ISOLATION OF *STREPTOMYCES TENDAE* MUTANTS WITH AN ALTERED NIKKOMYCIN SPECTRUM

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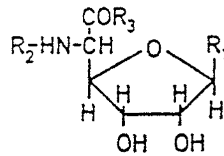
To isolate *Streptomyces tendae* mutants blocked in the biosynthesis of the nikkomycin nucleoside base 4-formyl-4-imidazoline-2-one, an assay was developed to detect the formation of nikkomycins containing this base during growth on solid medium. The assay is based on the reaction of the 4-formylimidazolone structure of nikkomycins with the aldehyde reagent barbituric acid leading to red-colored products. Among 18,000 *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treated clones tested in the barbituric acid assay, we isolated one mutant which was incapable of forming any nikkomycins containing the 4-formylimidazolone base (nikkomycins C_x, X and I) but instead produced nikkomycins containing uracil (nikkomycins C, Z and J). In addition, we isolated strains with mutations affecting the biosynthesis of 2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-methylbutyric acid, the unusual amino acid of nikkomycins Z, X, J and I. By analyzing colonies derived from single spores or protoplasts of *S. tendae* Tü901/395, a mutant producing besides nikkomycins Z, X, J and I, also nikkomycins K_z/K_x and O_z/O_x, we obtained strains which only formed nikkomycins K_z/K_x and O_z/O_x with 2-amino-4-hydroxy-4-(2-pyridyl)butyric acid and 2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-butyric acid as amino acids. Mutation of such a strain (Tü901/395-11) by $UV_{365\,nm}$ in the presence of 8-methoxypsoralen and selection of *S*-2-aminoethyl-L-cysteine-resistant clones led to the isolation of Tü901/AEC1 and AEC2 which produced exclusively nikkomycins K_z and K_x. According to their nikkomycin spectrum, these strains were blocked at the hydroxylation step occuring at the pyridyl residue during biosynthesis of the nikkomycin amino acid.

Nikkomycins belong to the nucleoside peptide antibiotics and act as potent inhibitors of chitin synthetases from fungi and insects[1~4]. *Streptomyces tendae* Tü901 produces a spectrum of various nikkomycins with 4-formyl-4-imidazoline-2-one and uracil as variable bases[5,6]. The nucleoside moiety of biologically active nikkomycins produced by the wild type as major components is peptidically linked to the unusual amino acid 2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-methylbutyric acid (nikkomycin D, Fig. 1). Studies on the biosynthesis of nikkomycins revealed that the imidazolone base is derived from L-histidine[7] and uracil from pyrimidine metabolism[8]. Furthermore, L-lysine was shown to be the precursor of the pyridyl residue and the attached hydroxymethylene carbon in nikkomycin D[9]. There is little known about enzymatic reactions and intermediate structures of nikkomycin biosynthesis. Therefore we decided to screen for mutants blocked in nikkomycin biosynthesis which could be employed as hosts for cloning experiments to isolate nikkomycin biosynthetic genes. We describe the isolation of strains of *S. tendae* with mutations affecting the biosynthesis of the imidazolone base and nikkomycin D.

Fig. 1. Structures of nikkomycins.
Biologically active (A) and inactive (B) structures.



(A)

Nikko-mycin	R_1	R_2	R_3
Z			OH
X			OH
J			Glu
I			Glu
K_z			OH
K_x			OH
O_z			OH
O_x			OH

(B)

Nikko-mycin	R_1	R_2	R_3
C		H	OH
C_x		H	OH
D			

Materials and Methods

Organisms and Mutagenesis

The wild type strain *S. tendae* Tü901/8c was obtained from the Streptomyces Culture Collection of the Institute of Microbiology I, University of Tübingen, FRG. The strain Tü901/395 was derived from Tü901/8c[10]. Spores of Tü901/8c were mutagenized by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) according to DELIĆ *et al.*[11]. 10^9 spores were incubated at 30°C for up to 60 minutes in 1 ml 0.05 M Tris-maleic acid, pH 9.0 containing 2 mg NTG.

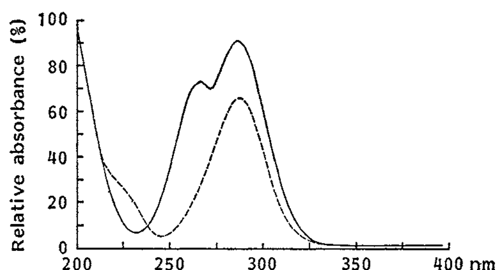
Mutants were selected among the 2% survivors. Mutagenesis with UV light at 365 nm in the presence of 8-methoxypsoralen (MOP) is described by TOWNSEND *et al.*[12]. Among the 0.1% survivors

analog-resistant mutants were selected on minimal medium[13] with 250 μg/ml S-2-aminoethyl-L-cysteine.

Culture Conditions

HA medium consisting of 0.4% yeast extract, 0.4% glucose, 1% malt extract and 2% agar, pH 7.2 was used for growth of strains on solid medium. To investigate the nikkomycin spectrum, batch cultures were grown in 10 ml nikkomycin-production medium in baffled 100-ml Erlenmeyer flasks on a rotatory shaker at 27°C. The production medium consisted of mannitol 3%, starch 1%, soy bean meal 2% and yeast extract 1%, pH 6.0.

Fig. 2. UV spectra of purified nikkomycin K_x (—) and nikkomycin X (---).



Nikkomycin Determination

The nikkomycin spectrum of the investigated strains was determined by analyzing culture filtrates by HPLC as described by FIEDLER[14]. UV spectra of eluted nikkomycins were recorded by a photodiode array detector during the HPLC run[15]. Nikkomycins in the culture broths were identified by their HPLC-retention times and their UV spectra which were compared to those of purified nikkomycins. Fig. 2 shows the UV spectrum of purified nikkomycin K_x which was corrected from that published by FIEDLER[15]. In contrast to all other known nikkomycins with the 4-formylimidazolone base which have a single absorption maximum at 287 nm (Fig. 2), nikkomycin K_X exhibit an additional absorption maximum at 267 nm.

Results

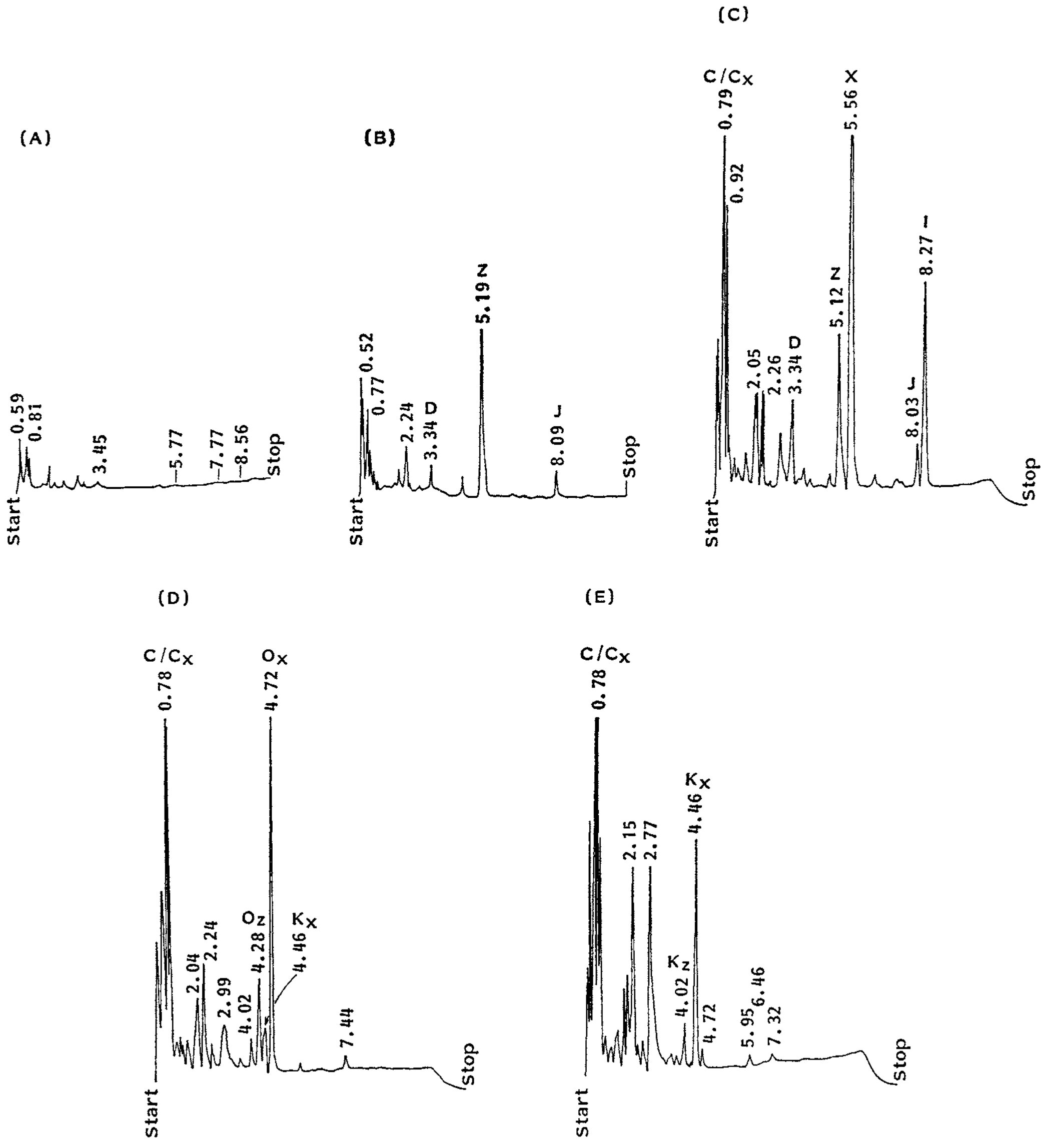
Isolation of Mutants Blocked in the Biosynthesis of the 4-Formyl-4-imidazoline-2-one Base

DELZER *et al.*[16] reported that barbituric acid reacts with nikkomycins containing the 4-formylimidazolone base leading to red-colored products having an absorption maximum of 440 nm in water. Similar to the reaction of dimedone with aldehydes, two molecules of barbituric acid react with one molecule of nikkomycin (determined for nikkomycin X). This reaction was used as a basis to develop a method to screen for strains with a mutation affecting the biosynthesis of the 4-formylimidazolone base; about 30~35 colonies derived from single mutagenized spores were streaked in patches on agar plates (85 mm in diameter) containing 30 ml HA medium and grown at 30°C for 2 days. Then, agar plugs of about 5 mm in diameter were cut out and incubated for 2 hours at 30°C on plates (85 mm in diameter) containing 10 ml 0.1% barbituric acid, 0.7% agarose, pH 7.0 adjusted with NaOH. Finally the developed color of the agar plugs was observed on the bottom of the barbituric acid plates. Clones producing nikkomycins with the 4-formylimidazolone base showed an orange color (wild type), while negative clones were yellow. Strains appearing to be negative after a second screening were cultivated in liquid production medium and their nikkomycin spectrum was analyzed by HPLC.

Among 18,000 clones tested, 26 showed a negative reaction with barbituric acid. As shown by HPLC, 25 of these strains did not produce any biologically active nikkomycins (Z, X, J and I) nor the inactive nucleoside structures, nikkomycins C and Cx, or the amino acid, nikkomycin D (Figs. 1 and 3A). In contrast, the mutant Tü901/L9 formed nikkomycins Z and J containing uracil as base at concentrations similar to those produced by the wild type (about 450 mg/liter nikkomycin Z; 150 mg/liter nikkomycin J) but did not form nikkomycins with the 4-formylimidazolone base (Fig. 3B).

Fig. 3. HPLC analyses of the culture broth of *Streptomyces tendae* Tü901 nikkomycin non-producing mutant (A), Tü901/L9 (B), wild type (C), Tü901/395-11 (D) and Tü901/AEC1 (E).

The strains were cultivated in nikkomycin-production medium for 7 days.
Retention times are indicated in minutes.



Tü901/L9 produced nikkomycin X, when resting cells were fed purified nikkomycin C_X (data not shown).

Isolation of Mutants Blocked in the Biosynthesis of Nikkomycin D

In a previous paper[10] we described the mutant Tü901/395 which produced nikkomycins Z, X (about 350 mg/liter), J/I (about 150 mg/liter) and also nikkomycin K (100 mg/liter) and O (250 mg/liter) which exhibit an altered amino acid[17] (Fig. 1). After isolating colonies derived from single spores or protoplasts of this mutant, only 6% among 120 investigated clones produced the complete

nikkomycin spectrum of the parent strain, while 3% were non-producers which did not form any known nikkomycin structures (Fig. 3A). The 15% of the clones had the nikkomycin spectrum of the wild type consisting of nikkomycin Z/X (800~900 mg/liter), J/I (300~400 mg/liter) and O (30 mg/liter) (Fig. 3C). In contrast, 76% of the strains formed only nikkomycin Kz/Kx (10~100 mg/liter) and Oz/Ox (50~300 mg/liter) as biologically active structures. In addition, relatively high amounts of the nucleoside moieties nikkomycin C/C_x (600~800 mg/liter) were excreted, while nikkomycin D could not be detected in their culture filtrates. Fig. 3D shows the HPLC analysis of the culture filtrate of Tü901/395-11, a clone belonging to this class of mutants.

Furthermore, we isolated two strains, Tü901/AEC1 and AEC2, with an additional mutation affecting the hydroxylation reaction at the pyridyl ring of the amino acid moiety of nikkomycins. They were derived from Tü901/395-11 (Fig. 3D) treated with $UV_{365\,nm}$/MOP and selected for resistance to the lysine analog *S*-2-aminoetyhl-L-cysteine. Tü901/AEC1 and AEC2 only formed nikkomycin K_z/K_x (200~300 mg/liter) and the nucleoside structures nikkomycin C/C_x (250 mg/liter) (Fig. 3E). In addition, two unknown substances were accumulated in the culture broths of Tü901/AEC1 and Tü901/AEC2 which eluted as significant peaks at 2.15 minutes and 2.77 minutes. The isolation and chemical characterization of these compounds are in progress, since they may be precursors in nikkomycin biosynthesis.

Discussion

The described barbituric acid plate assay for the detection of nikkomycins containing the 4-formylimidazolone base can be employed to screen specifically for strains of *S. tendae* unable to synthesize the 4-formylimidazolone base. After NTG mutagenesis the frequency of isolation of these mutants was relatively low (0.0055%). The mutant Tü901/L9, which only produced nikkomycins with an uracil base, could form nikkomycin X when resting cells were fed purified nikkomycin C_x. Obviously Tü901/L9 is able to form the peptide bond between the nucleoside moiety, nikkomycin C_x, and nikkomycin D. Although the enzymatic step blocked in the biosynthetic pathway from L-histidine to the 4-formylimidazolone base is not defined, Tü901/L9 is a suitable host for cloning nikkomycin biosynthetic genes which could then be subsequently screened in the barbituric acid assay.

The mutants producing only nikkomycins K and O are obviously blocked in the biosynthesis of the C_4-skeleton attached to the hydroxymethylene carbon of nikkomycin D. The alternative pathway leading to the 2-amino-4-hydroxy-4-pyridylbutyric acids seems to be less effective, since the mutants produced low amounts of nikkomycins K and O (400 mg/liter) and accumulated high levels of the nucleoside structures nikkomycins C and C_x. In contrast, revertants of Tü901/395 exhibiting the wild type nikkomycin spectrum (nikkomycins Z, X, J and I) synthesized up to 1,300 mg/liter of biologically active nikkomycins containing nikkomycin D. The hydroxylation occuring at the pyridyl residue during the biosynthesis of the amino acid moiety is specifically blocked in the mutants Tü901/AEC1 and Tü901/AEC2. As the MICs of nikkomycin K_z/K_x are about 100 times less than those of nikkomycins Z and X[10], these mutants can also be used as hosts in cloning experiments for the isolation of nikkomycin biosynthetic genes with a screening for antifungal activity.

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