glkA Is Involved in Glucose Repression of Chitinase Production in Streptomyces lividans

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Chitinase production in *Streptomyces lividans* is induced by chitin and repressed in the presence of glucose. A mutant of *S. lividans* TK24, strain G015, which was defective in glucose repression of chitinase production, was obtained by screening colonies for zones of clearing on colloidal chitin agar plates containing 1.0% (wt/vol) glucose. The transcriptional analysis of *chiA* in G015 with *xylE*, which encodes catechol 2,3-dioxygenase, as a reporter gene showed that the transcription from the *chiA* promoter of *S. lividans* TK24 occurred regardless of the presence of glucose. G015 was resistant to 2-deoxyglucose (2-DOG) and did not utilize glucose as a sole carbon source. When a DNA fragment containing *glkA*, a gene for glucose kinase, of *Streptomyces coelicolor* A3(2) was introduced into strain G015 on a low-copy-number plasmid, the sensitivity to 2-DOG, the ability to utilize glucose, and the glucose repression of chitinase production were restored. These results indicate that *glkA* is involved in glucose repression of chitinase production in *S. lividans* TK24.

Streptomyces species are saprophytic soil bacteria and are well known as decomposers of chitin, which is hydrolyzed by chitinase (EC 3.2.1.14). Many chitinase genes of *Streptomyces* have been cloned and sequenced (3, 7, 18–20, 23–25, 29). Their expression is induced by chitin and repressed by glucose (7, 19, 25). A pair of 12-bp direct repeats present in the promoter regions of all the known chitinase genes from *Streptomyces* (20) are implicated in the regulation of chitinase gene expression (5, 22). This suggests that there exists a common regulatory mechanism for induction and repression of chitinase genes in *Streptomyces*. Recently, *reg1*, a regulatory gene for amylase production in *Streptomyces lividans*, has been reported to be involved in the regulation of chitinase production as well (21). However, the mechanism of glucose repression of chitinase gene expression in *Streptomyces* is still not understood.

Mutants of *Streptomyces coelicolor* A3(2) that are resistant to 2-deoxyglucose (2-DOG) (8) are defective in glucose kinase activity (27) and relieved of glucose repression of several catabolite pathways, including sugar utilization (8), glycerol utilization (27), agarase production (10), and amylase production (31). These data indicate that glucose kinase plays a central role in glucose repression in *S. coelicolor* A3(2). The *glkA* gene of *S. coelicolor* A3(2), encoding glucose kinase (2), restores 2-DOG sensitivity and glucose repression of *dagAp₄* transcription (2) in 2-DOG-resistant mutants. However, Ingram and Westpheling (13) reported that *glkA* is not required for glucose repression of the *chi63* promoter of *Streptomyces plicatus* (25) in a *ccrA1-glkA* double mutant of *S. coelicolor* A3(2).

We isolated mutants of *S. lividans* TK24 defective in glucose repression of chitinase production by using colloidal chitin agar plates containing glucose. The mutants made clear zones around their colonies, while their parental strain, TK24, did not. G015, one of the mutants defective in glucose repression of chitinase production, was resistant to 2-DOG and did not utilize glucose as a sole carbon source, suggesting that it contained the mutation in *glkA*. After studying this mutant, we report here that *glkA* is involved in glucose repression of chitinase production in *S. lividans*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. Plasmid pEMX151, a derivative of the low-copy-number plasmid pXE4 (11), was used for transcriptional analysis of the *chiA* promoter from *S. lividans*. The *Bam*HI-*Hin*dIII fragment of pXE4 located upstream of the *xylE* reporter gene was replaced with a fragment of about 1 kb containing the DNA sequence extending from ca. 500 bp upstream to ca. 500 bp downstream of the transcription initiation site of *chiA* (17). pXE4 was used to construct pGA01 and pGA02. A 1.2-kb fragment including the coding region and one of the two promoters of *glkA* of *S. coelicolor* A3(2) was generated by digesting pIJ2423 (2) with *Bgl*II, ligated to the *Bam*HI site of pXE4, and introduced into *Escherichia coli* XL1-Blue by transformation. The orientations of *glkA* were determined from the digestion patterns with *Hin*dIII. The *glkA* gene was inserted in the same orientation as the *xylE* reporter gene in pGA01 and in the opposite orientation in pGA02. Plasmid pGAH01 was constructed by ligating the 1.2-kb *Bgl*II fragment containing *glkA* into the *Bam*HI site of the high-copy-number vector pIJ486 and introduced into *S. lividans* TK24 by transformation.

Manipulation of DNA. Protoplast preparation and transformation of *S. lividans* was performed according to the method of Hopwood et al. (9). Plasmids were prepared by the alkaline lysis method (1, 26). All procedures involving recombinant DNA were carried out as described by Sambrook et al. (26).

Isolation of *S. lividans* mutants. Spores of *S. lividans* TK24 were irradiated with UV light (wavelength, 302 nm) to 0.4% survival and spread in the dark (9) on ISCG agar medium [0.15% (wt/vol) colloidal chitin, 5 mM MgSO₄, 15 mM (NH₄)₂SO₄, 25 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; pH 7.2), 0.5 mM NaH₂PO₄, 0.5 mM K₂HPO₄, 0.1% (vol/vol) trace element solution (0.1 g each of ZnSO₄·7H₂O, FeSO₄·7H₂O, MnCl₂·4H₂O, and NaCl per liter), 1.0% (wt/vol) glucose, and 1.5% (wt/vol) agar]. Colloidal chitin was prepared by the method of Lingappa and Lockwood (16). After 4 weeks of incubation at 30°C in the dark, colonies with clear zones around them were picked up and streaked on SFM agar medium (30).

2-DOG sensitivity and glucose utilization. To test resistance to 2-DOG, spores formed on SFM agar medium were spotted on NMMB agar medium (8) containing 10 mM arabinose with or without 100 mM 2-DOG. For G015 containing derivatives of pXE4, the agar media were supplemented with 50 μg of thiostrepton/ml. After incubation for 5 days at 30°C, resistance to 2-DOG was judged from the sizes of the colonies. The ability to utilize glucose was determined from growth on NMMB agar medium containing 1.0% (wt/vol) glucose or in SMM liquid medium without polyethylene glycol 1000 and casamino acids (28) but containing 1.0% (wt/vol) glucose as a sole carbon source. On the agar plates, the sizes of colonies of G015 and its derivatives were compared with those of TK24 and its derivatives, respectively. Agar medium supplemented with 50 μg of

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TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Genotype and characteristics	Reference
S. lividans 66		
TK24	SCP1⁻ SCP2⁻ᵃ	9
G015	SCP1⁻ SCP2⁻ *glkA*; a derivative of TK24 mutagenized by UV irradiation	This study
S. coelicolor A3(2)	*hisA1 uraA1 strA1 pgl* NF SCP2⁻ *glkΔ119*; a spontaneous mutant of J1508 (10)	6
J1668		
E. coli XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA4 thi relA1 lac* F′[*proAB⁺ lacI*�q *lacZ ΔM15*::Tn*10* (Tetʳ)]	26
Plasmids		
pXE4	Low-copy-number plasmid with *xylE* as a reporter gene	11
pEMX151	pXE4 containing a 1.0-kb insert with the *chiA* promoter	17
pGA01	pXE4 containing a 1.2-kb insert with *glkA* of *S. coelicolor* A3(2); the orientation of *glkA* is the same as that of *xylE*	This study
pGA02	pXE4 containing a 1.2-kb insert with *glkA* of *S. coelicolor* A3(2); the orientation of *glkA* is opposite to that of *xylE*	This study
pIJ486	High-copy-number plasmid	9
pGAH01	pIJ486 containing a 1.2-kb insert with *glkA* of *S. coelicolor* A3(2)	This study
pIJ2423	pIJ2925 (13a) containing a 1.2-kb insert with *glkA* of *S. coelicolor* A3(2)	2

ᵃ SCP2⁻, the strain can be introduced with pXE4.

thiostrepton/ml was used for G015 containing derivatives of pXE4. Germinated spores (9) were inoculated and grown at 30°C with shaking at 200 rpm. One-milliliter portions of the culture fluid were sampled periodically, and the mycelia were harvested by centrifugation. Growth was monitored by the protein content of the mycelia, measured as follows. The pellet was washed with distilled water (MiliQ), resuspended in MiliQ, and sonicated on ice. One-half volume of 3 N NaOH was added to the sonicated lysate, and after incubation for 30 min at 100°C, the lysate was centrifuged at 10,000 rpm (M150; SAKUMA) (ca. 7,000 × *g*) for 10 min at room temperature. The protein concentration of the supernatant was measured by the method of Bradford (4) with bovine plasma gamma globulin as the protein standard, and the amount of protein per milliliter of culture was calculated.

Chitinase assay. Twenty milliliters of Luria-Bertani (LB) liquid medium (26) was inoculated with germinated spores and incubated at 30°C for 48 h with shaking at 150 rpm. For strains containing pXE4 derivatives or pIJ486 derivatives, the medium was supplemented with 2 or 5 µg of thiostrepton/ml, respectively. The culture was divided into three aliquots. Mycelia in each aliquot were harvested by centrifugation and washed with 10 ml of YE medium (0.7 g of K₂HSO₄, 0.3 g of KH₂SO₄, 0.5 g of MgSO₄, 0.01 g of FeSO₄, 0.3 g of NH₄NO₃, and 1.0 g of yeast extract per liter). The mycelia were resuspended in an equal volume of YE medium, YE medium with 0.05% (wt/vol) colloidal chitin, or YE medium with 0.05% (wt/vol) colloidal chitin and 1.0% (wt/vol) glucose. The protein content of 1 ml of culture was measured as described above, and the inocula were adjusted to about 100 µg of protein per ml. The cultures were grown at 30°C with shaking. A 0.5-ml portion of the culture fluid was sampled periodically and stored at −80°C. The samples were thawed and centrifuged, and chitinase activity was measured in the culture supernatant as described previously (19), using the fluorogenic substrate 4-methylumbelliferyl-*N,N′*,-diacetyl chitobioside [4MU-(GlcNAc)₂] or 4-methylumbelliferyl-*N,N′,N″*-triacetyl chitotrioside [4MU-(GlcNAc)₃] (Sigma). One unit of chitinase activity was defined as described by Miyashita et al. (19). Chitinase activity was expressed in units per microgram of mycelial protein.

Catechol dioxygenase assay. The transformants TK24(pEMX151) and G015 (pEMX151) were cultured as described in "Chitinase assay" above with the exception that G015(pEMX151) was precultured in LB medium containing 2 µg of thiostrepton/ml for 72 h. The longer preculture period of G015(pEMX151) was due to the slower growth rate of G015(pEMX151) compared to that of TK24(pEMX151). The cell extracts were prepared, and catechol 2,3-dioxygenase activity was measured spectrophotometrically as described previously (12, 32).

Catechol 2,3-dioxygenase activity was expressed as the rate of increase in optical density at 375 nm per minute per milligram of protein.

Glucose kinase assay. Glucose kinase activity measurements were done as described by Angell et al. (2).

Southern hybridization. Total DNA was prepared as described by Hopwood et al. (9). Two micrograms of the DNA was digested with *Bcl*I, electrophoresed in 0.8% (wt/vol) agarose gel, and transferred to a Hybond N+ membrane (Amersham). The 1.2-kb *Bgl*II fragment containing *glkA* from *S. coelicolor* A3(2), which was prepared from pIJ2423, was used as a probe. Hybridization was performed with digoxigenin (Boehringer Mannheim) by following the manufacturer's instructions.

RESULTS

G015 is defective in glucose repression of chitinase production. To isolate mutants of *S. lividans* TK24 defective in glucose repression of chitinase production, UV-irradiated spores were spread on ISCG agar medium. After 3 weeks of incubation at 30°C, several colonies among approximately 5,000 had clear zones around them and one, G015, was resistant to 2-DOG (see below). The phenotype of G015 on ISCG medium was confirmed after single-colony purification. Chitinase production by G015 was then compared with that of TK24 in liquid medium. Chitinase production by G015 was induced by the addition of 0.05% (wt/vol) colloidal chitin regardless of the presence of glucose, whereas TK24 did not produce chitinase in the presence of glucose (Fig. 1A). These results indicated that G015 was defective in glucose repression of chitinase production.
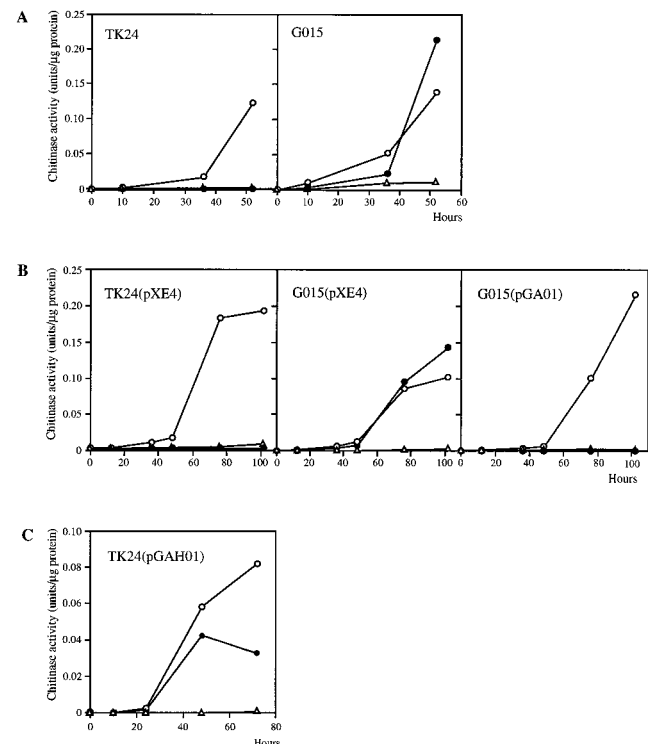

FIG. 1. Chitinase activity induced by colloidal chitin in the presence or absence of glucose in *S. lividans* TK24 and G015 (A); in TK24(pXE4), G015 (pXE4), and G015(pGA01) (B); and in TK24(pGAH01) (C). Chitinase activity was measured with 4MU-(GlcNAc)₂ for panel A and 4MU-(GlcNAc)₃ for panels B and C. At time zero, cultures grown in LB medium at 30°C for 48 h were divided into three aliquots, and the mycelia in each aliquot were suspended in YE medium (open triangles), YE medium plus 0.05% (wt/vol) colloidal chitin (open circles), or YE medium plus 0.05% (wt/vol) colloidal chitin plus 1.0% (wt/vol) glucose (solid circles).
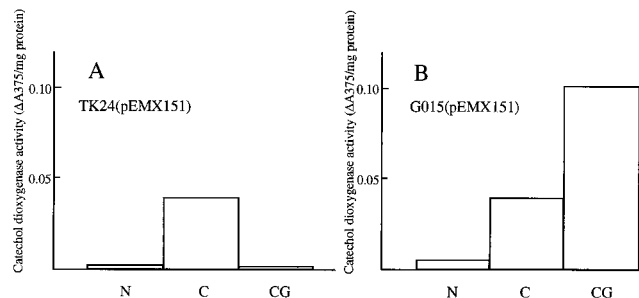
FIG. 2. Catechol dioxygenase activity of *S. lividans* TK24(pEMX151) (A) and G015(pEMX151) (B). Catechol dioxygenase activity was measured 45 h after subculturing (as described in the legend to Fig. 1) from LB medium supplemented with 2 μg of thiostrepton/ml. ΔA375 indicates the increase in optical density per minute at 375 nm. N, YE medium; C, YE medium plus 0.05% (wt/vol) colloidal chitin; CG, YE medium plus 0.05% (wt/vol) colloidal chitin plus 1.0% (wt/vol) glucose.

Glucose repression of *chiA* transcription is not observed in G015. To investigate the regulation of chitinase gene expression in G015, the levels of transcription from the *chiA* promoter in TK24 and G015 were compared by using *xylE*, which encodes catechol 2,3-dioxygenase, as a reporter gene. As shown in Fig. 2, in G015(pEMX151) transcription from the *chiA* promoter occurred in the presence of colloidal chitin regardless of the presence of glucose, whereas glucose strongly repressed the transcription of *chiA* in TK24(pEMX151). The level of expression of the *chiA* promoter in G015(pEMX151) in the presence of colloidal chitin and glucose was 2 to 3 times greater than that observed in the presence of colloidal chitin alone.

G015 was resistant to 2-DOG and did not utilize glucose. G015 grew on NMMB agar medium containing 10 mM arabinose regardless of the presence of 100 mM 2-DOG, whereas TK24 did not grow in the presence of 2-DOG. On NMMB agar medium containing 1.0% (wt/vol) glucose as a sole carbon source, G015 grew poorly compared to TK24. In SMM liquid medium containing 1.0% (wt/vol) glucose as a sole carbon source, G015 did not grow at all. The resistance to 2-DOG and the defect in glucose utilization suggested that the *glkA* gene was defective in G015 as shown in *S. coelicolor* A3(2) (2).

***glkA* restored 2-DOG sensitivity, glucose utilization, and glucose repression in G015.** Angell et al. (2) showed that integration of a wild-type *glkA* gene into the chromosome of a 2-DOG-resistant mutant of *S. coelicolor* A3(2) restored 2-DOG sensitivity, glucose utilization, and glucose repression of *dagAp_4*. To examine whether the *glkA* gene restored these three phenotypes in G015, the *glkA* gene of *S. coelicolor* A3(2) was introduced into G015. A low-copy-number plasmid, pGA01 (a derivative of pXE4), that contained *glkA* was constructed. G015 and TK24 were transformed with pGA01 or pXE4, and G015(pXE4), G015(pGA01), and TK24(pXE4) were obtained. The glucose kinase activity of G015(pXE4) was 1/10 that of TK24(pXE4), while G015(pGA01) had approximately the same level as TK24(pXE4) (Table 2). In contrast to G015(pXE4), G015(pGA01) grew well on NMMB agar medium containing 1.0% (wt/vol) glucose and showed no growth in the presence of 100 mM 2-DOG, like TK24(pXE4) (Table 2). These data indicate that G015 is a glucose kinase mutant of *S. lividans*. Moreover, G015(pGA01) did not produce chitinase when glucose was supplied together with colloidal chitin (Fig. 1B). When pGA02, with *glkA* in the opposite orientation, was introduced into G015, the same results were obtained (data

not shown). Thus, *glkA* restores glucose repression of chitinase production in G015, and it appears that *glkA* is involved in glucose repression of chitinase production in *S. lividans* TK24.

Introduction of *glkA* on a high-copy-number plasmid relieves glucose repression of chitinase production in *S. lividans*. Introduction of *glkA* on a high-copy-number plasmid relieves glucose repression in *S. coelicolor* A3(2) (1, 14). The same phenomenon might be expected in *S. lividans* if *glkA* is involved in glucose repression of chitinase production in that species. To assess this, the high-copy-number plasmid pGAH01, a derivative of pIJ486 with a 1.2-kb *Bgl*II fragment containing *glkA* of *S. coelicolor* A3(2), was constructed and introduced into *S. lividans* TK24 by protoplast transformation. Chitinase production in the presence of glucose was measured in liquid culture. Chitinase production in TK24(pGAH01) was relieved of glucose repression (Fig. 1C). This result strongly supports the involvement of *glkA* in glucose repression of chitinase production.

DISCUSSION

G015, a mutant of *S. lividans* TK24 that was defective in glucose repression of chitinase production was isolated. G015 showed 2-DOG resistance, no growth in medium that contained glucose as a sole carbon source, and low glucose kinase activity. The *glkA* gene of *S. coelicolor* A3(2) restored 2-DOG sensitivity, glucose utilization, and glucose kinase activity to approximately wild-type levels in G015 (Table 2). These data indicate that G015 possesses a mutated glucose kinase gene. The *glk* locus of *S. coelicolor* A3(2) is relatively unstable, with a high spontaneous mutation frequency. Spontaneous 2-DOG-resistant mutants show deletion in the *glk* locus (6). However, Southern analysis revealed that *glkA* was not deleted in G015, and the signal obtained with G015 was the same as those in *S. lividans* TK24 and *S. coelicolor* A3(2) (data not shown). Thus, the mutation of *glkA* in G015 is likely to be a UV-induced point mutation.

The fact that a *glk* mutant of *S. lividans* is relieved of glucose repression of chitinase production suggests the involvement of *glkA* in glucose repression in *S. lividans*. Consistent with this, the *glkA* gene of *S. coelicolor* A3(2) restored glucose repression of chitinase production in G015 when cloned on a low-copy-number plasmid (Fig. 1B). Moreover, introduction of *glkA* on a high-copy-number plasmid resulted in the relief of glucose repression of chitinase production in *S. lividans* TK24 (Fig. 1C). This is also consistent with the relief of glucose repression of *dagAp_4* transcription when *glkA* is overexpressed in *S. coelicolor* A3(2) M145 (1).

The expression level of the *chiA* promoter in G015

TABLE 2. Glucose kinase activity, glucose utilization,
and resistance to 2-DOG of transformants
of *S. lividans* TK24 and G015

Strain	Glucose kinase activity (U)	Glucose utilization[a]	Resistance to 2-DOG[b]
TK24(pXE4)	23	+	S
G015(pXE4)	2	−	R
G015(pGA01)	19	+	S

[a] Glucose utilization was estimated by growth on NMMB agar medium containing 1.0% (wt/vol) glucose and 50 μg of thiostrepton/ml. +, good growth; −, significantly poorer growth compared to that of TK24(pXE4).
[b] Resistance to 2-DOG was judged from growth on NMMB agar medium containing 10 mM arabinose, 100 mM 2-DOG, and 50 μg of thiostrepton/ml. S, sensitive to 2-DOG; R, resistant to 2-DOG.

(pEMX151) in the presence of colloidal chitin and glucose was about twice that observed on colloidal chitin alone (Fig. 2B). A similar stimulatory effect from loss of *glkA* was observed for *dagAp*$_4$ transcription in *S. coelicolor* (1). Hodgson (8) showed that most of the *glk* mutants of *S. coelicolor* A3(2) which do not utilize glucose as a sole carbon source can transport glucose. Considering that G015 is a *glk* mutant which cannot utilize glucose, although it is a derivative of *S. lividans* TK24, it is conceivable that the process of glucose transport or glucose itself stimulates the expression of *chiA* in G015.

Ingram and Westpheling (13) reported that *glkA* of *S. coelicolor* A3(2) is not required for glucose repression of the *chi63* promoter of *S. plicatus* (25), based on a *ccrA1-glkA* double mutant of *S. coelicolor* A3(2). This is in contrast to the conclusion obtained in this study of *S. lividans*. To confirm the involvement of the *glkA* gene in glucose repression of chitinase production in *S. coelicolor*, we examined the native chitinase production of a *glk* mutant of *S. coelicolor* A3(2) in liquid medium that contained colloidal chitin with or without glucose. Chitinase production in J1668, a *glkA* deletion mutant of J1508 (10), was repressed in the presence of glucose in liquid media (data not shown), consistent with the finding of Ingram and Westpheling (13). Thus, it seems that the mechanism of glucose repression of chitinase production in *S. coelicolor* A3(2) is different from that in *S. lividans*, which is surprising given that they are very closely related species (15) and possess nearly identical chitinase genes (unpublished data).

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