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Cloning of *whiG*, a Gene Critical for Sporulation of *Streptomyces coelicolor* A3(2)

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In *whiG* mutants of *Streptomyces coelicolor* A3(2), aerial hyphae do not show any sign of sporulation. A library of *S. coelicolor* DNA was prepared in a φC31 temperate phage vector (KC516), and one recombinant phage (KC750) that could restore the wild-type phenotype to a collection of *whiG* mutants when integrated into their genomes was found. Subcloning experiments with low- and high-copy-number *Streptomyces* plasmid vectors allowed partial localization of *whiG* in the cloned DNA and revealed that hypersporulation was associated with the presence of extra copies of *whiG*.

Members of the genus *Streptomyces* are gram-positive soil bacteria with a mycelial growth habit. Dispersal of streptomycetes growing on solid substrates is achieved by the metamorphosis of aerial hyphae into chains of uninucleate spores, a process involving the regular ingrowth of specialized sporulation septa and the formation of thickened cell walls (5). In *Streptomyces coelicolor* A3(2), at least eight genes (*whiA* to *whiI*) are involved in this metamorphosis. These genes were initially identified by genetic mapping (3) of *whi* mutations, which cause the surfaces of colonies to remain white upon prolonged incubation (12) instead of turning grey, as wild-type colony surfaces do during spore formation. Mutants for one of these genes, *whiG*, form abundant aerial hyphae, but these show no signs of further development (3, 18). A representative *whiG* mutation was subsequently found to be epistatic to representative *whiA*, *whiB*, *whiH*, and *whiI* mutations (4). Thus, *whiG* may be a key gene in triggering the onset of sporulation in aerial hyphae. Further studies would be greatly helped by the cloning of *whiG*.

To allow the cloning of developmental genes (such as *whiG*) at a low copy number, thereby avoiding potentially detrimental effects of placing such genes on multicopy plasmid vectors, a series of vectors has been developed from the temperate phage φC31 (10, 22). One of these, KC401 (10), was successfully used to clone the *bldA* (21) and *bldB* (J. M. Piret and K. F. Chater, unpublished results) genes of *S. coelicolor*, which are required for the formation of aerial hyphae (19). Here we report the use of another of the vectors, KC516 (22), to clone the *whiG* gene.

MATERIALS AND METHODS

Bacteria. The host for transfections was *Streptomyces lividans* 66 (14). *S. coelicolor* A3(2) derivatives were as follows: J1501 (*hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$ Pgl$^-$) (6) (the SCP2$^-$ status of J1501 facilitated the use of plasmid vectors based on SCP2 [16], and its Pgl$^-$ status [loss of phage growth limitation; 8] made it easier to use φC31-based vectors [22]); J1820, a *whiG71* derivative of J1501 (see Results); M145 (prototropic; SCP1$^-$ SCP2$^-$); J118 (*cysD18 strA1 whiG71* NF) (3); DL91 and DL93, spontaneous cysteine-requiring mutants of J1501 (D. J. Lydiate, personal communication); and a collection of *whiG* mutants isolated (12) from prototrophic wild-type *S. coelicolor* A3(2) and subsequently genetically analyzed (3). An *Escherichia coli* K-12 F$^-$ Z$^-$ ΔM15 *recA* strain (23) was used for subcloning experiments involving *E. coli* plasmid vectors.

Media and growth conditions. R2YE medium (11) was used for routine stock cultures, for the preparation of spores, and for protoplast regeneration. YEME medium (11) was used for liquid cultures for protoplast preparation. For optimal analysis of colony morphology, minimal medium (MM) (11) was supplemented with mannitol (0.5%) in place of glucose. Unless specified otherwise, thiostrepton (50 μg/ml) or hygromycin (200 μg/ml) was used to select transformants.

Phages. The *attP*-deleted φC31-derived vector KC516 (22) is shown in Fig. 1. Conditions for its assay, propagation, DNA isolation, and use in transfection were as described previously (11). The *attP*$^+$ *vph*-containing phage KC300 (φC31 *c*$^+$ΔMΔ23::pBR322 ΔW12::*vph*) (7) was used for *attP*-mediated lysogenization (11). Selection for phage integration used the resistance genes present in the phage; thiostrepton (50 μg/ml) or viomycin (40 μg/ml) was used as appropriate.

***Streptomyces* plasmids and transformation.** The *Streptomyces* plasmid vectors pIJ702 (13), pSCP103 (2), and pIJ941 (16) were prepared and used in cloning and transformation experiments as described previously (11). Mycelial cultures of the *whiG* mutant J1820 to be used in protoplast preparation were prepared as follows. J1820 aerial hyphal fragments were spread on a sterile dialysis membrane on R2YE medium and incubated at 30°C for 36 h. The resulting growth was scraped off with a spatula, suspended in 2 ml of 10.3% sucrose, and inoculated into two baffled 250-ml flasks containing 25 ml of YEME. These were incubated at 30°C on a rotary shaker for 2 days, and the resulting mycelium was used to produce protoplasts (11).

***E. coli* plasmids and transformation.** The *E. coli* plasmid vector pIJ2921 is a derivative of pUC18 (20) which was constructed by G. Janssen (personal communication) and which contains the polylinker region flanked by *Bgl*II sites. *E. coli* plasmid isolation and transformation were done as described previously (17).

In vitro manipulation of DNA. Standard procedures (11, 17) were used for restriction enzyme digestion, agarose gel electrophoresis, electroelution of DNA from agarose gels,

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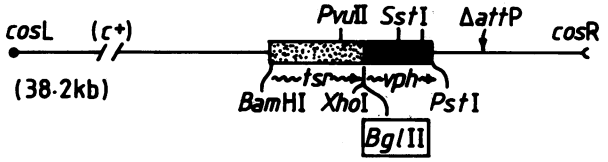

FIG. 1. Schematic representation of the φC31 cloning vector KC516. Sites for the restriction enzymes shown are absent from the rest of the DNA and can be used for cloning. The *Bgl*II site (boxed) was used in this paper for the cloning of *S. coelicolor* DNA. The *tsr* gene, encoding thiostrepton resistance, was used for the selection of lysogens carrying KC516 derivatives. (The *vph* gene, encoding viomycin resistance, was not used in the primary selection of lysogens because its expression is influenced by the nature of the DNA cloned in the *Bgl*II site [22].)

ligation, treatment with calf intestine alkaline phosphatase, end-filling with the Klenow fragment of DNA polymerase I, Southern blotting, nick translation, filter hybridization, and autoradiography.

Transduction of *S. coelicolor whiG* mutants. Replica plating of plaques from a library of *S. coelicolor* DNA on suitable indicator strains, as done in previous shotgun cloning experiments (6, 21), yielded very few transductants in our experiments with *S. coelicolor whi* mutants, partly because the morphological abnormalities of the *whi* mutants meant that only low numbers of plating units were obtainable and partly because the mutants were all Pgl^+ (i.e., did not allow extensive φC31 propagation) (8). After testing various conditions, we used the following two methods. In method 1, used in primary cloning experiments, aerial mycelium of the relevant *whi* mutant (harvested by scraping in sterile distilled water and stored in 20% glycerol at −20°C) was inoculated into test tubes containing 5 ml of YEME medium. The hyphal fragments were incubated at 30°C for 6 h on a rotary shaker (200 rpm). The actively growing hyphae were collected by centrifugation (bench centrifuge) and suspended in water before being mixed with a sample (about 10^7 PFU) from a phage library of *S. coelicolor* DNA and spread on R2YE. After 4 to 6 days of growth, the aerial growth of the cultures was harvested and replated on R2YE containing thiostrepton at the usual concentration (50 μg/ml). Colonies that grew were replica plated on MM containing only 5 μg of thiostrepton per ml. On this medium, colony morphology was readily scored. In method 2, used for transduction with defined phage constructs, a suspension of aerial hyphal fragments was subjected to several cycles of sedimentation (with a bench centrifuge) and suspension in water and then spread on R2YE plates with a phage suspension (about 10^7 PFU). Subsequent stages were as described for method 1.

Construction of a library of *S. coelicolor* DNA in a phage vector. DNA isolated from *S. coelicolor* M145 was partially digested with *Sau*3AI and size fractionated on a sucrose gradient (11). About 1.2 μg of DNA (1.8 to 8 kilobases) was ligated with 4.8 μg of KC516 DNA which had been cleaved with *Bgl*II and treated with calf intestine alkaline phosphatase (final DNA concentration, 200 μg/ml). The DNA was introduced into *S. lividans* protoplasts by the liposome- and polyethylene glycol-assisted procedure (11). Each transfection used 1.8 μg of DNA, and the protoplasts were distributed undiluted in six soft R2YE agar overlays containing *S. lividans* spores (11); 10^{-1} and 10^{-2} dilutions were also plated. The resulting libraries of plaques were harvested from the undiluted platings by adding 4 ml of nutrient broth (Difco Laboratories) to each plate and recovering the fluid after 2 h at room temperature. Each phage suspension was filter sterilized and stored at 4°C. The fraction of plaques containing inserts was calculated by replica plating of the 10^{-1} and 10^{-2} dilutions on R2YE plates spread with 10^7 spores of *S. coelicolor* DL91 or DL93. Plaques of phages with inserts gave rise to thiostrepton-resistant ($Thio^r$) transductants when the transduction mixtures were further replica plated on R2YE plus thiostrepton (exploiting insert-directed integration of the *attP*-deleted vector).

Microscopy. Impression preparations and cover slip cultures were used in phase-contrast microscopy as described previously (3).

RESULTS

Identification of a clone carrying the *whiG* gene. In two transfections, each with 1.8 μg of the ligation mixture, ca. 8,000 and ca. 5,300 plaques were obtained. About 67% of the plaques contained phages with inserts, as judged by the insert-directed transduction test with DL91 or DL93. The libraries were therefore expected to be fairly complete (assuming a *Streptomyces* genome size of 5×10^4 kb) (9). Indeed, several plaques carrying wild-type alleles for each of the three auxotrophic mutations in DL91 or DL93 were found.

To identify *whiG*-containing phages, we used a sample of the pooled libraries to transduce hyphal fragments of a *whiG* mutant (J118) to $Thio^r$ as described in Materials and Methods. Colonies were then replicated on MM containing mannitol and a low concentration of thiostrepton (5 μg/ml) to allow the scoring of colony morphology. Eight grey colonies were detected. These retained the cysteine requirement of J118. Phages recovered from each colony were purified by single-plaque isolation. DNA was prepared from a lysate of each phage and subjected to restriction analysis. All eight phages contained, in the same orientation, an identical insert of 5.2 kb, about 1 kb greater than the anticipated maximum size for inserts in KC516 (22). The phages were probably clonal in origin, and a representative one was termed KC750. All were able to retransduce J118 to yield $Thio^r$ colonies, most of which were light grey, like wild-type *S. coelicolor*. However, noticeable minorities of white and darker grey colonies were also observed (Fig. 2). We interpreted this as follows. The light grey colonies were probably the expected heterogenotes, carrying one wild-type and one mutant *whiG* allele (Fig. 3). The white colonies were either *whiG71* homogenotes, perhaps resulting from a gene conversion event associated with recombinational integration of KC750 (21, 25), or were the result of a deletion affecting the cloned *whiG* gene in KC750. The dark grey colonies were probably $whiG^+$ homogenotes, containing two copies of *whiG*, implying a striking gene dosage effect on colony morphology (see

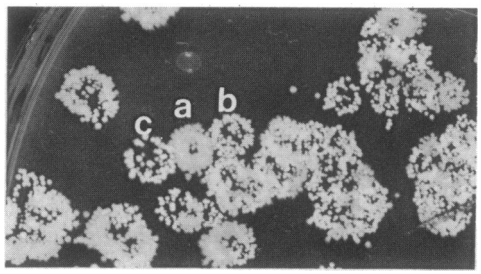

FIG. 2. Replica-plated colonies of the *whiG* mutant J118 after transduction with φC31 KC750. Most colonies were light grey (resembling wild-type *S. coelicolor*) (b). A few were darker grey (a) or white (c). A plausible explanation is given in Fig. 3.

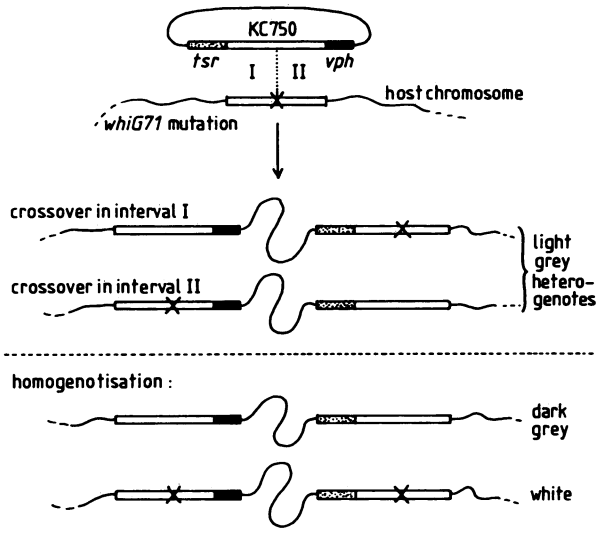

FIG. 3. Interaction of KC750 with the *whiG* region of strain J118. Heterogenotes (light grey colonies) were generated by crossovers in interval I or II. Homogenotes (*whi*$^+$/*whi*$^+$, dark grey; or *whi*/*whi*, white) were also found. Phages released from *whi*$^+$/*whi* or *whi*/*whi* colonies may contain the *whiG* mutant allele and can be used to generate *whi*/*whi* homogenotes upon introduction into a *whi*$^+$ strain (see the text).

below). (In agreement with this, lysogenization of a *whiG*$^+$ strain [J1501] by KC750 also produced mostly darker grey colonies, although some light grey colonies were also found with some KC750 preparations, presumably because of occasional deletions of the cloned *whiG* gene from the phage; see the section below on the stability of KC750.)

Another explanation for the occurrence of white transductants of J118 could be that KC750 contained only part of *whiG*, such that an intact wild-type gene could sometimes be generated during the recombinational integration of the phage (21). To eliminate this possibility, we integrated KC750 into a different region of the genome of a *whiG* mutant; a lysogen of J118 was constructed by using the *attP*$^+$ phage KC300 and then superinfected with KC750. In these circumstances, about 90% of the resulting double lysogens were expected to contain KC750 integrated into the KC300 prophage. Since the great majority of transductants were light grey, the cloned gene was confirmed to be intact and dominant over *whiG71*.

"Complementation" of other *whiG* mutations by KC750. Coarse genetic mapping by conjugation (3) suggested that a number of other *whi* mutants contained lesions in the same gene or gene cluster as *whiG71*. Twelve of these mutants (carrying *whiG* alleles *10*, *60*, *64*, *65*, *75*, *97*, *100*, *103*, *125*, *148*, *172*, and *209*) were transduced to Thio^r by KC750. In each case, a high proportion of the colonies were grey, confirming the genetic mapping results.

Stability of KC750. The 5.2-kb insert in KC750 resulted in a phage genome size of 43.4 kb, about 105% of that of wild-type ϕC31. Presumably because of this overpackaging, deletions were common in KC750 populations, particularly among the survivors in old phage preparations. This instability introduced uncertainty into the interpretation of some experiments and necessitated the use of phages from fresh single plaques and checking for the correct DNA structure and for complementation of *whiG71*. To allow detailed analysis of the cloned DNA (Fig. 4) and for the preparation of probes, we therefore subcloned the entire fragment (and part of the *vph* gene) into pIJ2921 (a pUC18 derivative in which the polylinker is flanked by *Bgl*II sites [Fig. 5; G. Janssen, personal communication]) to yield pIJ4400.

Southern blotting of *whiG* mutants. Samples of total DNA isolated from *S. coelicolor* M145 (*whiG*$^+$), from J118 (carrying *whiG71*), and from *whiG* mutant derivatives of strain A3(2) (alleles *10*, *60*, *64*, *75*, *97*, *100*, *103*, and *209*) were digested with *Bam*HI and used in Southern blotting experiments with pIJ4400 as a probe. In every case but J118, hybridization to bands of 8.2 and 5.1 kb was seen. In J118, the 8.2-kb band was absent, and a band of 8.7 kb was seen instead. Southern blotting analysis of M145 and J118 DNAs digested with various other enzymes was used to construct a map of the chromosomal region around *whiG* (Fig. 4). The difference between J118 and M145 was localized to a 2.6-kb *Bam*HI-*Pvu*II interval situated several kilobases from the region corresponding to the cloned DNA. It was therefore unlikely to be responsible for the WhiG$^-$ phenotype. Thus, it appeared that the nine *whiG* mutants tested all contained point mutations (or very small deletions or insertions). There was no evidence to suggest that the cloned DNA in KC750 was rearranged as compared with the same region of the *S. coelicolor* chromosome.

Construction of J1820 (a *whiG71* derivative of J1501) by homogenotization. All of the available *whi* mutants are relatively inconvenient for use as recipients in cloning experiments for two reasons: they do not support ϕC31 plaque formation (the Pgl$^+$ phenotype) (8), and they contain SCP2, a low-copy-number plasmid (1, 24) from which useful cloning vectors have been derived (16). We therefore used a two-step procedure to introduce a defined *whiG* mutation (*whiG71*) into a more suitable genetic background. First, phages released spontaneously from a light grey J118:: KC750 (presumptively *whiG71*/*whiG*$^+$; see above) lysogen were collected, on the assumption that the recombination event allowing excision of the prophage from the *whiG* region would sometimes result in acquisition of the mutant allele by the phage. Isolated phages were then used to transduce the *whiG*$^+$ Pgl$^-$ SCP2$^-$ strain J1501, yielding Thio^r transductants. Among these, a few proved to have the WhiG$^-$ phenotype, presumptively because they had been transduced by a phage carrying the *whiG71* allele, with

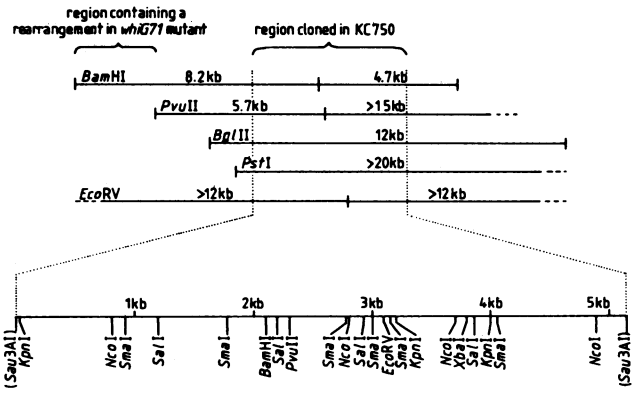

FIG. 4. Restriction map of the *S. coelicolor* DNA fragment cloned in KC750 and positions of restriction sites flanking it in the *S. coelicolor* chromosome. Flanking sites were determined by Southern blotting with ^{32}P-labeled pIJ4400 DNA as a probe. The approximate position of a DNA rearrangement present in the J118 chromosome is indicated.

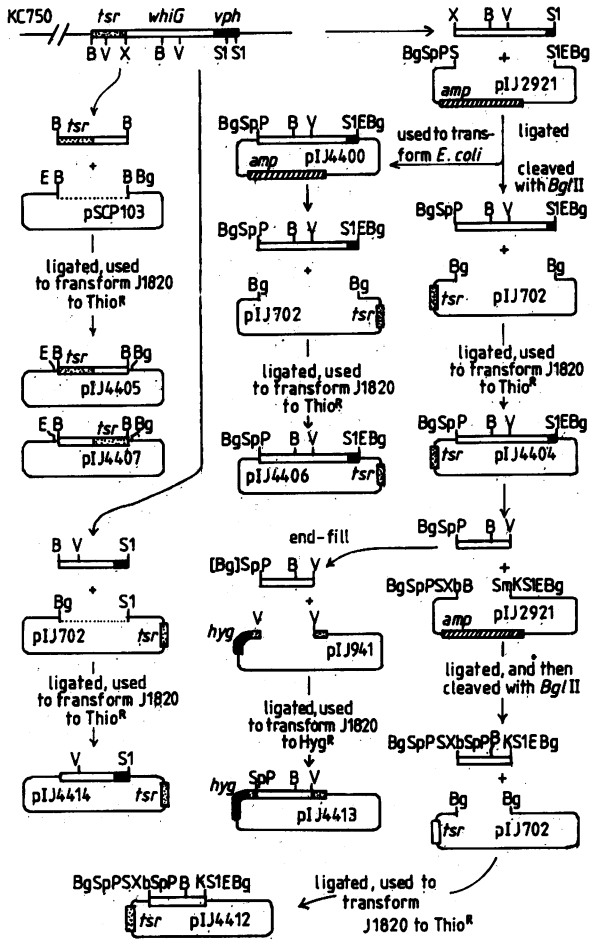

FIG. 5. Subcloning of the cloned *S. coelicolor* fragment from KC750.

homogenotization during the transduction to yield a *whiG71/whiG71* genotype. Restreaking of these colonies on medium lacking thiostrepton allowed the growth of rare Thio^r colonies that arose through the recombinational loss of the integrated phage (6). These were recognized by replica plating. One such colony (J1820) was used in most of the experiments described below. The *whiG* genotype of J1820 was verified as follows: J1820 was transduced by KC750 to yield a mixture of white, light grey, and dark grey colonies, and transduction of J1820 by a KC750 derivative released from a Whi$^-$ J118::KC750 colony (presumptively a *whiG71/whiG71* homogenote) yielded only Whi$^-$ colonies. The morphology of the aerial parts of J1820 colonies was typical of *whiG* mutants (determined by phase-contrast microscopy of impression preparations).

Recombination and complementation tests between *whiG* alleles. Using homogenotization, we prepared KC750 derivatives presumptively carrying each of nine other *whiG* alleles. They were used to transduce the *whiG71* mutant J1820, and the relative proportions of Whi$^-$ and Whi$^+$ colonies were examined. With *whiG10*- and *whiG65*-containing phages, all transductants were Whi$^-$. Rare Whi$^+$ colonies were seen when the transducing phage contained *whiG60*, *whiG64*, or *whiG100*, with a somewhat higher frequency for *whiG125* and *whiG148* and particularly for *whiG75* and *whiG172*. Although this experiment was not quantitative, because of the means by which the transductions were carried out (mixed growth of the host and phage followed by harvesting and replating on thiostrepton-containing medium), it indicated that the *whiG* alleles *10*, *60*, *64*, *65*, *71*, *100*, *125*, and *148* fell into a single complementation group (otherwise the great majority of colonies would have been grey). However, it remains possible that *whiG75* and *whiG172* fall outside this complementation group.

Localization of *whiG* in the cloned DNA by subcloning. Subcloning analysis of the cloned DNA to localize *whiG* was done in low- and high-copy-number *Streptomyces* plasmid vectors (Fig. 5 and Table 1). To avoid the restriction barrier between *E. coli* and *S. coelicolor*, we did these experiments entirely in *S. coelicolor*. (Nevertheless, a polylinker region from the *E. coli* plasmid pIJ2921 could still be added in vitro to the *Xho*I-*Sst*I fragment of KC750, allowing its insertion as a *Bgl*II fragment into the *Bgl*II site of pIJ702 to yield pIJ4404 [Fig. 5]; a similar strategy was used in the construction of pIJ4412.) Table 1 shows the results of introducing the various fragments into the *whiG* mutant J1820. Complementation was not observed with clones generated by cleavage at the *Bam*HI site, indicating that this site lay within or close to *whiG*. In the cases of pIJ4405 and pIJ4407, both of which carry the region to the left of the *Bam*HI site, a few light grey colonies were obtained when the aerial parts of the white primary transformant colonies were harvested and replated. These grey colonies were presumed to be the result of recombination between the mutant chromosomal gene and the partial *whiG* gene on the plasmids.

Effects of *whiG* copy number on differentiation. The various cloning and subcloning experiments in this work used vectors with low and high copy numbers, leading to changes in the *whiG* copy number (the relevant vector copy numbers were as follows: 1 for prophages; 1 to 2 [1] for pSCP103 and

TABLE 1. Results of subcloning the *whiG* region into low- and high-copy-number plasmids

Plasmid[a] present in J1820	Copy no.	Subcloned fragment[b]	Appearance of colonies	Interpretation
pIJ4404 and pIJ4406	High	Whole cloned region	Small, very dark grey	Entire *whiG* gene cloned; effects of increasing copy number seen
pIJ4412	High	Region to left of *Eco*RV site	Small, very dark grey	
pIJ4413	Low	Region to left of *Eco*RV site	Dark grey	
pIJ4405 and pIJ4407	Low	Region to left of *Bam*HI site	White, rare light grey	Part of *whiG* gene cloned containing wild-type sequence corresponding to *whiG71* allele
pIJ4414	High	Region to right of *Bam*HI site	White only	Subclone does not contain intact *whiG* gene

[a] See Fig. 5 for details of plasmid construction.
[b] See Fig. 4 for restriction map of the cloned *whiG* region.

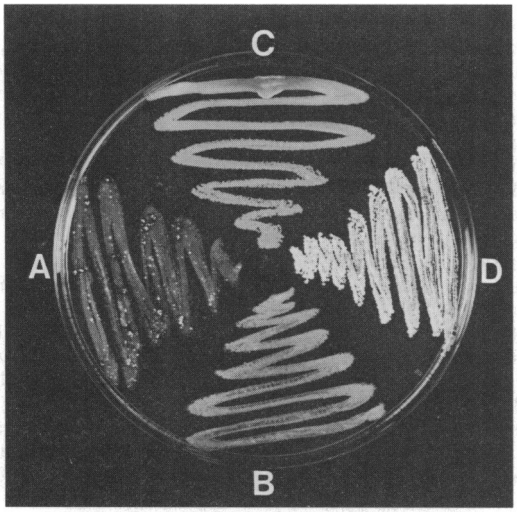

FIG. 6. Effect of introducing *whiG*$^+$ on plasmids into a *whiG* mutant. Transformants containing the high-copy-number plasmid pIJ4412 (A) were generally very dark grey and grew poorly, but occasional more-vigorous white outgrowths were seen. At a low *whiG* copy number (with plasmid pIJ4413) (B), the transformants were slightly darker grey than was a *whiG*$^+$ control strain (J1501) (C). The *whiG* mutant host strain (J1820) is also shown (D).

pIJ941; and 40 to 300 [13] for pIJ702). Striking effects of *whiG* copy number on colony morphology were observed (Fig. 2 and 6 and Table 1). The addition of one copy or a few extra copies of *whiG* caused the relatively early development of grey colonies, and the colonies became noticeably darker grey than with the *whiG*$^+$ strain J1501. When *whiG* was present on a high-copy-number plasmid, the grey color was even darker, and there was a pronounced effect on primary growth; colonies were smaller and flatter than usual. Faster-growing outgrowths were obtained from some colonies (Fig. 6). When J1820 was the host, these outgrowths were white, and when J1501 was the host, they were pale grey. We assume that these outgrowths contained mutant or deleted plasmids lacking a functional *whiG* gene and that the deleterious high-copy-number effects were the result of overproduction (or premature production) of an active *whiG* gene product.

The dark grey color was shown by phase-contrast microscopy to be correlated with early and abundant sporulation. Branched spore chains were frequent (Fig. 7). These were not seen with J1501 or J1501(pIJ702), and we have never previously observed them in *S. coelicolor*. No other abnormalities were seen.

The presence of *whiG* at a high copy number also influenced secondary metabolism. Whereas J1501(pIJ702) colonies on MM containing mannitol and a low concentration of thiostrepton (5 μg/ml) produced the red-blue antibiotic actinorhodine characteristic of *S. coelicolor*, J1501(pIJ4412) colonies produced little red or blue pigment, except when there were rapidly growing outgrowths.

DISCUSSION

Several pieces of evidence indicate that the recombinant phage KC750 carries the wild-type *whiG* gene rather than a suppressor of some kind: (i) all available mutants defective in *whiG* were restored to the Whi$^+$ phenotype by lysogenization with KC750; (ii) mutations in *whiG* could be transferred from the chromosome to the KC750 genome by recombination and from the KC750 genome into the host chromosome; and (iii) when several copies of the *whiG* gene were present, hypersporulation was observed.

Subcloning experiments localized *whiG* to a 3.1-kb segment of DNA and suggested that the single *Bam*HI site in this segment was internal to the gene. (One alternative hypothesis, that the *Bam*HI site is upstream of *whiG* in a polycistronic transcript, is unlikely, because at least one of the plasmids pIJ4405, pIJ4407, and pIJ4414 should have allowed expression by transcriptional readthrough from a vector promoter[s]). The *whiG71* mutation is apparently located close to and to the left of this *Bam*HI site.

The hypersporulation observed when additional copies of *whiG* were present suggests that *whiG* plays an important regulatory role in sporulation. Remarkably, it appears that overproduction of the *whiG* gene product not only promotes hypersporulation but also inhibits growth and the production of the antibiotic actinorhodine. These latter effects could be structural; more hyphae may be "sequestered" for sporulation and at an earlier stage (suggesting that sporulating hyphae do not make actinorhodine). Alternatively, a choice

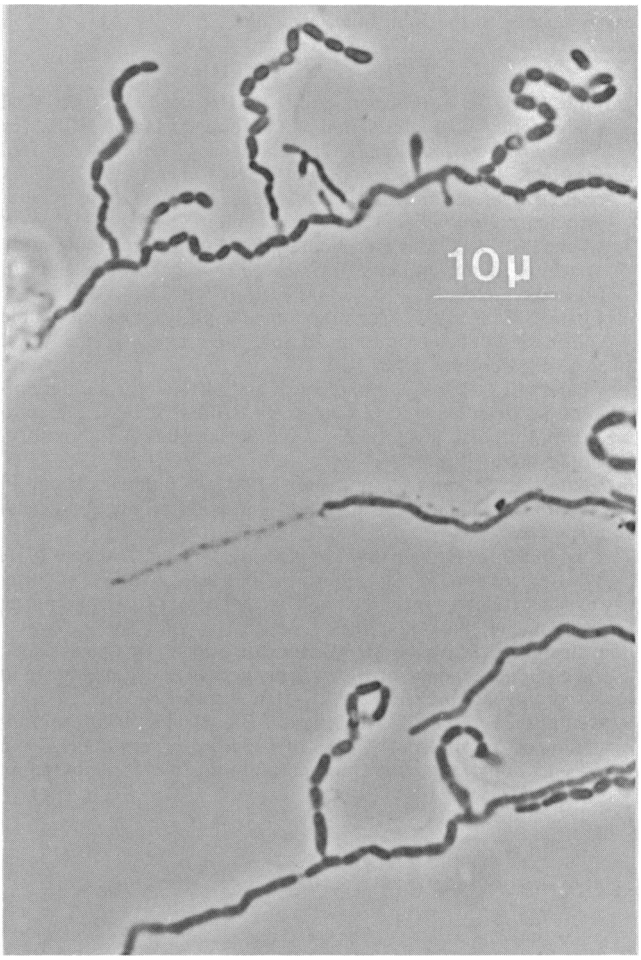

FIG. 7. Branched spore chains produced during surface growth of J1501(pIJ4412). The sample was examined by phase-contrast microscopy after 52 h of cover slip culturing (3) on MM containing mannitol. The photograph is typical of the upper parts of the culture. Little sporulation was observed in control cultures of J1501(pIJ702) or J1501 at 52 h. μ, Micrometers.

among alternative courses (growth, sporulation, or antibiotic production) may be importantly influenced at the physiological level by the concentration of the *whiG* gene product. Analogies might be found in the role of the concentration of the *cII* gene product of phage lambda in influencing the lysis or lysogeny decision (26) or in the accumulation of new RNA polymerase sigma factors during phage development or bacterial sporulation, leading to the redirection of the transcriptional apparatus (15).

The sequence of *whiG*, currently being determined, may help to reveal the function of this key developmental gene. Moreover, the availability of the cloned gene will allow studies of the temporal and spatial control of *whiG* expression in *S. coelicolor*, a multicellular, differentiating procaryote.

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