NOVEL MACROCYCLIC ANTIBIOTICS: MEGOVALICINS A, B, C, D, G AND H

I. SCREENING OF ANTIBIOTICS-PRODUCING MYXOBACTERIA AND PRODUCTION OF MEGOVALICINS

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New antibiotics belonging to macrocyclic were discovered and named megovalicins A, B, C, D, G and H. The antibiotics were accumulated endogeneously by a newly isolated myxobacterium identified as *Myxococcus flavescens*. Tank culture of the bacterium for 4 days gave 8.8 g cells/liter on a wet basis, and 4.8, 7.1, 20.0, 0.4, 3.75 and 15.0 μg of megovalicins A, B, C, D, G and H respectively were obtained from 1 g wet cells.

Myxobacteria are a rich source of new antibiotics. Recently new antibiotics such as myxin[1], sporocytophaga antibiotics[2], inhibitory lipid-factors[3], anbruticin[4], G1499-2[5], myxocidins[6], myxothiazol[7], pyrrolnitrin[8], althiomycin[9], antibiotic TA[10], myxovirescins[11], M-230B[12], myxovalargins[13], myxalamides[14], stigmatellins[15], myxopyronins[16], corallopyronins[17], angiolam A[18] and sorangicins[19] were reported to be produced by myxobacteria. Isolation of the bacteria from nature and cell purification, however, are very difficult due to their complicated life cycle. Using isolation techniques described previously[20], we obtained new strains and examined them or antibiotic production. Consequently, we discovered new antibiotics named megovalicins.

Materials and Methods

Microorganisms

Soil samples collected from forest area of Kanagawa, Japan were applied to isolation of myxobacteria. Twenty two strains thus purified and *Myxococcus fulvus* ATCC 25199, *Myxococcus virescens* ATCC 25203, *Myxococcus xanthus* IFO 13542 and *Myxococcus stipitatus* ATCC 29611 were examined for their ability to produce antibacterial substances. *Escherichia coli* 508, a temperature-sensitive cell-wall mutant was employed as an indicator strain in the antibacterial assay.

Culture Media

Myxobacteria isolated, cultivated and maintained in the following medium; raffinose 0.1%, sucrose 0.1%, galactose 0.1%, soluble starch 0.5%, Casitone 0.25%, yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 1.5%. Medium pH was adjusted at 7.0 with aq NaOH. The medium used for megovalicin production was as follows; Polypepton 0.5%, yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.025% and NaCl 0.5%. Medium pH was adjusted at 7.0.

Isolation of Myxobacteria

Filter paper was put on the STANIER's agar medium[21], then a small piece of soil sample was placed on it. Incubation was carried out at 30°C. After more than 7 days, fruiting bodies of myxobacteria could be observed. A small portion of fruiting bodies was scraped out with a platinum needle and

placed on the growth medium for cell purification. Pure cultures were obtained after repeated transfer on this medium.

Taxonomical Identification of Myxobacteria

Myxobacteria were grown on the growth medium at 30°C for 6 days, then their morphological observation including fruiting bodies, microcysts and vegetative cells was made. Congo red test was done according to the method described by McCURDY[22]. Quinone system was determined by the method of YAMADA *et al.*[23]. Other physiological tests were carried out according to our previous paper[20].

Culture Condition for Megovalicins Production

The microorganisms were grown on the growth medium at 27°C for 3 days and transferred to the production medium at the inoculum size of 10%. The fermentation was performed aerobically at 27°C for 4~12 days in 500-ml flask with working volume of 200 ml.

Extraction of Megovalicins

Cells for a 200-ml fermentation were harvested by centrifugation and then added with 10 ml MeOH. The MeOH suspension was agitated for more than 30 minutes at room temp and the MeOH extracts used for assay.

Antibacterial Assay

Quantitative determination of megovalicin produced by the organism was done with the disc assay method using *E. coli* 508. Diameter of inhibition zone was proportional to the logarithm of antibacterial activity (Fig. 1). One U of antibacterial activity was defined as purified megovalicin C concentration giving 12 mm of inhibition zone.

MIC test was conducted by the usual serial agar dilution method using a Nutrient agar. Test microorganisms of approximately 10^6 cells were streaked on agar plates containing 2-fold decremental dilutions of the antibiotics. The inoculated plates were incubated for 18~24 hours at 37°C.

Results and Discussion

Antibiotics Produced by Myxobacteria

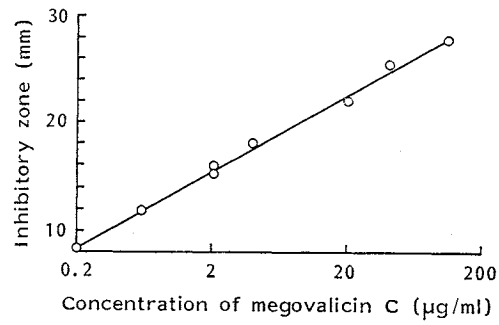
Twenty two strains of myxobacteria were newly isolated from nature including *M. fulvus*, *M. stipitatus*, *Myxococcus flavescens* and *Myxococcus coralloides*. In addition to these strains, four strains of ATCC and IFO cultures as listed in Table 1 were examined for their ability to produce antibacterial compounds. The activity of both culture liquids and methanol extracts of the cells was assayed.

Among them two strains, No. 53 and No. 154 were found to accumulate endogeneously antibacterial compound which could be extracted with methanol. The antibiotic was not detected at all in the culture liquids. Two compounds were considered to be similar to each other on the both antibacterial spectra and HPLC retention time.

Furthermore, these two compounds were considered to be similar to but not identical to the myxovirescins. The myxovirescins are not active against *Pseudomonas aeruginosa*.

No. 154 was applied to further detailed

Fig. 1. Standard curve of megovalicin C.



Escherichia coli 508, a temperature sensitive cell-wall mutant was employed as an indicator strain in the assay.

Table 1. Screening of new antibiotics-producing myxobacteria.

Myxobacteria	Antibacterial activity*	
	Culture filtrate	MeOH extract of cells
Myxococcus fulvus No. 2	−	−
M. fulvus No. 4	−	−
M. fulvus No. 5	−	−
M. fulvus No. 25	−	−
M. fulvus No. 27	−	−
M. fulvus No. 28	−	−
M. fulvus No. 29	−	−
M. fulvus No. 30	−	−
M. fulvus No. 33	−	−
M. fulvus No. 41	−	−
M. fulvus No. 50	−	−
M. fulvus No. 60	−	−
M. fulvus No. 64	−	−
M. fulvus ATCC 25199	−	−
M. virescens ATCC 25203	−	−
M. xanthus IFO 13542	−	−
M. stipitatus No. 18	−	−
M. stipitatus No. 21	−	−
M. stipitatus ATCC 29611	−	−
M. flavescens No. 9	−	−
M. flavescens No. 38	−	−
M. flavescens No. 53	−	+
M. flavescens No. 62	−	−
M. flavescens No. 63	−	−
M. flavescens No. 154	−	+++
M. coralloides No. 44	−	−

* Determination of antibacterial activity was done by the disc assay using *Escherichia coli* 508. *E. coli* 508 is a temperature-sensitive mutant of cell-wall biosynthesis.

−: Negative, +: positive, +++: stronger positive.

studies including its taxonomical identification, purification and structure analysis of the antibiotics.

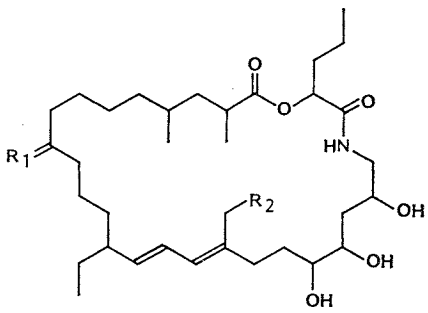
It will be reported elsewhere that the an-

Table 2. Characteristics of strain No. 154*.

Cultural and morphological characteristics	
Grows at pH	6~10
Maximum growth temperature	37°C
Vegetative cell	Gram-negative rod with tapering ends, move by gliding
Swarms	Stainable with Congo red
Microcyst	2.0~2.5×2.0~2.5 μm
Fruiting body	
Size:	60~600 μm
Color:	Deep yellow to orange
Physiological characteristics	
Enzyme:	
Catalase;	+
Urease;	+
Nitrate reduction;	−
Cellulose degradation;	−
Casein degradation;	+
Starch degradation;	+
DNA degradation;	+
Bacterial cell degradation;	+
Major quinone	MK-8
GC content	67.1%
Formation of diffusible pigment	+
	Light yellow

* Identified to those defined with *Myxococcus flavescens*.

Fig. 2. The structure of megovalicin.

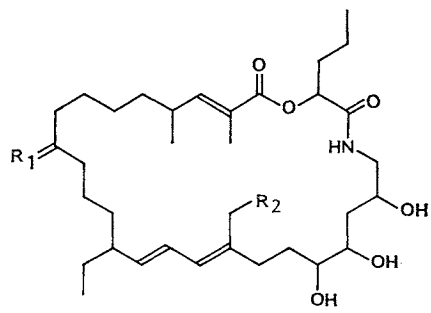


Megovalicin A R_1 = H, OH R_2 = OCH_3

Megovalicin C R_1 = O R_2 = OCH_3
(myxovirescin A_1)

Megovalicin D R_1 = O R_2 = H

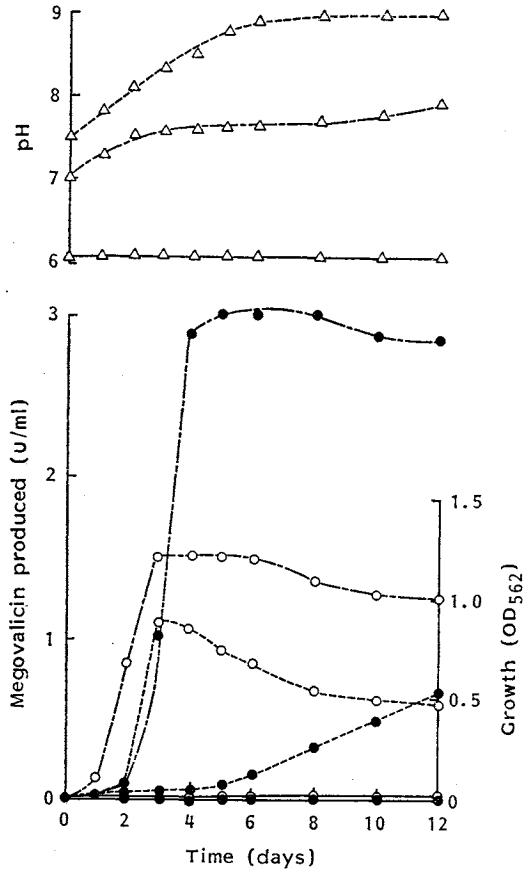
Megovalicin H R_1 = H, H R_2 = OCH_3



Megovalicin B R_1 = O R_2 = OCH_3
(antibiotic M-230B)

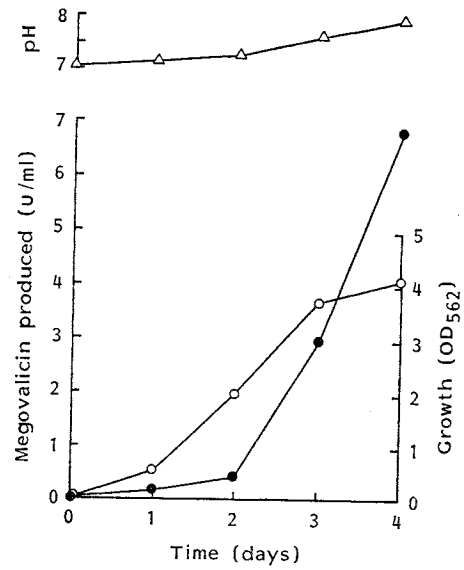
Megovalicin G R_1 = H, H R_2 = OCH_3

Fig. 3. Effect of initial pH of culture medium.
●: Megovalicin produced, ○: growth, △: pH, —: initial pH 6.0, –·–: initial pH 7.0, ---: initial pH 7.5.



Megovalicin producing *Myxococcus flavescens* was cultivated in the medium shown in Materials and Methods except that initial pH of the medium was varied at 27°C for 12 days.

Fig. 4. Time course of production of megovalicin by *Myxococcus flavescens* AJ12298.
●: Megovalicin produced, ○: growth, △: pH.



The fermentation was carried out in a 300-liter jar fermentor. *M. flavescens* AJ12298 was cultivated in the medium shown in Materials and Methods at 27°C.

Table 3. Effect of Polypepton concentration on the production of megovalicin.

Polypepton concentration (%)	Growth (OD_{562})	pH	Megovalicin produced (U/ml)
0	0.05	7.1	<0.1
0.25	1.20	7.9	3.0
0.50	2.20	7.9	6.4
1.00	4.10	7.9	6.9
2.00	4.00	8.0	6.0

Composition of the culture medium was indicated in Materials and Methods except that the concentration of Polypepton was varied.
Myxococcus flavescens was cultivated at 27°C for 4 days.

tibiotics consisted of six components, megovalicins A, B, C, D, G and H (Fig. 2). Among them, megovalicins D, G and H are new compounds. Megovalicins B and C were identical with antibiotic M-230B and myxovirescin A_1 respectively. Megovalicin A had been already derived from myxovirescin A_1 in the study of its structure analysis.

Taxonomic Identification of No. 154

Morphological and physiological characteristics of No. 154 are listed in Table 2 and compared to those of *M. flavescens* IAM 13189. The comparative study shows that No. 154 is identical with *M. flavescens*. No. 154 excreting light yellow pigment did not belong to *M. virescens*, producer of myxovirescin A_1, that formed green pigment.

Table 4. Antibacterial spectra of megovalicin.

Microorganism	MIC (μg/ml)					
	Megovalicin					
	A	B	C	D	G	H
Staphylococcus aureus FDA 209P	>25	>25	>25	>25	>25	>25
Bacillus subtilis ATCC 6633	3.1	0.8	0.8	1.6	1.6	1.6
Pseudomonas aeruginosa NCTC 10490	>25	>25	>25	12.5	6.3	1.6
Escherichia coli NIHJ JC-2	12.5	3.1	1.6	1.6	1.6	1.6
E. coli 508	0.8	0.8	0.8	0.4	0.4	0.2

MIC was determined by the serial agar dilution method using Nutrient agar medium.

Fermentative Production of Megovalicins

As shown in Fig. 3 presenting an example of the fermentation kinetics, megovalicins production occurred after sufficient cell growth. When the fermentation was carried out at the initial pH of 7.4, culture-pH began to go up in parallel with the cell growth. When culture-pH became more than 8.0, the cell growth ceased and gradual lysis of the cells took place.

Effect of initial pH on the cell growth as studied in Fig. 3 clarified the optimum of pH 7.0. Initial pH of 6.0 did not allow the cell to grow at all and the pH 7.5 caused the cell lysis.

Polypepton also had a marked effect on both cell growth and the product formation as shown in Table 3. Culture medium containing more than 10 g/liter of Polypepton supported both sufficient cell growth and megovalicin accumulation.

The fermentative medium containing 10 g/liter of Polypepton with an initial pH of 7.0 for the large scale performance of megovalicins production. An example of the production using a 300-liter fermentation tank is shown in Fig. 4. Tank culture of the bacterium for 4 days gave 8.8 g cells/liter on a wet basis, and 4.8, 7.1, 20.0, 0.4, 3.75 and 15.0 μg of megovalicins A, B, C, D, G and H were obtained from 1 g wet cells.

Biological Activity of Megovalicins

Each component of megovalicin fermentation was isolated and applied to MIC test as shown in Table 4. Antibacterial activity of six components of megovalicins was similar to each other in that the antibiotics were toxic to *Bacillus subtilis* ATCC 6633, *E. coli* NIHJ JC-2 and *E. coli* 508, but not to *Staphylococcus aureus* FDA 209P and *Pseudomonas aeruginosa* NCTC 10490. An exception was that only megovalicin H showed strong antibacterial activity to *P. aeruginosa* NCTC 10490. Megovalicin H was chosen for *in vivo* study and these structure are reported elsewhere.

Acknowledgment

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