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Transvalencin A, a Thiazolidine Zinc Complex Antibiotic Produced by a Clinical Isolate of *Nocardia transvalensis*

I. Taxonomy, Fermentation, Isolation and Biological Activities

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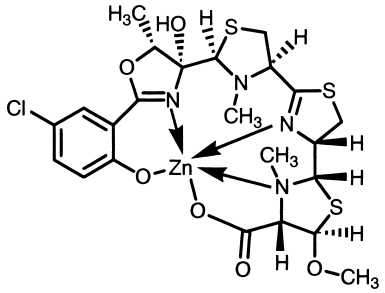
A new thiazolidine-type antibiotic with zinc in its structure, designated transvalencin A, was isolated from *Nocardia* sp. IFM 10065, a clinical isolate from a patient with actinomycotic mycetoma. The strain was identified as *Nocardia transvalensis* based on its morphological, phenotypic and phylogenetic characteristics. Transvalencin A showed antimicrobial activity against fungi such as *Trichophyton mentagrophytes* and *Cryptococcus neoformans*. The antibiotic is also active against Gram-positive bacteria such as *Micrococcus luteus*. We observed higher activity for fungi in an acidic medium than in a neutral medium.

Human nocardioses are caused by members of the *Nocardia asteroides* complex (*N. asteroides* sensu stricto, *N. farcinica*, and *N. nova*), *N. brasiliensis*, *N. otitidiscaviarum*, and *N. transvalensis*.[1,2] We have isolated several new bioactive compounds from clinical isolates of *Nocardia* strains.[3~5] Most bioactive compounds were produced by strains of *N. brasiliensis*.[4~9] However, we recently isolated new bioactive compounds from non-*N. brasiliensis* species such as *N. pseudobrasiliensis* and *N. asteroides*.[10~12] During our continuing studies of biologically active compounds from non-*N. brasiliensis* clinical isolates, we isolated a new antifungal compound, designated as transvalencin A (Fig. 1), from the mycelial cake of a clinical isolate of *Nocardia* sp. strain IFM 10065 obtained from a patient with actinomycotic mycetoma. This paper describes the taxonomy of the producing organism, fermentation, isolation, physicochemical properties, and biological activities of the antibiotic. Structural elucidation of the compound is reported in a subsequent paper.

Fig. 1. Structure of transvalencin A.



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Material and Methods

Taxonomic Studies

Nocardia sp. strain IFM 10065 was isolated in 2001 from a 65-year-old Japanese male patient with actinomycotic mycetoma. The strain IFM 10065 was cultured on a Mueller Hinton II agar slant (MHII; Difco Laboratories, USA) with 1% glucose and 1% glycerol for 1 week at 27°C. For extraction of DNA and its sequencing, the bacterial strain was cultured on brain heart infusion (BHI; Difco Laboratories, USA) broth for 4 days at 32°C.

Physiological and biochemical characterization of the strain was done by method described previously. Decomposition of various compounds such as adenine and casein were undertaken using methods of GORDON *et al.*[13] Acid production tests using various carbohydrates, utilization of organic acids, and growth temperature were determined by the modified method of POONWAN *et al.*[14] Whole cell hydrolysates were analyzed for diaminopimelic acid (DAP) isomers and for cell wall sugars using thin layer chromatography (TLC).[15,16] Menaquinones were extracted from freeze-dried biomass (500 mg) and analyzed as described by CHUN & GOODFELLOW.[17] Mycolic acids were analyzed using the procedure of MINNIKIN *et al.*[18] Genomic DNA samples were prepared using the guanidine thiocyanate method.[19,20] 16S rDNA was amplified by PCR and sequenced using prokaryotic 16S rDNA universal primers.[17] DNA sequences were determined using an automated sequence analyzer (ABI PRISM™ 3100; PE Applied Biosystems, USA). BLAST analysis was used to screen databases for strains related to the isolate.[2] The 16S rDNA sequence of the isolate was determined. These data were used for phylogenetic analysis. A phylogenetic tree was produced using the neighbor-joining method.[21,22]

Fermentation and Isolation

A slant culture of *Nocardia* sp. strain IFM 10065 grown on MHII medium supplemented with 0.2% glucose was used throughout this work. The seed broth was prepared by inoculating a slant culture of the producing strain IFM 10065 into a 100-ml Erlenmeyer shake flask containing 50 ml of BHI broth supplemented with 1% glucose and 1% glycerol. This broth was cultured at 32°C for 4 days on a rotary shaker at 300 rpm. We transferred 10% inoculum into a 500-ml Erlenmeyer flask containing 150 ml of the production medium comprising meat extract 0.5%, peptone 0.5%, glycerol 2.0%, and antifoam 0.05%. The culture was incubated on a rotary shaker at (250 rpm) at 32°C for 6 days.[10]

Antimicrobial Activity

Antimicrobial activities were determined using the micro-broth dilution method. The test used BHI broth media adjusted to pH 6.0 or 7.0 with the following microorganisms: *Micrococcus luteus* IFM 2066, *Escherichia coli* NIH JC-2, *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90112, *Aspergillus niger* IFM 5368, *Paecilomyces variotii* IFM 40913 and *Trichophyton mentagrophytes* IFM 10489. After inoculation, test bacteria and fungi were incubated at 37°C for 24 and 48 hours, respectively; thereafter, the MIC values were determined.

Results and Discussion

Taxonomy of Producing Strain IFM 10065

The strain IFM 10065 is an aerobic Gram-positive, acid fast, non-motile microorganism. It forms a grayish-orange to a faint-orange-colored substrate mycelium that fragments into irregular rod-shaped elements, which is characteristic of *Nocardia* (Fig. 2).

Chemotaxonomic characteristics of the strain IFM 10065 were consistent with its assignment to the genus *Nocardia*.[23,24] The strain contained galactose and arabinose as characteristic whole-cell wall sugars in addition to *meso*-diaminopimelic acid as the diagnostic cell-wall diamino acid. The strain IFM 10065 also contained mycolic acid, which co-migrates with that extracted from *Nocardia* type strains. The major menaquinone was MK-8($H_{4\omega\text{-cycl}}$); it was

Fig. 2. Scanning electron micrograph of *Nocardia transvalensis* IFM 10065 grown on MH II agar medium supplemented with 0.2% glucose at 30°C for 7 days.

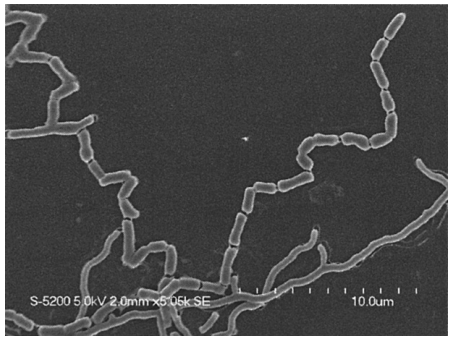
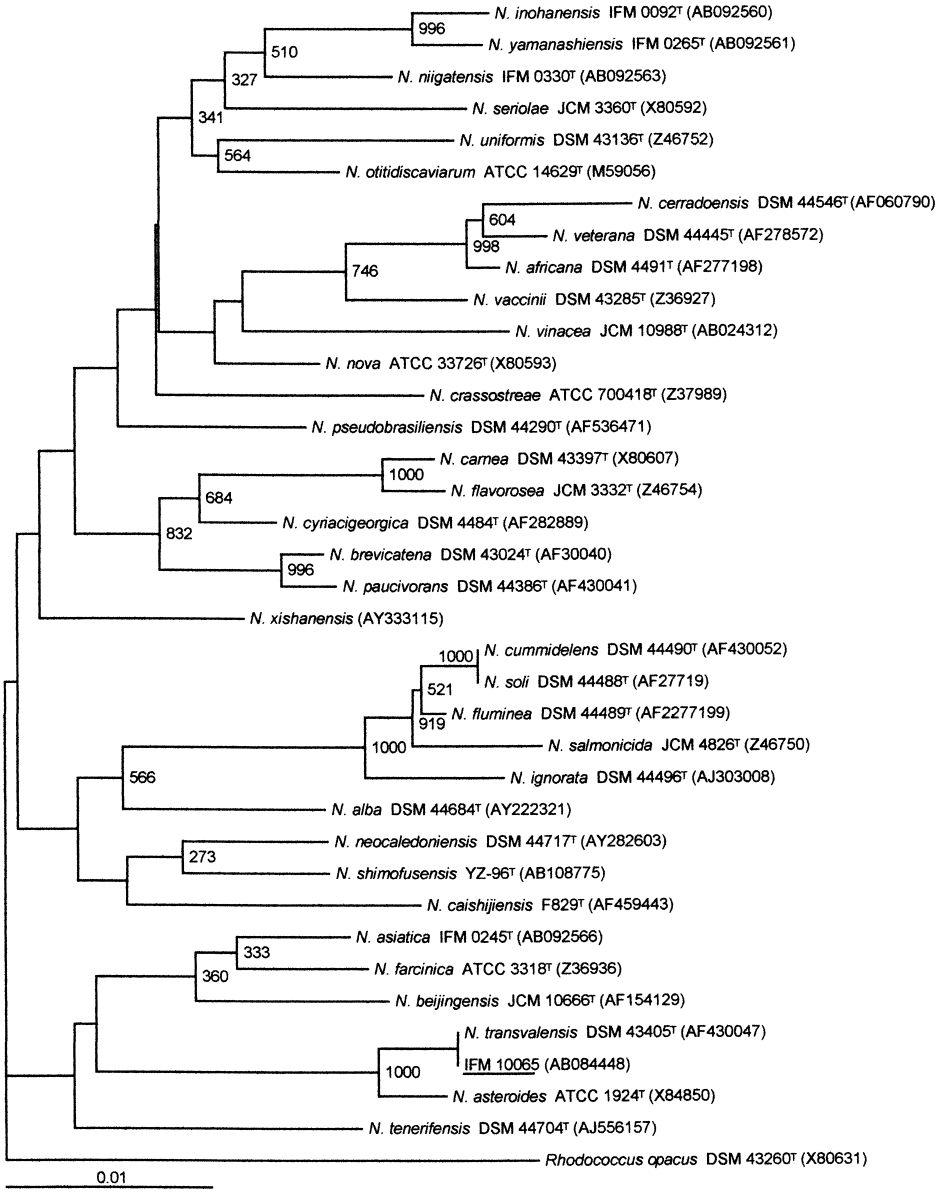


Fig. 3. The phylogenetic tree shows the position of *Nocardia* sp. IFM 10065 within the genus *Nocardia* species based on the 16S RNA gene sequences.



identical to that of a typical *Nocardia* strain. On the basis of typical nocardioform morphology with cell wall type IV, coupled with the chemotypes of mycolic acid and menaquinone, strain IFM 10065 was inferred to be a member of the genus *Nocardia*.

We determined the 16S rDNA sequence of the strain IFM 10065. A database search revealed that the strain belonged to the genus *Nocardia*, the suborder *Corynebacterineae* of the family *Nocardiaceae*.[25] The phylogenetic tree (Fig. 3) showed that the strain is highly associated with *N. transvalensis*.

A comparison of physiological characteristics of IFM 10065 with the type strain of *N. transvalensis* IFM 0333 indicated that it was a strain of *N. transvalensis* (Table 1). Thus IFM 10065 was identified as *N. transvalensis* based on these phylogenetic, physiological, and biochemical characteristics.

Table 1. Physiological characteristics of *Nocardia* sp. strain IFM 10065.

Characteristics	IFM 10065	*N. transvalensis* IFM 0333^T	Characteristics	IFM 10065	*N. transvalensis* IFM 0333^T
			maltose	−	−
Decomposition of			mannose	−	−
adenine	−[a]	−	rhamnose	−	−
casein	−	−	sorbitol	−	+
hypoxanthine	+[b]		Utilization of		
tyrosine	−	−	citrate	+	+
urea	+	+	Growth at		
xanthine	−	−	45℃	−	−
Acid from			Production of		
adonitol	+	+	β−lactamase	+	+
arabinose	−	−	Susceptibility to		
erythritol	+	+	imipenem	+++[c]	+++
galactose	+	+	tobramycin	−	−
glucose	+	+	5−fluorouracil	±[d]	±
inositol	−	+	kanamycin	−	−

a: negative reaction, b: positive reaction, c: highly susceptible, d: marginal growth inhibition around 30μg of 5-fluorouracil containing paper-disc can be observed

Fermentation, Isolation and Physico-chemical Properties

After 7 days' incubation, one half volume of methanol was added to the mycelial cake; it was incubated for another 3 hours to kill the *Nocardia* and extract the active compound. The extract was filtered and concentrated *in vacuo*. After addition of 100-ml water to the concentrated fraction, it was extracted with the same volume of ethyl acetate. The extract was concentrated *in vacuo*: a crude residue was subjected to silica gel column chromatography using $CHCl_3$. The combined active fractions were purified further using silica gel column chromatography with a mixture of toluene and ethyl acetate (3 : 1). The active fractions were further purified by preparative thin layer chromatography (TLC) using the mixture of toluene : ethyl acetate (3 : 1) as eluent. The active compound was applied to Sephadex LH-20 column chromatography ($CHCl_3$: methanol = 1 : 1). The purified compound was obtained by preparative reverse phase HPLC (ODS − 10×250 mm, 35~65% CH_3CN linear gradient, 5 ml/minute, 250 nm; Jasco Inc.). Final purification was carried out by HPLC (ODS − 10×250 mm; methanol, 5 ml/minute, 250 nm; Jasco Inc.) to obtain 7.2 mg of transvalencin A.

Table 2. Antimicrobial activity of transvalencin A against bacteria and fungi.

test organism	MIC values (μg/ml) pH 7.3	pH 6.0
Micrococcus luteus IFM 2066	16	0.5
Escherichia coli NIH JC-2	>32	>32
Candida albicans ATCC 90028	>32	>32
Cryptococcus neoformans ATCC 90112	4	>32
Aspergillus niger IFM 5368	>32	>32
Paecilomyces variotii IFM 40913	16	>32
Trichophyton mentagrophytes IFM 10489	2	8

MIC was determined by microplate dilution method using BHI medium.

Biological Properties

Table 2 shows *in vitro* antibacterial activities of transvalencin A. The antibiotic was active against fungi and Gram-positive bacteria, but was not active against the Gram-negative bacteria tested. MIC values against *C. neoformans* ATCC 92112, *T. mentagrophytes* IFM 10489, and *P. variotii* IFM 40913 were 4.0, 16.0, and 2.0 μg/ml on BHI medium (pH 7.3), respectively. However, lower activities against fungi such as *T. mentagrophytes* IFM

10489, *C. neoformans* ATCC 90112, and *P. variotti* IFM 40913 were observed in the acid medium with a pH of 6.0. The MIC value against *M. luteus* IFM 2066 is 16.0 μg/ml on BHI medium with a pH 7.3, but higher activity was observed in acidic BHI medium, such as pH 6.0.

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