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Phage-Mediated Cloning of *bldA*, a Region Involved in *Streptomyces coelicolor* Morphological Development, and Its Analysis by Genetic Complementation

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***Streptomyces coelicolor* bald (*bld*) mutants form colonies of vegetative substrate mycelium, but do not develop aerial hyphae or spore chains. The *bldA* strains form none of the four antibiotics known to be produced by the parent strain. With a vector derived from the temperate bacteriophage φC31, a 5.6-kilobase fragment of wildtype DNA was cloned which restored sporulation to five independent *bldA* mutants when lysogenized with the recombinant phage. The cloned gene(s) was dominant over the mutant alleles. Phage integration by recombination of the cloned *bldA*$^+$ DNA with the *bldA* region of each mutant produced mainly sporulating colonies, presumably heterozygous *bldA*$^+$/*bldA* partial diploids for the insert DNA. However, a minority of these primary transductants were bald and were apparently homozygous *bldA*/*bldA* mutant partial diploids, formed by some homogenetization process. The phages released from the bald lysogens carried *bldA* mutations and were used to show that *bldA*$^+$ sequences had been cloned and that fine mapping of the region could be performed.**

Streptomyces spp. are gram-positive procaryotes that grow vegetatively as elongating and branching hyphae. On solid medium, in response to poorly understood signals, this substrate mycelium sends up aerial branches, which septate to form spore chains. The morphological development of *Streptomyces* spp. offers a promising system in which to study differential gene expression (6, 11), i.e., the ordered activation and regulation of genes or gene groups.

Studies with *Streptomyces coelicolor* A3(2), a genetically well-characterized streptomycete (9), led to the isolation of a number of mutants blocked in aerial mycelium formation (termed *bld* mutants for their bald colony appearance) or in the septation and spore chain maturation process (termed *whi* mutants for their white colony coloration). The fourteen *bld* mutants found were characterized phenotypically and mapped to four locations in the *S. coelicolor* linkage map defining the *bldA*, *bldB*, *bldC*, and *bldD* classes (16, 29).

Although they are termed *bld*, microscopic examination of colonies of *bldA* mutants reveal that they are covered with malformed, prostrate hyphae (29), which are probably defective aerial hyphae (11). Most *bldA* mutants produce none of the four antibiotics known to be made by the parental strain (18, 29). The *bldA* mutant phenotype is carbon source dependent; on glucose or cellobiose, or in the presence of glucose in a mixture of carbon sources, colonies are bald, whereas on a number of "permissive" carbon sources, such as maltose, galactose, or mannitol, the bald phenotype is suppressed and sporulation occurs (29; J. Piret, unpublished results).

In a first approach to understanding the control of developmental genes in *S. coelicolor*, the cloning of the wild-type *bldA*$^+$ region was undertaken. Vectors based on the temperate bacteriophage φC31 were chosen for this work, since it was potentially important to clone the sequences of interest as a single copy, thus avoiding the possible disruptive effects of multiple gene copies on morphological development (4, 21, 26). Although derivatives of this phage were under development as cloning vectors, their application in the shotgun cloning of *Streptomyces* spp. chromosomal genes had not yet been attempted. Furthermore, relatively little is known of the molecular biology of the φC31 life cycle, and much of it is inferred by analogy with phage lambda biology, with which the φC31 system appears to share some similarities. φC31 integrates into a preferred location of the host chromosome (24) by site-specific recombination between the phage *attP* site and the host *attC* site (7). Lysogeny is maintained by the product of the *c* gene (24). Deletion mutants of φC31, providing cloning space for foreign DNA, were previously isolated (8). The deletion most relevant to our objectives was that of the phage *attP* site. *attP* phages cannot lysogenize a host alone; however host DNA inserts cloned into an *attP* phage vector provide homology with the host chromosome to allow integration. The resulting constructs are thought to be partial diploids for the cloned sequences where the two copies of the cloned DNA are separated only by the phage genome and are the products of the recombination event of integration. Such constructs were used in later stages of the work described here. However, for the initial cloning of the *bldA*$^+$ region from *S. coelicolor*, it was expected that this mode of integration might occur at a frequency too low to suffice in a shotgun cloning experiment. Therefore, the *bldA* mutant strains were first lysogenized with an *attP*$^+$ φC31 derivative (φC31 KC301 [7]) to provide an extensive (about 35-kilobase [kb]) region of homology for the integration of the cloning vector (the most suitable vector available was φC31 KC401; Fig. 1). The resulting constructs were again presumably double lysogens and partial diploids for the cloned DNA, but where the two copies lie far apart on the host chromosome.

Here we report on the use of the phage vector φC31 KC401 in the cloning of a DNA fragment that complements five *bldA* mutants to restore sporulation. Antibiotic production was also regained. Furthermore, this work revealed a method not previously applied to *Streptomyces* spp. to move

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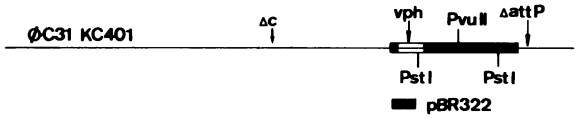

FIG. 1. Schematic representation of the cloning vector ϕC31 KC401. The phage is *c attP*. The pBR322 sequences cloned into the phage provided the unique *Pst*I and *Pvu*II sites used in the present work. The *vph* fragment carries the gene for the antibiotic resistance determinant viomycin phosphotransferase, which is not inactivated when the *Pst*I fragment is replaced. The phage is about 38 kb in length. Upon removal of the *Pst*I fragment (about 4 kb), virion packaging limits allow for insertion of foreign DNA of about 1 to 8 kb.

mutations between strains and to perform genetic complementation experiments.

MATERIALS AND METHODS

Bacterial strains. The *S. coelicolor* A3(2) derivatives are described in Table 1. The four *bldA* mutations all mapped close together at approximately 10:00 o'clock on the *S. coelicolor* circular linkage map (29). The mapping data for the strains carrying the *bld-34* and *bld-62* mutations were not sufficient to assign these alleles unequivocally to the *bldA* class. The construction of the single lysogens used as recipients in double lysogen experiments is described below. The Bld$^+$ phenotype is defined as aerial mycelium development and spore chain maturation covering the colony in 3 to 5 days on complex or defined plating medium at 30°C. The Bld$^-$ phenotype is defined as no further morphological development after formation of a bald, sculpted colony surface appearance under the same conditions. The Bld$^{+/-}$ phenotype refers to slow (10 to 14 days) formation of aerial hyphae and spore chains, never reaching the fully developed state of the Bld$^+$ colony.

Suspensions of mycelial fragments of *bldA* mutants were prepared from cultures (100 ml in 500-ml Erlenmeyer flasks) grown in yeast extract-malt extract broth containing 34% sucrose (5) and 5 mM $MgCl_2$ and supplemented with the auxotrophic requirements (16) of the individual strains. The flasks, containing coiled stainless steel springs to give dispersed growth, were inoculated with colonies from an R5 agar plate and shaken at 30°C for 5 to 7 days. The mycelium was concentrated by centrifugation about 10× and stored in 1- to 2-ml samples in 20% glycerol at −20°C.

Minimal medium salts agar was adapted from the minimal medium of Hopwood and Sermonti (17) by adding the following amounts of trace elements (per liter): $ZnCl_2$, 0.4 mg; $FeCl_3 \cdot 6H_2O$, 2.0 mg; $CuCl_2 \cdot 2H_2O$, 0.1 mg; $MnCl_2 \cdot 4H_2O$, 0.1 mg; $Na_2B_4O_7 \cdot 10H_2O$, 0.1 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1 mg. Glucose was replaced with other carbon sources, where required, at a final concentration of 0.5%. The trace elements and carbon source were autoclaved separately.

R2 yeast extract agar (37) was used for the regeneration of *Streptomyces lividans* protoplasts after transfection. R5 agar is a simplified version of R2 yeast extract agar where all but the $CaCl_2$, KH_2PO_4, and L-proline are added to the medium before autoclaving. R5 agar was routinely used to test the sporulation status of strains. Where appropriate, solid media were supplemented with auxotrophic requirements, thiostrepton (E. R. Squibb and Sons, Inc.) at 50 μg/ml and viomycin (Pfizer UK, Ltd.) at 30 μg/ml (final concentrations).

Streptomyces lividans strain 66 (25; John Innes strain 1326) was the primary recipient in *Streptomyces* spp. liposome-assisted protoplast transfections (33).

Streptomyces spp. colony morphology was observed under the light microscope by cover slip impression, whereby a glass cover slip was pressed gently to the surface of a colony and mounted in 20% glycerol on a microscope slide. For scanning electron microscopy, samples were prepared by the critical point drying method of Anderson (3). Colonies were coated with Pt and Au in a Balzers Union sputtering device and imaged in a Hitachi S-700 scanning electron microscope.

Escherichia coli ED8767 (30), a *recA* strain, was cultured in LB broth (27) or on LB agar supplemented, when appropriate, with tetracycline (Sigma Chemical Co.) at 10 μg/ml and carbenicillin (Sigma) at 100 μg/ml. Transformations were carried out by the procedure of Tabak et al. (36).

Phages. Derivatives of the temperate phage ϕC31 were used in cloning and studying the *bldA* region (Table 2). They were assayed and propagated on *S. lividans* 66 as described by Dowding (12). ϕC31 KC600 and ϕC31 KC601 are derivatives of ϕC31 KC401 carrying the cloned *bldA*$^+$ sequences. Double lysogens formed by superinfecting a host lysogenic for a *c*$^+$ *attP*$^+$ phage such as ϕC31 KC301 release phages that are often recombinants (7). This method was used to obtain *c*$^+$ derivatives of ϕC31 KC600 and ϕC31 KC601 (ϕC31 KC602 and ϕC31 KC603, respectively). Turbid plaques were purified from the mixture of turbid (*c*$^+$) and clear (*c*) plaques released from the double lysogens. Phages which could transduce viomycin resistance and *bldA*$^+$, but not thiostrepton resistance, were chosen for DNA isolation. By restriction enzyme mapping analysis, ϕC31 KC602 and ϕC31 KC603 differed from ϕC31 KC600 and ϕC31 KC601 only in the absence of the *c* gene deletion.

Transductions. Transductions were carried out in either of two ways. (i) Phage plaques produced in *S. lividans* lawns by transfection were replicated onto R2 yeast extract agar plates spread with spores or mycelial fragments of the recipient strain. Before replication, the *S. lividans* lawn was

TABLE 1. *S. coelicolor* strains[a]

Strain	Genotype[b]
M145	Prototroph
J650	*cysD18 mthB2 agaA1*
J668	*cysD18 mthB2 agaA1 bldA39*
J667	*cysD18 mthB2 agaA1 bldA35*
J673	*cysD18 mthB2 agaA1 bldA16*
J776	*nicA bldA1*
J394	*proA1 bld-34*
J395	*proA1 bld-62*

[a] All strains were originally derived from *S. coelicolor* A3(2).
[b] For marker designations, see reference 29.

TABLE 2. ϕC31 strains

Strain	Genotype[a]	Reference
ϕC31 KC301	*c*$^+$ *attP*$^+$ *tsr*	(7)
ϕC31 KC401	*c attP vph*	(14)
ϕC31 KC600/601[b]	*c attP vph* plus *bldA* insert	This paper
ϕC31 KC602/603[b]	*c*$^+$ *attP vph* plus *bldA* insert	This paper

[a] *c*, Repressor gene; *attP*, phage attachment sequence for site-specific integration; *tsr*, thiostrepton resistance; *vph*, viomycin resistance.
[b] Two orientations of the cloned insert.

killed by inverting the plates over chloroform held in glass petri dishes for 30 min, followed by evaporation of the chloroform in a laminar flow hood for 10 min. This procedure was used in the shotgun cloning experiment. (ii) Phage suspensions were mixed, with a glass spreader, with spores or mycelial fragments of the recipient strain directly on R2 yeast extract agar plates. The *bldA* mutant lysogens (ϕC31 KC301) used as recipients in double lysogen experiments were obtained by this method as were the transductants in the complementation experiments. After incubation at 30°C for at least 4 days, the growth was replicated onto medium containing the appropriate antibiotic(s) to select for transductants.

In vitro manipulation of DNA and isolation of *bldA*$^+$ sequences. The total DNA of *Streptomyces* spp. strains was isolated by the method of Hintermann et al. (15). Plasmid DNA was prepared by the method of Kieser (23). ϕC31 DNA was made by the small- or large-scale methods of Chater et al. (10). Digestion of DNA by restriction endonucleases was in a standard buffer (10 mM Tris-hydrochloride [pH 8.0], 10 mM $MgCl_2$, 5 mM dithiothreitol) supplemented with the required salt concentrations as described by the enzyme suppliers. Agarose gel electrophoresis was carried out with agarose type I (Sigma) and submerged horizontal gels (13). ϕC31 DNA was heated to 70°C for 10 min and cooled quickly in ice to separate the cohesive ends before electrophoresis. The electrophoresis buffer was TAE (27), and the DNA loading buffer contained 10% sucrose, 50 mM EDTA, and a small amount of orange G. DNA fragment sizes were measured with the DNAGEL program (22) on an Apple II microcomputer. Before ligation, the digested samples were extracted with an equal volume of water-saturated phenol-chloroform (1:1), and the appropriate DNAs were mixed and precipitated with 1/10 volume of 3 M sodium acetate (unbuffered) and an equal volume of isopropanol (1 h at −20°C). For DNA-DNA hybridizations, probe DNA (pIJ2151; see Fig. 3 legend) was labeled with [α-^{32}P]CTP by nick translation by the method of Maniatis et al. (27). Southern transfer and hybridization were done with GeneScreen membranes (New England Nuclear Corp.) as recommended by the manufacturer.

To construct the gene bank, *Pst*I-digested *S. coelicolor* M145 DNA was ligated with ϕC31 KC401 DNA digested with *Pst*I and *Pvu*II (*Pvu*II digestion reduced the frequency of regenerating the vector lacking inserts; Fig. 1). The ligation contained vector and donor DNA at 50 μg/ml each. Freshly prepared protoplasts of *S. lividans* 66 were transfected with the ligation mixture (1 μg of DNA). To screen for phages carrying the cloned *bldA*$^+$ DNA, the plaques produced were used to transduce the ϕC31 KC301 lysogen of the *S. coelicolor* strain J668 by replication. After growth of the double lysogens on R5 agar containing viomycin and thiostrepton, colonies were screened visually for the ability to sporulate. On R5 medium *bldA* mutant morphology is bald.

RESULTS

Shotgun cloning of *bldA*$^+$ sequences: double lysogens are formed. The *bldA*$^+$ region was identified in a gene bank of *S. coelicolor* wild-type DNA fragments cloned into a derivative of the bacteriophage ϕC31. The genomic library was produced by ligation of digests of *S. coelicolor* M145 DNA and ϕC31 KC401 DNA (as described above) and transfection of *S. lividans* 66 protoplasts with the ligation mixture. Phage plaques (2×10^4; 4×10^4 PFU/μg of vector DNA) were obtained. DNA extraction and restriction analysis revealed that 21 of 24 (87.5%) randomly chosen phages in the library carried foreign DNA inserts; the size range was 2.5 to 6 kb, with an average of 3.4 kb.

The cloning vector, ϕC31 KC401, is *attP* and thus incapable of establishing lysogeny alone. Therefore a lysogen of a representative *bldA* mutant, J668, which contains the *bldA39* mutation, was first constructed carrying an *attP*$^+$ ϕC31 derivative, ϕC31 KC301. The population of phages of the genomic library was used to transduce this recipient by plate replication and selection for antibiotic resistance carried by the vector. Lysogens were screened visually for those that had regained the ability to sporulate (Fig. 2). Ten separate sporulating (Bld$^+$) colonies were found among the transductants of the shotgun cloning experiment. The phages lysogenizing these transductants were isolated (since the phages are released from colonies at a significant frequency; see below), and their DNA was analyzed by restriction enzyme mapping. All 10 phages contained apparently identical 5.6-kb *Pst*I inserts. The orientation shown in Fig. 3 was found in six clones (a representative was named ϕC31 KC600), and the opposite orientation was found in four clones (named ϕC31 KC601).

The *bldA* phenotype was abolished by the presence of a *bldA*$^+$ transducing phage carrying the insert in either orientation: on minimal medium salts agar with glucose or with cellobiose the *bldA39* mutant double lysogen sporulated. In addition, the production of actinorhodin, a pigmented (red-blue pH indicator) antibiotic was restored. It was not determined whether production of the other three *S. coelicolor* antibiotics was restored.

Complementation and dominance tests for five *bldA* alleles: double and single lysogens. The genetic analysis of the cloned *bldA*$^+$ sequences was extended to the study of six independently isolated mutants carrying the *bldA39*, *bldA35*, *bldA16*, *bldA1*, *bld-34*, or *bld-62* allele. All were previously assigned to this region in mating experiments (29). Hybridizations of the cloned 5.6-kb fragment to total DNA prepared from the six mutants and the parent strain, J650, showed that they contained homologous sequences with the same restriction pattern as the parent (results not shown). Thus none of the *bldA* mutations was due to gross rearrangements or deletions of the DNA in this region and might be complemented by the cloned fragment. Such considerations are particularly important since chromosomal deletions and reiterated sequences, sometimes associated with bald morphology, are reportedly widespread and can occur at high frequencies in *Streptomyces* spp. (1, 19, 28, 32, 34).

Two different lysogen constructs (Fig. 4) were isolated for each of the six mutant strains to test for complementation by the cloned sequences and dominance. In no case did the orientation of the cloned insert have an effect on the results of the following experiments. In control experiments, the Bld$^+$ phenotype of the parent strain, J650, was unaltered when it was lysogenized with any of the *bldA*$^+$ transducing phages. Furthermore, strains of each mutant lysogenized with the phage vector lacking insert DNA were always bald.

The first arrangement was the same as that used in the initial shotgun experiment; a single lysogen (ϕC31 KC301) of each mutant was isolated and then superinfected with ϕC31 KC6000 or ϕC31 KC601 (Fig. 4a). In this situation, where the wild-type and mutant alleles were usually widely separated on the host genome, the five mutant strains carrying the *bldA39*, *bldA35*, *bldA16*, *bldA1*, or *bld-62* allele clearly regained the *Bld*$^+$ phenotype, sporulating in 3 to 5 days on R5 agar. They also produced abundant actinorhodin. This result for the *bld-62* mutant provided good evidence that this

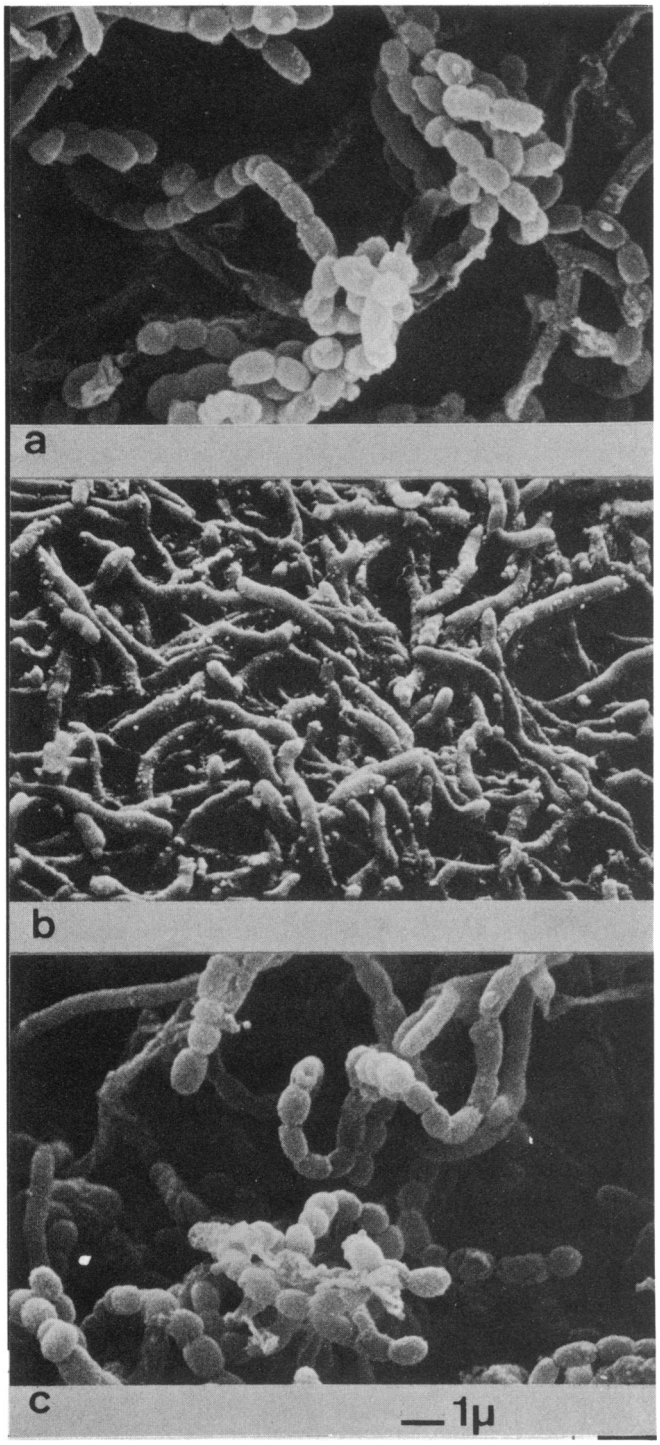

FIG. 2. Restoration of wild-type morphology to a *bldA* mutant lysogenic for a phage carrying wild-type *S. coelicolor* sequences. Electron micrographs were by Daniel Studer (Eidgenössische Technische Hochschule, Zürich). All micrographs are at the same magnification. The samples were from 6-day-old colonies grown on R5 agar with viomycin. (a) Parent strain (J650) double lysogen for ϕC31 KC301 and ϕC31 KC401 (the cloning vector). (b) *bldA* mutant (J668) double lysogen for ϕC31 KC301 and ϕC31 KC401. Only substrate mycelium is formed. (c) *bldA* mutant (J668) double lysogen for ϕC31 KC301 and ϕC31 KC600. Branching and septating aerial hyphae and spore chains are produced.

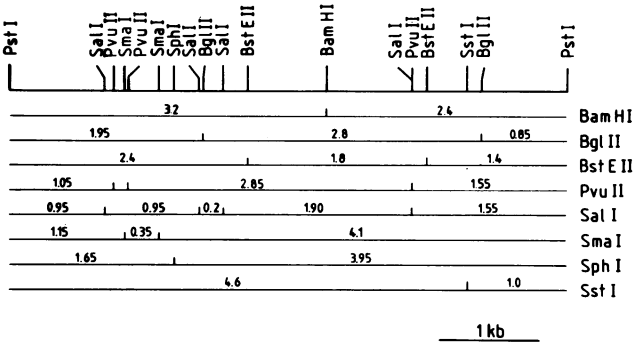

FIG. 3. Map of restriction endonuclease sites in the cloned DNA. The 5.6-kb fragment was ligated into the *Pst*I site of pBR322 and transformed into *E. coli* ED8767. Tetracycline-resistant, carbenicillin-susceptible colonies were selected, and plasmid isolations showed that the insert had integrated in each orientation. The resulting hybrid plasmids, pIJ2151 and pIJ2152, were mapped by single and double restriction enzyme digestions. The orientation shown, when inserted into ϕC31 KC401 left to right as drawn in Fig. 1, produced ϕC31 KC600.

mutation can be classified as *bldA* (it is not excluded that the *bld-62* allele is complemented by another gene on the 5.6-kb fragment). The response of the sixth mutant, *bld-34*, was inconclusive; it sporulated very slowly (10 to 12 days) and incompletely ($Bld^{+/-}$ phenotype) and produced little actinorhodin. Over 99% (Table 3) of all double lysogens had regained the Bld^+, or for *bld-34* the $Bld^{+/-}$, phenotype. The results suggested that the cloned insert carries the intact wild-type gene(s) corresponding to the *bldA39*, *bldA35*, *bldA16*, *bldA1*, and *bld-62* mutations and that the wild-type is dominant over these mutant alleles. (The case of the *bld-34* mutant was further analyzed as described below.) This conclusion rests on the assumption that the phage vector integrated into the resident prophage through their mutual homology (about 35 kb) more frequently than into the host chromosomal *bldA* region through homology with the cloned insert (5.6-kb homology). This assumption was tested by comparing the frequencies with which the double lysogens were formed (integration into the resident prophage plus integration into the *bldA* region) by infection of a *bldA* mutant lysogen versus the frequencies with which c^+ *attP* derivatives (described below) infecting a nonlysogen integrated into the *bldA* region only. The results supported the assumption; i.e., lysogeny by integration into a lysogen was 10^2 to 10^3 times more frequent than that into a nonlysogen.

TABLE 3. Phenotypes of transductants of *bldA* mutants[a]

Strain	*bld* mutation	Double lysogens			Single lysogens		
		No. Bld^+	No. Bld^-	% Bld^+	No. Bld^+	No. Bld^-	% Bld^+
J650	None	650	0	100	933	0	100
J668	*bldA39*	675	1	99.8	387	11	97
J667	*bldA35*	454	0	100	186	31	86
J673	*bldA16*	1,105	2	99.8	367	6	98
J776	*bldA1*	221	0	100	72	28	72
J394	*bld-34*	235[b]	2	99.2[b]	194	35	85
J395	*bld-62*	882	4	99.6	483	3	99

[a] The values given are sums of those obtained for lysogens carrying the cloned DNA in either orientation. The data for the two orientations were similar.

[b] $Bld^{+/-}$, Slow and incomplete sporulation.

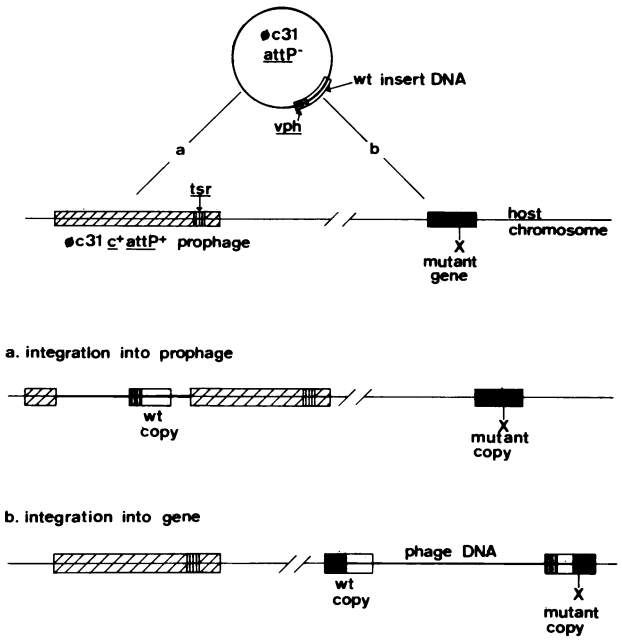

FIG. 4. Proposed structures for two modes of integration of an *attP* phage vector bearing cloned *bldA*$^+$ sequences (φC31 KC600 or φC31 KC601) into a *bldA* mutant host lysogenic for φC31 KC301 (*c*$^+$ *attP*$^+$). (a) The phage integrates into the resident prophage (itself integrated at the normal host *attC* site). (b) The phage integrates at the host region of homology with the cloned fragment, the *bldA* region, with a low frequency; see the text. The phage can only integrate at region of homology with cloned DNA when the host is a nonlysogen. wt, Wild type.

The Bld$^{+/-}$ response of the *bld-34* mutant as a double lysogen prompted the performance of complementation tests using a second configuration of host and *bldA*$^+$ transducing phage where phage integration could only occur into the host *bldA* region. *c*$^+$ derivatives (φC31 KC602 and φC31 KC603) of the *bldA*$^+$ transducing phages were isolated as described above. In this case the recipient strains used were nonlysogens, and the fact that these phages remained *attP* forced integration into the host *bldA* region, presumably resulting in direct repeats of the cloned DNA separated by the phage genome. Integration in this configuration was expected to lead to full Bld$^+$ expression even if the cloned wild-type sequences carried an incomplete transcriptional unit (which must include one intact end; Fig. 5) corresponding to this allele. However, when the *bld-34* mutant was transduced with either of these phages its sporulation was again very slow and incomplete (i.e., Bld$^{+/-}$), disputing the hypothesis of a truncated cloned gene for this allele. For the other five *bldA* mutants, sporulation was restored by φC31 KC602 or φC31 KC603 in the large majority of their transductants (Table 3; see below).

Lysogen stability. As single lysogens, the six *bldA* mutants sporulated more vigorously than as double lysogens. Integration of the phage at the *bldA* region places the cloned DNA adjacent to flanking chromosomal sequences (Fig. 4b), which may have some influence on the expression of the cloned sequences. Alternatively, the greater stability of the phage integrated at the *bldA* region versus its stability integrated into a resident prophage may explain this observation. Indeed, both single and double lysogens of complemented *bldA* mutants, since they contained tandem duplications, were unstable as would be expected (2). When lysogens were streaked out for single colonies on nonselective (antibiotic-free) medium, mixtures of sporulating and bald colonies, as well as sectored colonies of both phenotypes, were observed. The frequency of this segregation, quantitated for the *bldA39* mutant J668 and its lysogens, was higher with double lysogens than with single lysogens. After a round of growth and sporulation under nonselective conditions (on MMS-maltose), single colony sporulation status and loss of the phage-determined antibiotic resistance phenotype were scored. For double lysogens (35-kb homology with the resident prophage), sporulation and antibiotic resistance were simultaneously lost at a frequency of 3 to 4%. The single lysogens (5.6-kb homology) showed an instability of about 0.5%.

Homozygous *bldA* mutant partial diploids. Examination of the data in Table 3 reveals that, although the majority of the primary transductants lysogenizd with a phage bearing *bldA*$^+$ sequences are Bld$^+$, a significant number, particularly among the single lysogens, remain bald. This phenomenon was further analyzed. Bald colonies never arose among primary transductants of the parent strain, J650, indicating that alterations in the cloned sequences leading to *bldA* mutations during experimental manipulations were too infrequent to account for the observation. Phages released from the bald transductants and used to retransduce the same mutant could not restore sporulation. Restriction analysis of the DNA isolated from these phages showed no apparent alteration. Furthermore, on nonselective medium, all segregants of the bald primary transductants were bald while retaining the ability, carried by the phage vector, to grow under antibiotic selection. These observations suggested that such strains were only releasing phages carrying *bldA*

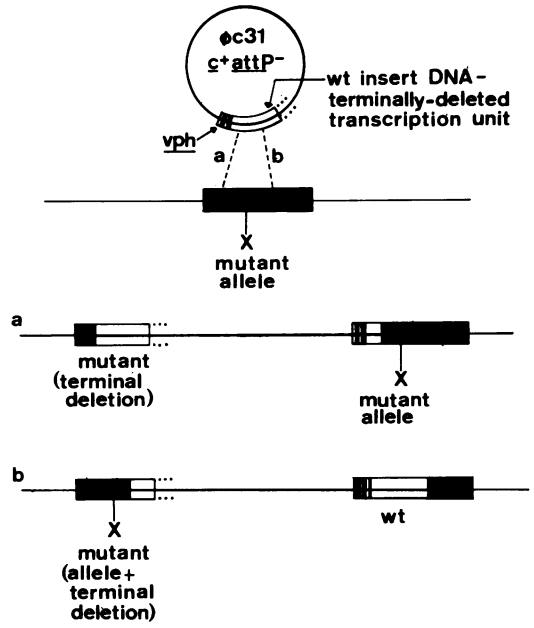

FIG. 5. Proposed structure for integration of *c*$^+$ *attP* derivative of phage vector into a mutant host chromosome by homology with cloned wild-type (wt) sequences where the cloned sequence is terminally deleted for the gene (functional unit) of interest. The location of the integration event determines the outcome. (a) Two mutant copies are formed, and the lysogen phenotype remains mutant. (b) A mutant copy and a wild-type copy are formed, and the lysogen phenotype is wild type.

TABLE 4. Complementation tests between *bldA* mutations

Lysogen	Recipient allele	*bldA* allele carried by phage: *bldA39* Bld+	*bldA39* Bld−	*bldA16* Bld+	*bldA16* Bld−	*bldA1* Bld+	*bldA1* Bld−	*bld-62* Bld+	*bld-62* Bld−
Single[a]	*bldA39*	0	119	85	50	4	539	117	34
	bldA16	17	235	0	184	15	108	0	78
	bldA1	2	220	6	88	0	362	44	57
	bld-62	45	72	0	120	67	52	0	212
	Wild type	220	2	20	0	532	6	63	0
Double[b]	*bldA39*	0	455	0	468	2	1,720	1	321
	bldA16	0	110	0	130	0	208	0	331
	bldA1	1	101	0	98	0	210	0	227
	bld-62	0	252	0	192	0	157	0	306
	Wild type	895	1	345	0	3,060	2	402	0

[a] Single lysogens for *bldA*-transducing phages integrated into the *bldA* region.

[b] Double lysogens for *bldA*-transducing phages integrated into the resident prophage.

mutations and that they behaved like homozygous partial diploids for the *bldA* mutations (*bldA*/*bldA*).

Using the phages that carry *bldA* mutations: moving mutations between strains and fine mapping. The bald colony morphology of presumably homozygous partial diploids for *bldA* mutations among primary transductants provided a simple means to isolate phages bearing *bldA* mutations. These alleles could then be moved from strain to strain. For example, when phages released from a bald transductant of the strain carrying *bldA39* were used to infect the parent strain J650, about 1% of the transductants were bald. This phenotype was stable and indistinguishable from the *bldA39* mutant in colony morphology and in sporulation pattern on different carbon sources. This result was strong evidence that *bldA*$^+$ DNA, rather than other sequences able to suppress *bldA* mutations, had been cloned.

By lysogenizing the *bldA* mutants with phages carrying the different *bldA* mutations, genetic complementation between the mutations could be tested. In the course of this work *c*$^+$ *attP* phages bearing the *bldA39*, *bldA16*, *bldA1*, and *bld-62* alleles were isolated and purified. Two configurations of lysogens were constructed, and the phenotypes of the transductants were scored (Table 4). For one configuration, the individual *bldA* mutants (nonlysogens) were transduced with each phage bearing a given *bldA* mutation to obtain integration into the *bldA* region. In any given combination one of two outcomes occurred: either the lysogens were a mixture of Bld$^+$ and Bld$^-$ colonies (e.g., *bldA39* versus *bldA16*, *bldA1*, and *bld-62*), or all lysogens were Bld$^-$ (e.g., *bldA16* versus *bld-62*). In control experiments no Bld$^+$ lysogens were found among the transductants resulting from the infection of a given *bldA* mutant with a phage bearing the same allele. For the second configuration, lysogens of each of the four *bldA* mutants were superinfected with each phage carrying a *bldA* mutation to place the two mutations far apart in the host genome. All, or the great majority, of the transductants produced were bald, indicating that the four mutations tested lie in one cistron. (The possibility is not excluded that they are in different genes and subject to polar effects.) In rare instances Bld$^+$ lysogens arose in these tests (e.g., *bldA39* versus *bld-62*). However, these few Bld$^+$ transductants are likely to be due to the rare event where the phage integrates into the *bldA* region rather than into the resistant prophage; indeed, Bld$^+$ transductants only arose in combinations of mutations that gave some Bld$^+$ transductants in tests for recombination. The results suggested that the four alleles tested are due to mutation events at different positions in the *bldA* region, with the possible exception that *bldA16* and *bld-62* may be at the same site or close together.

DISCUSSION

The isolation of the *bldA*$^+$ region of *S. coelicolor* by using a φC31 derivative demonstrated the feasibility of using this vector system in the shotgun cloning of *Streptomyces* spp. chromosomal genes. Subsequently, another region involved in morphological development (*bldB*$^+$; J. M. Piret, unpublished results) and the glucose kinase gene of *S. coelicolor* (20) were also isolated by using φC31 vectors. The phage system offered several analytical and technical benefits for the work reported here and for future experiments. The fact that φC31 can lysogenize about half of the *Streptomyces* spp. tested (10) suggests that φC31 vectors may be useful in these systems as well.

The cloned wild-type DNA was introduced into the mutants presumably as a single copy. Single copy cloning was potentially important in studying sequences affecting differentiation and could be important in other instances; in *Bacillus subtilis* high copy numbers of certain sequences involved in sporulation disrupted endospore formation (4, 21, 26). For the *bldA*$^+$ sequences this precaution proved to be unnecessary since the cloned DNA, subcloned on the *Streptomyces* spp. multicopy plasmid pIJ385 (a derivative of pIJ101; gift of T. Kieser) could also restore the wild-type phenotype to the *bldA39* mutant (J. M. Piret, unpublished results).

The DNA cloned in phages could be easily introduced into other strains via natural infection, without recourse to in vitro techniques such as transformation, which requires protoplast formation and regeneration in *Streptomyces* spp. The cloned sequences were stably maintained upon repeated phage propagation and extended storage. The lysogen stability also facilitated manipulations.

Lysogens formed by integrating the vector at a preestablished prophage allowed tests of complementation and dominance with the cloned wildtype DNA.

Lysogens formed by integration via the cloned DNA should have allowed the detection of a terminally deleted cloned gene (Fig. 5).

Tests of dominance and complementation were previously carried out in *Streptomyces* spp. by heteroclone analysis (16). This method, although sometimes useful in the analysis of nutritional markers, is not practical for the study of mutations involved in differentiation and antibiotic biosynthesis because these traits are not usually selectable (9). The bald colony morphology of apparently homozygous (*bldA*/*bldA*) mutant partial diploids arising at significant frequencies among the lysogens provided a simple and rapid way to recognize colonies releasing phages carrying *bldA* mutations. These phages were used for genetic fine mapping in easily selectable, stable partial diploids. This suggests a general method for such analysis of other *Streptomyces* spp. genes.

The isolation of *bldA*$^+$ sequences was a first step in our approach to the analysis of genes and gene expression involved in differentiation in *S. coelicolor* A3(2). The data indicate that the complete gene(s) corresponding to the *bldA39*, *bldA35*, *bldA16*, *bldA1*, and *bld-62* mutations is contained on the cloned DNA and that the wild type is dominant over these alleles. The results for the *bld-34* mutation remain to be clarified. The results counter the hypothesis that this allele corresponds to a terminally de-

leted transcriptional unit in the cloned DNA. Another possibility is that the *bld-34* allele exerts a partial dominance over the wild type. Alternatively, the *bld-34* mutant strain may carry other unrecognized mutations affecting morphological development.

Among the sporulating single lysogens of the *bldA* mutants, bald transductants occurred at frequencies of 1 to 28% and behaved like homozygous partial diploids for their mutations. One possibility is that phage integration and excision at opposite sides of a given mutation carried the mutation out of one genome and into another. However, the stability of these lysogens was tested, and phage markers were lost at a frequency of only 0.5%. Note also that these bald strains were primary transductants held under continuous antibiotic selection for the presence of the recombinant phage. It is not known at present to what extent ϕC31, once established as a prophage in its host and expressing the antibiotic determinant that it bears, can exist transiently as an autonomous molecule in the host cytoplasm while its host continues to grow under antibiotic selection. Another hypothesis is that the *bldA/bldA* mutant diploids are formed by a mechanism whereby the nonhomologous region between the two otherwise homologous sequences is corrected to give rise to two wild-type or two mutant copies. Nonreciprocal recombination or gene conversion is thought to involve mismatch repair synthesis in conjunction with recombination (31, 38). Shimkets et al. (35) reported that in *Myxococcus xanthus* developmental mutants containing an integrative plasmid carrying cloned developmental genes, gene conversion gave rise to homozygous partial diploids at a frequency of 7 to 33%. The elucidation of this homogenetization process in *Streptomyces* spp., apparently unreported to date, awaits further work. Notwithstanding, the phages that transduced *bldA* mutations were used in complementation experiments to assign four *bldA* alleles to one cistron. Furthermore, it was found that at least three of the four alleles are at different positions in this functional unit. The *bldA* mutations, isolated in phage derivatives, can be used in the future to analyze of the nature of the mutational events involved.

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