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# Cloning, Disruption, and Transcriptional Analysis of Three RNA Polymerase Sigma Factor Genes of *Streptomyces coelicolor* A3(2)

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**The *rpoD* gene of *Myxococcus xanthus* was used as a probe to isolate three *Streptomyces coelicolor* genes, *hrdB*, *hrdC*, and *hrdD*, which appear to encode RNA polymerase σ factors extremely similar to the $\sigma^{70}$ polypeptide of *Escherichia coli*. Gene disruption experiments suggested that *hrdB* is essential in *S. coelicolor* A3(2) but showed that *hrdC* and *hrdD* mutants are viable and are apparently unaffected in differentiation, gross morphology, and antibiotic production. S1 nuclease mapping showed that *hrdB* and *hrdD*, but not *hrdC*, were transcribed in liquid culture. The most upstream of two *hrdD* promoters is internal to an open reading frame (ORF X) on the opposite strand. The predicted product of this gene is homologous to the phosphinothricin acetyltransferases of *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes*. The possible significance of the overlapping and divergent transcription of *hrdD* and ORF X is discussed. A general method for in vivo gene replacement was developed that allowed a positive selection for the desired mutants even in the absence of a mutant phenotype; it was used to isolate a stable *hrdC* mutant.**

The promoter recognition specificities of eubacterial RNA polymerases are determined by the species of sigma (σ) subunit associated with them (see reference 25 for a review). In *Escherichia coli* and in vegetatively growing *Bacillus subtilis*, the vast majority of genes are transcribed by a holoenzyme containing the principal σ factor, referred to as $\sigma^{70}$ or $\sigma^{A}$, respectively (25). However, both of these bacteria synthesize a variety of alternative σ factors that have different promoter specificities and that are used to transcribe discrete regulons of physiologically related genes. The role of RNA polymerase σ factors in *B. subtilis* sporulation strikingly illustrates this point. Five σ factors, $\sigma^{E}$, $\sigma^{F}$, $\sigma^{G}$, $\sigma^{H}$, and $\sigma^{K}$, which have been identified by using both biochemical and genetic means, play key roles in changing the temporal program of transcription during endospore formation (34, 37, 55). Mutations in the genes encoding these σ factors (*spoIIGB*, *spoIIAC*, *spoIIIG*, *spo0H*, and *spoIVCB*::*spoIIIC*, respectively) block sporulation at characteristic stages.

The existence of multiple forms of RNA polymerase in *Streptomyces coelicolor* A3(2) is firmly established (recently reviewed in reference 12). Three different σ factors, $\sigma^{49}$, $\sigma^{35}$, and $\sigma^{28}$, have been identified biochemically by using holoenzyme reconstitution experiments (13, 62), and the existence of at least one further species has been inferred from in vitro transcription studies on the *S. coelicolor gal* operon (61). In addition, genetic studies have shown that the *whiG* gene of *S. coelicolor* A3(2) encodes another σ factor, $\sigma^{whiG}$, which plays a critical role in triggering the onset of sporulation in aerial hyphae (18). *whiG* mutants form abundant aerial hyphae, but the hyphae show no sign of further morphological development (15, 40). Conversely, overexpression of *whiG* causes early and hyperabundant sporulation, including the metamorphosis of subterranean vegetative hyphae into spore chains (18, 41). Thus, overexpression of *whiG* can actually change the developmental fate of hyphae, since vegetative hyphae are normally fated to lyse at the onset of aerial mycelium formation.

Remarkably, recent work by Tanaka et al. (57) has revealed features of the transcriptional apparatus of *S. coelicolor* A3(2) that have no parallel in *E. coli* or *B. subtilis*. Using an oligonucleotide probe, designed from a sequence of 10 amino acids that are completely conserved between $\sigma^{A}$ of *B. subtilis* and $\sigma^{70}$ of *E. coli*, they cloned four genes, each encoding a σ-factor highly similar to the $\sigma^{70}$ polypeptide of *E. coli*. These genes were designated *hrdA*, *hrdB*, *hrdC*, and *hrdD* (*hrd* stands for homolog of *rpoD*). The degree of similarity between the predicted products of the *hrd* genes and those of the *rpoD* genes (the principal σ factors) of *E. coli* and *B. subtilis* far exceeds that observed between σ factors for different promoter classes (12, 57).

Thus the potential for a high degree of transcriptional flexibility resulting from the capacity to synthesize different σ factors is now firmly established in *Streptomyces*. However, with the exception of $\sigma^{whiG}$, which has a specific role in controlling the development of aerial hyphae into spore chains, we have no clear idea of the biological roles of the σ factors so far identified. One of the most fruitful ways of determining the roles of individual σ factors in the control of gene expression is to clone the σ factor genes and to use the cloned DNA to generate mutants defective in σ factor synthesis. For instance, the gene encoding $\sigma^{D}$ (*sigD*) of *B. subtilis* was recently cloned and used to generate a *sigD* mutant that proved to be defective in flagellar biosynthesis (26). Further experiments showed that $\sigma^{D}$ controls the motility regulon of flagellar and chemotaxis genes in *Bacillus* species (26, 42) and led to the discovery of functionally analogous σ factors in the enteric bacteria (2, 24).

In a study initiated independently of that of Tanaka et al. (57), we used a different route to isolate the *hrdB*, *hrdC*, and *hrdD* genes. Here we describe this approach, the results of the disruption of these genes in vivo, and an analysis of their transcription.

## MATERIALS AND METHODS

**Bacteria, phages, and plasmids.** Bacteria, phages, and plasmids are listed in Table 1. The manual of Hopwood et al.

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TABLE 1. Strains, phages, and plasmids used

| Strain, plasmid, or phage | Genotype | Reference or source |
|---|---|---|
| *S. lividans* 66 | | |
| 1326 | Wild type | 36 |
| TK19 | SLP2$^-$ SLP3$^+$ | 31 |
| TK20 | SLP2$^+$ SLP3$^-$ | 31 |
| TK24 | *str-6* SLP2$^-$ SLP3$^-$ | 31 |
| TK38 | *his-2 spc-1* SLP2$^-$ SLP3$^-$ | T. Kieser, unpublished data |
| TK64 | *pro-2 str-6* SLP2$^-$ SLP3$^-$ | 31 |
| *S. coelicolor* A3(2) | | |
| 1147 | Wild type | 30 |
| M145 | SCP1$^-$ SCP2$^-$ | 30 |
| J1501 | *hisA1 strA1 uraA1* Pgl$^-$ SCP1$^-$ SCP2$^-$ | 17 |
| J1507 | *hisA1 strA1 uraA1* Pgl$^-$ SCP1$^{NF}$ SCP2$^+$ | 51 |
| J1668 | *hisA1 strA1 uraA1 glkΔ119* Pgl$^-$ SCP1$^{NF}$ SCP2$^-$ | 21 |
| J1955 | *hisA1 strA1 uraA1* Pgl$^-$ *hrdC*::KC925 SCP1$^-$ SCP2$^-$ | This paper |
| J1956 | *hisA1 strA1 uraA1* Pgl$^-$ *hrdD*::KC926 SCP1$^-$ SCP2$^-$ | This paper |
| J1957 | *hisA1 strA1 uraA1 glkΔ119* Pgl$^-$ *hrdC*::*ermE* SCP1$^{NF}$ SCP2$^-$ | This paper |
| φC31 phages | | |
| KC466 | *c*$^+$ Δ*attP* ::*vph* ::*gyl* | 51 |
| KC515 | *c*$^+$ Δ*attP* ::*tsr* ::*vph* | 48 |
| KC573 | *c*$^+$ Δ*attP* ::*tsr* ::*vph* ::*glk* | 21 |
| KC924 | *c*$^+$ Δ*attP* ::*tsr* ::*vph* ::!*hrdB*! | This paper |
| KC925 | *c*$^+$ Δ*attP* ::*tsr* ::*vph* ::!*hrdC*! | This paper |
| KC926 | *c*$^+$ Δ*attP* ::*tsr* ::*vph* ::!*hrdD*! | This paper |
| KC927 | *c*$^+$ Δ*attP* ::*tsr* ::*vph* ::!*gyl*! ::!*hrdB*! | This paper |
| KC928 | *c*$^+$ Δ*attP* ::*tsr* ::*vph* ::!*gyl*! ::!*hrdC*! | This paper |
| KC929 | *c*$^+$ Δ*attP* ::*vph* ::*glk* ::*hrdC*::*ermE* | This paper |
| *E. coli* TG2 | K-12 Δ(*pro lac*) *supE thi recA srl*::Tn*10 hsd*Δ*5* (r$^-$ m$^-$) F′ (*traD36 proAB lacI*$^q$ *lacZ* Δ*M15*) | T. Gibson, unpublished data |
| Plasmids | | |
| pIJ2032 | pIJ2925 containing the !*hrdB*! *Hin*fI fragment | This paper |
| pIJ2033 | pIJ2925 containing the !*hrdC*! *Sma*I-*Pvu*II fragment | This paper |
| pIJ2037 | pIJ2925 containing the *hrdC* gene on a 1.5-kb *Sph*I-*Sal*I fragment | This paper |
| pIJ2039 | Derivative of pIJ2037 containing *ermE* inserted at the *Bam*HI site internal to *hrdC* | This paper |
| pIJ2040 | pIJ2925 containing the !*hrdD*! *Fsp*I-*Sty*I fragment | This paper |
| pIJ2925 | Derivative of pUC19 having *Bgl*II sites at both ends of the polylinker | G. R. Janssen, unpublished data |
| pIJ4026 | pIJ2925 containing the *ermE* gene as a 1.7-kb *Kpn*I fragment | M. J. Bibb, unpublished data |
| λ phages | | |
| λMJB-B | λEMBL4 containing *hrdB* | This paper |
| λMJB-C | λEMBL4 containing *hrdC* | This paper |
| λMJB-D | λEMBL4 containing *hrdD* | This paper |

(30) contains details of conditions for growth of streptomycetes and their phages and for phage DNA isolation.

**Media and reagents.** NMM (28) is a defined minimal medium. 2-Deoxyglucose and erythromycin were purchased from Sigma Chemical Co., thiostrepton was a gift from S. J. Lucania of E. R. Squibb & Sons, and viomycin was a gift from E. J. Friend of Pfizer (UK) Ltd.

**Isolation of lysogens.** Lysogens were selected by replica plating onto NMM medium containing either 50 μg of thiostrepton per ml or 20 μg of viomycin per ml and/or 100 μg of erythromycin per ml. All carbon sources were added to a final concentration of 0.5% (wt/vol).

**Positive selection of directed mutations.** Mutants were isolated by plating serial dilutions of spores of lysogens on NMM containing 100 mM 2-deoxyglucose and 100 μg of erythromycin per ml.

**Southern blotting.** Southern blots (54) were performed as described by Hopwood et al. (30).

**DNA sequencing.** The nucleotide sequences of the *hrdB* and *hrdD* promoter regions were determined either by the method of Maxam and Gilbert (39) or by dideoxy-chain termination (49, 50). Oligonucleotide primers were used to facilitate completion of the dideoxy sequencing strategy.

**RNA isolation.** RNA was isolated from 36-h cultures of wild-type *S. coelicolor* A3(2) and from stationary-phase cultures of *E. coli* TG2 as described by Hopwood et al. (30). *S. coelicolor* was grown in YEME liquid medium (7) supplemented with 34% sucrose, 0.5% glycine, and 5 mM $MgCl_2$ or in NMMP liquid medium (30). *E. coli* was grown in 2xYT medium (3).

**S1 nuclease mapping.** High-resolution S1 nuclease mapping (52) was performed as described by Baylis and Bibb (4), except that 40 μg of total RNA was incubated overnight with approximately 0.1 pmol of probe (approximately $10^6$ cpm/pmol of probe) in sodium trichloroacetate-based buffer (44), and 100 U of nuclease S1 were used to digest the single-

stranded nucleic acid. The probes used (Fig. 1) were as follows: for *hrdB*, a 520-base-pair (bp) *Hin*dIII-*Lsp*I fragment uniquely labeled on the 5′ end at the *Lsp*I site; for *hrdC*, a 170-bp *Sph*I-*Xma*I fragment uniquely labeled on the 5′ end at the *Xma*I site and a 630-bp *Sma*I-*Not*I fragment uniquely labeled on the 5′ end at the *Not*I site; and for the two *hrdD* promoters, a 94-bp *Eco*RI-*Nar*I fragment uniquely labeled on the 5′ end at the *Nar*I site and a 360-bp *Hin*dIII-*Eco*RI fragment uniquely labeled on the 5′ end at the *Eco*RI site. The uniquely labeled probes were generated by sequential restriction digestion. The sizes of the RNA-protected fragments were assessed by comparison with Maxam and Gilbert sequencing ladders (39) generated from the corresponding end-labeled probes. Before assigning precise transcript start points, one to one and one-half nucleotides were subtracted from the length of the protected fragments to account for the difference in the 3′ ends resulting from S1 nuclease digestion and the chemical sequencing reactions (27).

## RESULTS

**Cloning of the *hrdB*, *hrdC*, and *hrdD* genes.** The *rpoD* gene of *E. coli*, which encodes the major σ factor of this species, has been used as a hybridization probe to isolate a homologous *rpoD* gene from *Myxococcus xanthus* (32). The relatively high G+C content of the myxobacterial gene (65 mol% G+C [32]) compared with that of *E. coli rpoD* (53 mol% G+C [11]) suggested that it might be a useful probe for similar experiments with *Streptomyces* DNA (average base composition, 74 mol% G+C [23]). When used as a probe in low-stringency (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50°C) Southern hybridization experiments, a mixture of restriction fragments that covered the entire *M. xanthus rpoD* gene (kindly provided by S. Inouye) hybridized to several restriction fragments in a variety of digests of *S. coelicolor* A3(2) total DNA (data not shown), indicating the presence of multiple hybridizing sequences. The same probe was subsequently used to isolate two recombinant phages carrying one of the hybridizing segments of the *S. coelicolor* A3(2) genome from a λEMBL4 library of *S. coelicolor* A3(2) M145 DNA (the kind gift of J. S. Feitelson). DNAs isolated from the two phages proved to have identical restriction maps, indicating that they were clonal in origin. One of the phage DNA preparations was digested completely with *Mbo*I; after dephosphorylation with calf intestinal alkaline phosphatase, the resulting fragments were ligated into *Bam*HI-cut M13mp18. *E. coli* TG2 was transfected with the ligation mix, and phages carrying the sequences responsible for hybridization to the *M. xanthus rpoD* probe were identified by plaque-lift hybridization. The nucleotide sequences of the *Mbo*I restriction fragments inserted into these M13 derivatives were determined directly by the dideoxy-chain termination protocol. Each of the recombinant phages carried the same *Mbo*I fragment, and the predicted sequence of amino acids encoded by this segment showed a very high level of similarity to region 2.3 (as defined in reference 25) of the σ factors encoded by the *rpoD* genes of *M. xanthus*, *B. subtilis*, and *E. coli* (14).

A 300 bp *Bam*HI-*Sst*I fragment, which contained the *Mbo*I fragment described above, was isolated from one of the two λEMBL4 clones and used in Southern hybridization experiments to probe digests of *S. coelicolor* A3(2) chromosomal DNA, again under low stringency (2× SSC, 65°C). In these conditions, it gave a hybridization pattern like that of the *M. xanthus rpoD* probe, but with a greater signal intensity. This probe was used to isolate 12 further recombinant phages from the λEMBL4 library of *S. coelicolor* A3(2) M145 DNA. Restriction mapping of DNA from the 12 phages identified three separate classes of clones in which the hybridizing genes were located on *Sma*I fragments of 3.55, 1.25, and 1.9 kilobases (kb), corresponding to *Sal*I fragments of 4.1, 3.2, and 7.6 kb, respectively. The sizes of these *Sma*I and *Sal*I fragments indicated that the three classes of clones corresponded to the *hrdB*, *hrdC*, and *hrdD* σ factor genes identified by Tanaka et al. (57). Restriction maps of the regions encoding *hrdB*, *hrdC*, and *hrdD* are shown in Fig. 1. Representative λEMBL4 clones of the *hrdB*, *hrdC*, and *hrdD* were designated λMJB-B, λMJB-C, and λMJB-D, respectively.

**Construction of KC924, KC925, and KC926: φC31-derived vectors for the insertional inactivation of *hrdB*, *hrdC*, and *hrdD*.** To investigate the roles of $\sigma^{hrdB}$, $\sigma^{hrdC}$, and $\sigma^{hrdD}$ in the control of gene expression in *S. coelicolor*, *hrdB*, *hrdC*, and *hrdD* were disrupted in vivo by the mutational cloning procedure of Chater and Bruton (16). The availability of the nucleotide sequences of the *hrd* genes (kindly provided by H. Takahashi and his colleagues) permitted the identification of restriction fragments internal to each coding region. Three *attP*-deleted, $c^+$ derivatives of φC31 were then constructed (Fig. 2A). A 1,010-bp *Hin*fI fragment (Fig. 1), internal to the *hrdB* gene (!*hrdB*!), was made blunt ended and cloned into the *Sma*I site of pIJ2925 (a derivative of pUC19 with *Bgl*II sites at both ends of the polylinker; G. R. Janssen, unpublished data) to create pIJ2032. A 910-bp *Sma*I-*Pvu*II fragment (Fig. 1) internal to the *hrdC* gene (!*hrdC*!) was cloned into the *Sma*I site of pIJ2925 to create pIJ2033. Similarly, a 620-bp *Fsp*I-*Sty*I fragment (Fig. 1) internal to the *hrdD* gene (!*hrdD*!) was made blunt ended and cloned into the *Sma*I site of pIJ2925 to create pIJ2040. These sequences were removed as *Bgl*II fragments and cloned into *Bam*HI-cut KC515 to create KC924 (*hrdB*) and KC925 (*hrdC*) or cloned into *Bgl*II-cut KC515 to create KC926 (*hrdD*).

Because these phages were *attP* deleted, they could form lysogens only by Campbell-type homologous recombination between the *hrd* DNA sequences of the phage and the chromosome. Because the restriction fragments used were internal to the *hrd* coding sequences, integration would result in gene disruption and loss of σ factor function (16).

***hrdC* and *hrdD* are dispensable for growth and differentiation.** For each of the three phages, attempts were made to select lysogens of *S. coelicolor* A3(2) strains J1501 and J1507 by virtue of their transduction to thiostrepton resistance (Thio$^r$). KC925 (containing *hrdC* DNA) and KC926 (containing *hrdD* DNA) both readily gave rise to lysogens, suggesting that KC925 and KC926 could integrate into *hrdC* and *hrdD*, respectively, to give rise to viable *hrdC* and *hrdD* mutant derivatives of J1501 or J1507. Disruption of *hrdC* and *hrdD* in the lysogens was confirmed by Southern blot analysis (data not shown). The *hrdC* and *hrdD* mutants were not visibly affected in gross colony morphology, differentiation, or production of the pigmented antibiotics undecylprodigiosin and actinorhodin. Representative *hrdC* and *hrdD* mutant derivatives of J1501 generated in this way were designated J1955 and J1956, respectively.

**Gene disruption experiments suggest that *hrdB* is an essential gene in *S coelicolor* A3(2).** In contrast to the results obtained with *hrdC* and *hrdD*, KC924 (containing the *hrdB* DNA) did not give rise to lysogens in J1501 or J1507, suggesting that integration of KC924, and hence disruption of *hrdB*, might be lethal in *S. coelicolor* A3(2). To test this further, a φC31 derivative was constructed that contained both the !*hrdB*! DNA and a 1.25-kb fragment of DNA

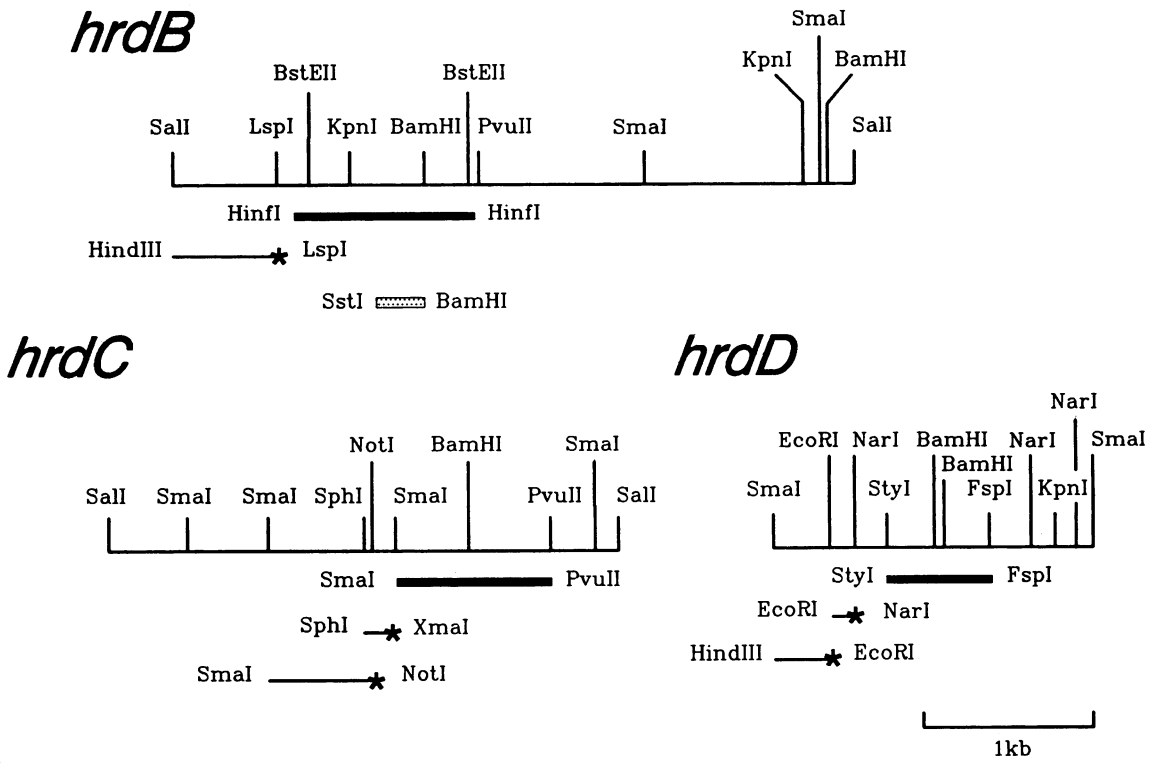


FIG. 1. Restriction maps of the fragments containing the *hrdB*, *hrdC*, and *hrdD* genes. The stippled box indicates the 300-bp *Sst*I-*Bam*HI fragment internal to *hrdB* used as a probe in the cloning of *hrdC* and *hrdD*. The black bars indicate the restriction fragments internal to the *hrd* genes used in the mutational cloning experiments. The lines below the restriction maps indicate the DNA probes used in S1 nuclease mapping experiments, and the asterisks identify the $^{32}$P-labeled 5′ ends.

internal to the *gyl* operon (!*gyl*!) to act as an internal control for lysogen formation (KC927; Fig. 2B). As an additional control, a similar phage derivative was made containing the !*hrdC*! DNA (KC928; Fig. 2B). The *Bgl*II fragments from pIJ2032 and pIJ2033 were separately ligated to the *Bam*HI-generated left arm of KC515 and the *Bam*HI-generated right arm of KC466 to create KC927 (*hrdB*) and KC928 (*hrdC*) (Fig. 2B), respectively. The right arm of KC466 carries the 1.25-kb fragment of DNA internal to the *gyl* operon of *S. coelicolor* A3(2). Integration of KC927 or KC928 into the *gyl* operon would give rise to lysogens with a functional glycerol kinase but without a functional glycerol-3-phosphate dehydrogenase (51). Such *gyl* mutants are glycerol sensitive (Gyl$^s$); that is, they are not only unable to grow on glycerol as a carbon source but are also unable to grow on a permissive carbon source in the presence of glycerol, probably because the accumulation of intracellular glycerol 3-phosphate is toxic (51).

Lysogens of J1501 or J1507 containing KC927 (*hrdB*) or KC928 (*hrdC*) were selected by virtue of their transduction to viomycin resistance (Vio$^r$). Twenty percent of KC928 lysogens (251 of 1,244 colonies) grew on plates containing either glycerol or glycerol plus arabinose (Gyl$^+$) and were presumably *hrdC* mutants, and 80% (993 of 1,244 colonies) were Gyl$^s$ mutants that were unable to grow on either glycerol or glycerol plus arabinose. It has been noted previously that the frequency of insert-directed integration is highly dependent on the size of the insert (51). It therefore seems likely that the excess of *gyl*-directed integration events over *hrdC*-directed integration events is a simple consequence of the relative sizes of the *gyl* DNA (1.25 kb) and *hrdC* DNA (0.9 kb) present in KC928.

In contrast, KC927, containing both *hrdB* and *gyl* DNA, gave rise to several hundred lysogens, all of which were Gyl$^s$. The absence of any Gyl$^+$ lysogens suggests that disruption of *hrdB* is a lethal event.

During this experiment, lysogens were sometimes selected by using thiostrepton instead of viomycin. Under these conditions, all colonies that failed to grow on glycerol as a carbon source grew in the presence of glycerol and arabinose. In other words, lysogens in which the phage had integrated into the *gyl* DNA behaved as Gyl$^s$ in the presence of viomycin but behaved as Gyl$^-$ (but Gyl$^r$) in the presence of thiostrepton. We are unable to account for this observation.

**Construction of a stable *hrdC* mutant strain of *S. coelicolor* A3(2).** To examine further the effects of *hrd* gene disruption on *Streptomyces* growth and development, it is desirable to have stable *hrd* mutants that are free of the phage φC31, because of potential problems with the slight instability of lysogens and also to permit the use of φC31-based vectors in further in vivo analysis. To construct such mutants, we extended the deletion-selection procedure of Fisher et al. (21) to give a φC31-based protocol for gene replacement in *Streptomyces* species, which allowed the easy identification of the desired, phage-free mutants, even in the absence of a selectable or easily detectable mutant phenotype. *hrdC* was chosen as the target for the gene replacement.

The construction of the φC31-derivative used in the gene replacement is illustrated in Fig. 3. A 1.5-kb *Sph*I-*Sal*I fragment (Fig. 1) carrying the entire coding region of the *hrdC* gene was cloned into pIJ2925 cut with *Sph*I and *Sal*I to create pIJ2037, which carries two *Bam*HI sites, one in the *hrdC* gene and a second in the polylinker. A 1.7-kb *Bgl*II

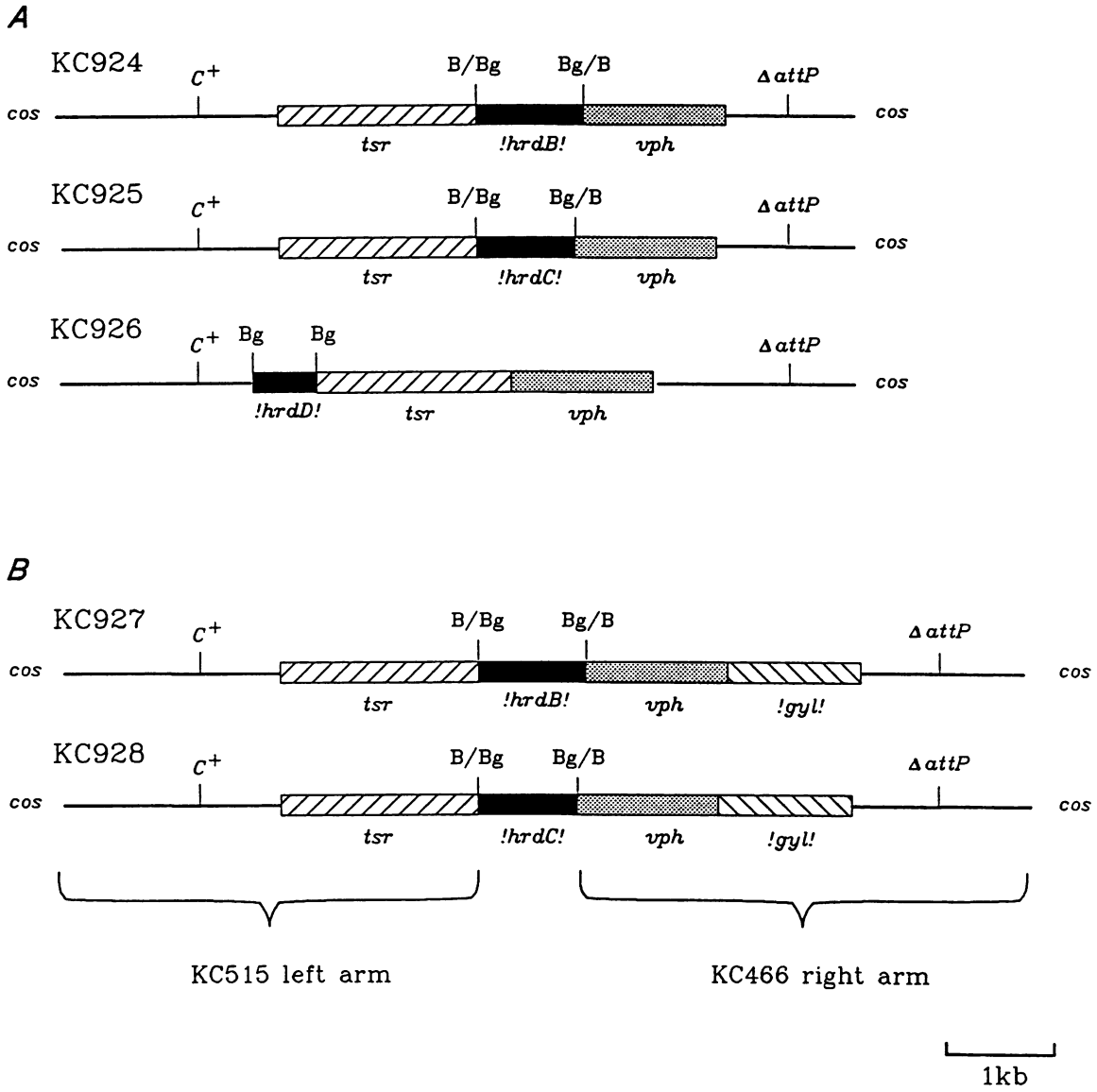


FIG. 2. Construction of ϕC31 derivatives for use in mutational cloning. The black bars marked !*hrd*! correspond to the restriction fragments internal to the *hrd* genes indicated in Fig. 1 after modification to give *Bgl*II ends (see the text). (A) Construction of KC515 derivatives used initially in attempts to disrupt the *hrdB*, *hrdC*, and *hrdD* genes in mutational cloning experiments. (B) Construction of ϕC31 derivatives capable of integrating at alternative sites in the chromosome for further mutational cloning analysis of *hrdB* and *hrdC*. The !*gyl*! DNA (an internal fragment of the *gyl* operon) serves as an internal control for lysogen formation. Restriction sites: B, *Bam*HI; Bg, *Bgl*II.

fragment carrying the erythromycin resistance (Ery$^r$) gene (*ermE*) (58) of *Saccharopolyspora erythraea* was isolated from pIJ4026 (M. J. Bibb, unpublished data) and cloned into pIJ2037 that had been linearized by partial digestion with *Bam*HI. A plasmid (pIJ2039) was recovered in which *ermE* was inserted into the *Bam*HI site in the *hrdC* coding sequence (the *ermE* coding sequence is in the opposite orientation relative to the *hrdC* coding sequence in this construct). The 3.2-kb *Bgl*II fragment carrying the *hrdC* gene interrupted by *ermE* was isolated from pIJ2039 and cloned into the ϕC31 derivative KC573 as a replacement for the *Bam*HI-*Bgl*II fragment carrying the *tsr* gene; the resulting phage, which carried the counterselectable *glk* gene, was designated KC929. *glk* encodes a glucose kinase that permits growth on glucose and that confers 2-deoxyglucose sensitivity (Dog$^s$) (21).

KC929 was used to replace the *hrdC* gene in the chromosome of *S. coelicolor* A3(2) with the disrupted copy of *hrdC* in the phage (Fig. 3). *S. coelicolor* J1668, in which the *glk* gene is deleted (and which is therefore Dog$^r$ and also unable to grow on glucose as the sole carbon source) was used for lysogen formation. This ensured that KC929 could integrate only via *hrdC* sequences and not through homologous recombination at the *glk* locus. KC929 lysogens of J1668 were selected by virtue of their transduction to Ery$^r$ Vio$^r$ and their ability to grow on glucose as a sole carbon source. Six lysogens (A through F) were picked and grown through one round of sporulation, selecting only for Ery$^r$. Putative *hrdC* mutants were then identified by selecting for Ery$^r$ Dog$^r$ derivatives on plates containing arabinose as the carbon source.

One of the six lysogens, lysogen B, persistently gave rise

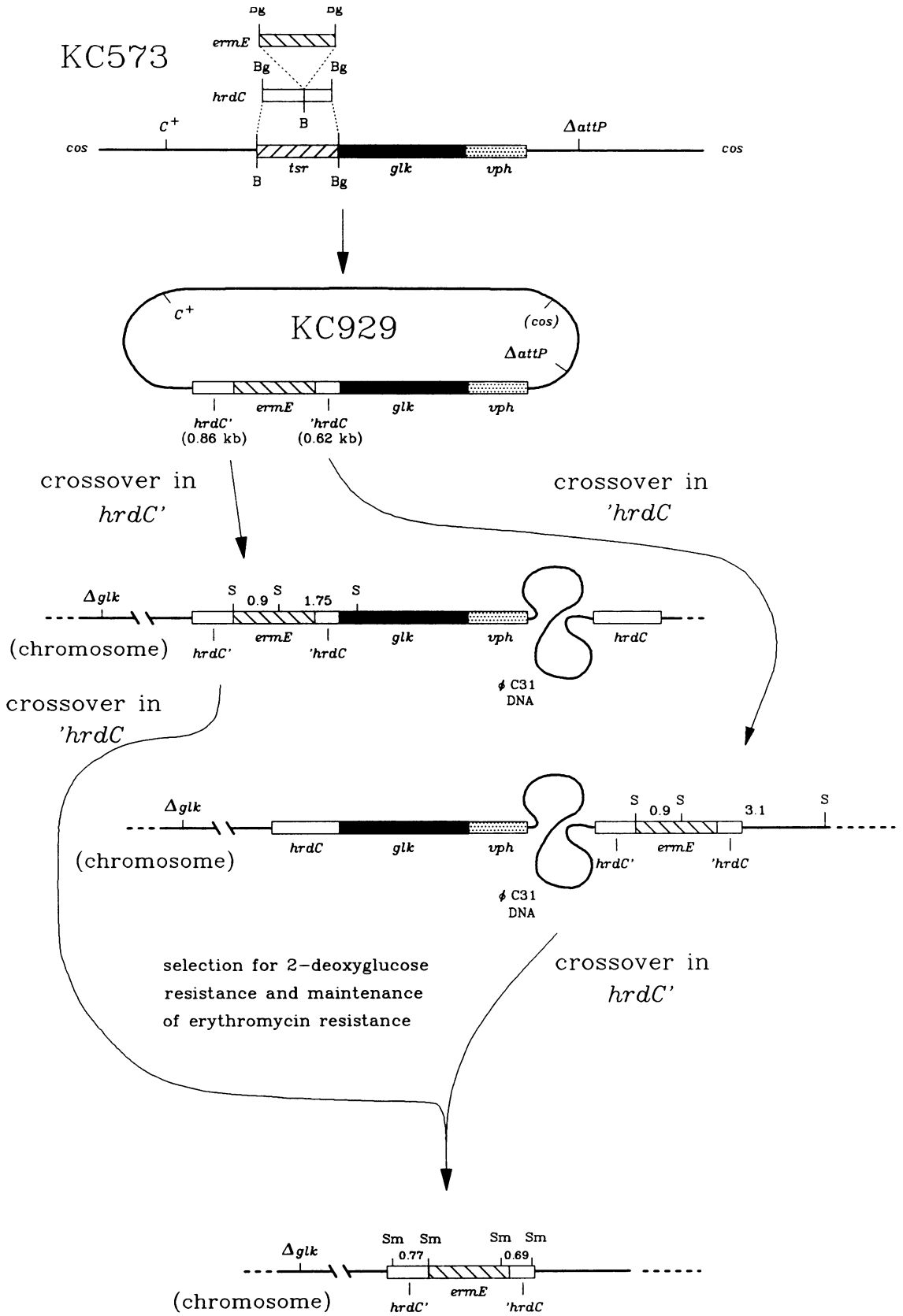


FIG. 3. Construction of KC929 and its use in the generation of a stable *hrdC* mutant. The diagram illustrates the alternative integration pathways of KC929 via either of the two intervals of *hrdC* DNA separated by the *ermE* gene in the phage. Selection for 2-deoxyglucose resistance and maintenance of erythromycin resistance identified phage-free *hrdC* mutant strains. In these strains the phage has excised through the alternative interval of *hrdC* DNA to that through which integration had occurred, resulting in replacement of the *hrdC* gene in the chromosome with the disrupted copy originally present in the phage. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; S, *Sal*I; Sm, *Sma*I.

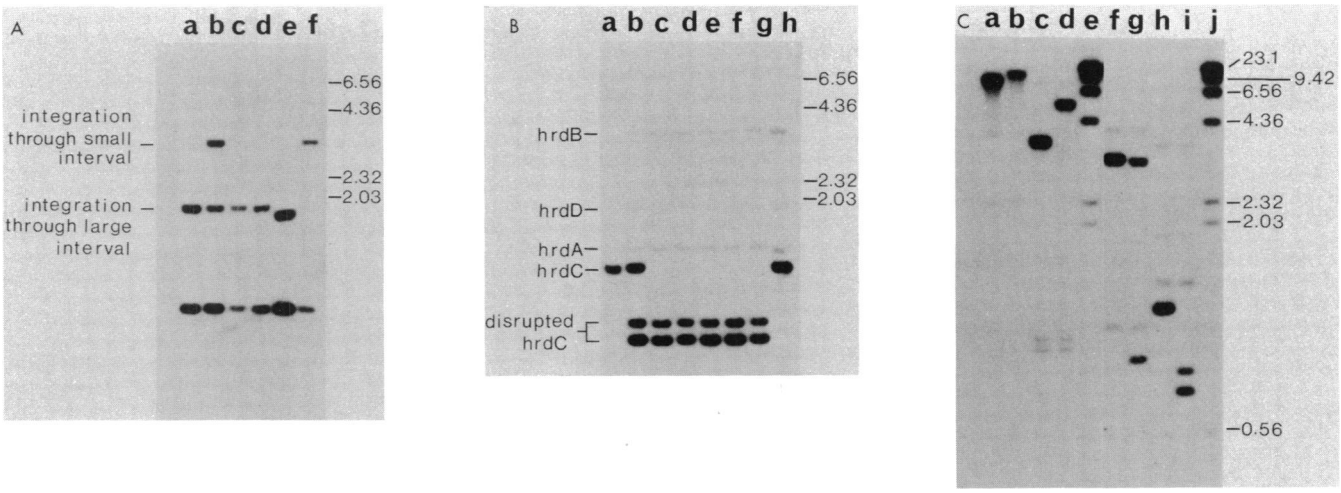


FIG. 4. Southern blot analyses of KC929 lysogens of J1668 and of *hrdC* mutants derived from the lysogens. (A) *Sal*I digests of DNA isolated from six J1668 lysogens containing KC929 probed with the 1.7-kb *ermE* fragment derived from pIJ4026. The origins of the hybridizing bands are shown in Fig. 3. (B) *Sma*I digests of DNA isolated from J1668 (a), a KC929 lysogen of J1668 (b), five putative *hrdC* mutants (c through g), and *S. lividans* 66 probed with a 0.9-kb *Sma*I-*Pvu*II fragment (Fig. 1) from the *hrdC* gene (h). The origins of the hybridizing bands are shown in Fig. 3. (C) DNA isolated from J1668 (a, c, f, and h) or from an *hrdC* mutant (b, d, g, and i) digested with *Bcl*I (a and b), *Bst*EII (c and d), *Sal*I (f and g), or *Sma*I (h and i) and probed with a 0.9-kb *Sma*I-*Pvu*II fragment (Fig. 1) from the *hrdC* gene. Lanes e and j show *Hin*dIII-digested lambda markers.

to Ery$^r$ Dog$^r$ colonies at a frequency approximately 200-fold lower than those of the other five. To investigate this observation, the structures of the lysogens were analyzed by Southern blotting (Fig. 4A). *Sal*I digests of DNA from the lysogens were probed with the 1.7-kb *ermE* fragment. This probe should hybridize to 0.9- and 3.1-kb *Sal*I fragments when integration has occurred through the small (0.62-kb) interval of *hrdC* and to 0.9- and 1.75-kb *Sal*I fragments when integration has occurred through the large (0.86-kb) interval of *hrdC* (Fig. 3). Integration occurred through the small interval to yield lysogen F and through the large interval to yield lysogens A, C, and D (Fig. 4A). The pattern for lysogen E cannot be interpreted because of an apparent deletion event. In the case of lysogen B, the presence of both the 1.75- and 3.1-kb bands indicates that this strain harbors two copies of the prophage. This interpretation is confirmed by the higher relative intensity of the 0.9-kb band in lysogen B; this 0.9-kb band would be present at twice the copy number of the 1.75- and 3.1-kb bands in a double lysogen. The presence of two copies of the prophage accounts for the lower frequency with which lysogen B gave rise to the desired *hrdC* mutants.

Lysogen A was chosen for further work. Chromosomal DNA preparations from lysogen A, from five putative *hrdC* mutants derived from lysogen A, and from the parent strain J1668 were digested with *Sma*I and probed with a 0.9-kb *Sma*I-*Pvu*II fragment (Fig. 1) from the *hrdC* gene. This probe should hybridize to a single 1.25-kb *Sma*I fragment in wild-type DNA and to 0.69- and 0.77-kb *Sma*I fragments in the desired *hrdC* mutants (Fig. 3). Lysogen A showed the presence of the intact and disrupted copies of the *hrdC* gene on either side of the prophage (Fig. 4B, lane b), but each of the DNAs isolated from the Ery$^r$ Dog$^r$ colonies showed the presence of only the disrupted *hrdC* gene (Fig. 4B, lanes c through g). Weaker hybridization to *Sma*I fragments corresponding to *hrdA* (1.4 kb), *hrdB* (3.5 kb), and *hrdD* (1.9 kb) was also observed.

DNAs isolated from one of the putative *hrdC* mutants and from the parent strain J1688 were subjected to further Southern blot analysis to confirm the structure of the mutant (Fig. 4C). The 0.9-kb *Sma*I-*Pvu*II fragment from *hrdC* (Fig. 1) was again used as the probe. All of the digests yielded the expected hybridizing bands and therefore confirmed the predicted structure. This was most graphically illustrated by digests with *Bcl*I and *Bst*EII (Fig. 4C, lanes a through d), which do not cut in *hrdC* or the *ermE* cassette used in the disruption. In these cases a single hybridizing band was observed in the J1668 digests, and the hybridizing band in the putative mutant was approximately 1.7 kb larger, corresponding to the size of the *ermE* cassette used in the gene disruption. Weaker hybridization to restriction fragments containing the *hrdA*, *hrdB*, and *hrdD* genes was also observed. A representative *hrdC* mutant of J1668 generated in this manner was designated J1957.

***Streptomyces lividans* possesses an *hrdC* gene.** It has been reported that *S. lividans* 66, a species closely related to *S. coelicolor* A3(2), carries homologs of *hrdA*, *hrdB*, and *hrdD* but does not carry a homolog of *hrdC* (56). However, Southern analysis with our *hrdC* probe showed the presence of homologs of all four *S. coelicolor hrd* genes in *S. lividans* 66 (compare lanes a and h, Fig. 4B) and some of its commonly used derivatives, TK19, TK20, TK24, TK38, and TK64 (data not shown). Thus, the *S. lividans* 66 isolate used by Takahashi et al. (57) may have undergone a deletion of the *hrdC* homolog. This would be consistent with the nonessential nature of the gene.

**Transcriptional analysis of the *hrdB*, *hrdC*, and *hrdD* genes.** Given the lack of obvious phenotypic consequences of mutations in *hrdC* and *hrdD*, we wished to know whether *hrdC* and *hrdD* were expressed. Therefore the transcription of these genes and of *hrdB* was investigated by using high-resolution S1 nuclease mapping. The RNA used was isolated from wild-type *S. coelicolor* A3(2), and the probes used are shown in Fig. 1 and described in Materials and Methods. For *hrdB* (Fig. 5A), an RNA-protected fragment was observed that corresponded to a transcript start approximately 353 bp upstream from the most likely translation initiation codon. For *hrdD*, RNA-protected fragments were

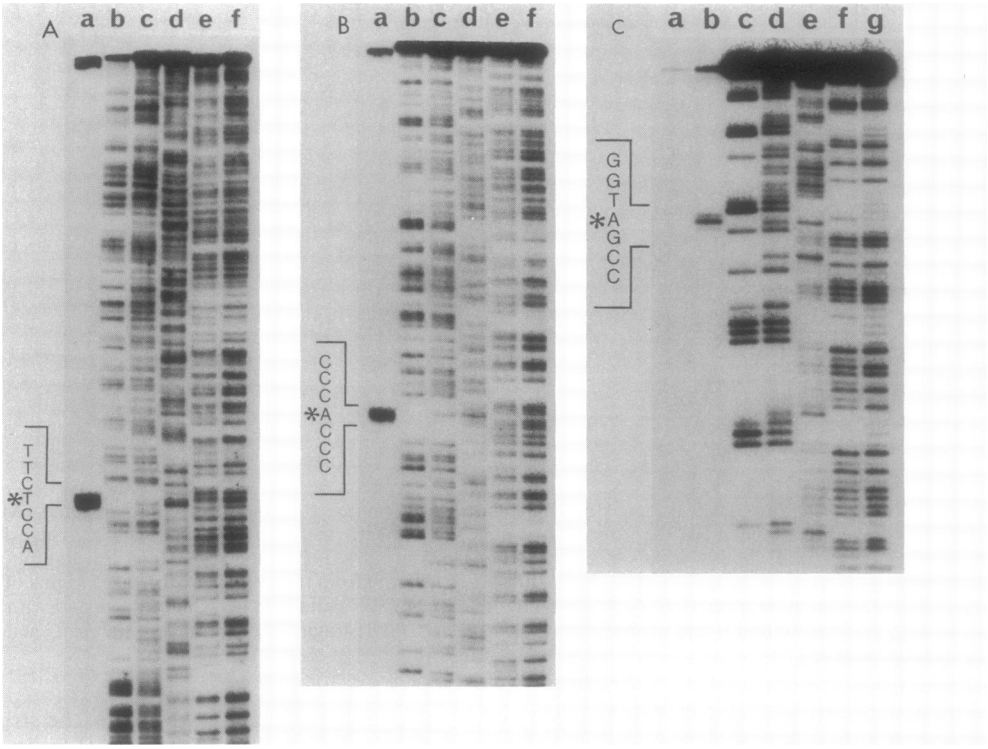


FIG. 5. High-resolution S1 nuclease mapping of the *hrdB* and *hrdD* promoters. Asterisks indicate the most likely start points. The RNA used was isolated from wild-type *S. coelicolor* A3(2) grown in supplemented YEME medium. In each panel the order of the Maxam and Gilbert sequence ladders is G, G+A, A>C, T+C, C. (A) The *hrdB* promoter: (a) RNA protected fragment, (b through f) Maxam and Gilbert sequence ladders. (B) *hrdDp1*: (a) RNA protected fragment, (b through f) Maxam and Gilbert sequence ladders. (C) *hrdDp2*: (a) Control S1 nuclease experiment with *E. coli* RNA, (b) RNA protected fragment, (c through g) Maxam and Gilbert sequence ladders.

observed that corresponded to transcripts with 5′ ends located approximately 365 bp (*hrdDp1*; Fig. 5B) and 117 bp (*hrdDp2*; Fig. 5C) upstream of the putative start codon. In each case these hybrids were not observed in control experiments with the same probes but with RNA isolated from *E. coli* (Fig. 5C and data not shown). However, in all S1 nuclease mapping experiments, we observed bands corresponding to full-length probe. Since these bands appeared in the nonhomologous RNA controls, at least some of the full-length protection must arise from probe reannealing. However, because of the presence of these bands we are unable to rule out the possibility of additional transcripts arising from promoters further upstream. The probes used for the S1 nuclease mapping experiments shown in Fig. 5 were radiolabeled at sites upstream of the *hrdB* and *hrdD* coding sequences. However, in preliminary experiments (data not shown) with probes radiolabeled at sites internal to the *hrd* genes, the three transcripts identified here were shown to extend into the coding sequences. The nucleotide sequences of the *hrdB* and *hrdDp1* and *hrdDp2* promoters (Fig. 6) were determined by Maxam-Gilbert sequencing of the probes used in the S1 nuclease mapping and by dideoxy-chain termination sequencing of the opposite strand. No *hrdC* transcripts were detected in RNA isolated from *S. coelicolor* A3(2) grown in either supplemented YEME or NMMP medium.

**The *hrdDp1* promoter is internal to a gene on the opposite strand, which is homologous with genes encoding phosphinothricin acetyltransferases from other streptomycetes.** The nucleotide sequence of the *hrdD* promoter region (Fig. 6) was analyzed for protein-coding character by using the program FRAME (6); this revealed a probable coding sequence on the opposite strand to the *hrdD* gene. An open reading frame (ORF X) extends leftward from a possible ATG start codon (nucleotides 294 through 296, Fig. 6) to the end of the available sequence. The sequence 5′-GAGG GAGG-3′, which occurs 7 bp upstream from the potential ATG start codon, shows perfect complementarity to a region close to the 3′ end of the 16S rRNA of *S. lividans* (5) and therefore has the potential to function as a ribosome-binding site (53). The amino acid sequence predicted from the 5′ end of this putative gene was compared with a six-phase translation of the EMBL nucleotide sequence data base (20 August 1989 release) with the program TFASTA (46). Significant homology was found to the predicted products of the *bar* (59) and *pat* (63) genes of *Streptomyces hygroscopicus* and *S. viridochromogenes*, respectively (Fig. 7). These two *Streptomyces* species produce the herbicidal antibiotic bialaphos, and the *bar* and *pat* genes encode phosphinothricin acetyltransferases that function as enzymes in the bialaphos biosynthetic pathway and are very likely also to function as resistance determinants in the producing organisms.

The *hrdDp1* promoter is internal to ORF X. Consequently, if ORF X is expressed in mRNA at the same time as *hrdD*, then the *hrdDp1* transcript and the ORF X transcript(s) must be complementary and overlapping for a minimum of 155 bases.

## DISCUSSION

Our cloning of three RNA polymerase σ-factor genes from *S. coelicolor* A3(2) made use of the *M. xanthus rpoD* gene as

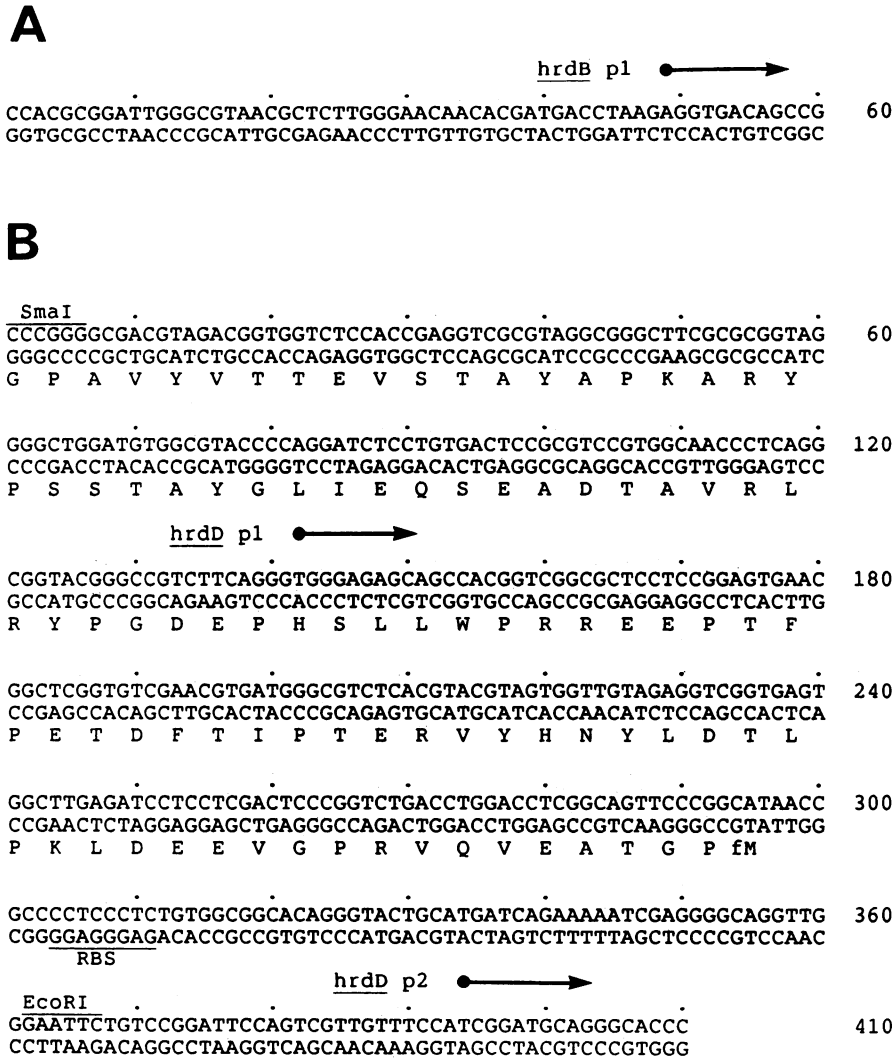


FIG. 6. Nucleotide sequences of the *hrdB* and *hrdD* promoter regions. Sequences were determined using the method of Maxam and Gilbert (39) on the end-labeled probes used in the nuclease S1 transcript mapping experiments and by dideoxy-chain termination sequencing (49, 50) of the opposite strand. Circles indicate the most likely transcription start points as determined by nuclease S1 mapping, and arrows indicate the direction of transcription. The putative primary translation product of ORF X on the opposite strand to *hrdD* is shown beneath the sequence. Restriction sites discussed in the text are indicated.

a heterologous probe. The *M. xanthus* gene was previously isolated by using the *rpoD* gene of *E. coli* as a probe (32). In earlier Southern blots of chromosomal DNA digests we failed to detect significant hybridization between the *rpoD* gene of *E. coli* and any *S. coelicolor* sequences, presumably because of the large differences in G+C content of *E. coli* DNA (50 mol% G+C [45]) and *Streptomyces* DNA (74 mol% G+C [23]). Our results suggest that it may be generally possible to overcome the barrier to the cloning of *Streptomyces* genes with heterologous probes derived from *E. coli*

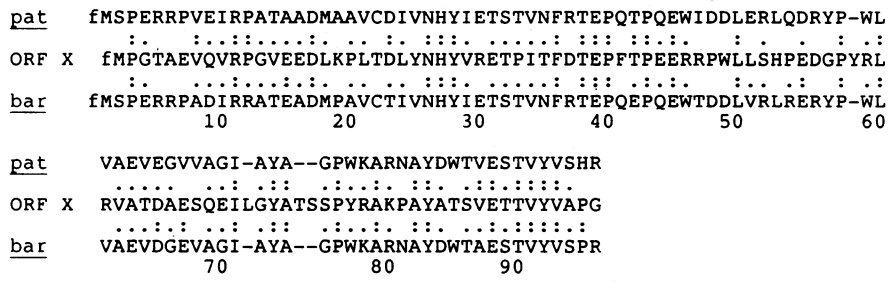


FIG. 7. Comparisons of the amino termini of the predicted products of ORF X and the *bar* and *pat* genes of *S. hygroscopicus* and *S. viridochromogenes*, respectively.

by using a two-step procedure that progresses across the G+C spectrum via species of genera of intermediate G+C content such as *Myxococcus* (69 mol% G+C), *Serratia* (60 mol% G+C) or *Pseudomonas* (67 mol% G+C) (45).

In gene disruption experiments with the *Streptomyces* phage φC31, *hrdC* and *hrdD* mutants were readily obtainable. They showed no obvious abnormalities in morphology, differentiation, or antibiotic production. Detailed analyses of the effects of the mutations in *hrdC* and *hrdD* are now in progress. In comparable phage-mediated gene disruption experiments, Takahashi and co-workers have demonstrated that *hrdA* mutants are viable and also have no obvious phenotype (H. Takahashi, personal communication [12]). In contrast, we were unable to isolate mutants in which φC31 had disrupted the *hrdB* gene. One interpretation of our data is that disruption of *hrdB* is lethal. This interpretation is consistent with the observation that, of the four *hrd* genes, only *hrdB* is present in all of the *Streptomyces* species so far examined; for example, *hrdC* is not present in *Streptomyces griseosporeus*, and neither *hrdA* nor *hrdC* is present in *S. griseus* (56). If *hrdB* is an essential gene in *S. coelicolor* A3(2), then it seems likely that $\sigma^{hrdB}$ is the functional homolog of the $\sigma^{70}$ of *E. coli*, responsible for the transcription of the majority of housekeeping genes in the cell. Alternative explanations for the failure to disrupt *hrdB* are possible. Conceivably, $\sigma^{hrdB}$ is responsible for transcription of both of the genes (*tsr* and *vio*) that were independently used in attempts to select for insertions in *hrdB* (an unlikely scenario in view of the unusual sequences of the *tsr* promoters [32a]). A more plausible possibility is that $\sigma^{hrdB}$ might be responsible for transcription of the φC31 repressor gene (*c*), such that φC31 would be unable to maintain lysogeny in *hrdB* mutants. Regardless of the details of interpretation, the *hrdB* disruption experiments illustrate the important point that the σ factors encoded by the other three *hrd* genes are unable to replace $\sigma^{hrdB}$ functionally in the cell.

Given the close similarity of each of the four *hrd* gene products to the $\sigma^{70}$ polypeptide of *E. coli*, especially in regions believed to be important in the recognition of the −10 and −35 regions of promoters (12, 57), it seems likely that they direct core RNA polymerase to transcribe from promoters with a strong similarity to the *E. coli* consensus sequence. Westpheling et al. (62) described an *S. coelicolor* holoenzyme, $E\sigma^{35}$, that transcribes from the *veg* promoter of *B. subtilis* and may also be responsible for transcription from the *dagAp4* promoter of *S. coelicolor* (13). Given that the *veg* promoter (43) conforms closely to the *E. coli* consensus sequence, it seems highly likely that $\sigma^{35}$ is encoded by one of the *hrd* genes. Here, we have shown that at least two of the *hrd* genes are expressed in mRNA. Recently, biochemical evidence has been obtained that suggests that more than one of the *hrd* genes is expressed in protein (J. Westpheling and M. Brawner, personal communication). In analyzing the in vitro transcription of several promoters, they demonstrated a clear separation of the RNA polymerase holoenzymes responsible for directing transcription from two *E. coli* consensuslike promoters, *veg* (from *B. subtilis* [43]) and *XP55* (the *XP55* gene encodes a major secreted protein of *S. lividans* [10]). The *veg* and *XP55* promoters differ from each other by only one base in the −10 region and one base in the −35 region, both having a spacing of 17 bp. There is no direct evidence to suggest that these two biochemically defined transcribing activities correspond to holoenzymes containing *hrd*-encoded σ factors. However, the availability of *hrdA*, *hrdC*, and *hrdD* mutants in which to test the activities of the *veg* and *XP55* promoters in vivo and from which to isolate RNA polymerase for in vitro transcription studies may shortly provide such a connection.

In this paper we describe a general method for gene disruption and gene replacement in *Streptomyces* species by using the phage φC31. The method permits the insertion of an antibiotic resistance determinant (or any other selectable marker) into the target gene and results in a mutant devoid of phage. This has at least two advantages over the conventional mutational cloning procedure: (i) φC31-derived vectors can be used subsequently in the in vivo analysis of the resulting mutant, and (ii) the mutation should, in principle, be completely stable. Several methods for gene replacement have been described by which mutations carried on phage or plasmid vectors have been introduced into *Streptomyces* chromosomal genes (1, 20, 47). However, these protocols relied upon the detection of a predictable mutant phenotype, either bald colonies, β-galactosidase deficiency, or the loss of antibiotic production. The use of the *glk* gene as the basis of a positive selection system permits the selection of the desired mutants even in the absence of a predictable or easily detectable mutant phenotype. However, the use of the *glk* gene requires that the mutant is first generated in a *glk* deletion strain, such that the mutation must be crossed subsequently into a $Glk^+$ background if that character is likely to affect further analysis. Since the counterselectable *S. coelicolor* genes for galactose kinase (22, 35) and sulfate utilization (38) have also been cloned, it should be possible to develop analogous positive selection systems for gene replacement based on 2-deoxygalactose resistance or selenate resistance.

Nucleotide sequencing of the *hrdD* promoter region showed that the *hrdDp1* promoter is internal to a gene (ORF X) on the opposite strand. This gene is predicted to encode a protein homologous with the products of the *bar* (59) and *pat* (63) genes of *S. hygroscopicus* and *S. viridochromogenes*, respectively, which encode phosphinothricin acetyltransferases that act as biosynthetic enzymes in the bioalaphos pathway and which are very likely also to confer resistance to the herbicidal antibiotic on the producing organism. Although transcription of ORF X has not been examined, any of its transcripts must overlap with the transcript originating at *hrdDp1* by at least 155 bases. The presence of a strong potential ribosome-binding site in front of the ORF X gene indicates that this overlap is likely to be greater. Since the start codon of ORF X and the −35 region of the *hrdDp2* promoter are only about 55 bp apart, it is also likely that the *hrdDp2* promoter region overlaps with the ORF X promoter region or with the transcripts originating from it. This situation is similar to the divergent and often overlapping transcription patterns observed around the promoter regions of several antibiotic resistance genes and the putative antibiotic biosynthetic genes that occur on the opposite strand. These include the genes for erythromycin resistance (*ermE*) in *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*) (8, 9), streptomycin phosphotransferase (*sph*) in *Streptomyces glaucescens* (60), methylenomycin resistance (*mmr*) in *S. coelicolor* (19), and aminoglycoside phosphotransferase (*aph*) in *Streptomyces fradiae* (33). It has been suggested that the transcriptional arrangement of these genes may permit coordination of the expression of the antibiotic biosynthesis and resistance genes (19, 29). From our data we speculate that the regulation and function of *hrdD* and ORF X may also be interconnected. If this is the case then *hrdD* disruption might perhaps lead to changes in the expression of ORF X if $\sigma^{hrdD}$ is involved in the transcription of ORF X. Moreover, the sequencing of

more genes around *hrdD* and the identification of the substrate of the ORF X gene product may help to identify the biological role of $\sigma^{hrdD}$.


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