EFFECTS OF LINCOMYCIN ON SYNTHESIS OF TEM β-LACTAMASE BY *ESCHERICHIA COLI*

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Sub-inhibitory concentrations of lincomycin slightly inhibit growth of *Escherichia coli* carrying plasmid RP4 and cause a 2-fold increase in TEM-2 β-lactamase. To analyze this effect, cultures were pulse-labeled with [^{3}H]leucine, chased with non-radioactive leucine and immunoprecipitated with anti-β-lactamase antiserum. The synthesis rate of β-lactamase was two times higher in inhibited cultures than in control cultures. No significant decrease of labeled enzyme occurred during the 30 minutes chase, indicating no degradation of β-lactamase. The rate of maturation of pre-β-lactamase was determined by measuring the decrease in the amount of pre-β-lactamase after a 1-minute labeling interval. There was no significant difference between the control and lincomycin-treated cultures, indicating that posttranslational translocation is not involved in the stimulation. Both plasmid encoded and chromosomally encoded TEM-1 β-lactamase increased in the presence of lincomycin. The effects of other protein synthesis inhibitors on the synthesis of TEM-1 β-lactamase were examined. The stimulation of β-lactamase synthesis by lincomycin appears to be specific for macrolide and related antibiotics and is not a general phenomenon resulting from partial inhibition of protein synthesis.

Lincomycin stimulates the production of a heat-labile enterotoxin by *Escherichia coli*[1,2] and of cholera toxin by *Vibrio cholerae*[3,4] at sub-inhibitory concentrations. We have previously described that the TEM-2 β-lactamase activity of *E. coli* carrying plasmid RP4 increased in the presence of low concentrations of lincomycin[5]. Indirect arguments have been used to show that stimulation of β-lactamase synthesis is responsible for the increase in β-lactamase activity.

To elucidate the mechanism of the stimulation, the step in β-lactamase synthesis which is stimulated must be identified. Previously we have suggested that stimulation could occur at i) translocation, ii) transcription, or iii) translation[5]. Recently, YOUNG and BROADBENT[6] reported a differential effect of lincomycin on the production of secretory proteins by *V. cholerae*. They suggested that the lincomycin effect might be specific for secretory proteins which are translocated posttranslationally. We have compared the rate of posttranslational translocation of β-lactamase in cultures with and without lincomycin and find no evidence for an involvement of posttranslational translocation in the stimulation by lincomycin.

The lincomycin effect may be due to the specific mode of action of the drug or a specific sequence in the *bla* gene. To investigate the first possibility, we examined the effects of other protein synthesis inhibitors on β-lactamase synthesis. The second possibility can be studied by gene manipulation, for which the *bla* gene of pBR322 is most suitable. We show that synthesis of the plasmid encoded TEM-1 β-lactamase is stimulated by lincomycin. Lincomycin also effects the chromosomally encoded TEM-1 β-lactamase (a preliminary report of this and related work was presented by A. OKABE *et al.* at the 22nd Joint U.S.-Japan Conference on Cholera, Toyama, Japan, July 20~23, 1986).

Materials and Methods

Microorganisms and Culture Conditions

All strains of *E. coli* used in this study were substrains of *E. coli* K-12: J53 (F^-, *pro*, *met*) carrying plasmid RP4 (Tra^+, Ap, Nm, Tc, Km)[7], YA21 (F^-, *met*, *leu*)[8] carrying plasmid pBR322, C600 (*thr*, *leu*, *thi*, *supE*, *lac*, *tonA*), C600 lysogenic for bacteriophage λcI857, S7, *xis*6, b515, b519 (abbreviated λbb) carrying plasmid pTY98 (colE1:: Tn3, Tn5)[9], and 112-2 (HfrC/*pur-leuS*, *metB*, *proA3*, *metD88*, *lac-3*, *tsx-76*, *relA*, Zaf:: Tn3). The strain C600 λbb/pTY98 was obtained from the Department of Microbiology, Chiba University, Chiba, Japan. *E. coli* 112-2 was obtained from the National Institute of Genetic Research Center, Mishima, Japan. *Staphylococcus aureus* Cowan I was obtained from the Department of Microbiology, Kawasaki Medical College, Kurashiki, Japan.

E. coli from overnight cultures in L broth were inoculated into fresh broth at a dilution of 1 to 100 and grown with shaking at 37°C as described previously[5], unless otherwise noted. Cell growth was monitored by measuring the optical density (OD) at 600 nm.

Lysogenization with λ:: Tn3

The strain C600 λbb/pTY98 grown in L broth at 28°C overnight was diluted 100-fold into fresh broth and incubated at 30°C with vigorous shaking. Cultures were heated at 45°C for 8 minutes when they grew to an OD of 0.3, and incubated at 37°C for 2 hours. Cells were lysed with chloroform and a cleared lysate after centrifugation was treated with DNase (10 μg/ml) at 25°C for 1 hour. The phage stock thus obtained was used to lysogenize strain C600. Lysogenization was carried out as described by BERG *et al.*[10]. Among colonies growing on a L agar plate containing 25 μg/ml of ampicillin, a colony sensitive to kanamycin (25 μg/ml) wes selected by replica plating. Thus obtained lysogen did not contain plasmids and phage induced from the lysogen yielded Amp^r cells upon lysogenization 10^5 times as often as did phage from strain C600 λbb/pTY98, indicating that Tn3 was transposed onto phage.

Labeling of Cultures with [^{3}H]Leucine

Strain J53/RP4 was precultured in L broth for 12 hours and then for 12 hours in a minimal medium (NaH_2PO_4 0.034 M, K_2HPO_4 0.064 M, $(NH_4)_2SO_4$ 0.02 M, $MgSO_4$ 0.3 mM, $ZnCl_2$ 1 nM, $FeSO_4$ 1 nM, glycerol 0.4%, thiamine-HCl 2 μg/ml, proline 500 μg/ml, methionine 300 μg/ml). Five ml of minimal medium with and without lincomycin (200 μg/ml) in 30-ml vials were inoculated with 50 μl of the last preculture. Cultures were grown by shaking on a reciprocal shaker (TC-1; Advantec Toyo Co., Tokyo, Japan) at 37°C. At both middle and late log phases, cultures were pulse-labeled with [4,5-^{3}H]leucine (20 μCi/ml; 53 Ci/mmol, Amersham, Bakinghamshire, England). At the end of a 5-minute labeling interval, non-radioactive leucine (300 μg/ml) and isoleucine (50 μg/ml) were added to the culture. Samples (0.3 ml) of each cultures were withdrawn immediately after addition of unlabeled leucine and processed as described below.

To measure the conversion of pre-β-lactamase, labeling with a short pulse was carried out as follows. One ml of the last preculture of strain J53/RP4 was inoculated into 100 ml of minimal medium in a 500-ml shake flask (Wheaton Scientific, Millville, New Jersey, U.S.A.) and grown at 37°C on a reciprocal shaker. When the culture reached an OD of 2, 0.5 ml were transferred to a 15-ml Corex tube (Corning Glass Works, Corning, New York, U.S.A.) which was prewarmed to 37°C. After the culture was labeled with [^{3}H]leucine (75 μCi/ml) at 37°C for 1 minute, 0.35 ml of the culture was removed and mixed with an equal volume of cold 10% TCA at 0°C. A 0.9-ml sample of the original culture was transferred to a separate Corex tube and pulse-labeled with [^{3}H]leucine (75 μCi/ml) for 1 minute followed by the addition of non-radioactive leucine (300 μg/ml) and isoleucine (50 μg/ml). Samples (0.35 ml) were removed at 1-minute intervals and added to TCA as before.

Immunoprecipitation and Fluorography

The TCA precipitated samples were chilled on ice for 30 minutes and centrifuged at 12,000 rpm at 4°C for 3 minutes. The pellets were washed with acetone at 4°C and dissolved in 20 μl of 50 mM Tris-HCl (pH 8) containing 1% SDS and 1 mM EDTA by heating in a boiling water bath for 2 minutes. Immunoprecipitation was carried out as described by ITO *et al.*[11]. Antiserum was prepared by im-

munizing a rabbit with TEM-2 β-lactamase purified as described previously[5]. Formalinated *S. aureus* Cowan I used as an immunosorbent was prepared by the method of KESSLER[12]. Immunoprecipitates adsorbed on *S. aureus* were solubilized with 60 μl of SDS sample buffer (Tris-HCl 65 mM, pH 6.8, SDS 3%, β-mercaptoethanol 2%, glycerol 10%, bromophenol blue 0.004%) by heating in boiling water for 2 minutes. After centrifugation at 15,000 rpm for 2 minutes at room temperature, the supernatants were transferred to small Eppendorf centrifuge tubes, capped, and heated in boiling water for 5 minutes. Fifteen μl of each sample were electrophoresed on a 12.5%-polyacrylamide gel (1 mm) as described previously[5]. Gels were fixed in 7% acetic acid for 30 minutes, soaked in Amplify (Amersham) for 25 minutes, dried and exposed to X-ray film (X-Omat AR-5, Eastman Kodak, Rochester, U.S.A.) at −80°C for 90 hours. Quantitative fluorography was carried out by the modified method of HORIUCHI *et al.*[13]. The intensities of the bands were measured by densitophotometry on a dual-wave length TLC scanner (CS-910; Shimadzu Seisakusho, Kyoto, Japan) followed by weighing the recorder paper containing the corresponding peak. A calibration curve of relative intensity was obtained from 8 different amounts of a sample prepared from a 28-hour lincomycin culture. The relative amounts of radioactivity incorporated into each band were determined by measuring 4 different amounts of each sample and calibrating the band intensity within the linear range of the calibration curve. Recovery of the antigen, β-lactamase, was determined by comparing the intensities of the β-lactamase band, before and after the immunoprecipitation of the periplasmic fraction which was prepared by extraction with polymyxin B as described previously[5]. The recovery was in the range of 86±2.6% in triplicate assays of three different samples.

Effects of Antibiotics on β-Lactamase Production

Strain 112-2 was grown in 10 ml of L broth with and without antibiotics in a 125-ml flask. The antibiotics used in this study were erythromycin, kanamycin sulfate, chloramphenicol, tetracycline, puromycin dihydrochloride (all purchased from Sigma Chemical Company, St Louis, U.S.A.), kasugamycin hydrochloride, streptomycin sulfate, oleandomycin phosphate (all purchased from Wako Pure Chemicals, Osaka, Japan), acetylspiramycin (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) and lincomycin (Japan Upjohn Limited, Tokyo, Japan). The concentration of each antibiotic was chosen so that the OD at 600 nm in late log phase (11-hour culture) was in the range between 65 and 75% of that of a control culture. Fractions (0.2 or 1 ml) of the cultures were withdrawn at 60-minute intervals after cultivation for 5 hours and kept at −80°C until they were used.

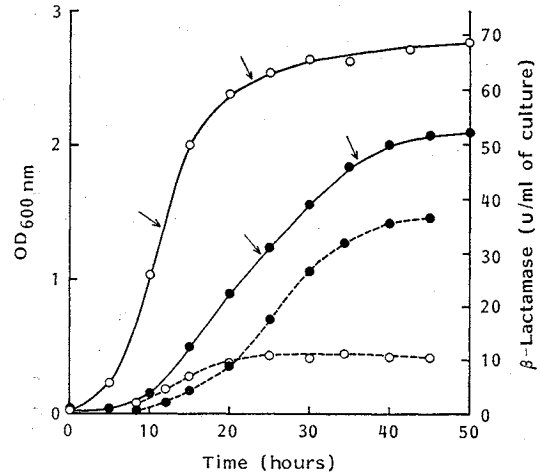
Assay Procedures

A fraction (0.2 or 1 ml) of the culture was added to 40 ml of cold PBS, sonicated and assayed for β-lactamase as described previously[5]. β-Galactosidase activity was assayed as described previously[5]. Incorporation of [^{3}H]leucine into cellular proteins was measured as follows. A 20-μl sample of the culture labeled for 5 minutes was spotted on a Whatman 3MM filter paper (2.5 cm-circle), soaked in cold 10% TCA for 1 minute, heated in 5% TCA at 95°C for 10 minutes, and rinsed with 5% TCA and 95% ethanol at 4°C. The filters were dried, and radioactivity was measured as described previously[5].

Results

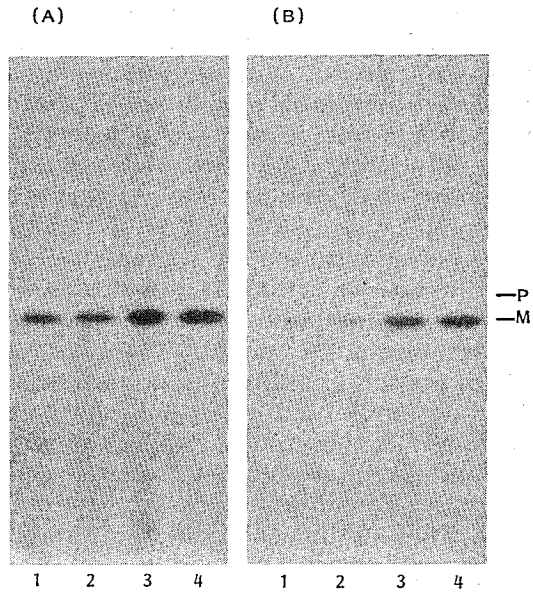
Fig. 1 shows the growth curves and β-lactamase activity of strain J53/RP4 grown in minimal medium with and without lincomycin (200 μg/ml). In middle and late log phases (indicated by arrows), cultures were labeled with [^{3}H]leucine (20 μCi/ml) for 5 minutes and chased with excess amounts of non-radioactive leucine and isoleucine for 30 minutes. Immunoprecipitates prepared with anti-β-lactamase antiserum were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography (Fig. 2). The 5-minute labeled samples only contained small amounts of pre-β-lactamase and most of the β-lactamase of both cultures was in the mature form. More β-lactamase was present in the lincomycin-treated culture than in the control. No decrease of β-lactamase was observed in either culture even after a 30-minute chase. The relative intensities of the labeled bands were estimated

Fig. 1. Effects of lincomycin on growth rate and TEM-2 β-lactamase production.



A 50-μl sample of the J53/RP4 preculture was added to 5 ml of minimal medium with (●) and without (○) lincomycin (200 μg/ml). Cultures were grown at 37°C. OD at 600 nm (——) and β-lactamase (------) were measured at the indicated times.

Fig. 2. Fluorogram of [^{3}H]leucine labeled β-lactamase from the pulse-chase experiment.



Strain J53/RP4 was grown as described in the legend to Fig. 1. At the time indicated by arrows in Fig. 1, cultures were pulse-labeled with [^{3}H]-leucine (20 μCi/ml) for 5 minutes and chased with excess non-radioactive leucine and isoleucine for 30 minutes. A 0.3-ml sample of the labeled culture was mixed with an equal volume of 10% cold TCA and immunoprecipitated with anti-β-lactamase antiserum as described in "Materials and Methods." The resulting ^{3}H-labeled β-lactamase samples were adjusted to 60 μl final volume. A fifteen-μl aliquots were loaded in each lane and electrophoresed on a 12.5%-polyacrylamide gel in the presence of SDS.

(A) Middle log phase cultures: Lanes 1 and 2, 0 and 30 minutes chase, respectively in the control culture; lanes 3 and 4, 0 and 30 minutes chase, respectively in the lincomycin culture. (B) Late log phase cultures: Lanes 1 and 2, 0 and 30 minutes chase, respectively in the control culture; lanes 3 and 4, 0 and 30 minutes chase, respectively in the lincomycin culture. The migration positions of pre-β-lactamase and mature β-lactamase are indicated by P and M, respectively.

by quantitative fluorography as described in Materials and Methods (Table 1). At mid log phase the amount of [^{3}H]leucine incorporated into cellular proteins per unit volume of the lincomycin culture was 81% of that of the control culture, while the relative amount of β-lactamase labeled in the former was two times as much as that in the latter. This difference was in good agreement with a difference calculated from the increase in the enzyme activity. In late log phase enzyme synthesis still continued at a high rate (72% of middle log phase) in the lincomycin culture. On the other hand, it decreased markedly in the control culture. No degradation occurred during a 30-minute chase in any sample. It should be noted that the ratio of pre-β-lactamase to β-lactamase in the lincomycin-treated culture was almost the same as that in the control, indicating that maturation of pre-β-lactamase was not activated by lincomycin. This was verified by measurement of the translocation rate.

The labeling conditions described above were not adequate for measuring the maturation of pre-β- to β-lactamase. When strain J53/RP4 was grown in a shake flask to improve aeration, the growth rates and maximal OD were high compared to those in labeling experiments with a long pulse length. Furthermore, the amount of β-lactamase synthesized per unit of time increased by 3 and

Table 1. Differences in the synthesis rate of β-lactamase between control and lincomycin cultures.

Growth phase	Lincomycin	Chase (minutes)	Relative amounts of [^{3}H]leucine labeled			
			β-Lactamase[a]	Pre-β-lactamase[a]	β- + pre-β-lactamase	Total protein[b]
Middle log	−	0	100	9.3	109.3	100
	−	30	106.9	—	106.9	ND
	+	0	199.5	23.1	222.6	81.0
	+	30	221.9	—	221.9	ND
Late log	−	0	29.5	3.5	33.0	47.4
	−	30	37.8	—	37.8	ND
	+	0	134.3	26.7	161.0	62.3
	+	30	166.7	—	166.7	ND

Cultures were pulse-labeled with [^{3}H]leucine and chased as described in the legend to Fig. 2.

[a] The relative amount of each labeled protein was measured as described under "Materials and Methods." The results are expressed as the percentage of the amounts of β-lactamase pulse-labeled in the control culture at middle log phase. The values represent the average of two determinations. The error range was 5.7%.

[b] The relative amount of labeled total protein is expressed as the percentage of the radioactivity of [^{3}H]-leucine incorporated into TCA precipitates in the control culture at middle log phase. The values represent the average of triplicates. The error range was 5.1%. 140,900±7,200 dpm was present in 20 μl of the control culture at middle log phase.

ND: Not determined.

Table 2. Effects of various protein synthesis inhibitors on the production of chromosomal TEM-1 β-lactamase.

Antibiotics	Concentration (μg/ml)	Growth[a] (%)	β-Lactamase[b] (%)
None	—	100	100
Lincomycin	90	67.1	263
Erythromycin	9	69.6	210
Oleandomycin	100	66.5	188
Acetylspiramycin	70	73.8	108
Chloramphenicol	1	70.5	124
Tetracycline	0.18	70.0	101
Streptomycin	1.25	73.9	95
Kanamycin	1	72.6	103
Puromycin	40	69.3	91
Kasugamycin	24	74.4	89

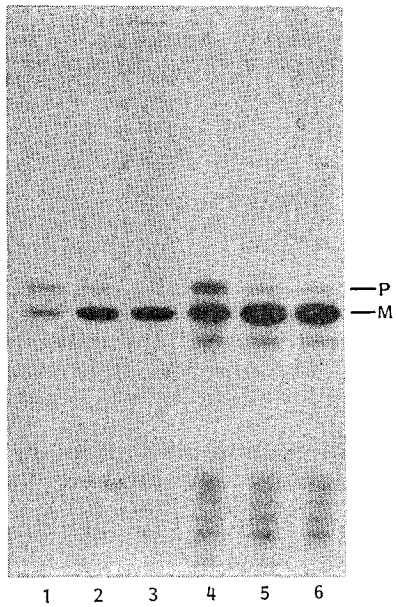
A 0.1-ml sample of the preculture of strain 112-2 was added to 10 ml of L broth and grown at 37°C. An effect of each antibiotic was examined in a separate experiment. Growth was determined by measuring OD at 600 nm in stationary phase. β-Lactamase activity was measured at hourly intervals after 5 hours of growth. The maximal activity of an antibiotic added culture was compared to that of a control culture.

[a] Values are expressed as the percentage of OD of the control culture. The mean OD±SE of the control culture was 4.91±0.057.

[b] Values are expressed as the percentage of the maximal activity in the control culture. The mean activity±SE of the control culture was 0.156±0.0035 u/ml of culture.

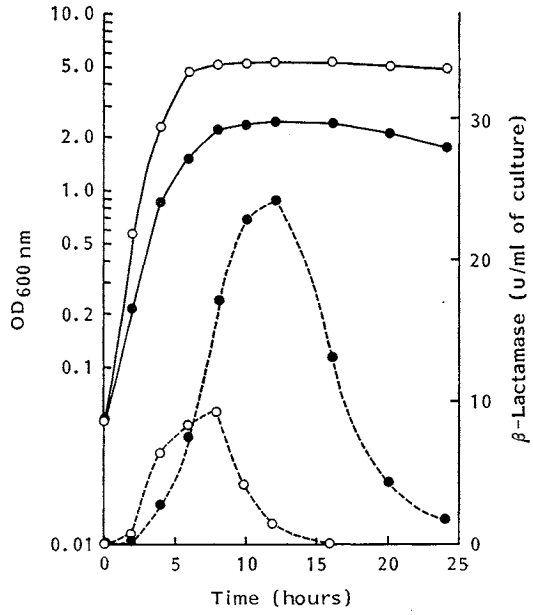
2.5-fold in the control and lincomycin culture, respectively (data not shown). At mid log phase (OD 2.0) cultures were labeled with [^{3}H]leucine (75 μCi/ml) for 1 minute and chased with excess amounts of non-radioactive leucine and isoleucine. Immunoprecipitates were electrophoresed and visualized as described above (Fig. 3). The relative amounts of radioactivity in each band were measured by densitometry. Comparison of the relative amount of total lactamase labeled in the culture chased

Fig. 3. Fluorogram of [^{3}H]leucine labeled pre-β-lactamase from a pulse-chase experiment.



A 1-ml sample of J53/RP4 preculture was added to 100 ml of minimal medium with and without lincomycin (200 μg/ml). At middle log phase (OD 2), cultures were pulse-labeled with [^{3}H]leucine (75 μCi/ml) for 1 minute and chased with excess non-radioactive leucine and isoleucine. Labeled cultures were treated with TCA, immunoprecipitated and electrophoresed in the same manner as described in the legend to Fig. 2. Lanes 1~3, control culture; lanes 4~6, lincomycin culture; lanes 1 and 4, 0 minute chase; lanes 2 and 5, 1 minute chase; lanes 3 and 6, 2 minutes chase. The migration positions of pre-β-lactamase and mature β-lactamase are indicated by P and M, respectively.

Fig. 4. Effects of lincomycin on growth rate and TEM-1 β-lactamase production.



A 1-ml sample of the preculture of strain YA21/pBR322 was added to 100 ml of L broth with (●) and without (○) lincomycin (150 μg/ml). OD at 600 nm (——) and β-lactamase (------) were measured at the indicated times. β-Galactosidase was also assayed. Its maximal activity in the control (10-hour culture) and in the lincomycin culture (16-hour culture) were 56.1 and 13.7 U/ml of culture, respectively.

for 1 minute with that in the culture chased for 2 minutes showed that such a chase completely stopped further incorporation of radioactive leucine into both pre-β- and β-lactamase. Therefore, the difference in the residual amounts of labeled pre-β-lactamase between 1 and 2 minutes chases should represent the rate of posttranslational translocation. The ratios of the residual amounts chased for 2 minutes to those chased for 1 minute in the control and lincomycin cultures were 0.72 and 0.66, respectively. These results rule out the possibility that lincomycin acts by stimulating the rate of posttranslational translocation.

To examine the effect of lincomycin on TEM-1 β-lactamase determined by plasmid pBR322, strain YA21/pBR322 was grown in L broth with and without 150 μg/ml of lincomycin. Fig. 4 shows the growth curves and the changes in β-lactamase activity of the two cultures. β-Lactamase activity reached its maximal level in late log phase with both of the cultures. It was 3 times higher in the lincomycin culture than in the control. Since pBR322 can be amplified by protein synthesis inhibitors[14], an increase in plasmid copy number might have been implicated in the lincomycin effect. Thus we examined two strains of *E. coli* which have a chromosomal *bla* gene. When strain C600 λbb::Tn3 and 112-2 were grown at 30 and 37°C, respectively, β-lactamase activity was stimulated by

lincomycin as in case of strain YA21/pBR322. These results show that TEM-1 β-lactamase is stimulated by lincomycin at sub-inhibitory concentrations wherever the gene is located.

The effects of various protein synthesis inhibitors on the synthesis of β-lactamase were examined. Since these inhibitors might affect the copy number of pBR322, strain 112-2 was used. Stimulation by lincomycin was observed at the concentration where cell growth was inhibited to 65 to 75%. Concentrations of antibiotics inhibiting cell growth to this same extent were used to compare their effects on β-lactamase activity with that of lincomycin. When cells were grown in L broth, β-lactamase decreased gradually during stationary phase. Therefore, β-lactamase activity was measured at hourly intervals after late log phase. The results are shown in Table 2. Two macrolides, oleandomycin and erythromycin, stimulated β-lactamase activity about 2-fold, similarly to lincomycin, while no other antibiotic caused significant stimulation.

Discussions

The present work demonstrates that lincomycin stimulates the activity of both TEM-1 and TEM-2 β-lactamase. The effect of lincomycin was also observed with a chromosomally encoded TEM-1 β-lactamase. From these results it can be concluded that the effect of lincomycin does not involve an increase in copy number of the *bla* gene.

The data presented here indicates that posttranslational translocation of β-lactamase across the membrane occurs in the lincomycin-treated culture at the same rate as in the control. The ratio of pre-β- to total β-lactamase (pre-β- plus β-lactamase) synthesized during 1 minute was approximately one third. However, some pre-β-lactamase should have matured to β-lactamase during 1 minute of labeling. Therefore, approximately one half of the β-lactamase can be assumed to be translocated posttranslationally. This does not seem to be an overestimate, since in a poor medium β-lactamase has been shown to be secreted predominantly by posttranslational translocation[15]. Cotranslational translocation does not seem to be stimulated as most of the periplasmic proteins have been shown to be secreted cotranslationally[16] and they decrease in the lincomycin-treated culture[5].

Macrolide antibiotics are similar to lincomycin in binding to sites on the 50S ribosome subunit[17] and in their mode of action[18,19]. Their effects on β-lactamase production were similar to that of lincomycin. This suggests that lincomycin exerts its "β-lactamase" effect by its interaction with ribosomes. It is possible that ribosomes in the presence of lincomycin or macrolide antibiotics bind *bla* mRNA preferentially or elongate more efficiently. However, there is still the possibility of activation at the transcriptional level even though these antibiotics are not known to effect transcription. To determine which step is activated, we are now examining the synthesis of and stability of *bla* mRNA.

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