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Isolation and Characterization of Mutants of *Haemophilus influenzae* Deficient in an Adenosine 5'-Triphosphate-Dependent Deoxyribonuclease Activity

KENT W. WILCOX* AND HAMILTON O. SMITH

Department of Microbiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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By a direct assay approach, mutants of *Haemophilus influenzae* Rd that are deficient in adenosine 5'-triphosphate-dependent deoxyribonuclease activity (*add*$^-$) were isolated and characterized. A large proportion (50 to 90%) of the cells in cultures of these mutants failed to produce visible colonies when plated. An extensive analysis of the recombination proficiency of these strains revealed that the transformation frequency (transformants per competent cell) in the mutants was similar to that found in the wild type, but that the transformation efficiency (transformants per microgram of irreversibly bound deoxyribonucleic acid [DNA]) was reduced approximately fourfold. Sensitivities of the mutants to gamma rays, ultraviolet radiation, and methyl methane sulfonate were only slightly greater than wild-type levels. The rate of degradation of host DNA after ultraviolet irradiation was significantly reduced in the mutants. It is suggested that the adenosine 5'-triphosphate-dependent deoxyribonuclease in *H. influenzae* plays a nonessential role in DNA recombination and repair.

Friedman and Smith (9-11, 27) have characterized an adenosine 5'-triphosphate (ATP)-dependent deoxyribonuclease (DNase) from *Haemophilus influenzae* Rd. The enzyme has hydrolytic activities on both ATP and deoxyribonucleic acid (DNA). The nucleolytic activity, which requires Mg^{2+} and ATP, is most active on double-stranded, linear DNA, reducing it ultimately to acid-soluble oligonucleotides (9). Investigation of the degradation reaction reveals that the DNA is initially converted to large fragments composed of a double-stranded region flanked by one or two single-stranded segments approximately 2,000 nucleotides long (11). It is thought that the single-stranded regions are created by "melting" apart long sections of duplex DNA, using the energy obtained from the hydrolysis of ATP (11). The specificity of the enzyme for linear DNA and the complex nature of the degradation reaction suggest that the nuclease serves a well-defined function in the cell. The occurrence of a similar nuclease activity among most bacterial strains tested, including *Bacillus subtilis* (6), *Diplococcus pneumoniae* (31), *Escherichia coli* (2), and *Micrococcus luteus* (29), suggests that the enzyme fulfills a need that is common to most bacterial species.

It has been postulated (7) that the ATP-dependent nuclease in *E. coli* (2) plays a role in recombination, because the *recBC* mutants of *E. coli* are deficient in this activity (23, 32).

The observation by Vovis and Buttin (31) that mutants of *D. pneumoniae* lacking ATP-dependent nucleolytic activity are apparently transformation deficient supports this hypothesis, as does the observation that among several recombination deficient strains of *B. subtilis* assayed for the ATP-dependent nuclease, two have been found with reduced levels of the nucleolytic activity (8). The properties of the *Haemophilus* enzyme are similar to those of the ATP-dependent nucleases from *E. coli* and *D. pneumoniae*, which encouraged us to postulate that mutants of *Haemophilus* deficient in the DNase would also be transformation deficient. We have isolated mutants deficient in this enzyme and characterized their recombination proficiencies. The results indicate that these mutants are recombination proficient when assayed by transformation of competent cells with purified DNA.

MATERIALS AND METHODS

Bacterial strains. The wild-type culture of *H. influenzae* Rd is that used in previous studies (9-11). A clone picked at random from this culture was used as the parent strain and is referred to in this paper as KW20. This and other bacterial strains used are listed in Table 1. Antibiotic-resistant strains were derived

TABLE 1. *Haemophilus influenzae Rd strains*[a]

Strain	Relevant characteristics	Derivation
KW3	*add-3*; Ery^R	KW21; clone from NTG vial 4
KW5	*add-5*; Ery^R	KW21; clone from NTG vial 8
KW14	*add-14*; Ery^R	KW21; clone from NTG vial 13
KW20	Wild type	R. M. Herriott
KW21	*add*$^+$; Ery^R	KW20; by transformation using Ery^R DNA
KW26	Thy$^-$	J. Setlow
KW30	*add-1*	KW20; clone from NTG stock vial 1
KW31	*add-1*	KW20; by transformation using DNA from KW30
KW38	*com-13*; Str^R	S. H. Goodgal (5)
KW40	Str^S, Nov^S; Kan^R	KW38; by transformation with Kan^R DNA
KW60	*add-18*; Str^R	J. Stuy; originally isolated by Greth and Chevallier (14) as Rd/mms-21
KW61	*add-18*; Str^S; Nov^S	KW60; by transformation with Kan^R DNA
DB116	Uvr^S	J. Setlow (26)
DB117	Uvr^S; Mms^S; *add*$^+$; Rec$^-$	J. Setlow (26)

[a] Abbreviations: NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; Str, streptomycin; Kan, kanamycin; Nov, novobiocin; Ery, erythromycin; Mms, methyl methane sulfonate; Uvr, ultraviolet radiation; Rec, recombination; Thy, thymine; R, resistance; S, sensitivity. The abbreviation *add* is the symbol for a gene involved in the production of the ATP-dependent DNase.

from isolated clones of cells transformed with the appropriate DNA. DNAs containing the Str^R, Nov^R, Ery^R, and Kan^R genetic markers were obtained from R. M. Herriott and conferred resistance to streptomycin (200 μg/ml), novobiocin (5 μg/ml), erythromycin (6 μg/ml), and kanamycin (6 μg/ml), respectively.

Media. *H. influenzae* was routinely grown in either 3.7% brain heart infusion (BHI; Difco) or 2.5% heart infusion (HI; Difco) supplemented with 2 μg of nicotinamide adenine dinucleotide/ml and 10 μg of hemin/ml. These media are referred to as sBHI and sHI, respectively. For plating on solid medium, a base layer of sBHI containing 1.5% agar was prepared. The agar for overlay contained 2.7% BHI, 0.75% Eugon broth (Baltimore Biological Laboratory), and 0.6% agar. It was supplemented just before use with nicotinamide adenine dinucleotide (2 μg/ml) and hemin (10 μg/ml), and is called sBE agar. For routine growth of colonies in solid medium, 0.1 ml of cells was added to 3 ml of sBE agar at 48 C and immediately poured onto a base layer of sBHI agar at 37 C. Cells were routinely diluted in 0.3% BHI-0.15 M NaCl (BHI-saline) for plating.

Enzymes and reagents. Pancreatic ribonuclease (RNase) (type II-A, 70 U/mg), pancreatic DNase (DN-EP grade, 3,000 U/mg), and egg white lysozyme (grade I, 23,000 U/mg) were purchased from Sigma Chemical Co. (units are those of the manufacturer). RNase T1 (B grade) was purchased from Calbiochem. Mitomycin C and Brij 58 were obtained from Sigma Chemical Co.

Preparation of DNA. Unlabeled *H. influenzae* DNA carrying particular antibiotic resistance markers for use in transformation experiments was extracted by the procedure of Marmur (21) from cultures grown in sBHI containing the relevant antibiotics.

H. influenzae DNA was ^{32}P labeled by growth of cells in low-phosphate HI broth prepared as described by Scocca et al. (25) and containing carrier-free [^{32}P]orthophosphate (25 μCi/ml). The DNA was extracted by the procedure of Marmur (21). Its initial specific activity was 4×10^4 counts/min per nmol.

^{3}H-labeled *H. influenzae* DNA was prepared by growing strain KW26 (a thymidine-requiring strain) in the synthetic medium MI$_c$-Cit (16) supplemented with streptomycin (100 μg/ml), uracil (80 μg/ml), 0.2% vitamin-free Casamino Acids (Nutritional Biochemical Corp.), and [^{3}H]thymidine (2.67 μg/ml, 166 μCi/ml, 20 Ci/mmol; New England Nuclear Corp.). The nucleic acids were extracted with chloroform. RNA was removed by digestion with T1 RNase (25 U/ml) and pancreatic RNase (45 μg/ml). The DNA was then extracted with phenol and dialyzed against 0.01 M tris(hydroxymethyl)aminomethane (Tris) (pH 8)-0.05 M NaCl. The specific activity of the DNA was 2.13×10^6 counts/min per μg.

Bacteriophage P22 DNA was labeled with ^{32}P as described by Friedman and Smith (9) and extracted as described by Smith and Wilcox (28). Its initial specific activity was 5×10^5 counts/min per nmol of nucleotides.

Isolation of mutants. The wild-type strain (KW20) and an erythromycin-resistant derivative (KW21) were independently mutagenized and used as a source for mutants. Mutagenesis was performed at 37 C according to the technique described by Adelberg et al. (1) using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Tris-maleate buffer, pH 6.0 (12), at 0.4 mg/ml for 10 min with KW20 and at 0.2 mg/ml for 4 min with KW21. Survival levels of 0.08% (KW20) and 0.25% (KW21) were obtained. The mutagenized cells were resuspended at a twofold dilution in sBHI, and samples (1.0 ml) were incubated in separate vials at 32 C overnight to permit phenotypic expression before being stored at −70 C in sBHI containing 17% glycerol.

Mutagenized cells were grown and assayed for ATP-dependent DNase activity by a modification of the microassay technique developed by Milcarek and Weiss (22). Cells derived from a mutagenized stock were plated on sBHI and incubated for 48 h at 33 C. A

12-prong radial inoculator was used to pick clones derived from mutagenized cells into depression plates (Linbro Plastics) containing 0.1 ml of sBHI per well. After incubation at 33 C for 18 h, replicas were made by using a 48-prong inoculator to transfer material from the wells to sBHI agar plates. The cells in the depression wells were then lysed by adding 0.025 ml of a solution containing 4.5% Brij 58; lysozyme (50 μg/ml); 5.0 mM ethylenediaminetetraacetate; and 150 mM Tris buffer, pH 8.0. After 30 min at 37 C, the depression plates were warmed to 42 C. To each well was added 0.025 ml of a reaction mixture cocktail containing ^{32}P-labeled phage P22 DNA, 2.0 to 6.0 μM; 50 mM $MgCl_2$; 50 mM Tris buffer, pH 8.0; and 5 mM ATP. Each well thus contained 0.05 to 0.15 nmol of ^{32}P-labeled phage P22 DNA (approximately 2×10^4 counts/min) and 125 nmol of ATP in a total volume of approximately 0.15 ml. After 30 min at 42 C, the depression plates were transferred to an ice bath and salmon sperm DNA was added as carrier DNA followed by trichloroacetic acid to precipitate the nonsolubilized DNA. The plates were centrifuged at 3,000 rpm for 25 min. A row of self-filling glass capillary pipettes was used to transfer 10 μl of supernatant from each well to a sheet of Norit-impregnated filter paper. Each sheet (5 by 7 inches; about 12.5 by 17.5 cm) of Norit, containing samples from two plates (192 wells), was dried and placed against a sheet of X-ray film for an overnight exposure. The film was developed in Kodak KLX developer and examined for blank spots, indicative of low levels of acid-soluble radioactivity. Under the above conditions, the wild-type strain converted 75 to 90% of the DNA to acid-soluble products. When ATP was omitted from the reaction mixture, only 1 to 4% of the DNA was made acid soluble. Because the background nuclease activity was so low under these conditions, control reactions lacking ATP were not necessary in order to identify potential mutants.

Nuclease assays. Crude extracts were prepared from stationary-phase cells grown at 33 C in 250 ml of sBHI. The cells were harvested by centrifugation, resuspended in 5 ml of 50 mM Tris buffer (pH 7.6)-1 mM glutathione and sonically disrupted at 0 C with 30 pulses of 5 s each from a Branson sonifier operated at maximum output (8 A). The suspension was centrifuged for 5 min at 12,000 $\times$ *g* to remove debris. The supernatant was mixed with an equal volume of glycerol and stored at -20 C.

ATP-dependent nuclease activity against double-stranded, linear DNA was determined by measuring the degradation of ^{32}P-labeled DNA to acid-soluble material. The standard reaction mixture (0.1 ml) contained 3.6 nmol of ^{32}P-labeled *H. influenzae* DNA, 100 nmol of ATP, 0.10 M Tris buffer (pH 8.0), 0.01 M $MgCl_2$, and 40 μg of protein from a cell extract. All solutions were mixed at 0 C and then incubated at 37 C for only 2 min in order to keep the reaction within a linear rate of digestion. Acid-soluble radioactivity was determined by chromatography of a 0.01-ml sample on polyethyleneimine-cellulose thin layer sheets (Brinkman Instruments, Inc.) as described by Friedman and Smith (9). Control reactions were carried out in the absence of ATP to determine background nuclease activity, and the values obtained were subtracted from those obtained with ATP. The net difference in degradation is referred to as the ATP-dependent nuclease activity.

Transformation and DNA uptake measurement. Competent cells were prepared by incubation in the non-growth, synthetic medium MIV as described by Herriott et al. (16). Cells were incubated with DNA at 37 C for 10 to 30 min. After appropriate dilution, the cells (0.1 ml) were added to 3 ml of sBE agar and immediately poured onto a base layer of sBHI agar. After 3 h of incubation at 37 C, a second sBE agar overlay was added containing sufficient streptomycin and/or novibiocin to give a final concentration in the plate of 100 and 1.1 μg/ml, respectively. Colonies were counted after 40 h at 37 C.

Irreversibly bound DNA is defined as DNA that co-sediments with cells after exposure to pancreatic DNase and 0.5 M NaCl. Competent cells exposed to ^{32}P-labeled DNA were centrifuged for 5 min at 4,000 $\times$ *g* and then resuspended in BHI-saline containing 10 mM $MgCl_2$ and 5 μg of pancreatic DNase/ml. After 5 min at 37 C, NaCl was added to 0.5 M. The cells were centrifuged for 5 min at 4,000 $\times$ *g* and then resuspended in 0.15 M NaCl. A sample of the suspension was added to Triton scintillation fluid and assayed for radioactivity.

Autoradiography. Competent cells containing irreversibly bound ^{3}H-labeled *H. influenzae* DNA were prepared as described above. A sample (5 μl, approximately 10^7 cells) of the suspension was placed on a 1-cm square of agar and air dried for 30 min. An acid-cleaned microscope slide was pressed against the surface of the block to imprint the cells onto the slide. After drying for 5 min, the slides were dipped into cold 5% trichloroacetic acid to fix the cells, rinsed in cold distilled water, and dried. This technique is similar to the one described by Lark and Lark (20).

The imprinted slides were gently dipped into melted, undiluted Kodak NTB 2 emulsion at 42 C in total darkness, air dried for 1 h, and then placed in Bakelite slide boxes containing anhydrous calcium sulfate (Drierite). Exposure was carried out in total darkness at 4 C. The slides were developed in Kodak D-19 at 25 C for 3 min, rinsed in water, fixed for 5 min, and then rinsed again in water.

The cells were stained with crystal violet (0.4%) for 30 s and then destained in 70% ethanol. A Zeiss photomicroscope with an $\times$100, 1.25 numerical aperture planar oil immersion objective and an $\times$12.5 ocular was used with bright-field illumination for routine examination of the slides.

Exposure of cells to lethal agents. In general, cultures were grown in sBHI to approximately 2×10^9 cells/ml, resuspended in the solution indicated, and exposed to a particular agent. Samples were removed at intervals, diluted in BHI-saline, and plated in sBE agar by the overlay method described above. Colonies were counted after incubation at 37 C for 36 to 40 h. Zero-time samples were taken immediately before exposure to the given agent.

For γ irradiation, cultures were resuspended to 2×10^8 cells/ml in cold BHI-saline in pre-irradiated Pyrex test tubes and exposed to γ rays (1.98 Krads/min)

from a Cs^{137} source. For ultraviolet irradiation, cultures were resuspended to 4×10^8 cells/ml in BHI-saline and irradiated in a petri dish using a General Electric 15-W germicidal lamp at a distance of 50 cm. This corresponded to an intensity of 8 ergs/mm^2 per s at a wavelength of 254 nm. For methyl methane sulfonate treatment, cultures were resuspended to 2×10^9 cells/ml in 0.05 M Tris-maleate buffer, pH 6.6 (10), at 37 C. Methyl methane sulfonate was added to a final concentration of 5.9 mM. The culture was placed in a water bath at 37 C and assayed at intervals for surviving cells.

RESULTS

Isolation of mutants. Primarily to avoid making any a priori assumptions about the growth or survival characteristics of mutants of *H. influenzae* deficient in the ATP-dependent DNase, we chose a screening technique that involved assaying extracts from clones of mutagenized cells for ATP-dependent nucleolytic activity. Using the basic microassay technique developed by Milcarek and Weiss (22) for the isolation of nuclease mutants, we assayed extracts from 10,000 heavily mutagenized clones picked at random as described in Materials and Methods. Thirteen mutants were identified, of which ten had less than 5% of the wild-type level of activity. We designated these ATP-dependent DNase negative mutants *add*$^-$.

The *add-1* genotype was shifted to a nonmutagenized genetic background by transforming a wild-type (KW20) culture of *Haemophilus* with DNA (1 μg/ml) obtained from the *add-1* mutant KW30. Among 500 clones chosen at random, an *add*$^-$ mutant was identified by the microassay technique and designated KW31.

Nuclease levels in mutants. The results of quantitative assays for ATP-stimulated nucleolytic activity in extracts from several strains are shown in Fig. 1. The addition of ATP to an extract from an *add*$^+$ strain increased the rate of nucleolytic degradation approximately 30-fold, whereas extracts from the *add*$^-$ mutants KW5, KW14, and KW31 showed no stimulation. No significant ATP-stimulated nucleolytic activity on linear, double-stranded DNA was detected in extracts from any of the *add*$^-$ strains described here. ATP-independent nucleolytic activities on double-stranded and single-stranded DNAs were not significantly altered in the *add*$^-$ mutants described (data not shown). None of the extracts was found to contain temperature-sensitive ATP-dependent nuclease activity when assayed at 30 C (unpublished data). To rule out the presence in mutant cells of an inhibitor of the ATP-dependent nuclease, extracts of mutant and wild-type cells were mixed and assayed for nucleolytic activity. No significant inhibition by any of the mutant extracts of the ATP-dependent nuclease activity in the wild-type extract was observed (data not shown). To insure that the *add*$^-$ characteristic was associated with cells at all stages of growth, we prepared log-phase, early stationary, overnight, and competent cultures of strain KW31 (*add-1*). When assayed, none revealed any significant ATP-stimulated DNase activity.

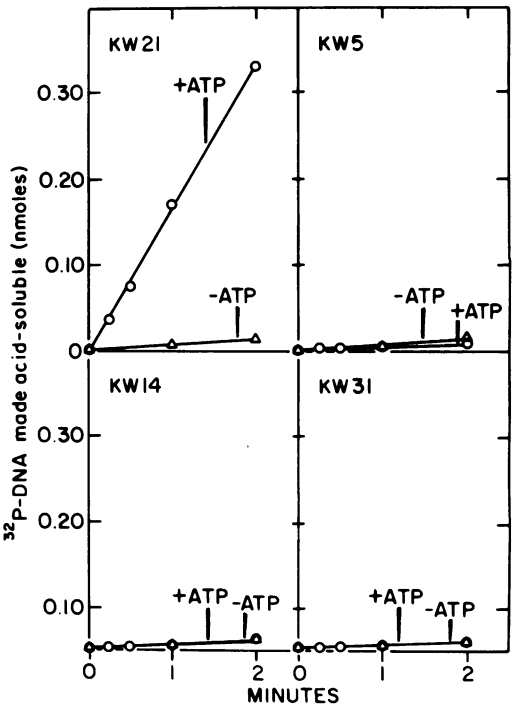

FIG. 1. *Degradation of ^{32}P-labeled H. influenzae native DNA by cell-free extracts (with and without ATP) from add$^+$ and add$^-$ strains of H. influenzae. Extracts were prepared by sonicating cells from an overnight culture as described in Materials and Methods. Each reaction mixture (0.10 ml) contained 40 μg of protein from the extract, 3.6 nmol of ^{32}P-labeled H. influenzae DNA, and, where indicated, 1.0 mM ATP. The assays were carried out as described in Materials and Methods. Symbols:* (○) *With ATP;* (△) *without ATP.*

Viability of the add$^-$ mutants. During the initial growth of the mutant strains, it was observed that their generation times (as defined by the time required for a doubling of the optical density at 650 nm [OD_{650}]) were generally 20 to 50% longer than those of the parent strain. In addition, the mutants yielded less than half the number of colonies produced by the parent strain when plated at a similar OD. To examine the basis for this phenomenon, the total number of cellular particles per milliliter and the number of colony-forming units per

milliliter were determined as a function of the OD_{650} in log-phase cultures of the parent strain KW20 and the *add-1* mutant strain KW31 (Fig. 2). The ratio of viable cells to total cells for the *add*$^+$ strain KW20 was close to 1.0 at all times sampled. In contrast, the ratio for the *add-1* strain KW31 was approximately 0.5, indicating a high rate of lethal sectoring (15) among the cells when plated in solid medium. Similar results obtained with several other *add*$^-$ strains indicated that decreased cell viability (as measured by colony-forming ability) was associated with the Add$^-$ phenotype.

Transformation frequency. The best means of determining the recombination proficiency of strains of *H. influenzae* is by transformation, a process involving (i) the uptake of DNA from the surrounding medium and (ii) the integration of that DNA into the host genome. To take up DNA from the medium, the cells must first become competent by exposure to particular culture conditions. Since only competent cells can be transformed, the recombination proficiency of a particular strain is usually expressed as the yield of transformants per competent cell, a value some refer to as the transformation frequency. Often, not all cells in a given culture are competent. To determine the fraction, f_c, of total cells, n_0, that are competent, one can use an equation proposed by Goodgal and Herriott (13) and later modified by Porter and Guild (24) to read $f_c = n_1 n_2 / n_{1,2} n_0 q$, where n_1 and n_2 are the number of cells transformed by the genetic markers 1 and 2, respectively, and $n_{1,2}$ is the number of cells transformed by both markers when a competent culture is exposed to a mixture of DNAs isolated from two different strains, one carrying the genetic marker 1 and the other carrying the genetic marker 2. The factor q allows for the multiplicity of transformable units (single DNA strands) per cell particle. This equation is generally used with the assumption that there is only one complete genome per cell particle ($q = 2$). Using the above notation, the transformation frequency (transformants per competent cell) for marker 1 is defined as $n_1/f_c n_0$. This expression of the transformation frequency is mathematically equivalent to the ratio $q\, n_{1,2}/n_2$, which is easily determined experimentally. If it is assumed that q is the same for all strains and that both populations of transformed cells ($n_{1,2}$ and n_2) are equally viable when plated, then for two DNAs (at saturation concentrations) containing either marker 1 or 2, the value obtained for the ratio $n_{1,2}/n_2$ is directly proportional to the yield of transformants per competent cell ($n_1/f_c n_0$) and thus is a measure of the recombination proficiency of that strain for marker 1.

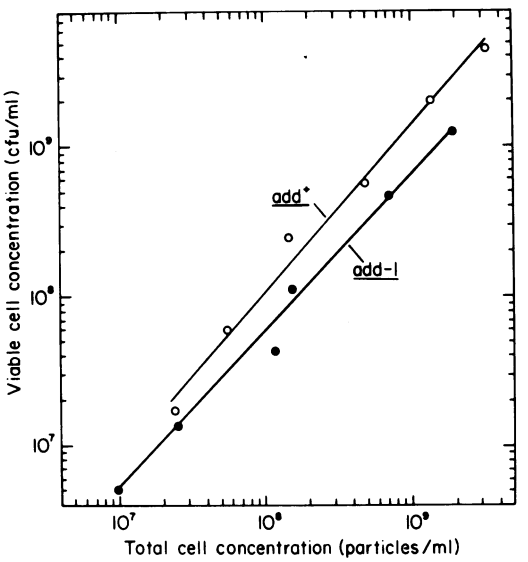

FIG. 2. *Number of viable cells as a function of total cell particles in log-phase cultures. The OD_{650} was determined with a Gilford 200 spectrophotometer. The colony-forming units (cfu) per milliliter were determined at intervals by serial dilution and duplicate plating so as to obtain 100 to 1,000 colonies per plate after 48 h of incubation at 37 C. The total number of cell particles per milliliter of culture was determined directly by examining samples in a Petroff-Hauser counting chamber at ×500, using a Zeiss microscope with a phase contrast optical system. Symbols: (○) KW20 (add⁺); (●) KW31 (add-1). Lines were drawn to fit the points based on linear regression analysis.*

To determine the recombination proficiency by this means, competent cultures of *add*$^+$ or *add*$^-$ cells were exposed to 1.0 μg each of Str^R and Nov^R DNAs per ml (on physically separate molecules, since each marker was obtained from a different strain), and the number of single and double transformants was determined. The fraction, f_c, of competent cells was calculated by the equation of Porter and Guild (24) with $q = 2$. For comparison, KW40, a derivative of the transformation-defective strain *com-13* (5), was included. The *add*$^-$ strains yielded 50 to 130% as many transformants per competent cell as did the wild type (Table 2), depending on the strain and marker assayed. KW31 (*add-1*) transformed quite well, yielding 10 to 20% more transformants per competent cell than did the wild type. KW5 (*add-5*) transformed nearly as well as the wild type, yielding 67 to 75% as many transformants per competent cell. KW14 (*add-14*) yielded 30% more Str^R transformants than did the *add*$^+$ strain but only 50% as many Nov^R

TABLE 2. *Transformation frequency for add⁻ mutants of Haemophilus influenzae Rd*[a]

Strain	f_c	Transformants/CFU[b]		Transformants/competent cell[c]	
		Str^R ($\times 10^2$)	Nov^R ($\times 10^2$)	Str^R ($\times 10^2$)	Nov^R ($\times 10^2$)
KW5 (*add-5*)	0.24	0.18	0.28	0.75	1.17
KW14 (*add-14*)	0.33	0.43	0.29	1.30	0.88
KW21 (*add⁺*)	1.10	1.12	1.93	1.02	1.75
KW31 (*add-1*)	0.30	0.36	0.58	1.20	1.93
KW40 (*com-13*)	0.42	0.0031	0.038	0.0072	0.091

[a] Freshly prepared competent cells at an OD_{650} between 0.7 and 1.0 were exposed to 1.0 μg each of Str^R and Nov^R DNA per ml for 15 min at 37 C and then plated. After incubation at 37 C for 3 h to permit maximum marker expression, the plates were overlaid with agar containing the appropriate antibiotics. f_c is the fraction of total cells that were competent. It was determined as described in the text.

[b] CFU, Colony-forming units.

[c] Str^R transformants per competent cell is equal to the value obtained when the yield (per milliliter) of Str^R-Nov^R colonies is divided by the yield of Nov^R colonies, as described in the text. Similarly, Nov^R transformants per competent cell is equal to Str^R-Nov^R/Str^R.

transformants. The Nov^R colonies from KW14 were variable in size and generally smaller than the Str^R colonies from KW14, indicating that the expression of the Nov^R marker in KW14 may in some way inhibit the growth and/or survival of the cell. In general, however, the results indicate that recombination, as measured by transformation, occurs at near-normal levels in the *add⁻* mutants.

In contrast to the results obtained with the *add⁻* strains, KW40 (*com-13*) transformed quite poorly, yielding only 0.7% as many Str^R transformants and 5% as many Nov^R transformants per competent cell as the wild-type strain. Because this strain is known to transform especially poorly for the Str^R marker (4), the transformation frequency for the Nov^R marker is probably the best measure of its general recombination proficiency.

Autoradiography of competent cells. Although the results presented in Table 2 indicate that the *add⁻* strains are recombination proficient, we realized that there was at least one other interpretation of the data. The yield of transformants per competent cell is dependent not only upon the recombination proficiency of the cell but also upon the amount of DNA bound per cell. Theoretically at least, one could have a strain with, say, a fourfold reduced recombination proficiency that compensated by binding four times as much DNA per cell, resulting in a normal yield of transformants per competent cell. To rule out this possibility, we determined the amount of DNA bound per cell by autoradiographic examination of competent cells that had been exposed to a saturating amount of ^3H-labeled *H. influenzae* DNA. Autoradiography has the advantage of yielding information not only on the amount of DNA bound per cell but also on the distribution of that DNA among the cells, and consequently what fraction of the cells bound DNA. Strains KW21 (*add⁺*) and KW31 (*add-1*) were compared. When exposed to the DNA, both cultures contained the same number of cell particles per milliliter (2×10^9) and both bound irreversibly an equal amount of DNA per ml (10^6 counts/min). In each case, roughly 86% of the irreversibly bound DNA was acid precipitable. Therefore, by gross measurement, the amount of DNA bound per cell was the same for both strains. To determine the actual distribution of the DNA among the cells, smears of each culture were exposed to photographic emulsion for varying periods of time, developed, and examined. A quantitative determination of the distribution of grains after a 68-h exposure among approximately 1,500 cells of each strain revealed the following facts (Table 3): (i) the average number of grains per cell was similar for both strains; (ii) the distribution of the grains among the cells was nearly the same for both strains; (iii) the distribution of the grains among the cells was roughly Poisson. Exposures for a period (693 h) long enough to accumulate approximately 10 grains per cell, on the average, indicated that roughly 90% of the cells in each culture bound DNA. This latter figure is approximate, since the presence of large masses of grains prevented an accurate count of the total number of cells in the field. Thus no significant differences were found in the ability of the *add⁺* and *add⁻* strains to become competent or to bind DNA.

Transformation efficiency. An alternative approach used to obtain a normalized index of recombination proficiency among strains is based on the yield of transformants per microgram of irreversibly bound DNA, a value we call the transformation efficiency. It relies on the assumption that if the DNA bound irreversibly by a given strain is integrated and expressed normally, then that strain will exhibit a normal transformation efficiency. This is the method followed by Kooistra and Venema (19) in their work with transformation-deficient mutants of *H. influenzae* and by Vovis and Buttin (31) in their work with mutants of *D. pneumoniae*.

The transformation efficiency in the *add*⁻ mutants was measured in the same experiment used to determine the transformation frequency. The Nov^R DNA was labeled with ^{32}P so that DNA uptake could be measured. A sample from each culture was diluted after exposure to DNA and plated in sBHI agar containing no antibiotic to determine the number of colony-forming units per milliliter.

The *add*⁻ strains yielded far fewer colonies than the wild type when plated at a roughly similar OD (between 0.7 and 1.0) (Table 4, column a). Assuming that each culture contained approximately the same number of cell particles, one can estimate that the fraction of cells in the *add*⁻ cultures capable of forming a colony varied from 0.1 to 0.5, depending on the particular strain (Table 4, column b). It is important to note that although the inviability of the *add*⁻ strains reduced the yield of transformed colonies, it did not reduce their ability to bind DNA irreversibly. The near-normal amounts of DNA bound by the *add*⁻ cells (Table 4, column c) implied that the so-called inviable cells could bind DNA, which was conclusively demonstrated by autoradiography (Table 3). Because the inviable cells bind DNA but make no contribution to the yield of transformed colonies, the transformation efficiency, as expressed by the ratio of Nov^R transformants per microgram of Nov^R DNA bound irreversibly (Table 4, column d), is not an accurate measurement of the recombination proficiency of the strain. We attempted to correct the measurement by normalizing the yield of Nov^R transformants per microgram of DNA bound to 100% viability (Table 4, column e). The "corrected" values for the *add*⁻ strains were still approximately fourfold lower than for the *add*⁺ strain. Whether this represents an actual recombination deficiency in the mutants or simply unknown artifacts in the technique is not clear at this time. In comparison, the value for the transformation efficiency of KW40 (the *com-13* strain used as a control) was 18-fold lower than the wild-type value, in good agreement with the 19-fold reduction shown for the transformation proficiency in Table 3.

TABLE 3. *Autoradiography of competent cells: distribution of silver grains over cells after exposure for 68 h*

r	% of total cells with r grains/cell			
	KW21 (*add*⁺)		KW31 (*add*⁻)	
	Observed	Calculated	Observed	Calculated
0	46.2	39.4	39.3	36.0
1	26.8	36.6	30.8	36.7
2	17.4	17.0	20.6	18.7
3	7.4	5.3	6.9	6.4
4	1.8	1.2	1.9	1.6
5	0.4	0.2	0.4	0.3

[a] Kodak NTB emulsion was exposed for 68 h to cells that had bound ^3H-labeled transforming DNA, developed, and then stained as described in Materials and Methods. Color photomicrographs of fields chosen to contain roughly 300 cells were prepared from an autoradiograph of each strain. The number of grains over each cell was recorded for all cells in a field. This information was used to determine the "observed" value. The "calculated" value was obtained by using the Poisson equation $P(r) = e^{-n}(n^r)/r!$ where r is the number of grains over a given cell, n is the average number of grains per cell, and $P(r)$ is the probability that a given cell will have r grains. For strain KW21, four fields were examined to yield a total of 1,503 cells and 1,393 grains; thus, $n = 0.93$ for KW21. For strain KW31, five fields were examined to yield a total of 1,434 cells and 1,469 grains; thus, $n = 1.02$ for KW31.

Responses of the add⁻ mutants to DNA-damaging agents. The established role of nucleases in DNA repair (17) led us to speculate that the ATP-dependent nuclease might carry out an essential function in the repair of certain

TABLE 4. *Transformation efficiency in add⁻ mutants of Haemophilus influenzae Rd*[a]

Strain	(a) CFU/ml ($\times 10^{-8}$)	(b) Fraction viable	(c) Nov^R DNA bound irreversibly (μg/ml)	(d) Nov^R transformants/μg of Nov^R DNA bound irreversibly ($\times 10^{-7}$)	(e) Corrected transformation efficiency $\{[(d)/(b)] \times 10^{-7}\}$
KW5 (*add-5*)	7.4	0.31	0.089	2.3	7.4
KW14 (*add-14*)	13.3	0.55	0.11	3.6	6.5
KW21 (*add*⁺)	24.1	1.00	0.16	29.2	29.2
KW31 (*add-1*)	2.6	0.11	0.19	0.69	6.3
KW40 (*com-13*)	19.2	1.00	0.046	1.6	1.6

[a] The data were obtained from the same set of experiments described in Table 2. Irreversibly bound DNA was determined as described in Materials and Methods, using ^{32}P-labeled Nov^R DNA. The fraction viable is an estimate based on the approximate OD at the time of plating and the expected cell number at that OD.

types of DNA damage. To test this idea, the ability of the *add*⁻ mutants to survive exposure to a variety of nucleic acid-destructive agents was measured and compared with that of the parental strain as well as strains with a known sensitivity to the particular agent. In general, log-phase cultures were prepared and exposed to a given agent. Samples were taken at intervals and plated to determine survival level. The results of exposing the *add*⁻ mutants to γ radiation, ultraviolet radiation, and methyl methane sulfonate are shown in Fig. 3. Although slightly sensitive to all three agents, the *add*⁻ mutants were not dramatically sensitive to any of the agents tested.

Degradation of the host genome after exposure to ultraviolet radiation. After exposure to large doses of ultraviolet radiation, cultures of both the wild-type and the *recBC* strains of *E. coli* contain acid-soluble breakdown products from their previously intact genomes (18, 32). However, the rate of production of the acid-soluble material is much slower (or "cautious") in the *recBC* strains, which are deficient in the ATP-dependent nucleolytic activity. The difference in degradative rates in the two strains implies that the ATP-dependent enzyme is involved in the production of acid-soluble breakdown products.

To determine whether the *add*⁻ mutants of *Haemophilus* also exhibit a "cautious" or slow rate of DNA degradation after irradiation by ultraviolet light, log-phase cells grown in a medium containing [^{3}H]thymidine were exposed to ultraviolet light and then resuspended in growth medium. At various times samples were removed and the total acid-soluble material was measured. After low doses of ultraviolet radiation, the rates of DNA degradation in the *add*⁻ cultures were slightly greater than in the *add*⁺ cultures (Fig. 4). However, after high ultraviolet doses, under conditions where nearly 37% of the DNA in the *add*⁺ culture was degraded within 1 h after irradiation, the rate of degradation was 3.5 to 6 times slower in the *add*⁻ mutants (Fig. 4). Thus, like the *recBC* mutants of *E. coli*, the *add*⁻ mutants of *Haemophilus* are "cautious."

Properties of Rd/mms-s 21. While this work was in progress, Greth and Chevallier (14) reported the isolation of a mutant of *H. influenzae* Rd that was sensitive to methyl methane sulfonate, deficient in ATP-stimulated nucleolytic activity, and reduced in transformation proficiency. We obtained a culture of this mutant, which they designated Rd/mms-s 21, in order to compare its transforming properties with those of the *add*⁻ mutants isolated in our laboratory. A colony picked at random from this culture was designated KW60. No detectable ATP-stimulated nucleolytic activity was found in an extract prepared from a culture of KW60. Because the Str^R phenotype of KW60 prevented measurement of the recombination proficiency using Str^R and Nov^R markers, a Str^S derivative of KW60 was obtained by transformation with Str^S Kan^R DNA and designated KW61. (Due to the close linkage of the Str and Kan markers, roughly 70% of the Kan^R clones obtained in this way are Str^S.) Like KW60, KW61 was also *add*⁻.

A freshly prepared competent culture of

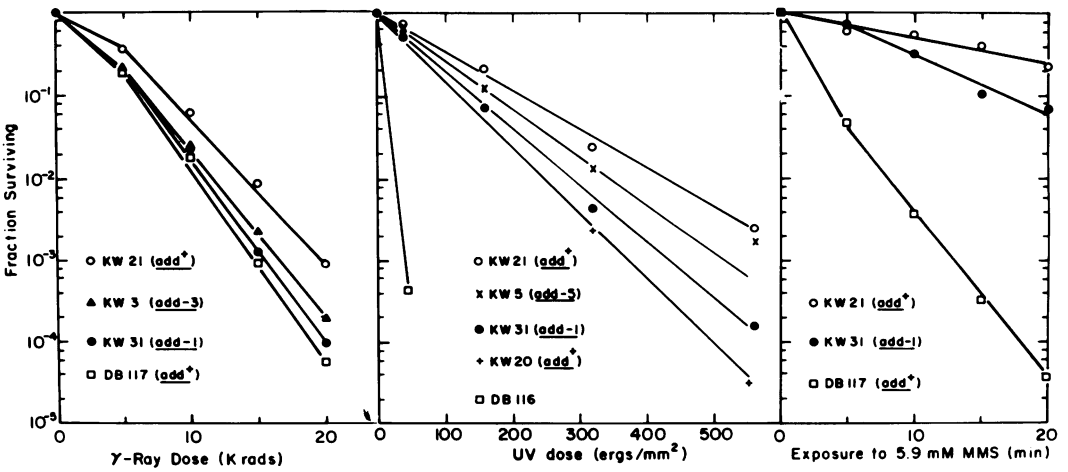

FIG. 3. *Survival of H. influenzae strains after exposure to cell-damaging agents. Fraction surviving was determined from the relative yield of colonies at zero time and at any subsequent time of exposure. The surviving fraction at zero time was assigned a value of 1.0 for each strain tested. KW20 is the parental strain for KW31. KW21 is the parental strain for KW3 and KW5. DB116 and DB117 are ultraviolet-sensitive mutants isolated by J. Setlow (26). Exposures were carried out as described in Materials and Methods.*

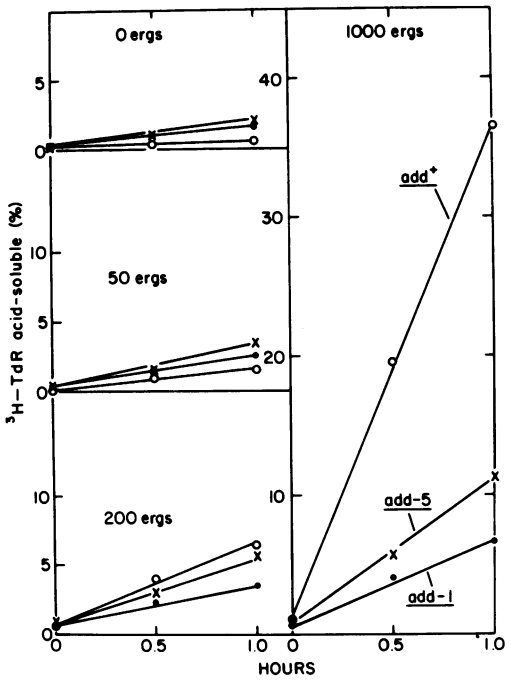

FIG. 4. *Degradation of tritium-labeled host DNA after ultraviolet irradiation. Cells were grown in sHI containing 1 mM inosine and 10 μCi of [^{3}H]thymidine per ml (20 Ci/mmol) to 10^8 cells/ml, followed by resuspension in label-free sHI, where they were shaken for 20 min at 37 C before being washed and then diluted fourfold into BHI-saline. Ten-milliliter samples were ultraviolet irradiated, resuspended in an equal volume of sHI, and shaken at 37 C. At intervals, the trichloroacetic acid-soluble material in a 0.5-ml sample was determined. Symbols:* (○) *KW21 (add$^+$);* (●) *KW31 (add-1);* (×) *KW5 (add-5).*

KW61 was transformed as described in the legend to Table 2. A comparison of the yield of colony-forming units per milliliter (2.5×10^8) with a microscopic count of cell particles per milliliter (1.22×10^9) indicated that the fraction of cell particles that were viable was approximately 0.20. The amount of Nov^R DNA bound irreversibly (0.18 μg/ml) was similar to that observed with competent cultures of both wild-type and other *add*$^-$ strains (Table 4). The yield of transformants per colony-forming unit (Str^R, 0.18×10^{-2}; Nov^R, 0.25×10^{-2}) was similar to that observed in the *add*$^-$ mutants KW5 and KW31 and roughly sevenfold lower than the value obtained with an *add*$^+$ strain (Table 2). The yield of transformants per competent cell (Str^R, 0.60×10^{-2}; Nov^R, 0.82×10^{-2}) was roughly twofold lower than the yield observed for the wild-type strain (Table 2). After correction for the fact that 80% of the cells bound DNA but never formed a colony, the yield of transformed colonies per microgram of Nov^R DNA bound irreversibly was 1.75×10^7, a figure that is 17-fold lower than that seen for the wild-type strain (Table 4). Thus, depending on the measurement one chooses, our derivative of Rd/mms-s 21 had a transformation proficiency relative to the wild-type strain somewhere between 0.06 and 0.50. For reasons presented below, we prefer the higher value.

DISCUSSION

Although laborious, the brute force technique we chose to identify *add*$^-$ clones assured us of a direct yield of enzyme-deficient mutants without our having to make any a priori assumptions about the phenotypic traits of such mutants. We isolated several independent mutants and transferred the *add-1* mutation to the parental strain so that we could correlate enzyme deficiency with any phenotypic traits observed in the *add*$^-$ mutants. The standard reaction mixture we used for assays of the *add*$^-$ mutants was sensitive enough to detect roughly 2.5% of the wild-type ATP-dependent nucleolytic activity. Although no ATP-stimulated nucleolytic activity was detected in the extracts from the *add*$^-$ mutants, we know that the *add*$^-$ mutants we have isolated may retain some residual activity that is sufficient to fulfill all required functions. The "cautious" phenotype of the mutants and their reduced viability indicate, however, that even in vivo there is less than wild-type activity in those *add*$^-$ strains tested.

Recombination proficiency. Two different approaches were used to measure the recombination proficiency of the *add*$^-$ mutants. One, which we call the transformation efficiency, measures the yield of transformants per microgram of DNA bound irreversibly during transformation. The results presented in Table 4 indicate that the *add*$^-$ mutants yield only 25% as many transformants per microgram of irreversibly bound DNA as does the wild type. Autoradiographic evidence (Table 3) indicates that the amount of DNA bound per cell is similar for the *add*$^+$ and *add*$^-$ strains. Consequently, this low value reflects either a reduced efficiency of incorporation of irreversibly bound DNA by the *add*$^-$ mutants, or else a reduced ability to express the incorporated Nov^R marker.

The second approach we used to evaluate the recombination proficiency of the *add*$^-$ mutants determined what proportion of those cells that were transformed by marker 1 and formed a colony were also transformed by marker 2. This measurement is independent of competence and survival levels and thus eliminates various correction factors. Our data (Table 2) indicate that among those cells which survive, the ratios

of Str^R-Nov^R transformants to Nov^R transformants are similar for all the *add*$^-$ strains tested and do not differ significantly from the wild-type value. Furthermore, using our transformation procedure, the ratio obtained with our derivative of Rd/mms-s 21 is only twofold lower than the wild-type value. Thus we feel that the *add*$^-$ mutants of *H. influenzae* Rd, including Rd/mms-s 21, are not significantly transformation deficient. The low yield of transformants obtained by Greth and Chevallier (14) with Rd/mms-s 21 may reflect low levels of cell competence and survival rather than a decreased recombination proficiency.

ATP-dependent nuclease mutants from at least two other transformable bacterial species have been isolated. Vovis and Buttin (31) have described a mutant of *D. pneumoniae* that has less than 2% of the wild-type level of nucleolytic activity and yields only 15% as many transformants. Doly et al. (8) recently reported on an ATP-dependent nuclease-deficient mutant of *B. subtilis* that yields only 5 to 10% as many transformants as the parental strain. Neither group adjusted their transformation results to account for the presence of inviable cells, if any, in cultures of the nuclease mutants. A further study of the *D. pneumoniae* mutant by Vovis (30) revealed no differences between the parental strain and the mutant in the fate of transforming DNA through the step of physical association of a single strand of donor DNA with the recipient DNA. This study indicates that the role of the enzyme in transformation occurs after the donor DNA has been physically integrated into the host genome. As proposed by Vovis (30) and discussed below, this role could involve degradation of nonintegrated fragments or "tails" of donor DNA.

Role of the enzyme in vivo. From the results presented, it appears that *Haemophilus* cells lacking the ATP-dependent nuclease differ from the wild-type strain only slightly, if at all, in transforming ability, γ-ray sensitivity, methyl methane sulfonate sensitivity, and ultraviolet sensitivity. Data not presented in this report also indicate that marker linkage, transfection, and phage recombination are not greatly affected in these *add*$^-$ mutants (K. W. Wilcox, Ph.D. thesis, Johns Hopkins Univ., Baltimore, Md., 1974). The decreased viability of the *add*$^-$ strains is the only property (other than lack of the enzyme) distinguishing them from the wild-type strain. Because the data reveal no specific deficiencies in the *add*$^-$ strains, it is possible that the enzyme is involved in several processes but essential for none. If one assumes that the preference of the nuclease for linear DNA observed in vitro holds true in vivo and that the bacterial chromosome is a covalent circle (3), then the normal substrates for the enzyme in vivo must be extrachromosomal DNA, such as phage and transforming DNA and/or linear DNA interacting with the genome during such processes as recombination and repair. One can easily draw models for recombination and repair that involve at some step a double- or single-stranded fragment of linear DNA having one end hydrogen bonded to the genome and the other end (which we shall refer to as the tail) hanging free (Fig. 5). The end of the tail is an obvious substrate for the ATP-dependent DNase. Failure to remove such a structure could result in a lower recombination frequency, faulty repair after exposure to DNA-damaging agents, or even cell death. It is conceivable that the ATP-dependent nuclease in *Haemophilus* plays a nonessential role in the removal of such tails. Strains lacking this enzyme could use alternate enzymatic pathways to remove tails as well as to carry out other functions normally performed by the ATP-dependent nuclease, although perhaps at a reduced efficiency. Clark (7) has presented evidence for the existence of multiple recombination pathways in *E. coli*, one of which requires the ATP-dependent nuclease. It may be that many bacterial species have several enzymatic pathways for recombination or re-

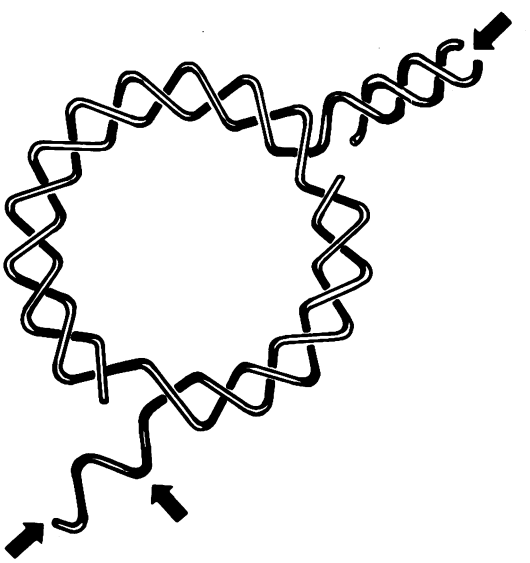

FIG. 5. *Schematic representation of a circular duplex DNA molecule with two interacting DNA "tails," one single stranded and the other double stranded. Such structures may represent intermediates in repair or recombinational processes and may also be substrates for the ATP-dependent DNase. Possible sites of enzymatic attack by the nuclease are indicated by the arrows.*

pair, including one requiring an ATP-dependent nuclease. Among mutant strains lacking the ATP-requiring nuclease, the efficiencies of the other pathways will determine to what extent phenotypic differences will be apparent.

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