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DOCTORADO EN CIENCIAS BIOMÉDICAS

"MODULACIÓN DE LOS NIVELES DE RECEPTORES GERMINANTES Y SU EFECTO SOBRE LA GERMINACIÓN DE *Bacillus subtilis*"

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS BIOMÉDICAS

PRESENTA:

M EN C ARTURO RAMÍREZ PERALTA

DIRECTOR DE TESIS: DR. MARCO ANTONIO LEYVA VÁZQUEZ

DIRECTOR EXTERNO: DR. PETER SETLOW

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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 21 días del mes de junio del dos mil trece, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Modulación de los niveles de receptores germinantes y su efecto sobre la germinación de *Bacillus subtilis*"**, presentada por el alumno Arturo Ramírez Peralta, 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

El trabajo experimental de esta tesis se realizó en el laboratorio del Dr. Peter Setlow en el departamento de microbiología molecular, estructural y fisiológica de la **Universidad de Connecticut "Health Center**" ubicada en Farmington, EUA. El proyecto estuvo bajo la dirección del:

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“Modulación de los niveles de receptores germinantes y su efecto sobre la germinación de *Bacillus subtilis*”

Modulación de los niveles de receptores germinantes y su efecto sobre la germinación de *Bacillus subtilis*

Anexos

RESUMEN

Las esporas de especies de *Bacillus* son extremadamente resistentes a una variedad de tratamientos y al germinar son causa de la descomposición de alimentos y enfermedades producidas por los mismos. La germinación inicia con la presencia e interacción de nutrientes con sus respectivos receptores presentes en la membrana interna de la espora. En el presente trabajo se evalúo el efecto de factores ambientales y genéticos sobre la expresión de receptores germinantes. En esporas preparadas en medio mínimo se observó una menor germinación en comparación a esporas obtenidas en medio enriquecido, esta diferencia es atribuida a una menor cantidad de receptores en esporas preparadas en medio mínimo. SpoVT reprime la síntesis de receptores, esto se comprobó por el aumento de receptores en esporas knock-out para este gen y por lo tanto de la germinación en comparación con las esporas silvestres. La nueva proteína GerKD en *B. subtilis* no afecta la expresión de receptores germinantes, sin embargo, en ausencia de esta proteína, la germinación de esporas con AGFK aumenta. Otra nueva proteína, YlyA regula la expresión de receptores que reconocen germinantes así como los canales de ácido dipicolínico. La composición del medio de esporulación así como diferentes factores de transcripción modularon la expresión de receptores que reconocen germinantes en poblaciones de esporas de *B. subtilis*.

INTRODUCCION

El género *Bacillus* incluye especies de bacilos Gram positivos, aerobios y con la principal característica de ser formadores de esporas, la mayoría de las especies se encuentran en el suelo o las raíces de las plantas. La aerobiosis estricta distingue a *Bacillus* de las especies de *Clostridium* que generalmente son anaerobios estrictos. Las especies de *Bacillus* son considerados como no patógenos u oportunistas en humanos y animales, excepto *Bacillus anthracis* (Priest FG,1993).

La espora es formada durante el proceso denominado esporulación, la cual en especies de *Bacillus* es inducida por la falta de carbono y/o nitrógeno. A nivel genético la esporulación es iniciada por la fosforilación de la proteína Spo0A que es tanto un activador como represor de expresión genética, donde su mayor función es activar la expresión de genes que codifican para los factores sigma (σ). La activación y/o síntesis de factores sigma dirige los diferentes patrones de expresión genética tanto en la célula madre (σ^E y σ^K) como en la espora (σ^F y σ^G) (Errington, 2003).

A nivel celular, la esporulación inicia con una división celular asimétrica que genera una célula madre y una estructura conocida como preespora. La membrana plasmática de la célula madre crece alrededor de la preespora, generando una envoltura que posteriormente formará tanto la membrana interna como externa de la espora (Piggot y Hilbert, 2004). En el núcleo de la espora se captan grandes cantidades del ácido 2-piridino-6-dicarboxílico (ácido dipicolínico o DPA) el cual es sintetizado en la célula madre. El DPA comprende del 5 al 15% del peso de las esporas y es preferentemente quelado con cationes divalentes, principalmente calcio. La acumulación de DPA en el núcleo es responsable de la reducción de la cantidad de agua durante esporulación y juega un papel importante en la fotoquímica del DNA de la espora (Setlow, 2001). Durante las etapas tardías de la esporulación, las proteínas sintetizadas en la célula madre se agregan sobre la superficie de la espora formando la cubierta; en algunas especies se sintetiza una capa de glicoproteínas con forma

de balón denominada exosporio. Finalmente, la célula madre se lisa, liberando la espora en el ambiente (Setlow y Johnson, 2007).

Las esporas pueden sobrevivir por largo tiempo sin nutrientes debido a que presentan una fase de latencia; las razones por las cuales se establece la latencia incluyen la nula cantidad de compuestos de alta energía como el ATP y NADH, acompañado de un metabolismo no detectable con escasa actividad enzimática, que puede ser explicado por la disminución del pH así como el bajo contenido de agua en el centro de la espora donde los metabolitos y las enzimas son localizados, por lo tanto, los bajos niveles de agua disminuyen la movilidad de las proteínas y la actividad enzimática (Setlow, 2005).

Durante la fase de latencia, las esporas adquieren características de resistencia, siendo la más importante la resistencia al calor; donde comúnmente resisten altas temperaturas (por arriba de 40˚C) en comparación con la célula madre. Además las esporas también son resistentes a otros tratamientos como la desecación, radiación UV, altas presiones, alteración mecánica y exposición a agentes químicos como los ácidos, alcaloides, aldehídos, agentes oxidantes y una variedad de agentes que dañan el DNA (Nicholson *et al*., 2000). Aun cuando son múltiples los factores que contribuyen a la resistencia de las esporas, la prevención y reparación del daño al genoma de la espora son de suma importancia (Setlow, 2005). El principal mecanismo de prevención de daño al DNA es la saturación del DNA de la espora con proteínas pequeñas solubles ácidas (SASP), las cuales sólo son sintetizadas durante la esporulación y son extremadamente abundantes en las esporas y comprenden de 3 al 6% del total de las proteínas de la espora. Estas se encuentran exclusivamente en el núcleo y al unirse al DNA alteran sus características químicas como enzimáticas; experimentalmente se ha demostrado que estas proteínas cambian la fotoquímica UV del DNA, lo protegen contra la degradación enzimática y disminuyen tanto la generación de sitios apurinicos como la desaminación de citosina a uracilo (Moeller R *et al*., 2008; Setlow B *et al*., 2006) .

Las esporas latentes monitorean el ambiente y cuando las condiciones son favorables para su crecimiento, las esporas germinan y se convierten de

nuevo en células en crecimiento. La germinación es importante en el sentido de que las esporas después de germinar son causa de la descomposición de alimentos y enfermedades transmitidas por alimentos (Setlow, 2003). En el caso de *Bacillus anthracis* puede invadir macrófagos y causar el ántrax pulmonar (Guidi-Rontani *et al* 1999).

El proceso de germinación se divide en dos etapas; la primera que comprende los tres primeros pasos del proceso: la liberación de la espora de H+, cationes monovalentes y Zn+, probablemente desde el centro de la espora; segundo, la liberación de DPA asociado a cationes divalentes, predominantemente de calcio; tercero, el remplazo de DPA por agua, resultando en el incremento de la hidratación de la espora y causando una disminución de la resistencia al calor. La segunda etapa de la germinación comprende los dos últimos pasos: cuarto, hidrólisis del peptidoglucano de la corteza de la espora favoreciendo el crecimiento de la espora después de la adquisición de agua y la expansión de la pared de la célula latente (Figura 1). El inicio de la actividad enzimática en el centro de la espora es después de haberse completado la segunda etapa, con el inicio de la síntesis macromolecular que convierte la espora germinada en una célula en crecimiento. Las dos etapas pueden ser separadas experimentalmente por tratamiento químico o mutaciones (Paredes-Sabja D *et al*., 2011).

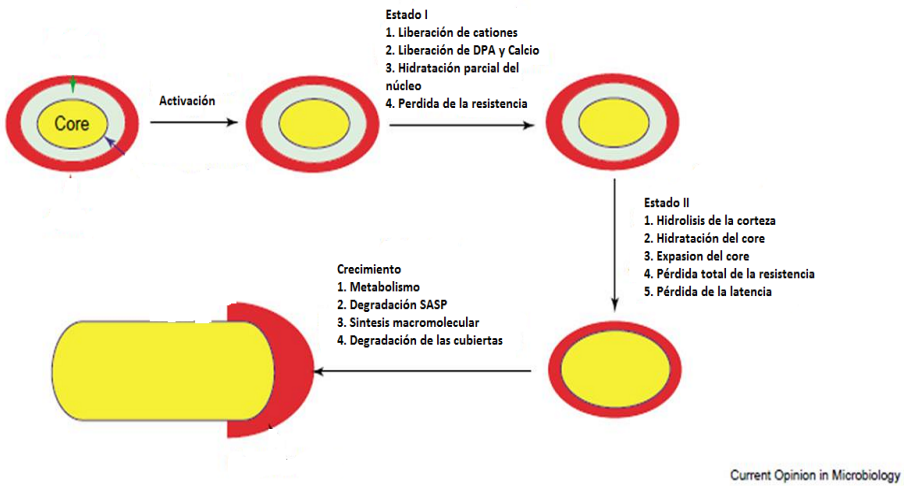

Figura 1. Etapas de la germinación de esporas de *Bacillus*

El sistema de germinación de la espora ha sido dividido en tres componentes principales: los receptores que responden a nutrientes germinantes; los canales a través de los cuales se mueven los iones y el DPA; y las enzimas líticas que degradan la corteza de la espora (Setlow, 2003).

El inicio de la germinación es determinado por la unión estereoespecífica de los nutrientes de bajo peso molecular a receptores que reconocen germinantes (GR). Generalmente, las esporas de *Bacillus* tienen múltiples receptores, siendo tres los de mayor relevancia en esporas de *B. subtilis* denominados GerA, GerB y GerK; cada receptor es codificado por un operón tricistrónico homólogo, siendo sus productos dos proteínas integrales de membrana (A y B) y una lipoproteína (C). Cada uno de esos receptores reconoce un nutriente germinante diferente o un grupo de germinantes, GerA solo responde a L- alanina o L- valina; mientras que GerB y GerK reconoce una mezcla de germinantes de L- asparagina, D- glucosa, D- fructosa y potasio (denominando a la mezcla como AGFK) (Stewart KA *et al*., 2012). Además de los receptores se ha descrito la participación de GerD, una lipoproteína codificada por un gen monocistrónico, que aun cuando se desconoce su función, su ausencia disminuye las tasas de germinación vía dependiente de receptores (Korza G y Setlow P, 2013). Griffiths y colaboradores en el 2011 demostraron que los receptores como GerD están localizados en cluster en la membrana interna de esporas latentes de *B. subtilis*, esta organización facilita la cooperación entre receptores (GerB y GerK responden a una mezcla de germinantes), así como la interacción con otras proteínas (SpoVA). A este conjunto de receptores y GerD se le denominó germinosoma; además, se demostró que la formación de esta estructura es dependiente de GerD, indicando que el papel de esta proteína puede ser la organización del germinosoma.

La unión del germinante con su receptor dispara la liberación del ácido dipicolínico del centro de la espora, sin embargo, el mecanismo es desconocido. El DPA al liberarse activa eventos subsecuentes de la germinación, en primera instancia, el aumento del contenido de agua en el centro de la espora, lo que

conlleva a la reactivación del metabolismo, síntesis macromolecular y eventualmente el crecimiento vegetativo (Setlow P, 2003). La liberación de cationes seguido por la liberación de DPA y Ca^{+2} durante las etapas tempranas de la germinación sugiere que uno o más de estos canales son abiertos en la membrana interna de la espora después de la unión del germinante a su receptor. Desafortunadamente, se desconocen las proteínas implicadas en el movimiento de estos iones, sin embargo, recientes trabajos describen que proteínas codificadas por el operon spoVA están implicadas en el movimiento del DPA (Tovar-Rojo *et al*., 2002). Se han descrito otras proteínas que tienen un papel importante en el movimiento de solutos durante la germinación, las cuales son codificadas por el operón gerP. Este operón hexacistrónico es transcrito en la célula madre aproximadamente al mismo tiempo que la síntesis de DPA. En *B. cereus* y *B. subtilis*, la germinación no ocurre en esporas con mutaciones en genes que forman parte de gerP. En estas mismas esporas, al retirar la cubierta de la espora la germinación ocurre, estos datos sugieren que las proteínas gerP son requeridas en algún aspecto de la cubierta de la espora que es crucial para el acceso de los germinantes a sus propios receptores (Behravan *et al*., 2000).

En *B. subtilis* se han descrito dos enzimas CwlJ y SleB, que participan en la degradación de la corteza de peptidoglucano durante etapas tardías de la germinación (Chirakkal *et al.,* 2002; Boland *et al.,* 2000; Atrih *et al.,* 2001). Estas enzimas para su acción requieren de lactama-δ-murámico en el peptidoglucano para la hidrólisis de la corteza, asegurando que la capa de la espora germinante que carece de esta modificación no sea degradada durante la germinación y forme la capa de una nueva célula vegetativa (Popham, 2002). Ambas enzimas son sintetizadas durante la esporulación, CwlJ en la célula madre mientras que SleB en la preespora. En la espora madura, CwlJ es localizada en la cubierta mientras SleB en diferentes capas de la espora como la cubierta, membrana externa y la corteza. Todavía se desconocen aspectos de estas enzimas, por ejemplo, cómo permanecen en estado inactivo durante la latencia, de lo que se sugiere que para su activación necesitan de Ca^{2+}, el cual es liberado durante la primera etapa de la germinación (Paidhungat *et al*., 2001).

La germinación de esporas ha sido de interés porque la latencia y sus propiedades de extrema resistencia se pierden en los primeros minutos de la germinación. Debido a que las esporas de algunas especies de *Bacillus,* así como especies cercanas son responsables de contaminación de alimentos así como enfermedades relacionadas por el consumo de éstos, se ha enfocado el interés en descubrir la manera de cómo disparar la germinación de esporas en alimentos y subsecuentemente eliminar las esporas ya germinadas (con una menor resistencia) con tratamientos menos agresivos a base de calor u otros agentes físicos o químicos (Setlow P y Johnson EA, 2010).

La estrategia del estudio de la germinación ha llevado a enfocarse al inicio del proceso, particularmente en los receptores que reconocen germinantes, debido a la relación que existe entre la cantidad de receptores y las tasas de germinación: 1) en la ausencia de un receptor en particular la germinación con su respectivo germinante se inhibe (Atluri S *et al.,* 2006), 2) o en su caso, la elevación de los niveles de receptores incrementan la tasa de germinación con el germinante correspondiente (Cabrera-Martinez R-M *et al.,* 2003). Además, se ha establecido que una desregulación en el nivel de receptores está asociado con la aparición de esporas superlatentes; las cuales no germinan con nutrientes o lo hacen en tiempos prolongados en comparación con las esporas latentes y presentan elevada resistencia a calor, características que las convierten en un potencial problema de salud publica en la industria alimentaria (Ghosh S y Setlow P, 2009).

El estudio de la modulación de receptores, en particular de factores que pueden afectar la síntesis de los mismos, permitirá comprender la desregulación que existe en las poblaciones de esporas superlatentes y en última instancia establecer estrategias para la erradicación total de esporas en alimentos. Por lo que en este estudio, se determinó el efecto de la composición del medio y temperatura de esporulación, factores de transcripción, fosfatasas así como otras proteínas sobre la variación de los receptores que reconocen germinantes en las esporas de *B. subtilis*.

Para el estudio de la esporulación y germinación en *B. subtilis,* las esporas se obtienen a partir de condiciones óptimas de esporulación: medio enriquecido y una temperatura de 37˚C; sin embargo, a nivel ambiental, las esporas que contaminan alimentos o que ocasionan enfermedad son producidas por lo regular bajo condiciones subóptimas e incluso extremas; en este ámbito, varios estudios han demostrado que la composición del medio y la temperatura de esporulación determinan las características de las poblaciones de esporas producidas, considerando la modulación de la expresión genética como el mecanismo responsable que determina las características de las esporas, generando la hipótesis de una posible modulación de receptores que reconocen germinantes por factores ambientales. Por lo que en el capitulo I se evalúa el efecto de las condiciones de esporulación en la germinación y proteínas de germinación de esporas de *B. subtilis.*

En esporas obtenidas en medio mínimo, se observó una disminución en la germinación así como en la cantidad de receptores en la membrana interna de la espora. Una posible explicación puede estar relacionada al papel de los factores de transcripción, los cuales por estímulos externos pueden activar o reprimir genes. Basándonos en esta teoría, se determinó a partir de genes reporteros los niveles del factor de transcripción SpoVT, quien dirige la expresión de los receptores que reconocen germinantes, encontrándose niveles elevados de este factor de transcripción en esporas preparadas en medio mínimo, generando la hipótesis de un posible papel represor de SpoVT en la expresión de receptores germinantes y considerándose como un factor importante en la modulación de los mismos. Para comprobar esta hipótesis, en el capitulo II se evalúo el efecto de la proteína reguladora SpoVT en la germinación y los niveles de proteínas de germinación de esporas de *B. subtilis*.

SpoVT reprime la síntesis de receptores que reconocen germinantes; sin embargo, en esporas preparadas en medio mínimo, la ausencia de este factor transcripcional no restaura o iguala la germinación de estas esporas a los niveles observados en esporas obtenidas en medio enriquecido; por lo que se podría inferir que otras proteínas, incluidos otros factores de transcripción

pueden modular la expresión de receptores. En este último punto, se ha descrito que en fases tardías de esporulación además de la expresión de SpoVT, otros factores de transcripción modulan la síntesis de genes mediados por sigma G: dentro de estos factores se encuentra YlyA, proteína de unión a RNA polimerasa, regulada por sigma G y SpoVT, la cual por estudios de mutación se ha demostrado que su ausencia está relacionada con una disminución en la germinación. Las características de esta proteína, la convierten en un potencial modulador de la expresión de receptores, por lo cual en el capitulo III se describe a una proteína de unión a RNA polimerasa que modula la expresión de genes involucrados en la germinación de esporas de *B. subtilis.*

Por análisis bioinformático, se ha intentado identificar otras proteínas que podrían estar relacionadas en la germinación, modulando los receptores que reconocen germinantes, tomándose en cuenta que la expresión de estas proteínas debe ser en la espora y en etapas tardías de la esporulación. Para evaluar estas características, se buscan proteínas con sitios para unión de ribosomas (asegurando que la proteína se expresa) con secuencias que son reconocidas por factores sigma. En este último punto, proteínas con sitios para sigma F o G determinan que la expresión es en la espora y no en la célula madre. A partir de estas características mencionadas; se analizó en *B. subtilis y B. megaterium*, un grupo de proteínas en su conjunto denominadas como subunidades D y en el capitulo IV se describen como nuevas proteínas que modulan la germinación de esporas de especies de *B. subtilis.*

Continuando con el papel de nuevas proteínas en la regulación de la síntesis de receptores, se ha descrito a PrpE como una proteína con dominios característicos de fosfatasa y al conocer que la esporulación es un proceso regulado por mecanismos de fosforilación, PrpE podría regular la síntesis de los receptores en etapas tardías de la esporulación, por lo cual se evaluó en papel de esta proteína en la germinación y proteínas de germinación en esporas de *B. subtilis*. No se encontró relación entre esta fosfatasa con la modulación de receptores, sin embargo, se incluye la información como anexo II, con el fin de aportar nuevos datos en relación a la germinación.

Como se mencionó, los niveles de receptores en la membrana interna de la espora determina las tasas de germinación, en este sentido, para comprender el mecanismo por el cual se establece esta relación; se ha intentado describir proteínas que podrían estar asociadas a la posible vía de señalización de estos receptores o incluso alteraciones en la agrupación de los receptores en la membrana. En base a este último punto, se ha establecido que la agrupación en cluster de los receptores, es una estrategia para aumentar la eficiencia de la germinación, debido a que la cantidad de receptores en la membrana es muy baja; en relación a esta idea, se podría establecer una posible relación entre la modulación de los receptores con los cluster: en condiciones normales, se han identificado la formación de uno a dos cluster por espora, por lo cual una alteración de receptores ya sea por factores fisiológicos o genéticos, podría aumentar la cantidad de cluster que son formados en la membrana interna de la espora. Por lo que se consideró importante evaluar el efecto de condiciones de esporulación en la formación de cluster en la membrana interna de la espora. Aun cuando se determinó que no existe relación, los datos son incluidos como anexo III, debido a que son los primeros datos que se obtienen acerca de variación en el número de receptores y la formación de cluster.

Además, se ha establecido que la cantidad de receptores por espora determina la tasa de germinación por alta presión: las esporas con un elevado número de receptores germinan más rápido en comparación a poblaciones de esporas con una menor cantidad. En este aspecto, durante el estudio se generaron poblaciones de esporas en condiciones fisiológicas como genéticas diferentes y todas presentaron una variación en el número de receptores, por lo cual se consideró importante evaluar la germinación por alta presión en poblaciones de esporas que presentan variaciones en el número de receptores que reconocen germinantes. Los resultados se presentan en el anexo IV, brindando información que podría ser importante en la industria alimentaria, donde la alta presión se considera como un importante mecanismo de esterilización.

Capítulo I

Efecto de las condiciones de esporulación en la germinación y los niveles de proteínas de germinación de esporas de *Bacillus subtilis*

Applied and Environmental Microbiology

Effects of Sporulation Conditions on the Germination and Germination Protein Levels of Bacillus subtilis Spores

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Effects of Sporulation Conditions on the Germination and Germination Protein Levels of *Bacillus subtilis* Spores

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Bacillus subtilis **spores prepared in rich medium germinated faster with nutrient germinants than poor-medium spores as populations in liquid and multiple individual spores on a microscope slide. Poor-medium spores had longer average lag times between mixing of spores with nutrient germinants and initiation of Ca-dipicolinic acid (CaDPA) release. Rich-medium spores made at 37°C germinated slightly faster with nutrient germinants than 23°C spores in liquid, but not when spores germinated on a slide. The difference in germination characteristics of these spore populations in liquid was paralleled by changes in expression levels of a transcriptional *lacZ* fusion to the *gerA* operon, encoding a germinant receptor (GR). Levels of GR subunits were 3- to 8-fold lower in poor-medium spores than rich-medium spores and 1.6- to 2-fold lower in 23°C spores than 37°C spores, and levels of the auxiliary germination protein GerD were 3.5- to 4-fold lower in poor medium and 23°C spores. In contrast, levels of another likely germination protein, SpoVAD, were similar in all these spores. These different spores germinated similarly with CaDPA, and poor-medium and 23°C spores germinated faster than rich-medium and 37°C spores, respectively, with dodecylamine. Since spore germination with CaDPA and dodecylamine does not require GerD or GRs, these results indicate that determinants of rates of nutrient germination of spores prepared differently are primarily the levels of the GRs that bind nutrient germinants and trigger germination and secondarily the levels of GerD.**

Spores of *Bacillus* species are dormant and extremely resistant to a variety of harsh treatments, including extremes of heat and radiation as well as high levels of toxic chemicals (33, 34). As a consequence of their dormancy and resistance, such spores can survive for years in the absence of nutrients. However, given the proper stimulus, generally the presence of specific nutrient molecules termed germinants that are sensed by spores' specific germinant receptors (GRs), spores can rapidly return to active growth in the process of germination followed by outgrowth (26, 32, 33). GRs are synthesized only in the developing forespore late in sporulation and are located in spores' inner membrane. Generally spores of *Bacillus* species have multiple GRs, with three major ones in *Bacillus subtilis* spores termed GerA, GerB, and GerK, with each GR having three subunits, designated A, B, and C. Each of these GRs recognizes a different nutrient germinant or subset of germinants, with GerA alone responding to L-alanine or L-valine and with GerB and GerK both being required for germination with a mixture of L-asparagine, D-glucose, D-fructose, and K^+ (termed AGFK). Catabolism of the nutrients plays no role in the triggering of spore germination. Rather, the binding of a nutrient germinant to its cognate GR or GRs triggers the release of the spore core's huge depot (~20% of core dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]), although the mechanism of this process is not known. DPA release then triggers subsequent germination events leading to resumption of metabolism, macromolecular synthesis, and eventually vegetative growth. GR levels in spores are an important determinant of rates of nutrient germination, as the absence of a particular GR eliminates germination with the cognate nutrient germinant, while elevation of a GR's level increases rates of germination with the cognate germinant (1, 6, 32).

Spore germination has been of significant interest because spores' dormancy and in particular their extreme resistance properties are lost in the early minutes of germination. Since spores of a number of *Bacillus* species as well as their close relatives are responsible for much food spoilage and food-borne disease, there has long been interest in somehow triggering germination of spores in foodstuffs and subsequently killing the less-resistant germinated spores by relatively mild treatments with heat or other agents. Unfortunately, this strategy has proven difficult to apply successfully due to the significant heterogeneity in the germination of individual spores in populations, as not all spores in populations germinate rapidly, with some small percentage, termed superdormant spores, taking many hours or even days to germinate (10, 11, 35, 40, 42).

Spores that are used for model studies of germination are generally prepared in relatively rich sporulation media at optimal temperatures, as such conditions give high spore yields. However, the precise sporulation conditions, in particular sporulation temperature and medium composition, can significantly influence the properties of the spores produced (3, 5, 8, 14, 18, 19, 20, 29). In particular, there are a number of reports of alterations in spore germination properties as the sporulation medium is altered (8, 14, 29). In one such recent study, both the rates and efficiency of spore germination were greatly decreased when *Bacillus cereus* spores were prepared in a poor medium compared to a rich one (14). This is obviously of interest, since spores from environments that contaminate foodstuffs have most likely been formed under less-than-optimal conditions of temperature and medium composition. Thus, spores prepared under such suboptimal condi-

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tions might actually be better models for analysis of spore germination as it might be used in an applied setting. In addition, it would be of great interest to determine the reason(s) that sporulation temperature and medium composition alter the germination properties of the resultant spores, since such information could shed light on mechanisms determining rates of spore germination. Consequently, in this work we prepared spores of *Bacillus subtilis* strains in both rich and poor liquid media and on rich-medium plates at 23°C and 37°C and examined these spores' germination with a variety of germinants. The expression of GRs in these spores as well of two other proteins likely involved in spore germination, GerD and SpoVAD, was also measured, both by measuring β-galactosidase expression from a *lacZ* fusion to an operon encoding a GR and by Western blot analysis with specific antisera against GR subunits, GerD, and SpoVAD.

MATERIALS AND METHODS

Spore preparation and purification. The *B. subtilis* strains used in this work are the following isogenic derivatives of strain PS832, a prototrophic laboratory derivative of strain 168: (i) PS533 (31) (wild-type for spore germination), which contains plasmid pUB110, conferring resistance to kanamycin (10 μg/ml); (ii) FB10 (*gerBB**) (23), carrying a specific mutation in the *gerB* operon (encoding the GerB GR) such that the GerB variant, termed GerB*, triggers spore germination in response to L-asparagine alone; (iii) PS767 (*gerA-lacZ*) (9) (wild type for spore germination), which is PS832 transformed with plasmid AAM81, so that the resultant strain carries a transcriptional *lacZ* fusion to the promoter of the *gerA* operon (encoding the GerA GR) as well as the wild-type *gerA* operon and its wild-type promoter; (iv) PS4209 (*gerA-lacZ gerBB**), generated in the present work by transforming strain FB10 with plasmid AAM81 (9, 36); and (v) PS4217 (Δ*gerD gerD*-Flag) (28), which lacks the wild-type *gerD* gene but expresses a functional Flag-tagged GerD protein ([GerD-Flag]. Spores of these strains were prepared either (i) on rich 2× Schaeffer's medium-glucose (2× SG) medium agar plates at 23°C or 37°C, (ii) in 2× SG liquid medium (rich medium) at 37°C (21), or (iii) at 37°C in Spizizen's minimal liquid medium (poor medium), which contains only citrate, glucose, NH_4^+, and inorganic salts (21, 22, 36). Spores were harvested, purified, and stored as described previously (21), and all spore preparations used in this work were free (>98%) of growing or sporulating cells, germinated spores, and cell debris, as determined by phase-contrast microscopy.

Spore germination. *B. subtilis* spore germination was monitored by two methods, one for spore populations and another for simultaneous monitoring of multiple individual spores. Germination of spore populations was monitored by measurement of the release of DPA (present in spores as a 1:1 chelate with Ca^{2+} [CaDPA]) by its fluorescence with Tb^{3+} using a multiwell fluorescence plate reader as described previously (38–40). For nutrient germination, spore germination was preceded by a heat shock of 30 min at 70 or 75°C to maximally activate spores for germination (both temperatures gave identical results), followed by cooling on ice for ≥15 min so that subsequent spore germination was not at an elevated temperature. Germination conditions were as follows, and all incubation mixtures also contained 50 μM $TbCl_3$: (i) at 37°C with 10 mM L-valine in 25 mM K-HEPES buffer (pH 7.4); (ii) at 37°C with 10 mM L-asparagine–10 mM D-glucose–10 mM D-fructose–10 mM KCl (AGFK) in 25 mM K-HEPES buffer (pH 7.4); and (iii) at 37°C with 10 mM L-asparagine in 25 mM K-HEPES buffer (pH 7.4). In these experiments, aliquots were also examined on a slide by phase-contrast microscopy to distinguish phase-bright (dormant) and phase-dark (germinated) spores at the end of germination incubations to determine the percentage of spores that had germinated. In germination experiments in which rates of germination were determined by measuring rates of CaDPA release, the relative rates for different spore preparations were corrected for any slight differences in spore DPA content. Total spore DPA content was determined by boiling samples of dormant spores for 15 min, cooling on ice, centrifuging, and measuring DPA in the supernatant fluid by its fluorescence with Tb^{3+} (39). All rates of spore germination were determined at least in duplicate and on two independent spore preparations, and differences were analyzed for significance by a two-tailed Student's *t* test.

In addition to nutrient germinants, spore populations were also germinated with two nonnutrient germinants, CaDPA and dodecylamine, both of which trigger spore germination in a GR-independent manner (32). CaDPA germination was at 30°C with a 1:1 mixture of 60 mM $CaCl_2$ and 60 mM DPA made to pH 7.4 with dry Tris base and with spores at ~10^8/ml. The progress of CaDPA germination was monitored solely by phase-contrast microscopy. Germination with dodecylamine was at 45°C with 0.8 mM dodecylamine in 25 mM K-HEPES buffer (pH 7.4) plus 50 μM $TbCl_3$, and DPA release was monitored by Tb-DPA fluorescence in a multiwell fluorescence plate reader as described above (30, 40).

Measurement of the nutrient germination of multiple individual spores simultaneously also used heat-shocked spores and the nutrient germination conditions described above but without $TbCl_3$. However, the spores were adhered to a microscope slide, and the germination of hundreds of individual spores was monitored by differential interference contrast (DIC) microscopy as described previously (16, 42). This method allows determination of the time between germinant addition and the initiation of rapid CaDPA release (T_{lag}), the time between germinant addition and completion of rapid CaDPA release ($T_{release}$), and the time for the rapid release of >85% of a spore's CaDPA ($\Delta T_{release} = T_{release} - T_{lag}$), since release of CaDPA from spores during germination is accompanied by a parallel fall in DIC image intensity which accounts for ~70% of the total fall in an individual spore's image intensity that takes place in spore germination (16, 38, 42). The remaining 30% of the fall in intensity is due to hydrolysis of the spore cortex peptidoglycan (PG), accompanied by core water uptake and swelling, and takes place following CaDPA release. The precise period in which the latter fall in a spore's DIC image intensity takes place is defined as ΔT_{lysis} and is the time between $T_{release}$ and the end of the fall in a spore's DIC image intensity. Previous work has shown that by far the major difference in the germination of individual spores is variation in T_{lag} values (16, 38, 41), and this was also the case in the present work (see Results). Consequently, differences in rates of germination of spore populations measured by analysis of multiple individual spores were analyzed for significance by comparing average T_{lag} values by a two-tailed Student's *t* test.

β-Galactosidase assays. Assay of β-galactosidase in spores used 4-methylumbelliferyl-β-D-galactoside (MUG) as the substrate and measured generation of 4-methylumbelliferone fluorometrically essentially as described previously (9). In brief, purified spores were first decoated to render them sensitive to lysis by lysozyme and disrupted with lysozyme plus DNase I and brief sonication treatment to reduce the extract's viscosity. Following centrifugation, the supernatant fluid was assayed for β-galactosidase using MUG as described previously (9). β-Galactosidase specific activities are expressed in arbitrary fluorescence units, and these were calculated with respect to the amount of DPA in various spore preparations. Analyses have shown that DPA levels are essentially identical in *B. subtilis* spores made on 2× SG medium plates at 23 and 37°C (18) and in rich and poor liquid media (data not shown). Assays of β-galactosidase in spores were carried out in duplicate on two independent spore preparations, and differences were analyzed for significance by a two-tailed Student's *t* test.

Isolation of superdormant spores. Superdormant spores were isolated from spore preparations made in rich and poor sporulation media by germination of 125 mg (dry weight) of spores for 5 h at 37°C in L-valine (10 mM) or AGFK (10 mM concentrations of each component) but without $TbCl_3$ as described above and previously (10, 11). Germination was followed by centrifugation to concentrate the spores, removal of germinated spores by buoyant density gradient centrifugation, germination of the remaining spores again for 5 h, and removal of any germinated spores again by buoyant density gradient centrifugation.

Antibody production and purification. The preparation of rabbit antisera against the *B. subtilis* GerBC and SpoVAD proteins was described previously, as was the source of the anti-Flag antiserum and all secondary antisera (17, 28, 37). For production of antisera against the *B. subtilis* GerAA, GerAC, and GerKA proteins and the *Geobacillus stearothermophilus* GerD protein, truncated *gerAA*, *gerAC*, *gerKA*, and *gerD* genes were amplified by PCR using genomic DNA from *B. subtilis* strain PS832 or *G. stearothermophilus* strain ATCC 7953 as the template; the 5′ primers introduced a NotI site, and the 3′ primers introduced a KpnI site. The *gerAA* and *gerKA* PCR products were cloned into a modified pET15b vector containing a His_6 tag and a tobacco etch virus (TEV) protease cleavage site, and the *gerAC* and *gerD* PCR products were cloned into a modified pGEX plasmid containing a TEV protease cleavage site between an N-terminal glutathione *S*-transferase tag and the target gene. The GerAA protein (residues 2 to 239), GerAC protein (residues 20 to 373), GerKA protein (residues 39 to 276), and GerD protein (residues 60 to 180) were expressed in *Escherichia coli* BL21 Star (DE3) (Invitrogen, Grand Island, NY) by induction with 1 mM isopropyl-β-D-thiogalactoside at 21°C for 16 h. The GerAA protein was soluble and was purified by nickel-nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography under native conditions followed by TEV protease cleavage to remove the His_6 tag, and subsequent cation exchange and gel filtration (GE Healthcare, Piscataway, NJ) chromatography. The GerKA protein was insoluble and was purified by Ni^{2+}-NTA affinity chromatography under denaturing conditions using 8 M urea in 100 mM sodium phosphate, 10 mM Tris-HCl (pH 8.0) as the solubilization buffer, the same buffer but at pH 6.3 as wash buffer, and finally the same buffer at pH 4.5 as the elution buffer. The GerD and GerAC proteins were soluble and were purified by glutathione affinity chromatography followed by TEV protease cleavage and cation exchange and gel filtration chromatography.

The purified proteins were dialyzed against PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.2), adjusted to a concentration of 1 mg/ml in PBS, and submitted for polyclonal antibody production in rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA). The GerAA, GerAC, and GerD proteins were supplied in solution and the GerKA protein as a suspension. The antibodies were detected in a bleed 2 months after the initial injection, and the GerAC and GerD antisera were used without further treatment, while the GerAA and GerKA antisera were affinity purified using a Pierce AminoLink Plus immobilization kit (Thermo Fisher Scientific, Rockford, IL) as per the supplier's instructions. Briefly, 1 mg GerAA protein was added to an AminoLink Plus resin column in 0.1 M sodium citrate, 0.05 M sodium carbonate coupling buffer (pH 10), or 1 mg GerKA protein was added in the same buffer also containing 4 M urea. The columns were rocked at room temperature for 4 h resulting in the formation of semistable Schiff base bonds, washed with 4 ml PBS, reduced with sodium cyanoborohydride overnight at 4°C followed by quenching with 1 M Tris-HCl (pH 7.4), resulting in stable secondary amine bonds. The stable antigen columns were washed with 10 ml 1 M NaCl and equilibrated in PBS, and then 2 ml of antiserum was added. After mixing by rocking for 1 h at room temperature, the columns were washed with 8 ml PBS. Bound antibody was eluted with 2 ml 0.2 M glycine·HCl (pH 2.5) into a tube containing 100 μl of 1 M Tris-HCl (pH 8.9) neutralizing buffer. The purified antisera were finally dialyzed against PBS and used for Western blot analysis. The antiserum against *G. stearothermophilus* GerD was used for Western blot analysis of *B. subtilis* GerD, because the anti-*G. stearothermophilus* GerD serum had become available in the laboratory and cross-reacted reasonably well with *B. subtilis* GerD.

Determination of GR subunit, GerD and SpoVAD levels in spores. Levels of various GR subunits in spores were determined by Western blot analysis of equal aliquots of spores' inner membrane proteins, since GRs, GerD, and SpoVAD are located in the spore's inner membrane (15, 25, 26, 32). The binding of the antisera to proteins on Western blots was detected with horseradish peroxidase coupled to goat anti-rabbit IgG and binding of the secondary antibody was detected by chemiluminescence as described previously (16). To quantitate differences in GR levels between spores prepared under different conditions, the intensities given by different amounts of appropriate inner membrane samples in Western blot analysis were compared on the same blot (see below). The GerD-Flag, GerD, and SpoVAD proteins were also detected by Western blot analysis on inner membrane samples using antiserum against the Flag tag, GerD, or SpoVAD as described above and previously (28, 37). In a number of cases, blots were stripped and then reprobed with a different antiserum.

For all Western blot analyses, since recoveries of the inner membrane fraction could well vary between spore preparations, a graded series of aliquots from inner membrane fractions that were to be compared were first run on denaturing polyacrylamide gel electrophoresis, the gels were stained with Coomassie blue, and equivalent amounts of inner membrane protein were determined by inspection of the stained gels and further similar analyses if needed. This allowed comparison of levels of GR subunits, GerD-Flag, GerD, and SpoVAD in samples with Western blots prepared with equivalent amounts of inner membrane protein. All analyses of levels of various spore germination proteins were carried out in duplicate on two independent spore preparations, and the variation in protein levels between different determinations was ≤15%. Differences in germination protein levels in spores made in the rich and poor medium and at 37 and 23°C were analyzed for significance using a two-tailed Student's *t* test.

RESULTS

Nutrient germination of spores prepared under different conditions. To begin to analyze the germination and other properties of spores prepared under different conditions, we chose to use strains carrying a *gerA-lacZ* fusion, since this would allow an eventual estimation of the expression of the *gerA* operon encoding the GerA GR in developing spores by measuring spore β-galactosidase levels (see below). We used two strains, a wild-type strain carrying *gerA-lacZ* (strain PS767) and a similar strain with a modified GerB GR, termed GerB*, that responds to L-asparagine alone (strain PS4209). These strains were sporulated either on rich-medium plates at 23 or 37°C (23°C and 37°C spores, respectively) or at 37°C in either a rich or poor liquid medium (rich-medium and poor-medium spores, respectively). Sporulation efficiencies were similar (±20%) under all these conditions as determined by microscopic examination (data not shown), although sporulation took about twice as long at 23 as at 37°C and cell growth was ~3-fold lower in the poor medium, which thus gave lower yields of spores.

The germination of these spores was monitored with a number of different nutrient germinants, using levels of these germinants well above those needed to saturate the relevant GRs. L-Valine was used to activate the GerA GR; the AGFK mixture was used to activate the GerB and GerK GRs together (32), since both of these receptors are needed for AGFK germination of wild-type spores; and L-asparagine was used to activate the GerB* GR. When germination of spore populations in liquid with nutrient germinants was assessed by monitoring CaDPA release, the rich-medium spores germinated better than the poor-medium spores, in particular with AGFK (Tables 1 and 2). Nutrient germination of populations of 23°C spores was also poorer than that of 37°C spores (Tables 1 and 2).

It was clear from the results noted above that sporulation conditions, in particular the medium composition, had significant effects on the rate of germination of spore populations. However, it was not clear which step in the germination process was affected, whether it was GR triggering or the CaDPA release process itself. To attempt to answer this question, the germination of multiple

TABLE 1 Rates of spore germination and levels of β-galactosidase in *B. subtilis* PS767 (*gerA-lacZ*) spores prepared under different conditions[a]

Sporulation condition	β-Galactosidase sp act[b]	Germination rate (RFU/min)[c]		Germination in 2 h (%)[d]	
		AGFK	Val	AGFK	Val
Rich liquid medium at 37°C[e]	1.1×10^5	77	245	68	97
Poor liquid medium at 37°C[e]	3.8×10^4	2	21	2	27
Rich-medium plates at 37°C[f]	9.1×10^4	83	157	70	99
Rich-medium plates at 23°C[f]	5.2×10^4	57	89	52	36

[a] Spores of strain PS767 (*gerA-lacZ*) were prepared under various condition and, purified, spore β-galactosidase specific activity was measured, spores were germinated, and germination rates and extents were measured as described in Materials and Methods. All values reported are averages of at least duplicate measurements with two independent spore preparations. Standard deviations for all values were <15%.
[b] Values for β-galactosidase specific activity from PS533 spores that do not carry a *lacZ* fusion were ≤5% of those in spores made in rich liquid medium.
[c] Values are given as relative fluorescence units (RFU) of DPA released/min and were determined as described in Materials and Methods.
[d] Values were determined by phase-contrast microscopy after 2 h of germination as described in Materials and Methods.
[e] The differences in values for spores made in rich and poor medium are highly significant ($P \leq 0.001$).
[f] The differences in values for spores made at 37 and 23°C are highly significant ($P \leq 0.001$).

TABLE 2 Rates of spore germination and levels of β-galactosidase in *B. subtilis* PS4209 (*gerA-lacZ gerBB**) spores prepared under different conditions[a]

Sporulation condition	β-Galactosidase sp act[b]	Germination rate (RFU/min)[c]		% germination in 2 h[d]	
		Asn	Val	Asn	Val
Rich liquid medium at 37°C[e]	6.2×10^4	220	200	100	100
Poor liquid medium at 37°C[e]	1.4×10^4	50	63	31	26
Rich-medium plates at 37°C[f]	6.2×10^4	100	134	100	100
Rich-medium plates at 23°C[f]	5.5×10^4	67	83	86	95

[a] Spores of strain PS4209 (*gerA-lacZ gerBB**) were prepared under various conditions and purified, spore β-galactosidase specific activity was measured, spores were germinated, and germination rates and extents were measured as described in Materials and Methods. All values are averages of at least duplicate measurements with two independent spore preparations. Standard deviations for all values were <15%.
[b] Values of β-galactosidase specific activity from PS533 spores that do not carry a *lacZ* fusion were ≤5% of those in spores made in rich liquid medium.
[c] Values are given as relative fluorescence units (RFU) of DPA released/min and were determined as described in Materials and Methods.
[d] Values were determined by phase-contrast microscopy after 2 h of germination as described in Materials and Methods.
[e] The differences in values for spores made in rich and poor medium are highly significant ($P \leq 0.001$).
[f] The differences in values for spores made at 37 and 23°C are highly significant ($P \leq 0.001$) for L-asparagine germination by microscopy and the rates of valine germination, marginally significant ($P \leq 0.06$) for rates of L-asparagine germination and valine germination by microscopy), and not significant ($P = 0.11$) for β-galactosidase specific activities.

individual spores adhered to a microscope slide was monitored by DIC microscopy (16, 42). This method can determine the lag time following germinant addition and prior to fast CaDPA release (T_{lag}), the time for the fast release of the great majority of a spore's CaDPA ($\Delta T_{release}$), which begins at T_{lag} and ends at $T_{release}$, and the time for spore cortex PG hydrolysis following CaDPA release (ΔT_{lysis}) (16, 42). This analysis showed that poor-medium spores of either strain PS767 or FB10 had 3- to 9-fold-longer average T_{lag} with nutrient germinants than rich-medium spores (Tables 3 and 4). However, the average ΔT_{lysis} were at most only 2-fold longer for the poor-medium spores than for the rich-medium spores, while the average $\Delta T_{release}$ differed ≤1.4-fold (Tables 3 and 4). It was surprising that the AGFK germination of the poor-medium PS767 spores was more efficient with individual spores adhered to a microscope slide than with spores suspended in liquid (compare Tables 1 and 3). We do not know the reason for this difference, although differences in the germination of spores adhered to a slide and in liquid have been seen previously, with the spores adhered to the slide germinating more efficiently (42).

In contrast to the slower nutrient germination of individual poor-medium spores, the effects of sporulation temperature on the germination of individual spores prepared on rich-medium plates were much smaller (Tables 3 and 4). Not only were the differences in $\Delta T_{release}$ between 23 and 37°C spores minimal, but the differences in T_{lag} and ΔT_{lysis} were minimal also. However, the individual 23°C spores germinated as well as or even faster than the 37°C spores when adhered to a slide (compare Tables 1 and 2 with Tables 3 and 4). As noted above, the poor-medium PS767 spores also exhibited better AGFK germination when adhered to a slide than suspended in liquid.

Nonnutrient germination of spores prepared under different conditions. There are a variety of reasons that spores made under different conditions would exhibit different rates of germination with nutrient germinants. However, one likely possibility is that levels of proteins essential for nutrient germination, including GRs and GerD, vary significantly in spores prepared differently, since GR levels in particular are known to affect rates of spores' germination with nutrients and GerD is also required for efficient GR-dependent germination (1, 6, 24, 27). To test if it is differences in levels of GRs or GerD that are responsible for different rates of germination of spores prepared differently, we first examined the germination of these spores with two nonnutrient germinants, CaDPA and dodecylamine, neither of which requires GRs or GerD to trigger spore germination (30, 32). Strikingly, the CaDPA germination of poor-medium spores was only slightly slower than that of rich-medium spores, while 23 and 37°C spores had identical CaDPA germination rates (Table 5). In addition, the dodecylamine germination of 23°C and poor-medium spores was actually faster than that of 37°C and rich-medium spores, respectively (Table 5), the opposite of the results with nutrient germination. The faster dodecylamine germination of 23°C spores has been seen previously and has been ascribed to differences in the inner membrane structure or composition of spores made at different temperatures (18).

Levels of GerA-LacZ in spores prepared under different conditions. That rates of CaDPA germination of spores made at different temperatures and in rich or poor media were essentially identical, and that dodecylamine germination was faster with 23°C and poor-medium spores, was striking, since these results were in contrast to results obtained when nutrient germination was measured. Since CaDPA and dodecylamine germination do not require GRs or GerD, this suggests that differences in rates of nutrient germination of spores prepared under these different

TABLE 3 Kinetic parameters of L-valine and AGFK germination of multiple individual PS767 (*gerA-lacZ*) *B. subtilis* spores prepared under different conditions[a]

Spore prepn and germinant	Time (min)				No. of spores		Observation period (min)
	T_{lag}	$T_{release}$	$\Delta T_{release}$	ΔT_{lysis}	Examined (% germinated)	Counted	
Rich liquid medium at 37°C							
10 mM L-Val[b]	12.5 ± 15.3	15.6 ± 17.7	3.2 ± 4.1	7.1 ± 2.1	329 (95)	93	120
10 mM AGFK[b]	6.6 ± 6.2	9.7 ± 6.8	3.0 ± 1.5	5.0 ± 2.7	432 (99)	90	90
Poor liquid medium at 37°C							
10 mM L-Val[b]	37.4 ± 33.4	41.0 ± 34.0	3.5 ± 1.6	8.3 ± 6.0	613 (47)	86	120
10 mM AGFK[b]	26.6 ± 23.0	30.4 ± 23.2	3.8 ± 1.6	5.9 ± 1.0	488 (37)	51	90
Rich-medium plates at 37°C							
10 mM L-Val[c]	7.2 ± 7.5	11.0 ± 8.3	3.8 ± 1.8	4.6 ± 1.9	404 (99)	97	120
10 mM AGFK[d]	6.6 ± 5.0	11.1 ± 6.9	4.5 ± 2.3	3.6 ± 1.7	329 (99)	91	90
Rich-medium plates at 23°C							
10 mM L-Val[c]	7.5 ± 11.6	11.5 ± 12.1	4.0 ± 2.0	5.9 ± 3.2	285 (96)	103	120
10 mM AGFK[d]	4.9 ± 5.5	8.9 ± 6.4	4.0 ± 2.5	4.3 ± 2.3	273 (99)	93	90

[a] Spores of *B. subtilis* strain PS767 were prepared under different conditions and the kinetic parameters of their germination were determined as described in Materials and Methods. All values for T_{lag}, $T_{release}$, $\Delta T_{release}$, and ΔT_{lysis} are means ± standard deviations.
[b] The differences in T_{lag} values between spores made in rich and poor media and germinating with either L-valine or AGFK are highly significant ($P < 0.001$).
[c] The differences in T_{lag} values between spores made at 37 and 23°C and germinating with L-valine are not significant ($P > 0.8$).
[d] The differences in T_{lag} values between spores made at 37 and 23°C and germinating with AGFK are slightly significant ($P < 0.05$).

conditions are due to differences in spore levels of GRs or GerD. To begin to critically examine this suggestion, the levels of β-galactosidase from a transcriptional *gerA-lacZ* fusion were determined in spores made under different conditions. Strikingly, levels of GerA-LacZ were ~3-fold lower in poor-medium spores, and levels of GerA-LacZ were slightly lower in 23°C spores (Tables 1 and 2). These differences were in the direction expected if differences in the expression of genes encoding GRs are responsible for

TABLE 4 Kinetic parameters of the germination of multiple individual FB10 (*gerBB**) *B. subtilis* spores prepared under different conditions[a]

Spore prepn and germinant	Time (min)				No. of spores		Observation period (min)
	T_{lag}	$T_{release}$	$\Delta T_{release}$	ΔT_{lysis}	Examined (% germinated)	Counted	
Rich liquid medium at 37°C							
10 mM L-Asn[b]	7.2 ± 10.0	10.6 ± 10.4	3.3 ± 1.0	6.6 ± 2.3	316 (96)	94	60
10 mM L-Val[b]	6.3 ± 7.5	9.6 ± 7.8	3.3 ± 1.1	7.1 ± 2.6	653 (97)	114	60
Poor liquid medium at 37°C							
10 mM L-Asn[b]	56.6 ± 53.6	60.0 ± 53.3	3.5 ± 1.6	14.0 ± 5.9	624 (23)	62	180
10 mM L-Val[b]	34.5 ± 34.7	38.3 ± 34.4	3.7 ± 1.8	12.5 ± 9.0	673 (15)	54	180
Rich-medium plates at 37°C							
10 mM L-Asn[c]	4.8 ± 3.1	7.5 ± 3.4	2.7 ± 0.8	7.8 ± 1.7	344 (100)	104	60
10 mM L-Val[d]	6.0 ± 4.2	8.5 ± 4.7	2.5 ± 0.9	8.5 ± 4.7	374 (99)	109	60
Rich-medium plates at 23°C							
10 mM L-Asn[c]	3.8 ± 3.5	7.5 ± 3.8	3.8 ± 1.5	7.2 ± 1.7	320 (100)	96	60
10 mM L-Val[d]	4.2 ± 4.2	7.9 ± 4.5	3.7 ± 1.5	6.2 ± 1.5	447 (99)	105	60

[a] Spores of *B. subtilis* strain FB10 were prepared under different conditions and the kinetic parameters of their germination were determined as described in Materials and Methods. All values for T_{lag}, $T_{release}$, $\Delta T_{release}$, and ΔT_{lysis} are means ± standard deviations.
[b] The differences in T_{lag} values between spores made in rich and poor media and germinating with either L-asparagine or L-valine are highly significant ($P < 0.001$).
[c] The difference in T_{lag} values between spores made at 37 and 23°C and germinating with L-asparagine are not significant ($P > 0.1$).
[d] The difference in T_{lag} values between spores made at 37 and 23°C and germinating in L-valine are highly significant ($P < 0.002$).

TABLE 5 Rates of CaDPA and dodecylamine germination of spores prepared under different conditions[a]

Sporulation condition	Relative % germination as a function of time	
	CaDPA	Dodecylamine
Rich liquid medium at 37°C	100[b]	15
Poor liquid medium at 37°C	85	50
Rich-medium plates at 37°C	75	20
Rich-medium plates at 23°C	75	100[c]

[a] Spores of strain PS767 (*gerA-lacZ*) were prepared under different conditions, purified spores were germinated, and germination rates were measured as described in Materials and Methods. All values are averages of duplicate measurements with two independent spore preparations, and standard deviations for all values were <15%.
[b] This value was set at 100 and denotes 100% germination in 8 h.
[c] This value was set at 100 and denotes 100% germination in 10 min.

the lower germination of spores, in particular 23°C and poor-medium spore populations.

Levels of GRs in spores made under different conditions. The general correlation between levels of *gerA*-driven β-galactosidase and the L-valine germination rates of spores made under different conditions was certainly consistent with differences in levels of GRs being a major reason for the differences in rates of germination of these spores. However, levels of β-galactosidase from *gerA-lacZ* may not be a precise reflection of levels of the GerA GR in spores or levels of the GerB, GerB*, and GerK GRs. Consequently, we carried out Western blot analysis of spore inner membrane proteins using a variety of antisera against GR subunits. These antisera exhibited no detectable cross-reaction with subunits from other GRs (data not shown), which is perhaps not surprising since there is generally <40% sequence identity between comparable subunits from the *B. subtilis* GerA, GerB, and GerK GRs. In all cases, the identities of GR subunit bands on Western blots were determined by their appropriate molecular weight, and their absence from the inner membrane fraction of spores carrying a deletion of the gene encoding the appropriate GR (15, 25) (data not shown). This Western blot analysis showed that poor-medium PS767 spores (*gerA-lacZ*) had lower levels of GerAA, GerAC, GerBC, and GerKA than the rich-medium spores (Fig. 1). Similarly, 23°C spores had slightly lower levels of these GR subunits than 37°C spores (Fig. 2). Quantitation of these differences (Table 6), showed that levels of the four GR subunits were 3- to 8-fold lower in poor-medium spores while 23°C spores had 1.6- to 2-fold-lower levels of GR subunits, and all of these differences were significant, with *P* values being below 0.05 and most being much lower.

Levels of GerD and SpoVAD in spores made under different conditions. In addition to GR subunits, there are other proteins that are important in spore germination. These proteins include GerD and the proteins encoded by the *spoVA* operon, which are essential for DPA uptake in sporulation and probably CaDPA release during germination as well (26, 27, 32, 38). Since these proteins are also almost certainly in the spore's inner membrane (12, 15, 25, 28, 37), the levels of GerD as well as a functional Flag-tagged GerD protein (note that this is the only GerD protein present in these spores [28]) and SpoVAD were determined by Western blot analysis of inner membrane fractions from PS4217 spores (Δ*gerD gerD*-Flag) prepared under different conditions (Fig. 3 and Table 6; also data not shown). In contrast to results obtained when GR subunit levels were measured in inner membranes of

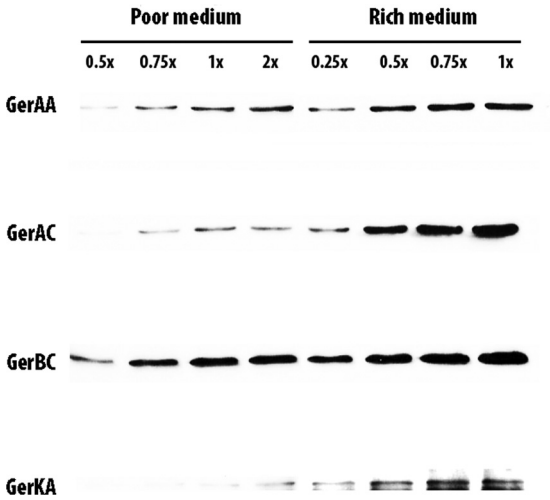

FIG 1 Levels of GR subunits in rich- and poor-medium spores. Rich- and poor-medium PS767 (*gerA-lacZ*) spores were prepared and purified, the inner membrane fractions were isolated, and aliquots were analyzed by Western blotting using various antisera as described in Materials and Methods. The values above the lanes refer to the amounts of inner membrane fractions run in the lanes. The 1× samples are from ~1 mg (dry weight) of spores, and the amounts of membrane protein in the 1× lanes from spores prepared differently were made essentially equal as described in Materials and Methods. The GerAA, GerAC, and GerBC lanes are all from the same blot, which was stripped and reprobed, while the GerKA lanes are from a separate blot.

spores prepared differently, levels of SpoVAD were at most very slightly higher in poor-medium and 23°C spores than in rich-medium and 37°C spores, respectively, and similar results were obtained with PS767 (*gerA-lacZ*) spores made under these different conditions; these slight differences were not significant (Table

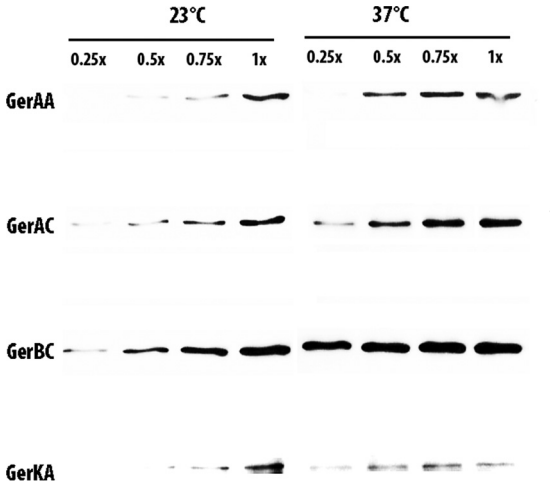

FIG 2 Levels of GR subunits in 23 and 37°C spores. The 23 and 37°C spores of strain PS767 (*gerA-lacZ*) were prepared and purified, the inner membrane fractions were isolated, and aliquots were analyzed by Western blotting using various antisera as described in Materials and Methods. The values above the lanes refer to the amounts of inner membrane fractions run in the lanes. The 1× samples are from ~1 mg (dry weight) of spores, and the amounts of membrane protein in the 1× lanes from spores prepared differently were made essentially equal as described in Materials and Methods. The GerAC, GerBC, and GerKA lanes are all from the same blot, which was stripped and reprobed, while the GerAA lanes are from a separate blot.

TABLE 6 Levels of GR subunits, GerD, and SpoVAD in spores prepared under different conditions[a]

Protein	Relative protein levels (arbitrary units) during sporulation			
	Rich medium[b]	Poor medium	37°C[c]	23°C
GerAA[d]	1.0	0.33	1.0	0.6
GerAC[e]	1.0	0.13	1.0	0.5
GerBC[e]	1.0	0.3	1.0	0.5
GerKA[e]	1.0	0.13	1.0	0.5
GerD[e,f]	1.0	0.3	1.0	0.25
SpoVAD[g]	1.0	1.2, 1.0	1.0	1.2, 1.0

[a] Spores of strains PS767 (*gerA-lacZ*) and PS4217 (Δ*gerD gerD*-Flag) were prepared in rich or poor liquid medium or on rich-medium plates at 23 or 37°C and purified, inner membrane fractions were isolated, and levels of various GR subunits, GerD, GerD-Flag, and SpoVAD were determined by Western blot analysis as described in Materials and Methods and shown in Fig. 1 to 3. All values are averages of at least duplicate determinations on two independent spore preparations, with differences between duplicates and different spore preparations being <15%. Values for GerAA, GerAC, GerBC, and GerKA are from PS767 spores. Values for GerD are from PS4217 spores, and the two values for SpoVAD levels in poor medium and 23°C spores are from PS4217 spores (first value) and PS767 spores (second value).
[b] Values for protein levels in spores made in the rich liquid medium were each set at 1.0.
[c] Values for protein levels in spores made at 37°C on plates were each set at 1.0.
[d] The difference between GerAA levels was significant ($P < 0.05$) for spores made in the rich and poor medium, but not significant ($P > 0.15$) for spores made at 37 and 23°C.
[e] The differences between this protein's level in spores made in rich and poor media or at 23 and 37°C are significant ($P < 0.05$) to highly significant ($P < 0.001$).
[f] Use of the anti-Flag serum to detect GerD-Flag gave essentially identical results.
[g] The differences between SpoVAD levels in spores made in rich and poor media or at 37 and 23°C were not significant.

6 and data not shown). However, levels of GerD and GerD-Flag were ~4-fold lower in poor-medium and 23°C spores than in rich-medium and 37°C spores, respectively (Fig. 3 and Table 6; also data not shown).

Amounts of superdormant spores in spore populations made in rich and poor media. The data above strongly suggested that the differences in rates of germination of spores made under different conditions, in particular in rich or poor media, were due largely to differences in GR or GerD levels, or levels of both proteins. Previous work has suggested that the amount of superdormant spores in spore populations is determined largely by that spore population's GR level, although effects of GerD levels on levels of superdormant spores have not been measured (10). The findings that have led to this suggestion are that spore populations with elevated average GR levels exhibit lower levels of superdormant spores, while spores lacking GRs exhibit minimal if any germination with all nutrient germinants (10, 24). However, spores with intermediate levels of GRs have not been examined for levels of superdormant forms. An obvious prediction from this previous work and the low levels of GRs in poor-medium spores is that these latter spores' populations would have an elevated percentage of superdormant spores. Consequently, we determined the levels of superdormant spores prepared using L-valine or AGFK in rich- and poor-medium PS533 spore populations. Similar to what has been found previously (10, 11), only a small percentage of rich-medium spores were superdormant following AGFK or L-valine germination (0.5% and 0.3% superdormant spores, respectively). However, 4- to 12-fold more of the poor-medium spores were superdormant for AGFK or L-valine germination (2.1% and 3.6% superdormant spores, respectively).

DISCUSSION

As seen in a number of previous studies of spores of *Bacillus* species (3, 8, 14, 18, 29), the present work also found that *B. subtilis* spores prepared under different conditions exhibited different germination kinetics with nutrient germinants. This was most pronounced for rich- and poor-medium spores, with the poor-medium spores germinating more slowly, and was seen with both spore populations and multiple individual spores. Germination of 23°C spore populations was also found to be slower than that of 37°C spore populations with nutrient germinants, but this difference was not seen when germination of multiple individual spores was examined. The reason for the difference in the germination of spores made at different temperatures with spore populations in liquid compared to multiple individual spores adhered to a microscope slide is not clear. However, poor-medium spores also germinated better adhered to a microscope slide with AGFK. Significant differences in the nutrient germination of spores germinated in liquid (populations) and adhered to a microscope slide (individual spores) have been seen previously (42), but the reason for this puzzling behavior is not clear.

Given that significant differences were seen between the nutrient germination of spores made under different conditions, the obvious question is that of what is responsible for these differences. Changes in levels of a number of proteins might be expected to alter spore germination significantly, including GRs, GerD, SpoVA proteins, and the redundant cortex-lytic enzymes (CLEs) CwlJ and SleB (25, 31). There were no significant differences in the levels of one SpoVA protein in spores prepared under different conditions, and thus the SpoVA proteins' levels seem unlikely to be responsible for the changes in spore germination with these spores. In addition, since CaDPA germination of rich-medium, poor-medium, 23°C, and 37°C spores was quite similar, it is likely that there is no notable change in CwlJ levels in these spores, since CaDPA triggers spore germination by activating CwlJ (32). That there is no major decrease in CwlJ levels in these different spores is further suggested by their very similar $\Delta T_{release}$ values for nutrient

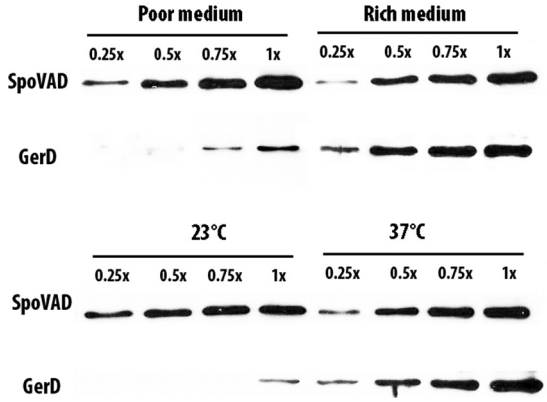

FIG 3 Levels of GerD and SpoVAD in spores prepared under different conditions. PS4217 spores (Δ*gerD gerD*-Flag) were prepared under different conditions, spores were purified, the inner membrane fractions were isolated, and aliquots were analyzed by Western blotting using various antisera as described in Materials and Methods. The values above the lanes refer to the amounts of inner membrane fractions run in the lanes. The 1× samples are from ~1 mg (dry weight) of spores, and the amounts of membrane protein in the 1× lanes from spores prepared differently were made essentially equal as described in Materials and Methods.

germination, since loss of CwlJ increases $\Delta T_{release}$ values ~10-fold (41, 42). There were, however, some small differences in average ΔT_{lysis} values for individual spores prepared under different conditions. While these differences could be due to changes in levels of CLEs, it is also possible that these spores have different cortex PG structures. Indeed, major alterations in cortex PG structure have significant effects on rates of nutrient germination, including both decreasing average T_{lag} and increasing $\Delta T_{release}$ (43). However, the effects of the different sporulation conditions used in the present work on average $\Delta T_{release}$ values were minimal (Tables 3 and 4). Indeed, while effects of sporulation medium richness on cortical PG structure have not been studied, at least different sporulation temperatures do not alter spore cortex PG structure notably (18).

While changes in levels of SpoVA proteins and CLEs seem unlikely to be responsible for the changes in nutrient germination of spores prepared under different conditions, the results in this work are certainly consistent with altered levels of GRs being a major factor in these changes in germination rates. Thus, spores prepared under different conditions that exhibited slower GR-dependent germination exhibited essentially identical germination with CaDPA, a GR-independent process, and faster germination with dodecylamine, again a GR-independent process (30, 32). More importantly, levels of a number of GR subunits and β-galactosidase levels from a *gerA-lacZ* fusion were significantly lower in spores that germinated more slowly, in particular in poor-medium spores. Given that all three of a GR's subunits are required for that GR's function (32), these data suggest that all GR function is greatly reduced in poor-medium spores. The present work is thus the first demonstration that significantly lower GR levels are correlated with lower rates of spores' nutrient germination. However, the correlation between rates of nutrient germination and GR levels is not linear, as an ~200-fold elevation in the GerBA level in *gerBB** spores gives only an ~3-fold elevation in the maximum spore germination rate with L-asparagine (1, 6), while 1.4- to 2-fold decreases in GR levels in 23°C spores compared to 37°C spores had only minimal effects on the rates of germination of multiple individual spores.

While it seems likely that lower GR levels play a significant role in determining lower rates of spore germination in spores prepared differently, in particular in rich or poor media, there are additional factors that could modulate rates of spore germination. One obvious possibility is the level of the GerD protein that is also essential for rapid GR-dependent spore germination (27) and is ~4-fold lower in 23°C and poor-medium spores. Indeed, the absence of GerD is known to increase average T_{lag} values for GR-dependent germination markedly and with no effect on average $\Delta T_{release}$ values (38). However, overexpression of GerD has no effect on rates of GR-dependent spore germination (27). In addition, the ~4-fold decrease in GerD levels in 23°C spores had less effect on the efficiency of spore germination than the 3.3-fold decrease in GerD levels in poor-medium spores. Given that GR levels were reduced much more in poor-medium spores than in 23°C spores, this suggests that GR levels are more important than GerD levels in determining overall rates and efficiency of spore germination with nutrients. Perhaps GerD is normally in excess in spores such that a 4-fold decrease in GerD level alone causes only a small decrease in GR-dependent germination. In order to critically examine this possibility, the effects on nutrient germination of decreasing GerD levels will need to be determined under conditions where there are no changes in GR levels.

Another possible factor in the differences in the altered germination of spores prepared differently could be levels of the spore coat GerP proteins that appear to be required for normal access of nutrient germinants to GRs in the spore's inner membrane (4, 7). Indeed, different sporulation temperatures result in altered profiles of spore coat proteins (18). However, the GerP proteins have also been reported to be essential for normal CaDPA germination, at least with *Bacillus anthracis* spores (7), and spores' CaDPA germination was reduced minimally if at all by the differences in sporulation used in the present work.

If, as seems most likely, changes in GR levels are a major factor determining changes in the germination of spores prepared under different conditions, a further obvious question is what causes changes in GR levels (and also GerD levels) as a function of sporulation conditions, in particular the richness of the sporulation medium. Since changes in the levels of β-galactosidase from a transcriptional *gerA-lacZ* fusion were generally similar to changes in levels of various GRs, this suggests that changes in transcription of operons encoding GRs as a function of sporulation conditions are responsible. Indeed, decreased levels of β-galactosidase from transcriptional fusions to promoters of operons encoding GRs in *B. cereus* spores prepared in a poor medium also generally reflected the slower germination of these poor-medium spores, although GR levels in these spores were not determined directly (14). The *spoVA* operon, *gerD*, and operons encoding GRs are transcribed by RNA polymerase containing the alternative sigma factor σ^G, and this transcription is further modulated by the transcription factor SpoVT, which has both positive and negative effects on σ^G-dependent gene expression (2, 32). There is, however, no information on how sporulation medium composition or temperature might modulate the transcription of these genes or their regulation by SpoVT. Analysis of the effects of sporulation conditions on *spoVT* expression could thus be informative.

There is also one report that a protein tyrosine phosphatase, PrpE, modulates expression of operons encoding GRs (13). Since how PrpE exerts this effect is not known, analysis of the effects of PrpE on GR levels would be worthwhile. It is also possible that there is translational modulation of GR levels. Indeed, the ~2.5-fold-higher relative level of GerAA (encoded by the first gene in the *gerA* operon) than GerAC (encoded by the third gene in the *gerA* operon) in poor-medium spores (Table 6) suggests that translational coupling of *gerA* mRNA may be less efficient during sporulation in a poor medium. Thus, analysis of the effects of sporulation conditions on expression of translational *gerA-lacZ* fusions could be worthwhile.

Given the slower nutrient germination of poor-medium than rich-medium spores, it was not surprising that the yields of superdormant spores were significantly higher from poor-medium spores. This finding as well as the lower GR levels in poor-medium spores is thus consistent with the suggestion that superdormant spores are likely those spores in a population that have the lowest GR levels (10). Since spores contaminating foodstuffs and medical products may well have formed in a nutrient-poor environment, it seems reasonable to suppose that levels of superdormant spores in such environmental spore populations will likely be significantly higher than if the spores were prepared in rich laboratory sporulation media. This also seems like a prediction

that would be well worth examining, given the importance of superdormant spores in a number of applied situations.

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Capítulo II

Efecto de la proteína reguladora SpoVT en la germinación y los niveles de proteínas de germinación de esporas de *Bacillus subtilis*

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Effects of the SpoVT Regulatory Protein on the Germination and Germination Protein Levels of Spores of *Bacillus subtilis*

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Bacillus subtilis **isolates lacking the SpoVT protein, which regulates gene expression in developing forespores, gave spores that released their dipicolinic acid (DPA) via germinant receptor (GR)-dependent germination more rapidly than wild-type spores. Non-GR-dependent germination via dodecylamine was more rapid with *spoVT* spores, but germination via Ca-DPA was slower. The effects of a *spoVT* mutation on spore germination were seen with spores made in rich and poor media, and levels of SpoVT-LacZ were elevated 2-fold in poor-medium spores; however, elevated SpoVT levels were not the only cause of the slower GR-dependent germination of poor-medium spores. The *spoVT* spores had ≥5-fold higher GerA GR levels, ~2-fold elevated GerB GR levels, wild-type levels of a GerK GR subunit and the GerD protein required for normal GR-dependent germination, ~2.5-fold lower levels of the SpoVAD protein involved in DPA release in spore germination, and 30% lower levels of DNA protective α/β-type small, acid-soluble spore proteins. With one exception, the effects on protein levels in *spoVT* spores are consistent with the effects of SpoVT on forespore transcription. The *spoVT* spores were also more sensitive to UV radiation and outgrew slowly. While *spoVT* spores' elevated GR levels were consistent with their more rapid GR-dependent germination, detailed analysis of the results suggested that there is another gene product crucial for GR-dependent spore germination that is upregulated in the absence of SpoVT. Overall, these results indicate that SpoVT levels during spore formation have a major impact on the germination and the resistance of the resultant spores.**

Spores of *Bacillus* species are dormant and resistant to a variety of environmental stress factors and can remain in this state for years (35). However, spores constantly sense their environment, and if nutrients become available, spores can rapidly return to vegetative growth through the process of germination followed by outgrowth (25, 34, 35). Major spore proteins that sense nutrients are the germinant receptors (GRs), each of which senses a distinct germinant or mixture of germinants. *Bacillus subtilis* spores contain three major GRs: GerA, which alone triggers germination with L-valine or L-alanine, and GerB and GerK, which together trigger germination with a mixture of L-asparagine–D-glucose–D-fructose–K^+ (AGFK). A GerB variant in *B. subtilis* termed GerB* that can trigger spore germination with L-asparagine alone has also been isolated (23). The GRs are located in the spore's inner membrane, and each one contains three subunits, A, B, and C (5, 18, 19, 28, 34). The levels of these GRs are a major factor in determining rates of spore germination with particular germinants, and elevated GR levels lead to faster germination, while nutrient germination is essentially abolished in spores lacking GRs (4, 24). GR levels vary significantly between individual spores in populations, probably for stochastic reasons, since levels of GR subunits in spores are generally only 10s of molecules per spore (7, 8, 34). Rates of nutrient germination of spores can also vary significantly depending on the medium used for sporulation (6, 28, 29). In particular, spores made in poor media tend to germinate more poorly with nutrient germinants than spores made in a richer medium (10, 28). Recent work has further shown that with *B. subtilis* spores made in a poor medium, their slow germination with nutrients is paralleled by 2- to 5-fold decreases in GR protein levels, as well as similar decreases in the level of the GerD germination protein that is required for normal GR-dependent spore germination (26, 28).

Given the relationship between rates of nutrient germination and GR levels in spores, it is of obvious interest to determine the factors that regulate spores' GR levels. The tricistronic operons encoding *B. subtilis* GRs are transcribed by RNA polymerase with the forespore-specific sigma factor σ^G (11, 34). However, there is no knowledge of how σ^G levels may vary between individual spores or how much stochasticity might affect an individual spore's σ^G levels. There is also a forespore-specific transcriptional regulatory protein, SpoVT, which modulates the expression of a number of σ^G-dependent genes (1, 3, 38, 41, 42). SpoVT can be either a positive or a negative regulator of gene expression, and in spores prepared by resuspension in a poor medium, SpoVT represses levels of expression of operons encoding GRs ~3-fold and slightly stimulates the expression of the forespore-specific *spoVA* operon that encodes a number of proteins likely involved in spore germination by allowing the spore's large depot (~20% of spore core dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) to leave the germinating spore, one of the earliest events in spore germination. In contrast to its effects on the expression of the *gerA*, *gerB*, *gerK*, and *spoVA* operons, SpoVT is reported to have a negligible effect on the transcription of the *gerD* gene. As with σ^G, there is no knowledge of if or why SpoVT levels may vary between individual sporulating cells, either by regulatory or by stochastic effects.

Given the significant repressive effects of SpoVT on *gerA*, *gerB*, and *gerK* transcription, with minimal effects on other genes in-

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volved in spore germination, it seemed likely that variation in SpoVT levels would have major effects on spores' GR levels and thus in effect modulate rates of spores' nutrient germination. However, it has been reported that a *spoVT* mutation results in spores that germinate extremely poorly (3). This finding is in contrast to what would have been expected on the basis of knowledge of SpoVT's effects on transcription of operons encoding GRs and effects of GR levels on rates of spores' nutrient germination. Consequently, we have examined the effects of a *spoVT* null mutation on rates of spore germination with both GR-dependent and GR-independent germinants and on the levels of a number of GR subunits, as well as GerD and a SpoVAD protein.

MATERIALS AND METHODS

***B. subtilis* strains used and spore preparation and purification.** The *B. subtilis* strains used in this work were isogenic derivatives of strain PS832, a laboratory *trp*$^+$ derivative of strain 168. Specific strains used were (i) PS533 (33), which is strain PS832 carrying plasmid pUB110 encoding resistance to kanamycin (10 μg/ml); (ii) PS4220 (*spoVT*), which was made in this work by transforming strain PS832 to spectinomycin resistance (Sp^r; 100 μg/ml) with chromosomal DNA from strain IB1 (3, 36) in which the *spoVT* gene has been largely deleted and replaced by a spectinomycin resistance cassette; the presence of the *spoVT* deletion was confirmed by PCR; (iii) FB10 (*gerB**) (23), which carries a point mutation in the *gerBB** cistron such that the resultant GerB GR, termed GerB*, triggers spore germination in response to L-asparagine alone; (iv) PS4222 (*spoVT-lacZ*), which was prepared by transforming strain PS832 to chloramphenicol (5 μg/ml) resistance with chromosomal DNA from strain IB5 (3, 36), which carries a transcriptional *spoVT-lacZ* fusion at the *amyE* locus; and (v) PS4225 (*spoVT gerBB**), which was prepared by transforming strain FB10 to Sp^r with chromosomal DNA from strain IB1; the *spoVT* genotype of this strain was also confirmed by PCR.

Spores of all *B. subtilis* strains were prepared by sporulation on 2× SG medium agar plates without antibiotics or in either rich liquid medium (2× SG) or poor liquid medium (Spizizen's minimal medium without Casamino Acids) as described previously (20, 22, 28, 36). Spores were harvested, purified, and stored in water at 4°C as described previously (20). All spores used in this work were free (>98%) of growing or sporulating cells, germinated spores, and cell debris, as determined by phase-contrast microscopy.

Spore germination and outgrowth. Germination of spores of all strains was preceded by a heat shock (30 min, 70°C), followed by cooling on ice for ≥15 min. Spore germination with the 1:1 chelate of Ca^{2+} and DPA was in 60 mM Ca-DPA at 23°C with spores at an optical density at 600 nm (OD_{600}) of 1.0. Spore germination with Ca-DPA was assessed by phase-contrast microscopy, with ~100 individual spores examined at each time point. Germination of spores with dodecylamine (31) and nutrient germinants was at either 45°C (dodecylamine) or 37°C (nutrient germinants) and with spores at an OD_{600} of 0.5. Routinely, germination was in 200 μl of 25 mM K-HEPES buffer (pH 7.4) plus 50 μM $TbCl_3$, and germination of spore populations was monitored by following DPA release by measurement of Tb-DPA fluorescence in a multiwell fluorescence plate reader as described previously (40, 43, 44, 46). Germinants used were (i) 0.8 mM dodecylamine; (ii) various concentrations of L-valine; (iii) various concentrations of L-asparagine alone; and (iv) various concentrations of L-asparagine plus 10 mM D-glucose, 10 mM D-fructose, and 10 mM KCl (GFK). Rates of spore germination were calculated as described previously (40, 43, 44, 46), and all values shown are averages of results of duplicate measurements on two independent spore preparations. Differences between rates of germination of spores of different strains or spores of the same strain prepared differently were assessed by the two-tailed Student's *t* test.

The germination of multiple individual heat-shocked spores with either 10 mM L-valine or 10 mM all AGFK components as described above was followed by differential interference contrast (DIC) microscopy of spores adhered on a microscope slide as described previously (12, 45, 46). The DIC images of hundreds of individual spores were recorded at a rate of 1 frame per 15 s for up to 120 min, and the image intensities of each individual spore were extracted (45). In these analyses, a spore's DIC image intensity remains relatively constant after mixing with a germinant until a time, T_{lag}, when rapid Ca-DPA release begins. The spore's DIC image intensity then falls rapidly in parallel with Ca-DPA release that ends at $T_{release}$. The parameter $\Delta T_{release}$, which is $T_{release} - T_{lag}$, defines the time for release of ≥90% of a spore's Ca-DPA pool. Following $T_{release}$, there is a further fall of ~30% in a spore's initial DIC image intensity due to hydrolysis of the spore cortex peptidoglycan (PG), with attendant water uptake and swelling of the spore core. The latter process ends at T_{lysis}, with $T_{lysis} - T_{release}$ giving ΔT_{lysis}, the period of cortex hydrolysis and core swelling. Following T_{lysis} there is little or no further change in the spore's DIC image intensity. The values for these kinetic parameters of the germination of individual spores were determined by analysis of between 86 and 271 individual spores that germinated.

The outgrowth of heat-shocked spores was carried out at 37°C in 2× yeast-tryptone (2× YT) medium containing 16 g tryptone–10 g yeast extract–5 g NaCl per liter plus 5 mM L-valine. Spores were added to an OD_{600} of ~0.8, and the OD_{600}s of the cultures were followed over time.

Measurement of levels of GR subunits GerD and SpoVAD. Levels of GR proteins GerD and SpoVAD, both of which are present largely or completely in spores' inner membrane (5, 18, 19, 27, 39), were determined in inner membrane fractions by Western blot analysis using primary rabbit antisera against the various proteins and a secondary antiserum as described previously (7, 13, 14, 28). The primary antisera are specific, and the anti-GR subunit antisera do not cross-react with the analogous GR subunits from heterologous GR subunits (7, 13, 14, 28). In brief, spores were decoated, ruptured by lysozyme digestion, and sonicated briefly to reduce the extract's viscosity and to shear the inner membrane from PG layers, and the inner membrane fraction was isolated by differential centrifugation. Twofold serial dilutions of inner membrane fractions in which levels of germination proteins were to be compared were first run on SDS-polyacrylamide gels and stained with Coomassie blue to determine how much of the different inner membrane fractions were needed to be run on SDS-polyacrylamide gels to load equal amounts of protein. Different amounts of inner membrane protein from different spore preparations were then run together on SDS-polyacrylamide gels, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Billerica, MA), and antigens on the membrane were detected by Western blot analysis using chemiluminescence. Following development of Western blots, the membrane was stripped and then reprobed with another antiserum as described previously (7, 28). Routinely, 8- to 16-fold ranges of inner membrane protein amounts were used to compare levels of germination proteins in different spore preparations, using both visual estimation and the ImageJ program as described previously (7, 28). Differences between levels of various germination proteins in spores of different strains or spores of the same strain prepared differently were assessed by a two-tailed Student's *t* test.

Other methods. Spores to be assayed for β-galactosidase were isolated, decoated, disrupted by lysozyme treatment, and sonicated as described above. After centrifugation in a microcentrifuge, β-galactosidase in the supernatant fluid was assayed fluorometrically using 4-methylumbelliferyl-β-D-galactoside as the substrate and measuring 4-methylumbelliferone as described previously (28). Specific activities of β-galactosidase are expressed in relative fluorescence units (RFU) obtained in a 40-min assay with 10^9 spores. Previous work has shown that DPA levels are essentially identical in spores made in the rich and poor media used in the current work (28). Assays for β-galactosidase were carried out in duplicate on two independent spore preparations, and differences between specific activities in spores prepared differently were analyzed for significance by a two-tailed Student's *t* test. The β-galactosidase specific activity

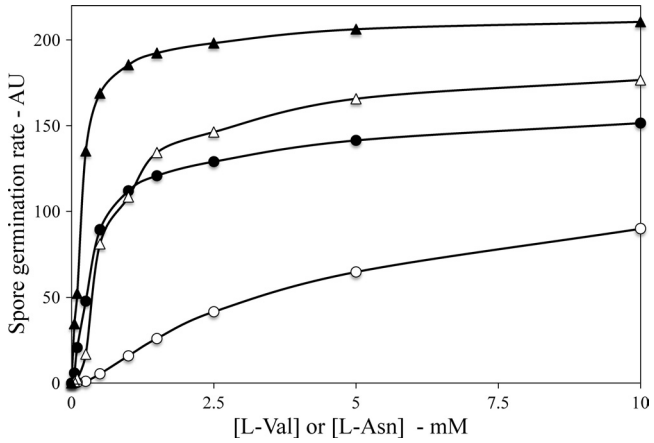

FIG 1 Rates of germination of wild-type and *spoVT* spores with L-valine and AGFK prepared in rich medium. Purified spores of *B. subtilis* strains PS533 (wild type) (○, △) and PS4220 (*spoVT*) (●, ▲) were prepared in a rich liquid medium as described in Materials and Methods. The spores were germinated with various concentrations of L-valine alone (○, ●) or L-asparagine plus GFK (△, ▲), DPA release was monitored to measure spore germination, and spore germination rates in arbitrary units (AU) were determined as described in Materials and Methods. The scales for arbitrary units were the same in all germination experiments. At between 0.05 mM and 10 mM germinant concentrations, differences in rates of germination with L-valine or AGFK between wild-type and *spoVT* spores were highly significant ($P < 0.0001$ to 0.02).

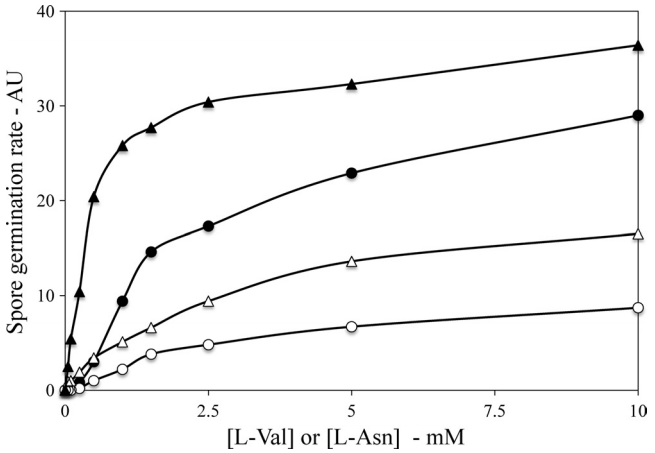

FIG 2 Rates of germination of wild-type and *spoVT* spores with L-valine and AGFK prepared in a poor medium. Purified spores of *B. subtilis* strains PS533 (wild type) (○, △) and PS4220 (*spoVT*) (●, ▲) were prepared in a poor liquid medium as described in Materials and Methods. The spores were germinated with various concentrations of L-valine alone (○, ●) or L-asparagine plus GFK (△, ▲), DPA release was monitored to measure spore germination, and spore germination rates in arbitrary units (AU) were determined as described in Materials and Methods. The scales for arbitrary units were the same as those in Fig. 1. At between 0.25 and 10 mM germinant concentrations, differences in rates of L-valine or AGFK germination between wild-type and *spoVT* spores were highly significant ($P < 0.002$).

of rich-medium spores without a *lacZ* fusion (PS533 spores) was ≤10% of that of PS4222 spores (data not shown).

The level of small, acid-soluble proteins (SASPs) in spores were determined by polyacrylamide gel electrophoresis (PAGE) at low pH of samples from dry ruptured spores (20) and subsequent densitometric analysis of various SASP bands on the stained gel using the program ImageJ. The acetic acid extracts from 6 mg dry ruptured spores were dialyzed, lyophilized, and dissolved in 50 μl of 8 M urea plus 25 μl of acid gel diluent, aliquots were run on polyacrylamide gels at low pH, and gels were stained with Coomassie blue.

The UV resistance of spores of various strains was determined by irradiation at 24°C with a UV lamp with maximum output at 254 nm (UVG-11; UVP, San Gabriel, CA) that was 35 cm from a 35-mm petri dish with 2 ml spores at an OD_{600} of 1.0 in water as described previously (20). Aliquots from unirradiated and irradiated samples were spotted on Luria broth agar plates (22) with the appropriate antibiotic, plates were incubated for 24 to 48 h, and colonies were counted.

RESULTS

Nutrient germination of wild-type and *spoVT* spores. It was reported that spores lacking SpoVT germinate very poorly with nutrients and that SpoVT is a repressor of operons that encode GRs (3, 41). Since GR levels appear to be directly related to spores' rates of nutrient germination (2, 4, 24, 28), these previous results with *spoVT* spores seem somewhat contradictory. One possible explanation for this apparent contradiction could be in the methods used to measure germination, as some methods measure an early event in germination such as DPA release, while others monitor the resumption of metabolism in spores, and this does not take place until both DPA release and cortex PG hydrolysis are complete and spore outgrowth has begun (34, 35). Since the germination of *spoVT* spores in previous work was monitored by measuring the resumption of spore metabolism (3), we examined the nutrient germination of wild-type and *spoVT* spores by monitoring DPA release, one of the earliest measurable events in spore germination (34, 35). In these experiments, spores made in both a rich and a poor liquid medium were examined, since previous work indicated that poor-medium spores germinated relatively poorly and had lower levels of GRs and GerD (28). Perhaps higher levels of SpoVT in developing spores prepared in the poor medium are involved in determining low levels of some germination proteins in poor-medium spores. To examine whether SpoVT levels might be involved in the determination of GR levels in spores made in rich and poor sporulation media, the specific activity of β-galactosidase from a *spoVT-lacZ* fusion was assayed in spores of strain PS4222 (*spoVT-lacZ*) prepared in rich and poor liquid media. Strikingly, the β-galactosidase specific activity in the poor-medium spores (2.1×10^4 RFU/10^9 spores) was twice as high as that in the rich-medium spores (1.1×10^4), and the latter value was ~10-fold higher than the β-galactosidase specific activity in spores of strain PS533, which does not contain a *lacZ* fusion (10^3). The difference in the levels of β-galactosidase in PS4222 spores made in a rich or a poor medium was highly significant ($P < 0.0001$).

When the levels of germination of *spoVT* and wild-type spores made in either the rich or poor medium were compared, the *spoVT* spores exhibited higher rates of germination with both L-valine via the GerA GR and with the AGFK mixture via the GerB plus GerK GRs (Fig. 1 and 2). The higher rates of germination of the *spoVT* spores were most pronounced at low nutrient germinant concentrations, as has been found previously when rates of germination of spores with elevated GR levels are compared to germination rates of wild-type spores (2, 4). Also as found previously (28), both the wild-type and *spoVT* spores prepared in the poor liquid medium germinated more poorly than rich-medium spores of the same genotype (Fig. 2).

Spores that contained a GerB GR variant, termed GerB*, that can trigger germination with L-asparagine alone and with either

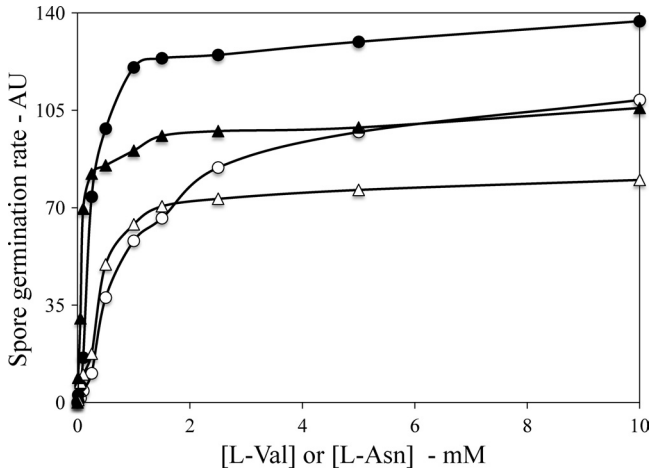

FIG 3 Rates of germination of *gerB** and *gerB* spoVT* spores with L-valine and L-asparagine prepared in a rich medium. Purified spores of *B. subtilis* strains FB10 (*gerB**) (○, △) and PS4225 (*gerB* spoVT*) (●, ▲) were prepared in a rich liquid medium as described in Materials and Methods. The spores were germinated with various concentrations of L-valine alone (○, ●) or L-asparagine alone (△, ▲), DPA release was monitored to measure spore germination, and spore germination rates in arbitrary units (AU) were determined as described in Materials and Methods. The scales for arbitrary units were the same as those in Fig. 1. At between 0.01 and 5 mM germinant concentrations, differences in rates of germination with L-valine or L-asparagine between wild-type and *spoVT* spores were highly significant ($P < 0.001$ to 0.02).

an otherwise wild-type or *spoVT* background were also prepared in a rich medium, and their germination with either L-valine or L-asparagine alone was also measured (Fig. 3). As seen with wild-type and *spoVT* spores prepared in a rich medium, *gerB* spoVT* spores (strain PS4225) germinated much faster than *gerB** spores both with L-valine and with L-asparagine alone, and again, the faster germination of the *gerB* spoVT* spores was most pronounced at lower germinant concentrations.

Kinetic parameters of germination of multiple individual wild-type and *spoVT* spores. During nutrient germination, the release of DPA was clearly faster with *spoVT* spore populations than with wild-type spores. Another way to more precisely determine the differences in the germination of *spoVT* and wild-type spores is to examine kinetic parameters of the germination of multiple individual wild-type and *spoVT* spores. This analysis can determine the average lag time, T_{lag}, between germinant addition and initiation of fast DPA release, the time, $\Delta T_{release}$, for release of ≥90% of a spore's DPA pool, and the time, termed ΔT_{lysis}, for PG cortex hydrolysis and core swelling (12, 40, 46). Consequently, we examined the L-valine and AGFK germination of large numbers of individual wild-type and *spoVT* spores (Fig. 4; Table 1). This analysis showed that T_{lag}s for germination with either AGFK or L-valine were significantly shorter with *spoVT* spores, $\Delta T_{release}$s were essentially identical for both wild-type and *spoVT* spores, and ΔT_{lysis}s were significantly longer for *spoVT* spores (Table 1).

Nonnutrient germination of wild-type and *spoVT* spores. The finding noted above, that *spoVT* spores, as both populations and individuals, germinated significantly faster than wild-type spores, as well as the knowledge that SpoVT represses operons encoding GRs, suggested that the faster nutrient germination of *spoVT* spores might be due to elevated GR levels. However, there are other ways in which spore germination rates can be increased, including alterations in SpoVA protein levels or in spore PG cortex structure (34, 39, 46). To obtain further evidence that the presence of SpoVT in sporulation had specific effects on spore germination via alterations in GR levels, we examined germination of wild-type and *spoVT* spores made in a rich medium with two nonnutrient germinants, dodecylamine and Ca-DPA, neither of which triggers germination via GRs (31, 34) (Fig. 5). As seen with nutrient germination, the germination of *spoVT* spores with dodecylamine was significantly faster than that of wild-type spores (Fig. 5A). However, Ca-DPA germination was significantly slower with *spoVT* spores than with wild-type spores (Fig. 5B). The same results were obtained with two independent sets of spore preparations (data not shown).

Levels of GR subunits, GerD, and SpoVAD in wild-type and *spoVT* spores. The results presented above showed that *spoVT* spores germinated significantly faster than wild-type spores with nutrients and dodecylamine, although not with one germinant, Ca-DPA, which does not trigger germination via GRs. These results plus the reported repression of the *gerA*, *gerB*, and *gerK* operons by SpoVT (3, 41) strongly suggested that *spoVT* spores might have higher GR levels than wild-type spores. To test this suggestion directly, the levels of a number of GR subunits, as well as two additional germination proteins, GerD and SpoVAD, were determined by Western blot analysis of inner membrane proteins from wild-type and *spoVT* spores (Fig. 6; Table 2). Strikingly, levels of the GerAA, GerAC, and GerBC GR subunits were 2- to 8-fold higher in *spoVT* spores, while levels of GerKA and GerD were similar in wild-type and *spoVT* spores and SpoVAD levels were ~2.5-fold lower in *spoVT* spores.

Germination and outgrowth of wild-type and *spoVT* spores. The results noted above indicated that *spoVT* and *gerB* spoVT* spores germinated significantly faster with nutrients than wild-type or *gerB** spores when germination was monitored by measuring DPA release. However, this result does not agree with the results described in a previous report that *spoVT* spores germinate slower than wild-type spores, although in this work, spore germination was followed by measurement of the resumption of spore metabolism (3). One possibility is that while DPA release is indeed faster during germination of spores of *spoVT* strains, perhaps some event later in germination is significantly slower with *spoVT* spores. Indeed, the average T_{lysis}s for *spoVT* spores germinating with either L-valine or AGFK were significantly longer than the T_{lysis}s for wild-type spores (Table 1). To test this suggestion more thoroughly, we measured the ability of wild-type and *spoVT* spores both to initiate germination and to return to active growth by germination of spores in a complete nutrient medium (Fig. 7). In this case, while the germination of the *spoVT* spores, as measured by the fall in the OD_{600} of the spore cultures, was faster than that of the wild-type spores, the return to vegetative growth was significantly slower than that with wild-type spores. This observation suggests that *spoVT* spores are much slower than wild-type spores in either an event in spore germination after DPA release or some early event in spore outgrowth.

UV resistance of and SASP levels in wild-type and *spoVT* spores. The elevated GR subunit levels in *spoVT* spores were consistent with SpoVT acting as a repressor of operons encoding GRs. SpoVT is also reported to affect the transcription of other genes expressed in the developing forespore, in particular, genes encoding the DNA protective α/β-type SASP, although it has minimal effects, if any, on transcription of the gene that encodes spores'

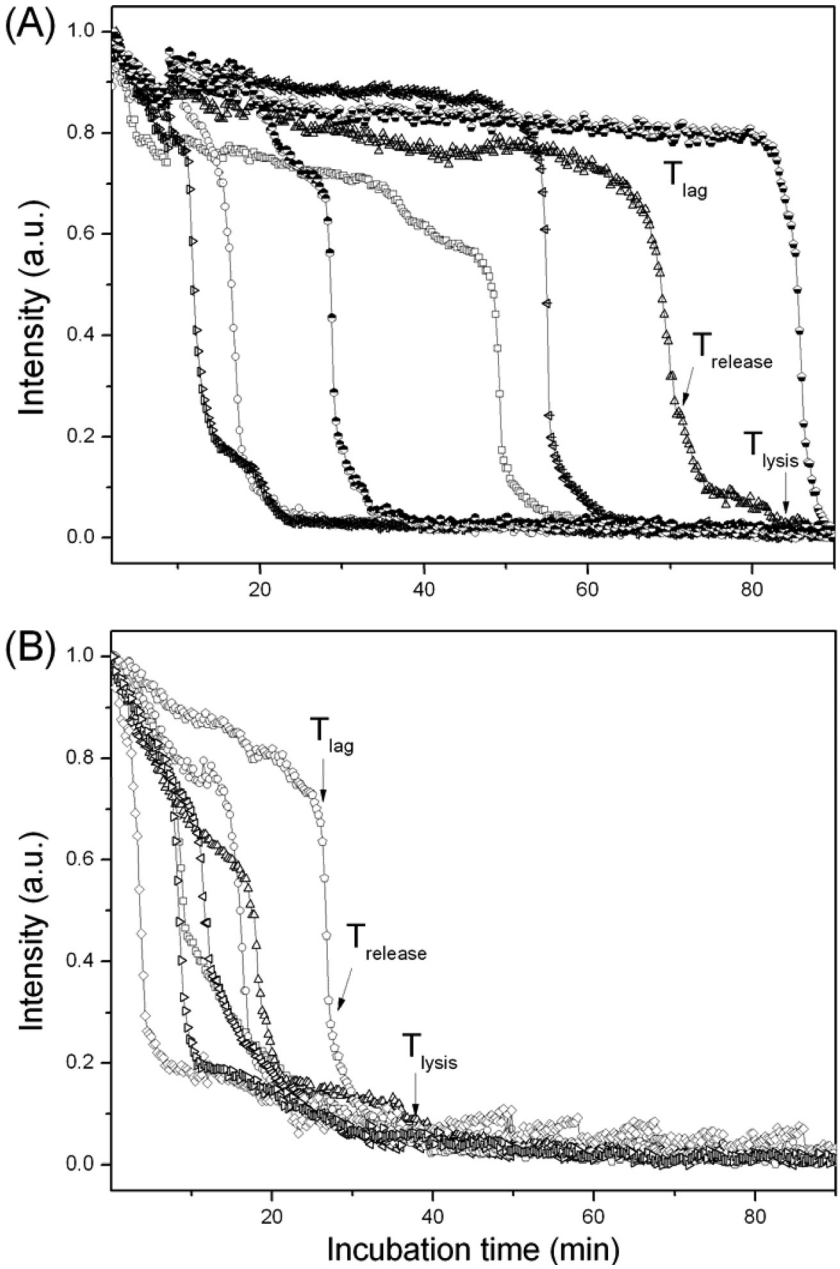

FIG 4 Germination of seven individual wild-type (A) and *spoVT* (B) spores with L-valine. Individual PS533 (wild-type) or PS4220 (*spoVT*) spores were germinated in 10 mM L-valine at 37°C, and DIC image intensities of spores were recorded every 15 s, were normalized to 1.0 at 0 min and to 0.0 after T_{lysis}, and are given in arbitrary units (a.u.). For one spore in each panel, the arrows denote the T_{lag}, $T_{release}$, and T_{lysis} for that spore.

single γ-type SASP, which plays no role in spore DNA protection (35, 41). Since spores with very low levels of SASPs exhibit slower outgrowth than wild-type spores (30, 32), we also examined the levels of SASPs in wild-type and *spoVT* spores (Fig. 8; Table 3). While levels of SASP-γ were essentially identical in wild-type and *spoVT* spores, as expected, levels of the DNA protective α/β-type SASP were ~30% lower in *spoVT* spores when the levels of the α/β-type SASP were expressed relative to the levels of the γ-type SASP (Table 3). Spore UV resistance in particular is very sensitive to spores' level of the α/β-type SASP, with even small decreases in these proteins' levels resulting in significant decreases in spore UV resistance (16). Indeed, *spoVT* spores were more UV sensitive than wild-type spores, consistent with the *spoVT* spores' lower apparent levels of α/β-type SASP, as shown above (Fig. 9).

DISCUSSION

The results reported in this communication provide new information on the reasons for the effects of the SpoVT regulator on the properties of *B. subtilis* spores, as follows. (i) In most cases, the reported effects of SpoVT on transcription of forespore-specific genes were mirrored by the generally similar effects on the levels of a number of spore proteins. Thus, SpoVT is reported to be a repressor of the *gerA*, *gerB*, and *gerK* operons (3, 41), and the levels of all these GRs' subunits except for those of GerKA were elevated

TABLE 1 Germination kinetic parameters of wild-type and *spoVT* spores[a]

Spore	Germinant	T_{lag} (min)	$T_{release}$ (min)	$\Delta T_{release}$ (min)	T_{lysis} (min)	ΔT_{lysis} (min)	No. (%) of spores examined
spoVT	L-Valine	6.6 ± 5.7[b]	8.6 ± 6.0	2.5 ± 0.8	25. 9 ± 13.5	17.4 ± 10.5[b]	279 (97.2)
spoVT	AGFK	8.7 ± 7.5[b]	10.9 ± 7.6	2.2 ± 0.7	23.7 ± 10.7	12.8 ± 7.6[b]	99 (86.7)
wt	L-Valine	25.1 ± 25.0[b]	27.2 ± 23.1	2.1 ± 0.7	36.1 ± 23.3	8.9 ± 4.5[b]	138 (84)
wt	AGFK	14.6 ± 11.8[b]	16.9 ± 11.8	2.4 ± 0.6	23.7 ± 11.9	6.8 ± 2.9[b]	117 (94)

[a] The germination of multiple individual spores of strains PS533 (wild type [wt]) and PS4220 (*spoVT*) with either 10 mM L-valine or 10 mM in all AGFK components was measured as described in Materials and Methods. All kinetic germination parameters are shown as averages ± standard deviations.
[b] The differences between T_{lag} and T_{lysis} values for wild-type and *spoVT* spores' germination with either L-valine or AGFK were highly significant ($P < 0.001$).

in *spoVT* spores. The reason that GerKA levels were not affected in the current work are not clear, although in the current work, *spoVT* spores were prepared by nutrient exhaustion in a rich medium, while in the work assessing the effects of SpoVT on forespore-specific gene expression, sporulation was induced by resuspension in a poor medium (3, 41). Levels of SpoVAD and the α/β-type SASP were also lower in *spoVT* spores, consistent with SpoVT's role as an activator of transcription of the *spoVA* operon and at least the *sspB* gene encoding SASP-β, although there is disagreement in the literature on whether SpoVT is also an activator of the *sspA* gene encoding SASP-α (3, 41). Finally, spores' levels of SASP-γ, encoded by the *sspE* gene, and GerD were essentially unaffected by the *spoVT* mutation, consistent with the minimal effects on *gerD* and *sspE* transcription in a *spoVT* strain (3, 41). (ii) Mutants with the *spoVT* mutation are reported to have a coat defect, including a grossly misassembled spore coat, and the spores are slightly lysozyme and chloroform sensitive (3). The *spoVT* spores are also reported to have slightly reduced wet heat resistance, although the spore coat alone does not generally play a significant role in spore wet heat resistance (35). However, *spoVT* spores' more rapid germination with dodecylamine and slower germination with Ca-DPA seen in the current work are similar to the effects of known coat defects on spore germination with these agents (21, 31), consistent with *spoVT* spores having a coat defect. An obvious question is how the action of SpoVT in the forespore affects spore coat assembly. One possibility is that normal coat assembly is dependent on normal forespore development, and there is recent evidence for a role for the forespore in modulating spore coat assembly (17). Another possibility is that it is reduced synthesis of α/β-type SASPs in the forespore that leads to a coat defect, as when α/β-type SASPs are not made in the forespore at levels sufficient to saturate forespore DNA, expression of other forespore-specific genes is altered and there is aberrant expression of at least several coat protein genes as well (32). (iii) The fact that

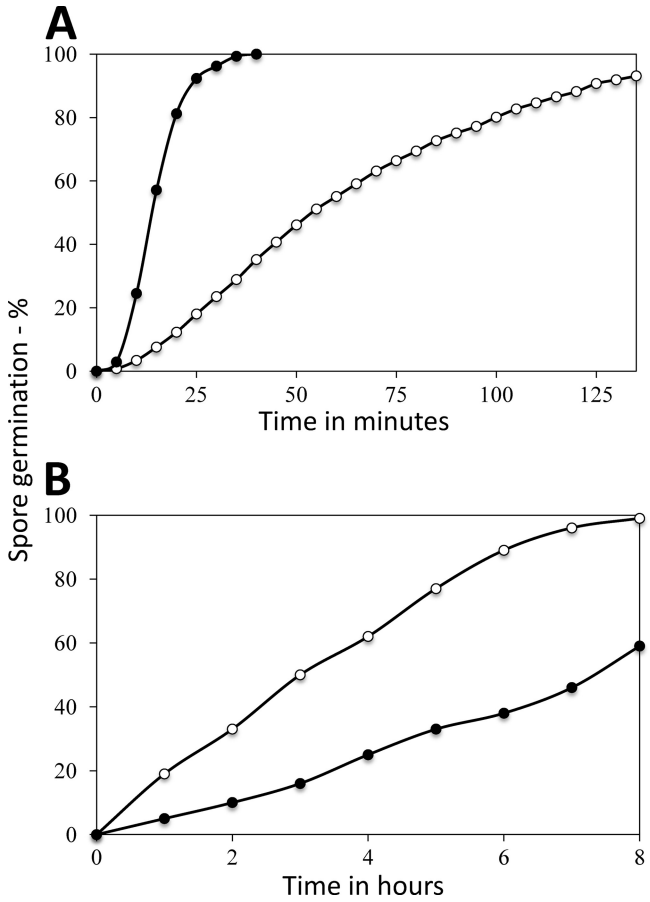

FIG 5 Rates of germination of wild-type and *spoVT* spores with dodecylamine and Ca-DPA. Purified spores of *B. subtilis* strains PS533 (wild type) and PS4220 (*spoVT*) prepared in a rich liquid medium were germinated with dodecylamine (A), for which DPA release was monitored to measure spore germination, and spore germination percentages in arbitrary units (AU) were determined as described in Materials and Methods, or Ca-DPA (B), for which spore germination percentages were determined by phase-contrast microscopy as described in Materials and Methods. Symbols: ○, PS533 (wild-type) spores; ●, PS4220 (*spoVT*) spores.

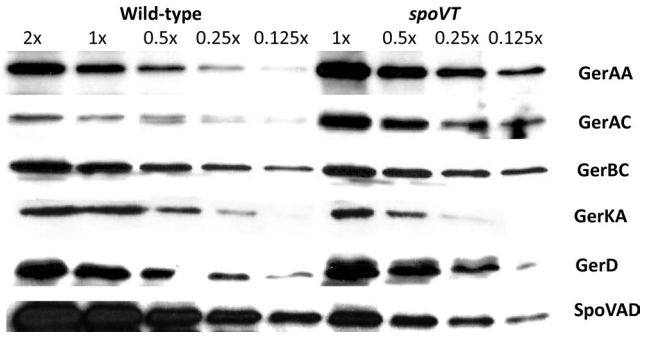

FIG 6 Western blot analysis of levels of germination proteins in wild-type (PS533) and *spoVT* (PS4220) spores. Spores of the two strains were prepared and purified, spores were disrupted, the inner membrane fractions were isolated, and aliquots of various amounts of inner membrane protein were subjected to Western blot analysis as described in Materials and Methods. The amounts of inner membrane protein in the 1× samples from wild-type and *spoVT* spores were identical. The GerAA, GerAC, GerBC, and SpoVAD strips are from the same Western blot that was stripped and reprobed with different antisera. The GerKA strip and GerD strips are each from different Western blots, but ones run with samples from the same spore inner membrane fraction analyzed for GerAA, GerAC, GerBC, and SpoVAD. Note that on the GerD strip there were bubbles on the 0.5× wild-type and 0.125× *spoVT* lanes that eliminated the right halves of these bands.

TABLE 2 Ratios of germination proteins in *spoVT* and wild-type spores[a]

Germination protein	Ratio of protein level in *spoVT* spores/ wild-type spores
GerAA	5
GerAC	8
GerBC	1.8
GerKA	1
GerD	1
SpoVAD	0.4

[a] Spores of strains PS533 (wild type) and PS4220 (*spoVT*) were prepared in rich liquid medium. Inner membrane fractions were isolated from these spores, and relative levels of various germination proteins were determined by Western blot analysis as described in Materials and Methods and in Fig. 6.

spoVT spores have lower levels of α/β-type SASPs was shown here directly and was indicated indirectly by the decreased UV resistance of these spores. Since decreased levels of α/β-type SASPs have been shown to result in decreased spore wet heat resistance (16, 35), this suggests that the decreased wet heat resistance of *spoVT* spores is also due to these spores' low α/β-type SASP levels. It is also possible that SpoVT may modulate the expression of the *spl* gene, involved in repair of specific UV damage in spore DNA, but there are no data available on this point (41).

Other new observations in this work included the finding that the elevated levels of GerAA, GerAC, and GerBC in *spoVT* spores, and thus, presumably, the GerA and GerB GRs, explain at least in part the more rapid GR-dependent DPA release during L-valine and L-asparagine germination of *spoVT* and *gerB* spoVT* spores, especially at low nutrient germinant concentrations (2, 4, 28). The elevated GerBC level in *spoVT* spores is also consistent with the increased AGFK germination seen with spores overexpressing only the GerB GR and not the GerK GR, although there was a much greater increase in the rates of AGFK seen in *spoVT* spores compared with those seen previously when the GerB GR alone was overexpressed ~3-fold (37). The more rapid DPA release from *spoVT* spore populations during nutrient germination appeared to be due to shorter T_{lag}s between nutrient germinant addition and the initiation of rapid DPA release, as the time for rapid release of the great majority of spore DPA during nutrient germination, $\Delta T_{release}$, was essentially identical in wild-type and *spoVT* spores. This is also what has been seen with spores with only a single overexpressed GR when germinated with nutrients that trigger either the overexpressed GerA or GerB* GR (45).

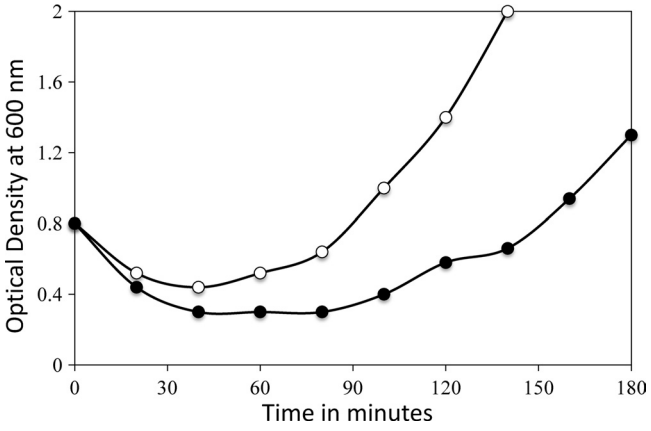

FIG 7 Germination and outgrowth of wild-type and *spoVT* spores. Spores of *B. subtilis* strains PS533 (wild type) and PS4220 (*spoVT*) prepared in a rich liquid medium were heat shocked, cooled, and incubated with shaking at 37°C and an initial OD_{600} of 0.8 in 2× YT medium plus 5 mM L-valine, and the OD_{600} of the cultures was measured over a 3-h period. Symbols: ○, PS533 (wild-type) spores; ●, PS4220 (*spoVT*) spores.

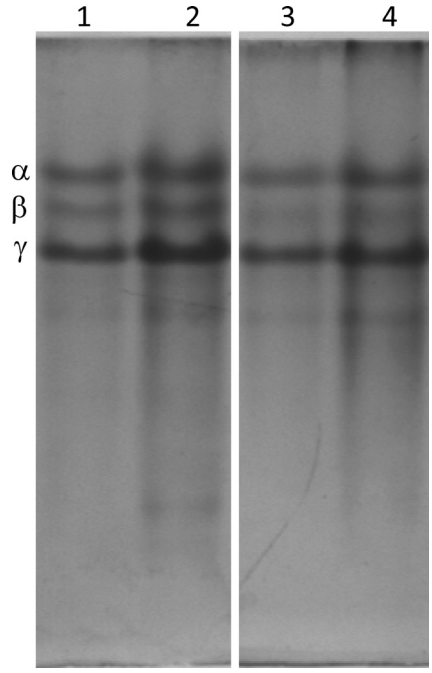

FIG 8 Levels of various SASPs in wild-type and *spoVT* spores. SASPs were extracted from PS533 (wild-type) (lanes 1 and 2) and PS4220 (*spoVT*) (lanes 3 and 4) spores, dialyzed, and lyophilized; aliquots (5 μl, lanes 1 and 3; 10 μl, lanes 2 and 4) of the dissolved lyophilized material were run on polyacrylamide gels at low pH; and the gels were stained with Coomassie blue as described in Materials and Methods. The symbols α, β, and γ adjacent to lane 1 denote the migration positions of SASP-α, -β, and -γ. Note that lanes 1 to 4 were from the same gel, but intervening lanes were removed for clarity.

The more rapid GR-dependent germination of *spoVT* spores with L-valine was not unexpected, as noted above. However, while the elevated rates of AGFK and L-asparagine germination of *spoVT* and *gerB* spoVT* spores, respectively, initially appeared to be consistent with the elevated GerBC level in *spoVT* spores, this result was actually quite surprising, because 8-fold higher levels of GerAA and GerAC in wild-type spores actually result in very strong inhibition of AGFK germination via the GerB plus GerK

TABLE 3 SASP levels in wild-type and *spoVT* spores[a]

Spore	Level of the following SASP (arbitrary units): α	β	γ	α + β/γ
Wild type	47	31	62	1.3
spoVT	33	19	58	0.9

[a] Spores of strains PS533 (wild type) and PS4220 (*spoVT*) were purified, disrupted, SASP extracted, dialyzed, and lyophilized; the dry powder was redissolved; aliquots were run on polyacrylamide gels at low pH; and gels were stained with Coomassie blue as described in Materials and Methods and shown in Fig. 8. Relative levels of various SASPs were determined by ImageJ analysis of the stained gel shown in Fig. 8. SASP levels are given in arbitrary units, but these units are identical for all SASPs in the spores of both strains.

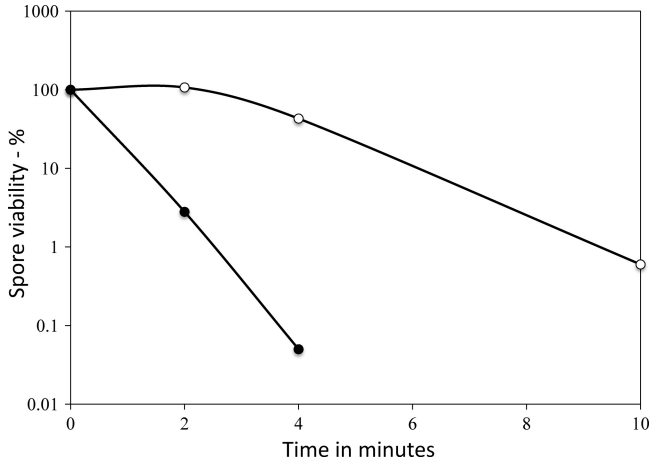

FIG 9 UV resistance of wild-type and *spoVT* spores. Spores of strains PS533 (wild type) and PS4220 (*spoVT*) were exposed to UV radiation, and spore viability was measured as described in Materials and Methods. All values shown are averages of duplicate determinations, and results similar to those shown here were obtained in two separate experiments. Symbols: ○, wild-type spores; ●, *spoVT* spores.

GRs or of L-asparagine germination via the GerB* GR (2, 37). The mechanism whereby the overexpressed GerA GR inhibits germination via other GRs is not known. However, one possible explanation is that all GRs compete for a low-abundance downstream signaling molecule in the spore germination pathway, such that with elevated GerA levels, GerA outcompetes other GRs for access to this signaling molecule, thus inhibiting germination via other GRs. If, however, the gene encoding the downstream signaling molecule is also repressed by SpoVT, then there will likely be more of this molecule in *spoVT* spores, thus allowing more rapid germination with all GRs, even if the GRs are not significantly overexpressed. Unfortunately, while the existence of this downstream signaling molecule has been proposed (2), it has not yet been identified.

The elevated rates of DPA release in GR-dependent germination of *spoVT* spores are in contrast to the report that *spoVT* spores germinate slower than wild-type spores (3). However, clearly, the return to vegetative growth of *spoVT* spores is much slower than that of wild-type spores. The time needed for PG cortex lysis, ΔT_{lysis}, in germination of *spoVT* spores is also longer than that for wild-type spores. The reason for this is not known, but SpoVT is reported to activate *cwlD* expression (41), and thus, *spoVT* cells may have less CwlD during spore formation. Since CwlD is essential for the generation of the cortex-specific modification, muramic acid δ-lactam (MAL) (35), it is possible that the cortical PG in *spoVT* spores has less MAL than that in wild-type spores. If this is the case, even though SpoVT appears to repress expression of one cortex-lytic enzyme (CLE), SleB (42), the degradation of cortex PG might be slower during germination of *spoVT* spores, since MAL is the recognition element for SleB as well as the other redundant CLE, CwlJ (34, 35). Indeed, *spoVT* spores have significantly longer ΔT_{lysis}s for both AGFK and L-valine germination, and SpoVT is also an activator of a spore cortex protein, CoxA (41), although the function of this protein is not known. However, the shorter T_{lag}s for *spoVT* spores make the actual T_{lysis}s for wild-type and *spoVT* spores rather similar, and thus, it seems unlikely that the slow outgrowth of *spoVT* spores is due only to slow cortex hydrolysis. Perhaps the slow outgrowth of *spoVT* spores is due to low levels of some proteins in *spoVT* spores that are needed for rapid spore outgrowth. Indeed, *spoVT* spores have lower levels of α/β-type SASPs than wild-type spores, and low SASP levels alone can slow spore outgrowth even in a rich medium (30), although whether the ~30% lower α/β-type SASP level in *spoVT* spores would cause this effect is not known.

Analysis of the germination of wild-type and *spoVT* spores made in rich and poor media indicated that a *spoVT* mutation increased rates of spore germination 1.5- to 10-fold, depending on the nutrient germinant used and its concentration. Spore levels of β-galactosidase expressed under the control of the *spoVT* promoter were ~2-fold higher in poor-medium spores than in rich-medium spores, suggesting that SpoVT levels are also ~2-fold higher in forespores developing in the poor medium. This is certainly consistent with the slower germination and lower GR levels of poor-medium spores (28), since SpoVT appears to decrease the levels of at least the operons that encode the GerA and GerB GRs. However, a difference in SpoVT level alone in forespores forming in a poor medium is not sufficient to explain the lower germination of poor-medium spores, since poor-medium *spoVT* spores had 2- to 3-fold lower maximal rates of germination than rich-medium *spoVT* spores and even lower rates at subsaturating nutrient germinant concentrations. The identity of other factors that might modulate spore GR levels in a medium-dependent fashion is not known, but a protein phosphatase, PrpE, has been suggested to modulate rates of spore germination and perhaps GRs in some fashion and also modulate SASP levels in spores (9, 15). It may therefore be worthwhile to examine rates of germination and GR levels in spores of a *prpE* strain.

Certainly one of the striking findings from this work is that the SpoVT regulatory protein can have drastic effects on spore properties. These properties include (i) spore germination by modulating the levels of GRs and perhaps other proteins involved directly or indirectly in spore germination and (ii) spore resistance properties by modifying the spore's level of α/β-type SASPs. Since the SpoVT protein appears to be encoded in all Gram-positive spore formers and its amino acid sequence is very highly conserved, these findings suggest that SpoVT levels during sporulation will be an important determinant of levels of at least α/β-type SASPs and germination proteins such as GRs in spores of both the *Bacillales* and *Clostridiales* orders. As a consequence, it may be important to understand the factors that modulate SpoVT expression and levels, as these may be important in modulating both the resistance and the germination of the resultant spores.

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Capítulo III

Una nueva proteína que se une a RNA polimerasa que controla genes involucrados en la germinación de esporas de *Bacillus subtilis*

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A novel RNA polymerase-binding protein controlling genes involved in spore germination in *Bacillus subtilis*

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Summary

A growing class of proteins regulates transcription through interaction with DNA-dependent RNA polymerase. Here we report that a recently identified, highly conserved sporulation gene *ylyA* encodes a novel RNA polymerase-binding protein that influences the expression of genes under the control of the late-acting, sporulation sigma factor σ^G in *Bacillus subtilis*. Spores from a *ylyA* mutant exhibited defects in germination corresponding to changes in the levels of membrane receptors for spore germinants and a protein channel governing the release of dipicolinic acid and hydration of the spore core during germination. Purified YlyA interacted with RNA polymerase and stimulated transcription from promoters dependent on σ^G but not promoters dependent on the housekeeping sigma factor σ^A. YlyA is a previously unrecognized RNA polymerase-binding protein that is dedicated to modulating the expression of genes involved in spore germination.

Introduction

Bacillus subtilis undergoes a complex process of cellular differentiation in response to changes in the environment that culminates in the formation of a dormant cell type known as the endospore (or simply spore). Spore formation takes place in a two-chamber sporangium consisting of a forespore and a mother-cell compartment. The forespore, which is nurtured by the mother cell, matures into the spore and is ultimately released by lysis of the mother cell. Through membrane-embedded receptors, mature spores monitor the environment, and are capable of rapidly germinating and resuming growth in response to small molecule germinants (Setlow, 2003). Spores of *B. subtilis* contain three germinant receptors (i.e. GerA, GerB and GerK), each produced from a single tricistronic mRNA. Germinant receptors are present at moderate abundance in spores, and their levels are a major determinant of the rate of germination (Paidhungat and Setlow, 2000; Cabrera-Martinez *et al.*, 2003). Binding of a germinant to one of the germinant receptors triggers the release of the large depot of pyridine-2, 6-dicarboxylic acid [dipicolinic acid (DPA)] from the spore, which results in initiation of the process of full hydration of the spore core, ultimately enabling the spore to resume enzymatic activity (Setlow, 2006). Many of the proteins encoded by the *spoVA* operon are required for the initial uptake of DPA during sporulation and are also involved in the release of DPA during germination (Tovar-Rojo *et al.*, 2002; Vepachedu and Setlow, 2007; Li *et al.*, 2012). Here we report the identification of a novel regulatory protein involved in the expression of genes governing germination.

The gene regulatory programme of sporulation is governed in part by the successive appearance of four sporulation-specific sigma factors, appearing in the order σ^F, σ^E, σ^G and σ^K. The activities of these sigma factors are confined to the forespore (σ^F and σ^G) or mother cell (σ^E and σ^K) compartments (Losick and Stragier, 1992). The late-acting forespore-specific sigma factor σ^G activates the expression of approximately 100 genes, including the gene for σ^G itself (*sigG*) and SpoVT (Wang *et al.*, 2006). SpoVT is a DNA-binding protein that can act as both a positive and a negative regulator of σ^G-dependent transcription (Bagyan *et al.*, 1996; Wang *et al.*, 2006). Genes under the control of σ^G and SpoVT include the operons coding for all three of the germinant receptors and the *spoVA* operon.

We and others recently used gene conservation as an alternative approach to identify previously unrecognized sporulation genes (Abecasis *et al.*, 2013; Traag *et al.*, 2013). One gene identified using this approach, *ylyA*, is expressed under the control of the forespore-specific transcription factors σ^G and SpoVT, and deletion of *ylyA* resulted in a defect in the efficiency of spore germination (Traag *et al.*, 2013). *ylyA* was previously suggested to

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Fig. 1. Alignment of the amino acid sequences of DksA-like proteins. CLUSTALW was used to align the amino acid sequence of *E. coli* and *A. tumefaciens* DksA, and *B. subtilis* YlyA, YocK and YteA. Identical residues (*), conserved substitutions of residues with similar properties (:), and semi-conserved substitutions of residues with similar steric confirmations (.) are indicated below the alignment. The N-terminal α-helix coiled-coil (light grey) and the C-terminal Cys-4 zinc finger motif (dark grey) are indicated above the alignment. The conserved residues of the DxxDxA motif, and the conserved first cysteine residue of the Cys-4 zinc finger motif are shaded in grey.

have a low level of sequence similarity to *Escherichia coli dksA* (Krasny and Gourse, 2004). Members of the DksA family transcriptional regulators, which are highly conserved among Gram-negative bacteria, are thought to interact directly with the secondary channel of RNA polymerase and modulate transcription in conjunction with the nucleotide alarmones guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (Srivatsan and Wang, 2008). This prompted us to investigate a potential role for *ylyA* in modulating sporulation-specific gene expression. Here we show that YlyA influences the expression of certain σ^G-controlled genes, including genes involved in germination and that a mutant lacking YlyA produces spores that exhibit specific defects in spore germination. We also show that YlyA binds to RNA polymerase and stimulates transcription by σ^G-containing RNA polymerase, but not RNA polymerase containing the housekeeping sigma factor σ^A. Thus, YlyA is a conserved sporulation-specific transcription factor that modulates the expression of a specialized subset of genes involved in spore germination through interaction with RNA polymerase.

Results

YlyA shares similarity with members of a family of RNA polymerase-binding proteins

ylyA, together with *yocK* and *yteA*, is one of the three closest homologues of *E. coli dksA* in *B. subtilis* (Krasny and Gourse, 2004; Traag *et al.*, 2013). Although the sequence similarity of YlyA to DksA is weak and mostly limited to the C-terminal part of YlyA (Fig. 1), structure prediction using the Homology Detection & Structure prediction by HMM-HMM comparison open-software programme (HHPred; Soding *et al.*, 2005) suggests that the structure of YlyA is highly related (probability score > 99.9) to the solved three-dimensional structures of DksA from *E. coli* (Perederina *et al.*, 2004) and *Agrobacterium tumefaciens* (DOI:10.2210/pdb2kq9/pdb). DksA consists of two characteristic domains: (i) an N-terminal coiled-coil region with two invariable acidic residues at its tip known as the DxxDxA motif and (ii) N-terminal and C-terminal globular regions including a conserved four cysteine (Cys-4) zinc finger motif (IPR012783) (Perederina *et al.*, 2004). The coiled-coil prediction programme COILS/PCOILS (Lupas

et al., 1991) predicts that the N-terminal region of YlyA indeed forms a coiled-coil. YlyA, however, lacks an N-terminal globular extension upstream of the coiled-coil, and has no acidic residues (and hence lacks the DxxDxA motif) near the predicted tip of the coiled-coil (Fig. 1; note that YocK and YteA each have one of the conserved aspartate residues). In a phylogenetic study of 965 DksA-like proteins, 677 homologues had an intact DxxDxA motif. The remaining sequences, however, contained variations in one or more of the conserved residues, suggesting a possible function distinct from the canonical motif (Furman *et al.*, 2013). Activity has previously only been demonstrated for DksA homologues with this motif intact.

An intact Cys-4 zinc finger motif was found in 561 out of 965 DksA homologues (Furman *et al.*, 2013). The C-terminal motif of YlyA has only a single conserved cysteine residue (Fig. 1), which is insufficient for a monomer of YlyA to co-ordinate zinc. One of the two DksA paralogues from *Pseudomonas aeruginosa* (named DksA2) lacks an intact Cys-4 zinc finger motif and zinc was not found in association with this protein. This variant, however, could functionally substitute for the canonical DksA *in vivo* and *in vitro* (Blaby-Haas *et al.*, 2011). Note that YocK, YteA, and *A. tumefaciens* DksA, to which YlyA was found to be structurally similar, also only contain the first of the four conserved cysteine residues in the motif (Fig. 1). We conclude that *ylyA* encodes a divergent homologue of the DksA family, lacking some of the features characteristic of canonical DksA proteins.

YlyA influences σ^G-directed transcription

The forespore line of gene expression is a hierarchical cascade in which σ^F, the earliest-acting regulatory protein in the forespore, turns on the synthesis of σ^G, which in turn activates the gene for the DNA-binding protein SpoVT. The *ylyA* gene is turned on at the end of this cascade by σ^G in conjunction with SpoVT (Traag *et al.*, 2013). Since YlyA resembles a transcription factor, we tested the effects of deleting and overexpressing *ylyA* on late gene expression in the forespore using fusions of *lacZ* to the promoters for *spoVT* (P_{spoVT}) (Bagyan *et al.*, 1996) and another σ^G-controlled gene *sspB* (P_{sspB}) (Sun *et al.*, 1991). Deleting *ylyA* increased β-galactosidase production from the P_{sspB}–*lacZ* and P_{spoVT}–*lacZ* reporters (Fig. 2A and B). To overexpress *ylyA*, we placed the gene under the control of P_{sspB} itself, which is a strong, σ^G-controlled promoter (Mason *et al.*, 1988). Using the P_{sspB}-*ylyA* overexpression construct, we found that β-galactosidase production from both P_{sspB}–*lacZ* and P_{spoVT}–*lacZ* was significantly decreased (Fig. 2A and B). As a test of whether the observed effects were due to an effect on host cell RNA polymerase, we used cells engineered to produce phage T7 RNA polymerase in the forespore (Camp and

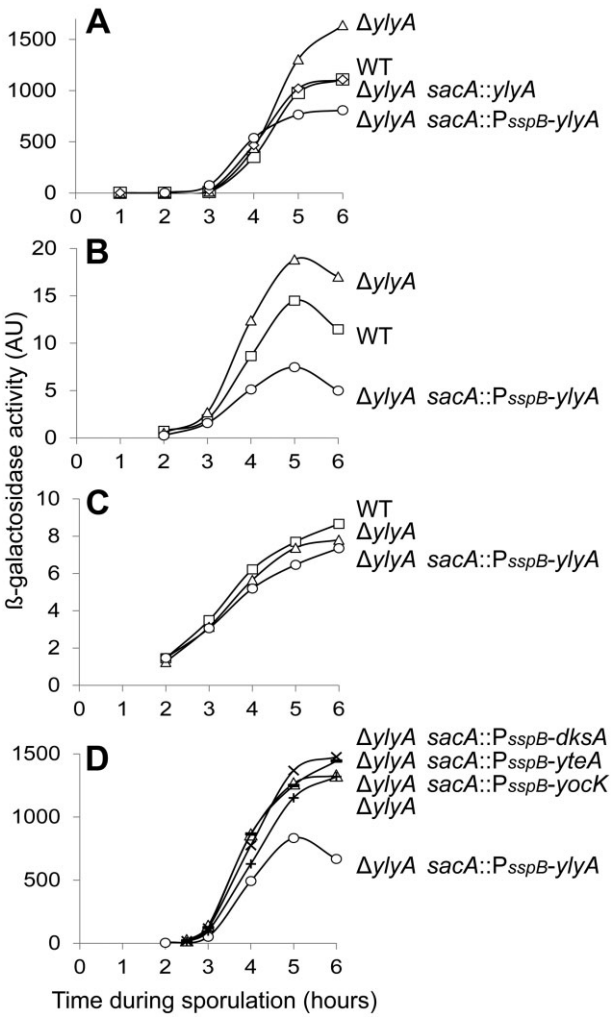

Fig. 2. YlyA exhibits a specific effect on σ^G-dependent transcription. β-Galactosidase activity (arbitrary units; AU) was monitored for samples taken at the indicated time points after sporulation was induced by resuspension.
A. P_{sspB}-directed *lacZ* activity was determined in wild-type cells (squares), *ylyA* mutant cells (triangles), *ylyA* mutant cells carrying a copy of *ylyA* at the ectopic *sacA* locus (diamonds), and *ylyA* mutant cells carrying a copy of *ylyA* expressed from the *sspB* promoter at the ectopic *sacA* locus (circles). All strains carry the P_{sspB}–*lacZ* reporter construct at the *amyE* locus.
B. P_{spoVT}-directed *lacZ* activity was determined in wild-type cells (squares), *ylyA* mutant cells (triangles), and *ylyA* mutant cells carrying a copy of *ylyA* expressed from the *sspB* promoter at the ectopic *sacA* locus (circles). All strains carry the P_{spoVT}–*lacZ* reporter construct at the *amyE* locus.
C. Forespore-specific T7 RNA polymerase-directed P_{T7}–*lacZ* activity was determined in wild-type cells (squares), *ylyA* mutant cells (triangles), and *ylyA* mutant cells carrying a copy of *ylyA* expressed from the *sspB* promoter at the ectopic *sacA* locus (circles). All strains carry the construct expressing T7 RNA polymerase (P_{spoIIQ}-*T7 RNAP*) at the *ylnF* locus, whereas the P_{T7}–*lacZ* reporter gene was integrated at the *ywrK* locus.
D. P_{sspB}-directed *lacZ* activity was determined in *ylyA* mutant cells carrying a copy of *ylyA* (circles), *yocK* (plus sign), *yteA* (minus sign) and *E. coli dksA* (cross) expressed from the *sspB* promoter at the ectopic *sacA* locus (circles).

Losick, 2009). Deleting or overexpressing *ylyA* in cells producing the phage RNA polymerase had little effect on β-galactosidase production from a fusion of *lacZ* to a phage T7 promoter (Fig. 2C).

To determine whether the observed effects were specific to YlyA, we tested the effect of overexpressing the two other *B. subtilis* homologues of *ylyA* (i.e. *yocK* and *yteA*), and *E. coli dksA* on β-galactosidase production from the P_{sspB}–*lacZ* reporter. Whereas overexpression of *ylyA* decreased β-galactosidase production approximately twofold as compared with the *ylyA* null mutant, constructs in which *yocK*, *yteA* or *dksA* were fused to P_{sspB} did not significantly affect P_{sspB}-directed β-galactosidase production (Fig. 2D). We conclude that the inhibitory effect of YlyA on σ^G-directed transcription is specific to YlyA itself.

YlyA influences germination protein levels

We previously reported that spores mutant for *ylyA* are defective in germination in LB medium (Traag *et al*., 2013). To investigate the basis for this germination defect, we examined the relative levels of several germination proteins in wild-type spores and spores from a *ylyA* mutant and from a *ylyA* overexpression strain (P_{sspB}-*ylyA*). *B. subtilis* spores contain three germinant receptors (i.e. GerA, GerB and GerK), which are present at moderate abundance in the inner membrane of the dormant spore (Hudson *et al*., 2001; Paidhungat and Setlow, 2001; Moir, 2006). To determine the relative levels of these proteins we used antibodies against the germinant receptor subunits GerAA, GerAC, GerBC and GerKA (Ramirez-Peralta *et al*., 2012b). We also determined the levels of SpoVAD, one of the seven proteins encoded by the *spoVA* operon, which is required for the accumulation of DPA in the spore core and is involved in its release during germination (Tovar-Rojo *et al*., 2002; Vepachedu and Setlow, 2007), and GerD, which plays a role in rapid response to nutrient germinants (Pelczar *et al*., 2007). GerAA, GerAC, GerKA and GerD levels were relatively unaffected by the *ylyA* deletion. Interestingly, GerBC levels were reduced fourfold in the mutant compared with the wild type, while the levels of SpoVAD were approximately 2.5 times higher (Fig. 3A, Table S2). Overexpression of *ylyA* resulted in decreased levels of all germinant receptor proteins, but did not affect SpoVAD and GerD levels (Fig. 3B, Table S2).

YlyA influences the response of spores to specific germinants

Germinant receptor levels are a major determinant of the rate of spore germination with particular nutrient germinants (Paidhungat and Setlow, 2000; Cabrera-Martinez *et al*., 2003). Our findings predict that *ylyA* mutant spores would be impaired when germination is induced through

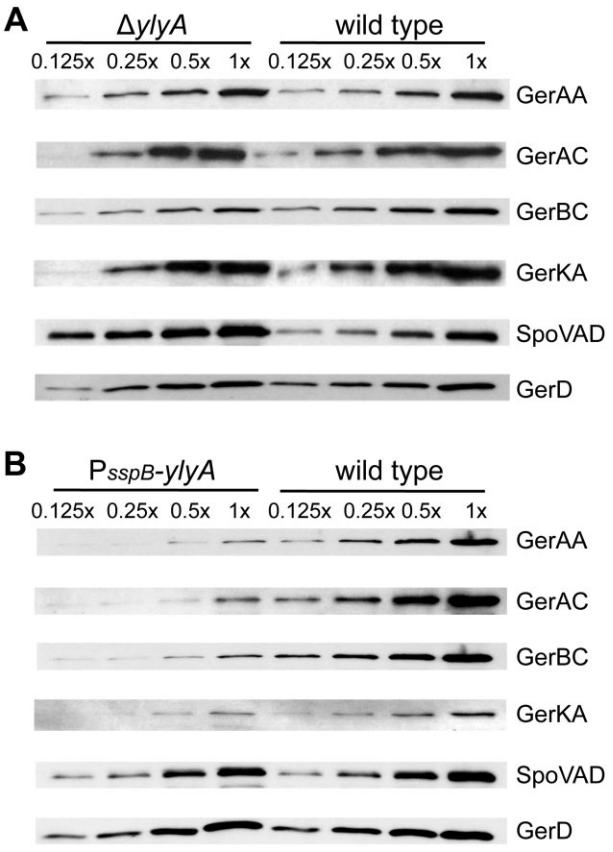

Fig. 3. YlyA influences the levels of germination proteins. Equal amounts of inner membrane protein fractions prepared from purified spores of the wild type, *ylyA* mutant and *ylyA* overexpression strains were subjected to SDS-PAGE and Western blot analysis. Western membranes were probed with antibodies against GerAA, GerAC, GerBC, GerKA, SpoVAD and GerD. For direct comparison, dilutions of samples prepared from wild type and *ylyA* mutant (A) or wild type and *ylyA* overexpression (B) spores were run on the same gel.

the GerB receptor, and that spores from the *ylyA* overexpression strain would be impaired in nutrient germination through any of the three germinant receptors. We induced germination of purified spores using germinants specific to the GerA receptor (i.e. L-valine) or the GerB and GerK receptors [i.e. a mixture of L-asparagine, glucose, fructose, potassium chloride (AGFK)], and measured the rate of germination as the release of DPA. *ylyA* mutant spores and spores from a *ylyA* overexpression strain showed reduced germination rates with both nutrient germinations (Fig. 4A and B). Spores from the *ylyA* overexpression strain in particular showed severe impairment in germination, consistent with the reduced levels of all germinant receptors in these spores. The reduced rate observed for *ylyA* mutant spores with AGFK is in agreement with the lower GerBC levels in these spores. GerAA and GerAC levels were not significantly changed in mutant spores (Fig. 3A, Table S2). The modest germination defect observed for mutant

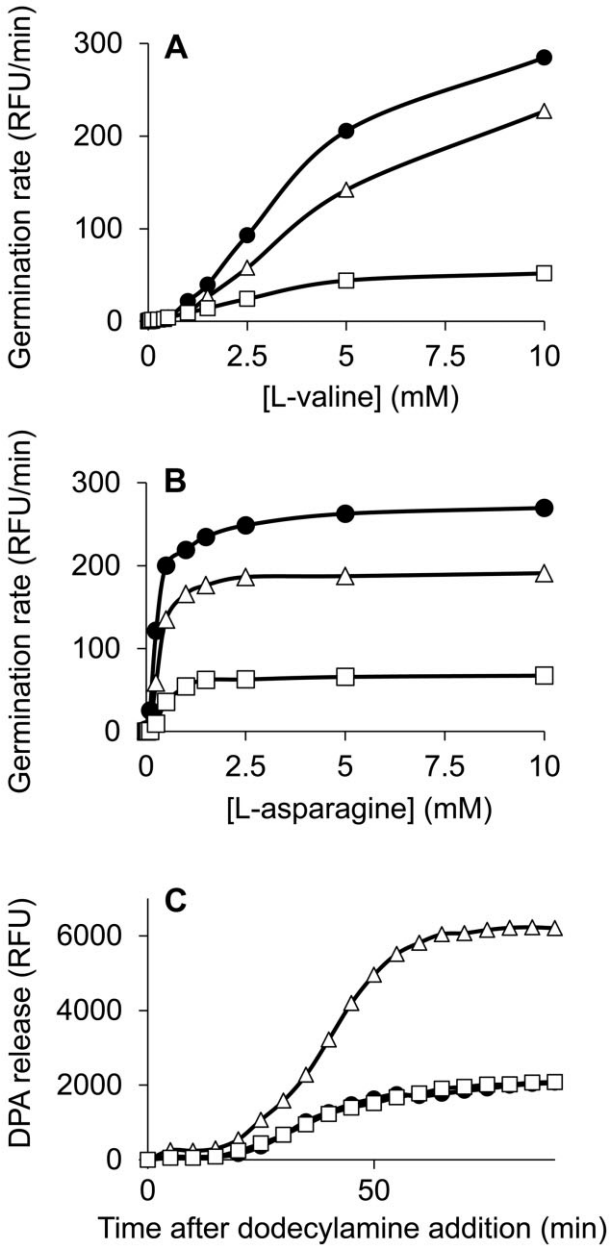

Fig. 4. YlyA affects the efficiency of spore germination.
Germination kinetics of purified spores were determined by measuring the formation of the fluorescent complex between terbium (Tb^{3+}) and the DPA released from spores.
A. Germination was induced with different millimolar concentrations of L-valine, which is specific for the GerA receptor. Results are plotted as the velocity of DPA release [relative fluorescence units per minute (RFU min^{-1})].
B. Germination was induced with different millimolar concentrations of L-asparagine in the presence of 10 mM each of glucose, fructose and potassium chloride (AGFK), which together are specific for the GerB and GerK receptors. Results are plotted as the velocity of DPA release (RFU min^{-1}).
C. Germination was induced with 0.8 mM dodecylamine, which acts on the SpoVA protein channel, and DPA release was measured over time. Results are plotted as the total release of DPA (RFU).
The symbols used are: (●) wild type; (△) *ylyA* mutant; and (□) *ylyA* overexpression.

spores in the presence of L-valine, which acts through the GerA receptor (Moir and Smith, 1990; Atluri *et al*., 2006), therefore possibly reflects changes in additional factors involved in nutrient germination.

Spores can also be induced to germinate through direct activation of the SpoVA protein channel for DPA by non-nutrient germinants such as dodecylamine, triggering the release of DPA, and bypassing the necessity for the germinant receptors. Elevated levels of SpoVA results in faster release of DPA during non-nutrient germination (Vepachedu and Setlow, 2007). We induced germination by addition of the cationic surfactant dodecylamine and monitored the release of DPA over time. Spores from the *ylyA* overexpression strains germinated as efficiently as wild-type spores, while the release of DPA by *ylyA* mutant spores was significantly enhanced (Fig. 4C). These results are in agreement with those expected from the observed effects of YlyA on the levels of SpoVAD (Fig. 3B). Taken together, these results indicate that YlyA modulates the levels of proteins involved in nutrient sensing and DPA release and ensures that spores can germinate efficiently.

Purified YlyA binds to RNA polymerase

Our results indicate that YlyA is a regulator of gene expression and that its activity depends on the host cell RNA polymerase. The similarity of YlyA to DksA, which interacts with RNA polymerase (Paul *et al*., 2004; Perederina *et al*., 2004), motivated us ask if YlyA too interacts with RNA polymerase. To this end, we created a C-terminal, hexa-histidine-tagged YlyA and then passed it over a metal affinity resin. We then applied a soluble protein lysate from sporulating cells of a *ylyA* mutant to the column. After the column was washed, YlyA was eluted from the column with imidazole. Bands seemingly corresponding to the molecular weight of the β, β' and α subunits eluted together with YlyA (Fig. 5A). Western blot analysis confirmed the identity of these bands as RNA polymerase subunits (Fig. 5B). In contrast, the RNA polymerase subunits were not present in the elution fractions when a control protein unrelated to YlyA, namely SinI, was bound to the column (Fig. 5A and B). RNA polymerase from a lysate from vegetatively grown cells was similarly co-immobilized with hexa-histidine-tagged YlyA, indicating that the RNA polymerase-YlyA interaction was not dependent on a sporulation-specific factor (Fig. S1).

YlyA stimulates transcription from σ^G-dependent promoters

The effects of *ylyA* deletion on σ^G-dependent expression *in vivo* and the interaction of YlyA with RNA polymerase *in vitro*, prompted us to investigate the effect of YlyA on transcription in multiple round, *in vitro* transcription assays.

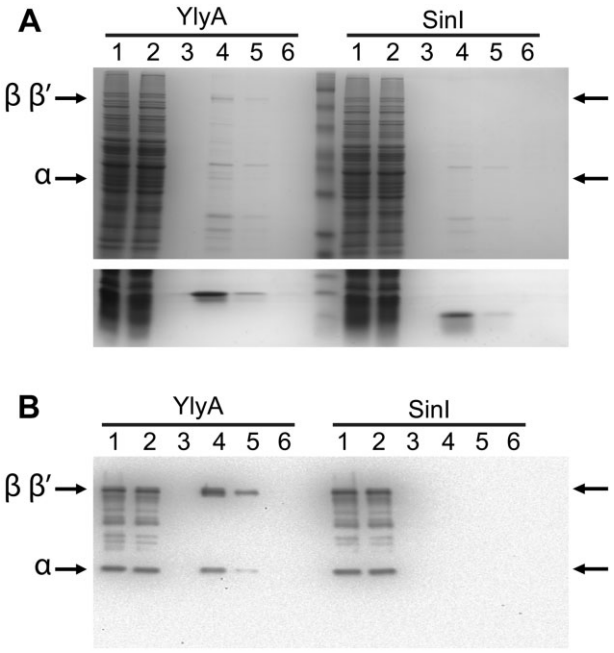

Fig. 5. YlyA interacts with RNA polymerase from a lysate. Recombinant hexahistidine-tagged YlyA or SinI was passed over a metal affinity resin. Next a soluble protein lysate from sporulating cells of a *ylyA* mutant was applied to the column. The column was washed and then eluted with imidazole. Lanes: soluble protein lysate (1), flow-through (2), wash (3), elution fractions (4–6).
A. Top panel: 10% SDS/PAGE gel showing bands corresponding to the β/β' and α subunits of RNA polymerase (indicated by arrows) which co-eluted with YlyA (left half of the gel) but not SinI (right half of the gel); Bottom panel: 15% SDS/PAGE gel showing the bands for YlyA (left) and SinI (right) eluted from the column.
B. Western blot analysis. Bands were transferred to a membrane, and the membrane was probed with antibodies raised against purified *Bacillus* RNA polymerase. Arrows indicate bands corresponding to the β/β' and α subunits of RNA polymerase which co-eluted with YlyA (left half of the gel) but not SinI (right half of the gel).

First, we tested the effect of YlyA on σ^G-dependent transcription using recombinant σ^G and three supercoiled plasmids carrying promoters that were found to be differentially affected by *ylyA in vivo*, namely P_{gerB}, P_{spoVA} and P_{sspB}. At 8 μM, YlyA stimulated the activity from all three σ^G-dependent promoters approximately sixfold (Fig. 6A and D). This effect on transcription was dose-dependent and non-linear, seemingly reaching near saturation at 8 μM YlyA (approximately 800-fold molar excess over RNA polymerase) under these conditions (Fig. 6B). In contrast, recombinant SinI did not have an effect on transcription from any of these promoters (Fig. 6A and D). As a further test of specificity, we investigated the effect of YlyA on transcription from promoters that are dependent on the housekeeping sigma factor σ^A. We tested this using recombinant σ^A and two plasmids carrying σ^A-promoters, namely P_{veg} and $P_{rrnB\,P1}$ [the *E. coli rrnB* P1 promoter was previously found to be inhibited by DksA (Paul *et al.*, 2004)]. Interestingly, YlyA had no effect on transcription using either template (Fig. 6B–D). We conclude that YlyA is a regulator of transcription, and that its activity appears to be specific to the alternative sigma factor σ^G.

Discussion

We describe an RNA polymerase-binding protein, YlyA, that is produced at a late stage of spore formation in *B. subtilis* and that modulates the expression of genes involved in spore germination. Our results suggest that YlyA fine-tunes forespore-specific transcription and indirectly prepares the spore for a future germination event.

YlyA shares some similarity with members of the DksA family of regulators, which are conserved among Gram-negative bacteria. YlyA, however, lacks two features characteristic of studied DksA homologues, namely the N-terminal DxxDxA motif at the coiled-coil tip and the C-terminal Cys-4 zinc finger motif. The zinc finger motif has previously been found to be dispensable for the function of other DksA homologues (Blaby-Haas *et al.*, 2011). Two residues in the DxxDxA motif, namely D74 and A76, are oriented towards the trigger loop in the β′ subunit of RNA polymerase (Lennon *et al.*, 2012). Deletion of the β′ trigger loop or substitution of these essential DksA residues eliminated DksA function in negative and positive control of transcription, without affecting the affinity of DksA for RNA polymerase (Rutherford *et al.*, 2009; Lee *et al.*, 2012; Lennon *et al.*, 2012). YlyA lacks both aspartate residues, and the region around the predicted coiled-coil tip of YlyA orthologues in different *Bacillus* species shows little sequence conservation (Fig. S2), strongly suggesting that these residues are not important for YlyA function, and that YlyA likely acts through a mechanism distinct from that of DksA. Further studies are required to identify residues or motifs important for YlyA activity. Preliminary work with truncated *ylyA* constructs indicates that the C-terminal region including the zinc finger motif is at least partially dispensable *in vivo*, highlighting the importance of the N-terminus for YlyA function (Fig. S3).

The similarity between YlyA and DksA, albeit weak, led us to the finding that recombinant YlyA co-immobilizes RNA polymerase from a crude cell lysate. In addition, YlyA stimulated transcription from three different σ^G-dependent promoters in a reconstituted *in vitro* system using purified RNA polymerase, suggesting that YlyA indeed interacts with RNA polymerase. One possibility is that YlyA, like DksA, interacts with the secondary channel of RNA polymerase. It is entirely conceivable, however, that the weak similarity of YlyA to DksA is adventitious and that it interacts with RNA polymerase at a site distinct from the DksA binding site. Interestingly, YlyA does not act in conjunction with the housekeeping sigma factor σ^A. Instead, YlyA seems to be specific to promoters under the control of the

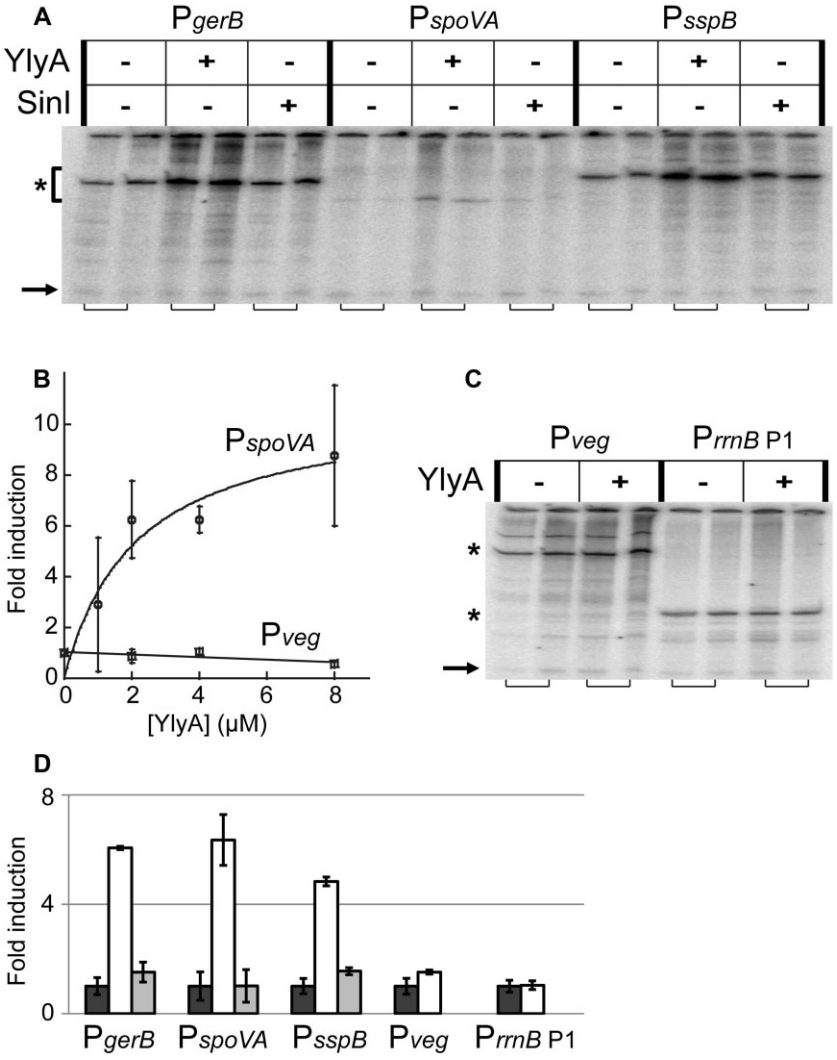

Fig. 6. YlyA stimulates σ^G-dependent, but not σ^A-dependent, promoters *in vitro*. Multiple round *in vitro* transcription assays using 10 nM purified *B. subtilis* RNA polymerase, recombinant sigma factors, and hexahistidine-tagged YlyA or SinI.
A. Experiments with purified σ^G and plasmid templates carrying the σ^G-dependent P_{gerB}, P_{spoVA} or P_{sspB} promoters, showing stimulation by YlyA. Dilution buffer, 8 μM YlyA or 8 μM SinI were included in the reactions. Specific signals are indicated by an asterisk to the left of the gel. Adjacent lanes (indicated by brackets below the gel) are from duplicate reactions. Arrow to the bottom left of the gel indicates the end-labelled probe included during phenol/chloroform extraction and ethanol precipitation (see *Experimental procedures*).
B. The effect of YlyA on transcription with σ^G-dependent P_{spoVA} is dose-dependent. Transcription with σ^A-dependent P_{veg} is unaffected by YlyA. Results are averages from three separate experiments. Error bars indicate standard errors (SEM).
C. Experiments with purified σ^A and plasmid templates carrying the σ^A-dependent P_{veg} or $P_{rrnB\ P1}$ promoters, showing these promoters are unaffected by YlyA. Dilution buffer, 8 μM YlyA or 8 μM SinI were included in the reactions. Specific signals are indicated by asterisks to the left of the gel. Adjacent lanes (indicated by brackets below the gel) are from duplicate reactions. Arrow to the left of the gel indicates end-labelled probe included during phenol/chloroform extraction and ethanol precipitation.
D. Quantification of the specific signals from gels from (A) and (B). Values are averages from the two duplicate experiments. Average signal from the lanes without YlyA or SinI added were set to one, and fold induction with protein added is given relative to that. Error bars indicate standard errors (SEM).

sporulation-specific sigma factor σ^G. (We cannot distinguish whether YlyA is truly specific to σ^G-containing RNA polymerase or whether the three promoters tested have some distinctive feature other than sigma factor recognition elements that distinguishes them from the σ^A-controlled promoters tested.) Importantly, then, YlyA is dedicated to the transcription of a specialized set of genes involved in spore germination. These include the operons coding for the GerB germinant receptor and the SpoVA channel for the uptake and release of DPA.

A complication in our analysis is that YlyA stimulated transcription from all σ^G-controlled promoters tested *in vitro*, even promoters of genes that were upregulated in *ylyA* mutant cells. Thus, and as expected, YlyA stimulated transcription from the *gerB* promoter, which correlates to the decrease in GerBC levels in the absence of the RNA polymerase-binding protein *in vivo*. On the other hand, YlyA also stimulated transcription from P_{sspB} and P_{spoVA}, whose levels of expression *in vivo* were higher in the absence of YlyA than in its presence. Conceivably, the precise biochemical conditions we used to carry out our *in vitro* transcription experiments did not faithfully mimic the conditions for RNA synthesis in the late-stage, forespore. Alternatively, however, the discrepancy between our *in vitro* and *in vivo* results may be apparent and reflect the complicated, multi-component, feedback circuit operating at the terminal stage of gene expression in the forespore (Fig. 7). This circuit involves σ^G, which stimulates the transcription of the gene (*sigG*) for σ^G itself, and the DNA-binding protein SpoVT (Wang *et al.*, 2006), which, in turn stimulates or represses the expression of other σ^G-controlled genes, including activating *ylyA* and repressing *spoVT* and *sigG* (Bagyan *et al.*, 1996; Wang *et al.*, 2006; Traag *et al.*, 2013). Further complicating this picture, deletion of *ylyA* results in elevated *spoVT* expression. This suggests that, in the absence of YlyA, SpoVT becomes the dominant modulator of σ^G-directed gene expression, resulting in upregulation of genes activated by SpoVT (e.g.

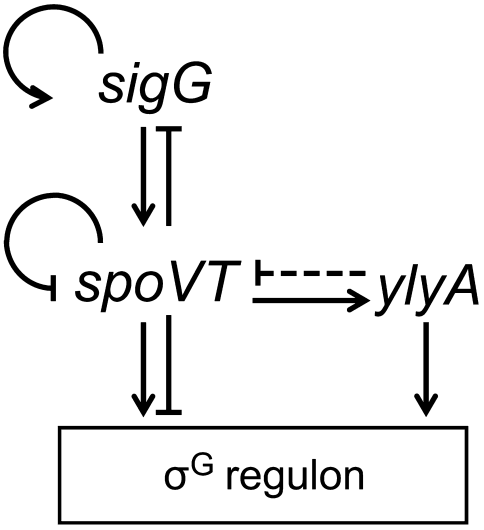

Fig. 7. The multi-component feedback circuit regulating the σ^G regulon. σ^G, encoded by the *sigG* gene, stimulates the transcription of *sigG* and *spoVT*, encoding the DNA-binding protein SpoVT. SpoVT in turn stimulates or represses the expression of other σ^G-controlled genes, including activating *ylyA* and repressing *spoVT* and *sigG*. YlyA interacts with RNA polymerase and stimulates σ^G-dependent gene expression. Through an unknown mechanism, *spoVT* gene expression is upregulated in cells lacking YlyA (indicated by the dashed repressor symbol from *ylyA* to *spoVT*), resulting in the upregulation of genes activated by SpoVT (e.g. *sspB* and *spoVA*) and downregulation of genes repressed by SpoVT (e.g. *gerB*).

sspB and *spoVA*) and downregulation of genes repressed by SpoVT (e.g. *gerB*). We therefore speculate that YlyA is a stimulator of σ^G-directed transcription (as we observed biochemically) but that the net *in vivo* output of individual promoters depends on the complex interplay among σ^G, SpoVT and YlyA.

In conclusion, YlyA is a novel RNA polymerase-binding protein that is conserved among endospore-forming bacteria and dedicated to the transcription of late-activated sporulation genes involved in germination of the mature spore.

Experimental procedures

General methods

Escherichia coli strain DH5α was used for propagating plasmids, and grown and transformed using standard procedures (Sambrook *et al*., 1989). *E. coli* BL21 (DE3) was used for the expression and purification of recombinant proteins. Details on plasmid sources and construction can be found in Supporting Information (SI) Experimental Procedures. *B. subtilis* strains used in this work are listed in Table S1. Transformation of *Bacillus* was done as previously described (Wilson and Bott, 1968). Spores were purified from liquid Difco sporulation (DS) medium as previously described (Nicholson and Setlow, 1990).

β-Galactosidase activity assays

Sporulation of strains carrying *lacZ* reporter constructs was induced by resuspension in Sterlini–Mandelstam (SM) medium (Nicholson and Setlow, 1990), and samples were collected at various time points after induction. Activity was measured in a Synergy 2 plate reader (BioTek) as previously described (Camp and Losick, 2009). β-galactosidase activity is reported in arbitrary (AU) units as the rate of 2-nitrophenyl β-D-galactopyranoside (ONPG) hydrolysis (i.e. V_{max}, with units of OD_{420} per minute) divided by the optical density at 600 nm (OD_{600}) of the culture at the time of collection.

Determination of the levels of spore germination proteins

Levels of germinant receptor subunits, GerD and SpoVAD were determined in the inner membrane fraction of spores of different strains by Western blot analyses using rabbit antibodies against these proteins and a secondary antibody as described previously (Paidhungat and Setlow, 2001; Ramirez-Peralta *et al*., 2012a,b). Quantitative comparisons of relative levels of various proteins were made using the ImageJ program, and the overall analysis was carried out with replicate blots on two independent spore preparations.

Spore preparation, purification and germination

Spores of various strains were prepared at 37°C on 2× Schaeffer's glucose agar plates, harvested, purified and stored as described previously (Nicholson and Setlow, 1990). All spores used in this work were free (> 98%) of growing or sporulating cells, germinated spores and cell debris as determined by phase-contrast microscopy. Spores at an OD_{600} of 5–10 were heat-activated at 70°C for 30 min, cooled on ice for at least 15 min, and germinated at an OD_{600} of 0.5 in the presence of various concentrations of L-valine or L-asparagine, or 0.8 mM dodecylamine, at 37°C (nutrient) or 45°C (dodecylamine), as previously described (Yi and Setlow, 2010). The incubations with L-asparagine additionally contained 10 mM each of D-glucose, D-fructose and KCl. The kinetics of spore germination in these incubations was followed by measurement of DPA release by Tb-DPA fluorescence in a multi-well fluorescence plate reader as described previously (Yi and Setlow, 2010). All germination experiments were repeated at least twice with two independent spore preparations with essentially identical results.

Protein expression and purification

Escherichia coli BL21 (DE3) derivative strains were used for the overexpression and purification of recombinant YlyA, SinI, σ^G and σ^A. The tagged variant of YlyA could functionally replace native YlyA *in vivo*. *B. subtilis* strain RL5493, which encodes a hexahistidine-tagged β′ subunit as the only copy in the cell, was constructed, and *B. subtilis* RNA polymerase was purified from crude lysates similar to previously described (Anthony *et al*., 2000). Protein purification was done using one-step Ni-NTA (Qiagen) affinity chromatogra-

phy purification protocols. Details on the construction of RL5493 and the purifications can be found in SI Experimental Procedures.

Multiple-round in vitro *transcription*

Supercoiled plasmids carrying promoter fragments of *gerB* (–120/+150 relative to the transcription start site), *spoVA* (–71/+92), *sspB* (–220/+151), *veg* (–334/+185), *rrnB* P1 (–58/+1), used as templates for *in vitro* transcription, are derivatives of pRLG770 (Ross *et al*., 1990). Multiple-round *in vitro* transcription was done similar to previously described (Lee *et al*., 2012), with some differences. In brief, 10 nM reconstituted RNA polymerase holoenzyme (*B. subtilis* RNA polymerase core and appropriate sigma factor in a 1:10 ratio) was incubated with buffer or micromolar concentrations of YlyA or SinI for 30 min at room temperature. Transcription was initiated by the addition of template (0.5 nM) and NTP (500 μM ATP, 200 μM GTP and CTP, 10 μM UTP/1 μCi [α-^{32}P]-UTP), and incubated for 15 min at 30°C. For *rrnB* P1 the initiating nucleotide is GTP, and therefore the concentrations of ATP and GTP were reversed. Reactions were terminated after 15 min by extraction with phenol/chloroform. At this stage a single-stranded end-labelled probe was included to ensure that the extraction and precipitation of all samples was similar. The aqueous phase was ethanol precipitated in the presence of glycogen, and the pellet was washed with 70% ethanol, dried, and resuspended in buffer containing 95% formamide and 20 mM EDTA. Samples were run on 7 M urea-6% polyacrylamide gels, and visualized and quantified by phosphorimaging. Multiple-round *in vitro* transcription experiments were repeated at least twice in duplicate for all tested promoter fragments.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Capítulo IV

Identificación de nuevas proteínas que modulan la germinación de esporas de especies de *Bacillus subtilis*

Identification of New Proteins That Modulate the Germination of Spores of Bacillus Species

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Identification of New Proteins That Modulate the Germination of Spores of *Bacillus* Species

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A number of operons encoding the nutrient germinant receptors (GRs) in dormant spores of *Bacillus megaterium* and *Bacillus subtilis* species have small open reading frames (ORFs) of unknown function within or immediately adjacent to the operons. Inactivation of the genes in these ORFs, encoding proteins now termed D proteins, either significantly increased or decreased spore germination via the associated GR but had no effects on germination via non-GR-dependent germinants. These effects on GR-dependent germination were complemented by ectopic expression of the appropriate *D* gene (gene encoding D protein). However, substitution of noncognate *D* genes in two GR operons resulted in inhibition of germination via the GR manipulated, although ectopic overexpression of a *D* gene had no effect on overall GR-dependent germination. The various *D* genes studied were expressed in the forespore during sporulation in parallel with the associated GR operon, and transcription of a *B. subtilis D* gene was controlled by RNA polymerase sigma factor σ^G. These results indicate that proteins encoded by small ORFs within or adjacent to operons encoding GRs play major roles in modulating GR function in spores of *Bacillus* species. In *B. subtilis*, deletion of a *D* gene (*B. subtilis gerKD* [*gerKDbs*]) adjacent to the *gerK* operon encoding the GerK GR or ectopic expression or overexpression of *gerKDbs* had no major effect on the levels of GR subunits or of two other germination proteins.

Spores of various *Bacillus* species are metabolically dormant and extremely resistant to a variety of harsh treatments (1). As a consequence, such spores can survive for years in the absence of exogenous nutrients. However, these spores constantly sense their environment, and if nutrients become available, spores can rapidly return to life in the process of germination followed by outgrowth (2, 3). Nutrients generally trigger spore germination through their interactions with proteins called germinant receptors (GRs) located in the inner membranes of spores, and *Bacillus* spores most often have multiple GRs, each with a different specificity for a nutrient germinant or nutrient germinant mixture.

GRs are thought to be composed of three protein subunits, termed A, B, and C, with the A and B subunits likely being integral membrane proteins and the C subunit likely being a lipid-anchored peripheral membrane protein. By far the best-studied *Bacillus* species is *Bacillus subtilis*, and spores of this species contain five operons, each encoding the three subunits of similar GRs. These operons are expressed most likely in parallel late in sporulation in the developing spore and under the control of the forespore-specific RNA polymerase sigma factor σ^G (2–6). The functions of two of these GRs, those encoded by the *yndEFG* and *yfkQRST* operons are not known, as deletions of either one or both of these operons have no effect on spore germination, even in the absence of the other three GRs (7). The GerA GR responds specifically to L-alanine or L-valine alone, while the GerB and GerK GRs are required together for germination with a mixture of L-asparagine (or L-alanine) plus D-glucose, D-fructose, and K^+ ions called AGFK (3, 8). In *B. subtilis*, all three GR subunits are required for a particular GR's function, and GRs are localized together in a cluster termed the germinosome in the inner membranes of spores, with this clustering essential for rapid spore germination (9).

Bacillus megaterium belongs to a deeply rooted phylogenetic lineage within the *Bacillus* genus, being quite distinct from members of the *Bacillus subtilis* and *Bacillus cereus* families. Its genome contains six GR operons, the best studied of which is the plasmid-borne *gerU* operon, which encodes a GR that can trigger spore germination in response to glucose, proline, leucine, or certain inorganic salts (10, 11). The *gerU* operon is unusual in that products of three different genes that encode GR B-subunit proteins have been shown to interact with the products of the *gerUA* and *gerUC* genes to produce GRs with overlapping germinant recognition patterns, demonstrating at the same time a rarely observed interchangeability between GR subunits. Of the five chromosomal GR operons, four have been characterized as being functional via mutagenesis analyses (36). However, whereas the GerU receptor can trigger germination in response to single germinant compounds, the chromosomally encoded GRs all appear to require combinations of germinants—typically glucose plus inorganic salts and certain amino acids—to promote efficient germinative responses.

Recently, the presence of a fourth protein that might be a component of at least some GRs was suggested in a bioinformatic analysis of genes encoding GRs in both *Bacillus* and *Clostridium* species (2). This putative GR component, termed a D protein, is a 60- to 85-amino-acid (aa) protein encoded by a gene either within or adjacent to a GR operon. In the current work, we have exam-

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TABLE 1 *Bacillus* strains used in this study

Bacillus strain	Relevant genotype or phenotype or description[a]	Reference or source
B. subtilis strains		
AM1247[b]	*gerB-lacZ*; MLS^r	12
PS533	Wild-type; Km^r	34
PS767	*gerA-lacZ*; MLS^r	4
PS832	Wild-type prototroph; laboratory strain	
PS3709	*gerB-lacZ*; MLS^r	This work
PS4256	Δ*gerKDbs*; Tc^r	This work
PS4290	*gerKDbs-lacZ*; Cm^r	This work
PS4293[c]	*gerKDbs-lacZ* Δ*sigE*; Cm^r MLS^r	This work
PS4294[c]	*gerKDbs-lacZ* Δ*sigF*; Cm^r	This work
PS4295[c]	*gerKDbs-lacZ* Δ*sigKbs*; Cm^r MLS^r	This work
PS4296[c]	*gerKDbs-lacZ* Δ*sigG*; Cm^r Km^r	This work
PS4313	Δ*gerKDbs amyE*::*gerKDbs*; Cm^r Tc^r	This work
PS4314	*amyE*::P*sspB-gerKDbs*; Cm^r	This work
PS4319	*gerA-lacZ amyE*::P*sspB-gerKDbs*; Cm^r MLS^r	This work
PS4320	*gerB-lacZ amyE*::P*sspB-gerKDbs*; Cm^r MLS^r	This work
PY79[c]	Wild-type	R. Losick
SC137[c]	Δ*sigE*; MLS^r	35
SC1159[c]	Δ*sigF*	35
SC64[c]	Δ*sigK*; MLS^r	35
RL831[c]	Δ*sigG*; Km^r	35
B. megaterium strains		
QM B1551	Wild-type	P. S. Vary
PV361	Plasmidless derivative of QM B1551; Δ*gerU*	P. S. Vary
GC614[d]	GR null (Δ*gerU* Δ*gerKbm* Δ$gerK_2$ Δ$gerA$ Δ$gerA_2$); Km^r MLS^r Cm^r Sp^r	36
GC615[e]	GR null pHT-*gerUD gerU**; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC630[e]	GR null pHT-Δ*gerUD gerU**; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC631[e]	GR null pHT-$gerUD^{M10stop}$ *gerU**; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC632[e]	GR null pHT-$gerUD^{M10stop}$ *gerU**, with *gerUD* located between *gerUC* and *gerVB*; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC633[e]	GR null pHT-*gerUD gerU**, with *gerKDbm* located between *gerUC* and *gerVB;* Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC634[e]	GR null pHT-$gerUD^{M10stop}$ *gerU**, with *gerKDbm* located between *gerUC* and *gerVB*; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC635[e]	GR null pHT-*gerKbm*; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC636[e]	GR null pHT-*gerKbm* Δ*gerKDbm*; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC637[e]	GR null pHT-*gerKbm*, with *gerUD* in place of *gerKDbm*; Km^r MLS^r Cm^r Sp^r Tc^r	This work
GC638[f]	*gerUA-lacZ*; Km^r	36
GC639[f]	*gerUD-lacZ*; Km^r	This work
GC640[f]	*gerKAbm-lacZ*; Km^r	36
GC641[f]	*gerKDbm-lacZ*; Km^r	This work

[a] Abbreviations for antibiotic resistance: Km^r, kanamycin resistance (5 and 10 μg/ml for *B. megaterium* and *B. subtilis*, respectively); Sp^r, spectinomycin resistance (100 μg/ml); Cm^r, chloramphenicol resistance (5 μg/ml); MLS^r, resistance to erythromycin (1 μg/ml) plus lincomycin (25 μg/ml); Tc^r, tetracycline resistance (12.5 μg/ml).
[b] This strain has the SC64 background.
[c] These strains have the PY79 genetic background.
[d] This strain is isogenic with PV361.
[e] These strains have the GC614 background and were transformed to Tc^r with plasmid pHT315t carrying the described receptor genes. *gerU**, GR operon comprising *gerUA*, *gerUC*, and *gerVB*.
[f] QM B1551 genetic background.

ined the expression, location, and function of the putative GR D proteins in both *Bacillus megaterium* and *B. subtilis* and present strong evidence that these new proteins can modulate rates of GR-dependent spore germination.

MATERIALS AND METHODS

***Bacillus* strains.** The *Bacillus* strains used in this work are listed in Table 1. *B. subtilis* strains are derivatives of strain 168 and are isogenic derivatives of strain PS832. The *B. subtilis* strain with a *gerB-lacZ* fusion in the PS832 background was generated by transformation of strain PS832 to MLS^r (resistance to erythromycin plus lincomycin) with chromosomal DNA from strain AM1247 (12), giving strain PS3709. A *B. subtilis* strain with much of the *B. subtilis gerKD* (hereafter termed *gerKDbs*) coding sequence replaced by a tetracycline resistance (Tc^r) cassette was constructed as follows. The Tc^r cassette was PCR amplified from plasmid pFE149 (7), and the upstream region of the *gerKDbs* gene (−500 to +19 relative to the *gerKDbs* translation start site [+1]) and the downstream region of the *gerKDbs* gene (+222 to +721) were PCR amplified from chromosomal DNA of strain PS832. The latter two PCR products plus the amplified Tc^r cassette were used for a three-way overlap PCR, and the product was purified and ligated to pGEM-T Easy vector (Promega Corp., Madison, WI) to generate plasmid pXY1223 in *Escherichia coli*. The *tet* gene's promoter is oriented in the direction opposite that of the *B. subtilis gerK* (hereafter termed *gerKbs*) operon to minimize the effects of transcription of the *tet* gene on transcription of the *gerKbs* operon. Plasmid pXY1223 was used to transform *B. subtilis* strain PS832 to Tc^r by a double-crossover event, giving strain PS4256; the expected chromosome structure in the *gerKDbs* region of this strain was confirmed by PCR.

A *B. subtilis* strain with a transcriptional fusion of the putative promoter region of *gerKDbs* to the *E. coli lacZ* gene was constructed as follows. The region between bp −447 to −1 relative to the putative *gerKDbs* translation start site (+1) was PCR amplified from *B. subtilis* PS832 DNA using primers containing EcoRI and BamHI sites (all primer sequences are available on request). The purified PCR product was digested with EcoRI and BamHI and then ligated to plasmid pDG268 (13) that was digested with the same restriction enzymes. The resulting plasmid, pPS4289, was isolated in *E. coli* and used to transform various *B. subtilis* strains to a chloramphenicol-resistant (Cm^r) amylase-negative phenotype by a double-crossover event at the *amyE* locus.

A *B. subtilis* strain expressing *gerKDbs* at *amyE* in a Δ*gerKDbs* background with the *gerKDbs* gene expressed from its own likely promoter (see Results) was constructed as follows. A DNA fragment from −500 to +350 bp relative to the *gerKDbs* translation start site (defined as +1), and encompassing the likely *gerKDbs* promoter (see Results) and its coding sequence, was amplified from strain PS832 chromosomal DNA with primers containing an upstream EcoRI site and a downstream BamHI site. The PCR product was purified, digested with EcoRI and BamHI, and ligated to similarly cut plasmid pDG364 (14). The recombinant plasmid was isolated in *E. coli* and used to transform strain PS4256 (Δ*gerKDbs*) to a Cm^r amylase-negative phenotype, giving strain PS4313. The double-crossover event leading to disruption of *amyE* by the *gerKDbs* gene plus the Cm^r cassette in strain PS4313 was confirmed by PCR.

To construct a *B. subtilis* strain in which *gerKDbs* expression was under the control of the strong forespore-specific P*sspB* promoter (4), the region from −500 to −1 bp relative to the translation start site of the *B. subtilis sspB* gene (this region has P*sspB* as well as a strong ribosome binding site [RBS]) was amplified from PS832 DNA using primers with an EcoRI site in the upstream primer and a region of overlap with the *gerKDbs* translation start site in the downstream primer. The upstream primer for *gerKDbs* amplification was complementary to the downstream primer for P*sspB*, and the downstream *gerKDbs* primer would amplify the complete *gerKDbs* coding region plus 112 downstream bp that should include the likely *gerKDbs* transcription terminator and a 3′ BamHI site. For overlap PCR, we amplified a product that has a P*sspB* promoter plus RBS just upstream of the *gerKDbs* coding region and between the EcoRI and BamHI sites, using the amplified P*sspB* promoter and *gerKDbs* fragments as the template. The overlap PCR product of the expected size was purified, digested with EcoRI and BamHI, and ligated to similarly cut plasmid pDG364 and the recombinant plasmid was isolated in *E. coli*. This plasmid was used to transform *B. subtilis* strain PS832 to a Cm^r amylase-negative phenotype by a double-crossover event, giving strain PS4314, and the expected genomic structure in the *amyE* region of this strain was confirmed by PCR. Chromosomal DNA from strain PS4314 was also used to transform strains PS767 and PS3709 to a Cm^r MLS^r amylase-negative phenotype, and the resultant strains were termed PS4319 and PS4320, respectively.

B. megaterium strain GC614, which is isogenic with the plasmidless QM B1551 derivative PV361 and lacks all functional GRs due to insertion-deletions in the A-cistrons of four respective GR loci and excision of the *gerU*-containing plasmid pBM700 (36), was the host strain for plasmid-based complementation analyses. A plasmid containing the entire *B. megaterium gerK* operon (hereafter referred to as *gerKbm*) plus upstream promoter sequence was prepared by ligating a 4.7-kb PCR fragment, amplified from QM B1551 genomic DNA using primers with BamHI sites at the 5′ ends (all primer sequences are available on request), with plasmid pHT315t (modified from the plasmid in reference [15]) digested with the same enzyme. The resulting plasmid, pHT-*gerKbm*, was purified from *E. coli* and used to transform *B. megaterium* GC614 to Tc^r, giving strain GC635. The same plasmid served as the template for an inverse PCR using primers designed to remove the entire *gerKDbm* open reading frame (ORF), which upon blunt-end religation would leave a 20-bp region between the stop codon of *gerKCbm* and the predicted translational start site of *gerKBbm*; the latter is preceded by an appropriately positioned RBS. The resultant plasmid, pHT-(*gerKbm* Δ*gerKDbm*), was isolated from *E. coli*, verified by DNA sequencing, and used to transform strain GC614 to Tc^r, giving strain GC636.

Construction of a pHT315-based plasmid containing the *gerU*⋆ (*gerUA*, *gerUC*, and *gerVB*) receptor operon has been described previously (16). The cloned locus contains approximately 400 bp of sequence upstream of the predicted *gerUA* translational start site, within which the putative *gerUD* ORF is located. This entire region was amplified by PCR using primers with BamHI sites and ligated with plasmid pHT315t digested with the same enzyme to prepare plasmid pHT-(*gerUD gerU*⋆), which was compatible with complementation experiments in the multi-antibiotic-resistant GC614 genetic background. A plasmid in which the entire *gerUD* ORF was deleted, but leaving the predicted *gerUA* promoter intact, was prepared by PCR amplification of the region comprising −132 bp relative to the *gerUA* translational start site to ~200 bp downstream of the predicted *gerVB* stop codon. This DNA fragment, which had flanking BamHI restriction sites, was digested and ligated with pHT315t digested with the same enzyme to prepare plasmid pHT-Δ*gerUD gerU*⋆ which was used to transform strain GC614 to Tc^r, giving strain GC630. Site-directed mutagenesis (SDM), conducted with a QuikChange Lightning SDM kit (Agilent Technologies, Wokingham, United Kingdom), was used to prepare plasmid pHT-$gerUD^{M10stop}$ *gerU*⋆, in which the methionine encoded by predicted codon 10 of the *gerUD* ORF, was changed to a stop codon. This plasmid, designed to result in the expression of a severely truncated GerUD protein while minimizing potential disruption to the *gerUA* promoter, was used to transform strain GC614 to Tc^r, giving strain GC631.

The Gibson Assembly technique (New England BioLabs, Hitchin, United Kingdom) was used to prepare plasmids in which either *gerUD* or *gerKDbm* were located between the *gerUC* and *gerVB* ORFs within the *gerU*⋆ operon. First, a PCR fragment spanning 444 bp with respect to the *gerUA* translational start site to position 2826, which included ORFs for $gerUD^{M10stop}$, *gerUA*, and *gerUC*, was prepared using plasmid pHT-$gerUD^{M10stop}$ *gerU*⋆ as the template DNA. A second PCR fragment comprising the *gerVB* ORF plus 104 bp upstream of the *gerVB* translational start site and 200 bp downstream of the stop codon was prepared from the same plasmid. Finally, PCR fragments spanning the *gerUD* and *gerKDbm* ORFs were prepared from genomic DNA. The appropriate fragments, including BamHI-linearized pHT315t vector backbone were subsequently purified and assembled using Gibson Assembly master mix (New England BioLabs, Hitchin, United Kingdom), and the reaction mixtures were used to transform *E. coli*. Purified and sequence-validated pHT-(*gerUD*^{M10stop} *gerUA gerUC gerUD gerVB*) was used to transform strain GC614 to Tc^r, giving strain GC632. Similarly, plasmid pHT-(*gerUD*^{M10stop} *gerUA gerUC gerKDbm gerVB*) was used to construct strain GC634. A similar approach was used to prepare a plasmid in which the *gerKDbm* ORF was located between *gerUC* and *gerVB* in a *gerU*⋆ operon with the intact *gerUD* gene, serving as the basis of strain GC633. The Gibson Assembly technique was also used to create a pHT315-based plasmid encoding a modified *gerKbm* operon, in which the *gerKDbm* ORF was replaced with the *gerUD* ORF. Essentially, PCR fragments encoding (i) *gerKAbm* and *gerKCbm* ORFs plus upstream promoter sequence, (ii) the *gerUD* ORF, and (iii) the *gerKBbm* ORF plus 200 bp of downstream sequence were amplified from *B. megaterium* QM B1551 genomic DNA. The PCR fragments, plus BamHI-linearized pHT315t vector, were purified and then assembled with Gibson Assembly master mix. Plasmid pHT-(*gerKAbm gerKCbm gerUD gerKBbm*) was isolated from *E. coli*, verified by PCR and sequencing, and used to transform *B. megaterium* GC614 to Tc^r, giving strain GC637.

B. megaterium strains with transcriptional fusions between the *E. coli lacZ* gene and either the *gerU* or *gerKbm* A and D genes (genes encoding the A and D proteins) were prepared essentially as described previously (17). PCR was used to amplify ~500-bp DNA fragments starting at the predicted translational start sites for *gerUA*, *gerUD*, *gerKAbm*, and *gerKDbm* using gene-specific primers with 5′ extensions to create *attB*-flanked PCR products compatible with Gateway cloning (Life Technolo-

gies Ltd., Paisley, United Kingdom) into pDONRtet (17) entry plasmids. Entry plasmids were isolated from *E. coli* and used in a series of LR reactions to create appropriate receptor gene-pNFd13-derived (17) destination plasmids. pNFd13-derived plasmids were then introduced into *B. megaterium* QM B1551 via polyethylene glycol (PEG)-mediated protoplast transformation. Colonies that had undergone homologous recombination, integrating the pNFd13-derived plasmid at the cloned locus and placing *lacZ* under the control of the promoter of the designated receptor gene, were isolated after incubation on solid medium at 42°C. The correct construction of the various strains was confirmed by PCR.

Spore preparation and purification. Spores of *B. subtilis* strains were prepared at 37°C on 2× Schaeffer's-glucose plates as described previously (18, 19). After incubation for 2 or 3 days, the plates were left an additional 1 to 3 days at 23°C, then scraped from plates, and washed with water by repeated centrifugation with intermittent sonication treatment. All *B. subtilis* spore preparations used in this work were free (>95%) from growing or sporulating cells and germinated spores as determined by phase-contrast microscopy.

B. megaterium spores were prepared by inoculating 200 ml of supplemented nutrient broth (SNB) (20) with 0.5 ml of a mid-log-phase SNB culture and incubated in 2-liter baffled flasks at 22°C (unless otherwise indicated) for 72 h. Spores were harvested and purified by repeated rounds of centrifugation (4,300 × *g* for 7 min at 4°C) and washing in sterile ice-cold deionized water, removing the upper layer of cellular debris with each cycle, until the spore pellet was observed by phase-contrast microscopy to comprise >99% dormant spores. Purified spores were stored on ice at an optical density at 600 nm (OD_{600}) of ~50.

Spore germination. *B. subtilis* spores were germinated following heat shock (30 min at 75°C) and cooling on ice. Spores at an 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 nutrient germinants added at various concentrations and with duplicate samples measured at all germinant concentrations. *B. subtilis* spore germination was monitored by measuring the release of the spores' large depot of dipicolinic acid (DPA) by inclusion of 50 μM $TbCl_3$ in all germination mixtures and measuring Tb-DPA fluorometrically in a multiwell plate reader as described previously (21). For all germination experiments, rates of germination were determined in arbitrary units (AU) by determining the maximum rates of increases in Tb-DPA fluorescence and correcting the values for slight differences in the DPA contents in different spore preparations; these corrections were always ≤10%. In a few experiments, spores 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, the supernatant fluid was made 50 μM in $TbCl_3$ and Tb-DPA fluorescence was measured as described previously (22). 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 (21).

In addition to nutrient germinants that trigger spore germination via GRs, there are also several agents that trigger spore germination without either GR involvement or a heat shock requirement, including the cationic surfactant dodecylamine and the 1:1 chelate of Ca^{2+} and DPA (CaDPA) (1, 3). *B. subtilis* spores germinated in dodecylamine at 45°C in 25 mM K-HEPES buffer (pH 7.4) plus 50 μM $TbCl_3$ with 1.2 mM dodecylamine and spores at an OD_{600} of 0.5, and germination was assessed by measuring the percentage of maximum Tb-DPA fluorescence obtained as described above. Germination of *B. subtilis* spores with CaDPA was at 23°C and in 60 mM CaDPA made to pH 7.5 with Tris base and with spores at an OD_{600} of 2. Spore germination with CaDPA was monitored by examining ~100 individual spores at various times of germination by phase-contrast microscopy, since germinated spores become phase dark.

For *B. megaterium* spore germination, concentrated spore suspensions (OD_{600} of ~50) were heat shocked (60°C for 10 min for *gerU*-associated experiments; 75°C for 30 min for *gerKbm*-associated experiments) and cooled on ice immediately prior to conducting germination assays. The physiological basis for apparent GR-specific heat shock regimens in *B. megaterium* has yet to be determined. Germination of *B. megaterium* spores was monitored by recording the decrease in optical density of 300-μl aliquots of spores suspended at an initial OD_{600} of 0.4 to 0.5 in 5 mM Tris-HCl (pH 7.5), supplemented with typically 5% (wt/vol) beef extract (for *gerKbm*-associated experiments) or 10 mM glucose or proline (for *gerU*-associated experiments), using a Perkin-Elmer EnVision-Xcite multilabel plate reader fitted with a 600-nm photometric filter. Germination assays were conducted typically for 90 min at 37°C, with 10 s of orbital shaking performed prior to OD_{600} measurements taken every minute. Experiments were conducted in triplicate, with at least two spore preparations for each strain.

Kinetic values associated with germination of *B. megaterium* spores were obtained by incubating heat-shocked spores at 37°C in 5 mM Tris-HCl (pH 7.5) containing various concentrations (0.1 mM to 1,000 mM) of glucose or proline. Germination data were plotted in SigmaPlot 11.0 (Systat Software Inc.), and the slope of the linear portion of curves was used to determine apparent K_m and V_{max} values using the program's ligand-binding macro. Plots of germinant concentration versus percent spore germination were analyzed with the same program to determine approximate concentrations of germinant required to stimulate the germination of 50% of the spore population in 60 min [$K_{0.5\ germ}$].

Analytical methods. For analysis of the levels of germination proteins of *B. subtilis* spores, spores of various strains were prepared, purified, decoated, ruptured with lysozyme, and subjected to brief sonication treatment, giving a spore lysate; in some cases, the spore inner membrane fraction was isolated by differential centrifugation of the spore lysate (all methods described previously [23–26]). The levels of germination proteins were determined by Western blotting analyses on equal amounts of both spore lysate and inner membrane protein as determined by analysis of samples run on gels by SDS-polyacrylamide gel electrophoresis (PAGE), and staining the gels with Coomassie blue as described previously (23–27). The antisera used have been shown to be specific for various germination proteins, including the various GR subunits, as well as the GerD protein essential for rapid GR-dependent spore germination and the SpoVAD protein essential for DPA movement into and out of spores (2, 3).

For measurement of *gerKDbs-lacZ* expression during sporulation, *B. subtilis* strains were sporulated at 37°C by resuspension in Sterlini-Mandelstam medium (28). At various times after resuspension (defined as time zero of sporulation), duplicate aliquots were centrifuged and washed with water, and pellets were frozen for subsequent analysis. β-Galactosidase activity in these samples was determined after lysozyme permeabilization, assaying equal aliquots of cell suspension using methylumbelliferyl-β-D-galactoside (MUG) as the substrate in 1.2 ml, with incubation at 30°C and measuring methylumbelliferone production fluorometrically after 40 min as described previously (4, 12). Note that this assay does not measure β-galactosidase in spores that have become lysozyme resistant, due to the assembly of the mature coat structure on the outer surfaces of the spores. DPA accumulation in the samples from the resuspension cultures was monitored by first boiling cells pelleted from 1 ml of culture in 1 ml of water, followed by centrifugation and measurement of DPA in supernatant fluids by its fluorescence with Tb as described previously (21).

In a few cases, equal amounts (4×10^9) of dormant *B. subtilis* spores of either *B. subtilis* PS533 (wild type) or strains carrying *gerA*- or *gerB-lacZ* fusions with or without *gerKDbs* overexpression were prepared on plates. The spores were purified, decoated, and permeabilized with lysozyme, and 8×10^8 spores were assayed for β-galactosidase as described above.

With dormant *B. megaterium* spores, β-galactosidase activity was assayed in lysates from spores with *lacZ* transcriptional fusions to GR *A* and *D* genes. Typically, equal amounts of spores (1 ml at an OD_{600} of 10) were suspended in 0.6 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol [pH 7.0]) and

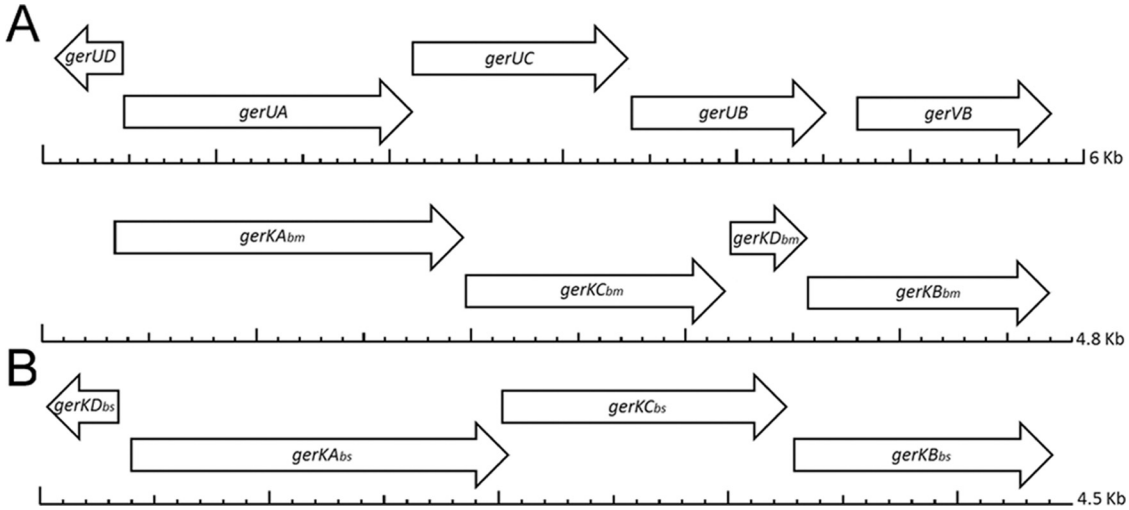

FIG 1 Locations of putative *D* genes in or adjacent to operons that encode GRs in *B. megaterium* (A) and *B. subtilis* (B). Scales below the genes are in kilobase pairs.

then lysed by two rounds of shaking (instrument setting 6 for 30 s) in a FastPrep FP120 cell disrupter (Fisher Scientific, Loughborough, United Kingdom), with chilling on ice for 5 min between cycles. Spore lysates were recovered from the FastPrep tubes and centrifuged for 1 min at 15,000 × *g*, and 0.2 ml of 40 μg/ml MUG was added to the supernatant fluid. Reaction mixtures were incubated at 30°C for 40 min and then terminated by the addition of 0.4 ml of 1 M Na_2CO_3. Fluorescence associated with methylumbelliferone production was recorded in triplicate using a Tecan Infinite 200 series plate reader, using excitation and emission filters set at 365 nm and 450 nm, respectively.

For reverse transcriptase PCR (RT-PCR) to determine the time of expression of various GR *A* and *D* genes during *B. megaterium* sporulation, wild-type *B. megaterium* cells were cultured at 30°C in 200 ml SNB, and duplicate samples, adjusted to an OD_{600} of 10, were collected on an hourly basis following entry into stationary phase (designated time zero in sporulation), with this point determined by the plateauing of OD_{600} values recorded every 30 min during the growth phase of the culture. Samples were immediately centrifuged (15,000 × *g* for 1 min) and washed with RNAprotect bacterial reagent (Qiagen Ltd.), and the cell pellets were stored at −80°C until further analysis. RNA was subsequently extracted and purified from thawed cell pellets using an RNeasy minikit (Qiagen Ltd.), and then stored at −80°C. Approximately 1 μg of RNA from each sample was converted to cDNA using a QuantiTect reverse transcription kit (Qiagen Ltd., Manchester, United Kingdom), using random hexamers (Life Technologies Ltd., Paisley, United Kingdom) as primers for cDNA synthesis. Finally, cDNA samples for each time point served as templates for PCRs employing NovaTaq DNA polymerase (Merck Chemicals Ltd., Nottingham, United Kingdom), and gene-specific primers were designed to amplify ≤300-bp fragments of the genes of interest.

RESULTS

Identification of genes encoding putative D proteins in *B. megaterium* and *B. subtilis*. Bioinformatic analysis of the *B. megaterium* QM B1551 genome revealed a number of ORFs encoding putative GR D proteins. The most conspicuous of these ORFs (BMQ_1272) is the third of four genes that comprise the *gerKbm* GR operon (Fig. 1) and is predicted to encode a 78-aa GerKDbm protein with two transmembrane (TM) domains. The GerKbm receptor has been characterized recently as responsible for triggering efficient germination in several rich undefined media, in particular solubilized beef extract, of *B. megaterium* spores that lack the GerU GR (36).

Several other putative GR *D* genes are evident on the *B. megaterium* chromosome, typically in the region of other GR-associated loci. These genes include BMQ_2235, predicted to encode a 78-aa protein with two TM domains located between two ORFs (BMQ_2236 and BMQ_2234) predicted to encode GR B-subunit proteins, which unusually do not appear to be organized in an operon with genes encoding associated A- and C-subunit proteins. Similarly, BMQ_3107, predicted to encode a 78-aa integral membrane protein, is immediately upstream of an apparently orphan GR B-subunit gene (BMQ_3108).

The best-studied *B. megaterium* GR, GerU, responsible for mediating spore germination with several single germinants, including either glucose or proline (11), also has a potential D protein encoded nearby. In this case, the putative *D* gene (BMQ_pBM70069) is located a short distance upstream of (133 bp) and on the opposite strand to the start codon of the first gene (*gerUA*) of the GR operon (Fig. 1). The provisionally entitled GerUD protein is predicted to comprise 76 aa and have two TM domains, similar to other putative D proteins identified in this work.

Three small genes encoding potential GR D proteins were also identified in *B. subtilis* as follows: (i) in the *yfkQRST* operon as the *yfkS* gene; (ii) as the *ynzB* gene immediately upstream of the *yndEFG* operon; and (iii) as the *yczF* gene (now termed *gerKDbs*; Fig. 1) immediately upstream of the *gerKbs* operon; note that the arrangement of *gerKDbs* and the *gerKbs* operon in *B. subtilis* is similar to that of *gerUD* and the *gerU* operon in *B. megaterium* (Fig. 1). All three of the putative *B. subtilis D* genes have a good RBS prior to the likely ATG translation initiation codon. YfkS and GerKDbs are also predicted to contain two TM segments by multiple prediction programs (data not shown). The expression of *yfkS* parallels that of *yfkQ*, *yfkR*, and *yfkT* as expected (29), given the presence of all four genes in an operon. In addition, *gerKDbs* transcription largely parallels that of *gerKA*, the first gene in the immediately adjacent *gerKbs* operon, although *ynzB* transcription does not parallel that of the *yndDFG* operon (29). These data

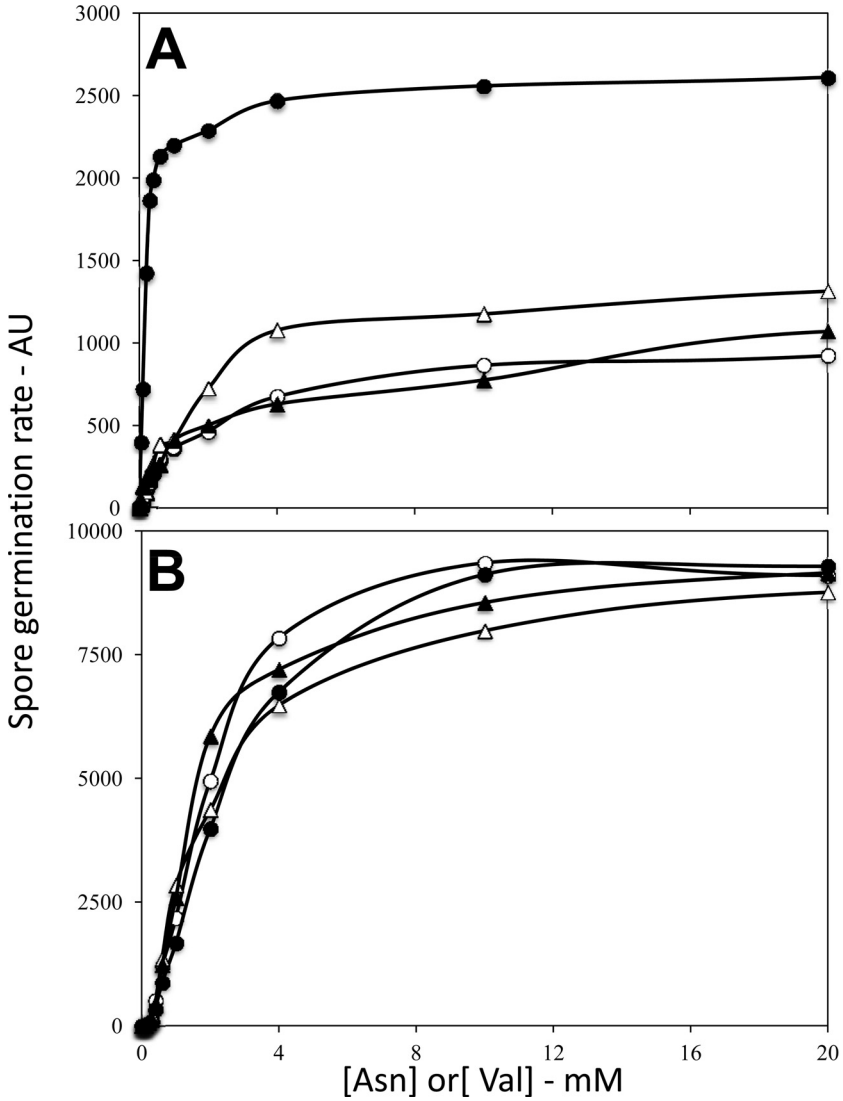

FIG 2 Rates of germination of wild-type and Δ*gerKDbs B. subtilis* spores with AGFK (A) and L-valine (B). Spores of *B. subtilis* strains PS533 (wild type) and PS4256 (Δ*gerKDbs*) were germinated with various concentrations of L-valine (A) or L-asparagine with D-glucose and D-fructose constant at 10 mM, and K^+ constant at ~15 mM (B), and rates of spore germination were assessed by Tb-DPA fluorescence and given in arbitrary units (AU) as described in Materials and Methods. Symbols: ○, wild-type (PS533) spores; ●, Δ*gerKDbs* (PS4256) spores; △, Δ*gerKDbs amyE*::*gerKDbs* (PS4313) spores; ▲, *amyE*::P*sspB-gerKDbs* (PS4314) spores.

together are consistent with at least the YfkS and GerKDbs proteins playing some role in the function of the YfkQRST and GerKbs GRs, respectively.

Since at least some of the D proteins described above might play roles in GR function, with *B. subtilis* we focused on analysis of the role and regulation of expression of GerKDbs in the function of the GerKbs GR, since the role of this GR in spore germination is known, in contrast to that of the GR encoded by the *yfkQRST* operon (7). With *B. megaterium* we examined the function and expression of GerKDbm and GerUD, since the functions of the GerKbm and GerU GRs in spore germination have been determined (10, 11).

Effects of deletion, complementation, and overexpression of *gerKDbs* on *B. subtilis* spore germination. To assess the function of the *B. subtilis gerKDbs* gene immediately upstream of the *gerKbs* operon, this small gene was largely deleted, and its coding sequence was replaced by a Tc^r cassette. The resulting Δ*gerKDbs* strain sporulated normally (data not shown). However, *B. subtilis* spore germination with the AGFK mixture, which requires both the GerB and GerKbs GRs, was significantly faster with Δ*gerKDbs* spores than with wild-type spores at multiple concentrations of L-asparagine and constant high GFK concentrations (Fig. 2A). In addition, the L-asparagine concentration needed for 50% maximal germination with AGFK was significantly higher for wild-type spores than for Δ*gerKDbs* spores (Fig. 2A). In contrast, germination of the Δ*gerKDbs* spores with L-valine alone via the GerA GR was essentially identical to that of wild-type spores at multiple L-valine concentrations (Fig. 2B). The similar germination of Δ*gerKDbs* and wild-type spores with L-valine and the faster germination of Δ*gerKDbs* spores with AGFK were seen with three independent spore preparations.

While AGFK germination was significantly affected by deletion of *gerKDbs*, AGFK triggers germination via GRs and there are other germinants that trigger germination in GR-independent

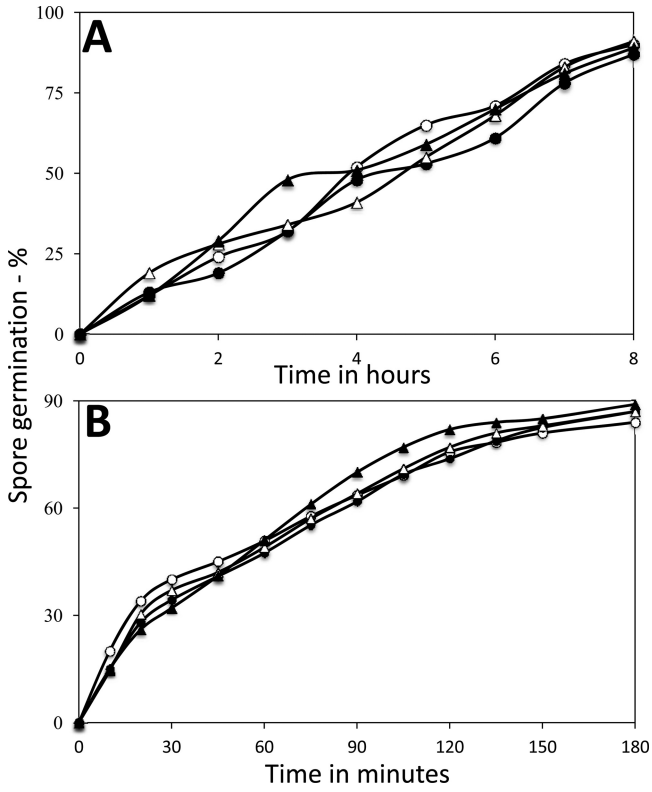

FIG 3 Germination of spores of various *B. subtilis* strains with CaDPA (A) and dodecylamine (B). Spores of *B. subtilis* strains PS533 (wild type), PS4256 (Δ*gerKDbs*), PS4313 (Δ*gerKDbs amyE*::*gerKDbs*), and PS4314 (*amyE*::P*sspB-gerKDbs*) germinated in the presence of CaDPA (A) or dodecylamine (B), and spore germination with these two agents was measured as described in Materials and Methods. The dormant spores of these four strains had essentially the same amounts of DPA. Symbols: ○, PS533 spores; ●, PS4256 spores; △, PS4313 spores; ▲, PS4314 spores.

processes (1, 3). Two germinants that do not require GRs for triggering germination are CaDPA and dodecylamine, with CaDPA directly activating hydrolysis of the peptidoglycan cortex of the spores, and dodecylamine most likely directly activating CaDPA release from the spore core (1, 3). In contrast to the significant effect of the *gerKDbs* deletion on AGFK germination, germination of Δ*gerKDbs* spores with either CaDPA or dodecylamine was essentially identical to that of wild-type spores (Fig. 3A and B).

Since the *gerKDbs* gene is located just upstream of the *B. subtilis gerKbs* operon, it was possible that the *gerKDbs* deletion could alter transcription of the *gerKbs* operon, even though the *tet* gene inserted in *gerKDbs* was oriented such that its transcription would be away from the *gerKbs* operon. To show that the effects of the *gerKDbs* deletion were indeed due to effects of the loss of the GerKDbs protein, we examined the effects of ectopic expression of *gerKDbs* on the germination of spores lacking the normal *gerKDbs* gene (Fig. 2A and B). Strikingly, expression of *gerKDbs* from its own promoter at the *amyE* locus in the Δ*gerKDbs* strain resulted in spores that exhibited significantly lower rates of AGFK germination, and this rate was comparable to that of wild-type spores, as was the dependence of the spore germination rate on L-asparagine concentration (Fig. 2A); this was also the case when L-valine germination was examined (Fig. 2B). In addition, when *gerKDbs* was overexpressed at *amyE* under P*sspB* control in an otherwise wild-type strain, the rates of both AGFK and L-valine germination of the spores were again very similar to those of wild-type spores (Fig. 2A and B). One trivial explanation for the effects on AGFK germination of *gerKDbs* expression at *amyE* in the Δ*gerKDbs* strain is that ectopic *gerKDbs* expression somehow interferes with sporulation such that spore coat synthesis or assembly is defective and since Tb^{3+} can strongly inhibit germination of coat-defective spores (29), perhaps this inhibition is more effective for germination of Δ*gerKbs* spores with AGFK than with L-valine. However, this was not the case, as the slower germination of spores of strains expressing or overexpressing *gerKDbs* at *amyE* was seen even if $TbCl_3$ was added only at multiple times after the initiation of germination to measure DPA release (data not shown).

In contrast to the slower AGFK germination of *B. subtilis* spores ectopically expressing *gerKDbs* in the Δ*gerKDbs* background, these spores exhibited essentially identical germination to wild-type spores with the GR-independent germinants CaDPA and dodecylamine (Fig. 3A and B). This was also the case with spores overexpressing GerKDbs in an otherwise wild-type background (Fig. 3A and B). Since spores with coat defects often exhibit major alterations in their germination with CaDPA and dodecylamine (2, 3), the normal germination of Δ*gerKDbs* spores ectopically expressing *gerKDbs* with these agents is further evidence that these spores do not have a coat defect as indicated above.

Effects of deletion and complementation of GR *D* genes on *B. megaterium* spore germination. In order to assess the roles of the putative GerKDbm and GerUD proteins on *B. megaterium* spore germination, a number of mutant constructs were generated in *B. megaterium* strain GC414, which lacks all five functional spore GRs (36). When complemented with intact *gerU*$^+$ (*gerUA gerUC* and *gerVB* plus the upstream *gerUD* gene) or the *gerKbm* operon genes plus native regulatory sequences on plasmids that are maintained at a low copy number (16), GC414-derived spores germinated efficiently (>95%) in response to appropriate germinant compounds. In both cases, germination rates were comparable to those of spores of appropriate parental strains (data not shown), indicating that any effects on germination resulting from differential expression of plasmid-borne GR operons was minimal. Spores of strain GC635, for example, which are complemented with the intact *gerKbm* operon, germinated almost completely in 60 min in 5% (wt/vol) beef extract (Table 2). Spores of strain GC636, which have plasmid-borne *gerKbm* Δ*gerKDbm*, also germinated fully and at an increased rate (Table 2).

Differences in germination efficiency between spores complemented with *gerKbm* or *gerKbm* Δ*gerKDbm* became even more pronounced as the beef extract concentration in the germination buffer was reduced (Table 2). Spores complemented with *gerKbm* minus the *gerKDbm* gene (strain GC636), for example, exhibited full germination in 0.1% (wt/vol) beef extract, whereas only ~12% of spores complemented with the intact *gerKbm* operon (strain GC635) germinated under the same conditions (Table 2). Indeed, a 10-fold-higher concentration of beef extract was required to trigger 50% germination of pHT-*gerKbm* spores (0.44%) versus spores complemented with pHT-*gerKbm* Δ*gerKDbm* (0.049%). These data indicate that GerKDbm has a negative effect on the function of the *B. megaterium* GerKbm GR, similar to the effect of the loss of GerKDbs in *B. subtilis*. However, spores of strain GC637, in which *gerKDbm* was replaced within the *gerKbm* operon by *gerUD*, did not show the same apparent in-

TABLE 2 Germination of *B. megaterium* GR null spores complemented with *gerKbm* and *gerKbm* Δ*gerKDbm* operons[a]

Strain	Relevant phenotype or genotype	Germination rate (%) with the following beef extract concn (wt/vol)[b]:				
		0.1%	0.5%	1%	5%	10%
GC614	GR null	<5	<5	<5	<5	<5
GC635	GR null pHT-*gerKbm*	12	53	73	98	83
GC636	GR null pHT-*gerKbm* Δ*gerKDbm*	99	98	94	97	89
GC637	GR null pHT-*gerKbm*, with *gerUD* in place of *gerKDbm*	11	41	67	83	64

[a] Heat-shocked spores were germinated at 37°C for 60 min in various concentrations of beef extract in 5 mM Tris-HCl (pH 7.5), and germination was measured as described in Materials and Methods.
[b] Rates of spore germination are given relative to the OD_{600} loss (55%) for spores of strain PV361 in 5% (wt/vol) beef extract, which was set at 100. This value is equivalent to 100% germination in 60 min. Values are the means of three independent experiments conducted with the same spore preparations, where standard deviations were <10% of the mean. Similar results were obtained with additional spore preparations.

crease in sensitivity to germinants, with a $K_{0.5\ germ}$ value of 0.67% (wt/vol) beef extract revealing a slight but significant ($P \leq 0.05$) reduction in sensitivity to beef extract compared to spores complemented with the native *gerK* operon ($K_{0.5\ germ}$ of 0.44% [wt/vol] beef extract) (where $K_{0.5\ germ}$ is the value required to trigger germination of 50% of the spore population).

Having examined the role of *gerKDbm*, we then turned our attention to the role of the *gerUD* gene that encodes a putative D subunit associated with the GerU GR. Two different mutants were engineered to examine the role of *gerUD*; first, a strain in which the entire predicted *gerUD* ORF was deleted from the pHT-*gerU*⋆ plasmid, leaving 132 bp upstream of the *gerUA* start codon. Although the latter region has been confirmed by 5′ RACE (rapid amplification of cDNA ends) experiments to contain the transcription start site of *gerUA* (S. Gupta and G. Christie, unpublished data) and putative SigG −35 and −10 consensus sequences remain intact upstream of *gerUA* on the modified plasmid (see below), it is possible that deletion of the *gerUD* gene may have compromised the integrity of the *gerU* promoter. Hence, a second plasmid was prepared in which a codon predicted to encode the 10th residue (methionine) of GerUD was modified by SDM to a stop codon (TAA), which should result in a severely truncated version of the protein being expressed while minimizing the likelihood of interfering with the *gerU* promoter. Preliminary experiments indicated that spores of strains complemented with either construct displayed essentially identical germination phenotypes (data not shown), and therefore the *gerUD*^{M10stop} variant was employed in subsequent analyses.

In contrast to observations concerning the impact of GerKDbm on GerKbm GR-mediated germination, kinetic analyses conducted with spores exposed to different concentrations of either proline or glucose, two compounds that trigger spore germination via GerU, revealed that the absence of *gerUD* had a deleterious effect on spore germination (Table 3). Indeed, for both glucose and proline, the concentration of germinant required to stimulate 50% germination was increased ~10-fold in spores lacking the *gerUD* gene (strain GC631) compared to spores complemented with the entire *gerU* or *gerUD* locus. This was also reflected in results from kinetic analyses, as similar V_{max} values were obtained for spore germination triggered by either proline or glucose via GerU with and without the GerUD protein, but the apparent affinity of the Δ*gerUD* spores for either germinant was decreased (Table 3).

Intriguingly, kinetic values for the germination of spores of strain GC632 in which the *gerUD* gene has been relocated from its native, divergently oriented position upstream of *gerUA* to a position within the *gerU* operon (encoded on the same strand between *gerUC* and *gerVB*) were similar to the benchmark pHT-*gerU gerUD* (strain GC615) spores (Table 3). These data demonstrate that the influence of the *gerUD* gene is not necessarily a *trans* effect, since the positive influence it appears to impart on GerU function was achieved when it is presumably cotranscribed as part of a GR operon. However, data in Table 3 also reveal that *gerKDbm* cannot be substituted for *gerUD* within a similarly organized *gerU* operon without adversely affecting GR function. This effect was most pronounced in the absence of functional GerUD, as evidenced by spores of strain GC634, which required ≥1,000-fold-higher germinant concentrations to stimulate 50%

TABLE 3 Kinetic parameters of germination of *B. megaterium* GC614 (GR null) spores complemented with GerU containing various combinations of GR D proteins[a]

Strain	Relevant genotype	Kinetic parameter of germination in spores incubated with:					
		Glucose			Proline		
		K_m[b]	V_{max}[c]	$K_{0.5\ germ}$[b,d]	K_m	V_{max}	$K_{0.5\ germ}$
GC615	pHT-*gerUD gerU*⋆[e]	1.7	0.05	0.24	1.7	0.05	0.24
GC631	pHT-*gerUD*^{M10stop} *gerU*⋆	7.4	0.04	2.49	5.6	0.04	2.49
GC632	pHT-*gerUD*^{M10stop} *gerU*⋆, with *gerUD* between *gerUC* and *gerVB*	1.6	0.08	0.22	1.5	0.07	0.24
GC633	pHT-*gerUD gerU*⋆, with *gerKDbm* between *gerUC* and *gerVB*	23.4	0.02	47	16.5	0.02	41
GC634	pHT-*gerUD*^{M10stop} *gerU*⋆, with *gerKDbm* between *gerUC* and *gerVB*	131	0.01	276	174	0.01	305

[a] Spores were germinated in 5 mM Tris-HCl, pH 7.5, for 90 min with concentrations of glucose and proline ranging from 0.1 to 1,000 mM. Germination was monitored, and kinetic parameters were calculated as described in Materials and Methods. The values for each strain are from three independent experiments conducted with the same spore preparation; similar values were obtained with at least one other spore preparation for each strain. Standard deviations from mean values are less than 10%.
[b] Values given in millimolar.
[c] Values represent the decrease in OD_{600} units/min, where starting values are normalized to 1 OD_{600} unit.
[d] Concentrations of glucose and proline required to stimulate 50% spore germination [$K_{0.5\ germ}$] were calculated after incubation for 90 min with the respective germinants. Values presented for each strain are from three independent experiments conducted with the same spore preparation; similar values were obtained with other spore preparations. Standard deviations from mean values are less than 10%.
[e] *gerU*⋆ comprises *gerUA*, *gerUC*, and *gerVB* and appropriate regulatory sequences.

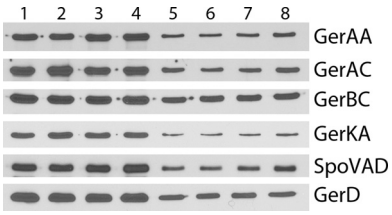

FIG 4 Western blot analysis of germination proteins in total lysates of spores of various *B. subtilis* strains. Total spore lysates from spores of *B. subtilis* strains PS533 (wild type) (lanes 1 and 5), PS4256 (Δ*gerKDbs*) (lanes 2 and 6), PS4213 (*amyE*::*gerKDbs* Δ*gerKDbs*) (lanes 3 and 7), and PS4314 (*amyE*::P*sspB-gerKDbs*) (lanes 4 and 8) were isolated, and samples with identical amounts of protein from ~7×10^6 spores (lanes 1 to 4) or 1.8×10^6 spores (lanes 5 to 8) (with amounts of protein adjusted appropriately based on preliminary SDS-PAGE and Coomassie blue staining) were run on SDS-polyacrylamide gels and subjected to Western blot analysis with antisera against various germination proteins as described in Materials and Methods. The strips probed for the various antigens were from several different blots.

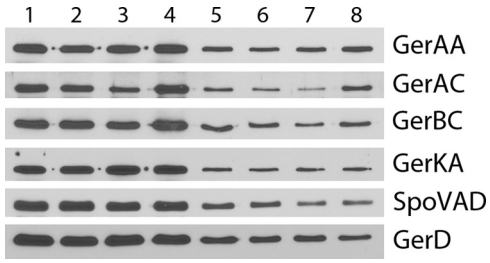

FIG 5 Western blot analysis of germination proteins in the isolated inner membrane fraction from spores of various *B. subtilis* strains. The inner membrane fraction was isolated from the total spore lysates analyzed in Fig. 4 from spores of *B. subtilis* strains PS533 (wild type) (lanes 1 and 5), PS4256 (Δ*gerKDbs*) (lanes 2 and 6), PS4213 (*amyE*::*gerKDbs* Δ*gerKDbs*) (lanes 3 and 7), and PS4314 (*amyE*::P*sspB-gerKDbs*) (lanes 4 and 8). Samples with identical amounts of inner membrane protein from ~2.3×10^8 spores (lanes 1 to 4) or 5.6×10^7 spores (lanes 5 to 8) (with amounts of protein adjusted appropriately based on preliminary SDS-PAGE and Coomassie blue staining) were run on SDS-polyacrylamide gels and subjected to Western blot analysis with antisera against various germination proteins as described in Materials and Methods. The strips probed for the various antigens were from several different blots.

spore germination. The presence of intact *gerUD* partially alleviated the adverse effects associated with the presence of *gerKDbm* within the *gerU* operon, although significantly increased germinant concentrations (>150-fold) were still required to stimulate 50% germination of spores of strain GC633 compared to GC615 spores (Table 3).

Effects of loss of *gerKDbs* or its ectopic expression or overexpression on the levels of germination proteins in *B. subtilis* spores. The results noted above indicated that alterations in levels of the various GR D proteins exert either positive or negative effects on GR function. Previous work has shown that rates of spore germination with particular nutrient germinants are often elevated by increases in levels of GRs that recognize the particular germinants (8, 23, 25, 26, 30). Consequently, it seemed possible that effects of alterations in D-protein levels on rates of spore germination might be mediated through effects on GR levels. Unfortunately, specific antisera are not available against *B. megaterium* GR subunits, but antisera against a number of *B. subtilis* GR subunits as well as several other *B. subtilis* germination proteins are available. To test whether the Δ*gerKDbs* mutation had resulted in alterations in GR subunit levels, we determined levels of such proteins as well as GerD (31) and SpoVAD in either spore lysates or inner membrane fractions, as these proteins are located in the inner membrane (1–3, 9, 23–26). Total spore lysates as well as isolated inner membrane fractions were used, because the lysates give much higher yields of inner membrane proteins, as spore fractionation by differential centrifugation recovers only ~10% of the inner membrane and inner membrane proteins in the final inner membrane pellet fraction (K.-A.V. Stewart and P. Setlow, unpublished results). Strikingly, the levels of SpoVAD, GerD, and the GerAA, GerAC, GerBC, and GerKA GR subunits showed less than a 25% difference in wild-type and Δ*gerKDbs* spores when either spore lysates or inner membrane fractions were analyzed (Fig. 4 and 5 and Table 4).

The slowed GR-dependent germination of Δ*gerKDbs B. subtilis* spores expressing *gerKDbs* from its own promoter was striking and suggested that this slow germination might be reflected in lower levels of the GerKbs GR, but perhaps not other GR proteins. However, ectopic expression of *gerKDbs* from its own promoter or ectopic overexpression of *gerKDbs* from P*sspB* also had no major effect on the levels of GR subunits, GerD, or SpoVAD when either total spore lysates or isolated inner membrane fractions were analyzed (Fig. 4 and 5 and Table 4). The lack of effect of ectopic overexpression of *gerKBbs* on the levels of various *B. subtilis* germination proteins was further assessed by examination of the levels of β-galactosidase expressed from either a *gerA-lacZ* fusion or a *gerB-lacZ* fusion in spores with and without ectopic *gerKDbs* overexpression (Table 5). As expected (4, 12), there was significant β-galactosidase in the spores carrying *gerA-lacZ* or *gerB-lacZ* fusions, and the level of this activity was much higher than in wild-type spores with no *lacZ* fusion. However, ectopic overexpression of *gerKDbs* had essentially no effect on the levels of β-galactosidase in spores from either the *gerA-lacZ* or *gerB-lacZ* fusions.

Expression of genes encoding putative GR D proteins during sporulation. Analysis of the regions upstream of *gerKDbs* and *gerUD* revealed sequences with good homology to the consensus sequences recognized by RNA polymerase with the forespore-specific sigma factor σ^G that transcribes the operons encoding the GRs, including GerKbs, as well as the *yndEFG* and *yfkQRST* operons that appear likely to encode GRs of unknown function (Fig. 6) (7). However, while transcription of *gerKDbs* has been observed during *B. subtilis* sporulation and correlates well with *gerKbs*

TABLE 4 Levels of germination proteins in total spores and the inner membrane fraction of spores of various *B. subtilis* strains[a]

Protein	Level in the following strain relative to that in wild-type spores[b]			
	Wild-type (PS533)	Δ*gerKDbs* (PS4256)	Δ*gerKDbs amyE*::*gerKDbs* (PS4313)	*amyE*::P*sspB-gerKDbs* (PS4314)
GerAA	1.0 (1.0)	0.8 (0.8)	1.0 (0.9)	1.0 (1.0)
GerAC	1.0 (1.0)	0.9 (0.8)	1.0 (0.6)	1.1 (1.2)
GerBC	1.0 (1.0)	0.9 (0.9)	0.8 (0.8)	1.0 (0.9)
GerKA	1.0 (1.0)	1.2 (0.9)	1.1 (1.0)	1.2 (1.0)
GerD	1.0 (1.0)	0.9 (1.0)	0.9 (0.8)	1.0 (0.8)
SpoVAD	1.0 (1.0)	0.9 (0.9)	1.0 (1.0)	1.2 (0.8)

[a] Levels of various proteins were obtained from Image J analysis of data in Fig. 4 and 5.
[b] Levels of each germination protein in wild-type spores from analysis of total spores or inner membrane fractions were set at 1.0. The first relative values (not in parentheses) are from total spores (Fig. 4), and the second values (values in parentheses) are from the inner membrane fraction (Fig. 5). Values are ±25%.

TABLE 5 Levels of β-galactosidase from *gerA-lacZ* or *gerB-lacZ* fusions in spores with or without *gerKDbs* overexpression[a]

Strain	Relevant genotype	β-Galactosidase activity (RFU)[b]
PS533	Wild-type	335
PS767	*gerA-lacZ*	10,700
PS4319	*gerA-lacZ amyE*::P*sspB-gerKDbs*	9,700
PS3709	*gerB-lacZ*	6,135
PS4320	*gerB-lacZ amyE*::P*sspB-gerKDbs*	5,250

[a] Spores of various strains were isolated, decoated, and permeabilized, and 8×10^8 spores were assayed for β-galactosidase as described in Materials and Methods.
[b] RFU, relative fluorescence units.

operon transcription (29, 32), the transcription of the *gerKDbs* gene alone has not been well studied, and expression of *gerKDbm* or *gerUD* has also not been well studied. Consequently, we prepared transcriptional *lacZ* fusions to these *D* genes in order to examine their expression during sporulation.

With *gerKDbs-lacZ* in an otherwise wild-type *B. subtilis* strain, significant β-galactosidase activity appeared ~2.5 h after the start of sporulation, although the β-galactosidase activity in this culture fell rapidly and almost to background levels after 6 h (Fig. 7). Note also that DPA accumulated ~2 h later than *gerKDbs-lacZ* accumulation. The latter timing as well as the fall in *gerKDbs*-driven *lacZ* expression late in sporulation is consistent with *gerKDbs* expression being confined to the developing forespore, as the forespore becomes refractory to lysozyme permeabilization as sporulation proceeds (33). If this is indeed the case, then high levels of β-galactosidase should be present in *gerKDbs-lacZ* spores. This was the case, as dormant *gerKbs-lacZ* spores had β-galactosidase activity similar to the maximum level accumulated during sporulation in liquid resuspension medium (data not shown).

To further assess the regulation of *gerKDbs* expression during *B. subtilis* sporulation, the *gerKDbs-lacZ* fusion was introduced into strains lacking an RNA polymerase σ factor (σ^E, σ^F, σ^G, or σ^K) needed for sporulation-specific transcription in either the mother cell or forespore compartment of the sporulating cell, and β-galactosidase was assayed during resuspension sporulation of these strains (Fig. 7). These assays showed that the absence of σ^E, σ^F, or σ^G eliminated β-galactosidase accumulation from *gerKDbs-lacZ*, while the absence of σ^K did not. However, in the absence of σ^K, the *gerKDbs*-driven β-galactosidase level did not decrease late in sporulation, consistent with this enzyme being accumulated only in the forespore, which does not become lysozyme resistant in a σ^K deletion. Together, these data indicate that *gerKDbs* is transcribed by RNA polymerase that contains σ^G, the σ factor that also directs the transcription of the operons encoding GRs, including *gerKbs*, as well as a number of other forespore-specific genes (1, 4–6, 12, 32).

To analyze expression of the *B. megaterium gerKDbm* and *gerUD* genes, *lacZ* translational fusions were generated in the wild-type background, fusing *lacZ* to the 3′ ends of the *gerKDbm* (creating a *gerKbm* null mutant in this case as a result of disrupting the *gerK* operon) and *gerUD* genes and then assaying extracts from disrupted spores for β-galactosidase activity. These data (Table 6) indicate that *gerKDbm* is expressed at a near identical level to *gerKAbm*, which is as expected, since both genes are located in the same operon and other GR operons have been shown to be transcribed as single mRNA molecules (4, 12). RT-PCR analyses conducted with cDNA prepared from sporulating wild-type cultures reinforced this observation, with transcription of both *gerKAbm* and *gerKDbm* being detected at the same time in sporulation (4 h after entry into sporulation on) in two apparent waves of expression (Fig. 8). The *gerUD* gene also appears to be expressed during sporulation, apparently at a higher level than *gerU* receptor genes encoded on the opposite strand according to analyses with *lacZ* fusion spores, although both *gerUD* and *gerUA* appear to be expressed at a lower level than *gerKbm* genes (Table 6). RT-PCR analyses supported the *lacZ* fusion data, indicating that despite their divergent genetic organization, *gerUD* and *gerUA* (and presumably the entire *gerU* operon) are expressed at the same time during sporulation (3 h after entry into sporulation on, although only as a single wave of expression) (Fig. 8), which is consistent with GerUD being associated with GerU receptor function.

DISCUSSION

The identification of the potential GR *D* genes by bioinformatic analysis was somewhat surprising, not the least because putative *D* genes were not found associated with the *B. subtilis gerA* and *gerB* operons encoding two of the major GRs of this organism. However, the location of a number of likely *D* genes immediately upstream of or within operons encoding GRs in both *B. megaterium* and *B. subtilis* suggests that these small genes may play roles in at least some functions of GRs. This suggestion is strongly supported by the findings for the *B. subtilis gerKDbs* gene and the *B. megaterium gerKDbm* and *gerUD* genes presented in this work. Thus, these three genes were expressed in parallel with their associated GR operon and only in the developing spore, and *gerKDbs* and the *gerKbs* operon are under the control of the same RNA polymerase σ factor, σ^G. Noteworthy also from an expression perspective was the observation that the *gerKbm* operon, including *gerKDbm*, is transcribed in two apparent waves of expression. Similar bimodal expression of GRs, and indeed other σ^G-dependent genes, has been observed previously in *B. subtilis*, although the biological relevance of this phenomenon has yet to be revealed (32). More importantly, deletion and subsequent ectopic expression of the deleted *D* genes had significant effects on GR-dependent spore germination. Notably, the effects of alteration of *D* gene expres-


```
                  -35                    -10          rbs
          ----GXATA------(17)------YAZAYTA-----AAAGGAGGTG-(7)-ATG
gerKDbs:TGCGGTATA------(18)------GACACTA-(2)-AGCGGAGGAT-(8)-ATG
gerUD:   TTATGTATC------(18)------AAACCTA-----AATGGAGCGT-(7)-ATG
```

FIG 6 Potential transcription and translation signals upstream of the *gerKDbs* and *gerUD* coding sequences. The perfect *B. subtilis* mRNA ribosome binding site (rbs) (28) and consensus −10 and −35 sequences recognized by *B. subtilis* RNA polymerase with σ^G and the rbs are shown above the *gerKDbs* sequence. The X in the −35 consensus sequence denotes a position that is any nucleotide, the Y in the −10 sequence denotes residues that are most often A or C, and the Z in the −10 sequence denotes a position that is most often A or T. The translation-initiating ATG codons are underlined. In the sequences shown, the values in parentheses between the −35 and −10 sequences, the −10 sequence and the rbs, and the rbs and the ATG codon are the consensus spacings between these elements (3, 27). The dashes indicate positions that are any nucleotide.

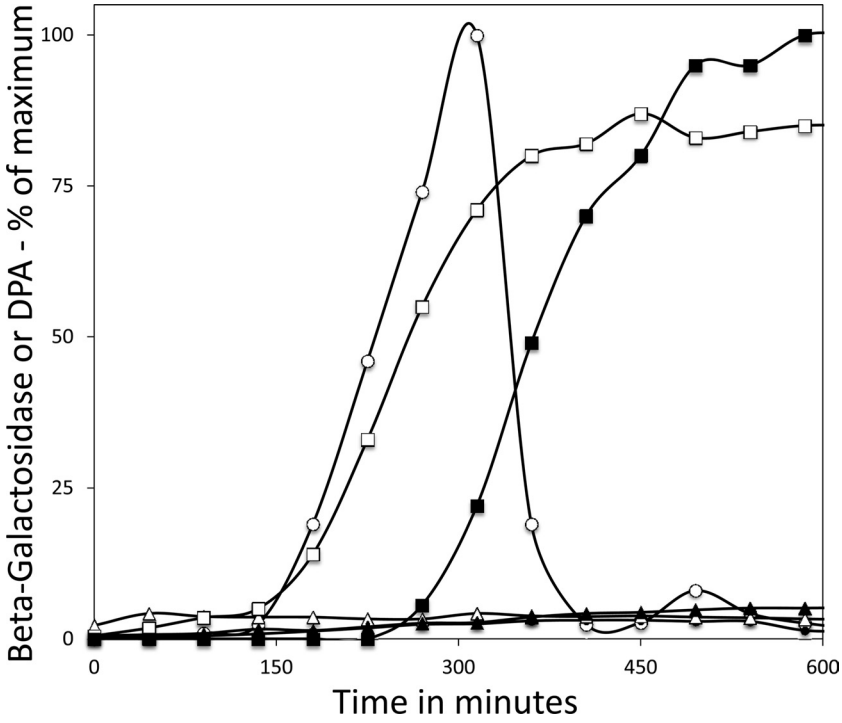

FIG 7 Levels of β-galactosidase and DPA during sporulation of strains with a *gerKDbs-lacZ* fusion and with or without sporulation-specific RNA polymerase σ factors. Various *B. subtilis* strains carrying *gerKDbs-lacZ* were sporulated by resuspension, and equal aliquots of cultures were assayed for β-galactosidase (○, ●, △, ▲, □) or DPA (■) as described in Materials and Methods. The strains analyzed were PS4290 (has all sigma factors) (○, ■), PS4293 (no σ^E) (●), PS4294 (no σ^F) (△), PS4295 (no σ^G) (▲), and PS4296 (no σ^K) (□). Strain PS533 that lacks a *lacZ* fusion was also sporulated by resuspension in parallel, and the levels of β-galactosidase from this strain were subtracted from those of other samples taken at the same time. The maximum value of these subtracted values was <10% of the maximum value obtained with the wild-type strain carrying *gerKDbs-lacZ*.

sion were only on GR-dependent germination, suggesting that the effects of these proteins on spore germination are not directly on either cortex peptidoglycan hydrolysis or the CaDPA release process.

The major question arising from the results noted above is how the D proteins exert their effects. The fact that the putative GR D proteins with documented effects on spore germination appear to be composed primarily of two membrane-spanning segments suggests that these proteins are located in the spore's inner membrane, where the GRs themselves are located. Thus, the D proteins could influence the organization of their GRs in the cluster of germination proteins, including GRs and GerD termed the germinosome, and there is evidence indicating that germinosome assembly is important for optimum GR function (9). In addition, the dramatic inhibition of the *B. megaterium* GerU function by substitution of *gerKDbm* for *gerUD* in the *gerU* operon suggests that the various D proteins are specific for their cognate GRs. A less pronounced but significant reduction in apparent receptor efficiency was also observed when *gerUD* was substituted for *gerKDbm* in the *B. megaterium gerKbm* operon, lending further support to this idea. D proteins can certainly affect the maximum germination rates via specific GRs, and this was achieved at least in *B. subtilis* spores with no significant alteration in the levels of subunits of the relevant GRs. In addition, the D proteins can markedly alter the affinity of their associated GRs for their cognate nutrient germinants, as exemplified by kinetic analysis of the effects of GerUD on the function of the *B. megaterium* GerU GR and GerKDbs on the function of the *B. subtilis* GerK GR. Thus, a D protein may somehow modulate either germinant access to GRs or alter GR conformation such that germinant binding affinities are either decreased or increased. Similarly, substitution of *gerKDbm* in the *gerU* operon resulted in a marked decrease in GerU function, both in terms of spores' apparent affinity for germinants and maximal rates of germination achieved. Hence, at least in this case, a noncognate D protein could not be tolerated as part of or associated with the GR complex, although the structural and/or physiological basis for this is not clear. Indeed, at this time, we do not understand how D proteins act to modulate spore germination rates.

In addition to the major question noted above on what exactly D proteins do, a second major question is whether D proteins are associated with all GRs. In particular, are there D proteins associ-

TABLE 6 Levels of β-galactosidase in *B. megaterium* spores with *gerU-lacZ* and *gerKbm-lacZ* fusions[a]

Strain	Relevant genotype	β-Galactosidase activity (RFU)[b]
QM B1551	Wild-type	500
GC638	*gerUA-lacZ*	16,300
GC639	*gerUD-lacZ*	29,200
GC640	*gerKAbm-lacZ*	43,000
GC641	*gerKDbm-lacZ*	42,500

[a] Spores of various strains (~10^9) were isolated, disrupted, and assayed for β-galactosidase activity as described in Materials and Methods. β-Galactosidase values are the average of triplicate measurements conducted with two different spore preparations. Standard deviations for all values are <10%.
[b] RFU, relative fluorescence units.

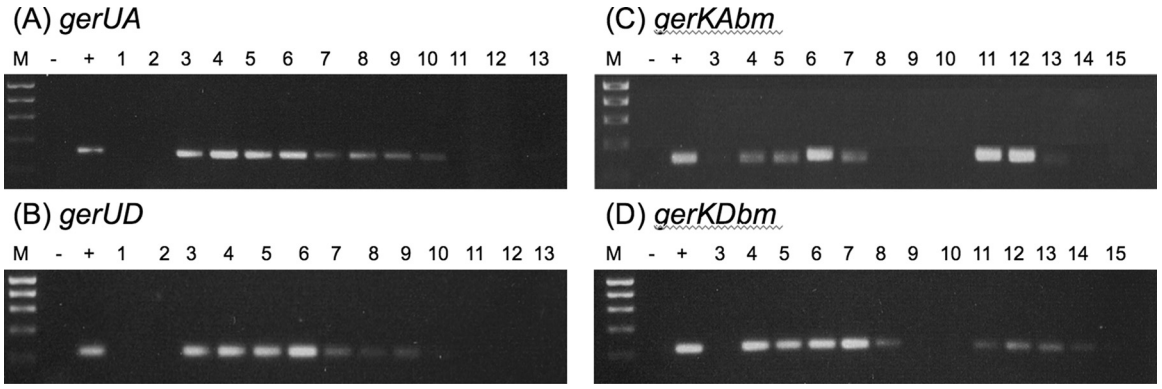

FIG 8 RT-PCR analysis of expression of *B. megaterium* GR A- and D-protein genes during sporulation. RT-PCR was conducted using gene-specific primers designed to amplify 230- to 300-bp fragments of *gerUA* (A), *gerUD* (B), *gerKAbm* (C), and *gerKDbm* (D) from RNA isolated from sporulating cultures as described in Materials and Methods. The numbers above the lanes refer to the time (in hours) after entry into sporulation. Positive-control reactions (+) (i.e., genomic DNA) and negative-control reactions (−) (i.e., no template DNA/RNA) reactions are indicated above the lanes. Lanes M, molecular weight markers (M).

ated with all GRs in *B. megaterium* and *B. subtilis*? In *B. megaterium* and *B. subtilis*, several operons encoding GRs have no obvious D genes in or adjacent to the operons. One possibility is that these GRs have no associated D proteins. However, since D proteins can clearly exert effects *in trans*, perhaps GRs encoded by operons without obvious nearby *D* genes utilize D proteins encoded far away, and there are potential *D* genes not associated with GR operons in *Bacillus* genomes. Clearly, there is much yet to be learned about these unexpected components of the spore germination apparatus.

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DISCUSION

La síntesis de los receptores que reconocen germinantes en esporas de *B. subtilis* está regulada por las condiciones de esporulación así como por factores de transcripción expresados durante la esporulación.

Se ha reportado anteriormente que las condiciones de esporulación alteran las características de resistencia de las esporas producidas (Baweja *et al.,* 2008; de Vries *et al.,* 2005; Hornstra *et al*., 2011; Rose *et al* 2007). Los autores plantean que los cambios son atribuidos a alteración de la expresión de genes; por ejemplo, Melly y colaboradores en el 2002 describe que la disminución en la resistencia a lisozima e hipoclorito está mediada por cambios en la expresión de proteínas de cubierta. Las condiciones de esporulación no solo podrían cambiar la expresión de estas proteínas, sino también modificar los perfiles de expresión de otras durante la esporulación, incluidos los receptores que reconocen germinantes. En este sentido, se prepararon esporas en medio mínimo y enriquecido así como a diferentes temperaturas de esporulación. Esporas preparadas bajo condiciones subóptimas (ya sea en medio mínimo sin aminoácidos o en temperaturas inferiores a 37˚ C) germinaron lentamente con nutrientes en comparación con esporas preparadas en medio enriquecido y óptima temperatura.

La disminución de la actividad del gen reportero GerA:lac Z así como de receptores en la membrana interna en esporas preparadas en medio mínimo, explican las diferencias en la germinación y confirman que las condiciones de esporulación modifican la expresión genética de la espora. Pero a su vez se puntualiza que esta modulación es específica; en ambas preparaciones de esporas (con cambios en la temperatura o en la composición del medio de esporulación) se disminuye la expresión de receptores germinantes pero no las proteínas SpoVA, las cuales constituyen los canales de ácido dipicolínico; en este punto, se ha descrito que variaciones en los niveles de SpoVA están relacionados con cambios en el tiempo de liberación de ácido dipicolínico cuando se germinan y monitorean esporas individuales; en el caso de las esporas preparadas, los tiempos de liberación fueron similares. Además, entre

ambas preparaciones de esporas no se observan diferencias en cuanto a la cantidad y actividad enzimas líticas; la germinación con Ca-DPA es una vía independiente de receptores basada en inducir la germinación activando las enzimas líticas por DPA externo (regularmente las enzimas líticas son activadas por el DPA que se libera desde el centro de la espora por la activación de receptores), lo cual permite evaluar cambios en estas enzimas, en las esporas preparadas no se observaron cambios significativos en la germinación con Ca-DPA.

Esta especificidad en la regulación de la expresión genética podría estar mediada por la activación o represión de factores sigma y/o factores de transcripción. En este sentido, se ha descrito que SpoVT es un factor de transcripción que se expresa en la esporulación tardía y regula la expresión de genes dependientes de sigma G (Asen *et al.,* 2009). Ambos eventos ocurren durante la síntesis de receptores germinantes, por lo que se sugiere que este factor transcripcional podría estar regulando su expresión. Nosotros encontramos que las esporas SpoVT germinaron mas rápido con los germinantes L-valina y AGFK, evento que es explicado por un aumento en la cantidad de receptores germinantes gerAA, gerAC, gerBC, gerK en la membrana de esporas SpoVT, datos que en su conjunto definen a la proteína SpoVT como un represor de la expresión de los receptores que reconocen germinantes. Sin embargo, algunos estudios también han descrito a esta proteína con un papel activador, de acuerdo a los autores este factor modula la expresión de las proteínas SASP durante la esporulación (Wang *et al* 2006; Dong *et al.,* 2004). Por lo que se determinó la cantidad de estas proteínas en esporas SpoVT y silvestres, siendo menor la cantidad de las proteínas SASP de tipo Beta en las esporas SpoVT, este cambio en la expresión de estas proteínas se ve reflejado en una disminución de la resistencia a UV por parte de las esporas SpoVT en comparación con las esporas de la cepa silvestre.

Además de describir el papel de SpoVT como activador y represor de genes en la esporulación, evaluamos su posible papel en la regulación de la expresión de receptores germinantes mediado por la composición del medio. En

células eucariotas los factores de crecimiento regulan la expresión de genes a partir de factores de transcripción, proceso que podría ser similar durante la esporulación y que tendría como punto final la expresión de SpoVT. En el estudio, se evalúo la expresión de este factor transcripcional por la generación del gen reportero SpoVT- lacZ. En esporas obtenidas en medio mínimo, la expresión de este gen reportero es mayor en comparación con las esporas obtenidas en medio enriquecido. Este aumento de la expresión de SpoVT en medio mínimo explica la baja germinación de las esporas por represión de la expresión de los receptores germinantes. Sin embargo, esporas de la cepa SpoVT preparadas en medio mínimo no recuperan completamente la germinación, por lo que otros factores podrían estar implicados en la regulación de los receptores, particularmente en un medio mínimo. En este sentido se ha descrito dentro de la regulación de SpoVT, la participación de la proteína YlyA, la cual se une al DNA y también está asociada a la activación de genes dependientes de sigma G (Wang *et al* 2006; Bagyan I, Hobot J, y Cutting S, 1996; Traag BA *et al*., 2013).

En el estudio, se generaron tanto la cepa knock-out como de sobre expresión para la proteína YlyA y se comparó tanto la germinación como los niveles de receptores germinantes con la cepa silvestre. Las esporas YlyA germinaron de manera similar con L-valina y AGFK, sin embargo, la germinación con dodecilamina fue mayor, estos cambios son explicados por un aumento de las proteínas SpoVAD en las esporas YlyA. Se ha descrito que la posible vía de la germinación con dodecilamina es la apertura de estos canales, por lo cual en esporas en donde la cantidad de estas proteínas es mayor, la germinación con dodecilamina será mayor. Al sobreexpresar YlyA, las esporas germinaron lentamente con los nutrientes L-valina y AGFK, pero con dodecilamina germinaron de manera similar a la silvestre , la disminución de la germinación con nutrientes se atribuye a una disminución de las subunidades de los receptores GerA, GerB y GerK. Estos resultados en su conjunto describen el papel de una nueva proteína, YlyA, en la regulación de la síntesis de los receptores. Esta regulación difiere en ausencia o sobre expresión de YlyA, se

sugiere además, que esta regulación implica la interacción con SpoVT, debido a que ambos están asociados a la transcripción de genes dependientes del factor sigma G. En este sentido, la ausencia de YlyA favorece la expresión de genes activados por SpoVT, por ejemplo las proteínas SpoVAD y las SASP de tipo beta, mientras que la sobre expresión mimetiza la actividad de SpoVT sobre los receptores germinantes al disminuir su expresión.

En las diferentes situaciones planteadas, los receptores juegan un papel importante en la germinación; cambios en su expresión se reflejan en alteraciones con la germinación con nutrientes. Sin embargo, algunos planteamientos aun quedan por resolver; por ejemplo, no se recuperó el 100% de la germinación de esporas preparadas en medio mínimo, o cambios en los niveles de GerD entre esporas preparadas en diferentes condiciones aun no son explicados. A pesar de haber estudiado factores de transcripción que regulan genes de esporulación tardía, los niveles de GerD no son afectados por la presencia o ausencia de factores como SpoVT o YlyA, por lo que se sugiere que otros factores podrían regular la expresión de los receptores o que su actividad puede ser regulada por otras proteínas aun no descritas en el germinosoma. En base a este último punto, por análisis bioinformático, se identificó la presencia de genes con función desconocida como proteínas que podrían participar en la germinación, proteínas que son expresadas y con promotores para el factor sigma G (expresados solo en la espora y por el mismo factor que codifica para receptores germinantes). En un caso particular, se identificó en el estudio a *gerKD*, un gen adyacente al operon GerK que por su cercanía cromosomal podría jugar un papel en la transcripción como promotor, además por su secuencia se predice que esta proteína es de carácter transmembranal considerándose como una posible proteína del germinosoma.

Para evaluar los posibles papeles de GerKD planteados, se generó el gen reportero GerKD- lacZ y se determinó que este gen es expresado en fases tardías de la esporulación (a la par de la síntesis de ácido dipicolínico) y que es regulado por sigma G. Este último dato se obtuvo por la generación de cepas knock-out para los diferentes factores sigma y la cuantificación de la expresión

del reportero descrito anteriormente. Al determinar que esta proteína es expresada en la espora, se determinó, por la generación de la cepa knock-out (GerKD) y su incorporación en cepas con genes reporteros GerA- lacZ y GerB-lacZ, que la proteína no participa en la regulación transcripcional de los receptores germinantes. Estos datos fueron confirmados por la determinación de receptores que reconocen germinantes en esporas GerKD y por la comparación con la cepa silvestre, los cuales mostraron diferencias menores entre esporas, sin embargo la germinación con AGFK es mayor en esporas GerKD, indicando que esta proteína está implicada en la germinación pero no en la regulación de la expresión de los receptores y al no observar cambios en la germinación con Ca-DPA y dodecilamina, se propone que GerKD solo está implicada en la germinación con nutrientes.

El intento de obtener respuestas, genera nueva información, por ejemplo, este es el primer estudio que describe la participación de las proteínas YlyA y GerKD en la germinación, sin embargo, genera nuevas interrogantes, como describir el posible mecanismo por el cual GerKD participa en la germinación o determinar los mecanismos de coregulación entre YlyA y SpoVT.

Además, este estudio realizado acerca de la modulación de receptores, plantea la forma en que factores ambientales y genéticos influyen en la síntesis de éstos y que finalmente definen la germinación de esporas individuales así como de una población (Figura 2). El conocer estos factores permitirá comprender la generación de esporas con diferentes tasas de germinación incluso en las mismas poblaciones, planteando un escenario donde las condiciones del medio, así como mutaciones generadas en genes importantes, propicia la diversificación de esporas y la aparición de esporas súper latentes, las cuales tienen como característica principal la baja o nula germinación, la cual es explicada por una desregulación de los niveles de receptores que reconocen germinantes. Comprender la aparición de este subgrupo de esporas permitirá conocer mecanismos de cómo son generadas y finalmente permitirá establecer mecanismos de erradicación.

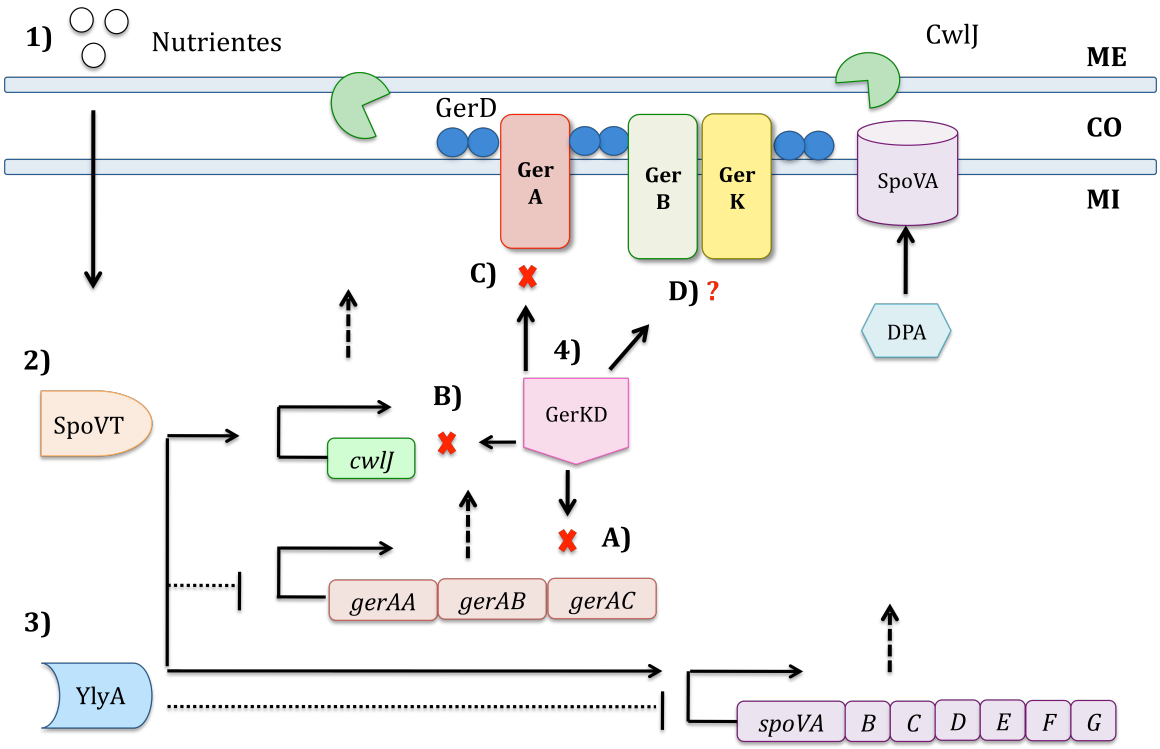

Figura 2. Modulación de receptores que reconocen germinantes en esporas de *B. subtilis* por factores ambientales y genéticos. En la membrana interna de la espora (**MI**) se encuentran localizados los receptores germinantes GerA, GerB y GerK así como la proteína GerD. La germinación es iniciada por la interacción de los nutrientes con su respectivo receptor dando como resultado la movilización del ácido dipicolínico (**DPA**) del centro de la espora hacia la corteza (**CO**) a partir de los canales **SpoVA**, una vez liberado el DPA activa la enzima lítica **CwlJ** y la germinación procede con la expansión del núcleo, la hidratación y el regreso de la actividad enzimática. En este estudio se demostró que **1**) la cantidad de nutrientes durante la esporulación afecta la actividad transcripcional de los receptores germinantes por lo tanto la cantidad de los mismos en la membrana y la germinación, **2)** se establece que uno de los mecanismos por los cuales los nutrientes afectan la actividad transcripcional es por el aumento de la proteína SpoVT, la cual es un factor que reprime la transcripción de los operones de los receptores que reconocen germinantes. Además, se describe que esta proteína es un factor transcripcional que activa genes como *cwlJ* y el operón *spoVA*. **3)** Se describió una nueva proteína, ylyA la cual reprime la síntesis de las proteínas *SpoVA* y en su sobreexpresión reprime los receptores que reconocen germinantes. **4)** Por último, se describió que la proteína gerKD: **A)** se expresa en la espora durante etapas tardías de la esporulación a la par de la síntesis de los receptores que reconocen germinantes; sin embargo, no modula la transcripción de los mismos, **(B)** no afecta la síntesis de enzimas líticas o proteínas de la corteza**, (C)** no esta relacionada con la germinación mediada por el receptor GerA pero **(D)** se plantea su participación en la germinación mediada por los receptores GerB y GerK**.**

El estudio contribuye a comprender el mecanismo de germinación de esporas de *B. subtilis* y poder extrapolarlo a microorganismos patógenos del mismo género como *B. cereus* e incluso de otros, como las especies de *Clostridium*. Por ejemplo, la baja cantidad de receptores observado en esporas

preparadas en medio mínimo y su germinación disminuida, podría explicar el caso de las esporas que contaminan alimentos (ejemplo, esporas de *B. cereus y C. botulinum)*; estas esporas aún cuando están en un medio rico de nutrientes, la germinación no ocurre y la explicación podría estar basada en que, por lo regular, las esporas son generadas en un medio subóptimo, generalmente el suelo, donde la cantidad de nutrientes es insuficiente y la temperatura es por debajo de la óptima, generando esporas con una cantidad disminuida de receptores, estas esporas contaminan alimentos, resisten las condiciones de esterilización y no germinan hasta llegar al lumen intestinal y producir enfermedad.

En este estudio se concluye que factores ambientales en los cuales se produce la esporulación como la composición del medio y la temperatura, así como factores genéticos, particularmente los factores de transcripción SpoVT y YlyA son los que regulan la expresión de receptores que reconocen germinantes.

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Anexos

Anexo I

Efecto de los niveles de Mn en la resistencia de esporas de *Bacillus megaterium* a calor, radiación y peróxido de hidrógeno.

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ORIGINAL ARTICLE

Effects of Mn levels on resistance of *Bacillus megaterium* spores to heat, radiation and hydrogen peroxide

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Bacillus megaterium, *Deinococcus*, heat resistance, Mn, radiation resistance, spore resistance, spores.

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Abstract

Aims: To determine the effects of Mn levels in *Bacillus megaterium* sporulation and spores on spore resistance.
Methods and Results: *Bacillus megaterium* was sporulated with no added $MnCl_2$ and up to 1 mmol l^{-1} $MnCl_2$. The resultant spores were purified and loosely bound Mn removed, and spore Mn levels were found to vary *c.* 100-fold. The Mn level had no effect on spore γ-radiation resistance, but *B. megaterium* spores with elevated Mn levels had higher resistance to UVC radiation (as did *Bacillus subtilis* spores), wet and dry heat and H_2O_2. However, levels of dipicolinic acid and the DNA-protective α/β-type small, acid-soluble spore proteins were the same in spores with high and low Mn levels.
Conclusions: Mn levels either in sporulation or in spores are important factors in determining levels of *B. megaterium* spore resistance to many agents, with the exception of γ-radiation.
Significance and Impact of the Study: The Mn level in sporulation is an important factor to consider when resistance properties of *B. megaterium* spores are examined, and will influence the UV resistance of *B. subtilis* spores, some of which are used as biological dosimeters.

Introduction

Spores of *Bacillus* species are extremely resistant to a variety of harsh treatments including heat, radiation and oxidizing agents (Setlow 2006). While there are multiple factors that contribute to spore resistance, the prevention and repair of damage to the spore's genome are of prime importance. A major mechanism preventing DNA damage in spores is the saturation of spore DNA with specific DNA-binding proteins, the α/β-type small, acid-soluble spore proteins (SASP), which protect DNA against damage by wet heat, dry heat, UV and γ-radiation and some oxidizing agents, including hydrogen peroxide (H_2O_2) (Setlow 2006; Moeller *et al.* 2008). The DNA protection given by the α/β-type SASP in spores is so great that a number of agents that might be expected to cause DNA damage, such as wet heat and H_2O_2, do not kill spores by damage to DNA. However, agents such as dry heat, UV radiation and γ-radiation do kill spores by DNA damage. Consequently, the other major factor in spore DNA resistance is the repair of DNA damage when spores return to life in germination followed by outgrowth, with this repair catalysed by a number of different enzymes, some of which are spore-specific (Setlow 2006; Wang *et al.* 2006; Moeller *et al.* 2007, 2008; Ibarra *et al.* 2008; Barraza-Salas *et al.* 2010).

Recent work in several biological systems has indicated that prevention of cell killing by agents like γ-radiation that can cause lethal damage by generation of reactive oxygen species (ROS) is in large part owing to the protection of proteins against such agents, in particular proteins involved in DNA repair (Daly *et al.* 2004, 2007, 2010; Daly 2009; Križko and Radman 2010; Slade and Radman 2011). One agent protecting proteins against ROS is Mn^{2+} ions complexed with low molecular weight species such as phosphate, amino acids, peptides or nucleosides,

and cells of several types of organisms exhibit heightened ROS resistance when their Mn content is increased (Daly *et al.* 2007, 2010; Barnese *et al.* 2008; McEwan 2009; McNaughton *et al.* 2010; Slade and Radman 2011). This work suggested that spores of *Bacillus* species might also exhibit increased ROS resistance if their Mn content was increased. However, a recent study found that *Bacillus subtilis* spore resistance to wet heat, dry heat, H_2O_2 or γ-radiation was unchanged over a ≥200-fold range of cytoplasmic Mn content (Granger *et al.* 2011). While this finding suggests that detoxification of ROS by Mn-containing complexes might not be important in bacterial spore resistance, *B. subtilis* spores are monogenomic. Consequently, haploidy might have masked potential gains in these spores' resistance owing to ROS scavenging by Mn-containing complexes, because recombination repair cannot be carried out efficiently in a haploid outgrowing spore. In contrast to *B. subtilis* spores, *Bacillus megaterium* spores are digenomic and can exhibit significant shoulders in their inactivation curves with both UVC and γ-radiation (Donnellan and Stafford 1968; Aoki and Slepecky 1974; Hauser and Karamata 1992), a general characteristic of radiation-resistant polyploid organisms. Consequently, in this work, we have examined the resistance of *B. megaterium* spores with very different Mn contents to wet and dry heat, H_2O_2 and UV and γ-radiation.

Materials and methods

Spores of *B. megaterium* QM B1551 (ATCC #12872; originally obtained from H.S. Levinson) were prepared at 30°C in liquid-supplemented nutrient broth (Goldrick and Setlow 1983) (125 ml in a 1 l flask) with no additional $MnCl_2$ added (supplemented nutrient broth with no $MnCl_2$ added has *c.* 0·3 μmol l^{-1} Mn) and with $MnCl_2$ added up to 1 mmol l^{-1}, and the spores were harvested and purified, including washing twice for 1 h with 10 mmol l^{-1} EDTA at 4°C to remove loosely bound Mn. The EDTA-treated spores were washed thoroughly with water, stored in water at 4°C and protected from light, and spores' Mn levels were determined as described (Nicholson and Setlow 1990; Granger *et al.* 2011). Spores of *B. subtilis* PS533 (Setlow and Setlow 1996) were prepared at 37°C in liquid 2xSG medium with different levels of $MnCl_2$ added, and the spores were isolated, purified and EDTA treated, and Mn levels were determined as described (Nicholson and Setlow 1990; Granger *et al.* 2011). All spores used in this work were free (>98%) of growing or sporulating cells and germinated spores.

Resistance of *B. megaterium* spores to wet heat [85°C in water with spores at an optical density at 600 nm (OD_{600}) of 1], dry heat (105°C with the lyophilized material from 1 ml of spores at an OD_{600} of 1) and H_2O_2 [5% in 50 mmol l^{-1} KPO_4 buffer (pH 7·4) with spores at an OD_{600} of 1] was measured as described (Popham *et al.* 1995; Granger *et al.* 2011). Spore resistance to γ-radiation in liquid (spores in water at an OD_{600} of 1) was measured using a ^{60}Co source with an output of 2·9 kGy h^{-1} as described (Granger *et al.* 2011). Spore resistance to γ-radiation in the dry state was measured by irradiation as for spores in liquid, but 1 ml of spores at an OD_{600} of 1 was centrifuged in a 1·5-ml microcentrifuge tube and the pelleted spores were freeze dried prior to irradiation. For measurements of spore viability following dry heat treatment or γ-irradiation of dry spores, samples were rehydrated with 1 ml water and treated briefly in a bath sonicator to disperse the spores, the OD_{600} was measured to quantitate spore recovery and spore viability was measured. UV resistance of *B. megaterium* and *B. subtilis* spores was measured at 23°C with spores at an OD_{600} of 1 in 2 ml water in a rotating 35-mm-diameter Petri dish exposed to 254 nm radiation (UVC) from a UVG-1 short wave UV lamp (UVP Inc., San Gabriel, CA, USA). The output of the lamp at the surface of the liquid was measured as 2×10^{-3} J min^{-1} cm^{-2} using a J-225 BLAK-RAY Ultraviolet Meter (UVP Inc.). All resistance properties were measured on two independent spore preparations with similar results.

Levels of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] that normally comprises *c.* 20% of the dry weight of the central core of spores of *Bacillus* species and is present in spores as a 1 : 1 chelate with divalent metal ions, generally Ca^{2+}, were determined in spores by laser tweezers Raman spectroscopy as described (Huang *et al.* 2007). Levels of the DNA-protective α/β-type SASP in spores were determined by dry rupture of 5 mg of purified dry spores, extraction of the ruptured spores twice with 0·75 ml of cold 3% acetic acid-30 mmol l^{-1} HCl, dialysis of the pooled extracts against cold 1% acetic acid for 18 h with one change and then lyophilization of the dialysates (Nicholson and Setlow 1990). The dry residue was dissolved in 30 μl of 8 mol l^{-1} urea plus 15 μl acid-gel diluent, aliquots run on polyacrylamide gel electrophoresis at low pH, the proteins on the gel were stained with Coomassie Blue, and the staining intensity of the bands because of SASP-α and SASP-β was compared (Nicholson and Setlow 1990).

Results

Sporulation of *B. megaterium* in supplemented nutrient broth with no $MnCl_2$ added and up to 1 mmol l^{-1} additional $MnCl_2$ was indistinguishable (data not shown), even though the Mn levels increased *c.* 100-fold in spores prepared with high $MnCl_2$ added to the sporulation

Table 1 Mn levels in *Bacillus megaterium* and *Bacillus subtilis* spores sporulated with different $MnCl_2$ concentrations*

[$MnCl_2$] added to sporulation (μmol l^{-1})	Mn (μg gm^{-1} dry wt) and DPA (attomol per spore) levels in spores		
	B. megaterium		*B. subtilis*
	Mn	DPA	Mn
0	52	505 ± 54	nd
0·3	–		28
1	–		261
3	757	547 ± 78	–
10	2922	533 ± 64	1811
100	5687	472 ± 56	2613
1000	6926	464 ± 39	3903

DPA, dipicolinic acid.

**B. megaterium* and *B. subtilis* were sporulated with different $MnCl_2$ concentrations added, spores were purified and EDTA treated twice, and the spores' Mn contents and DPA contents (±standard deviations for the 50 individual spores analysed) were determined as described in Materials and methods. The EDTA treatment only removed a significant amount of Mn (*c.* 60%) from the spores prepared with 1 mmol l^{-1} $MnCl_2$, as found previously (Granger *et al.* 2011).

medium (Table 1). This was seen with two independent preparations of *B. megaterium* spores (data not shown). The great majority of this spore Mn was tightly bound, because it was only with spores prepared with 1 mmol l^{-1} $MnCl_2$ that EDTA treatment removed any large percentage of the spores Mn (Table 1). While two EDTA treatments were routinely used to remove loosely bound Mn from spores, >95% of the Mn was removed in the first EDTA treatment (data not shown), as was found when *B. subtilis* spores were prepared in medium with up to 1 mmol l^{-1} $MnCl_2$ (Granger *et al.* 2011). The Mn levels in the EDTA-treated *B. megaterium* spores prepared with different $MnCl_2$ concentrations were also relatively similar to levels in *B. subtilis* spores prepared with the same added $MnCl_2$ (Table 1). It is most likely that the Mn remaining in spores after the EDTA washes is in the spore core and chelated to the spore's huge DPA pool that comprises *c.* 20% of the core's dry weight (Setlow 2006). However, even at the highest spore Mn levels obtained in this work, <5% of the spores' DPA was chelated with Mn, based on the amount of Mn in spores, and using 10% as the percentage of *B. megaterium* spore dry weight as DPA (Setlow 2006).

Previous work showed that increasing the Mn content *c.* 300-fold had no notable effect on *B. subtilis* spore resistance to wet heat, dry heat or hydrogen peroxide (Granger *et al.* 2011). However, with *B. megaterium* spores, increasing their Mn levels resulted in significantly elevated resistance to both wet heat and dry heat, and H_2O_2 was also increased when these spores' Mn levels were ≥750 μg g^{-1} (Fig. 1a–c). Increased Mn levels also greatly increased *B. megaterium* spores' resistance to UVC radiation, and this was also the case for *B. subtilis* spores but not as dramatically as with *B. megaterium* spores (Fig. 1d; Fig. 2). Note further that *B. megaterium* spores were significantly more resistant to UVC radiation than *B. subtilis* spores, perhaps because *B. subtilis* spores are monogenomic while *B. megaterium* spores are digenomic (Hauser and Karamata 1992). Previous work showed that *B. subtilis*

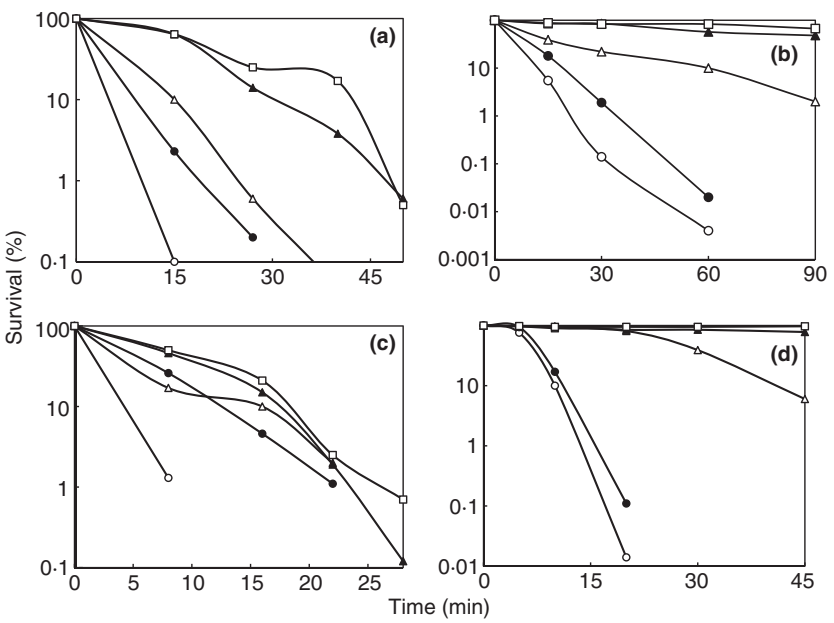

Figure 1 (a–d) Resistance of *Bacillus megaterium* spores with different Mn contents to heat, UVC radiation and H_2O_2. The resistance of *B. megaterium* spores with different Mn contents (Table 1) to (a) wet heat, (b) dry heat, (c) H_2O_2 and (d) UVC radiation were determined as described in Materials and methods. The symbols used to denote the concentrations of $MnCl_2$ added to the sporulation medium (Table 1) are (○) 0; (●) 3 μmol l^{-1}; (△) 10 μmol l^{-1}; (▲) 100 μmol l^{-1} and (□) 1 mmol l^{-1}.

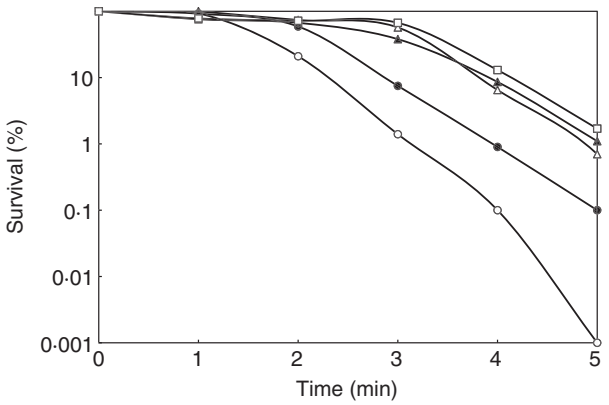

Figure 2 UVC radiation resistance of *Bacillus subtilis* spores with different Mn contents. The UVC resistance of *B. subtilis* spores with different Mn contents (Table 1) was determined as described in Materials and methods. The symbols used to denote the $MnCl_2$ concentrations added to the sporulation medium are (○) 0·3 μmol l^{-1}; (●) 1 μmol l^{-1}; (△) 10 μmol l^{-1}; (▲) 100 μmol l^{-1} and (□) 1 mmol l^{-1}.

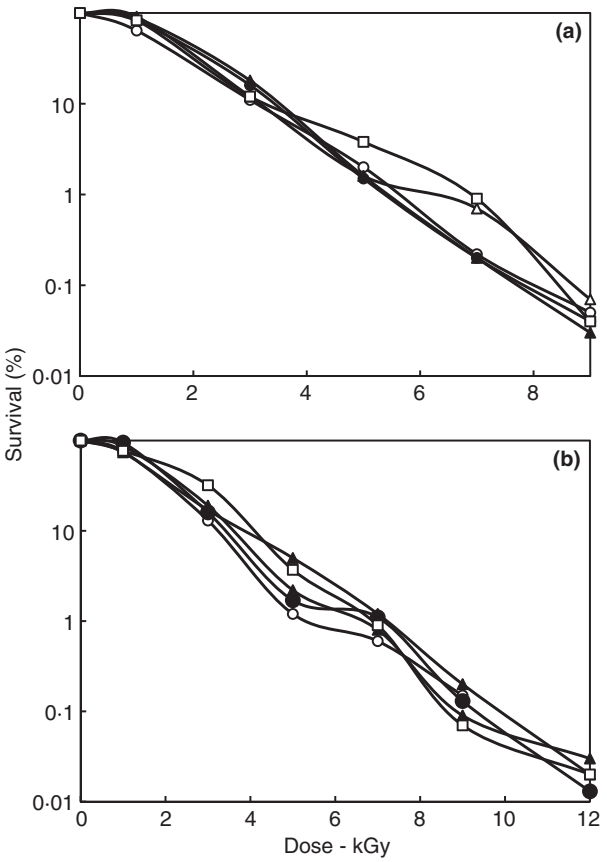

Figure 3 (a, b) The γ-radiation resistance of *Bacillus megaterium* spores with different Mn contents. The γ-radiation resistance of *B. megaterium* spores with different Mn contents (Table 1) was determined either (a) in liquid or (b) dry as described in Materials and methods. The symbols used to denote the $MnCl_2$ concentrations added to the sporulation medium are (○) 0; (●) 3 μmol l^{-1}; (△), 10 μmol l^{-1}; (▲), 100 μmol l^{-1} and (□) 1 mmol l^{-1}.

spore resistance to γ-radiation is independent of these spore's Mn content (Granger *et al.* 2011), and this was also the case with *B. megaterium* spores when irradiated either wet or dry (Fig. 3a,b). However, *B. megaterium* spores in liquid were slightly more resistant to γ-radiation than *B. subtilis* spores (Fig. 3a; and Granger *et al.* 2011).

While it seemed likely that the increased resistance of spores with high Mn levels was directly owing to the spores' high levels of Mn, it was certainly possible that elevated Mn levels during spore formation might have altered levels of some other component that protects spore proteins or DNA. One factor that certainly protects spore DNA from damage, and perhaps also spore protein, is DPA (Setlow 2006; Setlow *et al.* 2006; Magge *et al.* 2008). At least greatly elevated Ca^{2+} levels during sporulation have been shown to significantly alter the sporulating cell's transcriptome (Oomes *et al.* 2009). Interestingly, among the genes upregulated by elevated Ca^{2+} are the genes encoding the two subunits of DPA synthase. However, DPA levels in 50 individual *B. megaterium* spores prepared with $MnCl_2$ concentrations of 0–1 mmol l^{-1} were all essentially the same (Table 1).

A second major factor in spore DNA resistance is the α/β-type SASP, and spores with decreased α/β-type SASP levels are significantly less resistant to wet and dry heat, H_2O_2, and UVC and γ-radiation (Setlow 2006; Moeller *et al.* 2008). However, again, levels of α/β-type SASP were essentially identical in *B. megaterium* spores prepared with $MnCl_2$ concentrations of 0–1 mmol l^{-1} (data not shown). Another factor that could affect spore wet heat resistance is core water content (Setlow 2006). While we did not measure core water contents for *B. megaterium* spores, *B. subtilis* spores with large differences in Mn levels have identical core water contents (Granger *et al.* 2011).

Discussion

A number of observations made in the current work confirm reports made a number of years ago concerning the effects of preparation of spores of *Bacillus* species in media with elevated Mn concentrations. Thus, spores of *Bacillus fastidiosus* exhibit increased wet heat resistance when prepared in high-Mn media, and spores of *B. megaterium* prepared with high Mn exhibit significantly higher resistance to wet heat and UVC radiation (Donnellan and Stafford 1968; Aoki and Slepecky 1973, 1974). There is also one report that sporulation in medium with high Mn results in spores with very slightly increased

γ-radiation resistance (Aoki and Slepecky 1974), although this was not seen in the current work. In most of the work noted above, sporulation in media with high Mn resulted in increased spore Mn, although precautions were generally not taken to eliminate surface-bound Mn from spores. There have also been several studies in which the great majority of spores' divalent metal ions were replaced with Mn, by either sporulation with high Mn^{2+} concentrations and minimal levels of other divalent metal ions (Slepecky and Foster 1959) or removal of almost all divalent metal ions from spores by titration with acid and then back titration with Mn^{2+} in a basic solution (Marquis *et al.* 1981; Bender and Marquis 1985). In the latter experiments, almost all spore Ca and Mg were replaced by Mn, resulting in spore Mn levels 15–30 times higher than obtained with *B. megaterium* spores in this work. In contrast to the effects of increases in spore Mn levels seen by sporulation in normal media supplemented with up to 1 mmol l^{-1} $MnCl_2$, both in the current work and previously (Donnellan and Stafford 1968; Aoki and Slepecky 1973, 1974), replacement of almost all *B. megaterium* spore divalent cations with Mn generally resulted in slightly decreased wet heat resistance in the resultant spores (Slepecky and Foster 1959; Bender and Marquis 1985).

The current work showed clearly that increased Mn levels in sporulation and spores had major effects on the resistance properties of *B. megaterium* spores. For spore UV resistance, levels of two agents known to protect spore DNA against UVC radiation, DPA and α/β-type SASP, were essentially identical in spores with high and low Mn levels. Consequently, differences in DNA protection in spores with low and high Mn levels seem unlikely. Both UVC and γ-radiation can certainly kill spores by DNA damage, and dry heat kills at least *B. subtilis* spores by DNA damage (Setlow 2006; Moeller *et al.* 2007, 2008). In contrast, wet heat and H_2O_2 do not kill *B. megaterium* spores by DNA damage, but rather likely by damage to one or more spore proteins (Palop *et al.* 1998; Setlow 2006; Coleman *et al.* 2007, 2010), and at least *B. subtilis* spore inactivation by wet heat is independent of oxygen (Setlow and Setlow 1998). *Bacillus megaterium* spores prepared with high Mn levels exhibited elevated resistance to some but not all agents that kill spores by DNA damage, as well as to wet heat and H_2O_2. Consequently, it is difficult to ascribe the elevated resistance of spores with high Mn levels solely to either a general increase in DNA repair capacity, such as recombination repair to take advantage of the two complete genomes in *B. megaterium* spores or an increased ability to detoxify ROS. However, it is a logical possibility that the effects of high Mn are because of the ability of Mn complexes to inactivate specific ROS generated by some agents but not others. Indeed, Mn complexes from the radiation-resistant bacterium *Deinococcus radiodurans* strongly protect proteins against γ-rays, but protect DNA only poorly (Daly *et al.* 2007, 2010).

DNA in radiation-resistant and radiation-sensitive bacteria exhibits similar levels of DNA damage with the same dose of ionizing radiation (Daly *et al.* 2004; Daly 2009). In contrast, levels of protein damage in irradiated bacteria are dependent on their antioxidant status, and yields of radiation-induced protein oxidation can be >100-fold higher in radiation-sensitive bacteria (Daly 2009). Indeed in prokaryotes, the lethal effects of ionizing radiation appear to be mediated by oxidative protein damage, and for many oxidative stress conditions, including even UVA radiation, DNA may not be the major target of ROS (Daly *et al.* 2007; Leichert *et al.* 2008; Bosshard *et al.* 2010; Krisko and Radman 2010; Avery 2011; Espirito Santo *et al.* 2011; Sobota and Imlay 2011). In addition, levels of protein damage in γ-irradiated bacteria are linked to the accumulation of Mn^{2+}, such that as bacteria's Mn^{2+} concentrations decline, cells become more sensitive to protein oxidation, but with no effects on DNA damage levels (Daly *et al.* 2004, 2007). These findings led to the conclusion that proteins are the principal targets of γ-radiation in bacteria and that Mn^{2+} prevents γ-radiation toxicity by protecting protein function (Daly 2009).

Mn^{2+} protection of proteins from ROS appears to occur at two levels: (i) by replacing Fe^{2+} with Mn^{2+} in enzymes, active sites are protected from oxidative damage (Anjem *et al.* 2009; Sobota and Imlay 2011); and (ii) surplus Mn^{2+} forms complexes with metabolites which can scavenge superoxide, H_2O_2 and hydroxyl radicals (Daly *et al.* 2010). Therefore, when spores are prepared with elevated $MnCl_2$, spore enzymes that normally bind Fe^{2+} but are also capable of binding Mn^{2+} might be more resistant to oxidative stress (Sobota and Imlay 2011). In addition, DPA is present in spores at very high levels and can form potent ROS-scavenging complexes with Mn^{2+} (Granger *et al.* 2011).

UVC causes substantial direct (ROS-independent) damage to cellular macromolecules *in vivo*. However, antioxidants can still increase the survival of cells exposed to UVC (Chan *et al.* 2006). Consequently, antioxidants in cells help avert ROS-mediated toxicity that is secondary to the UVC itself. For example, UVC disrupts certain types of disulfide bonds and causes protein aggregation, which could uncouple metabolism from electron transport and lead to ROS production. In turn, metabolism-induced ROS production would be expected to damage other cellular processes including DNA repair (Krisko and Radman 2010; Slade and Radman 2011). Wet heat and exposure to H_2O_2 also can damage proteins

directly in spores and again could conceivably increase ROS production during spore outgrowth. A striking finding in this work was that UVC resistance but not γ-radiation resistance of *B. megaterium* spores was highly dependent on the spores' Mn content. This could be due at least in part to different modes of action of UVC and γ-radiation in cells. In contrast to UVC, most γ-radiation-induced lesions in cells are caused by ROS formed in the radiolysis of water during irradiation (von Sonntag 1987). As the water content of spores is lower than in vegetative cells (Setlow 2006), ROS production in γ-irradiated spores may be limited, and thus, ROS-scavenging Mn complexes might have less of an impact on spore survival. In addition, the limited molecular mobility of molecules in the spore core (Cowan *et al.* 2003) may decrease the ability of Mn complexes to scavenge long-lived ROS. Perhaps Mn complexes in outgrowing spores prevent oxidative inactivation of enzymes involved in the repair of UVC-damaged spore DNA. Alternatively, if enzymes that specifically repair UVC-induced DNA lesions in spores bind Mn^{2+} (note that such enzymes could not repair all the different lesions generated by γ-radiation), then elevated Mn levels during sporulation might selectively lead to the protection of the resultant spores against UVC but not γ-radiation. Because spore DNA lesions caused by wet heat, dry heat and H_2O_2 vary widely, and again most likely require different enzymes for their repair (Huesca-Espitia *et al.* 2002; Setlow 2006), the argument made above can also be made for differences observed in the effects of Mn accumulation on spore resistance to H_2O_2, dry and wet heat.

Another potential explanation for some results in the current work is that high Mn levels during sporulation selectively induce the synthesis of enzymes that can only repair DNA lesions generated in spores by UVC radiation (the spore photoproduct generated between adjacent thymine residues) and dry heat (abasic sites), but not the most dangerous lesions generated by γ-radiation (double-strand breaks). There are certainly repair enzymes that exhibit the appropriate specificity, although there is no information on the induction of synthesis of such enzymes by Mn and the packaging of such enzymes into spores. While induction of specific DNA repair enzymes by Mn could explain at least the elevated resistance to UVC radiation and dry heat of *B. megaterium* spores prepared with high Mn levels, such enzyme induction cannot explain such spores' elevated H_2O_2 and wet heat resistance, as these agents kill *B. megaterium* spores by protein damage (Palop *et al.* 1998; Coleman *et al.* 2010). However, we note that Mn^{2+} can form catalytic H_2O_2-decomposing complexes with amino acids and peptides, and Mn^{2+} can also selectively protect the function of metabolic pathways (e.g. pentose phosphate pathway), which could favour recovery from some stress conditions but not others (Berlett *et al.* 1990; Sobota and Imlay 2011).

Whatever the explanation for the increased resistance of *B. megaterium* spores with high Mn levels, this phenomenon will be important to consider when examining the resistance of spores of this species and perhaps spores of other species that form digenomic spores as well. In addition, the significant effects of Mn levels in sporulation and spores on the UVC radiation resistance of not only *B. megaterium* spores, but also *B. subtilis* spores and likely spores of all *Bacillus* species, will certainly need to be considered when preparing spores as indicators for UV inactivation regimens or UV dosimeters.

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Anexo II

Papel de prpE en la germinación y proteínas de germinación en esporas de *Bacillus subtilis*

Hinc K *et al* en el 2006 demostró que la expresión de los operones GerA y GerK es dependiente de la fosfatasa PrpE. Por lo que se consideró importante evaluar el papel de prpE en la modulación de receptores que reconocen germinantes en esporas preparadas en diferentes condiciones de esporulación para lo cual se utilizaron las cepas presentadas en la tabla IIa.

Tabla IIa. Cepas utilizadas para evaluar el efecto de PrpE en la modulación de receptores que reconocen germinantes.

Cepa	Fenotipo
PS767	*gerA:lacZ* MLS^R
PS4219	△*prpE gerA: lacZ* MLS^R Spc^R

En primera instancia, se evaluó el efecto de PrpE en la transcripción del operón GerA a partir de la fusión transcripcional *gerA:lacZ* e introduciendo una mutación en el gen *prpE* para generar la cepa knock-out y así compararla con la cepa silvestre. La actividad transcripcional fue determinada por la cuantificación del producto que emite fluorescencia a partir de la degradación de MUG por la beta galactosidasa producida del gen reportero *gerA:lacZ* en esporas preparadas bajo diferentes condiciones, la metodología se explica ampliamente en el capitulo I. No se encontraron diferencias entre la actividad transcripcional del gen reportero *gerA:lacZ* en esporas preparadas tanto en medio mínimo como enriquecido de la cepa silvestre PS767 y la mutante PS4219 (Figura IIa).

La fosfatasa PrpE podría estar modulado los receptores de manera post-transcripcional, por lo cual se determinaron por Western Blot los receptores que reconocen germinantes (GerAA, GerAC, GerBC y GerKA) así como las proteínas SpoVAD y GerD en la fracción membranal de esporas preparadas en medio enriquecido tanto silvestres como mutantes para *prpE*. Sin embargo, no se encontraron diferencias significativas entre esporas para los receptores que reconocen germinantes (Figura IIb)

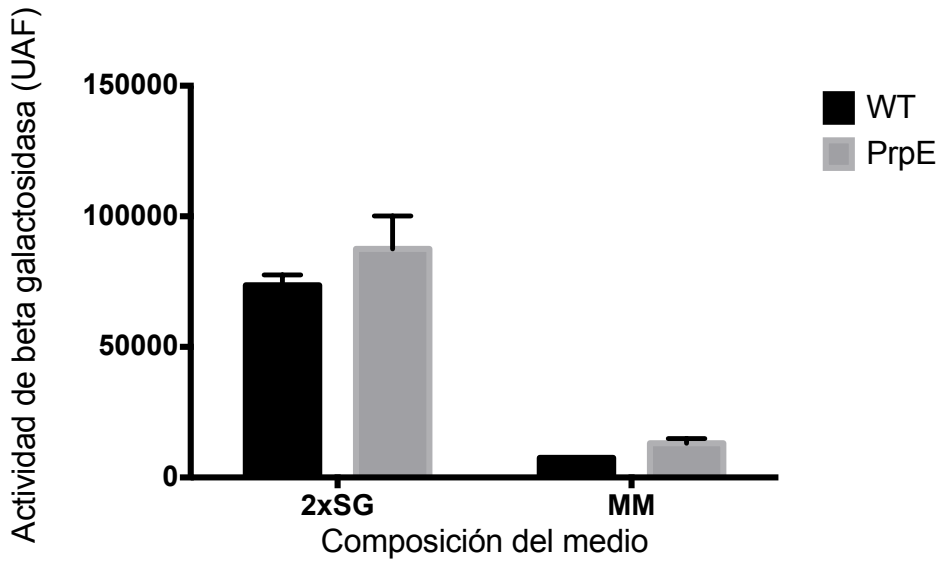

Figura IIa. Efecto de *prpE* en la actividad transcripcional del operón *gerA:lacZ*. Se determinó la actividad transcripcional del reportero *gerA: lacZ* en esporas de las cepas PS767 (WT *gerA:lacZ*) y PS4219 (*prpE gerA;lacZ*) preparadas en medio enriquecido (2xSG) y mínimo (MM).

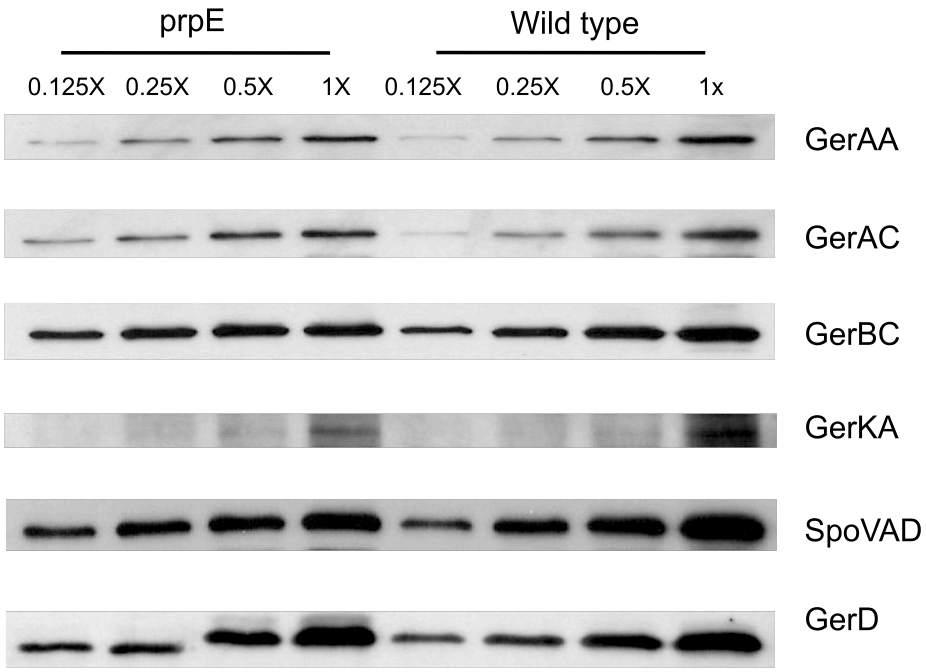

Figura IIb. Niveles de proteínas de germinación en esporas de las cepas PS767 (WT) y PS4219 (*prpE*). Las esporas de ambas cepas fueron preparadas en medio enriquecido, purificadas y se aisló la fracción membranal por centrifugación diferencial. La cantidad de fracción membranal estudiada es similar en ambas preparaciones. Los anticuerpos fueron probados en la misma membrana.

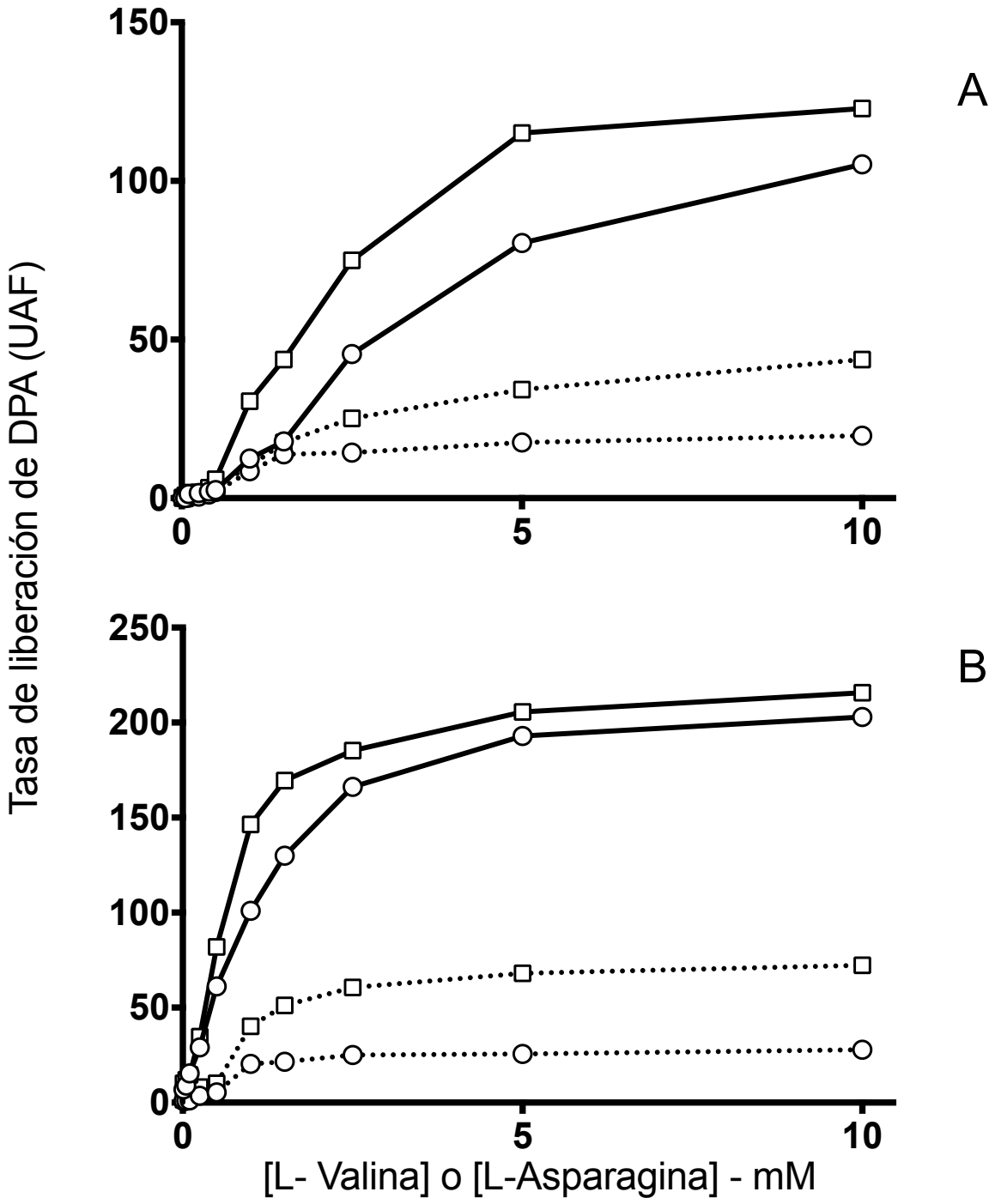

Figura IIc. Tasas de germinación con L- valina o L- asparagina preparadas en medio mínimo y enriquecido. Se prepararon esporas de las cepas PS767 (○) y PS4219 (□) en medio enriquecido (línea continua) y mínimo (línea discontinua) y se germinaron con varias concentraciones de L- valina (A) y L- asparagina (B).

La fosfatasa no está involucrada en la modulación de receptores germinantes pero podría tener un papel en la señalización de los receptores que reconocen germinantes, por lo cual se evaluó la germinación con nutrientes de esporas preparadas en medio enriquecido y mínimo tanto de la cepa silvestre como la mutante *prpE*. Sin embargo, no se encontraron diferencias significativas entre esporas para la germinación con L-valina o AGFK (Figura IIc). Aún cuando se siguió la metodología propuesta por Hinc K *et al* en el 2006 e incluso se repitieron los experimentos con la cepa mutante *prpE* utilizada en el estudio, no se relacionó esta fosfatasa con la germinación de esporas de *B. subtilis.*

Anexo III

Efecto de las condiciones de esporulación en la formación de cluster en la membrana interna de la espora de *Bacillus subtilis*

En el capitulo I se demostró que esporas preparadas en condiciones subóptimas de esporulación, sea de composición de medio o de temperatura germinan más lento que esporas preparadas en medio enriquecido y a 37˚C, además se estableció que estas diferencias son atribuidas a una disminución la cantidad de receptores germinantes presentes en la membrana de la espora. Griffiths *et al*., en el 2011 propusieron que la agrupación de receptores en cluster en la membrana interna de la espora es una estrategia para incrementar la respuesta en presencia de germinantes, debido a que la cantidad de receptores en la espora es baja. Bajo esta hipótesis, cambios en los niveles de receptores germinantes podrían alterar la formación de cluster en estos grupos de esporas. Para comprobar este supuesto se prepararon bajo diferentes condiciones de esporulación, esporas de cepas con fusiones entre receptores y proteínas fluorescentes y se evaluó la formación de cluster. Las cepas utilizadas son presentadas en tabla IIIa y las condiciones de esporulación que se trabajaron fueron relacionadas a la composición del medio: enriquecido y mínimo, así como de temperatura: 37˚C y 25˚C (temperatura ambiente).

Tabla IIIa. Cepas utilizadas para evaluar la formación de cluster en esporas preparadas bajo diferentes condiciones de esporulación.

Cepa	Fenotipo
KGB08	△ *cot E gerKB*:gfp Spc^R MLS^R
KGB174	△ *cot E gerAA*:mCherry Spc^R Km^R

Los cluster en las esporas fueron determinados por microscopía de epifluorescencia, para lo cual se colocaron 0.5 μL de la suspensión de esporas entre dos cristales que se limpiaron por sonicación en 10% de NaOH, 95% de etanol y posteriormente lavados con agua destilada. Las imágenes fueron tomadas usando un microscopio de fluorescencia equipado con un objetivo de 60X. El software para la adquisición de imágenes se compró en μManager platform

(http://micro-manger.org). En cada experimento se contaron 500 esporas y se determinó la frecuencia de foci o cluster. Las señales de fluorescencia fueron capturadas a partir de una cámara EM-CCD.

En esporas preparadas en diferentes condiciones de esporulación, aún cuando existe una variación en la cantidad de receptores así como en las tasas de germinación, la formación de cluster se mantiene (Figura IIIa y IIIb), por lo que en el estudio se establece que la cambios en la germinación de estas esporas no está relacionado con una alteración en la formación del germinosoma y es el primer estudio que presenta datos donde se evidencia que estos cluster no son dependientes de la cantidad de receptores presentes en la membrana.

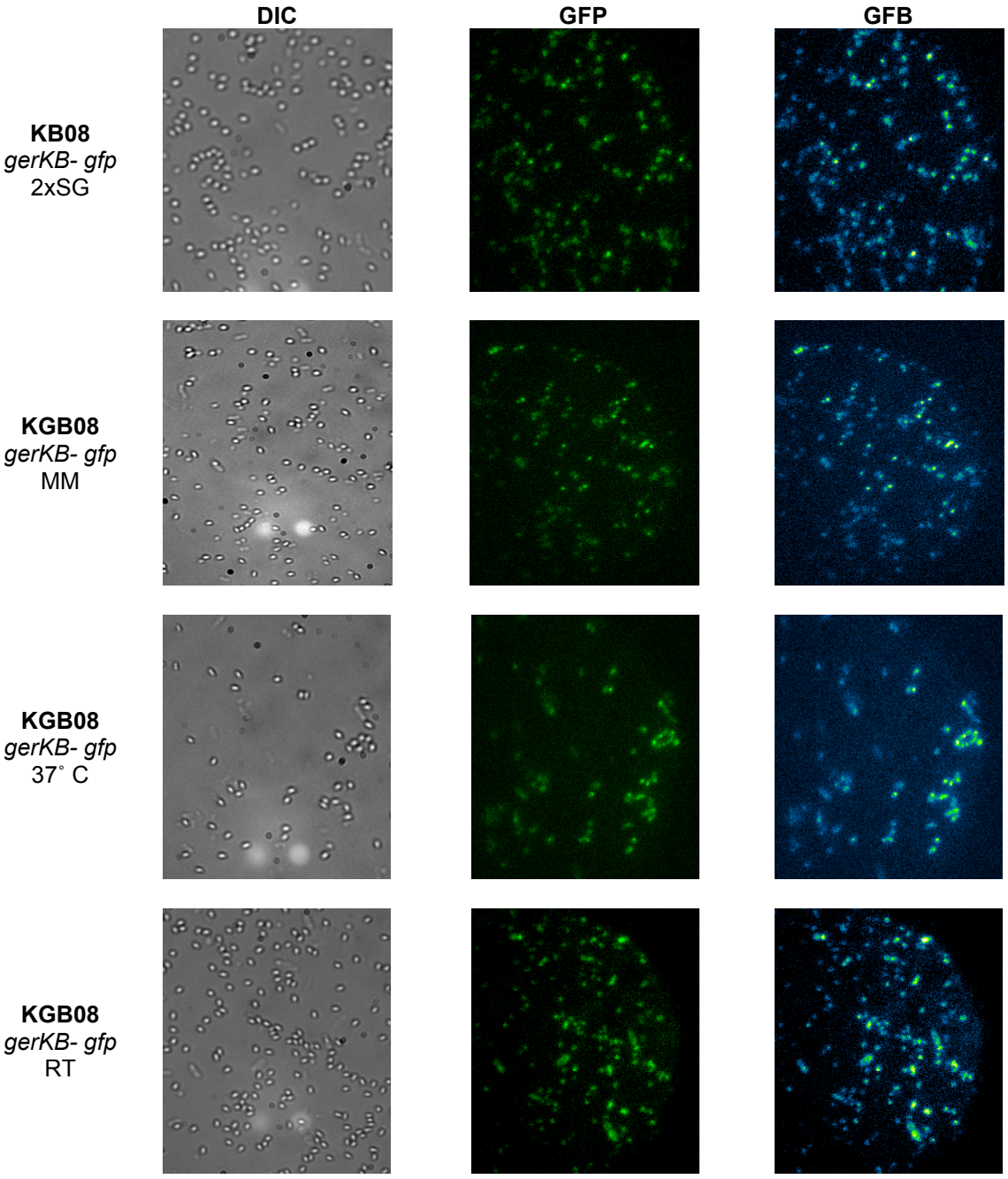

Figura IIIa. Identificación de foci en esporas de la cepa KGB08 (***gerKB*- gfp**) obtenidas en diferentes condiciones de cultivo. **2xSG** (medio enriquecido), **MM** (medio mínimo), **RT** (temperatura ambiente). **GFP** (canal proteína verde fluorescente), **GFB** (Green Fire Blue, filtro de tonalidad azul en fluorescencia es baja, y verde donde la fluorescencia es mayor.

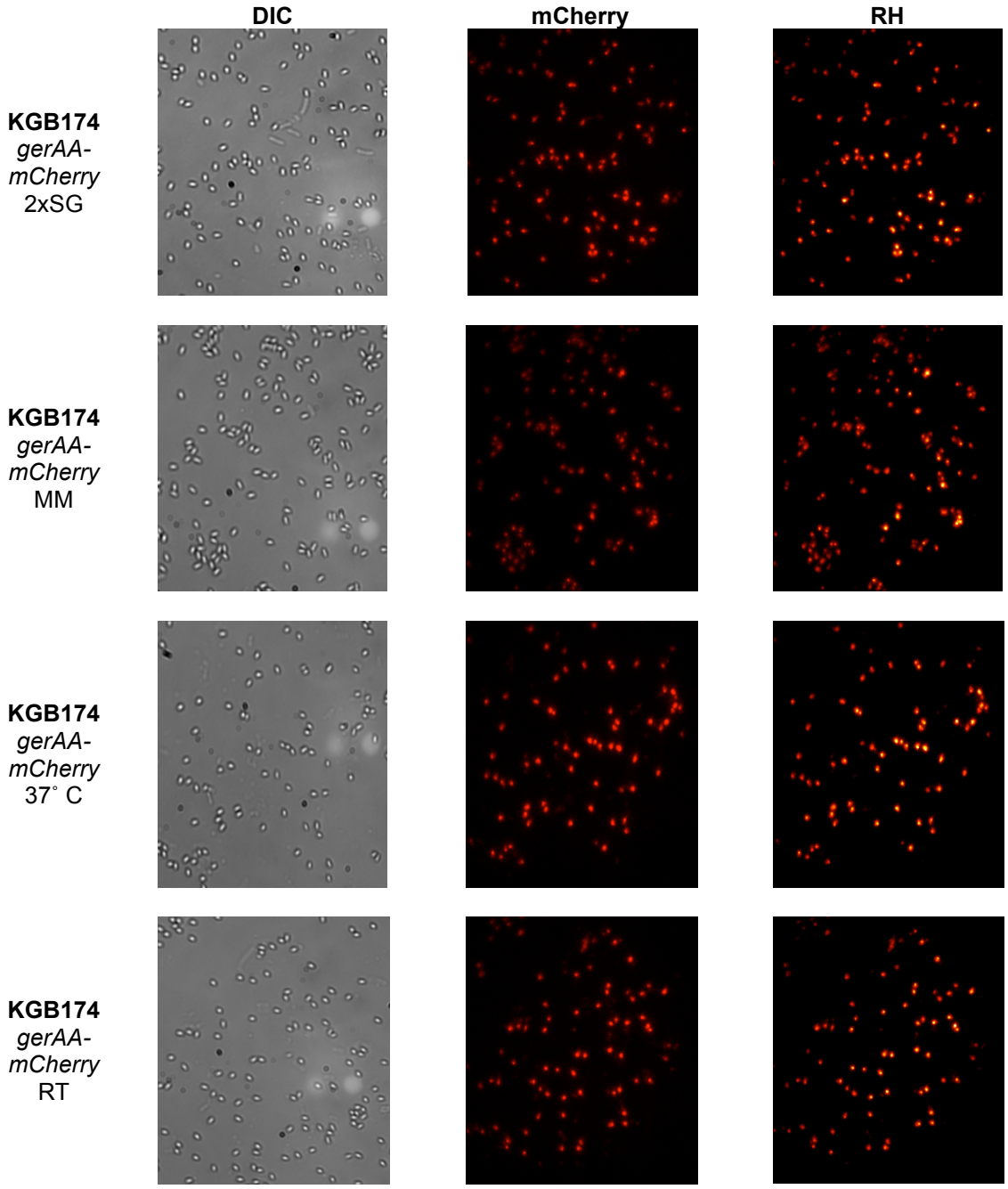

Figura IIIb. Identificación de foci en esporas de la cepa **KGB174** (*gerAA*- mCherry) obtenidas en diferentes condiciones de cultivo. **2xSG** (medio enriquecido), **MM** (medio mínimo), **RT** (temperatura ambiente). **GFP** (canal proteína verde fluorescente), **RH** (Red hot, filtro de tonalidad rojo en fluorescencia es baja, y amarilla donde la fluorescencia es mayor.

Anexo IV

Germinación por alta presión de poblaciones de esporas de *Bacillus subtilis* que presentan variaciones en el número de receptores que reconocen germinantes

Tanto en el capitulo I, II y III se generaron esporas con diferencias en el número de receptores que reconocen germinantes, estos cambios tienen efecto sobre la germinación con L- valina y AGFK. Black *et al.* en el 2009, demostraron que, además de la germinación con nutrientes, los receptores están involucrados en la germinación mediada con presión, esporas que sobreexpresan un receptor en particular germinan más rápido en comparación a la cepa silvestre. Por lo anterior, se germinaron con 250 mPa de presión las esporas generadas en el estudio (Tabla IVa) y los resultados se presentan en la figura IVa y IVb.

Cepa	Fenotipo
PS767	*gerA*:*lacZ* MLS^R
PS533	*wild type*
PS4250	△ *spoVT*
PS4238	*wild type* 186
PS4239	186 △ *ylyA* Erm^R
PS4240	186 △ *ylyA ssp*B: *ylyA* Erm^R Cm^R

Las diferencias entre esporas obtenidas en medio enriquecido y mínimo en cuanto a la cantidad de receptores que son observados en la germinación con presión, esporas preparadas en medio mínimo germinan más lento y se correlaciona con el bajo número de receptores que reconocen germinantes (Figura IVa). Estos resultados son importantes debido a que por lo regular las esporas que contaminan alimentos son generadas en el ambiente bajo condiciones subóptimas de esporulación y la presión se utiliza como un mecanismo para preservar la inocuidad de los alimentos, por lo que estas esporas pueden permanecer latentes (sin germinar) aún cuando son tratadas con presión y pueden producir enfermedad en personas que ingieren este tipo de alimentos. Esporas knock-out para *ylyA* no presentaron diferencias en cuanto

al número de receptores en comparación con la cepa silvestre, lo cual se observó también para la germinación con presión, sin embargo, la sobre expresión de este factor de transcripción se reportó que disminuye de 4 a 8 veces la cantidad de receptores en la membrana de las esporas, estos cambios disminuyen la germinación con nutrientes y en este caso particular también la germinación con presión (Figura IVba). En el capitulo II se describió que SpoVT reprime la síntesis de receptores que reconocen germinantes, por lo tanto en su ausencia, la cantidad de receptores aumenta así como la germinación con nutrientes, esta sobre expresión de receptores se correlaciona con un aumento en la germinación con presión (Figura IVbb).

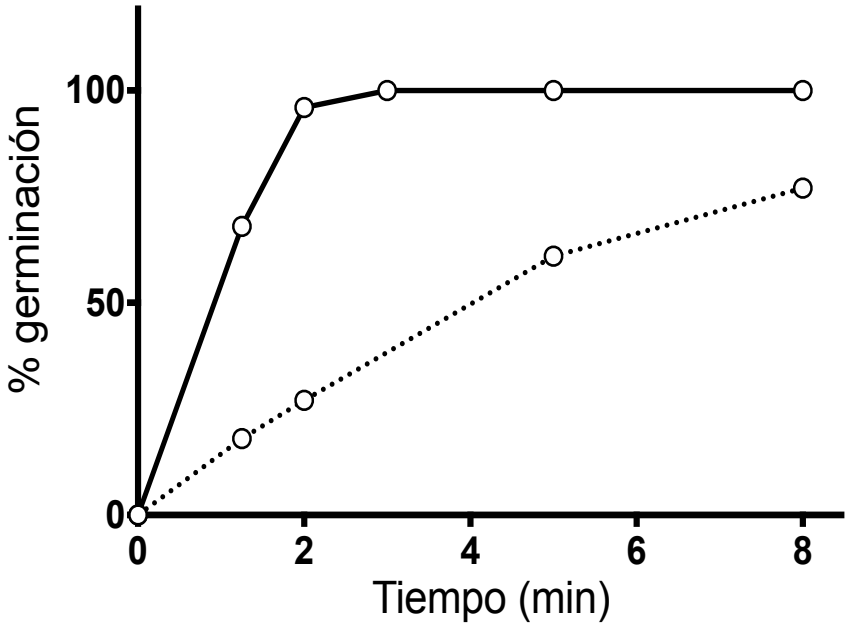

Figura IVa. Germinación con presión de esporas WT preparadas en diferentes condiciones de cultivo. Esporas de la cepa PS767 fueron preparadas en medio enriquecido (línea continua) y mínimo (línea discontinua) y se germinaron con 250 mPA de presión. La germinación fue monitoreada por el conteo de 100 esporas en microscopio de contraste de fases a diferentes tiempos, definiendo a esporas germinadas como esporas oscuras en comparación con las esporas latentes las cuales son observadas como refringentes.

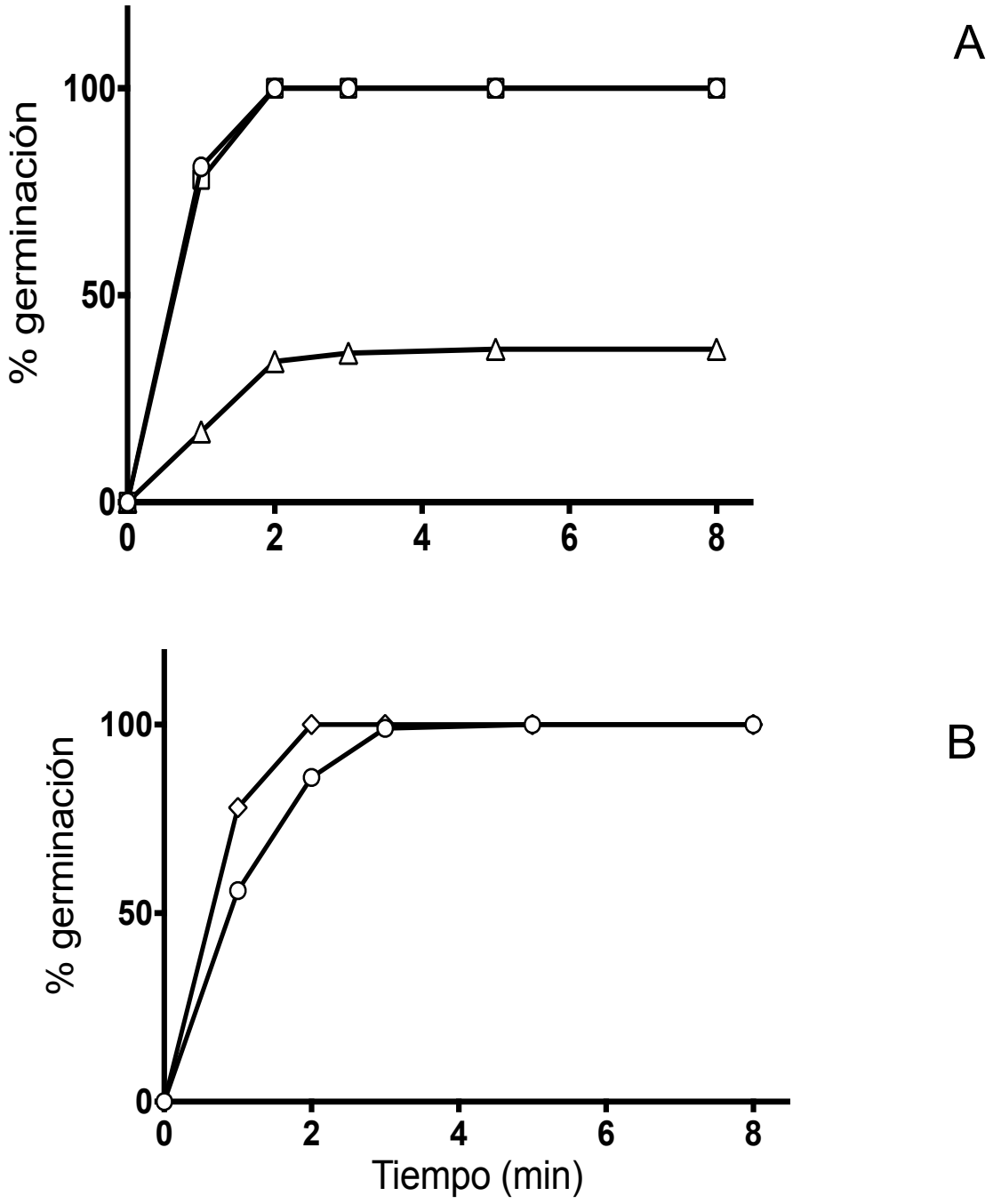

Figura IVb. Germinación con presión de esporas con diferentes niveles de receptores que reconocen germinantes. A) Esporas de las cepas PS4238 (○), PS4239 (□) PS4240 (△) y B) PS533 (○) y PS4210 (◇) fueron preparadas en medio enriquecido y se germinaron con 250 mPA de presión. La germinación fue monitoreada por el conteo de 100 esporas en microscopio de contraste de fases a diferentes tiempos, definiendo a esporas germinadas como esporas oscuras en comparación con las esporas latentes las cuales son observadas como refringentes.

Estos datos contribuyen a explicar la heterogeneidad en las poblaciones de esporas, no todas las esporas germinan igual con presión y estas diferencias son explicadas por un cambio en el número de receptores que reconocen germinantes el cual a su vez es explicado por cambios tanto en las condiciones de esporulación como en el propio genoma de *B. subtilis.* Esta heterogeneidad es un potencial problema de salud pública, debido a que mecanismos de erradicación como la presión no eliminarían el 100% de las esporas, debido a que algunos subgrupos dentro de la población podría tener alteraciones en el número de receptores explicada por alguno de los eventos mencionados anteriormente y esta alteración podría contribuir a que las esporas no germinen y no puedan ser eliminadas.