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Comparison of two transformation systems for the assay of the *Neurospora* photoreactivating enzyme

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SUMMARY

The *Neurospora crassa* photoreactivating enzyme has been assayed for by the *Hemophilus influenzae* and *Bacillus subtilis* transformation systems. In contrast to the *H. influenzae* system, u.v.-treated transforming DNA from *B. subtilis* did not give evidence of reactivation of u.v. lesions by crude enzyme extracts from *N. crassa* when exposed to photoreactivating light. The u.v. dose required to inactivate *B. subtilis* transforming DNA is about ten times that required to inactivate *H. influenzae* DNA to the same level of survival. This difference in dose required to inactivate DNA's of about the same base composition probably reflects the greater u.v. resistance of the *B. subtilis* recipient strains used. Hypotheses are considered which suggest that *N. crassa* crude enzyme extracts contain either nucleases which degrade *B. subtilis* transforming DNA excessively or an inhibitory factor which affects the transformation process itself.

1. INTRODUCTION

Photoreactivation is the reversal of the lethal effects of ultraviolet light (u.v.) by post treatment with long-u.v. and visible irradiation (Kelner, 1949). An enzyme has been extracted from *Escherichia coli* (Rupert, Goodgal & Herriott, 1958), *Saccharomyces cerevisiae* (Rupert, 1960) and *Neurospora crassa* (Terry, Kilbey & Howe, 1967; Terry & Setlow, 1967) which, when exposed to long-u.v. or visible light, is reported to be capable of reactivating u.v.-inactivated transforming DNA.

Terry *et al.* (1967) and Terry & Setlow (1967) assayed for the photoreactivating (PR) enzyme in crude enzyme extracts of *N. crassa* using u.v.-inactivated transforming DNA from *Hemophilus influenzae*. In a representative experiment they were able to obtain a tenfold increase in streptomycin resistant (*strep-r*) transformants from *H. influenzae* transforming DNA which had been inactivated to the 1 % survival level following treatment of the u.v.-DNA with crude *Neurospora* enzyme extracts and light. Tuveson & Mangan (1970) attempted to assay for the *Neurospora* PR enzyme using the reactivation of u.v.-inactivated transforming DNA from *Bacillus subtilis* for adenine independence as their assay. In these experiments, crude extracts from *Neurospora* were found not only incapable of reactivating the u.v. inactivated transforming activity, but also reduced the number of transformants below that of u.v.-DNA which had not been exposed to

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crude enzyme extracts. These results were explained on the basis of nucleases in the crude enzyme extracts from *Neurospora*.

In this paper we shall present evidence which resolves the apparent ambiguity between the results obtained with *H. influenzae* (Terry *et al.* 1967; Terry & Setlow, 1967) and *B. subtilis* (Tuveson & Mangan, 1970) based on the intrinsic u.v. sensitivity of the *H. influenzae* recipient strain when compared with the *B. subtilis* recipient.

2. MATERIAL AND METHODS

(i) *Fungal strains*. The *Neurospora crassa* strains used for the preparation of crude enzyme extracts were a wild type designated 74-OR23-1A (Fungal Genetics Stock Center no. 987) or an arginine-requiring strain (arg-4, 21502, a: Fungal Genetics Stock Center no. 778). The two microconidial strains used in the experiments testing for nuclease activity in *N. crassa* were a u.v.-sensitive carrying the *uvs-1* gene (Chang & Tuveson, 1967) and a double mutant derived directly from the *uvs-1* strain (*uvs-1 upr-1*; Tuveson & Mangan, 1970).

(ii) *Bacterial strains*. The streptomycin-sensitive *H. influenzae* recipient strain was kindly provided by Dr Jane K. Setlow, who had received it from R. M. Herriott, C. S. Rupert and S. H. Goodgal in 1960 (Setlow *et al.* 1968).

Dr J. Mangan provided the prototrophic DNA donor *B. subtilis* strain which was designated 168 P^-. The *B. subtilis* recipient strains were (1) Mu 8u 5u 1; -leu_8, -iso-leu, -met_5, which was provided by Dr J. Mangan; and (2) Mu 8u 5u 16; -ade_{16}, -leu_8, -met_5, which was provided by Dr K. Bott. Both of these *B. subtilis* recipients were derivatives of strains developed by N. Sueoka.

(iii) *Transforming DNA*. The transforming DNA carrying the *strep-r* marker used in the *H. influenzae* transformation experiments was provided by Dr Jane Setlow. The transforming DNA for the experiments involving *B. subtilis* was prepared from strain 168 P^- by the Marmur method (1961).

(iv) *Transformation*. The procedures used for transformation with *H. influenzae* transforming DNA were identical to those of Setlow *et al.* (1968). The procedures used for transformation with *B. subtilis*-transforming DNA were essentially those of Anagnostopoulos & Spizizen (1961) as modified by Bott & Strauss (1965) and Reiter & Strauss (1965).

(*v*) *Inactivation by u.v. of transforming DNA*. Both *H. influenzae* and *B. subtilis* transforming DNA were inactivated with a bank of six 8 W General Electric germicidal lamps (G 8 T 5) which delivered approximately 14 $ergs/mm^2$ at the level of the DNA solution. The DNA was dissolved either in 0·15M-NaCl (saline) or 0·15M saline +0·015M Na citrate and irradiated in 10 cm Petri dishes. Aliquots of transforming DNA were removed after varying periods of irradiation and used directly to assay for residual transforming activity or treated with crude enzyme extracts following which residual transforming activity was assayed.

(vi) *Inactivation of cells by u.v.* Exponentially growing cells of *H. influenzae* were washed once in 0·15M-NaCl (saline). Ten ml of washed cells diluted in saline to $3 \cdot 0 \times 10^8$ cells/ml were irradiated in a 10 cm Petri dish. Aliquots were removed

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after varying periods of irradiation, chilled in ice, diluted (where appropriate) in saline and pour plated in molten (50 °C) growth medium (Setlow *et al.* 1968). Viable counts were made following 48 h incubation at 37 °C.

Exponentially growing cells *B. subtilis* were washed in cold (ice-bath temperature) minimal broth Davis without dextrose (Difco). Ten ml of washed cells were diluted in minimal broth Davis without dextrose to $1{\cdot}6 \times 10^8$ cells/ml and irradiated in a 10 cm Petri dish. Aliquots were removed after varying periods of u.v. treatment, chilled in ice, diluted (where appropriate) in cold minimal broth Davis without dextrose and plated on the surface of solidified complex medium [TB Broth Base (Difco) + 1·5 % Bacto-Agar (Difco)]. Viable counts were made following incubation at 37 °C for 24 h.

(vii) *Preparation of crude enzyme extracts of* Neurospora. Mycelium was prepared by inoculating 2 l flasks containing 1 l of Vogel's minimal medium N with from 10^7 to 10^8 conidia and incubating in a shaker water-bath at 32 °C for 3–4 days. The resulting mycelium was harvested by filtration through cheesecloth, washed once in cold 0·066M phosphate buffer (Na–Na), pH 7·0, and extracted by grinding in a cold mortar and pestle in about 5 times its wet weight of cold buffer. The resulting extract was clarified by centrifugation in the Sorvall SS-34 rotor at 10^4 rev/min for 10 min. For those experiments in which nuclease activity in *Neurospora* crude enzyme extracts was being investigated, the mycelium was disrupted either by sonication (Tuveson, West & Barratt, 1967) or by pressing (West *et al.* 1967). Protein concentrations of the extracts were estimated by the method of Lowry *et al.* (1951) or the optical density of the solutions at 260 nm and 280 nm (Warburg & Christian, 1942). The extracts were sterilized by filtration through Metricel filters (pore size 0·45μ, Gelman Instrument Company, Ann Arbor, Mich.).

(viii) *Treatment of transforming DNA with crude enzyme extracts from* Neurospora. 0·2 ml of irradiated or unirradiated DNA was placed in a sterile 10 cm test-tube, following which 0·2 ml of crude *Neurospora* enzyme extract with a protein concentration of 1 mg/ml was added to the tube. Preparation of the reaction mixture was carried out in a room provided with KEN-RAD 40 W 'gold' fluorescent lights to prevent photoreactivation. For photoreactivation, the reaction mixture was placed in a beaker containing 37 °C water between the photoreactivating lamps which have been described previously (Tuveson & Mangan, 1970). The reaction was terminated by removing the tubes from the light and chilling in an ice bath.

(ix) *Nuclease activity assay.* To estimate the nuclease activity in the *Neurospora* crude enzyme extracts, a reaction mixture consisting of 0·2 ml tritium (^3H) labelled (sp. act. 16600 dpm/μg) u.v. irradiated (3×10^3 erg/mm^2) or unirradiated *B. subtilis* phage 2C DNA was incubated in 37 °C in the dark with 0·1 ml of crude enzyme preparation and 0·2 ml of 0·066M, pH 7·0, phosphate buffer (with or without 2·0 mM EDTA). The reaction mixture was assayed for cold TCA (trichloroacetic acid) precipitable counts in toluene scintillant. The results were expressed as the percentage dpm (disintegrations/min) solubilized per mg protein.

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Table 1. *Effects of* Neurospora *crude enzyme extracts on* Bacillus subtilis *u.v.-treated transforming DNA*

Competent* cells	0·06 γ DNA	0·06 γ† u.v.-DNA	0·06 γ‡ u.v.-DNA enzyme treated	*met*⁺ transformants/ml
	Components of the transformation tube			
+	−	−	−	0
−	+	−	−	0
−	−	+	−	0
−	−	−	+	0
+	+	−	−	$3\cdot1\times10^4$
+	−	+	−	$6\cdot1\times10^2$
+	−	−	+	$8\cdot5\times10^1$
+	−	−	+§	$7\cdot1\times10^1$

* Viable competent cells = $4\cdot5\times10^8$ = 0·007 % competence. It should be noted that the DNA concentration used is below saturation (saturation = 0·1 γ DNA/transformation tube or greater).

† U.v. dose = $3\cdot6\times10^4$ ergs/mm² = 2·5 % survival of met⁺ transforming activity.

‡ 0·2 ml of crude *Neurospora* enzyme extract (1 mg/ml protein) was mixed with 0·2 ml of u.v.-treated DNA (0·6 γ/ml). The mixture was exposed to PR light for 15 min at 37 °C. 0·2 ml of treated DNA was added to the transformation tube.

§ The u.v.-DNA-enzyme mixture was not exposed to light during the 15 min incubation period.

3. RESULTS

In a previous paper (Tuveson & Mangan, 1970) we reported that *Neurospora* crude enzyme extracts consistently reduced the transforming activity of *B. subtilis*-transforming DNA for adenine independence below that level attributable to u.v. irradiation. To ascertain that this result was not specific to the ade_{16} marker originally used, we repeated these results with the met_5 marker. The results of this experiment are presented in Table 1. As was true for the ade_{16} marker, treatment of *B. subtilis*-transforming DNA with *Neurospora* crude enzyme preparations results in a further decline in *met*⁺ transforming activity over and above that attributable to u.v. This experiment was carried out at a concentration of DNA (0·06 γ/transformation tube) well below saturation (0·1 γ/transformation tube) to test the effects of substrate concentration on detectable PR enzyme activity. In our previous experiments we had worked at DNA concentrations at or above saturation and it was considered possible that enzymic PR of *B. subtilis*-transforming DNA might have taken place, but that the repair was not being detected since repaired DNA was simply not incorporated due to excess substrate. The results in Table 1 rule out this possibility as an explanation for our inability to demonstrate enzymic photoreactivation of *B. subtilis*-transforming DNA.

The negative results obtained for *in vitro* PR of irradiated *B. subtilis*-transforming DNA are in contrast to the positive results reported by Terry *et al.* (1967) and Terry & Setlow (1967) with *Hemophilus influenzae*. One of several possible explanations for our results might be the presence of nucleases in our preparations

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Table 2. *Solubilizing of ^3H counts from labelled* Bacillus subtilis *2C phage irradiated and unirradiated DNA by crude enzyme preparations from* Neurospora crassa

DNA*	Enzyme preparation†	1 mM-EDTA in reaction mixture	Counts solubilized per mg protein	% dpm solubilized	% dpm solubilized / mg protein
U	1 S	−	3082	20·1	50·2
U	1 S	+	3462	22·6	56·5
I	1 S	−	10050	65·0	162·5
I	1 S	+	6732	43·9	109·8
U	1 P	−	2634	34·3	43·9
U	1 P	+	1612	21·0	26·2
I	1 P	−	6529	85·1	106·4
I	1 P	+	5570	72·6	90·8
U	2 S	−	816	13·3	13·3
U	2 S	+	650	10·6	10·6
I	2 S	−	3471	56·5	56·5
I	2 S	+	2804	45·7	45·7
U	2 P	−	1139	17·7	18·6
U	2 P	+	770	12·5	13·2
I	2 P	−	4142	64·3	67·7
I	2 P	+	3788	58·8	61·9
U	3 P	−	918	33·7	15·0
U	3 P	+	509	18·7	8·3
I	3 P	−	1593	58·5	26·0
I	3 P	+	1379	50·3	22·3

* I = irradiated (3×10^3 ergs/mm^2); U = unirradiated.
† 1 = *uvs-1 upr-1*$^+$, 2 = *uvs-1 upr-1*, 3 = FGSC 778; S = sonicated, P = pressed.

which mask any effective repair by the PR enzyme. Therefore, experiments were conducted to test the ability of the *Neurospora* enzyme extracts to solubilize ^{3}H counts from u.v.-irradiated and unirradiated tritium labelled *B. subtilis* phage 2C DNA. The crude enzyme preparations which had been shown to completely inhibit or reduce transformation were used in these experiments. The results of these experiments are presented in Table 2. It appears that all of the extracts tested are capable of solubilizing tritium counts from phage DNA. The sonicated preparations seem to be as effective as pressed preparations in liberating counts from both irradiated and unirradiated DNA. The presence of EDTA in the reaction mixture did not seem to influence the solubilization of counts in any consistent manner. The striking feature of these results is that irradiated DNA is substantially more sensitive to degradation than is unirradiated DNA. This result is consistent for the two u.v.-sensitive microconidial strains (*uvs-1 upr-1*$^+$ and *uvs-1 upr-1*) and the *uvs-1*$^+$ *upr-1*$^+$ macroconidial strain.

In Table 3 results are presented which confirm the findings of Terry & Setlow (1967), who reported the reactivation of u.v.-inactivated *H. influenzae*-transforming DNA by crude enzyme extracts from *N. crassa*. It should be noted that the reactivation seen in this experiment is apparently enzymic since heating the *Neurospora* extract before mixing with the u.v.-inactivated DNA results in the

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Table 3. *Effect of* Neurospora *crude enzyme extracts on* Hemophilus influenzae *transforming DNA*

Components of transformation tube				
Competent* cells	0·015 γ DNA	0·015 γ† u.v.-DNA	0·015 γ‡ u.v.-DNA enzyme treated	*strep-r* transformants/ml
+	−	−	−	0·5
−	+	−	−	0
−	−	+	−	0
−	−	−	+	0
+	+	−	−	$5 \cdot 3 \times 10^4$
+	−	+	−	$2 \cdot 5 \times 10^2$
+	−	−	+	$5 \cdot 0 \times 10^3$
+	−	−	+§	$1 \cdot 7 \times 10^2$
+	−	−	+‖	$2 \cdot 1 \times 10^2$

* Viable competent cells = $4 \cdot 3 \times 10^8$ = 0·012 %. It should be noted that the DNA concentration used is below saturation (saturation = 0·1 γ DNA/transformation tube or greater).

† U.v. dose = $1 \cdot 68 \times 10^3$ erg/mm² = 4·5 % survival of *strep-r* transforming activity.

‡ 0·2 ml of crude *Neurospora* enzyme extract (1 mg/ml protein) was mixed with 0·2 ml of u.v.-treated DNA (0·15 γ/ml). The mixture was exposed to PR light for 15 min at 37 °C. 0·2 ml of treated DNA was added to the transformation tube.

§ The crude *Neurospora* enzyme preparation was boiled for 5 min before exposure to the u.v.-DNA.

‖ The crude *Neurospora* enzyme preparation was incubated with DNA for 15 min at 37 °C in the dark.

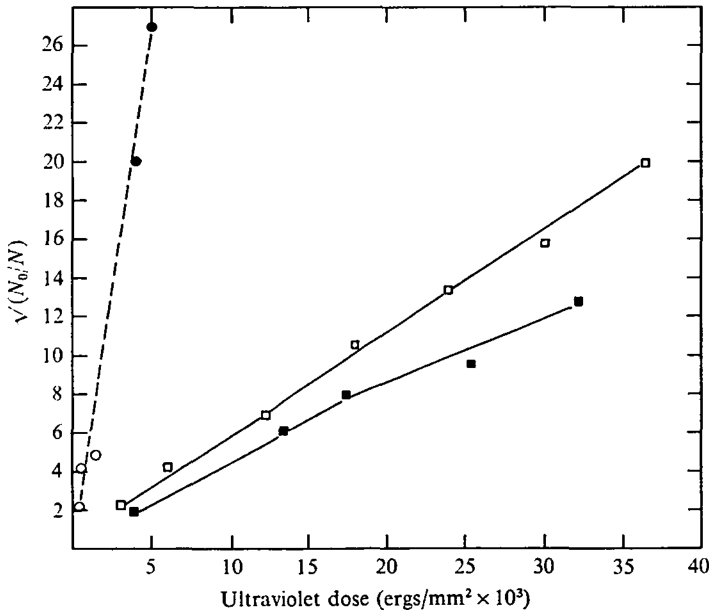

Fig. 1. U.v. inactivation of transforming DNA. ○, *strep-r* marker *H. influenzae*; ●, *strep-r* marker *H. influenzae* Setlow *et al.* (1968); □, ade_{16} marker *B. subtilis*; ■, met_5 marker *B. subtilis*.

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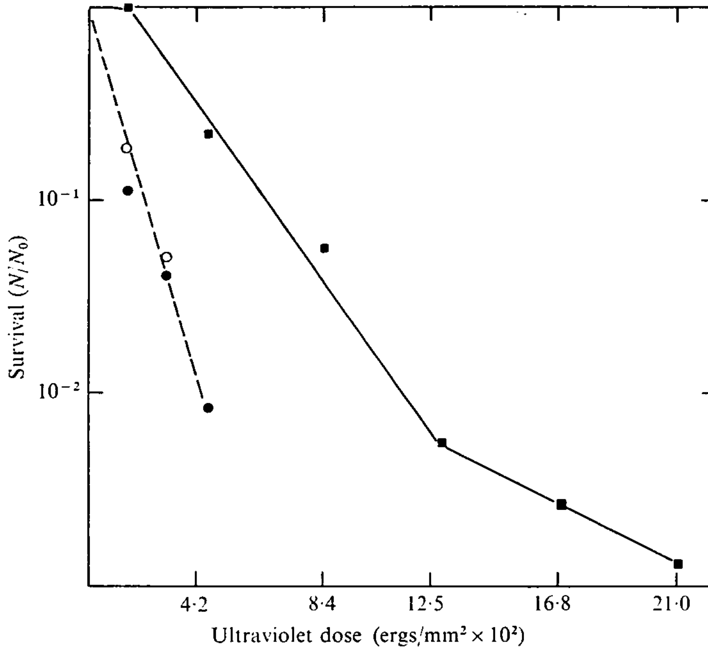

Fig. 2. U.v. inactivation of recipient cells used for transformation. ○, Experiment 1; ●, experiment 2 *H. influenzae*; ■, Mu 8u 5u 1 *B. subtilis*.

loss of the light-induced capability by the extract to reactivate u.v.-inactivated transforming DNA. It can be seen that incubation of u.v.-DNA in the dark with the crude enzyme preparation has no significant effect upon transforming activity.

The major difference in these two transformation systems is in the u.v. dose required to inactivate the transforming DNA to approximately the same level of surviving transforming activity. You will note from Tables 1 and 3 that it requires about ten times the dose of u.v. to inactivate *H. influenzae*-transforming DNA when compared to *B. subtilis*-transforming DNA to approximately the same survival level. In Fig. 1 we have presented a set of u.v. inactivation curves for transforming activity of markers in *B. subtilis* and *H. influenzae*. These data are presented on a square-root plot (Rupert & Goodgal, 1960) since the data tend to form straight lines the slope of which is a measure of u.v. sensitivity. The curve for the *strep-r* marker is a composite of our data together with data abstracted from results presented by Setlow *et al.* (1968). The results demonstrate that the ade_{16} and met_5 markers in *B. subtilis* are of the order of ten times more resistant to u.v. than is the *strep-r* marker in *H. influenzae*. It is well known that different markers within a transformable bacterial species can differ in their sensitivities to u.v. (Marmur *et al.* 1961; Munakata & Ikeda, 1969). The exact explanation for these marker differences in sensitivity to u.v. within a bacterial species is not known, but is believed to relate to the size of the particular marker and its base composition. It seems unlikely that the very large difference observed between the *strep-r* marker in *H. influenzae* and the auxotrophic markers in *B. subtilis* is simply the result of choosing an extremely sensitive *H. influenzae* marker since resistance

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to cathomycin at 25 γ/ml (C_{25} marker) is approximately as sensitive to u.v. as is the *strep-r* marker (Setlow *et al.* 1968). Since the base composition of *H. influenzae* versus *B. subtilis* does not differ greatly (38% versus 42% G C; Sueoka, 1961), it would seem most likely that the differences in marker sensitivities reflect differences in the u.v. sensitivities of the recipient cells of the two species. The idea that marker u.v. sensitivity is related to the sensitivity of the recipient strain was suggested by results obtained with *B. subtilis*-mutagen-sensitive mutants in which marker sensitivity is related to the sensitivity of the particular recipient (Munakata & Ikeda, 1969). To test this hypothesis, recipient cells of *H. influenzae* and *B. subtilis* were inactivated with u.v. The results of these experiments are presented in Fig. 2. The *H. influenzae* recipient appears to be inactivated exponentially while the inactivation of the *B. subtilis* recipient is not exponential since there is an initial shoulder in the inactivation curve followed by an exponential slope and a tail.

4. DISCUSSION

The results presented in Tables 1 and 3 confirm the original results of both Terry & Setlow (1967) and Tuveson & Mangan (1970). It is apparent that to assay for *Neurospora* enzymatic PR activity the *H. influenzae* transformation system is preferable since the transforming DNA from this species requires a much lower u.v. dose to attain the desired survival level. Although some fraction of the difference in marker sensitivity observed between the *step-r* marker in *H. influenzae* and the auxotrophic markers in *B. subtilis* (Fig. 1) may result from marker size and base composition, a significant fraction of the difference is a reflexion of the greater u.v. resistance of the *B. subtilis* recipient strain used in the transformation assay (Fig. 2). If the shoulder observed in *B. subtilis* u.v. inactivation curves (Fig. 2; Mahler, 1965; Strauss, Reiter & Searashi, 1966) reflects the operation of a 'dark repair' system it is possible to interpret the differences in marker sensitivity between these two transformable species as a manifestation of the more efficient 'dark repair' of transforming DNA in *B. subtilis*. That 'dark repair' can affect transforming DNA has been demonstrated (Munakata & Ikeda, 1969; Bresler, Kalinin & Perumov, 1970). Assuming that 'dark repair' can operate on transforming DNA, we can offer a plausible explanation for the apparent ambiguity between the results of Terry & Setlow (1967) and Tuveson & Mangan (1970). Since a heavy u.v. dose is required to inactivate *B. subtilis* transforming DNA, many lesions are available as substrate for the nuclease(s) present in the *N. crassa* crude enzyme extracts which are specific for irradiated DNA. These u.v.-DNA specific nucleases together with the usual nucleases might lead to such extensive DNA degradation that the number of detectable transformants might fall below that observed with u.v.-DNA which had not been exposed to enzyme. Although the absolute number of active transforming DNA molecules might decline, the photoreactivable sector should not decline. However, the number of detectable transformants does not differ significantly whether or not the u.v.-DNA plus crude enzyme reaction mixture is or is not treated with light (Table 1). This lack of

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detectable PR can most simply be accounted for by assuming that the efficient 'dark repair' in the *B. subtilis* recipient raises the transformation frequency in the dark to the level activated by enzymic PR. This model predicts that if one were able to obtain a transformable u.v.-sensitive strain of *B. subtilis* (lacking significant 'dark repair' capacity), enzymic PR might be demonstrable in *N. crassa* crude enzyme extracts in the face of competing nucleases. We are attempting to isolate transformable u.v.-sensitive mutants of *B. subtilis* to test this prediction.

Marmur *et al.* (1961) reported that u.v.-treated transforming DNA from both *Diplococcus pneumoniae* and *B. subtilis* could be reactivated by the PR enzyme from baker's yeast (*Saccharomyces cerevisiae*). The marker used in *D. pneumoniae* was streptomycin resistance, which required a u.v. dose of $9{\cdot}6 \times 10^4$ ergs/mm^2 for inactivation to the 1 % survival level. The sensitivity of this marker and the *B. subtilis* markers investigated was equivalent to the *B. subtilis* markers which we have investigated. This result might be taken to mean that the PR enzyme from yeast differs in some manner from the *Neurospora* enzyme since it can be demonstrated to repair *B. subtilis* transforming DNA *in vitro* when a recipient strain exhibiting 'normal dark repair' capacity is used to assay for transformation. If *B. subtilis* 'dark repair' were capable of raising the transformation frequency in the dark to the level activated by enzymic PR, one would not have expected demonstrable PR by the yeast enzyme since the sensitivity of the markers assayed indicates that the 'dark repair' capacity of the recipient used was equivalent to that of the recipient employed in our experiments. We must consider the possibility that the crude enzyme extracts are not acting directly on the DNA but rather on the transformation process itself in *B. subtilis*. We are forced to consider the hypothesis that the crude extracts of *Neurospora* contain an inhibitor of the competence factor produced by *B. subtilis* without affecting the competence factor in *H. influenzae* (Tomasz, 1969). Bresler *et al.* (1970) have reported repair of approximately 70 % of the inactivating lesions in *B. subtilis* transforming DNA by yeast photoreactivating enzyme. However, the enzyme had been 'partially' purified which might have eliminated the inhibitory factor in the crude enzyme preparations from yeast. We are currently attempting the purification of the *Neurospora* PR enzyme using the *H. influenzae* system as our assay. If during the purification process we eliminate the inhibitor(s) of transformation, then repair of u.v.-inactivated *B. subtilis* transforming DNA should be demonstrable using a recipient with 'normal' sensitivity to u.v. as has been reported by Marmur *et al.* (1961) and Bresler *et al.* (1970) with yeast enzyme.

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