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A moderate high pressure (mHP) of 150 megaPascals (MPa) triggers germination of Bacillus subtilis spores via germinant receptors (GRs), while germination by a very high pressure (vHP) of 550 MPa is GR-independent. The mHP and vHP germination of Bacillus subtilis spores with different levels of GRs and other germination proteins affecting GR-dependent germination has been measured, and the results showed that GR levels are the major factor determining mHP germination rates. However, other factors can modulate rates of mHP germination including: 1) the relative levels of individual GRs, as the GerA GR is more esponsive to mHP that are the GerB or GerK GRs; 2) the level of one recently identified small protein that has been suggested to be an additional GR subunit significantly modulated GRs' response to mHP; and 3) a dominant negative mutation in the gerD gene that largely eliminates GR-dependent nutrient germination but with no effects on spores' GR levels also eliminated mHP germination. In contrast, none of the alterations in germination proteins had any major effect on vHP germination, except for reduction of levels of the SpoVA proteins that comprise a spore membrane channel that is likely opened by vHP.

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ORIGINAL ARTICLE

High pressure germination of *Bacillus subtilis* spores with alterations in levels and types of germination proteins

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Keywords
Bacillus, germinant receptor, high pressure, spore germination, spores.

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Abstract

Aims: Examine effects of different levels and types of nutrient germinant receptors (GRs) and other germination proteins on *Bacillus subtilis* spore germination by a moderate high pressure (mHP) (150 megaPascals (MPa)) that triggers germination through GRs, and a very high pressure (vHP) (550 MPa) that triggers spore germination independent of GRs.

Methods and Results: The Moderate HP (mHP) and vHP germination kinetics of *B. subtilis* spores with large variations in levels of GRs and other germination proteins, including the GerD protein and the SpoVA proteins that comprise a spore membrane channel that is likely opened by vHP were measured.

Conclusions: GR levels were the major factor determining mHP germination rates. However, other factors modulated mHP germination rates including (i) relative levels of individual GRs (GerA, GerB, GerK), as mHP affected different GRs differently; (ii) levels of a recently identified small protein that may be a GR subunit; and (iii) a dominant negative mutation in *gerD* that eliminates GR dependent nutrient germination. In contrast, the alterations in germination proteins had no major effect on vHP germination, except for reduction of SpoVA protein levels.

Significance and Impact of the Study: With the increasing use of HP for food processing, this study provides new information on factors that modulate HP germination of spores for potential application of HP technology to achieve food sterility.

Introduction

Dormant spores of a number of *Bacillus* and *Clostridium* species are significant agents of food spoilage and food borne disease, and the extreme resistance properties of these spores make them a major concern for the food industry (Steyn *et al.* 2011; Logan 2012; Setlow and John son 2012). In particular, dormant spores are resistant to heat, which is commonly used in food sterilization meth ods such as retorting or canning, ultra high temperature, and potentially microwave irradiation. Dormant bacterial spores are also resistant to methods associated with emerging and established non thermal processing tech nologies, such as high hydrostatic pressure, UV light, cool plasma and γ irradiation. In most cases, these technolo gies can readily inactivate vegetative pathogens and to some extent bacterial spore populations, although not always to the point of achieving commercially sterile food products.

Spores lose their dormancy though the process of spore germination, through which they also lose their extreme resistance to lethal agents. Consequently, germinated spores are relatively easy to kill by treatments such as wet heat and high hydrostatic pressure. An important step in

spore germination is the release of the spore's store of dipicolinic acid (DPA) that contributes to spore resis tance to wet and dry heat. The loss of DPA from the spore core in germination is rate limiting en route to the inactivation of spores by high hydrostatic pressures com bined with elevated temperatures. These observations sug gest that it should be possible to eradicate spores by first stimulating their germination, then killing the germinated spores by a relatively mild heat treatment that causes a minimal decrease in a food's sensory quality and nutri tional value.

The usual technique for stimulating spore germination is to add one or more nutrient molecules that trigger spore germination by binding to specific nutrient germi nant receptors (GRs) (Paredes Sabja *et al.* 2011; Setlow 2013). *Bacillus subtilis* is the model spore former, and spores of this species have three major GRs: GerA that responds to L alanine or L valine, and GerB and GerK that together respond to a mixture of L asparagine, D glu cose, D fructose and K^+ (abbreviated AGFK). Rapid GR dependent germination also requires the GerD pro tein (Paredes Sabja *et al.* 2011; Setlow 2013). Unfortu nately, not all spores in populations germinate equally well with nutrient germinants. Small fractions of spores in populations have low levels of the various GRs in spores' inner membrane (IM) and germinate slowly, if at all, upon exposure to nutrients; these spores are termed superdormant (SD) for nutrient germination (Ghosh and Setlow 2009; Ghosh *et al.* 2012; Setlow 2013) with respect to the nutrient being used.

The reasons for the heterogeneity in GR levels in indi vidual spores in spore populations are not all known. Recent work has shown that alterations in the levels of at least two transcription factors, SpoVT and YlyA, that modulate the transcription of genes that encode GRs can drastically alter GR levels as well as rates of spore germi nation (Ramirez Peralta *et al.* 2012a; Traag *et al.* 2013). More recently, levels of small proteins termed D subunits that are associated with at least some GRs have also been shown to modulate GR function (Ramirez Peralta *et al.* 2013). Properties of sporulation media also have a signifi cant effect on both IM GR levels and spore germination rates, as spores made in rich complex media generally germinate faster than spores made in minimal media and have higher GR levels than spores made in a poor med ium (Hornstra *et al.* 2006; Ramirez Peralta *et al.* 2012b). An additional concern in using nutrient germinants to trigger the germination of spores prior to decontamina tion is that GRs are present in the spore's IM (Stewart *et al.* 2012; Setlow 2013; Stewart and Setlow 2013). Accordingly, nutrient germinants must pass through spores' outer layers, including the exosporium, if it is present, as well as the spore coats, outer membrane, peptidoglycan cortex and germ cell wall to reach the GRs. There is evidence that there is a permeability barrier in spores' outer layer that restricts passage of charged and hydrophilic molecules up to the IM (Gerhardt and Black 1961; Rode *et al.* 1962). However, spores may have a spe cial mechanism for ensuring that nutrient germinants can gain access to the IM, as *gerP* mutations in *Bacillus* spe cies appear to specifically impair nutrient germinant per meation to GRs (Behravan *et al.* 2000; Carr *et al.* 2010; Butzin *et al.* 2012).

Given the nature of the concerns regarding the effec tiveness of nutrient germination as a means for obtaining high levels of spore germination prior to spore inactiva tion, there has been significant interest in the food indus try in exploiting alternative methods for inducing spore germination, such as high hydrostatic pressure (HP) (Rastogi *et al.* 2007; Considine *et al.* 2008; Knorr *et al.* 2011; Reineke *et al.* 2013). High pressure processing (HPP) is a rapidly growing non thermal food processing technology that ensures the safety of meat, fruit juice and seafood products, extends product shelf life, and retains food freshness and natural tastes. According to the FDA, HPP for food processing can be run in two steps: first, to germinate the spores, and second, to inactivate the germi nated spores, with *Bacillus subtilis* and *Clostridium sporog enes* spores as candidates proposed as HPP indicators or surrogates for *Clostridium botulinum* spores (US Depart ment of Health and Human Services 2013), although there are certainly other candidates (Margosch *et al.* 2004; Sevenich *et al.* 2014). Interestingly, *Clostridium perfringens* spores are reported to germinate extremely poorly with HPP (Akhtar *et al.* 2009; Paredes Sabja *et al.* 2011).

Moderate HP (mHP) of 100 400 megaPascals (MPa) can trigger spore germination by directly activating GRs in the spore IM in a nutrient germinant independent fashion (Wuytack *et al.* 1998, 2000; Black *et al.* 2005; Setlow 2007), and thus such a HP treatment bypasses the need for nutrient germinants to permeate through spores' outer layers (Butzin *et al.* 2012). While mHP ger mination definitely involves GRs, there have been only a few studies examining the effects of decreased GR levels on mHP germination. Consequently, in this work, we have examined the mHP germination of spores of *B. subtilis* strains with changes in GR levels due to spore superdormancy, sporulation medium composition, alter ations in levels of various transcription factors involved in modulating the expression of genes encoding GR ope rons, as well as changes in levels of a recently described putative GR D subunit and a dominant negative muta tion in the *gerD* gene that greatly slows GRs' responses to all nutrient germinants (Li *et al.* 2014). The germina tion of the spores of these strains was also examined at

a very high HP (vHP) of 550 MPa, which is thought to trigger spore germination primarily in a GR independent fashion, perhaps by opening a spore IM channel and allowing the release of the spore core's huge depot of DPA (Wuytack *et al.* 1998; Black *et al.* 2007; Reineke *et al.* 2013).

Materials and methods

Bacillus subtilis strains used and spore preparation and purification

The *B. subtilis* strains used were isogenic derivatives of either strain PS832, a prototrophic 168 laboratory strain, or strain PY79 originally obtained from P. Youngman, and also a 168 strain; all strains are listed in Table 1. Strains isogenic with PS832 were as follows: PS533 (wild type) carrying plasmid pUB110 conferring kanamycin resistance; PS3486 (Δ*gerD amyE*::*gerD*) with wild type *gerD* at *amyE* in a *gerD* null background; PS4220 (Δ*spoVT*) with a deletion of the *spoVT* gene; PS4256 (Δ*gerKD*) with a deletion of the *gerKD* gene; PS4313 (Δ*gerKD amyE*::*gerKD*) with a deletion of the *gerKD* gene and with a wild type *gerKD* gene integrated at the *amyE* locus; PS4314 (*amyE*::P*sspB gerKD*) (termed ↑GerKD) overexpressing *gerKD* at the *amyE* locus from the very strong forespore specific promoter of the *sspB* gene (P*sspB*); PS4384 (*amyE*::*gerD*) with two copies of the *gerD* gene, one at the wild type locus and one at *amyE*; PS4385 (Δ*gerD amyE*::*gerD*^{F87C}) with a *gerD* gene with codon 87 that normally encodes phenylalanine changed to a cysteine codon (*gerD*^{F87C}) at *amyE* in a *gerD* null background; PS4387 (*amyE*::*gerD*^{F87C}) with wild type *gerD* at its normal locus and *gerD*^{F87C} at *amyE*; FB20 (Δ*gerA*) with a deletion of the *gerA* operon encoding the GerA GR; and FB62 (Δ*gerD*) with a deletion of the *gerD* gene. Strains isogenic with PY79 were as follows: a) BAT96 (Δ*ylyA*) with a deletion of the *ylyA* gene; and b) BAT264 (P*sspB ylyA*) (termed ↑YlyA), overexpressing the *ylyA* gene from P*sspB*.

Unless noted otherwise, spores were routinely pre pared on 2× Schaeffer's glucose (a rich complex med ium) plates at 37°C, and harvested, purified and stored as described previously (Nicholson and Setlow 1990; Paidhungat *et al.* 2000). Spores of strain PS533 (wild type) were also prepared at 37°C in rich or poor liquid media as described previously (Ramirez Peralta *et al.* 2012b), and purified and stored as described above. Spores of strain PS533 (wild type) that were SD for germination with 10 mmol l^{-1} L valine via the GerA GR were isolated as previously described (Ghosh *et al.* 2012). All spore preparations used in this work were free (>98%) from growing or sporulating cells and germinated spores as determined by phase contrast microscopy.

Table 1 Genotype and phenotype of *Bacillus subtilis* strains used*

Strain	Genotype (phenotype)	Reference
Isogenic with strain PS832		
PS533	Carries plasmid pUB110 (Km^r)	Setlow and Setlow (1996)
PS3486	Δ*gerD amyE*::*gerD*	Pelczar *et al.* (2007)
PS4220	Δ*spoVT*	Ramirez Peralta *et al.* (2012a)
PS4256	Δ*gerKD*	Ramirez Peralta *et al.* (2013)
PS4313	Δ*gerKD amyE*::*gerKD*	Ramirez Peralta *et al.* (2013)
PS4314	*amyE*::*PsspB gerKD* (↑*gerKD*)	Ramirez Peralta *et al.* (2013)
PS4384	*amyE*::*gerD*	Li *et al.* (2014)
PS4385	Δ*gerD amyE*::*gerD*^{F87C}	Li *et al.* (2014)
PS4387	*amyE*::*gerD*^{F87C}	Li *et al.* 2014
FB20	Δ*gerA*	Paidhungat and Setlow (2000)
FB62	Δ*gerD*	Igarashi *et al.* (2004)
Isogenic with strain PY79		
BAT96	Δ*ylyA*	Traag *et al.* (2013)
BAT264	P*sspB ylyA* (↑YlyA)	Traag *et al.* (2013)

*See text for more details.

HP treatment and quantitation of spore germination

Spores at an optical density at 600 nm ($O.D._{600}$) of 1 (approx. 10^8 CFU ml^{-1}) were diluted 1 : 10 in 25 mmol l^{-1} aqueous K Hepes buffer (pH 7·4), heat acti vated for 30 min at $T = 70$°C, dispensed as 1·5 ml volumes in flexible pouches that were subsequently double sealed, then germinated for various high pressure hold times by mHP (150 MPa) at 37°C or vHP at 550 MPa and 50°C (Paidhungat *et al.* 2002; Butzin *et al.* 2012). Samples were placed in ice immediately after HP treatment, frozen and kept frozen until analysed for ger mination. After thawing, samples were centrifuged in a microcentrifuge at 16 000 ***g*** for approx. 2 min, the pellet suspended in 50 μl water, and examined by phase con trast microscopy, counting three fields of approx. 100 spores each to determine the average percentage of spores that had become phase dark and thus had germinated. These data were then plotted to determine the percent spore germination as a function of time, and for spores of the wild type strain PS533 made in or on the rich complex medium this rate was set at 100; rates of germi nation of spores of all other strains were expressed rela tive to this value. The spores of all strains whose rates of spore germination were to be directly compared were prepared, purified and mHP or vHP treated together, and all except the experiment in Fig. 3 were carried out on two independent spore preparations with essentially identical results.

Results

Effects of sporulation medium and alterations in transcription factor levels on mHP spore germination

Previous work has shown that sporulation medium richness and levels of various sporulation specific transcription factors have significant effects on rates of spore germination with nutrient germinants, as well as on levels of various GRs in spores' IM (Ramirez Peralta *et al.* 2012a,b; Traag *et al.* 2013) (Table 2). Significant effects were also seen when the mHP germination of spores made in rich or poor liquid media or on rich medium plates with and without the transcription factors SpoVT or YlyA was compared (Fig. 1a c). Thus, mHP germination of poor medium spores was approx. fourfold slower than that of rich medium spores, spores lacking SpoVT germinated approx. fourfold faster with mHP than wild type spores, spores lacking YlyA germinated slightly slower than wild type spores, while spores over expressing YlyA germinated four to fivefold slower than wild type spores with mHP (Table 2). Notably, the effects of the alterations in transcription factors or sporulation medium on rates of mHP germination were generally similar to effects on rates of nutrient germination, and the effects on rates of germination generally were reflected in spores' total GR levels in the IM and total spore lysates, although there was certainly no linear relationship between IM GR levels and spore germination rates (Table 2) (Ramirez Peralta *et al.* 2012a,b; Traag *et al.* 2013; and S. Luu and P. Setlow, unpublished results).

Table 2 Nutrient and mHP germination rates and germinant receptor (GR) levels in spores of various strains*

Strain (genotype; preparation)	Relative nutrient germination rate†	Relative GR level‡	Relative mHP germination rate
	Spores made in liquid media		
PS533 (wild type; rich medium)	100§	100§	100§
PS533 (wild type; poor medium)	10	23	31
	Spores made on plates		
PS533 (wild type)	100§	100§	100§
PS533 (wild type, SD)¶	9	36	6
FB20 (*gerA*)	35	53	7
PS4220 (*spoVT*)**	150	380	225
PS4256 (*gerKD*)††	120	100	100
PS4313 (*gerKD amyE*:*gerKD*)	100	100	53
PS4314 (↑GerKD)	100	100	17
PS4387 ($gerD^{F87C}$)	<2	100	<2
FB62 (Δ*gerD*)	12	100	14
PY79 (wild type)	100§	100§	100§
BAT96 (*ylyA*)	75	65	75
BAT264 (↑YlyA)	20	18	15

*Rates of nutrient germination and GR levels in spores' inner membrane (IM) were calculated based on data from the literature (Ghosh *et al.* 2012; Ramirez Peralta *et al.* 2012a,b, 2013; Traag *et al.* 2013; Li *et al.* 2014). Rates of mHP germination were calculated from the data in Figs 1(a d) and 2(a b), and are given as relative rates compared to the mHP germination rate of PS533 spores made in or on the rich complex medium. All values for relative rates of mHP germination are ≤ ±20%.

†Rates of spore germination with nutrient germinants are the sum of values for germination via the GerA GR with L valine and the GerB plus GerK GRs with AGFK, all at saturating concentrations of the nutrient germinants. All values are expressed relative to values for the isogenic wild type spore populations prepared in/on rich medium, purified and treated at the same time, and these values were set at 100.

‡GR levels are the sum of the GerA, GerB and GerK GR levels based on the average numbers of molecules of these three GRs in the IM of spores of strain PS533 prepared on rich medium plates (Stewart and Setlow 2013). These values are expressed relative to the GR levels in the IM of isogenic wild type spores prepared under the same conditions, and these latter values were set at 100. Levels of individual GR subunits in all strains also changed in parallel, and in both the IM and total spore lysates.

§These values were set at 100.

¶In these SD spores prepared by germination with L valine as described in Materials and methods the GerA GR level in the IM is decreased approx. sevenfold, and IM levels of the GerB and GerK GRs are decreased approx. twofold (Ghosh *et al.* 2012).

**While IM GR subunit levels, in particular GerAA and GerAC are markedly higher in the IM of *spoVT* spores, levels of at least one SpoVA protein, SpoVAD, are decreased approx. 2·5 fold (Ramirez Peralta *et al.* 2012a).

††The increased rate of nutrient germination in these spores was due only to an increase in the rate of AGFK germination.

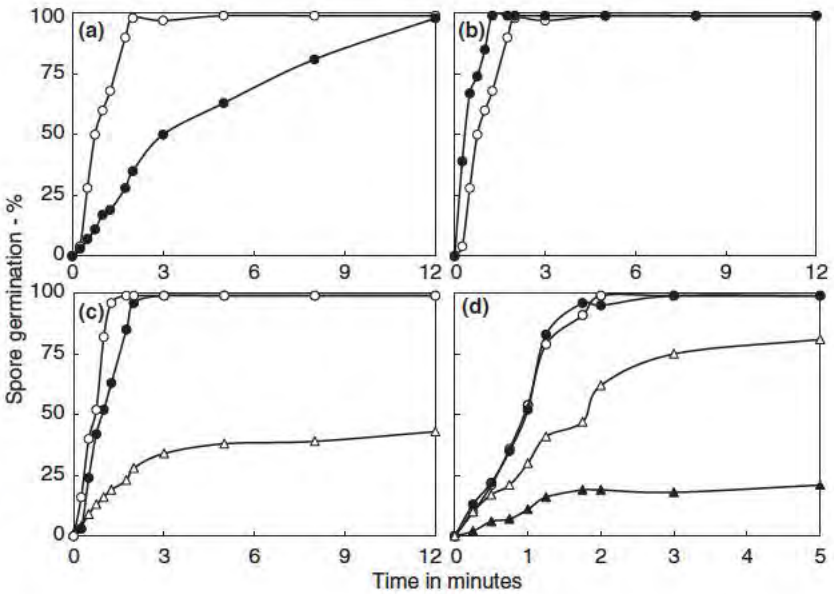

Figure 1 (a d) mHP germination of spores made in different media or with and without SpoVT or YlyA, and with various levels of GerKD. Spores of strains: (a) (○, ●) PS533 (wild type) made in rich (○) or poor (●) liquid media; and spores made on plates of strains; (b) (○) PS533 and (●) PS4220 (*spoVT*); (c) (○) PY79 (wild type), (●) BAT96 (*ylyA*), and BAT264 (↑YlyA); and (d) (○) PS533 (wild type), (●) PS4256 (*gerKD*), (△) PS4313 (*gerKD amyE::gerKD*), and (▲) PS4314 (↑GerKD) were germinated with mHP and the extents of spore germination at various times were determined as described in Materials and methods.

Effect of alterations in levels of a GR D subunit on mHP spore germination

Recent work has found that in addition to the A, B and C subunits that comprise GRs, some GRs, including the *B. subtilis* GerK GR, appear to have what may be D subunits, small proteins with two predicted membrane spanning segments (Ramirez Peralta *et al.* 2013). The loss of the D subunit of the *B. subtilis* GerK GR causes a marked increase in spore germination with the AGFK mixture that requires both the GerB and GerK GRs (Setlow 2013), although at least one GerK receptor sub unit's level in the IM and total spore lysates is relatively unchanged in *gerKD* spores (Ramirez Peralta *et al.* 2013; and S. Luu and P. Setlow, unpublished results) (Table 2). Germination via the GerA GR, as well as lev els of the GerA and GerB GRs were also unchanged in *gerKD* spores, and loss of *gerKD* had no significant effect on mHP germination (Fig. 1d). Complementation of a *gerKD* strain by introduction of a wild type *gerKD* gene plus its own promoter at the *amyE* locus again has no effect on the levels of these spores GRs in the IM or total spore lysates (Ramirez Peralta *et al.* 2013; and S. Luu and P. Setlow, unpublished results). However, the rate of mHP germination of these complemented spores was approx. 2 fold slower than that of wild type spores (Table 2; Fig. 1d). Overexpression of *gerKD* from a strong forespore specific promoter at the *amyE* locus also has no major effects on either nutrient germination rates or GR levels in the IM or total spore lysates (Ramirez Peralta *et al.* 2013; and S. Luu and P. Setlow, unpublished results), but spores of the strain overex pressing GerKD exhibited an approx. 5 fold slower rate of mHP germination than wild type spores (Table 2; Fig. 1d).

mHP germination of SD spores

Both the IM and total lysates of *B. subtilis* spores that are SD for germination with L valine have approx. 7 fold lower GerA GR levels and approx. 2 fold lower levels of other GRs, and these spores' germination with L valine and to a lesser extent AGFK is also greatly slowed (Ghosh and Setlow 2009; Ghosh *et al.* 2012; S. Ghosh and P. Set low, unpublished results) (Table 2). Previously, we reported that *B. subtilis* spores' SD for L valine germina tion germinated relatively normally with mHP (Wei *et al.* 2010). However, we decided to re examine these SD spores' mHP germination, because the procedure for the isolation of SD spores has been modified to become more stringent (Ghosh *et al.* 2012). Strikingly, the mHP germi nation of the spores SD for germination with L valine that were isolated by the new more stringent protocol was extremely slow compared to that of the total dor mant spore population (Fig. 2a). Indeed, the mHP ger mination of these SD spores was approximately the same as that of spores in which the GerA GR was absent due to mutation (Fig. 2a).

mHP germination of spores with a dominant negative *gerD* mutation

GerD is a small protein present in spores of *Bacillales* spe cies that is essential for rapid rates of nutrient germination, possibly because it is essential for organization of GRs in a complex in spores' inner membrane (Griffiths *et al.* 2011).

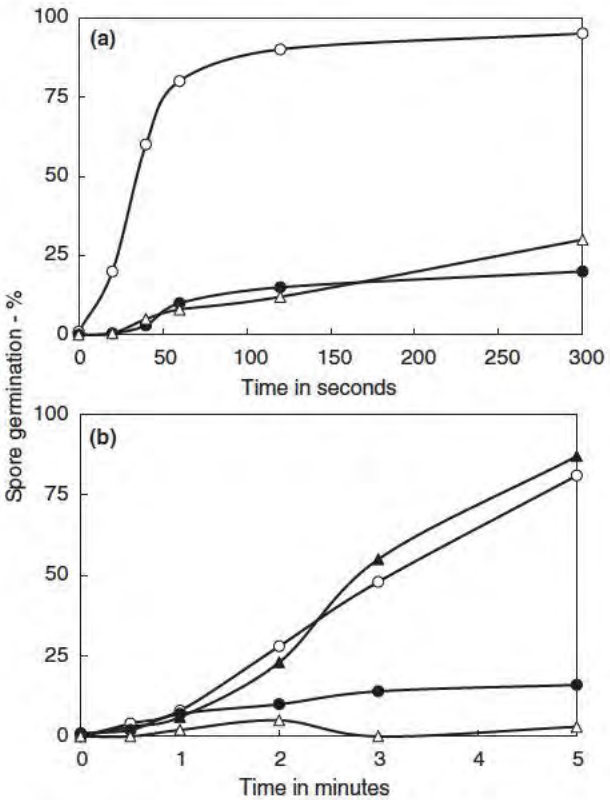

Figure 2 (a, b) mHP germination of (a) wild type, wild type SD and *gerA* spores, and (b) spores with various *gerD* alleles. (a) Spores of strains PS533 (wild type) (○, ●), either the original dormant spore population (○) or isolated from the latter population as SD for L valine germination (●), or FB20 (Δ*gerA*) (△); and (b) PS832 (wild type) (○), FB62 (*gerD*) (●), PS4386 (*gerD amyE::gerD*) (▲), or PS4385 (*gerD amyE::gerD*^{F87C}) (△) were germinated with mHP and the extents of spore germination at various times were determined as described in Materials and methods.

GerD is also essential for rapid germination of *B. subtilis* spores with mHP (Pelczar *et al.* 2007). Recently, a dominant negative *gerD* mutation in *B. subtilis* was identified (Li *et al.* 2014). This mutation is a Phe to Cys change in aa 87, and *gerD*^{F87C} acts much like a *gerD* null mutant alone but is dominant to wild type *gerD*. However, GR levels are unaffected by the *gerD*^{F87C} allele (Li *et al.* 2014). As expected spores with *gerD*^{F87C} alone exhibited minimal if any germination with mHP compared to that of wild type spores, and the mHP germination of spores of this *gerD* variant strain was even slower than that of spores lacking GerD altogether (Fig. 2b; Table 2).

vHP germination of spores with alterations in germination proteins

In contrast to the large effects on mHP germination of sporulation medium composition and levels of the YlyA transcription factor or the likely GR D subunit, these factors had no major effects on vHP germination (Fig. 3a c). This was not surprising, since the effects of these variables are, in so far as is known, only on GR lev els or GR function (Ramirez Peralta *et al.* 2012b; Traag *et al.* 2013). The dominant negative GerD allele also had no major effects on the vHP germination rate (Fig. 3d). Spores SD for germination with L valine also exhibited no major decrease in vHP germination compared to the initial dormant spores (Fig. 3e). In some of these experi ments, small decreases in rates of vHP germination were seen with spores that had altered GR levels or GR func tion, for example with SD spores (Fig. 3e). While the rea sons for these small differences are not clear and they may well not be very significant, a contributing factor may be a slight reduction in possible low rates of GR dependent germination even at vHP. The largest effect on vHP germination rates was seen with the *spoVT* spores that exhibited more than twofold lower rates of germina tion compared to wild type spores (Fig. 3f). The *spoVT* mutation results in increased levels of GRs in spores and faster rates of mHP germination (Ramirez Peralta *et al.* 2012a) (Table 2), so this cannot account for the decrease in vHP germination of these spores. However, in addition to its action as a repressor of operons that encode GRs, SpoVT is also an activator of the *spoVA* operon that encodes proteins involved in both sporulation and spore germination (Wang *et al.* 2006; Setlow 2013), and this may well explain the slow vHP germination of the *spoVT* spores (see Discussion). Indeed, levels of at least one SpoVA protein, SpoVAD are approx. twofold lower in the IM and total lysates of *spoVT* spores than in wild type spores (Ramirez Peralta *et al.* 2012a; and S. Luu and P. Setlow, unpublished results).

Discussion

The results on mHP germination obtained in this current work are certainly consistent with levels of total GRs being a major factor determining rates of *B. subtilis* spore germination with mHP. This was the case with spores in which GR levels were changed by alterations in either sporulation media or levels of several proteins that mod ulate transcription of operons encoding GRs. However, some qualification is needed, since it appears likely that different GRs respond differently to mHP; in particular, the GerA GR appears to be significantly more responsive to mHP than are the GerB and GerK GRs. This was seen previously (Black *et al.* 2005) and in the current work, as loss of GerA reduced spores' mHP germination much more than would be expected by the <50% decrease in total GRs in *gerA* spores. It is conceivable that mHP trig gers spore germination by inducing conformation

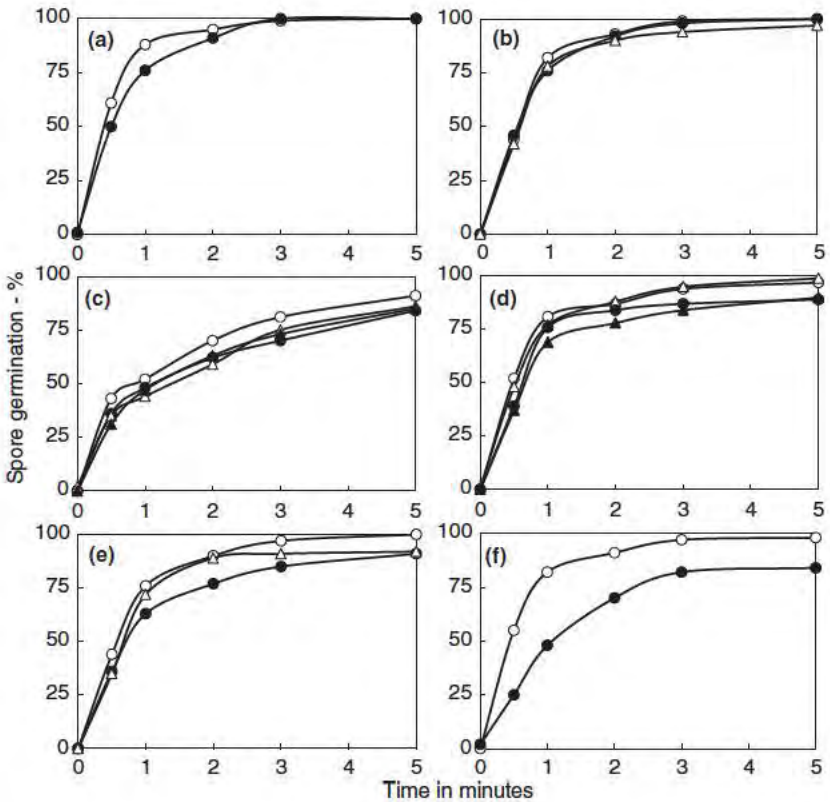

Figure 3 (a–e) vHP germination of spores made in different media or with and without SpoVT or YlyA, and with various levels of GerKD. Spores of strain: (a) (○, ●) PS533 (wild type) made in rich (○) or poor (●) liquid media; and spores made on plates of strains: (b) (○) PY79, (●) BAT96 (*ylyA*), and (△) BAT264 (↑YlyA); (c) (○) PS533 (wild type), (●) PS4256 (*gerKD*), (△) PS4313 (*gerKD amyE*::*gerKD*), and (▲) PS4314 (↑GerKD); (d) (○) PS832 (wild type), (●) FB62 (*gerD*), (△) PS4386 (Δ*gerD amyE*:*gerD*), (▲) PS4385 (*gerD amyE*:*gerD*^{F86C}); (e) (○, ●) PS533 either dormant (○) or SD with L valine (●), and (△) FB20 (*gerA*); and (f) (○) PS533 and (●) PS4220 (*spoVT*) were germinated with vHP and the extents of spore germination at various times were determined as described in Materials and methods. All values shown were ≤ ±15%.

changes that activate GRs, and it seems reasonable that different GRs would undergo such conformation changes differently in response to mHP. However, further study is needed to address this possibility.

While GR levels, in particular the levels of GRs' A, B and C subunits are clearly a major factor determining spores' rates of mHP germination, this is not the only such determining factor. This was seen most notably in the minimal if any mHP germination of spores with the *gerD*^{F87C} allele even though these spores have normal GR subunit levels, and the mHP germination of these spores was even lower than that of spores lacking wild type GerD. The presence of the GerD^{F87C} variant also essentially eliminates the nutrient germination of these spores (Li *et al.* 2014), indicating that this variant can completely block GR function, and that whatever role(s) GerD and GerD^{F87C} have in nutrient germination, these proteins have the same role(s) in mHP germination. In addition, the effects of variations in the level and perhaps also the location of expression of at least one putative GR D, subunit, GerKD, also altered rates of mHP germination independent of GR levels. Since the precise function of GR D subunits is not known, how defects in these genes alter rates of spore germination with either nutrients or mHP is unclear. One possible scenario is that GerKD is essential for normal function of the GerK GR, and while the loss of GerKD has a significant effect on AGFK germination, the contribution of the GerK GR to mHP germination is rather small, as seen previously (Black *et al.* 2005). However, the large decreases in mHP germination when *gerKD* is expressed ectopically at the *amyE* locus are more difficult to explain. Perhaps, when GerKD is expressed ectopically this small likely integral membrane protein can associate with and alter the response of the GerA GR to mHP, although not to nutrient germinants. Clearly, more work is needed to understand all the effects of these GR D subunits.

It is also clear that the quantitative relationship between GR levels and rates of mHP germination is not simple. As noted above, for total GRs this relationship is complicated since different GRs appear to have different responsiveness to mHP. However, it seems likely that even when this factor is taken into account, rates of mHP germination are not directly proportional to GR levels, and also may not directly track with rates of nutrient germination. Thus, spores prepared in a poor liquid medium exhibit less of a decrease in the rate of mHP germination than expected from their lower GR levels, and these spores' levels of the GerA GR decrease less than levels of the GerB and GerK GRs (Ramirez Peralta *et al.* 2012b). A likely explanation for this lack of a linear proportionality between GR levels and rates of mHP germi

nation is that mechanistically such proportionality is not to be expected because (i) there appear to be synergistic effects between GRs in triggering nutrient germination (Yi *et al.* 2011); and (ii) at high GR levels some factor other than GRs may become rate limiting for spore ger mination. Thus, there must be a factor or factors in addi tion to GR levels alone that determine rates of spore germination with mHP, and levels of GR D subunits and perhaps GerD are possible candidates.

In contrast to mHP germination where levels and func tionality of GRs are the major factor determining rates of this process, in almost all cases these variables played no role in affecting rates of vHP germination, consistent with previous work (Wuytack *et al.* 1998; Black *et al.* 2007; Reineke *et al.* 2013). The only result that was at odds with the latter statement was the approx. twofold decrease in the rate of vHP germination with *spoVT* spores. Since the *spoVT* spores have elevated GR levels, if anything this would might increase vHP germination slightly. However, SpoVT is not only a repressor of operons that encode GRs but is also an activator of the *spoVA* operon (Wang *et al.* 2006). Indeed, *spoVT* spores have significantly lower levels of at least one SpoVA protein, SpoVAD, in their IM and total spore lysates, while all other factors that affect mHP germination rates do not affect IM levels of SpoVAD (Ramirez Peralta *et al.* 2012a,b, 2013; Traag *et al.* 2013; and S. Luu and P. Setlow, unpublished results). The *B. subtilis spoVA* operon encodes seven proteins, most of which are likely integral IM proteins, and there is signifi cant evidence that these proteins form a channel in the IM that allows release of spores' huge DPA depot, with this release then leading to completion of spore germina tion (Setlow 2013; Velásquez *et al.* 2014). Given this information, it is tempting to speculate that it is this DPA channel that is activated somehow by vHP, since it is known that an early, and perhaps the earliest event in vHP germination is DPA release (Reineke *et al.* 2013). If this is indeed the case, then lower levels of SpoVA pro teins in *spoVT* spores might lead to a lower rate of vHP germination, although *ylyA* spores have elevated SpoVAD levels yet their vHP germination is like that of wild type spores (Traag *et al.* 2013). An obvious question then, is why do such changes in SpoVAD levels not have large effects on mHP germination as well, since DPA release is a normal event following triggering of GRs by either nutrients or mHP (Setlow 2013)? However, levels of SpoVA protein channels may not be rate limiting for mHP or even nutrient germination. Indeed, the crucial event in mHP germination appears to be extremely rapid GR activation with no apparent change in spores until DPA release at much later and variable times, and this later DPA release is not even dependent on continued mHP application (Kong *et al.* 2014). It may be informa tive in the future to examine how changes in GR levels and functionality affect what appears to be the commit ment to germinate that is rapidly induced by mHP treat ment, and also how changes in multiple SpoVA protein levels, either increases or decreases, alter spores' germina tion with both mHP and vHP.

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Conflict of Interest

No conflict of interest declared.

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