Common and Distinguishing Regulatory and Expression Characteristics of the Highly Related KorB Proteins of Streptomycete Plasmids pIJ101 and pSB24.2

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The conjugative plasmid pIJ101 of the spore-forming bacterium *Streptomyces lividans* contains a regulatory gene, *korB*, whose product is required to repress potentially lethal expression of the pIJ101 *kilB* gene. The KorB protein also autoregulates *korB* gene expression and may be involved in control of pIJ101 copy number. KorB (pIJ101) is expressed as a 10-kDa protein in *S. lividans* that is immediately processed to a mature 6-kDa repressor molecule. The conjugative *Streptomyces cyanogenus* plasmid pSB24.1 is deleted upon entry into *S. lividans* to form pSB24.2, a nonconjugative derivative that contains a *korB* gene nearly identical to that of pIJ101. Previous evidence that *korB* of pSB24.2 is capable of overriding pIJ101 *kilB*-associated lethality supported the notion that pIJ101 and pSB24.2 encode highly related, perhaps even identical conjugation systems. Here we show that KorB (pIJ101) and KorB (pSB24.2) repress transcription from the pIJ101 *kilB* promoter equally well, although differences exist with respect to their interactions with *kilB* promoter sequences. Despite high sequence and functional similarities, KorB (pSB24.2) was found to exist as multiple stable forms ranging in size from 10 to 6 kDa both in *S. lividans* and *S. cyanogenus*. Immediate processing of KorB (pIJ101) exclusively to the 6-kDa repressor form meanwhile was conserved between the two species. A feature common to both proteins was a marked increase in expression or accumulation upon sporulation, an occurrence that may indicate a particular need for increased quantities of this regulatory protein upon spore germination and resumption of active growth of plasmid-containing cells.

The gram-positive soil actinomycete *Streptomyces* is well known for having a developmentally complex life cycle as well as for producing a wide variety of medically and industrially important antibiotics and other compounds during this course of development. Vegetative growth of *Streptomyces* on solid media or in its natural soil environment occurs as a tangled mass of multigenomic hyphae termed the substrate mycelium; as nutrients become scarce, the bacteria differentiate into a vertically directed aerial mycelium, inside which regularly spaced cross walls form (4). It is during this stage of development that antibiotics and other secondary metabolites are produced, marking the onset of physiological differentiation of *Streptomyces* (3). Morphological development culminates with the formation of dormant, unigenomic spores within the now compartmentalized aerial hyphae, each of which is capable of germinating into a new substrate mycelium when reintroduced to favorable growth conditions (4). Both morphological differentiation and physiological differentiation occur when *Streptomyces* spp. grow on a solid substrate. In submerged culture, however, species such as *Streptomyces lividans* differentiate physiologically without concomitant morphological differentiation and instead grow as aggregates of mycelia that increase in density exponentially over time and then remain at a steady stationary level (3).

Streptomyces bacteria are notable not only for their life cycle and the production of secondary metabolites but also for the multitude of conjugative plasmids found throughout the group.

Streptomycete plasmids may mobilize DNA through novel transfer mechanisms, particularly considering the relatively few transfer functions that they encode (9). The 8,830-bp (11) high-copy-number *S. lividans* plasmid pIJ101 (14), for example, requires only two plasmid functions, the *tra* gene (12) and the *cis*-acting locus of transfer (*clt*), for intermycelial plasmid transfer to proceed from a donor to surrounding plasmid-free recipients (17). Besides *tra* and *clt*, pIJ101 also encodes three nonessential “spread” genes, *spdA*, *spdB*, and *kilB*, which function to increase the radial distance of plasmid dissemination within a population of potential recipients subsequent to the initial transfer act (11, 12), presumably through an intramycelial mechanism (14, 19). Of the three pIJ101 spread functions, only *kilB* is preceded by its own promoter (23), and expression of *kilB* from that promoter results in a lethal (*kil*) phenotype unless the corresponding plasmid-encoded *kil* override (*kor*) gene, *korB*, is present (12). *korB*, which regulates its own expression (23), may also control the copy number of pIJ101 (5) through interaction with the *sti* locus (25, 26), the site for second-strand initiation during rolling-circle replication of the plasmid (5, 7).

KorB appears to be initially expressed as a 10-kDa protein (22, 25, 28) that is apparently immediately processed towards its C-terminal end to produce a mature 6-kDa repressor (Fig. 1A) (25). In previous studies examining the expression of KorB, only the 6-kDa form was detected in *S. lividans*, while both the 10- and 6-kDa forms were present in the heterologous host *Escherichia coli*. The mature form of KorB was shown to have a 20-fold-higher binding affinity for the *kilB* promoter than the unprocessed form when both were individually isolated from *E. coli* (25).

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FIG. 1. KorB proteins encoded by plasmids pIJ101 and pSB24.2. (A) KorB encoded by pIJ101 as represented by open horizontal arrows in both unprocessed full-length 10-kDa and mature 6-kDa forms. A region previously identified (11) as a putative DNA-binding domain is indicated, as is the approximate position of posttranslational cleavage toward the C-terminal end. Amino acid positions Q3 (glutamine) and E30 (glutamic acid) are indicated on both the unprocessed and mature forms. (B) The full-length 10-kDa form of KorB encoded by pSB24.2. The putative DNA-binding domain, identical in sequence to the analogous region from KorB (pIJ101) with the exception of a lysine at amino acid position 30 (K30), is indicated. Amino acid position H3 (histidine) designates the only other sequence difference between the two KorB proteins. kDa, kilodaltons.

The conjugative plasmid pSB24.1, originally isolated from *Streptomyces cyanogenus*, undergoes spontaneous deletion when introduced into *S. lividans* and forms the stable 3.7-kb, nonconjugative derivative pSB24.2 (2). Although the parental plasmid has not been available for further study, pSB24.2 was found to be highly similar in sequence and organization to a portion of pIJ101 and includes analogous *korB* and *clt* functions, a potential *sti* locus, and the 3' end of a putative *kilB* gene (18). Such strong relatedness between the two plasmids, despite being derived from separate species, suggested a common ancestral origin and prompted a determination of whether transfer-related functions had remained conserved between them. Indeed, it was shown that the essential pIJ101 transfer locus *clt* could be complemented by the analogous (85% identical) region from pSB24.2 without significantly affecting plasmid transfer efficiency. Similarly, *korB* (pSB24.2), which shows a striking 97.5% identity to the pIJ101 *korB* gene, was shown to be capable of overriding pIJ101 *kilB*-associated lethality when, upon transformation, these genes were cointroduced on separate replicons into *S. lividans* (18).

From sequence comparisons, differences between the KorB protein of pIJ101 (Fig. 1A) and the putative KorB analog of pSB24.2 (Fig. 1B) are at only two amino acid positions: a glutamine at residue 3 of KorB (pIJ101) is a histidine in KorB (pSB24.2) (Q3 to H3 in Fig. 1), and a glutamic acid at residue 30 of KorB (pIJ101) is a lysine in KorB (pSB24.2) (E30 to K30 in Fig. 1). Based on their relative positions within the full-length KorB (pIJ101) protein, both the Q3 and E30 residues are predicted to be contained within the 6-kDa mature repressor form (25); interestingly, the E30 position is also located in a predicted α -helix-turn- α -helix DNA-binding domain (11) of that protein (Fig. 1A).

Since *korB* (pSB24.2) was capable of replacing *korB* (pIJ101) in overriding pIJ101 *kilB* gene lethality and since the two KorB proteins are nearly identical in sequence, we anticipated that they may share similar if not identical biological properties

with regard to their DNA-binding and expression characteristics. Using a reporter gene assay, we show here that KorB (pSB24.2) repression of the pIJ101 *kilB* gene promoter is as effective as that of KorB (pIJ101), although other DNA-binding properties as determined by using the electrophoretic mobility shift assay (EMSA) and DNase I footprinting show some differences. Using Western blotting involving antibodies that recognize both proteins, we also show that KorB (pSB24.2) surprisingly exists in both *S. lividans* and *S. cyanogenus* in several forms that range in size from 10 to 6 kDa; KorB (pIJ101) meanwhile persists in only its 6-kDa mature form in both species. Finally, expression patterns for both KorB proteins as seen in the presence (i.e., on solid surfaces) and in the absence (i.e., in liquid culture) of morphological differentiation demonstrate a strong correlation between sporulation and an obvious increase in concentration of both the 6-kDa KorB (pIJ101) protein and the multiple forms of KorB (pSB24.2). Our results thus reveal both common and distinguishing biological properties of these KorB proteins with respect to their repressor function, DNA-binding ability, processing, and linkage of expression or accumulation to streptomycete development.

MATERIALS AND METHODS

Strains, plasmids, and molecular biological methods. *E. coli*, *S. lividans*, and *S. cyanogenus* strains as well as plasmids used in this study are listed in Table 1. Ampicillin was included at 50 μ g/ml when plasmid-containing *E. coli* strains were being grown (8); when *Streptomyces* strains containing resistance-encoding plasmids were being grown, thiostrepton was included at a concentration of 50 or 5 μ g/ml in solid or liquid media, respectively, and hygromycin was included at a concentration of 200 or 20 μ g/ml in solid or liquid media, respectively. Standard molecular biology protocols (21) and enzymes purchased from New England BioLabs or Invitrogen were used to construct various pGSP and pSCON plasmids listed in Table 1. PCR performed as described by Pettis et al. (19) was used to construct plasmids pSCON20 and pSCON30, except that plasmid pGSP368 was the amplification template and that *Pfu* polymerase (Stratagene) was used. pSCON20 was made by first amplifying a 283-bp DNA fragment by using primers *korB*.eq-5 (5'-AAAAATCTAGAGCCCATGACGCAGGCTGACAC-3') and *revkorB242* (5'-AAAACTCGAGCTAGCTTCCGGAGCCCTTCTC-3'); the

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
Strains		
<i>E. coli</i>		
BL21(DE3)	F ⁻ <i>hsdS</i> (r _B ⁻ m _K ⁻) <i>gal</i> with T7 RNA polymerase gene in chromosome	24
BRL2288	<i>recA56</i> derivative of MC1061 [F ⁻ <i>araD139</i> Δ(<i>ara-leu</i>)7697 Δ <i>lacX74 galU galK hsdR2</i> (r _K ⁻ m _K ⁻) <i>mcrB1 rpsL</i>]	Gibco BRL
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> MD15 Δ <i>lacX74 deoR recA1 endA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL nupG</i>	Gibco BRL
<i>S. lividans</i>		
TK23	<i>spc-1</i>	13
<i>S. cyanogenus</i>		
NRRL B-12354	Type strain	ARS (NRRL) Culture Collection, Peoria, Ill.
Plasmids		
pBluescript II SK(+)	Ap ^r <i>E. coli</i> cloning vector ^a	Stratagene
pET30a+	Km ^r <i>E. coli</i> cloning vector with T7 promoter upstream of N-terminal His ₆ tag	Novagen
pGSP290	pSP72 with the 1.0-kb <i>BalI-PstI</i> region of pIJ101 that contains <i>kilB</i> and its promoter at <i>EcoRV</i>	18
pGSP311	pSP72 with the 74-bp <i>FspI-BstEII</i> pIJ101 region containing the <i>kilB</i> promoter cloned as a 0.1-kb <i>EcoRI</i> fragment	6
pGSP362	pBluescript II SK(+) with the 1.7-kb <i>hyg</i> gene (13) cloned as a <i>HindIII-BamHI</i> fragment	This study
pGSP368	pGSP362 with pSB24.2 linearized at <i>MluI</i> and inserted at <i>BssHII</i> (nucleotide 792 of GenBank accession no. X52328) so that <i>korB</i> is positioned near <i>BamHI</i> of the vector	This study
pHYG1	Conjugally deficient Hy ^r deletion derivative of pIJ101 lacking all known transfer functions	12
pHYG1:D8	pHYG1 derivative containing 1.2-kb <i>BclI</i> region of pIJ101 that includes <i>korB</i>	12
pIJ303	Conjugative Ts ^r derivative of pIJ101	14
pSB24.202	Ts ^r derivative of pSB24.2	18
pSCON2	pXE4 with the 351-bp <i>PstI-BstEII</i> region of pIJ101 at <i>HindIII-BamHI</i> so that transcription from the <i>kilB</i> promoter is in the direction of <i>xylE</i>	This study
pSCON20	pSP72 with the 0.3-kb pSB24.2 <i>korB</i> gene cloned as an <i>XbaI-XhoI</i> fragment at these same sites	This study
pSCON22	pSCON20 with pHYG1 linearized at <i>PstI</i> and inserted as a blunt-end fragment at <i>EcoRV</i>	This study
pSCON30	pET30a+ with the 0.3-kb pSB24.2 <i>korB</i> gene cloned as a <i>BamHI-XhoI</i> fragment at these same sites	This study
pSCON34	pGSP290 deleted from <i>SalI</i> in the vector to <i>SalI</i> in the pIJ101 <i>orf66</i> open reading frame	This study
pSCON38	pSP72 with the 191-bp <i>SalI-BstEII</i> pIJ101 region containing the <i>kilB</i> promoter cloned as an <i>XhoI-XbaI</i> fragment (derived from pSCON34) at these same sites	This study
pSCON57	pSP72 with the 0.3-kb <i>MluI-SpeI</i> fragment of pIJ101 containing <i>korB</i> cloned as a blunt-end fragment at <i>EcoRV</i>	This study
pSCON61	pSCON2 with the 0.3-kb <i>MluI-SpeI</i> region of pIJ101 containing <i>korB</i> cloned as a <i>BamHI-BglII</i> fragment (derived from pSCON57) at <i>BglII</i>	This study
pSCON95	pSP72 with the 0.3-kb <i>EcoRV-XhoI</i> fragment containing the pSB24.2 <i>korB</i> gene (derived from pSCON20) cloned as a blunt-end fragment at <i>EcoRV</i>	This study
pSCON98	pSCON2 with the 0.3-kb <i>EcoRV-XhoI</i> fragment of pSB24.2 containing <i>korB</i> cloned as a <i>BamHI</i> fragment (derived from pSCON95) at <i>BglII</i>	This study
pSP72	Ap ^r <i>E. coli</i> cloning vector	Promega
pXE4	Ts ^r SCP2* derivative with promoterless <i>xylE</i> reporter gene	10

^a Ap^r, Km^r, Hy^r, and Ts^r indicate resistance to ampicillin, kanamycin, hygromycin, and thiostrepton, respectively.

resulting product was extracted and precipitated as previously described (19) and was then digested to completion with *XbaI* and *XhoI* and was ligated into pSP72 at these sites. pSCON30 was constructed by amplifying a 266-bp DNA fragment by using primers BamKR242F (5'-AAAAAGGATCCATGACGACAGACACCG-3') and XhokrB242R (5'-AAAACTCGAGCTAGCTCCGAGCCCTTC-3'); subsequently, this product was treated in a manner similar to that for the fragment used to create pSCON20, except that digestion was with *BamHI* and *XhoI* and that ligation was into pET30a+ at these sites so that *korB* (pSB24.2) was in frame with the N-terminal His₆ tag. Chemical transformation of *E. coli* BRL2288 was according to the method described by Sambrook et al. (21), while electroporation of *E. coli* DH10B was performed by using a Bio-Rad Gene Pulser II along with the manufacturer's instructions. Transformation of *Streptomyces* bacteria was performed according to the method described by Kieser et al. (13). Unless otherwise indicated, cell extracts were prepared and their total protein concentrations were quantified as described previously (16), by using bovine serum albumin as a standard.

XylE analysis. For catechol dioxygenase (XylE assays), relevant *S. lividans* TK23 cultures were grown from spore suspensions inoculated into 25 ml of yeast extract-malt extract (YEME) media (13) with shaking (300 rpm) at 30°C. When culture densities reached 0.8 to 1.0 optical-density-at-600-nm (OD₆₀₀) units, 1-ml aliquots were harvested from which protein extracts were prepared, and measurement of catechol dioxygenase activity was determined as described by Ingram et al. (10). For three separate trials of each sample, the average change in absorbance at 375 nm over 6 min was recorded and was converted to milliunits of catechol dioxygenase per milligram of total protein (20).

EMSA and DNase I footprinting analysis of the pIJ101 *kilB* promoter. Relevant *S. lividans* TK23 cultures used to obtain KorB-containing cell extracts were grown as indicated above until culture densities reached 0.5 to 0.7 OD₆₀₀ units, whereupon they were harvested, and cell extracts containing active KorB protein were prepared as described earlier (27) in S30 cell lysis buffer, except that subsequent to lysis cell extracts were centrifuged twice for 30 min at 30,000 × *g*. *kilB* promoter binding by KorB proteins was performed as described by Tai and Cohen (25), with the following modifications: a 0.1-kb *EcoRI* fragment from pGSP311 containing the *FspI-BstEII* portion of the *kilB* promoter region was labeled on both ends by using [α-³²P]dATP (6,000 Ci/mmol; Amersham Biosciences) and the Klenow fragment of DNA polymerase I (New England Biolabs), and the labeled fragment was then purified from excess radionuclides by using an S300 column (Amersham Biosciences). In a total volume of 20 μl, approximately 17 μg of each relevant *S. lividans* TK23 cell extract was added to 30,000 counts of labeled fragment per min that had been preincubated in binding buffer (25), and following further incubation for 10 min at room temperature, these samples were electrophoresed on a 5% nondenaturing polyacrylamide gel in Tris-glycine buffer as described earlier (1). The gel was dried and analyzed by autoradiography. For EMSAs involving various concentrations of KorB (pSB24.2), serial twofold dilutions of *S. lividans* TK23 (pSCON22) extracts were made in S30 buffer prior to the addition of equal volumes to reaction mixtures.

DNase I footprinting was performed by using the same *S. lividans* TK23 cell extracts employed for EMSA. Here, a 231-bp *XbaI-XhoI* fragment from pSCON38 containing the 191-bp *SalI-BstEII* region of pIJ101 that includes the *kilB* promoter and the 3' end of the open reading frame *orf66* (11) was labeled

FIG. 2. The *kilB*-*xylE* fusion vector pSCON2. Plasmid pSCON2 contains the 351-bp *Pst*I-*Bst*EII *kilB*-promoter-containing pIJ101 region inserted upstream of the *xylE* structural gene in the orientation shown on the *E. coli*-*Streptomyces* shuttle vector pXE4 (10). The *Streptomyces* SCP2* replicon, *tsr* (thiostrepton resistance gene), *bla* (beta-lactamase gene), and pBR322 *ori* (origin of replication) regions of pXE4 are indicated, as is the position of inverted repeat sequences between the putative -35 and -10 determinants of the *korB*-regulated *kilB* promoter. Upstream of the *kilB* promoter within the inserted fragment is a pIJ101 open reading frame (*orf66*) of undetermined function (11). The line drawing for the 351-bp *Pst*I-*Bst*EII sequence of pIJ101 is not to scale.

at both ends by using [α - 32 P]dATP and the Klenow fragment of DNA polymerase I. The labeled fragment was digested at *Sal*I near the *Xho*I end, and this reaction mixture was then passed through an S400 column (Amersham Biosciences) in order to separate the desired, singly labeled 225-bp fragment from excess radionuclides and the 6-bp labeled *Sal*I-*Xho*I fragment. Binding reactions and subsequent DNase I digestion were performed as described by Tai and Cohen (25), except that approximately 72,000 counts of the 225-bp fragment per min and 17 μ g of each relevant *S. lividans* cell extract were included in reactions and that 50 ng of DNase I (Roche Diagnostics) was subsequently used. Samples were electrophoresed on an 8% LongRanger (BioWhittaker) sequencing gel alongside Maxam-Gilbert A+G reactions (15) of the same fragment. The gel was dried and analyzed by autoradiography.

Generation of anti-KorB antibodies and Western blot analysis of *Streptomyces* cultures. Expression of a His₆-tagged version of KorB (pSB24.2) from the T7 promoter of the pET30a+ derivative pSCON30 was induced in *E. coli* BL21 (DE3) grown in Luria-Bertani broth (21) as described previously (19). The fusion protein was purified by using an Ni²⁺-charged histidine-binding His trap column (Amersham Biosciences) according to the manufacturer, followed by electrophoresis on a preparative sodium dodecyl sulfate (SDS)-18% polyacrylamide gel electrophoresis (PAGE) gel. The 16-kDa band corresponding in size to the induced fusion protein was excised from the gel and was used to raise antibodies in a rabbit by Animal Pharm Services, Inc. Western blotting was performed as described previously (19), except that proteins were separated on an SDS-18% PAGE gel and that the primary antiserum was used at a dilution of 1:1,000. KorB protein bands on Western blots were quantified by using the ZERO-Dscan version 1.0 program from Scanalysis.

S. lividans TK23 plasmid-containing strains used to collect surface growth timepoints were inoculated as spores onto cellophane membranes placed on R5 plates as described earlier (19), and aliquots of these cultures were collected at the times indicated in the text. *S. lividans* and *S. cyanogenus* plasmid-containing strains used to collect submerged culture time points were inoculated as spores into YEME; these cultures were grown to a density between 0.1 and 0.3 OD₆₀₀ units, whereupon they were diluted into 25 or 50 ml of fresh YEME at a starting density of 0.05 OD₆₀₀ units. Subsequent to this dilution and at the times indicated in the text, aliquots were collected.

RESULTS

Equivalent repression of transcription from the pIJ101 *kilB* promoter by both *korB* genes. Since *korB* (pSB24.2) was capable of replacing its pIJ101 counterpart in the previous trans-

formation-based *kil* override assay involving the pIJ101 *kilB* gene, (18), we tested here the ability of *korB* from pSB24.2 to repress pIJ101 *kilB* promoter-directed expression of *xylE*, a *Pseudomonas putida* gene that encodes an enzyme (catechol dioxygenase) that breaks down the colorless substrate catechol to form a yellow product that absorbs light at 375 nm (20). Plasmid pSCON2 (Fig. 2) was constructed by inserting in the orientation shown a 351-bp *Pst*I-*Bst*EII fragment of pIJ101 containing the *kilB* promoter region upstream of the promoterless *xylE* gene on the low-copy-number vector pXE4 (10). Submerged cultures of *S. lividans* TK23 containing pSCON2 plus various individual *korB*-containing derivatives of the hygromycin-resistant pIJ101 plasmid pHYG1 (12) were grown to late exponential phase, and cell extracts were prepared and assayed for catechol dioxygenase activity as described in Materials and Methods.

The presence of pSCON2 allowed for the production of 22.9 ± 1.5 mU of catechol dioxygenase per mg of total protein in *S. lividans* TK23; this production was attributed to expression of *xylE* as directed by the *kilB* promoter, since cells containing the promoterless vector pXE4 showed no detectable catechol dioxygenase activity (Table 2). Addition of pHYG1 to pSCON2-containing cells had little if any effect on *kilB* promoter activity (18.5 ± 1.5 mU per mg of total protein), a result that was in contrast to when either plasmid pHYG1:D8 (12), which contains *korB* (pIJ101), or pSCON22, which contains the pSB24.2 *korB* gene, was present in cells in addition to pSCON2; in these latter cases, little or no catechol dioxygenase activity was detectable (Table 2). Similarly, complete repression by either *korB* function was also observed when, instead of expressing each from a separate high-copy-number plasmid, the *korB* (pIJ101) or *korB* (pSB24.2) gene was cloned directly onto pSCON2 in order to create plasmid pSCON61 or pSCON98, respectively (data not shown). Thus, in all of our

TABLE 2. Comparative repression of pIJ101 *kilB*-promoter-directed *xylE* transcription by the pIJ101 and pSB24.2 proteins

Traits for:				Catechol dioxygenase content (mU/mg of total protein) ^a
<i>kilB-xylE</i> plasmid		<i>korB</i> -containing plasmid		
Name	Feature	Name	Feature	
pXE4 ^b	No promoter			0
pSCON2	<i>kilB</i> promoter			22.9 ± 1.5
pSCON2	<i>kilB</i> promoter	pHYG1 ^c	No KorB	18.5 ± 1.5
pSCON2	<i>kilB</i> promoter	pHYG1:D8	KorB (pIJ101)	1.2 ± 1.2
pSCON2	<i>kilB</i> promoter	pSCON22	KorB (pSB24.2)	0

^a Numbers shown are based on the results from three separate assays (see Materials and Methods).

^b pXE4 is the parental plasmid of pSCON2 and contains the promoterless *xylE* gene.

^c pHYG1 is the parental plasmid of pHYG1:D8 and pSCON22 and contains no *korB* sequences.

assays, the pSB24.2 *korB* gene was equally effective at repressing transcription from the pIJ101 *kilB* promoter as the natural pIJ101 *korB* repressor function.

Disparate binding characteristics exist for the pIJ101 and pSB24.2 KorB proteins. KorB (pIJ101) expressed in either *S. lividans* or *E. coli* has been shown to bind and shift DNA fragments containing the *kilB* promoter to a single higher position in EMSAs (25, 28), and DNase I footprinting allowed the site of interaction of this protein with the *kilB* promoter to be determined (25, 28). To test for binding of KorB (pSB24.2) to the pIJ101 *kilB* promoter, we first utilized EMSA and compared the shift pattern of the pSB24.2 protein to that produced by KorB (pIJ101). Here, relevant cell extracts (approximately 17 µg of each) were incubated with the radiolabeled insert fragment of plasmid pGSP311, which contains the 74-bp *FspI*-*BstEII* region spanning the portion of the *kilB* promoter region (Fig. 2) that was shown by previous EMSA analysis to be bound by the pIJ101 KorB protein (25). Examination of the banding patterns following gel electrophoresis and autoradiography revealed that, as expected, *S. lividans* TK23 (pHYG1:D8) extracts containing KorB (pIJ101) shifted the *kilB* promoter fragment to a single position (Fig. 3A, lane 3) relative to the unshifted band, which was present when no extract (lane 1) or when a KorB⁻ extract (i.e., prepared from cells containing pHYG1) had been added (lane 2). In contrast, reactions involving TK23 (pSCON22) extracts containing KorB (pSB24.2) yielded two different shifted bands, including one that was near the KorB (pIJ101)-induced band but with a slightly lower mobility (band I in lane 4) and an additional, significantly more slowly migrating band (band II in lane 4), which appeared to be both less intense and distinct than either the KorB (pIJ101)-shifted band or band I for the pSB24.2 KorB protein.

From these initial data, it was not possible to distinguish the order of appearance of the KorB (pSB24.2)-DNA complexes represented by bands I and II. We therefore repeated the EMSA analysis by using the same labeled *kilB* promoter fragment along with serial dilutions of KorB (pSB24.2)-containing TK23 (pSCON22) extracts with total protein concentrations ranging from 0.53 to 17 µg. At the three lowest concentrations tested, only band I was observed (Fig. 3B, lanes 2 to 4), while at higher protein concentrations the more slowly migrating band II also appeared (lanes 5 to 7). In a parallel dilution analysis of KorB (pIJ101)-containing extracts, a second shifted band was never apparent even when up to 33 µg of total protein was tested (data not shown).

To compare the actual sites of interaction of the two KorB

proteins with the pIJ101 *kilB* promoter, DNase I footprinting was employed with the same cell extracts used for EMSA along with the radiolabeled *kilB* promoter-containing fragment derived from plasmid pSCON38, which includes the 191-bp *SalI*-*BstEII* region of pIJ101 (Fig. 2) used previously for DNase I footprinting involving KorB (pIJ101) (25). Though these experiments were performed by using extract amounts (i.e., 17

FIG. 3. EMSA analysis of the *korB*-regulated pIJ101 *kilB* promoter conducted by using the KorB proteins of pIJ101 and pSB24.2. (A) Approximately 17 µg of each relevant KorB-containing *S. lividans* TK23 extract (prepared as described in Materials and Methods) was incubated with 30,000 counts of a radiolabeled 0.1-kb fragment per min, which includes the 74-bp *FspI*-*BstEII* *kilB*-promoter-containing region of pIJ101 (Fig. 2), and reactions were then subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel followed by autoradiography. Lane 1, no extract added; lane 2, TK23 (pHYG1) extract added; lane 3, KorB (pIJ101)-containing TK23 (pHYG1:D8) extract added; and lane 4, KorB (pSB24.2)-containing TK23 (pSCON22) extract added. Two separate shifted bands (bands I and II) for KorB (pSB24.2) are indicated, as is the single KorB (pIJ101) shifted band. (B) Concentration-dependent characteristics of KorB (pSB24.2) binding to the pIJ101 *kilB* promoter. EMSA analysis was performed as described above, except that various amounts of KorB (pSB24.2)-containing TK23 (pSCON22) cell extracts were added to reactions. Total extract amount added: lane 1, none; lane 2, 0.53 µg; lane 3, 1.1 µg; lane 4, 2.1 µg; lane 5, 4.2 µg; lane 6, 8.4 µg; and lane 7, 17 µg.

FIG. 4. Comparative DNase I footprinting of the *korB*-regulated *kilB* promoter by the KorB proteins of pIJ101 and pSB24.2. A fragment containing the 191-bp *SalI*-*Bst*EII region of pIJ101 that includes the *kilB* promoter (Fig. 2) was radiolabeled on the coding strand, was incubated with 17 μ g of each relevant *S. lividans* cell extract, and was then assayed by DNase I footprinting as described in Materials and Methods. Lane 1, Maxam-Gilbert A+G reactions of the same fragment; lanes 2, 4, and 6, TK23 (pHYG1) extract added; lane 3, KorB (pIJ101)-containing TK23 (pHYG1:D8) extract added; and lane 5, KorB (pSB24.2)-containing TK23 (pSCON22) extract added. The putative -35 , -10 , and $+1$ elements of the *kilB* promoter as described previously (23) are indicated to the left of the lanes. Protected regions from -40 to $+21$ relative to the *kilB* transcription start site for KorB (pIJ101) and from -40 to $+3$ for KorB (pSB24.2) are based on the data presented here. Two independent trials of the DNase I footprinting analysis shown produced identical results.

μ g) that resulted in the appearance of multiple bands for KorB (pSB24.2) in EMSA analysis, this effect did not translate into an interaction of KorB (pSB24.2) with the *kilB* promoter more extensive than the one associated with KorB (pIJ101). As shown in Fig. 4, while KorB (pIJ101) protected a region from -40 to $+21$ relative to the *kilB* transcription start site (compare lane 3, which involves the KorB [pIJ101] extract, to lanes 2, 4, and 6, where a KorB⁻ extract was used), a result that was in close agreement with the previous determination (25), protection by KorB (pSB24.2) began at approximately the same

upstream position but extended to only about the $+3$ position (lane 5).

Expression and processing characteristics of the pIJ101 and pSB24.2 KorB proteins. When expressed in *S. lividans*, KorB (pIJ101) is apparently present in its unprocessed 10-kDa form only transiently, to be cleaved immediately at some unknown site nearer its C terminus in order to produce the mature 6-kDa repressor. The latter was the sole form of the protein observed previously in liquid cultures of this organism (25) although its expression or accumulation throughout morphological and physiological differentiation in *Streptomyces* was not studied. To examine in a temporal manner the appearance of both KorB (pIJ101) and KorB (pSB24.2) in *S. lividans*, we used Western blotting involving antibodies raised against a His₆-tagged version of the 10-kDa KorB (pSB24.2) protein expressed in *E. coli* (see Materials and Methods for details) and probed extracts of relevant *S. lividans* surface cultures that had been cultivated to various representative stages of growth. Cultures of *S. lividans* TK23 containing plasmid pIJ303 (14), a thiostrepton-resistant conjugative derivative of pIJ101, showed the expected 6-kDa mature form of KorB (pIJ101), which was initially present at barely detectable levels throughout the substrate mycelial (Fig. 5A, 18 and 24 h) and aerial mycelial (36 and 48 h) stages of growth, followed by a marked increase in concentration upon sporulation (144 h). In a parallel experiment involving strain TK23 containing plasmid pSB24.202 (18), a thiostrepton-resistant derivative of pSB24.2, multiple forms of KorB (pSB24.2) corresponding in size to 10, 8.5, 8, and 6 kDa were also present at barely detectable levels throughout the two mycelial growth stages (Fig. 5A, 18 to 48 h), which was followed again by an increase in concentration for all forms following sporulation (144 h). As expected, no proteins in the size range of 6 to 10 kDa were detectable in

FIG. 5. Appearance and processing of KorB (pIJ101) and KorB (pSB24.2) throughout differentiation of *S. lividans*. Spores of *S. lividans* TK23 containing either the pIJ101 derivative pIJ303 (14) or the pSB24.2 derivative pSB24.202 (18) were used to inoculate either R5 agar plates (A) or YEME broth (B), and cells were harvested at the indicated times (in hours). Protein extracts were separated on SDS-PAGE gels, transferred to nitrocellulose, and probed with antibodies raised against the His₆-tagged, unprocessed (10-kDa) form of KorB (pSB24.2). The sizes of KorB (pSB24.2) larger than 6 kDa are given on the right, while the positions of 15- and 6-kDa molecular mass standards (A) and 16- and 6-kDa molecular mass standards (B) are indicated on the left.

control TK23 extracts even when they were prepared from spores (Fig. 5A, 144 h). When the multiple forms of KorB (pSB24.2) were quantified by densitometric scanning (data not shown), there appeared to be nearly fivefold more of this protein in spores than the concentration of the sole 6-kDa form for KorB (pIJ101) at this same stage.

To examine the temporal pattern of expression or accumulation of the two KorB proteins under conditions where physiological but not morphological differentiation occurs, submerged cultures of *S. lividans* TK23 containing either pIJ303 or pSB24.202 were sampled at various times throughout exponential and stationary growth phases and were then probed again by Western blotting. Here, both proteins appeared overall in more gradually increasing amounts throughout growth as opposed to their distinctive biphasic patterns displayed in surface cultures. Specifically, the mature 6-kDa repressor form of KorB (pIJ101) was again present during early growth (Fig. 5B, 7 h) at a very low concentration but then steadily increased in amount as the cells progressed through late exponential phase (21 h) and then into early and later stationary-phase time periods (45 and 93 h, respectively). Similarly, for KorB (pSB24.2), gradually increasing amounts of the 10- and 8.5-kDa forms were apparent during these same growth periods, although the additional 6-kDa form appeared at a relatively steady concentration throughout (Fig. 5B, 7 to 93 h). Again, control extracts prepared from TK23 cells grown to late stationary phase showed no evidence of KorB-related bands (Fig. 5B, 93 h). Taken together, the Western blotting results indicate that, while KorB concentration may be affected under certain growth conditions (e.g., submerged culture) by as-yet-undetermined physiological parameters, the marked increase in KorB concentration observed in spores appears to be linked specifically to this morphological event.

Processing characteristics of both KorB proteins are conserved between species. The presence of KorB (pSB24.2) forms of sizes greater than 6 kDa raised the possibility that processing of this protein in the heterologous host *S. lividans* is inefficient or aberrant. To examine this possibility further, we used Western blotting to probe a stationary-phase submerged culture of *S. cyanogenus* strain NRRL B-12354 containing pSB24.202 for its KorB (pSB24.2) protein profile, since *S. cyanogenus* is the reported host of the parental plasmid pSB24.1 (2). Interestingly, KorB (pSB24.2) showed a profile in *S. cyanogenus* (Fig. 6) that was the same as that seen in *S. lividans* submerged cultures (Fig. 5B); this result thus argues that processing of KorB (pSB24.2) in the heterologous *S. lividans* host is proceeding relatively normally. In a parallel experiment, when the profile of KorB (pIJ101) was examined in *S. cyanogenus* cells containing plasmid pIJ303, only the 6-kDa mature repressor form of this protein was observed (Fig. 6), which extends the notion that processing for these KorB proteins remains conserved between the two streptomycete species tested.

DISCUSSION

In a previous study, the *clt* and *korB* loci of plasmid pSB24.2, which is a transfer-defective deletion derivative of the conjugative *S. cyanogenus* plasmid pSB24.1, were shown to be capable of complementing the corresponding loci of the well-studied *S. lividans* plasmid pIJ101. As measured by quantitative

FIG. 6. Processing of KorB (pIJ101) and KorB (pSB24.2) in *S. cyanogenus*. Spores of *S. cyanogenus* NRRL-B12354 containing either pIJ303 or pSB24.202 were used to inoculate YEME broth, and cells were harvested at the indicated times (in hours). Protein extracts were analyzed by Western blotting as described in the legend to Fig. 5. The sizes of KorB (pSB24.2) forms greater than 6 kDa are indicated on the right, while the positions of 16- and 6-kDa molecular mass standards are shown on the left.

mating assays, the *clt* locus of pSB24.2 functioned as efficiently as *clt* (pIJ101) did during plasmid transfer mediated by the pIJ101 *tra* gene. Likewise, the *korB* gene of pSB24.2 overrode the lethal effects of unregulated pIJ101 *kilB* expression in a cotransformation experiment (18). Here, using a quantitative reporter gene assay, we have extended upon the previous study by showing that *korB* (pSB24.2) is as effective as *korB* (pIJ101) at repressing transcription from the pIJ101 *kilB* gene promoter. These data thus provide additional evidence that the related plasmids pIJ101 and pSB24.1, though derived from different species, probably encode identical conjugation systems whose components are essentially interchangeable.

Although the two *korB* genes functioned interchangeably in vivo with respect to repression of the pIJ101 *kilB* promoter, there were significant differences in the processing of their nearly identical full-length 10-kDa KorB products. Because KorB (pIJ101) is observable only in the mature 6-kDa form in *S. lividans*, it has been presumed that once synthesized the 10-kDa form is immediately processed to the mature repressor form, and it was determined previously that this processing occurs towards the C-terminal rather than towards the N-terminal end of the protein (25). In the case of KorB (pSB24.2), the full-length molecule was often readily apparent in *S. lividans* along with up to three smaller forms of 8.5, 8, and 6 kDa. Although not investigated here, it is tempting to speculate that these multiple forms result from either sequential or possibly random processing of the 10-kDa precursor from the C-terminal end; in this case, the smallest product would be a 6-kDa form that retains the putative DNA-binding domain (Fig. 1) originally identified for KorB (pIJ101) and that, based on previous DNA-binding studies of KorB (pIJ101), might have the highest affinity for its operator sequences (25). The possibility that the multiple forms of KorB (pSB24.2) are instead the result of inefficient or aberrant processing exclusively in the heterologous *S. lividans* host was ruled out when the same KorB (pSB24.2) profile was observed in *S. cyanogenus*, the natural host of the parental plasmid, pSB24.1 (2).

Our data taken together thus support the notion that, in contrast to KorB (pIJ101), relatively stable multiple forms of KorB (pSB24.2) protein are naturally present during streptomycete growth. It is plausible that, even if the 6-kDa form does represent the primary repressor molecule, as is the case for KorB (pIJ101), the additional pSB24.2 forms may still participate in some if not all of the regulatory functions known to be associated with KorB repressor activity. For KorB (pIJ101) these include binding at its cognate operator sequences within the promoter regions of the pIJ101 *kilB* and *korB* genes as well as potentially interacting with the *sti* locus to control pIJ101 copy number.

What purpose is served by the relatively higher concentration of KorB seen in metabolically inactive spores? It is possible that KorB is at its highest level during the resting stage of the *Streptomyces* life cycle to ensure some aspect(s) of its regulation (i.e., repression of *kilB*, autoregulation of *korB*, or control of plasmid copy number) upon germination and outgrowth of newly formed plasmid-containing substrate hyphae. The specific regulatory role for *korB* that may be of particular importance for the germination process remains to be determined.

As demonstrated here, the onset of sporulation appears to serve as a morphological cue for increased expression or accumulation of the KorB proteins of both plasmids pSB24.2 and pIJ101. In contrast, it was previously found that pIJ101 KorA repressor concentration was reduced by about one-half following sporulation in *S. lividans* (16). Meanwhile, a temporally increasing pattern of KilB protein accumulation as well as the temporal disappearance of pIJ101 Tra protein during streptomycete differentiation was attributed to changes in as-yet-undetermined physiological parameters, since these same respective trends were observed in both surface and submerged *S. lividans* cultures (16, 19). Clearly, more study is needed to understand the complex interplay between the differentiating streptomycete life cycle and functions that mediate high-frequency transmission of *Streptomyces* conjugative plasmids.

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