Applied and Environmental Microbiology, Oct. 2011, p. 7147–7150 Vol. 77, No. 20
0099-2240/11/$12.00 doi:10.1128/AEM.00560-11

Molecular Basis of Sulfonamide and Trimethoprim Resistance in Fish-Pathogenic *Aeromonas* Isolates[▽]

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Received 11 March 2011/Accepted 14 June 2011

Sulfonamide-trimethoprim-resistant *Aeromonas salmonicida* and motile *Aeromonas* spp. from diseased fish of the GE*RM*-Vet study carried the *sul1* gene together with mostly cassette-borne trimethoprim resistance genes, including the novel gene *dfrA28*. The seven *dfrA* and *dfrB* genes identified were located mostly in class 1 integrons which commonly harbored other gene cassettes.

Aeromonads play an important role as pathogens not only in fish but also in humans and other animals (5). In humans and animals, members of the genus *Aeromonas* may be involved in a variety of intestinal and extraintestinal diseases (5, 14). In fish, the nonmotile *Aeromonas salmonicida* is considered an obligate pathogen and causes a typical furunculosis among salmonids characterized by deep abscesses and hemorrhages on the skin and mouth. Among nonsalmonids, *A. salmonicida* causes atypical furunculosis, also known as "goldfish ulcer" (23). Motile aeromonads, including *A. hydrophila*, *A. veronii*, *A. sobria*, *A. bestiarum*, and *A. caviae*, are considered facultative pathogens and mainly affect immunocompromised fish, causing superficial to deep skin lesions which may progress to ulcers, necrosis, or hemorrhagic septicemia (9). Infections caused by motile aeromonads are probably the most common bacterial disease of freshwater fish (12). Antimicrobial resistance genes, including cassette-borne resistance genes in class 1 integrons, have been described as occurring in *A. salmonicida* and in motile aeromonads (4, 8, 16–18).

Fish-pathogenic bacteria were included for the first time in the German national resistance monitoring program GE*RM*-Vet in 2005 to gain insight into their antimicrobial susceptibilities. A total of 186 fish-pathogenic aeromonads, 173 motile aeromonads and 13 *A. salmonicida* isolates, collected from all over Germany between January 2005 and October 2006 from different disease conditions of commercially reared fish and ornamental fish, were tested for their antimicrobial susceptibilities by broth microdilution according to CLSI document M49-A (2). Since only a combination of trimethoprim-sulfonamide is approved for antimicrobial therapy of fish in Germany, a particular focus was set on resistance to trimethoprim as well as trimethoprim-sulfamethoxazole (SXT) (1:19) and the molecular basis of SXT resistance. Sulfonamides alone were not included in the GE*RM*-Vet test panel. Resistance to trimethoprim is often associated with gene cassettes located in class 1 or class 2 integrons, and the sulfonamide resistance gene *sul1* is part of the 3′-conserved segment of class 1 integrons. Thus, all SXT-resistant isolates were screened by PCR for class 1 and class 2 integrons and associated gene cassettes (6). Same-size amplicons were compared by restriction analysis, and at least one representative of each amplicon type was cloned and sequenced completely. Plasmid pPCR-Script Amp SK(+) (Stratagene) or pCR-Blunt (Invitrogen) served as the cloning vector, and the *Escherichia coli* strain XL-10-Gold Kan or *E. coli* strain One Shot TOP10 served as the recipient. Sequence analysis started with the M13 reverse and forward primers and was completed by primer walking. In addition, the resi. stance phenotype conferred by the cassette-located genes was confirmed in the *E. coli* clones by susceptibility testing according to CLSI document M31-A3 (3). The species of the SXT-resistant isolates were determined by PCR amplification of an internal part of the *gyrB* gene with subsequent sequence analysis (24).

High SXT MIC values of >32/608 μg/ml were seen for 33 *Aeromonas* species isolates and two *A. salmonicida* isolates, all of which also exhibited trimethoprim MICs of >128 μg/ml. All 35 isolates carried the sulfonamide resistance gene *sul1*, as confirmed by PCR. Of these, 29 isolates (27 *Aeromonas* species and the 2 *A. salmonicida* isolates) harbored a single class 1 integron while 4 *Aeromonas* sp. isolates carried two class 1 integrons. No class 2 integrons were detected. Among the class 1 integrons, gene cassettes with the seven different trimethoprim resistance genes, *dfrA1*, *dfrA12*, *dfrA14*, *dfrA28*, *dfrB1*, *dfrB3*, or *dfrB4*, were identified by sequence analysis. PCR analysis confirmed the presence of the trimethoprim resistance gene *dfrA1* outside a class 1 integron in four SXT-resistant *Aeromonas* sp. isolates. Two of these isolates, however, carried class 1 integrons with *catB3-aadA2* gene cassettes. Twelve different gene cassette arrangements were detected (Fig. 1 and Table 1). The identified genes located in these gene cassettes matched the resistance phenotypes of the *Aeromonas* isolates and of the *E. coli* clones. The *dfrA* or *dfrB* gene cassettes were usually accompanied by gene cassettes conferring other resistance properties or by *orfV* or *orfF* cassettes whose functions are unknown (Table 1). The most frequently detected cassette combination was *dfrA12*-

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▽ Published ahead of print on 15 July 2011.

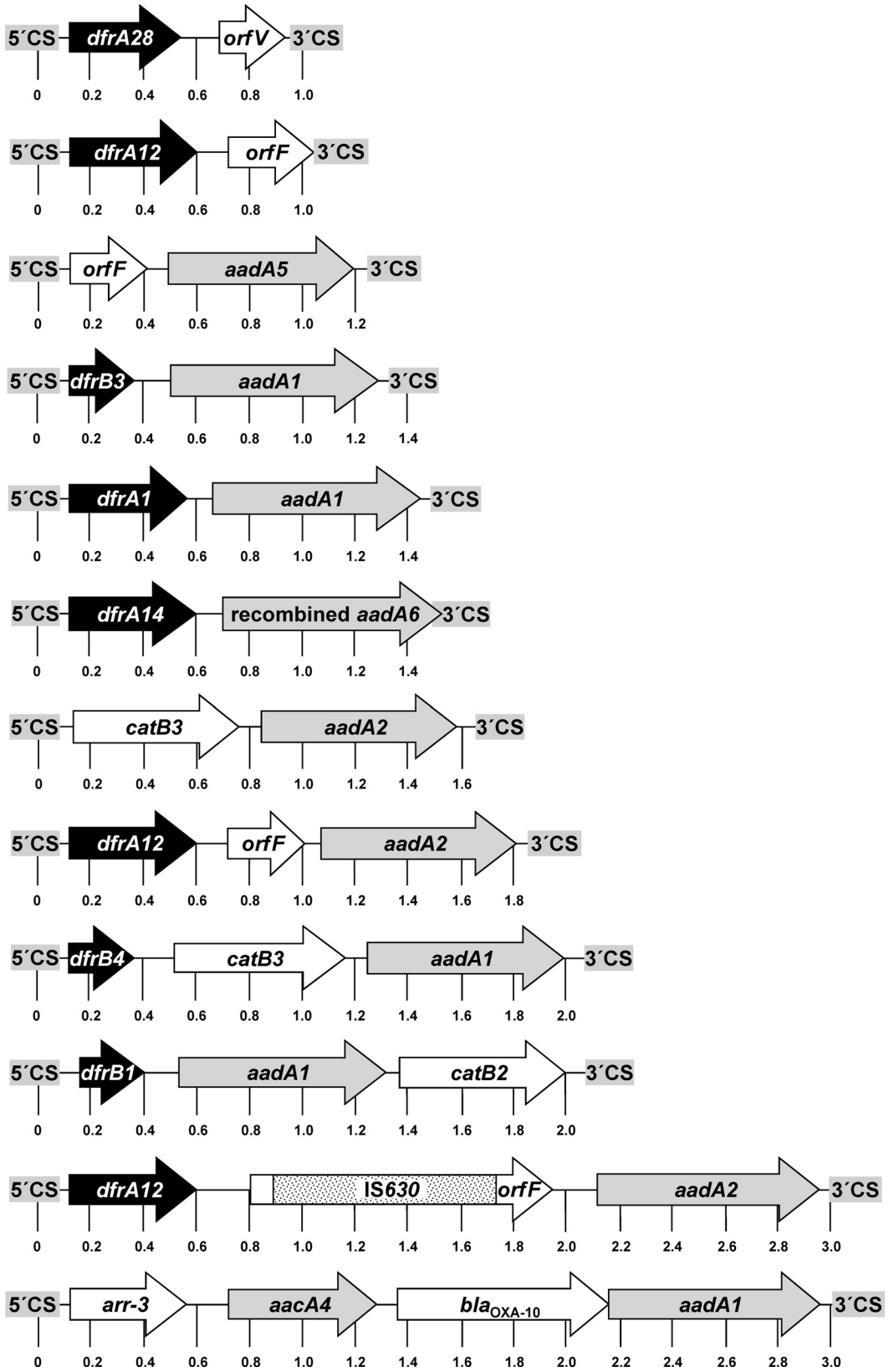

FIG. 1. Schematic presentation of the 12 gene cassette arrangements detected among the *A. salmonicida* and motile *Aeromonas* species isolates. The reading frames are shown as arrows, with the arrowhead indicating the direction of transcription. Trimethoprim resistance genes are shown in black, and aminoglycoside resistance genes are in gray. A distance scale in kb is given below each map. The 5′ conserved and the 3′ conserved segments of the class 1 integrons are depicted as "5′CS" and "3′CS" on a gray background.

TABLE 1. Presence of different gene cassettes within class 1 integrons among the 33 SXT-resistant motile aeromonads and the two *A. hydrophila* isolates

Gene cassette combination	Associated resistance phenotype	No. of isolates	Origin(s) (*n*)[a]	Aeromonad(s) in which combination was detected (*n*)[b]	EMBL accession no.
dfrA28-orfV	Trimethoprim	1	Koi carp	*A. veronii* biovar sobria	FM877476
dfrA12-orfF	Trimethoprim	1	Koi carp	*A. veronii* biovar sobria	FM877477
orf-aadA5	Streptomycin-spectinomycin	1	Koi carp	*A. veronii* biovar sobria	FM877481
dfrB3-aadA1	Trimethoprim, streptomycin-spectinomycin	1	Koi carp	*A. sobria*	FM877478
dfrA1-aadA1	Trimethoprim, streptomycin-spectinomycin	4	Koi carp (2), goldfish, salmon	*A. sobria*, *A. veronii* biovar sobria, *A. veronii* biovar veronii, *A. salmonicida*	FM877479
dfrA14-recombined *aadA6*	Trimethoprim, streptomycin-spectinomycin	1	Ram cichlid	*A. sobria*	FM877480
catB3-aadA2	Chloramphenicol, streptomycin-spectinomycin	3	Koi carp (3)	*A. hydrophila* (2), *A. bestiarum*	FM877482
dfrA12-orfF-aadA2	Trimethoprim, streptomycin-spectinomycin	19	Koi carp (13), goldfish, rainbow trout (2), nase, ND (2)	*A. hydrophila* (11), *A. bestiarum* (2), *A. caviae*, *A. veronii* biovar sobria (4), *A. veronii* biovar veronii	FM877483
dfrB4-catB3-aadA1	Trimethoprim, chloramphenicol, streptomycin-spectinomycin	1	Koi carp	*A. veronii* biovar veronii	FM877484
dfrB1-aadA1-catB2	Trimethoprim, streptomycin-spectinomycin, chloramphenicol	2	Rainbow trout, ND	*A. sobria*, *A. salmonicida*	FM877485
dfrA12-orfF with integrated IS*630-aadA2*	Trimethoprim, streptomycin-spectinomycin	1	Koi carp	*A. veronii* biovar veronii	FM877486
arr-3–aacA4–bla$_{\text{OXA-10}}$*–aadA1*	Rifampin, gentamicin, penicillins, streptomycin-spectinomycin	1	Koi carp	*A. hydrophila*	FM877487

[a] ND, no data available; *n*, no. of isolates; koi carp, *Cyprinus carpio*; goldfish, *Carassius auratus*; salmon, *Salmo salar*; ram cichlid, *Mikrogeophagus ramirezi*; clown loach, *Chromobotia makracanthus*; rainbow trout, *Oncorhynchus mykiss*; nase, *Chondrostoma nasus*.
[b] The species was determined by sequencing an internal part of the *gyrB* gene (24). *n*, no. of isolates.

orfF-aadA2, present in 19 isolates, followed by *dfrA1-aadA1*, present in 4 isolates.

The *dfrA28* gene was a novel *dfrA* gene identified for the first time during this study. The *dfrA28* gene cassette had a total size of 562 bp and a 59-base element of 82 bp. The *dfrA28* gene had a GTG start codon and comprised 474 bp. It coded for a dihydrofolate reductase of 157 amino acids. Identities at the nucleotide and amino acid sequence levels to the most-related *dfrA27* gene and the DfrA27 protein from *E. coli* (GenBank accession no. EU675686) were 96% and 97%, respectively. Downstream of the *dfrA28* cassette, a structure was detected which showed, at least in the 5′-terminal region, similarities to a gene cassette. The reading frame *orfV*, 249 bp in size, did not exhibit any similarities to known reading frames and ended in the 3′ conserved segment of the class 1 integron. A 59-base element was not detectable. The combination *dfrA12-orfF-aadA2* with the insertion sequence IS*630* integrated into *orfF* and the cassette combination *arr-3-aacA4-bla*$_{\text{OXA-10}}$*-aadA1* also have not been described before.

However, most of the other cassette combinations have been observed in class 1 integrons of bacteria from fish or other animals. Identical or closely related *dfrA12-orfF* and *catB3-aadA2* combinations have been found in *A. hydrophila* from foodborne outbreak-suspect samples and environmental sources in Taiwan (1). The *aadA5* cassette in the *orf-aadA5* combination was indistinguishable from an *aadA5* cassette found in a class 1 integron of *E. coli* from a catfish (11). The *dfrA1-aadA1* combination is widespread among Gram-negative bacteria, and very similar gene cassettes have been detected in *Salmonella enterica* serovar Infantis from swine (13), *E. coli* from swine (6), *Salmonella enterica* serovar Typhimurium from horses (21), and *E. coli* from a wastewater treatment plant (10). The combination of *dfrA14* and recombined *aadA6* cassettes has previously been detected in *E. coli* of porcine origin (6). The combination *dfrA12-orfF-aadA2* has been seen in *Salmonella* Typhimurium from imported seafood (7), *Salmonella enterica* serovar Schwarzengrund from chicken (22), and *A. hydrophila* (15) or *E. coli* (6) from swine. The combination *dfrB1-aadA1-catB2* was seen as part of larger *bla*$_{\text{VIM-1}}$-carrying integron structures in *Klebsiella pneumoniae* (19) and *E. coli* (20).

This study provided for the first time data on the SXT susceptibility status of fish-pathogenic aeromonads from Germany and the trimethoprim and sulfonamide resistance genes present. The association of SXT resistance with class 1 integrons, which in part carried gene cassettes for other resistance properties, bears the risk of coselection and persistence of other resistance genes under the selective pressure imposed by the use of trimethoprim-sulfonamide combinations. The observation that similar or even identical gene cassettes have been detected in bacteria from fish, humans, food-producing animals, and/or companion animals underlines the presence of a common resistance gene pool.

ACKNOWLEDGMENTS

We thank Anne-Kathrin Hinz und Katharina Stolz for excellent technical assistance.

This study was financially supported by the Bundesverband für Tiergesundheit e.V. (BfT).

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