CV-1, A NEW ANTIBIOTIC PRODUCED BY A STRAIN OF *STREPTOMYCES* SP.

I. FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES OF THE ANTIBIOTIC

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(Received for publication February 3, 1987)

A new antibiotic, CV-1, was isolated from the culture broth of a *Streptomyces* sp. by various chromatographies. CV-1 showed antibacterial activity against *Escherichia coli* in cooperation with spiramycin, a macrolide antibiotic. The mode of action of CV-1 seemed to be the inhibition of lipopolysaccharide synthesis.

In the course of our screening program for new antibiotics, we found a new antibiotic, CV-1, from *Streptomyces* strain CO-1. CV-1 exhibited cooperative antibacterial activity with spiramycin against *Escherichia coli*. Spiramycin alone, was not effective against *E. coli*. In this paper, the fermentation, isolation, antibacterial activity and mode of action of CV-1 are presented. Details of the physico-chemical properties and structure determination of CV-1 will be given in the following paper.[1]

Taxonomy of the Producing Strain

The taxonomic characterization was carried out according to the method used in the International Streptomyces Project (ISP),[2] and the CV-1 producing strain was classified as a species of *Streptomyces*. Details of the taxonomic studies will be published elsewhere.

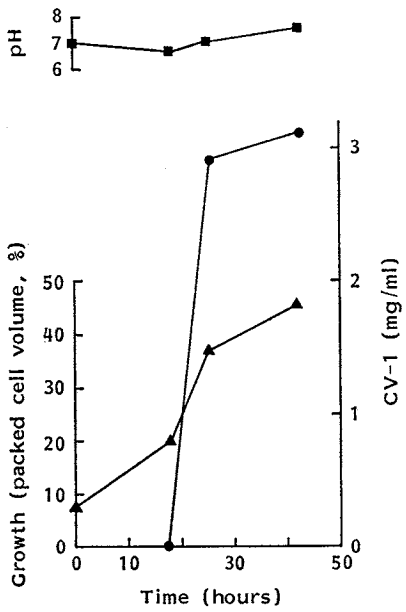
Fermentation

The seed flasks were inoculated with spores from a slant culture of the producing strain and incubated at 28°C for 48~72 hours. The seed medium consisted of glucose 10 g, soluble starch 10 g, beef extract 3 g, yeast extract 5 g, Bacto-tryptone 5 g and $CaCO_3$ 2 g per liter of deionized water. A 30-liter jar fermentor with 18 liters of the fermentation medium was inoculated to 3.4% volume with the seed culture. The fermentation medium consisted of dextrin 40 g, dry yeast 20 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 4H_2O$ 0.5 g and $CaCO_3$ 5 g per liter of deionized water. The fermentation was carried out at 28°C for 42 hours with aeration at 18 liters/minute and with agitation of 400 rpm. Antibiotic activity in the fermentation broth was determined by paper disc agar diffusion assay using *E. coli* ATCC 26 as the test organism in the presence of 40 μg of spiramycin (Kyowa Hakko Kogyo Co., Ltd.) per ml. Antibiotic activity appeared in the culture supernatant at about 24 hours and reached a maximum at 42 hours (Fig. 1). The antibiotic activity is expressed as the amount of CV-1 per ml of broth cultures. Growth is expressed as packed cell volume, determined by centrifugation at $1,200 \times g$ for 10 minutes.

Fig. 1. Time course of CV-1 fermentation.

Fermentation was carried out in a 30-liter jar fermentor using the medium indicated in the text at 28°C with agitation of 400 rpm and aeration of 18 liters/minute.

● CV-1, ▲ growth, ■ pH.



Purification and Isolation

The fermentation broth was filtered and the filtrate was adjusted to pH 8.0 with 0.5 N NaOH. The filtrate (15 liters) was applied to a column of Dowex 1-X2 (HCO_3^-, 1 liter). The effluent was applied to a column of an activated charcoal (2 liters). After the column was washed with H_2O (2 liters), the active compound was eluted with 20% aqueous acetone. The eluate was concentrated under reduced pressure at 20°C. Lyophilization of the active fractions provided a white powder (30 g). A sample of crude powder (1 g), thus obtained, was dissolved in H_2O (1 ml) and then chromatographed on a column of TSK-GEL Toyopearl HW-40 Fine (400 ml, Toyo Soda) using H_2O as the solvent. The active fractions were collected and lyophilized. The hygroscopic powder (100 mg) obtained was suspended in acetonitrile (500 μl) and chromatographed on a glass column (8 mm×20 cm) packed with Wakogel LC NH_2-10H (dry weight 20 g, Wako Junyaku) by the linear gradient elution method (100% acetonitrile to 70% acetonitrile) under pressure (about 5 kg/cm²). Active fractions containing only CV-1

Table 1. Cooperative effect of CV-1 and spiramycin against *Escherichia coli* by the disc diffusion method (inhibitory zone).

Amounts of CV-1/disc (μg)	Without spiramycin (mm)*	With spiramycin (mm)*
62.5	0	12.2
125	0	14.5
250	15.5	18.1

Disc: Paper disc with 8 mm diameter.

* Turbid inhibitory zone.

The medium consists of Nutrient broth (Difco) 8 g, NaCl 5 g and Bacto - agar (Difco) 12 g per liter of deionized water. The pH of the medium was adjusted to 7.0 before sterilization.

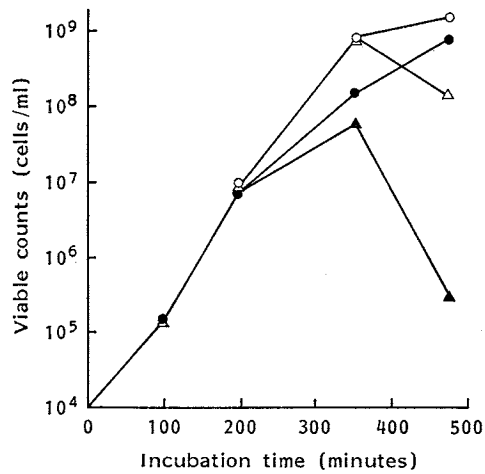
Spiramycin (sterilized by filtration) was added at 40 μg/ml.

E. coli ATCC 26 was inoculated at 1×10^7 cells/ml and was incubated at 37°C over night.

Fig. 2. Cooperative effect of CV-1 and spiramycin against *Escherichia coli* in the liquid medium with shaking.

E. coli ATCC 26 was cultured at 37°C with shaking under each of the conditions indicated in the figure. The medium consisted of Nutrient broth (Difco) 8 g and NaCl 5 g per liter of deionized water (pH 7.0 before sterilization). Viable cell numbers were counted on the above medium containing 15 g of agar after incubation for 20 hours at 37°C.

○ Control, △ spiramycin, ● CV-1, ▲ spiramycin +CV-1.



were collected, concentrated and lyophilized to yield pure CV-1 (10 mg).

Cooperative Effect of CV-1 and Spiramycin in Antibacterial Activity

CV-1 had very weak activity against *E. coli* by the agar diffusion method (Table 1). MICs of CV-1 were above 100 μg/ml against Gram-positive and Gram-negative bacteria by the agar dilution method at pH 7.0. However, antibacterial activity against *E. coli* was observed, when spiramycin was present together with CV-1. Spiramycin is a macrolide antibiotic and is not effective against Gram-negative bacteria. As shown in Table 1, in the presence of spiramycin (40 μg/ml) CV-1 elicited larger inhibitory zones than were observed in the absence of the macrolide. Furthermore a similar phenomenon was observed in the liquid shaken culture. When only CV-1 (125 μg/ml) or only spiramycin (25 μg/ml) was added to the liquid culture, the growth of *E. coli* was hardly affected. However when the both CV-1 (125 μg/ml) and spiramycin (25 μg/ml) were added, the viable counts of *E. coli* were decreased markedly at about 5 hours of cultivation (Fig. 2).

Effect of CV-1 on Macromolecular Synthesis

As described above, CV-1 and spiramycin exhibited a cooperative effect against *E. coli*. Spiramycin had no activity against Gram-negative bacteria. This is attributed to the inability of spiramycin to penetrate the cell wall barrier[8] (especially the outer membrane) of the bacterium. Thus, it was expected that CV-1 disorganized the cell wall barrier in some fashion.

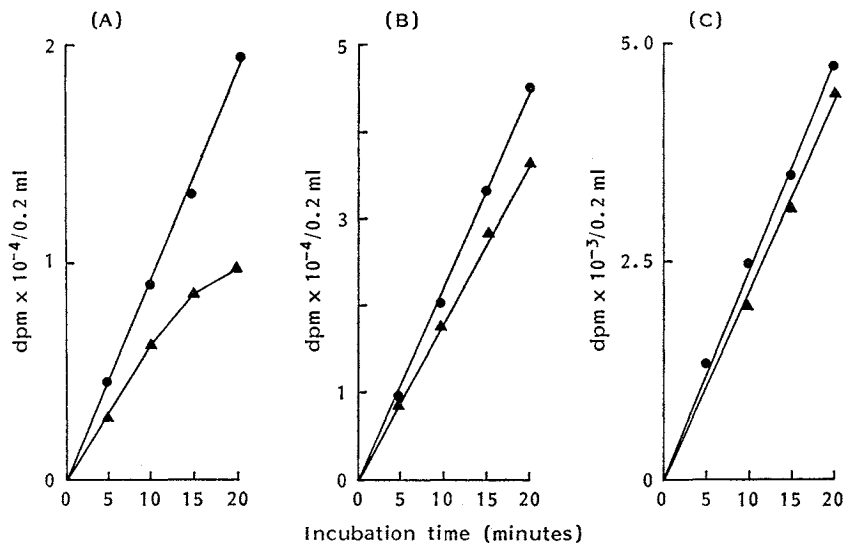
It is of interest to study the effect of CV-1 on the synthesis of macromolecules. The effect of

Fig. 3. Effect of CV-1 on macromolecular synthesis in *Escherichia coli* galE⁻.

When the cell density of *E. coli* galE⁻ in the medium consisted of Nutrient broth (Difco) 8 g and NaCl 5 g per liter of deionized water (pH 7.0 before sterilization) reached $OD_{680\,nm}=0.1$, radioactive precursors and CV-1 (0 or 250 μg/ml) were added. After addition of these compounds, 0.2 ml samples were removed and treated as described in the text. All the incubation were carried out at 37°C with shaking.

● CV-1 at 0 μg/ml, ▲ CV-1 at 250 μg/ml.

(A) Incorporation of D-[1-^{3}H]galactose into the alkali-insoluble fraction (lipopolysaccharide). D-[1-^{3}H]Galactose (1 μCi/ml) was added. (B) Incorporation of [8-^{14}C]adenine into the acid-insoluble fraction (nucleic acid). [8-^{14}C]Adenine (0.5 μCi/ml) was added. (C) Incorporation of L-[^{14}C]threonine into the acid-insoluble fraction (protein). L-[^{14}C]Threonine (0.25 μCi/ml) was added.



CV-1 on the synthesis of lipopolysaccharide (LPS) in growing cells of *E. coli* galE$^-$ is presented in Fig. 3A.[4] The effect of CV-1 on the synthesis of nucleic acid and protein in growing cells of *E. coli* galE$^-$ are presented in Figs. 3B and 3C. The synthesis of LPS was followed by measuring the incorporation of labeled [1-^{3}H]galactose into alkali-insoluble precipitates. The synthesis of nucleic acid and that of protein were followed by measuring the incorporation of labeled [8-^{14}C]adenine, and [^{14}C]threonine into acid-insoluble precipitates. After the addition of radioactive precursors, 0.2 ml samples were periodically removed and poured into 1.0 ml of ice-cold 1.32 N NH_4OH containing 6.6 mM $MgCl_2$ in the case of LPS synthesis and 1.0 ml of ice-cold 10% trichloroacetic acid in the case of nucleic acid and protein synthesis and allowed to stand for 90 minutes in the ice bath. The reaction mixtures were filtered through HA Millipore filters (0.45 μm) and washed with 2 ml of cold 10% trichloroacetic acid. The filters were dried and counted in vials containing scintillation fluid consisted of 4 g 2,5-diphenyloxazole and 0.1 g 2,2′-*p*-phenylene-bis-(5-phenyloxazole) per liter of toluene. As shown in Figs. 3B and 3C inhibition by CV-1 of nucleic acid and protein synthesis was slight at concentration of 250 μg/ml. On the other hand, LPS synthesis was inhibited in 5~10 minutes. These results suggested that CV-1 inhibits LPS synthesis.

Discussion

CV-1 itself is a very weak antibiotic. However, CV-1 showed a cooperative effect with spiramycin against *E. coli*. Spiramycin is not effective against Gram-negative bacteria. It is thought that spiramycin and the other antibiotics which are not effective against Gram-negative bacteria are excluded by the outer membrane of Gram-negative bacteria. The LPS of the outer membrane component seems to have an especially important role in the barrier function. As described above, CV-1 inhibited the synthesis of LPS and consequently the cooperative effect was probably due to the entry of spiramycin into *E. coli* by the virtue of the action of CV-1. It was previously reported that a polymyxin B nonapeptide derivative (PMBN) or polycations such as lysine polymer disorganized the outer membrane to facilitate the entry of antibiotics.[5,6] These agents disorganized the outer membrane physiologically in a short time.

On the other hand, the effect of CV-1 took much longer than those agents to emerge. Some turnover of the outer membrane in the presence of CV-1 appears necessary for the entry of spiramycin into the cell. Further studies of the details of the inhibition of LPS biosynthesis by CV-1 are now in progress.

Acknowledgments

We thank Dr. H. NAKANO for support and encouragement, and Dr. T. TAMAOKI, Dr. K. TAKAHASHI and Dr. M. OKII for encouragement and useful suggestions.

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