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ADP-Ribosylation of Proteins in *Bacillus subtilis* and Its Possible Importance in Sporulation

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Endogenous ADP-ribosylation was detected in *Bacillus subtilis*, as determined in vitro with crude cellular extracts. The ADP-ribosylated protein profile changed during growth in sporulation medium, displaying a temporary appearance of two ADP-ribosylated proteins (36 and 58 kDa) shortly after the end of exponential growth. Mutants resistant to 3-methoxybenzamide, a known inhibitor of ADP-ribosyltransferase, were obtained, and a significant proportion (15%) were found to be defective in both sporulation and antibiotic production. These mutants failed to ADP-ribosylate the 36- and 58-kDa proteins. The parent strain also lost the ability to ADP-ribosylate these proteins when grown in the presence of 3-methoxybenzamide at a concentration at which sporulation but not cell growth was severely inhibited. Results from genetic transformations showed that the mutation conferring resistance to 3-methoxybenzamide, named *brgA*, was cotransformed with the altered phenotypes, i.e., defects in ADP-ribosylation and sporulation. *spo0A* and *spo0F* mutants displayed an ADP-ribosylation profile similar to that of the parent strain, but a *spo0H* mutant failed to ADP-ribosylate any proteins, including the 36- and 58-kDa proteins. The significance of protein ADP-ribosylation in sporulation was further indicated by the observation that ADP-ribosylation of the 36-kDa protein could be induced by treatment with decoyinine, an inhibitor of GMP-synthetase, and by amino acid limitation, both of which resulted in an immediate decrease in GTP pool size eventually leading to massive sporulation. We propose that a new sporulation gene, which presumably controls sporulation via ADP-ribosylation of certain functional proteins, exists.

Sporulation of *Bacillus subtilis* usually starts when cells encounter adverse environmental conditions such as nutrient limitation. In contrast, cells do not sporulate in media containing excess glucose, ammonium, and phosphate ions. The process of *B. subtilis* sporulation involves seven morphological events (stage 0 to stage VII); mutations that block the earliest process from stage 0 to stage I are called *spo0* mutations and include at least eight known loci: *spo0A*, *spo0B*, *spo0E*, *spo0F*, *spo0H*, *spo0J*, *spo0K*, and *spo0L*. The original *spo0E* and *spo0L* mutants have been shown to be gain-of-function mutations and are in fact negative regulators of sporulation, in contrast to the other *spo0* genes (reviewed in references 7, 8, 18, 19, and 29). These *spo0* genes are of particular interest because they are considered to play an essential role as an engine for the initiation of the developmental process and because many *spo0* mutants exhibit typical pleiotropic effects, including an inability to produce sporulation-associated antibiotics and proteases, a failure to acquire genetic competence, and a lack of sensitivity to polymixin and certain phages. The degree of this pleiotropy depends on the particular *spo0* locus. Mutations in *spo0A* give rise to the most highly pleiotropic effects, while *spo0B*, *spo0E*, *spo0F*, and *spo0H* mutations cause numerous changes in the pattern of protein synthesis in both vegetative and early-stationary-phase cells (reviewed in references 7 and 11). Genes representing *spo0* loci have been cloned and characterized, and functional relationships between *spo0* genes and other *spo* genes have been postulated (17, 33, 34, 37). The Spo0A protein is a DNA-binding protein and belongs to a large family of bacterial proteins called response regulators (11, 36). Its binding activity to DNA is enhanced by phosphorylation, and this is considered to be a key step in its control of the initiation of sporulation (1, 28). Spo0F and Spo0B proteins participate in the phosphorylation of the Spo0A protein as intermediate carriers of phosphate in a phosphorelay system (11). *spo0H* also plays a key role in the initiation of sporulation. It encodes a sigma factor, σ^H, and transcription of spo0H is known to gradually increase with growth, reaching a peak at the end of exponential-phase growth (T_0) (4, 42). The Spo0A protein may interact with the sigma factor subunit σ^H of RNA polymerase (2).

Recently, results from our laboratory (26, 31) have demonstrated the potential significance of ADP-ribosylation of proteins in the developmental process (aerial mycelium formation) of *Streptomyces* spp. ADP-ribosylation is an enzymatic reaction by which the ADP-ribose moiety of NAD is covalently bound to specific acceptor proteins by ADP-ribosyltransferase (ADPRT) (reviewed in references 20, 32, and 41). ADP-ribosylation can be considered as a mode of metabolic regulation similar to phosphorylation, adenylylation, uridylation, and methylation; the ADP-ribosylated protein may lose its activity but may later be reactivated by enzymatic de-ADP-ribosylation. A possible role for ADP-ribosylation in the development of the prokaryote *Myxococcus xanthus* has also been suggested (5). Mono-ADP-ribosylation is detected in prokaryotes and eucaryotes, while poly-ADP-ribosylation occurs only in eucaryotes. The best studied ADPRTs are those for the bacterial toxins such as cholera toxin and diphtheria toxin, which catalyze the ADP-ribosylation of eucaryotic signal-transducing trimeric GTP-binding proteins (35). In contrast to the considerable knowledge of ADP-ribosylation in eucaryotes, little is known about prokaryotic ADP-ribosylation. The α-subunit of RNA polymerase of *Escherichia coli* is known to be ADP-

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ribosylated as a result of T4 and N4 phage infections (32). Nitrogenase activity in *Rhodospirillum rubrum* and *Azospirillum lipofernum* is inactivated by ADP-ribosylation of the iron-containing subunit of the enzyme (10), and two cytoplasmic proteins of *Pseudomonas maltophilia* are reported to be ADP-ribosylated (6). The aim of the present study was to demonstrate the significance of endogenous ADP-ribosylation of proteins in morphological and physiological differentiation of *B. subtilis* and its relation to previously known mechanisms regulating initiation of sporulation.

MATERIALS AND METHODS

Chemicals. 3-Methoxybenzamide (3-MBA; 3-anisamide) and 3-aminobenzamide (3-ABA) were purchased from Nakarai Tesque, Kyoto, Japan. Isonicotinic acid hydrazide (INH; isoniazid) and ADP-ribose were from Sigma Chemical Co., and [^{32}P-adenylate]NAD and [2,8-^{3}H-adenine]NAD were from Amersham and ICN Biochemicals, respectively. Decoyinine was a gift from the Upjohn Co., Kalamazoo, Mich. Labeled ADP-ribose was prepared in this laboratory from [2,8-^{3}H-adenine]NAD according to an unpublished method developed in the laboratory of J. C. Ensign. ^{3}H-labeled ADP-ribose thus prepared with partially purified *Streptomyces griseus* NAD glycohydrolase was more than 90% pure.

Bacterial strains. Strain 168 (*trpC2*), a standard strain of *B. subtilis* Marburg, was mainly used in the present study. Strain 61884 (*aspB66 trpC2*) is isogenic with strain 61883 (*aspB66 trpC2 relA1*) with the exception of *relA*, as described previously (23). The *relA1* mutation was originally found by Swanton and Edlin (38). The *spo0A* mutant (strain UOT-1611 [*trpC2 lys-1 nprR2 nprE18 spo0AΔHB*]), *spo0F* mutant (strain UOT-1279 [*trpC2 lys-1 nprR2 nprE18 spo0FΔS*]), and *spo0H* mutant (strain UOT-1281 [*trpC2 lys-1 nprR2 nprE18 spo0H17*]) were provided by F. Kawamura, Rikkyo University.

Preparation of mutants. The spontaneous benzamide derivative-resistant mutants were obtained as colonies that grew within 24 h after wild-type spores were spread on GYM agar (see below) containing 30 mM 3-MBA or 100 mM INH. Since *B. subtilis* was resistant even to 100 mM 3-ABA when incubated at 37°C, resistant mutants were obtained by increasing sensitivity to the drug by incubating at an elevated (50°C) temperature in the presence of 65 mM 3-ABA. The frequency of appearance of mutants resistant to each drug was 10^{-7} to 10^{-8}. The resistant isolates were purified by single-colony isolation before being used for experiments. Transformation was performed as previously described (23).

Media and sporulation conditions. GYM medium contained 0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.1% peptone (NZ-amine, type A), 0.2% NaCl (adjusted to pH 7.2 with NaOH), and supplements as required by auxotrophs. GYM agar contained 2% agar in addition to the above components. Sporulation medium contained 0.8% (wt/vol) nutrient broth (Difco), 1 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM $MnCl_2$, 10 μM $FeCl_3$, 10 mM potassium phosphate buffer (pH 7.0), 5 mM potassium acetate (pH 7.0), and supplements as required by auxotrophs. Synthetic medium (S7 medium) (22) contained 10 mM ammonium sulfate, 5 mM potassium phosphate (pH 7.0), 100 mM morpholinopropanesulfonate (adjusted to pH 7.0 with KOH), 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM $MnCl_2$, 5 μM $FeCl_3$, 2 μM thiamine, 55 mM glucose, and 20 mM L-glutamate (adjusted to pH 7.0 with KOH). Synthetic medium was filter sterilized before use. Auxotrophic requirements were satisfied by the addition of 0.25 mM L-tryptophan and L-lysine or 10 mM L-aspartate. Since 3-MBA, 3-ABA, and INH are all heat stable, media containing them were autoclaved with all components present.

Strains were inoculated from frozen cultures onto plates containing GYM agar supplemented as necessary and incubated for 10 to 15 h at 37°C. The cells were inoculated at a low optical density at 600 nm (OD_{600}) (0.01 to 0.02) into flasks containing medium (10% volume) plus any requirements and were incubated at 37°C for the indicated times with vigorous shaking. Growth was monitored by measuring the OD_{600}. Incubation was at 37°C unless otherwise specified. The spore titer was measured by heating the culture for 20 min at 75°C and then plating after appropriate dilution.

Assay of antibiotic activity. The amount of antibiotic produced in the medium was determined by bioassay using *Staphylococcus aureus* 209P as the indicator organism as described previously (25). Antibiotic activity was expressed as units per milliliter; 1 U/ml produced a halo of 1.5-mm width (diameter of clear area around disk, 11 mm).

Assay of ADPRT activity. Cells were harvested by centrifugation (1,500 × *g*) at 4°C for 20 min, washed with 10 mM triethanolamine · HCl solution (pH 7.5), and stored at −20°C until used for experiments. The cells could be stored without a loss of enzyme activity for at least 7 days when cells were frozen at −20°C, but activity decreased to less than 10% when crude extract prepared by disrupting the cells was left overnight at −20°C. The cells were suspended in 10 mM triethanolamine · HCl (pH 7.0), subjected to sonication (1 to 3 min) in an ice-cold bath, and immediately supplemented with phenylmethylsulfonyl fluoride (0.5 mM), leupeptine (25 μg/ml), and 9-laurylether (0.4%). After standing for 30 min on ice, crude extracts were obtained by centrifugation (5,000 × *g*, 15 min) and typically contained 20 to 30 mg of protein per ml. Reaction conditions described by Just et al. (13) were modified as follows. A 20-μl sample of crude extract containing 150 μg of protein was suspended in 20 μl of a solution containing 25 mM triethanolamine · HCl (pH 7.5), 3 mM $MgCl_2$, 300 μM GTP, 5 mM $CaCl_2$, 1 mM dithiothreitol, and 30 μM [2,8-^{3}H-adenine]NAD (3.7 kBq, as a donor of ADP-ribose). The presence of $CaCl_2$ was essential for ADPRT activity. Reaction mixtures (each 40 μl) were incubated at 30°C for 3.5 h before addition of 50 μl of ice-cold 20% (wt/vol) trichloroacetic acid. After 30 to 60 min at 0°C, the suspensions were collected on membrane filters (Millipore; 0.45-μM pore size), which were then washed with 5 ml of ice-cold 5% (wt/vol) trichloroacetic acid. Radioactivity on the filters was measured with a liquid scintillation counter. The specific activity of ADPRT was expressed as the amount (disintegrations per minute) of radioactivity incorporated into acid-precipitable material per milligram of protein per minute.

To obtain autoradiograms of ADP-ribosylated proteins, 30 μM [^{32}P-adenylate]NAD (111 kBq) was used instead of [2,8-^{3}H-adenine]NAD. After a 3.5-h reaction, 4 μl of sodium dodecyl sulfate (SDS)-urea solution (containing 4% [wt/vol] SDS and 4 M urea) and 50 μl of 20% (wt/vol) trichloroacetic acid were added sequentially to the reaction mixture. After standing on ice for 30 min, the mixture was centrifuged at 4°C (15,000 × *g*, 10 min), and the pellet was washed twice with 100 μl of diethylether and then resuspended in a small amount (20 μl) of SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer containing 0.5% sarcosyl and 10% (wt/vol) mercaptoethanol. SDS-PAGE was carried out as described by Laemmli (14). The acrylamide concentration used was 12.5% (wt/vol). The gel was dried and exposed to X-ray film. The typical exposure time was 2 days at −80°C with intensifying screens.

NAD-glycohydrolase was assayed as described previously (22).

Alkaline hydrolysis of ^{32}P-labeled proteins. Alkaline hydrolysis was carried out as described in detail previously (26).

RESULTS

ADPRT activity. ADPRT activity was detected in *B. subtilis* by incubating 30 mM [2,8-^{3}H-adenine]NAD with a crude extract, containing not only the enzyme protein but also substrate proteins to be ADP-ribosylated (endogenous ADP-ribosylation). Specific activity was 20 to 70 dpm/mg of protein per min throughout cell growth. Virtually no activity (7% or less) was detected when NAD was replaced with ^{3}H-labeled ADP-ribose at the same concentration. Unlike in *S. griseus* (22), NAD-glycohydrolase activity was not detected in *B. subtilis.*

ADP-ribosylation of cellular proteins. Changes in the ADP-ribosylation profile during growth in sporulation medium were examined by incubating crude extracts with [^{32}P]NAD. The results of SDS-PAGE analysis of labeled cellular proteins are shown in Fig. 1. The profile of ADP-ribosylated proteins changes markedly during culture development. It is noteworthy that the labeled 36- and 58-kDa proteins appear at the onset of the sporulation process (T_0), displaying a peak at T_1 to T_2, and later (T_3 to T_4) are absent. The labeling of these two proteins is therefore temporary. In contrast, labeling of the 42- and 60-kDa proteins is pronounced in early growth phase but becomes faint as the cultures develop.

In chase experiments, a 100-fold molar excess of unlabeled NAD but not ATP completely abolished the radioactive labeling of these proteins (Fig. 1), demonstrating that protein phosphorylation was not responsible for the labeling. Furthermore, the labeled modifying group was identified as ADP-ribose by subjecting a preparation of ^{32}P-labeled proteins to alkaline hydrolysis. Thin-layer chromatography analysis indicated that ADP-ribose was the major radiolabeled product released (Fig. 2). Accordingly, we propose that the appearance and disappearance of ADP-ribosylated proteins is age dependent and for some of them (36, 42, 58, and 60 kDa) is also correlated with the early stage of the developmental process.

Inhibition of sporulation by benzamide derivatives. There are several approaches to induce sporulation of *B. subtilis*. The best-known sporulation condition is to cultivate cells in a nutritionally poor medium such as sporulation medium originally developed by Schaeffer (30). Sporulation occurs 6 to 8 h after development has started at the end of exponential growth, when rapidly metabolizable carbon, nitrogen, or phosphate sources have been exhausted (briefly reviewed by Freese and

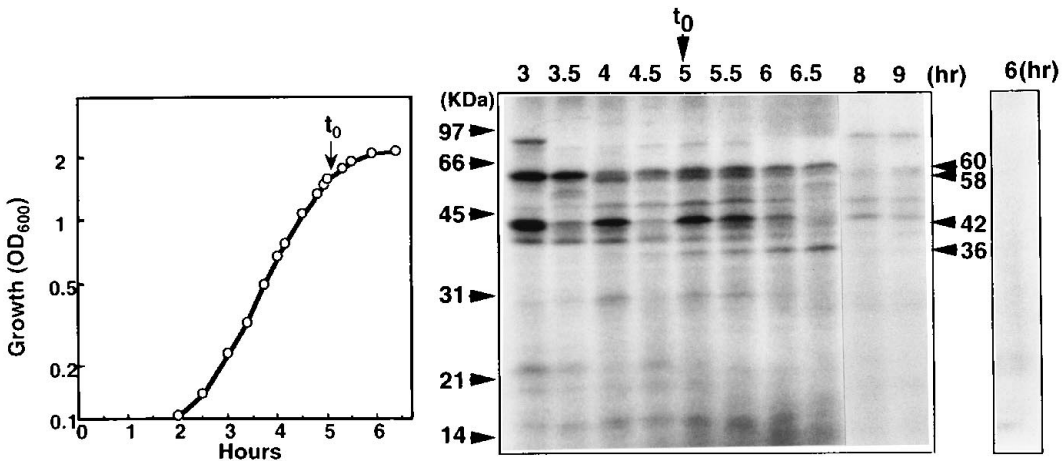

FIG. 1. Autoradiogram of an SDS-PAGE gel on which ADP-ribosylated proteins of parent strain 168 were separated. Crude extracts from cells grown at 37°C for various times in sporulation medium (left) were incubated at 30°C for 3.5 h with [^{32}P-adenylate]NAD. (Center) Equivalent amounts of protein from the assay mixtures were analyzed by SDS-PAGE. (Right) Reference experiment in which a 100-fold molar excess (3 mM) of unlabeled NAD was included in the assay mixture. The gel was dried and exposed to X-ray film with an intensifying screen for 2 days at −80°C. Positions of molecular mass standards are shown. t_0, the time point at which exponential growth ceased.

Heinze [8]). Another approach for the timely induction of sporulation is the use of decoyinine, an inhibitor of GMP-synthetase. It induces sporulation by decreasing intracellular GTP pool size without affecting ppGpp and pppGpp levels and is effective even in the nutritional conditions under which sporulation is normally suppressed (15, 16, 23). The stringent response represents another method by which timely sporulation can be induced in stringent (*rel*$^+$ [wild-type]) strains but not in relaxed (*rel* [mutant]) strains. Partial limitation of a required amino acid in suitable auxotrophs causes an immediate decrease in the GTP pool size (in *rel*$^+$ cells but not in *rel* cells) resulting in extensive sporulation (23, 24). (The *rel* strains can sporulate as well as *rel*$^+$ strains when cultivated in sporulation medium with a prolonged incubation [21]).

The benzamide derivatives 3-MBA, 3-ABA, and INH are the most commonly used inhibitors of ADPRT (40, 41), and it was therefore reasonable to believe that these drugs might interfere with sporulation more potently than with growth. In order to assess this, cells were grown in sporulation medium and a range of concentrations of each drug was added to the cultures when the OD_{600} reached 0.1. Cell densities and spore titers were measured 20 h after drug addition, and the results are shown in Fig. 3. It is evident that 3-MBA, 3-ABA, and INH all severely inhibited sporulation at concentrations at which cell density decreased only slightly. The ADP-ribosylation of proteins by crude extracts prepared from cells grown to late growth phase ($T_{1.5}$) in the presence of a concentration of 3-MBA (15 mM) that was sufficient to inhibit sporulation was examined. The cells exhibited a greatly reduced level of the ADP-ribosylation of the 36- and 58-kDa proteins (Fig. 4), confirming the correlation between sporulation and the ADP-ribosylation of the proteins.

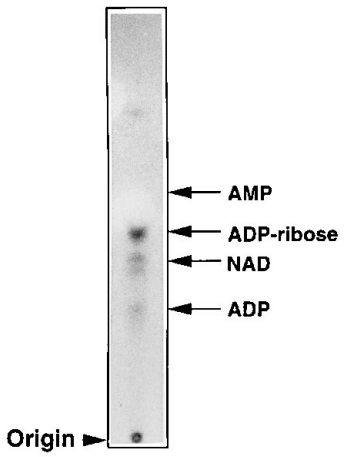

FIG. 2. Thin-layer chromatography analysis and autoradiography of alkaline hydrolysates of proteins previously incubated with 185 kBq of [^{32}P]NAD. ^{32}P-labeled proteins of a crude extract were precipitated with trichloroacetic acid, washed, and incubated in 0.1 M KOH at 30°C for 3 h.

Isolation and characterization of mutants. 3-MBA, 3-ABA, and INH, analogs of nicotinamide, all completely inhibited cell growth when added at high concentrations (Table 1). Since these drugs are potent inhibitors of ADPRT of *S. griseus* (27), it was reasoned that some drug-resistant mutants might possess an altered ADPRT affecting sporulation. This was assessed by isolating and analyzing mutants of *B. subtilis* 168 which developed spontaneously at a frequency of 10^{-7} to 10^{-8} when cultured on GYM agar containing each drug (see Materials and Methods). Of these mutants, 15% had a severely reduced ability to sporulate and produced spore titers of less than 10^6/ml in sporulation medium. They grew as well as, or slightly more slowly than, the parent strain on GYM agar. The impaired ability to sporulate was no longer restored by cultivation at a lowered temperature (down to 20°C) or by addition of decoyinine. Strikingly, these mutants were all defective in antibiotic production. Representative mutants are listed in Table 1. Analysis of their ADP-ribosylated protein profiles showed that these mutants were all lacking the labeled 36- and 58-kDa proteins. They also possessed cross-resistance to the other two drugs, indicating that the mutations occurred in the same gene, which is hereafter designated *brgA* (benzamide-derivative resistance gene). Another class of 3-MBA-resistant mutants with a normal ability to sporulate and produce antibiotics revealed a normal ADP-ribosylated protein profile, as examined in three strains (data not shown).

A *brgA1* mutation conferring resistance to 3-MBA was introduced into parent strain 168 from mutant 168-1 by transformation. 3-MBA-resistant transformants (more than 100

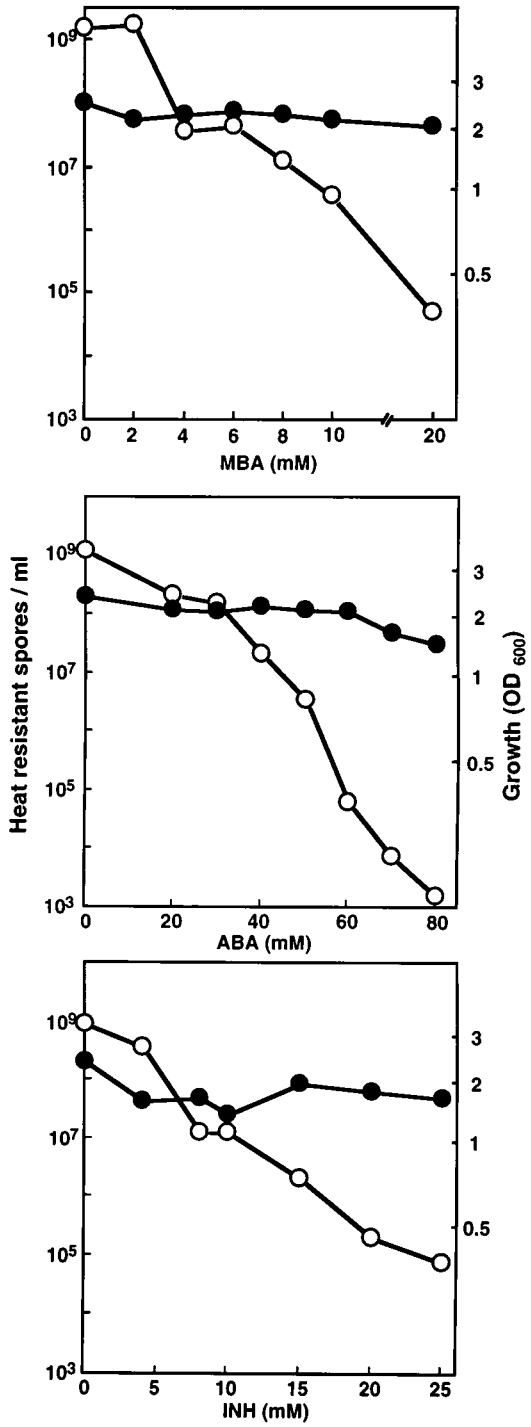

FIG. 3. Effects of benzamide derivatives on growth and sporulation. Strain 168 was grown at 37°C in sporulation medium to an OD_{600} of 0.1. The culture was subdivided into small flasks containing various amounts of 3-MBA, 3-ABA, or INH. The cultures were shaken at 37°C, and the cell density (OD_{600}) and heat-resistant spore titers were measured 20 h later. ○, heat-resistant spore titer; ●, cell density.

clones tested) were all defective in sporulation and antibiotic production and failed to ADP-ribosylate the 36- and 58-kDa proteins (5 clones tested). The pattern of ADP-ribosylated proteins in transformant 168-3 is a typical example (Fig. 5).

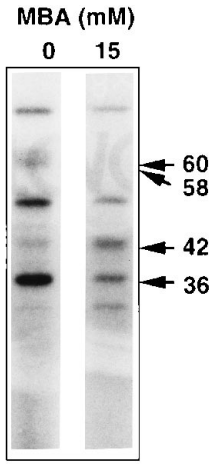

FIG. 4. Autoradiogram of an SDS-PAGE gel of ADP-ribosylated proteins. Crude extracts from cells of strain 168 grown in sporulation medium to late growth phase ($T_{1.5}$) in the presence or absence of 15 mM 3-MBA were prepared, and the ADP-ribosylation reaction was performed as stated in the legend to Fig. 1. Positions of molecular mass standards (in kilodaltons) are indicated on the right.

(Although the pattern of ADP-ribosylated proteins in the 168 control in this figure [and also that in Fig. 4] differs to some extent from that in Fig. 1, this difference was apparently due to the fact that endogenous ADP-ribosylation as determined in vitro with crude cellular extracts not only for the enzyme protein but also for substrate proteins is more complicated than ADP-ribosylation with ADP-ribosylating toxins.)

ADP-ribosylation following decoyinine addition. The sporulation of strain 168 was severely suppressed (spore titer, 10^5/ml or less) when cells were grown in sporulation medium supplemented with 1% (wt/vol) Casamino Acids, although growth was greatly enhanced. Addition of 0.4 mM decoyinine in the mid-exponential phase induced extensive sporulation, producing 1.5×10^8 spores per ml. Almost all cells (>90%) were spore forms 10 h after decoyinine addition, as determined microscopically. Changes in the ADP-ribosylation profile following addition of decoyinine were studied, and the results are shown in Fig. 6. It is evident that a labeled 36-kDa protein (and perhaps also a 58-kDa protein) appeared shortly after decoyi-

TABLE 1. Characterization of drug-resistant mutants

Strain	Resistance level[a]	No. of heat-resistant spores/ml[b]	Antibiotic produced (U/ml)[c]
168 (parent)	25 mM 3-MBA, 50 mM 3-ABA, 60 mM INH	1.5×10^9	8
168-1	50 mM 3-MBA	1.5×10^3	3
168-2	50 mM 3-MBA	1×10^2	0
168-3[d]	40 mM 3-MBA	7.5×10^4	1
168-4	70 mM 3-ABA	7×10^4	1
168-5	65 mM 3-ABA	2.5×10^4	0
168-6	120 mM INH	2.5×10^4	1
168-7	110 mM INH	1×10^2	0

[a] Resistance was determined by incubating cells at 37°C on GYM agar containing each drug, except for resistance to 3-ABA, which was determined at 50°C. Cells can grow (although slowly) at the indicated concentrations but cannot grow at higher concentrations.

[b] Heat-resistant spore titers were determined by cultivating cells in sporulation medium (without drug) for 24 h.

[c] One unit is defined as described in Materials and Methods.

[d] Transformant of 168 by DNA of 168-1.

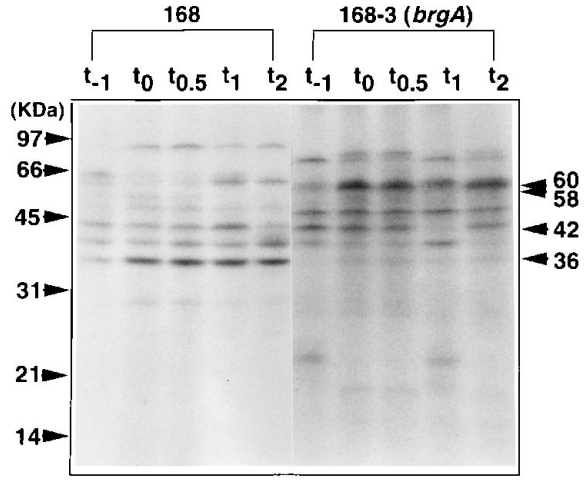

FIG. 5. Autoradiogram of an SDS-PAGE gel of ADP-ribosylated proteins. Parent strain 168 and 3-MBA-resistant (*brgA1*) transformant 168-3 were grown in sporulation medium to various growth phases (T_{-1} to T_2). Crude extracts from cells were prepared, and the ADP-ribosylation reaction was performed as stated in the legend to Fig. 1.

nine addition, while two other proteins (42 and 60 kDa) gradually disappeared. The 40-kDa protein band also showed an increase in intensity, but this band is visible at T_0. When cells were not treated with decoyinine, virtually no change in the band pattern was observed over a 4-h incubation period, and labeled bands corresponding to the 36- and 58-kDa proteins were not present (data not shown).

ADP-ribosylation following stringent response. Sporulation was induced in strain 61884 (*asp rel*$^+$) by transferring cells from

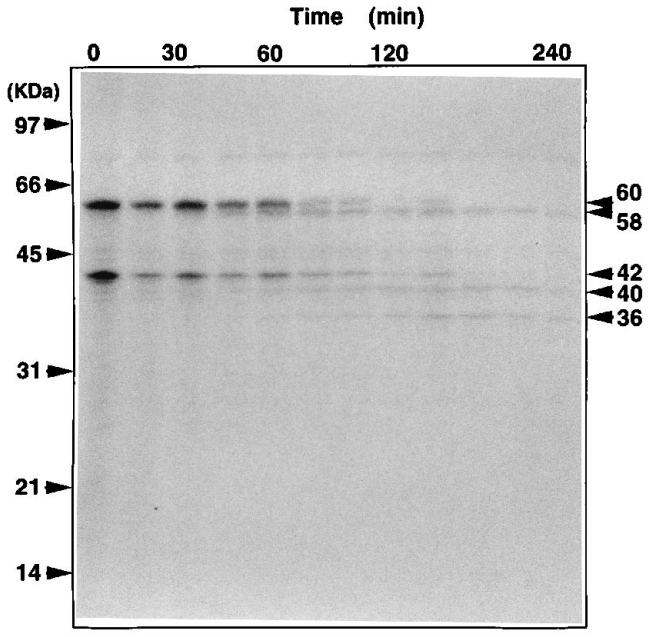

FIG. 6. Autoradiogram of an SDS-PAGE gel of ADP-ribosylated proteins following decoyinine addition. Strain 168 was grown in synthetic (S7) medium supplemented with 1% (wt/vol) Casamino Acids and 0.25 mM tryptophan. When the OD_{600} reached 2.3 (mid-exponential phase), decoyinine was added at a final concentration of 0.4 mM, and incubation was continued under shaking. At the indicated times after decoyinine addition cells were harvested, and crude extracts for the ADP-ribosylation reaction were prepared as stated in the legend to Fig. 1.

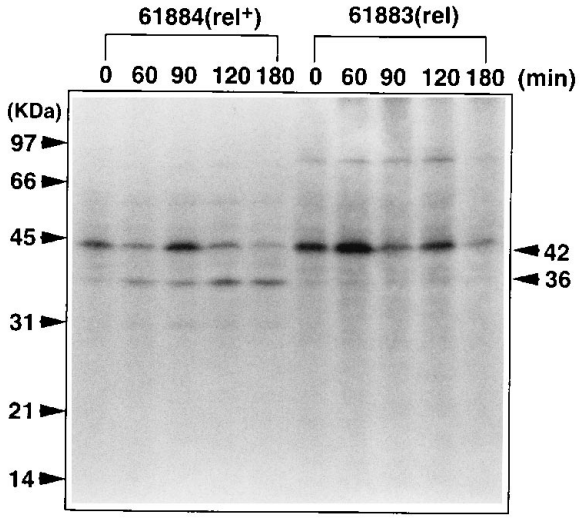

FIG. 7. Autoradiogram of an SDS-PAGE gel of ADP-ribosylated proteins after cell transfer of a stringent (61884) or a relaxed (61883) auxotroph to a limiting aspartate concentration. Cells were grown in synthetic (S7) medium containing 0.25 mM tryptophan and 10 mM aspartate. When the OD_{600} reached 0.5 (early exponential phase), the cells were rapidly washed with synthetic medium and transferred to the same volume of synthetic medium containing 0.25 mM tryptophan and a limiting concentration (1 mM) of aspartate. Incubation was continued under shaking. At the indicated times after cell transfer cells were harvested, and crude extracts for the ADP-ribosylation reaction were prepared as stated in the legend to Fig. 1.

synthetic medium containing an excess of aspartate to the same medium containing limiting aspartate (see legend to Fig. 7). Extensive sporulation was detected 10 h after cell transfer, producing about 3×10^7 spores per ml. In contrast, the isogenic *rel* strain 61883 produced only 10^1 to 10^2 spores per ml under similar experimental conditions, indicating a failure in timely sporulation. Changes in the ADP-ribosylated protein profile under the sporulation-inducible and noninducible conditions were investigated (Fig. 7). In *rel*$^+$ cells, labeled 36-kDa protein appeared soon after cell transfer, but *rel* cells failed to ADP-ribosylate this protein. In contrast to results when decoyinine was used to induce sporulation (Fig. 6), ADP-ribosylation of the 58-kDa protein was not detected (or was faint, if there was any) (Fig. 7). These results indicate a tight correlation between sporulation and ADP-ribosylation of the 36-kDa protein. (The marked difference in the ADP-ribosylated protein profiles between Fig. 7 and 1 was due to the cultivation media used.)

ADP-ribosylation in *spo0* mutants. Changes in the ADP-ribosylated protein profile in *spo0* mutants as represented by *spo0A*, *spo0F*, and *spo0H* were studied by cultivating cells in sporulation medium (Fig. 8). The *spo0A* and *spo0F* mutants both displayed the normal ADP-ribosylation of the 36- and 58-kDa proteins at late growth phase, while the *spo0H* mutant failed to ADP-ribosylate not only those two proteins but also any other proteins at late growth phase. This characteristic of the *spo0H* mutant was confirmed in three separate experiments. The *spo0A* mutant was characterized by an intensified 42-kDa protein band at late growth phase, a band which normally becomes faint in the wild-type strain and in the *spo0F* and *spo0H* mutants (Fig. 8).

None of the *spo0A*, *spo0F*, and *spo0H* mutants revealed resistance to 3-MBA.

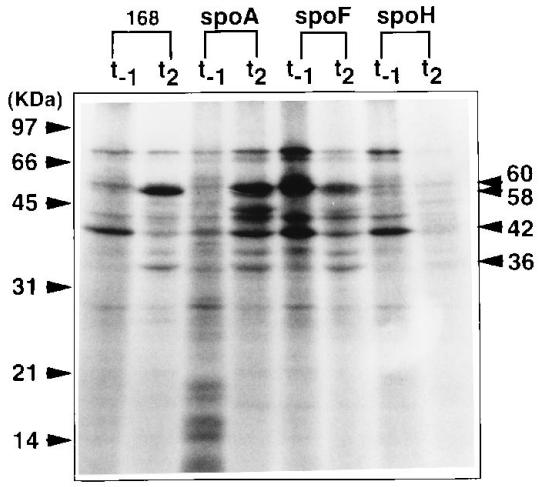

FIG. 8. Autoradiogram of an SDS-PAGE gel of ADP-ribosylated proteins in certain *spo0* mutants. Strains were grown in sporulation medium supplemented with 0.25 mM (each) tryptophan and lysine to the mid-exponential growth phase (t_{-1}) or to the late growth phase (t_2). Cells were harvested, and crude extracts for the ADP-ribosylation reaction were prepared as stated in the legend to Fig. 1.

DISCUSSION

In this paper we have demonstrated (i) the existence of ADPRT activity in *B. subtilis* and (ii) its possible role in the differentiation process of this organism. This offers an interesting new approach to the investigation of sporulation in *B. subtilis*. ADPRT activity has previously been found in members of the family *Bacillaceae*. A human pathogenic strain of *Bacillus cereus* produces an exoenzyme which selectively ADP-ribosylates GTP-binding proteins in platelet membranes (13), and *Bacillus sphaericus* produces a mosquitocidal toxin which possesses ADP-ribosylating activity (39). These data, however, do not provide any evidence for endogenous ADP-ribosylation, since the substrates to be ADP-ribosylated are from foreign organisms. Thus, to our knowledge, the present work is the first report of endogenous ADP-ribosylation in the genus *Bacillus*. Since labeled ADP-ribose was not an efficient substrate, NAD appears to be a direct substrate for ADP-ribosylation.

In the present study, ADP-ribosylation of two proteins and de-ADP-ribosylation of two other proteins was focused on in relation to cell developmental processes. Of these proteins, evidence for the significance of ADP-ribosylation of the 36-kDa protein is the most convincing, as demonstrated in four different studies: (i) sporulation in sporulation medium, (ii) sporulation upon decoyinine addition, (iii) sporulation upon amino acid limitation, and (iv) 3-MBA-resistant mutants with an impaired ability to sporulate. Although the labeled 36-kDa protein disappeared after T_3, it remains unknown whether this was a result of de-ADP-ribosylation by ADP-ribosyl glycohydrolase, as has been reported in nitrogenase of *Rhodospirillum rubrum* (9), or simply due to an absence or decrease in the amount of the protein. In *S. griseus* and *Streptomyces coelicolor*, ADP-ribosylation of certain proteins with apparent molecular masses of 37, 39, and 41 kDa has been suggested to be involved in the developmental processes of these organisms (26, 31), and the 36-kDa protein of *B. subtilis* may be homologous to one of these. Given results with *M. xanthus* (5), ADP-ribosylation of certain proteins may play an essential role in developmental processes in a wide range of microorganisms.

Although *brgA* mutants were selected by 3-MBA resistance and showed an altered ADP-ribosylation profile, it remains to be clarified, by cloning and subsequent analyses, whether the *brgA* gene actually codes for the ADPRT enzyme. It is also essential to demonstrate that proteins ADP-ribosylated in vitro are also so modified in vivo. This is important, because the ADPRT assay used in the present study is complicated by the fact that the amount of ADP-ribosylating enzyme in vivo will affect the amount of unribosylated target proteins available for radiolabeling in vitro. Namely, the paradoxically reduced ADP-ribosylation activity detected in the *brg* mutant might implicate an increase in the level of ADP-ribosylation enzyme, resulting in the near-complete ADP-ribosylation of all protein substrate in vivo before cell extraction and therefore leaving no substrate available for labeling. Alternatively, it is also possible that the substrate proteins are absent in the *brg* mutant. These criticisms apply to the analysis of various *spo0* mutants. In addition to gene cloning of *brgA*, it is also essential to clone the genes encoding ADP-ribosylatable proteins. In this regard we have recently succeeded in isolating and sequencing the 36-kDa protein, and the results indicate that it is a novel protein in *B. subtilis* (9a).

As stated in the introduction, the initiation of sporulation in *B. subtilis* is controlled by three *spo0* genes: *spo0A*, *spo0F*, and *spo0H*. It is evident that *spo0A* and *spo0F* have no significance in the ADP-ribosylation of at least the 36-kDa protein. In contrast, the *spo0H* mutant not only simply failed to ADP-ribosylate the 36-kDa protein but also failed to ADP-ribosylate any other proteins in the late growth phase. Interestingly, a *bldH* (*bld* = bald) mutant of *S. coelicolor*, originally isolated by Champness (3) and defective in early development, also exhibits a similar phenotype as to ADP-ribosylation (31). *spo0H* of *B. subtilis* encodes sigma factor σ^H, while *bldH* of *S. coelicolor* has not yet been cloned. Although it is premature at this time to argue about the role of *spo0H* in the light of a possible function for protein ADP-ribosylation, it can be suggested that *spo0H* exerts its effects on the initiation of the developmental process, at least in part via protein ADP-ribosylation. Alternatively, it is possible that the changes in protein ADP-ribosylation in the *spo0H* mutant are secondary effects. Jaacks et al. (12) have shown that a considerable number of genes are controlled by $E\sigma^H$ at late growth phase and that σ^H probably controls genes involved in a range of activities that have not yet been discovered.

brgA may be a new pleiotropic regulatory locus (13a) which affects the early developmental process of *B. subtilis*, conceivably by modifying the activity of proteins via ADP-ribosylation. Mapping of *brgA* on the chromosome is now under way.

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