Resistence to the Peptidyl Transferase Inhibitor Tiamulin Caused by Mutation of Ribosomal Protein L3

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The antibiotic tiamulin targets the 50S subunit of the bacterial ribosome and interacts at the peptidyl transferase center. Tiamulin-resistant Escherichia coli mutants were isolated in order to elucidate mechanisms of resistance to the drug. No mutations in the rRNA were selected as resistance determinants using a strain expressing only a plasmid-encoded rRNA operon. Selection in a strain with all seven chromosomal rRNA operons yielded a mutant with an A445G mutation in the gene coding for ribosomal protein L3, resulting in an Asn149Asp alteration. Complementation experiments and sequencing of transductants demonstrate that the mutation is responsible for the resistance phenotype. Chemical footprinting experiments show a reduced binding of tiamulin to mutant ribosomes. It is inferred that the L3 mutation, which points into the peptidyl transferase cleft, causes tiamulin resistance by alteration of the drug-binding site. This is the first report of a mechanism of resistance to tiamulin unveiled in molecular detail.

The antibiotic tiamulin is used exclusively in veterinary medicine to treat swine dysentery and respiratory diseases in pigs and poultry. In some countries, only a few antibiotics are approved for use in pigs, and some of the drugs have limited effectiveness due to increased bacterial resistance. Treatment is important not only for the eradication of the causative agent of swine dysentery, from infected herds. Resistance develops rapidly and is caused by a single point mutation in ribosomal protein L3 responsible for the tiamulin resistance phenotype. The mutation is located in a nonglobular domain of L3 that extends close to the site of peptide bond formation. We conclude that the mutation in ribosomal protein L3 confers the observed tiamulin resistance phenotype by affecting the binding and inhibitory action of tiamulin at the peptidyl transferase center.

MATERIALS AND METHODS

E. coli strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Isolation and characterization of tiamulin-resistant mutants in a strain with a plasmid-encoded rRNA operon. Antibiotic sensitivity testing showed that the growth of E. coli strain TA542 is completely inhibited at 60 μg of tiamulin per ml. Twenty-two spontaneous mutants with decreased tiamulin sensitivity were isolated by growing strain TA542 in the presence of 60 to 100 μg of tiamulin per ml. Plasmid DNA from the mutant strains was isolated and transformed into a strain with pFK1 (4). Tiamulin susceptibilities of strains containing the wild-type TA542 strain background and pHK-rrnC -5 from each of the mutant strains were determined by testing for growth on NZY plates containing tiamulin. In all cases, the tiamulin susceptibility of a strain containing pHK-rrnC -5 isolated from a mutant was indistinguishable from that of TA542.
RESULTS AND DISCUSSION

Search for tiamulin-resistant mutants with alterations in the rRNA. Several lines of evidence indicate that tiamulin binds in a cavity lined with rRNA nucleotides at the peptidyl transferase center. For many of the peptidyl transferase antibiotics, single nucleotide alterations in the peptidyl transferase loop have been found to confer antibiotic resistance. In order to select for mutations in rRNA that could lead to tiamulin resistance, an E. coli strain with a plasmid-borne rRNA operon and seven inactivated chromosomal rRNA operons was used (Table 1) (1). Plasmid DNA from the mutant strains was isolated and transformed individually into the parent strain, where pHK-rrnC\textsuperscript{+} was replaced with pFK1 (see Materials and Methods and Table 1). The isolated plasmid did not confer a tiamulin-resistant phenotype for any of the selected mutants. Thus, the tiamulin resistance determinants obtained do not reside on the plasmid and in these cases are therefore not due to mutations in rRNA. The reason that no tiamulin-resistant rRNA mutants were selected could be that the antibiotic binding pocket is lined with essential nucleotides that are necessary

TABLE 1. E. coli strains and plasmids

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant genotype and characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA542</td>
<td>ΔΔ (ΔEBHGC&lt;DeCc recA56 pTRNA66, pHK-rrnC\textsuperscript{+})</td>
<td>1</td>
</tr>
<tr>
<td>CN2476</td>
<td>[CN1709] ada::cam CN1709: F- ara Δ(codB-lac)3 thi</td>
<td>16, 17</td>
</tr>
<tr>
<td>JBS</td>
<td>[CN2476] pRLC</td>
<td>This study</td>
</tr>
<tr>
<td>CAG50051-55\textsuperscript{a}</td>
<td>nusA57::Tn10 btaB3191::Tn10 zrd-20::Tn10 trp88::Tn10 zed-3069::Tn10</td>
<td>19</td>
</tr>
<tr>
<td>CAG58209\textsuperscript{a}</td>
<td>gzi-3075::Tn10</td>
<td>19</td>
</tr>
<tr>
<td>CAG8160\textsuperscript{a}</td>
<td>thi-39::Tn10</td>
<td>19</td>
</tr>
<tr>
<td>CAG12162\textsuperscript{a}</td>
<td>gzi-3075::Tn10</td>
<td>13</td>
</tr>
<tr>
<td>CAG12072\textsuperscript{a}</td>
<td>zha-203::Tn10</td>
<td>13</td>
</tr>
<tr>
<td>CAG12075\textsuperscript{a}</td>
<td>zrd-3083::Tn10</td>
<td>13</td>
</tr>
<tr>
<td>PG201\textsuperscript{a}</td>
<td>kefB20::Tn10</td>
<td>I. Booth via EGSC\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Plasmids

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant genotype and characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFK1</td>
<td>rmb operon inserted into Tet\textsuperscript{r} gene of pBR322; Amp\textsuperscript{r}</td>
<td>4</td>
</tr>
<tr>
<td>pCN102A</td>
<td>lac promoter and polylinker inserted downstream of the fourth codon of the lacZ gene; Amp\textsuperscript{r}</td>
<td>15</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Only the position of Tn10 is given.

\textsuperscript{b} EGSC, E. coli Genetic Stock Center, Yale University, New Haven, Conn.
for cell growth. Another explanation is that such a plasmid-born mutation is not viable or frequent enough to be manifested as a resistant phenotype under the investigated conditions. Preliminary investigations (data not shown) indicate that the cause of decreased sensitivity to tiamulin may be nonribosomal in some cases, although these strains have not been characterized further.

**Isolation and genetic mapping of a tiamulin-resistant E. coli mutant.** A second selection was carried out with an *E. coli* strain containing all seven chromosomal rRNA operons and a gene conferring chloramphenicol resistance to facilitate genetic mapping (Table 1) (15, 16). Spontaneous mutants were isolated after growth on agar plates containing 150 or 200 µg of tiamulin per ml. One strain, JB5, was selected for further characterization due to its relatively rapid growth in the presence of tiamulin. Strain JB5 exhibits at least a fourfold decrease in sensitivity to tiamulin compared to the parental strain (Table 2). A two-step genetic mapping strategy, involving mating with a set of Hfr strains followed by P1 transductions, was used to identify the genetic determinant of the phenotype (19).

The Hfr mating experiment enabled localization of the mutation to the region bounded by min 69 to 83 on the *E. coli* chromosome. A series of P1 transductions using strains marked with tetracycline resistance in the relevant region indicated that the mutation is contained within min 73.9 to 74.6, a region of the genome that encodes approximately half of the ribosomal proteins. Sequencing of selected genes in the S10 operon including *rplC*, *rplD*, and *rplV* showed that JB5 contained a point mutation in *rplC*, the gene encoding ribosomal protein L3. The mutation, A445G, results in an Asn149Asp amino acid change. This result is consistent with an earlier report that associated tiamulin resistance with alterations in ribosomal proteins L3 and L4, although the molecular basis of this connection was never characterized (3).

The Asn149Asp mutation in ribosomal protein L3 confers the tiamulin resistance phenotype. Two approaches were used to obtain genetic evidence that the Asn149Asp mutation is

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**TABLE 2. Susceptibilities of strains to tiamulin**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tiamulin MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN2476 (parent)</td>
<td>None</td>
<td>100–125</td>
</tr>
<tr>
<td>CN102A</td>
<td></td>
<td>100–125</td>
</tr>
<tr>
<td>CN102A-wt L3</td>
<td>100–125</td>
<td></td>
</tr>
<tr>
<td>CN102A-mt L3</td>
<td>150–175</td>
<td></td>
</tr>
<tr>
<td>JB5 (L3 mutant)</td>
<td>None</td>
<td>&gt;400</td>
</tr>
<tr>
<td>CN102A</td>
<td></td>
<td>&gt;400</td>
</tr>
<tr>
<td>CN102A-wt L3</td>
<td>200–250</td>
<td></td>
</tr>
<tr>
<td>CN102A-mt L3</td>
<td>300–400</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> wt, wild type; mt, mutant.
responsible for the tiamulin resistance phenotype. First, a group of 30 transductants, composed of 15 tiamulin-resistant and 15 tiamulin-sensitive colonies, was sequenced in the region of the mutation. In every case, there was a 1:1 correspondence between the presence of the mutation and the tiamulin resistance phenotype. Second, complementation experiments were used to determine the effect of overexpressing wild-type and mutant copies of \textit{rplC} on the tiamulin resistance phenotype.

Two L3-\textit{lacZ} expression vectors were constructed by inserting a fragment of the S10 operon flanking \textit{rplC} containing either the wild-type or the mutant \textit{rplC} sequence downstream of the fourth codon of the \textit{lacZ} gene carried on a plasmid. The vectors were transformed into parent and mutant strains, and their subsequent tiamulin susceptibilities were determined.

The MIC for the wild-type strain increased upon plasmid-encoded expression of the mutated \textit{rplC} sequence together with chromosome-encoded expression of wild-type \textit{rplC}, whereas plasmid-encoded expression of the wild-type \textit{rplC} sequence did not measurably affect the MIC (Table 2). The marginal increase in the MIC may reflect reduced expression of the mutated \textit{rplC} sequence, lower incorporation of the mutant L3 protein into ribosomes, or poor usage of mutated ribosomes when in competition with those containing the wild-type genome-encoded L3 protein. Similarly, the MIC for the mutant strain was only slightly affected by plasmid-encoded expression of the mutated \textit{rplC} sequence together with chromosome-encoded expression of mutant \textit{rplC} but decreased significantly upon plasmid-encoded expression of the wild-type \textit{rplC} sequence (Table 2). In addition, the parent vector did not measurably affect the MIC in either the wild-type or mutant strain (Table 2). The data show that the degree of tiamulin sensitivity decreases in the wild-type strain by expression of the mutated sequence and increases in the mutant strain by expression of the wild-type L3 sequence. We conclude that the mutation yielding an Asn149Asp mutation in ribosomal protein L3 is responsible for the tiamulin resistance phenotype.

The mutation is located in a region of L3 close to the peptidyl transferase center. Like many ribosomal proteins, L3 has a globular domain on the ribosome surface plus an extended domain that stretches into the ribosome interior (Fig. 2A). L3 is one of four ribosomal proteins that come closest to the site of peptide bond formation through its extended domain (14). An alignment of selected L3 sequences was carried out to determine whether the mutation occurred at a conserved position in the sequence (Fig. 2B). Although Asn149 itself is not conserved, it lies within a conserved region of the protein with enough amino acid identity to obtain a good alignment between sequences representing the three domains of life. The L3 sequences of \textit{Halocarcula marismortui} and \textit{Deinococcus radiodurans} were included to enable localization of the mutation in the three-dimensional structures of the large ribosomal subunit solved recently through X-ray crystallography (2, 6).

Only the \textit{H. marismortui} structure provides the resolution needed to position the amino acid side chains. The positions in \textit{H. marismortui} and \textit{D. radiodurans} that are equivalent to Asn149 in \textit{E. coli} are Trp242 and Arg144, respectively. Trp242 is located at the tip of the extended domain and represents the position on L3 that comes closest to the catalytic center (Fig. 2C). The shortest distance between Trp242 and U2541 (U2506 in \textit{E. coli} numbering), one of the nucleotides in the tiamulin footprint, is about 7 Å. Although amino acid side chains are not included in the \textit{D. radiodurans} 50S structure, the backbone position of Arg144 is also the closest point on L3 to the peptidyl transferase site. The distance between the \(\alpha\)-carbon of Arg144 and the phosphorus atom of nucleotide U2485 (U2506 in \textit{E. coli} numbering) is approximately 10 Å. Interestingly, alteration of ribosomal protein L3 at the relevant amino acid also affects the action of another class of antibiotics, called the trichothecenes, that specifically target eukaryotic cells. The mutation Trp255Cys at the equivalent amino acid position causes resistance to the antibiotic trichodermin in \textit{Saccharomyces cerevisiae} (5).

Ribosomes containing the L3 mutation exhibit reduced tiamulin binding. Chemical footprinting was used to examine tiamulin binding to wild-type versus L3 mutated ribosomes. Ribosome-tiamulin complexes were probed with CMCT, which modifies the N-3 of uridine residues. Primer extension with reverse transcriptase was then used to identify alterations in protection of U2506 induced by tiamulin binding to the wild-type and mutant ribosomes at drug concentrations ranging between 0.1 and 10 μM. In the wild-type ribosomes, complete protection at U2506 was observed at tiamulin concentrations above 0.5 μM (Fig. 3). In contrast, complete protection at U2506 was not observed in the mutant ribosomes even at a tiamulin concentration of 10 μM. These results reveal that tiamulin binding to mutant ribosomes is significantly reduced relative to binding to wild-type ribosomes.

**Concluding remarks.** It is well known that single nucleotide or amino acid changes in ribosomal components can lead to antibiotic resistance. In this investigation, a point mutation in the gene encoding ribosomal protein L3 was mapped in a tiamulin-resistant mutant strain and shown to be a tiamulin resistance determinant in \textit{E. coli}. The mutation produces an Asn-to-Asp alteration at position 149 of ribosomal protein L3 that is in the vicinity of the peptidyl transferase site. We conclude that the mutation perturbs the drug-binding site at the...
peptidyl transferase center. As the mutated position seems accessible from the peptidyl transferase center according to the X-ray structures of the large ribosomal subunit, we believe it is a direct perturbation. The mutation could function by altering the tiamulin binding site either directly, by eliminating a specific interaction with tiamulin, or indirectly, by influencing the RNA structure at the peptidyl transferase center. This is the first report of a tiamulin resistance mechanism that has been characterized on a molecular level. In addition, it is the first account of a ribosomal protein L3 mutant in bacteria that confers antibiotic resistance. It will be interesting to learn if clinical isolates from the veterinary field also contain mutations in ribosomal protein L3.

ACKNOWLEDGMENTS

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REFERENCES