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A Single Mutation in the *oprF* mRNA Leader Confers Strict Translational Control by the Gac/Rsm System in *Pseudomonas fluorescens* CHA0

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Abstract The rhizobacterium *Pseudomonas fluorescens* CHA0 is able to antagonize fungal phytopathogens on a variety of crop plants, mainly due to the production of secondary metabolites that are coordinately upregulated by the global regulatory Gac/Rsm cascade. The two-component system GacS/GacA activates transcription of the three small regulatory RNAs RsmX, RsmY, and RsmZ, which counteract translational repression of target mRNAs by RsmA and RsmE proteins. In a search for novel Gac/Rsm targets based on the minimal sequence on mRNA leaders required for RsmA/RsmE control, the leader region of the major porin OprF emerged as a candidate. Although an isogenic CHA0 *oprF* mutant showed a reduced ability to attach to cucumber and tomato roots, suggesting a role for OprF in root colonization as a requisite for pathogen antagonism, a translational *oprF′-′lacZ* fusion was weakly regulated by Gac/Rsm despite its high sequence similarity to the *hcnA* leader. A single base substitution put the modified *oprF* 5′-UTR under strict control by Gac/Rsm. The results highlight the subtle sequence requirements of Gac/Rsm targets.

Introduction

Pseudomonas fluorescens CHA0 has biocontrol activity against several plant root pathogens [12]. The antibiotics 2,4-diacethylphloroglucinol (DAPG) and pyoluterin, HCN and exoprotease AprA, together contribute to the antifungal activity of CHA0 [12]. Notably, production of these compounds is posttranscriptionally activated by the global regulatory two-component system GacS/GacA [4, 27], which in turn promotes transcription of the small regulatory RNAs, RsmX, RsmY, and RsmZ [13]. These RNAs avidly bind the regulatory proteins RsmA and RsmE, which block translation of *hcnABC* (for HCN synthase), *phlABCDE* (for DAPG synthesis), and *aprA* mRNAs [21]. The GacS/GacA-dependent accumulation of RsmX/Y/Z RNAs results in the formation of RsmX/Y/Z-RsmA/E complexes and the concomitant relief of target mRNA translational repression [25].

In the *hcnA* mRNA leader a short stretch centered 13 nucleotides (nt) upstream of the start codon is required for Gac/Rsm control [4, 15]. The 5′-UTRs of the *P. aeruginosa* PAO1 *hcnA* and the *P. fluorescens* CHA0 *aprA* genes, both of which are also Gac/Rsm targets [4], contain a highly similar sequence (Fig. 1a). To search for new Gac/Rsm targets, we queried the genome of the closely related strain Pf-5 [20] for the presence of additional *hcnA* 5′-UTR-like sequences. Thus, *oprF* was picked up because the sequence around its ribosome binding site (RBS) is almost identical to the *aprA* and *hcnA* motifs required for Gac/Rsm control (Fig. 1a). OprF is the major porin of pseudomonads and it is considered a diagnostic molecule for this eubacterial group [5]. Besides facilitating the passage of polar solutes across the outer envelope [17], OprF has also been implicated in the maintenance of membrane integrity [11], and, most relevant to plant growth promotion, it has been reported as a root adhesive protein [8]. In this work, we characterized an *oprF* mutant and studied translational regulation of *oprF* by the Gac/Rsm system in *P. fluorescens* strain CHA0.

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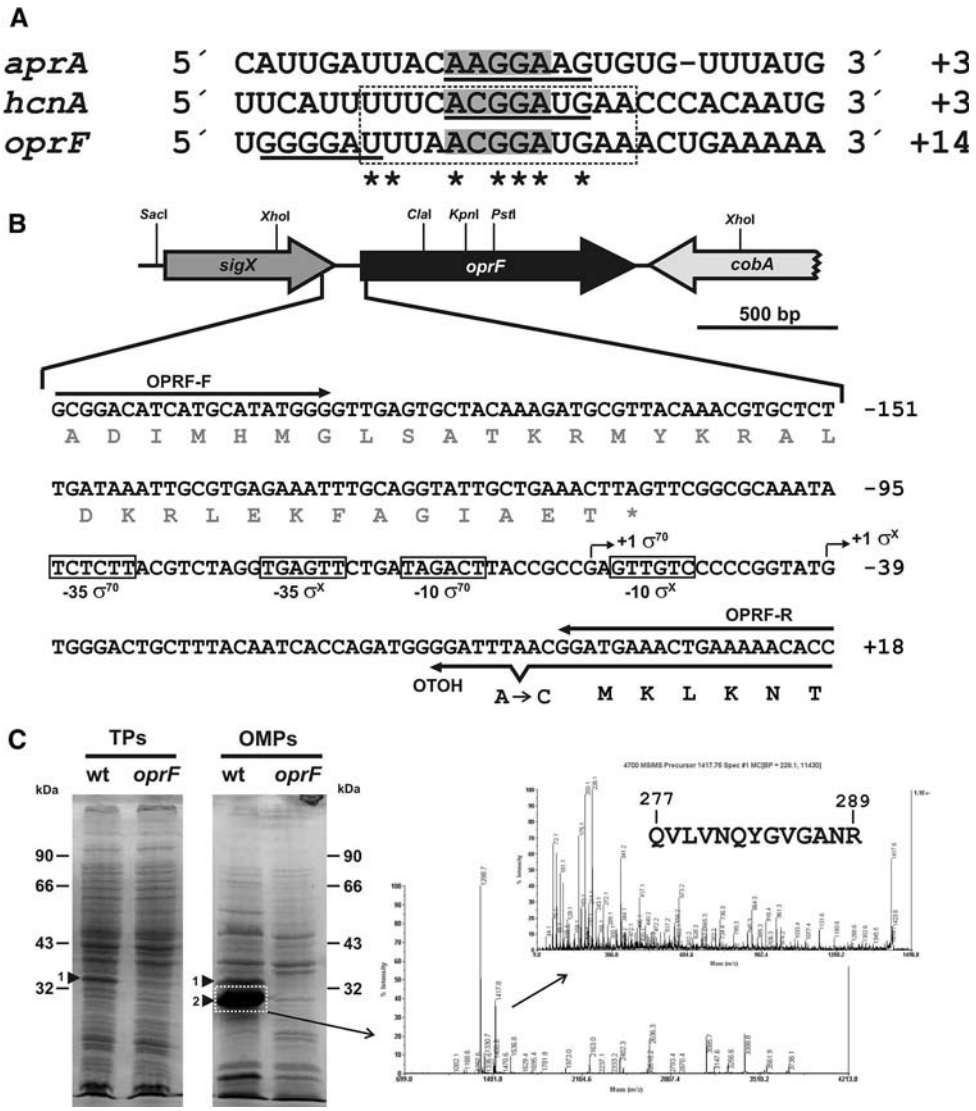

Fig. 1 *Pseudomonas fluorescens* CHA0 *oprF* locus. (**a**) Alignment of the Gac/Rsm-controlled *hcnA* and *aprA* 5′-UTRs and a highly similar motif present in the *oprF* 5′-UTR (dashed box). Putative RBSs are underlined. Potential RsmA/E binding sites (ANGGA) are highlighted with gray boxes. (**b**) Organization of the CHA0 *oprF* locus. The putative σ^{70}- and σ^{X}-dependent *oprF* promoters (boxed) were deduced from multiple alignments of *oprF* promoters of sequenced pseudomonads including the mapped promoter of *P. aeruginosa* PAO1 [6]. Oligonucleotide pairs OPRF-F/OPRF-R and OPRF-F / OTOH were used to construct translational *oprF′-′lacZ* and mut-*oprF′-′lacZ* fusions. Nucleotide positions are relative to the first coding base. (**c**) Coomasie blue-stained SDS-PAGE of total proteins (TPs) and outer-membrane proteins (OMPs) of *P. fluorescens* CHA0 (wt) and the isogenic *oprF* mutant ARQ22 (*oprF*). OprF bands are indicated by arrowheads. The amount of TPs loaded corresponds to $\sim 9 \times 10^9$ lysed cells, whereas the amount of OMPs in the gel corresponds to $\sim 1.2 \times 10^{11}$ cells. The prominent 32-kDa band was subjected to trypsin digestion and MS analysis (righthand panel). MS-MS fragmentation analysis was done with the monoisotopic 1417.8-Da peptide, which rendered the sequence corresponding to OprF residues 277–289 (service performed at TopLab GmbH, Germany)

Materials and Methods

Bacterial Strains, Growth Conditions, and DNA Manipulations

Strains (Supplementary Table 1) were routinely grown in nutrient yeast broth (NYB), with shaking, or on nutrient agar (NA) amended with the following antibiotics (μg mL^{-1}) when required: ampicillin, 100; gentamicin, 10; tetracycline, 25 (125 for *P. fluorescens*); and kanamycin, 50 [26]. When relevant, 0.1 g L^{-1} X-gal was added to plates. Routine incubation temperatures were 37°C for *E. coli* and 28°C for *P. fluorescens*. For transformation with heterologous DNA, *P. fluorescens* cells were grown at 35°C to reduce DNA restriction [26]. For determination of growth rate, triplicate cultures were grown in NYB or in

basal medium 2 (BM2) [10]. Small-scale plasmid preparations were done using the CTAB method [9] or the Nucleospin kit (Macherey-Nagel). *P. fluorescens* chromosomal DNA was prepared as previously described [26]. DNA manipulations were carried out using standard protocols [22]. DNA fragments were purified from agarose gels with Qiaex II (Qiagen Inc.). Bidirectional DNA sequencing was performed at Macrogen Inc. (Korea). PCRs were carried out as described [26].

Construction and Characterization of an Isogenic *oprF* Mutant

We cloned and sequenced a 2456-bp fragment encompassing CHA0 *oprF* (Fig. 1b; accession number EF592174). An *oprF* mutant (ARQ22) bearing a 930-bp internal deletion and replacement by a ΩKm cassette was generated in strain CHA0 by marker exchange using the replacement vector pME3087-UD-ΩKm (Supplementary Table 1) and established procedures [26]. The mutation was confirmed by PCR and Southern blot. Total protein extracts and a fraction enriched in outer membrane proteins (OMPs) were prepared using established procedures [18], fractionated in 10% acrylamide gels with Tris-glycine-SDS buffer, and stained with Coomasie brilliant blue [14]. HCN production, exoprotease activity, and antagonism against *Pythium ultimum* were studied as previously described [16, 19, 26]. Root adhesiveness was assessed on cucumber (*Cucumis sativa* var. Poinset 76) and tomato (*Solanum lycopersicum* L. Platense) roots. Briefly, fifteen 2-day-old axenic seedlings were incubated for 1 h at 28°C and 50 rpm with *P. fluorescens* cells in flasks containing 50 mL of mineral Fåhraeus solution (FS) [7]. After a gentle wash in 50 mL of FS, the roots were shaken in 20 mL of FS for 30 min at 200 rpm to detach cells loosely adhered to the root surface. The adhesiveness index A1 was calculated as the percentage of inoculated bacteria detached by this procedure. Washed roots were gently laid on the bottom of a petri dish, carefully covered with soft nutrient agar prewarmed at 45°C, and incubated overnight at 28°C to count the number of colonies developed on the root surface under bright-field microscopy (40×) for calculation of the adhesiveness index A2 [7].

Translational Fusions and β-Galactosidase Assay

A 244-bp PCR product containing the putative CHA0 *oprF* promoter, the RBS, and the first six codons was generated with oligonucleotides OPRF-F/OPRF-R (Fig. 1b and Supplementary Table 1) and inserted upstream of '*lacZ* in pME6015 [23] to create an *oprF*′-′*lacZ* translational fusion vector (pPIB-1). A modified *oprF*′-′*lacZ* fusion was created in pPIB-2 (Fig. 1b and Supplementary Table 1) as above but using reverse oligonucleotide OTOH instead of OPRF-R, to introduce an A → C substitution at −5 relative to the *oprF* start codon (Fig. 2). β-Galactosidase activities were quantified using cells permeabilized with 50 μL mL^{-1} toluene on cells grown in 20 mL of NYB with shaking at 28°C. Triton X-100 (1 g L^{-1}) was added to avoid cell aggregation [26].

Results

Identification of *oprF* as a Putative Gac/Rsm Target

We used the pattern search tool Fuzznuc [1] to query the genome of the *P. fluorescens* CHA0 related strain Pf-5 [20] with the 13-nt stretch of the 5′-UTR *hcnA* that is required for Gac/Rsm regulation (TTTCACGGATGAA; Fig. 1a). With only one mismatch allowed, 11 hits other than the *hcnABC* leader were retrieved; 7 were found within open reading frames (ORFs), 2 in intergenic regions, and the last 2 were located nearby the RBS of a putative sulfate transporter gene (Pfl_2632) and of the major outer membrane porin gene *oprF* [5]. The *oprF* hit matched the Gac/Rsm target of the *hcnA* leader in 12 of 13 bases (Fig. 1a). As OprF has been reported as a root adhesive protein in rhizobacterial pseudomonads [8], we were prompted to characterize the *oprF* locus in the root colonizing and biocontrol strain CHA0 and to study its regulation by the Gac/Rsm cascade [25].

Characterization of the *oprF* Locus of Strain CHA0

$oprF_{CHA0}$ (978 bp) lies downstream of *sigX* (encoding the sigma factor σ^X required for *oprF* transcription in *P. fluorescens* OE28.3 and *P. aeruginosa* H103 [6]) and upstream of the divergently transcribed siroheme synthase *cobA* (Fig. 1b). Two partially overlapped σ^{70}- and σ^X-dependent promoters (Fig. 1b) were deduced from an alignment of pseudomonad *oprF* loci. $oprF_{CHA0}$ encodes a polypeptide of 325 residues (34.5 kDa) and a mature monomer of 301 residues (32.2 kDa). A prominent protein band of ~32 kDa was detected in the CHA0 OMP-enriched fraction and confirmed as OprF upon tryptic digestion and MS/MALDI-TOF peptide analysis (Fig. 1c). Based on this surface exposure and its reported adhesive properties [2, 8], we thought that OprF would be important for early interactions of CHA0 cells with roots. The *oprF* deletion in the CHA0 isogenic mutant ARQ22 (Fig. 1c) reduced the growth rate in minimal medium BM2 ($\mu = 0.32\ h^{-1}$ for ARQ22 and $\mu = 0.40\ h^{-1}$ for CHA0; $p = 0.0044$) but had no effect in rich NYB medium ($\mu = 0.92\ h^{-1}$ for ARQ22 and $\mu = 0.89\ h^{-1}$ for CHA0; $p = 0.62$). Single copy and chromosomal *oprF* complementation [3] in strain ARQ24

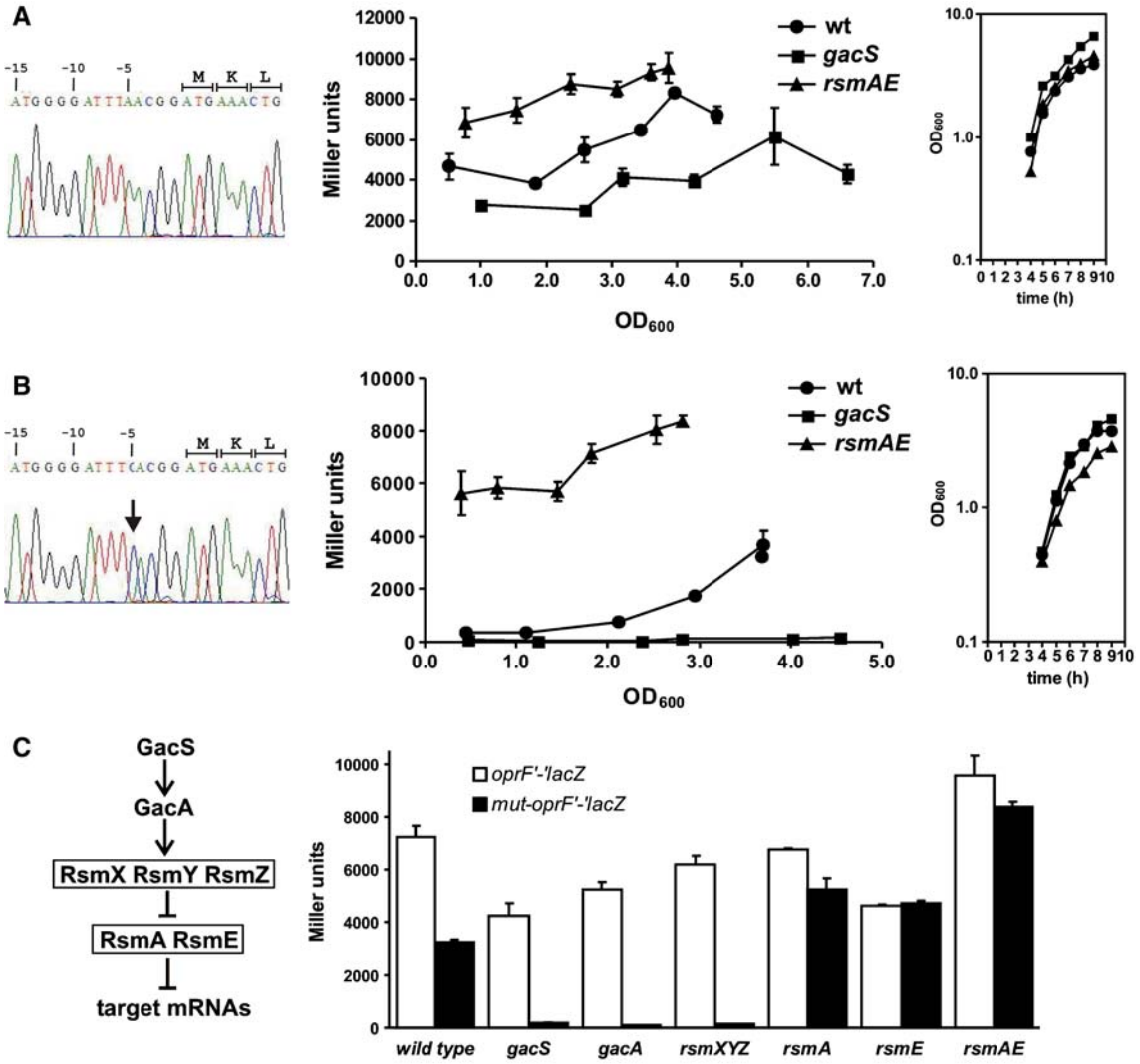

Fig. 2 Expression pattern of translational *oprF′-′lacZ* (**a**) and mut-*oprF′-′lacZ* (**b**) fusions in *P. fluorescens* CHA0 (wt), CHA19 (*gacS*), and CHA1009 (*rsmA rsmE*). The sequencing chromatograms of the *oprF* (A) and mut-*oprF* (B) leaders (–15 to 3rd codon) confirmed the $A_{-5} \rightarrow C_{-5}$ transversion. *β*-Galactosidase data correspond to averages of triplicate cultures ± SD. The growth curves are shown in the righthand panels. (**c**) Comparison of the translational activity of *oprF′-′lacZ* (white bars) and mut-*oprF′-′lacZ* (black bars) at the transition from exponential to early stationary phase (OD_{600} 4.0 to 5.0) in strains lacking different elements of the CHA0 Gac/Rsm cascade (shown at the left) [25]

restored the wild-type growth rate in minimal medium BM2 ($\mu = 0.39\ h^{-1}$). In transmission electron microscopy, exponential-phase ARQ22 cells were 26% shorter (2.1 ± 0.1 μm; $p < 0.0001$) than wild-type cells (2.8 ± 0.1 μm). Under the light microscope, ARQ22 cells were as motile as CHA0 cells and both strains showed polar flagella at the TEM (data not shown). The lack of OprF did not affect the activity of the inner membrane sensor GacS, as ARQ22 retained the ability to synthesize HCN, secrete exoprotease, and antagonize *Pythium ultimum* in dual plate assays (data not shown). The *oprF* mutant ARQ22 was handicapped regarding firmly attaching to tomato and cucumber roots (A2% in Table 1), particularly in the apical region of the root. This loss of firm attachment was reversed in the complemented strain ARQ24 (Table 1).

Regulation of $oprF_{CHA0}$ Expression by the Gac/Rsm System

A translational *oprF′-′lacZ* fusion was functional in CHA0 in rich NYB medium (5912 ± 502 MU at $OD_{600} = 3.0$) and was ~50% more active in minimal medium BM2 (9553 ± 664 MU). *oprF′-′lacZ* was already expressed in the early exponential phase at relative high levels (~4000 MU), and only a modest, 2× increase was observed in the stationary phase (Fig. 2a). In a *gacS* mutant the pattern was

Table 1 Adhesiveness of *Pseudomonas fluorescens* cells to cucumber and tomato roots

	CHA0 (wt)	ARQ22 (Δ*oprF*::ΩKm)	ARQ24 (Δ*oprF*::ΩKm, *att*Tn*7*-*oprF*)
Tomato			
A1 (%)	1.25 ± 0.06[a]	0.99 ± 0.01[b]	1.12 ± 0.37[ab]
A2 (%)	0.037 ± 0.006[a]	0.005 ± 0.009[b]	0.031 ± 0.007[a]
Cucumber			
A1 (%)	1.94 ± 0.53[a]	3.10 ± 0.10[b]	1.88 ± 0.45[a]
A2 (%)	0.095 ± 0.014[a]	0.008 ± 0.015[b]	0.276 ± 0.041[c]

Note: A1 (%), percentage of cells in the inoculum that adhered loosely to roots; A2 (%), percentage of cells in the inoculum that adhered tightly to roots. Values are the average ± 95% confidence interval. Significant statistical differences (p-value = 0.05) between treatment averages are denoted by different superscript letters. The data correspond to a representative experiment that was repeated three times

similar but with an overall 30–50% reduction in galactosidase activity (Fig. 2a). In an *rsmA/E* background, expression was up to 100% higher than for CHA0 along the growth curve (Fig. 2a). This is in line with the expected control by the Gac/Rsm system, but the extent of regulation is far below that previously reported for Gac/Rsm target genes such as *hcnA* and *aprA* (Fig. 3). When a single base substitution was introduced in the *oprF* 5′-UTR to make it identical to that of *hcnA* (Fig. 2), the mut-*oprF*′-′*lacZ* expression pattern was modified strikingly: it was poorly expressed in the exponential phase but activated ∼10× upon entry into the stationary phase in CHA0 (Fig. 2b). Remarkably, mut-*oprF*′-′*lacZ* expression was almost totally lost in the *gacS* background but strongly activated in the *rsmA/E* mutant (Fig. 2b). The gain of control of the mut-*oprF*′-′*lacZ* fusion by the Gac/Rsm system was confirmed in strains lacking other elements of the cascade (Fig. 2c). Whereas activity of the wild-type *oprF*′-′*lacZ* fusion was barely affected by *gacA*, *rsmA*, *rsmE*, or *rsmXYZ* mutations (Fig. 2c), mut-*oprF*′-′*lacZ* expression was strongly repressed in the *gacA* and *rsmXYZ* backgrounds (Fig. 2c). Single *rsmA* or *rsmE* mutations caused a minor activation of mut-*oprF*′-′*lacZ* (Fig. 2c) because the second intact *rsmE* or *rsmA* gene functions as a backup repressor [21].

Discussion

oprF was identified as a gene putatively controlled by the Gac/Rsm system in *P. fluorescens* CHA0 because its RBS region matched the RsmA/E binding site on the *hcnA* 5′-UTR in 12 of 13 nucleotides (Fig. 1a). As for other pseudomonads [5], OprF is the most abundant OMP of CHA0 cells (Fig. 1c). The lack of OprF provoked a reduction in growth rate in minimal medium and a smaller cell size but did not affect the Gac/Rsm cascade or secretion of biocontrol products. However, the OprF mutant had difficulties attaching to cucumber and tomato root surface (Table 1). This was not due to reduced swimming and it probably reflects the OprF requirement for root surface attachment, as suggested for other biotic surfaces [2, 8]. Thus, OprF-deficient CHA0 cells would be less proficient in root colonization and probably less competitive than wild-type cells.

Although the sequence around the *oprF* RBS is almost identical to that of *hcnA* (Fig. 1a), the *oprF* 5′-UTR cannot

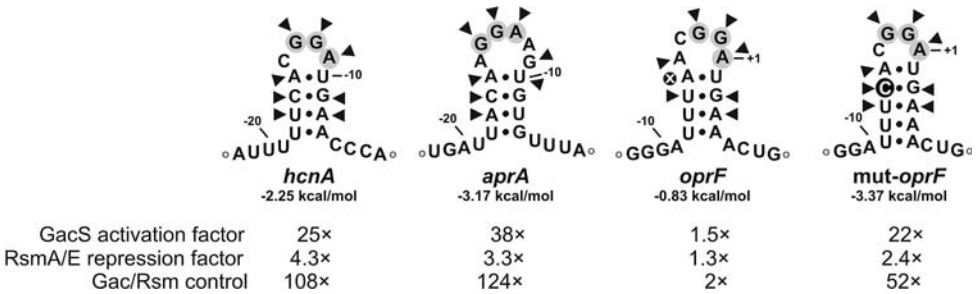

Fig. 3 Predicted secondary structure and Gac/Rsm control of *oprF* 5′ UTR. Local structure and folding energy were predicted with Mfold [28] set to default parameters at 30°C. Base positions are relative to the first coding nucleotide. The characteristic single-stranded GGA triplets regarded as RsmA/RsmE binding sites are highlighted with gray circles. Arrowheads point to nucleotide specific contacts with RsmE residues around the *hcnA* leader [24] and the corresponding deduced contacts for *aprA*, *oprF*, and mut-*oprF* leaders. The A_{-5} → C_{-5} *oprF* transversion is indicated by a black circle. For each translational fusion, the GacS activation factor is the CHA0/CHA19 expression ratio, the RsmA/E repression factor is the CHA1009/CHA0 expression ratio, and the Gac/Rsm control factor represents the CHA1009/CHA19 expression ratio. The factors were derived from early stationary phase data shown in Fig. 2. The *hcnA*′-′*lacZ* and *aprA*′-′*lacZ* factors were calculated from published data [21]

be considered a Gac/Rsm target, as a translational *oprF′-′lacZ* fusion was not significantly affected by mutations in elements of the Gac/Rsm system (Fig. 2a and c). Upon introduction of a point mutation in the *oprF* 5′-UTR (Fig. 2b), the expression pattern of the mut-*oprF′-′lacZ* fusion changed substantially (Fig. 2b and c): it gained cell-density-dependent expression and the magnitude of Gac/Rsm control approached that of true Gac/Rsm targets (Figs. 2 and 3). In light of the requirement of $OprF_{CHA0}$ to grow under low-saline conditions and to adhere to root cells, a tight *oprF* control by Gac/Rsm would compromise cells to compete in the soil and the rhizosphere because *oprF* would be poorly expressed at a low cell density (Fig. 2b). Under the same reasoning, it is expected that a spontaneous $A_{-5} \rightarrow C_{-5}$ transversion in the *oprF* RBS would put the *oprF* mRNA under strict control by Gac/Rsm (Fig. 2b and c and Fig. 3) and such disadvantaged cells would be outcompeted. Clearly, the $A_{-5} \rightarrow C_{-5}$ replacement transformed the *oprF* 5′-UTR into a very good recognition motif for RsmA/E proteins. This may be partly due to the formation of a more stable hairpin by the mut-*oprF* RBS (−3.37 kcal/mol) compared to the *oprF* leader (−0.83 kcal/mol) (Fig. 3). Note that the local folding energy for Gac/Rsm targets such as *hcnA* and *aprA* is comparable to that of mut-*oprF* (Fig. 3). Recently, the resolved structure of RsmE complexed with a piece of the *hcnA* 5′-UTR revealed that RsmE residues make base specific contacts within the consensus sequence 5′-$^{A}/_{U}$CANGGANG$^{U}/_{A}$-3′ (Fig. 3) [24]. One of these contacts is not possible at A_{-5} in the *oprF* 5′-UTR, whereas the contact is permitted in mut-*oprF* (Fig. 3). Thus, the lack of tight Gac/Rsm control on *oprF* and the gain of control on mut-*oprF* could be explained by the structural properties of the RsmE-*hcnA* complex [24]. Our results suggest that both sequence and local secondary structure are important for RsmA/E recognition of mRNA targets.

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