Susceptibility of ribosomes of the tetracycline-producing strain of *Streptomyces aureofaciens* to tetracyclines

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Ribosomes from cells of *Streptomyces aureofaciens* producing tetracycline antibiotics (Tc-ribosomes) differ in electrophoretic mobility of ribosomal proteins S2, S10 and L19 from those of the same strain, where the production of tetracyclines was suppressed by changed cultivation conditions (C-ribosomes). Purified tight vacant couples C- and Tc-ribosomes are equally active in the translation of poly(U). Both types of *S. aureofaciens* ribosomes are more sensitive to tetracycline and chlortetracycline than ribosomes of *Escherichia coli* in the Phe-tRNA binding and the translation of poly(U).

Tetracycline *Ribosomes* *Resistance*

1. INTRODUCTION

It is well established that the target site of tetracyclines in protein synthesis is localized on ribosomes [1,2]. The major inhibitory effect of the antibiotic is the interference with binding of the ternary complex aminoacyl-tRNA.EFTu.GTP to the acceptor site of the ribosome [3,4]. The resistance to tetracyclines is usually mediated by a mechanism preventing the permeation of the antibiotic into the cells [5,6]. We have shown previously that this type of resistance developed in the tetracycline-producing strain of *Streptomyces aureofaciens* [7]. Ribosomes and ribosomal subunits from these cells bind tetracycline similarly as reported earlier with other prokaryotic ribosomes [8,9]. Recently, a mutant strain of *Bacillus subtilis* has been isolated [10] bearing the tet-4 mutation specifying an altered ribosomal protein S10. Ribosomes from the mutant strain were more resistant in the translation of poly(U) to tetracycline than wild type ribosomes.

In the present communication we examined protein pattern of ribosomes from the cells of *S. aureofaciens* producing tetracycline and from the cells where the production of the antibiotics was suppressed by changed cultivation conditions. In addition, we have assayed differences in sensitivity to tetracycline between ribosomes of *Escherichia coli* and *S. aureofaciens* in the binding of Phe-tRNA to ribosomes and in the translation of poly(U).

2. MATERIALS AND METHODS

2.1. *Preparation of ribosomes and elongation factors Tu*

Ribosomes were isolated according to Hershey et al. [11]. Tight vacant couples were prepared by the method of Noll et al. [12]. Elongation factors Tu from *E. coli* and *S. aureofaciens* were isolated as described previously [13].

2.2. *Poly(U)-directed enzymatic and non-enzymatic binding of [^{14}C]Phe-tRNA to ribosomes*

For the enzymatic binding, EFTu.GTP complex was preformed in a reaction mixture (150 μl) containing 50 mM Tris–HCl (pH 7.4), 8 mM $MgCl_2$, 60 mM NHCl, 4 mM GTP, 40 mM phosphoenol pyruvate, 20 μg pyruvate kinase, 900 pmol EF-Tu.GDP. The incubation was carried out at 37°C for 10 min. The mixture was then cooled to 0°C.

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Samples of 100 μl containing 50 mM Tris–HCl (pH 7.4), 8 mM MgCl, 60 mM NHCl, 160 μg poly(U), 50 pmol 70 S ribosomes were preincubated with a mixture of deacylated tRNA (1 A_{260} unit) at 37°C for 5 min, 60 pmol EFTu.GTP were then added (for the non-enzymatic binding, EF-Tu.GTP was omitted). In inhibition studies samples were further incubated with tetracycline at 37°C for 5 min. The reaction was started by adding 50 pmol [^{14}C]Phe-tRNA. After a 10-min incubation at 37°C the samples were diluted with cold binding buffer and the amount of bound [^{14}C]Phe-tRNA was determined by the membrane filter assay. To control for a possible polypeptide synthesis, reaction mixtures were stopped with 1 ml 10% trichloroacetic acid (TCA) and heated at 95°C for 20 min. The mixtures were filtered through glass fibre discs, washed with 5% TCA, and radioactivity was determined. No significant synthesis of a polypeptide was demonstrated in the control experiments.

2.3. *Translation of poly(U)*

The poly(U)-directed polyphenylalanine synthesis was carried out in 100-μl reaction mixtures containing 50 mM Tris–HCl (pH 7.4), 8 mM $MgCl_2$, 70 mM NH_4Cl, 6 mM 2-mercaptoethanol, 25 pmol ribosomes, [^{14}C]phenylalanine (315 Ci/mol), 40 μg tRNA, 0.2 mM GTP, 5 mM phosphoenolpyruvate, 1 μg pyruvate kinase, 160 μg poly(U), tetracycline as indicated in the figures. The reaction mixture was incubated 20 min at 37°C. It was stopped with 1 ml 5% TCA and the tubes were heated for 20 min at 90–95°C. The precipitate was collected on Whatman GF/C filters and radioactivity was assayed.

2.4. *Isolation of ribosomal proteins and two-dimensional gel electrophoresis*

Ribosomal proteins were isolated in the presence of 0.2 mM phenylmethyl-sulfonyl fluoride [14]. The two-dimensional gel electrophoresis of ribosomal proteins was performed as previously described [15].

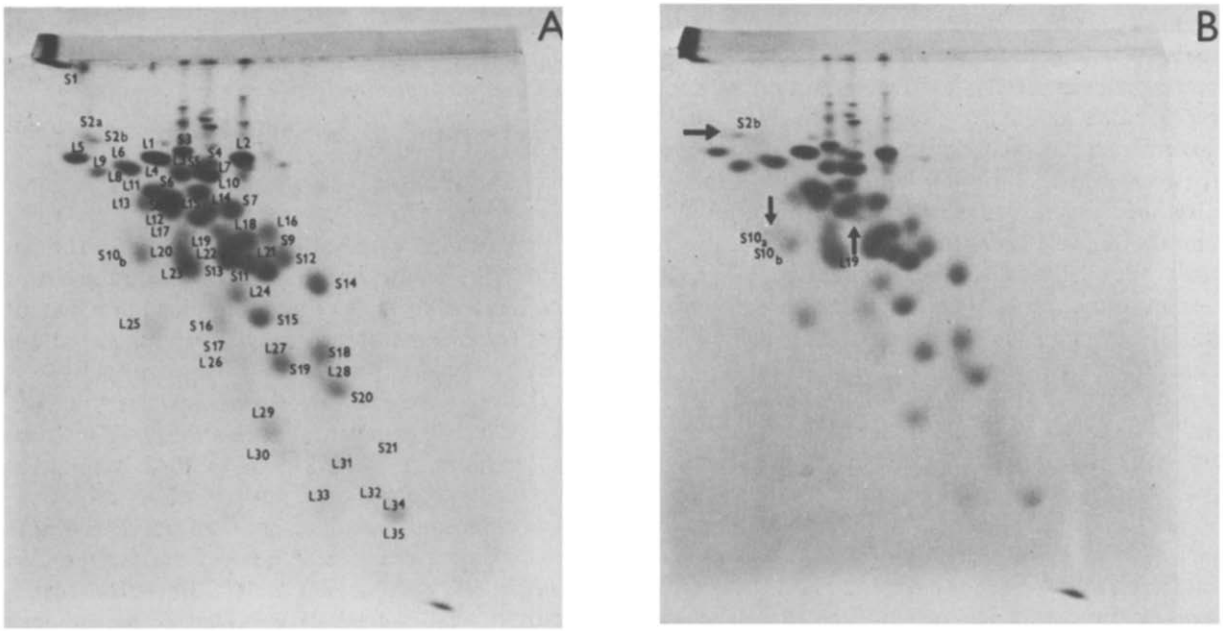

Fig.1. Comparison of 70 S ribosomal proteins from C-ribosomes (A) and Tc-ribosomes (B) of *Streptomyces aureofaciens*. Proteins (200 μg) from the two types of ribosomes were analyzed by two-dimensional polyacrylamide gel electrophoresis [15].

3. RESULTS AND DISCUSSION

As the wild strain of *S. aureofaciens* or a mutant lacking the ability to produce tetracycline or compounds belonging to the tetracycline family are not available, we have isolated ribosomes from young growing cells of *S. aureofaciens* producing tetracycline (Tc-ribosome) and from the same strain, where the production of the antibiotic was suppressed by changed cultivation conditions [16] (C-ribosomes). Tight couples 70 S ribosomes of both Tc- and C-ribosomes were used for functional tests and electrophoretic analysis of ribosomal proteins. As shown in fig.1, ribosomal protein patterns of C-ribosomes (fig.1A) differ significantly from those of Tc-ribosomes (fig.1B) in three respects. In C-ribosomes the protein S2 forms two spots designated *a* and *b*. Furthermore, two forms of S10 proteins *a* and *b* are present in Tc-ribosomes and the protein L19 changes its position near to the protein L15. The observed changes in positions of the spots are most probably due to posttranslational modification of the proteins. This possibility remains to be studied in more detail. Further data concerning nomenclature of ribosomal proteins from *S. aureofaciens* are presented in [15].

The rates of polyphenylalanine synthesis on ribosomes of *S. aureofaciens* and on those of *E. coli* are depicted in fig.2. Purified vacant couples C- and Tc-ribosomes from *S. aureofaciens* are equally active in the translation of poly(U). The results also show that polyphenylalanine-synthesizing activity of *E. coli* ribosomes is about three times higher than that of *S. aureofaciens* ribosomes. Similar results were obtained in experiments with ribosomal preparations of different streptomycete species [17–19].

Tetracycline antibiotics are best known inhibitors of aminoacyl-tRNA binding to the ribosomal acceptor site [1]. To demonstrate whether ribosomes of *S. aureofaciens* are resistant to the drug, we first examined the non-enzymatic and enzymatic binding of [^{14}C]Phe-tRNA to ribosomes. The enzymatic binding of [^{14}C]Phe-tRNA was estimated with preformed EFTu.GTP. Results of the binding experiments are shown in fig.3. Tetracycline inhibited both the non-enzymatic and EFTu-dependent binding to C-ribosomes of *S. aureofaciens* (fig.3A) as well as to those of *E. coli* (fig.3B). To obtain more specific information about the binding of [^{14}C]Phe-tRNA to the acceptor site, ribosomes were preincubated with 1.0 A_{260} unit of deacylated tRNA [2]. The non-enzymatic binding of [^{14}C]Phe-tRNA to ribosomes of *E. coli* was about twice higher than to ribosomes of *S. aureofaciens*. In the presence of 0.01 mM tetracycline the non-enzymatic binding to ribosomes of *E. coli* or *S. aureofaciens* was inhibited to about 40%. No significant increase of the inhibition was observed at concentrations higher than 0.1 mM tetracycline. These data are consistent with the previously published data [1,20]. The binding of [^{14}C]Phe-tRNA to ribosomes was markedly stimulated in the presence of EFTu.GTP. There are differences in the kinetics of inhibition of enzymatic binding of [^{14}C]Phe-tRNA to ribosomes of *S. aureofaciens* and *E. coli*. At lower tetracycline concentrations (from 10^{-6} M

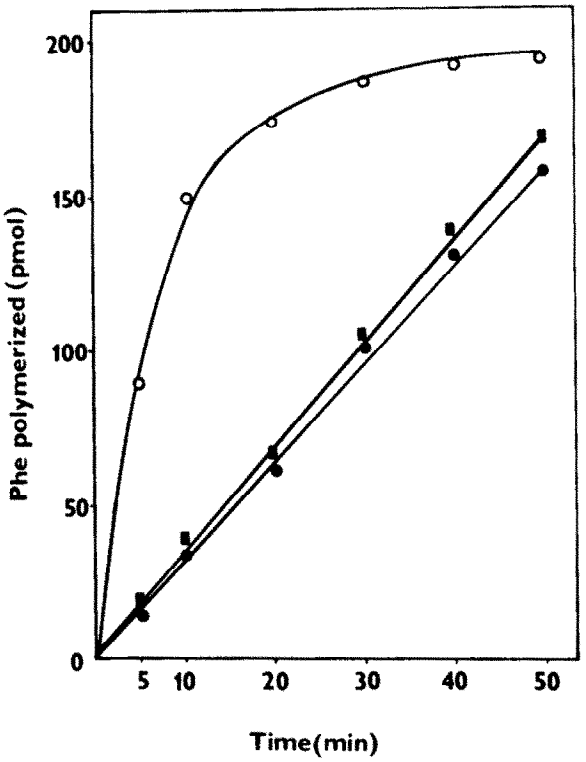

Fig.2. Kinetics of polyphenylalanine synthesis on ribosomes of *E. coli* and Tc- or C-ribosomes of *Streptomyces aureofaciens*. The assays were carried out as described in section 2 using 25 pmol of the 70 S tight couples from *E. coli* (—○—), C-ribosomes of *S. aureofaciens* (—■—), Tc-ribosomes of *S. aureofaciens* (—●—).

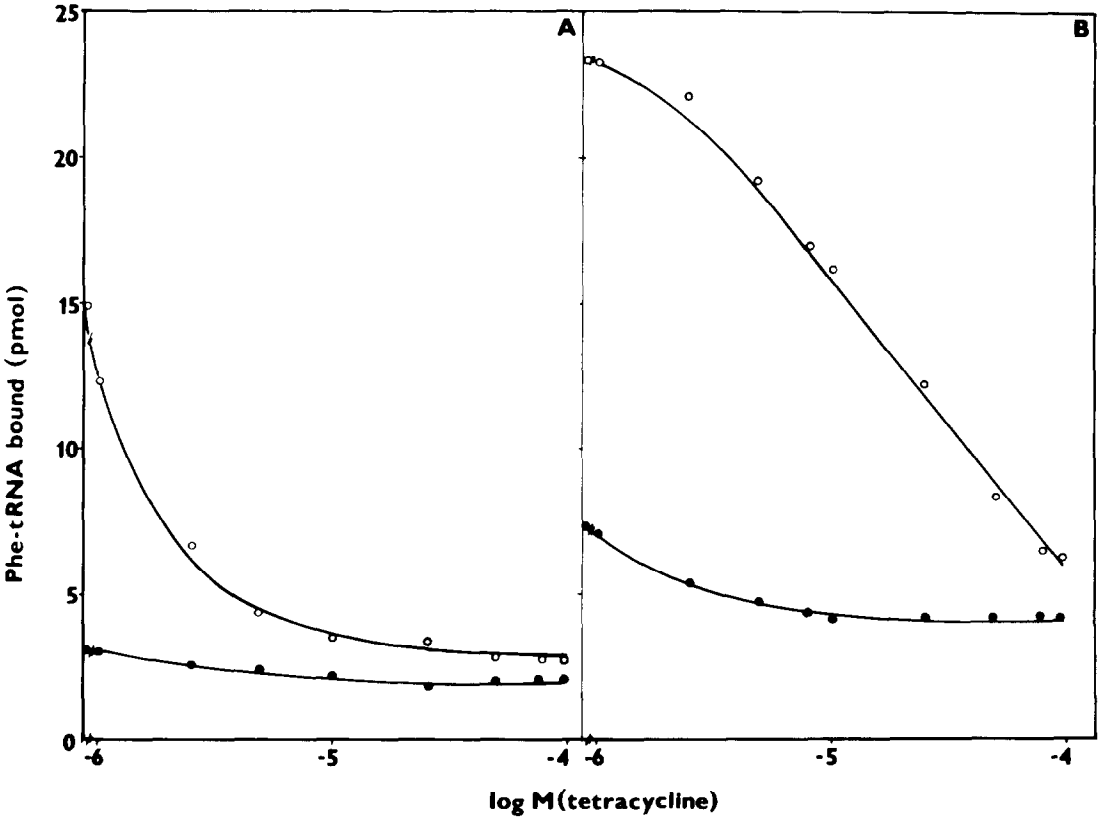

Fig.3. Effect of tetracycline upon the enzymatic and non-enzymatic binding of [^{14}C]Phe-tRNA to the 70 S couples from *E. coli* and *S. aureofaciens*. The experiments were performed as described in section 2. (A) C-ribosomes from *S. aureofaciens*; (B) ribosomes of *E. coli*. EFTu-dependent binding (—○—); non-enzymatic binding (EFTu.GTP omitted) (—●—).

to 5×10^{-6} M) the drug inhibits the binding of Phe-tRNA to ribosomes of *S. aureofaciens* by about 75%. A further increase of the tetracycline concentration (from 10^{-5} M to 10^{-4} M) results only in a 5% decrease of the Phe-tRNA binding. In addition, essentially the same results as in fig.3A were obtained with purified 70 S tight couples Tc-ribosomes of *S. aureofaciens*. Ribosomes of *E. coli* are more resistant to tetracycline at the lower concentration of the drug. At 10^{-6} M to 5×10^{-6} M concentration the antibiotic inhibits 10% Phe-tRNA binding to ribosomes and at the higher drug concentration (between 5×10^{-6} M to 5×10^{-5} M) more than 45% of the Phe-tRNA binding were inhibited.

In further experiments we examined the effect of chlortetracycline in the translation of poly(U). Ribosomes were preincubated with the antibiotic and polyphenylalanine synthesis was initiated by the addition of poly(U) and [^{14}C]phenylalanine. Results of these experiments are shown in fig.4. Translation of poly(U) on ribosomes of *E. coli* is resistant to lower concentrations of chlortetracycline (from 10^{-6} M to 5×10^{-6} M). About 75% of the polyphenylalanine synthesizing activity was inhibited by 10^{-5} M to 10^{-4} M concentration of the drug. The translation of poly(U) on both Tc- and C-ribosomes of *S. aureofaciens* is more sensitive to the antibiotic. At the low chlortetracycline concentration (between 10^{-6} M to 10^{-5} M) the synthesis of polyphenylalanine was inhibited by about 75%. The results obtained demonstrate that the ribosomes of *S. aureofaciens* are more sensitive to the low concentration of the drug than ribosomes of *E. coli*. This suggestion is also supported by the experiments in which homologous or heterologous

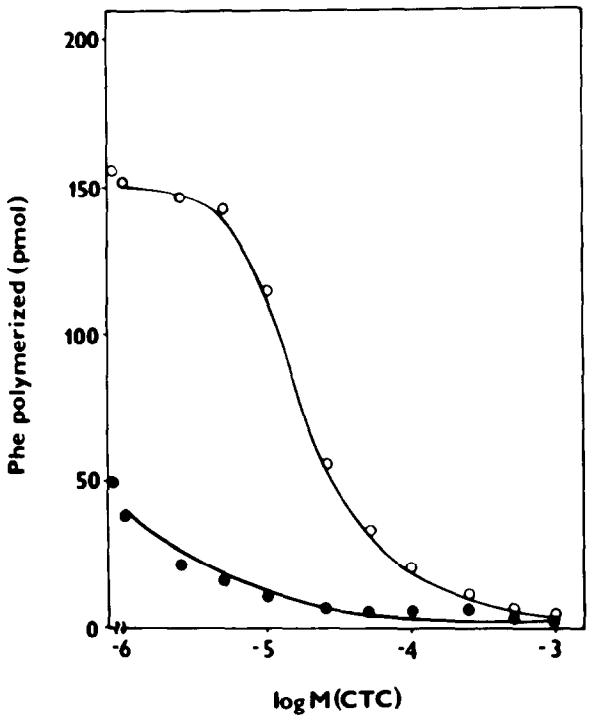

Fig.4. Inhibition of poly(U) translation with chlortetracycline (CTC). The assays were carried out as described in section 2 using 25 pmol of the 70 S ribosomes and saturation concentration of poly(U). In experiments with *E. coli* (—○—) 10 μg of poly(U) and with *S. aureofaciens* (—●—) 160 μg of poly(U) was used.

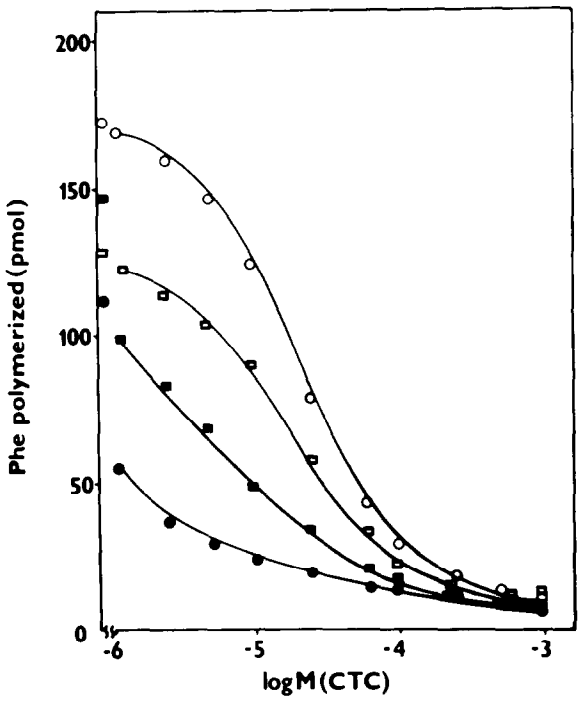

Fig.5. Effect of chlortetracycline on polyphenylalanine synthesis on the homologous and heterologous 30 S–50 S couples derived from ribosomal subunits of *E. coli* and *S. aureofaciens*. In vitro translation of poly(U) was estimated in 0.1 ml reaction mixtures containing 0.4 A_{260} unit of 30 S subunits and 1.2 A_{260} unit of 50 S subunits. The experiments were carried out as described in section 2. Ribosomes were preincubated for 5 min with chlortetracycline (CTC) and the reaction was started by poly(U) and [^{14}C]phenylalanine. Homologous 30 S–50 S couples of *E. coli* (—○—). Homologous 30 S–50 S couples of *S. aureofaciens* (—●—). Heterologous 30 S–50 S couples containing 30 S subunits of *E. coli* and 50 S from *S. aureofaciens* (—□—). Heterologous 30 S–50 S couples containing 30 S subunits of *S. aureofaciens* and 50 S from *E. coli* (—■—).

30 S–50 S couples derived from ribosomal subunits of *E. coli* and *S. aureofaciens* were incubated in the presence of chlortetracycline and poly(U) translation was estimated. Results of these experiments (fig.5) show that homologous 30 S–50 S couples of *S. aureofaciens* are most sensitive to chlortetracycline. Substitution of 30 S subunits of *S. aureofaciens* by analogous subunit from *E. coli* increased the resistance of the heterologous 30 S–50 S couples to the antibiotic substantially. Replacement of the 50 S subunits from *S. aureofaciens* with 50 S subunits of *E. coli* has a stimulatory effect on the translation of poly(U) but these heterologous couples containing 30 S from *S. aureofaciens* and 50 S of *E. coli* are more sensitive to the antibiotic. The data demonstrate again that homologous 30 S–50 S couples of *E. coli* are less susceptible to lower chlortetracycline concentration than those of *S. aureofaciens*.

REFERENCES

[1] Sarkar, S. and Thach, R.E. (1968) Proc. Natl. Acad. Sci. USA 60, 1468–1479.
[2] Watanabe, W. (1972) J. Mol. Biol. 67, 443–457.
[3] Shorey, R.L., Ravel, J.M., Garner, C.W. and Shire, W.J. (1969) J. Biol. Chem. 244, 4555–4564.
[4] Peters, M. and Yarus, M. (1979) J. Mol. Biol. 134, 471–491.
[5] Tait, R.C. and Boyer, H.W. (1978) Cell 13, 73–81.

[6] Mendez, B., Tachibana, Ch. and Levy, S.B. (1980) Plasmid 3, 99–108.
[7] Mikulík, K., Karnetová, J., Quyen, N., Blumauerová, M., Komersová, I. and Vaněk, Z. (1971) J. Antibiot. 24, 801–809.
[8] Day, L.E. (1966) J. Bacteriol. 91, 1917–1923.
[9] Maxwell, I.H. (1981) Mol. Pharmacol. 4, 25–37.
[10] Williams, G. and Smith, I. (1979) Molec. Gen. Genet. 177, 23–29.
[11] Hershey, J.W.B., Yanov, J., Johnston, K. and Fakunding, J.L. (1977) Arch. Biochem. Biophys. 182, 626–638.
[12] Noll, M., Hapke, B., Schreier, M.M. and Noll, H. (1973) J. Mol. Biol. 75, 281–294.
[13] Weiser, J., Mikulík, K. and Bosch, L. (1981) Biochem. Biophys. Res. Commun. 99, 16–22.
[14] Barritault, D., Expert-Bezançon, A., Guérin, M.F. and Hayes, D. (1976) Eur. J. Biochem. 63, 131–135.
[15] Mikulík, K., Janda, I., Weiser, J., Jiráňová, A. (1982) Biochim. Biophys. Acta, in press.
[16] Šťastná, J. and Mikulík, K. (1981) Zbl. Bakt. Suppl. 11, 481–486.
[17] Jones, G.H. (1977) J. Bacteriol. 129, 81–86.
[18] Thompson, J. and Cundliffe, E. (1980) J. Bacteriol. 142, 455–461.
[19] Yamamoto, H., Hotta, K., Okami, Y. and Umezawa, H. (1981) Biochem. Biophys. Res. Commun. 100, 1396–1401.
[20] Suarez, G. and Nathans, D. (1965) Biochem. Biophys. Res. Commun. 18, 743–750.