Dissecting interactions between nucleosides and germination receptors in *Bacillus cereus* 569 spores

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Bacillus cereus 569 spores germinate either with inosine as a sole germinant or with a combination of nucleosides and L-alanine. Whereas the inosine-only germination pathway requires the presence of two different germination receptors (GerI and GerQ) to be activated, the nucleoside/alanine germination pathway only needs one of the two receptors. To differentiate how nucleoside recognition varies between the inosine-only germination pathway and the nucleoside/alanine germination pathway, we tested 61 purine analogues as agonists and antagonists of the two pathways in wild-type, Δ gerI and Δ gerQ spores. The structure–activity relationships of germination agonists and antagonists suggest that the inosine-only germination pathway is restricted to recognize a single germinant (inosine), but can be inhibited in predictable patterns by structurally distinct purine nucleosides. *B. cereus* spores encoding GerI as the only nucleoside receptor (Δ gerQ mutant) showed a germination inhibition profile similar to wild-type spores treated with inosine only. Thus, GerI seems to have a well-organized binding site that recognizes inosine and inhibitors through specific substrate–protein interactions. Structure–activity analysis also showed that the nucleoside/alanine germination pathway is more promiscuous toward purine nucleoside agonists, and is only inhibited by hydrophobic analogues. *B. cereus* spores encoding GerQ as the only nucleoside receptor (Δ gerI mutant) behaved like wild-type spores treated with inosine and L-alanine. Thus, the GerQ receptor seems to recognize substrates in a more flexible binding site through non-specific interactions. We propose that the GerI receptor is responsible for germinant detection in the inosine-only germination pathway. On the other hand, supplementing inosine with L-alanine allows bypassing of the GerI receptor to activate the more flexible GerQ receptor.

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INTRODUCTION

Endospore-forming bacteria produce some of the most potent toxins known (Barth *et al.*, 2004) and are important pathogens in hospital-borne infections (*Clostridium difficile*), food contamination (*Bacillus cereus*, *Clostridium botulinum*), wound infestation (*Clostridium tetani*) and bioterrorism (*Bacillus anthracis*) (Cloud & Kelly, 2007; Edlich *et al.*, 2003; Ehling-Schulz *et al.*, 2004; Mock & Fouet, 2001; Ting & Freiman, 2004). Both *Bacillus* and *Clostridium* species form spores under unfavourable environmental conditions (Paredes *et al.*, 2005; Piggot, 1996; Piggot & Hilbert, 2004; Sauer *et al.*, 1995; Stephenson & Lewis, 2005). These spores return to normal vegetative growth, a process called germination, upon encountering favourable environmental conditions (Moir *et al.*, 2002; Setlow, 2003). In both *Bacillus subtilis* and *B. cereus*, the initial step in the germination pathway is believed to be the binding of specific germinants to a family of tripartite membrane

receptors (Ger proteins) (Moir *et al.*, 2002; Moir, 2006; Setlow, 2003). Following the activation of these receptors, spores release dipicolinic acid, calcium and amino acids (Moir, 2006; Setlow, 2003). Subsequently, the core of the spores becomes hydrated, and the spore cortex and spore-specific proteins are hydrolysed (Chirakkal *et al.*, 2002; Sanchez-Salas *et al.*, 1992; Setlow & Wailes, 1976). Approximately 30 min after addition of germinants, the newly formed vegetative cells start to divide (Levinson & Hyatt, 1966; Setlow, 2003).

While nucleosides and amino acids are recognized as germinants by many *Bacillus* species, there are differences in spore germination profiles (Barlass *et al.*, 2002; Hornstra *et al.*, 2006; Ireland & Hanna, 2002). There are two distinct nucleoside-dependent germination pathways in *B. cereus* 569 (Barlass *et al.*, 2002; Clements & Moir, 1998): the inosine-only pathway and the nucleoside/alanine pathway. The nucleoside/alanine germination pathway can be

activated by a larger variety of nucleoside analogues in the presence of L-alanine (Abel-Santos & Dodatko, 2007). These results are consistent with previous spore germination experiments in *B. cereus* NCIB 8122 and T strains (Preston & Douthit, 1988; Senesi *et al.*, 1991; Shibata *et al.*, 1986; Yousten, 1975). *B. anthracis*, a species closely related to *B. cereus*, can also germinate using this pathway (Ireland & Hanna, 2002; Weiner *et al.*, 2003). Analysis of structurally diverse nucleosides as co-germinants with L-alanine suggested that, in the nucleoside/alanine pathway, germination-inducing activity is governed by the structural properties of the sugar rather than the base moieties of the nucleosides (Senesi *et al.*, 1991; Shibata *et al.*, 1986). Thus, it seems that the germination receptor(s) that activate the nucleoside/alanine germination pathway do not recognize the purine ring of the nucleoside to trigger germination.

In addition to the nucleoside/alanine germination pathway, *B. cereus* has also developed a mechanism that allows it to germinate in the presence of inosine as a sole germinant. The inosine-only germination pathway has been previously described in *B. cereus* strains 569 and ATCC 14579 (Barlass *et al.*, 2002; Clements & Moir, 1998; Hornstra *et al.*, 2006).

The fact that *B. cereus* has two interrelated nucleoside-mediated germination pathways, and that the two pathways recognize nucleosides differently, points to possible adaptation to distinct environmental niches. Interestingly, two germination receptors, GerI and GerQ, have been linked to nucleoside-mediated germination of *B. cereus* 569 spores (Barlass *et al.*, 2002; Clements & Moir, 1998). A third germination receptor, GerL, is believed to be involved, together with GerQ, in detecting L-alanine as a germinant (Barlass *et al.*, 2002; Hornstra *et al.*, 2006). However, the relative contributions of these receptors to the inosine-only and nucleoside/alanine germination pathways have not been determined.

AgerQ *B. cereus* spores are unable to germinate in the presence of inosine alone, while *AgerI* *B. cereus* spores show greatly reduced germination rates in the presence of a single germinant (Barlass *et al.*, 2002; Clements & Moir, 1998). However, *AgerI* and *AgerQ* strains germinate efficiently in the presence of a combination of inosine and suboptimal concentrations of L-alanine (Barlass *et al.*, 2002; Clements & Moir, 1998). Thus, both the GerI and GerQ receptors are needed to activate the inosine-only germination pathway. The nucleoside/alanine germination pathway, on the other hand, seems to require only one of these two nucleoside receptors to synergize with L-alanine.

The ability of *B. cereus* 569 spores to use structurally similar compounds to trigger alternative germination pathways makes it an ideal model to study how different signals are used for spore germination. However, since even minute changes in the inosine structure severely impair the inosine-only germination pathway (Abel-Santos & Dodatko, 2007), few agonists have been found for structure–activity relationship analysis. In contrast, a number of nucleosides are competitive inhibitors of nucleoside-mediated germination

and probably bind to the same site as inosine. The inhibition constant (K_i) measures the affinity of receptors to inhibitors. Thus, changes in K_i correlate to structural features in the nucleoside-binding sites of germination receptors. Analysis of the resulting inhibitory structure–activity relationships can provide information on the specificity of a receptor, the physical and chemical architecture of binding sites, and the kinetic mechanism of the germination process (Gero, 1972; Segel, 1993).

In order to understand the requirements for nucleoside recognition by the GerI and GerQ receptors, we tested 61 purine analogues as germination agonists and antagonists for wild-type, *AgerI* and *AgerQ* spores, in the presence or absence of L-alanine. Systematically changing nucleoside functional groups allowed the determination of epitopes necessary for germination receptor activation and inhibition. We found that the inosine-only germination pathway has very narrow substrate specificity, but is inhibited in predictable patterns by a number of nucleoside analogues. A similar profile was observed for mutant *B. cereus* spores (*AgerQ*) that encoded GerI as the only nucleoside receptor. In contrast, the presence of L-alanine converted many nucleosides from antagonists to agonists of spore germination. In this case, only 6-substituted adenosine analogues were able to inhibit inosine/alanine-mediated spore germination. Mutant spores (*AgerI*) that encoded GerQ as the only nucleoside receptor mirrored this behaviour. We speculate that the more stringent GerI receptor is responsible for initial germinant detection in the inosine-only germination pathway. On the other hand, supplementing inosine with L-alanine allows bypassing of the GerI receptor and activates the more flexible GerQ receptor.

METHODS

Strains, reagents and chemicals. *Bacillus cereus* strain 569 (*trp-1* Str^r), *Bacillus cereus* strain AM1311 (*AgerQ*, Tn917-LTV1::*gerQA2* (*ino-2*) Ery^r *trp-1* Str^r) and *Bacillus cereus* strain AM1314 (*AgerI*, Tn917-LTV1::*gerIA5* (*ino-5*) Ery^r *trp-1* Str^r) were obtained from the *Bacillus* Genetic Stock Center (BGSC). Nucleosides were purchased from Sigma-Aldrich, Berry & Associates or Moravsek Biochemicals and Radiochemicals.

The structures of the purine analogues tested as agonists and antagonists are shown in Fig. 1. Molecular masses and ¹H-NMR data of the synthesized compounds agreed with reported values (Bressi *et al.*, 2000; Hecht *et al.*, 1971; Véliz & Beal, 2000; Vittori *et al.*, 2000).

Synthesis of 6-*N*-methyladenosine (10), 6-*N*-dimethyladenosine (11), 6-*O*-methylinosine (12), 6-*N*-ethyladenosine (15), 6-*N*-propyladenosine (16), 6-*N*-butyladenosine (17), 6-*N*-isopropyladenosine (18), 6-*N*-sec-butyladenosine (19) and 6-*N*-isobutyladenosine (20). These compounds were synthesized following published procedures (Véliz & Beal, 2000). Briefly, to a –20 °C solution of 2',3',5'-tri-*O*-acetylinoine and *N*-bromosuccinimide (NBS) in dry acetonitrile, hexamethylphosphoric triamide (HMPT) was added dropwise. After addition, the cold bath was removed, and the reaction mixture was stirred at room temperature for 0.5 h. LiBr was then added, and the mixture was heated to 70 °C for 5 h. The mixture was concentrated under reduced pressure and then purified

Fig. 1. Compounds tested as germinants and germination inhibitors: inosine (1), adenosine (2), 2-aminopurine riboside (3), 2,6-diaminopurine riboside (4), 6,8-diaminopurine riboside (5), 6-amino, 8-*N*-methylaminopurine riboside (6), 8-hydroxyadenosine (7), 8-bromoadenosine (8), 8-azaadenosine (9), 6-*N*-methyladenosine (10), 6-*N*-dimethyladenosine (11), 6-*O*-methylinosine (12), 6-methylmercaptapurine riboside (13), 6-thioinosine (14), 6-*N*-ethyladenosine (15), 6-*N*-propyladenosine (16), 6-*N*-butyladenosine (17), 6-*N*-isopropyladenosine (18), 6-*N*-sec-butyladenosine (19), 6-*N*-isobutyladenosine (20), 6-*N*-isopentyladenosine (21), 6-*N*-isopentenyladenosine (22), 6-*N*-cyclohexyladenosine (23), 6-*N*-phenyladenosine (24), 6-*N*-benzyladenosine (25), 6-*N*-(*o*-tolyl)adenosine (26), 6-*N*-(*p*-tolyl)adenosine (27), 6-*N*-(2-ethylphenyl)adenosine (28), 6-*N*-(2-isopropylphenyl)adenosine (29), 6-*N*-benzoyladenosine (30), guanosine (31), 6-chloroguanosine (32), 6-*O*-methylguanosine (33), 6-thioguanosine (34), isoguanosine (35), 2-chloroadenosine (36), xanthosine (37), 2-*N*-methylguanosine (38), 2-*N*-dimethylguanosine (39), 2-*N*-acetylguanosine (40), 1-methyladenosine (41), 1-methylguanosine (42), 1-deazaadenosine (43), 1,6-dimethyladenosine (44), 7-methylguanosine (45), 7-deazaadenosine (46), 7-deazaguanosine (47), 7-deaza-8-azaadenosine (48), 3-deazaadenosine (49), 3-deazaguanosine (50), 9-deazaadenosine (51), 9-deazaguanosine (52), formycin A (53), 2'-deoxyadenosine (54), 2'-*O*-methyladenosine (55), 5'-deoxyadenosine (56), 3'-deoxyadenosine (57), 3'-*O*-methyladenosine (58), α -adenosine (59), arabinoadenosine (60), β -L-adenosine (61). Rib represents 9- β -D-ribose; Ade represents the adenine (6-aminopurine) heteroring.

by flash column chromatography (1% MeOH/CH₂Cl₂) to afford 2',3',5'-tri-*O*-acetyl, 6-bromopurine riboside as the major product (69% yield) and compound (11) as a by-product (10% yield). Purified 2',3',5'-tri-*O*-acetyl, 6-bromopurine riboside was dissolved in MeOH supplemented with 2 equivalents of the corresponding alkyl amine to produce compounds (10) and (15)–(20). Purified 2',3',5'-tri-*O*-acetyl, 6-bromopurine riboside was also dissolved in MeOH/K₂CO₃ to yield compound (12) in 89% yield.

Synthesis of 6-*N*-isopentyladenosine (21). This compound was synthesized by a modification of published procedures (Hecht *et al.*, 1971). In brief, 6-*N*-isopentenyladenosine (22) was dissolved in 3.0 ml MeOH containing 0.3 g Raney nickel. The suspension was heated under reflux for 5 h. The mixture was then filtered through celite and dried under vacuum. The resulting residue was dissolved in 1% MeOH/CH₂Cl₂ and purified by flash chromatography (1% MeOH/CH₂Cl₂ to 10% MeOH/CH₂Cl₂) to yield compound (21) in 28% yield.

Synthesis of 1-deazaadenosine (43). This compound was synthesized following published procedures (Vittori *et al.*, 2000). Briefly, 2,6-dichloro-1-deazapurine riboside was dissolved in ethanolic ammonia. The mixture was refluxed overnight. The resulting mixture was purified by flash chromatography (CH_2Cl_2 to 50 % $\text{MeOH}/\text{CH}_2\text{Cl}_2$) to yield 1-deaza, 2-choroadenosine in 20 % yield. Catalytic hydrogenolysis of the chlorinated compounds with 10 % Pd/C in ethanol and 2 M NaOH afforded 1-deazaadenosine (43) in quantitative yield.

Spore preparation. Wild-type, ΔgerI and ΔgerQ *B. cereus* 569 cells were individually plated on Difco sporulating medium (DSM) agar (3.08 % solution) to yield single-cell colonies (Nicholson & Setlow, 1990). Single colonies were suspended on 0.1 ml DSM, replated and 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 five times with deionized water and stored at 4 °C. Spores were more than 98 % pure as determined by phase-contrast microscopy.

Activation of nucleoside-mediated germination. Spore germination was monitored spectrophotometrically, whereby the loss in light diffraction following addition of a germinant was reflected by decreased optical density. All germination experiments were carried out in a Labsystems iEMS 96-well plate reader (ThermoElectron) fitted with a 540 nm cut-off filter. The final volume of each reaction was 0.2 ml. Experiments were carried out in triplicate on two different days with two different spore preparations. Standard deviations of germination rates were calculated from these six independent assays. 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_{540} of 1. The spore suspension was monitored for auto-germination at OD_{540} for 1 h. No auto-germination was detected in any sample used. Lack of auto-germination was confirmed by phase-contrast microscopy on selected samples. Spore suspensions were individually supplemented with 2.5 mM purine analogues (1)–(61) to test for agonists of the inosine-only germination pathway or with 2.5 mM purine analogues (1)–(61) and 40 μM L-alanine to test for agonists of the nucleoside/alanine germination pathway (Abel-Santos & Dodatko, 2007). Germination was monitored by following the decrease in OD_{540} for 15 min. If slow or no germination was observed, the spore suspension was further monitored for 2 h to detect slower-germinating spores. Optical densities were normalized by dividing each data point by the optical density at the time of inosine addition. Germination rates were calculated from the initial linear region of the germination curves (Akoachere *et al.*, 2007). Germination rates were set to 100 % for wild-type spores treated with 2.5 mM inosine (for the inosine-only germination pathway) or wild-type spores treated with 2.5 mM inosine and 40 μM L-alanine (for the nucleoside/alanine germination pathway). These inosine and L-alanine concentrations represent the lowest concentrations that induce maximal germination rate. Under these conditions, the germination rates of wild-type, ΔgerI and ΔgerQ strains were indistinguishable and standard deviations were less than 2 %. Percentage germination for other nucleosides was calculated as the fraction of germination rate compared to these conditions.

Inhibition of nucleoside-mediated germination. To test for antagonists of spore germination, spore aliquots were individually supplemented with various concentrations of purine analogues (2)–(61) and incubated for 15 min at room temperature. Spore/nucleoside mixtures were then treated with 2.5 mM inosine to test for inhibitors of the inosine-only germination pathway, or with 2.5 mM inosine and 40 μM L-alanine to test for inhibitors of the nucleoside/alanine germination pathway. As above, optical densities were normalized by dividing each data point by the optical density at the

time of inosine addition. Germination curves were fitted using the four-parameter logistic function of SigmaPlot v.9 software to calculate the time point ($t_{1/2}$) when the optical density reached the halfway point between its initial and final values. As expected, germination $t_{1/2}$ values increased in the presence of active germination inhibitors. The $t_{1/2}$ values obtained were plotted against inhibitor concentrations to calculate IC_{50} values using SigmaPlot v.9 software. Inhibition constants (K_i) were determined from a modified Cheng-Prusoff equation (Cheng, 2001; Munson & Rodbard, 1988).

Inter-atomic distance calculations. To determine the distance between the exocyclic 6-nitrogen and the first branched carbon (carbon atom forming covalent bonds with at least three other carbons and/or heteroatoms), molecules were drawn in the ACD/ChemSketch software (Li *et al.*, 2004). The sketched molecules were optimized using the 3D viewer module. Distance was determined using the same software module.

RESULTS AND DISCUSSION

B. cereus 569 wild-type spores can germinate with a nucleoside alone or with a combination of a nucleoside and L-alanine. In contrast, mutant ΔgerI and ΔgerQ spores can only germinate efficiently with nucleoside/L-alanine mixtures (Barlass *et al.*, 2002; Clements & Moir, 1998). In this study, we tested 61 purine analogues (Fig. 1) as (i) agonists and antagonists of the inosine-only germination pathway in wild-type *B. cereus* spores, (ii) agonists and antagonists of the nucleoside/alanine germination pathway in wild-type *B. cereus* spores, and (iii) agonists and antagonists of *B. cereus* ΔgerQ and ΔgerI spore germination. These conditions were used to determine the epitopes necessary for nucleoside-mediated stimulation or inhibition of *B. cereus* spore germination. Agonists yielded information on functional groups necessary for both binding to germination receptors and activation of the germination pathway. On the other hand, antagonists yielded information on functional groups that allowed binding, but not activation, of germination receptors. Inhibition assays served as an indirect method to map the chemical and physical configuration of the GerI and GerQ receptor-binding sites. Purine analogues that complement the binding site in terms of hydrophobicity, hydrogen bonding pattern, size and shape will be strong germination inhibitors (low K_i), and will outcompete inosine for germination receptor binding. Finally, compounds that could neither activate nor inhibit spore germination yielded information on functional groups that interfered with germination receptor interaction. As expected, changing the functional groups of the nucleoside moieties affected germination efficiency.

Agonists of *B. cereus* spore germination

As previously reported, inosine (1) was the only nucleoside tested that induced a strong spore germination response in the absence of a co-germinant (Abel-Santos & Dodatko, 2007). In this study, a larger collection of purine analogues was tested for the ability to induce *B. cereus* spore germination as sole germinants. None of the compounds

tested was able to substitute for inosine as a germinant in the inosine-only germination pathway (data not shown).

All the nucleoside analogues were also tested for the ability to serve as co-germinant with suboptimal concentration of L-alanine. The presence of L-alanine allowed 25 structurally different purine nucleoside analogues to co-activate germination. Under these conditions, guanosine analogues, purine nucleosides with amino groups at the 2 and 6 positions, carbocyclic and N-methylated purine rings were also recognized as germinants, albeit less effectively than inosine (Table 1).

Only two compounds, inosine and adenosine, were able to act as co-germinants with L-alanine to activate Δ gerQ spore

germination. In contrast, Δ gerI spores were able to germinate in the presence of 15 structurally different purine nucleosides. Thus, spores expressing GerI as the only nucleoside receptor (Δ gerQ mutant) are activated by a narrower range of agonists than spores expressing only GerQ as the only nucleoside receptor (Δ gerI mutant).

Antagonists of *B. cereus* spore germination

Adenosine (2) is a nucleoside analogue that has an exocyclic amino group at position 6. Adenosine has been reported to be a weak germinant of *B. cereus* 569 spores (Clements & Moir, 1998). However, in our hands, adenosine was only a germinant in the presence of L-alanine. On the contrary, pre-incubation of *B. cereus* 569 spores with adenosine inhibited inosine-mediated germination with an inhibition constant (K_i) of 109 μ M (Abel-Santos & Dodatko, 2007). Thus, in the absence of L-alanine, changing the 6-carbonyl group of inosine to the amino group of adenosine is sufficient to change the nucleoside moiety from an agonist of the nucleoside/alanine germination pathway to an antagonist of the inosine-only germination pathway (Table 2).

2-Aminopurine riboside (3) is a positional adenosine isomer, while 2,6-diaminopurine riboside (4) has amino groups at both position 2 and position 6. Both compounds are better inhibitors than adenosine for wild-type spores germinated with inosine.

The presence of L-alanine as a co-germinant has a profound effect on nucleoside recognition. While compounds (2)–(4) inhibit the inosine-only germination pathway in wild-type spores, the same compounds are co-germinants for the nucleoside/alanine germination pathway. The shift from antagonist to agonist of spore germination indicates a shift in the nucleoside recognition mode when different pathways are activated. Compounds (2)–(4) also inhibited germination of Δ gerQ spores better than adenosine, suggesting that these compounds interact with, but do not activate, the GerI receptor. Compounds (2)–(4) do not inhibit or activate germination of Δ gerI spores, suggesting that they are not recognized by the GerQ receptor. Thus, compounds (2)–(4) are recognized differently by the GerI and GerQ receptors.

6,8-Diaminopurine riboside (5) is a positional isomer of 2,6-diaminopurine riboside (4) and has similar physical and chemical properties. However, 6,8-diaminopurine riboside (5) did not activate or inhibit spore germination under any conditions tested. Its inability to influence *B. cereus* spore germination could be due to steric hindrance at the 8-position or to the ability of the amino group to form hydrogen bonds. To distinguish between these possibilities, the 8-amino group was either methylated [8-N-methyladenosine (6)] or substituted with hydroxyl [8-hydroxyadenosine (7)] and bromide [8-bromoadenosine (8)]. All three compounds were inactive against germination under all conditions tested. Thus, bulky exocyclic groups at

Table 1. Agonists of *B. cereus* spore germination

Spore suspensions were individually supplemented with 2.5 mM purine analogues (2)–(61) and 40 μ M L-alanine. Values shown are percentage germination rates, determined by comparing each germination rate to the germination rate of spores germinated with 2.5 mM inosine and 40 μ M L-alanine. Purine analogues that did not induce germination under any condition tested are not shown. Data for germination with purine analogues alone are not shown, since only inosine was active in the absence of L-alanine.

Analogue	Wild-type	Δ gerQ	Δ gerI
Adenosine analogues			
1	100	100	100
2	85	26	0
3	22	0	0
4	65	0	0
8-Substituted purine ribosides			
9	69	0	38
6-Methylated purine nucleosides			
14	88	0	78
6-Substituted guanosine analogues			
31	21	0	6
32	12	0	0
33	14	0	3
34	19	0	3
35	28	0	8
36	8	0	3
N1-substituted purine ribosides			
41	38	0	29
42	5	0	0
43	22	0	8
44	28	0	1
N3-, N7- and N9- substituted purine ribosides			
45	6	0	11
46	61	0	15
47	17	0	0
48	33	0	0
49	29	0	34
50	13	0	0
51	62	0	16
52	2	0	0
53	21	0	0

Table 2. Antagonists of *B. cereus* spore germination

Spore suspensions were individually supplemented with various concentrations of purine analogues (2)–(61) and then treated with 2.5 mM inosine ('Inosine-only' column) or with 2.5 mM inosine and 40 μ M L-alanine ('Inosine/L-alanine' columns). The data are K_i values (μ M), with standard deviations in parentheses. NI, No inhibition of spore germination observed. Purine analogues that did not inhibit germination under any condition tested are not shown.

Analogue	Wild-type		Δ gerQ	Δ gerI
	Inosine-only	Inosine/L-alanine	Inosine/L-alanine	Inosine/L-alanine
Adenosine analogues				
2	109 (12.9)	NI	80 (1.6)	NI
3	80.3 (3.9)	NI	17.5 (0.3)	NI
4	17.3 (0.5)	NI	19.9 (1.7)	NI
8-Substituted purine ribosides				
9	34.4 (0.8)	NI	54 (3.7)	NI
6-Methylated purine nucleosides				
10	17.5 (2.3)	22.9 (2.6)	28.3 (1.4)	23.8 (2.9)
11	27.0 (1.3)	45.0 (3.2)	35.0 (3.2)	69.3 (8.2)
12	124 (9.3)	NI	98.0 (7.3)	NI
13	8.2 (0.5)	111 (9.2)	8.1 (0.1)	206 (17.6)
Linear and branched alkyl adenosine analogues				
15	6.2 (0.3)	10.2 (0.1)	12.8 (1.8)	44.9 (3.2)
16	5.8 (0.2)	9.7 (0.5)	5.4 (0.3)	4.9 (0.4)
17	5.5 (0.2)	9.3 (0.5)	15.8 (2.8)	33.3 (2.9)
18	50.2 (1.8)	46.8 (4.9)	66.9 (5.1)	98.0 (11.5)
19	40.8 (3.6)	121 (13.7)	54.9 (6.6)	NI
20	22.0 (3.1)	88.9 (9.2)	32.3 (2.2)	111 (8.5)
21	12.8 (0.9)	43.3 (3.1)	18.9 (3.1)	42.9 (0.4)
22	14.5 (1.1)	23.8 (2.3)	25.3 (4.8)	38.9 (2.3)
23	54.0 (0.3)	65.5 (0.4)	52.7 (9.9)	NI
Cycloalkyl and aryl adenosine analogues				
24	143 (0.9)	NI	NI	NI
25	28.1 (0.7)	42.9 (2.2)	66.3 (11.2)	89.2 (8.4)
26	32.2 (0.7)	45.2 (1.3)	78.0 (8.2)	95.5 (3.9)
27	30.4 (0.9)	48.3 (3.4)	154 (28)	72.6 (9.1)
28	5.0 (0.2)	87.3 (8.1)	37.2 (2.5)	133 (2.8)
29	93.6 (1.0)	122 (8.9)	42 (3.8)	154 (12.6)
6-Substituted guanosine analogues				
31	10.2 (0.6)	NI	15.3 (0.4)	NI
32	10.4 (1.3)	NI	2.9 (0.6)	NI
33	11.4 (0.9)	NI	21.9 (3.2)	NI
34	15.9 (1.4)	NI	13.2 (2.3)	NI
35	58.5 (0.1)	NI	NI	NI
N1-Substituted purine ribosides				
41	20.3 (1.9)	NI	49.0 (1.9)	NI
42	11.3 (0.2)	NI	28.5 (3.2)	NI
43	28.0 (0.5)	NI	NI	NI
Ribose-modified nucleoside analogues				
57	18.9 (0.4)	NI	33.9 (3.6)	NI
58	45.3 (2.8)	NI	122 (18.4)	NI

position 8 interfere with recognition of purine analogues by both the GerI and GerQ receptors of *B. cereus* spores.

8-Azaadenosine (9), where adenosine's C8 group is converted to N8, behaves similarly to adenosine in wild-type and Δ gerQ spores. Interestingly, while adenosine has no effect on the germination of Δ gerI spores, 8-azaadenosine acts as an agonist. This suggests that 8-azaadenosine is recognized as an antagonist by the GerI receptor and as an agonist by the GerQ receptor.

While exocyclic amines at the 6- and/or 2-positions are determinants for inosine-only spore germination inhibition in wild-type spores, other *sp3*-hybridized groups can

also inhibit this pathway. 6-*N*-methyladenosine (10) is an adenosine analogue that converts the exocyclic primary amine to a secondary amine, while 6-*N*-dimethyladenosine (11) has a tertiary amine at position 6. 6-*O*-Methylinosine (12) and 6-methylmercaptapurine riboside (13) are analogues of 6-*N*-methyladenosine (10) in which the amine nitrogen has been substituted by oxygen and sulfur, respectively. Interestingly, although inosine is a powerful germinant of *B. cereus* spores, 6-*O*-methylinosine blocks inosine-mediated germination in wild-type spores and inhibits Δ gerQ spore germination.

The strength of inhibition for compounds (10)–(13) is strongly dependent on the calculated electronegativity (Mullay, 1985) of the 6-substitution. Indeed, inhibitor-binding affinity increases with increased electronegativity for both inosine-germinated wild-type spores and Δ gerQ spores (Fig. 2). In these two cases, spores preferentially bind purine analogues with electron-rich atoms at the 6-position. This suggests that the GerI receptor must possess an electron-poor binding site.

In contrast to the inhibition trends found for the inosine-only germination pathway, compounds (10)–(13) show different behaviours in the nucleoside/alanine germination pathway in wild-type spores and Δ gerI spore germination. While 6-*N*-methyladenosine (10) and 6-*N*-dimethyladenosine (11) continue to behave as strong inhibitors, 6-*O*-methylinosine (12) becomes inactive and 6-methylmercaptapurine riboside (13) becomes a weak inhibitor. Thus, methylation of the adenosine amine is sufficient to change adenosine from a co-germinant to an inhibitor of the nucleoside/alanine germination pathway. These results also suggest that purine nucleoside-binding sites are similar in wild-type and Δ gerI spores germinated with inosine/

alanine. These similarities suggest that the GerQ receptor is prominently involved in the activation of the nucleoside/alanine germination pathway.

6-Thioinosine (14) is an inosine analogue in which the carbonyl group has been substituted by a sulfur atom. Although sulfur and oxygen have similar chemical properties, 6-thioinosine cannot activate or inhibit germination of wild-type or Δ gerQ spores on its own. In the presence of L-alanine, however, 6-thioinosine becomes a strong co-germinant for both wild-type and Δ gerI spores. This suggests that the GerQ receptor, but not the GerI receptor, is able to bind 6-thioinosine.

The observation that methylation of the 6-exocyclic amine resulted in increased inhibition of both the inosine-only and nucleoside/alanine germination pathways suggested that hydrophobic residues might be needed to optimize germination receptor binding. Indeed, substituting the methyl group by an ethyl chain [6-*N*-ethyladenosine (15)] results in a better germination inhibitor of the inosine-only germination pathway in wild-type spores. However, no substantial inhibition increase is observed with propyl [6-*N*-propyladenosine (16)] or butyl groups [6-*N*-butyladenosine (17)]. The inhibition profile for the germination of Δ gerQ spores follows a similar trend, suggesting that these compounds inhibit the GerI receptor.

Even though all the 6-alkyladenosine derivatives are inhibitors of inosine-mediated germination, their relative potency is dependent not only on the hydrophobicity of their *N*-substitutions but also on the distance between the first branched carbon in the substituents and the exocyclic nitrogen (Fig. 3a). Compounds that have a branched carbon close to the amino nitrogen are poor inhibitors of the inosine-only germination pathway in wild-type spores and of Δ gerQ spore germination. As the distance between the nitrogen and the first branched carbon increases, there is a corresponding increase in binding affinity. In all cases, linear analogues (15)–(17) were more effective than their branched isomers (18)–(23) in germination inhibition assays. Thus, it seems that the GerI receptor recognizes nucleoside inhibitors with linear alkyl chains better than nucleosides with branched alkyl chains.

Compounds (15)–(23) also acted as inhibitors of the nucleoside/alanine germination pathway in wild-type spores and of Δ gerI spore germination. In these cases, however, no clear relationship was observed between any physico-chemical property of the side-chain and binding affinity. This suggests that the GerQ receptor is less restrictive in the recognition of alkylated adenosine analogues.

The relationship between K_i and distance to branched carbon can also be observed for the inhibition of the inosine-only germination pathway by 6-*N*-aryladenosines (24)–(29) (Fig. 3b). Furthermore, benzoylation of the exocyclic amine [6-*N*-benzoyladenosine (30)] resulted in an inactive compound.

Fig. 2. Decreased electronegativity correlates with increased germination inhibition. Group electronegativity was calculated using the 'super-atom' approximation and is based on the Pauling electronegativity scale. The plot of the logarithm of the calculated K_i against group electronegativity can be fitted by a positive-sloped straight line for both Δ gerQ (●) and wild-type (○) spores. Side-chains and compound numbers are shown for reference.

In contrast to the 6-alkyladenosine series, no correlation was observed in the inhibition of $\Delta gerQ$ spore germination by compounds (24)–(29). It is possible that the presence of L-alanine required for $\Delta gerQ$ spore germination could affect binding of 6-aryladenosines by the GerI receptor. Although compounds (24)–(29) also acted as inhibitors of the nucleoside/alanine germination pathway and $\Delta gerI$ germination, no structure–activity analysis could be calculated for these two cases, confirming the lack of selectivity of the GerQ receptor.

The flexibility of substituent recognition at position 6 was further demonstrated by the inhibition characteristics of guanosine analogues. Compounds (31)–(34) differ only in the exocyclic group at position 6. Although the carbonyl oxygen of guanosine (31) was substituted with groups with various size and electronic properties, all the inhibition constants for the inosine-only germination pathway were comparable. Similarly, these compounds inhibited $\Delta gerQ$ spore germination.

Isoguanosine (35) is a positional isomer of guanosine. Although these two compounds have similar physical properties, isoguanosine is almost six times less effective as an inhibitor of the inosine-only germination pathway of wild-type spores. Isoguanosine (35) also failed to inhibit germination of $\Delta gerQ$ spores. Thus, the relative positions of the exocyclic groups play an important role in purine analogue recognition by the GerI receptor.

In contrast to the inhibitory properties of 6-*N*-alkyl and 6-*N*-aryl adenosines, guanosine analogues (31)–(36) activated the nucleoside/alanine germination pathway. Similarly, compounds (31) and (33)–(36) served as germinants of $\Delta gerI$ spores, but the germination rates were slower than for wild-type spores. Thus, guanosine analogues are inhibitors of the inosine-only germination pathway in wild-type spores and of $\Delta gerQ$ spore germination, but act as activators of the

nucleoside/alanine germination pathway in wild-type and $\Delta gerI$ spores. This suggests that guanosine analogues are recognized as antagonists by the GerI receptor and as agonists by the GerQ receptor.

Remarkably, even though 6-chloroguanosine (32) is a good inhibitor of the inosine-only germination pathway in wild-type spores and of $\Delta gerQ$ spore germination, its positional isomer, 2-chloroadenosine (36), does not inhibit germination under any conditions tested. Instead, chloroadenosine (36) is an agonist of the nucleoside/alanine germination pathway and of $\Delta gerI$ spore germination. Both compounds have the same chloride and amino functional groups, but they differ in their relative position in the purine nucleus. These positional differences are sufficient to change how these compounds are recognized. While the GerI receptor recognizes 6-chloroguanosine (32) as an antagonist, it does not detect 2-chloroadenosine (36). In contrast, the GerQ receptor recognizes both 6-chloroguanosine and 2-chloroadenosine as agonists.

Xanthosine (37) neither induced nor interfered with germination under any conditions tested. The failure of spores to recognize xanthosine could be due to the double amide structure that breaks the aromaticity of the purine ring. It is also possible that xanthosine does not affect spore germination because it cannot pass through the spore coats to interact with the germination receptors. This scenario is highly unlikely since the hydrophobicity of xanthosine and inosine are similar ($\log P = -1.62$ for xanthosine, -1.74 for inosine) (Wang *et al.*, 1997). Indeed, xanthosine failed to induce or inhibit germination even in decoated spores (data not shown).

Although functional groups at positions 2 and 6 can act synergistically to inhibit spore germination, compounds with bulkier substituents at the 2-amino group [2-*N*-

Fig. 3. Increased nitrogen to branched carbon distance results in diminishing K_i . Plotting the logarithm of the K_i values against the distance between the exocyclic nitrogen and the first branched carbon follows a general trend in which longer *N*-branched atom distance correlates with lower K_i values. (a) Inhibition by 6-*N*-alkyladenosines of $\Delta gerQ$ (●) and wild-type (○) spores. (b) Inhibition by 6-*N*-aryladenosines of wild-type (○) spores. The common name of the substituent and compound numbers are shown for reference.

methylguanosine (38), 2-*N*-dimethylguanosine (39) and 2-*N*-acetylguanosine (40)] were not able to serve as germination inhibitors or activators under any condition tested.

It is interesting to compare the effect of 2,6-diaminopurine riboside (4), guanosine (31), isoguanosine (35) and xanthosine (37) on *B. cereus* spore germination. All four compounds are substituted at positions 2 and 6. They differ in the number and positions of carbonyl and/or amino groups present. 2,6-Diaminopurine riboside (4) and guanosine (31) inhibit the inosine-only germination pathway in wild-type spores and Δ gerQ spore germination with similar strength. In contrast, isoguanosine is an approximately sixfold worse inhibitor and xanthosine has no effect on germination. This suggests that the GerI receptor can discriminate between functional groups located at these positions.

The purine ring has four different endocyclic nitrogens that have been shown to be important epitopes for recognition by purine transporters and enzymes involved in purine catabolism and anabolism (Wallace *et al.*, 2002). Methylation or substitution of the N1 position [1-methyladenosine (41), 1-methylguanosine (42) and 1-deazaadenosine (43)] results in purine analogues that show agonist and antagonist properties similar to their parent compounds (adenosine and guanosine) in wild-type spores.

For Δ gerQ spores, 1-methyladenosine (41) and 1-methylguanosine (42) also behave like their parent compounds. However, unlike adenosine, 1-deazaadenosine (43) does not inhibit Δ gerQ spore germination. Interestingly, 1-methyladenosine (41) and 1-deazaadenosine (43), but not 1-methylguanosine (42), act as co-germinants for Δ gerI spores; the opposite effect was observed for guanosine and adenosine. These results suggest that the recognition of the N1 position by both GerI and GerQ receptors is dependent on the presence of the other germination receptor and L-alanine.

Both 6-*N*-methyladenosine (10) and 1-methyladenosine (41) are good inhibitors of the inosine-only germination pathway and of Δ gerQ spore germination. On the other hand, 6-*N*-methyladenosine (10) is an antagonist and 1-methyladenosine (41) is an agonist of the nucleoside/alanine germination pathway and of Δ gerI spore germination. Interestingly, 1,6-dimethyladenosine (44) does not inhibit either of the two pathways in wild-type spores. Furthermore, 1,6-dimethyladenosine (44) has little effect on Δ gerQ or Δ gerI spore germination. This compound has methyl groups at both the N1 and N6 positions. It seems that N1 can tolerate substitutions, but only when the exocyclic atom at position 6 is not simultaneously derivatized.

In contrast to N1-substituted nucleosides, compounds with modifications at the N7 [7-methylguanosine (45), 7-deazaadenosine (46), 7-deazaguanosine (47) and 7-deaza-8-azaadenosine (48)], N3 [3-deazaadenosine (49) and 3-deazaguanosine (50)] or N9 [9-deazaadenosine (51), 9-

deazaguanosine (52) and formycin A (53)] positions fail to inhibit inosine-only germination in wild-type spores and Δ gerQ spore germination. Since these compounds neither activate nor inhibit Δ gerQ endospores, substitutions at the N3, N7 and N9 positions probably prevent binding to the GerI receptor. This suggests that the GerI receptor recognizes N3, N7 and N9 as epitopes for inhibition.

In contrast to the differential effect demonstrated by carbocyclic and methylated purine analogues on the inosine-only germination pathway, compounds (41)–(53) are agonists of the nucleoside/alanine germination pathway in wild-type spores. A subset of these compounds also act as germinants to Δ gerI, but not Δ gerQ spores. The fact that these compounds are agonists for Δ gerI spore germination suggests that the GerQ receptor does not recognize the N3 and N7 positions during receptor activation. On the other hand, the fact that these compounds cannot activate germination of Δ gerQ spores suggests that the N3 and N7 are necessary for the activation of the GerI receptor.

The ribose sugar has three hydroxyl groups that could form hydrogen bonds with the germination machinery. Removal [2'-deoxyadenosine (54)] or methylation [2'-*O*-methyladenosine (55)] of the 2'-hydroxyl or the 5'-hydroxyl [5'-deoxyadenosine (56)] results in inactive compounds under all conditions tested. This suggests that both GerI and GerQ use the 2'- and 5'-hydroxyl groups to recognize purine nucleosides.

In contrast to the deleterious effects of 2'- and 5'-substitutions, removal [3'-deoxyadenosine (57)] or methylation [3'-*O*-methyladenosine (58)] of the 3'-hydroxyl results in good inhibitors of the inosine-only germination pathway in wild-type spores and of Δ gerQ spore germination. In contrast, 3'-modified nucleosides [3'-deoxyadenosine (57) and 3'-*O*-methyladenosine (58)] do not activate or inhibit the nucleoside/alanine germination pathway in wild-type spores or Δ gerI spore germination. These results suggest that the 3'-hydroxyl group is necessary for recognition by the GerQ, but not the GerI receptor.

Ribose has four chiral centres. All the adenosine stereoisomers tested [α -adenosine (59), arabino-adenosine (60), and β -L-adenosine (61)] failed to inhibit or activate germination under any of the conditions tested. Thus, the correct stereochemistry is required for efficient recognition of purine analogues by both GerI and GerQ receptors.

Conclusions

Spore germination has been studied in different *Bacillus* and *Clostridium* species (Moir *et al.*, 2002; Setlow, 2003). *B. cereus* spores use nucleosides as a signal for germination, but nucleoside recognition varies depending whether a second germinant is present to co-activate germination (Abel-Santos & Dodatko, 2007). In *B. cereus* 569, two receptors necessary for inosine-mediated germination have been identified (Barlass *et al.*, 2002; Clements & Moir, 1998). However, the mechanism for differential purine

nucleoside recognition by germination receptors is not known.

We have previously shown that *B. cereus* spores recognize inosine and other nucleosides as agonists of spore germination (Abel-Santos & Dodatko, 2007). In this study, structure–activity relationship analysis points to a number of specific interactions between the germination machinery and the nucleoside moieties. For the inosine-only germination pathway, nucleoside–receptor interactions are independent of L-alanine, and inosine is the only germinant able to strongly activate germination on its own.

The presence of L-alanine allows a broader range of nucleosides to act as co-germinants of wild-type *B. cereus* spore germination. Thus, the nucleoside/alanine germination pathway is more promiscuous in recognition of purine analogues. However, only changes in the purine ring are tolerated. These results are consistent with previous work that showed the importance of the sugar moiety in nucleoside recognition (Preston & Douthit, 1988; Senesi *et al.*, 1991; Shibata *et al.*, 1986; Yousten, 1975).

Mutant spores (Δ gerQ) that express GerI as the sole nucleoside receptor are restricted to inosine and adenosine as germinants when supplemented with L-alanine as co-germinant. On the other hand, mutant spores (Δ gerI) that express GerQ as the sole germination receptor are able to germinate with structurally diverse nucleosides in the presence of L-alanine. However, only approximately half of the purine nucleosides that activate the nucleoside/alanine germination pathway in wild-type spores are able also to activate Δ gerI spore germination. Thus, even though GerQ is more flexible than GerI in nucleoside recognition, it seems that the presence of GerI increases the promiscuity of the GerQ binding site in wild-type spores.

Inhibition analysis of the inosine-only germination pathway indicates an extensive network of interactions that include the N3, N7 and N9 endocyclic nitrogens and the 2'- and 5'-hydroxyl groups in the sugar moiety. Modifications of any of these positions affect binding of inhibitory purine analogues. Furthermore, sugar recognition is finely tuned to detect only the correct ribose pentose isomer.

In the inosine-only germination pathway, position 6 of the purine base seems to be accommodated in a narrow hydrophobic pocket that allows preferential binding of analogues with linear chains. However, neither the ability to form hydrogen bonds nor amine basicity are important determinants for recognition of the 6-position. The binding pocket around position 6 also seems to contain an electron-poor patch that allows interaction with exocyclic heteroatoms.

In comparison to the binding flexibility around position 6, there seems to be greater steric constraint around positions 2 and 8 of the purine ring. For guanosine analogues, the amino group at position 2 becomes the most important epitope for the inhibition of the inosine-only germination pathway.

In contrast to the detailed molecular information derived from the inhibition study of inosine-only germination, only 6-*N*-substituted adenosine analogues are able to inhibit nucleoside/alanine spore germination. This suggests that binding of inhibitors in this case requires a basic exocyclic amine, but is mostly driven by hydrophobic interactions.

Mutant Δ gerI and Δ gerQ spores also show markedly different recognition patterns towards purine nucleoside inhibitors. Spores (Δ gerQ) encoding GerI as sole nucleoside receptor show a similar but not identical recognition pattern to wild-type spores germinated with inosine only. Indeed, two purine analogues, isoguanosine (35) and 1-deazaadenosine (43), out of the 61 tested are able to inhibit the inosine-only germination pathway in wild-type spores, but have no effect on Δ gerQ spores.

An analogous case is observed for spores (Δ gerI) encoding GerQ as the sole nucleoside receptor. These mutant spores mostly behave like wild-type spores germinated with inosine/alanine. However, the absence of the GerI receptor causes slight changes in the nucleoside recognition pattern of the mutant spores. Most notably, only 6-*N*-sec-butyladenosine (19) and 6-*N*-cylcohexyladenosine (23) inhibit the nucleoside/alanine germination pathway in wild-type spores, but do not affect Δ gerI spore germination.

The differences in nucleoside recognition between wild-type and mutant spores are not surprising. The small changes in germination patterns between wild-type spores germinated with inosine alone and Δ gerQ spores germinated with inosine/L-alanine could be attributed to either the absence of the GerQ receptor or the activation of the alanine-bound GerL receptor by the added L-alanine (Barlass *et al.*, 2002), or a combination of both. Similarly, the differences observed between wild-type and Δ gerI spores germinated with inosine/L-alanine could be attributed to the effect of the GerI receptor on the nucleoside-binding site of GerQ.

It is evident that germination receptor interactions can affect their functions. Both GerI and GerQ receptors are necessary for inosine-only germination, suggesting synergy between these two complexes. Indeed, GerI and GerQ probably interact with each other as well as with the alanine germination receptor, GerL (Barlass *et al.*, 2002; Clements & Moir, 1998). Hence, in the mutant spore, changes in germination receptor interactions could cause subtle alterations of the nucleoside-binding site in the GerI receptor.

The GerI receptor in Δ gerQ spores is tightly controlled to recognize a narrow range of germinants, but can be inhibited in defined patterns by a number of structurally distinct nucleosides. These are characteristics of well-defined binding sites containing specific substrate–protein interactions (Segel, 1993). This is also similar to the activation and inhibition pattern shown by wild-type spores germinated with inosine only. Thus, GerI activation

broadly correlates with the activation of the inosine-only germination pathway. On the other hand, the GerQ receptor in *ΔgerI* spores is more promiscuous toward nucleoside agonists, but is inhibited only by hydrophobic nucleosides. This is characteristic of flexible binding sites that use non-specific interactions to bind substrates (Segel, 1993).

In conclusion, the GerI and GerQ receptors are differentially activated. In the absence of amino acids, nucleoside-mediated germination seems to be mostly controlled by the more stringent GerI receptor, and only inosine and a few structural analogues are recognized as germinants. On the other hand, when both nucleoside and L-alanine are present in the medium, nucleoside recognition is relaxed. In this case, the more promiscuous GerQ receptor seems to control the germination process.

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REFERENCES

- Abel-Santos, E. & Dodatko, T. (2007). Differential nucleoside recognition during *Bacillus cereus* 569 (ATCC 10876) spore germination. *New J Chem* **31**, 748–755.
- Akoachere, M., Squires, R. C., Nour, A. M., Angelov, L., Brojatsch, J. & Abel-Santos, E. V. (2007). Identification of an in vivo inhibitor of *Bacillus anthracis* Sterne spore germination. *J Biol Chem* **282**, 12112–12118.
- Barlass, P. J., Houston, C. W., Clements, M. O. & Moir, A. (2002). Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of *gerL* and *gerQ* operons. *Microbiology* **148**, 2089–2095.
- Barth, H., Aktories, K., Popoff, M. R. & Stiles, B. G. (2004). Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. *Microbiol Mol Biol Rev* **68**, 373–402.
- Bressi, J. C., Choe, J., Hough, M. T., Buckner, F. S., Van Voorhis, W. C., Verlinde, C. L. M. J., Hol, W. G. J. & Gelb, M. H. (2000). Adenosine analogues as inhibitors of *Trypanosoma brucei* phosphoglycerate kinase: elucidation of a novel binding mode for a 2-amino-N6-substituted adenosine. *J Med Chem* **43**, 4135–4150.
- Cheng, H. C. (2001). The power issue: determination of K_B or K_i from IC_{50} . A closer look at the Cheng–Prusoff equation, the Schild plot and related power equations. *J Pharmacol Toxicol Methods* **46**, 61–71.
- Chirakkal, H., O'Rourke, M., Atrih, A., Foster, S. J. & Moir, A. (2002). Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. *Microbiology* **148**, 2383–2392.
- Clements, M. O. & Moir, A. (1998). Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. *J Bacteriol* **180**, 6729–6735.
- Cloud, J. & Kelly, C. P. (2007). Update on *Clostridium difficile* associated disease. *Curr Opin Gastroenterol* **23**, 4–9.
- Edlich, R. F., Hill, L. G., Mahler, C. A., Cox, M. J., Becker, D. G., Horowitz, J. H., Nichter, L. S., Martin, M. L. & Lineweaver, W. C. (2003). Management and prevention of tetanus. *J Long Term Eff Med Implants* **13**, 139–154.
- Ehling-Schulz, M., Fricker, M. & Scherer, S. (2004). *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. *Mol Nutr Food Res* **48**, 479–487.
- Gero, A. (1972). Determination of the inhibition constant in competitive inhibition combined with enzyme activation. *Enzymologia* **43**, 261–269.
- Hecht, S. M., Kirkegaard, L. H. & Bock, R. M. (1971). Chemical modifications of transfer RNA species. Desulfurization with Raney nickel. *Proc Natl Acad Sci U S A* **68**, 48–51.
- Hornstra, L. M., de Vries, Y. P., Wells-Bennik, M. H., de Vos, W. M. & Abee, T. (2006). Characterization of germination receptors of *Bacillus cereus* ATCC 14579. *Appl Environ Microbiol* **72**, 44–53.
- Ireland, J. A. & Hanna, P. C. (2002). Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* ΔSterne endospores: GerS mediates responses to aromatic ring structures. *J Bacteriol* **184**, 1296–1303.
- Levinson, H. S. & Hyatt, M. T. (1966). Sequence of events during *Bacillus megaterium* spore germination. *J Bacteriol* **91**, 1811–1818.
- Li, Z., Wan, H., Shi, Y. & Ouyang, P. (2004). Personal experience with four kinds of chemical structure drawing software: review on ChemDraw, ChemWindow, ISIS/Draw, and ChemSketch. *J Chem Inf Comput Sci* **44**, 1886–1890.
- Mock, M. & Fouet, A. (2001). Anthrax. *Annu Rev Microbiol* **55**, 647–671.
- Moir, A. (2006). How do spores germinate? *J Appl Microbiol* **101**, 526–530.
- Moir, A., Corfe, B. M. & Behravan, J. (2002). Spore germination. *Cell Mol Life Sci* **59**, 403–409.
- Mullay, J. (1985). Calculation of group electronegativity. *J Am Chem Soc* **107**, 7271–7275.
- Munson, P. J. & Rodbard, D. (1988). An exact correction to the “Cheng–Prusoff” correction. *J Recept Res* **8**, 533–546.
- Nicholson, W. L. & Setlow, P. (1990). *Molecular Biological Methods for Bacillus*. Chichester, UK: Wiley.
- Paredes, C. J., Alsaker, K. V. & Papoutsakis, E. T. (2005). A comparative genomic view of clostridial sporulation and physiology. *Nat Rev Microbiol* **3**, 969–978.
- Piggot, P. J. (1996). Spore development in *Bacillus subtilis*. *Curr Opin Genet Dev* **6**, 531–537.
- Piggot, P. J. & Hilbert, D. W. (2004). Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* **7**, 579–586.
- Preston, R. A. & Douthit, H. A. (1988). Functional relationships between L- and D-alanine, inosine and NH_4Cl during germination of spores of *Bacillus cereus* T. *J Gen Microbiol* **134**, 3001–3010.
- Sanchez-Salas, J. L., Santiago-Lara, M. L., Setlow, B., Sussman, M. D. & Setlow, P. (1992). Properties of *Bacillus megaterium* and *Bacillus subtilis* mutants which lack the protease that degrades small, acid-soluble proteins during spore germination. *J Bacteriol* **174**, 807–814.
- Sauer, U., Santangelo, J. D., Treuner, A., Buchholz, M. & Dürre, P. (1995). Sigma factor and sporulation genes in *Clostridium*. *FEMS Microbiol Rev* **17**, 331–340.
- Segel, I. H. (1993). *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems*. Chichester, UK: Wiley Interscience.
- Senesi, S., Cercignani, G., Freer, G., Batoni, G., Barnini, S. & Ota, F. (1991). Structural and stereospecific requirements for the nucleoside-triggered germination of *Bacillus cereus* spores. *J Gen Microbiol* **137**, 399–404.
- Setlow, P. (2003). Spore germination. *Curr Opin Microbiol* **6**, 550–556.

- Setlow, P. & Waites, W. M. (1976).** Identification of several unique, low-molecular-weight basic proteins in dormant spores of *Clostridium bifermentans* and their degradation during spore germination. *J Bacteriol* **127**, 1015–1017.
- Shibata, H., Ohnishi, N., Takeda, K., Fukunaga, H., Shimamura, K., Yasunobu, E., Tani, I. & Hashimoto, T. (1986).** Germination of *Bacillus cereus* spores induced by purine ribosides and their analogs: effects of modification of base and sugar moieties of purine nucleosides on germination-inducing activity. *Can J Microbiol* **32**, 186–189.
- Stephenson, K. & Lewis, R. J. (2005).** Molecular insights into the initiation of sporulation in Gram-positive bacteria: new technologies for an old phenomenon. *FEMS Microbiol Rev* **29**, 281–301.
- Ting, P. T. & Freiman, A. (2004).** The story of *Clostridium botulinum*: from food poisoning to Botox. *Clin Med* **4**, 258–261.
- Véliz, E. A. & Beal, P. A. (2000).** C6 substitution of inosine using hexamethylphosphorous triamide in conjunction with carbon tetrahalide or N-halosuccinimide. *Tetrahedron Lett* **41**, 1695–1697.
- Vittori, S., Lorenzen, A., Stannek, C., Costanzi, S., Volpini, R., Ijzerman, A. P., Kunzel, J. K. & Cristalli, G. (2000).** N-Cycloalkyl derivatives of adenosine and 1-deazaadenosine as agonists and partial agonists of the A1 adenosine receptor. *J Med Chem* **43**, 250–260.
- Wallace, L. J. M., Candlish, D. & De Koning, H. P. (2002).** Different substrate recognition motifs of human and trypanosome nucleobase transporters. Selective uptake of purine antimetabolites. *J Biol Chem* **277**, 26149–26156.
- Wang, R., Fu, Y. & Lai, L. (1997).** A new atom-additive method for calculating partition coefficients. *J Chem Inf Comput Sci* **37**, 615–621.
- Weiner, M. A., Read, T. D. & Hanna, P. C. (2003).** Identification and characterization of the *gerH* operon of *Bacillus anthracis* endospores: a differential role for purine nucleosides in germination. *J Bacteriol* **185**, 1462–1464.
- Yousten, A. A. (1975).** Germination of *Bacillus cereus* endospores: a proposed role for heat shock and nucleosides. *Can J Microbiol* **21**, 1192–1197.

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