Amylase and Chitinase Genes in *Streptomyces lividans* Are Regulated by *regI*, a Pleiotropic Regulatory Gene

JACQUELINE NGUYEN,* FRANCOIS FRANCOU, MARIE-JOËLLE VIROLLE,
AND MICHEL GUÉRINEAU

Institut de Génétique et Microbiologie, URA 2225, Université Paris XI, F-91405 Orsay, France

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A regulatory gene, *regI*, was identified in *Streptomyces lividans*. It encodes a 345-amino-acid protein (RegI) which contains a helix-turn-helix DNA-binding motif in the N-terminal region. RegI exhibits similarity with the LacI/GalR family members over the entire sequence. It displays 95% identity with MalR (the repressor of *malE* in *S. coelicolor*), 65% identity with ORF-SI (a putative regulatory gene of α -amylase of *S. limosus*), and 31% identity with CcpA (the carbon catabolite repressor in *Bacillus subtilis*). In *S. lividans*, the chromosomal disruption of *regI* affected the expression of several genes. The production of α -amylases of *S. lividans* and that of the α -amylase of *S. limosus* in *S. lividans* were enhanced in the *regI* mutant strains and relieved of carbon catabolite repression. As a result, the transcription level of the α -amylase of *S. limosus* was noticeably increased in the *regI* mutant strain. Moreover, the induction of chitinase production in *S. lividans* was relieved of carbon catabolite repression by glucose in the *regI* mutant strain, while the induction by chitin was lost. Therefore, *regI* can be regarded as a pleiotropic regulatory gene in *S. lividans*.

Streptomyces species, gram-positive soil bacteria with a high GC content, have the capacity to produce a vast array of secondary metabolites and extracellular proteins. The latter comprise many hydrolytic enzymes such as cellulases, chitinases, amylases, and xylanases allowing *Streptomyces* to grow on polymeric substrates (12). Genes encoding these enzymes are generally induced by degradation products of their polymeric substrates and repressed by readily metabolizable carbon sources such as glucose by a process known as catabolite repression. Several *cis*-acting sequences, displaying no apparent similarity, have been shown to be involved in the regulation of genes encoding such hydrolytic enzymes in various species of *Streptomyces* (10, 11, 44, 49). However, no specific regulatory gene among those encoding extracellular proteins has so far been characterized for *Streptomyces*.

α -Amylases are secreted by several species of *Streptomyces* and catalyze the cleavage of the α -1,4 linkage of starch, yielding short linear maltodextrins which are finally converted to glucose. The highest α -amylase production was obtained when the α -amylase gene *aml* of *Streptomyces limosus* was cloned in *S. lividans*, which has a negligible α -amylase activity (43). In *S. lividans*, *aml* expression was induced by maltose and repressed by glucose. The regulation occurs at the level of transcription from the unique promoter. This led to the assumption that a regulatory protein(s) in *S. lividans* controlling the expression of *aml* might exist. A putative repressor, called open reading frame R (ORFR) in the present work, which is homologous to the regulatory proteins LacI and GalR of *Escherichia coli* has been located upstream of the α -amylase gene of *S. venezuelae* (45) and is also present upstream of the α -amylase genes of *S. limosus* (27) and *S. griseus* (42). Therefore, it was assumed that an ORF homologous to ORFR might be present in *S. lividans*. During the preparation of this report, the existence of a gene encoding a regulatory protein homologous to ORFR in *S. coelicolor* was reported (41a). This gene, *malR*, plays a major

role in maltose utilization in *S. coelicolor*. The present report describes the molecular cloning of a regulatory gene of *S. lividans*, *regI*, homologous to the gene encoding ORFR of *S. limosus*. It encodes the protein RegI, which is a member of the LacI/GalR family. *regI* is unlinked to the α -amylase genes of *S. lividans*. The disruption of *regI* leads to changes in the regulation of the production of α -amylases and chitinases in *S. lividans*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. lividans* TK24 was used for the isolation of the regulatory gene *regI*, and TK21 was used for the mapping of *regI*. Strain TK24::pTS6000, containing the α -amylase gene of *S. limosus* integrated in the chromosome, was described by Virolle and Gagnat (44). *E. coli* DH5 α (14) was used for plasmid construction and isolation.

Plasmid pIJ2925, a derivative of pUC18 (23), was used for cloning the 3.5- to 4.0-kb *Asp*718/*Bgl*II fragments of *S. lividans* TK24. The thermosensitive plasmid pGM160 (30) deleted for the *Hind*III/*Hind*III fragment containing the apramycin resistance gene, *aac* (gift of J. L. Pernodet), was used for cloning the disrupted *regI* gene. Plasmid pIJ486 (50) was used as a multicopy vector.

Culture conditions. *E. coli* was grown on Luria-Bertani plates (36) supplemented with ampicillin (50 μ g per ml) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal; 40 μ g per ml) when appropriate. *Streptomyces* strains were grown on media as described by Hopwood et al. (18). They were grown in YEME liquid medium for preparation of protoplasts or in tryptic soy broth (TSB) for preparation of total DNA.

To test gene regulation, cultures were generated from a mycelium glycerol stock grown for 3 days at 30°C on an orbital shaker at 160 rpm in minimum medium NMMP (18) containing 0.5% Casamino Acids and 1% carbon source (mannitol or glycerol) plus 10 μ g of gentamicin per ml when appropriate. Identical amounts of mycelium (a pellet volume of 50 μ l after a 2-s centrifugation in a microcentrifuge) were grown in 8 ml of fresh NMMP containing 0.5% Casamino Acids and 0.5 or 1% carbon source as indicated. Cultures were carried out in 150-ml Erlenmeyer flasks with three indents in the lower part, in the presence of 12 glass beads 4 mm in diameter, on an orbital shaker at 160 rpm, thus providing a dispersed growth. Cultures were analyzed after 22 or 45 h of growth at 30°C for amylase or chitinase activity, respectively.

For RNA analysis, 10⁸ spores were pregerminated as described previously (18) and grown in 20 ml of NMMP containing 0.5% Casamino Acids and 1% glycerol in the presence of 30 glass beads on an orbital shaker at 160 rpm for 40 h at 30°C.

Growth was estimated by the increase in turbidity (1-cm cuvette, 450 nm). The relationship between optical density and cell protein content was estimated as 0.3 mg of protein per ml per OD₄₅₀ (optical density at 450 nm) unit.

DNA manipulations. Chromosomal DNA was isolated from *S. lividans* by the method of Ausubel et al. (2). Plasmids were extracted from *E. coli* by the Easy Prep method (3). Restriction, ligation, and other DNA modification enzymes were purchased from Boehringer, Biolabs, and Pharmacia. Agarose gel electro-

* Corresponding author. Mailing address: Institut de Génétique et Microbiologie, Université Paris XI, Bâtiment 400, F-91405 Orsay, France. Phone: 33 1 69 15 69 13. Fax: 33 1 69 15 72 96. E-mail: nguyenj@igmors.u-psud.fr.

phoresis and Southern blotting by capillarity were performed as described by Sambrook et al. (36). Labeling of DNA by [α - 32 P]dCTP was achieved by using a Quick Prime labeling kit (Pharmacia).

PCR amplification of chromosomal DNA of *S. lividans* was performed by using the two oligonucleotides 5'-GTCSGCSGACATCGCSGCCSAGGC-3' and 5'-ACCACCAGCTCCGGCTGGAAGACGAAC-3', the former corresponding to the sequence RLADIAAQA located within the helix-turn-helix motif of ORFR of *S. limosus* (41a) and the latter corresponding to a conserved sequence in the 3' region of the putative repressor genes of *S. limosus* and *S. venezuelae* (43, 45). Reactions were carried out under standard conditions (22) in the presence of 5% dimethyl sulfoxide in a total volume of 50 μ l. The mixture was covered with 30 μ l of mineral oil and submitted to 30 cycles of 1 min at 98°C, 1 min at 55°C, and 1 min at 72°C. Southern blots of PCR products were hybridized with ORFR of *S. limosus* at 45°C overnight and then washed with 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 45°C.

The partial gene library of *S. lividans* was constructed by ligating the 3.5-4 kb *Asp*718/*Bgl*II DNA fragments into plasmid pIJ2925 (23) digested by *Asp*718/*Bam*HI. White colonies were selected for further hybridization with the PCR product homologous to ORFR. Hybridization was done at 65°C overnight followed by washes with 0.1 $\times$ SSC–0.1% SDS at 65°C. One positive clone contained the plasmid pJN518.

Determination of the DNA sequence. DNA sequencing was performed on double-stranded DNA by the dideoxy-chain termination method of Sanger et al. (37), using a Deaza T7 sequencing kit (Pharmacia) and vector or specific primers. Deletion clones were constructed by using the exonuclease III-S1 method of the Pharmacia double-stranded nested deletion kit. The DNA and protein sequences were analyzed by using the PC/GENE package (IntelliGenetics, Inc., Mountain View, Calif.). ORFs were identified by using a program similar to the FRAME program developed by Bibb et al. (4). The predicted proteins were scanned for similarities to the Swissprot protein sequence data base by using the FASTA program (32).

Construction of *regI* insertion mutants. The *Asp*718/*Bgl*II fragment of *S. lividans* (3.7 kb) cloned in pIJ2925, yielding pJN518, was deleted of the *Asp*718/*Nru*I part (1.9 kb) after digestion by *Nru*I/*Sac*I. The *aacC4* cassette (5) was inserted as a *Sma*I fragment (1.8 kb) into the *Bst*EII site of *regI* after fill-in with Klenow enzyme. The resulting 3.6-kb *Bgl*II fragment was cloned in the *Bam*HI site of the thermosensitive shuttle vector pGM160 (30) deleted for the *aacC1* gentamicin resistance gene, thus yielding pJN295. Selection in *E. coli* was achieved in the presence of G418 at 30 μ g per ml.

Protoplasts of *S. lividans* TK24::pTS6000 and TK24 prepared as described by Hopwood et al. (18) were transformed with pJN295, regenerated, and selected by using a top soft agar containing 500 μ g of nisin per ml. Nisin-resistant colonies were ground in TSB and used to inoculate TSB liquid medium containing 10 μ g of nisin per ml. They were grown for 24 h at 30°C. The mycelium was then ground in TSB and used to inoculate TSB liquid medium containing 10 μ g of gentamicin per ml. Cultures were grown at a nonpermissive temperature (39°C) for 3 days. Mycelium was ground and gently sonicated. Aliquots containing around 100 CFU were plated on HT medium (18) containing 30 μ g gentamicin per ml. Colonies were then replicated on HT plates containing 50 μ g of nisin per ml. Gentamicin-resistant but nisin-sensitive colonies were picked out.

Complementation of the *regI* mutant. The 3.7-kb *Eco*RI/*Xba*I fragment of pJN518 was cloned into the *Eco*RI and *Xba*I sites of the multicopy plasmid pIJ486 (50), yielding p486JN. Protoplasts of JN241 were transformed with p486JN. Transformants were selected for nisin resistance.

PFGE. Pulsed-field gel electrophoresis (PFGE) analysis was done with chromosomal DNA of *S. lividans* TK21 or JN241 prepared and digested by either *Ase*I or *Dra*I as previously described (25). The DNA fragments were isolated by PFGE on gels at 1 or 1.3% agarose. The electrophoresis buffer contained 20 mM triethylamine (Fluka), 8.75 mM acetic acid, and 1.5 mM EDTA. Runs were carried out for 30 h with pulse times ranging from 40 to 110 s, depending on the sizes of the DNA fragments to be separated. After blotting of DNA fragments onto a nylon membrane (Hybond; Amersham) by using a vacuum transfer system, pJN518 containing *regI* was nick translated and used as a probe for hybridization of Southern blots overnight at 65°C in 6 $\times$ SSC–0.1% SDS. After removal of the hybridization solution, washes were performed with 0.1 $\times$ SSC–0.1% SDS solution at 65°C. Membranes were exposed to X-ray films.

RNA analysis. Total RNA of TK24::pTS6000 and JN914 was extracted and estimated as described by Hopwood et al. (18). RNA was denatured by the glyoxal-dimethyl sulfoxide method, electrophoresed, and blotted onto a Hybond nylon membrane (Amersham) as described elsewhere (2). RNA transferred onto the membrane was visualized by staining as described by Wilkinson et al. (54). Blots were hybridized with the 1.7-kb DNA fragment containing the α -amylase of *S. limosus* (43) randomly 32 P labeled by using a Pharmacia Quick Prime labeling kit in 10 ml of hybridization solution (8) for 20 h at 65°C. After two 15-min washes in 0.2 $\times$ SSC–0.1% SDS at room temperature and twice at 65°C, blots were either exposed to X-ray films for visualization of transcripts or placed overnight in a beta cassette for estimation of α -amylase transcripts by using a PhosphorImager 425 (Molecular Dynamics).

Enzyme activity and protein measurements. α -Amylase activity was measured by the turbidimetric method (46) carried out with a Beckman DU640 spectro-

photometer. It was estimated as the initial decrease in OD₃₀₀ of a 1% starch solution. One arbitrary unit corresponds to the amount of enzyme contained in 100 μ l of culture supernatant yielding a rate of decrease in OD₃₀₀ of 10⁻⁴ U per min at 37°C.

Chitinase activity was measured as described by Miyashita et al. (29) in the presence of 4-methylumbelliferyl diacetyl chitobiose as a substrate and using a Fluoroskan II photometer (Labsystems). It was estimated as units of fluorescence produced per minute at 37°C by 20 μ l of supernatant.

Protein contents were estimated by the Bradford method (6) by using the Bio-Rad dye reagent and bovine serum albumin as a standard.

Nucleotide sequence accession number. The nucleotide sequence is stored in the EMBL database under accession no. X98242.

RESULTS

Molecular cloning of *regI*. The presence of a putative regulatory gene has been observed upstream of the α -amylase gene in *S. limosus* (27), *S. venezuelae* (45), and *S. griseus* (42). The homologous gene of *S. lividans* was isolated by using PCR to amplify chromosomal DNA of *S. lividans* (see Materials and Methods). A 16-fold-degenerated 5' primer was designed from the sequence of the DNA-binding motif of the putative repressor ORFR of *S. limosus* (41a), and a 3' primer was designed from a region which exhibited an identical nucleotide sequence in the 3' region of ORFR of *S. limosus* and *S. venezuelae* (27, 45). Among the PCR products, a 1-kb fragment hybridized with the putative regulatory gene encoding ORFR of *S. limosus* (data not shown). Digestion of the 1-kb PCR product by *Bst*EII generated a 0.5-kb fragment. When used as a probe, this fragment hybridized a 3.7-kb *Asp*718/*Bgl*II fragment of chromosomal DNA of *S. lividans* as ORFR did. A minilibrary of 3.5- to 4.0-kb *Asp*718/*Bgl*II fragments was constructed in pIJ2925 (23). Upon screening with the 0.5-kb PCR fragment, a positive clone could be isolated. The plasmid contained a 3.7-kb insert and was named pJN518.

Nucleotide sequence and characterization of *regI*. The nucleotide sequence of the insert contained in pJN518 was determined from the *Bgl*II end (1,435 bp). FRAME analysis of the G+C content of the nucleotide positions within each codon (4) revealed the presence of an ORF having the typical codon usage of *Streptomyces* genes starting about 250 bp from the *Bgl*II site (Fig. 1). A GTG start codon was present at position 237, but no obvious Shine-Dalgarno sequence was found. Such an absence has already been reported for other *Streptomyces* genes for which transcription and translation were thought to start at the same nucleotide (40). Therefore, an ORF of 1,038 nucleotides beginning at position 237 and ending at position 1274 was analyzed. It is 73% G+C rich, a value close to the average G+C content reported for other *Streptomyces* genes (55). The deduced protein contains 345 amino acids with a calculated molecular mass of 36,391 Da. A helix-turn-helix motif found in many DNA-binding proteins (7) is present in the N terminus. As this motif contains three amino acids (Ala9, Gly13, and Val19 [Fig. 1]) identical to the most conserved residues of these regulators, it is likely that this ORF corresponds to a regulatory gene in *S. lividans*, and it was thus designated *regI*.

The comparison of the deduced amino acid sequence of *regI* (Reg1) to entries in the data bank by using the University of Wisconsin Genetics Computer Group FASTA program revealed that Reg1 is almost identical (95%) to MalR, the newly identified repressor of *malE* transcription of *S. coelicolor* (41a). It exhibits 65% identity to ORF-SI, the LacI/GalR-like protein of *S. limosus* (41a), and 55% identity to *Streptomyces* ORFs found in regions of chromosomal instability in *S. ambifaciens* and *S. lividans* (1, 33, 47). Reg1 has 31% identity with CcpA, the carbon catabolite repressor of *Bacillus subtilis* (16). It ex-

*Bgl*III 10 20 30 40 50 60 70 80 90 100 110 120
 GATCTCCGAAGTTATCCGGCGTGACCTCTCGGCGACCGTCATGAGACGCTTGTATTACGGGGCCCGAGCGGTATCGGGCGGGTTGTGCAAGACTCTGCAAGCTCTTGTCCGCCACTTTTCTC
 130 140 150 160 170 180 190 200 210 220 230 240
 GAGGGAGCGGATGACGACGAGCCCCGCCGGGCGTCCGACGGGCGCTCCAAACAGGCGGCCCGCGCGCGGATCGGTGTGCAAGGAGCGGCCGACAGGTACAGTCCACCCCTGTGA
 M
 250 260 270 280 290 300 310 320 330 340 350 360
 CCACACGGCTTGCCGACATCGCCGCCAGGCGGGGGTGAGCGAAGCGACCGTCAGCCCGCTCCTCAACGGGAAGCCGGGCGTCCGCCGCCACCCGCCAGTCCGTGCTCGCCGCCCTCG
 T T R L A D I A A Q A G V S E A T V S R V L N G K P G V A A T T R Q S V L A A L
 * * * * *
 370 380 390 400 410 420 430 440 450 460 470 480
 ACGTGTGGCTTACGAGCGCCCGGTGCGGCTGCGGCGGCGAGCGCGGCCCTGGTGGGCTCTGTCACCCCGAGCTGGAGAACCCGATATTCCCGGCTTCGCCCGAGTCATCGGGCAGG
 D V L G Y E R P V R L R R S A G L V G L V T P E L E N P I F P A F A Q V I G Q
 490 500 510 520 530 540 550 560 570 580 590 600
 CGCTGACCCGCCAGGCTACACGCCCTCTCGCCACCCAGACCGCGGGCGGTCCACGGAGGACGAGCTGACCGAGATGCTGCTGACCGCGGGGTGGCGCGCATCATCTACGCTCTCG
 A L T R Q G Y T P V L A T Q T P G G S T E D E L T E M L V D R G V A G I I Y V S
 610 620 630 640 650 660 670 680 690 700 710 720
 GGCTGACCGCGACACCCGCCGACATGACGCGCTACGAGCGGCTGGGGGGAGGGGGGTGCCCTTCTGCTCTGACGGCTTCTGCTGCGAGGTGACAGCGCGCTCATCTCCCGCG
 G L H A D T T A D M Q R Y E R L G G R G V P F V L V D G F S S Q V Q A P F I S P
 730 740 *Bst*EII 760 770 780 790 800 810 820 830 840
 ACGACCGGCGCGATGACCTTCGCGGTACCCACCTCTCTCGCTCGGCGCACACAGGATCGGACTGGGCCCTCGGGCCGAAAGCGGTTCGTGCGCGGTGACGCGCAAGATGAGGGCTTCG
 D D R A A M S L A V T H L V S L G H T R I G L A L G P K R F V P V Q R K I E G F
 850 860 870 880 890 900 910 920 930 940 950 960
 TACGACCGCTCCAGGAGCCAGTTGGGCTGAGCGCGGAGCACGGTGAGAAAGAGCTGGTCCAGCACTCGCTGTACACCTCGAGGGCGCGCCAGGCGCGCTCCCGCTCATCGGCG
 V R T V Q E P V G A E R R S T V E K E L V Q H S L Y T L E G G Q A A A S A L I G
 970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
 GGGACTGCACGGCGGTGTCTGCGCCAGCGACATGATGGCGCTGGGGGCTATACGGCGCGCGCGAGCTGGGCGCTGACGCTGCCTAAGGACGCTCTCGGTCTCGGCTTCGACGACTCGC
 R D C T A V V C A S D M M A L G A I R A A R Q L G L D V P K D V S V V G F D D S
 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
 CGCTGATCGGCTTACCCGACCCCGCGCTCACACGGTCCGCAAGCCGGTCCCGGCGATGGGGCAGGCGGGCGGTGCGCACCGCTCTGAGGAGATCGGCGGACGCCCGCGCGCACAGCG
 P L I A F T D P P L T T V R K P V P A M G Q A A V R T L L E E I G G T P A P H S
 1210 1220 1230 1240 1250 1260 *Not*I 1270 1280 1290 1300 1310 1320
 AGTTGCTGTTCATGCCGAGTTGGTGGTGGCGGCTGACGGCGTGGCGCGCGGCGAGCGCGCGCGCCCTGATTCCCGTCCGGGGCGCGGCTCCGGGGCGGATCGGGTCCGTTCCAC
 E F V F M P E L V V R G S T A S A P G E R G R P -
 1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430
 CTGAGGGATGATCGTGAAGAAGGCTTTTCTGCGAGACTTATGCTATGGGTGACGCGACCGTGAAGGACTCGGGAACGCCGAGGAGCGCGTTCGCCACCCCGTCCGAGGGA

FIG. 1. Nucleotide sequence of the *reg1* gene of *S. lividans*. Relevant restriction sites are italicized. The deduced amino acid sequence is presented; the helix-turn-helix domain is indicated by asterisks.

hibits 28% identity to the regulatory protein RegA of *Clostridium acetobutylicum*, which can complement a *B. subtilis ccpA* mutant (9). Reg1 also exhibits a similar identity to GalR (30%), the galactose repressor in *E. coli* (48), and PurR (27%), the purine repressor in *E. coli* (35).

Alignment of the Reg1 amino acid sequence with that of homologous proteins showed that the degree of homology varies along the amino acid sequence (data not shown). The highest conserved region lies at the N terminus (amino acids 1 to 50), with nearly 50% identity between Reg1 and CcpA or RegA. It corresponds to the DNA-binding domain of these regulatory proteins (51). The most conserved part of this domain appears as a motif of six residues, ATVSRV, constituting most of the recognition helix (amino acids 16 to 24) and binding specifically to the major groove of the operator. Another well-conserved region is present at the C terminus spanning amino acids 230 to 310, with nearly 50% identity between these regulatory proteins. This region is important for the function of

these proteins, as it contains sugar-binding sites and dimerization sites, the latter being essential in the DNA-binding activities of these regulatory proteins. The central part of these proteins (amino acids 100 to 250) displays weaker homology, but some residues are conserved. They correspond to α -helices and β -strands constituting both subdomains of the ligand-binding pocket (38).

Physical mapping of *reg1*. In *S. lividans*, two amylase genes have been isolated: *amy*, isolated by Tsao et al. (41); and *amlB*, isolated in our laboratory (56). Southern blot analysis showed that the 3.7-kb *Asp*718/*Bgl*III fragment containing the *reg1* gene was recognized neither by the 11-kb *Bam*HI fragment containing *amy* nor by the 7-kb *Bcl*II fragment containing *amlB* (data not shown). This result showed that no amylase gene of *S. lividans* was located close to *reg1*, i.e., within at least 10 kb around *reg1*. It indicated that the gene arrangement around *reg1* in *S. lividans* was quite different from that occurring around ORFR in *S. limosus*, *S. venezuelae*, and *S. griseus*, in

FIG. 2. Chromosomal inactivation of *regI* in *S. lividans* by the integration of the temperature-sensitive plasmid pJN295. Homologous recombination by a single crossover yields nosiheptide (*tsr*)- and gentamicin (*aacC4*)-resistant colonies. The elimination of vector is obtained after growth at the nonpermissive temperature, resulting in nosiheptide-sensitive, gentamicin-resistant colonies. Abbreviations for restriction sites: A, *Asp*718; Bg, *Bgl*II; Bs, *Bst*EII; D, *Dra*I; N, *Nru*I.

which a putative regulatory gene was located just upstream of the α -amylase genes.

The localization of *regI* was further investigated by PFGE. Chromosomal DNA from *S. lividans* TK21 was digested either by *Ase*I or *Dra*I. This strain exhibited the same restriction pattern as strain ZX7 (26). Southern blot analysis showed that *regI* was located in the *Ase*I B fragment (975 kb) and in the *Dra*I IV fragment (700 kb) (data not shown). A more precise location of *regI* was obtained by analyzing the genomic DNA of the *regI* mutant strain JN241 in which two additional and adjacent *Ase*I and *Dra*I sites were introduced together with the *aac* cassette used to inactivate *regI* (see below). The *Ase*I and *Dra*I restriction patterns of JN241 were the same as those of TK21 except for fragment B, which was slightly smaller and generated an additional *Ase*I fragment of 25 kb, and for fragment IV, which was split into 550- and 150-kb fragments (data not shown). Therefore, it could be deduced that *regI* was located at 150 kb from the right end of the *Dra*I IV fragment, namely, in the 11 o'clock region according to the physical map defined by Leblond et al. (26).

Chromosomal inactivation of *regI*. To determine the role of *regI*, mutant strains were constructed. By using the thermosensitive plasmid pJN295 derived from pGM160 (30), the wild-type *regI* was exchanged for a *regI* derivative interrupted by a gentamicin resistance cassette (*aac*) of 1.8 kb (Fig. 2). The insertion of the cassette at the *Bst*EII site (Fig. 1) located between the regions encoding the DNA-binding domain and the dimerization domain will inactivate the gene product, since the regulatory proteins of the LacI family are known to require both domains to be active, dimerization being a prerequisite of protein binding to the regulatory sites.

Two kinds of mutant strains were generated, one from *S. lividans* TK24::pTS6000, which contains the α -amylase gene of *S. limosus* integrated in its chromosome and exhibits a high level of α -amylase production, and another from *S. lividans* TK24. Southern blot analysis showed that the wild-type gene *regI* was replaced by the disrupted gene. In both types of mutants, a single *Bgl*II/*Nru*I band was detected when the genomic DNA was probed with *aac* (Fig. 3) or *regI* (data not shown). The size of this chromosomal fragment, which was 1.8 kb in length in the wild-type strains (Fig. 3A, lane 1; Fig. 3B,

lane 3) was increased by 1.8 kb (the size of *aac* cassette) in the mutant of TK24::pTS6000 designated JN914 (Fig. 3A, lane 2) and in the mutants of TK24 (Fig. 3B, lanes 4 to 9). One of these latter mutants, designated JN241, was used for further investigation of the functional role of *regI* in *S. lividans* TK24.

Effect of *regI* on the expression of α -amylase genes. As shown above, *regI* is not located in the vicinity of the α -amylase genes in *S. lividans*. Nevertheless, because of its homology with the putative α -amylase regulator ORFR located upstream of *aml*, the α -amylase gene of *S. limosus*, it was of interest to determine whether it could have a role in the regulation of the α -amylase genes of *S. lividans*. As α -amylases are exocellular proteins, regulation of the corresponding genes was mainly studied through the changes in the α -amylase activity of culture supernatants. Mycelium was grown in minimum medium NMMP (18) supplemented with various carbon sources for 22 h, as preliminary experiments showed that the α -amylase activity increased linearly from 5 to 24 h of culture (data not shown). In these conditions, growth of the *regI* mutant strains was 15 to 20% lower than that of the parental strains (data not shown).

The production of α -amylase in TK24 was weak in minimum medium and was eightfold higher in the presence of maltose (fully induced state). The presence of glucose together with maltose led to the repression of α -amylase production which was maintained at a low level as in glucose or glycerol (Fig. 4A). In contrast, in the *regI* mutant strain JN241, the production of α -amylases exhibited high levels similar to those in the fully induced state in all conditions, especially in minimum medium, glucose, or glycerol, while a slightly lower production occurred in the presence of maltose or maltose plus glucose (Fig. 4B). In minimum medium or in the presence of glucose or glycerol, the production of α -amylases was seven- to eightfold higher in JN241 than in TK24. However, in the presence of maltose, this production was significantly less in JN241 than in TK24. When glucose and maltose were present together, the production was about threefold higher in JN241 than in TK24. It should be stressed that the repressor effect of *regI* on α -amylase production was completely restored in JN241 when this strain was transformed with the wild-type gene cloned on the multicopy plasmid pIJ486 (data not shown). These results indicated that *regI* seems to act as a repressor gene of the

FIG. 3. Southern blot analysis of chromosomal DNA of *S. lividans*. DNA was restricted by *Bgl*II and *Nru*I and probed with *regI* (lanes 1 and 3) or *aac* (lanes 2 and 4 to 9). (A) TK24 strains containing the α -amylase gene of *S. limosus*. Lane 1, parental strain TK24::pTS6000; lane 2, *regI* strain JN914. (B) Strain TK24. Lane 3, wild-type strain; lanes 4 to 9, *regI* strains (lane 4, strain JN241).

FIG. 4. Effect of the *reg1* mutation on the production of α -amylase in *S. lividans*. Mycelium pregrown for 72 h in minimum medium NMMP with mannitol (1%) was transferred to NMMP supplemented with various carbon sources for 22 h. N, none; M, maltose; G, glucose; MG, maltose and glucose; Y, glycerol. Culture growth was approximately equivalent to an OD_{450} of 4. α -Amylase activity was expressed in arbitrary units (Au). Each value is the mean of four independent experiments. Bars indicate standard deviations. (A and B) *S. lividans* TK24 (wild-type strain) and JN241 (*reg1* strain), respectively; (C and D) *S. lividans* TK24 containing the α -amylase gene of *S. limosus* (TK24::pTS6000 [parental strain] and JN914 [*reg1* strain], respectively). Carbon sources were used at a final concentration of 0.5% (A and B) or 1% (C and D).

weakly expressed α -amylase genes of *S. lividans* and is required for their catabolite repression.

However, it might be asked whether *reg1* could affect the regulation of the highly expressed α -amylase gene of *S. limosus* (*aml*) integrated in the chromosome of *S. lividans*. It is induced in the presence of maltose and repressed in the presence of glucose (43). In TK24::pTS6000, the parental strain (Fig. 4C), α -amylase was produced at relatively low levels either in minimum medium, in the presence of glucose, or in the presence of glycerol. This production was sixfold higher in the presence of maltose. In contrast, in the *reg1* mutant strain JN914 (Fig. 4D), the production of α -amylase reached high levels regardless of the carbon source added. These results indicated that *reg1* also regulates the expression of the α -amylase gene *aml* of *S. limosus* integrated in *S. lividans*, as it controls the α -amylase genes of *S. lividans*. Since the production of *aml* in *S. lividans* is 20-fold higher than that of the α -amylases of *S. lividans*, the

strains containing the *aml* gene were used to extract total RNA and measure the transcription level of *aml* by Northern blot analysis. The results in Fig. 5 show that the α -amylase mRNA was more abundant in the *reg1* mutant strain JN914 than in the parental strain TK24::pTS6000. The ratio of *aml* transcripts between the mutant strain and the parental strain was equal to 10, while that of α -amylase activity was equal to 6. The results indicated that the α -amylase gene is regulated at the transcriptional level by the *reg1* gene product.

Effect of *reg1* on the expression of chitinases of *S. lividans*.

As *reg1* appeared to be involved in catabolite repression of α -amylase genes, it was of interest to determine whether it could play a role in the regulation of other genes whose expression is repressed by glucose. Chitinases are extracellular enzymes hydrolyzing chitin. Four genes encoding chitinases have been cloned from *S. lividans*. Expression of all of them is induced in the presence of chitin and subject to catabolite repression when they are cloned in a multicopy plasmid (29). Mycelium of *S. lividans* TK24 (wild type) or JN241 (*reg1*) was grown for 45 h in minimum medium supplemented with chitin, chitin and glucose, or glucose. As expected, in TK24 the production of chitinases was induced 17-fold in the presence of chitin and repressed to the same extent in the presence of chitin plus glucose (Fig. 6). In contrast, in JN241 the production of chitinases was not induced in the presence of chitin, indicating that the product of *reg1* has a positive, probably indirect, effect on chitinase induction. On the other hand, the production of chitinases was not repressed in the presence of chitin plus glucose, where it reached a level similar to that in TK24 in the presence of chitin, indicating that catabolite repression of chitinases in *S. lividans* is mostly relieved when *reg1* is disrupted.

DISCUSSION

In this report, the isolation of a regulatory gene, named *reg1*, from *S. lividans* is described. This gene encodes the Reg1 protein, which exhibits similarity with prokaryotic transcriptional regulators of the LacI/GalR family (51), both in the DNA-binding domain at the N terminus and in the ligand-binding and dimerization domain of the C terminus.

Reg1 is almost identical to MalR encoded by *malR* in *S. coelicolor* (41a), which is closely related to *S. lividans*. As the chromosomal mapping of both regulatory genes is the same in both species, we can consider that *reg1* and *malR* represent the same gene. *malR* is adjacent to the *malEFG* gene cluster. It acts as a repressor gene of *malE* and is required for induction

FIG. 5. Northern blot of the transcription of *S. limosus* α -amylase in *S. lividans*. Mycelium was grown for 40 h in NMMP supplemented with 1% glycerol; 30 μ g of total RNA was hybridized with the α -amylase gene of *S. limosus* used as a probe. Lane 1, parental strain TK24::pTS6000; lane 2, *reg1* strain JN914. The arrow indicates the α -amylase transcript.

FIG. 6. Effect of the *reg1* mutation on the production of chitinases of *S. lividans*. Mycelium pregrown for 72 h in NMMP with glycerol (1%) was transferred for 45 h to NMMP with no added carbon source (N) or with 0.15% chitin (C), 0.15% chitin and 1% glucose (CG), or 1% glucose (G). Culture growth was approximately equivalent to an OD₄₅₀ of 7. Chitinase activity was estimated as units of fluorescence (uFl). Each value is the mean of two independent experiments. Bars indicate the variations of experimental values. (A) *S. lividans* TK24 (wild-type strain); (B) JN241 (*reg1* strain).

and catabolite repression of this gene. In addition, inactivation of *malR* represses agarase production. Since *malR* regulates the transport of maltose in *S. coelicolor* and maltose is one of the starch degradation products inducing α -amylase genes, it would seem likely that MalR also regulates the amylase genes of a maltose regulon similar to that in *E. coli* (39).

Reg1 acts as a repressor of α -amylase genes in *S. lividans*. No other regulatory gene (repressor or activator) seems to control the expression of the α -amylase genes, since the production of α -amylases is at a maximum in minimal medium and was not further inducible by maltose when *reg1* is disrupted. In addition, Reg1 seems to be solely responsible for glucose catabolite repression of the α -amylase genes, which is completely lost upon *reg1* disruption. The dual role of Reg1 resembles that of GylR, the repressor of the *gyl* operon in *S. coelicolor* which is also involved in catabolite repression of this operon (17). However, the relative locations of the two regulatory genes are different, as *gylR* is adjacent to the *gyl* operon under its control in *S. coelicolor* whereas *reg1* is not adjacent to α -amylase genes in *S. lividans*. By analogy with *malR* (41a), it is likely that *reg1* is adjacent to the *malE* homolog of *S. lividans* and therefore also ensures a dual control of the expression of this gene in *S. lividans*.

Putative operator sites mediating induction and glucose repression have been reported for α -amylase genes of *S. limosus* and *S. lividans* (44, 56). Similar sequences have been identified upstream of *malE* in *S. coelicolor* (41a). They consist of 6 or 7 bp arranged in either direct or inverted repeats separated by a variable number of nucleotides (Fig. 7A). The locations of these sequences are also variable relative to the transcriptional start site of these genes. These motifs have been proposed to be the targets of regulatory proteins, but no evidence of their binding activity has been reported so far. On the other hand, these sequences have no similarity with the operator sites of the GN₄CGN₄C type, which are recognized by homologs of the

LacI/GalR family and contain a central CG pair essential for their ability of protein binding (13, 53), a G at the fifth position upstream of the CG pair, and a C at the fifth position downstream of the CG pair (15, 20, 52). Interestingly, several putative sites of this type could be identified in the upstream regions of the α -amylase genes regulated by Reg1. One of them overlaps the repeated sequences in both the α -amylase gene *aml* of *S. limosus* and its homolog *amlB* of *S. lividans* and is also found in *amy*, the other α -amylase gene of *S. lividans* (Fig. 7B). Further experiments will allow us to characterize the actual binding sites of Reg1 in these genes.

Reg1 seems also to be involved in the regulation of chitinase production in *S. lividans*. While the repression or deactivation of chitinase gene expression remains unchanged in the *reg1* mutant strain, the production of these extracellular enzymes normally subject to catabolite repression is no longer repressed in the presence of glucose plus chitin. This finding implies that putative operator sites for Reg1 similar to those observed in α -amylase genes should be present in chitinase genes. Such sequences are effectively found in *chiA* (Fig. 7B), the only chitinase gene sequenced so far in *S. lividans* (28). The presence of these sequences at locations similar to those observed for the α -amylase genes of *S. lividans* might account for the effect of *reg1* upon catabolite repression in both systems.

As chitin no longer induces the production of chitinases in the *reg1* mutant strain, the involvement of *reg1* in chitinase induction may raise the question of its role in sugar transport. Chitin induces the production of chitinases in *S. lividans*. Although no simple inducing molecule has been characterized (34), chitin must be hydrolyzed prior to the uptake of low-molecular-weight degradation products which are probably the

		Position (5' base)	References
A			
Direct repeat sequences			
<i>aml</i>	(<i>S. limosus</i>)	<u>CTTGCAGAGGCTTGCGG</u>	-124 (44)
<i>amy</i>	(<i>S. lividans</i>)	<u>CTTGCCGCAAGACTTGCGG</u>	-37 (41)
<i>amlB</i>	(<i>S. lividans</i>)	none	/ (56)
<i>malE</i>	(<i>S. coelicolor</i>)	<u>CTTGCAGAGTCTTGAC</u>	-161 (41a)
Inverted repeat sequences			
<i>aml</i>	(<i>S. limosus</i>)	<u>CTTGCAGTTTGTCTGCAAG</u>	+10 (44)
<i>amy</i>	(<i>S. lividans</i>)	none	/ (41)
<i>amlB</i>	(<i>S. lividans</i>)	<u>CTTGCAAGATTCTTGCTGCAAC</u>	+8 (56)
<i>malE</i>	(<i>S. coelicolor</i>)	<u>TCTTGACATTTTTCAGCAAGA</u>	-60 (41a)
B			
Putative Reg1 operators			
<i>aml</i>	(<i>S. limosus</i>)	GGCTTGCGGGCCCT	-116 (44)
<i>amlB</i>	(<i>S. lividans</i>)	CGCAACCGCTTGCA	-1 (56)
<i>amy</i>	(<i>S. lividans</i>)	CGGGTCTCGACCCG	+1 (41)
<i>chiA</i>	(<i>S. lividans</i>)	GGTGGCCGCCGTCC	+5 (28)
		GGCCGCCGTCCCC	+8

FIG. 7. (A) Comparison of putative regulatory sequences involved in induction and glucose repression of α -amylase and *malE* genes. The repeated motifs are underlined. The position of the 5'-base is given with respect to the transcription start site. (B) Putative Reg1 operator sites in α -amylase and chitinase genes which are similar to operators of members of the LacI/GalR family. The most-conserved bases are in boldface.

inducer molecules. These inducers are likely produced by a low level of extracellular chitinase activity. If we consider that the basal levels of chitinase production are the same in the wild-type and *regI* strains, we may assume that *regI* is required for some step of the uptake of chitinase inducers derived from chitin. As the presence of glucose together with chitin allows the production of chitinases in the *regI* mutant strain, it would seem that the increase in the energy balance of cells, especially ATP, due to glucose supply might restore inducer uptake in the absence of a functional *regI*. Since an ATP-binding protein (MsiK) is required in the transport of the putative inducers of xylanases and cellulases in *S. lividans* (21), Reg1 may regulate a similar transport system of chitinase inducers.

In conclusion, this study provides evidence of the pleiotropic role of the regulatory gene *regI* isolated from *S. lividans*. It acts either positively as shown for chitinase induction or negatively as shown for α -amylase and chitinase production in the presence of glucose. The mechanisms of catabolite repression in *Streptomyces* are poorly understood (31). The question of whether carbon catabolite repression in *Streptomyces* might be mediated by a general regulatory protein similar to CcpA in bacilli (19) is worthy of consideration, since MalR cross-reacts with CcpA antibodies (41a) and similar cross-reacting bands are observed in *S. lividans* and *S. reticuli* (24). This would imply that common regulatory sequences similar to the catabolite-responsive element (20) might be present in *Streptomyces* genes encoding carbon catabolic enzymes subject to catabolite repression. Interestingly, a sequence similar to those hypothesized for Reg1 and similar to the operator sites of CcpA has been identified in the cellulase gene *cell* of *S. reticuli* (49). Since this site binds a repressor protein present in glucose-repressed cultures, the question arises as to whether this repressor of *cell* in *S. reticuli* might be a homolog of Reg1 in *S. lividans*. Further investigation will allow us to determine whether Reg1 could be involved in catabolite repression of other glucose-repressed genes of *S. lividans* and whether Reg1 targets could be identified in genes whose regulation is *regI* dependent in order to determine whether Reg1 acts as a general carbon catabolite repressor in *Streptomyces*.

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