Differential nucleoside recognition during *Bacillus cereus* 569 (ATCC 10876) spore germination†

Ernesto Abel-Santos*[a] and Tetyana Dodatko[b]

Received (in Durham, UK) 15th November 2006, Accepted 7th March 2007
First published as an Advance Article on the web 20th March 2007
DOI: 10.1039/b616695d

We have tested a series of inosine analogs for their effect on germinating *B. cereus* 569 spores. Our results showed that although inosine (hypoxanthine nucleoside) causes spore germination by itself, the kinetic pathway exhibited complex and strongly cooperative character. Contrary to inosine's germinating effect, the purine pathway degradation products xanthine, xanthosine, uric acid, hypoxanthine, ribose, or ribose plus hypoxanthine failed to activate spore germination. Furthermore, even small modifications of inosine's nucleobase or sugar moieties have deleterious effects on germination efficiency. In contrast to previous work with the *B. cereus* 3711 strain, incubation of *B. cereus* 569 spores with adenosine (6-aminopurine riboside) did not trigger germination, but prevented inosine-mediated germination. The inhibitory effect was lost if adenosine was substituted with adenine alone, or ribose plus adenine. Although adenosine is able to block inosine-mediated germination, it acts as a co-germinant in the presence of alanine. Nucleosides that have substitutions in the purine base are not able to trigger germination by themselves, but can act as co-germinants in the presence of sub-germinant concentrations of alanine. In contrast, modifications of the sugar moiety precluded germination activity under all conditions tested. The data suggests that only inosine can activate germination by itself. However, when alanine is present as a co-germinant, different germination receptors are activated that recognize a larger subset of nucleoside structures.

Introduction

Endospore-forming bacteria produce some of the most potent toxins known and are important pathogens in hospital-borne infections (*Clostridium difficile*), food contamination (*Bacillus cereus, Clostridium botulinum*), wound infestation (*Clostridium tetani*), and bioterrorism (*Bacillus anthracis*).[1] These bacteria differentiate into spores to survive harsh environmental conditions.[2,3] Afterwards, when a spore finds a suitable milieu, it germinates into a replicating cell, thus completing its life cycle.[4]

Although spores are metabolically inactive, they must be able to detect nutrients in the surrounding environment.[5] The first step in the germination process is believed to be the binding and recognition of amino acids, nucleosides, and other small molecules (germinants) by a family of membrane proteins (Ger proteins) that act as germination receptors.[6] The *ger* families of receptors are encoded as tricistronic operons and are essential for the onset of germination. These operons encode for three distinct proteins that are assumed to form a receptor complex in the forespore membrane.[7,8]

Genome sequencing revealed at least eight *ger* operons in *B. cereus* strains (*gerB*, *gerH*, *gerI*, *gerK*, *gerL*, *gerQ*, *gerR* and *gerS*). Recently, direct phenotypic evidence for germination function was obtained for all genomically encoded germination receptors of *B. cereus* 3711 and *B. anthracis* Sterne strains.[9–11]

The kinetics of spore germination has been fitted to an exponential equation that describes the changes in optical density as spores germinate.[12] Previous reports postulated that spore germination kinetics could be described using a rapid equilibrium approach.[13] More recently, Ireland and Hannah demonstrated that *B. anthracis* germination with alanine follows complex kinetics.[14]

While nucleoside and amino acids are recognized as germinants by many *Bacillus* species, there are subtle differences in spore germination profiles. *B. cereus* 569 (ATCC 10876) germinates readily in the presence of inosine, but weakly with adenosine and guanosine.[15] Furthermore, alanine-mediated germination is only induced at concentrations above 60 mM.[16,17] However, alanine and inosine show strong synergy when added as co-germinant, even at sub-optimal concentrations. In contrast, *B. cereus* 3711 (ATCC 14579) germinates with either adenosine or inosine, as well as with low concentrations of alanine, cysteine and glutamine.[9] Other amino acid tested showed lower germination efficiency.

Two different inosine receptors (*GerI* and *GerQ*) have been detected in *B. cereus* 569.[16,17] Elimination of either of the two receptors is sufficient to disable inosine-mediated germination. Δ*gerI* mutants also showed reduced germination rates with alanine as sole germinant. However, this strain was able to germinate if both alanine and inosine are added together.[17] In contrast, Δ*gerQ* mutant spores were unable to germinate in

[a] *Department of Chemistry, University of Nevada-Las Vegas, 4505 Maryland Pkwy-Box 454003, Las Vegas, NV 89154, USA. E-mail: ernesto.abelsantos@unlv.edu; Fax: 702 895-4072; Tel: 702 895-2608*
[b] *Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461, USA*

† This article is dedicated to Prof. George W. Gokel on his 60th birthday.

the presence of inosine, but responded normally to alanine and inosine plus alanine.[16] Thus, it seems that there are at least two nucleoside-mediated pathways for germination in *B. cereus* 569. The first pathway only requires the presence of a nucleoside to activate spore germination. The second pathway needs both a nucleoside and an amino acid to function.

Although nucleoside receptors are required for the germination of *B. cereus* 569 spores, the molecular determinants for germinant binding and receptor activation are not known. In order to understand the requirements for nucleoside recognition in the two nucleoside-mediated germination pathways, we tested a series of inosine analogs for their ability to induce *B. cereus* 569 spore germination in the presence and absence of alanine (Fig. 1). Systematically changing nucleoside functional groups allowed determining epitopes necessary for germination activation. We determined that the two nucleoside-dependent germination pathways in *B. cereus* 569 recognize nucleosides differently. The nucleoside-only pathway is activated solely with inosine, while it is inhibited with adenosine. On the other hand, the nucleoside/alanine pathway can be activated by a larger variety of nucleoside analogs.

Results and discussion

B. cereus 569 spores germinate upon addition of inosine, but the germination shows a lag phase and non-linear kinetics

Spores have a highly dense structure and therefore scatter light strongly. Upon germination, the spore core hydrates and light scattering is reduced. As a result, a germinating spore suspension will show a large decrease in optical density when irradiated at 540 and 580 nm.[18] Analysis of optical density has been applied extensively to monitor the germination process.[12,13] As reported previously, addition of 0.06 to 1.5 mM inosine (**1**) to a suspension of *B. cereus* 569 spores was sufficient to trigger germination (Fig. 2(A)).[19]

Germinated spores lose the refractivity to heat, harsh chemicals, and UV light.[20,21] Heat treatment eliminates germinated cells, while it does not affect spore viability. Spores that were heat treated before inosine addition formed the expected number of colonies. On the other hand, heat treatment of spores that have been incubated with inosine resulted in 95% decrease in colony forming ability, thus confirming that inosine alone is sufficient to induce spore germination.

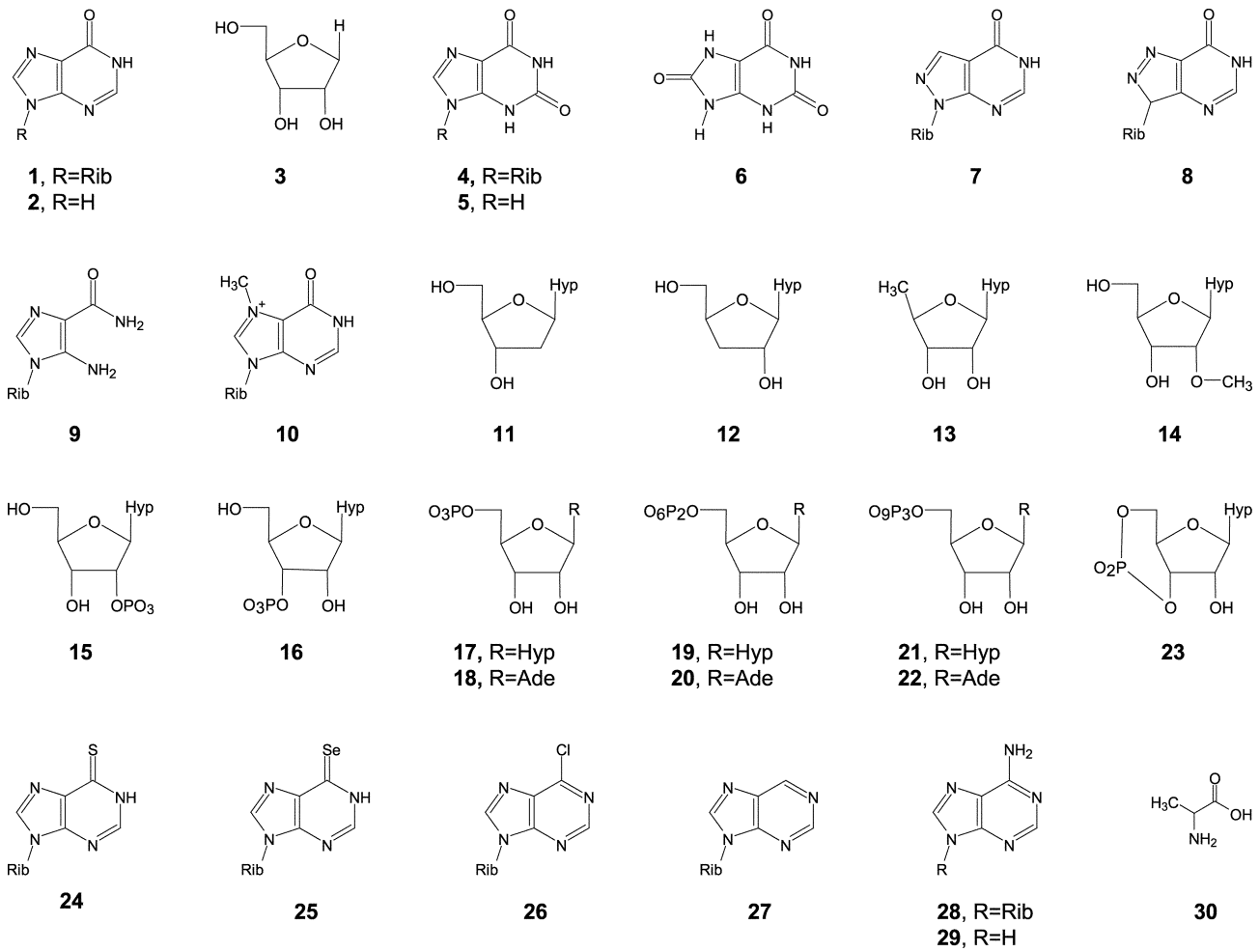

Fig. 1 Compounds tested as germinants and germination inhibitors: Rib = 9-β-D-ribose, H = hydrogen, Hyp = hypoxanthine (6-hydroxypurine), Ade = adenine (6-aminopurine), OPO_3 = phosphate, OP_2O_6 = diphosphate, OP_3O_9 = triphosphate and PO_2 = cyclic monophosphate.

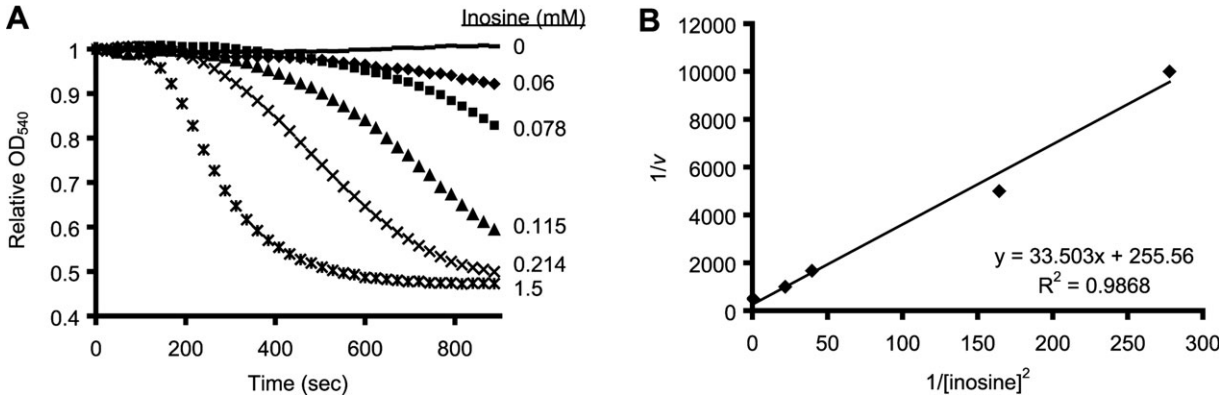

Fig. 2 (A) Germination of *B. cereus* spores as a function of inosine concentration. Suspensions of *B. cereus* spores were treated with 0 (lines), 0.06 (diamonds), 0.078 (squares), 0.115 (triangles), 0.214 (crosses) or 1.5 (stars) mM inosine. Germination was followed as the decrease of optical density at 540 nm for 35 min. Only the first 15 min of germination are shown for clarity. (B) Double reciprocal analysis of germination rate (v) *vs.* square of inosine concentration. The resulting plot was fitted to a linear equation with R^2 of 0.99.

For the kinetic analysis of the germination process, we hypothesized that *B. cereus* 569 spore germination simulates a unireactant enzyme system whereby the germination receptor represents the enzyme, inosine represents the substrate, and the germinated bacterium represents the product. Thus, germination rates (v) were calculated from the changes in optical density during the linear phase of the sigmoidal curves (Fig. 2(A)). The resulting germination rates were used for Michaelis–Menten analysis. We used double reciprocal Lineweaver–Burk plots to generate linear functions in order to calculate maximal germination rates (V_{max}) and the spores' affinity for inosine (K_m).[22]

Inosine-induced optical density changes are highly reproducible, with standard deviations between 0.010 and 0.035 absorbance units (Fig. 2(A)). However, we could not directly determine the apparent affinity of spores for inosine (K_m) or the maximum rate of germination (V_{max}) for inosine-mediated germination. Plotting the inverse of initial germination velocities ($1/v$) against the inverse of inosine concentrations (1/[inosine]) showed strong deviation from linearity (data not shown). The curvature of the double reciprocal plot shows that germination rates accelerate with increasing inosine concentration. This is characteristic of systems that show positive cooperativity.[22] Although the plot of $1/v$ *vs.* 1/[inosine] is curved, the plot of $1/v$ *vs.* $1/[\text{inosine}]^2$ is linear (Fig. 2(B)). Plotting $1/v$ *vs.* $1/[\text{inosine}]^3$ resulted in a parabolic graph (data not shown). These results suggest that the inosine-only germination machinery consists of no more than two strongly interacting binding sites. From the plot in Fig. 2(B) an apparent binding constant (K'_m) of 130 μM and V_{max} of 0.003 OD s^{-1} were calculated. It should be noted that K'_m is a constant that contains both the real inosine dissociation constant (K_m) and interaction factors that describe the cooperativity between different binding sites.

The cooperative nature of inosine-mediated germination was further probed by determining the Hill coefficient.[22] A Hill coefficient of 1 corresponds to systems where no cooperativity is observed. Hill coefficients higher or lower than one result from systems demonstrating positive or negative cooperativity, respectively.[23] Plotting of the logarithm of the inverse relative OD against the logarithm of inosine concentration yields a straight line with an R^2 value of 0.98 (data not shown). From the exponential equation fitting the data, we determined a Hill coefficient (k) of 1.7.

The derivation of the Hill equation assumes strong synergy between binding sites. As a result, it is expected that only unbound and completely saturated species are present in solution and that single bound species concentrations are negligible. Under these assumptions, the Hill coefficient would be an integer number. The fact that we calculated a non-integer Hill coefficient suggests that the fraction of spores where only one receptor is activated is not unimportant. However, since the Hill coefficient is close to a value of 2, it further suggests the existence of two inosine binding sites.[24] Thus, *B. cereus* inosine-only spore germination is a strongly cooperative process between two separate binding sites.

Double reciprocal and Hill plots suggest the presence of two distinct, strongly interacting binding sites. This data is consistent with previous reports that showed the existence of two different germination receptors that are required to respond to inosine as sole germinant.[16,17] Thus, our kinetic data support a mechanism where binding of inosine to one receptor (*e.g. gerI*) creates an activated spore with increased affinity for binding inosine at the second receptor (*e.g. gerQ*). Only when both receptors are complexed with inosine, the spore becomes competent for germination.

B. cereus 569 spores do not germinate with other purine degradation products

Inosine (**1**) is a nucleoside composed of hypoxanthine (**2**) and D-ribose (**3**). In mammalian cells, hypoxanthine is obtained directly from inosine through the purine degradation pathway.[25] Hypoxanthine is then converted to xanthine and eventually to uric acid by xanthine oxidase. As previously reported for *B. cereus* 3711,[26] addition of hypoxanthine (**2**), D-ribose (**3**), or an equimolar mixture of both, did not cause any changes in optical density or heat resistance in *B. cereus* 569 spores, even at 10 mM final concentrations.

Even though hypoxanthine (**2**) and D-ribose (**3**) are unable to activate germination, they might still interact with the germination receptors and inhibit germination by excluding inosine from the receptors' binding sites. However, addition of

2.5 mM inosine to spores pre-treated with a 10 mM of hypoxanthine (**2**) and D-ribose (**3**) resulted in spore germination at rates comparable with spores treated only with inosine. Addition of 10 mM xanthosine (**4**), xanthine (**5**) or uric acid (**6**) to spore suspension neither activated nor inhibited inosine-mediated germination.

Since the Ger proteins are located in the inner membrane,[7] germinant molecules have to diffuse across the exosporium, coat and cortex layers to activate the germination program. To test whether the inactivity of purine degradation products is due to coat impermeability, *B. cereus* spores were subjected to decoating. This process removes the exosporium and spore coats and places the germination receptors in contact with the extracellular milieu.[27] Decoated spores germinated in the presence of inosine, albeit at a slower rate than intact spores. Addition of 10 mM hypoxanthine (**2**), D-ribose (**3**), xanthosine (**4**), xanthine (**5**) or uric acid (**6**) did not trigger germination in decoated spores. Thus, except for inosine, none of the compounds formed during the purine degradation pathway seems to interact with the germination machinery.

Small changes in inosine structure affect germination efficiency

The purine nucleobase is a heterobicyclic ring system with endocyclic nitrogens at positions 1, 3, 7 and 9. Allopurinol riboside (**7**) is an inosine analog where the normal N7 position was substituted with a N8–N9 diaza group. Formycin B (**8**), on the other hand, substitutes N9 for a N7–N8 diaza group. Both of these compounds differ from inosine solely in the relative positions of N7, C8 and N9. These small differences, however, significantly affect spore germination rates. Addition of 2.5 mM allopurinol riboside (**7**) or formycin B (**8**) resulted in germination rates that were reduced by more than 90% compared to inosine-triggered germination. Lower concentrations of allopurinol riboside (**7**) or formycin B (**8**) did not trigger spore germination.

AICA riboside (**9**) is an inosine analog that lacks the C2 position, while 7-methylinosine (**10**) has an extra methyl group. None of these compounds is able to induce spore germination, even at 10 mM final concentration. The lack of germination activity of base-modified inosine analogs suggests that the hypoxanthine nucleobase is recognized as a major germination determinant.

Sugar oxygens are required for inosine-mediated germination

Ribose is a pentose sugar with three distinct hydroxyl groups. 2′-Deoxyinosine (**11**), 3′-deoxyinosine (**12**), and 5′-deoxyinosine (**13**) are inosine analogs that lack the hydroxyl group at the 2′-, 3′- and 5′-positions, respectively. 2′-*O*-methylinosine (**14**), on the other hand, substitutes the hydroxyl for a bulkier methoxy group. All four compounds failed to induce germination or to block inosine mediated germination, even at the highest concentrations tested. Thus, all three hydroxyl groups are important epitopes for inosine-mediated germination. Furthermore, since 2′-*O*-methylinosine (**14**) has no effect on germination, we inferred that the 2′-hydroxyl is a hydrogen bond donor when inosine interacts with the germination machinery.

Phosphorylated inosine analogs cannot induce spore germination

Nucleosides are intermediates in the biosynthesis of nucleotides. Thus, the intracellular concentration of nucleosides is diminishingly small. In turn, nucleotides are utilized for energy storage, nucleic acid metabolism, and signal transduction. Specifically, inosinic acid (5′-IMP) represents a branch point in purine nucleotide synthesis.[28] 2′-IMP (**15**), 3′-IMP (**16**) and 5′-IMP (**17**) have phosphate groups substituting their hydroxyl groups. 5′-IDP (**19**) and 5′-ITP (**21**) have diphosphate and triphosphate groups at the 5′-position, respectively. 3′,5′-cIMP (**23**) have a phosphate bridge linking the 3′- and 5′-hydroxyls of inosine. All phosphorylated inosine analogs were tested for their ability to induce spore germination. However, no effect was observed on spore optical density or heat resistance, even at 10 mM final concentrations. Furthermore, phosphorylated analogs were unable to block inosine-mediated germination. Decoated spores demonstrated the same lack of response to phosphorylated inosine analogs as intact spores. Since all phosphorylated inosine analogs tested were unable to induce germination even in permeabilized spores, it suggests that only a nucleoside structure is recognize by *B. cereus* 569 spores.

The 6-carbonyl oxygen in the inosine nucleobase is an important epitope for germination

Inosine (**1**) is a purine analog with an exocyclic 6-oxo group. To test the importance of the carbonyl oxygen, the 6-position was varied. 6-thioinosine (**24**) and 6-selenoinosine (**25**) replace oxygen with sulfur and selenium, respectively. Oxygen, sulfur, and selenium have similar chemical and physical properties. However, sulfur and selenium have larger atomic radii and lower electronegativity. Thus, sulfur and selenium atoms cannot form the strong hydrogen bonds that are hallmark of oxygen atoms. 6-Chloropurine riboside (**26**) replaces oxygen for an isosteric chlorine atom at the 6-position. Purine riboside (**27**), on the other hand, does not posses any exocyclic group. Of the 6-substituted compounds tested, only 6-chloropurine riboside (**26**) had marginal germination activity. Whereas addition of 2.5 mM inosine (**1**) caused the spores' optical density to change by 50%, addition of the same concentration of 6-chloropurine riboside (**26**) resulted in less than 10% reduction of optical density over the same period. The fact that none of the 6-substituted analogs is able to activate germination efficiently suggests that the inosine germination machinery is tuned to recognize the 6-oxo group.

Inosine-mediated spore germination is inhibited by adenosine

Adenosine (**28**) is a nucleoside analog that has an exocyclic amino group at position 6 and is involved in numerous cellular processes.[29] Adenosine (**28**) has been reported to be a weak germinant of *B. cereus* 569 spores.[17] However, in our hands adenosine (**28**) was unable to activate *B. cereus* 569 spore germination. On the contrary, pre-incubation of *B. cereus* 569 spores with adenosine (**28**) prevented inosine-mediated germination (Fig. 3(A)). Heat-resistance assay confirmed that adenosine-treated spores did not germinate upon inosine addition. Thus, adenosine (**28**) seems to be able to interact with the inosine receptor, but the binding is not productive for

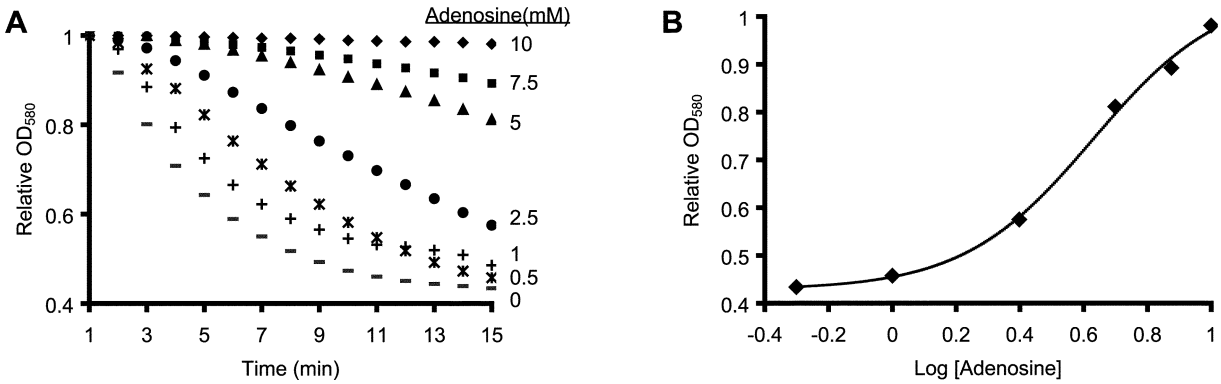

Fig. 3 (A) Inhibition of *B. cereus* spore germination as a function of adenosine concentration. Suspensions of *B. cereus* spores were treated with 2.5 mM inosine and 0 (lines), 0.5 (crosses), 1 (stars), 2.5 (circles), 5 (triangles), 7.5 (squares) or 10 (diamonds) mM adenosine. Germination was followed as the decrease of optical density at 580 nm. (B) Dose–response curves of germination *vs.* concentration of adenosine. The optical density values were measured 10 min after germination initiation and fitted to a four-parameter logarithmic function.

germination. Furthermore, if adenosine (**28**) is removed from the media, spores are able to resume their normal germination program upon inosine treatment.

Previous works that identified adenosine as a *B. cereus* spore germinant used spore preparation that were purified by washing extensively with water.[9,17] Although this procedure is sufficient for most experiments, we found great variability between different spore preparations. In some cases, adenosine behaved as germinant at low concentrations. Other preparations were only activated for germination if a large amount of adenosine was added. Yet other spores did not respond to adenosine at all. We attributed these erratic results to the inability of water washing to remove completely unreleased spores and germinating spores.[18] This contamination can serve as a source of trace amounts of amino acids, and trigger germination through the nucleoside/alanine germination pathway (see below). Due to these inconsistent results, we purified *B. cereus* 569 spores by centrifugation through a HistoDenz gradient followed by extensive washing. Spores prepared through this procedure responded reproducibly to the different nucleosides and amino acids tested. Thus, a clean spore preparation is needed to distinguish between inosine-only and nucleoside/alanine germination pathways. Similarly, previous reports have tested different nucleoside analogs with *B. cereus* spores that were purified by washing alone. These reports show that many nucleoside analogs are able to trigger germination by themselves.[30–32] In our hands, however, inosine is the only nucleoside capable of triggering spore germination on its own. Although some of these differences may be strain specific, they also are explained easily by improved purification procedures.

Differences in instrumentation could also affect germination results. A close look at the published data,[17] shows that addition of inosine to a spore suspension caused a maximum germination rate of 10% min^{-1}. For a spore suspension with an initial OD_{580} of 1.0, this represents a change of 0.1 OD min^{-1}. Substitution of inosine with adenosine slows the germination rate a hundred fold. Thus, under the same conditions, the OD_{580} change would only be 0.001 OD min^{-1}. Our instrumentation is unable to determine accurately such small changes in optical density. When observed in our laboratory, these minute optical density variations could be attributed to slow spore sedimentation.

Lower inhibitor concentrations increase germination rates

Since inosine-mediated germination follows non-linear kinetics, determining inhibition constants from double reciprocal plots is not trivial. However, the extent of germination inhibition is directly proportional to adenosine concentration. Thus, dose–response analysis yields a sigmoidal plot that can be fitted with a four-parameter logistic function (Fig. 3(B)).[33] The inflection point of the curve represents the concentration of adenosine (**28**) that causes 50% inhibition in optical density change (IC_{50}).

Use of the Cheng–Prusoff equation for determination of inhibition constant (K_i) from IC_{50} values is based on an assumption that simple bi-molecular interaction kinetics is strictly followed.[34] Recently, the Cheng–Prusoff equation has been modified to include a cooperative coefficient in cases where the agonist binds to the receptor in a synergistic manner.[35] Since the K'_m value calculated from Fig. 2(B) contains the affinity of spores for inosine as well as the interaction factors for cooperative inosine binding, this value can be used in the modified Cheng–Prusoff equation. The K_i for adenosine inhibition of *B. cereus* inosine-mediated germination was calculated to be 109 μM.

Adenosine-mediated inhibition requires the nucleobase and the ribose sugar forming a covalent nucleoside

Adenosine is a nucleoside composed of an adenine base (**29**) and a D-ribose sugar (**3**). Pre-incubation with adenine (**29**), D-ribose (**3**), or an equimolar mixture of both do not prevent inosine-mediated germination. Furthermore, neither, AMP (**18**), nor ADP (**20**), nor ATP (**22**) had any effect on inosine-mediated germination. Thus, just as for germination activation, germination inhibition requires an intact nucleoside structure. This suggests that the structural analogs, adenosine and inosine, are competing for the same sites in the germination receptor.

Adenosine is a co-germinant with alanine

Alanine (**30**) is an amino acid with strong germinant properties. Addition of alanine (**30**) alone at high concentrations is sufficient to trigger *B. cereus* 569 spore germination. In the presence of inosine (**1**), alanine (**30**) acts synergistically to accelerate spore germination three-fold (Table 1).[16,36,37] Although adenosine (**28**) alone is not a germinant, but inhibits inosine-mediated germination, *B. cereus* 569 spores incubated with adenosine (**28**) and alanine (**30**) germinate at the same rate as spores treated with inosine (**1**) and alanine (**30**). Thus, adenosine (**28**) inhibits inosine-induced germination, but is a co-germinant when alanine (**30**) is present.

Although in principle, the kinetics of *B. cereus* 569 spore co-germination could be treated as a rapid equilibrium bireactant enzyme, the alanine/nucleoside germination synergy proved too complex to analyze by double reciprocal plots (data not shown). Plotting $1/v$ *vs.* 1/[adenosine], 1/[adenosine]^2 or 1/[adenosine]^3 resulted in non-linear plots. This could be the result of multiplex interactions between the *GerI* and *GerQ* receptors (that are inhibited by adenosine), the *GerL* receptor (that is activated by amino acids only), and an unknown nucleoside receptor (that is activated by alanine and adenosine).

GerI and *gerQ*-null mutant spores cannot germinate with inosine (**1**) alone.[16,17] However, both mutants are able to germinate if inosine (**1**) or adenosine (**28**) are supplemented with alanine (**30**). Thus, it seems that there is a nucleoside/alanine germination pathway distinct from inosine-only triggered germination. Since the receptor in the nucleoside/alanine pathway can use both inosine (**1**) and adenosine (**28**) to trigger germination, the unidentified nucleoside receptor seems to be more flexible in recognizing purine nucleosides. Indeed, allopurinol riboside (**7**), formycin B (**8**), thioinosine (**24**), 6-chloropurine riboside (**26**), and purine riboside (**27**) were able to germinate *GerI*-null spores when supplemented with alanine, albeit at lower efficiency than inosine (Table 1). On the other hand, hypoxanthine (**2**) plus D-ribose (**3**), xanthosine (**4**), AICA riboside (**9**), 7-methylinosine (**10**), 2′-deoxyinosine (**11**), 3′-deoxyinosine (**12**), 5′-deoxyinosine (**13**), 2′-*O*-methylinosine (**14**) and 5′-IMP (**17**), failed as alanine co-germinants. Thus, even though position 6 and endocyclic nitrogen recognition is not necessary in the nucleoside/alanine germination pathway, there is still an absolute requirement for an intact nucleoside structure. It is also apparent that exocyclic groups, except at position 6, impede germination. Furthermore, all three sugar hydroxyls are needed also to trigger germination.

These results are consistent with previous reports that show flexibility of nucleoside recognition in the presence of saturating concentrations of alanine.[31] In this work, we were able to differentiate the binding mode of the two different pathways that use nucleosides as germination triggers (Fig. 4).

Table 1 Effect of selected nucleosides in *B. cereus* 569 spore germination rate

Relative germination rates[a]			
Compound[b]	Alone	+ Inosine[c]	+ Alanine[d]
1	1.0	N/A	3.1
2 + **3**	0	1.0	0
4	0	1.0	0
7	0.1	1.0	0.4
8	0.1	1.0	0.6
9	0	1.0	0
10	0	1.0	0
11	0	1.0	0
12	0	1.0	0
13	0	1.0	0
14	0	1.0	0
17	0	1.0	0
24	0	1.0	0.6
26	0.1	1.0	0.4
27	0	1.0	0.3
28	0	0.4	3.1
29 + **3**	0	1.0	0
30	0	3.1	N/A

[a] Germination rates for individual compounds are expressed as percentage of the germination rate at saturating concentrations of inosine. [b] All compounds were tested at 2.5 mM final concentration. [c] Inosine was at saturating concentration for germination (2.5 mM). [d] Alanine was at sub-optimal concentrations for germination (40 μM). N/A: not applicable.

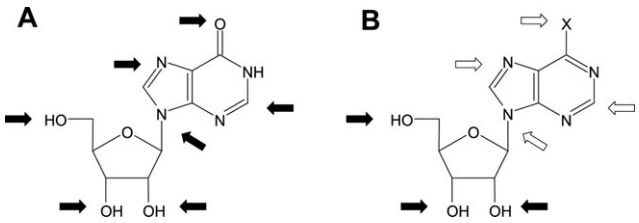

Fig. 4 Epitopes for the recognition of nucleosides in the (A) inosine-only and (B) nucleoside/alanine germination pathways. Solid arrows signal functional groups necessary for receptor activation. Open arrows signal positions that can be varied; X represents oxygen, sulfur, hydrogen, or chlorine atoms.

Conclusions

Due to its susceptibility to degradation by purine metabolism, inosine seems to be an unlikely candidate to activate spore germination. Yet, no other nucleoside tested was able to trigger *B. cereus* 569 spore germination without a co-germinant. In contrast, upon supplementation with sub-germinant concentrations of alanine, nucleoside analogs with diverse purine base structures are able to support spore germination. However, modifications of the sugar moiety inactivate germination effects on both the inosine-only and the nucleoside/alanine pathways.

The effect of alanine on spore germination is more striking in the presence of adenosine. Adenosine acts as a germination inhibitor of the inosine-only germination pathway. In contrast, addition of alanine not only lessens spore germination inhibition, but adenosine becomes a co-germinant in the nucleoside/alanine germination pathway. Adenosine/alanine-mediated germination functions even when the known inosine receptors, *GerI* and *GerQ*, are disabled. The data suggests two different nucleoside recognition modes during spore germination: *GerI* and *GerQ* receptors are activated by inosine in the absence of other co-germinants. However, if alanine is present

as a co-germinant a different nucleoside receptor(s), with wider substrate specificity, is activated.

Experimental

Materials and methods

Nucleosides were purchased from Sigma-Aldrich Corporation, (St. Louis, MO), Berry & Associates, Inc (Dexter, MI), or Moravek Biochemicals and Radiochemicals (Brea, CA). All other compounds were obtained from Sigma. All compounds were of the best purity available. *B. cereus* 569 (ATCC 10876) cultures were obtained from the America Type Culture Collection (Manassas, VA). The *B. cereus* 569 Δ*GerI* (AM1314, Tn917-LTV1::gerIA5 (ino-5) Eryr trp-1 Strr) strain was a generous gift from Prof. Anne Moir, University of Sheffield, UK. Spore germination was monitored on a Biomate 5 spectrophotometer at 580 nm or a Labsystems iEMS 96-well plate reader (ThermoElectron Corporation, Waltham, MA) fitted with a 540 nm cut-off filter.

Spore preparation

B. cereus 569 cells were plated in DIFCO sporulating media (DSM) agar. Plates were incubated for 72 h at 37 °C. The resulting bacterial lawns were scraped from the plates and resuspended in deionized water. Spores were purified by centrifugation through a 20–50% HistoDenz gradient. Purified spores were washed ten times with water and stored at 4 °C.

Analysis of nucleoside-mediated germination

Spore germination was monitored spectrophotometrically whereby the loss in light diffraction following addition of inosine alone or inosine and alanine was reflected by decreased optical density at 580 nm (for Biomate 5 spectrophotometer) or above 540 nm (for Labsystems iEMS 96-well plate reader). Spores were heat-activated at 70 °C for 30 min before resuspension in germination buffer (50 mM Tris-HCl pH 7.5, 10 mM NaCl) to an OD_{580} of 1. The spore suspension was monitored for auto-germination at OD_{580} for 1 h. Only spores that did not auto-germinate were used for subsequent assays in 96-well format. All germination experiments were carried out in 96-well plates, at room temperature, and with a total volume of 200 μl/well. Every experiment was carried in triplicate with at least two different spores preparation. Spores were allowed to germinate upon exposure to 10 mM nucleoside supplemented with 0 or 40 μM alanine. Compounds that showed germination activity were added to fresh spores at decreasing concentrations in the presence of 0 or 40 μM alanine. The concentration range for nucleosides was selected to avoid data clusters in the double reciprocal plots. Spore germination was evaluated based on decrease in OD_{540}. Data points were obtained every 20 s for 35 min. The germination extent of each well at each time point was expressed as a fraction of the actual OD divided by the OD obtained at the beginning of germination. Relative OD values were plotted against time. All measurements show standard deviations of less than 10%. Germination rates (v) were calculated as the slope of the initial linear portion immediately following the lag phase of relative OD values over time. All plots were fitted using the linear regression analysis from the SigmaPlot v.9 software to determine apparent K_m and V_{max} values.

Assay for heat resistance

Spore aliquots were serially diluted in water until about 10 CFU μl^{-1}. Aliquots were incubated at 80 °C for 10 min. A 100 μl sample from each aliquot was plated onto LB agar. Spores incubated with inosine were similarly heat-treated and plated. All plates were incubated overnight at 37 °C. The percentage of germinated cells was determined from the ratio of colonies formed before and after inosine addition.

Decoating of *B. cereus* spores

Decoating for *B. cereus* 569 was based on published procedures.[27] Spore suspensions were incubated at 37 °C for 90 min in 5 mM CHES buffer, pH 8.6, supplemented with 8 M urea, 70 mM dithiothreitol, and 1% (wt/vol) sodium dodecyl sulfate (SDS). The spores were then pelleted and washed five times with ice-cold distilled water. The permeability to lysozyme was checked by measuring the loss of OD at 580 nm of an aliquot of the spore suspension incubated in 50 mM NaCl supplemented with lysozyme (30 μg ml^{-1}). This gave 30 to 40% OD loss in less than 30 min, demonstrating that the extraction had removed coat layers sufficiently to allow this enzyme to penetrate to the cortex and induce cortex lysis. The permeabilized spores were heat activated for 30 min at 70 °C, then cooled and used within 2 h.

Calculation of inhibition constants (K_i)

Purified spores were diluted in 1 ml germination buffer. Nucleoside analogs were added to 10 mM final concentration. Spore suspensions were incubated for 15 min at room temperature while monitoring OD_{540}. If no germination was detected, inosine was then added to 2.5 mM final concentration. Germination was monitored every minute for 15 min. Compounds that showed inhibitory properties were added to fresh spore aliquots at decreasing concentrations. Inosine was then added to 2.5 mM concentration, as above. Relative OD_{540} obtained at arbitrary time points were plotted against the logarithm of inhibitor concentrations. The data was fitted using the four parameter logistic function of SigmaPlot v.9 software to obtain IC_{50} values.[33] Inhibition constants (K_i) were calculated from a modified Chang–Prusoff equation.[35]

Acknowledgements

This work was supported by the Ellison Medical Foundation Young Scholar Award in Global Infectious Diseases and by Public Health Service Grant 1R01AIGM053212 from the National Institute of Health.

References

1 M. Mock and A. Fouet, *Annu. Rev. Microbiol.*, 2001, **55**, 647.
2 C. J. Paredes, K. V. Alsaker and E. T. Papoutsakis, *Nat. Rev. Microbiol.*, 2005, **3**, 969.
3 A. Moir, B. M. Corfe and J. Behravan, *Cell. Mol. Life Sci.*, 2002, **59**, 403.
4 A. Driks, *Trends Microbiol.*, 2002, **10**, 251.

5 P. Setlow, *Curr. Opin. Microbiol.*, 2003, **6**, 550.
6 A. Moir, *J. Appl. Microbiol.*, 2006, **101**, 526.
7 M. Paidhungat and P. Setlow, *J. Bacteriol.*, 2001, **183**, 3982.
8 T. Igarashi and P. Setlow, *J. Bacteriol.*, 2005, **187**, 2513.
9 L. M. Hornstra, Y. P. de Vries, M. H. Wells-Bennik, W. M. de Vos and T. Abee, *Appl. Environ. Microbiol.*, 2006, **72**, 44.
10 N. Fisher and P. Hanna, *J. Bacteriol.*, 2005, **187**, 8055.
11 L. M. Hornstra, Y. P. de Vries, W. M. de Vos, T. Abee and M. H. Wells-Bennik, *Appl. Environ. Microbiol.*, 2005, **71**, 774.
12 C. R. Woese, J. C. Vary and H. O. Halvorson, *Proc. Natl. Acad. Sci. USA*, 1968, **59**, 869.
13 M. R. Massa, M. R. Cardillo, R. Tarsi and S. Burberi, *Nuov. Ann. Ig. Microbiol.*, 1975, **26**, 450.
14 J. A. Ireland and P. C. Hanna, *J. Bacteriol.*, 2002, **184**, 1296.
15 A. Moir, E. H. Kemp, C. Robinson and B. M. Corfe, *Soc. Appl. Bacteriol. Symp. Ser.*, 1994, **23**, 9S.
16 P. J. Barlass, C. W. Houston, M. O. Clements and A. Moir, *Microbiology*, 2002, **148**, 2089.
17 M. O. Clements and A. Moir, *J. Bacteriol.*, 1998, **180**, 6729.
18 W. L. Nicholson and P. Setlow, *Molecular Biological Methods for Bacillus*, ed. C. R. Hardwood and S. M. Cutting, John Wiley & Sons, Ltd, New York, NY, 1990, ch. 9, pp. 391–450.
19 R. A. Preston and H. A. Douthit, *J. Gen. Microbiol.*, 1988, **134**, 3001.
20 P. Setlow, *J. Appl. Microbiol.*, 2006, **101**, 514.
21 W. L. Nicholson, N. Munakata, G. Horneck, H. J. Melosh and P. Setlow, *Microbiol. Mol. Biol. Rev.*, 2000, **64**, 548.
22 I. H. Segel, *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems*, Wiley Classics Library Edition, Wiley Interscience Publication, New York, NY, 1993.
23 G. G. Hammes and C. W. Wu, *Annu. Rev. Biophys. Bioeng.*, 1974, **3**, 1.
24 S. J. Edelstein and W. G. Bardsley, *J. Mol. Biol.*, 1997, **267**, 10.
25 C. Scazzocchio, *Prog. Ind. Microbiol.*, 1994, **29**, 221.
26 A. A. Yousten, *Can. J. Microbiol.*, 1975, **21**, 1192.
27 J. Behravan, H. Chirakkal, A. Masson and A. Moir, *J. Bacteriol.*, 2000, **182**, 1987.
28 M. Pizzichini, A. Di Stefano, G. Pompucci and E. Marinello, *Adv. Exp. Med. Biol.*, 1986, **195 Pt B**, 381.
29 C. K. Mathews, K. E. v. Holde and K. G. Ahern, in *Biochemistry*, ed. B. Roberts, Addison Wesley, Longman Inc., San Francisco, CA, 2000.
30 H. Shibata, N. Ohnishi, K. Takeda, H. Fukunaga, K. Shimamura, E. Yasunobu, I. Tani and T. Hashimoto, *Can. J. Microbiol.*, 1986, **32**, 186.
31 S. Senesi, G. Cercignani, G. Freer, G. Batoni, S. Barnini and F. Ota, *J. Gen. Microbiol.*, 1991, **137**, 399.
32 S. Senesi, G. Freer, G. Batoni, S. Barnini, A. Capaccioli and G. Cercignani, *Can. J. Microbiol.*, 1992, **38**, 38.
33 D. Rodbard, *Clin. Chem.*, 1974, **20**, 1255.
34 P. J. Munson and D. Rodbard, *J. Recept. Res.*, 1988, **8**, 533.
35 H. C. Cheng, *J. Pharmacol. Toxicol. Methods*, 2001, **46**, 61.
36 K. P. McCann, C. Robinson, R. L. Sammons, D. A. Smith and B. M. Corfe, *Lett. Appl. Microbiol.*, 1996, **23**, 290.
37 S. C. Warren and G. W. Gould, *Biochim. Biophys. Acta*, 1968, **170**, 341.