An Unusual Response Regulator Influences Sporulation at Early and Late Stages in *Streptomyces coelicolor*^{▽†}

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WhiI, a regulator required for efficient sporulation septation in the aerial mycelium of *Streptomyces coelicolor*, resembles response regulators of bacterial two-component systems but lacks some conserved features of typical phosphorylation pockets. Four amino acids of the abnormal “phosphorylation pocket” were changed by site-directed mutagenesis. Unlike *whiI* null mutations, these point mutations did not interfere with sporulation septation but had various effects on spore maturation. Transcriptome analysis was used to compare gene expression in the wild-type strain, a D27A mutant (pale gray spores), a D69E mutant (wild-type spores), and a null mutant (white aerial mycelium, no spores) (a new variant of PCR targeting was used to introduce the point mutations into the chromosomal copy of *whiI*). The results revealed 45 genes that were affected by the deletion of *whiI*. Many of these showed increased expression in the wild type at the time when aerial growth and development were taking place. About half of them showed reduced expression in the null mutant, and about half showed increased expression. Some, but not all, of these 45 genes were also affected by the D27A mutation, and a few were affected by the D69E mutation. The results were consistent with a model in which WhiI acts differently at sequential stages of development. Consideration of the functions of *whiI*-influenced genes provides some insights into the physiology of aerial hyphae. Mutation of seven *whiI*-influenced genes revealed that three of them play roles in spore maturation.

Streptomyces are filamentous gram-positive soil bacteria that produce many useful antibiotics. Their vegetative substrate mycelia grow across and into agar media and contain relatively few cross walls. Older colonies produce a white aerial mycelium that grows vertically away from the substrate mycelium. Long chains of spores eventually form at the aerial hyphal tips. The spores are gray in the model species *Streptomyces coelicolor*. A number of developmental regulatory genes are specifically required for sporulation to take place in aerial hyphae of *S. coelicolor*. Mutations in many of these genes prevent the accumulation of the gray spore pigment, giving colonies that remain white upon prolonged incubation, hence the general designation “*whi*” for many of these genes (5).

The classification of *whi* genes as “early” and “late” sporulation genes depends on whether the relevant mutants undergo sporulation septation, the process by which the long multigenomic apical compartments of aerial hyphae become regularly and (within any one hypha) approximately synchronously divided into unigenomic prespore compartments. Thus, *whiABGHII* and *ssgB* are early genes, whereas *sigF*, *ssgA*, *ssgR*, and *whiD* are late genes. Where tested, most of the early genes are needed for the expres-

sion of late genes, and at least two independent early regulatory pathways (σ^{WhiG} dependent and σ^{WhiG} independent) converge to activate the late regulatory events (5).

The focus of the work described here is *whiI* (SCO6029 in the annotated genome sequence of *S. coelicolor*) (3). Transcription of *whiI* is directly and completely dependent on the sigma factor encoded by *whiG* (1). WhiI has an N-terminal domain similar to the well-characterized phosphorylation domains of response regulators in bacterial two-component systems and a putative helix-turn-helix DNA-binding domain at the C terminus (1). Despite relatively low amino acid identity (<30%), WhiI is predicted to be structurally similar to NarL of *Escherichia coli*, for which there is a high-resolution X-ray structure (1, 2). The putative phosphorylation domain of WhiI is unusual among response regulators in that the first of three highly conserved D residues is replaced by A, and the highly conserved K near the end of the domain is replaced by T. WhiI also contains a relatively long N-terminal extension. Unlike genes for typical two-component response regulators, *whiI* lacks a nearby cognate sensor kinase gene. The combination of this genetic and structural information suggests that changes of the active state of WhiI, if they occur, may be dependent on some atypical phosphorylation process or other posttranslational modification/activation mechanism rather than direct conventional phosphorylation by a cognate histidine sensor kinase.

In previous work, 7 of 15 mutagen-induced *whiI* alleles were found to cause single amino acid substitutions (1). Molecular modeling indicates that the mutant amino acids may all affect correct folding or the stability of the protein, either by changing the residue polarity or through side chain interference with spatially close amino acids (data not shown). To obtain func-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Parent strain	Reference
Strains			
<i>S. coelicolor</i> strains			
M145	Prototrophic; SCP1 ⁻ SCP2 ⁻ Pgl ⁺	A3(2)	23
J2450	M145 <i>whiI::hyg</i>	M145	1
J2660	J2450/pSET152:: <i>whiI</i>	J2450	This work
J2661	J2450/pSET152:: <i>whiI</i> (D27A)	J2450	This work
J2662	J2450/pSET152:: <i>whiI</i> (D69A)	J2450	This work
J2663	J2450/pSET152:: <i>whiI</i> (D69E)	J2450	This work
J2664	J2450/pSET152:: <i>whiI</i> (D27E)	J2450	This work
J2665	J2450/pSET152:: <i>whiI</i> (S96A)	J2450	This work
J2666	J2450/pSET152:: <i>whiI</i> (T118K)	J2450	This work
J2667	J2450/pSET152:: <i>whiI</i> (D27A D69A)	J2450	This work
J2670	M145 <i>whiI</i> ⁺	J2450	This work
J2671	M145 <i>whiI</i> (D27A)	J2450	This work
J2672	M145 <i>whiI</i> (D69A)	J2450	This work
J2673	M145 <i>whiI</i> (D69E)	J2450	This work
J2674	M145 <i>whiI</i> (T118K)	J2450	This work
J2675	M145 <i>whiI</i> (D27A D69A)	J2450	This work
J2676	M145 Δ <i>whiI</i>	J2450	This work
<i>E. coli</i> strains			
ET12567	<i>dam dcm hsdS cat tet</i>		25
BW25113	K-12 derivative; Δ <i>rhaBAD</i>		9
BT340	DH5 α /pCP20 (FLP recombination plasmid)		6
Plasmids			
pSET152	<i>oriT</i> RK2 <i>aac</i> (3) <i>IV</i> <i>int</i> ϕ C31		23
pIJ773	<i>aac</i> (3) <i>IV</i> <i>oriT</i>		17
pIJ778	<i>aadA</i> <i>oriT</i>		17
pIJ790	λ -RED (<i>gam bet exo</i>)		17
pUZ8002	<i>tra neo</i> RP4		33
SC1C3	<i>S. coelicolor</i> cosmid containing <i>whiI</i>		34
SCF12	<i>S. coelicolor</i> cosmid containing SCO0323		34
SC7H2	<i>S. coelicolor</i> cosmid containing SCO2008		34
SCD66	<i>S. coelicolor</i> cosmid containing SCO4172 and SCO4174		34
SC4A2	<i>S. coelicolor</i> cosmid containing SCO6375		34
2SC7G11	<i>S. coelicolor</i> cosmid containing SCO5249		34
SC7A1	<i>S. coelicolor</i> cosmid containing SCO5576		34

tional information about WhiI, therefore, new mutations were needed that were not expected to interfere with the protein folding process.

This paper shows that although WhiI has an early role in sporulation, the amino acids that correspond to residues conserved in phosphorylation pockets of typical response regulators are important mainly later in sporulation. This suggests that these residues are involved in the response of WhiI to a signal generated during development and that the resulting change in WhiI's state causes it to take on a role as a late sporulation regulator. Candidate stage-specific WhiI target genes were identified by DNA microarray analysis of selected *whiI* mutants, and gene disruption showed that some of these genes have late sporulation functions. We believe this to be the first evidence that a single response regulator-like protein exerts effects on different intermediate stages in bacterial development.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in this study are summarized in Table 1. Media and conditions for *Streptomyces* culture were as described by Kieser et al. (23). *S. coelicolor* strains were incu-

bated on MS (soya flour with mannitol) and MM (minimal medium containing 0.5% mannitol as the carbon source) agar media. For DNA extraction, *Streptomyces* strains were incubated in YEME (yeast extract-malt extract) liquid medium supplemented with glycine to 0.5%. The nonmethylating *E. coli* strain ET12567 containing pUZ8002 was used to mobilize unmodified plasmids or cosmids containing *oriT* from *E. coli* into *Streptomyces*, bypassing the methyl-sensing restriction system of *S. coelicolor* (23, 25). *E. coli* DH5 α was used for plasmid or cosmid construction and routine subcloning. Transformation of *E. coli* was carried out by electroporation as described previously (1).

Strategy for *whiI* mutagenesis and complementation with *whiI* mutant alleles in trans. Mutant versions of *whiI* were generated in *E. coli* by using a two-step PCR procedure with single codon changes in the intermediate primers (Table 2). Codon usage biased towards G+C was used for amino acid replacements (GAG for E, GCC for A, and AAG for K). Each mutant gene was confirmed by sequencing and cloned into the EcoRV site of pSET152, which can integrate efficiently into the *Streptomyces* chromosomal *attB* site for the ϕ C31 prophage. The cloned genes included the complete upstream intergenic region containing the *whiI* promoter, but they lacked the putative transcriptional terminator because the hairpin sequence could not be amplified by PCR. The seven mutant pSET152::*whiI* derivatives and wild-type *whiI* inserted into pSET152 were introduced by conjugation into the *whiI* null mutant J2450 (a derivative of the sequenced strain M145 containing a hygromycin resistance gene inserted into *whiI* between the phosphorylation and DNA-binding domains; J2450 showed a severe *whiI* phenotype, with white aerial mycelia and almost no sporulation septation) (1). For each of the seven mutant forms of *whiI*, at least four independent exconjugants were analyzed and found to be phenotypically identical in colony appearance and in sporulation, as visualized by phase-contrast microscopy.

PCR-targeted mutagenesis, including construction of *whiI* mutant alleles at the native *whiI* locus. Disruption and mutagenesis of genes in *S. coelicolor* were based on the PCR targeting method (9), as modified for *Streptomyces* (17, 18). Unless stated otherwise, pIJ773 was the template for PCR amplification of the cassette [*aac*(3)*IV-oriT*] used to disrupt the target genes in appropriate *S. coelicolor* cosmids in *E. coli* BW25113/pIJ790, which contains the arabinose-inducible λ -RED system (see Table 2 for PCR primers). Mutant cosmids were transferred into *E. coli* ET12567/pUZ8002 before conjugation into *S. coelicolor* M145 or its derivatives. Kanamycin-sensitive, apramycin-resistant double-crossover mutants with target genes replaced by the *aac*(3)*IV-oriT* cassette were confirmed by PCR amplification and Southern blot hybridization. For the disruption of potential WhiI target genes, the gene-specific extensions of PCR primers were designed to eliminate the whole of each target gene, excluding the translational start and stop codons, except when neighboring genes overlapped with the target genes, in which case the locations of the primers were shifted inwards in multiples of three nucleotides.

Mutations were created at the natural *whiI* chromosomal locus by using a modification of the PCR targeting method (Fig. 1). In outline, the *whiI* gene in cosmid SC1C3 (1, 34) was replaced by a cassette containing the selectable *aac*(3)*IV* gene and the counterselectable *sacB* gene, which confers sucrose sensitivity on the *E. coli* host strain [the *aac*(3)*IV-sacB* cassette was constructed by two-step PCR, using pIJ773 and *Bacillus subtilis* genomic DNA as the templates for amplifying *aac*(3)*IV* and *sacB*, respectively]. The cassette itself was then replaced by the desired mutant form of *whiI* by coelectroporating the *aac*(3)*IV-sacB*-containing cosmid, linearized via a unique restriction site in the cassette, together with the mutant *whiI* PCR product, selecting for carbenicillin and sucrose resistance; in these circumstances, the PCR product acted as a bridge for the recombinational recircularization of the cosmid. The kanamycin resistance determinant of the mutated cosmid was then replaced by an *aac*(3)*IV-oriT* cassette. The resulting cosmid was introduced by intergeneric conjugation into *S. coelicolor* J2450 (*whiI::hyg*). A normal frequency of apramycin-resistant transconjugants was obtained. In further propagation without apramycin selection, about 10% of progeny were sensitive to both hygromycin and apramycin, indicating that a second crossover had led to the loss of the Supercos 1 cosmid backbone and the replacement of *whiI::hyg* with the new mutant version of *whiI*. The mutant structures were confirmed by Southern hybridization, PCR amplification, and sequencing. The *whiI* null mutant (J2676) was made by PCR targeting but included subsequent removal of the apramycin resistance cassette by FLP recombinase in *E. coli* BT340 (17).

Complementation of SCO5576, SCO5249, and SCO0323 mutants. The primer pairs used to amplify SCO5576, SCO5249, and SCO0323 by PCR are listed in Table 2. For SCO5249 and SCO5576, after digestion with XbaI, PCR products with one blunt and one XbaI-cleaved end were ligated with the XbaI/EcoRV-digested integrative vector pSET152, and for SCO0323, the PCR product was directly inserted into pSET152 at the EcoRV site, and then the constructs were

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5' to 3') ^a
<i>whiI</i> mutagenesis primers	
IF (<i>whiI</i> forward outside primer)	CGTCGTTTCATCGTTCCGGGCAGGGG
IR (<i>whiI</i> reverse outside primer)	TCAGGTCAGTGAAGACCGGCTTCACGAGG
D27A(E) forward ^a	CGTGGCCG/(A)C/(G)CCGCGCGTCCGTTCCACCG
D27A(E) reverse ^a	CGCGCGGG/(C)G/(T)CGGCCACGACCACCATGGCGG
D69A(E) forward ^a	CGTCGCGGG/(A)C/(G)GTCCACCTTCCCGACGG
D69A(E) reverse ^a	GGTGGACG/(C)G/(T)CCGCGACGCAGATGTCGG
S96A forward	CGCCCTGGCCGCGCCGACGACATCGGC
S96A reverse	CGTCGCGGGCGGCAGGGCGAGCCCGTTGGGCC
T118K forward	CGTCGTCAAGGGCACCCGTACCAACGTC
T118K reverse	GGGTGCCCTTGACGACGTAGCCCTTG
<i>aac(3)IV-sacB</i> cassette primers	
IFAPR [<i>aac(3)IV</i> forward]	CGTCGTTTCATCGTTCCGGGCAGGGGGTCCGCACGTCCGGAGGGTTCATGTGCAGCTCCAT
SAH-APE [<i>aac(3)IV</i> reverse]	CTAAGAAGTGAAGTTCATGAGCTCAGCC
APE-SAH (<i>sacB</i> forward)	CATAAGTTCAGTCTTTAGGCCCGTAGTC
IRSAC (<i>sacB</i> reverse)	TCAGGTCAGTGAAGACCGGCTTCACGAGGGCGTTGTCGGATCGGCATTTTCTTTTGCCT
Primers for disrupting	
<i>WhiI</i> -dependent genes	
SCO0323F	TCCCTGCGGCTTCTACGAGACCGCTCCGACGCTACTATGTAGGCTGGAGCTGCTTC
SCO0323R	GGCCTGATCCTCGACCGGGTCGGACATCACGTGAGGTGATTCCGGGGATCCGTCGACC
SCO5249F	CGCAGCCGTCCGGCATGACCGGGAGGGCAATTCCTTATGATTCCGGGGATCCGTCGACC
SCO5249R	CGGGAGGGCGGGAACACGGCGTTCGGGACAGTGACGTATGAGGCTGGAGCTGCTTC
SCO5576F	GTGTCCCGCAGACATTCAGTGTAGGTTGACGTATGATTCCGGGGATCCGTCGACC
SCO5576R	GCTCCCGGCGCCCGCGGACCTCGCACGGAGCATCGTCATGTAGGCTGGAGCTGCTTC
SCO2008F	CGCGCTCGACTCGACCGCTCCACGGGGAAGCGGCCGTGATTCCGGGGATCCGTCGACC
SCO2008R	GCGCCCCGCGGGCTCGTGGGCGAGTGGTGGCGTCCGTCATGTAGGCTGGAGCTGCTTC
SCO4172F	CGACCGCTCGGCGAGACCGGGGTGGCGGTGTGACCGAGATTCCGGGGATCCGTCGACC
SCO4172R	AGGCCCTCGTCTACTGAGCGGTCCGCGCCACCCGATCATGTAGGCTGGAGCTGCTTC
SCO4174F	TTCGCCATGCCGAAGCGTAGCCCTCGCGGGCGGTGTCATGTAGGCTGGAGCTGCTTC
SCO4174R	GCGCCAGCACCAGCGCAAGGAGCAGGAGGATGACGTACATTCCGGGGATCCGTCGACC
SCO6375F	TGAGAGACGCTCAAGCAGTGCCTTGTCCCTGCGTCATGTAGGCTGGAGCTGCTTC
SCO6375R	GCCCAAGCCCTCCTCCACAGGGAACGAGGAATTGGTATGATTCCGGGGATCCGTCGACC
S1 mapping primers	
SCO0323c-forward	GAGCGCGGTGCACCGTGAAC
SCO0323c-reverse	CCGAGCGCCGAGTCGTC
SCO5249-forward	CGCGGACATGGGTGAGG
SCO5249-reverse	CGTGCCGAGGCTCTGCT
SCO6375-forward	CCCCAAGGCCACGACAA
SCO6375-reverse	AGCGACACGGGGAGGCT
Primers for amplification of target	
genes for complementation	
C0323-forward	GAGCGCGGTGCACCGTGAAC
C0323-reverse	CACGGCCCGACAGGTCTTCG
C5249-forward	CGCGGACATGGGTGAGG
C5249-reverse	GCTCTAGACATGGACTCACCTTCCCC
C5576-forward	GTCCGGAGCGAGCGGATC
C5576-reverse	GCTCTAGACGACTCCTGCGGGCCTCT
Primers for <i>aac(3)IV</i> change to	
<i>aadA</i> in the construction of	
pSET152::SCO0323 (SCO5249	
and SCO5576)	
P1	TGATGTCATCAGCGGTGGAGTGCAATGTCGTGCAATACGATGAGGGAAGCGGTGATCGC
P2	AGCGCGACCTTGCCCCCTCAACGTATCTCGTTCTCCGCTTATTGCGGACTACCTTGG

^a Primers designed for a change from aspartate to alanine. The parentheses in the sequence indicate the alternative nucleotide design for the change from aspartate to glutamate, separated by slashes. Underlined nucleotides indicate changes from the wild-type sequence.

introduced into *E. coli* DH5 α . Since the mutations of the three genes to be complemented were marked by apramycin resistance, the *acc(3)IV* (apramycin resistance) marker on the pSET152 derivatives was then replaced by the streptomycin/spectinomycin resistance gene *aadA* by PCR targeting. The newly constructed plasmids were transferred via *E. coli* ET12567/pUZ8002 into the relevant *S. coelicolor* mutants. Streptomycin/spectinomycin-resistant exconjugants were checked by PCR before phenotypic testing.

Phenotypic testing. All strains were incubated at 30°C on MM containing mannitol as the carbon source, and their growth was checked visually and using a phase-contrast microscope after 3 and 5 days. For *whiI* mutants, DNA-specific DAPI (4',6'-diamidino-2-phenylindole) staining was performed according to the method of Kieser et al. (23), but without poly-L-lysine treatment. Scanning electron microscopy was performed as described by Flardh et al. (13).

FIG. 1. Generation of *whiI* mutants at the correct chromosome location by gene replacement. (1) Elimination of the unique *Hind*III site in cosmid 1C3 by filling in and religation. (2) PCR targeting to replace the “phosphorylation domain”-encoding part of *whiI* on the cosmid by the *aac*(3)*IV-sacB* cassette containing a *Hind*III site. (3) *Hind*III digestion to linearize cosmid DNA. (4) Coelectroporation of the mutant *whiI* PCR product together with the linear cosmid into the λ -RED-expressing *E. coli* host strain to replace the *aac*(3)*IV-sacB* cassette with mutant *whiI* by recombination. Only the strains containing the recircularized cosmid obtained by recombination with *whiI* survived on carbenicillin-containing plates. Transformants were selected on sucrose-containing medium to reduce the background from religated cosmids by counterselection with *sacB*. (5) PCR targeting to replace *neo* with the *aac*(3)*IV-oriT* cassette. (6) Transfer of the cosmid with a *whiI* point mutant into *S. coelicolor* J2450 was done by conjugation, followed by screening for apramycin- and hygromycin-sensitive double-crossover exconjugants.

RNA isolation and S1 nuclease mapping. For RNA isolation, *S. coelicolor* strains were pregerminated in 2 $\times$ YT (yeast extract-Bacto tryptone) for 6 to 10 h, and about 3×10^6 pregerminated spores (or mycelial fragments in the case of the *whiI* null mutant) were inoculated on cellophane discs on MM containing mannitol. The cultures were harvested at 18, 24, 36, 48, 72, and 84 h for the wild type (three replicate experiments) and at 24, 48, and 72 h for the mutants, corresponding to early and late aerial mycelium formation and early spore formation in a wild-type time course (two replicate experiments). Mycelia were ground in liquid nitrogen and transferred to 50-ml Corning tubes. The culture powder was immediately homogenized in 20 ml Tri-reagent by vortexing and incubated at

room temperature for 15 min. Chloroform (4 ml) was added, vortexed vigorously for 20 s, and incubated at room temperature for 5 min. The sample was centrifuged (15 min, $8,000 \times g$, 4°C) for phase separation. The aqueous phase was transferred into a new Corning tube, and 10 ml isopropanol was added to precipitate RNA. The following steps were used as described by Kieser et al. (23): DNA was removed by RNase-free DNase I, and two additional chloroform extractions were carried out before the final precipitation of total RNA. RNAs were quantified by UV spectroscopy, and their quality was checked by agarose gel electrophoresis. S1 nuclease mapping of selected genes was used to verify the microarray results, as described by Kieser et al. (23). Sequencing ladders were generated with a Promega fmol cycle sequencing kit according to the product manual, using the same downstream labeled primer as that used to amplify the probe for S1 mapping.

Target labeling and array hybridization. The synthesis and labeling of cDNA and the array hybridization procedures were carried out according to the microarray hybridization protocol of Mersinias et al. (<http://www.surrey.ac.uk/SBMS/Fgenomics>). Cy3-labeled cDNA and Cy5-labeled genomic DNA were mixed before hybridization to allow all cDNA hybridizations to have the same competitor (wild-type M145 genomic DNA). Each slide hybridization used 12.5 μg of total RNA and 3.5 μg sonicated genomic DNA. *Streptomyces* microarray chips were designed as described by Bucca et al. (4; <http://www.surrey.ac.uk/SBMS/Fgenomics>). The probe set, totaling 8,736 spots, included 7,084 *S. coelicolor* genes, 254 SCP1 genes, and 1,398 positive and negative control spots. Each of the 36 cDNA mixtures was hybridized to two microarrays (technical duplicates).

The array slides were made in two different batches. One set of 36 was singly printed (S chips), and a second set of 18 was from a batch printed with two copies of the probe set (D chips). They were allocated as follows. For J2670 (*whiI*⁺), S chips were used for all hybridizations of biological replicates I and II, and D chips were used for all replicate III hybridizations (with technical duplication in this case being provided on a single D chip). For J2676 and J2671, all hybridizations of biological replicate I were done with S chips, and all those with replicate II were done with D chips. For J2673, all hybridizations were done with D chips (Fig. 2).

Array data analysis. After hybridization, array chips were scanned by a GenePix 4000B scanner, and the microarray images were quantified using GenePix Pro 5.0 (Axon Instruments, Inc.) to generate the data file. The data were analyzed using GeneSpring 5.0 (Silicon Genetics Inc.). Normalization of the raw data was performed by per spot and per chip intensity-dependent (Lowess) normalization. Candidate genes affected by *whiI* were first identified by comparing wild-type (J2670) data sets at 48 h and 72 h against the equivalent time points for each data set for the null mutant (J2676). In practice, hybridizations to D chips were treated as single combined data sets, so there were five wild-type data points and three null mutant data points for each time point. Genes showing at least a twofold expression difference (up or down) in at least four of five comparisons of the wild type with each of the three null mutant data points were listed, giving three gene lists for each time point. A final list of 45 genes with expression changes in all three reference comparisons was obtained using a Venn diagram (Fig. 2). The 45 genes were then examined in similar comparisons in which the wild-type data were used as the reference points for data obtained with two *whiI* point mutants.

Microarray accession number. The experimental details and data from the microarrays have been deposited in Streptobase, an online database established for *Streptomyces* microarray data (G. Velarde, unpublished; <http://www.streptobase.umist.ac.uk>), under accession number SB6.

RESULTS

Mutations in the “phosphorylation pocket”-related region only partially affect WhiI function. Alignment of WhiI with other response regulators (1) and modeling based on the crystal structure of NarL (2) indicated that four amino acids of WhiI, namely, D27, D69 (potential phosphorylation target), S96, and the nonconserved residue T118 (usually K, e.g., in NarL), correspond to the phosphorylation pocket of the canonical response regulator. Based on work on the response regulator NtrC (28), in separate experiments we changed D27 and D69 to the very similar residue E, in each case giving a protein possibly somewhat similar to a phosphorylated form of WhiI, which might be constitutively active and have only a minor effect on WhiI function. D27, D69, and S96 were also

FIG. 2. Assignment of array slides used for hybridization and flow chart for Genespring analysis. (A) Assignment of array chips for hybridization. (B) Genespring analysis and screening to obtain target gene lists.

changed to A, which was expected to prevent possible phosphorylation and therefore to have more severe effects on *WhiI* function. Conversion of T118 to a highly conserved lysine that was suggested to be important for autophosphatase activity in most response regulators (28) made the *WhiI* T118K “phosphorylation motif” potentially more similar to a consensus phosphorylation pocket; the effect of this mutation was not readily predictable. Finally, a double mutation of D27A and D69A was made in order to completely eliminate any conserved receiver aspartate in *WhiI*.

Each mutant form of *whiI* was inserted into pSET152, a vector that integrates into the chromosomal ϕ C31 *att* site of streptomycetes. The constructs were introduced into *S. coelicolor* J2450 (*whiI::hyg*). J2450 colonies have white, non-sporulating aerial hyphae with very few cross walls and lack sporulation-associated condensation of DNA, as visualized by staining with DAPI (1). The wild-type gene, introduced via pSET152, fully restored sporulation, sporulation-associated DNA condensation, and gray colony color to J2450. This strategy for introducing mutant forms of *whiI* had the benefit that any phenotypic change could be shown to be due to the introduced DNA simply by showing that every exconjugant had the same phenotype.

Remarkably, all of the J2450/pSET152 derivatives restored the ability to form spore chains, but in several cases the spores were defective in maturation, even when incubation was con-

tinued for up to 10 days. The D27A and D27A-D69A mutants (lacking residues that, in consensus phosphorylation pockets, would be directly involved in phosphorylation) were the most severely affected. Their colonies had very pale gray aerial mycelia (Fig. 3A) with many chains of spores (Fig. 4A). The spores were more transparent than wild-type spores under a phase-contrast microscope, and germination was often observed within spore chains (Fig. 4A). DAPI staining indicated that almost all of the spores contained normally condensed DNA, although a few were DNA-free (data not shown). Scanning electron microscopy revealed that irregularly sized spores were >10-fold more frequent than those in the wild type and again showed prematurely germinating spores in some spore chains (Fig. 4B). The T118K mutant was very similar to the D27A mutant, except that it had a slightly grayer appearance (Fig. 3A). The D27E and D69A mutants also had light gray colonies, but the spores appeared normal under a phase-contrast microscope (not shown). Two mutants, D69E and S96A, were indistinguishable from the wild type.

To avoid any possible artificial phenotypic changes in complementation of *whiI* caused by the use of pSET152, we developed a PCR targeting-based procedure (described in Materials and Methods) (Fig. 1) that permitted the efficient replacement of the inactive *whiI::hyg* cassette of J2450 by several of the point mutant forms of *whiI* at the correct position in the chromosome. Similar but slightly less severe phenotypes were

FIG. 3. Effects of substitution of conserved amino acids in the putative WhiI phosphorylation domain on morphological phenotype. (A) "Complementation" of J2450 ($\Delta whiI$) by *whiI* variants, using the integrative vector pSET152. The cultures were grown on MM containing mannitol for 5 days. (B) *whiI* mutants with substitutions inserted at the correct chromosome location, grown on MM containing mannitol for 3 days.

observed compared with those for the initial mutations introduced via pSET152 (Fig. 3B), and the order of severity remained unchanged, with the D27A, T118K, and D27A-D69A phenotypes being more severe than that of the D69A mutant.

The more severe phenotypic changes obtained with mutations introduced on pSET152 may have been caused by the additional wild-type *whiI* promoter on the chromosome in J2450, which may compete for the σ^{WhiG} RNA polymerase holoen-

FIG. 4. Microscopy of *whiI* point mutants. (A) Phase-contrast micrographs. Samples were taken from 3-day-old cultures grown against a coverslip inserted at an angle into MM containing mannitol. The triangles indicate the irregular phase-light spore chains and germinating spores in the mutant strains J2661, J2666, and J2667 (a, b, and c), which formed immature spores, in contrast to the phase-dark wild-type spore chains (J2660) (d). Bars, 5 μ m. (B) Scanning electron micrographs of the colony surfaces of *whiI* point mutant J2666 (a) and the wild-type strain J2660 (b). The triangles indicate enlarged spores, and the arrows indicate spores germinating within spore chains. Bars, 5 μ m.

FIG. 5. Model for the regulation of different stages of sporulation by WhiI in different active states. From the intermediate phenotypes of *whiI* point mutants, the regulatory role of WhiI in sporulation was separated into two parts. WhiI in state I (WhiI^I) is required for prespore formation, whereas changing the activity to another state (WhiI^{II}) by posttranslational modification or effector binding is needed for further spore maturation, probably including expression of the later genes *sigF* and *whiE*. The modification/effector is probably induced by a signal produced at a certain developmental stage. Solid lines indicate well-established (but not necessarily direct) regulatory steps, and dashed lines indicate the suspected regulation steps that have yet to be confirmed.

zyme, hence impairing the transcription of σ^{WhiG} -dependent genes (26, 37), including *whiI* alleles on pSET152.

In summary, these results were consistent with the more phenotypically severe of the mutations in the “phosphorylation pocket” (D27A and T118K) effectively preventing WhiI from making a transition from an initial form (state I) to a modified form (state II). The less severe mutations (D27E, D69A, D69E, and S96A) might permit state II to form (Fig. 5). However, we cannot eliminate the possibility that different *whiI* alleles result in changes in WhiI abundance, which could also account for phenotypic differences. This situation could happen either if different WhiI allelic forms have different half-lives or if they have altered autoregulatory properties.

Identification of 23 potentially WhiI-dependent target genes by transcriptome analysis, with two developmental stage-specific patterns of target gene expression. The diversity of *whiI* mutant phenotypes suggested that some genes might show *whiI* allele-specific changes in expression. To investigate this, we first used near-whole-genome microarray analysis of extracted RNA samples to identify genes dependent, directly or indirectly, on *whiI*. Each RNA sample was used to generate Cy3-labeled cDNA, which was then mixed with Cy5-labeled genomic DNA of wild-type J2670 before being hybridized with the microarrays. Thus, all RNA samples had the same reference (genomic DNA) for normalization.

In the initial stage of the microarray analysis, we obtained detailed time course data for the “wild-type” strain J2670, using RNAs isolated at six time points representing the following three developmental stages: before aerial mycelium formed (18 and 24 h); during aerial mycelium formation, but before spore chains were obvious (36 and 48 h); and after sporulation had become obvious (72 and 84 h). Previous S1 mapping studies had shown that *whiI* transcription started at 36 h and was strong from 48 to 72 h under conditions similar to those used here (1). Three separate sets of J2670 cultures were used as the source of RNA, and each hybridization was

done in duplicate. We then compared these results with those obtained with the *whiI* null mutant J2676 at 24, 48, and 72 h (representing time points equivalent to each of the three major stages of the wild type). Duplicate time courses were used for the extraction of RNA from the mutant, and hybridizations were again duplicated.

Using a threshold of a twofold difference at any time point, 23 genes were found to be underexpressed in the mutant and were therefore directly or indirectly dependent on *whiI* (Table 3; see Fig. S1 in the supplemental material for examples). As expected for possible targets of a sporulation gene, 17 were upregulated in the wild type at times after aerial development had been initiated: 8 were upregulated at 36 to 48 h, when aerial hyphae were abundant but before there was much sporulation (SCO0323, -0681, -0682, -2718, -4172, -4174, -4393, and -5315), and 9 were upregulated at 72 h, after sporulation was well established (SCO0834, -1366, -1367, -3899, -3900, -6375, -6744, -7510, and -7511). In general, the point at which *whiI* dependence became significant for these 17 genes coincided with the developmental upshift in their expression. The remaining 6 of the 23 (SCO2008, -2009, -2162, -4104, -4761, and -6197) showed no developmentally associated upshift in expression, but their expression declined earlier in the null mutant than in the wild type. As expected from the fact that *whiI* is not transcribed until aerial growth takes place (1), only the later time points (48 and 72 h) were affected by *whiI* mutation.

Apart from the fact that most of the WhiI-dependent genes were developmentally regulated, confidence in the meaningfulness of the data was reinforced by the following considerations: 10 of the 23 genes were in adjacent or nearly adjacent pairs (SCO0681/0682, SCO2008/2009, SCO3899/3900, SCO4172/4174, and SCO7510/7511) and were therefore possibly cotranscribed or functionally related, most of the WhiI-dependent genes upregulated at 48 h in the wild type were downregulated in the mutant at both that and the subsequent time point, subsequent experiments showed that 18 of the genes were also

TABLE 3. Transcripts reduced or enhanced more than twofold between J2670 (*whiI*⁺) and J2676 (Δ *whiI*) at 48 h and/or 72 h of growth

Open reading frame ^{a,f}	Gene ^a	Product ^a	Time of growth (h) ^b	Normalized intensity ratio ^c				Transcriptional ratio ^d		
				J2670 (<i>whiI</i> ⁺)	J2671 (D27A)	J2673 (D69E)	J2676 (Δ <i>whiI</i>)	J2670/ J2676	J2670/ J2671	
Gene expression reduced only in <i>whiI</i> null mutant ^d										
SCO0323*	<i>rdlA</i>	Unknown	48	4.3	4.0	6.8	0.8	5.4	1.1	
SCO2718*		Secreted protein	48	5.5	7.1	11.2	1.3	4.2	0.8	
SCO0834		Unknown	72	2.1	1.5	2.2	1.0	2.1	1.4	
SCO6375*		Secreted protein	72	1.6	1.1	3.2	0.6	2.7	1.4	
SCO6744		Unknown	72	1.4	1.2	1.5	0.6	2.3	1.2	
Gene expression reduced in both <i>whiI</i> null and D27A mutants ^d										
SCO4104	<i>groES</i> <i>whiE</i>	Integral membrane protein	48	2.0	1.3	1.5	1.0	2.0	1.5	
SCO4393		Phosphoheptose isomerase	48	3.6	2.0	2.5	1.6	2.3	1.8	
SCO4761		10-kDa chaperonin Cpn10	48	3.9	2.3	2.5	1.8	2.2	1.7	
SCO5315*		Polyketide cyclase	48	2.2	1.0	3.0	0.7	3.1	2.2	
SCO6197		Secreted protein	48	1.2	0.6	1.1	0.6	2.0	2.0	
SCO0681		Ferredoxin/ferredoxin-NADP reductase	72	3.7	2.4	2.2	1.5	2.5	1.5	
SCO0682		Unknown	72	3.6	2.2	2.7	1.6	2.3	1.6	
SCO1366*		PadR-like transcriptional regulator	72	7.2	3.2	3.5	2.4	3.0	2.3	
SCO1367		ABC transport system ATP-binding protein	72	2.6	1.2	2.7	0.7	3.7	2.2	
SCO2008*		Branched-chain amino acid binding protein	72	6.4	2.4	4.4	2.1	3.0	2.7	
SCO2009	Branched-chain amino acid transport permease	72	1.7	1.0	1.3	0.8	2.1	1.7		
SCO2162	<i>cypH</i>	Quinolinate synthetase	72	2.5	0.7	1.8	0.8	3.1	3.6	
SCO3899		<i>myo</i> -Inositol-1-phosphate synthase	72	3.0	1.0	1.8	1.3	2.3	3.0	
SCO3900		Conserved hypothetical protein	72	4.0	1.4	2.7	1.7	2.4	2.9	
SCO7510		Peptidyl-prolyl <i>cis-trans</i> isomerase	72	3.6	1.7	4.2	1.5	2.4	2.1	
SCO7511		<i>gap-2</i>	Glyceraldehyde 3-phosphate dehydrogenase	72	3.6	1.7	4.3	1.7	2.1	2.1
Gene expression reduced in <i>whiI</i> null mutant, with indeterminate response to other <i>whiI</i> alleles ^e										
SCO4172*			Membrane protein	48	1.7	0.7	0.7	0.4	4.3	2.4
SCO4174*		Integral membrane protein	48	1.3	0.4	0.7	0.3	4.3	3.3	
Gene expression enhanced only in <i>whiI</i> null mutant ^d										
SCO4187	<i>amtB</i> <i>glnK</i> <i>rtcB</i>	Membrane protein	48	1.0	1.1	1.2	2.0	2.0	1.8	
SCO5583		Ammonium transporter	48	1.3	0.8	0.8	3.2	2.5	4.0	
SCO5584		Nitrogen regulatory protein P-II	48	1.2	0.9	0.8	3.6	3.0	4.0	
SCO3299		Unknown	72	0.6	0.5	0.6	1.3	2.2	2.6	
SCO4002*		Secreted protein	72	1.2	2.5	2.0	4.5	3.8	1.8	
SCO5189*	<i>wblC</i>	Unknown	72	6.3	10.2	6.9	20.5	3.3	2.0	
SCO5190*		DNA-binding protein	72	3.8	4.6	3.7	10.9	2.9	2.4	
SCO5191		Unknown	72	6.9	10.0	5.5	23.5	3.4	2.4	
SCO7211		Secreted glycosyl hydrolase	72	0.6	1.0	1.0	1.7	2.8	1.7	
Gene expression enhanced in both <i>whiI</i> null and D27A mutants										
SCO5249*		Cyclic nucleotide-binding protein	48	2.0	2.9	2.0	4.4	2.2	1.5	
SCO0140*		MerR family transcriptional regulator	72	0.5	1.3	0.6	1.9	3.8	1.5	
SCO1648	<i>arc</i>	AAA ATPase	72	2.5	5.2	3.0	5.0	2.0	1.0	
SCO2922		Membrane protein	72	1.2	3.4	0.8	2.4	2.0	0.7	
SCO3123	<i>prsA2</i>	Ribose-phosphate pyrophosphokinase	72	1.0	1.4	1.2	2.1	2.1	1.5	
SCO3660	<i>nuoH2</i> <i>nuoK2</i>	Integrase/recombinase	72	0.5	0.8	0.5	1.0	2.0	1.3	
SCO4602		NADH dehydrogenase subunit	72	1.0	2.2	1.2	2.6	2.6	1.2	
SCO4605		NADH dehydrogenase subunit	72	2.4	4.8	3.1	7.0	2.9	1.5	

Continued on following page

TABLE 3—Continued

Open reading frame ^{a,f}	Gene ^a	Product ^a	Time of growth (h) ^b	Normalized intensity ratio ^c				Transcriptional ratio ^d	
				J2670 (<i>whiI</i> ⁺)	J2671 (D27A)	J2673 (D69E)	J2676 (Δ <i>whiI</i>)	J2670/J2676	J2670/J2671
SCO5124*		Hypothetical protein	72	2.6	6.5	2.8	6.9	2.6	1.1
SCO5125		Chitinase promoter binding protein 1	72	1.8	3.7	2.0	3.7	2.1	1.0
SCO5269		Unknown	72	0.9	1.9	0.9	1.8	2.0	0.9
SCO5576*	<i>acy</i>	Acylphosphatase	72	1.6	4.8	2.0	4.5	2.8	0.9
Gene expression enhanced in <i>whiI</i> null mutant, with indeterminate response to other <i>whiI</i> alleles ^e									
SCO1201		Reductase	72	1.0	1.7	1.5	2.0	2.0	1.2

^a The SCO numbers were taken from EMBL database accession no. AL645882. The possible function of each product was either known from previous work or was based on the genome annotation and further information given in the ScoDB database (<http://streptomyces.org.uk>). Genes in bold are also represented in *Streptomyces avermitilis*, based on reciprocal hits in BLAST searches.

^b Earliest culture age showing *whiI* influence.

^c The normalization process was performed by the per spot and per chip intensity-dependent (Lowess) normalization method, which performs an intensity-dependent (Lowess) normalization and divides the signal channel (Cy3 channel for cDNA) by the control channel (Cy5 channel for genomic DNA). The values in the table were averaged from all the biological and experimental replicates.

^d A J2670/J2676 ratio of >2 was the threshold for reduced expression in the *whiI* null mutant. If the J2670/J2671 ratio was <1.5, then the effect was considered *whiI* null specific. A J2676/J2670 ratio of >2 was the threshold for enhanced expression in the *whiI* null mutant, and if the J2676/J2671 ratio was >1.5, then the effect was considered *whiI* null specific.

^e For SCO4172 and -4174, expression in J2673 was not consistent with that in J2670 (wild type); for SCO1201, expression in both J2671 and J2673 was intermediate between that in J2670 and J2676.

^f Genes indicated with asterisks passed all filters during the screen for changes in expression by GeneSpring, whereas the others had differences in at least four of five comparisons (five wild-type replicates, including replicate cultures and experiments, compared with three null mutant individual samples).

affected in at least one of the *whiI* point mutants (see below), and genes representing each developmental expression pattern (SCO0323 and SCO6375) gave results consistent with the microarray data in S1 nuclease protection assays (Fig. 6). Some known *whiI*-dependent genes were not detected, indicating limitations in the sensitivity of the microarray analysis (see below), but this did not invalidate the conclusion of *whiI* dependence for the genes identified above.

Mutations changing the “phosphorylation pocket” of WhiI vary in their effects on *whiI*-dependent genes. Using the strategy outlined above, we also analyzed global gene expression in two constructed *whiI* point mutants, namely, J2671 (*whiI* D27A), which gave a phenotype intermediate between those of the wild type and a null mutant and was expected to freeze

WhiI in state I, and J2673 (*whiI* D69E), which had wild-type morphology and in which WhiI was expected to achieve state II. The expression of the 23 genes showing reduced expression in the *whiI* deletion mutant was then examined in the two *whiI* point mutants, with the expectations that some, but not all, of the 23 genes would also show reduced expression in J2671 and that a smaller number would show reduced expression in J2673.

Eighteen of the 23 *whiI*-dependent genes were indeed reduced in expression in the D27A mutant, and two of these (SCO4172 and -4174) were also reduced in expression in the D69E mutant, confirming the graded pattern of response predicted from overall phenotypic characterization. The five genes whose expression was reduced only in the *whiI* null mutant (see

FIG. 6. S1 nuclease protection assay. S1 mapping was done with candidate WhiI target genes in the wild-type strain (J2670), the null mutant (J2676), and the point mutants D27A (J2671) and D69E (J2673). RNA samples were taken from the second batch of all strains used in microarray analysis. *hrdB* was used as a control for RNA quality and loading.

Fig. S1A in the supplemental material) were candidates for expression before sporulation septation, but none of them encoded a product that might recognizably have a biochemical function in septation. Some of the other 18 (see Fig. S1B in the supplemental material) may be involved in spore maturation, but although several of them have functional annotations (see Discussion), there were no obvious connections to this process.

Some genes show increased expression in *whiI* mutants. Unexpectedly, the expression of 22 genes was significantly enhanced in the *whiI* null mutant (Table 3; see Fig. S1 in the supplemental material for examples). All 22 genes showed some evidence of increased expression in the wild type (J2670) after rapid growth had been completed (i.e., not before 36 h), and the increased expression in the mutant always occurred at the later time points (see Fig. S1C and D in the supplemental material). Thus, *WhiI* (directly or indirectly) has both positive and negative effects on global gene expression. All but 1 of these 22 genes fell into one of three classes with respect to both the wild-type profile and the time at which the *whiI* null mutation had its effect. Nine genes were maximally expressed around 48 h, with a subsequent decline that was much less severe in the null mutant (SCO1201, -3123, -3299, the possibly cotranscribed SCO4602 and -4605, the clustered but separately oriented SCO5189, -5190 [*wblC*], and -5191, and SCO7211). In three other cases, expression in the wild type increased sharply between 48 and 72 h and remained high (SCO4187 and the possibly cotranscribed genes SCO5583 and -5584), with enhanced expression in the mutant being specific to the 48-h time point. For nine other genes, expression in the wild type increased late (after 72 h), and enhancement in the mutant was seen only at 72 h. It is possible that the effects on the 12 genes in the last two classes were not *whiI* specific, but instead reflected differences in the rates of developmental progression caused by the differences in the nature of the wild-type and mutant inocula. The occurrence of early and late effects indicates that there are at least two kinds of repression, although it is not possible to conclude that any of the repression involves a direct effect of *WhiI*. For example, some of the 22 genes could be targets for a repressor encoded by a *WhiI*-activated gene (though only one regulatory gene was identified among these genes [Table 3]).

Thirteen of the 22 genes were also derepressed in the D27A mutant. These were all affected late in the null mutant. Only one gene, SCO1201, was also affected by the D69E mutation. Thus, the hierarchical pattern of *whiI* influence seen for “*WhiI*-dependent” genes also applied to “*WhiI*-repressed” genes.

Effects of several potential *WhiI* targets on spore maturation. Some of the *WhiI*-affected genes identified by microarray analysis have previously been subjected to mutational analysis (*rdlA/B* [8], *glnK* [12], *whiE* [10], and *wblC* [29; B. Gust and K. Chater, unpublished data]), but although each mutation had significant phenotypic consequences, in no case was sporulation septation affected, in contrast to the phenotype of a *whiI* null mutant. Furthermore, the late blocked phenotype of some of the new *whiI* mutants indicated that *WhiI* also mediates important aspects of spore maturation in addition to pigmentation. In the hope of understanding more about the causes of the phenotypes of *whiI* mutants, we deleted seven of the newly uncovered *whiI*-influenced genes, including examples of “*WhiI*-repressed” genes (Table 1). All of these but one (SCO4174) have

orthologues in *Streptomyces avermitilis* (Table 3), making it more likely that they have significant roles. Three mutants (SCO0323 [function unknown], -5249 [cyclic nucleotide binding protein], and -5576 [acyl phosphatase]) had pale gray aerial mycelia, but the severity of phenotypic changes was weaker than that for the D27A mutant (data not shown). All three mutants were complemented by the introduction of a copy of the relevant gene on pSET152 (data not shown). Thus, the three genes are all necessary for spore maturation, and presumably this contributes to the phenotypes of the new *whiI* point mutants, but none of them has a crucial involvement in the early stages of sporulation.

Deletions affecting SCO2008 (likely involved in transport of branched-chain amino acids), SCO4172 and -4174 (encoding unknown membrane proteins), and SCO6375 (encoding an unknown secreted protein) caused no obvious phenotypic changes. Thus, none of these four genes makes a major, non-redundant contribution to any known aspect of the *whiI* mutant phenotypes.

DISCUSSION

Atypical and “orphan” response regulators in streptomyces are often involved in processes associated with stationary phase, and modulation of their activity does not usually take place via phosphorylation of the central Asp of the receiver domain. There are more than 80 response regulator genes in the *S. coelicolor* genome (20). Thirteen are “orphan” genes that are not located close to a histidine protein kinase gene. Such orphan genes have also been found in other streptomyces. Some of their gene products resemble *WhiI* in having atypical “phosphorylation pockets.” In *RamR*, a key activator of development in which a histidine replaces the first residue of what would normally be a DD pair at about amino acid (aa) 11, D12A and D56E changes had no obvious phenotypic effect, and a D56A and D56N mutant allele permitted the growth of aerial hyphae in a *ramR* null mutant, albeit only at a high copy number (30, 31). It was also documented that unphosphorylated *RamR* bound to multiple sites in the *ramC* promoter in vitro and that this was dependent on *RamR* dimerization. This indicated that *RamR* function does not need phosphorylation (32), although the evidence that *ramR* is expressed for much longer than *ramC* could suggest that *RamR* undergoes some modification (30, 31). *DnrN*, the direct activator protein for the transcription of genes for daunorubicin biosynthesis in *Streptomyces peucetius*, has a pair of E residues instead of D residues near its N terminus, and there was no difference in *dnrI* promoter binding in vitro between wild-type *DnrN* and a D55N mutant form (16). *RedZ*, which activates the pathway-specific regulatory gene *redD* for undecylprodigiosin biosynthesis, lacks most of the conserved amino acids at the potential phosphorylation pocket (with only one D at position 10), yet it evidently functions effectively (19, 38). In the developmental protein *BldM*, which contains all the conserved amino acids of normal phosphorylation pockets, D54A and D54E substitutions had no phenotypic effect (27). Thus, adding the fact that the D27 and D69 substitutions in *WhiI* have only weak effects on sporulation, five of five *Streptomyces* orphan or aberrant response regulator-like proteins for which suitable information is available do not need conventional phosphorylation for activity,

and the central Asp residue does not seem to be a phosphoryl group receiver.

In contrast, none of 67 gene pairs encoding a response regulator and a sensor kinase have yet been found to be related to morphological differentiation in *S. coelicolor*, although some of them do influence secondary metabolism (20). It is possible that signals may be sensed through a more complex mechanism during development by the atypical or orphan response regulators, rather than via cognate sensor kinases, which are typically responsive to extracytoplasmic stimuli. Alternatively, the activities of these atypical or orphan response regulators may be independent of signals, but in that case, the function of the N-terminal "regulatory domain" would need to be explained.

How do *WhiI* mutations cause various phenotypes? All of the mutant *WhiI* proteins tested in this study were competent to bring about the early steps of sporulation, leading to sporulation septation and the rounding up of prespore compartments, but some of the mutations interfered with the maturation of spores. We can interpret this in two ways.

In one model, the developmental role of *WhiI* is determined mainly by its abundance: those genes related to early stages of spore formation would require a low (threshold) level of *WhiI*, and when *WhiI* accumulates to a higher level, it would activate later genes, including some involved in spore maturation. In this model, the different mutant phenotypes would result from differences in the effective concentrations of allelic forms of *WhiI*, reflecting either different half-lives or different effects of *whiI* expression on autoregulation. By way of precedent, different target genes of Spo0A, the response regulator-like key initiator of sporulation in *B. subtilis*, respond differentially to low and high (threshold) levels of Spo0A-P (14, 15).

In a second model, *WhiI* is changed from an unmodified state (form I) to a modified state (form II) in response to a signal. In this model, the different allelic forms differ in the ability to respond to this signal or in their similarity to one form or the other. Although the effects of equivalent amino acid substitutions may differ considerably between different response regulators (35), it is striking that all of the mutants with substitutions constructed in the *WhiI* "phosphorylation pocket" were able to achieve sporulation septation, including a D27A-D69A double mutant lacking any aspartates in the "pocket" and a T118K mutant that for conventional response regulators should increase autophosphatase activity. It seems unlikely (though not inconceivable) that these mutants all make *WhiI* proteins competent for conventional phosphorylation, so we concluded that at least the early sporulation functions controlled by *WhiI* can (probably) be carried out efficiently in the absence of phosphorylation. Although form II could correspond to a phosphorylated form, we think it is also possible that *WhiI* interacts directly and noncovalently with a molecule whose concentration changes during aerial hyphal development. Some of these questions could be clarified in future by examining the *in vivo* and *in vitro* binding activities of *WhiI* and its mutant forms to potential targets identified in our microarray analysis (but we emphasize that, at present, we do not know whether any of these genes are direct *WhiI* targets).

Implications of the microarray data for the physiology of sporulating aerial hyphae. An influence of *whiI* on the expression of a subset of genes can be taken to indicate changes in the importance of their functions during aerial growth and devel-

opment. This idea is independent of whether these genes are direct targets of *WhiI*. The genes affected by *whiI* were annotated to have a broad range of functions, encoding, for instance, membrane proteins or secreted proteins, metabolic enzymes, transcriptional regulators or DNA-binding proteins, possible signal molecule binding proteins, and transport systems (Table 3). Here we highlight some of these.

Only two of the genes, SCO2718 and SCO5315, were previously associated with aerial hyphal development. SCO2718 encodes RdlA, one of two previously identified hydrophobic rodlin proteins, major components of the rodlet layer that, along with chaplins, covers the surfaces of aerial hyphae in *S. coelicolor* (7, 8). Transcripts of the neighboring *rdlB* gene (SCO2719, encoding another rodlin) had a similar expression pattern to that of SCO2718, but *rdlB* was not included among the set of 23 genes because it showed a <2-fold change in microarray signal intensity. Both *rdl* genes were shown previously to be expressed specifically in growing aerial hyphae (7), and our data indicate that some degree of *WhiI* dependence helps to ensure that as each aerial hypha grows, it produces rodlin continuously to ensure complete coverage of the hyphal surface. However, some of this production is *WhiI* independent, since *rdlA* mRNA levels in the *whiI* null mutant continued to rise at 72 h, when expression in the wild type and the two point mutants had begun to decrease. In addition, rodlin accumulated in a *whiG* mutant (7), in which *whiI* would not have been expressed (1).

The second gene, SCO5315, is open reading frame VI in the *whiE* gene cluster, responsible for the biosynthesis of the gray spore pigment, consistent with the paler color of spores of the D27A mutant. Previously, Kelemen et al. (22) showed that the promoter of the operon containing SCO5315 was activated during sporulation and downregulated in a *whiI* mutant. Of four other *whiE* gene probes printed on the microarray slides, SCO5314 (*whiE orfVII*) showed the same expression pattern as SCO5315, but the difference between the wild type and the null mutant was <2-fold. The other three *whiE* genes (SCO5316, -5318, and -5321) showed no obvious temporal increase in expression nor any changes in the three *whiI* mutants. This was presumably due to limitations in the sensitivity of the microarray analysis, which also failed to detect another known severely *whiI*-dependent gene, *sigF* (21). Overall, a lack of synchrony of development in *S. coelicolor* and the presence of abundant vegetative mycelium throughout the time course probably contributed to this lack of sensitivity, so we presume that some other *whiI*-influenced genes were not detected in our analysis.

The *WhiI*-influenced SCO5249 gene (also verified in S1 nuclease protection assays) (Fig. 6) encodes a likely cyclic NMP (cNMP)-binding protein that appears to play a part in spore maturation, since deletion of SCO5249 caused spores to be pale. In *Streptomyces griseus*, a sporulation-specific protein (EshA) with a putative cNMP-binding domain was present at a high level during sporulation (including that induced in a submerged culture of this species by nutritional downshift) but absent from the vegetative mycelium. The abolition of EshA interfered with the growth of sporogenic hyphae, the localization of septation, and spore maturation (24). This implies that cyclic nucleotides may play a signaling role in aerial hyphal development as well as in the earlier stages of mycelium formation (11, 36). Note that the annotated *S. coelicolor* genome

database identifies genes for six proteins containing cNMP-binding domains, as follows: SCO0168, 190 aa; SCO3447, 227 aa; SCO3571 (*crp*, encodes a cAMP-binding protein), 224 aa; SCO5249, 468 aa; SCO7310, 151 aa; and SCO7699 (with high homology to *eshA*), 471 aa.

Inevitably, there must be changes in metabolism during the growth of aerial hyphae into a water- and nutrient-free environment and during their differentiation into spores. Indeed, at least one aspect of carbon storage metabolism, involving glycogen, is under the developmental control of *whiG* (39). Consideration of *WhiI*-influenced functions leads to other inferences. SCO4602 and -4605 are part of a gene cluster (SCO4599-4608) for the 10 subunits of NADH dehydrogenase. The other genes in this cluster showed similar expression patterns to those of SCO4602 and -4605, but the changes fell below the twofold threshold, indicating that the whole cluster is repressed during aerial development and therefore that there is reduced oxidative phosphorylation.

There are several pointers to further possible physiological changes associated with development. These include developmental repression of *amtB* (ammonium transporter) and the probably cotranscribed *glnK* gene (encodes the PII protein, which integrates several major aspects of nitrogen metabolism through functional coupling to AmtB [12]), the increase in expression of components of a likely branched-chain amino acid transport system, the higher-level expression of an inositol-1-phosphate synthase (encoded by SCO3899) and its possible regulator (encoded by SCO3900), and the developmental repression of SCO5190 (*wblC*, which plays a key role in the induction of multiple antibiotic resistances [29]) and its two flanking genes. However, the evidence for the *WhiI* dependence of these genes rests on a single time point in each case, so we do not discuss them further here. Investigation of the effects of eliminating other early sporulation regulators (*WhiA*, -B, -G, and -H) (5) by comparable microarray analyses may be needed to reinforce these results.

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