# DNA Repair and the Evolution of Transformation in *Haemophilus influenzae*

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ABSTRACT

Under certain environmental conditions, naturally transforming bacteria are induced to pick up DNA released into the environment by other cells of the same or closely related species and, by homologous recombination, integrate that DNA into their chromosome. The selective pressures responsible for the evolution and maintenance of this form of genetic outcrossing, or sex, in bacteria are not known. A prominent hypothesis is that transformation, and sex in general, evolved as a means of obtaining DNA templates to repair damaged regions of the chromosome. Previous results obtained with *Bacillus subtilis* were consistent with the repair hypothesis. In an effort to explore the generality of those results, I have tested the repair hypothesis with *Haemophilus influenzae*, a naturally transforming, gram-negative species of bacteria. The results of UV damage-survivorship experiments with *H. influenzae* were also consistent with that hypothesis. However, additional experiments demonstrate that the higher survival of transformed cultures cannot be accounted for by use of the transforming DNA as templates for repair. I consider alternative hypotheses for the means by which transformation can increase cell survival following UV exposure and discuss the implications of these results with respect to the DNA repair hypothesis and the evolution of transformation.

THE determination of the conditions responsible for the evolution and maintenance of sex is an intriguing, long-disputed, and essentially unresolved issue (for reviews see STEARNS 1987; MICHOD and LEVIN 1988). Although there is a plethora of theoretical studies on the subject, the hypotheses generated remain largely untested. Most support for or against these theories has been limited to retrospective interpretations of observed patterns in nature rather than experimental studies (reviewed in BELL 1982).

BERNSTEIN *et al.* (1984, 1985) presented the hypothesis that sex in both eukaryotes and prokaryotes evolved for the repair of DNA lesions. They suggested that outcrossing first evolved as an efficient means of acquiring an undamaged template for recovering lost genetic information without the cost of permanently retaining a redundant copy of the whole genome. BERNSTEIN *et al.* (1984) demonstrated theoretically that, when damage was prevalent and resources were limiting, transient sexual matings were evolutionarily beneficial. They proposed that, once large, unsegmented, duplex DNA genomes arose, simple reassortment of segments from two genomes was no longer sufficient to assemble an undamaged genome and intramolecular recombination became necessary. Although this repair hypothesis is difficult to test in eukaryotic species, bacterial sex is experimentally accessible and provides a useful model system.

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Natural transformation is a facultative sexual cycle which occurs in many eubacterial species (STEWART 1989; STEVENS and PORTER 1980). Although it is not linked to reproduction, it is truly a form of sex because it involves both outcrossing and genetic recombination. Chromosomal DNA is exchanged between cells by active uptake of DNA from the surrounding media and incorporation of this DNA into the recipient chromosome by homologous recombination. The mechanisms of transformation are highly evolved, in some cases involving the production of specialized organelles for processing the DNA (KAHN and SMITH 1984). The repair hypothesis (BERNSTEIN *et al.* 1985) proposes that the transforming DNA is used to provide templates for the recovery of damaged regions of the chromosome and that this capacity to repair DNA lesions is the selective pressure responsible for the evolution and maintenance of natural transformation.

MICHOD and his colleagues (MICHOD, WOJCIECHOWSKI and HOELZER 1988; WOJCIECHOWSKI, HOELZER and MICHOD 1989; HOELZER and MICHOD 1991) investigated the possible role of transformation in DNA repair in *Bacillus subtilis*. Because only 10% of a population of *B. subtilis* becomes competent for transformation (SMITH, DANNER and DEICH 1981), they were unable to measure directly the relative survival of all transforming cells and nontransforming cells. They therefore used the integration and expression of a genetic marker carried by the transforming DNA

to tag a fraction of the competent population. They observed that DNA damage resulting from exposure to ultraviolet light produced an increase in the proportion of the population that had acquired and expressed a genetic marker carried by the transforming DNA. They interpreted this result as support for the DNA repair hypothesis.

In an effort to explore the generality of the results obtained with *B. subtilis*, I have tested the repair hypothesis with *Haemophilus influenzae*, a naturally transforming, gram-negative species of bacteria. In contrast to *B. subtilis*, *H. influenzae* cultures reach high levels of induction where nearly the entire population is competent for transformation (HERRIOTT, MEYER and VOGT 1970; DEICH and HOYER 1982; STEWART 1989). Therefore, in *H. influenzae*, one can measure the effect of transformation on repair by observing the relative survivorship of whole populations with and without transforming DNA. Since there is no reliance on incorporation or expression of a particular genetic marker, this provides a more direct test of the role of transformation in DNA repair.

The results of these initial survivorship experiments are consistent with the repair hypothesis. Transforming DNA increases the survivorship of cultures which have been exposed to ultraviolet radiation. However, additional experiments demonstrate that this increase in survival cannot be accounted for by DNA serving as a template to recover genetic information which was lost due to damage, and therefore the results do not support the repair hypothesis for the evolution and maintenance of transformation in *H. influenzae*.

## MATERIALS AND METHODS

**Bacterial strains and media:** *H. influenzae* strains BC200 and JKS28, a BC200 derivative resistant to 2.5 μg/ml novobiocin, were obtained from J. K. SETLOW. BC200 was used as the recipient in these experiments and JKS28 was used as a DNA source. Cultures were grown in either 3.7% brain-heart infusion (Difco) broth (BHI) or 2.5% heart infusion (Difco) broth (HI) both supplemented with hemin (10 μg/ml) and nicotinamide adenine dinucleotide (2 μg/ml). Samples were diluted in 3% Eugenbroth (Difco) and plated on BHI with agar added (16 g/liter). To estimate the proportion of a population which had acquired the novobiocin marker by transformation, diluted samples were embedded in 10 ml BHI agar (12.5 g/liter) and, after incubating for 2 hr at 37° to allow expression of the new gene, an additional 10 ml of BHI agar with 5 μg/ml novobiocin were pipetted on top.

**Competence induction:** Cultures of BC200 were grown in HI to an optical density of approximately 0.25 at 675 nm which corresponds to a cell density of approximately $5 \times 10^8$ cells/ml. The cells were washed once with M-II and resuspended in M-IV artificial competence medium (HERRIOTT, MEYER and VOGT 1970). Cultures were incubated at 37° with shaking at 100 rev/min. The majority of experiments were started after only 30 min of incubation in M-IV. At this point, the cultures are not yet fully induced for transformation. Where indicated, the cultures were allowed to incubate for the full 100 min which is required for maximum induction of competence (HERRIOTT, MEYER and VOGT 1970; SMITH, DANNER and DEICH 1981).

**DNA preparation:** Genomic DNA was extracted from bacterial strain JKS28 using the method of SILHAVY, BERMAN and ENQUIST (1984) with the omission of the lysozyme and proteinase. The second source of transforming DNA was a 9-kb region of the plasmid pNov1 (SETLOW *et al.* 1981) which was constructed by the insertion of a chromosomal fragment into the plasmid RSF0885. This fragment is flanked by *Pvu*II restriction sites and carries two genes which together confer resistance to 25 μg/ml novobiocin (SETLOW *et al.* 1985). Plasmid pNov1 was obtained from R. J. REDFIELD. The plasmid DNA were extracted by the method of BIRNBOIM and DOLY (1979) and digested with *Pvu*II restriction endonuclease (New England Biolabs, Inc.). The digestion products were separated by electrophoresis on a 0.7% low melting point agarose gel. Part of the gel was stained with ethidium bromide to visualize the position of the 9-kb chromosomal fragment. This fragment was then cut from the unstained portion of the gel and the DNA recovered using GeneClean (Bio 101, Inc.). The third source of transforming DNA was a preparation of whole chromosome fragments of the same size as the cloned fragment. This was produced by first randomly shearing JKS28 genomic DNA using a VirTis homogenizer at 1/4 speed for 2 min. Fragments of approximately 9 kb were then extracted from agarose in the same manner as described for the pNov1 DNA.

The fourth source of DNA used in this study was not homologous to the *H. influenzae* chromosome. The plasmid pUC18 (Bethesda Reseach Laboratories) was used for this purpose. Since *H. influenzae* will only take up DNA which carries a specific 11-bp sequence (SISCO and SMITH 1979), a recombinant plasmid was constructed in the following manner. An oligonucleotide consisting of the 11-bp uptake sequence flanked by *Eco*RI and *Hin*dIII sites was synthesized (National Biosciences) and inserted into pUC18 between its *Eco*RI and *Hin*dIII sites. This region of the recombinant plasmid was sequenced using a standard double-stranded sequencing protocol and the Sequenase kit (U.S. Biochemical Corp.) to verify the presence and position of an intact copy of the uptake sequence. The recombinant plasmid DNA was linearized by digestion with *Eco*RI restriction enzyme following the protocol recommended by the manufacturer (New England Biolabs, Inc.).

**UV irradiation:** Cultures of BC200 in M-IV competence medium were irradiated with UV light in open glass Petri dishes with constant shaking. The UV light source was a germicidal Sylvania G1578 bulb. UV light intensity was determined using a Blak-Ray UV meter (UltraViolet Products, number J225). UV doses were varied by changing the exposure time.

## RESULTS

**Determination of the saturating DNA concentration:** The concentration of transforming DNA used in these experiments was the amount required to acheive the maximum amount of recombination for cells of a specific level of competence. To estimate these saturating concentrations of DNA, cultures of novobiocin sensitive cells (approximately $6 \times 10^8$ cells/ml), incubated in M-IV competence inducing medium for 30 min (partially induced) and 100 min (fully induced), were transformed with different concentra-

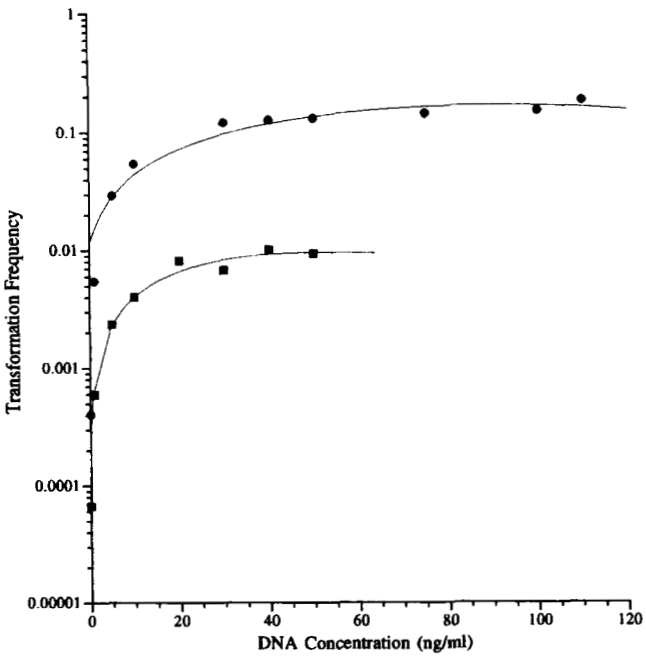


FIGURE 1.—Determination of saturating DNA concentrations. Transformation frequency ($Nov^R$ transformants/total cells) is plotted as a function of DNA concentration for fully competent cells (●) and partially competent cells (■).

tions of the 9-kb NovR fragment of pNov1. Seen in Figure 1, the frequency of novobiocin-resistant transformants increases with the concentration of DNA until it reaches a maximum at approximately 75 ng/ml for fully induced competent cultures. Partially induced cultures reached a maximum transformation frequency that was two orders of magnitude lower than fully competent cultures, and were saturated with 50 ng/ml DNA.

**Effect of transformation on survival:** Fully and partially induced competent cultures of BC200 were exposed to a range of UV radiation doses. Samples were transformed with either JKS28 chromosomal DNA (100 ng/ml) or buffer with no DNA added. The samples were incubated at 37° for 30 min at which time DNase I (20 μg/ml) was added to stop transformation in all samples. Dilutions were plated on BHI agar plates to estimate total cell density. Survival at each UV dose is defined as the ratio of the density of cells in that sample divided by the density of cells with the same treatment but no UV exposure. Survival data were log transformed to stabilize the variances (STEEL and TORRIE 1980). The survival of cultures treated with DNA was compared to those without DNA by a two-way analysis of variance (ANOVA). The statistical analysis was performed using the StatView (Abacus Concepts, Inc.) computer package. The raw means and 95% confidence intervals for the survival of partially competent (Figure 2a) and fully competent cultures (Figure 2b) are plotted. The survival of partially competent cultures treated with DNA

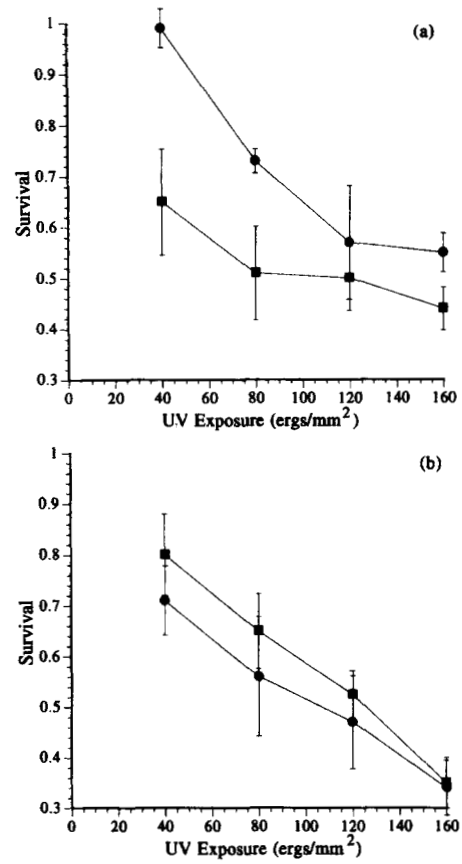


FIGURE 2.—Survival of cells as a function of UV dosage. Average survival is given for (a) partially induced competent cultures and (b) fully induced competent cultures. Error bars indicate 95% confidence intervals. Cells were incubated for 0.5 hr following UV exposure with (●) and without (■) homologous chromosomal DNA (100 ng/ml).

was significantly higher than untreated cultures (ANOVA: $F_{1,16} = 60$; $P \leq 0.0001$). This effect is most prominent at low doses of UV. Treatment with DNA following UV had no beneficial effect on fully competent cultures.

**Effect of the genetic content of the transforming DNA:** Implicit in the DNA repair hypothesis is the assumption that the DNA taken up during transformation serves as a genetic template for the repair of lesions. Therefore, when mortality is due to DNA damage at random sites around the chromosome but the DNA available for transformation is only homologous to a small fraction of the genome, the repair hypothesis predicts that transformation would not produce a detectable effect on survival.

To test this prediction, partially induced cultures were irradiated with UV light (80 ergs/mm$^2$) and then treated with (1) random fragments of approximately 9 kb representing the entire genome (60 ng/ml), (2) the 9-kb chromosomal fragment from the pNov1 plasmid (60 ng/ml) or (3) buffer containing no DNA as a control. Samples taken before irradiation were treated in the same manner. Survival is again calculated as the ratio of total cell density in the damaged samples

TABLE 1

**Effect of treatments on survival following UV damage**

| No DNA | Random genomic DNA | 9-kb cloned DNA |
|---|---|---|
| 0.52 ± .03 | 0.61 ± .03** | 0.61 ± .04* |

* $P \leq 0.05$, ** $P \leq 0.01$.

divided by total cell density in the undamaged samples for each treatment. The mean survival and standard errors for each of the three treatments are shown in Table 1. The survival rates for the two DNA treatments from 10 experiments were compared to the control treatment using the one-tailed Wilcoxon signed-ranks test (SIEGEL 1956). Survival was significantly higher for cultures treated with either DNA preparation as compared to the control without DNA. There was no significant difference in survival between cultures transformed with the full complement of the Haemophilus genome and those treated with only the small fraction of it.

**Effect of nonhomologous DNA, nucleotides and gyrase inhibition:** There are several alternative hypotheses which could explain the beneficial effect of DNA on cell survival. First, the transforming DNA could simply make cells healthier by providing a source of food (STEWART and CARLSON 1986). Second, uptake of DNA followed by the production of single-stranded tails on the transforming DNA could be a general inducer of recombination enzymes (SASSANFAR and ROBERTS 1990) resulting in higher levels of repair or otherwise improving survival by acting as a salve. Finally, integration of DNA itself could affect the cells in unknown ways. One could speculate, for example, that DNA integration might inhibit or otherwise affect DNA replication and thereby coincidentally allow repairs to be completed before lesions caused fatal replication problems.

One can distinguish between these alternatives since, in the first two cases, integration of DNA is not required. The first two hypotheses lead to the prediction that DNA which is picked up by the cells but not incorporated by homologous recombination should produce the same effect on survival as homologous DNA does. In the case of the food hypothesis, one would predict that even predigesting the DNA would not eliminate the effect. The third hypothesis suggests that recombination is necessary to produce the effect on survival and, therefore, only homologous DNA should result in higher survival. According to this hypothesis, one would also predict that a chemical agent such as oxolinic acid, which reversibly inhibits DNA gyrase and therefore temporarily halts DNA replication (PRUSS *et al.* 1986), might also increase survival.

To test these predictions, the UV survivorship experiments were repeated using partially induced cultures and the following treatments: (i) buffer with no DNA, (ii) chromosomal DNA (60 ng/ml), (iii) DNA (60 ng/ml) pretreated with DNase I, (iv) pUC18 DNA with the 11-bp uptake sequence (60 ng/ml) and (v) oxolinic acid (0.1 μg/ml). For treatment (iii), the DNA was degraded so that most of the DNA was less than 72 bp in length. No DNA larger than 400 bp was visible in a 10-μl sample run on an EtBr-stained agarose gel. Some fragments of this length would contain the 11-bp uptake sequence, but they would not be efficiently integrated since approximately 1.5 kb of the donor DNA is obligatorily degraded during the transformation process (PIFER and SMITH 1985). In treatments (i) and (v), the buffer included DNase I at a final concentration in the sample of 0.5 μg/ml to match the concentration in treatment (iii). The cultures were irradiated, transformed and plated to estimate survival as described above.

Uptake of the pUC18 DNA with the 11-bp sequence attached was demonstrated by its ability to compete with *H. influenzae* DNA for uptake sites on competent cells. The proportion of cells which were transformed to novobiocin resistance by JKS28 DNA was reduced from $3.82 \times 10^{-4} \pm 4.5 \times 10^{-5}$ to $4.80 \times 10^{-5} \pm 5.3 \times 10^{-6}$ when transformed with a mixture of pUC18 DNA with the uptake sequence and JKS28 DNA in a ratio of 10:1. The ratio of the number of copies of the uptake sequences in the two types of DNA was also approximately 10:1 since there is approximately one uptake sequence per 4 kb in the *H. influenzae* genome and the pUC18 plasmid is 3.8 kb in size. Cultures that were transformed with a mixture of pUC18 DNA without the 11-bp uptake sequence and JKS28 DNA in the same ratio also had a slightly lower transformation rate, $1.94 \times 10^{-4} \pm 1.5 \times 10^{-5}$ possibly indicating the presence of at least one degenerate copy of the uptake sequence on the pUC plasmid.

The results of the survival experiments using the five treatments described above are shown in Table 2. The data reported are the means of seven replicate experiments and the means were compared using the statistical test previously described. There was no significant increase in the survival of samples treated with digested DNA or nonhomologous DNA. There was a significant increase in survival for the chromosomal DNA treatment and oxolinic acid treatment.

## DISCUSSION

Under the conditions examined here, transforming DNA does increase the survival of *H. influenzae* suffering from UV-induced DNA damage. Previous experiments with *B. subtilis* (MICHOD, WOJCIECHOWSKI and HOELZER 1988; WOJCIECHOWSKI, HOELZER and MICHOD 1989; HOELZER and MICHOD 1991) demonstrated that the frequency of transformed cells, as

TABLE 2

**Effect of nonhomologous DNA, nucleotides and gyrase inhibition on survival following UV damage**

| No DNA | Genomic DNA | Nonhomologous DNA | Digested DNA | Oxolinic acid (0.1 μg/ml) |
|---|---|---|---|---|
| 0.63 ± 0.03 | 0.76 ± 0.06** | 0.68 ± 0.07 | 0.69 ± 0.06 | 0.78 ± 0.06* |

* $P \leq 0.05$, ** $P \leq 0.025$.

measured by genetic recombination, is increased when the cultures are given homologous DNA following UV exposure. Both sets of observations are consistent with the repair hypothesis, and the present results add generality to the observations since the gram-negative *H. influenzae* and gram-positive *B. subtilis* are distantly related species of bacteria (WOESE 1987) with highly divergent mechanisms for transformation (SMITH, DANNER and DEICH 1981).

However, the repair hypothesis states that the transforming DNA molecules are used as templates to restore genetic information lost due to damage. Several of the observations obtained with *H. influenzae* are inconsistent with this hypothesis. In these experiments, (1) as one increases the level of competence in the population, the effect of transforming DNA on survival is reduced, and (2) transformation with a small fraction of the *H. influenzae* chromosome is as effective in increasing survival as whole chromosomal DNA. This second observation was not predicted by the repair hypothesis since the DNA fragment supplied would be able to patch less than 1% of the possible sites of damage.

There are several ways in which DNA could affect UV survival without being directly involved in the repair of lesions. I have tested three possible alternatives. First, since the competence inducing medium, M-IV, is a minimal media with no nitrogen or carbon source, transforming DNA could be providing nutrition in the form of energy, carbon, nitrogen or DNA precursors which would be necessary for repair. However, this explanation for the results is rejected because digested DNA and nonhomologous DNA do not increase the survival of UV damaged cells (Table 2). Furthermore, fully competent cells do not survive better when treated with transforming DNA (Figure 2b) although they take up more DNA.

Second, although I am unaware of a specific mechanism, it could be that DNA physiologically affects cells so that repair is more efficient. One possibility is that the uptake of DNA, followed by the production of single-stranded tails, could induce higher levels of rec enzymes and thereby increase the extent of DNA repair. Single-stranded DNA is known to be the inducing signal of SOS repair in *Escherichia coli* (SASSANFAR and ROBERTS 1990) and it is possible that the analog of RecA in Haemophilus, Rec-1, could be induced in a similar manner. In accord with this hypothesis, the lack of an effect of DNA on the survival of fully competent cells could be explained by the fact that they already have high levels of rec enzyme activity (BOLING, SETLOW and ALLISON 1972). However, uptake of linear molecules of pUC18 DNA does not improve survival. Therefore, this hypothesis is not supported by the results obtained. This conclusion is subject to the caveat that the smaller size of the pUC18 molecules or lack of homology could in some way affect the processing of the DNA so that its effect on the cell is reduced. Also, in *B. subtilis*, the size of the transformant fraction did not increase following UV exposure when plasmid DNA was used (MICHOD, WOJCIECHOWSKI and HOELZER 1988).

Finally, DNA integration could be responsible for the observed effect in a general way that is not dependent on specific integration of the DNA at the sites of damage. For example, if DNA replication is inhibited while homologous recombination occurs, the time taken to integrate transforming DNA might allow excision repair to be more effective in removing the damages before they fatally interfere with DNA replication. With this interpretation, the lack of an effect of transformation on the survival of fully competent cells could be due to the fact that they have stopped dividing. This hypothesis is supported by the fact that only DNA which is integrated by homologous recombination during transformation produces a significant increase in cell survival. The small fragment of chromosomal DNA from the plasmid pNov1 improves cell survival (Table 1) whereas the nonhomologous pUC18 DNA does not (Table 2). Oxolinic acid, which reversibly inhibits DNA replication (PRUSS *et al.* 1986), also increased survival when added during the half hour incubation period following UV irradiation (Table 2). Since oxolinic acid may affect cells in a number of ways, this should only be viewed as corroborative evidence.

In conclusion, the results of these experiments seriously question the hypothesis that natural transformation in *H. influenzae* evolved and is maintained for the function of acquiring templates for the repair of DNA lesions. This study does not rule out the possibility that transformation might play a role in the repair of other forms of DNA damage than that considered here or that DNA which is naturally present in the environment when cells are competent may behave differently than experimentally added DNA.

To be sure, it also is possible that the template repair mechanism accounts for the results of MICHOD and his colleagues with *B. subtilis* transformation (MICHOD, WOJCIECHOWSKI and HOELZER 1988; WOJCIECHOWSKI, HOELZER and MICHOD 1989; HOELZER and MICHOD 1991). However, this study demonstrates that transformation can affect cells in ways that coincidentally alter the efficiency of repair and therefore it is necessary to directly test whether the transforming DNA is being used as a genetic template before concluding that DNA repair is the function of natural transformation.

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