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Genetic Recombination and Transformation in Protoplasts of *Thermomonospora fusca*

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Protoplasts were produced from the thermophilic actinomycete *Thermomonospora fusca* and were regenerated to 0.1% of the direct count on regeneration agar. Recombination after protoplast fusion was demonstrated with drug-resistant mutants of *T. fusca* YX. A single thiostrepton-resistant colony was isolated after transformation of *T. fusca* YX with the streptomycete vector pIJ702, providing the first evidence for transformation in the genus *Thermomonospora* and suggesting that some mesophilic streptomycete genes can be expressed in thermophilic actinomycetes. Of 20 thermophilic actinomycete strains isolated from self-heated composts, 3 were found to harbor native plasmid DNA, providing potential sequences for the development of *Thermomonospora-Streptomyces* shuttle vectors.

Thermophilic actinomycetes are of significant scientific and technological interest for their role in biomass degradation and as producers of bioactive metabolites. Several genera, found as major components of self-heated organic composts (9), produce hydrolytic enzymes, including cellulases. While some aspects of the molecular biology of thermophilic actinomycetes can be studied by gene cloning into exogenous hosts such as *Escherichia coli* (3) and the mesophilic actinomycete *Streptomyces lividans*, the development of endogenous cloning and genetic manipulation systems for thermophilic actinomycetes is required for detailed examination of their physiology and optimum exploitation of their potential for biotechnology.

The genus *Thermomonospora* includes thermophilic actinomycetes with type III cell walls (10) that produce single aleuriospores at the termini of aerial mycelia. Three species (*T. alba*, *T. curvata*, and *T. fusca*) were shown to secrete enzymes for hydrolysis of cellulose and xylans (12); in particular, the thermostable cellulases of *T. curvata* and *T. fusca* received attention for potential application in biomass conversion strategies (5, 6). To date, however, no system for genetic analysis or exchange has been described for this genus.

We report here (i) the development of procedures for production and regeneration of *T. fusca* protoplasts and evidence for genetic recombination after protoplast fusion, (ii) a method for screening thermophilic actinomycetes for the presence of covalently closed circular DNA (CCC DNA) and its application in the detection of native plasmids from field isolates, and (iii) transformation of *T. fusca* YX with a streptomycete cloning vector.

T. fusca YX (1) and *S. lividans* 3131 (8) were maintained as spore suspensions stored at −20°C in 20% glycerol. Thermophilic actinomycetes were grown at 55°C on a Luria-Bertani medium supplemented with 0.1% glucose and 10 ml of a mineral salts solution per liter (13), with 2% agar added for plates.

For *T. fusca* protoplast production, dense mycelial cultures were used as 5% inocula into complete broth containing 0.5% glycine. After incubation with shaking at 55°C for 6 h, mycelia from 25 ml of the glycine-exposed culture were washed and suspended in 5 ml of P medium (14) containing lysozyme (2 mg/ml), followed by further incubation for 2 h at 35°C. The suspension was passed through a glass fiber filter (GF/D; Whatman, Inc., Clifton, N.J.) to remove mycelial fragments. Protoplasts were recovered by centrifugation (1,000 × *g*, 20 min), washed twice in 5 ml of P medium, and resuspended in 1 ml of P medium. For regeneration, protoplasts were diluted through P medium, spread onto R2YE agar (15), and incubated for 7 days at 45°C. The presence of mycelial fragments and spores in a protoplast suspension was determined after dilution of the suspension through water before spreading onto regeneration agar.

T. fusca YX protoplast suspensions routinely yielded approximately 10^5 CFU/ml on R2YE regeneration agar from 10^8 protoplasts per ml by direct count, indicating 0.1% regeneration. Fewer than 10^3 CFU/ml survived dilution through water, indicating less than 1% contamination by mycelial fragments. The expansion of phase-bright figures followed by peripheral growth of mycelia (Fig. 1) was similar to the regeneration sequence reported previously for *Streptomyces* protoplasts (14), although at present the regeneration frequency obtained with *T. fusca* is lower than that reported for mesophilic actinomycetes.

To demonstrate genetic recombination in *T. fusca*, we isolated a variety of marked strains after mutagenesis with ethyl methanesulfonate or nitrosoguanidine. No recombinants were detected among progeny spores of mixed platings, suggesting that the YX strain of *T. fusca* is not self-fertile. We therefore decided to induce protoplast fusion with polyethylene glycol and screen regenerants for a recombinant genotype. Protoplasts were prepared from two drug-resistant strains (YD201 Rif^r; and YD202 Str^r). Mixed protoplasts were recovered by centrifugation and resuspended in 0.5 ml of P medium containing 40% polyethylene glycol 1000. After 3 min of incubation, fusion products were spread onto R2YE agar for regeneration. Progeny spores from these plates were recovered and plated onto selective media for detection of recombinants. Rif^r Str^r colonies were recovered at 4×10^{-5} of viable count, a frequency greater than 100-fold higher than spontaneous mutation rates, providing the first evidence for genetic recombination in the genus *Thermomonospora*.

Initial experiments aimed at transforming *Thermomonospora* used the plasmid-free YX strain of *T. fusca* and the streptomycete cloning vector pIJ702. This plasmid carries

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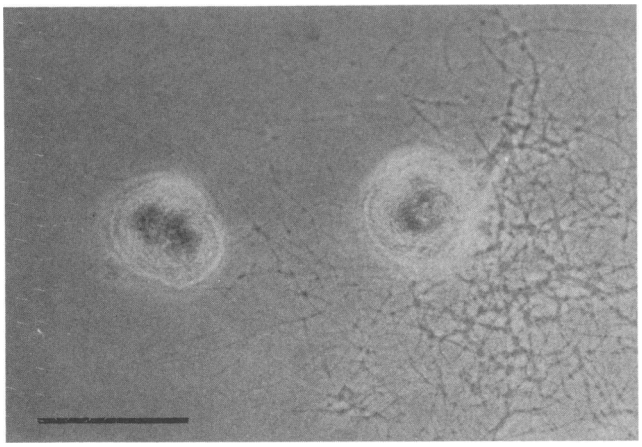

FIG. 1. Regenerant colonies from *T. fusca* YX protoplasts after 60 h of incubation on R2YE agar. Bar, 0.1 mm.

genes for thiostrepton resistance and tyrosinase (melanin production), replicates in a high copy number, and exhibits a wide host range among *Streptomyces* species (8).

When 10^5 viable *T. fusca* YX protoplasts were incubated in the presence of polyethylene glycol (2) with 30 μg of purified pIJ702 and regenerated on R2YE agar, and when the resulting confluent growth was replicated onto nutrient agar containing 50 μg of thiostrepton per ml, a single thiostrepton-resistant colony was detected. Agarose gel electrophoresis of CCC DNA from a pure liquid culture of this isolate revealed the presence of a plasmid that comigrated with pIJ702 (Fig. 2, lanes b and c). Double digestion of the plasmid with *Bam*HI and *Pst*I produced linear fragments of 4.9 and 0.8 kilobases, identical to fragments obtained from pIJ702 from *S. lividans* (Fig. 2, lanes d and e). Finally, the identity of the isolate as *T. fusca* was confirmed by the sensitivity of the transformant to two narrow-host-range *T. fusca* bacteriophages. All of this evidence is consistent with identification of the isolate as *T. fusca* YX(pIJ702). Plasmid DNA isolated from *T. fusca* YX(pIJ702) was able to transform plasmid-free *T. fusca* YX to thiostrepton resistance at a frequency of 50 transformants per μg of DNA, which corresponded to 1 transformant per 10^3 viable protoplasts.

T. fusca YX(pIJ702) grows at 55°C in the presence of 50 μg of thiostrepton per ml, whereas plasmid-free *T. fusca* YX is unable to grow in the presence of the drug at 0.25 μg/ml. Therefore, it is clear that the thiostrepton resistance gene of pIJ702 can be expressed in *T. fusca* and that the gene product, a 23S rRNA methylase (4), is active at 55°C. No pigment production was detected in *T. fusca* transformants, suggesting that active tyrosinase is not produced by pIJ702 in *T. fusca*. While initial observations suggested that pIJ702 may be unstable in *T. fusca*, subsequent experiments showed that >90% of CFU retain the thio^r phenotype after a single spore-spore growth cycle in the absence of thiostrepton.

To screen for naturally occurring plasmids in thermophilic actinomycetes, we isolated from self-heated composts containing manured straw a variety of thermophilic actinomycetes by using a membrane filter technique developed for mesophilic streptomycetes (7). We readily isolated strains that resembled *Thermomonospora* in morphology of aerial mycelia and exhibited sensitivity to *T. fusca* and *T. alba* narrow-host-range phages isolated from the same soils (manuscript in preparation). Thermophilic actinomycete field isolates and *Thermomonospora* laboratory cultures (*T. fusca* YX and the type strains *T. fusca* ATCC 27730 and *T. alba* IPV 1900) were screened for the presence of plasmid DNA with a rapid small-scale plasmid purification procedure. The purification procedure is essentially a combination of the neutral cleared-lysate procedure of Westpheling (cited in reference 2) and the acidic phenol extraction technique of Zasloff et al. (16), which removes linear DNA fragments from plasmid preparations. Plasmids prepared by this procedure are suitable substrates for cleavage with restriction endonucleases, as demonstrated for plasmid pIJ702 (Fig. 2, lane e). Plasmid DNA in 3 of 20 thermophilic actinomycetes isolated from compost is shown in Fig. 2, lanes g to i. The laboratory strains *T. fusca* ATCC 27730, *T. fusca* YX, and *T. alba* IPV 1900 were found to be plasmid free.

The results of these experiments suggest that techniques developed for gene cloning in *Streptomyces* can be applied, with modifications, to at least some thermophilic actinomycetes. While the efficiencies of protoplast production and regeneration obtained for *T. fusca* are less than those found by other workers for various mesophilic actinomycetes, they are sufficient to allow use of protoplasts for genetic manipulation and analysis of *T. fusca*. Recombination after protoplast fusion, demonstrated here with drug-resistant strains of *T. fusca*, may be a useful technique for investigating the organization of genes for hydrolytic enzymes secreted by *Thermomonospora*.

Using particular procedures reported by several separate laboratories, an overall procedure for the rapid isolation and purification of plasmid DNA from actinomycetes was developed and used to screen field isolates of thermophilic actinomycetes for the presence of plasmids. Approximately 10% of the isolates screened contained plasmid DNA. To date, all of these plasmids are cryptic; they do, however,

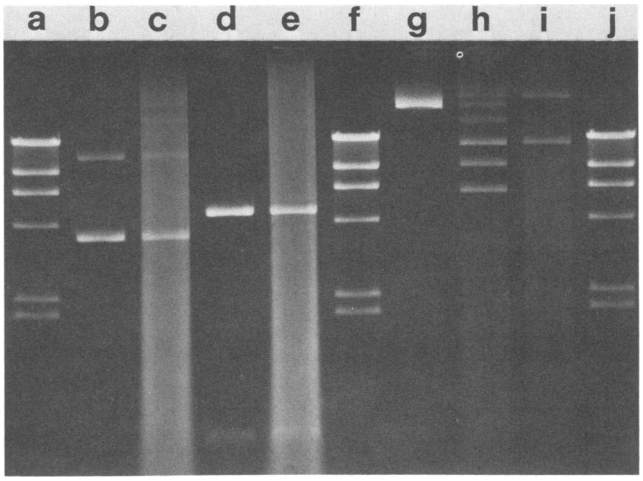

FIG. 2. Electrophoretogram of CCC DNA from thermophilic actinomycetes. Electrophoresis was carried out at 4 V/cm for 3 h in 0.8% agarose gels prepared in TBE running buffer (11). Gels were stained with ethidium bromide and viewed with a near UV transilluminator. Restriction endonuclease digests were carried to completion with buffers provided by the supplier (IBI, New Haven, Conn.). Lanes: a, f, and j, *Hin*dIII fragments of bacteriophage DNA; b, pI702 from *S. lividans* 3131; c, plasmid DNA from thiostrepton-resistant *T. fusca* YX transformant; d, as in lane b, but *Bam*HI/*Pst*I double digest; e, as in lane c, but *Bam*HI/*Pst*I double digest; g, CCC DNA from field isolate JL30d; h, CCC DNA from field isolate JL43b; i, CCC DNA from field isolate JL43c.

provide potential sequences that might be of future use in the construction of cloning vectors expressly tailored for cloning into thermophilic actinomycete hosts.

The most interesting result obtained in this study was evidence for the transformation of *T. fusca* by the streptomycete cloning vector pIJ702. The ability of pIJ702 to transform *T. fusca* YX to thiostrepton resistance indicates the following: (i) transformation is possible in *Thermomonospora* spp., (ii) *Streptomyces* plasmid origin of replication sequences can probably be recognized by *Thermomonospora* DNA polymerases, and (iii) at least one *Streptomyces* gene (i.e., thiostrepton resistance gene of *S. azureus*) can be functionally expressed by the *Thermomonospora* transcription and translation systems at a temperature (55°C) higher than those encountered by the mesophilic streptomycetes. These results suggest the feasibility of efficiently moving genes between mesophilic and thermophilic actinomycetes for the purposes of analyses of gene structure and regulation and their expression by actinomycetes over a fairly wide range of temperatures.

The *T. fusca* transformation frequency (50 transformants per μg of DNA) obtainable with pIJ702 isolated from *T. fusca* YX(pIJ702) is low, but this is mainly because of inefficient protoplast production and regeneration. Thus, if procedural modifications lead to significantly higher protoplast regeneration frequencies, it is possible that pIJ702 could be used as a vehicle for gene cloning into *Thermomonospora* spp.

The work described here thus provides a foundation for the development and use of gene cloning systems for *Thermomonospora* spp. and for the cloning and analysis of its genes for various hydrolytic enzymes, including cellulases. In particular, *Thermomonospora-Streptomyces* shuttle vectors are under development with DNA sequences from thermophilic actinomycete cryptic plasmids and phages.

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