

## Ribosomal Alterations Contribute to Bacterial Resistance against the Dipeptide Antibiotic TAN 1057

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**TAN 1057-resistant *Staphylococcus aureus* and *Escherichia coli* strains were selected to elucidate the mechanism of resistance and the mode of action of this dipeptide antibiotic. Cell-free translation with isolated ribosomes and S150 fractions from sensitive and resistant *S. aureus* strains demonstrated that alterations in the ribosomes contribute to the resistance of the bacteria.**

There is a critical need for new antibacterial compounds with no cross-resistance to commonly prescribed antibiotic classes (2, 15, 23). The antibiotic activity and the unique molecular architecture of TAN 1057 A/B prompted us to launch a chemical optimization program. TAN 1057 was first described by Hideo in 1989 (10) as a metabolite produced by the gram-negative soil bacterium *Flexibacter* sp. strain PK-74. The two diastereomers TAN 1057 A and B can be separated, but they spontaneously epimerize in aqueous solution (1, 3, 4, 14, 21, 22, 25). Therefore, all of the experiments described here were performed with a diastereomeric mixture (Fig. 1). TAN 1057 displays in vitro (5) and in vivo (9) antibacterial activity against staphylococci, including methicillin-resistant *Staphylococcus aureus* strains. Its in vitro activity is excellent in synthetic medium and pure fetal calf serum (FCS), whereas in a standard assay medium such as Mueller-Hinton (MH) broth, the MICs increase 10- to 256-fold (e.g., the MICs against *S. aureus* P209 in AOAC and MH medium are 0.1 and 3.1  $\mu\text{g/ml}$ , respectively) (3, 12). Preliminary mechanism-of-action studies revealed that TAN 1057 inhibits protein synthesis in whole-cell experiments and in cell-free translation assays (12). Further experiments demonstrated that the mechanism of action of TAN 1057 is complex. Cell-free translation is inhibited with a 50% inhibitory concentration ( $\text{IC}_{50}$ ) of 4.5  $\mu\text{g/ml}$ , and ribosome assembly is affected with almost equal potency ( $\text{IC}_{50}$ : 9  $\mu\text{g/ml}$ ) (6, 7). Boeddeker et al. (3), reported that TAN 1057 inhibits protein synthesis in cellular assays in *Escherichia coli* and *S. aureus* and in cell-free translation assays derived from both bacteria, probably by inhibiting the peptidyltransferase activity of the ribosomes.

The purpose of our study was to select bacteria resistant to TAN 1057 as a tool with which to investigate if the mechanism of resistance is mediated by alterations in the translational apparatus. Resistance selection was performed with the clinical isolate *S. aureus* 133 (SA133-TAN<sup>S</sup>, DSM11832) and the laboratory strain *S. aureus* RN4220 (SA4220-TAN<sup>S</sup>) by the broth dilution method in FCS (13). The resulting isolates were the product of six serial transfers over a 6-day period with increas-

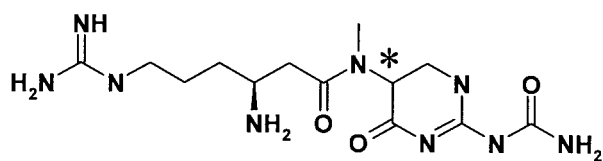
ing TAN 1057 concentrations (SA-TAN<sup>R</sup>-1 to -6). A stepwise increase in resistance was observed for both strains with MICs increasing by 1 or 2 dilution steps per day. At day 6, highly resistant strains for which the MICs were  $\geq 64 \mu\text{g/ml}$  were observed (Table 1). Clinical *E. coli* isolates are normally not sensitive to TAN 1057. However, this constant increase in TAN 1057 resistance was also observed in experiments with TAN 1057-sensitive *E. coli* HN 818 ( $\Delta\text{acrAB}$ ), demonstrating that this phenotype is observed in gram-positive and gram-negative bacteria. To examine the stability of resistance, SA133-TAN<sup>R</sup>-6 was subcultured in drug-free FCS for 6 days (SA133-TAN<sup>R</sup>-6a). No MIC difference between these two strains was observed, indicating that the resistance phenotype observed is stable.

Antibiotic susceptibilities of SA133-TAN<sup>S</sup> and SA133-TAN<sup>R</sup>-6a were determined for 19 reference compounds, including several translation inhibitors (Table 2). MICs of TAN 1057 increased >1,000-fold, and no significant change in susceptibility to any of the other compound tested was evident. This lack of cross-resistance indicates that TAN 1057 acts by a new mechanism.

In order to investigate if the mechanism of resistance is due to modifications in the bacterial translation machinery, cell-free S30 transcription-translation (TT) extracts were prepared from SA133-TAN<sup>R</sup> strains and the precursor strain SA133-TAN<sup>S</sup> and used for coupled cell-free TT experiments (16, 17). In these experiments, plasmid pKV48 was used as a template and incorporation of radioactive [<sup>35</sup>S]Met into the chloramphenicol acetyltransferase protein was measured by precipitation with potassium hydroxide, subsequent filtration, and detection of the filter-bound radioactivity in a scintillation counter (17). TAN 1057 inhibits TT of SA133-TAN<sup>S</sup> extract with high potency ( $\text{IC}_{50}$ , 1.45  $\mu\text{g/ml}$ ; Table 3). Extracts from strains isolated early during resistance selection were still sensitive, with  $\text{IC}_{50}$ s between 1.28 and 1.78  $\mu\text{g/ml}$ . Extracts isolated from SA133-TAN<sup>R</sup>-5 (day 5) became approximately 13-fold resistant in TT experiments ( $\text{IC}_{50}$ , 23.2  $\mu\text{g/ml}$ ). No further  $\text{IC}_{50}$  increase was detected for extracts isolated at day 6 (SA133-TAN<sup>R</sup>-6 and -6a). In control TT experiments, no change in sensitivity was observed for the translation inhibitor erythromycin, with  $\text{IC}_{50}$ s of 0.19 to 0.39  $\mu\text{g/ml}$  for all extracts (SA133-TAN<sup>S</sup> and SA133-TAN<sup>R</sup>).

In order to identify the component responsible for maintain-

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## TAN 1057 A/B

TAN 1057 A \* = R  
B \* = S

FIG. 1. Chemical structure of the dipeptide antibiotic TAN 1057 A/B.

ing TAN 1057 resistance in TT, S30 extracts from SA133-TAN<sup>S</sup> and the final isolate SA133-TAN<sup>R</sup>-6a were separated into S150 fractions (containing all of the soluble factors necessary for TT) and crude ribosomes (16, 17). Subsequently, TT experiments with various combinations of these S150 fractions and ribosomes were performed (Fig. 2A). Experiments in the absence of any inhibitor demonstrated that both *S. aureus* strains had comparable activities, and experiments in the presence of erythromycin indicated that both strains had comparable sensitivities to this control compound (Fig. 2B). All combinations of ribosomes and S150 fractions showed a dose-dependent decrease in activity in the presence of TAN 1057. TT was sensitive when ribosomes from SA133-TAN<sup>S</sup> were used and became resistant when ribosomes from SA133-TAN<sup>R</sup>-6a were tested, independently of the S150 fractions added. This suggests that the TAN 1057 resistance observed in the TT experiments results from alterations of the bacterial ribosome.

TAN 1057 resistance is complex. Boeddeker et al. (3) selected TAN 1057-resistant *S. aureus* by a one-step selection method in solid MH medium. Many of the colonies that grew at four times the MIC were small and grew slowly. At least one strain was resistant to TAN 1057 when tested in a liquid MIC test, with values increasing by a factor of >50 compared to the wild-type *S. aureus* strain. However, wild-type and mutant extracts supported cell-free poly(U)-dependent poly(Phe) syn-

TABLE 1. MICs for bacterial strains isolated during resistance selection

Isolate	TAN 1057 MIC ( $\mu\text{g/ml}$ )		
	<i>S. aureus</i> 133	<i>S. aureus</i> RN 4220	<i>E. coli</i> HN818
TAN <sup>S</sup>	0.125	0.06	4
TAN <sup>R</sup> -1	0.5	0.25	8
TAN <sup>R</sup> -2	1	0.5	16
TAN <sup>R</sup> -3	2	1	32
TAN <sup>R</sup> -4	8	4	32
TAN <sup>R</sup> -5	32	16	64
TAN <sup>R</sup> -6	>64	64	64
TAN <sup>R</sup> -6a	>64	ND <sup>a</sup>	ND

<sup>a</sup> ND, not done.

TABLE 2. MICs of different reference compounds for SA133-TAN<sup>S</sup> and SA133-TAN<sup>R</sup>-6a

Compound	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>	
	SA133-TAN <sup>S</sup>	SA133-TAN <sup>R</sup> -6a
TAN 1057	0.1	>100
Erythromycin	0.125	0.25
Clindamycin	0.03	0.03
Chloramphenicol	8	4
Linezolid	4	2
Streptomycin	8	4
Fusidic acid	1	1
Tetracycline	4	4
Neomycin	0.5	0.25
Kanamycin	2	1
Ampicillin	0.25	0.25
Penicillin G	0.015	0.03
Methicillin	2	4
Vancomycin	1	1
Teicoplanin	1	1
Fosfomycin	8	8
Moxifloxacin	0.03	0.03
Ciprofloxacin	1	0.25
Trimethoprim	2	2
Ethidium bromide	8	4

<sup>a</sup> All compounds were tested in ISO-Sensitest medium, except TAN-1057, which was tested in FCS.

thesis at similar rates, indicating that the translation machinery was not altered in this resistant strain. The authors concluded that alterations in a dipeptide transport mechanism might be responsible for this increase in resistance, because TAN 1057 resembles a dipeptide molecule and its activity can be antagonized in cellular assays by addition of dipeptides (3, 12). In our study, we used a multistep selection procedure over a 6-day period in liquid FCS. Different resistance mechanisms might contribute to the phenotypes observed in our experiments. Early, low-level resistance might be due to alterations in dipeptide transporters or alterations in efflux pumps, e.g., *norA*, as observed for quinolones (8, 11, 19). We have evidence that *norA* overexpression does not contribute to TAN 1057 resistance, because resistant strain SA133-TAN<sup>R</sup>-6a is sensitive to the *norA* substrates ciprofloxacin and ethidium bromide (Table 2). Since *norA* is not the only efflux pump in *S. aureus*, further

TABLE 3. Inhibition by TAN 1057 and erythromycin of coupled cell-free TT of extracts derived from different TAN-sensitive and TAN-resistant strains

Strains	Cell-free TT IC <sub>50</sub> ( $\mu\text{g/ml}$ ) <sup>a</sup>	
	TAN 1057	Erythromycin
SA133-TAN <sup>S</sup>	1.45	0.3
SA133-TAN <sup>R</sup> -1	1.4	0.19
SA133-TAN <sup>R</sup> -2	1.38	0.3
SA133-TAN <sup>R</sup> -3	1.28	0.31
SA133-TAN <sup>R</sup> -4	1.78	0.37
SA133-TAN <sup>R</sup> -5	23.2	0.37
SA133-TAN <sup>R</sup> -6	15.4	0.19
SA133-TAN <sup>R</sup> -6a	19.3	0.39

<sup>a</sup> [<sup>35</sup>S]Met incorporated into chloramphenicol acetyltransferase was detected by precipitation with potassium hydroxide, subsequent filtration, and quantification of the filter-bound radioactivity in a scintillation counter. IC<sub>50</sub> are mean values of at least two independent experiments.

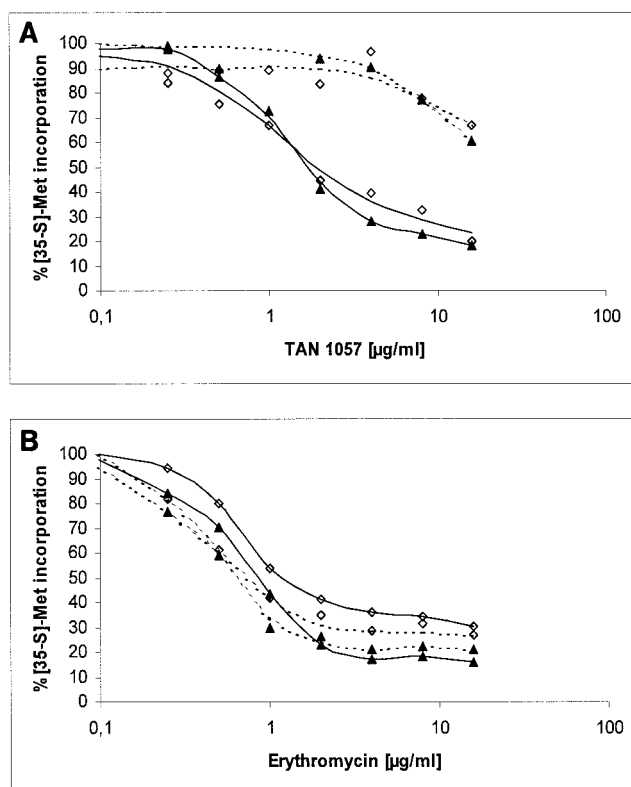


FIG. 2. Coupled cell-free TT assays with ribosomes (70S) and S150 fractions derived from SA133-TAN<sup>S</sup> and SA133-TAN<sup>R</sup>-6a were used to test for resistance to TAN 1057 (A) and erythromycin (B). Dotted lines, ribosomes isolated from SA133-TAN<sup>R</sup>-6a; solid lines, ribosomes from SA133-TAN<sup>S</sup>; diamonds, S150 fraction from SA133-TAN<sup>R</sup>-6a; triangles, S150 fraction from SA133-TAN<sup>S</sup>.

investigations are needed to demonstrate if other efflux pumps are involved during early development of resistance to TAN 1057. The early, low-level resistance mechanism is followed by an alteration in the ribosome. Ribosomal-protein genes are the most likely targets for these mutations, because these proteins are encoded only once in the genome and one mutation would lead to 100% mutated ribosomes. Another possible explanation for a stepwise MIC increase is the occurrence of mutations in the rRNA, as reported for the oxazolidinones. In *Enterococcus faecalis* and *E. faecium*, the level of resistance to linezolid is correlated with the number of 23S rRNA genes containing a G2576U mutation, providing one example for a clear gene dosage effect (6, 18, 20, 24). Five rRNA gene copies have been described in *S. aureus*. If one of these genes has been mutated during TAN 1057 selection, presumably 15 to 20% of the ribosome population would be resistant. Mutations of further rRNA genes would lead to further increases in the mutated ribosome population and, consequently, might lead to stepwise increases in resistance. However, these stepwise increases in resistance were not observed in the cell-free translation and further experiments are necessary to investigate the effect of TAN 1057 on protein synthesis in whole-cell assays with TAN<sup>S</sup> and TAN<sup>R</sup> strains.

In summary, a stepwise increase in resistance was observed during TAN 1057 resistance selection in different bacterial

species, and cell-free TT assays revealed that alterations in the ribosomes contribute to the bacterial resistance observed.

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