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COMMUNICATIONS TO THE EDITOR

Production of Selenohomocystine as an Antibiotic by a Marine *Bacillus* sp. No. 14 with Selenomethionine Resistance

Sir:

An antibiotic substance was produced in a minimal medium added with seleno-DL-methionine by a marine *Bacillus* sp. No. 14[1]. We characterized its activity, structure and production conditions. The antibiotic showed a specific antibiotic activity to *Micrococcus luteus* and was found to be selenohomocystine of which antibiotic activity has never been reported. The antibiotic activity was also formed from seleno-DL-ethionine but not from seleno-cystine or inorganic selenium compounds.

Marine microorganisms have been known to be a potential source of novel secondary metabolites[2]. Recently, we have preferentially screened marine bacteria with novel amino acid analogue (AAA) resistance because of the following consideration. In the biosynthesis of wide varieties of secondary metabolites, various amino acids are involved as precursors or regulators. It is obvious that an endogenous pool for supplying specific amino acids is necessary for the biosynthesis of amino acid related secondary metabolites such as peptide antibiotics. In other words, it seems likely that the producing organisms of these secondary metabolites may have some sort of deregulated amino acid metabolism so as to form an endogenous pool for specific amino acids. This reminded us of amino acid fermentations established by obtaining bacterial mutants with specific AAA resistance from wild type bacteria[3,4]. These mutants have deregulation of specific amino acid metabolisms that are strictly regulated in their parental strains, and therefore be capable of accumulating specific amino acids. Based on these, we speculated that producers of amino acid-related secondary metabolites show specific AAA resistance phenotypes. In this context, HOTTA *et al.*[5] reported that there are varieties of AAA resistance phenotypes among actinomycete strains that produce amino acid-related secondary metabolites. This finding prompted us to start a preferential screening of marine bacteria with AAA-resistance for the discovery of novel amino acid-related secondary metabolites with antibiotic activity.

We were successful in isolating marine bacteria with varieties of AAA-resistance profiles. Among them, strain No. 14 was found to produce an antibiotic activity against *Micrococcus luteus* when cultivated in a minimum medium containing seleno-DL-methionine[1]. Strain No. 14 is the only one selenomethionine resistant isolate identified as *Bacillus* sp. In this communication, we report the requirement of selenomethionine or selenoethionine by the bacteria for the antibiotic production, the structure of the antibiotic, and its anti-microbial activity.

Table 1 shows the effect of various selenium compounds on the production of antibiotic activity against *M. luteus* IFO 3333. The basal medium for cultivation of *Bacillus* sp. No. 14 with selenomethionine resistance consisted of the following: glucose 0.2%, $(NH_4)_2SO_4$ 0.1% and K_2HPO_4 0.14% in Jamarine S artificial seawater (Jamarine Laboratory, Osaka, Japan). After the strain No. 14 was shake-cultured at 27°C for 2 weeks in this medium added with various selenium compounds at different concentrations, antibiotic activity and growth were monitored by regular cup assay using Mycin Assay Agar Arei (Mikuni Chemicals, Tokyo) and turbidmetric assay at 620 nm, respectively. The antibiotic activity was produced when seleno-DL-methionine or seleno-DL-ethionine was added to the medium at concentrations ranging 0.063~0.5 mM. Antibiotic activity was relatively low at concentration of 0.125 mM or lower, and highest in the presence of 0.5 mM seleno-DL-methionine. No significant growth inhibition was observed except for the complete growth inhibition by 1 mM seleno-DL-methionine. The other selenium compounds induced neither antibiotic activity nor significant growth inhibition.

Figure 1 illustrated the time course of growth and antibiotic production when the strain No. 14 was cultivated in 100 ml of the medium containing 0.5 mM seleno-DL-methionine. Following 2 days of lag phase, the strain started to grow exponentially for about 3 days and then reached the stationally phase. The antibiotic activity almost paralleled the growth and reached the maximum level after 5 days incubation. No substantial deduction in both activity and growth was observed after 14 days incubation (data not shown).

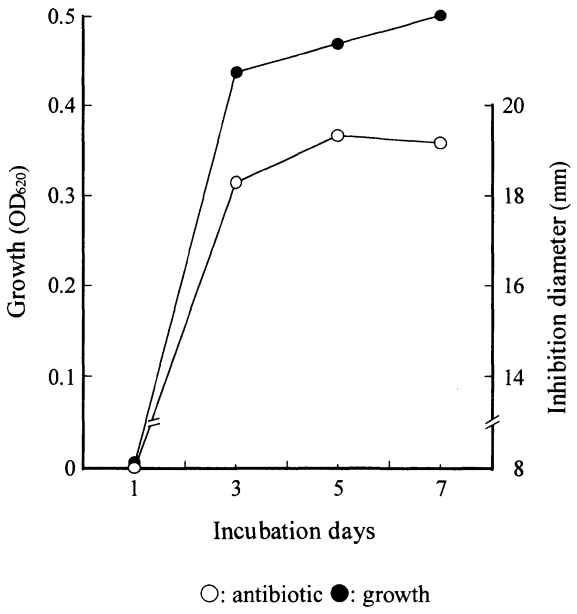
Isolation and chemical characterization of the antibiotic substance was carried out as follows. After 5 days incubation, the supernatant (total 2.5 liters) of the cultured broth was added with active carbon (50 g) to adsorb the antibiotic substance. After stirring for 1 hour at room

Table 1. Effect of selenium-containing compounds on the growth of and antibiotic production by strain No. 14.

Compound added	Concentration (mM)	Growth (OD_{620})	Inhibition* (mm)
None (Basal medium)	—	0.72	0
Seleno-DL-methionine	1.0	0.00	0
	0.5	0.65	22.7
	0.25	0.63	20.0
	0.125	0.56	20.6
	0.063	0.68	12.8
Seleno-DL-ethionine	0.5	0.43	22.0
	0.25	0.59	20.1
	0.125	0.40	16.7
Seleno-DL-cystine	0.125～0.5	0.43～0.59	0
α-methyl-DL-methionine	0.125～0.5	0.59～0.68	0
Na_2SeO_3	0.125～0.5	0.61～0.65	0
Na_2SeO_4	0.125～0.5	0.60～0.68	0

* Inhibition diameter against *Micrococcus luteus* IFO 3333 by the regular cup assay.

Fig. 1. Time course of antibiotic production by strain No. 14.



temperature, the carbon was packed in a column and washed with deionized water (2 liters). The antibiotic substance was eluted with 50% aqueous acetone (2 liters) and concentrated by evaporation. The resulting syrup was loaded on SP-Sephadex C-25 column (H^+ type) previously equilibrated with deionized water which was then eluted with a linear concentration gradient (0～1.2 M) of NaCl and collected as 15 ml fractions at the flow rate of 60 ml/hour. The active fractions were pooled and loaded on a Dowex 50W-X4 (H^+ type) column. After washing the column with deionized water, the active substance was eluted with 100 ml of 1 N-NH_4OH. Subsequently, the substance was purified by preparative HPLC using Pegasil column (Sensyu Kagaku, Japan) equilibrated with 5% acetonitrile - 0.1% trifluoroacetic acid. The antibiotic substance was eluted with the same solvent at a flow rate of 7 ml/minute. The active fractions were pooled and evaporated to yield 2.5 mg of the purified antibiotic substance which gave a single peak upon HPLC using Cupcelpack C_{18} column and 5 mM ammonium carbonate as the solvent.

The antibiotic substance was subjected to MS and NMR analyses using LC-MS mass spectrometer (Hitachi Type M-1200H) and Jeol JNM-GM400 using D_2O, respectively, in comparison with selenomethionine. The MS spectrum gave *m/z* 360, 362 and 364 (M^+) as the parent peaks due to the three isotopes of Se. Upon ^{1}H-NMR, the following signals were observed: 2.25～2.38 ppm, 2×2H, multiplet, indicating β-β'-CH_2; 2.98 ppm, 2×2H, triplet, indicating γ-γ'-CH_2; 3.81 ppm, 1x1H, triplet, indicating α-α'-CH_2,

whereas no signal of methyl protons was observed. On the other hand, the ^{13}C-NMR gave signal at 175 ppm due to –COOH. The substance gave a ninhydrin-positive purple spot on TLC (data not shown). Based on these spectral data, it was concluded that the antibiotic substance was selenohomocystine (m.w. 362; Fig. 1) formed from two molecules of selenomethionine.

The antibiotic activity of selenohomocystine against a variety of bacteria was examined by a regular cup assay. The concentrations necessary to give rise to >20 mm inhibition zone were 1.5 μg/ml (lowest concentration tested) against *M. luteus* IFO 3333 and 100 μg/ml or higher against the following organisms tested; *B. subtilis* PCI219, *Staphylococcus aureus* (3 strains; Smith, FDA 209P and MRSA No. 5), *Escherichia coli*. K12. No inhibition was observed with 100 μg/ml against *Mycobacterium smegmatis* ATCC6097, *M. vaccae* ATCC15483 and *Pyricularia oryzae*. Thus, so far the antibiotic activity was observed specifically against *M. luteus*. Selenohomocystine has never been recognized as an antibiotic.

The formation of antibiotic substance also occurred in the presence of seleno-DL-ethionine, but not in the presence of seleno-DL-cystine and inorganic selenium compounds. These results and structural similarity suggest that selenohomocystine was converted from seleno-methionine and seleno-ethionine (Fig. 2).

To our knowledge, selenomethionine formation has been known by the following 3 reactions: chemical reaction between 2-amino-4-chlorobutyric acid and disodium diselenate[6], an enzymatic reaction between *o*-acetyl-L-homoserine and disodium diselenate[7], and metabolic conversion in animal of selenomethionine *via* selenoadenosyl-methionine followed by adenosyl-selenohomocystine[8]. Based on these, it seems most likely that selenohomocystine formation by the strain No. 14 was due to the last pathway, although another possibility like the condensation of 2 molecules of selenomethionine might not be ruled out. In addition, molar based conversion rate from selenomethionine to selenohomocystine was estimated at 3% at the lowest.

In any case, the formation of selenohomocystine from both selenomethionine and selenoethionine will be the first finding in microorganisms. Its formation paralleling the growth is of indicative that enzyme(s) involved is constitutively expressed. At first we thought that the antibiotic activity of selenohomocystine might be expressed only in the minimum medium because selenohomocystine could be regarded as an AAA. However, the activity in a regular antibiotic assay medium containing varieties of amino acids and proteins indicated that selenomethionine was regarded as an antibiotic. *M. luteus* was inhibited by selenohomocystine as low as about 0.004 mM (=1.5 μg/ml), but not by seleno-DL-methionine as high as 0.5 mM. Therefore, selenohomocystine should be regarded as an amino acid analogue antibiotic such as D-cycloserine[9] and others[10].

The optical isomerism and action mechanism of selenohomocystine produced by strain No. 14 remain unsolved.

Fig. 2. Structures of selenium compounds.

$CH_3SeCH_2CH_2CH(NH_2)COOH$

Selenomethionine

$CH_3CH_2SeCH_2CH_2CH(NH_2)COOH$

Selenoethionine

$SeCH_2CH_2CH(NH_2)COOH$

$SeCH_2CH_2CH(NH_2)COOH$

Selenohomocystine

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