A Small Regulatory RNA Controls Cell Wall Biosynthesis and Antibiotic Resistance

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ABSTRACT  Small regulatory RNAs play an important role in the adaptation to changing conditions. Here, we describe a differentially expressed small regulatory RNA (sRNA) that affects various cellular processes in the plant pathogen Agrobacterium tumefaciens. Using a combination of bioinformatic predictions and comparative proteomics, we identified nine targets, most of which are positively regulated by the sRNA. According to these targets, we named the sRNA PmaR for peptidoglycan biosynthesis, motility, and ampicillin resistance regulator. Agrobacterium spp. are long known to be naturally resistant to high ampicillin concentrations, and we can now explain this phenotype by the positive PmaR-mediated regulation of the beta-lactamase gene ampC. Structure probing revealed a spoon-like structure of the sRNA, with a single-stranded loop that is engaged in target interaction in vivo and in vitro. Several riboregulators have been implicated in antibiotic resistance mechanisms, such as uptake and efflux transporters, but PmaR represents the first example of an sRNA that directly controls the expression of an antibiotic resistance gene.

IMPORTANCE The alphaproteobacterium Agrobacterium tumefaciens is able to infect various eudicots causing crown gall tumor formation. Based on its unique ability of interkingdom gene transfer, Agrobacterium serves as a crucial biotechnological tool for genetic manipulation of plant cells. The presence of hundreds of putative sRNAs in this organism suggests a considerable impact of riboregulation on A. tumefaciens physiology. Here, we characterized the biological function of the sRNA PmaR that controls various processes crucial for growth, motility, and virulence. Among the genes directly targeted by PmaR is ampC coding for a beta-lactamase that confers ampicillin resistance, suggesting that the sRNA is crucial for fitness in the competitive microbial composition of the rhizosphere.

KEYWORDS antibiotic resistance, gene regulation, plant-microbe interaction, posttranscriptional control, regulatory RNA

Small regulatory RNAs (sRNAs) or noncoding RNAs (ncRNAs) are versatile regulators crucial for bacterial adaptation to changing environments (1, 2). These small RNA molecules range between 50 and 500 nucleotides (nt) in length and usually remain untranslated. Most sRNAs bind to target mRNAs modulating their stability and/or translation, albeit protein activity can also be controlled by sRNAs (3). Thus, sRNAs are involved in the differential regulation of numerous cellular pathways, including cell division (4), stress responses (5), quorum sensing (6), and virulence (7, 8). Moreover, several sRNAs have an impact on antibiotic resistance by affecting the expression of genes coding for uptake or efflux systems, peptidoglycan biosynthesis, or biofilm formation (9). Two different types of sRNAs can be distinguished according to their location on the genome. While cis-antisense RNAs (asRNAs) are encoded on the opposite strand of their target gene (10), trans-encoded sRNAs are located in intergenic regions and usually interact with several targets from distinct genomic locations (11).
Since trans-encoded sRNAs share only limited base pair complementarity with their target mRNAs, their association is often promoted by RNA chaperones, such as Hfq (12).

Most of the previously described sRNAs have been studied in enterobacteria, such as *Escherichia coli* or *Salmonella* spp. (2, 3). However, by means of differential RNA sequencing, sRNAs have been identified in essentially all bacterial and archaeal species studied thus far (13, 14), including alphaproteobacteria, such as photosynthetic *Rhodobacter* species (15), plant-symbiotic rhizobia (16–18), and the mammalian pathogen *Brucella abortus* (19). The alphaproteobacterium *Agrobacterium tumefaciens*, also known as *Agrobacterium fabrum* (20), is a plant pathogen that has the unique ability to transfer part of its own DNA (T-DNA) into numerous eudicots (21). Integration of the T-DNA into the plant genome and subsequent expression of the involved genes leads to enhanced production of phytohormones and thereby to the formation of so-called crown gall tumors (22, 23). Through genetic engineering of Ti plasmids and their cognate T-DNA, *A. tumefaciens* has become the most important biotechnological agent for genetic manipulation of plant cells. As a member of the *Rhizobiaceae* family, *A. tumefaciens* is naturally resistant to certain β-lactam antibiotics, based on the chromosomally encoded beta-lactamase AmpC (24, 25). This enzyme is highly conserved among *Rhizobiaceae* and is possibly advantageous for microbial competition in the rhizosphere and the specific lifestyle of *A. tumefaciens*.

Recently, several RNA sequencing (RNA-seq) studies revealed more than 600 putative sRNAs in *A. tumefaciens*, suggesting a crucial role of sRNA-mediated regulation in this organism (26–29). At present, only a small number of *Agrobacterium* sRNAs have been functionally characterized. RepE was the first sRNA described in *A. tumefaciens* and controls Ti plasmid replication (30). The growth-phase-regulated sRNA AbcR1 targets multiple mRNAs of ABC transporter substrate-binding proteins, indicating an important role in nutrient acquisition during the transition to stationary phase (31). Importantly, AbcR1 was shown to regulate the uptake of γ-aminobutyric acid (GABA) (32), an amino acid derivative produced by wounded plants that stimulates degradation of a quorum sensing signal (33). The sRNA RNA1111 expressed from the Ti plasmid has an impact on the aggressiveness of the phytopathogen and affects the expression of several virulence genes (26).

To reveal the biological function of the countless bacterial sRNAs, one of the challenges in the field is the identification of their target genes due to the imperfect complementarity of the sRNA-mRNA pairs. Contemporary bioinformatic prediction programs can support target mRNA identification and currently work best for enterobacteria (34). The identification of sRNA targets in other species has remained difficult. Often, even experimentally verified sRNA-mRNA interactions are not predicted as top candidates by the available algorithms (35). In the present study, we used a combination of bioinformatic predictions and comparative proteomics by mass spectrometry to identify target mRNAs of the small regulatory RNA PmaR in *A. tumefaciens*. We identified PmaR as a crucial regulator for peptidoglycan biosynthesis, motility, and biotin synthesis. Moreover, PmaR regulates ampicillin resistance by modulating beta-lactamase levels. The major impact of PmaR on *Agrobacterium* physiology undermines the importance of sRNA-mediated regulation in this organism.

**RESULTS**

**Expression of PmaR is induced in stationary phase.** In previous work (29), PmaR (formerly C10) from the circular chromosome of *A. tumefaciens* was found to be transcribed under virulent and nonvirulent conditions (Fig. 1A). The gene of the trans-encoded sRNA is located between two hypothetical open reading frames of unknown function (Fig. 1B). Further RNA-seq data demonstrated that PmaR is highly expressed in complex medium at different growth phases (27). By Northern blot analysis, we observed differential expression of PmaR depending on growth conditions and growth phases (Fig. 1C). Transcript levels were highest during stationary phase in complex medium and in minimal medium (−Vir conditions). Furthermore, we observed higher expression of PmaR in minimal medium under acidic conditions (pH 5.5) than
with neutral pH and downregulation of the sRNA under virulence-mimicking conditions. Differential expression of a sRNA often is indicative of a regulatory role, which motivated us to study the physiological role of PmaR.

The structure of PmaR contains an accessible C-rich loop. The RNAfold-predicted secondary structure of PmaR comprises a spoon-like structure with a long stem and a single-stranded loop containing several C-rich regions (Fig. 2A). Enzymatic structure probing with RNases T1 (cuts single-stranded guanines) and T2 (cuts preferentially single-stranded adenines) and nuclease S1 (cuts the 3′ end of unpaired nucleotides) confirmed a highly stable structure, which was preferentially cleaved in the end-standing loop (Fig. 2B). As expected, the preferred substrate of RNase T1 was the accessible G62 residue. The poorest substrate was G47, which is predicted to close the loop (Fig. 2C). Further prominent cleavage sites for RNase T1 were identified for G18, G19, and G81, supporting the predicted structure.

Identification of targets by CopraRNA. PmaR is restricted to Agrobacterium species and Rhizobium sp. strain IRBG74, and both the sequence and structure of the sRNA are highly conserved (see Fig. S1B in the supplemental material). The target prediction tool CopraRNA (34, 36) generated a list with top candidates that are involved in peptidoglycan biosynthesis (murB and murI) and cell division (ftsQ) (Fig. 3A). We analyzed the transcript levels of these candidates in the presence or absence of PmaR by Northern blot analysis and found that murB and murI were both downregulated in the ΔPmaR mutant strain during exponential phase (Fig. 3B). The already low expression of these genes in early stationary phase was not further affected. PmaR had no influence on ftsQ mRNA amounts, and the same was true for the candidate xynA encoding a beta-xylanase (data not shown). Transcript levels of the candidate cheD encoding a methyl-accepting chemotaxis protein were downregulated in the ΔPmaR mutant during exponential phase (Fig. 3B). These results suggest that PmaR is involved in positive regulation of peptidoglycan biosynthesis, motility, and chemotaxis. Furthermore, prediction of the sRNA interaction region by CopraRNA strongly supported our hypothesis that the single-stranded loop of PmaR is responsible for target binding (Fig. S1A).

Impact of PmaR on Agrobacterium physiology. To correlate the influence of PmaR on the targets described above with Agrobacterium physiology, we tested for phenotypes of the deletion mutant. Growth of the ΔPmaR mutant strain in complex medium
was slightly delayed in comparison to the wild type (Fig. 4A). Furthermore, mutant cells were slightly elongated in stationary phase and tended to aggregate in both exponential and stationary phase (Fig. 4B). Motility assays on soft agar plates revealed enhanced motility of the mutant compared to the wild type (Fig. 4C). Complementation by plasmid-borne PmaR reduced motility to wild-type values, and overexpression of the sRNA in the wild-type background almost completely abolished motility. Virulence assays revealed that the sRNA mutant elicits an increased number of tumors on potato discs (Fig. 4D). These pleiotropic effects of PmaR deletion on *A. tumefaciens* physiology suggest a major regulatory impact of the sRNA and prompted us to experimentally search for more targets.

**Identification of targets by mass spectrometry.** To identify further targets of PmaR, we chose a gel-free mass spectrometry approach to compare the wild-type and mutant proteomes. Since PmaR expression is induced in stationary phase (Fig. 1C), we used samples obtained from early stationary phase (optical density at 600 nm [OD₆₀₀], 1.5). At least 10 proteins showed differential accumulation between the wild-type and ΔPmaR mutant strains. Seven of these putative targets were upregulated or exclusively detected in the ΔPmaR mutant, while three were downregulated in the mutant or exclusively found in wild-type samples (Table 1). Consistent with comparative transcript levels between the wild type and ΔPmaR mutant in stationary phase (Fig. 3B), MurB, Murl, and CheD were not found to be regulated using the proteomics approach.

We chose six of the 10 putative targets (marked in bold in Table 1) on the basis of their annotated functions. Given the high ampicillin resistance of *A. tumefaciens*, one of
the most interesting candidates was the beta-lactamase AmpC, the levels of which were 4-fold decreased in the absence of the sRNA. Other candidates are BioA and BioB, which are encoded in an operon and involved in biotin synthesis, and Atu3504, a substrate-binding protein of an ABC transporter of unknown function. Northern blot experiments demonstrated that the transcripts of all six targets identified by comparative proteomics were influenced by PmaR (Fig. 5D). Surprisingly, transcript levels of minD and pepF were downregulated in the ΔPmaR mutant, while the mass spectrometry data suggested upregulation of these targets in the PmaR mutant.

**Exchange of four nucleotides in the PmaR loop abolishes target regulation.** To experimentally validate the prediction that PmaR binds its targets through C-rich regions in the single-stranded loop, we designed a mutated variant with an exchange of four nucleotides (58 to 61 [CCCA-to-UUUU]; Fig. 5A) that did not alter the secondary
structure of the sRNA (see probing experiments in Fig. S2) and complemented the ΔPmaR mutant with this variant. The transcript amounts of PmaR were not affected by this mutation, and the plasmid-derived sRNA variants accumulated similarly to the endogenous wild-type PmaR (Fig. 5B). Target mRNA levels in exponential and stationary phase were compared by Northern blot analysis in the wild type and mutant (with an empty vector), as well as in the complemented ΔPmaR mutant strain with wild-type sRNA (Comp) and mutated variant (Mut) (Fig. 5C and D). The three targets murB, murI, and cheD exhibited similar transcript patterns in exponential phase, as shown in Fig. 3B.

FIG 4 Impact of PmaR on growth, motility, and virulence. (A) Growth curve of wild type and ΔPmaR mutant grown in YEB medium. (B) Samples were taken from cultures grown in YEB medium during exponential and early stationary phase and examined by bright-field microscopy. Cell size and aggregation of 100 cells per strain were measured. (C) Motility of A. tumefaciens wild type and ΔPmaR mutant, both supplemented with empty vector control (+EV) and plasmid-derived PmaR (+PmaR), was determined by soft agar plates with AB medium (pH 5.5). Average motility and mean standard deviation are indicated below the pictures. (D) Potato disc infection assay with wild type and ΔPmaR mutant. Developed tumors appear white on the greenish potato discs. Experiments were performed in triplicates with similar results.
In comparison to the strains without an empty vector (Fig. 3B), the transcript amounts of \textit{murI} and \textit{cheD} differed only slightly between the wild type and \( \Delta \text{PmaR} \) mutant (Fig. 5C). Complementation of the \( \Delta \text{PmaR} \) mutant with the wild-type sRNA restored elevated target mRNA levels, suggesting positive regulation of these targets by the sRNA. Consistent with an interaction via the C-rich motif around nucleotide 60, the target mRNA amounts in the \( \Delta \text{PmaR} \) mutant did not change in the presence of the Mut plasmid (Fig. 5C). The same pattern was observed for the target mRNAs \textit{ampC}, \textit{minD}, \textit{pepF}, \textit{bioA}, and \textit{bioB} in stationary phase, indicating positive regulation by the same

![Diagram](image)

\textbf{FIG 5} Nucleotide exchanges in the loop affect target regulation. (A) Schematic drawing of the mutated PmaR variant with four exchanged nucleotides (marked in blue). (B) Northern blot analysis of PmaR from stationary phase. Transcript levels were determined in the wild type and \( \Delta \text{PmaR} \) mutant supplemented with an empty vector as well as in \( \Delta \text{PmaR} \) mutant complemented with plasmid-derived wild-type PmaR (Comp) and the mutated variant (Mut). (C and D) Northern blot analysis of targets from exponential (C) and early stationary (D) phase. Transcript levels were determined in the wild type and different \( \Delta \text{PmaR} \) mutant strains as described above. Ethidium bromide-stained 16S rRNA served as a loading control.

\begin{table}
\centering
\caption{Putative targets identified by mass spectrometry}
\begin{tabular}{llc}
\hline
Protein & Annotated function & Regulation* \\
\hline
Atu1710 & Conserved hypothetical protein & 1.756 \\
Atu1883 & Conserved hypothetical protein & \( \Delta \text{PmaR} \) mutant only \\
MinD (Atu3248) & \textit{Cell division inhibitor} & \( \Delta \text{PmaR} \) mutant only \\
Atu3504 & \textit{ABC transporter substrate (sulfate) binding protein} & \( \Delta \text{PmaR} \) mutant only \\
Htp (Atu3604) & Hypoxanthine phosphoribosyltransferase & \( \Delta \text{PmaR} \) mutant only \\
PeplF (Atu3765) & \textit{Oligopeptidase F} & \( \Delta \text{PmaR} \) mutant only \\
Atu6048 & Conserved hypothetical protein & \( \Delta \text{PmaR} \) mutant only \\
AmpC (Atu3077) & Beta-lactamase & 0.259 \\
BioB (Atu3997) & Biotin synthetase & 0.622 \\
BioA (Atu4000) & Adenosylmethionine-8-amino-7-oxononanoate aminotransferase & WT only \\
\hline
\end{tabular}
\footnote{Regulation indicates detection of the protein either in wild type (WT) or \( \Delta \text{PmaR} \) mutant only or shows the ratio between the \( \Delta \text{PmaR} \) mutant and WT. Tested and validated targets on an RNA level are indicated in bold.}
\end{table}
sRNA region. As suggested by the proteomics data (Table 1) and in accordance with negative regulation by PmaR, \textit{atu3504} showed the opposite transcript pattern (Fig. 5D). The mRNA levels were low in the wild type and Comp strains but elevated in the ΔPmaR mutant and the complementation with the Mut plasmid, which shows that the same sRNA region is involved in positive and negative regulation.

The importance of the exchanged nucleotides was further corroborated by growth experiments (Fig. 6A) and motility assays (Fig. 6B) of the complemented mutant strain. Complementation with wild-type PmaR restored wild-type growth and motility, whereas complementation with the mutated variant resulted in reduced growth and enhanced motility comparable to those of the PmaR mutant. Taken together, these data confirm that the four mutated nucleotides in the sRNA loop are essential for target mRNA regulation.

**Regulation of target mRNAs in vivo.** In order to monitor target regulation by PmaR \textit{in vivo}, we constructed transcriptional (Fig. 7A) and translational (Fig. 7B) reporter fusions of targets in exponential (\textit{murB} and \textit{cheD}) or stationary phase (\textit{ampC} and \textit{atu3504}) and introduced them into the chromosome of the \textit{Agrobacterium} wild type and the ΔPmaR mutant under the control of the native promoter. Transcript levels were measured with a \textit{lacZ} fusion via β-galactosidase activity assays, and protein levels were monitored by Western blot analysis detecting a fused 3×FLAG epitope. Fully consistent
with the assumed positive regulation, the transcript levels of murB, cheD, and ampC were strongly reduced in the ΔPmaR mutant in comparison to the wild type (Fig. 7C). The response on an RNA level was mirrored by a reduction in the corresponding protein amounts (Fig. 7D). In accordance with negative regulation by the sRNA, atu3504 mRNA levels (Fig. 7C) and, in particular, Atu3504 protein levels (Fig. 7D), were induced in the ΔPmaR mutant.

Since both the transcript and protein levels of the examined fusions were altered in the ΔPmaR mutant, we assumed that PmaR might affect the stability of target mRNAs. We measured the half-lives of PmaR targets by adding rifampin to wild-type and ΔPmaR mutant cultures and taking samples before and up to 4 min after treatment with the transcription inhibitor. Northern blot analysis confirmed that transcript stability of the positively regulated murB transcript was decreased in the ΔPmaR mutant (Fig. 8A and B), whereas the stability of the negatively regulated atu3504 mRNA was elevated in the PmaR mutant (Fig. 8C and D). Consistent with the observed downregulation in the ΔPmaR mutant (Fig. 5C and D), the transcript stability of the PmaR targets cheD, ampC, minD, and bioA was decreased in the ΔPmaR mutant strain (Fig. 5E).

**Direct binding of target mRNAs by PmaR in vitro.** The CopraRNA program predicted sRNA-mRNA interactions in various regions of the PmaR targets (Fig. 3A). We chose murB and ampC, which are both predicted to bind PmaR in their 5′ untranslated region (UTR) (Fig. S3A and S4A) for monitoring a direct interaction with PmaR by electrophoretic mobility shift assays. Both targets bound PmaR resulting in a gel shift, whereas the mutated PmaR variant with the exchanged CCCA region did not produce
a shift (Fig. 9A and B). Remarkably, the binding affinities of murB and ampC to PmaR were vastly different. A hundredfold excess of murB was necessary to retard wild-type PmaR (Fig. 9C), whereas equimolar amounts of ampC were sufficient to shift the sRNA (Fig. 9D). We quantified the signals of Fig. 9C and D and plotted the binding affinity of both murB (Fig. 9E) and ampC (Fig. 9F) to PmaR. The calculated \( K_D \) (equilibrium dissociation constant) values for murB and ampC were around 700 and 1 \( \mu \)M, respectively (Fig. 9E and F).

**PmaR positively controls ampicillin resistance.** The beta-lactamase AmpC is highly conserved among Rhizobiaceae and confers high resistance to \( \beta \)-lactam antibiotics, such as ampicillin. Direct interaction of PmaR with the ampC transcript in vitro, as well as decreased ampC mRNA and AmpC protein levels in the PmaR mutant in vivo, strongly suggest a direct regulation of ampicillin resistance by the sRNA in A. tumefaciens. Hence, we examined the sensitivity of the PmaR mutant to ampicillin. The sRNA mutant indeed displayed higher sensitivity to the antibiotic at a concentration of 200 \( \mu \)g/ml than did the wild type (Fig. 10A and B). Moreover, complementation of the mutant with wild-type (WT) PmaR resulted in WT-like resistance to ampicillin. The sRNA deletion strain complemented with the Mut plasmid remained sensitive to the antibiotic. Sensitivity to the carboxypenicillin ticarcillin, which is widely used in plant biotechnology to kill A. tumefaciens after plant infection, was not affected by PmaR (data not shown).

**DISCUSSION**

**Deletion of PmaR leads to pleiotropic effects on A. tumefaciens physiology.** In this study, we examined the function of the small regulatory RNA PmaR in the plant
pathogen A. tumefaciens. We discovered a broad impact of PmaR on Agrobacterium physiology, including peptidoglycan biosynthesis, motility, and virulence. Using a combination of bioinformatic predictions with CopraRNA (34, 36) and comparative proteomics by mass spectrometry, we identified nine targets that are affected by PmaR at the RNA and protein levels. Several of these targets can explain the observed phenotypes of the PmaR deletion mutant (Fig. 11). The moderate growth defect of the ΔPmaR mutant may result from decreased expression of murB and murI leading to perturbations in peptidoglycan formation. Further, decreased levels of BioA and BioB in the mutant most likely cause lower biotin levels in the cell that can reduce bacterial growth. Biotin is important for fatty acid biosynthesis, and decreased biotin levels in the cell can cause a severe imbalance in the bacterial cell envelope. Already in 1933, biotin was described to enhance the growth of rhizobial isolates (37, 38). Although A. tumefaciens is able to synthesize profligate amounts of the vitamin, which might be beneficial in its ecological niche, biotin is needed for growth in minimal medium (39).

Regulation of the target cheD by PmaR is in accordance with the observed motility phenotype. PmaR-dependent regulation of cheD was dependent on the growth phase, and slightly enhanced cheD levels were found in ΔPmaR mutant during stationary phase (Fig. 3B). This regulation may well reflect the conditions during the motility assay where the cells reside in stationary phase after 48 h of incubation. Another interesting observation is the enhanced tumor formation of the PmaR mutant. We did not identify any direct virulence-related target of the sRNA and propose that the enhanced tumor formation is due to a secondary effect, for example, the enhanced motility of ΔPmaR.

### Figure 9

PmaR interacts with murB and ampC in vitro. (A and B) Electrophoretic mobility shift assays for murB (A) and ampC (B) with labeled PmaR and the mutated variant (Mut). Final concentrations of unlabeled RNA were added in 400- to 800-fold excess for murB and in 100- to 400-fold excess for ampC. Samples treated with water served as a control. (C and D) Concentration series for murB (C) and ampC (D) with labeled wild-type PmaR. Samples treated with water served as a control. (E and F) Binding kinetics and calculated Kd values for complex formation of murB (E) and ampC (F) with PmaR by pixel counting of panels C and D. Experiments were performed in triplicate, with similar results.
mutant promoting a more efficient infection of the potato discs. To the best of our knowledge, the only other previously described hypervirulent mutant of 
A. tumefaciens is impaired in the formation of ornithine lipids (40). In that mutant, the causal relationship between this process and tumorigenesis is not yet clear.

Small RNAs often negatively regulate ABC transporters, and a prominent example in alphaproteobacteria is the AbcR family (41). Based on the downregulation of atu3504, PmaR may have a related function in nutrient acquisition. Atu3504 is annotated as an ABC transporter substrate-binding protein for sulfate. Therefore, higher levels of this protein in the ΔPmaR mutant might result in more efficient sulfate uptake, although we found that growth of the wild type and the PmaR mutant was identical under sulfate-limiting conditions in minimal medium (data not shown). This can be attributed

\[ \text{FIG 10} \] Regulation of ampicillin resistance by PmaR. Serial dilutions of cultures from wild type and ΔPmaR mutant supplemented with an empty vector as well as ΔPmaR mutant complemented with plasmid-derived wild-type PmaR (Comp) and the mutated variant (Mut) were spotted (A) and plated (B) on YEB medium with different concentrations of ampicillin. CFU were counted and calculated per milliliter of culture. Experiments were performed in triplicate, with similar results.
Several sRNAs influence bacterial resistance to antibiotics by their impact on the biosynthesis of porins, efflux systems, or biofilm formation (9). We provide complementary evidence in vivo and in vitro that PmaR is an sRNA that directly controls a bacterial antibiotic resistance gene coding for the beta-lactamase AmpC, which is highly conserved in Rhizobiaceae (24, 25). PmaR is only present in Agrobacterium species and a closely related Rhizobium strain. Due to their short sequence, sRNAs are known to evolve rapidly. The even shorter seed sequences that are sufficient for target interaction allow an enhanced turnover of sRNAs in terms of their de novo emergence, frequent change of function, or loss from bacterial lineages (42, 43). It is possible that PmaR is a recently evolved feature of Agrobacterium to stabilize ampC transcripts, thereby ensuring sufficient amounts of beta-lactamase. This may provide a competitive advantage of A. tumefaciens in the microbe-rich environment of the rhizosphere.

**PmaR influences transcript stability and translation.** In contrast to the majority of previously studied sRNAs (44), PmaR positively regulates most of its targets. Not surprisingly, the interacting sequence of PmaR does not consist of an anti-Shine-Dalgarno region as in AbcR1 and many other sRNAs that regulate translation initiation (32), but it consists primarily of cytosine residues. Most of the predicted interaction regions of PmaR in the target mRNAs are not located close to the ribosome binding site or the start codon. Instead, the program CopraRNA (34, 36) predicted various interaction regions throughout the transcripts (Fig. 3A). Predictions with IntaRNA (36, 45, 46) for the nine experimentally validated targets suggested interactions both in the 5’UTR (murB and ampC) and the coding sequence (Fig. S3 and S4). Prediction of the secondary structures of murB and ampC by Mfold (47) revealed structures that might prevent ribosome binding due to base pairing of the Shine-Dalgarno sequence (data not shown). Structural rearrangement of the 5’UTRs upon binding of PmaR might facilitate ribosome binding and therefore promote translation and/or stabilize the mRNAs as shown in various other cases (48). A similar mode of action might apply to cheD, since
the interaction site was predicted immediately downstream of the start codon. These hypotheses are supported by the observed decrease in murB and cheD transcript stability in the ΔPmaR mutant (Fig. 8A, B, and E). For the other target mRNAs, we suggest that the binding of PmaR might either block RNase cleavage sites or open complex secondary structures to allow translation of the mRNA to proceed. Interestingly, the downregulated target atu3504 also does not conform to the standard mechanism, in which an sRNA binds at or around the Shine-Dalgarno region. Instead, the predicted sRNA-mRNA interaction site is far within the coding sequence (Fig. S4C), and PmaR seems to destabilize the atu3504 transcript (Fig. 8C and D), suggesting that other factors are involved in the regulatory process.

Growth phase-dependent competition of targets for PmaR binding. PmaR controls a complex network of at least nine targets (Fig. 11). The abundance of both the sRNA and the target mRNAs changes throughout growth, suggesting that the relative sRNA-mRNA concentrations vary constantly and that the targets compete for binding of the sRNA. Despite low expression of PmaR in exponential phase, it regulates several targets under this condition. Other targets are primarily regulated in stationary phase. Interestingly, the affinities of PmaR to murB (regulated in exponential phase) and ampC (regulated in stationary phase) are very different. However, the calculated dissociation constants are derived from in vitro experiments and may not reflect the actual conditions inside the cell, where mRNAs are probably targeted by several different sRNAs and RNA-binding proteins mediate sRNA-mRNA interactions. From a “target-centric” perspective, it is possible that targets in stationary phase, such as ampC, compete for PmaR binding, neutralizing the effect of the sRNA on targets from exponential phase, as reviewed previously (1, 49). The band shift results contradict the bioinformatic predictions by IntaRNA (36, 45, 46) that proposed stronger binding of PmaR to murB (Fig. S3A) than to ampC (Fig. S4A) and show how misleading predictions without experimental validation can be. Overall, our data suggest that under specific conditions, competition of target mRNAs for PmaR binding determines the sRNA function rather than the mere expression of the sRNA.

MATERIALS AND METHODS

Bacterial growth conditions. The bacterial strains used in this study are listed in Table S1. A. tumefaciens C58 was cultivated in yeast extract-beef extract (YEB) complex medium at 30°C to OD600 of 0.4 (exponential phase) and 1.5 (early stationary phase), respectively. Cultivation in minimal medium (AB) and subsequent virulence induction were performed as described previously (29). E. coli was grown in LB medium at 37°C. Media were supplemented with ampicillin (Amp, 100 µg/ml) or kanamycin (50 µg/ml) if required.

Ampicillin sensitivity assays were performed by cultivation of A. tumefaciens strains to an OD600 of 1.0 (10^8 cells). Serial dilutions in A. dest buffer were spotted or plated on YEB medium with or without ampicillin and incubated at 30°C.

Strain and vector construction. The oligonucleotides and plasmids used in this study are listed in Tables S1 and S2. Deletion of PmaR was performed as described previously (32, 50). Complementation of the ΔPmaR mutant was achieved by cloning PmaR into pSRK, as described previously (51), and the resulting construct was introduced into A. tumefaciens by electroporation.

Transcriptional lacZ reporter gene fusions were constructed by amplifying the complete open reading frame of a target gene plus 75 nt of the 5′ UTR by PCR using the corresponding primer pairs (Table S2). The fragments were inserted blunt end into pUC19. A lacZ-Gn-oriT cassette derived from pYP1411 (Y. Pfänder and B. Masepohl, unpublished data) was introduced in these pUC19 constructs via the primer-derived BamHI restriction site that was added to the 3′ end of the target genes. The resulting reporter constructs were introduced into the chromosome of the A. tumefaciens wild type and ΔPmaR mutant by single-crossover integration via electroporation and expressed from the native promoter.

Translational reporter gene fusions were constructed by insertion of target gene sequences without the stop codon into pUC19, as described above. A 3×FLAG-Kmr-oriT cassette derived from pYP247I (S2) was introduced in these pUC19 constructs via an Smal restriction site. The resulting reporter constructs were introduced into the chromosome of A. tumefaciens, as described above.

Runoff plasmids for in vitro transcriptions were constructed by amplifying specific target gene sequences (150 nt) by PCR using the corresponding primer pairs (Table S2) and ligation into pUC19. A primer-derived T7 promoter sequence was added to the 5′ end, while an EcoRV restriction site was added to the 3′ end of the target gene sequence.

Site-directed mutagenesis of PmaR in pSRK or runoff plasmids was achieved using the corresponding primer pairs (Table S2).
Motility test. Determination of motility was performed by spotting 3 μl of liquid overnight cultures on soft agar plates containing M9 minimal medium (pH 5.5) with an agar concentration of 0.5% (wt/vol). Plates were incubated at 30°C for 48 h.

Potato disc infection assays. Quantitative virulence assays with potato tuber discs were carried out as described previously (53).

β-Galactosidase activity assay. *A. tumefaciens* strains harboring chromosomal 3×FLAG fusions to target genes were grown at 30°C in YEB medium to indicated growth phases. Cells (1 ml) were harvested by centrifugation and resuspended in 800 μl of 10× Z-buffer. Cells were permeabilized with chloroform and 0.01% SDS. Enzymatic reactions were started by adding 200 μl o-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml) and stopped by adding 500 μl Na₂CO₃, o-Nitrophenyl (ONP) production at 420 nm was measured.

Western blot analysis. Protein samples were separated via SDS-PAGE (12%) gels and subsequently transferred onto nitrocellulose membranes (Hybond-C Extra; GE Healthcare, Munich, Germany) by tank blotting. Anti-3×FLAG M2 antibody (Sigma-Aldrich, Germany) and secondary goat anti-mouse horse-radish peroxidase (HRP) conjugate (Bio-Rad, Munich, Germany) were used in a 1:5,000 dilution. Detection by luminescence was performed using Luminata Forte Western HRP substrate (Merck, Darmstadt, Germany) and the Chemi Imager Ready system (Alpha Innotec, San Leandro, CA, USA).

Identification of target mRNAs by mass spectrometry. Tryptic digestion, mass spectrometry, and data processing for proteomic profiling were essentially performed as described previously (54), with the following changes: mass range, m/z 50 to 1,200, and scan time, 1 s/scan. For data processing using Protein Lynx Global Server (version 2.5.2; Waters), a nonredundant version of the UniProtKB/Swiss-Prot database containing 5,558 protein entries (including sequences for rabbit PhosB quantitation standard [Waters], trypsin, and keratin) was used for protein identification. Proteins were considered up- or downregulated when they were identified (i) in all three biological replicates in the mutant samples but not in the wild-type samples or vice versa, or (ii) in at least two of three biological replicates with P values below 0.05 and with the following ratios exceeding a threshold of 0.653/1.540. Thresholds were calculated using a confidence interval of 95% (mean ratio ± 1.96 × standard deviation).

RNA preparation. *A. tumefaciens* cells (10 ml) were harvested for RNA preparation, as described previously (32). Isolation of total RNA was performed using the hot acid phenol method (35). Stability assays were performed by cultivation of the wild type and ΔPmaR mutant to exponential or stationary phase and subsequent addition of rifampin to a final concentration of 250 μg/ml. Samples for RNA preparation were taken before and 1, 2, 3, and 4 min after rifampin treatment.

Northern blot analysis. PmaR transcript levels were detected by Northern blot analysis, as described before (32). Hybridization with a digoxigenin-labeled RNA probe (Roche, Mannheim, Germany) was performed at 42°C overnight. Washing steps and detection by using chemiluminescence substrate CDP-Star (Roche) were carried out as described previously (32). Detection of target mRNAs was performed using the vacuum blot technique, as described before (56).

Enzymatic RNA structure probing. To elucidate the RNA structure of PmaR, transcripts were synthesized in vitro by runoff transcription from EcoR-V-linearized plasmids (listed in Table S1) with T7 RNA polymerase. The sRNA was purified, dephosphorylated with calf intestinal alkaline phosphatase (CIP; Thermo Scientific, Waltham, MA, USA), and radioactively labeled at the 5′ end, as described before (57). Partial digestions with ribonucleases T1 (0.02 U) (Thermo Scientific) and T2 (0.45 U) (MoBiTec, Göttingen, Germany) and nuclease S1 (1 U) (Thermo Scientific) were performed at 30°C, as described previously (58).

Electrophoretic mobility shift assays. RNA transcripts were synthesized in vitro by runoff transcription, as described above. RNA band shift experiments were performed in 1× structure buffer (Ambion, Austin, TX, USA) using 5,000 cpm-labeled sRNA and unlabeled murB and ampC mRNA fragments. Final concentrations of mRNA fragments are given in Fig. 8A to D. Samples were incubated in the presence of 1 μg trRNA at 30°C for 30 min. Binding reactions were stopped with 3 μl native loading dye (50% glycerol, 0.5× Tris-borate-EDTA [TBE], 0.1% bromophenol blue, and 0.1% xylene cyanol) and separated on native 6% polyacrylamide gels in 0.5× TBE at 300 V for 1 h.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02100-18.

**FIG S1, TIF file, 90.4 MB.**

**FIG S2, TIF file, 74.5 MB.**

**FIG S3, TIF file, 82.4 MB.**

**FIG S4, TIF file, 93.4 MB.**

**TABLE S1, DOCX file, 0.02 MB.**

**TABLE S2, DOCX file, 0.02 MB.**

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