DC-52, A NOVEL ANTITUMOR ANTIBIOTIC

1. TAXONOMY, FERMENTATION AND BIOLOGICAL ACTIVITY

FUSAO TOMITA, KEIICHI TAKAHASHI and KEN-ICHI SHIMIZU

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd.,
Machida, Tokyo, Japan

(Received for publication January 31, 1983)

A novel antitumor antibiotic, DC-52 was found in the culture broths of Actinomycete DO-52. The producing organism was subsequently determined to be a new species and named *Streptomyces melanovinaceus* nov. sp. For the production of the antibiotic, soluble starch served as a good carbon source and soybean meal was a good nitrogen source tested. The antibiotic DC-52 is active against *Bacillus subtilis*, *Staphylococcus aureus* and *Klebsiella pneumoniae*, but not active against most Gram-negative bacteria. The antibiotic is also active against mouse leukemia P388.

In the course of our continuing search for new antibiotics, we found a novel antibiotic with antibacterial and antitumor activity from Actinomycete strain DO-52. As described in the following paper,[1] the antibiotics, DC-52* and the closely related DC-52-d** were isolated from the culture broths.

DC-52 and DC-52-d contain the novel skeleton, 8,11-iminoazepinoisoquinoline which has been discovered in nature for the first time. Details of the structure determination of the antibiotics will be given in separate papers.[1,2]

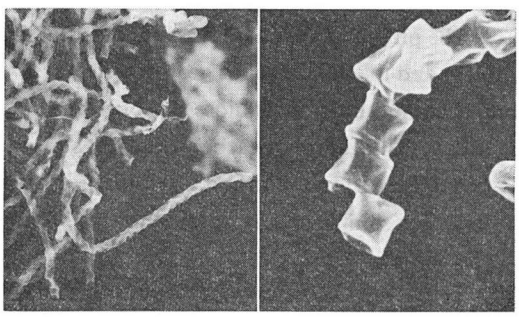
In this paper, taxonomic studies of the producing strain, fermentation studies and antibacterial activity of DC-52 and DC-52-d are presented.

Taxonomy

Actinomycete strain DO-52 was isolated from a soil sample collected in Machida-shi, Tokyo, Japan. The strain has been deposited at Northern Regional Research Laboratories, Peoria, Illinois, U.S.A. and has been assigned accession number NRRL 12388.

Taxonomic characterization was carried out according to the methods used in the International Streptomyces Project (ISP).[3] The various kinds of media were inoculated with washed mycelia suspended in 0.85% saline obtained from a culture shaken at 28°C for 72 hours in a liquid medium consisting of 10 g glucose, 24 g soluble starch, 3 g beef extract, 5 g yeast extract, 5 g peptone per liter of water, pH 7.0 prior to sterilization.

Fig. 1. Spores of strain DO-52 grown on oatmeal agar.



Cultural Characteristics

Strain DO-52 showed good growth on various media as shown in Table 1. Abundant aerial mycelia with simple branching and straight to flexious spore chains were observed by microscope. The mature spore chains were generally long with 10 to 30 or more spores per chain. The spores were cylindrical in shape, with smooth

* DC-52 has recently been named as quinocarcin.
** DC-52-d has recently been named as quinocarcinol.

Table 1. Cultural characteristics of strain DO-52.

Medium	Growth	Color of colony*		Growth and color of aerial mycelium	Pigment
		Surface	Reverse		
Czapeck agar (Waksman No. 1)	Poor, flat	Eggshell (2ca)	Eggshell (2ca)	Poor Covert gray (2fe)	Cloud pink (7cb)
Glucose - asparagine agar (Waksman No. 2)	Good, flat	Clove brown (3pl)	Clove brown (3pl)	Good Covert gray (2fe)	Golden brown (3pi)
Yeast extract - malt extract agar (ISP No.2)	Good, flat	Yellow maple (3ng)	Yellow maple (3ng)	Good Covert gray (2fe)	Cordovan (8pl)
Oatmeal agar (ISP No. 3)	Good, flat	Beaver gray (3ml)	Ebony teak (3po)	Good Covert gray (2fe)	Mauve wine (71/2ni)
Inorganic salts - starch agar (ISP No. 4)	Good, flat	Clove brown (3ni)	Golden brown (3pg)	Good Silver gray (3fe)	Rose wine (71e)
Glycerol - asparagine agar (ISP No. 5)	Good, flat	Sepia brown (3pn)	Golden brown (3pi)	Moderate Covert gray (2fe)	Cordovan (8pl)
Peptone - yeast extract - iron agar (ISP No. 6)	Good, flat	Bamboo (2gc)	Bamboo (2gc)	Poor White (a)	None
Tyrosine agar (ISP No. 7)	Good, flat	Sepia brown (3pn)	Clove brown (3pl)	Good Covert gray (2fe)	Cordovan (8pl)
Nutrient agar	Poor, flat	Bamboo (2gc)	Bamboo (2gc)	None	None

* Color designation from Color Harmony Manual, 4th Edition, Container Corporation of America, 1958

Table 2. Physiological properties of strain DO-52.

Liquefaction of gelatin	Negative
Liquefaction of milk	Positive
Peptonization of milk	Negative
Decomposition of cellulose	Weakly positive
Hydrolysis of starch	Positive
Formation of tyrosinase	Positive
Formation of melanoid pigments	Negative
Optimum growth temperature	28~38°C
Optimum growth pH	6.6~7.5

Table 3. Utilization of carbohydrates by strain DO-52.

D-Arabinose	±
D-Xylose	+
D-Glucose	++
D-Fructose	++
D-Mannitol	+
Sucrose	±
m-Inositol	++
Raffinose	+
L-Rhamnose	−

surface as seen in the electron microscope (Fig. 1). Cell wall analysis revealed the presence of LL-diaminopimelic acid.

The cultural characteristics of strain DO-52 shown in Table 1 were observed after two weeks of incubation at 28°C. The aerial mycelia were gray colored on agar media. Melanoid pigments were not produced on peptone - yeast extract - iron agar and on tyrosine agar.

Physiological Characteristics

The physiological characteristics of strain DO-52 are shown in Table 2. The temperature range for growth and the pH range for growth were observed after cultivation for 2 days and the action upon milk and decomposition of cellulose was observed after one month. All other observations were made after 20 days. Utilization of carbohydrate by strain DO-52 is shown in Table 3.

According to the method of KÜSTER's classification,[4] analysis of the above results indicated strain DO-52 was similar to *Streptomyces tendae, Streptomyces nodosus, Streptomyces cyanogenus* or *Streptomyces violaceolactis.*

In literature descriptions,[5~8] *S. tendae* and *S. nodosus* form similar spore chains to those of DO-52, but their ability to utilize carbohydrates and the characteristics of soluble pigments are different from those of DO-52. *S. cyanogenus* and *S. violaceolactis* form different spore chains and their ability to

Table 4. Taxonomic comparison.

	DO-52	*S. cyanogenus*	*S. nodosus*	*S. tendae*	*S. violaceolactis*
Aerial mycelium	Gray	Gray	Gray	Gray	Gray
Melanoid pigments	—	—	—	—	—
Reverse color	Brown	Brown to red	Reddish brown	Dark olive	Purple to red
Spore	Cylindrical	Oval	Oval to cylindrical	Oval	Oval
	Smooth	Smooth	Smooth	Smooth	Smooth
Spore chain	Straight to flexious	Spiral	Spiral	Straight to flexious	Spiral
Carbon sources					
D-Glucose	+	+	+	+	+
D-Xylose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
Inositol	+	+	+	+	+
D-Fructose	+	+	+	+	+
L-Rhamnose	−	+	+	+	+
Raffinose	+	+	−	−	+
Sucrose	+	+	−	+	+
Pigment	Mauve wine Brown (NaOH)	Brown Blue (NaOH)	Trace	Yellow Orange (HCl)	Red Blue (NaOH)

utilize carbohydrates and the characteristics of soluble pigments are different from those of DO-52. Comparison of these characteristics are listed in Table 4.

Besides the difference in characteristics shown above, DO-52 forms cylindrical spores and a unique pigment. The pigment, mauve wine color, produced on oatmeal agar medium is very characteristic for this strain and this color turned to cinnamon brown color under alkaline conditions. The pigment was also produced when DO-52 was grown in the fermentation medium. Accordingly, the strain DO-52 was assigned to a new Streptomyces species for which was chosen the name, *melanovinaceus*, to denote the color excreted in media.

Fermentation

Seed flasks were inoculated with stock cultures maintained in a deep freezer (−70°C) and grown for 48~72 hours at 28°C. The seed medium consisted of 10 g glucose, 24 g soluble starch, 3 g beef extract, 5 g yeast extract, 5 g Bacto-tryptone per liter of water. Basic inorganic salts of the fermentation media were 0.3 g KH_2PO_4, 0.4 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ per liter of water. The pH of the fermentation media was adjusted to 7.0 prior to sterilization.

Using the above basal medium, the effect of carbon and nitrogen sources in the fermentation medium was investigated in test tubes at 28°C for 5 days.

The amounts of DC-52 produced in the broths were determined by the agar diffusion method using conventional paper discs. Purified DC-52 obtained as described in the following paper[1] was used as the standard to determine the amounts in the samples. Antibacterial activity of DC-52-d against *Bacillus subtilis* is almost negligible and was not determined. Among the carbohydrates tested, soluble starch, maltose, fructose and glucose gave good production, while sucrose and sorbitol gave no production (Table 5). Soybean meal or soy casein gave good production, while yeast extract or peptone were poor nitrogen sources for the production (Table 6). Thus the optimum fermentation medium was determined

Table 5. Effect of carbon sources on production of DC-52.

Carbon source (g/liter)		DC-52 (Inhibitory zone, mm)		
		Day 3	Day 5	Day 7
Glucose	50	13.8	16.0	24.4
Soluble starch	50	23.3	21.6	24.6
Sorbitol	50	—	—	—
Maltose	50	—	17.8	25.8
Glycerol	50	—	16.6	23.8
Fructose	50	14.0	14.0	25.0
Sucrose	50	—	—	—
Lactose	50	—	—	—

Nitrogen source was 20 g soybean meal per liter of medium.

Table 6. Effect of nitrogen sources on production of DC-52.

Nitrogen source (g/liter)		DC-52 (Inhibitory zone, mm)		
		Day 3	Day 5	Day 7
Soybean meal	30	—	—	18.6
	20	16.8	18.0	23.0
	10	17.7	17.8	19.2
	5	15.6	14.6	14.4
Yeast extract	30	—	—	—
	20	—	—	9.0
	10	—	—	16.0
	5	—	—	—
Peptone	10	—	—	—
	5	12.4	14.1	—
Soy casein	30	—	—	—
	20	—	16.8	23.0
	10	13.8	13.0	19.5
Corn steep liquor	30	—	14.0	20.0
	20	—	9.0	17.5
	10	13.6	—	—
	5	13.2	—	—

Carbon source was 50 g glucose per liter of medium.

as follows: 50 g glucose, 20 g soybean meal, 0.3 g KH_2PO_4, 0.4 g K_2HPO_4 and 0.2 g $MgSO_4 \cdot 7H_2O$ per liter of water (pH 7.0).

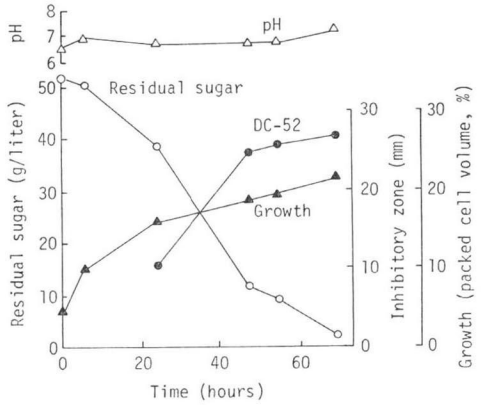
The time course of a typical fermentation is shown in Fig. 2. Antibiotic activity appeared in the culture supernatant at about 24 hours and reached maximum at about 64 hours. The active materials were isolated by sequential chromatography on non-ionic porous resin, cation exchange resin and silica gel. Details of the isolation and their physico-chemical characteristics are given in the following paper.[1)]

Biological Activity

Antibacterial activity was determined by the agar dilution method at pH 7.0. As shown in Table 7, DC-52 had moderate activity against *Staphylococcus aureus*, *B. subtilis* and *Klebsiella pneumoniae*, while no activity was observed against the Gram-negative bacteria tested. DC-52-d was almost devoid of antibacterial activity.

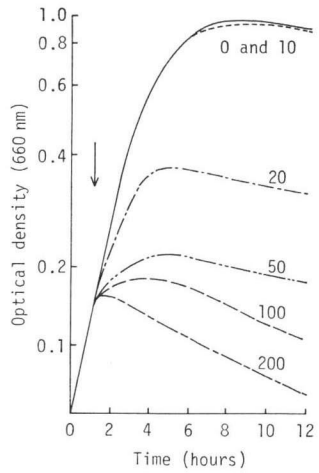
The effect of DC-52 on the growth of *B. subtilis* is shown in Fig. 3. The growth was inhibited at a concentration of 20 μg per ml and increase of the antibiotic showed more suppressive effect on the growth.

Fig. 2. Time course of the fermentation in a 30-liter jar fermentor.



Fermentation medium	(g/liter)
Glucose	50
Soybean meal	20
KH_2PO_4	0.3
K_2HPO_4	0.4
$MgSO_4 \cdot 7H_2O$	0.2
pH 7.0 with NaOH	

Fig. 3. Effects of DC-52 on the growth of *B. subtilis*.



Medium	(g/liter)
Casamino Acids	1
Yeast extract	2
Glucose	5
Citric acid	2
$MgSO_4 \cdot 7H_2O$	0.2
K_2HPO_4	10
$NaNH_4HPO_4 \cdot 4H_2O$	3.5
Tryptophan	0.05
Arginine	0.05
	pH 7.0

DC-52 was added at the time indicated by the arrow and the numbers in the Fig. indicate amounts of the drug added (μg/ml). Growth was automatically recorded with the Biophotorecorder (Toyo Kagaku Sangyo, Japan) at 37°C.

At a concentration of 50 μg/ml, lysis of cells was observed, indicating that DC-52 acts as a bactericidal antibiotic.

DC-52 was effective against mouse lymphocytic leukemia P388 and at a single injection of 12.5 mg/kg, it inhibited growth of P388 with 47% ILS (increase of life span). The LD_{50} value of DC-52 in mice was 27 mg/kg of body weight by intraperitoneal injection. Details on antitumor activity are described in a separate paper.[9]

Table 7. Antibacterial activity.

	Minimum inhibitory concentration (μg/ml)	
	DC-52	DC-52-d
Serratia marcescens	>100	>100
Pseudomonas cepacia	>100	>100
Escherichia coli	>100	>100
Proteus vulgaris	25	>100
Shigella sonnei	>100	>100
Salmonella typhosa	>100	>100
Klebsiella pneumoniae	25	>100
Staphylococcus aureus	12.5	100
Bacillus subtilis	12.5	100

Medium: Nutrient agar.

Acknowledgments

The authors are grateful to Miss REIKO HIGUCHI and Miss MITSUE AOYAGI for their technical assistance.

References

1) TAKAHASHI, K. & F. TOMITA: DC-52, a novel antitumor antibiotic. 2. Isolation, physico-chemical characteristics and structure determination. J. Antibiotics 36: 468~470, 1983
2) HIRAYAMA, N. & K. SHIRAHATA: X-Ray determination of the molecular structure of a novel antibiotic DC-52-d. J. Chem. Soc. Perkin II, in press
3) SHIRLING, E. B. & D. GOTTLIEB: Method for characterization of Streptomyces species. Intern. J. Syst. Bacteriol. 16: 313~340, 1966
4) KÜSTER, E.: Simple working key for the classification and identification of named taxa included in the International Streptomyces Project. Intern. J. Syst. Bacteriol. 22: 139~148, 1972
5) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of Streptomyces. II. Species description from the first study. Intern. J. Syst. Bacteriol. 18: 69~189, 1968
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of Streptomyces. III. Additional species descriptions from the first and second studies. Intern. J. Syst. Bacteriol. 18: 279~392, 1968
7) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of Streptomyces. IV. Species descriptions from the second, third and fourth studies. Intern. J. Syst. Bacteriol. 19: 391~512, 1969
8) BUCHANAN, R. E. & N. E. GIBBONS: BERGY's Manual of Determinative Bacteriology. 8th Ed., The Williams & Wilkins Co., Baltimore, 1974
9) FUJIMOTO, K. & M. MORIMOTO: DC-52, a novel antitumor antibiotic. 3. Antitumor activity. J. Antibiotics (in preparation)