TRANSFORMATION IN MICROCOCCUS RADIODURANS AND THE ULTRAVIOLET SENSITIVITY OF ITS TRANSFORMING DNA*

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Wild-type *Micrococcus radiodurans* possesses a very efficient mechanism for the dark repair of radiation damage in its DNA.[1] Consequently, of the vegetative bacteria so far investigated, it is the most resistant to both ultraviolet and ionizing radiation.[2, 3] For example, it excises UV-induced pyrimidine dimers from its DNA with such efficiency that inactivation of heavily irradiated bacteria appears to be as much a function of protein damage as of nucleic acid damage.[4]

The process of transformation provides a powerful tool for studying repair mechanisms, since it is possible to irradiate transforming DNA and to assess its reactivation in the bacterial host [5–7] without irradiation of the host and its repair system. We report the discovery of transformation in *Micrococcus radiodurans* and present some results indicating repair of UV-irradiated transforming DNA in this bacterium.

Materials and Methods.—Microorganisms: Wild-type *Micrococcus radiodurans*, which is sensitive to streptomycin, originally obtained from Dr. A. W. Anderson; a mutant of *M. radiodurans*, resistant to 200 μg/ml streptomycin; *Haemophilus influenzae*, strain Rd, and two UV-sensitive mutants of *H. influenzae*, DB112 and DB117, were used.[7]

Preparation of transforming DNA: Streptomycin-resistant *M. radiodurans* was grown to saturation in TGY (0.5% tryptone, 0.1% glucose, and 0.1% yeast extract), washed in SSC (0.15 *M* NaCl and 0.015 *M* Na_3 citrate), and resuspended in SSC at a concentration of about 10^{10} viable units/ml. The cells were gently shaken overnight at 37°C with 0.75 mg/ml lysozyme, a treatment which does not lyse them. They were then centrifuged, resuspended in SSC/10 at a concentration of about 2×10^{10}/ml, and lysed by addition of Duponol to a final concentration of 2%. The DNA was purified by the method of Marmur,[8] and was diluted in M/15 phosphate buffer, pH 7.

Nuclease treatment of purified transforming DNA: DNA, 400 μg/ml, was incubated at 37°C for 30 min with 0.25 mg/ml DNase or 1 mg/ml RNase. Although the Marmur procedure[8] includes an RNase step, some RNA could be present, hence an RNase control.

Preparation of crude extract of M. radiodurans for enhancing the frequency of transformation: Wild-type cells were grown to saturation, washed, and resuspended in M/15 phosphate buffer, pH 7, at a concentration of about 2×10^{10}/ml. This suspension was passed twice through a French press (pressure 15,000 psi), which reduced the viability of the cells by about 80%. Viable cells and cell wall debris were removed by centrifugation. During transformation procedures, this extract was diluted directly into the mixture of recipient cells and transforming DNA. For most of the experiments the extract was boiled for 5 min.

UV irradiation of transforming DNA: Solutions of DNA (20 μg/ml) in quartz cuvettes were exposed to 265 nm radiation from a Hilger quartz-prism monochromator. Doses were varied by changing the time of irradiation. Incident intensities were measured with a calibrated photocell and d-c amplifier. These were corrected for absorption by the DNA solutions.[9]

Photoreactivation and photoreactivating enzyme: DNA was treated with photoreactivating enzyme under blacklight illumination as previously described.[10] Photoreactivating enzyme was prepared from bakers' yeast according to Muhammed.[11]

Transformation of M. radiodurans: Wild-type cells (streptomycin-sensitive) grown in TGY, usually at a concentration of around 10^8/ml, were gently shaken at 37°C with

various concentrations of DNA from the streptomycin-resistant strain. Appropriately diluted samples from this mixture were plated after 2 hr incubation (unless stated otherwise) in 10 ml TGY containing 1.5% agar which was melted and cooled to about 40°C before being mixed with the cells. The plates were incubated at 37°C for 8 hr (unless stated otherwise) and then overlaid with 10 ml of the same melted agar medium containing 200 μg/ml streptomycin. Colonies derived from transformed bacteria were counted after at least 3 days' incubation at 37°C. The total number of cells was measured by counting colonies which grew in agar not containing streptomycin.

Transformation of H. influenzae: The preparation of competent cells and transforming DNA containing the streptomycin marker was as previously described.[7, 12] Tritium-labeled DNA was prepared by purifying DNA from cells grown with 250 μg/ml adenosine and 50 μc/ml H^3-thymidine (spec. act., 6.7 c/mmole) in the medium. The specific activity of the purified DNA was about 10^5 cpm/μg.

Measurement of DNA uptake by cells: M. radiodurans at about 10^8 viable units/ml was gently shaken at 37°C for 2 hr with 0.2–0.4 μg/ml H^3-DNA from *H. influenzae*, with and without the addition of *M. radiodurans* extract and calf thymus DNA. In some experiments the H^3-DNA at a concentration of 1.8 μg/ml in M/15 phosphate buffer, pH 7, was boiled for 5 min and quickly cooled just before it was added to the cells. DNase (0.1 mg/ml) was added after the 2-hr incubation, and the mixture was incubated further for 15 min. The cells were collected by centrifugation, washed four or five times with water, and lysed in hot formic acid in scintillation vials. The formic acid was boiled off, and 1 ml water was added while the vial was hot (which helped to resuspend the dried material). Dioxane scintillation fluid was added after the vials cooled, and the radioactivity in the vials was measured in a scintillation counter. The radioactivity in aliquots of the supernatant fluid from the first centrifugation was also measured. *M. radiodurans* that had been killed by being heated at 70°C for 10 min took up considerably less than 1% of the DNA taken up by unheated cells, as measured by this method, although the cells are not lysed by such heating. Uptake of DNA by *H. influenzae* was measured in essentially the same way, except that the concentration of H^3-DNA was 2.4 μg/ml, the incubation was 45 min, and the cells were washed with 3.5% brain-heart infusion instead of water.

Results and Discussion.—Stimulation of transformation in H. influenzae by an extract from M. radiodurans: During an unsuccessful attempt to reactivate UV-irradiated *H. influenzae* transforming DNA by incubating it with a crude extract of *M. radiodurans*, it was discovered that the extract stimulated transformation. The transforming ability of both UV-irradiated and unirradiated *H. influenzae* DNA was increased by a factor of up to 20, depending on the degree of competence of the *H. influenzae* cells. Some of the results obtained with the extract are shown in Table 1. When the extract was boiled for five minutes, the number of transformations was increased by about another

TABLE 1. *The effect of a crude extract of M. radiodurans on transformation in H. influenzae.*

Strain of *H. influenzae*	Streptomycin-Resistant Colony-Forming Units/ml			
	Without extract	With extract*	With boiled extract	With autoclaved extract (15 min, 20 psi)
Rd (competent)	2.2×10^5	2.6×10^5		
Rd (log phase)	5	56		
DB112 (low competence)	2.1×10^3	4.5×10^4	8.3×10^4	
DB112 (low competence)	33		9.3×10^2	1.2×10^3
DB117 (mutant defective in transformation)	2		1	
None		0		

* Diluted by factor of 30 into transformation medium.

factor of 2, presumably because of the heat inactivation of nucleases in the extract. In no case did the extract affect the viability of *H. influenzae* cells during the usual two-hour incubation of transforming DNA and cells. The presence or absence of magnesium chloride (10^{-3} *M*) in the mixture of cells, DNA, and extract did not affect the number of transformants. Table 1 shows that the extract has little or no effect on transformation when relatively competent cell preparations are used. Exponentially growing cells, which are poorly transformed, as well as other cultures of low competence, transform considerably better in the presence of the extract. However, transformation in DB117, a UV-sensitive mutant of *H. influenzae* which is deficient in transformability,[7] even though it takes up DNA normally when grown as for competence,[13] is not improved by the extract.

The effect of M. radiodurans extract and competing DNA on labeled DNA uptake by M. radiodurans: Table 2 shows that *M. radiodurans* takes up native and denatured DNA to the same extent and that the uptake of both is inhibited by the *M. radiodurans* extract. When the extract is treated with DNase and then autoclaved, there is much less inhibition of uptake. Thus the inhibiting effect of the extract on DNA uptake in *M. radiodurans* is apparently the result of competition between the denatured DNA present in the extract (approximately 40 μg/ml in these experiments) and the labeled DNA. The uptake of DNA into *H. influenzae* is unaffected by the extract. Under normal conditions *H. influenzae* takes up more than ten times as much native DNA as denatured DNA.[14] The lack of inhibition in the case of *H. influenzae* may therefore be explained in terms of the inability of denatured DNA to compete with native DNA during uptake.

Although the frequency of transformation can normally be correlated with DNA uptake, in the case of *M. radiodurans* the extract increases the frequency of transformation about 100-fold, while the DNA uptake decreases by an order of magnitude. The extract stimulates transformation in *H. influenzae* without affecting uptake. Therefore the extract may exert its stimulatory effect on transformation at the stage of DNA integration.

TABLE 2. *The effect of M. radiodurans extract and competing DNA on uptake of tritiated H. influenzae DNA by M. radiodurans.*

	Additions to mixture of cells and H^3-DNA	State of H^3-DNA	Cells (cpm)	Supernatant (cpm)
Expt. 1	None*	Native	1,750	41,000
	Extract (diluted ×8)	Native	100	46,000
	None*	Denatured	1,820	43,000
	Extract (diluted ×8)	Denatured	150	48,000
Expt. 2	None*	Native	5,200	56,000
	Calf thymus DNA (600 μg/ml)	Native	120	66,000
	Calf thymus DNA (600 μg/ml) boiled 10 min and quick-cooled	Native	44	67,000

* M/15 phosphate buffer, pH 7, was substituted for extract and calf thymus DNA in controls. Note that the counts which disappear from the supernatant are not all recovered in the cell fraction, presumably because of quenching by the cell fraction. The uptake of label into the bacteria is not caused by DNase-induced breakdown of DNA with consequent uptake of nucleotides, since counts obtained when DNase is not used are higher than when it is.

Transformation in M. radiodurans: Evidence is presented in Table 3 that wild-type cells which are sensitive to streptomycin may be transformed to streptomycin resistance by purified DNA extracted from a streptomycin-resistant strain. DNA is necessary for transformation, since in its absence, or after incubation with DNase, no transformants are obtained. RNase treatment of the DNA does not affect the number of transformants. The frequency of transformation is extremely low when only recipient cells and DNA are incubated together but is greatly enhanced by the addition of the extract from wild-type *M. radiodurans.* The addition of extract does not affect the total number of viable cells, for example, by increasing the growth rate during incubation.

The transformation of a culture requires a period of incubation of the recipient bacteria with the transforming DNA to allow the DNA to be taken up by the cells. A further period is required for the marker on the transforming DNA to be expressed. In the transformation of *M. radiodurans* to streptomycin resistance the best conditions include a two-hour incubation of cells, DNA, and extract in liquid culture, followed by eight hours of incubation on solid medium before addition of the streptomycin (see Table 4). With longer times of incubation in liquid culture, the number of transformants drops markedly. A possible explanation for this decrease is that the high cell concentration which precludes further growth in the liquid culture is unfavorable for the in-

TABLE 3. *Evidence for transformation in M. radiodurans.*

Streptomycin-sensitive *M. radiodurans* (2.5×10^8/ml)	Donor DNA (80 μg/ml)	Boiled *M. radiodurans* extract	Streptomycin-resistant colony-forming units/ml
+	+	+	1.32×10^4
+	(RNase-treated)	+	1.36×10^4
+	(DNase-treated)	+	0
+	+	−	1×10^2
+	−	+	0
−	+	+	0

Wild-type *M. radiodurans* (streptomycin-sensitive), 0.3 ml, were incubated for 2 hr at 37°C with 0.1 ml transforming DNA from a streptomycin-resistant *M. radiodurans* plus 0.1 ml crude extract from the wild type. Samples of 0.1 ml were assayed for transformants by plating in agar, incubating for 8 hr, and then adding agar containing streptomycin. Transformants were scored after 3 days.

TABLE 4. *Number of transformants as a function of the time of incubation in liquid and on solid media.**

Hours in liquid medium before plating in agar medium without streptomycin	Transformants/ml: Streptomycin agar layer added after 6 hr†	Transformants/ml: Streptomycin agar layer added after 10 hr†
1	1.5×10^3	7.5×10^3
2	3.6×10^2	1.1×10^4
3	1.0×10	5.2×10^3
6	1.0×10	1.0×10
8	—	0

Wild-type *M. radiodurans*, 0.3 ml, were incubated at 37°C with 0.1 ml transforming DNA plus 0.1 ml crude extract from wild type. Samples of 0.1 ml were plated and incubated at 37°C until a layer of agar containing streptomycin was added. Transformant colonies were scored after 3 days.

* Viable units/ml, 2.5×10^8.

† Hours from the mixing of Cells and DNA.

tegration of transforming DNA, in which case any unintegrated DNA in the bacteria may break down.

The amount of transformation as a function of the concentration of transforming DNA is shown in Figure 1, together with a similar titration curve for transformation to streptomycin resistance of *H. influenzae*, strain Rd, with *H. influenzae* transforming DNA. Approximately 100 times more DNA is required for the maximal transformation of *M. radiodurans*. The reason for this is not clear. The titration curve might be explained on the basis that only a few denatured molecules in the DNA preparation cause transformation. However, single-stranded *M. radiodurans* DNA apparently does not transform, since this DNA, denatured at 40°C at pH 11.7 by the method of Alberts and Doty,[15] has less than 10 per cent of the transforming ability of native DNA, a result similar

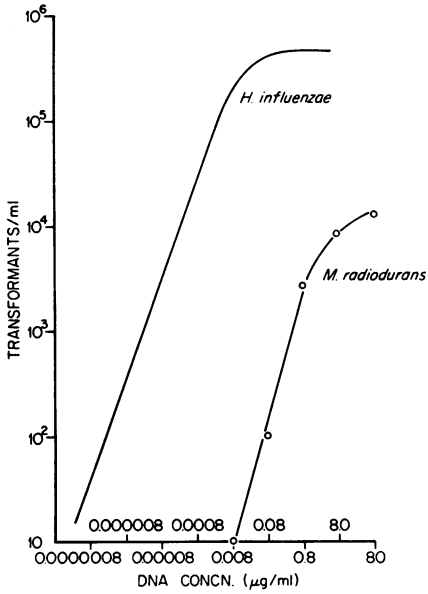

FIG. 1.—Number of cells of wild-type *M. radiodurans* transformed to streptomycin resistance as a function of transforming DNA concentration. The viable units/ml in the case of *M. radiodurans* were 2.5×10^8. A similar plot for transformation of *Haemophilus influenzae* strain Rd to streptomycin resistance is included for comparison.

to that found for *H. influenzae* DNA.[15] Another possibility is that only a few very long DNA molecules in the purified DNA preparation are capable of transforming *M. radiodurans*. However, a crude lysate of the streptomycin-resistant strain, which was gently handled so as to produce a minimum of breakage of the DNA molecules, did not transform appreciably better than the usual purified DNA at an equivalent DNA concentration. Another possible explanation for the low activity is that the excision mechanism, which is present in *M. radiodurans*,[1] may excise newly integrated DNA and thus prevent expression of the streptomycin marker in the majority of the DNA molecules that enter the cell.

The competence of bacterial cells is normally a transitory state in the growth of the culture, maximal competence developing just before the cells enter the resting phase.[16] However, in the case of *M. radiodurans*, competence does not appear to be dependent on the growth stage (see Table 5).

UV inactivation of M. radiodurans transforming DNA: The survival curves

of UV-irradiated transforming DNA's from *M. radiodurans* and *H. influenzae* are compared in Figure 2. The *M. radiodurans* DNA appears to be considerably more UV-resistant. The ratio of the dose necessary for 50 per cent inactivation of the *M. radiodurans* DNA relative to that of *H. influenzae* is about 24 (ref. 17). The *M. radiodurans* DNA does not appear to be inactivated exponentially, nor does the dose-effect relationship fit a square-root plot as observed for *H. influenzae* DNA.[7, 18] Thus a meaningful comparison of UV sensitivities of the two transforming DNA's is difficult, because of the different types of response to UV irradiation.

Pyrimidine dimers are apparently formed in UV-irradiated DNA from *M. radiodurans* since the DNA competes normally with irradiated *H. influenzae* transforming DNA for photoreactivating enzyme.[19] Nevertheless, Figure 3

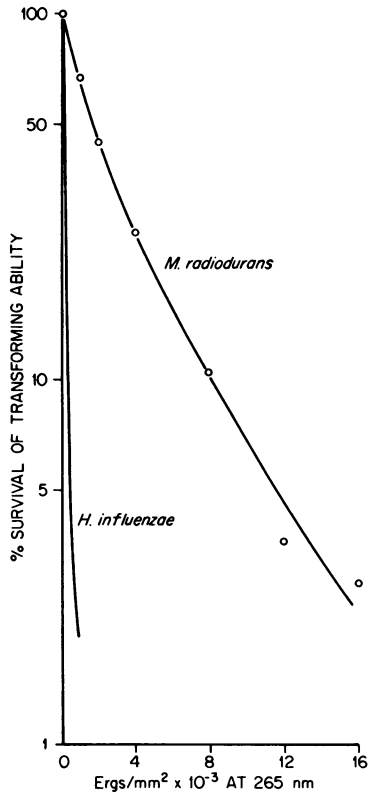

FIG. 2.—Survival of *M. radiodurans* transforming DNA as a function of UV dose at 265 nm. Cells were transformed in the presence of *M. radiodurans* extract. The survival of *H. influenzae* transforming DNA (streptomycin marker) is included for comparison.

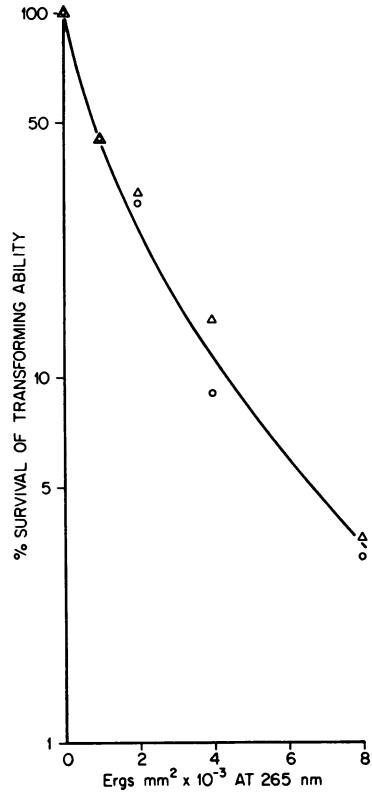

FIG. 3.—Survival of UV-irradiated *M. radiodurans* transforming DNA with and without photoreactivation of the DNA with yeast photoreactivating enzyme before transformation. DNA incubated in the dark (O) and in the light (Δ) with the enzyme. The conditions used would be expected to give maximum monomerization of pyrimidine dimers. Cells were transformed in the presence of *M. radiodurans* extract.

TABLE 5. *Number of transformants as a function of growth stage of the culture.*

OD_{675nm}*	Viable units/ml ($\times 10^{-8}$)	Transformants/ml ($\times 10^{-3}$)	Per cent transformation
0.50	1.37	2.8	0.0020
0.75	1.89	3.9	0.0021
0.90	2.23	4.7	0.0021
0.97	2.26	7.7	0.0034
1.05	3.80	5.5	0.0015

Exponential growth of wild-type *M. radiodurans* ceases at an OD of about 0.65. Thus at an OD of 0.75 or above, the culture is entering the resting phase. 0.3 ml of cultures at the various OD's were incubated with 0.1 ml transforming DNA and 0.1 ml crude extract from wild-type for 2 hr. Volumes of 0.1 ml were plated in agar, incubated for 8 hr at 37°C, and then agar containing streptomycin was added. Transformant colonies were scored after 3 days.

* OD: Optical density measured in Bausch and Lomb spectrophotometer.

shows that after treatment of the UV-irradiated *M. radiodurans* DNA with purified yeast photoreactivating enzyme, there is no increase in the survival of activity. The same enzyme preparation diluted tenfold more than in the *M. radiodurans* experiment was active and increased the transforming ability of a similar concentration of UV-irradiated *H. influenzae* DNA by a factor of 10. It is therefore assumed that almost all the pyrimidine dimers in the irradiated *M. radiodurans* transforming DNA were monomerized by the photoreactivating enzyme but that this was irrelevant to *M. radiodurans* cells, which have a mechanism for the repair of dimer damage. We consider it unlikely that the *M. radiodurans* extract contributes to the repair of transforming DNA, since it does not repair *H. influenzae* transforming DNA. Thus the efficiency of wild-type *M. radiodurans* at repairing its own irradiated DNA would appear to be matched by its ability to repair irradiated transforming DNA.

The inactivation of DNA shown in Figure 3 appears somewhat greater than that shown in Figure 2. The DNA is consistently slightly more sensitive to UV radiation when photoreactivating enzyme is added and irrespective of whether or not it is illuminated. This effect is probably due to the presence of ethylenediaminetetraacetate in the photoreactivating enzyme preparation.

Summary.—Evidence has been presented that *Micrococcus radiodurans* can undergo transformation. The frequency of transformation is increased about 100-fold in the presence of a heat-stable factor from a crude extract of wild-type *M. radiodurans*. This extract also increases the frequency of transformation in *Haemophilus influenzae* of low competence. The extract decreases DNA uptake in *M. radiodurans* because of competition by DNA in the crude extract.

Transforming activity in DNA from *M. radiodurans* appears to be UV-resistant, and is not increased by treatment with yeast photoreactivating enzyme. We conclude that the recipient cells efficiently repair pyrimidine dimer damage in transforming DNA as they do in cellular DNA when cells themselves are irradiated.

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