Optimization of *Streptomyces aureofaciens* transformation and disruption of the *hrdA* gene encoding a homologue of the principal σ factor

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(Received 11 February 1993; revised 19 April 1993; accepted 10 May 1993)

Conditions for the preparation of protoplasts from *Streptomyces aureofaciens* and transformation with several replicative and integrative plasmids were optimized. Of all the replicative plasmids used, carrying various *Streptomyces* replication origins, only plasmid pGM9 produced transformants, with a transformation efficiency of 2×10^3 ($\mu\text{g DNA}$)⁻¹. When plasmid DNA isolated from *S. aureofaciens* was used, the transformation efficiency increased to 10^5 transformants ($\mu\text{g DNA}$)⁻¹. Transformation of protoplasts with integrative plasmids containing part of the *hrdA* gene gave rise to transformants with an efficiency of approximately 30 transformants ($\mu\text{g DNA}$)⁻¹. Integration frequency was strongly reduced when plasmid DNA was modified by *dam* or *dcm* methylase. By integrative transformation, via a double crossover, a stable null mutant of the *hrdA* gene was prepared. This mutation appeared to have no obvious effect on growth, morphology, differentiation and production of chlortetracycline.

Introduction

In *Streptomyces aureofaciens* CCM3239 (ATCC 10762), four genes (*hrdA*, *hrdB*, *hrdD* and *hrdE*) encoding homologues of the principal σ factor of RNA polymerase were identified (Kormanec *et al.*, 1992).

Several methods have been developed for disrupting chromosomal copies of cloned *Streptomyces* genes. Phage Φ C 31 with a deleted *attP* site was used for disruption via homologous recombination in *S. coelicolor* A3(2) (Chater & Bruton, 1983). However, the use of the Φ C 31-based system is restricted to the phage host range (Chater, 1986). Another system based on a temperature-sensitive replicon was developed in *S. ghanaensis* (Muth *et al.*, 1989). Recently, non-replicative vectors were used for the disruption in several *Streptomyces* strains (Hillemann *et al.*, 1991).

To disrupt the *S. aureofaciens* *hrd* genes, first it was necessary to develop a plasmid DNA transformation system for the organism. The basic techniques of protoplast formation and regeneration, required for DNA transformation, initially developed by Okanishi *et al.* (1974), have been modified and adapted for a range of *Streptomyces* species (Shirahama *et al.*, 1981; Baltz &

Matsushima, 1981; Ogawa *et al.*, 1983; Hopwood *et al.*, 1985). However, we could not prepare protoplasts of *S. aureofaciens* CCM3239 by any of the techniques mentioned. Also, the recently published methods for preparation of protoplasts from industrial *S. aureofaciens* strains (Isaeva & Voeikova, 1990; Muchová *et al.*, 1991) were unsuccessful. Therefore, we had to optimize conditions for preparation and transformation of protoplasts of *S. aureofaciens* CCM3239.

Disruption of the *hrdA* gene, using a non-replicative plasmid containing a Thio^R marker, was performed by homologous recombination.

This paper also describes the phenotype of this disruption and compares the results with those obtained recently by disruption of homologous *hrd* genes in *S. coelicolor* A3(2) (Buttner & Lewis, 1992).

Methods

Bacterial strains and plasmids. *S. aureofaciens* CCM3239 wild-type (ATCC 10762) was from the Czechoslovak Collection of Micro-organisms (CCM), Brno, Czechoslovakia. Strain *S. lividans* TK24, and plasmids pIJ702 and pIJ486 (Hopwood *et al.*, 1985; Ward *et al.*, 1986) were gifts from Professor D. A. Hopwood, John Innes Institute, Norwich, UK. Plasmid pARC1 (Horinouchi & Beppu, 1985) was a gift from S. Horinouchi, University of Tokyo, Japan. Plasmid pFD666 (Denis & Brzezinski, 1992) was a gift from R. Brzezinski, Sherbrooke University, Canada, and plasmid pGM9 (Muth *et al.*, 1989) was a gift from W. Wohlleben, University Bielefeld, Germany.

Escherichia coli SURE (Stratagene) was used in cloning experiments. *E. coli* JM110 (Stratagene) was used for preparation of *dam*⁻ and *dcm*⁻

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Abbreviation: Thio^R, thiostrepton resistance.

plasmid DNA. *E. coli* plasmids pBluescript II SK and pUC19 were from Stratagene.

Media and cultivation conditions. About 10^9 spores of *S. aureofaciens* CCM3239 were inoculated into 100 ml TSB liquid medium (Hopwood *et al.*, 1985) containing 1% (w/v) maltose and, unless otherwise stated, 0.5% glycine in a 500 ml baffled flask. The culture was grown in a rotary shaker at 28 °C. *S. lividans* TK24 was grown as described by Hopwood *et al.* (1985). Solid media used for regeneration of protoplasts were R2YE (Hopwood *et al.*, 1985), R5, R6 and MR6 (Illing *et al.*, 1989), R4 (Ogawa *et al.*, 1983), M4 (Isaeva & Voeikova, 1990) and R3 (Shirahama *et al.*, 1981). Before use, they were dried to approximately 80% of their original weight. *E. coli* was grown in L-broth as described by Sambrook *et al.* (1989).

Preparation and transformation of protoplasts. The following protocol is a summary of our efforts to optimize the procedure. Cells of *S. aureofaciens* CCM3239 were cultured to stationary phase (30–34 h) as described above. Mycelium was recovered by 10 min centrifugation at 5000 g and washed twice with ice-cold 10.3% (w/v) sucrose. Two grams (wet wt) of the mycelium was suspended in 5 ml lytic medium P3 (Shirahama *et al.*, 1981) with 1 mg of lysozyme ml⁻¹. The mixture was incubated for 40–60 min at 28 °C with occasional trituration by pipette. Protoplast formation was monitored microscopically. After completion of lysis, an equal volume of PWP medium (Shirahama *et al.*, 1981) containing 0.1% bovine serum albumin (BSA) was added and the rest of the non-protoplasted mycelium was removed by 5 min centrifugation at 1000 g. Protoplasts in the supernatant were recovered by 20 min centrifugation at 2000 g and washed twice with PWP medium containing 0.1% BSA. The washed protoplasts were resuspended to approximately 10^9 protoplasts ml⁻¹ in PWP medium with BSA, and 0.1 ml aliquots were slowly frozen at –80 °C by inserting the tubes in an ice filled beaker before freezing.

For transformation, approximately 10^8 protoplasts were quickly thawed at 37 °C and spun in an Eppendorf centrifuge for 10 s. The pellet was gently suspended in 50 µl PWP medium containing 0.1% BSA, then 5 µl DNA in TE was added and the mixture was transferred to 200 µl T-buffer (Hopwood *et al.*, 1985). After mixing by pipetting up and down several times, the protoplast suspension was spread on two dried M4 plates. The regeneration plates were incubated at 28 °C for about 20 h, then overlaid with 1 ml water containing 4 µl 50 mg thio-strepton ml⁻¹ (Calbiochem) in dimethyl sulphoxide. Thio^R transformants were scored after 4–7 d.

DNA isolation and Southern blot hybridization. *S. lividans* TK24 plasmid DNA was isolated as described by Hopwood *et al.* (1985) and from *E. coli* as in Sambrook *et al.* (1989). Plasmid DNA from *S. aureofaciens* was isolated as described by Matsushima & Baltz (1985).

Chromosomal DNA from wild-type and disrupted *S. aureofaciens* CCM3239 strains was isolated according to Hopwood *et al.* (1985). Chromosomal DNA (1 µg) was digested with an appropriate restriction endonuclease, separated by electrophoresis in a 0.8% agarose gel in TBE, and transferred on Hybond N (Amersham) as described by Sambrook *et al.* (1989). The membrane was then hybridized with a random primed [α -³²P]dCTP-labelled DNA probe at 45 °C in 50% (v/v) formamide as described by Sambrook *et al.* (1989).

Recombinant DNA techniques. All DNA manipulations in *E. coli* were performed as described by Sambrook *et al.* (1989). DNA fragments were isolated from agarose gels with GeneClean (BIO101, LaJolla). Integrative vector pFK21 was prepared by inserting a 1.05 kb *Bcl*I fragment of plasmid pIJ486 containing the gene conferring resistance to thiostrepton, into the *Bam*HI site of pUC19. Plasmid pFK41 was constructed by cloning the 1050 bp *Sac*I–*Xba*I fragment bearing Thio^R from pFK21 into *Sac*I- and *Xba*I-cut pBluescript II SK.

Plasmid pRPO4-27 contains the 3.5 kb *Hind*III–*Pst*I fragment containing the full-length *hrdA* gene in pUC19 (Kormanec *et al.*,

1992). pHRDA2, used for disruption of *hrdA*, was prepared by insertion of an 800 bp *Bam*HI fragment, internal to *hrdA*, into the *Bam*HI site of pFK41. Plasmid pRPO4-28, used for gene replacement of *hrdA*, was constructed as follows. pFK21 was digested with *Eco*RI, filled with the Klenow fragment of DNA polymerase I (PolIk) and dNTPs, followed by *Sph*I digestion. The 1050 bp *Sph*I–*Eco*RI (blunt) fragment, containing the Thio^R marker, was ligated to *Not*I- (blunted with PolIk) and *Sph*I-cut pRPO4-27.

Antimicrobial activity. Bennet agar medium (Horinouchi & Beppu, 1985) with patches of wild-type and *hrdA*-disrupted *S. aureofaciens* CCM3239 strains in various differentiation stages was overlaid with 3 ml soft nutrient agar (Hopwood *et al.*, 1985) containing 0.1 ml overnight culture of *Bacillus amyloliquefaciens* in L-broth and allowed to solidify. The plate was examined for zones of inhibition after incubation for 16 h at 37 °C.

Results and Discussion

Optimization of protoplast formation and regeneration

To find conditions for preparation and regeneration of *S. aureofaciens* CCM3239 protoplasts, various combinations of cultivation and regeneration media (see Methods) were tested. In these preliminary experiments, the protoplasts were prepared by a standard procedure (Hopwood *et al.*, 1985). The best results were obtained when protoplasts of cells cultured in TSB medium with 1% maltose to stationary phase (30–34 h) were regenerated on M4 medium. The addition of glycine (0.5%) to TSB medium increased the yield of protoplasts (by about 80%). The regeneration efficiency of the protoplasts was almost unaffected (5%). A higher glycine concentration (1%) resulted in a better yield of protoplasts, but their regeneration efficiency was greatly reduced (0.6%).

Protoplasts prepared by this procedure were unstable and their viability rapidly decreased when stored at –80 °C. Therefore, using these cultivation conditions, the procedure for preparation of protoplasts was optimized with respect to the lytic and storage media. A combination of P3 medium for lysis of cells and PWP medium for storage of protoplasts was found to be satisfactory. The *S. aureofaciens* protoplasts displayed greater stability and an enhanced regeneration efficiency (about 30%) when 0.1% BSA was introduced into both media, as shown for *S. clavuligerus* (Illing *et al.*, 1989). Such protoplasts could be stored for at least 3 months at –80 °C without a significant decrease in viability. Using the optimum procedure detailed in Methods, the average yield of viable protoplasts was 2×10^8 (g wet mycelium)⁻¹, and their regeneration efficiency was 21%.

Transformation of protoplasts with replicative plasmids

About 10^8 *S. aureofaciens* CCM3239 protoplasts were transformed with several plasmid DNAs (pIJ486, pIJ702, pARC1, pFD666 and pGM9) in the range of 10–5000 ng. All the plasmids were isolated from *S. lividans* TK24.

Table 1. Transformation of *S. aureofaciens* with replicative and non-replicative vectors

Data are the means of three experiments. pGM9 (100 ng), pHRDA2 (1 µg) and pRPO4-28 (1 µg) were used to transform approximately 10^8 protoplasts from three independent preparations.

Plasmid	Transformation frequency [transformants (µg DNA) ⁻¹]
pGM9 (isolated from <i>S. lividans</i>)	$2.0 \times 10^3 \pm 0.5 \times 10^3$
pGM9 (isolated from <i>S. aureofaciens</i>)	$1.1 \times 10^5 \pm 0.2 \times 10^5$
pHRDA2 (isolated from <i>E. coli</i> SURE)	3.3 ± 1.5
pHRDA2 (isolated from <i>E. coli</i> JM110)	$3.1 \times 10^4 \pm 0.8 \times 10^4$
pRPO4-28 (isolated from <i>E. coli</i> JM110)	9.3 ± 2.5

All the transformations failed to produce transformants irrespective of the plasmid concentration used, except with pGM9. Each transformation experiment was performed three times with various preparations of protoplasts. Transformation frequency for pGM9 (Table 1) slightly decreased (by about 10%) when 1 µg DNA was used. Using general methods for isolation of plasmid DNA (Hopwood *et al.*, 1985), we could not detect any plasmid in the analysed Thio^R transformants. Only when an increased concentration of EDTA (100 mM) was used in the lytic solution, were weak bands of plasmid DNA detected by agarose gel electrophoresis. It seems likely that plasmid DNA was degraded by a strong nuclease activity present and still active in lytic solution. The successful isolation of pGM9 DNA, according to Matsushima & Baltz (1985), where all the initial steps of isolation are done at 0 °C, is in favour of this explanation.

Restriction analysis of the plasmid DNA isolated from the resulting Thio^R colonies showed that the DNA had the same structure as the original pGM9 isolated from *S. lividans*. Transformation frequency increased about 50-fold with a plasmid isolated from *S. aureofaciens* (Table 1).

A possible explanation for the failure to produce transformants by the other plasmids tested could be incompatibility between the transforming plasmid and an endogenous plasmid. To check this possibility, an attempt was made to examine the presence of such plasmid(s) by several methods (Hopwood *et al.*, 1985; Matsushima & Baltz, 1985), but no plasmid was detected. It is possible that only plasmid pGM9 has an origin of replication which is functional in *S. aureofaciens*.

Disruption of the *hrdA* gene by transformation with non-replicative plasmids

Our primary objective was to establish a method of gene disruption for *S. aureofaciens* strain CCM3239. Thio^R transformants were obtained with non-replicative plasmid pHRDA2 containing part of the *hrdA* gene (Fig. 1). In several *Streptomyces* strains, transformation frequency was strongly reduced if the plasmid DNA had been modified by *dam* or *dcm* methylase (MacNeil, 1988). Accordingly, when the plasmid pHRDA2 was prepared from *dam*⁻ and *dcm*⁻ *E. coli* JM110, the transformation efficiency of *S. aureofaciens* CCM3239 was about 10 times higher (Table 1).

Correct integration of plasmid pHRDA2 into the chromosome has been proved by Southern blot hybridization. The presence of the Thio^R marker was

Fig. 1. Chromosomal DNA comprising the *hrdA* gene (filled boxes) of (a) *S. aureofaciens* CCM3239 (wild-type strain) and (b) the strain disrupted via a double crossover. The 800 bp *Bam*HI fragment of pHRDA2 and the 3.7 kb *Pvu*II–*Pst*I fragment of pRPO4-28 are shown by stippled boxes. The black bars below the maps represent probes used for Southern hybridization analysis: probe 1, *Bst*EII–*Bam*HI fragment comprising part of *hrdA*; probe 2, *Xba*I–*Kpn*I fragment comprising the Thio^R gene (hatched box). Restriction sites relevant to the Southern hybridization experiment are indicated. Arrows indicate the direction of transcription.

Fig. 2. Southern hybridization analysis of chromosomal DNA from the gene replacement experiments. Experimental details are given in Methods. Lanes: 1, *Bst*EII-digested wild-type *S. aureofaciens* CCM3239 DNA; 2, *Bst*EII-digested DNA from the disrupted strain (*S. aureofaciens* CCM3239-A4); 3, *Pvu*II-digested wild-type DNA; 4, *Pvu*II-digested disrupted DNA; 5, *Bst*EII- and *Not*I-digested wild-type DNA; 6, *Bst*EII- and *Not*I-digested disrupted DNA; 7, *Bst*EII- and *Xba*I-digested wild-type DNA; 8, *Bst*EII- and *Xba*I-digested disrupted DNA. (a) Southern blot probed with probe 1 comprising part of *hrdA* (Fig. 1b). (b) The same blot was treated to remove radioactive probe 1 as described in Sambrook *et al.* (1989) and probed with radioactively labelled probe 2 comprising the *Thio*^R gene (Fig. 1b) under the same conditions. *Bst*EII-digested λ DNA was used as size marker.

shown in all characterized clones, but various deletions of the *E. coli* DNA were observed in all cases (data not shown). Similar instability of *E. coli* DNA was observed in *S. coelicolor* A3(2) (Davis & Chater, 1992).

The instability of the *E. coli* DNA led us to use a double crossover for preparation of stable mutants. The *Thio*^R marker was used to replace the 300 bp *Sph*I–*Not*I fragment internal to *hrdA* (Fig. 1). The loss of this fragment removes the sequences encoding region 3 and part of region 4 conserved in all of the σ factors (Helmann & Chamberlin, 1988) and should result in a null mutation. The resulting plasmid, pRPO4-28, isolated from *dam*[–] *dcm*[–] *E. coli* JM110, was used to transform *S. aureofaciens* protoplasts. Correct integration was confirmed by Southern blot hybridization (Fig. 2) using the probes indicated in Fig. 1. The disruption of the *hrdA* gene occurred via a double crossover as indicated in Fig. 1.

Phenotype of the *hrdA*-disrupted *S. aureofaciens* strain

The *hrdA*-disrupted strain of *S. aureofaciens* CCM3239-A4 was viable and stable. Growth of the disrupted strain in liquid TSB medium and minimal NMP medium (Hopwood *et al.*, 1985) was comparable with the wild-type. The disrupted strain grew on solid media, produced aerial mycelium and spores comparable to the wild-type

strain. The production of chlortetracycline was also not affected by the disruption as shown by the determination of antimicrobial activity (see Methods). Similar results were obtained by disruption of the homologous *hrdA* gene in *S. coelicolor* A3(2) (Buttner & Lewis, 1992). Our results indicate that the *hrdA*-encoded σ factor in *S. aureofaciens* CCM3239 is not required for growth, differentiation or antibiotic production. The only effect we have noticed is a small difference in the colour of spores. However, microscopically the two types of spores are indistinguishable. Further characterization of the mutation and the disruption of other *S. aureofaciens* *hrd* genes are in progress.

Based on these results, the biological function of the *hrdA* gene remains unknown. Absence of the *hrdA* gene in *S. griseus* (Takahashi *et al.*, 1988) indicates that the gene is dispensable in *Streptomyces* spp. However, its role can be substituted by other types of *hrd* homologue identified in *S. griseus* (Takahashi *et al.*, 1988). The expression of the *hrdA* gene into mRNA in *S. aureofaciens* and *S. coelicolor* (Kormanec *et al.*, 1992; H. Takahashi, personal communication cited by Buttner, 1989) indicates a biological role for the *hrdA*-encoded σ factor rather than the assumption that it is a cryptic gene. The identification of genes directed by RNA polymerase containing the *hrdA*-encoded σ factor would elucidate its physiological function.

We would like to thank Mrs Renáta Knirschová for excellent technical assistance, and Dr Štefan Kužela for critical reading of the manuscript. We are grateful to Professor D. A. Hopwood for providing us with *S. lividans* TK24 and plasmids pIJ702 and pIJ486; to S. Horinouchi for plasmid pARC1; to R. Brzezinski for plasmid pFD666 and to W. Wohlleben for plasmid pGM9. This work was supported by a Grant GA999135 from the Slovak Academy of Sciences.

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