Molecular Microbiology (2004) **54**(5), 1295–1306 doi:10.1111/j.1365-2958.2004.04373.x

Mutations in ribosomal protein L3 and 23S ribosomal RNA at the peptidyl transferase centre are associated with reduced susceptibility to tiamulin in *Brachyspira* spp. isolates

Märit Pringle,[1] Jacob Poehlsgaard,[2] Birte Vester[2] and Katherine S. Long[3]*

[1]*Department of Antibiotics, National Veterinary Institute, SE-75189 Uppsala, Sweden.*

[2]*Department of Biochemistry and Molecular Biology, University of Southern Denmark, DK-5230 Odense, Denmark.*

[3]*Institute of Molecular Biology, University of Copenhagen, DK-1307, Copenhagen K, Denmark.*

Summary

The pleuromutilin antibiotic tiamulin binds to the ribosomal peptidyl transferase centre. Three groups of *Brachyspira* spp. isolates with reduced tiamulin susceptibility were analysed to define resistance mechanisms to the drug. Mutations were identified in genes encoding ribosomal protein L3 and 23S rRNA at positions proximal to the peptidyl transferase centre. In two groups of laboratory-selected mutants, mutations were found at nucleotide positions 2032, 2055, 2447, 2499, 2504 and 2572 of 23S rRNA (*Escherichia coli* numbering) and at amino acid positions 148 and 149 of ribosomal protein L3 (*Brachyspira pilosicoli* numbering). In a third group of clinical *B. hyodysenteriae* isolates, only a single mutation at amino acid 148 of ribosomal protein L3 was detected. Chemical footprinting experiments show a reduced binding of tiamulin to ribosomal subunits from mutants with decreased susceptibility to the drug. This reduction in drug binding is likely the resistance mechanism for these strains. Hence, the identified mutations located near the tiamulin binding site are predicted to be responsible for the resistance phenotype. The positions of the mutated residues relative to the bound drug advocate a model where the mutations affect tiamulin binding indirectly through perturbation of nucleotide U2504.

Accepted 20 August, 2004. *For correspondence. E-mail long@mermaid.molbio.ku.dk; Tel. (+45) 35 32 20 30; Fax (+45) 35 32 20 40.

Introduction

The pleuromutilins inhibit protein synthesis by targeting the large subunit of the bacterial ribosome. This group of antibiotics is derived from the natural compound pleuromutilin, isolated originally from the basidiomycete *Pleurotus mutilus* (now *Clitopilus scyphoides*) (Kavanagh *et al*., 1951; Singer, 1986). Tiamulin is a semisynthetic pleuromutilin derivative containing an aliphatic thioether side-chain. Chemical footprinting experiments have demonstrated that tiamulin, and the related drug valnemulin, bind at the peptidyl transferase centre in the middle of the ribosome. Altered reactivities to base-specific modifying chemicals in the presence of the drugs are observed in *Escherichia coli* 23S rRNA, with protection effects at U2506 and U2584-5, and enhancement effects at A2058-9 (Poulsen *et al*., 2001). Puromycin-based assays have shown that the pleuromutilins inhibit peptide bond formation directly (Hodgin and Högenauer, 1974; Poulsen *et al*., 2001). This is supported by competitive footprinting studies with macrolide antibiotics in which tiamulin and valnemulin bind to the ribosome concurrently with erythromycin, but compete with carbomycin, a peptidyl transferase inhibitor (Poulsen *et al*., 2001).

Swine dysentery is a severe diarrhoeal disease among pigs with mortality up to 30%, caused by the anaerobic spirochaete *Brachyspira hyodysenteriae* (Harris *et al*., 1999). The disease is spread worldwide and causes distress for the pigs as well as major economic losses in affected herds. Porcine intestinal spirochaetosis is a milder diarrhoeal disease of growing pigs causing reduction in growth rate. The etiologic agent is *Brachyspira pilosicoli* (Trott, 1996), a species closely related to *B. hyodysenteriae.* The antibiotic arsenal available to treat these diseases is limited because of both the withdrawal of drugs authorized for use in pigs and reduced susceptibility to available drugs. Tiamulin is an important option in the treatment and eradication of *Brachyspira* spp. from infected herds. Recently, isolates of *B. hyodysenteriae* with decreased susceptibility to tiamulin have been reported in various parts of Europe, including Germany, Great Britain and the Czech Republic (Karlsson *et al*., 2004; Lobova *et al*., 2004). Tiamulin resistance in *B. pilosicoli* has been

reported in Finland (Fossi *et al*., 1999) and in a recent study, 14% of *B. pilosicoli* isolates from Swedish pig herds were resistant to tiamulin (M. Pringle, unpubl.).

Little is reported about resistance mechanisms to the pleuromutilin antibiotics. Tiamulin resistance develops in a slow, stepwise manner *in vitro* (Drews *et al*., 1975; Böck *et al*., 1982; Karlsson *et al*., 2001). In *E. coli*, resistance to tiamulin has been associated with mutations in ribosomal proteins (Böck *et al*., 1982). A recent investigation revealed that the tiamulin resistance phenotype in a single *E. coli* mutant was caused by mutation of ribosomal protein L3 (Bøsling *et al*., 2003). The mutation results in an Asn to Asp change at amino acid 149 (*E. coli* numbering), a position that is in close proximity to the peptidyl transferase centre (Bøsling *et al*., 2003).

The dearth of knowledge on tiamulin resistance is probably because the pleuromutilins have been used exclusively in veterinary medicine. The alarming situation with multiresistant bacteria in human medicine and the meager development of new antimicrobial agents has spawned a renewed interest in the pleuromutilins (Brooks *et al*., 2001; Bacqué *et al*., 2002; Springer *et al*., 2003). They are, for example, active against gram-positive bacteria including staphylococci (Drews *et al*., 1975; Boggs and Hecker, 2002), making them a potential treatment alternative for methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Moreover, the recent major advances in ribosomal structure determination and computational methods for drug design have made the ribosome more attractive as a target for the development of new drugs (Knowles *et al*., 2002).

Additional information on tiamulin resistance mechanisms and their development in clinically relevant organisms is necessary in preventing the spread of resistant isolates. In this work, we have searched for tiamulin resistance determinants by investigation of *Brachyspira* spp. isolates with reduced susceptibility to tiamulin. Portions of the genes encoding ribosomal protein L3 and 23S rRNA components neighbouring the peptidyl transferase centre have been sequenced. We find that mutations clustered near the peptidyl transferase centre are associated with decreased susceptibility to tiamulin. Chemical footprinting experiments show that ribosomes from *B. hyodysenteriae* isolates with decreased tiamulin susceptibility have a reduced ability to bind the drug. Taken together, the proximity of the mutations relative to the tiamulin binding site and the footprinting data explain how resistance in these isolates can be mediated by alteration of the drug-binding cavity.

Results

Selection of tiamulin-resistant Brachyspira *spp. mutants*

Acquired tiamulin resistance of clinical importance has thus far only been reported in *Brachyspira* spp. The resistance mechanisms that operate in this organism may be different from those found in laboratory strains of *E. coli*. In addition, resistance derived from selection in *E. coli* laboratory strains may not occur in the field. Therefore, we have chosen to investigate the pathogen itself, in spite of the difficulties involved because of the lack of available genetic information on *Brachyspira* spp. Three groups of *Brachyspira* spp. strains were used in the investigation. The first group are mutants derived in the laboratory from a susceptible type strain (K4) and a field isolate (K2), whose isolation via repeated passages on tiamulin agar has been described previously (Karlsson *et al*., 2001). The second group are mutants derived in the laboratory from different field isolates with a range of tiamulin susceptibilities (P2, P4, P5, P6). They were isolated via repeated passages on tiamulin agar for up to 10 months. The third group of strains are clinical *B. hyodysenteriae* field isolates from Great Britain (E) (Karlsson *et al*., 2004). The tiamulin minimal inhibitory concentrations (MICs) for each strain were determined using a broth dilution method (see *Experimental procedures*) and the results are presented in Table 1. An interval is presented for isolates where the MIC varied between several test rounds. As some of the strains showed correlated changes in chloramphenicol susceptibilities, these MICs are also presented (Table 1).

The increase in tiamulin MIC for the laboratory-derived mutants varies from 4 to >12 twofold dilutions. The highest tiamulin MICs (>128 $\mu g\ ml^{-1}$) are observed for the K4R and K2R strains. Strain K4p16 was saved as an intermediate stage during preparation of K4R from passage 16 on tiamulin agar. The tiamulin and chloramphenicol MICs increase 6 and 5 twofold dilutions, respectively, for K4p16 compared to K4S. Although the tiamulin MIC increases by >5 twofold dilutions from K4p16 to K4R, the two strains have the same chloramphenicol MIC. The observed increases in tiamulin MICs for the P2, P4, P5 and P6 strains are generally lower and range between 4 and 6 twofold dilutions. The British field isolates consist of a tiamulin-susceptible strain (E8), plus strains with intermediate (E7) and low tiamulin susceptibility (E1) with MICs that differ from E8 by 3 and 9 twofold dilutions respectively.

Sequencing of genes encoding ribosomal components at the pleuromutilin binding site

Mutations located at the pleuromutilin binding site on the ribosome constitute one possible class of tiamulin resistance mechanisms. To test this hypothesis, portions of ribosomal protein L3 and 23S rRNA genes encoding ribosomal components at the peptidyl transferase centre were amplified and sequenced. The identified mutations are summarized in Table 1. An alignment of ribosomal protein

Table 1. Mutations identified in laboratory-selected strains (original strains in italics) and field isolates.

		MIC (μg ml^{-1})		23S rRNA mutation						
	Species[a]	Tiamulin	Cam	2032 G-A	2055 C-A	2447 G-T	2499 C-A	2504 T-G	2572 A-T	Ribosomal protein L3 mutation[b]
Strain										
K4S (B78^T)[f]	*B.h.*	*0.063*	*4*							
K4p16[c]	B.h.	4	128					x		
K4R[c]	B.h.	>128	128	x				x		Asn148Ser
P2S (T20)	*B.h.*	*2–4*	*1*							
P2R	B.h.	64	2							Ser149Ile[d]
P4S (174:92)	*B.h.*	*0.25*	*2*							
P4R	B.h.	8	16			x				Asn148Lys
P5S (2554:02)	*B.h.*	*0.5*	*2*							*Asn148Ser*
P5R	B.h.	32	8	x			x			Asn148Ser
P6S (2420:97)	*B.h.*	*0.063*	*4*							
P6R	B.h.	4	16		x				x	
K2S (497:93)[c]	*B.p.*	*0.031*	*8*							
K2R[c]	B.p.	>128	256		x[d]			x[d]		Asn148Ser
Field isolate										
E1[e]	B.h.	16–32	4							Asn148Ser
E7[e]	B.h.	0.5	2							Asn148Ser
E8[e]	B.h.	0.063	8							

a. B.h., *Brachyspira hyodysenteriae*; B.p., *Brachyspira pilosicoli*.
b. The L3 mutations are numbered according to the *B. pilosicoli* sequence AF114845 (GenBank Accession No.).
c. Karlsson *et al.* (2001).
d. Mixed sequence, see text in the *Results* section.
e. Karlsson *et al.* (2004).
f. ATCC 27164^T.
Cam, chloramphenicol.

L3 sequences flanking the L3 mutations is shown in Fig. 1A. The positions of the rRNA mutations are shown in Fig. 1B on the *B. hyodysenteriae* 23S rRNA secondary structure, together with other resistance mutations at corresponding positions. In this paper, all 23S rRNA nucleotides and ribosomal protein L3 are given according to *E. coli* and *B. pilosicoli* numbering, respectively, unless otherwise indicated. The sequencing process was complicated by the fact that the complete sequences of *Brachyspira* spp. rRNA and ribosomal protein genes are not published. Primer sites on 23S rDNA were therefore chosen by comparison of *E. coli* and various spirochaete sequences. Ribosomal protein L3 gene primers were designed using the *B. pilosicoli* sequence. The primers used for PCR and sequencing reactions are summarized in Table 2.

Mixed sequences are observed in the L3 and 23S rRNA genes of the P2R and K2R isolates respectively. The presence of mixed sequences corroborates the slow stepwise development of tiamulin resistance *in vitro*, where a large number of passages are necessary to isolate highly resistant *Brachyspira* spp. mutants (Karlsson *et al.*, 2001). Hence, several clones with different mutations could evolve and persist on the plates during this procedure. Approximately 80% of the DNA is mutated in the L3 gene sequence of the P2R strain. Although *B. hyodysenteriae* (Zuerner and Stanton, 1994) and *B. pilosicoli* (R.L. Zuerner, pers. comm.) are known to contain single copies of the rRNA genes, the K2R 23S rDNA sequence has approximately 80% and 50% mutated DNA at nucleotide positions 2055 and 2504 respectively. In the case of K2R, the mixed sequences could also be attributed to the fact

Table 2. Primers used for PCR and DNA sequencing in this study.

Primer	Position[a]	Sequence (5′-3′)	Application
L3 forward	1269–1288	GGGTATGACAACAGTTTTCG	PCR/sequencing, L3 gene
L3 reverse	1802–1821	GCTCCAGGTATAGAACCYTT	PCR/sequencing, L3 gene
DV forward	1894–1914	CCAGTAAACGGCGGCCGTAAC	sequencing, 23S rRNA gene
2058Fo forward[b]	1858–1879	GAGAGGTTAGCGTAAGCGAAGC	PCR/sequencing, 23S rRNA gene
Spiro1 reverse	2653–2675	CCACTCCGGTCCTCTCGTACTA	PCR/sequencing, 23S rRNA gene
Spiro2 reverse	2745–2767	GCTTCCCACTTAGATGCRTTCAG	PCR/sequencing, 23S rRNA gene
DVpilo reverse	2656–2677	CGTTCATTTCGGTCCTCTCGTA	PCR/sequencing, 23S rRNA gene

a. Numbered according to the *B. pilosicoli* AF114845 L3 and *E. coli* 23S rRNA sequences.
b. Karlsson *et al.* (1999).

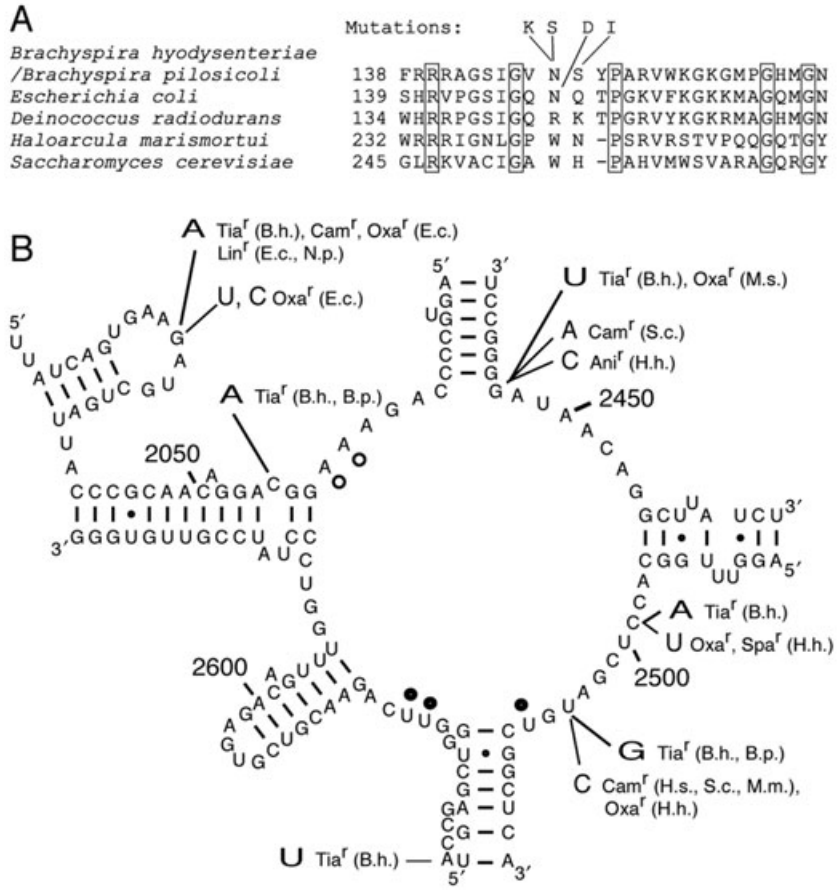

Fig. 1. Mutations found in *Brachyspira* spp. isolates with reduced susceptibility to tiamulin.
A. Alignment of various ribosomal protein L3 sequences in the regions flanking the mutations presented in Table 1. The positions and identities of the characterized mutations in *B. hyodysenteriae*, *B. pilosicoli* and *E. coli* are marked with lines. Positions of amino acid identity are boxed.
B. Secondary structure of the peptidyl transferase loop and adjacent regions of *B. hyodysenteriae* 23S rRNA. The secondary structure was modified from the *E. coli* secondary structure models from the Comparative RNA Web Site at URL: http://www.rna.icmb.utexas.edu/. Nucleotides that exhibit altered reactivities in the presence of tiamulin in *E. coli* are indicated with circles (Poulsen *et al*., 2001). Filled circles represent protections whereas open circles denote enhancements. Mutations observed in *Brachyspira* spp. isolates with reduced susceptibility to tiamulin (Table 1) are highlighted in broad letters. Mutations that confer resistance or decreased susceptibility to other antibiotics at the same nucleotides are indicated with the relevant organism(s) in parentheses. Abbreviations: B.h., *Brachyspira hyodysenteriae*; B.p., *Brachyspira pilosicoli*; E.c., *Escherichia coli*; H.h., *Halobacterium halobium*; H.s., *Homo sapiens*; M.m., *Mus musculus*; M.s., *Mycobacterium smegmatis*; N.p., *Nicotiana plumbaginifolia* (tobacco chloroplasts); S.c., *Saccharomyces cerevisiae*. The eukaryotic mutations have been characterized in chloroplast or mitochondrial rRNAs. Literature references to the indicated mutations are as follows: G2032A (Cseplö *et al*., 1988; Douthwaite, 1992; Xiong *et al*., 2000), G2032U and G2032C (Xiong *et al*., 2000), G2447A (Dujon, 1980), G2447U (Sander *et al*., 2002), G2447C (Hummel and Böck, 1987), C2499U (Tan *et al*., 1996; Kloss *et al*., 1999), U2504C in H.h. (Kloss *et al*., 1999), H.s. (Blanc *et al*., 1981a; Kearsey and Craig, 1981), M.m. (Blanc *et al*., 1981b) and S.c. (Weiss-Brummer *et al*., 1995).

that DNA was prepared from material that was subcultured on agar without tiamulin (Karlsson *et al*., 2001), where reversions could have occurred.

Correlation between the MICs and mutated positions

All of the laboratory-derived strains with an increased MIC relative to the parent strain contain mutations in the sequenced portions of either ribosomal protein L3 or domain V of 23S rRNA. Although this is not direct proof that the mutations cause the increase in MICs, it strongly predicts them as resistance determinants. The isolates selected in the laboratory contain different combinations of mutations and none of the combinations are identical. This illustrates that changes in susceptibility to tiamulin can be obtained in various ways. The strains with the highest tiamulin MICs contain three mutations, indicating that one mutation is not sufficient to cause a high level of resistance. The two strains with the highest tiamulin MICs (>128 μg ml^{-1}), K4R and K2R, plus K4p16, are the only strains with the T2504G mutation. These strains also have the highest chloramphenicol MICs, suggesting cross-resistance mediated by this mutation. The U2504C mutation leads to chloramphenicol resistance in *Halobacterium halobium* (Kloss *et al*., 1999), and human (Blanc *et al*., 1981a; Kearsey and Craig, 1981), mouse (Blanc *et al*.,

1981b) and yeast mitochondria (Weiss-Brummer *et al*., 1995), thus explaining the decreased chloramphenicol susceptibility for the 2504 mutant strains (Table 1). Another strain containing three mutations is P5R. The reduced tiamulin susceptibility from 0.5 (P5S) to 32 μg ml^{-1} (P5R) is associated with mutations at nucleotides G2032 and C2499 of 23S rRNA (Table 1). The parent strain, P5S, is less susceptible to tiamulin than K4S and K2S and this can be explained by the presence of the Asn148Ser change in ribosomal protein L3 in P5S. A similar trend is also observed in the MIC value for the E7 strain containing the same L3 mutation. Moreover, this correlates with the reduced susceptibility observed in *E. coli* containing a Asn to Asp change at the corresponding position (Bøsling *et al*., 2003).

None of the 23S rRNA gene mutations identified in the laboratory-derived strains are present in the field isolates. Only the L3 Asn148Ser mutation was found in the field isolates E7 and E1, and both of these strains have increased tiamulin MICs (0.5 and 16–32 μg ml^{-1}). The absence of mutations in 23S rRNA at the peptidyl transferase centre in the field isolates could be because both the high concentration of tiamulin and the long exposure time for the laboratory-derived mutants in this study have been extreme. Alternatively, the described mutations lead to decreased strain viability, with the consequence that such mutants cannot survive in the field and can only be isolated under more supportive conditions in the laboratory. However, the extensive use of tiamulin for prolonged periods in many pig herds could provide the environment needed for these mutations to also appear in the field.

A set of German field isolates with reduced susceptibility to tiamulin has also been examined. These strains have been investigated previously as to their possible relationship by pulsed-field gel electrophoresis and cross-resistance to other antimicrobials (Karlsson *et al*., 2004). The domain V fragment of 23S rRNA was sequenced for 10 of these isolates, as well as for eight tiamulin-susceptible German field isolates. A few sequence differences were detected, but they are in regions either associated with avilamycin resistance or unlikely to be significant for tiamulin binding (M. Pringle, unpubl. results). Additionally, no mutations were identified in the L3 fragment from 12 isolates with decreased susceptibility (M. Pringle, unpubl. results). Although neither ribosomal protein L3 nor 23S rRNA mutations are present in the German field isolates, it is possible to select for a mutation in ribosomal protein L3 *in vitro* (P2S and P2R, Table 1) that correlates with reduced susceptibility to tiamulin.

Binding of tiamulin to mutant ribosomes is reduced

Chemical footprinting was used to assess and compare tiamulin binding to ribosomes from five *B. hyodysenteriae* strains with different susceptibilities to the drug (K4S, K4p16, K4R, E7, E1). Standard ribosome purification methods were used as no isolation procedure has been reported in the literature for *Brachyspira* ribosomes. For *B. hyodysenteriae*, both the ribosome content and the overall yield of purified ribosomal particles is significantly reduced compared to a laboratory *E. coli* strain. Large ribosomal subunits were used in footprinting experiments, because only a minute amount of purified 70S ribosomes was obtained. Tiamulin-50S subunit complexes were modified with CMCT, which modifies the N3 of accessible uridine residues. This was followed by primer extension with reverse transcriptase to identify alterations in protection patterns induced by tiamulin. The results with three tiamulin concentrations are shown in Fig. 2. Tiamulin protects three uridine residues (U2506, U2584, U2585) from chemical modification when complexed with *E. coli* ribosomes (Poulsen *et al*., 2001). In contrast, only U2506 is protected when tiamulin is complexed with *B. hyodysenteriae* 50S subunits isolated from the tiamulin-susceptible strain, K4S (Fig. 2, lanes 6–9). Although U2584 and U2585 are not protected by tiamulin, these nucleotides are accessible to CMCT modification in the absence of drug (Fig. 2, lane 6). Comparison of K4S, K4p16 and K4R strains shows a significantly increased CMCT reactivity at U2584-5 in the tiamulin-resistant strain, K4R (Fig. 2, compare lane 16 with lanes 6 and 11).

In the strain with the lowest tiamulin MIC (K4S), there is nearly complete protection of U2506 at a tiamulin concentration of 2 μM (Fig. 2, lane 8). A significantly reduced level of tiamulin binding is observed in K4p16, as there is only a partial protection of U2506 at 10 μM tiamulin (Fig. 2, lane 14). In the strain with the highest tiamulin MIC, there is no protection of U2506 at 10 μM tiamulin (Fig. 2, lane 19), indicating that tiamulin is not bound to K4R ribosomes over the tested concentration range. These data show a progressive decrease in tiamulin binding to ribosomes from K4S, K4p16 and K4R strains, indicating that reduced tiamulin binding to ribosomes is the resistance mechanism in these strains. This is consistent with the mutations in 23S rRNA and ribosomal protein L3 being responsible for the reduced susceptibilities in K4p16 and K4R.

The chemical footprinting patterns obtained for the field isolates E7 and E1 show that the protection observed at U2506 is nearly identical for E7 and E1 (Fig. 2, compare lanes 20–24 with 25–29), despite the difference in tiamulin MIC between the two strains. A complete protection at U2506 is not observed (Fig. 2, lanes 24 and 29), indicating that tiamulin binding is reduced compared to the K4S control strain. These data suggest that an additional non-ribosomal resistance determinant is present in E1, together with the L3 mutation. Comparison of the tiamulin footprints from K4p16 and E7 strains shows that mutation

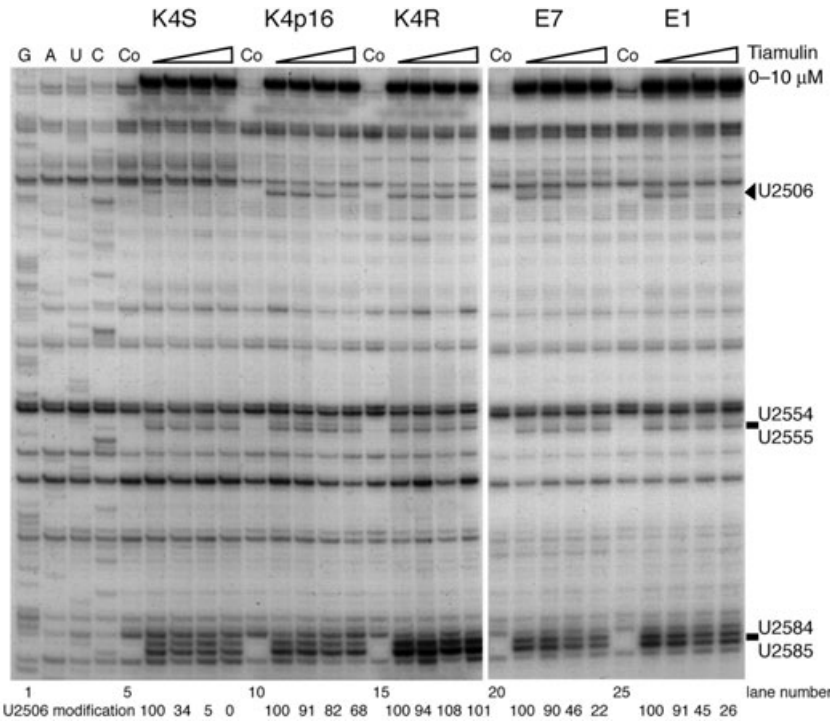

Fig. 2. Gel autoradiographs of the tiamulin footprint in domain V of 23S rRNA on *B. hyodysenteriae* 50S subunits, showing CMCT modifications detected by primer extension analysis. Control lanes (Co) for each strain contain unmodified 50S subunits. Wedges are used to indicate the increase in tiamulin concentration in the following sample order: modified 50S subunits in the absence of tiamulin and modified subunits in the presence of 0.5, 2 and 10 μM tiamulin. The altered reactivity at U2506 is indicated with an arrow. Lanes marked G, A, U and C denote dideoxy sequencing reactions. Reverse transcriptase stops one nucleotide before the corresponding nucleotide in the sequencing tracks. The primer extension stop at U2506 was quantified with a phosphorimager using the control stops at positions U2544–U2555 as reference bands. The degree of modification for each sample is given below the gel as a percentage relative to the sample with modified 50S subunits in the absence of tiamulin for each strain.

of 2504 decreases tiamulin binding more than mutation of ribosomal protein L3 (Fig. 2, lanes 14 and 24).

Model of the resistance mechanism

In order to rationalize the resistance patterns observed for the mutant strains (Table 1), the positions of the mutations were examined in the high-resolution structure of a bacterial 50S subunit. As data for the X-ray structure of the *Deinococcus radiodurans* 50S subunit soaked with tiamulin was obtained (Schlünzen *et al.*, 2004), it was possible to directly relate the positions of the mutated nucleotides to that of the bound drug (Fig. 3). Examination of the structure revealed that all mutations and the drug are placed close to the peptidyl transferase centre in the 50S subunit. All mutated nucleotides are in close proximity to the central core of tiamulin, that binds directly in the peptidyl transferase centre, but only U2504 is close enough for a direct contact. However, all of the mutated nucleotides are either in contact with or very close to nucleotide U2504. To visualize the area with the mutations, an appropriate cutting plane was placed in the ribosomal subunit (Fig. 3D). The positions of the mutations, footprints and drug relative to the cut plane are shown in Fig. 3E, where the view in Fig. 3D is rotated 90 degrees. This view also shows the positions of nucleotides 2058 and 2059 that are located in the peptide exit tunnel. These nucleotides exhibit enhanced reactivities in a footprinting assay in the presence of tiamulin (Poulsen *et al.*, 2001), but their distances to the bound drug suggest that this is because of indirect effects. Both Fig. 3D and E show that nucleotide U2504 can play a crucial role in tiamulin binding and that structural perturbations at this position can hinder tiamulin binding. Hence, the most straightforward explanation for the observed resistance mechanism is that the different mutations alter the position of U2504, which in turn impedes tiamulin binding.

Discussion

Tiamulin is one of the last available options for the treatment of swine dysentery. As resistance to tiamulin has been reported in *B. hyodysenteriae* and is potentially spreading, we have investigated resistance mechanisms in this organism. It is well known that resistance to many ribosomal drugs is caused by altered drug binding. The facts that tiamulin inhibits peptidyl transferase, footprints at nucleotides in the peptidyl transferase region (Poulsen *et al.*, 2001) and that a L3 mutation in *E. coli* confers reduced susceptibility to tiamulin (Bøsling *et al.*, 2003) pointed to where to search for mutations causing tiamulin resistance. The presence of a mutation in a strain with an altered tiamulin MIC is, by itself, not proof of a direct causal relationship. However, this kind of correlation has been seen with other ribosomal antibiotics such as avilamycin and macrolides (Vester and Douthwaite, 2001; Kofoed and Vester, 2002). Sufficient evidence is presented in this study to associate the identified mutations

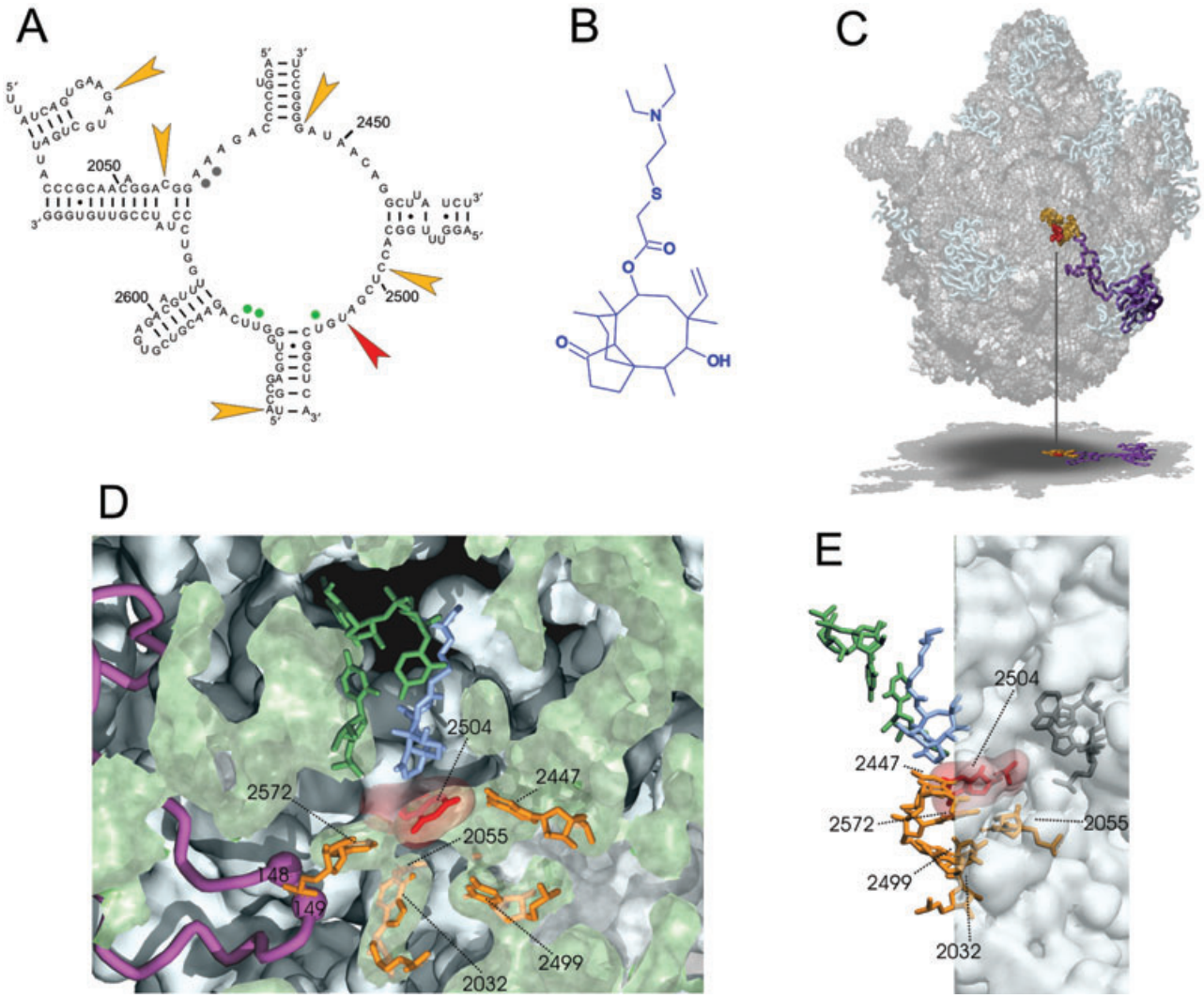

Fig. 3. The mutated positions and the tiamulin binding site at the ribosomal peptidyl transferase centre.
A. Secondary structure of the central loop region of domain V of *B. hyodysenteriae* 23S rRNA. Residues involved in resistance mutations are indicated by coloured arrows and footprints are indicated with dots (protections in green and enhancements in grey).
B. The chemical structure of tiamulin.
C. The 50S subunit from *D. radiodurans* (modified from Harms *et al*., 2001; PDB Accession No. 1NKW) with the mutated positions from A (same colouring) and L3 (purple ribbon). RNA is represented by grey spheres and proteins are shown as light blue ribbons. The subunit is rendered transparently to show the residues and the positioning inside the subunit is indicated by the shadow.
D. Part of a sliced 50S subunit of *D. radiodurans* showing the tiamulin binding site. Outer RNA surface is grey and cut surface is light green. Tiamulin is in blue and RNA residues protected by tiamulin are shown in green. RNA resistance determinants are coloured as in (A) and L3 mutations are shown as spheres on the purple ribbon. The surface of residue 2504 (*E. coli* numbering) is shown in transparent red. Co-ordinates were kindly provided by Schlünzen *et al*. (2004).
E. As in D, but turned 90 degrees to show the cut plane.

with reduced tiamulin susceptibility. First, the mutations are located near the tiamulin binding site (Fig. 3D and E). In addition, none of the mutations are found in highly susceptible strains. Furthermore, most of the mutations occur in more than one strain. Finally, most of the RNA mutations are at positions implicated in resistance to other ribosomal drugs (Fig. 1B) whose binding sites overlap with tiamulin (Schlünzen *et al*., 2004). The data presented in Table 1 show multiple tiamulin resistance mechanisms in *Brachyspira* spp. associated with mutations around the tiamulin binding site. No single mutation by itself is responsible for a high level of tiamulin resistance, but when various combinations of two or three mutations are present, high levels of resistance are obtained. This explains the stepwise decrease in tiamulin susceptibility observed in this study and by Karlsson *et al*. (2001).

L3 mutations and tiamulin susceptibility

The amino acid sequence around the L3 mutations char-

acterized in *Brachyspira* spp. can be aligned with L3 sequences from other organisms (Fig. 1A). Using this alignment and the X-ray structures of the 50S subunits from *D. radiodurans* (Harms *et al*., 2001) and *Haloarcula marismortui* (Ban *et al*., 2000), it can be seen that the two mutated positions are situated at the tip of an extension reaching close to the peptidyl transferase centre. As mentioned previously, a corresponding position in *E. coli* has been identified as a tiamulin resistance determinant (Bøsling *et al*., 2003). Furthermore, the Trp255Cys mutation at the corresponding position of ribosomal protein L3 in *Saccharomyces cerevisiae* confers both anisomycin and trichodermin resistance (Schindler *et al*., 1974; Jimenez *et al*., 1975). The same mutation also renders yeast ribosomes resistant to the ribosome-inactivating protein, pokeweed antiviral protein (Hudak *et al*., 1999). Moreover, mutations in this region of L3 (Trp255Cys, Pro257Ser and Ile282Thr) have been associated with decreased peptidyl transferase activities, increased levels of (–1) programmed ribosomal frameshifting, and viral maintenance defects in *S. cerevisiae*. (Peltz *et al*., 1999; Meskauskas *et al*., 2003). These lines of evidence demonstrate that mutations in L3 can affect the structure of the peptidyl transferase centre and thus also tiamulin binding.

23S rRNA mutations and tiamulin susceptibility

The three-dimensional arrangement of nucleotides surrounding the drug-binding cavity depicted in Fig. 3D shows that U2504 may play an important role in tiamulin binding. Among the mutated sites characterized in this investigation, U2504 is the only position that is accessible from the peptidyl transferase cavity, where it forms part of the electron density in the wall of the cavity (Fig. 3D). Nucleotide U2504 is post-transcriptionally modified to pseudouridine in *E. coli* (Bakin and Ofengand, 1993) and it is unknown whether this is also the case in *D. radiodurans* and *Brachyspira* spp. The conclusions presented here are probably not influenced by this modification because the pyrimidine ring at position 2504 forms a hydrophobic patch on the wall of the binding cavity which interacts with a hydrophobic part of tiamulin.

The other mutated 23S rRNA and L3 positions are clustered around nucleotide 2504, but are buried inside the 50S subunit according to the X-ray structures mentioned previously (Ban *et al*., 2000; Harms *et al*., 2001). As these positions are apparently inaccessible and too remote from the tiamulin binding pocket, it is unlikely that these nucleotides and amino acids contact tiamulin directly. Their close proximity to U2504 suggests that they function indirectly by perturbing the local conformation of the binding cavity and contribute to resistance specifically by altering the position of U2504. This view is reinforced by the enhanced reactivity to CMCT observed in the K4R strain, relative to the K4S and K4p16 strains (Fig. 2, lanes 6, 11 and 16). This data suggests that there is a structural alteration at the peptidyl transferase centre in K4R ribosomes that, in addition to the mutation at U2504, contain mutations at G2032 and L3 Asn148 (Table 1).

The precise positioning of nucleotide 2504 could be important for both direct interactions and metal binding at the tiamulin binding site. High-resolution structures of antibiotic-50S subunit complexes have revealed that nucleotide 2504 is an important component of the anisomycin (Hansen *et al*., 2003) and chloramphenicol (Schlünzen *et al*., 2001) binding sites. The hydroxyl group of anisomycin is hydrogen-bonded to the O1P of U2504 and co-ordinated to a potassium ion that is chelated by nucleotides 2061, 2447 and 2501 (Hansen *et al*., 2003). In the case of chloramphenicol, one of the oxygens of the p-nitro group forms a hydrogen bond with the 2′-hydroxyl group of U2504 and a bound magnesium ion mediates an interaction between the same oxygen atom and the carbonyl oxygen of U2504 (Schlünzen *et al*., 2001).

U2504 is a highly conserved nucleotide at the peptidyl transferase centre, emphasizing its importance for some aspect of ribosome assembly or function. Mutations at U2504 are considered lethal for *E. coli* (Triman *et al*., 1998), although the *in vitro* viabilities of the *B. hyodysenteriae* K4R, K4p16 and K2R strains are not significantly affected. Mutations at the corresponding position in *H. halobium* and mitochondrial 23S rRNAs confer oxazolidinone and chloramphenicol resistance (Fig. 1B and references therein). The binding sites of tiamulin and chloramphenicol are partially overlapping according to Schlünzen *et al*. (2004). It is thus not surprising that the highest levels of tiamulin resistance are observed in mutants containing a combination of 23S rRNA and ribosomal protein L3 mutations, where one of the mutated nucleotides is U2504 and that mutation of U2504 is sufficient to obtain high levels of chloramphenicol resistance (Table 1). The combination of 23S rRNA mutations at 2032 and 2499 plus the Asn148Ser L3 mutations also yields a relatively high resistance. The three mutations have previously and independently been identified as resistance determinants for chloramphenicol, lincosamides, oxazolidinones, sparsomycin or tiamulin (Fig. 1 and references herein). From the model presented in this study, lincosamide and oxazolidinone resistance may also be mediated by disturbance of the 2504 position. Mutations at nucleotide positions 2055 and 2572 of 23S rRNA are only identified in strains with multiple mutations (Table 1) and have not previously been associated with drug resistance. One explanation for this is that mutation of 2055 or 2572 alone is not enough to affect tiamulin binding, but together they can disturb the environment of 2504. Selection of two such mutations will probably take many generations as is typical for tiamulin resistance. The

2447 and Asn148Ser mutations are present in strain P4R. Nucleotide 2447 is close to the site of peptide bond formation (Nissen *et al*., 2000) and mutations at this position have been associated with anisomycin, chloramphenicol and oxazolidinone resistance (Fig. 1B and references herein). Not unexpectedly, the P4R strain has reduced susceptibilities to both tiamulin and chloramphenicol (Table 1). Hence, nucleotide positions in the peptidyl transferase region where mutations lead to cross-resistance to several antibiotics are strongly correlated with overlapping drug binding sites.

B. hyodysenteriae field isolates

The reduced susceptibility to tiamulin in the British isolates (E1, E7, Table 1) is correlated with mutation of ribosomal protein L3. The relatively high MIC and the absence of RNA mutations in the sequenced regions for strain E1, suggest that the L3 mutation is combined with an unknown resistance determinant. Furthermore, the similar protection patterns observed for E7 and E1 in chemical footprinting experiments, despite their different susceptibilities, also suggest that this additional resistance determinant does not affect binding of tiamulin to the ribosome. The high tiamulin MICs in the German field isolates can be explained neither by mutations in 23S rRNA nor ribosomal protein L3 at the peptidyl transferase centre. The resistance mechanisms operating in these strains appear not to function through alteration of the tiamulin binding site. Elucidation of the undefined resistance determinants in the two sets of field isolates must await further studies.

Conclusions

Mutations identified in ribosomal protein L3 and 23S rRNA in the peptidyl transferase region are associated with reduced tiamulin susceptibility in laboratory-selected *Brachyspira* spp. strains. In addition, mutation of ribosomal protein L3 contributes to tiamulin resistance in clinical British *B. hyodysenteriae* field isolates. Chemical footprinting experiments show that tiamulin binding to large ribosomal subunits with mutations in 23S rRNA or ribosomal protein L3 is reduced, suggesting that resistance is caused by alteration of the drug binding site. The close proximity of the mutated residues to U2504, and the abutting position of U2504 relative to bound tiamulin, suggest a model in which the mutations alter drug binding indirectly by perturbing the conformation of U2504 at the tiamulin binding site. The presence of additional resistance determinants in clinical field isolates that do not affect tiamulin binding to the ribosome indicates that other tiamulin resistance mechanisms function in *Brachyspira* spp.

Experimental procedures

Strains, isolates and in vitro *development of tiamulin resistance*

All bacteria were grown on fastidious anaerobe agar with 10% horse blood (National Veterinary Institute, Uppsala, Sweden) in an anaerobic atmosphere at 37°C. The *Brachyspira* spp. strains and isolates (Table 1) are stored in liquid nitrogen in a strain collection at the National Veterinary Institute, Uppsala, Sweden. The species differentiation was made biochemically as described previously (Fellström and Gunnarsson, 1995). Development of decreased susceptibility to tiamulin for the *B. hyodysenteriae* type strain B78^{T} (ATCC 27164^{T}) (K4S) and the *B. pilosicoli* field isolate 497:93 (K2S) has been described previously (Karlsson *et al*., 2001). Six field isolates of *B. hyodysenteriae* with different tiamulin MICs were chosen for development of tiamulin resistant strains (P1–P6) for this study. The method to provoke tiamulin resistance in those strains differed slightly from the K-strains. After thawing and subculturing isolates twice, a DNA preparation and second susceptibility test were made before tiamulin exposure. Twice a week the strains were subcultured on agar with antibiotic disks containing tiamulin. The concentration of tiamulin in the disks varied according to the tiamulin susceptibility of each strain. The tiamulin concentration was slowly increased and the subculturing was continued for approximately 10 months. The susceptibility was monitored during the subculturing and at the end of the experiment the strains were frozen and new DNA preparations were made from the last subculture on tiamulin agar. The concentration of tiamulin in the disks ranged from 64 μg to 3200 μg and the volume of agar in the plates was 20 ml. The pulsed-field gel electrophoresis pattern of each final (R) strain was compared to that of the original (S) strain to confirm the clonal identity of each resistant strain after the selection. Two of the strains, P1 and P3, were excluded from the study because the tiamulin susceptibility did not change. Of the remaining strains one originates from Germany and the other three from Sweden. A set of three *B. hyodysenteriae* field isolates from the UK (E1, E7 and E8) with varying tiamulin MICs were chosen for sequencing. Although the ordinary interstrain variation in growth is not insignificant, no marked differences in growth were observed between the mutants and any other field isolate.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was carried out using the broth dilution method described previously (Karlsson *et al*., 2003). Briefly, antimicrobial agents were dried in twofold serial dilutions in tissue culture trays, into which a suspension of the bacteria was dispensed (0.5 ml per well) and incubated. The medium for the susceptibility tests was brain heart infusion broth supplemented with 10% fetal calf serum. The MIC was read as the lowest concentration of the antimicrobial agent that prevented visible growth.

Amplification and sequencing of 23S rRNA and ribosomal protein L3 genes

The fragments of the 23S rRNA and ribosomal protein L3

genes were amplified with the PCR primers summarized in Table 2. To design the 23S rRNA gene primers, *Brachyspira* spp. sequences with GenBank Accession No. U72699, U72703 and *Brachyspira* spp. sequences previously determined at the National Veterinary Institute, Sweden, were used. When the reverse primers Spiro1 and Spiro2 were designed, this part of the 23S rRNA for *Brachyspira* spp. was unknown. The sequences for *E. coli* (J01695) *Borrelia burgdorferi* (M88330), *Treponema pallidum* (AE001204) and *Leptospira interrogans* (X14249) were used to design these primers. The *B. pilosicoli* sequence AF114845 was used for design of primers for the L3 gene. The PCR amplicons were purified with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) and the sequence was determined in an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, CA, USA). Internal primers were designed for better 23S rDNA domain V sequence quality (Table 2), whereas the L3 gene was sequenced with the PCR primers. Labelled terminators (Big Dye from Applied Biosystems) were used in the cycle sequencing reactions. Contigs and alignments were created using Vector NTI Suite (InforMax, Frederick, MD, USA). The sequence covering position 2032 and 2055 for strains K2S and P4S was determined as described previously (Karlsson *et al*., 1999). All mutations were confirmed by sequences determined in both directions.

It was difficult to obtain complete sequences of the 23S rRNA gene fragment and it was necessary to sequence many strains repeatedly. A product was not obtained from the *B. pilosicoli* strain K2S with the Spiro 1 reverse primer because it is not the exact complement of the *B. hyodysenteriae* or *B. pilosicoli* sequences. Therefore, the DV pilo reverse primer was made from the K2R-strain sequence and this primer, together with the DV forward primer, is the only combination that yields a product for the K2S strain. The correct sequence for a *B. hyodysenteriae* primer at the same location would be 5′-TCATTCCGGTCCTCTCGTACTA-3′. The complementary *Brachyspira* spp. sequence for the Spiro 2 reverse primer is still not known because this was the outermost primer at the 3′-end of the 23S rRNA gene.

Growth of cells and isolation of ribosomal subunits

To obtain material for ribosome preparations the bacteria were grown in 40 ml of BHIS broth, in flasks. The flasks were incubated in 7.5 l anaerobic jars, with 20 flasks in each, on a shaker for 48 h in 37°C. The cells were harvested by centrifugation of the broth in a refrigerated centrifuge. The pellet was kept on ice and washed twice in buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM NH_4Cl). Immediately after washing the cells were frozen and stored in liquid nitrogen. Cells were resuspended in TMN buffer (20 mM Tris-Cl, pH 7.75, 10.5 mM $MgCl_2$, 100 mM NH_4Cl, 0.5 mM EDTA) and stored at −80°C. After thawing on ice, the cells were opened by three freeze/thaw cycles using a dry ice/ethanol bath. Lysozyme (Sigma) and RNase-free DNase I (Roche) were added, followed by vortexing 6×30 s with an equal volume of glass beads. The lysate was recovered by centrifugation and layered on a 40 ml 10–40% sucrose gradient prepared in TMN buffer and centrifuged 16 h at 21 000 r.p.m. in an SW28 rotor (Beckman, Palo Alto). The gradient was analysed on a fraction collector (GradiFrac, Pharmacia). As only a small absorbance peak corresponding to 70S ribosomes was observed, the fractions corresponding to 50S ribosomal subunits were pooled. The subunits were recovered by precipitation with 0.7 volumes of ethanol, resuspended in TMN buffer and stored at −80°C.

Chemical modification and primer extension analysis

Large ribosomal subunits (2.5 pmoles) were incubated with 0, 0.5, 2, or 10 μM tiamulin in modification buffer (50 mM Hepes-OH, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 5 mM DTT) for 30 min at 37°C. The tiamulin-50S subunit complexes (12.5 μl) were modified with 12.5 μl CMCT [1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-P-toluene sulphonate, 42 mg ml^{-1} in modification buffer] for 20 min at 37°C. The reactions were stopped by precipitating the 50S subunits with ethanol. The 50S subunits were recovered by centrifugation, resuspended in 0.3 M sodium acetate, and extracted with phenol and chloroform. Ribosomal RNA was precipitated with ethanol, resuspended in water, and monitored by primer extension analysis with AMV reverse transcriptase (Life Sciences) (Poulsen *et al*., 2001 and references therein). The 5′-[^{32}P]-labelled deoxyoligonucleotide primer Bh2654 (5′-TCCG GTCCTCTCGTACT-3′), complementary to nucleotides 2654–2670 of *B. hyodysenteriae* 23S rRNA, was used. The cDNA primer extension products were separated on 8% polyacrylamide sequencing gels. The positions of the stops were visualized by autoradiography and identified by referencing to dideoxy sequencing reactions on 23S rRNA that were electrophoresed in parallel. The intensities of the modifications were quantified using a phosphorimager. Chemical footprinting experiments were performed two to three times with reproducible results.

Visualization of the tiamulin binding cavity

Figure 3C was created in MolMol (Koradi *et al*., 1996) and PovRay from the *D. radiodurans* crystal structure (Harms *et al*., 2001; PDB code 1NKW). Figure 3D and E were created using co-ordinates of the *D. radiodurans*-tiamulin complex (J. Harms, pers. comm.) in Discreet Gmax/3DSmax.

Acknowledgements

We thank Annica Landén, Marianne Persson and Sara Johansson (National Veterinary Institute, Sweden) for expert technical assistance. We acknowledge Novartis for providing tiamulin, K. Perry and J. Rohde for supplying field isolates from the UK and Germany, respectively, and J. Harms, F. Schlünzen and colleagues for sharing data and co-ordinates before publication. M.K. was supported by Formas, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning. B.V. was supported by the Nucleic Acid Center funded by the Danish National Research Foundation. K.S.L. was supported by a grant from the European Commission's Fifth Framework Program (Grant QLK2-CT-2002–00892).

References

Bacqué, E., Pautrat, F., and Zard, S.Z. (2002) A flexible strategy for the divergent modification of pleuromutilin. *Chem Commun (Camb)* 2312–2313.

Bakin, A., and Ofengand, J. (1993) Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application of a new sequencing technique. *Biochemistry* **32:** 9754–9762.

Ban, N., Nissen, P., Hansen, J., Moore, P.B., and Steitz, T.A. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* **289:** 905–920.

Blanc, H., Adams, C.W., and Wallace, D.C. (1981a) Different nucleotide changes in the large rRNA gene of the mitochondrial DNA confer chloramphenicol resistance on two human cell lines. *Nucleic Acids Res* **9:** 5785–5795.

Blanc, H., Wright, C.T., Bibb, M.J., Wallace, D.C., and Clayton, D.A. (1981b) Mitochondrial DNA of chloramphenciol-resistant mouse cells contains a single nucleotide change in the region encoding the 3′end of the large ribosomal RNA. *Proc Natl Acad Sci USA* **78:** 3789–3793.

Böck, A., Turnowsky, F., and Högenauer, G. (1982) Tiamulin resistance mutations in *Escherichia coli*. *J Bacteriol* **151:** 1253–1260.

Boggs, A.F., and Hecker, S.J. (2002) A tour of recent patent applications addressing antibacterial resistance. *Expert Opin Ther Pat* **12:** 1159–1172.

Bøsling, J., Poulsen, S.M., Vester, B., and Long, K.S. (2003) Resistance to the peptidyl transferase inhibitor tiamulin caused by mutation of ribosomal protein L3. *Antimicrob Agents Chemother* **47:** 2892–2896.

Brooks, G., Burgess, W., Colthurst, D., Hinks, J.D., Hunt, E., Pearson, M.J., *et al.* (2001) Pleuromutilins. Part 1. The identification of novel mutilin 14-carbamates. *Bioorg Med Chem Lett* **9:** 1221–1231.

Cseplö, A., Etzold, T., Schell, J., and Schreier, P.H. (1988) Point mutations in the 23 S rRNA genes of four lincomycin resistant *Nicotiana plumbaginifolia* mutants could provide new selectable markers for chloroplast transformation. *Mol Gen Genet* **214:** 295–299.

Douthwaite, S. (1992) Functional interactions within 23S rRNA involving the peptidyltransferase center. *J Bacteriol* **174:** 1333–1338.

Drews, J., Georgopoulos, A., Laber, G., Schutze, E., and Unger, J. (1975) Antimicrobial activities of 81.723 hfu, a new pleuromutilin derivative. *Antimicrob Agents Chemother* **7:** 507–516.

Dujon, B. (1980) Sequence of the intron and flanking exons of the mitochondrial 21S rRNA gene of yeast strains having different alleles at the omega and *rib*-1 loci. *Cell* **20:** 185–197.

Fellström, C., and Gunnarsson, A. (1995) Phenotypical characterisation of intestinal spirochaetes isolated from pigs. *Res Vet Sci* **59:** 1–4.

Fossi, M., Saranpää, T., and Rautiainen, E. (1999) *In vitro* sensitivity of the swine *Brachyspira* species to tiamulin in Finland 1995–97. *Acta Vet Scand* **40:** 355–358.

Hansen, J.L., Moore, P.B., and Steitz, T.A. (2003) Structures of five antibiotics bound at the peptidyl transferase center of the large ribosomal subunit. *J Mol Biol* **330:** 1061–1075.

Harms, J., Schlünzen, F., Zarivach, R., Bashan, A., Gat, S., Agmon, I., *et al.* (2001) High resolution structure of the large ribosomal subunit from a mesophilic eubacterium. *Cell* **107:** 679–688.

Harris, D.L., Hampson, D.J., and Glock, R. (1999) Swine dysentery. In *Diseases of Swine*. Straw, B.E., D'Allaire, S., Mengeling, W.L. and Taylor, D.J. (eds). Ames, IA: Iowa State University Press, pp. 579–600.

Hodgin, L.A., and Högenauer, G. (1974) The mode of action of pleuromutilin derivatives. Effect on cell-free polypeptide synthesis. *Eur J Biochem* **47:** 527–533.

Hudak, K.A., Dinman, J.D., and Tumer, N.E. (1999) Pokeweed antiviral protein accesses ribosomes by binding to L3. *J Biol Chem* **274:** 3859–3864.

Hummel, H., and Böck, A. (1987) 23S ribosomal RNA mutations in halobacteria conferring resistance to the anti-80S ribosome targeted antibiotic anisomycin. *Nucleic Acids Res* **15:** 2431–2443.

Jimenez, A., Sanchez, L., and Vazquez, D. (1975) Simultaneous ribosomal resistance to trichodermin and anisomycin in *Saccharomyces cerevisiae* mutants. *Biochim Biophys Acta* **383:** 427–434.

Karlsson, M., Fellström, C., Heldtander, M.U., Johansson, K.-E., and Franklin, A. (1999) Genetic basis of macrolide and lincosamide resistance in *Brachyspira* (*Serpulina*) *hyodysenteriae*. *FEMS Microbiol Lett* **172:** 255–260.

Karlsson, M., Gunnarsson, A., and Franklin, A. (2001) Susceptibility to pleuromutilins in *Brachyspira* (*Serpulina*) *hyodysenteriae*. *Anim Health Res Rev* **2:** 59–65.

Karlsson, M., Fellström, C., Gunnarsson, A., Landén, A., and Franklin, A. (2003) Antimicrobial susceptibility testing of porcine *Brachyspira* (*Serpulina*) species isolates. *J Clin Microbiol* **41:** 2596–2604.

Karlsson, M., Aspan, A., Landen, A., and Franklin, A. (2004) Further characterization of porcine *Brachyspira hyodysenteriae* isolates with decreased susceptibility to tiamulin. *J Med Microbiol* **53:** 281–285.

Kavanagh, F., Hervey, A., and Robbins, W.J. (1951) Antibiotic substances from basidiomycetes. VIII. *Pleurotus multilus* and *Pleurotus passeckerianus*. *Proc Natl Acad Sci USA* **37:** 570–574.

Kearsey, S.E., and Craig, I.W. (1981) Altered ribosomal RNA genes in mitochondria from mammalian cells with chloramphenicol resistance. *Nature* **290:** 607–608.

Kloss, P., Xiong, L., Shinabarger, D.L., and Mankin, A.S. (1999) Resistance mutations in 23S rRNA identify the site of action of the protein synthesis inhibitor linezolid in the ribosomal peptidyl transferase center. *J Mol Biol* **294:** 93–101.

Knowles, D.J., Foloppe, N., Matassova, N.B., and Murchie, A.I. (2002) The bacterial ribosome, a promising focus for structure-base drug design. *Curr Opin Pharmacol* **2:** 501–506.

Kofoed, C.B., and Vester, B. (2002) Interaction of avilamycin with ribosomes and resistance caused by mutations in 23S rRNA. *Antimicrob Agents Chemother* **46:** 3339–3342.

Koradi, R., Billeter, M., and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J Mol Graphics* **14:** 29–32, 51–55.

Lobova, D., Smola, J., and Cizek, A. (2004) Decreased susceptibility to tiamulin and valnemulin among Czech isolates

of *Brachyspira hyodysenteriae*. *J Med Microbiol* **53:** 287–291.

Meskauskas, A., Harger, J.W., Jacobs, K.L., and Dinman, J.D. (2003) Decreased peptidyltransferase activity correlates with increased programmed −1 ribosomal frameshifting and viral maintenance defects in the yeast *Saccharomyces cerevisiae*. *RNA* **9:** 982–992.

Nissen, P., Hansen, J., Ban, N., Moore, P.B., and Steitz, T.A. (2000) The structural basis of ribosome activity in peptide bond synthesis. *Science* **289:** 920–930.

Peltz, S.W., Hammell, A.B., Cui, Y., Yasenchak, J., Puljanowski, L., and Dinman, J.D. (1999) Ribosomal protein L3 mutants alter translational fidelity and promote rapid loss of the yeast killer virus. *Mol Cell Biol* **19:** 384–391.

Poulsen, S.M., Karlsson, M., Johansson, L.B., and Vester, B. (2001) The pleuromutilin drugs tiamulin and valnemulin bind to the RNA at the peptidyl transferase centre on the ribosome. *Mol Microbiol* **41:** 1091–1099.

Sander, P., Belova, L., Kidan, Y.G., Pfister, P., Mankin, A.S., and Böttger, E.C. (2002) Ribosomal and non-ribosomal resistance to oxazolidinones: species-specific idiosyncrasy of ribosomal alterations. *Mol Microbiol* **46:** 1295–1304.

Schindler, D., Grant, P., and Davies, J. (1974) Trichodermin resistance – mutation affecting eukaryotic ribosomes. *Nature* **248:** 535–536.

Schlünzen, F., Zarivach, R., Harms, J., Bashan, A., Tocilj, A., Albrecht, R., *et al.* (2001) Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature* **413:** 814–821.

Schlünzen, F., Pyetan, E., Fucini, P., Yonath, A., and Harms, J.M. (2004) Inhibition of peptide bond formation by pleuromutilins: the structure of the 50S ribosomal subunit from *Deinococcus radiodurans* in complex with tiamulin *Mol Microbiol* doi:10.1111/j.1365-2958.2004.04346.x

Singer, R. (1986) Clitopilus. In *The Agaricales in Modern Taxonomy*. Koenigstein, Germany: Koeltz Scientific Books, pp. 699–702.

Springer, D.M., Sorenson, M.E., Huang, S., Connolly, T.P., Bronson, J.J., Matson, J.A., *et al.* (2003) Synthesis and activity of a C-8 keto pleuromutilin derivative. *Bioorg Med Chem Lett* **13:** 1751–1753.

Tan, G.T., DeBlasio, A., and Mankin, A.S. (1996) Mutations in the peptidyl transferase center of 23S rRNA reveal the site of action of sparsomycin, a universal inhibitor of translation. *J Mol Biol* **261:** 222–230.

Triman, K.L., Peister, A., and Goel, R.A. (1998) Expanded versions of the 16S and 23S ribosomal RNA mutation databases (16SMDBexp and 23SMDBexp). *Nucleic Acids Res* **26:** 280–284.

Trott, D.J., Stanton, T.B., Jensen, N.S., Duhamel, G.E., Johnson, J.L., and Hampson, D.J. (1996) *Serpulina pilosicoli* sp. nov., the agent of porcine intestinal spirochetosis. *Int J Syst Bacteriol* **46:** 206–215.

Vester, B., and Douthwaite, S. (2001) Macrolide resistance conferred by base substitutions in 23S rRNA. *Antimicrob Agents Chemother* **45:** 1–12.

Weiss-Brummer, B., Zollner, A., Haid, A., and Thompson, S. (1995) Mutation of a highly conserved base in the yeast mitochondrial 21S rRNA restricts ribosomal frameshifting. *Mol Gen Genet* **248:** 207–216.

Xiong, L., Kloss, P., Douthwaite, S., Andersen, N.M., Swaney, S., Shinabarger, D.L., and Mankin, A.S. (2000) Oxazolidinone resistance mutations in 23S rRNA of *Escherichia coli* reveal the central region of domain V as the primary site of drug action. *J Bacteriol* **182:** 5325–5331.

Zuerner, R.L., and Stanton, T.B. (1994) Physical and genetic map of the *Serpulina hyodysenteriae* B78T chromosome. *J Bacteriol* **176:** 1087–1092.