Universidad de León

Departamento de Biología Molecular

Mecanismo molecular del control por fosfato de la cascada de expresión de los genes de biosíntesis de metabolitos secundarios en *Streptomyces coelicolor*

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INFORME DEL DIRECTOR DE LA TESIS

(Art. 11.3 del R.D. 56/2005)

Los Dres. D. Juan Francisco Martín Martín y D. Antonio Rodríguez García como directores[1] de la Tesis Doctoral titulada "Mecanismo molecular del control por fosfato de la cascada de expresión de los genes de biosíntesis de metabolitos secundarios en *Streptomyces coelicolor*" realizada por D. Fernando Santos Beneit en el Departamento de Biología Molecular, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

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A mi familia

"No estudio por saber más, sino por ignorar menos"

SOR JUANA INÉS DE LA CRUZ (1651 – 1695)

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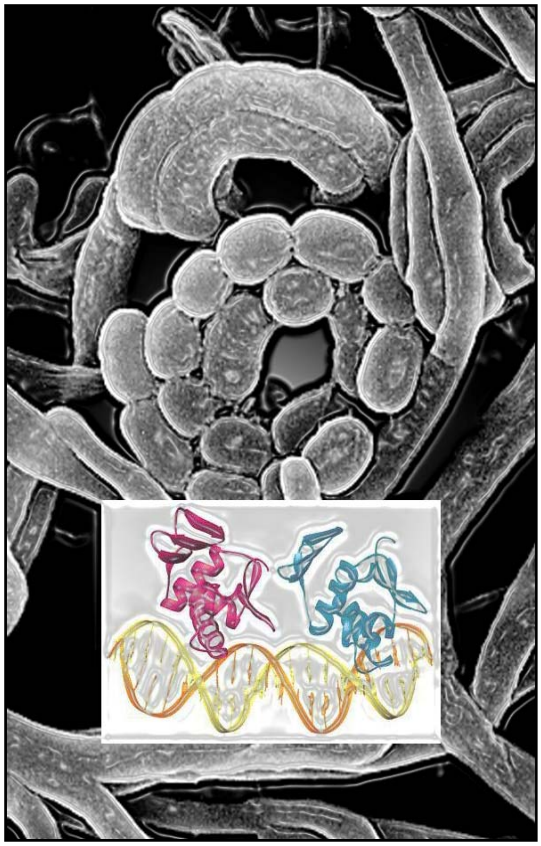

1. Introducción

Este capítulo está enfocado a dar una visión general y complementar las introducciones específicas de cada uno de los artículos adjuntados. Esta introducción general se divide en dos apartados. En el primer apartado se describe la importancia del género *Streptomyces* como productor de metabolitos secundarios de gran interés industrial y farmacológico y la utilización de *Streptomyces coelicolor* como organismo modelo. En el segundo apartado se analiza la respuesta a la escasez de fosfato dependiente del regulador PhoP, esto es, el regulón *pho*, objeto principal de esta tesis.

1.1. *Streptomyces coelicolor*: organismo modelo del género *Streptomyces*

1.1.1. Características generales del género *Streptomyces* y reseña histórica del grupo de los actinomicetos

Los microorganismos del género *Streptomyces* pertenecen al grupo de los actinomicetos. Los actinomicetos fueron descubiertos a finales del siglo XIX como los organismos causantes de algunas enfermedades mortales como la lepra o la tuberculosis (Hansen, 1874; Koch, 1982, citado por Hopwood, 2007). En las siguientes décadas se describieron otros muchos microorganismos con características semejantes. El grupo se reconoció oficialmente con el nombre latino de *Actinomycetales* en 1916 (Buchanan, 1916, citado por Hopwood, 2007). El primer *Streptomyces* descrito data del año 1875 (Cohn, 1875, citado por Hopwood, 2007). Fue denominado *Streptothrix* (que significa pelo enrollado) por su aspecto filamentoso y enmarañado. Posteriormente, tras la clasificación del grupo de los *Actinomycetales* realizada por Waksman y Henrici en 1943, el *nombre* de *Streptothrix* fue cambiado por el de *Streptomyces* (que significa hongo enrollado). En ese mismo año un estudiante llamado Albert Schatz descubrió la estreptomicina. El organismo productor de dicho antibiótico, *Streptomyces griseus*, fue

determinado como especie tipo del género *Streptomyces* recién creado (Waksman y Henrici, 1943). El alto poder antibiótico de la estreptomicina contra el agente etiológico de la tuberculosis se consideró de tal importancia que en 1952 se le otorgó a Waksman el premio Nobel de medicina. Durante las siguientes décadas, especialmente entre los años 1950 y 1980, se descubrieron muchos antibióticos en especies de actinomicetos. De este modo, los actinomicetos pronto se convirtieron en el grupo de microorganismos más utilizados en la industria farmacológica (Hopwood, 2007).

Taxonómicamente los actinomicetos forman un grupo coherente tanto fisiológicamente como por comparación de secuencia del RNA ribosomal 16S. Sus principales características son el alto contenido en G+C, la tinción Gram-positiva y la formación de filamentos ramificados en la mayoría de ellos (Garrity *et al*., 2004). De hecho, el contenido en bases G+C de los miembros que forman parte de este grupo es el más alto de entre todos los grupos de bacterias (63-78 %) (Chater y Hopwood, 1993).

El género *Streptomyces* pertenece al grupo de los actinomicetos aerobios estrictos, de morfología filamentosa, que desarrollan micelio aéreo y largas cadenas de esporas (Lechevalier, 1989). Se han caracterizado más de 500 especies (http://www.bacterio.cict.fr/s/streptomycesb.html), que se pueden clasificar según el tamaño, aspecto y color de la colonia. La taxonomía actual del género es la siguiente (Garrity *et al*., 2004):

- Dominio: Bacteria
- Filo: Actinobacteria
- Clase: Actinobacteria
- Subclase: Actinobacteridae
- Orden: Actinomycetales
- Suborden: Streptomycineae
- Familia: Streptomycetaceae
- Género: *Streptomyces*

1.1.2. Genética de *Streptomyces*

El tamaño medio del cromosoma de *Streptomyces* es de 8 Mb y el contenido medio de G+C es del 72 % (Volff y Altenbuchner, 1998). El cromosoma de *Streptomyces* presenta una estructura lineal (Lin *et al*., 1993), a diferencia de la mayoría de las bacterias, con proteínas unidas covalentemente a los extremos 5' del mismo (Chang y Cohen, 1994). Dichas proteínas teloméricas (Tpg) actúan como cebadores para la replicación de los telómeros del cromosoma (Bao y Cohen, 2001). La proteína con función helicasa Tap (proteína asociada al telómero) también intervine en este proceso (Bao y Cohen, 2004). Por lo general estas proteínas teloméricas están bien conservadas entre las especies del género *Streptomyces*, con algunas excepciones como *S. griseus* (Ohnishi *et al*., 2008). La replicación del cromosoma de *Streptomyces* tiene lugar bidireccionalmente desde un *oriC* localizado centralmente en el cromosoma (Musialowski *et al*., 1994; Jakimiwicz *et al*., 1998). El hecho de que el *oriC* se conserve centrado en el cromosoma sugiere una presión selectiva sobre dicho posicionamiento (Bentley *et al*., 2002). Por el contrario, los extremos del cromosoma están formados por secuencias repetidas e invertidas de entre 20 y 600 Kb denominadas TIRs (acrónimo inglés de repeticiones invertidas terminales) y por zonas de inserción de fagos, plásmidos y elementos transponibles que causan la conocida inestabilidad genética del cromosoma de *Streptomyces* (Volff y Altenbuchner, 1998).

En el momento actual han sido secuenciados y publicados los genomas completos de tres especies (Bentley *et al*., 2002; Ikeda *et al*., 2003; Ohnishi *et al*., 2008). La comparación de los cromosomas de *S. coelicolor*, *S. avermitilis* y *S. griseus* muestra un núcleo central de 6 Mb muy conservado en el que residen mayoritariamente genes relacionados con el metabolismo primario (Ohnishi *et al*., 2008). El núcleo central se sitúa ligeramente hacia la izquierda del cromosoma por lo que deja dos regiones terminales asimétricas de 1 y 2 Mb, aproximadamente (figura 1.1). En estas regiones, denominadas *brazos*, se encuentran mayoritariamente genes no esenciales, incluidos genes y agrupaciones del metabolismo secundario (Bentley *et al*., 2002).

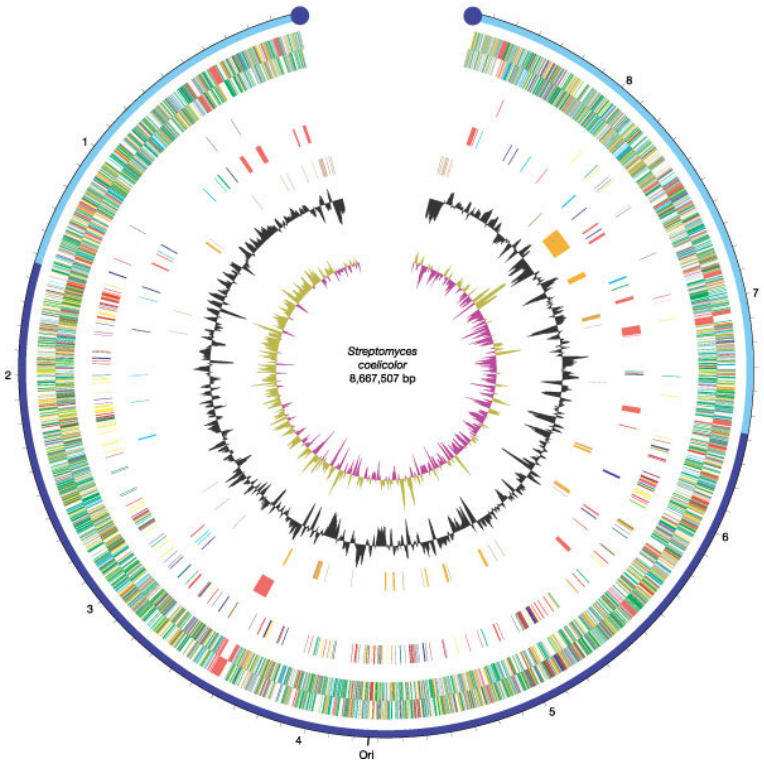

Figura 1.1: Representación circular del cromosoma de *Streptomyces coelicolor* tomada de Bentley *et al*. (2002). La línea azul oscura y azul clara representa el núcleo y los brazos del cromosoma, respectivamente. Los círculos en el extremo del cromosoma representan las proteínas teloméricas.

El núcleo central del cromosoma de *Streptomyces* presenta una gran sintenia con los cromosomas circulares de *Mycobacterium tuberculosis* y *Corynebacterium diphteriae*, lo que parece indicar un antecesor común para todos los actinomicetos (Cole *et al*., 1998; Bentley *et al*., 2002; Cerdeño-Tárraga *et al*., 2003). De hecho, los cromosomas de *Streptomyces* ocasionalmente sufren pérdidas espontaneas de sus extremos, tras lo cual son capaces de unirse y formar cromosomas circulares que se replican como los del resto de las bacterias (Hopwood, 2007). La transmisión horizontal de DNA en los extremos del cromosoma pudo provocar la expansión del cromosoma de *Streptomyces* durante la evolución reciente (Bentley *et al*., 2002).

Una gran cantidad de plásmidos han sido detectados en *Streptomyces*, entre los que se incluyen: plásmidos lineales, plásmidos integrativos, grandes plásmidos circulares de bajo número de copias y pequeños plásmidos circulares de alto número de copias (Hopwood y Kieser, 1993). Diversos fagos y transposones presentes en *Streptomyces* se utilizan en estudios de biología molecular; destacando el fago ΦC31 y el transposón IS*117*, utilizados como vectores integrativos, y los transposones IS*493* y Tn*4556*, utilizados para mutagénesis aleatorias (Kieser *et al*., 2000).

1.1.3. Ciclo de vida de *Streptomyces*

El hábitat principal de *Streptomyces* es el suelo (Hagedorn, 1976), aunque existen especies capaces de habitar el agua dulce ó marina (Cross, 1981) y el aire (Lloyd, 1969). Unas pocas son patógenas de plantas y animales (Bouchek-Mechiche *et al*., 2006; Quintana *et al*., 2008). *Streptomyces* sintetiza una gran cantidad de enzimas extracelulares, como amilasas, quitinasas, xilanasas, celulasas, proteasas, lipasas o nucleasas, que le confieren una gran versatilidad nutricional. Es capaz de utilizar como fuente de carbono azúcares, polisacáridos, alcoholes, ácidos orgánicos, aminoácidos y compuestos aromáticos (Williams *et al*., 1983). Su habilidad para colonizar el suelo se debe principalmente a la capacidad de formar esporas resistentes a la sequedad que en muchos casos presenta dicho sustrato. En laboratorio se ha llegado a crecer cultivos de *Streptomyces* a partir de muestras de suelo de más de 70 años de antigüedad conservadas en esterilidad (Morita, 1985). Las esporas pueden permanecer latentes en el suelo varias décadas hasta que las condiciones ambientales proporcionen la humedad y los nutrientes necesarios para que estas germinen.

La germinación comienza con la degradación del principal componente de reserva de la espora, el disacárido trehalosa (Ensign, 1978), y finaliza con la formación de uno o dos tubos germinales. Posteriormente crecen y se ramifican formando una densa y compleja red en el medio que recibe el nombre de micelio sustrato (Hardisson *et al*., 1978). A partir del micelio sustrato se desarrolla perpendicularmente el micelio aéreo gracias a los nutrientes que le aporta el primer micelio (Méndez *et al*., 1985;

Braña *et al*., 1986). Las hifas del micelio aéreo (ver figura 1.2) se diferencian en cadenas de esporas que darán con el tiempo comienzo a un nuevo ciclo biológico (Hardisson y Manzanal, 1976; Chater, 2001).

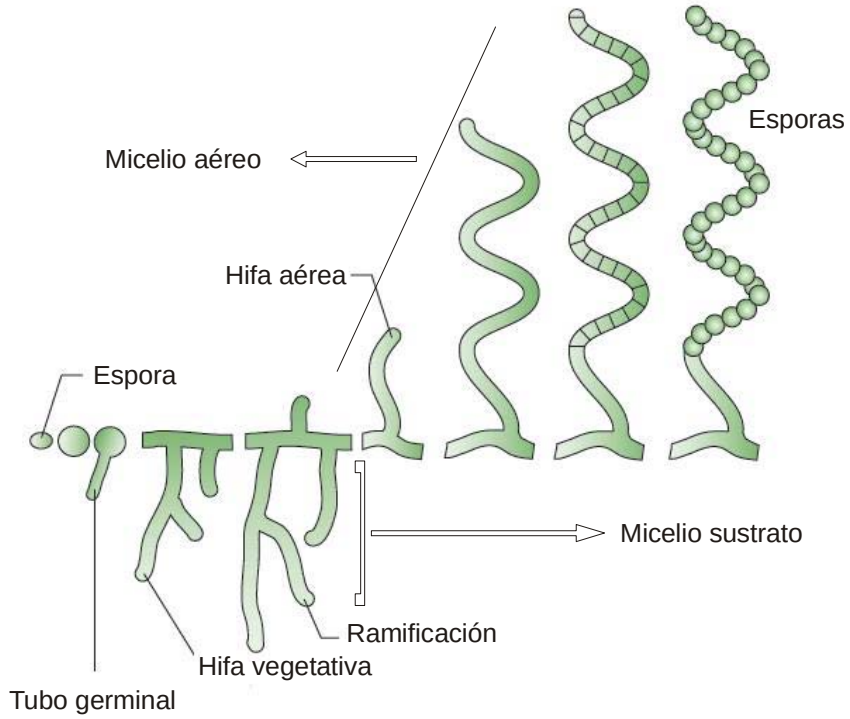

Figura 1.2: Representación de la diferenciación morfológica en el ciclo de vida de *Streptomyces*. Imagen modificada de Flärdh y Buttner, 2009.

El proceso de diferenciación morfológica que da lugar a la formación de esporas comienza con la septación de las hifas aéreas, en un principio multinucleoidales, para originar hifas con compartimentos mononucleoidales. Posteriormente, las presporas engrosan su pared y se desarrollan como esporas hidrofóbicas, generalmente pigmentadas y adaptadas a la dispersión aérea (McCarthy y Williams, 1992). La hidrofobicidad de las esporas permite que floten; así se evita que el agua las arrastre hacia las profundidades del suelo donde la concentración de oxígeno es baja y nula la posibilidad de dispersión aérea.

El proceso de diferenciación morfológica está coordinado con el proceso de diferenciación bioquímica (producción de metabolitos secundarios) por mecanismos reguladores comunes (Chater, 1993). Según este autor, la producción de antibióticos permitiría disminuir la cantidad de competidores del entorno aumentándose así las posibilidades de que el proceso de esporulación se desarrolle completamente. Por otro lado, la producción de ciertos metabolitos como pigmentos o aromas podría estar encaminada a la atracción de artrópodos que dispersen las esporas en un modo análogo a lo que sucede en las semillas de las plantas. Se ha comprobado que ciertos gusanos son atraídos por la geosmina, un metabolito producido por la mayoría de las especies de *Streptomyces*, que es responsable del típico olor a tierra mojada. Por tanto parece que las esporas de *Streptomyces* podrían estar adaptadas tanto para la dispersión aérea como zoogénica (Hopwood, 2007).

1.1.4. *Streptomyces* en la medicina, agricultura, ecología e industria farmacológica

Solo un par de especies pertenecientes al género *Streptomyces* (*S. somaliensis* y *S. sudanensis*) se han descrito como patógenos en humanos (causan micetoma, una infección progresiva y destructiva de la piel y del tejido subcutáneo), por lo que su importancia médica desde el punto de vista infeccioso es muy limitada (Gumaa y Mahgoub, 1975; Taha, 1983; Quintana *et al*., 2008). En la agricultura, la patogenicidad causada por especies del género *Streptomyces* afecta principalmente a la producción de patata. Diversas especies de *Streptomyces*, *S. scabies*, *S. turgidiscabies*, *S. acidiscabies*, *S. europaeiscabiei* y *S. stelliscabiei*, causan la roña de la patata (*common scab*) (Loria *et al*., 2006). Esta enfermedad ocurre en todas las zonas productoras de patata del mundo y afecta tanto a la cantidad como la calidad de la producción (Loria *et al*., 1997; Hiltunen *et al*., 2005). El biocontrol de estas especies patógenas es posible mediante la utilización de otras especies de *Streptomyces* no patógenas que crecen en el mismo nicho ecológico (Tahvonen, 1988; Minuto *et al*., 2006). Un ejemplo es la especie *S. griseoviridis* K61 que se comercializa con el nombre de “Mycostop” (Mohammadi, 1992).

Desde el punto de vista ecológico, *Streptomyces* es importante, además de por su función de saprófito en la naturaleza, como herramienta para procesos de biorremediación. La enorme versatilidad metabólica de *Streptomyces*, unido a su amplia distribución en el suelo, permite llevar a cabo este tipo de procesos (Albarracín *et al*., 2005). Un ejemplo del uso de *Streptomyces* en biorremediación, la provee la publicación de Polti *et al*. (2009), en la que se demuestra la capacidad de la especie *Streptomyces* sp. MC1 de reducir hasta un 94% la concentración de cromo hexavalente, Cr (VI), de muestras de suelo. El Cr (VI) es un carcinógeno humano que daña las estructuras celulares debido a su alto poder de oxidación. Debido a su gran incidencia en vertidos industriales y a su persistencia en el medio, la agencia de protección del medio ambiente en Estados Unidos de America lo ha declarado como uno de los principales contaminantes del suelo. La falta de métodos de descontaminación resalta la importancia de *Streptomyces* para este fin.

No obstante, el principal atractivo de las especies pertenecientes al género *Streptomyces* radica en la capacidad de producir metabolitos con numerosas aplicaciones biológicas. Alrededor del 50 % de ellas sintetizan compuestos con actividad antibiótica. De hecho, aproximadamente la mitad de los antibióticos naturales conocidos son sintetizados por bacterias del género *Streptomyces* (Bérdy, 2005). Además, producen también otros compuestos de gran interés para la medicina, la agricultura, la industria y la biotecnología; como antifúngicos, antivirales, antiparásitos, antitumorales, insecticidas, herbicidas, inmunosupresores, inmunoestimuladores, anticolesterolémicos, factores de crecimiento para plantas y animales, inductores de la diferenciación celular en eucariotas, pigmentos, aromas, inhibidores enzimáticos y enzimas (Demain, 1999). En la tabla 1.1 se expone los metabolitos producidos por *Streptomyces* más utilizados en la medicina, industria, ganadería y agricultura.

Metabolito	Especie productora	Diana biológica	Aplicación
Actinomicina	*S. antibioticus*	Transcripción	Anticancerígeno
Adriamicina	*S. peucetius*	Replicación del DNA	Anticancerígeno
Anfotericina	*S. nodosus*	Esteroles de membrana	Antifúngico
Avermectina	*S. avermitilis*	Neurotransmisores de invertebrados	Antiparásito
Avoparcina	*S. candidus*	Pared celular bacteriana	Promotor del crecimiento del ganado
Bialafos	*S. hygroscopius*	Metabolismo del nitrógeno	Herbicida
Bleomicina	*S. verticillus*	Replicación del DNA	Anticancerígeno
Candicidina	*S. griseus*	Esteroles de membrana	Antifúngico
Cloranfenicol	*S. venezuelae*	Ribosomas bacterianos	Antibacteriano
Clorotetraciclina	*S. aureofaciens*	Ribosomas bacterianos	Antibacteriano
Clavulánico (ácido)	*S. clavuligerus*	Inhibidor de penicilinasas	Antibacteriano combinado con penicilinas
Daunomicina	*S. peucetius*	Replicación del DNA	Anticancerígeno
Estreptotricina	*S. lavendulae*	Ribosomas bacterianos	Promotor del crecimiento del ganado
Estreptomicina	*S. griseus*	Ribosomas bacterianos	Antibacteriano
Filipina	*S. avermitilis*	Esteroles de membrana	Antifúngico
Fosfomicina	*S. wedmorensis*	Pared celular bacteriana	Antibacteriano
Kanamicina	*S. kanamyceticus*	Ribosomas bacterianos	Antibacteriano
Kasugamicina	*S. kasugaensis*	Síntesis proteica	Antifúngico (planta de arroz)
Lincomicina	*S. lincolnensis*	Ribosomas bacterianos	Antibacteriano
Mitomicina	*S. caespitosus*	Replicación del DNA	Anticancerígeno
Monensina	*S. cinnamonensis*	Membrana celular	Promotor del crecimiento del ganado
Neomicina	*S. fradiae*	Ribosomas bacterianos	Antibacteriano
Nikomicina	*S. tendae*	Síntesis de chitina	Antifúngico e insecticida
Novobiocina	*S. niveus*	Replicación del DNA bacteriano	Antibacteriano

Nistatina	*S. noursei*	Esteroles de membrana	Antifúngico
Oleandomicina	*S. antibioticus*	Ribosomas bacterianos	Antibacteriano
Oxitetraciclina	*S. rimosus*	Ribosomas bacterianos	Antibacteriano
Pimaricina	*S. natalensis*	Esteroles de membrana	Antifúngico
Polioxina	*S. cacoi*	Síntesis de chitina	Antifúngico
Pristinamicina	*S. pristinaespiralis*	Ribosomas bacterianos	Antibacteriano
Rapamicina	*S. hygroscopius*	Linfocitos	Inmunosupresor
Tacrolimus	*S. tsukubaensis*	Linfocitos	Inmunosupresor
Tetraciclina	*S. aureofaciens*	Ribosomas bacterianos	Antibacteriano
Tienamicina	*S. cattleya*	Pared celular bacteriana	Antibacteriano
Tilosina	*S. fradiae*	Ribosomas bacterianos	Promotor del crecimiento del ganado
Virginiamicina	*S. virginiae*	Ribosomas bacterianos	Promotor del crecimiento del ganado

Tabla 1.1: Principales metabolitos de aplicación humana producidos por especies del género *Streptomyces*. Modificada de Hopwood, 2007.

1.1.5. Control por fosfato de la biosíntesis de metabolitos secundarios en especies del género *Streptomyces*

La biosíntesis en microorganismos de muchos antibióticos y otros metabolitos secundarios se regula por la concentración de fosfato en el medio. Generalmente la producción de estos metabolitos se reprime por concentraciones altas de fosfato (Martín y Demain, 1980). Sin embargo, no todos los metabolitos son igualmente sensibles, de forma que, por ejemplo, la biosíntesis de los antibióticos β-lactámicos es muy poco sensible al fosfato (Liras *et al*., 1990). Entre los metabolitos regulados negativamente se incluyen estreptomicina, oxitetraciclina, ácido clavulánico, tilosina, cefalosporina, cefamicina C y tienamicina, entre otros (Martín, 2004). Desde un punto de vista biosintético, este grupo de metabolitos tienen poco en común, excepto que todos se sintetizan como metabolitos secundarios. El efecto negativo del fosfato en la producción

de metabolitos secundarios se ha observado en un amplio rango de microorganismos incluyendo bacterias Gram-negativas, Gram-positivas y hongos. Martín y Demain (1980) proponen que la regulación ejercida por la concentración de fosfato en el medio supone para los microorganismos un mecanismo de defensa frente a sus competidores cuando el fosfato en el medio es limitante para el crecimiento. Además, según estos autores los metabolitos secundarios podrían servir para la comunicación intercelular dentro de la colonia, o de las células productoras con otros organismos procariotas o eucariotas en el medio ambiente.

En un principio los trabajos existentes sobre la regulación ejercida por el fosfato en la producción de metabolitos se limitaron simplemente a estudios fisiológicos (revisados por Martín y Demain, 1980). Más tarde, se probó la hipótesis de que el fosfato inhibiese la actividad de las enzimas biosintéticas. En los casos de los metabolitos candicidina, oxitetraciclina y pimaricina, producidos por especies del género *Streptomyces*, se demostró que el exceso de fosfato repercutía sobre la transcripción de algún gen biosintético. En estudios pioneros en *S. griseus*, productor de candicidina, solo se observó transcripción del gen *pabS* en condiciones limitantes de fosfato (Asturias *et al*., 1990). Este gen codifica la enzima encargada de la síntesis de ácido p-aminobenzoico, precursor de candicidina (Martín y Aparicio, 2009). Lo mismo ocurrió con los genes de biosíntesis de oxitetraciclina *otcC*, *otcX* y *otcY* en *S. rimosus* (McDowall *et al*., 1999) y con al menos 17 genes de la agrupación génica de la pimaricina en *S. natalensis* (Mendes *et al*., 2007).

A pesar de esto, algunos autores destacan el desconocimiento que hay en la transducción de señales que va desde la captación de la concentración de fosfato en el medio hasta la regulación del metabolismo secundario en cuestión (Chater y Bibb, 1996). Es decir, no queda claro si el fosfato, como tal, es el último efector en la regulación transcripcional o participa en una cadena reguladora más compleja. Se sabe, por ejemplo, que los niveles de ATP aumentan rápidamente tras la adición de fosfato. Además, se ha observado que un exceso de estos niveles provoca la síntesis de cadenas de polifosfatos. Los experimentos realizados por Chouayekh y Virolle (2002) en *Streptomyces lividans* demuestran que en mutantes carentes de polifosfato quinasa, que

por ello no sintetizan polifosfatos, la producción de antibióticos aumenta significativamente en condiciones limitantes de fosfato. Concluyen que en condiciones abundantes de fosfato la síntesis de polifosfatos tiene un efecto negativo en la producción de metabolitos secundarios.

Otro mecanismo de la acción negativa del fosfato es la represión de fosfatasas necesarias para la rotura de intermediarios biosintéticos fosforilados de algunos antibióticos, como la espiramicina en *Streptomyces ambofaciens* (Lounes *et al*., 1996). La involucración del regulón *pho* (sistema que responde a la escasez de fosfato en el medio; se detalla ampliamente en el siguiente apartado), en la activación de los genes que codifican fosfatasas explica el efecto negativo del fosfato sobre la expresión de dichas enzimas (Torriani-Gorini, 1994; Hulett, 1996; Apel *et al*., 2007). Sin embargo, no se han descrito genes de biosíntesis de metabolitos secundarios o reguladores de la producción de estos pertenecientes a dicho regulón. Por tanto, es importante dilucidar si el control por fosfato de la producción de antibióticos se realiza por medio del regulón *pho* de forma directa o indirecta, y si incluye, o no, también otros sistemas.

1.1.6. La regulación de la biosíntesis de antibióticos incluye reguladores específicos y globales

Los metabolitos secundarios son compuestos con estructuras químicas complejas, no ensenciales para el crecimiento y la supervivencia del organismo, sintetizados generalmente al comienzo de la fase estacionaria (Martín y Demain, 1980). Los antibióticos representan el grupo más importante de dichos metabolitos secundarios. La mayoría son productos de complejas rutas biosintéticas que abarcan grandes agrupaciones de genes (Martín y Liras, 1989; Hopwood *et al*., 1995). Dichas agrupaciones generalmente contienen, además de los genes estructurales, genes de transporte, genes de resistencia y genes reguladores. Los genes reguladores actúan como activadores transcripcionales de los genes biosintéticos de la agrupación a la que pertenecen. Son denominados reguladores específicos de ruta. En *S. coelicolor* entre estos se incluyen; *actII*-ORF4 para actinorrodina (Fernandez-Moreno *et al*., 1991), *redD*

y *redZ* para undecilprodigiosina (Takano *et al*., 1992; White y Bibb, 1997) y *cdaR* para el antibiótico dependiente de calcio (Chouayekh y Virolle, 2002).

Los reguladores específicos son controlados a su vez por otros reguladores más generales. Al menos ocho reguladores distintos podrían estar uniéndose en los promotores de *actII*-ORF4 y *redD* según los estudios de DACA (ensayos de captura por afinidad al DNA) realizados recientemente por Park *et al*. (2009). Un nivel superior de reguladores incluye a los denominados reguladores pleotrópicos, los cuales son capaces de integrar una gran variedad de estímulos como señales ambientales, señales intercelulares, tasa de crecimiento, deficiencias nutricionales o estreses fisiológicos. Más de veinte reguladores pleotrópicos se han descrito como moduladores de la síntesis de antibióticos en *S. coelicolor* (Bibb, 1996). Entre estos, inicialmente se caracterizaron los genes *abs* (Adamidis *et al*., 1990; Champness *et al*., 1992)*, aba* (Fernández-Moreno *et al*., 1992) y *afs* (Horinouchi *et al*., 1983). Los productos de los genes *absA1-absA2* y *absB* (un sistema de dos componentes y una ribonucleasa III, respectivamente) suprimen la producción de los cuatro antibióticos producidos por *S. coelicolor* (Price *et al*., 1999; Anderson *et al*., 2001). Por el contrario, los productos de los genes *abaB*, *afsR* y *afsS* estimulan la producción de al menos dos de ellos, actinorrodina y undecilprodigiosina (Floriano y Bibb, 1996; Scheu *et al*., 1997; Lee *et al*., 2002). Otros reguladores pleotrópicos de la producción de antibióticos en *Streptomyces* son: CutR-CutS (Chang *et al*., 1996), RapA1-RapA2 (Lu *et al*., 2007) y AfsQ1-AfsQ2-SigQ (Ishizuka *et al*., 1992; Shu *et al*., 2009).

Finalmente, por encima de estas proteínas reguladoras se encuentran una variedad de efectores intracelulares relacionados con el metabolismo primario, como el guanosín-3´-5´-bispirofosfato (ppGpp), la S-adenosil-L-metionina y el AMP cíclico. Estos efectores responden a cambios en la disponibilidad de nutrientes desencadenando una serie de procesos de regulación que afectan la producción de metabolitos secundarios (Chakraburtty y Bibb, 1997; Kim *et al*., 2003; Susstrunk *et al*., 1998).

1.1.7. Regulación coordinada de la diferenciación morfológica y la producción de antibióticos en *Streptomyces*

La variabilidad de las condiciones nutricionales y ambientales del suelo hace que los microorganismos que viven en él hayan desarrollado una gran complejidad biológica en su ciclo de vida (McAdams *et al*., 2004). Dicha complejidad se manifiesta en el complejo proceso de diferenciación morfológica y bioquímica que presentan las bacterias del género *Streptomyces* (Chater, 1993). Seguramente, el "decidir" cuándo se pasa de un crecimiento vegetativo a un crecimiento dirigido a la formación de esporas sea el acto más importante para la supervivencia del microorganismo. En consecuencia, el proceso de diferenciación morfológica y bioquímica involucra una compleja red de regulación capaz de responder a una gran variedad de señales tanto extracelulares como intracelulares. Ambos procesos están coordinados, de hecho, hay una clara correlación temporal entre el comienzo de la producción de antibióticos y la esporulación (Chater, 1993). No obstante, los mecanismos reguladores globales que controlan estos procesos son prácticamente desconocidos (Bibb, 2005).

A lo largo de los estudios genéticos se han aislado diversos mutantes afectados en la diferenciación morfológica incapaces de formar esporas maduras. Entre estos destacan los mutantes blancos (en ingles *white*), los cuales tienen afectados el proceso de esporulación en diferentes niveles. En consecuencia el micelio aéreo adquiere siempre un fenotipo de coloración blanca, ya sea porque no se llegan a formar las esporas, o porque no se forma el pigmento gris asociado a la última etapa de maduración. La mayoría de los genes caracterizados responsables de este fenotipo (genes *whi*) codifican proteínas reguladoras (*whiG*, *J*, *A*, *B*, *H*, *I* y *D*). Los más estudiados son los siguientes: (i) *whiG* que codifica un factor sigma que gobierna el inicio de la esporulación (Chater *et al*., 1989), (ii) *whiH* que codifica un regulador transcripcional de la familia GntR que juega un papel clave en la septación del micelio aéreo (Ryding *et al*., 1999) y (iii) *whiD* que junto con el factor sigma *sigF* interviene en el engrosamiento de la pared de la espora y en el depósito del pigmento gris asociado a la esporulación que sintetiza WhiE (Chater, 1993; Potúcková *et al*., 1995).

Otros mutantes están afectados tanto en la diferenciación morfológica como en la producción de antibióticos. Son incapaces de formar micelio aéreo y, salvo alguna excepción, también de producir antibióticos. Por su fenotipo (carecen del aspecto de pelusa del micelio aéreo) han sido denominados mutantes calvos (en inglés, mutantes *bald*; ver figura 1.3). En la actualidad se han descrito más de 20 genes que generan un fenotipo calvo en *S. coelicolor* (genes *bld*). El primero fue el gen *bldA* que codifica el tRNA de leucina para el codón raro UUA, muy poco frecuente en bacterias de gran contenido en G+C (Lawlor *et al*., 1987). En *S. coelicolor* solamente 145 genes contienen el triplete TTA. La mayoría están relacionados con el metabolismo secundario, como el regulador *actII*-ORF4 (Fernández-Moreno *et al*., 1991), o con el proceso de diferenciación morfológica, como el factor transcripcional *bldH* (Nodwell *et al*., 1996). Otros genes *bld* destacables son *bldD*, *bldB* y *bldN* que codifican dos reguladores transcripcionales y un factor sigma extracitoplasmático, respectivamente (Elliot *et al*., 1998; Pope *et al*., 1998; Bibb *et al*., 2000).

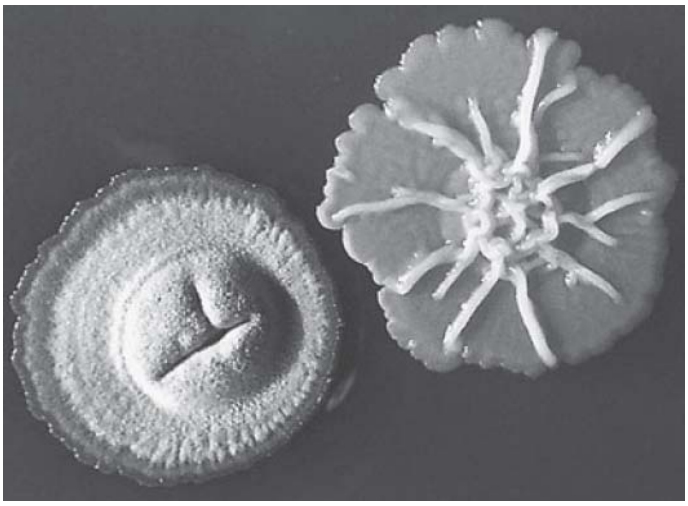

Figura 1.3: Comparación de una colonia silvestre de *S. coelicolor* (izquierda) con una colonia de *S. coelicolor* interrumpida en el gen *bldA* (derecha). No hay todavía una teoría que explique las estructuras que se desarrollan en la superficie de la colonia mutante en *bldA*. Imagen tomada de Hopwood, 2007.

Recientemente se ha demostrado que la transcripción de al menos cinco sRNA (RNA de pequeño tamaño, no codificante) en *S. coelicolor* depende de la fase de desarrollo y de los genes *bldA, bldB, whiG* y *whiB*. La función de estos sRNA es desconocida, aunque podrían tener un efecto regulador interaccionando con RNA mensajeros y/o proteínas (Swiercz *et al*., 2008).

1.1.8. Señales intercelulares en *Streptomyces*: γ-butirolactonas

Streptomyces sintetiza moléculas de bajo peso molecular llamadas γ-butirolactonas, las cuales se acumulan en el medio de forma gradual en relación al crecimiento. Dicha acumulación determina la densidad celular del microorganismo (quórum) e implica una respuesta de la maquinaria génica de la célula hacia dicha señal. Estas moléculas funcionan a modo de hormonas bacterianas e intervienen, tanto en el crecimiento vegetativo, como en la producción de antibióticos y la esporulación (Takano, 2006). La primera γ-butirolactona descubierta en *Streptomyces* fue denominada factor A (por factor de autorregulación). Fue encontrada en *S. griseus* como una molécula extracelular, difusible, capaz de complementar algunos mutantes *bald* no productores de micelio aéreo ni estreptomicina (Khokhlov *et al*., 1967). El gen *afsA* codifica la enzima responsable de su síntesis (Horinouchi *et al*., 1989; Kato *et al*., 2007). El factor A se acumula en el medio de forma gradual alcanzando su concentración máxima (100 nM) aproximadamente en la mitad de la fase exponencial (Ando *et al*., 1997). Cuando alcanza dicha concentración se une a su proteína receptora, el represor transcripcional ArpA, haciendo que se suelte de los promotores a los que se une (Onaka y Horinouchi, 1997). La diana principal de ArpA es el gen *adpA* (Kato *et al*., 2004). AdpA regula una gran cantidad de genes (al menos 72; Ohnishi *et al*., 2005; Hara *et al*., 2009), entre los que se incluyen: *strR* (activador específico de ruta de estreptomicina), *adsA* (factor sigma extracitoplasmático homólogo a *bldN* de *S. coelicolor*), *wblA* (regulador de la morfogénesis de la familia de WhiB), *ssgA* (regulador de la septación del micelio aéreo) y *amfR* (activador transcripcional del operón *amfTSBA* de síntesis de SapB). El péptido SapB es fundamental en la formación

del micelio aéreo debido a su efecto en la rotura de la tensión superficial que ejerce la interfase agua-aire del sustrato. La expresión constitutiva del operón homologo a *amfTSBA* en *S. coelicolor* (*ramCSABT*) restaura la formación del micelio aéreo en todos los mutantes *bald* (Willey *et al*., 2006). Esto sugiere que la acción de todos los genes *bld* culmina en la formación y transporte del péptido SapB, y que a su vez, todos están regulados por el mecanismo de *quorum sensing* dependiente de la proteína AdpA y el factor A. En *S. coelicolor* se han encontrado genes homólogos a *afsA* (*scbA*), *adpA* (*bldH*) y *arpA*. Aunque de este último hay dos homólogos (*crpA* y *crpB*; Onaka *et al*., 1998).

1.1.9. Un proceso de muerte celular precede a la producción de antibióticos en *Streptomyces*

A pesar de que la mayoría de los procesos de producción industrial de metabolitos secundarios tienen lugar en cultivos sumergidos, la mayor parte de los estudios de diferenciación morfológica y producción de metabolitos secundarios se han desarrollado sobre medio sólido. El hecho de que la mayoría de los *Streptomyces* no esporulen bajo estas condiciones de cultivos sumergidos ha llevado durante mucho tiempo a suponer que no ocurren cambios morfológicos claros en dichas condiciones (Daza *et al*., 1989; Novella *et al*., 1992). Sin embargo, el proceso de diferenciación morfológica y bioquímica parece no ser tan diferente en ambas condiciones (Manteca *et al*., 2008).

En medio sólido un primer micelio, llamado micelio sustrato, crece embebido en el sustrato. Posteriormente, sobre el micelio sustrato crece un segundo micelio, llamado micelio aéreo. La producción de antibióticos y la esporulación están condicionadas a la formación de este micelio aéreo, como se observa en los mutantes calvos incapaces de formar dicho micelio. A su vez, la formación del micelio aéreo está sujeta a la aportación de los nutrientes que provienen de la lisis del micelio sustrato. Por tanto, un paso previo de muerte celular precede a la diferenciación morfológica y bioquímica. Manteca y colaboradores (2008) han publicado recientemente ciertas similitudes entre

lo que sucede en cultivos de *S. coelicolor* en medio sólido y medio líquido. Así, al germinar las esporas en medio líquido se forma un primer tipo de micelio (tipo I), análogo al micelio sustrato de los cultivos sólidos. Las hifas del micelio tipo I se alargan y se ramifican formando una especie de agregados esféricos o glomérulos. El grado de crecimiento radial depende del medio y del inóculo, los cuales determinan el tamaño final de los glomérulos. Al mismo tiempo comienza un proceso de muerte celular en el centro de los glomérulos. Con los nutrientes liberados se nutre un segundo tipo de micelio (tipo II) que se desarrolla sobre el primero. Este proceso es semejante al crecimiento del micelio aéreo sobre el micelio sustrato en cultivos sólidos. El comportamiento bifásico de los cultivos líquidos de *S. coelicolor*, con dos fases de crecimiento rápido claramente separadas por una fase de decrecimiento, se corresponde, según estos autores, con la formación de los micelios tipo I y II, respectivamente. La interfase se corresponde en este caso con la fase de muerte del primer micelio. Estos autores han demostrado que únicamente el micelio tipo II sintetiza los antibióticos actinorrodina y undecilprodigiosina.

Por consiguiente, parece que un mecanismo programado de muerte celular, equivalente al mecanismo de apoptosis en organismos superiores, es el principal desencadenante de la producción de antibióticos en *Streptomyces* (Fernández y Sánchez, 2002; Bibb, 2005; Manteca *et al*., 2006; 2008). De hecho, la acumulación en el medio de *N*-acetilglucosamina (componente del peptidoglicano de las paredes celulares bacterianas), resultado de la muerte del micelio sustrato, es uno de los principales desencadenantes de la producción de antibióticos en *S. coelicolor* y otras especies de *Streptomyces* (Rigali *et al*., 2008).

1.1.10. *Streptomyces coelicolor*: características generales

Streptomyces coelicolor es el organismo modelo de los actinomicetos y más concretamente del género *Streptomyces* (Hopwood, 1999). Su genética molecular ha sido desarrollada durante las últimas cinco décadas especialmente gracias al esfuerzo del Dr. David Hopwood y colaboradores en el instituto John Innes en Norwich (Reino

Unido). Las técnicas han sido recopiladas en el libro *Practical Streptomyces Genetics* (Kieser *et al*., 2000). Además, la secuencia completa de su genoma está disponible con libre acceso desde hace varios años (Bentley *et al*., 2002).

El genoma de *S. coelicolor* revela las adaptaciones que dicho microorganismo ha sufrido para adaptarse a las condiciones de vida del suelo. Destaca el gran número de genes que contiene su genoma (7825) comparado con otras bacterias como *Escherichia coli* (4289) y *Bacillus subtilis* (4099); ó incluso con eucariotas, como *Saccharomyces cerevisiae* (6203). De hecho, las bacterias del género *Streptomyces* presentan los cromosomas más grandes y el mayor número de genes de entre las bacterias secuenciadas (Bentley *et al*., 2002). Ni siquiera otros actinomicetos presentan cromosomas tan grandes. Así, por ejemplo, los tamaños de los cromosomas de *Mycobacterium tuberculosis* (4,4 Mb), *Corynebacterium diphtheriae* (2,5 Mb) ó *Kocuria rhizophila* (2,7 Mb) quedan bastante lejos de los tamaños de los cromosomas de las cuatro especies secuenciadas de *Streptomyces*; *S. coelicolor* (8,7 Mb), *S. avermitilis* (9,0 Mb), *S. griseus* (8,5 Mb) y *S. scabies* (10,1 Mb). Además, los genomas de dichas especies contienen una proporción sin precedentes entre las bacterias de genes reguladores, factores sigma, proteínas transportadoras, enzimas secretadas y metabolitos secundarios. Las características generales del cromosoma de *S. coelicolor* se muestran en la tabla 1.2.

Tamaño total	8.667.507 pb
Contenido en G+C	72,12 %
DNA no codificante	11,1 %
Tamaño medio de los genes	991 pb
RNA ribosomales	6 x (16S-23S-5S)
RNA de transferencia	63
Otros RNA estables	3
Marcos de lectura	7825
Proteínas reguladoras	965 (12,3 %)
Proteínas transportadoras	614 (7,8 %)
Proteínas secretadas	819 (10,5 %)
Factores sigma	65
Sistemas de dos componentes completos	53
Metabolitos secundarios	22
Antibióticos	4

Tabla 1.2: Características generales del cromosoma de *S. coelicolor* A3(2). Modificado de Bentley *et al*. (2002).

La cepa silvestre A3(2) porta tres plásmidos de diferentes características. Un plásmido lineal de 356 Kb denominado SCP1 que contiene los genes de biosíntesis del antibiótico metilenomicina, un plásmido circular transmisible de 30 Kb y de pocas copias denominado SCP2, y un plásmido de 17 Kb normalmente integrado en el cromosoma denominado SLP1 (Chater y Hopwood, 1993). La cepa M145, utilizada normalmente en los laboratorios, es una cepa libre de los plásmidos SCP1 y SCP2, por lo que no produce metilenomicina (Kieser *et al*., 2000). *S. coelicolor* A3(2) produce 4 antibióticos estructuralmente distintos: actinorrodina (poliquétido aromático), undecilprodigiosina (antibiótico tripirrólico), antibiótico dependiente de calcio (antibiótico peptídico no ribosomal) y metilenomicina (antibiótico del tipo de las ciclopentanonas). Los dos primeros, además de antibióticos, son compuestos pigmentados. Su fácil extracción y posterior estabilidad permiten que su cuantificación se consiga fácilmente por espectrofotometría. Esto hace de *S. coelicolor* un buen organismo modelo para el estudio de la producción de antibióticos. En la figura 1.4 se muestra la estructura química de los cuatro antibióticos:

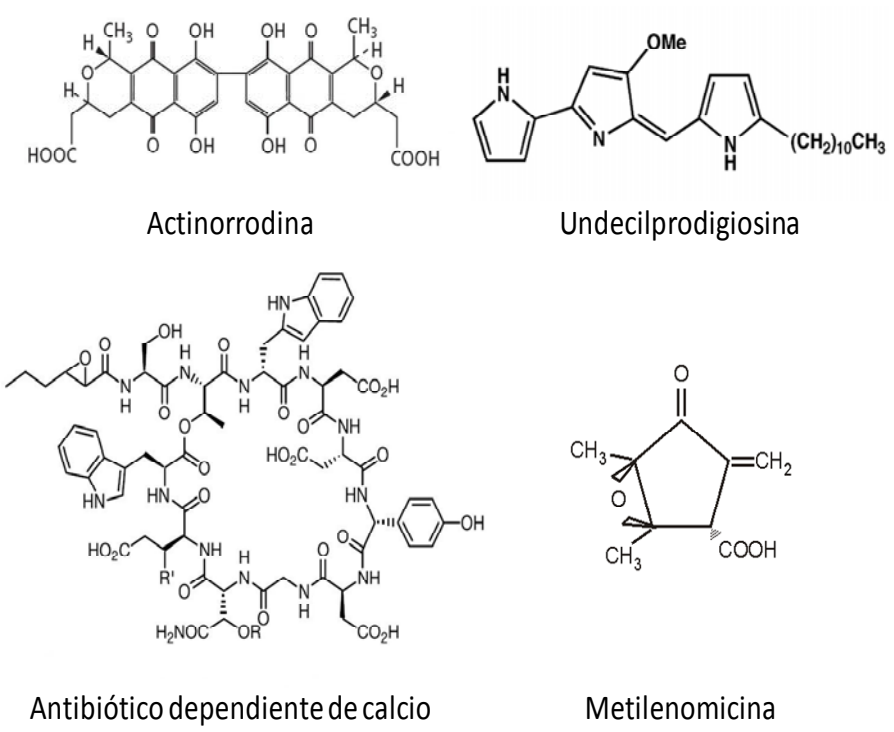

Figura 1.4: Estructura química de los cuatro antibióticos producidos por *S. coelicolor* A3(2).

a) Actinorrodina: es un antibiótico pigmentado, de color azul a pH básico y rojo a pH ácido y neutro, que fue descrito la primera vez como un indicador de ácido-base (Brockmann y Hieronimus, 1955). Es el responsable del color azul-púrpura característico de *S. coelicolor* (*color de cielo*). Se ha propuesto como un buen colorante para la industria alimentaria gracias a su solubilidad, estabilidad y nula toxicidad (Zhang *et al*., 2006). La actividad antibiótica es muy limitada en bacterias Gram-positivas y ausente en bacterias Gram-negativas (Wright y Hopwood, 1976a). Se suele ensayar frente a bacterias Gram-positivas como *B. subtilis* y *Staphylococcus aureus*. Se han descrito seis análogos de la molécula (α-, β-, γ-, ε-actinorrodina y ácido actinorrodínico), aunque el número podría ascender al menos hasta diez (Bystrykh *et al*., 1996). La γ-actinorrodina (presenta los grupos carboxilos de los extremos ciclados) es la forma principal de secreción del compuesto (Bystrykh *et al*., 1996; Kieser *et al*., 2000).

b) Undecilprodigiosina: es un compuesto muy insoluble en agua lo cual impide su difusión en el medio. Su localización es intracelular. Frecuentemente se denomina pigmento rojo (*red*) ya que se trata realmente de una mezcla de al menos cuatro prodigioninas, de entre las cuales, las más abundante es la undecilprodigiosina (Tsao *et al*., 1985). Las prodigioninas se forman por la condensación de tres anillos pirrólicos y se diferencian por el hidrocarburo presente en el tercero de ellos. Fueron descubiertas por primera vez en especies del género *Serratia* (y más tarde también en especies del género *Streptomyces*) por su actividad antibacteriana, antifúngica y antiprotozoos (Williams *et al*., 1971; Williams y Quadri, 1980; Rudd y Hopwood, 1980). Se las ha asignado además un efecto inmunosupresor y anticancerígeno (Williamson *et al*., 2007). Recientemente se ha demostrado su toxicidad selectiva frente a todas las líneas celulares responsables del cáncer de mama (Ho *et al*., 2007).

c) CDA (*calcium-dependent antibiotic*): el antibiótico dependiente de calcio es un lipopéptido cíclico de 11 aminoácidos con un hidrocarburo de 6 carbonos en su extremo N-terminal. Fue descrito por primera vez en *S. coelicolor* por Lakey *et al*.

(1983) y caracterizado químicamente por Kempter *et al*. (1997). Su actividad antibacteriana depende de la presencia de calcio en el medio. Su cuantificación se puede realizar mediante bioensayo utilizando organismos como *Bacillus mycoides* o *S. aureus* y añadiendo nitrato cálcico al medio (Kieser *et al*., 2000).

d) Metilenomicina: es un compuesto con un núcleo ciclopentanona, incoloro y lipofílico, que fue descubierto por primera vez en *Streptomyces violaceoruber* por Haneishi *et al*. (1974). Más tarde fue identificado también en *S. coelicolor* A3(2) (Wright y Hopwood, 1976b). Este antibiótico es un caso excepcional en cuanto que sus genes biosintéticos se localizan en un plásmido (Bentley *et al*., 2002). Su actividad antibacteriana se ha descrito en bacterias Gram-negativas y Gram-positivas. Es especialmente efectivo en especies del género *Proteus* y ambientes ácidos (Terahara, 1979). Su cuantificación se determina por HPLC o por bioensayo, generalmente, frente a cepas de *S. coelicolor* SCP1$^-$.

1.2. El regulón *pho*

El regulón *pho* se definió en *E. coli* como el conjunto de genes directamente controlados por un sistema de dos componentes que responde a la escasez de fosfato inorgánico en el medio (Wanner, 1993). Dicha respuesta se ha estudiado en microorganismos procariotas y eucariotas. Dentro de los procariotas, el mayor número de estudios corresponde a *E. coli* y *B. subtilis*, aunque también destacan los trabajos realizados en *Sinorhizobium meliloti* (Bardin *et al*., 1996; Voegele *et al*., 1997; Bardin y Finan, 1998; Parker *et al*., 1999; Ruberg *et al*., 1999; Krol y Becker, 2004; Yuan *et al*., 2006a; 2006b), *Synechocystis* (Hirani *et al*., 2001; Suzuki *et al*., 2004; Juntarajumnong *et al*., 2007a; 2007b; 2007c), *Corynebacterium glutamicum* (Ishige *et al*., 2003; Kocan *et al*., 2006; Schaaf y Bott, 2007) y *S. coelicolor* (ver anexo). Actualmente, el regulón *pho* de *E. coli* cuenta con 47 genes identificados (Lamarche *et al*., 2008), aunque el número de genes pertenecientes a dicho regulón seguramente sea mucho mayor como indican los estudios proteómicos realizados por VanBogelen *et al*. (1996).

1.2.1. Transporte de compuestos que contienen fósforo en las bacterias

La membrana citoplasmática de las bacterias forma una barrera física no solo para las grandes y pequeñas moléculas hidrofílicas, sino también para los iones, los cuales tienen que ser transportados por proteínas específicas de transporte embebidas en la membrana. El control del transporte de estas moléculas hacia el interior y el exterior de la célula es de vital importancia para el funcionamiento de la misma. Las bacterias utilizan tres tipos de transportadores: (i) transportadores primarios, los cuales utilizan la energía proveniente de la hidrólisis de ATP para conducir los iones y solutos a través de la membrana; (ii) transportadores secundarios, los cuales utilizan la energía libre generada por los transportadores primarios almacenada en forma de gradiente electroquímico, y (iii) transportadores acoplados a translocación de grupos, los cuales acoplan la entrada del sustrato a favor de gradiente con una modificación química que permite no romper dicho gradiente (dicha modificación química conlleva gasto de energía).

El transporte de compuestos que contienen fósforo en las bacterias es realizado por transportadores pertenecientes a las familias ABC (*ATP-binding cassette*) y MFS (*major facilitator superfamily*), que realizan un transporte primario y secundario, respectivamente. Los transportadores ABC están formados por una proteína de unión al sustrato, una o dos proteínas de membrana y una proteína citoplasmática con función ATPasa que aporta la energía libre necesaria para el transporte (Hyde *et al*., 1990; Higgins, 1992). Los transportadores MFS están constituidos por una proteína transmembrana formada por dos mitades simétricas que generan un poro en la membrana. Estos transportadores no usan proteína de unión al sustrato, ni bomba ATPasa (Marger y Saier, 1993). Funcionan mediante tres mecanismos: (i) uniporte, se transporta un solo tipo de sustrato debido al propio gradiente que este provoca; (ii) simporte, dos o más sustratos se transportan a la vez, en la misma dirección, utilizando el gradiente electroquímico producido por uno de ellos; y (iii) antiporte, dos o más sustratos se transportan en direcciones opuestas (Law *et al*., 2008). Aproximadamente el 25 % de las proteínas transportadoras conocidas en procariotas pertenecen a la superfamilia de transportadores MFS (Saier *et al*., 1999). En *E. coli* se han caracterizado

tres transportadores de tipo ABC (Pst, Ugp y Phn) y tres de tipo MFS (Pit, GlpT y UhpT) para compuestos que contienen fósforo (ver figura 1.5).

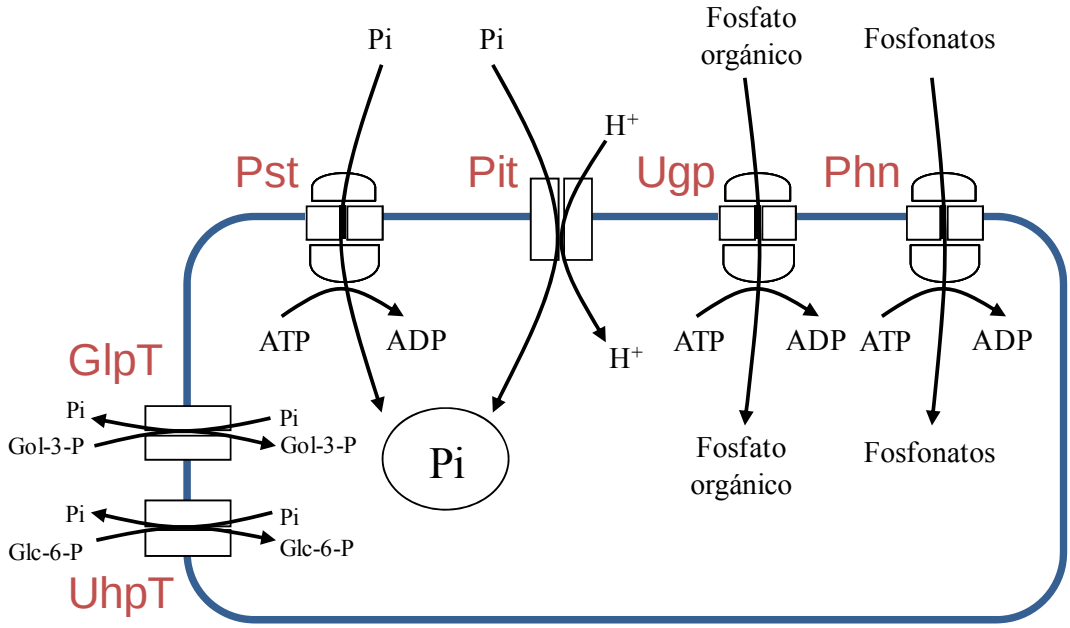

Figura 1.5: Esquema del transporte de compuestos que contienen fósforo en *E. coli*. Las figuras correspondientes a los transportadores Pst, Ugp y Phn representan las proteínas que configuran un transportador tipo ABC. Las figuras correspondientes a los transportadores Pit, GlpT y UhpT representan la proteína que constituye un transportador MFS tipo. El rectángulo que engloba la figura representa la membrana citoplasmática de la célula. Pi (fosfato inorgánico), Gol-3-P (glicerol-3-fosfato) y Glc-6-P (glucosa-6-fosfato). En la reacción de hidrólisis del ATP se ha omitido incluir el grupo Pi liberado por no sobrecargar la figura.

La forma principal de asimilación del fósforo en las bacterias es el ortofosfato (HPO_4^{2-}) que a menudo se abrevia con la expresión de fosfato inorgánico (Pi). El sistema Pit (*phosphate inorganic transport*) transporta el Pi quelado con un metal divalente como Ca^{2+}, Mg^{2+}, Co^{2+} y Mn^{2+} ($MeHPO_4$) mientras que el sistema Pst (*phosphate specific transport*) transporta el Pi en su forma monobásica ó dibásica ($H_2PO_4^-/HPO_4^{2-}$; van Veen *et al*., 1994). Cuando el Pi es abundante *E. coli* utiliza el sistema Pit, cuya constante de Michaelis Menten (K_m) se sitúa alrededor de 25 µM (Rosenberg *et al*., 1977). Si la concentración externa de Pi presenta una concentración

menor (en el rango μM) se induce el sistema de transporte de fosfato específico (Pst), cuya K_m es de alrededor de 0,25 μM (Rosenberg *et al*., 1977). Es decir, el sistema Pst tiene 100 veces más de afinidad por Pi que el sistema Pit. Por eso, generalmente al transportador Pit se le denomina transportador de baja afinidad y al transportador Pst transportador de alta afinidad (Higgins, 1992). El ión arseniato (AsO_4^{3-}) debido a su gran similitud con el ión fosfato (PO_4^{3-}) es también transportado por dichos sistemas (Willsky y Mallamy, 1980). El arseniato sustituye al fosfato en muchos de los procesos de fosforilación celular. Esto convierte al arseniato en un compuesto muy tóxico, por lo que las bacterias han tenido que desarrollar sistemas capaces de destoxificar dicho ión.

En *Vibrio cholerae* se ha descrito un tercer tipo de transportador de Pi (NptA). Al igual que el transportador Pit realiza un transporte secundario, pero en vez de utilizar un gradiente de protones (H^+) utiliza un gradiente de sodio (Na^+). NptA presenta una gran homología con el cotransportador de tipo II sodio-fosfato que se encuentra exclusivamente en eucariotas superiores. No se ha encontrado transportadores homólogos en otras bacterias (incluida otras especies del genero *Vibrio*) por lo que se supone que dicha bacteria lo ha adquirido por transmisión horizontal (Lebens *et al*., 2002). La afinidad de dicho transportador hacia el Pi es 10 y 1000 veces menor que la de los transportadores Pit y Pst (respectivamente) de la propia bacteria. Estos autores relacionan su actividad con la patogenicidad del microorganismo.

Las bacterias son capaces de transportar azucares fosforilados y otros compuestos que contienen fósforo como alternativa al Pi. De este modo, en *E. coli*, GlpT y UhpT transportan glicerol-3-fosfato y glucosa-6-fosfato, respectivamente. Ambos realizan un antiporte fosfato orgánico/fosfato inorgánico por lo que no generan un balance positivo de fosfato hacia el interior (Larson, 1987; Sonna *et al*., 1988). Su función principal es acumular fosfato orgánico en condiciones fisiológicas de exceso de Pi (Maloney *et al*., 1990). Por el contrario, Ugp transporta fosfato orgánico (glicerofosfodiésteres y glicerol-3-fosfato) para utilizarlo como fuente de fosfato cuando el Pi escasea (Brzoska y Boss, 1988). No obstante, el fósforo no se encuentra siempre en las moléculas orgánicas como ésteres de fosfato, es decir con el fósforo unido al carbono mediante un enlace con un átomo de oxigeno. En algunos casos el átomo de

fósforo está unido directamente al átomo de carbono, como en los fosfonatos (Metcalf y Wanner, 1991). Algunas bacterias son capaces de transportar y romper estos enlaces para utilizar estos compuestos como fuentes de fósforo. *E. coli* utiliza el transportador Phn y enzimas especiales llamadas C-P liasas para tal fin (Wanner, 1994).

1.2.2. El regulón *pho* está controlado por un sistema de dos componentes

Las bacterias captan multitud de señales extracelulares mediante sistemas de dos componentes. La función de estos sistemas es fundamental para la adaptación y la supervivencia del microorganismo (Hoch, 2000; Stock *et al*., 2000). Típicamente el sistema consiste en dos genes que codifican una quinasa sensora y un regulador de respuesta. La quinasa sensora se autofosforila en un residuo de histidina cuando capta una determinada señal y transfiere el grupo fosfato a un residuo de aspártico del regulador de respuesta, el cual se activa. Generalmente el regulador de repuesta es un regulador transcripcional, aunque también puede actuar afectando la actividad enzimática de una proteína concreta.

El sistema de dos componentes que responde a la escasez de fosfato en *E. coli* es denominado PhoR-PhoB (Wanner, 1993). Esta nomenclatura no se ha seguido en otras bacterias: PhoR-PhoP (*B. subtilis*), PhoS-PhoR (*C. glutamicum*), SphS-SphR (*Synechocystis*), SenX3-RegX3 (*Mycobacterium smegmatis*), PhosS-PhosR (*Campylobacter jejuni*) y PnpR-PnpS (*Streptococcus pneumoniae*). En *Myxococcus xanthus* el sistema de control por fosfato es más complejo, ya que al menos tres sistemas de dos componentes (PhoR1-PhoP1, PhoR2-PhoP2 y PhoR3-PhoP3) y un regulador transcripcional (PhoP4) intervienen en la regulación por fosfato (Whitworth *et al*., 2008). En eucariotas inferiores, como *Saccharomyces cerevisiae* el sistema está formado por tres componentes; dos histidina quinasas, Pho80p-Pho85p, y un regulador transcripcional, Pho4p (Kaffman *et al*., 1994).

1.2.3. Interacción de los sistemas PhoR-PhoB y PhoR-PhoP de *E. coli* y *B. subtilis* con otros sistemas

Muchos reguladores de respuesta son fosforilados por quinasas sensoras de otros sistemas de dos componentes distintos, lo cual indica la capacidad de dichos sistemas para integrar diferentes señales. El fenómeno es denominado regulación cruzada (*cross-talk*). Por ejemplo, en mutantes *phoR* de *E. coli*, la proteína de respuesta PhoB puede ser activada por la proteína sensora CreC del sistema implicado en la regulación de genes del metabolismo del carbono (Amemura *et al*., 1990). Lo mismo ocurre con al menos seis quinasas sensoras más en dicha bacteria (Fisher *et al*., 1995; Zhou *et al*., 2005). Por otra parte, en *B. subtilis* la quinasa sensora PhoR es capaz de activar el regulador de respuesta YycF (Howell *et al*., 2006). El sistema YycG-YycF juega un papel esencial en la división celular y la homeostasis de la membrana celular (Fukuchi *et al*., 2000; Howell *et al*., 2003). En esta bacteria al menos tres sistemas reguladores intervienen en la respuesta a la escasez de fosfato. Uno es el sistema PhoR-PhoP (responsable de la inducción del regulón *pho*), otro es SigB (un factor sigma responsable de la respuesta al estrés general de la célula) y otro desconocido, independiente a estos dos anteriores (Antelmann *et al*., 2000; figura 1.6). La interacción entre estos sistemas se pone de manifiesto por la inducción de genes del regulón *pho* en mutantes *sigB*, y la inducción de genes del regulón *sigB* en mutantes *phoPR* (Antelmann *et al*., 2000; Prágai y Harwood, 2002). Además, la existencia de un promotor dependiente de σ^B en el operón *phoPR* indica la implicación directa de σ^B en la regulación de dicho sistema (Paul *et al*., 2004).

La activación del regulón *pho* en *B. subtilis* necesita de dos sistemas reguladores, ResE-ResD y AbrB, (Hulett, 1996). Una mutación en el regulador de respuesta *resD* reduce un 80% la activación del regulón *pho*, y una doble mutación de *resD* y *abrB* anula completamente la inducción de dicho regulón (Sun *et al*., 1996). El sistema ResE-ResD está involucrado principalmente en la regulación de la respiración aeróbica o anaeróbica del bacilo (Nakano *et al*., 1996), mientras que AbrB es un regulador dependiente de la fase de desarrollo (Perego *et al*., 1988). La expresión de ResE-ResD requiere de PhoP, por lo que ambos sistemas establecen una

retroalimentación positiva (Birkey *et al*., 1998; figura 1.6). Por otro lado, la activación del regulón *pho* en *B. subtilis* está reprimida por el regulador transcripcional Spo0A, el cual reprime los genes *resDE* y *abrB* y activa los genes responsables de la esporulación (Jensen *et al*., 1993; Piggot, 1996; Hulett, 1996; Prágai *et al*., 2004; figura 1.6). Este antagonismo se justifica por una secuencia de respuestas del microorganismo. En un primer momento, el regulón *pho* se encarga de la respuesta inicial a la escasez de fosfato, pero si es insuficiente, Spo0A conmuta la respuesta ejercida por PhoR-PhoP, por medio de la represión de *abrB* y *resDE*, por el desencadenamiento de la esporulación (Hulett, 1996).

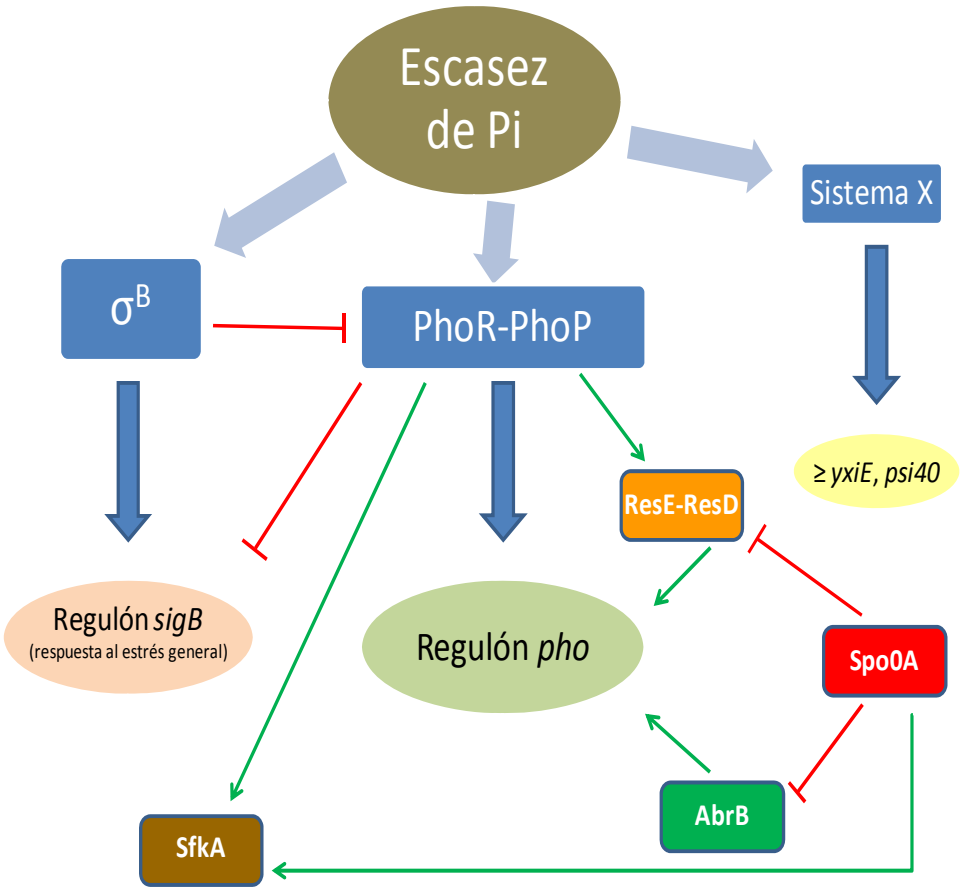

Figura 1.6: Esquema de los sistemas que intervienen en la respuesta a la escasez de Pi en el medio en *B. subtilis*. Basado en las publicaciones de: Antelmann *et al*. (2000); Paul *et al*. (2004); Sun *et al*. (1996); Birkey *et al*. (1998); Hulett (1996) y Allenby *et al*. (2006). Al menos dos genes (*yxiE* y *psi40*) son regulados por la escasez de Pi en el medio de una forma totalmente independiente de PhoR-PhoP y σ^B. El sistema que realiza dicha regulación es desconocido y por eso se le ha denominado sistema X. Las conexiones representadas en el esquema indican activación (líneas con punta de flecha y color verde) o represión (líneas rematadas con un segmento y color rojo).

La conexión de los procesos de regulación de la esporulación en *B. subtilis* con los procesos de regulación mediados por PhoR-PhoP, se pone de manifiesto una vez más con la doble regulación positiva ejercida por Spo0A y PhoP sobre el gen responsable del factor de muerte dependiente de esporulación, *skfA* (*sporulation killing factor*; Allenby *et al*., 2006). SkfA induce la lisis de células que no han sido capaces de iniciar el proceso de esporulación, lo cual le sirve al microorganismo como fuente de nutrientes y como control de la esporulación (Fawcett *et al*., 2000; figura 1.6).

1.2.4. Implicación del regulón *pho* en la respuesta al estrés y la patogenicidad microbiana

El número creciente de genes pertenecientes al regulón *pho* sin una función evidente en el metabolismo del fosfato apunta a una función más general del regulón *pho* que la de intervenir simplemente en la homeostasis de dicho nutriente. Diversos trabajos relacionan al regulón *pho* con la síntesis de inductores de diferentes respuestas al estrés, como el polifosfato (Kornberg *et al*., 1999; Brown y Kornberg, 2004) y el guanosín-3´-5´-bispirofosfato (ppGpp; Cashel y Kalbacher, 1970). De este modo, el gen (*ppk*), que codifica la enzima responsable de la síntesis de polifosfato (polifosfato quinasa), está regulado en muchas especies por la proteína PhoB/PhoP (Kato *et al*., 1993; Rao y Kornberg, 1999; Ghorbel *et al*., 2006; Yuan *et al*., 2006b). Por otro lado, la síntesis de ppGpp en *E. coli* disminuye en mutantes *pho*, y viceversa, mutantes con menor producción de ppGpp presentan una menor activación del regulón *pho* (Spira y Yagil, 1998; Spira *et al*., 1995). Además, los genes *sigB* y *rpoS*, que codifican factores sigma implicados en la respuesta al estrés, están regulados directa o indirectamente por PhoP/PhoB, en *B. subtilis* y *E. coli*, respectivamente (Antelmann *et al*., 2000; Schurdell *et al*., 2007).

Muchos trabajos relacionan también al regulón *pho* con la patogenicidad microbiana (revisado por Lamarche *et al*., 2008). Mayoritariamente los efectos del

regulón *pho* en la patogenicidad microbiana están relacionados con la respuesta al estrés ácido y la producción de biopelículas (*biofilms*). Varios autores sugieren que el sistema PhoR-PhoB es capaz de detectar la acidez del medio y posteriormente regular genes implicados en la respuesta al estrés por acidez (Tucker *et al*., 2002; Seputiene *et al*., 2006). La resistencia a la acidez es fundamental para organismos patógenos de ambientes ácidos, como el intestino. Así, el gen *asr*, inducible por pH ácido y relacionado con una función de protección de las proteínas periplásmicas a la acidez (Seputiene *et al*., 2003), está activado por PhoB en *E. coli* (Suziedeliene *et al*., 1999).

En los procesos de patogénesis la capacidad del microorganismo de producir biopelículas proporciona al microorganismo patógeno un ambiente de protección frente al entorno del hospedador. Además, las biopelículas sirven para incrementar la concentración del factor de virulencia producido por el microorganismo patógeno. Todo ello hace que el patógeno sea más virulento y más resistente a los mecanismos de defensa del hospedador. En el patógeno de plantas, *Agrobacterium tumefaciens*, la formación de biopelículas es inducida en condiciones de escasez de Pi por el sistema PhoR-PhoB (Danhorn *et al*., 2004). La implicación del regulón *pho* en la formación de biopelículas también ha sido descrita en *E. coli* y en varias especies de *Pseudomonas* (Ren *et al*., 2004; Parsek y Greenberg, 2005; Monds *et al*., 2001; 2007).

1.2.5. Activación y desactivación del regulón *pho*: modelo de Wanner

La concentración de Pi extracelular parece ser la señal que desencadena la activación del sistema PhoR-PhoB en la mayoría de los microorganismos estudiados, con la excepción de, al menos, la levadura *S. cerevisiae*, en la que la concentración de Pi intracelular también interviene en la activación de dicho sistema (Auesukaree *et al*., 2004). En *E. coli*, concentraciones de Pi en el medio inferiores a 4 μM desencadenan la activación del regulón *pho* (Wanner, 1997). La transducción de señales que van desde la captación de la señal de escasez a la activación del sistema PhoR-PhoB es un proceso que todavía no se conoce totalmente. Según el modelo propuesto por Wanner (1997), tres complejos de proteínas (PhoR-PhoB, PhoU y Pst) son los responsables de la

activación y desactivación del regulón *pho*. Curiosamente, en algunas bacterias, como *E. coli*, los genes que codifican las proteínas del transportador Pst (*pstSCAB*) y *phoU* forman un operón (Surin *et al*., 1986). Sin embargo, en otras, como *S. coelicolor* o *Aquifex aeolicus*, dichos genes se encuentran en operones distintos (Ghorbel *et al*., 2006; Oganesyan *et al*., 2005). Además, en el caso de *S. coelicolor*, *phoRP* y *phoU* se transcriben a partir de un promotor divergente (Sola-Landa *et al*., 2005). No se ha propuesto todavía ninguna explicación para esta variación en la sintenia de estos genes. Es muy probable que estas diferencias entre unas bacterias y otras den origen a distintos mecanismos de transmisión de las señales de activación.

Según el modelo de Wanner, en condiciones de escasez de Pi en el medio PhoR fosforila al regulador de respuesta PhoB, mientras que en condiciones de exceso muestra actividad fosfatasa sobre él (Carmany *et al*., 2003) para evitar la fosforilación del mismo por otras quinasas (Wanner y Wilmes-Riesenberg, 1992; McCleary y Stock, 1994). El modelo describe que en condiciones de exceso de Pi, PhoU interaccionaría con el complejo Pst y la proteína PhoR, formando lo que denominan el complejo represor, mientras que en condiciones de escasez de Pi se produciría un cambio conformacional en el sistema Pst que haría que PhoU se soltara del complejo y se pasara a lo que denominan complejo activador (ver figura 1.7). Estudios de transferencia de energía de resonancia de fluorescencia (FRET) demuestran que PhoU no interacciona con PhoR ni PhoB en condiciones de escasez de Pi, en las que el regulón *pho* está activo (Baek *et al*., 2007), en concordancia con lo expuesto en el modelo. Diversos trabajos demuestran que la desactivación del sistema PhoR-PhoB requiere de la proteína PhoU y del transportador Pst. Así, en *E. coli* cuando alguno de estos componentes son interrumpidos se genera un fenotipo de activación del regulón *pho* constitutivo (Muda *et al*., 1992; Steed y Wanner, 1993). Lo mismo se ha comprobado en otras bacterias como *M. smegmatis*, *Pseudomonas fluorescens* y *Synechocystis* (Kriakov *et al*., 2003; Monds *et al*., 2006; Juntarajummong *et al*., 2007c).

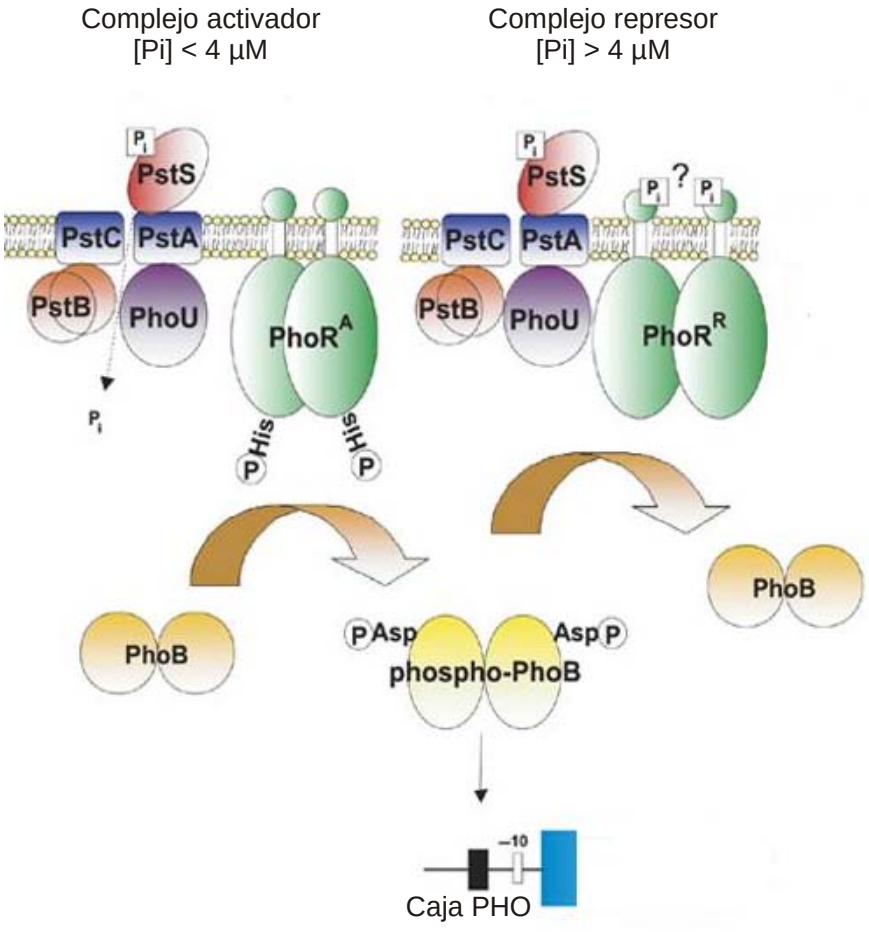

Figura 1.7: Control de la activación del regulón *pho* y de la transducción de señales desencadenada por la concentración de Pi en el medio según el modelo de Wanner, 1997. Imagen modificada de Lamarche *et al*. (2008).

1.2.6. Captación de la señal de escasez de fosfato: PhoR, PhoU y PstS

El modelo de Wanner no explica, entre otras cosas, que proteína capta la escasez de Pi en el medio y se lo transmite a PhoU para que esta se suelte de PhoR y se configure el complejo activador. La proteína PstS, de unión específica al Pi, y/o la proteína PhoR parecen ser las responsables (ver figura 1.7). PhoR presenta dos dominios transmembrana en su extremo N-terminal unidos por un dominio periplásmico. La mayor parte de la proteína se encuentra en el citoplasma, incluidos el subdominio de dimerización y autofosforilación (que contiene la histidina autofosforilable, His-213) y el subdominio catalítico y de unión a ATP (Shinagawa *et*

al., 1983). El dominio periplásmico podría ser el responsable de captar la señal de escasez de Pi y trasmitirlo al resto de la proteína haciendo que PhoU se suelte. Por otro lado, el sistema Pst podría sufrir un cambio conformacional al transportar el Pi y provocar el despegamiento de PhoU. En todo caso, la proteína PhoU tiene un papel esencial en el proceso de activación y desactivación del sistema PhoR-PhoB. Dos homólogos de la proteína PhoU de *E. coli* en *A. aeolicus* y *Thermotoga maritima* han sido cristalizados (Oganesyan *et al*., 2005; Liu *et al*., 2005). PhoU muestra una estructura novedosa consistente en dos dominios, de tres hélices α cada uno, prácticamente iguales. En ambos dominios existen dos lugares de unión de átomos de hierro junto con varios pares de un motivo conservado, E(D)XXXD, en posiciones equivalentes (Liu *et al*., 2005; figura 1.8).

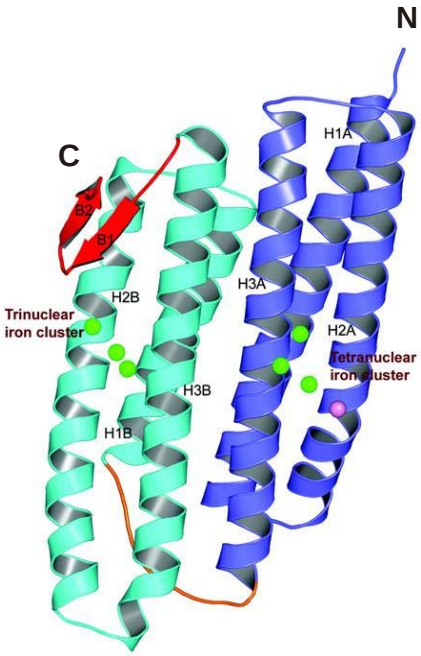

Figura 1.8: Estructura de PhoU de *Thermotoga mariritima* según los estudios cristalográficos de Liu *et al*. (2005). Los círculos representan átomos de hierro. La imagen está tomada de dicha publicación.

La delección de *phoU* causa una severa represión en el crecimiento de *E. coli*, mientras que la delección de *pstSCAB-phoU* no, a pesar de que en ambos casos se genera una activación constitutiva del regulón *pho* (Steed y Wanner, 1993). Además, en cepas de *E. coli* con expresión controlada del sistema PhoR-PhoB, se ha demostrado que la ausencia de PhoU provoca un aumento en la tasa de transporte de Pst (Rice *et al*., 2009). Se deduce, por tanto, que la expresión de PhoU es necesaria para controlar la

actividad de Pst, y evitar así un transporte incontrolado de Pi que genere una gran concentración de Pi intracelular dañina para la célula (Ugurbil *et al*., 1982). Estos datos parecen indicar una doble función de PhoU, una inhibiendo al transportador Pst en condiciones de exceso de Pi para mantener la homeostasis del fosfato, y otra modulando a PhoR en su dominio catalítico para promover la desfosforilación de PhoB y por tanto la desactivación del regulón *pho*.

El genoma de *B. subtilis* no presenta ningún miembro perteneciente a la familia PhoU, a pesar de que dicha familia está distribuida entre la mayoría de las bacterias estudiadas. Sin embargo, se ha comprobado en esta bacteria que ambientes reductores (generados por la adicción de DTT) inhiben la autofosforilación de PhoR y por tanto la inducción del regulón *pho* (Eldakak y Hulett, 2007). La capacidad de ResE-ResD de inducir ambientes oxidantes podría explicar el efecto positivo que ejerce este sistema en la activación del regulón *pho* (Schau *et al*., 2004). Estos autores proponen que las células creciendo en condiciones de exceso de Pi tienen quinonas reducidas en sus membranas que inhiben la autofosforilación de PhoR. En condiciones de escasez de Pi el sistema ResE-ResD se activa e induce la síntesis de oxidasas capaces de oxidar dichas quinonas reducidas liberando, por tanto, a PhoR de la inhibición de su autofosforilación. PhoP fosforilado induce la expresión de ResE-ResD produciéndose una retroalimentación positiva que genera la completa inducción del regulón *pho*. Esto está en concordancia con la existencia de un dominio PAS en la proteína PhoR justo al lado del dominio fosfotransferasa que contiene la histidina autofosforilable, His-360, en *B. subtilis* (Shi y Hulett, 1999). Los dominios PAS están relacionados con la captación de señales como el potencial rédox o la concentración de oxigeno y luz (Taylor y Zhulin, 1999).

1.2.7. Efecto de la fosforilación en la activación y unión de PhoB/PhoP al DNA

En los sistemas de dos componentes la captación de la señal es realizada por la quinasa sensora y transmitida al regulador de respuesta mediante fosforilación (Hoch, 2000; Stock *et al*., 2000). Los reguladores de respuesta actúan generalmente como

factores de transcripción. Están formados por un dominio receptor (DR), que recibe el grupo fosfato, y un dominio efector (DE), que se une específicamente al DNA. Las proteínas PhoB de *E. coli* y PhoP de *B. subtilis* pertenecen a la familia de reguladores transcripcionales tipo OmpR. Los miembros de esta familia presentan una estructura muy conservada con un DR en forma de ovillo y un DE con la estructura de tipo hélice-vuelta-hélice con ala. Ambos dominios están unidos por una cadena polipeptídica, flexible y de tamaño variable (Martínez-Hackert y Stock, 1997). La flexibilidad del conector ha dificultado la cristalización de la proteína entera en los miembros de esta familia. Solo en las proteínas DrrD y DrrB de *T. maritima* se ha determinado la estructura entera (Buckler *et al*., 2002; Robinson *et al*., 2003). En otros miembros, como PhoB, la cristalización se ha realizado en cada dominio de forma independiente (figura 1.9).

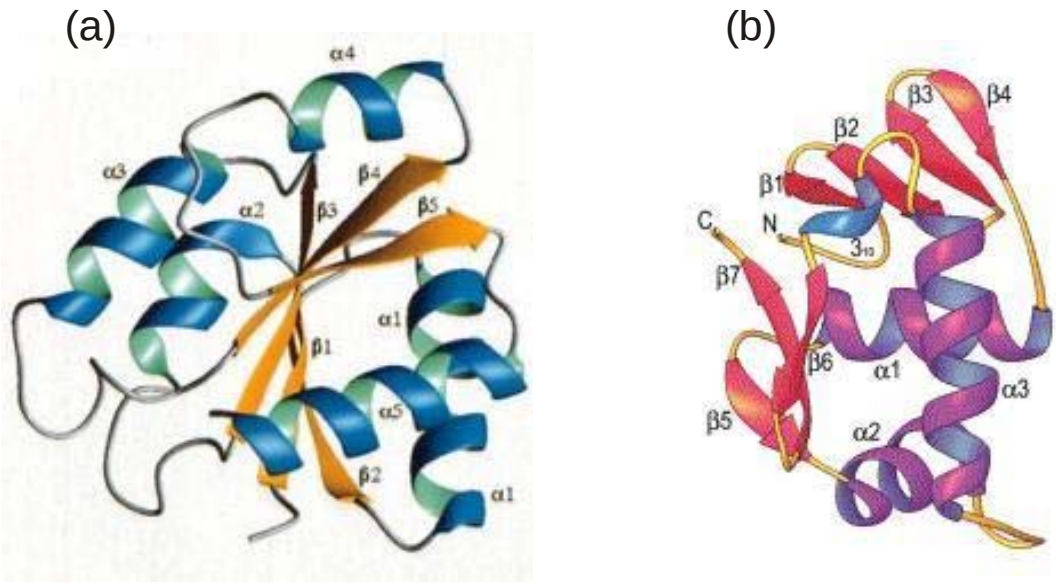

Figura 1.9: Estructura del dominio receptor (a) y del dominio efector (b) de PhoB de *E. coli* según los estudios cristalográficos de Solà *et al*. (1999) y Blanco *et al*. (2002). La estructura secundaria del DR está constituida por cinco cadenas β intercaladas con cinco hélices α desde el extremo N-terminal al C-terminal. La estructura terciaria adquiere una forma de ovillo con las cinco cadenas β formando una lamina β paralela central y con dos hélices α a un lado (α1 y α5) y tres a otro (α2, α3 y α4). La estructura secundaria del DE está constituida por 4 cadenas β (β1-β4) en su extremo N-terminal seguidas por tres hélices α (α1-α3), con una pequeña cadena β (β5) insertada entre las hélices α1 y α2, y por dos cadenas β (β6 y β7) en su extremo C-terminal. Las cadenas (β1-β4) y (β5-β7) forman dos laminas β antiparalelas a ambos lados de las tres hélices α que constituyen el motivo alado de la estructura terciaria.

Los estudios cristalográficos de $PhoB^{DE}$ unido a DNA realizados por Blanco *et al*. (2002) demuestran que dos protómeros se unen como un dímero, en tándem, en la misma cara del DNA. Los contactos proteína-proteína se realizan entre la lamina β del extremo C-terminal del protómero anterior y la lamina β del extremo N-terminal del protómero posterior. Por tanto la dirección de la unión es cabeza-cola/cabeza-cola. Esta unión al DNA de $PhoB^{DE}$ en dímeros está apoyada por los estudios realizados por Bachhawat *et al*. (2005) con $PhoB^{DR}$. Dichos estudios demuestran que $PhoB^{DR}$ se encuentra en forma dimérica antes y después de la fosforilación. Según estos autores, en su estado desfosforilado, $PhoB^{DR}$ está en equilibrio entre monómero y dímero (ver modelo en figura 1.10). En dicho estado de desfosforilación $PhoB^{DR}$ dimerizaría simétricamente usando las caras α1-α5, lo cual expondría, en la proteína entera, los DE de cada monómero en lados opuestos (forma inactiva); incompatible con la unión al DNA en tándem. La fosforilación del DR provoca un cambio conformacional en la estructura del dominio que hace que la superficie de contacto entre los dímeros se produzca de forma asimétrica por las caras α4-β5-α5 en vez de las caras α1-α5. Esta forma de dimerizar haría que los DE de cada monómero se expusieran hacia el mismo lado (forma activa); compatible con la unión en dímeros al DNA (figura 1.10). Sin embargo, al unirse por una misma superficie de contacto, la dirección de la unión del dímero del DR sería cola-cabeza/cabeza-cola, lo cual contrasta con la dirección de la unión cabeza-cola/cabeza-cola del dímero del DE descrita por Blanco *et al*. (2002). Esto implica la necesidad de un conector flexible entre los dos dominios (que está presente en la proteína entera) que permita el giro de un dominio con respecto al otro. De hecho, según Bachhawat *et al*. (2005), dicha flexibilidad permitiría a la proteína unirse al DNA tanto en una orientación en tándem como simétrica, o lo que es lo mismo, reconocer repeticiones directas o invertidas.

La misma forma de dimerización (α4-β5-α5) de $PhoB^{DR}$ ha sido descrita en dos mutantes distintos de PhoB capaces de activar el regulón *pho* en ausencia de fosforilación (Arribas-Bosacoma *et al*., 2007). Dímeros similares se han obtenido también en otros DR de miembros de la familia OmpR como MicA, DrrD, DrrB, PhoP, KdpE, TorR y ArcA (Bent *et al*., 2004; Buckler *et al*., 2002; Robinson *et al*., 2003; Bachhawat y Stock, 2007; Toro-Roman *et al*., 2005a; 2005b).

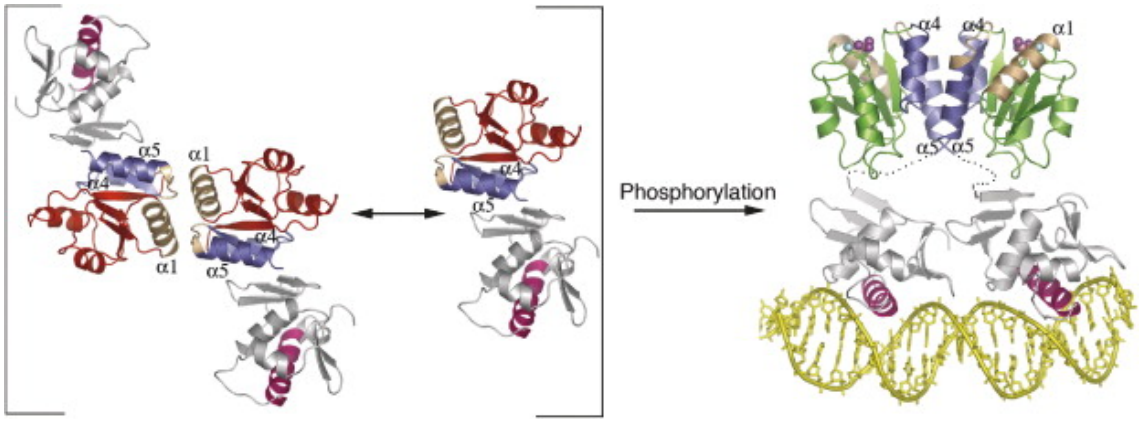

Figura 1.10: Modelo de activación por fosforilación de la proteína PhoB de *E. coli*. Imagen tomada de Bachhawat *et al*. (2005). PhoB desfosforilado se encuentra en equilibrio entre dímero y monómero (izquierda). En la forma fosforilada la configuración del dímero cambia permitiendo la unión de la proteína al DNA en tándem (derecha).

No obstante, la forma de dimerización dependiente o no de fosforilación difiere en algunas bacterias. Este es el caso del homologo de PhoB en *B. subtilis* (PhoP; Birck *et al*., 2003). La dimerización de $PhoP^{DR}$ en su forma desfosforilada se produce de distinto modo al descrito en $PhoB^{DR}$ de *E. coli*. PhoP presenta dos superficies de contacto, una cara A en la que participan las hélices α4, α5 y la cadena β5; y una cara B en la que participan las hélices α3, α4 y la cadena β4. La unión de dos monómeros deja dos caras libres de unión correspondientes a la cara A ó B de cada monómero, lo cual permite que se pueden unir sucesivos monómeros formando multímeros. La unión no es simétrica por lo que un monómero no tiene que rotar sobre el otro obligatoriamente para tener los dos DE hacia un mismo lado. Esto provoca una oligomerización de los monómeros de PhoP sobre el DNA sin la necesidad de fosforilación, lo cual contrasta con lo descrito en *E. coli*. De hecho una mutación en el residuo Arg113 de $PhoP^{DR}$, esencial para dicha unión asimétrica, impide la dimerización y la unión al DNA de la proteína tanto en su forma fosforilada como desfosforilada (Chen *et al*., 2003). Esto indica que la dimerización se produce de la misma forma independientemente de la fosforilación de la proteína, lo cual concuerda con lo observado *in vitro*; PhoP se une al

DNA tanto en su forma fosforilada como no fosforilada (Eder *et al*., 1999; Prágai *et al*., 2004). Este tipo de dimerización y unión al DNA no dependiente de fosforilación se ha descrito también en la proteína PhoP de *Salmonella entérica* (Perron-Savard *et al*., 2005). Sin embargo, el hecho de que la fosforilación de PhoP en *B. subtilis* sea necesaria para la activación de la transcripción (Liu y Hulett, 1997) indica un efecto de dicha fosforilación sobre la interacción de la proteína con los componentes de la RNA polimerasa y/o con el DNA. Se ha determinado *in vitro* que la fosforilación de PhoP aumenta 10 veces la afinidad de unión al DNA (Liu y Hulett, 1997).

Por tanto, el efecto de la fosforilación entre los miembros de la familia OmpR es muy variable. Así, en algunos casos, como en PhoB de *E. coli*, la fosforilación es esencial para la dimerización y unión al DNA (Allen *et al*., 2001; Bachhawat *et al*., 2005), aunque no para activar la transcripción (Makino *et al*., 1996), mientras que en otros, como en PhoP de *B. subtilis*, la fosforilación no es esencial para la dimerización y la unión al DNA (Birck *et al*., 2003; Prágai *et al*., 2004) pero sí para activar la transcripción (Liu y Hulett, 1997). Además, en algunos casos, como en OmpR de *E. coli*, la fosforilación incrementa la afinidad de unión al DNA pero no la formación de dímeros (Huang e Igo, 1996) y en otros casos, como en PhoP de *M. tuberculosis*, la fosforilación no aumenta la afinidad al DNA (Gupta *et al*., 2006) pero sí las interacciones proteína-proteína (Sinha *et al*., 2008). Por todo esto, se puede concluir que la fosforilación es esencial para el funcionamiento del regulador de respuesta, bien induciendo la formación de dímeros activos capaces de unirse al DNA, bien activando la transcripción, o bien incrementando en general las interacciones proteína-proteína y proteína-DNA.

1.2.8. Unión de PhoB al DNA: cajas PHO

La regulación de la transcripción de los genes pertenecientes al regulón *pho* está sujeta a la unión del regulador de respuesta (PhoB/PhoP) a secuencias específicas del DNA. Estas secuencias fueron definidas por primera vez en *E. coli* con el nombre de cajas PHO (Makino *et al*., 1986). Inicialmente se definieron como una secuencia de 18

nucleótidos comprendida por dos repeticiones directas de 7 nucleótidos separadas por una secuencia de 4 nucleótidos rica en pares AT (Makino *et al*., 1986). Posteriormente, tras los estudios cristalográficos de PhoB unido a DNA realizados por Blanco *et al*. (2002), se definió la caja PHO en *E. coli* como la suma de dos repeticiones directas de 11 nucleótidos cada una (correspondiendo cada repetición aproximadamente con una vuelta entera del DNA en conformación B). Dichas repeticiones están formadas por una parte conservada de 7 nucleótidos y una parte más variable de 4 nucleótidos. La secuencia menos conservada se corresponde con los nucleótidos contactados por el ala β6-β7 del dominio efector en el surco menor, mientras que la secuencia más conservada se corresponde con los nucleótidos contactados por la hélice de reconocimiento α3 en el surco mayor (figura 1.11). Un patrón similar de caja PHO se ha descrito también en *B. subtilis*, *Synechocystis*, *C. jejuni*, *C. glutamicum* y *S. meliloti* (Liu *et al*., 1998; Suzuki *et al*., 2004; Wösten *et al*., 2006; Schaaf & Bott, 2007; Yuan *et al*., 2006b).

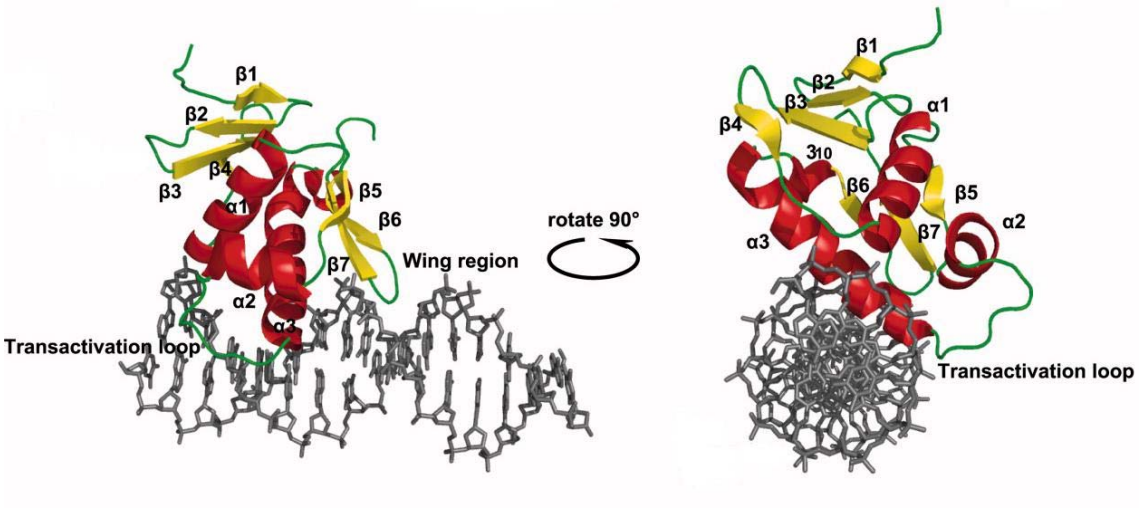

Figura 1.11: Estructura del dominio efector de PhoB de *E. coli* unido al DNA. Se muestran los contactos ejercidos entre el surco mayor del DNA y la hélice α3 (en ambas figuras), así como los ejercidos entre el surco menor del DNA y el ala β6-β7 (figura de la izquierda). La segunda imagen es la misma estructura rotada 90º. Tomado de Yamane *et al*. (2008).

La especificidad de la secuencia consenso de la caja PHO en cada microorganismo está determinada por la secuencia de aminoácidos del dominio de unión al DNA del respectivo regulador de respuesta. Dichos aminoácidos establecen contactos específicos e inespecíficos tanto con las bases nitrogenadas como con los esqueletos azúcar-fosfato de los nucleótidos. Blanco *et al*. (2002) demostraron que las dos únicas bases contactadas de forma directa por la hélice de reconocimiento de PhoB son las bases GT, situadas en el centro de la zona conservada de la repetición (CTGTCAT). Posteriormente, Yamene *et al*. (2008) han publicado que la quinta base (C) es también contactada directamente por la hélice α3. Además, han demostrado que una T del surco menor es contactada directamente por el ala de PhoB, indicando la importancia de los pares ricos en AT en dicha zona. Estos últimos autores han determinado la estructura de PhoB unido al DNA por métodos avanzados de RMN en ambientes acuosos, en los que las interacciones mediadas por moléculas de agua son resueltas con mayor fiabilidad. Según Blanco *et al*. (2002), las bases no contactadas de la repetición generan una estructura tridimensional concreta necesaria para la unión de PhoB. Dicho fenómeno, que había sido previamente denominado "indirect readout" (lectura indirecta) por Otwinowski *et al*. (1988), se ha explicado recientemente por la capacidad de las proteínas de distinguir cambios en el potencial electroestático debidos a la presencia de una secuencia de nucleótidos u otra (Rohs *et al*., 2009).

1.2.9. Regulación transcripcional ejercida por PhoB/PhoP: interacción con la RNA polimerasa

En *B. subtilis*, PhoP unido al DNA puede ejercer su función reguladora bien activando la transcripción o bien reprimiéndola (Liu *et al*., 1998). Para realizar el efecto represor no necesita interaccionar con la RNA polimerasa, basta con bloquear de forma estérica el proceso de transcripción (Liu *et al*., 1998; Makarewicz *et al*., 2006). Sin embargo, para activar la transcripción el regulador de respuesta no solo tiene que unirse al DNA, sino también interaccionar con la RNA polimerasa, como indican los estudios de mutagénesis realizados en PhoB y OmpR de *E. coli* y PhoP de *B. subtilis* (Pratt y Silhavy, 1994; Makino *et al*., 1996; Chen *et al*., 2004). Dichos estudios demuestran que la integridad del bucle de transactivación (localizado entre las hélices α2 y α3 del

dominio efector) es necesaria para que el regulador de respuesta interaccione con la RNA polimerasa y active la transcripción. La interacción del bucle de transactivación con los componentes de la RNA polimerasa difiere de unas proteínas a otras; PhoB y PhoP lo hacen con la subunidad σ mientras que OmpR lo hace con la subunidad α (Kondo *et al*., 1997).

La involucración del factor sigma principal (σ^{70}) de *E. coli* en la transcripción de los genes del regulón *pho* fue demostrada por Makino *et al*. (1993). Estos autores demostraron que los genes del regulón *pho* no se expresaban en mutantes alterados en dicho factor sigma, mientras que otros genes independientes de PhoB sí. Los genes *pho* presentan la caja -10 (TATAAT) reconocida por σ^{70} pero por el contrario no presentan la caja -35 (TTGACA). En sustitución a la falta de la caja -35 dichos genes presentan cajas PHO en dicha región. La unión de PhoB a las cajas PHO no solo permite la interacción con la RNA polimerasa sino que induce una curvatura en el DNA que favorece el reclutamiento de dicha enzima (Makino *et al*., 1996; Blanco *et al*., 2002). Por tanto, la expresión de los genes *pho* en *E. coli* está ligada a la unión de PhoB al DNA y a la interacción con σ^{70}. Algo similar ocurre en *B. sutilis* con su respectivo factor sigma principal (σ^{A}), aunque a diferencia de *E. coli*, posee al menos otro factor sigma capaz de interaccionar con PhoP, como es el caso del factor sigma específico del proceso de esporulación, σ^{E} (Paul *et al*., 2004).

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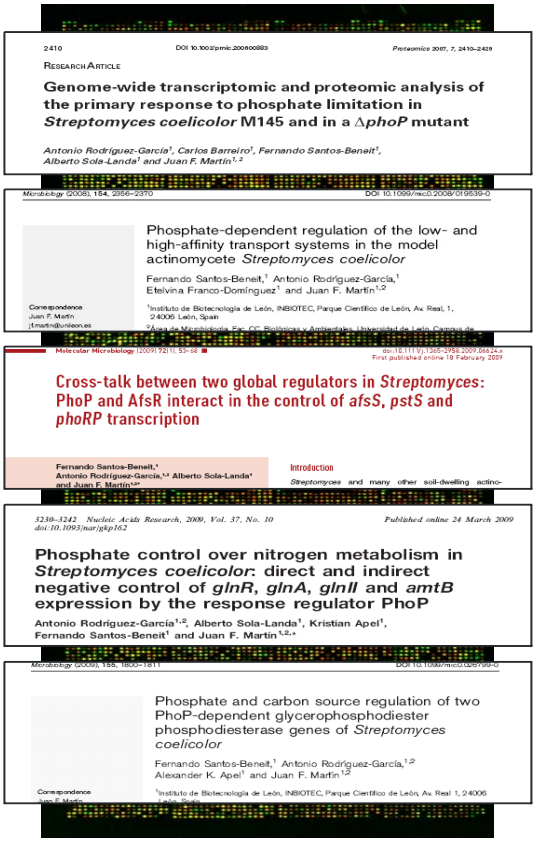
2. Publicaciones



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RESEARCH ARTICLE

Genome-wide transcriptomic and proteomic analysis of the primary response to phosphate limitation in *Streptomyces coelicolor* M145 and in a Δ*phoP* mutant

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Phosphate-dependent regulation of the low- and high-affinity transport systems in the model actinomycete *Streptomyces coelicolor*

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Cross-talk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription

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Introduction

Streptomyces and many other soil-dwelling actino-

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Phosphate control over nitrogen metabolism in *Streptomyces coelicolor*: direct and indirect negative control of *glnR*, *glnA*, *glnII* and *amtB* expression by the response regulator PhoP

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Phosphate and carbon source regulation of two PhoP-dependent glycerophosphodiester phosphodiesterase genes of *Streptomyces coelicolor*

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2. Publicaciones

El punto de partida de esta tesis es la publicación realizada por Sola-Landa *et al*. (2003) en la que se caracteriza el sistema de dos componentes PhoR-PhoP en *S. lividans* y *S. coelicolor* y se demuestra su involucración en la producción de antibióticos en *S. lividans*. De acuerdo con esto, los objetivos de la tesis fueron los siguientes:

1. Identificar genes pertenecientes al regulón *pho* en el organismo modelo *S. coelicolor* y caracterizar los mecanismos de regulación ejercidos por PhoP en dichos genes.
2. Determinar el efecto del sistema PhoR-PhoP y el fosfato en la producción de antibióticos en *S. coelicolor* y dilucidar los mecanismos moleculares de dicho control.

En esta memoria se presentan cinco publicaciones, tres de ellas como primer autor:

1. Antonio Rodríguez-García, Carlos Barreiro, **Fernando Santos-Beneit**, Alberto Sola-Landa y Juan F. Martín. Genome-wide transcriptomic and proteomic analysis of the primary response to phosphate limitation in *Streptomyces coelicolor* M145 and in a *ΔphoP* mutant. **Proteomics, 2007**.

2. **Fernando Santos-Beneit**, Antonio Rodríguez-García, Etelvina Franco-Domínguez y Juan F. Martín. Phosphate-dependent regulation of the low and high affinity transport systems in the soil actinomycete *Streptomyces coelicolor*. **Microbiology, 2008**.

3. **Fernando Santos-Beneit**, Antonio Rodríguez-García, Alberto Sola-Landa y Juan F. Martín. Crosstalk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription. **Molecular Microbiology, 2009**.

4. Antonio Rodríguez-García, Alberto Sola-Landa, Kristian Apel, **Fernando Santos-Beneit** y Juan F. Martín. Phosphate control over nitrogen metabolism in *Streptomyces coelicolor*: direct and indirect negative control of *glnR*, *glnA*, *glnII* and *amtB* expression by the response regulator PhoP. **Nucleic Acids Research, 2009**.

5. **Fernando Santos-Beneit**, Antonio Rodríguez-García, Kristian Apel y Juan F. Martín. Phosphate and carbon source regulation of two PhoP-dependent glycerophosphodiester phosphodiesterase genes of *Streptomyces coelicolor*. **Microbiology, 2009**.

En el caso de las dos publicaciones en las que el doctorando figura como coautor la aportación realizada por el mismo se indica a continuación.

Publicación 1:

- Figura 1: Se establecen las condiciones para la realización del análisis transcriptómico.
- Figura 7: EMSA de los promotores SCO1565 y SCO1968.

Publicación 4:

- Figura 4: Estudio de la actividad promotora de los genes *glnR*, *glnA*, *glnII* y *amtB* en la cepa silvestre y la cepa mutante en el gen *phoP*.

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RESEARCH ARTICLE

Genome-wide transcriptomic and proteomic analysis of the primary response to phosphate limitation in *Streptomyces coelicolor* M145 and in a Δ*phoP* mutant

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Phosphate limitation in *Streptomyces* and in other bacteria triggers expression changes of a large number of genes. This response is mediated by the two-component PhoR–PhoP system. A *Streptomyces coelicolor* Δ*phoP* mutant (lacking *phoP*) has been obtained by gene replacement. A genome-wide analysis of the primary response to phosphate limitation using transcriptomic and proteomic studies has been made in the parental *S. coelicolor* M145 and in the Δ*phoP* mutant strains. Statistical analysis of the contrasts between the four sets of data generated (two strains under two phosphate conditions) allowed the classification of all genes into 12 types of profiles. The primary response to phosphate limitation involves upregulation of genes encoding scavenging enzymes needed to obtain phosphate from different phosphorylated organic compounds and overexpression of the high-affinity phosphate transport system *pstSCAB*. Clear interactions have been found between phosphate metabolism and expression of nitrogen-regulated genes and between phosphate and nitrate respiration genes. PhoP-dependent repressions of antibiotic biosynthesis and of the morphological differentiation genes correlated with the observed Δ*phoP* mutant phenotype. Bioinformatic analysis of the presence of PHO boxes (PhoP-binding sequences) in the upstream regions of PhoP-controlled genes were validated by binding of PhoP, as shown by electrophoretic mobility shift assays.

Keywords:
Gene expression / *phoP* mutant / Phosphate control / Transcriptome

1 Introduction

A few years ago, several authors studying in *Escherichia coli* and *Bacillus subtilis* [1–4], proposed that expression of about 40 genes belonging to the so-called PHO regulon is controlled by the phosphate concentration [5].

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Abbreviations: DRu, direct repeats unit; **FDR,** false-discovery rate; **gDNA,** genomic DNA; **VA,** vector analysis;

In different *Streptomyces* species it is well known that the biosynthetic pathways of distinct types of secondary metabolites are regulated negatively by high-phosphate concentrations [6–11]. These metabolites belong to a variety of polyketide groups, including the anthracyclines and related compounds (*e.g.* actinorhodin), the tetracyclines, macrolides and other secondary metabolites such as aminoglycosides, amino acid-derived metabolites (*e.g.* prodigionins [12] and clavulanic acid). Depletion of phosphate in *Streptomyces coelicolor* and in other *Streptomyces* species triggers a poorly known "transition phase" in which primary metabolism is reorganized and genes inducing secondary metabolism are triggered [13]. However, the cascade involved in signal transduction from phosphate sensors to transcriptional factors

interacting with phosphate-regulated promoters is still poorly understood [14]. Knowledge of the genes that are induced by phosphate limitation during the transition phase in shift-down nutritional experiments is important to understand the interaction between primary and secondary metabolism.

We reported that expression of *S. lividans* genes involved in both phosphate-scavenging (*phoA*) and secondary metabolism (actinorhodin and undecylprodigiosin biosynthesis) is mediated by the two-component *phoR–phoP* system [15]. Mutants deleted in the *phoP* gene (Δ*phoP*) or in the *phoP* and *phoR* genes (Δ*phoR–phoP*) overexpress the actinorhodin and undecylprodigiosin biosynthesis genes [15, 16]. Recently, we showed that the PhoP response regulator binds to specific sequences (PHO boxes) in the promoter regions of several phosphate-regulated genes [17]. However, a wide analysis of phosphate regulation is required for understanding the differential effect of phosphate on primary and secondary metabolism and the possible involvement of other regulatory proteins in signal transduction mechanisms of control, as described in *B. subtilis* [18].

DNA microarrays [19, 20] and proteomic studies [4, 21, 22] have become extremely useful tools for analysis of gene expression. Since DNA microarrays of *S. coelicolor* were available, it was of utmost interest to use this tool for a global analysis of gene expression in *S. coelicolor* following phosphate shift-down experiments. Phosphate control affects many genes but only part of them will be directly PhoP dependent. Therefore, it was important to study the changes in gene expression in Δ*phoP* mutants when compared with the parental strain, and to compare these changes with those observed in the proteomics studies. In this work, the integration of transcriptomic and proteomic studies has allowed us to identify 551 genes that are significantly affected by phosphate limitation, by the *phoP* deletion or both; these have been classified into 12 types of expression profiles.

2 Materials and methods

2.1 Bacterial strains and plasmids

S. coelicolor strains M145 [23], M145[pLUX-*pstS*], and INB101 (Δ*phoP*, this work) were manipulated according to standard preservation and conjugation procedures [23], except that TBO medium [24] was used to obtain spore preparations. *E. coli* DH5α was used as the general cloning host.

pLUXAR+ is a promoter-probe vector constructed from the previously described plasmid pAR933a [25]. pLUX-*pstS* (E. Franco-Domínguez, personal communication), a derivative of pLUXAR+ containing the *pstS* gene promoter was introduced by conjugation in *S. coelicolor* M145 to obtain the strain M145[pLUX-*pstS*].

A *phoP* deletion mutant, strain INB101, was constructed by PCR targeting [26]. Two primers (CAR03 and CAR04; Supporting Information, Table S3) were designed for replacement of the *phoP* coding region by the apramycin resistance cassette of pIJ773. The cosmid SCD8A [27] allowed the introduction of the mutation into the M145 strain. The *phoP* deletion in the strain INB101 was verified by Southern hybridization using internal probes to the apramycin resistance gene and to the *phoP* gene.

2.2 Culture conditions

Cultures were performed at 30°C, 220 rpm, in defined MG medium containing starch (Scharlau; 50 g/L) and glutamate (60 mM) [28]. Four hundred milliliters of MG medium in 2-L-baffled flasks were inoculated with 10^6 spores/mL for reproducible dispersed growth. Cultures containing 15 mM potassium phosphate (MG-15 medium) were used for the phosphate-replete condition. For the phosphate shift down, the cells were centrifuged (10 min at 8000 × *g*), washed with MG medium containing 0.05 mM of phosphate (MG-005), and harvested again. Half of the cells were suspended in MG-005 medium and cultivated as above.

2.3 Luciferase assay, DNA and phosphate determinations

The *luxAB* gene expression was determined in a Luminoskan luminometer (Labsystems, Helsinki). Frozen cells from 1-mL culture samples were resuspended in 1 mL of 0.9% NaCl. Fifty microliters of 1% *n*-decanal was added to 0.1 mL of resuspended cells measuring the luminescence after 15 s of elapsed time (during 20 s of integration). Measurements were performed in triplicate. DNA content in culture biomass was determined with diphenylamine [29]. The colorimetric malachite green assay was employed to measure the phosphate in culture supernatants [30].

2.4 Nucleic acid extractions

Two to eight milliliters from liquid cultures were used to extract RNA using the RNeasy Protect Bacteria kit (Qiagen cat. no. 75552) and the protocol of V. Mersinias (http://www.surrey.ac.uk/SBMS/Fgenomics/Microarrays/html/Downloads.html). Total RNA concentration and quality were checked spectrophotometrically and by agarose gel electrophoresis. Total genomic DNA (gDNA) was isolated from a stationary-phase culture following the Kirby mix procedure [23].

2.5 2-D electrophoresis

Bidimensional electrophoresis was performed using the procedure adapted to *S. coelicolor* and described by Flores *et al.* [31]. Sixty micrograms of crude protein extract was used for IEF in 18-cm precast IPG strips with linear pH gradients of 3.0–10.0 and 4.0–7.0 using an IPGphor IEF unit (Amersham). The second dimension was run in SDS-PAGE gels of 12.5% acrylamide in an Ettan Dalt apparatus

(Amersham), and gels were subsequently silver stained following an MS-compatible protocol (Amersham). Precision Plus protein Standards (BioRad) were used as size markers. Image analyses were performed with biological triplicates by using the ImageMaster™ 2D Platinum v5.0 software (Amersham).

Protein spots were excised from gels and digested with modified trypsin (Promega). Peptide mass fingerprints were analyzed with the MASCOT software [32].

2.6 Labeling and hybridization reactions: Image quantification

S. coelicolor microarrays were obtained from the Functional Genomics Laboratory of the University of Surrey (UK). These arrays (SCp40 print run) contained duplicated PCR-probes for 7083 chromosomal genes. Four biological replicates were made for each condition. The Pronto! Universal Microarray Hybridization kit (Corning, # 40026) was used for prehybridization of the slides. Labeling reactions were performed according to the recommendations described by http://www.surrey.ac.uk/SBMS/Fgenomics. In brief, 4.2 μg of total RNA was labeled with Cy3-dCTP (Amersham), using random primers and Superscript II reverse transcriptase (Invitrogen). gDNA (3 μg) was labeled with Cy5-dCTP (Amersham), from random primers extended with the Klenow fragment of DNA polymerase (Roche). Final products were purified with MinElute columns (Qiagen) and labeling efficiencies were quantified spectrophotometrically. Two hundred picomoles of Cy3-cDNA and 20 pmol of Cy5-labeled gDNA were mixed and vacuum dried. Labeled samples were resuspended in 40 μL of the Pronto! Universal Hybridization Solution (Corning), and applied on the microarray surface. Hybridizations were carried out at 42°C for 16–20 h. After hybridization, slides were washed three times, with gentle agitation, at 42°C for 10 min in solution 1 (2X SSC, 0.5% SDS). Then, the slides were transferred to solution 2 (0.5X SSC, 0.1% SDS, 0.1 mM DTT) at room temperature, and washed three times (firstly for 15 min, twice for 5 min). TIFF images generated by the Agilent DNA Microarray Scanner G2565BA were processed with Agilent's Feature Extraction Software V7.5. The WholeSpot method was used for segmentation and signal intensities for each spot were calculated as the mean of the pixel fluorescences after interquartile range rejection of outlier pixels. Local background and spatial detrend corrections were applied, as well as the surrogate algorithm.

2.7 Data preprocessing

Data preprocessing for normalization, quality labeling, and outlier detection was performed in Microsoft Excel spreadsheets as follows. Spot data were flagged with quality values from 1 to 0 (seven categories), where 1 means that all the selected Feature Extraction quality controls were passed (see Supporting Information). Medians of $\log_2$ Cy3/Cy5 intensities (spots with flag ≥ 0.9) were calculated in each print-tip block for linear normalization. Since the *S. coelicolor* strains of this work lack the SCP1 plasmid, only data from chromosomal genes were considered in the analysis. The normalized $\log_2$ of Cy3/Cy5 intensities is referred in this work as the M_g value, and is a measure of the abundance of transcripts for a particular gene with respect to its genomic copies [33, and references therein]. To evaluate the consistency of the eight gene spot M_g values in each condition (four arrays with duplicated spots), the Grubb's test was applied following the instructions of the MADSCAN manual [34], with a significance level of 0.01. The percentage of spots detected as outliers in each quality category served to assign spot weights to the data (see Supporting Information).

2.8 Statistical analysis

To assess the statistical significance of the data we used two Bioconductor software packages run in the R environment [35]. Quantile normalization of the gDNA intensities was applied by using limma (*li*near *m*odels for *m*icroarray *a*nalysis) [36, 37]. The information from within-array spot duplicates [38], and the empirical array weights [39] were taken into account in the linear models. Five contrasts between the four experimental conditions (Fig. 2) provided the respective *p*-values for each gene – reflecting the probability that a specific gene is differentially transcribed between the two conditions compared – and five *M*-values of contrasts (M_c), as the quantification of the differential transcription. The *p*-values were corrected for multiple testing (false-discovery rate, FDR). Rank products [40], unlike limma, is a nonparametric method that allowed us to confirm and complement the results of limma, with the aim of reducing the number of false negatives. This method provided another probability value, *pfp* (*p*roportion of *f*alse *p*ositives), corrected for multiple tests in an equivalent manner to the FDR algorithm. Thus, a result from a contrast was considered as statistically significant if it passed at least one of these filters: (i) limma FDR-corrected *p*-value <0.05; (ii) Rank products *pfp* value <0.05. These *h*ypothesis-testing *r*esults were summarized with the notation H_{ri}, where i refers to the contrast number. When the contrast result was not significant $H_{ri} = 0$; if the result was significant and the respective M_c was positive, then $H_{ri} = 1$; or $H_{ri} = -1$, for a significant negative M_c value. A total of 529 genes showed statistically significant results in at least one contrast, and were selected for profile classification.

2.9 Transcription profile classification

The H_r values were used to determine the transcription pattern. The contrasts 2, 4, and 5 were expected to be more meaningful because they reflect the responses to the phosphate limitation of the wild-type strain, of the mutant strain, and the difference between both responses, respectively. The combinations of the H_r values of these contrasts that

are logically self-consistent served us to define a total of 12 profiles (Fig. 3), which comprise all the possible transcription patterns that can be discriminated. To completely assign profiles to the set of 529 selected genes these approaches were used: (i) previous H_{r5} were complemented with the results of the limma test "nestedF" (FDR-corrected *p*-values), and the gene results satisfying the logical constraints were classified into profiles of quality 1 (197 genes); (ii) when H_{r5} was null and logically inconsistent with H_{r2} and H_{r4}, only the last values were taken into account, and the profiles were assigned a quality label of 2 (295 genes); (iii) the rest of the genes (37) were classified with vector analysis (VA), described by Breitling *et al.* [41], and the profiles were given a quality label of 3. The VA approach classifies the transcription responses of two different backgrounds in eight patterns (Fig. 4). The pattern scheme is coincident with the H_r-based scheme in eight profiles (AA, DD, A0, D0, 0A, 0D, AD, and DA). The profiles aA, Aa, dD, and Dd are intermediate profiles that only the H_r-based method can discriminate. The limma-calculated M_{c2} and M_{c4} values were the input for VA.

2.10 Bioinformatics search of PhoP-binding sites

The individual information theory programs makebk, encode, rseq, dalvec, ri, multiscan, and delila [42, 43] were used to identify putative target sequences of the response regulator PhoP. PhoP-binding sites are formed with a minimum of two direct repeats units (DRus), 11 nucleotides in length, and tandemly arranged (one PHO box). An individual information weight matrix was constructed following the alignment of 16 DRus. Of those DRus, six were previously described [17] and were taken from the promoters of *pstS* (two DRus) and *phoU–phoR* (four DRus). The other DRus were identified in the promoter regions of genes SCO7697 (three DRus located between positions −165 to −133, on the coding strand from the annotated translational start site), SCO1565 (three DRus, positions −81 to −49) and SCO1968 (four DRus, positions −91 to −48). This weight matrix served to compute a measure of the individual information content (Ri) of each candidate sequence. Applying this matrix, the average Ri and SD of the above 16 DRus were 5.84 and 2.77 bits, respectively; and for the whole five binding sites, were 18.68 and 2.68 bits, respectively. These four values and the Student's *t*-distribution ($\alpha = 0.05$) served to determine thresholds for DRus (Ri > 1 bit), and for whole sites (Ri > 10 bits). Both strands of gene sequences (positions −300 to +101), obtained from the RSA tools web server [44], were screened with the multiscan program. The models that guided the multiscan search were designed to detect two, three, or four consecutive DRu. The above thresholds were used, except for sites composed of four DRu. In these cases, as occurs in the SCO1968 gene, one intermediate DRu was allowed to have a Ri > −1.

2.11 DNA binding to gene promoters

DNA-binding analyses were performed by electrophoretic mobility shift assays (EMSA) with the GST-PhoP^{DBD} protein (fusion of GST and the DNA-binding domain), as described previously [17]. DNA fragments of the following promoter regions were amplified by PCR (gene name, start, end; positions from the translation start site): (i) SCO0034, −279, −3; (ii) SCO1565, −242, −3; (iii) SCO1968, −253, −3; (iv) SCO2532, −285, +3; (v) SCO2878, −218, +22; (vi) SCO4261, −188, +9; (vii) SCO4879, −260, +14; (viii) SCO7697, −219, +4. Primers used are listed in Table S3 of the Supporting Information.

3 Results

3.1 Phosphate shift-down experimental design

Control experiments were initially performed to define the optimal conditions to observe expression changes in *S. coelicolor* cultures after phosphate shift down. The initial cultures were carried out in MG-15 medium (15 mM phosphate; see Section 2) to minimize differences between the wild-type *S. coelicolor* M145 strain and the Δ*phoP* mutant. The time (t_0) previous to the shift down was selected at 39 h because at that point the growth was still in the exponential phase. Moreover, at this time there was already enough biomass in a 400-mL culture for RNA and protein extractions and for the inoculum of the shift-down culture. The expression of the *pstS* gene (a phosphate-regulated gene) [17, 45] was used as reporter of the phosphate-deficiency response. The *pstS* expression remained at basal levels until the phosphate in the medium decreased below 0.1 mM (Fig. 1), a similar threshold than that reported for the PHO response in *B. subtilis* [2]. We chose 7.5 h for cell collection ($t_{7.5}$) since the induction factor of the *pstS* promoter was 140 at this time and the growth was still approximately exponential (Fig. 1). The 7.5 h phosphate-deprivation time is coincident with that used by Ishige *et al.* [19] in phosphate control studies in *Corynebacterium glutamicum*. The Δ*phoP* mutant and the parental strain showed similar growth patterns in the shift-down cultures until the sampling time. Growth, measured as DNA content, was exponential until 2 h of culture, and there was no increase in DNA content from 4 h to the sampling time in both strains (Supporting Information, Fig. S1). The continuous increment of the dry weight (Fig. 1) probably is due to accumulation of glycogen or another carbon storage polymer. The average dry weight increase in the four biological replicates was 35% (with respect to the weight at t_0) for the wild type, and 36% for the Δ*phoP* mutant strain during the 7.5 h interval. Therefore, the sampling time is a trade-off between induction of PHO response and limitation of secondary changes [46].

Therefore, the four experimental conditions for RNA and protein extraction were: (i) wild type at time 0 (Wt.t_0), (ii) wild type at time 7.5 h following phosphate shift down (Wt.$t_{7.5}$),

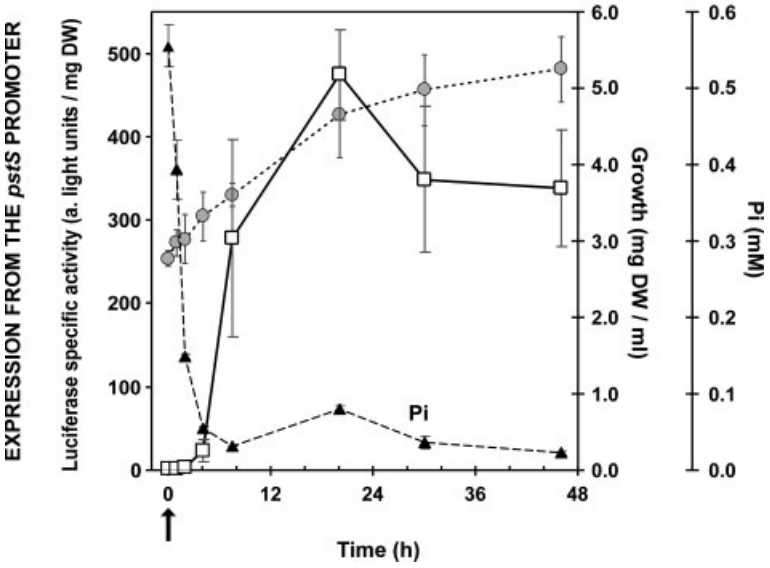

Figure 1. Induction of the *pstS* promoter after phosphate shift down. The wild-type strain carrying the *pstSp–luxAB* fusion was cultivated in MG-15 medium for 39 h. Half of the culture cells were washed (vertical arrow), and resuspended in MG-005 medium. Specific luciferase activity (squares, solid lines), growth (circles, dotted lines), and phosphate content in the medium (triangles, dashed lines) are shown as the mean of two replicates. Vertical bars show the SD. The expression of *pstS* in control phosphate-replete MG-15 medium continued at basal levels during the time course of this experiment.

(iii) Δ*phoP* mutant at time 0 (Mu.t_0), and (iv) Δ*phoP* mutant at time 7.5 h (Mu.$t_{7.5}$) (Fig. 2). The microarray approach chosen to evaluate the differential expression between those conditions involved the use of gDNA as the common reference [47, 33, and references therein]. These comparisons and contrasts are summarized in Fig. 2.

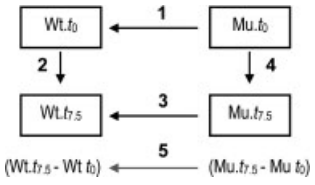

Figure 2. Linear model contrasts [36] of the microarray analysis. The experimental conditions are boxed; Wt refers to the wild type strain, and Mu, to the Δ*phoP* INB101 mutant strain. The samples were taken from cultures immediately before the shift down (t_0), and 7.5 h following shift down ($t_{7.5}$). The arrow orientation refers to the comparison between conditions. Differential transcription values M_c were obtained by subtracting the first condition M_g values from the last M_g (corresponding to the arrowhead) values, (*e.g.* Wt.$t_{7.5}$–Wt.t_0). A fifth comparison, or interaction contrast, was defined as the difference in the response to phosphate starvation between both strains (Wt.$t_{7.5}$–Wt.t_0)–(Mu.$t_{7.5}$–Mu.t_0).

3.2 A comprehensive set of transcription profiles define the wild type and the Δ*phoP* mutant primary responses to phosphate limitation

Following the statistical criteria, 529 genes showed differential transcription in at least one contrast, and were selected for further analysis. We have used the hypothesis-testing results for a logical classification of this set of genes. Considering that transcription differences between the two strains at t_0 (before phosphate shift down) are null, there are 12 transcription profiles that can be discriminated in response to phosphate limitation. A main set of eight profiles [AA, A0, AD, 0D, DD, D0, DA, and 0A; the first symbol corresponds to the wild type and the second to the Δ*phoP* mutant. A, stands for increased (*a*ugmented) expression and D for decrease; 0 indicates no change] reflect the major transcriptional responses (Fig. 3). These eight profiles were also used to summarize the pattern of protein synthesis observed in the proteomic studies (Table 2). In addition, four other profiles aA, Aa, dD, and Dd, correspond to differences between the strains when the responses are in the same direction, *i.e.* both increase or decrease, but of different intensity (small letters indicate a lower relative response).

Common regulatory inferences can be grouped in three categories of profiles. Firstly, the profiles AA and DD reflect an equal transcription response to phosphate limitation in both strains. Therefore, PhoP should not play a role in regulating those genes. More interestingly, the A0 profile indicates that expression of the genes showing this profile is PhoP dependent, since the increase in expression in the wild type following phosphate starvation is not observed in the Δ*phoP* strain. The *pstS* gene illustrates this profile since it showed the strongest response in the wild-type strain (10 times in linear scale), whereas no change in the mutant was detected (Fig. 4). That behavior fully agrees with the activity of the *pstS* promoter (Fig. 1) and with the data reported previously [17]. Other profiles that indicate a PhoP dependence were Aa, AD, 0D, and dD. In these cases, the expression of the corresponding genes was always lower in the Δ*phoP* mutant than in the wild type. On the other hand, the profiles Dd, D0, DA, 0A, and aA imply a higher expression of those genes in the Δ*phoP* mutant than that of the wild type. These higher levels can be related to a mechanism of PhoP-dependent repression.

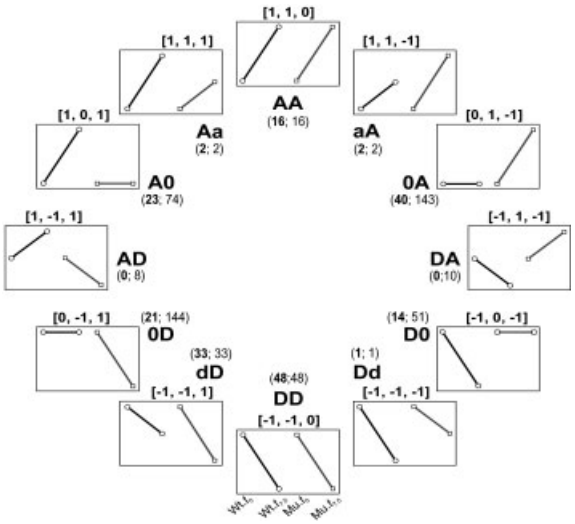

Figure 3. Transcription profiles. Each of the graphs in the figure depicts schematically the change in transcription (vertical axis) from time t_0 (immediately previous to shift down) to time $t_{7.5}$ (7.5 h) of both strains (as indicated under the DD profile at the bottom of the figure). The black line corresponds to the wild-type strain (M145), and the dark gray line to the Δ*phoP* mutant (INB101). Each profile is named by a two-character designation, in which the first character refers to the expression changes in the wild type and the second to the change in the mutant strain. "A" means increase (Augmentation), and "D," decrease. The lack of a detected change of expression is indicated by "0." Lower case letters refer to a relative smaller change in one strain. The three H_r values that define each profile are indicated at the top of each panel (see Section 2 for details). The total number of genes classified into a profile are indicated in round brackets (first number in bold refers to quality 1 profiles, second number is the total).

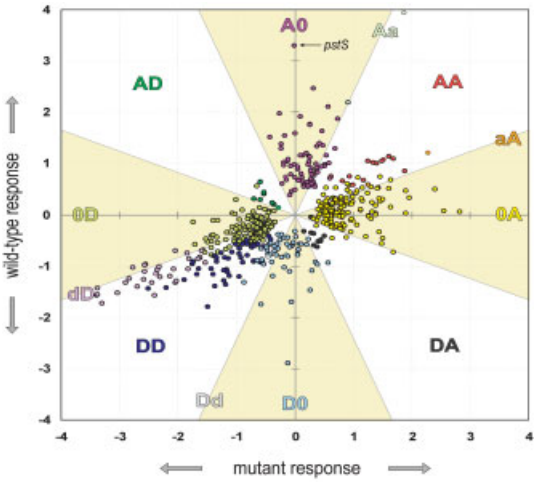

Figure 4. Vector analysis plot [41] showing the phosphate-limitation response of the statistically selected 529 genes that are differentially expressed. Vertical axis is the M_{c2} values and the horizontal axis is the M_{c4} values that define the wild type and mutant responses to phosphate limitation, respectively. The color of each circle corresponds to the color of the assigned profile, indicated in each sector.

3.3 Amino acids and protein synthesis genes

This group includes 62 genes related to translation that code for ribosomal proteins (47 genes), translation factors (5), aminoacyl-tRNA synthesis or modifying enzymes (5), proteins related to protein maturation (4), and rRNA modification (SCO1597) (Table 1A and Supporting Information). Of these genes, 50 are integrated in 11 clusters. These clusters also include genes involved in transcription (*rpoA*, *rpoB*, *rpoC*, *ssb*). Except in one case (*serS*), all these genes showed decreased transcription levels in the phosphate-depleted cultures, what is consistent with their role in protein synthesis. Indeed, the proteomic results detected the ribosomal protein S1, three translation factors [TU-1 (Fig. 6), Ts, ribosome recycling factor] and the Glu-tRNAGln amidotransferase subunit B with reduced synthesis after phosphate shift down (Table 2). Similar changes in transcription of this group of genes have been detected for the *C. glutamicum* and *E. coli* phosphate regulons [3, 19].

Differential transcription between strains was clearly detected. Most genes in this group showed profiles 0D or dD that indicate an upregulation of these genes mediated by PhoP (Table 1A; the zero in the 0D profile means lack of statistically significant change in the wild-type strain). Especially significant is the coincidence of profiles found in the 21 genes in the cluster *rpsJ* (SCO4701)-*rplO* (SCO4721). A *phoP*-dependent positive effect is also suggested by the profiles of amino acid transport (*gluABCD*, 0D profiles) and biosynthetic genes (*ask*, SCO4293, *sahH*, 0D profiles; *trpE*, A0; Table 1A). However, the *S*-adenosyl-L-homocysteine hydrolase protein (*sahH*) showed a different behavior (see Section 3.14).

On the other hand, a wild-type *vs.* mutant downregulation was observed for biosynthetic genes SCO0992 (putative cysteine synthase), *hisC2* and *aspC*, and for three genes encoding aminoacyl-tRNA synthetases (Table 1A).

3.4 Nucleotide metabolism genes

This group of genes includes seven genes for biosynthesis of nucleotides which showed strain-dependent expression (Table 1B). Three genes of the biosynthesis of purines, *purE* and the *purF–purM* operon, showed the 0A profile. On the other hand, the *purN* gene and the operon *purSQL* involved in the purine pathway (fourth step of the pathway), showed decreased transcription in both strains, but lower in the Δ*phoP* mutant strain (Table 1B).

Interestingly, the profile 0A of *pyrR*, the regulator of pyrimidine biosynthesis, can be correlated with the profile 0D of the structural *pyrH* gene. These profiles are in opposite positions in the diagram of Fig. 3. It is suggested that PhoP is responsible of a downregulation of *pyrR*, what in turn maintains the transcription of *pyrH*.

Table 1. Transcriptional responses to phosphate limitation[a)]

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
(A) Amino acids metabolism, transcription, and translation								
Wild type *vs.* mutant upregulation								
SCO1597		rRNA methylase	**0D**	**−0.09**	**−0.59**	0.529	**<0.001**	0.013
SCO1598	*rplT*	Ribosomal protein L20	**dD**	**−0.85**	**−1.63**	**<0.001**	**<0.001**	0.009
SCO1599	*rpmI*	Ribosomal protein L35	**dD**	**−1.06**	**−2.08**	**<0.001**	**<0.001**	0.002
SCO2117	*trpE*	Anthranilate synthase	**A0**	**1.38**	**−0.23**	**0.001**	0.555	0.006
SCO2563	*rspT*[b)]	Ribosomal protein S20	**DD**	**−1.42**	**−2.19**	**<0.001**	**<0.001**	0.027
SCO3023	*sahH*	*S*-adenosyl-L-homocysteine hydrolase	**0D**	**0.43**	**−0.53**	0.047	0.016	0.003
SCO3615	*ask*	Aspartokinase	**0D**	**−0.25**	**−0.66**	0.084	**<0.001**	0.045
SCO3906	*rpsF*	Ribosomal protein S6	**dD**	**−1.41**	**−2.70**	**<0.001**	**<0.001**	**<0.001**
SCO3907	*ssb*[b,c)]	Single-strand DNA-binding protein	**DD**	**−0.81**	**−1.37**	**<0.001**	**<0.001**	0.055
SCO4293		Threonine synthase	**0D**	**−0.26**	**−0.97**	0.180	**<0.001**	0.015
SCO4652	*rplJ*	Ribosomal protein L10	**0D**	**−0.69**	**−1.58**	0.013	**<0.001**	0.025
SCO4653	*rplL*	Ribosomal protein L7/L12	**dD**	**−0.70**	**−2.05**	**<0.001**	**<0.001**	**<0.001**
SCO4654	*rpoB*	RNA polymerase beta chain	**0D**	**−0.35**	**−1.12**	0.026	**<0.001**	0.001
SCO4655	*rpoC*	RNA polymerase beta' chain	**0D**	**−0.35**	**−1.17**	0.145	**<0.001**	0.022
SCO4659	*rpsL*	Ribosomal protein S12	**dD**	**−1.04**	**−2.78**	**<0.001**	**<0.001**	**<0.001**
SCO4660	*rpsG*	Ribosomal protein S7	**dD**	**−0.96**	**−2.55**	**<0.001**	**<0.001**	**<0.001**
SCO4661	*fusA*	Elongation factor G	**dD**	**−1.20**	**−2.14**	**<0.001**	**<0.001**	0.001
SCO4701	*rpsJ*[b,c)]	Ribosomal protein S10	**DD**	**−1.44**	**−2.50**	**<0.001**	**<0.001**	0.020
SCO4702	*rplC*	Ribosomal protein L3	**dD**	**−1.29**	**−2.04**	**<0.001**	**<0.001**	0.026
SCO4703	*rplD*[c)]	Ribosomal protein L4	**0D**	**−0.43**	**−0.98**	0.056	**<0.001**	0.079
SCO4704	*rplW*	Ribosomal protein L23	**dD**	**−1.25**	**−2.83**	**<0.001**	**<0.001**	**<0.001**
SCO4705	*rplB*	Ribosomal protein L2	**dD**	**−1.33**	**−2.44**	**<0.001**	**<0.001**	**<0.001**
SCO4706	*rpsS*	Ribosomal protein S19	**dD**	**−1.80**	**−4.15**	**<0.001**	**<0.001**	**<0.001**
SCO4707	*rplV*	Ribosomal protein L22	**dD**	**−1.57**	**−3.39**	**<0.001**	**<0.001**	**<0.001**
SCO4708	*rpsC*	Ribosomal protein S3	**dD**	**−1.26**	**−2.60**	**<0.001**	**<0.001**	**<0.001**
SCO4709	*rplP*	Ribosomal protein L16	**dD**	**−1.33**	**−2.90**	**<0.001**	**<0.001**	**<0.001**
SCO4710	*rpmC*	Ribosomal protein L29	**dD**	**−1.73**	**−3.31**	**<0.001**	**<0.001**	**<0.001**
SCO4711	*rpsQ*	Ribosomal protein S17	**dD**	**−1.56**	**−3.38**	**<0.001**	**<0.001**	**<0.001**
SCO4712	*rplN*	Ribosomal protein L14	**dD**	**−1.53**	**−3.05**	**<0.001**	**<0.001**	**<0.001**
SCO4713	*rplX*	Ribosomal protein L24	**dD**	**−1.44**	**−3.39**	**<0.001**	**<0.001**	**<0.001**
SCO4714	*rplE*	Ribosomal protein L5	**dD**	**−0.98**	**−2.58**	**<0.001**	**<0.001**	**<0.001**
SCO4715	*rpsN*	Ribosomal protein S14	**dD**	**−1.22**	**−2.31**	**<0.001**	**<0.001**	**<0.001**
SCO4716	*rpsH*	Ribosomal protein S8	**dD**	**−1.11**	**−2.58**	**<0.001**	**<0.001**	**<0.001**
SCO4717	*rplF*	Ribosomal protein L6	**dD**	**−1.09**	**−2.36**	**<0.001**	**<0.001**	**<0.001**
SCO4718	*rplR*	Ribosomal protein L18	**dD**	**−1.06**	**−1.83**	**<0.001**	**<0.001**	0.007
SCO4719	*rpsE*	Ribosomal protein S5	**dD**	**−0.86**	**−1.81**	**<0.001**	**<0.001**	**<0.001**
SCO4720	*rpmD*	Ribosomal protein L30	**0D**	**−0.64**	**−1.44**	0.001	**<0.001**	0.005
SCO4721	*rplO*	Ribosomal protein L15	**0D**	**−0.74**	**−1.54**	0.002	**<0.001**	0.014
SCO4727	*rpsM*[b,c)]	Ribosomal protein S13	**DD**	**−0.92**	**−1.74**	**0.001**	**<0.001**	0.029
SCO4728	*rpsK*	Ribosomal protein S11	**dD**	**−0.84**	**−1.63**	**<0.001**	**<0.001**	0.002
SCO4729	*rpoA*[c)]	RNA polymerase alpha chain	**0D**	**−0.63**	**−1.13**	0.002	**<0.001**	0.064
SCO4730	*rplQ*	Ribosomal protein L17	**dD**	**−1.02**	**−2.32**	**<0.001**	**<0.001**	**<0.001**
SCO4734	*rplM*	Ribosomal protein L13	**dD**	**−0.87**	**−1.65**	**<0.001**	**<0.001**	0.007
SCO4735	*rpsI*[b,c)]	Ribosomal protein S9	**DD**	**−0.81**	**−1.36**	**<0.001**	**<0.001**	0.022
SCO5595	*rplS*	Ribosomal protein L19	**0D**	**−0.55**	**−1.32**	0.004	**<0.001**	0.005
SCO5774	*gluD*[c,d)]	Glutamate permease	**0D**	**−0.16**	**−0.64**	0.374	**0.001**	0.061
SCO5775	*gluC*	Glutamate permease	**0D**	**−0.26**	**−0.95**	0.120	**<0.001**	0.005
SCO5776	*gluB*	Glutamate-binding protein	**0D**	**−0.54**	**−1.49**	0.038	**<0.001**	0.011
SCO5777	*gluA*	Glutamate uptake system ATP-binding protein	**0D**	**−0.29**	**−1.00**	0.101	**<0.001**	0.007
Wild type *vs.* mutant downregulation								
SCO0992		Cysteine synthase	**DA**	**−0.52**	**0.44**	0.003	0.014	**<0.001**
SCO3961	*serS*	Seryl-tRNA synthase	**0A**	**−0.21**	**0.36**	0.063	**0.003**	0.001
SCO3792[c)]		Methionyl-tRNA synthetase	**D0**	**−0.50**	**−0.26**	**0.001**	0.059	0.204

Table 1. Continued

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
SCO3795	*aspS*	Aspartyl-tRNA synthetase	**D0**	**−0.61**	**−0.06**	**0.001**	0.727	0.028
SCO3944	*hisC2*	Histidinol-phophate aminotransferase	**D0**	**−0.67**	**−0.29**	**<0.001**	0.034	0.043
SCO4645	*aspC*	Aspartate aminotransferase	**D0**	**−0.64**	**−0.09**	**<0.001**	0.544	0.013
SCO5571	*rpmF*	50S ribosomal protein L32	**D0**	**−0.54**	**−0.02**	**0.001**	0.885	0.021
(B) Nucleotide metabolism								
Wild type *vs.* mutant upregulation								
SCO4077	*purS*[b,e]	Phosphoribosylformylglycinamidine synthase	**DD**	**−0.49**	**−0.82**	**<0.001**	**<0.001**	0.066
SCO4078	*purQ*	Phosphoribosylformylglycinamidine synthase I	**dD**	**−0.76**	**−1.85**	**0.001**	**<0.001**	0.001
SCO4079	*purL*[d]	Phosphoribosylformylglycinamidine synthase II	**0D**	**−0.41**	**−0.66**	0.005	**<0.001**	0.199
SCO5626	*pyrH*[d]	Uridylate kinase	**0D**	**−0.20**	**−0.59**	0.154	**<0.001**	0.052
Wild type *vs.* mutant downregulation								
SCO1488	*pyrR*	Pyrimidine operon regulatory protein	**0A**	**−0.19**	**0.60**	0.244	**0.001**	0.001
SCO3059	*purE*	Phosphoribosylaminoimidazole carboxylase catalytic subunit PurE	**0A**	**0.15**	**0.57**	0.194	**<0.001**	0.012
SCO4086	*purF*	Amidophosphoribosyltransferase	**0A**	**0.32**	**1.13**	0.126	**<0.001**	0.008
SCO4087	*purM*[d]	Phosphoribosylformylglycinamidine cyclo-ligase	**0A**	**0.32**	**0.69**	0.124	**0.002**	0.210
(C) Respiration								
Wild type *vs.* mutant upregulation								
SCO0212		Hypothetical	**0D**	**0.03**	**−1.07**	0.921	**0.001**	0.012
SCO0213	*narK2*[f]	Nitrate/nitrite transporter	**0D**	**−0.04**	**−0.49**	0.766	**0.001**	0.020
SCO0216	*narG2*[f]	Nitrate reductase alpha chain	**0D**	**0.01**	**−1.03**	0.971	**<0.001**	0.007
SCO0217	*narH2*[c,d,f]	Nitrate reductase beta chain	**0D**	**0.00**	**−0.69**	0.990	0.009	0.055
SCO0922[c]		Succinate dehydrogenase iron–sulfur protein	**0D**	**−0.49**	**−0.74**	0.005	**<0.001**	0.299
SCO0923		Succinate dehydrogenase flavoprotein subunit	**dD**	**−1.15**	**−2.38**	**0.001**	**<0.001**	0.009
SCO0924		Cytochrome *b* subunit	**dD**	**−0.94**	**−1.53**	**<0.001**	**<0.001**	0.029
SCO4947	*narG3*[f]	Nitrate reductase alpha chain	**A0**	**1.33**	**0.38**	**<0.001**	0.176	0.018
SCO4950	*narI3*[f]	Nitrate reductase gamma chain	**A0**	**1.15**	**0.23**	**<0.001**	0.293	0.005
SCO6534	*narH*[f]	Nitrate reductase beta chain	**A0**	**0.47**	**−0.07**	0.008	0.694	0.029
SCO6535	*narG*[f]	Nitrate reductase alpha chain	**AD**	**0.22**	**−0.39**	0.165	0.021	0.01
Wild type *vs.* mutant downregulation								
SCO2149	*qcrA*	Rieske iron–sulfur protein	**D0**	**−0.57**	**0.08**	**0.001**	0.582	0.004
SCO2150	*qcrC*	Cytochrome *c* heme-binding subunit	**D0**	**−0.68**	**0.07**	**<0.001**	0.562	**<0.001**
SCO2155	*cox1*	Cytochrome *c* oxidase subunit I	**D0**	**−0.57**	**0.11**	**<0.001**	0.414	0.001
SCO3946	*cydB*	Cytochrome *bd* oxidase subunit II	**D0**	**−0.79**	**0.05**	**<0.001**	0.786	0.003
SCO3965		Conserved	**D0**	**−1.70**	**−0.05**	**<0.001**	0.811	**<0.001**
SCO3966[e]		Cytochrome *c* oxidase assembly factor	**D0**	**−1.03**	**−0.18**	**<0.001**	0.320	0.002
SCO3967		Conserved	**D0**	**−1.47**	**0.27**	**<0.001**	0.105	**<0.001**
SCO3968		Membrane protein	**D0**	**−0.93**	**0.36**	**<0.001**	0.073	**<0.001**
(D) Cell envelop biosynthesis								
Wild type *vs.* mutant upregulation								
SCO4873[e]		Possible *N*-acetylmannosamine-6-phosphate 2-epimerase	**A0**	**1.55**	**−0.18**	**<0.001**	0.141	**<0.001**
SCO4875[e]		Bactoprenol glucosyltransferase	**A0**	**1.16**	**0.09**	**<0.001**	0.698	0.003
SCO4879		Conserved	**A0**	**0.69**	**−0.15**	**<0.001**	0.343	**0.001**
SCO4880	*neuA*[e]	CMP-*N*-acetylneuraminic acid synthetase	**A0**	**0.79**	**0.28**	**<0.001**	0.062	0.017
SCO4881	*neuB*[e]	*N*-acetylneuraminic acid synthase	**A0**	**1.03**	**−0.19**	**0.003**	0.565	0.013
(E) Oxidative stress and iron metabolism								
Wild type *vs.* mutant upregulation								
SCO0379	*catA*	Catalase	**A0**	**1.16**	**0.14**	**<0.001**	0.414	**<0.001**
SCO0380		Hypothetical	**A0**	**1.00**	**0.36**	**<0.001**	0.066	0.022
SCO0560	*cpeB*	Catalase/peroxidase	**A0**	**0.99**	**0.22**	**<0.001**	0.208	0.003
SCO2113	*bfr*	Bacterioferritin	**A0**	**1.51**	**−0.43**	**0.001**	0.313	0.003

Table 1. Continued

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
SCO2114[e]		Bacterioferritin-associated ferredoxin	**A0**	**1.30**	**−0.24**	**0.001**	0.533	0.007
Wild type *vs.* mutant downregulation								
SCO0494	*cchF*	Iron-siderophore binding lipoprotein	**D0**	**−0.82**	**−0.12**	**<0.001**	0.533	0.011
SCO0495	*cchE*[d]	Iron-siderophore ABC-transporter ATPase	**D0**	**−0.46**	**−0.17**	**<0.001**	0.143	0.082
SCO0498	*cchB*	Peptide monooxygenase	**D0**	**−0.57**	**−0.13**	**<0.001**	0.365	0.032
SCO0885	*trxC*[c]	Thioredoxin	**0A**	**0.09**	**0.49**	0.598	0.009	0.115
SCO5032	*ahpC*	Alkyl hydroperoxide reductase	**0A**	**0.04**	**0.68**	0.821	**0.002**	0.029
SCO5217	*rsrA*	Anti-sigma factor	**0A**	**−0.01**	**0.69**	0.975	**<0.001**	0.009
SCO5254	*sodN*	Superoxide dismutase	**aA**	**1.20**	**2.28**	**<0.001**	**<0.001**	0.012
(F) Nitrogen metabolism								
Wild type *vs.* mutant upregulation								
SCO2197		Membrane protein	**0A**	**0.22**	**0.88**	0.232	**<0.001**	0.017
SCO2198	*glnA*[c,d]	Glutamine synthetase	**0A**	**−0.08**	**0.32**	0.693	0.112	0.158
SCO2199[d]		Membrane protein	**0A**	**0.25**	**0.65**	0.129	**<0.001**	0.085
SCO2210	*glnII*	Glutamine synthetase	**0A**	**0.06**	**2.82**	0.806	**<0.001**	**<0.001**
SCO5583	*amtB*	Ammonium transporter	**0A**	**0.01**	**1.38**	0.971	**<0.001**	**<0.001**
SCO5584	*glnK*	Regulator	**0A**	**0.08**	**2.54**	0.749	**<0.001**	**<0.001**
(G) Phosphate metabolism								
Wild type *vs.* mutant upregulation								
SCO1565		Glycerophosphoryl diester phosphodiesterase	**A0**	**1.05**	**−0.14**	**0.009**	0.727	0.036
SCO1968		Glycerophosphoryl diester phosphodiesterase	**A0**	**1.98**	**−0.27**	**<0.001**	0.158	**<0.001**
SCO4139	*pstB*	Phosphate transporter, ATPase component	**A0**	**1.21**	**0.19**	**<0.001**	0.215	**<0.001**
SCO4140	*pstA*	Phosphate transporter, permease component	**A0**	**1.08**	**−0.11**	**<0.001**	0.643	0.001
SCO4141	*pstC*	Phosphate transporter, permease component	**A0**	**1.75**	**0.26**	**<0.001**	0.057	**<0.001**
SCO4142	*pstS*	Phosphate transporter, periplasmic component	**A0**	**3.31**	**−0.01**	**<0.001**	0.959	**<0.001**
SCO4228	*phoU*[d]	PhoU regulator	**A0**	**0.70**	**0.27**	**0.001**	0.186	0.143
SCO4229	*phoR*	PhoR sensor kinase	**A0**	**1.59**	**0.00**	**<0.001**	0.984	**<0.001**
SCO4230	*phoP*[c,d]	PhoP response regulator	**A0**	**0.57**	**0.22**	**<0.001**	0.143	0.106
SCO7697		Phytase	**A0**	**0.73**	**0.00**	**<0.001**	0.979	0.001
Wild type *vs.* mutant downregulation								
SCO2532	*phoH*	PhoH-like protein	**0A**	**−0.19**	**1.06**	0.54	**0.002**	0.008
SCO2533		Conserved	**0A**	**−0.23**	**0.76**	0.292	**0.002**	0.003
(H) Morphological differentiation								
Wild type *vs.* mutant upregulation								
SCO2954	*sigU*[g]	Transcriptional regulator	**Aa**	**2.20**	**0.91**	**<0.001**	**0.001**	**0.001**
Wild type *vs.* mutant downregulation								
SCO1674	*chpC*	Chaplin C	**D0**	**−0.83**	**−0.22**	**<0.001**	0.198	0.016
SCO3579	*wblA*	Transcriptional regulator	**0A**	**0.74**	**1.45**	0.002	**<0.001**	0.030
SCO5189		Hypothetical	**0A**	**−0.72**	**1.68**	0.006	**<0.001**	**<0.001**
SCO5190	*wblC*	Transcriptional regulator	**0A**	**−0.33**	**1.45**	0.067	**<0.001**	**<0.001**
SCO5191		Hypothetical	**0A**	**−0.03**	**1.26**	0.855	**<0.001**	**<0.001**
SCO7251		Hypothetical	**0A**	**0.09**	**1.13**	0.662	**<0.001**	0.001
SCO7252		Transcriptional regulator	**0A**	**0.33**	**1.96**	0.13	**<0.001**	**<0.001**
(I) Secondary metabolites								
Wild type *vs.* mutant downregulation								
SCO5877	*redD*	Transcriptional regulator	**0A**	**0.43**	**1.24**	0.006	**<0.001**	**0.001**
SCO5878	*redX*	Polyketide synthase	**0A**	**0.17**	**0.84**	0.372	**<0.001**	0.020
SCO5880	*redY*	RedY protein	**0A**	**0.47**	**1.89**	0.021	**<0.001**	**<0.001**
SCO5887	*redQ*	Acyl carrier protein	**0A**	**0.49**	**2.39**	0.087	**<0.001**	**<0.001**
SCO5888	*redP*	3-Oxoacyl-[acyl-carrier-protein] synthase	**0A**	**0.30**	**1.50**	0.223	**<0.001**	0.001
SCO5890	*redN*	8-Amino-7-oxononanoate synthase	**0A**	**0.51**	**1.60**	0.042	**<0.001**	0.003

Table 1. Continued

Systematic name	Gene name	Product	Profile	M_{c2}	M_{c4}	*p*-value contrast 2	*p*-value contrast 4	*p*-value contrast 5
SCO5891	*redM*	Peptide synthase	**0A**	**−0.08**	**0.98**	0.683	**<0.001**	0.001
SCO5893	*redK*	Oxidoreductase	**0A**	**0.13**	**0.78**	0.448	**<0.001**	0.011
SCO5896	*redH*	Phosphoenolpyruvate-utilizing enzyme	**0A**	**0.54**	**1.31**	0.006	**<0.001**	0.006
SCO5897	*redG*[c,d)]	Oxidase	**0A**	**0.52**	**1.41**	0.166	**0.001**	0.095
SCO6273	*cpkA*[h)]	Polyketide synthase	**0A**	**−0.02**	**0.52**	0.895	**0.002**	0.016
SCO6282	*cpkI*[h)]	3-Oxoacyl-[acyl-carrier protein] reductase	**D0**	**−2.90**	**−0.12**	**<0.001**	0.718	**<0.001**
SCO6283	*cpkJ*[h)]	Nucleoside-diphosphate-sugar epimerase	**D0**	**−1.10**	**−0.09**	**<0.001**	0.642	**<0.001**
SCO6284	*cpkK*[h)]	Acyl-CoA carboxylase, beta-subunit	**D0**	**−0.97**	**0.40**	**<0.001**	0.064	**<0.001**

a) Transcription profile type, M_c and *p*-values are shown for 140 selected genes. The selection criteria were: (1) PhoP-dependent transcription (excluded profiles AA and DD), 2) *p*-value < 0.05 of contrast 5. Some genes that did not meet both criteria are also included (see footnotes[b, c,] and [d)]). Genes are ordered, firstly by functional class, secondly by the type of PhoP regulation, and then by chromosomal position with the aim of highlighting the coincidence of profiles among clustered genes. The primary annotation source is the ScoDB server (www.streptomyces.org.uk). For simplicity, designations "putative" have been removed. The *p*-values for contrasts 2, 4, and 5 are indicated in bold type when found significant after adjustment for multiple testing ($p<0.05$; see Section 2).
b) Despite being classified as profile DD, this gene is included since its M_c values reflect the dD profile of genes functionally related.
c) Despite a *p*-value >0.05 of contrast 5, the "nestedF" test is significant for this contrast.
d) Gene include because the profile shown match those of genes functionally related.
e) According to our own results of homology and motif searches.
f) According to ref. [48].
g) According to ref. [65].
h) According to ref. [68].

3.5 Respiration genes

Nineteen genes involved in oxidative phosphorylation were found to be differentially regulated between strains (Table 1C). Firstly, profile D0 was assigned for three genes (*qcrA*, *qcrC*, and *cox1*) coding for components of the cytochrome terminal oxidases, for the gene *cydB*, coding for the cytochrome *bd* oxidase subunit II, and for the genes SCO3965–SCO3968. The protein encoded by SCO3966 is a putative cytochrome *c* oxidase assembly factor, and a related function is assumed for the other gene products of the cluster (conserved and membrane proteins), because they share the same transcription pattern. Secondly, a possible PhoP-dependent positive effect (profiles 0D, dD, A0, and AD) was found for respiratory genes SCO0922, SCO0923 (succinate dehydrogenase subunits), and SCO0924 (cytochrome *b* subunit), and for components of nitrate reductase (Table 1C). There are three nitrate reductase clusters in *S. coelicolor*, each one including the structural genes *narG*, *narH* and *narI*, and the chaperone *narJ* [48]. In contrast to those genes, no significant differences between strains were found for six genes of the F_0F_1-ATP synthetase operon (*atpB*, *atpF*, *atpH*, *atpA*, *atpG*, and *atpD*) [49, 50], which showed a similar decreased transcription after phosphate shift down in both strains (Supporting Information). Indeed, a DD profile was found for the AtpA protein by 2-D analysis (Table 2).

Taken together all these results indicate that phosphate starvation produces a PhoP-dependent switch in energy metabolism, downregulating the aerobic respiration genes (terminal oxidases), and upregulating the nitrate reductase clusters (see Section 4).

3.6 Glycolysis and central metabolism

The only detected significant differences between the wild type and Δ*phoP* strain responses are those of the genes *gabD* (succinate-semialdehyde dehydrogenase, profile 0A), and SCO7040, one of the three paralogs coding for glyceraldehyde-3-phosphate dehydrogenases (profile 0D).

The gene SCO7630, coding for a putative phosphoglycerate mutase, shows a biologically significant A0 profile. In addition, the paralog phosphoglycerate mutase (SCO4209), which is not present in the used microarray slides, was also detected in 2-D gels under two protein variants showing the profiles AA and A0, respectively (Table 2). Both results suggest a PhoP-dependent activation of this activity to compensate for substrate limitation. Since this enzyme catalyzes the first reaction that needs inorganic phosphate as substrate, it has been suggested that this is a bottleneck for the glycolisis pathway under phosphate limitation [51]. Therefore, PhoP-mediated activation of this phosphoglycerate mutase gene could compensate for substrate limitation.

3.7 Genes involved in cell envelope biosynthesis

In the Gram-positive *B. subtilis*, PhoP regulates the cell wall secondary polymer replacement after phosphate limitation. PhoP represses the genes for teichoic acid synthesis that

Table 2. Protein changes in the proteome of the wild type and Δ*phoP*-lacking mutant of *S. coelicolor* under phosphate-limitation conditions

Spot[a)]	Systematic name	Gene name	Product	2-D profile	Variants[b)]
(A)					
1	SCO1773	–	Putative L-alanine dehydrogenase	AA	+
2	SCO4209	*pgm*	Phosphoglycerate mutase	AA/A0	+
3	SCO1947	*gap1*	Glyceraldehyde-3-phosphate dehydrogenase	AA	–
4	SCO4662	*tuf1*	Elongation factor TU-1	DD/(A0)	+
5	SCO5625	*tsf*	Elongation factor Ts	DD	–
6	SCO5627	*frr*	Ribosome recycling factor	DD	–
7	SCO1998	*rpsA*	30S ribosomal protein S1	DD	–
8	SCO4761	*groES*	Cochaperonin GroES	DD	–
9	SCO3671	*dnaK*	DnaK, heat-shock protein 70	DD	–
10	SCO5371	*atpA*	ATP synthase alpha chain	DD	–
11	SCO5501	*gatB*	Probable Glu-tRNA-Gln amidotransferase subunit B	DD	–
12	SCO3767	–	Hypothetical protein	DD	–
13	SCO4293	–	Putative threonine synthase	DD	–
14	SCO4230	*phoP*	Putative response regulator	A0	–
15	SCO2198	*glnA*	Glutamine synthetase I	0A	–
16	SCO3023	*sahH*	*S*-adenosyl-L-homocysteine hydrolase	0A	–
17	SCO6282	–	Putative 3-oxoacyl-[acyl-carrier protein] reductase	D0	–
18	SCO3899	–	Inositol-1-phosphate synthase	D0	–
(B)					
19	SCO2180	*pdhL*	Putative dihydrolipoamide dehydrogenase	AA	–
20	SCO7000	*idh*	Isocitrate dehydrogenase	AA	–
21	SCO4827	*mdh*	Malate dehydrogenase	AA	–
22	SCO3622	–	Putative aminotransferase	AA	–
23	SCO5743	–	Thymidylate synthase	AA	–
24	SCO0596	*dpsA*	DNA-binding protein	DD	–
25	SCO2901	–	Hypothetical protein	DD	–
26	SCO4411	–	Calcium binding protein	DD	–
27	SCO4771	–	Putative inosine-5′-monophosphate dehydrogenase	A0	–
28	SCO4809	*sucD*	Succinyl-CoA synthetase alpha chain	A0	+
29	SCO4366	–	Putative phosphoserine aminotransferase	0A	–
30	SCO4723	*adk*	Adenylate kinase	0A	–
31	SCO0179	–	Putative zinc-containing dehydrogenase	D0/00	+
32	SCO2158	–	Putative kinase	0D	–
33	SCO2440	–	Putative transcriptional regulator	0D	–
34	SCO4277	–	Putative tellurium resistance protein	0D	+
35	SCO4636	–	Hypothetical protein	0D	–

(A) Proteins which encoding genes belong to the group of 529 genes selected from the transcriptomic results. The proteins encoded by the *pgm*, *frr*, *rpsA*, *dnaK*, and *gatB* genes are also included because of these genes are clustered or are functionally related with other selected genes (see text for details).

(B) Proteins which encoding genes do not have statistically significant transcriptomic results.

a) Spot numbers are indicated in concordance with Fig. 6.

b) Detected protein variants are indicated.

forms the phosphate-rich secondary polymer, and activates the biosynthetic genes for the phosphorous-free teichuronic acid, as a way to obtain phosphate [52]. Teichoic acids as a phosphorous storage have also been reported in *Streptomyces* [53]. The biosynthetic genes for both cell wall polymers have not yet been identified in the *S. coelicolor* genome. However, we have identified five clustered genes with putative roles on the biosynthesis of cell wall components that showed the A0 profile (Table 1D), and includes the putative genes *neuA* and *neuB*, encoding enzymes involved in *N*-acetyl neuraminic acid biosynthesis. A putative PhoP-binding site (14.28 bits) has been located in the bidirectional promoter region SCO4873-SCO4874; and another PhoP-binding site has experimentally validated in the promoter region of SCO4879 (see Section 3.16).

3.8 Stress response genes

Universal stress proteins (Usp) have been found across genomes of Archaea, Bacteria, and Eukarya. Its synthesis is induced in response to a large number of stresses [54], including phosphate starvation. We have identified four *S. coelicolor* genes containing the PF00582 motif characteristic of the *E. coli* Usp proteins. These genes cluster in two close regions (SCO0180–SCO181 and SCO0198–SCO200; the last cluster including a zinc-dependent alcohol dehydrogenase). Their transcriptions decreased in phosphate limitation, but a greater transcription decrease was observed in the mutant strain.

A group of seven genes code for chaperones, heat-shock and cold-shock proteins. They are the *tig* gene (cell division trigger factor), chaperone *sugE*, chaperonins *groES* and *groEL1*, and cold-shock proteins *scoF1* and *scoF4*. All of them, except the chaperonin *groEL2*, showed decreased transcription in response to phosphate limitation without any significant differences between strains, as occurs in *Sinorhizobium meliloti* [20]. The same patterns were found for the DnaK and GroES proteins in the bidimensional electrophoresis, which are part of the *dnaK* operon (*dnaK*, *grpE*, *dnaJ*, *hspR*) and *groES-groEL1*, respectively [55].

3.9 Oxidative stress and iron metabolism genes

It is known that *E. coli* cells undergo oxidative stress after phosphate limitation and that the cells respond by increasing the catalase activity [56, 57]. Yuan *et al.* [58] have recently reported that the catalase gene is activated by phosphate limitation in *S. meliloti*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens*. In our results, the transcription of *catA*, for the major *S. coelicolor* vegetative catalase, and of a gene (SCO0380) located immediately downstream of *catA*, was activated under phosphate limitation only in the wild-type strain (profile A0). The same occurs with the catalase/peroxidase gene *cpeB* (Table 1E). However, bioinformatic searches in the *catA* promoter region of *S. coelicolor* did not reveal any putative PHO box sequence (see Section 3.16), suggesting that PhoP activates its transcription indirectly.

The two superoxide dismutases known in *S. coelicolor* [59] showed opposite responses to phosphate limitation. The transcription of the *sodF* gene, encoding the iron- and zinc-containing superoxide dismutase, decreased in both strains (DD profile), but the nickel-containing superoxide dismutase gene (*sodN*) was overexpressed and higher in the mutant strain (aA profile; Table 1E). In addition, the alkyl hydroperoxide reductase *ahpC* gene transcription was higher in the mutant strain (0A profile).

The bacterioferritin gene *bfr* and the downstream gene SCO2114 (bacterioferritin-associated ferredoxin) had the A0 profile, indicating that their expression is PhoP dependent, as in *S. meliloti* [20]. It has been suggested that the primary function of bacterioferritins is the detoxification of iron and protection against radical oxygen species [60]. Other iron-related genes are those of the coelichelin biosynthesis cluster genes *cchB* (peptide monooxygenase), *cchE* (iron-siderophore ABC-transporter ATPase), and *cchF* (iron-siderophore binding lipoprotein); their transcription decreases most in the wild type at t_0 (profiles D0). Although iron is an essential nutrient, it is also problematic due to its ability to catalyze Fenton reactions that generate reactive oxygen species. These data suggest that the PHO response includes the prevention of this source of oxidative damage.

3.10 Nitrogen metabolism genes

Several genes involved in nitrogen metabolism (Table 1F) showed a characteristic overtranscription in the Δ*phoP* mutant (0A profile), suggesting that they are modulated by PhoP. This group includes *glnA* (glutamine synthetase I, beta subtype), that is flanked by two genes encoding membrane proteins showing the same profile; *glnII* (glutamine synthetase II), *amtB* (ammonium transporter), and *glnK* (PII protein). The GlnA protein has been identified in the bidimensional gels and the same pattern was revealed (Fig. 6, enlarged). The *amtB* and *glnK* genes form part of the *amtB–glnK–glnD* operon [61–63], where *glnD* is another post-translational regulator (PII nucleotidyltransferase).

3.11 Phosphate metabolism-related genes

Several *S. coelicolor* genes are involved in phosphate scavenging from organic phosphate compounds in soil, phosphate transport, and regulation (Table 1G). The operon *pstSCAB* encodes the phosphate-binding protein PstS and the components of the high affinity phosphate transport system. As expected – since the *pstS* promoter is PhoP activated [17, 45] – all of them showed the A0 profile. The *phoR–phoP* system is self-regulated [17]. Both these genes showed an A0 profile, indicating that they are PhoP regulated in agreement with the proteomic profile (Fig. 6). The *phoRP* cluster is transcribed from a bidirectional promoter region that also expresses *phoU* in the opposite orientation as a monocistronic transcript [16]. The profile of *phoU* was also A0, but the downstream genes, *mtpA* and SCO4226, showed clear AA profiles (Supporting Information). This confirms that the metallothionein *mtpA* gene – putatively involved in oxidative stress response – is upregulated when P_i was limiting by a PhoP-independent mechanism, as proposed recently in *S. lividans* [16].

Seven genes were found encoding glycerophosphodiester phosphodiesterases, whereas in *E. coli* there are only two: one periplasmic enzyme, GlpQ, and another cytosolic one, UgpQ. The genes SCO1565, SCO1968, and SCO7550 encode secreted glycerophosphodiesterases [64]. Four other genes encode cytoplasmic proteins (SCO1090, SCO1419, SCO3976, and SCO5661). Of these genes, SCO1565 and SCO1968 showed an A0 profile, and both genes are members of the PHO regulon since their promoters contain PhoP-binding sites (see Section 3.16).

The putative phytase encoded by SCO7697 (myo-inositol hexakisphosphate 3-phosphorylase) is also a phosphate-scavenging secreted enzyme. The gene showed the A0 profile, and is indeed regulated by PhoP, since it contains a PhoP-binding site in the promoter region (see Section 3.16). In *Bacillus licheniformis*, but not in *B. subtilis*, phytase accumulates extracellularly in conditions of phosphate starvation [22].

A different behavior (0A profile; Table 1G) was observed in the genes *phoH* (SCO2532), and SCO2533 (conserved protein) that belongs to a cluster of four genes. The function of PhoH, a known member of the PHO regulon in *E. coli*, is not clear. The 0A profile indicates that these genes could be repressed by PhoP. This repression must be indirect since no PHO box was found in their promoter regions (see Section 3.16).

3.12 Morphological differentiation

Eight genes related to morphological differentiation are listed in Table 1H. The extracytoplasmic sigma factor *sigU* that causes a delay in aerial mycelium formation when overexpressed [65], showed increased transcription; the response is higher in the wild type (profile Aa). The results of six other genes, putatively involved in sporulation, reflected 0A profiles. The hypothetical gene SCO7251 contains a TTA codon that is the target of *bldA* regulation. Finally, the chaplin (cell-surface proteins involved in aerial mycelium formation [66]) gene *chpC* decreased its expression mainly in the wild type (profile D0).

3.13 Secondary metabolites

S. coelicolor in postexponential cultures produces several chemical classes of antibiotics including extracellular actinorhodin and cell-bound prodigiosins. Prodigiosins are synthesized in MG medium, just after the end of the exponential phase, and actinorhodin production is delayed several hours (data not shown). Consequently, no expression was expected in the actinorhodin cluster genes at t_0 time (39 h), and no significant change was observed. On the other hand, ten of the genes belonging to the prodigiosins red cluster were expressed and showed 0A profiles (Table 1I). This profile (Fig. 5) indicates that transcription of these secondary metabolism genes is derepressed following phosphate deprivation and that this derepression is higher in the Δ*phoP* mutant.

The gene SCO7221 encoding a polyketide synthetase for germicidin biosynthesis [67] was expressed at the time used in the experiment showing an AA profile, although the transcription response was higher in the mutant strain (Supporting Information). Four genes of the cryptic polyketide synthetase cluster *cpk* [68] were found to be downregulated in a PhoP-dependent manner (Table 1I). The *cpkI* gene encodes a 3-oxoacyl-[acyl-carrier protein] reductase which has been clearly detected by the proteomics approach substantiating the D0 microarrays profile (see below; Fig. 6, enlarged area).

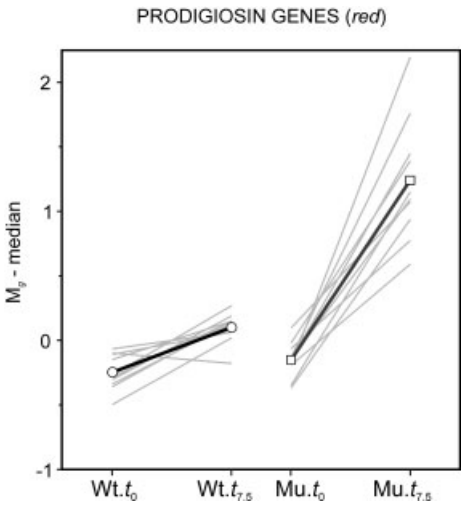

Figure 5. Transcription profiles of *red* genes. Ten prodigiosin biosynthesis genes, including the specific activator *redD*, showed significant changes. The vertical axis is the normalized M_g value. For each *red* gene, the mean of the four conditions M_g values was computed. Then, normalized M_g values were calculated as the subtraction. In this way, the coincidences between the transcription profiles are most evident. Gray lines are the plots of the transcription values in the wild-type strain (lines on the left), and in the mutant strain (on the right). The black line is the mean profile of the wild type, and the dark gray line corresponds to the mean profile of the mutant strain.

3.14 Proteomic analysis of phosphate deprivation

Proteomic studies to analyze the effect of phosphate deprivation on *S. coelicolor* M145 and in the *phoP*-lacking mutant *S. coelicolor* INB101 (Table 2) were made by using the same samples collected for RNA extraction. The *phoP*-gene deletion was confirmed by the proteomics studies, since no PhoP protein spot was observed in the Δ*phoP* mutant. Upregulation of the PhoP protein following phosphate limitation was only detected in the wild-type strain.

Changes in the levels of the first group of proteins (Table 2A) mostly agree with the microarray data and those have been explained in the microarrays section (see above). Therefore, only special behaviors will be explained in this section. Several proteins have been observed under two or more protein isoforms. The presence of protein variants has been described in different microorganisms; as an average, *E. coli* contains 1.18 variants *per* protein while *C. glutamicum* shows 1.15 variants, due to p*I* modifications or molecular mass variations [69]. Besides, in *S. coelicolor* 1.2 variants *per* protein have been reported, and several proteins involved in actinorhodin biosynthesis presented more than one form [21, 70]. A putative L-alanine dehydrogenase was detected in two different positions (spots) with similar molecular mass, but showing different p*I*. Both isoforms were induced by phosphate limitation and the most intensive induction effect was shown by the mutant strain (Fig. 6, enlarged). These

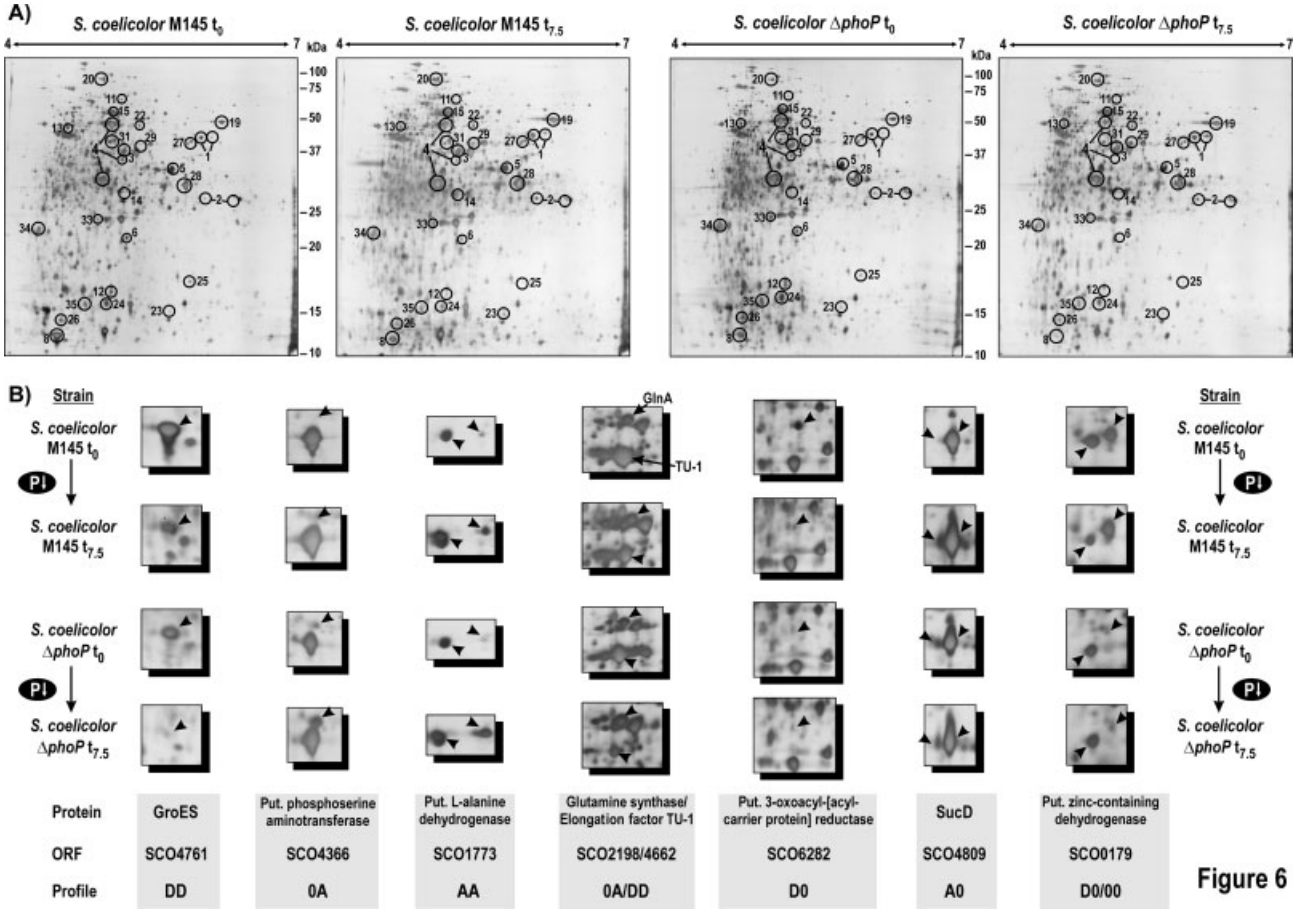

Figure 6. (A) Comparative 2-D gel analysis of phosphate-controlled cytoplasmic proteins of wild-type strain of *S. coelicolor* (M145) and the Δ*phoP* mutant (Δ*phoP*). Phosphate-replete (t_0) or phosphate-limited ($t_{7.5}$) conditions are shown. Encircled proteins correspond to those in Table 2. pH ranges (4.0–7.0) in first dimension are shown by arrows. (B) Some examples of protein profiles are enlarged. Protein spots are indicated by arrowheads. Protein name, gene identifier (ORF) and expression profile are shown in the lower part of the figure. Strain and phosphate conditions are specified (right and left). Put., putative protein.

variant forms suggest modifications in these proteins, probably affecting the activity of the protein, as proposed for SigH in *S. coelicolor* [71].

A tellurium resistance protein (SCO4277) was found as two protein variants with 0D profile (Table 2B). The tellurium resistance protein-encoding gene is not represented in the microarrays but one hypothetical protein (SCO3767, Table 2A) also related to tellurite resistance was found to be repressed under phosphate limitation. The biochemical function of tellurite resistance proteins is not clear but genes encoding these proteins are found in many pathogenic bacteria. The uptake of tellurite in *E. coli* occurs *via* P_i transport system [72]. It seems clear that tellurite-resistance proteins are regulated by phosphate.

The Glu-tRNAGln amidotransferase subunit B is downregulated following phosphate limitation (Table 2A). The encoding gene (*gatB*) is located in a gene cluster with the related genes *gatA* and *gatC*. This last gene showed a transcription profile DD in agreement with the observed shift down in proteome under phosphate deprivation in both strains.

The glyceraldehyde-3-phosphate dehydrogenase paralog named *gap1* showed discordance between proteomics and transcriptomics data (Table 2A), since that protein is upregulated under phosphate deficiency but the transcription pattern showed a D0 profile. A similar discordance was exhibited by *S*-adenosyl-L-homocysteine hydrolase. That differential gene and protein expression patterns has been frequently described [73, 74] and they appear to be due to posttranscriptional control mechanisms.

Some proteins that were not discussed in the microarrays section because their data were not statistically significant are included in Table 2B with their corresponding proteomic profiles. Three AA-profiled proteins: putative dihydrolipoamide dehydrogenase (SCO2180), isocitrate dehydrogenase (SCO7000), and malate dehydrogenase (SCO4827), directly involved in the TCA cycle have been detected by bidimensional gels. Moreover, a thymidylate

synthase (SCO5743) involved in the pyrimidine metabolism was induced under phosphate-limiting conditions reflecting the cellular response to obtain ATP from different monophosphate nucleotides.

A DD profile was shown by two proteins related with the oxidative damage protection: (i) a DNA-binding protein (SCO0596), which is a ferritin-like protein showing the presence of a "dps" motif (Pfam database [75]); these proteins bind to DNA without any apparent sequence specificity, to protect DNA from oxidative damage. (ii) A hypothetical protein (SCO2901) similar to members of the alkyl hydroperoxide reductase C/thiol-specific antioxidant family (Bcp, *M. tuberculosis* bacterioferritin comigratory proteins). Therefore, some proteins involved in oxidative stress protection show DD profiles indicating a PhoP-independent regulation, whereas other genes could be PhoP regulated (see Section 3.9).

Two calcium-binding protein paralogs have been found in *S. coelicolor* (SCO4411, SCO5464). SCO4411 was found by the proteome analysis to have a clear DD profile. These proteins are involved in signal transduction and their down-regulation could be related with the growth rate reduction.

Two other proteins listed in Table 2B were induced in the mutant strain under phosphate limitation. The first is a putative phosphoserine aminotransferase involved in vitamin B6 biosynthesis and in glycine, serine, and threonine metabolism pathways. Both pathways release phosphate providing it under phosphate-starvation conditions in the Δ*phoP* mutant. The other protein was adenylate kinase involved in purine metabolism that may be induced to provide energy (obtaining ATP).

The last group of proteins in Table 2B include proteins with a 0D profile. These include a putative kinase and a putative IclR-family transcriptional regulator.

3.15 Formation of some protein variants responds to PhoP control

Some protein variants exhibited distinct profiles, suggesting a differential regulation of expression, or PTMs. Thus, two phosphoglycerate mutase (SCO4209) variants showing the profiles AA and A0 were identified, while a paralog (SCO7630) phosphoglycerate mutase exhibited also an A0 profile (see Section 3.6). The existence of isoforms for the SCO4209 protein has been observed previously [21]. Another example of differences among protein variants was shown by the elongation factor TU-1, which was detected under three variant forms (Fig. 6, enlarged). Four variants of this protein have been found in *C. glutamicum* [69]. Interestingly, two TU-1 variants showed a DD profile (in concordance with the microarrays data), while one exhibited an A0 pattern.

Two other proteins in Table 2B showed the A0 profile. A putative inosine-5′-monophosphate dehydrogenase, which is involved in purine metabolism and the succinyl-CoA synthetase alpha chain (*sucD*), a component of the TCA cycle (identified under two protein variants). The major spot did not show variation in response to phosphate, while the acidic variant presents a clear A0 profile (Fig. 6, enlarged), *i.e.* succinyl-CoA synthetase modification responds to phosphate control. Isoforms of this protein have been reported previously [21].

A putative zinc-containing dehydrogenase (SCO0179) was identified under two proteins variants (Fig. 6, enlarged). First, a D0-profiled variant showing repression in the wild-type strain under phosphate-limited conditions. On the other hand, the second variant presents a special 00 profile, since it does not change by phosphate deprivation but it is poorly detected in the *phoP*-lacking strain when it is compared with the wild-type strain.

3.16 Validation of the PhoP-dependent transcriptional profiles: Identification of new PHO-regulon genes

We have previously described the target sequences of the regulator PhoP as composed of two or more DRus of 11 nucleotides [17]. Each two DRu constitute one PHO box. These DRus served to identify putative DRu in the promoter regions of the phytase gene SCO7697 (four DRu), and of glycerophosphodiesterase genes SCO1565 (three DRu) and SCO1968 (four DRu). The binding of PhoP to these promoter regions was demonstrated by EMSA (Fig. 7). These results validate the A0 transcriptional profiles of these genes and confirm that they are directly activated by PhoP.

To identify additional PhoP-binding sites, the sequences of 468 differentially transcribed genes, *i.e.* those which profiles were not AA or DD, and of seven genes which proteins were differentially synthesized (Table 2B), were scanned with a weight matrix (see Section 2). This search detected the known five PhoP sites and other 125 candidate sites (Supporting Information, Table S2). The sites found in the promoter regions of SCO0034 (containing three DRu; Ri = 21.37 bits), SCO2878 (five DRu; 18.57 bits), SCO4879 (three DRu; 13.23 bits), and SCO4261–SCO4262 (three DRu; 12.55 bits) were experimentally validated (Fig. 7). The highest affinity of PhoP was to the regulatory region of SCO4879 (Fig. 7). This gene encodes a conserved protein and is followed by the putative genes *neuA* and *neuB* (Table 1D), all of which showed A0 profiles. This suggests that the binding of PhoP to the SCO4879 regulatory region activates the transcription of the three clustered genes.

Transcription profiles of both SCO0034 and SCO2878 were of the type 0D which indicates a positive role for PhoP. Gene SCO0034 encodes a 159-amino acid protein that contains the helicase-associated domain, pfam03457, mainly found in *Streptomyces* proteins. Yeats *et al.* [76] have suggested a role for these proteins in the telomere formation of the linear chromosome.

The PHO box that controls two opposite genes, SCO4261 and SCO4262, separated by only 16 nucleotides, has a novel regulatory role and an unprecedented location. This PHO box is located in the coding region of SCO4262 (positions 26–

58). Both genes showed 0A profiles, what indicates a direct repressor function for PhoP. The SCO4261 gene encodes a response regulator of unknown function and SCO4262 encodes a hypothetical protein of 115 amino acids, and contains a TTA codon, target of *bldA* regulation.

We have also investigated the promoter region of the *phoH* gene, SCO2532 (see Section 3.11) for the presence of an undetected PhoP-binding site, since this gene is upregulated in the mutant strain (profile 0A). The electrophoretic mobility was unchanged (Fig. 7). It is possible that PhoP represses the transcription of *phoH via* an intermediate regulatory protein.

4 Discussion

In the soil-dwelling *Streptomyces* species, the concentration of inorganic phosphate in the culture media is one of the most important nutritional factors affecting both growth and differentiation [77], and secondary metabolite biosynthesis [6, 78]. Taking together the transcriptomic and proteomic results, we have observed that 551 genes of *S. coelicolor* had increased or decreased expression in at least one comparison between the experimental conditions (Fig. 2). Several factors account for the observed changes. Main factors are the growth arrest of the shift-down culture and the phosphate limitation that eventually triggers the PHO regulon. In addition, other media components varied in the half-spent medium at t_0 as compared to the fresh one at $t_{7.5}$. No major stress caused by manipulation of the cells during the shift down was observed, since the immediate growth after the shift down is exponential (Fig. S1). Moreover, general-stress genes are downregulated at $t_{7.5}$, thus excluding a stress-mediated induction of other genes. In order to discriminate these factors from the changes due to PhoP-dependent regulation, the responses of wild type and Δ*phoP* strains were compared following culture conditions that allowed a similar growth pattern. The statistical analysis lead us to distinguish eight major types of profiles. Four other intermediate profiles were also observed that allowed a fine-tuning in the classification of the responses (Figs. 3 and 4). We have focused on the analysis of genes with known biological function or with a plausible putative function. Data for the 368 genes not commented here are provided in the Supporting Information, Table S1. These set of genes also include 38 regulators, of unknown function at present, which are differentially transcribed in both strains.

The expression of a significant number of genes was the same in the wild type and in the Δ*phoP* mutant (profiles DD or AA). Therefore, these genes are not dependent upon PhoP and their expression is regulated by other factors. Alternatively, it is possible that other regulators of phosphate control can act as well in the shift-down cultures as suggested for the regulation of the *mtpA* gene in the close species *S. lividans* [16].

The results of this work confirmed that phosphate deprivation induces expression of the *phoR–phoP* cluster (A0 profile). The response regulator PhoP becomes phosphorylated following phosphate deprivation and the increased expression of PhoP allows the induction of a number of genes – the PHO regulon – by binding to the PHO boxes occurring in the promoters of these genes [17]. Matin *et al.* [46] have proposed that a two-step starvation process occurs in *E. coli*; the first step involves induction of enzymes to capture the scarce available phosphate. As shown in Table 1G, phosphate deprivation triggers a series of extracellular enzymes involved in phosphate scavenging from organic phosphates. These include a phytase gene and two glycerophosphodiester phosphodiesterase genes. These extracellular phosphodiesterases correspond functionally to the GlpQ of *E. coli*, a periplasmic enzyme that hydrolyzes glycerophosphodiesters into sn-glycerol-3-phosphate and the corresponding alcohol or amine. The PhoP-dependent induction of phytase is consistent with the fact that phytate is the major storage form of phosphate in some plant materials that eventually become part of the organic matter of the soil. The presence of PhoP-binding sites in these genes, validated by EMSA (Fig. 7), supports the conclusion that they belong to the PHO regulon.

One of the key proteins in phosphate scavenging is PstS, the extracellular phosphate-binding protein. The *pstS* promoter activity increased from basal levels up to 240-fold in the wild-type phosphate-limited cultures (Fig. 4). Indeed, PstS has also been found to be the most abundant protein induced in *B. subtilis* after phosphate starvation [4]. We

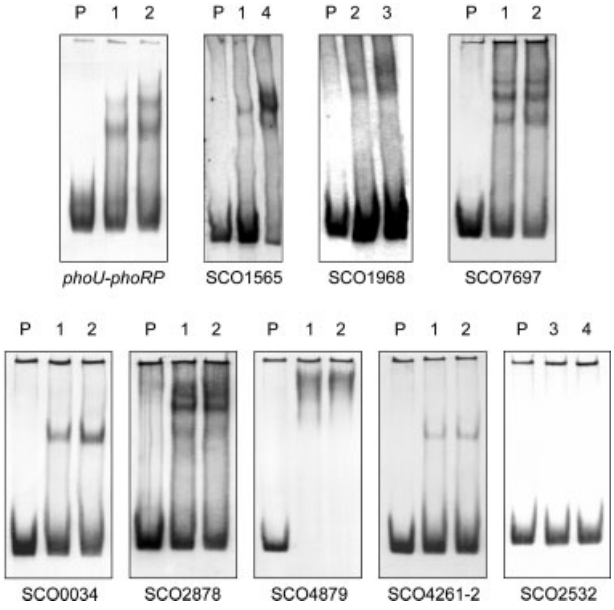

Figure 7. Electrophoretic mobility analysis (EMSA) of PhoP binding to different promoter regions. Lane P, probe without protein; Lane 1, 3.125 pmol of GST-PhoP^{DBD} protein; Lane 2, 6.25 pmol; Lane 3, 12.5 pmol; Lane 4, 25 pmol. The *phoU–phoRP* bidirectional promoter region was used as positive control.

reported previously that the *pstS* promoter is activated by PhoP binding in *S. coelicolor* [17]. This earlier finding validates the PhoP-dependent *pstS* profile A0 (Table 1G). Interestingly, PstS is induced by fructose and other sugars [45], indicating that an excess of these sugars triggers a phosphate response because of the high phosphate demand for sugar transport and phosphorylation deprives the cell of phosphate. The entire *pstSCAB* cluster, as expected for the components of the ABC-type phosphate transport system, is PhoP dependent, and probably is transcribed as an operon. This is also the case of the homologous genes of *E. coli*, *B. subtilis*, *S. meliloti*, and *Clostridium acetobutylicum*.

As a parallel strategy to survive hunger, *Streptomyces* cells may consume phosphorous storage material, such as polyphosphates, nucleotides, or teichoic acids [53, 79]. In *B. subtilis* phosphate-limited cells, teichoic acids are partially replaced by teichuronic acids, *via* the transcriptional repression/activation of the biosynthesis genes mediated by PhoP [52]. Two PhoP-binding sites, one of them validated by EMSA (Fig. 7), have been found in a cluster of genes putatively involved in the biosynthesis of cell wall polysaccharides. This activation indicates that also a replacement of phosphate-rich polymers with phosphate-free polymers could take place in the *Streptomyces* cell walls.

Analysis of the data obtained revealed interesting conclusions on other genes not obviously involved in phosphate metabolism. Most of the primary metabolism genes, particularly those involved in transcription, protein synthesis, cell division, and amino acid metabolism, were underexpressed in both the wild type and the Δ*phoP* mutant following phosphate deprivation. The growth arrest of the shift-down cultures accounts for the decreased expression. Remarkably, 58 of these genes showed a dD profile, *i.e.* their expression is upregulated in wild-type strain (Fig. 4). This regulation is also observed in the data of *S. meliloti*, although it was not discussed by the authors [20].

The cross regulation between phosphate deprivation and nitrogen metabolism is another interesting finding. *S. coelicolor* has an unusually large arsenal of glutamine synthases including a prokaryotic GS-beta subtype enzyme encoded by *glnA*, a eukaryotic-like glutamine synthetase II (encoded by *glnII*), and three annotated *glnA*-like genes [61]. The microarrays results of *glnA* regulation were validated by the proteomics findings (Fig. 6). The *amt* and *glnK* genes are part of the *amtB–glnK–glnD* operon [61], where *glnD* encodes a posttranslational regulator of the glutamine synthetase activity. The clustering of these genes suggests that their promoter might be phosphate regulated. The finding that *glnII* is also regulated by phosphate suggests that all those genes that are *glnR* and *glnRII* dependent [62, 63] are controlled by phosphate through a PhoP-mediated control of the *glnR* genes.

Eight genes of oxidative phosphorylation showed a D0 profile. Some of them encode subunits of terminal oxidases, whereas others are probably participating in this function (Table 1C). Related to this, the transcription response of the three nitrate reductase clusters is higher in the wild type than in the mutant strain. Transcription of genes of the second cluster remains constant in the wild type after the shift down (0D profile), whereas genes of the first and third clusters are upregulated in a PhoP-dependent manner (A0 or AD profiles; Table 1C). Taken together, those profiles indicate that phosphate-limiting conditions triggering the PhoP-mediated response result in a switch from aerobic to anaerobic respiration. Similarly, in *B. subtilis* several studies have revealed a regulatory loop between the PHO response and the cellular respiratory conditions [80].

The bioinformatics searches coupled to EMSA validation have revealed novel PhoP-binding sites in the promoter regions of SCO0034, SCO2878, and SCO4261–SCO4262. A putative role in the chromosome maintenance has been attributed to SCO0034, whereas SCO4261 is a putative response regulator. Although we do not know which genes depend on this regulator, it is an example of PhoP acting as a regulator of other regulators, what can explain expression responses of genes lacking PHO boxes in their promoter regions. An indirect cascade mechanism has also been suggested for the PhoP regulation of the *ppk* gene in *S. lividans* [79]. The complexity of the phosphate-limitation response is enriched by the observation of protein variants differently synthesized (Table 2).

This work provides also information on genes related to morphological differentiation and secondary metabolism. The transcriptomic data suggest a PhoP-dependent repression of genes related to differentiation at the time of culture collection ($t_{7.5}$). Most *Streptomyces* species do not sporulate in liquid culture, although the genetic program can be initiated [81]. All of the prodigiosin biosynthesis genes showed a 0A profile, indicating that they are derepressed in the Δ*phoP* strain (Fig. 5). These results correlate with the observation that the Δ*phoP* mutant grown on solid media showed increased pigment production and precocious sporulation, as observed previously in *S. lividans* [15]. Genes of cryptic cluster *cpk* were also derepressed in the Δ*phoP* strain (Table 1I). However, there is no evidence for direct binding of PhoP to the promoter of antibiotic biosynthesis genes [17, 82] indicating that the negative regulation of secondary metabolism genes is exerted by a PhoP-mediated signal transduction cascade that modulates pleiotropic regulatory genes that in turn activate pathway-specific regulators, rather than by direct binding of PhoP to the promoters of antibiotic biosynthesis genes. The alternative possibility that PhoP binds directly to the promoters of pathway-specific activators cannot be excluded at this time, but experimental support has not been found to substantiate this last hypothesis.

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Phosphate-dependent regulation of the low- and high-affinity transport systems in the model actinomycete *Streptomyces coelicolor*

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The transport of inorganic phosphate (P_i) is essential for the growth of all organisms. The metabolism of soil-dwelling *Streptomyces* species, and their ability to produce antibiotics and other secondary metabolites, are strongly influenced by the availability of phosphate. The transcriptional regulation of the SCO4138 and SCO1845 genes of *Streptomyces coelicolor* was studied. These genes encode the two putative low-affinity P_i transporters PitH1 and PitH2, respectively. Expression of these genes and that of the high-affinity transport system *pstSCAB* follows a sequential pattern in response to phosphate deprivation, as shown by coupling their promoters to a luciferase reporter gene. Expression of *pitH2*, but not that of *pap-pitH1* (a bicistronic transcript), is dependent upon the response regulator PhoP. PhoP binds to specific sequences consisting of direct repeats of 11 nt in the promoter of *pitH2*, but does not bind to the *pap-pitH1* promoter, which lacks these direct repeats for PhoP recognition. The transcription start point of the *pitH2* promoter was identified by primer extension analyses, and the structure of the regulatory sequences in the PhoP-protected DNA region was established. It consists of four central direct repeats flanked by two other less conserved repeats. A model for PhoP regulation of this promoter is proposed based on the four promoter DNA–PhoP complexes detected by electrophoretic mobility shift assays and footprinting studies.

INTRODUCTION

Streptomyces are soil-dwelling Gram-positive filamentous bacteria with complex regulatory networks that allow their adaptation to changing nutritional conditions (Chater & Bibb, 1997). *Streptomyces* species have a central role in soil ecology processes, and produce an impressive array of secondary metabolites with interesting biological properties and pharmacological activities (Bérdy, 2005). There are hundreds of genes in the sequenced genomes of *Streptomyces* that encode enzymes for the biosynthesis of secondary metabolites (Omura *et al.*, 2001; Bentley *et al.*, 2002). The biosynthesis of most secondary metabolites is negatively regulated by phosphate (Martín *et al.*, 1994).

Phosphorus is an essential component of bacterial nutrition and is one of the major constituents of the cell, making up ~1.5–2.1 % of the cell dry weight. Phosphorus plays an important role in cell metabolism and is a constituent of nucleic acids, phospholipids, phosphate-containing lipopolysaccharides, nucleotide cofactors and some post-translationally modified proteins.

Expression of phosphate-regulated genes in *Streptomyces* species is modulated by the two-component system PhoR–PhoP (Sola-Landa *et al.*, 2003). PhoR is the membrane sensor kinase, and senses phosphate scarcity; PhoP is the response regulator, and binds DNA and controls the transcription of genes belonging to the so-called PHO regulon. PhoP has been shown to modulate the expression of primary and secondary metabolism genes, including the actinorhodin and undecylprodigiosin biosynthesis genes (Sola-Landa *et al.*, 2003; Ghorbel *et al.*, 2006). Binding of PhoP to the promoter regions of three different genes of the PHO regulon, *pstS*, *phoU* and *phoRP*, has been shown in both *Streptomyces coelicolor* (Sola-Landa *et al.*, 2005) and *Streptomyces natalensis* (Mendes *et al.*, 2007). The PhoP operator sequences of these genes, as well as those present in the promoter regions of *phoA* and *phoD* of *S. coelicolor* (Apel *et al.*, 2007), are composed of direct repeat units (DRus) of 11 nt. Novel operator sequences have been

Abbreviations: DRu, direct repeat unit; EMSA, electrophoretic mobility shift assay; *Ri*, information content; TMS, transmembrane segment; TSP, transcription start point.

recently described in *S. coelicolor* (Sola-Landa *et al.*, 2008). Analysis of the novel and the previously known operators (up to 19) by means of electrophoretic mobility shift assays (EMSAs), footprinting, and information theory studies, has revealed the structure of the PhoP-binding sites. Two or three well-conserved DRus form the core of the binding site, in which each DRu is bound by a protein monomer. Complex sites have adjacent DRus that are bound subsequently (Sola-Landa *et al.*, 2008).

The transport of phosphorus sources is essential for the growth of all living organisms. The preferred source of phosphorus in bacteria is inorganic phosphate (P_i), which can enter the cell with the aid of at least two different transport systems (as described below), although there are other phosphorus-containing compounds that can enter the cell intact, such as organophosphates and phosphonates. However, numerous organic phosphate compounds have to be hydrolysed before being transported into the cell, such as nucleotides, some sugar phosphates, and phospholipids (Martín & Demain, 1977, 1980). *S. coelicolor* and other *Streptomyces* species contain at least three phosphatases and a phosphodiesterase system (Apel *et al.*, 2007) to hydrolyse different organic phosphates.

P_i is taken up in bacteria mainly by two different transport systems, the high-affinity phosphate-specific transporter (Pst) and the low-affinity phosphate inorganic transporter (Pit). Pst is an ABC transporter that has an ATP-driven high-affinity P_i uptake. In *Escherichia coli* this transporter is composed of four proteins. These are the PstS (periplasmic phosphate-binding), PstA and PstC (integral membrane proteins), and PstB (ATP-binding) subunits. The *pstSCAB* operon belongs to the PHO regulon and has been identified in *E. coli* and in other bacterial species (Rao & Torriani, 1990; Nikata *et al.*, 1996). The *pstS* gene has also been studied in *S. coelicolor* (Sola-Landa *et al.*, 2005; Díaz *et al.*, 2005), in which its expression is subject to a strict phosphate control.

Pit is a low-affinity, high-velocity phosphate-uptake system and is dependent on the proton motive force for energy. The Pit transport system has been studied in *E. coli* (Hoffer *et al.*, 2001; Harris *et al.*, 2001; van Veen *et al.*, 1994a; van Veen, 1997), *Acinetobacter johnsonii* (van Veen *et al.*, 1993, 1994b, c; van Veen, 1997) and *Sinorhizobium meliloti* (Voegele *et al.*, 1997; Bardin & Finan, 1998; Bardin *et al.*, 1998; Yuan *et al.*, 2006a). In *E. coli*, Pit is the major P_i uptake system when P_i is in excess (Rosenberg *et al.*, 1977, 1979) and consists of a single transmembrane protein (Elvin *et al.*, 1986). Divalent cations such Mg^{2+} and Ca^{2+} are essential for Pit activity in both *E. coli* (Russell & Rosenberg, 1980) and *A. johnsonni* (van Veen *et al.*, 1994b), since phosphate is symported with a proton, as a soluble and neutral metal complex ($MeHPO_4$; van Veen *et al.*, 1994a, c; van Veen, 1997).

E. coli contains two functional *pit* genes, *pitA* and *pitB* (Hoffer *et al.*, 2001; Harris *et al.*, 2001). Two or more paralogues can also be found in many other genomes. PitA and PitB have similar transport characteristics (Hoffer *et al.*, 2001) and have 90 % similar amino acid sequences. However, the regulation of the two genes appears to be different. Thus, *pitA* is expressed constitutively, while *pitB* appears to be repressed under conditions of phosphate limitation (Elvin *et al.*, 1986; Harris *et al.*, 2001).

The *pit* gene(s) have not been studied in *Streptomyces* species, but their characterization is important to establish their role in phosphate transport and their effect on the biosynthesis of secondary metabolites and on the ecology of these bacteria in soil. This work describes two paralogous *pit* genes in *S. coelicolor*, and elucidates their pattern of regulation by means of both *in vivo* assays, using wild-type *S. coelicolor* and Δ*phoP* mutant strains, and DNA-binding PhoP-footprinting analysis.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *S. coelicolor* strains M145 (Kieser *et al.*, 2000) and INB101 (Δ*phoP*) (Rodríguez-García *et al.*, 2007) were manipulated according to standard procedures (Kieser *et al.*, 2000). TBO medium (Higgens *et al.*, 1974) was used to obtain spore preparations. *E. coli* DH5α was the general cloning host. Cloning procedures were performed as described by Sambrook *et al.* (1989).

The *pap-pitH1* and *pitH2* promoter regions were amplified by PCR using total DNA as template, as follows. The primers FSC21 (5′-TGGTGGATCCCGCGTCCTCCAATAACGG) and FSC22 (5′-CAGAGGTACCATATGGTGGGGTCCTCGGGGTGCATGGGTTTC) amplified a 232 bp fragment encompassing the promoter region of *pap-pitH1* from −210 to −3 (positions from the ATG translation start triplet). The *Bam*HI (FSC21) and *Kpn*I/*Nde*I (FSC22) cloning sites (underlined) were introduced via the primer sequences. Primers FSC15 (5′-CTTCGGATCCTCCGGCCCGCGAATAC) and FSC16 (5′-GAGAGGTACCATATGTACCCAGGCAATCGTTCG) were used to amplify a 270 bp fragment encompassing the promoter region of *pitH2* from positions −250 to −3 (positions from the translation start) flanked by the above-mentioned cloning sites. *Bam*HI–*Kpn*I fragments were cloned into pBluescript II SK+ to obtain pFS-*pitH1* and pFS-*pitH2*, respectively. The inserts of both plasmids were checked by sequencing.

The *pstS* promoter region was cloned from pGEM-P*pstS* (Sola-Landa *et al.*, 2005). *Bam*HI–*Nde*I fragments of 222 bp from pFS-*pitH1*, 260 bp from pFS-*pitH2*, and 403 bp from pGEM-P*pstS* were cloned into the promoter-probe vector pLUXAR+ (Rodríguez-García *et al.*, 2007), yielding pLUX-*pitH1*, pLUX-*pitH2* and pLUX-*pstS*, respectively. In order to introduce the pLUX plasmids into the *S. coelicolor* INB101 strain (which is apramycin resistant), the *neo* gene was inserted to construct derivatives with neomycin resistance (Table 1).

Culture conditions. *S. coelicolor* cultures were performed in defined MG (maltose and glutamate) medium containing starch (Scharlau; 50 g l^{-1}) and glutamate (60 mM) (Doull & Vining, 1989). Aliquots (100 ml) of MG medium in 500 ml baffled flasks were inoculated with 10^6 spores ml^{-1} and incubated at 30 °C, 300 r.p.m. (25.4 mm orbit diameter) for reproducible and dispersed growth. For the phosphate-replete and the phosphate-limited conditions, cultures contained 18.5 and 3.2 mM potassium phosphate, respectively (MG-18.5 and MG-3.2 media; experimentally determined concentrations). The potassium concentrations in MG-18.5 and MG-3.2 were equalized by adding KOH to MG-3.2, instead of NaOH alone, when

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Reference or source
Strains		
S. coelicolor A3(2) M145	Wild-type	Kieser *et al.* (2000)
S. coelicolor A3(2) INB101	Δ*phoP*, Am^r	Rodríguez-García *et al.* (2007)
E. coli DH5α	F′ Φ80 *dLacZ* ΔM15	Hanahan (1983)
E. coli ET12567 (pUZ8002)	*dam dcm* mutant, Neo^r-Cm^r	MacNeil *et al.* (1992)
Plasmids		
pBluescript II SK+	Cloning vector, Amp^r	Stratagene
pFS-*pitH1*	PCR product carrying *pitH1* promoter cloned into pBluescript II SK+, Amp^r	This work
pFS-*pitH2*	PCR product carrying *pitH2* promoter cloned into pBluescript II SK+, Amp^r	This work
pLUXAR+	Integrative promoter-probe vector, *luxAB* genes, Am^r	Rodríguez-García *et al.* (2007)
pLUX-*pitH1*	*Bam*HI–*Nde*I pFS-*pitH1* fragment cloned into pLUXAR+, Am^r	This work
pLUX-*pitH2*	*Bam*HI–*Nde*I pFS-*pitH2* fragment cloned into pLUXAR+, Am^r	This work
pLUX-*pstS*	*Bam*HI–*Nde*I pGEM-P*pstS* fragment cloned into pLUXAR+, Am^r	This work
pLUX-*pitH1*-Neo	pLUX-*pitH1* with the *neo* gene inserted, Am^r-Neo^r	This work
pLUX-*pitH2*-Neo	pLUX-*pitH2* with the *neo* gene inserted, Am^r-Neo^r	This work

the pH was adjusted (sodium ions in both media are in excess, ~60–75 mM). Samples were taken after 36, 40, 44, 48, 60, 70 and 90 h of incubation.

Luciferase assay, and growth and phosphate determination. *luxAB* gene expression was determined in a Luminoskan luminometer (Labsystems). Culture samples (1 ml) were taken, spun down (4 °C, 10 min) and kept frozen until all samples from an experiment were available to be processed simultaneously; then the cells were resuspended in 1 ml NaCl (0.9 %, w/v), incubated at 25 °C for 15 min and measured in triplicate, as described in Rodríguez-García *et al.* (2007).

For dry weight determination, culture samples (2 ml) were washed twice with MilliQ water and dried for 4 days at 80 °C.

The phosphate concentration of MG medium and culture supernatants was measured using the malachite green assay (Lanzetta *et al.*, 1979).

RT-PCR. RNAs from *S. coelicolor* wild-type and Δ*phoP* strains were isolated using RNeasy Mini Spin columns (Qiagen). Before RNA isolation, one volume of each culture was treated with two volumes of RNAProtect Bacteria reagent (Qiagen) to provide immediate stabilization of RNA. Cell lysis and phenol pre-purification were carried out prior to column purification as described previously (Rodríguez-García *et al.*, 2007). RNA preparations were treated on-column with DNase I (Qiagen), and the eluted solution with the DNA-free kit (Ambion) to eliminate possible chromosomal DNA contamination. RNA concentration and quality were checked using a NanoDrop ND-1000 (Thermo Fisher Scientific) and RNA nano labchips in a 2100 Bioanalyser (Agilent).

Gene expression analysis by RT-PCR was performed with the SuperScript One-Step RT-PCR system with Platinum *Taq* (Invitrogen). Primers CAR65 (5′-ATTTCACGAACGGTTTCCAC) and CAR66 (5′-GATGCCCATGGTCTTCTGG) were used to amplify 542 bp of the *pitH2* coding region; primers CAR67 (5′-CGTACACCAACGGTTTCCAC) and CAR68 (5′-ATGCCCATCGTCTTCTGC) were used to amplify 541 bp of *pitH1*. The synthesis of a common transcript *pap-pitH1* was detected with the primer pair CAR73 (5′-ACCGCGAGGACATCTACAAC) and CAR74 (5′-ACCAGGTGATGAGGTTCCAG). RT-PCR cycling conditions were as follows: 50 °C for 60 min, 94 °C for 2 min; 11 cycles of 94 °C for 30 s, 65 °C for 30 s with touchdown of 1 °C per cycle, 72 °C for 60 s; 23–29 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s; finally 72 °C for 10 min. Total RNA (200 ng), 1.6 mM $MgSO_4$ (final concentration), and 5 % DMSO were added in all reactions. Platinum *Taq* was used in the control reactions to check for the absence of DNA contamination.

DNA–protein binding assays. DNA-binding analyses were performed by EMSA with the GST–$PhoP^{DBD}$ protein (PhoP DNA-binding domain fused to glutathione-*S*-transferase), as described previously (Sola-Landa *et al.*, 2005). The probes used were the *Bam*HI–*Kpn*I fragments of 222 and 260 bp obtained by PCR of the promoter regions of *pap-pitH1* and *pitH2* and purified after agarose-gel electrophoresis (GFX columns; GE Healthcare). Probes were labelled at both ends with DIG (DIG Oligonucleotide 3′-End Labeling kit, Roche Applied Science).

DNase I footprinting assays. DNase I footprinting assays based on the fluorescent label procedure (Rodríguez-García *et al.*, 1997) were performed as described in Sola-Landa *et al.* (2005). DNA probes were obtained by PCR using pFS-*pitH2* as template. For analysis of the coding strand, a fluorescently-labelled universal primer and a non-labelled reverse primer were used; for the complementary strand, the labelled primer was the reverse primer. The labelled probes (424 and 419 bp for the coding and complementary strand, respectively) were purified by agarose electrophoresis (GFX columns). DNase I footprinting was performed by incubating 0.28 pmol of the DNA probe with different concentrations of GST-$PhoP^{DBD}$ protein for 30 min at 30 °C. Nuclease digestions were carried out for 1 min at 30 °C. The reaction products were resolved in an ALF DNA sequencer (GE Healthcare) and analysed with the Fragment Manager program.

Primer extension analysis. For primer extension analysis, RNA samples were isolated as described above, but with the RNeasy Midi kit (Qiagen) and the on-column DNase treatment only. Total RNA concentration and quality were checked both spectrophotometrically and by denaturing agarose gel electrophoresis. Transcription start sites were determined by the fluorescent primer extension procedure (Altermann *et al.*, 1999; Fekete *et al.*, 2003), modified as follows. Primers LUX-FAM+47 [6-carboxyfluorescein- (6-FAM) 5′-

GATAGCTCAGGTGGCTGATAAG] and LUX-FAM + 135 (6-FAM 5′-GTGGTGCTCTAGCAACCAAAC), both obtained from MWG-Biotech, were complementary to the coding region of *luxAB* genes of the pLUXAR + vector (nucleotides +25 to +47 and +114 to +135 with respect to the translation start, respectively). These primers (20–40 pmol) were hybridized with total RNA (20–30 μg) in a 10 μl final volume by heating to 90 °C for 2 min and cooling to 30 °C at a rate of 2 °C min^{-1}. Then, cDNA synthesis was performed with either Superscript II or Superscript III reverse transcriptase (Invitrogen). The primer extension reaction (30 μl) contained 10 mM DTT, 500 μM dNTPs (NEB), 1.5 μg actinomycin D, 10 U SUPERase-In (RNase inhibitor; Ambion), and 200 U reverse transcriptase in 1 × First Strand buffer (Invitrogen). The samples were incubated at 42 °C for 5 or 15 h. Then, 10 μl NaOH (1 M) was added and the RNA was lysed by heat treatment (70 °C, 10 min). A 10 μl volume of HCl (1 M) was added to neutralize the solution; the cDNA samples were phenolized and precipitated with ethanol. The pellets were dissolved in a solution of 9.6 μl Hi-Di formamide (Applied Biosystems) and 0.4 μl GeneScan LIZ-500 internal size standard (Applied Biosystems). Samples were heated to 95 °C for 5 min, placed immediately on ice for 5 min, and loaded onto an ABI PRISM 3130 sequencer (Applied Biosystems). To determine the product size accurately, sequencing reactions were performed with the same primer as the reverse transcription step and with the Thermo Sequenase Primer Cycle Sequencing kit (GE Healthcare). Reactions contained 400 ng plasmid DNA as template, and 2 pmol of the corresponding primer. Cycling conditions were 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, for a total of 60 cycles. The EDTA/ethanol-precipitated products of the four sequencing reactions were combined with the GeneScan LIZ-500 size standard and analysed on the DNA sequencer. Electrophoretograms were aligned according to the size standards using the GeneMapper 3.7 software (Applied Biosystems).

Bioinformatic analysis. The phylogenetic studies were done using the BLAST tree option on the NCBI Web BLAST service with the Fast Minimum Evolution method (Desper & Gascuel, 2002) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa *et al.*, 2006). Comparative genomics was done using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; von Mering *et al.*, 2007). The percentages of sequence similarity between two proteins were calculated using the Needleman-Wunsch global alignment of the European Molecular Biology Open Software Suite (EMBOSS; Rice *et al.*, 2000). The putative transmembrane segments (TMSs) were determined using the TopPred server (Claros & von Heijne, 1994) and the TMpred server (Hofmann & Stoffel, 1993). Prediction of signal peptides was done with the SignalP server (Bendtsen *et al.*, 2004).

To identify and analyse putative binding sites of the response regulator PhoP, the information theory programs delila, makebk, encode, rseq, dalvec, ri, scan and lister (Schneider & Stephens, 1990; Schneider, 1996, 1997) were used. Model 1 of Sola-Landa *et al.* (2008) provided the information content matrix to assign the information content (*R*i) values.

RESULTS

Two putative low-affinity phosphate transporters are encoded in the *Streptomyces coelicolor* genome

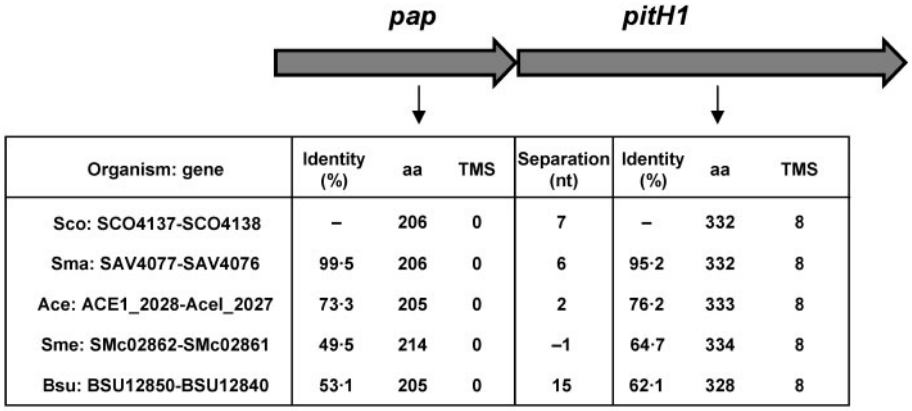
Homology searches identified two putative low-affinity phosphate transport genes in the *S. coelicolor* genome, SCO4138 and SCO1845 (Bentley *et al.*, 2002); they are designated in this work as *pitH1* and *pitH2*, respectively, following the annotation of the homologous genes of *Streptomyces avermitilis* (Ikeda *et al.*, 2003). The PitH1 and PitH2 proteins show 39 and 33 % amino acid sequence similarity, respectively, to the transporter PitA of *E. coli* and contain the Pfam motif characteristic of the phosphate transporter family (PF01384 PHO4). Their sizes and similarities with homologous proteins are shown in Fig. 1. Both PitH1 and PitH2 contain putative TMSs.

In *S. coelicolor*, both *pitH1* and *pitH2* are clustered with genes encoding other proteins that may participate in phosphate transport. Firstly, *pitH1* lies 8 bp downstream of a gene, SCO4137, that encodes a conserved protein containing a motif known as DUF47 (Pfam PF01865.7; Fig. 1). In *Sinorhizobium meliloti*, the homologous genes of SCO4137-*pitH1* are considered to form an operon (*orfA-pit*). The *orfA* gene is annotated as *pit*-accessory, although the possible role of the OrfA protein in P_i transport has not yet been investigated (Voegele *et al.*, 1997; Bardin *et al.*, 1998). In this work the SCO4137 protein is referred as Pit-accessory protein (Pap). On the other hand, downstream of *pitH2* there is a gene (SCO1846) that encodes a membrane protein. The amino acid sequences of homologous proteins in other bacteria are poorly conserved, as expected for membrane proteins, but in all cases they contain two putative TMSs (Fig. 1). Comparative genomics showed that whereas the *pap-pitH1* genetic organization is widespread in bacteria, the distribution of the *pitH2*-SCO1846 gene organization is confined to actinobacteria.

The upstream sequences of *pitH2* and *pap-pitH1* were selected for study of their transcriptional regulation by phosphate-limitation. Interestingly, bioinformatics analysis identified putative PhoP-binding sequences (PHO boxes) in the promoter region of *pitH2*, but no PHO boxes were found in the upstream region of *pap-pitH1*. The promoter of the *pstS* gene, which encodes the phosphate-binding transport protein, was also included in this study as a positive control PhoP-activated gene (Sola-Landa *et al.*, 2005).

Phosphate-limited and phosphate-replete cultures of wild-type and Δ*phoP* mutant *S. coelicolor* strains

In order to study phosphate regulation, P_i-limited and P_i-replete conditions were defined in initial experiments. Cultures of *S. coelicolor* wild-type and Δ*phoP* mutant strains were carried out in MG medium with initial P_i concentrations of 2.1, 3.2 and 18.5 mM (media designated MG-2.1, MG-3.2 and MG-18.5, respectively). The growth of both cultures was quite similar in MG-18.5 (Fig. 2a). Indeed, in MG-18.5, the P_i concentration in the medium was in excess throughout the time-course of the culture (always higher than 4 mM in both strains; Fig. 2a). For this reason, the MG-18.5 medium was chosen as the P_i-replete condition. On the other hand, an initial P_i concentration of 2.1 mM supported only poor growth of the Δ*phoP* mutant, which can be explained by the lack of both PhoP-dependent phosphate transport and a nutrient-limitation



Organism: gene	Identity (%)	aa	TMS	Separation (nt)	Identity (%)	aa	TMS
Sco: SCO4137-SCO4138	–	206	0	7	–	332	8
Sma: SAV4077-SAV4076	99·5	206	0	6	95·2	332	8
Ace: ACE1_2028-Acel_2027	73·3	205	0	2	76·2	333	8
Sme: SMc02862-SMc02861	49·5	214	0	–1	64·7	334	8
Bsu: BSU12850-BSU12840	53·1	205	0	15	62·1	328	8

Organism: gene	Identity (%)	aa	TMS	Separation (nt)	Identity (%)	aa	TMS
Sco: SCO1845-SCO1846	–	423	10	34	–	73	2
Sma: SAV6419-SAV6418	80·7	417	10	12	91·8	71	2
Lxx: Lxx08880-Lxx08890	67·4	404	10	–4	39·8	110	2
Mtc: MT0570-MT0569	66·4	417	9	–4	35·1	92	2
Cgb: cg0545-cg0544	63·8	425	10	–1	38·7	87	2

Fig. 1. Map of the organization of *pitH1* and *pitH2* genes of *S. coelicolor*. Below are tables summarizing the sequence similarity (including identical and functionally conserved amino acids) of the *S. coelicolor* (Sco) Pit proteins with Pit proteins of *S. avermitilis* (Sma), *Acidothermus cellulolyticus* (Ace), *Sinorhizobium meliloti* (Sme), *B. subtilis* (Bsu), *Leifsonia xyli* (Lxx), *Mycobacterium tuberculosis* (Mtc) and *C. glutamicum* (Cgb), the number of amino acids (aa) of the Pit proteins, the number of deduced TMSs and the separation (in nt) between the genes.

response; however, the growth of the wild-type strain was also severely reduced (results not shown). In contrast, MG-3.2 medium allowed a substantial biomass increase in the Δ*phoP* mutant during the first 44 h, although the growth did not follow the diauxic pattern of the wild-type (Fig. 2b). About 85 % of the initial P_i was utilized in the first 40 h (corresponding with the rapid growth), and after 44 h it was mostly spent (coinciding with the end of the rapid growth phase), falling below 0.1 mM in both wild-type and Δ*phoP* mutant cultures (Fig. 2b). The growth of the wild-type in MG-3.2 was also reduced as compared to the phosphate-replete condition (Fig. 2a, b). Therefore, MG-3.2 was chosen as the P_i-limited condition.

The *pitH2* and *pstS* promoters, but not the *pap-pitH1* one, are strongly induced under P_i-limited conditions

To investigate how expression of *pap-pitH1*, *pitH2* and *pstS* responds to P_i concentration in the growth medium, their promoter regions were amplified, sequenced and cloned in the pLUXAR(+) promoter-probe plasmid, as described in Methods. Derivative plasmids were introduced by conjugation into both *S. coelicolor* wild-type and Δ*phoP* mutant strains. The reporter activity was quantified in liquid cultures of phosphate-replete (MG-18.5) or phosphate-limited (MG-3.2) media (Fig. 3).

In the wild-type strain under conditions of phosphate limitation (MG-3.2), the activities of *pstS*, *pitH2* and *pap-pitH1* promoters followed a pattern of sequential induction that could be correlated with the decrease in P_i concentration of the medium (compare Figs 2b and 3a). As shown in Fig. 3(a), the maximum activity of *pitH2p* was reached at 40 h, when the P_i concentration had decreased to 0.5 mM; until 44 h of culture this was the major activity. This time (44 h) marked the end of the transition growth phase, as well as the maximum of *pap-pitH1p* activity, which showed a small delay with respect to the peak of *pitH2p* activity. Later, the *pstSp* activity became increasingly predominant. In fact, this is the only one of these promoter activities that can be

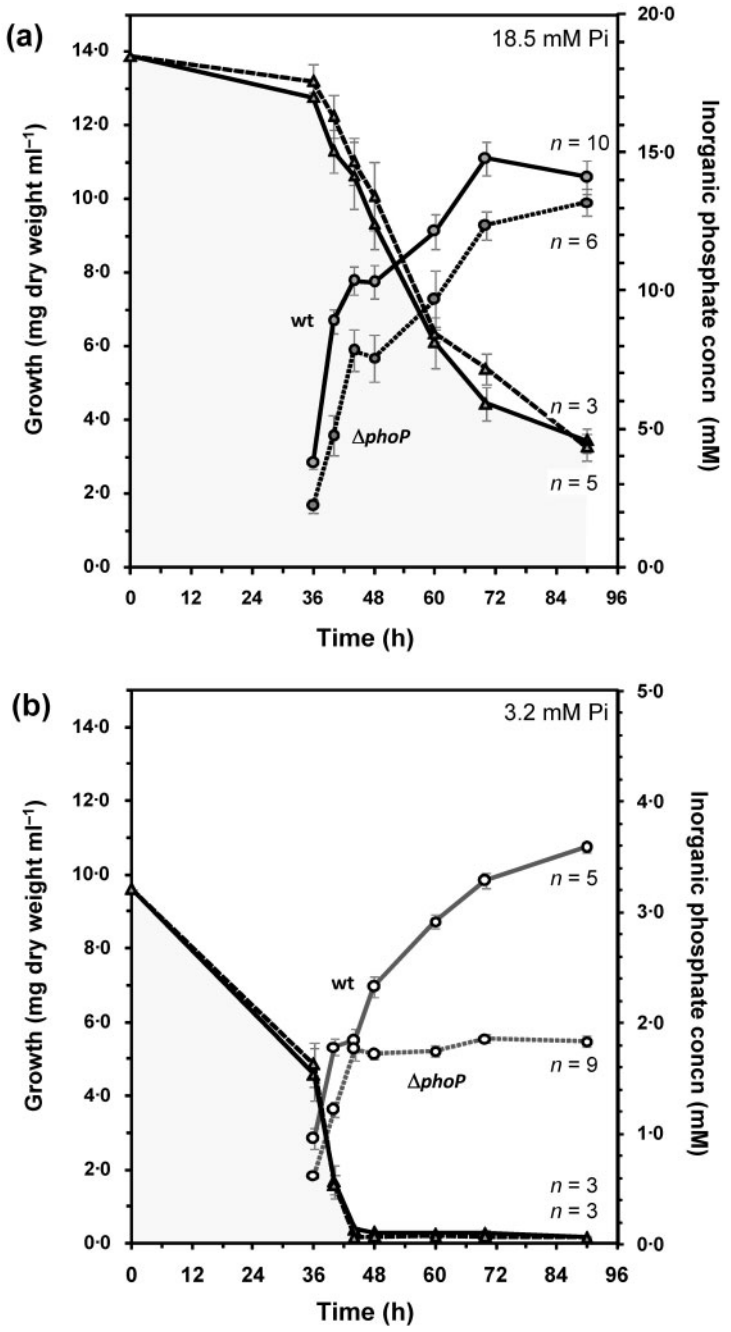


Fig. 2. Growth (○) and residual P_i concentration (△) for *S. coelicolor* M145 (wild-type) and INB101 (Δ*phoP* mutant) grown on MG-18.5 (a) and MG-3.2 (b). In all cases the dashed line represents the Δ*phoP* mutant strain. *n*, Number of independent experiments represented in each curve. Error bars, SEM.

detected up to 70 h. This is not surprising, since the Pst transporter is the high-affinity system that accounts for P_i transport under conditions of low concentration (Rao & Torriani, 1990). The highest induction of *pstSp* correlated with the drop of P_i below 0.1 mM (44–48 h; Figs 2b and 3a).

In MG-18.5, in which P_i is in excess throughout the course of growth (Fig. 2a), the pattern of expression changed dramatically. Firstly, *pstS* promoter activity remained at very low levels throughout the culture (Fig. 3b). Secondly, the activity of *pitH2p* was constant until the late growth phase, and lacked the high levels reached in MG-3.2 at 40 and 44 h (compare Fig. 3a, b). The predominant activity in cultures in MG-18.5 was that of *pap-pitH1p* throughout most of the culture. Indeed, its values increased 1.3–5.8-fold, as compared with that of the MG-3.2 culture, throughout the growth course. In conclusion, the *pitH2* and *pstS* promoters are induced by P_i limitation, while the *pap-pitH1* promoter is more active under P_i-replete conditions.

Expression of *pitH2*, but not that of *pap-pitH1*, is *phoP*-dependent

The transcriptional regulator PhoP controls expression of the *pstSCAB* transporter gene cluster by binding to the

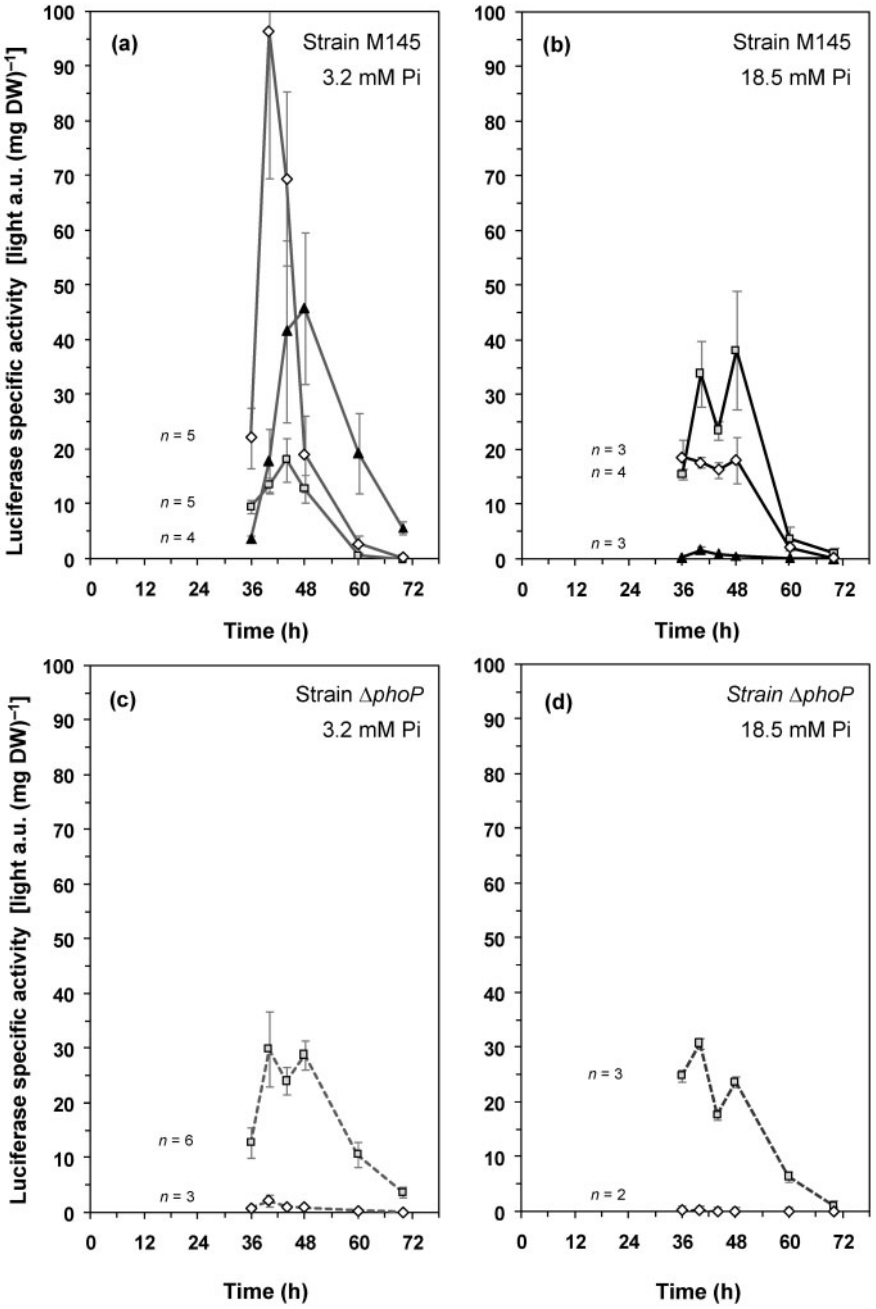


Fig. 3. Promoter activity of the exconjugants containing pLUX-*pitH2* (◇), pLUX-*pitH1* (□) and pLUX-*pstS* (▲) derived from the wild-type M145 grown in MG-3.2 (a) and MG-18.5 (b), and from the Δ*phoP* mutant INB101 grown in MG-3.2 (c) and MG-18.5 (d). The same scale is used in the four panels for comparison. In all cases the dashed line represents the Δ*phoP* mutant strain. *n*, Number of independent experiments represented in each curve. Error bars, SEM.

PHO box located in the *pstS* promoter (Sola-Landa *et al.*, 2005). In order to study whether the expression of *pap-pitH1* and *pitH2* genes depends on the PhoP response regulator, reporter expression studies were performed with the *S. coelicolor* Δ*phoP* mutant (Rodríguez-García *et al.*, 2007). Results (Fig. 3c, d) clearly showed that expression of *pitH2* was dependent upon the PhoP activator. In contrast, expression from the *pap-pitH1* promoter was not affected by the lack of PhoP in the Δ*phoP* mutant (Fig. 3c, d). Indeed, the *pap-pitH1* activity was higher in the mutant than in the wild-type strain in MG-3.2 (1.7 times higher when the maximum values are compared). This result may reflect a mechanism of adaptation of *pap-pitH1* to the lack of expression in the Δ*phoP* mutant of the other PhoP-

dependent transporters, Pst and PitH2. In the Δ*phoP* mutant grown in MG-18.5, the *pap-pitH1* promoter showed a similar behaviour to that in the wild-type strain, although the values at 36 and 60 h were higher in the mutant than in the wild-type strain.

Transcriptional analysis of *pitH2* and *pap-pitH1*

In order to confirm the promoter-probe results, RT-PCR was carried out as described in Methods using total RNA isolated from 40 h MG-3.2 cultures of both wild-type and Δ*phoP* mutant strains. Amplification of the *pitH2* transcript was clearly detected in the wild-type RNA; in contrast, only a low-intensity band was present in the Δ*phoP* mutant reaction, even after 40 cycles of PCR (Fig. 4a). The presence of a low level of *pitH2* transcriptin in the Δ*phoP* mutant correlates well with the very low, but significant, activity of the *pitH2* promoter at 40 h in the Δ*phoP* mutant (see Fig. 3c). On the other hand, both wild-type and mutant RNA gave rise to amplification products corresponding to *pitH1* and *pap-pitH1* transcripts (Fig. 4a). As expected, these results indicate that *pap* and *pitH1* form a bicistronic transcript and are PhoP-independent.

The expression profile of *pitH2* in the wild-type strain was studied in samples taken after 36, 40, 44 and 48 h of culture in MG-3.2 (Fig. 4b). Amplification of the cDNA from *pitH2* transcripts is higher at the earlier times (36 and 40 h) than the later ones (44 and 48 h). Interestingly, maximum luciferase activities from the *pitH2p-luxAB* fusion were observed ~4 h later (Fig. 3a).

PhoP binds to the *pitH2* promoter but not to the *pap-pitH1* promoter

In order to test whether the PhoP protein binds to the *pitH2* and *pap-pitH1* promoters *in vitro*, the PhoP DNA-binding domain ($PhoP^{DBD}$) fused to GST was used in binding assays, as described in Sola-Landa *et al.* (2005). The promoter DNA–PhoP interaction was studied by EMSA. A protein concentration of 0.25 μM was sufficient to produce up to four complexes with the *pitH2p* DNA fragment (Fig. 5, arrows). As the protein concentration increased, the amount of unshifted probe decreased. However, no shifted bands were observed with the *pap-pitH1* promoter under the same experimental conditions.

Characterization of the *pitH2* and *pap-pitH1* promoters

The transcription start site of the *pitH2* promoter was determined by primer extension, using RNA from a 42 h culture in MG-3.2 of the *S. coelicolor* [pLUX-*pitH2*] exconjugant that corresponds to conditions of high promoter activity (Fig. 3a). Extension products with an apparent size of 76 and 166 nt, primed with LUX-FAM + 47 and LUX-FAM + 135, respectively, were observed clearly (Fig. 6). The same transcription start

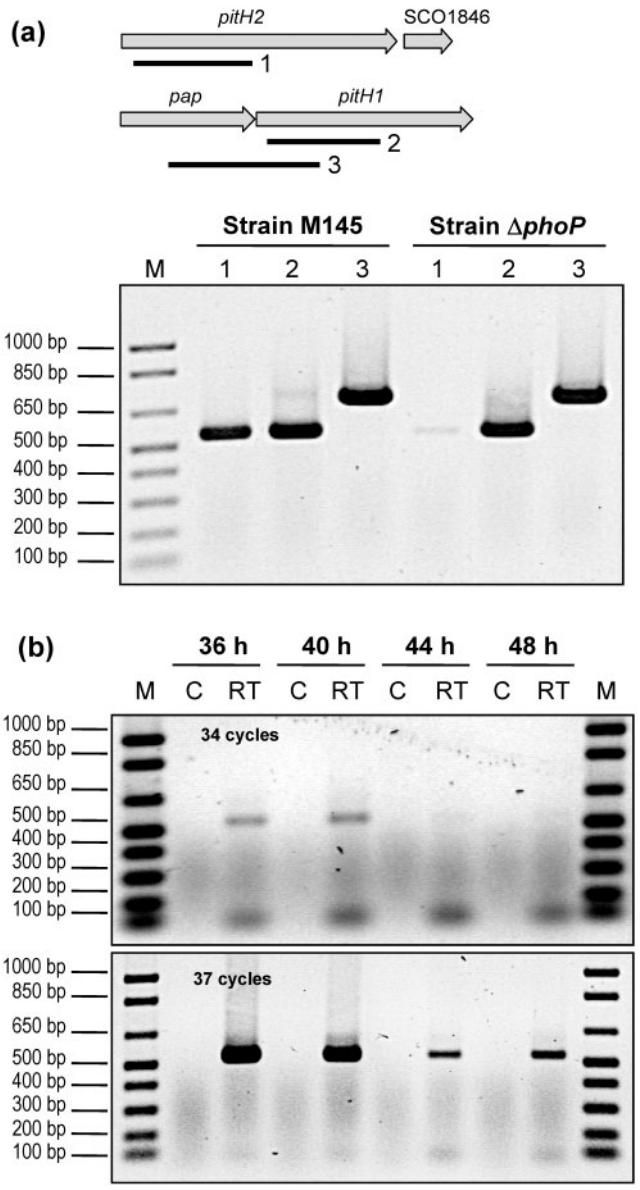


Fig. 4. RT-PCR analysis of the *pitH2*, *pitH1* and *pap-pitH1* transcripts in *S. coelicolor* M145 and *S. coelicolor* INB101 (Δ*phoP*). The analysis was carried out as described in Methods. (a) The expected RT-PCR products 1, 2 and 3 of the *pitH2* and *pap-pitH1* genes are depicted above. Below, RT-PCR results (40 cycles) using total RNA from wild-type and Δ*phoP* mutant cultures in MG-3.2 (40 h) as template. Lanes: M, 1 kb plus DNA ladder; 1, amplification of a 542 bp fragment internal to *pitH2*; 2, amplification of a 541 bp fragment internal to *pitH1*; 3, amplification of a 697 bp fragment covering both *pap* and *pitH1* coding sequences. The absence of contaminating DNA in the RNA samples was assessed using Platinum *Taq* (data not shown). (b) RT-PCR amplification of the *pitH2* transcript. Template total RNAs were isolated at 36, 40, 44 and 48 h from a wild-type MG-3.2 culture. Lanes: C, control reactions with Platinum *Taq*; RT, reverse trancriptase reactions. Upper panel, 34 cycles of amplification; lower panel, 37 cycles.

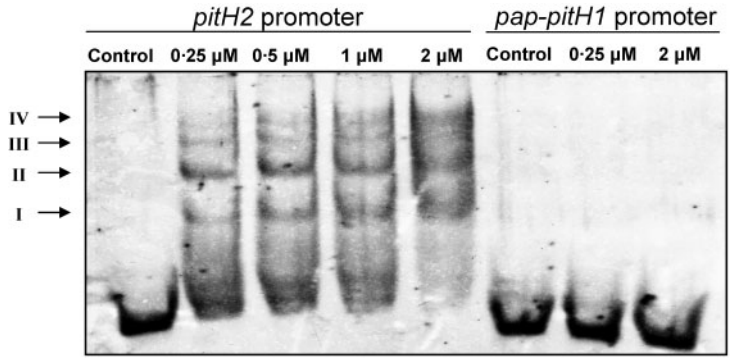


Fig. 5. EMSAs of the *pitH2* and *pap-pitH1* promoters. Control, probe without protein. Other lanes, increasing concentrations (0.25, 0.5, 1 and 2 µM) of the GST-PhoP^{DBD} protein (DNA-binding domain). For *pap-pitH1*, only 0.25 and 2 µM protein concentrations were used. Note the lack of binding to the *pap-pitH1* promoter.

point (TSP) was deduced from both products when compared with the respective sequencing reactions. This corresponds to a guanine located 32 bp upstream of the ATG codon (Fig. 6). Sequences resembling the −10 and −35 consensus (Strohl, 1992) were located at the proper distance from the TSP, centred at positions −7 and −33, respectively. These −10 and −35 boxes overlap the PhoP-binding site (Fig. 8). Although the distance between the −10 and −35 boxes (20 nt) is longer than usual (16–18 nt), this feature seems to be a characteristic of the PhoP-dependent activation mechanism of this and other genes (see Discussion).

Following the research of Strohl (1992), the *pap-pitH1* upstream sequences TTAGCA-N18-TAGCAT constitute plausible −35 and −10 promoter boxes (located at

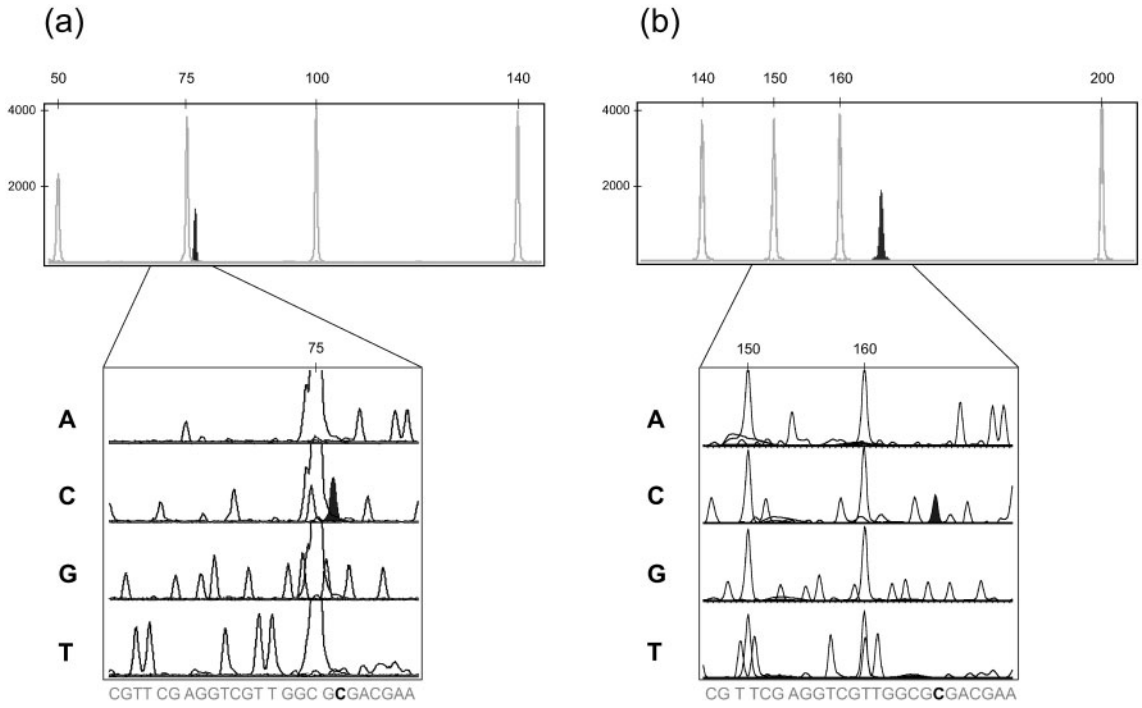


Fig. 6. Primer extension analysis of the *pitH2* promoter using automated fluorescent capillary electrophoresis. Primer extension reactions were done with both the LUX-FAM+47 (a) and LUX-FAM+135 (b) primers. In the upper fluorograms, the black-filled peaks represent the extension products (FAM-labelled cDNA) and the grey traces represent the LIZ-500 standards that were included in each sample. Standard sizes are marked on the upper axis, and fluorescence intensities are indicated as arbitrary units on the *y* axis. The expanded lower fluorograms correspond to the four sequencing reactions (A, C, G and T) obtained with the Thermo Sequenase kit using the respective primers. Size standards were also included and the apparent molecular sizes were determined using GeneMapper software. Each black-filled peak corresponds to the sequencing product with the same apparent size as the respective extension fragment. In both primer extension reactions the same base (the C shown in bold type; note that this is the non-coding strand) was established as the transcription start site of the *pitH2* gene.

positions −61 and −37, respectively, from the translation start codon). However, we have failed to obtain clear extension products with the *pap-pitH1* promoter, despite exhaustive trials. Retrotranscription reactions were done with five different RNA samples from wild-type and mutant cultures using two different enzymes (Superscript II and Superscript III, both from Invitrogen), two different primers (LUX-FAM+47 and LUX-FAM+135), varying the amounts of RNA (5–200 μg) and primer (5–50 pmol), varying reaction time and temperature (5–15 h; 42–50 °C), and with or without DMSO (5 %) or actinomycin D. The lack of detected extension products also occurs in the *Sinorhizobium meliloti* wild-type strain carrying only one copy of the *orfA-pit* gene region (Bardin *et al.*, 1998), and suggests that the *pap-pitH1* transcript may be rapidly degraded, thus precluding retrotranscription.

Analysis of the PhoP-binding sequence in the *pitH2* promoter

To determine the PhoP-binding sequence, DNase I footprinting of the *pitH2* promoter region in the presence and absence of $PhoP^{DBD}$ was performed as described previously (Rodríguez-García *et al.*, 1997; Sola-Landa *et al.*, 2005). $PhoP^{DBD}$ was found to protect a 69 bp region in the *pitH2* promoter. The protected sequence was of 53 nt in the *pitH2* coding strand and 64 nt in the complementary strand; 48 nt were coincident in both strands (Figs 7 and 8). Full protection of the coding strand was achieved at 0.5 μM GST-$PhoP^{DBD}$, and the protected region was not enlarged by increasing protein concentration (Fig. 7a). The protection of the complementary strand showed the same requirement for GST-$PhoP^{DBD}$ protein (0.5 μM), and the protected sequence was not enlarged by increasing protein concentration (Fig. 7b). However, when a protein concentration of 0.26 μM was used, the protected region was shortened in the upstream and downstream regions (see the marked traces of the fluorograms in Fig. 7b), suggesting that the core region of the protected region has a higher affinity for PhoP than the adjacent nucleotide sequences (see below).

The information theory (Schneider, 1996, 1997) analysis of this *pitH2* region revealed six DRus of 11 nt in length using the published matrix model I (Sola-Landa *et al.*, 2008). The central sequence of the *pitH2* protected region contains four consecutive DRus (DRu-1, DRu-2, DRu-3 and DRu-4), and the flanking sequences contained two additional repeats, DRu-A and DRu-B. Both DRu-A and DRu-B are separated from the central repeats by 1 and 2 bp, respectively (Figs 7 and 8). The individual *Ri* values indicate that three of the DRus in the *pitH2* operator (DRu-1, DRu-3 and DRu-4) are well conserved, whereas DRu-2 is poorly conserved (Fig. 8). The DRu-A and DRu-B sequences, in contrast, show a negative *Ri* that reflects a poor sequence conservation (Fig. 8). The binding of PhoP to these poorly conserved DRus can be explained by a cooperative interaction between consecutive protein monomers at high protein concentration. We have proposed elsewhere (Sola-Landa *et al.*, 2008), for these complex operator structures, that at lower PhoP concentrations only DRu-3 and DRu-4 are bound, and these form the core of the site. Higher PhoP concentrations would allow the sequential binding to DRu-1-DRu-2, DRu-A and DRu-B (Fig. 8; see Discussion). This explains the appearance of up to four retarded complexes in the EMSA assays (Fig. 5).

DISCUSSION

Phosphate deprivation in bacteria triggers the PHO regulon, which includes a series of extracellular enzymes involved in phosphate scavenging from organic phosphates. In *S. coelicolor* these include at least a phytase gene, two glycerophosphodiester phosphodiesterase genes (Rodríguez-García *et al.*, 2007) and four phosphatase genes (Apel *et al.*, 2007; Sola-Landa *et al.*, 2008). As a parallel strategy to survive P_i starvation, *Streptomyces* cells may consume phosphorous storage material, such as polyphosphates, nucleotides and teichoic acids. The *ppk* gene, which encodes an enzyme that catalyses the reversible polymerization of the γ phosphate of ATP into polyphosphate, is under the positive control of the PhoR–PhoP system in *Streptomyces lividans* (Ghorbel *et al.*, 2006). Moreover, two PhoP-binding sites have been found in a cluster of genes putatively involved in the biosynthesis of cell wall polysaccharides, so that phosphate-free polymers could substitute for phosphate-rich polymers in the cell wall (Rodríguez-García *et al.*, 2007).

Inorganic phosphate transport (Pit) proteins are found in all groups of living organisms, including bacteria, archaea, yeast, fungi, plants and animals. *pit* genes encoding proteins from 195 to 763 aa in length are found in different organisms (Saier *et al.*, 1999). In some bacteria the Pit system has been proposed to have a role in heavy metal tolerance; thus, in the presence of heavy metals, metal phosphates are transported out of the cell by Pit (Keasling, 1997; Beard *et al.*, 2000; Álvarez & Jerez, 2004).

Three P_i transport systems have been identified in the genome of *S. coelicolor*. Of these three, PitH1 is the major transporter when P_i is in excess, whereas PitH2 and Pst work when P_i is limited. In *E. coli* there are two *pit* genes as well, although PitA and PitB share greater sequence similarity (90 %) than PitH1 and PitH2 in *S. coelicolor* (53 %), and have the same topological structures (10 TMS; Harris *et al.*, 2001). Moreover, the *S. coelicolor pit* genes are clustered with distinct accessory genes (Fig. 1), which is not the case for *E. coli*. On the other hand, in *Acinetobacter johnsonii, Sinorhizobium meliloti* and other bacteria, there is only one *pit* gene.

The *pit* genes can also be classified according to their regulation. We have described the regulation of the *pap-pitH1* and *pitH2* genes above. The *pitH2* gene is PhoP-dependent, while the *pap-pitH1* one is not. In *E. coli* the

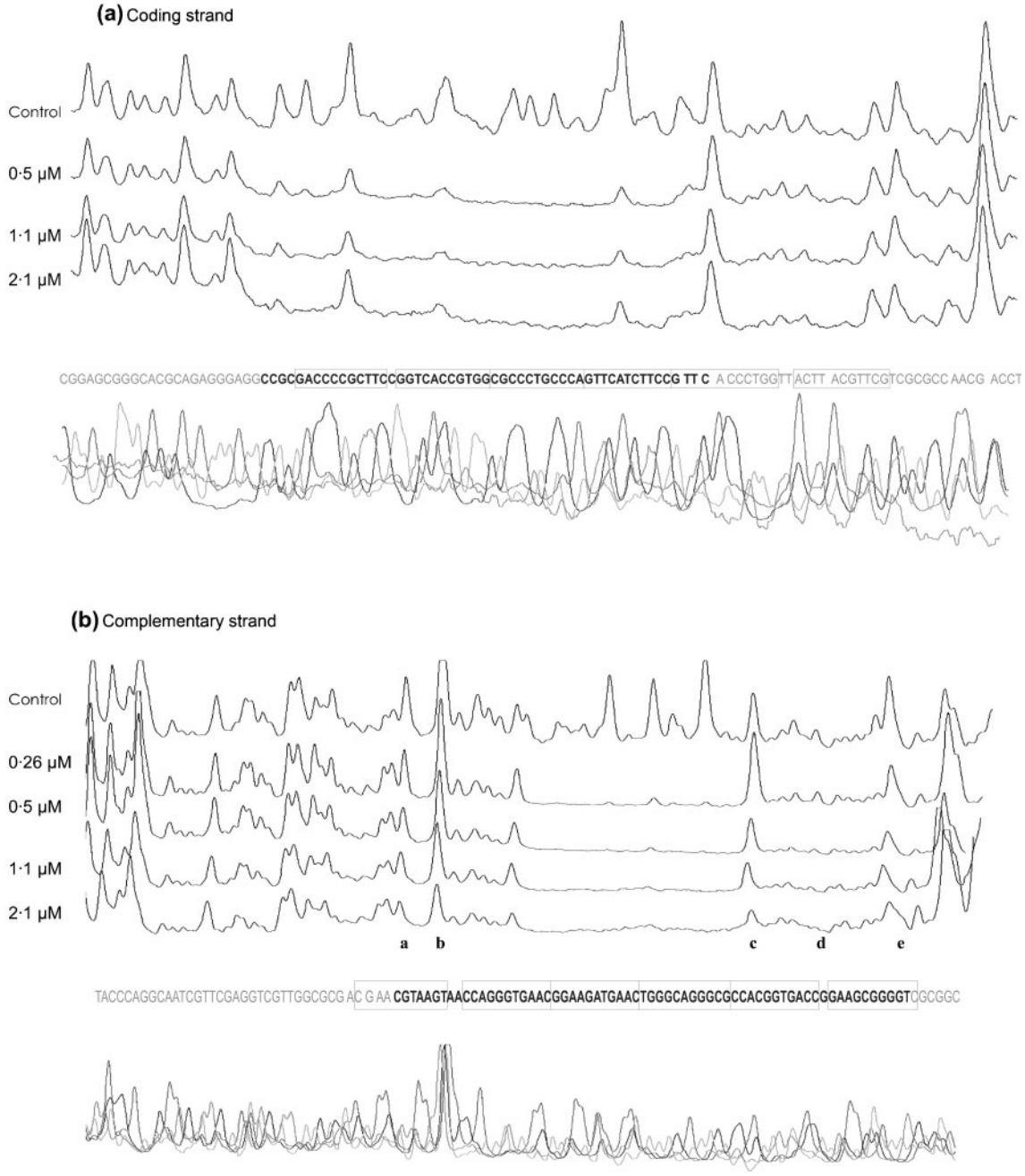


Fig. 7. Footprinting of the coding (a) and the complementary (b) strands of the *pitH2* promoter region using GST-PhoP^{DBD}. The upper fluorograms correspond to the control DNA (without protein) and to the protected reaction (with increasing concentrations of protein). The lower fluorograms correspond to the full nucleotide sequence used as the molecular mass marker. The protected nucleotide sequence is indicated in bold type, and the direct repeats that form the PHO operator are boxed. The letters a–e represent the main peaks that decrease as the protein concentration increases in the complementary strand. Note that the protection is hardly detected between peaks a and b, and not detected between peaks c and e when the lowest protein concentration (0.26 μM) is used.

pitA promoter is constitutive, but the *pitB* promoter appears to be repressed by PhoB (the PhoP orthologue) (Elvin *et al.*, 1986; Harris *et al.*, 2001). Both patterns of regulation were also found in different organisms with a single *pit* gene. In *A. johnsonii*, the *pit* gene is expressed constitutively (van Veen *et al.*, 1993; van Veen, 1997), whereas in *Sinorhizobium meliloti* it is repressed by PhoB (Bardin & Finan, 1998; Bardin *et al.*, 1998; Yuan *et al.*,

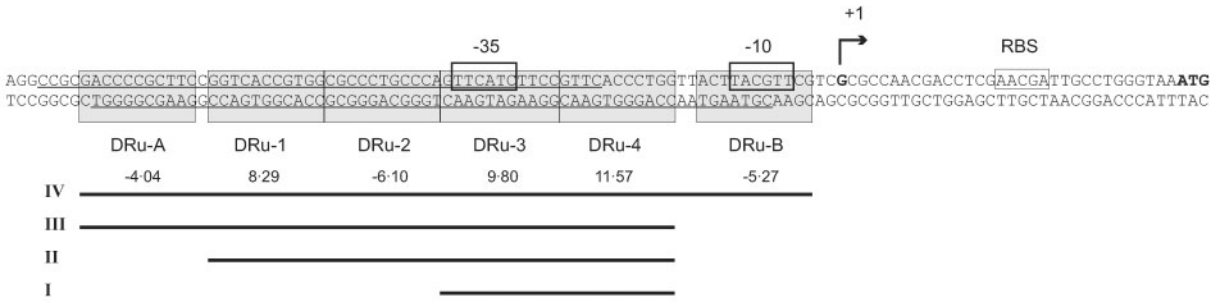


Fig. 8. Promoter region of *pitH2*. DRus are indicated by shaded boxes. The number below each DRu is the *R*i value. The region protected by $PhoP^{DBD}$ in DNase footprinting assays is underlined in the corresponding strand. The −10 and −35 hexanucleotides are boxed. The TSP is indicated by a bent arrow and bold type. Nucleotides showing homology with the 16S RNA, which could form a ribosome-binding site, are framed with a box labelled RBS. The start codon is shown in bold type. The bars with roman numerals represent the expected DRus that participate in each shifted band of the EMSA experiment.

2006a, b). In *Corynebacterium glutamicum* the *pit* gene has also been proposed to be repressed by PhoR, the response regulator of the two-component PhoS–PhoR (Schaaf & Bott, 2007). Similarly, the *Myxococcus xanthus pit* gene is repressed in phosphate-starved cells. This repression is not observed in a mutant in *phoP4*, which is one of the four response regulators involved in the PHO response in this organism (Whitworth *et al.*, 2008). In contrast, PhoP positively regulates *pitH2* of *S. coelicolor*. To our knowledge, this is the first *pit* gene to be described as PhoP-upregulated in bacteria, suggesting that the mechanism of action of the phosphate response regulator may be positive in some bacteria and negative in others.

As shown in this work, in the Δ*phoP* mutant only the *pap-pitH1* promoter is active, but both wild-type and mutant strains take up P_i from the medium with similar efficiency (Fig. 2). Gebhard *et al.* (2006) proposed that the loss of one or two P_i transport systems can be compensated for by increased activity of the remaining systems. It is reasonable to assume that the higher *pitH1* expression in the *S. coelicolor* Δ*phoP* mutant serves to compensate for the lack of the other two transport systems (Fig. 3). Additionally, another *pst* operon could exist in *S. coelicolor* (SCO6816–SCO6814), as suggested by Díaz *et al.* (2005). Two specific ABC P_i transport systems have been described in various bacteria, such as *Sinorhizobium meliloti* (Voegele *et al.*, 1997; Yuan *et al.*, 2006a) and some species of mycobacteria (Braibant *et al.*, 1996; Gebhard *et al.*, 2006).

The Δ*phoP* mutant did not grow after 44 h in phosphate-limited MG-3.2 medium, when the P_i concentration dropped below 0.1 mM (Fig. 2b). This is probably due to the fact that the Δ*phoP* mutant is defective in the expression of PhoP-dependent genes. The mutant strain has an altered expression of genes involved in central metabolism and protein synthesis, which are PhoP upregulated (Rodríguez-García *et al.*, 2007).

Although transcription from *pitH2* and *pstS* promoters is PhoP-dependent, the two genes showed different patterns of expression. Firstly, sequential activation and decay of the reporter activity at distinct levels of residual P_i are evident from the results. The first gene induced in MG-3.2 medium was *pitH2*, encoding the low-affinity transporter (Fig. 3a). It makes sense that when the P_i in the medium falls, the bacterium first responds with high induction of a low-affinity transporter (*pit*), since this system is energetically less expensive to the cell than the *pst* system. The expression of *pst* occurs when the P_i in the medium becomes scarce, which is consistent with the higher affinity of this transporter.

In phosphate-replete cultures the *pitH2* promoter was constantly active from 36 to 48 h, while *pstS* promoter activity was nearly absent (Fig. 3b). We propose two mechanisms to explain the differences between *pitH2* and *pstS* expression: (i) the different structure of the PhoP-binding sites, and (ii) the interaction of PhoP with specific sigma factors. These mechanisms, which can act together, are based on the operator structures and might involve the participation of alternative sigma factors.

The differences in structure of the PhoP-binding sites in the promoters of *pitH2* and *pstS* provide mechanisms to explain their expression patterns. The PHO box of *pstS* is composed of two well-conserved DRus located upstream of the promoter elements (Sola-Landa *et al.*, 2005). In contrast, EMSA, footprinting and information content analysis of the *pitH2* operator revealed a complex structure of six DRus. It is proposed, following the model of Sola-Landa *et al.* (2008), that phosphorylated PhoP (PhoP~P) binds first to the most conserved DRu-3 and DRu-4 sequences, which form the core of the binding site. Increasing concentrations of PhoP~P would result in the consecutive occupancy of DRu-1 and DRu-2. Since the last DRu shows a negative *R*i (Fig. 8), the binding here of a protein monomer can be explained by protein–protein interactions, as proposed previously for the *phoRP* operator (Sola-Landa *et al.*, 2005). Higher concentrations of PhoP~P would allow the binding of DRu-A and DRu-B,

although the relevance to the control of gene expression of the binding of PhoP to this sequence may be minor. The functionality of a DRu separated by 2 bp has already been demonstrated for the *phoD* promoter (Apel *et al.*, 2007) and the SCO1196 operator (Sola-Landa *et al.*, 2008). This stoichiometry matches the number of EMSA complexes observed (Figs 5 and 8).

The position of these DRus in relation to the promoter elements accounts for the dual role, positive and negative, of PhoP, as occurs in the *phyC* gene of *Bacillus amyloliquefaciens* (Makarewicz *et al.*, 2006). The main features of the *phyC* promoter are: (i) one PHO box overlapping the −35 element; (ii) an improper spacing with the −10 element (21 bp); and (iii) a direct repeat overlapping the −10 element. Makarewicz *et al.* (2006) have proved that while binding of PhoP at −35 is essential for activation of the promoter, binding of PhoP at −10 suppresses promoter activity. Similarly, the PhoP operator core in the *pitH2* promoter overlaps the −35 region (Fig. 8); the separation from the −10 element is 20 bp, which is larger than those of standard *Streptomyces* promoters (16–18 bp; Strohl, 1992), and DRu-B overlaps the −10 region of the *pitH2* promoter (Fig. 8). This is a characteristic feature of some promoters regulated by dual activator/repressor proteins (Hidalgo & Demple, 1997; Makarewicz *et al.*, 2006). With this model in mind, PhoP~P oligomerization on the DNA up to DRus A, 1, 2, 3 and 4 would result in the activation of the *pitH2* promoter. However, the binding of PhoP~P to DRu-B would cause repression. The repression at the highest PhoP~P concentrations is in agreement with the rapid activity drop of the *pitH2* promoter from 40 to 48 h, coincident with the rise in *pstSp* activity (Fig. 3a).

The homologous *pitH2* gene in *S. avermitilis* (SAV6419) also has a PhoP-binding site in its promoter region with a similar structure, which supports our proposed model. In this species, there are two highly conserved DRus with *R*i values of 9.8 and 11.6 bits, respectively. As in *S. coelicolor*, downstream of these DRus, separated by 2 bp, there is another DRu with an *R*i of 0.1 bits. This DRu should be the functional homologue of *S. coelicolor* DRu-B in the *pitH2* promoter.

The earlier activation of the *pitH2* gene with respect to the *pstS* gene can be also explained if its promoter is recognized by a sigma factor, active in the first phase of growth, that interacts with PhoP, whereas *pstS* is triggered by PhoP in combination with a phosphate-limitation-responsive sigma factor. This mechanism is supported by the finding that the *Bacillus subtilis* PhoP is able to interact with promoters of at least three different sigma factors σ^A, σ^E and σ^M (Paul *et al.*, 2004; Minnig *et al.*, 2005). The sequencing of the *S. coelicolor* genome has identified a plethora of genes that encode sigma-factors, including four homologues (*hrdA*, *hrdB*, *hrdC* and *hrdD*) of the principal sigma factors, nine homologues of *B. subtilis* stress-response σ^B, and 51 extracytoplasmic function sigma factors (Bentley *et al.*, 2002; Hahn *et al.*, 2003). Therefore, it will not be surprising if one or more sigma factors are dedicated to the phosphate-limitation stress response. Moreover, the sigma factor genes *hrdB* and *hrdD* of *Streptomyces griseus* are expressed differentially under P_i-rich or P_i-starved conditions, respectively (Marcos *et al.*, 1995).

Finally, the *pitH2* promoter activity in phosphate-replete cultures (Fig. 3b) indicates that unphosphorylated PhoP can bind and activate this promoter. *In vitro* DNA binding of the unphosphorylated PhoP (the PhoP form existing under high-phosphate conditions) proteins of *S. coelicolor* (Sola-Landa *et al.*, 2005) and *B. subtilis* has been reported, although in *B. subtilis*, the phosphorylated protein is required for full activation (Liu *et al.*, 1998; Eder *et al.*, 1999). Also, in *S. coelicolor*, the full *pitH2* promoter activity is achieved under conditions of phosphate limitation, i.e. when PhoP is phosphorylated.

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Cross-talk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription

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Summary

The regulatory proteins AfsR and PhoP control expression of the biosynthesis of actinorhodin and undecylprodigiosin in *Streptomyces coelicolor*. Electrophoretic mobility shift assays showed that PhoP^{DBD} does not bind directly to the *act*II-ORF4, *redD* and *atrA* promoters, but it binds to the *afsS* promoter, in a region overlapping with the AfsR operator. DNase I footprinting studies revealed a PhoP protected region of 26 nt (PHO box; two direct repeats of 11 nt) that overlaps with the AfsR binding sequence. Binding experiments indicated a competition between AfsR and PhoP; increasing concentrations of PhoP^{DBD} resulted in the disappearance of the AfsR–DNA complex. Expression studies using the reporter *luxAB* gene coupled to *afsS* promoter showed that PhoP downregulates *afsS* expression probably by a competition with the AfsR activator. Interestingly, AfsR binds to other PhoP-regulated promoters including those of *pstS* (a component of the phosphate transport system) and *phoRP* (encoding the two component system itself). Analysis of the AfsR-protected sequences in each of these promoters allowed us to distinguish the AfsR binding sequence from the overlapping PHO box. The reciprocal regulation of the *phoRP* promoter by AfsR and of *afsS* by PhoP suggests a fine interplay of these regulators on the control of secondary metabolism.

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Introduction

Streptomyces and many other soil-dwelling actinomycetes produce an impressive array of secondary metabolites, many of them having important biological activities (Crandall and Hamill, 1986; Von Döhren and Gräfe, 1997; Martín *et al*., 2000). Nevertheless, very little is known about the regulation of secondary metabolism in *Streptomyces*. Therefore, a better understanding of how regulatory genes act in the secondary metabolism is of great importance. The biosynthesis of antibiotics and other secondary metabolites is controlled by interactions of both global and pathway-specific regulators that trigger, or repress, expression of the genes encoding secondary metabolite biosynthetic enzymes. These regulators respond to a variety of environmental stimuli like stress-inducing agents or nutritional imbalance; i.e. phosphate starvation.

We reported previously that phosphate control of actinorhodin and undecylprodigiosin in *Streptomyces lividans* is mediated by the two-component PhoR–PhoP system (Sola-Landa *et al*., 2003). Purification of the *Streptomyces coelicolor* PhoP response regulator – 100% identical to that of *S. lividans* – allowed the initial characterization of its operators (Sola-Landa *et al*., 2005). These sequences, commonly known as PHO boxes, are formed of 11 nt direct repeat units (DRu). DNA binding of phosphorylated PhoP occurs following phosphate depletion in the culture media and this binding controls the expression of phosphate-regulated genes (Sola-Landa *et al*., 2005; Rodríguez-García *et al*., 2007).

PhoP acts as an activator of genes encoding phosphate scavengers, such as the PstSCAB phosphate transporter system, the alkaline phosphatase PhoA, and two glycerophosphodiesterases (Sola-Landa *et al*., 2005; Apel *et al*., 2007; Rodríguez-García *et al*., 2007). Other genes are repressed by PhoP, such as the *hrdA* sigma factor, the putative oxidoreductase gene SCO2262, and the putative regulator SCO4261 (Sola-Landa *et al*., 2008). Furthermore, the phospholipase *phoD* and the phosphate transporter *pitH2* genes possess complex operators that appear to be subjected to dual phosphate induction and repression mechanisms, depending upon the position of

the DRu with respect to the –35 and –10 regions of their promoters (Apel *et al*., 2007; Santos-Beneit *et al*., 2008; Sola-Landa *et al*., 2008). How does PhoP regulate antibiotic production? It is possible that PhoP interacts with global regulators (AfsR, AtrA), or that it controls cluster-specific regulatory genes (*redD*, *act*II-ORF4), or regulates structural genes directly (Martín, 2004).

The biosynthesis of actinorhodin and undecylprodigiosin is controlled by the global regulator AfsR, a member of the large-size SARP family (Horinouchi *et al*., 1983). Floriano and Bibb (1996) found that the stimulatory effect of AfsR on antibiotic production is exerted through the pathway-specific regulatory genes *act*II-ORF4 and *redD*. Nevertheless, the only known target of the AfsR regulator is a small gene located downstream of *afsR,* named *afsS* in *S. coelicolor* and *afsR2* in *S. lividans*. Amplification of *afsR2/afsS* on a high-copy-number plasmid conferred overproduction of actinorhodin and undecylprodigiosin in both *S. coelicolor* and *S. lividans* (Vögtli *et al*., 1994; Matsumoto *et al*., 1995; Floriano and Bibb, 1996). The binding sequence of AfsR to the *afsS* promoter region was identified by Lee *et al*. (2002); our analysis of this sequence identified a putative PHO box overlapping with the AfsR binding sequence suggesting that both PhoP and AfsR might interact in the regulation of *afsS*. This work demonstrates that both proteins compete for overlapping sequences in the *afsS* promoter, providing the first description of a secondary metabolism regulatory gene controlled by PhoP. In addition, AfsR turned out to regulate the *PhoR–PhoP* system and to repress the *pstS* gene, a major PHO regulon member involved in phosphate transport.

Results

Growth and antibiotic production in wild-type, ΔphoP*, and* ΔafsR S. coelicolor *strains*

Streptomyces coelicolor A3(2) produces several unrelated antibiotics, two of which are easily identified thanks to their colour. In liquid cultures, the red colour is caused by the cell-bound accumulation of undecylprodigiosin and other prodigionines (Tsao *et al*., 1985). At later times, cultures become blue due to the synthesis of actinorhodin, which is linked to the cells, and to its lactone form (γ-actinorhodin) that is exported to the medium (Bystrykh *et al*., 1996). We will refer to the mixtures of red and blue antibiotics as Red and Act respectively.

It is well known that inorganic phosphate (Pi) exerts a negative control on the biosynthesis of a great variety of antibiotics and other secondary metabolites (Martín, 2004). The published works on *S. coelicolor* physiology have limited their focus to Act (Doull and Vining, 1990; Ozergin-Ulgen and Mavituna, 1993; Bystrykh *et al*., 1996; Melzoch *et al*., 1997), but there is very limited information on phosphate control of Red biosynthesis (Hobbs *et al*., 1990). These authors reported the inhibitory effect of Pi on both Act and Red final yields, measured after 72 h of culture. Besides, our laboratory showed the positive effect of the *phoP* deletion on the Act and Red productions in *S. lividans* (Sola-Landa *et al*., 2003). Following these studies, we decided to test the effect of *phoP* and phosphate on antibiotic production in *S. coelicolor* cultures. For this we constructed a new Δ*phoP* mutant, INB201, derived from *S. coelicolor* M145(a plasmid-cured derivative of the wild-type A3(2) strain), as described in *Experimental procedures*. We included also in this study the Δ*afsR* mutant strain M513 (Floriano and Bibb, 1996). These strains were cultured in liquid MG, a defined medium used in previous works (Doull and Vining, 1989; Rodríguez-García *et al*., 2007; Santos-Beneit *et al*., 2008), supplemented with 3.2 mM Pi (MG-3.2) or with 18.5 mM Pi (MG-18.5). In MG-3.2, Pi is depleted (< 0.1 mM) from the medium at 45 h, while it is always in excess in MG-18.5 (Santos-Beneit *et al*., 2008).

Growth of the Δ*phoP* mutant was impaired in the Pi-limited condition (Fig. 1A), as described previously (Santos-Beneit *et al*., 2008). In contrast, the Δ*phoP* mutant and the parental strain growth curves overlapped in the Pi-replete medium. The M513 Δ*afsR* mutant growth was slightly higher throughout the entire time-course than that of the parental M145 strain, especially under Pi limitation (Fig. 1A). Floriano and Bibb (1996) reported that overexpression of *afsR* on strains grown on solid media caused smaller colonies, but these authors attributed this effect to the higher antibiotic production rather than to a deleterious effect of AfsR.

The Red and Act synthesis in the wild-type strain initiated during the transition phase (45–50 h; Fig. 1A–C). Act production in phosphate-replete MG-18.5 was reduced to 50% of that in MG-3.2, either comparing final Act titres or production rates (calculated from the slope of the three last time points; Fig. 1B). Red production was severely inhibited by the high Pi concentration (down to 4%, mean value of 50–75 h; Fig. 1C).

The deletion of *phoP* resulted in a lower and delayed production of both antibiotics under Pi limitation. The Act levels at 50 h, 55 h and 75 h were reduced to 12%, 10% and 51% of the levels in the parental strain; and Red levels decreased to 18%, 24% and 54% respectively.

Deletion of *afsR* affected more severely Act production (11% of that of the parental strain, mean value of 50–75 h) than Red production (51%, mean of 50–75 h) and did not delay the onset of Red in the Pi-limited condition. The Δ*afsR* strain cultures in Pi-replete MG-18.5 had strongly reduced antibiotic production; only residual Act levels

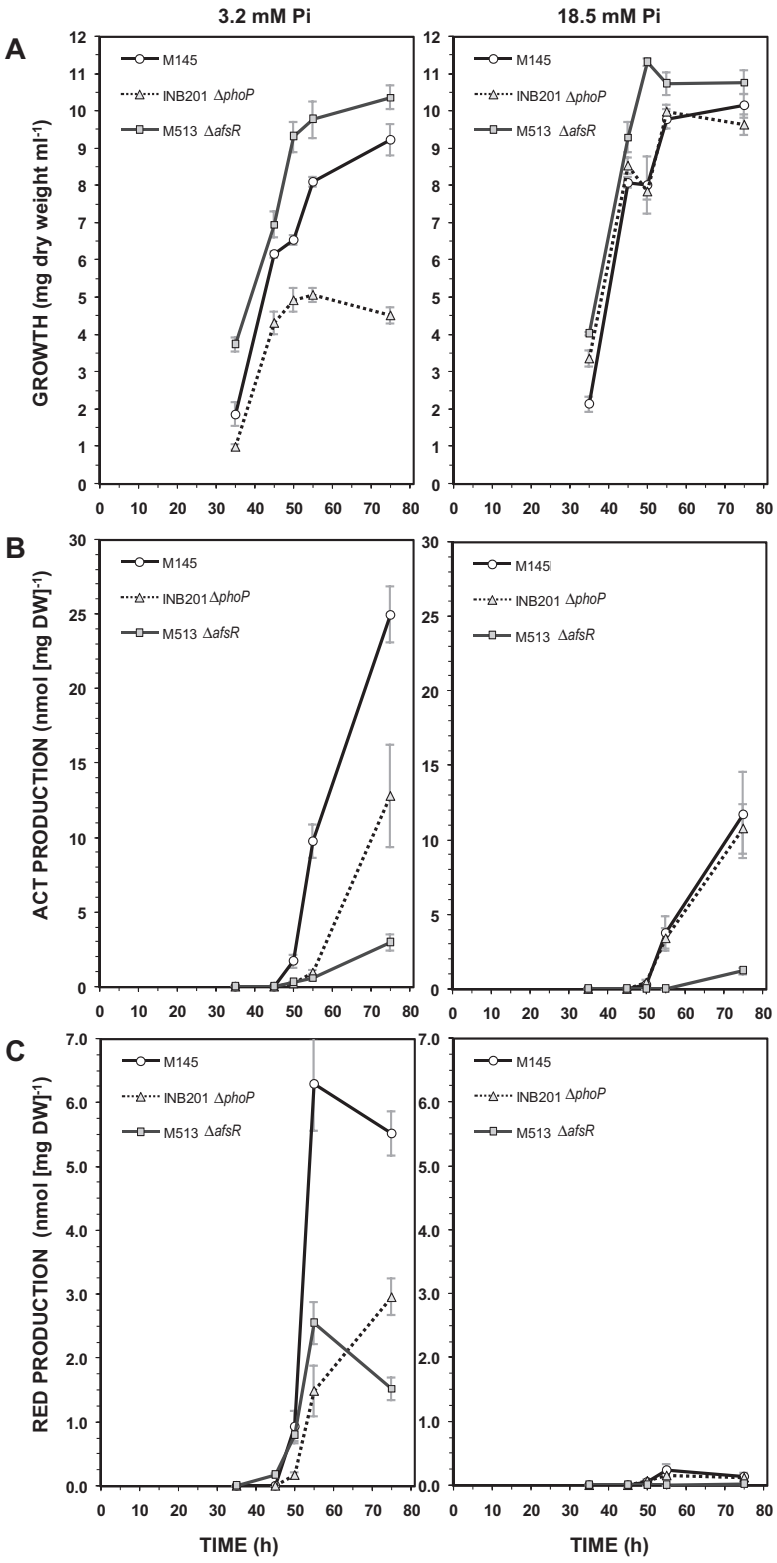

Fig. 1. Growth (A), Act production (B) and Red production (C) of *S. coelicolor* strains M145 (parental strain; ○), INB201 (Δ*phoP* mutant; ▲) and M513 (Δ*afsR* mutant; ■) grown on phosphate-limited MG-3.2 (left panels) and phosphate-replete MG-18.5 (right panels). Vertical error bars correspond to the standard error of the mean of four replicated cultures.

were detected at 75 h (Fig. 1B and C), in agreement with results of Floriano and Bibb (1996).

From all available evidence, we may conclude that (i) excess of Pi inhibits Act and Red productions, being Red especially sensitive to phosphate concentrations above 10 mM; (ii) PhoP has a positive role in both antibiotics production in *S. coelicolor*, because its deletion in an otherwise isogenic strain caused a delay and reduction of Act

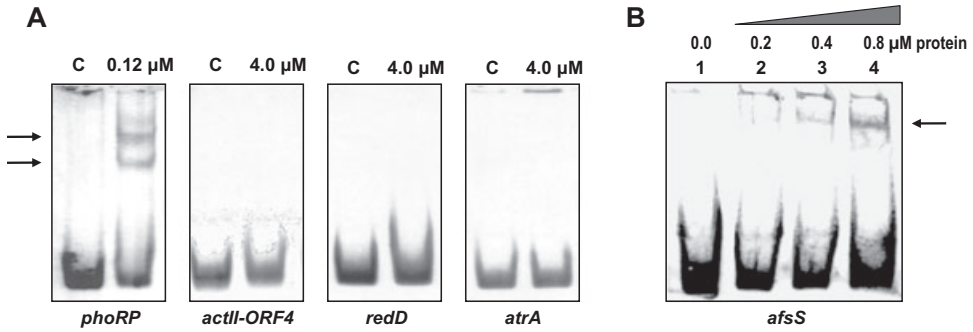

Fig. 2. A. EMSA of the *phoRP*, *act*II-ORF4, *redD* and *atrA* promoters with the GST-PhoP^{DBD} protein. The protein concentration used is indicated on top of each panel; no protein was added in the control (lane C) reaction.
B. EMSA of the *afsS* promoter with the GST-PhoP protein. Lane 1, DNA probe without protein; lane 2, 0.2 μM; lane 3, 0.4 μM, lane 4, 0.8 μM protein. Note the retarded DNA–protein complex (arrow).

and Red production; (iii) the positive effect of AfsR on antibiotic production is observed in limiting and sufficient Pi concentrations.

PhoP does not bind to the actII-*ORF4,* redD *and* atrA *promoters, but binds to the* afsS *promoter*

As an initial approach to elucidate the phosphate control of Act and Red biosynthesis, we investigated whether PhoP regulates directly the pathway-specific (cluster-located) regulators, *act*II-ORF4 and *redD*, as well as *atrA*, a transcriptional activator of *act*II-ORF4 (Narva and Feitelson, 1990; Arias *et al*., 1999; Uguru *et al*., 2005). In order to test if PhoP binds to these promoters, electrophoretic mobility shift assay (EMSA) analyses were done with the GST-PhoP^{DBD} protein, as described in *Experimental procedures*. The *phoRP* promoter known to bind PhoP (Sola-Landa *et al*., 2005) was used as positive control. Results (Fig. 2A) showed that the promoters of the three regulatory genes *act*II-ORF4, *redD* and *atrA* did not bind PhoP^{DBD}, even using 4 μM of protein, whereas the *phoRP* promoter shows retardation with 0.12 μM protein. Those results indicate that phosphate regulation of Act and Red is not exerted directly by binding of PhoP to the *act*II-ORF4 and *redD* promoters or by binding to the promoter of the *atrA* regulator.

The first characterization of PhoP operators (Sola-Landa *et al*., 2005) allowed us to detect a putative PHO box in the upstream region of the *afsS* gene by means of the 'information theory' programmes (Schneider, 1997). EMSA analyses were done to validate if this sequence is bound by PhoP. A unique retarded band was detected, even at PhoP protein concentrations as low as 0.4 μM (Fig. 2B).

Analysis of the PhoP operator in the afsS *promoter*

To determine the PhoP binding sequence in the *afsS* promoter, DNase I footprinting assays of the *afsS* promoter were carried out in the presence (0.5 μM to 1.1 μM) and absence of PhoP^{DBD} and analysed in an ALF sequencer, as described in *Experimental procedures*. These assays showed that there is a stretch of 26 nt protected both in the sense and the antisense strands of the *afsS* promoter region (Fig. 3A and B). This protected region coincides with the region reported by Lee *et al*. (2002) as the binding region of the AfsR regulator (see *Discussion*).

The PhoP binding sites are formed by two or more DRu of 11 nt.

Recently, the alignment of 37 DRu from 16 operators allowed us to build a weight matrix defined by the 'information theory' (Sola-Landa *et al*., 2008). This matrix (model I) serves to quantify the sequence conservation of new sites, given as their individual information content or R_i value (Schneider, 1997). The R_i values of model I DRu have a mean of 8.1 bits.

The PhoP-protected region in the *afsS* promoter contains two conserved DRu of R_i values 7.0 and 5.8 bits respectively (Fig. 3C). This PhoP operator belongs to class I (Sola-Landa *et al*., 2008). The two DRu are located at positions –39 to –18 with respect to the reported transcription start point of *afsS* (Lee *et al*., 2002). Thus, the –35 hexamer of the *afsS* promoter is included inside DRu-1. This same operator organization (overlapping with the -35 promoter region) is also found in the operator core of the *pitH2* gene (Santos-Beneit *et al*., 2008).

AfsR and PhoP compete for the afsS *promoter region*

To determine whether PhoP binding to the *afsS* promoter interferes or not with the binding of AfsR, both proteins were used together and separately in EMSA experiments. The H-AfsRΔC and the GST-PhoP^{DBD} proteins (both containing the DNA binding domain of the respective proteins; see *Experimental procedures*) were used. Both truncated proteins exhibited the same DNA-binding specificity as full-length proteins (Lee *et al*., 2002; Sola-Landa *et al*., 2005).

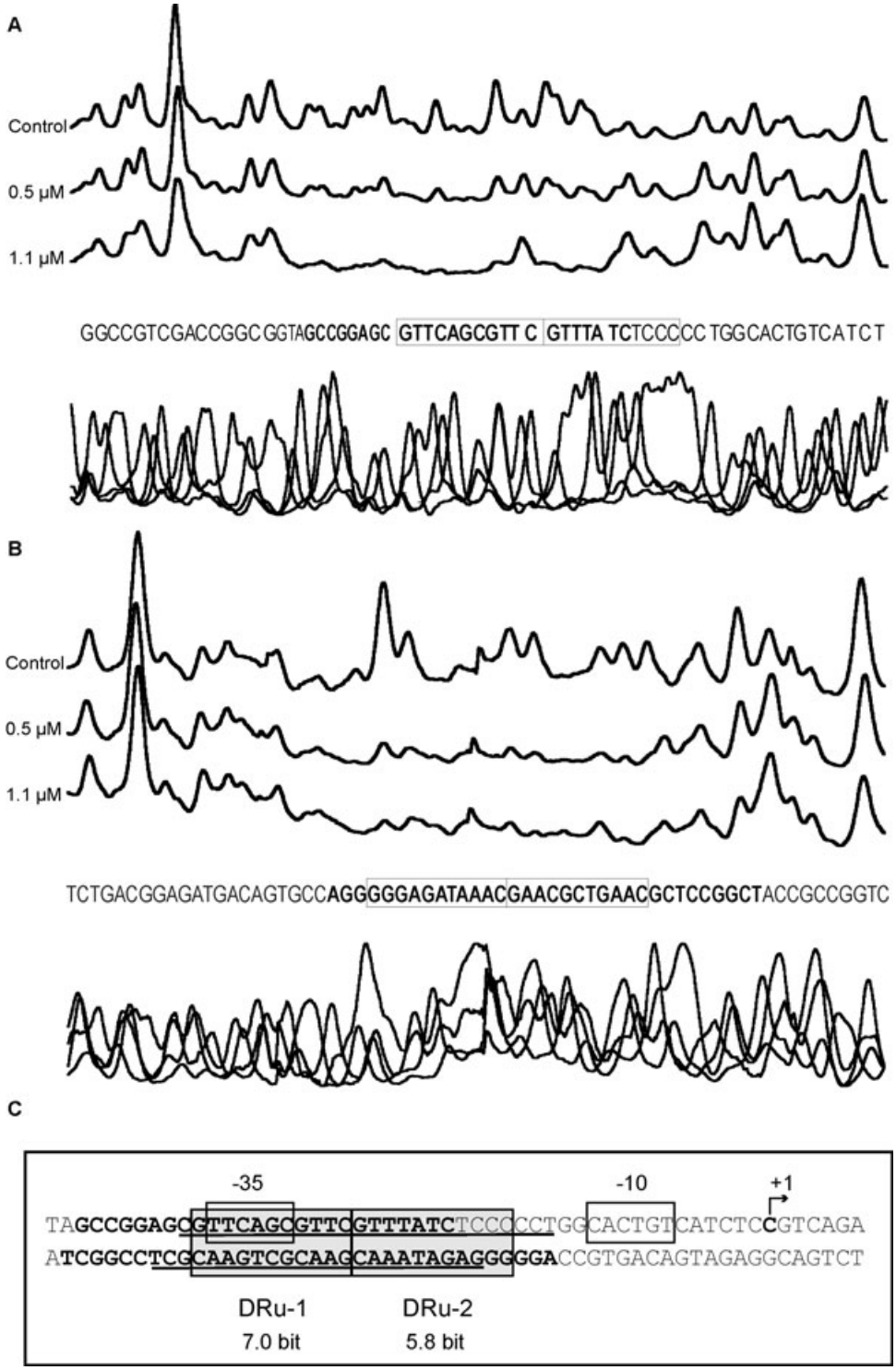

Fig. 3. DNase I footprinting of the coding (A) and the complementary (B) strands of the *afsS* promoter using GST-PhoP^{DBD}. The upper electropherograms correspond to control reaction (no protein), 0.5 μM protein and 1.1 μM protein respectively. The superimposed electropherograms correspond to sequencing reactions. The protected nucleotide sequence is indicated in boldface, and the direct repeat units (DRu) that form the PHO operator are boxed.
C. Nucleotide sequence of the *afsS* promoter. The DRu are indicated with shaded boxes; the information content of each DRu is indicated below. Sequence protected from DNase I digestion by GST-PhoP^{DBD} is in boldface; the sequence protected by AfsR (Lee *et al*., 2002) is underlined. The promoter –10 and –35 elements are boxed and the transcription start point is indicated by a bent arrow and a boldface letter (Lee *et al*., 2002).

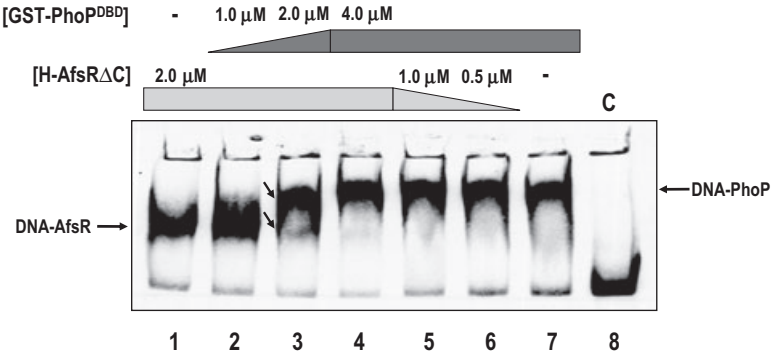

Fig. 4. Competitive EMSA of the *afsS* promoter with the H-AfsRΔC and GST-PhoP^{DBD} proteins. The end-labelled DNA fragment was incubated with the indicated concentrations of GST-PhoP^{DBD} (increasing from the left) or H-AfsRΔC (increasing from the right) proteins. Lane 8 is the control reaction with no protein added. The arrows point to the DNA–protein complexes. Note the two complexes in lane 3 (arrows). The binding buffer is that of Sola-Landa *et al.* (2005).

When applied separately, both proteins bind to the *afsS* promoter region (Fig. 4, lanes 1 and 7). In the presence of a constant H-AfsRΔC concentration, increasing the concentration of PhoP^{DBD} resulted in the disappearance of the AfsR–DNA complex and the formation of a PhoP–DNA complex (Fig. 4, lanes 1–4). Thus, PhoP can displace AfsR from the *afsS* promoter. When equimolecular protein concentrations were used the PhoP–DNA complex predominates over the AfsR–DNA complex (Fig. 4, lane 3; note the two inclined arrows). It must be noted that the binding buffer used in the experiment is that of Sola-Landa *et al.* (2005), suited for PhoP.

Transcription from the afsS *promoter is repressed by PhoP*

To investigate the role that PhoP plays on *afsS* expression, the promoter of *afsS* was coupled to the reporter luciferase gene (see *Experimental procedures*). The plasmid, pLUX-*afsS*, was introduced by conjugation in *S. coelicolor* M145 and INB201 strains and the expression of *luxAB* was followed in cultures in Pi-replete (MG-18.5) or Pi-limited (MG-3.2) media.

Luminescence data from MG-3.2 cultures revealed that the *afsS* promoter was between two and five times more active in the Δ*phoP* mutant than in the parental strain throughout the culture, except at the peak value (50 h), when both activities were similar (Fig. 5A). The expression pattern of the parental strain in MG-18.5 was similar to those of the Δ*phoP* mutant in both Pi-replete and Pi-limited cultures, except for the lower values of the mutant at 50 and 55 h in 18.5 mM (Fig. 5A). These results indicate that the phosphorylated form of PhoP controls negatively the *afsS* expression and that the lack of this regulator relieves the *afsS* promoter. This negative control may be due solely to the competition with the AfsR activator site.

As stated previously, the PHO box of *afsS* is located respect to the promoter elements in the same position as that of the *pitH2* gene, which is activated by PhoP (Santos-Beneit *et al.*, 2008). This raises the possibility that PhoP might itself act as an activator of *afsS*. To test this hypothesis, we examined the *afsS* promoter activity in a Δ*afsR* background. The pLUX-*afsS* plasmid was introduced in the Δ*afsR* mutant strain by conjugation and cultures were carried out in MG-3.2 and in MG-18.5. As expected, expression from the *afsS* promoter was impaired in the Δ*afsR* mutant, but not completely. Specific luciferase activities in Pi limitation were null at 35 and 75 h (Fig. 5B), and the maximum of 50 h was reduced to 18% of that of the wild-type peak (Fig. 5A and B). In the Pi-replete cultures, a null activity at 35 h was followed by a higher activity than in Pi-limited medium (twofold to threefold lower activities than in the wild-type values). It is interesting that the *afsS* promoter activities in the Δ*afsR* mutant were two to three times higher in Pi-replete than in the Pi-limited condition (Fig. 5B). In high Pi concentration PhoP is dephosphorylated, hence releasing its operators. Therefore, these results indicate that phosphorylated PhoP is a transcriptional repressor of *afsS*, in addition to the competitive effect with the AfsR activator.

Reciprocal regulation: AfsR also binds to the phosphate-controlled pstS *and* PhoR–PhoP *promoters*

As both global regulators PhoP and AfsR protect from DNase digestion the same sequence in the *afsS* promoter, we investigated if AfsR can bind to PHO sequences in other PhoP-regulated promoters as well. For this purpose we chose the *pstS* and *phoRP* promoter regions because they contain representative operators of class I and class III respectively (Sola-Landa *et al.*, 2008). Three versions of AfsR fusion proteins were used by Tanaka *et al.* (2007). They reported that H-AfsRΔTPR is much more stable than H-AfsRΔC or the full H-AfsR. For this reason the H-AfsRΔTPR fusion protein was used in the EMSA studies of *phoRP* and *pstS* promoters. Results of the EMSA analysis showed that *pstS* and *phoRP* promoters bind H-AfsRΔTPR even when a low (0.56 μM) concentration of protein was used. In control competition experiments, when increasing concentrations of unlabelled DNA of *pstS* or *phoRP* promoters were added, the

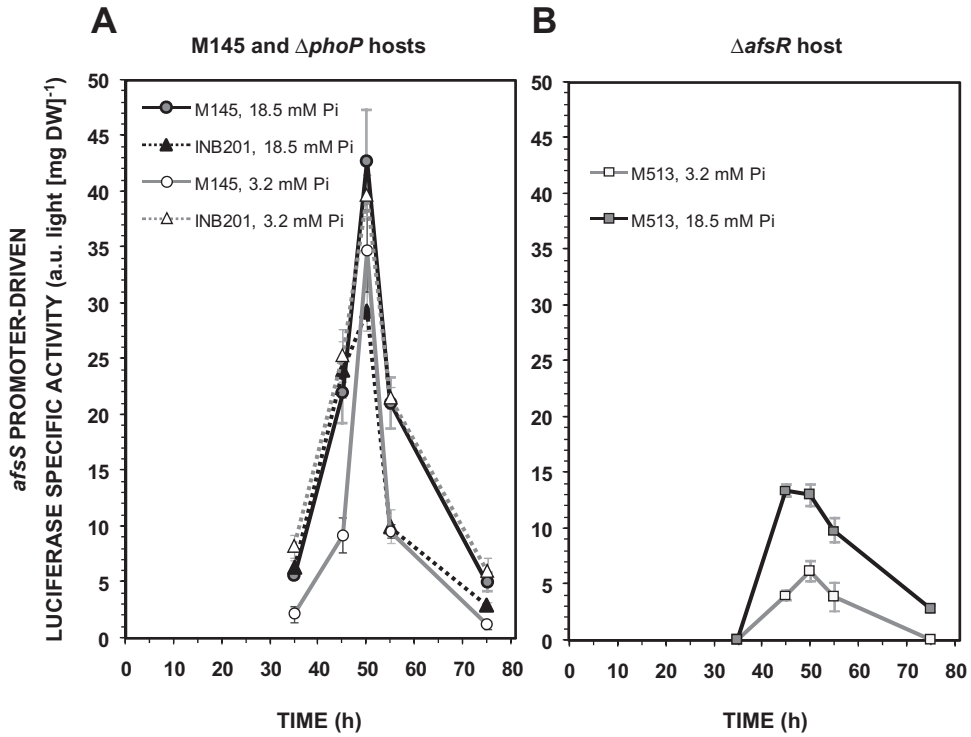

Fig. 5. Promoter activity of *S. coelicolor* exconjugants containing pLUX-*afsS* in parental strain (M145, ○) and Δ*phoP* (INB201, △) backgrounds (A), and in Δ*afsR* background (M513, □, B). The strains were grown in phosphate-limited MG-3.2 medium (grey lines and not-filled symbols), and in phosphate-replete MG-18.5 medium (black lines and filled symbols). Error bars correspond to the standard error of the mean of four culture replicates.

respective AfsR–DNA complexes were reduced in intensity (Fig. 6)., indicating that H-AfsRΔTPR binds specifically to these promoters.

Analysis of the AfsR operator sequences in the pstS *and* phoRP *promoter regions*

To determine the sequences bound by AfsR in these regions, we performed DNase I footprinting experiments using the H-AfsRΔTPR protein and a capillary sequencer to analyse the digestion fragments. As seen in Fig. 7A, the coding strand of the *pstS* promoter showed a main protected stretch of 20 nt (positions –127 to –108, respect to the translation start). To quantify the degree of protection, ratios of the areas under each fluorescence peak were calculated respect to the control reaction (i.e. a lower ratio implies a higher protection). Ratios of 81% ± 8%, 34% ± 14% and 30% ± 17% (mean ± SD) were observed at protein concentrations of 0.2, 0.8 and 1.7 μM respectively. DNase I hypersensitive positions flanked the protected sequence. H-AfsRΔTPR also bound to an upstream sequence (positions –151 to –176; Fig. 7A),

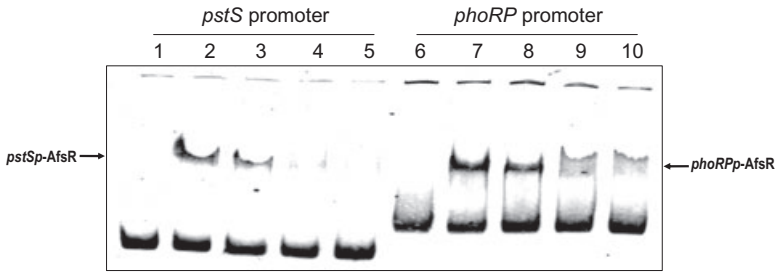

Fig. 6. EMSA of the *pstS* and *phoRP* promoters with the H-AfsRΔTPR protein and competition with unlabelled probe. Lanes 1–5, labelled *pstSp* probe; lane 1, control without protein; lane 2, 0.84 μM protein; lane 3, 0.56 μM protein; lane 4, 0.56 μM protein and 250× excess of unlabelled probe; lane 5, 0.56 μM protein and 500× excess of unlabelled probe. Lanes 6–10, labelled *phoRPp* probe; lane 6, control without protein, lane 7, 0.84 μM protein; lane 8, 0.56 μM protein; lane 9, 0.56 μM protein and 250× excess of unlabelled probe; lane 10, 0.56 μM protein and 500× excess of unlabelled probe. The arrows indicate the DNA–protein complexes.

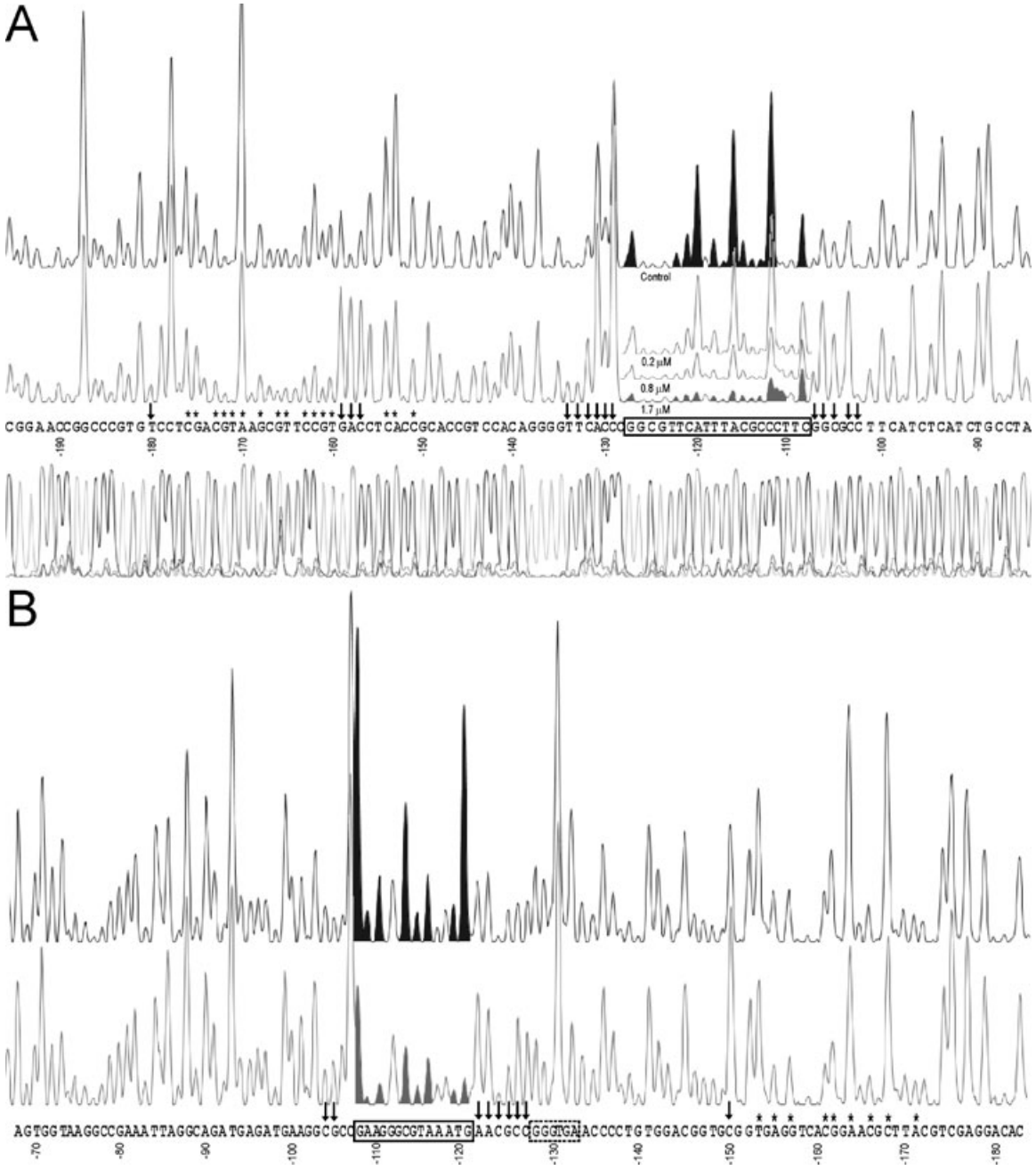

Fig. 7. DNase I footprinting of H-AfsRΔTPR binding to the *pstSp* coding (A) and complementary (B) strands, and to the *phoRPp* coding (C) and complementary strands (D). In all cases, the upper electropherogram is the control reaction without protein. In A, numbers above traces in the protected region indicate the concentrations of H-AfsRΔTPR; otherwise, 1.7 μM protein (B) and 4 μM protein (C and D) were used. The correspondence between fluorescence peaks and nucleotide bases was determined using sequencing reactions (only shown in A). Peak shadowed areas were used to calculate the degree of protection (see text). The protected sequence is boxed, or indicated by asterisks at the secondary site of *pstSp* (A and B). The vertical arrows indicate DNase I hypersensitive sites. The position respect to the translation start codon is indicated under the nucleotide sequence.
E. Summary of AfsR protection results on the *afsS* promoter (Lee *et al.*, 2002) and on the *pstS* and *phoRP* promoters. The protected sequence by AfsR in each strand is overlined or underlined. In the *pstSp*, the sequence protected to a lower extent is indicated with dotted lines. The 11 nt direct repeat units (DRu) that form the PhoP operator in *afsSp* and in *pstSp* are indicated (the sequence that contain the PHO boxes of the *phoU-phoRP* regulatory region is not included). The promoter elements (boxes) and the transcription start points (bent arrows) identified by Lee *et al.* (2002) and Sola-Landa *et al.* (2005), and the deleted nucleotide in the *pstS** mutant sequence (Δ) are also indicated.

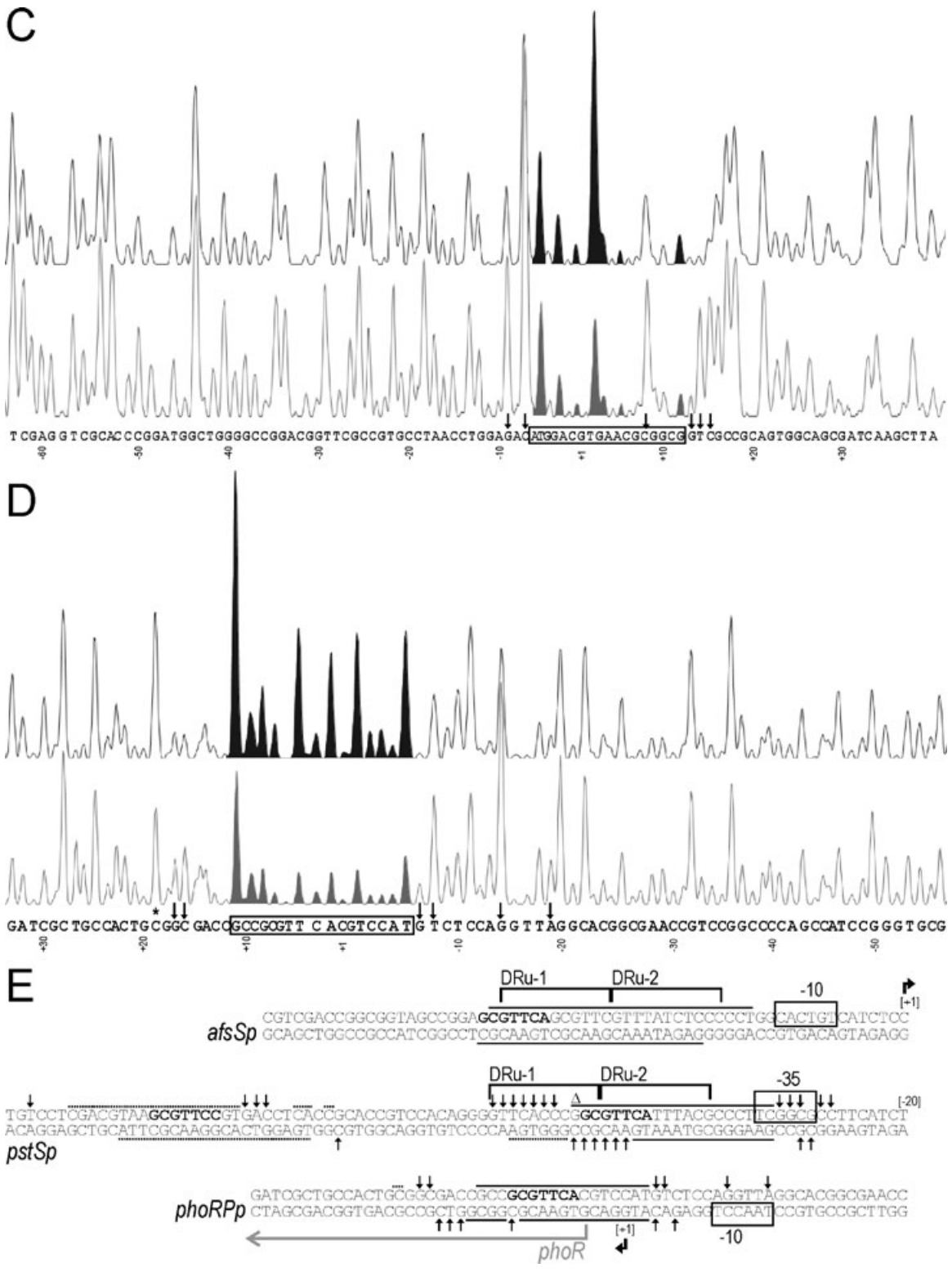

Fig. 7. *cont.*

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Table 1. Luciferase specific activity (light a.u. per mg dry weight) of *pstS* promoter in M145 (parental strain) and M513 (Δ*afsR*) strains in MG-3.2.

Time	M145	M513	Ratio
40 h	$2.15 \times 10^2 \pm 0.99 \times 10^2$	$7.91 \times 10^2 \pm 1.05 \times 10^2$	3.7
44 h	$8.99 \times 10^2 \pm 0.04 \times 10^2$	$11.7 \times 10^2 \pm 1.96 \times 10^2$	1.3
48 h	$13.2 \times 10^2 \pm 1.47 \times 10^2$	$20.1 \times 10^2 \pm 1.08 \times 10^2$	1.5
52 h	$2.78 \times 10^2 \pm 0.15 \times 10^2$	$2.30 \times 10^2 \pm 0.50 \times 10^2$	0.8
64 h	$0.45 \times 10^2 \pm 0.20 \times 10^2$	$1.28 \times 10^2 \pm 0.22 \times 10^2$	2.9

Mean and SD of two independent cultures are shown.

but with lower affinity, because area fractions were 95% ± 5%, 76% ± 7% and 58% ± 15% of that of the control at the above-indicated protein concentrations respectively.

Footprinting of the complementary strand confirmed the specific binding to both sites (Fig. 7B). Peak ratios of the main binding site (positions from –121 to –108), were 76% ± 7%, 42% ± 14% and 40% ± 23% at the above protein concentrations. DNase I hypersensitive sites surrounded this region. The adjacent hexamer (GGGTGA) appeared protected to a lower extent (peak ratios of 100% ± 4%, i.e. null protection; 91% ± 9% and 64% ± 17%, respectively, to the indicated protein range). Protection of the secondary binding site (positions –153 to –171) in the complementary strand was reflected by the peak ratios 99% ± 2%, 92% ± 5% and 79% ± 7% respectively.

The *phoRP* promoter was protected by H-AfsRΔTPR at a unique region located at positions –6 to +12 in the coding strand (Fig. 7C), and –6 to +11 in the non-coding strand (Fig. 7D). Also DNase I hypersensitive sites were present in the flanking positions. The protein affinity to this sequence was lower than the affinity to the main *pstSp* binding site because a higher protein concentration was required to obtain similar protection. Thus, 4 μM protein yielded peak ratios of 63% ± 19% in the coding strand (Fig. 7C), and of 37% ± 15% in the non-coding strand (Fig. 7D).

The summary of these results is shown in Fig. 7E. As indicated previously, the binding site of the AfsR regulator overlapped completely the PHO box in the *afsS* promoter. In the *pstS* promoter the AfsR binding site only covered the last DRu of the PhoP operator, which comprises two DRu, and in the *phoRP* promoter the AfsR site is completely separated from the PhoP operator.

Previously, we reported that deletion of the 10th bp of the first DRu disrupted the binding of PhoP to the *pstS* operator (Sola-Landa *et al.*, 2008; Fig. 7E). We used this mutant sequence (*pstS**) to check if the first DRu participates in the AfsR binding. The H-AfsRΔTPR protein retarded and protected similarly this *pstS** fragment as well as the wild-type sequence (data not shown). As the only difference was that the protection of the *pstS** coding strand lacked the deleted nucleotide (starting GCGT TCA . . . ; Fig. 7E), we may conclude that the first DRu of the PhoP is not involved in the AfsR binding. In summary, PhoP and AfsR recognize overlapping (or adjacent) but different nucleotide sequences.

AfsR represses the transcription of the pstS *gene*

The plasmid pLUX-*pstS* was used previously as a reporter of the *pstS* promoter activity in Pi shift-down experiments, because expression of this gene is strongly dependent upon PhoP and serves to monitor the PHO response *in vivo* (Rodríguez-García *et al.*, 2007). To determine the sign of the AfsR regulation on this gene, this plasmid was introduced into the M145 and M513 (Δ*afsR*) strains. Cultures were done only in MG-3.2 medium, because the *pstS* promoter is not activated in high Pi concentrations (Santos-Beneit *et al.*, 2008). The lack of AfsR caused consistently higher *pstS* promoter activities through most of the time-course of the experiment (Table 1). This negative effect of AfsR on the expression of *pstS* might explain why under Pi-limited conditions the growth of the Δ*afsR* strain was higher than the parental strain (Fig. 1).

Discussion

The two-component system PhoR–PhoP controls the response to Pi scarcity in *Streptomyces* species (Sola-Landa *et al.*, 2003; 2005; Ghorbel *et al.*, 2006). In this work we focused on the search of PhoP target sequences (PHO boxes) in the model organism *S. coelicolor* to explain the role of the PhoR–PhoP system in secondary metabolism. As a first approach, we cloned three promoters of antibiotic regulatory genes. PhoP failed to bind the promoters of *act*II-ORF4, *redD* and *atrA*, thus excluding a direct regulation of these pathway-specific and AtrA transcriptional regulators.

Bioinformatic searches allowed the identification of a putative PHO box in the *afsS* promoter that overlaps with the AfsR binding sequence. We concluded from gel-retarding and footprinting assays *in vitro*, and promoter–

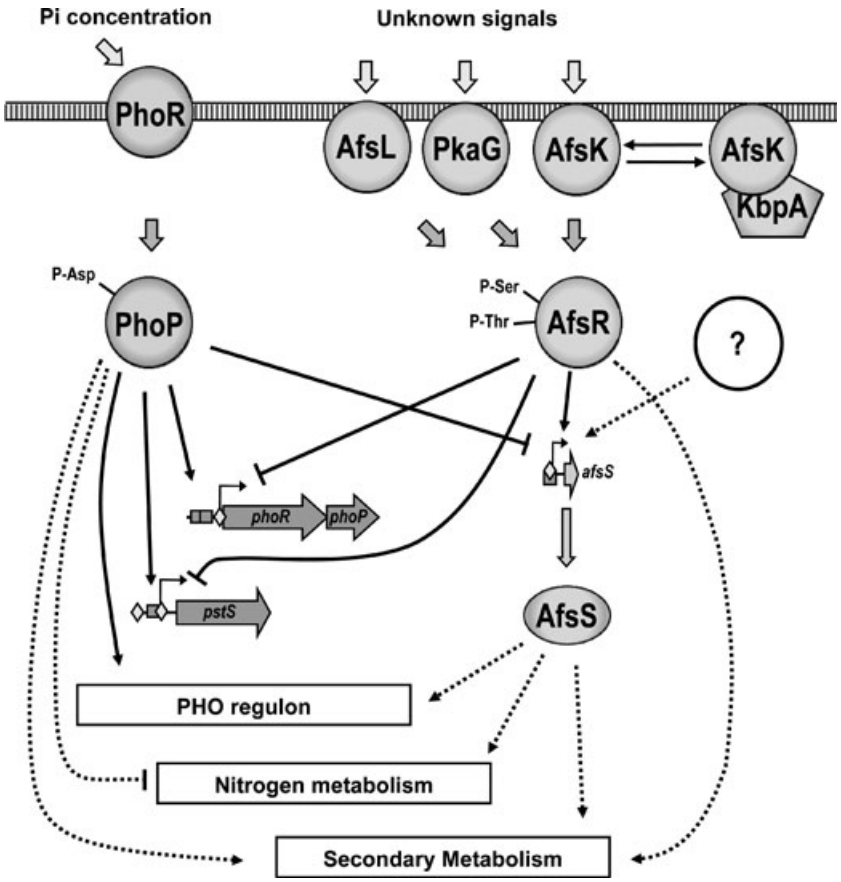

Fig. 8. Model of signal transduction and cross-talk regulation in PhoR–PhoP and AfsK–AfsR–AfsS systems. The model takes into account the data of Horinouchi (2003) and the results of Sola-Landa *et al.* (2005), Rodríguez-García *et al.* (2007), Lian *et al.* (2008) and this work. PHO boxes are represented by a small square and AfsR binding sites by a diamond.

luciferase genes fusions *in vivo*, that PhoP represses the transcription of *afsS*. Two mechanisms account for this repression: first, PhoP is an anti-activator (Browning and Busby, 2004) because it competes with AfsR; second, PhoP blocks the RNA polymerase binding to the promoter by steric hindrance. To our knowledge, this is the first description of a complete signal transduction cascade from a nutrient-sensing (Pi) to an antibiotic regulator (Fig. 8).

Very little is known about the signal transduction pathways that take place upstream of the specific antibiotic regulators. In bacteria, signal transduction is mediated by protein phosphorylation of two-component systems, and by serine/theonine protein kinases. One of the first regulatory cascades discovered in bacteria that involve serine/theonine kinases was indeed the AfsK–AfsR–AfsS system (Horinouchi, 2003). AfsK is a loosely attached membrane kinase that first phosphorylates itself and then the serine and threonine residues of the pleiotropic regulator AfsR (Matsumoto *et al.*, 1994). Another protein, KbpA, inhibits the autophosphorylation of AfsK (Umeyama and Horinouchi, 2001). Other two serine/threonine kinases, AfsL and PkaG, were also found to phosphorylate AfsR (Sawai *et al.*, 2004), which suggests that AfsR integrates multiple inputs. It is not yet known what kind of environmental signals trigger the system, although AfsK appears to bind S-adenosyl-L-methionine (Lee *et al.*, 2007). Phosphorylated AfsR binds the –35 region of the *afsS* promoter and activates its transcription (Lee *et al.*, 2002; Tanaka *et al.*, 2007). When introduced in a high-copy-number plasmid, *afsS* enhances the production of Act and Red in both *S. lividans* and *S. coelicolor* (Vögtli *et al.*, 1994; Matsumoto *et al.*, 1995; Floriano and Bibb, 1996). Although *afsS* stimulates the expression of the pathway-specific transcriptional regulators *act*II-ORF4 and *redD* respectively, the molecular mechanism of this activation is unknown (Horinouchi, 2003). In addition, the results of Floriano and Bibb (1996) and Lee *et al.* (2002) indicated that the positive effect of AfsR in secondary metabolism involves genes other than *afsS*. Indeed, we report herein the existence of three new AfsR binding sites in the promoters of key phosphate transport and regulatory genes.

DNase I footprinting assays showed that AfsR protects two separate sequences in the *pstS* promoter. The higher-affinity site overlaps partially with the –35 element (Fig. 7E), but does not meet the strict requirements to be an activator site (Tanaka *et al.*, 2007). Thus, when AfsR is bound at this sequence, it should prevent the binding of both the PhoP and the RNA polymerase. The lower-

affinity site is placed more than 40 nt upstream of the –35 element. Distal sites may act as repressors when the protein binding induces a loop in the DNA (Browning and Busby, 2004), and it is possible that this secondary site reinforces *pstS* repression at higher AfsR concentrations. Besides, luciferase reporter assays confirmed that AfsR represses the *pstS* gene.

The third new AfsR binding site was found at the *phoRP* transcription start (Fig. 7E). The position and the inverse orientation of the binding-site, compared with that of the *afsS* activator, strongly indicate that AfsR blocks the RNA polymerase. Repressors that bind downstream of the transcriptional start point are common (Babu and Teichmann, 2003). Blocking the transcription at this site is expected to affect the expression of the two components PhoR–PhoP of the phosphate-responsive system that appear to be cotranscribed in *S. coelicolor*.

Although both AfsR and PhoP proteins bind to the same region in the *afsS* promoter, they must have distinct sequence specifities, because AfsR protects sequences that PhoP does not bind (e.g. the site upstream of the *pstS* PHO box, the site that overlaps the start codon of *phoR*, and the mutant sequence *pstS**) and because AfsR does not protect the PHO boxes in the *phoU-phoRP* intergenic region (Fig. 7).

Tanaka *et al*. (2007) indicated that the AfsR binding site of the *afsS* promoter contains two direct repeats (consensus sequence CGTT(T/C)ATCGNN). However, there is no apparent conservation of a second repeat in the other three operators located in the *pstS* and *phoR* genes (Fig. 7E). This suggests that the clear sequence repetition found in the *afsS* promoter is due mostly to the PhoP binding site and not to the AfsR recognition sequence. Alignment of the four known AfsR binding sequences only revealed conservation of the sequence GCGTTC(A/C). Clarification of the sequence pattern recognized by AfsR will require detection of additional operators and directed mutagenesis studies.

As shown in flask liquid cultures of wild-type and mutant strains in defined MG medium, PhoP has an overall positive role in *S. coelicolor* Red and Act production. As PhoP represses *afsS*, which positively controls Red and Act production, it should influence other mechanisms to account for the overall positive effect.

This positive effect of PhoP contrasts with previous results of our group in *S. lividans* and in *Streptomyces natalensis* liquid cultures using complex media (Sola-Landa *et al*., 2003; Mendes *et al*., 2007). Disruptions of *phoRP* or *phoP* alone lead to Act and Red synthesis in R5 cultures of *S. lividans* (Sola-Landa *et al*., 2003). The pimaricin production of the *S. natalensis* Δ*phoRP* strain was higher than that of the wild-type strain in NBG medium (composed of NaCl, beef extract and glucose; Mendes *et al*., 2007). Indeed the regulation of antibiotic production in *S. lividans* is different from that of *S. coelicolor*, because *S. lividans* usually does not synthesize Act or Red in common laboratory media. Differences between *S. lividans* and *S. coelicolor* have been reported on glucose repression of Act (Kim *et al*., 2001), and on the stimulatory effect of multiple copies of *afsS* in *afsR* mutant backgrounds (Floriano and Bibb, 1996). Thus, the regulatory features of PhoP might vary across species producing different antibiotics.

As expected from the results of other laboratories, the absence of AfsR in the M513 mutant led to a lower antibiotic production. Our results add new information on the AfsR–AfsS regulation. Thus, the *afsSp*-driven specific luminescence is reduced, but not abolished, in the *afsR* mutant grown on liquid MG medium. This indicates that other regulatory factors may also activate the *afsS* promoter, such as one of the three AfsR-like proteins encoded in the *S. coelicolor* genome (Bentley *et al*., 2002).

The cross-regulation of PhoR–PhoP and AfsK–AfsR–AfsS systems that is described here at the molecular level complements the recent work of Lian *et al*. (2008). These authors compared the transcriptomic profiles of wild-type and *afsS* mutant strains in liquid cultures, and found that AfsS positively controls some PHO regulon genes, including the *phoRP* system itself. Thus, AfsS produces the opposite effect on the expression of the PHO regulon genes than AfsR. This intricate cross-regulation is summarized in Fig. 8. Growing evidence indicates that the main regulatory systems in the soil-dwelling bacteria *Streptomyces* are intertwined. In addition to Pi, the carbon source modulates the effect of AfsR and AfsS (Floriano and Bibb, 1996; Sekurova *et al*., 1999; Kim *et al*., 2001), as well as the expression of *pstS* (Díaz *et al*., 2005). Key genes of the nitrogen metabolism are upregulated in the *phoP* mutant strain (Rodríguez-García *et al*., 2007), but are downregulated in the *afsS* mutant strain (Lian *et al*., 2008). In summary, *Streptomyces* have developed intertwined mechanisms to adapt their metabolism to the changing nutritional conditions in the soil.

Experimental procedures

Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 2. As this work is part of the European systems biology project of *S. coelicolor*, we used the strain provided by Eriko Takano as the parental strain. This is a single colony isolate of the widely used M145 strain (Kieser *et al*., 2000). The *S. coelicolor* INB201 Δ*phoP* mutant was obtained for this work by the replacement procedure already used in Rodríguez-García *et al*. (2007), except that the parental of INB201 was the above mentioned strain. *S. coelicolor* M513, an in-frame deletion mutant of the *afsR* gene (Floriano and Bibb, 1996), was provided by Mervin Bibb. All strains were

Table 2. Bacterial strains and plasmids used in this work.

Strains/plasmids	Characteristics	Reference
Strains		
Streptomyces coelicolor A3(2) M145	Plasmid-free derivative of the Wild-type *S. coelicolor* A3(2) strain	Kieser *et al*. (2000)
Streptomyces coelicolor A3(2) INB201	Δ*phoP,* Am^r	This work
Streptomyces coelicolor A3(2) M513	Δ*afsR*	Floriano and Bibb (1996)
Escherichia coli DH5α	F′Φ80 *dLacZ* ΔM15	Hanahan (1983)
Escherichia coli ET12567 (pUZ8002)	*dam dcm* mutant, Neo^r -Cm^r	MacNeil *et al*. (1992)
Escherichia coli BL21 (DE3) pLysS	F$^-$ *ompT hsdS$_B$* (r_{B^-} m_{B^-}) *gal dcm* (DE3)pLysS (cam^R)	Novagen
Plasmids		
pGEM-T-easy	Cloning vector, Amp^r	Promega
pGEM-P*redD*	PCR product carrying *redD* promoter cloned into pGEM-T-easy, Amp^r	This work
pGEM-P*actIIORF4*	PCR product carrying *actIIORF4* promoter cloned into pGEM-T-easy, Amp^r	This work
pGEM-P*atrA*	PCR product carrying *atrA* promoter cloned into pGEM-T-easy, Amp^r	This work
pBS-P*phoU*	PvuI fragment carrying *phoU* promoter cloned into pBluescript KS+, Amp^r	Sola-Landa *et al*. (2005)
pGEM-P*pstS*	PCR product carrying *pstS* promoter cloned into pGEM-T-easy, Amp^r	Sola-Landa *et al*. (2005)
pGEM-P*pstS**	PCR product carrying *pstS** promoter cloned into pGEM-T-easy, Amp^r	Sola-Landa *et al*. (2008)
pBluescript II SK+	Cloning vector, Amp^r	Stratagene
pFS-*afsS*	PCR product carrying *afsS* promoter cloned into pBSIISK+, Amp^r	This work
pLUXAR-neo	Integrative promoter-probe vector, *luxAB* genes, Am^r, Neo^r	This work
pLUX-*afsS*	BamHI-NdeI pFS-*afsS* fragment cloned into pLUXAR-neo, Am^r, Neo^r	This work
pLUX-*pstS*	BamHI-NdeI pGEM-P*pstS* fragment cloned into pLUXAR+, Am^r	Santos-Beneit *et al*. (2008)
pET16-*afsR*ΔC	Cloning vector carrying His-tagged truncated AfsR (Met1 to Ala270), Amp^r	Lee *et al*. (2002)
pET16-*afsR*ΔTPR	Cloning vector carrying His-tagged truncated AfsR (Met1 to Glu618), Amp^r	Tanaka *et al*. (2007)
pGEX-PhoP	*S. coelicolor phoP* gene cloned into pGEX-2T	Sola-Landa *et al*. (2005)
pGEX-DBD	*S. coelicolor phoP*-DBD gene cloned into pGEX-2T	Sola-Landa *et al*. (2005)

manipulated and conjugated according to standard procedures (Kieser *et al*., 2000). *Escherichia coli* DH5α was the general cloning host.

The *afsS* promoter region was amplified by PCR using total DNA as template, as follows: the primers CAR21 (5′-ATGCGGATCCTCAGCCTCTACGAGCAGC) and CAR22 (5′-TGGGTACCATATGTCCGCGTCCTTCATCTTG) amplified a 339 bp fragment encompassing the intergenic region between *afsR* and *afsS*, the last 77 bp of *afsR* and the first 26 bp of *afsS*. The BamHI (CAR21) and KpnI/NdeI (CAR22) cloning sites were introduced in the primer sequences. A BamHI-KpnI fragment was cloned into pBluescript II SK+ obtaining pFS-*afsS*. The insert of the plasmid was checked by sequencing. A BamHI-NdeI fragment of 321 bp from pFS-*afsS* was cloned into the promoter-probe vector pLUXAR-neo, yielding pLUX-*afsS*. pLUXAR-neo was constructed from pLUXAR+ (Rodríguez-García *et al*., 2007) by insertion of the *neo* gene at the NheI site, downstream of the *aac(3)IV* gene.

redD, *act*II-ORF4 and *atrA* promoters were amplified by PCR using the primer pairs *redD*-Up (5′-CGCGGGATCC GATGATGTTGGAGTTG) and *redD*-Low (5′-CCCCTCTAG ACATATGCCGAACGATCGGATTC), *act*II-Up (5′-GGACGG ATCCGAGGACCCAGCCGTATCAG) and *act*II-Low (5′-AG TTTCTAGACATATGCGCCCCCGTC), and *atrA*-Up (GGATC-CGCTCGTCAAATTGTGTGACCGG) and *atrA*-Low (5′-TCTAGAATAGACAATCCCCCGGTAATGACG) respectively. Final products of PCR (533 bp, 241 bp and 353 bp respectively) were cloned into pGEM-T-easy (Promega) and the correct amplification was confirmed by sequencing.

Streptomyces coelicolor cultures were performed in defined MG medium containing starch (Scharlau; 50 g l^{-1}) and glutamate (60 mM; Doull and Vining, 1989). One hundred millilitres of MG medium in 500-ml baffled flasks were inoculated with 10^6 spores ml^{-1} and incubated at 30°C, 300 r.p.m. (25.4 mm orbit diameter) for reproducible and dispersed growth. For the phosphate-replete and the phosphate-limited conditions cultures contained 18.5 mM and 3.2 mM potassium phosphate respectively (MG-18.5 and MG-3.2 mediums; Santos-Beneit *et al*., 2008). Samples to measure *afsSp* activity, antibiotic production and growth were taken at 35 h, 45 h, 50 h, 55 h and 75 h of incubation. In all cases two different exconjugants were selected for the cultures. Duplicates of each exconjungant were used (four replicates of each condition). For *pstSp–luxAB* reporter studies samples were taken at 40 h, 44 h, 48 h, 52 h and 64 h of incubation. In this case, only one exconjugant was used for each strain. Both cultures were done in duplicate.

Luciferase assay, growth and antibiotic production determinations

The *luxAB* activity driven from the *afsS* promoter was determined in a Luminoskan luminometer (Labsystems, Helsinki) as reported in Rodríguez-García *et al*. (2007) and Santos-Beneit *et al*. (2008). Cell pellets from 1 ml culture samples were kept frozen until luminescence readings. The *pstSp–luxAB* gene fusion activity was measured as stated

above, but the samples were kept on ice and measured immediately.

Antibiotic assays were performed as described by Kieser *et al*. (2000). For dry weight determination, culture samples of 2 ml were washed twice with MilliQ water and dried during 4 days at 80°C.

Production and purification of H-AfsRΔC, H-AfsRΔTPR, GST-PhoP and GST-PhoP^{DBD} proteins

H-AfsRΔC (291 amino acids, 32 kDa; containing the N-terminal portion of AfsR from Met-1 to Ala-270; Lee *et al.,* 2002) and H-AfsRΔTPR (639 amino acids, 68 kDa; containing the N-terminal and central portions of AfsR from Met-1 to Glu-618; Tanaka *et al*., 2007) histidine-tagged proteins were purified from the soluble fractions of *E. coli* BL21 (DE3) pLysS harbouring the plasmids pET16-*afsR*ΔC and pET16-*afsR*ΔTPR respectively, as described by Lee *et al*. (2002). The purified samples were dialysed overnight against 10 mM Tris-HCl (pH 7) and 10% glycerol.

GST-PhoP (449 amino acids, 51 kDa; containing a full-length PhoP construct fused to the GST protein) and GST-PhoP^{DBD} (341 amino acids, 39 kDa; containing the C-terminal portion of PhoP from Ile-109 to Pro-223; Sola-Landa *et al*., 2005) proteins were purified from the soluble fractions of *E. coli* DH5α harbouring the plasmids pGEX-PhoP and pGEX-DBD respectively, as described by Sola-Landa *et al*. (2005).

Protein concentration was determined with the Bradford reagent (Bio-Rad).

Gel mobility shift assays

The promoters were excised from its respective plasmids by digestion with restriction endonucleases and labelled at both ends with digoxigenin using the DIG Oligonucleotide 30-End Labeling Kit, 2nd Generation (Roche Applied Science). The sizes of the DNA probes were the following: 321 bp (*afsS*), 315 bp (*phoRP*), 280 bp (*pstS*), 279 bp (*pstS**), 553 bp (*redD*), 261 bp (*act*II-ORF4) and 373 bp (*atrA*). The conditions for DNA–protein binding and detection were described previously by Sola-Landa *et al*. (2005). As the H-AfsRΔC solution differs from the GST-PhoP^{DBD} one, the binding reactions in the competitive EMSA were equalized adding different amounts of SAD (10% glycerol and 10 mM Tris-HCl pH 7.0) and SPD (40% glycerol, 25 mM Tris-HCl pH 8.0 and 5 mM reduced glutathione).

Samples were run in 0.5× TBE buffer on a 5% polyacrylamide native gel during 2 h at 80 V using a Bio-Rad Mini Protean III apparatus.

DNase I footprinting

The DNase I footprinting assays of *afsS* promoter were performed using the GST-PhoP^{DBD} protein as described in Sola-Landa *et al*. (2005). DNA probes for both the coding and complementary strands were obtained by PCR using pFS-*afsS* as template and the respective fluorescent-labelled primer. The labelled probes (495 bp and 490 bp for the coding and complementary strand respectively) were purified from agarose electrophoresis (GFX columns). DNase I footprinting was performed by incubating 0.28 pmol of the DNA probe with different concentrations of the GST-PhoP^{DBD} protein during 30 min at 30°C. DNase I digestions were carried out during 1 min at 30°C. Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare) and labelled primers were used to obtain reaction sequences. The reaction products were resolved in an ALF DNA sequencer (GE Healthcare) and analysed with the Fragment Manager program as described previously (Rodríguez-García *et al*., 1997).

The DNase I footprinting assays of *phoRP, pstS* and *pstS** promoters with the H-AfsRΔTPR protein were carried out as above, but using the SAD buffer instead of SPD. For these footprinting assays we used the 6-FAM-labelled primers CAGGAAACAGCTATGAC (reverse) and CGACGTTGTAAAACGACGGCCAGT (forward). Labelled PCR products were added to a final concentration of 9.3 nM. Each reaction was loaded into an ABI PRISM 3130 sequencer together with the molecular standard Gene-Scan® 500 LIZ™ (Applied Biosystems). Electrophoregrams were analysed with Peak Scanner software v1.0 (Applied Biosystems) and normalized against the control. For this purpose, the total peak area was calculated for the regions shown in Fig. 7, excluding protected peaks.

'Information theory' analysis of binding sites

To evaluate the potential binding of PhoP, we calculated the individual information content (Schneider, 1997) of each 11 nt stretch using the weight matrix of model I of Sola-Landa *et al*. (2008). This weight matrix gathers the information of 37 DRu that form the core of experimentally demonstrated PhoP operators.

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Phosphate control over nitrogen metabolism in *Streptomyces coelicolor*: direct and indirect negative control of *glnR*, *glnA*, *glnII* and *amtB* expression by the response regulator PhoP

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ABSTRACT

Bacterial growth requires equilibrated concentration of C, N and P sources. This work shows a phosphate control over the nitrogen metabolism in the model actinomycete *Streptomyces coelicolor*. Phosphate control of metabolism in *Streptomyces* is exerted by the two component system PhoR-PhoP. The response regulator PhoP binds to well-known PHO boxes composed of direct repeat units (DRus). PhoP binds to the *glnR* promoter, encoding the major nitrogen regulator as shown by EMSA studies, but not to the *glnRII* promoter under identical experimental conditions. PhoP also binds to the promoters of *glnA* and *glnII* encoding two glutamine synthetases, and to the promoter of the *amtB-glnK-glnD* operon, encoding an ammonium transporter and two putative nitrogen sensing/regulatory proteins. Footprinting analyses revealed that the PhoP-binding sequence overlaps the GlnR boxes in both *glnA* and *glnII*. 'Information theory' quantitative analyses of base conservation allowed us to establish the structure of the PhoP-binding regions in the *glnR*, *glnA*, *glnII* and *amtB* genes. Expression studies using *luxAB* as reporter showed that PhoP represses the above mentioned nitrogen metabolism genes. A mutant deleted in PhoP showed increased expression of the nitrogen metabolism genes. The possible conservation of phosphate control over nitrogen metabolism in other microorganisms is discussed.

INTRODUCTION

Bacterial growth requires an equilibrated concentration of carbon, nitrogen and phosphorus sources. Carbon, nitrogen and phosphate concentrations exert an important regulatory effect on primary and secondary metabolism in different bacteria including *Streptomyces* (1–5).

Phosphorus is an essential component of bacterial nutrition; expression of phosphate-regulated genes in *Streptomyces* species is modulated by the two-component system PhoR-PhoP (6). PhoR is the membrane sensor protein kinase which senses phosphate scarcity; PhoP is the response regulator which binds DNA and controls the transcription of genes belonging to the so-called *pho* regulon. PhoP was shown to control the expression of primary and secondary metabolism genes including actinorhodin and undecylprodigiosin biosynthesis genes (6,7). Binding of PhoP to the promoter regions of three different genes of the *pho* regulon *pstS*, *phoU* and *phoRP* was shown both in *Streptomyces coelicolor* (8) and *S. natalensis* (9). The PhoP-binding operator sequences of these genes as well as those present in the promoter regions of *phoA* and *phoD* of *S. coelicolor* (10) are composed of direct repeat units (DRu) of 11 nt. Operator sequences of other PhoP-regulated genes have been recently described in *S. coelicolor* and classified into three types of DRus organization with different degrees of complexity (11).

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In bacteria a central role in nitrogen metabolism is played by glutamine synthetases that assimilate ammonium into the cellular organic nitrogen (NH_3 + glutamate + ATP $\rightarrow$ glutamine + ADP + P_i) (2). The overall nitrogen metabolism is regulated in *S. coelicolor* by complex mechanisms that involve an apparent doubling of some structural and regulatory genes (12). There are two glutamine synthetase (GS) genes in *S. coelicolor*: *glnA* (13), that encodes the GS type I (GS-I), orthologous to that found in prokaryotes, and *glnII* (14), which encodes GS type II (GS-II), similar to eukaryotic GS. The *glnII* gene appears to be present in all *Streptomyces* species (15), but it is absent in the related actinomycetes *Mycobacterium* and *Corynebacterium* (12). The two types of GS enzymes differ in size, number of subunits, expression pattern and post-translational modification (2). In *S. coelicolor*, the adenylyltransferase GlnE down-regulates the GS-I enzyme by post-translational modification in response to excess of ammonium (16). The adenylated GS-I enzyme becomes inactive. GlnE can also deadenylate and restore the GS-I activity under ammonium limitation. This post-translational regulatory mechanism is widespread in bacteria. In *Escherichia coli* the GlnE activity depends on three proteins that are thought to be nitrogen sensing proteins. These are the GlnD protein, an uridylyltransferase/uridylyl-removing enzyme, and two proteins of the P_{II} signal transduction superfamily, GlnB and GlnK (2,17). Both GlnB and GlnK are covalently modified—and thus regulated—by GlnD, and both P_{II} proteins modulate the GlnE activity.

The GlnD and GlnK homologues of *S. coelicolor* were characterized by Hesketh *et al.* (18). In contrast to the enteric system, these proteins are not required for the GlnE-mediated regulation of the GS-I enzyme. The targets of the GlnK/GlnD system are not yet known (12). Genes encoding this system are clustered with the putative ammonium transporter gene *amtB* and form the operon *amtB-glnK-glnD* (19).

Two regulatory genes *glnR* and *glnRII* control expression of several nitrogen metabolism genes at the transcriptional level. The global nitrogen activator/repressor GlnR controls all the important routes for ammonium assimilation (19–22). GlnR in response to nitrogen limitation activates the transcription of *glnA* and *glnII* (encoding both GSs), as well as the transporter *amtB* and the putative nitrite reductase gene *nirB*. The binding motif of GlnR (GlnR box), has been characterized by footprinting assays of *glnA*, *gdhA* and *nirB* promoters, and by sequence analysis of 13 bound promoters (19,22).

The regulator gene *glnRII* is located 1 kb downstream of *glnII*, separated by two hypothetical coding sequences. The overall amino-acid sequence of GlnRII is similar to GlnR, and is nearly identical in the DNA recognition helixes. In fact, GlnRII also binds the promoter regions of *glnA*, *glnII* and *amtB* (19). Nevertheless, it is not strictly a functional homologue of GlnR and its role in nitrogen regulation is not yet clearly established (12).

Initial microarray studies (23) suggested that there might be a connexion between the phosphate control exerted by PhoP and the overall nitrogen regulation mediated by GlnR. It was, therefore, of great interest to study the relationships between these two pleiotropic regulators in the control of metabolism in *S. coelicolor*.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The *S. coelicolor* strain M145 (24), which is the standard strain for the European Union STREAM (*Streptomyces* analysis of metabolism) project was used as the wild-type strain. The M145-derivative mutant strain *S. coelicolor* INB201 was obtained by replacement of the *phoP* coding sequence by the apramycin resistance cassette (F. Santos-Beneit *et al.*, unpublished results). All strains were manipulated and conjugated according to standard procedures (24).

All the DNA materials are listed in Table 1. PCR using the indicated amplification primer pairs were done to clone promoter regions into adequate vectors. Fidelity of the PCR was checked by sequencing the entire insert. Vectors pGEM-T easy and pBS II KS + were used to clone the indicated amplicons (Table 1) for gel retarding and footprinting analyses. For luciferase reporter analysis, primers containing a NdeI-restriction site were designed in order to clone the promoters into the ATG codon of the *luxA* gene. Using this strategy, the cloned promoter sequences include the Shine-Dalgarno nucleotides and maintain the same distance to the start codon in the luciferase fusions than in the original gene.

Streptomyces cultures were carried out in defined MG-3.2 medium containing 50 g l^{-1} starch, 60 mM glutamate, and 3.2 mM potassium phosphate. Total 10^6 spores ml^{-1} were used to inoculate 100 ml of MG medium in 500 ml baffled flasks and incubated at 30°C, 300 r.p.m. as indicated previously (25).

Electrophoretic mobility assays (EMSA)

DNA–PhoP interaction was tested in electrophoretic mobility shift assays (EMSA) as described previously (8). The promoters were excised from plasmids by restriction digestion and labelled at both ends with digoxigenin using the DIG Oligonucleotide 3′-end Labeling Kit, Second Generation (Roche). DNA fragments were incubated with different GST-$PhoP^{DBD}$ concentrations and DNA–protein complexes were resolved by PAGE.

DNase I footprinting assays

Pure GST-$PhoP^{DBD}$ protein was used for DNase I footprinting assays as previously described (8). DNA probes for *glnRp*, *glnAp*, *glnIIp* and *amtBp* were obtained by PCR using a 6-FAM-modified primer for labelling just one strand. Labelled and unlabelled primer pairs correspond to the forward and reverse M13 sequences listed in Table 1. The reaction mixtures included 9.3 nM of probe DNA and 2 μM of GST-$PhoP^{DBD}$ protein.

The labelled primers were used also for Sanger sequencing with the Thermo Sequenase Primer Cycle Sequencing

Table 1. List of primers and plasmids

Primer	Sequence[a]	Promoter	Size of amplicon
PHO-37	TCTAGAGGCTACGACGAGCGGGAAC	*glnII*	316
PHO-38	GGATCCACGGGGCCACATCCTTCG		
PHO-39	TCTAGAGGAGAGCCACGATCCGATTG	*glnA*	284
PHO-40	GGATCCCGGCGTTCTGGAACATCC		
PHO-41	TCTAGATCCCGAACTGCCCGACTC	*amtB*	293
PHO-42	GGATCCATCGGCGTCTCCTCGTCG		
glnR-1	GCCGTACGGAGGAAGGTACG	*glnR*	362
CAR35	TCAGGAGCAGCAGAGAACTCATC		
CAR36	GGCGGTCGGTTGCTCATG	*glnR*	257
glnRII-1	GGATCCCCACGCACTGAGAGGAGTCTCCT	*glnRII*	321
glnRII-2	TCTAGAATGAGACGTCAGCTCTTTCGCG		
CAR57	GTCAGGATCCGTCTCGGGATGCGGACGATTGG	*glnR*	308
CAR58	ATGGTACCATATGCCCCACCTGCCTGGGACGGTTTG		
CAR59	GAACGGATCCGAGCCACGATCCGATTGC	*glnA*	273
CAR60	ATGGTACCATATGGCTCCTCCTACTCCCGACCGT		
CAR61	TTCTGGATCCGTCCCACTTCGGACCGCTGATC	*glnII*	307
CAR62	ATGGTACCATATGGCCACATCCTTCGGGGTGGGTCT		
CAR63	CCGTGGATCCGGCCGTACGCGATTTC	*amtB*	420
CAR64	CTGGTACCATATGCGTCTCCTCGTCGTTG		
6FAM-F	CGACGTTGTAAAACGACGGCCAGT	Various	
Reverse	GGAAACAGCTATGACCATG		
6FAM-R	CAGGAAACAGCTATGAC	Various	
Forward	GTAAAACGACGGCCAGT		

Plasmid	**Features**[b]	**Reference**
pGEM-T-easy	Cloning vector, Amp^r	Promega
pBS II KS+	Cloning vector, Amp^r	Stratagene
pBS II SK+	Cloning vector, Amp^r	Stratagene
pLUXAR+*neo*	Conjugative-integrative promoter-probe vector, *luxAB* genes, Am^r, Neo^r	Pérez-Redondo, unpublished
pGEM-P*glnII*	PCR product from PHO-37 and PHO-38 cloned into pGEM-T-easy	This work
pGEM-P*glnA*	PCR product from PHO-39 and PHO-40 cloned into pGEM-T-easy	This work
pGEM-P*amtB*	PCR product from PHO-41 and PHO-42 cloned into pGEM-T-easy	This work
pBS-P*glnR*	PCR product from glnR-1 and CAR35 cloned into pBS II KS+(EcoRV)	This work
pBS-P*glnR*-b	PCR product from CAR35 and CAR36 cloned into pBS II KS+(EcoRV)	This work
pBS-P*glnRII*	PCR product from glnRII-1 and glnRII-2 cloned into pBS II KS+(EcoRV)	This work
pAR-N1	PCR product from CAR57 and CAR58 cloned into pBS II SK+	This work
pAR-N2	*glnRp* from pAR-N1 (−289 to +3) cloned into pLUXAR+*neo* (BamHI, NdeI)	This work
pAR-N3	PCR product from CAR59 and CAR60 cloned into pBS II SK+	This work
pAR-N4	*glnAp* from pAR-N3 (−253 to +3) cloned into pLUXAR+*neo* (BamHI, NdeI)	This work
pAR-N5	PCR product from CAR61 and CAR62 cloned into pBS II SK+	This work
pAR-N6	*glnIIp* from pAR-N5 (−287 to +3) cloned into pLUXAR+*neo* (BamHI, NdeI)	This work
pAR-N7	PCR product from CAR63 and CAR64 cloned into pBS II SK+	This work
pAR-N8	*amtBp* from pAR-N7 (−401 to +3) cloned into pLUXAR+*neo* (BamHI, NdeI)	This work

[a]Restriction sites introduced in amplicons are indicated by underline.
[b]Promoter coordinates are referred to the translation start site.

Kit (GE Healthcare). Each reaction was loaded into an ABI PRISM 3130 sequencer together with the molecular standard Gene-Scan® 500 LIZ™ (Applied Biosystems). Electropherograms were analysed with PeakScanner v1.0 software (Applied Biosystems) to determine the protected sequence.

Luciferase assay and growth determination

The reporter luciferase activity was measured in a Luminoskan luminometer (Labsystems, Helsinki) as described previously (23,25). Riboflavine ($10\,\mu g\,ml^{-1}$) was added to the cell suspension in all samples to improve the sensitivity of luciferase assays, following the recommendation of Bachmann *et al.* (26). For dry weight determination, culture samples of 2 ml were washed twice with MilliQ water and dried at 80°C during 4 days.

'Information theory' analysis of binding sites

To calculate the information content (R_i value) of individual sequences (27), and to obtain logos and walkers for the analysis of binding sites we used the Delila package which include the makebk, encode, rseq, dalvec, makelogo, ri and lister programs (28,29). DNA sequences were scanned for binding sites using the RSA tools server (30) and R_i matrixes.

RESULTS

Transcriptional response of the nitrogen metabolism genes to phosphate limitation

We previously reported that phosphate limitation upregulated nitrogen metabolism genes in the Δ*phoP*

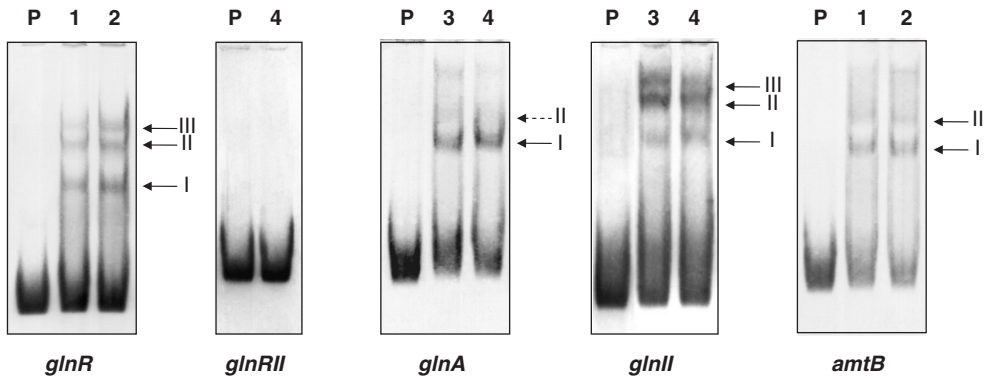


Figure 1. Analysis by EMSA of the promoters. Lane P, probe without protein; lanes 1–4, increasing concentrations of GST-PhoP^{DBD} (0.125, 0.25, 0.5 and 1 μM, respectively). An excess (more than 1000-fold) of poly[d(I-C)] is included in every lane as internal control to avoid an unspecific binding of the protein to the DNA. Controls with competing excess of unlabelled probe are shown as Supplementary Data (Figure S1). The different shift bands are indicated by arrows. The assays were repeated three times.

mutant (23). Microarray results showed that glutamine synthetase gene *glnII* and genes of the operon *amtB-glnK-glnD* increased their transcription in response to phosphate downshift in the Δ*phoP* mutant, while no change was detected in the wild-type strain. The GlnA protein, detected in 2D gels, was overproduced in the Δ*phoP* mutant. These expression responses suggested that PhoP might be exerting a negative effect on expression of those nitrogen metabolism genes.

Inspection of the nucleotide sequences of the promoter regions indicated the presence of PHO-like sequences in the above mentioned nitrogen structural genes and in the *glnR* regulator gene. This suggested that the previously observed transcriptomic profiles were due to both direct and indirect regulation by PhoP. The direct control was checked by DNA-binding analysis of the *glnA, glnII* and *amtB* promoters using the purified DNA-binding domain of the PhoP response regulator (GST-PhoP^{DBD}) (8). The promoter regions of the two regulatory genes, *glnR* and *glnRII*, were also analysed to explore a possible indirect control of the structural genes.

PhoP binds the *glnR* promoter but not the *glnRII* one

The *glnR* 5′-region was cloned by PCR as a fragment of 362 bp that also included 98 bp of the upstream coding sequence (CDS). Electrophoretic mobility shift assays (EMSA) of this fragment with the GST-PhoP^{DBD} protein revealed the formation of three retarded bands (Figure 1). As reported previously (11), each retarded DNA–protein complex correspond to a number of protein monomers bound to the DNA fragment. The established model of the PhoP-binding site indicates that each PhoP monomer binds a direct repeat unit (DRu) of 11 nt. Two or three consecutive DRus form the core of the binding site. Once the core is occupied, further protein monomers can bind adjacent DRus, what account for the DNA–protein complexes of lower electrophoretic mobility (see the detailed analysis below).

To locate the PhoP-binding site, we carried out DNase I footprinting experiments. Electrophoretic separation of digestion products was facilitated by using a smaller fragment of 257 bp that comprised only the *glnR* promoter sequence. The GST-PhoP^{DBD} protein at a concentration of 2 μM protected from DNase I digestion a stretch of 33 nt located at positions −139 to −107 in the coding strand (all coordinates are referred to the translation start site; Figure 2A). This stretch comprised a DRu with sequence matching the first seven PHO box consensus bases (GTTC ACC). In addition, some upstream and downstream nucleotides were protected to a lesser extent by protein binding. As previously observed in other PhoP footprintings (11), DNase I hypersensitive sites appeared next to the binding site at its 3′-end. The complementary strand showed protection from −112 to −140 nt, partial protections up to position −103, and hypersensitive sites at positions −144 and −145 (Figure 2B).

The promoter region of *glnR* contains three transcription start sites at positions −200, −170 to −168 and −119, which correspond to promoters P3, P2 and P1, respectively (21). Promoter P3 is active only during the exponential phase, whereas transcriptions originated from P2 and P1 promoters are detected at both exponential and stationary phases (31). Fink *et al.* (19) reported that only P2 and P3 are active in conditions of nitrogen limitation. The PhoP protection covers the distal P1 transcription start (Figure 3A). Thus, binding of PhoP probably blocks the *glnR* expression whatever promoter is active.

Although computer searches of the promoter region of the second nitrogen regulatory gene *glnRII* did not reveal any conserved PHO DRu, we examined it for PhoP binding. Even at 1 μM of protein, the EMSA results did not show any binding under conditions identical to those used for *glnR* (Figure 1; see 'Discussion' section).

PhoP-binding sites overlap the GlnR boxes in both glutamine synthetase genes *glnA* and *glnII*

The 5′ sequences of the two glutamine synthetase genes of *S. coelicolor* were amplified by PCR. The cloned *glnA* fragment comprised the intergenic sequence (−220 to +16) and 36 bp of the upstream opposite CDS. The *glnII* fragment contained the full promoter region of

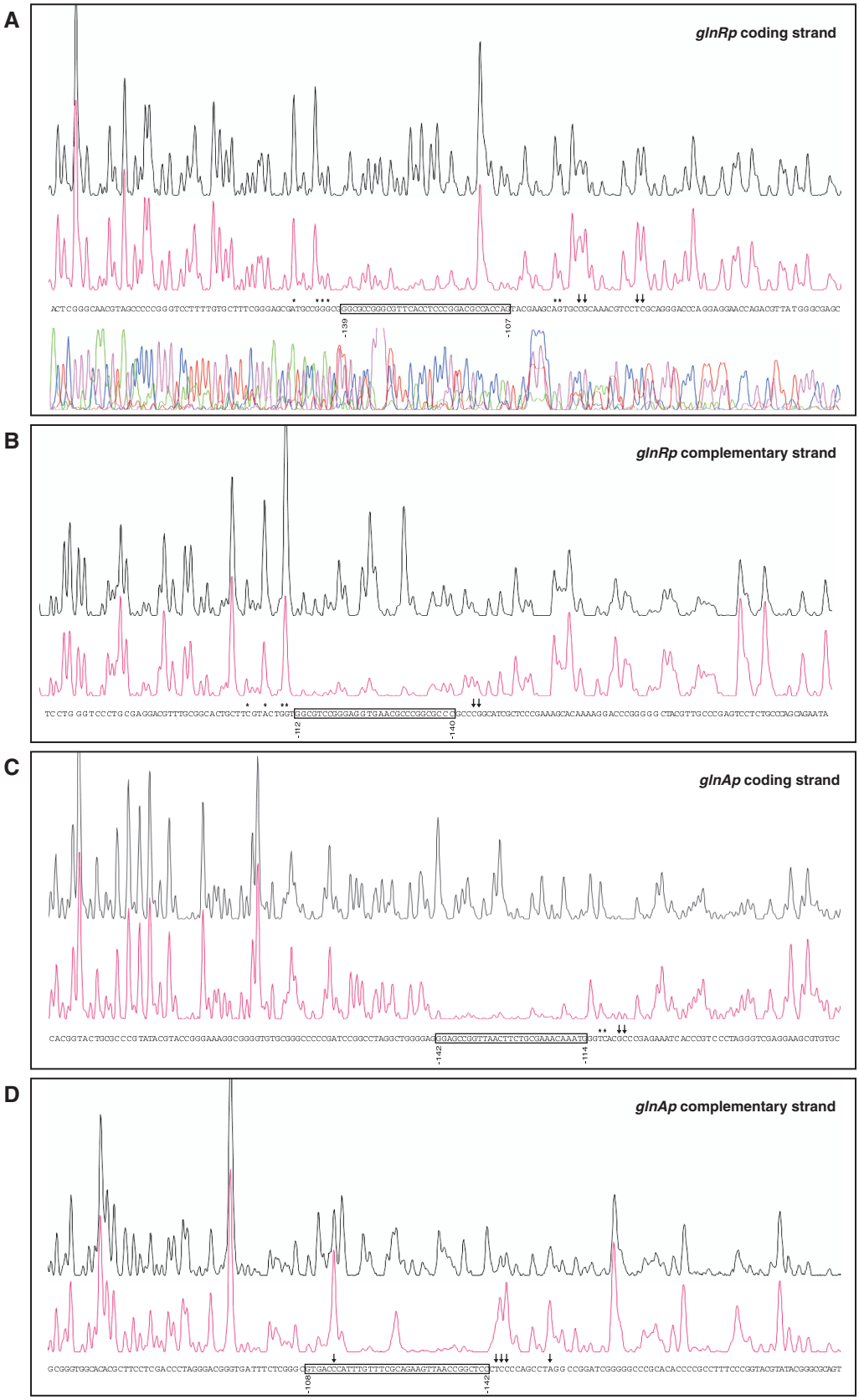


Figure 2. DNase I footprints of the GST-PhoP^{DBD} protein bound to the promoter regions of *glnR* (**A, B**), *glnA* (**C, D**), *glnII* (**E, F**) and *amtB* (**G, H**). In each panel, the upper electrophoregram (black line) is the control reaction. The protected nucleotide sequence is boxed; partially protected nucleotides (*), and hypersensitive sites (arrows) are also indicated. Sequencing reactions are not included except in panel A. Coordinates are from the translation start codon.

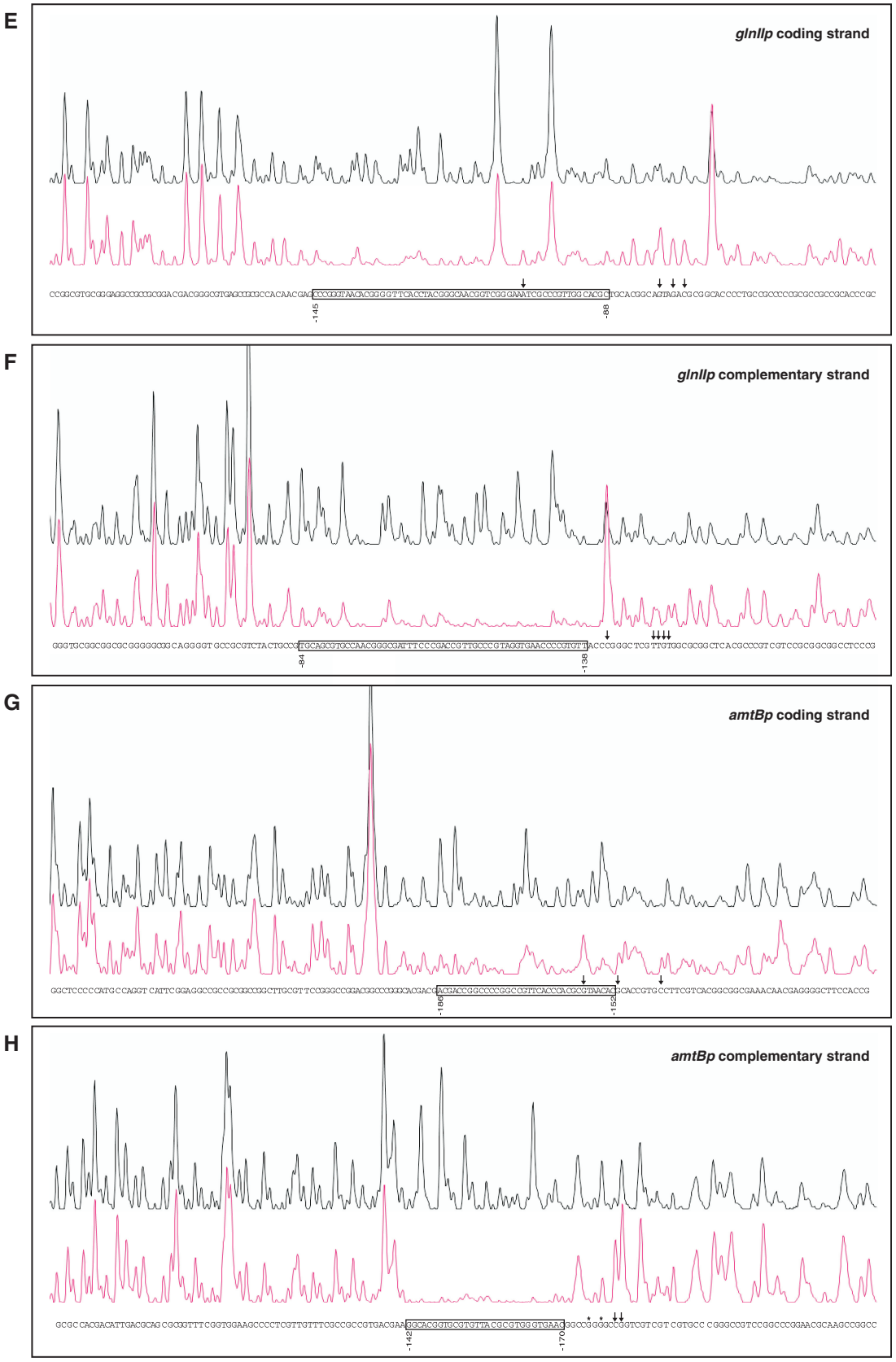


Figure 2. Continued.

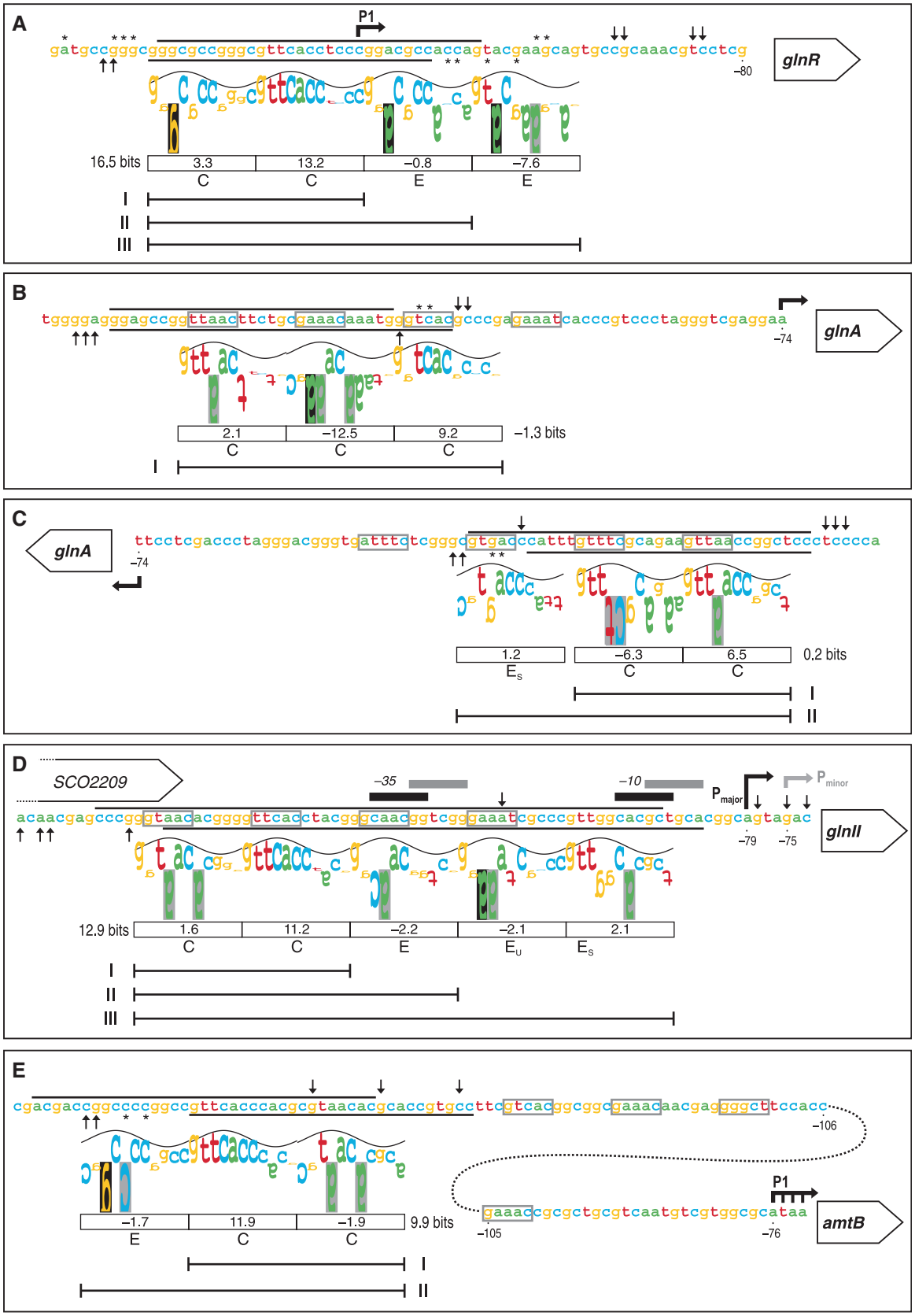


Figure 3. 'Information content' analysis of the new PhoP-binding sites identified in this work using the sequence walker method (29). The footprinting results of the promoter regions of *glnR* (**A**), *glnA* (**B**, **C**), *glnII* (**D**) and *amtB* (**E**) are summarized on the nucleotide sequences. The protected regions of the upper and bottom strands are indicated by the respective lines. Symbols for partially protected (asterisk) and hypersensitive sites

136 bp plus the last 105 bp of the upstream CDS. Both fragments were able to bind PhoP since they gave shifted bands in EMSA.

The *glnAp* gave a clear shifted band and a weak one of lower electrophoretic mobility (I and II, respectively; Figure 1). The footprinting assay of the *glnA* coding strand revealed a protected region extending from −142 to −114 and partial protection of nucleotides −111 and −110 (Figure 2C). The same region was protected in the complementary strand, from −142 to −108, although a hypersensitive site was evident at nucleotide −113 (Figure 2D). This protected region comprises the first and half of the second GlnR box that have been described in the *glnA* promoter (22). The GlnR boxes have been defined as composed of two conserved sequences of 5 nt separated by 6 variable nt (22) (see also Figure 3B–E). GlnR function as a transcriptional activator of *glnA* (19,22). Binding of PhoP at the *glnA* promoter region is likely to interfere with GlnR binding and possibly with RNA polymerase function since the *glnA* transcription start site is located at position −74 (20) (Figure 3B).

The gel mobility shift assays of the *glnII* promoter revealed three clear DNA–protein complexes. Complexes II and III showed stronger retardation (lower mobility) than complex I, and also showed a higher signal intensity (Figure 1).

As shown in Figure 2E and F, the GST-PhoP^{DBD} protein protected a large nt stretch of the *glnII* promoter from DNase I digestion, extending from −145 to −88 in the coding strand, and from −138 to −84 in the complementary strand. In contrast to the *glnA* sequence, the coding strand of *glnII* exhibited a well-conserved DRu (Figure 3D). Also, the large protected nt stretch completely covers the two GlnR boxes described by Tiffert *et al*. (22). Two closely placed promoters have been described in the *glnII* gene. The main promoter should correspond to the major vegetative σ factor and its transcription start site is the adenine −79. The minor promoter shows putative elements of σ^{31} (14). This sigma factor, which is more active at stationary phase, recognizes promoters of *actIII* (an actinorhodin structural gene) and of *hrdD* (a homologue of *Escherichia coli rpoD*), and also the *glnR* P2 promoter (31). As shown in Figure 3D, the overlapping location of promoters and binding sites indicate that PhoP may repress *glnII* transcription interfering with both GlnR binding and promoter activity.

The promoter for the *amtB-glnK-glnD* operon contains separated PhoP and GlnR operators

The *amtB* gene encodes a putative ammonium transporter and forms an operon with *glnK* and *glnD*, which encode the P_{II} protein and the P_{II} nucleotidyl transferase respectively (18,19). The latter two proteins may be considered nitrogen metabolism sensors. Using RT–PCR experiments and a *glnR* mutant, Tiffert *et al*. (22) showed that GlnR activates the transcription of the *amtB* gene.

EMSA analyses of the DNA fragment containing the *amtB* 5′-region (−285 to +4) showed a clear shifted band and a second weak one with lower mobility (Figure 1). The DNase I protection of this fragment extended from −186 to −152 in the coding strand what contained a conserved DRu (Figure 2G). Results for the complementary strand showed a protected stretch from −142 to −170, and partial protection of the nucleotides −175 and −177 (Figure 2H).

In contrast to what we have found for the GS genes, the PhoP-binding site at the *amtB* promoter is placed upstream and separated from the GlnR boxes (Figure 3E). Three separated promoters (P3 to P1) have been identified in the *amtB* upstream region by low resolution S1 analysis (19). In principle, the location of the P1 transcription start site (−76 to −73, Figure 3E) suggests that PhoP does not control this promoter but the upstream ones P3 and P2. In this case PhoP might act as a 'road-block' preventing the expression from the upstream promoters.

Analysis of the complex operator structures

The PhoP-binding sites are formed by direct repeats units (DRu) of 11 nt, each one bound by a protein monomer. We have recently described the structures of DRus based on the 'information theory' analysis and on combined EMSA and footprinting results (11). The differentiated structures served to classify the PHO operators into three groups. Operators that produce a unique retarded band in EMSA are either class I, if formed by 2 DRus (i.e. one PHO box), or class II, if composed of three DRus. More frequently (as occurs in all cases reported here) PhoP binding produces two or more retarded bands in EMSA. Those sites belong to class III and comprise two or three conserved DRus that form the core of the site, and one or several DRus that extend the protein occupancy beyond the core.

Figure 3 includes the summary of the footprinting results. The protected regions were analysed by the sequence walker method (29) using the Model 1 matrix (11). In each walker, the letter height represents the conservation of the base and its contribution to the information content of the site (R_i value). Base conservation correlates with the number of contacts to the protein (32), and, when displayed as walkers, serves to identify the binding sequence.

(upward arrow, downward arrow) are as in Figure 2. For the PhoP-binding site in *glnA*, the two possible walkers are shown in the coding (B) and complementary strands (C). The walker limits are 2 bits, which is also the top of the sine wave, and −3 bits at the bottom. The height of each letter is the individual information value, determined from the Model I weight matrix (11). Negative values are represented by upside down letters. When a base does not occur at a given position in the set of sequences which forms the model, the letter background is black; a grey background indicates that the letter extends beyond the lower limit (−3 bits). The sine wave has a periodicity of 10.6 bases (the helical twist of B-form DNA) and serves to indicate major/minor groove contacts (28). The following features are bellow the walkers: boxes containing the R_i values for the above 11 nt direct repeat unit (DRu), the total R_i for the core site, letter codes for the DRu structure [C for core, E for extension, E_U for extension unstable, E_S for extension support (11)], and line segments comprising the DRus bound by PhoP monomers in the retarded complexes observed in Figure 1. The conserved 5-mer sequences of the GlnR-binding sites are boxed (grey lines). Bent arrows are the transcription start points. The −35 and −10 elements of major and minor *glnII* promoters are also shown.

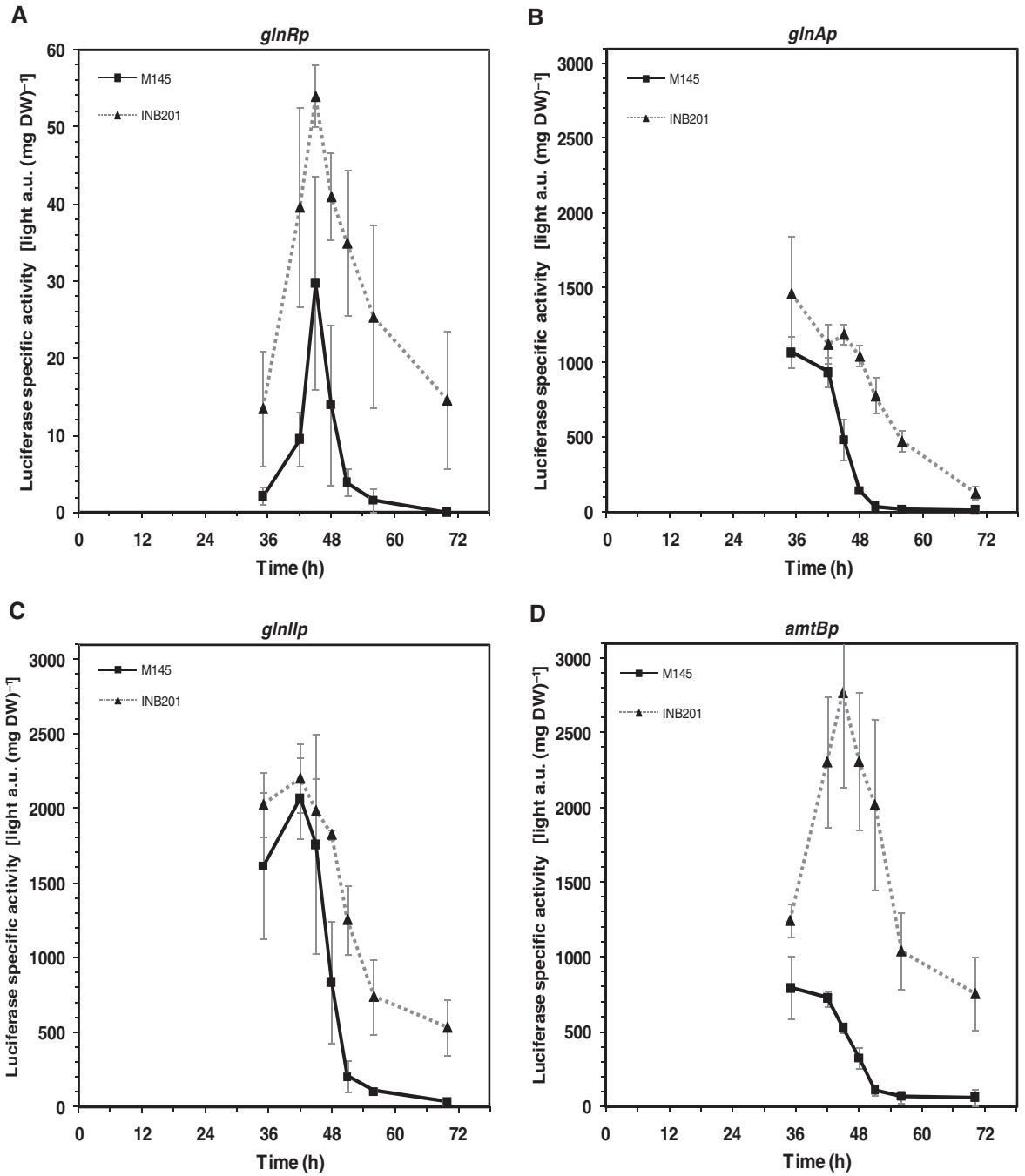


Figure 4. Promoter activity in *S. coelicolor* M145 (squares, solid lines) and Δ*phoP* mutant (triangles, dashed lines) of *glnR* (**A**), *glnA* (**B**), *glnII* (**C**) and *amtB* (**D**) using the *luxAB* genes as reporter. Error bars correspond to the standard error of triplicated cultures in MG-3.2.

The protected region in the *glnR* promoter comprised three DRus, and a partially protected fourth one (Figure 3A). The proposed DRu structure explains the formation of the three observed complexes in EMSA (Figure 1). A similar operator structure was observed in the phytase gene (SCO7697) (11).

The PhoP-binding site in the *glnA* promoter showed a less evident interpretation. In the coding strand a class II structure was suggested. This implies that the *glnAp* fragment should yield a unique retarded complex. Nevertheless, a second faint band appeared above the major retarded band in EMSA (Figure 1). Moreover, the highest conserved DRu is poorly protected, and the total R_i for this structure is negative (−1.3 bits, Figure 3B). When an accurate binding model is used, it has been demonstrated that the R_i value must be positive (29). In the complementary strand a more plausible structure was found because the core sequences show a low but positive R_i (0.2 bits), they were completely protected, and the two retarded bands can be explained

(Figure 3C). The large protected sequence in the *glnII* promoter included five DRus (Figure 3D) that account for the formation of the three EMSA complexes.

The PhoP protected region in the *amtB* promoter covered a well conserved DRu that is flanked by two poorly conserved DRus. The two observed EMSA complexes indicate a structure composed of a 2 DRu core plus an extension repeat. The degree of protection and the walkers served us to decide which of the flanking DRus forms part of the core (Figure 3E).

Reporter studies: PhoP represses the transcription of nitrogen genes

In order to quantify the effect of PhoP on expression of the nitrogen-regulated genes, *glnR, glnA, glnII* and *amtB* promoter regions were cloned in pLUXAR+*neo* driving expression of the *luxAB* reporter gene. The resulting integrative plasmids pAR-N2 (*glnRp*), pAR-N4 (*glnAp*), pAR-N6 (*glnIIp*), pAR-N8 (*amtBp*) were introduced by conjugation into the wild-type M145 strain and into the INB201 (Δ*phoP*) mutant. Plasmids are stably maintained by integration into the *Streptomyces* chromosome ΦC31 *attB* site.

Cultures were grown in MG-3.2 medium (containing 3.2 mM phosphate), using three independent replicates. The cultures showed a diauxic growth with transition phase from 45 to 51 h that triggered the onset of antibiotic production; the Δ*phoP* mutant showed reduced growth as described in Santos-Beneit *et al*. (25).

Using these reporter constructions it was clearly observed that the four promoter regions were repressed by PhoP since reporter enzyme higher activities were observed in the Δ*phoP* mutant with respect to parental strain at all sampling times (Figure 4A–D). The reporter expression patterns, varied among the promoters assayed. Thus, compared to the other assayed promoters (of phosphate transporter genes *pstS*, *pitH1* and *pitH2*, and of glycerophosphodiesterase genes *glpQ1* and *glpQ2*) [(23,25), Santos-Beneit, unpublished] the promoter region of the *glnR* gene produced usual or low activities across the time course of the culture. Indeed, in the wild-type strain the luminescence signals were below the instrument detection limit at 70 h (Figure 4A). In contrast, the structural *glnA*, *glnII* and *amtB* genes appeared to have very strong promoters. Among these, *glnIIp* showed the highest activities in the wild-type strain. Besides, maximum activities of the different promoters were reached at distinct growth phases. In both strains *glnRp* activities increased until the transition phase (45 h, Figure 4A), and decreased thereafter. For the *glnA* and *amtB* promoter activities, the highest values were at the exponential growth phase in the wild-type (35 h, Figure 4B and D). The *glnIIp* showed high activities during the first growth phase and a maximum activity at 42 h (at the initial transition phase, Figure 4C). In all cases, the wild-type activities dropped rapidly after the transition phase resulting in low values in the stationary phase. In contrast, deletion of the *phoP* gene caused slower promoter activity drops, as seen in the INB201 plots (Figure 4A–D).

In summary, we can conclude that PhoP represses directly the transcription of GS genes *glnA*, *glnII*, the putative ammonium transporter gene *amtB*, and the nitrogen regulatory genes *glnD* and *glnK*, which form part of the *amtB* operon. In addition, PhoP negatively controls these genes via repression of its activator gene *glnR*.

DISCUSSION

For many years it was known that optimal media for growth of *Streptomyces* species and secondary metabolite biosynthesis need to be equilibrated in their C/N/P/S ratios (1,33), but the molecular mechanism of the interactions between these major nutrients still remain obscure, although they are receiving increasing attention in some microorganisms (34–36).

Carbon and phosphate sources interact through several metabolic pathways, mainly in the glycolysis and different molecules serve as sensors of the C/P ratio in the cells. In enterobacteria carbon catabolite regulation is mediated by activation of the adenylate cyclase (forming cAMP) through interaction with the phosphorylated form of the IIA protein (IIA^{Glu}) of the glucose translocation systems. In *Bacillus subtilis*, the interaction between C and P sources is mediated by fructose 1,6-bisphosphate (FBP) that interacts with the global regulator CcpA [reviewed by Sonenshein (35)]. Interestingly, CcpA causes repression of the *phoPR* promoter by binding to a novel transcription start site (37,38). This is a good example of interaction between two regulatory networks. In *S. coelicolor* the level of extracellular phosphate-binding protein PstS responds to both phosphate limitation (8,23) and some carbon sources like fructose or glucose (39).

There are no similar detailed reports of the interaction between the nitrogen and phosphate regulatory circuits. In the model actinomycete *S. coelicolor* the mechanism of phosphate control, mediated by the two-component PhoR-PhoP system, has been widely studied (4,6–8,11). Phosphate control of many *pho* regulon genes is mediated by binding of PhoP to PHO operators formed by direct repeats of 11-nucleotides (11). In initial transcriptomic studies we observed that several genes involved in nitrogen metabolism are regulated by phosphate (23).

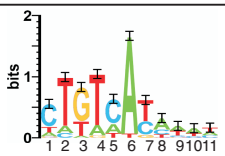
The nitrogen-source regulon in *S. coelicolor* is controlled by two related regulatory proteins—GlnR and GlnRII (20,22).

GlnR is a master regulator in *S. coelicolor* that controls several genes involved in nitrogen metabolism in response to ammonium limitation. This response includes activation of *glnA*, *glnII* and *amtB* genes, and repression of the putative NADP-specific glutamate dehydrogenase gene *gdhA* (22). The GlnR regulon appears to be conserved in *Mycobacterium* and other actinomycetes (22,40).

As shown in this article, PhoP binds to operators located in the upstream regions of *glnR*, *glnA*, *glnII* and *amtB*. The complex structures of these operators can provide a tuning mechanism for the PhoP regulation. Expression from these promoter regions was drastically increased in the Δ*phoP* mutant, demonstrating that

A

Gene	DRu	*Ri*, bits	
pstS	**CTGTCATAAAA**	12.9	**42.5**
	CTGTCATATTC	11.7	
	CTTACATATAA	9.3	
	CTGTCACCTGT	8.7	
ugpB	**CCGTCACCGCC**	3.3	**39.3**
	TTGTCATCTTT	9.1	
	CTGACACCTTA	7.9	
	CTATCTTACAA	2.4	
	ATGTAACAAAA	8.3	
	AAGTTATTTTT	2.9	
	CTGTAATTCGA	5.3	
phoB	**TTTTCATAAAT**	9.8	**23.0**
	CTGTCATAAAT	13.2	
phoH	**CTGTCATCACT**	10.4	**21.5**
	CTGTCATCTTT	11.2	
phoE	**CTGTAATATAT**	11.8	**20.1**
	CTTTAACAATC	8.3	
psiE	**GTTGAACAAAA**	1.1	**13.0**
	CATACACAAAA	5.9	
	AATATAGATCT	−2.5	
	CCGTCACATTT	8.4	
phoA	**CTGTCATAAAG**	9.9	**11.9**
	TTGTCACGGCC	2.0	
phnC	**CTGTTAGTCAC**	3.7	**9.5**
	TTTTAATTAAC	5.8	



B

Gene	Putative PHO box	*Ri*
glnH	ctggcacgatttttttcatatat (−71 … −50)	13.4 bits
glnL	ttgtcagctatctgtagcccat (−128 … −107)	10.5 bits

Figure 5. Putative PHO boxes in the *E. coli* genes *glnH* and *glnL*. (**A**) Alignment of the 25 direct repeat units (DRu) that form the known binding sites of PhoB (the orthologue regulator of *Streptomyces* PhoP), and the logo (28) that depicts the 'information theory' model. Information contents (R_i) of each DRu and site are shown. (**B**) Putative PHO boxes (composed of 2 DRu) found in the promoter regions of *glnHPQ* operon (glutamine permease) and *glnLG* (*ntrBC*) operon. Walkers and R_i values are determined by the model depicted above. Positions are referred to the translation start codon.

they are repressed by PhoP. The derepression was clearly observed using the *luxAB* reporter system throughout the time course of the culture and also in the transcriptomic analysis of the phosphate limitation response (23). These results revealed that there is a control of nitrogen metabolism by phosphate availability. This control is exerted indirectly by the binding of PhoP to the *glnR* promoter that in turn controls expression of the other nitrogen metabolism genes. However, PhoP also binds to the promoter regions of *glnA*, *glnII* and the *amtB-glnD-glnK* operon indicating that, in addition to the *glnR*-mediated regulation, PhoP also directly controls expression of the other five nitrogen metabolism genes. This double mechanism ensures a good degree of control of *glnA*, *glnII* and *amtB-glnD-glnK* by PhoP.

In *S. coelicolor* there is a second nitrogen regulator GlnRII that interacts with the *glnA*, *glnII* and *amtB* promoters (12,19). It is interesting that PhoP did not bind to the *glnRII* promoter and no PHO sequences were found in its promoter region. This result suggests that there is a nitrogen regulatory system that is independent of PhoP.

The PhoP negative control on nitrogen assimilation genes can be explained as a way to save the cell resources and to channel them to obtain phosphate from the medium when this nutrient is limiting. Indeed, the negative effect on growth of a high glutamine synthetase activity has been shown (41), and, as reported here, the *S. coelicolor glnA* and *glnII* promoters showed high activities (Figure 4). A response to phosphate limitation, mediated by an increased PhoP level, is to reduce expression of genes involved in N utilization.

An important question is how PhoP and GlnR interact with their respective operators in the nitrogen regulated promoters when the binding sequences are overlapping (e.g. *glnA* promoter) or non-overlapping (e.g. *amtB* promoter). We are further investigating if both operators in *glnA* are coincident or whether each of those two regulatory proteins recognize specific sequences in overlapping regions. Initial evidence suggests that in this case PhoP acts by preventing binding of GlnR to its specific operator in *glnA* (A. Sola-Landa *et al.*, unpublished work). In the case of the *amtB* promoter PhoP appears to act as a 'road-block' for the transcription originating from upstream transcription start sites. This may explain the results observed in the Δ*phoP* mutant.

Streptomyces coelicolor is a habitant of the soil, an oligotrophic medium that is frequently limited in phosphate (3). An open question is if the phosphate government over nitrogen control is conserved in other microorganisms. A proteomic study on the global analysis of protein synthesis showed that the phosphate limitation decreases the expression of *glnA* in *E. coli* (42). Microarray analysis provided evidence that *glnII* and *glnK* are repressed after phosphate starvation in *Shinorhizobium meliloti* (43). These results are in agreement with the conservation of a phosphate control over N metabolism regulation, similar to that of *S. coelicolor*. We have also explored the existence of PHO boxes in nitrogen metabolism genes in *E. coli*. For this purpose, an information model was built based on the alignment of 25 PHO DRu compiled by Blanco *et al.* (44). Although no clear PHO boxes were detected in the *glnA* promoter region, there were candidate sequences in the promoters of the *glnLG* (*ntrBC*) two-component system that

regulates *glnA* (2) and of the glutamine permease operon (*glnHPQ*) (45) (Figure 5).

In conclusion, the nitrogen source and phosphate regulatory networks interact in different bacteria to provide an adaptation to changes in the available nitrogen and phosphate nutrients. *S. coelicolor* provides the first description of the control of a nitrogen regulon (GlnR) by the phosphate regulatory protein PhoP, what allows a fine coordination of the utilization of those two nutrient sources.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Phosphate and carbon source regulation of two PhoP-dependent glycerophosphodiester phosphodiesterase genes of *Streptomyces coelicolor*

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Glycerophosphodiesters are formed by deacylation of phospholipids. *Streptomyces coelicolor* and other soil-dwelling actinomycetes utilize glycerophosphodiesters as phosphate and carbon sources by the action of glycerophosphodiester phosphodiesterases (GDPDs). Seven genes encoding putative GDPDs occur in the *S. coelicolor* genome. Two of these genes, *glpQ1* and *glpQ2*, encoding extracellular GDPDs, showed a PhoP-dependent upregulated profile in response to phosphate shiftdown. Expression studies using the *luxAB* genes as reporter confirmed the PhoP dependence of both *glpQ1* and *glpQ2*. Footprinting analyses with pure GST-PhoP of the *glpQ1* promoter revealed four protected direct repeat units (DRu). PhoP binding affinity to the *glpQ2* promoter was lower and revealed a protected region containing five DRu. As expected for *pho* regulon genes, inorganic phosphate, and also glycerol 3-phosphate, inhibited the expression from both *glpQ1* and *glpQ2*. The expression of *glpQ1* was also repressed by serine and inositol but expression of *glpQ2* was not. In contrast, glucose, fructose and glycerol increased expression of *glpQ2* but not that of *glpQ1*. In summary, our results suggest an interaction of phosphate control mediated by PhoP and carbon source regulation of the *glpQ1* and *glpQ2* genes involving complex operator structures.

INTRODUCTION

Glycerophosphodiester metabolism has been described in organisms spanning the biological spectrum (from bacteria to humans), supporting the fundamental nature of this metabolism (Patton-Vogt, 2007). Glycerophosphodiesters are formed by deacylation of phospholipids and differ according to their alcohol moiety; examples are glycerophosphoethanolamine, glycerophosphocholine, glycerophosphoinositol, glycerophosphoserine and glycerophosphoglycerol. These compounds are hydrolysed by glycerophosphodiester phosphodiesterases (GDPDs; EC 3.1.4.46), releasing *sn*-glycerol 3-phosphate (G3P) and the corresponding alcohol (Larson *et al.*, 1983).

The GDPD enzyme was first identified in *Escherichia coli* (Larson *et al.*, 1983). Two genes coding for GDPDs (*glpQ* and *ugpQ*) have been characterized in this organism. The *glpQ* gene encodes a periplasmic enzyme of 333 amino acids while *ugpQ* encodes a cytosolic enzyme of 247 amino acids (Tommassen *et al.*, 1991). The *glpQ* gene forms part of the *glpTQ* operon, which belongs to the glycerol regulon and is induced by G3P (Larson *et al.*, 1983, 1987). The *ugpQ* gene forms part of the *ugpBAECQ* operon (Brzoska & Boos, 1988; Overduin *et al.*, 1988). This operon includes genes coding for an ABC transporter system and its expression is induced by the *pho* regulon (Brzoska & Boos, 1988).

In *Bacillus subtilis* there is only one gene coding for a GDPD (namely *glpQ*). This gene encodes a secreted GDPD and forms an operon with the *glpT* gene, which codes for a G3P permease. The *B. subtilis glpTQ* operon belongs to the glycerol regulon and is homologous to the *E. coli glpTQ* operon (Nilsson *et al.*, 1994). Under phosphate starvation *B. subtilis glpQ* is transcribed monocistronically from a PhoP-dependent promoter located upstream of the gene (Antelmann *et al.*, 2000).

In the *Streptomyces coelicolor* genome seven genes encoding putative GDPDs have been identified (Bentley *et al.*, 2002).

Abbreviations: CCR, carbon catabolite regulation; DRu, direct repeat unit(s); EMSA, electrophoretic mobility shift assay; G3P, *sn*-glycerol 3-phosphate; GDPD, glycerophosphodiester phosphodiesterase; GST, glutathione *S*-transferase; TSP, transcription start point.

Three of those genes (SCO1565, SCO1968 and SCO7550) have been predicted to encode secreted GDPDs and the other four (SCO1090, SCO1419, SCO3976 and SCO5661) cytoplasmic GDPDs. Moreover, the SCO1565 protein is exported out of the cell by the twin-arginine translocation (Tat) pathway, which is the major route of protein export in *S. coelicolor* (Widdick *et al.*, 2006). As occurs commonly with some other GDPD-encoding genes (Brzoska & Boos, 1988; Antelmann *et al.*, 2000; McLoughlin *et al.*, 2004; Schaaf & Bott, 2007), two of them (SCO1565 and SCO1968) are members of the *pho* regulon (Rodríguez-García *et al.*, 2007). The wealth of GDPD-encoding genes in *S. coelicolor* is intriguing and their roles in the metabolism of this bacterium need to be studied.

In this work we show that the regulation of two PhoP-dependent GPDP-encoding genes in *S. coelicolor* depends not only on the phosphate concentration in the medium but also on the concentration of carbon sources such as sugars, alcohols and amino acids. Additional information about the PhoP operator structure in *S. coelicolor* is provided, showing that the PhoP-dependent genes *glpQ1* and *glpQ2* contain class III complex operators that bind several PhoP molecules.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *Streptomyces coelicolor* strains M145 (Kieser *et al.*, 2000) and INB201 (Δ*phoP*; Santos-Beneit *et al.*, 2009) were manipulated according to standard procedures (Kieser *et al.*, 2000). *E. coli* DH5α was the general cloning host.

The *glpQ1* and *glpQ2* promoter regions were amplified by PCR using total DNA as template, as follows. The primers FSC19 (5′-TGTTGGATCCACACGGCCCGGATCGAAG-3′) and FSC20 (5′-TGCGGTACCATATGGCCCTCCTGCCGTAGAC-3′) amplified a 263 bp fragment encompassing the promoter region of *glpQ1* from the −247 to the −2 position (positions from the ATG translation start triplet). *Bam*HI (FSC19) and *Kpn*I/*Nde*I (FSC20) cloning sites (underlined) were introduced into the primer sequences. Primers FSC17 (5′-CCGGGATCCTCCCGGATTCTCCTGATG-3′) and FSC18 (5′-CGCAGGTACCATATGTACTCCTCGCGTCGAAC-3′) were used to amplify a 274 bp fragment encompassing the promoter region of *glpQ2* from the −257 to the −1 position (positions from the translation start) flanked by the above-mentioned cloning sites. *glpQ1* and *glpQ2* *Bam*HI–*Kpn*I fragments were cloned into pBluescript II SK+, yielding pFS-*glpQ1* and pFS-*glpQ2*, respectively. The inserts of the plasmids were checked by sequencing. *Bam*HI–*Nde*I fragments from pFS-*glpQ1* and pFS-*glpQ2* were cloned into the promoter-probe vector pLUXAR-neo, yielding pLUX-*glpQ1* and pLUX-*glpQ2*, respectively.

Culture conditions. *S. coelicolor* cultures were performed in 100 ml defined MG-3.2 medium, containing 3.2 mM phosphate (Santos-Beneit *et al.*, 2008). The cultures were inoculated with 10^6 spores ml^{-1} and grown in 500 ml baffled flasks (three replicates) at 30 °C and 300 r.p.m. For the time series cultures, samples were taken at 36 h, 40 h, 44 h, 48 h and 60 h of incubation.

In order to test the effect of adding different nutrients, the cultures were supplemented at 36 h with 15 mM of phosphate ($KH_2PO_4 + K_2HPO_4$; Scharlau), phosphate plus glycerol (Merck), *rac*-glycerol 3-phosphate disodium salt hexahydrate (G2138, Sigma), L-serine (Sigma) or *myo*-inositol (Fluka); and with 2% (w/v) of D-glucose (Merck), D-fructose (Sigma), maltose (Sigma) or glycerol (Merck). Samples were taken at 44 h and 48 h of incubation. In all cases three replicates were used.

Luciferase assay and growth determinations. The reporter luciferase activity was measured in a Luminoskan luminometer (Labsystems, Helsinki) as described by Rodríguez-García *et al.* (2007) and Santos-Beneit *et al.* (2008). For dry weight determination, culture samples of 2 ml were washed twice with MilliQ water and dried for 4 days at 80 °C.

DNase I footprinting assays. Non-radioactive DNase I footprinting assays of *glpQ1* and *glpQ2* promoters were performed using the pure GST-PhoP^{DBD} protein (Sola-Landa *et al.*, 2005) as described previously (Santos-Beneit *et al.*, 2009). Final protein concentrations were 1 μM and 2 μM for the *glpQ1* and *glpQ2* promoters, respectively. In the latter case, polyethylene glycol 1000 (PEG) was added at various concentrations to increase the effective protein concentration. DNA probes for both promoters were obtained by PCR using pFS-*glpQ1* and pFS-*glpQ2* as template. Electropherograms were analysed with PeakScanner v1.0 software (Applied Biosystems) to calculate the area under each fluorescence peak as well as the peak size. An Excel spreadsheet served to normalize peak areas and to calculate the degree of DNase I protection. Normalization factors for

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Characteristics	Origin
Strains		
S. coelicolor A3(2) M145	Wild-type	Kieser *et al.* (2000)
S. coelicolor A3(2) INB201	Δ*phoP*, Am^r	Santos-Beneit *et al.* (2009)
E. coli DH5α	F′ ϕ80 d*lacZ* ΔM15	Laboratory stock
E. coli ET12567(pUZ8002)	*dam dcm* mutant, Neo^r Cm^r	MacNeil *et al.* (1992)
Plasmids		
pBluescript II SK+	Cloning vector, Amp^r	Promega
pFS-*glpQ1*	PCR product carrying *glpQ1* promoter cloned into pBSIISK+, Amp^r	This work
pFS-*glpQ2*	PCR product carrying *glpQ2* promoter cloned into pBSIISK+, Amp^r	This work
pLUXAR-neo	Cloning vector, Am^r Neo^r	Santos-Beneit *et al.* (2009)
pLUX-*glpQ1*	*Bam*HI–*NdeI* pFS-*glpQ1* fragment cloned into pLUXAR-neo, Am^r Neo^r	This work
pLUX-*glpQ2*	*Bam*HI–*NdeI* pFS-*glpQ2* fragment cloned into pLUXAR-neo, Am^r Neo^r	This work

each strand were obtained taking into account the regions shown in the respective figure, excluding the protected stretches, i.e. 50 nucleotides at both upstream and downstream sides.

Primer extension analysis. RNA samples were taken at 44 h from *S. coelicolor* M145 (containing pLUX-*glpQ1* or pLUX-*glpQ2*) cultures in MG-3.2 medium or in MG-3.2 supplemented with glucose or maltose. RNA was extracted with the RNeasy Midi kit (Qiagen). RNA concentration and quality were checked using a NanoDrop ND-1000 (Thermo Fisher Scientific) and a 2100 Bioanalyser (Agilent). Transcription start sites were determined as described by Santos-Beneit *et al.* (2008) using both LUX-FAM + 47 and LUX-FAM + 135 primers and Superscript III reverse transcriptase (Invitrogen). The primer extension reactions were incubated at 50 °C for 3 h.

Bioinformatic analysis. SCO1565 and SCO1968 orthologous genes were sought using the *Streptomyces* annotation server (http://strepdb.streptomyces.org.uk). For the analysis of the PhoP-binding sites, we calculated the individual information content (the R_i value; Schneider, 1997a) of each 11 nt direct repeat unit (DRu) using model 1 of the PhoP-binding site (an R_i matrix that gathers the information of 37 DRu that are part of operator cores; Sola-Landa *et al.*, 2008). This model also served to make the sequence walkers (Schneider, 1997b).

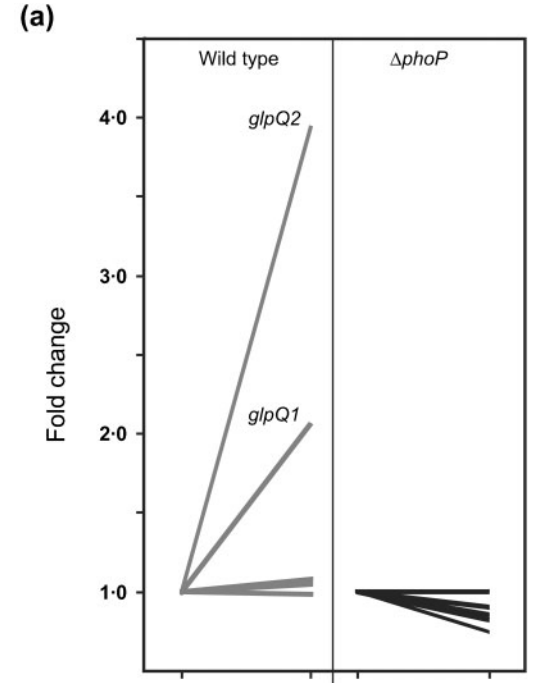
RESULTS

Analysis of the PhoP control of seven GDPD genes

There are seven genes encoding putative GDPDs in the *S. coelicolor* genome (Rodríguez-García *et al.*, 2007). All of these genes contain the Pfam motif characteristic of GDPDs. According to the presence or absence of signal peptide and transmembrane domains we propose that the genes SCO1565, SCO1968, and SCO7550 encode secreted proteins, and that the four other genes (SCO1090, SCO1419, SCO3976, and SCO5661) encode cytoplasmic proteins. Following the annotation of the *E. coli* GDPD, in this work, the three extracytoplasmic proteins were named GlpQ1, GlpQ2 and GlpQ3, respectively; and the four cytosolic ones UgpQ1, UgpQ2, UgpQ3 and UgpQ4, respectively.

Of these genes, only *glpQ1* (SCO1565) and *glpQ2* (SCO1968) showed a PhoP-dependent upregulated profile A0 (ascending transcription in the wild-type response to phosphate shift-down and null change in the Δ*phoP* mutant) in our previous transcriptomic analysis (Rodríguez-García *et al.*, 2007; Fig. 1). The binding of PhoP to these two promoter regions was demonstrated by electrophoretic mobility shift assay (EMSA) (Rodríguez-García *et al.*, 2007). Since the other five genes (SCO7550, SCO1090, SCO1419, SCO3976 and SCO5661) are not phosphate regulated and we did not find any PHO box in their promoter regions, we concluded that of the seven genes coding for GDPD in the *S. coelicolor* genome, only *glpQ1* and *glpQ2* belong to the *pho* regulon.

In order to confirm the PhoP-dependent expression of *glpQ1* and *glpQ2*, reporter studies were performed with the *S. coelicolor* wild-type and Δ*phoP* mutant strains grown in



(b)

		Wt·t_0	Wt·$t_{7·5}$	Mu·t_0	Mu·$t_{7·5}$
ugpQ1	SCO1090	-0·47 ±0·274	-0·36 ±0·312	-0·23 ±0·251	-0·65 ±0·151
ugpQ2	SCO1419	-0·83 ±0·167	-0·76 ±0·125	-0·77 ±0·225	-0·99 ±0·158
glpQ1	SCO1565	0·76 ±0·274	1·81 ±0·206	0·89 ±0·613	0·76 ±0·633
glpQ2	SCO1968	-0·46 ±0·344	1·52 ±0·399	-0·37 ±0·227	-0·64 ±0·096
ugpQ3	SCO3976	-0·22 ±0·128	-0·23 ±0·145	-0·14 ±0·135	-0·13 ±0·234
ugpQ4	SCO5661	-0·03 ±0·239	0·09 ±0·380	-0·29 ±0·280	0·03 ±0·171
glpQ3	SCO7550	0·31 ±0·195	0·34 ±0·298	0·51 ±0·514	0·33 ±0·343

Fig. 1. Transcriptomic profiles of the seven *S. coelicolor* GDPD-encoding genes in the wild-type and Δ*phoP* mutant backgrounds from the microarray studies of Rodríguez-García *et al.* (2007). The samples were taken from phosphate-replete MG cultures immediately before the shiftdown (t_0), and 7.5 h after phosphate shiftdown ($t_{7.5}$). (a) Fold changes were calculated from transcription values at t_0 (M_g values, see below). Grey lines, wild-type strain; black lines, Δ*phoP* mutant strain. Only *glpQ1* (SCO1565) and *glpQ2* (SCO1968) showed significant changes (A0 profiles). (b) M_g values (normalized $\log_2$ of Cy3/Cy5 intensities) and standard deviations of four biological replicates. The Cy3 signal corresponded to the labelled cDNA and the Cy5 signal to the labelled gDNA as the common reference.

MG-3.2 medium. This medium is limited in phosphate and thus is optimal for the expression of *pho* genes (Santos-Beneit *et al.*, 2008). As shown in Fig. 2, wild-type cultures yielded high levels of luciferase activity, while the Δ*phoP* mutant activities were almost null (less than 3 % of the wild-type), indicating that the transcription of both genes is PhoP-dependent.

Luciferase activities of *glpQ1* and *glpQ2* promoters were very low during the rapid growth phase (Fig. 2; 36–40 h) and reached maximum values during the transition growth

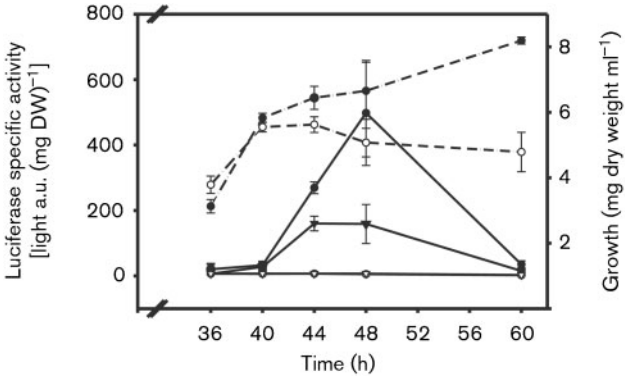


Fig. 2. Promoter activity and growth of *S. coelicolor* exconjugants containing pLUX-*glpQ1* and pLUX-*glpQ2* in the wild-type and Δ*phoP* mutant backgrounds. Cultures were grown in MG-3.2 medium at 300 r.p.m. and 30 °C. Three replicates were used. Samples were taken at 36, 40, 44, 48 and 60 h of culture. In all cases, the black symbols represent the wild-type strain and the open symbols the Δ*phoP* mutant strain. The discontinuous lines represent the growth of the wild-type (●) and Δ*phoP* mutant (○). The solid lines represent the promoter activities of the exconjugants containing pLUX-*glpQ1* (●, ○) or pLUX-*glpQ2* (▼, ▽). Error bars correspond to the SD.

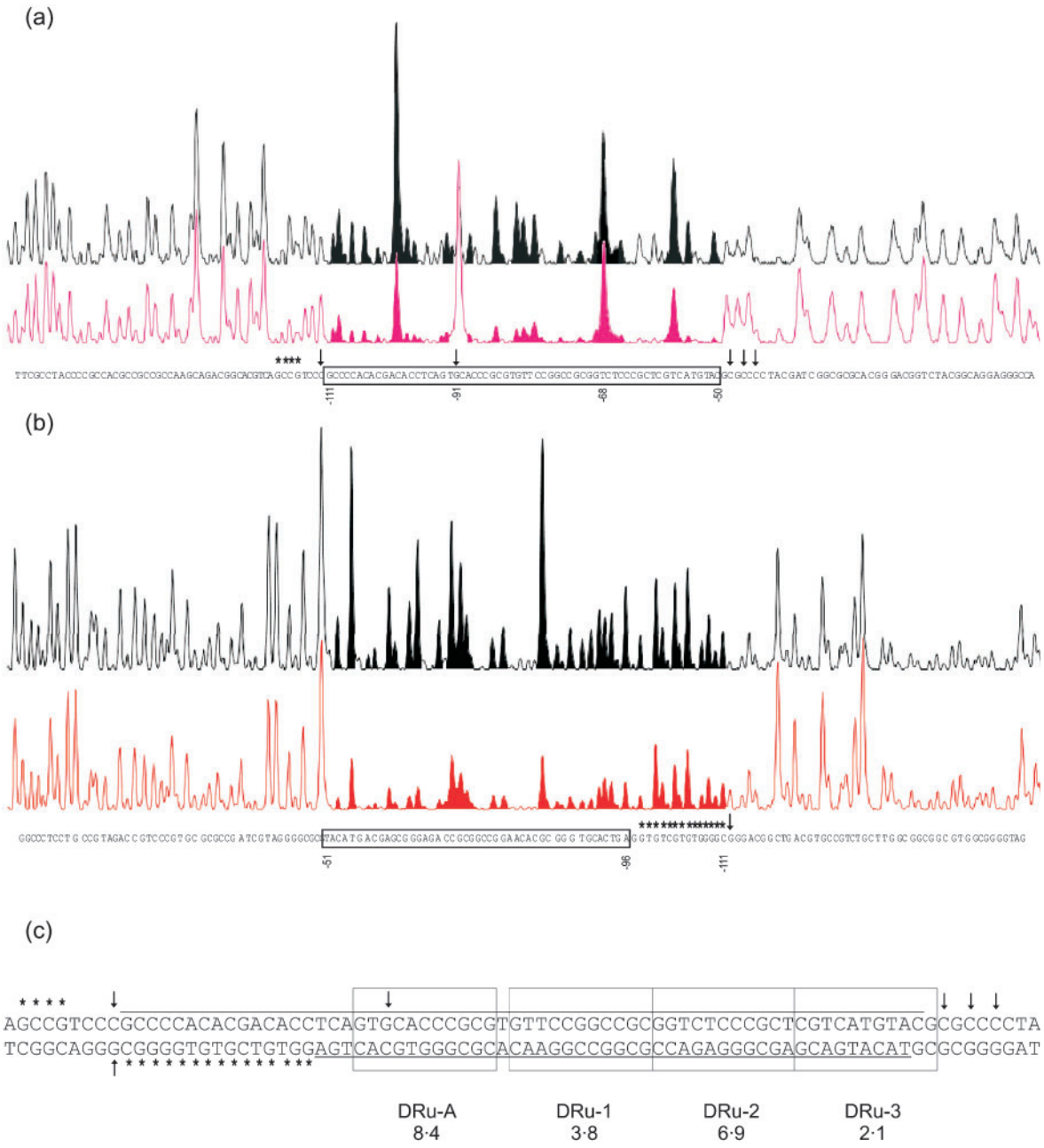
phase (44–48 h). When maximum promoter activities are compared, the *glpQ1*p activity is more than three times higher than that of *glpQ2*p (Fig. 2). The induction rates of both genes were highest at 44 h and very similar for both genes (fold ratios, referred to 40 h activities, of 8 and 6 for *glpQ1* and *glpQ2*, respectively). This pattern of expression correlates with the drop of inorganic phosphate (P_i) below 0.1 mM, which triggers the *pho* response, as is seen for the *pstS* gene (Rodríguez-García *et al.*, 2007; Santos-Beneit *et al.*, 2008).

Complex PhoP operator structures regulate *glpQ1* and *glpQ2* expression

In order to determine the nucleotide sequences where PhoP binds in the *glpQ1* and *glpQ2* promoters, footprinting studies were done using GST-PhoP^{DBD} as described in Methods. The *glpQ1* promoter was protected in a region located 50 nt upstream of the translation start codon (Fig. 3a, b). GST-PhoP^{DBD} protein at 1 μM produced 63 % ± 16 % protection (mean peak ratios and SD) against DNase I digestion in the coding strand, while in the complementary strand a stretch of 46 nt was protected to a higher extent (64 % ± 13 %) than the following sequence of 15 nt (34 % ± 11 %). This region was analysed by means of information theory tools (see Methods) to determine the structure of the direct repeat units (DRu) that are the 11 nt sequences bound by each PhoP monomer (Sola-Landa *et al.*, 2008). Four DRu were located from −93 to −49 with respect to the translation start codon and all showed positive R_i values, which indicate PhoP binding (Fig. 3c). Although upstream of DRu-A there are 18 protected nucleotides in both strands, this sequence lacked any detected DRu. There is one nucleotide separating DRu-1 and DRu-A. This gap hampers the cooperative binding of PhoP monomers to DNA. Thus, DRu-A may function as an extension support DRu (E_S; Sola-Landa *et al.*, 2008). Since we observed three retarded DNA–protein complexes in the EMSA analysis (Rodríguez-García *et al.*, 2007) and taking into account the R_i values of the four DRu, it is proposed that DRu-1 and DRu-2 form the core of the operator. The fastest-migrating complex probably involves the binding of two PhoP monomers to the core (DRu-1 and DRu-2). An additional PhoP monomer would bind DRu-3, named E for extension (Sola-Landa *et al.*, 2008), and would explain the second retarded complex. Finally, binding of DRu-A would result in the third complex observed in the EMSA analysis.

The GST-PhoP^{DBD} protein protected the *glpQ2* promoter from DNase I to a lesser extent than the *glpQ1* promoter because we needed to increase the protein concentration to 2 μM to obtain similar protection. PEG was included in the reaction mixture to favour the protein–DNA binding. In the coding strand two protected stretches of 39 nt and 14 nt were intercalated with hypersensitive sites (Fig. 3d). This bipartite pattern was also found in the complementary strand, although a partially protected sequence, instead of hypersensitive sites, separated the two main protected stretches of 27 nt each (Fig. 3e). Protection was 55 % ± 8 % in the coding strand, and 42 % ± 11 % in the complementary strand. The bound region encompasses five DRu, which are located from positions −105 to −48 with respect to the translation start codon. Four of the five DRu have positive R_i values: DRu-A (1.3 bits), DRu-1 (2.5 bits), DRu-2 (3.7 bits), and DRu-4 (3.9 bits). DRu-3, with a negative R_i (−8.0 bits), coincided with the region of hypersensitive sites in the upper strand and low protection in the bottom strand (Fig. 3f). As occurs in the *glpQ1* promoter, DRu-A is not contiguous with DRu-1. According to the model of Sola-Landa *et al.* (2008), we propose that DRu-1 and DRu-2 would form the core of the operator; binding of PhoP to the poorly conserved DRu-3 of E_U type (unstable extension) would be facilitated by cooperative interaction between PhoP monomers bound to DRu-2 and DRu-4 (E_S). Thus, DRu-3 and DRu-4 would be bound at the same time in a second stage. The two retarded complexes detected previously (Rodríguez-García *et al.*, 2007) might correspond to two PhoP monomers bound to the core (DRu-1 and DRu-2) and to four PhoP monomers bound together to DRu-1, DRu-2, DRu-3 and DRu-4. However, further binding to DRu-A would imply the existence of a third complex of lower migration, which was not observed.

Orthologous genes of *glpQ1* and *glpQ2* were found in other sequenced *Streptomyces* genomes. The operator structures described here are depicted in Fig. 4. These structures are conserved in the orthologous counterparts with the exception of the *S. griseus glpQ1* gene (SGR5973), which has a different operator structure. According to the



classification of Sola-Landa *et al.* (2008) these operators belong to class III (operators of complex structure).

Effect of inorganic phosphate, G3P, serine and inositol on *glpQ1 and glpQ2* expression

GPDP hydrolyses glycerophosphodiesters into G3P and the corresponding alcohol (Larson *et al.*, 1983). We tested the effect of adding G3P on *glpQ1* and *glpQ2* expression because G3P is the final product in all glycerophosphodiester hydrolysis reactions. Moreover G3P induces the expression of both *E. coli* and *B. subtilis glpQ* genes (Larson *et al.*, 1987; Nilsson *et al.*, 1994). The effect of adding inorganic phosphate (P_i) or P_i plus glycerol on the expression of both *glpQ* genes was also tested. Thus, 15 mM inorganic phosphate (or G3P) or 15 mM P_i plus 15 mM glycerol was added to 36 h cultures of *S. coelicolor* M145 in MG-3.2 medium. At this time cultures were in the fast growth phase and luciferase activities of both promoters were still below the detection level. Maximum activities from both promoters were detected at 44 h or 48 h in the unsupplemented cultures, before the beginning of the secondary growth phase (Fig. 2). Therefore, luciferase activities were measured 8 and 12 h after the

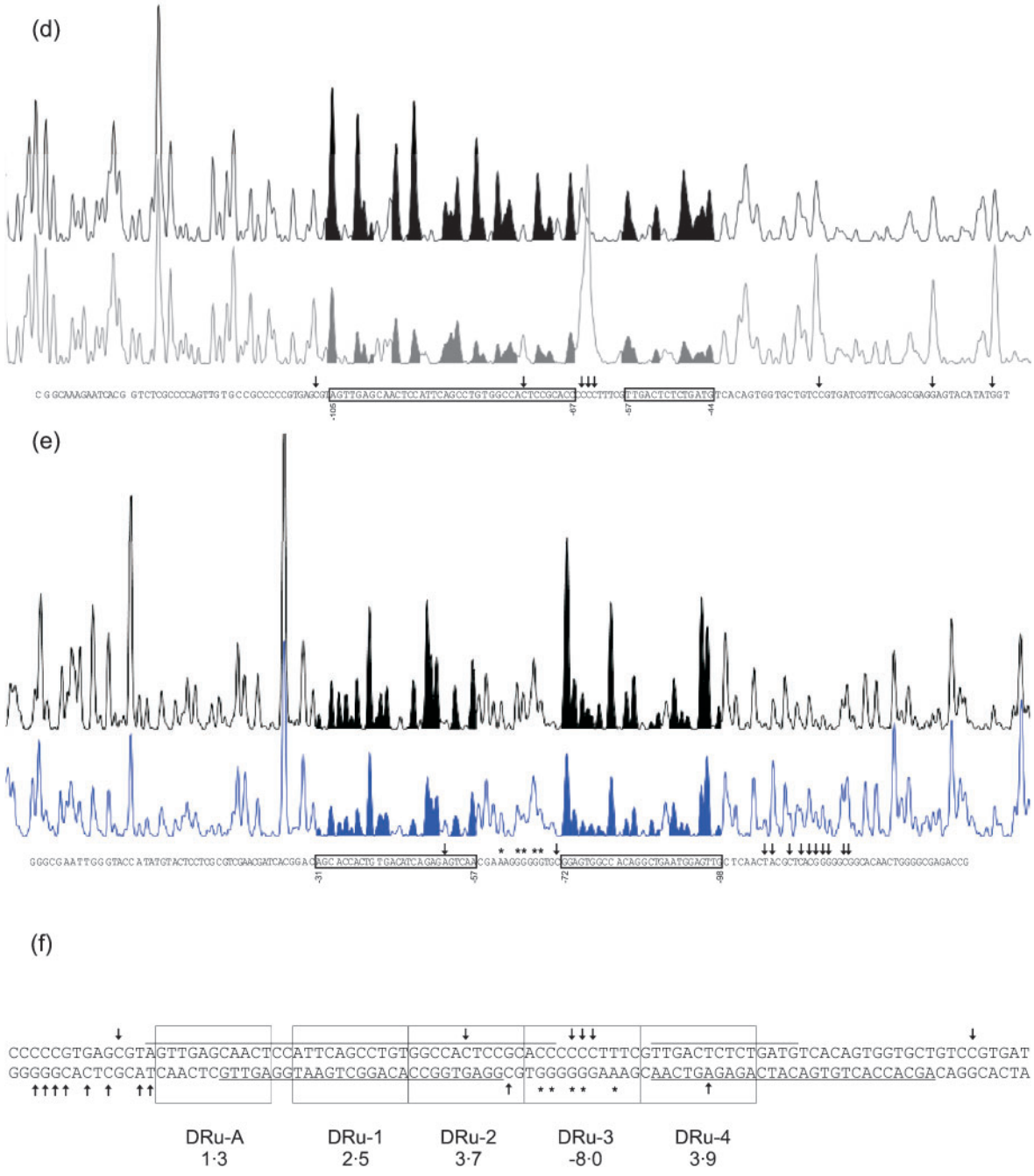


Fig. 3. DNase I footprinting of GST-PhoP^{DBD} binding to the *glpQ1*p coding (a) and complementary (b) strands, and to the *glpQ2*p coding (d) and complementary (e) strands. The upper fluorogram of each panel corresponds to the control reaction without protein and the lower one to the protected reaction (with 1 μM protein concentration for *glpQ1* and 2 μM for *glpQ2*). The correspondence between fluorescence peaks and nucleotide bases was determined using sequencing reactions. The shaded peak areas were used to calculate the degree of protection. The main protected sites are boxed and the secondary protected sites are indicated by asterisks. The vertical arrows indicate DNase I hypersensitive sites. The position with respect to the translation start codon is indicated under the nucleotide sequence. (c) and (f) Summary of GST-PhoP^{DBD} protection results for the *glpQ1* and *glpQ2* promoters, respectively. The sequence protected by GST-PhoP^{DBD} in each strand is overlined or underlined. The 11 nt DRu that form the PhoP operator are boxed and the R_i value of each DRu is indicated below.

(a)

Organism Gene	Individual information content (bit) and walkers* DRu-A	DRu-1	DRu-2	DRu-3	Core†	Structure‡	Distance to-10	-10 element§	Distance to ATG
S. coelicolor *glpQ1* (SCO1565)	8·4	3·8	6·9	2·1	**10·8**	**E_S[1]CCE**	7	TACGAT	49
S. avermitilis SAV6784	7·2	5·8	6·6	1·9	**12·3**	**E_S[1]CCE**	7	TACGAT	58
S. griseus SGR5973	-0·6	9·6	-4·4	6·5	**11·6**	**E_S[2]CCC**	50	TACGAT	102
S. scabies SCAB74351	2·3	2·6	8·5	5·2	**11·1**	**E_S[1]CCE**	7	TACGAT	29

(b)

Organism Gene	Individual information content (bit) and walkers* DRu-A	DRu-1	DRu-2	DRu-3	DRu-4	Core†	Structure‡	Distance to-10	-10 element§	Distance to ATG
S. coelicolor *glpQ2* (SCO1968)	1·3	2·5	3·7	-8·0	3·9	**6·3**	**E_S[2]CCE$_U$E$_S$**	6	CACAGT	48
S. avermitilis SAV6271	-6·3	-1·7	1·2	-5·6	1·5	**-0·5**	**E_S[2]CCE$_U$E$_S$**	6	CACTCT	48
S. griseus SGR5556	-5·7	6·8	3·0	-1·4	4·1	**9·8**	**E_S[2]CCE$_U$E$_S$**	6	CACTCT	48
S. scabies SCAB69411	-4·2	-1·4	8·1	-3·6	7·6	**6·8**	**E_S[2]CCE$_U$E$_S$**	6	CACTCT	87

Fig. 4. Individual information analysis of the PhoP-binding sites using the sequence walker method (Schneider, 1997b) and the model I weight matrix of Sola-Landa *et al.* (2008) for the *glpQ1* (a) and *glpQ2* (b) operators. * Boxes contain the individual information content (R_i, bits) of each 11 nt DRu. The height of the letters represents the R_i contribution of each position to the total information content. Letters extending downward represent unfavourable protein–DNA contacts (negative R_i values; a grey letter background indicates $R_i < -3$ bits; a black background indicates that the base is not found at that position in any of the 37 core DRu that are included in the model I matrix). The walker limits are 2 bits, which is also the top of the sine wave, and −3 bits at the bottom; 0 bits is at the middle of the walker. The sine wave depicts the accessibility of a face of the DNA double helix (10.6 bases of helical pitch) to a globular protein. The maximum R_i for each base is 2 bits when that base is contacted through the major groove (Schneider, 1996). Here the wave maximun is located at position 4 of each DRu. † The value comprises the sum of the R_i values of the DRu that form the core of the operator, which are indicated as 'C' in the operator structure. ‡ Each letter symbolizes the functional class of each DRu according to model I of Sola-Landa *et al.* (2008). Numbers in square brackets indicate the separation in base pairs between contiguous DRu. § The −10 element has been identified experimentally only for the *S. coelicolor glpQ1* and *glpQ2* genes.

addition (44 and 48 h of culture time). Compared to the unsupplemented cultures, *glpQ1* and *glpQ2* were barely expressed when P_i was added to the medium (down to 2 % and 1.5 % at 44–48 h, respectively, in *glpQ1*; down to 2.5 % and 9 % at 44–48 h in *glpQ2*; Fig. 5a, b). These results are in agreement with an expected PhoP-dependent activation. Both promoter activities were also severely inhibited by G3P (down to 27 % and 6 % at 44–48 h in *glpQ1*; down to 9 % and 17 % at 44–48 h in *glpQ2*) and by P_i plus glycerol (down to 13 % and 3 % at 44–48 h in *glpQ1*; down to 22 % and 16 % at 44–48 h in *glpQ2*). These results show that the effect of adding G3P is very similar to those of adding P_i plus glycerol or P_i alone, which strongly indicates that the G3P in the medium is hydrolysed by secreted phosphatases and used mainly as a phosphate source in *S. coelicolor*. The fact that the only two GDPDs activated by the *pho* regulon in this bacterium are secreted supports this hypothesis.

Phosphatidylinositol and phosphatidylserine are two of the major lipids of biological membranes (Divecha & Irvine, 1995). In addition to G3P, we also tested if other products GDPD hydrolysis inhibit *glpQ1p* and g*lpQ2p* activities. Besides G3P, GDPD decomposes glycerophosphoserine and glycerophosphoinositol, yielding serine and inositol, respectively. To test the effect of these compounds 15 mM serine or inositol was added to 36 h cultures of *S. coelicolor* M145 in MG-3.2 medium, as above. The *glpQ1p* activities were reduced more than twofold when serine or inositol was added (Fig. 5a). In contrast, *glpQ2p* activities did not significantly change (Fig. 5b). In summary, serine and inositol exert a repressive effect on *glpQ1* but not on *glpQ2* expression.

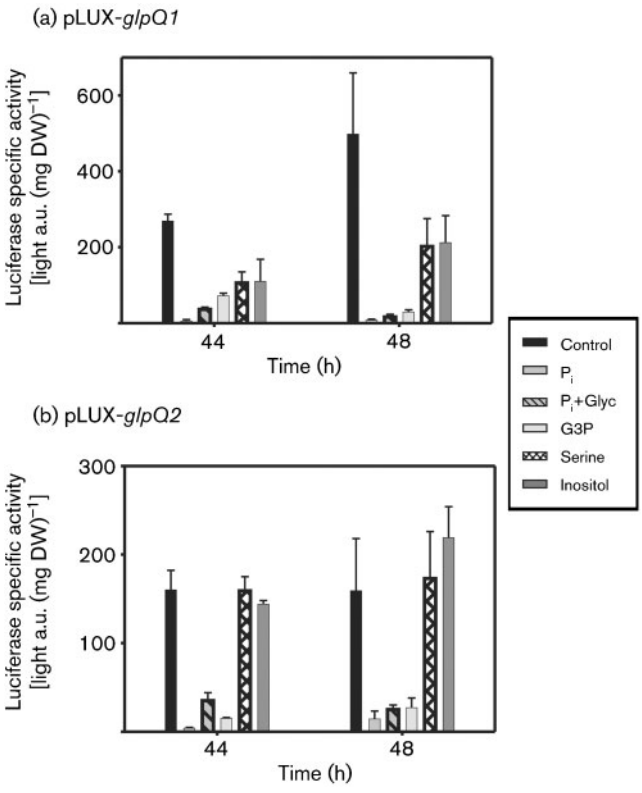


Fig. 5. Effect of the addition to the medium of phosphate, phosphate plus glycerol, G3P, serine and inositol on the promoter activity of *glpQ1* (a) and *glpQ2* (b). Cultures were grown in MG-3.2 medium at 300 r.p.m. and 30 °C and supplemented at 36 h with 15 mM P_i, P_i + glycerol, G3P, serine or inositol. Cultures were done in triplicate and samples were taken at 44 and 48 h of culture. The control cultures (no addition of any compound) represent the values at 44 and 48 h of *glpQ1* and *glpQ2* expression shown in Fig. 2. Error bars, SD.

Expression of the *glpQ1 and glpQ2 phoP*-dependent genes is also influenced by the carbon source

It is well known that expression of *pho* regulon genes depends on the P_i concentration in the medium, but to our knowledge the effect of carbon sources on the expression of these genes has not been investigated in *Streptomyces*, with the exception of the works of Díaz *et al.* (2005) and Esteban *et al.* (2008). A possible link between phosphate and carbon source regulation is of great interest.

The effects of different carbon sources on the promoter activity of the *glpQ1* and *glpQ2* genes were investigated. For this purpose glucose, fructose, maltose and glycerol were added to 36 h *S. coelicolor* M145 cultures in MG-3.2 medium at a final concentration of 2 % (w/v) and the promoter activity was measured at 44 h and 48 h. The addition of these carbon sources to the cultures at 36 h had no significant effect on the growth rates; in all cases the cultures were still at the transition growth phase when the promoter activity was determined and dry weights (44 h and 48 h) were similar (data not shown).

Of the four carbon sources added, only maltose produced a significant effect on *glpQ1p* (Fig. 6a). At 48 h the promoter activity of the maltose-supplemented culture was reduced to 45 % of the control. In contrast, *glpQ2p* activities were significantly increased when glucose, fructose or glycerol was added: the values of the reporter activities were twofold higher than those of maltose-supplemented or unsupplemented cultures (Fig. 6b). None of these carbon sources reversed the lack of expression from *glpQ2p* in the Δ*phoP* mutant background (data not shown). Thus, even in the presence of these inducing carbon sources the *glpQ2* gene depends on PhoP to be expressed. In other words, the positive effect of glucose, fructose and glycerol on *glpQ2* gene expression requires the presence of the PhoP activator protein and the effect of these carbon sources appears to be complementary to that of PhoP.

Characterization of the *glpQ1* and *glpQ2* promoters

The transcription start points (TSPs) of *glpQ1* and *glpQ2* were determined by primer extension using RNA samples

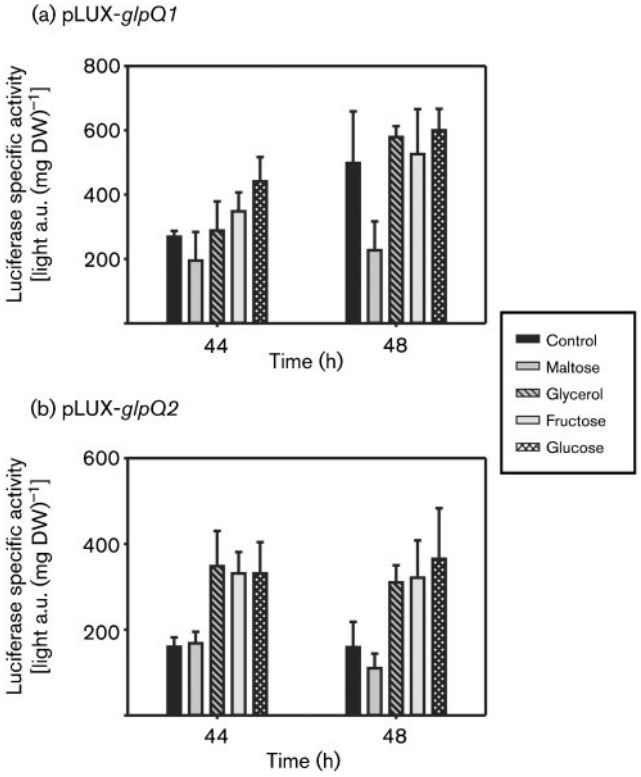


Fig. 6. Effect of the addition to the medium of different carbon sources on the promoter activity of *glpQ1* (a) and *glpQ2* (b). Cultures were grown in MG-3.2 medium at 300 r.p.m. and 30 °C and supplemented at 36 h with 2% (w/v) maltose, glycerol, fructose or glucose. Cultures were done in triplicate and samples were taken at 44 and 48 h of culture. Control cultures were the same as in Fig. 5. Error bars, SD.

extracted from control MG-3.2 cultures at the time of maximum promoter activities (44 h). RNAs were also extracted from glucose- and maltose-supplemented cultures (as in the expression studies, glucose and maltose addition was done at 36 h and samples were taken at 44 h). As shown above, glucose induced the expression of *glpQ2*, while maltose had no effect. In contrast, *glpQ1* expression was unaffected by glucose, but repressed by maltose. Thus, it was investigated if the carbon regulation relies on the use of different TSPs.

Extension products of the *glpQ1*p-*luxAB* fusion gene with sizes of 74 nt and 164 nt, primed with LUX-FAM + 47 and LUX-FAM + 135, respectively, were clearly detected (Fig. 7a). When compared with the sequencing reactions, the same TSP was deduced from both primer extension reactions, which corresponded to a guanine located 32 bp upstream of the ATG codon (Fig. 7a). The same TSP was determined with the three sugar conditions (control, glucose or maltose) used. Although an extra signal of apparent size 68 nt was detected with the LUX-FAM + 47 primer in the maltose condition, we discarded the idea that it represented a TSP because the corresponding product was not detected with the other primer.

The extension products of the *glpQ2*p-*luxAB* fusion gene were 74 nt and 164–165 nt in size for the LUX-FAM + 47 and LUX-FAM + 135 primers, respectively (Fig. 7b). A double peak of 164 and 165 nt (different in only one nucleotide) is observed with the LUX-FAM + 135 primer, and a single peak is detected with the LUX-FAM + 47 primer, suggesting the existence of a unique TSP. The 74 and 164 nt products correspond to a cytosine located 32 bp upstream of the ATG codon (Fig. 7b). The same TSP was determined using RNA samples from glucose- and maltose-supplemented cultures. We concluded that the glucose and maltose regulation of *glpQ1* and *glpQ2* genes is not executed from different promoters.

To identify promoter elements based on the identified TSPs, we used the frequency matrices of Strohl (1992), which include 29 streptomycete promoters that are recognized by the major sigma subunit (i.e. vegetative promoters). Clear sequences were found for the −10 element (Fig. 7), but both the *glpQ1* and *glpQ2* promoters lacked obvious −35 elements.

DISCUSSION

Streptomyces species constitute some of the most proficient producers of naturally occurring therapeutic molecules such as antibiotics, immunosuppressants and antitumour agents (Von Döhren & Gräfe, 1997; Challis & Hopwood, 2003). The regulation of biosynthesis of these compounds is influenced by phosphate, carbon and nitrogen sources (Martín & Demain, 1980; Doull & Vining, 1989). However, very little is known about the interaction between the metabolic pathways of these nutrients in *Streptomyces*.

There are some articles describing a cross-regulation between the carbon and phosphate sources (Wanner *et al.*, 1988; Sage & Vasil, 1997; Díaz *et al.*, 2005; Oh *et al.*, 2007), but only a few of them describe this regulation at the molecular level (Kasahara *et al.*, 1991; Puri-Taneja *et al.*, 2006; Esteban *et al.*, 2008). The involvement of carbon catabolite regulation (CCR) in the expression of the *phoPR* operon of *B. subtilis* provides an interesting link between the carbon regulation and the *pho* regulon (Puri-Taneja *et al.*, 2006). This operon is repressed by direct binding of CcpA (a transcriptional regulator mediating CCR) to a *cre* box consensus sequence localized upstream of the PhoP operator. On the other hand, the *ugp* operon of *E. coli* is induced under both phosphate- and carbon-limiting conditions from two separate promoters. The downstream promoter has multiple copies of the *E. coli* consensus PHO box and the upstream promoter has a consensus sequence for the promoters activated by the cAMP–CRP complex, which is formed only under starvation of preferred carbon sources, such as glucose (Kasahara *et al.*, 1991).

In *S. coelicolor* CCR is independent of the phosphotransferase system, which plays a central role in CCR in other

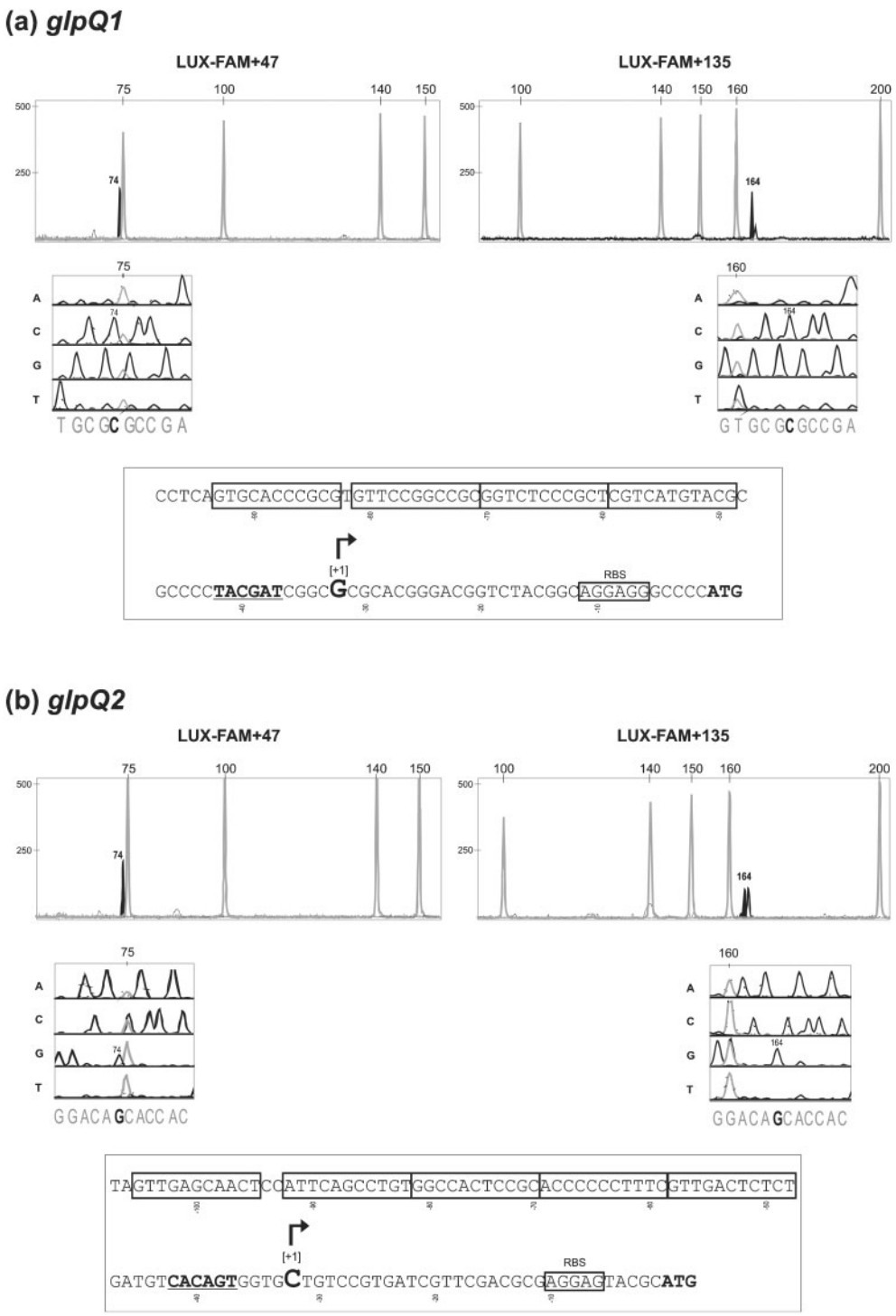


Fig. 7. Primer extension analysis of *glpQ1* (a) and *glpQ2* (b) promoters using fluorescent capillary electrophoresis. Primer extension reactions were done with both the LUX-FAM+47 and LUX-FAM+135 primers. In the upper fluorograms, the black-filled peaks represent the extension products (FAM-labelled cDNA) and the unfilled grey traces represent the LIZ-500 standards that were included in each sample. Standard sizes are marked on the upper axis, and the product sizes above the corresponding peak. The expanded lower fluorograms correspond to the four sequencing reactions (A, C, G, T) obtained with the Thermo Sequenase kit using the respective primers. Size standards were also included and the apparent molecular sizes were determined using GeneMapper software. The nucleotide corresponding to the extension fragment is indicated in bold. The box below summarizes the promoter elements of each gene. The translation start triplet is in boldface. The TSP (+1) is indicated with a bigger letter in bold and with an arrow above. The −10 element is shown in bold and underlined. The DRu that form the PhoP operator are boxed. The putative ribosome-binding site (RBS) is boxed.

bacteria (van Wezel *et al.*, 2007). Instead, glucose kinase (GlkA) is the key player of CCR (Angell *et al.*, 1994). In this work we have investigated the carbon regulation of two *pho* genes (*glpQ1* and *glpQ2*). Both genes are regulated by the carbon source but in a different manner. Thus, carbon sources exerting CCR in *S. coelicolor* such as glucose, fructose and glycerol (Kwakman & Postma, 1994) induce the expression of *glpQ2* but not that of *glpQ1*. However, *glpQ1* expression is reduced when maltose, serine or inositol is added to the medium, while *glpQ2* expression is not affected. A similar induction by high concentrations of certain sugars has been described for the *Streptomyces lividans pstS* gene (Díaz *et al.*, 2005; Esteban *et al.*, 2008).

Glycerophosphodiesters are used as phosphate or carbon sources according to the requirements of the cell (Brzoska & Boos, 1989). In this work, we have studied the effect of different phosphate and carbon sources on the expression of two GDPD-encoding genes. The *E. coli ugpQ* and *B. subtilis glpQ* genes, also encoding GDPDs, are regulated by the carbon and phosphate sources from different promoters (Kasahara *et al.*, 1991; Antelmann *et al.*, 2000). Our primer extension analyses showed that phosphate and carbon regulation of *S. coelicolor glpQ1* and *glpQ2* genes is not executed from different promoters. Both genes are regulated by PhoP in a similar manner but they are subject to a different control by the carbon source, so these two *glpQ* genes appeared to be under the control of additional regulatory systems which may be specific for the expression of each gene.

Recently, the structure of the direct repeat units (DRu) in the DNA-binding sequences of the response regulator PhoP in *S. coelicolor* has been reported (model I; Apel *et al.*, 2007; Sola-Landa *et al.*, 2008). According to this model the PhoP operator structures are divided into three classes: class I (simple operators with two well-conserved DRu), class II (operators with three conserved DRu) and class III (operators of complex structure). In this study using DNase I foorprinting experiments we have defined two new PhoP-binding sites, corresponding to the *glpQ1* and *glpQ2* genes. Both *glpQ1* and *glpQ2* have multiple DRu and belong to the class III PhoP operators. The structures of these operators are slightly different; however, the distances to the −10 element, TSP and translation start triplet are similar, suggesting a common mechanism of activation by PhoP. In fact, two identical features – (i) overlapping of the PhoP-binding sites with the −35 region and (ii) the exact location of the −10 element and TSP with respect to the translation start triplet – were found in the *glpQ1* and *glpQ2* promoters and also in that of *pitH2*, another *pho* regulon gene encoding a low-affinity phosphate transporter (Santos-Beneit *et al.*, 2008).

Bacterial GDPDs display broad catalytic activity on glycerophosphodiesters (Larson *et al.*, 1983). This study shows that inositol and serine affect *glpQ1* expression but not that of *glpQ2*, so it could be hypothesized that GlpQ1 has more hydrolytic activity for glycerophosphoinositol and glycerophosphoserine than GlpQ2. In contrast, the expression of *glpQ2* is significantly increased when preferred carbon sources such as glucose, fructose or glycerol are added to the medium. This could indicate a role for GlpQ2 in the link between carbon and phosphate assimilation. Interestingly, seven mammalian GDPDs homologous to bacterial GDPDs have been identified (Zheng *et al.*, 2003). Mammalian GDPDs are involved not only in phospholipid metabolism, but also in numerous physiological functions, such as signal transduction, cytoskeleton regulation and motor neuron differentiation (Zheng *et al.*, 2003; Nogusa *et al.*, 2004; Rao & Sockanathan, 2005). In view of the variety of GDPDs in *Streptomyces*, it will be interesting to determine if some of them (e.g. GlpQ2) also play roles in signal transduction.

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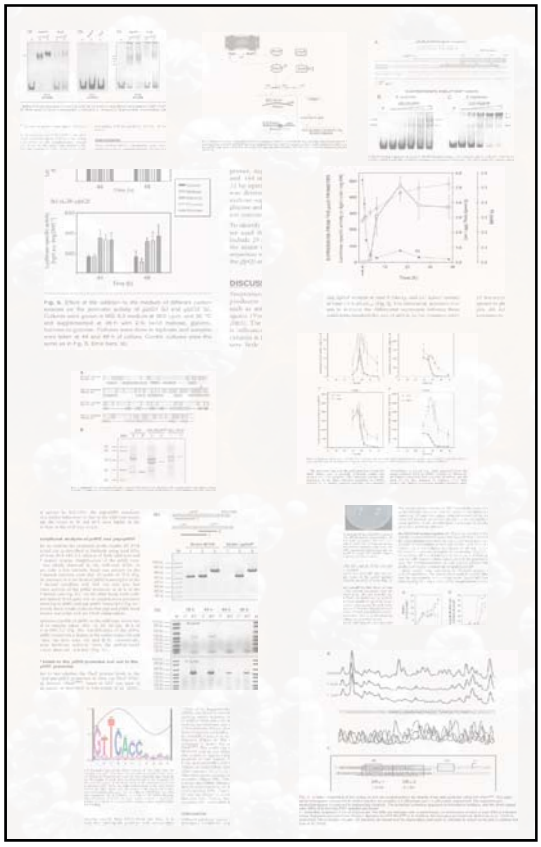
Edited by: H. L. Drake

3. Discusión



3. Discusión

Este capítulo comprende una discusión general según lo establecido para la realización de tesis doctorales por compendio de publicaciones. Los cinco apartados desarrollados en este capítulo no se corresponden directamente con los cinco artículos adjuntados en esta tesis, sino que cada uno de ellos integra los resultados de varios artículos. Además, para lograr una visión completa y de conjunto de los estudios realizados, se hace referencia a los resultados de otros trabajos de nuestro grupo. En cualquier caso, se ha puesto énfasis en los resultados de las publicaciones en las que el doctorando figura como primer autor.

3.1. Aportación de la estructura operadora de *pitH2*, *glpQ1*, *glpQ2* y *afsS* al conocimiento de la caja PHO en *Streptomyces coelicolor*

Las bacterias pertenecientes al género *Streptomyces* producen una gran cantidad de metabolitos secundarios, de los cuales, muchos de ellos son de gran interés industrial y farmacológico (Bentley, 1997; Demain, 1999). En dichas especies la concentración de fosfato inorgánico (Pi) en el medio es uno de los factores nutricionales más importantes que afectan tanto al crecimiento como a la síntesis de metabolitos secundarios (Martín, 2004). La biosíntesis de dichos metabolitos secundarios se produce generalmente bajo condiciones de escasez de Pi (Martín & Demain, 1980; Doull & Vining, 1990). Otros muchos procesos celulares están regulados por la disponibilidad de Pi en el medio. El sistema regulador principal que responde a la escasez de dicho nutriente se denomina regulón *pho*.

El regulón *pho* en *S. lividans* y *S. coelicolor* está controlado por el sistema de dos componentes PhoR-PhoP (Sola-Landa *et al*., 2003; Sola-Landa *et al*., 2005). Este sistema es homólogo al caracterizado en los organismos modelo *E. coli* y *B. subtilis* (Torriani-Gorini, 1994; Hulett, 1996). En todos los casos PhoR es la quinasa sensora

que bajo condiciones de escasez de fosfato fosforila al regulador de respuesta (PhoB en *E. coli* y PhoP en *B. subtilis* y *S. coelicolor*). PhoB/PhoP fosforilado ejerce su papel regulador uniéndose a secuencias de DNA específicas. La caracterización de los sitios de unión de PhoP (secuencias PHO) en el organismo modelo *S. coelicolor* ha sido publicada por nuestro grupo recientemente (Sola-Landa *et al*., 2008). Las secuencias PHO de *Streptomyces*, al igual que en otras bacterias (ver introducción), están formadas por repeticiones directas (DRu - *direct repeat units*) de 11 nucleótidos, de los cuales, los siete primeros están más conservados, mientras que los otros cuatro son más variables (ver figura 3.1). La secuencia consenso de la región más conservada (GTTCACC) presenta similitudes con la descrita en *B. subtilis* (TT-A/C/T-ACA; Liu y Hulett, 1998), mientras que difiere más de la descrita en proteobacterias como *E. coli* o *S. meliloti* (CTGTCAT; Makino *et al*., 1986; Yuan *et al*., 2006) o incluso en otros actinomicetos como *C. glutamicum* (CCTGTGA; Schaaf & Bott, 2007).

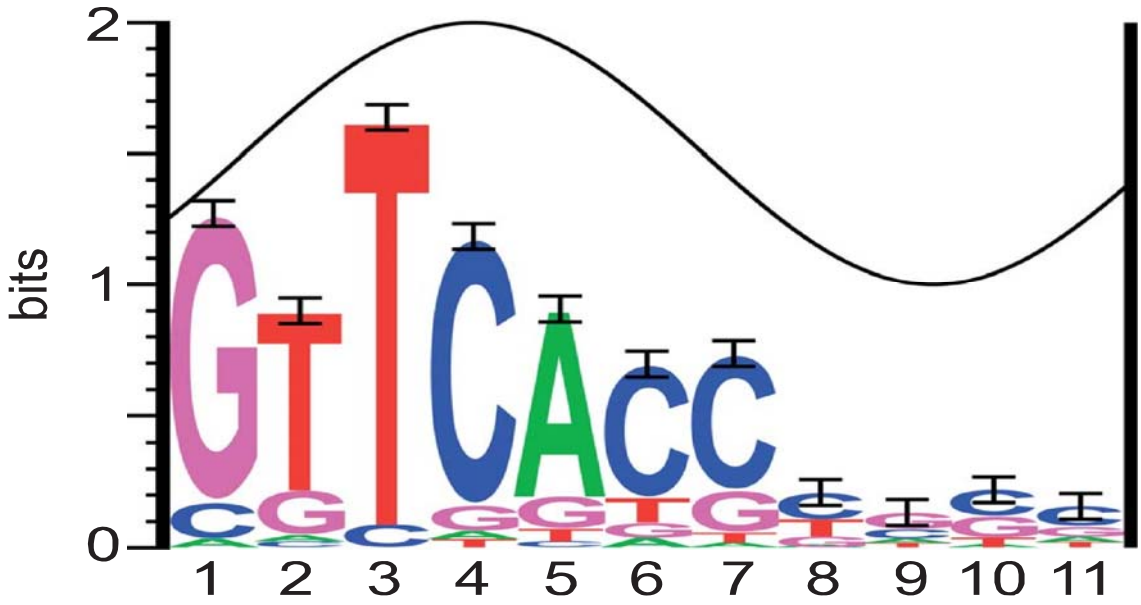


Figura 3.1: Logotipo de la secuencia de unión de PhoP al DNA en *S. coelicolor* correspondiente al modelo I publicado en Sola-Landa *et al*. (2008). La altura de cada letra es proporcional a la frecuencia de aparición en dicha posición. El valor de conservación en cada posición se cuantifica en bits. La onda representa una vuelta completa del DNA en su forma B.

Para la caracterización precisa de los operadores PHO se utilizó la metodología basada en la teoría de la información publicada por Schneider (Schneider, 1997). En una etapa inicial se alinearon las repeticiones directas demostradas mediante ensayos de protección de los genes *pstS*, *phoU* (Sola-Landa *et al*., 2005), *phoA*, *phoD* (Apel *et al*., 2007) y *pitH2* (Santos-Beneit *et al*., 2008). Con la información resultante se construyó una matriz de puntuación (modelo 0) que sirvió para rastrear la secuencia cromosómica de *S. coelicolor* e identificar posibles secuencias PHO. A partir de los resultados transcriptómicos y proteómicos obtenidos en experimentos de cambio a escasez de fosfato en el medio (Rodríguez-García *et al*., 2007), y teniendo en cuenta la posible función de dichos genes en el metabolismo del fosfato, se seleccionaron secuencias candidatas con el objetivo de ampliar el conocimiento del regulón *pho* y de enriquecer el modelo del sitio de unión. La unión de la proteína PhoP a dichos candidatos fue comprobada por estudios de EMSA y de protección frente a DNasa I. Finalmente, con un total de 37 DRus se definió el modelo I (Sola-Landa *et al*., 2008). Las cajas PHO de los promotores de *glpQ1*, *glpQ2* y *afsS* no fueron incluidas en el modelo I dado que la publicación de estos trabajos fue posterior.

Tras seis años de investigación en nuestro laboratorio se han determinado experimentalmente 27 sitios de unión de PhoP en el DNA de *S. coelicolor* (ver anexo). El número y la organización de las DRus que forman dichos operadores son muy variados. El análisis de la organización y conservación de las DRus se ha correlacionado con los complejos de retraso observados en los experimentos de EMSA para proponer un modelo de unión de PhoP a las secuencias de DNA. Así, se ha determinado que el sitio de unión debe estar formado por un mínimo de 2 DRus (Sola-Landa *et al*., 2008). En los operadores más complejos, existen unas DRus que forman el núcleo y otras la extensión de la zona de unión. Para su mejor descripción, se han clasificado estas secuencias operadoras en tres clases. La clase I comprende los operadores más sencillos formados por 2 únicas DRus. Ambas repeticiones están bien conservadas. En los estudios de EMSA del fragmento de DNA, la proteína PhoP solamente da lugar a un único complejo de retraso. La clase II incluye operadores con 3 DRus bien conservadas. Del mismo modo, se observa un único complejo de retraso en los estudios de EMSA. Por tanto, los operadores de las dos primeras clases se definen por tener un núcleo en el que cada una de las DRus es ocupada por un monómero de PhoP simultáneamente. La

tercera clase (clase III) comprende operadores de estructura compleja formados por una secuencia núcleo y secuencias adyacentes que son unidas por la proteína reguladora con menor afinidad. El núcleo puede estar formado por 2 ó 3 DRus bien conservadas. La unión de monómeros de PhoP a estas DRus permite la unión de otros monómeros a DRus con un valor de conservación más bajo. Se ha propuesto que esta unión es posible gracias a interacciones proteína-proteína entre los monómeros de PhoP consecutivos (Eder *et al*., 1999). Esta unión secuencial de la proteína explica la formación de dos o más complejos de retraso en los experimentos de EMSA cuando se utilizan operadores de esta clase.

El análisis de las secuencias operadoras junto con los estudios de EMSA y de protección de los cuatro genes descritos en esta tesis (*pitH2*, *glpQ1*, *glpQ2* y *afsS*) nos permite clasificar estos operadores de acuerdo con el modelo I. El operador de *afsS* se incluye dentro de la clase I al estar formado por dos DRus bien conservadas (CC) y presentar una sola banda de retraso en los estudios de EMSA (Santos-Beneit *et al*., 2009a). Los operadores de los genes *pitH2*, *glpQ1* y *glpQ2* se incluyen dentro de la clase III puesto que presentan estructuras complejas. El hecho de que aproximadamente el 70 % de los sitios de unión de PhoP caracterizados hasta la fecha en *S. coelicolor* pertenezcan a la clase III (operadores complejos) sugiere que PhoP no funciona como un simple interruptor de encendido y apagado. Evidencias de esto se tiene en la inducción del gen *pitH2* (ver Santos-Beneit *et al*., 2008). La estructura operadora de PhoP en dicho gen está formada por seis DRus dispuestas en la siguiente fórmula: E_S[1]$E_S E_U$CC[2]E_S (ver anexo para información sobre el significado de las letras presentes en la formula). Cabe destacar que el operador de *pitH2* junto con el del gen *phoD* (Apel *et al*., 2007) tienen el mayor número de DRus descrito hasta el momento en *S. coelicolor*. Los operadores de *glpQ1* y *glpQ2* presentan cuatro y cinco DRus con las siguientes estructuras operadoras respectivamente: E_S[1]CCE y E_S[2]CC$E_U E_S$. En total, estos cuatro operadores aportan para el estudio de secuencias de unión de PhoP la secuencia de 17 DRus y tres estructuras operadoras novedosas, correspondientes a *pitH2*, *glpQ1* y *glpQ2*.

3.2. Inducción y represión génica por la proteína PhoP

El sistema PhoR-PhoP responde a la escasez de fosfato en el medio induciendo o reprimiendo numerosos genes para adecuar su metabolismo de la forma más eficiente a la limitación de este nutriente básico. Un ejemplo característico es la sustitución de polímeros ricos en fosfato por polímeros libres de fosfato en la pared celular de *B. subtilis* en condiciones de limitación de fosfato en el medio. Así, PhoP reprime los genes de síntesis de los ácidos teicoicos (ricos en fosfato), mientras que induce los genes de síntesis de ácidos teicurónicos (libres de fosfato), como una forma de obtener y ahorrar fosfato (Liu *et al*., 1998).

La mayoría de los estudios clásicos con respecto a la expresión de genes del regulón *pho* incluyen aquellos que son inducidos por dicho sistema, como fosfatasas o transportadores de fosfato. Sin embargo, el avance en las técnicas "ómicas" ha provocado que el estudio de la expresión génica adquiera un nivel mucho más global. De este modo, en estudios transcriptómicos de *S. coelicolor*, hasta 207 genes, de un total de 468 afectados por la delección de PhoP, mostraron un perfil de represión (Rodríguez-García *et al*., 2007). Es decir, un 44 % de los genes fueron controlados negativamente, directa o indirectamente, por PhoP. Además, PhoP ejerce un efecto represor de forma directa en el 35 % de los sitios a los que se ha demostrado que se une en *S. coelicolor* (ver anexo).

La gran variabilidad con respecto a la organización, localización, número y conservación de las DRus en los operadores PHO de los distintos genes (ver Sola-Landa *et al*., 2008) podría ser suficiente para explicar porqué unos genes son más inducidos que otros, o porqué en algunos casos la unión de PhoP reprime y en otros activa. De hecho, esta complejidad en la estructura de dichos operadores sea posiblemente la que permita un control tan fino en la regulación de un número tan alto de genes. Aunque tampoco se puede descartar la actuación de otros mecanismos reguladores. Por tanto, es importante conocer el mecanismo molecular de la activación y represión génica debido a la proteína PhoP.

Fundamentalmente se observan dos mecanismos de represión por PhoP en genes de *S. coelicolor*. Un mecanismo consiste en el impedimento estérico causado por la unión de la proteína en el promotor de un determinado gen, y el otro en la competencia entre PhoP y otra proteína por un mismo sitio de unión. En el primero, la RNA polimerasa se ve obstaculizada tanto en el reconocimiento del promotor como en el avance de la misma. En el segundo, PhoP ocupa el lugar de unión del activador transcripcional de un determinado gen evitando, así, que el activador transcripcional ejerza su función. Como ejemplo de lo primero está el caso del gen *glnR* (reprimido por PhoP) que presenta cuatro DRus que solapan con la región -10 y el promotor P_1 del gen (Rodríguez-García *et al*., 2009), lo cual coincide con lo descrito por primera vez (para PhoP como represor) en los operones *tagAB* y *tagDEF* (responsables de la síntesis de ácidos teicoicos) de *B. subtilis*. En dichos casos la unión de PhoP fosforilado se extiende desde la región -10 de la zona promotora a la región 5' del marco de lectura del gen (Liu *et al*., 1998). Por otro lado, como ejemplos del segundo mecanismo mencionado se tienen los casos de los genes *afsS* y *glnA*. En el gen *afsS*, PhoP compite por el mismo sitio de unión que su activador transcripcional principal, la proteína AfsR (Santos-Beneit *et al*., 2009a). Nunca antes se había descrito un mecanismo de competencia en la proteína PhoP/PhoB. En el gen *glnA* PhoP compite con el activador transcripcional GlnR (Sola-Landa, comunicación personal).

En *E. coli* se ha descrito que PhoB ejerce su efecto activador uniéndose a la región -35 y supliendo la carencia de la caja -35 que presenta los genes que regula (Makino *et al*., 1996). De igual modo podría ocurrir en *S. coelicolor*. Los operadores de PhoP en los genes *glpQ1*, *glpQ2* y *pitH2* (dependen totalmente de PhoP para su activación) se localizan siempre en la región -35, con la excepción del gen *pitH2*, en el que una de las DRu que forma el operador solapa también con la región -10. En dicho gen PhoP podría ejercer una regulación dual (activación/represión) según se una a las DRus que se localizan en la región -35 ó -10, como se ha descrito en el gen *phyC* de *Bacillus amyloliquefaciens* (Makarewicz *et al*., 2006). Un caso específico es el del gen *afsS* en el cual PhoP también se une a su región -35, pero a diferencia de los anteriores, PhoP no es necesario para la activación del gen. No obstante, en contra punto con el

efecto represor que ejerce PhoP sobre la expresión de *afsS* cuando AfsR y PhoP están presentes, no se puede descartar que PhoP sea capaz de activar la transcripción del gen. Los mutantes con el gen *afsR* deleccionado muestran niveles de expresión muy significativos (Santos-Beneit *et al*., 2009a), los cuales podrían deberse a la proteína PhoP tanto en su estado fosforilado como desfosforilado (Santos-Beneit, no publicado). Es decir, el efecto de AfsR y PhoP sobre *afsS* podría ser positivo en ambos casos. Los niveles de expresión más altos de *afsS* en el mutante Δ*phoP* con respecto a la cepa silvestre se explicarían por la mayor capacidad de AfsR frente a PhoP de inducir la transcripción de *afsS*. De este modo cuando PhoP está presente (cepa silvestre), al competir con AfsR por el mismo sitio de unión, la inducción total de *afsS* es menor. El hecho de que la activación de *afsS* esté asegurada por dos proteínas, indica una función pleotrópica de la proteían reguladora AfsS.

3.3. Respuesta primaria ejercida por PhoP ante la escasez de fosfato en el medio

La respuesta primaria ejercida por PhoP en condiciones de escasez de fosfato en el medio comprende principalmente la inducción de enzimas capaces de obtener Pi a partir de compuestos orgánicos y la inducción de transportadores de Pi. En *S. coelicolor* se ha demostrado la activación por PhoP de cuatro fosfatasas (Apel *et al*., 2007; Sola-Landa *et al*., 2008), dos glicerofosfodiéster fosfodiesterasas (Rodríguez-García *et al*., 2007; Santos-Beneit *et al*., 2009b), una 5'-nucleotidasa (Rodríguez-García *et al*., 2007), una fitasa (Rodríguez-García *et al*., 2007; Sola-Landa *et al*., 2008) y dos transportadores de Pi, uno de alta afinidad (Pst; Sola-Landa *et al*., 2005) y otro de baja afinidad (PitH2; Santos-Beneit *et al*., 2008). Los genes que codifican estas enzimas son miembros clásicos del regulón *pho* en la mayoría de las bacterias estudiadas, a excepción de los genes que codifican la fitasa (SCO7697) y el transportador de baja afinidad (*pitH2*). De hecho, la activación por PhoP de un transportador de baja afinidad no ha sido descrita con anterioridad en ningún microorganismo.

Por otro lado, la expresión de una fitasa tiene sentido en bacterias que tengan su hábitat en suelos con abundantes restos vegetales, ya que los inositoles-fosfato (fitatos) son el mayor acumulo de fosfato en las plantas. En muchos suelos el 80 % del fosfato orgánico total se corresponde con fitatos (Quiquampoix y Mousain, 2005). Así, no sorprende la inducción de una fitasa por PhoP en bacterias del suelo como *Streptomyces* (Rodríguez-García *et al*., 2007; Sola-Landa *et al*., 2008) o *Bacillus* (Hoi *et al*., 2006; Makarewicz *et al*., 2006).

La respuesta a la escasez de fosfato en el medio también incluye el ahorro y la reutilización de dicho nutriente. La mayoría de los microorganismos acumulan largas cadenas de polifosfatos como reservas de energía y de fosfato. En *S. lividans* los polifosfatos son sintetizados a partir de ATP por medio de la enzima polifosfato quinasa codificada por el gen *ppk*. La enzima cataliza además la reacción reversa para generar ATP a partir del polifosfato acumulado (Chouayekh y Virolle, 2006). La proteína no es sintetizada en el doble mutante *phoR-phoP*, por lo que al igual que en otras bacterias su activación depende del sistema *pho* (Ghorbel *et al*., 2006). En *S. coelicolor* la cepa Δ*phoP* es capaz de retirar fosfato del medio al mismo nivel que la cepa silvestre; sin embargo el crecimiento de la cepa mutante se ve disminuido (Santos-Beneit *et al*., 2008). Esto podría ser debido en parte a la falta de activación del gen *ppk* en la cepa Δ*phoP*, que evitaría acumular y utilizar el fosfato transportado.

En *B. subtilis* es bien sabido que en condiciones de escasez de fosfato se produce un reemplazamiento en la pared celular de polímeros ricos en fosfato por otros libres de fosfato como una forma de ahorrar y reutilizar fosfato (Liu *et al*., 1998). En *S. coelicolor* PhoP activa genes que podrían estar involucrados en la síntesis de polímeros libres de fosfato, los cuales de un modo análogo al descrito en *B. subtilis*, sustituirían los polímeros ricos en fosfato de las paredes celulares (Rodríguez-García *et al*., 2007; Sola-Landa *et al*., 2008).

En los estudios transcriptómicos realizados por Rodríguez-García *et al*. (2007) otros genes que respondieron a la escasez de fosfato en el medio están relacionados con el metabolismo de nucleótidos y amino ácidos, la respiración, el metabolismo del

nitrógeno y el hierro, la diferenciación morfológica y las respuestas al estrés. En general, la mayoría de los cambios están dirigidos a una menor transcripción y síntesis proteica.

3.4. Interacción de las regulaciones por carbono, nitrógeno y fósforo

El crecimiento bacteriano está frecuentemente limitado por la escasez de nutrientes esenciales como carbono, nitrógeno y fósforo. Los sistemas que captan estas deficiencias nutricionales en el medio difieren de un nutriente a otro. Poco se sabe de la interacción entre estos sistemas y menos todavía de los mecanismos moleculares que intervienen en dicha interacción. No obstante, se sabe que las fuentes de carbono, nitrógeno y fósforo influyen notablemente en el metabolismo secundario (Martín & Demain, 1980; Doull & Vinning, 1990; McDowall *et al*., 1999; Rokem *et al*., 2007).

La glucosa es la fuente de carbono más comúnmente utilizada; sin embargo, en muchos casos ejerce un efecto negativo en la producción de antibióticos (Hobbs *et al*., 1990). La elección de la fuente de nitrógeno y su concentración también es crucial para la producción de los mismos. Así, el amonio es la fuente preferida para la mayoría de los microorganismos, aunque no la más apropiada para la producción de muchos de ellos (Merrick y Edwards, 1995). El fósforo se encuentra en la naturaleza de forma inorgánica, como ortofosfato (Pi), pirofosfato (PPi) ó polifosfato (poliPi), y de forma orgánica, principalmente, como nucleótidos, azucares fosforilados y fosfolípidos. La forma principal de asimilación del fósforo en las bacterias es el ortofosfato, también denominado fosfato inorgánico (Pi). Para obtener un buen crecimiento microbiano es necesario utilizar concentraciones no limitantes de fosfato; no obstante, la mayoría de los antibióticos se sintetizan en condiciones limitantes de dicho nutriente (Martín, 2004). Por tanto, es de gran importancia conocer los mecanismos moleculares de estas regulaciones y sus posibles interconexiones.

La regulación cruzada nitrógeno-carbono es la más estudiada y se detalla en la revisión de Commichau *et al*. (2006). Menos descrita está la interacción carbono-fosfato, aunque varios trabajos dejan entrever una interconexión entre ambas regulaciones (Wanner *et al*., 1988; Kasahara *et al*., 1991; Sage & Vasil, 1997; Matsuzaki *et al*., 2003; Díaz *et al*., 2005; Puri-Taneja *et al*., 2006; Oh *et al*., 2007; Esteban *et al*., 2008). De estos trabajos, dos de ellos relacionan directamente el mecanismo de represión catabólica por carbono (CCR) con la expresión de genes del regulón *pho* (Kasahara *et al*., 1991; Puri-Taneja *et al*., 2006). En ambos casos el mecanismo de CCR ejerce un efecto negativo en la expresión de dichos genes (ver Santos-Beneit *et al*., 2009b). Puri-Taneja propone que el hecho de que el sistema PhoR-PhoP esté regulado por el mecanismo de CCR en *B. subtilis* abre la posibilidad de que una de las funciones del regulón *pho* sea la de adecuar el metabolismo energético no solo en condiciones de escasez de Pi, sino también en condiciones de escasez de carbono.

En esta tesis se ha determinado el efecto de diversas fuentes de carbono y fosfato sobre la expresión de dos genes (*glpQ1* y *glpQ2*) pertenecientes al regulón *pho* de *S. coelicolor*. Las enzimas que codifican (glicerofosfodiéster fosfodiesterasas) están relacionadas con el metabolismo del carbono y del fosfato, ya que su producto (glicerol-3-fosfato) puede ser utilizado como fuente de ambos nutrientes. Como experimento inicial se comprobó que el glicerol-3-fosfato ejercía un efecto inhibidor semejante al Pi en la expresión de dicho genes. Aunque no se puede concluir si el responsable de dicho efecto es el propio compuesto en sí, o el grupo fosfato liberado tras su hidrólisis por fosfatasas. Por otro lado se comprobó que la adición de distintas fuentes de carbono al medio provocaba diferentes niveles de expresión en ambos genes. Esto demuestra que el fosfato no es el único nutriente que regula la expresión de genes dependientes de PhoP en *Streptomyces*, lo cual está en concordancia con lo publicado por el grupo de Santamaría en relación al gen *pstS* de *S. lividans* (Díaz *et al*., 2005; Esteban *et al*., 2008). Dicho gen se induce significativamente por concentraciones abundantes de ciertos azucares como la fructosa, galactosa o manosa. Por consiguiente, se deduce una interconexión carbono-fosfato en la regulación de genes del regulón *pho* en *Streptomyces*, semejante a lo descrito en *E. coli* y *B. subtilis*. No obstante, el mecanismo molecular de dicha interacción necesita ser elucidado todavía.

Recientemente nuestro grupo ha demostrado también una conexión entre las regulaciones por fosfato y nitrógeno (Rodríguez-García *et al*., 2009). En la publicación se demuestra, mediante estudios de EMSA y de protección frente a DNasa I, que PhoP se une de forma específica a los promotores de genes involucrados en el metabolismo del nitrógeno. Dicho metabolismo está regulado en *S. coelicolor* por mecanismos complejos que incluyen interacción de genes reguladores y genes estructurales (Fink *et al*., 2002; Reuther & Wohlleben, 2007). El regulador transcripcional GlnR responde a la limitación de nitrógeno activando la transcripción de diversos genes cuya función principal es transportar y asimilar amonio (Tiffert *et al*., 2008). Entre estos genes destacan dos glutaminas sintetasas (*glnA* y *glnII*) y un transportador de amonio (*amtB*). Estudios de expresión en la cepa silvestre y en el mutante Δ*phoP* indicaron que los cuatro genes están reprimidos por PhoP (Rodríguez-García *et al*., 2009). El hecho de que PhoP regule genes esenciales para la asimilación del nitrógeno otorga al sistema PhoR-PhoP un papel fundamental, no solo con respecto a la regulación del metabolismo del fosfato, sino también, con la de otros nutrientes vitales como el nitrógeno.

3.5. Efecto de PhoP en el metabolismo secundario

La formación de metabolitos secundarios en el género *Streptomyces* se correlaciona generalmente con el estado nutricional del microorganismo y la fase de desarrollo del mismo (Bibb, 2005). El conocimiento de las señales que van desde la percepción de dichos estados nutricionales hasta el comienzo de la producción de antibióticos es un objetivo de gran importancia para la investigación básica, además de para la industria farmacológica.

Como se ha mencionado anteriormente, las concentraciones abundantes de fosfato en el medio producen un efecto negativo en la producción de muchos metabolitos secundarios (Martín, 2004), entre los que se incluyen los antibióticos actinorrodina y undecilprodigiosina producidos por *S. coelicolor* (Hobbs *et al.*, 1990).

Precisamente sobre este último antibiótico es donde las concentraciones cuantiosas de Pi producen un mayor efecto represor (Santos-Beneit *et al*., 2009a). No obstante, los sistemas moleculares que intervienen en esta regulación son totalmente desconocidos.

Para determinar el efecto de *phoP* sobre la producción de antibióticos, se estudió el efecto de la inactivación génica en *S. lividans* (Sola-Landa *et al.*, 2003) y en *S. natalensis* (Mendes *et al*., 2007). De estos trabajos efectuados en nuestro laboratorio se observó un efecto positivo en las dos especies, del que se deduce que PhoP ejerce un control global negativo sobre la producción de antibióticos. En *S. coelicolor* el efecto de la deleción de *phoP* resultó opuesto al anteriormente citado, es decir, PhoP ejerce un control global positivo (Santos-Beneit *et al*., 2009a). El sistema PhoR-PhoP se activa en condiciones de Pi limitantes, en las cuales la producción de antibióticos se ve favorecida (ver Santos-Beneit *et al*., 2009a). Por tanto, en *S. coelicolor*, el efecto global de PhoP (positivo) y el efecto de la concentración de Pi en la producción de antibióticos (mayor producción cuando el Pi es limitante) se correlaciona. Recientemente se ha comprobado que en el organismo productor de geldanamicina, *Streptomyces hygroscopicus*, el sistema PhoR-PhoP ejerce también un efecto positivo sobre la producción de dicho antibiótico (Angelina Ramos Castro, comunicación personal). Se puede concluir que el efecto de PhoP sobre la producción de metabolitos secundarios difiere de una especie de *Streptomyces* a otra.

La regulación debida a PhoP y a la concentración de Pi en el medio también varía de un antibiótico a otro dentro de la misma especie. En Santos-Beneit *et al*. (2009a) se demuestra que el control negativo sobre la producción de actinorrodina, ejercido por las grandes concentraciones de Pi (en las que PhoP está en su forma inactiva) y por la delección de *phoP*, es de una magnitud semejante. Este dato indica que el efecto negativo de las grandes concentraciones de Pi sobre la producción de este antibiótico se podría deber simplemente a la carencia de PhoP en su estado activo. Sin embargo, no ocurre lo mismo con la undecilprodigiosina, ya que las concentraciones abundantes de Pi ejercen un efecto negativo de mucha mayor magnitud que la delección de *phoP* (ver Santos-Beneit *et al*., 2009a). Por tanto, al menos otro mecanismo diferente

al sistema PhoR-PhoP esta mediando el control de este antibiótico en relación a la concentración de Pi en el medio.

La búsqueda de genes pertenecientes al metabolismo secundario regulados por PhoP, que expliquen el efecto positivo de dicho regulador sobre la producción de antibióticos en *S. coelicolor*, ha sido uno de los objetivos principales de esta tesis. Las búsquedas bioinformáticas de secuencias PHO sobre estos genes no resultaron concluyentes excepto para el gen *afsS*. AfsS es un activador transcripcional de *actII*-ORF4 y *redD* y por tanto de la producción de actinorrodina y undecilprodigiosina (Floriano & Bibb, 1996). Sin embargo, PhoP lejos de activar dicho gen (lo cual explicaría, en parte, el efecto positivo de PhoP sobre la producción de actinorrodina y undecilprodigiosina) lo reprime (Santos-Beneit *et al*., 2009a). De esta manera, el efecto de PhoP sobre *afsS* no explica el efecto global positivo del sistema PhoR-PhoP sobre la producción de estos antibióticos en *S. coelicolor*. No obstante, dicha interacción abre un camino muy interesante en la implicación directa del sistema PhoR-PhoP sobre el metabolismo secundario; conectando la señalización de la escasez de un nutriente con la producción de antibióticos.

La regulación que ejerce el sistema PhoR-PhoP sobre el sistema AfsR-AfsS tiene un nivel superior de complejidad. Ambos sistemas se regulan de forma cruzada, como ponen de manifiesto los trabajos realizados por Lian *et al*. (2008) y Santos-Beneit *et al*. (2009a). El primer trabajo indica un efecto global positivo de *afsS* sobre la transcripción de genes *pho*. El segundo demuestra que la proteína AfsR reprime los genes *phoRP* y *pstS* mediante la unión directa a sus promotores. Por tanto, esta regulación cruzada conecta, de algún modo, el metabolismo primario y el secundario.

En consecuencia, se puede considerar a PhoP como un regulador global clave en *Streptomyces*, no solo por su papel en el metabolismo del fosfato, sino por su interacción con otros reguladores globales como GlnR o AfsR. La siguiente figura (3.2) resume los procesos de regulación en los que el sistema PhoR-PhoP interviene, según lo descrito en esta tesis.

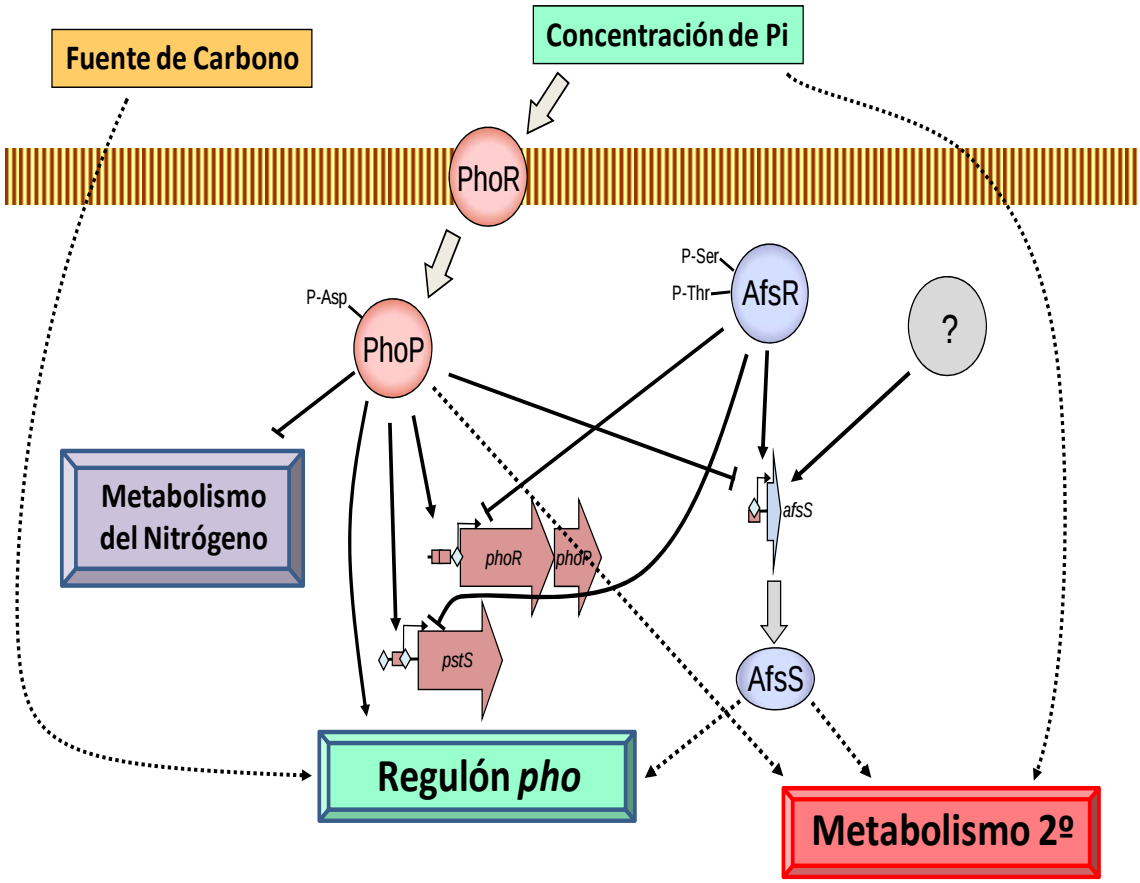


Figura 3.2: Efecto de PhoR-PhoP en el metabolismo de *S. coelicolor* según lo descrito en esta tesis y en los trabajos de: Lee *et al*., (2002); Sola-Landa *et al*., (2005) y Lian *et al*., (2008). Las líneas continuas y discontinuas indican regulación directa o indirecta, respectivamente. Las líneas que acaban en forma de flecha y cabeza roma indican activación o represión, respectivamente. Los cuadrados y los rombos en la zona promotora de los genes indican la unión de PhoP y AfsR al DNA, respectivamente.

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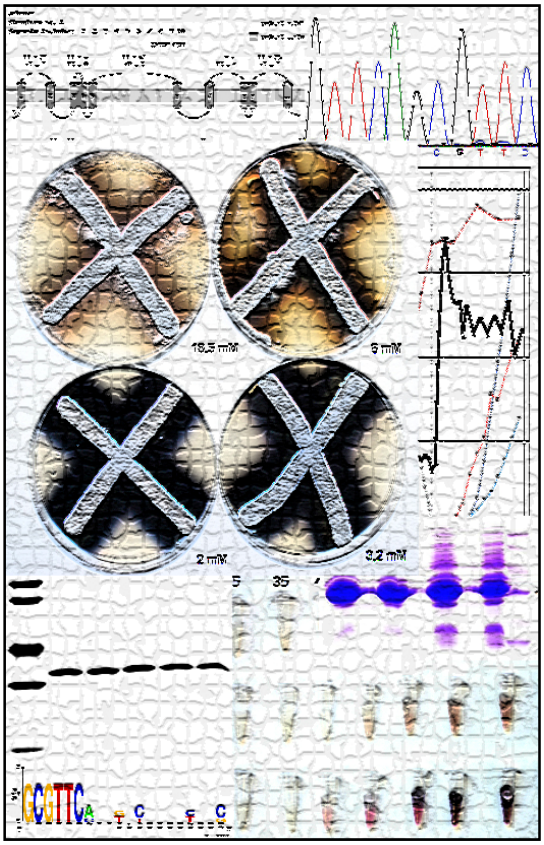
4. Conclusiones

4. Conclusiones

1. La cuantificación de la actividad promotora del gen *pstS* en un medio fresco con limitación de fosfato permitió definir las condiciones para la realización de un estudio global transcriptómico y proteómico de la respuesta a dicha escasez. La transcripción de un total de 468 genes fue afectada por la escasez de fosfato mediante un mecanismo dependiente de PhoP.
2. Estudios de expresión y de unión de PhoP al DNA de genes de *S. coelicolor* que codifican hipotéticos transportadores de fosfato (*pstS*, *pitH2* y *pap-pitH1*) han demostrado que el regulón *pho* de dicha bacteria incluye genes de dos transportadores distintos, uno de baja afinidad (PitH2) y otro de alta afinidad (Pst). La activación transcripcional de ambos sistemas en condiciones limitantes de fosfato es secuencial; primero se activa *pitH2* y luego *pst*.
3. La integridad del gen *phoP* es necesaria para el crecimiento de *S. coelicolor* en condiciones de fosfato inferiores a 0,1 mM. La disminución del crecimiento en la cepa Δ*phoP* no es debida a la falta de activación de los transportadores PitH2 y Pst, ya que la cepa mutante es capaz de retirar el fosfato del medio en similar cuantía a la cepa silvestre. La incapacidad de la cepa Δ*phoP* de sintetizar macromoléculas de reserva como polifosfatos puede ser la responsable de la falta de crecimiento cuando el fosfato del medio es agotado.
4. La producción de actinorrodina y undecilprodigiosina disminuye en cultivos con grandes concentraciones de fosfato (superiores a 5 mM). La producción de undecilprodigiosina es la más afectada.
5. La inactivación de *phoP* tiene un efecto negativo en la producción de actinorrodina y undecilprodigiosina en condiciones de fosfato limitantes. En condiciones de exceso de fosfato el efecto de la delección es nulo ya que tanto la cepa silvestre como Δ*phoP* tienen la misma producción.
6. El efecto de la inactivación del gen *afsR* sobre la producción de actinorrodina y undecilprodigiosina es negativo tanto en condiciones limitantes como no limitantes de fosfato.

7. Los reguladores PhoP y AfsR compiten por un mismo sitio de unión en el promotor del gen *afsS*. La expresión de *afsS* es mayor en la cepa Δ*phoP* que en la cepa silvestre en condiciones limitantes de fosfato. Por tanto, PhoP ejerce un efecto negativo sobre la expresión de *afsS*.
8. Al menos otro regulador diferente a AfsR activa la expresión de *afsS*, como muestran los estudios de cuantificación de la actividad promotora de *afsS* en la cepa Δ*afsR*.
9. AfsR se une a los promotores de *pstS* y *phoRP*. El alineamiento de las secuencias de los cuatro lugares de unión de AfsR en *pstS* (2), *phoR* (1) y *afsS* (1) revela la siguiente conservación en los 7 primeros nucleótidos: GCGTTCA. El efecto de la unión de AfsR en *pstS*, y seguramente también en *phoRP*, es negativo. Esto indica una función más global de AfsR que la de regular solo la producción de antibióticos. Indica además, una interconexión entre los sistemas PhoR-PhoP y AfsR-AfsS.
10. La expresión de los genes *glnR*, *glnA*, *glnII* y *amtB* es mayor en la cepa Δ*phoP* que en la cepa silvestre en condiciones limitantes de fosfato. Por tanto PhoP ejerce un efecto negativo sobre genes relacionados con el metabolismo del nitrógeno, lo cual sugiere una conexión en las regulaciones fosfato-nitrógeno.
11. PhoP activa dos (*glpQ1* y *glpQ2*) de los siete genes que codifican hipotéticas glicerofosfodiester fosfodiesterasas en *S. coelicolor*. La expresión de dichos genes responde tanto a la concentración de fosfato en el medio como a la fuente de carbono añadida. Esto indica una implicación de la regulación por carbono en la expresión de genes del regulón *pho*.

5. Anexo



5. Anexo

5.1. Sitios de unión de PhoP en *S. coelicolor*: modelo I

Hasta el momento actual este grupo ha determinado 27 sitios de unión de la proteína PhoP en el genoma de *S. coelicolor*. Todos los sitios a excepción de dos (ver tabla 5.1) pueden ser clasificados de acuerdo con el modelo I publicado por Sola-Landa *et al*. (2008). Este modelo tiene en cuenta el número de complejos de retraso observados en los estudios de EMSA, la secuencia protegida determinada en los estudios de protección frente a DNasa I y el valor de conservación de las distintas repeticiones, obtenido a partir de programas informáticos basados en la teoría de la información descrita por Schneider (1997). Según esta teoría la conservación de cada base y su contribución al contenido total de información de la repetición se mide en bites. De este modo el contenido de información máximo para cada base es dos.

Las premisas del modelo I son: 1) un monómero de PhoP interacciona con una secuencia de 11 nucleótidos mediante contactos específicos entre los aminoácidos y las bases nitrogenadas (Blanco *et al*., 2002; Yamane *et al*., 2008); 2) se necesita un mínimo de dos repeticiones directas de 11 nucleótidos para la unión de PhoP al DNA (Sola-Landa *et al*., 2008); 3) la unión de monómeros de PhoP a secuencias consecutivas es favorecida por las interacciones proteína-proteína (Birck *et al*., 2003; Chen *et al*., 2003; Perron-Savard *et al*., 2005; Sinha *et al*., 2008); 4) la unión de un monómero a una determinada repetición es más fuerte cuanto más conservada esté la secuencia.

El modelo distingue dos tipos de repeticiones en los operadores de PhoP: unas constituyen el núcleo del operador (simbolizadas por una C del inglés *core*) y otras constituyen la extensión (E). El núcleo comprende la estructura mínima requerida para la unión de PhoP y está siempre formado por 2 ó 3 repeticiones, dependiendo de la mayor o menor conservación de las mismas, respectivamente. En los estudios de EMSA, la unión de PhoP al núcleo resulta en una sola banda de retraso (todas las repeticiones son unidas al mismo tiempo). Las repeticiones de extensión (E) son las secuencias a las que se unen monómeros de PhoP tras la ocupación previa del núcleo. Esto tiene como consecuencia la formación de varios complejos de retraso en los

estudios de EMSA. Existen tres mecanismos que explican la unión secuencial de PhoP al DNA:

a) Las repeticiones de extensión (E) ubicadas adyacentes al núcleo presentan un grado de conservación menor, lo que hace que la unión sea más inestable.
b) Existe una repetición muy poco conservada, que por su efecto se denomina inestable (E_U, del inglés *unstable*), situada entre el núcleo y una repetición conservada. La repetición conservada tiene una función de apoyo (E_S, del inglés *support*) que permite la unión de forma conjunta de dos monómeros a ambas repeticiones.
c) Existe una separación de 1 ó 2 nucleótidos entre dos repeticiones que dificulta las interacciones proteína-proteína. La repetición separada tiene también una función de apoyo, por lo que es denominda (E_S).

Como ejemplo de acción de estos mecanismos se tiene el caso del operador del gen *pitH2* (Santos-Beneit *et al*., 2008) que incluye dos (b y c) de los tres mecanismos mencionados (véase figura 5.1).

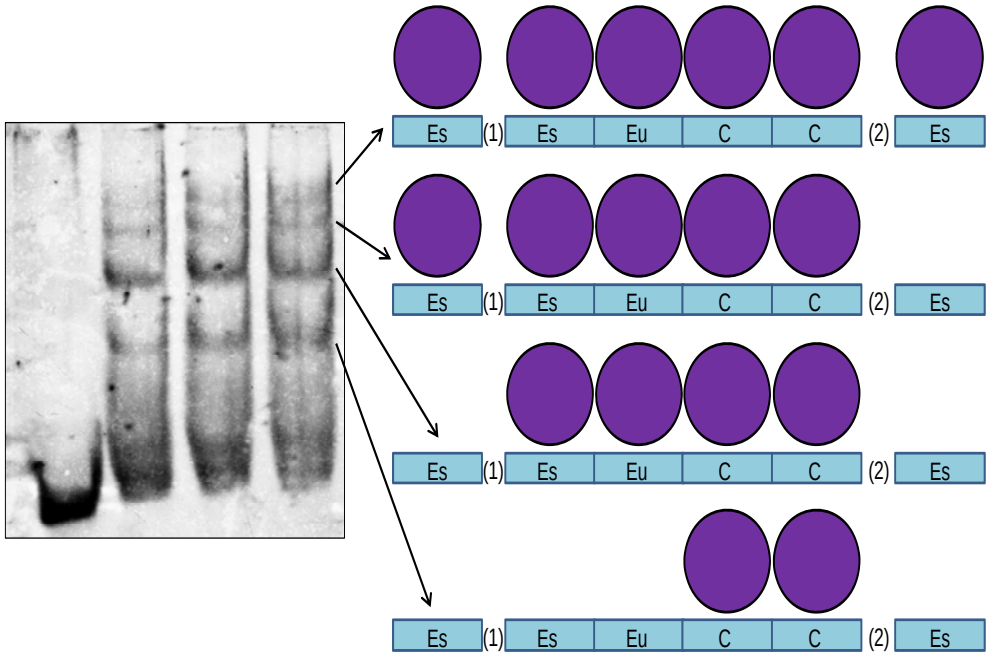


Figura 5.1: Representación de la unión de monómeros de PhoP al operador de *pitH2* según el modelo I.

	Gen	Estructura operadora	Clase	Distancia al ATG	Regulación	Cita
1	*phoA*	CC	I	128	Activación	Apel *et al*., 2007
2	*afsS*	CC	I	162	Represión	Santos-Beneit *et al*., 2009a
3	SCO3790	CC	I	79	Activación	Sola-Landa *et al*., 2008
4	SCO6169	CC	I	195	ND	Sola-Landa *et al*., 2008
5	SCO6372	CC	I	57	ND	Santos-Beneit (no publicado)
6	SCO0034	CCC	II	127	Activación	Sola-Landa *et al*., 2008
7	SCO1906	CCC	II	92	ND	Sola-Landa *et al*., 2008
8	SCO4261	CCC	II	43	Represión	Sola-Landa *et al*., 2008
9	*pstS*	EECC	III	114	Activación	Sola-Landa *et al*., 2005
10	*phoU*	CCEuEs	III	109	Activación	Sola-Landa *et al*., 2005
11	*pitH2*	Es[1]EsEuCC[2]Es	III	123	Activación	Santos-Beneit *et al*., 2008
12	*phoD*	CCC/Es[1]EE	III	52/10	Activación	Apel *et al*., 2007
13	*hrdA*	ECC	III	80	Represión	Sola-Landa *et al*., 2008
14	*glpQ1*	Es[1]CCE	III	49	Activación	Santos-Beneit *et al*., 2009b
15	*glpQ2*	Es[2]CCEuEs	III	48	Activación	Santos-Beneit *et al*., 2009b
16	*amtB*	ECC	III	149	Represión	Rodríguez-García *et al*., 2009
17	*glnR*	CCEE	III	108	Represión	Rodríguez-García *et al*., 2009
18	*glnII*	CCEEuEs	III	87	Represión	Rodríguez-García *et al*., 2009
19	*glnA*	Es[1]CC	III	103	Represión	Rodríguez-García *et al*., 2009
20	SCO1196	CCC[2]Es	III	83	Activación	Sola-Landa *et al*., 2008
21	SCO1394	ECC	III	30	Activación	Sola-Landa *et al*., 2008
22	SCO2262	ECC	III	70	Represión	Sola-Landa *et al*., 2008
23	SCO2878	CCCEuEs	III	42	Activación	Sola-Landa *et al*., 2008
24	SCO5447	ECCE	III	214	Activación	Sola-Landa *et al*., 2008
25	SCO7697	CCEE	III	122	Activación	Sola-Landa *et al*., 2008
26	SCO4878	4 DRu (NC)	NC	40	ND	Sola-Landa *et al*., 2008
27	SCO4879	5 DRu (NC)	NC	30	Activación	Sola-Landa *et al*., 2008

Tabla 5.1: Tabla con los 27 sitios de unión de PhoP determinados en *S. coelicolor* hasta el momento actual. Los operadores se clasifican en tres clases diferentes: I, II y III, de acuerdo con el artículo de Sola-Landa *et al*. (2008). Se clasifican en la clase I y II cuando están formados por dos y tres repeticiones nucleares, respectivamente. En la clase III se incluyen los operadores complejos formados por repeticiones nucleares y de extensión. El número entre corchetes en las estructuras operadoras representa la distancia de separación (en nucleótidos) entre las repeticiones de 11 nt. La nomenclatura NC significa estructuras no clasificadas, debido a que los resultados de los experimentos de EMSA no son concluyentes. En dichos casos se ha indicado el número de DRus obtenido según los ensayos de protección. La regulación ejercida por PhoP en los operadores determinados en la publicación de Sola-Landa *et al*. (2008) está determinada en relación a los estudios transcriptómicos publicados por Rodríguez-García *et al*. (2007). Cuando la regulación figura como ND significa que no ha podido ser determinada mediante dichos estudios transcriptómicos. La regulación ejercida por PhoP en los operadores del resto de las publicaciones ha sido determinada mediante dichos estudios transcriptómicos además de por estudios de la actividad promotora acoplada a genes testigo (*xylE* y *luxAB*).

5.2. Herramientas genéticas desarrolladas

5.2.1. Construcción de los vectores sonda de promotores: pLUXAR+ y pLUXAR-neo

El análisis directo de la expresión de genes mediante la determinación de la cantidad de transcrito o proteína es un método costoso y lento que presenta además bastantes limitaciones para estudios cuantitativos. El uso de genes testigo de fácil detección y cuantificación, es por tanto, de gran importancia para estudios de genética molecular. Un amplio número de genes testigo han sido utilizados en *Streptomyces*, entre los que destacan los siguientes: *amy*, *melC*, *neo*, *cat*, *xylE*, *luxAB* y *egfp*. Todos tienen ventajas e inconvenientes, lo cual ha provocado que ninguno de ellos haya predominado en el análisis de la expresión génica en *Streptomyces*. Uno de los más utilizados actualmente en biología celular es el gen que codifica la proteína verde fluorescente (*egfp*). Sin embargo, no es muy adecuado para los microorganismos que exhiben niveles significativos de autoflorescencia, como es el caso de *Streptomyces* (Sun *et al*., 1999). Por el contrario, *Streptomyces* y la mayoría de las bacterias no producen bioluminiscencia por lo que actividades promotoras muy bajas pueden ser detectadas con los genes testigo de la luciferasa.

La luz emitida por la reacción catalizada por la luciferasa ($FMNH_2$+O_2+aldehído de cadena larga → luz) puede ser detectada y cuantificada utilizando luminómetros. El sistema de la luciferasa reúne los requisitos necesarios de un buen testigo: rapidez, sensibilidad y amplio rango de linealidad. Dos tipos de luciferasas han sido utilizadas principalmente para estudios de genética molecular. La luciferasa de la luciérnaga (gen *luc*), utilizada únicamente en eucariotas y la luciferasa de *Vibrio harveyi* (genes *luxAB*), utilizada principalmente en procariotas. Recientemente el operón *luxCDABE* de la bacteria *Photorhabdus luminescens* se ha adaptado para su uso en bacterias de gran contenido en G+C como *Streptomyces*. Este operón codifica, además de la enzima luciferasa, la síntesis del sustrato aldehído necesario para la reacción (Craney *et al*., 2007).

En esta tesis se han desarrollado dos vectores sonda de promotores, pLUXAR+ y pLUXAR-neo, basados en la luciferasa de *Vibrio harveyi*. Los genes *luxAB* que

contienen han sido modificados genéticamente para eliminar el uso de codones raros en *Streptomyces* y fueron obtenidos de un fragmento extraído del vector pAR933a publicado por Rodríguez-García *et al*. (2005). En la figura 5.2 se muestra el vector pLUXAR-neo.

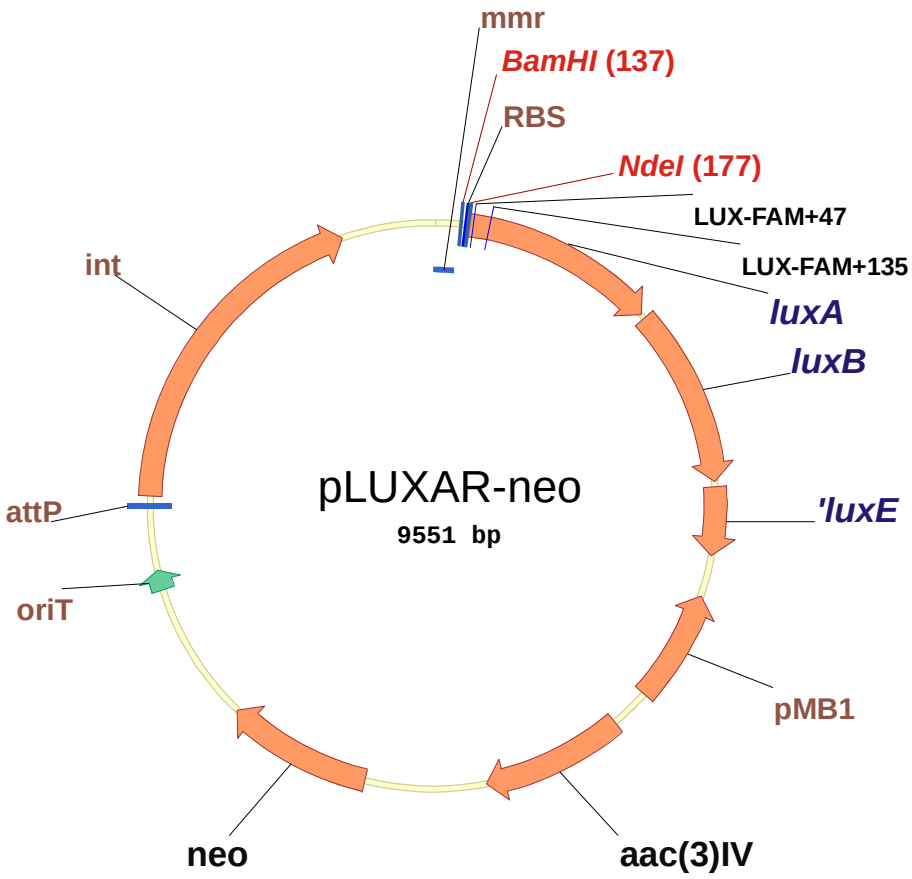


Figura 5.2: Representación del vector pLUXAR-neo obtenida a partir del programa *Vector NTI*. Lux-FAM+47 y Lux-FAM+135 representan el lugar de hibridación de dos oligos diseñados para realizar análisis de extensión del cebador (ver punto 5.2.2). '*luxE* es un fragmento del gen corriente abajo de *luxB*.

Las características de los vectores pLUXAR+ y pLUXAR-neo son:

- Portan el origen de replicación pMB1 que les hace ser funcionales en *E. coli*. Además, contienen un origen de transferencia (*oriT*) que permite introducir los vectores por conjugación.

- Contienen la integrasa del fago ΦC31 cuyo lugar de integración (*attP*) en el cromosoma de *S. coelicolor* es conocido (SCO3798; Combes *et al*., 2002).
- Contienen dos cortes de restricción para la clonación de promotores. El corte *NdeI* coincide con el ATG del codón de inició del gen *luxAB*, por lo que permite acoplar el promotor de estudio al gen testigo de forma que se consideran las características de la secuencia que influyen en la expresión, como el sitio de unión al ribosoma (RBS).
- Aguas arriba del sitio de clonación portan un terminador de la transcripción que evita la transcripción del gen testigo por actividades promotoras distintas a las del promotor de estudio. El terminador proviene del gen *mmr* de *S. coelicolor*.
- Como marcador de resistencia, pLUXAR+ utiliza el gen de resistencia a apramicina *aac*(3)IV. La versión pLUXAR-neo incluye además el gen *neo* de resistencia a neomicina.

Las ventajas de la herramienta desarrollada son las siguientes:

- ✓ El sistema informante de la luciferasa permite detectar pequeñas diferencias de expresión, lo cual convierte al vector en una buena herramienta para análisis cuantitativos.
- ✓ Al tratarse de un vector integrativo, no replicativo, el promotor de estudio acoplado al gen testigo se encuentra en una o dos copias (en la integración a veces se introducen dos copias en tándem en vez de una); lo cual evita el secuestro de proteínas reguladoras que puedan unirse a dicho promotor falseando los resultados, como cuando se usan vectores multicopia (Kieser *et al*., 2000).
- ✓ La herramienta se puede utilizar para cultivos en medio sólido y líquido.

o Medio sólido: el cultivo se hace directamente sobre los pocillos de una microplaca de 96 pocillos. El crecimiento se determina por densidad óptica (450 nm) y la luminiscencia añadiendo el sustrato aldehído (decanal) directamente sobre el pocillo. La desventaja del método es que solo se puede medir la actividad promotora a un tiempo ya

que el decanal hace que no sea viable la continuidad del cultivo. La ventaja es que permite probar muchas condiciones de cultivo y de cepas en una sola microplaca.

- Medio líquido: los cultivos líquidos permiten coger varias muestras en una serie temporal. La principal desventaja es que la disponibilidad de matraces o fermentadores limita el número de condiciones que se pueden ensayar. La gran ventaja es que se pueden estudiar series temporales y el efecto de la adición de determinados nutrientes o efectores. Los cultivos líquidos permiten, además, una medida precisa del crecimiento y de la producción de antibióticos.

En la tesis no se incluyen resultados de luminiscencia utilizando la técnica de cultivos en medio sólido. A continuación se muestra un ejemplo con los promotores *pstS* y *afsS* en condiciones limitantes y no limitantes de fosfato.

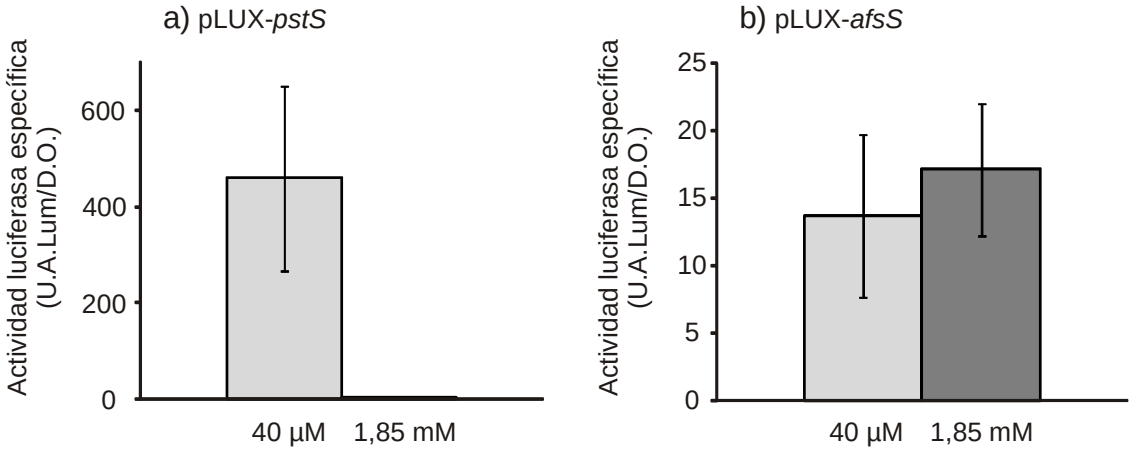


Figura 5.3: Los cultivos se realizaron sobre una microplaca de 96 pocillos con 0,3 mL de medio R5 (con 1,5 % de agar Difco) en cada pocillo. Las concentraciones de Pi para el exceso (1,85 mM) y el defecto (40 µM) fueron las mismas que las descritas en el artículo de Sola-Landa *et al*. (2005). En cada pocillo se añadieron 4 µL de una solución de esporas de $2{,}5 \times 10^8$ esp/mL de *S. coelicolor* M145 conteniendo el plásmido pLUX-*pstS* (a) ó pLUX-*afsS* (b). Las placas se incubaron a 30º C durante 24 horas (hasta una DO_{450} alrededor de 0,75). La luminiscencia se determinó añadiendo a cada pocillo 20 µl de decanal al 0,1 % y midiendo durante un tiempo de integración de 40 segundos. El valor de cada condición representa la media de los valores de luminiscencia específica obtenidos en 16 pocillos (2 columnas de pocillos por condición) y como estadístico se representa el error típico.

5.2.2. Diseño de los oligos marcados con fluorescencia, LUX-FAM+47 y LUX-FAM+135, para determinar el inicio de la transcripción mediante extensión de cebadores

Clásicamente la determinación del inicio de la transcripción por extensión de un cebador complementario del RNA mensajero (*primer extension*) ha implicado la necesidad de utilizar marcajes radiactivos y geles de electroforesis. Esta técnica se facilita utilizando oligonucleótidos marcados con fluoróforos y resolviendo los productos de la transcripción reversa por electroforesis capilar. Respecto del método radiactivo, además de eliminar el manejo de radiactividad, mejora la sensibilidad. Respecto al uso de geles de poliacrilamida mejora la resolución y posterior análisis de los datos.

A pesar de esto, apenas existen trabajos que fusionen ambas características. Para la puesta a punto del procedimiento nos hemos basado en la experiencia previa de nuestro laboratorio en ensayos de protección, y asimismo en ensayos de extensión del cebador, utilizando cebadores fluorescentes y un secuenciador ALF (Amersham Biosciences), que utiliza geles de poliacrilamida. Basándonos en los artículos de Altermann *et al*. (1999) y Fekete *et al*. (2003) se ha sustituido el uso de un secuenciador ALF por el de un secuenciador ABI de electroforesis capilar (Applied Biosystems). El funcionamiento del método se ha puesto a punto tanto para ensayos de extensión del cebador como de protección frente a DNasa I. Los protocolos desarrollados se detallan en las publicaciones de Santos-Beneit *et al*. (2008) y Santos-Beneit *et al*. (2009a), respectivamente.

Para poder determinar el inicio de transcripción de los genes estudiados de una forma eficiente, evitando tener que hacer nuevas construcciones en multicopia o diseñar numerosos oligos por cada gen de estudio, se diseñaron dos oligos complementarios a la cadena codificante del gen *luxA* de la siguiente forma:

LUX-FAM+47: 5´- GATAGCTCAGGTGGCTGATAAG

Complementario a la región codificante (25-47) del gen *luxA*. Está marcado en el extremo 5' con el fluoróforo 6-FAM, pedido a MWG-Biotech (Bonsai) y purificado por HPLC. Las características del oligo según las instrucciones del comerciante son las siguientes: longitud de 22 nt, contenido en G+C de 50%, Tm de 60.3º C y peso molecular de 7377 g/mol.

LUX-FAM+135: 5´- GTGGTGCTCTAGCAACCAAAC

Complementario a la región codificante (114-135) del gen *luxA*. Está marcado en el extremo 5' con el fluoróforo 6-FAM, pedido a MWG-Biotech (Bonsai) y purificado por HPLC. Las características del oligo según las instrucciones del comerciante son las siguientes: longitud de 21 nt, contenido en G+C de 52%, Tm de 59.8º C y peso molecular de 6953 g/mol.

Las ventajas de la herramienta desarrollada son las siguientes:

- ✓ Se puede determinar el inicio de transcripción de todos los promotores en estudio, utilizando la construcción correspondiente derivada de pLUXAR.
- ✓ El resultado adquiere fiabilidad al utilizarse dos oligos distintos.
- ✓ Se pueden correlacionar los valores de expresión de una serie temporal con el inicio de transcripción usando los mismos cultivos.

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6. Agradecimientos

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