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MECHANISMS OF RESISTANCE

A GC-Rich Prophage-Like Genomic Region of *Mycoplasma bovirhinis* HAZ141_2 Carries a Gene Cluster Encoding Resistance to Kanamycin and Neomycin

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ABSTRACT Recently, a complete genome sequence of *Mycoplasma bovirhinis* HAZ141_2 was published showing the presence of a 54-kB prophage-like region. Bioinformatic analysis revealed that this region has a more than 40% GC content and a chimeric organization with three structural elements—a prophage continuous region, a restriction-modification cassette, and a highly transmittable *aadE-sat4-aphA-3* gene cluster found in both Gram-positive and Gram-negative bacteria. It is known that *aadE* confers resistance to streptomycin, *sat4* governs resistance to streptothricin/nourseothricin, and *aphA-3* is responsible for resistance to kanamycin and structurally related antibiotics. An *aadE*-like (*aadE**) gene of strain HAZ141_2 encodes a 228-amino acid (aa) polypeptide whose carboxy-terminal domain (positions 44 to 206) is almost identical to that of a functional 302-aa AadE (positions 140 to 302). Transcription analysis of the *aadE*-sat4-aphA-3* genes showed their cotranscription in *M. bovirhinis* HAZ141_2. Moreover, a common promoter for *aadE*-sat4-aphA-3* was mapped upstream of *aadE** using 5′ rapid amplification of cDNA ends analysis. Determination of MICs to aminoglycosides and nourseothricin revealed that *M. bovirhinis* HAZ141_2 is highly resistant to kanamycin and neomycin (≥512 μg/ml). However, MICs to streptomycin (64 μg/ml) and nourseothricin (16 to 32 μg/ml) were similar to those identified in the prophageless *M. bovirhinis* type strain PG43 and Israeli field isolate 316981. We cloned the *aadE*-sat4-aphA-3* genes into a low-copy-number vector and transferred them into antibiotic-sensitive *Escherichia coli* cells. While the obtained *E. coli* transformants were highly resistant to kanamycin, neomycin, and nourseothricin (MICs, ≥256 μg/ml), there were no changes in MICs to streptomycin, suggesting a functional defect of the *aadE**.

KEYWORDS *Mycoplasma bovirhinis*, *aadE-sat4-aphA-3* gene cluster, aminoglycosides, horizontal gene transfer, kanamycin and neomycin resistance, prophage

Aminoglycosides are broad-spectrum bactericidal antibiotics, which are primarily used to treat infections caused by Gram-negative aerobic bacilli, staphylococci and some other Gram-positive bacteria, and *Mycobacterium* and *Mycoplasma* spp. (1, 2). Based on the identity of the aminocyclitol moiety, aminoglycosides are grouped into 4,6-disubstitued 2-deoxystreptamine (DOS)-containing kanamycin, tobramycin, amikacin, gentamicin, and others; 4,5-disubstitued DOS-containing neomycin, paromomycin, lividomycin, and others; and a group of compounds with alternative core rings or organization, such as streptomycin, spectinomycin, apramycin, and hygromycin B (3). Aminoglycosides impair protein synthesis by binding to different sites in the 16S rRNA of the 30S ribosomal subunit and to some ribosomal proteins (4). However, the drug binding site of 2-DOS aminoglycosides is composed mainly of nucleotides located within the decoding region A site of bacterial helix 44 of the 30S subunit (5). In

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addition, some 2-DOS aminoglycosides have a secondary binding site in helix 69 of the 50S subunit (6). Acquired resistance to aminoglycosides can arise via a number of various mechanisms, including modification of the ribosomal target, antibiotic uptake and accumulation, efflux of antibiotic, and inactivation of the drugs by aminoglycoside-modifying enzymes (7).

In the members of the class *Mollicutes*, a large group of wall-less bacteria (8), the main mechanisms of acquired resistance described until now are target modification with point mutation(s) and ribosome protection by the *tet*(M) determinant (9–11). A single study also reported that in ureaplasmas, macrolide resistance can be achieved through ribosomal methylation mediated by *ermB* and through drug efflux mediated by *msrA*, *msrB*, or *msrD* (12), but these data have not been confirmed by any other group. In addition, in *Mycoplasma hominis* mutants selected *in vitro* on ethidium bromide and showing increased MICs to ciprofloxacin, an active efflux mechanism has been demonstrated (13). Regarding aminoglycosides, it was shown previously that point mutations at positions 912 and 1192 (*Escherichia coli* numbering) of 16S rRNAs were associated with decreased susceptibility to streptomycin and spectinomycin, respectively, in several mycoplasmas (14, 15). However, to the best of our knowledge, no studies describing mechanisms of resistance to kanamycin and neomycin in mycoplasmas have been published.

Mycoplasma bovirhinis is a species that is frequently isolated from the upper and lower respiratory tracts of both healthy and diseased cattle and buffaloes all over the world, and it is often coisolated with other bacterial or mycoplasmal pathogens (16). Recently, complete genomes of three *M. bovirhinis* strains were released in databases, namely, the type strain PG43 (NCTC 10118/ATCC 27748; GenBank accession no. LR214972.1), the Japanese isolate HAZ141_2 (AP018135.1 [17]), and the Chinese isolate GS01 (CP024049.1 [18]). Initial reports describing genomes of the two latter strains mentioned a 53.5-kb DNA sequence uniquely present in HAZ141_2 (17, 18). Chen et al. (18) noticed that the HAZ141_2 strain-specific genomic region, besides bacteriophage-like DNA, contains three open reading frames (ORFs) resembling the following known genes: *aadE*, encoding 6′ adenyltransferase [AAD(6′)] conferring resistance to streptomycin, *sat4*, encoding streptothricin *N*-acetyltransferase conferring resistance to streptothricin/nourseothricin, and *aphA-3*, encoding a 3′ phosphotransferase [APH(3′)-III] conferring resistance to kanamycin/neomycin/amikacin. This information caught our attention and motivated the analysis of these genes never seen before in *Mollicutes*.

The *aadE-sat4-aphA-3* genes are one of highly transmittable and disseminated clusters found mainly on the plasmids in both Gram-positive (e.g., *Enterococcus* spp., *Streptococcus* spp., *Staphylococcus aureus*, etc.,) and Gram-negative bacteria (e.g., *Campylobacter* species). Indeed, two genes, *aadE* and *aphA-3*, were described as a part of the composite *S. aureus* transposon Tn*5405* (19). Later, the same group identified a pseudogene (truncated ORF) within Tn*5405* located between *aadE* and *aphA-3* (20), which shared a strong similarity to *sat4* described in Gram-negative *Campylobacter coli* (21). A comprehensive analysis of 50 *S. aureus* strains carrying the *addE-sat4-aphA-3* locus revealed that only 14 strains contained an intact *sat4* (22). The obvious transmittable character of the *aadE-sat4-aphA-3* cluster became apparent after the discovery of *Streptococcus* phages carrying these genes (23). Since genes encoding resistance to aminoglycosides and strepthotricin were identified for the first time in *Mollicutes*, the aim of this study was the preliminary characterization of these genes and analysis of their phenotypic expression in both *M. bovirhinis* HAZ141_2 and in antibiotic-susceptible *Escherichia coli* cells where the genes were cloned on a low-copy-number vector.

RESULTS

Characterization of the *M. bovirhinis* HAZ141_2 prophage-like region. Our *in silico* analysis of the *M. bovirhinis* HAZ141_2 prophage-like region (Fig. 1A) revealed its putative integration sites, *attR* and *attL*, to be a dinucleotide (TG) located within a 3′ end of the tRNA-His gene (MBVR141_t0068; nucleotides [nt] 924505 to 924506) and

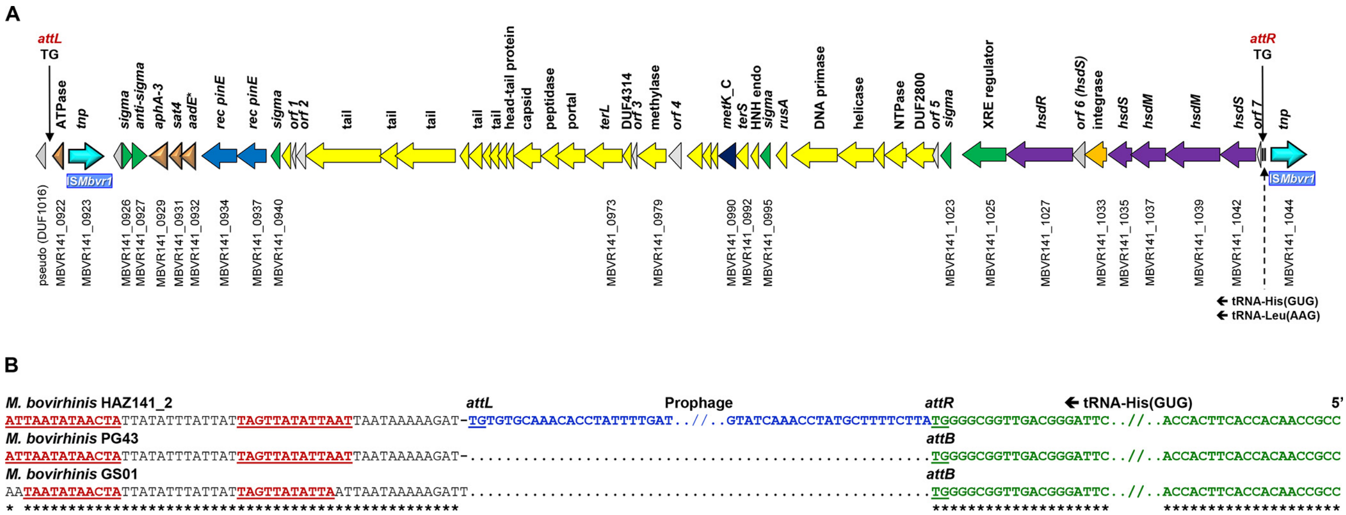

FIG 1 Genomic organization of *M. bovirhinis* HAZ141_2 prophage and its integration sites. (A) Schematic representation of the prophage-like genomic region. Arrows represent ORFs, with the arrowheads indicating the direction of transcription. The ORFs are color coded based on the putative function of the predicted encoded proteins. Brown, antibiotic resistance genes from the *aadE**-sat4-aphA-3* gene cluster; different shades of blue, IS*Mbvr1* encoding transposases and PinE-related recombinases; different shades of yellow, phage-associated genes; purple, restriction-modification enzymes; green, transcriptional regulation; gray, not annotated, hypothetical or unknown proteins. Only ORFs discussed in the text are tagged. The *attL* and *attR* sites are indicated by a vertical black line, and their sequences (TG) are presented. Schematic representation of the prophage-like genomic region prepared according to a scale using IS*Mbvr1* (2,010 bp) as a scale bar. (B) Analysis of the putative integration sites of the *M. bovirhinis* HAZ141_2 prophage. Putative integration sites *attL* and *attR* as predicted in *M. bovirhinis* HAZ141_2 are shown. The prophage integrated into a putative 2-bp target sequence (TG) at the 3′ end of the tRNA-His (anticodon GUG) gene (MBVR141_t0068); its duplication is present in the genome of strain HAZ141_2, but not in the *M. bovirhinis* PG43 and GS01 strains. Perfect inverted repeats of a putative transcriptional terminator of the tRNA-His gene are shown in red bold and underlined. Asterisks mark identical nucleotides.

within an intergenic region (nt 870582– to 870583), respectively (Fig. 1B). Moreover, this strain HAZ141_2-specific region was inserted precisely between a 3′ end of the tRNA-His gene and its putative transcriptional terminator, whose stable stem-loop structure (ΔG, 8.3 kcal/mol) is formed by two perfect 13-bp inverted repeats (IR), ATTAATATAACTA (nt 870531 to 870569; Fig. 1B). An identical terminator-like sequence was found downstream of orthologous tRNA-His genes in both prophageless genomes, PG43 and GS01, suggesting a remarkable expression variability of this gene in strain HAZ141_2.

An entire prophage-like region (including an *attP* site) was estimated to be 53,923 bp with 40.7% GC content, which is much higher than the average GC content of mycoplasmal genomes (28 to 29%) and *M. bovirhinis* HAZ141_2 (28.24% [17]). The codon usage in the *M. bovirhinis* HAZ141_2 prophage-like region is similar to that of *Firmicutes* bacteria and their phages (https://www.kazusa.or.jp/codon/). For example, analysis of prophage ORFs showed that tryptophan (Trp) residues are mainly encoded by the TGG codon, while in *Mycoplasma* species, Trp is encoded by either TGG or TGA codons (24, 25), suggesting a relatively recent acquisition of this region by *M. bovirhinis*. Remarkably, translation of TGA as Trp leads to some extension of translated polypeptides (e.g., MBVR141_0932, MBVR141_0995, MBVR141_0999, etc.; see below). The influence of such extension on protein stability and putative function is unknown.

Genome sequence analysis revealed 55 ORFs (48 were annotated in previous publications [17, 18] and 7 were newly annotated as ORFs 1 to 7), of which 52 were transcribed in the same direction and 3 (MBOV141_0923, 0926, and 0927) were transcribed in the opposite direction (Fig. 1A; see Table S1 in the supplemental material). The *M. bovirhinis* HAZ141_2 prophage-like region has a chimeric structural organization. The 5′ end of the prophage consists of a highly transmittable *aadE**-sat4-aphA-3* gene cluster (nt 875016 to 877062). *aadE** (MBVR141_0932) encodes a protein whose C terminus shares a strong similarity to 6′ adenyltransferase [AAD(6′)] enzyme AadE, conferring resistance to streptomycin. The *M. bovirhinis* AadE* deduced amino acid sequence contains 228 residues, which is nearly 25% less than a length of an enzymatically active AadE (302 amino acids [aa]; Fig. 2). Three features of the *M. bovirhinis* AadE* should be

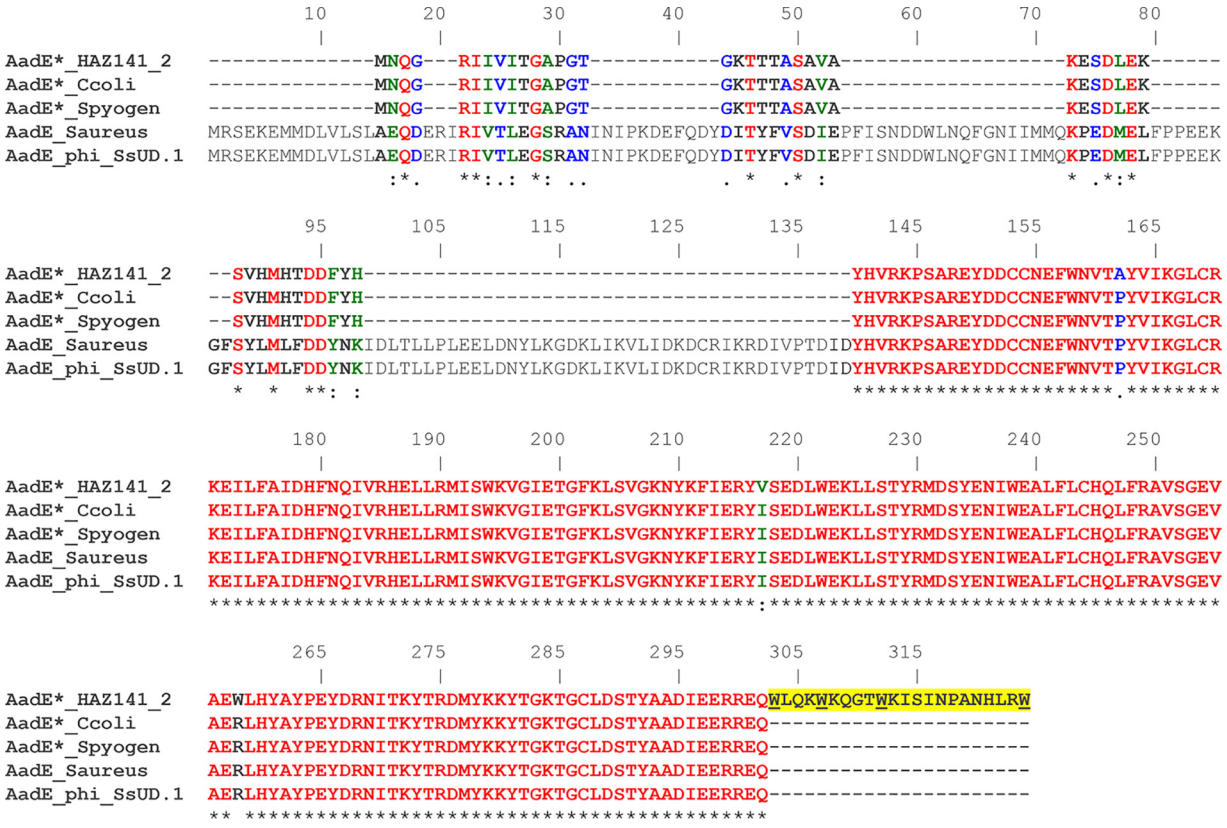

FIG 2 Amino acid sequence alignment of the *M. bovirhinis* strain HAZ141_2 AadE*-like protein with homologs from other bacterial species. The comparison includes AadE*_HAZ141_2 (MBVR141_0932, GenBank accession no. AP018135.1, 228 aa), *Mycoplasma bovirhinis* strain HAZ141_2; AadE*_Ccoli (accession no. KJ610809.1, 206 aa), *Campylobacter coli* strain SH-CCD11C145; AadE*_Spyogen (SD89_05780, accession no. CP010449.1, 206 aa), *Streptococcus pyogenes* strain NGAS322; AadE_Saureus (SA268_2518, accession no. AII57085.1, 302 aa), *Staphylococcus aureus* strain SA268; AadE_phi_SsUD1 (accession no. CBR26932.1, Orf62, 302 aa), *Streptococcus suis* phage phi-SsUD.1. The alignment length is 324 aa residues; the protein sequences were aligned using CLUSTAL W (70). Identical amino acids (53.39%) are shown in red and marked with an asterisk (*); strongly similar amino acids (2.78%) are shown in green and marked with a colon (:); weakly similar amino acids (2.47%) are shown in blue and marked with a dot (.); different amino acids (41.36%) are indicated in black. A 22-aa C-terminal extension of AadE*_HAZ141_2 is highlighted in yellow, and four Trp (W) residues encoded by TGA codons are underlined.

mentioned as follows. First, the amino (N)-terminal part of the *M. bovirhinis* AadE* (residues 1 to 43) does not show any similarity to amino acid sequences of known AadE enzymes with experimentally validated activity. Second, the carboxy (C)-terminal residues of AadE* (residues 44 to 206) are almost identical to the C terminus of a classic 6′ adenyltransferase AadE (residues 140 to 302 of a 302-aa polypeptide). Third, AadE* has a 22-aa C-terminal elongation (residues 207 to 228), as a result of a specific usage of the TGA translation stop codon by mycoplasmas; this sequence is absent from any other enzymatically studied AadE proteins (Fig. 2). The next gene of the cluster is *sat4* (MBVR141_0931), which encodes a 180-aa protein with 100% identity to streptothricin acetyltransferase Sat4, conferring resistance to aminoglycoside glycopeptide antibiotics of the streptothricin class in multiple Gram-positive and Gram-negative bacterial species (Table S1). In *M. bovirhinis*, there is a 70-bp overlap between the 3′ end of an extended *aadE** and the 5′ end of *sat4*. Next to *sat4* is *aphA-3*, encoding a 264-aa protein showing 100% identity to 3′ phosphotransferase [APH(3′)-III], conferring resistance to kanamycin as well as to other structurally related aminoglycosides (e.g., neomycin and amikacin) in both Gram-positive and Gram-negative bacteria (Table S1). The *aadE*-sat4-aphA-3* gene cluster is bordered downstream by two genes encoding a putative alternative sigma factor resembling YlaD (MBVR141_0926) and its cognate membrane-associated negative regulator (an antisigma, MBVR141_0927), while two genes coding for putative serine site-specific recombinases (MBVR141_0934 and 0937) are located upstream of the cluster (Fig. 1A and Table S1).

TABLE 1 *In vitro* susceptibility to aminoglycosides and nourseothricin of *M. bovirhinis* strains used in this study

Strain	MIC (μg/ml)[a]					
	Km	Gm	Nm	Sp	Sm	NTC
PG43	8	4	64	4	32	16–32
316981	16	8	64	8	32	16–32
HAZ141-2	≥512	8	≥512	8	64	16–32

[a]Km, kanamycin; Gm, gentamicin; Nm, neomycin; Sp, spectinomycin; Sm, streptomycin; NTC, nourseothricin.

The central part of the *M. bovirhinis* HAZ141 prophage-like region contains multiple *orf*s encoding tail- and capsid-associated components as well as both subunits of terminase, TerL (MBVR141_0973) and TerS (MBVR141_0992), an essential enzyme of bacteriophages responsible for packaging genomic DNA into proheads of future virions (Fig. 1A and Table S1). The 3′ end of the prophage consists of 9 genes (from MBVR141_1025 to MBVR141_1042 and two putative nonannotated genes, *orf6*, resembling *hsdS*, and *orf7*). Six out of those nine genes encode type I restriction and modification (RM)-related proteins. Finally, an insertion-like sequence, which we designated IS*Mbvr1* (MBVR141_0923), is located at the 5′ end of the prophage-like region, while an additional copy of IS*Mbvr1* (which encodes transposase MBVR141_1044) flanks the 3′ end of the prophage (Fig. 1A).

***In vitro* susceptibility of *M. bovirhinis* strains to aminoglycosides and nourseothricin.** Since the presence of the *aadE*-sat4-aphA-3* gene cluster, conferring resistance to aminoglycosides and strepthotricin in other bacteria, has been identified in *Mollicutes* for the first time, we tested their phenotypic expression in *M. bovirhinis* HAZ141_2 by determining the MICs. The results of *in vitro* susceptibility data of three *M. bovirhinis* strains to the aminoglycosides gentamicin, kanamycin, neomycin, streptomycin, and spectinomycin, as well as to nourseothricin, are presented in Table 1. While the MICs to gentamicin, spectinomycin, streptomycin, and nourseothricin were compatible among the strains, *M. bovirhinis* HAZ141_2 demonstrated high-level MICs to kanamycin and neomycin (≥512 μg/ml), which were at least 32 to 64 and 8 times higher, respectively, than those in *M. bovirhinis* 316981 and *M. bovirhinis* PG43 (Table 1).

The prophage-borne *aadE*-sat4-aphA-3* gene cluster expressed in *E. coli* provides resistance to kanamycin, neomycin, and nourseothricin, but not to streptomycin. Since we showed a significant difference in susceptibility to kanamycin and neomycin between *M. bovirhinis* HAZ141_2 and two other *M. bovirhinis* strains (Table 1), we first checked if there are any mutations present in the 16S rRNA-encoding genes (*rrs*) of strain HAZ141_2 in comparison to the type strain, PG43. The sequence analysis of 3 alleles of the 16S rRNA-encoding gene revealed that 7 positions differed between two strains, namely, 91, 138, 178, 494, 847, 987, and 1290 (*E. coli* numbering [Fig. S1]). None of the substitutions detected in *M. bovirhinis* HAZ141_2 were located within the helix 44 of 16S rRNA, which is known as a binding site for 2-DOS-containing aminoglycosides (5). In addition, since some 2-DOS aminoglycosides may have a secondary binding site in the ribosome—helix 69 of the 50S subunit (6)—the sequences of 23S rRNA-encoding genes (*rrl*) were compared between *M. bovirhinis* strains PG43 and HAZ141_2. No nucleotide difference was found at positions 1906 to 1930 (*E. coli* numbering) between two *M. bovirhinis* strains or between them and *E. coli* (data not shown).

Then, since the *aadE*-sat4-aphA-3* genes were identified only in *M. bovirhinis* HAZ141_2, we wanted to test whether this cluster was, indeed, active and might confer resistance to kanamycin and neomycin as well as to streptomycin and nourseothricin in a heterologous host. To that end, the *aadE*-sat4-aphA-3* cluster was cloned into the low-copy-number plasmid vector pACYC184 derivative and introduced by transformation into *E. coli* JM109 (see Materials and Methods). No increase in MIC values to gentamicin, spectinomycin, or streptomycin was observed in recombinant clone pAC10 in comparison to host *E. coli* JM109 and *E. coli* transformants carrying an empty pACYC184 plasmid vector (Table 2). In contrast, the MIC values for neomycin and kanamycin were at least 128-fold higher in *E. coli* JM109 transformants carrying pAC10 than

TABLE 2 *In vitro* susceptibility to aminoglycosides and nourseothricin of the *E. coli* JM109 recombinant plasmid pAC10 carrying the *aadE*-sat4-aphA3* gene cluster

Strain	MIC (μg/ml)[a]					
	Km	Gm	Nm	Sp	Sm	NTC
JM109	2	1	2	16	4	2
JM0109 (pACYC184)	2	1	2	16	4	2
JM109 (pAC10)	≥256	1	≥256	16	4	≥256

[a]Km, kanamycin; Gm, gentamicin; Nm, neomycin; Sp, spectinomycin; Sm, streptomycin; NTC, nourseothricin.

in *E. coli* JM109 and the plasmid-containing *E. coli* JM109 recipient, respectively (Table 2), and were comparable to that demonstrated in *M. bovirhinis* HAZ141_2 (Table 1). In addition, *in E. coli* JM109, the prophage-borne *aadE*-sat4-aphA-3* gene cluster provided resistance to nourseothricin, with the MIC value of ≥256 μg/ml in pAC10 in comparison to 2 μg/ml in *E. coli* JM109 and in its transformant carrying only the pACYC184 plasmid (Table 2). Notably, the MIC value for nourseothricin was at least 8-fold less in *M. bovirhinis* HAZ141_2 than in *E. coli* containing pAC10 (Tables 1 and 2).

Transcription analysis of the *M. bovirhinis* HAZ141_2 *aadE*-sat4-aphA-3* genes confirms their expression and cotranscription. Regardless of the presence of *aadE** and *sat4* in *M. bovirhinis* HAZ141_2, no significant difference in MICs to streptomycin and nourseothricin has been identified among the 3 *M. bovirhinis* strains tested in this study. In addition, no increase in MICs to streptomycin was evident in recombinant plasmid pAC10 (Tables 1 and 2). To explore this issue, reverse transcription PCR (RT-PCR) analysis was performed on total RNA of *M. bovirhinis* HAZ141_2 using primers complementary to the *aadE**, *sat4*, and *aphA-3* genes (Table S3). The results of RT-PCR showed expression of all three genes (Fig. 3A). Moreover, an RT-semiquantitative PCR analysis of each individual gene revealed the same pattern of expression (Fig. S2). In addition, RT-PCR analysis performed on cDNA, obtained with the aphA3-R2 primer,

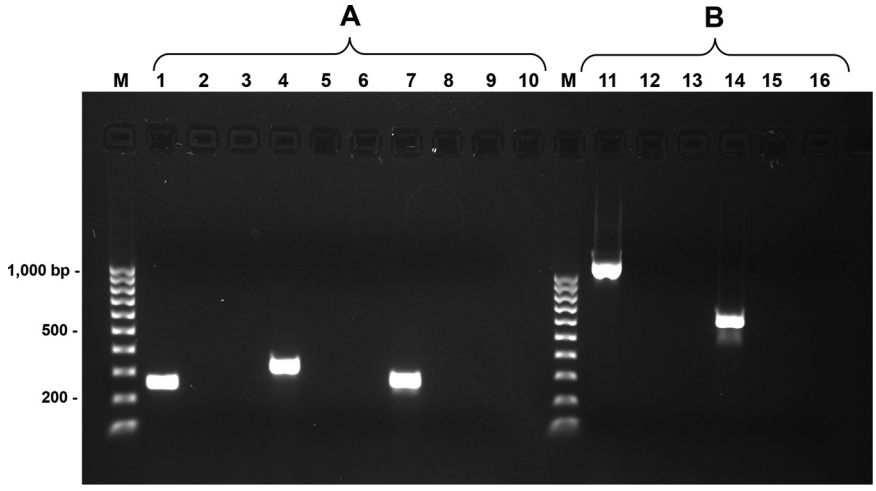

FIG 3 Transcription analysis of the *M. bovirhinis* HAZ141_2 *aadE*-sat4-aphA-3* genes. (A) RT-PCR analysis of the *aadE**, *sat4*, and *aphA-3* genes. Agarose gel electrophoresis of RT-PCR products. cDNAs, synthesized with either aadE-R1, sat4-R1, or aphA3-R2 primers complementary to the *aadE**, *sat4*, and *aphA-3* genes, respectively, were subjected to PCR amplifications to determine the expression of each individual gene. The PCR products were obtained using the aadE-F2 and aadE-R2 (lane 1), sat4-F1 and sat4-R1 (lane 4), and aphA3-F1 and aphA3-R2 (lane 7) primers. The control cDNAs (lanes 2, 5, and 8) were without RT in the reaction. Negative PCR controls are presented in lanes 3, 6, and 9. (B) Coexpression of the *M. bovirhinis* HAZ141_2 *aadE*-sat4-aphA-3* genes. PCR amplifications of the cDNA, obtained with the aphA3-R2 primer complementary to the *aphA-3* gene, were produced to determine cotranscription of the *aadE**, *sat4*, and *aphA-3* genes. The amplification products were obtained using the aadE-F3 and aphA3-R1 (lane 11) and the aadE-F3 and sat-R2 (lane 14) primers. The control samples (lanes 12 and 15) were those without RT in the reaction. Negative PCR controls are presented in lanes 13 and 16. The 100-bp ladder (Bio-Rad, CA, USA) is shown as M.

complementary to the *aphA-3* gene, revealed the cotranscription of the *aadE**, *sat4*, and *aphA-3* genes in *M. bovirhinis* HAZ141_2 (Fig. 3B).

In silico analysis of the DNA region upstream of the *aphA-3* gene revealed two putative promoters, one resembling both P1 and P1′, described in literature (in this study designated P1″), and another identical to P2 (Fig. 4) that was previously characterized (26–28). The *M. bovirhinis* P2 promoter is located within an intergenic region between the *sat4* and *aphA-3* genes and is totally identical to the sequence of P2, which was experimentally validated first on plasmid pJH1 of the Gram-positive *Enterococcus faecalis* (28) and on plasmid pIP1433 of the Gram-negative *Campylobacter coli* (27). The P1 promoter of *aphA-3* was identified within the encoding region of *sat4* of *C. coli* plasmid pIP1433 (27), and it was flanked by two 12-bp direct repeats (DRs) like those found in the corresponding region of the *M. bovirhinis* HAZ141_2 *sat4* (Fig. 4 and Fig. S3). In contrast to the P1 promoter of *aphA-3* in plasmid pIP1433, which contained a tandemly repeated 7-bp sequence, ATAATAT, located between the −35 and −10 elements, the *M. bovirhinis* HAZ141_2 P1″ promoter possessed only one ATAATAT repeat resembling the experimentally determined P1′ promoter in the pJH1 and pLG2 plasmids of *E. faecalis* (28, 29). An identical to P1″ promoter was previously described in strepthotricin- and kanamycin-resistant *C. coli* BE/G4 (21). It is noteworthy that a deletion of one of the ATAATAT repeats changed the −10 element of P1 from the optimal consensus TATAAT sequence to TATCTT (Fig. 4 and Fig. S3) and restored an intact ORF of the *sat4* gene that was truncated in plasmid pIP1433.

Using 5′ rapid amplification of cDNA ends (5′-RACE) analysis, we identified a promoter upstream of the *aadE** gene with a transcriptional start site (TSS) represented by two nucleotides, A and G, located 35 and 36 nucleotides upstream of the ATG start codon of the *aadE** gene, respectively. The −35 site, TTGAAA, and an extended −10 Pribnow box, TGaTATAAT, (30) were also mapped (Fig. 4 and Fig. S4). Notably, the TTS was identified on cDNA obtained with either *aphA-3*, *sat4*, or *aadE** complementary primers (see Table S2), supporting cotranscription of these genes demonstrated by RT-PCR (Fig. 3).

DISCUSSION

Bacteriophages are intracellular parasites of bacteria that are highly prevalent in nature (31) and can be categorized by their life cycle as either lytic or lysogenic. In the latter scenario, the phage genome (prophage) is usually integrated into the bacterial genome and transmitted vertically during replication. Prophages play an important role in bacterial evolution, population shaping, virulence, and genetic transfer through horizontal gene transfer (HGT). Indeed, many prophages carrying virulence as well as fitness factors (32, 33) influence bacterial traits and allow the bacteria to access new environmental niches (34–36). Recently, a unique 53.5-kb chromosomal insertion was identified in the genome of *M. bovirhinis* HAZ141_2 (17). Later, Chen et al. (18) reported that this region is missing in the genome of strain GS01 and provided its initial analysis. Many ORFs of the 53.5-kb region resembled genes of bacterial phages. Our *in silico* analysis deduced a more precise size of the prophage-like region, which is 53,523 bp, including a copy of a novel insertion element, IS*Mbvr1* (2,010 bp) (Fig. 1 and Table S1). To the best of our knowledge, it is the biggest prophage-like genomic region ever identified in *Mollicutes*. Its high GC content in comparison to the rest of the *M. bovirhinis* HAZ141_2 genome, as well as the almost sole use of the TGG codon (rather than TAG) to encode Trp, suggests the recent exogenous acquisition of this prophage by mycoplasma. BLAST analysis of amino acid sequences of proteins encoded by the *M. bovirhinis* HAZ141_2 prophage-like region revealed that most of the homologous polypeptides were detected in species of *Firmicutes* (Gram-positive), and several homologous ORFs were associated with both Gram-positive and Gram-negative bacteria (Table S1).

The *M. bovirhinis* HAZ141_2 prophage-like region has a modular organization similar to that of *Streptococcus suis* phage phi-SsUD.1 (23), with the phage-specific genes located in the middle of this region, while the aminoglycoside/streptothricin resistance

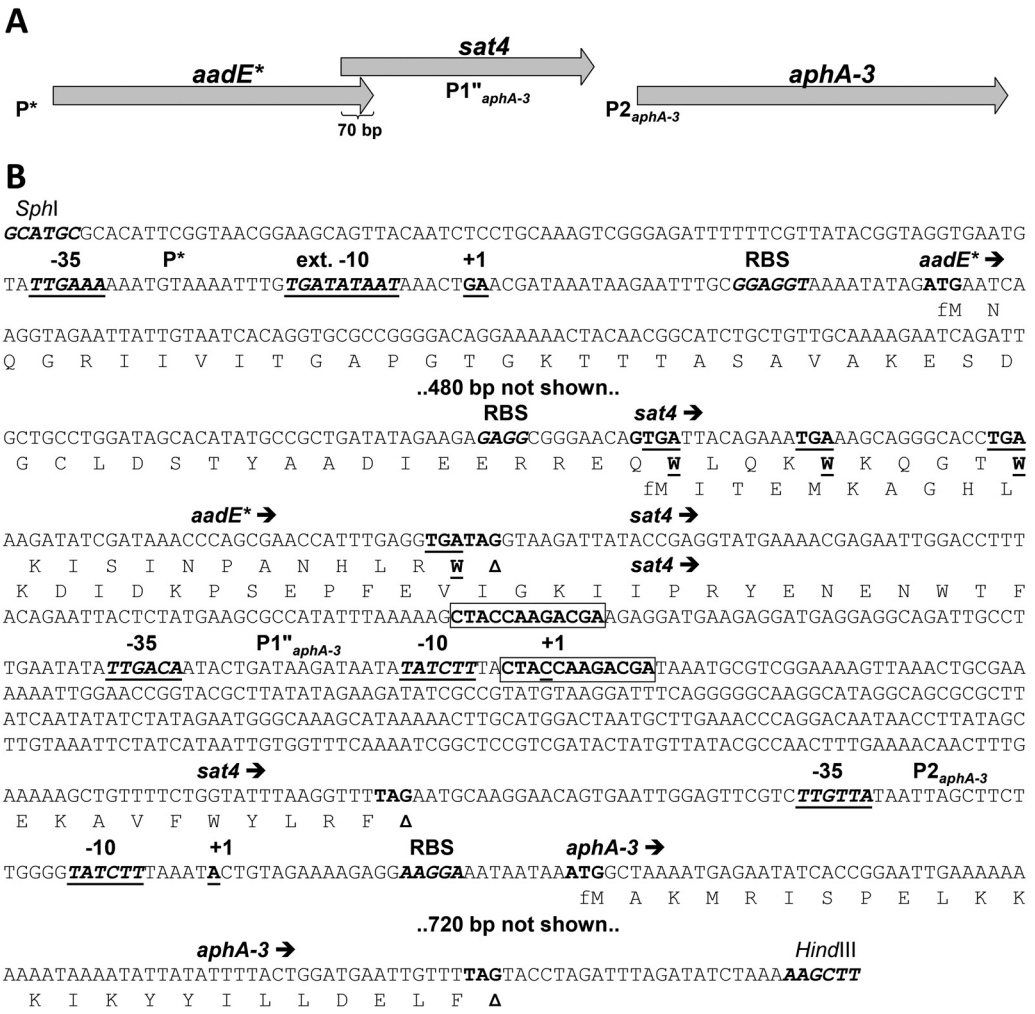

FIG 4 Genomic organization of the *aadE*-sat4-aphA-3* gene cluster of *M. bovirhinis* HAZ141_2 and its regulatory sites and features. (A) Schematic representation of the *aadE*-sat4-aphA-3* cluster. Gene designations: *aadE**, a partial homolog of *aadE* encoding streptomycin aminoglycoside 6-adenyltransferase (see the text for other details); *sat4* encoding streptothricin acetyltransferase; *aphA-3* encoding aminoglycoside O-phosphotransferase APH(3′)-IIIa (neomycin-kanamycin phosphotransferase type III). Promoters are designated P*, P1″$_{aphA-3}$, and P2$_{aphA-3}$. (B) Partial nucleotide sequence of the *aadE*-sat4-aphA-3* gene cluster (two hidden DNA stretches are indicated with the labels "480 bp not shown" and "720 bp not shown"). Deduced amino acid sequences of corresponding gene products are shown only for C- and N-terminal regions; fMet (formyl-methionine) is used for the first translated residue of each polypeptide. C-terminal tryptophane (W) residues of AadE* encoded by TGA (UGA) codons are shown as underlined boldface letters. The 5′- and 3′-terminal restriction sites, SphI (designed in this study) and HindIII (authentic), respectively, are those used for cloning and expression of the cluster in *E. coli*. Promoter elements are shown with −35 and −10 or ext. −10 (extended −10) above the underlined boldface italicized letters. Two 12-bp direct repeats flanking promoter P1″ are shown as boxed boldface letters. The experimentally validated two-nucleotide transcription start site is shown with +1 above the underlined boldface nucleotides. Predicted ribosome-binding sites are shown with RBS above the boldface italicized letters. Arrows indicate the positions and directions of transcription of the genes. Start and stop codons are shown in bold, with the latter marked with a triangle.

gene cluster *aadE*-sat4-aphA-3* and the stretch of 6 genes encoding type I RM enzymes were identified at the 5′ and 3′ ends of the prophage, respectively (Fig. 1). The *aadE-sat4-aphA-3* gene cluster, characterized so far in different Gram-positive and Gram-negative bacteria (22, 37–39), has never been described in *Mollicutes* (as of 20 September 2020). However, our BLASTN analysis identified the *aadE-sat4-aphA-3* locus in the recently published genome of the *Acholeplasmatales* human gut isolate UBA11505 (GenBank accession number DPOU01000014.1), but its neither presence nor its function were not discussed or mentioned by Parks et al. (40). Moreover, the *aadE* gene (GenBank accession number HCX08069.1) of the *Acholeplasmatales* isolate

encodes a full-size 302-aa AadE-like polypeptide, suggesting another gene-transferring source of this cluster.

The *aadE*-sat4-aphA-3* gene cluster confers high-level resistance to kanamycin and neomycin in *M. bovirhinis* HAZ141_2 and to kanamycin, neomycin, and nourseothricin *in E. coli* JM109 (pAC10) cells, while no expression of resistance to streptomycin was evident in either host system (Tables 1 and 2). The obtained phenotypic discrepancy between MIC values to nourseothricin in *M. bovirhinis* HAZ141_2 (16 to 32 μg/ml) and in *E. coli* transformants carrying pAC10 ($\geq$256 μg/ml) can be explained, at least partially, by the fact that in *M. bovirhinis* HAZ141_2, the extended 3′ end of *aadE** extensively (70 nucleotides) overlaps with a 5′ end of *sat4* and impairs the translational expression of the latter (Fig. 4). Moreover, a GC-rich GTG as a start codon for *sat4* might be an additional reason for translational silencing of this gene in mycoplasma cells. In addition, no significant difference in MICs to streptomycin was found among three *M. bovirhinis* strains (Table 1), and low MIC to streptomycin was identified in the recombinant *E. coli* pAC10 clone (Table 2). Actually, *M. bovirhinis* HAZ141_2 prophage-encoded AadE* is a chimeric protein with truncated N-terminal and extended C-terminal parts (Fig. 2). These changes, especially a lack of the regular AadE N-terminal part, apparently resulted in a loss of function, i.e., in a failure to confer resistance to streptomycin in either *M. bovirhinis* HAZ141_2 or *E. coli* (Tables 1 and 2). The presence of a similar *aadE**-like gene encoding a 206-aa polypeptide that did not provide resistance to streptomycin was previously identified on a chromosome of *C. coli* (41) and on conjugative plasmids from *Camplylobacter jejuni* (39) and *C. coli* (42).

Despite the fact that acquired resistance to kanamycin and neomycin mainly arises via inactivation of the drugs by aminoglycoside-modifying enzymes, the modifications of the aminoglycoside binding site (helix 44) located within the 16S rRNA by point mutations or methylation have also been reported (43–47). We did not identify any nucleotide differences within the primary and secondary 2-DOS aminoglycoside binding sites (4, 6, 48) comparing *rrs* and *rrl* genes, respectively, of *M. bovirhinis* HAZ141_2 and PG43 (Fig. S1).

Our analysis revealed the high identity (>99%) of the *M. bovirhinis aadE*-sat4-aphA-3* region to the genome of *Streptococcus pyogenes* strain NGAS322 (CP010449.1). *S. pyogenes* is almost exclusively restricted to humans, but several reports described identification of this pathogen from different mammals, including dogs, rabbits, sheep (49, 50), and even hedgehog (*Erinaceus europaeus* [51]), indicating the ability of *S. pyogenes* to colonize and infect these animals. One may hypothesize that *aadE*-sat4-aphA-3* cluster, as a part of some yet unknown *Streptococcus* phage, was transferred directly to *M. bovirhinis* HAZ141_2. Indeed, Palmieri et al. (23) described the *S. suis* phage phi-SsUD.1, which carries a related *aadE-sat4-aphA-3* cluster that encodes an intact 302-aa AadE. Another possible hypothesis is that this locus was first inserted in a prophage-like region present in other bacteria, whose sequences do still are not present in GenBank, and then transferred to *M. bovirhinis*. Such putative gene transfer could illustrate the exchange between evolutionarily distanced bacterial species and demonstrates the ability of HGT in *M. bovirhinis*. In any scenario, the *M. bovirhinis*-specific element IS*Mbvr1* (which encodes transposase MBVR141_0923) was then inserted between MBVR141_0922 and MBVR141_0925 (Fig. 1).

The distribution of the *aadE*-sat4-aphA-3* gene cluster among other *M. bovirhinis* strains is not known, and our preliminary attempts to detect this cluster by PCR in a cohort of strains isolated in Austria ($n = 17$) and Italy ($n = 32$) were not successful (data not shown). It might be that the prevalence of *M. bovirhinis* isolates carrying the *aadE*-sat4-aphA-3* gene cluster is low and/or restricted to the particular geographic region. The presence of *M. bovirhinis* with high MICs to kanamycin (>100 μg/ml) was previously reported from Japan (52, 53). In addition, *Mycoplasma bovis* strains with MICs of $\geq$64 μg/ml to kanamycin were also identified in Japan (52–54). Unfortunately, it was impossible to compare kanamycin susceptibility of bovine mycoplasmas isolated in Japan and that in other countries since kanamycin is usually not included in the panel

of antibiotics tested. Moreover, the susceptibility of *M. bovirhinis* isolates was almost solely tested in Japan.

The 3′-terminal module of the *M. bovirhinis* HAZ141_2 prophage-like region contains a cluster of 6 genes encoding type I RM enzymes (Fig. 1). Methylation is crucial in many aspects of cell life, such as metabolism, gene regulation, DNA replication and repair, and epigenetic programming, and it is often used as a defense system against invading foreign DNA (55). Therefore, such abundance of RMs within the prophage may limit other parasitic DNA from integrating into a prophage or/and into a chromosome of prophage-occupied mycoplasma. We can speculate that RMs may also help the prophage and themselves to establish a stable symbiosis between AT-rich mycoplasma genomes and GC-rich prophage regions or protect the prophage DNA from an auto-immune degradation. Moreover, RMs are known to cause genomic rearrangements by promoting homologous or site-specific recombination, generating bacterial diversity and regulating gene expression, for example, by phase variation as previously described in the case of *hsdS* genes in *Mycoplasma pulmonis* (56). The site-specific XerC family recombinase (MBVR141_1033) encoded by the RM cluster probably generates such recombinations and rearrangements (Fig. 1). In addition to the type I RM system, an ORF similar to *S*-adenosylmethionine synthetase MetK was identified in the *M. bovirhinis* HAZ141_2 prophage region (MBVR141_0990). The *metK*-like genes were annotated in certain genomes of *Firmicutes* viruses, including *S. suis* phage phi-SsUD.1 (23), *Staphylococcus* phage UPMK_1 (GenBank accession no. MG543995.1), *Faecalibacterium* phage FP_Brigit (GenBank accession no. MG711465.1), etc. The primary function of orphan MTases is to actively methylate phage DNA and protect it from multiple host-encoded restriction endonucleases (57), but more pleotropic regulatory roles have also been proposed (58, 59). We can also speculate that methyltransferases may play an additional role in conferring resistance to aminoglycosides in *M. bovirhinis*, as has been shown for other bacteria (60–62).

Conclusion. Our results revealed for the first time that *Mycoplasma* species can stably maintain a large composite GC-rich prophage-like genomic island that carries a highly conserved *aadE-sat4-aphA-3*-like gene cluster previously characterized in certain Gram-positive and Gram-negative pathogens. This cluster is expressed in mycoplasma cells conferring resistance to the aminoglycosides kanamycin and neomycin, but not to streptomycin and nourseothricin, although the *M. bovirhinis* HAZ141_2 *sat4* gene encoding streptothricin acetyltransferase is intact, and it does provide resistance to nourseothricin in *E. coli* recombinant clones. The expression of the *aadE-sat4-aphA-3* gene cluster in kanamycin/neomycin- and streptothricin-sensitive *M. bovirhinis* strain or other ruminant mycoplasma species would provide the final demonstration of the drug resistance phenotype conferred by this prophage-like genomic region. In addition, we suggest that other *Mycoplasma* species associated with both human and animal diseases be monitored for a presence of this transmittable gene cluster.

MATERIALS AND METHODS

***Mycoplasma bovirhinis* strains and growth conditions.** *M. bovirhinis* strain HAZ141_2, isolated from the nasal discharge of a coughing calf in Japan in 2008 (17), was kindly provided by Eiji Hata (National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan). *M. bovirhinis* strain 316981 was isolated from the lungs of cattle in Israel in 2018, and the *M. bovirhinis* PG43 type strain (NCTC 10118) was purchased from the National Collection of Type Cultures (NCTC; Public Health England, UK). All three isolates were propagated at 37°C and 5% CO_2 in modified Friis (FF) broth or agar medium (63). Stock cultures were aliquoted and maintained at −80°C. For each stock, the number of CFU per ml of was determined by performing serial 10-fold dilutions in FF broth and by plating each dilution on agar in triplicates.

Genomic DNA extraction and PCR amplification. *M. bovirhinis* genomic DNA was extracted from 10 ml logarithmic-phase broth cultures using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers were developed and commercially synthesized (Sigma-Aldrich, Rehovot, Israel) based on the nucleotide sequence of the *M. bovirhinis* strain HAZ141_2 genome (GenBank accession no. AP018135.1 [17]). The nucleotide sequences and locations of the oligonucleotide primers are given in Table S2. When needed, primers used in the PCRs or primers complementary to the internal sequences of the amplicons were used to complete the sequence of the PCR products (data not shown). PCRs were carried out in 50-μl volumes containing 50 to 100 ng of

template DNA, 1 μl of Phire Hot Start II DNA polymerase (Thermo Fisher Scientific, Waltham, MA), 5× Phire reaction buffer, 1 μl of 10 mM deoxynucleoside triphosphate (dNTP), and 0.4 μM each primer. PCR amplifications were carried out in a C1000 Touch thermal cycler (Bio-Rad, CA). PCR amplicons were purified using a PureLink quick PCR purification kit (Invitrogen, CA, USA). PCR amplicons used for cloning were extracted from the gels and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Sequencing was performed at the DNA Sequencing Unit of the Hebrew University (Jerusalem, Israel).

Enzymes and antibiotics. Restriction enzymes (SphI and HindIII) and T4 ligase were purchased from New England Biolabs (NEB) (MA, USA) and Promega, Inc., (Madison, USA), respectively, and used according to the manufacturer's recommendations. Antibiotics, chloramphenicol, gentamicin sulfate, kanamycin sulfate, neomycin trisulfate, spectinomycin hydrochloride, and streptomycin sulfate were purchased from Sigma (Sigma-Aldrich, Rehovot, Israel), and nourseothricin sulfate was purchased from ENCO Diagnostics Ltd. (Israel).

Cloning and transformation. To clone *aadE*-sat4-aphA-3* genes from *M. bovirhinis* HAZ141-2, forward primer aadE-F1-SphI containing the SphI site and reverse primer down-aphA3-R1-HindIII containing the HindIII site were used for PCR amplification of the genes (Table S2). The PCR amplification program was as follows: 35 cycles of denaturation at 98°C for 30 s, primer annealing at 58°C for 30 s, and extension at 72°C for 2 min. The resulting PCR fragment was purified, sequenced, cut with HindIII and SphI, and cloned into a derivative of the low-copy-number pACYC184 plasmid vector (gifted from M. Kolot, Tel Aviv University) cut with the same enzymes. The pACYC184 derivative was obtained by PCR using the pACYC184-F1-HindIII and pACYC184-R1-SphI primers (Table S2). The PCR amplification program was as follows: 35 cycles of denaturation at 98°C for 30s, primer annealing at 59°C for 30 s, and extension at 72°C for 2 min. The pACYC184 derivative product was 2,280 bp long and contained the p15A origin of replication as well as the *cat* gene encoding a chloramphenicol acetyltransferase responsible for chloramphenicol resistance (Cm^R). The recombinant clones and the pACYC184 plasmid itself were transformed into competent cells of *E. coli* strain JM109 (Promega Inc., Madison, USA). The transformants were plated on Luria-Bertani broth (LB) plates containing chloramphenicol (15 μg/ml). DNA of the recombinant plasmids was isolated using a PureLink quick plasmid miniprep kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The resulting recombinant plasmid pAC10 was completely sequenced on both strands (HU; Jerusalem, Israel).

Antimicrobial susceptibility testing. The *In vitro* susceptibility of *M. bovirhinis* strains to gentamicin, kanamycin, neomycin, spectinomycin, streptomycin, and nourseothricin was determined using the agar dilution method as previously described (64) with some modifications. Briefly, 2-fold dilutions of antimicrobials from 0.5 to 1,024 μg/ml were incorporated onto the FF agar plates, and 2.5 μl of each isolate, containing 1×10^5 to 1×10^6 CFU, was spotted onto the agar. Plates were incubated at 37°C with 5% CO_2 for 3 days. The procedure was repeated independently three times.

In vitro susceptibility of the host *E. coli* strain JM109 and that of its transformants carrying either the empty pACYC184 vector or the recombinant clone pAC10 containing the *aadE*-sat4-aphA-3* gene cluster from *M. bovirhinis* HAZ141_2 was tested using the agar dilution method following instructions of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (65). Briefly, overnight-grown cultures of *E. coli* JM109 and recombinant clones were diluted with LB broth to give 10^7 CFU/ml. The volume of the suspension drop was 2.5 μl, and it contained 10^4 CFU of *E. coli*. Bacterial growth of inoculates was determined after incubation at 37°C for 20 h.

RNA isolation, reverse transcription (RT), and RT-PCR. Total RNA was extracted from a mid-logarithmic-phase culture of *M. bovirhinis* strain HAZ141-2 using the RNeasy minikit (Qiagen, Hilden, Germany). The elimination of contaminating genomic DNA was performed during RNA isolation as specified by the manufacturer. In addition, after extraction, the RNA was treated with DNase I (NEB, USA) to remove any contaminated chromosomal DNA and then cleaned up using an RNA mini-prep column (Zymo Research, USA). The concentration of total RNA was determined using a NanoDrop system, and a ratio of about 2.0 was considered a good indication of purity. RT was performed with the RevertAid Moloney murine leukemia virus reverse transcriptase (M-MuLV; Promega, Inc., Madison, USA) using the aadE-R1, sat4-R1, and aphA3-R2 primers specific to the *aadE**, *sat4*, and *aphA-3* genes, respectively (Table S2). Briefly, about 1 μg total RNA and 20 μM oligonucleotide primer at the final volume of 15 μl were added to a microtube, incubated for 5 min at 70°C, and cooled on ice. dNTP (10 mM), M-MuLV buffer X1, RNase inhibitor (25 u; NEB, USA), and 200 u M-MuLV enzyme were added to the cocktail (25 μl final volume) and incubated for 60 min at 42°C. To inactivate the enzyme, the reaction was then incubated for 10 min at 70°C. Synthesized cDNA was stored at −20°C. For each RT-PCR, the negative control containing all reagent, except the M-MuLV enzyme, was included. The resultant cDNA products were subjected to PCRs using MyTaq DNA polymerase (Bioline) and different primers specific to the *aadE**, *sat4*, and *aphA-3* genes (Table S2). The PCR amplification program was as follows: 30 cycles of denaturation at 95°C for 15 s, primer annealing at 56°C for 15 s, and extension at 72°C for 45 s.

Rapid amplification of 5′ cDNA ends (5′-RACE) analysis. The 5′-RACE analysis was performed as previously described by Amram et al. (66) using a 2nd-generation 5′/3′-RACE kit (Roche Diagnostics GmbH, Mannheim, Germany) and according to the manufacturer's instructions. Briefly, total cellular RNA was extracted from mid-logarithmic-phase cultures of *M. bovirhinis* strain HAZ141-2 using the High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). The cDNA was obtained using 1 μg of DNA-free RNA of *M. bovirhinis* strain HAZ141-2 with either aadE-R1, sat4-R1, or aphA3-R2 primers complementary to the sequences of the *aadE**, *sat4*, and *aphA3* genes, respectively (Table S2). In the first 5′-RACE PCR, the cDNA products with a poly(A) tail were subjected to PCR using the forward

oligonucleotide(dT) anchor primer provided with the kit and either *aadE**-specific aadE-R2, *sat4*-specific sat4-R3, or *aphA3*-specific aphA3-R1 reverse primers (Table S2). In the nested 5′-RACE PCRs, an aliquot from the first 5′-RACE PCRs was amplified using the forward PCR anchor primer, provided with the kit, and the reverse aadE_R3-specific primer (Table S2). The final PCR products were purified using the MEGA quick-spin PCR and agarose gel extraction system (iNtRON Biotechnology, South Korea) and sequenced.

Computational analysis. BLAST analysis of protein and nucleotide sequences was performed using the NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The different functional domains were identified using the Pfam protein family database (http://pfam.xfam.org/), the Integrated Resource of Protein Domains (InterPro) (http://www.ebi.ac.uk/interpro/), and the database of protein families and domains PROSITE (https://prosite.expasy.org/). Primary DNA sequence analyses (GC content, DRs, dyad symmetries, etc.) were performed with either Clone Manager 9, professional edition, software (Scientific & Educational Software, Durham, NC) or DNASTAR software version 5.06/5.51, 2003 (Lasergene, Inc., Madison, WI). DNA promoter motif searches were performed with the Pattern Locator program (67) (http://www.cmbl.uga.edu/software/patloc.html). The search was carried out using the general motif for RpoD (SigA)-dependent bacterial promoters, which is TTGACA in the −35 element and TATAAT in the −10 element (or Pribnow box), with a spacer of 16 to 19 nucleotides between −35 and −10 (68, 69). Multiple sequence alignments were performed with the PRABI Lyon Gerland public servers for DNA sequences (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalwan.html) and for amino acid sequences (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html).

Data availability. The data supporting the findings of this study are available within the paper and its supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 2, PDF file, 3.3 MB.

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