An endonuclease-deficient strain of *Ustilago maydis*

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INTRODUCTION

There has recently been a growing realization that certain nucleases are likely to play a crucial role in the process of genetic recombination (e.g. Lehman, 1967). One way of obtaining evidence that this is indeed the case is to isolate mutants lacking particular DNases and then examine the effect of the deficiency on genetic recombination. The smut fungus *Ustilago maydis* is a convenient organism for studying the mechanism of recombination between eukaryotic chromosomes. This species produces one or more extracellular DNases, which perhaps are unlikely to be required for recombination. In this paper we describe a strain in which most of the extracellular enzyme activity is lost. By further mutation of this we hope to derive strains which have lost other nucleases which are more likely to be involved in the recombination process.

2. METHODS AND MATERIALS

Reference should be made to earlier papers for methods in *Ustilago* genetics which are not described here (Holliday, 1967, and references therein).

The method used for screening colonies for DNase activity is based on that of Jeffries, Holtman & Guse (1957). To induce mutations, wild-type log-phase cells growing in liquid complete medium were washed and suspended for 5 min at 32 °C in an aqueous solution of 100 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine, a treatment which kills about 25 % of cells. After washing, the cells were resuspended and incubated in liquid complete medium overnight, and then plated on Difco tryptic soy agar containing 0·3 % crude calf thymus DNA (British Drug Houses) at a colony density of 80–100/plate. After 3 days' incubation at 32 °C the colonies were rescued by replica plating to complete medium, before the masters were treated with acid to precipitate the DNA. Since flooding the plates with dilute HCl washes off the colonies, precipitation was achieved by placing a piece of filter paper in the lid of the dish and moistening it with concentrated HCl. After 10–15 min the lids were discarded and the plates examined for colonies producing weak or no haloes. (On this medium wild-type colonies produce clear haloes; with standard minimal or complete medium containing DNA the haloes are weaker and more diffuse.) In two experiments 11 000 colonies were scored. Of a number of possible mutants which were retested by streaking on the tryptic soy medium, only two were found to digest very little DNA; the others all had significant activity. Only one of these two mutants was examined further, since the other showed rather slow mycelial growth.

A standard viscometric method was used for following the digestion of highly polymerized DNA (McCarty, 1945). The assay mixture was as follows: 9 ml of 0·05 % calf thymus or staphylococcal DNA in 0·05 M Tris HCl, pH 7·5; 0·1 ml 0·5 M-MgCl₂ containing 1 % gelatine; 0·9 ml enzyme. The enzyme, either crude extract or culture supernatant, was added at zero time to the other components in a modified Ostwald viscometer, with a flow time for water of 56·0 s at the incubation temperature of 32 °C. Crude extracts were

prepared by breaking the cells in Tris HCl, pH 7·5, with glass beads in a Mickle disintegrator, and spinning the cell-free extract at 15000 rev/min for 10 min—all operations being carried out at 5 °C or less. A qualitative test for enzyme activity could be made by adding 0·2 ml enzyme to a standard well in tryptic soy DNA plates, incubating overnight before precipitating the DNA with acid.
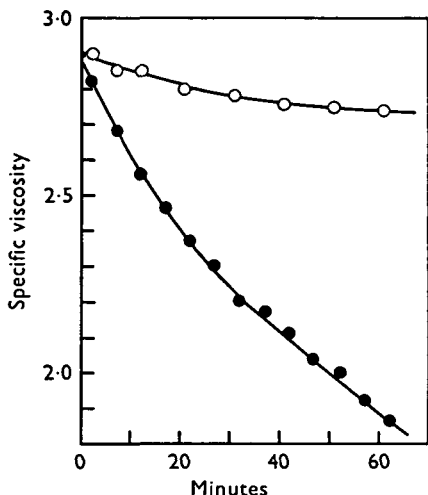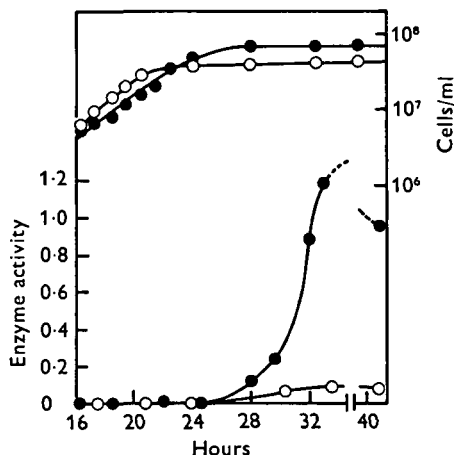

Fig. 1. The degradation of high molecular weight DNA by supernatants from stationary-phase cultures of the wild type (●) and the mutant strain (○).

Fig. 2. The development of nuclease activity in culture supernatants as the cells pass from log to stationary phase: ● wild type, ○ mutant. Equal inocula were added to tryptic soy broth medium at zero time. Cell counts were made with a Model A Coulter Counter. Enzyme activity is expressed as the drop in specific viscosity after 30 min incubation. (In this experiment 0·5 % denatured DNA was included in the growth medium, as plate assays had indicated that the nuclease might be inducible. In fact this is not the case—at least for the major activity—as a parallel experiment without DNA in the medium gave the same result.)

Figure 1 shows the digestion of DNA by supernatants from stationary phase-cultures of the mutant and wild type growing in Difco tryptic soy broth. The almost linear fall in viscosity shows that the enzyme is reducing the molecular weight of the DNA without any lag and must therefore be producing double strand breaks. The mutant has slight, although measurable, activity, as is also shown by the plate assay. Mixtures of mutant and wild-type supernatants retained the expected activity. Crude extracts of washed stationary-phase cells gave the same result, but the activity was always lower than that in the culture supernatants. The enzyme has a broad pH range with an optimum of 8·5; it has a requirement for Mg^{2+} which is not satisfied by Ca^{2+}; boiling for 1 min destroys its activity. Enzyme activity has not been demonstrated in log-phase cultures. Figure 2 shows that the enzyme is produced very rapidly 4–8 h after the cells enter stationary phase. The slight activity in the mutant appears at the same time. The enzyme was not leaking out of dead cells as there was no drop in viability at the time the enzyme appeared.

Apart from its endonuclease deficiency the mutant has other distinct and rather surprising phenotypic properties. The cells are shorter in length and greater in width than the wild type. In old cultures, in liquid or on agar, a proportion of the cells round off

further and may develop into thick-walled vegetative spores. The formation of spores is associated with the production of a brown pigment, some of which diffuses into the medium. Spores are particularly liable to form at 35 °C, a temperature which allows only slow growth of the mutant, but vigorous growth of the wild type.

In view of its complex phenotype, it is perhaps not surprising that crosses between the mutant and standard nuclease-positive strains have provided evidence that the nuclease-deficient strain carries two mutations. Analysis of meiotic tetrads and the scoring of nuclease activity by the qualitative plate assay method has shown that the normal 2:2 segregation expected of a single mutation is produced only in a proportion of cases; the other tetrads show a 3:1 or 4:0 ratio for nuclease positive to negative. The brown phenotype segregates 2:2 in every case. The scoring of the enzyme activity was not always clear-cut, but the results are consistent with the segregation of two unlinked genes each controlling a nuclease, one of which is also responsible for the brown phenotype. Only when both mutations are present is the enzyme activity largely abolished. This need not mean that the endonuclease activity shown in Figs. 1 and 2 is due to two enzymes: one of the enzymes may cause two strand breaks; the other, which would not be detected in the viscometric assay, might be an exonuclease or an endonuclease producing single strand breaks.

3. DISCUSSION

So far as we are aware, mutants deficient in nucleases have not previously been isolated in a eukaryotic organism. Ribonuclease I deficient mutants in *Escherichia coli* were first described by Gesteland (1966), and in the same organism Endonuclease I deficient mutants have also been reported (Dürwald & Hoffmann–Berling, 1968; Eigner & Block, 1968). Okubo, Nakayama, Sekiguchi & Takagi (1967) isolated a mutant of *Micrococcus lysodeikticus* which lacked a nuclease specific for u.v.-irradiated DNA.

It has recently been shown that *Ustilago sphaerogena* has four extracellular ribonucleases (Arima, Uchida & Egami, 1968). If *U. maydis* has more than one active DNase then it is perhaps not surprising that single mutants were not picked up by the method employed, since these would retain considerable activity. The thirty or so possible mutants that were discarded initially because of their considerable ability to digest DNA in the medium may in fact have been single mutants. Colonies grown 3 days on tryptic soy agar contain a considerable number of dead autolysed cells from which intracellular nucleases could leak out into the medium. However, the results with liquid media show that at least one of the enzymes is produced by living cells and is therefore extracellular. The genetic results indicate that the mutant strain lacks another enzyme, and that this has a function other than the digestion of extracellular DNA is indicated by the altered morphology of the mutant cells, their pigmentation and their sensitivity to temperature. From these preliminary results it would be premature to speculate about the possible basis of these pleiotropic effects.

The effect of the deficiency on genetic recombination is under examination. However, having abolished the major nuclease activity, a more promising approach would be to obtain further mutations in this strain. These might eliminate those exonucleases or endonucleases likely to be important in genetic recombination in fungi (Holliday, 1968). Finally, we wish to test the possibility, suggested in an earlier paper (Holliday, 1967), that the radiation-sensitive mutants, *uvs*-1, which severely upsets the normal processes of genetic recombination in *Ustilago*, may have excess or constitutive exonuclease activity. This can be done if the appropriate nuclease-deficient strain can be obtained.

SUMMARY

A strain of *Ustilago maydis* has been isolated, after treatment with nitrosoguanidine, which lacks an extracellular endonuclease capable of causing two strand breaks in DNA. Genetic analysis suggests that the strain carries two mutations and is probably also deficient in a second nuclease.

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