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Expression Characteristics of the Transfer-Related *kilB* Gene Product of *Streptomyces* Plasmid pIJ101: Implications for the Plasmid Spread Function

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Received 13 September 2000/Accepted 27 November 2000

Intermycelial transfer of *Streptomyces* plasmid pIJ101 occurs prior to cellular differentiation and is mediated by plasmid functions that are also required for production of zones of growth-inhibited recipient cells (i.e., "pocks") that develop around individual donors during mating on agar medium. Several other pIJ101 functions, including that of the *kilB* gene, whose unregulated expression on pIJ101 is lethal, are required for normal pock size and so have been postulated to mediate intramyecelial spread of the plasmid throughout recipient cells. Using antibodies raised against a KilB fusion protein expressed in *Escherichia coli*, native KilB protein was detected throughout development of pIJ101-containing *Streptomyces lividans* cells, with the concentration of KilB increasing dramatically and reaching a maximum during the final stages (i.e., sporulation and secondary metabolism) of cellular differentiation. Insertion of the *kilB* gene of pIJ101 into the *S. lividans* chromosome in cells lacking the pIJ101 KorB protein, which normally represses *kilB* gene transcription, resulted in elevated but still temporally increasing amounts of KilB. The increased expression or accumulation of the KilB spread protein throughout cellular differentiation of *S. lividans*, which leads to maximum KilB concentrations during developmental stages that occur far later than when intermycelial transfer of pIJ101 is mediated, supports the existence of a subsequent intramyecelial component to the pIJ101 spread function. The results also suggest that intramyecelial spread of pIJ101 molecules within the recipient extends beyond intercompartmental movements within the substrate mycelia and includes undetermined steps within the spore-yielding aerial hyphae as well.

*Streptomyces* bacteria are gram-positive actinomycete soil organisms that display complex cellular differentiation which involves both morphological and physiological changes (4). Although they persist vegetatively as an infrequently septated, multinucleoid substrate mycelium, this growth pattern ceases as nutrients become scarce, and substrate hyphal compartments begin to differentiate, yielding vertically directed aerial hyphae that appear fuzzy white. Concomitant with this morphological change, *Streptomyces* bacteria also begin producing a vast array of secondary metabolites, including antibiotics. Growth of aerial hyphae, which is fueled by organic material derived from the dying substrate layer, eventually also stops, and regularly spaced septation then divides the tips of these vertical structures into unigenomic sections that subsequently develop into grayish-colored spores. Submerged cultures of species such as *Streptomyces lividans* grow in a mycelial form that does not differentiate morphologically but does develop physiologically, with cells producing secondary metabolites as they enter stationary phase (3).

Conjugative plasmids in *Streptomyces* spp. can be detected when individual spores containing a plasmid germinate within a dense lawn of plasmidless potential recipient mycelia, and subsequent transfer of plasmids from donors to surrounding recipients leads to finite circular regions where aerial hypha development and sporulation are transiently delayed or prevented (1, 10). Since such zones or “pocks” correspond to cells that have received plasmid copies (1), pock formation depends on and is coincident with transmission of streptomyctye plasmids (1, 9). Presumably, such developmental inhibition in turn promotes the transfer process, perhaps by prolonging the growth period during which transmission can occur (9).

In marked contrast to plasmids from other bacteria, *Streptomyces* plasmids encode few transfer functions. Such loci can be divided into those that are essential for plasmid transfer and pock formation and others that are not required for transfer and pocking to occur but affect pock size and thus plasmid “spread” (9, 13). The first set of loci are undoubtedly required for the intermycelial transfer of plasmid molecules between donor and recipient hyphae, while the function of the latter group is less clear. Given the mycelial pattern of streptomycete growth, these loci may mediate intramyecelial spread of plasmids within recipient cells, such as movement across infrequent hyphal cross walls that separate the original point of transfer from other connected cell compartments (9, 13). Alternatively, it is possible that plasmid spread functions instead augment the initial intermycelial transfer step, for example, by increasing its efficiency or by extending the transfer period (9, 14).

The transmission properties of pIJ101 (Fig. 1), the 8,830-base pair (bp) (11), high-copy-number (i.e., up to 300 copies per chromosome) (13) *S. lividans* plasmid, have been among the most studied for a *Streptomyces* extrachromosomal element. Loci responsible for intermycelial plasmid transfer as well as pock formation include the pIJ101 *tra* gene (12, 13), which encodes a temporally expressed 70-kDa membrane protein of unknown function that is found only in the substrate mycelium of *S. lividans* cells (14), as well as *clt*, a *cis*-acting
FIG. 1. Physical map and genetic organization of Streptomyces plasmid pIJ101. The highlighted region includes previously determined genetic functions related to transmission of the plasmid (12, 13, 15), which are shown alongside their respective ORFs (filled arrows) or, in the case of clt, its locus (filled box). The remaining plasmid region includes functions involved in replication of the plasmid (2, 6, 13), and these are also indicated beside their respective ORFs (striped arrows) or loci (striped boxes). Small ORFs (i.e., orf56, orf66, orf79, and orf85) (11) whose functions remain undetermined are indicated in both regions. The sequence that is deleted (Δ) in plasmid pIJ303ΔkilB, which is a derivative of the conjugative, thiostrepton-resistant pIJ101 plasmid pIJ303 (13), is indicated.

There is currently no information available on the expression of proteins that mediate plasmid spreading in Streptomyces bacteria. To begin to elucidate the mechanism of plasmid spread, we have focused on the pIJ101 kilB gene, whose associated lethality function suggests that it may play a unique role in the process. Using antibodies raised against an *Escherichia coli* fusion protein comprised largely of KilB sequences, we found KilB protein in pIJ101-containing *S. lividans* cells throughout their differential growth, with concentrations reaching a maximum during the terminal sporulation and antibiotic production stages. A similar pattern of elevated KilB expression also appeared in the absence of KorB in an *S. lividans* strain containing the chromosomally integrated kilB gene. By demonstrating that the KilB spread protein is present throughout cellular differentiation of *S. lividans*, including morphological stages that occur considerably later than that during which intermycelial transfer of pIJ101 is completed, our data provide support for a KilB-mediated intracyclic component to spreading of pIJ101, which may be operative during all stages of *Streptomyces* development.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** *S. lividans* strain TK23 (spe−) has been described previously (8), while *E. coli* hosts for cloning were DH10B [F− mcrA Δ(mrr-hsdRMS-merBC) g680lacZAM15 Δaac574 deoR recA1 endA1 araD139 Δ(ara-leu)7697 galU galK λ− rpsL mK− mcrA− lamB−] (Life Technologies Inc., Gaithersburg, Md.) and BRL2288, which is a recA56 derivative of MC1061 [F− araD139 Δ(ara-leu)7697 Δaac574 galU galK hsdR2(rK− mK−) mcrB1 rpsL2] (Life Technologies Inc.). For overexpression of recombinant KilB protein, the *E. coli* host was BL21(DE3) (21). To construct plasmid pIJ303ΔkilB, the 3.5-kb PstI fragment of pIJ101 was first ligated into this site in the *E. coli* vector pSP72 (Promega, Madison, Wis.) in order to create pGSP304. This plasmid was partially digested with FspI and then digested to completion with MscI, and the vector-containing fragment lacking the 726-bp MscI-FspI (nucleotides 1772 to 2497) region of
pIJ101 (11) was ligated to itself. The 2.8-kb PstI insert of the resulting plasmid, pGSP305, was then ligated to the 7.3-kb PstI fragment of pIJ303 in the natural orientation to create pIJ303ΔkilB. A 481-bp DNA fragment containing the 444-bp kilB ORF (11) was amplified following 30 PCR cycles (1 cycle = 94°C for 30 s, 37°C for 1 min, and 72°C for 2 min) using the primers kilB5, 5′-CTCGAGTGCATGTGACTGAGTCAGGCGCCGAACCGGCGGGCCGCGGC, and kilB3, 5′-CTGACCCGCTAGCTGCAGTACTCGAGTCAGGCGCCGAACCGGCGGGCCGCGGC, both at 0.5 μM, 100 ng of the 6.1-kb BamHI-BamHI fragment of pIJ101 as a template, and ULTma DNA polymerase (Perkin-Elmer, Branchburg, N.J.) in the presence of 1% dimethyl sulfoxide. Following extraction with phenol-chloroform (10:90) and chloroform, DNA was ethanol precipitated, resuspended, and digested to completion with BamHI and XhoI. The 446-bp kilB-containing BamHI-XhoI digestion product was then isolated on a 1.5% agarose gel, purified using a GeneClean kit (Bio 101, Carlsbad, Calif.), and ligated to similarly digested pET30α (+) (Novagen, Inc., Madison, Wis.) vector DNA in order to create pGSP281. Plasmid pGSP290, a pSP72 derivative that contains the 1.0-kb PstI-BamHI region of pIJ101 that includes the kilB gene, has been described previously (16). To create pGSP295, plasmid pGSP290 was digested with BamHI and BglII and the 1.0-kb kilB-containing fragment was ligated to BamHI-digested pBeBal2 (15) in the orientation indicated below. Besides kilB, this pIJ101 fragment also contains orf66, which appears to lacks its own promoter (11, 14) and does not contain orf66, which appears to lacks its own promoter (11, 14) and does not

Bacteriological methods and molecular biology techniques. Transformation of S. lividans and E. coli was performed as described previously (8) using R5 agar (8) and Luria-Bertani agar (15), respectively, for plating of transformants. Transformant colonies of S. lividans TK23 were excised with a scalpel, macerated with a pipette tip, and spread in patches onto Streptomycetes spore growth agar (SIGA) (5) containing thiostrepton in order to obtain spores. Nonselective growth of NWR2 isolates was performed by patching spores onto SIGA in the absence of thiostrepton and then plating appropriate dilutions of the resulting spores onto either Luria-Bertani agar or SIGA; upon subsequent growth and sporulation, colonies were replica plated onto the same respective media either containing or lacking thiostrepton in order to score for loss of thiostrepton resistance. Submerged cultures of S. lividans were grown in yeast extract-malt extract medium (8). Thiostrepton was used in agar or liquid medium at the previously described (8) concentrations of 50 and 5 μg/ml, respectively.

Cloning was performed using standard procedures described previously (18). Genomic DNA was extracted and purified from S. lividans NWR2 spores using the method of Rainey et al. (17). Amplification of the 481-bp kilB-containing fragment from 100 ng of purified genomic DNA was performed using the kilB5 and kilB3 primers along with the PCR conditions described above.

Preparation of KilB antiserum and Western blotting of cell extracts. A 200-ml Luria-Bertani broth culture of E. coli strain BL21(DE3) containing plasmid pGSP261 was grown and induced for expression of recombinant KilB protein as described previously (21), except that induction occurred at an ratio of approximately 23,000 was excised and used as antigen (by Animal Pharm Services, Inc., Healdsburg, Calif.) to raise polyclonal antibodies in a rabbit. Lysed TK23 protoplasts from submerged cultures were used to preadsorb antibody-containing serum. To collect S. lividans surface cultures, spores were heat shocked and cooled as described previously (8) and then plated immediately onto cellophane (Bio-Rad, Hercules, Calif.) placed on R5 agar plates. Extracts of cells collected at the indicated times were prepared and quantified as described previously (14). SDS-PAGE analysis of proteins and Western blotting were also performed as previously described (14), except that proteins were electrophoresed on 15% polyacrylamide gels, unless otherwise indicated, and goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate was obtained from Bio-Rad and used at a 1:3,000 dilution.

RESULTS

KilB protein concentration increases temporally throughout S. lividans cellular differentiation. The KilB gene of plasmid pIJ101 encodes a 147-amino-acid protein (11) that has a predicted molecular mass of 15 kDa. Analysis by SDS-PAGE and Coomassie blue staining of extracts of S. lividans strain TK23 substrate mycelia containing conjugative pIJ101 derivatives versus equivalent amounts of extracts from plasmidless TK23 cells failed to reveal any additional protein bands in this molecular mass range that were specific for plasmid-containing cells. However, Western blotting of these same extracts using antibodies raised against a fusion protein expressed in E. coli that was encoded in part by the entire kilB ORF (see Materials and Methods) revealed a rather weakly expressed protein of 15 kDa (Fig. 2, lane 2) for S. lividans strain TK23 containing the conjugative pIJ101 derivative pIJ303 (13). This protein was absent in extracts either from strain TK23 cells alone (Fig. 2, lane 1) or from TK23-containing plasmid pIJ303ΔkilB (Fig. 2, lane 3), a derivative of pIJ303 that has a deletion (Fig. 1) of the entire kilB gene as well as a portion of orf56, a small ORF of undetermined function (11), which may encode a protein of approximately 6 kDa. As expected, the products of pIJ101 genes unaffected by the deletion in pIJ303ΔkilB, including the KorA protein, were present in approximately equivalent amounts (as judged by Western blotting) (22) in cells that contained either pIJ303 or pIJ303ΔkilB (data not shown).

The pIJ101 Tra protein, which mediates intermycelial transfer of the plasmid, was shown by previous Western blotting to be expressed temporally in S. lividans, with its cellular concentration being highest at the earliest point analyzed during growth of substrate mycelia and then rapidly decreasing in amount so that Tra was undetectable by about the time aerial hyphae began to form and antibiotic production began (14). To determine whether the KilB spread protein of pIJ101 shows a similar pattern of expression, spores of strain TK23(pIJ303) were seeded onto R5 agar plates, and following growth for various times, cells were collected and equivalent amounts of extracts were examined by Western blotting (Fig. 3A) as described above for KilB. Extracts from identically grown and harvested strain TK23 alone were also analyzed (Fig. 3A) to provide a control for nonspecific background unrelated to KilB in Western blots.
In contrast to the pattern observed for Tra, the 15-kDa KilB protein was present throughout cellular differentiation of *S. lividans* TK23(pIJ303), with its lowest concentration being found in the substrate mycelium (18 h after inoculation of plates with spores); at subsequent morphologically distinguishable points, including the first approximate indication of aerial hypha production (24 h), later times during aerial hyphal growth (36 and 48 h), and finally following sporulation (144 h), the amount of KilB increased steadily such that by sporulation the concentration was at its lowest concentration at the earliest time analyzed for TK23(pIJ303) during exponential growth (time point E1) and then showed a steady increase in concentration at subsequent time points during both exponential (E2 and E3) and stationary (S1, S2, and S3) growth phases. These results were again in contrast to those shown previously for the pIJ101 Tra protein, which was found only in exponentially growing broth cultures of Tra-producing *S. lividans* strains (14).

**Construction of an *S. lividans* strain containing the chromosomally inserted pIJ101 kilB gene in the absence of KorB repressor.** Binding of the pIJ101 KorB repressor to the *kilB* gene promoter controls the transcription of *kilB* and also suppresses the lethality phenotype associated with unregulated *kilB* expression on pIJ101 (9). To determine whether this critical regulation is the basis for the temporal increase in KilB protein levels during differentiation of *S. lividans*, we sought to monitor production of KilB in *S. lividans* cells that lacked KorB repressor. Since previous results raised the possibility that lower dosages (i.e., five copies or less per chromosome) of unregulated *kilB* might alleviate its effects on cell viability and thus circumvent the need for KorB (12), we inserted the pIJ101 *kilB* gene into the *S. lividans* chromosome, which therefore allowed unregulated *kilB* expression at a permissibly low gene dosage. As such construction also guaranteed that the copy number of *kilB* would remain invariant, we were simultaneously able to evaluate whether copy number increases in pIJ101 (and thus in the *kilB* gene), which are known to occur during the course of streptomycete growth (13), could be responsible for the temporally increasing pattern of KilB protein expression.

Using a gene replacement method (24) previously employed to integrate numerous exogenous genes, including the *tra* and *korA* genes of pIJ101 (15), into the *S. lividans* chromosome, we inserted the pIJ101 *kilB* gene into the cloned *S. lividans* chromosomal *afsR* locus present on the thiostrepton-resistant pUC19-based vector pBeBal2 (15), which lacks the ability to replicate in *Streptomyces* bacteria (24). Transformation of *S. lividans* TK23 using the resulting plasmid, pGSP295, yielded thiostrepton-resistant transformants in which single reciprocal (Campbell-like) recombination between homologous sequences present in the chromosome and on the plasmid led to integration of pGSP295 sequences at the chromosomal *afsR* locus (Fig. 5). Screening of transformants by PCR analysis of their chromosomal DNA using opposing primers specific for the 5′ and 3′ ends of the *kilB* gene resulted in an amplified product of the expected size (see Materials and Methods), which confirmed that *kilB* gene sequences had been retained upon integration of pGSP295 into the *S. lividans* chromosome (data not shown).

Following integration of pBeBal2 derivatives by this method,
FIG. 5. Genetic organization at the chromosomal afsR locus of *S. lividans* strain NWR2. A previously described (24) gene replacement procedure was used to integrate the pIJ101 *kilB* gene at the *S. lividans* afsR locus. A 1.0-kb *kilB*-containing pIJ101 fragment was inserted into the afsR gene contained within the cloned *S. lividans* chromosomal afsR locus present on the thioestrepton-resistant, pUC19-based *E. coli* plasmid pBeBal2 (15, 23). Upon transformation of *S. lividans* strain TK23 with the resulting plasmid, pGSP295, integration of the plasmid at the chromosomal afsR locus by single reciprocal recombination between homologous sequences present on the plasmid and in the chromosome led to the genetic organization shown. The relative location of the afsR2 gene (23) within the afsR locus is also indicated. *bla*, beta-lactamase gene present within pUC19 sequences (unfilled box); *tsr*, thioestrepton resistance gene; *afsR* and *afsR2*, interrupted portions of the *afsR* gene that resulted from insertion of the *kilB*-containing pIJ101 fragment.

Nonselective growth for two rounds of sporulation typically results in loss of the integrated thioestrepton resistance gene and associated pUC19 sequences by homologous recombination between duplicated afsR locus flanking sequences at a frequency of 1 to 10% (24); of these new thioestrepton-sensitive derivatives, 25% or more still retain a stably integrated copy of the exogenous gene(s) of interest (24). Here, however, thioestrepton-sensitive isolates were obtained following identical nonselective growth at the remarkably high rate of approximately 90% (i.e., 735 thioestrepton-sensitive isolates out of a total of 840 colonies analyzed). Additional screening of genomic DNA from 43 of these thioestrepton-sensitive derivatives by PCR amplification for potential *kilB* sequences as described above revealed that none of these isolates had retained the integrated *kilB* gene determinants that were present in the original thioestrepton-resistant strain, which we have named NWR2 (Fig. 5). Thus, due to the apparent high degree of instability of integrated *kilB*-containing pGSP295 sequences in the absence of thioestrepton selection, we used strain NWR2 itself grown continually in the presence of thioestrepton for subsequent studies on *KilB* protein expression in cells lacking *KorB* repressor.

*S. lividans* strain NWR2 shows elevated yet still temporally increasing concentrations of *KilB*. To examine the temporal profile of *KilB* in strain NWR2, spores were spread onto R5 agar containing thioestrepton, and following growth for various times and analysis of cell extracts by Western blotting (Fig. 3B), we found that, similar to the results seen earlier for strain TK23(pIJ303), *KilB* showed a steady temporal increase in concentration. Although the pattern of *KilB* expression or accumulation was similar in strain NWR2 compared to *KorB*-containing cells, the amount of *KilB* present at each time point following the plating of NWR2 spores was appreciably higher than the corresponding levels seen in equivalent amounts of TK23(pIJ303) cell extracts (e.g., Fig. 3B, compare NWR2 and TK23 containing pIJ303 at 144 h). The temporal increase in *KilB* concentration occurred in NWR2 despite the fact that little or no aerial hyphae formed and sporulation was not evident during the course of the experiment. Though the enhanced intracellular levels of *KilB* may have contributed to the observed inhibited development of strain NWR2, we were unable to rule out growth effects related to the presence of thioestrepton in the medium, since a TK23-based control strain containing a chromosomally integrated copy of the thioestrepton-resistant pBeBal2 integration vector was also somewhat inhibited for its development when grown identically on R5 agar containing thioestrepton (data not shown).

Western blots of NWR2 extracts prepared from submerged cell cultures (Fig. 4B) also showed temporally increasing amounts of *KilB* throughout exponential (E1 and E2) and stationary (S1 and S2) growth. Aside from elevated *KilB* concentrations (data not shown), the only detectable difference in profile from that seen for similarly grown TK23(pIJ303) cells was that *KilB* appeared to reach and maintain maximum levels earlier either in late log phase or just as cells entered stationary growth. We conclude that the overall pattern of *KilB* protein expression or accumulation seen during streptomyces cellular differentiation is not due to temporal changes in either *KorB* control of *kilB* gene expression or copy number of pIJ101 but rather to some additional, previously unknown regulatory mechanism.

**DISCUSSION**

It has been hypothesized that plasmids that contribute to the size of pocks elicited by transmission of *Streptomyces* plasmids mediate the movement or spread of plasmid molecules within recipient cells (9, 13). Following the initial intermycelial transfer step, plasmid spread through established cross walls that separate hyphal compartments within the substrate mycelial network of the recipient, for example, not only would theoretically increase pock size but also would enhance plasmid dissemination by allowing plasmid copies to reach more cell compartments and therefore more of the occasionally emerging aerial hyphae that ultimately yield dispersible spores. The presence of the *KilB* spread protein of pIJ101 at stages of streptomyces differentiation that are far subsequent to that when *Tra*-mediated intersubstrate mycelial transfer is completed and steady-state *Tra* protein expression ends (14) is consistent with the existence of a subsequent intramycelial component to plasmid spread. If *KilB* were instead only required for modulating some aspect of intermycelial plasmid transfer, as has been alternatively hypothesized (9), then its presence would no longer be required once growth in the substratum ceases. The intriguing temporal increase in *KilB*, which reaches its highest concentration following sporulation, raises the possibility that the *KilB* spread function may in fact be active (perhaps even most active) following the presumed intramycelial movement of plasmid molecules between vegetative substrate compartments and so during the latest stages...
of *Streptomyces* development; for example, KilB may somehow promote movement of plasmids within aerial hyphae either prior to or possibly following their systematic septation, a compartmentalization process that eventually leads to the formation of chains of individual spores.

The exact role of KilB protein in the spreading of pIJ101 remains undetermined. Previously, *kilB*-associated lethality raised the possibility that KilB may function to inhibit cell growth, which may then prolong the period during which intramyccelial spread and perhaps additional rounds of intermyccelial transfer can occur (12). As shown here, KilB’s presence in substrate mycelia may serve, for example, to keep open the initial “transfer window” during which Tra-mediated intermyccelial transfer between substrate compartments is known to occur (14), while the appearance of KilB throughout cellular differentiation may indicate that spread-promoting growth inhibition continues during the entire differentiation process, thereby leading to the retarded development of plasmid-containing aerial hyphae and spores that is a hallmark of pock formation. Alternatively, it is possible that KilB is directly required for intramyccelial plasmid spread (and possibly contributes to intermyccelial transfer as well) and that any associated growth inhibition is instead a consequence of its direct role in pIJ101 transmission.

The elevated concentrations of KilB seen here in nonmating *S. lividans* NWR2 cells may approach the transient levels thought to occur during transmission of pIJ101 when presumably single copies of the plasmid are transferred into recipients (or subsequently between recipient compartments); upon such transfer events, the absence of Kor proteins in recipient cells may induce temporary derepression of pIJ101 functions such as *kilB* that either direct plasmid transmission or inhibit recipient growth, and this induction, whose magnitude may be further enhanced as transferred pIJ101 molecules begin to replicate, may in turn stimulate additional plasmid transfer and spreading (12). Should such induction exist for *kilB* during mating, it will be interesting to determine whether this derepression affects the temporally increasing pattern of KilB protein expression or accumulation seen here under nonmating conditions for both pIJ101-containing cells and strain NWR2.

With further regard to the increased levels of KilB seen in strain NWR2, we have also observed variable reductions in growth rates and maximum cell densities achieved among NWR2 isolates grown in submerged culture (K. Schully and G. Pettis, unpublished results), despite the fact that the overall temporal pattern of elevated KilB protein expression remained invariant. While the basis for this variation in growth effects is currently unknown and under investigation, the results are nevertheless consistent with the notion that the higher KilB concentrations seen in strain NWR2 are growth inhibitory for *Streptomyces* cells.

We speculate that the instability observed for integrated, thioestrepton-resistant pGSP295 sequences in strain NWR2, which led to abnormally high numbers of thioestrepton-sensitive derivatives following nonselective growth, is another indication of toxic effects related to unregulated KilB protein expression. Upon repeated sporulation cycles, cells that had not undergone additional homologous recombination to remove integrated *kilB*-containing pGSP295 sequences (this should be the vast majority of cells) (24) apparently became nonviable at a high frequency so that most of the isolates recovered at this point (approximately 90%) were thioestrepton sensitive and had deletions of all of the integrated pGSP295 sequences, including *kilB*. Consistent with this argument, we found that even under constant selection for thioestrepton NWR2 isolates passed through multiple rounds of sporulation lost the ability to produce KilB protein as judged by Western blotting (data not shown); thus, unregulated expression of *kilB* apparently led to nonviability and selection for KilB-derivatives under all growth conditions tested.

It will be interesting to determine the KorB-independent mechanism that temporally regulates KilB protein levels in *Streptomyces* cells and whether this additional control is implemented during the course of *kilB* gene transcription or instead occurs posttranscriptionally. In any event, the temporal changes in KilB concentration for both submerged and surface-grown cell cultures indicate that physiological rather than morphological cues are involved in this regulatory process. Previously, the temporal decrease of pIJ101 Tra protein prior to cell differentiation in *S. lividans* was shown to be controlled by a posttranscriptional mechanism that also operates independently of morphological development (14).

Although temporal increases in KilB are not manifested by changes in KorB regulation per se, KilB concentrations were much greater in strain NWR2 compared to TK23 containing the pIJ101 derivative pJ303, despite a reduction of some 300-fold in copy number of the *kilB* gene in NWR2. These data indicate that KorB repression normally results in significantly reduced intracellular steady-state levels of KilB throughout *Streptomyces* growth. That KilB production is so tightly regulated is not surprising given the associated lethality and otherwise deleterious growth effects seen for this plasmid spread protein.

**ACKNOWLEDGMENTS**

We thank Sally Murphy for assistance with overexpression of recombinant KilB protein and Kevin Kendall for critical reading of the manuscript.

This work was funded by grant MCB-9604879 from the National Science Foundation (to G.S.P.). K.L.S. is the recipient of a Louisiana State University Board of Supervisors scholarship.

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