SHORT COMMUNICATION

Characterization of *Bacillus subtilis* Mutants Temperature-sensitive in the Synthesis of Ribonucleic Acid

By G. MAZZA¹* AND P. PLEVANI²

¹ Istituto di Genetica, Università di Pavia, Via S. Epifanio 14, 27100 Pavia, Italy

² Ente Universitario Lombardia Orientale, Corsi di Medicina, 25100 Brescia, Italy

(Received 2 May 1980)

Two mutants of *Bacillus subtilis* temperature-sensitive in RNA synthesis were isolated. One mutation (*rna-20*) was demonstrated to be an allele of a previously identified gene (Riva *et al.*, 1976). The other mutation (*rna-16*) identified a different gene and was mapped near *aroI*. The *rna-16* mutation at the permissive temperature affected the spore outgrowth process. Purified RNA polymerase from *rna-16* did not show any temperature sensitivity or structural defect.

INTRODUCTION

Different programmes of gene expression in bacteria and in phage-infected cells are based on the interaction of the RNA polymerase with regulatory factors or through the modification of its own structure. This regulation is particularly evident in *Bacillus subtilis* cells during sporulation (Losick *et al.*, 1970; Brevet, 1976; Losick & Pero, 1976). RNA polymerase present in vegetative cells undergoes, during sporulation, changes in subunit composition and template specificity. Analysis of messenger RNA molecules in sporulating cells shows striking differences with respect to those found in vegetative cells (Di Cioccio & Strauss, 1973; Sumida-Yasumoto & Doi, 1974). At present, little is known of the factors involved in the control of RNA synthesis. Identification of the genes coding for the β and β' structural subunits of RNA polymerase has been possible using specific antibiotics (Harford & Sueoka, 1970; Haworth & Brown, 1973; Sonenshein *et al.*, 1974, 1977; Halling *et al.*, 1978).

Isolation of mutants temperature-sensitive in RNA synthesis may offer an additional tool for identifying genes involved in the regulation of gene expression and in understanding regulatory mechanisms. We have previously described a temperature-sensitive mutant of *B. subtilis* affected in the regulation of RNA synthesis (Riva *et al.*, 1976). This paper describes the characterization of a new mutant temperature-sensitive in RNA synthesis.

METHODS

Bacterial strains. *Bacillus subtilis* strains used were: PB202 *hisB2 trpC2 tyrA1 aroB2* (SB202, J. Lederberg); PB1652 *lys-3 metB10 trpC2* (BR151, F. Young); PB1654 *trpC2 hisA1 cysB3* (SB3, E. Nester); PB1715 *metB5 dal aro1906 sacA321* (QB820, J. A. Lepesant).

Isolation of mutants. Temperature-sensitive mutants were obtained from strain PB202 following treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Adelberg *et al.*, 1965). After washing, the mutagen-treated culture was dispensed in tubes with NB and incubated with shaking at 28°C overnight. Portions of these cultures were stored under liquid nitrogen. Temperature-sensitive mutants were isolated from these cultures

by the replica-plating method on agar plates, testing for growth at permissive (35°C) and restrictive temperatures (47°C). To avoid sibling selection each mutant was obtained from independently mutagenized cultures. The *rna-16* and *rna-20* mutations were transferred by DNA congression into strain PB1652 in order to avoid the study of mutants with multiple defects, as are often produced after nitrosoguanidine treatment (Guerola *et al.*, 1971). Selection was made for Lys⁺ transformants which were then scored for the presence of the temperature-sensitive phenotype. In these constructed strains the temperature sensitivity in RNA synthesis was confirmed.

Media. The minimal medium of Davis & Mingioli (1950) supplemented by the appropriate amino acid requirements (25 µg ml⁻¹) was used for selection of transformants and transductants. Penassay broth (antibiotic medium no. 3, Difco) containing 0.5% (w/v) glucose (PY) was used for RNA synthesis determinations. Nutrient broth (Difco) containing 0.5% (w/v) glucose (NB) was used for spore germination.

RNA synthesis. An overnight culture in PY grown at 30°C was diluted in the same medium to an A_{560} of 0.1 and incubated at 35°C. The rate of RNA synthesis was determined by 2 min pulse-labelling with [³H]-uridine as described by Albertini & Galizzi (1975). At an A_{560} of 0.2 to 0.4, part of the culture was shifted to 47°C and the rate of RNA synthesis was measured at both temperatures.

RNA polymerase purification. RNA polymerase holoenzyme from strain PB1652 and the *rna-16* mutant was purified from vegetative cells according to the method of Plevani *et al.* (1977). The two enzymes had specific activities of 584 and 609 U mg⁻¹, respectively. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was performed as described by Plevani *et al.* (1977).

Genetic mapping. The linkage relationship of *rna* mutations with other markers of known location on the genetic map was obtained by DNA transformation and PBS1 phage-mediated transduction, according to the methods described by Hoch *et al.* (1967). Distances between markers (*D*) are expressed as percentage of recombination, according to the convention: % recombination = (1 - cotransfer) × 100. The distance between *rna-20* and *rna-53* mutations was evaluated by the recombination index method of Lacks & Hotchkiss (1960).

RESULTS

Isolation of mutants temperature-sensitive in RNA synthesis

A total of 139 temperature-sensitive mutants unable to grow at the restrictive temperature (47°C) on nutrient agar plates were obtained from mutagen-treated cultures of strain PB202. The ability of the vegetative cells, in the exponential phase of growth, to synthesize RNA after a shift to the restrictive temperature was followed by [³H]uridine pulse-label incorporation. Seven mutants showed a sharp decrease in the rate of uridine incorporation at 47°C. Five of these mutants were not directly affected in RNA synthesis since at the restrictive temperature there was a rapid decrease in the A_{560} of the culture and in the induction of phage PBSX. The two remaining mutants, *rna-16* and *rna-20*, were specifically temperature-sensitive in RNA synthesis; DNA synthesis and protein synthesis were not affected until later. Results for the *rna-16* mutant are shown in Fig. 1(a); the *rna-20* mutant behaved in a similar manner.

Genetic mapping

The *rna-16* and *rna-20* mutations were mapped by PBS1 transduction using the temperature-sensitive mutants as donors in crosses with a set of recipient strains. Prototrophic recombinants for a given nutritional marker were selected and scored for ts phenotype. The *rna-20* mutation was found to be linked to *hisA* and *cysB* in the order *cysB hisA rna-20* (Table 1). In the same chromosomal region we have previously located another temperature-sensitive mutant (Riva *et al.*, 1976) affecting RNA synthesis (*rna-53*). The mutations *rna-20* and *rna-53* were probably alleles of the same gene since the recombination index between the two markers was 0.02.

The *rna-16* mutation was closely linked to *aroI* (Table 1). Several other temperature-sensitive mutants, *tscB*, *F*, *G*, *H* (Galizzi *et al.*, 1976b), have been located in the same region. These mutants probably belong to different, closely linked genes since RNA synthesis in these strains was not affected at the restrictive temperature.

Fig. 1 (a) Effect of shift to 47°C on [3 H]uridine incorporation and culture turbidity in the *rna-16* mutant. RNA synthesis was followed by 2 min pulse-labelling. At the time indicated by the arrow (A_{560} between 0.2 and 0.4) a portion of the culture at 35°C was shifted to 47°C and RNA synthesis was determined at both temperatures. A_{560} of the culture grown at 35°C (●) and 47°C (○); [3 H]-uridine incorporation at 35°C (■) and 47°C (□).

(b) Germination and outgrowth in NB of the spores of strain PB1652 (parental) and the *rna-16* mutant at 35 and 47°C. Spores in distilled water were heat-activated at 75°C for 15 min before inoculation. A_{560} of PB1652 at 35°C (○) and 47°C (□); A_{560} of *rna-16* mutant at 35°C (●) and 47°C (■).

Table 1. Two- and three-factor crosses for mapping *rna-20* and *rna-16* mutations

Donor strain	Recipient strain	Recombinants			Order implied by results and map distance (D)
		Selected phenotype	Classes	No. scored	
PBS1 transduction					
PB1652(<i>rna-20</i>) <i>metB10 trpC2 rna-20</i>	PB1654 <i>trpC2 hisA1 cysB3</i>	His ⁺	His ⁺ Cys ⁺ ts-Rna ⁺	20	<i>cysB3-hisA1-rna-20</i> <i>(hisA-cysB3) D=75</i> <i>(hisA-rna-20) D=63</i> <i>(cysB3-hisA) D=71</i> <i>(cysB3-rna-20) D=91</i>
			His ⁺ Cys ⁺ ts-Rna ⁻	6	
			His ⁺ Cys ⁻ ts-Rna ⁺	46	
			His ⁺ Cys ⁻ ts-Rna ⁻	33	
		Cys ⁺	Cys ⁺ His ⁺ ts-Rna ⁺	19	
			Cys ⁺ His ⁺ ts-Rna ⁻	8	
			Cys ⁺ His ⁻ ts-Rna ⁺	66	
			Cys ⁺ His ⁻ ts-Rna ⁻	0	
PB1652(<i>rna-16</i>) <i>metB10 trpC2 rna-16</i>	PB1715 <i>metB5 dal aroI sacA321</i>	Aro ⁺	Aro ⁺ ts-Rna ⁺	2	<i>(aroI-rna-16) D=2</i>
			Aro ⁺ ts-Rna ⁻	120	
DNA transformation					
PB1652(<i>rna-16</i>) <i>metB10 trpC2 rna-16</i>	PB1715 <i>metB5 dal aroI sacA321</i>	Aro ⁺	Aro ⁺ ts-Rna ⁺	24	<i>(aroI-rna-16) D=16</i>
			Aro ⁺ ts-Rna ⁻	124	

Spore germination in rna-16 mutant

The *rna-16* mutation did not affect spore formation at the permissive temperature. However, spores of this mutant showed an altered pattern of germination. Outgrowth at the permissive temperature was delayed with respect to the parental strain (Fig. 1*b*) or to a revertant derivative (*rna-16* ts⁺) (results not shown). This result was not due to a general defect in the growth rate, since in the exponential phase of growth the mutant had the same mean germination time as the parental strain. The possibility of an indirect effect due to the presence of a fraction of non-germinating spores was ruled out.

In vitro assay with purified RNA polymerase

RNA polymerase holoenzyme was purified from the wild-type and the *rna-16* mutant. The structure of the two enzymes was analysed by SDS gel electrophoresis; in both cases the enzymes separated into four major bands with molecular weights of 155 000, 145 000, 53 000 and 44 000, suggesting an identical structure (β , β' , 2α , σ). The RNA polymerase holoenzymes purified from the parental strain and *rna-16* mutant showed the same thermal stability *in vitro* as judged by the kinetics of inactivation at 47 °C (performed as described by Galizzi *et al.*, 1976*a*).

DISCUSSION

Among 139 temperature-sensitive mutants of *B. subtilis* produced by independent mutagenic treatments, we have identified two mutants in which the rate of RNA synthesis decreases sharply after a shift to 47 °C. One mutation (*rna-20*) was demonstrated by genetic analysis to be an allele of a previously identified gene affecting RNA synthesis (Riva *et al.*, 1976). The other mutation (*rna-16*) identifies a different gene involved in the control of RNA synthesis which mapped near the *aroI* marker. At the permissive temperature of 35 °C the *rna-16* mutation affects the spore outgrowth process but not vegetative growth, suggesting that the altered gene product could be involved in the regulation of spore outgrowth.

Purified RNA polymerase from the mutant strain (*rna-16*) appears to have neither a different structure nor greater heat lability compared with the enzyme purified from the parental strain. In contrast to DNA synthesis, where several different genes have been described and located on the genetic map (*dna* mutants), the identification of the proteins involved in RNA synthesis and in the regulation of transcription is at present unsatisfactory. The *in vitro* RNA polymerase assay may be unable to detect structural alterations of the enzyme or RNA polymerase factors may be removed from the core structure during the purification procedures (Fukuda *et al.*, 1975). The use of a well-defined DNA template to test the transcribing ability of RNA polymerase (Jaehning *et al.*, 1979) may offer additional information on the purified enzyme from RNA mutants. The mutants described here could be useful in the study of the functions involved in the regulation of RNA synthesis.

REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788–795.
- ALBERTINI, A. M. & GALIZZI, A. (1975). Mutant of *Bacillus subtilis* with a temperature-sensitive lesion in ribonucleic acid synthesis during spore germination. *Journal of Bacteriology* **124**, 14–25.
- BREVET, J. (1976). Evolution of the transcription complex during sporulation of *Bacillus subtilis*. *Journal of Bacteriology* **125**, 74–83.
- DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of Bacteriology* **60**, 17–28.
- DI CIOCCIO, R. A. & STRAUSS, N. (1973). Pattern of transcription in *Bacillus subtilis* during sporulation. *Journal of Molecular Biology* **77**, 325–336.
- FUKUDA, R., KEILMAN, G., MCVERY, E. & DOI, R. H. (1975). RNA polymerase pattern of sporulating *Bacillus subtilis*. In *Spores VI*, pp. 213–220. Edited by P. Gerhardt, R. N. Costilow & H. L. Sadow. Washington D.C.: American Society for Microbiology.

- GALIZZI, A., ALBERTINI, A. M., PLEVANI, P. & CASSANI, G. (1976a). Synthesis of RNA and protein in a mutant of *Bacillus subtilis* temperature sensitive during spore germination. *Molecular and General Genetics* **148**, 159–164.
- GALIZZI, A., SICCARDI, A. G., MAZZA, G., CANOSI, U. & POLSINELLI, M. (1976b). A recombination test to classify mutants of *Bacillus subtilis* of identical phenotype. *Genetical Research* **27**, 47–58.
- GUEROLA, N., INGRAHAM, J. L., CERDO-OLMEDO, E. (1971). Induction of closely linked multiple mutations by nitrosoguanidine. *Nature New Biology* **230**, 122–125.
- HALLING, S. M., BURTIS, K. C. & DOI, R. H. (1978). β' subunit of bacterial RNA polymerase is responsible for streptolydigin resistance in *Bacillus subtilis*. *Nature, London* **272**, 837–839.
- HARFORD, N. & SUEOKA, N. (1970). Chromosomal location of antibiotic resistance markers in *Bacillus subtilis*. *Journal of Molecular Biology* **51**, 267–286.
- HAWORTH, S. R. & BROWN, L. R. (1973). Genetic analysis of ribonucleic acid polymerase mutants of *Bacillus subtilis*. *Journal of Bacteriology* **114**, 103–113.
- HOCH, J. A., BARAT, M. & ANAGNOSTOPOULOS, C. (1967). Transformation and transduction in recombination defective mutants of *Bacillus subtilis*. *Journal of Bacteriology* **93**, 1925–1937.
- JAEHNING, J. A., WIGGS, J. L. & CHAMBERLIN, M. J. (1979). Altered promoter selection by a novel form of *Bacillus subtilis* RNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 5470–5474.
- LACKS, S. & HOTCHKISS, R. D. (1960). A study of the genetic material determining an enzyme activity in *Pneumococcus*. *Biochimica et biophysica acta* **39**, 508–517.
- LOSICK, R. & PERO, J. (1976). *Bacillus subtilis* RNA polymerase and its modification in sporulating and phage-infected bacteria. *Advances in Enzymology* **44**, 165–185.
- LOSICK, R., SHORENSTEIN, R. G. & SONENSHEIN, A. L. (1970). Structural alteration of RNA polymerase during sporulation. *Nature, London* **227**, 910–913.
- PLEVANI, P., ALBERTINI, A. M., GALIZZI, A., ADAMOLI, A., MASTROMEI, G., RIVA, S. & CASSANI, G. (1977). RNA polymerase from *Bacillus subtilis* isolation of core and holo enzyme by DNA-cellulose chromatography. *Nucleic Acids Research* **4**, 603–623.
- RIVA, S., VILLANI, G., MASTROMEI, G. & MAZZA, G. (1976). *Bacillus subtilis* mutant temperature sensitive in the synthesis of ribonucleic acid. *Journal of Bacteriology* **127**, 679–690.
- SONENSHEIN, A. L., CAMI, B., BREVET, J. & COTE, R. (1974). Isolation and characterization of rifampicin resistant and streptolydigin resistant mutants of *Bacillus subtilis* with altered sporulation properties. *Journal of Bacteriology* **120**, 253–265.
- SONENSHEIN, A. L., ALEXANDER, H. B., ROTHSTEIN, D. M. & FISHER, S. H. (1977). Lipiarmycin-resistant ribonucleic acid polymerase mutants of *Bacillus subtilis*. *Journal of Bacteriology* **132**, 73–79.
- SUMIDA-YASUMOTO, C. & DOI, R. H. (1974). Transcription from the complementary deoxyribonucleic acid strands of *Bacillus subtilis* during various stages of sporulation. *Journal of Bacteriology* **117**, 775–778.