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Transformable mutants of a biopesticide strain *Streptomyces griseoviridis* K61

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SUMMARY

The aim of this work was to isolate transformable mutants of *Streptomyces griseoviridis* K61 without affecting the secondary metabolism of this strain. *S. griseoviridis* K61 produces an antifungal aromatic heptaene polyene antibiotic, and is used as a biological control agent. In protoplast transformation experiments using plasmid pIJ702 DNA, the few spontaneous transformants were phenotypically bald and their secondary metabolism was pleiotropically affected. By mutagenizing K61 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) a highly transformable variant K61-42 was obtained. Protoplasts of *S. griseoviridis* K61-42 could be transformed by several model plasmids producing 10^4–10^5 transformants/μg plasmid DNA. The polyene synthesis of K61-42 was normal, making this strain a useful tool in genetic studies on the mechanism of biopesticide action.

INTRODUCTION

The role of antibiosis in the biocontrol is currently a much studied but still controversial topic [6]. *Streptomyces griseoviridis* strain K61, isolated from Finnish *Sphagnum* peat, is used as a biological fungicide effective against *Fusarium*, *Alternaria*, and other phytopathogenic fungi [18,19]. K61 produces an aromatic heptaene polyene antibiotic, which was of the candicidin type according to HPLC analysis [16]. The production of this antibiotic has been detected in steam-sterilized peat after inoculation with *S. griseoviridis* K61 (unpublished observation). One of the mechanisms of action of K61 has been suggested to be antibiosis. However, it is known that actinomycetes have numerous activities in the soil, such as degradation of organic material or interaction with the growth of other micro-organisms, which might directly (hyperparasitism, toxins) or indirectly (competition, siderophore production) cause the fungicidic activity observed. The research reported here is a part of a study on the actual antifungal mechanism of K61. One of our aims has been to develop and apply genetic techniques for this purpose, a method for protoplast transformation being the first step in this direction.

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Our first attempts to transform K61 protoplasts with antibiotic resistant plasmids from other *Streptomyces* species were unsuccessful, probably because of the presence of a potent restriction–modification system (R-M) in K61 (unpublished results). R-M systems are common among *Streptomyces* [1,10,13], and they can greatly reduce the efficiency of transformation or inhibit it totally [10]. On the other hand, inactivation of R-M systems can make *Streptomyces* strains highly transformable [14].

Transformation experiments were continued in order to obtain transformable K61 variants, either spontaneously or through mutagenization. The spontaneous K61 transformants detected were nonsporulating, and their antifungal activity was apparently also affected. A highly transformable K61 variant with intact polyene synthesis was obtained by chemical mutagenization. The properties of both these classes of transformable K61 mutants are described in this report.

MATERIALS AND METHODS

Microorganisms, plasmids and media

The bacteria and plasmids used in this study are listed in Table 1. *S. griseoviridis* K61 was obtained from Dr. R. Tahvonen (Department of Plant protection, The Agricultural Research Center, Jokioinen). The *S. lividans* strains harboring the model plasmids were kindly donated by Professor David A. Hopwood (John Innes Institute, Nor-

TABLE 1

Streptomyces strains and plasmids used in this study

Strain	Relevant plasmid (kbp)[a]	Plasmid-associated phenotype	Source or Ref.
S. griseoviridis			
K61 (wild-type)			R. Tahvonen [19]
Transformable mutants of K61			
K61 Bald			This study
K61-4	pIJ702 (5.6)	Mel^+, Tsr^r	This study
K61-42			This study
S. lividans			
Hosts of the model plasmids			
TK24	pIJ702 (5.6)	Mel^+, Tsr^r	[11]
	pIJ680 (5.3)	Aph^r, Tsr^r	[9]
	pIJ385 (6.0)	Aph^r, Tsr^r	[9]
JT46	pIJ699 (5.8)	Tsr^r	[12]

[a] Plasmid size in kilobase pairs; Mel^+ = melanin production; Tsr^r = thiostrepton resistance; Aph^r = neomycin resistance.

wich, UK). A wild strain of a plant pathogenic mold *Alternaria brassicola* was used as a bioassay organism for the detection of antifungal activity of *Streptomyces* strains. Potato Dextrose Agar (PDA, Difco Laboratories, MI, USA) was routinely used for cultivation of both *S. griseoviridis* strains and *A. brassicola*. The media for protoplasting and regeneration were Yeast Extract-Malt Extract broth (YEME), and R2YE agar, respectively, as described by Hopwood et al. [9]. Minimal medium salts agar (MM) [9] supplemented (1% w/v) with different carbon sources was used for the phenotypic analysis and tests for fungicidic activity with certain mutant strains isolated during the study.

Isolation of DNA

For DNA preparation the strains were grown in YEME containing 0.5% (w/v) glycine and 25 μg per ml thiostrepton (Sigma Chemical Co., St. Louis, MO, USA). The cells were grown in baffled flasks at 32 °C for 36 h in a rotary shaker (225 rpm). Plasmid and chromosomal DNA were isolated from the mycelium using the standard techniques described by Hopwood et al. [9]. In some experiments plasmids were also isolated using a Qiagen^R kit (Diagen GmbH, Germany) according to the instructions of the manufacturer, with slight modifications, the most relevant of which was the use of 1 mg of lysozyme (Sigma Chemical Company, St. Louis, USA) per ml of the incubation buffer P1.

The restriction enzymes used were purchased from Boehringer Mannheim Biochemicals, Germany. Digestions were done according to the instructions of the manufacturer. DNA was electrophoresed in 0.8% agarose in TBE-buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3).

Protoplast transformation

For protoplasting the cells were grown as for DNA isolation, with the exception that the growth medium did not contain any antibiotics. The protoplasting and transformation procedures were essentially those described by Hopwood et al. [9]. The protoplasts were generated with lysozyme (1 mg/ml) in P-buffer for 30–45 min at 30 °C. Most of the nonprotoplasted mycelial material was removed by filtration through a sterile cotton plus, before plating of the protoplasts on R2YE regeneration plates. As a result, <5% of colonies were derived from osmotically stable, nonprotoplasted units.

Transformations were performed using 100 μl of protoplast suspension (about 10^8 protoplasts), 10 μl plasmid DNA in 10 mM Tris-HCl/1 mM EDTA (TE) buffer (pH 8.0), and 200 μl of 25% polyethylene glycol (PEG) 1450 (Koch-Light Laboratories) in P-buffer. PEG treatment was terminated after 2 min by addition of 700 μl of P-buffer. Samples (100 μl) were plated on R2YE plates. After an incubation for 24 h at 30 °C, the regeneration plates were overlayed with 1 ml of sterile water containing thiostrepton (500 μg/ml), and dried in a laminar flow cabinet. After 5 days' incubation at 30 °C the plates were scored for transformants.

Mutagenization

Spores of *S. griseoviridis* K61 were suspended in TM-buffer (0.05 M Tris, 0.05 M maleic acid, pH 9.0) containing (3 mg per ml) *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

(MNNG, Sigma Chemical Co.). The treatment was at 30 °C for 1 h. The spores were then centrifuged, suspended in TM-buffer, and plated on R2YE plates with and without streptomycin (Orion Oy, Helsinki, Finland, 100 μg/ml). The mutant frequency was calculated by dividing the number of streptomycin-resistant colonies by the total number of viable colony-forming units per ml. A sample having about 1% survivors, when compared to untreated control suspension, and with a frequency of streptomycin-resistant mutants of about 10^{-5}, was subjected to two protoplast formation and regeneration cycles. Subsequently a spore suspension harvested from a regeneration plate was grown, protoplasted and transformed with pIJ702 plasmid DNA isolated from *S. lividans*.

Plasmid curing

Ethidium bromide (EtBr), as a DNA intercalating agent, was used to eliminate plasmids from *S. griseoviridis* K61 transformants [4]. Spores from freshly prepared YEME plates were serially diluted in water and spread on YEME agar plates containing 10 μmol EtBr per liter. After an incubation of 4 days at 32 °C the EtBr plates were replica plated on YEME-agar plates with and without thiostrepton. Thiostrepton-sensitive colonies were picked up, purified, and analyzed for plasmid content.

Antifungal activity

For the bioassay of heptaene production the *S. griseoviridis* variants were grown for 120 h at 32 °C at a high density on either PDA, YEME agar or MM agar. Agar plugs (diameter 8 mm, height 5 mm) from confluent plates were then placed on PDA plates previously seeded with conidial suspension of *Alternaria brassicola*. After 48 h incubation at 32 °C the presence or absence of inhibition zones around the plugs was recorded.

Analysis of secondary metabolites

Heptaene production in either YEME of MM agar cultures was screened by ultraviolet spectrometry. Several (four to six) agar plugs were homogenized in acetonitrile/water (60:40) and the mixture was centrifuged at 15000 rpm in an Eppendorf centrifuge for 10 min. The ultraviolet spectrum (310–450 nm) of the supernatant was measured by a double beam ultraviolet spectrometer (Hitachi, Tokyo, Japan). The typical ultraviolet spectrum of heptaenes was indicated by the presence of three absorption maxima at about 360, 380 and 400 nm.

The qualitative analysis of the heptaene antibiotic was confirmed by HPLC [16,17]. Briefly, individual components of the heptaene complex were separated on ODS Hypersil 5 phase (column size 125 × 4.6 mm) purchased from Polymerlabs (Copenhagen, Denmark) and monitored at 380 nm with HP 1040 photo diode array detector (Hewlett Packard, Waldbronn, Germany). The isocratic elution was performed at 1 ml/min (Hewlett Packard 1050 pump) by using 0.05 ammonium acetate/acetonitrile (62:38) as the mobile phase.

RESULTS

Spontaneous transformable mutants of S. griseoviridis K61

Although the protoplasting conditions applied were optimized for *S. griseoviridis* K61 as judged by both the formation of protoplasts (>99% of the cells as protoplasts in microscopic examination) and their regeneration, no

TABLE 2

Transformant frequencies of *Streptomyces griseoviridis* K61 and its derivatives with plasmid DNA isolated from different hosts

Original host	Plasmid	*S. griseoviridis* transformants/μg of plasmid DNA		
		Recipient strain		
		K61	K61 Bald[a]	K61-42[b]
S. lividans				
TK24	pIJ702	ND	5×10^2	1.5×10^5
	pIJ385	–	–	4.2×10^4
	pIJ680	–	–	5.5×10^4
JT46	pIJ699	ND	3×10^4	5.8×10^4
S. griseoviridis				
K61 Bald	pIJ702	ND	–	–
K61-42	pIJ702	ND	–	4.7×10^5

[a] = Representative experiment; [b] = Mean of three experiments; ND = No transformants detected; – = Not tested.

transformants were obtained in the initial experiments with plasmids pIJ702, pIJ680 or pIJ385. Finally a few transformants were obtained with pIJ702, their frequency being $< 10/\mu g$ DNA. These transformants were thiostrepton resistant and produced melanin, but did not sporulate on either R2YE or PDA plates. They were found to contain plasmids with the size and restriction patterns similar to pIJ702. In preliminary experiments it appeared that the antifungal properties (in vitro) of these transformants were also affected.

After the initial detection of nonsporulating transformants, the K61 plates were screened for the presence of nonsporulating colonies. In some cultures they were relatively easy to find, segregating at a frequency of about 10^{-3}. The colonies were hard, flat and nonpigmented. Near vicinity of a wild-type growth did not restore the sporulation, thus excluding the possibility of a diffusible sporulation inducing agent absent from the nonsporulating variants. One isolate, K61 Bld, was arbitrarily chosen for further studies. Protoplasts of this strain were transformable by plasmids pIJ702 and pIJ699 (isolated from *S. lividans*), the transformant frequency with the latter plasmid being nearly 100-fold higher than with pIJ702 (Table 2). It can be seen that wild-type K61 could not be transformed with plasmid DNA isolated from K61 Bld transformants.

The pleiotropic nature of nonsporulating K61 mutants

To test whether the phenotype of the spontaneous nonsporulating K61 mutants was dependent on the carbon source, as is the case with some other bald variants of *Streptomyces* (see Discussion), K61 Bald was plated on MM agar supplemented either with fructose, galactose, glucose, glycerol, maltose or mannose. The confluent cultures were subsequently checked both for the sporulation and for fungicidic activity and polyene synthesis, as described in Materials and Methods. Fructose, glycerol, and in particular maltose effectively restored both the sporulation and the synthesis of antifungal polyene in K61 Bald, while with the other sugars all of these wild-type characteristics were suppressed. The carbon source did not have any observable effect on the phenotype or secondary metabolism of the wild-type K61. The characteristic ultraviolet spectra of heptaene, measured from culture extracts of K61 and K61 Bald grown on either glucose or maltose are shown in Fig. 1.

Transformable mutants obtained after MNNG treatment

Transformation of MNNG-mutagenized K61 protoplasts with pIJ702 DNA produced about 10^2 thiostrepton-resistant colonies/μg DNA. Most of them failed to express the melanin synthesis coded by pIJ702. Eight isolates were chosen for further studies and their plasmid DNA was

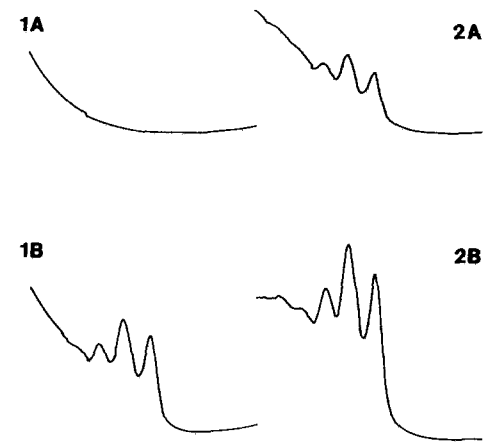

Fig. 1. The ultraviolet spectra (absorption maxima at ca. 360, 380 and 400 nm) of heptaene polyene produced by *S. griseoviridis* K61 Bald (1) and the wild parent strain K61 (2) grown on MM-agar, supplemented with glucose (A) or maltose (B).

isolated. Gel electrophoresis revealed plasmids with sizes and restriction patterns identical to pIJ702. This plasmid DNA failed to transform wild-type *S. griseoviridis*.

Plasmid pIJ702 was eliminated by EtBr from one of the transformants, designated K61-4, which had a diminished ability to produce melanin, possibly because of some mutation in the plasmid. Five cured variants, designated K61-41 to K61-45, with general appearance and polyene production corresponding to those of the wild-type, were chosen for further studies. These isolates, when transformed with pIJ702 obtained from either *S. griseoviridis* K61 strains or *S. lividans* TK24, produced transformants at a frequency of 10^4–10^5 per μg of DNA, depending on the experiment. Isolate K61-42 was chosen as the final recipient strain. K61-42 has a general wild-type appearance, although the intensity of sporulation on plates with high cell densities, while clearly higher than that of K61 Bald, appears reduced compared to the wild-type. The expression of the melanin production gene present in pIJ702 is also good in this background. Several other plasmids isolated from *S. lividans* could transform K61-42 with high efficiency (Table 2).

The polyene production of K61-42

In bioassays K61-42 was as active against *A. brassicola* as the original K61 (data not shown). According to ultraviolet spectroscopy and HPLC analysis, the ability of K61-42 to synthesize polyene was equal to that of the wild-type (Fig. 2). This indicates that neither the MNNG-treatment nor the plasmid curing by EtBr had affected the heptaene-producing properties of K61-42. In contrast to the nonsporulating mutants there was no indication that

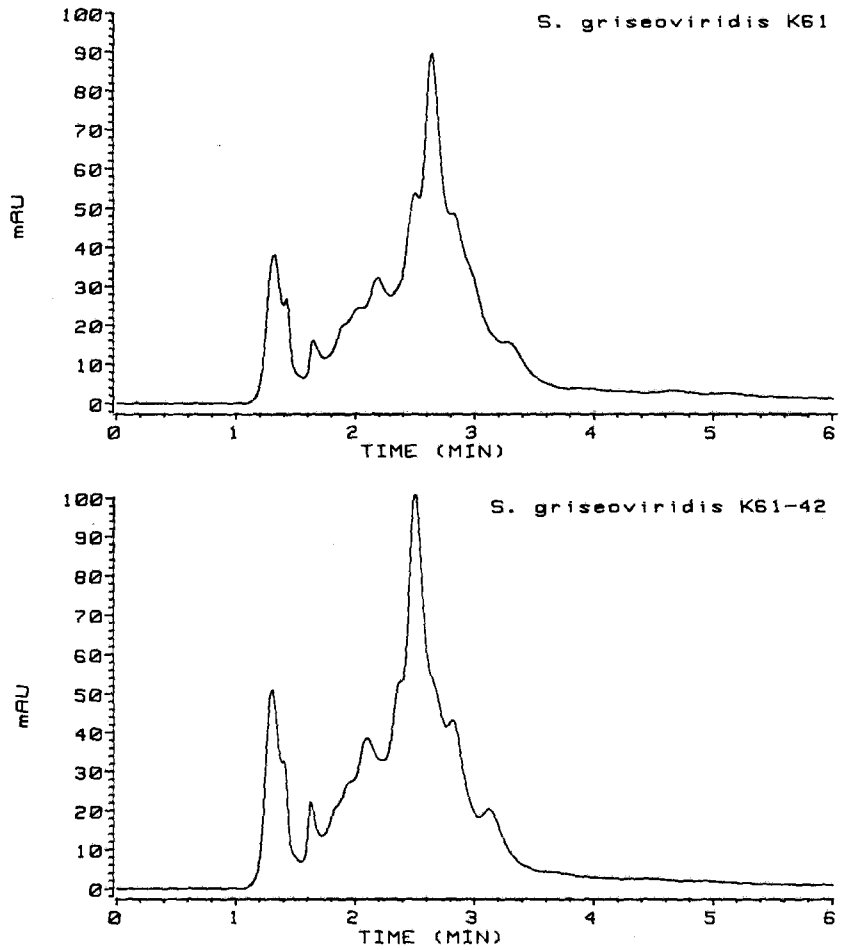

Fig. 2. HPLC-chromatograms of the heptaene components produced by *S. griseoviridis* K61 (upper panel) and its transformable MNNG-mutant K61-42 (lower panel).

the carbon source had affected the secondary metabolism of this strain.

DISCUSSION

Two classes of transformable *S. griseoviridis* K61 strains were obtained in this study. The spontaneous transformable mutant K61 Bald was nonsporulating, and its secondary metabolism was also apparently affected. In contrast, except for the high efficiency of transformation, the mutagenized strain K61-42 was, in most other observable respects, like the wild-type.

Nonsporulating or bald (Bld) mutants are well known in other species of *Streptomyces*. Typically these mutants are pleiotropic, their secondary metabolism being also defective in comparison to that of the wild strains. Seven different *bld* loci have been characterized in *S. coelicolor* [3,7,8,15]. Four of these loci (*bldA*, *D*, *G*, and *H*) are conditional, sporulation and, with *bldH*, also antibiotic production depending on the carbon source. The *bldA* gene has been cloned and found to encode a species of leucine tRNA for the rare codon UUA. A hypothesis has been formulated according to which the *bldA* gene has a key function in the pleiotropic genetic control system by enabling the translation of UUA containing mRNA. The protein product of this mRNA in turn is involved in the general transcriptional activation of several genes controlling both sporulation and secondary metabolism [7].

In addition to sporulation and antibiotic synthesis, some bald mutants of *S. griseus* also show an increased transformability by plasmid DNA in comparison to the wild parent strain [5]. This might indicate an inactivation of some restriction-modification system(s).

It is apparent that the bald mutants described here resemble in many respects those detected previously in other *Streptomyces* species. In particular, the carbon source dependent antibiotic production appears analogous to the situation with the *bldH* mutation in *S. coelicolor*. At

present, however, there are no indications of the molecular nature of the mutation in K61 Bld. Because of the defective secondary metabolism, and because even the enhanced transformation rate of this strain is too low for efficient gene cloning, the practical usefulness of K61 Bld is, at present, limited.

The transformation frequencies (10^4–10^5 transformants/μg of plasmid DNA) observed with the MNNG-induced mutant *S. griseoviridis* K61-42 are adequate for most cloning strategies. The fact that there was no practical difference in transformant yields with plasmid DNA isolated either from K61-42 hosts or from *S. lividans* indicate reduced restriction barriers between K61-42 and other species of *Streptomyces*.

Since the strain K61-42 appears to be stable and produces high amounts of candicidin when cultivated under the proper conditions, it should be an excellent strain for studying the genetics of the antagonistic mechanism of K61. Further isolation of mutants defective in different aspects of the antifungal activity and sporulation, combined with the transformability of the strain, should make the isolation and characterization of the corresponding wild-type genes possible.

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