DNACINS, NEW ANTIBIOTICS

I. PRODUCING ORGANISM, FERMENTATION, AND ANTIMICROBIAL ACTIVITIES

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Dnacins are new antibiotics produced by an actinomycete, strain No. C-14482 (N-1001). The characteristic features of the organism are: the formation of coremia on solid media, production of rod-shaped motile spores with peritrichous flagella from mature aerial mycelia, fragmentation of the mature organism (at later stage of growth) in liquid media in which some fragmented elements have motility, lysozyme resistance, *meso*-diaminopimelic acid in the cell wall, and a guanine-cytosine content of 71 ± 1 mol%. The organism has been designated as *Nocardia* sp. No. C-14482 (N-1001). Dnacins show strong activity against various Gram-negative, Gram-positive, and acid-fast bacteria, but slight activity against fungi. The antibiotics hardly affect the growth of *Escherichia coli* K-12 under anaerobic condition even at concentrations more than five times that of the minimum inhibitory concentrations under aerobic conditions.

In our search for antibiotics that preferentially inhibit the growth of a thermosensitive mutant of *E. coli*,[1] strong activity was detected in the culture fluid of an actinomycete strain No. C-14482 (N-1001). The active materials were found to be new antibiotics and named dnacins A_1 and B_1.

The present paper deals with the properties of the producing organism, fermentation studies and the antimicrobial activities of dnacins A_1 and B_1.

Materials and Methods

Characterization of the Producing Organism

The actinomycete strain No. C-14482 (N-1001) was isolated from a grass blade collected in Shiga Prefecture, Japan. Working stock of the organism was maintained at $-20°C$.[2] This was prepared by growing the organism on yeast extract-malt extract agar (ISP-2) at 28°C for 4 days; the mycelia were harvested, suspended in 40% glycerol (v/v) and stored at $-20°C$.[2] The cultural and physiological characterizations were carried out by the method described previously.[2]

Electron Microscopic Observation

An agar block of a sporulating culture was dehydrated through an ethyl alcohol series. For scanning electron microscopy, the block was critical-point dried, coated with gold and viewed with a MSM4C-101 scanning electron microscope (Hitachi-Akashi Co.) The sporulating culture growing on agar medium was immersed in TYG medium (1% tryptone (Difco), 0.6% yeast extract, 1% glucose, pH 7.0) and incubated for 60 minutes at 28°C. Motile spores produced from the aerial mycelia were gently diluted with distilled water and deposited on formvar-coated grids. The grids were air-dried and shadowed with platinum-paladium, then the specimen was examined with a JEM-T7S transmission electron microscope (Japan Electron Optics Laboratory Co., Ltd.).

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Formation of glistening and leathery colonies on solid media, coremia formation (Fig. 1), fragmentation of some branched mycelia at a later stage of growth in liquid culture and motility of some polymorphic fragments, release of rod-shaped motile spores ($0.5\sim0.8\times1.5\sim2$ μm) having peritrichous flagella ($3\sim5$ μm in length, Fig. 2) upon immersion of the mature aerial mycelia into liquid media, contain *meso*-diaminopimelic acid in the cell wall, growth in lysozyme broth, no growth in 5% NaCl, hydrolysis of starch, casein and gelatin, production of nitrate reductase and alkaline phosphatase, and a guanine-cytosine content of 71 ± 1 mol%. Based on the above properties, the strain was designated as *Nocardia* sp. No. C-14482 (N-1001).

Fermentation Studies

Fermentation studies on the organism were carried out in five kinds of media (Table 1). The antibiotic production was assayed using *Proteus mirabilis* ATCC 21100 as the test organism; this was selected because the materials active against this bacterium were confirmed to be identical with the desired components by thin-layer chromatography.

The antibiotic yields are shown in Table 2; strain N-1001 was a poor antibiotic producer while the mutant N-1020 produced as high as 24 μg/ml in medium 3. The fermentation patterns of the 2 strains in medium 3 are shown in Fig. 3. After 72 hours of fermentation, the yield of antibiotics reached a maximum of about 25 μg/ml with the N-1020 strain; the amount of dnacin B_1 also increased to about 50 times higher than that of the strain N-1001 (data not shown).

Details of the isolation and physicochemical properties are described in a separate paper.[5]

Antimicrobial Activities

Dnacins show strong antimicrobial activity against various Gram-negative, Gram-positive, and acid-fast bacteria, but weak antifungal activity (Table 3); dnacin B_1 is more potent than A_1 against susceptible bacteria. There was a marked difference in the activity of dnacins against *E. coli* when assayed under aerobic and anaerobic conditions. As shown in Fig. 4A, dnacin B_1 suppressed the aerobic growth of *E. coli* K-12 at 0.1 μg/ml whereas the antibiotic hardly affected the anaero-

Table 1. Culture media.

Component	Media (%, w/v)				
	1	2	3	4	5
Glucose					2
Dextrin	5	5	5	5	
Soluble starch					3
Soybean flour	3				1
Corn-steep liquor			3	3	1
Proflo (Traders Oil Mill Co.)		3			
Peptone	0.1			0.1	
Meat extract		0.1			0.5
$(NH_4)_2SO_4$			0.1		
NaCl					0.3
$CaCl_2$			1		
$CaCO_3$	0.5	0.5	0.5	0.5	0.5

Table 2. Fermentation results in various media.

Producer	Medium	Potency (μg/ml)	Specific production (μg/mg DNA)
N-1001	1	<0.5	—
	2	<0.5	—
	3	1.2	3.8
	4	<0.5	—
	5	<0.5	—
N-1020	1	2.3	6.6
	2	0.5	2.5
	3	24.5	66.9
	4	2.2	8.5
	5	1.4	5.6

The potency was determined by the paper disk method described in Materials and Methods. After 3-days cultivation at 28°C, growth and potency were measured. Specific production is the potency divided by the growth (DNA, mg/ml).

Fig. 3. Time course of the fermentation of dnacins by wild type and mutant strains.
Potency was determined by a paper disk method using *Proteus mirabilis* ATCC 21100 as the test organism. Growth was measured as the DNA content in 1 ml culture as described in Materials and Methods. A, wild type strain N-1001; B, high producing mutant N-1020.

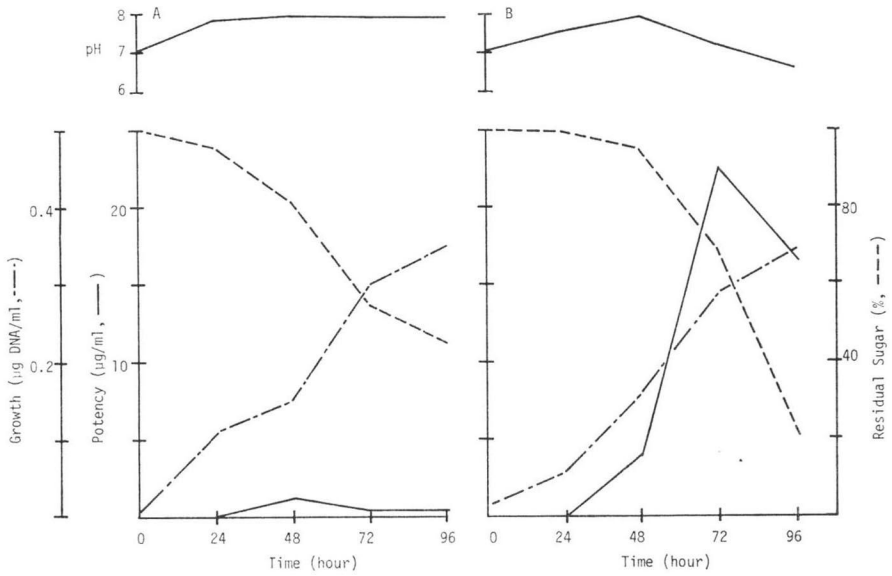


Table 3. Antimicrobial activity.

Test organism	MIC (μg/ml)		Test organism	MIC (μg/ml)	
	A_1	B_1		A_1	B_1
Escherichia coli K-12	2	0.2	*B. cereus* IFO 3514	2	0.2
E. coli NIHJ JC2	5	0.2	*B. megaterium* IFO 12108	1	0.05
Proteus vulgaris IFO 3045	10	2	*B. brevis* IFO 3331	0.2	0.1
P. morganii IFO 3168	50	2	*Staphylococcus aureus* FDA 209P	1	0.01
P. mirabilis ATCC 21100	10	0.5	*Micrococcus luteus* IFO 12708	1	0.2
Pseudomonas aeruginosa IFO 3080	2	0.2	*Mycobacterium avium* IFO 3143	10	0.5
Salmonella typhimurium IFO 12529	2	0.2	*M. vaccae* ATCC 15483	5	1
S. enteritidis IFO 3313	10	0.5	*Aspergillus niger* IFO 4066	>100	100
Alcaligenes faecalis IFO 13111	1	0.05	*Penicillium chrysogenum* IFO 4626	>100	50
Enterobacter cloacae IFO 12009	20	0.2	*Trichophyton rubrum* IFO 5467	>100	50
Serratia marcescens IFO 3046	50	0.5	*Saccharomyces cerevisiae* IFO 0209	>100	>100
Bacillus pumilus IFO 3813	2	0.2	*Candida albicans* IFO 0583	>100	>100
B. subtilis PCI 219	1	0.05			

The activity was determined by the agar dilution method. Trypticase soy agar (BBL) was used as the assay medium for common bacteria. In the assay for acid-fast bacteria and fungi, the medium was supplemented with 3 % glycerol and 1 % glucose, respectively. Bacteria were grown for 18 hours at 37°C whereas fungi were grown for 2 days at 28°C.

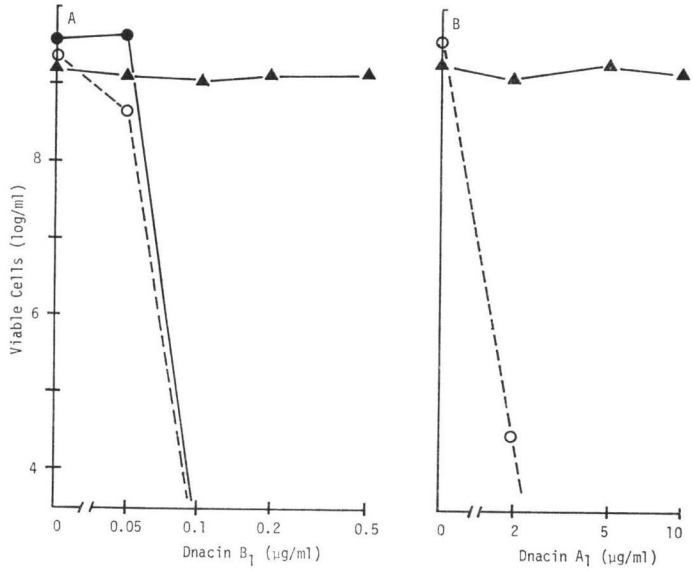
bic growth at levels up to 0.5 μg/ml. Similar results were also obtained using dnacin A_1 (Fig. 4B).

In an acute toxicity test by intravenous administration to mice, the estimated LD_{50} of dnacins A_1 and B_1 was about 5 and 0.625 mg/kg, respectively.

Fig. 4. Effect of dnacins on growth of *E. coli* K-12 under aerobic and anaerobic conditions.

The assay was carried out by the broth dilution method with antibiotic medium 3 (Difco) as the assay medium. The anaerobic conditions were obtained by using an anaerobic jar. After incubation for 18 hours at 37°C, surviving cells were determined with trypticase soy agar (BBL) plates.

●, Aerobic condition with shaking; ○, aerobic condition without shaking; ▲, anaerobic condition without shaking.



Discussion

Dnacins were detected from a screening program using a thermosensitive strain of *E. coli* for prescreening.[1] This *E. coli* strain is integratively suppressed[6] and is able to grow at 42°C in spite of its dna A thermosensitive mutation. This organism was designated to detect various agents that interact with the DNA molecule,[1,6,7] some of which have curing effect on plasmids, and with antitumor properties. Therefore, it is possible that dnacins interact with DNA resulting in inhibition of DNA and/or RNA synthesis, elimination of plasmid molecules, or inhibition of tumor cell proliferation. Results from preliminary studies which support the above speculation will be presented in the near future.

Oxygen metabolism is involved in the activation of several agents in cells. In this study, dnacins were found to be potent antibacterial agents, but the inhibitory effects were markedly decreased under anaerobic condition. This suggests that oxygen may be necessary for the antibiotics to act upon cellular targets. Some of the antitumor antibiotics that interact with cellular DNA are known to act as generators of superoxide radical.[8~16] The activity of dnacins may also involve the generation of the radical in susceptible cells, resulting in the killing of the cells. On the basis of this possibility, studies are under way.

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