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Isolation of a Degeneration-Resistant Mutant of *Clostridium acetobutylicum* NCIMB 8052

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Unless periodically grown from germinated spores, *Clostridium acetobutylicum* tends to degenerate (that is, to spontaneously lose the capacity both to produce solvents and to develop into spores). To obtain mutants that are deficient in degeneration, *C. acetobutylicum* NCIMB 8052 was mated with *Enterococcus faecalis* BM4110 harboring transposon Tn*1545*. We developed a degeneration resistance assay based on a secondary effect of degeneration, the production of toxic levels of acetic and butyric acids. Erythromycin-resistant transconjugant clones were tested individually for longevity by repeated and timely subculturing. One long-lived mutant, A10, survived 18 ± 3 transfers (mean ± standard deviation; $n = 20$) before extinction, while the wild type (parental cells) survived 6.6 ± 1.5 transfers ($n = 11$). The three-fold difference in longevity is statistically significant. In a batch culture in a rich medium, the wild-type cells degenerated within 24 h after inoculation with 1% of an overnight culture derived from germinated spores. In contrast, A10 cells were able to switch to solventogenesis and to sporulate. In a minimal medium with greater buffering capacity, both cell types produced solvents and spores. Southern blots of *Eco*RI and *Hin*dIII restriction digests of A10 chromosomal DNA (but not parental DNA) showed that only one copy of Tn*1545* was inserted into the clostridial chromosome. Our findings are consistent with the hypothesis that there was an alteration at a regulatory locus that was effected by the insertion of the transposon.

When the saccharolytic spore-forming anaerobic bacterium *Clostridium acetobutylicum* is growing in the exponential phase in a batch culture, it converts sugars to acetic and butyric acids and produces large quantities of H_2 and CO_2 (reviewed in references 13 and 20). In this way the medium is acidified to pH values as low as 5 or lower, depending on the strain (12, 21). At this point, the cells usually switch metabolism and convert the organic acids to nonacidic solvents, primarily butanol and acetone, thus raising the pH of the culture. Concomitantly, the cells start to develop endospores. It has also been known for 100 years (10) that saccharolytic clostridia often lose the ability to produce solvents and to sporulate; i.e., they degenerate (1, 7, 8, 11, 13, 15, 16, 25). To preserve highly solventogenic strains for industrial use, it has been necessary to routinely start with fresh cultures derived from heat-treated spores (19). The mechanisms responsible for clostridial degeneration and the regulatory mechanisms involved in the initiation of solventogenesis and sporulation are not understood yet.

In this paper we describe the isolation of a mutant of *C. acetobutylicum* NCIMB 8052 that is resistant to degeneration and the methods used to obtain it. Strain NCIMB 8052 was chosen as the parental strain because it seems to be highly unstable (9) compared with other commonly used strains of *C. acetobutylicum* (25).

MATERIALS AND METHODS

Growth of cells. For long-term storage, sporulated cultures of *C. acetobutylicum* NCIMB 8052 (formerly NCIB 8052) were dried onto a sterilized mixture consisting of equal volumes of sand, commercial African violet potting soil, and $CaCO_3$. For short-term storage, overnight inoculum cultures (see below) were diluted $1:10^6$, and 100-μl portions were plated onto plates containing medium T6 (see below) supplemented with 1.8% agar. Each plate contained approximately 10 colonies, which grew large enough (i.e., about 1 cm in diameter) within 1 to 2 weeks so that spores could be picked easily from the brownish centers of the colonies. (The most efficient solvent-producing cells are found in the centers of Adler-Crow type I colonies [1].) Inoculum cultures were prepared by placing a visible amount of spores or soil containing spores into 10 ml of medium T6 in thick-walled 20-ml tubes (Wheaton Co., Millville, N.J.) fitted with rubber septa and sealed with crimped aluminum seals. The tubes were heated in a water bath at 70°C for 10 min to help spore germination and then incubated at 37°C overnight.

For batch culture experiments 0.5- to 1.5-ml aliquots of the overnight inoculum cultures were injected into 120-ml sealed serum bottles (Wheaton) containing 50 ml of medium by using CO_2-flushed syringes for the transfers and incubated at 37°C. The tops of the bottles were sterilized by wetting the caps with alcohol and then flaming them. The bottles were vented with needles to relieve excess gas pressure before the cultures were sampled for optical density and pH measurements and for gas chromatography samples. CO_2-flushed syringes were used for transfers. The cell suspensions were diluted with 0.1 M sodium phosphate buffer (pH 7.0) so that the optical density at 600 nm was measured at <0.1. The fermentation end products were measured as described previously (21) with a model 9A gas chromatograph (Shimadzu Scientific Instruments, Columbia, Md.) equipped with a flame ionization detector.

Culture media. Medium T6 was a modification of medium TYA (18) and contained (per liter) 6 g of tryptone (Difco Laboratories, Detroit, Mich.), 2 g of yeast extract (Difco), 0.5 g of KH_2PO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 3 g of ammonium acetate (38.9 mM), and 0.5 g of cysteine hydrochloride. The pH was adjusted to 6.5 with NaOH. Minimal medium DMM was a modification of the

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minimal medium of Andersch et al. (2) [$Na_2S_2O_4$ was omitted and $(NH_4)_2SO_4$ (2 g/liter) and $CH_3COONa \cdot 3H_2O$ (8.16 g/liter) were added]. The media were autoclaved to sterilize them, and the vitamin solutions were filter sterilized. Glucose was autoclaved separately and was added to both media at a final concentration of 60 g/liter. Erythromycin was used at a final concentration of 10 μg/ml. Liquid media and plates were deoxygenated overnight under an atmosphere containing 5% H_2, 5% CO_2, and 90% N_2 in an anaerobic glove box (Forma Scientific Co., Marietta, Ohio), which was also used for anaerobic manipulations.

Isolation of transposon insertion mutants of *C. acetobutylicum* NCIMB 8052. To generate clostridial Tn*1545* insertion mutants, *C. acetobutylicum* NCIMB 8052 cells were mated with *Enterococcus faecalis* BM4110::Tn*1545* (obtained from P. Courvalin, Institut Pasteur, Paris, France) as described by Woolley et al. (26). Tn*1545* is a 25.3-kb conjugative shuttle transposon that confers resistance to kanamycin (*aphA-3*), erythromycin (*ermAM*), and tetracycline (*tetM*) (5). Transconjugants were selected by plating the mated mixtures onto agar plates containing medium DMM supplemented with 10 μg of erythromycin per ml and then isolating bacterial clones by streaking out on agar plates containing medium T6 supplemented with erythromycin. After several days most colonies were raised and glossy and had well-defined edges. One week later most colonies had developed flat, pale, translucent outgrowths and resembled the colonies of degenerate or asporogenous clostridia that have been described previously (1, 13, 25). When examined by phase-contrast microscopy, the centers of the colonies contained numerous phase-bright spores, whereas the offshoots contained short rod-shaped cells which were not culturable, which was consistent with the hypothesis that the latter cells were degenerate cells. The centers of the colonies became brown after 2 to 3 weeks and contained mostly phase-bright spores. Transconjugant colonies that did not produce diffuse offshoots were selected for further testing for degeneration resistance.

Measurement of degeneration resistance. The assay used to measure degeneration resistance was based not on the loss of solventogenesis and sporogenesis per se, but on the loss of viability during transfer. This secondary effect of degeneration is the result of the production of toxic levels of acetic and butyric acids by cells that do not switch to solvent production. To determine whether transconjugant colonies were longer lived than the parental cells, the cells were repeatedly subcultured and the number of generations reached, starting from germinated spores, was determined. In this procedure 1 μl of an overnight inoculum culture was transferred to 1 ml of medium in a 2-ml well of a 24-well microtiter plate and incubated at 35°C. The cells were subcultured every 2 days by using the same 1:1,000 dilution. Thus, each transfer was equivalent to approximately 10 generations. Within 2 days the cells grew to a high optical density and produced gas, which caused the medium to foam. The turbidity and froth were easily observed through the vinyl front of the anaerobic glove box. After a number of transfers the cultures grew to a lower density than previously seen and there was less foaming due to gas production. Once this stage was reached, one more transfer usually resulted in no visible growth. The last well showing growth was defined as the end point of the assay. Each potential mutant was tested at least three times in triplicate, and the mean number of generations of growth was compared with the mean number of generations of growth of parental cells grown under the same conditions.

Detection of Tn*1545* in chromosomal DNA from the mutant. Chromosomal DNAs from wild-type (parental) and mutant cells were isolated from protoplasts prepared as described by Williams et al. (24) by using the method of Noirot (17), as described by Woolley et al. (26). The DNAs were digested with restriction endonucleases (New England Biolabs, Beverly, Mass.), the fragments were separated by agarose gel electrophoresis, and Southern analyses were carried out by using standard methods (14). The probe used, an intragenic 1.7-kbp *Hin*dIII-*Kpn*I fragment that was located in the *tetM* gene of Tn*1545* (4), was labelled with digoxygenin and was detected by using a commercial kit (Genius; Boehringer Mannheim, Indianapolis, Ind.). Tn*1545* was also detected with a probe prepared by using pAT187, a plasmid containing the *aphA-3* gene (23).

RESULTS

Isolation of degeneration-resistant mutants of *C. acetobutylicum* NCIMB 8052. There is no known selection pressure which favors nondegenerating clostridia, and in fact degenerate cells appear to have a growth advantage (13). It was necessary, therefore, to seek degeneration-deficient mutants by surveying a large number of transconjugants. We obtained more than 460 colonies of Tn*1545*-containing transconjugants from 16 matings of *C. acetobutylicum* NCIMB 8052 with *E. faecalis* BM4110::Tn*1545* and selected 220 transconjugants for growth on medium T6 plates containing erythromycin. A total of 26 colonies were selected for degeneration assays from the colonies that did not produce pale outgrowths after 3 to 5 days of growth; these colonies contained degenerate cells.

Degeneration assay. Clostridial degeneration is typically observed after cells are subcultured from the exponential phase of growth. In the medium which we used degenerate cells produce toxic concentrations of acetic and butyric acids as they fail to switch to solvent production. Since the acids can diffuse into both wild-type and degenerate cells, both types of cells are overacidified and killed at a low medium pH (3, 22). Loss of viability during transfer is thus an indirect effect of degeneration. We developed a subculturing protocol to identify long-lived mutant cells; this protocol was based on the rationale that such cells would not overacidify their media. The numbers of generations achieved by selected transconjugants (i.e., potential degeneration-resistant mutants) were compared with the numbers of generations observed for the parental cells. In this procedure cultures were always initiated from germinated spores. We found that subculturing the cells by using a 1:1,000 dilution every 2 days in medium T6 at 35°C allowed us to identify four putative degeneration-deficient mutants. In addition to these mutants, one transconjugant degenerated after approximately 120 generations, and the rest of the transconjugants were as short lived as the parental type. One transconjugant seemed to be an amino acid auxotroph, because it required the addition of Casamino Acids (Difco) for growth when the assay was performed in minimal medium (data not shown).

Mutant A10. Under the conditions described above, mutant A10 survived three times longer in subculture than the wild type (Fig. 1). The number of transfers before extinction for mutant A10 was 18 ± 3 (mean ± standard deviation; n = 20), compared with 6.6 ± 1.5 transfers (n = 11) for the parental cells. A comparison of the data by using Student's t test showed that the means were significantly different ($P < 0.005$).

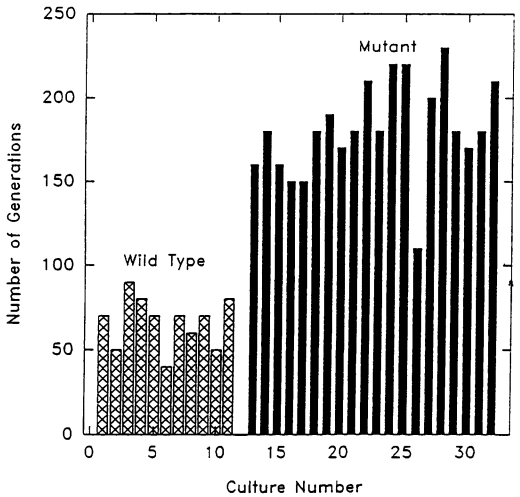

FIG. 1. Survival of serially transferred wild-type *C. acetobutylicum* NCIMB 8052 and degeneration-resistant mutant A10. The assay conditions used are described in the text.

Growth of A10 and the wild type in batch cultures in rich medium T6. The different behaviors of the two cell types were evident when the cells were grown in batch cultures in medium T6 (Fig. 2). Both cell types grew rapidly, but the wild type reached a final optical density of 1.6, while A10 grew to an optical density as high as 4.4 (Fig. 2A). The wild type degenerated within 24 h of inoculation with an overnight culture derived from germinated spores, but the mutant was able to switch to solventogenesis and sporulation. The parental cells acidified the medium to pH 4.8 by producing acetic and butyric acids. At this low pH value the interior of the cells presumably became acidified below the pH that is tolerated by these organisms (21). In contrast, the pH of the mutant culture exhibited the typical biphasic response observed in batch cultures of nondegenerated *C. acetobutylicum* (Fig. 2B). Thus, mutant A10 acidified the medium to only pH 5.08 during the acetogenic phase, and the medium pH increased to 6.2 as the cells initiated the conversion of acids to solvents. In contrast, the pH of the wild-type culture was 4.89.

The parental culture contained only 0.4 g of total solvents (butanol and acetone) per liter and 4.9 g of acetic acid (51 mM) plus butyric acid (21 mM) per liter after 100 h. In contrast, mutant A10 cultures contained 18.7 g of total solvents per liter (13.0 g of butanol per liter [176 mM] and 5.2 g of acetone per liter [89 mM]) and much less acid than the wild-type cultures (1.2 g/liter). The presence of erythromycin in the medium had no effect on the growth of A10 or its pH profile (data not shown). Under those conditions the A10 cultures contained 17.4 g of total solvents per liter and 1.38 g of acetic acid plus butyric acid per liter when they were assayed at the end of the experiment.

Growth of A10 and the wild type in minimal medium. In minimal medium both strains formed solvents and sporulated (Fig. 3). Since medium DMM contains 80 mM acetate, compared with 40 mM acetate in medium T6, it has a greater buffering capacity than the rich medium. This was confirmed by titrating the two media (data not shown). The wild type and the mutant both grew to an optical density of approximately 3.0, but acidified the medium to only pH 5.5 to 5.6. These values are within the pH range tolerated by the cells in the presence of permeable organic acids, as the cells were

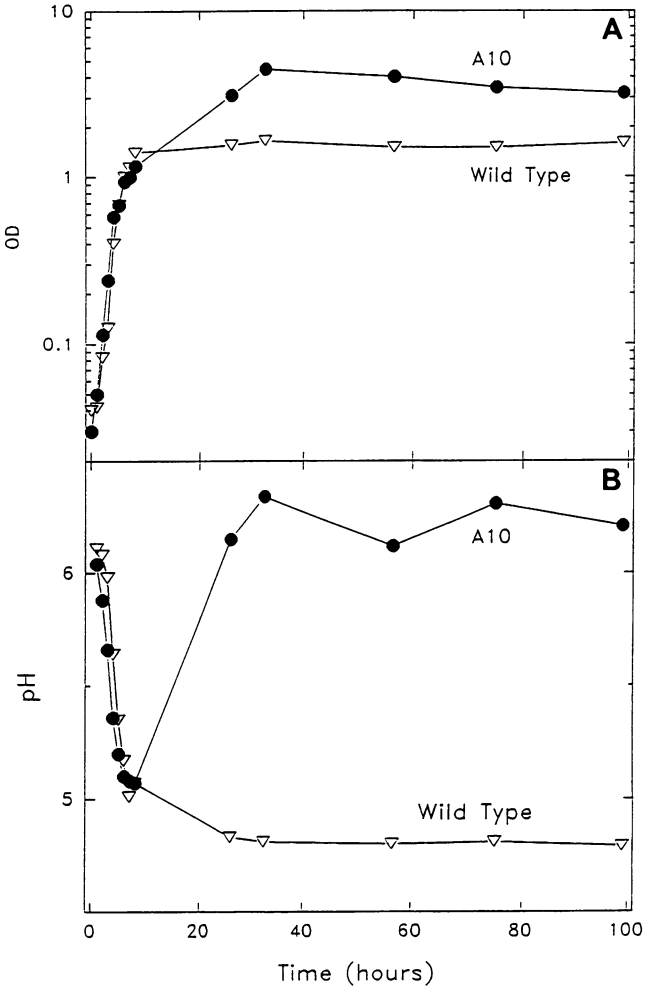

FIG. 2. Growth of mutant A10 and wild-type *C. acetobutylicum* NCIMB 8052 in batch cultures in rich medium T6. Growth of the cells (A) and the pH profiles of the medium (B) were determined as described in the text. OD, optical density.

able to switch to solventogenesis. After 16 days the final pH values for the wild type, mutant A10, and A10 grown in the presence of erythromycin were 6.13, 6.07, and 5.93, respectively. These high pH values correlated with the low acid levels (2.0, 1.36, and 1.4 g/liter) present in the cultures. No significant differences were observed in the relative concentrations of acetic and butyric acids in the mutant and the wild type, suggesting that the carbon flow in the various pathways was not affected by the mutation. The solvent concentrations were high (14.5, 14.0, and 16.0 g of total solvents per liter for the three cultures).

Mutant A10 contains one copy of Tn*1545*. It has been observed that multiple inserts of Tn*1545* in the clostridial chromosome occur frequently (4, 26). Degeneration-resistant mutant A10, however, contained only one copy of the transposon, as only one restriction endonuclease fragment of the chromosomal DNA of mutant A10 gave a positive signal when it was probed with an intragenic fragment of the *tetM* gene (Fig. 4). The *Eco*RI fragment that was ca. 30 kb long was large enough to contain the entire transposon, which lacks internal *Eco*RI recognition sites, together with flanking clostridial DNA. Tn*1545* contains three *Hin*dIII recognition

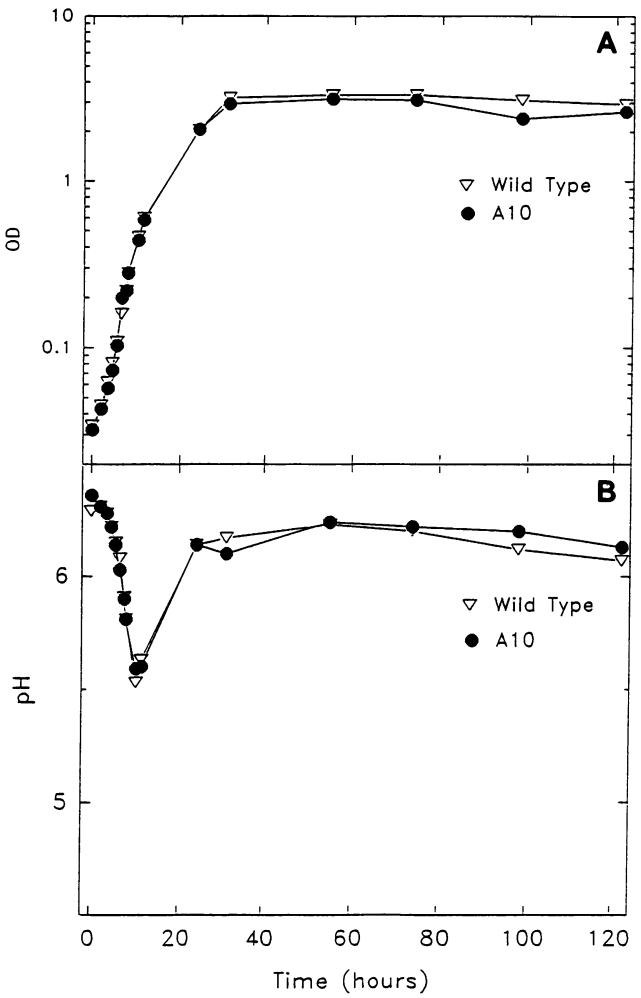

FIG. 3. Growth of *C. acetobutylicum* NCIMB 8052 and mutant A10 in minimal medium DMM. Growth (A) and pH profiles (B) were determined as described in the text. OD, optical density.

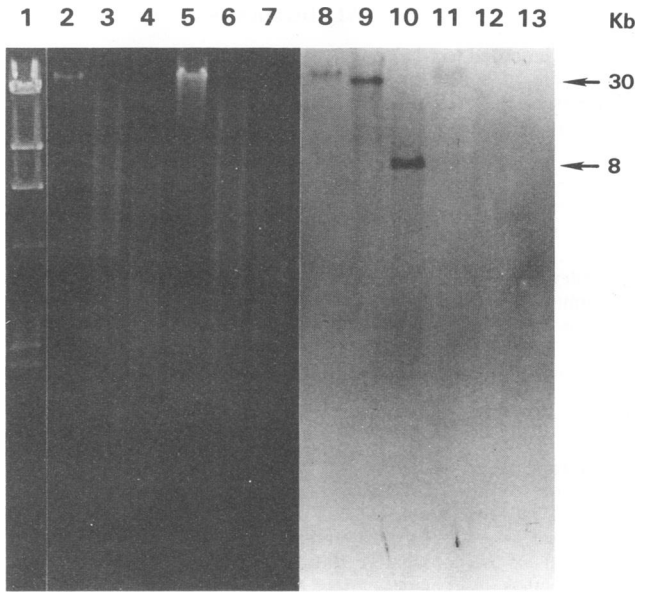

FIG. 4. Agarose gel electrophoresis (lanes 2 through 7) and Southern blots (lanes 8 through 13) of chromosomal DNAs from mutant A10 and parent *C. acetobutylicum* NCIMB 8052. Lane 1 contained a molecular size marker (*Hin*dIII-digested lambda). Lanes 2 and 8, mutant DNA before digestion; lanes 3 and 9, mutant DNA after digestion with *Eco*RI; lanes 4 and 10, mutant DNA after digestion with *Hin*dIII. The following lanes contained ethidium bromide-stained DNA but show no hybridization: lanes 5 and 11, undigested wild-type DNA; lanes 6 and 12, DNA digested with *Eco*RI; lanes 7 and 13, DNA digested with *Hin*dIII.

sites, and the *tetM*-containing 8-kb fragment of A10 chromosomal DNA corresponds to the right end of Tn*1545* (5) plus flanking clostridial DNA. When tested with a probe containing the *aphA-3* gene, which is located near the left end of Tn*1545* (5), A10 chromosomal DNA digested with *Eco*RI and *Hin*dIII again produced only one signal for each digest (data not shown). As expected, the *Eco*RI fragment was large (ca. 30 kb), while the *Hin*dIII fragment was approximately 7.4 kb long. Again, the wild-type DNA did not hybridize with the probe.

DISCUSSION

One current view concerning clostridial degeneration is that it is the result of progressive overgrowth by phenotypic variants which are deficient to varying degrees in the capacity to form solvents and the capacity to sporulate (1, 13, 16, 25). The culture conditions, the composition of the growth medium, and other unknown factors appear to determine the nature of the final population (9, 11, 13, 16, 25). The gradual change in the population over time is observed most clearly in chemostat cultures (26) and in colonies on agar plates (1), as we also found.

Different strains of *C. acetobutylicum* degenerate at different rates, which is consistent with the heterogeneity in colonial morphology, biochemical characteristics, and chromosomal size observed for the different isolates (23, 25). For example, Woolley and Morris (25) found that in chemostat cultures *C. acetobutylicum* ATCC 824 and DSM 1731 produced asporogenous cells that continued to form either solvents or acids, depending on the pH of the medium. In contrast, strain NCIMB 8052 cultures invariably degenerated to asporogenic populations incapable of solvent production. However, the degenerate NCIMB 8052 cells reverted to the wild-type phenotype at a frequency of about 5×10^{-6}. This suggested to Woolley and Morris that a single mutational event at a global regulatory locus determines the degenerate phenotype in this strain. In fact, the report of Woolley and Morris was the impetus for the work described in this paper, and strain NCIMB 8052 was chosen as the parental strain because of its instability.

Our intention was to use transposon insertion to obtain stable mutants that are degeneration deficient and that would permit us to the identify the gene supposedly involved in degeneration. Our approach was based on the assumption that there is at least one gene that is negatively regulated in the normal switch from acidogenesis to solventogenesis and sporulation. Precedent for both positive and negative regulation of initiation of sporulation exists in the genus *Bacillus* (reviewed in reference 6).

We succeeded in isolating mutant A10, which is significantly more resistant to degeneration than its parent. We also found that the mutant DNA possesses only one copy of the transposon. As far as we know, this is the first report of a laboratory-generated mutant deficient in degeneration.

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