Centro de Investigación en Alimentación y Desarrollo, A.C.

EVOLUCIÓN Y BIFUNCIONALIDAD DEL RECEPTOR DE QUORUM SENSING NprR DE *Bacillus thuringiensis*

Por:

M. C. Jorge Gustavo Rocha Estrada

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Dra. Mayra de la Torre Martínez
Directora de Tesis

Dr. Gabriel Guarneros Peña
Codirector de Tesis

Dr. Alfredo Herrera Estrella
Asesor

Dra. Luz Vázquez Moreno
Asesor

Dra. Gloria Yépiz Plascencia
Asesor

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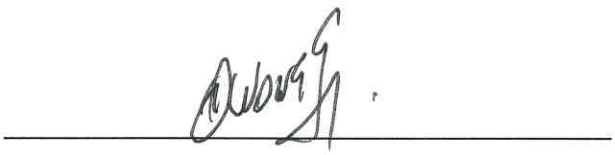

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CONTENIDO

Nota: La numeración al interior de los Capítulos I y II corresponde al formato de la revista donde fueron publicados; el Capítulo III, de acuerdo al formato requerido por la revista a la que será enviado.

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RESUMEN

Quorum sensing (QS) es la regulación de la expresión genética bacteriana en respuesta a la densidad celular, a través de moléculas de señalización autoinductoras y proteínas receptoras. En este trabajo se estudiaron la evolución y funciones del receptor de QS NprR y su péptido de señalización maduro NprRB. Se utilizó a la bacteria *Bacillus thuringiensis* (Bt), que produce proteínas insecticidas. NprR pertenece a la familia RNPP de receptores de QS (nombrada por sus miembros **R**ap, **N**prR, **P**lcR y **P**rgX). Se realizó una revisión sobre esta familia de proteínas y los péptidos de señalización que activan sus funciones en bacterias Gram positivas. Esta revisión fue publicada y se muestra en el Capítulo I. Posteriormente se analizó la evolución de *nprR* y *nprRB* a través de herramientas de bioinformática encontrándose que hubo una coevolución de ambos genes dentro del grupo *Bacillus cereus*. Con base en la secuencia del posible péptido de señalización maduro, se encontraron 6 ferotipos que podrían permitir o limitar la comunicación cruzada dentro del grupo de bacterias. Para estudiar las funciones de NprR en Bt, se diseñaron péptidos de señalización putativos codificados en el gen *nprRB;* éstos péptidos sintéticos se añadieron a cultivos de Bt8741 observando que tienen efecto en esporulación y expresión de *cry1Aa*, gen que codifica para la proteína insecticida del mismo nombre. En el Capítulo II se detallan los hallazgos publicados sobre la evolución del sistema y el efecto de los péptidos sobre Bt8741. La parte final del trabajo se enfocó en la participación de NprR en la esporulación de Bt. La modelación de la estructura de NprR sugirió que además de ser un activador transcripcional, podría interactuar con el regulador de respuesta Spo0F, proteína del *phosphorelay* que lleva al inicio de la esporulación. Utilizando ingeniería genética se demostró que NprR participa en la regulación temporal del inicio de la esporulación probablemente a través del *phosphorelay* y que esta función no requiere de la unión de NprR a ADN, definiendo que NprR es una proteína bifuncional. El Capítulo III, se describen los experimentos para definir a NprR como una proteína bifuncional que participa en la regulación del inicio de la esporulación en Bt.

Palabras clave: *quorum sensing*, NprR, *Bacillus thuringiensis*, esporulación**.**

ABSTRACT

Quorum sensing (QS) is the regulation of bacterial gene expression in response to cell-population density, using auto-inducer signaling molecules and receptor proteins. In this work, we studied the function of the QS receptor NprR and its mature signaling peptide NprRB. We used *Bacillus thuringiensis* (Bt), a bacteria that produces insecticidal proteins. NprR is a member of the RNPP family of QS receptors (named after its members **R**ap, **N**prR, **P**lcR, and **P**rgX). The literature regarding this family of proteins and the signaling peptides that activate their functions was reviewed. This review was published and is shown in the Chapter I. Next, we analyzed the evolution of *nprR-nprRB* genes using bio-informatic tools, showing that both genes co-evolved in the *Bacillus cereus* group. Based on the putative sequence of mature NprRB, we found 6 pherotypes that could allow or limit communication among this group of bacteria. In order to study the functions of NprR in Bt, putative signaling peptides coded in *nprRB* were designed. These synthetic peptides were added to Bt8741 cultures and affected the sporulation and expression of *cry1Aa*, gene that codes for an insecticide protein. Chapter II shows the results published on the evolution of the system and the effect of peptides on Bt8741. The last part of the project was focused on the role of NprR in the sporulation process of Bt8741. Structure modeling suggested that besides being a transcriptional activator, NprR could bind the response regulator Spo0F, a protein that participates in the phosphorelay system that leads to sporulation initiation. Genetic engineering experiments showed that that NprR participates in the temporal regulation of sporulation initiation, probably through the phosphorelay; additionally, we proved that this function does not require the NprR capacity of binding DNA, indicating that NprR is a bi-functional protein. In Chapter III describes the findings regarding NprR as a bi-functional protein that participates in the regulation of sporulation initiation in Bt.

Keywords: quorum sensing, NprR-NprRB, *Bacillus thuringiensis*, sporulation.

INTRODUCCIÓN

Quorum sensing (QS) es la regulación de la expresión genética de bacterias en respuesta a la densidad celular. Las bacterias producen y secretan moléculas de señalización que se difunden en el ambiente y cuando las poblaciones llegan a densidades celulares altas, dichas moléculas alcanzan los umbrales de concentración necesarios para unirse a sus respectivas proteínas receptoras localizadas en la membrana celular o en el espacio intracelular. Las bacterias Gram-negativas usan acil-homoserina lactonas como moléculas de señalización de QS, mientras que las bacterias Gram-positivas generalmente usan oligopéptidos. Existe una alta especificidad entre las moléculas de señalización y sus receptores, y la interacción de ambos da a lugar funciones que la población bacteriana lleva a cabo de manera coordinada. Algunas de éstas funciones son el desarrollo de virulencia, formación de *biofilms*, esporulación y bioluminiscencia (1-4).

Bacillus thuringiensis (Bt) es una bacteria Gram-positiva perteneciente al grupo *Bacillus cereus*. Bt es utilizada ampliamente en control biológico de plagas agrícolas y de vectores transmisores de enfermedades para el hombre, porque produce proteínas Cry, que son tóxicas para los insectos. Durante la esporulación, las proteínas Cry forman un cristal paraesporal en la bacteria; las esporas y los cristales son liberados al final del proceso de esporulación. Cuando son ingeridas, las proteínas Cry en forma de protoxinas son activadas matando al insecto por un mecanismo que aún no se conoce completamente, pero que podría involucrar la formación de poros en la membrana de células epiteliales del tracto digestivo de insectos susceptibles, o bien por el desencadenamiento de vías de señalización que provocan la muerte de las células. Finalmente, la espora bacteriana utiliza al insecto como medio de cultivo para su reproducción (5-7). La producción de la mayoría de las proteínas Cry está ligada al proceso de esporulación, ya que su transcripción está dirigida por factores sigma de

esporulación. Por ejemplo, las regiones promotoras de los genes *cry1A* contienen los promotores BtI y BtII reconocidos por los factores sigma de esporulación σ^E y σ^K (8). Por lo tanto, conocer el proceso de esporulación es fundamental para entender y manipular la producción de proteínas Cry en la bacteria. Aunque existen pocos estudios que aborden la esporulación de Bt, el proceso ha sido explicado detalladamente en la bacteria *Bacillus subtilis,* que ha servido como modelo para este proceso de diferenciación celular (9-10).

En *B. subtilis,* se sabe que receptores del tipo Histidina Cinasas (HC) participan en la transducción de señales que regula el inicio de la esporulación. Estos receptores tienen un dominio extracelular, otro transmembranal y un tercero intracelular y responden a señales endógenas y exógenas. Esencialmente una HC se autofosforila en respuesta a una señal y posteriormente transfiere el grupo fosfato a un Regulador de Respuesta (RR) del sistema de transducción de señales denominado en inglés *phosphorelay*. El *phosphorelay* consiste en la fosforilación secuencial de las proteínas Spo0F→Spo0B→Spo0A. Spo0A fosforilada (Spo0A~P) es el regulador maestro del inicio de la esporulación y regula la transcripción de 121 genes (10-11). La esporulación es un proceso de diferenciación celular irreversible y endergónico, por lo tanto las células bacterianas que inician el proceso de esporulación sin la energía suficiente, mueren. Para asegurar que las bacterias que inicien el proceso de esporulación lo terminen, el inicio es regulado por múltiples mecanismos, entre ellos el QS (12-13). Se infiere que los mecanismos de regulación del inicio de esporulación en Bt, son similares a los descritos en *B. subtilis*; sin embargo, muchos de los genes importantes para la regulación del inicio de la esporulación de *B. subtilis* no están conservados en los genomas de bacterias del grupo *B. cereus.* Entre estos, destacan genes de algunos sistemas de 2 componentes como los formados por HC y RR, o bien los genes de proteínas Rap, reguladores de QS que actúan en el *phosphorelay* (14).

En bacterias Gram-positivas existe la familia de proteínas receptoras de QS denominada RNPP (15), integrada por las proteínas **R**ap, **N**prR, **P**lcR y **P**rgX. Estas proteínas están relacionadas filogenéticamente y conforman sistemas de quórum sensing con algunas características particulares, entre ellas: 1) son proteínas receptoras intracelulares; 2)

utilizan como molécula de señalización a péptidos reimportados a través de proteínas Oligo-Péptido-Permeasa, y sus funciones están relacionadas directamente con la activación/inhibición por éstos; 3) normalmente los genes de la proteína receptora y del pro-péptido de señalización correspondiente están codificados en casets transcripcionales; 4) en su estructura contienen varias repeticiones tetratricopéptido (TPRs) responsables de interacciones proteína-proteína y proteína-péptido; 5) tres de los cuatro miembros (NprR, PlcR y PrgX) tienen en su extremo N-terminal un domino Hélice-vuelta-Hélice (HTH) para unirse al ADN.

Aunque se activan por mecanismos similares, las funciones de las proteínas receptoras de la familia RNPP son diversas. Las proteínas Rap y sus péptidos de señalización llamados Phr fueron descritos en *B. subtilis* como reguladoras del *phosphorelay* y por lo tanto del inicio de la esporulación (16-17). Estos receptores de QS se unen a Spo0F e impiden el flujo de fosfatos en el *phosphorelay*, bloqueando alostéricamente o defosforilando a este RR. Algunas proteínas Rap pueden unirse a RRs como ComA y DegU, inhibiendo su función como activadores transcripcionales (18). En alta concentración celular (*quorum*), el péptido maduro Phr interactúa con la proteína Rap correspondiente impidiendo que Rap defosforile al RR (19). En *B. subtilis* se han encontrado 11 proteínas Rap y 8 péptidos Phr: RapA, RapB y RapH son proteínas Rap que defosforilan a Spo0F. RapC, RapF y RapH son ejemplos de proteínas Rap que se unen a activadores transcripcionales. Es importante recalcar que la proteína RapH juega un papel regulatorio en esporulación defosforilando a Spo0F y en competencia inhibiendo a ComA (20-22).

PrgX y PlcR son reguladores transcripcionales que responden a QS. PlcR fue descrito en bacterias del grupo *B. cereus* como un regulador pleiotrópico de virulencia que responde a la activación por el péptido de señalización maduro PapR (23). En alta concentración celular, el péptido maduro ocasiona la oligomerización de PlcR y en dicha conformación es capaz de unirse al ADN en cajas específicas (caja PlcR), regulando así la transcripción de 45 genes (24). El sistema PlcR-PapR está conservado en todo el grupo *B. cereus* formando 4 ferotipos (25). Por su parte, PrgX fue descrito en la bacteria *Enterococcus faecalis* como un regulador de la conjugación del plásmido pCF10 que

responde a quórum sensing. La función de PrgX depende de 2 péptidos maduros diferentes: cCF10 es el activador e iCF10 es el inhibidor; el primero está codificado en el genoma de *E. faecalis* y el segundo en el plásmido pCF10. Este sistema permite que las células acarreadoras del plásmido pCF10 transfieran dicho plásmido por conjugación a células receptoras (26-27).

NprR es el cuarto miembro de la familia RNPP de receptores de QS. Esta proteína fue descrita en *B. subtilis* como un elemento genético que interviene en la expresión de la proteasa neutra NprE (28-29). Posteriormente, el gen del activador NprR (nombrado *nprA*) y el de la proteasa neutra (nombrado *nprS)* de *Bacillus stearothermophilus* fueron clonados y secuenciados (30). Sin embargo, fue hasta el trabajo de Pottathil y Lazazzera (31) que se propuso que NprR de Bt era una proteína de QS tipo Rap, porque corriente abajo del gen *nprR* se encontró un marco de lectura abierta que codifica para un pro-péptido de señalización tipo Phr. El gen fue denominado *nprRB* y se propuso que la secuencia del péptido de señalización maduro era SKPDT. A diferencia de la mayoría de los péptidos de señalización del grupo RNPP, la secuencia putativa de NprRB maduro no pertenece a los 5 residuos a partir del C-terminal del propéptido; por tanto, variantes de este péptido con diferente secuencia y longitud podrían ser la forma madura del péptido de señalización en la bacteria. Posteriormente, se definió a NprR como un miembro de la familia de receptores de QS RNPP (15), aunque no se sabía si además de ser el regulador transcripcional de la proteasa neutra *nprA*, tenía otras funciones en *B. thuringiensis*. El trabajo de Aceves-Diez et al. (32) fue el primero en estudiar experimentalmente las funciones de NprR como proteína de QS, demostrando que el péptido sintético SKPDT tenía un efecto sobre la esporulación y expresión de *cry1Aa* en Bt serovar *kurstaki*.

El conocimiento de mecanismos de QS que afectan esporulación en la bacteria Bt, tiene implicaciones de importancia en biotecnología, porque podrían ser la base para la manipulación y optimización de los procesos fisiológicos por los cuales la bacteria desarrolla su patogenicidad hacia insectos. Además, los sistemas de QS de bacterias patógenas de humanos son un blanco para la generación de fármacos no bactericidas que bloqueen dichos sistemas y eviten el desarrollo de virulencia, sin generar resistencia por

presión selectiva como sucede con los antibióticos comunes (2-3). Finalmente, el QS es considerado uno de los primeros pasos en el desarrollo de la multicelularidad, por lo cual su estudio tiene implicaciones evolutivas (33). El estudio sobre un nuevo sistema de QS y sus funciones es de importancia para las áreas mencionadas.

Este trabajo se centra en estudiar las funciones que NprR lleva a cabo en respuesta a la activación por el péptido de señalización maduro NprRB. Con el escaso conocimiento que se tenía sobre el sistema NprR-NprRB al inicio del presente proyecto de tesis (2010), se tomaron en cuenta las siguientes evidencias: a) el receptor de QS NprR contiene un dominio de unión a ADN (dominio HTH); b) la adición del péptido sintético SKPDT, codificado en *nprRB* tiene efectos sobre la esporulación y actividad de una fusión transcripcional *cry1Aa'lacZ*, procesos que dependen de muchos genes. Originalmente se propuso que NprR era un regulador transcripcional pleiotrópico relacionado con esporulación y expresión de proteínas Cry en Bt. Se estudió la evolución de los genes *nprR* y *nprRB* así como el efecto de péptidos sintéticos sobre la esporulación y la expresión de *cry1Aa* en Bt8741. Posteriormente, partiendo de análisis *in silico*, se propuso que NprR es un regulador de QS bifuncional y proyecto se enfocó en encontrar las funciones que NprR tiene sobre la esporulación a través de su posible participación en el *phosphorelay*, uniéndose a un RR de manera similar a las proteínas Rap. Se utilizaron estrategias de ingeniería genética para eliminar los genes *nprR-nprRB* de Bt8741 y expresar variantes del sistema *nprR-nprRB* en la mutante. De esta manera se logró evaluar tanto la participación del gen *nprRB*, como del requerimiento de unión a ADN, para las distintas funciones de NprR.

SINOPSIS

Del grupo de receptores de QS RNPP, al inicio del proyecto (año 2010) las proteínas Rap, PlcR y PrgX habían sido ampliamente estudiadas y se conocía su evolución, estructura, funciones y secuencias de los péptidos de señalización maduros. Por otro lado, había escaso conocimiento acerca del receptor NprR. Se revisó ampliamente la literatura concerniente a estos receptores y se publicó un artículo de revisión que englobó el estado del arte en el tema de los receptores Rap, PlcR y PrgX que además permitió proponer funciones de NprR, así como secuencias probables del péptido NprRB maduro y el mecanismo de activación. El Capitulo I de la presente tesis corresponde a la revisión "The RNPP family of quorum-sensing proteins in Gram-positive bacteria", en el cual se analizaron y compararon los sistemas de QS de la familia RNPP; de igual manera se discutieron las posibles aplicaciones biotecnológicas que pueden surgir a partir del estudio de dichos sistemas.

La investigación experimental se inició con el estudio de la evolución de los genes *nprR* y *nprRB* en el grupo *B. cereus.* Se secuenció una región genómica que incluye a *nprR-nprRB* de algunas cepas de la colección del laboratorio y se incluyeron otras secuencias de distintas cepas que estaban depositadas en el GenBank. El estudio demostró que los genes *nprR* y *nprRB* estaban presentes en al menos un representante de cada especie del grupo *B. cereus* y que habían coevolucionado, formando 6 grupos o ferotipos, cada uno caracterizado por una secuencia del péptido maduro de señalización NprRB putativo. Esta parte del trabajo permitió sugerir la existencia de comunicación cruzada en el grupo *B. cereus* a través del sistema NprR-NprRB. Lo anterior es de especial importancia porque la virulencia en bacterias regularmente es activada por sistemas de QS y dentro del grupo *B. cereus* se incluye a las bacterias patógenas de humanos *Bacillus anthracis* (causante del ántrax) y *B. cereus* (causante de enfermedades transmitidas por alimentos) (34).

Para estudiar las funciones del sistema NprR-NprRB, se eligió la cepa *Bacillus thuringiensis* serovar. *thuringiensis* 8741 (Bt8741). A partir del péptido sintético SKPDT reportado en trabajos anteriores (31-32) se diseñaron secuencias de péptidos de señalización para ensayos *in vivo*, tomando en cuenta la secuencia de *nprRB* de la cepa Bt8741 (Fig. 1). Se midió el efecto de 8 péptidos sintéticos sobre la expresión de *cry1Aa* y de 5 péptidos sobre la esporulación. La longitud de los péptidos fue la variable más importante en el estudio. Los péptidos de 5 aminoácidos provocaron un incremento sutil en la expresión de *cry1Aa*, mientras que los péptidos de 7 y 8 aminoácidos provocaron una disminución. Por su parte, el péptido de 9 aminoácidos no tuvo ningún efecto. En cuanto al efecto de los péptidos sobre la esporulación, se encontró que solamente los péptidos de 7 y 8 aminoácidos (SKPDIVG y SSKPDIVG) indujeron una aparición temprana de esporas y un aumento en la eficiencia de esporulación de Bt8741. La importancia de estos resultados radica en que permitieron establecer las hipótesis de que el sistema NprR-NprRB podría ser activador y represor de genes. Los resultados de los análisis bioinformáticos de coevolución y de los experimentos *in vivo* de la adición de péptidos al cultivo de la bacteria fueron publicados en el artículo de datos originales "Evolution and some functions of the NprR-NprRB quorum-sensing system in the *B. cereus* group", presentado como Capítulo II de esta tesis.

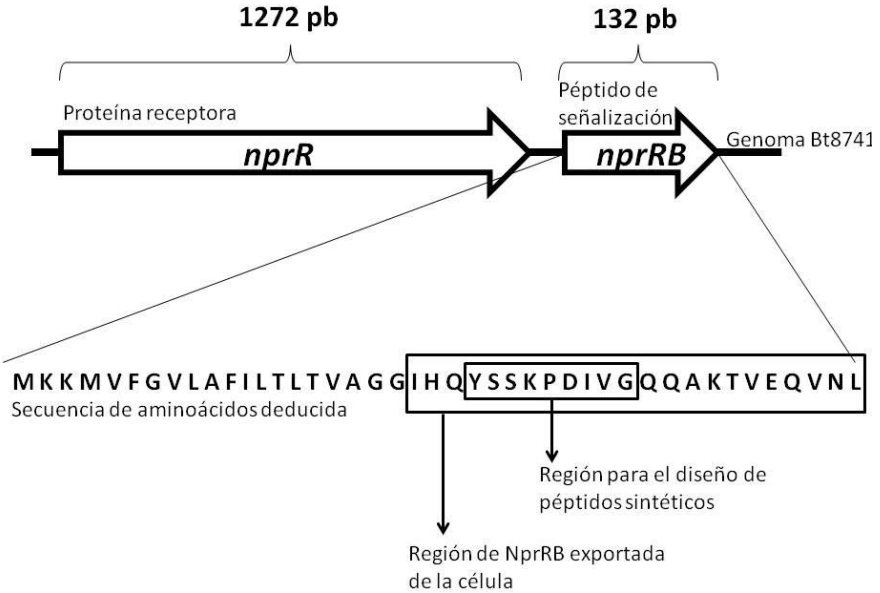

Figura 1. Caset transcripcional *nprR-nprB* de *Bacillus thuringiensis* Bt8741. Se muestra la región de *nprRB* a partir de la cual se seleccionaron variantes de péptidos sintéticos para añadir a los cultivos de Bt 8741; pb: pares de bases.

Mientras en nuestro grupo de investigación se observó el efecto de los péptidos codificados en *nprRB* sobre esporulación y Cry1Aa, así como la coevolución de *nprR* y *nprRB,* otros autores reportaron funciones de NprR como regulador de QS. Primeramente, Perchat et al. (35) describieron el mecanismo de QS por el cual el péptido maduro NprRB se une y activa a NprR, permitiendo la unión del complejo NprR-NprRB a regiones promotoras de DNA y activando la transcripción del gen de la proteasa neutra *nprA*. Dicho mecanismo coincide con el mecanismo propuesto en nuestro artículo de revisión que fue publicado antes (Capítulo I). En el mismo trabajo también se describe la coevolución de *nprR-nprRB* y la presencia de 7 ferotipos, 6 de los cuales fueron encontrados y publicados simultáneamente por nuestro grupo (Capítulo II).

Por otra parte, Yang et al. (36) describieron la aparición de colonias papilas de *B. anthracis* con mutaciones en *nprR*, que presentaron actividad proteolítica extracelular disminuida y no llevaban a cabo esporulación en medio LB. El efecto en esporulación de *B. anthracis* coincide con el efecto de algunos péptidos de incrementar la esporulación en Bt8741 (Capítulo II) y Bt serovar kurstaki (32). Posteriormente, Dubois et al. (37) utilizando mutantes Δ*nprR-nprRB*, demostraron que este sistema de QS se requiere para que la bacteria se desarrolle después de la muerte de insectos infectados, y que hay 41 genes regulados diferencialmente en la cepa mutante, comparada con la silvestre. En este trabajo también se encontró una esporulación disminuida en la mutante de Bt Δ*nprR-nprRB* durante la infección, sin embargo los autores no discutieron el mecanismo por el cual NprR podría participar en este proceso. Entre los 41 genes, regulados por NprR, no se identificaron genes conocidos relacionados con esporulación. Por lo tanto, aunque muchas de las funciones de NprR fueron descritas, el mecanismo por el cual NprR-NprRB estaba involucrado en la esporulación era desconocido y se consideraba que la función NprR era la de regulador transcripcional pleiotrópico.

Para responder a la pregunta ¿como el sistema NprR-NprRB está involucrado en la esporulación de Bt? con el apoyo del grupo de trabajo del Dr. Gabriel Guarneros, se modeló *in silico* la estructura de NprR, encontrando que se asemeja a la de RapH. El

sistema conformado por la proteína RapH y su péptido de señalización PhrH está involucrado en la esporulación a través de su unión con el regulador de respuesta Spo0F (22, 38). Por lo tanto, se estableció la hipótesis de que NprR podría ser una proteína bifuncional, que además de activar la transcripción del gen de la proteasa neutra extracelular *nprA* uniéndose directamente a ADN, podría estar involucrada en la regulación del inicio de la esporulación uniéndose al regulador de respuesta Spo0F.

Los experimentos *in vivo* incluidos en el artículo del Capítulo II presentaban el problema de que la cepa Bt8741 producía naturalmente su péptido de señalización NprRB, que en su forma madura podría actuar sobre NprR ocultando el efecto de los péptidos sintéticos. Aunque los péptidos sintéticos se añadían después de eliminar el sobrenadante y diluir a las células en medio fresco, y los resultados se normalizaban contra un control sin péptido, los efectos observados eran sutiles. Por lo tanto, se generó una cepa Bt8741 mutante en la que se eliminaron los genes *nprR-nprRB* (cepa BtΔRB). La cepa mutante presentó actividad de proteasa extracelular disminuida y un retraso en el inicio de la esporulación.

Para comprobar que el efecto de la esporulación era a nivel del *phosphorelay* (función tipo Rap), se evaluó la activación de la transcripción de los genes *spoIIG* y *spoIIA*, que está dirigida por Spo0A~P, el producto final del *phosphorelay*. Dado que Spo0A~P activa directamente la transcripción de estos genes en la hora 2 después del inicio de esporulación (t_2), diversos autores han validado la medición de su expresión como un indicador del estado de fosforilación de Spo0A (11, 39-41). Se extrajo RNA de células en t_0 (fase de transición) y t_2 (2 h después de la fase de transición) de esporulación y se encontró que en la cepa Bt8741 en el t_2 de esporulación, los niveles de transcrito de *spoIIG* y *spoIIA* aumentaban 280 y 422 veces, respectivamente comparado con t_0. Por su parte en la cepa mutante BtΔRB, el mRNA de ambos genes aumentó 21 veces (*spoIIA*) y 3 veces (*spoIIG*) en el mismo lapso. Estos resultados sugieren que el *phosphorelay* está afectado por la mutación Δ*nprR-nprRB*.

A pesar de la alta similitud en la estructura de NprR y Rap encontrada en nuestros análisis *in silico* y descrita también por otros autores (21, 35), es importante notar que las proteínas Rap no contienen el dominio de unión a DNA (dominio HTH). De tal

manera que si NprR tiene una relacionada con el *phosphorelay* a través de la unión a Spo0F (de manera similar a Rap), el dominio HTH de NprR no debería participar en esta función. Por lo tanto, se construyó un gen *nprR(ΔHTH),* en el cual se truncó el dominio HTH por lo que codifica para una variante de NprR que no tiene la capacidad de unirse al ADN. Se expresaron los genes *nprR-nprRB* y sus variantes en la cepa mutante BtΔRB, para realizar ensayos de complementación de los fenotipos de proteasa extracelular y esporulación. En la tabla 1 se muestran los nombres y construcciones genéticas de las cepas utilizadas en los ensayos de complementación. La actividad de proteasas extracelulares fue complementada a niveles similares a los de Bt8741 solamente en la cepa Bt[R-B], indicando que tanto el gen *nprRB* como el dominio HTH son indispensables para llevar a cabo la activación de la transcripción de *nprA*, lo cual había sido demostrado anteriormente por otros autores (35).

Tabla 1. Cepas utilizadas en los ensayos de complementación.

Nombre	**Plásmido**	*nprR*	*nprR(ΔHTH)*	*nprRB*
Bt8741	pMAD*	En el genoma	-	En el genoma
BtΔRB	pMAD*	-	-	-
BtΔRB[R-B]	pR-B	En plásmido	-	En plásmido
BtΔRB[R]	pR	En plásmido	-	-
BtΔRB[RΔHTH-B]	pRΔHTH-B	-	En plásmido	En plásmido
BtΔRB[RΔHTH]	pRΔHTH	-	En plásmido	-

*Las cepas Bt8741 y BtΔRB se transformaron con el plásmido pMAD sin inserto y se utilizaron como control en los experimentos de complementación.

Se evaluó la esporulación en las cepas que expresan variantes del sistema NprR-NprRB. Las cepas Bt[R-B] y Bt[R(ΔHTH)-B] fueron capaces de complementar la esporulación a niveles similares a los encontrados en Bt8741; esto indica que el dominio HTH no es necesario para la función de NprR en la regulación de la esporulación y soporta la hipótesis de que la función depende de la interacción con una proteína. Como lo muestran los análisis *in silico,* esta función podría ser similar a las proteínas Rap, a través de la unión a Spo0F, pero con un efecto positivo sobre la esporulación. Finalmente, las cepas Bt[R] y Bt[R(ΔHTH)] presentaron conteos de esporas similares a

los de la cepa mutante BtΔRB, lo cual indica que el gen *nprRB* tiene un papel importante en esta nueva función de NprR.

Se preparó el manuscrito "The bi-functional quorum sensing receptor NprR from *B. thuringiensis* regulates sporulation through a DNA-binding-independent mechanism", con formato para enviar a la revista *Journal of Bacteriology*. En éste se incluye el estudio *in silico* de la estructura de NprR y su similitud con las proteínas Rap de *B. subtilis*, los fenotipos de la mutante BtΔRB y los estudios de complementación utilizando las cepas que expresan los genes de variantes del sistema NprR-NprRB: *nprR*, *nprRΔHTH* y *nprRB*. Los resultados de dichos análisis permiten proponer un modelo por el cual el sistema NprR-NprRB está involucrado en la regulación del inicio de la esporulación. Este manuscrito conforma el Capítulo III de la tesis.

CONCLUSIONES Y PERSPECTIVAS

La parte experimental la presente tesis aportó conocimiento acerca de la evolución, funciones y finalmente un mecanismo alternativo del sistema de QS NprR-NprRB para regulación del inicio de la esporulación a través del *phosphorelay*, para definirla como una proteína bifuncional de QS. Integrando los resultados del proyecto, las conclusiones generales son:

Los genes que codifican para el sistema NprR-NprRB, han coevolucionado porque tienen una función conjunta como sistema de QS. Ambos genes se encontraron en cepas de todas especies del grupo *B. cereus*, formando 6 ferotipos caracterizados por diferentes péptidos NprRB maduros putativos. Durante la evolución probablemente se dieron fenómenos de transferencia genética horizontal.

El sistema NprR-NprRB está involucrado en la esporulación y expresión de *cry1Aa* en la bacteria Bt8741; algunos péptidos codificados en NprRB tienen efectos sobre la esporulación (adelantando el proceso y aumentando la eficiencia) y la expresión de *cry1Aa* (aumentando o disminuyendo la expresión). Estos resultados indicaron que el sistema podría llevar a cabo funciones de activador y represor transcripcional y probablemente regular temporalmente la esporulación.

NprR está involucrado en la regulación temporal de la esporulación, afectando el *phosphorelay*. Esta función es independiente de su dominio de unión a ADN, por lo tanto podría darse a través de un mecanismo de interacción proteína-proteína. Los análisis *in silico* sugieren que NprR podría ser capaz de proteger al regulador de respuesta Spo0F de la defosforilación por parte de proteínas Rap.

De acuerdo a los resultados obtenidos, los esfuerzos futuros dentro del proyecto deben enfocarse a obtener evidencia directa para demostrar la bifuncionalidad de NprR-NprRB, el mecanismo alternativo y el papel del péptido de señalización. A continuación

se enumeran recomendaciones específicas para fortalecer el proyecto y probar las hipótesis propuestas:

1. Para evaluar el papel del sistema NprR-NprRB sobre la transcripción de *cry1Aa*, es necesario obtener por PCR la región promotora de *cry1Aa* y evaluar in vitro la interacción de NprR o NprR-NprRB con dicha región de ADN. Un ensayo de movilidad electroforética retardada (EMSA) podría dar indicios acerca de la interacción, mientras que con un ensayo de protección de nucleasas (*footprinting*) se pueden definir las regiones de ADN importantes para la interacción. Añadiendo un ensayo de expresión de *cry1Aa´lacZ* en las cepas Bt8741, BtΔRB y cepas que expresan las variantes de *nprR-nprRB* se puede definir el papel de NprR como activador o represor de la transcripción de *cry1Aa* en respuesta al péptido NprRB en Bt8741.
2. Si se logran obtener regiones específicas de interacción de NprR con regiones promotoras de *cry1Aa*, *nprA* y otros genes, se podría identificar una secuencia consenso de unión de NprR (caja NprR) para buscar otros genes regulados transcripcionalmente por el sistema NprR-NprRB en genomas de bacterias del grupo *B. cereus*.
3. Dado que las evidencias obtenidas en estudios *in silico* sugieren que el proceso de esporulación en Bt es sustancialmente diferente al proceso en *B. subtilis*, es necesario entender estas diferencias, lo cual es de importancia para el estudio de la producción proteínas Cry en Bt, y esto sería de impacto biotecnológico además de contribuir al conocimiento sobre diferenciación celular y regulación por transducción de señales en bacterias. Para investigar a profundidad la regulación del inicio de esporulación en Bt, es recomendable iniciar un estudio para entender las funciones de las proteínas Rap identificadas en el genoma de Bt.
4. Se requieren plásmidos para medir expresión de genes por fusiones transcripcionales con el gen reportero *lacZ*, que permitan la cuantificación de mRNA de indicadores como *spoIIG* y *spoIIA* utilizados en este trabajo, pero evitando la complejidad técnica de medir expresión genética por RT-PCR en bacterias. Asimismo, se requieren plásmidos de fusiones transcripcionales con genes de proteínas fluorescentes para medir la expresión de genes en células individuales

por citometría de flujo, que permitan estudiar los cambios en la biestabilidad característica de la esporulación y procesos relacionados que son regulados por QS. Dichos plásmidos se deben construir o modificar de tal manera que sean sistemas compatibles a los que se han construido anteriormente en el laboratorio (basados en el plásmido pMAD).

5. Se requiere construir o modificar un plásmido para lograr la integración de construcciones genéticas en regiones específicas del genoma de Bt, similares a los que existen para integración en el gen de la enzima Amilasa en *B. subtilis,* que permiten el intercambio genético utilizando como marcador la pérdida de actividad de amilasa. De esta manera se obtendrán sistemas más estables para estudios de complementación.
6. Para demostrar el mecanismo por el cual NprR regula el inicio de la esporulación en Bt, es necesario estudiar a los componentes del *phosphorelay*. La clonación y sobreexpresión de las proteínas Spo0F y Spo0A es el primer paso para el estudio in vitro del *phosphorelay* en Bt y el papel de NprR. Adicionalmente, se necesitan anticuerpos específicos para Spo0F, Spo0F~P, Spo0A y Spo0A~P para estudiar las dinámicas de fosforilación del sistema, que ha sido extensamente estudiado en *B. subtilis* pero parece ser sustancialmente diferente en *B. thuringiensis*.

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CAPÍTULO I

The RNPP family of quorum sensing receptors in Gram-positive bacteria

Jorge Rocha-Estrada, Ángel E. Aceves-Diez,
Gabriel Guarneros & Mayra de la Torre

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RESUMEN

El quorum sensing es uno de los mecanismos que las bacterias usan para interactuar unas con otras y coordinar ciertos procesos fisiológicos en respuesta a la densidad celular. Este mecanismo esta mediado por moléculas de señalización extracelulares; una vez que la concentración alcanza un umbral, se activa (o reprime) una enzima Cinasa sensora o reguladores de respuesta, facilitando la expresión de genes dependientes de quorum sensing. Las bacterias Gram positivas usan oligopéptidos como moléculas de señalización. En estas bacterias, se ha descrito un grupo de sistemas de quorum sensing, en el cual la proteína receptora interactúa directamente con su péptido de señalización correspondiente. Los receptores son Rap fosfatasas o reguladores transcripcionales, e integran a la familia RNPP, nombre asignado por sus 4 miembros: Rap, NprR, PlcR y PrgX. Estos sistemas de quorum sensing controlan una variedad de procesos microbianos como la esporulación, virulencia, formación de *biofilms*, conjugación y producción de enzimas extracelulares. El estudio detallado del mecanismo de unión de proteína-péptido de señalización así como la interacción molecular entre el receptor, el péptido y el ADN blanco ha diferido de los modelos clásicos. A pesar del conocimiento adquirido y el potencial de aplicaciones biotecnológicas de estos sistemas de quorum sensing, solamente se han publicado unos cuantos ejemplos de la ingeniería de dichos sistemas. Las aplicaciones reales surgirán cuando los investigadores del área de microbiología aplicada y biotecnología noten la importancia de los sistemas de quorum sensing para aplicaciones de salud y bioprocesos.

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MINI-REVIEW

The RNPP family of quorum-sensing proteins in Gram-positive bacteria

Jorge Rocha-Estrada · Angel E. Aceves-Diez · Gabriel Guarneros · Mayra de la Torre

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Abstract Quorum sensing is one of several mechanisms that bacterial cells use to interact with each other and coordinate certain physiological processes in response to cell density. This mechanism is mediated by extracellular signaling molecules; once a critical threshold concentration has been reached, a target sensor kinase or response regulator is activated (or repressed), facilitating the expression of quorum sensing-dependent genes. Gram-positive bacteria mostly use oligo-peptides as signaling molecules. These cells have a special kind of quorum-sensing systems in which the receptor protein interacts directly with its cognate signaling peptide. The receptors are either Rap phosphatases or transcriptional regulators and integrate the protein family RNPP, from Rap, Npr, PlcR, and PrgX. These quorum-sensing systems control several microbial processes, like sporulation, virulence, biofilm formation, conjugation, and production of extracellular enzymes. Insights of the mechanism of protein-signaling peptide binding as well as the molecular interaction among receptor protein, signaling peptide, and target DNA have changed some earlier perceptions. In spite of the increased knowledge and the potential biotechnological applications of these quorum-sensing systems, few examples on engineering for biotechnological applications have been published. Real applications will arise only when researchers working in applied microbiology and biotechnology are aware of the importance of quorum-sensing systems for health and bio-process applications.

Keywords Quorum sensing · Signaling peptides · RNPP protein family · Gram-positive bacteria

J. Rocha-Estrada · A. E. Aceves-Diez · M. de la Torre (✉)
Centro de Investigación en Alimentación y Desarrollo, A. C.,
Carretera a la Victoria Km. 0.6,
83304 Hermosillo, Sonora, México
e-mail: mdelatorre@ciad.mx

G. Guarneros
Departamento de Genética y Biología Molecular,
Centro de Investigación y de Estudios Avanzados
del Instituto Politécnico Nacional,
Av. Instituto Politécnico Nacional 2508 Col.
San Pedro Zacatenco,
07000 México, DF, México

Introduction

Bacteria communicate with one another and with their eukaryotic hosts, using different means. Communication as well as decision-making capabilities enable bacterial populations to coordinate growth, movement, and biochemical activities for species survival and differentiation, as well as for communication with their symbionts and competitors. *Quorum sensing* represents one of several mechanisms that bacterial cells use to interact with each other. This mechanism depends on synthesis, secretion, and recognition of small molecules that diffuse in and out of the cells. As bacterial population density increases, the concentration of signal molecules in the external environment rises. Once a critical threshold of concentration has been reached, a target sensor kinase or response regulator is either activated or repressed, facilitating the expression of quorum sensing-dependent genes (for review, see Bassler and Losick 2006; Dunny and Leonard 1997; Kleerebezem and Quadri 2001; March and Bentley 2004; Shapiro 1998; Williams 2007; Williams et al. 2007).

Several bacterial processes such as virulence, sporulation, development of genetic competence, production of secreted proteolytic enzymes, synthesis of peptide antibiotics, formation of biofilms, and fluorescence are coordinated by quorum sensing. Gram-negative quorum-sensing bacteria predomi-

nantly communicate through acyl-homoserine lactones. When this autoinducer binds to its cognate cytoplasmic protein, the protein–auto-inducer complex binds the DNA and activates transcription of target quorum-sensing genes. Contrastingly, Gram-positive bacteria use oligopeptides as intercellular signals in the same way as eukaryotic organisms do. Signaling peptides elicit a response when they are recognized by their cognate membrane-bound, two-component sensor histidine kinase or by binding directly to their corresponding intracellular receptor in the responder cell (for review, see Bassler and Losick 2006; Pottathil and Lazazzera 2003; Shapiro 1998).

In *Bacillus* species, the quorum-sensing proteins that bind directly to their cognate signaling peptides consist of certain aspartyl phosphate phosphatases (Rap phosphatases or Rap proteins), the neutral protease regulator (NprR and its orthologues), and the phospholipase C regulator (PlcR). In *Enterococcus faecalis*, the pheromone receptor is also a quorum-sensing protein that binds to its signaling peptide. Although these proteins control diverse processes in different bacterial species, they share a similar structure. Phylogenetic analysis suggested that they descend from a common ancestor. Declerck et al. (2007) proposed that they form a protein family named RNPP which stands for Rap, NprR, PlcR, and PrgX. This family appears to comprise all Gram-positive quorum-sensing systems which bind directly to their signaling peptide in the recipient cell. Usually, genes encoding for the proteins of the RNPP family and its cognate pro-signaling peptide form a cassette located in the bacterial chromosome or in plasmids. The receptor proteins remain intracellular, but a fragment corresponding to the C-terminus of the pro-signaling peptide is exported and processed by proteases to the mature signaling peptide. The mature peptide is internalized by an oligopeptide permease to bind the intracellular receptor protein (Gohar et al. 2008; Pottathil and Lazazzera 2003).

Research on the RNPP quorum-sensing systems has focused on amino acid sequence and assessment of the biological relevant form of signaling peptides, determination of the critical amino acid residues in the signaling peptide, molecular mechanism of the interaction protein-signaling peptide and protein-signaling peptide-target DNA, as well as molecular evolution. Although only a few examples on the manipulation of these quorum systems have been published, the potential applications in biotechnological process, drug discovery, and health are great.

The RNPP quorum-sensing systems have been mainly investigated in *Bacillus subtilis*, *Enteroccocus faecalis*, and the *Bacillus cereus* group (Dunny 2007; Gohar et al. 2008; Pottathil and Lazazzera 2003). All these bacterial species are important in biotechnology since *B. subtilis* is commonly used for the production of extracellular enzymes, *E. faecalis* is a multi-antibiotic resistant-opportunist pathogen that causes nosocomial infections, and the *B. cereus* group includes *B. cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis*. The first species causes food borne illness, the second species causes anthrax, and the last is an insect pathogen that has been used for biological control for over four decades.

This review provides a cursory overview of the mechanisms and functions of the RNPP quorum-sensing systems, with a more in-depth discussion in the determinants of signaling peptide specificity. We also review the molecular mechanism of the protein–signaling peptide–target interaction and highlight the relevance of these systems in biotechnology.

Rap proteins

In *B. subtilis*, the opposing activities of Rap proteins and histidine kinases determine whether cells should sporulate or continue with vegetative growth, by means of the phosphorelay signal transduction system (Jiang et al. 2000). The 11 Rap phosphatases of *B. subtilis* have a tandem of six tetratricopeptide repeats (TPR) domains that promotes protein–protein interactions (Core and Perego 2003). These Rap proteins are approximately 375 amino acids in length, share >25% identity with each other, and have different functions. RapA, RapB, and RapE work as negative regulators of the phosphorelay signal transduction system which leads to the initiation of sporulation, by promoting dephosphorylation of the response regulator Spo0F. RapC regulates competence development by modulating the activity of the response regulator and transcriptional factor Com A (Pottathil and Lazazzera 2003).

Open reading frames (ORFs) encoding small proteins (Phr), which are precursors of signaling peptides, are situated immediately downstream of the genes encoding Rap phosphatases. Phr proteins do not share significant amino acid identity, but all are approximately 40 amino acids in length and have a signal sequence for export, followed by a polar segment consisting of between 13 and 20 amino acids in the C-terminus that is secreted. The secreted peptide is processed by proteolysis to produce the mature signaling peptide, which can be imported by the oligopeptide permease (Opp) transport system (Perego 1996; Perego et al. 1991) (Fig. 1). If the mature signaling peptide corresponds to the last five C-terminal amino acids of the secreted peptide, only a proteolytical cleavage is required in order to release a C-terminal penta-peptide. Two or more cleavages are required to release an internal peptide; in this case, genome analysis is not enough to establish the sequence of the relevant biological signaling peptide. Although Phr displays a variety of sequences, they commonly have a positively charged amino acid at the second position (Pottathil and Lazazzera 2003; Stephenson et al. 2003).

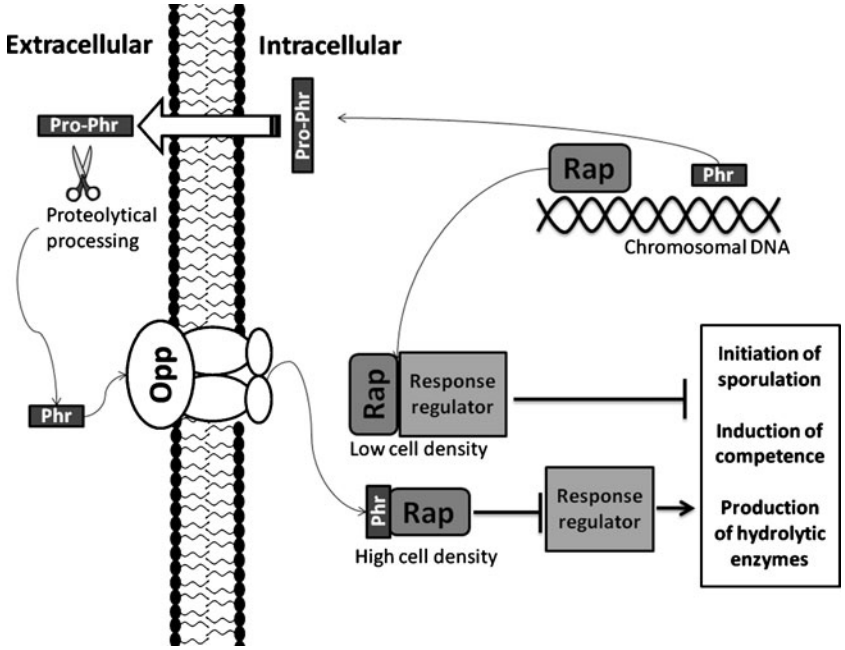

Fig. 1 Model of the mechanism of Rap–Phr systems. The pro-Phr peptide is synthesized and exported (*white arrow*). After proteolytical processing, the mature peptide is reinternalized via the Opp system. At low cell density, Rap binds to the response regulator inactivating it. At high cell density, binding of Phr prevents Rap interaction with the response regulator. *Dark arrows* indicate induction; *truncated lines* indicate repression

Regarding Rap phosphatases mechanism, Tzeng et al. (1998) found that the β1-α1 loop and N-terminal half of helix α1 of Spo0F surface has the largest number of residues in which an alanine substitution leads to resistance or decreased sensitivity to RapB phosphatase activity. Other mutations desensitizing Spo0F to RapB are also located near the site of phosphorylation on the β3-α3 and β4-α4 loops, suggesting that Rap phosphatases catalyze acyl phosphate hydrolysis by inducing conformational changes in phosphorylated Spo0F, which results in increased autodephosphorylation. In the case of RapA, RapB, and RapC, Core and Perego (2003) never identified a phosphorylated intermediate in the Rap-dependent dephosphorylation of SpoOF~P. All these findings support that Rap is an allosteric effector of SpoOF~P and its binding stimulates the dephosphorylation activity of the response regulator. On the other hand, binding of RapC to the response regulator ComA inhibits the regulator ability to bind its promoter, but rather than affecting ComA phosphorilation state RapC acts as an allosteric effector. Ogura et al. (2003) found that, in *B. subtilis*, RapG inhibits binding of the response regulator DegU to the target promoters; the inhibition was counteracted by its signaling peptide (EKMIG). Thus, the molecular mechanism in these cases is related to allosteric changes induced by Rap binding and the signaling peptide inhibits Rap union to the response regulator.

Another major issue concerns what the determinants for the interaction Rap–signaling peptide are. The competence and sporulation factor (CSF) was the first signaling peptide from the culture supernatant of *B. subtilis* to be purified and characterized (Solomon et al. 1996). It is encoded by *phrC* and has the amino acid sequence ERGMT which matches the C-terminal five amino acids of the PhrC proteins from *B. subtilis* subsp. *subtilis* str. 168, *Bacillus mojavensis*, and *Bacillus amyloliquefaciens* FZB42 (Solomon et al. 1996).

CSF stimulates competence by inhibiting RapC; it stimulates sporulation by inhibiting RapB and probably also inhibits competence by interacting with another quorum-sensing protein (Lazazzera et al. 1997). However, the determinants of the peptide specificity are different for each activity. The entire sequence (ERGMT) is essential for stimulating competence, but only R and M in the second and fourth positions are required to inhibit competence. The third residue (G) is essential for stimulating sporulation. It is worthy to mention that the glycine-to-alanine mutant peptide (ERAMT) stimulates higher levels of sporulation than the wild-type peptide (Lazazzera et al. 1997). These data suggest that, although the same signaling peptide may bind to several quorum sensing proteins, the need to have a specific amino acid in a certain position varies in the case of each cognate protein and function.

Most of the putative mature peptides coded for by *phr* genes in *B. subtilis* have arginine and threonine residues in the second and fifth positions, respectively. When the other residues (first, third, and fourth positions) of the mature penta-peptides corresponding to PhrA (ARNQT) or CSF (ERGMT) were substituted, ARNMT restored about 50% efficiency in sporulation of the wild-type peptide in a *phrA*

mutant strain, but manifested only 1% to 6% efficiency in the case of ARGMT, ERNMT, ERGQT, ARGQT, and ERNQT. In other experiments, ARNQT and the hexa-peptide AARNQT were equally efficient for complementing a *phrA* mutant in vivo. In vitro ARNQT inhibited about 50% RapA phosphatase activity and AARNQT inhibited no more than 10% (Perego 1997). These results not only suggest a certain flexibility in the specificity of the interaction of Rap protein–signaling peptides in vivo but also suggest that binding of different peptides does not modify the structure of the protein to the same extent. Another possibility is that each peptide has a different affinity for the cognate protein, and therefore lower or higher concentrations are needed for the same effect.

The number of *rap* and *phr* genes in the genomes of *B. subtilis* and the bacteria of the *B. cereus* group is different. *B. subtilis* genome encodes 11 putative Rap phosphatases and eight putative Phr proteins (Hayashi et al. 2006; Kunst et al. 1997). In the *B. thuringiensis* serovar *israelensis* genome, there are putative sequences for eight Rap and four Phr; in *B. anthracis* A2012, six Rap and five Phr; and in *B. cereus*, five Rap and five Phr (Anderson et al. 2005). These data suggest that some signaling peptides in these species may interact with more than one Rap, as in the case of CSF in *B. subtilis*, or equally that some Rap are not quorum-sensing proteins. Out of the six Rap encoded in the *B. anthracis* genome, only Rap BA3790 is involved in the regulation of sporulation initiation, together with Rap BXA0205, which is encoded in the virulence plasmid pX01. The signaling peptide for Rap BXA0205 corresponds to the last C-terminal five amino acids (GHTGC) in the protein encoded by *phr* BXA0205, but for Rap BA3790 the active penta-peptide is internal (Phr BA3791) and its sequence has not yet been identified (Bongiorni et al. 2006).

Concerning Rap proteins in the *B. cereus* group, major issues remain unsolved, for example: as well as controlling the initiation of sporulation, do Rap–Phr systems have other biological functions? What are the sequences of signaling peptides? Does cross-talk take place among different species and strains? This question has not been addressed in *B. subtilis* either.

A practical example of application of Rap–Phr systems is the production of alkaline protease (Apr). The expression of *apr* gene in *B. subtilis* is modulated by Rap–Phr systems encoded either in the bacterial chromosome or in plasmids (Koetje et al. 2003; Tjalsma et al. 2004). Disruption of *rapA*, *rapE*, and *rapF* in *B. subtilis* stimulated *aprE* transcription, suggesting that Rap–Phr systems are negative regulators. Indeed, disruption of *rap* increases the production of proteases in the strain (Tjalsma et al. 2004). Therefore, the authors proposed that quorum-sensing systems, which are important for bacterial adaptation under natural conditions, may decrease yields when used in production processes.

Moreover, since Rap–Phr systems regulate the levels of SpoOA~P, they are part of the regulatory networks that ensure the appropriate timing of sporulation, toxins, antibiotics and other secondary metabolites production in *Bacillus* and *Clostridium* species (Bongiorni et al. 2006). Thus, a better understanding of Rap–Phr systems in *B. subtilis* and other bacilli, as well as its interaction with other regulatory systems, will permit the establishment of strategies for engineering and control processes such as sporulation, genetic competence, and protease production.

Sex pheromone receptor (PrgX)

The sex pheromone receptor of *E. faecalis* (PrgX) is encoded in the antibiotic resistance plasmid pCF10 and participates in the regulation of the expression of the conjugative transfer genes encoded in the plasmid. This is a rather complex phenomenon regulated by a network of proteins and RNAs (Kozlowicz et al. 2006).

The PrgX quorum-sensing system is different from the other ones described in this review, as the signaling occurs between two different cell types, permitting the plasmid donor cells to regulate the expression of conjugation ability, in response to the density of the recipient cell (Kozlowicz et al. 2006). PrgX regulates its own expression. Furthermore, the transcriptional regulator PrgX binds two different signaling peptides: the pheromone cCF10 (codified in the bacterial chromosome) and the inhibitor iCF10 (encoded in the plasmid pCF10 in the donor cells). The hepta-peptide cCF10 (LVTLVFV) serves as a specific inducer of pCF10 conjugation genes, while iCF10 (AITLIFI) represses their expression. Donor cells, as well as recipient cells, carry genes for cCF10 pheromone production. Thus, a mechanism was selected along evolution to avoid self-induction by the endogenous pheromone in the donor while allowing the response to the pheromone from the recipient cell (Kozlowicz et al. 2006). Chandler et al. (2005) proposed that the extracellular domain of the membrane protein PrgY interacts with nascent cCF10 by sequestering, modifying, or degrading the peptide, reducing the production of endogenous pheromone activity by donor cells. The residual excreted cCF10 activity is neutralized by iCF1O. A considerable proportion of cCF10 remains associated with the cell wall. In the donor cell, the induction state depends on the ratio cCF10/iCF10 (Buttaro et al. 2000; Dunny 2007) (Fig. 2).

The X-ray crystal structure of PrgX and PrgX/cCF10 complex revealed that the protein has three functional domains: an N-terminal binding domain, a large central dimerization-signaling peptide binding domain, and a C-terminal regulatory domain. The oligomeric state of PrgX in crystals is a tetramer formed by two dimers. Since dimeric PrgX binds to two different operators in pCF10, a PrgX

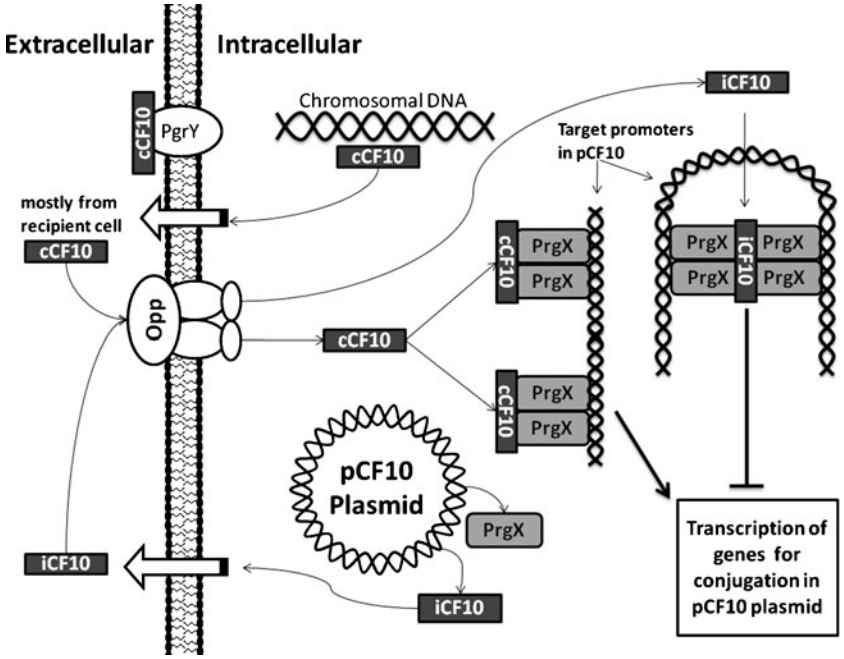

Fig. 2 Model of the mechanism of PrgX and its signaling peptides: pheromone cCF10 and inhibitor iCF10 in *E. faecalis*. Both donor and recipient cells synthesize cCF10 (encoded in the bacterial chromosome); most of cCF10 from the donor is sequestered by PrgY in its membrane, but the free peptide is transported to the cytoplasm by Opp, as well as iCF10. PrgX dimer specifically binds pCF10 at two promoters in plasmid pCF10. iCF10 stabilizes PrgX tetramer, forming a DNA loop restricting access of RNA polymerase. cCF10 competes with iCF10 for the binding pocket of PrgX. When cCF10 binds to PrgX, it causes disruption of the tetramers and eventually releases; dimer binding to DNA promoters allow access of RNA polymerase (Dunny 2007). *Dark arrow* and *truncated lines* indicate induction and repression, respectively. *White arrows* indicate export to the citoplasm

tetramer/DNA loop model for controlling *prgX* operon transcription was proposed. In this model, a dimer binds to site O1 and another dimer to site O2. Both dimers interact, forming a tetramer that provokes a stable DNA loop which restricts RNA polymerase access to the prgQ promoter. As the experimental results indicate, cCF10 binding to PrgX induces conformational changes in the C-terminal domain. The model implies that the binding of this signaling peptide might break up tetramers, opening the loop and allowing the polymerase access. iCF10 may compete with cCF10 for the binding groove, but iCF10 does not induce the same conformational changes; thus, its binding should not destabilize the tetramer (Fig. 2) (Shi et al. 2005). Binding of iCF10 to PrgX increases protein DNA binding capacity (Dunny GM, personal communication). Because the gene encoding iCF10 precursor is in *prgQ* locus, as the transcription from *prgQ* operon increases so does the concentration of iCF10, and therefore *prgQ* transcription is reduced to un-induced levels (Kozlowicz et al. 2006).

Since cCF10 and iCF10 bind to PrgX, this quorum-sensing protein interacts with two different cognate peptides. However, given that cCF10 is encoded in the bacterial chromosome and PrgX in the plasmid pCF10, the corresponding genes do not form a cassette. On the other hand, the gene conferring iCF10 production is encoded at 5′ end of the pheromone-inducible operon *prgQ* adjacent to *prgX* in the plasmid, so they may form part of an ancient quorum-sensing protein–signaling peptide cassette. In fact, the region-encoded stable plasmid maintenance and pheromone-inducible conjugation functions were assembled in a modular fashion from evolutionary distinct sources (Kozlowicz et al. 2006).

Both cCF10 and iCF10 correspond to the C-terminus end of the lipoprotein that is proteolytically processed to release the active peptide, but they do not have a positively charged amino acid at the second position from the N-terminus as most of the mature signaling peptide of Rap proteins have. Indeed, the pheromone and the inhibitor signaling peptides have a non-polar residue in this position. Investigating determinants of signaling peptides and specifically in the case of PgrX, we were unable to find experimental reports where variants of the hepta-peptides sequences, or either longer or shorter peptides, coded for in the pro-peptide genes has been tested to determine amino acid residues critical for biological activity. Furthermore, it would be important to determine whether distinct PrgX–cCF10/iCF10 phenotypes exist in *E. faecalis*, as is the case with PlcR–PapR, and whether cross-talk occurs in this case.

E. faecalis forms robust biofilms in host tissues and on abiotic surfaces, such as catheters and heart valves. These biofilms are likely to play a major role in the pathogenesis and in the transfer of antibiotic-resistance genes to bacteria (Hendrickx et al. 2009). Ballering et al. (2009) identified 68

candidate biofilm genes in the bacteria, some of which have functions already associated with biofilm formation, i.e., stress response and polysaccharide metabolism, and other are novel determinants. Building a biofilm requires a series of discrete and well-regulated steps, including aggregation of cells (O'Toole 2003). In *E. faecalis*, the protein Asc10 (aggregation substance) is encoded in plasmid pCF10, inside the locus encoding genes for replication and pheromone-inducible aggregation, where *prgX* is also encoded (Dunny 2007). Since biofilm formation requires quorum-sensing, blocking quorum-sensing systems may help to prevent biofilm formation in in-dwelling devices and human tissues, as well as facilitating the design of drugs destined to act on specific targets (Schachter 2003). However, it is important to highlight that the regulation of the expression of genes encoded in pCF10 from *E. faecalis* represents a very complex system. Furthermore, when *E. faecalis* is growing in mammalian bloodstream, a host factor selectively sequesters or degrades iCF10, leading to induction of conjugation proteins including Asc10 (Dunny 2007). Thus, the host is an active partner in the regulation of the genes that code for these proteins. Besides this, the physiology of single bacterial cells (plankton cells) and cells in biofilms is different. Therefore, all these facts have to be taken into account when developing and testing drugs directed towards specific targets intended to either inhibit the formation of *E. faecalis* biofilms or to destroy them.

PlcR transcriptional regulator

PlcR is a pleiotropic transcriptional regulator specific to the *B. cereus* group, which includes *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. wiehenstephanensis* (Agaisse et al. 1999). It was originally described as a pleiotropic regulator of extracellular virulence factors such as phospholipase C (encoded by *plcA* gene) (Lereclus et al. 1996). PlcR positively controls 45 genes mainly coding for extracellular proteins. The functions of the quorum sensing-regulated proteins are associated to food supply, cell protection, and signals integration. PlcR regulon includes enzymes, toxins, bacteriocins, sensors, and transporters (Gohar et al. 2008). As with PrgX, PlcR positively regulates its own expression (Lereclus et al. 1996), but its activity only depends on the presence of the signaling peptide derived from PapR, encoded immediately downstream of PlcR (Fig. 2). Interestingly, the spores of a Δ*papR* mutant of *B. thuringiensis* were less virulent against *Galleria mellonela* larvae than those of the wild-type strain (Slamti and Lereclus 2002), suggesting that genes regulated by PapR are involved in the insecticidal activity of the bacteria.

Slamti and Lereclus (2002) investigated whether the synthetic peptides corresponding to the C-terminus of the PapR polypeptide which have three to five, seven, or nine amino acids would be capable of activating the expression of *lacZ* in *B. thuringiensis* 407 Cry$^-$ A′Z Δ*papR*. They found that those with five, seven, or nine residues activated it, and they concluded that the signaling peptide is at least five amino acids long (LPFEP). In order to address whether PlcR and PapR displayed strain specificity, the same authors carried out complementation studies among the *B. thuringiensis* 407 Cry$^-$A′Z Δ*papR* mutant strain and on different strains of *B. thuringiensis* (serotypes 1-14 and 34), *B. cereus*, *B anthracis*, and *B. mycoides*. Also, the *papR* genes of the strains were sequenced and the mature signaling peptides were deduced. The penta-peptides of the strains that activated *lacZ* in the mutant had a leucine residue in the first position, in the same way as the mutant peptide (LPFEP), but the non-activating strains manifested methionine or valine. Thus, the activating mechanism is strain specific and the activating capacity of peptides turns out to cause very subtle regulation, whereas the N-terminal first residue of the penta-peptide determines the specificity.

The same group carried out in vivo complementation assays using the *B. thuringiensis* 407 Cry$^-$ (Δ*papR plcA'-lacZ*) and 29 wild-type strains of *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. weihenstephanensis*, and *B. anthracis*. They also compared PlcR–PapR sequences of the strains and found four classes of PlcR–PapR couples, defining for different phenotypes in the *B. cereus* group. Also worth to mention is that strains belonging to different species are found in the same group and strains from the same species may belong to different clusters. The penta-peptides pertaining to the four groups are LPFE(F/Y), VP(F/Y)E(F/Y), MPFEF, and LPFEH. The homologous penta-peptide LPFEF was at least ten times more efficient than LPFEH for activating PlcR in *B. thuringiensis* 407 Cry$^-$ (Δ*papR plcA′–lacZ*), suggesting that the fifth amino acid was involved in the specificity of the interaction PlcR–PapR, in the same way as the first. A low-positive cross-reactivity of heterologous penta-peptides was observed, suggesting that these heterologous penta-peptides are able to interact with the heterologous PlcR (Slamti and Lereclus 2005).

Bouillaut et al. (2008) purified the extra- and intracellular forms of PapR, using a mutant of *B. thuringiensis* 407 Cry$^-$ strain, which over-expresses the PlcR regulon and belongs to Group I. The mature and active peptide in the extracellular and intracellular environments was the hepta-peptide ADLPFEF, corresponding to the last seven residues of the PapR C-terminus. Thus, at least for this strain, the biologically relevant form is the hepta-peptide rather than the penta-peptide. In steady-state fluorescence anisotropy binding titrations using PlcR protein and fluorescein-labeled peptides, the hepta-petide was found to be a slightly better competitor than the four, five, or nine amino acids PapR. In vivo, penta- and hepta-peptides corresponding to PlcRI, PlcRIII, and

PlcRIV groups were able to activate their cognate PlcR in a similar extension. In the case of PlcRII, the hepta-peptide activated the *plcA* (a gene belonging to PlcR regulon) expression up to 6.5-fold than the penta-peptide. Furthermore, the activity of PlcRI and PlcRIV was strictly dependent on their cognate peptides, but PlcRII and PlcRIII were less restrictive and may be activated by a heterologous peptide, although the activity was lower. Therefore, cross-talk between strains belonging to these two phenotypes is possible, i.e., among *B. thuringiensis*, *B. cereus*, and *B. weihenstephanensis* strains, and peptides produced by one species may activate virulence in other species. In *B. subtilis* strain 168, the introduction of *plcR* and *papR* genes activated the expression of PlcR-regulated genes, indicating that PapR processing also occurs in this heterologous group.

The mature PapR penta-peptides have proline, a non-polar amino acid, in the second position from N-terminus instead of a positively charged amino acid as is the case with most of the mature Phr peptides of Rap proteins. Indeed, neither those PapRII hepta-peptide have a positively charged amino acid in this position; the amino acid is glutamate, a negatively charged amino acid. From a structural analysis of the interaction PlcR–PapR, Bouillaut et al. (2008) speculate that the proline residue may be required for the peptide to fit into the binding groove on PlcR, and lysines 87 and 89 of PlcR function as gatekeepers, selecting PapR from other oligopeptides, by means of ionic interactions with the glutamic acid. In the crystal structure of PlcR Group I, the groove that accommodated the cognate penta-peptide continues after the N-terminus residue and the two additional amino acids of the hepta-peptide fitted well in the groove extension.

The structure and mechanism of PlcR is similar to the structure to that of PrgX. The binding of the cognate peptide also induces a conformational change, with the consequent oligomer formation and binding to DNA (Fig. 3), but in this case the formation of a DNA loop as occurs in the case of PrgX–iCF10 has not been proposed. Both the cognate penta-peptide and the hepta-peptide of PlcRI triggered the same specific oligomerization of the protein (Declerck et al. 2007).

Since the PlcR–PapR system regulates a large number of processes involved in the pathogenesis of the *B. cereus*, which includes human and insect pathogens, the understanding and engineering of this system will have impact in biotechnology and human health. Additionally, *B. cereus* forms biofilms on plastic, glass wool, and stainless steel, affecting the industrial processes and product quality, and PlcR is also related to biofilm formation (Hsueh et al. 2006).

However, although PlcR regulon comprises a wide variety of processes related to signaling integration, food supply, and cell protection, it does not represent the only factor affecting virulence in bacteria (Gohar et al. 2008). As observed in *B. thuringiensis*, disruption of PlcR decreases but does not eradicate virulence, because other factors such as flagella seem to have a pleiotropic role in virulence (Bouillaut et al. 2005). Furthermore, the oligopeptide permease system is also necessary for PlcR expression (Gominet et al. 2001). It seems that manipulation of bacterial virulence, either for biotechnological, health-related, or economical purposes, requires adequate and integral engineering of PclR–PapR-regulated genes and other quorum-sensing systems, as at least proteases are also regulated by Rap–Phr and NprR–Phr.

NprR protein

The quorum-sensing NprR protein was originally described as a regulator of the neutral protease in *B. subtilis* (Uehara et al. 1974). In the *B. subtilis* genome, *nprR* is closely linked to the structural gene *nprE*, which codes for the neutral protease. Two alleles of this regulator, *nprR1* and *nprR2*, were found in various *B. subtilis* strains; those *bacilli* harboring *nprR2* produced 20 to 50 times more neutral proteases than the strains carrying *nprR1* (Toma et al. 1986; Uehara et al. 1979). Nishiya and Imanaka (1990) cloned orthologues of both genes from *B. stearothermophilus*. As in *B. subtilis*, the regulatory gene *nprA* is located upstream of *nprS*, which is the neutral protease structural gene.

Pottathil and Lazazzera (2003), searching for putative Rap–Phr cassettes on the chromosome of non-*B. subtilis* Gram-positive spore-forming bacteria, found genes whose encoded proteins shared a tenuous similarity to Rap phosphatases of *B. subtilis* in *Clostridium acetobutylicum*, *B. stearothermophilus*, *B. thuringiensis*, and *B. anthracis*. These proteins contain, as in the case of Rap proteins, multiple TPR motifs for protein–protein interaction but have a helix-turn-helix (HTH) motif for DNA binding in the N-terminus region, suggesting that rather than Rap phosphatases these are transcriptional regulators. These putative Rap phosphatases are NprA, NprR, and a homologue of NprA in *B. stearothermophilus*, *B. thuringiensis*, and *B. anthracis*, respectively. Thus, the regulators of neutral proteases may be quorum-sensing proteins.

In the same work, the authors identified the cognate mature signaling peptide for each protein (Phr). They searched in the secreted domain corresponding to each Phr protein for a penta-peptide that has a positively charged amino acid at the second position from N-terminus. The putative mature peptides are SKPDT for the NprR of *B. thuringiensis* and AKDEH for the NprA of *B. stearothermophilus* and for the protein encoded by contig 344 in the same bacterium. In the case of *B. stearothermophilus*, the penta-peptide is repeated twice. The *phr* corresponding to contig 4777 of *B. anthracis* codes for two putative pentapeptides: SKPDI and AKTVQ, and in the Phr

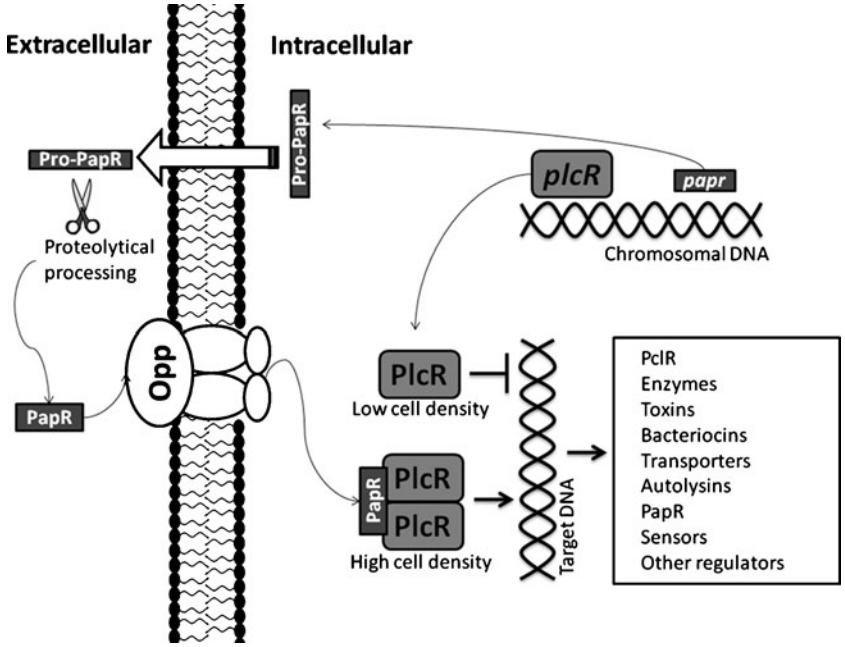

Fig. 3 Model of the mechanism of PlcR and its cognate peptide PapR. PapR is synthesized and exported (*white arrow*), after proteolytical processing the mature peptide is reinternalized via Opp system. At high cell density, PapR binds to PlcR, promoting a conformational change, oligomerization, and DNA binding capacity. The polymers bind to DNA in PlcR boxes stimulating transcription of the PlcR virulence regulon (Declerck et al. 2007). *Dark arrows* indicate induction; *truncated line* indicates repression

putative-secreted domain of *C. acetobutylicum*, there are no charged residues. All the putative penta-peptides were derived from the internal portions of the Phr-secreted domain and not from the C-terminal residues. Therefore, the secreted peptide has to be cleaved at least twice in order to release the penta-peptide, and it is very difficult to assess the real length and sequence of the biologically active peptide.

We analyzed the sequences immediately upstream and downstream of *nprR* in several *Bacillus* species and found a sequence that codes for bacillolysin (thermolysin-like metalloprotease) in *B. thuringiensis* var. konkukian (GenBank accession No. NC_005957) and *B. cereus* E33L (GenBank accession No. NC_006274). Likewise, we found an orthologue of *nprE* in *B. thuringiensis* strain Al Hakam (GenBank accession No. NC_008600) and in *B. cereus* Q1 (GenBank accession No. NC_011969). These ORFs were located about 300 bp downstream of *nprR*, suggesting that NprR may regulate these protease genes as in *B. subtilis* and *B. stearothermophilus* (Nishiya and Imanaka 1990; Toma et al. 1986; Uehara et al. 1979).

Little is known about the regulation and function of the system NprR–Phr. In the *B. stearothermophilus* genome, three palindromic sequences for NprA were found in the region 5′ of *nprA*, suggesting that *nprA* self-regulates its expression (Nishiya and Imanaka 1990) in the same way that PlcR and PrgX do (Kozlowicz et al. 2006; Lereclus et al. 1996). In an acrystalliferous mutant of *B. thuringiensis* bearing a *cry1Aa–lacz* transcriptional fusion, the synthetic peptide SKPDT was able to stimulate β-galactosidase activity, sporulation, and spore release (Aceves-Diez et al. 2007). Since this penta-peptide is the cognate putative signaling peptide of NprR (Pottathil and Lazazzera 2003), the NprR–Phr system may be involved in the regulation of several genes, but further studies are required in order to assess this.

We investigated whether some amino acid residues were critical for the activity of the peptide SKPDT in an acrystalliferous mutant of *B. thuringiensis* serovar. *thuringiensis* bearing a *cry1Aa–lacz* transcriptional fusion. For this purpose, we substituted each amino acid residue, one by one, with alanine. The addition of 200 nM of synthetic peptides SAPDT or SKPDA to low-density cultures did not stimulate *cry1Aa* whereas AKPDT, SKADT, and SKPAD did, suggesting that lysine and threonine may somehow participate in the interaction with NprR (Ramirez, unpublished data).

Unfortunately, there are no published data on the conformational changes induced by the cognate signaling peptides in NprR. Preliminary experiments on the part of our group indicate that the protein in isolation does not join with the neutral protease promoter of *B. thuringiensis* serovar *thuringiensis* and that SKPDT induces a conformational change in NprR but SAPDT does not (Islas and Sotelo, unpublished results). Further studies are needed to define the molecular mechanism of the pair NprR–Phr.

Proteases are the second most important group of industrial enzymes; in bacilli, neutral and alkaline proteases are regulated by RNPP quorum-sensing systems (Koetje et

al. 2003; Tjalsma et al. 2004); therefore, engineering of these systems may serve to improve yields in industrial processes. Neutral proteases are mainly used in leather industry to remove hair from fur, in textile industry as a degumming agent for silk, and in the food industry to break down and increase solubility, dispersibility, and digestibility (http://www.specialtyenzymes.com) concerning other functional proteins; alkaline proteases are mostly used in detergents.

Neutral proteases, as previously mentioned, seem to be closely related to the production and integrity of insecticidal Cry proteins in *B. thuringiensis*. The sporulated cultures of a mutant strain, where the gene *nprA3* was interrupted, contained a higher concentration of full-length insecticidal protein than those of the isogenic counterpart which manifests the wild gene. Also, the crystals were more stable in the mutant, but growth and sporulation were not affected (Donovan et al. 1997). Furthermore, the putative signaling peptide of the neutral protease regulator NprR stimulates sporulation and *cry* expression in *B. thuringiensis* BtpHTcry1A2 (Aceves-Diez et al. 2007). These results suggest that neutral proteases and neutral proteases regulator may play an important role in production of Cry protein bioinsecticides.

PlcR–PapR system also regulates proteases expression (Gohar et al. 2008); therefore, an increased understanding of the role of quorum-sensing NprR–Phr system and its interaction with the other quorum-sensing systems like PlcR–PapR and Rap–Phr may lead to an improvement at least in bacilli–proteases processes. Unfortunately, research concerning the NprR quorum-sensing protein and its cognate peptide is very limited; for example, important questions such as which genes are included in the NprR regulon, what is the relevant biological form of the mature peptide for each *Bacillus* species, and whether cross-talk among different bacilli species exist have still not been answered.

Conclusions and perspectives

In recent years, the research on quorum-sensing systems where the receptor protein interacts directly with its cognate signaling peptide has diversified from the Rap proteins of *B. subtilis* and the pheromone receptor of *E. faecalis* to other quorum-sensing proteins and bacteria species, mainly those of the *B. cereus* group. It seems that the mature signaling oligopeptides, which are effectors of transcriptional regulators, do not quite follow the general rules concerning the length and sequence of signaling peptides established for Rap phosphatases. Some of them are hexa- and hepta-peptides and do not have a positively charged amino acid in the second position from the N-terminus. Furthermore, there is certain flexibility in the peptide specificity of these quorum-sensing systems. It seems that, as more profound studies on the RNPP family are conducted in other bacteria, more diverse sequences and peptide lengths will be discovered, and more data on flexibility of the signaling peptide-protein interaction will emerge.

Insights into the mechanism of the protein-signaling peptide pair, as well as understanding the molecular interaction among receptor protein, signaling peptide, and target DNA, have changed some earlier perceptions. For example, the quorum-sensing activity of Rap proteins may not be related to its phosphatase activity; the interaction between signaling peptide and receptor protein seems not to be as specific and as stringent as earlier believed. Therefore, cross-talk between different species takes place at least in the PlcR–PapR system in the *B. cereus* group. It is possible that cross-talk occurs in the real world where different species share the same habitat and one species may activate the quorum system responses of others, e.g., interaction between different bacterial species, where their response as a population to different stimuli may take place in nature. Beyond this, the participation of these proteins in the regulation of genes associated with the virulence and production of proteases suggests that interaction may take place among these quorum systems and that, in some way, the cell may integrate these and other regulatory systems in its response.

The PlcR–PapR system has been extensively investigated regarding different topics, and the PrgX–cCF10–iCF10 is in second place. The molecular mechanism of Rap–Phr interaction has not been fully elucidated and the NprR–Phr system has been practically ignored. Although it is highly speculative to establish analogies among the four systems, the information available suggests that the mechanisms of interaction between the signaling peptide and its cognate receptor protein share several characteristics, and we speculate that, as in the case of PlcR–PapR system, variants of the mature signaling peptide may bind to the same cognate receptor protein in the other RNPP systems. Besides this, peptides of different lengths may bind together but the magnitude of the activation or inactivation of the receptor protein would vary.

In his 1997 review on cell–cell communication in Gram-positive bacteria, G. Dunny mentions that an increasing understanding of the extracellular signaling mechanism may lead to many useful applications. Thirteen years later, we do have better understanding of these systems, but hardly any biotechnological application have been made involving their manipulation in Gram-positive bacteria. Real applications will emerge only when researchers working in applied microbiology and biotechnology are aware of the necessity to manipulate quorum-sensing systems.

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CAPÍTULO II

Evolution and some functions of the NprR-NprRB quorum sensing system in the *Bacillus cereus* group

Jorge Rocha, Víctor Flores, Rosina Cabrera, Adriana Soto-Guzmán, Giovana Granados, Eusebio Juaristi, Gabriel Guarneros & Mayra de la Torre

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RESUMEN

El Quorum sensing (QS) son mecanismos que las bacterias utilizan para regular la expresión genética en respuesta a la densidad celular. Las baterías Gram positivas utilizan oligo péptidos como moléculas de señalización de QS. La familia de proteínas RNPP (Rap, NprR, PlcR, PrgX) está conformada por receptores intracelulares de QS que se unen directamente a su péptido de señalización específico para regular la transcripción de genes. NprR es activador de una proteasa neutra en *Bacillus subtilis* que recientemente se relacionó con esporulación, transcripción de genes *cry* y actividad proteolítica extracelular en cepas del grupo de bacterias *B. cereus*. En el genoma de *B. thuringiensis*, corriente abajo del gen *nprR*, se encontró un gen que codifica para un pro-péptido de señalización de QS (*nprRB*). Se planteó que *nprR* y *nprRB* coevolucionaron gracias a su función coordinada, dentro del grupo *B. cereus*. Un árbol filogenético construido con secuencias de nucleótidos de *nprR* reveló que existen seis ferotipos, cada uno correspondiendo a un péptido de señalización putativo maduro NprRB. El árbol de *nprR* no concuerda con el agrupamiento taxonómico actual del grupo *B. cereus*, ni con el arreglo filogenético obtenido con MLST utilizando marcadores moleculares de las mismas cepas. SKPDI y otros péptidos codificados en el gen *nprRB* de *B. thuringiensis* serovar *thuringiensis* cepa 8741 tuvieron un efecto en la regulación temporal de la esporulación y en la expresión de la fusión transcripcional *cry1Aa'lacZ,* pero los péptidos que ocasionaron la detección temprana de esporas, disminuyeron la expresión de *cry1A*, sugiriendo que NprR podría activar o reprimir la transcripción de diferentes genes.

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Evolution and some functions of the NprR–NprRB quorum-sensing system in the *Bacillus cereus* group

Jorge Rocha · Victor Flores · Rosina Cabrera · Adriana Soto-Guzmán · Giovana Granados · Eusebio Juaristi · Gabriel Guarneros · Mayra de la Torre

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Abstract Quorum-sensing (QS) is a bacterial mechanism for regulation of gene expression in response to cell density. In Gram-positive bacteria, oligopeptides are the signaling molecules to elicit QS. The RNPP protein family (Rap, NprR, PlcR, and PrgX) are intracellular QS receptors that bind directly to their specific signaling peptide for regulating the transcription of several genes. NprR is the activator of a neutral protease in *Bacillus subtilis*, and it has been recently related to sporulation, *cry* genes transcription and extracellular protease activity in strains from the *B. cereus* group. In the *B. thuringiensis* genome, downstream *nprR,* a gene encoding a putative QS signaling propeptide (*nprRB*) was found. We hypothesized that the *nprR* and *nprRB* co-evolved because of their coordinated function in the *B. cereus* group. A phylogenetic tree of nucleotide sequences of *nprR* revealed six pherotypes, each corresponding to one putative mature NprRB sequence. The *nprR* tree does not match the current taxonomic grouping of the *B. cereus* group or the phylogenetic arrangement obtained when using MLST markers from the same strains. SKPDI and other synthetic peptides encoded in the *nprRB* gene from *B. thuringiensis* serovar *thuringiensis* strain 8741 had effect on temporal regulation of sporulation and expression of a *cry1Aa'Z* transcriptional fusion, but those peptides that stimulated earlier detection of spores decreased *cry1Aa* expression suggesting that NprR may either activate or repress the transcription of different genes.

Keywords *Bacillus thuringiensis* · Quorum-sensing · Sporulation-regulation · *cry1Aa* expression

J. Rocha · R. Cabrera · A. Soto-Guzmán · M. de la Torre (✉)
Centro de Investigación en Alimentación y Desarrollo,
A. C. Carretera a La Victoria Km 0.6, Hermosillo,
Sonora 83304, Mexico
e-mail: mdelatorre@ciad.mx

V. Flores · G. Guarneros
Departamento de Genética y Biología Molecular,
Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional,
Av. Instituto Politécnico Nacional 2508,
Col. San Pedro Zacatenco,
07360, Mexico City, Mexico

G. Granados · E. Juaristi
Departamento de Química, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional,
Av. Instituto Politécnico Nacional 2508,
Col. San Pedro Zacatenco,
07360, Mexico City, Mexico

Introduction

Communication as well as decision-making capabilities enables bacterial populations to coordinate growth, movement, and biochemical activities for species survival and differentiation, as well as for communication with their symbionts and competitors. Quorum-sensing (QS) is one of several mechanisms that bacterial cells use to interact with each other, regulating gene expression and controlling several microbial processes like sporulation, virulence, biofilm formation, conjugation, and production of extracellular enzymes (for review, see Bassler and Losick 2006; Williams 2007; Williams et al. 2007). For example in the *B. cereus* group, which includes *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, and *B. wiehenstephanensis*, the QS system PlcR–PapR regulates the expression of 45 genes including those encoding the most known virulence factors (i.e. phospholipases, enterotoxins, and hemolysins) (Gohar et al. 2008).

PlcR belongs to the RNPP family of intracellular QS-receptors of Gram-positive bacteria, which interacts directly with its cognate signaling peptide to be activated or deactivated. The other QS-receptors that integrate this family are Rap (Rap phosphatases involved in the regulation of sporulation onset), NprR (neutral protease regulator), and PrgX (sex-pheromone receptor) (Declerck et al. 2007; Rocha-Estrada et al. 2010). The genes encoding for the receptor and a small protein, which is processed to the mature signaling peptide, form a cassette located in the bacterial chromosome or in plasmids. The small protein has a putative signal sequence for the Sec-dependent export pathway and a secreted domain (Lazazzera 2001). The exported peptide is processed by extracellular proteases to the signaling peptide, which in the Rap phosphatase sensors usually corresponds to the last five amino acid residues from the C-terminus of the secreted peptide and has a basic amino acid in the second position from the N-terminus. The signaling peptide is internalized by an oligopeptide permease (Gohar et al. 2008; Pottathil and Lazazzera 2003).

PlcR, NprR, and PrgX are transcriptional regulators. PlcR is activated when it binds its mature signaling peptide (PapR). The complex protein/peptide binds to DNA to activate the target genes. The activating mechanism is strain specific being the N-terminal first and fifth residue of the penta-peptide which determine the specificity (Agaisse et al. 1999; Gohar et al. 2008; Lereclus et al. 1996; Okstad et al. 1999; Slamti and Lereclus 2002). For *B. thuringiensis* 407 Cry–strain, the mature and active peptide in the extracellular and intracellular environments was the hepta-peptide ADLPFEF, corresponding to the last seven residues of the PapR C terminus (Bouillaut et al. 2008), and the second amino acid residue from the N-terminus is not basic, like usually happens in Rap sensors. Thus, the length and sequence of mature signaling peptides may differ in the RNPP family of QS receptors.

Because of their cooperative role, *plcR* and *papR* genes have co-evolved by positive selection in the *B. cereus* group. Slamti and Lereclus (2005) identified four pherotypes based on the diversity of mature PapR peptide. A low-positive cross-reactivity of heterologous penta-peptides with heterologous PlcR was observed (Bouillaut et al. 2008; Slamti and Lereclus 2005).

NprR shares a structural similarity to PlcR (Declerck et al. 2007). NprR was originally described as the activator of a neutral protease in *Bacillus subtilis* (Uehara et al. 1979, 1974). Recently, it was related to sporulation and extracellular protease activity in *B. anthracis* Sterne 7702 (Yang et al. 2011) and also to *cry* genes transcription and sporulation in *B. thuringiensis* (Aceves-Diez et al. 2007; Wang et al. 2010). NprRB is the small protein that includes the mature signaling peptide of NprR (Pottathil and Lazazzera 2003). We aimed to determine if the genes encoding the signaling peptide (*nprRB*) co-evolved with their respective receptor gene (*nprR*) in the *B. cereus* group. We also tested the effect of synthetic peptides encoded in the exported region of *nprRB* on sporulation and *cry1Aa'Z* fusion transcription in *B. thuringiensis* serovar *thuringiensis* str. 8741 (Bt8741).

Materials and methods

Bacterial strains and growth conditions

The strains used in this work were the following: *B. thuringiensis* serovar *kurstaki* strains 4D22 and 4D11; *B. thuringiensis* serovar *israelensis* strains 4Q1 and 4Q7; and *B. thuringiensis* serovar *thuringiensis* strain 4A2 from Bacillus Genetic Stock Center (Ohio State University, Columbus, OH, USA). We also used a plasmidless mutant of *B. thuringiensis* serovar *thuringiensis* harboring the plasmid pHTcryIA2 (kindly provided by D. Lereclus) named strain 8741 and deposited in a microbial collection (Colección Nacional de Cepas Microbianas y Cultivos Celulares, CINVESTAV). The plasmid is a shuttle vector that includes the replication origins *oriC* and *ori*1030, which are functional in *Escherichia coli* and *B. thuringiensis* respectively, the gene *ermC* for erythromycin resistance, and a fusion of *lacZ* with a 362-bp fragment containing the promoter region of *cry1Aa* that includes promoters BtI and BtII (Bravo et al. 1996). Sterile paper disks containing 10^8 spores from nutrient-agar Petri dishes were prepared and maintained at 4°C for strain preservation. Unless different growth conditions are indicated, bacterial cultures were grown at 30°C in LB medium; the medium was supplemented with 15 μg mL^{-1} of erythromycin for Bt8741.

DNA manipulations

Chromosomal DNA was extracted from the *B. thuringiensis* cells using the method described by Msadek et al. (1990), with minor modifications. The cassettes encoding NprR and NprRB (signaling peptide precursor) were amplified in each strain with oligonucleotide primers NprR-1: 5′-GGGCATTTGTTCTGTCTC-3′ and NprR-2 5′-GCTAACACTAACGCTAAAC-3′, which were designed from *B. thuringiensis* serovar *konkukiani* str. 97–27 genome (GenBank Accession No. AE017355.1). Oligonucleotides were purchased from Sigma Genosys (USA). PCR was performed following the next steps: 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 40 s, and 72°C for 1:30 min, followed by one cycle of 72°C for 10 min in a DNA Engine thermal cycler (Bio Rad). After electrophoresis, PCR products were extracted and purified from the 1% agarose gels using the QIAEX II kit (QIAGEN) following the instructions of the

manufacturer. The DNA sequences were determined from the PCR products as a service from the University of Arizona. The nucleotide sequences obtained in this work were compared with sequences retrieved from databases in a pairwise mode, with the BLAST2 sequences tool (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi). After the confirmation of their identity, sequences were deposited in GenBank (accession numbers JN637468 to JN637473).

Phylogenetic analyses

In order to determine the co-evolution of *nprR* and *nprRB* in the *B. cereus* group, sequences from 26 strains of the *B. cereus* group were analyzed. We included the sequences from the six strains mentioned previously and complemented the study with 20 sequences obtained from the GenBank or the GenBank draft genome server database (http://www.ncbi.nlm.nih.gov/genbank/ and ftp.ncbi.nlm.nih.gov/genomes/Bacteria_DRAFT) (Benson et al. 2010), corresponding to strains from the *B. cereus* group (Table 1). Nucleotide sequences of *nprR* from all the strains were used to build a maximum likelihood tree under the general time reversible (GTR) substitution model using Phyml (Guindon et al. 2010). Tree topology was optimized with nearest neighbor interchanges and sub-tree pruning and re-grafting approaches. Statistical support was determined by 10,000 bootstrap replicates. Analysis of the peptide NprRB was performed using the software SignalP 3 (Emanuelsson et al. 2007), which incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models. Alignment of the secreted region was made in ClustalX, to identify putative mature signaling penta-peptides, based on the sequence SKPDT which was first identified by Pothathil and Lazzazzera (2003) and the criteria established by these authors. Information on the putative mature NprR peptide of each strain was compared to the grouping obtained in the *nprR* tree.

In order to determine the associations of the *B. cereus* group, we used a phylogenetic approach with nine Multi Locus Sequence Typing (MLST) markers (*adk*, *ccpA*, *ftsA*, *gmk*, *pta*, *pur*, *recF*, *sucC*, and *tpi*) based on previously reported schemes (Priest et al., 2004; Helgason et al.

Table 1 Strains used for the phylogenetic analysis

Strain	Abbreviation	Genbank Accession No.
Bacillus anthracis str. Australia	BaA	NZ_AAES01000045.1
Bacillus anthracis str. A0465 BAM	BaA0465	ABLH01000019.1
Bacillus anthracis str. Vollum	BaV	NZ_AAEP01000034.3
Bacillus cereus NVH0597-99	BcNVH0597	NZ_ABDK02000018.1
Bacillus cereus ATCC 10876	Bc10876	ACLT01000017.1
Bacillus cereus Rock3-29	BcR329	NZ_ACMJ01000017.1
Bacillus mycoides Rock3-17	BmR317	NZ_ACMW01000016.1
Bacillus pseudomycoides DSM 12442	Bp12442	NZ_ACMX01000014.1
Bacillus thuringiensis serovar *huazhongensis* BGSC 4BD1	Bth4BD1	NZ_ACNI01000018.1
Bacillus thuringiensis serovar *monterrey* BGSC 4AJ1	Btm4AJ1	NZ_ACNE01000016.1
Bacillus thuringiensis serovar *pakistani* str. T13001	Btpt13001	NZ_ACNC01000023.1
Bacillus thuringiensis serovar *pulsiensis* BGSC 4CC1	Btp4CC1	NZ_ACNJ01000017.1
Bacillus thuringiensis serovar *sotto* str. T04001	BtsT04001	NZ_ACNB01000029.1
Bacillus thuringiensis serovar *tochigiensis* BGSC 4Y1	Btt4Y1	NZ_ACMY01000020.1
Bacillus thuringiensis strain 407	Bt407	ACMZ01000019.1
Bacillus thuringiensis serovar *andalousiensis* BGSC 4AW1	Bta4AW1	ACNG01000020.1
Bacillus thuringiensis serovar *berliner* ATCC 10792	Btb10792	NZ_ACNF01000019.1
Bacillus thuringiensis serovar *kurstaki* str. T03a001	BtkT03a001	NZ_ACND01000019.1
Bacillus thuringiensis serovar *pondicheriensis* BGSC 4BA1	Btp4BA1	NZ_ACNH01000016.1
Bacillus thuringiensis serovar *thuringiensis* str. T01001	BttT01001	NZ_ACNA01000020.1
Bacillus thuringiensis serovar *israelensis* 4Q1	Bti4Q1	JN637469[a]
Bacillus thuringiensis serovar *israelensis* 4Q7	Bti4Q7	JN637470[a]
Bacillus thuringiensis serovar *thuringiensis* 4A2	Btt4A2	JN637471[a]
Bacillus thuringiensis serovar *thuringiensis* 8741	Bt8741	JN637473[a]
Bacillus thuringiensis serovar *kurstaki* 4D11	Btk4D11	JN637468[a]
Bacillus thuringiensis serovar *kurstaki* 4D22	Btk4D22	JN637472[a]

[a]Sequences of *nprR–nprRB* for these strains were obtained in this study and are not included in MLST tree. Underlined abbreviations are strains with truncation in *nprR* or *nprRB* gene

2004). Sequences were extracted from the draft genome sequences of 20 *Bacillus* strains (Table 1) using an in-house script and BLAST searches. We then constructed a codon-based alignment of the concatenated sequences. This alignment was used to build the phylogenetic tree with the same procedure used for the *nprR* tree. A third tree was built with *nprR* sequences from the 20 strains used in the MLST markers tree to infer if this gene was acquired horizontally. Finally, *nprR* and *nprRB* genes were tested for selection using MEGA5 (Tamura et al. 2011) and start2 (Jolley et al. 2001).

In vivo activity of synthetic signaling peptides in Bt8741

Peptides SKPDT, SAPDT, SKPDI, YSSKPDI, and SKPDIVG were obtained from GenScript (NJ, USA). Peptides SSKPDIV, SSKPDIVG, and YSSKPDIVG were synthesized at CINVESTAV peptide facility according to Lloyd-Williams et al. (1997). The purity of the peptides as estimated from analytical RP-HPLC was ≥95%.

Effect on β-Galactosidase activity

Bt8741 cells were incubated until early exponential growth phase (DO_{600}=0.4). Then, 60 mL of the culture was centrifuged at 5000 × *g*, and the supernatant was discarded. The pellet was diluted in 300 mL of the CDGS medium for sporulation (Nickerson and Bulla 1974) supplemented with 5 μg mL^{-1} of erythromycin. This culture was fractionated in 125-mL flasks containing 25 mL of the culture and either 20 nM or 100 nM of each peptide (DO_{600}=0.08). Control groups with no peptide added were included. Cultures were further incubated in a shaker at 30°C, and β-Galactosidase assays were performed 4 h after peptide addition. At least two independent experiments with four replicates were done for each peptide on different days.

For the β-Galactosidase assay, 400 μL of cell samples were lysed with 45 μL of chloroform and 30 μL of 0.1% SDS, followed by 10 s mixing in a vortex and addition of 600 μL of Z buffer (Sambrook et al. 1989). After treatment, samples were assayed for β-Galactosidase activity using Miller's method (Miller 1992; Sambrook et al. 1989). β-Galactosidase activity was expressed as times-fold increase or decrease in respect to basal conditions.

Effect of putative synthetic peptides on sporulation

Erlenmeyer flasks containing 50 mL of LSM medium (3.0 gL^{-1} KCl, 0.2 gL^{-1} $MgSO_4$ $7H_2O$, 40 mg L^{-1} $MnSO_4$, 30 mg L^{-1} $CoCl_2$ $6H_2O$, 7.5 mg L^{-1} $CuSO_4$ $5H_2O$, 5.8 mg L^{-1} $ZnSO_4$ $7H_2O$, 1.35 mg L^{-1} $FeSO_4$ 7H2O, 12 gL^{-1} glucose, 4 gL^{-1} starch, 5.86 gL^{-1} yeast extract, 9.5 gL^{-1} soybean peptone) and 15 μg mL^{-1} of erythromycin were inoculated with a spore disk of Bt8741 and incubated in a shaker at 30°C and 300 rpm. The growth kinetics was assessed every 5 h during 40 h. When the cells were in the transition growth phase (5 h after incubation), synthetic peptides (SKPDI, SKPDIVG, SSKPDIVG, SKPDT, and SAPDT) were added to a final 100-nM concentration. Bacilli, sporulated bacilli and spores counts were determined microscopically with three replicates in a Neubauer chamber. Sporulation efficiency (E_s) was calculated using Eq. 1:

$$E_s = \frac{A_{sb} + A_s}{A_b} \quad (1)$$

where A_{sb} is the integrated area of sporulated bacilli concentration, from time of detection to the time when the maximal concentration of sporulated bacilli was reached (t_{max}); A_s is the integrated area of free spores concentration, from the time of detection to t_{max}; and A_b is the integrated area of bacillus (in vegetative phase, i.e. without spores), from t_0 to t_{max}. Three independent experiments for each peptide were done in different days, and for each experiment a control without peptide was run. Results of sporulation efficiency are expressed as times-fold in respect to control groups.

Statistics

Statistical analyses were performed using one-way ANOVA followed by Duncan's multiple comparison test to identify differences among groups using the software NCSS 2007 (Hintze, 2006). Statistical probability of $p<0.05$ was considered significant.

Results

Co-evolution of *nprR* and *nprRB* genes

From the *nprR–nprRB* sequences, we found that some strains contained a truncated version of *nprR*, while Bc10876 contained a 7-bp deletion on *nprRB* gene causing a premature stop codon. In the phylogenetic analyses, we included all the strains because nucleotide sequences still gave useful information on the evolution of these genes. The phylogenetic tree based on nucleotide sequence of *nprR* from the 26 strains of the *B. cereus* group (Table 1) showed a perfect correlation with six putative mature NprRB pentapeptide sequences or pherotypes (Fig. 1). In the *nprR* tree, we observed that all *B. anthracis* strains grouped with all *B. thuringiensis* strains serovar *thuringiensis*, *israelensis*, and *pondicheriensis*, being SKPDI the putative mature NprRB sequence. WKPD [T/L] group was formed by one strain

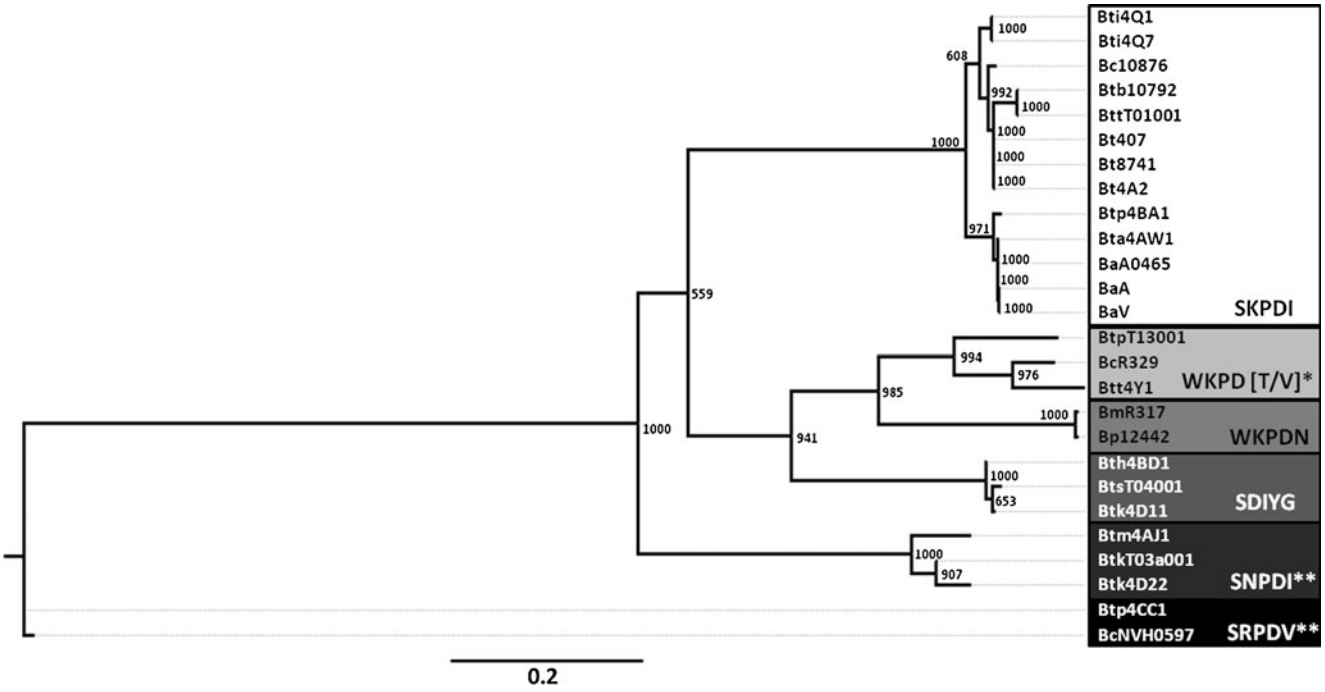

Fig. 1 Maximum likelihood tree based on the *nprR* sequences of 26 strains from the *B. cereus* group (Table 1) under the GTR substitution model. *Numbers* in each node indicate the number of bootstrap replicas that support each group (from a total of 1,000). *Bar* indicates the number of substitutions per site. Strains whose gene is interrupted are *underlined*. One *asterisk* represents sequences of NprRB that are lowly predicted to be exported from the cell. Two *asterisks* represent NprRB sequences that are not predicted to be secreted by the cell

of *B. cereus* and one of *B. thuringiensis* serovar *pakistani*. Pherotype WKPDN included one strain of *B. mycoides* and one of *B. pseudomycoides*. Groups corresponding to mature NprRB with the sequences SDIYG and SNPDI were formed by *B. thuringiensis* strains from the serovar *mycoides*, *kurstaki*, *huazhongensis*, and *sotto*. Finally, SRPDV included two strains from *B. cereus* and *B. thuringiensis* serovar *pulsiensis* (Fig. 1). From all the NprRB sequences analyzed, SignalP 3 analysis showed that those peptides containing the putative mature peptides WKPD [T/V] have low probability of being exported from the cell, whereas those containing the sequences SNPDI and SRPDV do not contain a signal sequence for the Sec dependent export pathway. We also built a tree based on sequences of nine MLST markers from the same strains, excluding the six strains from which we obtained the sequence of *nprR–nprRB* in the laboratory. We observed that NprR–NprRB pherotypes are not consistent with the MLST analysis, as the topology of *nprR* and MLST trees was different (Figs. 1 and 2). This analysis showed that a strain could be closer to another from different species than to a strain from the same species (for example Btm4AJ1 is close to Ba strains and far from BtpT13001, Fig. 1). Finally, selection tests showed that *nprR* and *nprRB* dN/dS ratios were 1.1425 and 1.1288 respectively (positive selection).

Effect of synthetic peptides on β-Galactosidase activity from the *cry1Aa-Z* fusion

In preliminary tests we observed that the effect of the synthetic peptide on β-Galactosidase activity varies according to its final concentration in the media under the assay conditions used (data not shown); thus, we assayed 20 and 100 nM for each peptide. Figure 3 shows that pentapeptides SKPDT, SAPDT, and SKPDI induced the expression of the *cry1Aa-Z* fusion at 20 nM. SKPDT was the peptide that induced a higher level of activity up to 1.28 times-fold relative to the control. SKPDT also had a positive effect on β-Galactosidase activity when added at 100 nM, but the other two peptides did not.

We also assayed heptapeptides encoded in *nprRB* from Bt8741, by adding one or two amino acids to the amino or carboxyl terminus of SKPDI. β-Galactosidase levels decreased when heptapeptides YSSKPDI, SKPDIVG, and SSKPDIV were added at a final 100-nM concentration. SSKPDIV also had a negative effect when added at 20 nM. The strongest effect was observed with the octapeptide SSKPDIVG at 20 nM that reduced β-Galactosidase activity to 65% of the basal conditions. Finally, addition of the nonapeptide YSSKPDIVG did not affect β-Galactosidase activity relative to the control (Fig. 3).

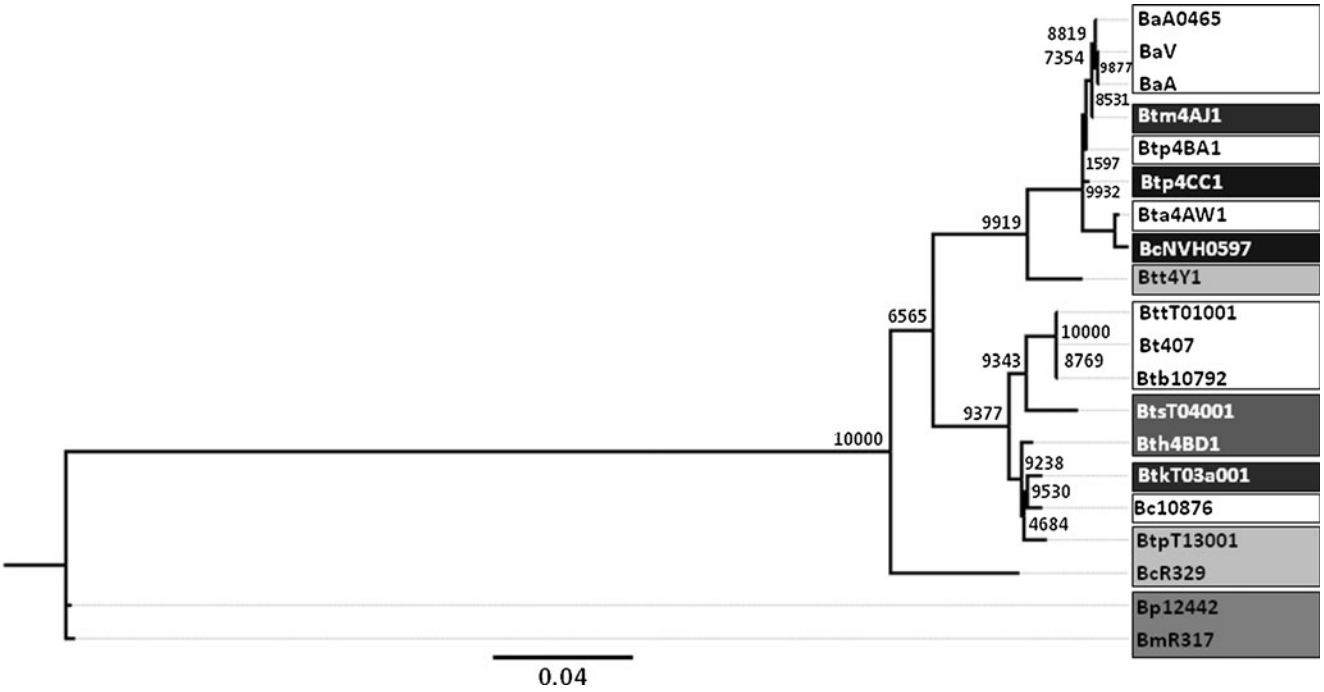

Fig. 2 Maximum likelihood MLST tree of the *B. cereus* group. The tree was constructed from the aligned sequences of nine MLST markers from 20 strains of the *B. cereus* group (Table 1). Phylogenetic reconstruction was carried out under the GTR substitution model. *Numbers* in each node indicate the number of bootstrap replicas that support each group (from a total of 10,000). *Bar* indicates the number of substitutions per site. *Grayscale squares* indicate the putative NprRB pentapeptide from Fig. 1

Effect of putative synthetic peptides on sporulation

We tested the effect on sporulation of three synthetic peptides encoded in the sequence of *nprRB* of Bt8741 and had an effect in cry1Aa'Z transcription. Peptides SKPDI and SKPDIVG decreased 20% the time of sporulated bacilli detection. Also, these two peptides caused an early apparition of free spores (28% and 14%, respectively), while SSKPDIVG diminished 14% the time of free spore detection. In addition, SKPDIVG and SSKPDIVG increased the sporulation efficiency 2.1 and 1.6 times respectively (Table 2). In Fig. 4, we show the bacillus growth and sporulation kinetics of a control group and the SKPDIVG induced group, which had the most noticeable effect on both.

We also assayed the peptide SKPDT, previously proposed as signaling peptide of NprR in *B. thuringiensis* (Pottathil and Lazazzera 2003; Aceves-Diez et al. 2007)

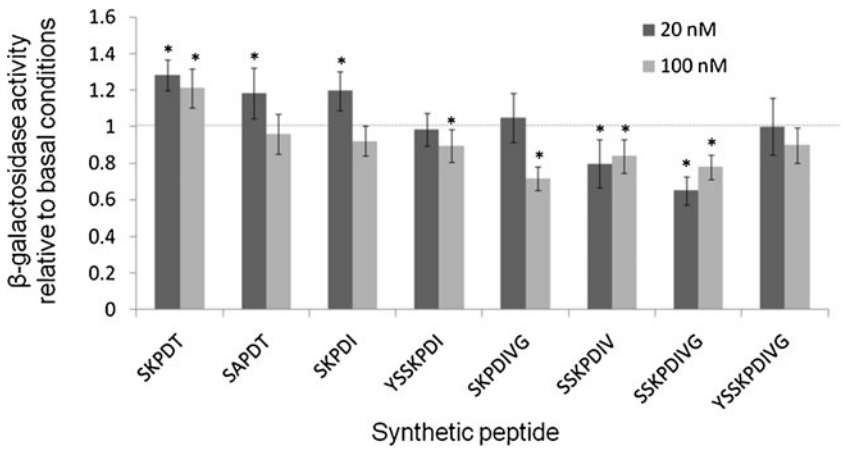

Fig. 3 Effect of synthetic peptides (20 nM and 100 nM) on *cry1Aa-lacz* expression of Bt8741, after 4 h of induction of sporulation. The peptide sequences are contained in internal region of the exported portion of NprRB. Activity is reported as times-fold of control value without synthetic peptides (represented by *dotted line*). *Columns* are means of four replicates, and *vertical bars* are standard deviations. *Asterisks* indicate significant difference ($p \leq 0.05$) between the treatment and the control group

Table 2 Effects of synthetic peptides on sporulation of Bt8741

Synthetic peptide	Time-decrement in sporulated-bacilli detection (%)	Time-decrement in free spores detection (%)	Sporulation efficiency (times-fold of control value)
SKPDI	20	28	1.05
SKPDIVG	20	14	2.061*
SSKPDIVG	0	14	1.623*
SKPDT	0	0	1.211
SAPDT	0	0	0.896

Asterisks (*) denote statistical difference compared to control ($p<0.05$)

and not encoded in Bt8741. We found that SKPDT had no effect on sporulation. Since previous studies in Rap proteins showed that a basic amino acid was essential for a functional signaling peptide, we substituted K for A. SAPDT effect was not significant ($p>0.05$) (Table 2). None of the peptides tested affected the growth of the strain (data no shown).

Discussion

The grouping in the *nprR* tree does not match the current classification of the *B. cereus* group, which is based on phenotypic traits (Didelot et al. 2009; Slamti and Lereclus 2005) (Fig. 1). The correct classification of the *B. cereus* group is an active field of study because these bacteria present many phenotypes and broad pathological effects, some of them concerning human health. Several methods have been used in order to find a correct classification of this group, but the results depend on the method, the strains, and the molecular marker used for each study. For example, multi-locus enzyme electrophoresis and analysis of nine chromosomal genes showed that *B. cereus*, *B. anthracis*, and *B. thuringiensis* are the same species (Helgason et al. 2000). On the other hand, analysis of 16S, 23S, and *gyrB* genes from *B. cereus* group strains indicated that *B. anthracis* can be distinguished from *B. cereus* and *B. thuringiensis* strains (Bavykin et al. 2004). MLST analysis showed that the group is divided in three clades (Didelot et al. 2009; Priest et al. 2004) with heterogeneous species. For this reason, we used the available information on GenBank to build a phylogenetic tree using nine MLST markers, from the same strains used in the *nprR* tree. Although we could not distinguish the three clades mentioned (probably due to the low number of strains), the analysis was definitive for understanding the possible evolution of NprR–NprRB system.

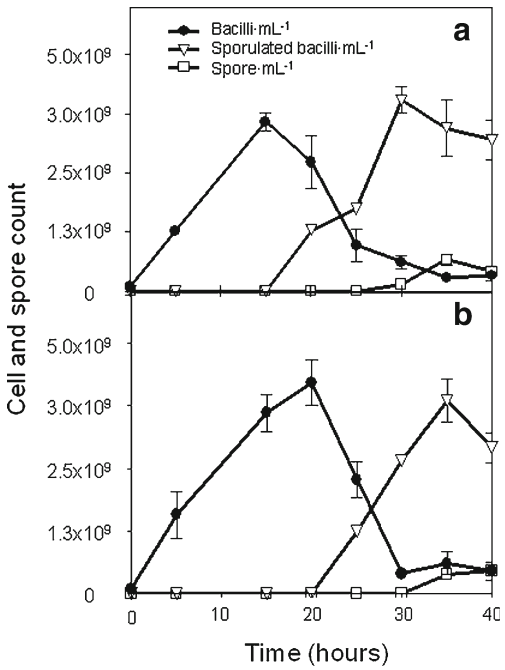

Fig. 4 Effect of peptide SKPDIVG on the growth and sporulation of Bt8741 (**a**). Synthetic SKPDIVG (100 nM) was added to a culture at cells in transition phase (**b**) control culture without synthetic peptide added. *Vertical bars* ± SD

The incongruence between both trees is probably caused by efficient horizontal transfer of one or both elements of the *nprR–nprRB* cassette accompanied by positive selection, as shown by the dN/dS value and neutrality tests. A similar phenomenon was suggested in a study of the pherotypes of PlcR–PapR in the *B. cereus* group (Slamti and Lereclus 2005). It was proposed that they co-evolved by positive selection. The fact that *nprR* and *nprRB* genes have co-evolved in this group of bacteria suggests that they have a coordinated role.

Regardless that NprR is not a molecular marker for evolution, it is worthy to note that the *nprR* tree/NprRB analysis provides significant biological information, as it constitutes a system that controls functions like extracellular enzyme productions, and as we propose in this study, it plays a role in sporulation and *cry1Aa* transcription in Bt8741. Of high relevance is the fact that some *B. cereus* and *B. thuringiensis* strains share the same signaling peptide (SKPDI) with the strains of *B. anthracis* included in this study. As virulence of *B. anthracis* is highly related to germination receptors functional at the time of infection (Carr et al. 2010) and sporulation is an essential step for *B. anthracis* persistence and infection ability (White et al. 2006), NprR system is now a candidate regulator of sporulation/virulence-related processes activation in this species. Additionally, this system could even permit cross talk among strains of different species.

We tested the effect of some synthetic peptides on Bt8741 cultures, which belongs to the SKPDI pherotype. From the full exported sequence expected for NprRB of

Bt8741 strain (IHQYSSKPDIVGQQAKTVEQVNL), the pentapeptide SKPDI was the first candidate because it has a positive-charged amino acid in the second position (Pottathil and Lazazzera 2003). However, as in the case of PlcR, its signaling peptide could have a different length (Bouillaut et al. 2008). For this reason, we designed a series of peptides of 5, 7, 8, and 9 amino acids to test in these experiments. Most of these peptides are encoded in *nprRB* gene, and although SKPDT and SAPDT are not encoded, the first was previously described by Pottathil and Lazazzera (2003) as the signaling QS peptide of NprR receptor in *B. thuringiensis*, and previous reports indicate that it has an effect on sporulation and *cry1Aa* expression (Aceves-Diez et al. 2007). Peptide SAPDT was tested because the signaling pentapeptides commonly have a positively charged amino acid at the second position (Pottathil and Lazazzera 2003), and therefore this peptide was not expected to be functional. Peptides SKPDI, SKPDT, and SAPDT at 20 nM had a positive effect on β-Galactosidase activity; thus, a positively charged amino acid in the second position from N-terminus is not essential in the mature NprRB sequence. Since the signaling pentapeptides of the four groups of PlcR have proline, a non-polar amino acid in the second position (Slamti and Lereclus 2005) and signaling heptapetides of PrgX have either valine or isoleucine (Kozlowicz et al. 2006), a basic amino acid in the second position from N-terminus is not a general rule for signaling peptides of the QS receptors of the RNPP family. Further, our data suggest that in the system NprR–NprRB, cross talk among bacteria belonging to different pherotypes and species is possible in the *B. cereus* group, just like in PlcR–PapR (Bouillaut et al. 2008). Future experiments are needed to clear this specific issue.

Although the biologically relevant sequence of the mature NprRB peptide is unknown, we observed that peptides of five, seven, and eight amino acids have an effect on *cry1aA'lacZ* expression, suggesting a degree of flexibility in specificity of peptide-receptor binding. In fact, synthetic peptides of five, seven, and nine amino acids encoded in *papR* were able to restore *plcA'lacZ* expression in *B. thuringiensis* 407 cry$^-$Δ*papR* (Slamti and Lereclus 2002). Therefore, certain flexibility may be common to the RNPP-family receptors.

Our results show that pentapeptides increased *cry1Ac'lacZ* expression, while longer peptides decreased it. It may be that pentapeptides are the true effectors, and longer peptides act as competitive inhibitors. Recently, it was reported that in a Δ*nprR* mutant of *B. thuringiensis* HD73, the expression of *cry1Ac'Z* was increased compared to the wild type strain (Wang et al. 2010). Therefore, it may be that pentapeptides inhibited NprR and longer peptides restored its function. Negative or positive effect on PrgX receptor, a member of the RNPP family, is induced depending on the peptide attached in the receptor peptide binding site. When this protein binds cCF10 (LVTLVFV), the complex receptor–peptide serves as a specific inducer of the pCF10 conjugation genes, but when it binds iCF10 (AITLIFI) the expression of these genes is repressed (Kozlowicz et al. 2006).

To date there are no reports about interaction between NprR and mature NprRB, and the mechanism by which the synthetic peptides affect β-Galactosidase activity of the Bt8741 strain carrying a *cry1Aa-lacZ* fusion is unknown. We do not exclude the possibility that more than one variant of the signaling peptide could be produced in nature, acting either in a synergistic or competitive manner, since SKPDI is an internal sequence of the exported peptide encoded in *nprRB*.

Sporulation was also affected by the addition of peptides. Initiation of sporulation is a well-studied process in *B. subtilis*. In this bacteria, multiple factors like some Rap proteins, Spo0A transcription factor and a series of RNA polymerase σ factors, among others, add up to regulate this differentiation process (Piggot and Hilbert 2004). However, full characterization of initiation of sporulation is yet to be studied in the *B. cereus* group. In this work we showed that putative mature NprRB peptides encoded in the *nprRB* sequence of Bt8741 (SKPDI, SKPDIVG, and SSKPDIVG) induced earlier detection of sporulated bacilli and increased sporulation efficiency, while SKPDT and SAPDT had no effect. Thus, NprR may regulate sporulation. Recently, a strain of *B. anthracis* containing specific mutations in *nprR* gene was unable to sporulate in rich medium; authors suggested that this protein is an early sensor for sporulation (Yang et al. 2011).

Those peptides that have an effect on sporulation in Bt8741 decreased β-Galactosidase activity from the fusion *cry1Aa-lacZ*, and those that stimulate this activity did not diminish the time when sporulated bacilli or free spores occur. Further studies are needed to elucidate if some genes are activated and others inactivated by NprR.

From the widely studied receptors that belong to the RNPP family, the mechanisms of activation and the functions of NprR as a quorum sensor in the *B. cereus* group remain elusive. Early reports showed that it is an activator of the neutral protease in *B. subtilis* and *B. stearothermophilus* and that it is related to sporulation and Cry toxins in strains from the *B. cereus* group; however, these works did not consider the role of the signaling peptide NprRB. Our results indicate that the genes encoding NprR and NprRB co-evolved in the *B. cereus* group, thus suggesting a coordinated function. In concordance with their possible coordinated role, addition of synthetic peptides affected *cry1Aa'z* and sporulation in Bt8741. We suggest that synthetic peptides added in cultures can interact with NprR in the intracellular space, and the effects observed are a consequence of gene regulation by NprR. The reason for some peptides having positive effect on sporulation but negative effect on *cry1Aa'Z* is not known. Further studies are needed in order

to asses if some peptides may activate and other inactivate NprR, or if NprR can act as an activator and repressor of genes, simultaneously.

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CAPÍTULO III

The bi-functional quorum sensing receptor NprR from *B. thuringiensis* regulates sporulation through a DNA-binding-independent mechanism

Jorge Rocha, Víctor Flores, Mayra de la Torre,
Gabriel Guarneros & Gabriela Olmedo

(manuscrito)

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RESUMEN

NprR pertenece a la familia RNPP de receptores de *quorum sensing*, un grupo de proteínas intracelulares activadas directamente por oligopéptidos de señalización. En *Bacillus thuringiensis* (Bt), *nprR* se encuentra en un caset transcripcional con *nprRB*, que codifica para el precursor del péptido de señalización NprRB. En respuesta a la unión con el péptido NprRB procesado y reimportado, NprR activa la transcripción de la proteasa neutra *nprA*. Existen evidencias que indican que NprR participa en el proceso de esporulación, sin embargo no se conoce el mecanismo. Los análisis *in silico* realizados en este trabajo indican que NprR de Bt tiene similitud estructural con RapH de *Bacillus subtilis*, y sugieren que NprR podría unirse a Spo0F. Encontramos que en la cepa Bt8741, la eliminación del caset *nprR-nprRB* causó una disminución en la actividad de proteasa extracelular y un retraso en la esporulación probablemente relacionado con una alteración en la fosforilación de Spo0A. En la cepa mutante, la expresión de NprR(ΔHTH) carente del dominio de unión al ADN, mostró que este dominio es necesario para la restauración de las proteasas extracelulares pero no para la restauración de la esporulación. Por otra parte, encontramos que la presencia del gen *nprRB* es crucial para ambas funciones. Nuestros resultados muestran que NprR es una proteína bifuncional que además de ser un activador transcripcional, regula positivamente a la esporulación, utilizando un mecanismo independiente de su capacidad de unir ADN, probablemente a través del *phosphorelay* en respuesta al péptido de señalización NprRB.

Title:

The bi-functional quorum sensing receptor NprR from *Bacillus thuringiensis* regulates sporulation through a DNA-binding-independent mechanism

Running title: DNA-binding-independent function of NprR in Bt.

Authors:

Jorge Rocha[1], Víctor Flores[2], Mayra de la Torre[1], Gabriel Guarneros[2], Gabriela Olmedo[3]#

Affiliation**:**

[1] Centro de Investigación en Alimentación y Desarrollo A. C. Km 0.6 Carretera a La Victoria 83304, Hermosillo, Sonora, México.

[2] Departamento de Genética y Biología Molecular. Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. Av. Instituto Politécnico Nacional 2508 Col. San Pedro Zacatenco 07000 México, D.F., México.

[3] Departamento de Ingeniería Genética. Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Irapuato. Km. 9.6 Libramiento Norte Carretera Irapuato-León 36821 Irapuato Gto., México.

#Corresponding author

Email: golmedo@ira.cinvestav.mx

Abstract

NprR belongs to the RNPP family of quorum sensing receptors, a group of intracellular proteins activated directly by signaling oligopeptides. In *Bacillus thuringiensis* (Bt), *nprR* is found in a transcriptional cassette with *nprRB*, that codes for the precursor of the signaling peptide NprRB. After binding with the re-imported processed peptide NprRB, NprR activates the transcription of the neutral protease *nprA*. There is growing evidence that NprR participates in the sporulation process, but the mechanism is unknown. *In silico* analyses performed in this work show that NprR from Bt has structural similarity to RapH from *Bacillus subtilis*, and suggest that NprR could also bind Spo0F. We found that in strain Bt8741, deletion of *nprR-nprRB* cassette caused a decrease in extracellular protease activity and a delay in sporulation probably due to defective Spo0A phosphorylation. In the mutant, expression of NprR(ΔHTH) lacking the DNA-binding domain showed that this domain is necessary for restoration of extracellular protease but not for restoration of sporulation. Moreover, the presence of *nprRB* gene is crucial for both functions. Our results show that NprR is a bifunctional protein because besides being a transcriptional activator, it positively regulates sporulation using a mechanism independent of its DNA-binding capacity, probably through the phosphorelay in response to the NprRB peptide.

Introduction

Bacterial regulation of gene expression in response to cell density is known as quorum sensing (QS). Bacteria use several mechanisms in order to sense cell density and perform coordinated functions. Diffusible molecules produced by the bacteria accumulate in the environment and when they reach a threshold concentration, serve as signals for cognate membrane-bound or intracellular receptor proteins. Bioluminescence, virulence, biofilm development, conjugation, and sporulation are bacterial processes regulated by quorum sensing (1-4).

Bacillus thuringiensis (Bt), a member of the *Bacillus cereus* group, is widely used in crops as an alternative to chemical insecticides, because it produces parasporal inclusions (Cry proteins) with entomotoxic activity (5). The transcription of some Cry proteins is directed by sporulation-specific sigma factors (6). The sporulation process has been studied using *Bacillus subtilis* as model (7-8). In *B. subtilis*, membrane-bound kinases respond to multiple endogenous and exogenous inputs, activating the phosphorelay associated to initiation of sporulation (9). This phosphorelay consists in the sequential phosphorylation of proteins Spo0F→Spo0B→Spo0A. Spo0A~P regulates over 100 genes related to the cell differentiation process (10). Several regulatory mechanisms, including QS, act on the phosphorelay in order to ensure that cells commit to sporulation only under the right conditions (11). However, some genes that are important for regulation of sporulation initiation in *B. subtilis* are not conserved in the *B. cereus* group bacteria; for example eleven Rap phosphatases are coded in the *B. subtilis* genome while only six are found in *Bacillus anthracis*, and eight in Bt (12);

consequently, sporulation in Bt is not well understood and most related mechanisms are only inferred to occur in a similar fashion to that in *B. subtilis*.

The RNPP family of QS receptors was described in Gram-positive bacteria (13). The members of this family (Rap, NprR, PlcR, and PrgX) are intracellular proteins activated directly by cognate signaling peptides. (14). Typically, RNPP receptors are coded in operons together with a small ORF that codes for the specific signaling pro-peptide. The structure of the four receptors is conserved, and they share tetratricopeptide repeats (TPRs) essential for protein-protein and protein-peptide interactions (15). PlcR and PrgX are helix-turn-helix (HTH)-domain-containing transcriptional regulators that bind DNA to activate virulence and conjugation, respectively (16-17). On the other hand, Rap proteins lack the HTH-domain and bind response regulators. They regulate the initiation of sporulation by binding to Spo0F and preventing the phosphate flux in the phosphorelay (9, 18). At high cell density, the Phr signaling peptides bind to Rap proteins, preventing their binding to Spo0F and releasing their inhibitory effect on the phosphorelay (19). Other response regulators inhibited by Rap proteins are the transcriptional activators ComA and DegU (20). Some Rap proteins bind more than one response regulator, such is the case of RapH that binds and inhibits ComA activity allosterically but also binds and dephosphorylates Spo0F, thereby regulating competence and sporulation in *B. subtilis* (21). NprR contains the HTH domain and is a transcriptional regulator that in response to activation by its signaling peptide, the mature NprRB (also known as NprX), activates transcription of the extracellular neutral protease NprA in the *B. cereus* group (22). The NprR-NprRB system is necessary for the necrotrophic lifestyle of Bt when it infects insects, and in this model, 41 genes (the NprR

87 regulon) are differentially expressed in a Δ*nprR-nprRB* mutant (23). Additionally, some
88 reports show that NprR is involved in the sporulation process of bacteria from the *B.*
89 *cereus* group. Yang et al. (24) described that papillae colonies of *B. anthracis* with
90 mutations in the *nprR* gene had decreased sporulation; and we found that addition to Bt
91 cultures of hepta- and octa-peptides derived from the amino acid sequence of the
92 exported region of the *nprRB* gene product, increased sporulation efficiency, caused
93 early detection of spores, and decreased expression of a *cry1Aa'lacZ* fusion (25). How
94 the NprR-NprRB system regulates both sporulation and the NprR regulon? NprR is
95 thought to represent an evolutionary step between Rap and the other members of the
96 RNPP group (22, 26). Other authors have noted a resemblance of NprR structure to that
97 of *B. subtilis* RapF bound to ComA response regulator (27). Thus, it is feasible that
98 NprR could participate in the phosphorelay to regulate the initiation of sporulation,
99 similar to Rap proteins.

100 In this work, we aimed to evaluate NprR functions related to sporulation and the
101 phosphorelay, which should be independent of its DNA-binding capacity. We proposed
102 by *in silico* modeling that NprR could bind to Spo0F. We assayed the production of
103 extracellular proteases and sporulation phenotypes in a BtΔ*nprR-nprRB* mutant.
104 Expression of *spoIIA* and *spoIIG* at the mRNA level was used to evaluate the Spo0A
105 phosphorylation state. Finally, we expressed in the mutant, genes coding for NprR or a
106 variant lacking the HTH domain (ΔHTH) for complementation studies. Our results
107 suggest that NprR is indeed a bifunctional protein since besides activating transcription
108 of *nprA*, positively regulates sporulation probably in response to its peptide NprRB;

however, only the transcriptional function requires the DNA-binding domain of NprR. A model for sporulation regulation by NprR is proposed.

Materials and Methods

Strains, culture media, and growth conditions

We used *Bacillus thuringiensis* serovar. *thuringiensis*, strain 8741 (25). Plasmid pHTCryIA2 (28), carried by this strain was eliminated by growth in nutrient agar (Difco, Franklin Lakes, NJ, US) at 42°C for 48 h. We used *Escherichia coli* strain Top10 and *E. coli* strain MC1061 for sub-cloning plasmid constructions. For cultures of Bt8741 and its variants, we used nutrient broth (Difco) at 30°C and agitation at 125 rpm (if not otherwise specified). When needed, erythromycin (5 $\mu g \cdot ml^{-1}$) or spectinomycin (250 $\mu g \cdot ml^{-1}$) was added. For protease activity assays, milk agar plates were prepared with nutrient agar (23 $g \cdot l^{-1}$) and 5% of skim milk powder. SSM agar was prepared as described earlier (29). BHI (Fluka, Buchs SG, CH) was used in the preparation of competent Bt cells. For *E. coli* strains, we used LB agar or LB broth at 37°C and agitation at 125 rpm. When needed, ampicillin (100 $\mu g \cdot ml^{-1}$) was added.

Competent cells and transformation

Electro-competent Bt8741 cells were prepared by growing in BHI to OD_{600} of 0.9. Then, cells were chilled on ice and washed 3 times with chilled milliQ water. The cell pellet was resuspended in chilled 40% PEG 600 (Fluka) and 400 µl aliquots were prepared.

Electro-competent Bt8741 cells were immediately electroporated with a single pulse of 2.3 kV, 475 Ω, and 25 μF (30) in an ECM 630 Electro Cell Manipulator (Harvard Apparatus, Holliston, MA, US). Cells were recovered in BHI for 3 h and then selected in BHI agar plates with the required antibiotic. Electro-competent Top10 cells were prepared by growing in LB to OD_{600} of 0.8 and subsequent washings (x3) with chilled 15% glycerol. Cells were resuspended in 15% glycerol and 50 μl aliquots were prepared. Aliquots were frozen in liquid nitrogen and stored at -80°C until use. Electro-competent Top10 cells were electroporated at 2.5 kV, 200 Ω, and 25 μF. Chemically competent MC1061 cells were prepared by growing for 4 h, washing with cold 0.1 M $CaCl_2$ and re-suspending in cold 0.1 M $CaCl_2$/15% glycerol. 300 μl aliquots were frozen in liquid nitrogen and stored at -80°C until use. Competent MC1061 cells were transformed applying a treatment of 5 min in ice, 45 s at 42°C, and 5 min in ice. Transformed *E. coli* cells were recovered in LB medium and selected on LB agar with ampicillin.

Plasmids and DNA manipulations

We used plasmid pMAD through the study (31) and pIC333 for obtaining the spectinomycin resistance cassette gene (*spcR*) (32). Plasmids were extracted by alkaline lysis using the method described by Birnboim and Doly (33) and purified using the Plasmid Midi Kit (Qiagen, Valencia, CA, US). DNA fragments were amplified by PCR using the Dream Taq PCR Master Mix 2x (Thermo Scientific, Waltham, MA, US). Restriction enzymes *Bam*HI (Sigma, St. Louis, MO, US), *Sal*I, *Bgl*II, and *Eco*RI (Thermo Scientific) were used for DNA constructions. PCR products and restriction

reactions were purified using the Purelink Quick PCR Purification Kit (Invitrogen, Carlsbad, CA, US). For DNA ligations we used T4 DNA ligase (Thermo Scientific).

In silico analyses

In order to obtain structural information we inferred the possible three dimensional structure of NprR from its sequence using the I-TASSER server (34). Next, the obtained models were used to find homologues in the PDB structure database using TM-align (35). Structure visualization and analysis were performed using PyMOL v1.6 (The PyMOL Molecular Graphics System, Version 1.6.0.0 Schrödinger, LLC). Identification of the possible functional residues in NprR was carried out by examination of the three dimensional structures and the sequence alignments. Comparison of the sequences of different Spo0F binding Raps and NprR was performed using JalView v2.8

Sequences corresponding to the 11 Raps from *B. subtilis* were used to search for homologues in the genome of Bt407, which is phylogenetically close to Bt8741. Similarly, the sequence of NprR from Bt407 was used to search for homologues in the genome of *B. subtilis*168 (Bs168). Sequence alignments were constructed using Muscle v.3.8.31(36) and visualized using JalView v2.8 (37). Phylogeny was constructed by Maximum Likelihood under the JTT+G+F model. Tree topology was refined using nearest neighbor interchange and subtree pruning and rebranching. Associations were statistically validated by approximate likelihood ratio tests and 1000 bootstrap replicates.

Deletion of *nprR-nprRB* cassette from Bt8741

Genes *nprR-nprRB* were deleted from Bt8741 strain and replaced with a spectinomycin resistance gene (*spcR*). We used the plasmid pMAD and inserted a construction consisting of the *spcR* gene, flanked by regions upstream and downstream from the *nprR-nprRB* genes. A 658 bp DNA fragment (extending from positions -793 to -135 with respect to the *nprR* translation initiation codon) was PCR amplified (Fragment R5') using primers R5F and R (Table 1). R5' was inserted between the *Bam*HI and *Sal*I sites of pMAD to generate pMAD-R5'. The *spcR* gene was amplified from plasmid pIC333 using primers SpcF and R primers (Table 1) (31). The 1.3 kb fragment was inserted between the *Sal*I and *Bgl*II sites of pMAD-R5'. Finally, we amplified a 798 pb region (R3') extending from positions 13 to 811 from the stop codon of *nprRB* gene using R3F and R primers (Table 1). R3' was inserted in the *Bgl*II site of pMAD-R5-*spcR*. In each step, the plasmid was transformed into *E. coli* TOP10 cells. The final construction was transformed into *E. coli* MC1061 cells. Plasmid pMAD with the construction R5'-*spcR*-R3' was transformed in Bt8741. Several transformant colonies were PCR-checked for the presence of pΔ*nprR-nprRB*. A transformant was chosen and three growth-cycles were carried out to stationary phase with subsequent dilution in fresh LB medium at 37°C. Dilutions of the last culture were plated on LB agar with spectinomycin and multiple individual colonies were checked for erythromycin sensitive phenotype that indicates the loss of the pMAD plasmid after the gene replacement. We confirmed by PCR that two erythromycin sensitive colonies had the *nprR-nprRB::spcR* gene replacement. One colony of the Bt8741 mutant strain (BtΔRB) was grown in SSM

medium with spectinomycin to stationary phase and was then stored at -80°C in 15% glycerol.

Genetic constructions for complementation experiments

Plasmid pMAD was used to carry the *nprR-nprRB* genes and its variants into BtΔRB strain. The *nprR-nprRB* cassette was amplified with primers cNprR-F and cNprRB-R (Table 1). The resulting DNA fragment was inserted between the *Eco*RI and *Bam*HI sites of pMAD to obtain pR-B. Gene *nprR* was also amplified alone using cNprR-F and cNprR-R primers (Table 1). The fragment was inserted into pMAD to obtain the pR plasmid. We generated a truncated version of *nprR* in which 37 amino acids (from V12 to E48) in the HTH domain were deleted, denominated *nprR(ΔHTH*). To construct this mutation we amplified the 5' region that included the *nprR* promoter and the 5' end of the *nprR* ORF, using primers cNprR-F and V12-R (Table 1). This fragment was inserted between *Eco*RI and *Sal*I sites of pMAD to generate p5'NprR(ΔHTH). Then, two versions of the 3' end of the construction were amplified, including or excluding the *nprRB* gene, using E48-F as forward primer and cNprRB-R/cNprR-R as reverse primers. Each 3' fragment was inserted into p5'NprR(ΔHTH) to generate pRΔHTH-B, containing *nprR(ΔHTH*)-*nprRB* and pRΔHTH containing *nprR(ΔHTH*) alone. Sequences from all the constructions were obtained. Plasmids pR-B, pR, pRΔHTH-B and pRΔHTH were transformed into the BtΔRB strain to generate BtΔRB[R-B], BtΔRB[R], BtΔRB[RΔHTH-B] and BtΔRB[RΔHTH] strains (Table 2), respectively. The forward primer at the 5' end of the constructions, cNprR-F, which contains the *Eco*RI restriction

site (Table 1) was located at the position -498 from the translation initiation codon of *nprR*; however, an *Eco*RI site located in the -450 position was cut and ligated in the corresponding site of pMAD, representing the start of all constructions. Strains Bt8741 and BtΔRB were transformed with plasmid pMAD without an insertion. These two variants were used as controls in complementation experiments (Table 2). All transformed strains were stored at -80°C in 15% glycerol.

Protease activity

Frozen aliquots of strains, Bt8741, BtΔRB, BtΔRB[R-B], BtΔRB[R], BtΔRB[RΔHTH-B], and BtΔRB[RΔHTH] (Table 2) were thawed on ice and used to inoculate 30 ml of nutrient broth with erythromycin. Cultures were grown for 8 h; next, cultures were streaked on a milk agar plates with erythromycin and grown for 48 h. Protease activity was observed as a clear area around the streaks.

Growth and Sporulation

Strains of Bt8741 and variants were inoculated in triplicate in flasks containing 30 ml of SSM broth with erythromycin. Growth was monitored each hour in a Klett-Summerson photoelectric colorimeter with red filter until the transition phase, defined as the end of exponential growth (t_0 of sporulation). The transition phase for all the strains was detected constantly at 80-90 Klett Units throughout the experiments. For sporulation of Bt8741 and BtΔRB, 300 µl samples of cultures were taken at 6, 9, 12, 16, 24, and 48 h

238 after t_0. For complementation assays we measured spores at 10 h after t_0. For spore
239 quantification samples were heated at 80°C for 10 min and thermo-resistant CFU were
240 quantified by pour plate using 100 µl of serial dilutions. Sporulation counts were
241 expressed as thermo-resistant CFU; complementation of sporulation was expressed as
242 thermo-resistant CFU in reference to Bt8741.

243

244 RNA samples

245 Cultures for RNA extraction were the same as that of sporulation experiments. When the
246 transition phase in SSM broth was detected (t_0), 10 ml of culture were sampled from
247 each strain (duplicate flasks). Two hours after t_0, a second sample was taken (t_2).
248 Samples were immediately chilled on ice for 10 min and then centrifuged at 3000 x *g* for
249 10 min at 4°C. The supernatant was discarded carefully. The cell pellet was frozen in
250 liquid nitrogen and then stored at -30°C until use. For RNA extraction, samples were
251 thawed in ice. We used the GeneJET RNA purification Kit (Thermo Scientific),
252 following the instructions provided for Gram positive bacteria. RNA was quantified by
253 spectrophotometry (Nanodrop ND-100) and integrity confirmed in 1% agarose gels
254 containing 200 mM of formaldehyde. Samples were treated with DNAse I (Invitrogen)
255 and absence of genomic DNA was confirmed by PCR, using primers gatB_YqeyF and R
256 (Table 1).

257

258

259 Quantitative PCR

260 Quantification of *spoIIA* and *spoIIG* mRNA was performed by Real-time qRT-PCR
261 using the One-step Brilliant II SYBRGREEN QRT-PCR Master Mix kit (Stratagene, La
262 Jolla, CA, US) in a Step One system (Applied Biosystems, Foster City, CA, US).
263 Primers for *spoIIA* (spoIIA-F and R) and *spoIIG* (spoIIG-R and F) (Table 1) amplifying
264 regions of 189 and 251 bp respectively, were designed from the Bt strain 407 genome
265 (38). Primers gatB-Yqey-F and R (Table 1) amplify a 175 bp fragment of the gene
266 coding the gatB/Yqey-protein; this gene was evaluated by Reiter et al. (39) as
267 normalization gene for expression experiments in *B. cereus* group bacteria, when large
268 expression differences are quantified . Duplicate mRNA samples from t_0 and t_2 were
269 used for each strain. For the coupled RT-PCR reaction, the program was the following:
270 50°C for 30 min and 95° for 10 min, then 40 cycles of 95°C for 10 s, and 57°C for 30 s.
271 The melting curve was performed from 95°C for 15 s, 60°C for 15 s, and 3°C increases
272 each 10 s until 95°C. Threshold cycle values were obtained and relative expression at t_2
273 was obtained for *spoIIA* and *spoIIG* using the double ΔCT method (40) normalizing
274 against the reference gene and the t_0 value.

275

276 **Results**

277 NprR structure resembles the *B. subtilis* RapH regulator

278 After predicting the possible structure of NprR, we obtained five models from the I-
279 TASSER server. We selected the first model since it had the highest C-score (-1.79 in a
280 range from -5 to +2). Predictably, the I-TASSER server detected structural similarity

between the first model and the crystal structure of *Bacillus subtilis* RapH (PDB accession number: 3Q15). The published crystal structure of NprR was excluded from this study, since it is in association with a putative NprRB peptide, which induces major conformational changes (PDB accession number 4GPK) (26); moreover, the crystal structure corresponds to a truncated version of NprR lacking the HTH domain. However, it was included in the structure based analysis of NprR.

Superimposition of the predicted model of NprR and the crystal structures of RapH (with amino acid identity of 19.63%) in PyMOL resulted in a root mean squared deviation (RMSD) of 0.578, which is indicative of a highly conserved structure between the two proteins. On the other hand, comparison between modeled NprR and crystallized NprR (PDB accession number 4GPK), did not result in significant similarity.

Multiple sequence alignment, indicated that most of the residues responsible for RapH binding and dephosphorylation of Spo0F (41) are conserved or semi-conserved in NprR: Asp-46, Leu-50, and Leu-55 from RapH match Asp-114, Ile-118, and Leu-123 from NprR (Fig. 1a), suggesting that NprR could be able to bind Spo0F. Moreover, the Spo0F binding residues in RapH form a protruding loop which is conserved in both the predicted and the crystal structure of NprR (Fig. 1b). Moreover, similarity between NprR and Spo0F-binding Raps at binding residues is 85% (6 out of 7), meanwhile similarity of whole sequences ranges in 40-45% (Figure 1a). Two residues in RapH are required for Spo0F dephosphorylation: a catalytic glutamine at position 47, and a Mg^{2+} binding tyrosine at position 175. Whereas the catalytic glutamine is missing in NprR, the tyrosine is conserved and located in the surface according to our model (Tyr 230, Fig.

1b); however, Tyr230 is buried far from the binding residues in the tetrameric crystal structure (Fig. 1c) suggesting that NprR could not be able to dephosphorylate Spo0F.

As no studies have been published regarding Rap proteins in Bt, we built a phylogenetic tree for assessing similarity among Rap proteins from Bs168 Bt, NprR from Bt8741, and putative Rap proteins from Bt407. Rap proteins are approximately 380 amino acids long, and consist mainly of 9 tandem TPRs, NprR protein also possesses an N-terminal HTH domain. As shown in Figure 2, we found 9 rap-like proteins in Bt407. In the phylogenetic tree, Spo0F-binding Raps from Bs168 are grouped together, as well as Bt407 Raps. The Non-Spo0F-binding Raps from Bs168 are distant from Spo0F-binding Raps. Finally, it is worth to notice that NprR is closer to Bs168 Raps than Bt407 Raps.

The BtΔRB mutant exhibits a delay in sporulation and defective trigger of expression of sporulation stage II genes

We obtained an *nprR-nprRB* mutant strain from Bt8741 through gene replacement with a spectinomycin resistance gene (*spcR*). Extracellular protease activity, growth, and sporulation of BtΔRB mutant and the parental strain Bt8741 were evaluated. A decrease in extracellular protease activity was observed as a smaller activity halo around BtΔRB colonies, compared to Bt8741 colonies (Fig. 3a). Growth kinetics were not affected (Fig. 3b) but sporulation was decreased in the BtΔRB mutant at times 9, 12, and 16 h after the transition phase, with respect to Bt8741 (Fig. 3c). After 24 and 48h, however, the spore-count of both strains was the same, indicating that the effect on sporulation was a delay probably at the initiation of the process (Fig. 3c).

To test if the sporulation delay in the mutant was caused by an effect in the phosphorelay, we quantified *spoIIA* and *spoIIG* mRNA, which serve as indicators of Spo0A phosphorylation (42-44). In the wild type strain Bt8741 the expression increase of *spoIIA* and *spoIIG* genes from t_0 to t2 was 280- and 422-fold, respectively. On the other hand, in the mutant, the expression of *spoIIA* and *spoIIG* genes from t_0 to t_2 increased only 21- and 3-fold, respectively (Fig. 4).

Complementation of extracellular protease activity in the BtΔRB mutant requires the intact *nprR* gene, but sporulation can be restored with an NprR variant lacking the HTH domain

We evaluated the ability of the different constructions to complement extracellular protease activity and sporulation (Table 2). As shown in Figure 5, only the plasmid carrying both wild type *nprR* and *nprRB* (strain BtΔRB[R-B]) was able to complement the extracellular protease activity. Extracellular protease activity of strains BtΔRB[R], BtΔRB[RΔHTH-B], and BtΔRB[RΔHTH] was similar to that of the BtΔRB mutant, indicating that presence of *nprRB* gene as well as the HTH domain in *nprR* were necessary for this function.

In figure 6, we show the results regarding sporulation of strains carrying gene constructions. Spore-counts were made 10 hours after transition phase in each strain and were expressed as mutant/Bt8741 ratio. Sporulation level of BtΔRB[R-B] was similar to that of Bt8741, indicating that the function was restored by the expression of both genes. The spore count of BtΔRB[R] and BtΔRB[RΔHTH] was similar to that of the BtΔRB

mutant, indicating that presence of the *nprRB* gene was essential for this function. Finally, strain BtΔRB[RΔHTH-B] exhibited a spore-count similar to that of BtΔRB[R-B]. This result indicated that the HTH domain was not necessary for the complementation of the sporulation phenotype by NprR.

Discussion

Several works have assessed the functions of NprR as a transcriptional regulator that responds to QS (22-23, 25). However, evidences show that NprR also has a role in sporulation and sporulation-related processes of bacteria from the *B. cereus* group (23-25, 45) but the mechanism by which it participates in sporulation has not been uncovered. Although an early work suggested that NprR was a Rap-like protein in Bt (46), only recently has it been noticed that the structure of NprR from Bt resembles the structure of some Rap proteins from *B. subtilis* (22, 27).

The hypothesis of a Rap-like function of NprR was first supported by the *in silico* structure modeling performed in this work (Fig. 1). Despite the low identity in amino acid sequence between NprR and Rap proteins (19.63% of identity), we found high structural similarity between the NprR model and the RapH crystal structure (Fig. 1b); of high relevance is the fact that Spo0F-binding residues from RapH are conserved in NprR (Fig 1a), which is a common feature of protein-protein interacting residues in signal transduction systems (47). Thus, according to our structure model, NprR could be able to bind to Spo0F in a similar region than RapH. Also, in the tetrameric, peptide-bound crystal structure of NprR (26), most of these residues are exposed (Fig 1c) and

not involved in formation of NprR tetramer or NprRB peptide binding, suggesting that NprR in this conformation could also be able to bind Spo0F.

The information about Rap proteins in Bt is null and the functions of the 9 Rap-like proteins found in Bt407 are unknown. We found that they are grouped together in a similar fashion than the Spo0F-binding Raps from Bs168. Interestingly, NprR grouped closer to the Spo0F binding proteins of Bs168 than to Raps from Bt407 or Non-Spo0F-binding proteins. Thus, besides resembling RapH structure, NprR is more closely related to Spo0F-binding Rap proteins from *B. subtilis* than to any Rap from Bt407 (Fig. 2).

To test the hypothesis regarding a bifunctional NprR receptor, as a transcriptional regulator and as regulator of the phosphorelay, we obtained a *ΔnprR-nprRB* mutant from Bt8741 strain (BtΔRB). Using the *ΔnprR-nprRB* background, we expressed variants of the NprR-NprRB system (Table 1). The variables in the complementation studies were the presence of the HTH domain in *nprR* and the presence of *nprRB*. Thus we assessed the requirement of DNA binding for NprR functions as well as role of the NprRB signaling peptide. It was previously known that after activation by mature NprRB, NprR binds to a promoter region of *nprA,* activating its transcription (22). Therefore, a decrease in extracellular proteases was expected for the BtΔRB mutant and this was indeed observed (Fig. 3a). Complementation experiments also agreed with the previous report, as both HTH domain of NprR and *nprR* gene were essential for recovering the extracellular protease phenotype (Figure 5).

When we assessed the phenotype of sporulation we observed a delay in the initiation of sporulation (Fig. 3c). We measured the expression of genes *spoIIA* and *spoIIG* that have

been used as indicator of Spo0A phosphorylation and initiation of sporulation through the phosphorelay (10, 42-44). The increase in both transcripts in Bt8741 indicates that the cells have committed to sporulation; we suggest that the absent acute regulation of stage II genes in the mutant is related to the delay in sporulation observed. (Fig. 4).

According to our observations regarding NprR structure model and comparison to RapH-Spo0F crystal and the crystal of tetrameric NprR(ΔHTH) bound to the signaling peptide NprRB, we hypothesized that NprR could bind to Spo0F to regulate the phosphorelay. If the effect observed in sporulation by NprR deletion is related to its binding to Spo0F, the HTH domain is not expected to be essential for the function. Thus we measured spore-count at hour 10 after the transition phase in the strains carrying the constructions (Table 2), to assess the percentage of recovery of each strain with respect to the Bt8741 strain. We found that the HTH domain is not necessary for the complementation of the sporulation phenotype (Figure 6). As complementation of sporulation by NprR is not related to its DNA-binding capacity, it is feasible to account the functions to protein-proteins interactions using its TPR domain. This observation supports our initial hypothesis that NprR could participate in the regulation of sporulation initiation in a Rap-like function. We also found that presence of *nprRB* is necessary for the complementation of sporulation by NprR. It seems that NprRB peptide collaborates with NprR to carry out the regulation of sporulation initiation; i. e. both genes are needed for complementation of sporulation (Fig. 6).

Previous works had reported effects in sporulation related phenotypes due to mutations or deletion of the gene coding NprR. Yang et al. (24) reported that papillae colonies of *B. anthracis* containing mutations in *nprR* gene exhibited a decreased in extracellular

protease activity and failed to initiate sporulation in LB medium, but sporulated normally in SSM medium; however, these authors did not asses spore-count at different times. Dubois et al. (23) found that a Bt*ΔnprR* strain has decreased sporulation in relation to the wild type strain when the bacteria infects an insect host, but these results were not discussed by the authors. Finally, in a previous report we showed that addition to Bt cultures of synthetic peptides (SKPDIVG and SSKPDIVG) encoded in *nprRB* induced increase of sporulation efficiency, early appearance of spores, and decreased *cry1Aa* expression (25). The same peptides were also found to bind to NprR in a later report (26). Although all these data showed that NprR is involved in sporulation, none of the reports discussed this function of the protein or suggested a possible mechanism. We now suggest that the effects on sporulation caused by the *nprR* mutations might be an effect in the phosphorelay that leads to a delay in sporulation initiation (Fig. 3c).

As observed in figure 3c, figure 4, and in previous reports (23-24), *nprR-nprRB* mutants exhibit decreased sporulation; this suggests that the NprR-NprRB system acts on this process in a positive manner. This is the opposite of what happens in *B. subtilis,* where Rap act negatively on the phosphorelay; i. e. Rap mutants exhibit increased sporulation relative to their parental strains (44, 48). Recently, it was noted that the Rap proteins which are able to dephosphorylate Spo0F share a Glutamine residue at position 47 (Gln-47) (49); a point mutation of Gln-47 to asparagine caused the loss of phosphatase activity in RapH (41), indicating that this residue is essential for the activity. Gln-47 is not present in NprR (Fig 1b). Additionally, Tyr175 from RapH is also necessary for its phosphatase activity (27). In our NprR model, Tyr230 seems to be exposed in the surface and could interact with Spo0F. However, in the crystal structure, Tyr230 is

buried and may be incapable of interacting with Spo0F. It may be possible that if NprR binds Spo0F, it is not be able to carry out dephosphorylation.

As discussed above, mutants in *rap-phr* genes have increased sporulation, because Rap are inhibitors of the phosphorelay (44, 48). However, when *rap* genes alone are expressed in Δ*rap-phr* mutants, sporulation level is lower than the wild type strain (48). This is because in the quorum state, Phr peptides have an antagonistic effect with respect to Rap: Phr peptides bind their cognate Rap protein preventing its binding to Spo0F and allowing the phosphate transfer to Spo0A (50). The presence of *nprRB* is necessary for NprR function in regulation of sporulation, probably due to the necessity of the activation by mature NprRB. Thus, opposite to Phr peptides, the function of the NprRB peptide is not likely to be antagonistic. Further experimentation should reveal the actual role of the *nprRB* gene in the system.

The model presented in figure 7 explains how the Δ*nprR-nprRB* mutation could cause delayed sporulation compared to the parental strain. It seems that in response to the mature NprRB, NprR is a positive effector of the phosphorelay. The complex could be able to bind Spo0F, but unable to dephosphorylate the response regulator. Further experimentation should be aimed at testing the ability of NprR to bind Spo0F, in a possible competitive relationship with Rap proteins and the phosphorilation dynamics of the systems *in vitro* and *in vivo*.

Conclusion

Based on domain architecture and sequence similarity among the QS receptors of the RNPP family, previous reports noted the similarity between Rap and NprR, the latter being an evolutionary step from Rap proteins to the transcriptional activators PlcR/PrgX (14, 22, 26, 46). We now show that NprR-NprRB is involved in the phosphorelay and affects sporulation in a positive manner. We propose that NprR is a bifunctional QS receptor, not only acting on two different processes, but using contrasting molecular mechanisms, in response to activation by the mature NprRB signaling peptide. These mechanisms may be DNA binding for transcriptional regulation and binding to a response regulator of the phosphorelay system, as a part of the signaling network that decides when *B. thuringiensis* cells commit to sporulation.

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Figure 1.

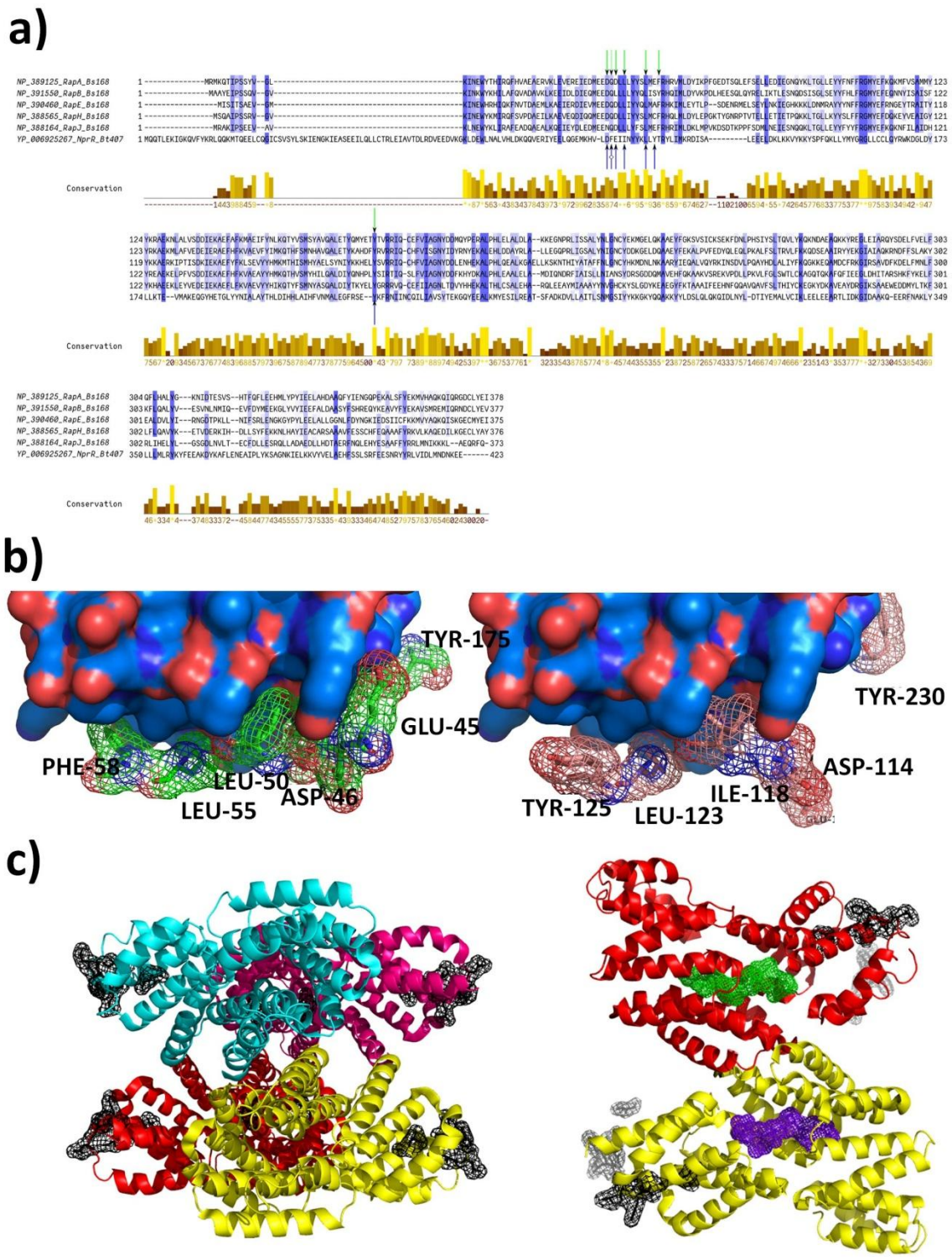

a)
NP_389125_RapA_Bs168
NP_391550_RapB_Bs168
NP_390460_RapE_Bs168
NP_388565_RapH_Bs168
NP_388164_RapJ_Bs168
YP_006925267_NprR_Bt407
Conservation
b)
TYR-175
GLU-45
PHE-58
LEU-50
ASP-46
LEU-55
TYR-230
ASP-114
ILE-118
TYR-125
LEU-123
c)

Figure 1: a) Multiple sequence alignment constructed from the amino acid sequences of several Spo0F binding Raps proteins and NprR. Columns colored blue indicate total conservation in the sequences at any given position. Green arrows above the alignment show the residues involved in Spo0F-binding by RapH (D46, D48, L50, L55, and F58) or Spo0F-dephosphorylation (Q47 and Y175). Blue arrows at the bottom of the alignment indicate the corresponding residues in NprR. Six out of seven residues are conserved in NprR corresponding to 85%, compared to a global sequence similarity of 42, 42, 40, 45, and 44% (NprR vs RapA, RapB, RapE, RapH and RapJ, respectively). Yellow bars indicate similarity among the proteins at any given position. b) Residues of RapH (left) and NprR (right) that interact with Spo0F (blue, PDB acc. no. 3Q15). The residues of RapH responsible for Spo0F binding and dephosphorylation are depicted as green mesh; the equivalent residues of NprR are shown as salmon mesh. c) Left: tetrameric structure of NprR(ΔHTH) (PDB accession 4GPK). Right: NprRB peptide (purple and green mesh) bound to tetrameric NprR(ΔHTH). Only two monomers of the tetramer are shown. Residues of NprR that may interact with Spo0F are shown in black mesh.

Figure 2.

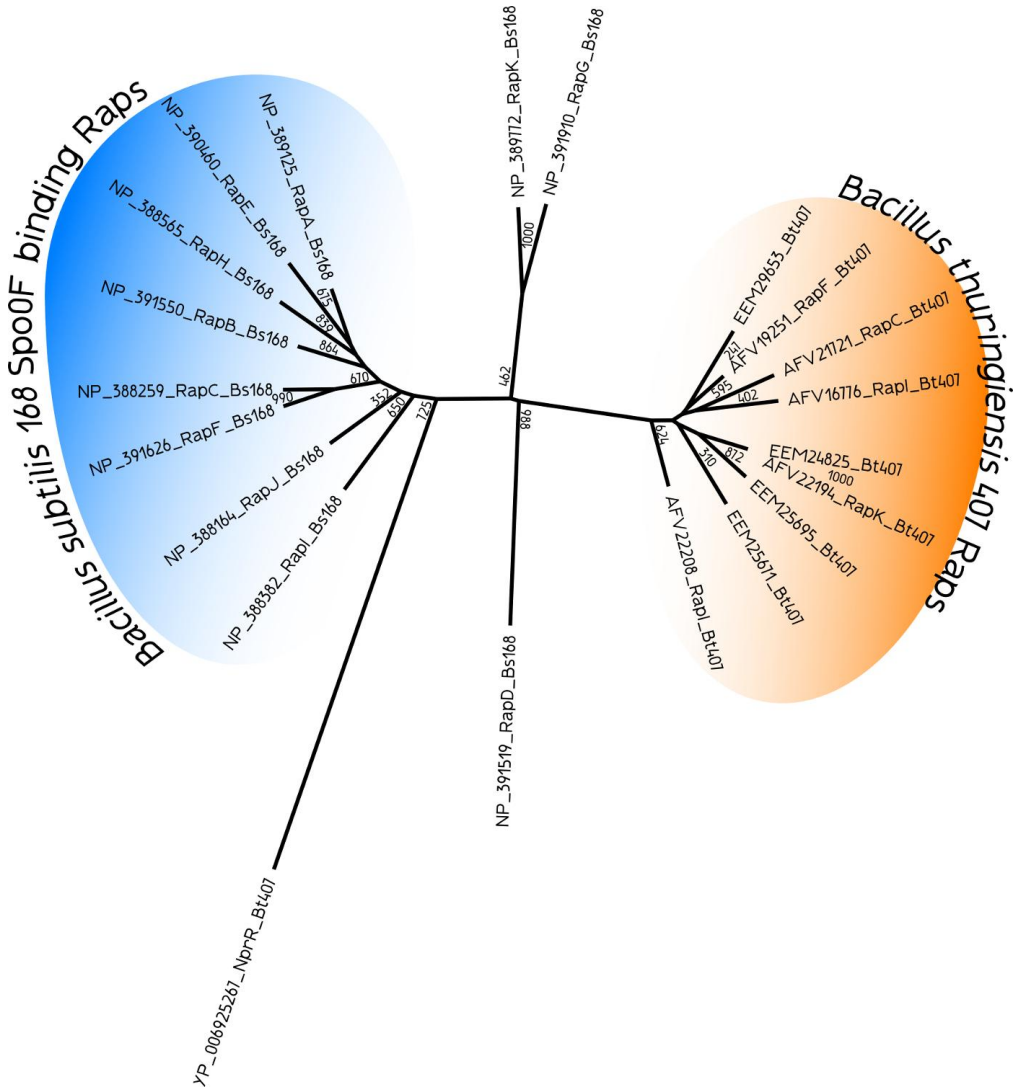


Figure 2: Phylogenetic tree of the response regulator aspartate phosphatases from *B. subtilis* 168 (Bs168) and Bt407. Based on the aligned sequences of each protein, the tree was constructed under the JTT+G+F model, 1000 bootstrap replicas were performed for statistical validation (numbers in nodes, indicate bootstrap replicas in which each grouping was found).

Figure 3.

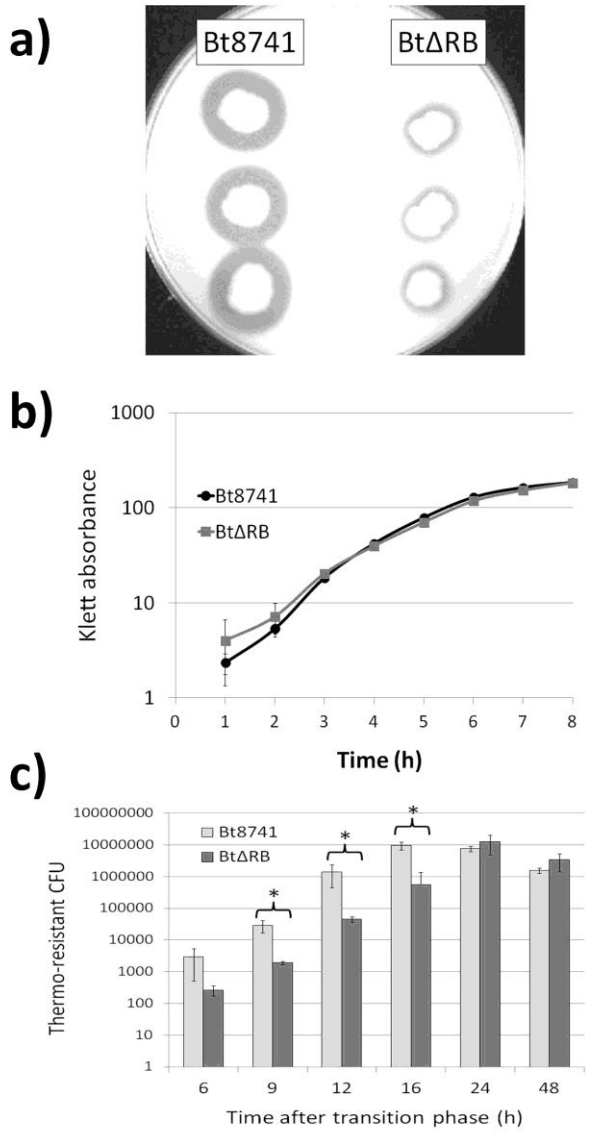


Figure 3. Mutation of *nprRB* causes diminished protease activity and delay in sporulation, but has no effect on growth. a) Extracellular protease activity of Bt8741 and BtΔRB on milk agar plates; b) Growth kinetics of Bt8741 and BtΔRB; c) Sporulation of Bt8741 and BtΔRB. Asterisks indicate significant differences between spore count of the strains at each time.

Figure 4.

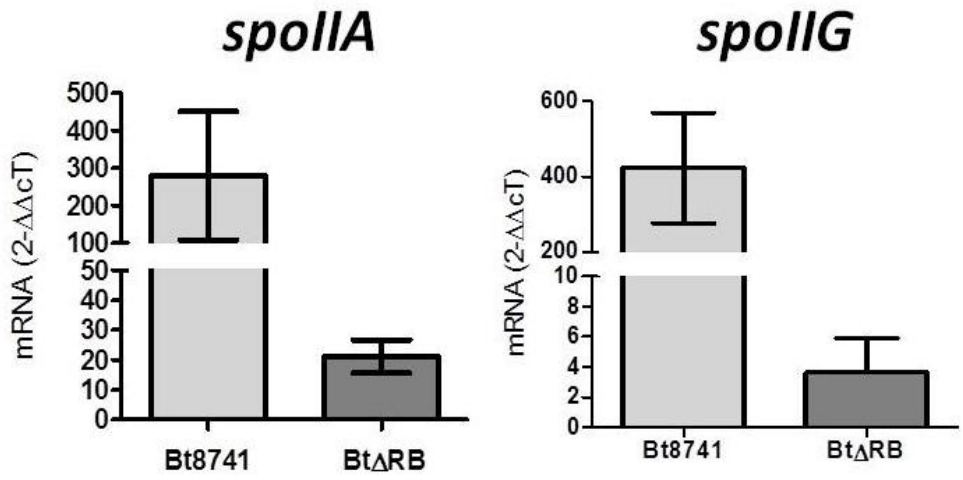


Figure 4. Deletion of *nprR-nprRB* drastically affects expression of *spoIIA* and *spoIIG*. Quantification of *spoIIA* and *spoIIG* mRNA in strains Bt8741 and BtΔRB. The values in the graph correspond to expression t_2 normalized to expression at t_0 and to the reference gene.

Figure 5.

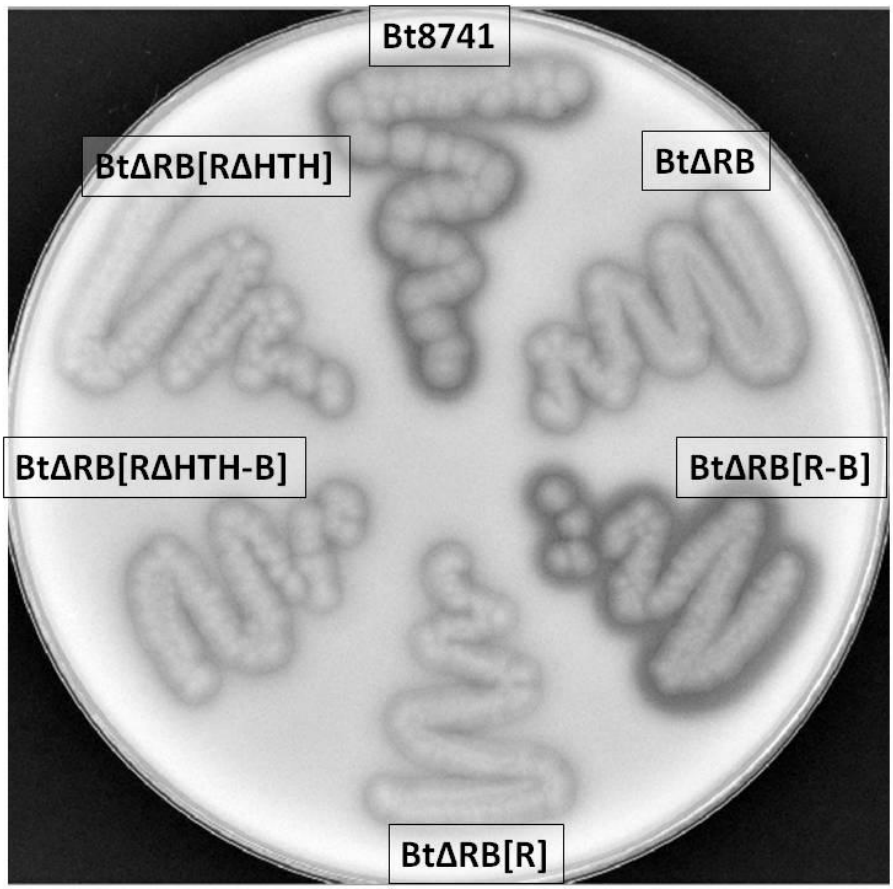


Figure 5. Loss of protease activity of the NprRB mutant can be recovered by complementation with a wild type *nprR* gene plus *nprRB* signaling peptide gene [R-B], but not with a similar construction lacking the DNA binding domain [RΔHTH-B]. Streaked Bt8741, BtΔRB and transformed strains grown on milk agar plates for 48 h. The clear zone around the bacteria streak is the result of hydrolyzed milk proteins by extracellular proteases.

Figure 6.

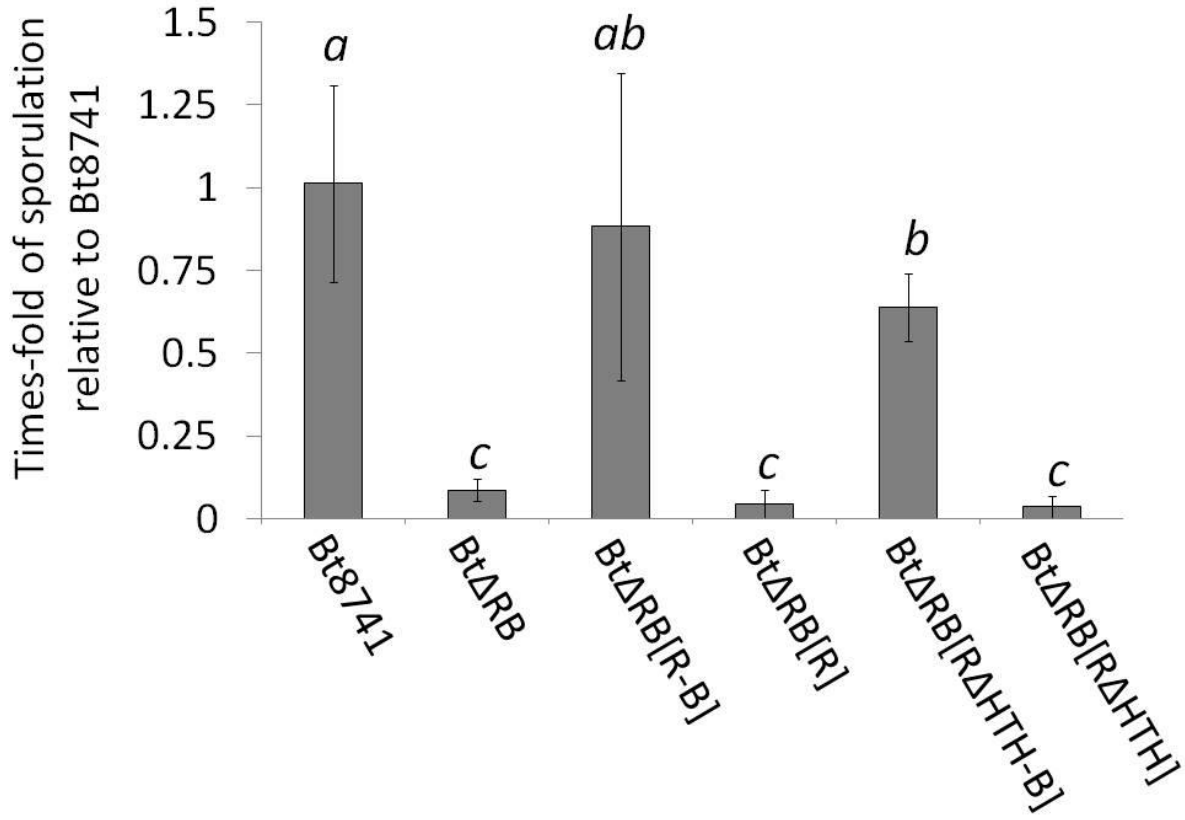
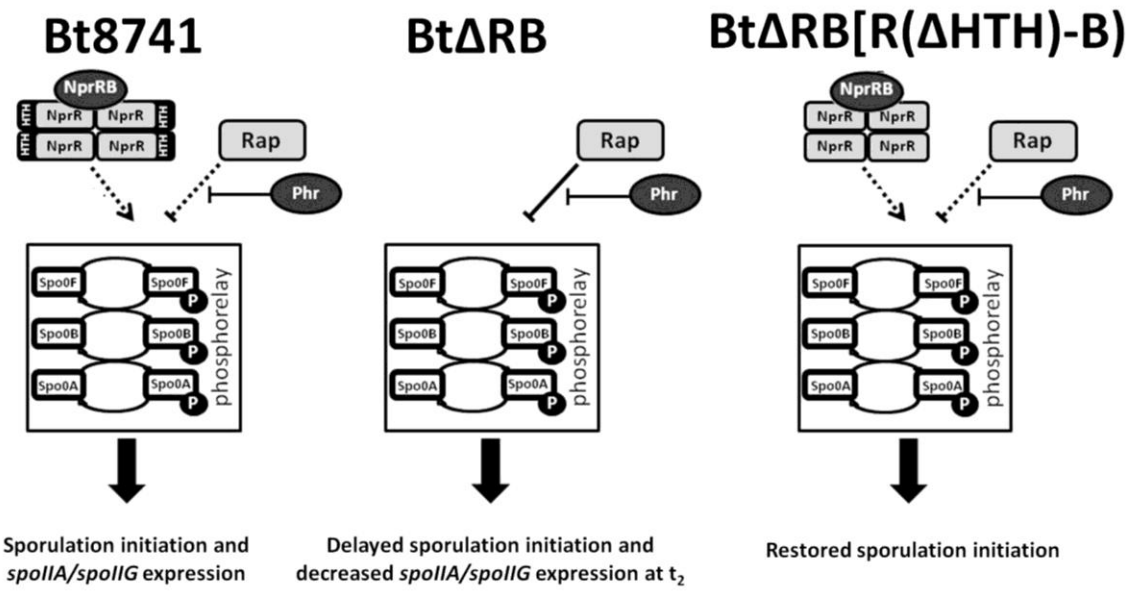


Figure 6. Delay in sporulation of the Δ*nprR-nprRB* mutant (BtΔRB) can be recovered by expressing a wild type *nprR-nprRB* [R-B] or a similar construction lacking the DNA-binding domain of *nprR* [RΔHTH-B]. The graphic shows sporulation of the BtΔRB mutant and strains transformed with the different constructions, relative to the wild type Bt8741 strain; spore-count of each strain was assessed at hour 10 after the transition phase. Different letters indicate significant differences among spore-count of strains. Bars show standard error of 3 independent experiments.

706 Figure 7.



707

708 Figure 7. Proposed model for the regulation of sporulation initiation by NprR. Left: In
709 the wild type strain, NprR and Rap compete for binding to Spo0F. NprRB is required for
710 NprR-tetramer formation, which binds to Spo0F allowing the phosphate flux trough the
711 phosphorelay. Rap binds to Spo0F dephosphorylating it but Rap-Phr is not functional.
712 The outcome of the phosphorelay is the normal timing of sporulation initiation and
713 *spoIIA/spoIIG* expression. Center: the deletion of *nprR-nprRB* results in a delay in
714 sporulation and a decrease in expression sporulation stage II genes at t_2 caused by the
715 elimination of a positive effector of the phosphorelay. Right: expression of the truncated
716 version of NprR(ΔHTH) and *nprRB* results in the restoration of sporulation initiation
717 time. Squares represent proteins; dark segments represent the HTH domain of NprR;
718 ovals represent signaling peptides; tick arrows represent the outcome of sporulation; thin
719 straight arrows represent binding; dashed arrows in NprR and Rap represent the
720 competitive binding to Spo0F.

721

722 Table 1. Primers used in this study

Primer	Sequence (from 5' to 3')	Restriction site
R5-F	CAGCTACAGGGATCCCAAAG	BamHI
R5-R	GTCGTCGACCTTCTTTGCTACGAATGGTG	SalI
Spc-F	GTCGTCGACAAAGTAAGCACCTGTTATTGC	SalI
Spc-R	AGAAGATCTCACCTAGATCCTTTTGACTC	BglII
R3-F	AGAAGATCTCAATCGGAAAGGGTTTTTTC	BglII
R3-R	GGAGGATCCCTTTACCGCTGTCTGCTTC	BamHI
cNprR-F	GAAGAATTCCATACTACGAATCGTCCCAG	EcoRI
cNprRB-R	GGAGGATCCGAAAAAACCCTTTCCGATTG	BamHI
cNprR-R	GGAGGATCCGTTTATTCCTCCTTATTATCATTC	BamHI
V12-R	GTCGTCGACTTGTTTGCCTATTTTTTCT	SalI
E48-F	GTCGTCGACGAAATTCTACAATTGCTCTGC	SalI
spoIIA-F	GGTTGTACAGCGGTTTCTTAATCG	NA
spoIIA-R	CTAACTTTCACTGATCCATCATCGC	NA
spoIIG-F	GTCTTGTACTGAGCGTCATC	NA
spoIIG-R	CTTCTCTCTTACTTGCAAAGC	NA
gatB-Yqey-F	AGCTGGTCGTGAAGACCTTG	NA
gatB-Yqey-R	CGGCATAACAGCAGTCATCA	NA

723 *Underlined sequences indicate restriction sites; NA: not applicable

724 Table 2. Strains used in this study.

Name	**Plasmid**	*nprR*	*nprR(ΔHTH)*	*nprRB*
Bt8741	pMAD	genome	-	genome
BtΔRB	pMAD	-	-	-
BtΔRB[R-B]	pR-B	in plasmid	-	in plasmid
BtΔRB[R]	pR	in plasmid	-	-
BtΔRB[RΔHTH-B]	pRΔHTH-B	-	in plasmid	in plasmid
BtΔRB[RΔHTH]	pRΔHTH	-	in plasmid	-

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