

DNA repair and the evolution of transformation IV. DNA damage increases transformation

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Abstract

Natural genetic transformation in the bacterium *Bacillus subtilis* provides a model system to explore the evolutionary function of sexual recombination. In the present work, we study the response of transformation to UV irradiation using donor DNAs that differ in sequence homology to the recipient's chromosome and in the mechanism of transformation. The four donor DNAs used include homologous-chromosomal-DNA, two plasmids containing a fragment of *B. subtilis* *trp*⁺ operon DNA and a plasmid with no sequence homology to the recipient cell's DNA. Transformation frequencies for these DNA molecules increase with increasing levels of DNA damage (UV radiation) to recipient cells, only if their transformation requires homologous recombination (i.e. is *recA*⁺-dependent). Transformation with non-homologous DNA is independent of the recipient's recombination system and transformation frequencies for it do not respond to increases in UV radiation. The transformation frequency for a selectable marker increases in response to DNA damage more dramatically when the locus is present on small, plasmid-borne, homologous fragments than if it is carried on high molecular weight chromosomal fragments. We also study the kinetics of transformation for the different donor DNAs. Different kinetics are observed for homologous transformation depending on whether the homologous locus is carried on a plasmid or on chromosomal fragments. Chromosomal DNA- and non-homologous-plasmid-DNA-mediated transformation is complete (maximal) within several minutes, while transformation with a plasmid containing homologous DNA is still occurring after an hour. The results indicate that DNA damage directly increases rates of homologous recombination and transformation in *B. subtilis*. The relevance of these results and recent results of other labs to the evolution of transformation are discussed.

Introduction

Sex involves recombination between homologous DNA sequences that come from separate individuals, such as occurs during the process of natural transformation in some bacteria. Natural transformation has recently come to occupy a key position in testing theories for the evolution of sex (Michod, Wojciechowski and Hoelzer, 1988; Levin, 1988; Wojciechowski, Hoelzer and Michod, 1989; Michod, Wojciechowski and Hoelzer, 1989; Maynard Smith, 1990; Maynard Smith, Dowson and Spratt, 1991; Hoelzer and Michod, 1991; Redfield, 1993a, b; and Mongold, 1992). According to the DNA repair hypothesis for the evolution of sex, sexual recombination functions to repair DNA damage (for review see Bernstein, Hopf and Michod, 1987). In light of this hypothesis, the response of transformation to damaging agents such as UV radiation becomes of special interest.

There has been interest in the effects of DNA damage by UV radiation on genetic recombination in a variety of organisms (for review see Bernstein, 1983). Recombination is stimulated by UV in bacteriophage T4 (Epstein, 1958), phage λ (Baker and Haynes, 1967; Huskey, 1969), conjugational crosses in *Escherichia coli* using UV-irradiated donors and unirradiated recipients (Howard-Flanders et al., 1968; R. Devoret, personal communication), the yeast *Saccharomyces cerevisiae* (references in Kunz and Haynes, 1981), and in *Drosophila* during meiosis (Prudhommeau and Proust, 1973) and mitosis (Martensen and Green, 1976). Further, in *E. coli*, it has been shown that homologous recombination between the chromosomal *lacZ* gene and an unirradiated λ lacZ phage can be stimulated by co-infection with a second, UV-irradiated λ phage. This suggests that recombination can be stimulated by interactions at sites that are distant from the actual sites of damage, a phenomenon which has been termed indirect stimulation or teleactivation (Golub and Low, 1983). However, in spite of considerable study, the nature and actual mechanism of the stimulatory effect of DNA damage on genetic recombination is still poorly understood. Critical questions remain concerning the relationship between the type and the physical location of the damaged site and the site of the subsequent recombination events (Clark and Low, 1988).

Recent studies (Michod, Wojciechowski and Hoelzer, 1988; Wojciechowski, Hoelzer and Michod, 1989; Hoelzer and Michod, 1991) with the naturally competent bacterium *Bacillus subtilis* show that DNA damage by UV irradiation of recipient cells increases the frequency of genetic transformation, if the donor DNA is homologous-chromosomal-DNA and if it is added after the UV irradiation of recipient cells (termed "UV-DNA" experiments). There is no corresponding increase in transformation in a control experiment in which the transforming DNA is administered before the UV irradiation of recipient cells (termed "DNA-UV" experiments). The increase in transformation frequency in UV-DNA experiments could not be attributed to competent cells having enhanced forms of the commonly studied forms of DNA repair in bacteria: SOS repair, post-replication recombinational repair, or excision repair. For these reasons, we hypothesized that transformation functions in DNA repair.

The increase in transformation frequency in *B. subtilis* with UV irradiation of recipient cells observed in UV-DNA experiments could be caused by any combination of three factors. (i) UV radiation may directly increase recombination during transformation. (ii) Competent cells (those physiologically capable of the binding and uptake of exogenous DNA) may use the donor transforming DNA as a template for recombinational repair resulting in a survival (fitness) advantage over non-competent cells. (iii) UV radiation, and DNA damage in general, may directly increase the binding and uptake of donor DNA.

In the present work, we use fragments of homologous-chromosomal-DNA that are cloned in integrative (i.e., non-replicating) plasmid vectors as donor DNA for transformation of *B. subtilis*. These cloned fragments contain two contiguous *B. subtilis* chromosomal genes, *trpC* and *trpF*. For comparison we study transformation with a non-homologous plasmid, which replicates but does not integrate in *B. subtilis* (pMK4), and with homologous chromosomal DNA obtained from the whole *B. subtilis* genome (YB1011). We show that UV irradiation of *B. subtilis* cells increases the frequency of plasmid DNA-mediated transformation if the plasmid contains (as cloned inserts) small regions of homology to the recipient chromosome. If the plasmid does not contain any sequence homology to the recipient cell, transformation frequencies do not respond to increases in UV. These results rule out hypothesis (iii) above and indicate that UV radiation directly stimulates homologous recombination during transformation, perhaps by providing single-strand gaps at which the RecA protein binds and catalyzes recombination. A possible survival (fitness) advantage of transformation in competent cells cannot be ruled out by our results, but is unlikely to be a dominant factor.

Throughout this paper we use the term "homologous-plasmid-DNA" to refer to the non-replicative (in *B. subtilis*) plasmids, either pTRPH3 or pJH101-*trp1*, into which *B. subtilis* *trp*⁺ chromosomal DNA has been cloned. In contrast, we use the term "non-homologous-plasmid-DNA" to refer to the plasmid pMK4, which carries no homology to the *B. subtilis* chromosome but which encodes a selectable marker (chloramphenicol resistance, Cm^R) and replicates in *B. subtilis* as an extra-chromosomal element. The important distinction between these two kinds of plasmids is that transformation of recipient cells with the homologous-plasmid-DNA, like chromosomal DNA, requires homologous recombination functions (i.e., is *recA*⁺-dependent) for integration into the chromosome, whereas transformation of cells with nonhomologous-plasmid DNA does not. It should be noted that although the end result of the transformation process may differ, plasmids and chromosomal donor DNAs are brought into the cell by a common pathway of binding and uptake (Dubnau, 1982, 1991).

Material and methods

1. Bacterial strains and plasmids

The *B. subtilis* recipient strain used in this study is YB886 (*trpC2*, *metB5*, *amyE*, *sigB*, *xin-1*, SP β), a derivative of the naturally competent, DNA recombination-

and repair-proficient *B. subtilis* strain 168 (Yasbin, Fields and Anderson, 1980). Strain YB1011, a prototrophic (Met^+ , Trp^+) derivative of YB886 (B. Friedman and R. Yasbin, personal communication), was used as the source of high molecular weight homologous-chromosomal-DNA for the transformation experiments and was purified as described previously (Michod, Wojciechowski and Hoelzer, 1988).

Plasmid pMK4 (Sullivan, Yasbin and Young, 1984) is a 5.6 kb chloramphenicol-resistant and ampicillin-resistant bifunctional plasmid which replicates and encodes resistance in both *B. subtilis* and *E. coli*, respectively (Fig. 1). Since plasmid pMK4 contains no region of homology to the *B. subtilis* chromosome, the transformation of cells with pMK4 DNA does not require homologous recombination functions (i.e., is not *recA*⁺-dependent). Plasmid pTRPH3 (Henner, Band and Shimotsu, 1984), an 8.0 kb plasmid which replicates and encodes resistance to ampicillin in *E. coli*, is a derivative of pBR322 containing a 3.5 kb *Hind* III fragment carrying a portion of the *trp*⁺ operon from the *B. subtilis* chromosome cloned into the unique *Hind* III site (Fig. 1). Like the transformation of cells with homologous-chromosomal-DNA (YB1011), the ability of pTRPH3 DNA to transform the *trpC2* mutation, and other *trp* mutations to Trp^+ prototrophy requires homologous recombination (unpublished results). The approximately 2.5 kb segment of the *trp* operon contained in the plasmid pTRPH3 includes the *trp* promoter distal portion of the *trpD* gene, the entire *trpC* and *trpF* genes and the promoter proximal end of the *trpB* gene (Henner, Band and Shimotsu, 1984).

The plasmid pJH101-*trp1* (Fig. 1) was constructed by subcloning the *trp*⁺ DNA containing fragment from pTRPH3 into the integrative vector pJH101, which contains the chloramphenicol resistance gene (*cat*) from the *Staphylococcus aureus* plasmid pC194 inserted into the unique *Pvu* II site of pBR322 (Ferrari et al., 1983), as an additional selectable marker in *B. subtilis*. The 3.6 kb *Hind* III *trp* fragment from pTRPH3 was gel purified using low melting temperature agarose and Elutip-d chromatography (Schmitt and Cohen, 1983) and ligated to pJH101 DNA that had been previously digested with *Hind* III and dephosphorylated with calf intestinal phosphatase (Maniatis, Fritsch and Sambrook, 1982). The ligated DNA was used to transform *E. coli* strain MM294 (*recA*⁺) with simultaneous selection for ampicillin resistance and chloramphenicol resistance. The orientation of the *B. subtilis* *trp*⁺ operon sequences in pJH101-*trp1*, relative to the direction of transcription of the tetracycline resistance gene (*tet*) in pJH101-*trp1*, is parallel as determined by restriction endonuclease analysis of plasmid DNA from several ampicillin/chloramphenicol resistant transformants. Plasmid pJH101, which does not contain an origin for replication in *B. subtilis*, does not transform *B. subtilis* cells to chloramphenicol resistance (Ferrari et al., 1983; our unpublished results). However, unlike pJH101, the recombinant derivative pJH101-*trp1* carrying the *trp*⁺ operon fragment from plasmid pTRPH3 which provides a region of homology to the *B. subtilis* chromosome, is able to transform competent cells to either *trp*⁺ prototrophy and/or chloramphenicol resistance. The transformation of *B. subtilis* cells by pJH101-*trp1* for either selectable marker requires homologous recombinant functions (our unpublished results).

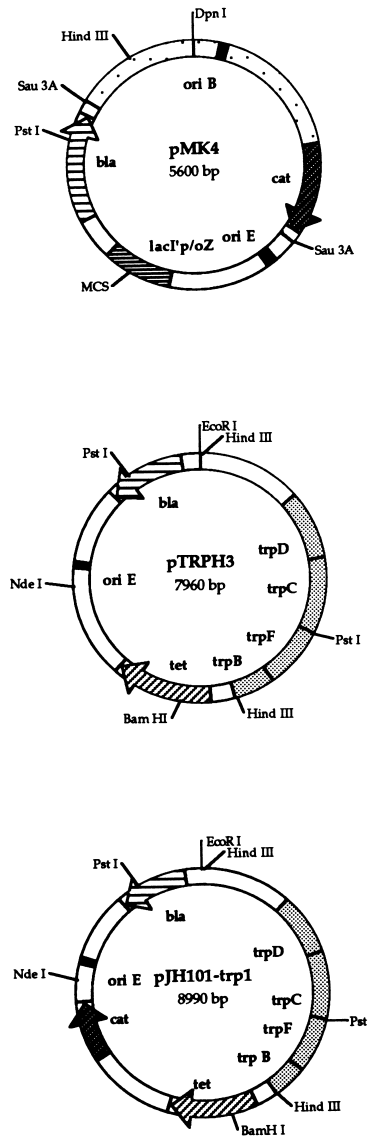


Fig. 1. Structure of plasmids pMK4, pTRPH3 and pJH101-trp1. *bla* and *tet*, genes that confer resistance to ampicillin and tetracycline, respectively, and the replication origin "*oriE*" (in *E. coli*) are derived from plasmid pBR322 (Bolivar et al., 1977). *trpD*, *trpC*, *trpF* and *trpB* gene sequences of the *trp*⁺ operon fragment contained in pTRPH3 (Henner, Band, and Shimotsu, 1984) are indicated. *cat*, encoding chloramphenicol resistance, and the replication origin "*oriB*" (in *B. subtilis*) are derived from the plasmid pC194 (Ehrlich, 1977). MCS is the location of multiple cloning (restriction) sites in the *lacZ* region contained in pMK4 that was derived from plasmid pUC9 (Sullivan, Yasbin and Young, 1984).

The plasmids pMK4, pJH101-trp1 and pTRPH3 were propagated in a *recA*⁺ *E. coli* host strain to increase the frequency of multimeric DNA molecules, and purified by CsCl-ethidium bromide density gradient centrifugation as described in Maniatis, Fritsch, and Sambrook (1982).

2. Media

The growth media used for plating in our experiments have a distinct effect on cell survival (see, for example, Figs 2 and 5 below). For this reason, comparisons between donor DNAs are best made between cells plated on the same kind of medium. In experiments using the homologous plasmid pJH101-trp1 and the non-homologous plasmid pMK4 as transforming DNAs, we selected for the same marker (chloramphenicol resistance) so that transformants and total cells were both plated on the same medium, LB medium with and without chloramphenicol for total cells and transformed cells, respectively (Michod, Wojciechowski and Hoelzer, 1988), for the two kinds of donor DNAs. In experiments using pTRPH3 DNA as donor and measuring transformation to Trp⁺ prototrophy, we plated both total and transformed cells on minimal glucose medium (MG), supplemented as required, so as to compare results with our previous work using chromosomal DNA (Michod, Wojciechowski and Hoelzer, 1988; Wojciechowski, Hoelzer and Michod, 1989; Hoelzer and Michod, 1991).

Spizizen minimal salts, MG (Spizizen, 1958) and competence media (GM1 and GM2) were prepared as described previously (Michod, Wojciechowski and Hoelzer, 1988). Dilutions and suspensions of cells were done in Spizizen minimal salts (1 × SS) unless otherwise indicated. LB medium was prepared as described in Miller (1972).

3. Genetic procedures

Liquid cultures of *B. subtilis* YB886 were grown in GM1/GM2 media to maximize competence and transformed with chromosomal and plasmid DNAs as described previously (Michod, Wojciechowski and Hoelzer, 1988). Essentially, cells were grown at 37° C with aeration in GM1 until 90 min. following the end of exponential growth (designated as "T₉₀"), then diluted 10⁻¹ into warm GM2 medium and incubated as above for 60 min. before irradiation with UV light (UV-DNA treatment) or the addition of transforming DNA (DNA-UV treatment). Competent cultures were incubated with transforming DNA (concentrations of 0.1 µg/ml or 1.0 µg/ml) at 37° C for 30 to 45 min., after which DNase I was added to 100 µg/ml and the incubation was continued for an additional 10 min. Cells were transformed to tryptophan prototrophy (Trp⁺) with either chromosomal DNA from strain YB1011 or plasmid pTRPH3 DNA, or to chloramphenicol resistance with plasmids pMK4 or pJH101-trp1 DNAs, and plated accordingly for numbers of total viable cells and genetic transformants, as described previously (Michod,

Wojciechowski and Hoelzer, 1988; Wojciechowski, Hoelzer and Michod, 1989). The UV irradiation of competent cultures was performed as described previously (Michod, Wojciechowski and Hoelzer, 1988).

At $T_{90} + 60$ min., aliquots of competent cultures were either collected by centrifugation, resuspended in $1 \times$ SS, UV irradiated, then recollected and resuspended in warm GM2 just before the addition of transforming DNA ("UV-DNA"), or incubated directly with transforming DNA, treated with DNase I, collected by centrifugation, resuspended in $1 \times$ SS and then UV irradiated, at approximately $T_{90} + 100$ min. ("DNA-UV"). Following UV irradiation in DNA-UV experiments, cells were collected again by centrifugation, resuspended in warm GM2 at the same density and incubated with aeration at 37°C for an additional 40 min. before plating (termed "delay" DNA-UV experiments in Wojciechowski, Hoelzer and Michod, 1989). As discussed previously (Wojciechowski, Hoelzer and Michod, 1989), this delay is necessary to control for differences between UV-DNA and (non-delay) DNA-UV experiments in the period of growth after UV and before plating.

In the "time before DNase" (TBD) experiments described below, cultures were incubated with transforming DNA and at the indicated time points, one ml aliquots were removed, treated with DNase I for 10 min. as described above, then diluted appropriately and plated for total cells and transformants. In the "time after DNase" (TAD) experiments described below, cultures were incubated with transforming DNA for 30–45 min. and treated with DNase I as described above, then collected by centrifugation and resuspended at the same density in either GM2 or $1 \times$ SS and incubated at 37°C as before. At the indicated time points thereafter, aliquots were taken and plated accordingly for total cells and transformants.

4. Statistical analysis of data

At a given UV dose, X , the survival of total cells, survival of transformed cells and transformation frequency are defined as,

$$\frac{N_{\text{TOT},X}}{N_{\text{TOT},0}}, \frac{N_{\text{TRA},X}}{N_{\text{TRA},0}}, \frac{N_{\text{TRA},X}}{N_{\text{TOT},X}}, \quad (1)$$

respectively. The variables $N_{\text{TOT},X}$ and $N_{\text{TRA},X}$ are the numbers of total and transformed cells which survive a UV treatment of $X \text{ J/m}^2$, respectively. Log transformations were taken of the survivals, transformation frequencies, and factor changes in transformation frequency for the purpose of statistical analysis, since this made the variances more homoscedastic. The factor change, or deviation, in transformation frequency at $X \text{ J/m}^2$ is defined as the transformation frequency at $X \text{ J/m}^2$ divided by the transformation frequency at 0 J/m^2 . In addition to the means and standard errors of the mean, regression curves are also plotted in some of the Figures. When regression curves are compared to each other in the following discussion, they were compared using the indicator variable technique discussed in Neter, Wasserman and Kutner (1985, Chapter 10). This technique involves defining

binary variables for each of the following qualitative variables: cell type (total cell or transformed cell) and order of the UV and DNA treatments (UV-DNA and DNA-UV). To test whether these factors had significant effects on survival and transformation frequency, an F statistic was constructed using the error sum of squares for the full model (SSE_F) and for a reduced model (SSE_R). The full model contains the indicator variable and its interactions with UV, whereas the reduced model is obtained by deleting the indicator variable and its interactions with UV from the full model. Thus the reduced model pools the data in a way which ignores the qualitative variable of interest. The F statistic used in the test is

$$\frac{SSE_R - SSE_F}{df_R - df_F},$$

in which df_F and df_R are the degrees of freedom for the full and reduced models, respectively. Regression analyses were performed using the REG procedure of the SAS (Statistical Analysis System) computer package.

Results

1. Transformation frequencies for plasmid and chromosomal DNAs

Frequencies and properties of transformation for each of the four donor DNAs are given in Table 1 for our recipient strain, *B. subtilis* YB886 (*recA*⁺). Two donor

Table 1. Properties of transformation for chromosomal and plasmid DNAs^a.

Donor DNA	Marker locus	Marker loci per incoming fragment ^c	Homology to recipient ^d	Transformation frequency ^b	
				1.0 μ g/ml	0.1 μ g/ml
pTRPH3	<i>trpC</i>	≈ 1	<i>trpC</i> - <i>F</i> region	2.7×10^{-3}	6.9×10^{-4}
pJH101- <i>trpI</i>	<i>trpC</i>	≈ 1	<i>trpC</i> - <i>F</i> region	7.3×10^{-3}	9.9×10^{-4}
pJH101- <i>trpI</i>	<i>cat</i>	≈ 1	<i>trpC</i> - <i>F</i> region	4.3×10^{-4}	8.0×10^{-5}
pMK4	<i>cat</i>	≈ 1	none	7.8×10^{-3}	8.6×10^{-4}
YB1011	<i>trpC</i>	$\approx 0.001-0.01$	genome wide ^e	4.7×10^{-4}	1.1×10^{-4}

^a Maximum transformation frequencies for recipient strain YB886 grown to competence and transformed with DNA at 0.1 and 1.0 μ g/ml as described in Material and Methods: pTRPH3 (plasmid with cloned *trpC* locus), pJH101-*trpI* (plasmid with cloned *trpC* and *cat* locus), pMK4 (plasmid with cloned *cat* locus) and YB1011 (chromosomal fragments).

^b Results are averages of between four and eight experiments.

^c For plasmid donor DNA, each transforming plasmid or fragment should, in principle, carry the selected marker. For chromosomal donor DNA, between 1 in 1000 and 1 in 100 fragments will carry the selected locus.

^d Homology to chromosome of *B. subtilis* recipient strain.

^e A particular chromosomal DNA fragment will be homologous to a tiny fraction of the recipient's genome, but the region of homology will differ for each fragment. For this reason, any region of the recipient's chromosome could be homologous to an incoming fragment.

DNA concentrations were used, 0.10 $\mu\text{g/ml}$ and 1.0 $\mu\text{g/ml}$, which are non-saturating and saturating, respectively, for transformation. (In other words, DNA concentrations above 1.0 $\mu\text{g/ml}$ do not result in higher transformation frequencies, whereas concentrations 0.1 and 1.0 $\mu\text{g/ml}$ produce a linear response to transformation frequency.) The four donor DNAs used were (i) pTRPH3, a non-replicative (in *B. subtilis*) plasmid containing as a cloned insert *B. subtilis trp*⁺ operon DNA, (ii) pJH101-trp1, a non-replicative (in *B. subtilis*) plasmid containing as a cloned insert *B. subtilis trp*⁺ operon DNA and the selectable *cat* locus (chloramphenicol resistance), (iii) pMK4, a replicative plasmid with no sequence homology to the recipient strain but containing the selectable *cat* locus, and (iv) homologous-chromosomal-DNA from *B. subtilis* strain YB1011. For the homologous donor DNAs pTRPH3 and YB1011, transformation frequencies were measured at the *trpC* locus. For the non-homologous-plasmid-DNA pMK4, transformation frequencies were measured for the *cat* marker carried on the plasmid. For the homologous-plasmid-DNA pJH101-trp1, transformation frequencies were measured for both the *cat* and *trpC*⁺ markers.

Transformation frequencies at the *trpC* locus were considerably higher, by almost an order of magnitude, when the transforming marker was carried as a cloned DNA insert in the plasmids pTRPH3 or pJH101-trp1, than when carried by chromosomal DNA (YB1011). This is likely due to the fact that for the plasmid donor DNAs, almost every transforming fragment carries the selectable *trpC*⁺ marker. In contrast, with YB1011 chromosomal DNA, only a small proportion, probably 0.1% to 1.0%, of the transforming fragments, carries the selectable *trpC*⁺ marker (column 3 of Tab. 1). Although the transformation frequency for a locus is considerably increased when it is cloned into a plasmid, every competent cell in the culture is probably not being transformed. When the proportion of competent cells were not measured directly, about 10% of a culture is normally competent (Dubnau, 1991), and pTRPH3 and pJH101-trp1 transformation frequencies were considerably less than 10⁻¹ (Tab. 1).

Transformation with chromosomal DNA involves recombination between the resident chromosome and a homologous single-strand linear DNA fragment (Dubnau, 1982). Could it matter whether the homologous single-strand originated outside the cell as a double-stranded circular plasmid or as a double-stranded linear fragment? After binding to the competent cell surface, it is thought that circular plasmid DNA is cut and single-strands of DNA enter the cell (Dubnau, 1982). For multimeric plasmids, complementary single strands could enter the cell and anneal into duplex DNA and undergo circularization. If this were to occur *before* recombining with the recipient chromosome, the mechanism of recombination might be different for homologous regions carried on plasmids and homologous chromosomal DNA. Whether this occurs is not, at present, known.

The plasmid pJH101-trp1 carries both the homologous *trpC-F* region and the non-homologous *cat* locus. Since this plasmid cannot replicate in *B. subtilis*, successful transformation for either marker requires homologous recombination via the *trpC-F* region carried by the plasmid. For pJH101-trp1, transformation frequencies at the *cat* locus are lower than for the *trpC* locus, probably as a result

of the fact that the *cat* gene may not always be integrated when there is recombination in the *trpC-F* region.

Transformation frequencies at the *cat* locus are highest for the replicative plasmid pMK4. Successful transformation using pMK4 donor DNA does not require homologous recombination but depends upon establishment of the plasmid as an autonomously replicating extra-chromosomal element in the recipient cell.

2. UV survival of transformed and total cells

Changes in cell density in response to UV dose

The transformation frequency is defined as the ratio of two cell numbers (the number of transformed cells divided by the number of total cells), each of which may respond differently to UV dose. Consequently, to understand the response to UV of the transformation frequency for a given donor DNA, one needs to consider the response of cell numbers and survival to UV dose. Changes in cell density for total cells and transformed cells in a culture in response to UV dose were determined for the following three plasmid donor DNAs: pTRPH3 at DNA concentrations of 1.0 and 0.1 $\mu\text{g/ml}$, and pMK4 and pJH101-*trp1* at the concentration of 1.0 $\mu\text{g/ml}$. The absolute cell numbers as a function of UV radiation for transformed and total cells are presented in Fig. 2 (UV-DNA and DNA-UV experiments pooled). Because total cell number is not affected by the kind or concentration of donor DNA used in an experiment, the numbers of total cells on LB medium is pooled for the pMK4 and pJH101-*trp1* experiments and total cell number on MG medium are pooled for the different concentrations of pTRPH3 donor DNA. Initial total cell densities in all experiments reported here vary around 10^8 cells/ml. Differences between experiments in initial cell densities translate into differences in survival at the UV doses tested making for large variances in absolute cell numbers at the tested UV doses. For this reason, statistics that are not standardized to initial cell density, such as absolute cell numbers or transformation frequency, are highly variable (for example, Figs 2 and 6), while statistics that are expressed relative to the initial cell density for each experiment, such as survival and the deviation in transformation frequency (Equation (1)), are less variable (for example, Figs 3, 4, 5, and 7).

Cell survival may be calculated for the data in Fig. 2. At a given UV dose, cell survival is defined as the number of cells surviving the UV dose divided by the number of cells at no UV (0 J/m^2) dose (Equation 1).

pJH101-trp1 transformants survive better than total cells

Cells transformed to chloramphenicol resistance by the homologous plasmid pJH101-*trp1* survive UV treatment better than total cells (Fig. 2). The regressions in Fig. 3 are different at the $P < 0.0001$ level (Tab. 2).

pMK4 transformants and total cells survive the same

In contrast to pJH101-*trp1* experiments, cells transformed to chloramphenicol resistance by the non-homologous plasmid pMK4 survive UV treatment the same

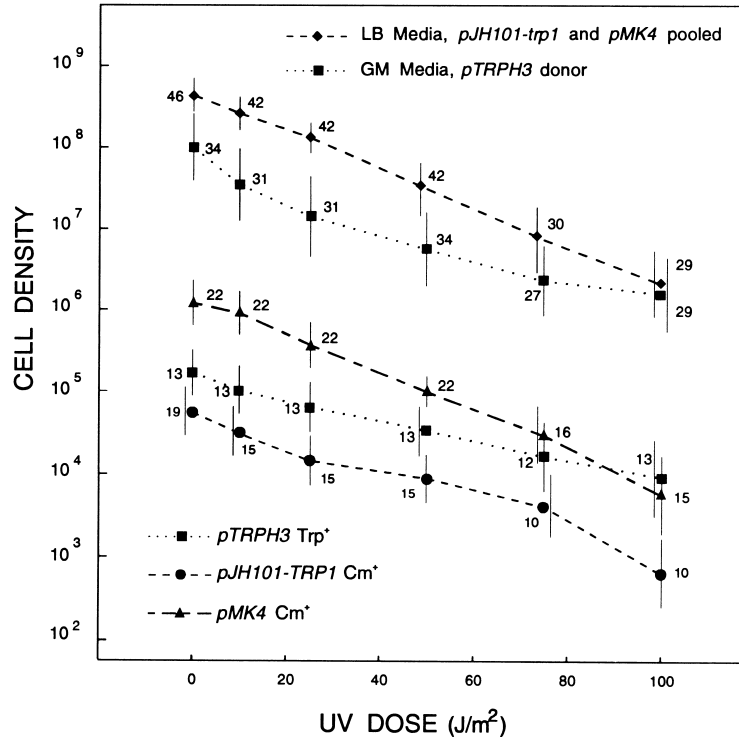


Fig. 2. Densities (numbers/ml) of plasmid transformants (bottom curves) and total cells (top curves) in response to UV dose. Points plotted are the averages connected by lines. Error bars give the average \pm the standard error. Sample size given near each average. Order of UV treatment (UV-DNA and DNA-UV) and DNA pooled for reasons explained in text. Transformant numbers are for 1.0 $\mu\text{g/ml}$ donor DNA. Total cells for pTRPH3 include both 1.0 and 0.1 $\mu\text{g/ml}$ experiments as there was no difference in total cell density for the two treatments. See text for further explanation.

as total cells. A separate figure showing this result is not present here for reasons of space. However, the relevant data are shown in Figs 5 and 3. Total cell survival in pJH101-trp1 and pMK4 experiments is the same (data not shown), so the pooled data for total cells given in Fig. 3 can be used to compare pMK4 transformation results. The pMK4 transformants survive curve presented in Fig. 5 is almost identical to the total cells survival curve in Fig. 3.

pTRPH3 transformants survive better than total cells

Cells transformed to Trp⁺ prototrophy by the homologous plasmid pTRPH3 exhibit greater survival to UV treatment better than total cells (Fig. 4). The regressions in Fig. 4 are different at the $P < 0.0001$ level (Tab. 2). The survivals of total cells shown in Figs 3 and 4 are different. This is because total cells (and transformants) are plated on a rich complex medium (LB) when selection is for Cm^R (Fig. 3), whereas total cells are plated on a minimal medium (MG) when

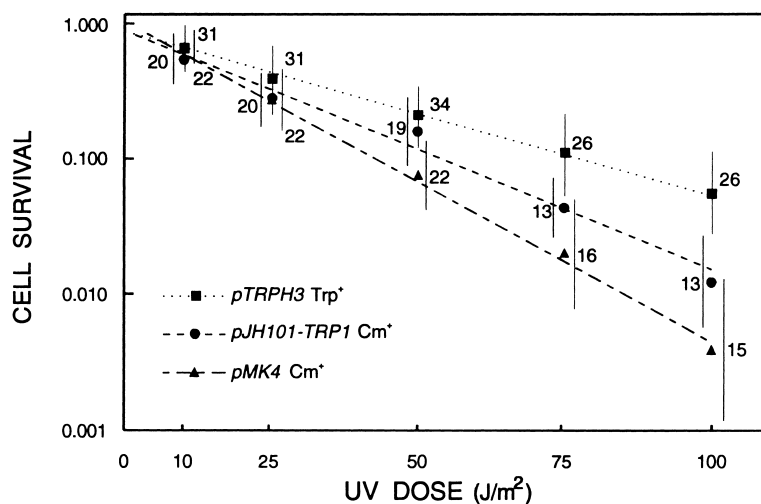


Fig. 3. Cell survival for Cm^R transformants in pJH101-trp1 donor DNA experiments is greater than survival of total cells. Donor DNA concentration used as 1.0 $\mu\text{g}/\text{ml}$. Order of UV treatment and DNA (UV-DNA and DNA-UV) pooled. Total cell survival in pJH101-trp1 and pMK4 donor experiments pooled. Points plotted are the averages. Error bars give the average \pm the standard error. Sample size given near each average. Regression analyses given in Table 2.

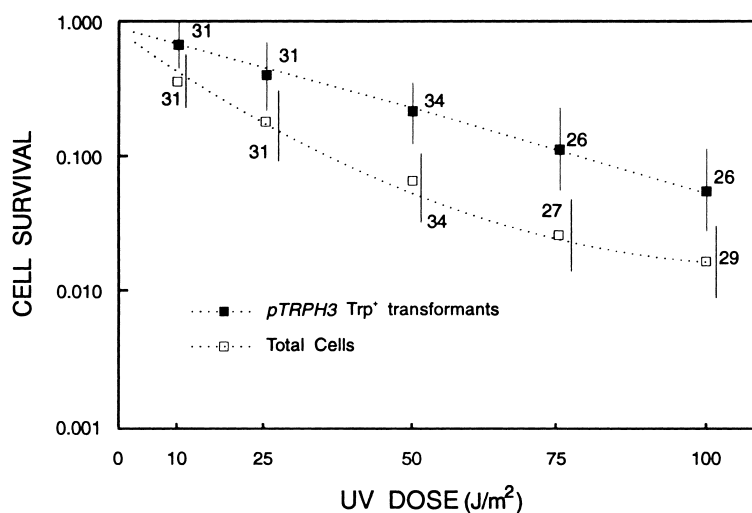


Fig. 4. Cell survival for Trp⁺ transformants and total cells in pTRPH3 donor DNA experiments. Donor DNA concentration is 1.0 $\mu\text{g}/\text{ml}$, and order of UV treatment and DNA addition (UV-DNA and DNA-UV) are pooled. Points plotted are the averages. Error bars give the average \pm the standard error. Sample size given near each average. Regression analyses given in Table 2.

selection is Trp⁺ prototrophy (Fig. 4). The survival of total cells on LB medium (pMK4 and pJH101-trp1) is linear on a semi-log plot (Fig. 3), while survival on MG medium (pTRPH3) is best described by a quadratic regression (Fig. 4).

Cell survival of transformants: pTRPH3 > pJH101-trp1 > pMK4

Competent cells transformed to Trp⁺ using homologous plasmid pTRPH3 donor DNA survived UV treatment better than Cm^R transformants using the homologous plasmid pJH101-trp1, while pJH101-trp1 transformants survived UV treatment better than pMK4 transformants (Fig. 5). Pairwise comparisons of the three regressions for transformant survival in Fig. 5 are significantly different at the $P = 0.0001$ level or below. However, the comparison between the pTRPH3 survival curve and the other curves is confounded by the fact that the plating medium for Trp transformants is different from the plating medium for Cm^R transformants (see methods).

Regression analysis of data

The regression analyses for the data presented in Figs 3 through 9 are described in Table 2. The differences in cell survival as a function of UV dose observed here are not large by the standards commonly employed in molecular biology, which usually require order of magnitude effects instead of the two or three fold effects considered here. Nevertheless, these differences are highly significant statistically and are meaningful when considered within the context and from the perspective of

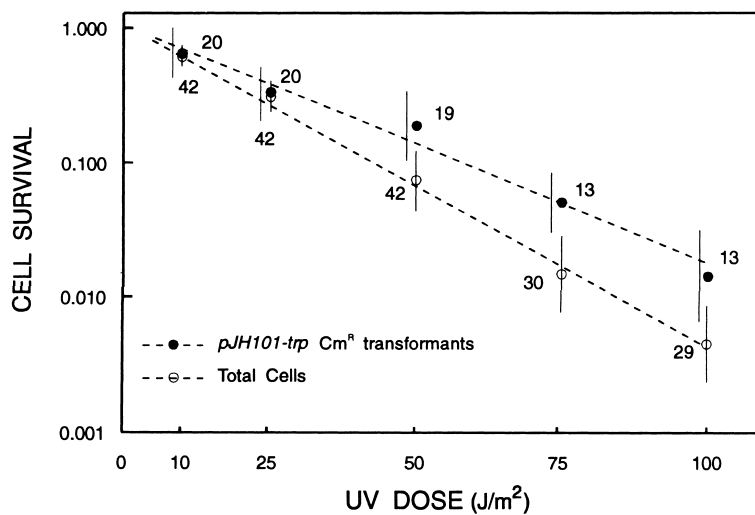


Fig. 5. Survival of transformed cells in plasmid DNA transformation experiments in response to UV dose. Donor DNA concentrations and order of UV treatment and DNA (UV-DNA and DNA-UV) are pooled. Points plotted are the averages. Error bars give the average \pm the standard error. Sample size given near each average. Regression analyses given in Table 2. Transformants for Cm resistance are plated on a different medium than transformants for Trp prototrophy.

Table 2. Regression analysis of cell survival data in Figs 3–9.

	Intercept ^a	Coefficients ^a			<i>r</i> ²
		UV	UV ²	UV ³	
Figure 3					
pJH101-trp1	0.00154	−0.0176*****			0.87
Total cells, LB	0.0261	−0.0240			0.94
Figure 4					
pTRPH3	−0.0317	−0.0119*****			0.78
Total cells, MG	−0.0666*	−0.0314*****	0.0001*****		0.87
Figure 5					
pTRPH3	−0.0317	−0.0119*****			0.78
pJH101-trp1	0.00154	−0.0176*****			0.87
pMK4	0.0726	−0.0237*****			0.88
Figure 7					
pJH101-trp1	−0.0386		0.0003*****	3.8×10^{-7} *****	0.58
pTRPH3	0.0400	0.0174*****	−0.0001*****		0.46
YB1011 UV-DNA	0.0398	0.0104***	−0.0001*		0.16
YB1011 DNA-UV	−0.00018	0.0046	−0.0001*		0.38
Figure 8 (bottom panel)					
YB1011					
time ≤ 60'	3.4101	0.0211*****			0.50
time > 60'	4.6005	0.0051			0.05
total cells					
time ≤ 45'	6.8725	0.0326*****			0.41
time > 45'	8.3352	0.0036***			0.15
pTRPH3	3.5892	0.0431*****	−0.0002*****		0.82
Figure 9					
pTRPH3	0.0466	0.0077***			0.52
YB1011 + pMK4	0.0111	−0.0011			0.02

Tests for differences between regressions are discussed in the text.

^a Intercept and coefficients are for regressions of log transformed data. For Figs 8 and 9, coefficients are “Time” instead of “UV”.

^b Value is complete piecewise regression involving both pieces for time ≤ 60' and time > 60'.

P* < 0.1, *P* < 0.05, ****P* < 0.01, *****P* < 0.001, ******P* < 0.0001.

evolutionary biology. It is such differences between types, on the order of just a few percent, that can have large effects over evolutionary time. Two or three fold differences matter in competition and in evolution.

3. UV increases frequency of transformation by homologous DNA donors

The response of the absolute transformation frequency (Equation 1; defined as the numbers of transformants divided by the numbers of total cells) to UV dose for

the three plasmid donor DNAs with strain YB886 is shown in Fig. 6. For each of the three plasmids data from the DNA-UV and UV-DNA experiments are pooled, as there is no difference between the DNA-UV and UV-DNA experiments in the response of the transformation frequency for each donor DNA to UV. The deviation in transformation frequency or "DTR", defined as the transformation frequency at a given UV dose divided by the transformation frequency at no UV, 0 J/m^2 (Equation (1) above), is plotted against UV dose for each of the different plasmid and chromosomal donor DNAs and shown in Fig. 7. If the DTR is greater (lesser) than the value of one at a specified UV dose, then the transformation frequency has increased (decreased) at that dose from what it was with no UV. The results in Figs 6 and 7 show that the level of UV dose to the recipient cells had relatively little effect on the transformation frequency for pMK4, except, for the initial response to 10 J/m^2 . This slight increase in transformation frequency at 10 J/m^2 becomes convincing in light of the smaller variance associated with the result. Nevertheless, for the rest of the doses, the transformation frequency for this donor DNA shows no apparent response to UV treatment.

In contrast, the transformation frequency for both of the homologous DNA-containing plasmids increases in response to UV dose. Transformation to chloramphenicol resistance increases with increasing dose of UV to recipient cells, when transformation requires homologous recombination (pJH101-trp1 curve in Fig. 7). However, no such increase is observed for the same locus when it is carried on a non-homologous plasmid (pMK4 curve in Fig. 7). The difference in transformation

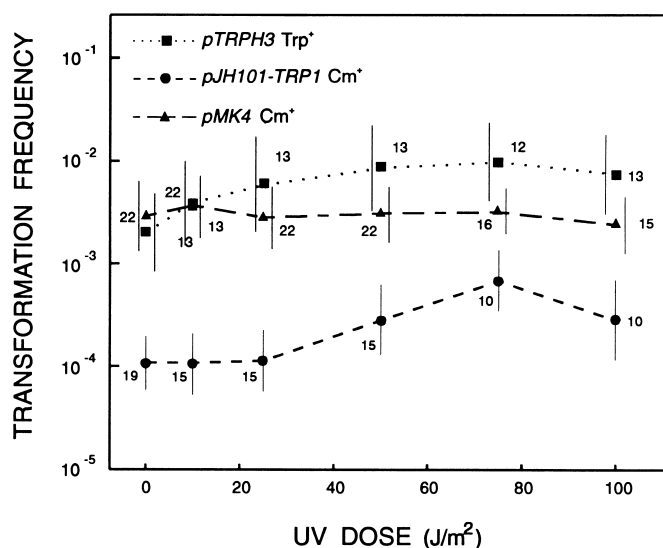


Fig. 6. Transformation frequency for the three plasmid DNAs (at $1.0 \mu\text{g/ml}$) as a function of UV dose to strain YB886, with data from DNA-UV and UV-DNA experiments pooled. Points plotted are the averages. Error bars give the average \pm the standard error. Sample size given near each average.

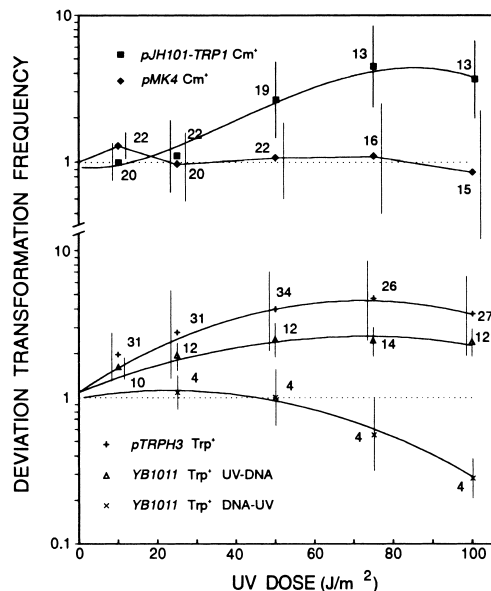


Fig. 7. Deviation in transformation frequencies for plasmids pTRPH3, pMK4, pJH101-trp1 and YB1011 chromosomal DNA as a function of UV dose. Transformation frequency measured for Trp⁺ at the *trpC* locus in strain YB886 for pTRPH3 and YB1011 donor DNAs, and for Cm^R marker for pMK4 and pJH101-trp1 donor DNAs. Data from UV-DNA and DNA-UV treatments pooled for pTRPH3, pJH101-trp1 and pMK4 experiments. DNA concentration for 1.0 and 0.1 $\mu\text{g}/\text{ml}$ experiments for pTRPH3 pooled. DNA concentration for pMK4 and pJH101-trp1 is 1.0 $\mu\text{g}/\text{ml}$. UV-DNA and DNA-UV results for YB1011 donor DNA shown for comparison (Wojciechowski, Hoelzer and Michod, 1989; 0.01 and 1.0 $\mu\text{g}/\text{ml}$ donor DNA experiments pooled). The scale of the y-axis is the same in all regions of the figure. Points plotted are the averages. Error bars give the average \pm the standard error. Sample size given near each average. Regression analyses given in Table 2.

frequencies between the three DNAs is primarily due to the different survivals of the transformants as discussed previously (Fig. 5).

The increase in transformation frequency at the *trpC* locus as a function of UV dose can be compared for the different sources of homologous *trpC* donor DNA, pTRPH3 and YB1011 (Fig. 7). In UV-DNA experiments, the *trpC* transformation frequency increased more dramatically when the homologous DNA donor marker was carried on plasmid-borne fragments than when it was carried on high molecular weight chromosomal fragments ($P = 0.0001$ level, comparing the regression for pTRPH3 pooled data with that for YB1011 UV-DNA). With chromosomal donor DNA, the transformation frequency did not increase in DNA-UV experiments (Fig. 7), whereas the transformation frequency increased in DNA-UV experiments for pTRPH3 (Trp⁺ transformants) and pJH101-trp1 (Cm^R) donor DNAs.

Since the response of the transformation frequency as a function of UV dose is similar for the two pTRPH3 DNA concentrations (separate data not shown here), the DTRs for the two DNA concentrations were pooled and their regression is

shown in Fig. 7. No regression for pMK4 is given in Table 2 since its (DTR) slope is not significantly different from zero. The deviation of transformation frequency with UV for pJH101-trp1 and pTRPH3 are each significantly different ($P < 0.0001$, Tab. 2) from the regression for pMK4.

In a previous paper (Wojciechowski, Hoelzer and Michod, 1989, Fig. 2, Tab. 3), we first reported transformation frequencies for UV-irradiated cells transformed with pMK4 donor DNA. Results from that work for UV-DNA treatments are almost identical to those presented in Fig. 7, and, also showed a slight increase in transformation frequency at 10 J/m^2 . However, for the (non-delay) DNA-UV experiments used previously, the results with pMK4 DNA showed a decrease in transformation frequency with increasing dose of UV radiation to the recipient cells (Wojciechowski, Hoelzer and Michod, 1989, Fig. 2, Tab. 3) similar to that observed for DNA-UV treatments with YB1011 donor DNA (Fig. 7). In contrast to these previous results, there is no noticeable trend in the transformation frequency with increasing UV dose in the delay DNA-UV experiments reported here and for this reason the UV-DNA and DNA-UV experiments were pooled in Figs 6 and 7. This discrepancy with our previous DNA-UV results can be explained on the basis of the fact that our previous DNA-UV protocol with pMK4 DNA did *not* employ a 45-minute delay after UV treatment before plating, whereas our present protocol does employ this delay. UV-DNA experiments naturally have a 45 minute time period after UV treatment during which time the culture is incubated with transforming DNA. Thus, for UV-DNA and DNA-UV experiments to be comparable, DNA-UV experiments must incorporate this delay so that the cultures have equal time for cell growth and division following UV and before plating (Wojciechowski, Hoelzer and Michod, 1989). The time in growth medium after UV treatment affects survival, because DNA replication expresses unrepaired DNA lesions. Since total cells (predominately non-competent) in competent cultures are generally dividing more rapidly than the competent cells (Dabnau, 1991), time spent in growth medium after UV treatment may cause greater killing of the total cells than competent cells just because competent cells have longer for excision repair to operate before DNA replication. This effect also explains why transformation frequencies in DNA-UV experiments are higher when a delay is incorporated than when cells are plated immediately after UV treatment (Wojciechowski, Hoelzer and Michod, 1989; Fig. 2 and Tab. 3).

4. Why does the order of DNA and UV not matter for plasmid transformation?

Donor DNA is administered after UV treatment in UV-DNA experiments and before UV in DNA-UV experiments. For the three plasmids studied here (pJH101-trp1, pMK4 and pTRPH3), the results of UV-DNA and DNA-UV experiments did not differ in any significant way. For example, the survivals of transformants were similar in both treatments and the transformation frequency responded to UV radiation in a similar fashion in both UV-DNA and DNA-UV experiments. For these reasons, the results were pooled in Figs 2, 3, 4, 5, 6, and 7. In contrast, the

results of UV-DNA and DNA-UV experiments with *chromosomal* DNA-mediated transformation differed dramatically (representative results are given here in Fig. 7; Michod, Wojciechowski and Hoelzer, 1988; Wojciechowski, Hoelzer and Michod, 1989; Hoelzer and Michod, 1991). Why do plasmid and chromosomal transformation behave differently in this regard?

For non-homologous pMK4 transformation, there is no response of the transformation frequency to UV in either UV-DNA or DNA-UV experiments. Consequently, we focus on the question of why homologous plasmid transformation frequencies (for example, pTRPH3) increase in both UV-DNA and DNA-UV experiments.

Homologous plasmid transformation continues after chromosomal transformation is complete

We hypothesized that homologous plasmid transformation frequencies increase in both UV-DNA and DNA-UV experiments because transformation is still occurring at the time of UV in a DNA-UV experiment when homologous-plasmid-DNA (pTRPH3 or pJH101-trp1) is used, but that transformation is complete at this time when either of the other two kinds of DNA is used: homologous-chromosomal-DNA (YB1011) or non-homologous-plasmid-DNA (pMK4). To test this idea we studied the kinetics of transformation for three different donor DNAs: pTRPH3, YB1011 and pMK4. We find that transformation by homologous-plasmid-DNA (pTRPH3) is still occurring at the time of UV treatment in DNA-UV experiments (Fig. 8), whereas transformation by either chromosomal or pMK4 donor DNAs is essentially complete at this time (i.e., ca. $T_{90} + 100$ min.; Fig. 8). We do not report the pMK4 data here for reasons of space and since it didn't show any response to UV in either treatment. In conclusion results reported below show that DNA-UV and UV-DNA experiments do not differ with regards to the order of treatment of UV and homologous-plasmid-DNA, as they do for homologous-chromosomal-DNA and as they do for non-homologous-plasmid-DNA.

First, we studied how the numbers of transformants depends on time of incubation with donor DNA. In these experiments, saturating amounts of donor DNA (1 μ g/ml) were added to a competent culture, and then at the indicated times thereafter aliquots of the transformed culture were taken, treated with DNase I and plated as described above. For this reason, we refer to these experiments as "Time Before DNase" or "TBD" experiments. The cell densities for transformants and total cells in TBD experiments for chromosomal-DNA (YB1011) and homologous-plasmid-DNA (pTRPH3) transformation experiments are shown in the bottom panel of Fig. 8. A quadratic regression fit the pTRPH3 transformants, however, for chromosomal-DNA transformants or total cell density, a quadratic or linear regression did not describe the data well. Linear piecewise regressions were fitted to these cell densities as shown in Fig. 8. The effect of time being less than 45 min., or greater than 45 min., is significant at the $P < 0.05$ level for chromosomal transformants and the effect of time being less than 60 min., or greater than 60 min. is significant at the $P < 0.0001$ level for total cell numbers. Notice in Table 2 for

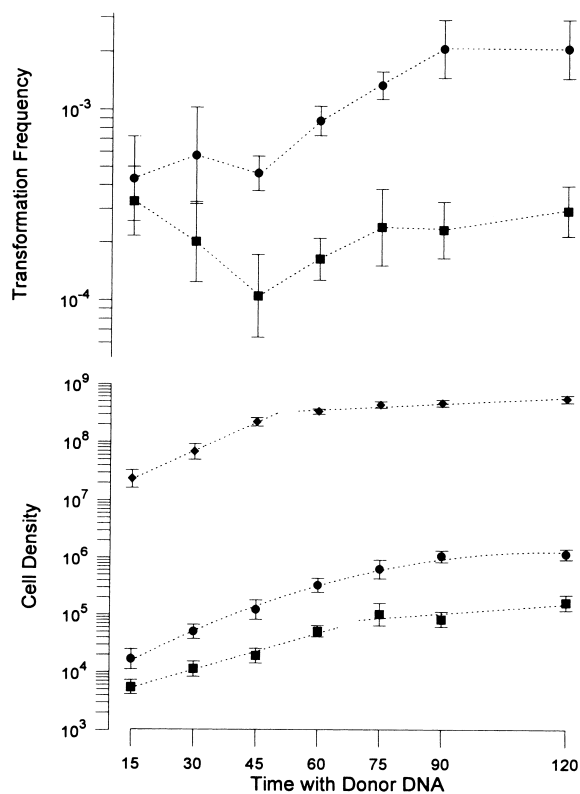


Fig. 8. Effects of time of incubation with homologous donor DNAs on numbers of cells (bottom panel) and transformation frequency (top panel). Transformation at *trp* locus. Squares are for chromosomal YB1011 transformants (both panels), circles for plasmid PTRPH3 transformants (both panels) and diamonds for total cell density (bottom panel). Average values and error bars (standard error of the mean) are plotted. Regression analyses for cell densities in bottom panel are given in Table 2. In top panel, average values are simply connected by a line.

YB1011 donor DNA that the slope of the time > 60 min. portion is not significantly different from zero, while the slope for time < 60 min. is highly significant. In other words, density of transformants for chromosomal DNA does not increase past about 60 minutes.

The transformation frequency is the density of transformants divided by total cell density. For chromosomal-DNA, the transformation frequency decreased with time (Fig. 8, top panel), since total cell density increases faster than transformant density (Fig. 8, bottom panel). However, for homologous-plasmid-DNA, the transformation frequency continues increasing up to 90 min. at which time it levels off. Since competent cells are dividing more slowly than non-competent cells, if they divide at all (Dooley, Hadden and Nester, 1971; Nester and Stocker, 1963), the increase in transformation frequency observed for pTRPH3 as a function of time should result

from newly transformed cells. In conclusion, transformation is still occurring after 60 minutes with homologous-plasmid-DNA (pTRPH3), whereas it is complete by this time with chromosomal-DNA (or pMK4 DNA, data not shown).

Homologous plasmid transformation continues after DNase I treatment

Several factors could contribute to the difference in the kinetics of plasmid pTRPH3 and chromosomal DNA-mediated transformation. Differences may exist in copy number or efficiency of binding, uptake and/or integration of the transforming fragment at the recipient's *trpC* locus when the marker is carried on plasmids as compared to when it is carried on chromosomal fragments. To better understand what factors might be responsible, we conducted separate experiments in which the transformation frequency was measured after subjecting the culture to DNase I treatment ("Time after DNase" or "TAD" Experiments). DNase I effectively stops all further binding and subsequent uptake of exogenous DNA in competent cells (Dubnau, 1982). However, DNase has no effect on the processing and/or recombination of DNA that is already in progress inside a cell. Therefore, any differences observed in TAD experiments between chromosomal- and plasmid-DNAs should not be caused by differences in binding of the two DNAs or copy number of the selected marker outside the cell, but rather by differences inside the cell.

In the TAD experiments, following the standard procedure for incubation with transforming DNA and DNase I, cells from a competent culture were collected and

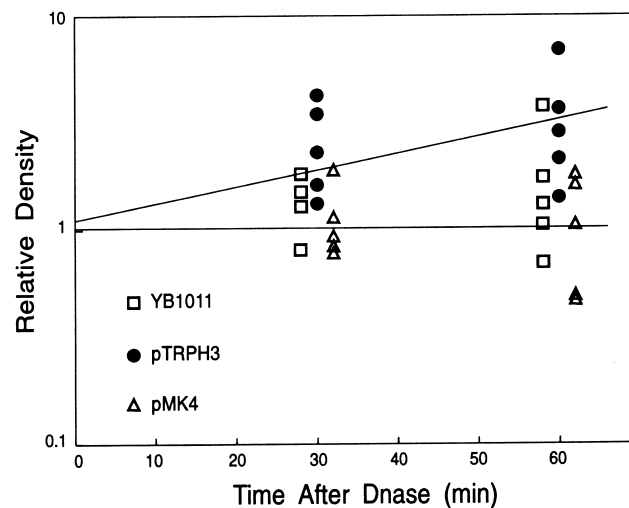


Fig. 9. Effect of time after DNase on Relative Density of transformed cells. After addition of transforming DNA and DNase, cells are collected and resuspended in GM2 and incubated at 37° C for the indicated times before plating. Transformants were counted at 0, 30 and 60 min. after DNase treatment for all three donor DNAs. For ease of presentation, the data points for YB1011 and pMK4 DNA transformants are offset at the 30 and 60 min. times. The regressions for pTRPH3 and YB1011 + pMK4 (pooled) are significantly different ($P = 0.0001$). Regression analyses given in Table 2.

resuspended at the same density in GM2 and incubated again at 37° C. As shown in Fig. 9, numbers of pTRPH3 transformants (Trp⁺) continued to increase after DNase treatment and resuspension in fresh growth medium, while numbers of chromosomal DNA (Trp⁺) and pMK4 DNA (Cm^R) transformants did not increase following DNase I treatment of the culture. Since there was no difference in the regression for the chromosomal and pMK4 transformation results, the data was pooled (the regression shown in Fig. 9 and Tab. 2 is for the pooled data, and the individual regressions for pMK4 and YB1011 donor DNAs are indistinguishable for the pooled regression).

In conclusion, the results of the TBD and TAD experiments shown in Figs 8 and 9 indicate that significant transformation (generation of transformants) is still occurring for homologous-plasmid-DNA (pTRPH3) at the time of UV treatment in DNA-UV experiments, whereas transformation for chromosomal DNA (YB1011) and non-homologous-plasmid-DNA (pMK4) is complete by this time.

Discussion

Rationale of protocol

There are two internal controls present in our experiments. First, the numbers and survival of total cells (primarily non-competent cells) are compared to the numbers and survival of transformed cells. These comparisons are made at the same time, in the same treatment, in the same population. Second, we compare the relative numbers (or survival) of these two classes of cells for two treatments that differ in the order of administration of UV and DNA: the so-called UV-DNA and DNA-UV treatments. By comparing the response of cell densities to UV in the two treatments, we control for possible effects of the many differences that exist between competent and non-competent cells, since these differences should be present in both treatments. Background levels of DNA should also be similar in the two treatments. Thus, if different results are obtained in the UV-DNA and DNA-UV treatments (as reported for homologous chromosomal donor DNA in Fig. 7), the difference must be due to the order of administering UV and donor DNA.

Since only about 10% of a *B. subtilis* population of cells are actually competent, the majority of non-transformed cells are non-competent, and, therefore, have not bound or taken up exogenous DNA. These non-transformed, non-sexual, cells serve as an internal control for the effect of experimentally added DNA on competent cells. Since bacterial cells release DNA, either actively or upon death, there is always homologous DNA present in a bacterial culture, even if no additional DNA is provided in the experiment. Therefore, it is not possible to have a no-DNA control by simply not adding additional DNA (as done, for example, in Mongold, 1992 and Redfield, 1993b). A nice feature of the *B. subtilis* system for testing theories of sex is precisely that *not* all cells are competent. Consequently, both sexual and asexual cells are present in the same population at the same time in the same treatment so that non-competent cells may serve as an internal comparison for competent cells.

Recent work on transformation in other labs

Recent papers claim to show that transformation did not evolve for the function DNA repair (Redfield, 1993a, b and Mongold, 1992). In the work reported above, a population of cells was first grown to competence – that is the life stage and conditions during which competence is maximal – and then the response of the transformation frequency to the amount of DNA damage given to the competent cells is measured. Redfield (1993a) has conducted similar experiments on *B. subtilis* and *H. influenzae*, but in her experiments the transformation frequency was studied as a function of time after the administration DNA damage. This was done for populations that were either grown to stationary phase (and competence) or were at log phase and rapidly dividing. In no case did she find an effect of DNA damage on the transformation frequency to time after administration of the damage up to a period of four hours after the time of damage. She interpreted these results as showing that DNA damage does not induce competence and as evidence against the hypothesis that transformation evolved for DNA repair. We do not believe Redfield's results refute the repair hypothesis for several reasons.

There are reasons that are specific to the bacterial systems under study. In the case of rapidly growing noncompetent populations (Fig. 2 of Redfield, 1993a), individual cells are basically diploid for most loci. In bacteria, DNA replication is not precisely coordinated with cell division, and so most genes are present in multiple copies in rapidly dividing cells. A bacterial cell does not need to become competent to obtain extra templates from outside the cell, if it is already diploid at many or most loci. In the case of populations that are already induced for maximal competence (Fig. 3 of Redfield, 1993a), we don't see why Redfield expected that competence could be increased further by damage? After all, maximal competence is maximal competence.

Transformation frequencies are quite variable. To measure two- and three-fold effects like those reported above, we performed statistical analyses of multiple replicates. We doubt that effects of these magnitudes could have been detected by Redfield (1993a), since multiple replicates were not reported in her studies. Although her aims were different, certain data points of Redfield's (1993a) study are directly comparable to the studies reported here. For example, she found no initial increase in the transformation frequency following UV irradiation of 10, 30 or 60 J/m² in *B. subtilis* cultures when compared to the transformation frequency with no UV ("0 min. after damage" points of the "+DNA" curves in panel D, Fig. 3, of Redfield, 1993a). This observation is based on a single data point for each UV dosage (0, 10, 30 and 60 J/m²). However, from the data set reported in Fig. 7 above, in which over ten replicates at each UV dosage were performed, we found that transformation frequencies do show a significant increase (the YB1011 UV-DNA curve in Fig. 7) over the range of UV studied by Redfield (1993a).

However, there is a more general issue concerning how one infers the function of a process from its regulation. As mentioned above, Michod, Wojciechowski and Hoelzer (1988) and Wojciechowski, Hoelzer and Michod (1989) also concluded that DNA damage did not induce competence (the binding and uptake of DNA)

based on their observation that DNA damage does not increase non-homologous plasmid transformation (results shown in Fig. 7 above). But we did not view this result as counter to the repair hypothesis, while Redfield (1993a, b) does. The reasons we do not view this observation as counter to the repair hypothesis are as follows.

Transformation is a multi-step process involving binding, uptake, protection, export and recombination of DNA. It is not required that all steps in the pathway respond to DNA damage for DNA repair to be the function of transformation, only that the overall process be regulated in such a way that transformation (sex) responds adaptively to damage. The overall process of transformation, as measured by transformation frequency in *B. subtilis*, does respond adaptively to DNA damage as shown in Fig. 7. The development of the competence stage is likely a response to starvation and low genetic redundancy inside the cell. There is no reason for a cell to bring in template for repair from outside, if the cell is already diploid for most loci – as bacterial cells are during phases of rapid growth. Starvation often stimulates sex in facultatively sexual organisms probably because low resources indicate a high local density of cells. We doubt that starvation stimulates sex because sex provides nutrition, as Redfield (1993b) argues. The reason, we think, is simply that starvation indicates there are others about. Why have sex if there are not others about?

Our point is that the regulation of any process is usually a complex interaction of different factors. The *lac* operon in *E. coli* functions in metabolism of lactose, yet lactose does not induce β -galactosidase if glucose is present. Furthermore, in studies of the *lac* operon, there is no relation between the inducing capacity of a compound and its affinity for β -galactosidase. Likewise, why should DNA damage induce competence, if cells are already diploid for most loci, or if there are not other cells about? Quite simply, there is usually not a simple direct relationship between the factors that induce each step in a process and the overall function of the process. For these reasons, we do not believe that experiments of the kind conducted by Redfield (1993a) refute the repair hypothesis.

Experiments of the kind conducted by Mongold (1992) and Redfield (1993b) should, in principle, be able to test the repair hypothesis. However, we believe that more work and more careful controls need to be done for their negative results to be interpreted as refuting the repair hypothesis. Mongold (1992) reported that added homologous DNA increases the survivorship of partially, but not fully, competent populations of *H. influenzae* in the face of UV irradiation. The apparently beneficial effect of added DNA in partially competent populations could not be attributed to DNA repair. We do believe there is a transient effect of DNA in Mongold's (1992) experiments that cannot be explained by recombinational repair using transforming DNA as template. Unfortunately, Mongold was not able to clarify the cause of this transient effect. In any event, the relevance of this transient effect to the evolution of transformation is unclear, since the effect disappears when cells are fully competent. For this reason, we believe the main result of Mongold's paper to be a negative one – no benefit of added homologous DNA was observed in fully competent populations in the face of UV irradiation. Redfield (1993b) also

reports no effect of homologous DNA on survival of fully competent *H. influenzae* populations in the face of mitomycin C.

There are several reasons why a negative result could be obtained in Mongold's (1993) and Redfield's (1993b) experiments. (i) The repair hypothesis may be incorrect. This is the interpretation Mongold (1992) favors, but Redfield (1993b) is more cautious. We would accept this interpretation, if the following alternative interpretations could be addressed.

(ii) UV radiation used by Mongold (1992) is not an appropriate damaging agent for *H. influenzae* and it may be that *H. influenzae* has not evolved a means of responding to UV. For an organism, like *B. subtilis*, that lives in the soil, UV radiation is a constant challenge. However, *H. influenzae* lives in the respiratory system and UV radiation is probably not a naturally occurring problem. Oxidative damage is more relevant for an organism that lives in the respiratory system, and, consequently, an oxidative damaging agent like hydrogen peroxide would be more appropriate. The relevance of the agent mitomycin C used by Redfield (1993b) to the life style of *H. influenzae* is also unclear.

(iii) The background DNA, likely present in Mongold's (1992) and Redfield's (1993b) cultures, was sufficient template for repair in fully competent cultures and, consequently, there was no effect of the added DNA. As we pointed out in the discussion of our protocol above, there is probably always background DNA available in a culture of bacterial cells because of active export of DNA (as is known to occur in *B. subtilis*) and/or cell lysis. Consequently, there is not a "no DNA" control in Mongold's (1992) and Redfield's (1993b) experiments – only a "no added DNA" control. Fully competent cells may be especially good at using their partner's resident DNA. Mongold (1992) and Redfield (1993b) use saturating levels of added DNA, however, this simply means that enough DNA was added to the cultures to out compete the resident DNA. There still could be plenty of resident DNA available for repair in a fully competent culture, *even when no DNA is added to the culture*. This explanation is testable by diluting competent cells into fresh media that contain no DNA and measuring survival in the face of a damaging agent, while making sure to control for the absorbing effect that the resident DNA may have on the effective dose of the damaging agent. If cells are using resident DNA for repair, cell survival should decrease when the resident DNA is diluted out.

(iv) Transforming DNA functions in DNA repair, however, the effect on survivorship is too small to be measured in Mongold's (1992) and Redfield's (1993b) experiments. We do not like this interpretation, but it could well be true. During the 30 or so minutes typical of most transformation experiments only a small fraction of the genome can be brought into the cell. Only rarely would incoming fragments be homologous to any particular damage site, unless the uptake of DNA fragments is somehow targeted to damage sites. However, over longer periods of time, the probability of obtaining a needed fragment could be much higher. Michod, Wojciechowski and Hoelzer (1988) and Redfield (1993b) discuss other matters bearing on this interpretation.

Relation of results to hypotheses

Previously, we demonstrated in homologous-chromosomal-DNA-mediated transformation of *B. subtilis* that transformed cells have higher survival than total cells in UV-DNA experiments but not in DNA-UV experiments (Michod, Wojciechowski and Hoelzer, 1988; Wojciechowski, Hoelzer and Michod, 1989; Hoelzer and Michod, 1991). This higher survival of transformants results in increasing transformation frequencies with increasing UV dose in UV-DNA experiments, but not in DNA-UV experiments (a typical result is shown here in Fig. 7, YB1011 DNA curves). Further, these results could not be attributed to induced excision repair or SOS repair in competent cells or to more general differences between competent and non-competent cells.

Three hypotheses have been considered as explanations for the observed increase in chromosomal DNA-mediated transformation in UV-DNA experiments but not in DNA-UV experiments (Michod, Wojciechowski and Hoelzer, 1988; Wojciechowski, Hoelzer and Michod, 1989; Hoelzer and Michod, 1991). First, competent cells may have higher fitness in a damaging environment as a result of a greater capacity for recombinational repair (postreplicational) of DNA damage afforded by the homologous transforming DNA. Second, UV-induced damages in DNA may directly stimulate the frequency, efficiency, or localization of homologous recombination (Clark and Low, 1988) and, hence, transformation. Third, UV treatment may result in the increased binding or uptake of exogenous DNA. Any of these hypotheses could explain the increase in frequency of genetic transformants (chromosomal donor DNA) with increasing UV dose in UV-DNA experiments.

The results from experiments using the three donor DNAs in this study have helped to resolve which of the three hypotheses discussed above is likely to be correct. First, consider the hypothesis that UV radiation directly increases genetic recombination (Clark and Low, 1988; Devoret, 1988), as measured here by transformation. UV radiation could increase transformation frequencies by either increasing the rate or efficiency of binding and uptake of DNA, or UV radiation could increase transformation by increasing rates of recombination directly. The finding that pJH101-*trp1* transformation frequencies increase in response to UV, while pMK4 transformation frequencies are unaffected by UV, indicate that DNA damage increases transformation by directly increasing rates of recombination and not by increasing the binding or uptake of transforming DNA (Figs 3, 5 and 7). Although experiments with these two different plasmids utilize the same non-homologous marker for selection of transformed cells (i.e., Cm resistance), the successful transformation of cells with pJH101-*trp1* DNA requires the sequence homology provided by the *trpC-trpF* region cloned in pJH101-*trp1*, while transformation with pMK4 DNA does not. Since pMK4 transformation is independent of a cell's ability for homologous recombination, UV irradiation could increase the frequency of pMK4 transformation only if DNA damage somehow increased the efficiency or rate of binding and/or uptake of exogenous donor DNA.

Second, consider the hypothesis that transforming DNA may provide a source of homologous template for the recombinational repair of damages in the recipient's

genome. Plasmid pMK4 donor DNA can not provide any homologous template for the repair of damages to the recipient's chromosome, whereas the plasmids pTRPH3 and pJH101-trp1 can only provide homologous template for repair of damages that are located in the region of the recipient's *trpC* and *trpF* genes. For this reason, the homologous DNA sequences present in pTRPH3 and pJH101-trp1 should be of only limited utility for repair of damages that are distributed elsewhere throughout the genome. Homologous-chromosomal-DNA can, in principle, provide template molecules for recombinational repair of damages anywhere in the recipient's genome. For this reason, chromosomal DNA should be of more general use in repair of DNA damages that occur randomly throughout the genome. This reasoning leads us to conclude that the observed increase in transformation frequency using pTRPH3 and pJH101-trp1 (Fig. 7) is primarily caused by direct stimulation of recombination by UV and not by fitness differences between competent and non-competent cells.

However, in all experiments, whether utilizing chromosomal, pTRPH3, or pJH101-trp1 as donor DNA, we are monitoring competent cells that have undergone a recombination event at the *trpC* locus. Thus, for both chromosomal (UV-DNA experiments), pTRPH3, and pJH101-trp1 donor DNA experiments, damages may have been repaired in the region of the recipient's *trpC* gene. For chromosomal donor DNA, damages may have been repaired at other sites in the genome, but whether recombination (and recombinational repair) has occurred elsewhere in a *trpC*⁺ transformant's genome, depends on the number of additional recombination events (in addition to the event that transforms the *trpC* locus). During the typical transformation experiment just a few DNA fragments, amounting to approximately 0.1% of the genome, are brought into the cell (Dubnau, 1982) so that multiple recombination (transformation) events per competent cell are probably infrequent. For this reason, we wonder whether chromosomal or homologous-plasmid-DNA provides widely different repair advantages *in our experiments*. Nevertheless, the most parsimonious hypothesis to explain our results is that UV directly increases rates of homologous recombination during transformation, possibly by providing additional single-stranded regions for the RecA protein to bind. Indeed, activation (and increased synthesis) of RecA is believed to result from the binding of RecA molecules to exposed regions of single-stranded DNA that are produced as a consequence of DNA damage (Devoret, 1988; Sassanfar and Roberts, 1990).

Cells transformed with chromosomal, pTRPH3 or pJH101-trp1 donor DNAs (to Trp⁺ prototrophy) are similar in that they have undergone homologous recombination in the region of the *trpC* locus. A critical issue in determining the utility of homologous donor DNA (whether carried on plasmids or chromosomal fragments) in providing template for recombinational repair in *trpC* transformants, is the question of why a cell becomes transformed at the *trpC* locus in the first place (or any locus for that matter). We consider two points of view as far as the role of DNA damages are concerned: either recombination is targeted to sites of pre-existing damage or sites of recombination bear no relationship to sites of DNA damage.

If recombination (transformation) events are targeted to sites of DNA damage, then cells transformed at specific sites, such as the *trpC* locus monitored in our experiments, may have previously suffered a damage nearby. Support for this hypothesis was provided in a previous paper (Hoelzer and Michod, 1991). The targeting hypothesis suggests that transformed cells should have previously undergone a damage in the region of the marker locus. How many cells are expected to be damaged within an approximately 2500 base pair region around the *trpC* gene and, hence, repairable by the homologous fragment contained within pTRPH3 or pJH101-*trpI* DNA. The answer to this question depends on the level of UV dose and kinetics of UV inactivation. Assuming 1-hit-1-target kinetics, the mean lethal fluence to cells (Harm, 1980) is about 10 J/m^2 in our experiments (Figs 3, 4 and 5), which yields between 0 and 10 lethal hits for the range of UV fluences studied (0 to 100 J/m^2). With these assumptions, about 0.001 of the cells would be damaged in the *trpC* region, a frequency similar to the observed transformation frequency (Fig. 6). Under the targeting hypothesis, one may wonder why cells undergo transformation at 0 J/m^2 , since there should be no damages in the recipient cell. However, cells without at least some basal level of DNA damage probably never exist (even at 0 J/m^2) due to the many endogenous sources of agents which damage DNA.

By what mechanism(s) might UV-induced DNA damage lead to an increase in the frequency of homologous DNA-mediated transformation observed here? UV irradiation could stimulate homologous genetic recombination in competent cells by the production of additional single-stranded regions (in the proximity of damaged sites) in the chromosome which efficiently promote the activation of existing RecA molecules. Although the levels of RecA protein are normally amplified in competent cells, the UV-mediated activation of RecA may result in an increase in either its synthesis or in its ability to catalyze DNA strand exchange. This first appears unlikely given RecA levels in competent cells are already near maximal (Lovett, Love and Yasbin, 1989) and any subsequent increase resulting from further SOS induction in competent cells (Wojciechowski, Hoelzer and Michod, 1989) would probably be negligible. Since transformation proceeds by the internalization and recombination of single-stranded molecules into duplex chromosomal DNA (Dubnau, 1982, 1991), it seems more likely that the observed increase in homologous transformation frequency is the result of an increase in both the binding of RecA to incoming single-stranded DNA and a promotion of the RecA-mediated strand exchange reaction between these molecules and regions of gapped DNA in the chromosome (Roca and Cox, 1990). This possibility is consistent with the proposed repair function for RecA protein during competence in *B. subtilis* and is currently the subject of our investigation. There are, of course, other enzymes involved in homologous recombination in addition to RecA, such as helicases, resolvases, etc., and these other enzymes may also be responding to UV irradiation.

The results of the present paper indicate that UV treatment directly increases transformation in *B. subtilis* by directly increasing homologous recombination, possibly because recombination is targeted to sites of prior DNA damages. A possible fitness advantage of competent cells over non-competent cells cannot be

ruled out by our results, although it is unlikely to be the major cause of the results given here.

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