Phosphoinositides Are Involved in Control of the Glucose-Dependent Growth Resumption That Follows the Transition Phase in *Streptomyces lividans*\(^\text{\textsuperscript{\textregistered}}\)

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The interruption of the *sblA* gene of *Streptomyces lividans* was previously shown to lead to relief of glucose repression of the normally strongly glucose-repressed α-amylase gene. In addition to this relief, an early entry into stationary phase was observed when cells were grown in a minimal medium containing glucose as the main carbon source. In this study, we re-established that this mutant does not resume growth after the transition phase when cultured in the complex glucose-rich liquid medium R2YE and sporulates much earlier than the wild-type strain when plated on solid R2YE. These phenotypic differences, which were abolished when glucose was omitted from the R2YE medium, correlated with a reduced glucose uptake ability of the *sblA* mutant strain. *sblA* was shown to encode a bifunctional enzyme possessing phospholipase C-like and phosphoinositide phosphatase activities. The cleavage of phosphoinositides by SblA seems necessary to trigger the glucose-dependent renewed growth that follows the transition phase. The transient expression of *sblA* that takes place just before the transition phase is consistent with a regulatory role for this gene during the late stages of growth. The tight temporal control of *sblA* expression was shown to depend on two operator sites. One, located just upstream of the −35 promoter region, likely constitutes a repressor binding site. The other, located 170 bp downstream of the GTG *sblA* translational start codon, may be involved in the regulation of the degradation of the *sblA* transcript. This study suggests that phosphoinositides constitute important regulatory molecules in *Streptomyces*, as they do in eukaryotes.

In spite of a large number of studies, the molecular basis of the phenomenon of glucose catabolite repression in the genus *Streptomyces* has yet to be established. The mutants isolated as resistant to glucose repression mainly affect the uptake of glucose via the DasABC transporter in *Streptomyces griseus* (39) or via the major facilitator GICP1 in *Streptomyces coelicolor* (39, 49) or affect the first step of glucose metabolism by the glucose kinase encoded by *glkA* (2) or, perhaps, affect the regulation of both glucose uptake and glucose kinase activity by the product of *SCO2127* (16). However, other mutants whose default in relation to glucose repression was less obvious were also characterized (8, 17, 20, 33). Our scientific interest for this subject led us to isolate mutants of *Streptomyces lividans* TK24 resistant to glucose catabolite repression by using transposon mutagenesis. We isolated and characterized a mutant that showed the expected relief of glucose repression of the expression of a normally strongly glucose-repressed alpha-amylase gene (14). In this mutant, the gene *sblA* (equivalent to SCO0479 of *S. coelicolor*) was interrupted. This gene was predicted to encode a protein of 274 amino acids bearing some similarities to phosphatases of the inositol phosphate monophosphatase family (14). Furthermore, the *sblA* mutant strain was shown to enter prematurely into stationary phase when grown in a minimal medium containing glucose as a main carbon source. In contrast, very little or no difference in the growth profile of the wild-type and mutant strains was observed when cells were grown in the presence of glycerol (14). In order to get a better understanding of the role of *sblA* in the phenomenon of glucose repression, we assessed the growth characteristics and glucose uptake abilities of the wild-type and the *sblA* mutant strains of *S. lividans* TK24 grown in the complex liquid or solid medium R2YE, rich or poor in glucose. We determined the enzymatic function and the substrate specificity of SblA, using in vitro and in vivo approaches. In addition, we identified some cis-acting regulatory sequences that play a role in the tight temporal control of *sblA* expression, using appropriate transcripational fusions of its 5′ region with the reporter genes *luxA* and *luxB* from *Vibrio harveyi*.

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**Materials and Methods**

**Strains and plasmids.** The Streptomyces strains used were the wild-type strain *S. lividans* TK24 (19), the *S. lividans* TK24 sblA::aac strain (14) and the *S. lividans* TK24 EcoRI::sblac sblA (this strain) derived from it as well as the *S. lividans* TK24 carrying the replicative vector pHCI002 or the integrative vectors pHCI013, pHCI023, pHCI053, pHCI063, and pHCI073 (Fig. 1).

(i) **Construction of the sblA gene by its disrupted version using the usual procedure (29).** The chromosomal structure and/or in the AGGAGG sequence, the original 521-bp SphI-BamHI DNA fragment was replaced by the “equivalent” 2,053-bp SphI-AgeI DNA fragment, yielding pHCI010 (in which the sequence of the stem-loop [SL*] and the AGGAGG sequence was intact).

(ii) **Introduction of various mutations in the stem-loop structure containing the sequence AGGAGG present 170 bp downstream of the SblA translational start codon.** The different primers used are listed in Table 2. In one case, the stem-loop was destroyed and an Xbal site (TCTAGA) was created as described below. The following two PCRs were performed using pHCI002 as the matrix:

- In reaction 1 primer D and primer M harboring an XhoI site were used; in reaction 2, primer J and primer N harboring an XhoI site were used. The PCR fragment 1 was cleaved by Agel (internal to sblA) and XhoI, and PCR fragment 2 was cleaved by XhoI and BamHI (internal to sblA).
- Both the resulting 145-bp AgeI-XhoI and 106-bp XhoI-BamHI fragments were cloned into pHCI010 in place of the original 251-bp AgeI-BamHI DNA fragment yielding pHCI060 (in which the sequence of the stem-loop [SL*] and the AGGAGG sequence were changed).

In this last case, the sequence AGGAGG was replaced by the sequence CT CGAG constituting an XhoI site, and compensatory mutations were introduced in the right part of the stem of the stem-loop in order to preserve the possibility of formation of the secondary structure. Two PCRs were performed using pHCI002 as the matrix: in reaction 1 primer D and primer M harboring an XhoI site were used; in reaction 2, primer J and primer N harboring an XhoI site were used. The PCR fragment 1 was cleaved by Agel (internal to sblA) and XhoI, and PCR fragment 2 was cleaved by XhoI and BamHI (internal to sblA). Both the resulting 145-bp AgeI-XhoI and 106-bp XhoI-BamHI fragments were cloned into pHCI010 in place of the original 251-bp AgeI-BamHI DNA fragment yielding pHCI060 (in which the sequence of the stem-loop [SL*] and the AGGAGG sequence were changed).

(iii) **Construction of the transcriptional fusions of the wild-type and the mutant sblA 5’ regions with the reporter genes lacZ and luxB.** First, the 2,794-bp partial BamHI-SalI DNA fragment from M13lacAB12 carrying the V. harveyi lacZ reporter genes and the 2,802-bp SalI-Asp718 DNA fragment from PlaB were inserted into the SalI site of the thermo-sensitive vector pGM160, a *Streptomyces* thermosensitive replicative plasmid by deletion of an HindIII fragment containing aacC1*.

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TABLE 2. Sequence of the PCR primers used in this study and their utilization

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Description or purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal reverse (−48)</td>
<td>5′-AGCGGATAACAATTTCAACAGGA-3′</td>
<td>Moving of 7 of the 9 DRs of the sequence 5′-C(C/G)GAGGG(C/T)-3′, located upstream of the −35 promoter sequence, away from the sblA promoter region. The EcoRI sites are underlined.</td>
</tr>
<tr>
<td>Universal (−47)</td>
<td>5′-CCAGAATTCTCCACCGGGCTCCCGACCT-3′</td>
<td>Destruction of the stem-loop and creation of an XhoI site. The AGGAGG sequence is intact. The XbaI sites are underlined.</td>
</tr>
<tr>
<td>L</td>
<td>5′-CGCCAGGTTTTTCCAGTCAGA-3′</td>
<td>Replacement of the sequence AGGAGG by the sequence CTTCAG, constituting an XhoI site, and introduction of compensatory mutations into the right-hand part of the stem-loop in order to preserve the formation of the secondary structure. The XhoI sites are underlined.</td>
</tr>
<tr>
<td>R</td>
<td>5′-CCGGAATTCGCCCGAGGCGCGGAAGCT-3′</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5′-CTGACCATATGCAACTTGGCTGTC-3′</td>
<td>Replacement of the sequence AGGAGG of the stem-loop by the sequence CTTCAG, constituting an XhoI site. These changes led to an impairment of the formation of the stem-loop. The XhoI sites are underlined.</td>
</tr>
<tr>
<td>I</td>
<td>5′-GGGATATCTCATAGAGATCCTCGGCGTCATC-3′</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>5′-GGGGTGAATCCAGTGGACCCGCCGGA-3′</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>5′-GGGATATCTCATAGAGATCCTCGGCGTCATC-3′</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5′-CTGACCATATGCAACTTGGCTGTC-3′</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>5′-TCGCGCTAGCGCGGCCACCGGAGGCCCGGAAAGCT-3′</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>5′-GGGGTGAATCCAGTGGACCCGCCGGA-3′</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>5′-GCCGCTCGAGCGACGCGGATCGTGGCTCCGCGTCATC-3′</td>
<td>Amplification and cloning of the sblA gene into pJF1423 yielding pHCl002. The Ndel (ATATGAT) and EcoRI (GAATTC) sites are underlined.</td>
</tr>
<tr>
<td>E</td>
<td>5′-AAGGGGAATTTCTTACCAGTTGTCGAG-3′</td>
<td></td>
</tr>
</tbody>
</table>

DNA fragment from pHC1011 was replaced by the equivalent 521-bp Sphl-BamHI DNA fragment from pHC1050, pHCl060, and pHC1070, yielding pHC1051, pHCl061, and pHC1071, respectively. Then the original 2,171-bp Sphl-NsiI fragment (site present in luxA and luxB) from pHC1012 was replaced by the equivalent 2,171-bp Sphl-NsiI fragment from pHCl051, pHCl061, and pHCl071, respectively. Then the original 2,171-bp Sphl-NsiI fragment from pHC1012 was replaced by the equivalent 2,171-bp Sphl-NsiI fragment from pHCl051, pHCl061, and pHCl071 to yield pHCl052, pHCl062, and pHCl072, respectively. Then the 5,777-bp BglII inserts from pHCl052, pHCl062, and pHCl072 were cloned into pTO1 (42) cut by BamHI to give pHCl053, pHCl063, and pHCl073, respectively.

The various pTO1 constructs were inserted as one copy into the chromosomes of S. lividans TK24, resulting in the S. lividans TK24 strains carrying the following plasmids with the indicated stem-loop structures and sequences: S. lividans TK24(pHC1013) (SL*; AGGAGG), S. lividans TK24(pHC1023), S. lividans TK24(pHC1053) (SL*; AGGAGG), S. lividans TK24(pHC1063) (SL*; CTCGAG), and S. lividans TK24(pHC1073) (SL*; CTTCGAG).

(iv) Construction of the expression plasmid pHCl002. In order to overexpress and purify the protein SblA fused to an His tag, the expression of sblA was placed under the control of the nosiheptide-inducible tipA promoter (28). To do so, sblA was amplified by PCR using primer D harboring an NdeI site and primer E harboring an EcoRI site. The PCR fragment was cleaved with NdeI and EcoRI and cloned into pJF1423 in place of the red D gene (43) to yield pHCl002.

Media. The growth media used in this study were either the classical R2YE in which 1% (wt/vol) glucose was added before autoclaving (18) or the same medium with no glucose added. The assay of the concentration of glucose in the glucose-containing R2YE medium used in this study was surprisingly found to be 2.7% (150 mM instead of the 55 mM expected). We believe that the extra glucose arises from the presence of glucose in the autoclave and/or different cooling times, e.g.) or that sucrose might vary under slightly different autoclaving conditions (different positions of the flasks in the autoclave and/or different cooling times, e.g.) or that the presence of glucose promotes sucrose hydrolysis during autoclaving. The latter medium is called the glucose-poor R2YE medium in the text.

Determination of growth, glucose consumption, and glucose transport abilities. In order to follow growth and glucose consumption of S. lividans TK24 and of the sblA mutant (see Fig. 2A), the sblA mutant was inoculated (10⁶ spores/ml) in liquid glucose-rich R2YE medium and grown with shaking at 30°C. One-milliliter samples were taken every 3 h, centrifuged at 13,000 rpm for 5 min, and assayed for glucose (GAGO20 kit; Sigma). Mycelial pellets, suspended in 1 ml...
of sterile water, were broken in the presence of glass beads, using a Bio 101 Savant Prep system, and used for protein quantification with the Bradford reagent.

For growth curve and glucose transport assays (see Fig. 2A and B), spores of *S. lividans* TK24 and of the sblA mutant were pregerminated and used at 7.5 × 10^7 ml^-1 to inoculate 50 ml of R2YE medium without sucrose and grown for 15 h at 30°C. Cells were then washed twice in R2YE medium, and 100 mg of mycelia was inoculated in 1 liter of R2YE medium without sucrose in Erlenmeyer flasks supplemented with 1% (wt/vol) glucose. Cells were grown with vigorous shaking at 30°C, and aliquots of 10 ml were taken every 4 h and centrifuged at 13,000 rpm for 5 min. Pellets were weighed after drying in a Speed Vac concentrator for 30 min at 50°C using an analytical scale (Sartorius). Transport assays were performed following a method described previously (37). Mycelia were harvested every 4 h by centrifugation, washed twice in ice-cold transport buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM KCl), and adjusted to 1.0 to 1.5 mg of dry weight ml^-1 (for determination of dry weight ml^-1, see above). Cell suspensions were prewarmed to 30°C for 10 min prior to initiation of uptake assays. Uptake was initiated by the addition of [14C]glucose (Amersham) at a final concentration of 20 μM and a specific radioactivity of 6.1 mCi mmol^-1. Samples were taken every 20 s up to 120 s in order to determine the optimal length of the incubation. Incubation of 1 min was shown to correspond to a steady state; thus 0.5-ml samples were taken after 1 min of incubation at 30°C, rapidly filtered through nitrocellulose filters (NC45; Schleicher & Schuell), and promptly washed twice with 3 ml of chilled 0.1 M LiCl. Radioactivity was determined by scintillation oscillography with 3 ml of scintillation solution per sample. Uptake was initiated by the addition of [14C]glucose (Amersham) at a concentration of 10 μM ml^-1 to induce protein production, and growth was continued for a further 10 h. Mycelium was centrifuged for 15 min at 8,000 rpm, resuspended in 80 ml of buffer A (60 mM Tris-HCl, pH 7.5, 250 mM KCl, 15 mM imidazole, 10% glycerol) and broken with a French press. Soluble fractions obtained after centrifugation for 20 min at 12,000 rpm were filtered with a 0.45-μm-pore-size filter and loaded on an XK 16/20 chromatography column (Pharmacia) packed with chelating Sepharose Fast Flow, charged with 100 mM NiCl₂ (pH 7.2), and equilibrated with buffer A. Purification was automated with an AKTA FPLC purifier (Pharmacia). The column was washed with buffer A, and His-tagged SblA was eluted with an imidazole gradient of 15 mM (buffer A) to 1.0 to 1.5 mg of dry weight ml^-1 (for determination of dry weight ml^-1, see above). Protein concentration of purified SblA was determined by incubating SblA (1 μg, or 35 pmol) for 20 min at 37°C under shaking with 1.5 μg of fluorescent di-C6-NBD (λ excitation) = 400 nm and λ emission) = 510 nm) of lipids was extracted by sonication. After extensive sonication, the spotted quantity of phosphatidylinositol. The phosphatidylinositol peak was identified by simultaneous injection of a standard. Peak areas were quantified using ChromStar integrator software (version 4.14; Iatron Laboratories). These areas were found to be proportional to the spotted quantity of phosphatidylinositol.

**Determination of the luciferase activity.** In order to assay the luciferase activity resulting from the sblA::aac and sblA::lac transpositional fusions, 10^8 spores of the various *S. lividans* TK24 strains were spread on the surface of cellulose disks (Cannings Packaging Limited, United Kingdom) laid down on the surface of glucose-rich R2YE agar plates (18). Squares of mycelium of a defined area were cut out at regular intervals during growth, suspended in 250 μl of H₂O containing a mixture of protease inhibitors (Sigma), and thoroughly homogenized. Luciferase activity was measured with a Berthold LUMAT LB 9501 luminometer by mixing instantaneously 100 μl of the homogenized mycelium and 100 μl of 0.1% N-decyl aldehyde emulsified by sonication. After extensive sonication of the mycelial samples, protein concentration, as a measure of concentration, was determined with the Bradford reagent (Bio-Rad). Luciferase activity was then expressed in arbitrary units of luciferase (AUL) mg of protein^-1.

**RESULTS**

Growth characteristics, glucose consumption, and phenotype of *S. lividans* TK24 and of the sblA mutant strain. The early growth arrest that characterizes the *sblA* mutant strain was previously established in a liquid minimal medium containing glucose as main carbon source (14). To further explore the phenotype of the *sblA* mutant, we grew cultures of *S. lividans* TK24 and TK24 sblA::aac in the more complex and rich R2YE liquid medium with or without added glucose. Proteins and glucose consumption were assessed during growth. Results shown in Fig. 2A indicate that growth of *S. lividans* TK24 showed the expected profile (15): a first phase of rapid growth up to 28 h, a transition phase between 28 h and 31 h, and then a second phase of renewed growth continuing for a few hours before entry into stationary phase. Surprisingly, the sblA mutant strain did not resume growth after the transition phase. Figure 2A shows that the wild-type and the sblA mutant strains consume glucose at approximately the same rates, up to the 30th hour of growth. Then, from the 30th hour of growth, when the concentration of glucose in the growth medium approximates 10 g liter^-1 (approximately 2/3 of initial glucose was consumed), glucose consumption slowed down considerably in the wild type and completely stopped in the sblA mutant strain. In the glucose-poor R2YE medium, both strains showed a similar growth pattern and consumed glucose at the same
clearly SblA dependent since it is not observed in the glucose-poor R2YE medium (Fig. 2B). This growth resumption is also the presence of glucose since it is not observed in the glucose-rich liquid R2YE medium. Plates were incubated for 72 h at 30°C. Brown and smooth aspect, vegetative mycelium; white and fluffy aspect, nonsporulating aerial mycelium; gray and fluffy aspect, sporulated aerial mycelium.

FIG. 3. Lawns of S. lividans TK24 and TK24 sblA::Ωaac grown on glucose-rich or glucose-poor R2YE medium. Plates were incubated for 72 h at 30°C. Brown and smooth aspect, vegetative mycelium; white and fluffy aspect, nonsporulating aerial mycelium; gray and fluffy aspect, sporulated aerial mycelium.

rates (Fig. 2B). Curiously, both strains entered prematurely into stationary phase when grown in this amino acid-rich but glucose-poor R2YE medium. They stopped growing when they reached a level of biomass comparable to that of the wild-type strain grown in the glucose-rich R2YE medium, at the transition phase (Fig. 2B). These observations suggest that the growth resumption that follows the transition phase relies on the presence of glucose since it is not observed in the glucose-poor R2YE medium (Fig. 2B). This growth resumption is also clearly SblA dependent since it is not observed in the sblA mutant strain (Fig. 2A).

Similarly, when both strains were plated on glucose-rich solid R2YE medium, the sblA mutant sporulated much earlier than the original strain S. lividans TK24 (Fig. 3). The early growth arrest of the sblA mutant strain observed in glucose-rich liquid R2YE medium obviously correlates with an early triggering of the sporulation process on glucose-rich solid R2YE. The “omission” of glucose from the R2YE medium almost completely abolished the phenotypic differences existing between the two strains, with both sporulating early. These observations suggest that a high glucose level represses or delays the sporulation process and that SblA plays a role in this repression. Since the phenotypes of the sblA mutant strain might be explained by a default in glucose uptake, the glucose uptake abilities of the two strains were assessed during growth.

Glucose uptake abilities of S. lividans TK24 and of the sblA mutant strain. The relative abilities to take up glucose (see Materials and Methods) of cultures of each strain grown for 24, 28, 32, 36, and 40 h are shown in Fig. 4A, and the detailed kinetics of glucose uptake of cultures grown for 40 h in glucose is given in Fig. 4B. Figure 4A shows that the kinetics of glucose uptake of both strains are comparable at 24, 28, and 32 h, whereas at 36 and 40 h (time points where sblA expression is switched on in the wild-type strain), the ability of the sblA mutant strain to transport glucose is approximately 1.5-fold lower than that of the wild-type strain (Fig. 4B). Altogether, these observations suggest that SblA has a positive effect on glucose uptake. In order to clarify the regulatory role that SblA might exert on glucose uptake, SblA was overproduced as a histidine-tagged fusion protein in S. lividans TK24; the protein was purified, and its enzymatic function and substrate specificity were determined.

Enzymatic function and substrate specificity of SblA. Since sblA was predicted to encode a protein bearing similarities to phosphatases of the inositol phosphate monophosphatase family (14), we assayed its putative phosphatase activity using the chromogenic substrate para-nitro-phenyl phosphate and then different phosphorylated substrates, including various phosphoinositides (phosphatidylinositol, phosphatidylinositol-4-P, and phosphatidylinositol-4,5-biP), in addition to other phospholipids, such as phosphatidylethanolamine and phosphatidylglycerol (data not shown). SblA was shown to liberate phosphate specifically from phosphoinositides, and this activity was confirmed using fluorescent di-C6-NBD phosphoinositides (Echelon Research Laboratories) in which the fluorescent dye is linked to the acyl chain of the molecule. Figure 5A shows that SblA is able to hydrolyze the phosphodiester bond between the diacylglycerol (DAG) moiety and the inositol moiety, leading to the production of a compound migrating at the front of the solvent of the thin-layer chromatography at the expected position of DAG. Thus, SblA appears to possess a phospholipase C-like activity. Moreover, the detection of phosphoinositides containing less phosphate than the original substrates used (Fig. 5A, PI and PIP) and migrating slightly faster than the latter strongly suggested that SblA also possesses a phosphoinositide phosphatase activity. This enzyme, therefore, can be considered a bifunctional enzyme with phospholipase C-like and phosphoinositide phosphatase activities.
In order to confirm, in vivo, the enzymatic function of SblA, we checked the effect of the interruption or overexpression of sblA on the phosphoinositide (PI) content of the bacteria. PIs were prepared as described in Materials and Methods from S. lividans TK24, the wild-type strain (14), and S. lividans S. lividans TK24 EcoRI::Δaac sblA (TK24, the wild-type strain (14), and S. lividans TK24 via the attachment site of PhiC31. Growth of this mutant strain (14), and S. lividans TK24 EcoRI::Δaac sblA (TK24 via the attachment site of PhiC31. Growth of this mutant strain was confirmed that PIs were more abundant in the wild-type and TK24 (pHC1023), which also lasted longer (10 to 12 h) compared to the wild-type situation. This site is thus pressed to the middle of the first growth phase and switched off just before the transition phase. Since the expression of sblA is tightly temporally regulated, we inspected the 5' region of sblA for putative operator-like sequences likely to play a role in sblA regulation. A putative operator-like structure was found upstream, and another was found within the sblA promoter region. The two putative operator regions are represented by nine arrowheads upstream of the sblA promoter region and by two inverted arrowheads downstream of the transcriptional start site, represented by an arrow. (B) Growth curves of S. lividans TK24 (pHC1013) (black diamonds) and TK24 (pHC1023) (gray squares) and luciferase activity (expressed as AUL mg of protein−1) from samples of S. lividans TK24 (pHC1013) (black bars) and TK24 (pHC1023) (gray bars) grown on glucose-rich R2YE solid medium.

Role of putative operator sites in the regulation of sblA expression. In order to assess the role of the upstream operator-like structure, the latter was moved away from the promoter region by cloning a cassette conferring resistance to apramycin after the seventh repetition (plasmid pHC1023). Growth of S. lividans TK24(pHC1013) and S. lividans TK24(pHC1023), as well as the luciferase specific activity, was followed throughout the developmental cycle as described above. The growth profiles of the two strains were comparable and showed the expected pattern. Figure 6B (gray bars), clearly indicates that in S. lividans TK24(pHC1023), the displacement further upstream from the −35 promoter of the putative operator sequence led to a two- to threefold enhancement of the expression of sblA, which also lasted longer (10 to 12 h) compared to the wild-type situation. This site is thus putative operator-like sequences likely to play a role in sblA regulation. A putative operator-like structure was found upstream, and another was found within the sblA promoter region. The two putative operator regions are represented by nine arrowheads upstream of the sblA promoter region and by two inverted arrowheads downstream of the transcriptional start site, represented by an arrow. (B) Growth curves of S. lividans TK24 (pHC1013) (black diamonds) and TK24 (pHC1023) (gray squares) and luciferase activity (expressed as AUL mg of protein−1) from samples of S. lividans TK24 (pHC1013) (black bars) and TK24 (pHC1023) (gray bars) grown on glucose-rich R2YE solid medium.

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likely to constitute a transcriptional repressor binding site. However, the higher and longer expression of sblA in *S. lividans* TK24(pHC1023) was still strongly reduced before the start of the second growth phase (Fig. 6B), suggesting the existence of another regulatory mechanism that might be specifically involved in the switch off of sblA expression.

Therefore, we investigated a possible regulation of sblA through the operator-like sequence located within the sblA coding region. We constructed three plasmids (pHC1053, pHC1063, and pHC1073) in which the putative internal operator site was altered (Fig. 7A). In pHC1053 (dark gray bars) the stem-loop was destroyed and in pHC1063 (light gray bars), the sequence AGGAGG was replaced by the sequence CTCGAG, and compensatory changes were introduced in the right-hand part of the stem in order to preserve the possibility of the formation of a stem-loop. In pHC1073 (white bars), the sequence AGGAGG was replaced by the sequence CTCGAG, and the possibility of formation of a stem-loop activity (expressed in AUL mg of protein $^{-1}$) from samples of *S. lividans* TK24 containing either pHC1013 (black bars), pHC1053 (dark gray bars), pHC1063 (light gray bars), or pHC1073 (white bars) grown on glucose-rich R2YE solid medium.

FIG. 7. (A) Schematic representation of the modifications introduced by site-directed mutagenesis into the stem-loop structure (SL, SL, and SL*) including the AGGAGG sequence present 170 bp downstream of the sblA GTG translational start codon. (B) Growth curves of *S. lividans* TK24 containing either pHC1013 (black squares), pHC1053 (dark gray squares), pHC1063 (light gray squares), or pHC1073 (white squares) grown on glucose-rich R2YE solid medium and luciferase activity (expressed in AUL mg of protein $^{-1}$) from samples of *S. lividans* TK24 containing either pHC1013 (black bars), pHC1053 (dark gray bars), pHC1063 (light gray bars), or pHC1073 (white bars) grown on glucose-rich R2YE solid medium. more precisely, for the switch off of sblA expression toward the end of the first growth phase.

### DISCUSSION

The *Streptomyces* are a group of high-GC DNA, gram-positive, filamentous bacteria living in the superficial layers of the soil. In liquid, as in solid medium, the growth of *Streptomyces* was reported to be multiphasic (15, 52), starting with a phase of active growth that ends with a transitory growth arrest called either the “transition phase” (15) or the “diauxic lag” (32, 50, 51). Then growth resumes for a few hours before entry of the bacteria into stationary phase. The transitory growth arrest is thought to result from some nutritional limitations (52) and is characterized by extensive degradation of cellular macromolecules (15) and by the induction of the expression of proteins associated with specific stress-induced regulons (32). This short pause in growth is considered a key regulatory transition since it precedes a phase of renewed growth and the triggering of secondary metabolite production (51). However, very little is known about the regulatory mechanisms underlying the complex changes that are taking place during this transition phase. This study suggested that the previously characterized sblA gene of *S. lividans* (14) might be involved in the regulation of this process since the *sblA* mutant strain was shown to be unable to pursue growth beyond the transition phase in a minimal medium containing glucose as a main carbon source (14) and even in the complex medium R2YE rich in glucose (this study). The second phase of renewed growth is not observed when the wild-type strain is grown in a minimal medium containing glycerol as the main carbon source (14) or in the rich and complex medium R2YE poor in glucose (this study), suggesting a key role for glucose in this growth resumption.

On the glucose-rich solid R2YE medium, the *sblA* mutant develops aerial mycelium and sporulates much earlier than the wild-type strain, whereas the omission of glucose from the R2YE medium abolishes the phenotypic differences between the two strains that both sporulate early. An acceleration of aerial mycelium development and sporulation was previously reported to be conferred by the interruption of the gene *cvnD9* of *S. coelicolor* encoding a G protein of the Ras family usually found in eukaryotes (25) or by the overexpression of the *ram* genes (24, 27) or of the mutant alleles Y21A and F75A of the *bldB* gene of *S. coelicolor* (12), which has been described as involved in carbon source sensing and/or utilization (33).

The early growth arrest that characterizes the *sblA* mutant strain grown in the glucose-rich R2YE medium was shown to correlate with a reduction in its capacity to take up glucose when the concentration of glucose reaches approximately 10 g liter$^{-1}$. However, it is difficult to know whether the reduction in the glucose uptake ability is the cause or the consequence of the observed growth arrest of the *sblA* mutant strain. It was recently reported that in *S. coelicolor*, a close relative of *S. lividans*, glucose is mainly transported via the major facilitator GlcP1 (49). We can thus propose that during the first phase of growth, when the concentration of glucose is still high, glucose would be transported by GlcP1 (49) whereas when the concentration falls below a certain threshold, this uptake (and thus the continuation of growth) may necessitate either an increase...
in the abundance and/or in the affinity of GlcP1 for glucose (perhaps via a posttranslational modification of the latter?) or the induced synthesis of an alternative glucose transporter with a higher affinity for glucose than GlcP1. These hypothetical processes, aimed at stimulating glucose uptake, might be under the indirect control of sblA. However, since the sblA mutant was obviously shown to be able to take up glucose when the latter was present at a low concentration (less than 10 g liter⁻¹) in the growth medium (Fig. 2B), it seems more reasonable to propose that SblA governs a complex regulatory cascade controlling, in addition to glucose uptake, many other processes necessary for growth resumption after the transition phase. The ability of SblA, to cleave or dephosphorylate signaling phospholipids of the phosphoinositide family suggests that it may play a rather complex regulatory role. In eukaryotes, phosphoinositides are very important signaling and regulatory molecules (40). Their cleavage by enzymes of the phospholipase C family leads to the production of secondary messengers, inositol-phosphate and DAG, involved in the regulation of various cellular processes including the modulation of actin cytoskeleton organization (38, 44), calcium flux (4, 23, 41), and phosphorylation of regulatory proteins (9, 34) as well as glucose transport (13, 26). The topology prediction for SblA using the TMPRED software (http://www.ch.embnet.org/software/TMPRED_form.html) suggests that this protein is indeed likely to be a membrane protein with three predicted transmembrane helices. A membrane localization for SblA is consistent with its enzymatic activity since phosphoinositides are membrane components.

The temporal expression of sblA is consistent with a role of this gene during the late stages of growth since we established that the transient expression of sblA mainly occurred towards the middle of the first phase of growth and was switched off just before the transition phase. Tight temporal control of transitory sblA expression was shown to involve at least two cis-acting negative operator sequences: one constituted by nine DRs of the sequence 5′-(C/G)GGAGG(C/T)-3′ present just upstream of the −35 promoter region (the last copy overlapping the putative −35 sequence) and the other constituted by a 23-nucleotide-long putative stem-loop structure containing AGGAGG, resembling an RBS, present 170 bp downstream of the GTG start codon. The nine DRs are likely to constitute a transcriptional repressor binding site, but the precise molecular mechanisms by which the stem-loop–RBS structure achieves its negative regulatory role remain mysterious. However, it is noteworthy that this structure strongly resembles the binding site of the CsrA protein of Escherichia coli (36). CsrA is a small, RNA binding, regulatory protein that interacts with RBS-like sequences, inhibiting the binding of ribosomes and thus translation of the target transcript (10). The “naked” untranslated transcripts are thus more vulnerable to endo- and exo-ribonucleolytic attack and are more rapidly degraded (10, 11, 53). By analogy with this system, we may speculate that the abrupt switch off of sblA expression just before the transition phase might involve the specific degradation of the sblA transcript indirectly promoted by a CsrA-like RNA-binding protein interacting with the stem-loop–RBS structure.

In conclusion, this study strongly suggests that the cleavage of the signaling lipids, phosphoinositides, by SblA is necessary to trigger the glucose-dependent phase of renewed growth that follows the transition phase in S. lividans TK24. The previous characterization of genes encoding phospholipase C-like activities in Streptomyces species (21), as well as the production by several Streptomyces species of inhibitors of phosphatidylinositol turnover in eukaryotes (1, 30, 31, 46, 48), already suggested the likely existence of a eukaryotic-like phosphoinositide signaling pathway in Streptomyces. However, this study constitutes the first evidence that phosphoinositides are indeed playing important signaling and regulatory roles in Streptomyces, as they do in eukaryotes. Nevertheless, more work is obviously needed to clarify the regulatory role of phosphoinositides and of SblA in Streptomyces. Attempts will be made to elucidate the possible relationships of sblA with other genes playing a role in the regulation of the morphological differentiation process by glucose. It is noteworthy that some of these genes encode, similar to sblA, “eukaryotic-like” functions, such as cvnD9 mentioned above (25) or asfK encoding a eukaryotic-like protein serine/threonine kinase essential for morphogenesis in the presence of glucose (47). Altogether, these observations suggest that eukaryotic-like signaling and regulatory networks might be involved in the regulation by glucose of the late growth stages and of the morphological differentiation process in Streptomyces.

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