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Deletion of *ku* homologs increases gene targeting frequency in *Streptomyces avermitilis*

Xiaojuan Zhang · Wei Chen · Yang Zhang · Libin Jiang · Zhi Chen · Ying Wen · Jilun Li

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Abstract *Streptomyces avermitilis* is an industrially important soil bacterium known for production of avermectins, which are antiparasitic agents useful in animal health care, agriculture, and treatment of human infections. *ku* genes play a key role in the non-homologous end-joining pathway for repair of DNA double strand breaks. We identified homologs of eukaryotic *ku70* and *ku80* genes, termed *ku1* and *ku2*, in *S. avermitilis*. Mutants with deletion of *ku1*, *ku2*, and both genes were constructed and their phenotypic changes were characterized. Deletion of *ku* genes had no apparent adverse effects on growth, spore formation, or avermectin production. The *ku* mutants, in comparison to wild-type strain, were slightly more sensitive to the DNA-damaging agent ethyl methanesulfonate, but not to UV exposure or to bleomycin. Gene targeting frequencies by homologous recombination were higher in the *ku* mutants than in wild-type strain. We conclude that *ku*-deleted strains will be useful hosts for efficient gene targeting and will facilitate functional analysis of genes in *S. avermitilis* and other industrially important bacterial strains.

Keywords *Streptomyces avermitilis* · *ku* gene · Non-homologous end-joining (NHEJ) · Homologous recombination (HR) · Gene targeting frequency

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X. Zhang · W. Chen · Y. Zhang · L. Jiang · Z. Chen · Y. Wen (✉) · J. Li
State Key Laboratories for Agro-biotechnology and College of Biological Sciences, China Agricultural University, Beijing 100193, People's Republic of China
e-mail: wen@cau.edu.cn

Abbreviations

DSBs	Double strand breaks
HR	Homologous recombination
NHEJ	Non-homologous end-joining
HPLC	High-performance liquid chromatography
EMS	Ethyl methanesulfonate
MMS	Methyl methanesulfonate

Introduction

The repair of DNA double strand breaks (DSBs), a crucial process for genome stability [30], takes place in eukaryotic cells by two pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ) [13]. The HR pathway requires interaction between homologous sequences and leads to accurate damage repair. In the NHEJ pathway, DSBs are repaired by direct ligation of two broken ends of DNA without regard to their sequence homology, leading to inaccurate connections. NHEJ depends on the Ku protein, which binds to DNA ends and has end-bridging activity. Ku is a heterodimer consisting of two subunits of ~70 and 80 kDa, termed Ku70 and Ku80, respectively [22]. These two repair mechanisms act independently and function competitively in eukaryotes [27], and the rate of HR in a given host determines the efficiency of gene targeting. Yeast uses mainly the HR system, with highly efficient gene targeting, for DSB repair [10]. In contrast, humans, other mammals, nematodes, plants, and filamentous fungi use primarily NHEJ for DSB repair, and gene targeting efficiency in these organisms is generally lower than in yeast. Deletion of the *ku* gene could increase HR activity, thereby facilitating the study of gene targeting and gene function. This approach has been well utilized in studies of filamentous fungi, including *Neurospora crassa*

[20], *Hypocrea jecorina* [31], *Claviceps purpurea* [8], *Magnaporthe grisea* [28], and *Aspergillus* spp. [14, 17, 19, 24, 25].

Homologs of Ku protein are also found in archaea and bacteria and help elucidate their evolutionary conservation. Aravind and Koonin [1] found a highly conserved Ku core domain that formed the predicted DSB repair system with ligase and primase in the genomes of *Bacillus subtilis*, *Archaeoglobus fulgidus*, *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, and *Mesorhizobium loti*. Functional NHEJ systems have been identified experimentally in *B. subtilis* [18, 29], *M. tuberculosis* [7], and *Sinorhizobium meliloti* [12]. However, the function of Ku homologs in *Streptomyces* has not been reported previously.

Streptomyces is a genus of Gram-positive, filamentous soil bacteria known for their complex morphological differentiation and production of a broad range of useful secondary metabolites such as antibiotics, immunosuppressants, and cholesterol-lowering agents [6]. In the present study, we identified *ku70* and *ku80* homologs, termed *ku1* and *ku2*, in avermectin producer *S. avermitilis*. Avermectins are a series of 16-membered macrocyclic lactones with potent anthelmintic properties and are widely used in agriculture, veterinary medicine, and human medicine [3, 21]. *ku1*-, *ku2*-, and *ku1ku2*-deleted mutants were constructed in *S. avermitilis* and their phenotypes were investigated. These mutants displayed enhancement of gene targeting frequency without obvious phenotypic defects.

Materials and methods

Strains, plasmids, and growth conditions

S. avermitilis ATCC 31267 (wild-type strain) was grown at 28°C and used as a host strain for gene propagation and gene disruption. Solid YMS medium [9] and liquid YEME [11] medium with 25% sucrose were used for sporulation and growth of mycelia for extraction of DNA and preparation of protoplasts, respectively. Seed medium and fermentation medium [4] were used for avermectin production. RM14 [16] was used for regeneration of protoplasts and selection of transformants. *E. coli* DH5α was used as cloning host. *E. coli* ET12567 (*dam dcm hsdS*) [16] was used to propagate non-methylated DNA for transformation in *S. avermitilis*. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium and transformed as described by Sambrook et al. [23]. Antibiotics used were described previously [32]. pKC1139, a temperature-sensitive *E. coli*–*Streptomyces* shuttle vector [2], was used to construct gene deletion mutants via homologous recombination. pIJ963 [11] was used to provide hygromycin resistance gene (*hyg*).

Construction of vectors for deletion of *ku1* and *ku2*

To construct the *ku1* deletion vector, two fragments flanking *ku1* were prepared by PCR from genomic DNA of ATCC 31267. A 1,104-bp 5′ flanking region was amplified with primers ku1hz (CGGGGTACCAGTACGGCGTGTC CATCTG, *Kpn*I) and ku1hf (CTAGAATTCCAGTCGAC GAACACCTTGC, *Eco*RI), and a 890-bp 3′ flanking region was amplified with primers ku1qz (CCCAAG CTTACGGACATGTGGACATCGC, *Hin*dIII) and ku1qf (AAACTGCAGAGACCACGGCGAAGAAGAC, *Pst*I). The two PCR fragments were digested with *Eco*RI/*Kpn*I and *Pst*I/*Hin*dIII, respectively. The 1.7-kb *hyg* gene was excised from pIJ963 by *Kpn*I/*Pst*I digestion. The above three fragments were simultaneously ligated into the *Eco*RI/*Hin*dIII-digested vector pKC1139 to generate *ku1* deletion vector pKU1 (Fig. 2a).

For *ku2* deletion, a 1,374-bp fragment upstream of the *ku2* start codon was amplified with primers ku2HBqz (CTA GAATTCTCAGCCTACGAACCACTCC, *Eco*RI) and ku2HBhf (GTAGGATCCATGCCTCGAGGAAGCTGAC, *Bam*HI), and a 1,104-bp fragment downstream of the *ku2* stop codon was amplified with primers ku2QBqz (CCCAAGCT TGAGTCGATGGAGTGTGCAC, *Hin*dIII) and ku2QBhf (CTAGAATTCTGTCGCGGATGTCCTGTAC, *Eco*RI). The two PCR fragments were digested with *Eco*RI/*Bam*HI and *Hin*dIII/*Eco*RI, respectively, and were simultaneously ligated into the *Hin*dIII/*Bam*HI-digested vector pKC1139 to generate *ku2* deletion vector pKU2 (Fig. 3a).

Construction of vectors for gene targeting analysis

S. avermitilis has a linear 9.02-Mb chromosome, which consists of a highly conserved 6.5-Mb "core" internal region and two variable "auxiliary" telomeric regions: a 2.0-Mb left arm and a 0.5-Mb right arm [21]. Three gene loci on the *S. avermitilis* chromosome were selected for gene targeting: SAV741 (*sig8*, pos: 890067–890906 nt) is on the left arm, SAVt57 (*trn57*, pos: 4362610–4362695 nt) is in the central region, and SAV5775 (putative MarR-family transcriptional regulator, pos: 6981481–6981912 nt) is close to the right arm.

The vector pKC57, which contains a 0.5-kb homolog of the upstream region of SAVt57, was constructed as follows. A 509-bp fragment upstream of the SAVt57 start codon was amplified by PCR with primers 57QZ (GTCGGATC CCATCGAGGTTCCGAGCCTATC, *Bam*HI) and 57QF (ATCGAATTCGTCTCGCCCTGTCCGTAGAC, *Eco*RI). The PCR fragment was digested with *Bam*HI and *Eco*RI and was ligated into the *Bam*HI/*Eco*RI-digested vector pKC1139 to generate vector pKC57 (Fig. S1).

The vector pKCD5775 contains a 0.86-kb flanking region of SAV5775. A 456-bp fragment upstream of the

SAV5775 start codon was amplified with primers uP1 (CGGGATCCCCTCGGCGTTGGA, *Bam*HI) and uP1-2 (CCCAAGCTTATCGGGGCGGGTCTTCTG, *Hin*dIII), and a 403-bp fragment downstream of the SAV5775 stop codon was amplified with primers dP2-1 (CCCAAGCTTGCGGGACTGTCCCACGAG, *Hin*dIII) and dP2 (CGGAATTCACCGGACCGCTTCGTTGC, *Eco*RI). The two PCR fragments were digested with BamHI/*Hin*dIII and *Hin*dIII/*Eco*RI, respectively, and were simultaneously ligated into the *Bam*HI/*Eco*RI-digested vector pKC1139 to generate vector pKCD5775 (Fig. S2).

The vector pKCD741 contains a 2.1-kb flanking region of SAV741. Two fragments flanking SAV741 were prepared by PCR. A 1,019-bp 5′ flanking region was amplified with primers B5 (AAGCTTGTGGATCGCTGTCATCGTG, *Hin*dIII) and B6 (CTGCAGTGCCTCGGTCACTGTCATC, *Pst*I), and a 1,035-bp 3′ flanking region was amplified with primers B7 (GGATCCTCTCCCAGATGCACGTGTC, *Bam*HI) and B8 (GAATTCAGTACGACTGTGCCCAGAC, *Eco*RI). The two PCR fragments were digested with *Hin*dIII/*Pst*I and *Bam*HI/*Eco*RI, respectively. The 1.7-kb *hyg* gene was excised from pIJ963 by *Pst*I/*Bam*HI digestion. The above three fragments were simultaneously ligated into the *Hin*dIII/*Eco*RI-digested vector pKC1139 to generate vector pKCD741 (Fig. S3).

DNA damage sensitivity assays

S. avermitilis wild-type ATCC 31267 and *ku* deletion mutants were assayed for sensitivity to UV radiation and to the DNA-damaging agent ethyl methanesulfonate (EMS) and bleomycin. For assessment of UV sensitivity, a fresh spore suspension of *S. avermitilis* was exposed to UV light (20 W) irradiation for various durations. The distance between the plate and the UV light source was 30 cm. After UV treatment, serial dilutions of spores were spotted or spread onto YMS plates and incubated at 28°C in the dark for 5 days. For calculation of lethality rate, colonies were counted and normalized with respect to the number of colonies without UV treatment.

Sensitivity to bleomycin and EMS was assessed by both spot tests and lethality rate analysis. For spot tests, serial dilutions of spores were spotted onto YMS plates containing bleomycin (15 or 20 μg/ml) or EMS (0.5 or 0.6 μl/ml) and incubated at 28°C for 5 days. For lethality rate analysis, spores were treated with various concentrations of bleomycin for 5 min, or of EMS for 1 h, plated onto YMS plates, and incubated at 28°C in the dark for 5 days. Lethality rate was calculated as described for UV treatment.

Fermentation and HPLC analysis of avermectins

Fermentation of *S. avermitilis* ATCC 31267 and its mutants was performed as described previously [4]. Avermectins in fermentation culture were identified by HPLC analysis as described by Chen et al. [4].

Results

Analysis of *ku* genes in *S. avermitilis* and other *Streptomyces* species

The complete *S. avermitilis* genome has been sequenced [21] and the annotation of gene function reveals two genes encoding homologs of eukaryotic Ku70 and Ku80 proteins, Ku1 (SAV2945) and Ku2 (SAV879) (http://avermitilis.ls.kitasato-u.ac.jp). Ku1 consists of 366 aa and Ku2 consists of 319 aa. Both Ku1 and Ku2 have a possible functional Ku70/Ku80 beta-barrel domain. Homologs of Ku1 and Ku2 in *Streptomyces* were searched for by the BLAST program. Sequences were aligned using the MEGA5 program [26], and phylogenetic trees were constructed by the neighbor-joining method (Fig. 1). Although homologs of Ku2 are not commonly present in *Streptomyces*, homologs of Ku1 are widely distributed, suggesting the biological significance of Ku protein in *Streptomyces*.

Isolation of *ku* deletion mutants

To investigate the function of *ku* genes in *S. avermitilis*, mutants in which *ku1*, *ku*2, or both genes were deleted were constructed by homologous recombination using plasmids pKU1 and pKU2. Introduction of pKU1 into the wild-type strain ATCC 31267 and selection of double-crossover recombinant strains were performed as described previously [32]. pKU1 was introduced into protoplasts of ATCC 31267 and transformants were confirmed by colony PCR. Spores of transformants containing pKU1 were harvested and spread on YMS agar containing apramycin. These cultures were grown for 2 days at 28°C, then for 7–10 days at 39°C. Vector pKC1139 has a temperature-sensitive replicon and cannot replicate itself in *Streptomyces* at temperatures above 34°C; therefore, only mutants in which pKU1 was inserted into the chromosome of ATCC 31267 by a single crossover could grow on YMS containing apramycin at 39°C. Insertion mutants were confirmed by PCR analysis and inoculated on nonselective YMS plates at 28°C to generate double-crossover gene replacement mutants. After four passages on nonselective medium, the putative *ku1*-deleted strains (termed Δku1) were selected by both apramycin sensitivity and hygromycin resistance and confirmed by PCR analysis using primers ku1up (TGGTTGCAGGTGTGAGACG), ku1down (CCATCTCGTACGGCATCATC), hqf (CGGGATCGCCAATCTCTAC), hhz (CCATCCCAGCTCGGCAAC), ku1A (CTTGGCGGTGGTCTTCTTC), and ku1B (ACACGCCGTACTACCTGTC) (Fig. 2a).

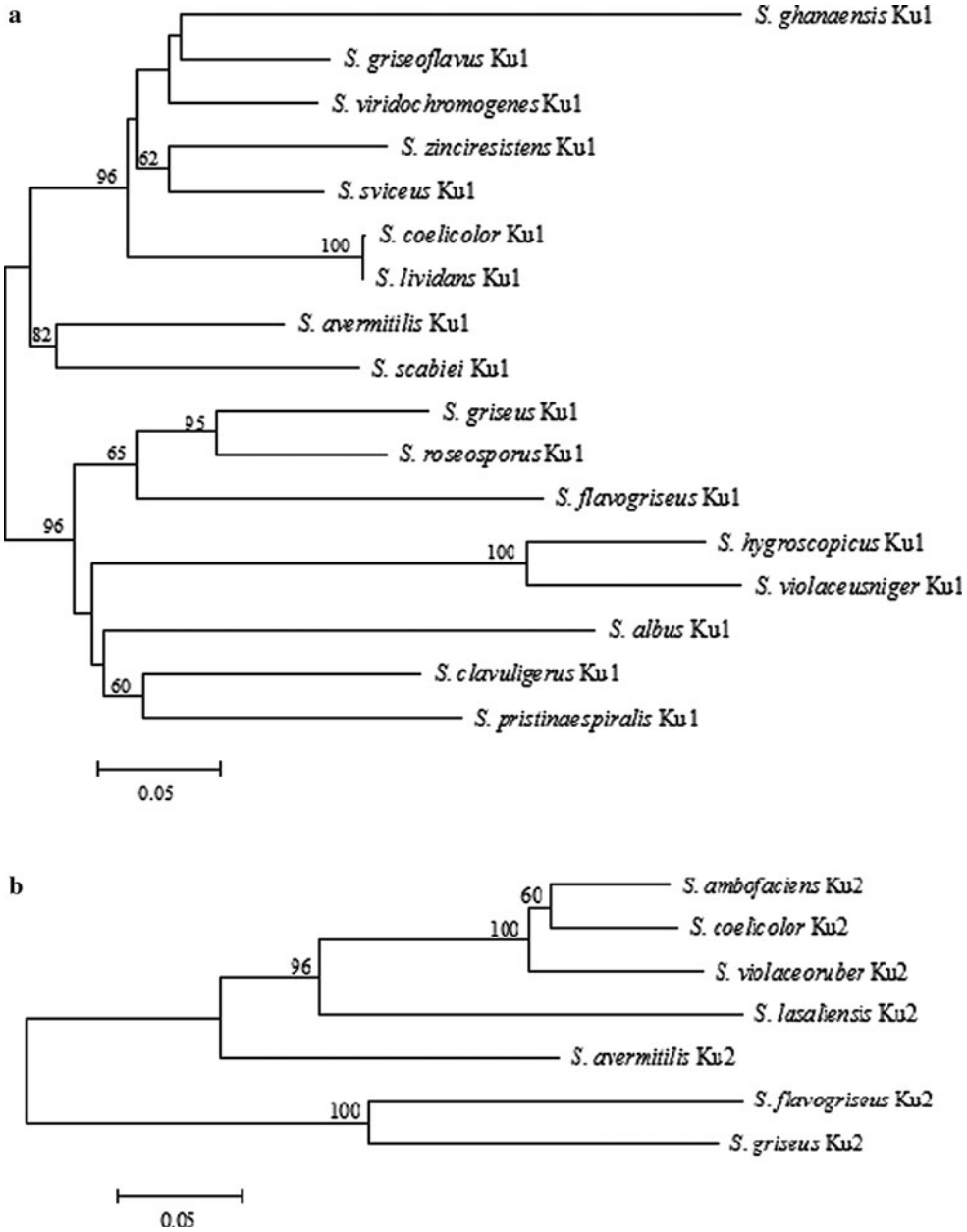

Fig. 1 Phylogenetic relationship among Ku proteins in *Streptomyces*. **a** Phylogenetic tree of Ku1 proteins. **b** Phylogenetic tree of Ku2 proteins. Amino acid sequences were analyzed and trees were calculated using the MEGA5 program [26]. The *number* at each *node* indicates the percentage of 1,000 bootstrap replicates required for statistical significance. The *bar* in the *lower left corner* represents 0.05 amino acid substitution per amino acid for the branch length

Primers ku1up and ku1down flank the exchange region, primers hhz and hqf are specific for *hyg*, and primers ku1A and ku1B are located within the deletion region of *ku1*. When primer pairs ku1down/hqf and hhz/ku1up were used for Δku1 total DNA, a 1,786-bp and a 1,121-bp band appeared, respectively, whereas such bands were not detected from genomic DNA of the wild-type strain. In contrast, when primers ku1A and ku1B were used, only the wild-type strain produced a 698-bp fragment as predicted (Fig. 2b). There results indicate that Δku1 was a *ku1* gene deletion mutant in which the *ku1* gene was replaced by *hyg*.

pKU2 was transformed into the wild-type strain to produce a *ku2* gene deletion mutant with apramycin sensitivity. The putative *ku2* mutant was termed Δku2 and was confirmed by PCR using primers ku2qz (GGTGAT GGCTGACGTGATC), ku2qbm (TGCTCGTCGTCTTCA CTCC), ku2hf (GTGCTCCATGTCGTCGAAC), ku2A (CT TCTTCGTGGGCTCCTTC), and ku2B (CAAGTACTGC GAGCTGGAG) (Fig. 3a). Primers ku2qz and ku2hf flank the exchange region, ku2A and ku2B are located within the deletion region of *ku2*, and ku2qbm is inside the 3′ exchange region. When primer pairs ku2qz/ku2hf and ku2qbm/ku2hf were used for Δku2 total DNA, a 2,957-bp and a 1,493-bp band appeared, respectively, whereas such bands were not detected when genomic DNA of ATCC 31267 was used. In contrast, when primers ku2A and ku2B were used, only the wild-type strain produced a 757-bp fragment as predicted (Fig. 3b). These results indicate that

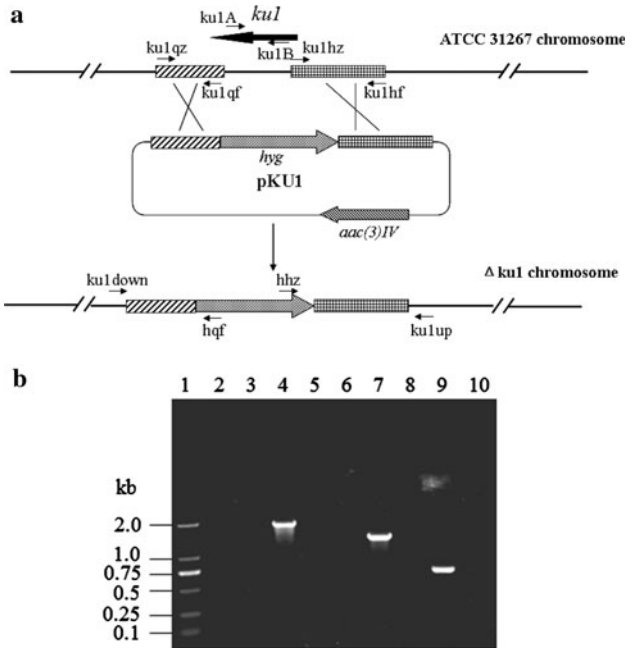

Fig. 2 Construction of *ku1* gene deletion mutant. **a** Strategy for deletion of *ku1* gene. *Long broad arrows* indicate genes and their directions. *Short small arrows* indicate positions of primers used for cloning exchange regions and confirming gene deletions, as described in "Materials and methods". Double-crossover recombination led to *ku1* deletion. **b** PCR analysis to confirm deletion of *ku1* in mutant Δku1. *Lane 1* DL2000 marker, *Lanes 2, 5, 8* PCR system without template DNA used as negative control, *Lanes 3, 6, 9* PCR products from ATCC 31267, *Lanes 4, 7, 10* PCR products from Δku1, *Lanes 2–4* using primer pair ku1down/hqf, *Lanes 5–7* using primer pair hhz/ku1up, *Lanes 8–10* using primer pair ku1A/ku1B

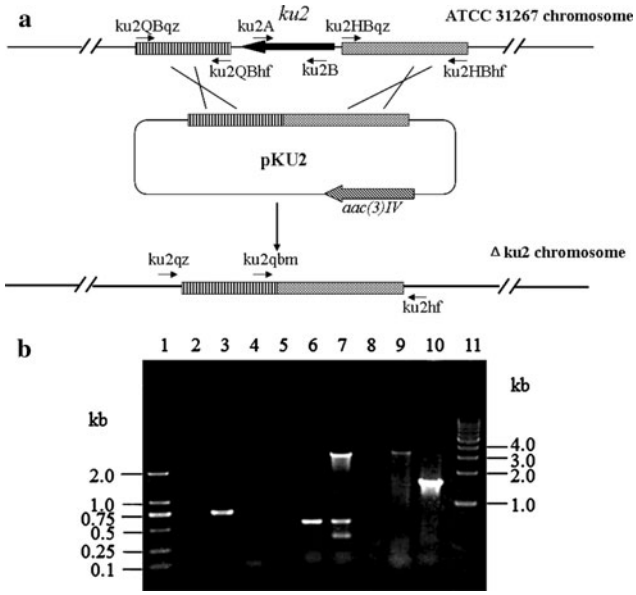

Fig. 3 Construction of *ku2* gene deletion mutant. **a** Strategy for deletion of *ku2* gene. **b** PCR analysis to confirm deletion of *ku2* in mutant Δku2. *Lane 1* DL2000 marker, *Lanes 2, 5, 8* PCR system without template DNA used as negative control, *Lanes 3, 6, 9* PCR products from ATCC 31267, *Lanes 4, 7, 10* PCR products from Δku1, *Lane 11* 1-kb marker, *Lanes 2–4* using primer pair ku2A/ku2B, *Lanes 5–7* using primer pair ku2qz/ku2hf, *Lanes 8–10* using primer pair ku2qbm/ku2hf

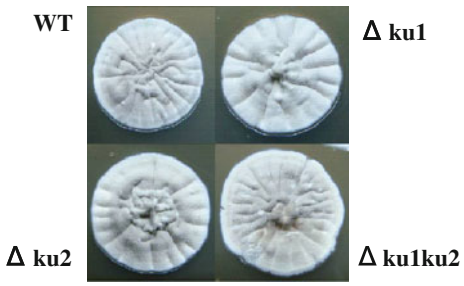

Fig. 4 Growth of *ku* mutants and wild-type strain (WT). Strains were grown for 7 days on YMS agar

ku2 gene was deleted in Δku2 by double-crossover recombination.

To construct the *ku1ku2* double deletion mutant, Δku1 was transformed with the *ku2* deletion vector pKU2. The expected mutant, termed Δku1ku2, was isolated using the same strategy as described for selection of the *ku2* deletion mutant Δku2 and was confirmed by PCR analysis using the same primer pairs (data not shown).

Phenotypic characterization of *ku* deletion mutants

Deletion of *ku* genes did not cause alteration of growth behavior of the *S. avermitilis ku* mutant strains as compared to wild-type strain in cultivation on YMS plates (Fig. 4). Spore formation and pigment production also occurred normally in the mutant strains.

To determine the role of *ku* genes in avermectin production, the *ku* mutants and wild-type strain were cultured separately in fermentation medium for 10 days. HPLC analysis of the fermentation products revealed no marked difference in avermectin yield between the *ku* mutants and wild-type strain (data not shown), suggesting that *ku* genes are not involved in avermectin biosynthesis in *S. avermitilis*.

In the assays for sensitivity to UV and DNA-damaging agents EMS and bleomycin, spot tests did not show any marked differences in sensitivity among the various mutant and wild-type strains (Fig. 5a). In lethality rate analysis, the *ku* mutants showed slightly higher sensitivity to EMS in comparison to wild type at concentrations higher than 3% (v/v). Sensitivity of the mutants to bleomycin treatment and to UV exposure was the same as that of wild type (Fig. 5b).

Increased gene targeting frequency in *ku* mutants

To examine the effect of *ku* deletion on gene targeting in *S. avermitilis*, the *ku* mutants and wild-type strain were used as recipients and various chromosomal loci were selected as targets. Since homologous recombination

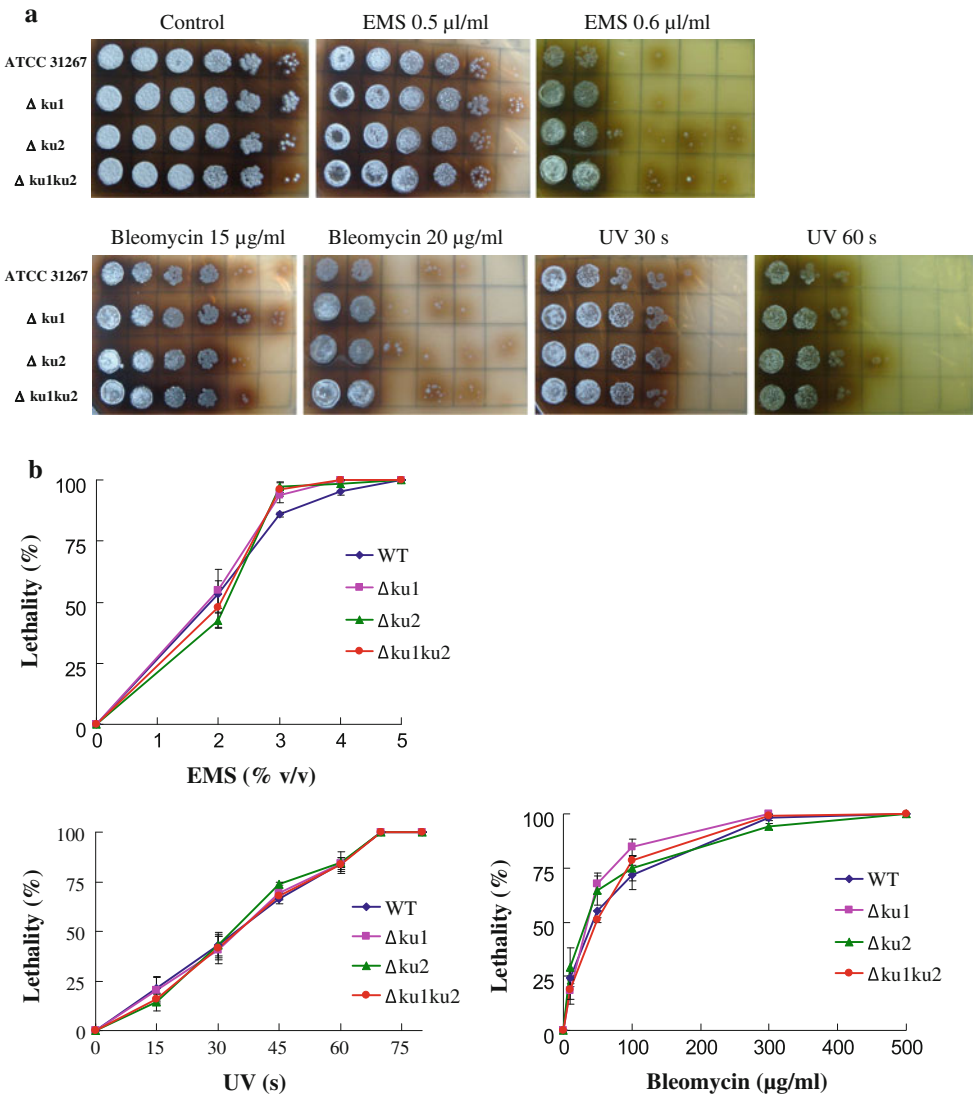

Fig. 5 Sensitivity of *ku* mutants and wild-type strain to EMS, bleomycin, and UV radiation. **a** Spot tests were performed to measure survival of various strains on YMS plates containing EMS (0.5 or 0.6 µl/ml) or bleomycin (15 or 20 µg/ml). UV irradiation was for 30 or 60 s. **b** Lethality of various strains after treatment with EMS, bleomycin, or UV exposure

frequencies are often low in *Streptomyces*, it is difficult to isolate double-crossover recombination mutants from primary transformants in one step through antibiotic resistance selection. It is easier to isolate single-crossover intermediates and grow them on nonselective plates for several rounds, and then isolate the desired second crossover product. When transformants containing the temperature-sensitive pKC1139-based vector inserted by the homologous fragment are grown at 39°C, one can generate single-crossover mutants and calculate the homologous integration frequency because only mutant in which the vector is integrated into the target locus by a single crossover can grow at this temperature. We therefore examined the relative rate of homologous integration by single crossover for gene targeting analysis in the *ku* mutants and wild-type strain.

The *S. avermitilis* strains were first transformed with plasmid pKC57 containing a 0.5-kb homolog of the upstream region of SAVt57 and the resulting transformants were screened at 39°C on YMS plates containing apramycin. Normal colony growth indicates that pKC57 is integrated into the SAVt57 locus. Of the 963 transformants tested in Δku1, 132 colonies displayed normal growth, suggesting that 13.7% of the transformants resulted from a single-crossover HR event. The targeting frequencies in Δku2 and Δku1ku2 were 11.6 and 13.6%, respectively. In contrast, with wild-type strain as recipient, only 77 colonies out of 1,099 tested transformants grew normally (targeting frequency 7.0%). In PCR analysis using primers 88q (TG TCTGCATTCCACCATCC) and RV-M (GAGCGGA TAACAATTTCACACAGG) (Fig. S1), a 0.7-kb band specific for homologous integration appeared in all the normally growing colonies.

In the case of SAV5775 locus, the *ku* mutants and wild-type strain were separately transformed with pKCD5775 containing a 0.86-kb homologous region flanking the SAV5775 gene. It was assumed that a single homologous crossover occurring on the 3′ flanking region would result in amplification of a 1,001-bp PCR fragment using primers

Table 1 Homologous integration frequencies at the SAVt57, SAV5775, and SAV741 loci in wild-type and *ku* mutant strains

Gene locus	Length of homology (kb)	Strains	Single-crossover mutants	Transformants	Targeting frequency (%)
SAVt57	~0.5	ATCC 31267	77	1,099	7.0
		Δku1	132	963	13.7
		Δku2	120	1,031	11.6
		Δku1ku2	156	1,147	13.6
SAV5775	~0.86	ATCC 31267	242	842	28.7
		Δku1	287	751	38.2
		Δku2	308	805	38.3
		Δku1ku2	377	904	41.7
SAV741	~2.1	ATCC 31267	823	1,019	80.8
		Δku1	892	984	90.7
		Δku2	883	983	89.8
		Δku1ku2	1,065	1,098	97.0

Targeting frequency single-crossover mutants/transformants

vp2 (CGAGCGTGTACGACCGGA) and up1 (CGGGAT CCCCTCGGCGTTGGA), and that such crossover occurring on the 5′ flanking region would result in a 894-bp PCR fragment using primers vp1 (CAGCCCAGGAAGTG CAGG) and dp2 (CGGAATTCACCGGACCGCTTCGT TGC) (Fig. S2). Of the 751 transformants tested, 287 showed a single crossover on either side of the target gene SAV5775 in Δku1, giving a targeting frequency of 38.2%. Similar targeting frequencies were found for Δku2 (308/805; 38.3%) and Δku1ku2 (377/904; 41.7%), whereas the targeting frequency in wild-type stain was much lower (242/842; 28.7%).

In the case of SAV741 locus, the strains were transformed with pKCD741 containing a 2.1-kb homologous region flanking the SAV741 gene. It was assumed that a single crossover occurring on the 5′ flanking region would result in amplification of a 1.4-kb PCR fragment using primers B17 (CTCTGATCCTCTTCGGTGC) and hqf (CGGGATCGCCAATCTCTAC), and that such crossover occurring on the 3′ flanking region would result in a 1.3-kb PCR fragment using primers B18 (GCGTAGATGG GAGTGGTGC) and hhz (CCATCCCAGCTCGGCAAC) (Fig. S3). The single-crossover HR frequency on either side of SAV741 was 90.7% (892/984) in Δku1, 89.8% (883/983) in Δku2, 97.0% (1,065/1,098) in Δku1ku2, and 80.8% (823/1,019) in wild-type strain.

Taken together, these results clearly show that deletion of *ku* genes in *S. avermitilis* increased the frequency of gene targeting by homologous integration.

Relationship between length of homologous region and gene targeting frequency

We further examined the relationship between gene targeting frequencies and the length of the homologous sequence. As shown in Table 1, when the homologous region was 2.1 kb, targeting frequencies were high in both wild-type strain and *ku* mutants (80.8–97.0%); the targeting frequency decreased to 28.7–41.7% for homology length 0.86 kb and to 7.0–13.7% for homology length 0.5 kb. Compared to wild-type strain, gene targeting frequencies for each homology length were higher in all *ku* mutants. These results indicate that homology length is directly correlated with targeting frequency and that *ku* deletion enhances homologous integration at various homology lengths.

Discussion

We present here the functional analysis of *ku* homologs in *S. avermitilis* and demonstrate that deletion of *ku* genes increases the frequency of homologous integration. Compared to results in filamentous fungi, the degree of increased HR frequency is smaller in *ku* deletion mutants of *S. avermitilis* (Table 1). In *N. crassa* with disruption of *ku* genes, the HR frequency increased to over 90% when homologous arm length was 500 bp (compared to 9% in wild type) and reached 100% when homologous arm length was 1 kb (compared to 21% in wild type) [20]. In *A. niger*, deletion of the *kusA* gene (*ku70* homology) increased HR efficiency to 88% when 500-bp homologous flanks were used (compared to 7% in wild type) and to 95% with 1-kb homologous flanks (compared to 19% in wild type) [17]. This difference is probably due to the predominant role of the NHEJ pathway in DSB repair in filamentous fungi. Deletion of *ku* genes blocks the NHEJ pathway and leads to greatly increased HR frequency for gene targeting. In contrast, HR plays the predominant role in DSB repair in prokaryotes. Therefore, the effect of *ku* deletion on HR frequency in prokaryotes is smaller than in filamentous fungi.

The homologous integration frequency of the *ku1ku2* double mutant of *S. avermitilis* was little different from that of the *ku1* or *ku2* single mutant (Table 1), and the phenotypes of these mutants were almost the same. These results suggest that Ku1 and Ku2 are dependent on each other and act in a heterodimeric manner, similarly to Ku70 and Ku80 in eukaryotes. The HR frequency in the *ku* mutants did not reach 100% even with 2.1-kb homology (1-kb homologous flanks). In the *ku1ku2* double mutant, chromosomal rearrangements could also occur through non-homologous recombination, as fusion sequences resulting from the rearrangements still displayed non- or micro-homology (our unpublished data). These findings suggest the existence of a *ku*-independent NHEJ pathway in *S. avermitilis*.

Differential sensitivity to UV and to DNA-damaging agents has been reported in filamentous fungi. In *N. crassa*, *ku* mutants were hypersensitive to methyl methanesulfonate (MMS), EMS, and bleomycin, but were not sensitive to UV [20]. A *ku* mutant of *Aspergillus fumigatus* showed increased sensitivity to MMS [5], but its sensitivity to bleomycin was similar to that of wild-type strain [14]. A *ku* mutant of *A. niger* showed increased sensitivity to UV [17], but this mutant and *ku* mutants of *A. nidulans* and *A. oryzae* did not show increased sensitivity to bleomycin or MMS [17, 19, 25]. In *S. avermitilis*, *ku* mutants were slightly more sensitive than wild type to EMS, but their sensitivity to UV was similar. Sensitivity of *ku* mutants and wild type to bleomycin was also similar in this species, suggesting that DSBs caused by bleomycin may be repaired by an as yet unknown *ku*-independent NHEJ pathway.

Conclusion

Deletion of *ku* genes in *S. avermitilis* did not result in adverse phenotypic changes such as a low growth rate, poor spore formation, or low avermectin production. On the other hand, gene targeting frequencies were higher in the *ku1* or *ku2* single mutants and the *ku1ku2* double mutant than in wild-type strain. These findings will lead to a more efficient gene targeting system in *S. avermitilis*. Because the complete genome sequence is known, this system will promote genomewide functional characterization of genes and improve the capacity of *S. avermitilis* for commercial production of avermectins and their derivatives such as ivermectins [15] and doramectins [33]. Deletion of *ku* homologs provides a useful general strategy for facilitating gene targeting and has the potential to be extended to other industrially valuable bacterial strains.

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