Gene, 142 (1994) 225–230

GENE 07750

Three selectable markers for transformation of *Ustilago maydis*

(Nourseothricin; phleomycin; benomyl; corn smut; phytopathogen; expression vectors; fungi

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Received by J.A. Gorman: 30 July 1993; Revised/Accepted: 19 October/8 November 1993; Received at publishers: 6 December 1993

SUMMARY

Although *Ustilago maydis* is readily amenable to molecular genetic experimentation, few antibiotic-resistance markers are available for DNA-mediated transformation. This poses constraints on experiments involving targeted gene disruption and complementation. To address this problem, we constructed vectors using one of three additional genes as dominant selectable markers for transformation. Two genes, *sat-1* (encoding streptothricin acetyltransferase) and Sh-*ble* (encoding a phleomycin-resistance polypeptide), are of bacterial origin and have been engineered for expression in *Ustilago sp.* The third gene encodes an allele of *U. maydis* β-tubulin that confers resistance to the fungicide benomyl.

INTRODUCTION

Members of the genus *Ustilago* are phytopathogens which exhibit dimorphic growth under the control of two mating-type loci designated *a* and *b* (Froeliger and Kronstad, 1990; Banuett, 1992). We are presently analyzing genes involved in dimorphism and pathogenesis (Barrett et al., 1993). Such genes are likely to encode components of complex developmental pathways and may be members of gene families. The analysis of these genes would be facilitated by the availability of a variety of dominant selectable markers to allow construction of mutants containing multiple gene disruptions. The construction of mutants with two or more mutations would be greatly facilitated by selection of progeny of known mutant genotype based on drug-resistance phenotypes. Dominant markers allow selection in prototrophic strains, thus avoiding potential problems associated with selection by complementation of auxotrophic defects. In *U. maydis*, such defects are known to affect pathogenicity (Holliday, 1974), thus complicating the use of auxotrophic strains for experimentation.

To facilitate the construction of mutant strains with multiple gene disruptions and to allow complementation in such strains, we developed vectors with three new dominant selectable markers for *Ustilago* transformation. These markers supplement the genes currently used for transformation of *U. maydis*; the Hy^R gene (Wang et al., 1988; Tsukuda et al., 1988), a mutant allele of the *U. maydis* succinate dehydrogenase gene, which confers resistance to the fungicide carboxin (Keon et al., 1991), and several genes encoding metabolic enzymes (Kronstad et al., 1989; Fotheringham and Holloman, 1989).

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Abbreviations: Ap, ampicillin; *ARS*, autonomously replicating sequence; Ben, benomyl; Ble, bleomycin/phleomycin; DCM, double complete medium; *gap*, glyceraldehyde-3-phosphate dehydrogenase-encoding gene; Hpt, Hy phosphotransferase; *hsp70*, heat-shock protein 70-encoding gene; Hy, hygromycin; Hy^R, Hpt-encoding gene; kb, kilobase(s); *ori*, origin of DNA replication; MCS, multiple cloning site(s); *nat1*, *Streptomyces noursei* Nst-resistance-encoding gene; Nst, nourseothricin; nt, nucleotide(s); PCR, polymerase chain reaction; *P*, promoter; ^{R}, resistant/resistance; ^{S}, sensitive; *sat-1*, streptothricin acetyltransferase-encoding gene 1; Sh-*ble*, *Streptoalloteichus hindustanus* Ble-resistance-encoding gene; SSC, 0.15 M NaCl/0.015 M Na_3-citrate pH 7.6; *T*, terminator; *Tub*, gene encoding β-tubulin; *U.*, *Ustilago*; wt, wild type.

SSDI 0378-1119(94)E0746-Z

EXPERIMENTAL AND DISCUSSION

(a) Isolation of a Ben^R gene from *U. maydis*

Ben resistance in several fungi is conferred by mutant alleles of *Tub* (Gold et al., 1991; Orbach et al., 1986; Weatherbee and Morris, 1984). The minimum inhibitory concentration of Ben for *U. maydis* strains 518 and 521 on double complete medium (DCM; Holliday, 1974) is 0.75–1.0 μg Ben/ml. Ben^R mutants of strain 518 were isolated following irradiation with ultraviolet light and direct selection on medium containing 1.5 or 5 μg Ben/ml. Several colonies appeared on medium containing 1.5 μg Ben/ml, while a single mutant colony was found on medium containing 5 μg Ben/ml. The latter mutant (518BEN1) was capable of growth at a concentration of 10 μg Ben/ml, the highest level tested. In a cross of mutant 518BEN1 with wt strain 521, a similar frequency of Ben^R (82) and Ben^S progeny (62) resulted, indicating segregation of a single locus. All resistant progeny were capable of growth on medium containing 10 μg Ben/ml.

The Ben^R gene was isolated from a cosmid library constructed in pJW42 (Wang et al., 1989) with genomic DNA from strain 518BEN1. The vector pJW42 carries the Hy^R gene for selection of *U. maydis* transformants. Recombinant cosmid DNA from a pool of approx. 10^4 *E. coli* transfectants was introduced into *U. maydis* strain 518. Twelve transformants were selected on medium containing 1.5 μg Ben/ml and ten were studied further. All of the Ben^R transformants were also Hy^R, indicating the presence of vector sequences (pJW42). Nine of these strains (BEN1 T1–T9) grew on medium with 2 μg Ben/ml; BEN1 T10 was capable of growth on medium with 10 μg Ben/ml. Growth of transformants BEN1 T1–T9 on medium without Ben resulted in frequent loss of resistance, indicating that these strains contained autonomously replicating cosmid DNA. However, it was not possible to cure transformant BEN1 T10 of the cosmid; apparently this strain contains integrated cosmid DNA.

A cosmid (pBEN1) was isolated from transformant BEN1 T1 by preparation of total DNA and electroporation into *E. coli*. Re-introduction of this cosmid into *U. maydis* confirmed that it conferred both Hy and Ben resistance. Southern hybridization was carried out to confirm that pBEN1 carried a *Tub* gene and to determine the number of *Tub* genes in *U. maydis* (Fig. 1). Strong hybridization signals were found to fragments of the insert of pBEN1 (lanes a–c) upon hybridization with a *Neurospora crassa Tub* probe (Vollmer and Yanofsky, 1986) and a 3.9-kb *Pst*I-*Eco*RI fragment (subcloned from pBEN1) was found to confer Ben resistance; this fragment was used to construct the Ben resistance vector pBEN102 (Fig. 2A). The 3.9-kb *Pst*I-*Eco*RI insert of pBEN102 was also used as a hybridization probe to digests of *U. maydis* genomic DNA (Fig. 1, lanes d–f). A single band was found to hybridize for each digest suggesting that *U. maydis* contains one *Tub* gene.

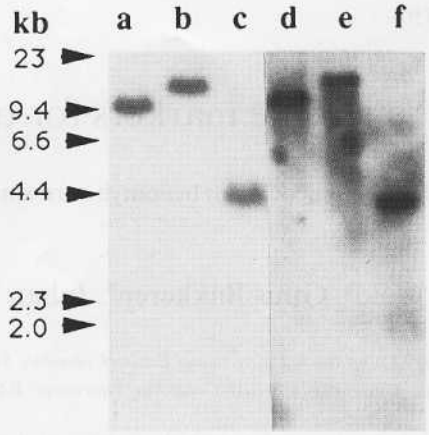

Fig. 1. Hybridization with *Neurospora crassa* and *U. maydis Tub* gene probes. Following agarose gel electrophoresis, restriction fragments were transferred to Zetabind (AMF Cuno, Meriden, CT, USA) and hybridized as previously described (Gold et al., 1991). Lanes **a–c** contain cosmid pBEN1 digested with: **a**, *Pst*I; **b**, *Eco*RI; **c**, *Pst*I + *Eco*RI. The probe for these lanes was the 2.6-kb *Sal*I fragment of plasmid pSV50 containing the *N. crassa Tub* gene (Vollmer and Yanofsky, 1986). Lanes d–f contain *U. maydis* genomic DNA digested with: **d**, *Pst*I; **e**, *Eco*RI; **f**, *Pst*I + *Eco*RI. The probe for these lanes was the 3.9-kb *Pst*I-*Eco*RI fragment of pBEN102 (Fig. 2A). The blot for lanes **d–f** was washed under reduced stringency conditions (2 × SSC, 25°C).

(b) Transformation with vectors conferring Ben resistance

Resistance to the microtubule depolymerizing agent Ben has proved to be a useful selectable marker in a number of fungal species. Ben is an attractive selective agent because it is relatively inexpensive compared to the commonly used Hy, and because it is a systemic fungicide which, like carboxin (Keon et al., 1991), may provide in planta selection of plasmid bearing fungal pathogens. The *Ustilago* Ben^R gene allowed the construction of vectors that confer resistance upon transformation (Fig. 2A,B; Table I). We have noted that the difference in the selective level between wt cells and those carrying pBEN102 (Fig. 2A) is approx. 1 μg Ben/ml. Although this poses no significant technical difficulties, we speculate that this narrow window for selection may occur because the Ben^R *Tub* gene acts in a codominant fashion with the endogenous wt copy of the gene. Transformation experiments with a Ben^R vector lacking an *ARS* (pBEN103, not shown) indicate that the Ben^R gene does not provide effective selection of random integrants.

(c) Transformation with vectors conferring Nst resistance.

The antibiotic nourseothricin is a member of the streptothricin group of antibiotics (Krügel et al., 1993). Resistance to this antibiotic is generally mediated by ace-

TABLE I

U. maydis transformation frequencies with various plasmids[a]

Plasmid	Transformants/μg DNA
pCM54	452
pSAT103	0
pSAT104	132
pSAT112	275
pSATX1	98
pBEN102	1200
pBENX1	235
pUBLE10	335
pBLEX1	398
pCM54+pSAT112	326

[a]Transformation of *U. maydis* strain 521 was performed using established methods (Wang et al., 1988; Tsukuda et al., 1988) and 2×10^6 protoplasts per experiment. Values represent averages of two independent transformation experiments. Growth conditions for *U. maydis* were as previously described (Barrett et al., 1993). All antibiotics were dissolved in water, except Ben which was dissolved in dimethylsulfoxide, and tested on DCM (Holliday, 1974). Antibiotics were employed at the following concentrations for selection of transformants: Ble (phleomycin; Sigma, St. Louis, MO, USA), 10 μg/ml; Hy (Calbiochem, La Jolla, CA, USA), 150 μg/ml; Ben (Dupont; generously provided by Noel Keen, University of California, Riverside, CA, USA), 1.5 μg/ml; and Nst (generously provided by Udo Gräfe, Hans-Knöll-Institut für Naturstoff-Forschung e.V., Beutenbergstrasse 11, 07708 Jena/Thüringen, Germany), 150 μg/ml. Nst is available from Dr. Albert Hinnen, Hans-Knöll-Institut für Naturstoff-Forschung e.V., Jena. For cotransformation with pCM54 and pSAT112, protoplasts were plated on medium containing both Hy and Nst. pCM54 serves as a control vector with an *ARS* and a Hy^R cassette (Tsukuda et al., 1989).

tylation; genes encoding this activity include the *nat1* gene of *S. noursei* (Krügel et al., 1993) and the *sat-1* and *sat-2* genes of *E. coli* (Heim et al., 1989; Tietze and Brevet, 1990). The minimum inhibitory concentration for *U. maydis* on DCM is 30 μg Nst/ml. To test *sat-1* gene function in *U. maydis*, vectors were constructed to place the gene downstream from the *U. maydis hsp70* or *gap* promoter (Wang et al., 1988; Holden et al., 1989; Smith and Leong, 1990; Kinal et al., 1991). The *ARS*-containing plasmids pSAT103 and pSAT104 (not shown) were constructed as intermediates for other vectors. pSAT104 carries the *sat-1* gene in the sense orientation with respect to the *U. maydis hsp70* promoter, and is expected to produce a functional product in *U. maydis*. Plasmid pSAT103 carries the *sat-1* gene in the antisense orientation relative to the promoter. The vector pSAT112 (Fig. 2C) has the same *sat-1* resistance cassette in a vector with a MCS and the blue/white screening capability of pBluescript KS^+. Vectors pSAT104, pSAT112 and pSATX1 (Fig. 2D) are capable of transforming *U. maydis* to Nst resistance (Table I). As expected, pSAT103 fails to transform cells to Nst resistance (Table I). It should be noted that after prolonged incubation (one week), a significant number of background colonies appear when plates contained 50 μg Nst/ml or less. Transformants containing pSAT104, pSAT112 or pSATX1 are able to grow upon transfer to medium containing 150 μg Nst/ml, a concentration which is inhibitory to nontransformed *U. maydis* cells. Surprisingly, the Nst^R transformants generally grow poorly in liquid medium containing Nst, even at low concentrations (50 μg Nst/ml). The presence of the *sat-1* gene in eight resistant transformants was confirmed by PCR amplification (data not shown). Overall, these results indicate that the *sat-1* gene is a useful selectable marker in *U. maydis*. We have also found that integrative vectors carrying the *sat-1* gene successfully transform *U. maydis* to Nst resistance.

Resistance to Nst, conferred by the *sat-1* gene, has also been used for the selection of transformants in mammalian cells (F. Tufaro, personal communication) and *Leishmania* (R. McMaster, personal communication). Nst and the *sat-1* gene may prove to be generally useful for transformation of eukaryotic cells.

(d) Transformation with vectors conferring Ble resistance

Vectors have also been constructed to make use of the Sh-*ble* and Tn5-*ble* genes (Drocourt et al., 1990; Gatignol et al., 1990) which confer resistance to Ble and other antibiotics (zeocin and tallysomycin). Ble resistance has been used for transformation of *U. hordei* (Bakkeren and Kronstad, 1993); in addition, the Sh-*ble* expression cassette constructed for *U. hordei* has been used in *U. maydis* expression vectors, as described by Kinal et al. (1993). The coding region of the Sh-*ble* gene was cloned downstream from the *gap* gene promoter (Smith and Leong, 1990) in the *ARS*-containing expression vector pUXV1, which confers Hy resistance (Kinal et al., 1991). *U. maydis* transformants carrying the resulting plasmid (pUXVble1) were first selected on Hy and then tested for resistance to various levels of Ble. These transformants were resistant to 20 μg Ble/ml on DCM; the minimum inhibitory concentration for Ble on this medium is 5 μg/ml.

Vectors specifying Ble^R and containing MCS sequences are shown in Fig. 2E and F. Table I shows transformation frequencies of *U. maydis* strain 521 with the vectors pUBLE10 and pBLEX1 (Fig. 2E,F) by direct selection on medium containing 10 μg Ble/ml. Transformants harboring pUBLE10 grew on medium containing up to 50 μg Ble/ml, the highest level tested. Integrative vectors also have been found to confer Ble^R upon transformation (data not shown).

It should be noted that both sensitive and resistant strains of *U. maydis* often show an altered cellular morphology when grown on sub-inhibitory concentrations of Ble (L. Giasson, personal communication). Specifically, the cells are elongated and exhibit cell wall distortions. None of the other antibiotics employed in this study have this influence on cell morphology.

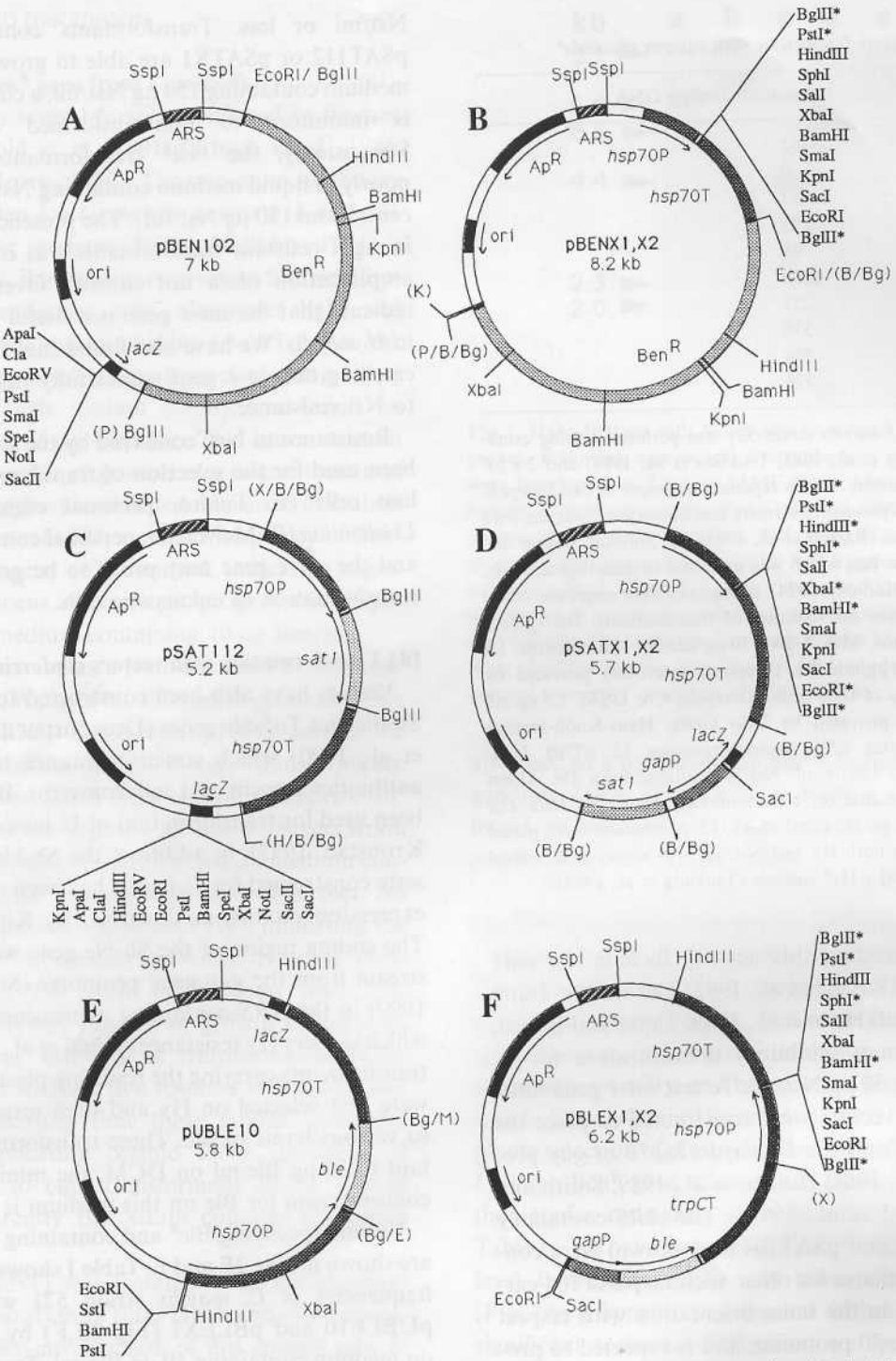

Fig. 2. Restriction maps of transformation and expression vectors. The vectors are based on *U. maydis* expression signals (Wang et al., 1988, Holden et al., 1989; Smith and Leong, 1990; Kinal et al., 1991) and an *ARS* (Tsukuda et al., 1988). The remaining sequences of the vectors are from common cloning vectors, pUC18 (Yanisch-Perron et al., 1985) and pBluescript KS$^+$ (Stratagene, La Jolla, CA, USA). Standard methods were used for DNA manipulations (Sambrook et al., 1989). The MCS sequences are from the *Bgl*II fragment of plasmid pIJ2925 (Janssen and Bibb, 1993) (**B**, **D** and **F**), from pBluescript KS$^+$ (**A**, **C**) and from pDWH10 (**E**; Wang et al., 1988; Tsukuda et al. 1988; Holden et al., 1989; originally from pUC12). Only unique MCS sites are shown in **A**, **C** and **E**; for **B**, **D** and **F**, an asterisk denotes unique sites. Expression vectors designated X1/X2 differ in the orientation of the MCS; the X1 orientation is shown. (**A,B**) pBEN102 has a 3.9-kb *Pst*I-*Eco*RI fragment from the cosmid pBEN1 (Ben^R) in a pBluescript KS$^+$ vector with a *U. maydis ARS* sequence (Tsukuda et al., 1988). Plasmid pBEN103 is identical to pBEN102, except that it lacks the *U. maydis ARS* (not shown). To construct pBENX1/X2, the *U. maydis hsp70* promoter and terminator sequences, separated by a MCS, were added to a vector containing the fragment conferring Ben^R from pBEN102. (**C,D**) The pSAT vectors contain nt 244 to 784 of the *sat-1* gene (Heim et al., 1989) amplified by PCR using the primers (5′-TG*AGATCT**GCGTTAGGCGTCATA**TG**AAG) and (5′-AA*AGATC***T*G**TTA**GGCGTCATCCT GTG). Bold sequences are the *sat-1* gene start and stop codons; italicized sequences are *Bgl*II sites and sequences before the asterisk are not from the *sat-1* gene. Plasmids pSAT101 and pSAT102 (not shown), which are intermediates of pSAT112, contain the *sat-1* PCR product cloned in plasmid pDWH10 in anti-sense and sense orientations, respectively. pSAT112 (**C**) was constructed from pSAT102 to include the MCS, a *U. maydis ARS*

(e) Conclusions

We have constructed a set of vectors which demonstrate that an allele of the *U. maydis Tub* gene, the *sat-1* gene and the Sh-*ble*/Tn*5*-*ble* genes are effective selectable markers for the transformation of *U. maydis*. We find that there are minor variations in the efficacy of these genes and the corresponding antibiotics for transformation. For example, the transformants selected on Nst and Ben appear more slowly than those on Ble, and Nst^R transformants grow poorly in liquid medium containing Nst. However, these genes generally are as effective as the commonly used Hy^R gene for the selection of transformants.

ACKNOWLEDGEMENTS

The authors thank Luc Giasson for comments on the manuscript, Yigal Koltin, Hai Kinal and Jeremy Bruenn for the Sh-*ble* and Tn*5*-*ble* genes, and Erhard Tietze for the *sat-1* gene. We also thank Noel Keen for providing benomyl, Udo Gräfe for providing nourseothricin and Tom Davis for technical help. This work was supported by strategic and operating grants from the Natural Sciences and Engineering Research Council (to J.W.K.).

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(Tsukuda et al. 1988) and the *lacZ* sequence from pBluescript KS^+. Expression vectors, pSATX1/X2 (**D**), use the *U. maydis gap* promoter (Smith and Leong, 1990; Kinal et al., 1991) for *sat-1* transcription and contain the *hsp70* promoter and terminator sequences separated by an MCS. (**E,F**) Ble^R vectors were constructed with the Sh-*ble* gene from the phleomycin-producing Actinomycete *Streptoalloteichus hindustanus* (in pUT771; Drocourt et al., 1990; Cayla Laboratories, Toulouse, France). The Sh-*ble* coding region was expressed using the *hsp70* promoter and terminator sequences from pDWH10. For vector pUBLE10 (**E**), the *hsp70*-Sh-*ble*-resistance cassette was obtained as a 2.7-kb *Hin*dIII fragment and used to replace the 3.1-kb *Hin*dIII fragment containing the Hy^R cassette in plasmid pCM54 (Tsukuda et al., 1988, 1989). The Sh-*ble* gene was also cloned as a 1.5-kb *Eco*RV-*Bgl*II fragment (which includes a transcription terminator from yeast) into the *Bam*HI-site of pUXV1 (Kinal et al., 1991) to create the intermediate plasmid pUXVBLE1. A 3.1-kb *Hin*dIII fragment of pUXVBLE1, containing the Hy^R cassette, was replaced by the 2.1-kb *Hin*dIII fragment of plasmid pDWH10. The resulting vectors, pBLEX1/X2, are missing a 650-bp *Xba*I fragment (upstream from the promoter) and contain a MCS. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RV; H, *Hin*dIII; K. *Kpn*I; M, *Mlu*I; P, *Pst*I. The restriction sites in parentheses were employed in the construction of the vectors; these sites are no longer available for digestion with the indicated enzymes.

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