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Mutations in 16S rRNA and Ribosomal Protein S5 Associated with High-Level Spectinomycin Resistance in *Pasteurella multocida*[▽]

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Pasteurella multocida **isolates with high-level spectinomycin resistance in which no adenyltransferase genes could be demonstrated exhibited a C1192G transversion in the 16S rRNA of all six or five of the six rRNA operons and/or two different types of 3-bp deletions in the *rpsE* gene that codes for the ribosomal protein S5.**

The most frequently detected mechanism of bacterial resistance to spectinomycin is the inactivation of the drug by adenyltransferases. Although adenyltransferase genes are widely distributed among bacteria isolated from animals, only one such gene, *aadA14*, in a bovine *Pasteurella multocida* isolate has so far been identified (11). Further studies of *P. multocida* isolates with high-level spectinomycin resistance showed that the *aadA14* gene is not widespread (11), and none of the other known adenyltransferase genes could be detected among such isolates (11, 19). Besides mechanisms of enzymatic inactivation, mutations in 16S rRNA conferring spectinomycin resistance on a wide variety of bacteria (1–3, 5, 8, 10, 13, 17), but also on chloroplasts of *Chlamydomonas reinhardtii* (9) and *Nicotiana* spp. (6, 20), have been described previously. All these 16S rRNA mutations are located in a specific region of helix 34. This region, encompassing the cross-linked positions 1063 to 1066 and 1190 to 1193 and known to be involved in spectinomycin binding, is tentatively referred to as the spectinomycin resistance-determining region (SRDR) within 16S rRNA. Furthermore, mutations in the protein S5, also known to be involved in the binding of spectinomycin to the bacterial ribosome, have been described previously (4, 7).

In the present study, we investigated 13 spectinomycin-resistant and 4 susceptible isolates for possible mutations in the *rpsE* gene and in the SRDR of 16S rRNA. All isolates were collected between 1999 and 2004 in individual cases of bovine respiratory tract infections and were investigated by SmaI macrorestriction analysis (12) and confirmed to be epidemiologically unrelated. MIC determination by broth macrodilution followed the specifications given in the document M31-A2 of the Clinical and Laboratory Standards Institute (CLSI) (15, 16) and confirmed that all 13 resistant isolates were highly resistant, with MICs of spectinomycin for these isolates ranging from 4,096 to >8,192 μg/ml, while MICs for the susceptible isolates were 16 μg/ml (Table 1).

Based on the knowledge of the whole genome sequence of *P. multocida* strain Pm70 (14), PCR assays which enabled the separate amplification of part of the 16S rRNA of each of the six rRNA operons of *P. multocida* were established. For these procedures, a common reverse primer was combined with forward primers specific for the six operons, designated A to F according to their order of occurrence in the Pm70 sequence (14). The primer sequences, annealing temperatures, and amplicon sizes are given in Table 2. A standard protocol was applied which consisted of an initial denaturation step for 1 min at 94°C followed by 34 cycles, each comprising 1 min of denaturation at 94°C, 2 min of annealing at the respective annealing temperature, and 3 min of primer extension at 72°C, and a final extension step for 7 min at 72°C. For the sequence analysis of the SRDRs of the six operons, the 20-mer primers 5′-GTAAGGAGGTGATCCAACCG-3′ and 5′-GGTAGTCC ACGCTGTAAACG-3 were used. The primers PmS5-fw and PmS5-rv (Table 2) served for the PCR amplification of an 862-bp fragment encompassing the entire 501-bp *rpsE* gene, which codes for the ribosomal protein S5. The *rpsE* amplicons were cloned into pCR-Blunt II-TOPO (Invitrogen, Groningen, The Netherlands) and sequenced completely by using standard M13 universal and reverse primers.

Comparative analyses of 16S rRNA and *rpsE* gene sequences revealed the presence of four different types of mu-

TABLE 1. MICs of spectinomycin for and mutations detected in the 13 spectinomycin-resistant and the 4 spectinomycin-susceptible *P. multocida* isolates

Isolate no.	MIC (μg/ml) of spectinomycin	Operons with C1192G transversion in 16S rRNA (no. of mutated operons/no. of operons present)	Mutation in S5 protein
1	>8,192	A, B, C, D, E, F (6/6)	
2	>8,192	A, B, C, D, E, F (6/6)	
3	>8,192	A, B, C, D, E, F (6/6)	
4	>8,192	A, B, C, D, E, F (6/6)	
5	>8,192	A, B, C, D, E, F (6/6)	
6	4,096	A, B, C, D, E (5/6)	
7	8,192	A, B, C, D, E (5/6)	
8	4,096	A, B, C, D, E (5/6)	
9	4,096	B, C (2/6)	32-SF-33 → 32-I
10	>8,192	B, C (2/6)	32-SF-33 → 32-I
11	>8,192	B, C (2/6)	32-SF-33 → 32-I
12	>8,192	B, C (2/6)	32-SF-33 → 32-I
13	4,096	None (0/6)	Loss of 23-K
14	16	None (0/6)	
15	16	None (0/6)	
16	16	None (0/6)	
17	16	None (0/6)	

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TABLE 2. PCR primers used for the separate amplification of parts of the six 16S rRNA genes and the *rpsE* gene

Gene and region	Primer designation	Sequence (5′ → 3′)	Amplicon size (bp)	Annealing temp (°C)
16S rRNA, operon A	16S RNA PmA-fw	GAGATAGTAGATACACCTCGCGTCG	1,740	60
16S rRNA, operon B	16S RNA PmB-fw	TGGATAGAGCGTTGGCCTCC	1,976	62
16S rRNA, operon C	16S RNA PmC-fw	CGCCTTGGCAGTCAATTCAG	2,179	58
16S rRNA, operon D	16S RNA PmD-fw	TCACAGGTGGAGAAACAGATACCA	2,055	60
16S rRNA, operon E	16S RNA PmE-fw	TGTGGTCAATTGAGTAATGCCTG	2,091	62
16S rRNA, operon F	16S RNA PmF-fw	CTATGTATTAGAGTCCATTGCGGATCT	1,935	60
16S rRNA, operons A to F	16S RNA Pm-rv	AGGAGGTGATCCAACCGCAG		
Gene for 30S ribosomal protein S5	30S RNA PmS5-fw	TGCATATGGCGAAGACCAAG	862	55
	30S RNA PmS5-rv	AAGTGATTGCACCGAACGG		

tations. The five isolates with the first type of mutation exhibited a C1192G transversion in all six operons, while all other positions so far known to be associated with spectinomycin resistance remained unchanged (Fig. 1a). The three isolates carrying the second type of mutation had this alteration in five of the six operons (Table 1). These eight *P. multocida* isolates did not reveal any structural alteration in the *rpsE* gene (Table 1). The third type of alteration, represented by four isolates, was characterized by the presence of the aforementioned transversion in only two of the six operons and an additional 3-bp deletion in the *rpsE* gene which resulted in the change of Ser32Ile and the loss of the subsequent amino acid Phe33 (Fig. 1b). In the single isolate representing the fourth type of alteration, no mutation in the SRDR of any of the six rRNA operons was detectable. However, a different 3-bp deletion in the *rpsE* gene which caused the loss of the amino acid Lys at position 23 was present (Fig. 1b). In contrast to the highly resistant isolates, all four susceptible isolates did not exhibit mutations in the SRDR of any rRNA operon or in the *rpsE* gene.

Studies of *Chlamydophila psittaci* revealed that mutations at position 1192 have only very minor effects on the organism's biological fitness (1). A similar observation for *Escherichia coli* has also been reported previously (17). The growth curves determined in this study confirmed that *P. multocida* isolates exhibiting the C1192G mutation did not differ in their generation time from isolates not exhibiting this mutation (data not shown). The amino acids 19 to 33 in the N terminus of the

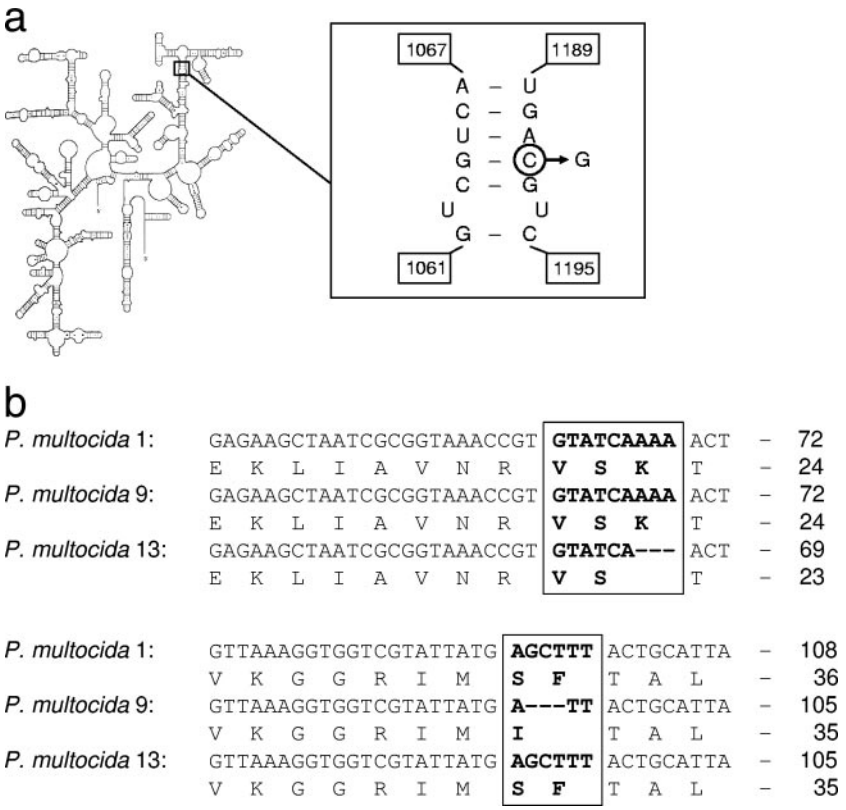

FIG. 1. (a) Secondary structure of 16S rRNA with the site of mutation in helix 34 depicted in detail. (b) Nucleotide and amino acid alignment of the 5′ ends of the *rpsE* genes and the corresponding amino acid sequences of ribosomal proteins S5 of the three representative *P. multocida* isolates 1, 9, and 13. Amino acids are given in the single-letter code. Boxes and boldface type indicate the altered regions.

ribosomal protein S5 form a loop structure which represents the RNA-binding region (4). Thus, the loss of the highly conserved Phe at position 33 accompanied by the exchange of Ser at position 32 for Ile may have an impact on the interaction of the S5 protein with helix 34 of the 16S rRNA and thus contribute to spectinomycin resistance. Early studies of spectinomycin resistance, which were based on the determination of net charges of peptides, identified the exchange of Val for Glu at position 21 or the exchange of Ser for Pro at position 22 (7). More recent studies using matrix-assisted laser desorption ionization–time of flight analyses also identified the Ser22Pro alteration (21). The amino acid positions 20 to 22 are known sites of mutations that produce spectinomycin resistance in *E. coli*, while the conserved Lys at position 23 is believed to interact with 16S rRNA (18). Thus, the loss of this conserved Lys residue detected in a single *P. multocida* isolate in this study is believed to have a negative impact on the binding of the mutated S5 protein to 16S rRNA. The observed high MIC of spectinomycin of 4,096 μg/ml for the respective *P. multocida* isolate may support this assumption.

In summary, this is to the best of our knowledge the first report of mutations in 16S rRNA and the ribosomal protein S5 associated with spectinomycin resistance in *P. multocida*. These findings indicate that in addition to enzymatic inactivation, mutations in 16S rRNA and/or ribosomal protein S5 are an efficient way to render *P. multocida* isolates highly resistant to spectinomycin.

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