The 1-kb-repeat-encoded DNA-binding protein as repressor of an α -glucosidase operon flanking the amplifiable sequence AUD1 of *Streptomyces lividans*

Jean-Nicolas Volff† and Josef Altenbuchner

Author for correspondence: Josef Altenbuchner. Tel: +49 711 685 7591. Fax: +49 711 685 6973.
e-mail: Josef.Altенbuchner@po.uni-stuttgart.de

Institut für Industrielle
Genetik, Universität
Stuttgart, Allmandring 31,
70569 Stuttgart, Germany

High-copy-number amplification of the AUD1 element is frequently associated with the large chromosomal deletions responsible for genetic instability in *Streptomyces lividans* TK64. Five ORFs were found in a 7 kb region directly adjacent to AUD1. The putative products of ORF1, ORF2 and ORF3 showed similarities to ATP-binding cassette (ABC) sugar transporters, the deduced protein sequence of ORF4 displayed similarities to α -glucosidases whilst no homology to proteins with known functions was found for ORF5. ORF4 (renamed *aglA*) was expressed in *Escherichia coli* and the protein purified and characterized. An α -glucosidase activity was detected using the synthetic α -glucoside *p*-nitrophenyl α -D-glucopyranoside. Of the many oligosaccharides tested, only sucrose was hydrolysed at a measurable rate [specific activity 32.4 units (mg protein)⁻¹] but no growth of *S. lividans* TK64 on sucrose was observed. A strain in which *aglA* was disrupted showed the same low α -glucosidase activity as strain TK64 and in both strains no stimulation of activity was seen by sucrose, trehalose or maltose; dextrin increased α -glucosidase activity about 10-fold. This probably resulted from induction of a second α -glucosidase-encoding gene. The AUD1 element contains three 1 kb repeats which encode DNA-binding proteins necessary for high-frequency amplification. In strains with a unique 1 kb repeat, disruption of the repeat led to a significant increase in the α -glucosidase activity. These results strongly suggest that the 1-kb-repeat-encoded proteins of AUD1 have a dual function: they are the repressors of the *agl* genes and they promote amplification of AUD1.

Keywords: *Streptomyces*, α -glucosidase, ABC transporter, repressor, amplification

INTRODUCTION

High-copy-number amplification of the chromosomally located AUD1 (amplifiable unit of DNA no. 1) is frequently observed in spontaneous mutants generated by genetic instability in *Streptomyces lividans*

(Altenbuchner & Cullum, 1984, 1985). The mutants are sensitive to chloramphenicol (Cml^s) and auxotrophic for arginine (Arg⁻). This double mutant phenotype is due to the deletion of two chromosomal markers included in a large deletion bordering the amplified DNA sequence (ADS).

† **Present address:** Biozentrum/Physiologische Chemie I, Am Hubland, 97074 Würzburg, Germany.

Abbreviations: ABC, ATP-binding cassette; AUD, amplifiable unit of DNA; LDR, left direct repeat; LIP, left imperfect palindrome; MDR, middle direct repeat; PBS, potential binding sequence; *p*-NPG, *p*-nitrophenyl α -D-glucopyranoside; RDR, right direct repeat; RIP, right imperfect palindrome. The GenBank accession number for the sequence reported in this paper is U22894.

AUD1 is composed of three 1 kb and two 4.7 kb repeats alternately arranged in direct orientation (Fig. 1; Altenbuchner & Cullum, 1985). The 4.7 kb repeats are identical and encode putative proteins having some similarities to chitinases (Volff *et al.*, 1996). They serve as substrate for the *recA*-dependent recombination event leading to amplification. When they are replaced by direct repeats generated from *Escherichia coli* DNA the

Fig. 1. Physical map of the *S. lividans* TK64 AUD1 region with the AUD1 sequence consisting of the three 1 kb and two 4.7 kb direct repeats (open arrows) and the various ORFs (below) found inside AUD1 and in the flanking region. The location of the binding sites (LIP and PBS) of the DNA-binding protein, encoded by the right 1 kb direct repeat (RDR), as well as a further imperfect inverted repeat (RIP) are shown. The orientation of the genes with respect to the chromosomal end, *argG* (responsible for arginine auxotrophy), *cml* (chloramphenicol resistance gene) and replication origin are indicated.

modified AUD1 structure is still able to amplify (Volff *et al.*, 1996). The left and middle 1 kb repeats are identical (LDR and MDR, 1009 bp in length) and differ from the right 1 kb repeat (RDR, 1012 bp in length) at 63 positions (6.2% divergence; Piendl *et al.*, 1994). The 1 kb repeats encode DNA-binding proteins displaying similarities to repressors of *E. coli* inducible operons, such as LacI, CytR and GalR. The RDR-encoded protein binds upstream and downstream of the right 1 kb repeat and upstream of the middle 1 kb repeat (Volff *et al.*, 1996). The two identical upstream binding sites named LIP (for left imperfect palindrome) are 36 bp long and are located 8 nt upstream of the start codon of MDR and RDR (Volff *et al.*, 1996). The downstream binding site is located in a 147 nt fragment flanking RDR on the right side. This fragment contains another imperfect palindromic sequence, RIP (for right imperfect palindrome). RIP is 32 bp long and is found 90 nt downstream from RDR. However, RIP is not a binding site for the 1-kb-repeat-encoded proteins (Volff *et al.*, 1996). A 34 nt sequence with similarities to LIP, called PBS (for potential binding sequence), is also present in the 147 nt fragment 32 nt downstream of RDR. The presence of both up- and downstream binding sites as well as a functional protein encoded by the RDR is important for efficient amplification of AUD1 (Volff *et al.*, 1996).

The homology with several bacterial operon repressors suggested a similar function for the 1-kb-repeat-encoded proteins in *S. lividans*. This prompted us to search for the presence of an operon by sequencing the regions flanking AUD1. No ORFs with the typical *Streptomyces* codon usage were found in the 2 kb DNA to the left of AUD1 (Volff *et al.*, 1996). In contrast, we report in this paper the presence of five ORFs to the right of AUD1 which are probably part of an operon negatively regulated by the proteins encoded by the 1 kb repeats.

METHODS

Strains, cultures and transformations. *S. lividans* TK64 is streptomycin-resistant and auxotrophic for the amino acid proline (Hopwood *et al.*, 1985). Strains Jni1, WP and AJ100

are independent spontaneous *Cml*^r *Arg*⁻ mutants derived from TK64 (Altenbuchner *et al.*, 1988; Volff *et al.*, 1996; Fig. 3a). Strain Jni1 possesses a high-copy-number amplification of AUD1. The AUD1 sequence of strain AJ100 (Altenbuchner & Eichenseer, 1991) is composed of a partially deleted 4.7 kb repeat flanked on its right side by a 1 kb repeat (MDR/RDR) which was the product of a recombination event between MDR (80%) and RDR (20%) (unpublished data). *S. lividans* WP has AUD1 and the flanking *agl* genes completely deleted. Mutants Jni26 (*aglA::tsr*), Jni29 (LDR, MDR, RDR::*tsr*) and Jni30 (LDR, MDR/RDR::*tsr*) were constructed by gene replacement in strain TK64. Jni27 (MDR/RDR::*tsr*) was obtained from strain AJ100 (Fig. 3a).

S. lividans strains were cultivated at 30 °C on Hickey-Tresner (HT) agar medium (Pridham *et al.*, 1957) or M40 minimal solid medium without asparagine but plus the required amino acids (Polsinelli & Beretta, 1966). For protoplast regeneration, R2 plates were used (Hopwood *et al.*, 1985). For genomic DNA extraction, strains were grown in HT liquid medium. To determinate the α -glucosidase activity, *S. lividans* strains were cultivated in 50 ml modified HT liquid medium without dextrins. Maltose, maltooligosaccharides (C₂–C₈), dextrin, trehalose or sucrose were added at a final concentration of 0.5%. *S. lividans* protoplast preparation, transformation and regeneration were done according to Hopwood *et al.* (1985). Genetic instability was analysed as described by Altenbuchner & Cullum, 1985).

E. coli JM109 (Yanisch-Perron *et al.*, 1984) was used as host in cloning experiments. All *E. coli* cultures, including those for selective expression, were done in dYT medium or on dYT agar plates (Sambrook *et al.*, 1989). λ phage were propagated on *E. coli* NM538 (Frischauf *et al.*, 1983) in dYT liquid medium or dYT soft agar supplemented with 10 mM Mg²⁺ and 0.2% maltose. For *E. coli* transformation, competent cells were prepared as described by Chung *et al.* (1989) and transformants were selected with ampicillin (100 mg l⁻¹).

DNA manipulations and sequencing. *S. lividans* and *E. coli* plasmid preparations were done using the alkaline lysis procedure of Kieser (1984). Genomic DNA was isolated according to Hopwood *et al.* (1985). Southern blot experiments and DIG labelling of DNA were done as described by Volff *et al.* (1996). Construction of a genomic library of *S. lividans* TK64 in λ EMBL4, plaque hybridization with [α -³²P]dCTP-labelled DNA and isolation of λ DNA were done as described by Eichenseer & Altenbuchner (1994).

Sequencing reactions were performed on double-stranded DNA using the Autoread Sequencing kit and an automated laser fluorescent ALF Sequencer (Pharmacia Biotech). Nucleotide sequences were analysed using programs of the GCG Wisconsin package (Devereux *et al.*, 1984). For the codon preference program (Gribskov *et al.*, 1984), a codon usage table from eight *Streptomyces* genes was generated as described by Pfeifer *et al.* (1992). Database searches were run with the programs BLASTN, BLASTX and TBLASTN (Altschul *et al.*, 1990) on the BLAST e-mail server from the National Center for Biotechnology Information, Bethesda, MD, USA.

Gel retardation assays. LDR was amplified from plasmid pMT680 as described for RDR (Volff *et al.*, 1996) using the primers S485 (5'-GCG CTA GTC TTG CGC ATA TGA CGC GAC GAC TTG C-3') and S1014 (5'-AAA AGG ATC CCG CAA GGA ACT TGC GGT-3'). The restriction sites for *Bam*HI and *Nde*I in the primer sequences allowed insertion of the PCR fragment into the L-rhamnose-inducible vector pJOE2702 (Volff *et al.*, 1996). The resulting plasmid, pJOE2935, and plasmid pJOE2797 (RDR in pJOE2702) constructed earlier (Volff *et al.*, 1996), were transferred into *E. coli* JM109. Expression of the genes was induced by growing the strains at 30 °C in dYT medium to an OD₆₀₀ of 0.5, followed by addition of 0.2% rhamnose and incubation for 3 h before preparation of crude extracts. Gel mobility shifts were done in 5% polyacrylamide gels using the LIP-containing *Hind*III fragment of plasmid pJOE2201 (Volff *et al.*, 1996) or the PBS-containing *Hind*III fragment of pJOE2864-2. The plasmid pJOE2864-2 contains the two complementary oligonucleotides S973 (5'-GATCCATTTACGAAATCAGCGCG-AGATATTGCGCCACTTGCATG-3') and S974 (5'-CAAG-TGGGCGCAATATCTCGCGCTGATTTCGTAAATG-3'), inserted between the *Sph*I and *Bam*HI site of pIC20H. The two *Hind*III fragments of pJOE2201 and pJOE2864-2 were labelled by filling in the ends with [α -³²P]dATP and Klenow polymerase as described by Volff *et al.* (1996). Sugars (sucrose, maltose, C₂-C₈ maltooligosaccharides and trehalose) were included in the binding assay at a final concentration of 1 mM.

Selective expression of *aglA*. *aglA* was PCR-amplified in the presence of 10% DMSO (30 cycles, annealing temperature 58 °C) from plasmid pJOE683. Primers (S1069, 5'-AAA ACA TAT GGC AGC CCC CAA CTC GCA CC-3'; and S1070, 5'-AAA AAG ATC TGG CGC GCA GCC ACA CCG TGT-3') were designed to introduce a *Nde*I site overlapping the ATG start codon of *aglA* and a *Bgl*II site directly flanking its last C-terminal codon. For selective expression in *E. coli*, *aglA* was inserted as an *Nde*I-*Bgl*II fragment into pJOE2775 (Krebsfänger *et al.*, 1998) under the control of the *rhaB* promoter. The six histidine codons present in the vector were fused to *aglA*. The correct sequence of the amplified *aglA* gene was verified by DNA sequencing of the resulting plasmid, pJOE2951. *E. coli* JM109 cells transformed with pJOE2951 were induced with 0.2% rhamnose for 3 h at 30 °C.

Determination of α -glucosidase activities with *p*-nitrophenyl α -D-glucopyranoside (*p*-NPG). Enzyme activity was measured spectrophotometrically by monitoring the release of *p*-nitrophenol from *p*-NPG. Cells of *S. lividans* and *E. coli* were grown in 50 ml medium, washed twice and resuspended in enzyme buffer (20 mM Tris, 100 mM NaCl, 0.02% NaN₃, pH 8.0). Crude extracts (20 μ l, prepared either by sonication or by French press) or purified enzyme were added to 600 μ l 0.1 M sodium phosphate pH 7.0 and 400 μ l of a 13 mM *p*-NPG solution in the same buffer and incubated at 25 °C until a yellow colour appeared. The reaction was stopped by addition of 500 μ l 0.4 M Na₂BO₃ pH 9.8 and the absorbance at 405 nm was measured. For *K_m* determination, the final *p*-NPG

concentration was varied from 0.1 to 10 mM. Protein concentration was determined using the method of Bradford (1976). One unit of α -glucosidase activity was defined as the amount of enzyme hydrolysing 1 μ mol *p*-NPG under these conditions. The temperature stability of *Ag*IA was determined by incubation of the enzyme for 15 min at temperatures between 25 °C and 60 °C and cooling on ice for several minutes before adding it to the reaction mixture. The pH optimum of *Ag*IA was determined by using 600 μ l 0.66 M potassium/sodium phosphate buffer pH 5.0–9.0 or 0.05 M Tris/HCl pH 7.0–9.0 as reaction buffer.

Gene disruption experiments. To disrupt the chromosomal *aglA* gene in *S. lividans* TK64, the *tsr* gene conferring resistance to thiostrepton (Ts^R, Thompson *et al.*, 1980) was inserted into the *Bam*HI site of *aglA* contained in the 3.8 kb insert of pJOE683 (this work). This insert ligated with *tsr* was then cloned into the *E. coli*-*Streptomyces* shuttle vector pJOE1082. This temperature-sensitive vector carries the minimal replicon and kanamycin resistance gene of *Streptomyces* plasmid pGM11 (Muth *et al.*, 1989) ligated to the *E. coli* plasmid pUC19 (Vieira & Messing, 1982). The resulting plasmid, pJni5, was used to disrupt *aglA*. To inactivate the 1 kb repeat(s), the *tsr* gene was inserted into the *Pst*I site of the right 1 kb repeat RDR (Piendl *et al.*, 1994). The resulting disrupted repeat was cloned into pJOE1082 and the resulting plasmid was called pJOE2369. Protoplasts of *S. lividans* were transformed with pJni5 and pJOE2369. After selection of the transformants with thiostrepton (50 μ g ml⁻¹) at 30 °C, spores were separated from mycelium by filtration (Hopwood *et al.*, 1985) and plated on HT medium at 40 °C to inhibit the replication of the temperature-sensitive vector pJOE1082. After a further plating of spores on HT medium plus thiostrepton at 40 °C, single colonies were tested for their growth on HT medium plus kanamycin (15 μ g ml⁻¹). Thiostrepton-resistant and kanamycin-sensitive colonies were retained and further cultivated. The success of the gene disruption and the loss of vector pJOE1082 were confirmed by Southern blot hybridization using pJni5, pJOE2369, the *tsr* gene and pJOE1082 as probes (data not shown).

Construction of plasmids pJOE3072-9, pJOE3073-1 and pJOE3076-2. These three plasmids are derivatives of the *E. coli*-*S. lividans* shuttle vector pJOE3069, which is similar to pE116 described previously (Volff *et al.*, 1996). The plasmid pJOE3069 contains the SCP2 origin of replication on a *Sal*I-*Xho*I fragment and the SCP2 stability region on a *Bam*HI-*Sac*I fragment, both inserted into pUC13. Plasmid pJOE3076-2 contains the *tsr* gene inserted into pJOE3069 as a *Hind*III fragment from plasmid pJOE803 (Altenbuchner & Eichenseer, 1991). The *agl* genes, together with the C-terminal end of RDR, were obtained from λ 559-1 as an *Eco*RI-*Pst*I fragment and inserted into pJOE803 (pJOE3063-2). The *agl* genes were isolated again from pJOE3063-2 together with the *tsr* gene as a *Hind*III-*Pst*I fragment and combined with a *Hind*III-*Pst*I fragment from pJOE2434 (Volff *et al.*, 1996) containing the N-terminal end of RDR together with 73 bp upstream sequence. This *Hind*III fragment was inserted into pJOE3069 to give pJOE3072-9. For pJOE3073-1, the *Pst*I site in the RDR was destroyed by Klenow polymerase. Nucleotide sequencing of the deleted region showed that instead of a 4 bp deletion, actually 11 bp were missing.

Substrate specificity of the α -glucosidase. To 30 μ l of reaction buffer (100 mM sodium phosphate pH 7.0) 10 μ l substrate (500 mM stock solution in 100 mM sodium phosphate) and 10 μ l enzyme (usually 2.4 μ g purified enzyme, but when sucrose was used as substrate, the enzyme solution was tenfold diluted) were added and incubated for 60 min at 25 °C. To determine

the K_m for sucrose, the final concentration of sucrose in the reaction mixture was varied from 10 mM up to 900 mM. The reaction was stopped by heating to 90 °C for 2 min. Granutest 250 (E. Merk; 1 ml) was added and after 45 min at 25 °C the absorbance at 340 nm was determined on a Kontron spectrophotometer. The test is based on reduction of NAD^+ by glucose dehydrogenase oxidizing the glucose which is generated by the enzyme reaction to gluconolactone. The amounts of glucose were obtained from a calibration curve generated with defined glucose concentrations. In addition, the products of enzyme reactions were determined and quantified by HPLC using a Hamilton RCX-10 column (250 × 4.1 mm) and an electrochemical detection unit, CoulochemII (ESA), with the analytical cell 5040. The mobile phase was 0.1 M NaOH and the flow rate was 0.75 ml min⁻¹. For tri- and tetrasaccharides and phosphorylated disaccharides, the mobile phase was 0.22 M NaOH/0.02 M sodium acetate.

Purification of AglA. *E. coli* JM109(pJOE2951) was grown in 50 ml dYT at 37 °C to an OD₆₀₀ of 0.5, the culture shifted to 30 °C, L-rhamnose added to a final concentration of 0.1 % and incubation continued for a further 3 h. The cells were harvested by centrifugation (Sorvall SS34, 10 min, 6000 r.p.m., 4 °C), washed in enzyme buffer (20 mM Tris, 100 mM NaCl, 0.02 % NaN₃, pH 8.0) and resuspended in 3 ml enzyme buffer. The cells were disrupted using a French press (two cycles at approx. 800 bar), the crude extract clarified by centrifugation (Sorvall SS34, 30 min, 20000 r.p.m., 4 °C) and the supernatant (1 ml) applied to a metal affinity spin column (Talon, Clontech Laboratories) equilibrated with enzyme buffer. After centrifugation at 600 r.p.m. for 2 min in a Heraeus Megafuge with a swingout rotor, the column was washed twice with 1 ml enzyme buffer and the enzyme eluted with 2 × 1 ml enzyme buffer supplemented with 50 mM imidazole. Finally, the two fractions were combined and dialysed twice against enzyme buffer.

Gel filtration of AglA. The native molecular mass of AglA was determined by gel filtration on a Superdex 75, PC 3.2/30 column (Smart system, Pharmacia), calibrated with ribonuclease A (13700 Da), chymotrypsin (25000 Da), ovalbumin (43000 Da) and bovine serum albumin (67000 Da). The column was equilibrated with enzyme buffer and the enzyme eluted with this buffer at a flow rate of 0.04 ml min⁻¹. The elution profile was monitored at 280 nm and the presence of active enzyme in the fractions by enzyme assays with *p*-NPG as substrate.

RESULTS

Nucleotide sequence of the region flanking AUD1 on the right side

The AUD1 region, isolated on a 15 kb *Bcl*I fragment, has been completely sequenced (Volff *et al.*, 1996). To extend this sequence to the right side, a genomic library of *S. lividans* TK64 in λ EMBL4 (Frischauf *et al.*, 1983) was screened by plaque hybridization with pMT682, a pEMBL8 plasmid containing the right end of the 15 kb *Bcl*I fragment (Altenbuchner & Cullum, 1985). Two recombinant phages were obtained (λ 559-1 and λ 559-5) which carried DNA from beyond the right *Bcl*I site. This DNA was inserted into the *E. coli* pIC20H vector (Marsh *et al.*, 1984) and sequenced by subcloning restriction fragments or by transposon tagging. About

7 kb of DNA was sequenced in this way, extending the nucleotide sequence of the AUD1 region to 20996 bp. The sequence was examined by codon usage analysis and similarity searches in various databases, which identified five new ORFs, all with the same orientation. The deduced gene products of the first three ORFs showed similarities to the periplasmic-binding-protein-dependent ABC transporters and the product of the fourth ORF to α -glucosidases. To the last one, no possible function could be attributed.

The similarities indicated that the ORFs may form an operon for uptake and degradation of an α -glycosidic sugar and were named tentatively *aglE*, *aglF*, *aglG*, *aglA* and *aglX* (Fig. 1 and Table 1). The first ORF, *aglE*, begins 151 bp downstream of the stop codon of RDR (right 1 kb repeat of AUD1) and encodes a protein of 471 amino acids. The deduced protein shows similarities to periplasmic sugar-binding proteins. The N-terminal amino acid sequence is in accordance with consensus sequences of signal peptides of secreted proteins with a hydrophilic region containing six arginine residues followed by a hydrophobic region of 14 aa and a signal peptide cleavage site, LAAC, which is recognized by signal peptidase II (van Heijne, 1989). This indicates that AglE is a lipoprotein. The next two predicted gene products, AglF and AglG, have sizes of 231 and 291 aa, respectively, and show similarity to the MalF-MalG proteins, integral membrane components of the *E. coli* maltose transport system (Boos & Lucht, 1996). AglF and AglG are hydrophobic proteins according to a Kyte-Doolittle plot as expected (data not shown). AglA (534 aa) is an α -glucosidase, as indicated by high aa sequence identity with a maltooligosaccharide hydrolase (61% identity, Table 1) and the *E. coli* trehalose-6-phosphate hydrolase (38% identity), and later proved by enzyme assays. The *aglX*-encoded protein of 547 aa shows significant similarities to a putative mycobacterial protein of unknown function.

Expression of *aglA* in *E. coli*, and purification and biochemical analysis of the enzyme

The homology of the putative product of *aglA* with different bacterial α -glucosidases and eukaryotic maltases predicted that AglA would have a similar enzyme activity. For further biochemical characterization, the gene was amplified by PCR and placed under the control of the L-rhamnose-inducible promoter in the plasmid pJOE2775 (Krebsfänger *et al.*, 1998). In addition, the C-terminal end of the gene was fused to six histidine codons in the vector for purification of the gene product. The resulting plasmid pJOE2951 was transferred into *E. coli* JM109 and the α -glucosidic activity was measured spectrophotometrically by monitoring the release of *p*-nitrophenol from *p*-NPG. After 3 h induction with rhamnose at 30 °C, a 60-fold increase in enzyme specific activity from 0.07 U mg⁻¹ to 4.03 U mg⁻¹ was determined. The protein was purified (5.6-fold) from crude extract in one step by immobilized metal affinity chromatography, taking advantage of the

Table 1. ORFs on the right side of AUD1

ORF	Size (aa)	Putative RBS*	Similar protein (database: accession number)	Identity (%)	Putative function
AglE	471	<u>cgaagggg</u> accacccatg	<i>S. coelicolor</i> maltose-binding protein (GB: Y07706)	23.9	Extracellular sugar-binding protein
AglF	231	cccc <u>aaggag</u> cgacgatg	<i>Synechocystis</i> sp. lactose transport protein (DDBJ: D90905)	31.9	Membrane protein of ABC transport complex
AglG	291	cggggg <u>aa</u> accacccgatg	<i>Synechocystis</i> sp. lactose transport protein (DDBJ: D90910)	32.1	Membrane protein of ABC transport complex
AglA	534	ccgaaaggacgcgtcacgtg	<i>Thermomonospora curvata</i> α -glucosidase (GB: U17917)	60.6	α -Glucosidase
AglX	547	gggaggagcgacgcgtg	<i>Mycobacterium tuberculosis</i> protein (SP: Q11034)	29.3	Unknown

* Putative RBS are underlined; start codons are in italics.

Fig. 2. SDS-PAGE analysis of the purification of AglA from *E. coli* JM109 pJOE2951. Lanes: 1, protein size standard; 2, crude extract of *E. coli* JM109 pJOE2951 induced with rhamnose; 3, protein fraction which did not bind to the metal affinity spin column; 4, first washing fraction of the metal affinity spin column; 5, purified AglA eluted with 50 mM imidazole.

His-tag attached to the end of the enzyme. The protein was more than 95% pure as estimated by densitometrical scanning of an SDS-polyacrylamide gel (Fig. 2). The molecular mass of the enzyme determined by SDS-PAGE was 57 000 Da, which is in good agreement with the calculated molecular mass of 59 148 Da. Gel fil-

tration of the protein in comparison with known standards showed a molecular mass of 56 000 Da for the native enzyme, which indicates that AglA has a monomeric structure. AglA activity was tested from pH 5.0 to 9.0, raising the pH in steps of 0.5. The pH optimum was at 7.5; there was no activity below pH 5.5 and at pH 9.0 the residual activity was about 15%. The enzyme was stable up to 35 °C; incubation for 15 min at 40 °C reduced activity to 60% and 15 min at 45 °C abolished activity completely.

Substrate specificity of AglA

To find out more about the biological function of *aglA* and the neighbouring ORFs, AglA was incubated with a series of natural products containing an α -glucosidic bond, and with some phosphorylated disaccharides which might be intermediates in the catabolism of disaccharides. The reaction was stopped by heating, and the release of glucose was monitored by HPLC analysis using an electrochemical detection unit and also by measuring the NADH produced by glucose dehydrogenase in a second reaction using a spectrophotometer. Furthermore, a series of sugars linked by an α -glycosidic bond with *p*-nitrophenol were tested to see if sugars different from glucose might be accepted as substrate. The results are summarized in Table 2 and demonstrate a high substrate specificity of AglA. From the *p*-nitrophenol-containing compounds only *p*-nitrophenyl α -D-glucopyranoside, and from the oligosaccharides only sucrose, were hydrolysed at a fairly high rate. No activity was seen with phosphorylated sucrose or other phosphorylated disaccharides. With *p*-NPG or sucrose as substrate the specific activity of AglA was nearly the same (22.60 and 14.90 U mg⁻¹, respectively) under standard conditions (5.2 mM *p*-NPG or 100 mM sucrose). When the K_m values for the two substrates were determined, a nearly 100-fold difference was found (K_m for *p*-NPG = 1.5 mM and K_m for sucrose = 110 mM).

Table 2. Substrate specificity of AgIA

Trivial name	Structure	Activity (U mg ⁻¹)*
Cyclodextrin	Cyclohexaamylose α -(1→4)	<0.01
Kestose	α -D-Glcp-(1↔2)- β -D-Fruf-(6←2)- β -D-Fruf	<0.01
Isokestose	α -D-Glcp-(1↔2)- β -D-Fruf-(1←2)- β -D-Fruf	0.05
Maltose	α -D-Glcp-(1→4)-D-Glcp	0.43
Maltotriose	α -D-Glcp-(1→4)- α -D-Glcp-(1→4)-D-Glc	1.05
Maltotetraose	α -D-Glcp-[(1→4)- α -D-Glcp] ₂ -(1→4)-D-Glc	0.29
Maltose 1-phosphate	α -D-Glcp-(1→4)-D-Glcp-1-P	0.03
Maltitol	α -D-Glcp-(1→4)- α -D-Sor	0.03
Maltulose	α -D-Glcp-(1→4)-D-Fruf	0.32
Melibiose	α -D-Galp-(1→6)-D-Glcp	<0.01
Melizitose	α -D-Glcp-(1→3)- β -D-Fruf-(2↔1)- α -D-Glcp	<0.01
Palatinose	α -D-Glcp-(1→6)-D-Fruf	<0.01
Panose	α -D-Glcp-(1→6)- α -D-Glcp-(1→4)-D-Glcp	0.01
Trehalose	α -D-Glcp-(1↔1)- α -D-Glcp	<0.01
Trehalose 6-phosphate	α -D-Glcp-6-P-(1↔1)- α -D-Glcp	<0.01
Trehalulose	α -D-Glcp-(1→1)- β -D-Fruf	0.59
Turanose	α -D-Glcp-(1→3)-D-Fruf	0.53
Sucrose	α -D-Glcp-(1↔2)- β -D-Fruf	14.90
Sucrose 6-phosphate	α -D-Glcp-(1↔2)- β -D-Fruf-6-P	<0.01
<i>p</i> -Nitrophenyl α -L-arabinopyranoside		<0.01
<i>p</i> -Nitrophenyl α -L-fucopyranoside		<0.01
<i>p</i> -Nitrophenyl α -D-mannopyranoside		<0.01
<i>p</i> -Nitrophenyl α -D-xylopyranoside		<0.01
<i>p</i> -Nitrophenyl α -D-glucopyranoside		22.60

* Sugar concentrations in the enzyme reactions were 100 mM, except for kestose and isokestose, which were used at 20 mM, and the *p*-nitrophenyl compounds, which were used at 5.3 mM (all final concentrations). Each value represents the mean of at least three different experiments.

Table 3. α -Glucosidase activity in *S. lividans* TK64 and Jni26 grown in modified HT medium with different carbon sources

Values are the means of at least three different measurements.

Strain	Specific activity (U mg ⁻¹)				
	Glycerol	Dextrin	Maltose	Trehalose	Sucrose
TK64 (wild-type)	0.086	0.911	0.138	0.106	0.095
Jni26 (<i>aglA::tsr</i>)	0.063	0.803	0.088	0.144	0.162

At non-limiting sucrose concentrations (600–900 mM), the specific activity of AgIA was 32.4 U mg⁻¹.

Disruption of *aglA* in *S. lividans*

Release of *p*-nitrophenol from *p*-NPG was detected in the presence of crude extracts of *S. lividans* TK64 after culturing in M40 liquid minimal medium plus glycerol

or in modified HT medium where dextrin was replaced by glycerol. To see if this activity was due to AgIA, the *aglA* gene was disrupted by insertion of the thiostrepton resistance gene, *tsr* (Thompson *et al.*, 1980), and the α -glucosidase activity in the resulting strain Jni26 was compared with TK64. In addition, to see if the α -glucosidase activity could be increased by induction with α -glucosides, both strains were grown in modified

Fig. 3. Effect of disruption, deletion and amplification of the 1 kb repeats on the basal α -glucosidase activity. (a) Structure of AUD1 in the mutants studied. The black arrow represents the *tsr* gene and its transcriptional orientation. (b) Relative α -glucosidase activity of the mutants compared to TK64. The specific activity of TK64 (0.086 ± 0.029 U mg⁻¹) was set to 100%; values are the mean of at least three independent measurements having a standard error of <25%.

HT-medium supplemented with either glycerol, dextrin, maltose, sucrose or trehalose for 24 h and the α -glucosidase activity determined in crude extracts with *p*-NPG as substrate. No difference was found between *S. lividans* TK64 and Jni26. Both strains exhibited about the same basal α -glucosidase activity, and the presence of maltose, sucrose or trehalose did not lead to a significant increase in activity. Only with dextrin was an about 10-fold higher activity observed in both Jni26 and TK64 (Table 3). This indicated that there is a second, dextrin-inducible α -glucosidase in *S. lividans*.

The 1-kb-repeat-encoded protein as repressor of the *agl* genes

The 1 kb repeat RDR was disrupted in TK64 by the *tsr* gene and the resulting strain was called Jni29 (Fig. 3). In strain Jni30, which was also constructed from TK64, one event of homologous recombination necessary for disruption had occurred in RDR and the second in MDR. The resulting strain had an intact LDR, a disrupted second 1 kb repeat consisting of the recom-

bined MDR and RDR, and a deletion of one 4.7 kb repeat. Jni1 and AJ100 are two Cml^S Arg⁻ spontaneous mutants isolated from TK64. Strain Jni1 contains a high amplification of AUD1 and therefore presumably of MDR. Crude extracts of this strain retarded DNA fragments containing the binding sites of the 1-kb-repeat-encoded proteins, whilst crude extracts of strain TK64 did not (Volff *et al.*, 1996). Strain AJ100 contains a total deletion of one 4.7 kb repeat, a partial deletion of the other, a deletion of LDR and one unique 1 kb repeat recombined from the MDR sequence (about 80%) and the RDR sequence (20%). Strain Jni27 was constructed by disruption of the unique 1 kb repeat of AJ100 (Fig. 3). All strains were grown in modified HT medium with glycerol and the α -glucosidase activity determined from crude extracts with *p*-NPG as substrate. Jni27 showed an α -glucosidase activity higher than that of TK64 (about 10-fold) and of its more closely related parent AJ100 (about 17-fold). This strongly suggested that the 1 kb repeat encodes the repressor of the *agl* genes. Strains Jni29, Jni30 and AJ100 showed no increased activity, indicating that LDR, MDR and the composite repeat of AJ100 control the expression of *aglA*. No reduction in enzyme activity was observed in the amplified strain Jni1, probably indicating that most of the activity observed derives from the second gene present in *S. lividans* strains (Fig. 3).

Cml^S Arg⁻ mutants of *S. lividans* are highly unstable and therefore the possibility that there were additional rearrangements in strain Jni27 leading to increased expression of the *agl* genes could not be completely excluded. A further experiment was performed to definitively corroborate the role of the RDR-encoded protein as repressor of the *agl* genes. All five *agl* genes together with RDR and a thiostrepton resistance gene were inserted into a low-copy SCP2 plasmid derivative to give plasmid pJOE3072-9. A second plasmid was generated by deleting the *Pst*I site in pJOE3072-9, which introduced a frameshift mutation in RDR (pJOE3073-1), and for a control, the *tsr* gene alone was inserted into the SCP2 vector (pJOE3076-2). All three plasmids were introduced into *S. lividans* WP, a Cml^S Arg⁻ mutant of *S. lividans* which has AUD1 and the *agl* genes deleted. After growth in modified HT medium without dextrin, the α -glucosidase activity was determined. As can be seen from Fig. 4, there was a slight increase in enzyme activity in *S. lividans* WP with pJOE3072-9 compared to the vector control and a 10-fold increase when RDR was inactivated, which confirms the repressor function of the RDR-encoded protein.

To demonstrate a binding of the protein encoded by the left and middle 1 kb direct repeat, LDR was amplified by PCR from a plasmid containing only LDR (pMT680; Altenbuchner & Cullum, 1985) and inserted into a rhamnose-inducible vector, pJOE2702, as described for RDR (Volff *et al.*, 1996). Crude extracts from rhamnose-induced cultures of *E. coli* JM109 containing the vector pJOE2702, pJOE2797 (RDR) and pJOE2935 (LDR) were incubated with a radioactively labelled fragment containing the already identified binding site LIP, which

Fig. 4. Restriction map of the plasmids pJOE3072-9, pJOE3073-1 and pJOE3076-2. The α -glucosidase activities (mean values $\pm$ standard deviations) obtained after transformation of *S. lividans* WP with these plasmids are shown on the right side [$\text{U (mg protein)}^{-1}$]. From each transformation, three colonies were grown separately in modified (without dextrin and other additional carbohydrates) HT medium with thio-strepton ($10 \mu\text{g ml}^{-1}$) for 3 d. Enzyme activity was determined with *p*-NPG as substrate. Activity of each colony was measured at least twice in independent experiments.

Fig. 5. (a) Mobility shift assay with the LDR- and RDR-encoded proteins and the DNA-binding site LIP located upstream of the 1 kb repeats. A *Hind*III fragment from plasmid pJOE2201 containing the LIP sequence was labelled with [α - ^{32}P]dATP and incubated with crude extract from *E. coli* JM109(pJOE2702) (vector control; lanes 1, 4), *E. coli* JM109(pJOE2797) (RDR-protein; lanes 2, 5), and *E. coli* JM10(pJOE2935) (LDR-protein; lanes 3, 6). In lanes 4, 5 and 6, 10 mM of a mixture of maltooligosaccharides (maltose–maltooctaose) was added to the binding reaction. (b) Mobility shift assay with the DNA-binding site PBS obtained from plasmid pJOE2864-2 as a *Hind*III fragment. The labelled fragment was incubated with crude extract from *E. coli* JM109(pJOE2702) (vector control; lane 1) and from *E. coli* JM109(pJOE2797) (RDR-encoded protein; lane 2).

was bound by the protein encoded by RDR (Volff *et al.*, 1996) and analysed by mobility shift assay (Fig. 5a). To determine whether the 1-kb-repeat-encoded proteins

respond to maltose, maltooligosaccharides or sucrose, binding was done in the presence or in the absence of these sugars (in Fig. 5 only the results of binding without sugar and with 10 mM maltooligosaccharides are shown). Fig. 5 shows that both proteins bind to LIP. Interestingly, despite the very low difference in amino acid sequence between the LDR- and RDR-encoded proteins, there is a clear difference in migration of the retarded bands. The presence of the sugar sources did not modify the binding of the 1-kb-repeat-encoded proteins. This is consistent with the results previously obtained and reinforces the conclusion that sucrose, maltose and maltooligosaccharides are not inducers of the putative *agl* operon.

LIP is located upstream of RDR. A second binding site was located in the intercistronic region between the end of RDR and the beginning of *aglE*. It was shown previously that a palindromic sequence called RIP in this region was not bound by the 1-kb-repeat-encoded protein. Another candidate for binding was a 36 bp sequence which showed homology to LIP. This nucleotide sequence, called PBS (potential binding site), was synthesized as two complementary oligonucleotides and inserted into the polylinker sequence of pIC20H (pJOE2864-2). From there it was cut out again by *Hind*III, labelled with Klenow polymerase and [α - ^{32}P]dCTP and used in mobility shift experiments. As can be seen in Fig. 5(b), the sequence is indeed shifted by the RDR-encoded protein.

DISCUSSION

We report here that the amplifiable element AUD1 of *S. lividans* TK64 is on one side flanked by five ORFs. The *S. lividans* AUD1 right 1 kb repeat and the following

ORFs show similarity to the maltose operon of *S. coelicolor* A3(2) (van Wezel *et al.*, 1997a, b). There is a *lacI*-like repressor gene, an intercistronic promoter/operator region, where the repressor binds, genes for an ABC transport system (composed of an extracellular sugar-binding protein and two membrane proteins for the uptake of the sugar) and a gene encoding an α -glucosidase. The lack of a gene encoding a MalK-homologous ATP-binding protein, which is necessary to provide energy to the sugar transport systems, is not unusual. It is also missing in the *S. coelicolor* maltose operon (van Wezel *et al.*, 1997a). It seems that there is a *malK*-homologous gene *msiK* in *S. lividans* (and *S. coelicolor*) which is used by different ABC transport systems responsible for uptake of xylobiose, cellobiose and other disaccharides (Hurtubise *et al.*, 1995; Schlösser *et al.*, 1997).

The 1-kb-repeat-encoded protein binds 8 nt upstream of its start codon (for MDR and RDR) at a sequence called LIP which would allow autoregulation of its own synthesis. It also binds in the region between RDR and *aglE* at a sequence called PBS which shows significant similarity to LIP. A nucleotide sequence fitting the consensus sequence of a *Streptomyces* vegetative promoter was identified within the 151 bp intercistronic region (Fig. 6). PBS overlaps the -35 sequence of this putative promoter sequence and therefore the binding of the RDR-encoded protein to PBS would inhibit binding of the RNA polymerase to this promoter. When the *agl* genes are transcribed by this promoter the mRNA would start at the beginning of RIP. The function of RIP remains unclear. It could be the target of a global regulatory system. As a part of the leader region of the *agl* mRNA it could also stabilize the mRNA by a stem-and-loop secondary structure as described by Deng *et al.* (1990).

in crude extracts of *S. lividans* was due to a second gene since inactivation or even deletion of *aglA* had no influence on this activity. This second gene might be part of a genuine maltose operon. The gene *reg1*, which was isolated from *S. lividans* as a regulatory gene of α -amylase and chitinase activity, is nearly identical to the *malR* gene of the *S. coelicolor* maltose operon (Nguyen *et al.*, 1997) and might be in fact the regulator of the *S. lividans* maltose operon. *S. coelicolor* and *S. lividans* grow very poorly on maltose (van Wezel *et al.*, 1997b and our own results). The reason might be that maltooligosaccharides, i.e. degradation products of dextrin, but not maltose are the inducers of the maltose operons in the two strains. Indeed, inactivation of MalR in *S. coelicolor* improved growth on maltose considerably (van Wezel *et al.*, 1997b) and addition of maltooligosaccharides (C_2 – C_8) to the growth medium of *S. lividans* had about the same effect on α -glucosidase activity as dextrin (data not shown).

According to its amino acid sequence, AglA clearly belongs to the family 13 of glycoside hydrolases. Members of this family retain the configuration at the anomeric centre of the substrate during hydrolysis and the catalytic domain of the enzymes shows a characteristic (β/α)₈ barrel structure. Secreted and intracellular enzymes with various substrate specificities, such as α -glucosidases, α -amylases, pullulanases, cyclodextrinases or trehalose-6-phosphate hydrolase, belong to this family (Henrissat, 1991; Henrissat & Bairoch, 1993; Warren, 1996). For the α -glucosidase MalL of *Bacillus subtilis*, a member of this family, sucrose was also the preferred substrate (Schönert *et al.*, 1998). But the specific activity of MalL was about 10-fold higher and the K_m for sucrose 10-fold lower than for AglA. Despite the preference for sucrose, the expression of the corresponding gene was induced by maltose and not by sucrose and the biological function of MalL seems to be in the breakdown of internal storage polysaccharides (Schönert *et al.*, 1998).

931

high K_m of AglA for sucrose (100 mM) might point in this direction.

Another reason might be that AUD1 and the *agl* genes, which are located in the unstable region of the *S. lividans* chromosome, were accidentally fused. This would mean that the inducer is different from the substrate of AglA. A further possibility would be that there was originally only one regulatory gene for the *agl* genes. This regulatory gene was duplicated twice during the evolution of the strain and by mutagenesis evolved a different function, for example to protect the chromosome in unstable mutants from further deletions by promoting amplification of the AUD1 sequence. When strain Jni27 with the inactivated regulatory gene was plated on agar plates with minimal medium and sucrose as carbon source there was no improvement in growth compared to TK64, AJ100 or Jni26, which makes the latter two possibilities unlikely. Alternatively, the *agl* genes may have lost their function by mutation in a similar manner to pseudogenes in higher eukaryotes.

ACKNOWLEDGEMENTS

J.-N. Volff was a recipient of a long-term fellowship from the European Molecular Biology Organization (EMBO). We thank Hilde Watzlawick and Gisela Kwiatkowski for their help in purification and biochemical characterization of AglA, Mervin Bibb for critical reading of the manuscript and Ralf Mattes for support and helpful discussions.

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- Received 25 October 1999; revised 31 December 1999; accepted 14 January 2000.