UNIVERSIDAD AUTÓNOMA DE GUERRERO

UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA

DOCTORADO EN CIENCIAS BIOMÉDICAS

"PAPEL DE LAS PROTEÍNAS DE GERMINACIÓN EN ESPORAS DE *Bacillus subtilis*"

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTORADO EN CIENCIAS BIOMÉDICAS

PRESENTA:

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CHILPANCINGO, GRO., ABRIL DE 2016.

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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 20 días del mes de enero del dos mil dieciséis, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Papel de las proteínas de germinación en esporas de *Bacillus subtilis*"**, presentada por el alumno José Cruz Mora, para obtener el Grado de Doctor en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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DIRECCIÓN 2014 - 2018

Esta tesis se realizó en el Departamento de Biología Molecular y Biofísica, del Health Center de la Universidad de Connecticut, Farmington, Connecticut, Estados Unidos de América y en el Laboratorio de Biomedicina Molecular de la Unidad Académica de Ciencias Químico Biológicas en Chilpancingo, Guerrero, Mexico.

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Durante el período en que cursó el Doctorado en Ciencias Biomédicas, el C. José Cruz Mora, recibió beca del CONACYT.

The experiments in this thesis were realized in the **Department of Molecular Biology and Biophysics**, **University of Connecticut Health Center**, Farmington, Connecticut, United States of America.

Under supervision of:

Ph.D. Peter Setlow

Acknowledgment/Agradecimientos

Thank you to members of Setlow laboratory, I want to give my gratefulness to:

Ph.D Peter Setlow and his wife Barbara, for their great support and patience when I was stayed with them. Particularly, for their support when I worked in their laboratory. Sincerely thank you.

Theacher George Korza, for his support and patience when I worked with him. Thank you.

Dra. Abigail Perez-Valdespino, por su apoyo y amistad que me brindo cuando estuve en Connecticut, por su gran ayuda en el laboratorio del Dr. Peter. Una gran amiga. Muchisimas gracias.

Muchas gracias a **Leo**, por su apoyo y amistad que me brindó durante mi estancia en los Estados Unidos.

Muchas gracias a los integrantes del Laboratorio de Biomedicina, especialmente al:

Dr. Marco Antonio Leyva Vázquez, por todo su apoyo brindado durante el transcurso del doctorado, por su apoyo en la escritura de la tesis y por su amistad. Muchas gracias doctor.

Gracias a la Dra. Natividad Castro Alarcón, Dra. Eugenia Flores Alfaro y a la **Dra. Berenice Illades Aguiar,** por sus revisiones y aportaciones durante el desarrollo de mi tesis.

A mis compañeros de la generación del Doctorado 2013-2016.

A todos y cada una de las personas que de forma directa o indirecta ayudaron en el desarrollo este proyecto. Muchas gracias.

José Cruz Mora

Dedico esta tesis a
mis padres y hermanos,
quienes me apoyaron todo el tiempo.

ÍNDICE GENERAL

RESUMEN

La germinación de esporas del género *Bacillus* inicia cuando los germinantes nutrientes se unen a los receptores germinantes (RG) en la membrana interna (MI) de la espora en un proceso estimulado por la activación subletal térmica; esta interacción dispara la liberación de ácido dipicolinico y cationes del núcleo de la espora y es remplazado por agua. La esporas de *B. subtilis* contienen tres RG funcionales; GerA, GerB, y GerK. El RG GerA dispara la germinación con L-valina o L-alanina, mientras que GerB y GerK actúan cooperativamente para disparar la germinación con una mezcla de L-asparagina, D-glucosa, D-fructosa y KCl (AGFK). En el 2013 se reportó que en *B. subtilis* el gen *gerW* es esencial para la germinación con L-alanina en esporas de *B. subtilis,* pero no para la germinación con AGFK. Sin embargo en este trabajo se encontró que la perdida del gen *gerW* en *B. subtilis* no tuvo efectos significativos en: 1) Las tasas de germinación de espora con L-alanina; 2) Los niveles de las proteínas de germinación, incluyendo a las subunidades del RG GerA; 3) Las tasas de germinación de espora con AGFK; 4) La germinación de espora por vía independiente de los RG y 5) El crecimiento de las esporas germinadas. Además la disrupción del gen *gerW* en *B. megaterium* no tuvo efectos en la germinación vía RG o en la germinación independiente de los receptores germinantes.

Las tasas germinación máxima de las esporas de *B. subtilis* vía diferentes RG requieren diferentes tiempos de activación térmica a 75°C, 15 minutos para la germinación con L-valina vía RG GerA y 4 horas para la germinación con AGFK vía RG GerB y GerK; pero GerK requiere de más tiempo de activación térmica para alcanzar la tasa máxima de germinación. En algunos casos la activación térmica óptima disminuyó a la mitad la concentración de los nutrientes necesarios para alcanzar la tasa máxima de germinación de las esporas. La germinación vía varios RG con presión alta de 150 MPa exhibió requerimientos similares de la activación térmica en la germinación con nutrientes. Interesantemente la perdida de GerD no eliminó estos requerimientos para obtener una optima germinación.

Los resultados son consistentes con que la activación térmica actúa principalmente en los RG. Sin embargo; 1) La activación térmica no tuvo efecto en la conformación de los RG o en la proteína GerD probada por la biotinilación con un agente externo, 2) Las esporas preparadas a baja y altas temperaturas que afectan las propiedades de la MI, mostraron diferencias significativas en los requerimientos de la activación térmica para la germinación con nutrientes y 3) La germinación de esporas con alta presión de 550 MPa también fue afectada por la activación térmica, pero los efectos fueron relativamente independiente de los RG. Interesantemente, la activación térmica estimula la germinación de las esporas de *Bacillus amyloliquefaciens* con presión alta de 150 y 550 MPa, estas esporas son utilizadas como modelo de estudio de las esporas de *Clostridium botulinum* en tratamientos con alta presión en alimentos.

ABSTRACT

Germination of dormant spores of *Bacillus* species is initiated when nutrient germinants bind to germinant receptors in spores' inner membrane a process stimulated by sublethal heat activation and this interaction triggers the release of dipicolinic acid and cations from the spore core and their replacement by water. *Bacillus subtilis* spores contain three functional germinant receptors encoded by the *gerA*, *gerB*, and *gerK* operons. The GerA germinant receptor alone triggers germination with L-valine or L-alanine, and the GerB and GerK germinant receptors together trigger germination with a mixture of L-asparagine, D-glucose, D-fructose and KCl (AGFK). Recently, it was reported that the *B. subtilis* gerW gene is expressed only during sporulation in developing spores, and that GerW is essential for L-alanine germination of *B. subtilis* spores but not for germination with AGFK. However, we now find that loss of the *B. subtilis* gerW gene had no significant effects on: i) rates of spore germination with L-alanine; ii) spores' levels of germination proteins including GerA germinant receptor subunits; iii) AGFK germination; iv) spore germination by germinant receptor-independent pathways; and v) outgrowth of germinated spores. Also, disruption of gerW again had no effect on the germination of *B. megaterium* spores, whether germination was triggered via germinant receptor-dependent or germinant receptor- independent pathways.

Bacillus subtilis spores maximum germination rates via different GRs required different 75°C heat activation times: 15 min for L-valine germination via the GerA GR and 4 h for germination with the AGFK mixture via the GerB and GerK GRs, with GerK requiring the most heat activation. In some cases, optimal heat activation decreased nutrient concentrations for half-maximal germination rates. Germination of spores via various GRs by high pressure (HP) of 150 MPa exhibited heat activation requirements similar to those of nutrient germination, and the loss of the GerD protein, required for optimal GR function, did not eliminate heat activation requirements for maximal germination rates.

These results are consistent with heat activation acting primarily on GRs. However, (i) heat activation had no effects on GR or GerD protein conformation, as probed by biotinylation by an external reagent; (ii) spores prepared at low and high temperatures that affect spores' IM properties exhibited large differences in heat activation requirements for nutrient germination; and (iii) spore germination by 550 MPa of HP was also affected by heat activation, but the effects were relatively GR independent. The last results are consistent with heat activation affecting spores' IM and only indirectly affecting GRs. The 150- and 550-MPa HP germinations of *Bacillus amyloliquefaciens* spores, a potential surrogate for *Clostridium botulinum* spores in HP treatments of foods, were also stimulated by heat activation.

I. INTRODUCCIÓN

Bacillus subtilis tiene dos ciclos de vida alternativos. En el ciclo vegetativo, en presencia de condiciones favorables, replica su cromosoma y se divide por fisión binaria en dos células hijas equivalentes cada 30 minutos (Angert, 2005) y el ciclo de esporulación. La espora se forma, mediante el proceso de esporulación en respuesta a la limitacion de nutrientes; este proceso involucra interacciones íntimas entre las dos células y una serie de cambios morfológicos que llevan a la formación de la espora en 8 a 10 horas aproximadamente, como se muestra en la Figura 1 (Higgins y Dworkin, 2012). La espora presenta una alta resistencia al entorno capaz de sobrevivir a temperaturas extremas, desecación, agentes químicos tóxicos y a radiación ionizante; estas propiedades de resistencia y el metabolismo inactivo de la espora le permiten permanecer largos periodos de tiempo en latencia en su entorno (Paredes-Sabja *et al*., 2011).

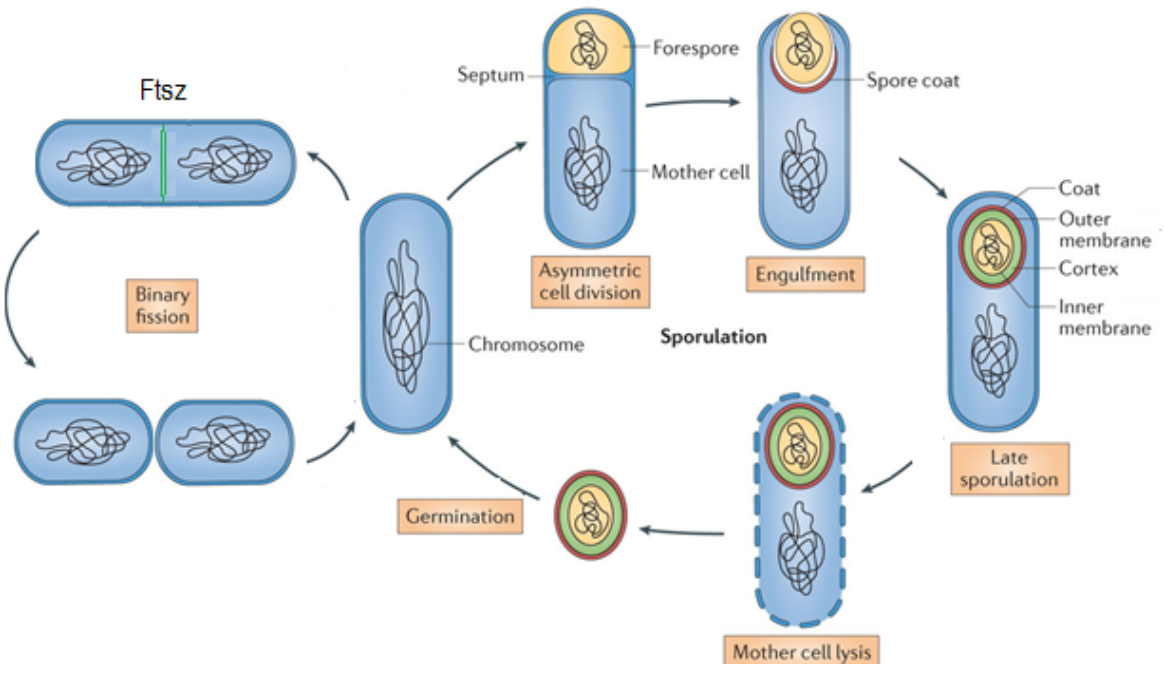

Figura 1. Ciclo de vida de *Bacillus subtilis* (Modificada y adaptada de Argent, 2005; McKenney *et al.*, 2013).

La esporulación de *B. subtilis* es iniciada por la fosforilación del regulador maestro de la esporulación Spo0A. Un evento temprano de la esporulación es una división asimétrica del citoplasma, generando dos tipos celulares, la pre-espora (compartimento pequeño) y la célula madre. En estos dos tipos celulares la expresión de genes en la esporulación es controlada por los factores sigma (σ) de la RNA polimerasa, con σ^F y σ^G en la pre-espora y σ^E y σ^K en la célula madre (Errington, 1993; Levdikov *et al*., 2012). La progresión del desarrollo de la pre-espora involucra una serie de eventos (de Hoon et al 2010) incluyendo (1) la célula madre engulle a la pre-espora en un proceso similar a la fagocitosis, (2) la síntesis del peptidoglicano (PG) en la pared celular bacteriana de la espora y el PG de la corteza; (3) una gran disminución del contenido de agua en el núcleo de la pre-espora, seguida por el almacenamiento de grandes cantidades de calcio unido al ácido dipicolínico (CaDPA), el cual es sintetizado en la célula madre y que favorece la disminución del contenido de agua del núcleo de la espora y (4) recubrimiento de la superficie externa de la pre-espora con un complejo de proteínas de cubierta. Finalmente la célula madre se lisa para permitir la liberación de la espora madura en el ambiente.

La estructura de la espora es muy diferente a la célula vegetativa, el núcleo de la espora contiene un bajo contenido de agua (25–50% de peso húmedo), altos niveles de CaDPA y el DNA esta saturado con proteínas pequeñas solubles ácidas (SASP), que contribuyen a las propiedades de resistencias en la espora (Setlow, 2007). La membrana interna (MI) rodea al núcleo y es impermeable aún a pequeñas moléculas sin cargas e incluso el agua tiene difícil acceso, propiedad que probablemente protege de daño al DNA por agente químicos. Alrededor de la MI esta la pared celular bacteriana compuesta de PG, esta es importante en el crecimiento de la espora después de iniciarse la germinación. En la parte externa de la pared celular esta la corteza de PG, este PG presenta tres principales modificaciones: 1) un 25% de los residuos ácido *N*-acetilmurámico (NAM) son sustituidos con péptidos cortos; 2) un 25% de los residuos NAM llevan un residuo de L-alanina; 3) un 50% de los residuos de ácido murámico son convertido a

murámico-δ-lactama; éstas dos últimas modificaciones no son encontradas en la pared celular bacteriana. Posteriormente esta la membrana externa, aunque esta no actúa como una barrera permeable pero es esencial para la formación de la espora. La siguiente capa es la cubierta que sirve como una barrera inicial a moléculas grandes, tales como la lisozima, enzima que degrada al PG y por último está el exosporium, el cual podría tener un papel importante en la patogénesis de la espora (Leggett *et al*., 2012; Paredes-Sabja *et al*., 2011).

Las esporas permanecen en un estado de latencia en el entorno y además son altamente resistentes a condiciones extremas, sin embargo son sensitivas a moléculas en el medio ambiente y si moléculas apropiadas o condiciones son sensados, la latencia y la resistencia de la espora es rápidamente perdida cuando la germinación es iniciada, este proceso presenta varias etapas como se muestra en la Figura 2 (Setlow, 2013).

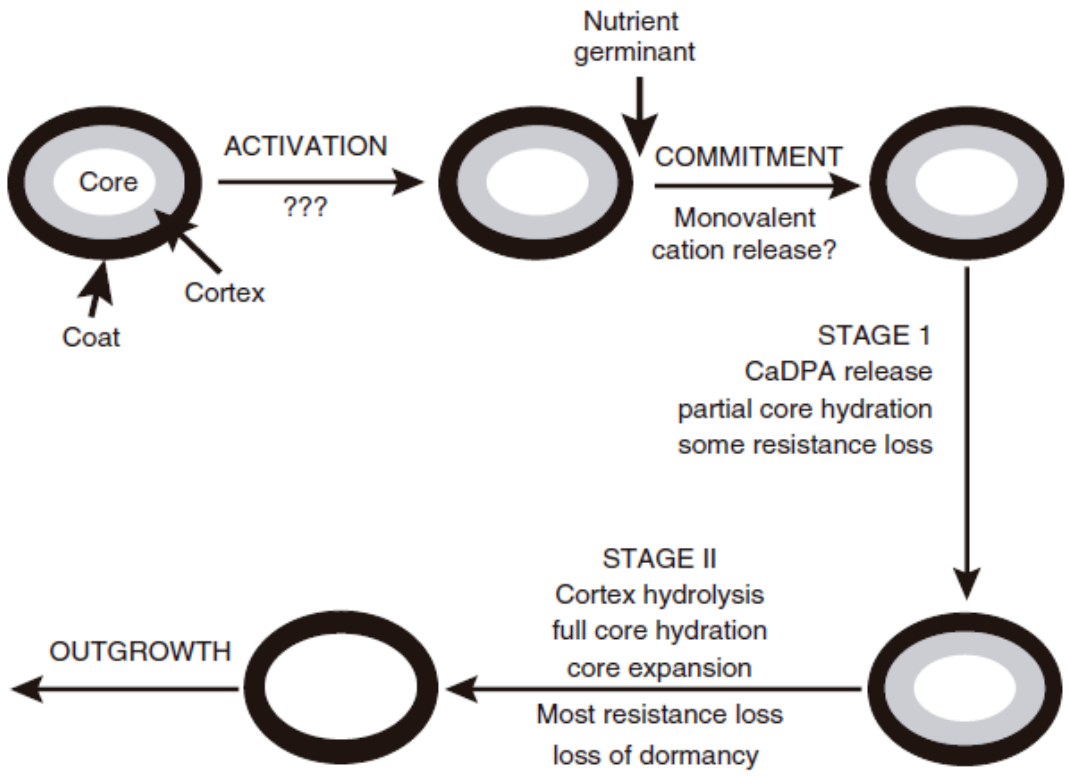

Figura 2. Proceso de germinación con nutrientes en esporas de *Bacillus* (Setlow, 2013).

Los eventos en la activación de la espora antes de la adición de los nutrientes germinantes no son completamente claros. En la germinación *in vitro* los germinantes nutrientes son adicionados después de la activación de la espora por choque térmico (75 °C por 30 minutos y 10 minutos en hielo) y el primer evento observado se ha denominado como "commitment", incluso si los germinantes nutrientes son removidos, la germinación no es bloqueada; la liberación de cationes monovalentes también es iniciada en una etapa muy temprana en la germinación y solo se ha sugerido que la liberación de cationes monovalentes se lleva a cabo al mismo tiempo en el que se establece el commitment. Posterior a la liberación de los cationes monovalente se establece la etapa 1, la cual se caracteriza por la liberación de grandes cantidades de CaDPA (~10% del peso seco de la espora) del núcleo de la espora. Mientras que en la etapa 2 se lleva a cabo la hidrólisis del PG de la corteza de la espora, debido a la activación enzimática por la liberación de CaDPA (Setlow, 2013). La acción enzimática en el núcleo de la espora inicia después de terminar la etapa II, permitiendo el inicio del metabolismo de la espora, seguida por la síntesis macromolecular que convierte a la espora germinada en una célula en crecimiento (Setlow, 2013).

Los germinantes comprenden una amplia variedad de compuestos generalmente de bajo peso molecular, así como también al menos una condición ambiental, la presión hidrostática (PH) (Setlow, 2013). Los germinantes de moléculas pequeñas son divididos en germinantes nutrientes y no nutrientes los cuales disparan la germinación por métodos diferentes. Sin embargo, esto es un poco impreciso porque algunos germinantes no nutrientes disparan la germinación por la misma vía que lo hacen los germinantes nutrientes, además los germinantes nutrientes solo actúan como moléculas de señalización. Los germinantes nutrientes incluyen un gran número de nutrientes específicos como aminoácidos, azúcares o derivados de purina. Los germinantes nutrientes son estéreoespecífico como la L-alanina es un germinante muy usual, mientras que la D-alanina es un fuerte inhibidor de la germinación con L-alanina. Generalmente los germinantes nutrientes disparan la germinación a través de interacciones con receptores

germinantes (RG) localizados en la membrana interna en la espora (Setlow, 2013, Setlow, 2014). Por otra parte los germinantes no nutrientes mejor estudiados son el CaDPA y los surfactantes catiónicos, en particular la dodecilamina. Estos compuestos disparan la germinación de esporas por un mecanismo independiente de los nutrientes germinantes donde los RG no están involucrados (Setlow, 2013). Además existe otros germinantes no nutrientes como los fragmentos de PG que se ha sugerido que disparan la germinación a través de la activación de proteínas cinasas (Shah *et al*., 2008); sales biliares específicas que disparan la germinación de esporas de *C. difficile* (Burns *et al*., 2010); y la lisozima u otras hidrolasas de PG, pero solo si la cubierta de la espora es destruida (Setlow, 2013). El último germinante no nutriente es la alta presión hidrostática. La exposición a niveles bajos de HP de 100–400 MPa y a moderadas temperaturas (20–50°C), se ha observado que dispara la germinación a través de los RG en esporas de *B. subtilis* y *B. cereus.* Sin embargo cuando las esporas son expuestas a una HP de mayor de 500 MPa y a temperatura de 25°C no disparan la germinación mediante los RG. Bajo estas condiciones se ha sugerido que los canales de CaDPA son abiertos directamente a partir de estudios realizados en esporas de *B. subtilis* knock out para los genes que codifican los principales RG (Reineke *et al*., 2013).

Existen varias proteínas requeridas en el proceso de germinación de esporas y estas proteínas no están presentes en células vegetativas, además solo son sintetizados durante la esporulación, algunas en la pre-espora y otros en la célula madre (Setlow, 2013), las cuales son las siguientes:

- **Proteína GerP**

Las proteínas GerP son un grupo de proteínas pequeñas codificadas en un operón y expresadas solo en la esporulación en el compartimento de la célula madre, estas proteínas están localizadas en la cubierta de la espora (Carr *et al.,* 2010). La secuencia de las proteínas GerP no presentan similitud con proteínas conocidas, además la pérdida de una o todas las proteínas GerP en *B. anthracis, B. cereus* y *B. subtilis* reduce significativamente la germinación con germinantes nutrientes y

con CaDPA, pero no tiene efecto en la germinación con dodecilamina. Además la esporas deficientes en GerP no parecen tener defectos en la cubierta y el fenotipo mutante de estas esporas es eliminado cuando se retira la cubierta de la espora, químicamente o genéticamente (Butzin *et al.,* 2012; Setlow, 2013). Estos hallazgos han sugerido que las proteínas GerP facilitan el movimiento de algunos germinantes a través de las capas externas de la espora para poder acceder a los RG presentes en la MI. Como soporte a esta sugerencia, el fenotipo de la esporas *gerP*-deficientes se puede suprimir por concentraciones elevadas de nutrientes germinantes y estas esporas germinan normalmente con presiones de aproximadamente 150 MPa que desencadenan la germinación mediante la activación de los RG (Setlow, 2013).

- **Proteína GerD**

La proteína GerD es de aproximadamente 180 aminoácidos es sintetizada solo en la pre-espora en paralelo con los RG y las proteínas SpoVA 1–2 horas antes de la acumulación de CaDPA en el núcleo de la espora. GerD es una proteína periférica en la MI de la espora, anclada por una porción diacilglicerol (Pelczar y Setlow, 2008). La función de la proteína GerD es desconocida, sin embargo la ausencia de este gen, disminuye significativamente la tasa de germinación dependiente de RG (Pelczar *et al*., 2007). El papel esencial de la proteína GerD en la germinación dependiente de RG parece ser solo en el género de *Bacillus*; GerD no está presente en las esporas del género de *Clostridium* (Paredes-Sabja *et al*., 2011). En el 2011, Griffiths *et al.,* mostraron que los RG y GerD están colocalizados en un mismo clúster en las esporas, además observaron que todas las subunidades del RG, así como también de la proteína GerD son requeridas para formar un clúster, sugiriendo que los “clúster” representan una unidad de germinación funcional o germinosoma en la MI de la espora, necesaria para una respuesta rápida y cooperativa a los nutrientes. En *B. subtilis*, existen ~4000 moléculas de GerD por espora y estas son constantes en diferentes medios de esporulación, aunque puede variar entre esporas individuales (Setlow, 2013).

- **Receptores germinantes**

Los RG estan compuestos por las subunidades A, B y C que interactúan para formar un RG funcional; además recientemente una cuarta proteína ha sido identificada en el RG en especies de *Bacillus* y *Clostridium* (Setlow, 2013). La subunidad A y B son proteínas transmembranales (TM), la primera presenta cinco o seis dominios TM, y los dominios hidrofílicos N y C terminal, mientras que la segunda presenta 10 a 12 hélices TM, la cual puede estar involucrada en el reconocimiento del germinante nutriente. Finalmente la subunidad C, una proteína periférica, la cual está anclada a la superficie externa de la membrana a través de una fracción lípidica N terminal (Ross y Abel-Santos, 2011).

Las esporas de *B. subtilis* contienen tres principales RG funcionales codificados por los operones *gerA, gerB* y *gerK* (Ross y Abel-Santos, 2011). Estos operones son expresados paralelamente en la esporulación tardía en el desarrollo de la espora, bajo el control del factor sigma σ^G (Wang *et al*., 2006). GerA es el único receptor en *B. subtilis* que es activado por un solo germinante, L-alanina. No se ha identificado un germinante para los RG GerB o GerK. El receptor GerK parece reconocer glucosa, mientras que GerB parece reconocer aminoácidos y fructosa, sin embargo, no es suficiente para inducir la germinación (Yi y Setlow, 2010; Ross y Abel-Santos, 2011). GerB y GerK actúan cooperativamente para inducir la germinación con L-asparagina suplementada con glucosa, fructosa y potasio (GFK) o L-alanina suplementada con GFK (Atluri *et al*., 2006; Griffiths *et al*., 2011). Es importante señalar que la activación de GerK estimula la germinación mediada por GerA con L-alanina. Así como también GerB puede interactuar con GerA para inducir la germinación en esporas en respuesta a nutrientes apropiados (Atluri *et al*., 2006).

La cuarta proteína en el RG que modifica la función del RG, llamada “subunidad D” al menos en esporas de *B. megaterium* y *B. subtilis*. Los genes para esta posible subunidad D están dentro o adyacente a los operones que codifican a las

subunidades del RG, son transcriptos en paralelo a las otras subunidades del RG, y codifican pequeños residuos de proteínas ~75 aa con una predicción de dos dominio TM. En esporas de *B. megaterium* y *B. subtilis*, la perdida de estos genes D tiene efectos negativos y positivos en función del RG, aunque no afecta los niveles de las proteínas de germinación (Ramirez-Peralta *et al*., 2013).

- **Proteína GerW**

La proteína GerW (tambien llamada YtfJ) es una proteína muy conservada de 151 aminoácidos encontrada en esporas de *Bacillus* y *Clostridium,* la cual es expresada bajo la regulación del factor σ^F en la pre-espora y fue reportada que está involucrada en los estados tempranos de la germinación bajo condiciones experimentales. La predicción de la secuencia de *gerW* (Bioomsbury Center for Bioinformatic), sugiere que es una proteína transmembranal con la parte N-terminal en el espacio extracelular y la porción C-terminal en el núcleo. Mediante estudios de fusión con la proteína verde fluorescente en la porción C-terminal de la proteína GerW, se mostró que está concentrada en el núcleo de la espora y que podria interactúar con las proteínas de germinación presentes en la membrana interna de la espora. Además la frecuencia de germinación de las esporas deficientes en *gerW* fue mucho más bajo que las esporas de tipo silvestre en presencia de L-alanina, mientras que la frecuencia de la germinación con AGFK fue similar a las esporas de tipo silvestre, concluyendo que la proteína GerW está involucrada en la germinación de esporas estimuladas con L-alanina. Además en las esporas deficientes en *gerW* cuando son incubadas con L-alanina no llevan a cabo la liberación del CaDPA; sin embargo cuando estas esporas son incubadas con AGFK muestran una liberación de cantidades comparable de CaDPA con las esporas de tipo silvestre (Kuwana y Takamatsu, 2013).

- **Proteínas SpoVA**

Las proteínas SpoVA son expresadas en el desarrollo de la pre-espora en paralelo con los RG y GerD, la predicción sugieren que son proteínas membranales, localizadas en la superficie externa de la MI (Paredes-Sabja *et al*., 2011; Xiao *et*

al., 2011, Setlow, 2013). En *B. subtilis*, existen siete proteínas, SpoVAA, B, C, D, Eb, Ea y F, los cuales son codificados en un solo operón en este orden. En otras especies de *Bacillus* y *Clostridium* pueden tener menos proteínas (aunque SpoVAC, SpoVAD y SpoVAEb siempre están presente) y en algunos casos con más de una unidad transcripcional. Se ha sugerido que las proteínas SpoVA forman un canal para la acumulación de CaDPA durante la esporulación y así como también la liberación del CaDPA en la germinación de la espora (Setlow, 2013; Perez-Valdespino *et al*., 2014). Además las proteínas SpoVA pueden asociarse con los receptores germinantes y unirse con el germinante nutriente para inducir la germinación de la espora (Vepachedu y Setlow, 2007).

- **Enzimas líticas de la corteza**

La bacteria *B. subtilis* presenta dos enzimas que cortan los enlaces muramico δ-lactama presentes en el PG permitiendo la degradación selectiva de la corteza de la espora durante la germinación llamadas CwlJ y SleB (Paredes-Sabja *et al*., 2011). CwlJ es sintetizado en el compartimento de la célula madre en el desarrollo de la espora y se localiza entre la cubierta y la corteza; para su localización CwlJ requiere asociarse con la proteína GerQ (llamada tambien YwdL). SleB es sintetizada en la pre-espora y se localiza en la MI, sin embargo, SleB también es encontrada entre la cubierta y la corteza; para su localización en la espora también requiere asociarse con la proteína YpeB (Setlow, 2013). Las esporas con mutaciones en genes para CwlJ y SleB no son capaces de degradar al PG de la corteza y no puede progresar más allá de la etapa I de la germinación. Además la ausencia de CwlJ resulta en un incremento en el tiempo requerido para la completa liberación de CaDPA durante la germinación dependiente de RG (Setlow *et al*., 2009: Zhang *et al*., 2012).

En los años recientes se ha logrado conocer mucho del proceso de germinación, así como también la identificación de varias proteínas involucradas en este proceso en espora de *B. subtilis*. Sin embargo aún no se han descrito todos los mecanismos implicados en las etapas de la germinación o el papel de las

proteínas de germinación identificadas recientemente, como son el caso de la activación por choque térmico en las esporas que incrementa su eficiencia en la germinacion vía RG y el mecanismo de la proteína GerW que recientemente fue reportada por Kuwana y Takamatsu, 2013.

El entendimiento detallado del proceso de germinación de la espora continua siendo esencial para el desarrollo de una estrategia que permita obtener un adecuado método para inducir la germinación de las esporas presentes en alimentos y en la instrumentación médica para finalmente destruir a las esporas germinadas que son menos resistentes. La eliminación de las esporas es importante debido que representan un alto impacto médico; intoxicaciones causadas por el consumo de alimentos contaminados por toxinas (Dembek *et al.,* 2007) y las enfermedades postquirúrgicas causadas por *C. botulinum*, *C. perfringens*, *C. difficile* y *B. cereus* (Surowiec *et al*., 2006; Bottone, 2010; Finsteres y Hess, 2007), economico; pérdidas económicas para la industria alimentaria por la contaminación de alimentos y gastos en sector salud por el tratamiento de intoxicaciones y enfermedades causadas por estas bacterias e incluso táctico como el bioterrorismo por *B. anthracis* (Wang *et al*., 2013). En este trabajo se examinó los efectos de la proteína GerW en las tasas de germinación y los niveles de las proteinas de germinación en esporas de especies de *Bacillus*, asi como tambien se analizaron los efectos de la activación térmica en: 1) la germinación con nutrientes de esporas de *B. subtilis* con varios RG y con o sin la proteína GerD. 2) la germinación de esporas obtenidas a diferentes temperaturas, que difieren en su composicion lipidica de la MI. 3) la germinación de espora con presión hidrostática a 150 y 550 Mpa.

II CAPITULOS

2.1 Capitulo 1

"La proteína GerW no está involucrada en la germinación de esporas de especies de *Bacillus*"

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RESEARCH ARTICLE

The GerW Protein Is Not Involved in the Germination of Spores of *Bacillus* Species

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Citation: Cruz-Mora J, Pérez-Valdespino A, Gupta S, Withange N, Kuwana R, Takamatsu H, et al. (2015) The GerW Protein Is Not Involved in the Germination of Spores of *Bacillus* Species. PLoS ONE 10(3): e0119125. doi:10.1371/journal.pone.0119125

Academic Editor: Eric A. Johnson, University of Wisconsin, Food Research Institute, UNITED STATES

Received: August 2, 2014

Accepted: January 9, 2015

Published: March 19, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by a Department of Defense Multi-disciplinary University Research Initiative through the U.S. Army Research Laboratory and the U.S. Army Research Office under contract number W911F-09-1-0286 (PS/YQL), and by a grant from the Army Research Office under contract number W911NF-12-1-0325. SG was the recipient of an R. A. Fisher Bursary Award from Gonville and Caius College, University of Cambridge. The funders had no role in study design, data collection and

Abstract

Germination of dormant spores of *Bacillus* species is initiated when nutrient germinants bind to germinant receptors in spores' inner membrane and this interaction triggers the release of dipicolinic acid and cations from the spore core and their replacement by water. *Bacillus subtilis* spores contain three functional germinant receptors encoded by the *gerA*, *gerB*, and *gerK* operons. The GerA germinant receptor alone triggers germination with L-valine or L-alanine, and the GerB and GerK germinant receptors together trigger germination with a mixture of L-asparagine, D-glucose, D-fructose and KCl (AGFK). Recently, it was reported that the *B. subtilis gerW* gene is expressed only during sporulation in developing spores, and that GerW is essential for L-alanine germination of *B. subtilis* spores but not for germination with AGFK. However, we now find that loss of the *B. subtilis gerW* gene had no significant effects on: i) rates of spore germination with L-alanine; ii) spores' levels of germination proteins including GerA germinant receptor subunits; iii) AGFK germination; iv) spore germination by germinant receptor-independent pathways; and v) outgrowth of germinated spores. Studies in *Bacillus megaterium* did find that *gerW* was expressed in the developing spore during sporulation, and in a temperature-dependent manner. However, disruption of *gerW* again had no effect on the germination of *B. megaterium* spores, whether germination was triggered via germinant receptor-dependent or germinant receptor-independent pathways.

Introduction

Bacillus species have two alternative life cycles. In the vegetative cycle with abundant nutrients, these organisms replicate their chromosome and divide by binary fission into two equivalent daughter cells [1]. However, in response to nutrient limitation, a morphologically distinct cell

analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Peter Setlow is indeed a member of the PLoS One Editorial Board. This does not alter the authors' adherence to PLoS One Editorial policies and criteria.

type called a spore is formed through a process termed sporulation [2]. Spores of *Bacillus* and *Clostridium* species are metabolically dormant with extreme resistance to environmental stresses, and are capable of surviving extreme temperatures, desiccation, chemical agents, and UV- and γ-radiation [3].

Spores are dormant and by themselves cannot cause deleterious effects. However, spores sense their environment and when specific signaling molecules, most often specific nutrients are again present, spores can return to life rapidly through germination. An early event in germination is the release from the spore core of large amounts of the 1:1 chelate of Ca^{2+} and dipicolinic acid (CaDPA) through inner membrane (IM) channels composed at least in part of SpoVA proteins. This is followed by hydrolysis of spore cortex peptidoglycan and expansion of the spore core. Finally metabolism and macromolecular synthesis convert the dormant spore into a growing cell in outgrowth [4]. Nutrients generally trigger spore germination through interactions with proteins called germinant receptors (GRs) located in spores' IM. *Bacillus* spores most often have multiple GRs, each with a different specificity for a nutrient germinant or nutrient germinant mixture. GRs are generally encoded by tricistronic operons encoding GRs' A-, B-, and C-subunits. The A and B subunits are likely integral IM proteins and the C subunit is a lipid-anchored peripheral IM protein [5,6]. By far the best-studied *Bacillus* species is *Bacillus subtilis*, and this species' genome contains five tricistronic operons encoding GRs. The GerA GR responds to L-alanine or L-valine alone, while the GerB and GerK GRs are both required for germination with a mixture of L-asparagine plus D-glucose, D-fructose, and K^+ ions (termed AGFK) [6,7]. The GRs encoded by the other two operons have no known function.

Another protein involved in triggering of spore germination is GerD, a peripheral IM lipoprotein. GerD colocalizes with GRs in a single cluster in dormant spores. These clusters represent a functional germination unit or "germinosome", facilitating spores' rapid and cooperative response to nutrients [8]. Recently, the GerW protein made in the developing spore was reported to be important in triggering of *B. subtilis* spore germination with L-alanine, as rates of L-alanine germination of GerW-deficient spores were > 10-fold lower than those of wild-type spores [9]. In contrast, rates of AGFK germination of *gerW*-deficient spores were almost identical to those of wild-type spores [9]. In the current work, we have examined the effects of the GerW protein on rates of germination in spores of two *Bacillus* species and levels of germination proteins in *B. subtilis* spores. Surprisingly, the absence of the GerW protein had no significant effects on *B. subtilis* spore germination rates with either L-alanine or AGFK or the levels of germination proteins. *B. megaterium* QM B1551 *gerW*-deficient spores also germinated efficiently in response to nutrient and non-nutrient stimulants. Collectively, these results indicate that GerW has no role in the germination of spores of the two species examined in this work, and in all likelihood *Bacillus* spores in general.

Materials and Methods

B. subtilis strains used in this work are isogenic derivatives of strain PS832 (wild-type), a prototrophic laboratory derivative of strain 168 (Table 1). To obtain the *gerW B. subtilis* strain PS4389 most of the *gerW* coding sequence was replaced by a chloramphenicol resistance (Cm^r) cassette as follows. The region between bp-124 to +115 relative to the *gerW* translation start (+1) was PCR amplified from *B. subtilis* PS832 DNA using primers containing *BamH*I and *Pst*I sites (Start Forward and Start Reverse primers; all primer sequences are available upon request). The purified PCR product was digested with *BamH*I and *Pst*I and ligated to a similarly digested modified pBluescript II KS plasmid that has a Cm^r cassette between *Eco*RI and *Eco*RV sites. The ligation reaction was used to transform *Escherichia coli* DH5α to ampicillin resistance (Amp^r) giving plasmid pJCM1. The presence of the appropriate *gerW* fragment in

Table 1. Bacterial strains and plasmids used in this study.

Strains	Relevant genotype, phenotype or description[a]	Reference or source
Bacillus subtilis		
PS832	Wild-type prototroph	Lab strain
PS4389	*gerW*1 (encodes 38 aa of GerW) Cm^r	This work
PS4399	*gerW*2 (encodes 6 aa of GerW) Cm^r	This work
Bacillus megaterium		
QM B1551	Wild-type	Pat Vary
GC618	*gerU*::pNFD13 Km^r	10
GC900	*gerW* pHT-GerU* Zn^r Er^r	This work
GC918	*gerW*::pNFD13 Km^r	This work
GC919	*gerD*::pNFD13 Km^r	This work
Escherichia coli		
DH5α	Competent cells	Lab strain
Plasmids		
Modified pBluescript II KS	Amp^r Cm^r	Lab plasmid
pJCM 1	*gerW* (-124 to +115) Amp^r Cm^r	This work
pJCM 2	Plasmid with Δ*gerW*1 Amp^r Cm^r-	This work
pJCM 3	Plasmid with Δ*gerW*2 Amp^r Cm^r	This work
pGEM-3Z	Amp^r	Promega
P7Z6	Amp^r Zn^r	BGSC[b]
pUCTV2	Amp^r Tc^r ts replication	11
pDONRtet	Gateway entry plasmid Tc^r	12
pNFD13	Vector to create *lacZ* fusions Km^r	12
pUC-Δ*gerW*::Zn	*B. megaterium* Δ*gerW* Amp^r Tc^r Zn^r	This work
pHT-GerU*	encodes GerU* GR genes Er^r	13

[a]Abbreviations used are: ts, temperature sensitive; Amp^r, ampicillin resistance (100 μg/ml); Cm^r, chloramphenicol resistance (5 μg/ml); Er^r, erythromycin resistance (1 μg/ml); Km^r, kanamycin resistance (10 μg/ml); Tc^r, tetracycline resistance (12.5 μg/ml); Zn^r, zeocin resistance (20 μg/ml).
[b]Bacillus Genetic Stock Center

doi:10.1371/journal.pone.0119125.t001

pJCM1, as well as in all other plasmid constructs was confirmed by PCR and restriction enzyme digestion. The region between bp +290 to +891 relative to the *gerW* translation start codon of the *gerW* gene coding and downstream region was PCR amplified from *B. subtilis* PS832 DNA using primers containing *Hind*III and *Kpn*I sites (EndP Forward and EndP Reverse primers). The purified PCR product was digested with *Hind*III and *Kpn*I, and ligated to *Hind*III and *Kpn*I digested plasmid pJCM1. This ligation reaction transformed *E. coli* DH5α to Amp^r giving plasmid pJCM2. Plasmid pJCM2 transformed *B. subtilis* strain PS832 to Cm^r giving strain PS4389 (*gerW*1). The expected genome structure in the *gerW* region of strain PS4389, as well as in the other *B. subtilis gerW* mutant strain described below, was confirmed by PCR and DNA sequencing (data not shown). *B. subtilis* strain PS4399 encoding only the first 6 aa of the GerW protein was constructed as follows. The region between bp-124 to +18 relative to the *gerW* translation start codon was PCR amplified from *B. subtilis* PS832 DNA using primers containing *BamH*I and *Pst*I sites (Start Forward and Start 2 Reverse primers). The purified PCR product was digested with *BamH*I and *Pst*I and ligated to similarly digested plasmid pJCM2 from which bp-124 to + 115 of *gerW* had been removed. The ligation reaction

transformed *E. coli* DH5α to Amp^r giving plasmid pJCM3. This plasmid transformed *B. subtilis* strain PS832 to Cm^r giving strain PS4399 (*gerW*2).

The *B. megaterium gerW* strain (GC900) was constructed using PCR to initially amplify a 1075 bp DNA fragment encompassing the *gerW* ORF (BMQ_4796), using primers with 5′-*EcoR*I restriction sites. The purified *EcoR*I digested PCR product was ligated with *EcoR*I linearised pGEM-3Z, giving plasmid pGEM-*gerW* in *E. coli*. An inverse PCR using pGEM-*gerW* as template and with appropriate primers incorporated a 60 bp deletion towards the middle of the *gerW* ORF and with 5′-*Nco*I sites. The purified inverse PCR product was ligated with a zeocin resistance (Zn^r) cassette (excised from plasmid p7Z6). Plasmid pGEM-Δ*gerW*::Zn was isolated from *E. coli*, and used as a template for a PCR reaction amplifying the Δ*gerW*::Zn cassette, using primers adding 5′-*Mfe*I sites. This cassette was digested with *Mfe*1 and ligated with *EcoR*I digested pUCTV2 [10], giving plasmid pUC-Δ*gerW*::Zn. This plasmid was used to transform *B. megaterium* QM B1551 protoplasts to tetracycline resistance (Tc^r), using the polyethyleneglycol (PEG)-mediated procedure described previously [14]. A colony that had replaced the native *gerW* locus with the Δ*gerW*::Zn cassette via allelic exchange, conferring a Tc^s Zn^r phenotype, was isolated after repeated sub-culture of single-crossover cells at 42°C in the absence of antibiotic. PCR was used to verify the correct construction of the mutant strain. These analyses revealed that the native pBM700 plasmid, which carries the GerU GR structural genes, had been excised during mutant construction procedures. To circumvent this loss, plasmid pHT-GerU∗, which is stable in *B. megaterium* at low copy number, and which encodes functional *gerU* GR genes plus regulatory sequences [13], was introduced by PEG-mediated transformation into the *B. megaterium gerW* strain.

A *B. megaterium* strain bearing a transcriptional fusion between the *gerW* ORF and *E. coli lacZ* was constructed essentially as described previously, using the Gateway cloning technique [10,12]. The *gerW* ORF was amplified by PCR using primers designed to introduce 5′ attB sites, and then purified and cloned into pDONRtet using the Gateway BP reaction mix (Life Technologies, Paisley, UK). Purified intermediate plasmid DNA was isolated from transformed *E. coli* and then employed in a Gateway LR reaction to create a pNFD13 derived *gerW-lacZ* plasmid. *B. megaterium* QM B1551 was transformed to kanamycin resistance (Km^r) with this plasmid using the PEG-mediated transformation method. A colony that had undergone homologous recombination to create a *gerW-lacZ* fusion strain (GC918) was isolated after incubation at 42°C, and its correct construction was verified by PCR. Lysates of spores (10^9) of strain GC918 were used in subsequent β-galactosidase assays as described previously [10] along with the same amounts of spores of *B. megaterium* strains carrying *gerU-lacZ* (strain GC618) or *gerD-lacZ* (strain GC919) for comparative purposes. The *B. megaterium gerD-lacZ* strain (*gerD* is encoded by BMQ_0176) was created in a similar manner to the *gerW-lacZ* strain.

Spores of *B. subtilis* strains were routinely prepared at 37°C on 2x Schaeffer's-glucose plates without antibiotics as described previously [15,16]. After incubation for ~ 5 d, the spores were scraped from plates, and washed with water by repeated centrifugation with intermittent sonication treatment. In one experiment, *B. subtilis* spores were prepared in liquid Schaeffer Sporulation medium as described previously [17], and spores were purified as described above. *B. megaterium* spores were prepared at 30°C in supplemented nutrient broth (SNB), and purified by repeated rounds of centrifugation and washing with ice-cold deionized water as described previously [11]. All spore preparations used in this work were free (~ 95%) from growing or sporulating cells and germinated spores as determined by phase-contrast microscopy.

B. subtilis spores were germinated following heat activation (30 min; 75°C) and cooling on ice for 10 min. Spores at an optical density at 600 nm (OD_{600}) of 0.5 were germinated for 2.5 h at 37°C in 200 μl of 25 mM K-Hepes buffer (pH 7.4) with various concentrations of L-alanine

or 10 mM L-valine, or with 10 mM of each AGFK component. All germination experiments were carried out in duplicate. Spore germination was routinely monitored by measuring the release of the spores' large depot of DPA by inclusion of 50 μM $TbCl_3$ in germination mixtures and measuring Tb-DPA fluorometrically in a multiwell plate reader as described previously [18]. Germination of spores with a coat defect due to chemical decoating is very strongly inhibited by $TbCl_3$ [19]. Consequently, decoated spores prepared as described previously [19] were germinated as described above, but without Tb^{3+} present from the initiation of germination. Instead, at various times after germination was initiated, aliquots of the germinating culture were centrifuged in a microcentrifuge, the supernatant fluid made 50 μM in $TbCl_3$ and Tb-DPA fluorescence was measured as described previously [19,20]. Spore germination was also routinely monitored at the end of germination incubations by phase-contrast microscopy. The total amount of DPA present in spores was assessed by Tb-DPA fluorescence after DPA had been released from spores by boiling as described previously [18,19]. In some experiments, spore germination was also monitored by the fall in optical density of spore cultures as described previously [9]. All *B. subtilis* GR-dependent spore germination experiments were carried out on multiple independent spore preparations prepared in different laboratories with essentially identical results.

B. subtilis spores that were not heat-activated were also germinated with GR-independent germinants as follows: i) at 50°C in 25 mM K-Hepes buffer (pH 7.4) and 1 mM dodecylamine, with spores at an OD_{600} of 2; and ii) at 23°C in 60 mM CaDPA made to pH 7.5 with Tris base, with spores at an OD_{600} of 2 [5]. Germination of spores with CaDPA and dodecylamine was monitored by examining ~ 100 individual spores at various times by phase-contrast microscopy [7].

Outgrowth of heat-activated *B. subtilis* spores was carried out at 37°C in 2x yeast tryptone (2x YT) medium containing 5 mM L-alanine and (per L) 16 g tryptone, 10 g yeast extract, 5 g NaCl. Spores were added to an OD_{600} of 0.5 and the OD_{600} of cultures were followed over time [21]. Finally, to determine spore viability, both PS832 and PS4389 spores at an OD_{600} of 1.0 were heat activated, cooled, spores spotted on LB medium plates with the appropriate antibiotic, plates incubated for 24 h at 37°C and colonies were counted [21].

B. megaterium spore germination was followed by monitoring the absorbance at 600 nm of heat-shocked (60°C, 10 min) spores suspended at an OD_{600} of ~ 0.4 in 5 mM Tris-HCl, pH 7.8, plus 0.1–25 mM glucose or proline. Germination assays were conducted in triplicate, in 96-well plates incubated at 30°C in a PerkinElmer EnVision-Xcite multilabel plate reader fitted with a 600 nm photometric filter. Similar absorbance-based assays were conducted with non-heat shocked spores incubated in either 60 mM CaDPA at 30°C or 1 mM dodecylamine at 40°C. All experiments were conducted with at least two independently prepared batches of spores with essentially identical results. Spore viability was assessed by plating serial dilutions of heat-shocked spores on solid LB medium plates which were incubated at 30°C overnight before determining the percent viability of *gerW* spores compared to wild-type spores in which 1 OD_{600} unit is equal to ~ 10^8 CFU ml^{-1}.

Levels of GR, GerD and SpoVAD proteins, which are present largely or completely in spores' IM [22–26], were measured in lysates of spores by western blot analysis using polyclonal rabbit antisera against the various proteins and a secondary antiserum as described previously [7,21,27,28]. In brief, spores were decoated, ruptured by lysozyme digestion, and sonicated briefly with glass beads present to obtain spore lysates. Aliquots of the lysates were then run on SDS-polyacrylamide electrophoresis (SDS-PAGE) and the gels were stained with Coomassie blue to determine how much of the lysates needed to be run to load equal amounts of protein. Equal amounts of the lysates were run on SDS-PAGE, proteins transferred to a polyvinylidene-difluoride (PVDF) membrane, and antigens on the membrane were detected as described

previously [10,21,27,28]. Following development of these western blots, the membrane was stripped and then reprobed with another antiserum as described previously [21,28].

Results

It was reported recently that the rate of L-alanine germination of *gerW B. subtilis* spores was > 10-fold slower than that of the wild-type spores, although AGFK germination of *gerW* spores was normal [9]. To further investigate the effect of a *gerW* mutation on spore germination, we replaced the great majority of the *gerW* coding sequence in *B. subtilis* strain PS832 (wild-type) by a Cm^r cassette giving *B. subtilis* strains PS4389 (*gerW*1) (retains 38 N-terminal GerW aa) and PS4399 (*gerW*2) (retains 6 N-terminal GerW aa). Strain PS4389 was generated first, and when spores of this strain were found to germinate normally with L-alanine, we also prepared strain PS4399 to eliminate the possibility that the N-terminal 38 aa of GerW were sufficient for its function. Multiple germination experiments with wild-type and PS4389 and PS4399 spores indicated that rates of *gerW* mutant spores' germination with AGFK were essentially identical to those of wild-type spores (Table 2), as reported previously [9]. Surprisingly the PS4389 and PS4399 spores also germinated like wild-type spores with either L-alanine or L-valine via the GerA GR (Table 2). Based on these experiments, there were no statistically significant differences between rates of wild-type and *gerW* spore germination with saturating levels of different nutrient germinants. In addition, wild-type and *gerW* spores prepared in liquid Schaeffer's-glucose sporulation medium or in liquid Spizizen's minimal medium [28,29] also germinated essentially identically with L-alanine (data not shown). Chemically decoated wild-type and PS4389 spores prepared as described previously [19] also germinated identically with L-alanine (data not shown), and the intact *gerW* spores' germination with different L-alanine concentrations was also essentially identical to that of wild-type spores (Fig. 1).

In addition to nutrient germinants that trigger spore germination via GRs, we also measured the germination of wild-type and *gerW B. subtilis* spores with CaDPA and dodecylamine, two agents that trigger spore germination without GR involvement. Again, we observed no significant difference between *gerW* and wild-type spores in their germination with either CaDPA or dodecylamine (data not shown).

We also measured the ability of wild-type and *gerW* spores to return to active growth after spore germination in a complete nutrient medium with L-alanine added to ensure rapid spore germination, and observed that both types of spores had similar rates of outgrowth (Fig. 2). In addition, the viability of wild-type and *gerW* spores was essentially identical when heat activated spores were spotted on LB medium plates (data not shown).

The levels of GR subunits, GerD and SpoVAD proteins were also determined in lysates of spores by western blot analysis. Some modest differences were observed between levels of these

Table 2. Rates of germination of wild-type and *gerW B. subtilis* spores with L-alanine, L-valine or AGFK.

Germinants: Spores	10 mM L-alanine	10 mM L-valine Spore germinationrate—%/min	10 mM AGFK
PS832 (wild-type)	2.6	2.5	0.75
PS4389 (*gerW*1)	2.4	2.3	0.8
PS4399 (gerW2)	2.5	2.4	0.78

Spores of various strains were germinated with saturating levels of different germinants, and spore germination was monitored by DPA release as described in Methods. Rates of spore germination were determined from plots of DPA release as a function of time. Values shown are averages of values in two independent experiments with the same spore preparations that differed by < 12%.

doi:10.1371/journal.pone.0119125.t002

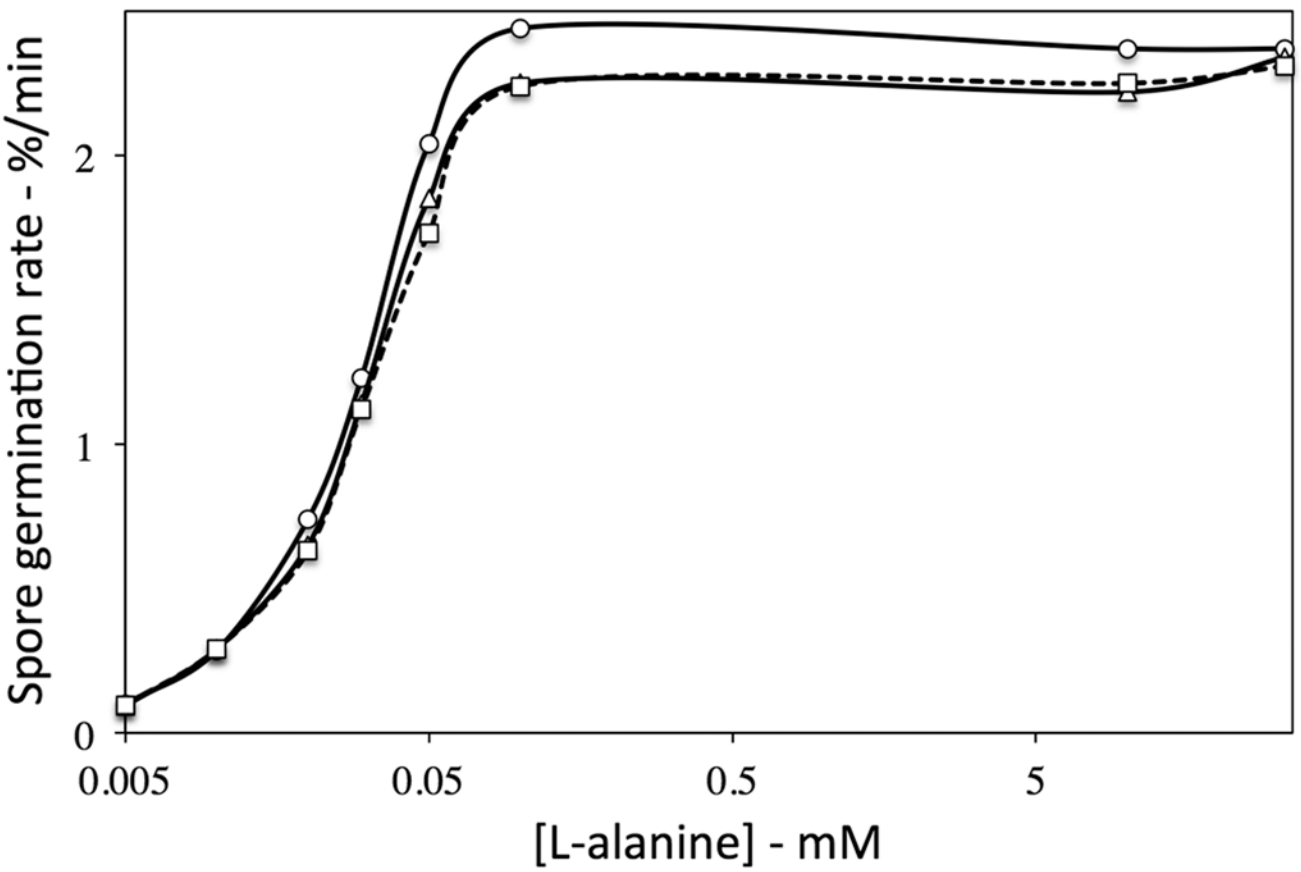

Fig 1. Rates of germination of wild-type and *gerW B. subtilis* spores with various L-alanine concentrations. Heat shocked wild-type (PS832, ○), *gerW*1 (PS4389, □) and *gerW*2 (PS4399, Δ) *B. subtilis* spores were germinated with various L-alanine concentrations and DPA released was monitored as described in Methods. Rates of spore germination are averages of values determined from maximum slopes of DPA release curves in two separate experiments, and these values varied by < 15%.

doi:10.1371/journal.pone.0119125.g001

proteins in PS832 (wild-type), PS4389 (*gerW*1) and PS4399 (*gerW*2) spores in some experiments, although these were generally ≤ 2-fold (Fig. 3). In addition, when blots from multiple replicate experiments were compared, there were on average ≤ 25% differences in the intensities of different germination proteins from wild-type and *gerW* spores (data not shown). In general, the similar levels of GR proteins and GerD in wild-type and *gerW* spores was consistent with the similar rates of germination of wild-type and *gerW* spores with all GR-dependent germinants (Table 2).

Bioinformatic analyses revealed that the *B. megaterium* QM B1551 genome also contains a single orthologue of *B. subtilis gerW*, encoded at locus BMQ_4796. The predicted protein shares 70% amino acid identity with its *B. subtilis* counterpart, with most variance occurring in an ~20 aa sequence towards the central region of the protein (data not shown). Analysis of lysates from disrupted purified *B. megaterium gerW-lacZ* spores revealed β-galactosidase activity, levels of which were dependent upon the temperature of sporulation (Table 3). Spores bearing a *lacZ* fusion to the GR gene, *gerUA*, also showed temperature dependent levels of expression as reported previously [10]. Collectively, the β-galactosidase assays indicate that GerD is expressed at a higher level than GerW or GerU at 22°C, while GerW and GerD are expressed at similar levels at 30 and 37°C.

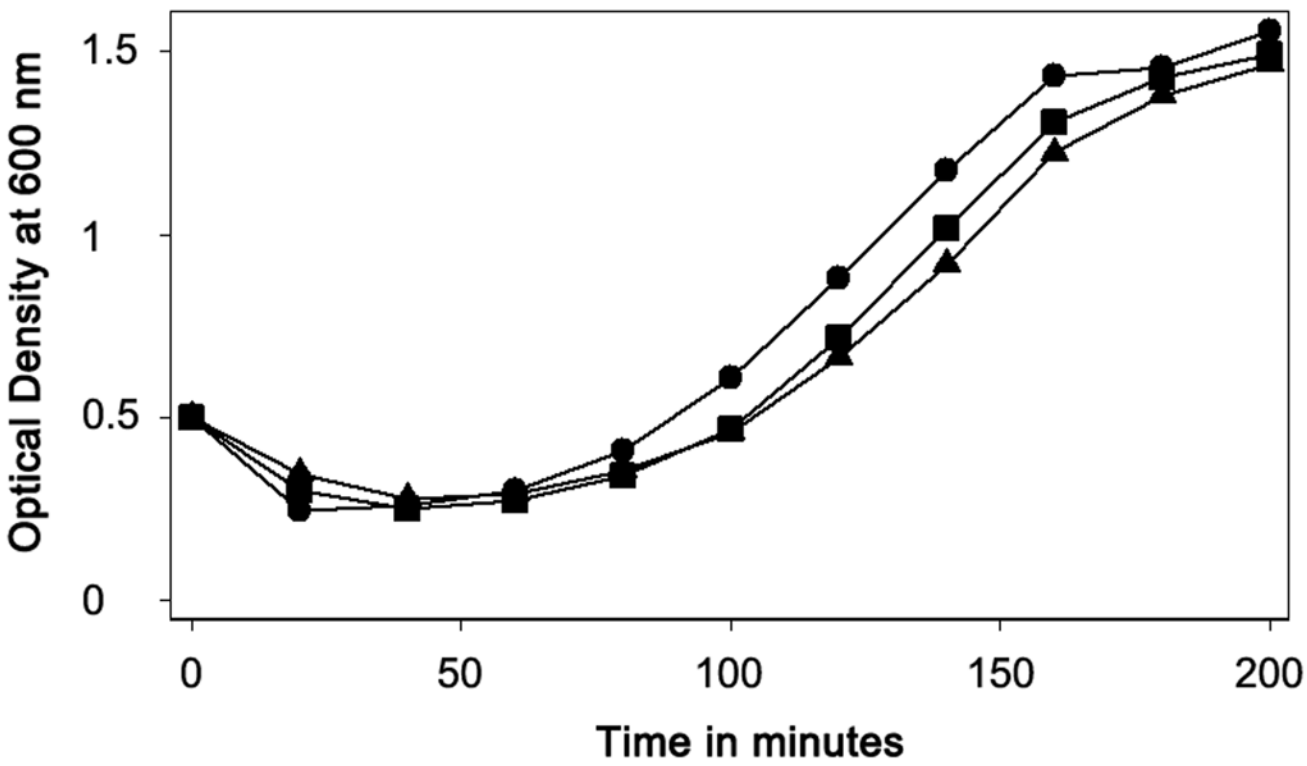

Fig 2. Germination and outgrowth of wild-type and *gerW B. subtilis* spores. Spores of *B. subtilis* strains PS832 (wild-type; ●), PS4389 (*gerW*1; ■) and PS4399 (*gerW*2, ▲) were heat shocked, cooled, and incubated with shaking at 37°C and an initial OD_{600} of 0.5 in 2xYT medium plus 5 mM L-alanine, and the OD_{600} of the cultures was measured.

doi:10.1371/journal.pone.0119125.g002

Spores were prepared at three different temperatures for each strain as described in Methods, and the β-galactosidase activity in lysates from 10^9 spores of each strain was measured in triplicate. Similar values were obtained with at least one other independently prepared batch of spores. Values have been corrected for wild-type spore background levels of fluorescence which were always < 500 relative fluorescence units. Standard deviations for all values were $< 15\%$.

Overall, in *B. megaterium*: i) the expression of GerW-LacZ in spores; ii) the identification of a putative σ^F promoter sequence upstream of BMQ_4796 with sequence homology to the *B. subtilis gerW* promoter region and approximately the same spacing between the two promoters and the translation start sites [9] (data not shown); and iii) the lack of detection of *gerW* mRNA in vegetative cells by RT-PCR (data not shown) are all consistent with forespore-specific expression of *gerW*, as observed previously in *B. subtilis* [9]. However, there were differences in the expression of the *gerW*, *gerD* and *gerUA* genes as a function of sporulation temperature (see Discussion).

In order to also investigate the role, if any, of GerW in the germination of *B. megaterium* spores, the *gerW* gene was disrupted with a Zn cassette by allelic exchange, which introduced a short deletion in the *gerW* ORF. The resultant strain was found to have excised plasmid pBM700 during strain construction, hence plasmid pHT-GerU∗ was introduced by PEG-mediated transformation to restore the *gerU* GR genes and their regulatory sequences. The *B. megaterium gerW* pHT-GerU∗ strain (GC900) sporulated normally (data not shown) and the spores were then examined for germination efficiency in response to nutrient and non-

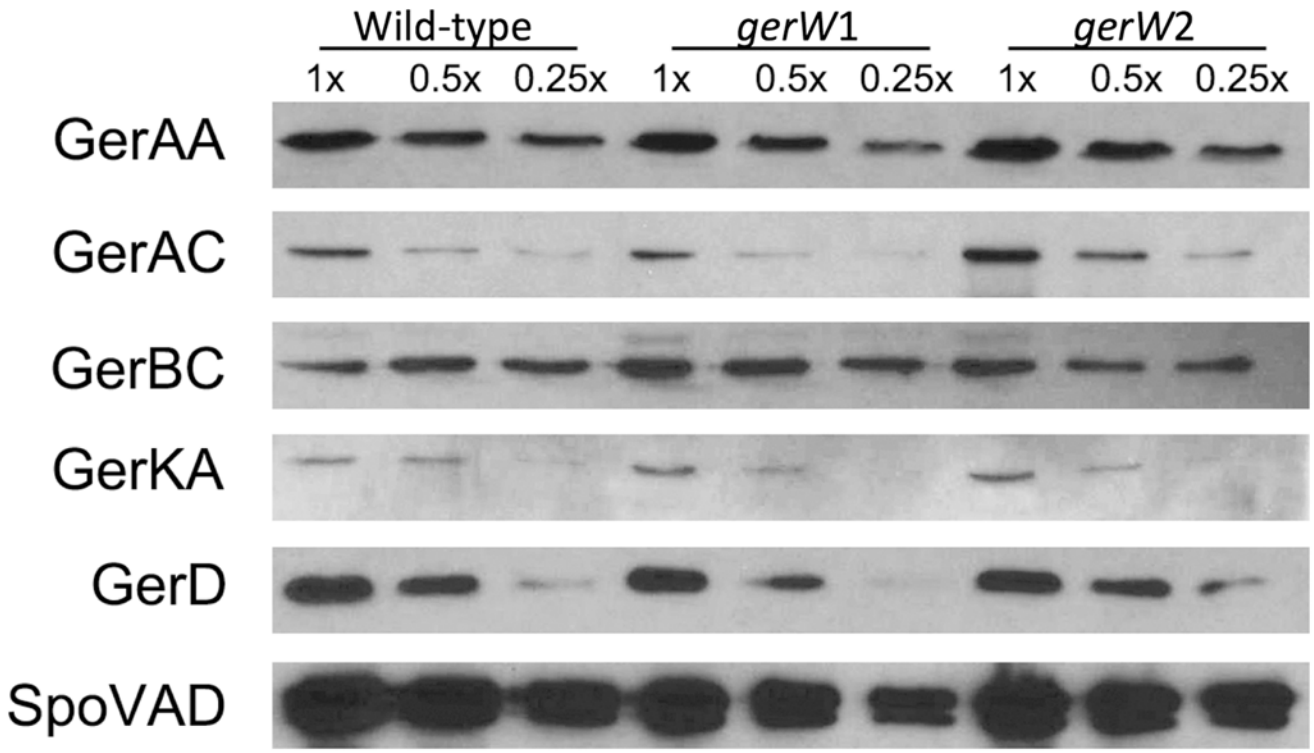

Fig 3. Levels of GR subunits, GerD and SpoVAD in wild-type and *gerW B. subtilis* spores. Aliquots of equal amounts of protein in lysates from spores of *B. subtilis* strains PS832 (wild-type), PS4389 (*gerW*1) or PS4399 (*gerW*2) were run on SDS-PAGE, proteins transferred to PVDF paper, and these western blots were probed with antisera against various proteins as described in Methods. The amount of protein in lysates in the 1x samples from wild-type and *gerW* spores was identical.

doi:10.1371/journal.pone.0119125.g003

nutrient stimuli. *B. megaterium gerW* spores were observed to germinate essentially with an identical efficiency to wild-type spores in response to either glucose or proline, including at sub-optimal germinant concentrations (Fig. 4A,B; Table 4). Similarly, spores with disrupted *gerW* displayed essentially wild-type germination with the GR-independent germinants CaDPA and dodecylamine (Fig. 4C,D). Additionally, *B. megaterium* GerK$^+$ spores, in which only the *gerK* GR operon is intact, germinated normally when plated on solid LB medium whether *gerW* was disrupted or not, as did the wild-type spores containing only the *gerK* GR operon (data not shown).

Table 3. β-Galactosidase activity from *gerW-*, *gerD- and gerUA-lacZ* transcriptional fusions in *B. megaterium* spores prepared at different temperatures.

Strain	Genotype	β-Galactosidase activity (relative fluorescence units)[1]		
		Sporulation temperature [1]		
		22°C	30°C	37°C
GC918	*gerW-lacZ*	9.5E+03	3.3E+04	2.9E+04
GC919	*gerD-lacZ*	3.6E+04	3.6E+04	3.0E+04
GC618	*gerU-lacZ*	4.2E+03	3.2E+03	7.9E+02

doi:10.1371/journal.pone.0119125.t003

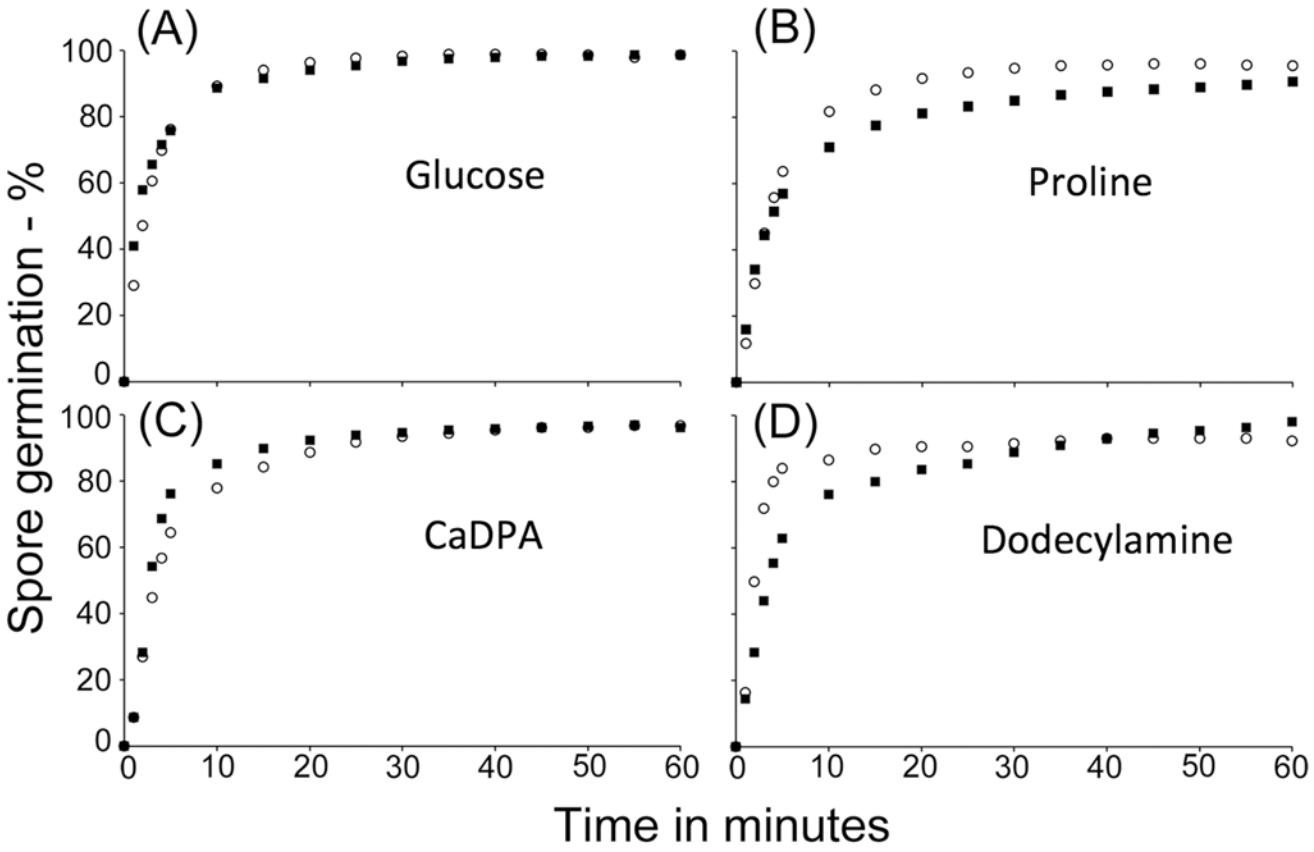

Fig 4. Germination of wild-type and *gerW B. megaterium* spores with various germinants. *B. megaterium* spores were germinated in 5 mM Tris-HCl, pH 7.8 plus (A) 10 mM glucose, (B) 10 mM proline, (C) 60 mM CaDPA, or (D) 1 mM dodecylamine. Spores were heat shocked at 60°C for 10 min prior to incubation in glucose or proline-supplemented germination buffer, but not for CaDPA or dodecylamine germination, and the progress of spore germination was monitored as described in Methods. Symbols used are: (◯), wild type spores; and (■), *gerW* spores.

doi:10.1371/journal.pone.0119125.g004

Table 4. Maximum rates of germination of wild-type and *gerW B. megaterium* spores in response to varying concentrations of glucose or proline.

Germinant:	Glucose mM				Proline mM			
	0.1	1	10	25	0.1	1	10	25
Strain	**Maximum rate of germination—% OD_{600}/min**							
QM B1551 (wt)	0.67	3.5	15.3	14.1	0.68	3.5	10.4	10.7
GC900 (*gerW*)	0.59	3.5	14.6	11.8	0.50	2.3	11.1	11.2

B. megaterium spores were heat shocked and incubated in 5 mM Tris-HCl, pH 7.8 plus the designated concentration of glucose or proline, and germination was monitored by measuring OD_{600} loss as described in Methods. The maximum % OD_{600} loss per min was calculated from plots of OD_{600} versus time. Values shown are the averages of results from three independent experiments conducted with the same spore preparations, and the standard deviations were <15% of the averages.

doi:10.1371/journal.pone.0119125.t004

Discussion

Clearly, the major conclusion from the current work is that GerW is not essential for the germination of spores of two *Bacillus* species, either *B. subtilis* spores with L-alanine or AGFK, and *B. megaterium* spores with either GR-dependent or GR-independent germinants. The obvious question based on the new findings is why GerW was previously found to be essential for normal *B. subtilis* spore germination with L-alanine via the GerA GR [9]. The answer to this question is not completely clear. However, it seems most likely that the original transformation to generate a *gerW* deletion mutation used a laboratory *B. subtilis* 168 strain [30], while the wild-type strain was strain 1A1 from the *Bacillus* Genetic Stock Center (BGSC). Unfortunately, the *B. subtilis* laboratory 168 strain used in the initial communication now appears to have had one or more mutations that significantly reduce its spores' germination with L-alanine even without deletion of *gerW*. In contrast, spores of the PS832 168 strain germinate very rapidly with L-alanine.

In addition to the major conclusion discussed above, there are several other notable points pertinent to the current work as follows. 1) Expression of *gerW* in *B. megaterium* and *B. subtilis* is forespore-specific during sporulation, as is that of many genes involved in spore-specific properties. However, in *B. megaterium*, *gerW* expression displayed a rather different response to sporulation temperature than did two other forespore-specific genes, *gerD* and *gerUA*. Differences in the regulation of these three genes as a function of temperature may reflect differences in the RNA polymerase σ factors that recognize these genes, σ^F for *gerW* and σ^G for *gerD* and *gerUA*, as well as likely additional regulators of σ^G-dependent genes such as SpoVT [14,31,32]. 2) While GerW does not play an obvious role in *Bacillus* spore germination, at least in *B. megaterium* and *B. subtilis*, an important question is what does this protein do. The GerW amino acid sequence suggests the protein is soluble, and GerW is present in the soluble fraction of disrupted *B. subtilis* spores [9]. In addition, a Blast search of the NCBI microbial genomes database readily detects obvious GerW homologs in the spore forming members of the order *Bacillales*, but also in the order *Clostridiales*. The latter information, as well as that *gerW* is expressed only in the developing spore suggests that GerW plays some important role in dormant spore properties. However, this role remains to be determined.

Author Contributions

Conceived and designed the experiments: PS SG HT GC APV. Performed the experiments: JCM SG NW RK. Analyzed the data: APV RK HT GC PS JCM. Contributed reagents/materials/analysis tools: RK. Wrote the paper: HT GC PS JCM.

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2.2 Capitulo 2

"El efecto de la activación térmica en la germinación de esporas de *Bacillus* con nutrientes o con alta presión, con o sin varias proteínas de germinación"

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The Effects of Heat Activation on *Bacillus* Spore Germination, with Nutrients or under High Pressure, with or without Various Germination Proteins

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Nutrient germination of spores of *Bacillus* species occurs through germinant receptors (GRs) in spores' inner membrane (IM) in a process stimulated by sublethal heat activation. *Bacillus subtilis* spores maximum germination rates via different GRs required different 75°C heat activation times: 15 min for L-valine germination via the GerA GR and 4 h for germination with the L-asparagine–glucose–fructose–K^+ mixture via the GerB and GerK GRs, with GerK requiring the most heat activation. In some cases, optimal heat activation decreased nutrient concentrations for half-maximal germination rates. Germination of spores via various GRs by high pressure (HP) of 150 MPa exhibited heat activation requirements similar to those of nutrient germination, and the loss of the GerD protein, required for optimal GR function, did not eliminate heat activation requirements for maximal germination rates. These results are consistent with heat activation acting primarily on GRs. However, (i) heat activation had no effects on GR or GerD protein conformation, as probed by biotinylation by an external reagent; (ii) spores prepared at low and high temperatures that affect spores' IM properties exhibited large differences in heat activation requirements for nutrient germination; and (iii) spore germination by 550 MPa of HP was also affected by heat activation, but the effects were relatively GR independent. The last results are consistent with heat activation affecting spores' IM and only indirectly affecting GRs. The 150- and 550-MPa HP germinations of *Bacillus amyloliquefaciens* spores, a potential surrogate for *Clostridium botulinum* spores in HP treatments of foods, were also stimulated by heat activation.

Spores of *Bacillus* species can remain dormant for long periods in the absence of suitable growth conditions (1, 2). However, if specific nutrients are sensed, spores can rapidly become metabolically active in the process of germination followed by outgrowth. The specific nutrients that trigger spore germination are termed germinants, and these molecules are sensed by germinant receptors (GRs) located in spores' inner membrane (IM). *Bacillus subtilis* spores have three functional GRs: GerA, which responds to L-alanine or L-valine alone, and GerB and GerK, which together are essential for germination with a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (termed AGFK), with all four components of the mixture required; neither GerB nor GerK alone triggers germination with any nutrient germinant (1, 3). There is also a variant of the GerB GR, termed GerB*, that responds to L-asparagine alone, although GerB* action can be stimulated by glucose via GerK (3). All GRs in *B. subtilis* spores appear to be located together in a small cluster in the IM termed the germinosome, and formation of this structure is dependent on the GerD protein, which is also in the IM (2, 4). Since *gerD* spores do not form a germinosome and exhibit extremely slow GR-dependent germination (4), germinosome formation may be essential for rapid GR-dependent germination. GR function and germinosome assembly may also depend on the precise structure of the IM, which appears to be quite different than that of the growing cell or germinated spore plasma membrane (2, 5). In particular, despite having a lipid composition similar to that of growing cells, the lipids in the spore IM are relatively immobile. In addition, the overall IM seems to be compressed somewhat, as the IM bounded volume increases 1.5- to 2-fold early in spore germination and occurs without new membrane synthesis.

GR-dependent spore germination can be potentiated or activated by pretreatment with chemicals or sublethal heat, with the latter being most commonly used (6). The process of heat activation increases the rate and extent of germination of spores of a number of *Bacillus* and related species (6–15). The effect of heat activation is observed primarily as decreasing the time, defined as T_{lag}, between the addition of germinant to the initiation of the rapid release of most of the spore core's large depot (~20% of core dry weight) of a 1:1 complex of Ca^{2+} and dipicolinic acid (DPA), with heat activation decreasing T_{lag} values for spores of a number of species (14, 15). However, heat activation has little or no effect on actual rates of rapid Ca-DPA release or the subsequent hydrolysis of spores' peptidoglycan cortex.

The molecular effect whereby heat activation increases rates of spore germination is not known, although there are several reports of effects accompanying heat activation such as changes in spore protein structure and the release of various spore molecules

Received 22 January 2015 **Accepted** 10 February 2015

Accepted manuscript posted online 13 February 2015

Citation Luu S, Cruz-Mora J, Setlow B, Feeherry FE, Doona CJ, Setlow P. 2015. The effects of heat activation on *Bacillus* spore germination, with nutrients or under high pressure, with or without various germination proteins. Appl Environ Microbiol 81:2927–2938. doi:10.1128/AEM.00193-15.

Editor: D. W. Schaffner

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00193-15.

doi:10.1128/AEM.00193-15

TABLE 1 *Bacillus subtilis* strains used in this study

Strain	Phenotype	Antibiotic resistance[a]	Reference
FB10	*gerB**	None	59
FB20	Δ*gerA*	Sp^r	60
FB61	Δ*gerA* Δ*gerB*	Cm^r Sp^r	60
FB62	Δ*gerD*	Sp^r	61
FB87	Δ*gerB* Δ*gerK*	Cm^r MLS^r	60
PS533	Wild type	Km^r	30
PS3476	P*sspD*::*gerA*	MLS^r	45
PS3521	Δ*gerA gerB**	Sp^r	45
PS3651	Δ*gerA* Δ*gerK*	Km^r MLS^r	3
PS3665	Δ*gerA gerB** Δ*gerK*	MLS^r Sp^r	3
PS4150	Δ*cotE* Δ*gerE*	Sp^r Tc^r	36

[a] Abbreviations for antibiotics: Cm, chloramphenicol (5 μg/ml); Km, kanamycin (10 μg/ml); MLS, erythromycin (1 μg/ml) and lincomycin (25 μg/ml); Sp, spectinomycin (100 μg/ml); Tc, tetracycline (10 μg/ml).

(16–18). However, heat activation appears only to stimulate nutrient germination via GRs, as heat activation does not stimulate germination by agents that act by a GR-independent mechanism, including Ca-DPA and long-chain alkylamines such as dodecylamine (1, 2). Spore germination triggered by high pressures (HPs) of ~150 or ~550 MPa is also reported not to be stimulated markedly by heat activation, even though an HP of 150 MPa clearly triggers spore germination by activating GRs (19, 20).

Spores of a number of *Bacillus* and *Clostridium* species are agents for food spoilage, food-borne disease, and other human diseases, and thus, there is continued interest in novel ways to kill such spores. One strategy is to first germinate spores and then kill the much less resistant germinated spores—the strategy that has been called "germinate to exterminate" for decontaminating spores of *Clostridium difficile* (21–23) and spores of *Bacillus anthracis*. Indeed, spore germination is a crucial mechanistic step in the inactivation of spores by HP processing, which uses conditions of elevated temperature (90 to 121°C) and pressure (≥600 MPa) to greatly reduce spore loads in certain foodstuffs (24–27), such as baby food purées (28). Consequently, since heat activation can be very important in determining the rates and efficiency of spore germination, the current study has analyzed the effects of heat activation on (i) nutrient germination of spores of *Bacillus subtilis* with various GRs and with or without GerD, (ii) germination of *B. subtilis* spores made at various temperatures that differ in their IM lipid compositions, and (iii) spore germination by HPs of 150 and 550 MPa, including spores of *B. subtilis* as well as *Bacillus amyloliquefaciens* spores, which have been suggested for use as a surrogate for *Clostridium botulinum* spores in HP treatments of foods (28, 29).

MATERIALS AND METHODS

***B. subtilis* strains used and spore preparation.** All *B. subtilis* strains used in this study are listed in Table 1 and are derivatives of strain PS832, a prototrophic laboratory 168 strain. The wild-type strain is PS533 (30), which contains plasmid pUB110, encoding resistance to kanamycin (Km^r; 10 μg/ml). *B. subtilis* spores of various strains were routinely prepared at 37°C on 2× Schaeffer's medium-glucose plates and in some cases at other temperatures as described previously (31, 32). Plates were incubated at sporulation temperatures until >90% of spores had been released from sporangia, generally 3 to 6 days, and were then incubated for several days at 23°C to allow lysis of growing cells and large fragments of cell debris. Spores were then scraped from plates or harvested from liquid media and purified as described previously (31, 32). All spores used in this study were free (>98%) from growing or sporulating cells, germinated spores, and cell debris as determined by phase-contrast microscopy.

The *B. amyloliquefaciens* strain used was TMW 2.479 Fad 82 isolated from ropy bread and maintained on standard nutrient 1 (ST-1) agar (33). Spores were prepared by growing cells aerobically in ST-1 broth, plating them onto ST-1 agar plates supplemented with 10 mg/liter of $MnSO_4 \cdot H_2O$, and then incubating them for 2 to 3 days at either 30 or 37°C (33). Spores were harvested from plates by gently scraping and rinsing with water and then cleaned by repeated centrifugations and resuspension in distilled water. Remaining sporangia and vegetative cells were removed by suspending washed pellets in 200 ml of 0.05 M potassium phosphate (pH 7.0) containing 100 μg/ml of lysozyme and stirring for 1 h at 37°C, with subsequent centrifugation and washing with distilled water (34). Final spore suspensions were examined with phase-contrast microscopy and then frozen in 1-ml aliquots. Spores prepared at 30°C contained 98% phase-bright (ungerminated) and 2% germinated spores, and spores prepared at 37°C contained 87 to 90% ungerminated spores. Cells incubated at 23°C lysed and failed to sporulate.

Spore germination. Prior to germination, spores of various strains at an optical density at 600 nm (OD_{600}) of ~10 were heat activated in water at 75°C for various times (0 to 6 h) and then cooled in an ice bath for ≥15 min. Spore germination with nutrient germinants was measured by monitoring the release of spore DPA by its fluorescence with Tb^{3+} in a multiwell fluorescence plate reader, as described previously (35). Germination took place at 37°C with spores at an OD_{600} of 0.5 in 200 μl of 25 mM K-HEPES buffer (pH 7.4) containing 50 μM $TbCl_3$. Germinants used were various concentrations of either L-valine, the mixture of L-asparagine–D-glucose–D-fructose and K^+ (AGFK), or L-asparagine alone, and the Tb-DPA fluorescence was read every 5 min for 100 to 150 min and expressed as relative fluorescence units (RFU). The maximum germinant concentration that was used routinely was 10 mM, because germination with 40 mM germinants in initial experiments was found to give <15% increases in germination rates. In most cases the percentage of spores that had released DPA at various germination times was determined from RFU measurements and knowing the total DPA in spores, which was determined after DPA was released from spores by boiling (35). The approximate extent of germination was also monitored at the end of all experiments by phase-contrast microscopy examining ~100 individual spores. The rates of spore germination were determined from linear portions of plots of RFU versus time as described previously (3, 35). All experiments assessing germination by fluorescence measurements were carried out with least two replicates for each time point analyzed and in at least two separate experiments, always with very similar results.

Analysis of levels of germination proteins and germination protein biotinylation. To determine levels of various germination proteins in unactivated or heat-activated (4 h at 75°C) spores, we used PS4150 spores with a severe coat defect that makes these spores lysozyme sensitive (36). This meant that spore lysis did not have to be preceded by a decoating step at high temperature that might also activate spores. Total lysates of unactivated or heat-activated PS4150 spores were prepared by lysozyme treatment followed by brief sonication and then incubation with SDS and 2-mercaptoethanol to extract all germination proteins as described previously (37). Levels of germination proteins in the lysates were then determined by Western blot analysis using specific antisera to germination proteins (37–40).

Biotinylation in unactivated and heat-activated (4 h at 75°C) PS4150 spores was carried out essentially as described previously for 1 h at 23°C using 2 mM EZ-Link Sulfo-NHS-SS-Biotin reagent (Pierce Chemical Co., Rockford, IL), which modifies lysyl amino groups in proteins (41). Unreacted reagent was quenched by the addition of 2 M glycine and 1 M Tris-HCl buffer (pH 7.4), followed by incubation for 30 min at 23°C and two washes with water. The IM and soluble and integument fractions from biotinylated PS4150 spores were obtained essentially as described previously (37, 41) by disruption in 0.5 ml of TEP buffer (50 mM Tris-HCl buffer [pH 7.4], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride

[PMSF]) containing 1 mg of lysozyme, 1 μg each of RNase A and DNase I, and 20 μg of $MgCl_2$ for 5 min at 37°C. After being held on ice for 20 min, the disrupted suspensions were sonicated briefly with 100 mg of glass beads (~5 15-s bursts) and centrifuged at 4°C for 5 min in a microcentrifuge at maximum speed, and the supernatant fluid was saved. The pellet fraction was suspended in 0.5 ml of TEP buffer, sonicated for 15 s, and centrifuged; the final pellet was saved as the integument fraction, and the two supernatant fluids were pooled. The pooled supernatant fluids were centrifuged at 4°C for 1 h at 100,000 × *g* to give a supernatant (soluble fraction), and the pellet fractions (IM) were suspended in 160 μl of TEP buffer containing 1% Triton X-100. The integument fraction was suspended in 400 μl of TEP buffer plus 1% Triton X-100 and left to stand for 2 h at 23°C with intermittent vortexing and bath sonication, and aliquots were analyzed as described below.

Biotinylated and unbiotinylated germination proteins were separated by adsorption to NeutrAvidin agarose beads (Pierce), giving the bead eluate (E; biotinylated) and bead flowthrough (F; unbiotinylated) fractions as described previously (41). Western blot analyses as described above were performed following SDS-PAGE of equal percentages of the total biotinylated spore lysate (T fraction) and the F and E fractions, all run on the same Western blot.

HP germination of unactivated and heat-activated spores. Spores at an OD_{600} of either 1 (*B. subtilis* spores) or 10 (*B. amyloliquefaciens* spores) were germinated by treating samples in 1.5 ml of 25 mM K-HEPES buffer (pH 7.4) with an HP of 150 MPa at 37°C or 550 MPa at 50°C for various periods (0 to 5 min), essentially as described previously (42). *B. amyloliquefaciens* spores were HP treated at a higher spore concentration because these spores did not pellet as tightly as *B. subtilis* spores upon centrifugation, but rather tended to form thin films of spores on the side of the centrifuge tube that significantly decreased *B. amyloliquefaciens* spore recovery (see below). The temperatures for HP treatments were chosen from previous work with *B. subtilis* spores that showed that (i) 37°C is near optimal for 150-MPa germination, with germination slower at 50°C, and (ii) 500-MPa germination is quite slow at 37°C, and while the temperature optimum for 500-MPa germination is ~60°C, it is difficult to measure the rates of the more rapid germination at this temperature (43). Samples were frozen immediately after HP treatment and kept frozen until analyzed for germination. After thawing on ice, HP-treated *B. subtilis* spore samples were centrifuged in a microcentrifuge at top speed for ~2 min, the pellet was suspended in 20 μl of water, and ~100 spores were examined by phase-contrast microscopy to determine the percentages of spores that had become phase dark or phase gray and thus had germinated. Aliquots of HP-treated *B. amyloliquefaciens* spores were analyzed directly by microscopy as described above, as their concentration prior to microscopy was not needed.

HP activation of *B. amyloliquefaciens* spores. Samples of sterile chicken baby food purée (Gerber baby food) were inoculated to ~10^7 CFU/ml with unactivated *B. amyloliquefaciens* spores, sealed in sterile pouches, and either not HP treated or treated with various combinations of HP (448 to 690 MPa) and exposed to high temperature (65 to 121°C) in a PT-1 high-pressure unit with bioglycol heat transfer fluid (Dynalene, Whitehall, PA) as the heat- and pressure-transmitting medium. At the end of the come-up time (30 to 45 s), pressure was released, and samples were diluted with sterile buffer solution, mixed with a masticator, spread plated onto ST-1 agar plates, incubated at 30 or 37°C for 18 to 22 h, and enumerated using a New Brunswick colony counter.

RESULTS

Effects of heat activation on germination triggered by different GRs. As noted in the introduction, heat activation can increase the germination of spores of various *Bacillus* species, and at least one report indicates that germination via different GR-dependent germinants exhibits different requirements for heat activation (10). This suggests that different GRs may exhibit different responses to heat activation. To test this suggestion, we examined the germination of *B. subtilis* spores via different GRs after various heat activation times (Fig. 1 and 2). Rates of L-valine germination of wild-type spores via GerA were increased ~40% with optimal heat activation, which required ~15 min for spores made at 37°C, while heat activation for 4 or 6 h resulted in significantly slower germination (Fig. 1A and 2A and data not shown). Heat activation for 4 h also decreased the extent of L-valine germination after 100 min, although heat activation for ≤2.5 h had no effect (Fig. 1A and 2B). Rates of germination of wild-type spores with the mixture of L-asparagine, D-glucose, D-fructose, and K^+ (AGFK) and the L-asparagine germination of *gerB** spores were stimulated >20-fold and ~5-fold, respectively, by optimal heat activation that required 4 h for the AGFK germination of wild-type spores and 2 h for L-asparagine germination of *gerB** spores. Heat activation for 6 h did not further increase AGFK germination of wild-type spores or L-asparagine germination of *gerB** spores (data not shown).

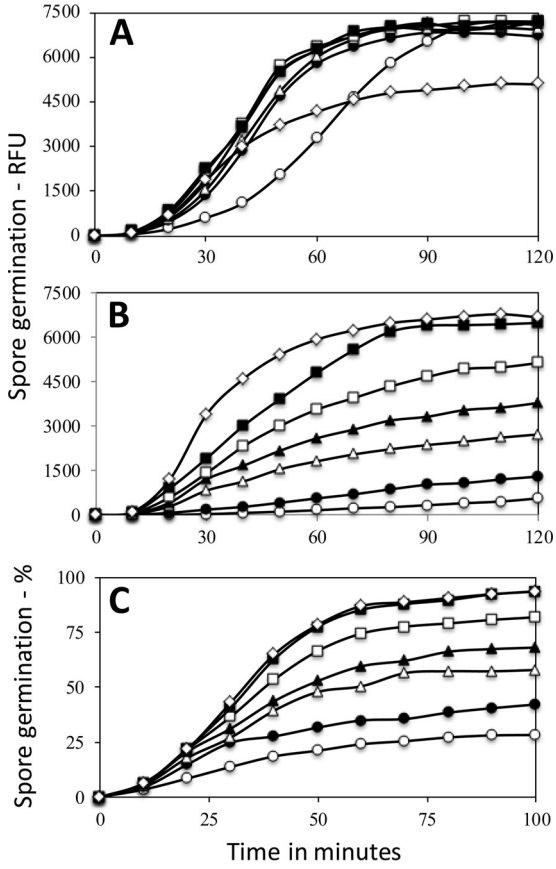

FIG 1 Effects of heat activation on germination of spores via various GRs. Spores of strains PS533 (wild type) (A and B) or FB10 (*gerB**) (C) were germinated with 10 mM L-valine (A), 10 mM (each) AGFK (B), or 10 mM L-asparagine (C) after various heat activation times as described in Materials and Methods. Spore germination was monitored by Tb-DPA fluorescence, with values given either in relative fluorescence units (RFU) or as percent spore germination as described in Materials and Methods. Values shown are the averages of results from measurements on duplicate germinations done simultaneously, and the individual measurements differed by ≤6% from average values. The symbols representing the heat activation times are as follows: ○, 0 min; ●, 5 min; △, 15 min; ▲, 30 min; □, 1 h; ■, 2.5 h; and ◇, 4 h. A 6-h heat activation did not increase AGFK germination further (data not shown). For the samples analyzed in panels A and B, the maximum percentages of spore germination at 100 min were 92 and 88%, respectively.

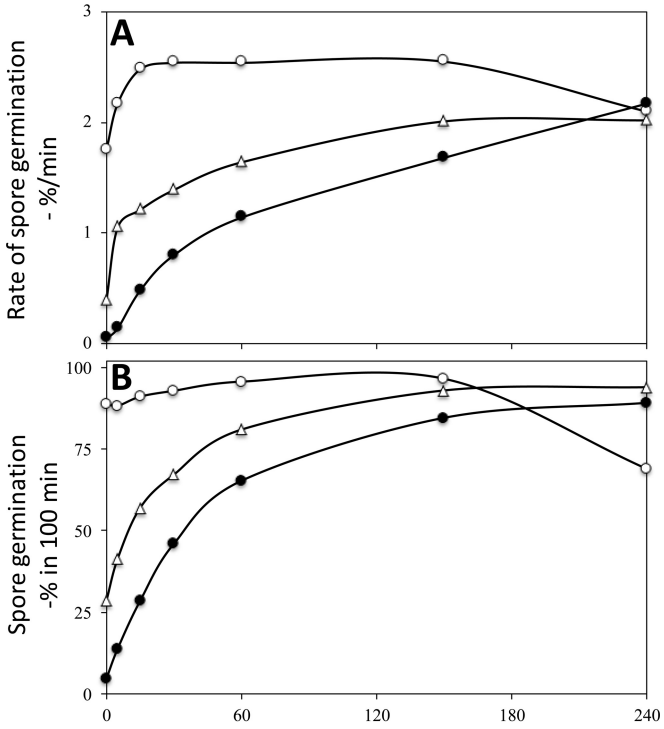

FIG 2 Effects of heat activation times on rates and levels of spore germination. Spores of strain PS533 (wild type) or FB10 (*gerB**) were prepared at 37°C and heat activated at 75°C for various times; spores were germinated in duplicate with either 10 mM L-valine, 10 mM (each) AGFK, or 10 mM L-asparagine as described in Materials and Methods. Spore germination was measured and germination rates (A) and percentages of spore germination (B) after 100 min were determined as described in Materials and Methods. Values shown are averages of duplicate determinations in two experiments with the same spore preparations and are ≤±12%. The symbols used are follows: ○, PS533 spores, L-valine germination; ●, PS533 spores, AGFK germination; and △, FB10 spores, L-asparagine germination.

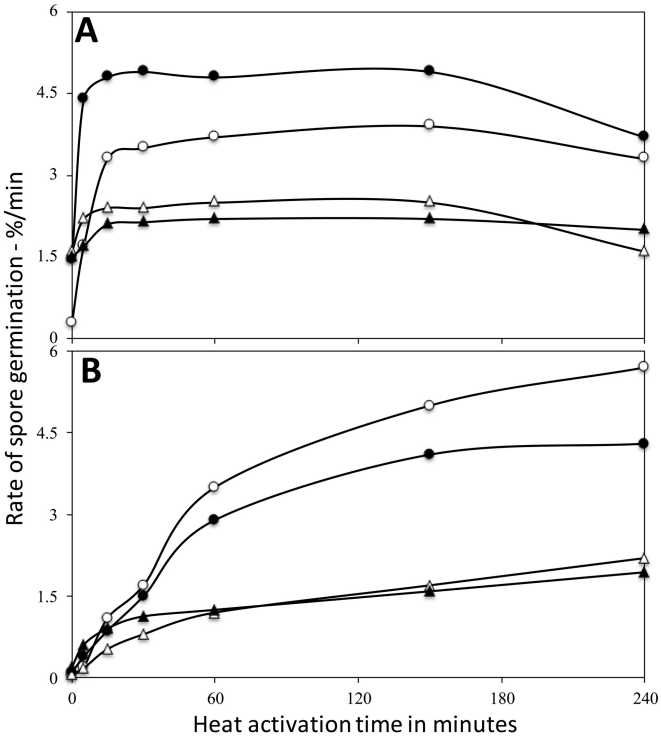

FIG 3 Effects of heat activation on germination of spores made at various temperatures. Spores of strain PS533 (wild type) were prepared at 23°C (○), 30°C (●), 37°C (△), or 43°C (▲) and heat activated at 75°C for various times, and spores were germinated in duplicate with either 10 mM L-valine (A) or 10 mM (each) AGFK (B) as described in Materials and Methods. Spore germination was measured and germination rates were determined as described in Materials and Methods. Values shown are averages of duplicate determinations in two experiments with the same spore preparations and were ≤±19%.

GRs are located in spores' IM, and it is possible that IM composition might also alter effects of heat activation on GR-dependent spore germination. One variable that greatly alters *B. subtilis* spore IM fatty acid composition is sporulation temperature, which can also affect rates of spore germination (38, 44). Wild-type spores prepared at temperatures from 23 to 43°C did exhibit differences in rates of spore germination with various germinants as expected (37) (Fig. 3). However, the optimal heat activation times for L-valine or AGFK germination did not differ appreciably for the spores made at the different temperatures, although heat activation caused greater stimulations in rates of L-valine or AGFK germination of spores made at lower temperatures (Fig. 3). Heat activation for 6 h did not further increase the rates of AGFK germination of the spores made at different temperatures (data not shown).

Effects of heat activation on germinant concentration dependence of spore germination. Heat activation clearly increased the rate and sometimes the extent of nutrient germination via GRs, and one possible reason was that heat activation reduces germinant concentrations needed to trigger spore germination. To test this possibility, we determined the germinant concentration dependence of spore germination via various GRs with and without heat activation (Table 2). Notably, optimal heat activation decreased L-asparagine concentrations needed for half-maximal rates of germination via GerB plus GerK or GerB* alone 1.5- to 2-fold. However, the effect of optimal heat activation on the concentration dependence of L-valine germination was small and not significant, likely because of the significant L-valine germination

TABLE 2 Effect of heat activation on germinant concentrations giving half-maximal germination rates[a]

Spores	Variable germinant	Germinant concn giving half-maximal germination rate (mM)	
		No heat	Heat
PS533 (wild type)	L-Valine[b]	3.2 ± 0.5	2.5 ± 0.4[b]
PS533 (wild type)	L-Asparagine[c]	2.7 ± 0.5	1.1 ± 0.26[d]
PS3665 (Δ*gerA gerBB** Δ*gerK*)	L-Asparagine	0.63 ± 0.1	0.40 ± 0.1[e]
PS3521 (Δ*gerA gerBB**)	L-Asparagine	0.64 ± 0.13	0.48 ± 0.1[b]
PS3521 (Δ*gerA gerBB**)	L-Asparagine[f]	0.62 ± 0.12	0.41 ± 0.09[b]
PS3521 (Δ*gerA gerBB**)	L-Asparagine[f]	0.62 ± 0.12	0.31 ± 0.08[e]
PS3521 (Δ*gerA gerBB**)	L-Asparagine[f]	0.62 ± 0.12	0.18 ± 0.05[d]

[a] Spores of various strains prepared at 37°C were germinated with variable germinant concentrations from 0.05 to 10 mM, and germination rates were determined in duplicate in 2 independent experiments to allow calculation of germinant concentrations giving 50% of the maximum germination rates.
[b] Heat activation for 30 min.
[c] GFK were also present, each at 10 mM.
[d] Heat activation for 4 h.
[e] Heat activation for 2 h.
[f] Glucose and K^+ were also present, each at 10 mM.

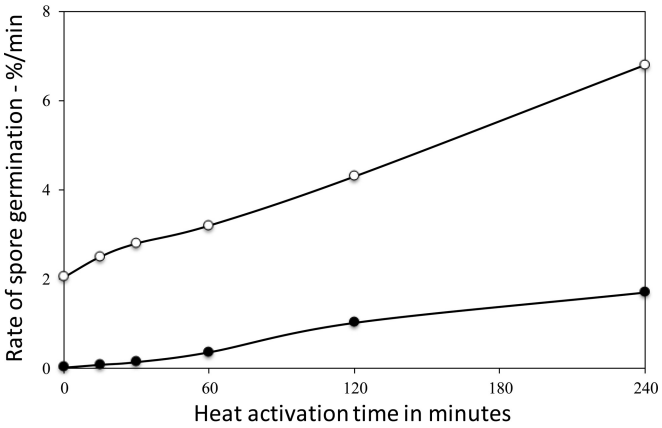

FIG 4 Effects of heat activation on germination of spores with overexpressed GerA. PS3476 (P*sspD*::*gerA*) spores were heat activated for various times and germinated in duplicate with either 10 mM L-valine (○) or 10 mM AGFK (●), spore germination was measured, and germination rates were determined as described in Materials and Methods. Values shown are averages of duplicate determinations in two experiments with the same spore preparation and were ≤±22%, with the largest variations in AGFK germinations at short heat activation times.

even with no heat activation; this was also seen using FB87 spores, which contain only the GerA GR (data not shown).

To examine the effects of heat activation on nutrient germination requiring GerK, we examined the effects of D-glucose on L-asparagine germination of PS3521 spores (Table 2). These spores lack GerA but have GerB* and GerK, and GerK is almost certainly the GR that mediates D-glucose stimulation of L-asparagine germination via GerB* (3). D-Glucose had minimal effects on the L-asparagine concentration dependence of the germination of PS3521 spores left unactivated or heat activated for 30 min. However, with PS3521 spores heat activated for 4 h, D-glucose decreased the L-asparagine concentration needed for half-maximal rates of germination even more than heat activation alone, while with spores heat activated for 2 h, there was essentially no effect. Overall, these data are consistent with GerK having a more stringent heat activation requirement for nutrient germination than GerA, GerB, or GerB*. This was also seen when effects of heat activation on spore germination triggered by 150 MPa of HP were examined (see below).

Effect of GR overexpression on the heat activation required for spore germination. Previous work has shown that overexpression of GerA from a strong forespore-specific promoter increases rates of spore germination with L-valine (45, 46). The levels of GerB and GerK do not decrease in spores overexpressing GerA, although these spores' germination with AGFK is significantly slowed (46). Since the inhibition of AGFK germination by elevated GerA levels might be due to altered heat activation requirements for the GRs in these spores, we examined the effects of heat activation on L-valine and AGFK germination of PS3476 spores overexpressing GerA ~8-fold (37, 45, 46) (Fig. 4). As expected (45), L-valine germination of PS3476 spores was faster than that of wild-type spores (compare Fig. 2A and 4). However, PS3476 spores' germination rate with L-valine was highest with 4 h of heat activation, in contrast to the ~15 min needed for spores with wild-type GerA levels, although 6 h of heat activation decreased L-valine germination of PS3476 spores markedly (data not shown). As seen previously (46), rates of AGFK germination of PS3476 spores were minimal with no or 30 min of heat activation and were below rates of AGFK germination seen with wild-type spores (compare Fig. 2B and 4). Heat activation for up to 4 h increased rates of AGFK germination of PS3476 spores to values close to those for wild-type spores (compare Fig. 2B and 4), but a 6-h heat activation caused no further increase (data not shown).

Effects of heat activation on synergy between GRs responding to different germinants. One striking behavior seen in GR-dependent germination is that with low concentrations of mixtures of germinants recognized by different GRs, for example, L-valine recognized by GerA and AGFK recognized by GerB plus GerK, the germination rate is higher than the sum of the germination rates with each germinant alone (47). To examine the effects of heat activation on this apparent synergy between different GRs, we determined extents of germination of wild-type spores with various concentrations of L-valine plus AGFK and of *gerB** spores with various concentrations of L-valine plus L-asparagine alone. These values were defined as actual (*a*) values. In addition, extents of germination were also determined for wild-type spores germinating with various concentrations of L-valine, AGFK, or L-asparagine alone (with *gerB** spores), and these values allowed calculation of values predicted (*p*) for extents of germination by the various concentrations of the germinant mixtures if there was no synergy. The value of *a*/*p* at any particular mixture of germinant concentrations has been defined as the degree of synergy (D_s), and values of ≥1 indicate synergy between GRs (47). D_s values were invariably larger at lower germinant concentrations (Fig. 5), as seen previously (47). Notably, unactivated spores exhibited the highest D_s values, and spores heat activated for 4 h exhibited only very small changes in D_s values as a function of germinant concentrations. Thus, much of the synergy between different GRs is abolished by optimal heat activation.

Effect of loss of GerD on heat activation required for GR germination. One defining feature of GR germination is that loss of the GerD protein greatly decreases GR-dependent germination, perhaps because the germinosome does not assemble in *gerD* spores (1, 2, 4). To test if *gerD* spores require much greater heat activation than wild-type spores, we examined the effects of heat activation on the extents of L-valine and AGFK germination of FB62 spores, which lack GerD (Fig. 6). As expected, with *gerD* spores made at 23 to 43°C, rates of L-valine and AGFK germination were much lower than for germination of wild-type spores made at these temperatures (compare Fig. 3 and 6). In contrast, just as with wild-type spores, heat activation for ~15 min or ~4 h gave maximal rates of germination of *gerD* spores with L-valine and AGFK, respectively (Fig. 6), and heat activation for 6 h gave no further increase in AGFK germination (data not shown). However, optimal heat activation gave less stimulation of *gerD* spore germination with L-valine than of wild-type spores, and perhaps also with AGFK, although this was more difficult to quantitate because of the very low rates of AGFK germination of *gerD* spores given no or short heat activation treatments. Interestingly, (i) *gerD* spores prepared at 23°C exhibited much faster germination with either L-valine or AGFK than did spores prepared at 37°C (Fig. 6), and (ii) with spores made at 43°C in particular, long heat activation times resulted in significant decreases in the rates of L-valine germination, suggesting that the GerA GR may be quite heat labile in these spores.

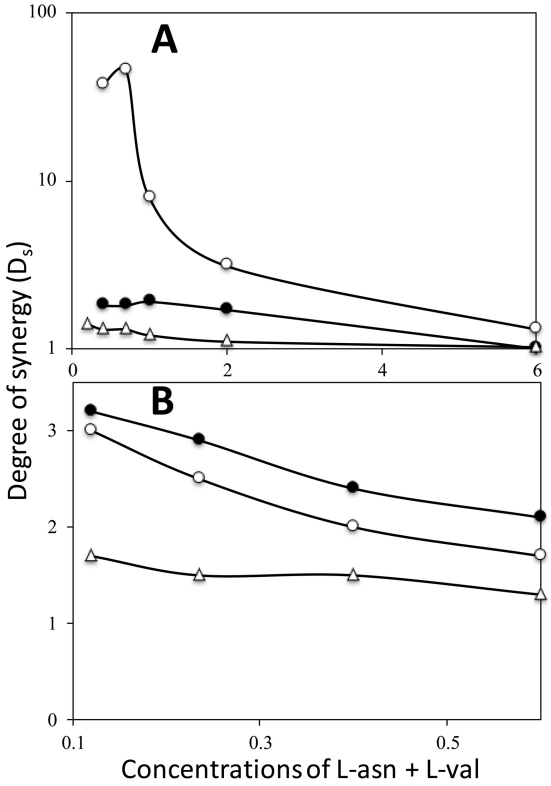

FIG 5 Effects of heat activation on the synergy between GerA and GerB plus GerK and GerA and GerB* in spore germination. Spores of strain PS533 (wild type) (A) or FB10 (*gerB**) (B) were either left unheated (○), heat activated for 30 min (●), or heat activated for 4 h (△) (A) or 2 h (B). These spores were germinated in duplicate with various concentrations of L-valine or L-asparagine (plus 10 mM [each] GFK) (A) or L-valine or L-asparagine (B) as described in Materials and Methods. The extents of spore germination at various times were determined as described in Materials and Methods and added together to give the predicted extents of spore germination, p, if there was no synergy. The spores were also germinated in duplicate with various concentrations of both L-valine and L-asparagine (plus 10 mM [each] GFK) (A) or both L-valine and L-asparagine (B), and the actual extents of spore germination with the germinant mixtures, *a*, were also determined. The degree of synergy (D_s) in germination at various concentrations of L-valine and L-asparagine was calculated as described previously (44) as $D_s = a/p$, and D_s values of >1 indicate synergy. In panel A the concentrations of L-valine and L-asparagine were equal, and in panel B the L-valine concentrations were 5-fold higher than the L-asparagine concentrations. The germination times selected for calculation of D_s values were the same for all data points for a particular germinant mixture, and these germination times gave the highest D_s values throughout the germinant concentration range. D_s values shown are averages from *a* and *p* values determined from duplicate measurements of extents of spore germination in two experiments with the same spore preparations and differed by ≤±32%.

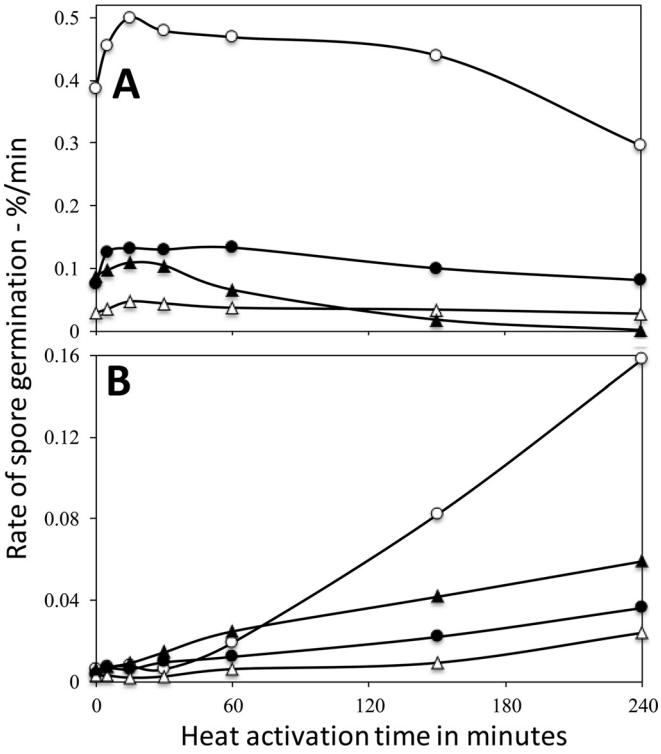

FIG 6 Effects of heat activation on the germination of *gerD* spores made at various temperatures. Spores of strain FB62 (*gerD*) made at 23°C (○), 30°C (●), 37°C (△), and 43°C (▲) were heat activated for various times and germinated in duplicate with either 10 mM L-valine (A) or 10 mM AGFK (B) as described in Materials and Methods. Rates of spore germination were also determined as described in Materials and Methods. Values shown are averages of duplicate determinations in two experiments with the same spore preparations and were ≤±22%.

Effects of heat activation on GR levels and accessibility. The facts that germination via different GRs exhibited different requirements for heat activation and that loss of GerD did not affect heat activation requirements for germination by different GRs suggested that heat activation affects GRs. One trivial, albeit extremely unlikely, possibility is that heat activation somehow alters spores' GR levels. However, this was not the case, as levels of GerD and multiple GR subunits differed by ≤15% in total lysates from wild-type spores left unactivated or heat activated for 4 h (data not shown).

A second possibility is that heat activation alters GR conformation such that these proteins are more responsive to their nutrient ligands. To attempt to obtain evidence consistent with this possibility, the EZ-Link Sulfo-NHS-SS-Biotin biotinylation reagent used to monitor accessibility of GR subunits and GerD in decoated *B. subtilis* spores (41) was used to examine whether biotinylation of these proteins differed in spores left unactivated and spores heat activated for 4 h. PS4150 spores that lack most of their spore coat were used in this experiment, allowing access of the biotinylation reagent to regions of germination proteins on the outer surface of these spores' IM (36, 41). Control experiments showed that germination of PS4150 spores exhibited the same heat activation requirements as germination of wild-type spores (data not shown). The results of the biotinylation experiment showed that levels of biotinylation of GR subunits and GerD in total PS4150 spore lysates and in isolated IM, integument, and soluble fractions were essentially identical in the unactivated and heat-activated spores (see Fig. S1 in the supplemental material).

Effects of heat activation on HP germination at 150 and 550 MPa. Previous work had indicated that in addition to nutrient germinants, HP of 150 MPa can also trigger spore germination via GRs, although heat activation of this HP germination has generally not been observed (19, 20). However, 150-MPa HP germination of unactivated or heat-activated (30 min at 70°C) *B. subtilis* spores is dominated by germination via GerA, with minimal contributions from GerB and GerK (20, 48). While it is possible that GerB and GerK are not especially responsive to HP of 150 MPa, it

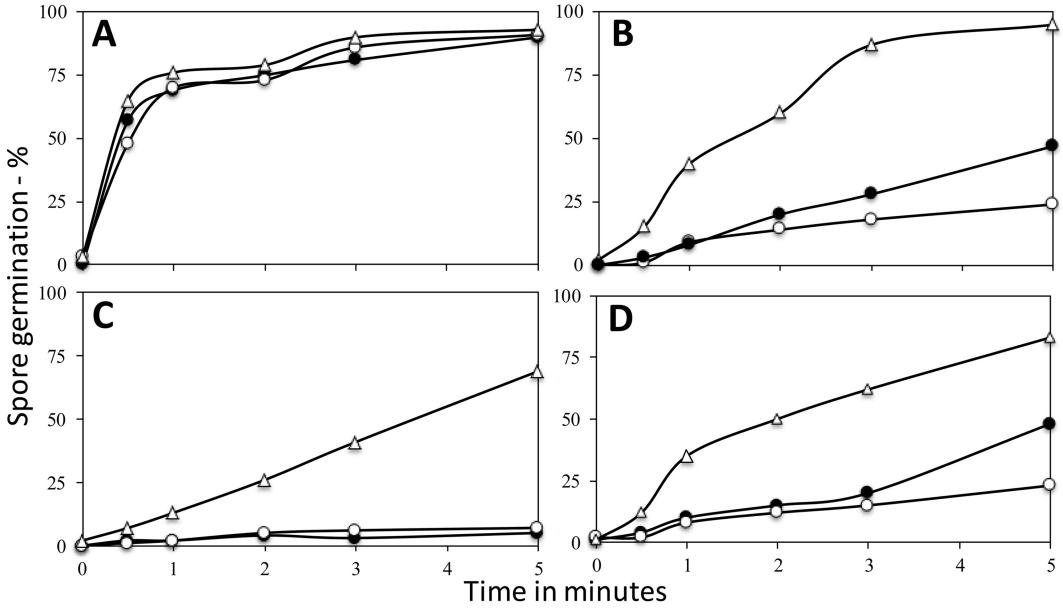

FIG 7 HP germination (150 MPa) of spores of various *B. subtilis* strains with and without heat activation. Spores of *B. subtilis* strains PS533 (wild type) (A), FB20 (Δ*gerA*) (B), FB61 (Δ*gerA* Δ*gerB*) (C), and PS3651 (Δ*gerA* Δ*gerK*) (D), without heat activation (○) or heat activated at 75°C for 30 min (●) or 4 h (△), were germinated in one experiment for various times with an HP of 150 MPa at 37°C; the extents of spore germination were measured as described in Materials and Methods.

is also possible that HP germination via GerB and GerK exhibits the extreme requirement for heat activation seen with nutrient germination. Consequently, we determined rates of 150-MPa HP germination of spores of *B. subtilis* strains containing various GRs, alone or in combination, and examined both unactivated spores and spores heat activated at 75°C for 30 min or 4 h (Fig. 7). As found previously (20, 48), wild-type spores left unactivated or heat activated for 30 min germinated rapidly and similarly with an HP of 150 MPa, while germination of unactivated spores via GerB plus GerK or GerB, or in particular via GerK alone, was extremely slow (compare Fig. 7A and D). However, a 4-h heat activation markedly stimulated 150-MPa HP germination via GerB and/or GerK, something that has not been seen previously (19, 20, 43), but had minimal effects on wild-type spore germination. In addition, 30 min of heat activation had only small effects on the 150-MPa HP germination of spores via GerB and particularly via GerK, and this result is consistent with effects of heat activation times on nutrient germination via these GRs.

While an HP of 150 MPa gave 7 and 23% germination of unactivated *B. subtilis* spores via the GerK and GerB GRs, respectively (Fig. 7C and D), in 5 min, an HP of 550 MPa at 50°C gave 33 and 60% germination of unactivated spores in 5 min via GerK and GerB, respectively (Fig. 8C and D). However, while 30 min of heat activation stimulated *B. subtilis* spore germination by an HP of 550 MPa, even that of wild-type spores, 4 h of heat activation decreased 550-MPa HP germination during the first 3 min of HP treatment (Fig. 8), as if this long heat activation treatment had significantly damaged some essential component involved in 550-MPa HP spore germination, similar to what was seen in some instances with nutrient germination via GerA (Fig. 1A and 2A). In contrast to the kinetics observed with nutrient germination, the extent of germination observed in the 3- to 5-min region with 550-MPa HP treatment of spores heat activated for 4 h reached about 95%, a level comparable to that of spores heat activated at 75°C for 30 min and also HP treated. These effects of heat activation on *B. subtilis* spore germination by HP were surprising, since this has not been reported previously, as noted above.

HP treatment leading to bacterial spore germination and inactivation is used in a number of applications to reduce spore burdens in foodstuffs (24–27). As a consequence, methods to increase spore germination by HP are of significant applied interest. To determine if heat activation might affect the HP germination of spores of applied interest, we used spores of *B. amyloliquefaciens*, which have been suggested as a good surrogate for spores of *C. botulinum* in analyzing the efficacy of regimens for spore inactivation by HP (28, 29). Strikingly, heat activation for 4 h at 70°C markedly increased the germination of *B. amyloliquefaciens* spores at HPs of both 150 and 550 MPa (Fig. 9), although it is possible that less than 4 h at 70°C would have sufficed for maximal germination.

HP sterilization of foods typically involves preheating to up to 90°C and then pressurizing to ≥600 MPa over some finite come-up time, during which adiabatic heating of compression causes the product temperature to increase typically ≥121°C (49). As an example, the pressure chamber containing only bioglycol oil at an initial temperature (T_i) of 75 to 95°C and HPs of 552 to 690 MPa induced adiabatic heating, giving 20 to 35°C temperature increases in the HP chamber that decayed to T_i during the ensuing hold time (see Table S1 in the supplemental material). Spores in a food matrix would experience similar treatment conditions for appreciable periods during such an HP process. The effects of temperature and HP in activating *B. amyloliquefaciens* spores in chicken purée were determined at various combinations of temperature and pressure. With a T_i of 75 to 95°C and pressure of 449 to 690 MPa, the viable counts increased after the come-up time (30 to 45 s) required to reach the target HP compared to the

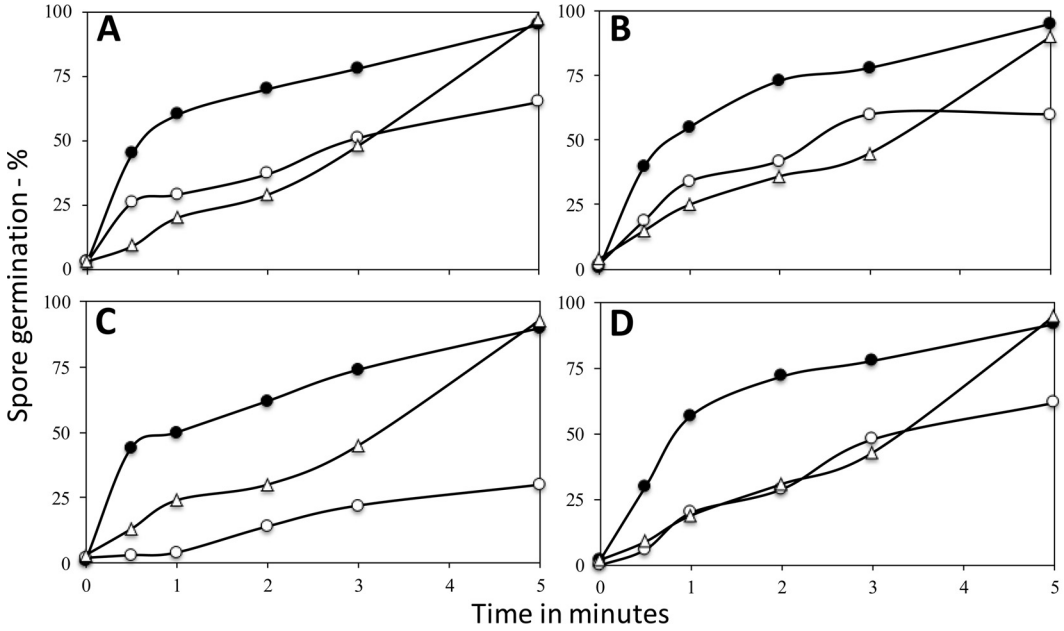

FIG 8 HP germination (550 MPa) of spores of various *B. subtilis* strains with and without heat activation. Spores of *B. subtilis* PS533 (wild type) (A), *B. subtilis* FB20 (Δ*gerA*) (B), *B. subtilis* FB61 (Δ*gerA* Δ*gerB*) (C), and *B. subtilis* PS3651 (Δ*gerA* Δ*gerK*) (D), without heat activation (○) or heat activated at 75°C for 30 min (●) or 4 h (△), were germinated in one experiment for various times with an HP of 550 MPa at 50°C; the extents of spore germination were measured as described in Materials and Methods.

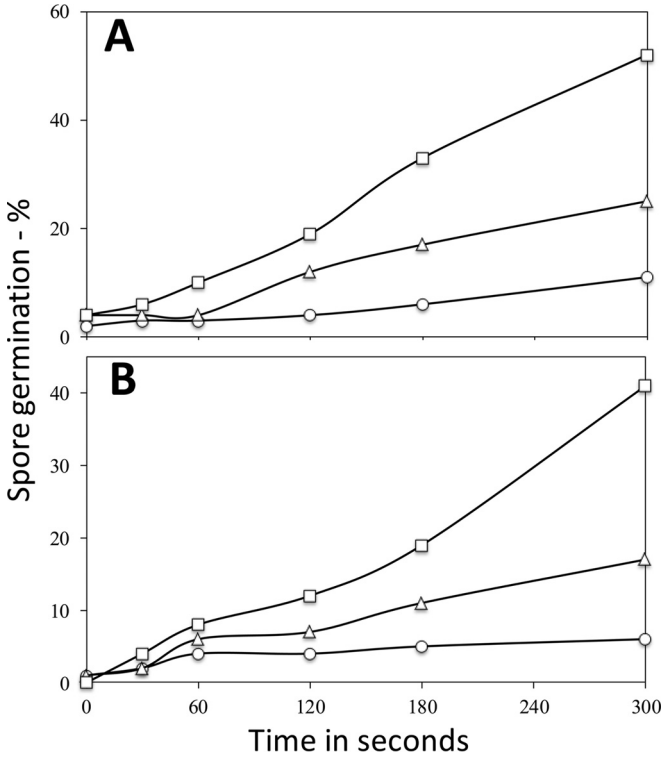

FIG 9 HP germination of *B. amyloliquefaciens* spores with and without heat activation. Spores of *B. amyloliquefaciens* prepared at 37°C and without heat activation (○) or heat activated at 70°C for 30 min (△) or 4 h (□) were germinated in one experiment for various times with an HP of 150 MPa (A) or 550 MPa (B); the extents of spore germination were measured as described in Materials and Methods. Similar results were obtained with two independent spore preparations, and HP germination of spores prepared at 30°C was affected similarly by heat activation (data not shown).

untreated samples, indicative of spore activation prior to plating and incubation (Table 3). Heating samples to 75 to 121°C at ambient pressure (1 atm ≈ 0.1 MPa) for as long as 15 min also induced activation of *B. amyloliquefaciens* spores, with the exception of significant inactivation by treatments at 112.5 and 121°C for 15 min. Significant inactivation was also seen with spores heated at 95°C and then exposed to 690 MPa for 3 min plus come-up time. Consequently, an HP process with these pressures and temperatures can activate, germinate, and inactivate individual spores sequentially, and these processes can occur concurrently for spore populations, given the heterogeneous distribution of resistances of spores within large populations. It is also possible that GR-dependent germination occurs during the pressurization process, although this contribution would likely be relatively minor, due to the transience of the come-up time.

DISCUSSION

Heat activation of germination of spores of *Bacillus* species was described more than 45 years ago (6, 11), although it has been studied relatively little recently. Overall, heat activation has been shown to be (i) temperature dependent, as extended incubation at relatively low temperatures also activates spores, although activation is faster at high temperatures, and (ii) reversible to some degree by incubation at low temperatures. These observations, as well as thermodynamic studies, are consistent with heat activation causing a reversible conformational change in one or more proteins (6, 11, 17, 50). However, such proteins have not been identified, although a recent study found reversible changes in global protein structure accompanying spore heat activation (17).

Since heat activation affects only GR-dependent germination and not germination by GR-independent germinants (1, 2), GRs are attractive as heat activation targets, perhaps via temperature-dependent conformational changes. However, other proteins, no-

TABLE 3 Activation of *B. amyloliquefaciens* spores by heat with or without HP[a]

Inoculum (CFU/ml, $\times 10^{-7}$)	Treatment		Time (s)	No. of survivors (CFU/ml)	% change
	Temp (°C)	P (MPa)			
			Come-up		
3.51 ± 1.03	65	552	36	$(3.41 \pm 0.32) \times 10^7$	−2.8
5.21 ± 1.20	75	448	29	$(8.27 \pm 4.28) \times 10^7$	+58.7
2.78 ± 0.61	75	552	36	$(7.09 \pm 1.55) \times 10^7$	+155
3.04 ± 1.59	75	690	45	$(7.07 \pm 0.95) \times 10^7$	+132.6
1.87 ± 0.46	95	690	45	$(2.72 \pm 0.06) \times 10^7$	+45.5
			Hold		
1.87 ± 0.46	95	0	180	$(2.96 \pm 0.71) \times 10^7$	+58.3
2.72 ± 0.06	95	690	180	$(1.08 \pm 0.13) \times 10^4$	−99.9[b]
2.50 ± 0.54	112.5	0	180	$(9.30 \pm 0.99) \times 10^7$	+372
1.56 ± 0.47	121	0	180	$(4.50 \pm 0.42) \times 10^7$	+188.5
3.40 ± 0.28	75	0	420	$(5.50 \pm 0.85) \times 10^7$	+61.8
3.40 ± 0.28	85	0	420	$(1.14 \pm 0.24) \times 10^8$	+235.3
5.35 ± 0.92	95	0	420	$(1.21 \pm 0.18) \times 10^8$	+126.2
2.50 ± 0.54	105	0	420	$(1.39 \pm 0.08) \times 10^8$	+456
2.50 ± 0.54	112.5	0	420	$(1.59 \pm 0.04) \times 10^8$	+536
3.45 ± 0.07	121	0	420	$(4.95 \pm 0.21) \times 10^7$	+43.4
2.50 ± 0.54	95	0	900	$(1.18 \pm 0.13) \times 10^8$	+372
2.50 ± 0.54	105	0	900	$(1.06 \pm 0.26) \times 10^8$	+324
2.50 ± 0.54	112.5	0	900	$(1.12 \pm 0.05) \times 10^7$	−55.2
3.45 ± 0.07	121	0	900	$(1.45 \pm 0.08) \times 10^7$	−58.0

[a] Activation of spores was measured after heat treatment with or without subsequent HP treatments, as increases in viable counts before (inoculum) and after (no. of survivors) heat and HP treatments. All viability measurements are mean plate counts and standard deviations from 2 to 12 replicates.
[b] Representative data indicating inactivation by HP treatment perhaps due to adiabatic heating.

tably GerD, are also involved in GR-dependent germination. The current study with *B. subtilis* spores provided evidence consistent with GRs being the major heat activation target as follows. (i) Heat activation times to get maximal germination via different GRs varied between 15 min and 4 h, with GerA exhibiting the lowest requirement, GerK the highest, and GerB* an intermediate requirement. It is reasonable that high temperatures, and thus heat activation, could have different effects on different GRs, as the same subunits in different GRs from the same species exhibit <35% amino acid sequence identity (51, 52) and thus could have different temperature requirements for structural changes. However, what such a conformational change might do is not clear. (ii) The different heat activation times for optimal stimulation of nutrient germination via different GRs were generally similar to the effects of heat activation times on 150-MPa HP germination of spores containing various GRs. Thus, HP germination via GerK was simulated most by heat activation, and germination predominantly by GerA was stimulated least. One difference between effects of heat activation on germination by an HP of 150 MPa and nutrients is that there was essentially no effect of heat activation on 150-MPa germination by GerA, in contrast to an ~40% stimulation in L-valine germination rate via GerA by heat activation. However, since HP can cause conformational changes in proteins (53–55) in addition to triggering GR-dependent germination, an HP of 150 MPa could also activate GRs, with GerA being most responsive to activation by this HP. (iii) While the loss of GerD greatly reduces rates of GR-dependent germination, *gerD* spores required heat activation times similar to those of wild-type spores for maximal germination rates. This rules out heat activation as affecting GerD or germinosome assembly or function rather than GRs directly. Indeed, the germinosome appearance in spores carrying functional GerA-mCherry or GerD-green fluorescent protein fusions (4) was not altered by 30 min or 4 h of heat activation (A. J. Troiano and P. Setlow, unpublished data). Interestingly, the *gerD* mutation had a much smaller effect on the germination of spores prepared at 23°C, something that has not been seen previously. Perhaps this is due to the likely greater IM fluidity in spores made at 23°C (44), which may allow GRs not in the germinosome in *gerD* spores to more readily move in the IM and interact. (iv) Heat activation decreased the nutrient germinant concentrations for 50% of the maximum germination rate, in particular in germination via GerB plus GerK or GerB* with or without GerK. Previous work showed that elevated GR levels decrease germinant concentrations needed for the same rates of spore germination of wild-type spores (3, 45), and heat activation may make more GRs functional or responsive to their nutrient germinants, thus decreasing germinant concentrations needed for a given spore germination rate.

While the results noted above suggest that GRs are the target for heat activation, heat activation may affect GRs only indirectly and directly affect the state of the spore IM, which then alters GR structure and responsiveness. It is difficult to rule out the latter mechanism of heat activation, and the lack of effect of heat activation on the biotinylation of GR proteins seen in this study was inconsistent with heat activation directly affecting GRs. However, several results described in this communication as well as one from the literature are less consistent with the IM as the heat activation target compared with GRs, as follows. (i) As shown in the current work, long heat activation times reduced GerA germination, while GerB and GerK function was increased, and this may reflect lower GerA thermal stability. (ii) The IM fatty acid compositions differ greatly in spores made at temperatures from 23 to 43°C (44). Such changes in IM lipid composition, and thus presumably in the degree of lipid mobility in the IM, could well in-

fluence the behavior of proteins embedded in the IM, as the A and B subunits of GRs are. This altered IM lipid mobility, in turn, could also modify the effects of heat activation on these proteins. However, as shown in the current work, different sporulation temperatures did not alter optimal heat activation times for germination via GerA or GerB plus GerK. (iii) In contrast to spores of *Bacillus* and *Clostridium* species which exhibit heat activation of GR-dependent germination, with *C. difficile* spores that have no GRs in the IM but germinate in response to specific bile salts that activate a protease that activates a cortex peptidoglycan lytic enzyme, heat activation has no effect on bile salt germination (56, 57).

In addition to the major findings noted above, other observations on effects of heat activation on spore germination are as follows. (i) Spores made at different temperatures had the same optimal heat activation times for maximum rates of nutrient germination, but germination of spores made at lower temperatures, in particular via GerA, was stimulated more by heat activation. The latter effect could be due either to less heat activation, in particular of GerA, during sporulation at lower temperatures, or to differences in the IM of spores made at different temperatures. (ii) Unactivated spores exhibited much greater synergy between germinants that act via different GRs than heat-activated spores. This suggests that GR-GR interaction is essential for this synergy, whether direct or indirect, and this interaction can be stimulated by heat activation. (iii) Overexpressing GerA from a moderately strong forespore-specific promoter gives spores with 8-fold-increased GerA levels, although with no changes in GerB and GerK levels (46). When these spores were not heat activated, rates of L-valine and AGFK germination were higher and lower, respectively, than rates with wild-type spores. Heat activation of these spores increased GerA-dependent germination ≥3-fold but required 4 h for this effect instead of the 15 min for wild-type spores, for reasons that are not clear. However, even 4 h of heat activation did not restore AGFK germination rates of spores overexpressing GerA to those in wild-type spores. Thus, inhibition of AGFK germination by elevated GerA levels affects GR function directly, as suggested previously (46). (iv) Germination of *B. subtilis* spores by an HP of 550 MPa was stimulated by 30 min of heat activation, something that has not been seen previously. This result was surprising since spore germination at 550 MPa is thought to be GR independent (19, 20, 48). However, effects of heat activation on 550-MPa germination of *B. subtilis* spores differed considerably from effects on GR-dependent 150-MPa HP germination as follows. (a) Heat activation affected 550-MPa HP germination of wild-type spores and spores containing only GerB plus GerK, only GerB, or only GerK relatively similarly, although germination of unactivated spores containing only GerK was a bit slower than that of other spores; (b) a 4-h heat activation decreased 550-MPa HP germination of all spores markedly over that following a 30-min activation. Thus, heat activation may have effects on spores' germination by 550 MPa of HP that are largely, but perhaps not completely, GR independent, as suggested previously (43). The target modified by heat activation leading to increased 550-MPa HP germination is not clear, but two possibilities are (a) the spore IM, where many germination proteins, including the SpoVA proteins comprising the Ca-DPA channel thought to be activated by HPs of ≥500 MPa, are located (1, 2, 19, 24, 43), and (b) one or more of the SpoVA proteins themselves. (v) Finally, the observation that heat activation also markedly stimulated HP germination of *B. amyloliquefaciens* spores may have applied implications, since HP germination and subsequent heat inactivation of less resistant germinated spores can minimize spore loads in shelf-stable foods (24–27), and *B. amyloliquefaciens* spores have been proposed for use as a surrogate for *C. botulinum* spores in analysis of regimens for spore inactivation by HP (28, 29). HP treatment for the commercial sterilization of foodstuffs is almost always carried out at temperatures much higher than the 50°C used in our study, and even with short processing times, temperatures and HPs may be sufficient to cause significant spore heat activation during the HP treatment. However, it is also possible that a pretreatment step at high temperature might increase the efficacy of subsequent HP treatments in food processing regimens; this possibility certainly seems to merit further study.

ACKNOWLEDGMENTS

This communication is based upon work supported by a Department of Defense Multi-Disciplinary Research Initiative through the U.S. Army Research Laboratory and the U.S. Army Research Office under contract number W911NF-09-1-0286.

We thank Haiqing Chen and Jonathan Huang for use of high-pressure equipment at the University of Delaware.

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III DISCUSION

Kuwana y Takamatsu, en el 2013 sugirieron que la proteína GerW en *B. subtilis* podría participar en el ensamblaje de los RG en la membrana interna de la espora o podría sensar las señales transmitidas por el RG GerA y esta proteína podría transmitir estas señales a proteínas involucradas en eventos posteriores en la germinación, en particular la liberación del CaDPA. Sin embargo en este estudio se encontró que las esporas de *B. subtilis* que codifican pocos aminoácidos de la proteína GerW germinan normalmente con germinantes dependientes del RG GerA diferente a lo reportado por Kuwana y Takamatsu, 2013, donde reportaron que las esporas deficientes en el gen *gerW* no son capaces de germinar o liberar el CaDPA en presencia de L-alanina. Además en este estudio se observó que las esporas de *B. subtilis* Δ*gerW* presenta niveles similares de las proteínas de germinación, incluyendo las subunidades del RG GerA a las esporas de tipo silvestre. Así como también los niveles de SpoVAD, principal proteína involucrada en la formación del canal para la acumulación del CaDPA durante la esporulación y su liberación del núcleo de la espora en la germinación fueron similares en las esporas Δ*gerW* y las esporas de tipo silvestre. Además en esporas Δ*gerW* en *B. megaterium* no se encontraron diferencias significativas entre las tasas de germinación con germinantes nutrientes o agentes no nutrientes comparadas con las cepas de tipo silvestre.

La activación térmica en las esporas es un aspecto importante en el proceso de germinación, debido que incrementa la eficiencia en la germinación de las esporas vía RG, sin embargo este proceso aun no es completamente claro (Setlow, 2013; Setlow, 2014). En general la activación térmica ha mostrado que: i) es dependiente de la temperatura, largos tiempos de incubación a bajas temperatura también puede activar a las esporas, aunque la activación es rápida a altas temperaturas; y ii) es reversible por incubación a bajas temperaturas. Estas observaciones, así como también los estudios termodinámicos son consistentes con que la activación térmica causa cambios conformacionales reversibles en una

o más proteínas (Zhang *et al*., 2009; Lodval *et al*., 2013). Debido que la activación térmica afecta solo la germinación dependiente de los RG y no la germinación vía independiente de los RG (agentes no nutrientes) (Setlow, 2013; Setlow, 2014), esto hace atractivo candidatos a los RG como blancos de la activación, quizás cambios conformacionales dependiente de la temperatura. Sin embargo en este trabajo la falta de efecto de la activación térmica en la biotinilación de los RG fue inconsistente con la posibilidad de que la activación afecte directamente en la accesibilidad de los RG, así como también que no hay diferencias significativas en los niveles de las proteínas de germinación como son los RG, GerD y SpoVAD en esporas con o sin activación térmica. Debido a estos resultados sigue siendo incierto como la activación térmica afecta a las proteínas de germinación presentes en la membrana interna.

Sin embargo, este trabajo provee de varias evidencias que los RG son al menos unos de los principales blancos de la activación térmica: 1) es diferente el tiempo requerido para la activación térmica para obtener la tasa máxima germinación vía RG, donde la germinación dependiente de RG GerA necesita de 15 minutos activación mientras que los RG GerB y GerK necesitan de 4 horas de activación; 2) los diferentes tiempos requeridos de la activación de las esporas para la germinación vía diferentes RG fue consistente con el efecto de los tiempos usados para la activación en la germinación de esporas con PH a 150 Mpa, donde se observó que se necesita de mayor tiempo de activación para la germinación vía RG GerB y GerK en comparación a la germinación vía RG GerA; 3) la activación térmica afectó la eficiencia de la germinación de las esporas *gerD* y los tiempos requeridos para la máxima germinación vía RG fueron similar a las observadas por las esporas de tipo silvestres y 4) la activación térmica disminuye la concentración de germinante nutriente requerido para alcanzar el 50% de la tasa máxima de germinación y en particular tuvo un efecto mayor en la germinación vía RG GerB y GerK comparado a la germinación vía RG GerA.

En conclusión, la proteína GerW no tiene un papel importante en el proceso de germinación en esporas de *B. subtilis* o *B. megaterium*. Por consiguiente, la precisa función de la proteína GerW en las esporas permanece incierto; aunque su posible asociación con el metabolismo y la presencia de ortólogos de gen *gerW* en *Bacillus* quizás indique que esta proteína tiene un rol importante en otras especies del genero *Bacillus*. Por otra parte, los resultados en este trabajo sobre la activación térmica en esporas de *B. subtilis* son consistentes que la activación actúa principalmente en los RG, quizás por inducción de cambios conformacionales, aunque la activación no tuvo efectos en la accesibilidad de las proteínas de los RG o GerD probados por biotinilación por un reagente externo.

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