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ATP-Binding Cassette Transport System Involved in Regulation of Morphological Differentiation in Response to Glucose in *Streptomyces griseus*

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Streptomyces griseus **NP4, which was derived by UV mutagenesis from strain IFO13350, showed a bald and wrinkled colony morphology in response to glucose. Mutant NP4 formed ectopic septa at intervals along substrate hyphae, and each of the compartments developed into a spore which was indistinguishable from an aerial spore in size, shape, and thickness of the spore wall and in susceptibility to lysozyme and heat. The ectopic spores of NP4 formed in liquid medium differed from "submerged spores" in lysozyme sensitivity. Shotgun cloning experiments with a library of the chromosomal DNA of the parental strain and mutant NP4 as the host gave rise to DNA fragments giving two different phenotypes; one complementing the bald phenotype of the host, and the other causing much severe wrinkled morphology in the host. Subcloning identified a gene (*dasR*) encoding a transcriptional repressor belonging to the GntR family that was responsible for the reversal of the bald phenotype and a gene (*dasA*) encoding a lipoprotein probably serving as a substrate-binding protein in an ATP-binding cassette (ABC) transport system that was responsible for the severe wrinkled morphology. These genes were adjacent but divergently encoded. Two genes, named *dasB* and *dasC*, encoding a membrane-spanning protein were present downstream of *dasA*, which suggested that *dasRABC* comprises a gene cluster for an ABC transporter, probably for sugar import. *dasR* was transcribed actively during vegetative growth, and *dasA* was transcribed just after commencement of aerial hypha formation and during sporulation, indicating that both were developmentally regulated. Transcriptional analysis and direct sequencing of *dasRA* in mutant NP4 suggested a defect of this mutant in the regulatory system to control the expression of these genes. Introduction of multicopies of *dasA* into the wild-type strain caused ectopic septation in very young substrate hyphae after only 1 day of growth and subsequent sporulation in response to glucose. The ectopic spores of the wild type had a thinner wall than those of mutant NP4, in agreement with the observation that the former was sensitive to lysozyme and heat. Disruption of the chromosomal *dasA* or *dasR* in the wild-type strain resulted in growth as substrate mycelium, suggesting an additional role of these genes in aerial mycelium formation. The ectopic septation and sporulation in mutant NP4 and the wild-type strain carrying multicopies of *dasA* were independent of a microbial hormone, A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), that acts as a master switch of aerial mycelium formation and secondary metabolism.**

The gram-positive, soil-inhabiting, filamentous bacterial genus *Streptomyces* shows complex morphological differentiation, which makes this genus one of the model prokaryotes for studying multicellular differentiation (7, 9). On agar medium, one or more substrate hyphae formed from a germinating spore branch frequently and grow rapidly by cell wall extension at the hyphal tips. Filamentous development is strong, and septation in the substrate mycelium occurs very rarely. Subsequently aerial hyphae emerge by reuse of material assimilated into the substrate mycelium, such as DNA, proteins, and storage compounds. Many cells in substrate hyphae thus lyse and die (67). When apical growth of aerial hyphae stops, in contrast to substrate mycelium, septa are formed at regular intervals along the hyphae to form many unigenomic compartments within a sheath composed of elongated hollow or grooved elements, finer fibrillar elements, and amorphous material.

The sporulation septa consist of two membrane layers separated by a double layer of cell wall material, which permits the eventual separation of adjacent spores (14, 17, 38, 68). Spore chains usually consist of many tens of spores. The aerial spores thus formed are resistant to heat treatment and lysozyme digestion. Some *Streptomyces* strains produce spores in submerged culture when critical nutritional and environmental conditions are met (10, 12, 32, 41). *Streptomyces griseus* B-2682, for example, produces abundant submerged spores in nutrient-depleted media (32). The submerged spores of *S. griseus* are similar but never identical to aerial spores and are sensitive to lysozyme digestion, probably because of the thinness of the spore wall.

The combination of morphological mutants and gene cloning techniques is useful for the study of morphological differentiation, giving insight into structural and regulatory genes important for the developmental processes. Many *bld* mutants lacking an aerial mycelium and *whi* mutants lacking spores

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have facilitated isolating such genes from *Streptomyces coelicolor* A3(2) (7, 40). *whiG,* which encodes an RNA polymerase σ factor resembling the motility σ factor of *Bacillus subtilis,* is one of those genes and determines the timing of sporulation (8, 39). Interestingly, overexpression of *whiG* causes the host to form septa even in substrate mycelium, resulting in occasional ectopic sporulation in the substrate hyphae. Ectopic sporulation also results from deletion of a region close to the *glkA* locus (31), from overexpression of *ssgA* encoding a small acidic protein (29, 62), and from a mutation in *ssfR* encoding an *iclR*-type transcriptional regulator for *ssgA* (26). Thus, an increase and decrease of expression of the genes involved in regulation of the programmed, ordered developmental processes yields morphologically deprogrammed mutants.

We have isolated an *S. griseus* mutant, NP4, that shows marked ectopic sporulation in substrate hyphae in response to glucose both on agar and in liquid media. Shotgun cloning experiments with this mutant as the host revealed a gene cluster encoding an ATP-binding cassette (ABC)-type transporter and a transcriptional factor. Some ABC transporters have been adapted to transport specific regulatory molecules and, hence, regulate cell physiology. Examples are the Spo0K system of *B. subtilis* (47, 50), which mediates the uptake of a regulatory peptide and is required for the initiation of sporulation; the BldK system of *S. coelicolor* A3(2) (43), which probably imports an oligopeptide and is required for aerial mycelium formation; and the AmfAB system, which is probably involved in transport of some substance and aerial mycelium formation in *S. griseus* (61) and *S. coelicolor* A3(2) (34).

In the present study, overexpression of one of the cloned ABC transporter genes in the wild-type *S. griseus* strain caused ectopic sporulation in the substrate hyphae in response to glucose. The ectopic spores of the wild-type strain harboring multicopies of one of the transporter genes differed in various aspects from those formed by mutant NP4 on glucose-containing medium. The ectopic sporulation in the wild type was A-factor independent, which shows that the ectopic septation and subsequent development into spores occur independently of the A-factor regulatory cascade. A-factor is a representative of γ-butyrolactones serving as microbial hormones in a wide variety of *Streptomyces* spp., and the chemical structure of A-factor in *S. griseus* is 2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone (20–22). It switches on aerial mycelium formation and secondary metabolism at a concentration as low as 10^{-9} M in *S. griseus*. An A-factor-deficient mutant *S. griseus* strain neither forms aerial mycelium nor produces streptomycin.

We thus expected that the *S. griseus* mutant showing ectopic sporulation would give useful information on morphological development. The purposes of this study were (i) to characterize the *S. griseus* mutant strain forming ectopic spores by septation in substrate hyphae, (ii) to clone and characterize the genes that altered the phenotype of the mutant strain, (iii) to characterize the ectopic spores formed by the mutant and similar but not identical ectopic spores formed by the wild-type strain carrying multicopies of one of the cloned ABC transporter genes by scanning and transmission electron microscopy, and (iv) to analyze transcription of the ABC transporter gene cluster.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. griseus* IFO13350 (13) was obtained from the Institute of Fermentation (IFO), Osaka, Japan. *S. griseus* HH1 is A-factor deficient due to the deletion of *afsA* (23). *S. griseus* NP4 was derived from the wild-type strain IFO13350 by UV mutagenesis. *S. griseus* Δ*adpA*, which had a deletion in *adpA,* encoding an A-factor-dependent transcriptional activator (45), and *S. griseus* Δ*adsA*, which has a deletion in *adsA,* encoding an extracytoplasmic function σ factor of RNA polymerase (70), were described previously.

Streptomyces strains were grown in YMPD medium (yeast extract [Difco], 0.2%; meat extract [Wako Pure Chemicals], 0.2%; Bacto-peptone [Difco], 0.4%; NaCl, 0.5%; $MgSO_4 \cdot 7H_2O$, 0.2%; glycine, 0.5%; and glucose, 1%). YMPD agar medium contained 2% agar. Phenotypes of *S. griseus* strains were examined at 28°C on YMP agar and in YMP liquid medium containing various sugars at a concentration of 1% instead of glucose. Minimal medium for *S. griseus* has been described (42). Thiostrepton (50 μg/ml) and neomycin (20 μg/ml) were added when necessary. R2YE medium (19) was used for regeneration of protoplasts. A-factor production was assayed by the streptomycin cosynthesis method with the A-factor-deficient *S. griseus* mutant HH1, as described (23).

As *Streptomyces* plasmids, high-copy-number plasmids pIJ486, containing the kanamycin and thiostrepton resistance genes (66), and pIJ702, containing the melanin production and thiostrepton resistance genes (28), both with copy numbers of 40 to 100 per genome, were used. A low-copy-number plasmid, pKU209, with a copy number of 1 to 2 per genome, containing the ampicillin and thiostrepton resistance genes (27), was also used.

Escherichia coli JM109 and pUC19 (71) for DNA manipulation were purchased from Takara Shuzo. *E. coli* JM110 containing *dam* and *dcm* mutations was used for preparing nonmethylated *Streptomyces* DNA used for gene disruption. Media and growth conditions for *E. coli* were those described by Maniatis et al. (35). Ampicillin (50 μg/ml) and kanamycin (20 μg/ml) were used when necessary.

General recombinant DNA studies. Restriction enzymes, T4 DNA ligase, reverse transcriptase, and other DNA-modifying enzymes were purchased from Takara Shuzo. [α-^{32}P]dCTP (110 TBq/mmol) for DNA labeling with the Takara *Bca*Best DNA labeling system and [γ-^{32}P]ATP (220 TBq/mmol) for end labeling at 5′ ends with T4 polynucleotide kinase were purchased from Amersham Pharmacia Biotech. DNA was manipulated in *Streptomyces* (19) and in *E. coli* (3, 35), as described earlier. Nucleotide sequences were determined by the dideoxy chain termination method (51) with the Termo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham) or the DNA sequencing kit (ABI Prism) on an automated DNA sequencer. Open reading frames in the nucleotide sequence were predicted by Frame Plot analysis (25).

Shotgun cloning. Chromosomal DNA of the wild-type *S. griseus* IFO13350 was partially digested with *Sau*3AI and ligated with either *Bam*HI-digested pIJ486 or *Bgl*II-digested pIJ702. The ligation mixture was introduced by protoplast transformation into *S. griseus* NP4 cells that showed a bald phenotype. Thiostrepton-resistant transformants were selected on YMPD medium, and seven sporulating colonies were isolated. A colony showing a severely wrinkled morphology was also isolated. The seven sporulating colonies contained a common region, and one of them (plasmid pNS5), which carried a 6-kb insert on pIJ702, was chosen for further study. The wrinkled colony contained a plasmid, named pES1, which carried a 2.7-kb insert on pIJ486.

Subcloning. A 1,379-bp *Hin*cII fragment containing *dasR* (see Fig. 4A) was cloned in the *Hin*cII site in the multilinker of pUC19. The *dasR* sequence was excised as a *Hin*dIII-*Bam*HI fragment and placed between the *Hin*dIII and *Bam*HI sites of pIJ486 to construct pHR3. The *dasR* sequence, excised as a *Hin*cII fragment, was cloned into the *Eco*RI site of pKU209 (plasmid pLR3) after the *Eco*RI site had been flush-ended with Klenow fragment. A 1,801-bp *Eco*RI fragment containing the whole *dasA* sequence was placed in the *Eco*RI site of pKU209 to construct pLA1. A 3.2-kb *Kpn*I-*Sac*I fragment containing the truncated *orf1*, *orf2*, and *orf3* was cloned between the *Kpn*I and *Sac*I sites of pUC19. This fragment was excised as an *Eco*RI-*Hin*dIII fragment and placed between the *Eco*RI and *Hin*dIII sites of pIJ486 to construct pKS3. A 3-kb *Sac*I fragment containing *dasB* and *dasC* was flush-ended with Klenow fragment and cloned in the *Hin*cII site of pUC19. The *dasBC* sequence was excised as a *Bam*HI-*Hin*dIII fragment and placed in pIJ486 to construct pHBC1. A 1.7-kb *Nru*I fragment containing the *dasB* sequence was similarly cloned in pIJ486 to construct pHB1.

Gene disruption. For disruption of the chromosomal *dasA* gene, the upstream and downstream regions of *dasA* were amplified by PCR with the following primers. The upstream region of about 2 kb in size (see Fig. 4A) was amplified with 5′-GAAGCTTGCTGAAGAGACAGCGCCGTCA-3′ (the underlining in-

dicates a *Hin*dIII site) and 5′-GGATCC*GTTAAC*ACGATGAGCTTGCGCTT **CAC**G-3′ (the underlined and italic letters indicate a *Bam*HI and *Hpa*I site, respectively; the boldface letters indicate the start codon of *dasA*). The downstream region was amplified with 5′-*GTTAAC*GATCAACGCGCTGATCAAC AACAAG-3′ (corresponding to the region 6-bp upstream of the stop codon of *dasA*) and 5′-GGATCCGTCAGGGTGTCCGTGGTGGAAACG-3′. The *Hin*dIII-*Hpa*I fragment from the upstream region, the *Hpa*I-*Bam*HI fragment from the downstream region, and pUC19 DNA digested with *Hin*dIII plus *Bam*HI were ligated by three-fragment ligation. The *Hin*cII-*Sma*I fragment containing the kanamycin resistance gene (4) was then inserted in the *Hpa*I site of the pUC19 recombinant plasmid. In the resultant plasmid, most of the *dasA* sequence was replaced by the kanamycin resistance gene. The plasmid was linearized by *Dra*I digestion, alkali denatured with 0.1 M NaOH, and introduced by transformation into *S. griseus* IFO13350, as described (44). Correct insertion of the kanamycin resistance gene by homologous recombination was checked by Southern hybridization with the 0.6-kb *Hin*cII fragment containing the *dasA* sequence and the 1.3-kb *Hin*cII-*Sma*I fragment containing the kanamycin resistance gene as probes against the *Eco*RI-digested chromosomal DNA.

For disruption of the chromosomal *dasR* gene, the kanamycin resistance gene was inserted in the *Kpn*I site within the *dasR* sequence (see Fig. 4A). The disrupted *dasR* sequence, together with the 1.7-kb upstream and 3.2-kb downstream regions, was placed in pUC19. The pUC19 plasmid linearized by *Dra*I digestion was similarly introduced in *S. griseus* IFO13350. Correct disruption of the *dasR* gene was checked by Southern hybridization with the 1.4-kb *Hin*cII fragment containing the *dasR* sequence and the 1.3-kb *Hin*cII-*Sma*I fragment containing the kanamycin resistance gene as probes against the *Hin*cII-digested chromosomal DNA.

S1 nuclease mapping. Methods for RNA isolation from cells grown on cellophane on the surface of agar medium and S1 nuclease mapping were described by Kelemen et al. (30). Hybridization probes were prepared by PCR with a pair of ^{32}P-labeled and nonlabeled primers. For *dasA*, 5′-GGTCACGGGTCGTCG CCCTCG-3′ (corresponding to positions −105 to −85, taking the transcriptional start point of *dasA* as +1, which was determined later) and 5′-GGTCTTCCTT GCGGTCCTTCG-3′ (corresponding to positions +212 to +196) were used. For *dasR*, 5′-GGTCTTTAATGGTTTAGACCAGTACCG-3′ (corresponding to positions −142 to −115, taking the transcriptional start point of *dasR* as +1) and 5′-GTCCGTCATGTCGAGGAGATGGC-3′ (corresponding to positions +167 to +144) were used. *hrdB,* encoding a σ factor of RNA polymerase, was used to check the purity and amount of RNA used, as described previously (70). Protected fragments were analyzed on 6% polyacrylamide sequencing gels by the method of Maxam and Gilbert (37).

RT-PCR. Transcription of the *dasABC* region was analyzed by reverse transcription (RT)-PCR with pairs of the following primers (see Fig. 8C): A1 (5′-**GTG**AAGCGCAAGCTCATCGTGG-3′; the boldface letters indicate the start codon of *dasA*) and A2 (5′-G**TCA**GGATTCCTTGTTGATCAGCG-3′; the boldface letters indicate the stop codon of *dasA*); AB1 (5′-GCCGCTACTGGT ACGCCGCGATG-3′, corresponding to Arg-203 to Met-209 of DasA) and AB2 (5′-GTTCCTTGGGGACCTGGGTGAGG-3′, corresponding to Leu-228 to Glu-222 of DasB); and B1 (5′-**GTG**TCTGCCGCTGATACCAAGGCCG-3′; the boldface letters indicate the start codon of *dasB*) and BC2 (5′-GCA**CTA**TCCC TTCACAGCGCCGGAAG-3′; the boldface letters indicate the stop codon of *dasC*).

Microscopy. For light microscopy, *S. griseus* strains IFO13350 and NP4 were cultured at 28°C in YMPD medium. Cells were harvested at intervals and placed with oil immersion under a phase-contrast light microscope (Zeiss Axiophot 2).

For scanning electron microscopy (59), *S. griseus* strains were grown on YMPD agar medium at 28°C for 4 days, and agar blocks containing the spores and hyphae were cut. For preparation of the specimens, the agar blocks were fixed with 2% osmium tetroxide for 24 h and then dehydrated by air-drying for 1 h. Each specimen was sputter coated with platinum-gold and examined with a Hitachi S4000 scanning electron microscope.

For transmission electron microscopy, *S. griseus* strains were grown on YMPD agar medium at 28°C for 2 to 4 days. A colony was scraped and prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 2 to 5 h. The samples were washed three times with 0.05 M potassium phosphate buffer (pH 7.0) and fixed with 1% osmium tetroxide in 0.05 M potassium phosphate buffer for 12 to 14 h. The samples were washed with water three times, prestained with 0.5% uranyl acetate for 2 h, and dehydrated with 70%, 80%, 90%, and absolute ethanol and finally with acetone. The samples were encapsulated in Spurr's resin, and sections were stained with 3% uranyl acetate and then with lead citrate for 2 h. The sections were placed under a JEOL 2010 electron microscope at 100 kV.

Heat and lysozyme resistance tests. *S. griseus* strains were precultured at 28°C for 24 h in YMPD liquid medium. About 1 mg of mycelium was inoculated to a petri dish (diameter, 9 cm) containing YMPD agar medium and further incubated at 28°C. At day 2, when spores were not yet formed, mycelium was transferred to 1 ml of 20 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (pH 7.2) and suspended. The mycelium suspension was divided into three parts: one was a control with no treatments, the second was heated at 55°C for 30 min, and the third was incubated at 37°C for 1 h in the presence of 20 μg of egg white lysozyme (Seikagaku Kogyo) per ml. After the treatments, portions of the suspensions were homogenized with a glass homogenizer and spread on YMPD medium, and colonies were counted after incubation at 28°C for 2 days. At day 5, when abundant spores were formed, heat and lysozyme resistance was similarly examined.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited to the DDBJ, EMBL, and GenBank DNA databases under accession no. AB061860.

RESULTS

Properties of *S. griseus* mutant NP4. We have long studied the regulation of morphological development and secondary metabolism in streptomycin-producing *S. griseus* IFO13350, especially in relation to a chemical signaling molecule, A-factor. During these studies, we found mutant NP4, showing a wrinkled and ragged colony shape among UV-mutagenized colonies (Fig. 1A). The defect in aerial mycelium formation of mutant NP4 did not result from A-factor deficiency, since NP4 was found to produce A-factor when assayed by the streptomycin cosynthesis method (23) (data not shown). Mutant NP4 produced little streptomycin when assayed by the bioassay with *B. subtilis* ATCC 6633 as an indicator. This is probably due to the cessation of development. The morphological defect in mutant NP4 was glucose dependent, because the defect was observed only on YMPD medium containing glucose as the carbon source and not on YMP medium containing maltose, galactose, mannitol, or glycerol instead of glucose (data not shown). Mutant NP4 showed normal morphological development culminating in sporulation on the latter media. As will be described in detail below, the effects of *dasA* overexpression on the wild-type strain were also glucose dependent.

Scanning electron microscopy revealed that mutant NP4 grew as substrate mycelium for 4 days, in which septa were subsequently formed at regular intervals (Fig. 1B). Septation in substrate mycelium occurs very rarely in the genus *Streptomyces*. In the visible field, some aerial hyphae and chains of spores developed normally from the substrate hyphae were also seen. Each compartment separated by the septa in substrate hyphae developed into a spore, which was indistinguishable in size, shape, and thickness of the spore coat from the aerial spore of the parental *S. griseus* strain IFO13350, as determined by transmission electron microscopy (Fig. 1B). The spores examined were those formed in the substrate hyphae that penetrated the agar. Consistent with the morphology of the ectopic spores from mutant NP4, they were resistant to heat and lysozyme to nearly the same extent as the aerial spores (Fig. 2). The septum is therefore supposed to consist of two separate cross walls, as is observed in sporulation septum formation and arthrospore maturation in *S. griseus* (14).

S. griseus IFO13350 rarely forms submerged spores in YMPD liquid medium, although a different *S. griseus* strain, B-2682, forms abundant submerged spores (32). When mutant NP4 was cultured in YMPD liquid medium, it grew with the same time course as the wild-type strain, but at the beginning of the stationary phase the cell mass suddenly dropped (Fig. 3A). The sudden decrease in the cell mass coincided with the

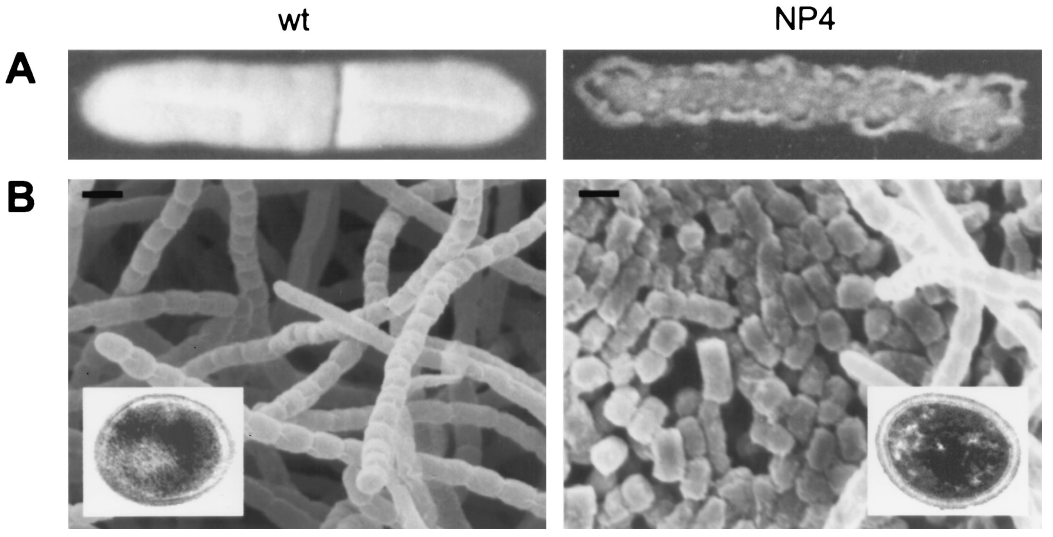


FIG. 1. Morphology of *S. griseus* NP4 on solid medium. (A) Mycelium of mutant NP4 and the wild-type (wt) strain *S. griseus* IFO13350 was spread on YMPD agar medium and grown at 28°C for 4 days. The wild-type strain forms white spores, but mutant NP4 shows a wrinkled and ragged morphology. (B) Scanning electron micrographs of the colony surfaces of the wild-type and NP4 strains grown at 28°C for 4 days. Bars, 1 μm. Transmission electron micrographs of a single spore of both strains are also shown.

septation in the growing mycelium and formation of apparent "submerged spores" (Fig. 3B). The decrease in cell mass was probably due to the breakdown of the sheath. Submerged spore formation was glucose dependent, as was found for substrate spore formation on solid medium. The spores of mutant NP4 formed in liquid medium were resistant to lysozyme, since the rod-like cells observed 40 h after inoculation gave nearly the same CFU after incubation at 37°C for 1 h in the presence and absence of 20 μg of lysozyme. This is in vivid contrast to the submerged spores of *S. griseus* B-2682, which are sensitive to lysozyme digestion (32). We assume that the submerged spores of mutant NP4 are formed in a different way from those of several other *Streptomyces* strains (10, 12, 32, 41), but in the same manner as the substrate spores are formed on solid medium. The growth of NP4 in liquid medium resembles that of "nocardioform organisms," which produce branching hyphae that sooner or later break up into a bacillary or coccoidal form.

Cloning and nucleotide sequencing of DNA fragments conferring morphological changes on *S. griseus* mutant NP4. We constructed a bank of partially *Sau*3AI digested chromosomal fragments of *S. griseus* IFO13350 by using multicopy plasmids, pIJ486 and pIJ702, as the cloning vectors. After transformation of mutant NP4 by the library, we found two types of colonies with different morphology. One showed the normal morphological development and formed spores, as does the wild-type strain. The other showed a more severely wrinkled colony morphology than mutant NP4 (Fig. 4). We isolated seven colonies of the former type and one colony of the latter type.

Restriction mapping and partial nucleotide sequencing of the DNA fragments in the transformants showed that they all derived from the same stretch of DNA. The largest DNA fragment (plasmid pNS5) was contained in the colony of the former type, and its nucleotide sequence was determined. The Frame analysis of the nucleotide sequence predicted four complete open reading frames, *orf2* to *orf5*, and two truncated frames, *orf1* and *orf6*. The seven colonies showing wild-type morphology carried *orf4* as a common region, and the colony showing the severe wrinkled morphology carried *orf5* as a single complete open reading frame.

We also cloned the neighboring regions and determined the whole nucleotide sequence in a total of 8,741 bp. The predic-

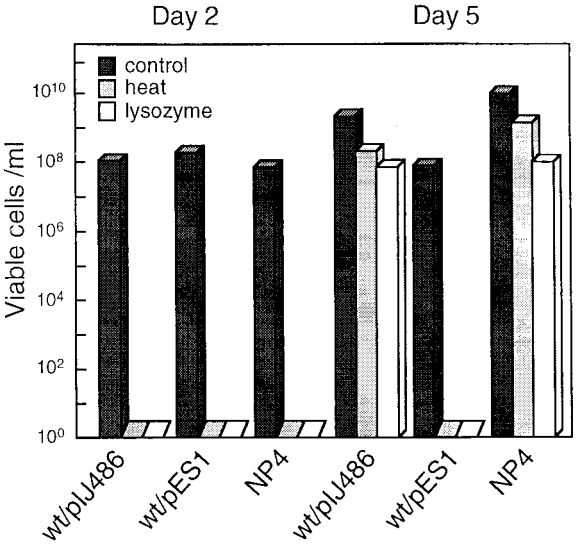


FIG. 2. Heat and lysozyme resistance of ectopic, substrate spores of *S. griseus* mutant NP4 and the wild-type strain *S. griseus* IFO13350 harboring pES1. The three strains were grown on YMPD agar for the indicated days and spread on the same medium after challenges by heat treatment at 55°C for 30 min and by lysozyme treatment at 20 μg/ml and 37°C for 1 h. At day 2, mutant NP4 and the wild-type strain grew as substrate mycelium, as did the wild-type strain harboring pIJ486 as a control, but the wild type carrying pES1 showed a wrinkled colony morphology, indicative of ectopic septation. At day 5, mutant NP4 formed substrate spores, whereas the wild-type strain formed arthrospores.

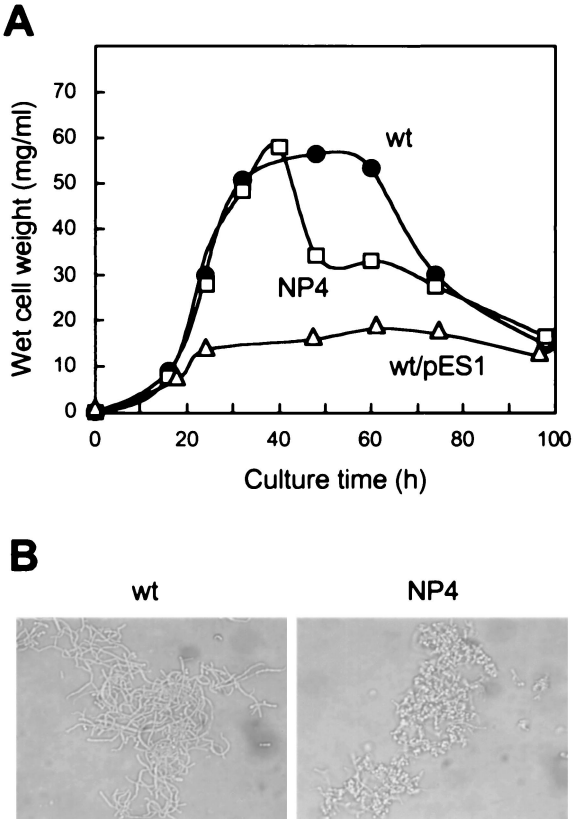


FIG. 3. Morphology of *S. griseus* NP4 in liquid medium. (A) The wild-type strain *S. griseus* IFO13350 (wt), mutant NP4, and the wild-type strain carrying multicopies of *dasA* on pES1 were grown at 28°C in YMPD liquid medium, and their wet cell weights were monitored. (B) Phase-contrast photomicrographs of the wild-type and NP4 strains grown at 28°C for 4 days in YMPD medium.

tion from a homology search by the computer was as follows. Orf2 (400 amino acids [aa]), hypothetical protein found in *Deinococcus radiodurans* (DNA database accession no. G75219); Orf3 (341 aa), oxidoreductase in *Streptomyces fradiae* (accession no. AF147704); Orf4 (258 aa), transcriptional repressor belonging to the GntR family (15); Orf5 (432 aa), sugar-binding protein; and Orf6 (335 aa) and Orf7 (278 aa), integral membrane proteins. Orf1, homologous with a putative sugar transferase (2), and Orf8, homologous with β-glucosidase (16), were truncated.

On the basis of this information, we subcloned the DNA fragment to identify the gene(s) responsible for the phenotypes of the NP4 transformants. As a result, *orf4* on either a high-copy-number or a low-copy-number plasmid was sufficient to reverse the wrinkled morphology and *orf5* was sufficient to cause mutant NP4 to show the severe wrinkled morphology and to form substrate spores (Fig. 4). Introduction of pKS3 containing *orf1* to *orf3*, pHB1 containing *orf6*, or pHBC1 containing *orf6* and *orf7* did not change the morphology of mutant NP4. *orf4* was named *dasR* (deficient in aerial mycelium and spore formation) and *orf5* was named *dasA*, since a mutation or overexpression of these genes affected morphological development even in the wild-type strain, as described below.

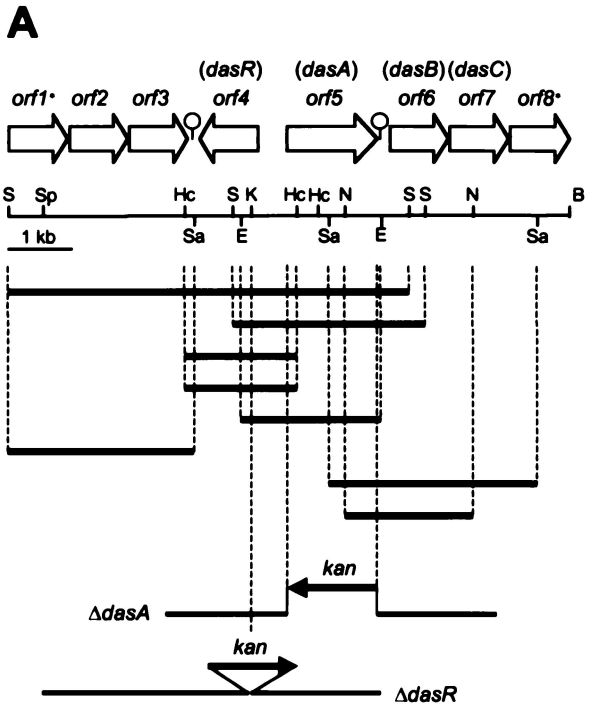
Cloned genes are members of the gene cluster for an ABC transporter. Amino acid alignment of DasR with transcriptional repressors in the GntR family is shown in Fig. 5A. They contain a helix-turn-helix DNA-binding motif at the NH_2-terminal portions. DasR shows 23% identity and 46% similarity to the transcriptional repressor TreR for the trehalose operon in *Pseudomonas fluorescens* (36), 31% identity and 50% similarity to the repressor HutC for the histidine utilization genes in *Pseudomonas putida* (1), and 32% identity and 49% similarity to the repressor FarR, responsible for the fatty acyl-responsive regulator in *E. coli* (48).

The NH_2-terminal portion of DasA shows features typical of a signal peptide (65), i.e., positively charged residues at the NH_2 terminus followed by a stretch of hydrophobic residues (Fig. 5B). In addition, the amino acid sequence around Cys-21 resembles the consensus, L(S, A)(A, G)*C(S, G), of the sites (indicated by the asterisk) cleaved by lipoprotein-specific signal peptidases in gram-positive bacteria (58). A more degenerate and longer consensus is (L, V, G, S)(L, I, V, A)(S, A, M)(A, G)*C(S, G), and the sequence including Cys-21 perfectly matches this consensus, suggesting that Cys-21 is at the NH_2 terminus of the mature form and is covalently modified by the typical ester-linked and amide-linked acylation of lipoproteins.

The sequence from Val-54 to Val-72 shows similarity to the signature sequence (cluster 1) of binding proteins specific for maltooligosaccharides, multiple sugars, α-glycerol phosphate, and iron (60). These include MalE, essential for import of maltose in *S. coelicolor* A3(2) (63), and CebE, essential for import of cellobiose and cellotriose in *Streptomyces reticuli* (53). Therefore, DasA seems to serve as a substrate-binding protein of the components comprising an ABC-type transport system. ABC transporters consist of multisubunit permeases that transport various molecules across the cytoplasmic membrane (6, 11, 18, 55, 60). They consist of two hydrophobic membrane-spanning domains (MSDs) associated with two cytoplasmic ATP-binding domains (ABDs) and a high-affinity extracytoplasmic substrate-binding protein (SBP). Lipoproteins serving as SBPs are anchored in the outer leaflet of the cell membrane by the lipid, usually palmitic acid, attached to the NH_2-terminal Cys residue.

The MSD and SBP genes of an ABC transport system are generally found organized together as an operon. The termination codon (TGA) of DasB, which is encoded by the region just downstream of *dasA*, overlaps the start codon (ATG) of DasC. Both DasB and DasC were predicted to span the membrane six times when their hydrophobicity was analyzed with the PSORT WWW server (http://psort.ims.u-tokyo.ac.jp). Furthermore, they contain the consensus sequence $EAAX_2DGAX_8IXLP$, conserved in MSDs of ABC transporters (52) (Fig. 5C). MalFG of *S. coelicolor* A3(2) and CebFG of *S. reticuli* also contain a sequence similar to the consensus. Thus, it is probable that DasABC are components of an ABC transport system (Fig. 5D), although no ATP-binding proteins as ABDs are encoded in the vicinity of this operon. DasR seemed to be a regulator for this *das* operon because of the gene organization and the transcriptional analysis (see below). *malEFG* and *cebEFG* are also accompanied by a gene encoding a repressor belonging to the LacI-GalR family, which is adjacent but divergently encoded (53, 64).

Ectopic sporulation of wild-type *S. griseus* containing multiple copies of *dasA*. We introduced pES1 and pHR3 into the



plasmid	vector	phenotype: NP4	phenotype: wt
—		esp	normal
pNS5	pIJ702	normal	normal
pES1	pIJ486	esp*	esp
pHR3	pIJ486	normal	normal
pLR3	pKU209	normal	normal
pLA1	pKU209	esp	normal
pKS3	pIJ486	esp	normal
pHBC1	pIJ486	esp	normal
pHB1	pIJ486	esp	normal

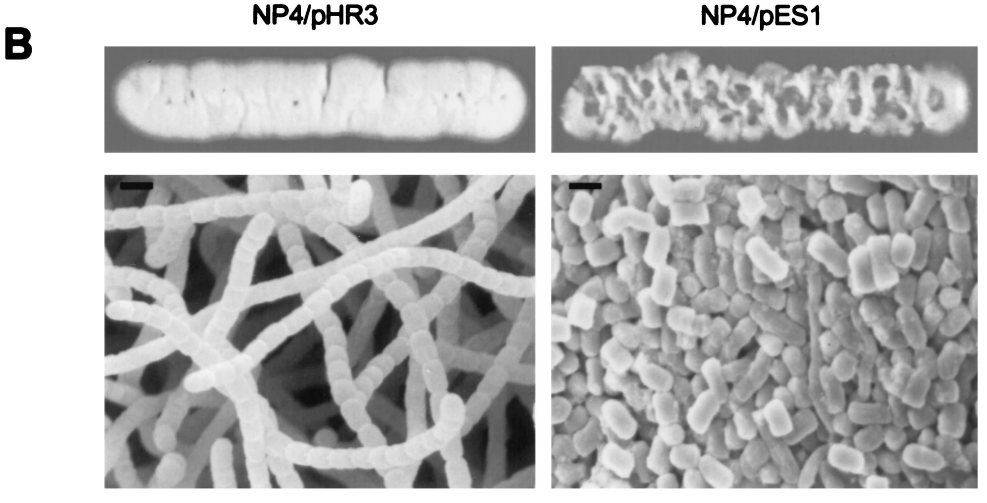


FIG. 4. Restriction map and subcloning of the cloned fragment. (A) The extents and directions of open reading frames on the cloned fragments are indicated by arrows. *orf1** and *orf8** are truncated. Plasmid pNS5 was harbored in one of the seven NP4 transformants showing normal development culminating in sporulation. Plasmid pES1 was harbored in the NP4 transformant showing a severe wrinkled morphology (esp*). pES1 caused the wild-type strain *S. griseus* IFO13350 to form ectopic, substrate spores (esp). An inverted repeat sequence downstream of *dasR* is TGA (termination codon of *orf3*)-$(N)_{10}$-GGGCGGTGGCCCGGGGA-(N:loop)$_5$-TCCCCGGGCCACCGGCCC-$(N)_{46}$-TCA (termination codon of *dasR*). An inverted repeat sequence downstream of *dasA* is TGA (termination codon of *dasA*)-$(N)_{67}$-TCCGGGGC-(N:loop)$_2$-GCCCCGGA-$(N)_{22}$-GTG (start codon of *dasB*). The insertion of the kanamycin resistance determinant (*kan*) into *dasA* and *dasR* is shown schematically. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; Hc, *Hin*cII: K, *Kpn*I; N, *Nru*I; S, *Sau*3AI; Sa, *Sac*I; and Sp, *Sph*I. (B) Mutant NP4 containing only *dasR* on either the high-copy-number or the low-copy-number plasmid developed normally and formed spores after growth at 28°C for 4 days on YMPD agar medium, whereas NP4 containing *dasA* on pIJ486 showed a more severe wrinkled morphology. Scanning electron micrographs reveals the formation of normal arthrospores by mutant NP4 harboring pHR3 and substrate spores by NP4 harboring pES1.

wild-type strain *S. griseus* IFO13350 to see the effects of multiple copies of *dasA* and *dasR*. No detectable phenotypic changes were observed for *S. griseus* IFO13350 harboring pHR3. However, the wild-type strain harboring pES1 showed a wrinkled colony morphology on YMPD medium, as did mutant NP4 (Fig. 6A). The effect of *dasA* on the wild-type strain was observed only on YMPD medium containing glucose and not on YMP medium containing other sugars (Fig. 6B), as was

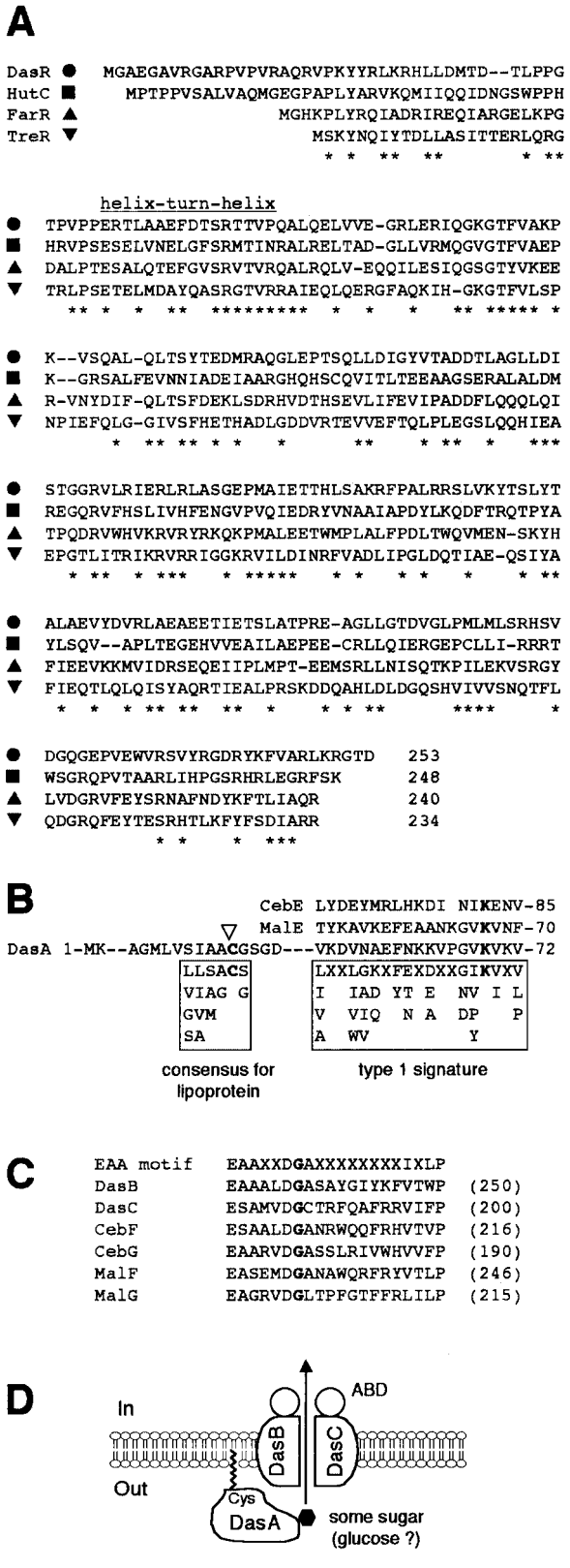


FIG. 5. Homologies of DasRABC products with components of the ABC-type transport systems. (A) Amino acid alignment of DasR with the transcriptional repressors in the GntR family. TreR from *P. fluorescens* (DNA database accession number AAG31030), HutC from *P. putida* (P22773), and FarR from *E. coli* (S04645) are shown. Asterisks indicate similar amino acids that are conserved in three of the four proteins. (B) Probable cleavage site of the signal peptide (indicated by a triangle), the signature sequence in SBPs specific for maltooligosaccharides, multiple sugars, α-glycerol phosphate, and iron (60), and amino acid alignment of this region of DasA with MalE of *S. coelicolor* A3(2) and CebE of *S. reticuli* are shown. X represents any amino acid. The Lys residue in boldface is the highly conserved amino acid in this family. (C) The consensus sequence conserved in MSDs of ABC transporters (52) and amino acid alignment of this region of DasBC with MalFG and CebFG are shown. X represents any amino acid. The Gly residue in boldface is the highly conserved amino acid in this family. (D) Predicted topology of DasABC as components of an ABC transporter. DasA, a substrate-binding protein, is anchored to the outer surface of the membrane by a lipid attached to Cys-21. DasBC are integrated in the membrane, to each of which an ATP-hydrolyzing subunit (ABD) is associated.

observed for mutant NP4. Vertical sections of the colony showed that the cells far below the agar surface were a mixture of short substrate hyphae with septa and spore-like cells (Fig. 6C). Sporulation of *Streptomyces* strains rarely occurs in substrate hyphae that have penetrated the agar (8, 67). The ectopic septa in the substrate hyphae and the cell wall of the wild-type strain harboring pES1 are thicker and have a lower electron density than those in the aerial hyphae of the wild-type strain. The envelope and septa of the aerial mycelium of the wild-type strain are electron dense. However, the main wall of an aerial spore of the wild-type strain is apparently thicker than that of the ectopic spore, which is enclosed by a thinner, electron-dense envelope (Fig. 6D).

Consistent with the apparent difference in the main spore wall, the ectopic spores were susceptible to both heat treatment and lysozyme digestion (Fig. 2). As described above, the ectopic spores of mutant NP4 were almost the same in morphology and susceptibility to heat and lysozyme as the aerial spores. The difference can probably be ascribed to the difference in the texture and thickness of the spore walls. Nevertheless, the two different ectopic spores were found to germinate at nearly the same frequency, as high as more than 90%, when the CFU were measured.

The wild-type strain carrying pES1 appeared to show the wrinkled morphology earlier than mutant NP4. We then examined the courses of the ectopic septation of the wild type carrying pES1 and mutant NP4 in relation to the development of the wild-type strain by scanning and transmission electron microscopy. A lump of mycelium on a petri dish was picked with a toothpick and streaked on YMPD agar medium. The wild-type strain grew as substrate mycelium for 2 days and then as a mixture of aerial and substrate mycelia and at day 4 formed spores. Mutant NP4 grew as substrate mycelium for 4 days and abruptly formed septa in the substrate hyphae. On the other hand, the wild-type carrying pES1 formed septa in the substrate hyphae as early as day 1. Because of the very early septation, the spread of mycelium of the wild type carrying pES1 was poor. The early septation and sporulation of the wild type carrying pES1 also occurred in YMPD liquid medium; no increase in the cell mass, indicative of sporulation, 20 h after inoculation was observed (Fig. 3A). The thin spore wall and sensitivity to lysozyme of the ectopic spores formed by the wild-type strain carrying pES1 likely result from this very early septation.

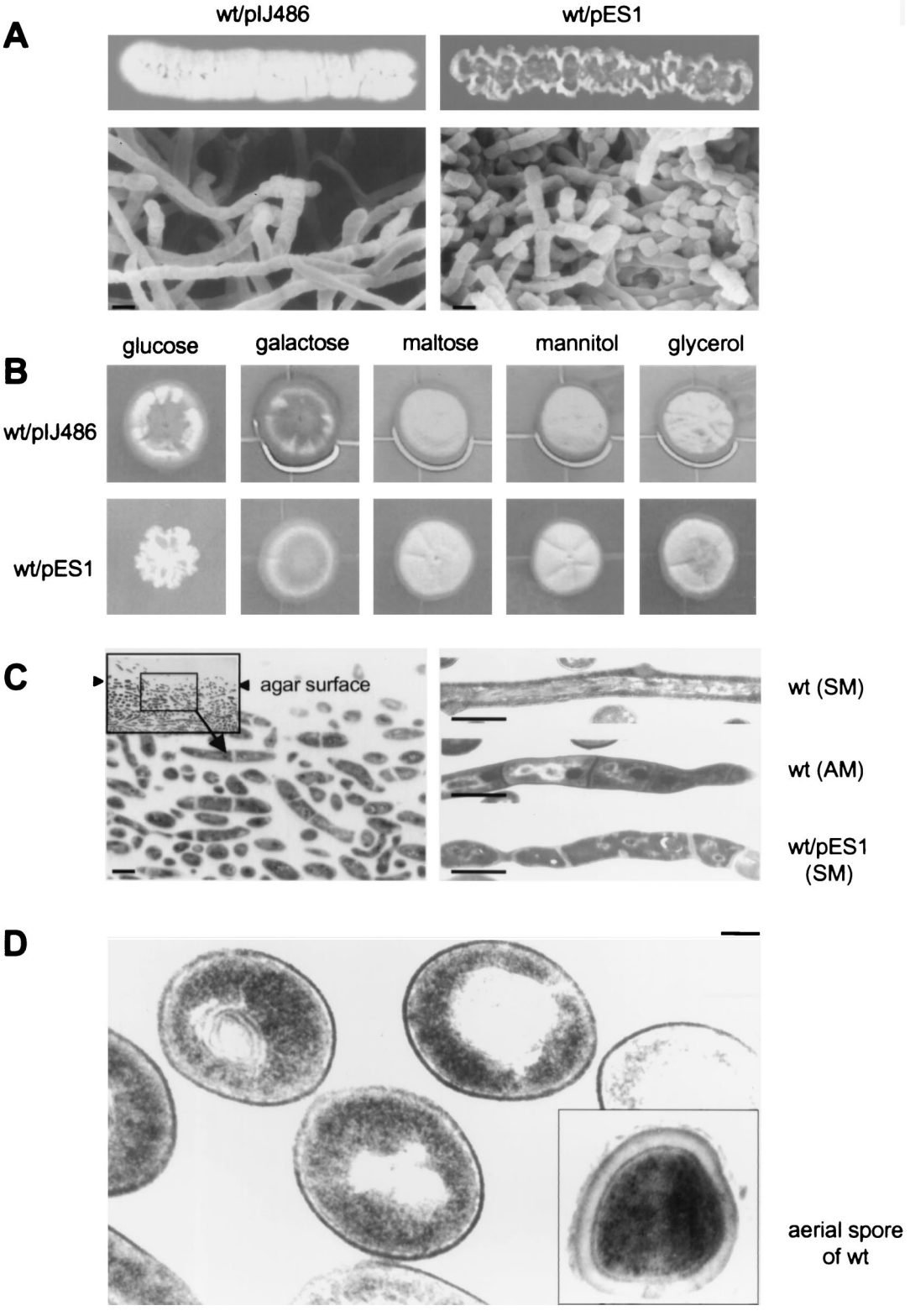


FIG. 6. Morphology of the wild-type *S. griseus* strain IFO13350 containing extra copies of *dasA*. (A) *S. griseus* IFO13350 harboring pES1 shows a wrinkled colony morphology on YMPD agar medium, as does mutant NP4, and forms substrate spores, as examined by scanning electron microscopy. Photographs were taken after growth at 28°C for 4 days. Bars, 1 μm. (B) The morphological defect of *S. griseus* IFO13350 harboring pES1 depends on glucose as a carbon source, because on YMP medium containing other sugars at a concentration of 1% instead of glucose, the wild-type strain harboring pES1 shows normal sporulation. Photographs were taken after growth at 28°C for 5 days. (C) Sporulation in the substrate mycelium of agar-grown *S. griseus* IFO13350 harboring pES1. Bars, 1 μm. An ultrathin section containing substrate mycelium penetrated into agar after growth at 28°C for 2 days was examined at different magnifications (left panel). A substrate spore chain of the wild type harboring pES1 is shown, together with the substrate (SM) and aerial (AM) mycelium of the wild-type strain as controls (right panel). (D) Ectopic, substrate spores of *S. griseus* IFO13350 harboring pES1, together with the aerial spore of the wild-type strain as a control, is shown. The samples were prepared after growth at 28°C for 4 days. Bar, 0.1 μm.

A-factor independence of ectopic sporulation of *S. griseus* carrying *dasA*. In *S. griseus*, A-factor serves as a master switch for aerial mycelium formation and secondary metabolism; the development of an A-factor-deficient mutant, *S. griseus* strain HH1, remains in substrate mycelium. We introduced pES1 into mutant HH1 to see the effect of A-factor on ectopic septation in substrate hyphae caused by overexpression of *dasA*. Mutant HH1 harboring pES1 apparently spread the mycelium poorly on YMPD agar and showed wrinkled colony morphology. Scanning electron microscopic analysis of the wrinkled colony of mutant HH1 harboring pES1 showed ectopic septation as early as day 1 (data not shown), indicating that the ectopic septation in substrate hyphae and subsequent sporulation were independent of A-factor. Consistent with this idea, Δ*adpA* and Δ*adsA* mutants, both containing a mutation in the respective regulatory step essential for normal development in the A-factor regulatory cascade, also showed a wrinkled colony morphology at day 1 on YMPD agar. The poor spread of mycelium of these strains carrying pES1 is presumably due to the very early septation. The septa in the substrate hyphae seem to be formed immediately after the hyphae have elongated.

Aerial mycelium-defective phenotype in *dasA* and *dasR* disruptants. We disrupted the chromosomal *dasA* gene of *S. griseus* IFO13350 so that almost the whole *dasA* coding sequence was replaced by the kanamycin resistance gene. The growth rate of the Δ*dasA* mutant on YMPD medium was almost the same as on minimal medium containing maltose, mannitol, or glycerol instead of glucose, suggesting that *dasRABC* are not so actively involved in glucose import, if at all, that loss of the function results in a decrease in growth rate. This is consistent with the observation that *dasA* is transcribed at the commencement of aerial mycelium formation and during sporulation (see below). However, the Δ*dasA* strain showed a bald phenotype on YMPD agar medium (Fig. 7A). Introduction of *dasA* on the low-copy-number plasmid pKU209 (plasmid pLA1) into this mutant reversed the defect, indicating that the inability to form aerial mycelium of the Δ*dasA* mutant was due solely to the disruption of *dasA* (Fig. 7B). The aerial mycelium formation and sporulation of this mutant were also greatly reduced on YMP medium containing mannitol, maltose, or glycerol, but sparse spores were still formed.

The Δ*dasR* mutant also showed a bald phenotype (Fig. 7A), irrespective of carbon source. Scanning electron microscopic analysis of the Δ*dasR* mutant revealed growth as substrate hyphae and occasional septation in the substrate hyphae (Fig. 7C). Comparison of the substrate hyphae between the Δ*dasA* and Δ*dasR* mutants showed that the hyphae of the former were apparently rough and those of the latter were rather smooth, suggesting some difference in wall structure. Consistent with this, the colony surface of the Δ*dasA* mutant was rough and that of the Δ*dasR* mutant was smooth and lustrous (Fig. 7A). The rough hyphae was usually observed for the A-factor-deficient mutant HH1. Introduction of *dasR* on the low-copy-number plasmid pKU209 (plasmid pLR3) into this mutant reversed the defect.

Transcriptional analysis of ABC-type transporter gene cluster. The transcriptional start points of *dasR* and *dasA* in the wild-type strain *S. griseus* IFO13350 were determined by high-resolution S1 nuclease mapping with RNA prepared from cells

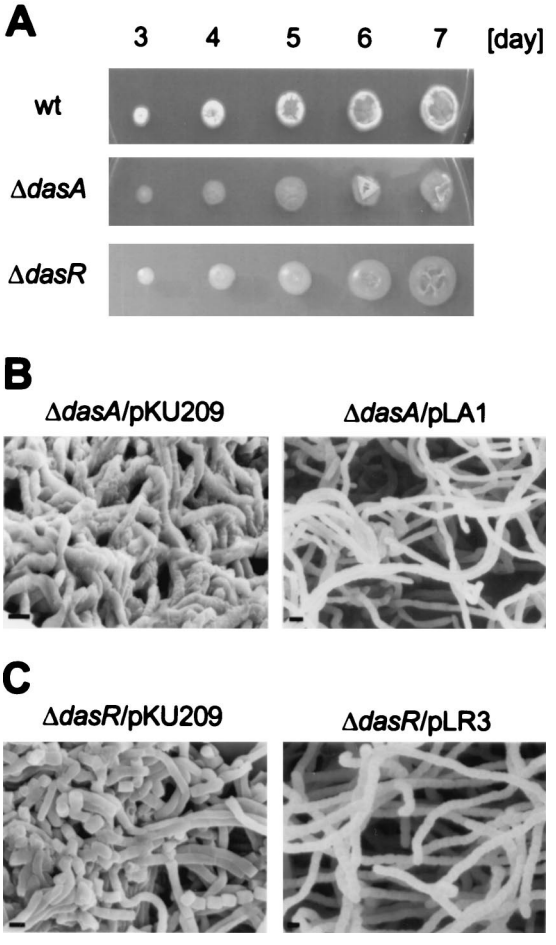


FIG. 7. Bald phenotypes of the Δ*dasA* and Δ*dasR* mutants derived from *S. griseus* IFO13350. (A) Colonies of the Δ*dasA* and Δ*dasR* mutants were formed with a toothpick on YMPD medium. The growth of the mutants is the same as the wild-type strain, as seen by the diameters of the colonies, but the mutants show a bald phenotype. (B) Scanning electron micrographs of the Δ*dasA* mutant harboring the vector plasmid pKU209 or pLA1, containing the intact *dasA* gene. (C) Scanning electron micrographs of the Δ*dasR* mutant harboring the vector plasmid pKU209 or pLR1, containing the intact *dasR* gene.

that were grown on YMPD agar medium (Fig. 8A). Both genes were transcribed from a single start point. In front of the start point of *dasA*, a TTGACA sequence, the same as the −35 consensus sequence for many bacteria, including *Streptomyces* spp. (57), and a CAAGCT sequence, somewhat similar to a typical −10 sequence TATAAT, are present (Fig. 8B). However, no such sequences are present at appropriate positions in front of the transcriptional start point of *dasR*.

dasBC was hypothesized to be transcribed by a promoter in front of *dasB*, since an inverted repeat sequence, probably serving as a rho-independent transcriptional terminator, was found downstream of the termination codon of *dasA* (Fig. 4A). We performed RT-PCR to examine transcription of *dasBC* by using the four primers indicated in Fig. 8C. A pair of primers for detection of the mRNA stretch corresponding to fragment AB yielded no amplified DNA fragments, and a pair of primers for mRNA corresponding to fragment BC yielded a DNA

FIG. 8. Determination of the transcriptional start points of *dasA* and *dasR* by S1 nuclease mapping. (A) RNA was prepared from cells grown on YMPD medium at 28°C for 1 day for *dasR* and 3 days for *dasA*. The sequence ladders, derived from the same primer, are shown with the A+G and T+C reactions. The deduced transcriptional start points are shown by arrows. (B) The nucleotide sequence covering the promoter regions of *dasA* and *dasR* is shown, together with their NH_2-terminal amino acid sequences. Probable Shine-Dalgarno (SD) sequences and probable −35 and −10 sequences for *dasA* are indicated. (C) RT-PCR for determination of transcriptional linkages of *dasABC*. Pairs of primers were used to amplify the indicated four DNA fragments. No amplification occurred when reverse transcriptase was omitted from the reaction mixture, indicating the absence of DNA in the mRNA samples.

fragment with the expected size of 1.9 kb. We did not further characterize the transcription of *dasBC*.

Developmentally regulated expression of *dasA* and *dasR*. The courses of transcription of *dasR* and *dasA* in the wild-type strain and mutant NP4 were examined by low-resolution S1 nuclease mapping with RNAs prepared from cells grown on YMPD agar medium. For preparation of cells in large amounts, cells cultured in liquid medium were collected, concentrated by centrifugation, and spread on cellophane on the surface of the agar medium. *hrdB,* which encodes σ^{HrdB} and is transcribed throughout growth (56), was used to monitor the quantity and quality of the RNAs used. At day 1 under these conditions, the wild-type strain grew as substrate mycelium. After 2 days, it grew as a mixture of aerial and substrate hyphae, and after 3 days, it grew as a mixture of spores and aerial hyphae. Mutant NP4 began to form septa in substrate hyphae after 3 days. In both the wild-type and mutant strains, *dasR* was mainly transcribed during vegetative growth, whereas *dasA* was transcribed just after commitment of aerial mycelium formation and during spore formation (Fig. 9A). The timing of *dasA* expression contravenes the bald phenotype caused by the *dasA* deletion. DasA in basal amounts may play a role in the initiation of aerial mycelium formation.

The gene organization of *dasRABC* and the homology of DasR with transcriptional repressors belonging to the GntR family suggested that DasR might repress the expression of at least *dasA*. The above-described course of *dasA* and *dasR* expression is in agreement with this idea. In addition, in the wild-type strain harboring multicopies of *dasR*, transcription of *dasA* was severely repressed (Fig. 9B), suggesting that DasR represses the promoter of *dasA*. This is also in agreement with the observation that the Δ*dasR* mutant, in which *dasA* is not repressed by DasR, shows occasional septation in substrate hyphae (Fig. 7C).

dasR and *dasA* were both transcribed more actively in mutant NP4 than in the wild-type strain. It is likely that the active transcription of *dasA* in NP4 results in the ectopic sporulation, because, as described above, introduction of multicopies of *dasA* into the wild-type strain caused ectopic sporulation. We determined the nucleotide sequence of the *dasR-dasA* region, i.e., the *Sac*I-*Eco*RI fragment covering the whole *dasR* and *dasA* genes in mutant NP4 (Fig. 4A), and found no base changes. This excluded the possibility that the mutation(s) in mutant NP4 lay in the coding sequences or promoter regions of *dasRA*.

DISCUSSION

S. griseus mutant NP4, which was isolated by UV mutagenesis, showed a bald and wrinkled colony morphology because

of ectopic septation in substrate hyphae and subsequent spore formation. The ectopic spores were the same as aerial spores in size, thickness of the spore wall, and shape, as determined by transmission and scanning electron microscopy, and in heat and lysozyme susceptibility. Mutant NP4 also formed abundant spores in liquid medium, whereas the parental strain IFO13350 rarely forms submerged spores under these conditions. The wall of the ectopic spores is supposed to be thicker than those of the submerged spores formed by several *Streptomyces* spp., including *S. griseus* B-2682 (32), under specific conditions, because the spores of NP4 were resistant to lysozyme. We therefore assume that both on solid and in liquid medium, mutant NP4 forms two separate cross walls in the vegetative hyphae and matures each compartment into a spore indistinguishable from aerial spores in many aspects, as is observed in aerial spore formation in *S. griseus* (14).

The frequency of septation in substrate mycelium in mutant NP4 is much higher than in *S. coelicolor* A3(2) strains harboring multicopies of *whiG* (7, 40) or having a deletion of a region close to the *glkA* locus (31). These strains form abundant aerial spores and only occasional septa in substrate mycelium and subsequent sporulation. The deprogrammed sporulation of NP4 implies that once septa are formed, even in substrate hyphae, in response to some signal, each compartment is inevitably destined to develop into a spore. In the substrate hyphae of the wild-type *S. griseus* strain, some signals must block the commitment to septation and subsequent sporulation. A-factor does not release the block, because exogenous addition of an appropriate amount of A-factor to the substrate hyphae of the A-factor-deficient mutant HH1 causes no septum formation but normal formation of aerial spores. An excess amount of DasA appears to release the block, since introduction of pES1 into mutant HH1 results in ectopic septation.

Shotgun cloning of genes on the wild-type chromosome into a mutant is a useful approach for identifying the mutated gene and genes closely related to the mutant phenotype. We at first expected that the mutation(s) responsible for the ectopic sporulation of NP4 was in *dasR* or *dasA*, since the ectopic sporulation of mutant NP4 was completely reversed by *dasR* encoding a transcriptional factor belonging to the GntR family, and *dasA* made the wrinkled morphology of mutant NP4 more severe. However, nucleotide sequencing of the *dasR-dasA* region in mutant NP4 revealed no base changes.

On the basis of the observations that introduction of *dasA* into the wild-type strain caused ectopic sporulation and that the amount of *dasA* transcript was greater in mutant NP4 than in the wild type, we assume that NP4 has a mutation in the regulatory pathway to control the expression of *dasA*. Because ectopic sporulation appears to result solely from an increase in the amount of DasA, the regulatory pathway in mutant NP4 seems to lack the ability to repress *dasA*. The elevated expression of *dasR* in NP4 suggests that the putative regulatory pathway controls *dasR* too. The increase in the amount of DasR, which may repress not only *dasA* but also some other genes involved in programmed development, is a possible explanation for the difference in timing of ectopic septation between the wild-type strain carrying *dasA* and mutant NP4; an increase in the amount of only DasA in the same background as in the wild type results in early commitment of septation, whereas an increase in the amount of DasR during early growth and the existence thereafter (Fig. 9A) bring forth a different background, resulting in septation at the programmed time.

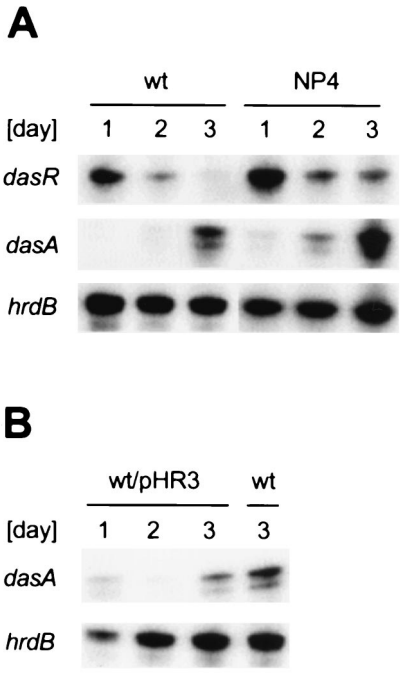


FIG. 9. Transcription of *dasRABC*. (A) RNA was prepared from colonies formed on YMPD agar medium. The wild-type *S. griseus* IFO13350 strain grew as substrate mycelium at day 1, as a mixture of substrate and aerial hyphae at day 2, and as a mixture of aerial hyphae and spores at day 3. (B) Transcription of *dasA* is repressed in the wild-type strain harboring pHR3.

Is DasRABC involved only in sugar import as an ABC transporter, as predicted by the homology of each of the components? The gene organization *dasR-dasA-dasB-dasC* and their predicted functions are the same as those for the maltose and cellobiose/cellotriose import systems in *Streptomyces* spp., *malR-malE-malF-malG* (63) and *cebR-cebE-cebF-cebG* (53), respectively. In addition, a gene encoding a glucosidase-like protein is encoded downstream of all three of these gene clusters. Although no ATP-binding proteins as ABDs are encoded in the vicinity of the *das* operon, the absence of the gene encoding ABD also holds for the *malEFG* and *cebEFG* operons. As pointed out by van Wezel et al. (64), MsiK or an MsiK-like ATP-binding protein (24, 53, 54), which is encoded elsewhere on the chromosome and homologous to the ATP-hydrolyzing subunit MalK in the maltose import system in *E. coli* (5, 11), may serve as a general ATP-hydrolyzing subunit for "orphan" ABC transporters. MalK is an essential component in the *E. coli* maltose import system, forming $MalEFGK_2$.

The effect of glucose on the ectopic sporulation of mutant NP4 and on the wild type containing multicopies of *dasA* tempted us to speculate that DasA recognizes and binds to glucose or a glucose derivative and imports it via the Das system. However, *dasA* is developmentally regulated, and its transcription is enhanced just after commitment of aerial mycelium formation and during spore formation. This means that DasA is not produced until the glucose in the medium is almost consumed. Conceivably, DasA binds a certain sugar compound other than glucose which is needed for septation in

aerial hyphae at the programmed point. The bald phenotype of the Δ*dasA* mutant suggests an additional role of DasA in aerial mycelium formation, although we have no plausible explanation for it. In addition, the bald phenotype, but accompanied with occasional septation in substrate hyphae, of the Δ*dasR* mutant suggests that the concentration of DasA controlled by *dasR* is critical for aerial mycelium formation.

In considering the function of DasA, we would like to point out the multiple functions of some substrate-binding proteins of the ABC transporters. For example, ChvE is a multifunctional glucose/galactose-binding protein which participates in the uptake of specific monosaccharides, chemotaxis to these sugars, and virulence gene induction in *Agrobacterium*. For induction of the virulence genes to form crown gall tumors, monosaccharide-bound ChvE interacts with the periplasmic region of VirA, a sensor kinase in the VirA-VirG two-component signal transduction system (46, 69). For chemotaxis, ChvE is supposed to interact with chemotaxis receptors such as Tar and Trg (33). The maltose-binding protein MalE of *E. coli*, the oligopeptide-binding protein OppA of *E. coli*, and the galactose-binding protein MglB of *Salmonella enterica* serovar Typhimurium are other examples that function as a chaperone for protein folding and protection from stress in the periplasm, in addition to their function in import and chemotaxis (49). These examples, together with the developmentally regulated expression of *dasA* and involvement in septum formation of DasA, present a possibility that substrate-bound or free DasA interacts with other regulatory proteins in the membrane, thus commencing a regulatory pathway for morphological development.

The ectopic septation and subsequent sporulation of *S. griseus* triggered solely by overexpression of *dasA* was independent of A-factor, because the A-factor-deficient mutant HH1 harboring pES1 showed ectopic sporulation. This is consistent with the observation that the wild-type strain harboring pES1 formed ectopic septa at day 1, when the concentration of A-factor is still low (Fig. 3A and 6). In *S. griseus*, A-factor at a critical concentration triggers aerial mycelium formation and streptomycin biosynthesis by binding a repressor-type receptor protein, ArpA, and dissociating it from the promoter region of *adpA,* encoding a transcriptional activator (45). A-factor is produced in a growth-dependent manner (20–22).

One of the targets of AdpA is *adsA,* encoding an extracytoplasmic function sigma factor, σ^{AdsA}, essential for the initiation of aerial mycelium formation (70). AdpA and AdsA supposedly activate many structural genes required for aerial mycelium formation. A-factor thus determines the timing of programmed and ordered development by acting as a master switch for turning on many genes at several hierarchic regulatory steps. The ectopic spores which are triggered by an excess amount of DasA and independently of A-factor germinate at the same frequency as aerial spores, although they are sensitive to lysozyme and heat because of a thinner spore wall. The difference in lysozyme and heat resistance of the aerial spores and ectopic spores of the wild-type harboring multicopies of *dasA* implies that some of the gene products necessary for the architecture of aerial spores are absent in the maturation of the ectopic spores.

Comparison of transcription of genes necessary for normal morphological and physiological development in the wild-type, A-factor-controlling background and in the *dasA*-overexpressing background will reveal the difference in the genetic network between the programmed septation in aerial hyphae and ectopic septation in substrate hyphae triggered by an excess of DasA. It will also be useful to study their counterparts in different *Streptomyces* spp. *S. coelicolor* A3(2) contains a very similar gene cluster, open reading frames CAB94616 to -94619, in cosmid SC7E4 (www.sanger.ac.uk/Projects/S_coelicolor/), each gene of which shows 33 to 91% identity to the corresponding gene in the *dasRABC* cluster.

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