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Substrate induction and glucose repression of maltose utilization by *Streptomyces coelicolor* A3(2) is controlled by *malIR*, a member of the *lacI–galR* family of regulatory genes

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Summary

*malIR* of *Streptomyces coelicolor* A3(2) encodes a homologue of the LacI/GalR family of repressor proteins, and is divergently transcribed from the *malEFG* gene cluster, which encodes components of an ATP-dependent transport system that is required for maltose utilization. Transcription of *malE* was induced by maltose and repressed by glucose. Disruption or deletion of *malIR* resulted in constitutive, glucose-insensitive *malE* transcription at a level markedly above that observed in the parental *malIR*⁺ strain, and overproduction of MalR prevented growth on maltose as carbon source. Consequently, MalR plays a crucial role in both substrate induction and glucose repression of maltose utilization. *malIR* is expressed from a single promoter with transcription initiating at the first G of the predicted GTG translation start codon.

Introduction

Members of the genus *Streptomyces* are Gram-positive, mycelial soil bacteria with a high genomic G+C content, and undergo a complex process of morphological development that normally results in sporulation (Chater and Losick, 1996). They also produce a wide variety of secondary metabolites, many of which are used as antibiotics in human medicine and agriculture (Miyadoh, 1993).

Streptomyces are saprophytes, obtaining nutrients and energy by solubilizing organic material in the soil through the production of extracellular hydrolytic enzymes that include amylases, xylanases, cellulases and proteases (McCarthy and Williams, 1992). Alpha-amyase genes (aml) have been cloned from a variety of streptomycetes, and the regulation of α-amyase production has been studied in *Streptomyces limosus* (Long et al., 1987; Virolle et al., 1988), *Streptomyces venezuelae* (Virolle and Bibb, 1988), *Streptomyces thermoviolaceus* (Bahri and Ward, 1990), *Streptomyces hygroscopicus* (Graefe et al., 1986) and *Streptomyces kanamyceticus* (Flores et al., 1993). Transcription of at least some of these genes is induced by starch-derived di- and oligosaccharides, such as maltose and maltotriose, and *aml* genes probably belong to the maltose regulons of most, if not all, streptomycetes. While induction of *aml* transcription in *S. venezuelae* is repressed by glucose, it is not repressed by this sugar in *S. limosus* (Virolle et al., 1988) and *S. thermoviolaceus* (Bahri and Ward, 1990); in both of the latter strains, mannnitol acts as a repressing carbon source. However, when cloned in *Streptomyces coelicolor* A3(2) or in *Streptomyces lividans*, *aml* of *S. limosus* adopts the regulatory characteristics of its surrogate host, with induction of *aml* transcription being repressed by glucose and not by mannnitol (Virolle et al., 1988).

The mechanism of glucose repression in streptomycetes is not understood. While phosphoenolpyruvate (PEP)-dependent fructose phosphotransferase systems occur in some streptomycetes (Tiltgemeyer et al., 1995), attempts to identify PEP-dependent glucose phosphotransferase systems in several *Streptomyces* species have failed (Sabater et al., 1972; Novotná and Hostálek, 1985). This, and the absence of fluctuations in cAMP levels with changes in carbon source in *S. coelicolor* (Hodgson, 1980) and in *S. venezuelae* (Chatterjee and Vining, 1982), suggest that the mechanism of glucose repression is markedly different from that in *Escherichia coli* (Postma et al., 1993; 1996). In other Gram-positive bacteria with genomic DNA of lower G+C content, such as *Bacillus subtilis*, *Bacillus megaterium* and *Staphylococcus xylosus*, CcpA, a homologue of the LacI–GalR family of regulatory proteins, acts
as a pleiotropic effector of glucose repression (Hueck and Hillen, 1995; Henkin, 1996; Egeter and Brückner, 1996). In S. coelicolor, the most genetically characterized streptomycete, inactivation of an ATP-dependent glucose kinase encoded by \( glkA \) results in the inability to utilize glucose and in a pleiotropic loss of glucose repression (Hodgson, 1982; Seno and Chater, 1983; Kwakman and Postma, 1994), but has no effect on glucose transport (Hodgson, 1982). Moreover, replacement of GlkA with an unrelated glucose kinase from \( Zymomonas \) mobilis, or with a normally cryptic glucose kinase of S. coelicolor, conferred glucose utilization, but not glucose repression (Angell et al., 1994). Thus, \( glkA \) plays a key regulatory role in mediating glucose repression in S. coelicolor, and its homologue in \( S. \) xylosus appears to have a similar function (Wagner et al., 1995).

Earlier studies of the \( aml \) genes of \( S. \) limosus (Long et al., 1987) and \( S. \) venezuelae (Virolle et al., 1988) identified the 3′ end of a gene located immediately upstream of \( aml \) in both strains that appeared to encode a member of the LacI–GalR family of regulatory proteins. In this study we report the sequence of this upstream gene from \( S. \) limosus, and its use to isolate and characterize a homologue from \( S. \) coelicolor, \( malR \), that is required for both substrate induction and glucose repression of maltose utilization.

### Results

**Isolation and sequence analysis of members of the lacI–galR family of regulatory genes from \( S. \) limosus and \( S. \) coelicolor**

Sequence analysis of the \( aml \) genes of \( S. \) limosus and \( S. \) venezuelae revealed the 3′ end of an upstream open reading frame (ORF) whose predicted product showed significant amino acid sequence identity to the LacI–GalR family of regulatory proteins. The sequence (EMBL, Accession no. Y08304) of a 2.3 kb region upstream of \( aml \) of \( S. \) limosus was determined and revealed a complete ORF (ORF-\( Sl \)) that would encode a protein of 351 amino acids (39 kDa) with a significant degree of sequence identity to members of the LacI–GalR family (e.g. 29% and 30% amino acid sequence identity to LacI and GalR, respectively; Fig. 1A). Upstream of ORF-\( Sl \) lies the 3′ end of \( aglA \), a homologue from \( S. \) limosus that appears to have a similar function (Wagner et al., 1995).

Fig. 1. A. Alignment of the amino acid sequences of a selection of MalR homologues: \( spnmalr \), MalR of \( S. \) pneumoniae (SWISSPROT Q08511); \( sxymalr \), MalR of \( S. \) xylosus (PIR S44187); \( samalr \), MalR of \( S. \) ambifaciens (PIR P33360); \( sams1r \), MalR of \( S. \) ambofaciens (PIR S33361); \( slilmdr \), MalR of \( S. \) lividans (PIR S21351); \( slirdr \), MalR of \( S. \) lividans (PIR S21353); \( sccomalr \), MalR of \( S. \) coelicolor (this study; EMBL Accession no. Y07706); \( bmeccpa \), CcpA of \( B. \) megaterium (EMBL L26052); \( bsuccpa \), CcpA of \( B. \) subtilis (EMBL Accession no. M85182); \( icaccpa \), CcpA of \( L. \) casei (GenBank Accession no. M85182); \( spnccpa \), CcpA of \( S. \) pneumoniae (EMBL Accession no. X95439); \( ecyct \), CytR of \( E. \) coli (SWISSPROT P06964); \( ecyogalr \), GalR of \( E. \) coli (SWISSPROT P03024); \( ecomalr \), MalR of \( E. \) coli (SWISSPROT P18811).

B. Phylogenetic tree of the sequences aligned in (A). The number of supporting bootstrap replicates (out of 100) is shown for each internal branch.

As \( S. \) limosus is not a genetically well-characterized and...
manipulable strain, further attempts to analyse the role of ORF-Sl were carried out by isolating and studying its homologue in S. coelicolor. To obtain the ORF-Sl homologue of S. coelicolor, a 32-fold degenerate oligonucleotide corresponding to the C-terminal segment of the helix-turn-helix motif of ORF-Sl (see below) was used to screen the ordered S. coelicolor cosmid library (Redenbach et al., 1996) by colony hybridization. DNA was isolated from 16 positive clones, digested with BamHI and SalI, and subjected to Southern analysis using the 1300 bp AatII fragment (EMBL, Accession no. Y08304) containing most of ORF-Sl and part of aglA as probe. One cosmid (10B7) gave a strong hybridization signal, which was subsequently localized to a 13 kb BamHI fragment. This fragment was cloned in the BamHI site of pBR329, yielding pJJ2564. Double-strand sequencing of this plasmid using the oligonucleotide used to probe the ordered cosmids revealed a close homologue of ORF-Sl. Sequencing of appropriate subcloned fragments from pJJ2564 revealed an ORF (malR) that would encode a protein of 344 aa (39 kDa). The cosmid maps at approx. 11 o’clock, on AseI fragment C, of the combined physical and genetic map of the S. coelicolor chromosome (Redenbach et al., 1996).

A restriction map of the 2.2 kb FokI fragment containing malR is shown in Fig. 2A. Upstream of and in the opposite orientation to malR lies a gene (malE) encoding a homologue of the maltose-binding protein found in other bacteria (Duplay et al., 1984; Puyet and Espinosa, 1993).
Disruption of malE prevented the utilization of maltose as carbon source (G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted). The sequence of the malR–malE intergenic region and the predicted translation start sites for malR and malE are shown in Fig. 2B.

Relationship of MalR to other members of the LacI–GalR family

In addition to ORF-Sl, the complete nucleotide sequences of five other streptomycete homologues of malR are available. Three occur as part of the amplifiable element AUD1 of S. lividans (Piendl et al., 1994), where they appear to play a role in DNA amplification (Volff et al., 1996), and two occur as part of the amplifiable element AUD6 of Streptomyces ambofaciens (Aubert et al., 1993); pairwise comparisons between the products of the six ORFs reveal 92–100% amino acid sequence identity (two of the homologues in AUD1 are identical). MalR and the product of ORF-Sl show a higher level of similarity to each other (63% identity) than to the products of the S. ambofaciens and S. lividans ORFs (53–55% identity). An alignment of the Streptomyces MalR homologues with members of the LacI–GalR family of proteins from other bacteria is shown in Fig. 1A, and a phylogenetic tree is shown in Fig. 1B. The Streptomyces homologues all group closely together, with MalR and the product of ORF-Sl forming a separate branch from the S. lividans and S. ambofaciens homologues. Both the protein sequence comparison and the phylogenetic tree indicate that MalR and the product of ORF-Sl are not significantly more similar to specific regulatory proteins for maltose utilization (e.g. MalR from Streptococcus pneumoniae (Puyet et al., 1993) and Sta. xylosus (Egeter and Brückner, 1995), and Mall from E. coli (Reidl et al., 1989)) than they are to the pleiotropic regulatory proteins CcpA (from B. subtilis (Henkin et al., 1991), B. megaterium (Hueck et al., 1994), Sta. xylosus (Egeter and Brückner, 1996) and Lactobacillus casei (GenBank, Accession no. U28137), and CytR (from E. coli; Valentin-Hansen et al., 1986). A feature shared by all members of this family of repressor proteins is a well-conserved N-terminally located helix-turn-helix motif responsible for DNA binding (Weickert and Adhya, 1992); such a motif is also present in the products of malR (Fig. 1A, scomalr, amino acid residues 5–24) and ORF-Sl (Fig. 1A, slimorf, amino acid residues 16–35).

The malR transcript lacks an untranslated leader sequence

To determine the transcription start site of malR, RNA was isolated from S. coelicolor M145 grown in liquid minimal medium (SMM) containing glucose as carbon source and subjected to S1 mapping. A single transcription start site was located corresponding to the first G of the predicted GTG translation start codon (Fig. 3). Thus, the malR transcript appears to lack a conventional ribosome-binding site, a property shown by several other streptomycete mRNAs (Janssen, 1993; Strohl, 1992). In vitro transcription assays using S. coelicolor RNA polymerase with a 564 bp SalI–BglII fragment and a 549 bp polymerase chain reaction (PCR) product (Fig. 2A; fragments b and c, respectively), each containing the malR promoter region as templates, gave the expected run-off transcripts of ~330 nucleotides (nt) and 120 nt, respectively. The transcriptional start site of malR is preceded by sequences (Fig. 2B, 5’TGTGCA–17 bp–TAGAGT–3’) that are similar to the proposed consensus sequence (5’TGTGAC–16–18 bp–TAGAPuT–3’; Strohl, 1992) for promoters recognized by the major RNA polymerase holoenzyme of Streptomyces.

S1 nuclease protection studies using RNA isolated from M145 grown in SMM containing glucose revealed malR transcripts throughout growth, but with maximal levels during mid- and late-exponential phases (Fig. 4).

Inactivation of malR causes constitutive, enhanced and glucose-insensitive transcription of malE

To determine the function of malR and its possible role in the regulation of malE, an in-frame deletion was made that removed the C-terminal two-thirds of the malR-coding region (corresponding to amino acid residues 112–341.)
out of 344). Such a mutation should not have a polar effect on the expression of genes 3' of malR. S. coelicolor J1501 was transformed with single-stranded DNA derived from pIJ2591 (Fig. 5), which carries tsr conferring thioestrepton-resistance (ThioR). One ThioR isolate, which had probably arisen by single cross-over integration of pIJ2591 into the malR region of the chromosome, was subjected to three rounds of sporulation on non-selective SFM agar plates to allow a second cross-over to occur, resulting in loss of tsr. DNA was isolated from 40 ThioS colonies, digested with NotI and analysed in two Southern blots. In the first hybridization, using the 564 bp SalI–BglII fragment corresponding to the N-terminal part of malR as probe (Fig. 2A, fragment b), the 2.2 kb NotI fragment containing malR should have been 700 bp smaller in the deletion mutant, while in the second hybridization, using the 477 bp FspI–SalI probe (Fig. 2A, fragment a) recognizing the part of malR that should have been deleted, no hybridization signal was expected in the mutant. One of the ThioS colonies (M542) gave the correct patterns. A second malR mutant (M541) was made by integrating pIJ2587 containing the 477 bp FspI–SalI fragment internal to malR (Fig. 2A, fragment a) in the chromosome of M145; disruption of malR was confirmed by Southern analysis.

In agreement with data obtained for other S. coelicolor strains (Hodgson, 1980), maltose proved to be a poor carbon source for S. coelicolor M145 and J1501, giving growth rates and final biomass accumulations that were consistently lower than those obtained with glucose (data for M145 are shown in Fig. 6A). In contrast, the growth rates of the malR mutants M541 and M542 on maltose were comparable to those for glucose-grown cultures and significantly higher than the congenic malR+ parental strains (data for M542 are shown in Fig. 6B). Furthermore, the final biomass accumulation of M541 and M542 grown on maltose approached that obtained with glucose (Fig. 6B), indicating an increased ability to use maltose as carbon source.

To assess whether this might reflect derepression of malEFG and elevated levels of maltose uptake, transcription of malE in M145 and in M542 was analysed by S1 nuclease protection assays using RNA isolated from liquid minimal medium (NMMP) cultures containing maltose, glucose or a combination of maltose and glucose (earlier studies had shown that transcription of malE was barely detectable when mannitol was used as a non-repressing carbon source, but markedly induced on addition of maltose (G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted)). In M145, transcription of malE was strongly induced by maltose, and this induction was completely repressed when glucose was present as an additional carbon source (Fig. 7). However, in M542 transcription of malE was constitutive and occurred at levels much greater than the induced level in M145. Moreover, transcription of malE was no longer repressed by...
glucose, indicating a role for MalR in both substrate induction and catabolite repression of maltose utilization. Similar results were obtained with M541. To confirm that the enhanced ability of M542 to utilize maltose was due solely to deletion of malR, the 2.2 kb FokI fragment containing the malR-coding region and promoter (Fig. 2A) was cloned in the conjugative vector pSET152 yielding pIJ2593, which was subsequently integrated at the chromosomal øC31-attachment site, resulting in M543. The growth rate and biomass accumulation of M543 in SMM containing maltose were essentially the same as those of M145, confirming restoration of the wild-type phenotype.

Inactivation of malR represses agarase production

Expression of the agarase gene (dagA) of S. coelicolor is subject to carbon catabolite repression (Hodgson, 1980; Bibb et al., 1987; Kwakman and Postma, 1994), and dagA transcription is strongly repressed by glucose (Servín-González et al., 1994; Angell et al., 1994). Agarase production by M145, the glkA mutant J1915, and the malR mutants M541 and M542, was readily detected on MM plates containing agar as sole carbon source (Fig. 8; upper left plate); as expected, agarase production by all except J1915 was effectively repressed by glucose (Fig. 8; upper right plate). Unexpectedly, while agarase production by M145 and J1915 was readily detected on plates containing 1% (w/v) maltose, agarase production by M541 and M542 was undetectable (Fig. 8; bottom left plate). This apparent repression of agarase activity in the malR mutants might reflect higher levels of intracellular glucose which could arise from elevated levels of maltose uptake upon the observed derepression of malEFG (Fig. 7). Agarase production in glucose-grown agar cultures of M543 was restored, confirming that repression did indeed result from inactivation of malR.

Overexpression of malR prevents maltose utilization

The 2.2 kb FokI fragment containing malR was cloned in the multicopy vector pIJ486 (c. 50–100 copies per genome), yielding pIJ2592. Introduction of pIJ2592 into M145 prevented growth in liquid minimal medium (NMMP lacking casamino acids) containing maltose as sole carbon source, presumably because of overexpression of...
malR and enhanced repression of malE. No growth inhibition was detected when glucose, glycerol, mannitol, arabinose or galactose were used as carbon sources.

Identification of MalR using antibodies raised against CcpA from B. megaterium

As MalR and CcpA from B. megaterium are 30% identical (Fig. 1A), we assessed whether antibodies raised against CcpA would cross-react with MalR. S30 supernatants derived from total-protein extracts prepared from 36 h TSB-grown cultures of S. coelicolor were analysed by Western blotting using antibodies raised against CcpA of B. megaterium (Küster et al., 1996). Two proteins of approx. 55 kDa and 43 kDa were detected in extracts from M145; bands of a similar mobility were noted in extracts of S. coelicolor DSM 40233 by Küster et al. (1996). The 43 kDa band is approx. the same size as that predicted for MalR (39 kDa). It was not observed in extracts from the malR deletion mutant M542, but reappeared in an extract from M542 containing pIJ2592 at a level several times higher than in M145. We therefore believe that the 43 kDa protein is MalR. The 55 kDa protein (=500 amino acids) is significantly larger than any known member of the LacI–GalR family of proteins.

Discussion

Members of the lacI–galR family of regulatory genes were identified in S. limosus (ORF-SI) and S. coelicolor (malR), located upstream of aml (encoding an α-amylase; Long et al., 1987) and malE (probably encoding a maltose-binding protein; G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted), respectively. The degree of similarity of the two gene products (63% amino acid sequence identity) is consistent with the notion that they are functionally homologous proteins. As attempts at phage-mediated disruption of ORF-SI in S. limosus failed (J. White, unpublished results), we focussed on its homologue from the genetically more amenable S. coelicolor.

Transcription of malE (and probably of malFG, both of which appear to be required for maltose uptake; G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted) was induced by maltose in S. coelicolor M145 (G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted; this study), but was constitutive in the malR mutants M541 and M542. Presumably, in the wild-type strain, maltose or a maltose metabolite binds to MalR and prevents it from repressing transcription initiation at the malE promoter. Disruption or deletion of malR also relieved glucose repression of malE transcription. Thus, MalR is required for both substrate induction and glucose repression of malE expression. While S. coelicolor grows poorly on maltose as sole carbon source (Hodgson, 1982; G. P. van Wezel, J. White, M. J. Bibb and P. O. Postma, submitted), the malR mutants grew much better than the parental strain, perhaps because of elevated levels of maltose uptake. Consistent with this, the level of malE transcription in the malR mutant M542 far exceeded the induced level observed in M145 on all carbon sources tested. Furthermore, in contrast to M145, agarase production by the malR mutants was repressed by maltose, perhaps reflecting higher levels of intracellular glucose, a potential consequence of improved maltose uptake. While compatible with its role as a repressor, the ability of 50–100 copies of malR (in the form of pIJ2592) to prevent growth of S. coelicolor M145 on maltose is surprising. This may reflect the ability of enhanced levels of MalR to completely repress malE transcription, preventing inducer uptake.

Notwithstanding the ability of antibodies raised against CcpA of B. megaterium to cross-react with MalR, disruption of malR had no apparent pleiotropic effect on carbon source utilization, and we have no evidence to suggest that MalR is a functional homologue of CcpA. Homologues of MalR occur in Sta. pneumoniae (Puyet et al., 1993) and in Sta. xylosus (Egeter and Brückner, 1995). While MalR of S. coelicolor and Sta. pneumoniae acts to repress maltosaccharide and maltose utilization, respectively, inactivation of malR in Sta. xylosus reduces the level of maltose.
transport and utilization. The role that these latter two homologues play in glucose repression of maltose metabolites and maltose catabolism in their respective hosts was not reported.

The ability of malR to mediate both substrate induction and catabolite repression of malE transcription resembles the role of the phylogenetically unrelated GyIR, the repressor of the glycerol operon glyCABX of *S. coelicolor* (Hindle and Smith, 1994). The level of glyCABX transcription in glyR null mutants is markedly increased in both uninduced and glucose-grown cultures, and the presence of glyIR on a multicopy plasmid prevents glycerol utilization (F. Amini, M. S. B. Paget and C. P. Smith, personal communication). Whether the dual roles that GyIR and MalR play in the regulation of catabolic pathways in *S. coelicolor* is a common occurrence in streptomycetes remains to be determined.

The existence of a regulatory protein that mediates both substrate induction and glucose repression of malE transcription predicts the existence of cis-acting sequences that are required for both aspects of regulation. Direct and inverted repeats that might fulfill such a function occur 5′ and 3′ of the malE promoter (Fig. 2B). The 5′ direct repeat CTTGC and the inverted repeat 5′-TCTTGC–11 bp–GCAAGA–3′, which occurs just upstream of the putative −35 region of malE, are also found in the promoter region of aml of *S. limosus*. The direct repeat appears to play a role in the induction of aml transcription by maltose, and deletion of the inverted repeat resulted in constitutive expression that was insensitive to glucose repression (Virolle and Gagnat, 1994). Consequently, this inverted repeat is a probable binding site for MalR. Direct repeats that show no sequence similarity to the direct and inverted repeats present in the malE promoter region are also required for both substrate induction and glucose repression of a chitinase gene (chi63) of *Streptomyces plicatus* (Delic et al., 1992), again consistent with the notion of a single regulatory protein with dual functions in chitinase gene regulation.

The role of a repressor in both induction and glucose repression of malE transcription could be readily explained if glucose repression was mediated by inducer exclusion, i.e., if glucose, directly or indirectly, prevented the uptake of maltose. Interestingly, glucose repression of lactose utilization during growth of *E. coli* on both sugars appears to be totally attributable to inducer exclusion and to the level of active LacI, the repressor of the lac operon, with cAMP and CRP (cyclic AMP receptor protein) playing no role (Inada et al., 1996). Although there is no evidence either for or against the role of inducer exclusion in regulating malE expression, constitutive expression of aml of *S. lividans*, achieved by cloning the gene on a high-copy-number plasmid, was still subject to glucose repression (Virolle and Bibb, 1988), i.e., under conditions where aml transcription was inducer independent, glucose repression was still operative. If applicable to the closely related *S. coelicolor* and to other genes in the maltose regulon, this suggests that inducer exclusion does not play a major role in glucose repression of malE transcription (similar conclusions were drawn for glucose repression of dagA expression in *S. coelicolor* (Servín-González et al., 1994)). How else might glucose repression of malE transcription be mediated in a MalR-dependent manner? There is evidence in *Bacillus* species that glucose-6-phosphate acts as an anti-inducer of XylR, the repressor of the xylene-utilization operon, both in vivo and in vitro, by competing with xylose for binding to XylR (Scheler and Hillen, 1993; Dahle et al., 1995); although inactivation of xylR reduces glucose repression only about twofold in *B. megaterium* (Schmiedel and Hillen, 1996), much less than the apparent effect of deleting malR on glucose repression of malE transcription (Fig. 7). It is possible that a similar mechanism operates in *S. coelicolor*. Alternatively, perhaps an unidentified pleiotropic regulatory protein responsible for glucose repression, and functionally analogous to CcpA, requires the presence of MalR to bind to the malE promoter region. Finally, as the glucose kinase gene (*glkA*) of *S. coelicolor* plays a pleiotropic role in carbon catabolite repression, and because GlkA is required for glucose repression of the aml genes of *S. limosus* and *S. venezuelae* when cloned in *S. coelicolor* (Virolle and Bibb, 1988; Virolle et al., 1988), glucose repression of malE may be mediated by MalR through interaction with, or modification by, GlkA.

malR is transcribed constitutively during growth of *S. coelicolor* in liquid culture, with transcript levels peaking during mid- to late-exponential phase. The transcription start site of the malR promoter coincides with the first G of the predicted GTG translational start codon, and thus the malR transcript lacks a conventional untranslated leader sequence and ribosome-binding site, consistent with the absence of a purine-rich Shine–Dalgarno sequence complementary to the 3′ end of the 16S RNA upstream of the malR-coding region. Although several streptomycete mRNAs lack untranslated leader sequences (Janssen, 1993; Strohl, 1992), the malR transcript appears to be only the second example of a leaderless mRNA involved in primary metabolism, the other being that derived from the histidase gene of *Streptomyces griseus* (Wu et al., 1995).

**Experimental procedures**

**Bacterial strains, culture conditions, plasmids and phages**

*E. coli* K-12 strains JM101 and JM109 (Messing et al., 1981), and ET12567 mini-F′ Km (MacNeil et al., 1992; M. J. Bibb, unpublished) were used for routine subcloning and for the preparation of single-stranded DNA, respectively, and were
grown and transformed by standard procedures (Sambrook et al., 1989); transformants were selected with carbenicillin at a final concentration of 200 μg ml⁻¹. Luria (L) broth containing 50 μg ml⁻¹ kanamycin was used to grow ET12567 mini-F'Km to isolate single-stranded DNA using M13KO7 as helper phage (Sambrook et al., 1989). S. coelicolor A3(2) strains containing the internal 477 bp Fsp I–Sal I fragment of malR were used to make agarase production and transformation were as described by Hopwood et al. (1985). SFM medium (mannitol, 20 g l⁻¹; soya flour, 20 g l⁻¹; agar, 20 g l⁻¹; dissolved in tap water and autoclaved twice) is a modified version of that reported by Hobbs et al. (1989) and was used to make spore suspensions. Liquid minimal medium (NMMP; Hopwood et al., 1985) containing 1% (w/v) glucose, mannitol, maltose or maltose plus glucose, and unless otherwise stated 0.05% (w/v) casamino acids, or SMM (Strauch et al., 1992) containing 0.1% (w/v) casamino acids and 1% (w/v) glucose or maltose, were used to assess carbon-source utilization and for RNA isolation. MM plates (Hopwood et al., 1985) were used to assess agarase production, which was detected as zones of clearing of the agar. TSb (Oxoid Tryptone–Soya broth powder; 30 g l⁻¹) was used to grow S. coelicolor strains for Western analysis.

pUC18 (Yanisch-Perron et al., 1985), pBluescript-II SK⁺ (Stratagene), and pSET152 (Bierman et al., 1992) were used for cloning experiments. pJ486 (Ward et al., 1986) was used as a high-copy-number vector (≈50–100 copies per chromosome; T. Kieser, personal communication) in S. coelicolor. The 2.2 kb Fok I fragment (Fig. 2A) containing malR and part of malE of S. coelicolor was cloned in pUC18, pJ486 and pSET152, resulting in pJ2588, pJ2592, and pJ2593, respectively. Standard procedures were used to isolate plasmid DNA from E. coli (Sambrook et al., 1989), and to isolate plasmid and total DNA from S. coelicolor (Hopwood et al., 1985).

pJ2564 was made by cloning a 13 kb BamHI fragment containing malR from cosmid 1087 in pBR329 (Covarrubias and Bolivar, 1982). pJ2587, which was used to make the malR disruption mutant M541, is a pUC18 derivative containing the internal 477 bp FspI–SalI fragment of malR (Fig. 2A, fragment a) and tsr, plJ2591 (Fig. 5), which was used to make the malR deletion mutant M542, is a pBluescript-II SK⁺ derivative containing tsr and a 3.5 kb segment from which the internal SalI–NotI region of malR (Fig. 2A) had been deleted. Double- and single-stranded DNAs derived from pJ2587 and pJ2591, respectively, were used to transform protoplasts of S. coelicolor M145 and J5101, respectively, and integrants selected with a final concentration of 50 μg ml⁻¹ Thio. Southern analyses were performed to confirm the mutations present in M541 and M542 using the appropriate 32P-labelled probes (Sambrook et al., 1989) and previously described hybridization conditions (van Wezel et al., 1991).

DNA sequence analysis

The nucleotide sequence of malR was determined using the Promega TaqTrack and Pharmacia T7 sequencing kits and double-stranded DNA templates derived by subcloning DNA fragments from pJ2588 and pJ2564 in pUC18. For ORF-SI and aglA, sonicated fragments of the 2.2 kb EcoRI–BcII chromosomal segment containing the genes (Viroille and Bibb, 1988) were cloned in the Smal site of M13mp18, and nucleotide sequences determined using single-stranded DNA templates and the Klenow fragment of DNA polymerase I (Sanger et al., 1977). Synthetic oligonucleotides were used to close gaps in the sequences. The sequences of S. coelicolor malR (Accession no. Y07706) and S. limosus ORF-SI with part of aglA (Accession no. Y08304) were deposited in the EMBL nucleotide sequence database.

Nuclease S1 protection assays

RNA was purified as described by Hopwood et al. (1985), except that DNase I treatment was used in addition to salt precipitation to eliminate DNA from the nucleic acid preparations. For each nuclease S1 protection assay, approx. 0.02 pmol (=10⁴ Cerenkov counts min⁻¹) of labelled probe was hybridized to 20 μg of RNA in Na-TCA buffer (Murray, 1986) at 45°C overnight after denaturation at 65°C for 15 min. All subsequent steps were carried out as described previously (Strauch et al., 1991), using an excess of probe. All of the nuclease S1 protection experiments were carried out at least twice using RNA isolated from independent cultures, and the results presented were shown to be reproducible. The probes used are shown in Fig. 2A. The 564 bp SalI–BglII fragment of pIJ2588 (Fig. 2A, probe b), 32P-end-labelled at the SalI site, and the 549 bp polymerase chain reaction (PCR) product (Fig. 2A, probe c) made using oligonucleotides mal02 and 32P-end-labelled mal01 (Fig. 2B), were used for mapping malR transcripts. The same PCR product, but made using unlabelled mal01 and 32P-end-labelled mal02, was used for determining the level of malE transcripts. PCRs contained 1x PCR buffer (Boehringer Mannheim), 0.2 mM of each dNTP, 25–50 pmol of each primer, 10 ng of pJ2564, 5 U Taq polymerase (Boehringer Mannheim) and 5% (v/v) glycerol in a total volume of 100 μl. Samples were subjected to 30 cycles of 60 s at 94°C, 60 s at 54°C and 60 s at 72°C.

In vitro transcription analysis

RNA polymerase was isolated from cultures in the transition phase between exponential growth and stationary phase, as described previously (Buttnner and Brown, 1985). In vitro run-off transcription experiments were performed as described by Buttnner et al. (1987) using the 564 bp SalI–BglII fragment and the 549 bp PCR product (Fig. 2A, fragments b and c) as templates. Products were analysed on denaturing 6% (w/v) polyacrylamide gels using 32P-end-labelled HpaII fragments of pBR322 as size markers.

Western blots

Western analyses were conducted as described by Vijgenboom et al. (1994) using a 1:1000 dilution of antibodies raised against CcpA of B. megaterium.

Amino acid sequence analysis

The program CLUSTALW (Thompson et al., 1994) at the SEQNET facility (Daresbury Laboratory, Cheshire, UK) was
used to align the sequences, calculate distances using the Kimura correction, and construct the tree by the neighbour-joining method (Saitou and Nei, 1987) with 100 bootstrap replicates. TreeView (R. D. M. Page, University of Glasgow) was used to display the tree.

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