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CORRELATION BETWEEN GENETIC TRANSFORMABILITY AND NONPHOTOREACTIVABILITY IN *BACILLUS SUBTILIS*

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ABSTRACT

KELNER, ALBERT (Brandeis University, Waltham, Mass.). Correlation between genetic transformability and nonphotoreactivability in *Bacillus subtilis*. J. Bacteriol. **87**:1295-1303. 1964.—Photoreactivation after ultraviolet irradiation was studied in the transformable *Bacillus subtilis* SB-1. Moderate photoreactivability (maximal increase in survival due to photoreactivating light, five- to tenfold) was found in (i) noncompetent vegetative cells produced in Brain Heart Infusion broth, and in (ii) the total viable cells of a competent culture grown in special competency-producing medium and tested either just before or after transformation with deoxyribonucleic acid. However, no, or only trace, photoreactivation (maximal increase in survival, 1.5- to 2-fold) was found in that fraction of a competent culture actually able to be transformed. This nonphotoreactivability was a temporary property of transformable cells, since clones derived from them were normally photoreactivable. Nonphotoreactivability is not explained by injury to transformants caused by photoreactivating light, although transformants are killed by very large doses of light. The findings in *B. subtilis* strengthen the idea that transformability is generally correlated with nonphotoreactivability.

Diplococcus pneumoniae and *Haemophilus influenzae*, the two most thoroughly studied and highly transformable organisms (Ravin, 1961), are among the few bacteria which cannot be photoreactivated (Goodgal, Rupert, and Herriott, 1957; Ellison and Beiser, 1960; Rebeyrotte and Latarjet, 1960). [See Jagger (1958) for a review of photoreactivation.] The transformable genus, *Streptococcus* (Pakula and Walczak, 1963), is also nonphotoreactivable (Bellamy and Germain, 1955). Transformable *Neisseria* species have never been tested for photoreactivability.

The occurrence of transformability and nonphotoreactivability in the same cells suggests a possible relationship between these phenomena. That their occurrence in the same cells might be coincidental is unlikely because of the rarity of both transformability and nonphotoreactivability. A correlation, if it exists, is especially interesting because both transformation and photoreactivation concern the cell's genetic material: one, genetic incorporation of exogenous deoxyribonucleic acid (DNA); the other, healing of ultraviolet radiation-caused lesions in the DNA.

The recent discovery by Spizizen (1958) of transformation in some strains of *Bacillus subtilis* originating from Burkholder and Giles (1947) offers a chance to obtain further evidence of a possible correlation. These strains and their derivatives are very transformable. *B. subtilis* differs from *D. pneumoniae*, *Streptococcus*, and *Haemophilus* taxonomically and physiologically. Species within the genus *Bacillus* vary in photoreactivability. Some, e.g., *B. cereus* (Stuy, 1956*a*), are reported to be photoreactivable; others, including *B. subtilis* (Stuy, 1956*a*), are not. Strains within a single species also vary in photoreactivability. Thus, one cannot predict whether any particular strain of *Bacillus* is photoreactivable.

The present study was initiated on the hypothesis that a transformable *B. subtilis* strain would, like *Haemophilus* and pneumococcus, be nonphotoreactivable.

MATERIALS AND METHODS

Cultures. All experiments were made with *B. subtilis* SB-1 (Mahler, Neumann, and Marmur, 1963), a derivative of strain 168 used by Spizizen (1958). *B. subtilis* SB-1 is histidine- and tryptophan-dependent. DNA for transformation came from *B. subtilis* 168^6, a prototroph made resistant to erythromycin, Bryamycin, and micrococcin.

For maintenance, stocks of *B. subtilis* SB-1 were grown to late log phase (about 4×10^8 per ml) in Brain Heart Infusion (Difco) broth, and then mixed with 10% glycerol, frozen, and stored for 6 months at −20 C.

Production of competent cells. Methods and

media used were based on those of Anagnostopoulos and Spizizen (1961) as modified by Mahler et al. (1963). In summary, our method was as follows.

Three loopfuls of an overnight culture on Tryptose Blood Agar Base (Difco) slants (inoculated from the frozen stock culture) were seeded into 10 ml of growth medium, and incubated with bubbling air for 4 hr at 37 C. The suspension was chilled to 0 C, 10 to 12% glycerol was added, and the cells were frozen on solid carbon dioxide, and stored at −20 C up to 1 month.

The growth medium contained minimal medium salts, composed of 0.2% ammonium sulfate, 1.4% dipotassium phosphate, 0.6% monopotassium phosphate, 0.1% sodium citrate·$2H_2O$, and 0.02% magnesium sulfate·$7H_2O$, at pH 6.8 to 7.0. To this minimal salts solution were added aseptically after autoclaving: glucose, to a final concentration of 0.5%; L-tryptophan, 50 μg/ml; acid casein hydrolysate, vitamin-free, 0.02%; and yeast extract (Difco), 0.1%. Just before use were added: arginine, 0.8%; ornithine, 0.4%; and histidine, 0.04%.

For the daily production of competent cells, the thawed frozen culture (in growth medium) was diluted 1:10 in transformation medium. The transformation medium was composed of the minimal salts solution, as described for the growth medium, to which were added aseptically after autoclaving: glucose, to final concentration of 0.5%; yeast extract, 0.1%; L-tryptophan, 5 μg/ml; and casein hydrolysate, 0.01%. Immediately before use were added: 0.01 ml/ml of 0.05 M spermine tetrahydrochloride, 0.025 ml/ml of 0.1 M $CaCl_2$, and 0.025 ml/ml of 0.1 M $MgCl_2$.

The cells were incubated in transformation medium with bubbling air for 110 min at 37 C; 1 μg/ml of transforming DNA was added, and incubation was continued 20 min longer. The magnesium content of the culture was then raised to 0.005 M, 10 μg/ml of deoxyribonuclease were added for 5 min, and the culture was cooled to 0 C. Cells were either assayed immediately or were kept at 0 C in the dark while being manipulated for as long as ca. 90 min with little change in competency.

Assay. All plates were incubated at 37 C. The diluent for plating was cold minimal salts solution. For solid media, the agar dissolved in water was autoclaved separately, and mixed with the rest of the medium just before pouring, to eliminate the toxicity for many bacilli of agar autoclaved in alkaline medium (Kelner, *unpublished data*).

Transformants. The Bryamycin marker was used. The transformed culture was mixed in a bottom layer of melted (47 C) brain heart agar. The layer was incubated at 37 C for 90 min to allow expression of the marker. A top layer of brain heart agar containing 3 to 6 μg/ml of Bryamycin was then added. The Bryamycin dissolved in iso-amyl alcohol was added to melted agar just before use. Colonies were scored after 2 to 3 days. Frequency of transformants was about 0.1%.

Transformation to histidine independence was studied in a few experiments, by methods adapted from Marmur (*personal communication*). Assay medium contained: minimal salts solution, 100%; glucose to a final concentration of 0.1%; histidine assay medium (Difco), 0.2%; glutamic acid, 0.15%; arginine, 0.005%; tryptophan, 0.001%; $MnSO_4$, 3×10^{-8} M; $CuSO_4$, 2×10^{-8} M; $ZnCl_2$, 10^{-7} M; $FeCl_3$, 10^{-8} M; and agar, 2%.

Total viable cells. Colonies growing on Brain Heart Infusion Agar are called "total viable cells." In most experiments, the cells were treated exactly as in the Bryamycin transformation assay, except that a second layer of plain brain heart agar was added. Colonies were scored after 1 day of incubation.

Irradiation. The ultraviolet (UV) source was a 15-w low-pressure Hg lamp (General Electric germicidal) emitting chiefly 2,537 A. The intensity was about 58 ergs $\times$ sec^{-1} $\times$ mm^{-2}. Cells were irradiated at 0 to 5 C by the method of Stuy (1955). Photoreactivating light came from a medium-pressure Hg lamp (General Electric H250-A-5) 17 cm from the bacterial suspension. The light passed through a Schott WG-3 filter (Fish-Schurman Corp., New Rochelle, N.Y.), 0.5 cm of water, and Pyrex glass. This combination transmitted less than 10% radiation below 330 mμ. The lamp emitted strong lines at 365, 405, and 436 mμ. During photoreactivation, the cell suspension was in test tubes submerged in water at an appropriate temperature.

Typical experiments. Reliable UV-photoreactivation experiments require conditions where cells, UV-irradiated or not, are stable in the dark for the duration of an experiment, generally 1 hr. *Bacillus* vegetative cells, especially UV-irradiated competent cells, are quite delicate and sensitive to diluents and temperature. In phosphate buffer and many other diluents, particularly at 37 C,

cells die rapidly. Cells were more stable in buffer containing dilute peptone or Brain Heart Infusion broth, or in the diluent finally adopted for most of the experiments, transformation medium with or without catalase.

Three kinds of cells were studied.

(i) *Standard vegetative cells.* Brain heart broth (10 ml) was inoculated with 0.2 ml of the stock, frozen brain heart broth culture, and incubated for about 4 hr at 37 C, with bubbling air (titer about 6×10^7 to 4×10^8 per ml). The suspension was chilled to 0 C, and diluted 1:1,000 with cold minimal salts solution, or transformation medium, before UV-irradiation.

Immediately after irradiation, the cells were diluted 1:1 with either cold minimal salts solution, or transformation medium containing 0.1 mg/ml of a catalase preparation ("lyophilized"; from General Biochemicals, Inc., Chagrin Falls, Ohio) to minimize peroxide effects possibly induced by subsequent illumination with photoreactivating light (Latarjet, 1956). The culture was assayed immediately, and then one part was kept at 19 C in the dark and another was exposed to photoreactivating light at 19 C. The cells were then chilled to 0 C, and kept cold during assay for total viable cells.

(ii) *Competent culture just before treatment with DNA.* The culture was chilled to 0 C, diluted 1:1 with sterile, cold transformation medium, UV-irradiated, and then immediately diluted again 1:1 with transformation medium containing 10^{-4} (w/v) catalase. It was photoreactivated and assayed as were standard vegetative cells.

(iii) *Transformed culture immediately after treatment with DNA.* The culture was cooled to 0 C, and treated as described for competent cells before transformation. After photoreactivation, the frequency, in the same suspension, of total viable cells (assay on brain heart agar) and of Bryamycin-resistant transformants was determined.

RESULTS

Photoreactivation in standard vegetative cells. Vegetative cells grown in Brain Heart Infusion broth were chosen as representative *B. subtilis* cells. Figure 1 shows that they were definitely photoreactivable. Although the level of photoreactivation, a maximum of 10- to 25-fold increase in survival, is less than that of *B. cereus* (Stuy, 1955, 1956*a*), it is high enough to indicate photoreactivability.

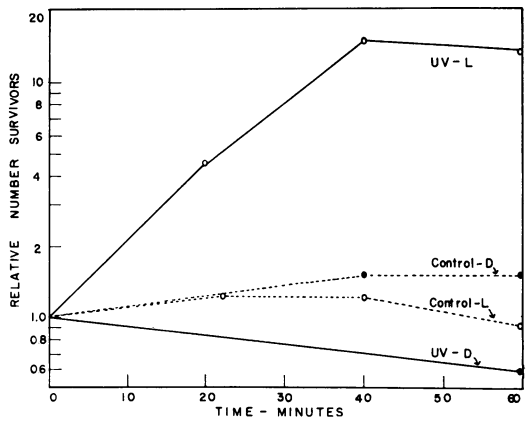

FIG. 1. *Photoreactivation of noncompetent, standard vegetative cells of Bacillus subtilis. UV-L and UV-D: cultures were grown in brain heart broth to a titer of 5.7×10^7 per ml, chilled to 0 C, and diluted with an equal quantity of transformation medium. They were irradiated with 1,100 ergs/mm² of UV light; survival was 2.5×10^{-2}. After irradiation, they were diluted again with an equal quantity of transformation medium containing catalase, warmed to 22 C, and illuminated with photoreactivating light (L), or stored in the dark (D). Control: the culture was not UV-irradiated, otherwise treated exactly as the UV-irradiated suspension. Survival at time zero was higher than usual for this UV dose; otherwise this experiment is typical.*

Cells grown in Brain Heart Infusion were photoreactivated whether diluted in minimal salts solution or transformation medium, and in the presence or absence of catalase.

B. subtilis SB-1 cells grown in Brain Heart Infusion were not transformable; e.g., after incubation with DNA for 60 min, no Bryamycin-resistant transformants were demonstrable. As Spizizen (1959) showed, competency in *B. subtilis* requires a sequence of growth in two specially designed media. A competent cell is almost certainly different physiologically and possibly cytologicallly from "normal" cells. Perhaps among the physiological changes accompanying competency was the loss of photoreactivability. The experiments were therefore repeated with a culture brought to the competent state.

Photoreactivation in total viable cells of a competent culture. In a typical experiment, a competent culture, grown 110 min in transformation medium, had a total viable count on brain heart agar of 1.8×10^8 per ml. It was chilled, diluted, UV-irradiated, and photoreactivated as described in

Materials and Methods. The UV dose was 1,100 ergs/mm^2; survival at time zero was 3.6×10^{-3}. Storage of UV-irradiated cells for 60 min in the dark at 19 C increased the titer 1.5-fold; in the light, there was a 8.3-fold increase due to photoreactivation.

A control (not UV-irradiated) was treated with photoreactivating light similarly. Storage for 60 min in the dark at 19 C increased the titer 1.4-fold; in the light, 1.5-fold.

Thus, a competent culture, too, was photoreactivable.

There was considerable variation day to day in photoreactivability of cultures. On some days there was only about a threefold increase in survivors. Nevertheless, for the purposes of this investigation, a competent culture can be assumed to be photoreactivable.

Since the level of photoreactivation was low, a fairly detailed study was made of factors influencing it in order to be reasonably sure the photoreactivation was real.

Temperature. In early experiments, the recipient competent culture was photoreactivated under conditions that had been successful for cells grown in Brain Heart Infusion at 0 C. At this temperature, the degree of photoreactivation was very low. At 37 C, cells were stable, but photoreactivation was again low and variable.

The optimal temperature was about 15 to 20 C. With increasing temperature, the cells were perfectly stable, but photoreactivation decreased.

UV dose. Photoreactivation after UV doses from 175 to 1,900 ergs/mm^2 (survival from about 4.5×10^{-1} to 2×10^{-4}) was studied. At doses above about 1,100 ergs/mm^2, survival and photoreactivation became variable. The higher the UV dose, the greater was the photoreactivation, up to a maximum of about a tenfold increase in survival. Because of the low photoreactivability, the dose-reduction was not evaluated, although the dose-reduction rule seemed to be followed.

Period of incubation in transformation medium. Incubation in transformation medium from 15 to 170 min (110 min being optimum for competency) was compared. Some inconsistent variation in photoreactivability of cells was seen, with no clear-cut trend; certainly, at no stage in the development and loss of competency within this time period was there photoreactivation distinctly greater than that at 110 min of incubation.

Comparison with standard vegetative cells. In paired experiments with brain heart-grown cells, the competent culture always showed lower photoreactivation.

In conclusion, a competent culture of *B. subtilis* shows a low, but consistent, photoreactivability.

B. subtilis SB-1 thus apparently differs from *Haemophilus* and pneumococcus in that it is photoreactivable.

The maximal frequency of transformation for the histidine or Bryamycin marker in our cultures is 0.2%. The low frequency is probably mainly caused by the low probability that a competent cell receives a piece of chromosome bearing an individual marker. An additional reason may be that only a fraction of the cells are in the proper physiological condition for transformation. If only a minority of a so-called competent culture is actually able to be transformed, then its photoreactivability (positive or negative) would go unnoticed in the experiment just described, in which total viable cells were assayed on brain heart agar. The photoreactivation shown by the competent culture might mostly be due to photoreactivation of the nontransformable fraction of the culture.

More conclusive evidence for a correlation between transformability and nonphotoreactivability would come from specific study of cells known to have actually just undergone transformation.

Photoreactivability in cells which have just been transformed. A competent culture was transformed with DNA. Immediately after the end of the DNA-contact period and just before the culture was ready for plating, it was subjected to UV and photoreactivation, and then assayed on selective medium on which only transformed cells could grow; changes in number of such cells showed whether they could photoreactivate. As a control, the culture was at the same time assayed on nonselective brain heart agar, to detect photoreactivation among all the cells.

Since competency might be a very short-lived state, cells were irradiated as soon as DNA was irreversibly taken up. And, to avoid complications due to UV-photoreactivation effects on expression of the incorporated gene, a period of incubation in a nonselective medium was interposed after photoreactivation to allow full expression of the marker before challenge by the selective agent.

A theoretically better experiment would be to treat the cells with UV and photoreactivating light immediately before they are exposed to DNA, rather than after. Changes in frequency of transformants would more accurately measure photoreactivability of the "competent" cell, one actually able to take up DNA and be transformed. However, interpretation of such an experiment would be difficult, for UV irradiation and photoreactivation might affect not only the viability of the competent cell itself, but also adsorption of DNA, incorporation into the cell, and into the genome. Also, the various manipulations involved in a UV-photoreactivation experiment take 40 to 60 min, during which period competency, labile at best, might change. It was thought best, therefore, to bring the cell up to the stage at which DNA has been taken up irreversibly, before UV irradiation and photoreactivation. This problem is discussed below.

Total viable cells of the transformed culture were photoreactivable; but, under the identical conditions where the total viable cells increased tenfold, the transformants increased only about 1.5-fold.

Of all the cell types studied, only the transformants showed no (or only trace) photoreactivation. Since these were clearly directly derived from transformable cells, this fact apparently establishes a correlation between the physiological condition conferring transformability, and nonphotoreactivability.

Since the evidence for this correlation came from the experiments with transformants, we considered whether there was some explanation for the results other than intrinsic nonphotoreactivability.

Specific light sensitivity of transformants. In Fig. 2, the survival of transformants rises slightly the first 40 min, and then falls. In the nonirradiated controls in Fig. 3, the transformants are specifically injured by high doses of photoreactivating light (over about 40 min). The transformants are, however, quite stable to doses of photoreactivating light for fewer than 30 or 40 min. Figure 3 shows that the slopes of the survival curves for photoreactivating light doses over 40 min are similar for nonirradiated and UV-irradiated cells. The change in direction of the curve comes at about the same point (at about 40 min of light) in both. We assumed, therefore, that both UV-irradiated and nonirradiated transformants have the same sensitivity to photoreactivating light, a

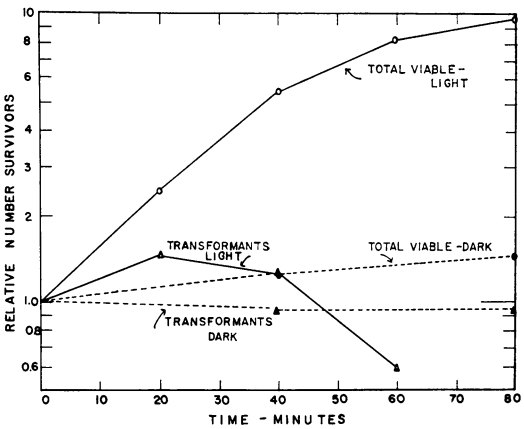

FIG. 2. *Comparison of photoreactivation of transformants and of total viable cells. After transformation with DNA, the Bacillus subtilis culture was chilled to 0 C, diluted with an equal quantity of transformation medium, irradiated with 1,220 ergs/mm² of UV light, and then diluted again with an equal quantity of transformation medium containing catalase. It was warmed to 19 C and illuminated with photoreactivating light (light), or stored in the dark (dark). Assay for Bryamycin-resistant transformants and total viable cells was done as described in Materials and Methods. The titers of culture before dilution or irradiation were: for total viable cells, 2.8 × 10⁸ per ml; and for Bryamycin-resistant transformants, 4.4 × 10⁵ per ml. Survival after UV treatment was: for total viable cells, 5.2 × 10⁻³; and for transformants, 6.7 × 10⁻³.*

sensitivity shown only at high doses. Any influence on photoreactivation caused by the lethality of photoreactivating light for transformants can be ruled out by correcting the UV-photoreactivation curve for transformants for the sensitivity of cells not UV-irradiated to high doses of photoreactivating light (Fig. 3).

This correction was calculated by dividing the change in survival of transformants of the UV-irradiated culture by the corresponding change in the nonirradiated control. For example, at 20 min of photoreactivating light, the change in the UV-irradiated culture is 1.49 (Fig. 2); in the control, 0.88 (Fig. 3, bottom): 1.49/0.88 = 1.70 = corrected change (Fig. 3, top). Similarly, the corrected change for 40 min of light is 1.27/0.74 = 1.72; for 60 min, 0.59/0.34 = 1.74.

Figure 3 shows that the increase in survival due to photoreactivating light reaches a plateau of less than twofold within about 20 to 30 min, and remains unchanged until about 60 min. Above 60 min of photoreactivating light, results

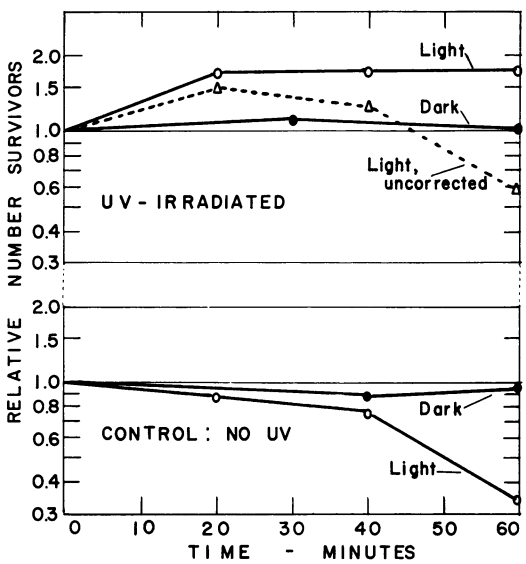

FIG. 3. *(top) Correction of dose-response photoreactivation curve of UV-irradiated transformants for light sensitivity. A competent culture of Bacillus subtilis was UV-irradiated and photoreactivated as described in Fig. 2 (same experiment). Dashed line indicates the actual change in survival due to photoreactivating light (data from Fig. 2). Solid line indicates changes in survival corrected for control, not UV-irradiated (shown in bottom graph). The corrected curve shows survival increases to a maximum of only 1.7-fold, remaining at this level a full 60 min. See the text for calculations. (bottom) Effect of photoreactivating light on transformants not UV-irradiated. Two experiments are averaged. The culture was treated exactly as the UV-irradiated suspension, except that UV irradiation was omitted.*

become variable; thus, the analysis is confined to a 60-min period. Unless one assumes that after 30 min of photoreactivating light the increase due to photoreactivation and the decrease due to light lethality exactly balance each other, it is a fair conclusion that for the first 60 min of photoreactivating light the transformants do not increase in survival more than twofold. Certainly, their photoreactivation is very much less than that of nonselected, total viable cells in the same suspension.

This analysis cannot be conclusive, since we do not know that the sensitivity to visible light of UV-irradiated transformants is really the same as that of nonirradiated cells. In any experiment involving photoreactivation, the changes in survival are due to a balance between photoreactivation and any other factors influencing survival.

Occasional experiments have shown threefold increase in survival of transformants; others have shown no increase at all. Where survival increased more than twofold, the nonirradiated control also increased in the photoreactivating light; correction for the control gave the same twofold maximum.

Our conclusion must be simply that under conditions showing photoreactivation in a competent culture of total unselected viable cells, the transformants in the same culture show trace or no photoreactivation.

UV dose. Photoreactivation of transformants was compared after UV doses ranging from 750 to 1,520 ergs/mm^2 (survival varying from about 1.5×10^{-2} to 1.0×10^{-3}). Above about 1,200 ergs/mm^2, survival became variable and, because of the low absolute number of transformant survivors, accurate assays were difficult.

At the lower UV doses, light scarcely increased survival. After any UV dose tested, the increase in survival due to light never exceeded 1.5 to 2-fold. This low, barely significant, level of photoreactivation made an accurate calculation of dose-reduction factor unfeasible.

The total viable cells of a just-transformed culture resembled in their UV-photoreactivation reactions a competent culture just before DNA addition.

The UV sensitivity of transformants was about the same as that of the total viable cells, so that lack of photoreactivability cannot be ascribed to unusual UV sensitivity or resistance.

Intensity and absolute dose of photoreactivating light. Prolongation of exposure to photoreactivating light to 100 min did not cause greater photoreactivation. Of course, sensitivity to the lethal action of visible light made results with high photoreactivating doses difficult to evaluate (Fig. 3).

In experiments with photoreactivating intensities one-half normal, the transformants still photoreactivated maximally about 1.8 times, reaching this level in 40 min instead of the usual 20 to 30 min, and maintaining it with little change to 80 min.

There was no sign of increased photoreactivation on the basis of absolute dose, with reduced light intensity down to one-fourth, as might have been expected for example if the maximum of twofold represented a balance between light

lethality and high intrinsic photoreactivability. Such increased maximum might have been expected, for example, if the lethal action of visible light had a higher threshold than photoreactivation.

Temperature. Even allowing for varying dark stability, there was no evidence that at any temperature between 0 and 37 C photoreactivation caused more than a twofold increase in survival of transformants.

It is noted that, for the transformant, photoreactivation varied less with temperature than for total viable cells. For the latter, photoreactivation was generally less at 37 than at 20 C.

Diluent. The diluent used had been carefully developed to prevent changes other than those due to photoreactivation. Since during photoreactivation cells were diluted in the same medium used for developing competency, perhaps the transformants lost their photoreactivability by metabolism during the sojourn at 19 C under photoreactivating light, or changed in other ways. Omission of glucose from the diluent made no significant change in photoreactivation of either total viable cells or transformants.

Period in transformation medium and competency. Photoreactivation of transformants was checked on cultures grown for different periods from 40 to 150 min in transformation medium; competency ranged from 5.2×10^{-5} to 1.5×10^{-3}. There was little consistent or significant difference in photoreactivability of transformants, indicating that the transformant was a characteristic, uniform type, whenever it appeared.

There was also little consistent difference in photoreactivability of total viable cells.

Filtration. For the bulk of the experiments, it was thought important to manipulate cells as little as possible, so as not to disturb the delicately balanced competent condition. To determine whether cell aggregation might have caused abnormal radiation responses, cultures were filtered six times through tightly packed absorbent cotton, removing all but one- or two-celled chains as confirmed by microscopy. Such cultures showed substantially the same competency and UV-photoreactivation reactions, except for slightly greater UV sensitivity of the total viable cells.

Miscellaneous other aspects. Many experiments were done with transformation to histidine independence. In all basic ways, this system showed the same results as the Bryamycin transformation, a maximum of twofold photoreactivation. However, in the histidine system the control for photoreactivation of total viable cells was considered less satisfactory than in the Bryamycin system. If total viable cells were assayed on brain heart agar, photoreactivation was, of course, normal. But photoreactivation was low and variable if they were assayed on histidine assay agar made complete by addition of histidine.

To check whether the nonphotoreactivability of the transformants was a temporary physiological state characteristic of the competent cell as such, rather than of the Bryamycin-resistant phenotype, clones for Bryamycin-resistant transformants were isolated, grown in the same way as the normal Bryamycin-sensitive culture, and subjected to UV and photoreactivating light. Assays were made on brain heart agar with and without Bryamycin. Photoreactivation was identical on both.

An important question is the nature of the cell we call the transformant. UV irradiation and photoreactivation were given after completion of the transformation process, but before expression. This meant the cell had incubated 25 min at 37 C after the first contact with DNA. What is actually the competent state depends on definition; nevertheless, the cell when we gave it UV irradiation was 25 min removed from the cell when it first took up DNA. Suppose one assumes that between the moment of first contact with DNA and the moment of UV irradiation the transformant rapidly loses photoreactivability; then, shortening the DNA-contact time and keeping all else constant should increase photoreactivability.

With 10 min of DNA-contact time instead of 20, photoreactivation of transformants was almost identical, showing that, if there is a change in photoreactivability of a competent cell, it occurs less than 15 min after initial contact with DNA.

DISCUSSION

The experiments with *B. subtilis* do suggest a connection between transformability and nonphotoreactivability, or trace photoreactivability, if a twofold increase in survival is thought significant.

Absolute proof is not furnished. Some method may be found to photoreactivate a *B. subtilis*

transformant: e.g., use of a marker other than histidine$^+$ or Bryamycin^R, use of another transformable strain, or development of competency in a different way.

The data obtained with *B. subtilis* emphasize the correlation in a particularly strong, meaningful way. The photoreactivability of (noncompetent) vegetative cells, and, to a lesser extent, the total viable cells of a competent culture, makes an excellent control for the nonphotoreactivability of transformants. In *B. subtilis*, nonphotoreactivability is apparently specific for the transformable state.

The correlation, if valid, does not mean that any nonphotoreactivable organism is necessarily transformable. Photoreactivability may be abolished in ways unconnected with transformation. One would expect, however, that no transformable cell is photoreactivable. It would be interesting to know whether transformable *Neisseria* spp. are photoreactivable.

Very high doses of photoreactivating light specifically injure transformants. Rebeyrotte and Latarjet's (1960) suggestion that the transformant is light sensitive is supported. This interesting specificity is under current investigation. However, for reasons outlined in the present paper, it seems unlikely that the sensitivity of transformants to photoreactivating light accounts for their nonphotoreactivability.

Photoreactivation shown by total viable cells in *B. subtilis* was variable, low, and had unusual temperature requirements. *B. subtilis* photoreactivation may differ from the usual *Escherichia coli-Streptomyces griseus* type. Work is in progress on this possibility.

Transformants clearly differed from total viable cells in their reactions to light, a fact indicating heterogeneity of the competent culture. Jensen and Haas (1963) found heterogeneity in competent *B. subtilis* cultures in regard to filterability through membrane filters. Nester and Stocker (1963) found that in *B. subtilis* transformants were temporarily resistant to penicillin in contrast to total viable cells, and in the period immediately after transformation delayed in growth and expression of the incorporated marker. These authors found much more heterogeneity in cultures made competent at 32 C than at 37 C (the temperature used in our experiments.)

What might be the reasons transformants do not photoreactivate? Perhaps they have temporarily a defective wall, and so suffer nonphotoreactivable, UV-induced membrane damage, while their nuclear damage is really photoreactivable. However, if transformants had UV-induced lesions in addition to those possessed by "viable" cells, transformants might be more UV-sensitive. This is apparently not so.

In *B. subtilis*, nonphotoreactivability may result from a general enzyme deficiency. Nester and Stocker's (1963) important evidence suggests such a state. The photoreactivating enzyme would be among those absent, reduced in concentration, or inactivated. The newly germinated spore of *Bacillus* may be a model for such a state (Stuy, 1956*b*). However, preliminary experiments attempting to connect a postgermination, or similar, hypothetical, presporulation state with transformants have been unsuccessful.

Instead of overall enzyme deficiency, we can postulate for the generally transformable state, in both *B. subtilis* and other transformable species, specific deficiency of the photoreactivating enzyme. Why such deficiency should be correlated with transformability is not known, unless nonphotoreactivability may result in instability of the genome (*see* Kelner, 1961) which makes transformation easier.

It may be possible to isolate transformable cells of *B. subtilis*. One would look for attributes common to all known highly transformable organisms, on the theory that there does exist a well-defined, characteristic physiological or cytological state conferring competency. One might thus find means of rendering diverse organisms transformable.

At present, the only feature common to the transformable bacteria, *D. pneumoniae*, *Haemophilus*, *Streptococcus*, and *B. subtilis*, is their nonphotoreactivability.

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