Transformation in *Micrococcus radiodurans*: Measurement of Various Parameters and Evidence for Multiple, Independently Segregating Genomes per Cell

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(Received 6 February 1980)

Transformation frequencies greater than 1 % for some single markers were obtained in *Micrococcus radiodurans* when bacteria in the exponential phase of growth were resuspended in fresh growth medium containing 0.03 M-Ca²⁺ before being incubated with transforming DNA. Mg²⁺, Sr²⁺ or Zn²⁺ could not replace Ca²⁺ in giving high frequencies of transformation. The time required for the maximum expression of transformed markers was 2 to 3 h for resistance to rifampicin and acriflavin and 6 to 8 h for resistance to erythromycin, kanamycin and streptomycin. The comparative frequency of transformants at maximum expression for each resistance marker was: kanamycin, 1; streptomycin, 1; acriflavin, 4; erythromycin, 25; rifampicin, 64.

Cultures were competent during all stages of exponential growth, the frequency of transformants only falling during stationary phase. The minimum time for DNA to be taken up by *M. radiodurans* into a DNAase-resistant form was between 3 and 6 s. From 6 s to 10 min exposure to DNA, the number of transformants increased non-linearly with time as though the process was inducible. The transformation frequency was directly proportional to the DNA concentration up to 1 µg ml⁻¹, although even at 88 µg ml⁻¹ the bacteria were not saturated. Attempts to measure the fraction of cells which were competent, using the unlinked marker technique, gave values well in excess of one. These were interpreted in terms of multiple genome copies and approximate values of between 0.25 and 0.72 were derived for the competent fractions.

INTRODUCTION

Micrococcus radiodurans is characterized by its extreme resistance to the lethal effects of ionizing and ultraviolet radiations (Anderson *et al.*, 1956; Duggan *et al.*, 1959) and to chemical damage to its DNA (Moseley, 1967; Sweet & Moseley, 1976). It is also resistant to mutation induced by these treatments, although it is sensitive to the mutagenic action of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sweet & Moseley, 1974; Kerszman, 1975). Transformation was first demonstrated in *M. radiodurans* by Moseley & Setlow (1968) and it remains the only method by which DNA repair and mutation in this organism can be studied genetically. However, the transformation frequencies reported to date have been low, and although transformation has been used to investigate the repair of irradiated transforming DNA (Moseley & Setlow, 1968), the assignment of mutations affecting DNA synthesis and repair to various genes (Moseley *et al.*, 1972*b*; Moseley & Copland, 1978) and as an indicator of recombinational activity (Moseley *et al.*, 1972*a*; Moseley & Copland,

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1975), very little effort has been made to increase the transformation frequency to those levels found in other systems, e.g. *Diplococcus pneumoniae* (Hotchkiss, 1954) and *Haemophilus influenzae* (Spencer & Herriot, 1965). An improved transformation frequency would be of great benefit since in a bacterium where transformation is the only means of genetic exchange the possibilities for strain construction would be considerably enhanced. Thus, a non-selectable mutation could be transformed into a recipient with an excellent chance of the transformant being detected by replica-plating, e.g. a Rec⁻ mutation into a recipient defective in some other repair pathway.

This paper describes a procedure which gives high transformation frequencies and some of the properties of the *M. radiodurans* transformation system.

METHODS

Bacteria. Organisms used were a wild-type *Micrococcus radiodurans*, originally isolated by Anderson *et al.* (1956), sensitive on TGY agar to 5 µg kanamycin ml⁻¹, 4 µg rifampicin ml⁻¹, 1 µg acriflavin ml⁻¹, 2 µg streptomycin ml⁻¹ and 2 µg erythromycin ml⁻¹, and a multiple mutant of *M. radiodurans* (Krase), resistant to 30 µg kanamycin ml⁻¹, 100 µg rifampicin ml⁻¹, 5 µg acriflavin ml⁻¹, 100 µg streptomycin ml⁻¹ and 30 µg erythromycin ml⁻¹.

Media. TGY medium for growth contained 5 g Bactotryptone (Difco), 1 g glucose and 3 g yeast extract (Difco) in 1 l distilled water. TGY agar was made by solidifying this medium with 15 g Bactoagar l⁻¹. TY medium was as TGY medium but without glucose. Phosphate buffer (0.067 M, pH 7.0) contained 4.73 g Na₂HPO₄ and 4.54 g KH₂PO₄ in 1 l distilled water.

Chemicals. All inorganic chemicals and the amino acids (tryptophan, arginine, alanine, tyrosine and glutamic acid) were from B.D.H.

Growth of bacteria. Samples from 18 h cultures were diluted into 20 ml quantities of TGY medium and the cultures were swirled in 250 ml conical flasks at 30 °C. Turbidity readings were made in a nephelometer (EEL, Halstead, Essex) with an orange filter. A turbidity of 0.30 was equivalent to 1.0×10^8 viable units ml⁻¹.

Preparation of transforming DNA. Two 1 l cultures of the antibiotic-resistant strain of *M. radiodurans* were incubated at 30 °C with shaking until they reached the stationary phase. The cultures were centrifuged, the bacteria washed in 50 ml SSC buffer (0.15 M-NaCl/0.015 M-sodium citrate; pH 7.0) resuspended in 50 ml butanol-saturated phosphate buffer pH 7.0 and left at room temperature for 45 min. The suspension was centrifuged, the bacteria were resuspended in 50 ml tenfold-diluted SSC buffer, lysozyme (EC 3.2.1.17, Sigma) was added to a final concentration of 20 mg ml⁻¹ and the mixture was incubated at 37 °C until a small sample (0.1 ml) removed to a test tube was completely lysed when one drop of 20 % (w/v) sodium dodecyl sulphate (SDS) in water was added (10 to 20 min). Then 5 ml 20 % SDS was added to lyse the whole bacterial suspension. The subsequent procedure followed that described by Marmur (1961). The DNA was finally dissolved in SSC buffer at a concentration of 0.6 to 2 mg ml⁻¹. The DNA concentration was calculated by its absorbance at 260 nm.

Procedure for transformation experiments. To 0.05 ml quantities of DNA (at various concentrations depending on the experiment) in test tubes held in ice 0.3 ml amounts of bacterial culture (treated in various ways as required) were added and the tubes were incubated with gentle shaking at 30 °C in a water bath. At the end of this period (usually 90 min), DNAase (EC 3.1.21.1; 0.05 ml; 2.5 mg ml⁻¹; Sigma) was added and incubation was continued for a further 15 min. The contents of each tube were then diluted appropriately in TGY medium, 1 ml samples were placed in sterile Petri dishes and 9 ml amounts of molten TGY agar at 46 °C were added to each. The plates were swirled several times to ensure even distribution of the bacteria, the agar was then allowed to solidify and the plates were incubated at 30 °C. After varying periods of time, determined experimentally, a second 10 ml layer of molten TGY agar was added containing the appropriate antibiotic (40 µg kanamycin ml⁻¹, 4 µg rifampicin ml⁻¹, 2 µg acriflavin ml⁻¹, 2 µg streptomycin ml⁻¹ or 10 µg erythromycin ml⁻¹), allowed to solidify and the plates were incubated at 30 °C for 4 d before transformant colonies were counted. Control plates from tubes lacking DNA were included in each experiment to measure the spontaneous mutation frequencies and the DNA was also checked for sterility.

Time required for maximum expression of transformed markers. Cultures prepared for transformation were added to transforming DNA in the ratio 0.3 ml culture to 0.05 ml DNA (1.5 mg ml⁻¹) and incubated for 90 min before DNAase (0.05 ml) was added for a further 15 min. The cultures were diluted appropriately, 1 ml samples were distributed into sterile Petri dishes and 9 ml amounts of molten TGY agar were added. When set, the plates were incubated at 30 °C. Agar overlays (10 ml) containing the antibiotics or acriflavin were added after various times of incubation, from 0 to 6 h, and the number of transformants resistant to each inhibitor was assayed.

Transformation frequency as a function of culture turbidity. Cultures of *M. radiodurans* were set up so that they were in exponential phase growth at a turbidity of 0.15 (5×10^7 viable units ml⁻¹) after overnight incubation. As the culture turbidity increased samples of decreasing size were removed so that when centrifuged and resuspended in a standard volume of fresh TGY medium containing Ca²⁺ approximately the same bacterial concentration was obtained. Samples of 0.3 ml were incubated with 0.05 ml transforming DNA (1.5 mg ml⁻¹) in SSC buffer for 90 min, then DNAase was added for 15 min and the culture was assayed for transformants resistant to rifampicin.

DNA saturation curve. DNA (620 µg ml⁻¹) in SSC buffer was serially diluted in SSC buffer and 0.05 ml of each dilution was added to test tubes at 30 °C. A 0.3 ml sample of culture of *M. radiodurans* prepared for transformation was added to each tube and incubation was continued for 90 min before 0.05 ml DNAase (2.5 mg ml⁻¹) was added to each tube for 15 min. The cultures were diluted appropriately, 1 ml samples were plated in agar and a rifampicin agar overlay was added after a further 4 h expression time.

Kinetics of DNA uptake. An exponential phase culture of *M. radiodurans* was prepared for transformation. Samples (0.3 ml) were added to 0.05 ml amounts of transforming DNA (see Fig. 4 legend for concentrations) in test tubes and incubated at 30 °C. At increasing times, from 3 s to 90 min, 0.05 ml DNAase (2.5 mg ml⁻¹) was added to a different tube and incubation was continued for a total of 105 min. As a control on the effectiveness of the DNAase, 0.3 ml culture was added to 0.05 ml DNAase, then 0.05 ml DNA was added immediately and the mixture was incubated for 105 min. Each tube was assayed for the number of transformants resistant to rifampicin or, in one experiment, acriflavin.

The unlinked marker technique for assaying competence. Transforming DNA prepared from the Krase strain of *M. radiodurans* was used to transform populations of the wild-type, prepared for transformation as described. The inhibiting agar overlay to select transformants was added at an appropriate time to give maximum expression of the transformed marker (4 h for rifampicin and acriflavin, 6 to 8 h for streptomycin and erythromycin). When two inhibitors were added together to select double transformants, the overlay was added at a time to give maximum expression to both markers.

RESULTS

Effects of divalent cations on the frequency of transformation

Various treatments were compared for their effect on the frequency of transformation. Cultures were grown in TGY or TY medium and transformed with no further treatment or the bacteria were resuspended in fresh TGY or TY medium or they were resuspended in TGY or TY medium with CaCl₂ added to a final concentration of 0.03 M, before being transformed (Table 1). The only treatment which caused a large increase in the frequency of transformation was the addition of CaCl₂. The presence or absence of glucose in the growth or transformation medium had no effect. The presence, singly, of the amino acids tryptophan, arginine, alanine, tyrosine and glutamic acid at a concentration of 100 µg ml⁻¹ did not increase the transformation frequency (results not shown).

Table 1. Comparison of various treatments to cultures of *M. radiodurans* on the transformation frequencies of two markers, rifampicin- and erythromycin-resistance

Growth medium	Resuspending medium	Ca ²⁺ added	Frequency of rifampicin-resistant transformants	Ratio	Frequency of erythromycin-resistant transformants	Ratio
TGY	—	—	6.1×10^{-4}	1.0	4.2×10^{-4}	1.0
TY	—	—	9.2×10^{-4}	1.5	7.0×10^{-4}	1.7
TY	TY	—	5.9×10^{-4}	1.0	5.2×10^{-4}	1.2
TY	TY	+	1.4×10^{-2}	23	5.7×10^{-3}	13.6
TGY	—	—	1.4×10^{-4}	1.0	6.4×10^{-5}	1.0
TY	TGY	+	5.9×10^{-3}	42	2.6×10^{-3}	41
TY	TY	+	2.4×10^{-3}	17	1.2×10^{-3}	19
TGY	TGY	+	5.0×10^{-3}	36	2.0×10^{-3}	31
TGY	TY	+	1.8×10^{-3}	13	5.5×10^{-4}	8.6

Table 2. *Effect of CaCl₂ concentration on the frequency of transformation of M. radiodurans to rifampicin-resistance*

CaCl ₂ concn in culture (M)	Transformation frequency	
	Expt 1	Expt 2
0		1.3×10^{-3}
0.003		1.6×10^{-3}
0.01		1.4×10^{-3}
0.02		2.0×10^{-3}
0.03	2.1×10^{-2}	8.0×10^{-3}
0.09	1.6×10^{-2}	8.0×10^{-3}
0.17	2.3×10^{-2}	
0.28	2.4×10^{-2}	2.2×10^{-3}
0.57	1.4×10^{-2}	
0.86	6.2×10^{-3}	
1.43	2.3×10^{-3}	2.7×10^{-3}

Fig. 1. Transformation frequencies in *M. radiodurans* for five resistance markers as a function of the time of addition of the inhibitory agar overlay following incubation at 30 °C. Resistance markers: ○, rifampicin; ●, erythromycin; □, acriflavin; △, streptomycin; ■, kanamycin.

A culture of *M. radiodurans* was grown in TGY medium, resuspended in the same medium and different concentrations of CaCl₂ were added to samples of the culture before they were exposed to transforming DNA. The frequencies of transformation obtained at the various concentrations are shown in Table 2. Maximum transformation frequencies were obtained between 0.03 and 0.28 M-CaCl₂. Routinely we now use 0.03 M.

The ability of the divalent cations Mg²⁺, Sr²⁺ and Zn²⁺ to replace Ca²⁺ was tested by using MgCl₂, SrCl₂ and ZnCl₂ at 0.03 M. Neither Mg²⁺ nor Sr²⁺ increased the transformation frequency while Zn²⁺ completely inhibited transformation.

Time required for maximum expression of transformed markers

The frequency of transformants resistant to each antibiotic as a function of the time of addition of the inhibitory overlay is shown in Fig. 1. The frequency of transformants

Fig. 2. Frequency of rifampicin-resistant transformants of *M. radiodurans* (●) as a function of culture age. At 24 and 72 h, samples of the original culture at a turbidity (○) of 1.4 were diluted into fresh medium to give turbidities of 0.13 (*a*) and 0.20 (*b*) and transformation frequencies and turbidities were measured in the original and diluted cultures.

resistant to rifampicin and acriflavin was already relatively high when the inhibitory layer was added immediately after exposure to DNA. The maximum frequency was obtained after 2 to 3 h incubation and was about three times that at time zero. The frequencies of transformants resistant to erythromycin, streptomycin and kanamycin were relatively low at time zero and increased at least 100-fold during post-transformation incubation, maximum frequencies being obtained only after at least 6 h. These results made rifampicin- and acriflavin-resistance useful markers because of the relatively short expression time required.

DNA from the strain of *M. radiodurans* resistant to kanamycin, rifampicin, acriflavin, streptomycin and erythromycin was used to transform a wild-type culture and each of the markers was selected by adding the appropriate antibiotic at the time giving maximum expression for each marker. The ratio of frequencies (averaged from a large number of experiments) was: kanamycin-resistance, 1; streptomycin-resistance, 1; acriflavin-resistance 4; erythromycin-resistance, 25; rifampicin-resistance, 64. Rifampicin-resistance was thus the most useful marker because of its high frequency and short expression time and it was used to examine some features of transformation in *M. radiodurans*.

Transformation frequency as a function of the growth phase

While growth of a culture of *M. radiodurans* continued through the exponential phase and into the stationary phase, samples were removed, treated as described in Methods and assayed for transformants (Fig. 2). A transformation frequency of approximately 1% was obtained when the culture was in the exponential phase of growth. The frequency of transformants decreased during the stationary phase and had fallen 5000-fold after 72 h. After 24 and 72 h, samples of the stationary phase cultures were diluted into fresh TGY medium to give turbidities of 0.13 and 0.20, respectively, and the increase in turbidity and transformation frequency was measured. The latter began to increase during the first hour during which time there was no increase in turbidity.

Fig. 3

Fig. 3. Number of rifampicin-resistant transformants of *M. radiodurans* as a function of transforming DNA concentration. The viable count of bacteria exposed to DNA was 6.4×10^8 ml $^{-1}$, the transformation frequency at the maximum concentration of DNA tested ($88 \mu\text{g ml}^{-1}$) being 3.6×10^{-2} .

Fig. 4

Fig. 4. Number of transformants of *M. radiodurans* as a function of the time of exposure to DNA. Since the results are from different experiments with different concentrations of DNA the number of transformants is plotted as a fraction of those obtained at 90 min, the maximum incubation time tested. ●: DNA concentration $88 \mu\text{g ml}^{-1}$; number of rifampicin-resistant transformants at 90 min $3.9 \times 10^6 \equiv$ transformation frequency of 6.5×10^{-3} . ○: DNA concentration $8.8 \mu\text{g ml}^{-1}$; number of rifampicin-resistant transformants at 90 min $4.0 \times 10^6 \equiv$ frequency of 6.5×10^{-3} . □: DNA concentration $8.8 \mu\text{g ml}^{-1}$; number of rifampicin-resistant transformants at 90 min $4.7 \times 10^6 \equiv$ frequency of 7.8×10^{-3} . ■: DNA concentration $0.34 \mu\text{g ml}^{-1}$; number of acriflavin-resistant transformants at 90 min $1.1 \times 10^4 \equiv$ frequency of 5.0×10^{-5} .

Table 3. Frequency of rifampicin-resistant transformants in a culture of *M. radiodurans* exposed to transforming DNA ($8.8 \mu\text{g ml}^{-1}$) for different periods of time

Time of incubation with DNA	Rifampicin-resistant clones	
	No. ml $^{-1}$	Frequency
0 s	2.0×10^1	3.2×10^{-8}
3 s	2.0×10^1	3.2×10^{-8}
6 s	3.3×10^2	5.0×10^{-7}
10 s	1.6×10^3	2.5×10^{-6}
30 s	1.6×10^3	2.5×10^{-6}
1 min	5.4×10^3	8.7×10^{-6}
2 min	2.3×10^4	3.7×10^{-5}
20 min	5.6×10^5	9.0×10^{-4}
90 min	4.7×10^6	7.6×10^{-3}

DNA saturation curve

Samples from a culture of *M. radiodurans* prepared for transformation (6.4×10^8 viable units ml $^{-1}$) were exposed to different concentrations of transforming DNA, and the number of rifampicin-resistant transformants was determined at each DNA concentration (Fig. 3). There was a linear increase in the number of transformants as a function of DNA concentration until the latter reached $1 \mu\text{g ml}^{-1}$. In excess of this concentration the linear relationship failed and for a further 100-fold increase in concentration there was a 5.3-fold increase

Table 4. Application of the unlinked marker technique to transformation of *M. radiodurans* in an attempt to measure the competent fraction of the population

Antibiotic-resistance markers* and antibiotic concn ($\mu\text{g ml}^{-1}$) used to select transformants				$10^8 \times$ Frequency of transformation for single markers		$10^5 \times$ Frequency of doubly transformed cells		Competent fraction of population [(a $\times$ b)/c]
1st marker		2nd marker		1st marker (a)	2nd marker (b)	Calculated† (a $\times$ b)	Observed (c)	
Expt 1								
Rif	2	Ery	5	19	4.6	8.7	2.1	4.1
Rif	2	Ery	10	19	3.5	6.7	1.4	4.8
Rif	2	Ery	20	19	2.6	4.9	1.2	4.1
Rif	10	Ery	5	17	4.6	7.8	1.9	4.1
Rif	10	Ery	10	17	3.5	6.0	1.5	4.0
Rif	10	Ery	20	17	2.6	4.4	1.1	4.0
Rif	50	Ery	5	15	4.6	6.9	1.6	4.3
Rif	50	Ery	10	15	3.5	5.3	0.6	8.8
Rif	50	Ery	20	15	2.6	3.9	0.6	6.5
Expt 2								
Rif	2	Ery	5	28	12	34	5.3	6.4
Ery	5	Acr	1	12	7.4	8.9	0.78	11.4
Rif	2	Acr	1	28	7.4	21	1.3	16.2
Expt 3								
Rif	2	Str	2	55	0.87	4.8	0.28	17.1
Rif	2	Str	10	55	0.71	3.9	0.27	14.5
Rif	2	Str	100	55	0.57	3.1	0.27	11.5
Rif	2	Acr	1	55	13	71	3.1	22.9
Rif	2	Acr	2.5	55	1.9	10	1.5	6.7

* Rif, Rifampicin; Ery, erythromycin; Acr, acriflavin; Str, streptomycin.

† Assuming the cultures to be 100 % competent.

in the number of transformants. Even at $88 \mu\text{g DNA ml}^{-1}$ there was no evidence for saturation.

Kinetics of DNA uptake

The frequency of transformation as a function of time of incubation with DNA was determined as described in Methods. When the DNA concentration was $8.8 \mu\text{g ml}^{-1}$ in the assay tubes the first transformants appeared after the bacteria had been exposed to DNA for between 3 and 6 s (Table 3). The number of transformants at 6 s was 16 times greater than the number of spontaneous mutants. The results from several experiments, using different concentrations of DNA and different markers, are shown in Fig. 4. It might be expected that the number of transformants would be linearly related to the time of exposure to DNA but this was not the case over the first 10 min when for a 10-fold increase in exposure time, e.g. 1 to 10 min, the number of transformants increased 100-fold, i.e. from 1.3×10^{-3} to 1.3×10^{-1} of the final number at 90 min. Only from about 10 to 90 min did the number of transformants increase in direct proportion to the exposure time. The addition of chloramphenicol ($100 \mu\text{g ml}^{-1}$) to cultures prepared for transformation 15 min before adding to transforming DNA did not significantly reduce the transformation frequency.

Attempted measurement of competence

Attempts were made to measure the competent fraction of the population using the unlinked marker technique (Goodgal & Herriot, 1961; Nester & Stocker, 1963). This method is based on the idea that in a population in which every cell is competent the frequency of cells transformed for two unlinked markers should be equal to the product of the transformation frequencies of each of the two markers individually. In a culture in which the

competent cells form only a fraction of the population the number of doubly transformed cells will be greater than the product of the individual frequencies so that the ratio of expected to observed frequencies of doubly transformed cells should be equal to the fraction of competent cells.

Table 4 gives values for the observed transformation frequencies for different individual markers selected at various concentrations of inhibitor, for the calculated transformation frequencies for doubly transformed cells expected, assuming the whole population to be competent, for the actual frequencies of double transformants and for the fraction of the population calculated to be competent. In all cases the final values exceeded one, i.e. more than 100 % of the cells in the culture were calculated to be competent, the values being from 4.0 to 22.9 (400 to 2290 %).

DISCUSSION

The transformation frequency in *M. radiodurans* is considerably enhanced by the presence of 0.03 M-Ca²⁺ during the uptake of DNA, levels in excess of 10⁻² being obtained routinely for the rifampicin-resistance marker. Ca²⁺ has a similar effect on other bacteria such as *Escherichia coli*, *Diplococcus pneumoniae* and *Staphylococcus aureus* (Mandel & Higa, 1970; Fox & Hotchkiss, 1957; Thompson & Pattee, 1977) but the biochemical mechanism remains obscure. The transformation frequencies now obtained compare favourably with those in the classical transformable species *Bacillus subtilis* (Anagnostopoulos & Spizizen, 1961), *Diplococcus pneumoniae* (Hotchkiss, 1954) and *Haemophilus influenzae* (Spencer & Herriot, 1965).

An unusual feature of transformation in *M. radiodurans* is that competence does not occur as a transitory state during the growth of a culture as it does, for example, in the three species mentioned above. A culture of *M. radiodurans* is competent at all stages of exponential growth and in this resembles *Neisseria meningitidis* (Catlin, 1960). This relative stability of the transformation frequency during exponential growth means that it can be used, for example, to measure changes in recombinational ability (Moseley *et al.*, 1972*a*).

The minimum time required for uptake of DNA into a DNAase-insensitive state of between 3 and 6 s is similar to that found in *H. influenzae* by Stuy & Stern (1964). They calculated that 4 to 5 s is required for the penetration of 8 × 10⁶ to 9 × 10⁶ daltons of a DNA molecule. In *B. subtilis* the minimum time required is 1 to 1.5 min (Levine & Strauss, 1965). The reasons for these variations are not known.

A second unusual feature of the *M. radiodurans* transformation system is that although the number of transformants is linearly related to the exposure time between 10 and 90 min this relationship does not hold during the incubation period up to 10 min. During this period the number of transformants produced per unit time increases as though the process were becoming more efficient with time. This can best be explained in terms of at least one of the processes involved in transformation being inducible. In *B. subtilis* genetic transformation can be stimulated by prior exposure of the bacteria to homologous DNA or polyphosphate and Kohiyama & Saito (1960) suggested that induction of a DNA permeating system was required. However, they could not reconcile this with the general concept of inducible enzymes since compounds which inhibited the formation of amylase, e.g. 2,4-dinitrophenol, did not influence the increased rate of formation of transformants. Levine & Strauss (1965) showed that although during 1.5 to 4 min incubation with DNA there was a gradually increasing rate of appearance of transformants, chloramphenicol did not inhibit transformation. This result also argues against an inducible protein being synthesized as a result of exposure of *B. subtilis* to transforming DNA. Our results support such a conclusion for *M. radiodurans* also.

The DNA saturation curve (Fig. 3) is unusual in that, in spite of very high concentrations of DNA, the number of transformants did not become constant but continued to rise with

increasing DNA concentrations above $1 \mu\text{g ml}^{-1}$, albeit at a slower rate. The molecules of DNA containing the rifampicin marker were obviously becoming less efficient at transforming as a result of competition with other DNA molecules but the recipient cells appear not to have been saturated with DNA even at concentrations up to $88 \mu\text{g ml}^{-1}$.

Attempts to measure the competent fraction of populations of *M. radiodurans* prepared for transformation, using the unlinked marker technique, led to values greatly in excess of one. Values greater than one have been obtained previously, e.g. 1.45 was obtained by Hadden & Nestor (1968) in *B. subtilis*. Porter & Guild (1969) explained a similar result in *Diplococcus pneumoniae* by assuming that because transformation involves the integration of transforming DNA into only one of the two strands of a DNA helix then the two unlinked markers could either be integrated into the same or opposite strands of the helix. Although in the latter case the cell would be doubly transformed, replication of the DNA and its subsequent segregation into different daughter cells would mean that a doubly transformed cell on dividing would score as two singly transformed cells. Thus, there would be less doubly transformed cells than the calculated frequency from data from singly transformed cells would predict. If all the cells in a population were competent, a value of 2.0 would indicate that there were two transformable units per cell (assuming that there is an equal chance of transforming DNA being integrated into the same or alternate strands of the DNA helix).

The results for *M. radiodurans* with average values from each experiment (Table 4) of 5.0, 11.3 and 14.5 cannot be assumed to be the number of transformable units since the competent fraction of the population is not known. The highest value (14.5) must be equal to, or less than, the real value. Of course, as the competent fraction of cells rises to one, the value for transformable units must approach its real value. Certainly in the three experiments described, as the competent fraction rises, indicated by the increasing frequency of rifampicin-resistant transformants (1.9×10^{-2} , 2.8×10^{-2} and 5.5×10^{-2}), the value for transformable units rises. Recently, the genome complexity of *M. radiodurans* was calculated from DNA renaturation data, and from measurements of DNA per cell it was estimated that resting phase cells have four genome equivalents per cell while cells growing exponentially with a doubling time of 80 min have ten genome equivalents (Hansen, 1978). This would mean that the latter would contain 20 transformable units (single strands of DNA) and this is borne out by our results. Assuming an average of 20 transformable units per cell then the competent fraction would be the values in the final column of Table 4, viz. the calculated number of transformable units per cell assuming a competent fraction of 1.0, divided by 20 (the real value), giving values of 0.25, 0.57 and 0.72. From this it can be calculated that if all the cells were competent the maximum value for transformation using a single marker would be about 7.6 % for rifampicin-resistance.

The data of Hansen (1978) did not distinguish between a single chromosome containing four genome equivalents, two chromosomes of two genome equivalents, or four independent chromosomes per resting cell. Our results strongly suggest that the genome copies must be independent and capable of independent segregation. Very recent studies of the amount of DNA (estimated chemically) per nucleus (as revealed by Giemsa staining) indicate that all four genomes are contained within a single nucleus (D. M. Evans & B. E. B. Moseley, unpublished observations). It seems likely, therefore, that recombination repair is possible between homologous regions of chromosomes while they are in this conformation, accounting for the extraordinary resistance of this bacterium to radiation damage, for example, although the four chromosomes eventually segregate as independent units.

We thank Mr H. J. R. Copland for his precision in carrying out many of the experiments described. This work was supported by a grant from the Medical Research Council to B.E.B.M. and by a Scholarship from the Iranian Ministry of Education to S.T.

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