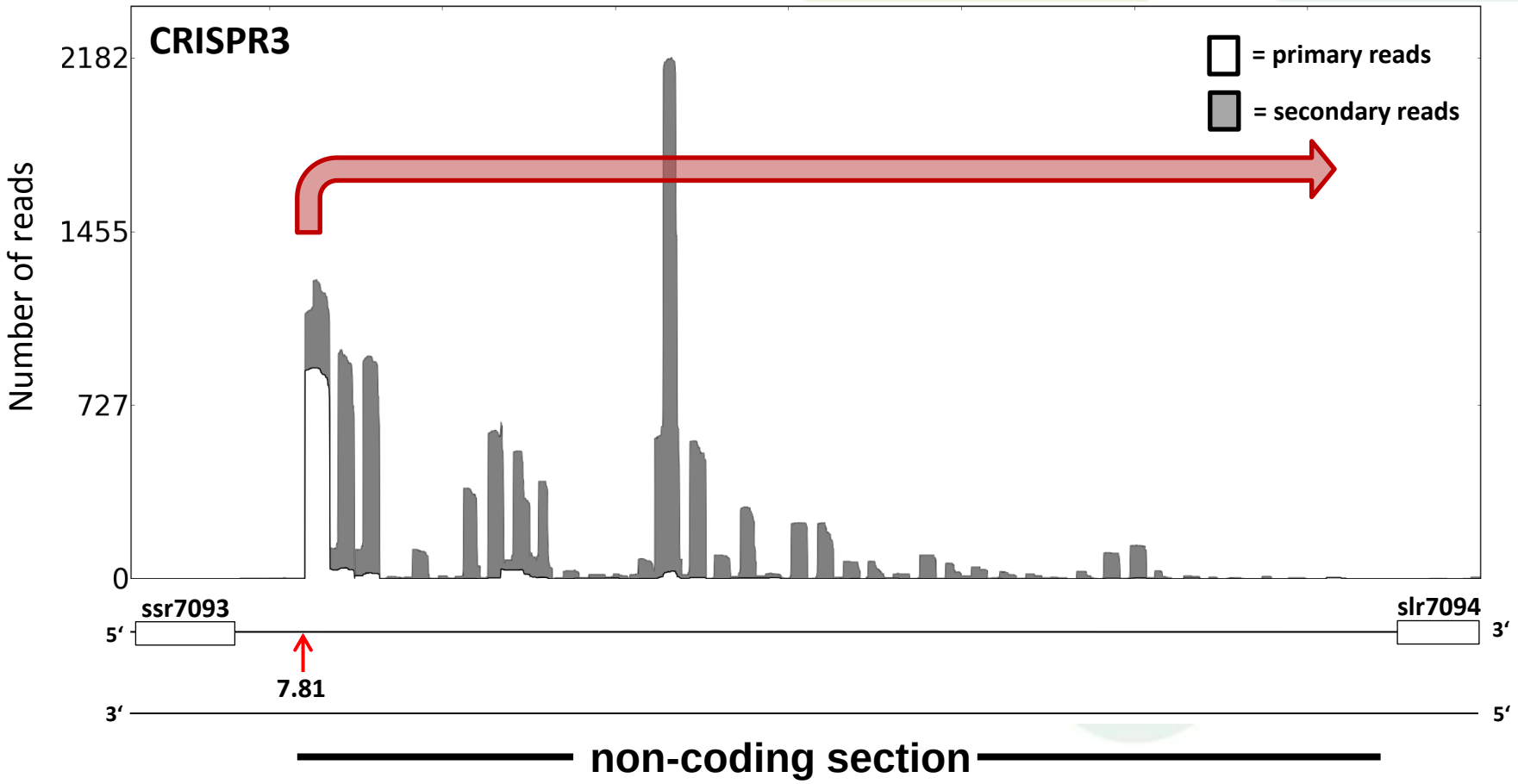


Accelerated Publications:

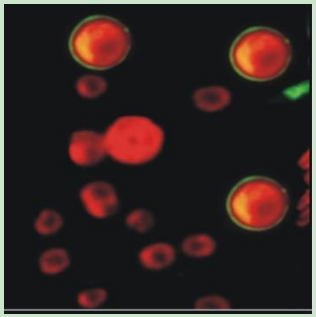
An Intron-encoded Protein Assists RNA Splicing of Multiple Similar Introns of Different Bacterial Genes

Qing Meng, Yanfei Wang and Xiang-Qin Liu
J. Biol. Chem. 2005, 280:35085-35088.
doi: 10.1074/jbc.C500328200 originally published online September 7, 2005

Interaction with bacteriophages: Identification of antiviral CRISPR expression (*Synechocystis*)



CRISPR in *Synechocystis* PCC6803



ssl7004 antitoxin

sll7006 toxin/antitoxin system

sll7003 toxin

slr7010 (cas3)

slr7011 (csc3)

slr7012 (csc2)

slr7013 (csc1)

slr7014 (cas6)

slr7015 (cas4)

slr7016 (cas1)

ssr7017 (cas2)

ssr7093 (cas2)

slr7092 (cas1)

sll7090 (Cas10, Cmr2)

sll7089 (Cmr3)

sll7087 (Cmr4)

sll7085 (Cmr6)

CRISPR RAMP module 1

slr7081 (UvrD helicase)

slr7080 (Csx3)

CRISPR3 constitutive, most highly expressed

CRISPR1 constitutive expression

Plasmid pSYSA

103,307 bp

xbase viewer: http://xbase.ac.uk/

CRISPR2 responds to environmental stimuli

sll7075 (cas6)

ssr7072 (cas2)

slr7071 (cas1)

slr7068 (cas6)

sll7067 (cmr2)

ssl7046 toxin

GC 0.5 0.45 0.4 0.35

number of reads in transcriptome analysis: >500 >200 >60 <60

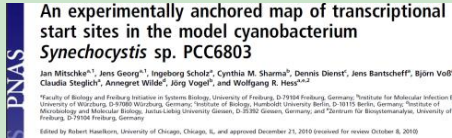
sll7063, sll7065, sll7066 (CRISPR system related protein, RAMP superfamily)

slr7061 (cas-protein TM1812 family)

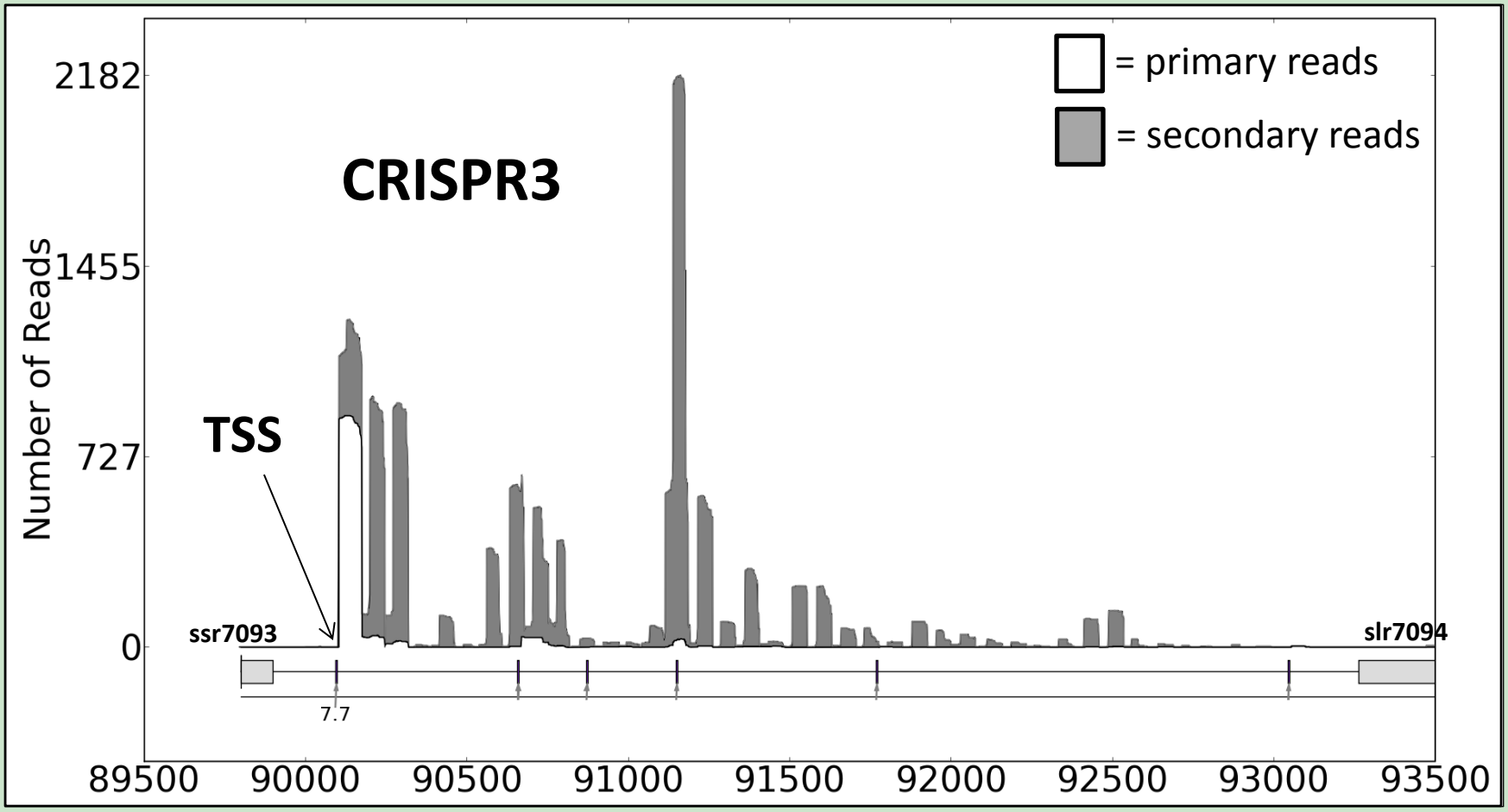
sll7062 (cas-protein APE2256 family)

CRISPR RAMP module 2

***Synechocystis* sp. PCC 6803 has three CRISPR arrays located on plasmid pSYSA**

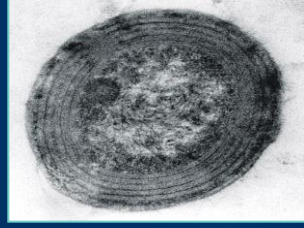


454-sequencing results

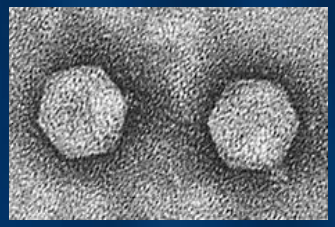


CRISPR-derived crRNAs are among the most highly expressed transcripts in the cell

Cyanophages

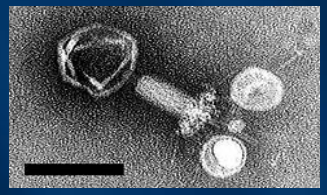


Podoviridae: - short tails that are non contractile
- linear double stranded genome (~ 40 kbp, ~ 55 genes)



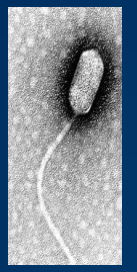
cyanophage P-SSP7, picture Bin Ni, Chisholm Lab

Myoviridae: - non enveloped viruses that consist of a head and a tail separated by a neck
- linear double stranded genome (30-170 kbp, 200-300 genes)



cyanophage P-SSM4, picture Bin Ni, Chisholm Lab

Siphoviridae: - consist of a head and non contractile tail
- linear double stranded genome (~ 50 kbp, ~ 70 genes)



cyanophage MIT9313-4, picture Matt Sullivan, Chisholm Lab

slide: C. Steglich

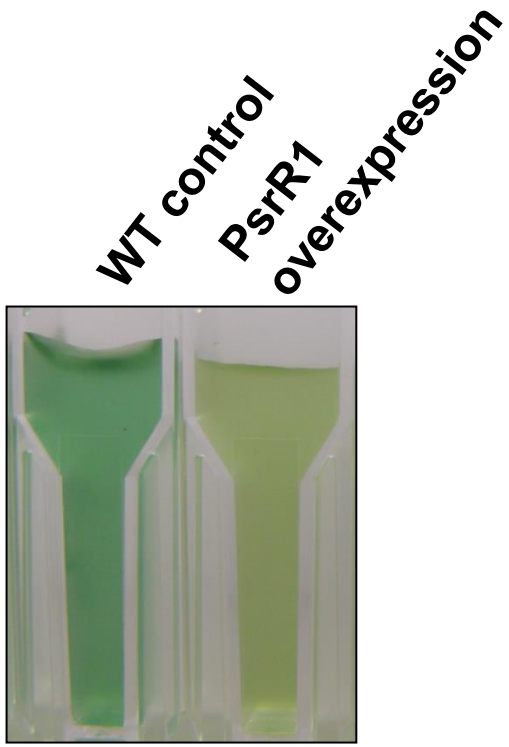
- **asRNAs modulate gene expression through the protection of mRNAs from RNase E cleavage**

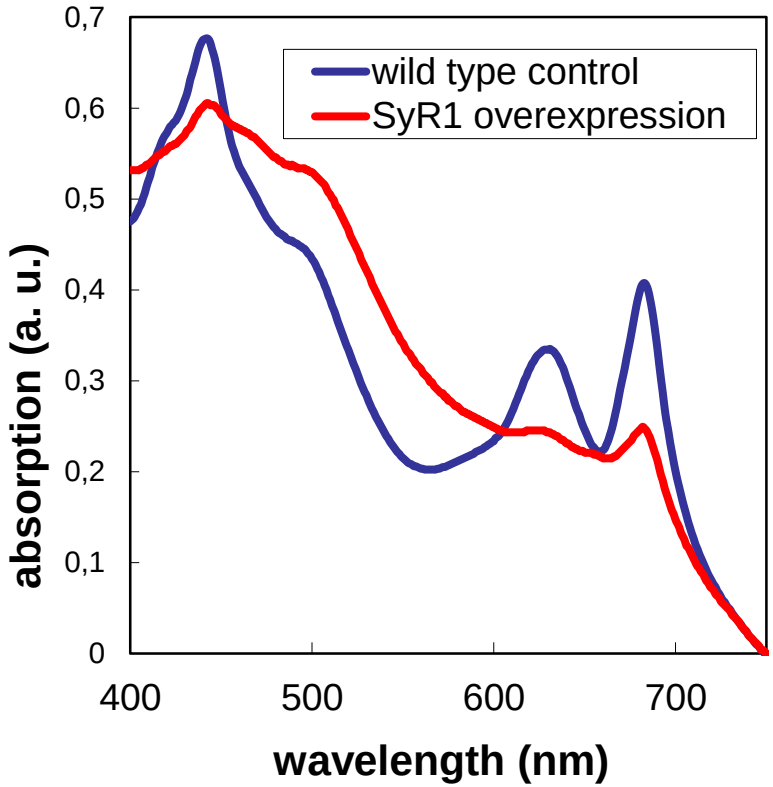
- **in T7 and T4 phage RNase E activity is regulated by phosphorylation of the C-terminus (not present in *Prochlorococcus* RNase E)**

- **indirect regulation of RNase E activity in *Prochlorococcus* by RNA duplex formation reflects an alternative mechanism of regulation of enzyme activity**

slide: C. Steglich

Pigmentation phenotype of PsrR1 overexpression in *Synechocystis*



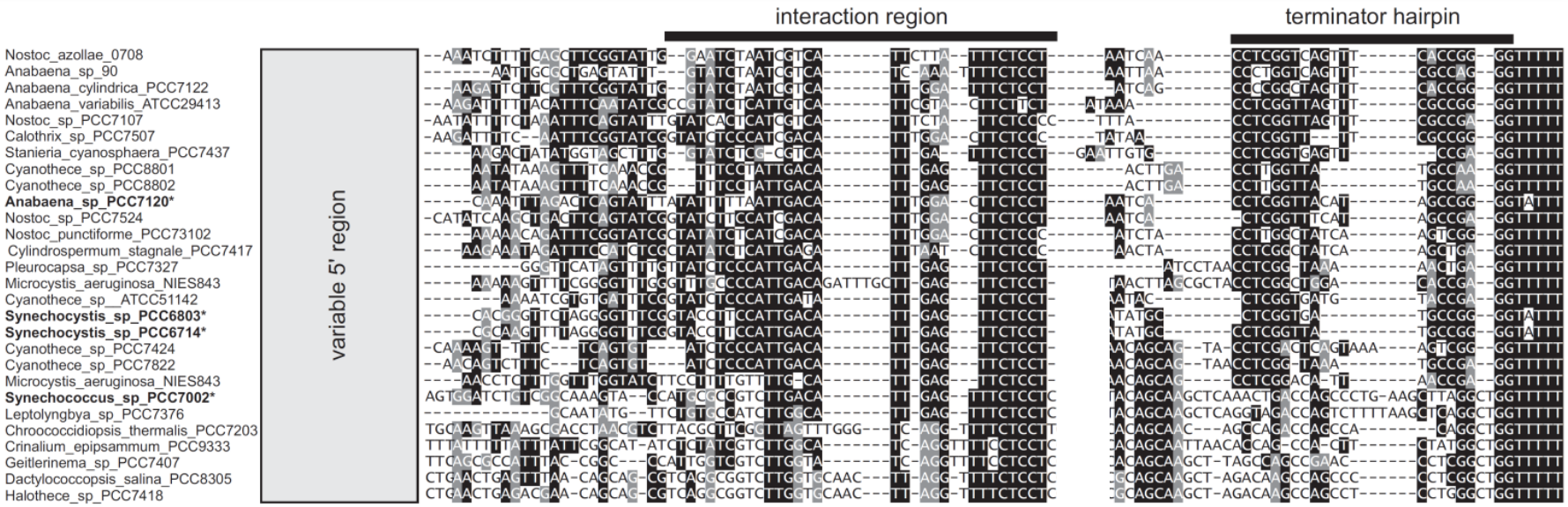


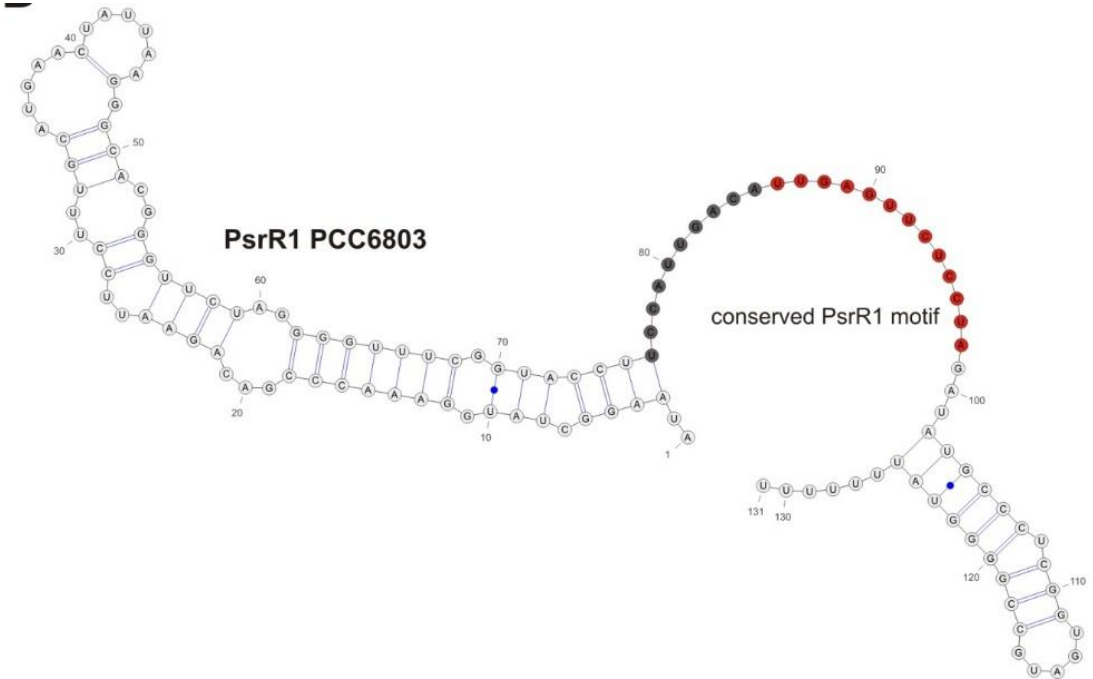
PNAS

An experimentally anchored map of transcriptional start sites in the model cyanobacterium *Synechocystis* sp. PCC6803

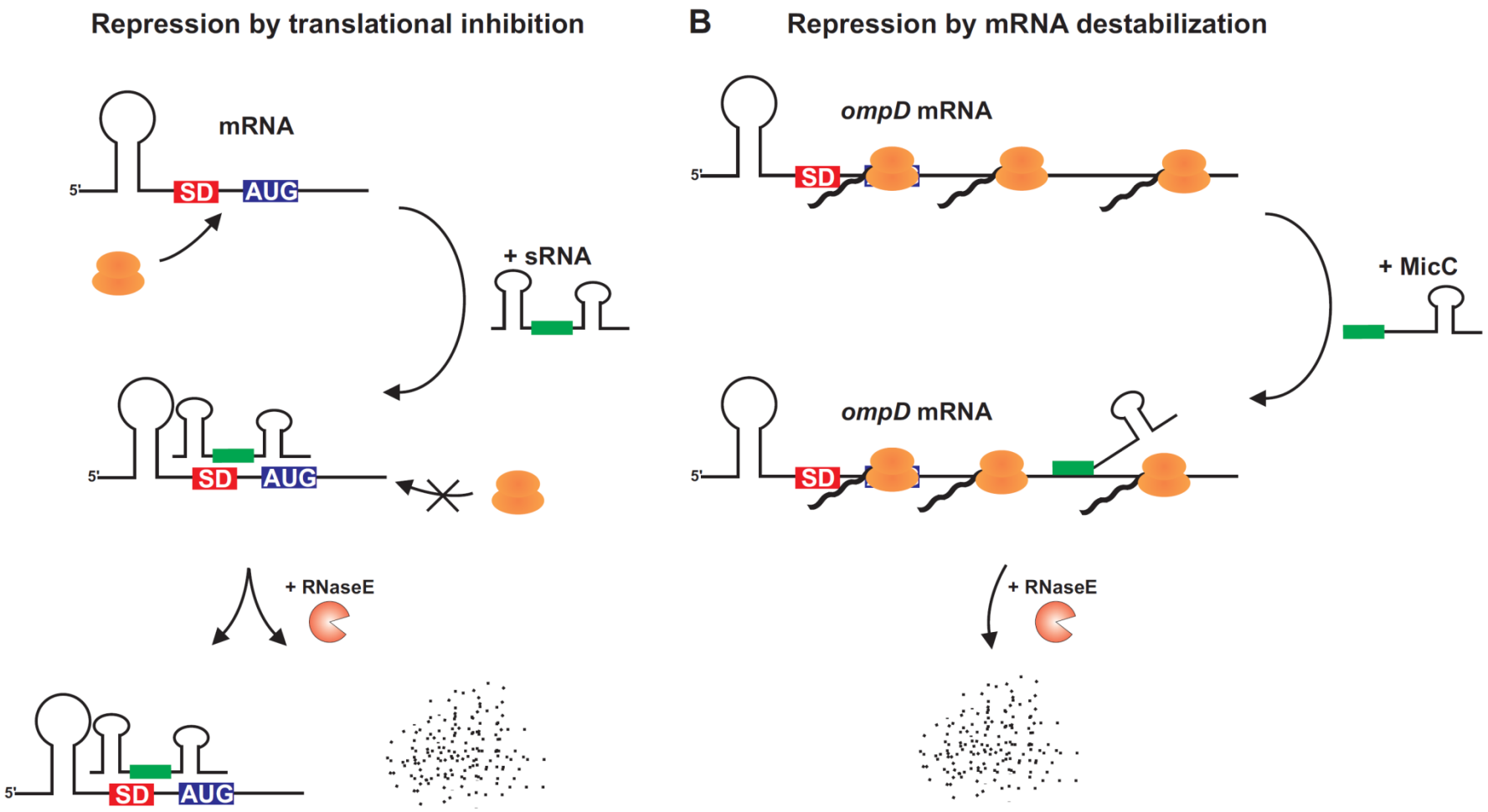
Jan Mitschke[a,1], Jens Georg[a,1], Ingeborg Scholz[a], Cynthia M. Sharma[b], Dennis Dienst[c], Jens Bantscheff[a], Björn Voß[a], Claudia Steglich[a], Annegret Wilde[d], Jörg Vogel[b], and Wolfgang R. Hess[a,e,f]

PsrR1 is a conserved sRNA



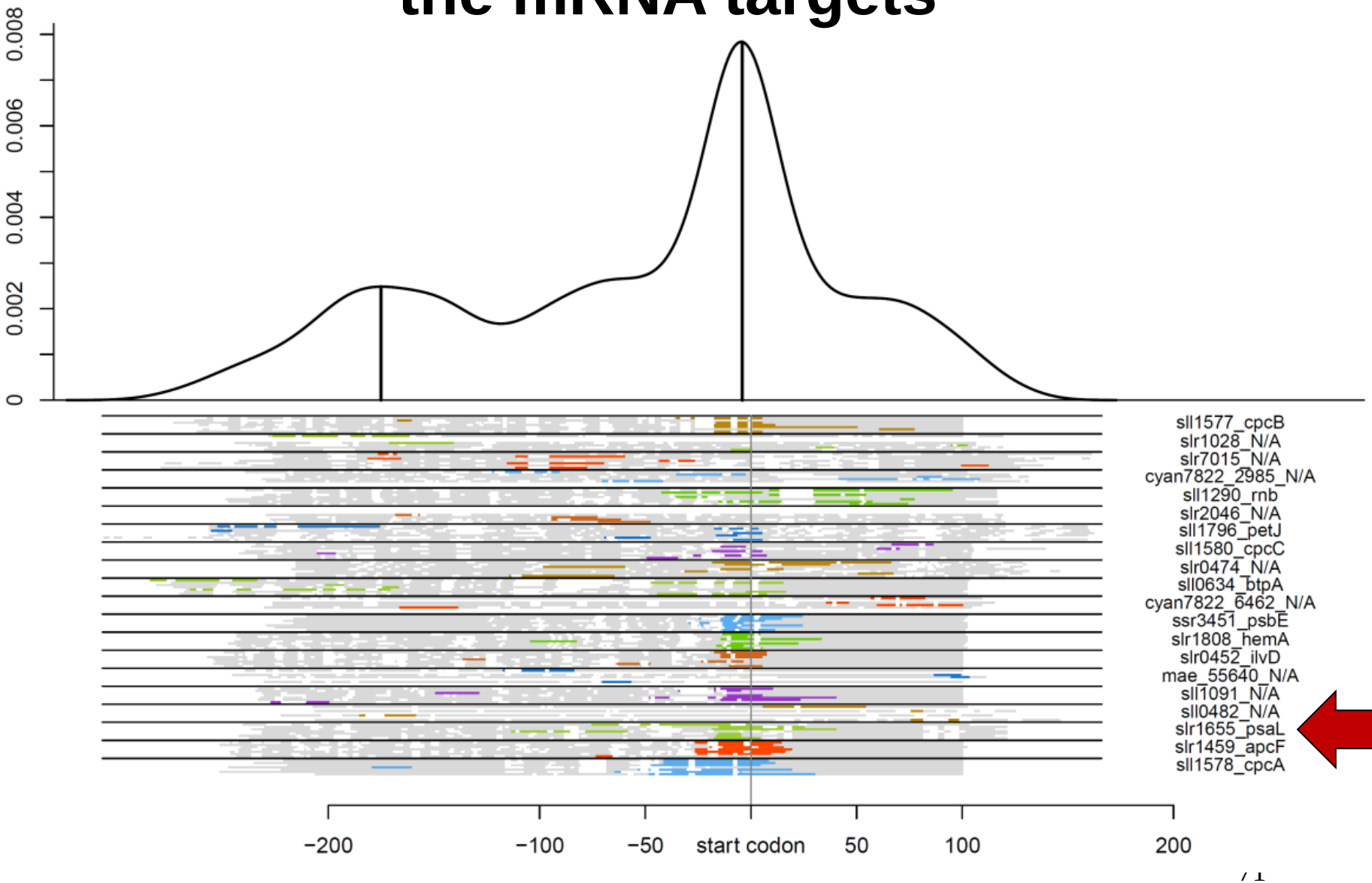


How bacterial sRNAs work: repression of gene expression



From: Corcoran *et al.* (2011), Hfq-associated regulatory small RNAs. In: Hess W.R. & Marchfelder A. (Editors) Regulatory RNAs in Prokaryotes. Springer-Verlag Wien New York.

Prediction of PsrR1 interaction sites within the mRNA targets



CopraRNA: Comparative Prediction Algorithm for sRNA Targets: *PNAS Plus* (2013) 110 (37), E3487-E3496.

The combination of different transcriptomic approaches allows to:

- measure gene expression in a comparative mode
- map precisely the suite of active promoters
- infer regulatory sequence elements
- derive excact information on transcript boundaries, i.e. define operons and transcript isoforms
- find highly transcribed regions of special importance, e.g. CRISPRs, the prokaryotic immune system
- identify previously unknown transripts, such as non-coding sRNAs, and
- PsrR1 is an sRNA regulator of *psaL* and likely of several more photosynthesis-associated genes in cyanobacteria

MaCuMBA

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Ilana Berman-Frank (Bar-Ilan-Univ. Israel)

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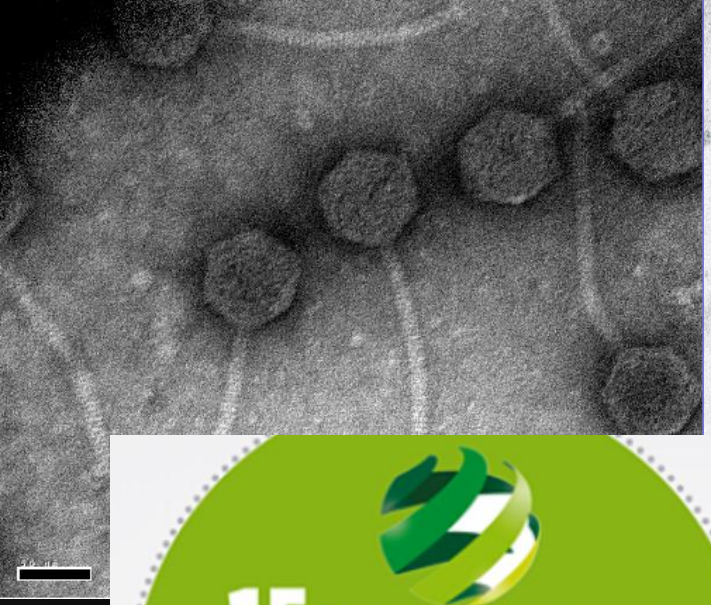
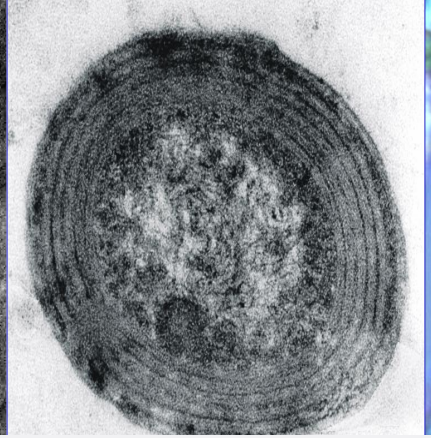


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