Sequences involved in growth-phase-dependent expression and glucose repression of a *Streptomyces* α -amylase gene

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In glycerol-grown, but not in glucose-grown cultures, expression in *Streptomyces lividans* TK24 of the cloned α -amylase gene (*aml*) of *Streptomyces limosus* is switched on toward the end of exponential growth. During this period, *aml* expression is further inducible by maltotriose. We showed that a 378 bp fragment, extending from position –204 through to +174 (relative to the transcriptional start site), included *cis*-acting sequences involved in *aml* regulation. When this fragment was present on a multicopy plasmid, the growth-phase-dependent *aml* expression conferred by a DNA fragment cloned on a compatible low-copy-number plasmid was greatly enhanced, as if negative regulators were being titrated. A study of the regulation of *aml* expression in variants with deletions in the *aml* promoter region indicated that a direct repeat (DR) between positions –124 and –106 (relative to the transcriptional start site) and an inverted repeat (IR) between positions +9 and +24 were good candidates for secondary and primary operator sites, respectively. Deletion of a 29 bp fragment containing the IR rendered *aml* expression partly growth-phase-independent, resistant to glucose repression, and insensitive to maltotriose induction.

Keywords: *Streptomyces lividans*, α -amylase gene, glucose-repression, growth-phase-dependent expression

INTRODUCTION

In *Streptomyces*, growth-phase (or growth-rate) control of gene expression is a regulatory feature shared by many genes of secondary metabolism, such as antibiotic-production genes (Takano *et al.*, 1992; Gramajo *et al.*, 1993). Several authors have recently reported the identification of genes involved in regulating production of antibiotics: *absA* (Adamidis *et al.*, 1990), *absB* (Adamidis & Champness, 1992), *abaA* (Fernandez-Moreno *et al.*, 1992), *afsR* and *afsK* (Horinouchi & Beppu, 1992), *afsQ1* and *afsQ2* (Ishizuka *et al.*, 1992) and *dnrI* and *dnrJ* (Stutzman-Engwall *et al.*, 1992). Some of these regulators (AfsR/AfsK, AfsQ1/AfsQ2 and DnrI/DnrJ pairs) show similarities with elements of signal transduction pathways (consisting of sensor/modulator pairs) described in other bacteria (Stock *et al.*, 1989). Whether these genes play a role in growth-phase-dependent regulation of the production of extracellular enzymes such as α -amylase (Aml),

or whether genes encoding these enzymes have a specific regulatory network, is not known. However, Daza *et al.* (1990) have cloned a gene from *Streptomyces griseus* that increases production of at least five extracellular enzymes when it is overexpressed in *Streptomyces lividans* (Daza *et al.*, 1990, 1991). This gene encodes a small protein of 110 amino acids possessing a putative DNA-binding domain. It shows some similarity in amino acid sequence with the *sen* gene of '*Bacillus natto*', which is involved in regulating several genes encoding extracellular proteins (Wong *et al.*, 1988).

Expression of genes encoding extracellular enzymes such as agarase (Buttner *et al.*, 1987), chitinase (Delic *et al.*, 1992) and Aml (Virolle & Bibb, 1988), or of operons such as those for galactose and glycerol assimilation (Fornwald *et al.*, 1987; Smith & Chater, 1988), is glucose repressible at the transcriptional level. Preliminary data seem to indicate that the molecular mechanisms mediating glucose repression in *Streptomyces* differ from those described in *Escherichia coli* (Angell *et al.*, 1992; Postma *et al.*, 1993). These mechanisms are still incompletely understood in Gram-positive bacteria. Nevertheless, deletion or mu-

Abbreviations: Aml, α -amylase; CS, cibacron starch; DR, direct repeat; i.r., induction ratio; IU, international units; IR, inverted repeat; NR, negative regulators; PR, positive regulators.

tation of sequences thought to be involved *in cis* in glucose repression of many genes of *Bacillus* or *Staphylococcus*, leads to derepressed expression of the corresponding genes (Stewart, 1993; Rygus & Hillen, 1992; Sizemore *et al.*, 1992). In those bacteria, glucose repression seems to operate through true negative regulation, and not, as in the Gram-negative *E. coli*, through the absence of a positive regulatory component; the CRP-cAMP transcriptional activator (Botsford & Harman, 1992).

Sequences involved *in cis* in glucose repression of *Streptomyces* gene expression are not yet clearly elucidated. Nevertheless, recent reports have described the possible involvement in glucose repression of direct repeats (DRs) present in the promoter region of chitinase genes in *Streptomyces plicatus* (Delic *et al.*, 1992), and of the galactose operon in *Streptomyces coelicolor* A3(2) (Mattern *et al.*, 1993).

Regulation of the expression of the *aml* gene of *S. limosus* cloned in *S. lividans* constitutes a good model to elucidate molecular mechanisms involved in the growth-phase dependence and glucose repression of gene expression in *Streptomyces*. In glycerol-grown cultures, *aml* expression is spontaneously switched on toward the end of exponential growth. This expression lasts for 4–6 h. During the interval when it occurs, *aml* expression is further inducible by maltotriose (Virolle & Bibb, 1988). The presence of glucose in the growth medium represses growth-phase-dependent *aml* expression and maltotriose induction (Virolle & Bibb, 1988).

In this paper, we describe marked changes in the regulatory pattern of *aml* expression (when *aml* is present on a low-copy-number vector) caused by the presence in the same cell of various subfragments of the *aml* promoter region cloned on high-copy-number vectors and by deletions in the *aml* promoter region. This work points to sequences probably involved *in cis* in the complex regulation of *aml* expression.

METHODS

Bacterial strains and plasmids. *S. lividans* TK24 (Hopwood *et al.*, 1983) was the host for all plasmids. pIJ487 (Thio^R, copy number 100–300 per chromosome; Ward *et al.*, 1986), pIJ941 (Thio^R and Hyg^R, copy number 1–2 per chromosome; Lydiate *et al.*, 1985) and pTS43Ωfd (Spc^R derivative of the integrative plasmid pSAM2; Smokvina *et al.*, 1990, and unpublished) were used as cloning vectors. A Thio^S derivative of pIJ941 was made by digesting the plasmid with *EcoRV* and *ClaI*, blunt-ending with the Klenow fragment and religating. We needed this *tsr*-disrupted derivative (pOS6000) to select concomitantly for pIJ941 derivatives and derivatives of the multicopy plasmid pIJ487.

DNA isolation, manipulation and characterization. These were performed by established procedures (Hopwood *et al.*, 1985; Sambrook *et al.*, 1989). Restriction endonucleases and T4 DNA ligase (Boehringer-Mannheim) were used according to the manufacturer's recommendations.

Culture and transformation conditions. Standard media, methods of culture, transformation and cloning procedures were as described by Hopwood *et al.* (1985). After trans-

formation, *S. lividans* protoplasts were plated on R2YE medium and incubated at 30 °C for 17 h to allow expression of the resistance determinant carried by the vector. They were then overlaid with 2.5 ml SNA (Hopwood *et al.*, 1985) containing 2 mg spectinomycin ml⁻¹ for pSAM2 derivatives, 0.5 mg thiostrepton ml⁻¹ for pIJ487 derivatives, 2 mg hygromycin ml⁻¹ for pIJ941 derivatives and, to detect Aml activity, 2% (w/v) cibacron starch (CS). Culture and transformation of *E. coli* DH5α with pUC18 derivatives were carried out by established procedures (Sambrook *et al.*, 1989).

Preparation of CS and media containing it. We made CS using a modification of the method of Klein *et al.* (1969) described by Virolle & Bibb (1988). Plates containing a 4 mm-thick basal layer (20 ml) of 1.5% (w/v) agar, 50 mM K₂HPO₄/KH₂PO₄, pH 7, and 50 mM KCl received a 1 mm-thick top layer (5 ml) of the same medium containing 2% (w/v) CS.

Assays for Aml activity. The theoretical basis and methodology of the turbidimetric assay are described by Virolle *et al.* (1990).

For the CS plate test, 5 µl drops of the supernatant from a 500 µl culture sample were spotted on the CS agar plates described above, which were then incubated for 24 h at 37 °C. The surface and intensity of the zones of starch hydrolysis were scanned using a densitometer (BIOPROFIL apparatus from Vilber Lourmat) with a threshold of 40. A standard curve was established with dilutions of Aml from *Bacillus licheniformis* (Sigma) prepared in a buffer containing BSA (500 µg ml⁻¹), K₂HPO₄/KH₂PO₄ (50 mM, pH 7) and KCl (50 mM). The surfaces and intensities of the clearing zones increased linearly with the log of the enzyme concentrations (Ceska, 1971) from 0.02 to 0.16 IU ml⁻¹.

Calculation of the induction ratio (i.r.). Maltotriose was added as an inducer (0.005%, w/v) at time $t = 0$ to one of two matching glycerol-grown cultures. The i.r. at a time t was calculated as follows: $(At^m - At_0)/(At - At_0)$, where At_0 and At are Aml activities [IU (µg protein)⁻¹] at times $t = 0$ (t_0) and t , respectively, in 1 ml of the supernatant of a culture to which no maltotriose was added. At and At_0 represent Aml accumulation due to growth-phase-dependent *aml* expression. At^m is the Aml activity at time t in 1 ml of the supernatant of a culture to which maltotriose was added at t_0 . At^m represents Aml accumulation due to growth-phase-dependent *aml* expression and maltotriose induction. $(At - At_0)$ represents the increment of Aml activity due only to growth-phase-dependent *aml* expression during the time interval $t - t_0$. $(At^m - At_0)$ represents the increment of Aml activity due to growth-phase-dependent *aml* expression and to maltotriose induction during the time interval $(t - t_0)$. The ratio between these two increments represents the i.r. due to maltotriose addition.

Total-cell-protein assay. A modification of the biuret method of Herbert *et al.* (1971) was used (Virolle & Bibb, 1988).

Regulatory studies of *aml* expression in liquid minimal medium. In all regulatory studies, modified 'minimal medium' (NMMP) was used and spores were pregerminated, as described in Hopwood *et al.* (1985). The pregerminated spores (at 10⁷ ml⁻¹) were used to inoculate 150 ml Erlenmeyer flasks with four indents containing 20 ml of NMMP supplemented at 1% (w/v) with glycerol (non-repressing carbon source) or glucose (repressing carbon source). Cultures were grown at 30 °C under agitation at 160 r.p.m. on a Braun Certomat R orbital shaker until Aml activity was detected in the culture supernatant. From this point, Aml activity and growth were measured hourly until Aml activity suddenly became undetectable in the culture supernatant. Aml production in *S. lividans* carrying *aml* cloned in a derivative of the integrative vector pSAM2 (pTS6000) was

Fig. 1. (a) Sequence of the *aml* promoter region from position -344 to $+192$ relative to the transcriptional start site. Amino acids corresponding to the codons in the coding regions are shown in single letter code. The two vertical arrows indicate transcriptional start sites. Restriction sites as well as the putative transcriptional terminator of the ORF located upstream of *aml* are underlined; -35 and -10 sequences are double-underlined. The Shine-Dalgarno sequence (S/D) is underlined with a dotted line. IR and DR are indicated in bold letters and are underlined by arrows. (b) Scheme of the organization of the region between *aml* and the upstream ORF. Numbers 1–8 refer to restriction sites. The arrow above the line represents the *aml* transcript. T is the putative transcriptional terminator; O1 and O2 are the IR and DR putative operators, respectively. Solid lines are drawn to scale; dotted lines are not. Lines above and below the scheme show the inserts carried by the different plasmids used.

10–15-fold lower than when *aml* was cloned in pIJ941 (pOS6001). Low expression was partly attributed to the very clumpy growth of the cultures, but was also attributed to a difference in copy number and DNA topology between pIJ941 and pSAM2. The latter vector is integrated at one copy per genome; the copy number of the former was not accurately determined in our experiments and could vary with growth phase and growth rate. When 12–14 g of 4 mm-diameter glass beads were added to each culture flask to achieve dispersed growth, cultures took more than 48 h to reach stationary phase. Despite this modification, Aml production by TK24(pTS6000) was too low to be reliably detectable by the turbidimetric assay. We thus used the more sensitive CS plate test. Each experiment was carried out at least three times to confirm the reproducibility and validity of the observations.

RESULTS

Multiple copies of the *aml* promoter region in trans increase expression of *aml*

Operator-like structures have often been described as a complex array of tandem- or inverted-repeat (IR) sequences (Collado-Vides *et al.*, 1991). A computer search of the *aml* promoter region pinpointed several repeats involving the sequence CTTGCAG: a DR between positions -124 and -106 (one mismatch) and a perfect IR between positions $+9$ and $+24$, relative to the transcriptional start site (Fig. 1).

To check the *in vivo* ability of the potential operators to bind and thus to titrate regulatory proteins, and to

determine the type of regulation (positive or negative) of *aml* expression in *S. lividans* TK24, we cloned sub-fragments of the *aml* promoter region (Fig. 1b) into multicopy vectors and introduced them into a *S. lividans* TK24 strain containing pOS6000. pOS6000 is a Hygro^R Thio^S derivative (see Methods) of the low-copy-number vector pIJ941 (Lydiat *et al.*, 1985) carrying in its *Bam*HI site a 5.85 kb *Bcl*I fragment from *S. limosus* chromosomal DNA (Virolle & Bibb, 1988). This fragment carries the *aml* gene and confers regulated *aml* expression on *S. lividans*. The 378 bp *Alu*I–*Aat*II fragment containing both the DR and the IR, extending from position -204 through to $+174$ (sites 3–8), the 181 bp *Nar*I–*Nar*I fragment containing only the DR, extending from position -267 to -86 (sites 2–4) and the 260 bp *Nar*I–*Aat*II fragment containing only the IR, extending from position -86 to $+174$ (sites 4–8) relative to the transcriptional start site, were inserted into the *Bam*HI site of the multicopy plasmid pIJ487 (Ward *et al.*, 1986) giving, respectively, pOSAB, pOSA' and pOSB (Fig. 1b).

To examine the effect of increased copy number on *aml* expression, we grew TK24(pOS6000) containing either pOSAB, pOSA', pOSB or pIJ487 (as the control) in NMMP plus glycerol. We maintained selective pressure for both plasmids by adding thiostrepton at $5 \mu\text{g ml}^{-1}$ and hygromycin at $10 \mu\text{g ml}^{-1}$. This double selection led to a very low growth rate of the cultures. Aml activity started to be detectable only after 40–42 h. Late production starting toward the end of exponential growth, in the

Fig. 2. Assay of Aml activity [$\text{IU } (\mu\text{g protein})^{-1}$] in glycerol-grown cultures of *S. lividans* TK24(pOS6000) carrying plJ487, pOSA', pOSB or pOSAB. $\circ$, Uninduced Aml activity; $\bullet$, maltotriose-induced Aml activity. At time 0, glycerol or glycerol+maltotriose were added at respective final concentrations of 1% (w/v) and 1%+0.005% (w/v) to glycerol-grown matching cultures in (a), (b), (c) and (d). Aml activity and total protein assays were assayed every hour for 4–7 h after 40–42 h of growth (time 0). (a) TK24(pOS6000+plJ487); i.r. values at time points 2, 3 and 4 are 5.1, 1.6 and 1.8. (b) TK24(pOS6000+pOSA'); i.r. values at time points 2, 3, 4 and 5 are 3.7, 1.1, 0.6 and 0.6. (c) TK24(pOS6000+pOSB); i.r. values at time points 2, 3, 4 and 5 are 3.8, 1.2, 0.8 and 0.7. (d) TK24(pOS6000+pOSAB); i.r. values at time points 2, 3, 4, 5, 6 and 7 are 1.2, 1, 0.8, 0.7, 0.7 and 0.7. (a) is repeated to scale in (d).

absence of any external inducer, suggested a growth-phase-dependent regulation of *aml* expression. This was confirmed by later experiments. At this stage, we added 1% (w/v) glycerol and 1% (w/v) glycerol+0.005% (w/v) maltotriose to glycerol-grown matching cultures. From this point, we estimated total protein and Aml activity hourly for 4–7 h (Fig. 2). We assayed Aml activity until it dropped suddenly (within 1 h) to an undetectable level. Whether this rapid disappearance was due to specific or unspecific proteolysis, or due to the production of an inhibitor, was not investigated. TK24(pOS6000) containing either pOSA', pOSB or plJ487 showed approximately the same growth rate ($0.4 \mu\text{g ml}^{-1} \text{h}^{-1}$) while TK24(pOS6000) containing pOSAB had a slightly inferior growth rate ($0.25 \mu\text{g ml}^{-1} \text{h}^{-1}$). The control

strain (Fig. 2a) showed reproducible induction of Aml activity when maltotriose was added to glycerol-grown cultures. Two hours after maltotriose addition, the i.r. (defined in Methods) was 5.1. Previous results showed that this induction takes place at the mRNA level (Virolle & Bibb, 1988).

The level of Aml production and the pattern of *aml* regulation were quite different in TK24(pOS6000) containing pOSAB (Fig. 2d). Aml production (uninduced or induced) was 10–20-fold higher than in the control strain, and there was no significant maltotriose induction. Production started 1–2 h earlier, and lasted 2–3 h longer, than in the control strain. The large Aml production of this strain might explain its slower growth rate. No Aml activity was detected during the exponential phase of growth (data not shown), meaning that *aml* expression was still subjected to strong growth-phase-dependent control. We attributed the very large increase in Aml production, and the insensitivity of *aml* expression to maltotriose induction, to the binding, and thus titration, of negative regulators (NRs) by the putative operator sequences carried by pOSAB.

The level of Aml production and the pattern of *aml* regulation were the same in TK24(pOS6000) containing pOSA' or pOSB (Fig. 2b, c). Two hours after maltotriose induction, these two strains gave i.r. values of 3.7 and 3.8, respectively, slightly inferior to that of the control strain (5.1). They produced 3–4-fold higher levels of Aml than the control strain, and lower levels than TK24(pOS6000) containing pOSAB. Thus, we supposed that pOSA' and pOSB did not bind NRs as efficiently as pOSAB.

In all the cultures one notices that the i.r. falls reproducibly below 1 (except in the control strain, see Fig. 2 legend) 4, 5 and 6 h after maltotriose addition, meaning that in the long term the presence of maltotriose reduces *aml* expression.

Deletion analysis of the *aml* promoter region

To refine analysis of the *aml* promoter region, and to confirm the role of the putative operators in negative regulation, we have studied the production of Aml in TK24 containing deletions (Fig. 1b) within the initial 5.85 kb *Bcl*I fragment cloned in the *Bam*HI site of plJ941. pOS6001 carried the initial 5.85 kb *Bcl*I insert; pOS6002 carried a fragment with its 5' end at the *Pvu*II site (site 1) 344 bp upstream of the *aml* transcriptional start site; pOS6003 carried a fragment with its 5' end at the *Nar*I site (site 4) located 86 bp upstream of the *aml* transcriptional start site. The latter deletion removes the entire upstream ORF as well as the DR located between –124 and –106. The three fragments have the same 3' endpoint. We maintained selective pressure for the plasmids by including hygromycin ($20 \mu\text{g ml}^{-1}$) in the medium. Aml activity was first detected after 20–22 h growth (time point 0). Glycerol, or glycerol and maltotriose, were then added to the glycerol-grown cultures. The three cultures showed approximately the same growth rate of $0.4 \mu\text{g ml}^{-1} \text{h}^{-1}$ but reached the Aml production phase much faster (20–22 h versus 40–42 h) than the cultures

Fig. 3. Assay of Aml activity [$\text{IU } (\mu\text{g protein})^{-1}$] in glycerol-grown cultures of *S. lividans* TK24 carrying pOS6001, pOS6002 or pOS6003. $\circ$, Uninduced Aml activity; $\bullet$, maltotriose-induced Aml activity. At time 0, glycerol or glycerol + maltotriose were added at respective final concentrations of 1% (w/v) and 1% + 0.005% (w/v) to glycerol-grown matching cultures in (a), (b) and (c). Aml activity and total protein were assayed every hour for 4–7 h after 20–22 h of growth (time 0). (a) TK24(pOS6001); i.r. values at time points 1, 2, 3, 4, 5 and 6 are 5.5, 2.4, 2.7, 1.5, 1.2 and 0.9. (b) TK24(pOS6002); i.r. values at time points 1, 2, 3, 4, 5 and 6 are 4.8, 5.5, 3, 1.8, 1 and 0.8. (c) TK24(pOS6003); i.r. values at time points 1, 2, 3, 4 and 5 are 27, 17, 6.6, 2.3 and 0.7.

studied in the previous section. The presence of hygromycin in addition to thiostrepton in the growth medium in the previous experiments may explain this difference, which prevents a direct comparison of the two sets of cultures.

Both of the partially deleted promoter constructs led to lower levels of Aml production than those occurring in TK24(pOS6001) (Fig. 3). The i.r. calculated 1 or 2 h after maltotriose addition for TK24(pOS6003) was reproducibly 4–5-fold larger than the i.r. calculated for TK24(pOS6001) and TK24(pOS6002). The higher i.r. mainly reflected a higher induced expression, notwithstanding a reduced basal *aml* expression (at least for time points 1, 2 and 3). This suggests that sequences between –344 and –86 play a negative role in determining induction levels. Without inducer, at time points 4 and 5, TK24(pOS6003) produced significantly more Aml than TK24(pOS6002); thus the –86 deletion also seems to have a slight enhancing effect on *aml* basal expression.

These observations are consistent with sequences between –344 and –86 (including the DR sequence) contributing to, but not playing the major role in, the repression of *aml* expression. Again one notices that 5 or 6 h after maltotriose addition the i.r. in all the cultures fell below 1 (see Fig. 3 legend).

To investigate the role of the IR downstream of +1, we deleted the interval between the *Nru*I (–2) and *Eco*RI (+29) sites of the 5.85 kb *Bcl*I fragment, rejoining the plasmid after blunt-ending with the Klenow fragment. This created a new *Eco*RI site and should not have changed +1. We made this construct in a pUC18 derivative and transferred it into the low-copy-number *Streptomyces* vector pIJ941 (Lydiat *et al.*, 1985). The resulting plasmid was very unstable, so we cloned the

Fig. 4. Restriction map of pTS6000. This plasmid contains the following five modules in clockwise direction: the omega interposon conferring spectinomycin resistance (Prentki & Krisch, 1984); pBR322 (Bolivar *et al.*, 1977); the 5.85 kb *Bcl*I fragment from *S. limosus* conferring Aml production upon *S. lividans* (Virolle & Bibb, 1988); the transcriptional terminator of the phage fd (Tfd; Gentz *et al.*, 1981); and the *Bam*HI–*Eco*RI fragment of pSAM2 containing all the DNA sequences necessary for site-specific integration via the *att* attachment site (Boccard *et al.*, 1989). MCS, multiple cloning sites. In pTS6000^A, the 5.85 kb *Bcl*I fragment bears an internal *Nru*I–*Eco*RI deletion of 29 bp.

fragment with the deletions into pTS43 Ω fd, giving pTS6000^A. pTS43 Ω fd is a derivative of a pSAM2-based integrative vector developed in our laboratory (Smokvina *et al.*, 1990; M. J. Virolle & J. Gagnat, unpublished). We also cloned the 5.85 kb *Bcl*I fragment containing *aml* into pTS43 Ω fd, giving pTS6000 (Fig. 4), to use as a reference. TK24 strains carrying either plasmid clearly produced

Table 1. Aml activity assay

Aml activity [$\text{IU} (\mu\text{g protein})^{-1}$] was assayed in the supernatant of glycerol-grown cultures of *S. lividans* TK24 carrying pTS6000 or pTS6000^A 1 h after the addition of glycerol (Gly), glycerol + maltotriose (Gly^m) or glucose + maltotriose (Glu^m) at respective final concentrations of 1% (w/v) and 1% + 0.005% (w/v). The values reported are means of at least three assays carried out with each strain.

Addition	TK24(pTS6000)	TK24(pTS6000 ^A)
Gly	0.025	0.154
Gly ^m	0.100	0.152
Glu ^m	0.034	0.200

Aml on R2YE agar plates containing CS; a control strain containing pTS43Ωfd did not. We confirmed the presence of the constructs in the TK24 chromosome by Southern blot analysis (data not shown). We determined the characteristics of *aml* regulation in TK24(pTS6000) and TK24(pTS6000^A) cultures under the growth conditions used previously, and assayed Aml activity by the CS plate test.

The results (Table 1) showed that *aml* expression in glycerol-grown cultures of TK24(pTS6000) was maltotriose inducible, and that induction was prevented by the concomitant addition of glucose, as previously found by Virolle & Bibb (1988) when the same insert was cloned in pIJ941. With the culture conditions used, and based on assays for Aml activity, glucose repression of maltotriose induction was not absolute. Uninduced *aml* expression with TK24(pTS6000^A) was higher than with TK24(pTS6000); maltotriose addition had no stimulatory effect, but glucose had a clear stimulatory effect on the already high *aml* expression (see Table 1).

When the CS plate test was used to follow Aml production throughout growth (Fig. 5a, b), glycerol- and glucose-grown cultures of TK24(pTS6000^A) began production at the start of growth. However, a distinct increase in Aml production occurred in glycerol-grown cultures between 48 and 51 h (Fig. 5b, c). This increase was not seen in glucose-grown cultures. Aml production was higher throughout growth in the glucose-grown cultures, and was thus detected earlier.

In contrast (if we disregard the very weak amylolytic activity observed between 24 and 33 h in glycerol- and glucose-grown cultures), TK24(pTS6000) produced Aml only in glycerol-grown cultures (and never in glucose-grown cultures) and only at the approach of stationary phase. Production was 6–8-fold lower than by TK24(pTS6000^A). The growth rate of TK24(pTS6000^A) was much slower than that of TK24(pTS6000). This might be due to the drain exerted on cell metabolism, or to blockage of the export system by high Aml production. The deregulated *aml* promoter may be very strong, and read-through into adjacent vector sequences could explain why this construct was not stable in pIJ941.

Fig. 5. Assay of Aml activity [$\text{IU} (\mu\text{g protein})^{-1}$] in cultures of *S. lividans* TK24 carrying pTS6000 or pTS6000^A. Every 3 or 6 h, from the 15–57 h of growth, a 500 μl sample of each culture was centrifuged. The mycelial pellet was assayed for total protein to measure growth, shown in (a). ○, Glucose-grown TK24(pTS6000) cultures; ●, glycerol-grown TK24(pTS6000) cultures; □, glucose-grown TK24(pTS6000^A) cultures; ■, glycerol-grown TK24(pTS6000^A) cultures. A 5 μl drop from each supernatant was placed on a CS plate and incubated for 24 h at 37 °C; a photograph of the plate is shown in (b). The zones of hydrolysis could be detected visually after 18 h of growth for TK24(pTS6000^A) cultures, whereas the densitometer (BIOPROFIL apparatus from Vilber Lourmat) reliably detected them only after 30 h of growth. A scan of the photograph with the densitometer using a threshold of 40 is shown in (c).

DISCUSSION

Experiments using high-copy-number effects and deletion mutations in the *aml* promoter region suggested that expression of the *S. limosus aml* gene cloned in *S. lividans* was negatively regulated. Two regions were involved in negative *aml* regulation. Both contain pairs of repeats of the sequence CTTGCAG. The two regions do not contribute equally to negative regulation of *aml*. Deletion of sequences upstream of –86, including a DR of

CTTGCAG, mainly enhanced inducible *aml* expression, whereas deletion of a 29 bp fragment between -2 and +29, including an IR of CTTGCAG, dramatically increased *aml* expression. Expression became partly growth-phase independent and insensitive to maltotriose induction and glucose repression. Based on the results of the deletion experiments, we propose that the IR may be a primary operator (O1) and the DR may be a secondary operator (O2). The results of the titration experiments (pOSAB had much greater titrating ability than pOSA' and pOSB) suggest that complete repression of *aml*, which implies a tight binding of the repressors, may involve repressor-mediated formation of a DNA loop (Matthews, 1992). This loop may be formed between the DR and the IR bracketing the *aml* promoter region, blocking access of the RNA polymerase to the promoter region. The repressors would disengage from the operator site in the presence of the inducer maltotriose. Future experiments to verify this model will include site-directed mutagenesis of the putative operators and band-shifting experiments.

Expression of *aml* was growth-phase dependent and, during the period of expression, further inducible by maltotriose. Glucose repressed both aspects of *aml* expression. Glucose strongly repressed growth-phase-dependent *aml* expression in TK24(pTS6000), and we never detected Aml activity in the supernatant of 40 h glucose-grown cultures of TK24(pOS6000) containing pOSAB (and a fortiori in none of the other cultures; data not shown). Virolle & Bibb (1988) have shown, and we confirmed using enzyme assays, that the concomitant addition of glucose and maltotriose, at respective final concentrations of 1% (w/v) and 0.005%, to glycerol-grown cultures actively producing Aml represses maltotriose induction at the transcriptional level. In glycerol-grown cultures maltotriose addition invariably had a positive effect on *aml* expression at first, but in the long run reduced *aml* expression (Figs 2 and 3). Maltotriose is probably metabolized via glucose; the minute amount of glucose formed might be sufficient to cause some level of glucose repression.

We have shown that deletion of a 29 bp fragment containing the IR downstream of +1 removes both maltotriose induction and glucose repression of *aml* expression. This is reminiscent of the situation with *chi63* operators of *Streptomyces plicatus*. Delic *et al.* (1992) showed that a single base mutation within a perfect DR between -47 and -15 in the promoter region of the *S. plicatus* chitinase gene *chi63* abolishes induction as well as glucose repression of this gene. These structures might be recognized by two types of transcriptional regulators: a pleiotropic catabolite repressor and a specific repressor. Alternatively, they might interact only with a specific repressor which itself also mediates catabolite repression. The latter regulatory strategy has been clearly demonstrated in the case of the glycerol operon of *S. coelicolor* (Z. Hindle & C. P. Smith, unpublished results). The authors have shown that the creation of a null mutation in the GylR repressor of the *gyl* operon of *S. coelicolor* concomitantly abolishes specific regulation and catabolite

repression of this operon. This result suggests that in *Streptomyces*, catabolite repression might be mediated by specific repressors and not by a pleiotropic transcriptional regulator.

However, on the basis of mutagenesis or deletion experiments of the *chi63* and *aml* operator-like sequences we cannot exclude that these sequences interact with two different proteins. The overlapping of sites involved in catabolite and specific repression was described for the lactose operon of *Staphylococcus aureus* (Oskouian & Stewart, 1990), but for most of the studied genes or operons of *Bacillus* and *Staphylococcus* species the Catabolite Responsive Elements (CRE) and the specific operator-like sequences are clearly distinct (Fisher & Sonenshein, 1991). The latter is the target of specific regulators whereas the protein interacting with the former is unknown, but a putative candidate for this role would be the CcpA protein (Chambliss, 1993).

The experiments described here demonstrate that *aml* expression is negatively regulated. However, Daza *et al.* (1990) suggest that a positive regulator (PR) regulates production of several extracellular proteins in *S. lividans*, and some of our results implicate PRs in the complex regulation of *aml* expression. For instance, the lack of titrating ability of pOSAB during exponential growth in glycerol-grown cultures, or throughout growth in glucose-grown cultures (data not shown), could be attributed to the absence of PRs. The presence of these putative PRs would be necessary to reveal (by the titration ability of pOSAB) the existence of the NRs. Similarly, the reduced Aml production in TK24(pOS6002) and TK24(pOS6003) cultures compared with TK24(pOS6001) cultures could be attributed to the deletion of some upstream activating sequences (even though at this stage we cannot exclude that it is due to trivial topological effects). These sequences would be located upstream of the *PvuII* site, more than 300 bp upstream of the -35 region. Furthermore, the quantitative jump in Aml production between 48 and 51 h in glycerol-grown cultures in TK24(pTS6000^A), which is approximately coincidental with the start of Aml production in cultures of TK24(pTS6000), argues as well in favour of PRs.

In summary, we propose the following working model for growth-phase-dependent regulation and glucose repression of *aml*. During the exponential phase in glycerol-grown cultures, NRs prevail and the PRs are ineffective; consequently *aml* will not be expressed, even if the inducer maltotriose is added to the growth medium. At the approach of stationary phase, switching on of the synthesis of PRs, and perhaps a decrease in the synthesis and/or in the DNA-binding ability of NRs, lead to *aml* expression. In glucose-grown cultures, synthesis of PRs would be repressed and the synthesis and/or DNA-binding ability of NRs would be enhanced, leading to the silencing of *aml*.

In glycerol-grown cultures of TK24(pTS6000^A), we assume that *aml* is expressed throughout growth because the NRs have little or no affinity for the *aml* promoter region after their main operator site has been deleted.

Nevertheless the quantitative jump in Aml production between 48 and 51 h in glycerol-grown cultures indicates that the mutant promoter is still capable of activation. Consistent with the model, this jump does not occur in glucose-grown cultures. In TK24(pTS6000^A), *aml* expression is higher throughout growth in glucose- than in glycerol-grown cultures. This might be due to a growth-rate effect (all strains grow faster with glucose than with glycerol) or to other trivial effects.

Our data suggest that *aml* regulation involves a complex interplay of multiple genes with contradictory effects on gene expression. To further our insight into the complex regulation of *aml*, we aim to identify regulatory protein(s) interacting with the *aml* promoter region and mediating the regulatory features described above.

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