

Disruption of *sbIA* in *Streptomyces lividans* permits expression of a heterologous α -amylase gene in the presence of glucose

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In a transposition mutant of *Streptomyces lividans* TK24, the usually glucose-repressible expression of a heterologous α -amylase gene (*aml*) became resistant to glucose repression. The transposon had inserted into an ORF called *sbIA* which encodes a 274 aa product sharing significant sequence similarities with various phosphatases that act on small phosphorylated substrates. *sbIA* was transcribed as a monocistronic mRNA and its transcription was enhanced at the transition phase. Because its transcriptional and putative translational start points coincide, *sbIA* is likely to be translated in the absence of a conventional RBS. The *sbIA*-disrupted mutant is characterized by early growth arrest in glucose-grown cultures and by partial relief of glucose repression of *aml* expression.

Keywords: glucose repression, induction, inositol monophosphatase, transition phase

INTRODUCTION

Streptomycetes are saprophytic Gram-positive bacteria living in the soil. Their saprophytic life style is made possible by their ability to degrade many kinds of organic molecules, thanks to their extremely large and diverse range of hydrolytic enzymes, including proteases, nucleases, lipases and enzymes hydrolysing polysaccharides (Williams *et al.*, 1983). We are currently studying the regulation of one of these hydrolytic enzymes, an α -amylase from *Streptomyces limosus* encoded by *aml* and stably integrated as one copy in the chromosome of *Streptomyces lividans* TK24 via the attachment site of the integrative vector pSAM2. In that strain, called *S. lividans* TK24(pTS6000), expression of *aml* was shown to be under the control of a LacI-like negative transcriptional regulator encoded by *reg1* (Nguyen *et al.*, 1997). Reg1 is a homologue of the *malR* gene product of *Streptomyces coelicolor*. In *S. coelicolor*, MalR negatively regulates transcription of the maltose transport operon encoded by *malEFG* (van Wezel *et al.*, 1997). Interruption of *reg1* in *S. lividans*, and of *malR* in *S. coelicolor*, concomitantly abolishes both malto-

dextrin induction and glucose repression of *aml* (Nguyen *et al.*, 1997) and *malEFG* expression (van Wezel *et al.*, 1997), respectively. In the streptomycetes, as in *Escherichia coli*, the genes encoding the secreted α -amylase and those encoding elements of the maltose transport system are in the same regulon. However, this regulon is controlled by the positive transcriptional regulator MalT in *E. coli* (Boos & Shuman, 1998), whereas in the streptomycetes it is controlled by a LacI-like negative transcriptional regulator.

When cultures of *S. lividans* TK24(pTS6000) are grown in the presence of a 'non-repressive' carbon source such as glycerol or mannitol, expression of *aml* is switched on at a low level at the end of the exponential phase of growth, even in the absence of exogenous inducer. Adding a low level of exogenous inducer (10^{-4} M maltotriose) to cultures growing exponentially in a glycerol medium does not induce *aml* expression, whereas adding the exogenous inducer after the growth-phase-dependent *aml* expression has started clearly enhances *aml* expression. Both growth-phase-related and maltotriose-inducible *aml* expression are strongly repressed in the presence of glucose (Virolle & Gagnat, 1994). The molecular mechanisms responsible for growth-phase-dependent *aml* expression are not yet elucidated, but could be related to growth-phase-dependent degradation of the internal storage compound glycogen. This degradation might provide the

Abbreviations: Amp, ampicillin; Apr, apramycin; CBS, Cibachron blue starch; IMP, inositol monophosphatase; Nos, nosiheptide.

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maltodextrins inducing, at a low level, the different components of the maltose regulon. Low level induction of the maltose transport system, and thus the transport of external inducer, could account for the growth-phase-related pattern of *aml* inducibility.

For more insight into the molecular mechanisms involved in glucose repression of *aml* expression, we developed a transposon mutagenesis procedure yielding *S. lividans* mutants in which *aml* expression had become insensitive to glucose repression. In this paper, we report the characterization of one such mutant. The interrupted gene, called *sblA*, was cloned and sequenced and its transcription was investigated. Effects of the *sblA* null mutation on growth and *aml* expression in different culture conditions are reported.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Culture conditions. All physiological studies of regulation were carried out in triplicate in modified liquid minimal medium (NMMP; Hopwood *et al.*, 1985). Spores were pre-germinated as described by Hopwood *et al.* (1985) and used at 10^7 ml⁻¹ to inoculate 20 ml NMMP supplemented at 1% (w/v) with glucose or glycerol. The cultures were grown in 150 ml Erlenmeyer flasks with four indents; each flask contained 12–14 g 4 mm diameter glass beads to achieve dispersed growth. Cultures were grown at 30 °C under constant agitation (160 r.p.m.) on a Braun Certomat R orbital shaker. Growth and α -amylase production were measured every 3 h. In the induction studies, maltotriose was added at 10^{-4} M to one of a set of two glucose- and glycerol-grown cultures as soon as Aml was detected in culture supernatants. The theoretical basis and methodology of the turbidimetric assay used to follow Aml production were described by Virolle *et al.* (1990).

Transposon mutagenesis. The delivery plasmid pCZA168 (Solenberg & Baltz, 1991), carrying the *tsr* gene that confers resistance to nosiheptide (Nos) in *Streptomyces* and the transposon Tn5096 harbouring the *aacC4* gene that confers resistance to geneticin (G418) in both streptomycetes and *E.*

Table 1. Strains and plasmids used

Strains	Description	References	
<i>S. lividans</i> TK24(pTS6000)	Wild-type strain with one copy of the <i>aml</i> gene from <i>S. limosus</i> present on a 5.85 kb <i>BclI</i> fragment and integrated into the chromosome via a derivative of the integrative plasmid pSAM2	Virolle & Gagnat (1994)	
<i>S. lividans</i> TK24(pTS6000) <i>sblA::Ωaac</i>	Derivative of <i>S. lividans</i> TK24(pTS6000) where <i>sblA</i> was interrupted with the Ω aac cassette	This study	
Plasmids	Relevant characteristics	Insert	References
pBR322 derivatives			
pHP45 Ω aac	Amp ^R Apr ^R	The Ω aac cassette conferring resistance to Apr	Blondelet-Rouault <i>et al.</i> (1997)
p6998	Amp ^R Apr ^R	The <i>Bam</i> HI– <i>Bgl</i> III fragment (Fig. 1a) carrying part of Tn5096 and flanking chromosomal DNA	This study
pIJ2925 derivatives*			
p7000	Amp ^R	The 2 kb <i>Sph</i> I– <i>Bgl</i> III chromosomal DNA fragment from <i>S. lividans</i> carrying <i>sblA</i> (Fig. 1b)	This study
p7001	Amp ^R Apr ^R	Insert of p7000 bearing the Ω aac cassette cloned into the <i>Bam</i> HI site of <i>sblA</i>	This study
pGM160 ^A derivatives†			
pCZA168 (used in transposition mutagenesis)	Amp ^R Apr ^R in <i>E. coli</i> , Nos ^R Apr ^R in <i>Streptomyces</i>	Tn5096	Solenberg & Baltz (1991)
p7002 (used in gene replacement)	Amp ^R Apr ^R in <i>E. coli</i> , Nos ^R Apr ^R in <i>Streptomyces</i>	Same insert as p7001	This study

* Plasmid derived from pUC18 (Janssen & Bibb, 1985).

† *E. coli*/*Streptomyces* shuttle vectors derived from pGM160 (Muth *et al.*, 1989) by deletion of the *Hind*III–*Hind*III fragment carrying the *aacC4* gene (a generous gift of J.-L. Pernodet, Université Paris-Sud, France); thermosensitive replication in *Streptomyces*.

coli, was used in transposon mutagenesis. Protoplasts of strain TK24(pTS6000) were transformed by pCZA168, plated on R2YE medium (Hopwood *et al.*, 1985) and overlaid with SNA (Hopwood *et al.*, 1985) containing Nos (50 $\mu\text{g ml}^{-1}$). Spores from the transformants were spread on solid defined agar to obtain separate colonies. This minimal agar consisted of a basal layer (20 ml) of minimal agar (Hopwood *et al.*, 1985) containing 1% glucose, 0.25% (w/v) Casamino acids and G418 (15 $\mu\text{g ml}^{-1}$) to maintain selective pressure for the presence of Tn5096, and a top layer (5 ml) of the same medium containing 2% (w/v) Cibachron blue starch (CBS), as a chromogenic substrate for α -amylase (Klein *et al.*, 1969). These cultures, called 'CBS plates', were incubated for 3 d at 30 °C (no haloes were seen), then for 2–3 weeks at 40 °C. At this non-permissive temperature for pCZA168 replication, colonies stopped growing uniformly in diameter, but after 1 week, most of them had grown sectors (a mean of four per colony), 1–2% of which were surrounded by white haloes of CBS hydrolysis and were considered to be potentially interesting mutants. Mycelium from these sectors was streaked on the same medium to confirm their phenotype.

Chemicals and enzymes. G418 was purchased from Sigma and Nos was a generous gift from Rhône-Poulenc. Restriction enzymes, DNA ligase and T4 polynucleotide kinase were used according to the recommendations of the suppliers (Boehringer Mannheim and Biolab).

DNA manipulation. Total genomic DNA and plasmid DNA were isolated from *S. lividans* TK24(pTS6000) as described by Hopwood *et al.* (1985). Methods for plasmid isolation from *E. coli*, purification of DNA fragments, preparation of DNA probes, Southern blotting or colony hybridization were as described by Sambrook *et al.* (1989).

Procedure used to clone the transposon bordering sequences

Total DNA of the chosen mutant was cut by *Bam*HI [a *Bam*HI site is located downstream of the 3' end of the *aacC4* gene of Tn5096] and *Bgl*III (this site is absent in Tn5096). Fragments were ligated into the *Bam*HI site of pBR322 (Bolivar *et al.*, 1977) and the ligation mix was used to transform competent *E. coli* DH5 α cells. Transformant colonies were selected on agar media containing G418 (30 $\mu\text{g ml}^{-1}$). Plasmid DNA was extracted from 24 independent transformants and was digested with *Pst*I (site located near one end of Tn5096, Fig. 1a) and *Sma*I or *Sal*I (two enzymes that cut streptomycete DNA frequently). Analysis of restriction patterns by agarose gel electrophoresis revealed two different types of bordering sequences. They were sequenced using the oligonucleotide OTN 1 (5'-TCAAGTCCTGGCGACTCCTTCGAGGCTCCGTTGCTCGAC-3'), which is complementary to the sequence downstream of the *Nru*I site of Tn5096 (Fig. 1a). One type of bordering sequence was shown to be pCZA168 DNA whereas the other was *bona fide* chromosomal DNA. The pBR322 derivative carrying the chromosomal DNA was called p6998 (Fig. 1a). To clone the gene interrupted by the transposon, a mini-library of the approximately 2 kb *Sph*I–*Bgl*III chromosomal DNA fragments from *S. lividans* was constructed in pIJ2925 (Janssen & Bibb, 1985) and probed with the 440 bp *Sal*I–*Sal*I fragment I of p6998.

DNA sequencing. p7000 (Table 1) was used to generate a set of nested deletions from each end of the insert. Appropriate deleted derivatives were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the *Taq* DNA polymerase Prism Ready Reaction Dye Primer Cycle Sequencing kit from Perkin Elmer with the universal or reverse primers and an ABI373A sequencer (Applied Biosystems).

RNA isolation and transcriptional analysis. RNA was prepared as described by Hopwood *et al.* (1985) except that a

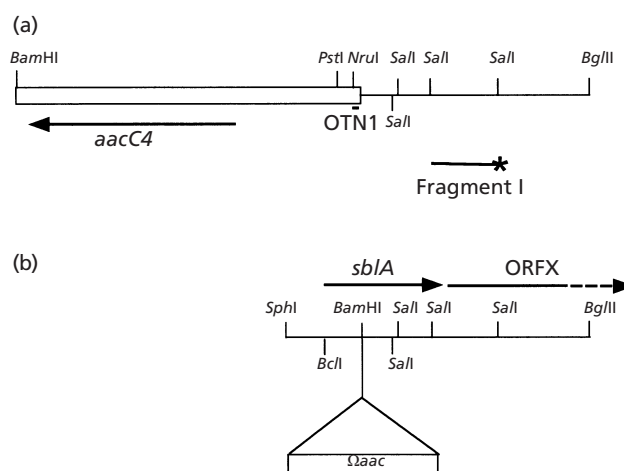


Fig. 1. (a) Restriction map of the 3.5 kb *Bam*HI–*Bgl*III chromosomal fragment cloned from the chosen transposition mutant of *S. lividans* TK24(pTS6000) including part of Tn5096 (white box) and chromosomal flanking sequences. (b) Restriction map of the 2 kb *Sph*I–*Bgl*III chromosomal fragment carrying *sbIA* and a truncated downstream ORF (ORFX). The arrows indicate the transcriptional directions of the two ORFs. OTN1 is the 40 nt long oligonucleotide showing complementarity to a region located downstream of the *Nru*I site of the transposon that was used to sequence the Tn5096 bordering sequence. The 440 bp *Sal*I–*Sal*I fragment I was used to probe the mini-library of the approximately 2 kb *Sph*I–*Bgl*III chromosomal DNA fragments from *S. lividans* TK24.

DNase I treatment was used in addition to salt precipitation. RNA used for Northern blot analysis was prepared from transition-phase cultures grown in NMMP with 1% (w/v) mannitol as the carbon source. RNA used for high-resolution nuclease S1 mapping was kindly provided by Eriko Takano (John Innes Institute, Norwich, UK). It was prepared from cultures grown in SMM medium (Takano *et al.*, 1992) and sampled at intervals during the exponential, transition and stationary phases.

For Northern blot analysis, total RNA was denatured with glyoxal/dimethylsulfoxide (DMSO), fractionated on 1% agarose gels, transferred to Hybond-N membrane and hybridized at 65 °C with the 563 bp *Bcl*I no. 3–*Not*I no. 10 fragment labelled with [α - 32 P]dCTP using the T7 quick prime kit (Pharmacia). Molecular mass standards from BRL were treated in the same way as the RNA samples and labelled as DNA probes.

The DNA fragment used in high-resolution nuclease S1 mapping was the PCR fragment synthesized using the –180 reverse primer (5'-ATGCAGCTGGCAGCAGGT-3') showing complementarity to pUC18, and the 5'-CGATCGCGACTTCGGTGTCTCGTGTG-3' oligonucleotide showing complementarity to the *sbIA* region boxed in Fig. 2. It was phosphorylated at its 5' end with [γ - 32 P]ATP; 40 μg total RNA was hybridized with 50 nmol labelled probe (corresponding to approximately 10⁴ Cerenkov counts min⁻¹) in NaTCA buffer (Murray, 1986) at 45 °C overnight after denaturation at 70 °C for 10 min. All subsequent steps were carried out as described by Strauch *et al.* (1991) using S1 nuclease from Pharmacia. As a sequencing template, p7000 double-stranded DNA was used with the oligonucleotide mentioned above phosphorylated at its 5' end with [γ - 32 P]ATP. The sequence ladders were run in parallel with the S1 protected products. The resulting poly-

acrylamide gel was dried, exposed for 72 h and scanned with a phosphor imager.

sbIA gene disruption. To replace *sbIA* with its disrupted allele, we used p7002, a derivative of the thermosensitive vector pGM160 (Muth *et al.*, 1989) carrying the *sbIA* gene interrupted by the Ω aac cassette (Blondelet-Rouault *et al.*, 1997) conferring resistance to G418. The Nos^R colonies of *S. lividans* TK24 and TK24(pTS6000) transformed with p7002 were grown for 24 h at 30 °C in TSB medium (Hopwood *et al.*, 1985) containing G418 (15 µg ml⁻¹). Mycelium was then harvested and homogenized for use as a low-density inoculum in the same medium. These cultures were grown for 72 h at 40 °C to eliminate the autonomously replicating plasmid. Mycelium was harvested, homogenized and sonicated to obtain small mycelial fragments. These were plated on HT agar (Pridham, 1957) containing G418 (30 µg ml⁻¹). Well-separated, sporulating colonies were replica-plated on HT agar containing G418 (30 µg ml⁻¹) as controls and on HT agar containing Nos (50 µg ml⁻¹) to screen for G418^R Nos^S colonies. Twenty-five per cent of the G418^R colonies were Nos^S. These had lost the replicative plasmid and had arisen from double crossover events between sequences carried by p7002 and homologous chromosomal sequences. Chromosomal DNA was isolated from two independent G418^R Nos^S colonies of each strain and the presence of the Ω aac cassette confirmed by cutting with *Nco*I or *Sph*I followed by Southern blot analysis.

Determination of the chromosomal location of sbIA by PFGE. Chromosomal DNAs of *S. lividans* TK24(pTS6000) and *S. lividans* TK24(pTS6000) *sbIA::* Ω aac were digested with *Ase*I or *Dra*I as described by Leblond *et al.* (1990). The DNA fragments were separated on 1 or 1.3% agarose gels by PFGE. Electrophoresis buffer contained 20 mM triethanolamine (Fluka), 8.75 mM acetic acid and 1.5 mM EDTA. Electrophoresis was carried out for 30 h with pulse times of 60 s for the *Ase*I digests and 200 s for the *Dra*I digests.

RESULTS

Isolation of mutants of S. lividans TK24(pTS6000) producing α -amylase in the presence of glucose

In *S. lividans* TK24(pTS6000), the regulatory features of *aml* expression (Virolle & Gagnat, 1994) described in the Introduction were established in liquid minimal media (NMMP, Hopwood *et al.*, 1985) and observed on solid minimal medium plates in the presence of CBS. With glycerol as the carbon source, *S. lividans* TK24(pTS6000) colonies were surrounded by large haloes of CBS hydrolysis, indicating α -amylase production, whereas much smaller haloes were seen with glucose-containing medium.

Mutants of TK24(pTS6000) producing α -amylase on glucose plates were isolated by transposon mutagenesis using Tn5096 carried by pCZA168 (Solenberg & Baltz, 1991). Tn5096 is derived from the insertion sequence IS493 (Solenberg *et al.*, 1989) present at three copies in the *S. lividans* TK24 chromosome. All the mutants isolated were apramycin (Apr)-resistant (Tn5096 marker) and Nos^R (plasmid marker), suggesting that, besides true transposition events, integration of pCZA168 into the chromosome via homologous recombination between Tn5096 and one of the three chromosomal copies of IS493 had occurred.

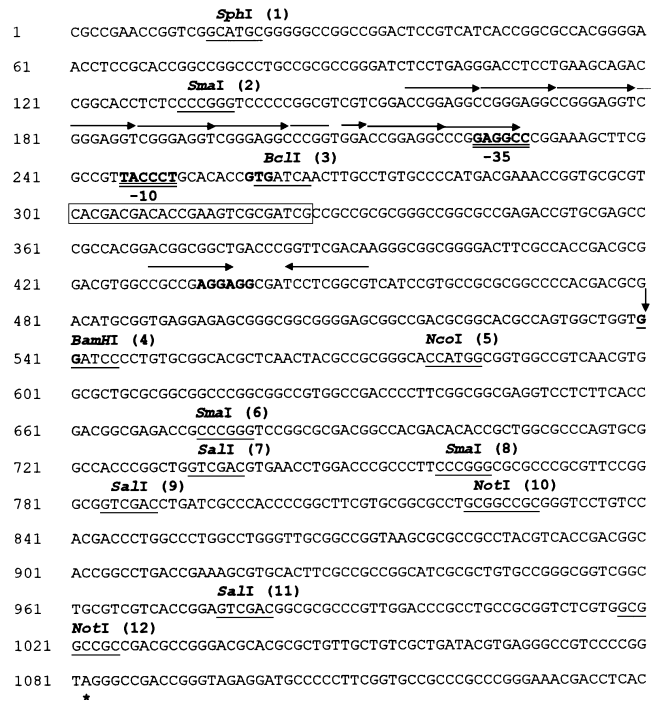


Fig. 2. Nucleotide sequence of *sbIA*. The numbers 1–12 refer to relevant restriction sites. Putative –35 and –10 regions are shown in bold letters and are double underlined. Direct and inverted sequence repeats are indicated by arrows. A Shine–Dalgarno-like sequence overlapping a 22 bp dyadic symmetry element is shown in bold letters. A vertical arrow at position 540 indicates the exact insertion point of the transposon. The asterisk represents the translation stop codon.

Cloning of the gene interrupted by Tn5096: nucleotide sequence and similarities between the deduced protein and database proteins

For the chosen mutant, the Tn5096 bordering sequences were first cloned into pBR322 by taking advantage of the presence of *aacC4* in the transposon. Of the two types of bordering sequences obtained, one was, as expected, pCZA168 DNA, whereas the other was *bona fide* chromosomal DNA. The pBR322 derivative carrying the chromosomal sequences flanking the Tn5096 insertion on one side was called p6998 (Fig. 1a). The 440 bp *Sal*I–*Sal*I fragment I of p6998 was used as a probe to screen an *E. coli* mini-library of the approximately 2 kb *Sph*I–*Bgl*III chromosomal DNA fragments from *S. lividans*. Two positive clones with the same 2 kb *Sph*I–*Bgl*III insert were isolated. The restriction map of the insert is shown in Fig. 1b. The resulting plasmid was called p7000 and its insert was sequenced.

The sequence of the ORF interrupted by Tn5096 is shown in Fig. 2. Comparison of this sequence with the transposon flanking sequence identified the exact insertion point (position 540) of Tn5096. The interrupted ORF starts with a GTG in position 259 and ends with a TGA in position 1080. No typical *Streptomyces* RBS could be found in the vicinity of the putative GTG start codon. The 72% G + C content of this ORF is in good

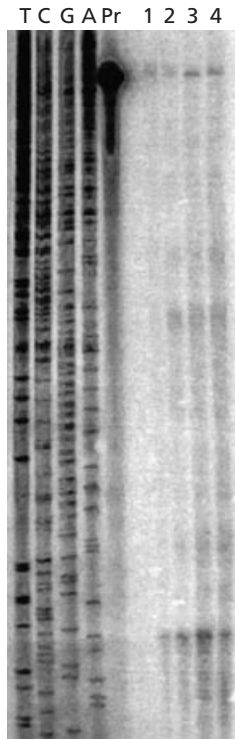


Fig. 5. Determination of the transcriptional start site of *sbIA* and transcriptional regulation of *sbIA* during growth in SMM medium (Takano *et al.*, 1992) using high-resolution nuclease S1 mapping. DNA-protected fragments following incubation with RNA isolated from *S. lividans* TK24 grown in SMM medium and stopped at mid-exponential phase (lane 1), late exponential phase (lane 2), transition phase (lane 3) and stationary phase (lane 4). Lanes T, C, G and A, *sbIA* nucleotide sequence ladder; lane Pr, full length probe. The dried gel was exposed for 72 h and fragments visualized with a phosphor imager. The transcriptional start site is located in the close vicinity of the putative GTG translational start codon.

The IMP I signature would comprise residues involved in binding metallic ions such as Mg^{2+} whereas the IMP II signature would be more specifically involved in substrate binding (Neuwald *et al.*, 1991). Fig. 3 shows that IMP signature I (from residue 92 to 105) is fairly well conserved in SblA whereas IMP signature II (from residue 227 to 244) is not.

Transcriptional regulation of *sbIA* expression

Northern blot analysis indicated that *sbIA* was transcribed as unique monocistronic mRNA of approximately 840 bp (Fig. 4). Therefore, the mutant phenotype linked to the interruption of *sbIA* was likely to be due to the inactivation of the gene itself and not to a polar effect on downstream sequences.

Furthermore, S1 nuclease protection studies (Fig. 5) showed that *sbIA* was weakly transcribed throughout growth, but with a net increase at the transition phase, indicating growth-phase-dependent regulation. Taking into account the occurrence of small differences in migration of the S1-protected products depending on the volume of the samples loaded, the transcript was

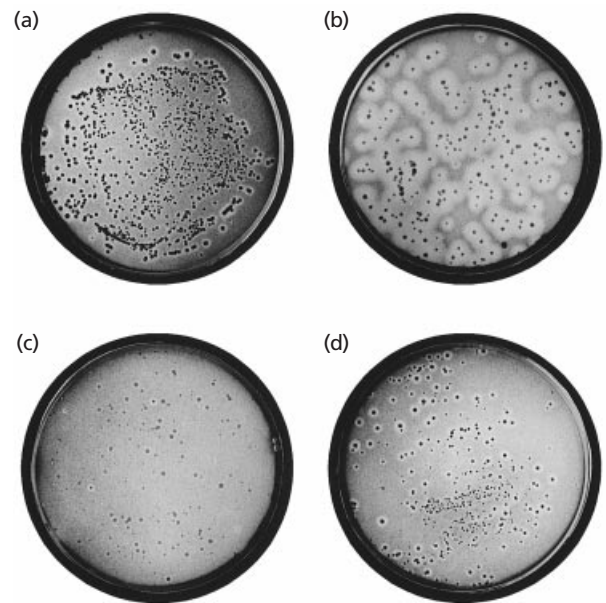


Fig. 6. Isolated colonies of *S. lividans* TK24(pTS6000) (a), *S. lividans* TK24(pTS6000) *sbIA*:: Ω aac (b), *S. lividans* TK24 (c) and *S. lividans* TK24 *sbIA*:: Ω aac (d) plated on NMMP (Hopwood *et al.*, 1985) in the presence of 1% glucose as carbon source and 2% CBS, a chromogenic substrate for α -amylase, to reveal α -amylase production.

thought to start in the close vicinity of the GTG translational start codon. Transcriptional and translational start points are usually very close for proteins translated in the absence of a conventional RBS (Strohl, 1992). From the existence of this leaderless transcript, -35 (GAGGCC) and -10 (TACCCT) promoter sequences with a spacing of 17 bp could be proposed. Noteworthy is the presence of eight direct repeats of the sequence $5'$ -C(C/G)GGAGG(C/T)- $3'$ upstream of the -35 region, with the last copy overlapping the putative -35 region. Moreover, a 23 bp sequence with dyad symmetry and including an RBS-like sequence is present within the coding sequence, 167 bp downstream of the GTG start codon.

Disruption and chromosomal localization of *sbIA*

In *S. lividans*, a convenient way to map genes on the circular chromosome is to interrupt them by introducing a cassette that contains rare restriction sites. Disruption of *sbIA* was achieved by cloning the *aac* cassette conferring resistance to Apr (Blondelet-Rouault *et al.*, 1997) into the *Bam*HI site no. 4 close to the insertion point of Tn5096. The wild-type chromosomal copy of *sbIA* was replaced by this interrupted derivative. Southern blot analysis confirmed that the wild-type copy of *sbIA* has been replaced by the interrupted copy (data not shown). The *S. lividans* TK24(pTS6000) *sbIA*:: Ω aac mutant strain (Fig. 6b) had the same phenotype as the initial transposition mutant (Fig. 6a).

The presence in the resistance cassette of sites for *Ase*I and *Dra*I, enzymes cutting *Streptomyces* DNA rarely, allowed the approximate location of the interrupted

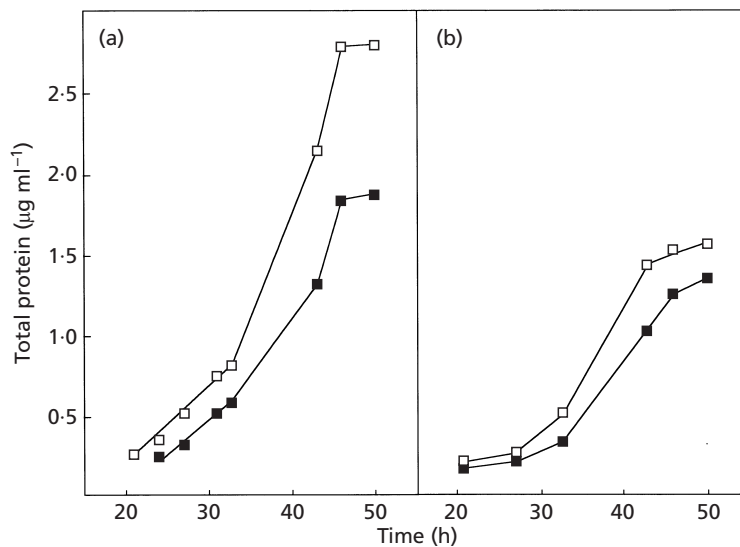


Fig. 7. Growth of *S. lividans* TK24(pTS6000) (□) and *S. lividans* TK24(pTS6000) *sbIA::Ωaac* (■) in NMMP medium supplemented with 1% glucose (a) or 1% glycerol (b) as carbon source. Values are means of triplicate experiments and the SD never exceeded 15% of the mean.

gene on the *S. lividans* TK24 chromosome to be determined by PFGE. When the chromosomal DNA of *S. lividans* TK24(pTS6000) *sbIA::Ωaac* was digested with *AseI*, the original 615 kb *AseI* fragment D (Leblond *et al.*, 1993) was split into two fragments of 355 kb (co-migrating with the *AseI* fragment F) and 260 kb (data not shown). When a *DraI* digestion was carried out, the original 1700 kb *DraI* fragment III (Leblond *et al.*, 1993) was split into two fragments of 450 kb and 1250 kb (data not shown). *sbIA* is thus located at approximately 450 kb from a chromosomal end at roughly 150 kb on the left of AUD1 in a chromosome region known for its high genetic instability (Volff *et al.*, 1996). Disruption of this gene did not lead to any detectable chromosomal rearrangements. Recently a BLAST search carried out at the Sanger centre (<http://www.Sanger.ac.uk>) revealed the presence of a protein bearing over 95% amino acid sequence identity with SblA on the overlapping cosmids StF80 and StF76. These cosmids are located on the *AseI*-F fragment of the chromosome of *S. coelicolor* (Redenbach *et al.*, 1996) close to a chromosomal end.

Effects of *sbIA* disruption on growth

S. lividans TK24(pTS6000) *sbIA::Ωaac* had the same mutant phenotype as the initial transposition mutant (Fig. 6). Colonies of TK24(pTS6000) *sbIA::Ωaac* were smaller on glucose plates than those of the corresponding wild-type strain, whereas no such growth difference was seen on glycerol plates (data not shown). Growth curves of *S. lividans* TK24(pTS6000) and TK24(pTS6000) *sbIA::Ωaac* were established in NMMP (Hopwood *et al.*, 1985) in the presence of 1% glucose or glycerol as carbon sources (Fig. 7). Glucose-grown cultures of the wild-type strain showed higher biomass production than glycerol-grown cultures. Growth rates of glycerol- or glucose-grown cultures of the mutant were comparable to those of the wild-type during exponential growth. However, glucose-grown cultures of the *sbIA* mutant reproducibly entered earlier into stationary phase than the glucose-grown wild-type

cultures. This premature growth arrest led to a 25% deficit in biomass compared with the wild-type strain.

Effects of *sbIA* disruption on the heterologous *aml* gene and on expression of the endogenous amyolytic genes

To assess more precisely the way the regulatory characteristics of *aml* were changed in the *sbIA* mutant, cultures of *S. lividans* TK24(pTS6000) and TK24(pTS6000) *sbIA::Ωaac* were grown in NMMP containing glycerol or glucose as carbon sources. Growth and Aml activity were measured every 3 h. Aml activity was detectable in the supernatant of glycerol- and glucose-grown cultures of both strains at the end of exponential growth in the absence of any exogenous inducer. In glycerol-grown cultures of the wild-type strain, maximal Aml activity was approximately 0.045 IU (mg protein)⁻¹, and the *sbIA* mutant reproducibly contained 1.3–1.5-fold more Aml than the wild-type (Fig. 8a). In glucose-grown cultures of the wild-type strain, maximal Aml activity was extremely low [approx. 0.01 IU (mg protein)⁻¹] and the *sbIA* mutant reproducibly contained 2.5–3-fold more Aml than the wild-type strain (Fig. 8a). The relative enhancement of growth-phase-related *aml* expression, in the absence of exogenous inducer, was more pronounced for the *sbIA* mutant in the glucose- than in the glycerol-grown cultures.

It was previously shown that *aml* expression is not inducible by maltotriose in glucose-grown cultures of TK24(pTS6000) (Virolle & Gagnat, 1994); therefore, the inducibility of *aml* expression in glucose-grown cultures of the *sbIA::Ωaac* mutant was tested. Two sets of two cultures of TK24(pTS6000) and two cultures of TK24(pTS6000) *sbIA::Ωaac* were grown in the same conditions as above. When Aml activity was just detectable in culture supernatants, 10⁻⁴ M maltotriose was added to one set of glycerol- and glucose-grown cultures. Aml activity was assayed in the culture

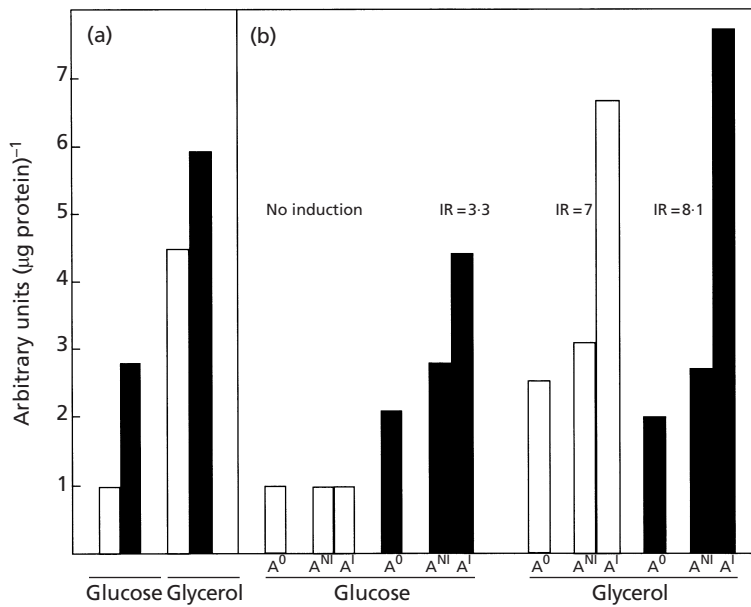


Fig. 8. Effect of the disruption of *sbIA* on the production of α -amylase. (a) Maximal growth-phase-related α -amylase activity detected in the supernatant of glucose- and glycerol-grown cultures of *S. lividans* TK24(pTS6000) (□) and *S. lividans* TK24(pTS6000) *sbIA::* Ω aac (■). (b) Maltotriose-induced α -amylase production detected in the supernatant of glucose- and glycerol-grown cultures of *S. lividans* TK24(pTS6000) (□) and *S. lividans* TK24(pTS6000) *sbIA::* Ω aac (■). A⁰, α -amylase activity at $t = 0$; A^{NI}, α -amylase activity at $t = 1$ h; A^I, α -amylase activity at $t = 1$ h after the addition of 10^{-4} M maltotriose. The induction ratio (IR) was calculated as follows: $(A^I - A^0)/(A^{NI} - A^0)$. α -Amylase activity values are means of triplicate experiments and the SD never exceeded 15% of the mean.

supernatants 1 h after maltotriose addition (Fig. 8b). *aml* expression proved to be maltotriose-inducible in glycerol-grown cultures of the wild-type and the mutant strains with rather similar induction ratios of 7 and 8, respectively. In glucose-grown cultures of the wild-type strain, the extremely low *aml* expression was not increased upon maltotriose addition, whereas the enhanced *aml* expression of the *sbIA* mutant strain was further inducible upon maltotriose addition (induction ratio of 3). These results were consistent with the phenotype of the TK24(pTS6000) *sbIA::* Ω aac mutant seen on the CBS plates in Fig. 6.

Similarly, the very weak amyolytic activity of *S. lividans* TK24 was clearly enhanced in *S. lividans* TK24 *sbIA::* Ω aac on the CBS plates in the presence of glucose (Fig. 6). The interruption of *sbIA* thus had a similar effect on the expression of endogenous amyolytic genes of *S. lividans* to that on the heterologous *aml* gene.

DISCUSSION

This paper describes the isolation of an *S. lividans* transposition mutant in which a usually strongly glucose-repressed gene, the heterologous *aml* gene from *S. limosus* encoding an α -amylase (Virolle & Gagnat, 1994), was expressed in the presence of glucose. The gene interrupted by the transposon Tn5096 was called *sbIA*. It is transcribed as a monocistronic RNA from a unique promoter and its transcription is enhanced toward the end of the exponential phase of growth. Its putative -10 region is similar to the consensus sequence (TAGAPuT) recognized by the major RNA polymerase holoenzyme of streptomycetes whereas the -35 (TTGACN) sequence is not. Eight direct repeats of the sequence $5'$ -C(C/G)GGAGG(C/T)- $3'$ are found upstream of the *sbIA* promoter region, the last copy overlapping the putative -35 sequence. These repeats exhibit similarities to the operator site ($5'$ -GGGGA-(T/G)GAGG- $3'$) recognized by MalT, the activator of

the maltose regulon in *E. coli* (Boos & Shuman, 1998). Furthermore, a hairpin loop with a 9 bp stem [$\Delta G = -10.90$ kcal mol⁻¹ (45.8 kJ mol⁻¹)], and a 4 bp loop is present 167 bp downstream of the GTG start codon. The loop is bordered by a Shine–Dalgarno-like sequence (AGGAGG) and comprises a GNRA tetraloop motif that can adopt a characteristic very stable fold (Jucker *et al.*, 1996). These structures are likely to play a role in regulating expression of *sbIA*. Translation of *sbIA* probably occurs in the absence of a conventional Shine–Dalgarno sequence, as its transcriptional start site and the GTG translational start codon coincide.

sbIA constitutes the fourth characterized locus involved in the regulation of *aml* expression. The three other loci are the *glkA* gene encoding the glucose kinase in *S. coelicolor* (Ikeda *et al.*, 1984; Virolle & Gagnat, 1994), the LacI-like negative transcriptional regulator called Reg1 in *S. lividans* (Nguyen *et al.*, 1997) and the Reg1 operator sites located in the *aml* promoter region (Virolle & Gagnat, 1994; Nguyen, 1999). In a strain of *S. coelicolor* mutated for the *glkA* gene, glucose repression of *aml* expression is abolished but the derepressed *aml* expression is further inducible by a low level of exogenous inducer (Virolle & Bibb, 1988). In *S. lividans*, the interruption of *reg1* (Nguyen *et al.*, 1997) or the deletion of operator-like sequences located in the *aml* promoter region and constituting the Reg 1-binding site (Virolle & Gagnat, 1994; Nguyen, 1999) concomitantly abolishes both maltodextrin induction and glucose repression of *aml* expression. Similarly, in *S. coelicolor*, the interruption of *gylR* and *malR* encoding the negative transcriptional regulators of the glycerol operon *gylX-ABC* (Hindle & Smith, 1994) and the maltose transport operon *malEFG* (van Wezel *et al.*, 1997), respectively, and the mutation of operator-like sequences located in the promoter region of *chi* (encoding chitinase) from *Streptomyces plicatus* (Delic *et al.*, 1992; Ni & Westpheling, 1997) concomitantly abolish both substrate induction and glucose repression of these genes and

operons. These observations suggest either that inducer exclusion is the main mechanism contributing to catabolite repression in *Streptomyces* or that the specific negative transcriptional regulators are mediating the process. The latter situation would be unusual since in *E. coli* or *Bacillus subtilis* catabolite repression is mainly mediated by the presence or absence of positive or negative pleiotropic transcriptional regulators, the cAMP-CRP (cAMP receptor protein) complex and CcpA, respectively (Saier *et al.*, 1995). Nevertheless, the contribution to catabolite repression of specific regulators containing a phosphotransferase system regulation domain (PRD regulators) has been demonstrated in *B. subtilis* (Stulke *et al.*, 1998).

The apparent relief of catabolite repression of *aml* expression observed in the *sbIA* mutant strain is not easy to rationalize. It could simply be due to an absence of glucose transport/metabolism resulting from the early growth arrest characteristic of this mutant. Nevertheless, in the *sbIA* mutant, Reg1, the negative transcriptional regulator of *aml* expression has obviously less affinity for its binding sites than in the wild-type strain. The affinity of transcriptional regulators for their target sites could be modulated by many mechanisms. A classical way is the binding of an effector molecule inducing a conformational change leading to the reduction or the enhancement of the affinity of the transcriptional regulators for their target sites (Matthews & Nichols, 1998). Alternatively, a post-translational modification (phosphorylation) of a regulator or its interaction with another protein could modulate its affinity for target sites. For instance, in *B. subtilis*, the phosphorylation state of PRD regulators governs their DNA-binding ability (Martin-Verstraete *et al.*, 1998), and interaction of the pleiotropic catabolite repressor CcpA with a phosphorylated form of the phosphotransferase Hpr, Hpr Ser 46P, modulates its affinity for the catabolite-responsive elements present in the promoter regions of genes sensitive to glucose repression (Deutscher *et al.*, 1995). The SblA protein bears similarities to various phosphatases of the IMP family. These enzymes are usually involved in the dephosphorylation (or the phosphorylation) of small phosphorylated molecules. Considering these structural similarities, as well as the enhancement of *aml* expression in the absence of exogenous inducer in the *sbIA* mutant, we propose that SblA is usually involved in the degradation of an internal inducer of *aml* expression or of a precursor of such an inducer. For instance, a phosphorylated glucose derivative (e.g. glucose-1-P as in *E. coli*; Decker *et al.*, 1993) might accumulate in the *sbIA* mutant and be used as a precursor for the intracellular synthesis of an inducing maltodextrin. Alternatively, SblA might be involved in the production of a signalling molecule playing a role in catabolite repression in *Streptomyces*. In the glucose-grown cultures of the *sbIA* mutant, an internal inducer of *aml* expression would be made (or would not be degraded) or the synthesis of a signalling molecule for catabolite repression would not occur, leading to the observed enhanced *aml* expression.

The identification of the SblA substrate should allow a better understanding of the biological role of this protein and of its connection with the phenomenon of catabolite repression in *Streptomyces*.

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REFERENCES

- Ahlert, J., Distler, J., Mansouri, K. & Piepersberg, W. (1997). Identification of *stsC*, the gene encoding the L-glutamine:scyllo-inosose aminotransferase from streptomycin-producing streptomycetes. *Arch Microbiol* **168**, 102–113.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Bibb, M. J., Findlay, P. R. & Johnson, M. W. (1984). The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. *Gene* **30**, 157–166.
- Blondelet-Rouault, M. H., Weiser, J., Lebrhi, A., Branny, P. & Pernodet, J. L. (1997). Antibiotic resistance gene cassettes derived from the omega interposon for use in *E. coli* and *Streptomyces*. *Gene* **190**, 315–317.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. & Boyer, H. W. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.
- Boos, W. & Shuman, H. (1998). Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiol Mol Biol Rev* **62**, 204–229.
- Decker, K., Peist, R., Reidl, J., Kossmann, M., Brand, B. & Boos, W. (1993). Maltose and maltotriose can be formed endogenously in *Escherichia coli* from glucose and glucose-1-phosphate independently of enzymes of the maltose system. *J Bacteriol* **175**, 5655–5665.
- Delic, I., Robbins, P. & Westpheling, J. (1992). Direct repeat sequences are implicated in the regulation of two *Streptomyces* chitinase promoters that are subject to carbon catabolite control. *Proc Natl Acad Sci USA* **89**, 1885–1889.
- Deutscher, J., Kuster, E., Bergstedt, U., Charrier, V. & Hillen, W. (1995). Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in gram-positive bacteria. *Mol Microbiol* **15**, 1049–1053.
- Hindle, Z. & Smith, C. P. (1994). Substrate induction and catabolite repression of the *Streptomyces coelicolor* glycerol operon are mediated through the GylR protein. *Mol Microbiol* **12**, 737–745.
- Hopwood, D. A., Bibb, M. J., Chater, K. F. & 7 other authors (1985). *Genetic Manipulation of Streptomyces: a Laboratory Manual*. Norwich: John Innes Foundation.
- Ikeda, H., Seno, E. T., Bruton, C. J. & Chater, K. F. (1984). Genetic mapping, cloning and physiological aspects of the glucose kinase gene of *Streptomyces coelicolor*. *Mol Gen Genet* **196**, 501–507.
- Janssen, G. R. & Bibb, M. J. (1985). Derivatives of pUC18 that have *Bgl*III sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. *Gene* **124**, 133–134.

- Jucker, F. M., Heus, H. A., Yip, P. F., Moors, E. H. & Pardi, A. (1996). A network of heterogeneous hydrogen bonds in GNRA tetraloops. *J Mol Biol* **264**, 968–980.
- Klein, B., Foreman, J. A. & Searcy, R. L. (1969). The synthesis and utilization of Cibachron Blue-amylose: a new chromogenic substrate for determination of amylase activity. *Anal Biochem* **31**, 412–425.
- Leblond, P., Francou, F. X., Simonet, J. M. & Decaris, B. (1990). Pulsed-field gel electrophoresis analysis of the genome of *Streptomyces ambofaciens* strains. *FEMS Microbiol Lett* **60**, 79–88.
- Leblond, P., Redenbach, M. & Cullum, J. (1993). Physical map of the *Streptomyces lividans* 66 genome and comparison with that of the related strain *Streptomyces coelicolor* A3(2). *J Bacteriol* **175**, 3422–3429.
- Martin-Verstraete, I., Charrier, V., Stulke, J., Galinier, A., Erni, B., Rapoport, G. & Deutscher, J. (1998). Antagonistic effects of dual PTS-catalysed phosphorylation on the *Bacillus subtilis* transcriptional activator LevR. *Mol Microbiol* **28**, 293–303.
- Matsuhisa, A., Suzuki, N., Noda, T. & Shiba, K. (1995). Inositol monophosphatase activity from the *Escherichia coli* *subB* gene product. *J Bacteriol* **177**, 200–205.
- Matthews, K. S. & Nichols, J. C. (1998). Lactose repressor protein: functional properties and structure. *Prog Nucleic Acid Res Mol Biol* **58**, 127–164.
- Murray, M. G. (1986). Use of sodium trichloroacetate and mung bean nuclease to increase sensitivity and precision during transcript mapping. *Anal Biochem* **158**, 165–170.
- Muth, G., Nussbaumer, B., Wohlleben, W. & Pühler, A. (1989). A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. *Mol Gen Genet* **219**, 341–348.
- Neuwald, A. F., York, J. D. & Majerus, P. W. (1991). Diverse proteins homologous to inositol monophosphatase. *FEBS Lett* **294**, 16–18.
- Nguyen, J. (1999). The regulatory protein Reg1 of *Streptomyces lividans* binds the promoter region of several genes repressed by glucose. *FEMS Microbiol Lett* **175**, 51–58.
- Nguyen, J., Francou, F., Virolle, M. J. & Guerinéau, M. (1997). Amylase and chitinase genes in *Streptomyces lividans* are regulated by *reg1*, a pleiotropic regulatory gene. *J Bacteriol* **179**, 6383–6390.
- Ni, X. & Westpheling, J. (1997). Direct repeat sequences in the *Streptomyces chitinase-63* promoter direct both glucose repression and chitin induction. *Proc Natl Acad Sci USA* **94**, 13116–13121.
- Parish, T., Liu, J., Nikaido, H. & Stoker, N. G. (1997). A *Mycobacterium smegmatis* mutant with a defective inositol monophosphatase gene homolog has altered cell envelope permeability. *J Bacteriol* **179**, 7827–7833.
- Pridham, T. G., Anderson, P., Foley, C., Lindenfelser, L. A., Hesseltine, C. W. & Benedict, R. C. (1957). A selection media for maintenance and taxonomic study of *Streptomyces*. *Antibiot Annu* 947–953.
- Redenbach, M., Kieser, H. M., Denapaité, D., Eichner, A., Cullum, J., Kinashi, H. & Hopwood, D. A. (1996). A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol Microbiol* **21**, 77–96.
- Saier, M. H., Jr, Chauvaux, S., Deutscher, J., Reizer, J. & Ye, J. J. (1995). Protein phosphorylation and regulation of carbon metabolism in Gram-negative versus Gram-positive bacteria. *Trends Biochem Sci* **20**, 267–271.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Solenberg, P. J. & Baltz, R. H. (1991). Transposition of Tn5096 and other IS493 derivatives in *Streptomyces griseofuscus*. *J Bacteriol* **173**, 1096–1104.
- Solenberg, P. J., Burgett, S. G. & Murray, M. G. (1989). Method for selection of transposable DNA and characterization of a new insertion sequence, IS493, from *Streptomyces lividans*. *J Bacteriol* **171**, 4807–4813.
- Strauch, E., Takano, E., Baylis, H. A. & Bibb, M. J. (1991). The stringent response in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **5**, 289–298.
- Strohl, W. R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res* **20**, 961–974.
- Stulke, J., Arnaud, M., Rapoport, G. & Martin-Verstraete, I. (1998). PRD – a protein domain involved in PTS-dependent induction and carbon catabolite repression of catabolic operons in bacteria. *Mol Microbiol* **28**, 865–874.
- Takano, E., Gramajo, H. C., Strauch, E., Andres, N., White, J. & Bibb, M. J. (1992). Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **6**, 2797–2804.
- Tercero, J. A., Espinosa, J. C., Lacalle, R. A. & Jimenez, A. (1996). The biosynthetic pathway of the aminonucleoside antibiotic puromycin, as deduced from the molecular analysis of the *pur* cluster of *Streptomyces alboniger*. *J Biol Chem* **271**, 1579–1590.
- Virolle, M.-J. & Bibb, M. J. (1988). Cloning, characterization and regulation of an alpha-amylase gene from *Streptomyces limosus*. *Mol Microbiol* **2**, 197–208.
- Virolle, M.-J. & Gagnat, J. (1994). Sequences involved in growth-phase-dependent expression and glucose repression of a *Streptomyces* α -amylase gene. *Microbiology* **140**, 1059–1067.
- Virolle, M.-J., Morris, V. J. & Bibb, M. J. (1990). A simple and reliable turbidimetric and kinetic assay for alpha-amylase that is readily applied to culture supernatants and cell extracts. *J Ind Microbiol* **5**, 295–302.
- Volff, J. N., Eichenseer, C., Viell, P., Piendl, W. & Altenbuchner, J. (1996). Nucleotide sequence and role in DNA amplification of the direct repeats composing the amplifiable element AUD1 of *Streptomyces lividans*. *Mol Microbiol* **21**, 1037–1047.
- van Wezel, G. P., White, J., Young, P., Postma, P. W. & Bibb, M. J. (1997). Substrate induction and glucose repression of maltose utilization by *Streptomyces coelicolor* A3(2) is controlled by *malR*, a member of the *lacI-galR* family of regulatory genes. *Mol Microbiol* **23**, 537–549.
- Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M., Sneath, P. H. & Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* **129**, 1743–1813.
- Yano, R., Nagai, H., Shiba, K. & Yura, T. (1990). A mutation that enhances synthesis of sigma 32 and suppresses temperature-sensitive growth of the *rpoH15* mutant of *Escherichia coli*. *J Bacteriol* **172**, 2124–2130.

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