JOURNAL OF BACTERIOLOGY, Aug 1977, p. 438-445
Vol. 131, No. 2

Isolation and Characterization of a *Bacillus subtilis* Mutant with a Defective *N*-Glycosidase Activity for Uracil-Containing Deoxyribonucleic Acid

FUMIKO MAKINO* AND NOBUO MUNAKATA

Radiobiology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan

Received for publication 7 March 1977

Crude cell extracts of *Bacillus subtilis* 168T exhibit enzyme activity capable of releasing free uracil from phage PBS1 deoxyribonucleic acid (DNA) in the presence of ethylenediaminetetraacetate. By measuring the enzyme activity in 300 clones that emanated from mutagenized cells, we obtained a mutant strain that did not show this *N*-glycosidase activity. The mutant strain, designated as TKJ6901 (*urg-1*) exhibited no physiological abnormalities. We observed the intracellular action of the enzyme by following the fate of uracil-containing DNA in cells from wild-type and mutant cultures. When infection with phage PBS1 was allowed in the presence of chloramphenicol, extensive degradation of phage DNA was observed only in the wild-type cells. When bromouracil residues were converted to uracil residues by ultraviolet light irradiation in the presence of cysteamine, the DNA was extensively fragmented in the wild-type cells. These single-strand breaks were rejoined upon postirradiation incubation. In contrast, such fragmentation of the DNA was not observed in the mutant cells, indicating that the uracil residues were not removed from the DNA. This demonstrated that the *N*-glycosidase activity was involved in the excision of uracil in DNA. A transformation assay with four types of recipient strains with combinations of *N*-glycosidase and DNA polymerase I deficiencies indicated that DNA polymerase I was involved in the later steps of this base excision repair pathway initiated by the action of the *N*-glycosidase.

Recently, several authors described a new class of deoxyribonucleic acid (DNA)-degrading enzymes that liberate unusual bases from DNA by catalyzing the hydrolysis of the *N*-glycosidic bond between a base and a deoxyribose sugar. Such an *N*-glycosidase specific for uracil residues in DNA has been observed in extracts of *Escherichia coli* (8), *Bacillus subtilis* (2, 4, 6), and human (15) cells. Another type of *N*-glycosidase activity specific for 3-methyladenine and/or O^6-methylguanine residues produced in DNA by alkylation has been detected in extracts of *E. coli* cells (7). A product of the *N*-glycosidase reaction, DNA with apurinic or apyrimidinic sites, can be recognized and attacked by apurinic or apyrimidinic endonuclease, which also exists in some organisms (5, 17). In view of these findings, Lindahl (9) and Duncan et al. (2) hypothesized an alternative mode of excision repair that could function as a repair mechanism to eliminate or correct unusual bases. This model of "base excision repair" calls for an *N*-glycosidase to release a base, an endonuclease to nick a phosphodiester bond adjacent to the apurinic or apyrimidinic site, and an exonuclease to remove nucleotides, including the apurinic or apyrimidinic one. The gaps thus produced could be filled by the action of DNA polymerase and DNA ligase, as in the classical mode of excision repair for ultraviolet (UV) light-induced pyrimidine dimers. To determine the biological role of the *N*-glycosidase and to shed light on this possible base excision repair mechanism, we attempted to isolate a mutant strain of *B. subtilis* defective in the *N*-glycosidase activity specific for uracil-containing DNA. This paper describes the successful isolation of such a mutant, which then made it possible to study the action of the enzyme in vitro and in vivo on phage PBS1 DNA and uracil-containing cellular DNA prepared by 5-bromodeoxyuridine incorporation and UV irradiation.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains employed in this work are listed in Table 1.

Phage. Propagation of phage PBS1 was routinely carried out by infecting it on cells of strain TKJ5214 at the late logarithmic growth phase in VY broth (25 g of veal infusion broth [Difco] and 5 g of yeast

TABLE 1. *B. subtilis strains used*

Designation	Genotype	Source
168T	*thyA thyB*	*trp*$^+$ derivative of 168 *thy trp* (3)
TKJ6901	*thyA thyB urg-1*	This paper
W23BUT	*thy his but*	Obtained from N. Sueoka (1)
TKJ5214	*ade-6 lys-21 trpC2 met-5 thr-5 ura-26 uvrA10*	Derivative of HLL3G obtained from H. Takahashi and constructed by H. Tanooka
TKJ5532	*hisA1 leu-8 met-5 lys-21 arg-15*	UVSSP42-1 (13) × HLL3G
TKJ5573	*hisA1 leu-8 met-5 urg-1*	TKJ6901 × TKJ5532
TKJ5572	*hisA1 leu-8 met-5 lys-21 polA151*	UVS151 (13) × TKJ5532
TKJ5581	*hisA1 urg-1 polA151*	UVS151 (13) × TKJ5573

extract [Difco] per liter). To obtain isotopically labeled phage, cells of strain TKJ5214 were grown at 37°C to the late logarithmic growth phase in Spizizen glucose minimal medium (16) supplemented with 0.2% casein hydrolysate (Difco) and required nutrients. Phage PBS1 was added at a multiplicity of 2 to 5, and the mixture was diluted 10-fold in the same medium containing 1 μCi of [^{3}H]deoxyuridine per ml (27 Ci/mmol, Radiochemical Centre). After lysis, the phage was collected and purified by differential centrifugation and by sedimentation in cesium chloride solution (20).

DNA. DNA of [^{3}H]deoxyuridine-labeled phage PBS1 was phenol extracted as described by Yamagishi and Takahashi (20). The DNA carried a tritium radioactivity of 2,600 cpm/μg.

To prepare 5-bromodeoxyuridine-substituted DNA (BU-DNA), cells of strain W23BUT were grown to the early stationary phase in Spizizen glucose minimal medium (16) supplemented with histidine (100 μg/ml), 5-bromodeoxyuridine (20 μg/ml), and 5 μCi of [*methyl*-^{3}H]thymidine per ml (18 Ci/mmol, New England Nuclear Corp.). The DNA was extracted by the method of Marmur (12) and dialyzed against SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.2). In this BU-DNA, 67% of the thymidine residues were substituted by 5-bromodeoxyuridine. It contained a tritium radioactivity of 29,000 cpm/μg.

Crude cell extracts and assay of the *N*-glycosidase. The preparation of crude cell extracts and the assay of *N*-glycosidase activity, using phage PBS1 DNA, were essentially as described by Duncan et al. (2).

Cells grown to the late logarithmic growth phase in nutrient broth medium (Difco, 1%) were harvested, washed, and suspended in a solution consisting of 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) and 1 mM ethylenediaminetetraacetate (EDTA). The cells were broken by sonic treatment with a Sonore 150 (Umeda Electronics). The sonic extract was centrifuged at 18,000 × *g* for 15 min at 4°C, and the supernatant fluid was then stored at 4°C and used as a crude cell extract. The amount of protein in the crude cell extract was estimated by the method of Lowry et al. (11).

The assays of *N*-glycosidase activity were carried out at 37°C in 0.2 ml of Tris-EDTA solution, with the addition of isotopically labeled DNA. After the reaction, the DNA was subjected to three types of analysis. (i) To measure release of radioactivity into the acid-soluble fraction, the reaction was terminated by the addition of 0.3 ml of trichloroacetic acid (10%) and 0.1 ml of bovine serum albumin (1 mg/ml) at 0°C. After centrifugation in the cold, the radioactivity of the supernatant fluid was determined. (ii) To observe the sedimentation pattern of the treated DNA, the reaction mixture was placed on 0.15 ml of lysing solution (0.25 N NaOH) that had been layered on top of a 4.7-ml linear alkaline sucrose gradient solution (5 to 20% [wt/vol], pH 12.0). The tube was centrifuged in a Hitachi RPS 40-T2 rotor at 40,000 rpm at 20°C for the times indicated. After centrifugation, fractions were collected on filter paper (AP1002500, Millipore Corp.), and the radioactivity was determined. A DNA sample carrying 5,000 to 10,000 cpm was layered in each gradient; the recovery was more than 85%. (iii) To measure transforming activity of the DNA, the reaction mixture was shaken with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol), and the upper phase obtained after centrifugation was dialyzed overnight against SSC. Recovery of the DNA after the assay reaction and the reextraction was >90%.

To follow the uracil-containing DNA in vivo, cells of strain 168T or TKJ6901 were grown for 3 h at 37°C in Spizizen glucose minimal medium supplemented with 2 μg of thymine per ml, 10 μg of 5-bromodeoxyuridine per ml, and 5 μCi of [^{3}H]thymidine per ml. The cells were collected by centrifugation and suspended in Spizizen salts (16) containing 10 mM cysteamine. The suspension was kept at 37°C for 5 min before UV irradiation. After irradiation, the cells were collected, suspended in the original volume of nutrient broth medium, and shaken at 37°C for the times indicated. Cells were collected and lysed for 5 min at 37°C in a saline-EDTA solution (0.15 M NaCl and 0.1 M EDTA, pH 8.0) containing 1 mg of lysozyme per ml. DNA was extracted, treated with the crude cell extracts, and analyzed by sedimentation in an alkaline sucrose gradient as described above.

Acid-soluble products of the reaction were analyzed by paper chromatography in the solvent system isopropanol-HCl-water (170:41:39, vol/vol) (19).

Transformation. A competent culture of strain TKJ5532, TKJ5573, TKJ5572, or TKJ5581 obtained in the transformation medium (16) was mixed with DNA at a concentration of 0.1 μg/ml and shaken at 37°C for 90 min. On proper selective medium, His$^+$ transformant colonies were scored (16).

Irradiation. As a mutagenizing treatment, spores of strain 168T were irradiated with electron beams from a Varian linear accelerator at a dose of 1.3 Mrad.

UV irradiation was carried out with a germicidal lamp (Matsushita Electric Co.) at a dose rate of 5 W/m², measured by a UV intensity meter (Toshiba, C-254). One milliliter of DNA solution in SSC was irradiated in a 35-mm-diameter petri dish at a concentration of 15 μg/ml, and 10 ml of cell suspension in Spizizen salts (16) was irradiated in a 90-mm-diameter petri dish at a concentration of 5×10^7 cells per ml. Cysteamine was included at a final concentration of 10 mM. The reduction of UV fluence due to absorption and shielding was not corrected for.

RESULTS

Mutant isolation. A suspension of *B. subtilis* 168T spores was bombarded with electron beams produced by a linear accelerator. This treatment reduced the number of colony formers on Schaeffer nutrient agar by a factor of 5×10^{-6}. After incubation of the plate at 33°C, each surviving colony was inoculated into 1 ml of nutrient broth medium. Cells grown at 33°C to saturation were collected by centrifugation, washed once, and suspended in 0.2 ml of Tris-EDTA solution with 5 μg of lysozyme and 0.5 mg of Brij-58. The cells were allowed to lyse at 0°C for 30 min. ³H-labeled PBS1 DNA was added, and the reaction mixture was incubated at 45°C for 30 min. In this screening assay, about 45% of the radioactivity in PBS1 DNA became acid soluble. Among 300 colonies tested, one strain that failed to release the radioactivity into the acid-soluble fraction was isolated. After several cycles of single-colony isolation and retesting, the mutant strain was established and designated TKJ6901. The mutation, which was defined by the loss of the *N*-glycosidase activity, was designated *urg-1*. Since no method other than direct enzyme assay is available to detect the loss of *N*-glycosidase activity, little is known about the molecular basis of this mutation. However, our success in transferring the defect by a congression technique to isolate strain TKJ5573 (Table 1) demonstrated that the mutation probably resides in one piece of transforming DNA. Crude cell extracts prepared from parental strain 168T and mutant strain TKJ6901 were compared for *N*-glycosidase activity for phage PBS1 DNA. The cell extracts of strain 168T liberated acid-soluble radioactivity from either single- or double-stranded PBS1 DNA. In contrast, the extracts of strain TKJ6901 at a concentration of 175 μg of protein per ml did not exhibit any detectable activity. The mixture of strain 168T and TKJ6901 extracts exhibited an activity that could be accounted for solely by the former cell extracts (Fig. 1). This indicates that the deficiency of the TKJ6901 extracts was not due to the presence of inhibitory substances antagonizing *N*-glycosidase. Under the same assay conditions, neither of the extracts liberated radioactivity from [³H]thymidine-labeled DNA of *B. subtilis*. Essentially all of the radioactive material released into the acid-soluble fraction from [³H]deoxyuridine-labeled PBS1 DNA was identified as free uracil by paper chromatography. The extracts did not possess activity capable of degrading deoxyuridine or deoxyuridine monophosphate in the assay employing the paper chromatography. These data, in concurring with those of Duncan et al. (2), argue against the possibility that the *N*-glycosidic cleavage and liberation of uracil occur after the degradation by nucleases.

Infection with phage PBS1. As shown above, phage PBS1 DNA is a very good substrate for the *N*-glycosidase and is degraded extensively in vitro by the crude cell extracts of strain 168T but not by the extracts of TKJ6901. Therefore, some differences might be expected in the growth behavior of the phage in host cells 168T and TKJ6901. When a culture of

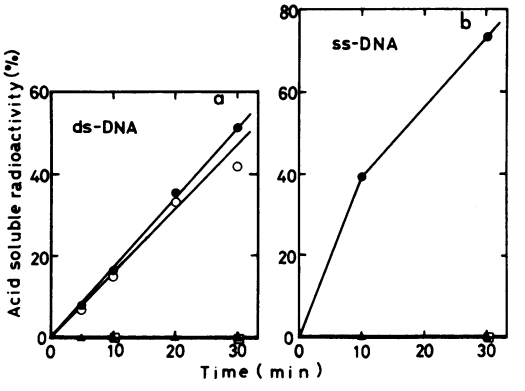

FIG. 1. *Degradation of PBS1 and B. subtilis [³H]DNA by extracts of 168T (urg⁺) and TKJ6901 (urg-1) cells. The incubation mixture contained 1.7 μg of PBS1 DNA (4,500 cpm) or 0.12 μg of B. subtilis DNA (7,500 cpm), and 35.5 μg of protein in crude extracts in Tris-EDTA solution (total volume, 0.2 ml). When the extracts were mixed, 35.5 μg of protein from each extract was added. Native DNA (a) or heat-denatured (at 100°C for 10 min) DNA (b) was used as a substrate. Incubation was at 37°C for the times indicated. Termination of the reaction and determination of acid-soluble radioactivity were performed as described in the text. Symbols: ●, PBS1 DNA with 168T extracts; ▲, PBS1 DNA with TKJ6901 extracts; ○, PBS1 DNA with a mixture of 168T and TKJ6901 extracts; □, B. subtilis DNA with 168T extracts. ds, Double stranded; ss, single stranded.*

either strain was infected with the phage in VY broth at a multiplicity of 5, lysis began after 45 min and was complete within 1 h. The average number of the progeny was about 250 per infected cell for both strains. The sizes of the progeny DNA obtained from either of the hosts were the same and were slightly larger than phage T4 DNA in alkaline sucrose gradients. Thus, the relative plaque-forming efficiencies, the kinetics of one-step growth, the burst sizes, and, the lengths of the progeny single-stranded DNAs were the same in these two hosts. These results can be explained in terms of the findings of several workers (2, 4, 6, 18) that phage PBS1 produces an inhibitor of the host *N*-glycosidase shortly after infection. In our own experiments, the *N*-glycosidase activity of the 168T host cells decreased more than 50% within 5 min and diminished completely within 10 min after infection. To observe the role of synthesis of this phage-directed inhibitor, we followed the acid solubilization of the infecting phage [^{3}H]deoxyuridine-labeled DNA in the presence of chloramphenicol (Fig. 2). Extensive degradation of the PBS1 DNA occurred only when 168T cells were infected in the presence of chloramphenicol. In contrast, in TKJ6901 cells, the extent of acid solubilization of the infecting phage DNA was negligible. The results demonstrated that the *N*-glycosidase activity was expressed not only in cell extracts, but also intracellularly.

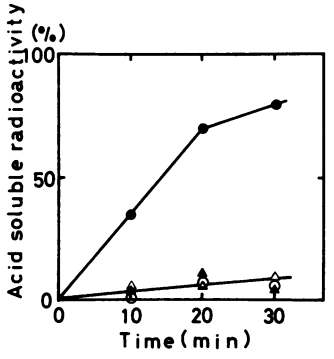

FIG. 2. *Effect of chloramphenicol (100 μg/ml) on degradation of PBS1 [^{3}H]DNA during infection of strain 168T or TKJ6901. Phage was added a multiplicity of 5 to cells grown to the early stationary phase in VY broth. After incubation for 5 min at 37°C, the cells were spun down and resuspended in the same medium. At times indicated, an equal volume of 10% trichloroacetic acid was added at 0°C to each 1-ml culture containing 500 cpm, and acid-soluble radioactivity was determined as described in the text. Symbols: ▲, host strain 168T; ●, host strain 168T in the presence of chloramphenicol; △, host strain TKJ6901; ○, host strain TKJ6901 in the presence of chloramphenicol.*

Action on uracil-containing DNA of *B. subtilis*. When BU-DNA is irradiated with UV light, BU residues are converted to uracil through debromination, and, concomitantly, single-strand breaks are produced. But when the irradiation occurs in the presence of a radical scavenger like cysteamine, uracil is produced without breakage of the DNA backbone (10). To measure the production of sites susceptible to the *N*-glycosidase, UV-irradiated BU-DNA was sedimented in alkaline sucrose gradients before and after incubation with crude cell extracts. BU-DNA sedimented as a high-molecular-weight species in the gradient and was not attacked by either of the extracts (Fig. 3a). When BU-DNA was irradiated with UV light in the presence of cysteamine, the size of the DNA decreased. This DNA was extensively fragmented by extracts of strain 168T (*urg*$^+$). In contrast, as seen from the patterns of sedimentation, DNA was not degraded by extracts of strain TKJ6901 (*urg-1*) (Fig. 3b).

DNA that had received similar treatment was used in the transformation assay with competent cultures of strain TKJ5573. In this assay, BU-DNA, with or without UV irradiation, was incubated with various amounts of crude extracts of strains 168T and TKJ6901. The number of His$^+$ transformants obtained with BU-DNA before UV irradiation was taken as 100% (Fig. 4). This DNA, incubated even with the highest concentrations of extracts, retained the original level of the marker activity. When the DNA was irradiated with UV light in the presence of cysteamine, the activity declined to 39%. Furthermore, when this irradiated DNA was incubated with the extracts of 168T cells, at concentrations >1 μg of protein per ml, additional reduction of marker activity was observed. Treatment at a concentration of 100 μg of protein per ml reduced the surviving marker activity to 2%. In contrast, the extracts of TKJ6901 did not destroy the marker activity even at a concentration as high as 100 μg of protein per ml. From the curves in Fig. 4, it can be deduced that the TKJ6901 extracts contained <1% of the activity of the 168T extracts.

Fate of uracil-containing DNA in vivo. Next, we tried to follow the fate of the uracil residues produced in DNA in strains 168T and TKJ6901 in situ. The existence of uracil residues in DNA was monitored by determining the presence of the sites susceptible to the *N*-glycosidase in vitro. When the cells of strain TKJ6901, which had incorporated 5-bromodeoxyuridine, were lysed immediately after UV irradiation in the presence of cysteamine, high-molecular-weight DNA was obtained. This DNA was extensively fragmented by the ex-

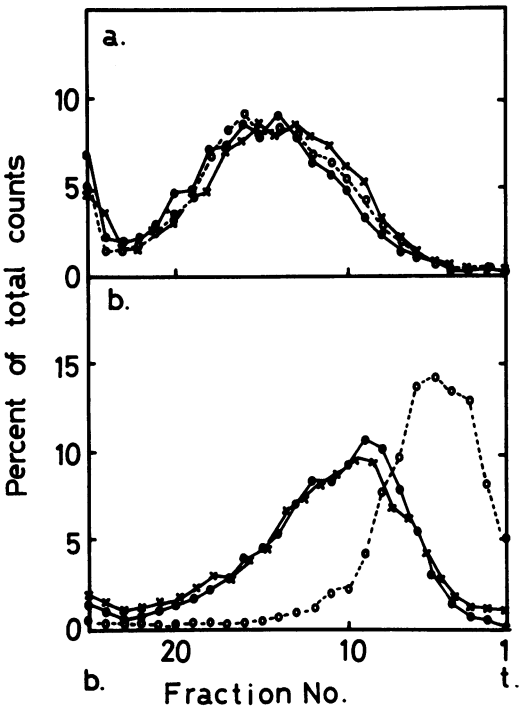

FIG. 3. *Action of crude cell extracts on UV-irradiated BU-DNA. BU-DNA (15 μg) was irradiated with UV light for 100 s in the presence of 10 mM cysteamine in 1 ml of SSC. Irradiated DNA was dialyzed overnight against SSC at 4°C. The reaction mixture, consisting of 0.75 μg DNA, and extracts, containing 71 μg of protein from strain 168T or TKJ6901 in 0.2 ml of Tris-EDTA solution, were incubated at 37°C for 30 min. The mixture was placed directly on top of a lysing solution on an alkaline sucrose gradient and centrifuged at 40,000 rpm for 2 h for unirradiated BU-DNA or for 5 h for UV-irradiated BU DNA. (a) Sedimentation patterns of unirradiated BU-DNA. (b) Sedimentation patterns of UV-irradiated BU-DNA. Symbols: ●, untreated DNA; ○, DNA treated with 168T extracts; ×, DNA treated with TKJ6901 extracts. Top and bottom of gradient are indicated by "b" and "t."*

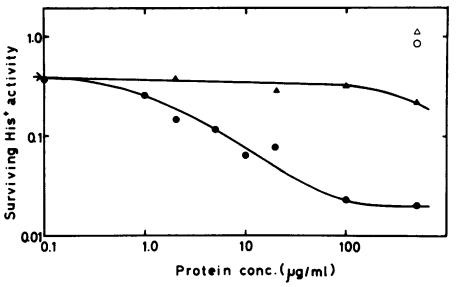

FIG. 4. *Inactivation of transforming activity of UV-irradiated BU-DNA by crude cell extracts from strains 168T and TKJ6901. BU-DNA, irradiated for 100 s with UV light in the presence of 10 mM cysteamine in SSC, was incubated at a concentration of 1.5 μg/ml with various amounts of crude cell extract in 0.5 ml of Tris-EDTA for 30 min at 37°C. Termination of the reaction and reextraction of DNA were performed as described in the text. The concentration of the reextracted DNA was adjusted by determining the ^3H radioactivity and used to transform competent cells of strain TKJ5573. The number of His$^+$ transformants with unirradiated DNA was taken as 1.0; this decreased to 0.39 with irradiation (arrow). Symbols: ●, irradiated DNA incubated with 168T extracts; ▲, irradiated DNA incubated with TKJ6901 extracts; ○, unirradiated DNA incubated with 168T extracts; △, unirradiated DNA incubated with TKJ6901 extracts.*

tracts of 168T cells, but no such fragmentation occurred with extracts of TKJ6901 cells (Fig. 5a). Thus, the procedure employed above produced sites susceptible to the *N*-glycosidase, presumably uracil residues in the DNA. The patterns of DNA sedimentation did not change when the irradiated cells were incubated for 60 min in nutrient broth medium at 37°C (Fig. 5b). The results demonstrated that the uracil residues formed in the DNA of TKJ6901 cells remained intact upon further incubation. In contrast, DNA in strain 168T subjected to the identical treatment exhibited quite different patterns of sedimentation. The DNA extracted immediately after UV irradiation appeared far smaller than that from TKJ6901 cells, and this DNA did not contain the sites susceptible to the 168T cell extracts in vitro (Fig. 6b). The activity of the intracellular *N*-glycosidase may be quite high, and it appears that the release of uracil takes place during and after UV irradiation and DNA extraction. In support of this, DNA from UV-irradiated TKJ6901 cells also fragmented if the TKJ6901 cells were mixed with 168T cells during lysis and DNA extraction (data not shown). When the irradiated cells were incubated in nutrient broth medium for 30 min at 37°C, the DNA sedimented faster (Fig. 6c). After 60 min of post-irradiation incubation, the DNA size distribution was similar to that of the unirradiated DNA and did not contain sites susceptible to the action of the 168T extracts (Fig. 6d).

Transformation assay of uracil-containing DNA. It was reported that the marker activity of BU-DNA irradiated with UV in the presence of cysteamine is significantly lower in DNA polymerase I-defective cells (14). To study the interaction of *N*-glycosidase activity and DNA polymerase I activity on uracil-containing DNA, BU-DNA of *B. subtilis* was UV irradiated in the presence of cysteamine and used to transform competent cells from strains TKJ5532 (*urg*$^+$ *pol*$^+$), TKJ5573 (*urg-1 pol*$^+$),

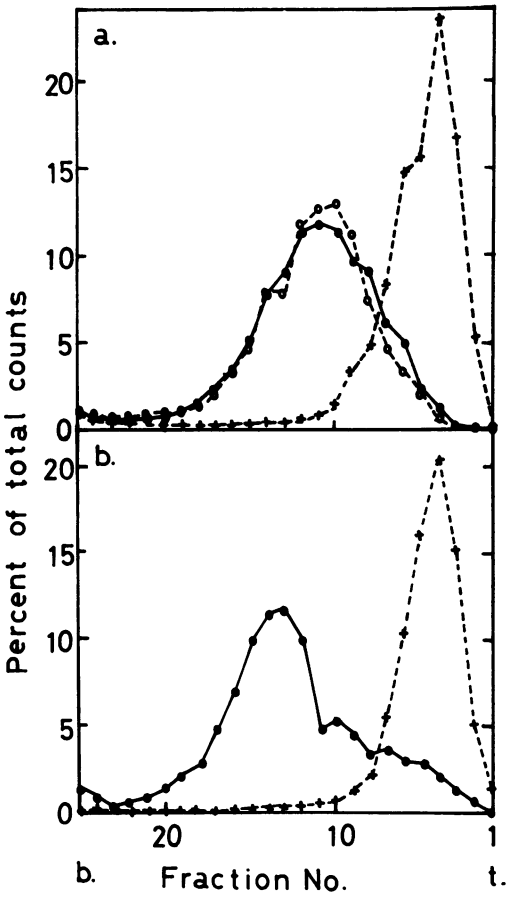

FIG. 5. *Sedimentation patterns of uracil-containing DNA extracted from TKJ6901 cells. 5-Bromodeoxyuridine-substituted cells were irradiated with UV light for 15 s in the presence of 10 mM cysteamine. DNA was extracted from the cells either before irradiation, immediately after irradiation, or after post-irradiation incubation in nutrient broth medium. Extracted DNA was incubated at 37°C with crude cell extracts containing 100 μg of protein from strain 168T or TKJ6901 in 0.2 ml of Tris-EDTA solution for 30 min. The reaction mixture was placed directly on a lysing solution on an alkaline sucrose gradient and was centrifuged at 40,000 rpm for 2 h. (a) DNA extracted from UV-irradiated cells. (b) DNA extracted from UV-irradiated cells after the incubation in nutrient broth medium for 1 h. Symbols: ●, untreated DNA; ×, DNA treated with 168T extracts; ○, DNA treated with TKJ6901 extracts.*

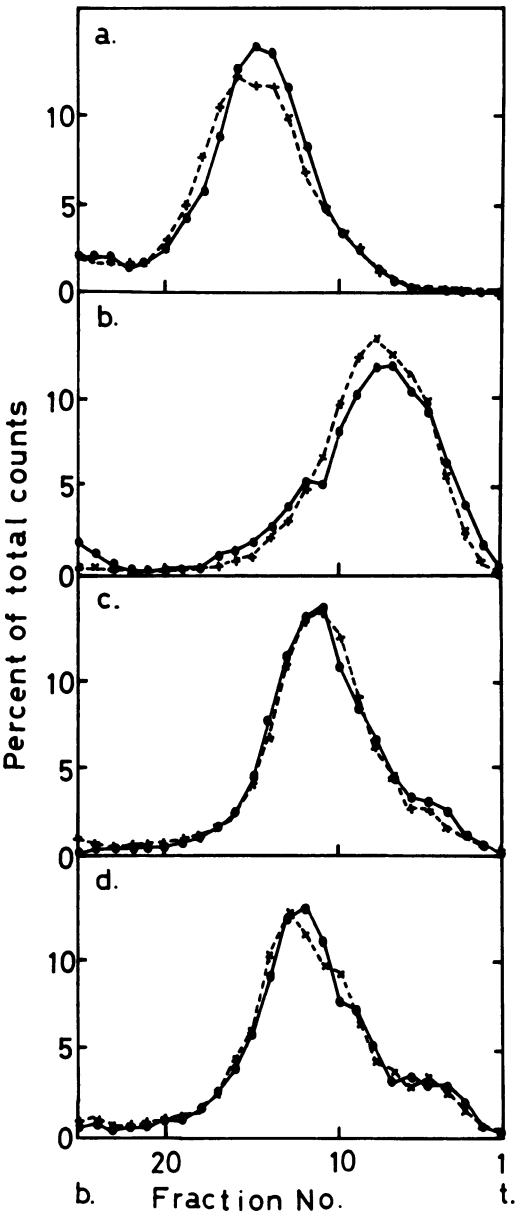

FIG. 6. *Sedimentation patterns of uracil-containing DNA extracted from 168T cells. Procedures were the same as described in the legend of Fig. 5. (a) DNA extracted from unirradiated cells. (b) DNA extracted from UV-irradiated cells. (c) DNA extracted from UV-irradiated cells after incubation in nutrient broth medium for 30 min. (d) DNA extracted from UV-irradiated cells after incubation for 1 h. Symbols: ●, untreated DNA; ×, DNA treated with 168T extracts.*

TKJ5581 (*urg-1 polA151*), and TKJ5572 (*urg*$^+$ *polA151*). The UV-survival curves of the marker activity essentially coincide for the first three strains, but the curve for TKJ5572 is significantly lower (Fig. 7). These results indicate that inactivation of the marker activity of BU-DNA by UV irradiation is enhanced in DNA polymerase I-defective cells and that this effect is abolished by the concurrent defectiveness of the *N*-glycosidase.

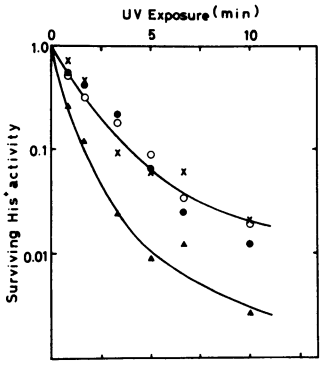

FIG. 7. *UV survival of transforming activity of BU-DNA in four recipient strains. BU-DNA was irradiated with UV light in the presence of 10 mM cysteamine in SSC for various times indicated and was employed to transform competent cells of strains TKJ5532* (○), *TKJ5573* (×), *TKJ5581* (●), *and TKJ5572* (▲).

DISCUSSION

An *N*-glycosidase activity capable of attacking uracil-containing DNA has been detected in various organisms. Our success in isolating a mutant strain of *B. subtilis* lacking most, if not all, of the activity indicates that this enzyme may be dispensable for this bacterium. Moreover, the cells of the *urg-1* mutant strain grew at 33, 37, or 45°C as well as the wild-type parental cells, sporulated normally, and exhibited no physiological abnormalities under the growth conditions used. They also displayed sensitivities similar to the parental *urg*$^+$ cells in response to the lethal actions of UV light, X rays, mitomycin C, methyl methane sulfonate, and sodium nitrite (data not shown). Hence, this *N*-glycosidase should possess rather limited, if any, function.

Possibly this enzyme is involved in a defense mechanism against invading DNA containing uracil. Phage PBS1 and several similar phages that can infect *B. subtilis* form a unique group of phages carrying DNA in which thymine residues are entirely replaced by uracil residues. DNA from phage PBS1 was extensively degraded by the enzyme in vitro. As reported by Tomita and Takahashi (18) and others (2, 4, 6), the phage manages to multiply in a *B. subtilis* cell by producing an inhibitor that binds and inactivates the *N*-glycosidase. In agreement with this, phage PBS1 grew in either *urg-1* or *urg*$^+$ cells with similar one-step growth kinetics, resulting in an equal burst size. In addition, we have shown that the infecting phage DNA was extensively degraded in *urg*$^+$ cells, but not in *urg-1* cells, when the infection was carried out in the presence of chloramphenicol. Thus, the phage depends on the synthesis of the inhibitor protein to cope with the host *N*-glycosidase for successful reproduction. This mechanism could be studied by isolating a PBS1 mutant that can grow in *urg-1* cells but not in *urg*$^+$ cells.

It also seems possible that the *N*-glycosidase could play a role in a correction mechanism that can eliminate unusual bases in DNA. This possibility has been examined in detail by following the fate of 5-bromodeoxyuridine-substituted DNA in which uracil residues were produced by UV irradiation in the presence of cysteamine (10). A sensitive test for detecting sites recognized and attacked by the *N*-glycosidase consists of incubating the irradiated DNA with *urg*$^+$ cell extracts and analyzing the resulting DNA by alkaline sucrose gradient sedimentation and genetic transformation. Cell extracts from the *urg*$^+$ strain, but not from the *urg-1* strain, contained activity that reduced the size of DNA on alkaline sucrose gradients and, at the same time, destroyed the marker activity of such DNA. Since a close association of the *N*-glycosidase and apyrimidinic endonuclease has been noted in *B. subtilis* extracts (2), these results with crude cell extracts could most likely be explained by successive actions of the *N*-glycosidase and apyrimidinic endonuclease, although it is not presently certain whether extracts of the *urg-1* cells contain the latter activity or not. A definitive answer to this question requires separation and purification of the two enzymes.

Upon UV irradiation in the presence of cysteamine to 5-bromodeoxyuridine-substituted *urg-1* cells, a large number of sites susceptible to the action of the *urg*$^+$ cell extracts were accumulated in the DNA. These sites, presumably uracil residues produced in cellular DNA, remained unaltered after 1 h of reincubation. It thus seems that this strain fails to release uracil from uracil-containing DNA in vivo, in agreement with the in vitro observation. In contrast, the DNA extracted from *urg*$^+$ cells subjected to the identical treatment was extensively fragmented even before incubation with the cell extracts. Thus, the *N*-glycosidase in *urg*$^+$ cells demonstrated its activity strongly during the UV irradiation and DNA extraction. To date, we have been unable to block the activity completely while lysing the cells for analysis. When the UV-irradiated cells were reincubated, their DNA recovered the size distribution of unirradiated cells. These results imply that the uracil residues produced in the

DNA of *urg*$^+$ cells were removed by intracellular *N*-glycosidase, which resulted in strand breaks in the DNA, and, upon post-irradiation incubation, these single-strand breaks were sealed. These observations substantiate the proposed mechanism for *N*-glycosidase-initiated base excision repair for uracil-containing DNA.

The transformation assay employing uracil-containing DNA illustrates another point of interest concerning this repair pathway. The UV survival of the marker activity was the same whether the uracil-containing DNA was used to transform *urg-1* or *urg*$^+$ recipient cells. It was expected that the transforming DNA would be attacked by the host *N*-glycosidase upon entry into *urg*$^+$ cells. Hence, the results discussed above may indicate that DNA containing uracil residues substituted for thymine could still display a normal genetic activity whether they were left unrepaired as in *urg-1* cells or were properly repaired as in *urg*$^+$ cells. In addition, the transforming activity was significantly lower in DNA polymerase I-defective cells as reported by Negishi et al. (14). This seems to support the contention made by these authors that, once uracil is liberated and the resultant apyrimidinic nucleotide is excised, resynthesis and joining of the gap should take place. The later processes are indispensable for restoration of the transforming activity and are dependent on the action of DNA polymerase I. The plausibility of this sequence of action was strengthened by the fact that in a doubly mutated *urg-1 polA151* strain inactivation due to the defectiveness of DNA polymerase I was not observed.

In the present investigations, uracil was derived solely from thymine and, therefore, paired with adenine. This type of substitution may not affect the functioning of the DNA. It would be of interest to determine if this same type of repair pathway could also operate on DNA that contained mispaired uracil.

ACKNOWLEDGMENTS

We thank Hiroshi Tanooka and Leon N. Kapp for critically reading the manuscript.

LITERATURE CITED

1. **Bishop, R. J., and N. Sueoka.** 1972. 5-Bromouracil-tolerant mutants of *Bacillus subtilis*. J. Bacteriol. 112:870–876.
2. **Duncan, J., L. Hamilton, and E. C. Friedberg.** 1976. Enzymatic degradation of uracil-containing DNA. II. Evidence for *N*-glycosidase and nuclease activities in unfractionated extracts of *Bacillus subtilis*. J. Virol. 19:338–345.
3. **Farmer, J. L., and F. Rothman.** 1965. Transformable thymine-requiring mutant of *Bacillus subtilis*. J. Bacteriol. 89:262–263.
4. **Friedberg, E. C., A. K. Ganesan, and K. Minton.** 1975. *N*-glycosidase activity in extracts of *Bacillus subtilis* and its inhibition after infection with bacteriophage PBS2. J. Virol. 16:315–321.
5. **Hadi, S. M., and D. A. Goldthwait.** 1971. Endonuclease II of *Escherichia coli*. Degradation of partially depurinated deoxyribonucleic acid. Biochemistry 10:4986–4993.
6. **Katz, G. E., A. R. Price, and M. J. Pomerantz.** 1976. Bacteriophage PBS2-induced inhibition of uracil-containing DNA degradation. J. Virol. 20:535–538.
7. **Kirtikar, D. M., and D. A. Goldthwait.** 1974. The enzymatic release of O^6-methylguanine and 3-methyladenine from DNA reacted with the carcinogen N-methyl-N-nitrosourea. Proc. Natl. Acad. Sci. U.S.A. 71:2022–2026.
8. **Lindahl, T.** 1974. An N-glycosidase from *Escherichia coli* that releases free uracil from DNA containing deaminated cytosine residues. Proc. Natl. Acad. Sci. U.S.A. 71:3649–3653.
9. **Lindahl, T.** 1976. New class of enzymes acting on damaged DNA. Nature (London) 259:64–66.
10. **Lion, M. B.** 1968. Search for a mechanism for the increased sensitivity of 5-bromouracil-substituted DNA to ultraviolet light. Biochim. Biophys. Acta 155:505–520.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
12. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 35:208–218.
13. **Munakata, N., and C. S. Rupert.** 1975. Effects of DNA polymerase defective and recombination deficient mutations on the ultraviolet sensitivity of *Bacillus subtilis* spores. Mutat. Res. 27:157–169.
14. **Negishi, K., H. Hayatsu, and H. Tanooka.** 1976. *PolA* dependent repair of 5-bromouracil-labeled *Bacillus subtilis* transforming DNA irradiated with UV in the presence of cysteamine. Int. J. Radiat. Biol. 30:491–494.
15. **Sekiguchi, M., H. Hayakawa, F. Makino, K. Tanaka, and Y. Okada.** 1976. Human enzyme that liberates uracil from DNA. Biochem. Biophys. Res. Commun. 73:293–299.
16. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S.A. 44:1072–1078.
17. **Strauss, B. S., and M. Robbins.** 1968. DNA methylated *in vitro* by a monofunctional alkylating agent as a substrate for a specific nuclease from *Micrococcus lysodeikticus*. Biochim. Biophys. Acta 161:68–75.
18. **Tomita, F., and I. Takahashi.** 1975. Changes in DNase activities in *Bacillus subtilis* infected with bacteriophage PBS1. J. Virol. 15:1073–1080.
19. **Wyatt, G. R.** 1951. The purine and pyrimidine composition of deoxypentose nucleic acids. Biochem. J. 48:584–590.
20. **Yamagishi, H., and I. Takahashi.** 1968. Transducing particles of PBS1. Virology 36:639–645.