Tn4563 Transposition in *Streptomyces coelicolor* and Its Application to Isolation of New Morphological Mutants

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The Tn3-like transposon Tn4556 (and its derivatives Tn4560 and Tn4563) has been used for insertion mapping of genetic loci cloned on plasmids, but it has been difficult to obtain chromosomal insertions, largely because of the lack of a strong selection against transposon donor molecules. In this communication, we report two efficient selection techniques for transposition and their use in the isolation of chromosomal insertion mutations. A number of independent *Streptomyces coelicolor* morphological mutants (*bld* and *whi*) were obtained. Two of the *bld* mutations were mapped to locations on the chromosome by SCP1-mediated conjugation; at least one mutation, *bld-5ml*, appears to define a novel locus involved in control of *S. coelicolor* morphogenesis and antibiotic production.

The *Streptomyces* spp. are gram-positive soil bacteria whose multicellular life-style resembles that of the filamentous fungi. A sophisticated program of gene expression regulates temporal and spatial changes in colony morphology as well as the activities of a prolific secondary metabolism (8, 18). Mature *Streptomyces* colonies contain at least two basic types of cells. The first are branching, multinucleoid hyphae which comprise the substrate mycelium. These cells form a network that secretes a large variety of hydrolytic exoenzymes and anchors the growing colony to a solid substrate. Nutrient depletion then induces production of a second cell type which makes up the aerial mycelium; these aerial hyphae are connected to the substrate mycelium but project upward from the colony surface.

Both morphological and physiological differentiation occur along a path leading to spore production. This differentiation process is triggered, at least in part, by nutritional starvation (8); thus, aerial mycelium synthesis is dependent on energy and material stored in the substrate mycelium. Physiological differentiation leads to production of unique secondary metabolites, some of which have antibiotic activity. Antibiotic production is thought to provide a chemical defense barrier around a colony as substrate hyphae are cannibalized to provide energy for the developing aerial hyphae (10). *Streptomyces coelicolor*, the most studied species, synthesizes four known antibiotics. One, methylenomycin, is plasmid encoded (9, 27), and two others are easily observed on plates because they are pigmented (undecylprodigiosin is red and actinorhodin is blue).

Recent advances in our understanding of the molecular controls over *Streptomyces* development have come from studies of sporulation mutants. The mutants fall into two major phenotypic classes. The first class is the bald (*bld*) mutants, which do not produce aerial hyphae or spores. The other class is white (*whi*) mutants, which synthesize the white-colored aerial hyphae but fail to make the gray-pigmented spores (with the exception of *whiE*, which produces nonpigmented spores). Most of the *whi* mutants retain their ability to produce antibiotics, but some *bld* mutants fail to produce any (18). This fact, along with the observation that antibiotic synthesis commences at about the same time that aerial hyphae first appear, implies partially overlapping genetic regulation of morphological and physiological differentiation (8). However, there are clearly separate genetic controls over the two processes as well: in addition to those *bld* mutants which retain antibiotic production, there are also mutants (*abs*) that globally block antibiotic production but have no effect on morphological differentiation (1).

In addition to the evidence for separate genetic controls over morphological and antibiotic production, there also appear to be two parallel regulatory pathways at an early stage in morphological differentiation itself. While *bld* mutants do not produce aerial mycelium or spores on glucose minimal medium, most of them can be phenotypically suppressed by growth on alternative carbon sources such as mannitol (the exceptions are *bldB* and *bldC* mutants, which remain bald on all carbon sources). Thus, there may be two independent control pathways that can initiate aerial hyphae production, one of which is repressed by glucose.

Tn4556 was the first transposon found to transpose to apparently random sites on the *Streptomyces* genome (11-13, 31). Originally isolated from *Streptomyces fradiae*, Tn4556 is a member of the *Tn3* family. The entire DNA sequence has been determined, and by comparison with Tn3, the putative transposase and resolvase genes have been identified (35). No known drug or metal resistance genes are carried by Tn4556, but genes encoding viomycin resistance or lincomycin resistance have been added in vitro (12, 13).

Experience in other microbial systems has shown that transposon mutagenesis can lead to the identification of previously undescribed developmental genes. For example, despite the large number of classical *spo* mutants of *Bacillus subtilis*, introduction of Tn917 and Tn917-lac interrupted hitherto unknown sporulation genes (34, 38). Similar results were obtained in *Myxococcus xanthus* when Tn5-lac was used (28). This success is likely due to the nature of the transposition process itself; insertional mutagens can efficiently create null alleles while minimizing the general lethality that results from chemical mutagenesis.

Drug-resistant Tn4556 derivatives have been reported to hop from plasmid to chromosome in some species (12, 37). However, using the same procedures, we were unable to
obtain chromosomal insertions in *S. coelicolor*. Cells carrying the temperature-sensitive plasmid (used as the transposon source) could not be cured; this persistence of the delivery vehicle at high temperature masked the ability to select for the relatively low frequency of transposition.

In the work reported here, we have found two simple ways to select against the transposon donor molecule in order to isolate chromosomal insertions of *Tn*563 (*Tn*4556-vph). The selections were used to generate a set of morphological insertion mutants ( *bd* and *whi*) in *Streptomyces coelicolor*. Two of the *bd* insertions have been genetically mapped. At least one appears to define a gene that had not been identified previously.

### MATERIALS AND METHODS

**Bacterial strains.** All of the *S. coelicolor* strains used were derived from *S. coelicolor* A3(2) and are described in Table 1. *U*TA1501 is an NF derivative of *J*1501 made by crossing *J*650 with *J*1501 and screening for loss of agarase production (integration of SCP1 interrupts the *dagA* gene; 17) and then for the presence of the original *J*1501 auxotrophic markers *hisA1*, *uraA1*, and *strA1*. *Tn*563 (*Tn*4556-vph) was delivered from plasmid pUC1172 (13). The vector plasmid pUC1172 is SCP1+ (NF), a temperature-sensitive derivative of the pJ702 multicopy plasmid cloning vector (23).

**Bacterial growth.** Standard *Streptomyces* media and methods have been published elsewhere (19). *R* and HT (16) were used for phenotypic characterization, cross-feeding studies, and some antibiotic resistance tests. Minimal medium (19) was used for phenotypic characterization and genetic mapping of the insertions with the modifications that NH₄₂(SO₄)₂ (2 g/liter) was substituted for L-asparagine and trace elements solution (19) was added in some experiments. When alternative carbon sources were used in the minimal medium, they replaced glucose at a final concentration of 1% (wt/vol). For spore preparations, matings, and some cross-feeding studies, R₄ medium was used. R₄-base contains (per liter) 103 g of sucrose, 4 g of yeast extract, 4 g of peptone, 0.25 g of K₂SO₄, 20 g of agar, and 950 ml of H₂O. After autoclaving, the following sterile solutions are added to the base: 10 ml of 1 M Tris (pH 7.0), 15 ml of 1 M MgCl₂, 15 ml of 1 M CaCl₂, 20 ml of 50% glucose, and 3.5 ml of trace elements solution (19). M7M medium was used for the initial selection of viomycin-resistant (*Vio*) colonies because it reduced the number of *Vio* colonies that would leak through the selection when impure viomycin preparations were used. M7M contains (per liter) 10 g of dextrin, 2 g of NZ-amine (type A; Sheffield/Kraft), 10 mg of CoCl₂, and 22 g of agar. Subsequent screenings for *Vio* colonies employed R₅, HT, or minimal medium.

**Selection of transposon hops to the chromosome by conjugation.** NF strains (e.g., *2612*) owe their Hfr-like fertility properties to a ~350-kb conjugal, linear plasmid (SCP1) integrated at 9 o’clock on the *S. coelicolor* chromosome. The origin of transfer lies with the integrated plasmid, and the donor’s chromosome is mobilized to the recipient bidirectionally; i.e., the probability of marker transfer decreases both clockwise and counterclockwise from 9 o’clock. When an NF strain is crossed with a bacterium that does not contain SCP1 (the UF mating type), the transfer gradient results in a nonrandom inheritance of parental genetic markers by the recombinants (markers around 9 o’clock would be inherited most frequently). However, when two NF parents are crossed, the progeny inherit the parental markers in a random fashion, presumably because the mating is two-way (both parents are donors), and as a result, the two gradients cancel each other out. Although NF × NF crosses are preferred for their lack of bias, the NF × UF matings are very useful for initial mapping studies because of the large number of recombinants that can be obtained: greater than 80% is not uncommon (20).

**Selection of transposon hops to the chromosome on the basis of SCP1 incompatibility.** Spores of *2612* carrying *Tn*4563 on pUC1172 (Fig. 1) was plated and allowed to sporulate on HT medium containing thiostrepton (30 µg/ml) to maintain the plasmid. Spores were then mixed with an equal number of *J*1501 spores and replate on the same medium but without thiostrepton present. Spores were again collected, and dilutions were plated onto M7M plates containing histidine, uracil, and thiostrepton (60 µg/ml) to kill *J*1501 (kindly provided by S.-C. Tomich, The Upjohn Co., Kalamazoo, Mich.) or florimycin (purified viomycin; 20 to 50 µg/ml; a generous gift of V. Lanzov, Institute of Nuclear Physics, Leningrad, USSR) along with streptomycin (50 µg/ml) to kill the *2612* donor. *Vio* colonies were allowed to develop for 7 to 10 days, after which time morphological mutants were identified by inspection. Thiostrepton-resistant transconjugants were not detected among 300 *Vio* colonies tested. No spontaneous, *Vio* mutants arose on M7M plates among approximately 10⁹ spores plated from *2612* or *J*1501.

**Selection of transposon hops to the chromosome on the basis of SCP1 incompatibility.** Spores of *2612* carrying pUC1172 were prepared from solid media containing thiostrepton as described above. They were then either (i) grown in YEME liquid medium (19) until late log phase and subcultured twice in the same medium ( *bd*-5m1 and -5m2) or (ii) plated onto HT plates ( *bd*-5m3, -5m4, and -5m5).

A *2612*/pUC1172 transformant was grown in YEME medium containing thiostrepton to amplify cell mass. After the culture had reached late log phase, it was subcultured into drug-free YEME medium to permit segregational loss of the plasmid. The subculture was repeated two more times, and then dilutions of mycelium were spread onto M7M agar medium containing thiostrepton. Colonies 5M1 and 5M2 (*U*TA5001 and -2) were chosen on the basis of (i) their failure to produce either of the pigmented antibiotics (actinorhodin and undecylprodigiosin), (ii) lack of aerial mycelium production, and (iii) sensitivity to thiostrepton. Because *S. coelicolor* is coenocytic and does not sporulate in liquid media, single-genome purification of 5M1 and 5M2 was achieved by
protoplast formation and regeneration on R2YE medium (19).

Additional 2612/pUC1172 transformants were grown in YEME medium containing thiostrepton to amplify cell mass. After each culture had reached late log phase, it was subcultured into drug-free YEME medium to permit segregational loss of the plasmid. When this culture reached late log phase, concentrated mycelium was spread onto drug-free HT plates and allowed to sporulate. The harvested spores were then plated onto M7 medium containing viomycin. Mutants 5M3, 5M4, and 5M5 were identified as described above for 5M1 and 5M2; three bald mutants (5M3 to 5M5) were found among approximately 6,000 colonies. The advantage of this method over that used for 5M1 and 5M2 is the single-genome purification that results from sporulation. A possible drawback might be the inability to isolate strongly dominant morphological mutations which cannot be phenotypically complemented by neighboring, wild-type genomes in a sporulating aerial hypha.

Phenotypic complementation tests. Mycelium from a mutant to be tested was streaked in six lines, radiating from the center of an agar plate. A short line of mycelium from strain 2612 was streaked orthogonally to one of the radiating lines, near the edge of the plate and 1 cm from the end of the center radiating lines. Another short, orthogonal streak was then laid down for each other mutant and also one for the mutant being tested. After incubation for 7 to 10 days on HT plates, a positive result is indicated by sporulation and pigmented antibiotic production of each mutant to be tested in the area of growth that is adjacent to another strain.

Physical analysis of insertions. Southern hybridization analysis of chromosomal insertions was performed as described previously (2, 33) except that in some experiments Immobilon-N (Millipore) charge-modified polyvinylidene difluoride membrane was used as recommended by the manufacturer. Chromosomal DNA was digested with a restriction enzyme, electrophoresed on agarose, transferred to a membrane, and probed with a 334-bp BstXI-BamHI DNA fragment from the right arm of Tn4536 (Fig. 1). The probe was labeled with [α-32P]dATP by primer extension using random, 14-mer oligonucleotides (22). The primer-to-probe ratio was 1:150, and the reaction was carried out at pH 7.6.

Backcrosses. The bld::Tn4563 mutants were tested for cosegregation of the bald and Vio' phenotypes after crossing with bld' Vio' strains. Each mutant was crossed with J1501 and with UTA1501. Individual recombinants were selected as described below. Individual colonies on the selective medium were patched onto 50-position grids of the same medium. The grids were then replica plated to R5 medium and to R6 containing florimycin (50 μg/ml). For all mutants, patches that grew on the antibiotic medium retained the original Bld' phenotype on both R5 media. Those which did not grow on the viomycin plates developed and sporulated normally on R5 (data not shown).

Genetic mapping. SCP1-mediated conjugation experiments were performed as described previously (19). NF × UF mapping experiments using J1501 were performed first, followed by NF × NF crosses between each bald mutant and UTA1501. Mycelium from each bld::Tn4563 mutant was mixed with spores of the recipient strain on an R4 plate. After 7 to 10 days, spores were collected from this plate and filtered (spores carrying bld mutations are produced by intramyccelium complementation; 29). Spore suspensions were diluted and plated onto minimal medium with selection for one marker from each parent. Individual recombinant colonies were then picked and patched to 50-position grids of the same selective medium. After about 3 days, the patches on these grids were replica plated to various other media in order to score nonselected markers.

To test for complementation of 5M1 by bldB', a lysogen of 5M1 was constructed by applying dilutions of KC625 (φC31-att bldB'), along with a helper (φC31-cts), to a lawn of UTA5001 on R5 plates. The phage did not restore sporulation to 5M1, whereas sporulation was restored to a control bldB strain (J669).

RESULTS

Development of methods for transposon delivery. Initial attempts to obtain transposition of Tn4563 to the S. coelicolor chromosome employed a protocol that had been used to demonstrate transposition in S. fradiae and S. lividans (11). We used the same source of Tn4563 in S. coelicolor as that used in the earlier work (pUC1172; Fig. 1). The vector portion of pUC1172 contains a temperature-sensitive replicon. However, we were unable to cure the plasmid from S. coelicolor J1501 at an acceptable rate, even with repeated subculturing at the elevated temperature (>39°C). To obvi-
ate this problem, we performed conjugation experiments in which a plasmid-containing bacterium (2612/pUC1172) served as chromosome donor (as a result of the presence of SCPl on the chromosome of 2612). Thus, if a transposon had hopped from the plasmid to the donor chromosome, it would be possible to select for its appearance in a recipient cell after mating. Selection against donor cells would eliminate the donor plasmid from the culture, provided the pUC1172 plasmid is not also mobilized to the recipient. Conjugation of pUC1172 would not be expected since its replicon lacks any known transfer functions (24, 25).

An S. coelicolor NF strain (2612) was transformed with pUC1172 and grown in the presence of thiostrepton. A mating with an SCPl- recipient was performed on solid medium, and the mixture was allowed to sporulate. Transconjugants carrying Tn4563 were isolated by selecting for both viomycin and streptomycin resistance. Streptomycin kills the donor, and viomycin selects for those recipients that have acquired the Tn4563 \( vph \) gene, which encodes viomycin phosphotransferase (3). Approximately \( 4 \times 10^{10} \) Uio' Str' colonies were screened (in three independent experiments) for morphological phenotypes. Thirty were chosen for purification; of these, six had reproducible morphological defects (one Bld- and five Whi- phenotypes). A total of 250 recombinants were screened for resistance to thiostrepton, encoded by the plasmid-borne \( \text{tsr} \) gene, and all were sensitive to the drug. Apparently, pUC1172 was not mobilized into recipient bacteria, but we cannot rule out the possibility that it was transferred and then quickly lost.

During the study described above, we discovered that pUC1172 was rapidly lost from S. coelicolor 2612 in the absence of thiostrepton selection. In a typical experiment, only \( 4\% \) of spores harvested from a drug-free medium gave rise to thiostrepton-resistant colonies upon subsequent replating. In contrast, the plasmid curing rate in strain J1501 was extremely low; only one in \( 10^4 \) spores lost pUC1172 during this procedure.

We therefore exploited this poor maintenance of pUC1172 to counterselect against transposon donor molecules. 2612 carrying pUC1172 was allowed to sporulate in drug-free medium, and the spores were spread onto viomycin plates. Five Bld- insertion mutants were isolated by screening for colonies which did not produce the S. coelicolor pigmented antibiotics actinorhodin and undecylenic acid and then, upon replating, checking for the absence of aerial mycelium production. In one experiment, three such mutants were found among a total of 6,000 colonies screened. Additional mutants were isolated in other experiments. The six pigment-free mutants studied in detail (1M1 and 5M1 through 5M5) also failed to form the white or gray colony colors characteristic of aerial mycelium and spore production, respectively (the Bld- phenotype).

If the Bld phenotype for each mutant is a direct result of Tn4563 insertion, then the viomycin resistance encoded by the transposon should cosegregate with the morphological defect in a conjugation experiment. Each mutant (2612 \( bld::\text{Tn4563} \)) was therefore crossed with J1501 (as described in Materials and Methods). In each case, the Bld- and Uio' phenotypes were inseparable.

pUC1172 is lost and each Tn4563 insertion is unique. A direct donor transposon Tn4563 can be effective only if the plasmid delivery vehicle can be easily eliminated from the cell after a transposition event. Although the mutants were thiostrepton sensitive, we physically analyzed the mutant chromosomal DNA to exclude the possibility that the \( \text{tsr} \) gene had been deleted from a still-resident pUC1172 donor molecule. Chromosomal DNAs were digested and probed with \( ^{32}P \)-labeled pIJ702 DNA (pIJ702 is the vector portion of pUC1172, minus the temperature sensitivity). The mutants showed no hybridization to the radiolabeled plasmid probe (data not shown).

The mutant chromosomal DNAs were also probed with transposon sequences to determine whether Tn4563 had inserted at a single locus in each case and whether the locus was different for each mutant. In these experiments, chromosomal DNAs were digested with \( BglII \) and probed with a \( ^{32}P \)-labeled 334-bp fragment taken from the extreme right-end of Tn4563 (see Fig. 1A). The enzymes chosen for digestion of the chromosomal DNA do not cut the transposon within the segment covered by the probe. (A) Chromosomal DNA from each mutant was digested with \( BglII \). DNAs from 5M1 to 5M5 (UTA5001 to -5) are shown in lanes 1 to 5. The junction fragment from \( BglII \)-digested pUC1172 (not shown) migrated at 1.4 kb. (B) Chromosomal DNA was digested with KpnI. Lanes: 1, pUC1172; 2 and 3, 5M1 and 5M2. No hybridization was detected between the transposon probe and chromosomal DNA prepared from 2612 in any of the experiments (data not shown). Molecular weights were determined from restriction digests of phage \( \lambda \) DNA (digested with HindIII) and plasmid pBR322 (digested with MspI) that were run on the same gels that were used for the Southern hybridizations.

**FIG. 2.** Southern hybridization of \( \text{bld::Tn4563} \) mutant chromosomal DNA with a junction fragment probe. Chromosomal DNA was digested and probed with a \( ^{32}P \)-labeled, 334-bp DNA fragment taken from the extreme right-hand end of Tn4563 (see Fig. 1A). The enzymes chosen for digestion of the chromosomal DNA do not cut the transposon within the segment covered by the probe. (A) Chromosomal DNA from each mutant was digested with \( BglII \). DNAs from 5M1 to 5M5 (UTA5001 to -5) are shown in lanes 1 to 5. The junction fragment from \( BglII \)-digested pUC1172 (not shown) migrated at 1.4 kb. (B) Chromosomal DNA was digested with KpnI. Lanes: 1, pUC1172; 2 and 3, 5M1 and 5M2. No hybridization was detected between the transposon probe and chromosomal DNA prepared from 2612 in any of the experiments (data not shown). Molecular weights were determined from restriction digests of phage \( \lambda \) DNA (digested with HindIII) and plasmid pBR322 (digested with MspI) that were run on the same gels that were used for the Southern hybridizations.
TABLE 2. Phenotypes of bld::Tn4563 mutants on various minimal media

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>5M1</th>
<th>5M2</th>
<th>5M3</th>
<th>5M4</th>
<th>5M5</th>
<th>2612</th>
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<td>A</td>
<td>M</td>
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<tr>
<td>Glucose</td>
<td>-</td>
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<td>Red</td>
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<td>Galactose</td>
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<tr>
<td>Glycerol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Red</td>
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<tr>
<td>Maltol</td>
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<tr>
<td>Mannitol</td>
<td>-/+</td>
<td>-/+</td>
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<td>Red</td>
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* M, morphology. Symbols: −, bald phenotype; +, sporulation occurred; −/+; some colonies produced small amounts of aerial hyphae. A, pigmented antibiotic production. Symbols: +, both undecylprodigiosin (red) and actinorhodin (blue) were produced; Red, only undecylprodigiosin was produced; −/+; only actinorhodin was produced, and only in discrete patches. The other two *S. coelicolor* antibiotics, methylenomycin and calcium-dependent antibiotic, were not assayed.

are bright yellow and have a soft, elevated surface. A trace of actinorhodin forms after 5M4 has been growing for more than 1 week on R4 plates. 5M3 colonies are soft and sometimes produce a nondiffusible orange pigment that has not been biochemically characterized.

The classical bld mutants have been characterized by their ability to be phenotypically suppressed on minimal media with different carbon sources. Table 2 summarizes the results of a similar study performed on the Tn4563 insertion mutants. Mutants were also tested for phenotypic suppression of sporulation or antibiotic production by diffusible substances provided by wild-type *S. coelicolor* (2612), or by each other, and none were detected.

Genetic mapping of the bld::Tn4563 alleles. Preliminary mapping was done by mating each mutant with J1501 and selecting for His<sup>+</sup> Str<sup>+</sup> recombinants. These two markers are at 12 and 6 o'clock, respectively. Because this is an NF × UF cross (see Materials and Methods), all of the recombinants also inherited the 9 o'clock region from the mutant donor bacterium. These crosses placed the transposon-encoded viomycin resistance marker on one half of the chromosome, and in the case of 5M1, they also provided additional detail (see below). After we mated 5M3 and 5M4 with J1501 and carried out different selections, bld-5M3 and bld-5M4 were roughly localized between 6 and 12 o'clock (data not shown). These two insertion mutations, along with bld-5M2, have not yet been more precisely mapped.

The results of crossing 5M1 (UTA5001) with J1501 are shown in Fig. 3A. Viomycin resistance was transferred from the donor relatively infrequently. The allele ratio (Vio<sup>+</sup>/Vio<sup>-</sup>) places bld-5M1 between 2 and 4 o'clock. No statistical linkage to adjacent auxotrophic markers was found.

Figure 3B shows an analogous mapping except that both the recipient and the donor are NF (i.e., carry an integrated SCP1; see Materials and Methods); thus, the selection bias toward 9 o'clock is relieved. The allele ratio for viomycin resistance was similar to that observed for Cys, refining the location of bld-5M1 toward 4 o'clock.

To be able to measure linkage to cysD, the same mating was subjected to a Ura<sup>+</sup> Arg<sup>+</sup> selection (Fig. 3C). Once again, the frequency of viomycin resistance among the recombinants places the insertion mutation in the 3 to 4

FIG. 3. Allele frequencies and linkage analysis for crosses between bld::Tn4563-5M1 and the SCP1<sup>-</sup> recipient J1501 (A) and the SCP1<sup>+</sup> (NF) recipient UTA1501 (B and C). In each diagram, the genome carrying the mutation to be mapped is drawn as the outer circle. Integrated copies of SCP1 are indicated by the box at 9 o'clock. Selected markers in each cross are denoted by triangles. Numbers around each marker are the number of recombinants having the indicated genotype; their ratio gives an index of gene order on the map. The dotted line indicates the approximate location of the viomycin resistance marker being mapped. Probabilities of linkage were calculated from χ<sup>2</sup> as described previously (19).
o'clock region. Linkage to cysD was strong, but two of the seven Vio' recombinants apparently resulted from a crossover between cysD and Tn4563. In addition, some linkage was observed between viomycin resistance and the streptomycin resistance locus strA.

The nearest, previously mapped, bld loci are bldB (15, 29) and bldH (6), but both of these loci are at 5 o'clock. These data strongly argue against the possibility that 5m1 maps to one of these two genes. But because 5m1 behavior on nonglucose carbon sources resembles the bldB phenotype (Table 2), a 5m1 lysogen of dC31-bldB+ was constructed (as described in Materials and Methods). The 5m1 lysogen was phenotypically indistinguishable from the parental nonlysogen, indicating that bld-5m1 is not a mutation in the bldB locus.

The bld-5m5 insertion was mapped in an analogous manner (Fig. 4). In the initial cross with J1501 (Fig. 4A), all of the 72 recombinants retained viomycin resistance. Because two different regions of the donor chromosome (the selected marker, plus SCP1 at 9 o'clock) are inherited by all recombinants in such a cross, the 5m5 allele could map either near 9 o'clock or around hisA at 12 o'clock.

In the NF × NF cross shown in Fig. 4B, the selection for SCP1 was removed and the inheritance of viomycin resistance clearly placed bld-5m5 near hisA. When the selected markers were changed (Fig. 4C), the viomycin allele ratio placed bld-5m5 to the left of hisA, near 11 o'clock.

A genetic map of S. coelicolor showing the bld and whi loci is given in Fig. 5.

**DISCUSSION**

A powerful approach to identifying key developmental genes in microbial systems is to use insertional inactivation by a transposable element to yield a mutant phenotype. The advantages of the method are numerous and include the tendency to generate null alleles, minimization of damage to other genes, and ease of cloning the interrupted loci. Despite the large number of genetic tools that have become available for S. coelicolor, it was only recently that a randomly inserting, composite transposable element for streptomycetes was serendipitously found during analysis of an unstable plasmid in S. fradiae (11). Tn4556 and its antibiotic-resistant derivatives, such as Tn4563, are now well-characterized members of the Tn3 family (35). All 6,625 bp of Tn4563 have been sequenced, target specificity appears to be random, and transposition immunity prevents secondary hops within a replicon (11-13).

Although random Tn4563 insertions into the chromosome have been reported for some Streptomyces species, the temperature-sensitive phenotype of the delivery plasmid (pMT660) used in these experiments proved to be too unreliable for use in S. coelicolor J1501, our standard genetic background. We do not know whether intrinsic leakiness of the temperature-sensitive phenotype is the problem or...
whether pMT660 exhibits a high frequency of reversion to temperature resistance. To generate chromosomal insertion mutations with Tn563, a more stringent selection against the donor molecule was required. No other temperaturesensitive plasmids were available, and Tn563 is too large to be encapsidated in the λ-like phage φC31.

In work by Davis and Chater (14), cloned genes to be further localized by insertional mutagenesis were cloned on a vector derived from the conjugal plasmid SCP2* (4). The transposon donor replicon (pUC1169, which is identical to pUC1172 except for the orientation of vph; 12) is compatible with SCP2* vectors and is not capable of conjugal transfer. After a period of growth in the presence of pUC1169, a mating was performed and SCP2* plasmid transconjugants carrying a copy of the transposon (Tn560) were selected with viomycin.

Thus, rather than select against the donor molecule by a temperature shift, we selected against the entire donor bacterium in a conjugation experiment. If a transposon has hopped to the chromosome of a bacterium which carries an integrated copy of SCP1 (NF mating type), it should be possible to mate the insertion to a recipient while leaving the donor plasmid behind. We obtained one bald mutant (Iml) and several white colony mutants with this selection. The donor plasmid for Tn563, pUC1172, was not transferred to the recipient during the mating at a detectable frequency.

Southern analysis on bld-Iml, UTA1492, showed a single junction fragment for the right end of the transposon which was not the same size as the junction fragment in pUC1172, the donor plasmid, indicating that a single transposition to the chromosome had occurred (data not shown). No plasmid DNA could be detected in the mutant (on agarose or in an ethidium bromide-CsCl density gradient), nor could viomycin resistance be transformed into a recipient from a UTA1492 DNA preparation. We also isolated several whi mutants by this method but have not further characterized them.

During the course of this work, we observed that the amount of pUC1172 was unstable in 2612. We do not know the mechanism responsible for the pUC1172 instability in 2612, but by comparison with strain J1501 in which pUC1172 is stable, it appears that SCP1 is responsible for the effect. Similar results, implicating SCP1, have been obtained by Chapanis (7). The replicon for pUC1172 is derived from the natural, circular, high-copy number plasmid pJJ101 (23) and is not thought to be related to the low-copy-number giant linear plasmid SCP1 (26).

The use of pUC1172 instability as a way of selecting against plasmid donor molecules has advantages over the conjugation method. First, the manipulations involved in conjugation are not necessary. Second, the original auxotrophic markers are retained in the mutants, making the genetic mapping easier (e.g., only the proA marker was still present after the conjugation that yielded bld-Iml).

Both methods, conjugation and plasmid exclusion, eliminate the requirement for pMT660, the temperature-sensitive pJJ702 derivative that is the pUC1172 vector backbone. We have used plasmid exclusion by SCP1 to deliver transposons from wild-type pJJ702 vehicles as well (30). A disadvantage to either delivery scheme is the possibility of isolating sister mutants, unless each mutant is isolated from a separate pUC1172 transformant, since a hop could occur at any time thereafter.

The insertion mutation in 5M5 maps at 11 o’clock on the S. coelicolor chromosome. The possibility that bld-5m5 is an allele of bldF awaits the cloning of the 5m5 insertion, which should be a straightforward task because of the associated viomycin drug resistance. Superficially at least, the two mutants appear to be different; 5M5 does not produce the bright red-orange pigment that is reportedly characteristic of the bldF1 mutant (32).

The bld-5m5 insertion mutation maps at 3 to 4 o’clock, a region of the chromosome that is relatively devoid of genetic markers (21). 5M1 cannot be phenotypically suppressed for morphological development by changing the carbon source, although limited actinorhodin production does occur on some media. This phenotype is essentially the same as that reported for bldB mutants (15), and the bldB locus maps nearby at 5 o’clock. However, 5m1 is most likely to be allelic to either of the two genes that make up the bldB locus because a φC31-bldB* transducing phage cannot restore sporulation or antibiotic production to 5M1.

The 5m1 insertion also does not appear to be located in bldH (6) nor in the gene identified by the JW21 bld mutation, based on map position (36). Also unlike JW21 or bldH, 5M1 does not produce aerial mycelium on minimal mannitol medium. Therefore, we believe that bld-5m1::Tn563 defines a previously undescribed locus that is required for aerial mycelium development and normal pigmented antibiotic production.

It should be noted that the NF strain used in this work (2612) also carries the circular, single-copy, conjugal plasmid SCP2* and that this plasmid is entirely dispensable for both morphological and physiological differentiation. Nevertheless, if a second copy of Tn560 is superimposed on SCP2*, its drug resistance gene could introduce noise into the genetic mapping of the chromosomal insertions. In the mutants described here, SCP2* insertions do not appear to be present because only a single transposon-junction fragment was detected by Southern analysis.

All of the bld mutations except bldB and bldC are suppressible for sporulation by growth on a non-glucose carbon source such as mannitol. This nutritional switch in phenotype has led to the proposal that there are two different regulatory pathways that lead to aerial mycelium production, one of which is glucose repressible (8). Among the set of bld insertion mutants described here, only one (5M4) is phenotypically suppressible by carbon source. The addition of non-repressible insertion mutants to the collection of classical bld mutants suggests that we have identified additional genes that are required by both the glucose-repressible and the glucose-independent pathways.

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