

COMMUNICATIONS TO THE EDITOR

**Enhanced Production of Fattiviracins by
Bacitracin-resistant Strains of
Streptomyces microflavus Strain No. 2445**

Sir:

Streptomycetes are soil microorganisms that produce a high proportion of medically important antibiotics. Many antibiotics with a variety of chemical structures and biological activities have been found from streptomycetes as their secondary metabolites. The improvement of *Streptomyces* strains with respect to antibiotics production is industrially and economically important for clinical use. Many mutations have been introduced into *Streptomyces* strains to enhance the production of a particular antibiotic.

Fattiviracins produced by *Streptomyces microflavus* strain No. 2445 isolated in our laboratories are potent antiviral agents effective for herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), human immunodeficiency virus type 1 (HIV-1) and influenza A and B viruses *in vitro*^{1,2}. Fattiviracins belong to sugar-fatty acid lactone, which have macrocyclic diesters formed by the binding of two trihydroxyfatty acids and four D-glucose residues in the molecule. They are divided in thirteen components by difference of the length of fatty acids and stereoisomer³. The purification procedure for thirteen components of fattiviracins is conducted finally with the repeated preparative HPLC. To obtain large quantity of the compounds efficiently, the improvement of their productivity and purification is required. If this producing strain produced only a few components of fattiviracins, the purification procedure would become simple. In this study we demonstrate that *Streptomyces microflavus* strain No. 2445 acquired resistance for bacitracin (BC) shows significantly quantitative and qualitative alterations in the production of fattiviracins.

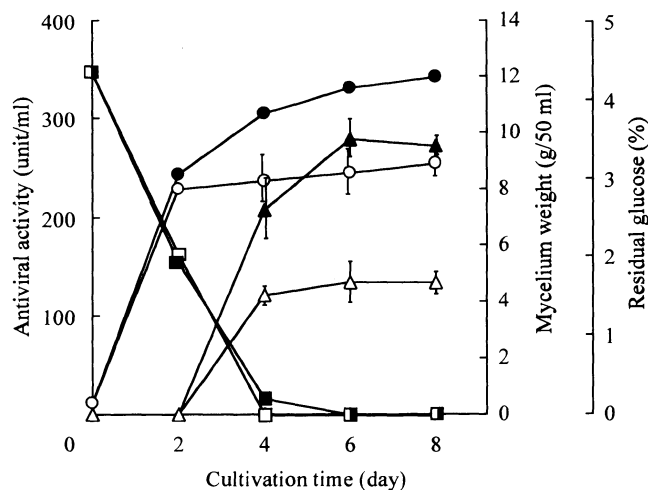
Minimum inhibitory concentrations (MIC) of BC to *Streptomyces microflavus* strain No. 2445 (the wild strain) was determined by Waksman's agar dilution streak method⁴ using MGA agar plates consisting of glucose 1.0%, asparagine 0.05%, K₂HPO₄ 0.05%, yeast extract 0.05%, and agar 1.5%, at pH 7.0. MIC of the wild strain to BC was 10 µg/ml. The spontaneous BC-resistant mutants of the wild strain were obtained as resistant colonies that grew within 10 days, after spores were spread on MGA agar

plates containing BC. Spores generated on the MGA agar plate containing the highest concentration of BC were transferred onto MGA agar plates containing the higher concentrations of BC. This cultivation was repeatedly carried out to generate BC resistance. After 5 to 6 transfers onto MGA agar plates containing the raising concentrations of BC, BC-resistant strains (BCR) designated as 40-BCR, 120-BCR and 350-BCR grown on the plates containing 40, 120 and 350 µg/ml of BC, respectively, were obtained. Spore mass color of the strain grown on MGA agar containing BC upto 20 µg/ml was observed as the same brownish gray as that of the wild strain, however, that of 40-BCR was gray, and those of BCR grown on MGA agar containing 60 to 350 µg/ml of BC turned to white. BC-resistant strains were stored at 5°C after cultivation on MGA agar slants containing the same resistant concentration of BC at 28°C for 2 weeks.

Comparison of the wild strain and 40-BCR with respect to fattiviracins production was examined. The antiviral activities of culture filtrate against HSV-1 were measured by a plaque reduction assay⁵. The residual glucose in the cultures was determined by Somogyi-Nelson method⁶. As shown in Fig. 1, the antiviral activities of the wild strain and 40-BCR were 134 and 280 unit/ml after 6 days cultivation, respectively. It showed that 40-BCR produced fattiviracins 2.1-fold more than the wild strain. The mycelium growth of 40-BCR was 1.3-fold higher than that of the wild strain after 6 days cultivation (Fig. 1). The utilization of glucose in the cultures was no difference between them. The patterns of antiviral activity of culture filtrate and mycelium growth among 40-BCR, 120-BCR and 350-BCR were almost the same (Fig. 2). From this result, 40 µg/ml of BC-resistant concentration was thought to be enough for the acquisition of higher fattiviracins production.

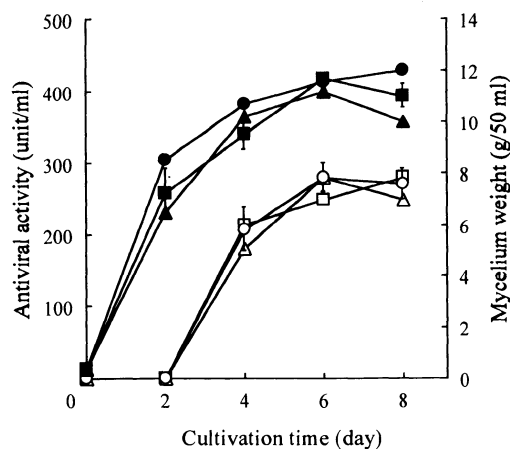
To investigate the fattiviracin components produced by the wild strain and 40-BCR, 500 ml of the 6 days cultures were partially purified and 167 mg and 34 mg of crude samples containing fattiviracin components were obtained, respectively. Purification of fattiviracins was described in our preceding paper³. Fattiviracin components in the crude sample were analyzed with an analytical HPLC (column: µBondapak C₁₈, Waters Associates, 4.6×150 mm, mobile phase: 90% MeOH, flow rate: 0.42 ml/minute, detection: OD at 210 nm). As shown in Fig. 3, the different patterns of

Fig. 1. Antiviral activity and cell growth of wild strain and BC-resistant strain (40-BCR) of strain No. 2445.



Wild strain and 40-BCR were incubated at 28°C on a rotary shaker set up at 180 rpm. Mycelium weight: ○, wild strain; ●, 40-BCR. Antiviral activity: △, wild strain; ▲, 40-BCR. Residual glucose: □, wild strain; ■, 40-BCR.

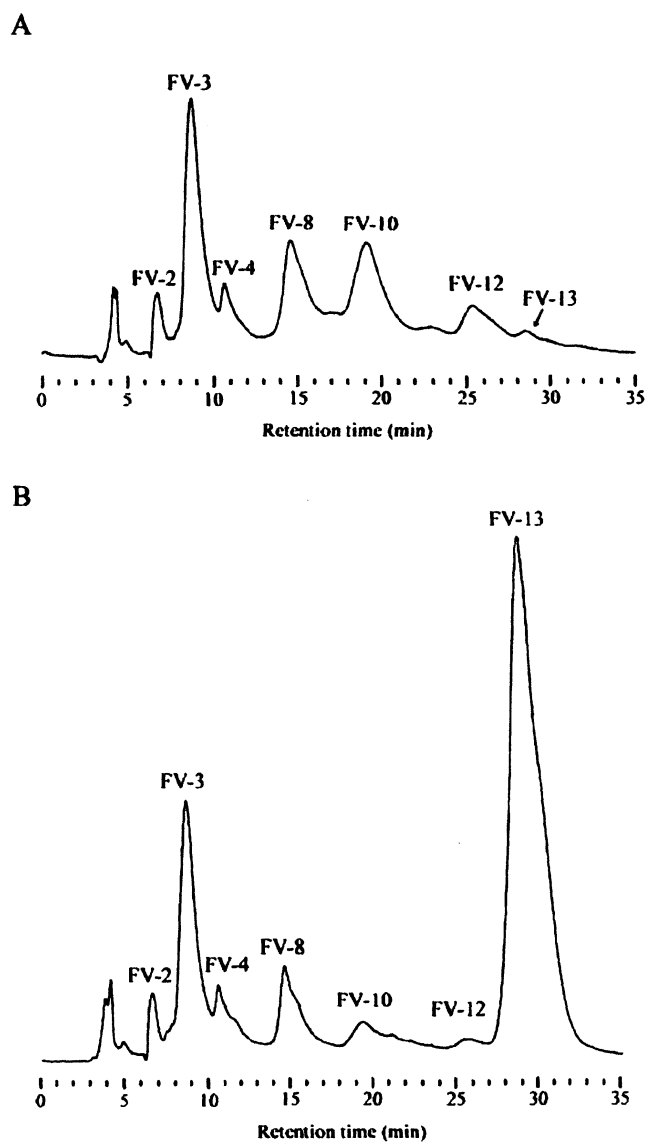
Fig. 2. Antiviral activity and cell growth of 40-BCR, 120-BCR and 350-BCR.



Mycelium weight: ●, 40-BCR; ▲, 120-BCR; ■, 350-BCR. Antiviral activity: ○, 40-BCR; △, 120-BCR; □, 350-BCR.

fattiviracin components produced by them were observed. Especially, 40-BCR produced exclusively fattiviracin FV-13, which accounted for approximately 68% of the total

Fig. 3. HPLC profiles of the fattiviracin components in the culture filtrate from wild strain (A) and 40-BCR (B).



fattiviracin components. On the other hand, the amounts of fattiviracin FV-10 and FV-12 were decreased in the 40-BCR culture. These results suggest that the fattiviracins-producing strain acquired resistance for bacitracin has the ability of the higher and astringent productivity of fattiviracin FV-13.

The regulation of the length of fatty acids moiety in fattiviracin molecule is important for the astringent production of fattiviracins. We considered BC-resistant mutation which might regulate the membrane lipid metabolism in the fattiviracins-producing strain, would

influence the production of fattiviracins. The development of fattiviracins as an antiviral drug was difficult for the large-scale preparation because of the low productivity and the existence of thirteen derivatives of fattiviracin. However, BC-resistant strain (40-BCR) exhibits the higher productivity and, in addition, the astringent productivity of fattiviracin FV-13 which has the highest molecular weight among fattiviracin components. The large scale preparation of fattiviracin FV-13 is now undergoing with the 40-BCR. Also the strains acquired resistance for higher concentration of bacitracin (120-BCR and 350-BCR) will be studied on their astringent productivity.

The mechanism of resistance of the fattiviracins-producing strain to BC has not been studied. Bacitracin is one of the inhibitors of cell wall biosynthesis of bacteria. By binding to the C₅₅-isoprenyl pyrophosphate in the presence of a divalent cation, bacitracin prevents the formation of C₅₅-isoprenyl phosphate. C₅₅-isoprenyl pyrophosphate functions as a lipid carrier for the transport across the membrane of the disaccharide-pentapeptide subunits of the peptidoglycan cell wall⁷⁾. In the bacitracin-resistant strains of *Escherichia coli*, *Streptococcus pneumoniae* and *Staphylococcus aureus*, when membrane-associated isoprenol kinase encoded in the *bacA* gene is overproduced, it is able to produce sufficient supplies of C₅₅-isoprenyl phosphate from C₅₅-isoprenol to overcome the effects of C₅₅-isoprenyl pyrophosphate sequestration, thereby causing bacitracin resistance^{8,9)}. In the bacitracin-resistant strain of *Streptomyces microflavus* strain No. 2445, the possibility of an increase in C₅₅-isoprenyl phosphate or the activation of isoprenol kinase that may be related with the biosynthesis of fatty acid moieties in FV-13 molecule is now under investigation.

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