Mutations in the *β*-Subunit of the RNA Polymerase Impair the Surface-Associated Motility and Virulence of *Acinetobacter baumannii*

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ABSTRACT *Acinetobacter baumannii* is a major cause of antibiotic-resistant nosocomial infections worldwide. In this study, several rifampin-resistant spontaneous mutants obtained from the *A. baumannii* ATCC 17978 strain that differed in their point mutations in the *rpoB* gene, encoding the *β*-subunit of the RNA polymerase, were isolated. All the mutants harboring amino acid substitutions in position 522 or 540 of the RpoB protein were impaired in surface-associated motility and had attenuated virulence in the fertility model of *Caenorhabditis elegans*. The transcriptional profile of these mutants included six downregulated genes encoding proteins homologous to transporters and metabolic enzymes widespread among *A. baumannii* clinical isolates. The construction of knockout mutants in each of the six downregulated genes revealed a significant reduction in the surface-associated motility and virulence of four of them in the *A. baumannii* ATCC 17978 strain, as well as in the virulent clinical isolate MAR002. Taken together, our results provide strong evidence of the connection between motility and virulence in this multiresistant nosocomial pathogen.

KEYWORDS *Acinetobacter baumannii*, surface-associated motility, *rpoB*, rifampin resistance

*A*cinetobacter baumannii is a Gram-negative bacterium often responsible for nosocomial infections, based on its capacity to acquire and develop antimicrobial resistance (1). It is one of the most frequently encountered pathogens in intensive care, neonatal, and burn units, where it causes pneumonia and infections involving the central nervous system, skin, soft tissues, and bone (1). However, despite the increasing clinical importance of *A. baumannii*, little is known about the virulence factors that contribute to its pathogenetic properties.

In previous studies of DNA damage-mediated mutagenesis in *A. baumannii* (2–5), we observed an atypical motility pattern in several spontaneous rifampin-resistant (Rif[r]) mutants derived from the wild-type (WT) strain ATCC 17978 growing on semisolid medium. Although *Acinetobacter* spp. lack flagella, they exhibit surface-associated motility (6, 7). In a recent study, hypermotile *A. baumannii* derivatives with increasing virulence were isolated, but whether a lack of motility negatively affects the virulence and pathogenesis of *A. baumannii* is unclear (8, 9).

The point mutations conferring rifampin resistance are located in the *rpoB* gene (encoding the *β*-subunit of the RNA polymerase), and they produce substantial changes in the transcriptional profile of bacterial cells by affecting several promoters (3, 10, 11). Nonetheless, not all mutations conferring rifampin resistance give rise to global modifications in the transcriptional profile of the respective bacterium (12, 13). In this

A

B

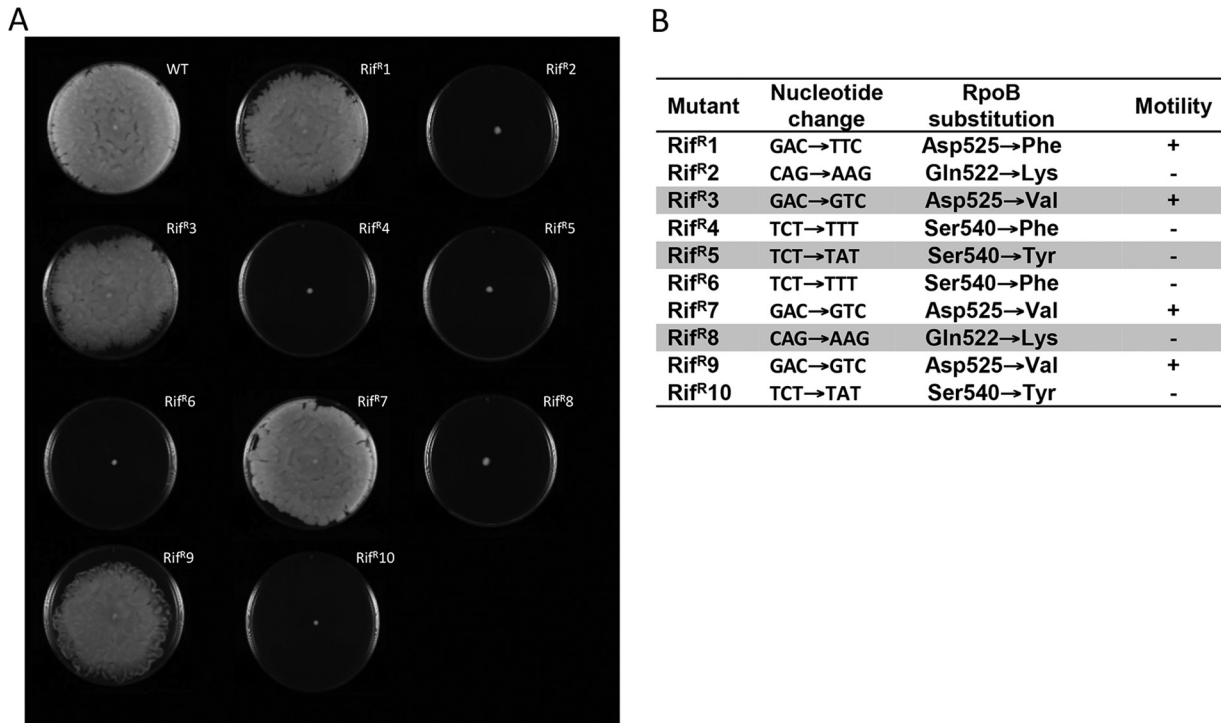| Mutant	Nucleotide change	RpoB substitution	Motility
Rif^{R}1	GAC→TTC	Asp525→Phe	+
Rif^{R}2	CAG→AAG	Gln522→Lys	-
Rif^{R}3	GAC→GTC	Asp525→Val	+
Rif^{R}4	TCT→TTT	Ser540→Phe	-
Rif^{R}5	TCT→TAT	Ser540→Tyr	-
Rif^{R}6	TCT→TTT	Ser540→Phe	-
Rif^{R}7	GAC→GTC	Asp525→Val	+
Rif^{R}8	CAG→AAG	Gln522→Lys	-
Rif^{R}9	GAC→GTC	Asp525→Val	+
Rif^{R}10	TCT→TAT	Ser540→Tyr	-

FIG 1 (A) Surface-associated motility assays of *A. baumannii* ATCC 17978 (WT) and 10 rifampin-resistant mutant derivatives (Rif^{r}1 to Rif^{r}10). (B) RpoB amino acid substitutions in the rifampin-resistant mutant strains and effects on motility (+, presence of motility; −, absence of motility). Mutants used in subsequent experiments are shaded.

study, *A. baumannii* rifampin-resistant mutants with altered surface-associated motility were isolated. Using DNA microarray technology, several genes involved in the motility and virulence of the bacterial species were identified.

RESULTS

Motility and virulence of *A. baumannii* rpoB mutants. By plating saturated cultures of *A. baumannii* ATCC 17978 in the presence of rifampin (50 mg/liter), we isolated 10 spontaneous mutants (Rif^{r}1 to Rif^{r}10) resistant to the antimicrobial. In subsequent motility assays, impaired surface-associated motility was confirmed in 60% of the isolated rifampin-resistant clones (Fig. 1A). Sequencing data for the *rpoB* gene revealed that rifampin-resistant clones with altered motility patterns had an amino acid substitution at either Gln522 or Ser540 of the wild-type RpoB protein (Fig. 1B). Moreover, wild-type motility was detected in rifampin-resistant mutants harboring an amino acid change at position Asp525 of RpoB (Fig. 1B). In several additional experiments, independent *rpoB* mutants that contained an amino acid substitution at either Gln522 or Ser540 were isolated; all of the mutants were similarly impaired in their surface-associated motility (data not shown).

To analyze whether motility plays a role in the pathogenesis of *A. baumannii*, the virulence of three different rifampin-resistant mutants, two with altered motility (Rif^{r}5 and Rif^{r}8) and the third with wild-type motility (Rif^{r}3), was determined in the *Caenorhabditis elegans* fertility model, recently validated for studies of the virulence of this bacterial species (14). The results showed that the virulence of the Rif^{r}5 and Rif^{r}8 mutant strains (both with impaired motility), but not of the Rif^{r}3 mutant (wild-type motility), was significantly lower than that of the wild-type strain (Fig. 2A). Since the growth kinetics of the Rif^{r}5 (growth rate [μ] = 0.86 ± 0.03 h^{-1}) and Rif^{r}8 (μ = 0.86 ± 0.02 h^{-1}) mutants in liquid medium were comparable to those of the wild-type strain (μ = 0.83 ± 0.05 h^{-1}), the defects in the motility and virulence of the two mutants cannot be attributed to any reduction in the bacterial growth rates.
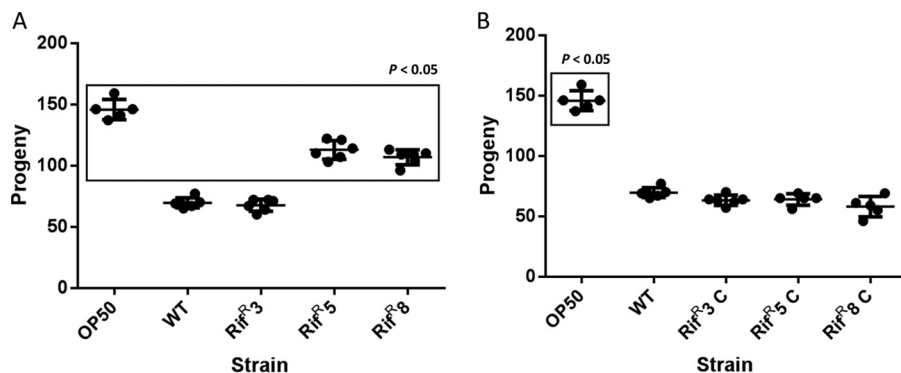
FIG 2 *C. elegans* fertility assay with the Rif^{r}3, Rif^{r}5, and Rif^{r}8 strains (A) or the complemented mutants (Rif^{r}3C, Rif^{r}5C, and Rif^{r}8C) carrying the vector pBAV1K-T5-gfp with the wild-type *rpoB* gene (B). Significant ($P < 0.05$) differences between the indicated strains and the *A. baumannii* wild-type strain ATCC 17978 (WT) are boxed. The error bars represent the standard deviations of the means (horizontal lines) from six replicates.

Introduction of the wild-type *rpoB* gene into Rif^r mutants completely restores the wild-type phenotype. It had been reported that a wild-type *rpoB* gene present in a high-copy-number plasmid restored rifampin susceptibility in a resistant mutant (15). For this reason, and to rule out the possibility that a secondary mutation different from those encountered in the *rpoB* gene was responsible for the phenotypes of the Rif^{r}5 and Rif^{r}8 mutants, the *A. baumannii* wild-type *rpoB* was cloned into the high-copy-number expression vector pBAV1K-T5-gfp (16) and introduced into Rif^{r}3, Rif^{r}5, and Rif^{r}8 *A. baumannii* mutants. As expected, the presence of the wild-type *rpoB* gene in the three resistant mutants restored rifampin susceptibility, evidenced by a reduction in the MIC from >512 mg/liter to 4 mg/liter in all three complemented mutants.

In addition, expression of the wild-type *rpoB* gene was able to restore both the virulence (Fig. 2B) and the surface-associated motility (Fig. 3A) of the Rif^{r}5 and Rif^{r}8 *A. baumannii* mutant strains. The vector pBAV1K-T5-gfp without the *rpoB* gene had no effect on rifampin susceptibility (MIC, >512 mg/ml in all cases), virulence (data not shown), or surface-associated motility (Fig. 3A) when introduced into the three Rif^r mutants of *A. baumannii* strain ATCC 17978.

Introduction of the pBAV1K-T5-gfp vector containing the *rpoB* gene isolated from any of the three Rif^r mutants into the wild-type strain conferred rifampin resistance (MIC, >512 mg/ml in all cases). However, only the pBAV1K-T5-gfp plasmid constructions containing the *rpoB* genes amplified from the Rif^{r}5 and Rif^{r}8 mutants, and not that from the Rif^{r}3 mutant, were able to impair motility when introduced into the *A. baumannii* ATCC 17978 wild-type strain (Fig. 3B). Together, these findings definitively demonstrated that the *rpoB* gene mutations of *A. baumannii* strains Rif^{r}5 and Rif^{r}8 were responsible for the lack of both surface-associated motility and virulence in the nosocomial pathogen.

Global transcriptional analysis of rifampin-resistant mutants of *A. baumannii* with impaired motility and virulence. As mentioned above, mutations in the *rpoB* gene alter gene expression patterns in several bacterial species (17). To obtain insights into the effect of the *rpoB* mutations on *A. baumannii* motility and virulence, the gene expression profiles of *A. baumannii* strains Rif^{r}5 and Rif^{r}8 were examined in cDNA microarrays (see Materials and Methods). Among the 3,431 cDNA sequences examined, the expression of 30 from strain Rif^{r}5 and 221 from strain Rif^{r}8 differed by more than 2-fold compared to the parental strain. In Rif^{r}5, 12 sequences were upregulated and 18 were downregulated, while in Rif^{r}8, 59 were upregulated and 162 were downregulated.

Six downregulated genes were shared by the two strains: two encoding putative membrane transporters and four encoding metabolism-related enzymes (Table 1). Decrease in the expression of these genes was validated by reverse transcription-quantitative real-time PCR (RT-qPCR) (Fig. 4). Furthermore, introduction of the expression vector pBAV1K-T5-gfp carrying a copy of the wild-type *rpoB* gene was able to
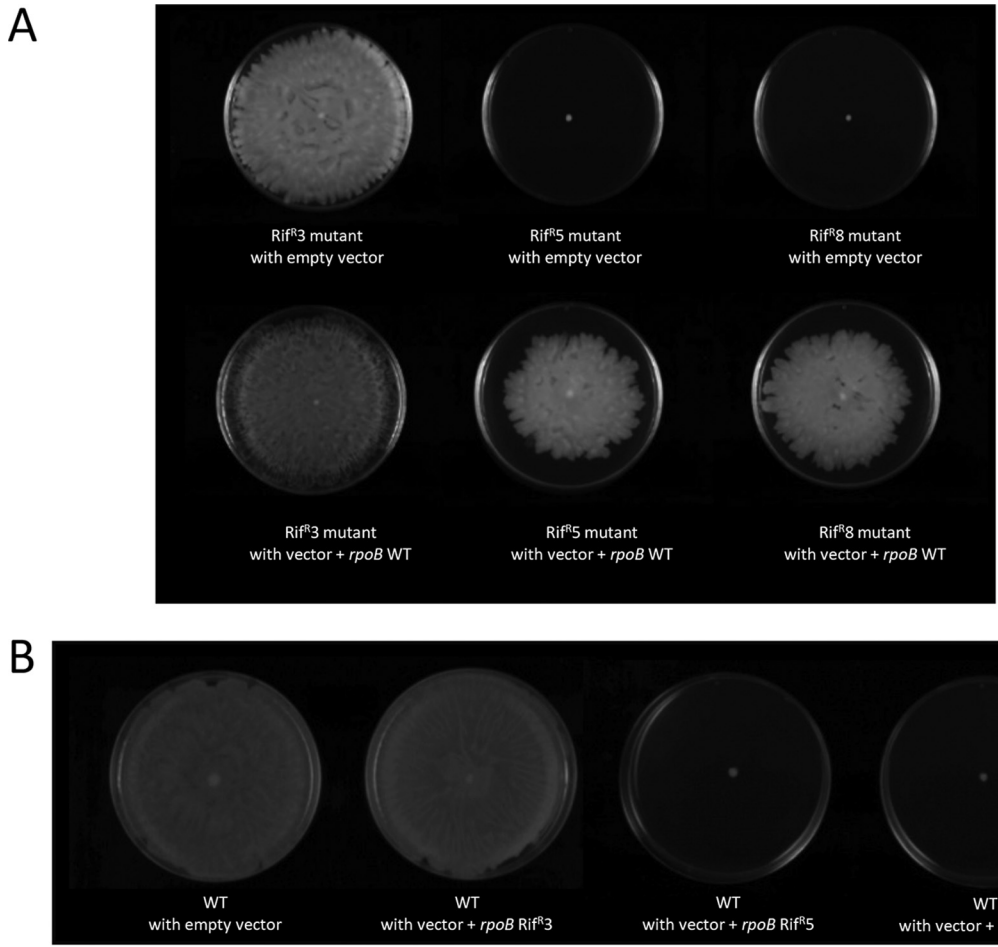
FIG 3 (A) Surface-associated motility assays of the indicated *A. baumannii* ATCC 17978 Rifʳ derivative mutants carrying either the empty expression vector pBAV1K-T5-gfp (empty vector) or the same vector containing the wild-type *rpoB* gene (vector + *rpoB* WT). (B) Surface-associated motility assays of the wild-type *A. baumannii* ATCC 17978 (WT) strain carrying the empty expression vector pBAV1K-T5-gfp (empty vector) or the same vector containing the *rpoB* gene amplified from the indicated Rifʳ strain.

increase the expression of these six downregulated genes in both the Rifʳ5 and Rifʳ8 *A. baumannii* mutants to levels similar to those of the wild-type parental strain (Fig. 4).

To further understand the genetic organization of the six downregulated genes, a transcriptional analysis of their surrounding regions using RT-PCR was carried out. The six genes are organized into four different transcriptional units, three of them polycistronic (Fig. 5) and one monocystronic. Two of the polycistronic units, designated *RS17040-RS17030* and *RS14730-RS14740*, are constituted of *RS17040*, *RS17035*, and *RS17030* and *RS14730*, *RS14735*, and *RS14740*, respectively. Another polycistronic unit

TABLE 1 Downregulated genes in the Rifʳ5 and Rifʳ8 mutants of *A. baumannii*

Gene	Product description	Fold change[a]	
		Rifʳ5	Rifʳ8
RS14730	Taurine ATP-binding transport system component	−2.7	−2.2
RS16805	Aldehyde dehydrogenase	−5.6	−4.7
RS17030	Aldehyde dehydrogenase	−5.6	−5.6
RS17035	Fumarylacetoacetate hydrolase	−6.6	−5.4
RS17040	Major facilitator superfamily permease	−4.7	−4.3
RS17045	Dihydroxy-acid dehydratase	−5.4	−5.5

[a]Fold change in gene expression in the indicated mutants with respect to the wild-type parental strain (ATCC 17978).
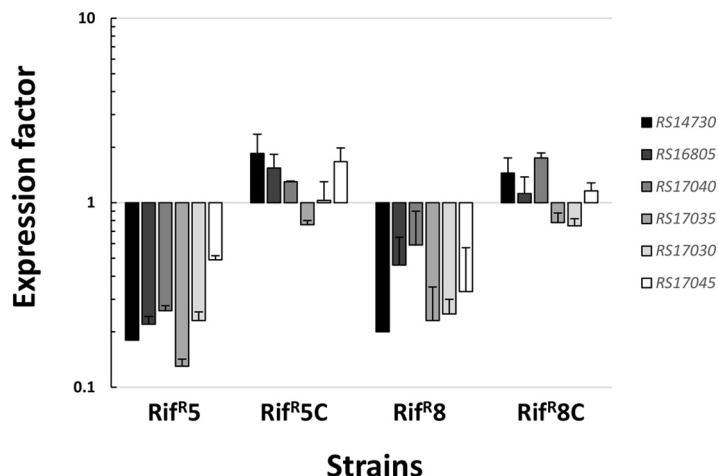
FIG 4 Expression factors of the indicated genes in *A. baumannii* strains. Rif^{r}5C and Rif^{r}8C represent the complemented rifampin-resistant strains Rif^{r}5 and Rif^{r}8, respectively, carrying the vector pBAV1K-T5-gfp with the wild-type *rpoB* gene. The expression factor is the ratio of the mRNA concentration of each gene from the indicated strain with respect to the wild-type *A. baumannii* parental strain (ATCC 17978). The error bars represent the standard deviations of the mean of at least two independent experiments, each carried out in duplicate.

contains two genes, *RS17045* and *RS17050*, whereas the *RS16805* gene is a single monocistronic transcriptional unit.

Effect of inactivation of the downregulated genes shared by *A. baumannii* Rif^{r}5 and Rif^{r}8 mutants on motility and virulence. To determine whether all the genes belonging to either the *RS17040-RS17030*, *RS14730-RS14740*, or *RS17045-RS17050* transcriptional unit are essential for *A. baumannii* motility, each of the open reading frames (ORFs) of these units was systematically inactivated, and the behavior of the resulting knockout mutants with respect to motility and virulence was studied. The results showed that mutations in four genes (*RS14730*, *RS16805*, *RS17040*, and *RS17045*) abolished motility, whereas inactivation of the remaining genes did not (Fig. 6). As expected, complementation of any of the four nonmotile mutants with the pBAV1TIC-T5-gfp plasmid carrying the corresponding wild-type gene restored wild-type motility (Fig. 6).

It is worth noting that the constructions used to obtain the insertional mutations were designed so that the gene immediately downstream of the one to be inactivated
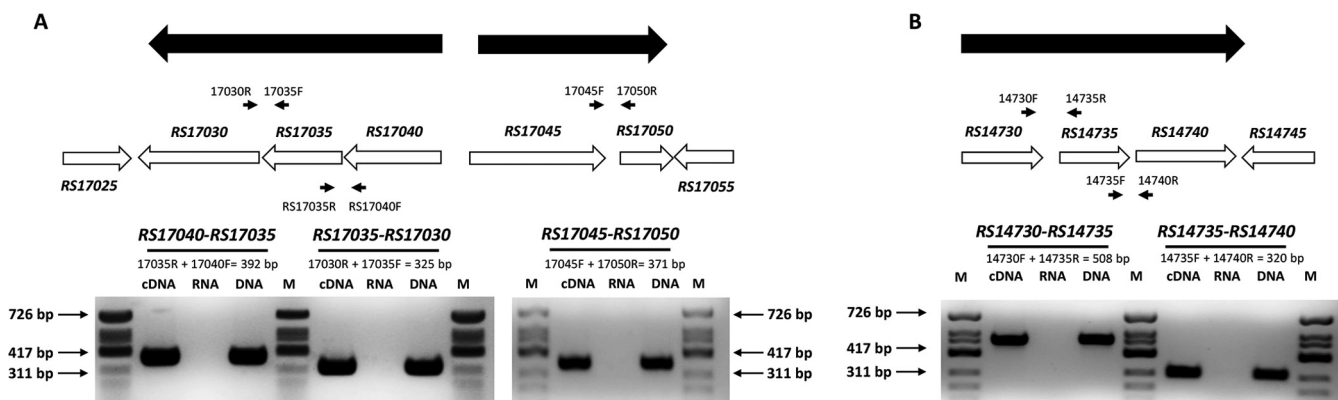

FIG 5 Organization and direction of transcription of the downregulated genes in the Rif^{r}3 and Rif^{r}5 mutants inactivated in this work and their surroundings according to the annotation of the genome of *A. baumannii* strain ATCC 17978. (A) *RS17025* to *RS17055*. (B) *RS14730* to *RS14745*. The small arrows indicate the oligonucleotides used (listed in Table S2 in the supplemental material). The large black arrows represent genes belonging to the same transcriptional unit, determined by RT-PCR analysis. The RT-PCRs were carried out in the presence of cDNA, total RNA, and DNA using the indicated combination of oligonucleotides amplifying a region of overlap between the specified genes of *A. baumannii* strain ATCC 17978. φX174 DNA/HinfI marker (Biotools) was used as a molecular size marker (lanes M). The lengths of the products amplified by the indicated oligonucleotide pairs are reported with respect to some of the marker bands.
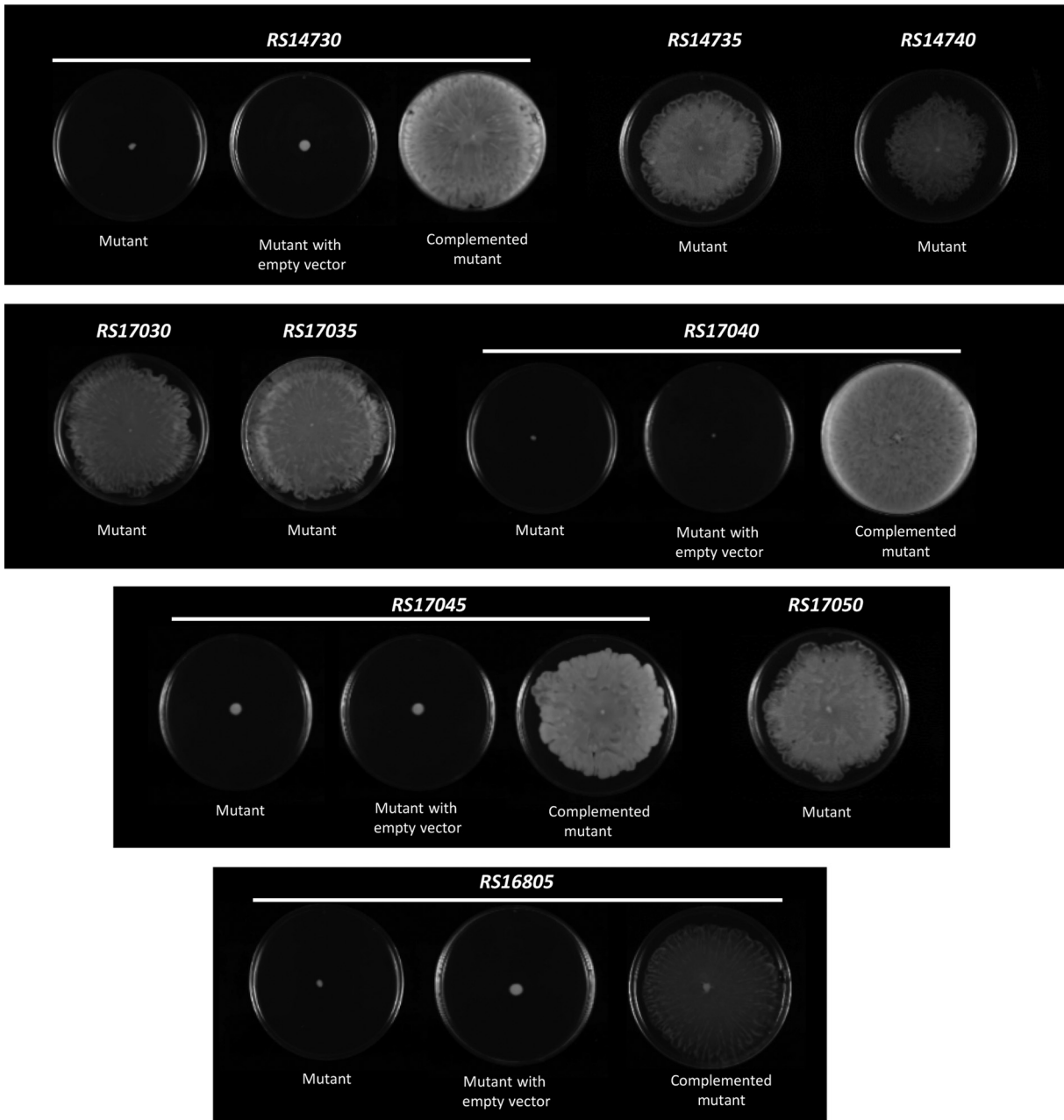
FIG 6 Surface-associated motility assays of the indicated *A. baumannii* strain ATCC 17978 derivative mutants and mutants carrying either the empty expression vector pBAV1TIC-T5-gfp (empty vector) or the same plasmid containing the corresponding wild-type gene (complemented mutants).

was under the T7 promoter present in the pCR-BluntII-TOPO vector (Fig. 7A). To confirm any putative polar effect of the inactivated genes upon distal genes affecting motility, the expression of genes placed downstream of the inactivated gene was confirmed through RT-PCR assays for each strain. The results demonstrated the absence of any polar effect of the genes being inactivated by the insertion of the pCR-BluntII-TOPO plasmid upon the expression of downstream genes (Fig. 7B). Furthermore, all of the *A. baumannii* gene knockout mutants constructed were stable, as confirmed by their culture for 10 passages without selective pressure (data not shown).

The virulence of four mutants lacking motility (*RS14730*, *RS16805*, *RS17040*, and *RS17045*) was also examined in the *C. elegans* fertility assay. The data showed that all of them were significantly less virulent than the wild-type strain (Fig. 8A). Furthermore,
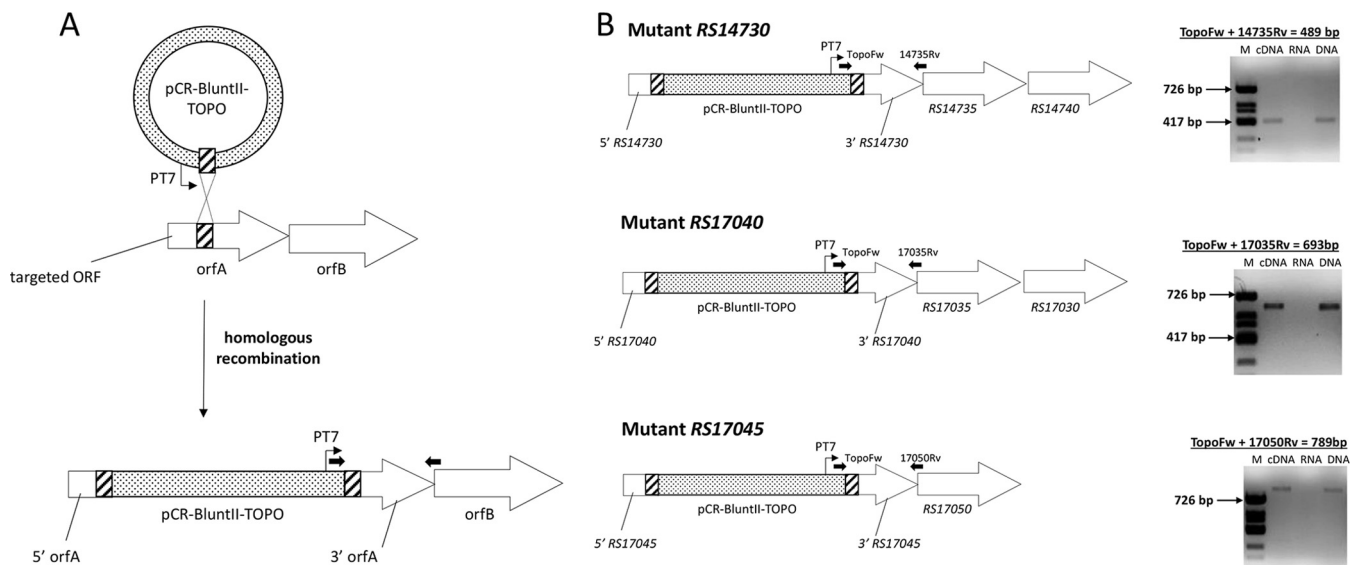
FIG 7 Strategies for plasmid insertion mutagenesis and assessment of the transcription of downstream genes used in this work. (A) Integration of pCR-BluntII-TOPO plasmid into a target gene. The hatched boxes correspond to the internal segment of the target gene. The vector is integrated into the targeted ORF by a single-crossover event. The direction of transcription from the T7 promoter (PT7) is indicated by the small thin arrows. The small black arrow pair represents the oligonucleotides used to assess the transcription of downstream genes. (B) RT-PCRs were carried out in the presence of cDNA, total RNA, and DNA using the indicated combinations of oligonucleotides amplifying overlapping regions between the T7 promoter of the pCR-BluntII-TOPO plasmid and the specified downstream genes of the indicated *A. baumannii* ATCC 17978 mutant derivatives. HinfI-digested Φ DNA was used as a molecular size marker (lanes M). The lengths of the products amplified by the indicated oligonucleotide pairs are reported with respect to some of the marker bands.

the presence in each of the four knockout mutants of the pBAV1TIC-T5-gfp plasmid carrying the corresponding wild-type gene restored virulence to the level of strain ATCC 17978 (Fig. 8B). In concordance with the data obtained in the motility assays (Fig. 6), inactivation of *RS17035*, *RS17030*, *RS14735*, *RS14740*, and *RS17050* did not affect the virulence of strain ATCC 17978 (Fig. 8A). The impaired motility and virulence conferred by these mutations on wild-type *A. baumannii* strain ATCC 17978 were not attributable to any putative effect on the growth kinetics of the respective mutants, which remained comparable to those of the wild-type parental strain (μ [mean] = 0.83 ± 0.05 h^{-1}).

The results of the above-described motility and virulence experiments (summarized in Fig. 9), together with the fact that inactivation of the *RS14730*, *RS17040*, or *RS17045* gene did not give rise to any polar effect on the rest of the genes belonging to their respective polycistronic transcriptional units (Fig. 7), clearly demonstrated that inacti-
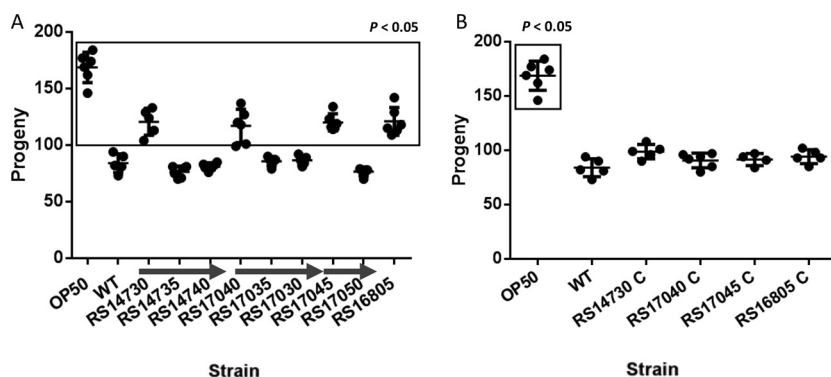

FIG 8 *C. elegans* fertility assay with the indicated *A. baumannii* strain ATCC 17978 derivative mutants (A) and the corresponding complemented mutants (B). Significant (*P* < 0.05) differences between the indicated strains with respect to the *A. baumannii* wild-type strain ATCC 17978 (WT) are boxed. *E. coli* strain OP50 was also included as a nonvirulent control. The arrows indicate genes belonging to the same transcriptional unit.
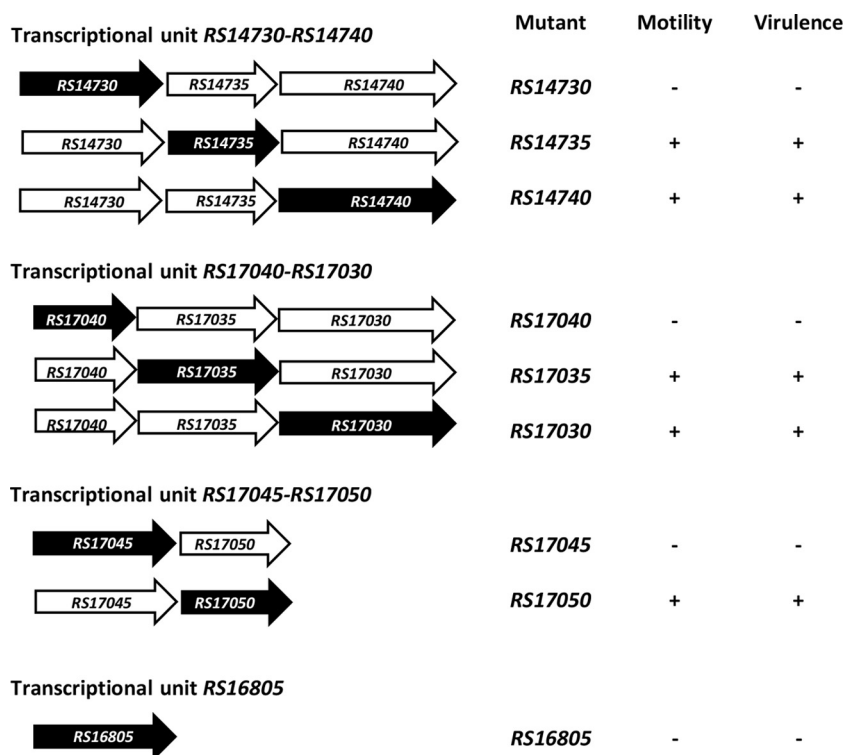
FIG 9 Summary of the effect on *A. baumannii* motility and virulence of the systematic inactivation of the genes studied in this work. The black arrows represent the inactivated genes.

vation of any of these three genes alters the motility and virulence of *A. baumannii* strain ATCC 17978.

Downregulated genes of *A. baumannii* impairing motility are widely distributed among hospital isolates of the species. Given the significant impact on the behavior of *A. baumannii* strain ATCC 17978 following the inactivation of genes *RS14730*, *RS16805*, *RS17040*, and *RS17045*, an *in silico* search of the sequences of the bacterial species deposited in the NCBI database was carried out to determine the presence of these genes in the genomes of other strains of the microorganism. This search involved 68 *A. baumannii* strains with complete chromosome sequences (see Table S1 in the supplemental material). The presence of the four genes of *A. baumannii* ATCC 17978, as well as the genes immediately upstream and downstream, was detected in 61 of the analyzed strains (see Table S1). These data demonstrate the strong conservation of the *RS14730*, *RS16805*, *RS17040*, and *RS17045* genes in *A. baumannii* species.

To further corroborate the relevance of these four genes in other *A. baumannii* strains, knockout mutants were constructed in *A. baumannii* strain MAR002, a virulent strain recently isolated from a wound sample collected in the Hospital del Mar in Barcelona, Spain (18). Assays of the mutants indicated that inactivation of *RS14730*, *RS16805*, *RS17040*, and *RS17045* homologues in *A. baumannii* MAR002 also caused a significant reduction in virulence in *C. elegans* (Fig. 10). In addition, as in strain ATCC 17978, the *A. baumannii* MAR002 strain defective in either the *RS17035* or *RS17030* homologue did not exhibit defective virulence (Fig. 10). These results clearly indicate the significant role of the four genes identified in this work in the virulence of *A. baumannii*.

DISCUSSION

Bacteria are able to live not only as single-celled organisms exhibiting planktonic growth, but also as communities of sessile cells embedded in extracellular matrices, in
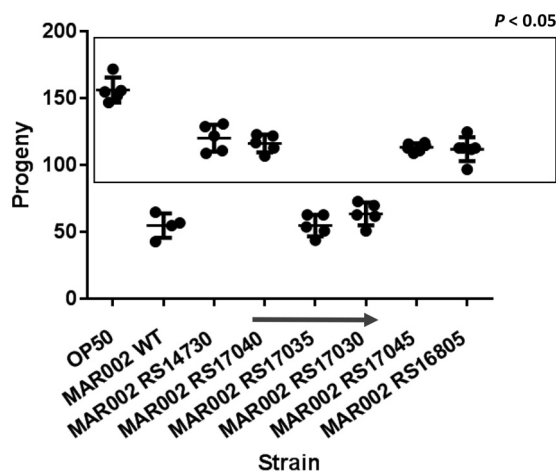
FIG 10 *C. elegans* fertility assay with the WT *A. baumannii* strain MAR002 and derivative mutants. Significant ($P < 0.05$) differences between the specified strains with respect to *A. baumannii* strain MAR002 (WT) are boxed. The *E. coli* strain OP50 is also indicated as a nonvirulent control. The arrow indicates genes belonging to the same transcriptional unit.

which they adhere to one another and to the surfaces on which they reside to form biofilms (19). Bacteria can expand their environments by moving along surfaces through various types of motility that differ in their required components and mechanisms (20). Motility has been linked to the infective capacity of pathogens, such as *Pseudomonas aeruginosa* (21), *Escherichia coli* O157:H7 (22), *Haemophilus influenzae* (23), *Neisseria meningitidis* (24), *Clostridium perfringens* (25), *Legionella pneumophila* (26), and *Proteus* spp. (27). Motility is also associated with enhanced antibiotic resistance (28), protection against host macrophages (29), and the first stage of biofilm generation (30).

The motility of *A. baumannii* is responsive to many of the same environmental and cellular signals involved in bacterial virulence (9). Our results clearly demonstrate that *A. baumannii* mutants with an amino acid substitution at position 522 or 540 of the RpoB protein lack surface-associated motility, which must be attributed to the decreased expression of several genes. The inactivation of some of these genes in *A. baumannii* also caused a reduction in its virulence. Introduction of the wild-type *rpoB* copy restored the wild-type phenotype in the Rif^r5 and Rif^r8 strains (rifampin susceptibility, surface-associated motility, and virulence). Since the wild-type gene encoding the RpoB subunit was cloned in a high-copy-number expression plasmid, recovery of the phenotype in these merodiploids was probably due to the presence of a high number of wild-type RpoB copies, which displaced the mutated RpoB proteins and thereby restored the wild-type phenotype. Similarly, the introduction into the wild-type strain of an expression vector containing the *rpoB* gene amplified from each rifampin-resistant mutant strain reproduced the same phenotype observed in the strain from which it was amplified.

In *E. coli* and *Streptomyces* spp., point mutations in the *rpoB* gene that confer rifampin resistance give rise to stringent RNA polymerase-like behavior normally associated with nutrient starvation (17). The resulting amino acid substitutions induce a conformational change in the β-subunit of the RNA polymerase that mimics the effect of guanosine tetraphosphate (ppGpp), an effector of the stringent response. The pattern of expression is similar to that seen during this response, but ppGpp is not required, as the same changes are produced in strains defective for the synthesis of the nucleotide (17). Substitutions at position Ser531 of the β-subunit of the *E. coli* RNA polymerase (which corresponds to Ser540 of the *A. baumannii* β-subunit reported here) specifically alter the interaction of *E. coli* RpoB with certain stringent promoters (31). In *N. meningitidis*, mutations in *rpoB* cause global transcriptional changes that functionally mimic the stringent response (32); in *Pseudomonas* spp., a phylogenetic neighbor of

Acinetobacter spp., stringent RNA polymerase is involved in twitching motility (33, 34). Thus, by isolating mutants arising from point mutations in the *A. baumannii rpoB* gene, we were also able to identify genes involved in the surface-associated motility of the bacterium (Table 1).

Six downregulated genes widespread among *A. baumannii* clinical isolates were shared by the two analyzed Rif^r *A. baumannii* spontaneous mutants. Inactivation of four of these genes (*RS14730*, *RS16805*, *RS17040*, and *RS17045*) significantly reduced *A. baumannii* surface-associated motility. Importantly, this loss of motility was associated with attenuation of virulence, not only in *A. baumannii* strain ATCC 17978, but also in the biofilm-hyperproducing *A. baumannii* clinical isolate strain (MAR002). The transcriptome response of *A. baumannii* strain ATCC 17978 during bacteremia was recently analyzed (35). Data obtained in that work confirmed that during this step of the *A. baumannii* infective process, the bacterial cells are in a planktonic state (35). Moreover, the expression levels of the *A. baumannii* genes identified in our study (*RS14730*, *RS16805*, *RS17040*, and *RS17045*) do not increase during bacteremia (35). These results are in concordance with the role of these genes in surface-associated motility. Similar scenarios, in which genes involved in surface-associated motility were not induced under planktonic conditions, have been reported for other bacterial species (36).

In *A. baumannii* Rif^{r}5 and Rif^{r}8 mutants, all of the downregulated genes described in this work encode putative proteins that in other bacterial species are related to either transporters or metabolic enzymes and play important roles in motility and virulence. For instance, *RS17040* is annotated as a major facilitator superfamily (MFS) permease. MFS proteins are involved in chemotaxis, which requires bacterial appendages, such as *pili*, for directed movement toward or away from attractants and repellents, respectively. In *Pseudomonas putida*, chemotaxis to the aromatic acid 4-hydroxybenzoate (4-HBA) is mediated by the MFS permease PcaK, a membrane-bound protein that also functions as a 4-HBA transporter (37).

Similarly, *Vibrio cholerae* possesses a chemoreceptor that mediates the response to taurine, a major constituent of human bile, and thus plays an important role in the chemotaxis and virulence of the pathogen (38). The *A. baumannii RS14730* gene encodes a putative taurine ATP-binding transport system component involved in the transport of taurine as a sulfur source. Thus, during the *A. baumannii* infection process, there may be competition for taurine between the nosocomial pathogen and its host (39).

Two other downregulated genes identified in the Rif^{r}5 and Rif^{r}8 mutants, *RS16805* and *RS17045*, encode a putative aldehyde dehydrogenase and a putative dihydroxy-acid dehydratase, respectively. In enterohemorrhagic *E. coli*, the aromatic aldehyde dehydrogenase FeaB is involved in chemotaxis to norepinephrine (40), whereas in *Pseudomonas syringae*, inactivation of the GDP-D-mannose dehydratase decreases the motility of the plant pathogen (41). A previous study showed that, in *A. baumannii*, genes encoding metabolic enzymes (involved in the synthesis of 1,3-diaminopropane) are also involved in the surface-associated motility of the bacterium (6).

To our knowledge, this is the first report clearly associating loss of motility with the attenuation of virulence in the pathogen *A. baumannii*. The data obtained in this work provide the basis for further investigations of the cellular components and mechanisms involved in the motility of this multiresistant microorganism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *A. baumannii* ATCC 17978, *A. baumannii* MAR002, and *E. coli* (strains DH5α and OP50) were grown in Luria-Bertani (LB) medium and incubated at 37°C with shaking at 180 rpm. When necessary, kanamycin (50 mg/liter) or ticarcillin (80 mg/liter) was added to the growth medium. The plasmids pCR-BluntII-TOPO (Invitrogen) and pBAV1K-T5-gfp (a gift from Ichiro Matsumura; Addgene plasmid 26702), were used for mutant construction and complementation, respectively. Growth was monitored by measuring the absorbance at 600 nm (OD$_{600}$). The growth rate (μ) was calculated based on the exponential segment of the growth curve and defined as ln $2g^{-1}$, where *g* is the doubling time of an exponentially growing culture. For the isolation of rifampin-resistant mutants, saturated cultures of *A. baumannii* ATCC 17978 were plated on LB agar containing rifampin (50

mg/liter) and then incubated at 37°C for 24 h. The MICs for rifampin were determined by broth microdilution.

Sequencing of *rpoB* mutants. Colony PCR was carried out using the oligonucleotides *rpoB*-1441F and *rpoB*-2095R (42) (Invitrogen), which in *rpoB* amplify a 654-bp region, the site of frequent Rif^r^-inducing base pair substitutions in the species (42). The PCR products were purified using the GFX PCR DNA and gel band purification kit (GE Healthcare) according to the supplier's protocols and sequenced (Macrogen) using the same oligonucleotide set. The data were analyzed using DNAStar Lasergene software (DNAStar).

Motility assays. Fresh LB agar motility plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% glucose, and 0.5% Difco agar) were prepared the day of the assay. After sterilization, the medium was deposited on 9-cm petri dishes with constant agitation to ensure the homogeneous spread of the agar. The inoculum was applied with a sterile toothpick. A single colony was picked and inoculated in the center of the plate, avoiding medium penetration. The inoculated plates were incubated at 37°C, usually for 16 to 20 h, previously determined to be the time required for the wild-type strain to reach the plate border under the experimental conditions. All assays were carried out a minimum of three times in independent experiments. Representative images of the motility of each bacterial strain on a semisolid agar surface are shown.

RNA extraction and RT-PCR. An overnight culture of the corresponding strain in LB medium was diluted 1:50 in fresh medium and then grown until the mid-exponential growth phase ($OD_{600} = 0.6$) was reached. The cells in 10 ml of each culture were pelleted by centrifugation at $13,000 \times g$, resuspended in Tris-EDTA (TE) buffer, and treated with lysozyme (50 mg/ml) for 10 min at 37°C. Total RNA was extracted using an RNeasy minikit (Qiagen). DNA contaminants were removed from the RNA by digestion with DNase Turbo (Ambion). The absence of DNA in the PCR mixtures was confirmed by incubating the RNA samples without reverse transcriptase. RT-PCR was performed using a first-strand cDNA synthesis kit (Nzytech) and the oligonucleotides listed in Table S2 in the supplemental material, following the manufacturer's instructions.

Microarrays. The microarrays were designed specifically for *A. baumannii* strain ATCC 17978 by Bioarray Diagnóstico Genético (Alicante, Spain) and were performed using eArray (Agilent). Labeling was carried out following the two-color microarray-based prokaryote analysis Fair Play III Labeling v. 1.3 (Agilent). Three independent RNA extractions per condition (biological replicates) were used for each experiment. The quality of the RNA was determined using Bioanalyzer (Bioarray Alicante, Spain). Statistical analysis was carried out using Bioconductor in the software package RankProd for the R computing environment. A gene was considered to be induced when the ratio of the treated preparation to the nontreated preparation was ≥ 2 and the *P* value of the difference compared to the control was <0.05. The resulting microarray data sets (WT versus Rif^r^5 and WT versus Rif^r^8) were submitted to the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/). During the revision of the manuscript, the genome of *A. baumannii* strain ATCC 17978 was updated. Consequently, in this article, the new annotation (NZ_CP018664.1) has been introduced. The correlation between the old and new annotations of the genes described in this work is presented in Table S3 in the supplemental material.

Gene expression. Gene expression was determined by RT-qPCR using Lightcycler RNA Master SYBR green I (Roche) on a Lightcycler 480 instrument (LC480; Roche) as previously described (43). Specific oligonucleotides (see Table S2 in the supplemental material) were used to validate the expression of the selected genes shown in the microarray to be deregulated. The relative mRNA concentrations of the genes of interest were determined according to a standard curve generated by amplifying an internal fragment of the *gyrB* gene that was not induced under the tested growth conditions. The expression factor was calculated as the ratio of the mRNA concentration of the target gene as expressed in the wild-type strain versus the studied strain.

Mutant construction. Plasmids were inserted into the target genes as previously described (44). Briefly, the kanamycin and zeocin resistance plasmid pCR-BluntII-TOPO, which is unable to replicate in *A. baumannii*, served as a suicide vector. An internal fragment (~500 bp) of the target gene was PCR amplified with the appropriate primers (see Table S2 in the supplemental material) using genomic DNA from *A. baumannii* as the template. The PCR products were cloned into the pCR-BluntII-TOPO vector and propagated in *E. coli* DH5α. The recombinant plasmids (0.1 μg) were then introduced into *A. baumannii* by electroporation as previously described (44). Mutants were selected on kanamycin-containing plates. Gene inactivation by plasmid insertion via single-crossover recombination was confirmed by sequencing the amplified PCR product using the appropriate oligonucleotides (see Table S2). All the mutants used in this work were assessed for stability after 10 passages (every 24 h) by incubating cultures of the mutants without selective pressure and then counting the colonies plated on LB plates with and without kanamycin.

Mutant complementation. The plasmid pBAV1K-T5-gfp was used to complement the Rif^r^3, Rif^r^5, and Rif^r^8 mutants by blunt-end cloning the wild-type *rpoB* gene from the *A. baumannii* ATCC 17978 wild-type genome into the XbaI site of the vector, whose overhangs were filled with T4 DNA polymerase (Roche). As this plasmid carries a gene encoding kanamycin resistance, complementation of the mutants constructed using the pCR-BluntII-TOPO vector, which also encodes kanamycin resistance, was carried out using a variant of the pBAV1K-T5-gfp vector (pBAV1TIC-T5-gfp), constructed in this work as follows. The β-lactamase-coding region from the pGEM-T vector (Promega) was PCR amplified, and the blunt-ended PCR product was cloned into the pBAV1K-T5-gfp plasmid digested by EcoRV, whose unique site in the plasmid is present in the coding region of the kanamycin resistance gene. All the genes were cloned into the XbaI site of the pBAV1TIC-T5-gfp vector, except *rpoB*, which contains an XbaI site in its coding region and was cloned into the original vector (pBAV1K-T5-gfp) as described above. The

recombinant plasmids were propagated in *E. coli* DH5α and introduced into the corresponding *A. baumannii* strain by electroporation as previously described (44). Transformants were selected on plates containing kanamycin (for cells carrying pBAV1K-T5-gfp derivatives) or ticarcillin (for cells carrying pBAV1TIC-T5-gfp derivatives). All of the resulting recombinant plasmids were verified by both PCR and sequencing (Macrogen). The oligonucleotides used in this study are listed in Table S2 in the supplemental material.

***In vivo* nematode model of virulence.** The virulences of the different *A. baumannii* strains used in this work were analyzed in a nematode fertility assay, as previously described (14). Briefly, *C. elegans* strain N2 was fed on the low-virulence *E. coli* OP50 strain grown as a lawn on NGM (nematode growth medium) plates. The nematode eggs were recovered and hatched in M9 medium (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.08 M NaCl, and 0.001 M $MgSO_4$), grown to stage L1, and growth arrested overnight at 16°C to physiologically synchronize the worms. The L1 larvae were cultivated to the L4 stage on lawns of the appropriate bacterial strain on NGM plates. A single L4 nematode was then inoculated on a peptone-glucose-sorbitol plate containing the same strain and incubated at 25°C. During the next 3 days, adult nematodes were removed daily to a fresh plate seeded with the same bacterial strain. To determine fertility, nematode progeny were counted daily using a stereomicroscope (Olympus SZ51) 48 h after removal of the parent. Six independent replicates were established for each bacterial strain, and each fertility assay was performed in triplicate. Representative experiments from the *C. elegans* fertility assay are shown. Statistical analysis consisted of a two-tailed one-way analysis of variance (ANOVA), followed by the Tukey test for *post hoc* multiple-group comparisons. A *P* value of < 0.05 was considered to indicate statistical significance.

Accession number(s). The microarray data sets (wild-type versus Rif^{r}5 and WT versus Rif^{r}8) were submitted to the NCBI GEO database under accession number GSE73193.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI .00327-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.2 MB.

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