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The *rpoZ* Gene, Encoding the RNA Polymerase Omega Subunit, Is Required for Antibiotic Production and Morphological Differentiation in *Streptomyces kasugaensis*

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The occurrence of pleiotropic mutants that are defective in both antibiotic production and aerial mycelium formation is peculiar to streptomycetes. Pleiotropic mutant KSB was isolated from wild-type *Streptomyces kasugaensis* A1R6, which produces kasugamycin, an antifungal aminoglycoside antibiotic. A 9.3-kb DNA fragment was cloned from the chromosomal DNA of strain A1R6 by complementary restoration of kasugamycin production and aerial hypha formation to mutant KSB. Complementation experiments with deletion plasmids and subsequent DNA analysis indicated that *orf5*, encoding 90 amino acids, was responsible for the restoration. A protein homology search revealed that *orf5* was a homolog of *rpoZ*, the gene that is known to encode RNA polymerase subunit omega (ω), thus leading to the conclusion that *orf5* was *rpoZ* in *S. kasugaensis*. The pleiotropy of mutant KSB was attributed to a 2-bp frameshift deletion in the *rpoZ* region of mutant KSB, which probably resulted in a truncated, incomplete ω of 47 amino acids. Furthermore, *rpoZ*-disrupted mutant R6D4 obtained from strain A1R6 by insertion of Tn*5 aphII* into the middle of the *rpoZ*-coding region produced neither kasugamycin nor aerial mycelia, similar to mutant KSB. When *rpoZ* of *S. kasugaensis* and *Streptomyces coelicolor*, whose deduced products differed in the sixth amino acid residue, were introduced into mutant R6D4 via a plasmid, both transformants produced kasugamycin and aerial hyphae without significant differences. This study established that *rpoZ* is required for kasugamycin production and aerial mycelium formation in *S. kasugaensis* and responsible for pleiotropy.

Members of the genus *Streptomyces* produce an enormous variety of biologically active antibiotics and morphologically differentiate in a similar manner to eukaryotic fungi. Mature colonies of these gram-positive prokaryotic bacteria show an upper layer of aerial mycelia bearing spores that covers a lower layer of vegetative mycelia growing on the surface of agar medium. The production of antibiotics and formation of aerial hyphae, which appear to be independent events, have been considered genetically correlated based on the occurrence of pleiotropic mutants that neither produce antibiotics nor form aerial mycelia (8, 17). Hence, the pleiotropic regulation has provided a subject of special interests in *Streptomyces* genetics.

A variety of pleiotropic mutants such as *bldA, bldB*, and *bldD* mutants have been obtained from *Streptomyces coelicolor* together with many other types of *bld* mutants that fail to form aerial hyphae. Molecular cloning of the responsible genes has demonstrated transcriptional and translational regulation of pleiotropy. The *bldA* gene encodes leucyl tRNA for a rarely used UUA codon in streptomycetes (24, 26). Since the rare TTA codon is present in the regulatory genes for actinorhodin and undecylprodigiosin syntheses in *S. coelicolor* and for streptomycin and aerial mycelium productions in *Streptomyces griseus*, *bldA* mutations result in the pleiotropic phenotype in the streptomycetes (12, 23). Furthermore, the findings that *bldB* and *bldD* encode 98 and 167 amino acids, respectively, with putative motifs of helix-turn-helix, support transcriptional regulation of pleiotropy (10, 11, 36). Meanwhile, studies on A-factor, the pleiotropic autoregulator that controls streptomycin biosynthesis and resistance together with the morphogenesis in *S. griseus*, have revealed the regulatory cascade triggered by A-factor (34). The binding of A-factor to its receptor protein ArpA relieves its repression to *adpA* that regulates the streptomycin-specific transcriptional activator *strR*, and the derepression leads to the activation of the genes for streptomycin biosynthesis and resistance. In addition, *adsA* that is involved in aerial mycelium formation is under the regulation of the cascade (42).

We present here evidence that *rpoZ*, the gene that is known to encode the RNA polymerase (RNAP) subunit omega (ω), is closely related to pleiotropy in *Streptomyces kasugaensis*, i.e., the production of kasugamycin, an aminoglycoside antibiotic that protects rice plants from infection with the fungus *Piricularia oryzae*, and the formation of aerial mycelium. *S. kasugaensis* MB273, isolated from soil, carries three pock-forming plasmids (1, 2), and the host-vector system for gene cloning and analysis has been established (31, 35). We isolated pleiotropic mutant KSB that produces neither kasugamycin nor aerial mycelium after mutagenesis of *S. kasugaensis* A1R6, a

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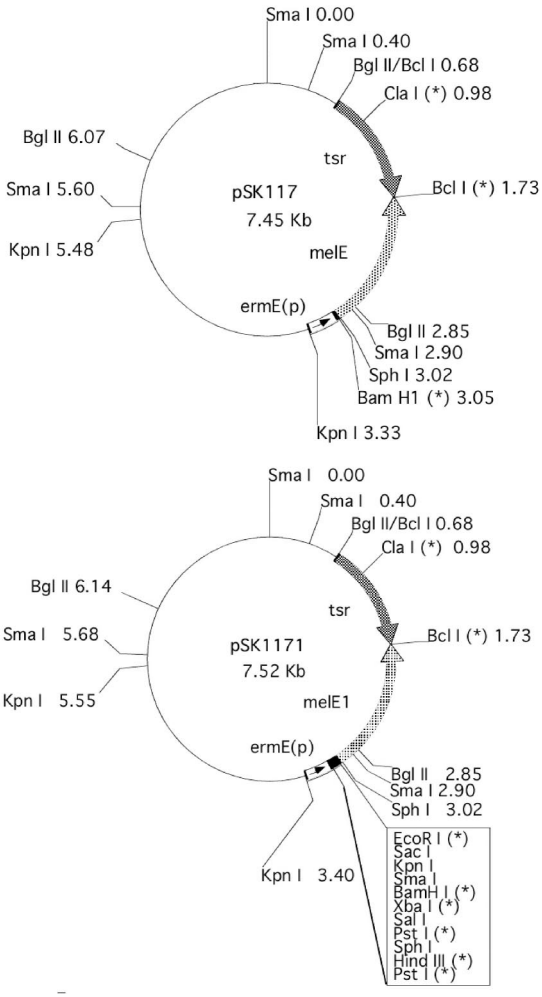

FIG. 1. Restriction map of pSK117 and pSK1171. Asterisks represent unique restriction sites in the plasmid, and ermE(p) indicates the promoter region of *ermE* harboring *ermE*p1 and *ermE*p2 (4). Both plasmids carry the hybrid tyrosinase genes *melE* and *melE1* as described in Materials and Methods.

wild-type strain, and subsequently cloned a 9.3-kb DNA fragment by phenotypic complementation. Our results establish that *rpoZ* and consequently its product ω play a physiologically pivotal role on antibiotic production and morphogenesis in *S. kasugaensis*.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *S. kasugaensis* A1R6, a wild-type, plasmid-free derivative of strain MB273 isolated from soil, and GMY medium were described previously (1, 35). *S. coelicolor* M145 and *Streptomyces lividans* TK21 were kindly provided by D. A. Hopwood. Multicopy *Streptomyces* plasmids pSK117, pSK1171 (as illustrated in Fig. 1), and pSK1172 and the *E. coli* plasmid pUC18 were used for construction of plasmids using *S. lividans* TK21 and *Escherichia coli* JM109 as host strains. Plasmid pSK117 was constructed by insertion of a hybrid tyrosinase gene (*melE*) and the thiostrepton resistance gene (*tsr*) into pSK11-ΔSK10 (31), a derivative of *S. kasugaensis* multicopy plasmid pSK1 (1). The *melE* gene consists of the promoter of the erythromycin resistance gene (*ermE*) (4) and the tyrosinase gene (*melC1* and *melC2*) (3, 25). The *melE1* gene of pSK1171 was constructed by insertion of the 72-bp multiple-cloning site in pIJ486 (39) into *melE* between the *ermE* promoter and the tyrosinase gene. Plasmid pSK1172 is identical to pSK1171 except that the multiple-cloning site in the hybrid tyrosinase gene is opposite in direction. DNA manipulations in *Streptomyces* were carried out as described in the manuals of Hopwood et al. (16) and Kieser et al. (21).

Aerial mycelium formation and kasugamycin production. Aerial mycelium formation was observed after *S. kasugaensis* strains were incubated on GMY medium at 30°C for 7 to 10 days. Kasugamycin production was assayed by the agar diffusion method with *Pseudomonas fluorescens* IFO15334, which is particularly susceptible to kasugamycin. Shaken cultures of *S. kasugaensis* strains grown in GPY medium (31) were inoculated onto agar plugs consisting of maltose, 25 g/liter; Bacto Yeast extract (Difco), 2 g/liter; and Phytone peptone (BBL), 10 g/liter (pH 7.0). The plugs were incubated at 30°C for 5 days and transferred onto *P. fluorescens*-seeded kasugamycin bioassay medium consisting of glucose, 1 g/liter; Bacto Yeast extract (Difco), 2 g/liter; Polypepton (Wako Chemical Co., Ltd.), 5 g/liter; and agar, 8 g/liter (pH 7.0). Subsequently, the medium was incubated at 30°C for about 40 h for detection of kasugamycin production.

Construction of deletion plasmids: pAK3521, pAK3451, pAK3371, pAK3621, and pAK3631. The respective desired fragment was isolated from the cloned 9.3-kb *Kpn*I-*Bcl*I fragment by digestion with appropriate restriction enzymes and inserted into pUC18. As no *Hin*dIII site was present in the cloned region, the resulting pUC18-derivative plasmid was digested by *Hin*dIII and ligated into a unique *Hin*dIII site of pSK1171 as described above. Of two possible plasmids regarding the insertional direction of the pUC18-derivative, the plasmid in which the desired fragment was located in the downstream region of the *ermE* promoter plus the whole pUC18 DNA was selected. DNA manipulations were conducted using *E. coli* JM109 as a host.

DNA sequencing and protein homology search. Nine deletion mutants of the 3.9-kb *Bam*HI-*Sma*I region subcloned in pAK3541 were obtained using a deletion kit (Deletion Kit for Kilo-Sequence) from Takara Shuzo Co., Ltd., for DNA sequencing. Specifically, an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Perkin-Elmer Corp.) was used, and the samples were analyzed on an Applied Biosystems model 377 sequencer. DNA sequence was analyzed for open reading frames with FramePlot 2.3.2 (www.nih.go.jp/~jun/cgi-bin/frameplot.pl) (19), and database searches were performed with BLAST (www.ncbi.nlm.nih.gov/BLAST/) for identification of homologs to deduced amino acid sequences. In addition, BLAST searches of the *S. coelicolor* database were conducted using The Sanger Institute website at www.sanger.ac.uk/Projects/S_coelicolor/blast_server.shtml.

Construction of pAK522 and pAK531. For amplification of the 1.57-kb fragment covering the regions of *orf3* to *orf5*, a PCR was performed with the upstream and downstream primers containing *Bam*HI adaptor sequences at 5′ ends (underlined), i.e., 5′-GCGGATCCTGTTCTCGCTGACTCCGGCGCG-3′ and 5′-GCGGATCCCCGAGCCTCGGTCCGTACGTAT-3′, and the chromosomal DNA of *S. kasugaensis* as a template. Using a reaction kit (KOD-plus-DNA polymerase) from Toyobo Co., Ltd., the reaction mixture was prepared according to the manufacturer's recommendations. The reaction program consisted of 3 min of initial heating at 98°C and 40 subsequent cycles of heating for 15 s at 98°C, 30 s at 65°C, and 60 s at 72°C. For construction of pAK522, the PCR product was digested with *Eco*RI and *Bam*HI, and the resulting 0.79-kb fragment containing *rpoZ* with 5′-truncated *orf4* was inserted into pSK1171.

For amplification of the 0.82-kb *rpoZ*-encoding region of *S. coelicolor*, which corresponded to the 0.79-kb *Eco*RI-*Bam*HI subcloned in pAK522, PCR amplification was carried out with the upstream and downstream primers containing *Hin*dIII and *Eco*RI adaptor sequences at 5′ ends (underlined), i.e., 5′-ATAAGCTTACAAGCTGATCGCCAACGGC-3′ and 5′-ACGAATTCTTCGGACTCGCTCTACGACC-3′, and the chromosomal DNA of *S. coelicolor* M145 as a template. The primers were designed based on information on *S. coelicolor* cosmid 9C5 (AL357523), whose sequence data were produced by the *S. coelicolor* Sequencing Group at The Sanger Institute. PCR amplification was performed under the same conditions as described above, and the PCR product was digested with *Hin*dIII and *Eco*RI and inserted into pSK1172 for construction of pAK531. DNA manipulations were carried out using *S. lividans* TK21 as a host.

Construction of *E. coli-Streptomyces* vector pAK3741 for disruption of *rpoZ*. Plasmid pAK364, a pUC18 derivative carrying the 1.2-kb *rpoZ*-encoding *Eco*RI-*Sma*I region where a *Sal*I site was uniquely present in the middle, was digested with *Sal*I. A 1.5-kb *Sal*I-digested fragment carrying Tn*5 aphII* was inserted into the *Sal*I site of pAK364 opposite in direction for generation of pAK374. Subsequently, *Eco*RI-digested pAK374 was ligated into a unique *Eco*RI site of pSK21-K2 (31) for construction of pAK3741 (see Fig. 6). DNA manipulations were performed using *E. coli* JM109 as a host.

Isolation of *rpoZ*-disrupted mutants. Transformants of strain A1R6 with pAK3741 were incubated with constant shaking in GPY medium supplemented with kanamycin (50 μg/ml) at 30°C for 4 days. An aliquot of the culture was

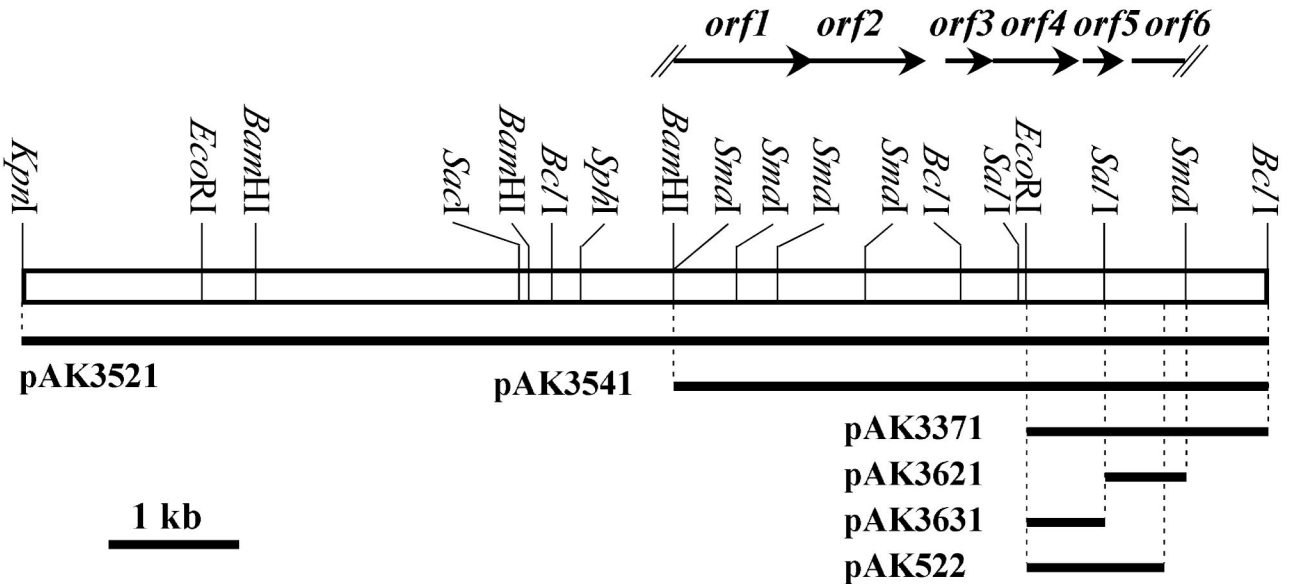

FIG. 2. Restriction map of 9.3-kb cloned DNA fragment. Solid arrows above the map indicate open reading frames predicted by FramePlot analysis (19) on the 3.9-kb *Bam*HI-*Sma*I sequenced region. Solid lines below the map denote the DNA regions subcloned into the respective deletion plasmids.

transferred to fresh GPY medium and cultivated for another 4 days. The same cultivation procedure was repeated three times. Kanamycin-resistant and thiostrepton-susceptible colonies were isolated on GMY medium from the resulting culture and subjected to colony PCR (20) for detection of disruptant mutants of *rpoZ*. PCR was performed with a reaction kit (*TaKaRa La Taq* with GC buffer) from Takara Shuzo Co., Ltd. The reaction mixture containing the same primers used for construction of pAK522 was prepared according to the manufacturer's recommendations. The reaction program consisted of 3 min of initial heating at 98°C and 40 subsequent cycles of heating for 20 s at 98°C, 30 s at 55°C, and 2 min at 72°C.

Nucleotide sequence accession no. The nucleotide sequence data for the 3,911-bp region containing *orf1* through *orf6* from strain A1R6 and 271-bp *rpoZ*-encoding region from mutant KSB have been deposited in DDBJ, EMBL, and GenBank under the accession numbers AB081073 and AB081074, respectively.

RESULTS

Isolation of pleiotropic mutant KSB. Pleiotropic mutant KSB that neither produces kasugamycin nor forms aerial mycelium was isolated after mutagenesis of wild-type strain A1R6 with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG). The mutant KSB retained its aerial mycelium-minus phenotype when grown on modified GMY medium containing mannitol instead of glycerol or in close proximity to wild-type strain A1R6 on the same agar medium. Hence, the mutant turned out to be genetically different from a group of *S. coelicolor bld* mutants (e.g., *bldA*, *bldC*, *bldD*, *bldG*, and *bldH* mutants) as explained in detail in Discussion.

Cloning of DNA fragment that restores aerial mycelium and kasugamycin production to mutant KSB. From the chromosomal DNA of strain A1R6, we cloned an approximately 9.3-kb *Kpn*I-*Bcl*I DNA fragment that complemented the antibiotic- and aerial mycelium-minus phenotype of the mutant and subsequently examined the phenotype of KSB transformants with five deletion plasmids, pAK3521, pAK3541, pAK3371, pAK3621, and pAK3631 (Fig. 2). The transformants with pAK3521, pAK3541, and pAK3371 restored both kasugamycin production (Fig. 3A) and aerial mycelium formation (Fig. 4A), whereas those with pAK3621 and pAK3631 showed no complementation. Specifically, however, the restored kasugamycin production varied with the plasmids, probably because of the mutations in mutant KSB as detailed in Discussion.

Determination of base sequence of cloned DNA and homology search of *orf5*. We determined the nucleotide sequences of the *Bam*HI-*Sma*I fragment subcloned in pAK3541, and FramePlot analysis of the sequences predicted six open reading frames, i.e., 5′-truncated *orf1*, *orf2* (the deduced sequence of 283 amino acids), *orf3* (107), *orf4* (205), and *orf5* (90) and 3′-truncated *orf6* as illustrated in Fig. 2.

BLAST searches revealed that the predicted 90-amino-acid product of *orf5* showed a similarity to bacterial RNAP subunit ω. We concluded therefore that *orf5* was *rpoZ* in *S. kasugaensis*. *S. kasugaensis* ω exhibited 32% identity with the amino acid sequence (91 amino acids) of *E. coli* ω, and as presented in Fig. 5, the protein was highly homologous with ω from actinomycetes, i.e., the 90-amino-acid *S. coelicolor* ω (99% identity), the 110- and 133-amino-acid *Mycobacterium tuberculosis* ω for strains H37Rv and CDC1551 (72% identities), and the 110-amino-acid *Mycobacterium leprae* ω (73% identity). In particular, *S. coelicolor* ω differed from *S. kasugaensis* ω in only the sixth amino acid, i.e., serine for *S. coelicolor* and threonine for *S. kasugaensis*.

Complementation of pleiotropic phenotype of mutant KSB by *rpoZ*. We amplified the 1.57-kb segment carrying *orf3* through *rpoZ* by PCR, digested the product with *Eco*RI and *Bam*HI, and inserted the resulting 0.79-kb segment coding for 5′-truncated *orf4* and *rpoZ* into pSK1171 to construct pAK522 (Fig. 2) as detailed in Materials and Methods. The transformants of KSB with pAK522 produced both of kasugamycin (Fig. 3A) and aerial hypha (Fig. 4A), which indicated that *rpoZ* was defective in mutant KSB. For construction of pAK522, the *rpoZ*-coding fragment was inserted in the opposite direction

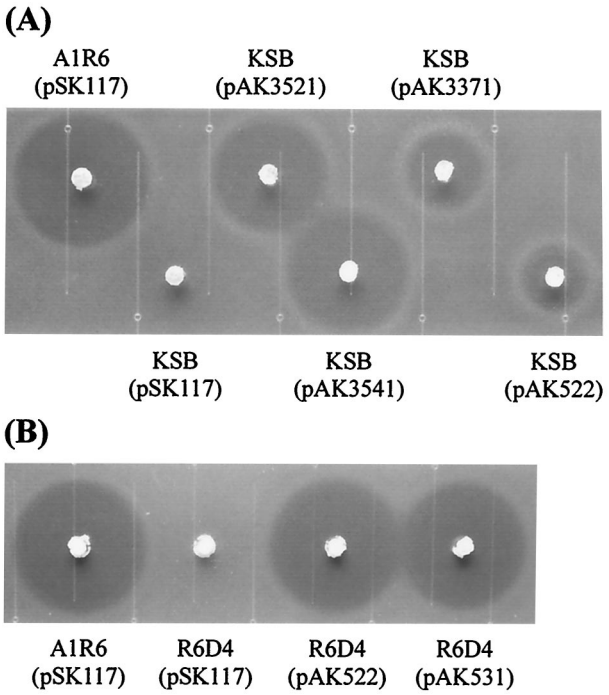

FIG. 3. Kasugamycin production by transformants of mutant KSB (A) and disruptant mutant R6D4 (B). Bioassays using agar plugs were performed for detection of kasugamycin production by the transformants as detailed in Materials and Methods. The halos formed around the plugs indicate the accumulation of kasugamycin by the transformants. The transformants of wild-type strain A1R6 with pSK117 were used as a positive control. (A) From left to right, strain A1R6 carrying pSK117; and mutant KSBs harboring pSK117, pAK3521, pAK3541, pAK3371, and pAK522. (B) From left to right, strain A1R6 carrying pSK117 and mutant R6D4s harboring pSK117, pAK522, and pAK531.

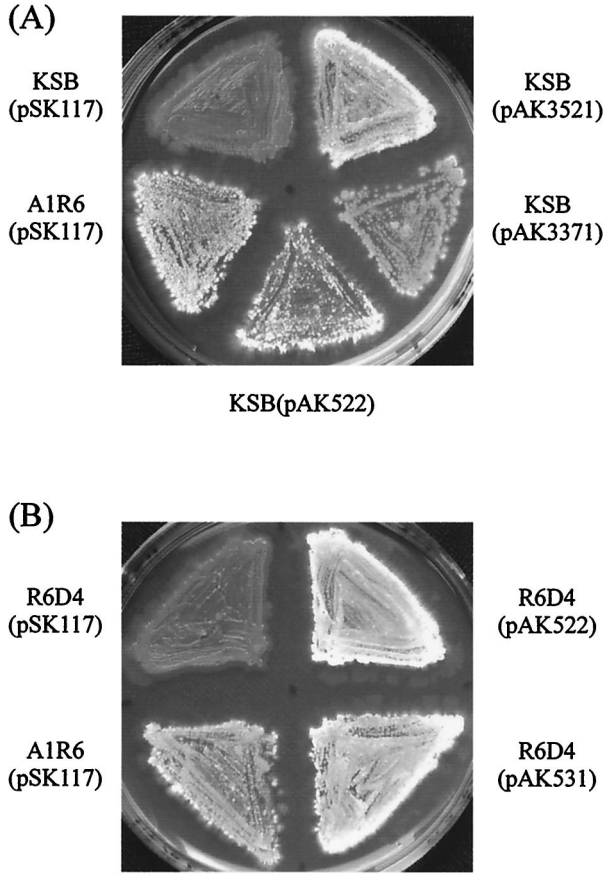

FIG. 4. Aerial mycelium formation by transformants of mutant KSB (A) and disruptant mutant R6D4 (B). Aerial hypha formation was examined qualitatively by inoculation of the transformants on GMY media as described in Materials and Methods. The transformants of wild-type strain A1R6 with pSK117 were used as a positive control. (A) Clockwise, from top left, mutant KSBs carrying pSK117, pAK3521, pAK3371, and pAK522 and strain A1R6 harboring pSK117. (B) Clockwise, from top left, disruptant mutant R6D4s carrying pSK117, pAK522, and pAK531 and strain A1R6 harboring pSK117.

downstream of the *ermE* promoter containing *ermE*p1 and *ermE*p2 in order that possible influences of the promoters should be avoided. Despite the complementary expression of *rpoZ* in the transformants, we have failed to identify any typical streptomycete promoter sequences in the upstream region of the gene. However, there still remained the possibility of transcriptional readthrough from the *tsr* promoters.

Isolation of *rpoZ*-disrupted mutant R6D4 and phenotypic complementation by *rpoZ*. We constructed pAK3741 by inserting Tn*5 aphII* conferring resistance to kanamycin into a unique *Sal*I site located in the middle of *rpoZ* from *S. kasugaensis* (Fig. 6). As described in Materials and Methods, we isolated disruptant mutant R6D4, in which the kanamycin resistance gene was inserted into the *Sal*I site of the chromosomal *rpoZ* of wild-type strain A1R6 in the opposite direction.

We found that disruptant mutant R6D4 neither produced kasugamycin nor formed aerial mycelium, in a very similar manner to strain KSB. In addition, as similarly observed with mutant KSB, mutant R6D4 retained its aerial mycelium-minus phenotype when grown on modified GMY medium containing mannitol as a sole carbon source or in close proximity to wild-type strain A1R6. The transformants of mutant R6D4 with pAK522 carrying *rpoZ* exhibited kasugamycin synthesis (Fig. 3B) and morphological differentiation (Fig. 4B). It should be noted that the antibiotic accumulation by the transformant harboring pAK522 was similar to that by wild-type strain A1R6 carrying pSK117, in contrast to that by the KSB transformant with the same plasmid.

Sequencing analysis of *rpoZ* region in mutant KSB. We determined the nucleotide sequence of the *rpoZ* region in mutant KSB and found a 2-bp (GC) deletion in the middle of the gene, which probably resulted in a truncated, incomplete ω protein of 47 amino acids (Fig. 7). Taken together with the observation on *rpoZ*-disrupted mutant R6D4, we conclude that a defective *rpoZ* results in the pleiotropic phenotype of the *S. kasugaensis* mutants. In other words, *rpoZ* is crucially related to antibiotic production and morphogenesis in *S. kasugaensis*.

Complementation of *S. kasugaensis rpoZ*-disrupted mutant R6D4 by *S. coelicolor rpoZ*. We amplified the 0.82-kb *rpoZ*-encoding region of *S. coelicolor* M145 by PCR and inserted the PCR product into pSK1172 to produce pAK531 as detailed in Materials and Methods. The cloned region corresponded to the 0.79-kb *rpoZ*-encoding region of *S. kasugaensis* subcloned in pAK522. The transformants of disruptant mutant R6D4 with pAK531 harboring *S. coelicolor rpoZ* formed aerial my-

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S.kasugaensis       ----------MSSSIT----------APEGIINPPIDELLEAT  23
S.coelicolor        ----------MSSSIS----------APEGIINPPIDELLEAT  23
M.tube.H37Rv        ----------MSISQSDASLAAVPAVDQFDPSSGASGGYDTPLGITNPPIDELLDRV  47
M.tube.CDC1551      MTHVVTSQYLAFQAALRRQEKFHVSISQSDASLAAVPAVDQFDPSSGASGGYDTPLGITNPPIDELLDRV  70
M.leprae            ----------MSIPQSNTSLSAVIAVDQFDPSSGGQGVYDTPLGITNPPIDELLDRV  47
                              :* . :                    :* ** ********: .

S.kasugaensis       DSKYSLVIYAAKRARQINAYYSQLGEGLLEYVGPLVDTHVHEKPLSIALREINAGLLTSEAIEGPAQ   90
S.coelicolor        DSKYSLVIYAAKRARQINAYYSQLGEGLLEYVGPLVDTHVHEKPLSIALREINAGLLTSEAIEGPAQ   90
M.tube.H37Rv        SSKYALVIYAAKRARQINDYYNQLGEGILEYVGPLVEPGLQEKPLSIALREIHADLL--EHTEGE--  110
M.tube.CDC1551      SSKYALVIYAAKRARQINDYYNQLGEGILEYVGPLVEPGLQEKPLSIALREIHADLL--EHTEGE--  133
M.leprae            SSKYALVIYAAKRARQINDHYNQLGEGILEYVGPLVEPGLQEKPLSIAMREIHADLL--EHTEGE--  110
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FIG. 5. Multiple alignment of the amino acid sequences of subunit of ω in *S. kasugaensis*. With the ClustalX program (available at innprot.weizmann.ac.il/software/ClustalX.html), sequences of ω subunits from the following genome-sequenced actinomycetes were analyzed: *S. coelicolor* (CAB93358), *M. tuberculosis* H37Rv (CAB02173), *M. tuberculosis* CDC1551 (AAK45700), and *M. leprae* (CAC30050). Asterisks indicate fully conserved amino acids, colons represent that one of the "strong" groups of amino acids (such as STA, MILV, NDEQ, HY, and NHQK) is fully conserved, and dots indicate that one of the "weak" groups of amino acids (such as STPA, ATV, and SGND) is fully conserved.

celium (Fig. 4B) and produced kasugamycin, similar to transformants of parental strain A1R6 with pSK117 and mutant R6D4 with pAK522 carrying *S. kasugaensis rpoZ* (Fig. 3B). These results indicate that *rpoZ* of *S. coelicolor* is functionally expressed and substitutes for the indigenous *rpoZ* in *S. kasugaensis*.

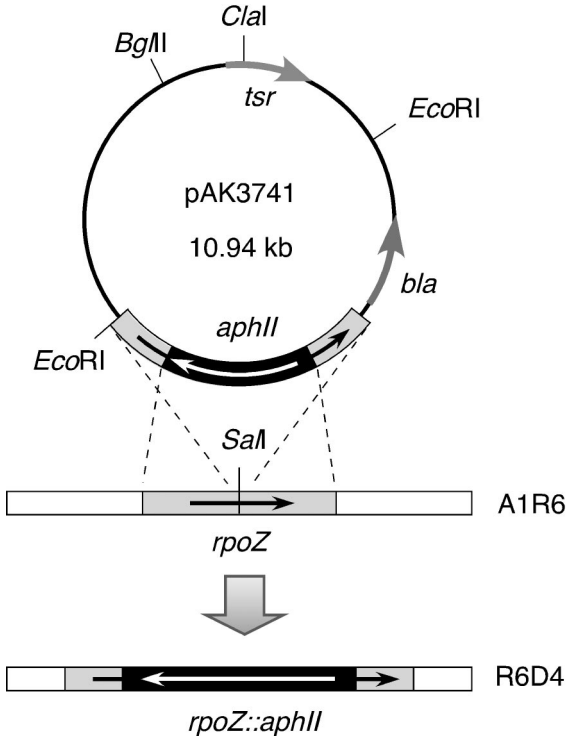

FIG. 6. Restriction map of pAK3741 used for generation of disruptant mutant R6D4. A shuttle vector pAK3741 composed of 5.57- and 5.37-kb *Eco*RI digests of *S. kasugaensis* plasmid pSK21-K2 carrying *tsr* (31) and pAK374, whose construction is detailed in Materials and Methods. In the plasmid, *bla* indicates β-lactamase originating from pUC18. As illustrated, a homologous recombination was expected to occur between *aphII*-disrupted *rpoZ* and chromosomal *rpoZ* of wild-type strain A1R6.

DISCUSSION

We have discovered in this study that the *rpoZ* gene that encodes the RNAP ω subunit is required for antibiotic production and morphogenesis in *S. kasugaensis*. The *rpoZ* genes have been found in all the sequenced genomes of free-living bacteria. In *E. coli*, ω binds to the β′ subunit to form the RNAP core enzyme, consisting of four subunits (2α, β, β′, and ω) (13). Moreover, bacterial ω subunits (including *S. coelicolor* ω) which are structural and functional homologs of archaeal RNAP subunit RpoK and eukaryotic RNAP I, II, and III subunit RPB6, are suggested to facilitate association with the $\alpha_2\beta$ assembly through binding to β′ (29). Apart from the physical findings, however, little has been known about a physiological effect of bacterial ω except that an ω-null mutant of *E. coli* exhibits the slow-growth phenotype and induces the molecular chaperonin GroEL to structurally maintain RNAP devoid of ω (30).

By cloning and subsequent complementation experiments using pleiotropic *S. kasugaensis* mutant KSB whose phenotype is aerial-mycelium- and kasugamycin-minus, we found that the responsible gene is *orf5*, which encodes 90 amino acids. On the basis of the results with BLAST searches, we conclude that *orf5* is the *rpoZ* encoding RNAP subunit ω in *S. kasugaensis*. Disruption of *rpoZ* of wild-type strain A1R6 by insertion of *aphII* produced mutant R6D4, which neither produced kasugamycin nor formed aerial mycelium in a phenotypically similar manner to mutant KSB. Furthermore, we showed that the pleiotropic phenotype of mutant KSB is due to a 2-bp frameshift deletion in the *rpoZ*-coding region, which probably results in a truncated, incomplete ω subunit of 47 amino acids. These results verify the involvement of *rpoZ* in the antibiotic and aerial hypha production in *S. kasugaensis*. Subunits ω of *S. kasugaensis* and *S. coelicolor* are conserved in constitutive amino acids except for the sixth amino acid residue, and *rpoZ* from *S.*

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          M  S  S  S  I  T  A  P  E  G  I  I  N  P  P  I  D  E  L  L  E  A  T  D  S  K  Y  S  L  V    30
A1R6  GTGTCCTCTTCCATCACCGCACCCGAGGGCATCATCAATCCGCCAATTGATGAGCTTCTCGAGGCCACCGACTCGAAGTACAGCCTCGTG   90
KSB   GTGTCCTCTTCCATCACCGCACCCGAGGGCATCATCAATCCGCCAATTGATGAGCTTCTCGAGGCCACCGACTCGAAGTACAGCCTCGTG   90
          M  S  S  S  I  T  A  P  E  G  I  I  N  P  P  I  D  E  L  L  E  A  T  D  S  K  Y  S  L  V    30

          I  Y  A  A  K  R  A  R  Q  I  N  A  Y  Y  S  Q  L  G  E  G  L  L  E  Y  V  G  P  L  V  D    60
A1R6  ATCTACGCCGCCAAGCGCGCGCGCCAGATCAACGCGTACTACTCGCAGCTCGGTGAGGGCCTGCTCGAGTACGTCGGCCCCCTCGTCGAC  180
KSB   ATCTACGCCGCCAAGCGCGCGC--CAGATCAACGCGTACTACTCGCAGCTCGGTGAGGGCCTGCTCGAGTACGTCGGCCCCCTCGTCGAC  178
          I  Y  A  A  K  R  A     P  D  Q  R  V  L  L  A  A  R  *                                   47

          T  H  V  H  E  K  P  L  S  I  A  L  R  E  I  N  A  G  L  L  T  S  E  A  I  E  G  P  A  Q  *    90
A1R6  ACGCACGTCCACGAGAAGCCGCTGTCGATCGCGCTCCGCGAGATCAACGCGGGCCTGCTGACCTCCGAGGCCATCGAGGGCCCGGCCCAGTAA  270
KSB   ACGCACGTCCACGAGAAGCCGCTGTCGATCGCGCTCCGCGAGATCAACGCGGGCCTGCTGACCTCCGAGGCCATCGAGGGCCCGGCCCAGTAA  268
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FIG. 7. Comparison of nucleotide sequences of *rpoZ* from strain A1R6 and mutant KSB. Hyphens in the *rpoZ*-encoding region from mutant KSB indicate gaps in the nucleotide sequence.

coelicolor rescued defective *rpoZ* of *S. kasugaensis* mutant, which may indicate the possible involvement of *rpoZ* in the pleiotropic events of *S. coelicolor* as well. Previously, a gene designated *rpoZ* that encodes the sporulation-controlling σ factor of 278 amino acids, a homolog of *S. coelicolor whiG*, was cloned from *Streptomyces aureofaciens* (22). Neither ω of *S. kasugaensis* nor that of *S. coelicolor* show any similarity to the *rpoZ*-encoding product of *S. aureofaciens*. Southern hybridization analysis indicated no highly homologous DNA region with *rpoZ* in *S. kasugaensis* genome (data not shown) and a homology search of *S. coelicolor* database revealed no homologs of *S. kasugaensis* ω except that from the indigenous gene, which might indicate that *rpoZ* is solely present in the genomes of *S. kasugaensis* and *S. coelicolor*.

The complementary restoration of kasugamycin production as observed in Fig. 3 clarified a genetic difference between NTG-derived mutant KSB and *rpoZ*-disrupted mutant R6D4. Introduction of *rpoZ* alone via pAK522 rescued the disruptant mutant to accumulate the antibiotic to a level of kasugamycin accumulation similar to that of wild-type strain A1R6. Meanwhile, pAK3541 or pAK3521 returned mutant KSB to produce kasugamycin to a similar level of kasugamycin accumulation to strain A1R6, but pAK522 or pAK3371 to accumulate the antibiotic to a far lower level. Plasmids pAK3451 and pAK3521 bore three complete *orf2*, *orf3*, and *orf4* in common together with *rpoZ*, and *rpoZ* resided 5.8 kb and 10.5 kb downstream from the *ermE* promoter, respectively. Furthermore, a plausible terminator located in the 193-bp region between *orf2* and *orf3* (data not shown) was positioned upstream of *rpoZ* in both plasmids. The *rpoZ* expression was therefore thought to remain unaffected by transcriptional readthrough from the *ermE* promoter. Additionally, in pAK522 and pAK3371, *rpoZ* resided downstream of the *tsr* promoter in the same orientation. Taken together, it is likely that other gene(s) was impaired in mutant KSB together with *rpoZ* and that a combination of *orf2*, *orf3*, or *orf4* with *rpoZ* turned the mutant into a wild-type producer of kasugamycin.

Many genetic studies have been conducted on the pleiotropy in streptomycetes, but none has reported the importance of *rpoZ* and its product ω. Moreover, on the possible involvement of ω in bacterial physiology, the slow-growth phenotype of an *E. coli* ω deletion mutant (30) is the only indication presented to date. We believe that the results presented here provide a new insight into studies on bacterial *rpoZ* and RNAP ω.

Initially, we speculated that mutant KSB might be included in a group of *bld* mutants isolated from *S. coelicolor*, such as *bldA*, *bldB*, *bldC*, *bldD*, *bldG*, and *bldH* mutants, that lack glucose catabolite repression of galactose utilization (37). Of the *bld* mutants, *bldA*, *bldC*, *bldD*, *bldG*, and *bldH* mutants display the aerial-mycelium-plus phenotype when mannitol is replaced by glucose in minimum agar medium. Extracellular molecules such as SapB relate to the aerial mycelium formation in *S. coelicolor* (32, 40, 41), and according to the extracellular complementation, the *bld* mutants except for *bldB* mutants can be sorted into a hierarchical grouping (33). The *bldB* mutants that are catabolite derepressed for genes for glycerol utilization and agar decomposition fail to fit into the grouping (33, 40) and neither sporulate nor produce antibiotics, regardless of carbon sources (7, 28). The phenotypic observations on *bldB* mutants are very similar to mutants KSB and R6D4. However, we found no homology between the 90-amino-acid ω of *S. kasugaensis* and 98-amino-acid BldB of *S. coelicolor.* Although the presence of *bld* genes has not been examined in *S. kasugaensis*, these results indicate that *rpoZ* is involved in the pleiotropy of *S. kasugaensis* in a manner different from that of the *bld* genes of *S. coelicolor*.

Intracellular accumulation of ppGpp above certain limits correlates with the onset of antibiotic biosynthesis in various *Streptomyces* species (6, 18, 27). The disruption of *relA* coding for ppGpp synthetase turns the wild-type phenotype of *S. coelicolor* into the pleiotropic phenotype on some agar media (5). Physically, ppGpp binds to the N-terminal portion of β′ (38) together with the C-terminal of β in *E. coli* RNAP (9). Although ω binds to β′, we are skeptical about a possible physiological connection of ppGpp with ω in the pleiotropy of *S. kasugaensis*, because an ω-null mutant of *E. coli* is known to exhibit a normal wild-type stringent response (14).

Although an ω-less mutant of *E. coli* grows to a lower cell density than the normal strain (30), mutant R6D4 reached the same levels of cell growth as wild-type strain A1R6 in GPY and other liquid media (data not shown), which may indicate that ω is not required for growth of *S. kasugaensis*. However, as reported with *E. coli* RNAP, the N-terminal domain of ω with 52 of 91 amino acids is capable of assembling a functional RNAP core enzyme (15). To further study the relation of *rpoZ* and its product ω with the growth of *S. kasugaensis*, we need to construct ω-null mutants.

As the *rpoZ* genes are present in all bacteria, it seems unlikely that *rpoZ* of *S. kasugaensis* controls directly and simultaneously both kasugamycin production and aerial mycelium formation. It is of great importance therefore to examine whether a functional role of *S. kasugaensis* ω is fundamentally

equivalent to *E. coli* ω. We will study the detailed mechanism how *rpoZ* is involved in the streptomycete-specific events in *S. kasugaensis* and investigate possible involvement of the gene in the pleiotropy in *S. coelicolor* that produces polyketide antibiotics, a class structurally different from the aminoglycoside antibiotics.

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