A Putative Enolpyruvyl Transferase Gene Involved in Nikkomycin Biosynthesis

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The nikO gene encoding a putative enolpyruvyl transferase has been identified within the Streptomyces tendae Tu901/nikkomycin gene cluster. nikO encodes a deduced protein of 471 amino acid residues which exhibits significant sequence similarity to UDP-7-β-acetyl-glucosamine enolpyruvyl transferase and 5-enol-pyruvylshikimate 3-phosphate synthase from various origin. The nikO gene was inactivated by inserting a kanamycin resistance cassette; the mutant did not produce biologically active nikkomycins I, J, X, and Z nor the nucleoside moieties, nikkomycins Cx and Cz, but accumulated the novel component RT 2.0. RT 2.0 has been isolated from culture filtrate and its structure was determined by using mass spectrometry and NMR analyses as ribofuranosyl-4-formyl-4-imidazolone which represents a novel nucleoside. The putative activity of the nikO gene product in nikkomycin biosynthesis will be discussed.

Streptomyces tendae Tu901 produces nikkomycins which belong to the group of peptidyl nucleoside antibiotics (reviewed by Fiedler et al., 1993†). They act as potent competitive inhibitors of chitin synthetases of fungi and insects. Major components of the culture filtrate of S. tendae Tu901 are nikkomycins X and Z consisting of a nucleoside moiety and the peptidically bound unusual amino acid hydroxypyridylhomothreonine (nikkomycin D). The nucleoside part is formed by an aminohexuronic acid that is N-glycosidically linked to 4-formyl-4-imidazolin-2-one forming nikkomycin Cx or to uracil forming nikkomycin Cz. Minor components of the culture filtrate are nikkomycins I and J which are analogous structures to nikkomycins X and Z that contain glutamic acid peptidically bound to the 6'-carboxyl group of the aminohexuronic acid. Polyoxins, other members of the peptide nucleoside antibiotics that have intensively been investigated by Isono and coworkers (reviewed by Isono²) also exhibit the aminohexuronic acid moiety with N-glycosidically bound uracil or a substituted uracil residue as nucleoside, while the peptidyl moiety of polyoxins is different from that of nikkomycins. Biosynthesis of nikkomycins³ and polyoxins² can be divided in two parts: the nucleoside and peptidyl moieties are synthesized in separate pathways and then are linked by peptide bonds. Incorporation studies with labeled precursors revealed that carbon-6' of the polyoxin nucleoside arises from carbon-3 of phosphoenolpyruvate⁴. Isono and coworkers⁴ proposed that the aminohexuronic acid moiety is synthesized by condensation of uridine and phosphoenolpyruvate to give octofuranosulosearonic acid nucleoside as the intermediate, subsequent oxidative elimination of the terminal two carbons, C-7' and C-8', and introduction of an amino group on carbon-5'. Isolation of octosyl acids, shunt metabolites that derive from the postulated intermediate supported the proposed biosynthetic pathway²). As analogs of the octosyl acids, nikkomycins Sx and Sz, have been isolated from the culture filtrate of nikkomycin-producing S. tendae⁵, the same biosynthetic pathway was suggested for nikkomycin nucleosides, nikkomycins Cx and Cz.

Recently, the S. tendae Tu901 nikkomycin biosynthetic gene cluster has been cloned⁶. Two-dimensional gel electrophoresis has led to the identification of ten proteins that are synthesized when nikkomycins are produced. N-terminal sequences of six of the ten proteins were obtained, and those of P1/P2 and P6 led to cloning of the entire set of
nikkomycin (nik) genes\(^7\)). Molecular analysis of seven co-
transcribed nik genes that all are involved in hydroxy-
pyridylhomothreonine biosynthesis led to a proposal for a
novel biosynthetic pathway\(^8\)\(^9\)). Here, we describe the
molecular analysis of the nikO gene encoding a putative
enolpyruvyl transferase and show that its gene product is
involved in the nucleoside biosynthetic pathway.

**Materials and Methods**

**Bacteria, Plasmids, and Culture Conditions**

*S. tendae* Tu901/8c was obtained from H. Zähner
(University of Tübingen, Germany). *S. lividans* TK23\(^10\)
was provided by D. A. Hopwood (John Innes Institute,
Norwich, UK). Subcloning was performed in *Escherichia
coli* JM83\(^12\)) using the vector pUC19\(^12\)). The Streptomyces-
*E. coli* shuttle vector pWHM3\(^13\)) was obtained from C. R.
Hutchinson (University of Wisconsin, Madison, USA),
and vector pIJ702 was used for gene expression in
*Streptomyces*\(^u\). The aph\(^ll\) cassette was isolated from
plasmid pUC19aph\(^9\)). The 5.7-kb *NcoI* fragment carrying
the nikO gene was isolated from cosmid p9/43\(^7\)), blunt-
ended by Klenow enzyme, and ligated into the *SmaI* site of
pUC19 yielding pVM18.

*Streptomyces tendae* Tu901 strains were grown in liquid
production soy-peptone medium\(^8\)\(^n\) on a rotary shaker at
27°C and on solid medium HA\(^8\). *S. lividans* strains were
incubated at 30°C. For the selection of plasmid-containing
*Streptomyces* strains, 30 μg thiostrepton/ml or 10 μg
kanamycin/ml was added to solid medium, and 10 μg
thiostrepton/ml or 10 μg kanamycin/ml was added to liquid
soy-peptone medium. Thiostrepton and kanamycin were
purchased from Sigma.

**General DNA Techniques**

DNA manipulation techniques, DNA sequencing, and
transformation of *Streptomyces* strains were carried out by
standard procedures\(^15\)) and by methods described by
Bruntner et al.\(^9\))

**Construction of the nikO Insertion Mutant**

pVM18 was digested with *Sall* and religated to yield
pBL181. pBL181 contained the 2.9-kb *NcoI-Sall* fragment
of pVM18 carrying the nikO gene (Fig. 1). The 1.3-kb
*HindIII-SmaI* kanamycin (*aphII*)-resistance cassette from
pUC19aph was ligated into the blunted *BglII* site of
pBL181, yielding pBL188 that contained nikO and aphII in
the same direction. Plasmid pBL188 was digested with
EcoRI and *HindIII*, and the insert was ligated into pWHM3
digested with *EcoRI* and *HindIII*. The resulting plasmid
pNO1 was transferred into *S. lividans* TK23 protoplasts,
reisolated, and transferred into *S. tendae* Tu901/8c
protoplasts. *S. tendae* carrying pNO1 was cultivated in
CRM medium\(^6\) containing 5 μg kanamycin/ml for 36 hours
at 27°C; protoplasts were prepared and regenerated on non-
selective regeneration medium. For complementation
experiments the nikO gene was amplified in a standard PCR
reaction (annealing temperature 55°C, Vent polymerase,
New England Biolabs) using oligonucleotides BL201 (5'-
CCTCACCCTCTGATCAGGAGGTACCACCGTG-3') and
BL202 (5'-CCAGAGCCTCTCTTGATCAGGAGGAAGGAGGA-
CCTCGTGCG-3'), containing a restriction site for *BglI*
(underlined). After digestion with *BglI*, the PCR product
was ligated into the *BglII* site of the *Streptomyces* plasmid
pIJ702 and the ligation mixture was transformed into *S.
lividans* TK23. The resulting construct pIJ702nikO that
contained the nikO gene under the control of the *mel*
promoter was used to transform into protoplasts of *S.
tendae* Tu901/8c and *S. tendae* Tu901 nikO::aphII mutants.

**Nikkomycin Analysis**

Nikkomycin structures in the culture supernatant were
determined by HPLC analysis with photodiode array
detection according to Schüz et al.\(^5\)). The Thermo
Separation Products Spectra System consisted of pump
P2000, a vacuum degasser, detector UV3000HR,
autosampler AS3000, controller SN4000, and PC1000
software v3.0.

**Isolation and Structure Elucidation**

Compound RT 2.0 was isolated from 1 liter culture
filtrate of *S. tendae* Tu901 nikO::aphII mutant cultivated in
soy-peptone medium according to Schüz et al.\(^5\)).

Mass spectra were recorded on an API III triple
quadrupole (Perkin-Elmer Sciex, Thornhill, Ontario,
Canada) equipped with an electrospray ionization (ESI)
source. NMR spectra (TOCSY, HMBC, and HSQC) were
recorded on a Bruker AMX 600 spectrometer at 300 K with
\(d_7\)-methanol as the solvent.

**Results**

Cloning and Sequence Analysis of the nikO Gene

In order to isolate a DNA fragment adjacent to the
previously described 8-kb *BamHI* fragment carrying the
gene for proteins P1/P2\(^6\)), the 1.6-kb *NcoI-BamHI* fragment
of one end of this fragment was used as a probe to
hybridize *NcoI*-digested cosmid p9/43 containing a part of
Fig. 1. Localization of the *nikO* gene on the 5.7-kb *NcoI* fragment and construction of *nikO* insertion mutants.

(A) Restriction map of the 5.7-kb *NcoI* fragment containing the *nikO* gene. The DNA fragment indicated as a black solid bar was sequenced. The DNA sequence is available from EMBL database under accession number AJ244016. The arrow below corresponds to *nikO*. The insertion of the kanamycin resistance cassette containing the *aphIII* gene which is indicated by an arrow, and sizes of genomic *NcoI*-fragments of *nikO*: *aphIII* mutants are shown. *pBL181* indicates the insert of plasmid *pBL181* that was used to clone the *aphIII* cassette into the *nikO* gene. B, *BamHI*; Bg, *BglII*; K, *KpnI*; N, *NcoI*; S, *SacI*.

(B) Southern hybridization of *NcoI*-digested chromosomal DNA of *S. tendae* Tu901/8c (lane 1) and of six *nikO*: *aphIII* mutants (lanes 2 to 7) using the 5.7-kb *NcoI* fragment as probe. Lambda *HindIII* size markers (lane 8) are shown on the right, and the calculated sizes of the hybridizing bands on the left.

The hybridizing 5.7-kb *NcoI* fragment was cloned in *pUC19* and mapped for restriction enzymes shown in Fig. 1. The *Tfil*-SacI has been subcloned and sequenced, and a 1413-bp reading frame, designated *nikO*, was found. According to the CODON-PREFERENCE analysis of the nucleotide sequence, *nikO* is assumed to start with the GTG codon at position 59–61 that is preceded by a potential ribosome binding site (GGAGG; nt 47–51) with a good complementarity to the 3′ end of the 16S rRNA and terminates at a *TGA* stop codon in position 1472–1474. *nikO* had a typical Streptomyces codon usage (73.5 mol G+C/100 mol) and encoded a protein of 471 amino acids and a deduced molecular mass of 50.3 kDa. Downstream of *nikO* coding region, there is an inverted repeat sequence beginning at nt 1542 (−15.6 kcal/mol).

Comparison of the deduced NikO sequence with data base protein sequences revealed significant similarity (26% identity, 40% similarity) along the entire protein to UDP-N-acetylglucosamine enolpyruvyl transferases that are similar in size (419 to 444 amino acids) to NikO. These enzymes catalyze the first committed step in murein biosynthesis of the bacterial cell wall transferring the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 3′-OH of UDP-N-acetylglucosamine (UDP-GlcNAc) to yield enolpyruvyl UDP-GlcNAc. A cysteine residue that has been implicated as the active site nucleophile and covalently binds the PEP analog phosphomycin16–18 is conserved in most of these enzymes and is also present in NikO (Cys-128; Fig. 3). In addition, NikO displayed similarity to 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthetases from different organisms in the range of 19% identity and 33% similarity over 390 amino acids. EPSP synthetase catalyzes the transfer of the enolpyruvyl group from PEP to the 5′-hydroxyl of shikimate 3-phosphate in the aromatic amino acid pathway19.

**Construction and Characterization of *nikO* Insertion Mutants**

To investigate the function of NikO in nikkomycin biosynthesis, *nikO* was inactivated by inserting a
kanamycin resistance gene via double-crossover homologous recombination. S. tendae/8c protoplasts were transformed with pNO1, which contains the 2.9-kb NcoI-SacI fragment with the aphII cassette cloned into the unique BglII site within nikO (Fig. 1). After protoplasting and regeneration of S. tendae Tü901/8c (pNO1) under non-selective conditions, about 95% of the tested colonies were kanamycin resistant and thiostrepton sensitive. Southern blot analysis using the 5.7-kb NcoI fragment as the probe showed that each of the six kanamycin-resistant, thiostrepton-sensitive clones had integrated the aphII cassette in the nikO gene, since hybridizing bands appeared with the expected 2.4-kb and 4.6-kb genomic fragments (Fig. 1). The nikO:aphII mutants and S. tendae Tü901/8c were grown in nikkomycin production medium, and the culture filtrates were analyzed by HPLC for the presence of nikkomycins (Fig. 2). The nikO::aphII mutants produced neither the biologically active nikkomycins X, Z, I, and J nor the nucleosides nikkomycins Cx and Cz, but accumulated the compound RT 2.0. The ultraviolet spectrum of RT 2.0 revealed an absorption maximum of 290 nm and was very similar to that of nikkomycin Cx (data not shown)\textsuperscript{20}. In addition, RT 2.0 reacted with the aldehyde reagent barbituric acid to form red-colored reaction compounds; this reaction is characteristic for nikkomycins containing the 4-formyl-imidazolone base\textsuperscript{21}).

For a complementation experiment the nikO gene was cloned into the BglII site of plasmid pJ702 under the control of the promoter of the tyrosinase gene. However, all transformation experiments to introduce this construct into protoplasts of the nikO::aphII mutants failed.
Isolation and Physicochemical Properties of the Biosynthetic Intermediate

Compound RT 2.0 was isolated from fermentation broth (1 liter) containing approximately 100 mg L⁻¹ by chromatography on Dowex 50 WX 2, Lewatit MP 64 Z, and Biogel P2. Compound RT 2.0 was obtained at more than 90% purity and used for structure elucidation.

Electrospray mass spectrometry gave a [M-H]⁻ signal at m/z 243.1 for RT 2.0. The complete structure elucidation was based on the following interpretation of NMR spectra (Table 1). The six signals in the range of 3.7 to 5.61 ppm in the proton spectrum could be easily assigned to the sugar moiety of the compound. The signal at 5.61 ppm is typical for a glycosidic alpha-proton in a sugar ring. According to the HSQC experiment, the two protons at 3.71 and 3.81 ppm are part of the CH₂-group at position 5' of the sugar ring. The other signals were assigned according to ¹H-¹H coupling constants and comparison with those of uridine and nikkomycin C₅. The ¹H-¹H coupling constants of RT 2.0 are very similar to those of the reference compounds uridine and nikkomycin C₅ (Table 2). The signal multiplicities of RT 2.0 and uridine are almost identical. Furthermore, the ¹H-¹H coupling constants J₁',₂', J₂',₃', J₃',₄' of RT 2.0 are very similar to those of nikkomycin C₅, both compounds contain the same formylimidazolone base. The ¹H-¹H coupling constants J₄',₅'(H₁'), J₄',₅'(H₂'), J₅'(H₁)', J₅'(H₂)' of RT 2.0 are very similar to those of uridine due to the identity of this structural element. These data led to the conclusion that RT 2.0 contains a ribofuranosyl moiety. For the proton with a chemical shift of 9.29 ppm the HSQC experiment showed a correlation to a carbon atom with a chemical shift of 179.0 ppm, which is characteristic of a carbonyl group. Thus, the presence of a formyl group in the molecule was confirmed. The remaining signal at 7.81 ppm was assigned to the aromatic proton in the heterocyclic ring at C-5. According to these results compound RT 2.0 has the structure shown in Fig. 4. Comparison of the NMR results with published data of similar compounds supported the proposed structure.

### Table 1. ¹H and ¹³C chemical shifts (ppm) of RT 2.0.

| ¹H      | 5.781 (5-H), 9.29 (6-H), 5.61 (1'-H), 4.33 (2'-H), 4.20 (3'-H), 4.01 (4'-H), 3.71 (5'-H), 3.81 (5'-H)³⁸
| ¹³C     | 126.0 (5-C), 179.0 (6-C), 88.9 (1'-C), 75.5 (2'-C), 71.7 (3'-C), 86.5 (4'-C), 62.6 (5'-C)³⁸

³⁸Numbering of atoms see Figure 4.

### Table 2. Coupling constants J_HH [Hz] determined for RT 2.0 and uridine and for nikkomycin C₅.

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<th>RT 2.0</th>
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<th>nikkomycin C₅</th>
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⁹Numbering of atoms see Figure 4.

Discussion

In this study we have identified the nikkomycin
biosynthesis gene \textit{nikO}. The deduced NikO protein has striking similarity to UDP-GlcNAc enolpyruvyl transferase and EPSP synthase catalyzing the transfer of the intact enolpyruvyl moiety of PEP to a substrate. The former enzyme catalyzes the first committed step in the peptidoglycan biosynthesis. It is the target of the antibiotic phosphomycin which acts as an PEP analog and binds covalently to a cysteine residue inactivating the enzyme\textsuperscript{16,18}. Increased synthesis of UDP-GlcNAc enolpyruvyl transferase by cloning the encoding gene on a multicopy plasmid has led to a phosphomycin resistant phenotype in \textit{Escherichia coli}\textsuperscript{24}. A similar result has been obtained for NikO that has a cysteine residue at the relevant position (Cys-128); \textit{E. coli} transformed with the multicopy plasmid pUC19 containing the \textit{nikO} gene under the control of the \textit{lac} promoter exhibited a ten times increased resistance towards phosphomycin compared to \textit{E. coli} carrying pUC19 alone (data not shown). The reason that we could not transform the multicopy plasmid pIJ702 carrying the \textit{nikO} gene under the control of the \textit{mel} promoter into \textit{S. tendae} Tu901 protoplasts could be that an immediate high expression of the \textit{nikO} gene inhibits peptidoglycan synthesis by titrating PEP and preventing regeneration of protoplasts.

The phenotype of the \textit{nikO} insertion mutant was due to the inactivated \textit{nikO} gene. A polar effect of the inserted kanamycin cassette on downstream located genes can be excluded, as \textit{nikO} represents the terminal gene of a transcription unit (paper in preparation). Based on the presented data the NikO protein is suggested to catalyze the initial reaction in nucleoside biosynthesis transferring enolpyruvate from PEP to ribofuranosyl-4-formyl-4-imidazolone (Fig. 4) (or 5'-phosphoribofuranosyl-4-formyl-4-imidazolin-2-one) and uridine (or UMP) to give the intermediate octofuranoseuronic acid nucleosides. Isono et al.\textsuperscript{25} proposed an aldol-type condensation of the 5'-aldehydes with PEP. This enzymatic condensation would be similar to that catalyzed by 3-deoxyheptulosonate 7-phosphate (DAH 7-P) synthase in which the C-3 of PEP is condensed with erythrose 4-phosphate to give DAH 7-P, the precursor of the shikimate pathway\textsuperscript{25}, and that catalyzed by 3-deoxyoctulosonate 8-phosphate (KDO 8-P) synthase in which PEP reacts with the C-1 of D-arabinose to give KDO 8-P, a sugar moiety of the lipopolysaccharide\textsuperscript{26}. By contrast, UDP-GlcNAc enolpyruvyl transferase and EPSP synthase reaction involve an attack of a hydroxyl nucleophile of a cosubstrate on the C-2 of PEP to yield a tetrahedral intermediate containing a phospholactoyl moiety and form a carboxyvinyl ether by the release of inorganic phosphate\textsuperscript{16,17,19}. To investigate the reaction
mechanism of NikO, enzyme studies with the over-
expressed protein are in progress.
Ribofuranosyl-4-formyl-4-imidazolone produced by the
nikO insertion mutant represents a novel nucleoside that
is easily isolated in large amounts. It is an analog to
uridine that might act as inhibitor of nucleotide-
metabolizing enzymes and might be used as moiety for the
synthesis of new antiviral or anticancer agents.

Acknowledgments

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