GREATLY IMPROVED ACTIVITY OF STAPHYLOCOCCAL RIBOSOMES IN POLYADENYLATE DIRECTED POLYLYSINE SYNTHESIS: AS AN ASSAY SYSTEM FOR INVESTIGATING THEIR SENSITIVITY TO MACROLIDE ANTIBIOTICS

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In polyadenylate directed polylysine synthesis, homologously cell-free extracts containing ribosomes and S-100 (105,000 × g supernatant) from staphylococcal cells have less than one-half (one-tenth, when the extracts were stored at -80 °C within a few weeks) of the activity of the extracts from *Escherichia coli* Q13.

The present study is concerned with further improving the activity of staphylococcal ribosomes. The polylysine-synthesizing ability by staphylococcal ribosomes increased up to about two times as much as that by *E. coli* Q13 ribosomes, when S-100 from *E. coli* Q13 was mixed with staphylococcal ribosomes which had been washed with a high salt HEPES buffer containing 10 mM HEPES, 1 mM EGTA, 16 mM magnesium acetate, 1.0 M ammonium chloride and 0.1 mM dithiothreitol (pH7.6).

Polylysine synthesis by the heterologous extracts has an advantage over polyuridylate-directed polyphenylalanine synthesis in the analysis of ribosome sensitivity for macrolide antibiotics, especially erythromycin.

KEYWORDS — *Staphylococcus aureus*; ribosome; macrolide antibiotic; poly(A); poly(U)

INTRODUCTION

Since Mao¹⁾ has obtained cell-free *Staphylococcus aureus* extracts which actively incorporate amino acids into protein, and found that the activity of the extracts was improved (1) by substituting dithiothreitol for

mercaptoethanol as a sulfhydryl protecting reagent and (2) by giving a much higher magnesium concentration required to obtain the maximal incorporation of amino acids, little attention has been given to any reproducible and practical study on polypeptide synthesis by the cell-free extracts.

Nakajima et al.²⁾ reported that polyuridyate [poly(U)]-directed poly-phenylalanine [poly(Phe)] synthesis by *S. aureus* ribosomes washed in 1.0 M NH_4Cl increased linearly up to 900 μg poly(U)/ml. The stimulated poly(Phe) synthesis by staphylococcal ribosomes reached the same level as that by *E. coli* Q13 ribosomes in the presence of about 800 μg poly(U)/ml. The poly(Phe) synthesis, however, is insensitive or poorly sensitive to erythromycin (EM) or oleandomycin (OL).^{3, 4, 5)}

The present paper therefore describes a method to obtain highly active, staphylococcal ribosomes which can serve as a test for the inhibitory action of macrolide, lincosamide, and streptogramin B (MLS) antibiotics.

MATERIALS AND METHODS

Chemicals — EM was obtained from Japan Upjohn Co., Ltd. Uniformly [^{14}C]-labeled L-lysine (330 mCi/mmol) and L-phenylalanine (522 mCi/mmol) were purchased from Amersham Japan Co., Ltd.; other chemicals from commercial sources.

Bacteria and Preparation of Subcellular Fractions — *S. aureus* ISP447 (formerly U9⁶⁾), ATCC6538P (formerly 209P¹⁾) and *E. coli* Q13 (Hfr, Met⁻, Tyr⁻, RNase⁻) were used.

Subcellular fractions were prepared according to the method of Mao,¹⁾ except that 10 mM potassium-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) was substituted for tris(hydroxymethyl)aminomethane (Tris)-acetate,²⁾ and that 1 mM ethylene glycol bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), i.e. an effective chelating agent for Ca^{2+} ion which can activate staphylococcal nuclease,^{7, 8)} was further added to a HEPES buffer (pH7.6) containing 16 mM magnesium acetate, 50 mM NH_4Cl and 0.1 mM dithiothreitol. This EGTA-supplemented medium was hereafter referred to medium SA. In all experiments, unless otherwise indicated, the washed ribosomes were prepared by washing in the same component medium (medium SB) as medium SA⁹⁾ except that 50 mM NH_4Cl was replaced with 1.0 M NH_4Cl .

Poly(A)-Directed Poly(Lys) Synthesis — The standard reaction mixture consisted of 1.0 mM adenosine triphosphate, 0.05 mM guanosine triphosphate, 5 mM phosphoenol pyruvate, 4 μg of pyruvate kinase, 2.5 A_{260} units of ribosomes, 12.5 μg of poly(A) and 10 μl of S-100 fraction (105,000 $\times$ g super-

natant) in medium SA. The assay was started by adding 0.05 μ Ci of [14 C]-lysine and carried out at 37 °C for 30 min in a total volume 100 μ l. The incorporation of [14 C]-lysine into hot Na tungstate (0.25%)-trichloroacetic acid (5%) precipitable material¹⁰⁾ was assayed by a filter paper disk method.¹¹⁾

RESULTS AND DISCUSSION

A 15-min preincubation (37 °C) of S-30 (a supernatant obtained by conducting a duplicate centrifugation at 30,000 $\times$ g for one hr from disrupted bacterial cells) was skipped, because endogenous incorporation was substantially low in the presence of a single amino acid, L-lysine (not exceeding 10%).

Table I. Poly(A)-Directed Poly(Lys) Synthesis by Homologously Cell-Free Extracts Containing Ribosomes and S-100 from either *S. aureus* or *E. coli*

Bacteria	Incorporation of [14 C]lysine into polylysine ^{a)} (pmol/mg)	
	Storage at	
	-80 °C	-196 °C
<i>S. aureus</i> ATCC6538P	1.0	—
<i>S. aureus</i> ISP447	8.5	192.7
<i>E. coli</i> Q13	84.6	435.5

a) The incorporation of [14 C]lysine into the polylysine fraction in the absence of poly(A) was subtracted from the corresponding total incorporation in the presence of the polynucleotide.

The poly(A) absent incorporations were less than 10% of the respective total incorporations. The unit "mg" represents mg of the weight of ribosomal proteins.

Table I shows that the poly(Lys) synthesizing activity by S-100 and ribosomes from *S. aureus* —when the extracts were stored at -80 °C— possesses only one-tenth or less of that from *E. coli* Q13, and that the activity of the staphylococcal extracts is fairly improved when stored in liquid nitrogen. However, the former was inferior to the latter in the activity of their extracts, even when they had been preserved at -196 °C. Therefore we inquired which of the staphylococcal ribosomes and S-100 caused the reduction in the synthesis of polylysine. When S-100 (equivalent to amounts ranging from 50 to 500 μ g protein) from *S. aureus* was added to a poly(Lys) synthesizing mixture composed of homologous cell-free extracts from *E. coli* Q13, the poly(Lys) synthesis decreased progressively to one half of the synthesis in

the absence of staphylococcal S-100 (data not shown). Although there was also a decrease in the poly(Lys) synthesis by adding staphylococcal ribosomes, the extent of the decrease due to the ribosomes was less than that due to the S-100 (data not shown). From this result, it was assumed that the ribosomes from *S. aureus* might be contaminated with some component of S-100 from the microorganism. The ribosomes washed with medium SB exhibited a great activity in the poly(Lys) synthesis, when the washed ribosomes and S-100 from *E. coli* Q13 were mixed together, in spite of only being washed once (Table II).

Table II. Poly(A)-Directed Poly(Lys) Synthesis by Cell-Free Extracts Containing Indicated S-100 and either *E. coli* Q13 or *S. aureus* ISP447 Ribosomes Washed with Medium SA or SB

S-100	Ribosomes washed with medium		[¹⁴ C]lysine incorporated into polylysine (pmol/mg of ribosomal proteins)		
	SA ^{a)}	SB ^{b)}	Once	Washed ^{c)} Twice	Thrice
Q13	ISP447		229.6	242.4	236.1
Q13		ISP447	718.1	771.9	NT
ISP447	Q13		318.5	344.0	NT
ISP447	ISP447		192.7	204.0	NT
Q13	Q13		435.5	477.2	389.7

a) Medium SA contains 10 mM HEPES, 50 mM NH₄Cl, 16 mM Mg(OAc)₂, 1 mM EGTA, and 0.1 mM dithiothreitol (pH7.6).

b) Medium SB had the same composition as the medium SA except for 1.0 M NH₄Cl.

c) The ribosomes pelleted by a centrifugation were suspended by an indicated medium (once). A part of the ribosome suspension was centrifuged again, so that the pelleted ribosomes were suspended (twice), and the similar procedure was further repeated (thrice). The ribosome suspensions obtained in each step were dialysed overnight against medium SA.

Abbreviation:NT, not tested.

The activity of the heterologously combined system is only dependent on poly(A) under our experimental conditions (Fig. 1).

On the contrary poly(Phe) synthesis by the 1.0 M NH₄Cl-washed ribosomes was increased steadily and smoothly with increasing amount of poly(U), and in the presence of 800 μ g poly(U)/ml, it reached the same level as that by similarly prepared ribosomes from *E. coli* Q13 at 40 μ g poly(U)/ml – at which the latter ribosomes gave the most synthesizing activity.²⁾ Moreover, staphylococcal ribosomes washed with medium SB did not always require the *E. coli* S-100 instead of the *S. aureus* S-100.²⁾

Fig. 1 Effect of Poly(A) Concentration on Poly(Lys) Synthesis by Homologous and Heterologous Systems Composed of Washed Ribosomes and S-100 of either *S. aureus* ISP447 or *E. coli* Q13

The radioactivity in the absence of poly(A) was less than about 10% of that in the presence of the polynucleotide and was subtracted from the latter radioactivity (pmol/mg of ribosomal protein).

Symbols: (○), cell-free extracts containing ISP447 ribosomes and S-100; (●), ISP447 ribosomes and Q13 S-100; (△), Q13 ribosomes and ISP447 S-100; (▲), Q13 ribosomes and Q13 S-100.

Consequently the behavior of the *S. aureus* ribosomes in poly(Lys) synthesis appears to be greatly different from that in poly(Phe) synthesis.²⁾ The cause of such a dissimilarity between those polypeptide syntheses is unknown at present.

The washed ribosome from *S. aureus*, when the poly(A)-directed poly(Lys) synthesis is attained by mixing its ribosome and S-100 from *E. coli* Q13, was very sensitive to not only EM, a 14-membered ring macrolide which can hardly inhibit poly(U)-directed poly(Phe) synthesis,^{3, 4, 5)} but also other macrolide (Fig. 2), lincosamide, and streptogramin B antibiotics (data not shown).

Fig. 2 Effect of Macrolide Antibiotics on Poly(Phe) and Poly(Lys) Synthesis by Cell-Free Extracts Containing *S. aureus*'s Washed Ribosomes and *E. coli*'s S-100

The extent of incorporation activity was determined by calculating the ratio of polymerized amino acid in the presence to the absence of an antibiotic.

Symbol: poly(Phe) synthesis against erythromycin (○), and leucomycin (●); poly(Lys) synthesis against erythromycin (△), and leucomycin (▲).

In conclusion, this poly(Lys) synthesis appears to be a useful system for analyzing a practical, reproducible, and distinct response of staphylococcal ribosomes to the ribosomal inhibitor such as MLS, since the extent of a 50% inhibitory dose of the drug in the poly(Lys) synthesizing system is comparable with that of the corresponding drug in bacterial growth (data not

shown).

The factor responsible for inhibiting the poly(Lys) synthesis, the factor which is present in staphylococcal S-100, remains to be investigated.

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