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Mechanism of Additive Genetic Transformation in *Haemophilus influenzae*

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Transforming deoxyribonucleic acid (DNA) preparations from *Haemophilus influenzae* Rd strains carrying a chromosomally integrated, conjugative, antibiotic resistance transfer (R) plasmid were exposed to ultraviolet radiation and then assayed for antibiotic resistance transfer on sensitive wild-type Rd competent suspensions and on similar suspensions of a *uvr-1* mutant unable to excise pyrimidine dimers. No host cell reactivation of resistance transfer (DNA repair) was observed. Parallel experiments with ethanol-precipitated, heated, free R plasmid DNA preparations gave much higher survival when assayed on the wild-type strain compared to the survival on the *uvr-1* strain. These observations indicate that additive genetic transformation (in this case, the addition of the integrated R plasmid to the recipient genome) involves single-strand insertion.

Additive genetic transformation describes the addition of long segments of donor DNA to the recipient cell genome through the process of DNA uptake by genetically competent cells. It was first reported for *Haemophilus influenzae* by Bendler (4), who studied the transfer of ampicillin resistance from some hospital isolates to strain Rd. He speculated that the donor strains could have possessed a chromosomally integrated plasmid. It was recently demonstrated that many antibiotic-resistant *H. influenzae* isolates (including one of the strains studied by Bendler) do indeed carry chromosomally integrated, conjugative, antibiotic resistance transfer (R) plasmids (26). Such integrated plasmids can be transferred—in the integrated state—to *rec*$^+$ but not to *rec* recipients. DNA from strains with free R plasmids, on the other hand, is equally active on both kinds of recipients. Thus, the transfer of an integrated plasmid can be seen as the uptake of a donor DNA fragment containing the plasmid, flanked by chromosomal DNA segments. This is followed by "synapsis" of the flanking segments with the corresponding homologous segments in the recipient genome, thus "looping out" the plasmid DNA segment. A crossover in both synapsed segments will then result in insertion of the plasmid DNA segment. One can now ask whether this process involves double-strand or single-strand insertion. This article describes experiments that were aimed at answering that question.

MATERIALS AND METHODS

Strains. Recipient Rd strains were derivatives of Barnhart's BC200 (3), into which the *uvr-1* mutation (Setlow's DB112; reference 20) or the mismatch repair deficiency mutation *hex-1* (2; H. Bagci and J. H. Stuy, Mutat. Res., in press) or both had been transformed. The antibiotic-resistant donor strains with free or with integrated plasmids are listed in Table 1. The *recA1* mutation is Setlow's DB117 (reference 20; for a detailed phenotypic description, see reference 14). The absence or presence of plasmids was determined by agarose gel electrophoresis (16) of ethanol-precipitated DNA extracts prepared from cleared sucrose-sodium dodecyl sulfate lysates (12). Other genetic markers were described previously (23).

Media. sBHI broth is 3% brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 μg each of hemin chloride and nicotinic acid amide adenine dinucleotide. Agar is sBHI with 1.3% Noble agar (Difco).

DNA preparations. Crude transforming DNA lysates were prepared by lysing 0.2 ml of a 15-h-old culture at 38°C in sBHI with 0.02 ml of a 1% sodium dodecyl sulfate–0.1% pronase solution in 0.15 M NaCl–0.015 M sodium citrate for 2 h at 42°C. A 5-ml amount of 0.1 M NaCl was then added, and the mixture was swirled gently.

Free plasmid DNA preparations were made by heating RNase- and phenol-treated cleared lysates (12) at 84 to 87°C for 5 min, followed by rapid cooling. Heating completely denatures the remaining chromosomal DNA while leaving the plasmid DNA entirely intact. The DNA of a 30-ml late logarithmic growth phase culture was ultimately dissolved in 100 μl of electrophoresis buffer (25). A total of 10 μl of this was added to 2 ml of 0.1 M NaCl containing 0.1 mM EDTA.

UV irradiation. UV irradiation was carried out in open petri dishes with 5 ml of DNA lysate in a 9-cm dish or with 2 ml of ethanol-precipitated DNA in a 5-cm dish. The dose rate was about 0.1 J/m^2 per s.

Transformation. Competent cells at 2×10^8 per ml were made as described earlier (23). To 1.0 ml of suspension was added 0.1 ml of DNA solution, and the mixture was swirled gently at 30°C for 30 min. Then 1.0 ml of sBHI was added, and the incubation was continued for exactly 2 h at 38°C. Samples were plated directly in agar with either 3 μg of tetracycline per ml or 6 μg of ampicillin and 0.1 μg of tetracycline per ml (26a). Colonies were counted after 24 h of incubation

at 38°C. One clone of each cross made with damaged DNA was checked for the presence of a plasmid. These checks established that adsorbed free plasmid DNA remained free in the recipient and that integrated plasmids transferred in the integrated state.

Assuming that the original donor cell suspension with integrated plasmids contained 2×10^9 cells/ml and that each cell possessed two chromosomes with a molecular weight of 80×10^7 (6), the DNA-to-competent cell ratio was about 0.15 chromosome equivalent or one 120-megadalton DNA fragment per cell. In the case of donor cells with free plasmids (about 10^9 per ml), the ratio of free plasmid DNA per competent cell was about 1.5 copies (assuming two plasmids per cell and quantitative extraction). The molecular weights of the plasmids studied range between 30×10^6 and 40×10^6 (J. H. Stuy, manuscript in preparation).

TABLE 1. *DNA donor strains with free or integrated, conjugative, antibiotic resistance transfer plasmids (25 and 26).*

Strain	Plasmid	Phenotype	State
Rd1318	pJS1245	Amp^r	Integrated
Rd1809	pJS1245	Amp^r	Free
Rd1322	pJS1261	Tet^r	Integrated
Rd1802	pJS1261	Tet^r	Free
Rd1900	pLEB1	Amp^r Cam^r Tet^r	Integrated
Rd1898	pLEB1[a]	Amp^r Cam^r Tet^r	Free
Rd1821	pUB702[b]	Amp^r	Free

[a] See reference 8.
[b] Small, nonconjugative plasmid.

RESULTS

DNA preparations from the seven antibiotic-resistant donor strains were exposed to UV and then assayed on the wild-type strain Rd1271 *hex-1* and on the DNA repair-deficient strain Rd1748 *hex-1 uvr-1*. The survival curves of the biological activity of the DNA (i.e., transfer of antibiotic resistance residing in the plasmid) are shown in Fig. 1 and 2. The survival curves for integrated plasmid pJS1261 and for free plasmid pLEB1 measured on wild-type Rd1287 *hex*$^+$ and Rd1747 *hex*$^+$ *uvr-1* were identical to those ob-

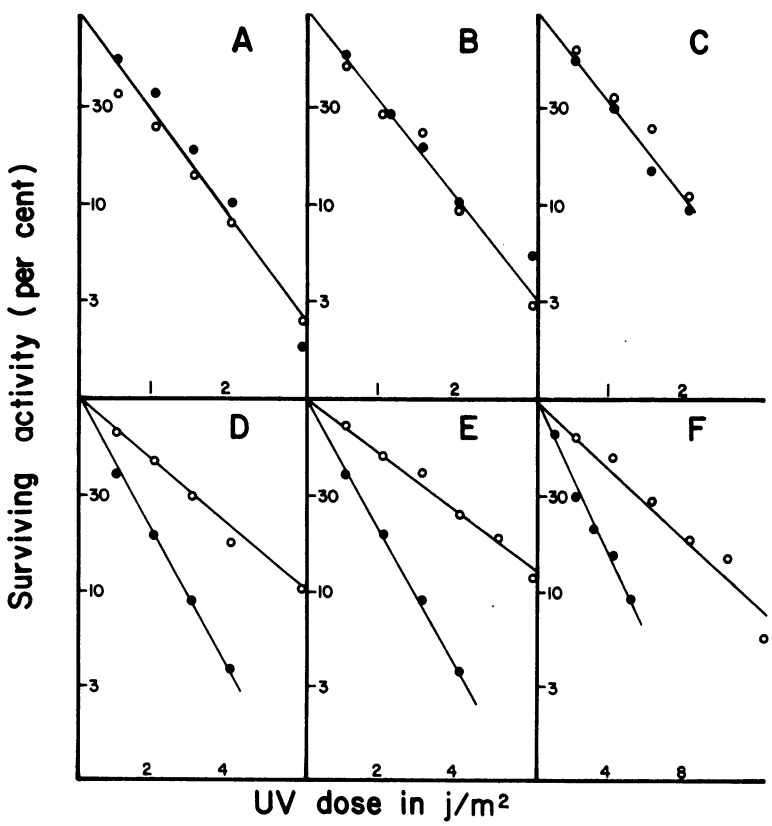

FIG. 1. *UV inactivation of the transforming activity of integrated, antibiotic resistance, transfer plasmid DNA (A, B, and C) and of free plasmid DNA (D, E, and F). Symbols: ○, measured on wild-type Rd hex-1; ●, measured on Rd hex-1 uvr-1. (A and D) Plasmid pJS1245; (B and E) plasmid pJS1261; (C and F) plasmid pLEB1. Zero time titers (transformants per milliliter) for wild-type and uvr-1, respectively: A, 8.8×10^3 and 3.2×10^3; B, 6.3×10^3 and 2.0×10^3; C, 1.6×10^4 and 5.0×10^3; D, 8.8×10^2 and 9.4×10^2; E, 5.6×10^2 and 1.0×10^3; F, 1.1×10^3 and 1.0×10^3.*

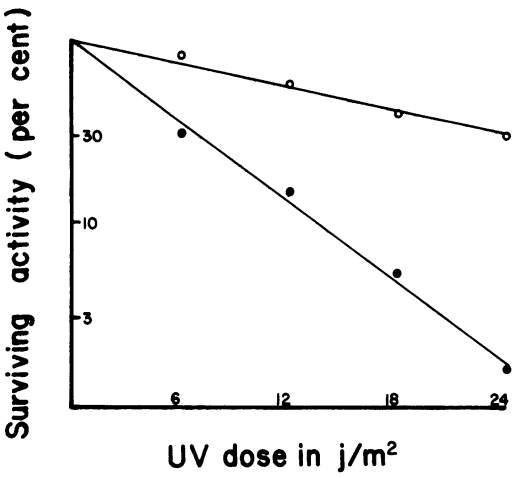

FIG. 2. *UV inactivation of the transforming activity of the small, free plasmid pUB702 measured on wild-type Rd hex-1 (●) and on Rd hex-1 uvr-1 (●). Zero time titers (transformants per milliliter): wild type, 8.8 × 10⁴; uvr-1, 3.8 × 10⁴.*

tained with the corresponding *hex* mutant recipients (data not shown). This indicates that the *H. influenzae* mismatch repair system does not significantly affect the integration of added DNA segments. It can be seen that only free plasmid DNA preparations gave higher survival values when assayed on the repair-proficient wild-type strain. The survival of the chromosomal high-efficiency *nov-rA1* marker and the low-efficiency *rif-rA2* marker (2) was measured (for experiment 1-A) by mixing in DNA with these two markers at a 1:100 ratio. After a dose of 66 J/m², the *nov-rA1* marker gave 71% survival on the *hex*⁺ *uvr*⁺ strain and 3.3% on the *hex*⁺ *uvr-1* strain. The values for the *rif-rA2* marker were 11 and 2.3%, respectively.

DISCUSSION

The addition to the genome of recipient cells of very long DNA segments by genetic transformation has been reported for *Bacillus subtilis* (1, 10), *Streptococcus pneumoniae* (21), and *H. influenzae* (4, 26). This transfer is characterized by its low efficiency and by its DNA shear sensitivity. Heterologous transformation is similarly characterized (*B. subtilis*, references 13, 27; *S. pneumoniae*, references 5, 21), and it has been proposed that both additive and heterologous transformation occurs through synapsis between regions in the chromosome and homologous regions in the donor DNA flanking the added segment (5, 13, 21). Since it has been established that adsorbed donor DNA is converted to single strands by *S. pneumoniae* (15) and by *B. subtilis* (9) and inserted as single strands (7, 11), the proposed models for additive and heterologous transformation speculate that single donor DNA strands either loop out (in additive transformation) or span the heterologous recipient DNA segment (13, 21). Duncan et al. (10), however, prefer a Campbell-like model to explain the integration into the *B. subtilis* chromosome of a chimeric plasmid containing foreign, as well as homologous, DNA sequences.

Adsorbed donor DNA is not degraded to single strands after uptake by competent *H. influenzae* cells (17, 24), although eventually only one strand is inserted into the recipient chromosome (17). It is thus possible that additive transformation in this bacterium occurs through double-strand insertion. The observations reported here do not support that possibility, however. UV-irradiated free plasmid DNA showed the phenomenon of host cell reactivation (DNA repair) when assayed on wild-type Rd and on a repair-deficient *uvr-1* recipient. UV-damaged, chromosomally integrated plasmid DNA, on the other hand, survived equally on both recipients, indicating that it is not repaired. Strike et al. (22) reported similar observations for the small, free plasmid NTP16, which they exposed to UV light and then assayed on $CaCl_2$-treated suspensions of wild-type and a *uvr* mutant of *Escherichia coli*. Thus, the most plausible model for the transfer of integrated plasmids by genetic transformation is that the plasmid-flanking DNA regions invade the recipient chromosome at homologous sites as single strands, thus looping out the plasmid DNA as a single strand. Fusing of the flanking single-stranded segments into the recipient chromosome is then followed by chromosome duplication and segregation of a transformed daughter cell. UV damage in the single plasmid DNA strand cannot be repaired out.

In their extensive review on bacterial transformation, Notani and Setlow (18) have listed the various studies that indicate very strongly that in *H. influenzae*, UV-damaged transforming DNA is repaired only after integration into the recipient chromosome (as a single strand). If integrated plasmids are inserted as double strands into the recipient genome, one would expect that repair of UV lesions could thereafter take place. The absence of such repair caused by the saturation of the repair system as a consequence of the uptake of other damaged DNA fragments is not likely because (i) a very much higher UV dose still allowed considerable repair of the chromosomal *nov-rA1* and *rif-rA2* markers, and (ii) the ratio of homologous DNA per competent cell was only about one 120-megadalton fragment. This does not seem enough for saturating the repair system. Double-strand insertion is thus not likely.

One may argue that the transfer of integrated plasmids does not necessarily occur according to the single-strand insertion hypothesis. Instead, the plasmid is excised after adsorption by the recipient cell and then either remains in the free state or is integrated. A UV lesion, blocking this excision, inactivates the plasmid DNA fragment (19). The inability of (wild-type) cells to repair DNA that is not chromosomally integrated would explain the observations in Fig. 1. This speculation is not believed to be viable because one would then also expect that transfer of integrated plasmids would result in some transformed cells' having a free plasmid and that transfer of free plasmids would result in transformed cells having an integrated plasmid. This was never observed in any of the test crosses performed in this laboratory on such transformants.

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