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Multiple Species of *Bacillus subtilis* DNA Alkyltransferase Involved in the Adaptive Response to Simple Alkylating Agents

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Three molecular species of methyl-accepting proteins exist in *Bacillus subtilis* cells, which collect methyl groups from methylated DNA. A 20-kilodalton (kDa) protein was constitutively present in the cells of the *ada*$^+$ (proficient in adaptive response) strain as well as in those of six *ada* (deficient in adaptive response) mutant strains and was assigned to the O^6-methylguanine:DNA methyltransferase. Another species of O^6-methylguanine:DNA methyltransferase, which had a molecular size of 22 kDa, emerged after adaptive treatment of the *ada*$^+$ but not any of the *ada* mutant cells. A 27-kDa methyl-accepting protein, which preferred methylated poly(dT) to methylated calf thymus DNA as a substrate, was assigned to the methylphosphotriester:DNA methyltransferase. It was produced, after adaptive treatment, in the cells of *ada*$^+$, *ada-3*, *ada-4*, and *ada-6* strains but not in the cells of *ada-1*, *ada-2*, or *ada-5* strains. These results support and extend our proposition that *ada* mutants can be classified into two groups; one (the *ada-4* group) is defective only in the inducible synthesis of O^6-methylguanine:DNA methyltransferase (22-kDa protein), and the other (the *ada-1* group) is deficient in the adaptive response in toto. The finding that inducible and constitutive methyltransferases reside in different molecular species of methyl-accepting proteins is intriguing compared with the regulatory mechanisms of the adaptive response to simple alkylating agents in other organisms.

During the course of studies on the "adaptive response" to simple alkylating agents in *Escherichia coli* cells, a novel repair enzyme, O^6-methylguanine:DNA methyltransferase, was discovered (1, 6, 18). The enzyme, which is inactivated by the reaction, transfers a methyl group from the O^6-methylguanine residues in the DNA to one of its own cysteine residues (18, 19). Growing cells in low concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induces the synthesis of this enzyme, and it has been shown that acquisition of resistance by the cell to mutagenesis after MNNG challenge depends on this induction (19). More recently, the activity of another DNA methyltransferase, which transfers a methyl group from one of two isomeric forms of methylphosphotriesters in DNA, has been described (9–11, 25).

Several *E. coli* mutants deficient in the adaptive response have been isolated, and the gene controlling the response was termed *ada* (4). The *ada* gene has now been cloned, and its product and the regulatory mechanism of the adaptive response have been elucidated (2, 8, 16, 17, 21, 23, 24). A 39-kilodalton (kDa) protein product of the *ada* gene possesses O^6-methylguanine:DNA methyltransferase activity and also functions as a positive regulator of the adaptive response. Two cysteine moieties, present in the protein, act as methyl group receptors; one, in the amino-terminal domain, accepts a methyl group from methylphosphotriesters, and the other, in the carboxy-terminal domain, accepts a methyl group from O^6-methylguanine (2, 24). Methylated Ada protein binds to the promoter regions of the *ada* and an *alkA* gene coding for 3-methyladenine-DNA glycosylase II, thereby activating the transcription of these genes. This activation seems to be perfected by the acceptance of a methyl group from a methylphosphotriester (24).

A similar response in *Bacillus subtilis* cells has been observed (3, 13). As in *E. coli*, growing cells in the presence of low concentrations of MNNG or methyl methanesulfonate renders them resistant to the lethal and mutagenic actions of the MNNG challenge. It has also been demonstrated that the O^6-methylguanine:DNA methyltransferase level is elevated in adapted cells. We have isolated and characterized six mutant strains deficient in the adaptive response to MNNG, and they all turned out to be deficient in the induction of O^6-methylguanine:DNA methyltransferase as well (14). Thus, it appears that in *B. subtilis* too, the DNA methyltransferase plays an important role in the response. We have proposed that *B. subtilis ada* mutants should be classified into two groups: one totally deficient in response, the other defective only in the inducible synthesis of O^6-methylguanine:DNA methyltransferase (15). The latter type of mutation has not been identified in *E. coli*.

To investigate further the biochemical mechanism of the adaptive response in this bacterium and to compare the results with those obtained for *E. coli*, we attempted to separate methyl-accepting proteins of different molecular weights by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (7). In the present paper, we report the recognition of three molecular species of methyltransferases in *B. subtilis* cells and the deduction of their substrates. Their patterns of appearance in the mutant cells confirmed our previous classification of the *ada* mutants. Furthermore, since the amounts of these methyltransferases in cell extracts can be readily determined by this method, to clarify the process of induction, we have followed the time course for the synthesis and its dependence on the MNNG concentration used.

MATERIALS AND METHODS

Strains. The isolation and characterization of *B. subtilis* mutant strains used in this study have been described previously (14, 15).

Alkylation of DNA. Calf thymus DNA (2.5 mg), dissolved in 1 ml of 20 mM cacodylate buffer (pH 7.2)–1 mM EDTA, was incubated at 37°C for 3 h after the addition of either 200 μCi of ^{3}H-labeled *N*-methyl-*N*-nitrosourea ([^{3}H]MNU) (1.0

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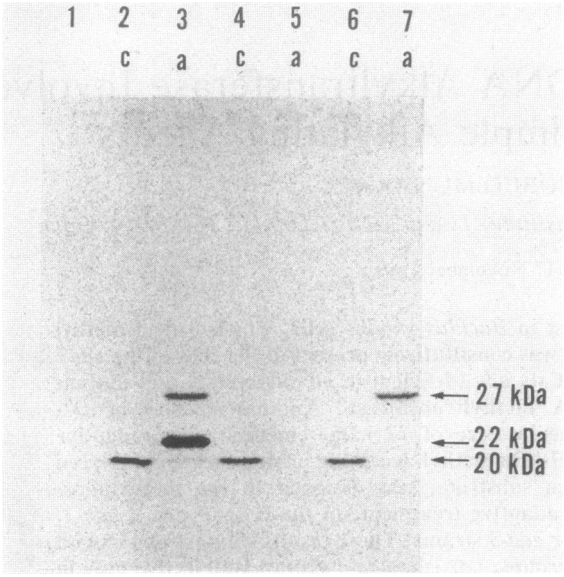

FIG. 1. SDS-polyacrylamide gel electrophoresis of cell extracts after incubation with methylated calf thymus DNA. Each extract, containing 150 μg of protein, was incubated with [^{3}H]MNU-treated and heated DNA carrying 4,900 cpm at 37°C for 20 min. As indicated above each lane, the extract was prepared from cells pretreated (lanes a) or not pretreated (lanes c) with MNNG (6.8 μM for 70 min). Lanes: no extract (lane 1) and extracts from cells of strains TKJ1922 (*ada*$^+$) (lanes 2 and 3), TKJ2925 (*ada-5*) (lanes 4 and 5), and TKJ2924 (*ada-4*) (lanes 6 and 7).

Ci/mmol; New England Nuclear Corp., Boston, Mass.) or 100 μCi of ^{14}C-labeled *N*-ethyl-*N*-nitrosourea ([^{14}C]ENU) (16 mCi/mmol; New England Nuclear). To the mixture, one-tenth volume of sodium acetate (2.5 M) was added, and the DNA was precipitated with three times the amount, by volume, of ethanol. The precipitate was washed three times with 70% ethanol and dissolved in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. The treated DNA was dialyzed extensively in the same buffer. The methylated DNA was heated at 80°C for 18 h to remove most of the methylated bases, except O^6-methylguanine (6). One microgram of [*methyl*-^{3}H]DNA and [*ethyl*-^{14}C]DNA carried 120 and 100 cpm, respectively, of radiolabel. Poly(dT) (12.5 U; Pharmacia P. L. Biochemicals, Uppsala, Sweden) was dissolved in 0.5 ml of 20 mM cacodylate buffer (pH 7.2)–1 mM EDTA and incubated at 37°C for 4 h after the addition of 250 μCi of [^{3}H]MNU. The treated poly(dT) was dialyzed extensively with 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA. It was annealed to 12.5 U of poly(dA) (Pharmacia P. L. Biochemicals) in 10 mM Tris hydrochloride (pH 7.5)–0.1 M NaCl–1 mM EDTA for 30 min at 45°C. One unit of [*methyl*-^{3}H]poly(dT):poly(dA) carried 8,800 cpm of radioactivity. The amounts of O^6-methylguanine and methylphosphotriester were determined by high-pressure liquid chromatography as described previously (10, 14).

Preparation of cell extract. Cells were grown in minimal medium supplemented with 0.02% of vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) (22). Control and MNNG-pretreated (adapted) cells at the logarithmic growth phase were harvested, washed once, and suspended in one of the buffer solutions described below. The standard buffer solution consisted of 70 mM HEPES {*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.5), 1 mM EDTA, and 5% glycerol as described previously (6). In addition, buffer solutions A and B described previously (16) were used. Buffer A contained 20 mM Tris hydrochloride (pH 8.5), 1 mM EDTA, and 1 mM 2-mercaptoethanol, and buffer B contained 70 mM HEPES-KOH (pH 7.4), 1 mM EDTA–7 mM 2-mercaptoethanol, and 0.2 M NaCl. The cells were sonicated five times for 10 s each with a sonifier (Cell Disruptor 200; Branson Ultrasonics Co., Danbury, Conn.) in an ice bath, and the sonicate was centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant portions were stored in small tubes at −70°C prior to use.

Gel electrophoresis. The cell extract was incubated with the labeled DNA in 70 mM HEPES-KOH (pH 7.8)–1 mM EDTA, 1 mM dithiothreitol–5% glycerol as described previously (13). After the reaction, one-fourth volume of a solution containing 0.1 M Tris hydrochloride (pH 6.8), 5% SDS, 8% 2-mercaptoethanol, 8% glycerol, and 0.015% bromophenol blue was added to the sample. The mixture was heated at 100°C for 3 min and subjected to electrophoresis in a 12.5% polyacrylamide gel containing 0.1% SDS as described previously (7). A low-molecular-weight electrophoresis calibration kit (Pharmacia) was used for molecular weight determination. After electrophoresis, the gel was fixed, stained, and immersed in an autoradiography enhancer (En^{3}Hance; New England Nuclear). After the gel was dried, it was exposed to X-ray films (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.) at −70°C for several weeks. The intensities of the bands in the fluorogram were determined with a densitometer (FD-AIV, Fujiriken, Tokyo).

Assay of methyltransferase. Methyltransferase activity was assayed by solubilizing methylated proteins with proteinase K by the method described previously (11).

RESULTS

Separation of methyl-accepting proteins. Logarithmically growing cells of strains TKJ1922 (*ada*$^+$), TKJ2924 (*ada-4*), and TKJ2925 (*ada-5*) were cultured in the presence (pretreated) or absence (control) of 6.8 μM MNNG for 70 min. The cells were collected, and sonic extracts were prepared. The extract containing 150 μg of protein was mixed with heated, [^{3}H]MNU-treated calf thymus DNA, and the mixture was incubated at 37°C for 20 min. The reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 1). The presence of a protein which had received a methyl group from the methylated DNA was indicated by a band of radioactivity. In lane 1, to which only DNA was applied, no band was seen. In lanes 2, 4, and 6, the reaction mixtures containing the extracts from the control cells of the *ada*$^+$, *ada-5*, and *ada-4* strains, respectively, were separated, a relatively weak band being detected in a position corresponding to 20 kDa. In lane 3, the extract was taken from the pretreated *ada*$^+$ cells, and the fluorogram exhibited two bands in positions corresponding to 27 and 22 kDa, the 22-kDa band being more intense than the 27-kDa band. In lane 5, the extract was taken from the pretreated *ada-5* cells, and no band was detectable. In lane 7, the extract was taken from the pretreated *ada-4* cells, and a 27-kDa band, but no 22-kDa band, was present. Similar separations were performed with the extracts from the other four *ada* mutant strains; for *ada-1* and *ada-2* the patterns were identical to that of *ada-5*, whereas those for *ada-3* and *ada-6* were identical to that of *ada-4*.

It has been shown that 39 kDa of the Ada protein of *E. coli*, having O^6-methylguanine:DNA methyltransferase and methylphosphotriester:DNA methyltransferase activity, is proteolytically cleaved, resulting in smaller proteins (16, 23).

This cleavage seems to occur during extraction and incubation and to depend on the conditions pertaining in the assay procedure. It has been shown (16) that a high salt concentration and a low pH promoted a similar degradation, and the 39-kDa protein was far less stable in buffer B than in buffer A (see Materials and Methods). To answer whether some of these species of methyl-accepting proteins in *B. subtilis* cells were produced by proteolysis, the extraction and incubation were carried out in buffer A and in buffer B.

However, the fluorogram patterns indicated no qualitative or quantitative differences from those obtained in the standard buffer. Incubation of the mixture for shorter periods was also tried, and the same pattern was obtained for an incubation of 1 min (data not shown). Thus, if these proteins were produced by proteolysis, this conversion must have occurred completely in vivo or very rapidly in vitro; otherwise they would have been synthesized as separate molecules.

Our previous assay of O^6-methylguanine destruction demonstrated the presence of a low level of O^6-methylguanine:DNA methyltransferase activity in the control extracts of *ada*$^+$ as well as *ada* mutant strains. This activity was enhanced approximately ninefold in the extract from the pretreated *ada*$^+$ cells, while it was nullified in the extracts from the pretreated *ada* mutant cells (15). Considering that the substrate DNA used here contained O^6-methylguanine as a major methylation product (about 74% of the total radioactivity remaining in the methylated calf thymus DNA after the heat treatment was associated with O^6-methylguanine residues), it can be deduced that the 20-kDa methyl-accepting protein corresponds to the activity of O^6-methylguanine:DNA methyltransferase constitutively present in the *ada*$^+$ and *ada* mutant cells, while the 22-kDa protein corresponds to the activity of O^6-methylguanine:DNA methyltransferase induced by MNNG pretreatment in the *ada*$^+$ but not in the six *ada* mutant strains.

Substrate specificities. Similar extracts from control and pretreated cells of strains TKJ7501 (*ada*$^+$), TKJ0902 (*ada-2*), and TKJ0903 (*ada-3*) were incubated with [^{14}C]ENU-treated calf thymus DNA. The fluorogram (Fig. 2) exhibited radioactive bands at positions similar to those seen in Fig. 1. All the control extracts contained the 20-kDa protein only. In the *ada*$^+$ extract, MNNG pretreatment caused the appearance of 22- and 27-kDa proteins. In the extract from the pretreated *ada-3* cells, only the 27-kDa protein was detected. Thus, it has been shown that all these species of methyl-accepting proteins can also accept an ethyl group from ethylated DNA.

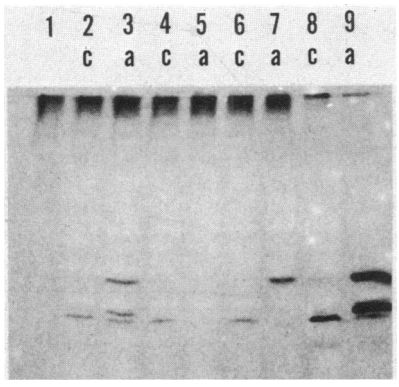

FIG. 2. SDS-polyacrylamide gel electrophoresis of cell extracts after incubation with ethylated calf thymus DNA. Each extract, containing 250 μg of protein, was incubated with [^{14}C]ENU-treated DNA carrying 3,000 cpm at 37°C for 40 min. As indicated above each lane, the extract was prepared from cells pretreated (lanes a) or not pretreated (lanes c) with MNNG (13.6 μM for 70 min). Lanes: no extract (lane 1) and extracts from cells of strains TKJ7501 (*ada*$^+$) (lanes 2 and 3), TKJ0902 (*ada-2*) (lanes 4 and 5), TKJ0903 (*ada-3*) (lanes 6 and 7), and TKJ7501 (*ada*$^+$) cells reacted with [^{3}H]MNU-treated and heated calf thymus DNA (lanes 8 and 9).

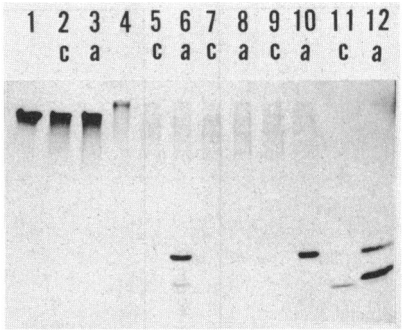

FIG. 3. SDS-polyacrylamide gel electrophoresis of cell extracts after incubation with methylated poly(dT). Each extract, containing 250 μg of protein, was incubated with [^{3}H]MNU-treated poly(dT) (5,600 cpm) (lanes 1 to 3) or with the methylated poly(dT) (2,300 cpm) after annealing to poly(dA) (lanes 4 to 10). As indicated above each lane, extracts were prepared from cells pretreated (lanes a) or not pretreated (lanes c) with MNNG (13.6 μM for 70 min). Lanes: no extract (lanes 1 and 4) and extracts from cells of strains TKJ7501 (*ada*$^+$) (lanes 2, 3, 5, and 6), TKJ0902 (*ada-2*) (lanes 7 and 8), TKJ0903 (*ada-3*) (lanes 9 and 10), and TKJ7501 (*ada*$^+$) reacted with [^{3}H]MNU-treated and heated calf thymus DNA (lanes 11 and 12).

Poly(dT) treated with [^{3}H]MNU was next used as a substrate. It was necessary to anneal the methylated poly(dT) to poly(dA) to activate it for use as a substrate for the methyl-accepting proteins, and when it was used as the substrate, 22- and 27-kDa proteins were detected in the extract from the pretreated *ada*$^+$ cells (lane 6, Fig. 3). The 27-kDa band was more intense than the band of 22-kDa protein, in contrast to when methylated calf thymus DNA was used as the substrate. Thus, it seems that the 22-kDa protein prefers *methyl*-^{3}H-calf thymus DNA to [*methyl*-^{3}H]poly(dT):poly(dA), the opposite being true for the 27-kDa protein. The 27-kDa protein was also detected in the extract from the pretreated *ada-3* cells but not in the extract from the pretreated *ada-2* cells.

Quantitative assay of alkyl-accepting activities. The assay of the methyl-accepting activities of proteins was performed by a previously described method (11). In this assay, crude extract was mixed with [*methyl*-^{3}H]DNA, and after incubation, the proteins in the mixture were digested by proteinase K, and the solubilized radioactivity was measured. The results obtained with various concentrations of *ada*$^+$ and *ada* mutant cell extracts with methylated calf thymus DNA plus methylated poly(dT) and methylated poly(dT) alone as substrates are shown in Fig. 4. The amounts of radioactivity shown in each part of Fig. 4 can be compared directly, since the [^{3}H]MNU used to methylate the DNAs had equal specific activity. The extracts from the control cells of the *ada*$^+$ and *ada* mutant strains showed very little activity with [*methyl*-^{3}H]poly(dT):poly(dA). Thus, it can be concluded that the activities are mostly due to the existence of O^6-methylguanine:DNA methyltransferase in the control (unadapted) cells. The activities with ^{3}H-methylated poly(dT) were approximately equal for extracts from both pretreated *ada*$^+$ and *ada-3* cells (Fig. 4b), whereas the

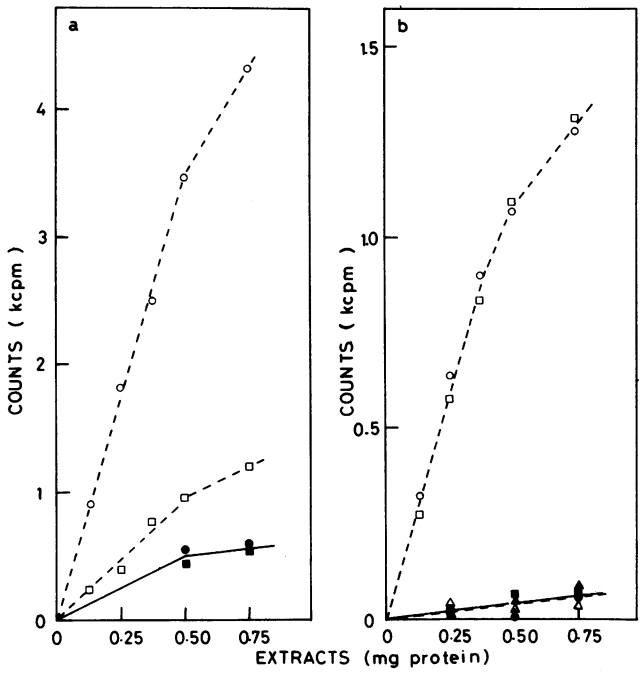

FIG. 4. Methyltransferase activity of *B. subtilis* cell extracts. Crude cell extracts were incubated either (a) with a mixture of *methyl*-^{3}H-labeled calf thymus DNA (4,900 cpm) and [*methyl*-^{3}H]poly(dT):poly(dA) (4,600 cpm) or (b) with [*methyl*-^{3}H]poly(dT):poly(dA) alone. The reaction was for 40 min at 37°C, and activities were determined by the amount of radioactivity transferred to proteins. Extracts were prepared from strains TKJ7501 (*ada*$^+$) (circles), TKJ0902 (*ada-2*) (triangles), and TKJ0903 (*ada-3*) (squares) which had (open symbols) or had not (closed symbols) been pretreated with MNNG (20.4 μM for 70 min).

activities with the DNA mixture in the extract from the pretreated *ada*$^+$ cells was about fourfold that from the pretreated *ada-3* cells (Fig. 4a). The activities in the pretreated *ada-3* cells were approximately equal in both cases. It follows that the rest of the activity existing in the pretreated *ada*$^+$ cells was probably caused by the activity of the O^6-methylguanine:DNA methyltransferase induced by adaptive treatment.

By high-pressure liquid chromatographic analysis, it was determined that the [*methyl*-^{3}H]poly(dT):poly(dA) used as a substrate in this assay contained 83% of the total radioactivity as methylphosphotriester. In the reaction with the extract (0.8 mg of protein) from the pretreated *ada*$^+$ cells, 1,250 cpm of ^{3}H radioactivity was transferred to the protein (Fig. 4b), whereas under the same conditions, 880 cpm in the form of methylphosphotriester was lost from the substrate DNA. These results together indicate that the major activity on [*methyl*-^{3}H]poly(dT):poly(dA) is due to the methylphosphotriester:DNA methyltransferase which resides in a 27-kDa protein.

Induction kinetics. The above results validated the notion that the band intensity in the fluorogram of the SDS-polyacrylamide gel reflects the content of each alkyl-accepting protein. Furthermore, the intensity of the 22- and 27-kDa bands was found to be proportional to the amount of extract from the pretreated *ada*$^+$ cells, up to a concentration of 250 μg of protein in the reaction mixture containing 4,900 cpm of [^{3}H]MNU-treated and heated DNA (data not shown).

MNNG was added to the logarithmically growing culture of the *ada*$^+$ strain at a concentration of 20 μM, and after various amounts of time, the cells were collected. The extracts obtained were incubated with heated, [^{3}H]MNU-treated calf thymus DNA and analyzed by SDS-polyacrylamide gel electrophoresis to determine the content of alkyl-accepting proteins (Fig. 5). Before the addition of MNNG, only the 20-kDa protein was present, as shown above (Fig. 1). After 5 min, a small amount of the 22-kDa protein appeared, but at this point the 27-kDa protein was undetectable and the amount of 20-kDa protein had decreased. After 15 min, the amount of the 22-kDa protein had increased, and the 27-kDa protein had made an appearance, while the amount of 20-kDa protein had become quite low. Thereafter, the amounts of the 22- and 27-kDa proteins increased steadily for 35 min and then gradually for 70 min. The question arose whether the delay in the appearance of the 27-kDa protein compared with the 22-kDa protein could have been attributable to the fact that the substrate used was not the one preferred by the 27-kDa protein. Therefore, the assay for the extract after 5 min was also performed with [*methyl*-^{3}H]poly(dT):poly(dA), and the presence of the 22-kDa but not the 27-kDa protein was confirmed (data not shown). It also can be seen that once the induction of both proteins had taken place, their proportion was almost constant, with three or four times as much 22-kDa as 27-kDa protein (Fig. 5).

Next, the dependence of 22- and 27-kDa protein induction on the concentration of MNNG during a 70-min pretreatment was studied. Significant amounts of both proteins were present when pretreatment was carried out with a concentration of MNNG as low as 1.4 μM (Fig. 6). The maximum amounts of protein were obtained at 20 μM MNNG. When 68 μM MNNG was used for the pretreatment, the amount of the 27-kDa protein became negligible, but that of the 22-kDa protein did not decline much.

DISCUSSION

Three molecular species of methyl-accepting proteins have been detected in the fluorogram of the extracts prepared from *B. subtilis* cells by SDS-polyacrylamide gel

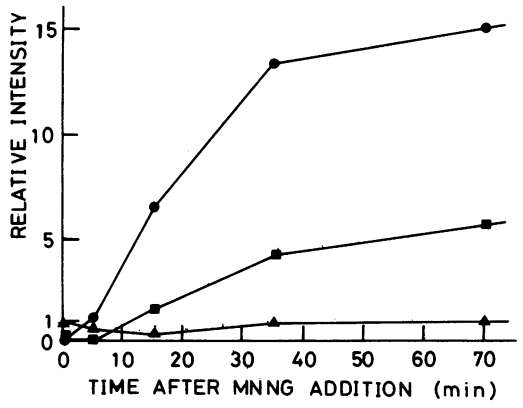

FIG. 5. Time course for the induction of methyl-accepting proteins after treatment with MNNG. To a culture of log-phase cells of strains TKJ7501 (*ada*$^+$), MNNG was added at 20.4 μM, and incubation was continued at 37°C. Samples were taken at the times indicated, and crude extracts were prepared. Each extract, containing 125 μg of protein, was incubated with *methyl*-^{3}H-calf thymus DNA (4,900 cpm) at 37°C for 20 min and subjected to SDS-polyacrylamide gel electrophoresis. The intensities of the three bands corresponding to the 20-kDa (▲), 22-kDa (●), and 27-kDa (■) proteins were determined by densitometry.

electrophoresis. The assignments of the proteins as DNA methyltransferases were made after comparing the patterns obtained with those from assays of enzymatic activities on different substrates. The 20-kDa protein, which was present constitutively in all the strains, was considered to be O^6-methylguanine:DNA methyltransferase. The 22-kDa protein was produced in large amounts only in the *ada*$^+$ cells after MNNG pretreatment and was also considered O^6-methylguanine:DNA methyltransferase. This protein was capable of accepting a methyl group from [*methyl*-^{3}H]poly(dT):poly(dA); however, the substrate in the methylated poly(dT) has not been identified. Both these species of methyl-accepting protein were capable of accepting an ethyl group, probably from O^6-ethylguanine residues in ethylated DNA. The 27-kDa protein accepted a methyl group from methylated poly(dT) and was produced in *ada*$^+$ as well as *ada-3*, *ada-4*, and *ada-6* mutant cells after adaptive treatment. This activity coincided with the loss of the methylphosphotriester from the methylated poly(dT), and it was assigned to methylphosphotriester:DNA methyltransferase.

With all the *ada* mutant cells, the 20-kDa protein disappeared when they were grown with MNNG. This, in accordance with the previous results concerning the disappearance of enzymatic activity (15) and the sensitization of *ada* cells to subsequent challenge with MNNG (14), indicated that the 20-kDa protein was exhausted by the reaction taking place during the pretreatment. For the *ada*$^+$ cells, the results were somewhat variable, and in some cases (Fig. 5) it looked as though the activity had resumed during the pretreatment. This may suggest a different metabolism of this enzyme in *ada*$^+$ and *ada* cells; however, the concomitant appearance of a large amount of the 22-kDa protein may have interfered with the 20-kDa protein in the gel. Further efforts will be required to substantiate this notion.

A distinction was made between *ada-1*, *ada-2*, and *ada-5* mutants on the one hand and *ada-3*, *ada-4*, and *ada-6* mutants on the other in the production of the 27-kDa methyltransferase after adaptive treatment; the former mutants were deficient and the latter were proficient. This corroborates our previous classification of the six *ada* mutants into two groups (15). After adaptive treatment, the cells of the latter class (*ada-4* group) produced methylpurine-DNA glycosylase as well as the 27-kDa methyltransferase and acquired resistance to propyl and butylnitroso compounds, whereas none of these characteristics were manifested in the cells of the former class (the *ada-1* group). Since sensitivity to the lethal and mutagenic effects of MNNG is the same in these groups irrespective of pretreatment, it is unlikely that either the glycosylase or the 27-kDa methyltransferase is responsible for the adaptation to MNNG. Induction of the 22-kDa methyltransferase would appear to explain totally the adaptive response to MNNG. This was also substantiated by the fact that its synthesis depended on the concentration of MNNG used for adaptation (Fig. 6) to an extent showing good correlation with acquisition of resistance (13). On the other hand, it remains to be found which of these two enzymes (or another yet to be revealed) is responsible for the acquisition of resistance to propyl and butylnitroso compounds. We have thus substantiated our previous proposition that mutants belonging to the *ada-4* group are specifically defective in the inducible synthesis of O^6-methylguanine:DNA methyltransferase (the 22-kDa protein), while those belonging to the *ada-1* group are deficient in the adaptive response in toto.

We recognized several features which may differ from those observed in *E. coli* cells. In contrast to the finding for

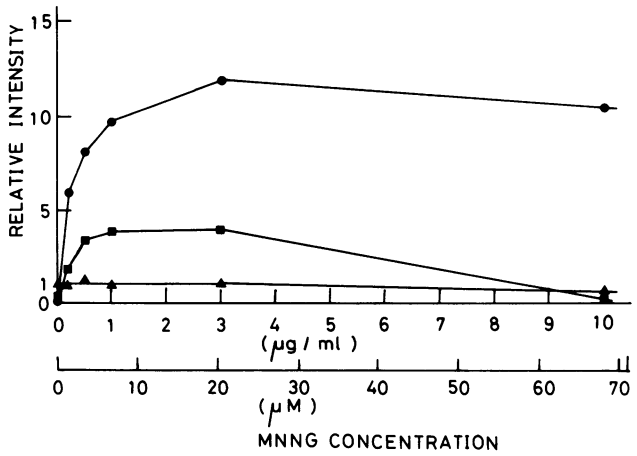

FIG. 6. Dependence of the induction of methyl-accepting proteins on the concentration of MNNG used for pretreatment. Pretreatment of strain TKJ7501 (*ada*$^+$) was performed with the indicated concentrations of MNNG at 37°C for 70 min. Each extract, containing 80 μg of protein, was incubated with *methyl*-^{3}H-calf thymus DNA (7,400 cpm) at 37°C for 20 min and subjected to SDS-polyacrylamide gel electrophoresis. The intensities of the three bands corresponding to the 20-kDa (▲), 22-kDa (●), and 27-kDa (■) proteins were determined by densitometry.

E. coli cells which harbor a plasmid carrying the cloned *ada* gene (16, 23), we have not obtained any evidence for in vitro cleavage of methyl-accepting proteins in *B. subtilis*. Some may be produced from precursor proteins by proteolysis, but if this had been the case production would have been completed before the analysis. As for the 22- and 27-kDa methyltransferases which were produced after adaptive treatment, our results seem to be unfavorable to the hypothesis that these two proteins are cleaved from a common precursor because (i) at the earliest phase of induction, only the 22-kDa protein was detected and (ii) the ratio of O^6-methylguanine:DNA methyltransferase and methylphosphotriester:DNA methyltransferase activity in the crude extracts from the adapted cells was approximately 3:1, not 1:1 as in *E. coli* cells (11). At this point, it is speculated that the synthesis of these two proteins is independently controlled.

Another rather unexpected finding was that the activity of O^6-methylguanine:DNA methyltransferase occurred in two different molecular species of methyltransferase, constitutive and inducible, although no difference in substrate specificity has so far been noticed. It has been estimated that a *B. subtilis* cell constitutively contains about 240 molecules of the enzyme, which is more than 10-fold the amount found in an *E. coli* cell (3, 12). This higher level of constitutive activity has now been shown to result from a different molecular species of methyltransferase from the one with inducible activity. A simple assumption may be made that these two species of the methyltransferase could be coded by two different genes, but clearly more complex possibilities should be kept in mind; for example, they could be derived from the same gene utilizing different promoters for the transcription, or they could be produced through different processing. Cloning the *ada* gene and isolating the mutants defective in the constitutive methyltransferase should help to answer this question. It is notable that in *E. coli*, constitutive and inducible activities of 3-methyladenine-DNA glycosylases reside in different molecular species (5). This type of dual defense mechanism against the assault of alkylating chemicals is intriguing from a phylogenic point

of view, particularly as mammalian cells and tissues generally contain DNA methyltransferase constitutively.

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