



# The RNase YbeY Is Vital for Ribosome Maturation, Stress Resistance, and Virulence of the Natural Genetic Engineer *Agrobacterium tumefaciens*

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**ABSTRACT** Riboregulation involving regulatory RNAs, RNA chaperones, and ribonucleases is fundamental for the rapid adaptation of gene expression to changing environmental conditions. The gene coding for the RNase YbeY belongs to the minimal prokaryotic genome set and has a profound impact on physiology in a wide range of bacteria. Here, we show that the *Agrobacterium tumefaciens ybeY* gene is not essential. Deletion of the gene in the plant pathogen reduced growth, motility, and stress tolerance. Most interestingly, YbeY is crucial for *A. tumefaciens*-mediated T-DNA transfer and tumor formation. Comparative proteomics by using isobaric tags for relative and absolute quantitation (iTRAQ) revealed dysregulation of 59 proteins, many of which have previously been found to be dependent on the RNA chaperone Hfq. YbeY and Hfq have opposing effects on production of these proteins. Accumulation of a 16S rRNA precursor in the *ybeY* mutant suggests that *A. tumefaciens* YbeY is involved in rRNA processing. RNA coimmunoprecipitation-sequencing (RIP-Seq) showed binding of YbeY to the region immediately upstream of the 16S rRNA. Purified YbeY is an oligomer with RNase activity. It does not physically interact with Hfq and thus plays a partially overlapping but distinct role in the riboregulatory network of the plant pathogen.

**IMPORTANCE** Although *ybeY* gene belongs to the universal bacterial core genome, its biological function is incompletely understood. Here, we show that YbeY is critical for fitness and host-microbe interaction in the plant pathogen *Agrobacterium tumefaciens*. Consistent with the reported endoribonuclease activity of YbeY, *A. tumefaciens* YbeY acts as a RNase involved in maturation of 16S rRNA. This report adds a worldwide plant pathogen and natural genetic engineer of plants to the growing list of bacteria that require the conserved YbeY protein for host-microbe interaction.

**KEYWORDS** *Agrobacterium tumefaciens*, Hfq, RNase, YbeY, iTRAQ, rRNA, ribosome, virulence

The highly conserved *ybeY* gene belongs to the postulated minimal bacterial genome and encodes an RNase containing the UPF0054 protein motif (1, 2). The human protein C21orf57 is predicted to be a homologue of bacterial YbeY proteins, suggesting a wide phylogenetic distribution of this protein (3). The structure of YbeY partly resembles the MID domain of Argonaute proteins, which are essential for RNA-mediated regulation in eukaryotes (4–6). YbeY is an endoribonuclease involved in processing of rRNAs (especially 16S rRNA) in a broad range of bacteria, including *Escherichia coli* (7–9), *Vibrio cholerae* (10), *Thermus thermophilus* (11), and *Yersinia enterocolitica* (12), and also in the chloroplast (13). In concert with RNase R,

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YbeY is further involved in quality control of 70S ribosomes in *E. coli* (8) and, together with RNase E/G, it is involved in the maturation of the 4.5S RNA of the signal recognition particle in *Corynebacterium glutamicum* (14). Aside from these specific functions, YbeY plays a global role in gene regulation and influences expression of more than 1,000 genes in *E. coli* (9) and up to 100 genes in *T. thermophilus* (11), but it is presently unclear how.

Due to its role in RNA metabolism, deletion of *ybeY* usually impacts viability and susceptibility toward various stresses, e.g., heat stress in *E. coli* or certain antibiotics in *V. cholerae* (10, 15, 16). In some bacteria, for example *V. cholerae*, *ybeY* is an essential gene that can be deleted only in the presence of a plasmid-carried copy (10). In bacteria interacting with eukaryotes such as the plant symbiont *Sinorhizobium meliloti* or the human pathogens *V. cholerae*, *Y. enterocolitica*, and *Brucella abortus*, YbeY is critically important for successful host interaction (4, 10, 12, 17).

Another striking observation is that YbeY affects sRNA regulation and physiology in *S. meliloti* very much like the RNA chaperone Hfq (4). Hfq is a widespread RNA-binding protein (18). It forms a homohexamer exhibiting proximal, lateral, and distal RNA-binding surfaces (19–24). Hfq supports duplex formation of sRNAs and mRNAs by increasing their local concentration, mediating structural rearrangements, and annealing of both RNA molecules (25, 26). Hfq also influences the stability of interacting RNAs by inducing or inhibiting their degradation. Deletion of *hfq* usually results in a severe reduction of viability, including reduced growth, motility, and stress tolerance (27–34).

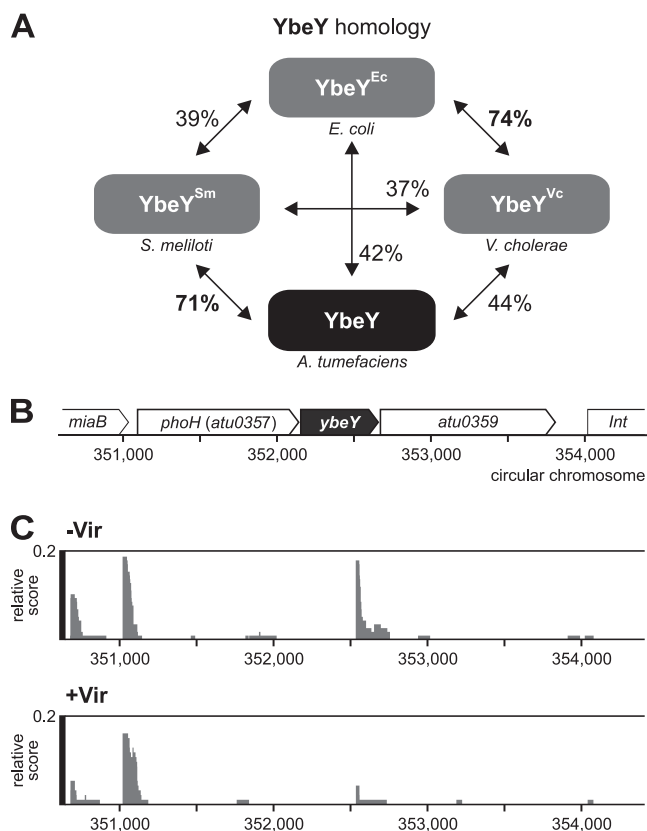
*Agrobacterium tumefaciens* is the causal agent of crown gall disease on mainly dicotyledonous plants. Its unique ability to transfer part of its own DNA (i.e., transfer DNA [T-DNA]) into eukaryotic hosts is responsible for *Agrobacterium* being the most valuable biotechnological tool for genetic manipulation of agronomically important plants like maize (35), soybean (36), and cotton (37) with an ever-increasing number of susceptible plants (38). The genome of the natural genetic engineer *A. tumefaciens* is divided into four replicons: a circular chromosome, a linear chromosome, the At plasmid, and the Ti (tumor-inducing) plasmid (39). Upon perception of signal molecules from plant wounds (e.g., phenolic compounds, sugars, and low pH), the VirAG two-component system initiates the infection process by activating virulence gene (*vir*) expression (40). The *vir* gene-encoded VirB/D4 type IV secretion system (T4SS) transfers a single-stranded portion of the Ti plasmid (T-DNA), along with several Vir proteins, into the plant cell. Integration of the T-DNA into the plant chromosome reprograms plant gene expression resulting in phytohormone and opine biosynthesis.

Recent RNA-sequencing studies in *A. tumefaciens* revealed more than 600 potential sRNAs, suggesting a large posttranscriptional network involved in the coordination of the unique physiology of this phytopathogen (41–44). Hfq plays a fundamental role in riboregulation in *A. tumefaciens* since it was shown by RNA coimmunoprecipitation-sequencing (RIP-Seq) analysis to bind more than 200 sRNAs and nearly one-third of all mRNAs (41). In an *hfq* mutant, the abundance of 136 proteins, many of them involved in ABC transport systems and motility, was altered in stationary phase. Finally, an *A. tumefaciens hfq* mutant exhibited severe defects in nutrient acquisition and motility and was inefficient in tumor formation (41, 45).

Here, we classify the *A. tumefaciens* YbeY ortholog Atu0358 as another chromosomally encoded virulence factor with a major impact on *Agrobacterium* physiology and tumor formation on plants. The absence of YbeY affects the biosynthesis of many Hfq-dependent proteins, albeit in an opposing direction. Cumulative evidence suggests that both proteins have different biochemical functionalities. While Hfq is an RNA chaperone, YbeY is a RNase involved in rRNA processing.

## RESULTS

**Atu0358 is the *A. tumefaciens* YbeY homologue.** The fundamental influence of riboregulation on the fitness and virulence of plant-associated alphaproteobacteria (46) motivated us to examine the impact of YbeY in the phytopathogen *A. tumefaciens*. The Atu0358 protein shares significant homology with other YbeY homologs (Fig. 1A). This

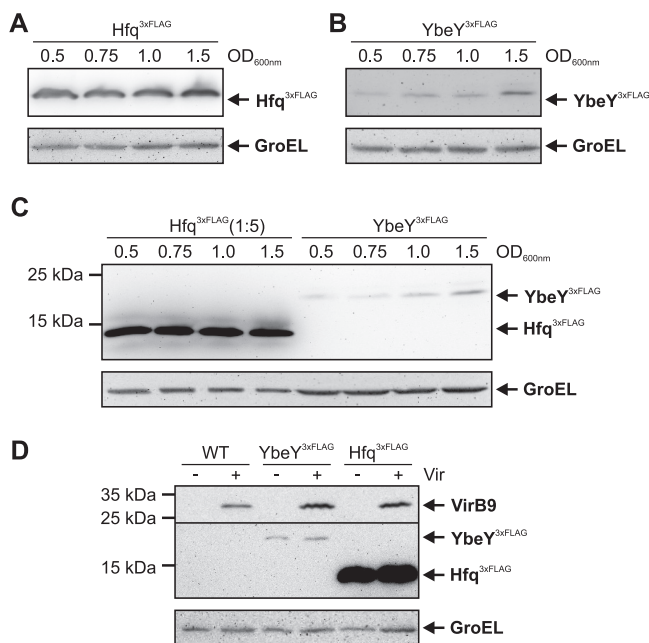


**FIG 1** Genetic context and transcription of the *A. tumefaciens ybeY*. (A) *Atu0358* shares high sequence identity with the YbeY orthologues from *S. meliloti*, *V. cholerae*, and *E. coli*. (B) The *ybeY* gene cluster in *A. tumefaciens* consists of the phosphate starvation-inducible protein gene *phoH (atu0357)*, *ybeY (atu0358)*, and the hemolysin gene *atu0359*. (C) Data from the differential RNA-Seq analysis by Wilms' et al. (42). The transcription start site of *ybeY* lies upstream of *phoH*. cDNA libraries were generated from cultures grown without (-Vir) and with (+Vir) virulence induction by acetosyringone. Screenshots of the results after treatment with terminator 5'-phosphate-dependent exonuclease (TEX) to enrich for primary 5' ends are shown.

assumption is further supported by the shared synteny of the *ybeY* locus in *A. tumefaciens*, *S. meliloti*, *B. abortus*, *V. cholerae*, and *E. coli*, where the *ybeY* gene is flanked upstream by the phosphate starvation-inducible gene *phoH (atu0357)* and downstream by a hemolysin-encoding gene (*atu0359*) (Fig. 1B). Transcriptome sequencing (RNA-Seq) data (42) show that *ybeY* is expressed under normal and virulence conditions and that it is part of a polycistronic operon with a transcription start site downstream of *atu0357* (Fig. 1C).

**Expression of *ybeY* is growth phase regulated, and YbeY is less abundant than Hfq.** To assess the cellular amounts of the YbeY protein, we fused the chromosomal *ybeY* gene to a 3×Flag sequence at the 3' end, resulting in a C-terminally tagged YbeY<sup>3×Flag</sup> protein. We compared the abundance of the YbeY<sup>3×Flag</sup> protein in complex medium from the exponential growth phase (optical density at 600 nm [OD<sub>600</sub>] = 0.5) to the stationary growth phase (OD<sub>600</sub> = 1.5) with that of a chromosomally encoded Hfq<sup>3×Flag</sup> protein, which as previously shown (41) was present in equal amounts throughout all growth phases (Fig. 2A). In contrast, YbeY<sup>3×Flag</sup> was least abundant in the exponential phase and gradually increased throughout growth reaching a maximum in the stationary phase (Fig. 2B). Detection of the Flag-tagged proteins on one gel revealed that Hfq exceeded YbeY amounts at least 10-fold even in the stationary phase (Fig. 2C).

Neither YbeY nor Hfq are virulence regulated because YbeY<sup>3×Flag</sup> and Hfq<sup>3×Flag</sup> amounts were unaffected in minimal medium under noninduced (-Vir) and virulence-



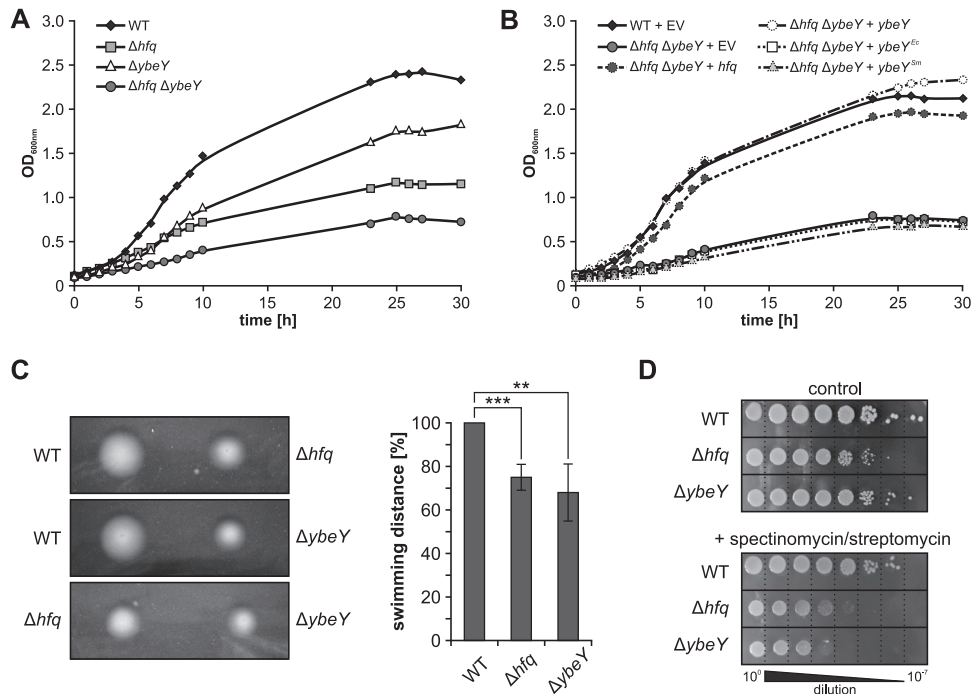
**FIG 2** Growth-phase-dependent production of YbeY<sup>3×Flag</sup>. (A and B) The *A. tumefaciens* YbeY<sup>3×Flag</sup> and Hfq<sup>3×Flag</sup> strains were cultivated in YEB medium and cells were harvested from the exponential (OD<sub>600</sub> = 0.5) to the stationary (OD<sub>600</sub> = 1.5) growth phases. Proteins were separated by SDS-PAGE and detected via a monoclonal anti-Flag M2 antibody after Western blotting. GroEL served as a loading control. (C) Direct comparison of Hfq<sup>3×Flag</sup> (1:5-diluted protein extracts) and YbeY<sup>3×Flag</sup> amounts on one Western blot. (D) WT, Hfq<sup>3×Flag</sup>, and YbeY<sup>3×Flag</sup> strains were cultivated under noninduced (–Vir) and virulence-induced (+Vir) conditions. Protein amounts were independent of the virulence inducer acetosyringone (AS). The membrane was cut (line), and the T4SS protein VirB9 was detected via a VirB9-specific antibody (upper part) to verify successful virulence induction.

induced (+Vir, in the presence of acetosyringone) (Fig. 2D). As in complex medium, Hfq<sup>3×Flag</sup> was much more abundant than YbeY<sup>3×Flag</sup>. Successful virulence induction was validated by immunodetection of the virulence-specific VirB9 protein, which was exclusively produced under +Vir conditions.

**Growth, motility, and stress resistance depend on YbeY.** The successful construction of a *ybeY* mutant ( $\Delta ybeY$ ) by deleting the *ybeY* open reading frame (ORF) from the chromosome demonstrated that the *A. tumefaciens* gene is not essential. We also obtained a  $\Delta hfq \Delta ybeY$  double mutant. However, this strain could not be propagated over longer time periods (see below). To determine the impact of YbeY on *A. tumefaciens* physiology, we analyzed the growth behavior of the wild type (WT) and  $\Delta hfq$ ,  $\Delta ybeY$ , and a  $\Delta hfq \Delta ybeY$  deletion strains in YEB complex medium (Fig. 3A) and observed the previously described severe growth defect of the *hfq* mutant (45). Deletion of *ybeY* alone resulted in a less pronounced growth defect and the  $\Delta hfq \Delta ybeY$  double mutant exhibited the most severe growth defect, which could be rescued by ectopically expressed *hfq* or *ybeY* from *A. tumefaciens* (Fig. 3B). In contrast, *ybeY* orthologues from *E. coli* (YbeY<sup>Ec</sup>) and *S. meliloti* (YbeY<sup>Sm</sup>), as well as the empty vector control (EV), were unable to complement the growth defect of the  $\Delta hfq \Delta ybeY$  strain. Successful expression of both *ybeY*<sup>Ec</sup> and *ybeY*<sup>Sm</sup> in the double mutant was confirmed by Northern blot analysis (Fig. S1).

Presumably due the severe growth defect, the double mutant could not be maintained and was therefore excluded from all subsequent experiments. Like the *hfq* mutant, the *ybeY* single mutant showed reduced motility (Fig. 3C). The swimming distance of both strains on low-concentration agar plates reached only ~70% of the WT radius.

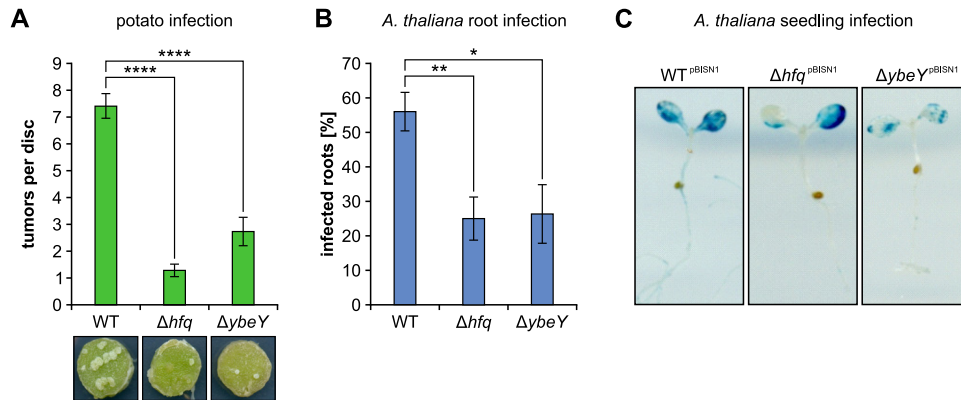
The stress sensitivity of the WT,  $\Delta hfq$  and  $\Delta ybeY$  strains was examined on agar plates in the presence of diverse stressors. Cultures were grown to exponential phase



**FIG 3** YbeY influences *A. tumefaciens* growth and motility. (A) Growth experiments with WT,  $\Delta hfq$ ,  $\Delta ybeY$ , and  $\Delta hfq \Delta ybeY$  strains revealed reduced growth of the *ybeY* mutant compared to the WT. Still, growth deficiency was less severe than in the *hfq* mutant. Deletion of both genes ( $\Delta hfq \Delta ybeY$ ) led to a severe growth defect. (B) Growth deficiencies of the  $\Delta hfq \Delta ybeY$  mutant were rescued by ectopic expression of *hfq* or *ybeY* from *A. tumefaciens*. Homologues from *E. coli* (*ybeY<sup>Ec</sup>*) or *S. meliloti* (*ybeY<sup>Sm</sup>*) did not complement the growth defect. (C) Swimming of *A. tumefaciens* WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains on low concentration agar. Swimming distance of  $\Delta hfq$  (\*\*\*,  $P = 0.001$ ) and  $\Delta ybeY$  (\*\*,  $P = 0.009$ ) strains was reduced to about 70% compared to WT. (D) Cultures of WT,  $\Delta hfq$  and  $\Delta ybeY$  strains were grown to exponential phase ( $OD_{600} = 0.5$ ), and serial dilutions were applied to LB agar plates. WT growth and  $\Delta ybeY$  strain growth were comparable, whereas the growth of the  $\Delta hfq$  strain was reduced  $\sim 10$ -fold compared to WT growth (upper panel). Addition of  $10 \mu\text{g/ml}$  spectinomycin plus  $3 \mu\text{g/ml}$  streptomycin had the strongest effect on the *ybeY* deletion strain but also inhibited growth of the *hfq* mutant (lower panel). EV, empty vector.

( $OD_{600} \sim 0.5$ ) and serially diluted ( $10^0$  to  $10^{-7}$ ) prior to spotting on the plates. The growth of all strains on Luria-Bertani (LB) agar (Fig. 3D, upper panel) was comparable to growth in liquid media, e.g., the growth of the *hfq* mutant was reduced more severely than the growth of the *ybeY* mutant. The commonly observed sensitivity of *ybeY* mutants toward heat stress was not observed in *A. tumefaciens* when incubated at  $42^\circ\text{C}$  (data not shown). A role of YbeY in translation was monitored on agar plates supplemented with the four ribosome-targeting antibiotics tetracycline (30S ribosome), gentamicin (30S), chloramphenicol (50S ribosome), and a combination of spectinomycin and streptomycin (30S). The addition of spectinomycin and streptomycin had the strongest effect on the *ybeY* mutant (Fig. 2C). The addition of tetracycline and chloramphenicol affected the *hfq* and *ybeY* mutants equally, whereas gentamicin impaired growth of only the  $\Delta hfq$  strain (see Fig. S2 in the supplemental material). The presence of the ribonucleotide reductase inhibitor hydroxyurea reduced growth of the *hfq* and *ybeY* mutants about 100- and 10-fold, respectively.

**YbeY, a virulence factor of *A. tumefaciens*.** Infection assays with potato tuber discs, *Arabidopsis thaliana* root segments, and *A. thaliana* seedlings were used to monitor tumor formation as well as T-DNA transfer of *A. tumefaciens*. WT,  $\Delta hfq$ , and  $\Delta ybeY$  cultures were diluted, and  $10^6$  bacteria were applied to 100 potato tuber discs each. After incubation for 4 to 6 weeks, the WT induced seven to eight tumors per disc, whereas the *hfq* or *ybeY* mutant strains produced about one or maximally three tumors, respectively (Fig. 4A). Similar results were obtained in the *A. thaliana* root infection assay (Fig. 4B). The WT elicited tumors on more than half of all root segments, whereas the  $\Delta hfq$  and  $\Delta ybeY$  strains infected only a quarter of the roots.

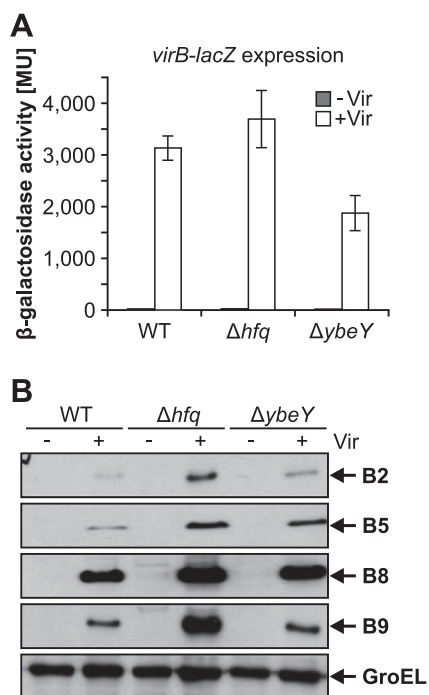


**FIG 4** YbeY is crucial for *A. tumefaciens* virulence. (A) Infection of potato tuber discs with  $10^6$  cells of WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains. After incubation for 6 weeks (16-h light/dark cycle, 23°C), tumors were counted per disc, and the average of 100 infected potato discs per strain was calculated. WT infection led to an average of seven to eight tumors per disc. The infection efficiency of  $\Delta hfq$  (one to two tumors/disc) and  $\Delta ybeY$  (two to three tumors/disc) strains was drastically reduced (\*\*\*\*,  $P < 0.0001$ ). (B) Similar results were obtained from *A. thaliana* root infection assays. About 25% of the 300 roots were infected by either the *hfq* (\*\*,  $P = 0.003$ ) or the *ybeY* (\*,  $P = 0.015$ ) mutant in contrast to the ~55% infection efficiency of *A. tumefaciens* WT. (C) Qualitative measurement of T-DNA transfer was performed using WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains harboring a plasmid-carried GUS-reporter gene system (pBISN1). Successful transfer of T-DNA (*gus*) resulted in  $\beta$ -glucuronidase expression in the infected plant tissue. Cleavage of the substrate X-Glc by the  $\beta$ -glucuronidase stained the corresponding plant tissue (blue). Infection with WT<sup>pBISN1</sup> led to extensive staining of the leaves, whereas seedling infection by *hfq*<sup>pBISN1</sup> or *ybeY*<sup>pBISN1</sup> mutants was less efficient.

To investigate T-DNA transfer efficiency and functionality of the T4SS, we infected *A. thaliana* seedlings with WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains by the AGROBEST method (47, 48). Successful T-DNA transfer resulted in blue staining of infected plant tissue due to the transient expression of the T-DNA encoded *gusA* gene. Sixty plant seedlings each were infected with the three *A. tumefaciens* strains carrying the plasmid required for this assay. Infection with WT<sup>pBISN1</sup> cells led to extensive staining of the leaves (Fig. 4C). Consistent with the reduced tumor formation, less plant tissue was stained upon infection with  $\Delta hfq$ <sup>pBISN1</sup> or  $\Delta ybeY$ <sup>pBISN1</sup> strains.

Finally, an ectopically expressed *virB-lacZ* fusion (49) was used to test for successful signal transduction and *vir* gene expression (Fig. 5A). Expression of *virB-lacZ* was induced by addition of 0.1 mM acetosyringone. Compared to the WT, *virB* expression in the  $\Delta hfq$  strain was slightly increased, whereas expression was decreased about one-third in the  $\Delta ybeY$  strain, indicating attenuated *vir* gene expression in the *ybeY* mutant. The amounts of VirB2, VirB5, VirB8, and VirB9 were not markedly reduced in the *ybeY* mutant and slightly increased in the *hfq* mutant (Fig. 5B), suggesting that the observed defects in tumor formation are not due to reduced production of the T4SS.

**Identification of YbeY-affected proteins by mass spectrometry.** The various phenotypes of the *ybeY* mutant suggest a broad impact of the YbeY protein on gene expression. To identify proteins that are affected by the presence or absence of YbeY, we compared the proteomes of *A. tumefaciens* WT and  $\Delta ybeY$  strains by using isobaric tags for relative and absolute quantitation (iTRAQ) labeling prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Total proteins were extracted from strains grown to the stationary phase ( $OD_{600} = 1.5$ ), and previously obtained protein samples of WT and  $\Delta hfq$  strains (41) were used as references for quantitative comparison of the WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains. In total, 2,544 proteins were uniquely identified by LC-MS/MS in three biological replicates, and the  $\Delta ybeY$ /WT ratios were used to calculate a confidence interval of 95% (Fig. 6A). Proteins with  $\Delta ybeY$ /WT ratios of  $>1.35$  (upper bound) were considered upregulated, and proteins with a ratio of  $<0.75$  (lower bound) were downregulated. Fifty-nine proteins were differentially expressed upon deletion of *ybeY*; 26 of these proteins were upregulated, and 33 were downregulated (see Table S1 in the supplemental material). Proteins were clustered according to KEGG ontology enrichment (Fig. 6B) as transporters ( $n = 12$ ), enzymes ( $n = 9$ ), transcription proteins ( $n = 8$ ), motility and chemotaxis proteins ( $n = 3$ ),



**FIG 5** YbeY affects *vir* gene expression and Vir protein amounts. (A) WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains harboring a plasmid-carried *virB-lacZ* fusion were cultured under virulence inducing (+Vir) and non-inducing conditions (–Vir). Under +Vir conditions, the  $\beta$ -galactosidase activity was reduced in the *ybeY* mutant, whereas deletion of *hfq* did not affect *virB-lacZ* expression significantly compared to WT activity. (B) Protein amounts of VirB2 (12.3 kDa), VirB5 (23.3 kDa), VirB8 (26.3 kDa), and VirB9 (32.2 kDa) under the conditions described for panel A were detected via Western blotting. Vir proteins were detectable only under +Vir conditions. Vir protein amounts were slightly increased in the  $\Delta hfq$  strain but did not show significant differences in the  $\Delta ybeY$  strain. GroEL (57.4 kDa) served as a loading control.

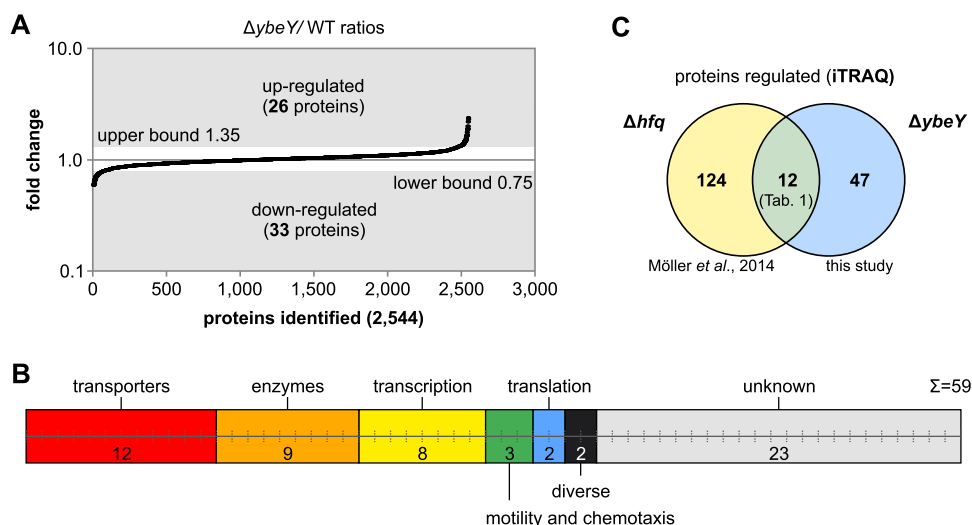
translation proteins ( $n = 2$ ), proteins with diverse cellular functions ( $n = 2$ ), and proteins with unknown functions ( $n = 23$ ).

In accordance with the overlapping phenotypes of  $\Delta hfq$  and  $\Delta ybeY$  strains, the influence of Hfq and YbeY on the *A. tumefaciens* proteome was partly overlapping (Fig. 6C). Twelve proteins, including nine ABC transporters, were influenced by the deletion of either *hfq* or *ybeY*. Interestingly, Hfq and YbeY had opposing effects on the coregulated proteins, e.g., proteins upregulated in  $\Delta hfq$  were downregulated in  $\Delta ybeY$  and vice versa (Table 1).

**YbeY is involved in 16S rRNA processing in *A. tumefaciens*.** To assay the *in vivo* function of YbeY in the processing of rRNAs, we isolated total RNA from the WT plus empty vector (v), the  $\Delta ybeY$  mutant plus empty vector (v), and a complemented  $\Delta ybeY$  mutant plus *ybeY* strain from the exponential and stationary growth phase and separated the RNA via agarose gel electrophoresis. An additional rRNA fragment in the  $\Delta ybeY$  strain migrated more slowly than the 16S rRNA and was designated 17S rRNA (Fig. 7A). The additional band disappeared when the mutant was complemented with plasmid-carried *ybeY*.

In our second global approach, we performed RIP-seq experiments with YbeY<sup>3×Flag</sup> analogous to the Hfq<sup>3×Flag</sup> RIP-Seq experiments performed previously (41). In contrast to Hfq<sup>3×Flag</sup>, YbeY<sup>3×Flag</sup> was unable to significantly enrich cellular RNAs (data not shown). However, the region immediately upstream of the 16S rRNA was enriched (Fig. 7B). When the 17S rRNA was isolated from an agarose gel and subjected to 5' rapid amplification of cDNA ends (RACE), the 5' end corresponded exactly to the region enriched by YbeY<sup>3×Flag</sup>, suggesting that YbeY interacts with the 173 nucleotides at the 5' end of 16S rRNA during ribosome biogenesis.

**Purification and coelution of YbeY and Hfq.** To characterize the YbeY protein, we constructed plasmids for heterologous production of C-terminally and N-terminally



**FIG 6** Global proteome analysis of *A. tumefaciens* WT and  $\Delta ybeY$  strains. Quantification of the WT and  $\Delta ybeY$  proteomes was performed using isobaric tags for relative and absolute quantitation (ITRAQ) and LC-MS/MS. (A) A total of 2,544 proteins were identified in three biological replicates. Based on a confidence interval of 95%, 26 proteins were upregulated ( $\Delta ybeY$ /WT ratio of  $>1.35$ ) and 33 proteins were downregulated ( $\Delta ybeY$ /WT ratio of  $<0.75$ ) in a *ybeY* deletion strain. (B) Proteins regulated by YbeY were clustered according to KEGG orthology enrichment into transporters, enzymes, transcription, motility and chemotaxis, translation, diverse cellular functions, and proteins with unknown function. (C) Overlap between Hfq- and YbeY-dependent proteins. Twelve YbeY-dependent proteins were previously identified to be influenced by Hfq (41).

tagged YbeY<sup>6×His</sup> in *E. coli* and purified the proteins via an Ni-nitrilotriacetic acid (Ni-NTA) column from total protein extracts. Subsequent to affinity purification, pooled elution fractions were subjected to gel permeation chromatography (GPC; YbeY<sup>6×His</sup> in Fig. 8A and <sup>6×His</sup>YbeY in Fig. S3B in the supplemental material). Comparison of the elution fraction with the elution of standard proteins (Fig. S3A) suggested an oligomeric organization of two to four YbeY proteins (20.6 kDa per monomer).

To provide evidence for RNase activity of YbeY *in vitro*, 50 to 250  $\mu$ M GPC-purified YbeY<sup>6×His</sup> was incubated with 5  $\mu$ g of total RNA from *A. tumefaciens* WT for 60 min at 37°C (Fig. 8B). As negative and positive controls for RNase activity, equal amounts of RNA were treated with buffer containing bovine serum albumin (BSA) or RNase A, respectively. The addition of increasing amounts of YbeY<sup>6×His</sup> (or <sup>6×His</sup>YbeY; data not shown) resulted in degradation of the RNA supporting an RNase activity of YbeY *in vitro*.

Since Hfq and YbeY influence similar phenotypes and affect the abundance of the same proteins, we sought to determine whether both proteins interact. Hfq and YbeY protein variants with either 6×His or 3×Flag tags at the C terminus were mixed and purified using Ni-NTA chromatography (Fig. 8C). As expected, the Flag-tagged proteins did not bind to Ni-NTA-Sepharose and eluted at low imidazole concentrations. Both His-tagged versions bound the Ni-NTA column and were eluted at an imidazole concentration of 250 mM. Importantly, the Flag-tagged proteins were not retained by the His-tagged proteins, suggesting that YbeY and Hfq do not interact.

## DISCUSSION

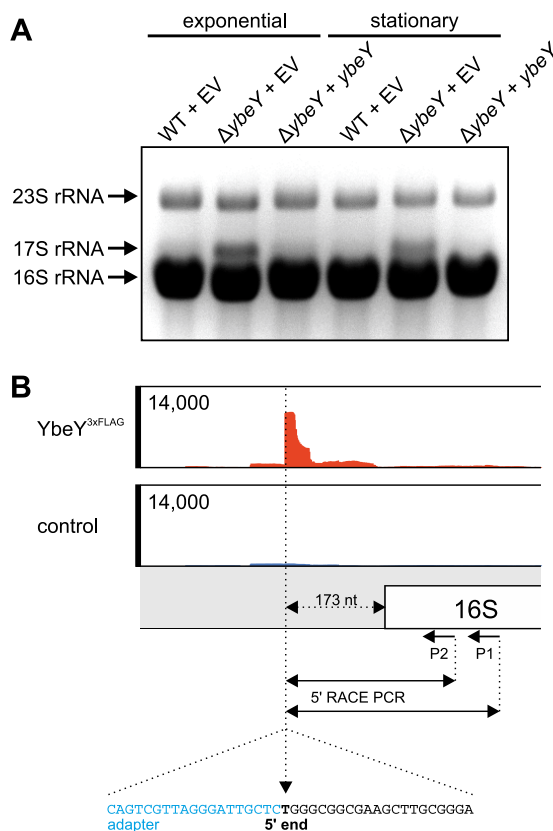
**The *A. tumefaciens ybeY* gene is differentially expressed, not essential, and a virulence factor critical for plant transformation.** As member of the prokaryotic core genome, the *ybeY* gene is essential in many bacteria, among them the human pathogen *V. cholerae* (10). The endoribonuclease activity in chloroplasts renders YbeY essential in the model plant *Arabidopsis thaliana*, further supporting an ancient function of the protein (13). In many bacteria, however, deletion of *ybeY* is tolerable, although associated with numerous physiological defects (see Table 2). This is also the case in the plant pathogen *A. tumefaciens*, where deletion of the gene caused pleiotropic pheno-



**TABLE 1** Proteins influenced by Hfq and YbeY

| GenBank accession no. | Annotation | Protein | Function   | Up- or downregulation <sup>a</sup> |                     |
|-----------------------|------------|---------|--|------------------------------------|---------------------|
|                       |            |         |  | $\Delta ybeY$ mutant               | $\Delta hfq$ mutant |
| AAK88993              | Atu4447    |         | Sorbitol ABC transporter substrate-binding protein                                 | Up (2.0)                           | Down (0.4)          |
| AAK90221              | Atu3165    |         | Sorbitol/mannitol ABC transporter substrate-binding protein                        | Up (1.5)                           | Down (0.4)          |
| AAK89922              | Atu3472    | BkdA2   | 2-Oxoisovalerate dehydrogenase subunit beta  | Up (1.4)                           | Down (0.3)          |
| AAK86355              | Atu0542    | Fla     | Flagellin  | Up (1.5)                           | Down (0.3)          |
| AAK88868              | Atu4577    |         | ABC transporter substrate binding protein  | Down (0.7)                         | Up (12.9)           |
| AAK90320              | Atu3063    |         | ABC transporter permease   | Down (0.6)                         | Up (11.9)           |
| AAK88755              | Atu4695    |         | Oligopeptide ABC transporter substrate-binding protein                             | Down (0.7)                         | Up (10.1)           |
| AAK88128              | Atu2391    |         | ABC transporter, substrate binding protein (nitrate/sulfonate/taurine/bicarbonate) | Down (0.7)                         | Up (9.8)            |
| AAK88731              | Atu4719    | DppA    | Dipeptide ABC transporter substrate-binding protein                                | Down (0.7)                         | Up (7.7)            |
| AAK90716              | Atu5343    |         | ABC transporter substrate binding protein (oligopeptide)                           | Down (0.7)                         | Up (6.4)            |
| AAK90608              | Atu5237    |         | ABC transporter substrate binding protein (amino acid)                             | Down (0.7)                         | Up (5.1)            |
| AAK89127              | Atu4312    | SoxA    | Sarcosine oxidase alpha subunit  | Down (0.7)                         | Up (3.0)            |

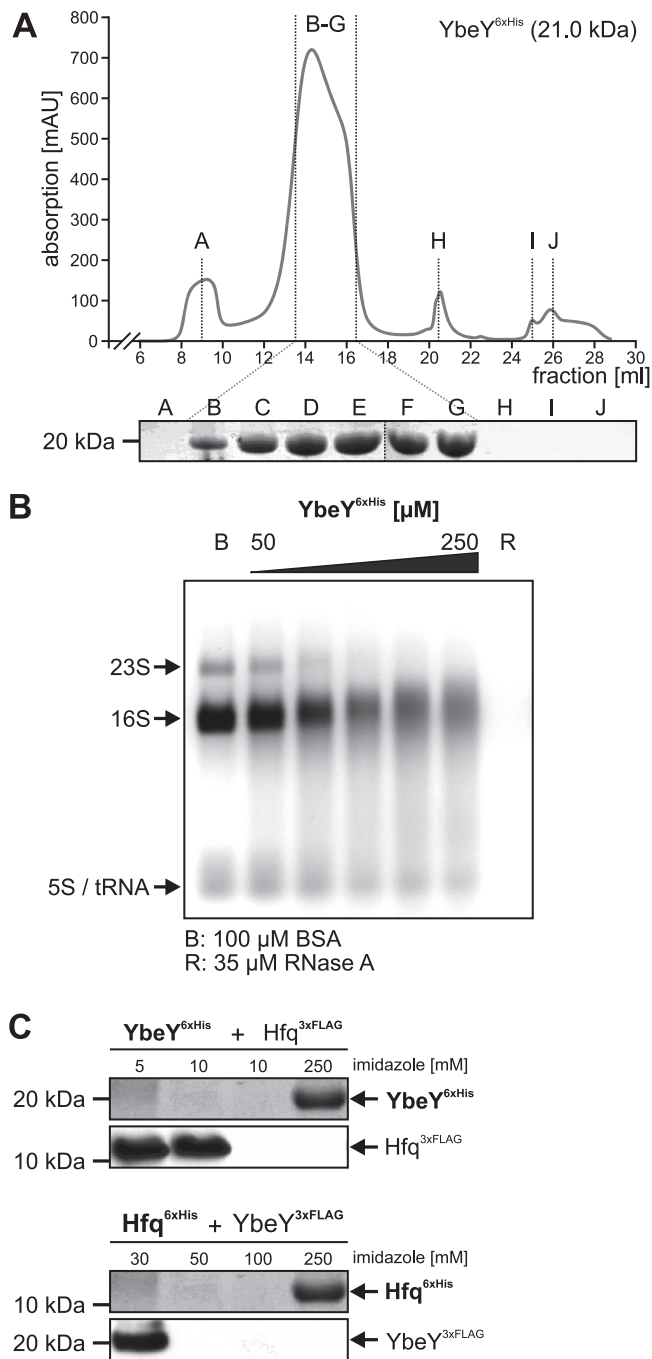
<sup>a</sup>The relative abundances of proteins in the  $\Delta ybeY$  or  $\Delta hfq$  mutants in comparison to the wild-type strain is given in parentheses. For a complete list, see Table S1 in the supplemental material. Source for  $\Delta hfq$  mutant data: Möller et al. (41).



**FIG 7** YbeY participates in rRNA processing. (A) Deletion of *ybeY* resulted in an additional rRNA fragment (17S rRNA). The fragment was most prominent in the exponential growth phase. Complementation (+*ybeY*) of the  $\Delta ybeY$  strain resulted in WT-like rRNA profile. (B) RIP-Seq experiments revealed binding of YbeY<sup>3xFLAG</sup> to the 5' upstream region of the 16S rRNA (upper panel). The 17S rRNA was isolated from an agarose gel, and the 5' end was identified via 5' RACE (lower panel). Sequencing of the RACE PCR fragment revealed extension of the 16S rRNA by 173 nucleotides in the *ybeY* mutant.

types. Similar to the closely related plant symbiont *S. meliloti* (4), most defects were reminiscent of the ones in an *hfq* mutant but generally less severe. The defects were additive in a combined *A. tumefaciens hfq ybeY* double mutant that was first difficult to obtain and second unable to survive prolonged cultivation, which suggests that at least one of these riboregulators is necessary for survival. Strikingly similar phenotypes in the individual mutants do not necessarily mean that Hfq and YbeY have redundant functions, as suggested previously (4). Although both proteins play a principle role in RNA-mediated coordination of cellular processes, they do so by different activities. Hfq rather acts as RNA chaperone facilitating RNA-RNA interactions whereas YbeY—at least predominantly—acts as catalytic enzyme involved in rRNA processing (see below). Due to their global impact on RNA functionality, it will be a challenge to separate direct from indirect effects of these proteins.

Quantitative proteomics further supported a functional relationship between YbeY and Hfq and revealed Hfq-dependent and -independent proteins affected by the presence of YbeY. The abundance of 59 proteins was dysregulated in the *ybeY* mutant. The nature of these proteins suggests a role of YbeY in general metabolism and nutrient uptake. It is remarkable that YbeY and Hfq had opposing effects on a number of proteins, in particular on ABC transporters (Table 1). A similar response was observed in *S. meliloti*, where 41 genes were upregulated in the *ybeY* mutant and downregulated in the *hfq* mutant (50). The mechanistic basis of this regulatory interplay is presently unclear. Our pulldown experiments did not provide any evidence for a stable, direct interaction between Hfq and YbeY, which is consistent with previous observations (4, 10, 51). Moreover, protein levels of YbeY were not influenced by deletion of *hfq* and



**FIG 8** Purification and biochemical analyses of *A. tumefaciens*. (A) GPC with YbeY<sup>6xHis</sup> (20.6 kDa, C-terminally tagged) resulted in a distinct protein peak with a maximum absorption of 719.5 mAU at 14.3 ml, indicating elution of 34- to 86-kDa large protein complexes, which corresponded to homooligomers of two to four YbeY<sup>6xHis</sup> monomers. Protein abundance in fractions B to G was verified by SDS-PAGE. (B) RNase activity of purified YbeY. Various amounts of N-terminally tagged YbeY<sup>6xHis</sup> protein (50 to 250 μM) were incubated with 5 μg total RNA from *A. tumefaciens* WT. Lane B, 100 μM BSA; lane R, 35 μM RNase A. (C) Pull-down experiments of YbeY<sup>6xHis</sup> and Hfq<sup>3xFlag</sup> protein variants from cell extracts after incubation for 30 min on ice. YbeY<sup>6xHis</sup> was bound by the Ni-NTA column, whereas Hfq<sup>3xFlag</sup> was washed off the column by low imidazole concentrations (5 to 10 mM). YbeY<sup>6xHis</sup> was eluted with 250 mM imidazole. Western blot analysis with specific His- or Flag-tagged antibodies did not show coelution of Hfq<sup>3xFlag</sup> protein with YbeY<sup>6xHis</sup>. Similar results were obtained when protein tags were switched between both proteins (YbeY<sup>3xFlag</sup> and Hfq<sup>6xHis</sup>).

**TABLE 2** Commonalities and differences between bacterial YbeY proteins<sup>a</sup>

| Feature                                      | Presence or absence of feature in: |                    |                    |                   |                       |
|--|------------------------------------|--------------------|--------------------|-------------------|-----------------------|
|  | <i>E. coli</i>                     | <i>V. cholerae</i> | <i>S. meliloti</i> | <i>B. abortus</i> | <i>A. tumefaciens</i> |
| Essential <i>ybeY</i> gene                   | No                                 | Yes                | No                 | No                | No                    |
| Pleiotropic phenotypes of <i>ybeY</i> mutant | Yes                                | Yes                | Yes                | Yes               | Yes                   |
| Virulence or symbiosis defect                | ND                                 | Yes                | Yes                | Yes               | Yes                   |
| Ribonuclease activity                        | Yes                                | Yes                | Yes                | Yes               | Yes                   |
| 16S rRNA maturation                          | Yes                                | Yes                | No                 | ND                | Yes                   |
| Oligomeric state of YbeY                     | ND                                 | ND                 | Monomer            | ND                | Oligomer              |

<sup>a</sup>For additional details, see Discussion. ND, not determined.

vice versa (data not shown), suggesting that both proteins act independently of each other.

In *E. coli*, *ybeY* is a heat shock gene and the *ybeY* mutant is temperature sensitive (15). Tracking *A. tumefaciens* YbeY levels throughout the growth curve showed that it was present under all conditions but accumulated toward stationary phase. This finding suggests a more important role of YbeY under starvation and stress conditions, which is fully reflected by the low cell densities of the mutant in stationary phase and its sensitivity to various noxious compounds.

YbeY is required for full virulence of *Y. enterocolitica*, *V. cholerae*, and *B. abortus* (10, 12, 17), which motivated us to assess virulence of the *ybeY* mutant in the tumor-inducing plant pathogen *A. tumefaciens*. Several plant infection assays looking at different stages of plant transformation placed YbeY among the virulence factors of *A. tumefaciens* because T-DNA transfer and concomitantly tumor formation were severely compromised in the *ybeY* mutant. This might be a cumulative effect of the phenotypic alterations in the *ybeY* mutant rather than an effect of YbeY on an individual virulence gene. Such a cumulative negative effect of the absence of *ybeY* on virulence was also postulated for the human pathogen *B. abortus* (17).

In enterohemorrhagic *E. coli* (EHEC), YbeY is required for normal type 3 secretion (T3SS) levels (52). This effect was not due to specific binding to or processing of the corresponding T3SS mRNA by YbeY. Instead, it was explained by an overall translation defect in the *ybeY* mutant caused by an accumulation of not fully matured ribosomes that leads to destabilization of untranslated mRNAs. In *A. tumefaciens*, the T4SS responsible for T-DNA transfer into the plant was present at normal levels in the *ybeY* mutant, and the signal transduction cascade responsible for plant transformation was operative. Expression of *virA* and *virG* coding for the virulence inducing two-component system was not affected (see Fig. S4 in the supplemental material), and the VirAG system was active as shown by almost normal *virB-lacZ* expression and WT-like VirB protein levels (Fig. 5). Although it is possible that barely noticeable changes in virulence protein levels elicit defects in T-DNA transfer, we favor the idea that translational defects and the dysregulated protein pool in the *ybeY* mutant are responsible for the virulence defect of the *A. tumefaciens ybeY* mutant. Among the suspicious proteins that might at least in part contribute to this defect are catalase CatE (underrepresented in the *ybeY* mutant) and the small heat shock protein HspL (overrepresented). CatE and the bifunctional peroxidase-catalase KatA protect *A. tumefaciens* from the plant defense molecule H<sub>2</sub>O<sub>2</sub> during infection. Deletion of the *kata* gene results in avirulent bacteria (53). Since CatE supports KatA activity, downregulation of CatE might increase sensitivity toward H<sub>2</sub>O<sub>2</sub>. The small heat shock protein HspL is one of four small heat shock proteins encoded in *A. tumefaciens* and is a VirB8-specific chaperone promoting functionality of the T4SS (54, 55). VirB8, together with VirB7, VirB9, and VirB10, is located in the core channel across the double membrane and HspL prevents its aggregation (56, 57). Misregulated HspL levels might influence function of VirB8, thus hindering efficient T-DNA export via the T4SS. The imbalance in several nutrient acquisition systems in the *ybeY* mutant might be yet another reason for the attenuation in plant infection.

**Different functionalities of YbeY in different organisms?** Although there is no doubt that YbeY plays a crucial role in bacterial physiology, the reported details on YbeY properties and activities are still controversial. As an isolated protein, YbeY is a nonspecific RNase. We and others showed that purified YbeY degrades bulk RNA (8, 13, 50). It is evident that such an activity must be counteracted *in vivo*, which led to the proposal that cellular factors restrict this indiscriminate activity and coordinate the molecular activity of YbeY. This assumption is supported by the observation that expression of the human *ybeY* homologue *C21orf57* (*huYBEY*) results in uncontrolled activity and is deleterious in the yeast *Saccharomyces cerevisiae*, which does not have a YbeY-related protein itself (3). A recent screen for YbeY interactors in *E. coli* revealed the association with ribosomal protein S11 and the two GTPases Era and Der involved in ribosome biogenesis (51). Elevated levels of Era partially suppressed the growth defect of the *ybeY* mutant and improved 16S rRNA maturation (58), suggesting that this protein serves as “specificity factor” for YbeY.

One of the functions ascribed to *E. coli* YbeY is an endoribonuclease activity responsible for processing of the 3' terminus of the 16S rRNA (8), a critical step in ribosome maturation that might explain why *ybeY* belongs to the prokaryotic core genome. A similar 3' processing activity was recently reported for YqfG, the essential YbeY orthologue in *Bacillus subtilis* (59). Chloroplast with reduced YbeY levels displayed maturation defects in both the 5' and 3' ends of rRNAs, which resulted in reduced plastid translational activity (13). Structural and biochemical data conclusively support a metal-dependent RNase activity of YbeY (2, 8, 50), but the target specificities vary. *E. coli* YbeY was shown to be a single-strand-specific endoribonuclease (8), whereas the *S. meliloti* enzyme cleaved single- and double-stranded RNAs (50), an unprecedented activity among bacterial endoribonucleases.

Maturation of rRNAs is a multifactorial process involving various RNases required for trimming of the different intermediates derived from the long precursor transcript. Typically, several RNases, which can have redundant functions, cooperate in these reactions (60). For instance, RNase G in combination with RNase E cleaves the 5' end of the 16S rRNA precursor in *E. coli* (61). On the other end of the transcript, YbeY and RNase R function together in 16S rRNA 3' maturation (8). Despite the functional relationship between YbeY and RNase R, a direct interaction between the proteins was not detected, suggesting that the association with other proteins guides the processing events (51). The fact that the repertoire of cellular endo- and exoribonucleases involved in the rRNA processing varies substantially between bacteria might explain discrepancies in our current understanding of the YbeY activities in different organisms. *Agrobacterium*, for example, does not encode RNase G, and it is conceivable that some other RNase, which might be YbeY, fills this gap.

Surprisingly, even in closely related bacteria, the YbeY-associated activities can differ, as observed in two alphaproteobacteria associated with plants (Table 2). While YbeY is not required for 16S rRNA maturation in *S. meliloti* (50, 62), we found clear evidence for a 16S rRNA processing defect in the *A. tumefaciens ybeY* mutant (Fig. 7). Moreover, there was no indication that YbeY binds strongly to mRNAs or sRNAs in *A. tumefaciens*, again in contrast to reports in *S. meliloti* (4, 50). Instead, our RIP-Seq experiments revealed binding of YbeY to the immature 5' end of 16S rRNA precursor. This is reminiscent of findings in EHEC, although different positions within the 16S rRNA were occupied (52). Another unsettled issue pertains to the oligomeric state of YbeY. While the *Agrobacterium* protein assembled into an oligomeric state after affinity chromatography and gel filtration, the *Sinorhizobium* protein behaved as a monomer in cross-linking experiments (50). Finally, distinct features of YbeY proteins from different organisms are supported by complementation experiments. Expression of the *E. coli* homologue complemented the symbiosis defect of the *ybeY* mutant in *S. meliloti* (7, 63), whereas the phenotypic defects of the *A. tumefaciens* mutant could not be rescued by *E. coli* or *S. meliloti ybeY*. It is presently unclear how these controversial findings can be

reconciled, but it is evident that YbeY as a member of a limited set of universal bacterial proteins is an important protein worthy of further comparative investigations.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** All bacterial strains and plasmids used in this study are listed in Table S2 in the supplemental material. *Agrobacterium tumefaciens* C58 strains were cultivated at 30°C in YEB complex medium or AB minimal medium (pH 5.5, supplemented with 1% [wt/vol] glucose) (64). *Escherichia coli* BL21 strains were cultivated in LB medium at 37°C (65). *A. tumefaciens* virulence was induced in AB medium inoculated to an OD<sub>600</sub> of 0.1 and grown for 6 h at 30°C. Virulence gene expression was induced by addition of 0.1 mM acetosyringone (Sigma-Aldrich, Germany) and further cultivation (23°C, 16 h). Non-virulence-induced cultures were treated with equal volumes of dimethyl sulfoxide (DMSO). For cultivation during mutagenesis, *A. tumefaciens* cells were grown in LB medium (65) supplemented with either 10% (wt/vol) sucrose or 50 µg/ml kanamycin (Km).

**Deletion of chromosomal ybeY.** Deletion of *ybeY* (*atu0358*) was performed by homologous recombination analogous to the deletion of *hfq* described by (45). A 300-bp up- and downstream region of the *ybeY* ORF was amplified using the primers *ybeY*\_up\_PstI\_fw, *ybeY*\_up\_SmaI\_rv, *ybeY*\_down\_SmaI\_fw, and *ybeY*\_down\_EcoRI\_rv listed in Table S3 and ligated into a pK19*mobsacB* suicide plasmid. The resulting *ybeY*\_up\_dwn was transformed into *Agrobacterium* via electroporation. Single-crossover events were selected on LB agar plates containing 50 µg/ml Km. Km-tolerant colonies were further inoculated overnight in LB medium and subsequently selected for Km sensitivity and tolerance of 10% (wt/vol) sucrose. Chromosomal deletion of *ybeY* was confirmed by colony PCR and Southern blot analyses.

**Chromosomal integration of ybeY<sup>3×Flag</sup>.** Chromosomal integration of 3×Flag tag sequence at the *ybeY* 3' end was performed analogous to the construction of *hfq*<sup>3×Flag</sup> described previously (41). The *ybeY* upstream region, including the *ybeY* ORF without the TAA stop codon was amplified using primers *ybeY*\_up\_PstI\_fw, *ybeY*\_up\_SalI\_rv. The downstream region was amplified using primers *ybeY*\_down\_Acc65I\_fw and *ybeY*\_down\_EcoRI\_rv. Both fragments were cloned into a pK19*mobsacB* suicide plasmid. The 3×Flag tag sequence was amplified from an *A. tumefaciens* *hfq*<sup>3×Flag</sup> strain using the primers 3×Flag\_SalI\_fw and 3×Flag\_Acc65I\_rv. The fragment was inserted between *ybeY* up- and downstream fragments via SalI and Acc65I restriction sites. The resulting *ybeY*\_up\_3×Flag\_down plasmid was transformed into *A. tumefaciens* via electroporation. Integration of the *ybeY*<sup>3×Flag</sup> into the chromosome was performed via homologous recombination equally to *ybeY* mutagenesis described before.

Successful integration of the *ybeY*<sup>3×Flag</sup> into the *A. tumefaciens* chromosome was confirmed by colony PCR and Southern blot analyses.

**Arabidopsis thaliana root infection.** Infection of *A. thaliana* Col-0 roots was performed as described previously (66). Roots of 2- to 3-week-old *A. thaliana* plants were cut into 0.5-cm segments and inoculated with *A. tumefaciens* strains for 30 min. Infected root bundles were inoculated for 2 days on MS agar plates at 23°C (16 h light/8 h dark). Root bundles were washed in H<sub>2</sub>O plus 100 µg/ml ticarcillin and root segments (300 roots per strain) were separated on MS agar plates plus 100 µg/ml ticarcillin. After incubation for 2 to 3 weeks at 23°C (16 h light/8 h dark), infected roots were counted for tumor formation.

**A. thaliana seedling infection.** To assess *A. tumefaciens* T-DNA transfer capability, we performed *A. thaliana* seedling infection assays based on the AGROBEST (*Agrobacterium*-mediated enhanced seedling transformation) method described previously (48), with minor modifications. The pBISN1 plasmid harboring *gusA* was transformed into *A. tumefaciens* WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains via electroporation (47). *A. thaliana efr-1* seeds (elongation factor Tu [EF-Tu] receptor mutant) were washed with 96% ethanol (30 s), 6% (vol/vol) NaOCl, and water (five times) and then transferred to 6-well plates with 1 ml of 1/2 MS liquid medium (1/2 MS salt, 0.5% [wt/vol] sucrose; pH 5.5). Three-day-old *A. thaliana efr-1* seedlings were inoculated with 1 ml of culture grown under virulence-induced conditions (AB plus 0.1% glucose [wt/vol] plus 0.1 mM acetosyringone), followed by incubation at 23°C (16 h light/8 h dark) for 3 to 4 days. Seedlings were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) at 37°C for 3 h. Successful T-DNA transfer resulted in blue staining of the corresponding plant tissue.

**Potato infection.** Infection of potato tuber discs was performed as described earlier (54). Bacterial cells were cultivated to late exponential phase (OD<sub>600</sub> = 0.7 to 1.0), harvested, and washed in phosphate-buffered saline (PBS) buffer. The bacterial cultures were diluted in PBS buffer to a final OD<sub>600</sub> of 10<sup>8</sup> and 10<sup>6</sup> cells/ml. Prepared potato discs (50 per strain and dilution) were placed on water agar and inoculated with 10 µl of the bacterial solution prior to incubation for 2 days (23°C, 16 h light/8 h dark). After 2 days, potato discs were placed on water agar plates supplemented with 100 µg/ml ticarcillin to kill the bacteria. After ongoing incubation of the potato discs for 3 weeks, the tumors were scored.

**$\beta$ -Galactosidase assay.** *A. tumefaciens* cells harboring the pAC01\_virB-lacZ (*virB* transcriptional *lacZ* fusion) plasmid (67) were cultivated under noninduced (+DMSO) and virulence-induced (+0.1 mM acetosyringone) conditions in AB minimal medium. The activity of the  $\beta$ -galactosidase was measured according to standard protocols (68).

**Motility assay.** Swimming was assessed by inoculation of 5 µl of bacterial solution (OD<sub>600</sub> = 0.5) on AB minimal medium agar (+0.1% [wt/vol] glucose) plates containing 0.5% (wt/vol) agar (Difco). Cells were incubated for 2 to 3 days at 30°C in the dark. Swimming rings were measured (diameter), and the distance was calculated relative to WT swimming.

**Stress sensitivity assays.** *A. tumefaciens* WT,  $\Delta hfq$ , and  $\Delta ybeY$  strains were grown to an OD<sub>600</sub> of 0.5, and cells were harvested at a final OD<sub>600</sub> of 1.0 in distilled water. Serial 10-fold dilutions (10<sup>0</sup> to 10<sup>-7</sup>) were prepared, and 3 µl was spotted onto LB/AB agar plates supplemented with 10 µg/ml chloram-

phenicol, 4  $\mu\text{g}/\text{ml}$  gentamicin, 10  $\mu\text{g}/\text{ml}$  spectinomycin plus 3  $\mu\text{g}/\text{ml}$  streptomycin, 1  $\mu\text{g}/\text{ml}$  tetracycline, or 20 mM hydroxyurea prior to incubation for 2 to 3 days at 30°C.

**Isolation of total RNA and Northern blot analysis.** Total RNA was isolated as described previously (45). Northern blot analysis was performed as described previously (69). For transcript analysis, 8  $\mu\text{g}$  of total RNA was separated using agarose gels, blotted onto Hybond-N membranes (GE Healthcare), and hybridized with digoxigenin-labeled (Roche Applied Science, Germany) RNA probes. The oligonucleotides used for probe synthesis via *in vitro* transcription are listed in Table S2 in the supplemental material. Signals were detected using the Hyperfilm ECL (GE Healthcare) system.

**SDS-PAGE and Western blot analysis.** For protein analysis, 1 ml of bacterial solution was harvested and resuspended in 1 $\times$  SDS loading dye as described by Klüsener et al. (67). Proteins were incubated for 10 min at 95°C and separated using a 12.5% sodium dodecyl sulfate polyacrylamide gel and subsequent electrophoresis. Proteins were either stained by Coomassie blue or subjected to Western blot transfer. Identification of proteins was performed using either 1:2,000 His-conjugated (His-tagged proteins; Qiagen), monoclonal 1:3,000 VirB9, or monoclonal Flag 1:3,000 M2 (Sigma-Aldrich) antibodies. Fluorescence was detected via a Hyperfilm ECL (GE Healthcare) system.

**Proteome analysis by iTRAQ.** Cultivation of *A. tumefaciens* Wt and  $\Delta ybeY$  strains, protein isolation, trypsin digestion, labeling with iTRAQ reagents, subsequent LC-MS/MS and data analysis were performed as described (41). Protein abundances significantly altered in the *ybeY* mutant compared to the WT were selected as previously described (70) with minor modifications. A confidence interval of 95% (Z score, 1.96) was used to determine protein ratios with a distribution outside the main distribution. The confidence interval for downregulated proteins was 0.0093 (mean ratio) – 1.96  $\times$  1.4289 (standard deviation [SD]) and corresponded to a protein ratio of 0.76. Proteins upregulated were similarly defined (mean ratio + 1.96  $\times$  the SD) and corresponded to a protein ratio of 1.34. Proteins were considered upregulated with  $\Delta ybeY/\text{WT}$  ratios of  $>1.35$  and downregulated with ratios of  $<0.75$ . Protein abundances were considered significantly altered when they reached the cutoff criteria and a variance between the three biological replicates of  $<40\%$ . For protein ratios reaching the cutoff but failing the variance criterion, a combined ratio was calculated (combined ratio = ratio  $\pm$  ratio  $\times$  variance) as described earlier (41). Considering combined ratios includes proteins that explicitly reach the cutoff criteria for up- or downregulation.

**Protein purification and GPC.** Hfq<sup>6 $\times$ His</sup> and YbeY<sup>6 $\times$ His</sup> fusion proteins were expressed in *E. coli* BL21(DE3) cells, using pET-plasmid-based expression from a T7 promoter (Novagen, Madison, WI). The proteins were purified as described previously (71). To assess the oligomeric state, we performed gel permeation chromatography. After Ni-NTA chromatography, purified His-tagged fusion proteins were separated by size via a Superdex 200 10/300 GL column (HEPES buffer [pH 7.5]) on an ÄKTA system (GE Healthcare). Additional standard proteins (Blue-Dextran [2,000 kDa], apoferritin [443 kDa],  $\beta$ -amylase [200 kDa], and conalbumin [67 kDa; Sigma-Aldrich]) were loaded to calculate a standard curve and estimate the molecular weights of the YbeY<sup>6 $\times$ His</sup> proteins.

**In vitro RNA degradation.** For RNA degradation experiments, 5  $\mu\text{g}$  of total RNA was incubated with 50 to 250  $\mu\text{M}$  protein in 20- $\mu\text{l}$  reaction mixtures for 60 min at 37°C prior to separation on a 1.5% morpholinepropanesulfonic acid-agarose gel. In parallel, 100  $\mu\text{M}$  BSA was incubated under the same conditions with 5  $\mu\text{g}$  of RNA to determine the interaction of RNA with unspecific proteins. RNase A (35  $\mu\text{M}$ ) was added as a positive control for RNase activity. After separation the RNA was stained with EtBr and visualized under UV light.

**5' RACE.** For the RACE analyses, total RNA from the *ybeY* mutant grown to the stationary phase was isolated and separated via agarose gel electrophoresis. The 17S rRNA fragment was eluted from the gel using the NucleoSpin gel and a PCR Clean-Up kit (Macherey-Nagel). RACE reactions to determine the 16S\* rRNA 5' end were performed as described previously (72) with minor modifications (73). The adapter oligonucleotides and gene-specific primers used are listed in Table S2 in the supplemental material.

**YbeY<sup>3 $\times$ Flag</sup> RIP-Seq of 16S rRNA.** RNA-Seq of YbeY<sup>3 $\times$ Flag</sup>-bound 16S rRNA was performed analogous to the Hfq<sup>3 $\times$ Flag</sup> RIP-Seq published previously (41) and based on the procedure described earlier (74, 75). The YbeY<sup>3 $\times$ Flag</sup> strain was cultivated in AB medium to stationary phase (OD<sub>600</sub> = 0.8). YbeY<sup>3 $\times$ Flag</sup> proteins were bound to monoclonal anti-3 $\times$ Flag M2 antibody (Sigma-Aldrich) coupled to protein G Dynabeads (Thermo Fisher Scientific) and specifically enriched from cell lysates. RNA bound by YbeY<sup>3 $\times$ Flag</sup> was isolated by using phenol-chloroform-isoamyl alcohol and precipitated by ethanol and sodium acetate. Remaining DNA was digested by DNase I (Thermo Fisher Scientific) prior to the final precipitation. cDNA libraries were prepared at Vertis Biotechnology AG (Germany) as described previously (41) and sequenced using a HiSeq 2500 (Illumina) machine in single-read mode, running 100 cycles.

Final reads (FASTQ) were trimmed by the fast\_quality\_trimmer program from FASTX toolkit (v0.0.13; [http://hannonlab.cshl.edu/fastx\\_toolkit](http://hannonlab.cshl.edu/fastx_toolkit)) and further processed using the READemption tool (v0.2.6) (76), as described previously (41).

The normalized reads (based on the total number of reads aligned from the respective library) from the 16S rRNA gene were enriched 9.5-fold (reads per million, 9,734; raw read count, 11,562) in the the YbeY<sup>3 $\times$ Flag</sup> sample compared to YbeY<sup>WT</sup>. Enrichment was visualized using the Integrated Genome Browser (77).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00730-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 2.3 MB.

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