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## Homology of Bacterial Antimicrobial Resistance Genes From Different Origins

Romet® is an antimicrobial agent frequently used to treat enteric septicemia of catfish caused by *Edwardsiella ictaluri* and furunculosis in salmonid fishes caused by *Aeromonas salmonicida*. In recent years, Romet®-resistant bacterial strains of *E. ictaluri* and *A. salmonicida* have become more prevalent, rendering antimicrobial therapy with this agent useless. Furthermore, because of the development of multiple resistances, some bacterial strains may also be resistant to the only other approved and available agent, Terramycin®.

### Background

Our earlier studies have shown that, in some strains, resistance to Romet® is afforded by presence of a transferable R-plasmid (~55 kilobases). Furthermore, we have demonstrated that R-plasmids from resistant strains of *E. ictaluri* and *A. salmonicida* are very similar, if not identical, to each other as well as to R-plasmids from a previously described *Escherichia coli* 1898. The *E. coli* R-plasmids were from a 1986 equine cystitis case and were originally shown to be resistant to Tribrissen®, a potentiated sulfonamide (as is Romet®). Thus, three different bacterial species--each from different hosts and geographic origins--apparently possess the same molecular basis for resistance but do not share an obvious means for transfer among themselves. We believed that they may share a common source for their R-plasmid resistance.

### Isolation of Antimicrobial Resistance Genes

In our study, we isolated the R-plasmid genes conferring resistance to Romet® from strains of *E. ictaluri, A. salmonicida,* and *E. coli* using DNA primers constructed from sulfonamide resistance gene sequences sul I and sul II cataloged in GenBank (National Institutes of Health National Center for Biotechnology Information database). Homology of isolated genes was compared among the three species and to sul I and sul II by determining the DNA sequences and subsequently performing sequence analysis comparisons.

Each bacterial strain (n = 10) was grown in 125 mL of Mueller Hinton Broth spiked with 30 mg Romet®/mL; incubation was for 48 h at 37 °C for *E. coli* and 26 °C for all other strains. A modified maxi prep protocol (Qiagen Inc., Chatsworth, California) was used for isolation of R-plasmid DNA. Cells were centrifuged, the supernatant discarded, and 10 mL buffer P1 (50 mM Tris, 10 mM EDTA, 100 mg Rnase/mL, pH 8.0) was added to resuspend the cells. Ten mL of buffer P2 (200 mM NaOH, 1% SDS) was added to lyse the cells and release DNA; after incubating 5 min, the reaction was stopped with 10 mL of buffer P3 (3M $KC_2H_3O_2$, pH5.5). Samples were incubated on ice for 10 min,

then centrifuged and 0.7 volumes of isopropyl alcohol added to the supernatant to precipitate the DNA. Bacterial DNA from each isolate was used in polymerase chain reactions (PCR) with DNA primers constructed from sul I and sul II gene sequences. GenBank was searched for sulfonamide resistance gene sequences, yielding two consensus sequences, sul I and sul II. These sequences were imported into DNA sequence analysis software (PCGene, IntelliGenetics, release 6.70), and suitable PCR primers were developed.

Gene sequences of *Enterobacteriaceae* (Accession no. X12869) and *E. coli* (Accession no. X15024) were used as a primer source for sul I, and plasmid pGS05 (Accession no. M36657) was used for sul II. Standard PCR reactions were done with 34 cycles, and resulting PCR products were subjected to electrophoresis in 1% agarose gels at 66 V for 70 min. A 100-base pair DNA ladder served as a reference marker (Bayou Biolabs, Harahan, Louisiana). Positive PCR products (i.e., bands in the gel) that were sul I and sul II gene fragments were removed and purified using PCR purification columns (Qiagen).

## DNA Sequence Determination

The PCR denaturation, annealing, and elongation cycle for the sequencing reactions was repeated 25 times. A standard control sequencing reaction (pGem) was used as a positive control. To end the sequencing program, the thermocycler was programmed to "soak" (4 °C for infinite). Sequence mixtures were purified to remove excess dNTPs using Centrisep columns (Princeton Separations, Adelphia, New Jersey) and the DNA sequences determined using the ABI 310 Automated Sequencer (Applied Biosystems, Perkin Elmer, Foster City, California). Sequences obtained from the isolated resistance genes from our test strains were analyzed to obtain consensus sequences for both sul I and sul II. To determine homology, these sequences were compared to each other and to sequences from the GenBank Accession sequences using Sequence Navigator (Applied Biosystems, Perkin Elmer, Foster City, California).

## Resistance Genes from Various Origins Shared Highly Conserved DNA Sequences

All Romet®-resistant strains yielded positive PCR products for both sul I and sul II, with the exception of *A. salmonicida* strain MI1; this strain was indeed resistant to Romet® but lacked demonstrable sul I or sul II genes. In agarose gels, sul I gene PCR products were about 900 bp (basepairs) and sul II genes were approximately 400 bp. None of our test strains possessed only one of the genes--they had either both or none.

The sul I PCR fragments from our strains of *A. salmonicida, E. ictaluri*, and *E. coli* demonstrated 100% identity to each other. When a sul I consensus from these strains was further compared to the sul I GenBank sequence, they were calculated to be 99.8% homologous, differing at only one of the 603 bases sequenced. Similar results were obtained from evaluation of sul II genes. Sul II PCR products of our test strains showed a 95%-100% range in homology. When we compared our calculated sul II consensus sequence to that of GenBank, a 97.5% homology resulted from different nucleotides at only 10 of 407 bases. The significance of these sequence similarities is that both sul genes are apparently conserved not only within one bacterial species, but also across different species and even across different hosts and geographic origins. We postulate that these two genes are being transferred via conjugation from R-factors (e.g., R-plasmid) present in environmental bacteria. With a source of an R-factor, along with use or perhaps overuse of an antimicrobial to create selection pressure, bacterial pathogens might readily acquire resistance, resulting in a situation where there is no option for therapy.

**For further information, contact:**

Scott Rodgers or Clifford E. Starliper
National Fish Health Research Laboratory
USGS Leetown Science Center
1700 Leetown Road
Kearneysville, West Virginia 25430
(304)724-4433
cliff_starliper@usgs.gov

or

Richard Cooper
Department of Veterinary Science
Louisiana Agricultural Experimental Station
Louisiana State University
Baton Rouge, Louisiana 70803
(504)388-5421
rcooper@agctr.lsu.edu

***For additional information on the BIT Note series, contact***

Terry D'Erchia, Managing Editor
USGS Center for Biological Informatics

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