Structural and stereospecific requirements for the nucleoside-triggered germination of *Bacillus cereus* spores

SONIA SENESI,^{1*} GIOVANNI CERCIGNANI,² GIULIA FREER,¹ GIOVANNA BATONI,¹ SIMONA BARNINI¹ and FUSAO OTA^{1†}

¹Department of Biomedicine, Medical Microbiology Section, Via S. Zeno 39, and ²Department of Physiology and Biochemistry, Via S. Maria 55, University of Pisa, 56100 Pisa, Italy

(Received 16 July 1990; revised 24 September 1990; accepted 25 October 1990)

A selection of adenosine analogues was tested for their ability to trigger germination of *Bacillus cereus* NCIB 8122 spores. The germination-inducing activity was governed by the structural properties of the sugar rather than the base moieties of the nucleosides. Among the sugar-modified analogues, only those containing a 2'-deoxy-D-ribose moiety promoted spore germination. Requirements for a specific molecular structure of the base were not clearly identified, although the highest activity was observed when substituents were inserted at position 6 of the purine ring. All the base-modified analogues, even those such as coformycin and 2'-deoxycoformycin with an expanded base ring, retained the germination-inducing activity of adenosine. However, of the two 2'-deoxycoformycin diastereoisomers characterized by an asymmetric carbon atom at position 8 of the homopurine ring, only the 8*S*-isomer induced germination, thus indicating that stereospecific configuration of the inducer, at least in the case of 2'-deoxycoformycin, appears to be essential for the initiation of spore germination. The differences in the germination-inducing activity of the various analogues tested were not affected significantly by spore activation at different temperatures, although the higher the activation temperature, the lower was the concentration of each analogue required for maximum germination.

Introduction

The molecular mechanism of bacterial spore germination is still the subject of intense investigation. Much is known of the conditions under which spores germinate and detailed information has been accumulated on the biochemical changes that accompany the sequential loss of spore properties during germination (Keynan, 1978; Setlow, 1983). On the other hand, very little is known of the nature of the reaction responsible for the initiation of germination, although there is increasing evidence to suggest that the 'trigger reaction' might be an allosteric change involving the binding of an inner-membrane receptor to exogenous germinants, rather than a metabolic energy-requiring event (Keynan & Sandler, 1983; Foster & Johnstone, 1990).

Purine nucleosides induce germination of spores of several bacterial species (Hills, 1949*a*; Lawrence, 1955*b*; Powell & Hunter, 1955, 1956), but the mechanism of

their action is far from fully understood. For *Bacillus cereus* spores only certain purine compounds induce germination (Hills, 1949*a*; Powell & Hunter, 1956; Warren & Gould, 1968) and their effectiveness is greatly influenced by prolonged heat treatment (Powell & Hunter, 1955, 1956). Inosine, adenosine and guanosine induce spore germination to a variable extent, but xanthosine and purine and pyrimidine nucleotides are inactive, as are the constituents of purine nucleosides, free bases and sugars tested individually and in combination (Hills, 1949*a*; Lawrence, 1955*b*; Warren & Gould, 1968).

Intact spores of *B. cereus* contain enzymes which catalyse the breakdown of purine nucleosides as soon as germination is induced (Lawrence, 1955*a, b*; Powell & Hunter, 1956; Senesi *et al.*, 1977; Weinberger *et al.*, 1980), which suggested that only metabolizable nucleosides would induce germination even though their cleavage did not appear essential to trigger germination (Lawrence, 1955*a*; Powell & Hunter, 1956; Weinberger *et al.*, 1980). More recently, non-metabolizable analogues of purine nucleotides have also been reported to induce germination of *B. cereus* spores although the extent of

† Permanent address: Department of Food Microbiology, School of Medicine, University of Tokushima, Tokushima 770, Japan.

Abbreviation: DPA, dipicolinic acid.

germination varied with the differently modified purine nucleosides (Shibata *et al.*, 1985).

In this study, the specific nucleoside requirements for the germination of *B. cereus* spores were investigated using a selection of adenosine analogues. The effect of varying heat-activation of spores was also studied to provide further insight into an environmental influence on the ability of the analogues to trigger germination.

Methods

Preparation of spore suspensions. *Bacillus cereus* NCIB 8122 was used throughout this study. Spores were produced on fortified nutrient agar (Senesi *et al.*, 1974) supplemented with sporulation salts: NaCl (5.0 mg ml⁻¹), CaCl₂ (0.1 mg ml⁻¹), and MgSO₄ · 7H₂O (2.0 mg ml⁻¹). Roux bottles, containing 150 ml of the above medium, were inoculated with 3.5 ml of spore suspension (5.0×10^7 spores ml⁻¹ in distilled water). After incubation at 36 °C for 72 h, the spores were scraped from the agar with a Teflon-coated magnetic stirrer, washed five times by centrifugation (10000 *g* for 10 min) with ice-cold distilled water and kept as a suspension containing more than 99% free refractile spores and no visible vegetative cells. The freshly harvested spores were stored at 4 °C after being diluted to about 3.0×10^9 spores ml⁻¹ (OD₆₂₅ 8.0). The stock spore suspensions were used within 6–10 d for germination experiments.

Heat activation of dormant spores. Two-millilitre aliquots of the stock spore suspensions, in 5 ml thin-walled glass tubes, were activated at 70, 80 and 90 °C for 10, 7.5 and 3.5 min respectively in a water bath; heat-up time was 0.25 min. The spores were then cooled rapidly in an ice-water bath, washed once as above, resuspended in ice-cold distilled water and used within 3 h.

Spore germination. This was continuously monitored at 36 °C by recording the decrease in OD₆₂₅ of spore suspensions in a Hitachi Perkin-Elmer DB 124 spectrophotometer: 0.1 ml of heat-activated spore suspension (3.0×10^8 spores) was diluted in 0.8 ml 67 mM-potassium phosphate buffer (pH 8.2) and equilibrated in 1.0 cm light-path semimicrocuvettes at 36 °C for 10 min. Germination was started by adding 0.1 ml prewarmed L-alanine/adenosine solution to final concentrations of 10 mM and 0.1 mM respectively. Typically, the initial OD₆₂₅ of 0.8 decreased within 15 s of the induction. The germination rate was the maximum rate of decrease of OD₆₂₅ min⁻¹ expressed as a percentage decrease relative to the initial OD₆₂₅. The extent of germination was measured by the total decrease in OD₆₂₅ expressed as a percentage of the initial value; since complete loss of spore refractility at 625 nm corresponded to a 60% decrease of the initial OD₆₂₅, this percentage value was taken as 100% extent of germination (i.e. germinated spores) and all intermediate values were expressed according to the above proportion.

Induction of germination with adenosine analogues. When the germination-inducing activity of these analogues was assayed, the conditions were as described above, except that adenosine was replaced by the compound to be tested. Experiments were carried out at least in triplicate. The maximum rate and extent of germination were taken directly from each time-course curve. The activity of the analogues was established by determining the lowest concentration stimulating 50% spore germination within 120 min (C₅₀). The purity of all analogues was tested by HPLC (Beckman) on C18-reversed-phase columns. Whenever contaminating nucleosides were present, only samples from the peak of authentic substance were used for germination. Samples of 8R-deoxycoformycin [pentostatin; 8R-3-(2-

deoxy-β-D-erythro-pentofuranosyl)-3,6,7,8-tetrahydroimidazo(4,5-d)(1,3)diazepin-8-ol] and 8S-deoxycoformycin [8S-3-(2-deoxy-β-D-erythropentofuranosyl)-3,6,7,8-tetrahydroimidazo(4,5-d)(1,3)diazepin-8-ol] were supplied by Dr David C. Baker (Department of Chemistry, University of Alabama, Tuscaloosa, USA). Coformycin [8R-3-β-D-ribofuranosyl-3,6,7,8-tetraimidazo(4,5-d)(1,3)diazepin-8-ol] was obtained from Calbiochem-Behring, 2,6-diaminopurine riboside from Pfaltz & Bauer and all other analogues from Sigma.

Determination of heat-resistance and of dipicolinic acid (DPA) during germination. Germination conditions were as described for the spectrophotometric assay, except that the final volume of germination mixtures was 20.0 ml and phosphate buffer was replaced by 0.9% NaCl plus 10 mM-Tris/HCl (pH 8.2). Loss of heat-resistance during germination was measured at 5 min intervals from the onset of germination in 1.0 ml samples which were either heated at 85 °C for 20 min or unheated. Heat-resistant spores were enumerated at 30 °C by plating, in triplicate, 0.1 ml samples of appropriate dilutions (Warth, 1978). Survival was expressed as a percentage of the total population. DPA release by germinating spores was measured by removing 1.0 ml samples at 5 min intervals, adding 1.0 ml cold 40 mM-D-alanine to block any further germination (Kutima & Foegeding, 1987) and after centrifugation determining the amount of DPA in the supernatants by either the method of Janssen *et al.* (1958) or that of Scott & Ellar (1978), depending upon the amount of DPA to be measured. Purified DPA (Aldrich) was used as a standard for calibration curves. The amount of released DPA was expressed as percentage of that extracted from ungerminated spores, for which the mean value for eight separate samples was 172.0 ± 6.3 (mean $\pm$ SD) nmol per 10⁸ spores.

Results

Heat-activation conditions and inducer concentrations to optimize spore germination

The ability of adenosine to trigger spore germination of most *B. cereus* strains can be enhanced greatly by L-alanine as well as by prolonged heat treatment (Powell & Hunter, 1955; Warren & Gould, 1968; Sogin *et al.*, 1972; Yousten, 1975), but it was important to establish standard conditions in which the germination-inducing activity of the analogues could be measured.

The effects of activation at 70, 80 or 90 °C for varying times and induction by L-alanine (10 mM) + adenosine (0.1 mM), adenosine (1 mM) and L-alanine (100 mM) were tested (Table 1). The optimum conditions for germination were activation at 70 °C for 10 min in 10 mM-L-alanine + 0.1 mM-adenosine. No increase in germination rate was obtained when heat activation was extended beyond 10 min, and the germination appeared bimodal, particularly when heating was extended beyond 30 min, as already described by Hashimoto *et al.* (1972), Sogin *et al.* (1972) and Shacter & Hashimoto (1975). Hence, when spores were activated at 70 °C, the germination-inducing activity of adenosine was amplified by adding L-alanine to the germination medium, since adenosine alone induced germination only when spores were activated for at least 45 min at that temperature (Table 1). By contrast,

Table 1. Heat-activation conditions required for maximum germination of *B. cereus* NCIB 8122 spores in L-alanine + adenosine, or adenosine and L-alanine aloneValues are given as the mean $\pm$ SD of seven separate experiments.

Heat activation		Inducer concns (mM)*		Germination rate†	Germination extent†	<i>t</i> _{50%} (min)‡
<i>T</i> (°C)	<i>t</i> (min)	L-Alanine	Adenosine			
70	10.0	10.0	0.1	23.5 $\pm$ 1.2	98.5 $\pm$ 0.8	2.2 $\pm$ 0.1
80	3.0	10.0	0.1	21.9 $\pm$ 0.8	97.6 $\pm$ 1.0	2.4 $\pm$ 0.2
90	1.5	10.0	0.1	20.5 $\pm$ 1.3	96.8 $\pm$ 0.7	2.6 $\pm$ 0.1
70	45.0	0	1.0	6.9 $\pm$ 0.4	58.6 $\pm$ 1.8	24.1 $\pm$ 1.4
80	7.5	0	1.0	13.6 $\pm$ 0.7	89.2 $\pm$ 1.5	4.6 $\pm$ 0.6
90	3.5	0	1.0	14.2 $\pm$ 0.9	90.4 $\pm$ 1.8	5.2 $\pm$ 0.3
70	25.0	100.0	0	2.3 $\pm$ 0.3	45.2 $\pm$ 1.2	ND
80	5.0	100.0	0	4.8 $\pm$ 0.7	70.2 $\pm$ 1.6	10.8 $\pm$ 1.3
90	2.5	100.0	0	5.8 $\pm$ 0.9	78.8 $\pm$ 1.8	8.2 $\pm$ 0.9

ND, Not detectable.

* Inducer concentrations for maximum germination rate and extent, as determined with spores activated at 70 °C.

† Germination rate is the maximum rate of decrease in OD₆₂₅ min⁻¹ and is expressed as a percentage decrease relative to the initial OD₆₂₅. Extent of germination is the total decrease of OD₆₂₅ expressed as a percentage of the total decrease of OD₆₂₅ (60%) corresponding to 100% loss of spore refractility (i.e. 100% germinated spores) (see Methods).

‡ Time required to reach 50% germination.

Table 2. Germination-inducing activity of adenosine analogues on *B. cereus* NCIB 8122 sporesValues are given as the mean $\pm$ SD of seven separate experiments.

Compound added (0.1 mM)*	Germination rate†	Germination extent†	<i>t</i> _{50%} (min)‡
None	< 0.05	7.8	ND
Adenosine	23.5 $\pm$ 1.2	98.5 $\pm$ 0.8	2.2 $\pm$ 0.1
<i>Ribose-modified analogues</i>			
2'-Deoxyadenosine	5.4 $\pm$ 0.3	60.2 $\pm$ 0.6	16.8 $\pm$ 0.3
3'-Deoxyadenosine	2.1 $\pm$ 0.2	14.8 $\pm$ 1.7	ND
5'-Deoxyadenosine	0.9 $\pm$ 0.1	11.9 $\pm$ 1.5	ND
5'-Chloro-5'-deoxyadenosine	0.7 $\pm$ 0.1	25.4 $\pm$ 1.6	ND
5'-Amino-5'-deoxyadenosine	0.8 $\pm$ 0.2	16.8 $\pm$ 1.9	ND
<i>Base-modified analogues</i>			
Inosine	21.5 $\pm$ 0.9	95.5 $\pm$ 0.6	2.6 $\pm$ 0.1
6-Methoxypurine riboside	24.5 $\pm$ 1.1	98.5 $\pm$ 0.7	2.0 $\pm$ 0.1
6-Hydroxylaminopurine riboside	26.2 $\pm$ 1.3	98.5 $\pm$ 0.7	1.6 $\pm$ 0.6
Purine riboside	14.2 $\pm$ 0.7	72.8 $\pm$ 1.5	6.5 $\pm$ 0.5
1-Methyladenosine	7.6 $\pm$ 0.5	73.1 $\pm$ 1.6	10.4 $\pm$ 0.4
2,6-Diaminopurine riboside	12.3 $\pm$ 0.7	94.5 $\pm$ 0.8	7.8 $\pm$ 1.1
2-Chloroadenosine	5.0 $\pm$ 0.4	58.3 $\pm$ 1.8	14.4 $\pm$ 2.1
Formycin B	8.5 $\pm$ 0.7	85.0 $\pm$ 0.9	8.2 $\pm$ 1.6
Formycin A	5.2 $\pm$ 0.3	56.4 $\pm$ 1.6	15.4 $\pm$ 2.1
<i>Expanded-base-ring analogues</i>			
8 <i>R</i> -Coformycin	18.2 $\pm$ 1.2	95.4 $\pm$ 0.6	2.8 $\pm$ 0.1
8 <i>R</i> -2'-Deoxycycoformycin	0.1 $\pm$ 0.01	12.3 $\pm$ 0.5	ND
8 <i>S</i> -2'-Deoxycycoformycin	5.7 $\pm$ 0.5	68.6 $\pm$ 1.3	16.5 $\pm$ 0.5

ND, Not detectable.

* Germination was in 10 mM-L-alanine plus the compound indicated, using spores heated at 70 °C for 10 min.

†,‡ As for Table 1.

Table 3. Dependence of germination-inducing activity of adenosine analogues on the temperature of heat-activation of *B. cereus* NCIB 8122 spores

Compound	C ₅₀ (μM)* with L-alanine			C ₅₀ (μM)* without L-alanine	
	70 °C	80 °C	90 °C	80 °C	90 °C
<i>Ribose-modified analogues</i>					
2'-Deoxyadenosine	42.0	30.0	24.0	375.0	135.0
3'-Deoxyadenosine	†	†	†	†	†
5'-Deoxyadenosine	†	†	†	†	†
<i>Base-modified analogues</i>					
Adenosine	17.5	12.5	8.7	150.0	62.5
6-Methoxypurine riboside	15.0	10.0	7.5	125.0	50.0
6-Hydroxylaminopurine riboside	12.5	8.7	6.2	75.0	37.5
1-Methyladenosine	40.5	31.5	22.5	360.0	120.0
2-Chloroadenosine	45.0	36.0	27.0	405.0	225.0
Formycin A	50.0	40.5	31.5	480.0	270.0
<i>Expanded-base-ring analogues</i>					
8-R-Coformycin	26.0	15.0	10.0	175.0	62.5
8R-2'-Deoxycoformycin	†	†	†	†	†
8S-2'-Deoxycoformycin	40.5	27.0	17.5	315.0	125.0

* Lowest inducer concentration allowing 50% spore germination within 120 min (C₅₀). In the presence of 10 mM-L-alanine, C₅₀ was determined with spores activated at 70 °C for 10 min, 80 °C for 3 min and 90 °C for 1.5 min; in the absence of L-alanine, C₅₀ was determined with spores activated at 80 °C for 7.5 min and 90 °C for 3.5 min.

† Maximum germination was 30% at an inducer concentration as high as 5 mM.

after heat activation at 80 or 90 °C, optimum germination was obtained with adenosine alone (although at a lower rate than in the presence of L-alanine) by heating spores for a very short time (7.5 and 3.5 min at 80 and 90 °C, respectively). Such heating did not appear to stimulate bimodal germination even in presence of L-alanine. It was therefore possible: (i) to analyse the germination-inducing activity of adenosine analogues with respect to adenosine alone and (ii) to compare the enhancing effect of L-alanine or of heating on the activity of the analogues as inducers of spore germination.

Effects of sugar- or base-modified adenosine analogues on spore germination

Most of the experiments to screen adenosine analogues for their ability to induce spore germination were done in 10 mM-L-alanine with spores activated at 70 °C for 10 min (Table 2). The nucleosides modified in their sugar moiety were generally strongly impaired as germinants. Although the ribose-modified analogues tested were very closely related compounds, only 2'-deoxyadenosine actively triggered germination, but to a lesser extent than adenosine (Table 2). This suggests that the germination-inducing activity of purine nucleosides is strictly regulated by the properties of the ribosyl moiety, the unmodified structure of which is essential for

the initiation of *B. cereus* spore germination. By contrast, none of the base-modified analogues was totally inactive as a germinant. In fact, those with modifications at C-6 were even better inducers of germination than adenosine. Only 2-chloroadenosine and formycin A resulted in sluggish germination, but they stimulated 50–60% germination. Collectively, these results show that the structural properties of the base moiety of purine nucleosides do not greatly affect germination induction, although there are minor differences in both the rate and the extent of germination.

In order better to quantify the germination-inducing activity of the different analogues, the lowest concentration resulting in 50% spore germination in 120 min (C₅₀) was measured. To determine the heat-activation dependence of their germination-inducing activity, spores were activated at 70, 80 or 90 °C and germinated in L-alanine (Table 3). To rule out the possibility that L-alanine might affect the activity of the different analogues, experiments were also performed in the absence of L-alanine using spores activated at 80 °C for 7.5 min or at 90 °C for 3.5 min. Germination of 50% of the spores was achieved at different concentrations of the inducer, depending upon the activation temperature: in general, the higher the activation temperature the lower the C₅₀ of the inducer, in both the presence and absence of L-alanine. Nevertheless, the differences in

Fig. 1. Molecular structure of expanded base ring analogues of adenosine. Position 8 is marked with a star.

Table 4. DPA release and loss of heat-resistance by *B. cereus* NCIB 8122 spores during germination in either $8R$ -2'-deoxycoformycin or $8S$ -2'-deoxycoformycin

Spores were activated at 70 °C for 10 min. The germination medium (0.9% NaCl + 10 mM Tris/HCl, pH 8.2) contained 3.0×10^8 spores ml⁻¹ and 10.0 mM-L-alanine.

Sampling time (min)	Germination in the presence of			
	0.1 mM- $8R$ -2'-deoxycoformycin		0.1 mM- $8S$ -2'-deoxycoformycin	
	DPA release*	Loss of heat-resistance†	DPA release*	Loss of heat-resistance†
5	0.1	0.9	10.0	15.0
10	2.3	5.8	25.0	36.0
15	4.2	13.7	47.0	52.0
30	6.7	28.6	56.0	79.0

* DPA release is expressed as a percentage of the total DPA content of ungerminated spores in the spore suspension (516.0 ± 12.6 nmol).

† Expressed as the percentage of the total population sensitive to heating at 85 °C for 20 min.

ability to trigger germination, based on comparisons of the maximum germination rate at a given analogue concentration (Table 2), did not appear to be modified significantly when expressed as C_{50} . As expected, such values were lower for each analogue in the presence of L-alanine.

Effects of adenosine analogues with an expanded base ring on spore germination

As can be seen in Fig. 1, 2'-deoxycoformycin diastereoisomers and coformycin have a partly saturated expanded base dihydrohomopurine ring characterized by the presence of an asymmetric carbon atom at position 8. In addition, the 2'-deoxycoformycins also lack the hydroxyl group at position 2 of the ribose moiety. Coformycin was nearly as active as adenosine in triggering spore germination, with or without L-alanine and at any activation temperature tested (Tables 2 and 3). These

results further support the conclusion that the germination-inducing activity of purine nucleosides depends only slightly, if at all, on the structural properties of the base ring, since the dihydrohomopurine nucleosides are effective in rapidly triggering germination.

When 2'-deoxycoformycins were used as germinants, $8S$ -2'-deoxycoformycin was as active as 2'-deoxyadenosine, the most active ribose-modified nucleoside tested, but the $8R$ -diastereoisomer was almost devoid of germination-inducing activity (Table 2). Similar results were obtained when germination was monitored by different procedures (Table 4). The stringent requirement for the stereospecific configuration of nucleosides acting as germinants cannot be compared with that of D-alanine (vs L-alanine) in the same process. Indeed, while D-alanine strongly inhibits L-alanine-triggered germination (Hills, 1949b), no inhibitory effect was observed on the germination rate even when the R -isomer was tested at a concentration twice that of the S -diastereoisomer.

Conclusions

Two major conclusions can be drawn from these results, as follows.

(i) If signal transduction results from the interactions of the analogues with a putative receptor, the varying affinity for purine nucleosides could relate to their different germination-inducing activities. However, the relationship between the structural feature and the activity of the various analogues has not been clearly established, except that the ability to promote germination appears most likely to be related to the structural properties of the sugar rather than the base moiety of the nucleosides. The requirement for an unmodified ribosyl structure to trigger germination rapidly has already been suggested (Shibata *et al.*, 1985), but their germination experiments were carried out with distantly related compounds and the spores were subjected to prolonged heat treatment (70 °C for 30 min and in the presence of L-alanine), conditions resulting in much interference with *B. cereus* spore germination (Sogin *et al.*, 1972; Shacter & Hashimoto, 1975).

(ii) The stereospecific configuration of the inducer appears to be critical for the rapid induction of the germination of *B. cereus* spores, at least in the case of 2'-deoxycoformycin. Whatever the nature of the spore structure interacting with nucleosides, it must also discriminate between opposite configurations at the C-8 of 2'-deoxycoformycin. As confirmation, it would be interesting to assay the germination-inducing activity of 8*S*-coformycin, since the natural compound has the *R* configuration at position 8. Unfortunately, its 8*S*-diastereoisomer is not yet available, so one can only speculate that, if the *S* configuration at position 8 contributes to the enhancement of the germination induction by the 2'-deoxyribonucleosides, it could also influence the inducing activity of the ribonucleosides.

This work was supported by grants from the 'Ministero dell'Università e della Ricerca Scientifica e Tecnologica', Rome. We are very grateful to Dr David C. Baker for his generous gift of the 2'-deoxycoformycin diastereoisomers.

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