Pseudomonas

Juan-Luis Ramos · Alain Filloux
Editors

Pseudomonas

Volume 6: Molecular Microbiology, Infection and Biodiversity

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Preface

Paris is a cosmopolitan city where roaring life, wonderful museums and excellent science can be found. It was during the XI IUMS conference held in this city that the *Pseudomonas* book series was first envisaged. On the first row of the auditorium sat a group of outstanding scientists in the field, who after devoting much of their valuable time, contributed in an exceptional manner to the first three volumes of the series, which saw the light simultaneously. The volumes were grouped under the generic titles of "Vol. I. *Pseudomonas*: Genomics, Life Style and Molecular Architecture", Vol. II. *Pseudomonas*: Virulence and gene regulation; Vol. III. *Pseudomonas*: Biosynthesis of Macromolecules and Molecular Metabolism.

Soon after the completion of the first three volumes, a rapid search for articles containing the word *Pseudomonas* in the title in the last 10 years produced over 6,000 articles! Consequently, not all possible topics relevant to this genus were covered in the three first volumes. Since then two other volumes were published: *Pseudomonas* volume IV edited by Roger Levesque and Juan L. Ramos that came to being with the intention of collecting some of the most relevant emerging new issues that had not been dealt with in the three previous volumes. This volume was arranged after the *Pseudomonas* meeting organized by Roger Levesque in Quebec (Canada). It dealt with various topics grouped under a common heading: "*Pseudomonas*: Molecular Biology of Emerging Issues".

Yet the "*Pseudomonas* story" was far from complete and a new volume edited by Juan L. Ramos and Alain Filloux was deemed necessary. The fifth volume was conceived with the underlying intention of collecting new information on the genomics of saprophytic soil *Pseudomonas*, as well as on the functions related to genomic islands and was published in 2006.

At the request of a number of scientists and colleagues working in the field, we have collected a new set of chapters that are called on to provide further views on the biology of *Pseudomonas*. Chapters in *Pseudomonas* volume VI have been grouped under the following topics: *Regulation and control of virulence, Life styles, Physiology and Metabolism*. The chapters under the heading *Life Styles* constitute an in-depth analysis of the genome of *Pseudomonas stutzeri*, a denitrifier *par excellence*, and the behaviour and life style of *P. aeruginosa* in the human lung. The *Physiology Metabolism and Markers* section collects four chapters that deal with the

metabolism of acyclic terpenes by *Pseudomonas*, the biodiversity of siderophores, resistance to heavy metals and the role of relevant second messenger, a c-di-GMP, as a signalling molecule. Finally under *Regulation and control of virulence* we find several chapters dealing with sensing at the level of cell surfaces and quorum sensing, as well as the role of small RNAs in the control of gene expression.

It would not be fair not to acknowledge that this sixth volume would never have seen the light if it were not for a group of outstanding scientists in the field who have produced enlightening chapters to try to complete the story that began with the five previous volumes of the series. It has been an honour for us to work with them and we truly thank them.

The review process has also been of great importance to ensure the high standards of each chapter. Renowned scientists have participated in the review, correction and editing of the chapters. Their assistance is immensely appreciated. We would like to express our most sincere gratitude to:

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Granada, Spain — Juan L. Ramos
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Obituary

Yoshifumi Itoh, Professor of Microbial Biotechnology at Tohoku University (Sendai, Japan), suddenly passed away on October 4, 2008. He was well-known to the *Pseudomonas* community as a specialist of amino acid metabolism and plasmid replication. In this book series, he contributed two chapters, one on arginine and polyamine metabolism (in volume III) and one on histidine catabolism and catabolite repression (in volume V). He published almost 100 papers and book chapters during his career as a microbiologist.

Yoshi, as he was known to his friends, was born in a small city near Sendai (Miyagi Prefecture) on August 5, 1951. Here he grew up with his elder sister and younger brother. After studies at Tohoku University, he obtained a Master of Agricultural Science in 1976 and a PhD of Agricultural Science in 1979. His PhD thesis was on the mode of action of a bacteriocin in *Erwinia carotovora*. In

1980, he was hired as a Research Associate at Shinshu University (Matsumoto, Japan), where he worked with Yoshiro Terawaki and Hideki Matsumoto. He soon left to spend two years (1981–1983) as a postdoctoral fellow at the Department of Microbiology, ETH Zurich (Switzerland) where he worked in the group of Thomas Leisinger and Dieter Haas. He characterized the replication and partition functions of the *Pseudomonas* plasmid pVS1. Many years later, in 2000, he extended this work and, in collaboration with the group of Dieter Haas, made use of the minimal pVS1 replicon in the construction of a series of stable plasmid vectors, which are widely used and cited today. Upon return to Japan in 1983, he again joined the School of Medicine at Shinshu University. In 1988, he moved to the National Food Research Institute in Tsukuba where he eventually became the head of the Applied Bacteriology Laboratory. He developed an interest in metabolic functions of *Pseudomonas aeruginosa* and their regulation. Simultaneously, he also studied *Bacillus subtilis* as an organism used for natto (fermented soybean) production. In 2004, he became Director General of the Akita Research Institute of Food and Brewing. At that time he was somewhat reluctant to take up this position, as it did not allow him to spend much time on research. So he was very pleased that two years later he was appointed to his home University at Sendai as a Professor. Although he had substantial teaching commitments, he was finally able to return to his favourite research topics.

Probably the most significant scientific contribution of Yoshifumi Itoh was his discovery of the CbrAB two-component system in *P. aeruginosa*. This system regulates the activity of sigma-54 RNA polymerase during the utilization of numerous carbon and nitrogen sources. Yoshifumi Itoh was a dedicated scientist and always enthusiastic about his research. He had a fine sense of humour. His untimely death came as a complete and very sad surprise to everyone. He is sorely missed by his wife Junko, his daughter, his son and his friends and colleagues.

Dieter Haas

Part I
Regulation and Control of Virulence

Chapter 1
Small RNAs of *Pseudomonas* spp.

Elisabeth Sonnleitner, Nicolas González and Dieter Haas

1.1 Introduction

Small RNAs (sRNAs) of prokaryotes are 40–600 nucleotides in length and most have regulatory functions. As a rule, sRNAs do not encode polypeptides but important exceptions exist. Some bacterial sRNAs were discovered and characterized in the last two decades of the twentieth century, mostly with emphasis on sRNAs that regulate functions of plasmids, bacteriophages or transposable elements. Although it was already clear at that time that sRNAs could have diverse functions, their roles as cellular regulators were not fully appreciated [1]. This picture has changed since then with the discovery of many novel sRNAs, made possible by the introduction of new computational and genomic approaches. To date, close to 100 sRNAs have been reported in *Escherichia coli* and more than 150 sRNAs in prokaryotes altogether [2]. In this chapter, we will review the current status of sRNAs encoded by chromosomal genes in *Pseudomonas* species, with emphasis on *P. aeruginosa* where most genomic information is available. To date, > 40 sRNAs have been detected in this organism; however, for many of them, the functions have not yet been uncovered.

Why has it been relatively difficult to find sRNAs and their genes in prokaryotes by classical genetical and biochemical approaches? There are several reasons for this. (i) Mutational inactivation of a particular sRNA gene often does not cause

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Note added in proof:
After completion of this review in December 2008, Brencic et al. (Mol. Microbiol. 73:434–445, 2009) reported that the GacS/GacA two-component system achieves virtually all of its regulatory effects by positively controlling the expression of the *rsmY* and *rsmZ* genes in *P. aeruginosa,* implying that the GacA protein binds exclusively to the *rsmY* and *rsmZ* promoters. Moreover, the global H-NS-type regulator MvaT was recognised as a repressor of the *rsmZ* gene. Sonnleitner et al. (Proc. Natl. Acad. Sci. USA, doi: 10.1073/pnas. 0910308106) described a novel sRNA, CrcZ, mediating catabolite repression in *P. aeruginosa.* CrcZ is specified by the *cbrB-pcnB* intergenic region.

J.L. Ramos, A. Filloux (eds.), *Pseudomonas*, DOI 10.1007/978-90-481-3909-5_1,

an easily observable phenotype. (ii) Numerous sRNAs are functionally redundant and therefore loss of one element does not have important consequences. (iii) In transposon mutagenesis, sRNA genes may be spared because they are small. (iv) Point mutations are much better tolerated in sRNA genes than in coding genes where missense, nonsense or frameshift mutations usually lead to loss of function. (v) Overexpression of sRNAs, while providing a useful tool for probing sRNA function, is a technically demanding method in that it often requires a strong external promoter to be fused precisely to the transcription start site of an sRNA gene.

There are several strategies that have proved useful in the elucidation of prokaryotic sRNAs. These approaches have been explored mostly in *E. coli* and several have also been applied to *Pseudomonas* spp. (i) A few sRNAs are sufficiently abundant so that they can be isolated from cells in pure form and sequenced. Historically, this has been the case for 4.5S RNA (the product of the *ffs* gene, the RNA component of the signal recognition particle), 6S RNA (the *ssrS* gene product, a regulator of RNA polymerase), 10Sa RNA (the *ssrA* gene product, termed tmRNA today) and 10Sb RNA (the *rnpB* product, the RNA component of RNase P) in *E. coli* [1]. However, most sRNAs are present in low amounts that preclude this approach. (ii) Computational prediction of sRNAs, followed by experimental verification using Northern blotting, has proved a broadly applicable strategy. Various algorithms have been developed to this end. They usually assume that sRNA genes are located in intergenic regions (IgRs) rather than within open reading frames and that putative promoter and rho-independent terminator sequences must be within a short distance of each other (< 500 nucleotides). Furthermore, computational predictions often include the criterion that sequences as well as secondary structures of sRNAs should be conserved in closely related bacterial species [3]. The principal limitation of this approach is that it is difficult to predict bacterial promoters except for those that have highly conserved RpoD, RpoN or RpoS recognition elements. Another limitation is that in some cases no typical rho-independent terminator can be identified. (iii) sRNAs that have a high affinity for an RNA-binding protein can often be co-purified with the protein. Typically, the protein is isolated by immunoprecipitation or affinity chromatography and cDNAs are prepared from the sRNAs attached to the protein [4]. This strategy has been exploited successfully with proteins of the Hfq and CsrA families. Here, an inconvenient feature is the fact that a large proportion of non-specifically bound rRNAs and mRNA fragments are also enriched in the purified protein fraction. At any rate, the roles of sRNAs recognized in (ii) or (iii) have to be verified by genetic tests. (iv) Genetic screens – often involving multicopy expression of certain phenotypes – can be a fruitful strategy, especially when post-transcriptional regulation of gene expression is suspected, as for example is the case of the *rpoS* gene and genes encoding outer membrane proteins in *E. coli* [5–7]. Here, a caveat is that some overexpression effects might be missed because they are slight and transient or toxic to the cell [8, 9]. (v) Transcripts emanating from IgRs can be detected by microarrays when these cover the IgRs entirely; tiling microarrays are particularly useful in this respect. However, this approach can only be a

first step towards the elucidation of sRNAs and needs to be combined with other methods [10]. (vi) Direct shotgun cloning of cDNAs that are derived from size-fractionated RNAs can also be a useful first step, but this approach yields a high background of clones that are isolated due to RNA degradation products [11]. (vii) Finally, once an sRNA gene is well established in one bacterium, homologous sRNA genes may be found in related bacteria by BLAST searches. Such an approach may be facilitated when recognition sites for activators or repressors are conserved in promoter regions of sRNA genes or when neighbouring coding genes are conserved [12, 13]. It has to be pointed out, however, that sRNA genes diverge more strongly than do coding genes [2]. For this reason, for example, only few sRNA genes of *Pseudomonas* spp. could be deduced from sRNA genes of *E. coli* by homology searches.

1.2 Overview of Observed sRNAs in *P. aeruginosa*

To date, three genomic surveys of sRNAs in *P. aeruginosa* strain PAO1 have been published. The first study by Livny et al. [14] used a computational tool termed sRNAPredict2, which attempts to identify sRNA genes in IgRs for which conservation of sRNA sequence and secondary structure is found among multiple species. Furthermore, appropriately located rho-independent terminators are taken into consideration [3, 14]. From 38 predicted candidate genes, about half were found to specify transcripts that were detectable in Northern blots, in addition to four genes (*rsmY*, *rsmZ*, *prrF1* and *prrF2*) that had previously been identified (Table 1.1). A second survey by González et al. [15] took a similar approach, but used the QRNA program [23] instead of sRNAPredict2. From a preliminary list of 162 candidate IgRs, 14 were found to specify short transcripts and of these, eight were new. The latter number probably represents an underestimate because not all candidate IgRs were subjected to Northern blot analysis [15]. In a third study by Sonnleitner et al. [16], total RNA was extracted from *P. aeruginosa*, size-fractionated in the range of 50–500 nucleotides, and incubated with Hfq protein. After addition of anti-Hfq antibodies, the immunoprecipitate was recovered and RNA bound to Hfq was converted to cDNA, which was cloned and sequenced. After elimination of clones derived from mRNA fragments, tRNAs and rRNAs, eight candidate genes were recovered and sRNAs were verified by Northern blotting or RT-PCR. In addition, two sRNAs were predicted by RNAz, a bioinformatics tool based on the conservation of RNA structures, and verified by RT-PCR. In total, five new sRNAs were found. The consolidated data of the three studies [14–16] and an early publication describing 6S RNA [20] are summarized in Table 1.1, which shows observed transcript sizes, the orientation of the sRNA genes, map coordinates according to the genomic sequence of strain PAO1 [24], and flanking genes. Clearly, the total of 41 sRNA genes or candidate sRNA genes observed (Table 1.1) represents a preliminary estimate and this number is likely to grow as more studies will become available.

Table 1.1 Experimentally found sRNAs of *Pseudomonas aeruginosa* PAO1

Coordinates of intergenic regions	sRNAs							Flanking genes	
	Designation according to							5′ gene	3′ gene
	Livny et al. (2006) [14]	González et al. (2008) [15]	Sonnleitner et al. (2008) [16]	Gene	Location[a]	Size (nt)[b]	Orientation[c]	Name	Name
334456-334733	P1					300	<< >	PA0296	PA0297-*spuA*
586664-587016	RsmY		RsmY	*rsmY*[d]	586867-586990	124	<><	PA0527-*dnr*	PA0528
785174-785969			PhrD		785498-785570	73	>>>	PA0714	PA0715
901047-901933			tmRNA	*ssrA*	901520-901872	353[n]	<<<	PA0826	PA0827
912780-913085	P5	491				90	< >>	PA0836	PA0837 - *slyD*
971625-972166	P7 (?)[e]					140	>>>	PA0887-*acsA*	PA0888-*aotJ*
1117391-1118157	P8					130	>>>	PA1030	PA1031
1140860-1141267		622				200, 300	>>>	PA1052	PA1053
1204782-1205770		645			1205031-1205330	300	>< >	PA1112	PA1113
1436397-1436663	P9		–/96[o]		1436491-1436618	128	>>>	PA1324	PA1325
1668833-1669085		888–4.5S RNA	25/102[o]	*ffs*[f]	1668911-1669081	171 (113[f])	>><	PA1530	PA1531
1928627-1928893	P11		26/–[o]			100	<<<	PA1781-*nirB*	PA1782

Table 1.1 (continued)

Coordinates of intergenic regions	sRNAs							Flanking genes	
	Designation according to			Gene	Location[a]	Size (nt)[b]	Orientation[c]	5′ gene	3′ gene
	Livny et al. (2006) [14]	González et al. (2008) [15]	Sonnleitner et al. (2008) [16]					Name	Name
1996807-1997508		1059				150, 200, 300	<?<	PA1838-*cysI*	PA1839
2918212-2918965		1466[e]	–/118[o]			300	>?<	PA2581	PA2582
3106752-3107002	P13 (?)[g]				3106919-3106994	76	<<<	PA2744-*thrS*	PA2745
3112151-3112876		1559				250, 300	>?>	PA2750	PA2751
3206253-3206915	P14 (?)[h]					300	< >>	PA2852	PA2853-*oprL*
3298922-3299492	P15		–/132[o]			180	< >>	PA2942	PA2943
3318657-3318881	P16	1698	40/129[o]	*rgsA*	3318747-3318868	122	>> <	PA2958	PA2959
3360654-3360873		1714				200	< ? >	PA3001	PA3002-*mvf*
3702951-3703166	P18					100	< < <	PA3304	PA3305
3705161-3705888	P20	1887	PhrS		3705309-3705521	213	< < <	PA3305	PA3306
3778000-3778265			*amiE* leader	*amiL*	3778034-3778133	100	<<>	PA3366-*amiE*	PA3367
4057483-4057910	RsmZ			*rsmZ*[i]	4057543-4057658	116	> <<	PA3621-*fdxA*	PA3622-*rpoS*

Table 1.1 (continued)

Coordinates of intergenic regions	sRNAs							Flanking genes	
	Designation according to			Gene	Location[a]	Size (nt)[b]	Orientation[c]	5′ gene	3′ gene
	Livny et al. (2006) [14]	González et al. (2008) [15]	Sonnleitner et al. (2008) [16]					Name	Name
4444597-4444977	P24		–/94[o]			300	> < >	PA3964	PA3965
4536493-4536919		2315	50/88[o]			180	< < <	PA4055-*ribC*	PA4056-*ribD*
4780618-4780838	P26		54/72[o]			250	< < <	PA4270-*rpoB*	PA4271-*rplL*
4781786-4781985	P27 (?)[e]					90	< > <	PA4272-*rplJ*	PA4273-rplA
4939188-4939299			72/101[l,o]		4939194-4939277	84	< > <	PA4406-*lpxC*	PA4407-*ftsZ*
4956029-4956732	P28	2510	PhrW	*rnpB*		350	< < <	PA4421	PA4422
4985731-4985846	P35				4985782-4985843	62	< < <	PA4451	PA4452
5196833-5197184		2626	–/34			200	> > >	PA4628-*lysP*	PA4629
5283906-5284368	PrrF1	2667	90/31[o]	*prrF1*[j]	5283995-5284110	115	> > <	PA4704	PA4705
	PrrF2		92/32[o]	*prrF2*[j]	5284207-5284319	113	> > <	PA4704	PA4705
5308425-5309326	P30 (?)[k]		–/29[o]			180	> < >	PA4726-*cbrB*	PA4727-*pcnB*
5344904-5345085	P32		–/27[o]			80	< < <	PA4758-*carA*	PA4759-*dapB*

Table 1.1 (continued)

Coordinates of intergenic regions	sRNAs							Flanking genes	
	Designation according to							5′ gene	3′ gene
	Livny et al. (2006) [14]	González et al. (2008) [15]	Sonnleitner et al. (2008) [16]	Gene	Location[a]	Size (nt)[b]	Orientation[c]	Name	Name
5835071-5835481	P34					150	> < >	PA5181	PA5182
5836402-5836909			PhrX[l]		5836429-5836579	151	> < >	PA5183	PA5184
5859457-5859792			PhrY[l]		5859480-5859674	195	< > >	PA5204-*argA*	PA5205
5884286-5884508			101/26[o]	*ssrS*[m]	5884320-5884502	183	> > >	PA5227	PA5228-*ygfA*
5986120-5986474			102/16[l,o]		5986120-5986170	51	< > >	PA5316-*rpmB*	PA5317

[a] A location is given when the 5′ and 3′ ends can be deduced with reasonable certainty from available data or when the predicted and observed lengths are in agreement [14, 15].
[b] Experimental values found in Northern blots.
[c] The middle arrow indicates the orientation of the sRNA, while the flanking arrows indicate the orientation of the adjacent genes. Unknown orientation of sRNAs is indicated by a question mark.
[d] Identified by Kay et al. [17].
[e] Signal intensities observed for these transcripts were particularly weak and the possibility of non-specific detection remains [14, 15].
[f] Identified by Toschka et al. [18]. The mature form is reported to consist of 113 nt [18].
[g] Transcript size and intensity observed differed using two distinct probes [14].
[h] Due to its size, the P14 transcript could correspond to the mRNA of the upstream PA2853 gene.
[i] Identified by Heurlier et al. [19].
[j] sRNAs encoded in tandem, identified by Wilderman et al. [13].
[k] In the authors' laboratory, P30 has not been detected but there is evidence for sRNAs including CrcZ encoded by the opposite strand.
[l] Detection by RT-PCR.
[m] 6S RNA gene identified by Vogel et al. [20].
[n] Mature tmRNA according to sequence homologies [21, 22].
[o] Predicted by a bioinformatic approach (NcDNAlign/multiz) in Sonnleitner et al. [16].

Not all transcripts listed in Table 1.1 are necessarily sRNAs *sensu stricto*. Some may be processed untranslated 5′ or 3′ leader sequences and two such examples (2315 sRNA, *amiL*) will be discussed in Section 1.3.5. It is interesting to note that three sRNAs that had been known for many years were sighted in at least one of the three screens: tmRNA (*ssrA*), 4.5S RNA (*ffs*) and the RNA component of RNase P (*rnpB*), whereas a forth traditional RNA, 6S RNA (*ssrS*), was not revealed although it is sufficiently abundant to be isolated directly and can be predicted by bioinformatics [16, 20]. One strongly expressed sRNA (P20/1887/PhrS) was found in all three genomic surveys and its properties as a quorum sensing regulator will be described in Section 1.3.9. Another distinctly expressed sRNA (P16/1698), which was termed RgsA [15], was seen in two studies and was investigated in some detail (see Section 1.3.7). A further sRNA (P5/491), which was also revealed in two studies, has not yet been characterized with respect to its function.

1.3 Examples of sRNAs in *Pseudomonas* spp.

An overview of several *Pseudomonas* sRNAs with known functions and the methods leading to their identification are presented in Table 1.2. In the following, we will discuss the major properties of these characterized sRNAs. Clearly, this should be seen as an initial appraisal of a rapidly growing area of research.

1.3.1 6S RNA

6S RNA has been mainly studied in *E. coli*. It has about 10,000 copies at the end of exponential growth, is about 180 nucleotides in length and adopts a large hairpin-like secondary structure with several bulges. 6S RNA specifically binds to the σ^{70} RNA polymerase holoenzyme whereby the central major bulge of about 15 nucleotides mimics the open complex structure of promoter DNA. Thus, by interacting with σ^{70} RNA polymerase, 6S RNA inhibits the transcriptional expression of certain σ^{70} promoters in stationary phase, whereas transcription from σ^{s} (σ^{38}) promoters appears to be favoured in vivo (Fig. 1.1). Mutants devoid of 6S RNA (*ssrS*) do not have any pronounced growth defect, but are affected in survival during late stationary phase. When *E. coli* cells are transferred from stationary phase cultures to fresh medium, 6S RNA is used as a template for transcription of a short RNA (< 20 nucleotides), which frees the enzyme of 6S RNA and restores RNA polymerase activity at σ^{70} promoters [33, 34]. 6S RNA occurs in many bacterial species. Owing to its conserved sequences bordering the central bubble and those in the closing stem formed by the 5′ and 3′ terminal segments, 6S RNA genes can be annotated with relative ease in a variety of bacteria including *Pseudomonas* spp. Furthermore, the conserved presence of a downstream gene, *ygfA*, facilitates the annotation of the *ssrS* gene in α- and γ-proteobacteria [12, 31]. In *P. aeruginosa*, 6S RNA was experimentally demonstrated by Vogel et al. [20]. These authors purified the RNA, which was associated with 70S ribosomes, labelled it at the 3′ end and

Table 1.2 Approaches used to detect and characterize sRNAs in *Pseudomonas* spp

Experimental approach	Examples of sRNAs	Species[a]	Gene	References
Direct isolation of abundant sRNA	6S RNA: regulator of RNA polymerase	*Pa*	*ssrS*	[20]
Similarity with known sRNA of *E. coli*	4.5 RNA: RNA in signal recognition particle	*Pa*	*ffs*	[18]
	10Sa RNA: tmRNA	*Pa*	*ssrA*	[22]
	10Sb RNA: RNaseP RNA	*Pf*	*rnpB*	[25]
Computational prediction of sRNA	PrrF1 and PrrF2: functional RyhB homologs	*Pa*	*prrF1, prrF2*	[13]
	RsmY: GacA-dependent regulator of exoproducts	*Pf*	*rsmY*	[26]
	RgsA: GacA-dependent regulator of oxidative stress response	*Pf*	*rgsA*	[15]
Co-purification of sRNA with a protein	RsmX, RsmZ: GacA-dependent regulators, binding to RsmA protein	*Pf*	*rsmX, rsmZ*	[27, 28]
	PhrS: regulator of PQS biosynthesis, binding to Hfg protein	*Pa*	*PhrS*	[16][6]
Genetic screen	TRR: RsmY homolog, multi-copy suppressor of temperature control of toxin synthesis	*Psp*	*rsmY*	[7, 29]
	PrrB: RsmZ homolog, multi-copy suppressor of *gacA*	*Pf*	*rsmZ*	[30]

[a] *Pa*, *P. aeruginosa*; *Pf, P. fluorescens; Psp, P. syringae* pv. *phaseolicola*
[b] E. Souuleitner et al., unpublished data

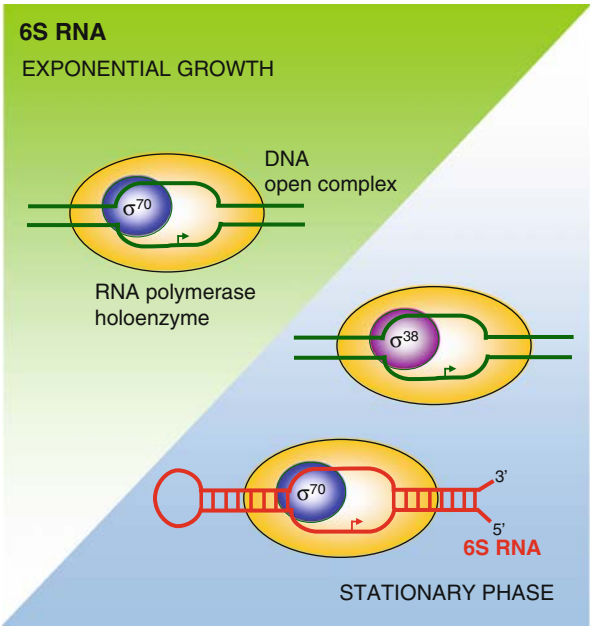

Fig. 1.1 During exponential growth (*shown in green*) bacterial cells strongly express housekeeping genes. These are usually under the control of σ^{70} promoters, which are recognized by σ^{70} RNA polymerase holoenzyme. At the beginning of the stationary phase (*shown in blue*) the expression of 6S RNA is increased. 6S RNA binds specifically to σ^{70} RNA polymerase holoenzyme and mimics the open complex structure of a σ^{70} promoter. This interaction inhibits the transcription from certain σ^{70} promoters and favors the transcription from σ^{38} promoters

used it as a probe to clone the chromosomal *ssrS* gene, which was then sequenced. The predicted secondary structure of 6S RNA from *P. aeruginosa* is very similar to that of 6S RNA from *E. coli* and there is 60% sequence identity between the 6S RNAs of both bacterial species [20], suggesting that these RNAs have similar functions in both organisms. The *ssrS* gene of *P. aeruginosa* appeared in the bioinformatic screen of Sonnleitner et al. [16], whereas neither Livny et al. [14] nor González et al. [15] detected this gene (Table 1.1). This is probably due to the fact that the latter screens rely on the presence of rho-independent terminators. However, 6S RNA does not have a rho-independent terminator and is formed by processing from a longer precursor, at least in *E. coli* [12].

1.3.2 4.5S RNA

The bacterial signal recognition particle (SRP), which consists of a protein (the *ffh* gene product) and 4.5S RNA (the *ffs* gene product), serves essentially to target proteins to the cytoplasmic membrane, immediately following translation. This is

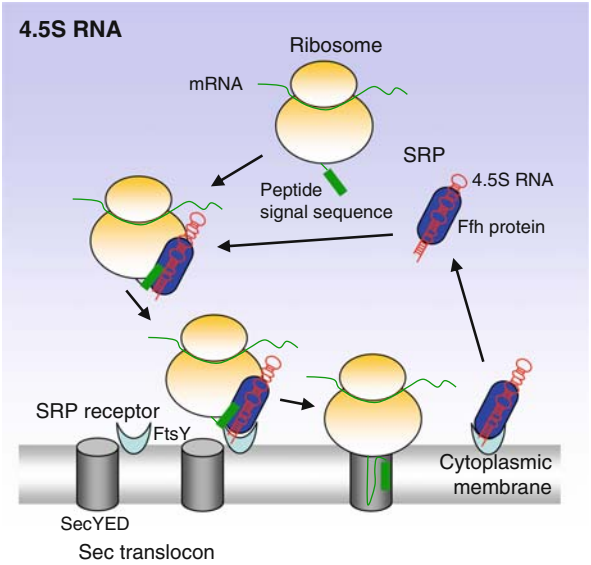

Fig. 1.2 4.5S RNA and the Ffh protein form together the signal recognition particle (SRP). This complex is essential for targeting membrane proteins to the cytoplasmic membrane. The SRP recognizes a hydrophobic signal sequence in the nascent protein and binds to it. The ribosome-SRP complex moves to the cytoplasmic membrane where the SRP interacts with the SRP receptor, FtsY. The peptide signal sequence is transferred to the Sec translocon and the membrane protein is co-translationally inserted into the cytoplasmic membrane. At the end of this process, the SRP dissociates from its receptor and can be recovered

achieved by an interaction between the SRP and the hydrophobic signal sequence of the nascent membrane protein, which is thus prevented from folding (or misfolding) in the cytoplasm (Fig. 1.2). SRP is essential for viability [35]. The bacterial 4.5S RNA adopts a long hairpin structure with several small bubbles; they are smaller than the open complex-like bulge of 6S RNA. The loop region of 4.5S RNA contains a strongly conserved motif of about 20 nucleotides. This motif is useful to identify the *ffs* gene in bacteria [36] and has served to clone the *P. aeruginosa ffs* gene [18]. In this organism, 4.5S RNA (113 nucleotides) is formed from a larger precursor by processing of the 5′ region, possibly by RNase P, as in *E. coli*. The 3′ end of 4.5S RNA might be generated by a poorly conserved rho-independent terminator or by processing of a transcript having 34 extra nucleotides at the 3′ end [18]. Although the function of the *ffs* gene has not been verified experimentally in *P. aeruginosa*, there can be little doubt that it has the same function as in *E. coli*, given the 75% sequence identity of the *ffs* genes between both organisms. The fact that the *P. aeruginosa ffs* gene was not revealed by the screen of Livny et al. [14] but was found in those of González et al. [15] and Sonnleitner et al. [16] (Table 1.1), probably reflects a less stringent terminator definition in the latter studies.

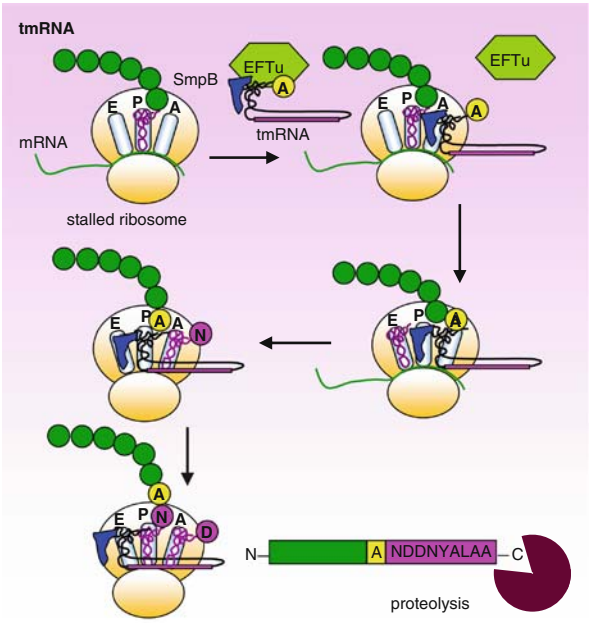

Fig. 1.3 Under certain conditions (e.g., premature termination of transcription or truncation of mRNA) a translating ribosome can be stalled. The release of the unfinished polypeptide from the ribosome is accomplished with the help of tmRNA, which has features of both tRNA and mRNA. The tRNA part of tmRNA is charged with alanine and enters the ribosome A (acceptor) site in a complex with the SmpB and EF-Tu proteins. The nascent polypeptide is transferred to the alanyl-tmRNA. After transfer of the peptidyl-tmRNA-SmpB complex to the P (peptidyl) site, a small open reading frame is provided by the mRNA part of tmRNA. This allows further elongation and termination of translation. The resulting peptide is tagged, which provides a signal for proteolysis

1.3.3 tmRNA

The function of tmRNA (for transfer-messenger RNA, the product of the *ssrA* gene, formerly termed 10Sa RNA) has been elucidated primarily in *E. coli* [37]. When translating ribosomes stall because of premature termination of transcription or truncation of mRNA, a special *trans*-translation mechanism elongates and releases the unfinished polypeptides from the ribosomes (Fig. 1.3). In this process, the tRNA-like part of tmRNA is charged with alanine. Alanyl-tmRNA enters the ribosome A (acceptor) site as a complex with the SmpB and EF-Tu proteins. The stalled nascent polypeptides are transferred to alanyl-tmRNA; upon translocation of the peptidyl-tmRNA-SmpB complex to the P (peptidyl) site, the mRNA part of tmRNA provides a small open reading frame that allows continuation and termination of translation. This results in the addition of a C-terminal peptide tag (ANDENYALAA) to the stalled nascent polypeptides. Subsequent termination of translation releases the ribosomes, and tagged proteins are targeted for proteolytic degradation (Fig. 1.3). The

presence of tmRNA appears to be universal in bacteria. Null mutations in *ssrA* are tolerated but result in reduced fitness of the organisms [37]. The 5′ and 3′ termini of tmRNA are conserved. This enabled Williams and Bartel [22] to clone the *ssrA* gene (353 bp) from *P. aeruginosa* and other proteobacteria. The deduced tag sequence of *P. aeruginosa* tmRNA (ANDDNYALAA) is very similar to that of *E. coli*. A tmRNA fragment was found to be bound to Hfq in the screen of Sonnleitner et al. [16] (Table 1.1). However, it is not clear whether Hfq binding to tmRNA occurs in vivo.

1.3.4 RNase P RNA

RNase P is an essential enzyme required for processing tRNA precursor molecules at the 5′ end. It contains a catalytic RNA (the *rnpB* product consisting of 276 to ∼500 nucleotides, depending on the organism) and has been found in bacteria, archaea and eukaryotes. In *E. coli* and other bacteria, a small protein encoded by the *rnpA* gene is needed for in vivo activity of RNase P. The RNA of RNase P has several highly conserved nucleotides in its core sequence of about 200 nucleotides. This information can be used to identify the *rnpB* gene in various microorganisms [38, 39]. The first *Pseudomonas* species from which the gene for RNase P RNA was isolated, was *P. fluorescens* [25]. More recently, RNase P RNA was detected in *P. aeruginosa* in three independent screens [14–16] (Table 1.1).

1.3.5 Processed Leader Transcripts

Some sRNAs do not have regulatory functions per se, but are products of regulatory transcription termination processes. For example, the expression of the *P. aeruginosa amiLEBCRS* operon is regulated by aliphatic amides via an antitermination mechanism, as discussed and illustrated in volume 2 [40]. In this operon, the *amiE* gene encodes aliphatic amidase and the *amiB* and *amiS* genes are thought to function in amide transport [40]. The AmiR protein, a positive regulator, acts as an antiterminator by allowing transcription to proceed beyond the *amiL* (5′ untranslated leader) sequence. Mutation in *amiR* leads to premature termination of transcription at the rho-independent terminator downstream of *amiL*, producing the 100-nucleotide AmiL leader RNA [40]. It is assumed that the AmiR protein binds to the leader sequence and thereby interferes with the formation of the stem–loop structure of the *amiL* terminator. The AmiC protein acts as a negative regulator and senses aliphatic amides by binding them. Accordingly, *amiC* disruption leads to constitutive amidase synthesis. In the absence of inducing aliphatic amides, AmiC forms a complex with AmiR, which occludes the antitermination activity of AmiR. In the presence of an inducer, the AmiR-AmiC complex is dissociated, which allows antitermination activity of AmiR [40, 41]. AmiL RNA was recovered from non-induced *P. aeruginosa* cells as an sRNA bound to Hfq [16]. It is possible that Hfq binds to the AU-rich sequence upstream of the *amiL* terminator; however, the biological significance of the observed Hfq-AmiL interaction is not clear. Artificial overexpression of *amiL*

did not reveal significant effects on the transcriptome and proteome in *P. aeruginosa* (E. Sonnleitner, unpublished data), suggesting that AmiL does not have global regulatory functions.

The 2315 sRNA of *P. aeruginosa* (Table 1.1) is a homologue of SroG of *E. coli* [11, 15]. These sRNAs are part of a riboswitch mechanism, in which premature termination of transcription in a 5′ untranslated leader mRNA results in the formation of the sRNAs. The particular riboswitch producing SroG is characterized by a conserved RNA structure element termed RFN [42]. Flavins such as FMN repress the expression of riboflavin biosynthetic (*rib*) genes by binding to the RFN element and thereby causing premature termination of transcription in the 5′ untranslated *rib* leader mRNAs. In *P. aeruginosa*, the 2315 sRNA (∼180 nucleotides) is generated from the *ribC* leader sequence [15].

1.3.6 sRNAs Sequestering Proteins of the RsmA Family

RsmY and RsmZ are two sRNAs of *P. aeruginosa* whose roles have been studied extensively in the context of quorum sensing regulation and virulence factor expression. The sequences and predicted secondary structures of RsmY and RsmZ of strain PAO1 have been given in volume 2 [43] and aspects of their expression via the GacS/GacA two-component system have been discussed in volume 5 [44, 45]. In the following, we will briefly review the salient features of the regulatory network involved. In *P. aeruginosa*, quorum sensing involves at least three signal molecules, i.e. *N*-(3-oxododecanoyl)-homoserine lactone (the reaction product of the LasI enzyme and principal activator of the LasR transcription factor), *N*-butanoyl-homoserine lactone (the reaction product of the RhlI enzyme and principal activator of the RhlR transcription factor), and the *Pseudomonas* quinolone signal (PQS), which activates the PqsR transcription factor [44, 46]. Quorum sensing positively regulates biofilm polysaccharide biosynthesis and the expression of a range of extracellular virulence factors such as exotoxin A, lytic enzymes, and toxic secondary metabolites. In addition, quorum sensing negatively controls the type III secretion system (TTSS) and pilus formation. Several global regulators including the GacS/GacA two-component system influence the expression of quorum sensing [44, 47]. Mutations in either *gacS* (sensor kinase) or *gacA* (response regulator) strongly attenuate virulence of *P. aeruginosa* for different host organisms including burnt mice, nematodes, and insects [48, 49]. The activity of the GacS/GacA system is modulated by two sensor kinases, RetS and LadS [50, 51, 52]. RetS acts as an antagonist of GacS and evidence for the inhibitory activity of RetS has been obtained both in vivo and in vitro [50, 51]. A *retS* null mutant exhibits a small colony phenotype due to strong cell-cell aggregation, overproduction of extracellular polysaccharides, enhanced biofilm formation, lack of the TTSS and reduced twitching motility due to downregulation of type IV pili [50, 53, 54]. By contrast, when LadS is mutationally inactivated, biofilm formation is reduced and TTSS expression is enhanced [52]. Evidence for cooperation between LadS and GacS comes from studies in a related bacterium, *P. fluorescens* CHA0 [55, 56]. These

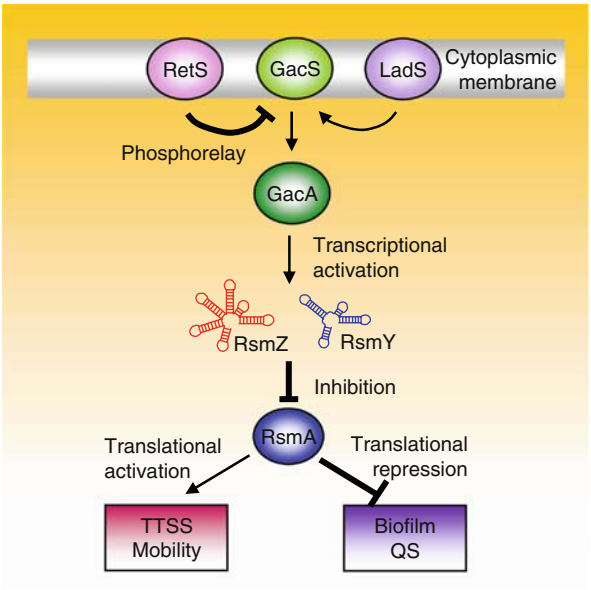

Fig. 1.4 In *P. aeruginosa*, RsmA, a translational regulator, acts as an activator of the type III secretion system (TTSS), pilus formation and mobility and as a repressor of biofilm formation and quorum sensing (QS). Under conditions of high cell population densities and slow growth, the expression of two sRNAs, RsmZ and RsmY, is elevated. These two sRNAs bind to the RsmA protein and inhibit its function. Both sRNAs are under the control of the GacS-GacA two-component system, where GacS is the sensor protein and GacA the response regulator. Phosphorylation of GacS is regulated by two membrane-bound sensors, RetS and LadS, which physically interact with GacS. RetS blocks GacS autophosphorylation, whereas LadS appears to stimulate this activity

findings can be rationalized by a model (Fig. 1.4) which stipulates that RetS and LadS interact directly with GacS and that the combined input from the three sensors determines the level of phosphorylation of the response regulator GacA. The signals and environmental cues that activate the three sensors are largely unknown [45, 57].

In *P. aeruginosa*, RsmY and RsmZ are expressed under strict positive control by GacA, which is an activator when it is in the phosphorylated state [51]. An *rsmY rsmZ* double mutant has the same phenotype as a *gacA*-negative mutant. Both RsmY and RsmZ antagonize the action of the RsmA protein, by avidly binding to it [17, 19]. RsmA (acronym for **r**egulator of **s**econdary **m**etabolism) is a small dimeric RNA-binding protein that represses translation of target mRNAs, many of which are involved in virulence factor expression [58, 59]. RsmA is a member of a large protein family found in > 150 bacterial species, including *E. coli* where the homologue is called CsrA (acronym for **c**arbon **s**torage **r**egulator [60]). In *P. aeruginosa*, RsmA also indirectly activates the promoters of the *rsmY* and *rsmZ* genes via an unknown mechanism [17, 19]. Hfq binds to RsmY and stabilizes it [61, 62].

A proteomic analysis suggests that the RsmA/RsmY/RsmZ triad mediates virtually all important functions of the GacS/GacA two-component system [17]. Loss of virulence in *gacS/gacA* mutants correlates with markedly diminished biofilm formation and with reduced expression of extracellular virulence factors that are positively regulated by quorum sensing [17, 63]. On the one hand, the GacS/GacA system positively influences the expression of the quorum sensing machinery, in particular that of the *rhlI* gene, which encodes the enzyme for the biosynthesis of the quorum sensing signal *N*-butanoyl-homoserine lactone [17, 59, 63]. On the other hand, the GacS/GacA system regulates the expression of certain virulence factors directly at the level of translation [64], as has been shown for the Gac/Rsm signal transduction pathway in other γ-proteobacteria [65].

The first observation of RsmY sRNA was not made in *P. aeruginosa*, but in *P. syringae* pv. *phaseolicola*, a producer of phaseolotoxin and pathogen of bean (Table 1.2). Some phaseolotoxin-negative mutants, which were not mapped but presumably carried mutations in *gacS* or *gacA*, were functionally restored by multicopy suppression with a 0.4-kb locus termed TRR (for **t**hermo**r**egulatory **r**egion) [29]. Although the TRR product was not identified at the time of publication (1993), in retrospect it is evident that TRR codes for an RsmY-like sRNA [26]. Overexpression of TRR not only restores phaseolotoxin production in the mutant background but also overrides temperature control of phaseolotoxin production. The wild type synthesizes the toxin at 20°C, but not at 28°C, whereas the suppressed mutants produce the toxin at both temperatures [29].

Similarly, initial evidence for RsmZ sRNA came from multi-copy suppression of *gacA* and *gacS* mutations (Table 1.2) in *P. fluorescens* F113, a soil bacterium that colonizes the roots of different crop plants and protects these from fungal pathogens [30]. The suppressor locus (termed *prrB*) specified an sRNA closely related to RsmZ. Overexpression of PrrB restored the production of typical antifungal GacA-dependent metabolites, i.e., 2,4-diacetylphloroglucinol (DAPG) and hydrogen cyanide (HCN), to *gacS* and *gacA* mutants of strain F113. However, mutation of the *prrB* gene had relatively minor effects on DAPG and HCN production, suggesting that PrrB may not be the only GacA-dependent regulatory sRNA in strain F113 [30].

A more complete picture of GacA-dependent sRNAs has emerged from studies of *P. fluorescens* CHA0. Like strain F113, strain CHA0 has biocontrol properties; they depend on a blend of antifungal secondary metabolites (including DAPG, HCN, pyoluteorin and pyrrolnitrin), extracellular lytic enzymes and poorly characterized elicitors of induced systemic resistance, i.e., a mechanism that renders the host plant less susceptible to pathogens [57, 66]. Biocontrol activity of strain CHA0 is lost in *gacS* or *gacA* null mutants, because most secondary metabolites and extracellular enzymes are produced at very low levels in such mutants, by comparison with the wild type [67–69]. In strain CHA0, the GacS/GacA two-component system is required for the expression of RsmY (118 nt) and RsmZ (127 nt) as well as that of a third sRNA termed RsmX (119 nt). A conserved upstream sequence element (consensus $TGTAAGN_6CTTACA$), which might be a GacA binding site, is found in the *rsmX*, *rsmY* and *rsmZ* promoters as well as in promoters of