INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Characterization and regulation of a gene involved in sporulation of *Streptomyces griseus*

Babcock, Martin John, Ph.D.

The Ohio State University, 1990
CHARACTERIZATION AND REGULATION OF A GENE INVOLVED IN SPORULATION OF STREPTOMYCES GRISEUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

Martin J. Babcock, B.S., M.S.

The Ohio State University

1990

Dissertation Committee

C. J. Daniels
K. E. Kendrick
J. N. Reeve
W. R. Strohl

Approved By

[Signature]

Adviser

Department of Microbiology
To My Wife, Shellie
ACKNOWLEDGEMENTS

I owe my deepest gratitude to Dr. Kathleen E. Kendrick for her years of support and guidance throughout this research. I express thanks to the other members of my committee, Dr. C. J. Daniels, Dr. J. N. Reeve and Dr. W. R. Strohl for their support and critical evaluation. To my wife, Shellie, daughter, Meghan, and family, I offer my sincere thanks for your faith and endurance during these endeavors.
VITA


1984  .................. B. S. in Microbiology, University of Illinois, Urbana, Illinois

1987  .................. M. S. in Microbiology, The Ohio State University, Columbus, Ohio

1984-1989 .............. GTA, Department of Microbiology, The Ohio State University, Columbus, Ohio

1989-Present ............. GRA, Department of Microbiology, The Ohio State University, Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Microbiology

Studies in Microbial Development and Molecular Biology
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>Overview and Objectives</strong></td>
<td>1</td>
</tr>
<tr>
<td>Physiological Studies of <em>Streptomyces</em> Sporulation</td>
<td>5</td>
</tr>
<tr>
<td>Diffusible Factors Associated with <em>Streptomyces</em> Sporulation</td>
<td>7</td>
</tr>
<tr>
<td>Sporulation Loci in <em>S. coelicolor</em></td>
<td>14</td>
</tr>
<tr>
<td>Sporulation in <em>S. griseus</em></td>
<td>21</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>31</td>
</tr>
<tr>
<td><strong>Strains</strong></td>
<td>31</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>31</td>
</tr>
<tr>
<td><strong>Media</strong></td>
<td>34</td>
</tr>
<tr>
<td><strong>Growth Conditions</strong></td>
<td>35</td>
</tr>
<tr>
<td>Induction of Sporulation in Submerged Culture</td>
<td>36</td>
</tr>
<tr>
<td>Isolation and Manipulation of DNA</td>
<td>36</td>
</tr>
<tr>
<td>DNA Fragment Isolation and Purification</td>
<td>38</td>
</tr>
<tr>
<td>Bacterial Transformations</td>
<td>39</td>
</tr>
<tr>
<td>Creating Nested Deletions Using Exonuclease III</td>
<td>41</td>
</tr>
<tr>
<td>DNA Sequencing</td>
<td>42</td>
</tr>
<tr>
<td>Nick-translation of Plasmid DNA</td>
<td>44</td>
</tr>
<tr>
<td>End-labelling of DNA Fragments</td>
<td>44</td>
</tr>
<tr>
<td>Southern Hybridization</td>
<td>45</td>
</tr>
<tr>
<td>Isolation of RNA</td>
<td>46</td>
</tr>
<tr>
<td>RNA Slot Blots</td>
<td>47</td>
</tr>
<tr>
<td>Northern Hybridization</td>
<td>48</td>
</tr>
<tr>
<td>High Resolution S1 Nuclease Experiments</td>
<td>48</td>
</tr>
<tr>
<td>Cell Lysate Preparation</td>
<td>50</td>
</tr>
<tr>
<td>Catechol Dioxygenase Assay</td>
<td>51</td>
</tr>
</tbody>
</table>
### RESULTS

- Sequence of the Cloned Fragment .............................................. 52
- Subcloning Analysis ..................................................................... 61
- Transcriptional Start Site Determination .................................. 71
- Level of Transcript Measured in Early Sporulation ..................... 71
- Promoter-Probe Studies ............................................................... 74
- Expression in *E. coli* ................................................................. 88
- Transcriptional Termination of ORF1590 .................................... 97
- Differential Expression of an Upstream ORF .............................. 100

### DISCUSSION .............................................................................. 103

### APPENDIX A ............................................................................... 125

### APPENDIX B ............................................................................... 132

### LIST OF REFERENCES .................................................................. 139
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bald mutant classes of <em>S. coelicolor</em> A3(2)</td>
<td>15</td>
</tr>
<tr>
<td>2. White mutant classes of <em>S. coelicolor</em> A3(2)</td>
<td>16</td>
</tr>
<tr>
<td>3. Phenotypic classification of sporulation-deficient isolates of <em>Streptomyces griseus</em></td>
<td>23</td>
</tr>
<tr>
<td>4. List of strains used</td>
<td>32</td>
</tr>
<tr>
<td>5. List of plasmid vectors used</td>
<td>33</td>
</tr>
<tr>
<td>6. Plasmids constructed for this study</td>
<td>133</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Life cycle of <em>Streptomyces</em></td>
<td>3</td>
</tr>
<tr>
<td>2. Chemical structure of A-factor</td>
<td>9</td>
</tr>
<tr>
<td>3. Crossfeeding of strain SKK1011</td>
<td>26</td>
</tr>
<tr>
<td>4. Cloning strategy used to isolate pKK200</td>
<td>28</td>
</tr>
<tr>
<td>5. Creating nested deletions in pKK209 using Exonuclease III</td>
<td>54</td>
</tr>
<tr>
<td>6. Diagram of DNA sequencing strategy</td>
<td>56</td>
</tr>
<tr>
<td>7. Nucleotide sequence of the 2756 bp BamHI fragment cloned from <em>S. griseus</em> B-2682 which restores sporulation to Class III sporulation-deficient mutants</td>
<td>58</td>
</tr>
<tr>
<td>8. Computer generated open reading frame analysis</td>
<td>60</td>
</tr>
<tr>
<td>9. Two inverted repeats located in the sequenced 2756 bp BamHI fragment</td>
<td>63</td>
</tr>
<tr>
<td>10. Amino acid homology in the C-terminal regions of ORF1590 and the nusA gene product of <em>E. coli</em></td>
<td>65</td>
</tr>
<tr>
<td>11. Helix-turn-helix motifs found in the amino acid sequence of ORF1590</td>
<td>67</td>
</tr>
<tr>
<td>12. Subclone analysis of pKK200</td>
<td>70</td>
</tr>
<tr>
<td>13. Determination of transcription initiation sites by S1 nuclease mapping</td>
<td>73</td>
</tr>
<tr>
<td>14. Time-course analysis of transcript abundance</td>
<td>76</td>
</tr>
<tr>
<td>15. Localization of in vivo promoter activity</td>
<td>78</td>
</tr>
</tbody>
</table>
16. Catechol is converted to 2-hydroxymuconic semialdehyde by the \textit{xylE} gene product, catechol 2,3-dioxygenase .... 81

17. Construction of \textit{pKK226} ........................................ 83

18. Catechol 2,3-dioxygenase plate assay for promoter activity of transformants of \textit{S. griseus} B-2682 .......... 85


20. Specific activity of catechol 2,3-dioxygenase in \textit{S. griseus} transformants containing \textit{pXE4} derivatives ... 90

21. Construction of the translational fusion plasmid \textit{pKK216} . 92

22. DNA and amino acid sequence in the translational fusion . 94

23. Protein fusion analysis ................................. 96

24. Measurement of transcript length by Northern analysis .. 99

25. Northern analysis of \textit{S. griseus} B-2682 following the induction of sporulation in liquid culture .......... 102

26. Summary diagram of \textit{ORF1590} .............................. 105

27. Survey of \textit{E. coli}-like promoters found upstream of \textit{Streptomyces} genes ................................. 116

28. Nucleotide sequence of a new class of \textit{Streptomyces} promoter ................................. 119

29. Characteristics of two potential polypeptides encoded by the \textit{ORF1590} gene ................................. 121

30. Circular map of \textit{pUC18} ........................................ 127

31. Circular map of \textit{pIJ702} ........................................ 129

32. Circular map of \textit{pXE4} ........................................ 131
Overview and Objectives

Streptomycetes are Gram-positive soil bacteria capable of undergoing cellular differentiation in response to environmental signals. When nutrients are abundant, Streptomyces grows as filamentous mycelia containing relatively few cross-walls. Nutrient limitation triggers the developmental process in which these mycelia form cross walls and long chains of spores (Fig. 1). Differentiation in Streptomyces is unique in that development is a reproductive process resulting in the dispersal of hundreds of free spores from a single mycelial unit. Streptomycete spores have thickened cell walls and are relatively resistant to desiccation but not to heat or other extreme environmental conditions (16). When these spores encounter suitable growth conditions they germinate and again grow as vegetative mycelia.

The focus of this study has been to identify and characterize genes which are necessary for the developmental transition from filamentous mycelia to spores. Streptomyces has a unique and interesting biology worthy of study. (i) Streptomyces is likely to make use of both spatial and temporal control mechanisms. Septation and
Figure 1. Life cycle of *Streptomyces*. A, mature spores; B, germinating spores; C, vegetative mycelia; D, reproductive mycelia. Bald mutants are blocked in the transition from vegetative mycelia to mature spores.
Figure 1.
spore formation occur at the tips of the mycelia and would require differential expression of genes whose products function specifically at this location. Furthermore, genes required for spore formation must be expressed at a precise time in the sporulation process. As researchers continue to identify genes involved in *Streptomyces* sporulation, transcriptional, translational, and post-translational regulatory mechanisms will undoubtedly be defined. (ii) The streptomycetes synthesize a wide variety of antibiotics and antimetabolites. Production of secondary metabolites frequently coincides with the transition from mycelia to spores. Therefore, the identification of regulatory mechanisms functional during sporulation is significant in that at least some of these mechanisms also seem to be necessary for secondary metabolite production. (iii) An understanding of morphological differentiation in *Streptomyces* could be applied to other developmental processes. Fundamental processes observed in *Streptomyces* may be present and tested for in genetically and physiologically complex higher organisms. Thus, a more thorough understanding of streptomycete sporulation may be applicable to the analysis of other morphological processes as well to the production of important secondary metabolites.

There is one major advantage to studying the regulation of sporulation in *Streptomyces griseus*. Unlike other species of *Streptomyces*, which sporulate abundantly only on solid surfaces such as agar media, *S. griseus* B-2682 can be induced to sporulate in liquid culture by phosphate or nitrogen starvation (55) or nutritional limitation (62, 76). More recently, Daza et al. (20) demonstrated that *Streptomyces acrimycini*, *Streptomyces albus*, and three additional
strains of *S. griseus* sporulate abundantly in liquid culture following nutritional downshift. Using this system, biochemical and regulatory events can be detected as early as 15 minutes after induction of sporulation (62). Thus, temporal features of short duration can be studied at the molecular level.

**Physiological Studies of *Streptomyces* Sporulation**

Using the submerged sporulation system of *S. griseus*, work has been undertaken to understand the biochemical events characteristic of the onset of sporulation after nutrient limitation. Sporulation of *S. griseus*, like that of *Bacillus subtilis*, is nutrient-dependent, and the stringent response may trigger its initiation. In *S. griseus* the pool size of ppGpp increased immediately following a nutritional downshift and was accompanied by a decrease in intracellular GTP (76). The concentration of ppGpp following the nutritional downshift was sufficient to inhibit IMP dehydrogenase and thus elicit the drop in GTP levels (76). Relaxed (rel) mutants of *S. griseus* have been isolated and characterized. The rel mutant had a severely reduced ability to accumulate ppGpp during a nutritional downshift, a less extensive decrease in the GTP pool, and was unable to induce enzymes necessary for streptomycin production (77). GTP levels as well as sporulation were restored to rel mutants by the addition of decoyinine, a specific inhibitor of GMP synthetase (77). The increase in ppGpp level and decrease in GTP pool accompanying the onset of sporulation seem to be
independent of a diffusible substance, A-factor, which is necessary for spore formation and streptomycin production by \textit{S. griseus} (77). It appears that morphological differentiation of \textit{S. griseus} results from a decrease in the pool of GTP, whereas physiological differentiation results from a more direct function of ppGpp (77).

We would expect that a number of new gene products, especially structural proteins associated with the spores, would be detected following the induction of sporulation of \textit{S. griseus}. Valu \textit{et al.} have demonstrated changes in protein patterns of \textit{S. griseus} as a consequence of the initiation of sporulation (94, 96). There were no differences in protein patterns of mature spores from submerged and solid media (94). In a similar comparison, I detected the presence of phosphorylated proteins and protein kinase activities associated with early sporulation (M. J. Babcock, M. S. thesis, 1987). In \textit{S. griseus} B-2682, two phosphoproteins of ca. $M_r$ 112K and 162K were identified in mycelial extracts made two hrs after the initiation of sporulation; neither phosphoprotein was found in vegetative cells. The 162K protein was phosphorylated \textit{in vitro} only in the presence of 1 mM CaCl$_2$. Presumably either these target proteins are synthesized only during sporulation or the protein kinase is functional only during differentiation. This result suggests that regulation at the post-translational level may occur during sporulation of \textit{S. griseus}. 


Diffusible Factors Associated with *Streptomyces* Sporulation.

Several substances have been identified in streptomycetes which seem to act as primary activator signals for differentiation to spores and in some cases also for antibiotic production. These include factor C, a protein that induces sporulation of *S. griseus* (90); pamamycin, an antibiotic associated with aerial mycelium formation in *Streptomyces alboniger* (69, 70); and A-factor (2-isocapryloyl-3R-hydroxy-methyl-gamma-butyrolactone; Fig. 2), a small molecule necessary for streptomycin production and sporulation in *Streptomyces bikiniensis* and *S. griseus* (58, 60). Of these, A-factor has been studied in greatest detail.

Mutants deficient in A-factor biosynthesis were detected by their inability to produce streptomycin. Colonies unable to produce A-factor were also unable to sporulate (58). A decrease in streptomycin resistance of the A-factor mutants was also evident and was shown to be the consequence of the loss of streptomycin-6-phosphotransferase activity (30). All of these characteristics can be restored to A-factor mutants by the exogenous addition of A-factor. A-factor accumulates in the culture broth of wild-type strains, reaching a maximum concentration after one day of growth, whereas streptomycin levels are maximal after three days of growth. The time at which the exogenous A-factor is added is critical: A-factor mutants will produce streptomycin only if the exogenous A-factor is added at an early stage of growth (5). Many species of *Streptomyces* and related genera produce A-factor (60), but production seems to be strain-specific rather than species-specific (5).
Figure 2. Chemical structure of A-factor.
This could be a consequence of ambiguous streptomycete taxonomy. In *Streptomyces* a new species is commonly classified and identified by the type of antibiotic produced rather than by standard taxonomic methods (65).

The genetic determinant of A-factor production in *S. bikiniensis* and *S. griseus* is thought to be associated with a plasmid. Although the precise location of the A-factor gene has not been identified, the A-factor gene shows no tight linkage with the chromosome (43) and is easily transferred upon protoplast fusion (31). A-factor mutants of *S. bikiniensis* and *S. griseus* were obtained at a high frequency by growth in the presence of acridine orange (30). Hara and Beppu (30) postulated that in these mutants, the strain was cured of a plasmid carrying the A-factor determinant. Although the *afsA* gene is thought to be contained on a plasmid, the authors have neither isolated the plasmid nor addressed the possibility that acridine orange acts as a mutagen in this case.

Chemical mutagenesis with N-methyl-N’-nitro-N-nitrosoguanidine was necessary to isolate A-factor mutants of *Streptomyces coelicolor*, detected by their inability to produce A-factor (31). Unlike A-factor mutants of *S. griseus* and *S. bikiniensis*, which lost their ability to sporulate simultaneously with the loss of A-factor production, A-factor nonproducers of *Streptomyces coelicolor* sporulated normally. This suggests that A-factor is not essential for sporulation in this species. Several of the *S. coelicolor* mutants simultaneously lost the ability to produce two red pigments, actinorhodin and undecylprodigiosin, characteristic of the wild-type organism. Two loci were mapped for
these mutants on the S. coelicolor chromosome: afsA, deficient in A-factor production, and afsB, deficient in both A-factor and red pigment production (5). The afsB mutants of S. coelicolor were found to be deficient in production of several secondary metabolites including actinorhodin, undecylprodigiosin, methylenomycin, and a calcium-dependent antibiotic (H44).

The afsA gene of S. bikiniensis was cloned by complementation of A-factor mutants of the same strain (45). The cloned gene restores streptomycin production, streptomycin resistance, and spore formation to A-factor mutants of S. bikiniensis and S. griseus (45). The afsA gene from S. griseus has recently been sequenced and is thought to encode a protein of Mr ca. 32,600. No apparent function has been identified for this protein, but it has been postulated that that afsA gene product is key for A-factor biosynthesis and that all Streptomyces require only a single structural gene (afsA) for A-factor synthesis (5). I think it is unlikely that afsA is solely responsible for A-factor production.

Rather than being a structural gene, the cloned afsA gene may be a regulatory gene necessary for the expression of silent genes required for A-factor synthesis. In S. lividans, the phenoxazinone synthase gene (54) and the genes encoding the biosynthetic pathway for actinorhodin production (44) are normally silent or expressed at very low levels. Jones and Hopwood (54) cloned two fragments of S. antibioticus DNA, unrelated to the structural gene for phenoxazinone synthase, which restored phenoxazinone synthase activity to mutants of S. lividans (54). Similarly, introduction of the afsB gene into S. lividans stimulated transcription of the actinorhodin biosynthetic genes (44).
A spontaneous A-factor mutant of *Streptomyces lividans* was used to clone the *afsB* gene of *S. coelicolor*. Genomic DNA of *S. coelicolor*, ligated into a high copy number vector, was transformed into the *S. lividans* mutant. A positive clone was identified by the ability of the transformant to produce a large quantity of red pigments, actinorhodin and undecylprodigiosin, which are normally produced in low amounts and after prolonged growth by *S. lividans*. The *afsB* gene restores synthesis of A-factor as well as actinorhodin and undecylprodigiosin in *afsB* mutants of *S. coelicolor* (43).

The nucleotide sequence of the *afsB* gene predicts a gene product of 243 amino acids. Inferred from the amino acid sequence are two regions that resemble the alpha helix-turn-alpha helix motif of a DNA binding protein. Northern analysis demonstrated that in *S. lividans* the mRNA was constitutively transcribed throughout growth (43). DNA homologous to the *afsB* gene was not detected in *S. bikiniensis* or *S. griseus*. The authors concluded that AfsB seems to be a positive regulator of the production of A-factor and secondary metabolites, although A-factor is not essential for differentiation or antibiotic production in *S. coelicolor* and *S. lividans* (46). In contrast, AfsA alone is necessary and sufficient for sporulation and streptomycin production in *S. bikiniensis* and *S. griseus*. No *afsB* homolog could be detected in either of these two species.

The *afsB* gene product appears to be a positive regulator which acts at the level of transcription (44). The transcripts from the actinorhodin gene cluster are not present in *afsB* mutants of *S. coelicolor*. Introduction of the *afsB* gene into this mutant restored the
production of actinorhodin transcripts. Transformation of *S. lividans* with the *afsB* gene also stimulated transcription of the *act* genes under conditions in which they are normally silent in this strain (44).

Similarly, the *afsB* gene product may enhance transcription of genes encoding synthesis of undecylprodigiosin, methylenomycin, and a calcium-dependent antibiotic -- secondary metabolites produced at low levels as a consequence of the *afsB* mutation in *S. coelicolor* (44).

Recently it has been demonstrated that the *afsB* gene cloned by Horinouchi *et al.* (42) suppresses the *afsB* phenotype by pleiotropic regulatory effects and does not correspond to the mutated chromosomal locus originally identified in *S. coelicolor*. The corresponding suppressor gene from *S. lividans* has been cloned and renamed *afsR* (88). The original *afsB* mutation maps near the *ilv* gene on the *S. coelicolor* chromosome. Transfer of the *Ilv* phenotype was used to select *S. lividans* exconjugants that had also received the *afsB101* allele from *S. coelicolor*. The chromosomally mapped *afsB* locus was transferred to *S. lividans* recipients with the transfer of the *Ilv* phenotype. The transferred *afsB101* allele did not hybridize with the cloned *afsB* gene. Therefore the *afsBafsR* gene cloned from *S. coelicolor* and *S. lividans* is a second gene which appears to suppress the *AfsB* phenotype (88). A disadvantage to "cloning by complementation" in *Streptomyces* appears to be the possibility of isolating suppressors of chromosomal mutations rather than the complementing genes.
Sporulation Loci in *S. coelicolor*.

The greatest number of genetic loci specific to sporulation has been identified and characterized by Chater, Hopwood, and colleagues, studying *S. coelicolor*. Two broadly defined classes of sporulation-deficient mutants have been isolated. The bald mutants (*bld*) produce neither aerial mycelium nor spores and thus obtain their name from their bald colony appearance (40, 72). The white mutants (*whi*), which produce aerial mycelia but no spores, remain white instead of developing the characteristic grey color of mature *S. coelicolor* spores (15). Eight *bld* (Table 1; 14, 17, 72) and eight *whi* loci (Table 2; 15, 40) have been identified and representative mutants characterized.

At least five *bld* mutant classes are conditionally defective in sporulation (*bldA, bldD, bldE, bldG, and bldH*). These mutants will not sporulate on complex medium but will differentiate on minimal medium if glucose is replaced by other carbon sources (72). Of these mutant classes, *bldA, bldD, bldG, and bldH* mutants are pleiotropically defective in antibiotic production (14, 72). It is evident that the mechanisms that control antibiotic production and sporulation may be both independent and interrelated.

The characteristics of the gene that complements the *bldA* phenotype, which has been cloned and well characterized, have revealed a regulatory mechanism unique to streptomycete sporulation. The *bldA* gene was cloned from *S. coelicolor* by complementation using a phage vector derivative of φC31 (79). The nucleotide sequence of the DNA encoding the *bldA* gene revealed no obvious protein-coding region (64). Instead,
TABLE 1. Bald mutant classes of *Streptomyces coelicolor* A3(2) (taken from [17]).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype of Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{bldA} (72), \textit{bldD} (72), \textit{bldG} (14)</td>
<td>Sporulation on mannitol salts agar but not on glucose salts agar; no synthesis of antibiotics under any condition</td>
</tr>
<tr>
<td>\textit{bldB} (72)</td>
<td>Weak aerial mycelium and antibiotic development after prolonged incubation</td>
</tr>
<tr>
<td>\textit{bldC} (72)</td>
<td>Production of normal levels of antibiotics; sporulation restored by a diffusible factor from sporulating cultures.</td>
</tr>
<tr>
<td>\textit{bldE} (17)</td>
<td>Mutants able to use agar as sole carbon source in the presence of homoserine; sporulation on mannitol salts agar</td>
</tr>
<tr>
<td>\textit{bldF} (17)</td>
<td>No production of actinorhodin, methylenomycin, or calcium-dependent antibiotic; undecylprodigiosin produced abundantly; no carbon source effect</td>
</tr>
<tr>
<td>\textit{bldH} (14)</td>
<td>No aerial mycelium formation or antibiotic production on glucose; restored on mannitol</td>
</tr>
</tbody>
</table>
TABLE 2. White mutant classes of *Streptomyces coelicolor* A3(2) (taken from [17]).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype of Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>whiA, whiB</em></td>
<td>Aerial mycelia coil; no sporulation septa are detectable</td>
</tr>
<tr>
<td><em>whiC</em></td>
<td>Aerial mycelia are mostly uncoiled; some spores are produced</td>
</tr>
<tr>
<td><em>whiD</em></td>
<td>Chains of spores are produced with thin spore walls</td>
</tr>
<tr>
<td><em>whiE</em></td>
<td>Chains of unpigmented spores are produced</td>
</tr>
<tr>
<td><em>whiF</em></td>
<td>A low level of rod-shaped unpigmented spores is produced</td>
</tr>
<tr>
<td><em>whiG</em></td>
<td>Straight aerial mycelia; no sporulation septa are detectable</td>
</tr>
<tr>
<td><em>whiH</em></td>
<td>Aerial mycelia are loosely coiled and partially fragmented</td>
</tr>
<tr>
<td><em>whiI</em></td>
<td>Aerial mycelia are tightly coiled and partially fragmented</td>
</tr>
</tbody>
</table>
the sequence suggested that this fragment of DNA had the potential to encode an RNA with secondary structure consistent with a tRNA molecule. Five bldA mutant alleles were cloned and sequenced, and point mutations were localized to a 16 bp region in the anticodon loop of the potential tRNA.

By dot-blot analysis of RNA isolated at various times in the growth of *S. coelicolor* on an agar medium, bldA transcript was detected after 36 hrs of growth immediately before aerial mycelium formation (64). To confirm the temporal expression of bldA and to visualize gene expression in vivo, a transcriptional fusion of the bldA gene with the luxA and luxB operon of *Vibrio harveyi* was constructed (85). An analysis of temporal expression was carried out by measuring light emission from colonies carrying this fusion. Schauer et al. (85) found that bldA was expressed at day 3 entirely in the substrate mycelium. From these two studies we can conclude that the bldA gene product is expressed late in vegetative growth at a time corresponding to the initiation of differentiation and antibiotic production.

On the basis of sequence analysis, the bldA gene product seems to be a leucyl-tRNA recognizing the UUA codon (64). The UUA codon is extremely rare in the G+C rich DNA of *Streptomyces* and has not been found in any genes involved in primary metabolism. The UUA codon has been found only in the hyg (80), carB (25), and spc (19) transcripts involved in antibiotic resistance and production in *Streptomyces*.

Four TTA-containing genes have been tested for expression in a bldA mutant (19). Two of these genes, the lacZ gene encoding beta-galactosidase in *E. coli* and the carB gene encoding resistance to
various macrolide antibiotics in *Streptomyces thermotolerans*, showed complete dependence on the *bldA* gene product for expression (17). Both of these genes have in-frame TTA’s in the center of the open reading frame. Two genes containing an in-frame TTA at the amino terminal portion of the coding sequence show a partial dependence on the *bldA* gene product. These are the spectinomycin resistance gene (*spc*) from plasmid R100 and the hygromycin resistance gene (*hyg*) from *Streptomyces hygroscopicus*. These genes have potential ribosome binding sites a short distance downstream of the TTA and may initiate translation downstream of the UUA codon when the *bldA* gene product is not present. The authors suggest that the *bldA* gene controls expression of TTA-containing genes at the level of translation, although they have not examined transcript levels of the regulated genes (C4). However, the target genes may not be absolutely required for morphological differentiation because *bldA* mutants can sporulate on mannitol-containing medium. It follows that if the *bldA* gene encodes the sole UUA-recognizing leucyl-tRNA and is expressed only after vegetative growth, then genes required for vegetative growth would lack in-frame TTA’s.

A grey pigment is associated with spores produced by *S. coelicolor* on agar medium. *S. coelicolor* whi mutants are so named because they have the white colony appearance associated with aerial mycelium formation. These whi mutants are blocked in the transition from aerial mycelium to mature spores. Eight whi loci have been identified in *S. coelicolor*. On the basis of epistatic determination the whiG gene seems to be the first whi gene essential for spore formation and therefore is
likely to be essential for later stages of morphogenesis (15). It is unlikely that whi mutants could be isolated from *S. griseus* because aerial mycelia and spores both have the same white pigmentation.

The *whiG* gene was cloned from *S. coelicolor* using a φC31 derivative vector (71). *whiG* mutants were constructed by homologous recombination between a fragment internal to the open reading frame and the functional gene in the chromosome. Mutants containing two truncated *whiG* genes in their chromosome grew on minimal medium and were phenotypically indistinguishable from prototypical *whiG* mutants (18). Therefore, the *whiG* gene is essential for sporulation but not for vegetative growth.

As determined from the nucleotide sequence of the cloned DNA, *whiG* is capable of encoding a polypeptide of ca. Mr 30,917. The *whiG* protein shows significant amino acid sequence similarity with sigma factors of *Escherichia coli* and *Bacillus subtilis* (18). One might expect that sigma*whiG* would show greater homology to sigmaH, the sporulation sigma factor expressed earliest in *B. subtilis* differentiation. Instead sigma*whiG* closely resembles sigma28, the sigma factor of *B. subtilis* associated with control of expression of genes for motility, chemotaxis and autolysis, but not sporulation (18, 33). Further evidence that sigma*whiG* functions in a manner similar to sigma28 is that promoter sequences recognized by sigma28, when introduced into *S. coelicolor* at high copy, inhibit sporulation. The binding of holoenzyme containing sigma*whiG* to these additional promoter sequences presumably would dilute out the number of molecules available for the transcription of genes essential for sporulation (18).
Introduction of whiG on a high copy number plasmid in *S. coelicolor* results in early and increased sporulation (71). *S. coelicolor* containing whiG at high copy can sporulate in submerged culture. This is a feature uncharacteristic of *S. coelicolor* and resembles the submerged sporulation of *S. griseus* (18). It is tempting to speculate that the ability of *S. griseus* wild-type strains to sporulate in submerged culture, unlike other streptomycetes, may therefore be due to increased expression of the whiG gene.

It would be expected that regulation of sporulation would include differential expression of a number of sigma factors. This morphogenic regulation has been demonstrated in *B. subtilis*. Streptomyces genes typically have more than one promoter; as many as four promoters have been identified upstream of the agarase gene of *S. coelicolor* (13). Streptomycete promoter regions also have a marked heterogeneity (13). On the basis of *in vitro* transcription assays, at least four sigma factors have been identified in *S. coelicolor* (13, 18). By hybridization techniques, Tanaka *et al.* (91) have identified four homologs of the gene product of rpoD, the principal sigma factor of various eubacteria, in *S. coelicolor*. A cascade of sigma factors may play a role in the temporal regulation of streptomycete differentiation as is known to occur in endospore formation by *Bacillus subtilis*.

As a complementary approach to the analysis of genes involved in spore formation in *S. coelicolor*, Guijarro *et al.* (29) have isolated and cloned a gene that encodes a spore coat-associated protein, SapA. In this approach two proteins associated with the spore coat were identified. The amino-terminal amino acid sequence was determined for
the SapA protein, and a complementary oligonucleotide was synthesized and used as a probe to clone the gene. The sapA gene was isolated, sequenced and found capable of encoding a 19 kd protein having a signal peptide of 37 amino acids. Thus, the SapA protein is likely to be secreted and to remain associated with the surface of the developing spore (29).

Transcription of sapA seems to be under temporal control in that the appearance of the sapA transcript coincided with the appearance of aerial mycelium formation (29). SapA mRNA was found in all S. coelicolor developmental mutants tested, but was significantly reduced in bldC, bldD, and whiH mutants (29). Use of a sapA-luxAB transcriptional fusion confirmed that transcription of sapA was temporally associated with aerial mycelium formation. Moreover, transcription was spatially regulated in that the fusion was localized to the differentiating mycelium (29, 85). Cloning by this method is useful in identifying genes which encode structural components of the mature spore. However, this approach is limited by the inability to identify and characterize regulatory genes necessary for sporulation.

Sporulation in Streptomyces griseus.

When I first became interested in streptomycete sporulation, relatively little was known about the genes involved in this morphogenic process. Sporulation-deficient mutants had been isolated from S. coelicolor (bld and whi) and S. griseus (afsA). The afsB and bldA genes
had been cloned but not sequenced. We decided to use *S. griseus* as a model organism to study streptomycete development for two reasons. First, a great advantage in studying sporulation in *S. griseus* is that, unlike other streptomycetes, this organism will sporulate in submerged culture under nutrient-limiting conditions. Therefore, we would be able to study with greater precision the temporal regulation of biochemical or regulatory events involved in *S. griseus* sporulation. Secondly, a parallel study of sporulation in *S. griseus* and *S. coelicolor* would elucidate regulatory mechanisms common to streptomycete sporulation as well as those which are species-specific. My specific aims when I began my studies of sporulation in *S. griseus* were to: 1) isolate sporulation-deficient mutants of *S. griseus*, 2) phenotypically characterize and classify these mutants, 3) isolate DNA which restores sporulation to a class of sporulation-deficient mutants of *S. griseus*, and 4) characterize this sporulation gene and study its regulation using the submerged sporulation system of *S. griseus*.

Sporulation-deficient mutants of *S. griseus*, obtained after chemical mutagenesis, were detected by their inability to sporulate on a complex medium, SpM. I classified these sporulation-deficient mutants of *S. griseus* into four phenotypic groups based in part on the ability to sporulate on glucose-ammonia minimal agar medium and in submerged culture following phosphate starvation (Table 3). Class I mutants did not sporulate under any condition. Class III and IV mutants demonstrated conditional sporulation: these isolates sporulated, although to a lesser extent than the wild-type strain, on glucose-ammonia minimal agar and in submerged culture. Further characterization
TABLE 3. Phenotypic classification of sporulation-deficient isolates of *Streptomyces griseus*.

<table>
<thead>
<tr>
<th>Class</th>
<th>SpM</th>
<th>GNH₄</th>
<th>Crossfed By</th>
<th>Streptomycin</th>
<th>Red Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>w.t.</td>
<td>+</td>
<td>+</td>
<td>n.a.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
<td>none</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>-</td>
<td>Class III, IV and w.t.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>+</td>
<td>none</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>-</td>
<td>+</td>
<td>none</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* Glucose-ammonia minimal agar medium; mutants that sporulate on glucose-ammonia minimal agar medium also form spores in submerged culture, but to a lesser extent than the wild-type strain.

*b* +, produces streptomycin at wild-type levels (ca. 3-5 ug/ml); -, streptomycin is not produced.

*c* +, produces a faint red pigment when grown on glucose-ammonia-histidine minimal agar medium; -, the red pigment is not produced.
relied on the ability of the strain to excrete diffusible factors that induce other bald mutants to sporulate, and to produce wild-type levels of streptomycin and a red pigment. Only Class II mutants could sporulate when cross-fed by diffusible substances secreted into the agar by mutants of Class III or Class IV, or by the wild-type strain (Fig. 3). Class IV mutants differ from Class III mutants in that they produced streptomycin and also a red pigment characteristic of the wild-type strain when grown on glucose-ammonia minimal agar medium (2).

None of these mutant classes seems to be phenotypically identical to A-factor mutants isolated after treatment with acridine orange (30). Since the afsA clone was not available, several bld mutants of S. griseus were isolated by the procedure of Hara and Beppu (30; M. K. Osman, unpublished results). One of these mutants, bld5, neither sporulated nor produced streptomycin, and was therefore phenotypically identical to an A-factor mutant. This putative A-factor mutant could be crossfed to sporulate and produce streptomycin by Class III mutants. Class II mutants differ from A-factor mutants in that streptomycin production is not restored by crossfeeding.

A Class III mutant, SKK1016, was chosen as the recipient for initial cloning studies. Because this class of mutants formed spores on glucose-ammonia minimal medium, these strains must contain the genetic determinants necessary for spore structure and might therefore be defective in the regulation of sporulation. A 2.8 kb BamHI fragment was cloned from S. griseus genomic DNA prepared from the wild-type strain; this fragment restored sporulation to SKK1016 after growth on complex medium (Fig. 4). The isolated plasmid, pKK200, was associated with the
Figure 3. Crossfeeding of strain SKK1011. Strains were streaked on a complex medium, SpM. After four days of incubation at 30°C, sporulation of the central strain was determined as white powdery growth covering the vegetative mycelia. Sporulation was verified in each case by microscopic observation of chains of spores. In this figure, strain SKK1011 (Class II) is crossfed by B-2682 (wild-type) and SKK1016 (Class III), but not by itself, SKK1017 (Class II), or SKK1002 (Class I).
Figure 3.
Figure 4. Cloning strategy used to isolate pKK200. (A) BamHI-digested genomic DNA from the wild-type strain, B-2682, was ligated to BglIII-digested pIJ702 which had been dephosphorylated with alkaline phosphatase. Protoplasts of a Class III mutant, SKK1016, were transformed with this ligation mixture. Among approximately 2000 thiostrepton-resistant transformants, one was able to sporulate on complex medium. This plasmid containing a 2.8 kb insert was designated pKK200. Photograph of cultures of SKK1016 containing pIJ702 (B) and pKK200 (C), showing the bald phenotype of the former and the restoration of sporulation in the latter.
Figure 4
sporulation phenotype of the transformant. The vector, pIJ702, alone had no effect on the mutant phenotype, whereas retransformation of SKK1016 and other Class III mutants with pKK200 restored the sporulation phenotype. Hybridization studies indicated that related fragments are present in the genomes of all four *Streptomyces* species tested including *S. griseus*, *S. coelicolor*, *S. lividans*, and *Streptomyces viridochromogenes* (5). Hence this 2.8 kb fragment seems to be involved in sporulation of *S. griseus*; moreover, because related DNA is present in distantly related species, perhaps the cloned DNA is important for sporulation of other streptomycetes as well.

My approach to study sporulation in *S. griseus* is similar to that of Chater and colleagues in the analysis of *S. coelicolor* sporulation. *S. griseus* sporulation mutants are similar to those of *S. coelicolor* in that sporulation can be restored on minimal agar medium, but differ in that *S. griseus* Class III and IV mutants will sporulate on glucose-ammonia minimal agar whereas *bid* mutants of *S. coelicolor* will not. Some *bid* mutants of *S. coelicolor* will sporulate and produce wild-type levels of antibiotics on mannitol minimal agar medium. Class II mutants of *S. griseus* are similar to A-factor mutants in that these strains can be crossfed to sporulate, but unlike A-factor mutants streptomycin production is not restored to Class II mutants. By hybridization and complementation studies, the 2.8 kb *BamHI* fragment, cloned from *S. griseus*, is not related to any of the *Streptomyces* sporulation genes cloned thusfar.
Several goals were set to characterize further the 2.8 kb BamHI fragment which restores sporulation to Class III sporulation mutants of S. griseus.

1. Determine the nucleotide sequence of the 2.8 kb fragment.

2. By subclone analysis, determine the boundaries of the sporulation phenotype contained on the 2.8 kb fragment.

3. Study regulation at the transcriptional level and assess temporal regulation of the gene.
Materials and Methods

Strains

Table 4 lists all of the bacterial strains used in this study. The wild-type strain of Streptomyces griseus used, NRRL B-2682, is a soil isolate obtained from the Northern Regional Research Laboratory, Peoria, Illinois. It produces the antibiotics streptomycin and cycloheximide. Sporulation-deficient mutants were derived by chemical mutagenesis from the wild-type strain and from strain SKK832, a histidine auxotroph derived from strain B-2682 (2).

Plasmids

The plasmid vectors used in this study are presented and described in Table 5. Circular maps of these vectors are shown in Appendix A. Appendix B is a list of all plasmids constructed for this study. Representative examples of plasmid constructions appear in the results section.
TABLE 4. List of strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Phenotype</th>
<th>Parent</th>
<th>Mutagen</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces griseus:</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-2682</td>
<td>wild-type</td>
<td>B-2682</td>
<td>EMS</td>
<td>NRRLa</td>
</tr>
<tr>
<td>SKK832</td>
<td>His</td>
<td>B-2682</td>
<td>EMS</td>
<td>this lab</td>
</tr>
<tr>
<td>SKK1000–SKK1017,</td>
<td>Bld</td>
<td>B-2682</td>
<td>EMS</td>
<td>(2)</td>
</tr>
<tr>
<td>SKK1019</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKK1020–SKK1022</td>
<td>Bld</td>
<td>SKK832</td>
<td>EMS</td>
<td>(2)</td>
</tr>
<tr>
<td>SKK1028, SKK1029</td>
<td>Bld</td>
<td>B-2682</td>
<td>MNNG</td>
<td>(2)</td>
</tr>
<tr>
<td><em>Streptomyces lividans:</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TK24</td>
<td>wild-type</td>
<td></td>
<td></td>
<td>D. A. Hopwood</td>
</tr>
<tr>
<td><em>Escherichia coli:</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM101</td>
<td></td>
<td></td>
<td></td>
<td>C. J. Daniels</td>
</tr>
<tr>
<td>TB1</td>
<td></td>
<td></td>
<td></td>
<td>C. J. Daniels</td>
</tr>
</tbody>
</table>

a Northern Regional Research Laboratory, Peoria, IL.
b Ethylmethane sulfonate
c N-methyl-N’-nitro-N-nitrosoguanidine
TABLE 5. List of plasmid vectors used.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant Phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptomyces plasmid:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIJ702</td>
<td>Mel$^a$ Tsr$^b$</td>
<td>D.A. Hopwood</td>
</tr>
<tr>
<td><strong>Escherichia coli plasmid:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>Amp$^c$</td>
<td>C.J. Daniels</td>
</tr>
<tr>
<td><strong>Bifunctional plasmid:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pXE4</td>
<td>XylE$^d$ Tsr Amp</td>
<td>J. Westpheling</td>
</tr>
</tbody>
</table>

$^a$ tyrosinase  
$^b$ thioestreptone resistance  
$^c$ ampicillin resistance  
$^d$ promoterless catechol 2,3-dioxygenase
Media

Cultures of *S. griseus* were maintained on a sporulation medium, SpM, containing 0.5% yeast extract, 0.5% glucose, 0.5% NaCl, and 1.5% agar, buffered with 10 mM 3-(N-morpholino)-propane sulfonic acid (MOPS)-KOH, pH 7.3 (56). Unless otherwise stated, all values are listed as % wt/vol. This medium allowed sporulation of *S. griseus* after 4-5 days of incubation at 30°C. *Streptomyces lividans* cultures were grown and stored on R2YE (39).

Trypticase soy broth (TSB; Microbiology Systems, Cockeysville, MD) supplemented with 5.0 mM MgCl₂ and 0.5% glycine was used to grow both *S. griseus* and *S. lividans* for protoplast preparation. *S. lividans* protoplasts were regenerated on R2YE plates, whereas protoplasts of *S. griseus* were regenerated on plates of SpMR medium (2). Both *S. griseus* and *S. lividans* strains were grown in TSB for plasmid and total DNA isolation. Cultures of *S. griseus* for the promoter probe analysis using xylE were grown in CAA medium (2).

*Escherichia coli* was grown routinely in Luria broth (LB) or on Luria agar plates (67) supplemented with 100 µg/ml ampicillin when appropriate. The color indicator 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal, Bethesda Research Laboratories Inc., Gaithersburg, MD) was included at 40 µg/ml when necessary (67).
Growth Conditions

Spore suspensions of \textit{S. griseus} were prepared from 50 ml cultures grown in medium C (55). The medium was inoculated from a single sporulated colony and incubated for 4-6 days at 30°C in a rotary shaker. Spores were harvested by centrifugation, washed and stored in sterile distilled H\textsubscript{2}O at 4°C.

Spore suspensions of \textit{S. lividans} were prepared from R2YE plate cultures. Plates spread for confluent growth were incubated for 5 days at 30°C. Five ml of sterile distilled H\textsubscript{2}O was added to the plate. The spores were gently scraped off with a sterile bent glass rod, poured into a sterile tube, centrifuged, washed and stored in sterile distilled H\textsubscript{2}O.

Liquid cultures were inoculated with heat-activated spores according to conditions described by Kendrick and Ensign (55). All liquid cultures of \textit{S. griseus} were grown at 30°C in a shaking incubator at 250 rpm and contained 5\% polyethylene glycol (PEG; molecular weight, 8000 (Sigma Chemical Co., St Louis, MO); added as a 10\% solution after germination of the spores) to improve the dispersion of the mycelia (55). \textit{S. lividans} cultures were grown in a final concentration of 15\% PEG.

Prior to induction of sporulation of \textit{S. griseus} in submerged culture, the organism was grown to early exponential phase in CAA medium (minimal medium containing 20 mM glucose, 20 mM NH\textsubscript{4}Cl, 50 mM NaK\textsubscript{2}PO\textsubscript{4}, pH 7.3, and 1.0\% casein hydrolysate) (2).
Induction of Sporulation in Submerged Culture

*S. griseus* was induced to sporulate in submerged culture by phosphate starvation based on the procedure of Kendrick and Ensign (55). A 3 ml TSB starter culture, inoculated from a spore suspension of the wild-type strain or from mycelial fragments of bald mutants of *S. griseus*, was grown for 4-8 hrs at 30°C. This starter culture was then used as the inoculum for 250 ml of CAA medium containing 5% PEG in a 1.0 liter flask. When the culture had grown to late exponential phase, the mycelia were harvested by filtration through a 0.45 um cellulose nitrate filter (Micro Filtration Systems, Dublin, CA) and washed with starvation medium. The filter containing the mycelia was then immersed in 250 ml of starvation medium prewarmed to 30°C. Starvation medium is the same as CAA medium except that 50 mM Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid-KOH (TES; United States Biochemical Corp., Cleveland, OH), pH 7.4, was substituted for the phosphate buffer and casein hydrolysate. Periodic microscopic examinations confirmed the virtually complete sporulation of the starved cells after 24 hrs and the absence of microbial contaminants.

Isolation and Manipulation of DNA

*Streptomyces* strains used for genomic or plasmid DNA isolation were grown in 3 ml of TSB (with 5.0 ug/ml thiostrepton where appropriate) in a test tube containing a 6.0 mm diameter glass bead (P.
Bartel, personal communication). The glass bead aided in the growth and dispersal of the mycelia which were easier to lyse and resulted in cleaner DNA minipreparations.

Isolation of streptomycete genomic DNA was performed by a modification of the method of Hopwood et al. (39). Cultures were grown for 48 hrs in TSB as above. A 1.5 ml sample of culture was centrifuged and suspended in a total volume of 0.5 ml TE buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0). Lysozyme was added to a final concentration of 2.0 mg/ml and the mycelia were incubated for 10 min at 37°C. Aliquots of 120 ul of 0.5 M EDTA and 13 ul of a 10 mg/ml pronase stock were added, mixed, and incubated for 5 min at 37°C. A 70 ul volume of 10% sodium dodecyl sulfate (SDS) was then added and the mixture incubated for 10 min. The DNA was mixed gently with an equal volume of phenol/chloroform (1:1, vol/vol) and centrifuged. The aqueous phase was removed to a clean tube and the extraction repeated until no white precipitate remained at the interface. The DNA was then mixed gently with an equal volume of chloroform, centrifuged, and the aqueous phase removed to a clean tube containing a 0.1-fold volume of 3.3 M sodium acetate (NaOAc). A 2.5-fold volume of absolute ethanol was added and the DNA was precipitated by placing the tube at -20°C for 15 min. The DNA was centrifuged for 7.0 min in a microcentrifuge, washed with 200 ul of ice-cold 70% ethanol and dried under vacuum for 5.0 min. The DNA was then dissolved in 200 ul TE buffer, incubated with 0.4 ug/ml RNase (Sigma Chemical Co., St. Louis, MO) for 1 hr at 37°C, and precipitated at -20°C by adding 20 ul of 3.3 M NaOAc and 220 ul of isopropanol. The DNA was pelleted by centrifugation, washed with ice cold 70% ethanol, dried, and
resuspended in 100 μl TE buffer. It was critical not to spool the precipitated DNA in order to recover total genomic DNA that included plasmid DNA.

Plasmid DNA was isolated by a modification of the procedure of Kieser (57), the details of which have been described by Babcock and Kendrick (2).

A standard buffer (10 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 5 mM dithiothreitol (DTT)) supplemented with the required salt at the concentration recommended by the enzyme supplier, was used for the digestion of DNA with restriction endonucleases (BRL). DNA fragments were resolved by electrophoresis through submerged horizontal agarose gels in GGB buffer (40 mM tris-acetate [pH 8.3], 20 mM sodium acetate, 0.2 mM EDTA; J. Gardner, personal communication).

DNA Fragment Isolation and Purification

DNA fragments used for cloning, as probes, and for S1 experiments were isolated by two methods. In both procedures the first step involved cutting the ethidium bromide-stained fragment out of an agarose gel. In the first method the DNA was electrophoresed out of the gel slice onto a DEAE membrane (NA-45, Schleicher and Schuell, Keene, MD) cut slightly larger than the gel slice. This was done at a constant voltage of 45V for one hour in Tris-borate buffer (10.8 g trizma base and 5.5 g boric acid per liter) using an electrophoretic transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA). The
membrane containing the DNA was rinsed in low salt buffer (150 mM NaCl and 10 mM Tris-HCl, pH 8.0) and incubated at 50°C for 45 min in a microcentrifuge tube containing 200 ul high salt buffer (1.0 M NaCl, 10 mM Tris-HCl, pH 8.0). During this incubation the DNA was eluted from the membrane into the high salt buffer. The buffer was then removed to a fresh tube and the filter was washed once with 200 ul of high salt buffer to improve recovery. The DNA fragment was precipitated upon the addition of 1.0 ml 95% ethanol and incubation at -20°C for 2 hrs.

In the second method the DNA fragment was electrophoresed from the gel slice down a V-shaped well and trapped in a high-salt cushion (3.0 M sodium acetate), using an IBI electroeluter (International Biotechnologies Inc., New Haven, CT) according to the manufacturer’s instructions. The electrophoresis buffer contained 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, and 5 mM NaCl. After 20 min at 80 V the DNA fragment was removed from the V-shaped well in a volume of 400 ul and precipitated with the addition of 1.0 ml 95% ethanol and incubation at -20°C for 2 hrs.

Bacterial Transformations

Competent *E. coli* cells were prepared on ice with CaCl₂ according to the procedure of Mandel and Higa (66) modified by Maniatis *et al.* (67). Transformations of *E. coli* with plasmid DNA were performed as described by Maniatis *et al.* (67) and the transformants grown on Luria agar containing 100 μg/ml ampicillin.
Protoplasts were prepared for the transformation of *S. griseus* and *S. lividans* in slightly different manners. Spores or mycelial fragments were inoculated into 50 ml of TSB supplemented with 5 mM MgCl₂. After 6-8 hr of incubation at 30°C, glycine and 5.0% polyethylene glycol (PEG) were added to *S. griseus* cultures. Routinely 1.0% glycine was added to cultures of bald mutants of *S. griseus*. Following an incubation of 6-8 hrs, 15% PEG was added to cultures of *S. lividans*. The 0.5% glycine was added after overnight incubation since it seemed to inhibit growth of *S. lividans* mycelia. Cultures were grown for 20-24 hrs. Protoplasts were prepared according to the procedure of Hopwood *et al.* (39) and were stored at -20°C at a concentration of 4 x 10⁹ protoplasts/ml. These protoplasts were stable for approximately 1 month.

Transformation of streptomycete protoplasts with plasmid DNA was performed essentially as described by Hopwood *et al.* (39), except that the T-medium contained 55% PEG-1000 (Sigma) instead of 25% (2). Optimal transformation frequency was achieved using 2 x 10⁹ protoplasts and 10 ng of plasmid DNA. Transformed protoplasts of *S. griseus* were plated on SpMR medium whereas *S. lividans* was plated on R2YE medium. After incubation at 30°C for 20 hrs, the plates were overlaid with 125 ug of thio strepton for *S. griseus* (final concentration of 5.0 ug/ml) or 1.0 mg of thio strepton for *S. lividans* (final concentration of 40 ug/ml) contained in 1.0 ml of H₂O.

The wild-type strain of *S. griseus* seemed to have a restriction system which prevented an efficient frequency of transformation of DNA originating from other species. For this reason, DNA ligations were transformed and screened in *S. lividans* before transfer to *S. griseus*. 
S. griseus protoplasts transformed with plasmid DNA isolated from S. lividans or E. coli resulted in 1,000-10,000-fold fewer transformants per ug DNA compared to the same DNA transformed into S. lividans protoplasts. The transformation frequency also decreased with increasing size of the plasmid DNA. Therefore, all ligated DNA was first screened in S. lividans and the appropriate plasmid was subsequently transformed into S. griseus. The protoplasts were heated for 10 min at 45°C to partially inactivate the restriction system of S. griseus (4).

Creating Nested Deletions Using Exonuclease III

Exonuclease III (BRL) was used to make deletions in pUC18 for subcloning and DNA sequencing (35). This enzyme hydrolyzes DNA with 5' overhanging ends at a uniform rate but is not active against 3' overhanging ends. Plasmid DNA (7.5 ug) was digested with an enzyme leaving a 5' end and an enzyme leaving a protected 3' end to ensure that the vector would not be digested. The restricted DNA was extracted twice with phenol/chloroform, then twice with chloroform, and was precipitated in 0.3 M NaOAc and 70% ethanol. The pellet was dissolved in Exonuclease III buffer (0.66 mM MgCl₂, 66 mM Tris-HCl, pH 8.0) at a DNA concentration of 100 ug/ml and incubated at 37°C. The reaction was started by the addition of 500 units of Exonuclease III (BRL), and at 2.0 min intervals 5.0 ul aliquots were removed to chilled microcentrifuge tubes containing 25 ul S1 nuclease buffer (0.25 M NaCl,
1.0 mM ZnSO₄, 5% glycerol and 30 mM potassium acetate, pH 4.6). When all the samples had been collected, 25 ul S1 nuclease (BRL) diluted to 0.5 units/ul in S1 nuclease buffer was added to each tube. The DNA was incubated at room temperature for 30 min and the reaction stopped with the addition of 6.0 ul S1 Stop Buffer (125 mM EDTA, 0.5M Tris-HCl, pH 8.0). For ligation, the DNA was extracted with phenol/chloroform once, twice with chloroform, and precipitated with 0.3M NaOAc and 70% ethanol. The DNA from each sample was dissolved in 40 ul T4 DNA ligase buffer (BRL), and 1.0 unit T4 DNA ligase (BRL, 1.0 unit/ul) was added. The blunt-end ligation was complete following 3 hrs of incubation at room temperature. The ligation mixtures were used to transform competent E. coli strain TB1. Plasmid DNA was isolated, digested with restriction enzymes, and the size and endpoints of the deletion determined by agarose gel electrophoresis and DNA sequencing.

DNA Sequencing

DNA was sequenced by the Sanger dideoxynucleotide method (84) using a modified bacteriophage T7 DNA polymerase (Sequenase; United States Biochemicals). Sequencing was performed using double stranded template, usually plasmid mini-preparation DNA isolated according to Babcock and Kendrick (2). The plasmid DNA in the vector pUC18 was denatured with alkali and hybridized with reverse or universal primer by the procedure of DiDonato et al. (22). The procedures for the extension and termination reactions were followed as provided by the manufacturer
Deoxyadenosine-5'-alpha-(^{35}S)-thiotriphosphate (alpha-^{35}S-dATP) and deoxyadenosine-5'-alpha-(^{32}P)-triphasphate (alpha-^{32}P-dATP) (Amersham Corporation, Arlington Heights, IL) were used in the sequencing reactions to label the synthesized fragments. If a region of sequence close to the primer needed to be resolved, ^{32}P-deoxyadenosine triphosphate was used as the radioactive label in the reactions. Both dGTP and dITP reactions were performed to resolve compressions in the sequencing gels.

Sequencing ladders used in the S1 nuclease experiments were performed by chemical means as reported by Maxam and Gilbert (68) and modified by Maniatis et al. (67). Sequencing reactions were resolved by electrophoresis through 6.0% denaturing acrylamide gels containing per liter: 57 g acrylamide, 3.0 g bis-acrylamide, 500 g urea, and 1X TBE buffer (10.8 g Tris-HCl, 0.93 g Na₂EDTA and 5.5 g boric acid per liter distilled H₂O). The gels were run on an M2 sequencing apparatus (BRL) at constant voltage of 1800 V. Prior to autoradiography the urea was removed from the gels containing ^{35}S by soaking in a bath of 10% acetic acid and 12% methanol for 10 min. The gels were then dried and placed in a folder with a piece of X-OMAT film (Eastman Kodak Co, Rochester, NY) for two days at room temperature. Gels containing ^{32}P were not dried, but were covered with plastic film and autoradiographed with an intensifying screen and X-OMAT film overnight at -20°C.
Nick-Translation of Plasmid DNA

Plasmid DNA was labeled by a variation of the nick-translation procedure published by Rigby et al. (81; C. J. Daniels, personal communication). The $^{32}$P-labeled DNA served as a probe for Southern and Northern hybridizations. Two ug of DNA, circular or linear, was dissolved in 45 ul of 10 mM Tris-HCl, pH 7.5, and 50 ul of 2X nick-translation mixture (10 mM MgCl$_2$, 2.0 mM DTT, 2.0 uM dCTP, 2.0 uM dTTP, 2.0 uM dGTP, and 100 mM Tris-HCl, pH 7.5). Ten pmol of alpha-$^{32}$P-dATP (3000 Ci/mmol, Amersham), 0.5 ng DNase (IBI), and 4.0 units of DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN) were added to begin the labeling reaction. The reaction mixture was incubated at 37°C for circular DNA or 15°C for linear DNA for 30 min, terminated by boiling for 5 min and chilled quickly on ice. Unincorporated alpha-$^{32}$P-dATP was separated by passing the plasmid through a spun column containing Sephadex G-50 (Pharmacia Inc., Piscataway, NJ) for 4.0 min at 1600 x g (67). Approximately 30% of the input label was incorporated into the nick-translated plasmid DNA.

End-labeling of DNA Fragments

End-labeled DNA fragments were used in high-resolution S1 experiments and for Maxam-Gilbert sequencing (67). Ten ug of plasmid DNA were digested with an appropriate restriction enzyme in a total volume of 25 ul. To this mixture 27 ul distilled H$_2$O, 6.0 ul 10X CIAP
buffer (10 mM MgCl₂, 1.0 mM ZnCl₂, 10 mM spermidine and 0.5M Tris-HCl, pH 9.0), and 2.0 ul calf-intestine alkaline phosphatase (4 units/ul, Boehringer-Mannheim) were added and the mixture incubated at 56°C for 30 min. To stop the reaction, phenol was added and the mixture incubated an additional 10 min. After centrifugation, the aqueous layer was extracted with chloroform twice and the DNA precipitated with isopropanol.

To melt the ends slightly, the DNA was dissolved in 20 ul of distilled H₂O (dH₂O) and 20 ul 2X denaturation buffer (10 mM spermidine, 1.0 mM EDTA and 1.0 M Tris-HCl, pH 9.5), incubated for 10 min at 65°C, and then placed on ice. The DNA was incubated at 37°C for 30 min following the addition of 3.0 ul dH₂O, 6.0 ul 10X kinase buffer (100 mM MgCl₂, 50 mM DTT, 50% glycerol, and 0.5 M Tris-HCl, pH 9.5), 1.0 ul gamma-³²P-ATP (10 uCi/ul, Amersham) and 1.0 ul T4 polynucleotide kinase (BRL). The reaction was stopped by extraction with phenol/chloroform, followed by extraction with chloroform and ethanol precipitation.

Southern Hybridization

Restricted DNA that had been separated on 1.0% agarose gels was transferred to nitrocellulose membranes (Schleicher and Schuell) by the procedure of Southern (87) as modified by Hopwood et al. (39). All prehybridizations and hybridizations were performed at 55°C in the presence of 50% formamide overnight. Membranes were washed at 70°C twice with 2X SSC and 0.1% SDS and twice with 0.2X SSC and 0.1% SDS. At
this stringency the probe should remain bound to DNA that is more than 80% identical. Autoradiography was performed as described above.

Isolation of RNA

Isolation of RNA was performed by a modification of Kirby et al. (59) as developed by H. Baylis and C. Smith (personal communication). All aqueous solutions and glassware used in the isolation of RNA were incubated at room temperature (RT) for 12 hrs with 0.01% diethylpyrocarbonate (DEPC, Sigma) and autoclaved to guard against RNase contamination. Gloves were worn at all times when handling RNA preparations. RNA samples were isolated from an *S. griseus* culture grown to exponential phase in CAA medium and transferred to phosphate starvation medium. *E. coli* strain JM101, containing derivatives of pUC18, was grown in LB supplemented with 100 ug/ml ampicillin. The beta-galactosidase gene was induced by the addition of 2.0 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, BRL) 3.0 hrs prior to harvest. A 1.5 ml aliquot of culture was centrifuged, the supernatant removed and the cells suspended in 250 ul SET buffer (5.0 mg/ml lysozyme, 0.3 M sucrose, 25 mM EDTA, and 25 mM Tris-HCl, pH 8.0). After incubation at 37°C for 5 min the cells were lysed by the addition of an equal volume of 2X modified Kirby mix (39) and vigorous mixing. Protein was removed by repeated extractions with phenol/chloroform until there was no precipitate at the interface following centrifugation. Nucleic acids were precipitated by the addition of 0.1 volume of 3.0 M sodium acetate,
pH 6.0, and 1.0 volume of isopropanol and incubation for at least 30 min at -20°C. The total nucleic acid was collected by centrifugation, washed with ice cold 70% ethanol, dried in a vacuum for 5 min and dissolved in 100 ul RQ1 DNase buffer as directed by the manufacturer (Promega International, Madison WI). DNA was removed by digestion with 2.0 ul of RQ1 DNase (1.0 unit/ul; Promega) and incubation for 20 min at 37°C. The reaction was stopped and the enzyme removed by two successive phenol/chloroform extractions. Following the addition of 10 ul 3M sodium acetate and 110 ul isopropanol, the RNA was stored at -20°C as an isopropanol precipitate until use.

The RNA samples were analyzed for degradation on standard 1.0% agarose gels stained with ethidium bromide. To guard against RNase contamination, the comb, gel apparatus, and gel support were soaked for at least 1.0 hr in 50 mM NaOH, and the agarose and electrophoresis buffer were autoclaved prior to electrophoresis.

RNA Slot Blots

RNA was applied to 0.2 um nitrocellulose (Schleicher and Schuell) by filtration through a dot blot apparatus (Bio-Rad Laboratories) or through a slot blot apparatus (Hoefer) according to the method described by the manufacturer. The RNA was first denatured by the addition of formaldehyde and SSC to final concentrations of 7.4% and 6X, respectively. The RNA was washed with three volumes of 6X SSC and fixed
to the nitrocellulose by baking 2 hrs under vacuum at 80°C. Hybridizations were performed as described above.

**Northern hybridization**

Northern hybridization was used to determine the size of mature RNA from total RNA preparations. RNA was separated using a denaturing formaldehyde agarose gel as described by Ausubel et al. (1). RNA was electrophoresed through a 1.0% agarose gel containing formaldehyde. The RNA was transferred by capillary action to Zeta Probe membrane (Bio-Rad) overnight using 10X SSC as the transfer buffer. To ensure that the RNA had not denatured during the RNA isolation and northern blotting procedure, the membrane was stained using 0.02% methylene blue in DEPC-treated water according to the method of Herrin and Schmidt (34). The methylene blue was removed by destaining in dH2O and the membrane baked under a vacuum at 80°C for 2 hrs to fix the RNA to the membrane. The hybridizations were performed according to the manufacturer’s instructions (BioRad).

**High Resolution S1 Nuclease Experiments**

To map transcriptional initiation sites and to determine the relative amounts of the transcripts present in cells in the early stages of sporulation, high resolution S1 nuclease experiments were performed.
on RNA samples of *S. griseus*. The method used is a modification of C. P. Smith as adapted by T. Clayton (personal communication). In this method the RNA:DNA hybridization takes place in 3.0 M sodium trichloroacetate (NaTCA); under these conditions RNA:DNA hybridization is very efficient but DNA:DNA duplex formation is inhibited (73).

The appropriate end-labeled DNA fragment (100,000 cpm) was combined with 40 ug RNA in a microcentrifuge tube and lyophilized using a Spin-Vac. More consistent results were obtained by concentration using lyophilization rather than ethanol precipitation. The dried nucleic acid was dissolved in 20 ul hybridization buffer (3.0 M NaTCA, 5.0 mM EDTA and 50 mM Pipes buffer, pH 7.0) and denatured at 70°C for 15 min. Keeping the tubes in the same water bath, the temperature was lowered slowly to 45°C and the samples were incubated an additional 5 hrs to allow time for complete hybridization. Single stranded nucleic acid was then hydrolyzed by the addition of S1 nuclease (200 units; BRL) in 0.3 ml S1 Digestion Buffer (0.28 M NaCl, 5.0 mM zinc acetate, 20 ug/ml denatured salmon sperm DNA and 30 mM sodium acetate, pH 4.4) and incubated for 45 min at 37°C. The reaction was stopped by adding 75 ul S1 termination buffer (2.5 M ammonium acetate [pH 4.4], 50 mM EDTA and 267 ug/ml tRNA). An equal volume of isopropanol was added and the remaining nucleic acid was precipitated at -20°C. The hybrids were pelleted by centrifugation, washed with ice cold 70% ethanol and suspended in 5.0 ul dH2O. These samples were electrophoresed in 6.0% denaturing acrylamide gels and compared with Maxam-Gilbert sequencing reactions (67) carried out on the end-labeled DNA fragment. The products of Maxam-Gilbert sequencing, which contain 5' OH-groups,
migrate slightly faster than fragments containing the same number of nucleotides and 5' phosphate groups.

Cell Lysate Preparation

To assay for catechol dioxygenase activity as a measure of promoter function, cell lysates were prepared every hour following the induction of sporulation. Cultures of the wild-type strain of *S. griseus* containing pXE4 derivatives were grown to exponential phase (200 Klett units using a green filter) in CAA medium containing 5 ug/ml thioestrepton. The cells were harvested by centrifugation or filtration, washed and suspended in prewarmed phosphate starvation medium containing 5 ug/ml thioestrepton. The culture was then incubated at 30°C with shaking. Samples of 25 ml were removed at various times after induction. The mycelia were harvested by filtration through a 0.45 um cellulose nitrate filter (Micro Filtration Systems, Dublin, CA), washed twice with 20 ml of chilled 0.1 M potassium phosphate buffer, pH 6.8, and suspended in 4.0 ml of the same buffer. The cells were disrupted by sonication (S-125, Branson Instruments, Danbury, CN), by applying three 5 sec bursts, or at 12,000 lb/in² using a French Pressure Cell. The second method gave more reproducible results in duplicate cultures. The intact cells and cell debris were removed by centrifugation at 4°C for 10 min in an Eppendorf microcentrifuge. The supernatant was removed to a clean tube and held at 4°C until assayed.
Catechol Dioxygenase Assay

To detect the xylE gene product, transformants of \textit{S. griseus} were grown on complex agar medium for 4 days at 30°C. These plates were sprayed with an aqueous solution of 0.5 M catechol and incubated for 30 min at 30°C. Colonies which expressed the gene turned yellow due to the decyclizing conversion by catechol 2,3-dioxygenase (101). Cell lysates were assayed for catechol dioxygenase activity in a 1.0 ml reaction mixture containing 50-200 ul cell lysate (ca. 0.1-0.4 mg protein) and 75 uM catechol in 0.1 M potassium phosphate buffer, pH 6.8. The rate of the reaction was measured spectrophotometrically at 375 nm in a thermostatically regulated spectrophotometer (Kontron; 83). One unit of activity corresponds to the formation of 1 nmol of 2-hydroxymuconic semialdehyde per min (101). Protein concentration was measured according to the spectrophotometric method of Ehresmann \textit{et al.} (24), with bovine serum albumin as a standard.
RESULTS

Sequence of the Cloned Fragment

Nested deletions of the 2756 bp BamHI fragment in pKK207 and pKK209 were generated using Exonuclease III (Fig. 5; Appendix B). These plasmids were sequenced using the dideoxynucleotide nucleotide sequencing method (Fig. 6). The sequence of the 2756 bp BamHI fragment cloned from S. griseus B-2682 is presented in Fig. 7.

An open reading frame analysis was performed on the sequence using a computer program developed by Bibb et al. (9). This program takes advantage of the fact that Streptomyces protein coding regions have a characteristic percentage of guanine + cytosine (G+C) residues in each position in the open reading frame. Streptomyces open-reading frames typically have 60-80% G+C in the first, 50-60% G+C in the second, and 90-97% G+C in the third position of the codon (9). Moreover, the asymmetrical G+C composition within the open reading frame enables one to predict the direction of transcription (9). By this procedure, an open reading frame of 1590 nucleotides (nts) was predicted, extending from nt 984 to nt 2574 in the sequence (Fig. 8).

Nine nucleotides upstream of the putative coding sequence is a potential ribosome binding site (GAGGG) (8, 38). Within the putative
Figure 5. Creating nested deletions in pKK209 using Exonuclease III.

ExonucleasIII Deletions

Cut with XbaI and PstI

Delete from 5' overhang with ExonucleaseIII

Blunt end with S1 nuclease

Ligate population of deletion subclones

Figure 5.
Figure 6. Diagram of DNA sequencing strategy. Arrows represent the direction and extent of sequence determined from a single Exonuclease III subclone plasmid by the dideoxy-chain termination method of Sanger et al (84). Restriction sites are indicated as: B, BamHI; E, Eco RV; S, SalI.
Figure 6.
Figure 7. Nucleotide sequence of the 2756 bp BamHI fragment cloned from *S. griseus* B-2682 which restores sporulation to Class III sporulation-deficient mutants. The proposed internal translation initiation site and the in-frame TTA are underlined. Two inverted repeats and four direct repeats located immediately downstream of the open reading frame are designated by arrows. Two putative helix-turn helix motifs are indicated by lower case letters.
Figure 7.
Figure 8. Computer-generated open reading frame analysis. This program determines the percentage of G+C nucleotides in each codon position. Streptomyces protein coding regions characteristically have 60-80% G+C in the first position, 50-60% G+C in the second position, and 90-97% G+C in the third position of the codon (9). All possible start (arrows) and stop (straight lines) codons in each of the six reading frames are denoted. By this method an open reading frame of 1590 bases was evident in frame N3>, which is transcribed from left to right. The figure was provided by M. J. Bibb.
Figure 8.
protein coding sequence is an in-frame TTA (nt 1770-1772) which would require the presence of the UUA-recognizing leucyl tRNA (bldA gene product) for translation.

Two inverted repeats are present in the sequence of the BamHI fragment (Fig. 9). The first, located at nt 530 to nt 558, would be capable of forming a secondary structure characterized by a calculated free energy of -21.7 kcal/mol (92). The second inverted repeat directly follows the ORF1590 stop codon (UGA) and has a free energy of -13.0 kcal/mol (92).

As determined from the sequence, ORF1590 would encode a protein having a Mr ca. 55,465, with a basic amino terminal region that would give the polypeptide a pI of 9.0. There was limited similarity between this polypeptide and one other sequence in the GenBank and PIR libraries. Although a function could not be ascertained from comparisons at the protein level, the carboxyl-terminal region did show 40% similarity with the carboxyl-terminal region of the NusA protein of E. coli (Fig. 10). This region is thought to be involved in protein binding. Two potential helix-turn-helix motifs from aa 12 to 32 and 303 to 323 were identified in the ORF1590 protein sequence (Fig. 11) (23, 78).

Subcloning Analysis

Subclones of pKK200 were constructed and analyzed to determine the minimum length of DNA required for sporulation in a Class III mutant
Figure 9. Two inverted repeats located in the sequenced 2756 bp BamHI fragment. The values for the free energies of these possible RNA secondary structures were determined by the method of Turner et al (92).
Figure 9.
Figure 10. Amino acid homology in the C-terminal regions of ORF1590 and the nusA gene product of *E. coli* (49, 82).
ORF1590 (420) A A D A A E C Y R D L K A P R Q V R R F T E Q A L S R P
NUSA (307) A V E A G N L P Q A I G R N G Q N V R L A S Q L S G W E

(448) T E E F - V R S H G R L V V S A V A E L E S - G N - L
(335) L N V M T V D D L Q A K H Q A E A H A A I D T F T K Y L

(473) D A A C A A G T R A V E V A G R I S S A R T T E Y V - -

(499) R D L L H R L E P Y G D E P R V A E L R E R A R P L L V
(389) K E L L E - I E G L - D E P T V E A L R E R A K N A L A

(527) T P G O (530)

(467) A D I E G L T D E K A G A L I M A A R N I C W G D B A O (496)

Aligned 115, Matches 31, Conserved 16, Homology 40%

Figure 10.
Figure 11. Helix-turn-helix motifs found in the amino acid sequence of ORF1590. The strength of these possible DNA-binding regions was evaluated according to the method of Dodd and Egan (23).
GENE (position)                      SCORE

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
ORF1590 (12) LGTVLAGISNAGLARRVN 535
ORF1590 (303) QEEAARYYIQLRLARA AA 727

Figure 11.
(Fig. 12). Introduction of pKK200, pKK210, or pKK236 restored sporulation to various Class III mutants. This indicated that the DNA from nt 798 to nt 2756 was necessary for the sporulation phenotype. A small deletion was made in ORF1590 at the NcoI site. The resulting constructs, pKK428 and pKK429, did not restore the sporulating phenotype. Copy number did not seem to have an effect on the ability of the 2.8 kb BamHI fragment to restore sporulation to Class III mutants (pKK240).

Two general ligation strategies were used for the subcloning analysis using the vector pIJ702, a high copy number streptomycete plasmid. In the first method, BamHI fragments were ligated into the BglIII site of pIJ702. The BglIII site is downstream of a strong promoter for the tyrosinase gene, melP (6, 38). By the second method in which SacI-PstI fragments were ligated into SacI-PstI digested pIJ702, this promoter was deleted. ORF1590 could not be obtained in the orientation in which transcription would proceed from the mel promoter through ORF1590. I was able to isolate readily plasmids in which ORF1590 was transcribed in the opposite orientation from the tyrosinase promoter (e.g., pKK200, pKK210). When the tyrosinase promoter was deleted by ligating ORF1590 as a SacI-PstI fragment, the transformants were readily obtained in either orientation. Alternatively, when a deletion was made in ORF1590 (e.g., pKK428 and pKK429), the inserted DNA was stable in either orientation (Fig. 12).
Figure 12. Subclone analysis of pKK200. All subclones were constructed in the vector pIJ702. The direction of transcription of ORF1590 is from left to right in pKK200. The direction and presence of the tyrosinase promoter (mel P) is designated by a dashed arrow. The portion of the 2756 bp BamHI fragment cloned into pIJ702 is designated by number according to the sequence (Fig. 7). Transcriptional start sites are designated by P1> and P2>. The deletion in pKK428 and pKK429 is designated as an X. The presence (+) or absence (-) of sporulation was recorded after examination by phase contrast microscopy.
Figure 12.
Transcriptional Start Site Determination

A 594 bp SacII-NarI fragment or a 299 bp SacII-NdeI fragment uniquely labeled on the 5’ end at the NarI or NdeI site was used in S1 nuclease mapping experiments. RNA was isolated from *S. griseus* B-2682 during vegetative growth and 2 hrs after sporulation had been initiated in submerged culture by phosphate limitation. The analysis using the SacII-NarI fragment as hybridization probe identified S1 hybrids, the shorter of which corresponded to a transcript designated P2, starting at approximately nt 1118 (Fig. 13). The location of the transcription initiation site corresponding to the longer transcript was determined by hybridizing RNA with the SacII-NdeI fragment. The upstream transcript, designated P1, initiated at nt 858 or 859 (Fig. 13). The amount of P1 transcript appeared to be constant, whereas the level of P2 transcript appeared to decrease two hrs after sporulation was initiated (Fig. 13).

Level of Transcript Measured in Early Sporulation

Because different amounts of the P2 transcript was detected in RNA isolated from sporulating cells, a more extensive analysis was performed to determine the level of transcript present during early sporulation. The SacII-NarI fragment labeled at the 5’ end was used in S1 nuclease experiments with RNA isolated from *S. griseus* during vegetative growth and at several times following the initiation of sporulation by phosphate starvation. This fragment was used because it hybridizes to
Figure 13. Determination of transcription initiation sites by S1 nuclease mapping. Left panel, origin of transcript P1, using as probe the \textit{SacII-NdeI} fragment labeled at the \textit{NdeI} site. Right panel, origin of transcript P2, using as probe the \textit{SacII-NarI} fragment labeled at the \textit{NarI} site. RNA was isolated from \textit{S. griseus} strain B-2682 growing as veg, vegetative mycelia, or spo, cells that had been induced to sporulate for two hours. Approximately equal amounts of RNA were applied to each lane. The sequencing ladders indicate the sequence of the antisense strand, and the numbers indicate the nucleotide in the sense strand.
Figure 13.
both transcripts P1 and P2. The analysis of sporulating mycelia demonstrated that the level of P2 transcript decreased significantly after the initiation of sporulation (Fig. 14). Densitometric scans of the bands on the autoradiogram indicated that the ratio of band intensity (P1:P2) began to increase within the first hour of sporulation, reaching 40 by 4 hrs into sporulation (Fig. 14).

Promoter-Probe Studies

A 676 bp Sau3A fragment from nt 514 to nt 1190 was shown to have in vivo promoter activity in S. lividans using two promoter probe vectors, pARCl (41) and pIJ487 (98; K. E. Kendrick, unpublished data). This fragment directed pigment production in pARCl and kanamycin resistance in pIJ487. Plasmids containing deletions of the Sau3A fragment were constructed to determine the boundaries of promoter activity. I made some of the preliminary constructs but the final constructs and bioassays were done by K. E. Kendrick. The levels of kanamycin resistance of the resulting S. lividans transformants were consistent with the presence of two promoters within this region. The promoter proximal to the open reading frame conferred a relatively high degree of kanamycin resistance, whereas the promoter distal to the open reading frame resulted in only a low level of resistance during vegetative growth (Fig. 15).

There are several drawbacks to using pIJ487 to study the activity of S. griseus promoter fragments. Because of the toxicity of kanamycin
Figure 14. Time-course analysis of transcript abundance. RNA was hybridized to the SacII-NarI fragment as in Fig. 13, right panel, and was analyzed by S1 nuclease mapping. RNA was isolated from S. griseus vegetative mycelia (veg), or mycelia induced to sporulate for 1, 2, 3, 4, or 6 hrs. With the exception of the 2 hr sample (in which the amount of RNA applied was significantly less than that from other samples), RNA was applied to obtain approximately equal intensities of the P1 band in each case. Ratios of abundance of transcript P1 to that of P2, determined by densitometric scanning, are shown at the bottom of the figure.
Figure 14.
Figure 15. Localization of in vivo promoter activity. Deletions of a 676 bp Sau3A fragment which showed promoter activity in vivo were inserted into pIJ487 and introduced into S. lividans to measure their capacity to confer kanamycin resistance. The highest concentration of kanamycin at which each transformant grew is indicated, as are the endpoints of the S. griseus DNA (open bar) and the nptII gene (closed bar).
Figure 15.
in the absence of promoter activity, this kanamycin-resistance detection system does not reveal promoters that are active only during sporulation. Another drawback of this experiment is that derivatives of pIJ101, such as pIJ487, are high copy number. Also, these promoter subclones were assayed in *S. lividans*, since the vector is unstable in *S. griseus* (personal observation).

For these reasons promoter fragments were cloned into a third vector, pXE4 (48), which recently became available. Promoter-active fragments are detected in pXE4 by their ability to direct catechol 2,3-dioxygenase activity from the promoterless *xylE* gene (48; Appendix A). The *xylE* gene encodes catechol 2,3-dioxygenase which oxidizes catechol to 2-hydroxymuconic semialdehyde (Fig. 16). Advantages to the use of this vector include: 1) a low copy number (ca. 1-2 per chromosome; K. E. Kendrick, unpublished) characteristic of plasmids with the SCP2 origin of replication, 2) stability in *S. griseus*, 3) a simple spectrophotometric assay system, and 4) the ability to detect temporally regulated promoters. The *Sau3A* fragment from nt 515 to nt 1190 carrying both promoters was cloned into pXE4 in both orientations relative to the promoterless *xylE* gene (Fig. 17). When these transformants of *S. griseus* were assayed by adding catechol to four day plate cultures, catechol dioxygenase activity was detected only in transformants which contained the fragment in the orientation consistent with the direction of transcription of ORF1590 (Fig. 18).

Promoters for the two transcripts P1 and P2 were also cloned separately into pXE4 by a similar method (Fig. 19). These plasmids were
Figure 16. Catechol is converted to 2-hydroxymuconic semialdehyde by the \textit{xyIE} gene product, catechol 2,3-dioxygenase (CatDase).
Figure 16.
Figure 17. Construction of pKK226. The Sau3A fragment from nt 515 to nt 1190 was isolated from a BglIII digest of pKK143 and ligated into the BamHI site of pXE4 upstream of the promoterless xylE gene. P1 and P2 are designated as the region of DNA upstream of the initiation sites of transcripts P1 and P2. Restriction sites are indicated as: B, BamHI; Bg, BglIII; H, HindIII; RI, EcoRI. DNA regions were indicated as: tsr, thiostrepton resistance; kan, kanamycin resistance; amp, ampicillin resistance; xylE, catechol dioxygenase (promoterless); colE1, E. coli origin of replication for colE1; SCP2 rep, Streptomyces origin of replication from the SCP2 plasmid.
Figure 18. Catechol 2,3-dioxygenase plate assay for promoter activity of transformants of *S. griseus* B-2682. Transformants containing the promoter fragment in correct (top, pKK226) and opposite (bottom, pKK225) orientation were grown on complex (SpM) agar medium for four days at 30°C. The colonies were sprayed with a 0.5 M aqueous solution of catechol and incubated for an additional 0.5 hrs. Promoter activity is detected by the conversion of the colorless catechol to a yellow semialdehyde in the mycelia.
Figure 19. Strategy for cloning into pXE4. *BglII* fragments containing
P1 and P2 promoters (from pKK143), P1 promoter (P1; from
pKK162), and P2 promoter (P2; from pKK160) were ligated into
the *BamHI* site of pXE4 as described in Fig. 17. The
resulting constructs, pKK226, pKK241, and pKK242
(respectively), contained promoter fragments from ORF1590,
the endpoints of which are designated by nt number from the
sequence. The orientation of the fragments was confirmed by
restriction digests, such that transcription from P1 or P2
would proceed through the *xyle* reporter gene on pXE4. The
*BglII* sites are indicated as *Bg*. 
Figure 19.
used in a more extensive analysis of promoter activity during early sporulation in S. griseus.

Cell lysates were generated from S. griseus transformants during vegetative growth and at intervals following the initiation of sporulation in submerged culture. Assays for catechol 2,3-dioxygenase were performed, and the results are summarized in Figure 20. Promoter activity for the two promoters in tandem (pKK226) dropped during the first six hrs of sporulation. This decrease is likely to be a consequence of a decrease in P2 promoter activity that initiates 1 hour after sporulation is induced. The promoter for P2 transcript is responsible for the majority of activity during vegetative growth according to studies in S. lividans. P1 promoter activity appears to be constitutively expressed. These results are consistent with those obtained by S1 nuclease analysis and by promoter probe analysis in pIJ487.

Expression in E. coli

A translational gene fusion was constructed between the -peptide of B-galactosidase and ORF1590 in pUC18 (Fig. 21). As analyzed by DNA sequencing, the first eleven amino acids of the α-peptide of B-galactosidase (99) were fused to aa 56 (met) of ORF1590 (Fig. 22). Transcription of the fusion protein initiated at and was under control of the lac promoter. The fusion protein of Mr ca. 51,000 (Fig. 23) was
Figure 20. Specific activity of catechol 2,3-dioxygenase in *S. griseus* B-2682 transformants containing pXE4 derivatives. Cell lysates were prepared from mycelia during vegetative growth (-1 Hr), when the mycelia were incubated under phosphate-limiting conditions to initiate sporulation in submerged culture, and at 1, 2, 3, 4, 6, and 7 hrs of sporulation. Results are presented as the average of at least three determinations. Only spores were present after incubation for 24 hours at 30°C under nutrient-limiting conditions.
Transcriptional Fusion Analysis
(xyle)

- + pKK226
  (P1 + P2)
- o pXE4
  (Vector)
- o pKK241
  (P1)

Figure 20.
Figure 21. Construction of the translational fusion plasmid pKK216. An in-frame fusion was made between the first 11 amino acids of the K-peptide of β-galactosidase (B-gal) and the ATG (nt 1149) of ORF1590. Restriction sites are indicated as: B, BamHI; Bs, BstXI; S, SalI; Sm, SmaI.
Figure 21.

Digest with *Bst*XI

Blunt ends with S1 nuclease

Digest with *Bam*HI

Digest with *Sma*I and *Bam*HI

Ligate

*pKK216*

*pUC18*

*pKK209*
Figure 22. DNA and amino acid sequence in the translational fusion.

The fusion protein of pKK216 fuses the first 11 amino acids of β-galactosidase to the 56\textsuperscript{th} amino acid of ORF1590.
FUSION PROTEIN from pKK216

\*peptide start\*---\>

MET THR MET ILE THR ASN SER SER SER VAL PRO MET VAL PRO GLN GLY

ACA GCT ATG ACC ATG ATT ACG AAT TCG AGC TCG GTA CCC ATG GTG CCG CAG GGC |

nt 1149

Figure 22.
Figure 23. Protein fusion analysis. SDS polyacrylamide gel electrophoresis of *E. coli* JM101 cell lysates containing pUC18 (lanes 2 and 3) and pKK216 (lanes 4 and 5) grown under repressed conditions (lanes 2 and 4; LB + 10mM glucose) and inducing conditions (lanes 3 and 5; LB + 2.0 mM IPTG). Molecular weight standards (lanes 1 and 6) are β-galactosidase (116,000), phosphorylase B (97,400), bovine serum albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000) and are indicated in Mr.
Figure 23.
expressed in *E. coli* strain JM101 under inducing conditions (2.0 mM IPTG), but not under glucose repression (20 mM glucose).

**Transcriptional Termination of ORF1590**

Despite repeated attempts, I was unable to obtain an estimate of transcript length of the ORF1590 region in the wild-type strain of *S. griseus*. Evidence from both Northern analysis and low-resolution SI nuclease studies indicated that transcripts from ORF1590 accumulate to only a low level during either vegetative growth or sporulation.

Deng *et al.* have demonstrated that streptomyocete DNA containing the classical stem-and-loop structure functions in transcription termination in both *Streptomyces* and *E. coli* (21). Because I was unable to map the 3' end of the ORF1590 transcript by the SI nuclease method, I studied the termination of transcription of the translational fusion in *E. coli* using the translational fusion in pKK216. There are two possible sites of transcriptional termination of the *Streptomyces* ORF1590 in *E. coli*. The first is in the neighborhood of the hairpin structure from nt 2577 to nt 2594 immediately downstream of ORF1590 (Fig. 7). The second possible transcriptional termination site is downstream of the α-peptide of β-galactosidase in pUC18. By northern analysis, the *EcoRV-BamHI* fragment of pKK200 hybridized with one transcript of ca. 1540 bp (Fig. 24). Therefore, transcription termination in *E. coli* appears to occur in the region immediately downstream of ORF1590.
RNA isolated from *E. coli* JM101 containing pKK216 was separated on a denaturing formaldehyde-agarose gel, transferred to nylon membrane, and hybridized to the 2.8 kb *BanHI* fragment. Markers indicate positions of DNA standards in nt. +, induced with 2.0 mM IPTG; -, without IPTG.
Differential Expression of an Upstream ORF

Evidence suggests that at least a portion of a second gene was cloned in pKK200. Two transcripts of ca. 470 and 700 bp were shown by northern analysis to hybridize with the BanHI-EcoRV fragment (nt 1-408) of pKK200 (Fig. 25). This region of the cloned DNA did not define any obvious open-reading frame when analyzed by the method of Bibb and al. (9), although there could be an open-reading frame ending just inside the BanHI site. These transcripts appear to be temporally regulated. Following the induction of sporulation in submerged culture, the amount of both transcripts increased at 1.5 hrs and decreased by 2.5 hrs of sporulation (Fig. 25). These results were consistent with RNA dot-blot analysis (data not shown).
Figure 25. Northern analysis of *S. griseus* B-2682 following the induction of sporulation in liquid culture. RNA was isolated from *S. griseus* at various times following phosphate starvation: vegetative (lane 1), 0.5 hr (lane 2), 1.0 hr (lane 3), 1.5 hrs (lane 4), 2.0 hrs (lane 5), 2.5 hrs (lane 6), 3.0 hrs (lane 7), 4.0 hrs (lane 8), 5.0 hrs (lane 9), and 6.0 hrs (lane 10). The RNA was separated electrophoretically on a denaturing formaldehyde-agarose gel. The RNA was transferred to nylon membrane and hybridized with pKK209 containing the entire 2756 bp *BamHI* fragment. Transcripts produced from ORF1590 are not sufficiently abundant to distinguish by this method. Two other transcripts of ca. 470 and 700 bp were detected and mapped (by subsequent experiments) to the *BamHI-EcoRV* fragment from nt 1–407.
DISCUSSION

The approach I undertook in this study was to characterize a gene which restores sporulation to a class of sporulation-deficient mutants of *S. griseus*. From an examination of the nucleotide sequence of the 2756 bp *Bam*HI fragment and putative protein coding region, it was apparent that this gene was distinct from other streptomycete sporulation genes thus far identified. Figure 26 is a diagram summarizing results obtained from the nucleotide sequence and transcriptional analysis. An open-reading frame of 1590 bp (ORF1590), extending from nt 984 to nt 2574, was determined using the "Frame" computer program of Bibb et al. (9). ORF1590 contains an average %G+C content in each codon position characteristic of streptomycete protein coding regions.

Nine nucleotides upstream of the putative translational start site is a potential ribosome binding site (GAGGGG; 8, 38). The sequence of the 3' end of the 16S ribosomal RNA of *S. lividans* and *S. griseus* is 5' G-A-U-C-A-C-C-U-C-U-U-C-U-C-\text{OH}3' (8). The ribosome binding sequence of ORF1590 is typical of that for other streptomycete genes, allowing for G-U base pairing and single base mismatches. For *Streptomyces*, binding to ribosomes does not seem to require a high degree of complementarity (38). Four nucleotides following the translational stop
Figure 26. Summary diagram of ORF1590. Two transcription initiation sites were detected for ORF1590 (P1 and P2). Restriction sites are indicated as: A, Sau3A; B, BamHI; D, DdeI; N, NcoI; S, SacII.
Figure 26.
(TGA) is an inverted repeat with a G of -13.0 kcal (92). This structure may function in the termination of transcription of ORF1590 in *E. coli*. Just downstream of this structure are four direct repeats, each of approximately 29 nt in length. A second inverted repeat is located upstream of the protein coding region, from nt 530 to nt 558, which would be capable of forming a secondary structure with a G of -21.7 kcal/mol (92).

Within the putative protein coding sequence is an in-frame TTA (nt 1770-1772) which is extremely rare in streptomycete coding sequences (17, 19). Expression of ORF1590 would therefore presumably depend on the presence of the bldA gene product, a UUA-recognizing leucyl tRNA. The *S. coelicolor* bldA gene is expressed in late vegetative growth of that organism (64, 85). Although the bldA gene homolog from *S. griseus* has been cloned and sequenced (L. A. McCue, personal communication), it is not yet known at what stage in development the bldA gene of *S. griseus* is expressed.

As determined from the nucleotide sequence, ORF1590 would encode a polypeptide of ca. Mr 55,500, with a basic amino terminal region that would give the protein a pI of 9.0. A thorough search of the GenBank and PIR libraries revealed a region of partial amino acid identity with the NusA protein of *E. coli*. ORF1590 and NusA show 25% similarity over the entire proteins but 40% similarity in the C-terminal regions of the proteins (49, 82). The nusA gene product of *E. coli* functions in the regulation of termination and antitermination of transcription (74, 91). NusA protein binds to core RNA polymerase after release of the sigma factor upon transcription initiation (28) and also interacts with RNA
and lambda N protein (91). Recently seven monoclonal antibodies have been made to this protein (75). Six of the seven antibodies map to a hydrophobic region (from aa 171-224) which may be responsible for the \textit{nusA} protein binding to RNA polymerase or other regulatory proteins (75). Because the region of greatest homology of NusA to ORF1590 is not essential for antitermination activity (82), the homologous region in ORF1590 may be necessary for protein folding or protein-protein interactions. The monoclonal antibody N2 recognizes a region of the \textit{nusA} protein from aa 360-412 in the C-terminal region (75). Because ORF1590 has 52% similarity to that region of the NusA protein, the N2 antibody may be useful in the detection and isolation of the ORF1590 gene product.

The putative ORF1590 protein has two possible regions of DNA-binding. Helix-turn-helix motifs of proteins are known to bind double stranded DNA in a sequence-specific manner (78). These DNA binding regions consist of two short alpha-helical segments connected by a three amino acid turn. The turn is usually initiated by a glycine, although other amino acids have been identified at that position. The consensus sequence for the turn is glycine/valine or isoleucine/serine (32). Numerical positions of the 20 amino acids comprising the helix-turn-helix region have been assigned according to the scheme of Dodd and Egan (23). The first position of the turn has been designated to be the ninth residue in the motif (32, 78). The first alpha helix comprises amino acids in positions 1-8, which are associated with the binding of the protein to the major groove of the DNA. This aligns the second alpha helix with the major groove and allows binding of the second
helical segment to specific nucleotide sequences in the DNA (78).
According to Helmann and Chamberlin (32), an important feature of the
helix-turn-helix unit is the "hydrophobic brace" of amino acids from
each helix which binds to the DNA. Bulky hydrophobic amino acids
(isoleucine, leucine, valine) are typically found at positions 4, 8, 10,
15, and often 18 or 19 (32). At position 5 is a highly conserved alanine (23, 78).

The first putative DNA-binding domain of ORF1590 (aa 12-31) meets
many of the criteria established by the analysis of DNA binding proteins
(23, 32, 78). This helix-turn-helix region has a turn (G-I-S)
characteristic of most procaryotic DNA binding proteins that have been
identified to date. Bulky hydrophobic amino acids are present in the 4,
10, 15, and 19 positions. Unlike most verified DNA binding proteins,
however, ORF1590 does not have an alanine at the 5 position. The second
helix-turn-helix region has a less conventional turn (Y-I-Q) with
hydrophobic residues at position 10, 15, and 19 and the characteristic alanine at position 5.

A quantitative method for determining the likelihood that a
protein has a lambda cro-like binding domain was devised by Dodd and
Egan (23). The primary sequences of 37 proteins with known or suspected
DNA binding domains were aligned and analyzed. The probability of a
specific amino acid occurring at each of the 20 positions was
determined. Favored amino acids were assigned a positive weight,
whereas amino acids not found at a specific position in any of the 37
sequences were given a negative value. A drawback to this method is
that it assigns an equal weight to each position of the amino acid
sequence, and does not compensate positively for the positions essential for hydrophobic interactions (100). It appears that the Dodd and Egan score is useful, but a closer look at the structure is very important in assessing helix-turn-helix DNA binding regions.

The Dodd and Egan (23) score for the putative DNA binding regions in ORF1590 are 535 (aa 12-31) and 727 (aa 303-322). The average score for a 20 amino acid block in ORF1590 is -600 and the standard deviation is 401. These helix-turn-helix regions are significant based on the method of Dodd and Egan (23), but the scores are not as high as those of cro-like DNA binding regions. On closer inspection of the potential helix-turn-helix regions in ORF1590 and in conjunction with the promoter analyses presented below, it becomes apparent that the first DNA binding region (aa 12-31) deserves further investigation as a potentially significant feature of the ORF1590 polypeptide.

By subcloning analysis, the boundaries of the region of DNA responsible for restoring sporulation to the mutants of S. griseus were determined. The region from nt 798 to nt 2756 was responsible for the sporulation phenotype. A high copy number vector, pIJ702, was used for the subcloning analysis. In this vector, ORF1590 did not seem to be stable when cloned downstream of and in the same orientation with the mel promoter. The BamHI fragment could be cloned in this orientation in pIJ702 if the mel promoter was deleted or if a frameshift mutation was introduced into ORF1590 at the NcoI site. This evidence suggests that the ORF1590 gene product may be lethal to the organism when under regulation of the strong mel promoter.
The ability of the BamHI fragment to restore sporulation to Class III sporulation-deficient mutants of *S. griseus* did not depend on the copy number of the plasmid vector. The original clone was isolated in pIJ702, a high copy number plasmid vector (50-200 per genome). The BamHI fragment cloned into pXE4, a single copy vector, restored sporulation to the same extent as the high copy vector.

ORF1590 transcripts are present at low levels during vegetative growth and sporulation of *S. griseus*. Northern analysis and low-resolution S1 nuclease mapping of RNA isolated from the transformant containing the 2.8 kb BamHI fragment at a high copy number suggest the presence of a small amount of transcript of ca. 1600 nts, consistent with the role of the downstream stem-loop structure in transcription termination. Similarly, transcript homologous to ORF1590 made by an *E. coli* transformant containing the fusion construct, pKK216, terminated in the region of *Streptomyces* DNA. Thus, the hairpin structure may also function in transcription termination in *E. coli* as was found by Deng *et al.* (21).

Two small transcripts of ca. 470 and 700 nts were identified by their ability to hybridize to DNA corresponding to nt 1 to 410 of the BamHI fragment. No obvious streptomycete open reading frame has been detected in this area of the DNA. The amounts of these transcripts increased significantly during early sporulation, suggesting the identification of a second region of DNA which is differentially expressed during sporulation of *S. griseus*. The region bounding the left end of the 2756 bp BamHI fragment has been cloned (M. J. Babcock;
unpublished results); its nucleotide sequence may reveal a gene product necessary in early sporulation of *S. griseus*.

Transcription initiation sites of ORF1590 were determined by S1 nuclease mapping. Analysis of RNA isolated from vegetative mycelia of *S. griseus* revealed two transcripts of ORF1590 with origins separated by 259 nts. It appeared that two promoters might have been involved in transcription of ORF1590. The transcript P1 initiated at nt 858 or 859, and the P2 transcript initiated at nt 1118. RNA isolated from *S. griseus* two hours after submerged sporulation was initiated contained similar levels of P1 transcript but very little P2 transcript as compared to RNA isolated from vegetative mycelia. The presence of more than one promoter and transcription initiation site is common to most *Streptomyces* genes.

To examine further the temporal regulation of the abundance of P2 transcript, RNA was isolated from *S. griseus* during vegetative growth and from points following the initiation of sporulation in submerged culture. S1 analysis of the RNA confirmed that the abundance of P2 decreased significantly following phosphate starvation. Densitometric scans of the autoradiogram indicated that the ratio of P1:P2 transcript increased forty fold by 4 hrs into sporulation.

The identification of a transcript initiation site at 1118 was unexpected since the open reading frame analysis identified the likely translational start at nt 984. Further inspection of the DNA sequence revealed a second possible translational initiation site at nt 1149 or 1152 in the same reading frame as ORF1590 and downstream of a potential ribosome binding site (AAGGGG).
Promoter probe studies were initiated to determine the boundaries of promoter activity and whether DNA upstream of the transcriptional initiation sites had \textit{in vivo} promoter activity. A Sau3AI fragment (nt 514-1190) was shown to have \textit{in vivo} promoter activity in \textit{S. lividans} using two promoter probe vectors, pARC1 and pIJ487. To test the possibility that two promoters were present, deletions of this fragment were made in both directions. The resulting fragments were cloned into pIJ487. The levels of kanamycin resistance of the resulting \textit{S. lividans} transformants were consistent with the presence of two promoters in this region. The promoter directly upstream of the P1 transcript resulted in a low level of resistance to kanamycin, whereas the promoter directly upstream of P2 transcript resulted in a relatively high level of resistance.

There are four drawbacks to assessing \textit{S. griseus} promoter activity using the pIJ487 vector. Because pIJ487 is a high copy number plasmid, regulation of the promoter \textit{in vivo} would not necessarily be accurate if regulatory factors are necessary for faithful transcriptional control. Secondly, pIJ487 is not stable in \textit{S. griseus}. Therefore, the promoter activity was measured in \textit{S. lividans}, a useful cloning host for \textit{Streptomyces} recombinant DNA work. This approach is not uncommon to the assessment of \textit{Streptomyces} promoter activity, but it is also preferable to measure promoter activity in the homologous system. Third, it is difficult to measure kanamycin phosphotransferase activity \textit{in vitro}. The assay is a coupled reaction measuring the oxidation of NADH to NAD and would probably not be sufficiently sensitive to evaluate weak promoters. Lastly, promoters identified by this method must be active
in vegetative growth for the transformants to survive on the kanamycin
selective medium. Promoters active only during sporulation cannot be
detected by this method unless transformants are first grown on a
nonselective medium.

Recently a promoter probe vector, pXE4, was constructed by Ingram
et al. (48) which uses a promoterless copy of the xyle gene of
Pseudomonas putida as a reporter gene. The xyle gene product, catechol
2,3 dioxygenase, converts the colorless substrate catechol to a yellow
product, hydroxymuconic semialdehyde. Promoter studies were undertaken
using this vector for several reasons. This vector is stable in S.
griseus and contains the SCP2 origin of replication and stability region
from SCP2, which maintains a low copy number in Streptomyces. The
plasmid is bifunctional, containing the ColE1 origin of replication.
This facilitates subcloning since S. griseus transformants must
regenerate from protoplasts, typically taking four or more days.
Catechol dioxygenase is easily assayed spectrophotometrically and does
not appear to have detrimental effects in Streptomyces transformants
(48). Promoters cloned into pXE4 need not be active during vegetative
growth.

The Sau3A promoter fragment from ORF1590 (nt 515-1190) as well as
fragments containing P1 and P2 alone were cloned into pXE4. These
constructs were assessed for promoter activity following the initiation
of sporulation as measured by catechol dioxygenase activity. Following
the initiation of sporulation by phosphate starvation, promoter activity
from the two promoters in tandem (pKK226) decreased during the first six
hours of sporulation. This decrease appears to be due to a decrease in
P2 activity since P1 is constitutively expressed at low levels. I expected to see high levels of catechol dioxygenase activity from P2 during vegetative growth, and decreased levels during the early stages of sporulation. However, not even background levels of catechol dioxygenase activity expected from the vector alone were detected in transformants containing pKK242. Background levels of catechol dioxygenase produced by transformants of \textit{S. griseus} containing pXE4 were consistent with the levels found in equivalent transformants of \textit{S. lividans} (48). Perhaps in the cloning of the P2-containing fragment, some secondary structure was formed, terminating transcription before the \textit{xylE} gene.

Relatively little is known about the nucleotide sequences of \textit{Streptomyces} promoters necessary for the initiation of transcription. Jaurin and Cohen (51) identified a class of A+T-rich promoters from \textit{S. lividans} which promote gene expression in both \textit{E. coli} and \textit{S. lividans}. From 11 of these promoter sequences, Hopwood \textit{et al.} (38) and Bibb (7) developed a consensus sequence for these \textit{E. coli}-like \textit{Streptomyces} promoters.

Since 1985 many more \textit{Streptomyces} genes have been cloned and sequenced. In an effort to classify streptomycete promoters further, W. Strohl and I tabulated 69 promoter sequences from 43 \textit{Streptomyces} genes for which the transcriptional initiation site had been determined by S1 nuclease mapping, primer extension analysis, or \textit{in vitro} transcription assays. Of the promoters surveyed, 24 were similar to and could be classified with the \textit{E. coli}-like \textit{Streptomyces} promoters (Fig. 27). The consensus sequence determined from these 24 promoters was similar to
Figure 27. Survey of *E. coli*-like promoters found upstream of
*Streptomyces* genes. Promoter regions from +1 to -50
relative to the transcriptional start site determined by S1
nuclease mapping or primer extention of 43 *Streptomyces*
genomes were collected and surveyed. Of the 69 promoters
surveyed, 24 were determined to be similar to the consensus
sequence of *E. coli*. From these promoter sequences a
*Streptomyces* consensus sequence was determined. Capital
letters denote a base which was present in >75% of the
sequences surveyed. Lower case letters denote nucleotides
found in 50-75% of these cases. Below each base is the
percentage of the 24 promoters which had the consensus
nucleotide in that position. The sequence of the upstream
promoter of ORF1590 (P1) was similar to this group of *E.
coli*-like *Streptomyces* promoters.
<table>
<thead>
<tr>
<th></th>
<th>-35 region</th>
<th>-10 region</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli/</strong> B. subtilis</td>
<td>T T G A C a</td>
<td>T a t A a T</td>
</tr>
<tr>
<td>Streptomyces Consensus</td>
<td>T T G a c a</td>
<td>t A G g a T</td>
</tr>
<tr>
<td>Hopwood et al. (H6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomyces Consensus</td>
<td>T T G a C n</td>
<td>t A g n a T</td>
</tr>
<tr>
<td>%</td>
<td>88 79 88 67 75 -</td>
<td>63 86 71 -</td>
</tr>
<tr>
<td>ORF1590 P1</td>
<td>T T T A C T</td>
<td>G G G A C T</td>
</tr>
</tbody>
</table>

Figure 27.
that reported by Hopwood et al. (38) and Bibb (7; Fig. 27). On the basis of similarity in the -35 region and to a lesser extent in the -10 region, the P1 promoter of ORF1590 may belong to this class of *Streptomyces* promoters. The P1 promoter is constitutively expressed during vegetative growth and sporulation, as one would expect for promoters having an *E. coli* -like consensus.

The promoter region of the P2 transcript of ORF1590 does not fit into the *E. coli* -like class of *Streptomyces* promoters. By comparing the nucleotide sequences of all non-*E. coli* -like *Streptomyces* promoters, a second class of potential streptomycete promoters was identified by W. Strohl strictly on the basis of sequence homology (personal communication). The ORF1590 P2 belongs to this potential new class of streptomycete promoters, which has a relatively divergent -10 region, but a conserved -38 region (Fig. 28). The spacing between the -10 and -38 regions is uniform at 23 nucleotides for all six promoters.

From the identification of two promoters associated with ORF1590, the possibility exists that two polypeptides can be generated from this region of DNA. Translation of the P1 transcript could initiate from either the AUG starting at nt 984 or from an in-frame AUGCUG starting at nt 1149-1152 in the sequence. Translation from the P2 transcript could initiate only from nt 1149-1152. The larger polypeptide, gpORF1590, having a pI of 9.0 and containing a putative amino-terminal DNA binding domain, would have an expected function distinct from the smaller polypeptide, gpORF1425, which would have a pI of 7.1 and lack the amino-terminal helix-turn-helix motif (Fig. 29). From the subcloning
Figure 28. Nucleotide sequence of a new class of *Streptomyces* promoters. The -10 and -38 regions are separated by 23 nucleotides in each of the promoters. Capital letters in the conserved sequence denote nucleotides present in at least five of the six promoter sequences. Small letters denote nucleotides present in at least three of the six promoter sequences. The underlined base indicates the first base of the transcript.
Figure 28.
Figure 29. Characteristics of the two potential polypeptides encoded by the ORF1590 gene. ORF1590 extends from nt 984 to 2574. ORF1425 is an in-frame truncated protein which starts at nt 1139 or 1142.
<table>
<thead>
<tr>
<th></th>
<th>ORF1590</th>
<th>ORF1425</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>55,500</td>
<td>49,600</td>
</tr>
<tr>
<td>pI</td>
<td>9.0</td>
<td>7.1</td>
</tr>
<tr>
<td>DNA-binding domains</td>
<td>aa 12-31; 303-322</td>
<td>aa 303-322</td>
</tr>
<tr>
<td>Expression</td>
<td>Vegetative and Sporulation</td>
<td>Primarily Vegetative</td>
</tr>
</tbody>
</table>

Figure 29.
analysis, I can conclude that the ORF1590 protein is essential for the restoration of sporulation in Class III mutants of S. griseus.

Translation of both open reading frames may depend on the expression of the bldA gene product of S. griseus since the in-frame TTA codon is present in both. In S. coelicolor bldA is expressed late in vegetative growth and is essential for expression of TTA-containing genes. It is not clear whether the lack of expression of the TTA-containing genes in bldA mutants is due to translational termination or premature transcriptional termination or both. If bldA is temporally controlled in S. griseus, then the possibility exists that four polypeptides could be produced from the ORF1590 region of DNA: two during vegetative growth (terminating at the UUA), and two during sporulation (terminating at the UGA).

To test the possibility of multiple gene products from ORF1590, antibodies need to be made to portions of the ORF1590 protein. The first step in this process, the production of antigen at high levels, was addressed by constructing a fusion protein which contains the first 11 amino acids from B-galactosidase and the entire ORF1425 polypeptide. Antibodies made to this fusion protein would be able to bind to both the ORF1590 and ORF1425 polypeptides. The monoclonal antibody N2 developed by Nakamura et al. (75), which recognizes a region from aa 360-412 of NusA, may also recognize the homologous region in ORF1590 and ORF1425, but not truncated proteins made in the absence of the bldA gene product. In either approach the antibody would be essential for the detection of the presence and relative abundance of ORF1590 gene products during sporulation in S. griseus.
Examples of two polypeptide products of the same coding sequence are known. (i) The gene II-X locus of bacteriophage f1 encodes two polypeptides that differ only in their amino terminal sequences (26). The gene II protein is required for phage DNA synthesis. The product of gene X is an in-frame truncated version of the gene II protein identical to the C-terminal third of the gene II protein. The gene X protein is a powerful inhibitor of gene II phage-specific DNA synthesis in vivo (27). (ii) Genetic analysis of plasmid ColI suggests that the sog locus encodes not only a DNA primase but also a shorter polypeptide which shares its carboxyl-terminal coding sequence with the primase gene but lacks primase activity (11). (iii) Genetic and biochemical evidence indicates that the cheA locus of E. coli encodes two polypeptides having the same reading frame but differing in amino terminal sequences (86). The nucleotide sequence of the cheA gene from Salmonella typhimurium (89) confirms this conclusion. Hess et al. (36) suggest that phosphorylation of CheA plays a central role in signal transduction in chemotaxis. The truncated CheA protein is not phosphorylated and its function in vivo has not yet been demonstrated (36). (iv) The transposase and transposase inhibitor of Tn5 share a carboxyl-terminal coding sequence, but the transposase is approximately 40 amino acids longer at the amino terminus, and two distinct promoters govern expression of each gene product (52, 53, 61). On the basis of the similar configuration of the ORF1590 coding sequence and the locations of the transcription initiation sites, it is tempting to speculate that the ORF1590 locus might encode a catalyst/regulator pair of polypeptides involved in the temporally controlled sporulation process.
In summary, two regions of DNA have been identified which are differentially expressed during the life cycle of *S. griseus*. The regions of DNA from nt 1 to nt 410 and ORF1590 show distinct patterns of temporal regulation. In ORF1590, P1 transcript was constitutively expressed, whereas P2 transcript decreased after the initiation of sporulation. An interesting feature of ORF1590 is the potential for the production of multiple polypeptides from one gene. The number of proteins produced and the effects of the *bldA* gene product on regulation of expression of ORF1590 deserve further study. The submerged sporulation system of *S. griseus* facilitated the study of temporal regulation during differentiation, permitting the identification of transcripts whose abundance changed rapidly as a consequence of the induction of sporulation. *S. griseus* may therefore prove to be a preferred species for the study of the regulation of *Streptomyces* sporulation.
APPENDIX A. Circular maps of plasmid vectors. Restriction sites are those employed in this study.
Figure 30. Circular map of pUC18. This *E. coli* plasmid contains the ampicillin resistance gene (Amp<sup>R</sup>) and the alpha peptide of *β*-galactosidase (lacZ<sup>+</sup>).

126
Figure 30.
Figure 31. Circular map of pIJ702. This plasmid contains the tyrosinase gene (mel) and is selected for by thiostrepton resistance (tsr). This vector is a derivative of pIJ101, a high copy number *Streptomyces* plasmid.
Figure 31.
Figure 32. Circular map of pXE4. This bifunctional plasmid contains the SCP2 origin of replication conferring a copy number of 1-2 per genome and is selected for by thiostrepton resistance (tsr) in Streptomyces. This vector is selected for by ampicillin resistance (amp) and replication originates from the colE1 origin in E. coli.
Figure 32.
APPENDIX B. Plasmids constructed for this study.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK200</td>
<td>pIJ702</td>
<td>2.8 kb <em>BamHI</em> genomic fragment from <em>S. griseus</em> B-2682 ligated into the <em>BglII</em> site of pIJ702</td>
</tr>
<tr>
<td>pKK206</td>
<td>pUC18</td>
<td><em>EcoRV-PstI</em> fragment from from pKK200 ligated into <em>SmaI-PstI</em> digested pUC18</td>
</tr>
<tr>
<td>pKK207</td>
<td>pUC18</td>
<td>The 2756 bp <em>BamHI</em> fragment from <em>S. griseus</em> B-2682 ligated into the <em>BamHI</em> site of pUC18 RI--B-----S-S------------------RV----B-H3</td>
</tr>
<tr>
<td>pKK209</td>
<td>pUC18</td>
<td>The same fragment as in pKK207 ligated into the <em>BamHI</em> site in the opposite orientation</td>
</tr>
<tr>
<td>pKK210</td>
<td>pIJ702</td>
<td>The <em>EcoRV-PstI</em> fragment containing ORF1590 was isolated from pKK206 on a <em>SacI-PstI</em> fragment and ligated into <em>SacI-PstI</em> digested pIJ702.</td>
</tr>
<tr>
<td>pKK213</td>
<td>pIJ702</td>
<td><em>SacI-PstI</em> fragment from pKK209 ligated into the <em>SacI-PstI</em> site of pIJ702</td>
</tr>
<tr>
<td>pKK216</td>
<td>pUC18</td>
<td>Translational fusion with the alpha-peptide of beta-galactosidase of pUC18. pKK209 was cut with <em>BstXI</em>, blunt ended with S1 nuclease, and cut with <em>BamHI</em>. The ORF1425 containing fragment nt 1149-2756 was ligated in-frame to <em>SmaI-BamHI</em> digested pUC18.</td>
</tr>
<tr>
<td>pKK217</td>
<td>pUC18</td>
<td>pKK207 was digested with <em>NcoI</em>, blunt-ended with S1 nuclease and religated. This creates a +1 frameshift mutation in ORF1590.</td>
</tr>
<tr>
<td>pKK218</td>
<td>pUC18</td>
<td>pKK209 was digested with <em>NcoI</em>, blunt-ended with S1 nuclease and religated. This creates a +1 frameshift mutation in ORF1590.</td>
</tr>
<tr>
<td>pKK219</td>
<td>pUC18</td>
<td>Deletion of the 250 bp <em>SalI</em> fragment of pKK216</td>
</tr>
<tr>
<td>pKK220</td>
<td>pUC18</td>
<td>Deletion of the 700 bp <em>SalI</em> fragment of pKK216</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Vector</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>pKK225</td>
<td>pXE4</td>
<td><em>Bgl</em>III fragment from pKK143 (containing nt 515-1190 of ORF1590) was ligated into <em>BamHI</em> digested pXE4. The promoters for ORF1590 were in the opposite orientation of the <em>xylE</em> gene of pXE4.</td>
</tr>
<tr>
<td>pKK226</td>
<td>pXE4</td>
<td><em>Bgl</em>III fragment from pKK143 (containing nt 515-1190 of ORF1590) was ligated into <em>BamHI</em> digested pXE4. The promoters for ORF1590 were in the correct orientation for transcription of the promoterless <em>xylE</em> gene of pXE4.</td>
</tr>
<tr>
<td>pKK236</td>
<td>pIJ702</td>
<td><em>SacI</em>-<em>PstI</em> fragment from pKK286 (containing nt 798-2756 of ORF1590) ligated into <em>SacI</em>-<em>PstI</em> digested pIJ702</td>
</tr>
<tr>
<td>pKK237</td>
<td>pIJ702</td>
<td><em>SacI</em>-<em>PstI</em> fragment from pKK287 (containing nt 930-2756 of ORF1590) ligated into <em>SacI</em>-<em>PstI</em> digested pIJ702</td>
</tr>
<tr>
<td>pKK238</td>
<td>pIJ702</td>
<td><em>SacI</em>-<em>PstI</em> fragment from pKK288 (containing nt 1074-2756 of ORF1590) ligated into <em>SacI</em>-<em>PstI</em> digested pIJ702</td>
</tr>
<tr>
<td>pKK239</td>
<td>pIJ702</td>
<td><em>SacI</em>-<em>PstI</em> fragment from pKK289 (containing nt 1169-2756 of ORF1590) ligated into <em>SacI</em>-<em>PstI</em> digested pIJ702</td>
</tr>
<tr>
<td>pKK240</td>
<td>pXE4</td>
<td>2756 bp <em>BamHI</em> fragment from pKK207 ligated into the <em>BamHI</em> site of pXE4.</td>
</tr>
<tr>
<td>pKK241</td>
<td>pXE4</td>
<td><em>Bgl</em>III fragment from pKK162 (containing nt 515-979 of ORF1590) was ligated into the <em>BamHI</em> site of pXE4. The P1 promoter from ORF1590 is in the correct orientation for transcription of the promoterless <em>xylE</em> gene of pXE4.</td>
</tr>
<tr>
<td>pKK242</td>
<td>pXE4</td>
<td><em>Bgl</em>III fragment from pKK160 (containing nt 930-1143 of ORF1590) ligated into the <em>BamHI</em> site of pXE4. The P2 promoter from ORF1590 is in the correct orientation for transcription of the promoterless <em>xylE</em> gene of pXE4.</td>
</tr>
</tbody>
</table>
Table 6 (continued)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKK251</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-2570 of ORF1590.</td>
</tr>
<tr>
<td>PKK252</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-2375 of ORF1590.</td>
</tr>
<tr>
<td>PKK254</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-2309 of ORF1590.</td>
</tr>
<tr>
<td>PKK255</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-2119 of ORF1590.</td>
</tr>
<tr>
<td>PKK261</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-1665 of ORF1590.</td>
</tr>
<tr>
<td>PKK262</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-1478 of ORF1590.</td>
</tr>
<tr>
<td>PKK264</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-995 of ORF1590.</td>
</tr>
<tr>
<td>PKK265</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-1240 of ORF1590.</td>
</tr>
<tr>
<td>PKK266</td>
<td>pUC18</td>
<td>PKK209 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1-1098 of ORF1590.</td>
</tr>
</tbody>
</table>
Table 6 (continued)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK270</td>
<td>pUC18</td>
<td>pKK209 was digested with <em>XbaI</em> and <em>PstI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 1-888 of ORF1590.</td>
</tr>
<tr>
<td>pKK272</td>
<td>pUC18</td>
<td>pKK209 was digested with <em>XbaI</em> and <em>PstI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 1-636 of ORF1590.</td>
</tr>
<tr>
<td>pKK274</td>
<td>pUC18</td>
<td>pKK209 was digested with <em>XbaI</em> and <em>PstI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 1-157 of ORF1590.</td>
</tr>
<tr>
<td>pKK275</td>
<td>pUC18</td>
<td>pKK209 was digested with <em>XbaI</em> and <em>PstI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 1-1253 of ORF1590.</td>
</tr>
<tr>
<td>pKK277</td>
<td>pUC18</td>
<td>pKK209 was digested with <em>XbaI</em> and <em>PstI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 1-1408 of ORF1590.</td>
</tr>
<tr>
<td>pKK280</td>
<td>pUC18</td>
<td>pKK122 was digested with <em>BamHI</em> and <em>KpnI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 170-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK281</td>
<td>pUC18</td>
<td>pKK122 was digested with <em>BamHI</em> and <em>KpnI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 263-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK282</td>
<td>pUC18</td>
<td>pKK122 was digested with <em>BamHI</em> and <em>KpnI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 545-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK284</td>
<td>pUC18</td>
<td>pKK122 was digested with <em>BamHI</em> and <em>KpnI</em>, digested with exonucleaseIII, blunt-ended with SI nuclease, and religated. This plasmid contains nt 646-2756 of ORF1590.</td>
</tr>
</tbody>
</table>
Table 6 (continued)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK286</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 798-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK287</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 930-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK288</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1074-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK289</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1169-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK290</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 170-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK292</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1328-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK293</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1489-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK294</td>
<td>pUC18</td>
<td>pKK122 was digested with BamHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 1902-2756 of ORF1590.</td>
</tr>
</tbody>
</table>
Table 6 (continued)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK295</td>
<td>pUC18</td>
<td>pKK122 was digested with BanHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 2026-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK296</td>
<td>pUC18</td>
<td>pKK122 was digested with BanHI and KpnI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 2124-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK298</td>
<td>pUC18</td>
<td>pKK207 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 2375-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK299</td>
<td>pUC18</td>
<td>pKK207 was digested with XbaI and PstI, digested with exonucleaseIII, blunt-ended with S1 nuclease, and religated. This plasmid contains nt 2508-2756 of ORF1590.</td>
</tr>
<tr>
<td>pKK428</td>
<td>pIJ702</td>
<td>BanHI fragment from pKK218 containing the frameshift mutation of ORF1590 ligated into BgIII digested pIJ702. In the same orientation as pKK209.</td>
</tr>
<tr>
<td>pKK429</td>
<td>pIJ702</td>
<td>BanHI fragment from pKK218 containing the frameshift mutation of ORF1590 ligated into BgIII digested pIJ702. Opposite orientation as pKK428.</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES


