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Extracellular sugar phosphates are assimilated by *Streptomyces* in a PhoP-dependent manner

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Abstract Filamentous microorganisms of the bacterial genus *Streptomyces* have a complex life cycle that includes physiological and morphological differentiations. It is now fairly well accepted that lysis of *Streptomyces* vegetative mycelium induced by programmed cell death (PCD) provides the required nutritive sources for the bacterium to erect spore-forming aerial hyphae. However, little is known regarding cellular compounds released during PCD and the contribution of these molecules to the feeding of surviving cells in order to allow them to reach the late stages of the developmental program. In this work we assessed the effect of extracellular sugar phosphates (that are likely to be released in the environment upon cell lysis) on the differentiation processes.

We demonstrated that the supply of phosphorylated sugars, under inorganic phosphate limitation, delays the occurrence of the second round of PCD, blocks streptomycetes life cycle at the vegetative state and inhibits antibiotic production. The mechanism by which sugar phosphates affect development was shown to involve genes of the Pho regulon that are under the positive control of the two component system PhoR/PhoP. Indeed, the inactivation of the response regulator *phoP* of *Streptomyces lividans* prevented the 'sugar phosphate effect' whereas the *S. lividans ppk* (polyphosphate kinase) deletion mutant, known to overexpress the Pho regulon, presented an enhanced response to phosphorylated sugars.

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Introduction

Streptomyces species belong to the soil-dwelling bacterial genus responsible for the production of about two-thirds of useful natural products of microbial origin, including antibiotics (Hopwood 2007). These mycelial bacteria follow a complex developmental program that includes two types of interrelated differentiation i.e., (i) a morphological differentiation where aerial hyphae, whose tips ends will differentiate

into spores, arise from the substrate (vegetative) mycelium and (ii) a physiological/metabolic differentiation characterized by the production of the so-called secondary metabolites. The onset of both types of differentiation correlates with one of the most sophisticated examples of a programmed cell death (PCD) process in prokaryotes (Granozzi et al. 1990; Miguelez et al. 1999). The latest model suggests two rounds of PCD during *Streptomyces* life cycle (Manteca et al. 2011). After spore germination, a compartmentalized mycelium grows until it undergoes a first PCD round that is followed by the development of a multinucleated mycelium still devoted to the vegetative life style. The second round of PCD starts when this mycelium produce surfactants-like peptides/proteins necessary for aerial growth (Claessen et al. 2006). This round of PCD is thought to correspond to the short period of growth cessation called the transition phase that takes places during the exponential growth and is followed by a slower regain of growth before entry into stationary phase (Granozzi et al. 1990; Puglia et al. 1995; Manteca et al. 2005). The cell death paroxysm at the transition phase causes intensive release of intracellular compounds into the extracellular environment (Manteca et al. 2006). The latter are thought to serve as nutritive sources for surviving vegetative filaments to grow and differentiate in the aerial structures (Mendez et al. 1985). This form of 'altruistic cannibalism' or 'programmed autophagy' is now commonly admitted but almost nothing is known about the assimilation of the cellular compounds released during PCD (lipids, nucleic acids, proteins, phosphorylated sugars, polyphosphate, ions, etc.). In addition, beyond a nutritive role, the sensing of these cellular compounds by the surviving cells might also constitute a decisive signal for the developmental program.

In this work we demonstrated that, on the specific carbon rich and inorganic phosphate (P_i) limited medium R2YE, the exogenous supply of phosphorylated sugars, which are most likely released in the course of the PCD process, leads to a delay in the differentiation process that we called the 'extracellular sugar phosphate (exSP) effect'. In contrast, exogenous addition of their cognate un-phosphorylated sugar had no effect on development whereas the addition of free P_i had a similar effect than addition of exSPs suggesting a major contribution of the phosphate moiety. Indeed we demonstrated that the 'exSPs effect' requires the response regulator PhoP that governs the adaptation of the bacteria to P_i limitation.

Materials and methods

Bacterial strains, media, and culture conditions

Streptomyces standard techniques were performed as described in (Kieser et al. 2000). Strains were cultivated on the carbon rich and P_i limited medium solid R2YE medium. Glucose, fructose, glycerol, glucosamine, glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, glucosamine-6-phosphate, and glycerol-3-phosphate were purchased from Sigma-Aldrich. 100 Mm NaH_2PO_4/K_2HPO_4 buffer (pH 7.2) was used as source of inorganic phosphate (P_i). *Streptomyces* strains used in this study are listed in Table 1.

Table 1 List of *Streptomyces* strains used in this study

Streptomyces strains	Genotype	Origin or reference
Streptomyces coelicolor M145	SCP1⁻, SCP2⁻, parental strain	Gift John Innes Center (UK)
Streptomyces lividans TK24		Gift John Innes Center (UK)
Streptomyces lividans Δppk	ppk::Ωhygro	Chouayekh and Virolle (2002)
Streptomyces lividans ΔphoP	phoP::Ωaac	Ghorbel et al. (2006)
Streptomyces avermitilis		Gift Leiden Institute of Chemistry (NL)
Streptomyces cacaoi DSM 40057		Gift National Defense Research Institute (S)
Streptomyces clavuligerus		Gift Leiden Institute of Chemistry (NL)

Scanning electron microscopy

Morphological studies of surface-grown vegetative and aerial hyphae of *S. coelicolor* M145 by cryo-scanning electron microscopy were performed as follows. Samples were dehydrated through an ethanol series, critical-point dried and platinum coated (20 nm) in a Balzers SCD-030 sputter-unit before observation using SEM (JEOL JSM-840A) at 20 kV accelerating voltage (Corbari et al. 2012).

Confocal microscopy

Surface-grown *Streptomyces* cultures were processed for microscopy as described previously (Manteca et al. 2005). The permeability assay previously described for *Streptomyces* was used to stain all samples. Staining of dead and viable cell populations were performed with the cell-impermeant nucleic acid stain (propidium iodide, PI) and with SYTO 9 green fluorescent nucleic acid, respectively, and according to the manufacturer's instructions (LIVE/DEAD Bac-Light Bacterial Viability Kit, Molecular Probes, L-13 152). Samples were then examined under a Leica TCS-SP5 confocal laser-scanning microscope at a wavelength of 488 and 568 nm for excitation and 530 nm (green) or 630 nm (red) for emission. Images were mixed using the Leica confocal software.

Results

Effect of extracellular sugar-phosphates on *Streptomyces* development

We assessed the possibility that exSPs could be used as nutrients by supplying exponentially growing vegetative mycelia of *S. coelicolor* M145 and *S. lividans* TK24 with glucose-6-phosphate (Glc-6-P), glycerol-3-phosphate (Gly-3-P), and their non-phosphorylated forms i.e., glucose (Glc) and glycerol (Gly). As shown in Fig. 1a, Gly and Glc had no effect on development when added after 20 h of growth to the carbon rich and phosphate limited medium, R2YE. In contrast, Glc-6-P, Gly-3-P as well as P_i retarded the timing of production of pigmented antibiotics prodiginines (Red) and actinorhodin (Act) (Fig. 1a). The inhibition of antibiotic production in *S. lividans* correlated with a strong delay of sporulation when observed at latter time points (Fig. 1b). Similar developmental delays were observed with other *Streptomyces* spp. (*S. avermitilis*, *S. clavuligerus*, and *S. cacaoi*; Fig. 1b) in the presence of all exSPs tested (data not shown).

Scanning electron microscopy after 40 h of growth on R2YE confirmed that the delay in *S. coelicolor* pigmented antibiotic production (physiological differentiation), induced by exSPs, is also accompanied by a delay in morphological differentiation. Indeed, filaments in contact with Fru-1,6-BP are still at the vegetative state (top part of Fig. 2, zone 3) whereas filaments out of influence of the exSP or in contact with fructose already switched to the aerial life style with their hyphal tips pointing vertically towards the surface of the plate (middle part of Fig. 2, zones 1, 2, and 4). Consistently, staining viable or dying filaments of *S. coelicolor* revealed that only the zone under the influence of Fru-1,6-BP still showed dense accumulation of viable cells at the culture surface while other zones of the plates presented almost exclusively dying cells (bottom part of Fig. 2). These observations indicate a clear delay in the onset of the second round of PCD in hyphae influenced by Fru-1,6-BP. Similar retardation of the occurrence of the second round of PCD was observed in *S. clavuligerus* (supplementary Fig. S2) and *S. lividans* (data not shown) using Gly-3-P as exSP source. These results suggest that phosphate limitation is a main trigger factor of the PCD-mediated developmental process on this P_i limited medium.

Interestingly, the model strain *S. coelicolor* M145 showed, as other species tested, a delay in development after 44 and 64 h of incubation (upper part of Fig. 2 and Fig. 1a, respectively), whereas it presented an accelerated onset of sporulation after 96 h of incubation (Fig. 1b). This peculiar behaviour of *S. coelicolor* was confirmed by further tests with glucosamine-6-phosphate, fructose-6-phosphate (Fru-6-P) and fructose-1,6-bisphosphate (Fru-1,6-BP) (Supplementary Fig. S1). This intriguing 'biphasic' (delayed then accelerated development) will be discussed later on (see "Discussion" section).

The utilization of sugar phosphates is PhoP-dependent

Theoretically, exSPs could be up-taken as such by a specific transporter similar to the *uhp* (uptake of hexose phosphate) system of enteroenvasive *Escherichia coli* and *Salmonella enterica* (Götz and Göbel 2010).
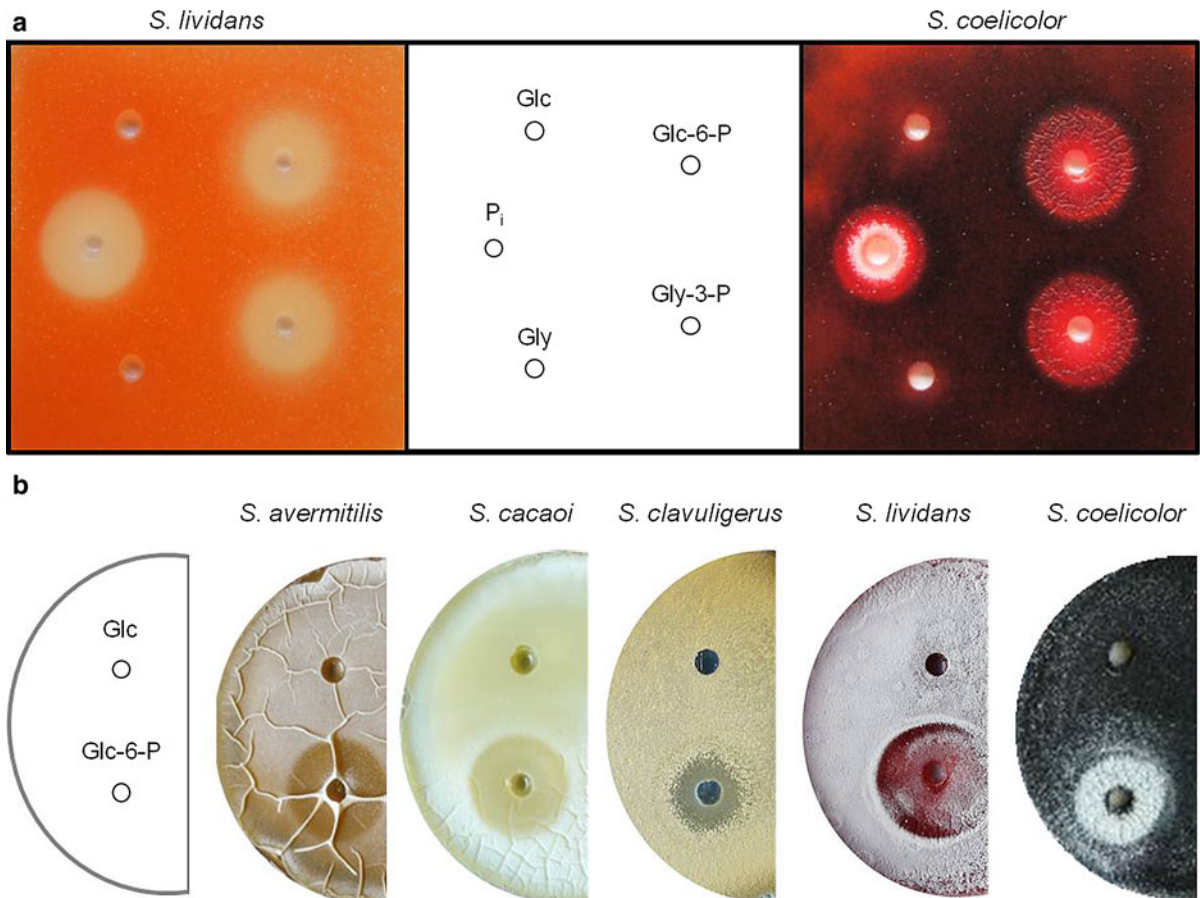
Fig. 1 Sugar phosphates delay development in *Streptomyces*.
a 10^7 spores of *S. lividans* TK24 and *S. coelicolor* M145 were
plated on R2YE solid medium. After ∼20 h of growth, wells
(5 mm diameter) were made into the agar and 100 mM of
glycerol (Gly), glucose (Glc), glycerol-3-phosphate (Gly-3-P),
glucose-6-phosphate (Glc-6-P), and inorganic phosphate (P_i)
were deposited into them. Pictures were taken after 64 h of
incubation at 28 °C. Note the retarded *Red* production in
S. lividans and *Act* production in *S. coelicolor*. **b** Glc-6-P blocks
the onset of sporulation in all *Streptomyces* species tested while
it accelerates aerial hyphae formation in *S. coelicolor* M145
after 96 h of incubation at 28 °C. The exogenous supply
100 mM of Glc (*top well*) and Glc-6-P (*bottom well*) was made
as described above

Alternatively the sugar phosphates can be dephosphorylated by extracellular phosphatases, the carbon moiety being taken-up by the usual transport system of the cognate sugar and the P_i moiety by known low or high affinity phosphate uptake systems (PitH1 and PitH2, or PstSCAB, (Esteban et al. 2008; Santos-Beneit et al. 2008). Since all the un-phosphorylated carbon sources tested had no impact on development whereas exogenous supply of P_i led to a developmental delay similar (although not identical) to that observed with exSPs (Fig. 1a), we postulated that the effect of exSPs was mostly due to their P moiety. Indeed, the exSPs effect extends to a wider area when *S. coelicolor* was subjected to exogenous supply of di-phosphorylated

fructose compared to its mono-phosphorylated form (Supplementary Fig. S1) suggesting a dose effect of phosphate.

In order to test this hypothesis, the effect of exogenous supply of exSPs and of P_i on development was assessed in the *phoP* mutant of *S. lividans*. PhoP is the transcriptional response regulator of the two component system (TCS) *phoR/phoP* that governs the adaptation of the bacteria to a limitation in P_i. Upon P_i limitation the kinase PhoR autophosphorylates and then transfers its phosphate to the response regulator PhoP. Phosphorylated PhoP is then able to drive the expression of genes of the very large *pho* regulon which includes, amongst others, all genes
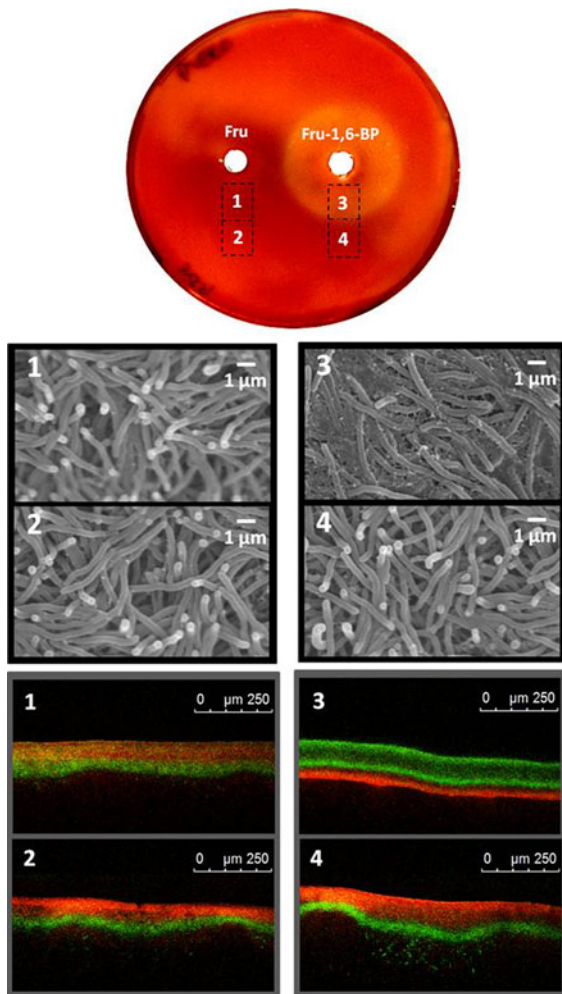
Fig. 2 Sugar phosphates delay the onset of aerial hyphae and the second round of PCD. *S. coelicolor* M145 was plated on R2YE and exposed to fructose (Fru) or fructose-1,6-biphosphate (Fru-1,6-BP) as described in Fig. 1. The picture on the *top* was taken after 44 h of growth (transition phase). Note the reduced production of *Red* around the well containing the exSP. *Zones 1, 2, 3,* and *4* were analyzed by cryo-scanning electron microscopy (*middle part*). Note the absence of aerial hyphal tips within the exSP diffusion area (*zone 3*). Thin slices of mycelium were cut from the plate and stained with the LIVE/DEAD Bac-Light Bacterial Viability Kit (Invitrogen). Samples were observed under a Leica TCS SP5 II confocal laser-scanning microscope at wavelengths of 488 and 568 nm for excitation and 530 nm (*green*, living cells) or 630 nm (*red*, dying cells) for emission (*bottom part*). Note that the surface of the bacterial culture treated with Fru-1,6-BP (*zone 3*) essentially contains viable cells, while dying cells are mostly present at the surface of zones treated with fructose (*zone 1*) or outside the zone influenced by the exogenous supplied compounds (*zones 2* and *4*)

required for phosphate sources scavenging, uptake, utilisation and storage (Sola-Landa et al. 2003; Sola-Landa et al. 2005). This TCS was shown to be highly and constitutively expressed on the carbon rich and P_i limited medium (R2YE) used in this study (Darbon et al. 2012).

We observe that the exogenous addition of P_i was still able to block antibiotic production in the *S. lividans* $\Delta phoP$ mutant (Fig. 3a) whereas exogenous supply of Glc-1-P or Gly-3-P was not able to inhibit antibiotic (prodiginines) production anymore in that background (Fig. 3a, b). These observations suggest that P_i is likely to be transported by the PhoP independent PitH1 system (Santos-Beneit et al. 2008) (Fig. 4) whereas exSPs utilisation involves genes of the Pho regulon under the positive control of PhoP. Interestingly, the polyphosphate kinase *ppk* mutant strain of *S. lividans*, in which the TCS PhoR/PhoP (and thus genes of the PhoP regulon) was shown to be over-expressed (Ghorbel et al. 2006), displays higher sensitivity to the exSPs than the parental strain (Fig. 3b).

Discussion

Numerous reports in the literature mention morphogenic molecules that are specifically involved in signalling networks governing metabolite production as well as aerial hyphae and spore formation in *Streptomyces* (Willey and Gaskell 2011). Although the role of ubiquist molecules in signalisation has been poorly investigated, several studies confirmed that these molecules are likely to signal specific metabolic state and thus also play important regulatory roles (Viollier et al. 2001a, b; Li et al. 2008; Rigali et al. 2008; Luti and Mavituna 2011). For instance, we have previously demonstrated that the peptidoglycan aminosugar building block *N*-acetylglucosamine (GlcNAc) has a different impact on development depending on the nutritional context and its concentration (Rigali et al. 2006, 2008; Nothaft et al. 2010; Craig et al. 2012; Swiatek et al. 2012).

Besides our studies on compounds emanating from the cell wall, we investigated here the possible role in development of cytoplasmic phosphorylated sugars spilled into the extracellular environment. We demonstrated the existence of a general mechanism that

Fig. 3 Sugar phosphates modulate the onset of development in a PhoP-dependent manner. **a** Spores of *S. lividans* TK24 (parental strain) and Δ*phoP*, were streaked on R2YE solid medium and the following addition of 100 mM of glycerol-3-phosphate (Gly-3-P, *top left well*), glucose-1-phosphate (Glc-1-P, *top right well*) or inorganic phosphate (Pi, *bottom wells*) were made as described in the legend of Fig. 1. Pictures were taken after 48 h of incubation at 28 °C. Note that inactivation of *phoP* prevents the 'exSPs effect' but not that of P_i on development. **b** The inactivation of the polyphosphate kinase encoding gene *ppk* renders *S. lividan*s more sensitive to the exSP
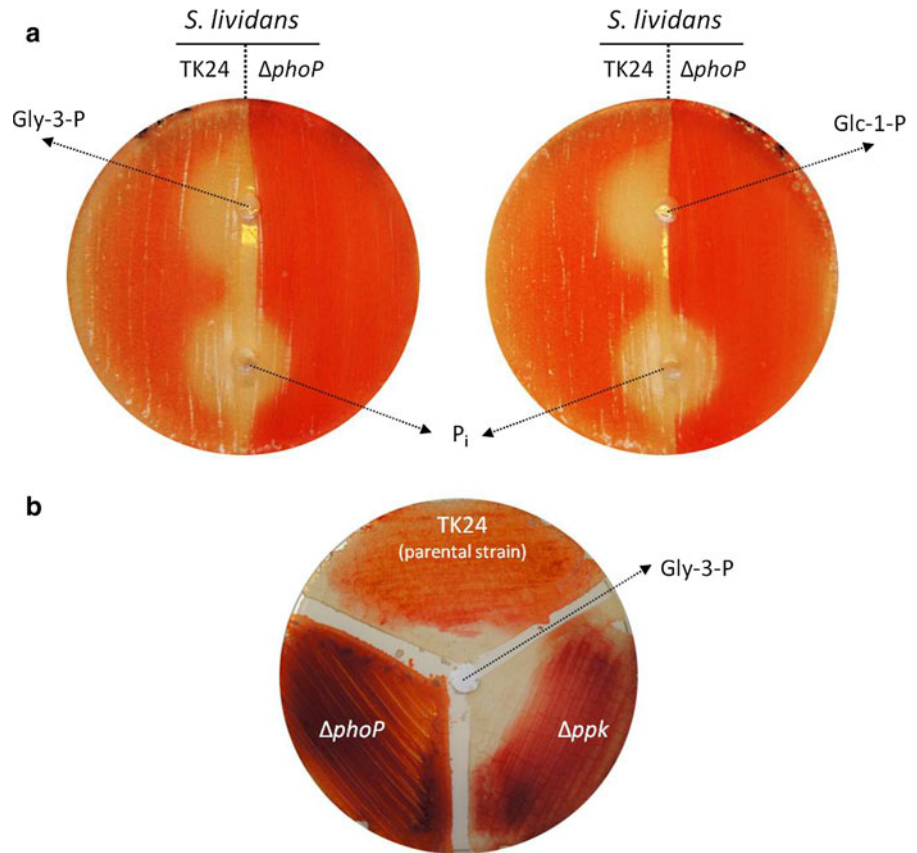
allows the utilization of sugar phosphates as nutrient leading to a delay in the timing of *Streptomyces* development (PCD, antibiotic production and sporulation). In the R2YE medium limited in P_i, where the expression of *phoR/phoP* was shown to be very high and constitutive (Darbon et al. 2012), the deletion of *phoP* in *S. lividans* suppresses the 'exSPs effect' on development but not that of exogenous addition of P_i. In order to rationalize these observations, we propose that the exSPs would be first dephosphorylated by PhoP-dependent secreted phosphatases (such as PhoA and PhoD) (Apel et al. 2007) and the resulting inorganic phosphate would enter the cell by classical medium or high affinity phosphate transporters (PitH2 or PstSCAB; Fig. 4).

However, at this stage of the work, we cannot totally exclude the possibility that exSPs would directly enter the cell via specific transporters similar to the *uhp* system (Götz and Göbel 2010) and controlled by PhoP. This hypothesis seems unlikely since a genomic scale analysis of possible carbohydrate transporters (Bertram et al. 2004) did not

identified, in *S. coelicolor*, an *uhp*-like uptake system. Alternatively, exSPs might be directly sensed by a specific still unknown sensory kinase that would also be able to activate PhoP (see Fig. 4 that summarizes the hypothetical ways exSPs could be sensed, uptaken and assimilated in streptomycetes.)

Interestingly, besides their nutritional function at high concentration, some of our results suggest that, at low concentration, the exSP might act as signalling molecules. The sensing of low level of such cellular compounds by the surviving cells might constitute, at the beginning of the second round of PCD, an inducing signal to trigger a cannibalistic behavior in those surviving cells and induce secondary metabolite production and sporulation (Claverys and Havarstein 2007; Gonzalez-Pastor 2011). Indeed, a sporulation ring is observed around the zone of delayed development of lawns of *S. coelicolor* and *S. lividans* under the influence of the exSP (zone 2 in Fig. 5). This observation suggests a dual 'nutrient/signal' function of exSPs. At a distance from the wells, where the concentration of the exSP is likely to be low and thus
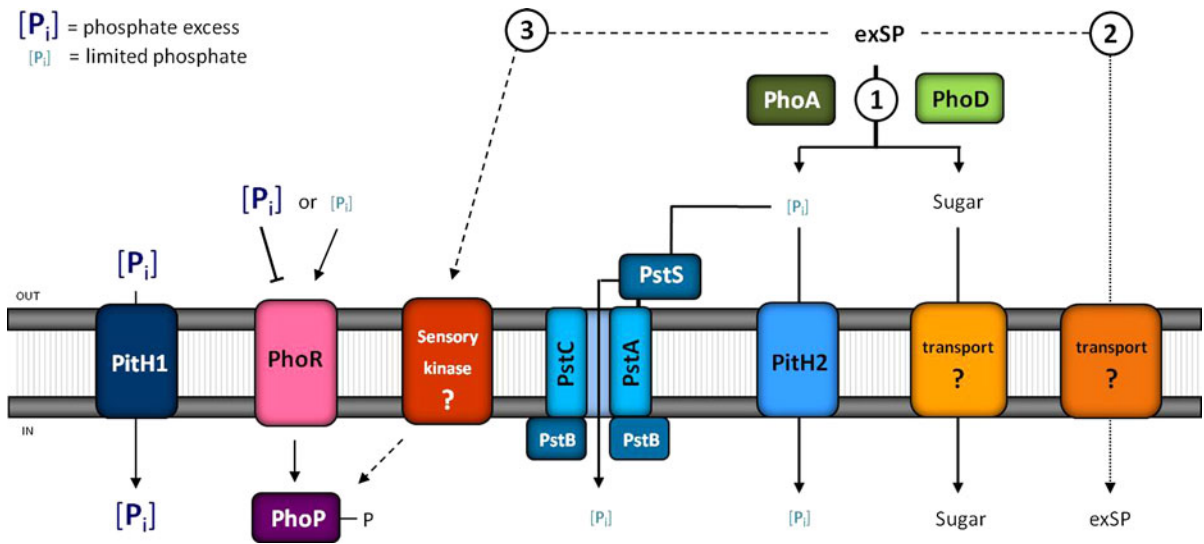
Fig. 4 Favored model (*continuous lines*, ①) of PhoP-dependant sugar phosphate assimilation. On R2YE, the two components system *phoR/phoP* and thus genes of the Pho regulon under its control, including secreted phosphatases (such as PhoA and PhoD), are highly and constitutively expressed. The secreted phosphatases would dephosphorylate exSPs and the released phosphate would be transported via the PhoP dependent medium and high affinity transporter PitH2 and PstSABC, respectively. Alternative model of putative PhoP-dependant direct sugar phosphate uptake (*doted lines*, ②): A direct transport into the cell of exSPs via a putative specific sugar-phosphates uptake system controlled by PhoP. Alternative model of putative sugar phosphate sensing (*dashed lines*, ③): The exSPs might be sensed by an unknown sensory kinase able to phosphorylate PhoP. P_i, inorganic phosphate, *PhoR* phosphate limitation two-component histidine kinase, *PhoP* phosphate limitation response regulator associated to PhoR, *PitH1* low-affinity P_i transporter under inorganic phosphate excess, *PitH2* P_i low-affinity transporter under phosphate limitation conditions, *PstABC* P_i high-affinity ABC-transporter. The model is deduced from the compilation of previous works (Sola-Landa et al. 2003, 2005; Diaz et al. 2005; Ghorbel et al. 2006; Apel et al. 2007; Esteban et al. 2008; Santos-Beneit et al. 2008)
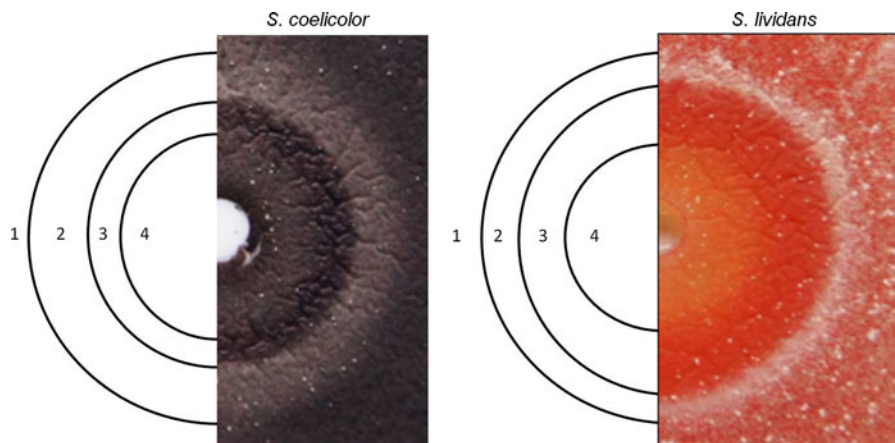

Fig. 5 Concentration dependence of the sugar-phosphates effect on *Streptomyces* development. *Close up* on the well of Fig. 1 with exogenous supply of Gly-3-P but taken after 72 h of incubation at 28 °C. In the plates, a gradient of exSP concentration is established around the wells, with the highest concentration around the wells (*zone 4*) and the lowest concentration at a distance from the wells in *zone 1*. In *S. coelicolor*, Gly-3-P has moderate enhancing effect on sporulation in *zone 4*, inhibits sporulation in *zone 3*, and accelerates sporulation in *zone 2*. In *S. lividans*, Gly-3-P inhibits *Red* production and sporulation in *zone 4*, inhibits sporulation but not *Red* production in *zone 3*, and accelerates sporulation in *zone 2*

when the nutritious effect is negligible, exSPs might signal the occurrence of an intensive autolysis linked to PCD that would inform *Streptomyces* that it is time to start its differentiation program. These observations are reminiscent from that made by Li and collaborators that have previously reported a similar effect of extracellular ATP on the physiology of *S. coelicolor* (Li et al. 2008). Furthermore, this effect might be related to the peculiar behaviour observed for *S. coelicolor* (Fig. 1b). Indeed, the accelerated onset of sporulation after 96 h of incubation might indicate that this strain assimilates exSPs at a higher rate than *S. lividans* and the other *Streptomyces* strains, reaching more quickly than the latter the critical low concentration of exSPs that triggers the developmental process.

Studies on the concentration-dependent effect of exSPs on development and on the molecular mechanisms by which exSPs might signal a metabolic/physiological state are currently under investigation.

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