JOURNAL OF BACTERIOLOGY, July 1996, p. 3785–3790 Vol. 178, No. 13
0021-9193/96/$04.00+0

Changes in Patterns of ADP-Ribosylated Proteins during Differentiation of *Streptomyces coelicolor* A3(2) and Its Developmental Mutants

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Received 6 February 1996/Accepted 23 April 1996

Mutants resistant to 3-aminobenzamide, a known inhibitor of ADP-ribosyltransferase, were obtained from *Streptomyces coelicolor* A3(2). One (strain 27) was analyzed in detail. Mutant 27 had a reduced ADP-ribosyltransferase activity, exhibited substantial changes from the wild type in ADP-ribosylated protein profile during cell aging, and was defective in producing aerial mycelium and antibiotics. A 92-kDa ADP-ribosylated protein disappeared at the onset of differentiation in the parent strain but was present in mutant 27. Four ADP-ribosylated proteins (39, 41, 43, and 46 kDa) appeared at the onset of differentiation in the parent strain but were missing in mutant 27. Failure to ADP-ribosylate these four proteins was detected when the parent strain was grown in the presence of subinhibitory amounts of 3-aminobenzamide. Genetic analysis showed that the mutation, named *brgA*, conferring resistance to 3-aminobenzamide, cosegregated with the altered phenotypes (i.e., defects in ADP-ribosylation and aerial mycelium formation) and was mapped to a new locus near *uraA*. The *brgA* mutants were nonconditionally deficient in producing aerial mycelium and antibiotics, as determined by using various media, and had a morphological and physiological phenotype quite different from that of a *bldG* mutant carrying a mutation which was previously mapped near *uraA*. Among the known *bld* mutants, *bldA*, *bldD*, and *bldG* mutants exhibited a ADP-ribosylated protein profile similar to that of the wild type, while like mutant 27, *bldB*, *bldC*, and *bldH* mutants failed to ADP-ribosylate certain proteins.

The genus *Streptomyces* contains mycelial soil bacteria that are of interest because they produce antibiotics (physiological differentiation), usually in the process of aerial mycelium formation (morphological differentiation) when cultured on agar. *Streptomyces griseus* and *S. coelicolor* A3(2) are both well studied physiologically or genetically. The mechanism of coupling of morphological and physiological differentiation, however, remains to be clarified (for reviews, see references 2 and 3). The results of isolating and analyzing relaxed (*relC*) mutants of several *Streptomyces* spp. led Ochi (16) to propose that morphological differentiation results from a decrease in the GTP pool, whereas physiological differentiation results from a function of ppGpp (guanosine 5′-diphosphate 3′-diphosphate), whose synthesis depends on the *rel* gene function. More recently, Ochi et al. (19) stressed the potential significance of ADP-ribosylation of proteins in *S. griseus* differentiation.

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 12, 22, and 24). ADP-ribosylation can be considered a mode of metabolic regulation along with phosphorylation, adenylylation, uridylation, and methylation; the ADP-ribosylated protein may lose its activity but may later be reactivated by enzymic de-ADP-ribosylation. A possible role of ADP-ribosylation during development of the prokaryote *Myxococcus xanthus* has also been suggested (4). Mono-ADP-ribosylation is detected in prokaryotes and eukaryotes, while poly-ADP-ribosylation occurs only in eukaryotes. The best-studied ADPRTs are those for bacterial toxins such as cholera toxin and diphtheria toxin that catalyze the ADP-ribosylation of eukaryotic signal-transducing trimeric GTP-binding proteins (23). In contrast to the considerable knowledge of ADP-ribosylation in eukaryotes, little is known of prokaryotic ADP-ribosylation. The α subunit of RNA polymerase of *Escherichia coli* is known to be ADP-ribosylated as a result of T4 and N4 phage infections (22). Nitrogenase activity in *Rhodospirillum rubrum* and *Azospirillum lipofernum* is inactivated by ADP-ribosylation of the iron-containing subunit of the enzyme (7). Two cytoplasmic proteins of *Pseudomonas maltophilia* are reported to be ADP-ribosylated (5). The aim of the present study was to demonstrate the significance of endogenous ADP-ribosylation in morphological and physiological differentiation of *S. coelicolor* A3(2), genetically the most-studied streptomycete.

MATERIALS AND METHODS

Chemicals. 3-Aminobenzamide (ABA) and 3-methoxybenzamide (3-anisamide) were purchased from Nakarai Tesque, Kyoto, Japan. [*adenylate*-^{32}P]NAD and [*adenine*-2,8-^{3}H]NAD were from Amersham and ICN Biochemicals, respectively. ADP-ribose was from Sigma.

Bacterial strains and preparation of mutants. All strains used were derivatives of *S. coelicolor* A3(2) (Table 1). Strain 1147, mainly used in this study, is the wild-type, prototrophic, antibiotic-producing strain and harbors plasmids SCP1 and SCP2 (9). The strains used in genetic analysis were derivatives of strain 1147. The spontaneous ABA-resistant mutants were obtained as colonies that grew within 20 days after wild-type spores were spread on GYM agar (see below) containing 80 mM ABA. The parent strain was able to grow, although slowly, with 20 to 60 mM ABA, but its growth was completely suppressed with 80 to 100 mM. The growth-inhibitory effect of ABA was greatly affected by the amount of cells inoculated. The resistant clones were purified by sequential (three times) single-colony isolation.

Media and growth conditions. GYM medium contained (per liter) 4 g of glucose, 4 g of yeast extract, 10 g of malt extract, 1 g of peptone (NZ-amine, type A), and 2 g of NaCl (adjusted to pH 7.2 with NaOH). GYM agar contained 2% agar in addition to the above-specified components. For genetic crosses, R2YE medium (9), a complex buffered medium, was used. Minimal medium (9) was used for analyzing nutritional markers and for phenotypic analysis of *brg* mutants. In these experiments, glucose was replaced by other carbon sources at a final concentration of 1%. Since ABA and 3-methoxybenzamide are both heat stable, media containing them were autoclaved with all components present. All incubations were carried out at 30°C. Cells were grown on GYM agar plates covered with cellophane sheets, and at the indicated times the mycelia were

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TABLE 1. Strains of *S. coelicolor* A3(2) used in this study

Strain	Relevant genotype[a]	Source (reference)
1147	SCP1$^+$ SCP2$^+$ (prototrophic wild type)	D. A. Hopwood (9)
1258	*argA1 cysD18 hisA1 proA1 strA1 uraA1* SCP1^{NF} SCP2*	D. A. Hopwood (9)
J1700	*bldA39 hisA1 strA1 uraA1* SCP1$^-$ SCP2$^-$	K. Chater (13)
J669	*bldB43 agaA7 cysD18 mthB2* SCP1^{NF}	K. Chater (13)
J660	*bldC18 agaA7 cysD18 mthB2* SCP1^{NF}	K. Chater (13)
J774	*bldD53 cysA15 mthB2 pheA1 strA1* SCP1^{NF}	K. Chater (13)
C103	*bldG103 hisA1 strA1 uraA1* SCP1$^-$ SCP2$^-$	W. Champness (1)
C109	*bldH109 hisA1 strA1 uraA1* SCP1$^-$ SCP2$^-$	W. Champness (1)
27	*brgA1* SCP1$^+$ SCP2$^+$	Spontaneous ABA-resistant isolate from 1147 (this study)
27-1	*brgA1 proA1 strA1*	Conjugation between strains 27 and 1258 (this study)
1	*brgB1* SCP1$^+$ SCP2$^+$	Spontaneous ABA-resistant isolate from 1147 (this study)

[a] Abbreviations: SCP1, *S. coelicolor* plasmid 1; SCP2, *S. coelicolor* plasmid 2; NF, SCP1 is integrated into the chromosome; SCP2*, variant of SCP2 with enhanced sex factor activity; *aga*, inability to use agar as a carbon source; *brg*, resistance to ABA.

scraped off the cellophane layer. Nutritional requirements were added to GYM agar at 100 μg/ml when auxotrophic strains were cultivated.

Determination of antibiotics. Although massive aerial mycelium formation was detected on both GYM agar and R2YE agar, the latter medium was solely used for detection of the pigmented antibiotics (undecylprodigiosin and actinorhodin), since antibiotic production was higher on R2YE agar than on GYM agar.

Genetic mapping techniques. Crosses and analysis of data were carried out as described by Hopwood and Chater (10). Chromosomal transfer was mediated primarily by plasmid SCP1. The designation NF refers to the integrated state of SCP1, at 9 o'clock on the genetic map. Recombinants arose at a frequency of about 10^{-3}, as determined by analyzing spore progenies developed after 7 days of mixed culture on R2YE agar medium supplemented with 100 μg of each nutritional requirement per ml. Resistance to streptomycin was determined at a concentration of 5 μg/ml.

Assay of ADPRT activity. ADPRT was assayed with [*adenine*-2,8-^{3}H]NAD as described previously (19) except that the concentration of NAD used was 3 μM instead of 30 μM. The incubation time was 30 min, since apparent ADPRT activity decreased gradually after 30 min, perhaps because of enzymic de-ADP-ribosylation. The enzyme was stored without loss of activity for at least 3 days when cells were frozen at −20°C, but the activity decreased to less than 10% when crude extracts were left overnight at −20°C. The specific activity of ADPRT is expressed as the amount (disintegrations per minute) of radioactivity incorporated into acid-precipitable material per milligram of protein per minute. Labeled ADP-ribose was prepared in this laboratory from [*adenine*-2,8-^{3}H]NAD according to an unpublished method developed by J. C. Ensign. ^{3}H-labeled ADP-ribose thus prepared with partially purified *S. griseus* NAD-glycohydrolase was more than 99% pure.

To obtain autoradiograms of ADP-ribosylated proteins, 3 μM [*adenylate*-^{32}P]NAD (37 kBq) was used instead of [*adenine*-2,8-^{3}H]NAD. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out as reported by Penyige et al. (20). The gel was dried and exposed to X-ray film. The typical exposure time was 3 days at −80°C, using intensifying screens.

NAD-glycohydrolase was assayed as described previously (15).

Alkaline hydrolysis of ^{32}P-labeled proteins. Alkaline hydrolysis was carried out as described in detail previously (19).

Assay of nucleotide pools. Intracellular concentrations of ppGpp were assayed by high-pressure liquid chromatography as described earlier (15).

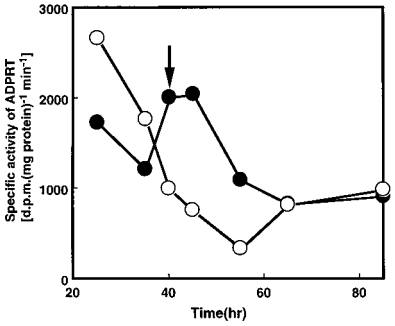


FIG. 1. Changes in ADPRT activity during growth of the parent strain 1147 (●) or mutant 27 (○) on GYM agar medium. The arrow indicates the time of onset of aerial mycelium formation. ADPRT activity was assayed by using [*adenine*-2,8-^{3}H]NAD (as a donor of ADP-ribose) as described previously (19) except that the final concentration of NAD was 3 μM instead of 30 μM.

RESULTS

ADPRT activity. ADPRT activity was detected in *S. coelicolor* A3(2) by incubating [*adenine*-2,8-^{3}H]NAD with a crude cell extract, which provides not only the enzyme but also substrate proteins to be ADP-ribosylated (endogenous ADP-ribosylation) (Fig. 1). Its activity was maximal in cells just beginning the developmental process, i.e., aerial mycelium formation. Virtually no activity (10% or less) was detected when NAD was replaced with ^{3}H-labeled ADP-ribose at the same concentration.

Unlike *S. griseus* (15), *S. coelicolor* A3(2) showed no activity of NAD-glycohydrolase.

Isolation and characterization of mutants. In preliminary experiments, only slight growth inhibition of *S. coelicolor* was observed on GYM agar and R2YE agar containing 20 to 50 mM ABA, whereas aerial mycelium formation and antibiotic production were both severely depressed (Fig. 2). ABA, an analog of nicotinamide, is a potent inhibitor of ADPRT of *S. griseus* (20). The possibility that some ABA-resistant mutants have an altered ADPRT affecting differentiation was assessed by isolating and analyzing such mutants, which developed spontaneously at a frequency of 10^{-8} to 10^{-9} when *S. coelicolor* 1147 was incubated at 30°C for 20 days on GYM agar medium containing 80 to 100 mM ABA. Of these mutants,

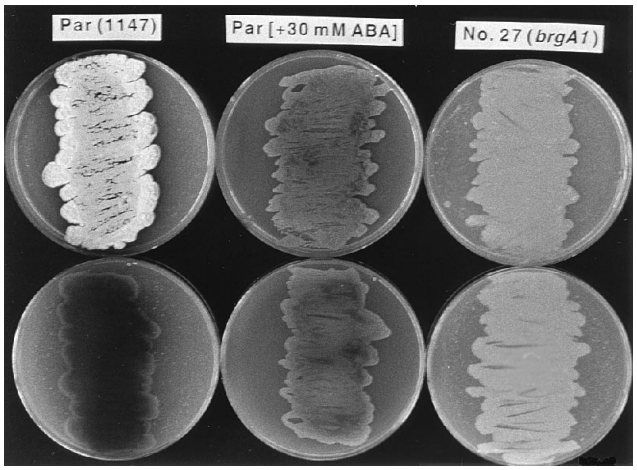


FIG. 2. Ability to produce aerial mycelia and antibiotic (actinorhodin) by strains of *S. coelicolor*. Spores or mycelia were spread on R2YE agar (plus or minus 30 mM ABA) and then incubated at 30°C for 5 days. The lower row shows the reverse sides of the plates. Par, parental strain.

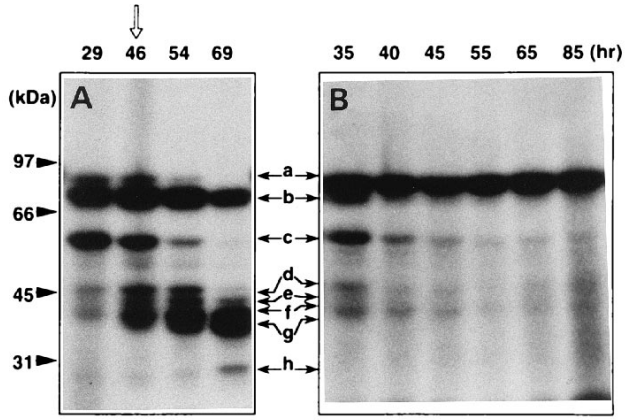


FIG. 3. Autoradiogram of SDS-polyacrylamide gels on which ADP-ribosylated proteins of parent strain 1147 (A) and mutant 27 (B) were separated. Crude extracts from cells grown for various times at 30°C on GYM agar medium covered with cellophane were incubated at 30°C for 30 min with [*adenylate*-^{32}P]NAD. Equivalent amounts of protein from the assay mixtures were analyzed by SDS-PAGE on a gel containing 8% (wt/vol) acrylamide. The gel was dried and exposed to X-ray film with an intensifying screen for 3 days at −80°C. Positions of molecular mass standards are shown at the left. Apparent molecular masses of labeled proteins designated by arrows a to h were 92, 78, 57, 46, 43, 41, 39, and 28 kDa, respectively. The numbers at the top indicate the ages of the cells examined. The open arrow indicates the time of aerial mycelium formation.

30% had a severely reduced ability to form aerial mycelium as well as the pigmented antibiotics undecylprodigiosin and actinorhodin. Mutants 27, 4, 7, and 10 were used for most further analyses since these mutants totally lacked aerial mycelium and antibiotic production (Fig. 2). They were resistant to up to 100 mM ABA. Also, the mutants showed resistance to another benzamide derivative, 3-methoxybenzamide; the growth of the parent strain was completely inhibited in the presence of 30 mM 3-methoxybenzamide, but the mutants were resistant up to 50 mM. As expected, mutant 27 revealed a pattern of changes in ADPRT activity during growth which is quite different from that of the parent strain (Fig. 1). The altered phenotype was defective regardless of cultivation temperature (between 20 and 37°C) or medium used (examined for 10 different media). Also, no restoration of aerial mycelium or antibiotic production was detected when glucose in the minimal medium was replaced by arabinose, galactose, glycerol, maltose, or mannitol. Mutant 27 accumulated as much ppGpp (487 pmol/mg [dry weight]) as the parent strain following a nutritional shift-down (17) but failed to produce A-factor and nucleotide-like substance as described earlier (17). Mutants 1, 9, 12, and 18 were resistant to up to 100 mM ABA, but unlike mutant 27, these mutants revealed normal phenotypes with respect to aerial mycelium and antibiotic production. Mutant 1 was used for further study as a reference for mutant 27.

ADP-ribosylation of cellular proteins. Changes in the ADP-ribosylation profile during growth on GYM agar medium were examined by incubating crude extracts with [^{32}P]NAD. The results of SDS-PAGE analyses of labeled cellular proteins are shown in Fig. 2. The profile of ADP-ribosylated proteins changes dramatically in the parent strain 1147 during culture development (Fig. 3A). It is noteworthy that proteins of 46 kDa (band d), 43 kDa (band e), 41 kDa (band f), and 39 kDa (band g) appear at the onset of differentiation (46 h). A 28-kDa protein (band h) was detected only at a late developmental stage. Strikingly, none of these labeled proteins was detected in mutant 27 (Fig. 3B), reflecting the observed low ADPRT activity in the mutant (Fig. 1). Failure to ADP-ribosylate these proteins was detected in mutants 4, 7, and 10, which were impaired in the ability to produce aerial mycelium and antibiotic. In contrast, ABA-resistant mutants 1, 9, 12, and 18, with normal phenotypes like that of the parent strain, revealed a labeled protein profile of the parental type (data not shown), indicating a good correlation between the pattern of labeled protein profile and developmental ability.

The significance of protein ADP-ribosylation for development was further stressed by the fact that the cells grown with subinhibitory amounts (20 to 60 mM) of ABA failed to ADP-ribosylate certain proteins (Fig. 4), in addition to having defects in producing aerial mycelium and antibiotics (see above). Also, de-ADP-ribosylation (or absence of protein per se) of a 92-kDa protein was detected in the parental strain but not in mutant 27.

Identification of a labeled modifying group. As shown by chase experiments, a 1,000-fold molar excess of unlabeled NAD but not ATP completely abolished the radioactive labeling of any proteins, eliminating a possibility of phosphorylation (data not shown). Furthermore, the labeled modifying group was identified as ADP-ribose by subjecting a preparation of ^{32}P-labeled proteins to alkaline treatment and thin-layer chromatography analysis (Fig. 5). Accordingly, we propose that the appearance of ADP-ribosylated proteins is age dependent and, for some of them (46, 43, 41, and 39 kDa), correlated with early stages of the developmental processes.

Mapping of mutants. An ABA resistance mutation was previously designated *aba* (16), but this symbol had already been used for mutations of an antibiotic biosynthesis activator gene (6). Therefore, we assigned a new symbol, *brg* (benzamide-derivative resistance gene), to the ABA resistance mutation. The *brg* mutation in mutant 27 was genetically analyzed. Preliminary experiments showed that it is tightly associated with all of the altered phenotypes. To map *brg* on the chromosome, a cross was performed between mutant 27 (*brgA1*) and strain 1258 (*proA1 hisA1 argA1 cysD18 uraA1 strA1*). After selection for the recombinants carrying *strA* and *his*$^+$, the other markers were scored. Allele frequencies (Fig. 6) indicated that *brgA1* mapped either close to *uraA* or near *cysD*. A position clockwise of *uraA* is clearly indicated by the 2-by-2 tabulations showing that segregation of *brgA* was highly dependent on that of *uraA* (with a marked deficiency of the *ura*$^+$ *brg*$^+$ genotype that would require multiple crossing-over) but clearly independent of that of *cysD*.

We also attempted to cross strain 27-1 (*proA1 strA1 brgA1*) with other ABA-resistant mutants with an impaired ability to differentiate (mutants 4, 7, and 10). All of the recombinants

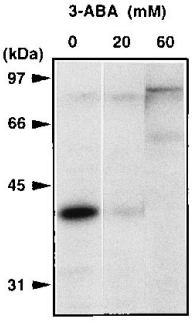


FIG. 4. Autoradiogram of an SDS-polyacrylamide gel of ADP-ribosylated proteins. *S. coelicolor* A3(2) parent strain 1147 was grown for 60 h on GYM agar or for 66 h on GYM agar containing 20 or 60 mM ABA. Crude extracts from cells were prepared, and the ADP-ribosylation reaction was performed as described in the legend to Fig. 3.

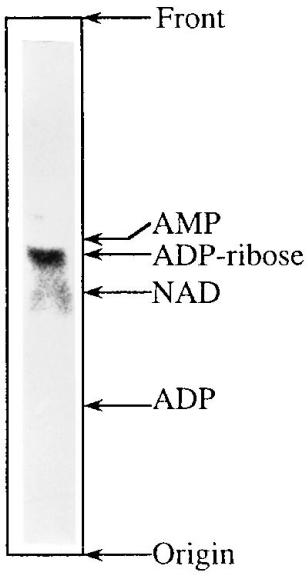


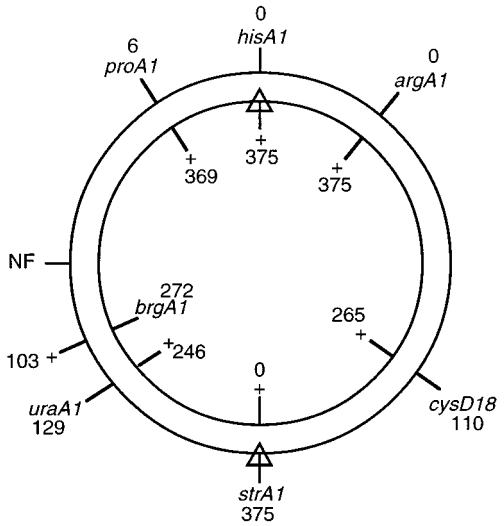
FIG. 5. Thin-layer chromatography analysis and autoradiography of alkaline hydrolysates of proteins previously incubated with [^{32}P]NAD. ^{32}P-labeled proteins of a crude cell extract were precipitated with trichloroacetic acid, washed, and incubated in 0.1 N-KOH at 27°C for 3 h.

tested (more than 150 for each cross), selected for *strA* and *pro*$^+$, were defective in aerial mycelium formation and antibiotic production. Thus, all or most *brg* mutations appear to be closely linked and may be in the same locus *brgA* (although this result could also be obtained if the ABA resistance mutations were located between *proA* and *brgA*). In contrast, another class of ABA-resistant mutant (mutant 1), which produced aerial mycelium and antibiotics as well as the parent strain, had a mutation in a different gene because a cross between strain 27-1 and mutant 1 gave rise to ABA-sensitive recombinants at a frequency of 25%. This mutation therefore identified a second locus, *brgB*. Mapping of *brgB* was not done.

In a cross between mutant 27 (*brgA1*) and strain 1258 (*proA1 hisA1 argA1 cysD18 uraA1 strA1*), ABA-resistant recombinants all exhibited an impaired ability to produce aerial mycelium and antibiotics, while ABA-sensitive recombinants all exhibited a normal phenotype. The ABA-resistant recombinants (examined for four strains) revealed a failure to ADP-ribosylate the proteins of 46, 43, 41, and 39 kDa, which were ADP-ribosylated normally in the ABA-sensitive recombinants (examined for two strains). These results strengthen further the correlation between the altered phenotypes and failure to ADP-ribosylate certain proteins.

Comparisons of *brgA* mutants with other bald mutants. In *S. coelicolor* A3(2), mutants which fail to form aerial mycelium have been isolated, and some of them also have defects in antibiotic production. Such mutations, named *bld* (for bald), include *bldA*, *bldB*, *bldC*, *bldD*, *bldG*, and *bldH*, which have all been mapped (1, 2). Although the *brgA* mutants have several characteristics in common with these *bld* mutants, the map position of *brgA* is clearly different from those for *bldA*, *bldB*, *bldC*, *bldD*, or *bldH*. Only *bldG* (mapping near 9 o'clock) is near *brgA*. However, *brgA* and *bldG* mutants differed in phenotype as compared in parallel experiments. The *brgA* mutants were blocked in aerial mycelium formation irrespective of carbon source, while aerial mycelium formation of *bldG* mutant depended on the carbon source as reported previously (1). Moreover, the colony surface morphology of *bldG* mutants was soft and smooth (1), while *brgA* mutants had a hard, yellowish morphology. Location of *brgA* and *bldG* to distinct map positions was confirmed by the fact that a cross between strains 27 (*brgA*) and C103 (*bldG103 hisA1 strA1 uraA1*) (selected for *strA* and *ura*$^+$) gave rise to recombinants with normal ability to produce aerial mycelium and antibiotics at a frequency of 15 to 20%. On the other hand, the incidence of such parental type recombinants was low (1% or less) when selection was for *strA* and *his*$^+$, indicating the gene order *uraA-bldG-brgA-hisA*. None of the *bldA*, *bldB*, *bldC*, *bldD*, *bldG*, and *bldH* mutants was resistant to ABA. It is therefore concluded that *brgA* is a new pleiotropic regulatory locus which plays an essential role in the early developmental process.

ADP-ribosylation in various bald mutants. Since *brgA* mapped near *bldG*, we first investigated the ADP-ribosylated protein profile of a *bldG* mutant. As Fig. 7 shows, the *bldG* mutant (strain C103) displayed the ADP-ribosylated protein profile of the parent type, ADP-ribosylating the 46-, 43-, 41-, and 39-kDa proteins. Interestingly, *bldA* and *bldD* mutants also displayed the profile of the parent type, while *bldB*, *bldC*, and *bldH* mutants failed to ADP-ribosylate certain proteins (Fig. 8). Instead, the *bldB* mutant showed a highly intensified band with a molecular size of 25 kDa. This band may represent phosphorylation of a protein, since use of ^{32}P-labeled ATP instead of NAD resulted in a labeled band at that position. Failure of ADP-ribosylation in *bldB*, *bldC*, and *bldH* mutants was confirmed by using cells of various growth phases (45 to 70 h of age). It should be noted that the *bldH* mutant failed completely to ADP-ribosylate any proteins throughout the cell aging process (data not shown).



	uraA1	ura$^+$		cysD18	cys$^+$
brgA1	30	242	brgA1	72	200
brg$^+$	99	4	brg$^+$	38	65

FIG. 6. Mapping of *brgA*. Strain 27 (*brgA1*; inner circle) was crossed with strain 1258 (*proA1 hisA1 argA1 cysD18 uraA1 strA1* SCP1^{NF} SCP2*; outer circle). Selection was for recombinants with insertions of *strA* and *his*$^+$ (triangles). Numbers around the circles indicate allele frequencies among the 375 recombinants scored. Segregation of *brgA* with respect to *uraA* and *cysD* is tabulated.

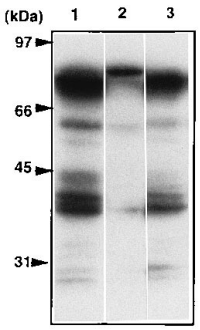


FIG. 7. Comparison of ADP-ribosylated protein profile between parent strain 1147 (lane 1), *brgA* mutant 27 (lane 2), and *bldG* mutant C103 (lane 3). Strains were grown on GYM agar for 60 h, and the ADP-ribosylation reaction was performed as described in the legend to Fig. 3.

DISCUSSION

The data presented in this report provide evidence for the significance of ADP-ribosylation of certain proteins for morphological and physiological differentiation of *S. coelicolor* A3(2), as was pointed out in an earlier report (19) on *S. griseus*. Apparently, *brgA* is a new pleiotropic regulatory locus which plays an essential role in the early developmental process, conceivably by modifying the activity of proteins via ADP-ribosylation. This is an interesting new angle on *Streptomyces* differentiation.

In *S. griseus*, ADP-ribosylation of three proteins (37, 39, and 44 kDa) has been suggested to be involved in the developmental process (19). These proteins may be homologous to the *S. coelicolor* proteins with similar molecular sizes (39, 41, 43, and 46 kDa), which were ADP-ribosylated at the onset of development (Fig. 3A). As will be reported elsewhere, certain ABA-resistant mutants of *Bacillus subtilis* also fail to ADP-ribosylate several proteins in addition to being defective in forming endospores and antibiotics (10a). De-ADP-ribosylation of a 92-kDa protein at the onset of differentiation in the parental strain should also be noted, since de-ADP-ribosylation of the protein was not detected in developmental mutant 27 (Fig. 3). Thus, ADP-ribosylation or de-ADP-ribosylation of certain proteins may play a role in developmental process of many microorganisms.

Although *brgA* mutants were selected as ABA resistant and had a reduced ADPRT activity, it remains to be clarified, by cloning and subsequent analyses, whether the *brgA* gene encodes the ADPRT enzyme. 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. Specifically, 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 substrates 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 *bld* mutants. In addition to cloning *brgA*, it is essential to clone the genes encoding ADP-ribosylatable proteins. We are now isolating the ADP-ribosylated proteins with molecular masses of 39, 41, and 92 kDa (21a). High activity of NAD-glycohydrolase, which hydrolyzes NAD, releasing nicotinamide and ADP-ribose, was detected in *S. griseus* (15), but ADP-ribose was not an efficient substrate (21a). *S. coelicolor* A3(2) not only showed a lack of NAD-glycohydrolase activity but also failed to use ADP-ribose as an efficient substrate (this study). Therefore, even if NAD-glycohydrolase enzyme existed in *S. coelicolor* A3(2), it would not play a role in ADP-ribosylation.

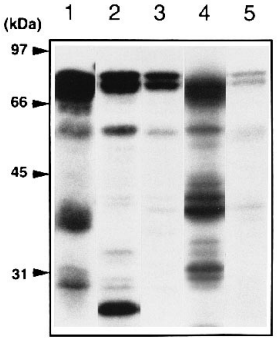


FIG. 8. ADP-ribosylated protein profiles of various *bld* mutants. Strains were grown on GYM agar for 60 to 70 h, and the ADP-ribosylation reaction was performed as described in the legend to Fig. 3. Lanes: 1, strain J1700 (*bldA*); 2, strain J669 (*bldB*); 3, strain J660 (*bldC*); 4, strain J774 (*bldD*); 5, strain C109 (*bldH*).

Of particular interest is the fact that *bldB*, *bldC*, and *bldH* mutants failed to ADP-ribosylate several proteins, which were normally ADP-ribosylated in the parent strain and *bldA* and *bldD* mutants (Fig. 8). *bldB* mutants show weak aerial mycelium and antibiotic production only after prolonged incubation, irrespective of carbon source (13), and the *bldB* gene was cloned previously (21). *bldC* mutants produce antibiotics normally but are deficient in aerial mycelium formation (13). The ability to form aerial mycelium is restored by a diffusible factor from sporulating cultures (13). *bldH* mutants are deficient in both aerial mycelium and antibiotic production in the presence of glucose, but both defects are corrected by replacing glucose with mannitol (1). Although it is difficult at present to discuss comprehensively the roles of such genes in light of a possible role of protein ADP-ribosylation, it can be proposed that certain *bld* genes (*bldB*, *bldC*, and *bldH*) exert their effects on the initiation of the developmental process, at least in part via protein ADP-ribosylation. Alternatively, it is also possible that the changes in protein ADP-ribosylation in some *bld* mutants are secondary effects.

Aerial mycelium formation by *S. coelicolor* A3(2) is suppressible in the presence of excess nutrients, but its suppression is released by adding decoyinine, a specific inhibitor of GMP-synthetase, leading to a lowered GTP pool (18). Also, the existence of GTP-binding proteins in *S. coelicolor* A3(2) (14) and *S. griseus* (11) was reported recently. It will therefore be intriguing to clarify how the GTP-regulated system and the ADP-ribosylation system cooperate to promote the developmental processes.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Basic Research Core System.

We express our sincere thanks to D. A. Hopwood and J. C. Ensign for valuable discussions throughout the study and K. Chater and W. Champness for the generous gift of *S. coelicolor* strains.

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