Deoxyribonucleoside-requiring Mutants of *Bacillus subtilis*

By B. K. RIMA* AND I. TAKAHASHI

Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

(Received 14 February 1978)

A number of deoxyribonucleoside-requiring mutants (*dns*) of *Bacillus subtilis* were isolated and their growth characteristics and ribonucleotide reductase activities were compared with those of the wild type and of a *dna* mutant (*tsA13*). Both *tsA13* and *dns* mutants required the presence of a mixture of deoxyribonucleosides for growth at 45 °C but not at 25 °C. All the mutant strains tested contained ribonucleotide reductase activity which showed heat sensitivity similar to that of the enzyme from a wild-type strain. The reductase in *B. subtilis* seemed to reduce ribonucleoside triphosphates in a similar manner to the enzyme in *Lactobacillus leichmannii*.

INTRODUCTION

In bacterial and mammalian cells, ribonucleotides are converted to corresponding deoxyribonucleotides by ribonucleotide reductase (O'Donovan & Neuhaard, 1970). In *Escherichia coli*, ribonucleoside diphosphates are the substrate for this enzyme (Larsson & Reichard, 1966), and in *Lactobacillus leichmannii*, ribonucleoside triphosphates are reduced to the corresponding deoxyribonucleotides (Goulian & Beck, 1966).

Fuchs & Neuhaard (1973) described a mutant of *E. coli* which is affected in ribonucleoside diphosphate reductase. Other mutants affected in this enzyme have been isolated as temperature-sensitive *dna* mutants of *E. coli* (Fuchs *et al.*, 1972) and of *B. subtilis* (Bazill & Karamata, 1972). Since *E. coli* lacks deoxyribonucleoside kinase (Karlström, 1970), the addition of deoxyribonucleosides does not support growth of mutants affected in ribonucleotide reductase. In contrast, *B. subtilis* contains deoxyribonucleoside kinase (Bazill & Karamata, 1972; Rima & Takahashi, 1973, 1977) and therefore mutants of *B. subtilis* affected in ribonucleotide reductase could possibly be obtained by isolating deoxyribonucleoside-requiring clones.

In the present paper, the isolation and growth characteristics of a number of such mutants is described and ribonucleotide reduction in the mutants is compared with that in the wild type and in one of the temperature-sensitive *dna* mutants of Bazill & Karamata (1972).

METHODS

Symbols and abbreviations. Genetic symbols used are: *dns*, mutants requiring deoxyribonucleosides; *pyr*, blocked in the *de novo* synthesis of UMP; *tsA*, temperature-sensitive DNA synthesis; *cdd*, deoxycytidine-cytidine deaminase; *dck*, deoxycytidine kinase; *ddd*, deoxycytidylate deaminase (Rima & Takahashi, 1978); *thyA*, thymidylate synthetase; *thyB*, an unknown enzyme involved in thymidylate synthesis (Wilson, Farmer & Rothman, 1966; Rima & Takahashi, 1978). Other abbreviations are: Ade, adenine; Gua, guanine; Thy, thymine; Cyt, cytosine; Ura, uracil; Cyd, cytidine; dCyd, deoxycytidine; dUrd, deoxyuridine; dGuo, deoxyguanosine; dAdo, deoxyadenosine; dThd, thymidine; NdR, dAdo + dGuo + dCyd + dThd.

Bacterial strains. The bacterial strains, their growth characteristics and the media have been described before (Rima & Takahashi, 1977, 1978).

* Present address: Department of Biochemistry, The Queen's University of Belfast, Belfast BT9 7BL, Northern Ireland.

Assay of ribonucleotide reductase. Crude extracts for the assay of ribonucleotide reductase were prepared as follows. Cells of *B. subtilis* were grown to late exponential phase in Difco Penassay Broth and were harvested by centrifugation. The cells were resuspended in 50 mM-potassium phosphate buffer (pH 7.4) containing 0.2 mM- β -mercaptoethanol. The cell suspension was treated twice with a French pressure cell at 103 MPa. Unbroken cells and debris were removed by centrifuging at 30000 *g* for 30 min and the supernatant fluid was used as crude extract. The concentration of protein in the extracts was determined by the method of Lowry *et al.* (1951).

The reaction mixture for the assay of UTP reductase contained (in 1.0 ml): 25 mM-potassium phosphate buffer (pH 7.4), 1 mM-dithiothreitol, 1.2 mM- CaCl_2 , 0.285 μM -[5- ^3H]UTP (5 μCi) and crude extract corresponding to 3.5 mg protein. The reaction mixture was incubated at 37 °C and the reaction was terminated after 10 min by heating at 100 °C for 3 min. The resulting precipitates were removed by centrifugation and 30 μl of the supernatant fluid was spotted on Whatman no. 1 paper together with unlabelled reference nucleotides and nucleosides. Products of the reaction were separated by paper chromatography using a mixture of 95 % ethanol/saturated sodium tetraborate/5 M-ammonium acetate/0.5 M-EDTA (220:80:20:0.5, by vol.) as solvent. The enzyme activity was calculated from the amount of product formed after 10 min at 37 °C.

The reaction mixture for CTP reductase contained (in 1.0 ml): 25 mM-potassium phosphate buffer (pH 7.4), 15 mM- MgCl_2 , 30 μmol creatine phosphate, 0.05 mg creatine phosphokinase, 0.255 μM -[5- ^3H]CTP (5 μCi) and crude extract corresponding to 0.5 to 1.0 mg protein. The amount of product formed in the reaction mixture during 10 min incubation at 37 °C was determined as described for UTP reductase.

RESULTS AND DISCUSSION

Isolation of dns mutants

Cells of *B. subtilis* contain deoxyribonucleoside kinase (Bazill & Karamata, 1972; Rima & Takahashi, 1977) and therefore mutants affected in deoxyribonucleotide synthesis might be obtained as deoxyribonucleoside-requiring clones (*dns* mutants). In order to isolate such mutants, strains SB19E (prototrophic) and A26 (*pyr-1*) were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described earlier (Rima & Takahashi, 1977). Mutagenized clones were plated on MMC medium supplemented with dAdo, dCyd, dGuo and dThd (NdR), each at 20 $\mu\text{g ml}^{-1}$. With *B. subtilis* A26, Ura was also added to the above synthetic medium (MMC) at 10 $\mu\text{g ml}^{-1}$. Colonies developed and were replica-plated on MMC agar without NdR and those that failed to grow were tested for NdR requirement. By this technique, eight mutants which required NdR for growth at 37 °C were obtained. This type of mutant occurred at a frequency of about 0.07 % after mutagenesis with nitrosoguanidine. The minimum requirement for growth of these strains was a combination of dAdo or dGuo plus dCyd. dThd could not replace dCyd as it cannot be converted to dCTP (see Fig. 1). In agreement with Bazill & Karamata (1972), we also found that any deoxyribonucleoside plus three bases (for example, dCyd + Ade + Gua + Thy) did not fulfil the growth requirement of these strains. This indicated that *B. subtilis*, unlike *L. leichmannii*, does not contain *trans-N*-deoxyribosylase (Beck & Levin, 1963).

The response of the *dns* mutants to addition of NdR was compared with that of the *tsA13* mutant at 25, 30 and 45 °C (Table 1). The *dns* mutants and *tsA13* required NdR for growth at 45 °C and strain *tsA13 cdd-1 dck-3*, which lacks deoxycytidine kinase, could not grow at 45 °C even in the presence of NdR. This confirms that the *tsA13* mutation affects the synthesis of deoxyribonucleotides. At 30 °C, mutants *dns-2* and *dns-3* grew in the absence of NdR and they resembled the *tsA13* mutant in the temperature sensitivity of the NdR requirement. At 25 °C, all *dns* mutants grew in the absence of NdR. The growth response of *dns-5*, *dns-6*, *dns-7* and *dns-8* (not shown) was similar to *dns-1*. Fuchs & Neuhaud (1973) reported that the dUrd-requiring mutants of *E. coli* grew in the absence of dUrd at low temperatures and under other conditions which resulted in slow growth. It is possible that the *tsA* and *dns* mutants of *B. subtilis* are similarly 'leaky' in their requirement for deoxyribonucleosides.

Table 1. *Effect of temperature and deoxyribonucleosides on the growth of dns and tsA mutants*

Strain	At 25 °C	At 30 °C		At 45 °C	
	MMC	MMC	MMC+NdR	MMC	MMC+NdR
<i>tsA13 cdd-1</i>	++	+	+	—	+
<i>tsA13 cdd-1 dck-3</i>	++	+	+	—	—
A26 (<i>pyr-1</i>)	++	++	++	++	++
A26 <i>dns-1</i>	±	—	+	—	+
SB19E (wild type)	++	++	++	++	++
SB19E <i>dns-2</i>	++	+	++	—	++
SB19E <i>dns-3</i>	++	+	++	±	+
SB19E <i>dns-4</i>	±	—	++	—	+

Ribonucleotide reductase of *B. subtilis*

Ribonucleotide reductase of *B. subtilis* has not been characterized previously and the following experiments were carried out to establish whether the enzyme reduces nucleoside di- or triphosphates. In *B. subtilis* strain SB19E, the reductase activity was determined with [³H]CMP, [³H]CDP and [³H]CTP as substrate. No activity was detectable with [³H]CMP. The activities with [³H]CDP and [³H]CTP were 13 and 62 pmol h⁻¹ (mg protein)⁻¹, respectively. The product of the reaction was dCTP and practically no radioactivity was found in dCDP, dCMP and dCyd. The rate of product formation was not constant with time and the reaction stopped after 10 min incubation. This appeared to be due to breakdown of the substrate, as [³H]CTP in the reaction mixture was rapidly converted to CMP (Fig. 2). Therefore, in later experiments, CTP in the reaction mixture was protected by addition of creatine phosphate and creatine phosphokinase. This modification led to a 10-fold increase in the level of CTP reduction in crude extracts of *B. subtilis*.

Fig. 2. Breakdown of [^3H]CTP in the reaction mixture for CTP reductase. The composition of the reaction mixture was as described in Methods except that creatine phosphate and creatine phosphokinase were absent. ▲, CTP; △, dCTP; ●, CDP; ■, CMP.

Fig. 3. Formation of dUTP and dUDP in the reaction mixture for UTP reductase. The concentrations of protein added to the reaction mixture in (a) and (b) were 7.0 and 1.75 mg ml $^{-1}$, respectively. No dUMP was synthesized. △, dUTP; ○, dUDP; ●, total dUTP+dUDP.

With uridine ribonucleotides, substrate stability was also found to be a problem in ribonucleotide reductase assays. The addition of creatine phosphate and creatine phosphokinase did not alter the rate of uridine ribonucleotide reduction [about 7 pmol h $^{-1}$ (mg protein) $^{-1}$ with UDP or UTP as substrate] as markedly as for CTP reduction. No dUMP was synthesized. The formation of dUTP and dUDP from the reduction of UTP is illustrated in Fig. 3. At a relatively high protein concentration, the formation of dUTP stopped after 10 min (Fig. 3a). At lower protein concentration, however, dUTP was produced continuously at a higher rate than dUDP (Fig. 3b). The apparent cessation of dUTP formation and the presence of dUDP in the reaction mixture are probably due to the action of dUTPase which produced predominantly dUDP in *B. subtilis* (Tomita & Takahashi, 1976).

In the only other study on the ribonucleotide reductase in a *Bacillus* species, Yau & Wachsman (1973) were unable to determine whether di- or triphosphates are the substrate for the enzyme, but they did not protect the substrate and got similar activity with either CTP or CDP as substrate. Our results suggest that ribonucleoside triphosphates are the substrate for reduction in *B. subtilis* and the enzyme may therefore resemble the ribonucleotide reductase of *L. leichmannii* (Goulian & Beck, 1966).

Table 2. Growth inhibition of *dns* and *tsA* mutants by hydroxyurea at 30 °C

NdR were added to MMC agar at 10 µg ml⁻¹ where indicated (—, absent; +, present). Symbols for growth are as defined in Table 1.

Hydroxyurea concn (mM)	NdR	SB19E (wild type)	<i>tsA13 cdd-1</i>	<i>tsA13 cdd-1 dck-3</i>
0	—	++	++	++
1	—	++	++	++
3	—	++	++	++
10	—	++	+	±
30	—	±	—	—
60	—	—	—	—
100	—	—	—	—
60	+	+	+	—
100	+	+	+	—

The ribonucleotide reductase activity in *L. leichmannii* and *B. megaterium* is stimulated by the addition of cobalamin (vitamin B₁₂) (Goulian & Beck, 1966; Yau & Wachsmann, 1973). However, we found that optimum activity was obtained with the reaction mixture described and cobalamin addition to crude extracts did not enhance reductase activity.

The enzymes in *E. coli* and *L. leichmannii* differ in sensitivity to hydroxyurea (HU). The reductase in *E. coli* is inhibited by HU *in vitro* and *in vivo* (Elford, 1968; Sinha & Snustadt, 1972). The enzyme in *L. leichmannii* is not sensitive to HU *in vitro* and the *in vivo* effect was not reported (Elford, 1968). We found that CTP and UTP reductase activity in *B. subtilis* was not inhibited *in vitro* by HU at concentrations ranging from 0.1 to 10 mM. *In vitro*, HU inhibits ribonucleotide reductase in *B. megaterium* only slightly if at all (Yau & Wachsmann, 1973). Therefore, the enzyme in *B. subtilis* appeared to respond *in vitro* to HU in a similar way to the other ribonucleoside triphosphate reductases. However, *in vivo*, the growth of *B. subtilis* was inhibited by HU (Table 2). Our results confirm and expand those of Bazill & Karamata (1972). Strains carrying the *tsA13* mutation appeared to be inhibited by lower concentrations of HU and the addition of NdR to the medium overcame the inhibitory effect. However, in a strain containing the *dck* mutation, which lacks dCyd kinase, NdR was unable to overcome the effect of HU. This indicates that NdR counteract the effect of HU after they have been phosphorylated to deoxyribonucleotides, suggesting that HU interferes with the supply of deoxyribonucleotides. These results indicate that, *in vivo*, ribonucleotide reduction in *B. subtilis* is sensitive to HU, even though we were unable to demonstrate this effect *in vitro*.

Ribonucleotide reductase in mutant strains

In order to investigate whether ribonucleotide reductase activity was affected by the presence of the *tsA13* mutation, CTP and UTP reductase activity was assayed in strains 167 *thyA thyB* and 167 *tsA13 thyA thyB* obtained from Bazill & Karamata (1972). There was no significant difference in reductase activity between the two strains grown either at 37 °C in the absence of NdR or at 45 °C in the presence of NdR (10 µg ml⁻¹).

To compare the temperature sensitivity of the reductase *in vitro*, the above *ts⁺* and *tsA13* strains were grown at 37 °C in Penassay Broth and CTP and UTP reductase activities were assayed at 37 and 45 °C. The activity of UTP reductase in the *ts⁺* strain assayed at 45 °C was 54 % of the activity at 37 °C. The activity of UTP reductase in the *tsA13* strain assayed at 45 °C was 59 % of that assayed at 37 °C. The presence of the *tsA13* mutation therefore had no appreciable effect on the temperature sensitivity of the enzyme *in vitro*. In the case of CTP reduction, enzyme activities in both *ts⁺* and *tsA13* strains at 45 °C were not significantly different from those determined at 37 °C.

Table 3. Activity of CTP reductase in *dns* and *tsA* mutantsEnzyme activities are expressed as nmol product formed h⁻¹ (mg protein)⁻¹.

Strain	CTP reductase activity	CTP reductase (% of parent strain)
SB19E (prototrophic)	1.26	100
<i>dns-2</i>	0.77	61
<i>dns-3</i>	0.29	23
<i>dns-4</i>	1.24	98
<i>tsA13 cdd-1</i>	1.06	85
A26(<i>pyr-1</i>)	1.61	100
A26 <i>dns-1</i>	0.76	44

Table 4. Phenotypic reversion of the *tsA13* mutationNdR were added to MMC agar at 10 µg ml⁻¹. Thy was added at 10 µg ml⁻¹ for *thyA thyB* mutants. Symbols for growth are as defined in Table 1.

Strain	MMC at 37 °C	MMC at 45 °C	MMC+NdR at 45 °C
167 <i>tsA13 thyA thyB</i>	++	—	+
167 <i>tsA13 thyA thyB</i> ⁺	++	—	+
167 <i>tsA13 thyA</i> ⁺ <i>thyB</i>	++	—	+
167 <i>tsA13 cdd-1 thyA thyB</i>	++	+	+
167 <i>tsA13 cdd-1 thyA</i> ⁺ <i>thyB</i>	++	+	+
167 <i>tsA13 cdd-1 thyA thyB</i> ⁺	++	—	+
<i>tsA13 cdd-1</i>	++	—	+
<i>tsA13 cdd-1 ddd-3</i>	++	+	+

To investigate whether the requirement of *dns* mutants for NdR was reflected in reduced levels of ribonucleotide reductase activity in the mutants, CTP reductase was assayed in extracts of the *dns* mutants. They showed a considerable amount of CTP reductase activity varying from 23 to 98 % of the activity in the parent strains (Table 3). The assayable reductase activities found in crude extracts of *B. subtilis* were very low and they may not be representative of the activity of the enzyme *in vivo*. This might explain why the requirement for NdR of the *tsA* and *dns* mutants did not correlate with the levels of assayable reductase activity in these strains, and the fact that all these strains were able to grow at low temperature in the absence of NdR may indicate that all our mutants can reduce ribonucleotides to some degree.

Suppression of *tsA* phenotype

Further evidence for the complex nature of the NdR requirement in *B. subtilis* mutants (i.e. none of them appeared to be ribonucleotide reductase negative) was obtained from studies on the effect of other mutations on the *tsA* phenotype. The growth response to NdR at various temperatures was determined for a number of mutants carrying the *tsA13* mutation together with other mutations that affect the metabolism of pyrimidine nucleotides and nucleosides such as *ddd* (dCMP deaminase), *cdd* (dCyd-Cyd deaminase), *thyA* (dTMP synthetase) and *thyB* (dTMP synthetase) (Fig. 1). It was found that mutants carrying the *tsA13* mutation in combination with *thyB* and *cdd-1* were able to grow at 45 °C without NdR (Table 4). A mutant carrying the *tsA13* mutation together with *ddd-3* and *cdd-1* was also able to grow at 45 °C without NdR (Table 4).

The finding that the *tsA* phenotype can be reversed by *cdd* and *ddd* or *cdd* and *thyB* mutations may be explained by the following observation. Bazill & Karamata (1972) showed that dCTP but not dTTP disappeared rapidly when cells of *tsA13* were shifted up to 45 °C. Therefore, mutations such as *cdd* and *ddd* which make cells unable to deaminate

deoxycytidine compounds would suppress the *tsA* phenotype. It has been suggested that the *thyA* and *thyB* mutations may have a different effect on the sizes of various deoxypyrimidine nucleotide pools (Wilson *et al.*, 1966) and this may explain the different effect of these two mutations on the *tsA* phenotype.

Financial support for this work was provided by a grant from the National Research Council of Canada (A-1996). We wish to thank Darryll Bradford for excellent technical assistance.

REFERENCES

- BAZILL, G. W. & KARAMATA, D. (1972). Temperature-sensitive mutants of *Bacillus subtilis* defective in deoxyribonucleotide synthesis. *Molecular and General Genetics* **117**, 19–29.
- BECK, W. S. & LEVIN, M. (1963). Purification, kinetics and repression control of bacterial *trans*-*N*-deoxyribosylase. *Journal of Biological Chemistry* **238**, 702–709.
- ELFORD, H. (1968). Effect of hydroxyurea on ribonucleotide reductase. *Biochemical and Biophysical Research Communications* **33**, 129–135.
- FUCHS, J. A. & NEUHARD, J. (1973). A mutant of *Escherichia coli* defective in ribonucleoside diphosphate reductase. *European Journal of Biochemistry* **32**, 451–456.
- FUCHS, J. A., KARLSTRØM, H. O., WARNER, H. R. & REICHARD, P. (1972). Defective gene product in *dnaF* mutant of *Escherichia coli*. *Nature New Biology* **238**, 69–71.
- GOULIAN, M. & BECK, W. S. (1966). Purification and properties of a cobamide dependent ribonucleotide reductase from *Lactobacillus leichmannii*. *Journal of Biological Chemistry* **241**, 4233–4242.
- KARLSTRØM, H. O. (1970). Inability of *Escherichia coli* B to incorporate added deoxycytidine, deoxyadenosine and deoxyguanosine into DNA. *European Journal of Biochemistry* **17**, 68–71.
- LARSSON, A. & REICHARD, P. (1966). Enzymatic synthesis of deoxyribonucleotides. X. Reduction of purine ribonucleotides: allosteric behaviour and substrate specificity of the enzyme system from *Escherichia coli* B. *Journal of Biological Chemistry* **241**, 2540–2543.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- O'DONOVAN, G. A. & NEUHARD, J. (1970). Pyrimidine metabolism in microorganisms. *Bacteriological Reviews* **34**, 278–343.
- RIMA, B. K. & TAKAHASHI, I. (1973). The synthesis of nucleic acids in *Bacillus subtilis* infected with phage PBS1. *Canadian Journal of Biochemistry* **51**, 1219–1224.
- RIMA, B. K. & TAKAHASHI, I. (1977). Metabolism of pyrimidine bases and nucleosides in *Bacillus subtilis*. *Journal of Bacteriology* **129**, 574–579.
- RIMA, B. K. & TAKAHASHI, I. (1978). Synthesis of thymidine nucleotides in *Bacillus subtilis*. *Canadian Journal of Biochemistry* **56**, 158–160.
- SINHA, N. K. & SNUSTADT, D. P. (1972). Mechanism of inhibition of deoxyribonucleic acid synthesis in *Escherichia coli* by hydroxyurea. *Journal of Bacteriology* **112**, 1321–1334.
- SPIZIZEN, J. (1958). Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National Academy of Sciences of the United States of America* **44**, 1072–1078.
- TOMITA, F. & TAKAHASHI, I. (1976). Changes in enzyme activities in *Bacillus subtilis* infected with bacteriophage PBS1. In *Microbiology—1976*, pp. 315–318. Edited by D. Schlesinger. Washington: American Society for Microbiology.
- WILSON, M. C., FARMER, J. L. & ROTHMAN, F. (1966). Thymidylate synthesis and aminopterin resistance in *Bacillus subtilis*. *Journal of Bacteriology* **92**, 186–196.
- YAU, S. & WACHSMAN, J. T. (1973). The *Bacillus megaterium* ribonucleotide reductase: evidence for a B₁₂ coenzyme requirement. *Molecular and Cellular Biochemistry* **1**, 101–105.