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ORIGINAL PAPER

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# Site-directed mutagenesis of conserved inverted repeat sequences in the xylanase C promoter region from *Streptomyces* sp. EC3

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**Abstract** *Streptomyces* sp. EC3, a strain which was originally isolated from cattle manure compost, was shown to possess a strong xylanolytic activity. One of the genes responsible for this activity, *xlnC* , encodes a secreted xylanase. In the native strain, as in the heterologous host *S. lividans*, expression of *xlnC* was detectable in the presence of xylan but not in the presence of glucose. Induction by xylan was shown to take place at the transcriptional level. The transcriptional start site of *xlnC* was mapped and likely –35 (5′-TTGACA-3′) and –10 (5′-GAGAAC-3′) motifs were identified. In order to localise putative conserved regulatory sequences, the promoter regions of xylanase-encoding genes from various *Streptomyces* species were aligned. This alignment revealed the existence of three sets of quite well conserved palindromic AT rich sequences called boxes 1, 2 and 3. Box 3 (5′-CGAAA N TTTCG-3′) is the farthest away from the promoter region (150–200 bp). A shorter version of this palindrome (5′-GAAA NN TTTC-3′) or (5′-CGAAA-3′) constitutes box 1, which is located just upstream of the putative –35 promoter sequence. Box 2, located 5–7 bp upstream of box 1, comprises a shorter palindrome than box 3, with inverted polarity [5′-(G/C)TTTC (N) GAAA(G/C)-3′]. The putative regulatory role of the conserved inverted repeats in boxes 2 and 3 in the promoter region of the *xlnC* gene from *Streptomyces* sp. EC3, was assessed. These boxes were modified by site-directed mutagenesis, and the mutant promoter regions, as well as the wild-type promoter region, were separately fused to a β-lactamase reporter gene. Analysis of the expression patterns of these fusions in cultures grown in the presence of glucose, xylan or both carbon sources demonstrated that these motifs were *cis* -acting negative regulatory elements, each playing a specific role in the regulation of *xlnC* expression. Box 3 was shown to be critical for the establishment of repression of *xlnC* expression by glucose, whereas box 2 was shown to play an important role in the induction of *xlnC* expression by xylan.

**Keywords** S1 mapping · DNA-binding protein · Catabolite repression · Transcriptional regulation · Operator sequence

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## Introduction

Members of the genus *Streptomycetes* are soil bacteria that rely primarily on complex carbohydrates originating from plants for their carbon supply. These bacteria possess numerous genes that code for secreted hydrolytic enzymes involved in the degradation of cellulose, starch, chitin or xylan. The genes encoding several of these enzymes have been cloned and partially characterised. These genes share common regulatory features: their expression is usually inducible by the corresponding substrate and repressible by glucose. In some instances, the inducing substrate has been shown to be taken up into the cell by ABC transporters such as those encoded by the *cebEFG* operon for the cellobiose/cellotriose uptake of *Streptomyces reticuli* (Schlösser et al. 1999), by the *malEFG* operon for the uptake of maltose and maltodextrin by *S. coelicolor* (van Wezel et al. 1997) or *S. lividans* (Schlösser et al. 2001) or by the *ngcEFG* operon for the import of N-acetylglucosamine and chitobiose by *S. olivaceoviridis* (Xiao et al. 2002). Some of these transporters

share a common energy provider, the ATPase MsiK (Hurtubise et al. 1995), the gene for which is not linked to the transport operon (Schlösser et al. 1997, 1999). MsiK has been shown to be involved in the transport of numerous disaccharides including xylobiose, cellobiose, maltose and trehalose (Hurtubise et al. 1995; Schlösser et al. 1997; Schlösser 2000). The expression of specific transporters was shown to be inducible by the substrate of the cognate degradative enzyme, and repressible by glucose. The *malEFG* operon of *S. coelicolor*, and the *cebEFG* operon of *S. reticuli*, have been shown to be controlled by negative regulators of the LacI-GalR family encoded by loci located upstream of the operons and named *malR* and *cebR*, respectively. In *S. lividans*, the MalR/Reg1 regulator was shown also to control the expression of the *aml* gene for an α-amylase (Nguyen et al. 1997). Disruption of *malR*/*reg1* led to the concomitant abolition of substrate induction and catabolite repression of the expression of the *malEFG* operon in *S. coelicolor* (van Wezel et al. 1997) and of the *aml* gene in *S. lividans* (Nguyen et al. 1997). Similarly, the inactivation of GylR, the repressor of the glycerol operon *gylXABC*, had a comparable effect on the expression of this operon (Hindle and Smith 1994). The promoter regions of *malEFG* and *aml* share similar operator-like sequences, which have been shown to interact in vitro with purified Reg1 (Nguyen 1999) or MalR (Schlösser et al. 2001). The deletion of operator-like sequences present in the *aml* promoter region was shown to have similar effect than the interruption of *reg1* (Virolle and Gagnat 1994). Similarly, site-directed mutagenesis of direct repeats present in the *chi63* promoter region from *S. plicatus* was shown to abolish both substrate induction and glucose repression of the expression of this gene (Ni and Westpheling 1997; Delic et al. 1992).

We are studying the regulation of the expression of secreted enzymes from *Streptomyces* species specifically involved in the degradation of xylan, the second most abundant carbon polymer in plant cell walls after cellulose. Xylan is a complex low-molecular-weight polysaccharide with a backbone of β-1,4-linked β-D-xylose residues that carries various substituents. Complete breakdown of this complex polymer requires the action of several enzymes, including β-1,4-xylanases, which cleave the β-1,4-glycosidic bonds are thus crucial for its depolymerisation. On the basis of amino acid sequences and hydrophobic cluster analysis, xylanases have been divided into two families (Henrissat and Bairoch 1993). Family 10 contains acidic xylanases of high-molecular-mass (30 kDa) with an $(\alpha/\beta)_8$ barrel fold. Members of Family 11 are basic, have lower molecular-masses, and display a folding pattern characterised by one α-helix and two β-sheets which form a large cleft that can accommodate the xylan polymer (Törrönen et al., 1994). Family 10 enzymes show greater catalytic versatility and hydrolyse short xylo-oligosaccharides more efficiently than do family 11 enzymes (Biely et al. 1997). It was previously reported that *S. lividans* secretes three xylanases, encoded by the *xlnA*, *xlnB* and *xlnC* genes, respectively (Shareck et al. 1991). XlnA, XlnB and XlnC are thought to act sequentially on xylan to produce xylo-oligosaccharides of various lengths (Kluepfel et al. 1992). XlnB and XlnC, which belong to Family 11, cleave xylan to yield large xylo-oligosaccharides (degree of polymerisation $> X_{11}$), whereas XlnA (Family 10) yield predominantly small xylo-oligosaccharides ($X_2$–$X_5$). Furthermore, it was shown that large xylo-oligosaccharides, the products of XlnB and XlnC action, could stimulate the expression of *xlnC* and *xlnB*, whereas the small xylo-oligosaccharides ($X_2$–$X_5$) produced by XlnA activity did not (Arhin et al. 1994).

In order to extend our understanding of the regulation and glucose catabolite repression of genes encoding hydrolytic enzymes in *Streptomyces*, we cloned and sequenced a gene encoding a xylanase sharing more than 80% of strict identities with XlnC of *S. lividans* (Georis et al. 1999) from an unidentified *Streptomyces* species (strain EC3) selected for its high xylanolytic activity (Mazy-Servais et al. 1996). We have now investigated the role played by operator-like sequences, made up by three well conserved inverted repeat sequences called boxes 1, 2 and 3, found in the promoter region of the xylanase gene *xlnC*.

Here, we report the effects of alterations in box 2, box 3, or both, on the regulation of the expression of a convenient reporter system directed by the *xlnC* promoter region in the host strain *S. lividans*.

## Materials and methods

### Bacterial strains and media

*Streptomyces* sp. strain EC3, isolated from cattle manure compost, was a gift of Dr. M. Penninckx (Ceria-ULB, Brussels). *S. lividans* TK24 and *S. parvulus* IMET 41380 were obtained from the John Innes Centre (Norwich, UK).

Spores were used to inoculate YEME liquid medium (Kieser et al. 2000).

Basal Minimal Medium (BMM; pH 7.5) contained the following ingredients: $(NH_4)_2SO_4$ (2g/l), $MgCl_2$ (0.2 g/l), $CaCl_2$ /11 mg/l), $K_2HPO_4$ (2 g/l), and $KH_2PO_4$ (0.6 g/l).

### Xylanase assay

Endo-β-1,4-xylanase activity was measured by the dinitrosalicylic acid method (Miller 1959). Appropriately diluted 40-µl enzyme samples were mixed with 360 µl of a 1% suspension of birchwood xylan (Sigma Chemical) in 50 mM phosphate buffer (pH 7.0) and incubated for 10 min at 50°C. One international unit (IU) is defined as the amount of enzyme that releases 1 µmol of reducing sugar per min per ml. Biomass estimation was performed as follows. Cells were harvested from 5 ml of culture and washed with 5 ml of 10 mM TRIS-HCl (pH 7.5). Cells were lysed by sonication; lysates were centrifuged at 4°C for 10 min, and protein concentration was measured by the bicinchoninic acid method using a BCA Protein Assay Kit (Pierce).

### β-Lactamase assay

Some $10^6$ spores of *S. lividans* harbouring a transcriptional fusion were grown for 36 h in YEME medium at 28°C. Then 100 ml of fresh BMM containing thiostrepton (25 µg/ml) and either 2%

glucose, 1% oat spelt xylan (Sigma Chemical) or 0.5% xylan plus 1% glucose as sole carbon source was inoculated with 1 ml of the YEME preculture. Biomass estimation was carried out as described above. β-Lactamase activity was assayed using 20-µl samples of culture medium, after removal of cells by cenrtifugation, in the presence of 300 µl of 100 µM nitrocefin, at 30°C and pH 7.0. One unit represents the amount of enzyme that hydrolyses 1 µmol of nitrocefin per min per ml (Matagne et al. 1990).

## S1 nuclease protection assay

RNA for S1 mapping analysis was isolated from a culture of *Streptomyces* sp. EC3 grown under the conditions described below. Some $10^9$ spores of that strain were used to inoculate a YEME preculture, which was grown for 36 h at 28°C. Then 100 ml of fresh BMM, containing either 2% glucose or 1% birchwood xylan (Sigma) as sole carbon source, was inoculated with 1 ml of the preculture. The isolation of RNA was carried out using the Qiagen RNA/DNA Midi Kit. The integrity of the RNA was checked by agarose gel electrophoresis. The DNA used for the RNA-DNA hybridisation experiment was an 870-bp DNA fragment encompassing the 5′ end of the *xlnC* gene, amplified by PCR from *Streptomyces* sp. EC3 genomic DNA using the Cy-5 coupled oligonucleotide 5′-CGGTGCCGGCGCCGCCGAGGAAGCCTC-3′, which is complementary to the 5′-end of the *xlnC* gene, and with the oligonucleotide 5′-CCGCCCCTGCGCCCCCC-3′. The PCR product was purified by electroelution from an agarose gel. Then 0.1–0.2 pmol of the Cy-5 labelled probe was hybridised with 40-µg aliquots of RNA in a formamide buffer (40 mM PIPES pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% deionised formamide) at 45°C overnight, after denaturation at 65°C for 15 min. The hybridised products were treated with 200 U of S1 nuclease. A DNA sequencing ladder was generated from the Cy-5 coupled primer, using pXLNI as the template (Table 1 and Fig. 1). The protected fragment and the sequencing products were subjected to electrophoresis and analysed on an ALF Express DNA Sequencer (Amersham Bioscience) by the method outlined by Brans et al. (1997).

## DNA computer analysis

Multisequence alignments were performed using PILEUP and PRETTY software from the Genetics Computer Group program package.

## Construction of an *xlnC* promoter- *blaL* fusion

In order to place the *blaL* gene from *S. cacoi*, which encodes a β-lactamase, under the control of the *xlnC* promoter from *Streptomyces* sp. strain EC3, we adopted the strategy described below. A 1.1-kb DNA fragment containing the *blaL* gene was amplified by PCR from *S. cacaoi* genomic DNA. Convenient restriction endonuclease cleavage sites for directional cloning into pXLNI (Table 1) were incorporated into the primers used. The upstream primer (5′-GGTCCGGGCATGCGTATCCGTCC-3′) included a *Sph* I site (underlined) containing the ATG initiation codon of *blaL*, and a *Hin* dIII site was incorporated into the downstream primer (5′-GGGGAAGCTTACCGGGATGTTACCAGGGG-3′). PCR was carried out in a Biometra thermocycler (Eurogentec), using 100-µl reactions containing 1 U of *Pwo* and 0.5 U of *Tth* DNA polymerase (Eurogentec). The reaction conditions used were: 4 min at 96°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s at 72°C. The PCR product was digested with *Sph* I and *Hin* dIII, then ligated into pXLNI cleaved with the same enzymes. The resulting construct was named pDML619 (Table 1 and Fig. 1).

## Oligonucleotide-directed mutagenesis

pXLNI (Table 1) was used as the template for site directed mutagenesis. Mutant oligonucleotides were synthesised with two specific base changes within the palindromic sequence of the *xlnC* -boxes. The primer used to mutate box 2 (mB2) was 5′-CGGACGCTCC-GAAAGAGTGTCG-3′, so that the initial TT was replaced by GC (underlined). The primer used for or mutation of box 3 (mB3), 5′-CAGTGGTTCGGGACTTTCGATCG-3′ replaces the initial AA by GG (underlined). The external primers were 5′-CGTGCTGGGCAGCAGCAGTCC-3′ and 5′-GGTCT-AGACCGCCCCTGCGCCCCCC-3′ (*Xba* I site underlined), which hybridized 900-bp upstream and just downstream of the *xlnC* start codon, respectively. Introduction of the desired mutations was performed by a double PCR, according to the method of Higuchi et al. (1988). The mB23 mutation was obtained in the PCR product generated by using the mB3 fragment as primer and the mB2 promoter as template. All fragments containing the native and the mutagenised promoter regions were cleaved with *Xba* I (site incorporated into the external primer) and *Sph* I (site encompasses the *xlnC* ATG) (see the map in Fig. 1), and ligated into pUC 18 digested with the same enzymes. Their sequences were checked and they were subcloned into the *Kpn* I/*Sph* I-digested pDML619. Each reporter plasmid, containing the convenient fusion with *blaL*, was inserted by blunt-end ligation into pIJ702, after linearisation with *Bgl* II. The final constructs, named pDML639 (containing the wild-type promoter sequence), pDML639mB2, pDML639mB3 and

**Table 1** List of plasmids used in this study

| Plasmid | Host | Relevant characteristics | Source |
|---|---|---|---|
| pXLN1 | *E. coli* | pUC20 derivative containing a 1.3-kb *Nco* I fragment of genomic DNA from *Streptomyces* sp. EC3 comprising the *xlnC* structural gene and 770 bp of upstream sequence | This work |
| pDML619 | *E. coli* | pXLN1 derivative carrying the *blaL* reporter gene n place of the *xlnC* gene | This work |
| PDML639 | *Streptomyces* / *E. coli* | Blunt-ended fusion between pIJ702 and pDML619; contains the wild-type *xlnC* promoter from *Streptomyces* sp. EC3 | This work |
| pDML639mB2 | *Streptomyces* / *E. coli* | pDML639 derivative containing a mutation in Box 2 of the *xlnC* promoter | This work |
| pDML639mB3 | *Streptomyces* / *E. coli* | pDML639 derivative containing a mutation in Box 3 of the *xlnC* promoter | This work |
| pDML639mB23 | *Streptomyces*/*E. coli* | pDML639 derivative containing mutations in Boxes 2 and 3 of the *xlnC* promoter | This work |
| pIJ702 | *Streptomyces* | Vector containing *tsr* and *mel* markers | Kieser et al. (2000) |

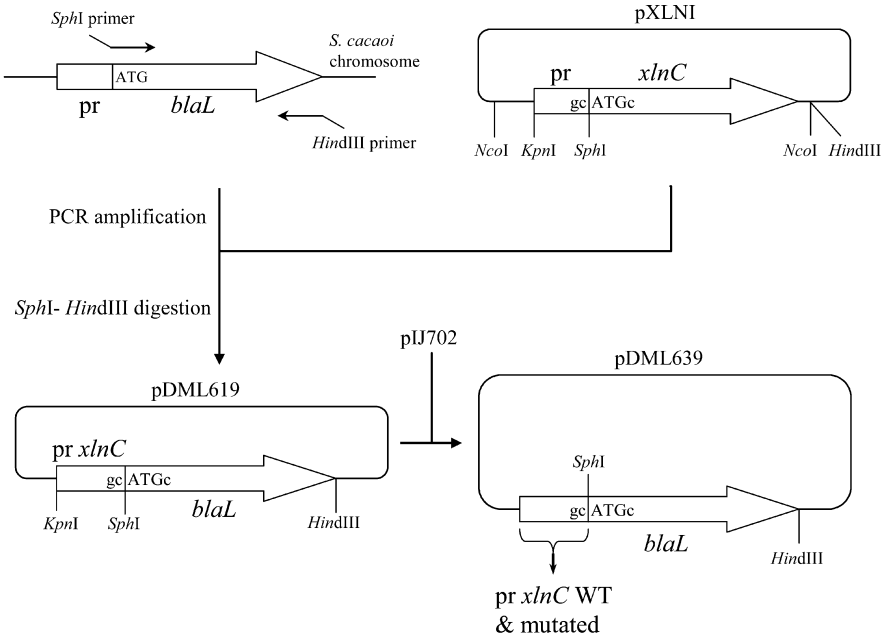


**Fig. 1** Construction of the *xlnC* - *blaL* transcriptional fusions in pDML619 and pDML639. pr, promoter of the *xlnC* gene

pDML639mB23 (containing the mutagenised promoters; Table 1), were introduced into *S. lividans* TK24. The resulting strains were used to carry out regulation studies.

## Results

### Regulation of the overall xylanolytic activity of the strains *Streptomyces* sp. EC3 and *S. lividans*

The xylanolytic strain *Streptomyces* sp. EC3 and the host strain *S. lividans* TK24 were grown on BMM with xylan, xylan and glucose, or glucose alone, in order to investigate the effect of the inducing substrate on the overall xylanolytic activity present in the supernatants of cultures of these strains. A high xylanolytic activity was detected in the culture supernatants of both strains when xylan alone was present. This activity was four- to five-fold higher in *Streptomyces* sp. EC3 than in *S. lividans* culture supernatants (Fig. 2). When xylan and glucose were both present in the growth medium only very weak xylanolytic activity was detectable in the culture supernatants after 96 h ( *Streptomyces* sp. EC3, 1.3 IU/mg; *S. lividans*, 0.42 IU/mg) whereas no enzyme activity was detected when glucose alone was present in the growth medium. These results indicated that the expression of genes encoding xylanolytic enzymes was inducible by their substrate and repressible by glucose.

### Identification of the transcriptional start site of the *xlnC* gene by S1 mapping

RNA was isolated from 48-h cultures of *Streptomyces* sp. EC3 grown in BMM supplemented with 1% birchwood xylan or 2% glucose. An 870-bp DNA fragment encompassing the 5′-end of the *xlnC* gene was obtained by PCR, and used in RNA-DNA hybridisation experiments. Hybrids were then subjected to digestion with S1 nuclease. A protected fragment was only observed with the RNA prepared from *Streptomyces* sp. EC3 grown in the presence of xylan (Fig. 3A). This result demonstrated that the *xlnC* gene was induced by xylan and that this regulation took place at the transcription level.

The observed transcriptional start site lies at an A residue, indicated by +1 in Fig. 3B. The *xlnC* transcript thus has a 163-nt untranslated leader sequence. The hexamer 5′-TTGACA-3′ is identical to the consensus proposed for the –35 promoter sequence [5′-TTGAC (Pu)-3′] recognised by $\sigma^{70}$, the main sigma factor of *E. coli* (Strohl 1992) and is likely to represent the –35

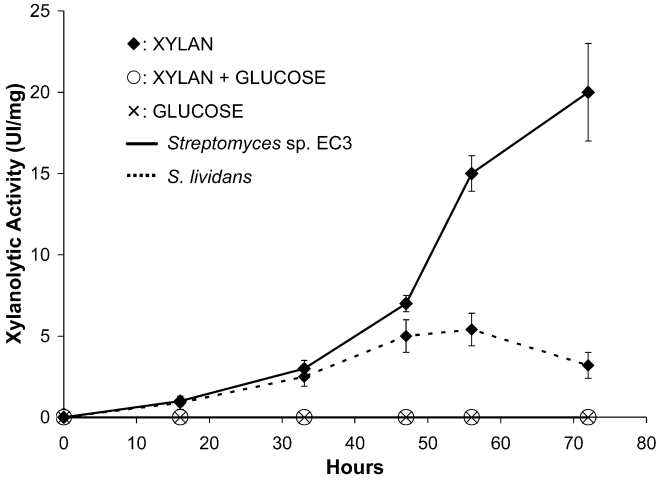


**Fig. 2** Xylanolytic activity (IU per mg of cell protein) of the strains *S. lividans* and *Streptomyces* sp. EC3 in the presence of 2% w/v glucose, 1% w/v oat spelt xylan, and both 0.5% xylan and 1% glucose

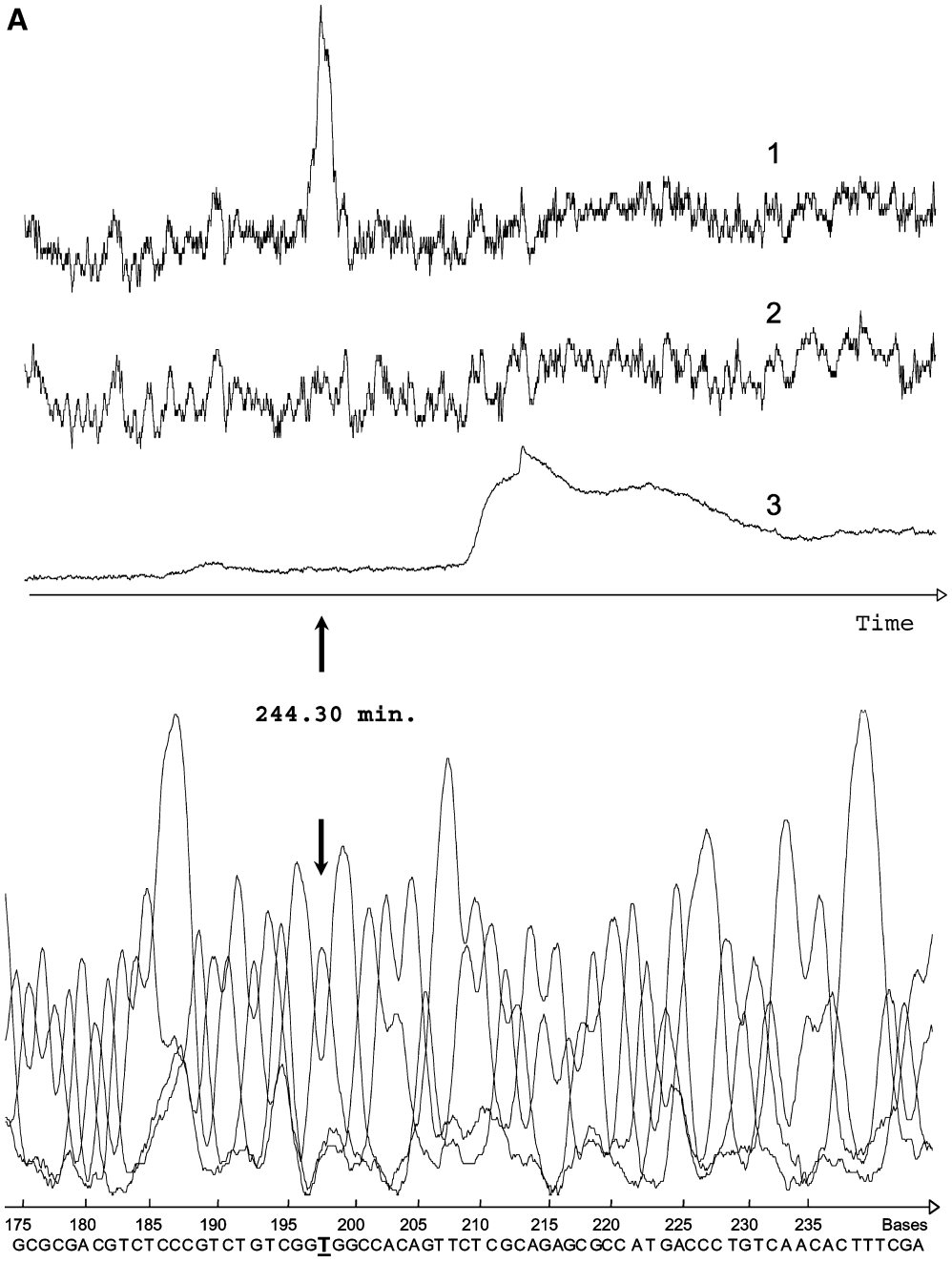


**Fig. 3A, B** Determination of the *xlnC* transcriptional start site by S1 mapping analysis (**A**) and DNA sequence of the promoter region of the *xlnC* gene (**B**). **A** (*upper panel*) Trace 1 shows electrophoretic mobility of the protected fragment derived from RNA isolated from *Streptomyces* sp. EC3 grown on xylan-containing culture medium, which migrates at 244.30 min (*arrow*). Trace 2 shows the result obtained with RNA isolated from a glucose-containing culture. Trace 3 shows the double stranded DNA template not treated with S1 nuclease. The traces in the *lower panel* are derived from the probe sequence (nucleotides +38 to −45, of panel **B**). **B** DNA sequence and predicted transcriptional initiation site of the *xlnC* gene from *Streptomyces* sp. EC3. The three palindromic *xlnC* promoter boxes are indicated in *bold* and *underlined with arrows*. The transcriptional start site is indicated at position +1. The translation start site is *boxed*. Putative –10 and –35 hexamers are *underlined*. RBS, ribosome binding site

promoter motif of *xlnC* (Fig. 3B). A double mutation was introduced upstream of and within this proposed –35 sequence, changing the sequence 5′-GTGTTGACA-3′ to 5′-GTTTCGACA-3′. This alteration completely abolished the activity of the promoter even in the presence of xylan (data not shown), supporting the idea that these bases have crucial role in the activity of this promoter. Seventeen base pairs downstream of the –35 sequence (i.e. the usual distance between –10 and –35 sequences in *Streptomyces* promoters) is the sequence 5′-GAGAAC-3′, which is a likely candidate for the –10 promoter motif (Fig. 3B). Four of these six bases match the –10 consensus sequence [5′-TAg(Pu)(Pu)T-3′] recognized by $\sigma^{70}$ in *E. coli* (Strohl 1992).

## Operator-like regulatory motifs in the promoter regions of xylanase-encoding genes from *Streptomyces* species

Multisequence alignments of the promoter sequences of genes from *Streptomyces* species encoding fourteen different xylanases belonging to Families 10 (6 genes) and 11 (8 genes) were performed using PILEUP and PRETTY software. These alignments are shown in Table 2. Note that three fairly well conserved AT rich palindromes, called boxes 1, 2 and 3 were found in all promoter sequences. Box 3 is the most distant one (150 to 200-bp) from the promoter region, its consensus sequence 5′-CGAAA (N) TTTCG-3′ is well conserved in all of the genes analysed. A shorter version of this palindromic sequence (5′-GAAA NN TTTC-3′) is found in box 1 of genes for Family 10 enzymes, whereas only the left portion of this palindrome (5′-CGAAA-3′) is found in box 1 of genes for Family 11 enzymes just upstream the very conserved putative –35 sequence 5′-TTGAC(Pu)-3′ (Strohl 1992). Box 2 is located 5–7 bp upstream of box 1, its consensus motif 5′-(G/C)TTTC N GAAA(G/C)-3′ (N could be either present or absent) has an inverted polarity compared to box 3. This motif is found in the promoter regions of all genes for Family 11 enzymes, but is usually absent from those for the Family 10 enzymes. However, AT rich palindromes are found at the position of box 2 in the promoter regions of the *xlnA* genes (for Family 10 enzymes) from the very closely related *Streptomyces* species *S. lividans* and *S. coelicolor* (Table 2). These sequences can be viewed either as a typical box 2 or box 3, as the 5′-GAAA-3′ motif is flanked by 5′-TTTC-3′ and 5′-CTTT-3′ motifs.

## Effects of alteration of the inverted repeats in the *xlnC* promoter on gene regulation

In order to determine the precise roles of box 2 and box 3 in the regulation of *xlnC* expression, box 2 (mB2), box 3 (mB3), and both boxes (mB23) were altered in such a way as to destroy the symmetry of the palindromic structure but leave one half-operator site intact. It was previously shown that such mutated boxes are unable to form DNA-protein binding complexes in vitro (Giannotta et al. 1996). Transcriptional fusions were con-

**Table 2** Alignments of promoter regions of genes encoding xylanases belonging to Families 10 and 11

| Class[a] | Gene[b] | Box 3 | B3-B2 [c] | Box 2 | B2-B1 [c] | Box 1 [d] | ATG[c] |
|---|---|---|---|---|---|---|---|
| I | Acti | CGAAA CGTTTCG | - | - | - | - | 315 |
| | XlnA (coe) | CGAAA C ATTCA | 105 | TTTC GAAA C TTTC [e] | 10 | GAAA CT TTTC CGGCGCC | 95 |
| | XlnA (liv) | CGAAA C ATTCA | 89 | TTTC GAAA C TTTC [d] | 10 | GAAA CT TTTC CGGCGCC | 95 |
| | XysA | CGAAA C TTTCG | 153 | - | - | GAAA TC TTTC GGAAACA | 197 |
| | XynA | CGAAA G TTTCG | - | - | - | - | 317 |
| | StxI | CGAAA G TTTCG | - | - | - | - | 317 |
| II | XlnC (coe) | CGAAA G TTTCG | 125 | GTTTC C GAAAG | 5 | CGAAA CTA TTGACA GGGTC | 170 |
| | XlnC (liv) | CGAAA G TTTCG | 125 | GTTTC C GAAAG | 5 | CGAAA CTG TTGACA GGGTC | 165 |
| | EC3 | CGAAA C TTTCG | 140 | CTTTC C GAAAG | 7 | CGAAA GTG TTGACA GGGTC | 187 |
| | XlnB (coe) | CGAAA T TTTCG | 125 | GTTTC GAAAC | 6 | CGAAA CTG TTGACG CTTGA | 57 |
| | XlnB (liv) | CGAAAAT TTTCG | 142 | GTTTC GAAAC | 6 | CGAAA CTG TTGACG CTTGA | 58 |
| | Xyl1 | CGAAA C TTTCG | 142 | GTTTC GAAAT | 5 | GGAAA CTG TTGACT GTTGA | 88 |
| | StxII | CGAAA C TTTCG | 142 | CTTTC GAAAT | 6 | CGAAA CTG TTGACA GTTGT | 64 |
| | No. 36a | ? [f] | ? [f] | GTTTC C GAAAG | 5 | CGAAA CTG TTGACA CGCTC | 146 |
| Consensus | | **CGAAA** N TTTCG | | G/CTTTC **GAAA** G/C | | **CGAAA** CTg TTGAC(Pu) | |

[a]Class I genes encode enzymes of Family 10, class II genes specify Family 11 enzymes

[b]Gene sources and Accession Nos. are as follows: Acti, *Actinomadura*sp. FC7 xylanase II (U08894); XlnA (coe),*S. coelicolor* xylanase A (AL021411); XlnA (liv), *S. lividans* xylanase A (M64551); XysA, *S. halstedii* beta-1,4-endoxylanase (U41627); XynA, *S. thermocyaneoviolaceus* (AF194024); StxI and StxII, *S. thermovolaceus* xylanases (D85896, D85897); EC3, *Streptomyces* sp. EC3 xylanase C (X81043); XlnC (coe),*S. coelicolor* xylanase C (AL109949); XlnC (liv), *S. lividans* xylanase C (M64553); XlnB (coe),*S. coelicolor* xylanase B (AL133220); XlnB (liv), *S. lividans* xylanase B (M64552); No. 36a, *Streptomyces* sp. No. 36a (E02180); Xyl1, *Streptomyces* sp. S38 xylanase 1 (X98518)

[c]The *numbers* indicate the distances (in bp) between Box 3 and Box 2 (B3-B2), Box 2 and Box 1 (B2-B1), and Box 1 and the initiator codon (ATG)

[d]Putative -35 hexamers in the promoters of xylanase genes for Family 11 enzymes are *underlined*

[e]Palindromic elements are *underlined*; the *double underline* indicates that the motif forms part of two separate palindromes

[f]?, sequence not available

structed between the native and mutated promoter regions of *xlnC* and the *S. cacaoi* β-lactamase gene, *blaL* (Lenzini et al. 1987). These fusions were then inserted into the multicopy plasmid pIJ702 to yield pDML639, pDML639mB2, pDML639mB3 and pDML639mB23 (Table 1). Like the xylanases, β-lactamase is secreted, and is easily detectable with high sensitivity in culture supernatant. The expression of the β-lactamase directed by these different promoter regions was followed during growth under different nutritional conditions. The experiments were conducted four times and the same kinetics of enzyme production were observed in each case, although the maximal activity level attained could differ by 1.5- to 2-fold from one assay to another. Mean values are shown in Fig. 4.

As expected, β-lactamase synthesis under the control of the native *xlnC* promoter (pDML639) was indeed induced by xylan and repressed by glucose, as was the overall xylanolytic activity of the wild-type strain *Streptomyces* sp. EC3 and of the host strain *S. lividans* (Fig. 2). However, in medium containing both xylan and glucose, the level of *blaL* expression was five-fold higher than on xylan alone. This finding is not well understood, but will be considered further in the Discussion.

In the presence of glucose alone, the alterations in box 2 had no effect, indicating that this box is not involved in the establishment of catabolite repression. In the presence of xylan, the mutation led to a strong increase in *blaL* expression, compared with the wild-type *xlnC* promoter fusion, indicating that, even in the presence of xylan, a repressor may remain bound to this box and maintain some level of repression. Mutations in box 2 would reduce the affinity of the repressor for its target site, thus allowing the latter to dissociate more easily from it in the presence of inducer. In the presence of xylan and glucose, the mutations in box 2 do not have much effect on *blaL* expression, except that the later lasted longer than with the native construct.

Base changes in box 3 led to strong expression in the presence of glucose, indicating that this box is important for the establishment of catabolite repression and represents the main repressor binding site in the presence of glucose. In the presence of xylan, this mutation led to only a moderate increase in *blaL* expression, confirming that, in the presence of inducer, box 2 rather than box 3 is occupied by a putative repressor. In the presence of xylan and glucose, the mutation in box 3 has an extremely strong effect, leading to 5- to 10-fold higher *blaL* expression than that observed with the native construct, confirming the previous assertion that as long as glucose is present the *xlnC* repressor primarily interacts with box 3.

In the presence of glucose, base changes in both boxes 2 and 3 led to a much smaller derepression effect than changes in box 3 alone, implying that the mutation in box 2 alleviated the effect of the mutations in box 3. In the presence of xylan, these mutations together behave like a box 2 mutation, again supporting the notion that, in the presence of xylan, box 2 is the main repressor binding site. In the presence of xylan and glucose, mutations in boxes 2 and 3 behave similarly to a box 2 mutation, although a slightly higher level of *blaL* expression was observed, as one would expect again if the mutation in box 2 cancelled the effect of the mutation in box 3 in the presence of glucose.

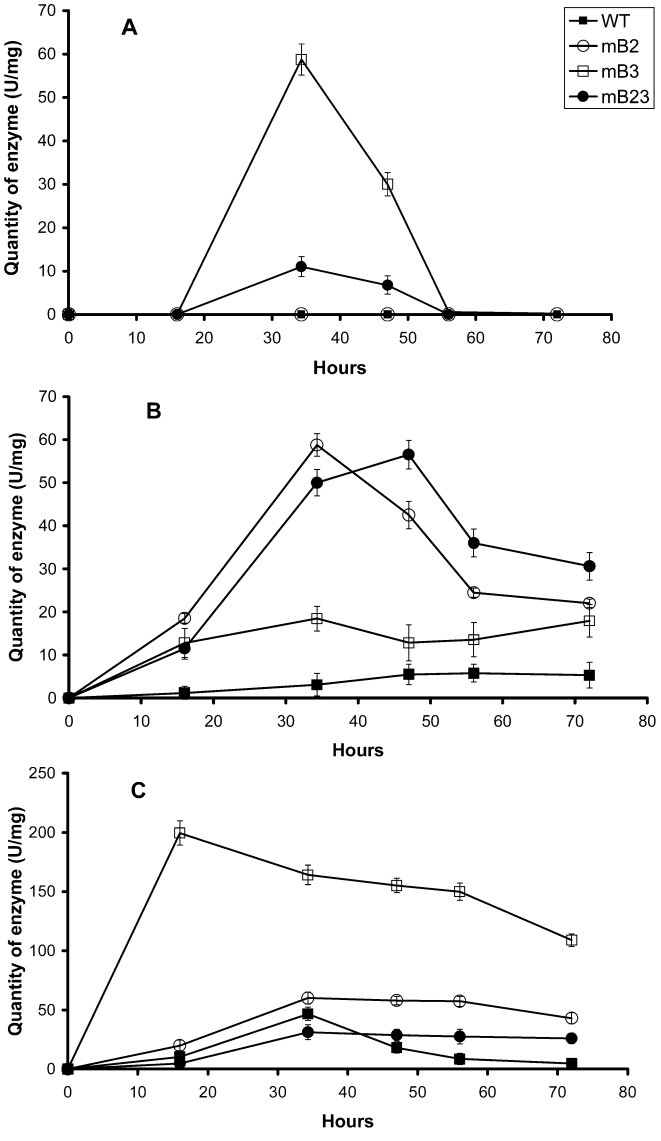


**Fig. 4A–C** Analysis of the expression of *blaL* under the control of the wild-type *xlnC* promoter or mutated derivatives thereof, in the presence of glucose ( **A**), xylan ( **B**), or both xylan and glucose ( **C**). The curves show the levels of β-lactamase activity expressed from the wild-type (WT, *filled squares*) and various mutant *xlnC* promoters [mB2 (box 2 mutated), *open circles*; mB3 (box 3 mutated), *open squares*; mB23 (boxes 2 and 3 mutated), *filled circles*]. Enzyme activity is expressed as units of β-lactamase activity per mg of total cellular protein

## Discussion

In this report we have shown that the gene *xlnC*, which encodes a secreted xylanase in *Streptomyces* sp. EC3, is inducible by xylan at the transcriptional level. We

localised the *xlnC* transcriptional start site and found probable –35 (5′-TTGACA-3′) and –10 (5′-GAGAAC-3′) motifs in the *xlnC* promoter. In order to find putative conserved regulatory sequences, several alignments, optimized by single gap insertion, of the promoter regions of genes encoding xylanases belonging to Families 10 and 11, revealed a great deal of similarity in the organisation of palindromic operator-like sequences in the promoters of genes for enzymes of Family 11 and a different organisation in the promoter regions of genes encoding Family 10 enzymes. In both families, box 3 is quite distant from the promoter region (150–200 bp) and its consensus sequence (5′-CGAAA N TTTCG-3′) is well conserved. In the promoter region of family 10 enzymes, box 1, located just upstream of the putative –35 promoter sequence, is represented by the palindromic motif 5′-GAAA-NN TTTC-3′, whereas the motif 5′-GAAA-3′ is found at a similar position in the promoter regions of genes for Family 11 enzymes. These differences suggest that in the Family 10 genes, the –35 promoter sequences are either different or located further downstream of box 1 than in the genes for Family 11 enzymes. The consensus sequence for box 2 is 5′-(G/C)TTTC N GAAA(G/C)-3′ (N could be either present or absent). Box 2 is found in the promoter regions of all genes for Family 11 enzymes, but is usually absent in those for Family 10 enzymes (with the exception of *xlnA* from *S. coelicolor* and *S. lividans*) and shows inverted polarity relative to box 3. These sequences could either be seen as a typical box 2 or a typical box 3, as the 5′-GAAA-3′ motif is flanked by 5′-TTTC-3′ and 5′-CTTT-3′ motifs (Table 2). In summary, in promoters of class I xylanase genes box 2 is often missing or when present shows a complex organisation, whereas class II promoter regions exhibit excellent conservation in sequence and position of the three boxes. These two different classes of promoter regions correspond to the two different enzymes families: class I promoters direct the expression of Family 10 enzymes, whereas class II promoters control the expression of Family 11 enzymes. These two enzyme families, which have slightly different catalytic characteristics, are thus likely to be regulated in different ways. As a matter of fact, it has been shown that large xylo-oligosaccharides, products of the activity of XlnB and XlnC (Family 11), can stimulate the expression of *xlnC* and *xlnB*, whereas small xylo-oligosaccharides ($X_2$–$X_5$), products of XlnA (Family 10) activity, did not (Arhin et al. 1994).

In order to test the putative regulatory role of boxes 2 and 3 defined in silico, the two-fold symmetry of these palindromic sequences in the promoter region of *xlnC* was disturbed by site-directed mutagenesis. We have previously reported gel retardation experiments on the *xlnC* promoter region. Protein extracts originating from xylanolytic strains retard DNA fragments including any of the three boxes. The binding protein(s) had greater affinity for the palindromic sequence constituting box 3 than for the operator site constituting box 2 and box 1. However, the affinity for box 2 was shown to vary depending upon the culture conditions (presence or absence of xylan). When the two-fold symmetry of these palindromic sequences was altered by site-directed mutagenesis, no protein binding was observed (Giannotta et al. 1996). Here, we have studied the levels of expression driven these mutant promoter regions and the wild-type promoter region. Disruption of the two-fold symmetry of box 3 or box 2 led to an enhancement of reporter gene ( *blaL*) expression, indicating that these sequences play a negative regulatory role. These operator-sequences are thus likely to constitute the binding site for a transcriptional repressor. However, these two boxes are not functionally equivalent. Mutagenesis of box 3 has a strong influence on gene expression, especially in the presence of glucose, indicating that, under these conditions, box 3 is the main repressor binding site. Mutagenesis of box 2, on the other hand, has a strong influence on gene expression mainly in the presence of xylan, indicating that under these conditions box 2 is the main repressor binding site. Since box 3 is located quite far away from the promoter region, we think that it might exert its negative regulatory role via the formation of a repressing loop with box 2, or more probably with box 1, which is slightly closer to the promoter region than is box 2. These observations are reminiscent of those made by Chen and Westpheling (1998), who found that deletion of the sequence –318 to –268 relative to the transcriptional start site in the *S. lividans xlnB* -promoter region relieved glucose repression. This region contains a sequence that shows 91% identity to the *xlnC* box 3 in *Streptomyces* sp. EC3.

Mutagenesis of box 2 led to enhanced *blaL* expression, but mainly in the presence of xylan. This observation suggests that, in the native promoter, a repressor molecule is still strongly interacts with box 2 despite the presence of the inducer. The repressor that interacts with the native box 2 is likely to have a reduced affinity for the mutated box 2; in consequence, it will be more easily displaced from its target site in the presence of the inducer. The mutated box 2 promoter will still be subject to glucose repression because a repressing loop might still be able to form between box 3 and box 1 and/or with the remaining half of the mutated box 2. In the double mutant, curiously, the mutation in box 2 partially cancelled out the derepressing effect associated with the mutation in box 3 when glucose was present in the growth medium. This finding suggests that an efficient repressing loop might still be able to form between the remaining half operator site of box 3 and the remaining half operator site of box 2 or box 1. Under inducing conditions these promoter mutations would behave like a box 2 mutation, as the repressor is mainly bound to box 2 in that case. Even though we cannot totally exclude the possibility that two different proteins bind to the different operator sequences within the *xlnC* promoter—the first responding to substrate induction while the second is responsible for glucose repression—we propose, by analogy with what is known of the regulation of the *araBCD* operon in *E. coli* (Harmer et al. 2001), that the negative regulatory effect of the

specific repressor of *xlnC* relies, in the presence of glucose, on the formation of a strong repressing loop involving box 3 and box 1 or perhaps part of box 2. Upon addition of xylan, this repressing loop would be undone and the repressor binding site would be shifted to box 2 slightly farther upstream, thus allowing some *xlnC* expression. However, the distance between two operator sites, and thus the probability that they come together in a repressing loop, varies with the degree of DNA supercoiling. The degree of supercoiling of a replicative plasmid is likely to vary with the growth conditions. We noticed that the growth rates of the strains were higher in cultures containing glucose and xylan cultures than in cultures containing xylan alone. We thus propose that in rapidly growing cultures the degree of supercoiling of a replicative plasmid is less favourable to the formation of a stable repressing loop than in slowly growing cultures. This "weak" repressing loop would then be more easily destabilized in the presence of the inducer. This model could account for the fact that higher xylanolytic activity is detected in the culture supernatants of all strains grown in the presence of glucose plus xylan (Fig. 4C) than in the presence of glucose or xylan alone (Fig. 4A and B, respectively). However, this model also implies that the phenomenon of inducer exclusion, which is often linked to the presence of glucose, is not absolute in *Streptomyces*; despite the presence of glucose, a sufficient amount of inducer can enter the cell to destabilise the weak repressing loop.

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## References

Arhin F, Shareck F, Kluepfel D, Morosoli R (1994) Effects of disruption of xylanase-encoding genes on the xlanolytic system of *Streptomyces lividans*. J. Bacteriol 176:4924–4930

Biely P, Vršanská M, Tenkonen M, Kluepfel D (1997) Endo-$\beta$-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151–166

Brans A, Loriaux L, Thamm I, Joris B, Dusart J (1997) Use of an automatic DNA sequencer for S1 mapping: transcriptional analysis of the *Streptomyces coelicolor* A3(2) *dnaK* operon. FEMS Microbiol Lett 149:189–194

Chen CC, Westpheling J (1998) Partial characterization of the *Streptomyces lividans xlnB* promoter and its use for expression of a thermostable xylanase from *Thermotoga maritima*. J Appl Environ Microbiol 64:4217–4225

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

Georis J, Giannotta F, Lamotte-Brasseur J, Devreese B, Van Beeumen J, Granier B, Frère J-M (1999) Sequence, overproduction and purification of the family 11 endo-$\beta$-1,4-xylanase encoded by the *xyl1* gene of *Streptomyces* sp. S38. Gene 237:123–133

Giannotta F, Georis J, Moreau A, Mazy-Servais C, Joris B, Dusart J (1996) A sequence-specific DNA-binding protein interacts with the *xlnC* upstream region of *Streptomyces* sp. strain EC3. FEMS Lett 142:91–97

Harmer T, Wu M, Schleif R (2001) The role of rigidity in DNA looping-unlooping by AraC. Proc Natl Acad Sci USA 98:427–431

Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781–788

Higuchi R, Krummel B, Saiki RK (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 16:7351–7367

Hindle Z, Smith CP (1994) Substrate induction and catabolite repression of the *S. coelicolor* glycerol operon are mediated through the GylR protein. Mol Microbiol 5:737–745

Hurtubise Y, Shareck F, Kluepfel D, Morosoli R (1995) A cellulase/xylanase-negative mutant of *Streptomyces lividans* 1326 defective in cellobiose and xylobiose uptake is mutated in a gene encoding a protein homologous to ATP- binding proteins. Mol Microbiol 17:367–377

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Pratical *Streptomyces* genetics. John Innes Foundation, Norwich, UK

Kluepfel D, Daigneault N, Morosoli R, Shareck F (1992) Purification and characterization of a new xylanase (xylanase C) produced by *Streptomyces lividans* 66. Appl Microbiol Biotechnol 36:626–631

Lenzini MV, Nojima S, Dusart J, Ogawara H, Dehottay P, Frère J-M, Ghuysen J-M (1987) Cloning and amplified expression in *S. lividans* of the gene encoding the extracellular $\beta$-lactamase from *Streptomyces cacaoi*. J Gen Microbiol 133:2915–2920

Matagne A, Misselyn-Bauduin A-M, Joris B, Erpicum T, Granier B, Frère J-M (1990) The diversity of catalytic properties of class A $\beta$-lactamases. Biochem J 265:131–146

Mazy-Servais C, Moreau A, Gérard C, Dusart J (1996) Cloning and nucleotide sequence of a xylanase-encoding gene from *Streptomyces* sp. strain EC3. J Seq Map 6:147–158

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:436–428

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, Guérineau 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

Schlösser A (2000) MsiK-dependent trehalose uptake in *Streptomyces reticuli*. FEMS Microbiol Lett 184:187–192

Schlösser A, Kampers T, Schrempf H (1997) The *Streptomyces* ATP-binding component MsiK assists in cellobiose and maltose transport. J Bacteriol 179:2092–2095

Schlösser A, Jantos J, Hackmann K, Schrempf H (1999) Characterisation of the binding protein-dependent cellobiose and cellotriose transport system of the cellulose degrader *Streptomyces reticuli*. Appl Environ Microbiol 65:2623–2643

Schlösser A, Weber A, Schrempf H (2001) Synthesis of the *Streptomyces lividans* maltodextrin ABC transporter depends on the presence of the regulator MalR. FEMS Microbiol Lett 196:77–83

Shareck F, Roy C, Yaguchi M, Morosoli R, Kluepfel D (1991) Sequences of three genes specifying xylanases in *Streptomyces lividans*. Gene 107:75–82

Strohl WR (1992) Compilation and analysis of DNA sequences associated with apparent *Streptomyces* promoters. Nucleic Acids Res 20:961–974

Törrönen A, Harkki J, Rouvinen J (1994) Three-dimensional structure of *endo* -1,4-*β*-xylanase II from *Trichoderma reesei*: two functional states in the active site. EMBO J 13:2493–2501

Van Wezel GP, White J, Young P, Postma PW, Bibb MJ (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

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

Xiao X, Wang F, Saito A, Majka J, Schlösser A, Schrempf H (2002) The novel *Streptomyces olivaceoviridis* ABC transporter Ngc mediates uptake of *N* -acetylglucosamine and *N,N'*-diacetylchitobiose. Mol Genet Genomics 267:429–439