Isolation of the Acetyl-CoA Synthase Gene from the Corn Smut Pathogen, *Ustilago maydis*

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The acetyl-CoA synthase (acuA) gene from *Ustilago maydis* has been isolated using a fragment of the *acu*5 gene from *Neurospora crassa* as a heterologous probe. The *U. maydis* acuA gene transformed Acu− mutants of *U. maydis* to acetate utilization. Transformation was accomplished by integration of the vector sequences into the chromosomal DNA.

**INTRODUCTION**

Isolation of the genes expressed during the interaction of a fungal pathogen with its host plant and a knowledge of their regulation will be central to the elucidation of mechanisms unique to the parasitic habit. However, before molecular genetic analysis of such genes can be done, a selection of gene markers needs to be available for gene transfer systems and to aid manipulation of the expression of particular genes of interest.

The basidiomycete maize pathogen, *Ustilago maydis*, has many advantages for studying both genetic and molecular genetic aspects of pathogenicity. It is amenable to manipulation in the laboratory, growing on defined media as small uninucleate haploid colonies. In addition, the genetics and sexual cycle of this pathogen are relatively well understood, allowing strains to be crossed and recombinants to be recovered (Banuett & Herskowitz, 1988; Holliday, 1974).

Integrative and multicopy plasmid vectors have been described for the transformation of *U. maydis* (Tsukuda et al., 1988; Wang et al., 1988). In both cases, the *Escherichia coli* hygromycin B phosphotransferase (*hygBR*) gene was employed as a selectable marker. However, unlike many other fungi used in molecular genetic studies, homologous wild-type or mutant genes have not yet been developed as selectable markers for gene transfer. The only homologous selectable gene described so far for *U. maydis* is the *pyr3* gene encoding dihydroorotase (Banks & Taylor, 1988).

Here we report the isolation of the acetyl-CoA synthase (acuA) gene of *U. maydis* and its use as a selectable marker in the transformation of *U. maydis* mutants unable to utilize acetate as the sole carbon source.

**METHODS**

*Escherichia coli* strain and plasmids. *E. coli* strain DH1 (recA palA hsr hmt+ endo 1B relA1) was transformed according to Hanahan (1983). pC2Bl.0 carrying a BamHI fragment of the coding region of the *acu*5 gene of *Neurospora crassa* was provided by I. Connerton, University of Reading, UK. The plasmid pUC9 was used as the cloning vector throughout.

*U. maydis* strain and culture conditions. All manipulations were done using an isolate obtained from the CAB International Mycological Institute, Kew, UK, no. 103761. Cultures were maintained on, and sporidia prepared from, a glucose (10 g l⁻¹)/yeast extract (2 g l⁻¹) medium (GYE). Minimal medium was as described by Holliday (1974), except that the carbon source (5 g l⁻¹) and 50 mM-MOPS (final concentration) were added after autoclaving. Mutants unable to utilize acetate as a carbon source were isolated by selection for resistance to fluoroacetate on minimal medium containing 5 g fluoroacetate l⁻¹ and 1 g glucose l⁻¹. All solutions were adjusted

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to pH 6.5 and solid media contained 2% (w/v) agar. Cultures were incubated at 25°C and liquid cultures were shaken at 150 r.p.m.

Isolation and manipulation of DNA. Total U. maydis DNA was isolated from sporidia grown in GYE medium for 48 h. The sporidia were harvested from 100 ml cultures by centrifugation at 1000 g for 20 min, then washed with 0.6 M-KCl. Protoplasts were prepared by incubating sporidia with 10 ml Novozyme 234 (10 mg ml⁻¹) and Cellulase R10 (10 mg ml⁻¹) in 0.6 M-KCl, for 45-60 min at 30°C. The protoplasts were centrifuged at 1000 g for 20 min and then washed once with 0.6 M-KCl. DNA was isolated from the protoplasts as described by Rothstein (1985), with the exception that phenol/chloroform extraction was performed twice. This procedure yielded approximately 20 µg DNA.

Standard methods were used for other DNA manipulations (Maniatis et al., 1982).

Conditions for Southern transfer and colony hybridization. The transfer of U. maydis genomic DNA from agarose to nylon filters (Hybond-N, Amersham) was in accordance with the manufacturer's instructions. Conditions to obtain specific hybridization between the N. crassa fragment and U. maydis DNA were optimized as described by Howley et al. (1979). Colony hybridization was as described by Maniatis et al. (1982) and the conditions used were similar to those for Southern hybridization. Analysis of DNA from transformants was done using standard procedures (hybridization in 5 × SSC at 65°C and washes to 0.1 × SSC, 0.1% SDS; 1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate).

Preparation and transformation of U. maydis protoplasts. Sporidia were grown overnight in GYE medium (100 ml) and protoplasts were isolated and transformed as described by Wang et al. (1988), with some minor modifications. Sporidia were incubated with Novozyme 234 (5 mg ml⁻¹) for 20–30 min and protoplasts were recovered by centrifugation at 1000 g for 10 min. Following transformation, the protoplasts were plated out directly on to minimal medium containing sodium acetate and 1 M-sorbitol. On this medium it was not possible to clearly differentiate the transformed colonies from background growth, since sorbitol supports limited growth of U. maydis. After 5 d the colonies that grew were replica plated on to minimal medium containing sodium acetate alone and acetate-utilizing colonies appeared 4–5 d later.

RESULTS AND DISCUSSION

A random primed labelled (Feinberg & Vogelstein, 1983) 1.0 kb BamHI fragment of the N. crassa acu5 gene (Thomas et al., 1988) was used as a hybridization probe against Southern blots of U. maydis genomic DNA (5 µg), digested with a number of different restriction enzymes. Using a stringency allowing hybridization of sequences with approximately 75% homology (30% formamide in the hybridization buffer at 35°C and washes at 56°C in 2 × SSC, 0.5% SDS), the N. crassa fragment hybridized to a single HindIII fragment (7.5 kb). At this stringency, a number of weaker background signals were also observed. U. maydis DNA (25 µg) was digested with HindIII and fragments 6.0–8.0 kb in size were isolated. A partial library (Nicholls, 1986) containing this HindIII fragment was constructed in the plasmid pUC9, and positive clones were identified by hybridization to the N. crassa acu5 gene fragment. One clone (pUCH2) was recovered which contained a U. maydis HindIII fragment that gave a strong hybridization signal with the N. crassa probe. A partial map of this U. maydis HindIII fragment is shown in Fig. 1.

Acetate non-utilizing (Acu⁻) mutants were isolated by positive selection for resistance to fluoroacetate as described by Apirion (1965). Resistant colonies (fac mutants) appeared after incubation for 6–8 d. Only fac mutants unable to grow on media containing acetate as the sole carbon source were selected for transformation. Four separate Acu⁻ mutants (A7615, A7617, A76120 and A76121) were transformed with 1 µg of either pUC9 or pUCH2. Colonies capable of growing on acetate medium were obtained with all the mutants transformed with pUCH2, at frequencies ranging from 5 to 20 colonies per µg DNA. No growth occurred with any of the mutants when pUC9 was used as the transforming DNA. The transformants were mitotically stable when grown in the absence of acetate.

Six transformants from three different mutants (A7615, A7617 and A76121) were selected for Southern blot analysis. In the lanes containing undigested DNA from the transformants (Fig. 2a), radiolabelled pUC9 hybridized, with one exception (lane 5), to bands corresponding to undigested DNA. This indicates that transformation had occurred by integration of the vector sequences into the chromosomal DNA. No homology was detected between pUC9 sequences and U. maydis DNA (Fig. 2a, lane C). Digests of U. maydis DNA with SstI were also probed with either pUC9 or an SstI/HindIII fragment (6.8 kb) of the cloned U. maydis HindIII fragment.
Ustilago maydis acetyl-CoA synthase gene

Fig. 1. A restriction endonuclease map of the U. maydis HindIII fragment carrying the acuA gene.

Fig. 2. Southern blot hybridization analysis of U. maydis Acu− mutant A7615 (C) and six selected acetate utilizing transformants (1, AT76151; 2, AT76152; 3, AT76171; 4, AT76172; 5, AT761211; 6, AT761212). DNA from the Acu− mutant and the transformants was probed undigested with radiolabelled pUC9 (a) or after SstI digestion, with either pUC9 (b) or the 6.8 kb SstI/HindIII fragment (c). The hybridization signals obtained were consistent with the interpretation that integration of one copy or, in one case, two copies of the vector had occurred by either homologous or heterologous recombination. In the transformant where no hybridization to pUC9 was detected a double cross-over event seems to have led to the mutant gene being replaced by the wild-type gene, with the rest of the vector sequences being lost (Fig. 2b, lane 5). Although it is possible that this transformant arose as a result of a reversion of the acuA mutation in the recipient strain, this would seem unlikely since no reversions were observed when pUC9 was used alone as the transforming vector. The type of integration events observed here are in general consistent with those reported for other homologous gene transfer systems in fungi (Ballance & Turner, 1986; Banks & Taylor, 1988; de Graaff et al., 1988; van Hartingsveldt et al., 1987).

These results indicate that pUCH2 contains all the necessary control and regulatory sequences to gain expression of the acetyl-CoA synthase gene in Acu− mutants of U. maydis. The availability of this and other homologous selectable genes for the transformation will aid the manipulation of gene expression in U. maydis using gene replacement or gene disruption techniques.

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REFERENCES


