Vol. 81, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1978 Pages 50-57

THE CORRELATION BETWEEN ANTIBIOTIC SYNTHESIS, TRANSCRIPTION AND SPORULATION IN BACILLUS POLYMYXA*

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Received January 10,1978

SUMMARY

A class of spo^+ revertants of B. polymyxa isolated from Rif^r, Ab^- and Spo^- mutants, pleiotropically acquired the ability to synthesise antibiotic and the sensitivity to rifampicin. An interesting observation was the appearance of RNA polymerase activity in the membrane fractions of cells committed for sporulation. A correlation of antibiotic synthesis and transcription in sporulation was speculated to involve membrane-DNA associated proteins, one of which was the multi-enzyme complex involved in the biosynthesis of polymyxin.

Sporulation, a cellular differentiation phenomenon operative in prokaryotes, is regulated by temporal gene expressions involving altered transcriptional systems (1,2,3,4 & 5). Events that precede the onset of sporogenesis have been suspected to play a determinative role in this process.

In view of the reported correlation between antibiotic synthesis and sporulation (6), a study of the multi-enzyme complex involved in the synthesis of polymyxin, a peptide antibiotic elaborated by B. polymyxa was undertaken. Our earlier studies have indicated that this protein complex was located in the membrane fractions (7) and which also contained DNA (8). The nature of changes in these fractions was moni-

*Studies reported here involving the RNA polymerase activity were carried out in the laboratory of Dr J.Szulmajster, Laboratoire d'Enzymologie, CNRS, Gif-sur-Yvette.

Abbreviations: Ab^- - Antibiotic synthesis; Rif^r - resistance to rifampicin; DAB - L-2,4-Diamino butyric acid; NTG - N'methyl N'nitro N'nitroso guanidine

0006-291X/78/0811-0050$01.00/0

tored during growth and sporulation. In addition, we have also assayed the RNA polymerase activities of the cell fractions prepared from the vegetative and sporulating cells. The effect of polymyxin on the *in vitro* translational process was investigated.

MATERIALS & METHODS

Microorganisms: The organism used in this study was *Bacillus polymyxa* 2459, obtained through the courtesy of Pfizer & Co., USA. Stock cultures were maintained as heat shocked spores. *E.coli* K 12 3000, was the sensitive organism used for the turbidimetric method of assay of polymyxin (9).

Media: For experiments involving antibiotic synthesis, the synthetic medium of Katznelson and Lockhead (10) was used. For other studies, the rich medium of Schaeffer was used (11).

Preparation of membrane fractions: The cells harvested at different stages of growth were suspended in 0.1M Tris-HCl buffer pH 7.5 containing 0.1M NaCl 10 mM EDTA and 20% sucrose. Lysozyme (1 mg/ml) was added and the cells were kept for 60' at 0°C. The spheroplasts were spun, resuspended in TKM buffer (Tris-HCl, 0.1M, pH 7.5, containing 0.01M KCl, 0.01M $MgCl_2$) and ruptured by passage through French pressure cell. The extracts were centrifuged at 39,000 g in a Sorvall RC 2-B centrifuge for 30 minutes. The particulate fraction was resuspended in a small volume and homogenized in the same buffer without sucrose. This was layered on a column of 60% sucrose in TKM buffer and centrifuged at 20,000 rpm for 20' in the ultra centrifuge (Janetzki VAC 601) in the swing out rotor. The particulate fraction floated on 60% sucrose and the whole cells and debris settled at the bottom. The floating membrane fraction was removed with a Pasteur pipette, diluted with TKM buffer and centrifuged at 40,000 g for 30'. The pellet was used as a source of membrane fraction.

Enzyme assays: The DAB activating enzyme was assayed as reported (7). Protease and alkaline phosphatase were measured as outlined (12). RNA polymerase activities were determined as outlined (13).

Isolation of Rif^r mutants: Log-phase cultures of *B. polymyxa* mutagenized with NTG (100 µg/ml; 30' at 37°C). After overnight growth in nutrient broth, the cultures were plated on to nutrient agar plates containing 10 µg/ml rifampicin. Resistant colonies which grew up after 3 days were picked and screened for sporulation specific characters. Spontaneous Rif^r mutants were isolated by the procedure of Schaeffer *et al* (14) which insures the enrichment of stage 0 mutants.

Isolation of Revertants: Revertants of the Rif^r mutants were

obtained by growing them on nutrient agar plates for a period of 5-6 days. The heat resistant revertants were purified and checked for antibitic production and sensitivity to rifampicin.

RESULTS AND DISCUSSION

Nature of Rif^r mutants: The characteristics of the Rif^r mutants and their revertants are outlined in Table 1. All the Rif^r, stage 0 mutants isolated by us were deficient in antibiotic production, substantiating our earlier observation that polymyxin production is linked to sporulation (6). That revertants from these mutants were feasible argued for a single site mutation, probably regulating multiple events. The revertants pleiotropically acquired all three wild-type traits, viz, antibiotic production, sensitivity to rifampicin and normal sporulation. All these stage 0 mutants had high protease levels. Several Rif^r, Spo^- mutants blocked at later stages also reverted to Rif^s and Spo^+ characteristics. This suggested that regulation at transcriptional level may well operate at different stages of sporulation as suggested earlier (1 & 5).

Studies on Antibiotic Production: Both the production of antibiotic and levels of the enzyme involved in the synthesis were monitored to ascertain their role in the process of sporulation in *B. polymyxa*. Production of polymyxin was maximal at the end of growth phase (insert in Fig.1). It was shown earlier that the enzyme complex having DAB activating enzyme activity as the chief component, plays a critical role in polymyxin synthesis and was predominantly localised in the membrane fraction (6). A closer look at the distribution of soluble and membrane bound DAB activating enzyme activity during growth revealed that the membrane-bound

Table 1 Characteristics of Rif^r Asporogenous mutants and their revertants

Mutants	Revertants from	Ab	Pro	APase	Ca^{++} Uptake	Heat Resistance	Rif
RS 01, 02, 04, 05, 06, 07	-	-	+	-	-	-	Resistant
	RS 01, 04, 05, 06	+	+	+	+	+	Sensitive
NRS 12	-	+	+	±	-	-	Resistant
	NRS 12	+	+	+	+	+	Sensitive
NRS 6, 18, 20 & 21	-	+	+	+	±	-	Resistant
	NRS 6	+	+	+	+	+	Sensitive
NRS 2,3,5,14	-	+	+	+	+	-	Resistant
	NRS 2	+	+	+	+	+	Sensitive

RS mutants - spontaneous mutants, NRS mutants - obtained with NTG mutagenesis
Ab - Antibiotic synthesis, Pro - Extracellular Protease, APase - Alkaline Phosphatase
Rif - Sensitivity to Rifampicin

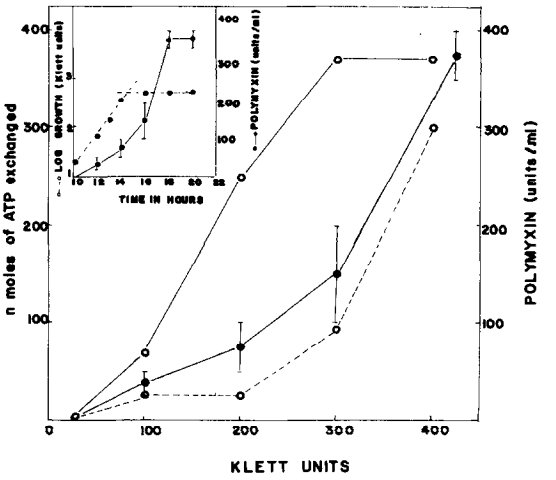

Figure 1: The correlation between antibiotic production and the distribution of soluble and membrane bound DAB - activating enzyme activity in *B. polymyxa*

——o——o—— membrane bound DAB activation

----o----o---- soluble DAB activation

—|—|— extracellular polymyxin (units/ml) as assayed by turbidimetric method (8)

Table 2 RNA polymerase activity of B. polymyxa at different stages of growth

		Activity*		
		ϕe DNA	ϕeDNA + Rif	ϕe DNA + Polymyxin
Vegetative Cells	Soluble	100	4	85
	Membrane	6	ND	ND
T_2 Stage Cells	Soluble	100	2	95
	Membrane	20	8	20

*Expressed as % of the soluble activities of the preparation concentrated by precipitation with 60% saturated $(NH_4)_2SO_4$. The template-DNA (5 µg/assay) used in these assays has DNA prepared from the phage ϕe lysates (13). Rifampicin 10 µg/assay and polymyxin 30 ug/assay were added at the start

ND - not detectable

activity was detectable earlier than the soluble activity (Fig.1). A sharp increase in the soluble activity was noticeable coinciding with a similar rise in the extra-cellular antibiotic production. It was also possible to effect release of the membrane-bound DAB activating enzyme activity by externally added polymyxin using isolated membrane preparations. This suggested that under *in vivo* conditions polymyxin could play a role in the de-localization of the membrane-bound activity.

We have also observed that these membrane fractions contained DNA (8). Using cells grown in the presence of ^{3}H-thymidine it was found that at least 10% of the total DNA was located in the membrane fractions, of which 2% was DNase resistant.

Studies on RNA polymerase activity: In view of the genetic

Table 3 Properties of membrane bound RNA polymerase activity in B. polymyxa at T_2 stage of sporulation

	Activity*	
	No DNA	DNA
No Pre-incubation:		
Membranes	2,515	2,910
+ Rif**	760 (30%)	800 (28%)
Pre-incubation:		
Membranes	1,423	1,800
+ Rif**	233 (16%)	210 (12%)

*Expressed as acid precipitable counts/mg protein. For preincubation membranes were kept at 27°C for 10' prior to the addition of ^{3}H ATP.

**10 µg/assay

evidence linking transcription, antibiotic production and sporulation, it was of interest to investigate the effect of polymyxin on the transcriptional activities of the cells at different stages of growth. Such a role for the peptide antibiotic has been strongly advocated earlier (15). The soluble RNA polymerase activity of B. polymyxa at vegetative and sporulating stages was sensitive to rifampicin, but was unaffected by the addition of polymyxin (Table 2). The appearance and the observed properties of the membrane bound polymerase activity was an unexpected and revealing phenomenon. The transcriptional activity in the membrane fraction appea-

red only in the sporulating cells. It was somewhat resistant to rifampicin but again remained unaffected by polymyxin (Table 2). The lack of the necessity of externally added template-DNA and the pre-incubation experiments outlined in Table 3 clearly indicated that RNA polymerase found in the membrane fraction was possibly complexed with the DNA at the sporulation stages and hence the observed resistance to rifampicin. This argues for an additional transcriptional programme involving membrane-bound DNA in sporogenesis.

The role of DNA-associated proteins in the regulation of gene expression is well-known. Whether the de-localization of membrane associated antibiotic synthesizing complex is a prerequisite for spore-specific transcription is currently under investigation.

ACKNOWLEDGEMENT

This research programme was supported by the Department of Science & Technology

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