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Molecular Basis of Macrolide, Triamilide, and Lincosamide Resistance in *Pasteurella multocida* from Bovine Respiratory Disease▽

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The mechanism of macrolide-triamilide resistance in *Pasteurella multocida* has been unknown. During whole-genome sequencing of a multiresistant bovine *P. multocida* isolate, three new resistance genes, the rRNA methylase gene *erm*(42), the macrolide transporter gene *msr*(E), and the macrolide phosphotransferase gene *mph*(E), were detected. The three genes were PCR amplified, cloned into suitable plasmid vectors, and shown to confer either macrolide-lincosamide resistance [*erm*(42)] or macrolide-triamilide resistance [*msr*(E)-*mph*(E)] in macrolide-susceptible *Escherichia coli* and *P. multocida* hosts.

Pasteurella multocida is one of the economically most relevant bacterial pathogens involved in bovine respiratory disease (9, 14). Antimicrobial agents are commonly applied to control respiratory tract infections in cattle (9, 14). Based on the available susceptibility data, most bovine *P. multocida* strains are still susceptible to newer antimicrobial agents, including the 15-membered ring macrolide tulathromycin (4, 7, 16). Since tulathromycin has a unique chemical structure characterized by the presence of three amine groups, it has been referred to in the literature as the first representative of the triamilide subclass among the macrolides (3). Tulathromycin was approved in 2005 for use in the treatment and control of bovine respiratory disease and the treatment of swine respiratory disease in the European Union and the United States (3). Since its approval, continuous surveillance of the susceptibility status of the target pathogens has been conducted by Pfizer Animal Health, and from 2005 on, single tulathromycin-resistant strains have been detected. Previous analyses, which included bioassays for drug inactivation, efflux pump inhibitor assays, PCR assays for the known macrolide resistance genes, and sequencing of the genes for ribosomal proteins L4 and L22 as well as the separate amplification of part of the six rRNA operons with subsequent sequencing of the domain V of the 23S rRNA, did not elucidate the mechanism of tulathromycin resistance in these *P. multocida* strains (15).

To identify the mechanism(s) of macrolide resistance in *P. multocida*, the plasmid-free, tilmicosin- and tulathromycin-resistant *P. multocida* strain 36950, obtained from a case of bovine respiratory tract infection in a Nebraska feedlot in 2005, was subjected to whole-genome sequencing. For this, genomic DNA was prepared as previously described (10). A shot-gun library and a long-tag paired-end library were produced using the 454 Life Sciences (Roche) GS-FLX system (Eurofins MWG, Ebersberg, Germany). A matched assembly against the sequence of the largely susceptible *P. multocida* strain Pm70 (11) was performed to identify contigs that differed from the Pm70 sequence. These contigs, which were the most likely ones to contain resistance genes, were analyzed with the BLAST programs available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The matched assembly identified ca. 220 small contigs of 1.5 to 25.7 kb that differed from the Pm70 sequence. One of these contigs contained a gene for an Erm-like rRNA methylase, while another contig harbored two genes organized in an operon structure: a gene for an Msr-like ABC transporter and a gene for a Mph-like macrolide phosphotransferase. These three genes received the novel designations *erm*(42), *msr*(E), and *mph*(E) from the macrolide-lincosamide-streptogramin (MLS) nomenclature center (http://faculty.washington.edu/marilynr/).

The *erm*(42) gene codes for a protein of 301 amino acids (aa) which is only distantly related (28% identity) to the 284-aa Erm(X) proteins of *Bifidobacterium thermophilum* (19) and *Corynebacterium diphtheriae* (18). However, during the course of this study, part of a sequence of a resistance plasmid from a fish-pathogenic *Photobacterium damselae* subsp. *piscicida* isolate has been released (DDBJ accession number BAJ34818). This sequence contained a gene for an erythromycin resistance protein of 303 aa which showed 99.3% identity to the Erm(42) protein from *P. multocida*. The *msr*(E) gene codes for a protein of 491 aa which showed identities of 60.6% to Msr(D) (GenBank accession number AF227521), 36.8% to Msr(C) (GenBank accession number AY004350), and 22.3% to Msr(A) (EMBL accession number X52085). The *mph*(E) gene coded for a protein of 294 aa, which proved to be 37.8 and 38.1% identical to Mph(A) variants (GenBank accession number U36578 and DDBJ accession number D16251, respectively), 27.2% identical to Mph(B) (DDBJ accession number D85892) and 26.7% identical to Mph(C) (GenBank accession number AB013298). The *msr*(E) and *mph*(E) genes are separated by a spacer sequence of 55 bp that contains the *mph*(E)-associated ribosome binding site, but no other regulatory elements. As a consequence, both genes are likely to be

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TABLE 1. PCR primers and amplicon sizes[a]

Gene region	Primer direction[b]	Primer sequence (5′ → 3′)	Amplicon size (bp)
erm(42)	fw	GGGTGAAAAGGGCGTTTATT	1,254
	rv	ACGTTGCACTTGGTTTGACA	
msr(E)-*mph*(E)	fw	TACCGGAACAACGTGATTGA	3,277
	rv	GAAGGGTTACGCCAGTACCA	

[a] The annealing temperature for both gene regions was 55°C.
[b] fw, forward; rv, reverse.

transcribed from a promoter upstream of *msr*(E). A putative promoter structure with a −35 sequence (TTGGAC), a −10 sequence (TTTAAT), and an A at position +1 is available in this *msr*(E) upstream region. A BlastN search revealed that these two genes (including the spacer) have previously been found on plasmids in *Klebsiella pneumoniae* (17), *Acinetobacter baumannii* (12, 20), *Citrobacter freundii* (5), *Escherichia coli* (6), and an uncultured bacterium from a sewage treatment plant (13). It should be noted that the *msr*(E) and *mph*(E) genes have been named *mel* and *mef*(E) or *mph* or *mph2*, respectively, in the aforementioned publications.

To confirm their role in macrolide resistance, the *erm*(42) gene as well as the *msr*(E)-*mph*(E) genes and their flanking regions were amplified by PCR (Table 1) and resequenced to confirm identity with the sequence obtained by whole-genome sequencing. Different approaches were conducted for cloning these amplicons in plasmid vectors that replicate well in *E. coli* and in *P. multocida*. For expression in the macrolide-susceptible *E. coli* AS19, vector pCR2.1-TOPO and the TA cloning kit (Invitrogen, Groningen, Netherlands) were used. For expression in the pan-susceptible *P. multocida* B130, the blunt-ended *erm*(42) amplicon was cloned into the EcoRV site of plasmid pLS88 (2), while the *msr*(E)-*mph*(E) amplicon was inserted into the EcoRV site within the tetracycline resistance gene *tet*(L) of plasmid pCCK3259 (8). The selection of *E. coli* AS19 and *P. multocida* B130 transformants occurred overnight on Luria-Bertani agar supplemented with 10 μg/ml erythromycin and 5% (vol/vol) sheep blood (for *P. multocida* B130). The transformants were confirmed by plasmid profiling. Antimicrobial susceptibility testing of the original *P. multocida* strain 36950 and of the transformants in both recipient strains was performed at least three times on independent occasions by broth microdilution according to document M31-A3 of the Clinical and Laboratory Standards Institute (1) using custom-made microtiter plates (MCS Diagnostics, Swalmen, Netherlands). *Staphylococcus aureus* ATCC 29213 served as the quality control strain. For erythromycin, concentrations from 0.015 to 32 μg/ml were tested, whereas for tilmicosin, tulathromycin, and clindamycin, the test concentrations ranged from 0.03 to 64 μg/ml.

Comparative analysis of the MIC values of the recipient strains with and without the cloned *erm*(42) gene (Table 2) showed up to 128-fold increases in the MICs of erythromycin, tilmicosin, and clindamycin when the *erm*(42) gene was present in either one of the two recipient strains. In contrast, expression of *erm*(42) resulted in only moderate 8- or 4-fold increases in the MICs of tulathromycin in the *P. multocida* and *E. coli* recipients, respectively. As expected, *erm*(42) conferred a mac-

TABLE 2. MICs of the original *P. multocida* 36950 and *P. multocida* B130 and *E. coli* AS19 with and without a cloned *erm*(42) or *msr*(E)-*mph*(E) amplicon

Antimicrobial agent	MIC (μg/ml) in the *P. multocida* host					MIC (μg/ml) in the *E. coli* host			
	P. multocida 36950	*P. multocida* B130	*P. multocida* B130 + pLS88	*P. multocida* B130 + pLS88 + cloned *erm*(42)	*P. multocida* B130 + pCCK3259	*P. multocida* B130 + pCCK3259 + cloned *msr*(E)-*mph*(E)	*E. coli* AS19	*E. coli* AS19 + pCR2.1	*E. coli* AS19 + pCR2.1 + cloned *erm*(42)	*E. coli* AS19 + pCR2.1 + cloned *msr*(E)-*mph*(E)
Erythromycin	≥64	4	4	≥64	4	≥64	0.5	0.5	≥64	≥64
Tilmicosin	≥128	4	4	≥128	4	≥128	1	1	32	8
Tulathromycin	≥128	2	2	16	1	≥128	8	8	32	≥128
Clindamycin	≥128	16	16	≥128	16	16	8	8	≥128	8

rolide-lincosamide resistance phenotype. Clones carrying the *msr*(E)-*mph*(E) amplicons exhibited up to 128-fold increases in the MICs of erythromycin, tilmicosin, and tulathromycin, whereas the clindamycin MIC remained unchanged in both hosts (Table 2). This observation strongly suggested that the substrate spectrum of *msr*(E)-*mph*(E) includes macrolides and triamilides, but not lincosamides.

In conclusion, to the best of our knowledge, this is the first report on the genetics of macrolide, triamilide, and lincosamide resistance in *P. multocida*. The observation that the three genes identified have—even only rarely—also been seen in other bacteria supports the assumption that *P. multocida* is able to acquire mostly plasmid-borne resistance genes from other Gram-negative bacteria. Further work is warranted to elucidate the exact mechanisms by which these resistance genes have become integrated into the chromosomal DNA of *P. multocida* strain 36950.

Nucleotide sequence accession numbers. The sequence of the *erm*(42) gene has been deposited in the EBI database under accession number FR734406, and the sequence of the *msr*(E)-*mph*(E) genes has been deposited in the EBI database under accession number FR751518.

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