Broth versus Surface-Grown Cells: Differential Regulation of RsmY/Z Small RNAs in *Pseudomonas aeruginosa* by the Gac/HptB System

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Two-component systems are capable of profoundly affecting genetic regulation in bacteria by detecting environmental stimuli, allowing them to quickly adapt. In *Pseudomonas aeruginosa*, the small RNAs (sRNAs) RsmY and RsmZ are under the control of the GacS/A system. They have been described as ones of the major key players in the control of planktonic and surface-associated behaviors. Genetic regulation by these sRNAs is achieved by the titration of the negative post-transcriptional regulator RsmA which affects the expression of over 500 genes. There is increasing evidence pinpointing the importance of RsmY and RsmZ in the planktonic-sessile *P. aeruginosa* lifestyles switch control. Using swarming motility as a model, we show here that these sRNA are differentially regulated depending on the selected growth conditions (i.e., planktonic versus surface grown-cells). Also, we report that opposite to planktonically grown cells, *rsmZ* regulation does not implicate the response regulator GacA in swarming cells. Furthermore, we present data indicating that RsmY/Z expression influence swarming motility via the protein HptB which acts as a negative regulator of these sRNAs and that they do not strictly converge to RsmA as previously reported.

**Keywords:** swarming, broth-surface, small RNAs, surface motility, genetic regulation, histidine phosphotransfer protein, HptB

**INTRODUCTION**

Bacterial survival in the environment relies on their capacity to quickly adapt to changing conditions by either inducing or repressing specific sets of genes (Boor, 2006). Some bacteria have the ability to colonize a broad range of hosts using virulence functions (Furukawa et al., 2006). One adaptation mechanism used by bacteria consists of two-component systems (TCSs) (Hoch and Varughese, 2001), membrane-bound sensors coupled to cytoplasmic response regulators that permit the integration of external stimuli and induce global gene expression shifts (Beier and Gross, 2006). The heterotrophic opportunistic pathogen *Pseudomonas aeruginosa* exemplifies such a remarkable capacity to adapt to changing environments by encoding more than 60 TCS on its genome (Rodrigue et al., 2000). The GacS/GacA TCS has been extensively described over the years and is central in regulating the expression of the two small RNAs (sRNAs) RsmY and RsmZ. Mainly controlled by the response regulator GacA, these sRNAs titrate the availability of RsmA, a post-transcriptional regulator modulating the expression of functions implicated in the transition...
between the surface-associated and the planktonic *P. aeruginosa* lifestyles (Hoch and Varughese, 2001; Brencic and Lory, 2009; Brencic et al., 2009).

Swarming motility is a surface-associated type of bacterial motility characterized by a rapid and coordinated movement of a bacterial population on a viscous surface (Kearns, 2010). In *P. aeruginosaa*, this type of bacterial movement necessitates the presence of functional flagella and the production of rhamnolipids (and other RhlABC products) responsible for both modulation of the surface behavior and lowering surface tension (Déziel et al., 2003; Tremblay et al., 2007). Over the years, swarming motility has been linked to many phenotypes, such as increased antibiotic resistance, and to be inversely regulated to another surface bacterial behavior, biofilm formation (Caiazza et al., 2007; Lai et al., 2009). Given that swarming motility represents a distinct bacterial lifestyle from planktonic and sessile cells, many studies have aimed at understanding how this surface behavior is regulated. Over the years, the secondary messenger molecule, c-di-GMP has been shown to have a profound impact over the motile-sessile behavior. Over the years, swarming motility has been linked to many surface tension (Déziel et al., 2003; Tremblay et al., 2007). Interestingly, Bordi et al. (2010) determined that HptB mediates its effect through the modulation of the sRNA RsmY exclusively.

In the present study, we demonstrate how HptB affects swarming motility via modulation of the expression of both RsmY and RsmZ and that the swarming default of an ΔhptB mutant is not associated with flagellar malfunction nor insufficient biosurfactant production. Comparing ΔhptB mutant cells cultivated in planktonic versus surface conditions, we show that both rsmY and rsmZ are negatively regulated by HptB. Furthermore, HptB-mediated rsmZ expression control is stronger in swimming cells and, unexpectedly, not under the absolute control of the GacA response regulator.

**MATERIALS AND METHODS**

**Strains, Plasmids, and Growth Conditions**

Bacteria used in this study are all derived from the parental strain PA14 (Rahme et al., 1995; Lee et al., 2006) and are listed in Table 1. Bacteria were cultivated at 37°C in Tryptic Soy Broth (TSB) (Difco) with shaking (240 rpm) in a TC-7 roller drum (New Brunswick) or on TSB plates solidified with 1.5% agar. For swarming motility assays, overnight cultures were washed in phosphate-buffered saline (PBS) and diluted to the desired OD600. For transcriptomic analyses, the bacteria were cultivated in M9 minimal medium supplemented with 11 mM dextrose and 0.5% casamino acids (Difco) (M9DCAA) broth at 34°C (Tremblay and Déziel, 2008). OD600 was measured with a Nanodrop ND-1000 (Thermo Fisher Scientific) and pathlength correction was applied by multiplying the given value by a factor of 10.

**Construction of the ΔhptB Knock-Out Mutant**

Deletion of the Hpt domain of the *hptB* gene (PA3345 in PAO1 and PA14_20800 in PA14) was performed as follows. Two fragments of DNA were amplified from PA14 genomic DNA using pairs of primers PA3345_Left_FWD and PA3345_Left_REV for the left fragment and PA3345_Right_FWD and PA3345_Right_REV for the right fragment. These fragments
were ligated together using a complementary engineered overhang sequences of 15 nucleotides common to PA3345 _Left_REV and PA3345_Right_FWD. The DNA fragment was overhang sequences of 15 nucleotides common to PA3345

RNA Preparation
Total RNA was extracted from liquid bacterial cultures grown in triplicate in M9DCAA cultivated to late exponential phase (OD$_{600}$ = 1.3) at 34°C. The cells were then centrifuged for 5 min at 12,000 × g and the supernatant was discarded. Cells were resuspended in PureZOL (BioRad) and RNA extraction was performed following the manufacturer’s recommendations. For surface-grown bacteria, RNA was collected from the cells located at the migrating tip of a swarming colony grown for 12 h at 34°C using 8 μl of RNAlater (Qiagen) that was put directly on each surface-grown bacteria, RNA was collected from the cells located at the migrating tip of a swarming colony grown for 12 h at 34°C using 8 μl of RNAlater (Qiagen) that was put directly on each

Motility Assays
Swarming motility assays were performed using the same medium as for swimming motility tests but with 0.5% agar. Once autoclaved, the semi-solid agar was dried in a laminar biological safety cabinet for 75 min. Swarming plates were inoculated with 5 μl of a bacterial subculture grown in TSB to early stationary phase and adjusted to an OD$_{600}$ of 3.0 in sterile 1X PBS. The plate were then incubated overnight at 34°C (Tremblay and Déziel, 2008). Time-lapse image analysis was done using Photoshop CS3 Extended (Adobe).

Quantitative Real-Time PCR (qRT-PCR)
Quantitative real-time PCR (qRT-PCR) was done with the qScript™ One-Step SYBR Green kit and a RotorGene 6000 (Corbett) thermocycler. Primers were designed in order to obtain amplicons of 80–150 bp. The $nadB$ gene was used as control. Each cycle of qRT-PCR was done in triplicate. The threshold cycle (Ct) was normalized to $nadB$ Ct amplified in each corresponding samples. Variation in expression was calculated using the $-2^{ΔΔCt}$ method (Livak and Schmittgen, 2001). Assessment of variation in expression was performed using biological triplicates on two different days. Statistical analysis was done using Prism version 6.0 (GraphPad). Gene expression variation is shown as Relative expression variation (log2) to the wild-type PA14 strain.

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LC/MS Rhamnolipids Quantification

Concentrations of rhamnolipids were determined by liquid chromatography/mass spectrometry (LC/MS) (Abdel-Mawgoud et al., 2014). A 400 µl sample of a liquid overnight culture was collected and the OD_{600} was measured. Then the samples were centrifuged at 16,000 × g for 10 min. to remove bacterial cells. To 300 µl of supernatant, 300 µl acetonitrile containing 20 mg/L 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d4) as the internal standard were added. Samples were analyzed by high-performance liquid chromatography (HPLC; Waters 2795, Mississauga, ON, Canada) equipped with a C8 reverse-phase column (Kinetex, Phenomenex) using a water/acetonitrile gradient with a constant 2 mmol l⁻¹ concentration of ammonium acetate. The detector was a mass spectrometer (Quattro Premier XE, Waters). Analyses were carried out in the negative electrospray ionization (ESI-) mode.

Surface biosurfactant production was measured by inoculating the ΔhptB mutant and the wild-type strain on swarm plates. After overnight incubation at 34°C, all the agar was recovered from the Petri dishes and transferred to an Erlenmeyer flask containing 1:1 (v/v) KHCO₃ (pH 9.0) (i.e., for five 20 ml swarm plates, 100 ml of KHCO₃ was added). The collected agar was vigorously mixed for 1 h at room temperature then filtered through Whatman no. 1 covered with silica sand (Fisher). The filtrate was acidified at pH 4.0 with concentrated HCl then centrifuged at 16,000 × g for 10 min. to remove bacterial cells. Total biomass was quantified by resuspending bacteria on the swarm plates twice with 1 ml PBS then transferred to pre-cooled plates, 100 ml of KHCO₃ buffer (pH 9.0) (i.e., for five 20 ml swarm plates, 300 ml of KHCO₃ was added). The collected agar was vigorously mixed for 1 h at room temperature then filtered through Whatman no. 1 covered with silica sand (Fisher). The filtrate was acidified at pH 4.0 with concentrated HCl then extracted three times with 50 ml ethyl acetate. The organic phases were pooled, evaporated, and the residue analyzed as described above. Total biomass was quantified by resuspending bacteria on the swarm plates twice with 1 ml PBS then transferred to pre-weighed aluminum boats, dried at 65°C for 2 h and then weighed again. Biomass was measured by preparing a parallel swarm set in identical conditions for rhamnolipids quantification. Rhamnolipids quantification was done in technical triplicate on two different days. Statistical analysis was done using Prism version 6.0 (GraphPad).

RESULTS

HptB-Mediated Swarming Regulation Does Not Implicate Flagellar Malfunction nor a Default in Rhamnolipids Production

There is increasing evidence for the implication of the HptB phosphotransfer protein in the modulation of several *P. aeruginosa* behaviors (Hsu et al., 2008; Bordi et al., 2010; Kong et al., 2013). Recently, swarming motility has been identified as being under the control of the HptB regulon, which was determined to include flagella-related genes (Bhuwan et al., 2012); however, surprisingly, no effect was seen on flagellar functionality. To further characterize the role of HptB in the regulation of the swimming surface-associated behavior, we engineered a markerless ΔhptB mutant by deleting the entire histidine phosphotransfer domain (Hsu et al., 2008). As previously noted by Hsu et al. (2008), for strain PAO1, inactivation of the hptB gene in strain PA14 results in the loss of swarming motility (Figure 1A). A functional flagellum and the production of RhlABC products (mostly rhamnolipids) are elements required for swarming motility (Köhler et al., 2000; Déziel et al., 2003; Caiazza et al., 2005; Tremblay et al., 2007). Thus, we verified whether or not flagellar functionality is responsible for the ΔhptB mutant swimming phenotype by performing swimming motility assays. As shown in Figure 1B, the ΔhptB mutant still has a functional flagellum in both tested conditions. The production of rhamnolipids was also verified both in planktonic cultures and directly on swarm plates. The ΔhptB mutant produces an equivalent amount of biosurfactant compared to the wild-type strain when incubated in M9DCAA broth (Figure 1C). When looking at production of rhamnolipids by swarming cells directly from plates the ΔhptB mutant is not impaired and it actually displays increased biosurfactant production (Figure 1D). Absence of a growth defect by the ΔhptB mutant (Supplementary Figure S1) excludes known factors for the observed deficient swarming phenotype. We conclude that HptB positive control on swarming motility does not go through the known required factors.

Swarming Motility is Linked to sRNAs Expression

There are indications that expression of the swarming phenotype is positively regulated by RsmA and antagonized by the small RNAs, RsmY and RsmZ (Heurlier et al., 2004; Kay et al., 2006). In cells cultivated as broth cultures, HptB negatively affects the expression of RsmY specifically, but with no effect on RsmZ (Bordi et al., 2010). Since bacterial growth mode (i.e., cells grown planktonically versus on a surface) can affect the output of genetic regulation (Overhage et al., 2008; Tremblay and Déziel, 2010), we monitored the expression of both *rsnY* and *rsnZ* in bacteria grown in both planktonic and swarming cultures. In agreement with what was seen in strain PAK (Bordi et al., 2010), the RsmY sRNA is also overexpressed in the PA14 ΔhptB mutant grown in broth cultures while a small (and not previously reported) increase in expression of RsmZ is also observed (Figure 2A). Surprisingly, when the same experiment is carried out on swarming cells sampled at the migrating tip of a swarming colony, a marked increase of 2 log₂ in rsmZ expression is observed when compared to its planktonic counterpart (Figure 2A), suggesting specific upregulation by surface growth. We therefore looked at the swarming phenotypes of ΔrsnY, ΔrsnZ, and ΔrsnYZ mutants and observed that they all exhibit a hyperswarming phenotype (Figure 2B), when compared to the wild-type (Figure 1A). To precisely confirm this hyperswarmer behavior, we measured the area covered by the swarming colony using time-lapse image analysis: ΔrsnY and ΔrsnZ behave the same way while the increased swarming phenotype of the double mutant is even more pronounced than both simple mutants (Figure 2C; Supplementary Figure S2).

To verify the hypothesis that the swarming defect of the ΔhptB mutant is directly caused by the overexpression of *rsnY* and *rsnZ*, we constructed double ΔhptBrsnY, ΔhptBrsnZ and triple ΔhptBrsnYZ mutants. Significantly, the introduction of either a *rsnY* or a *rsnZ* mutation in a ΔhptB background resulted in rescue of the swarming phenotype, yet not identical as the
pattern observed for the simple ΔrsmY and ΔrsmZ mutants (Figure 3A). Finally, swarming motility of the triple ΔhptBΔrsmYZ exhibited exactly the same phenotype as the double ΔrsmYZ (Figure 3B). Taken together, these results support a model where HptB promotes swarming motility through the RsmA pathway, likely by decreasing the expression of both inhibitory sRNAs RsmY and RsmZ.

**Regulation of rsms Differences in Surface-Grown Cells Compared to their Planktonic Counterpart**

To understand if the regulation of rsmsY and rsmsZ by HptB is exerted through the GacS/GacA TCS, which has been characterized as being responsible for the transcription of these two sRNAs in liquid cultures (Brencic et al., 2009), we engineered a double ΔhptBgacA mutant (Supplementary Material). In that mutant background, we monitored the expression of rsmsY and rsmsZ in planktonic and surface-associated (swarming) cells. When the cells are cultivated in broth, the expression levels of both rsmsY and rsmsZ are indeed negatively affected in the double ΔhptBgacA in a comparable way to the simple ΔgacA mutant, supporting the hypothesis that the control of HptB over rsmsY and rsmsZ occurs downstream of GacA (Figure 4A). Similarly, when the same experiment was performed on swarmer cells (surface-growing bacteria), a similar negative effect of HptB on rsmsY was confirmed to be mediated through GacA. On the other hand, unexpectedly, the overexpression of rsmsZ that was seen in the ΔhptB mutant background (Figure 2A) is still observed when gacA is also abrogated (Figure 4B), indicating that HptB regulates rsmsZ expression via an unidentified alternative regulatory pathway independent of GacA, specific to bacteria growing on a surface.

**DISCUSSION**

Swarming motility is a complex multicellular phenomenon that has been extensively investigated over the years. However, the underlying genetic regulatory pathways controlling that surface-associated type of motility still remain to be fully characterized.
FIGURE 2 | Swarming motility is linked to sRNA expression. (A) Expression of the sRNAs RsmY and RsmZ in the ΔhptB mutant strain grown in broth or on a surface (swarming condition) determined by qRT-PCR. (B) Swarming motility of various sRNA mutants. (C) Time-lapse analysis of the ΔrsmY/Z mutants. Error bars represent the standard error of the mean for experiments carried out at least twice with three biological replicates per experiment. Student’s t-test analysis was applied on two independent experiments with *** p < 0.001; ns, not significant. For (C), the data correspond to one single plate per strain. Gene expression variation is shown as relative expression variation (log$_2$) to the wild-type PA14 strain.

In *P. aeruginosa*. In the present study, we have identified that the inactivation of the *hptB* gene renders *P. aeruginosa* incapable of such a type of motility even though this mutant still expresses the necessary propelling and wetting tools to exert a normal movement on a semi-solid (0.5% agar) medium.

The HptB protein has been well-described and novel pathways implicating this protein have been identified. Bhuwan et al. (2012), saw that transcription of many flagella-related genes was affected in a ΔhptB mutant grown as swarming cells and that these effects were mediated via a novel regulatory
cascade implicating the PA3346 and PA3347 gene products. Surprisingly, they reported no differences in flagellar morphology and swimming motility of the ΔhptB mutant, indicating that the functionality of this propelling appendage was apparently not affected. Thus, a regulatory imbalance in that mutant was possibly responsible for such a phenotype. However, Lin et al. (2006), observed that the absence of HptB provoked an important decrease in swimming motility in the PAO1 strain. In contrast to these studies, we looked at flagellar functionality and did not observe a defect in swimming motility of the ΔhptB mutant that could explain the dramatic decrease in swimming motility. The discrepancies observed between these two studies and ours could be due to differences in experimental design and strains. Bhuwan et al. (2012), incubated their swarming plates at 30°C for 36 h before proceeding to their transcriptomic analyses. Furthermore, they looked at their swarming phenotypes by incubating their plates at 37°C for 36 h. Our experiments were all carried out using plates incubated at 34°C for 12–16 h, when the cells are still metabolically active (Tremblay and Déziel, 2010).

Also, previous studies looking at the implication of HptB in motility never addressed the question of RhlABC products (biosurfactants). To express the swarming phenotype, P. aeruginosa needs the production of the wetting agent rhamnolipids (Déziel et al., 2003; Caiazza et al., 2005). A ΔrhlA mutant is incapable of swarming motility thus looking at the production of the biosurfactants is imperative in studies investigating this type of surface-associated motility. Here, we looked at the production of rhamnolipids in the ΔhptB mutant and did not see differences in production compared to the wild-type strain when the cells were cultivated in liquid cultures. Interestingly the same mutant produced more rhamnolipids than wild-type PA14 under swarming conditions. We hypothesize that rhamnolipid production is upregulated to overcome the absence of swarming. Accordingly, a 1:1 co-culture of the ΔhptB and rhlA− mutants results in a rescue of swimming motility of a ΔrhlA mutant strain (data not shown) and therefore we have no reason to believe that overproduction of these wetting agents would prevent such a type of motility, quite the opposite. Thus, hptB was considered an interesting gene to investigate how swarming motility is regulated.

The membrane sensor RetS is capable of phosphorylating the HptB protein (Hsu et al., 2008). Transcriptomic analyses performed on planktonic bacteria have revealed that the HptB and RetS regulons are partially overlapping but consist of two separate signaling pathways that both converge to the GacS/GacA system through different mechanisms (Bordi et al., 2010). Interestingly, HptB was seen to have an effect on the regulation of the small RNA RsmY specifically and implicated an alternative pathway including the PA3346 and PA3347 gene products whereas no effect of the phosphotransfer protein was observed on RsmZ (Bordi et al., 2010). Thus, since HptB seems to control many phenotypes via RsmY...
regulation we investigated the effect of a ΔhptB mutation on small RNA regulation in both planktonic and swarming cells and confirmed the increased transcription of rsmY in both conditions at the same extent. However, in contrast with that study, we observe a moderate increase in rsmZ expression in a planktonic culture (Figure 2A). Since swarming is a surface-associated bacterial behavior, we also looked at the expression of both rsmY and rsmZ on cells that were collected at the tip of a migrating colony. Unexpectedly, we observed a 2 log₂ increase in expression of rsmZ in swarming cells compared to their planktonic counterparts. This result indicates that rsmZ is differentially regulated when cells are grown on a surface specifically. Such a different regulation on sRNAs is not unusual. For instance, Petrova and Sauer (2010) found that the inactivation of bfiS, implicated in biofilm formation, resulted in increased rsmY and rsmZ expression strictly in cells cultivated as biofilms but not in planktonic ones. Here, we found only rsmZ to be upregulated when comparing cells cultivated in broth versus a surface. This intriguing result guided us toward asking whether RsmZ could be the main mediator of swarming motility. Thus, we decided to look at the swarming phenotype of the simple and double ΔrsmYZ mutants. We expected to see an effect on swarming only for the ΔrsmZ mutant. Contrary to what has been observed by other groups (Heurlier et al., 2004; Kay et al., 2006), we saw an increase in swarming motility of both single mutants. The inactivation of both genes resulted in an even better capacity to swarm (Figures 2B,C). These results indicate that both RsmY and RsmZ act as negative regulatory elements of swarming motility and that the observed surface motility defect of the ΔhptB mutant is explained by the overexpression of these two sRNA. As a matter of fact, we have also observed that the overexpression of either RsmY or RsmZ in the wild-type PA14 background results in a decrease in swarming motility (data not shown). Interestingly, other reporter phenotypes such as increased exopolysaccharide production and hyperbiofilm formation seen in the PAKΔhptB (Bordi et al., 2010) was not detected in a PA14ΔrsmB mutant (Supplementary Figures S3 and S4), indicating that these strains behave differently, as previously reported for swarming motility for instance (Tremblay and Déziel, 2008).

To associate without any further doubt the implication of HptB in the regulation of swarming motility via the downregulation of both rsmY and rsmZ, we created a triple ΔhptBΔrsmYZ mutant. Swarming motility assay of that mutant resulted in a complete rescue of the phenotype equivalent to that of the double ΔrsmYZ strain. Swarming assessment of both double ΔhptBΔrsmY and ΔhptBΔrsmZ mutants somehow resulted in an intermediate surface motility phenotype, further confirming that both sRNAs are important for this type of surface-associated movement and have a cumulative effect. Furthermore, Bordi et al. (2010) demonstrated a corresponding sRNA summative effect, as the abolishment of either rsmY or rsmZ in a PAKΔhptB mutant background resulted in the production of intermediate biofilm phenotypes, whereas a triple ΔhptBΔrsmYZ mutant strain behaved exactly like a double ΔrsmYZ mutant. Together, these findings validate that the investigated phenotypes affected by the deletion of the hptB gene (in PA14 and PAK strains) are linked to rsmY and rsmZ overexpression.

Interestingly, an rsmA⁻ mutant is not as defective in swarming motility as the ΔhptB mutant (Supplementary Figure S5). Also, the inactivation of rsmA leads to impairment of rhamnolipid synthesis thus explaining its incapacity to swarm properly (Heurlier et al., 2004) while it is not the case for the ΔhptB mutant. Recently, the novel post-transcriptional regulator, RsmN (an RsmA ortholog) has been described as a positive regulator of swarming motility (Morris et al., 2013). However, it was shown that the mutation of the rsmN gene did not abolish swarming motility, but rather decreased it. Investigating the effect of a double ΔrsmArsmN mutant on swarming motility remains to be further studied. However, since the only known major target of both rsmY and rsmZ is the RsmA post-transcriptional repressor (Brencic and Lory, 2009), our data strongly suggest that these sRNA have alternative targets, yet to be identified.

As there is increasing evidence that sRNA control by HptB can be due to the alternative PA3346/PA3347 regulation pathway and to understand how that phosphotransfer protein can have an effect of sRNA regulation, we created a double ΔhptBΔgacA mutant. Knowing that GacA is the main positive regulator of rsmY and rsmZ expression (Brencic et al., 2009), we expected to see a loss of sRNA upregulation in that double mutant. As anticipated, we observed a downregulation of both rsmY and rsmZ in a planktonic culture of the ΔgacA mutant as it has already been reported by other groups (Kay et al., 2006; Brencic and Lory, 2009) as well as in swarming cells of that same genotype (Figure 4). Furthermore, the introduction of an hptB deletion in the ΔgacA mutant strain resulted in the abolishment of both
FIGURE 5 | Model for broth-surface differential sRNA regulation. We propose a regulation model where the control of the expression of \textit{rsmY} and \textit{rsmZ} by HptB is under the exclusive control of the GacS/GacA system and converges to the post-transcriptional regulator RsmA for both planktonic and swarming cells. However, in cells grown on a surface such as in swarming motility, the regulation of both \textit{rsmY} and \textit{rsmZ} is differential and does not strictly mediate their output via RsmA. The obtained data in our study indicates that other key-players allows for regulation of \textit{rsmZ} by HptB and does not implicate the Gac system. This regulation most likely involves other membrane sensors that can modulate the activity of the HptB protein. Presented model integrates previously published data (Hsu et al., 2008; Brencic et al., 2009; Bordi et al., 2010). Full arrows represent direct positive regulation. Dashed arrows represent indirect positive regulation. Dashed bars represent indirect negative control. Full bars represent direct negative regulation. ? = unknown contribution. X = Unknown regulating factor.

\textit{rsmY} and \textit{rsmZ} upregulation that was observed in the simple \textit{ΔhptB} mutant in liquid cultures. Interestingly, Bordi et al. (2010) observed that the hyperbiofilm phenotype of a PA1Δ\textit{hptB} strain was indeed abolished in a double \textit{ΔhptB\textit{gacA}} mutant suggesting that both sRNAs are implicated in the control of such a phenotype in strain PA1. Surprisingly in swarming cells, we observed that \textit{rsmZ} was still upregulated when compared to a simple \textit{ΔgacA} mutant whereas \textit{rsmY} overexpression was lost in a double \textit{ΔhptB\textit{gacA}} mutant. This result reveals a differential regulation of both sRNAs in swarming cells, where \textit{rsmZ} transcription does not require the presence of GacA as it has been seen for planktonic cultures, but rather necessitates the contribution of unknown regulator(s) that are specifically active in surface-grown cells.

In addition of GacA being the main activator of \textit{rsmY} and \textit{rsmZ} transcription (Brencic et al., 2009), there are other global regulators that can influence the expression levels of these sRNAs in planktonic cultures. Accordingly, the DNA-binding global negative regulators MvaT and MvaU, can specifically repress \textit{rsmZ} expression without affecting \textit{rsmY} (Castang et al., 2008) thus possibly being the reason why the upregulation was observed in surface-grown cells. We assessed the swarming motility of both \textit{ΔmvaT} and \textit{ΔmvaU} mutant strains and did not observe any difference in surface movement when compared to the wild-type (Supplementary Figure S6), indicating that any effect on \textit{rsmZ} regulation does not strictly exert an output on swarming motility.

To rule out the possibility of sRNA regulation by the PA3346/PA3347 pathway, we monitored the expression of \textit{rsmY} and \textit{rsmZ} in both swarming cells and their planktonic counterpart of mutants in these genes. Opposite to Bordi et al. (2010), we observed a slight increase in \textit{rsmY} expression in the PA3346\textsuperscript{−} mutant background but no effect for both sRNAs in PA3347\textsuperscript{−} in planktonic cultures (Supplementary Figure S7). Also, the inactivation of either PA3346 or PA3347 did not affect \textit{rsmY} and \textit{rsmZ} expression in surface-grown cells (Supplementary Figure S8). These results indicated us that \textit{rsmZ} overexpression in a \textit{ΔhptB} genetic mutant background is somehow independent of both GacS/GacA and PA3346/47 regulation pathways and that an unknown surface-activated regulator is responsible for the observed effect. Recently, a study by Wang et al. (2014), characterized a novel swarming regulator, BswR, capable of controlling the expression of \textit{rsmZ} directly in PAO1. We verified whether BswR could be responsible for \textit{rsmZ} regulation in swarming cells, but, surprisingly, we did not observe any effect on \textit{rsmZ} expression in a \textit{ΔbswR} mutant in both planktonic and swarming cells (Supplementary Figure S9), indicating that BswR does not play a role in the regulation of this sRNA under our conditions. Earlier this year, a study published by Xu et al. (2016) characterized the HapZ adaptor protein in a PAO1 strain and observed that it could act as an intermediate between the membrane sensor SagS and the HptB protein (Xu et al., 2016). However, it was reported that a mutation in the sagS gene does not affect swarming motility in PA14 (Petrova and Sauer, 2011), making HapZ unlikely to participate in swarming motility regulation, at least, in a PA14 genetic background.
The discrepancies observed between our study and other ones (Bordi et al., 2010; Bhawan et al., 2012; Wang et al., 2014) stress out the growing evidence that there are regulatory differences between the various P. aeruginosa strains (Tremblay and Déziel, 2008; Mikkelsen et al., 2011). Furthermore, we certainly cannot rule out the implication of the numerous previously characterized elements found to be implicated in the regulation of swarming motility. However, here we present a model where small RNA regulation is dependent on the conditions in which we study P. aeruginosa (i.e., broth versus surface) which favor different bacterial lifestyles. Furthermore, RsmW a novel RNA under the control of the Gac system has been identified to be strongly upregulated in biofilm conditions compared to planktonic cultures which further strengthens the existence of differential genetic regulation depending on the selected growth conditions (Miller et al., 2016). Our data confirm that the GacS/GacA system is not the only one responsible for the control of rsmZ expression and implicates the presence of unidentified key factors that are important when cells are grown on a surface rather than in planktonic cultures, as exemplified by the use of swarming motility as a model for surface behavior (Figure 5). Further downstream in the swarming regulatory cascade, we also show evidence that RsmY and RsmZ can modulate swarming motility not only via RsmA inhibition. Further experiments focusing on identifying these regulatory players specific to surface-grown bacteria will unveil a whole misunderstood genetic regulation portrait of P. aeruginosa.

REFERENCES


AUTHOR CONTRIBUTIONS

FJ-P, JT, and ED conceived and designed the experiments. FJ-P and JT performed the experiments. FJ-P, JT, and ED analyzed the data. FJ-P, JT, and ED contributed to manuscript preparation and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.02168/full#supplementary-material


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