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Regulation of the Synthesis of the Angucyclinone Antibiotic Alpomyacin in *Streptomyces ambofaciens* by the Autoregulator Receptor AlpZ and Its Specific Ligand[∇]

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Streptomyces ambofaciens produces an orange pigment and the antibiotic alpomyacin, both of which are products of a type II polyketide synthase gene cluster identified in each of the terminal inverted repeats of the linear chromosome. Five regulatory genes encoding *Streptomyces* antibiotic regulatory proteins (*alpV*, previously shown to be an essential activator gene; *alpT*; and *alpU*) and TetR family receptors (*alpZ* and *alpW*) were detected in this cluster. Here, we demonstrate that AlpZ, which shows high similarity to γ -butyrolactone receptors, is at the top of a pathway-specific regulatory hierarchy that prevents synthesis of the *alp* polyketide products. Deletion of the two copies of *alpZ* resulted in the precocious production of both alpomyacin and the orange pigment, suggesting a repressor role for AlpZ. Consistent with this, expression of the five *alp*-located regulatory genes and of two representative biosynthetic structural genes (*alpA* and *alpR*) was induced earlier in the *alpZ* deletion strain. Furthermore, recombinant AlpZ was shown to bind to specific DNA sequences within the promoter regions of *alpZ*, *alpV*, and *alpXW*, suggesting direct transcriptional control of these genes by AlpZ. Analysis of solvent extracts of *S. ambofaciens* cultures identified the existence of a factor which induces precocious production of alpomyacin and pigment in the wild-type strain and which can disrupt the binding of AlpZ to its DNA targets. This activity is reminiscent of γ -butyrolactone-type molecules. However, the AlpZ-interacting molecule(s) was shown to be resistant to an alkali treatment capable of inactivating γ -butyrolactones, suggesting that the AlpZ ligand(s) does not possess a lactone functional group.

Streptomycetes are gram-positive, filamentous, soil-dwelling bacteria that undergo a complex program of morphological differentiation. In addition to this singular multicellular morphogenesis, the members of the *Streptomyces* genus are well known for their ability to produce a variety of secondary metabolites with important uses in medicine and in agriculture. Recent advances in the sequencing of *Streptomyces* genomes, and more generally those of actinomycetes, have highlighted the underestimated potential of these organisms to synthesize secondary metabolites. For example, bioinformatic analysis of the *Streptomyces coelicolor* genome revealed the presence of 22 gene clusters with predicted roles in the production of secondary metabolites; only half a dozen of them had previously been identified experimentally, despite decades of study (4, 8). Similarly, 30 secondary metabolite gene clusters have now been identified using the *Streptomyces avermitilis* genome sequence

(18). More recently, the genome sequences of non-*Streptomyces* actinomycetes such as the biotechnologically important *Rhodococcus* sp. RHA1 (27), the marine strain *Salinispora tropica* that is responsible for producing the potent anticancer agent salinosporamide A (48), or the erythromycin-producing bacterium *Saccharopolyspora erythraea* (31) added, in total, more than 70 secondary metabolite biosynthesis clusters to the ever-growing list. From this wealth of sequences, a genome-mining approach has already led, for instance, to the discovery of the *tris*-hydroxamate tetrapeptide siderophore coelichelin from *S. coelicolor* A(3)2 (22).

In *Streptomyces ambofaciens* ATCC 23877, a known producer of congocidin (11) and spiramycin (36), the sequencing project of the left (1,544 kb; accession number AM238663) and right (1,367 kb; AM238664) arms of the linear chromosome has unveiled 11 novel secondary metabolite gene clusters (<http://www.weblgm.scbiol.uhp-nancy.fr/ambofaciens/>) (10). With the exception of the *des* cluster identified in the core region of the chromosome that directs the synthesis of desferrioxamine B and E, only the coelichelin-encoding *cch* cluster (2) and clusters presumably involved in the synthesis of carotenoids were found to be redundant compared with clusters present in the phylogenetically close relative *S. coelicolor*. This highlights the powerful ability of different streptomycete strains to produce distinct arrays of secondary metabolites.

In the course of deciphering the role of the remaining cryptic clusters in *S. ambofaciens*, the function of the type II polyketide

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synthase (PKS) gene cluster *alp*, located in the terminal inverted repeats (TIRs) and thus present in two copies, has been unraveled. This cluster was shown to be responsible for the synthesis of at least two distinct compounds: an antibiotic of the angucyclinone class, alpomycin, and also a diffusible orange pigment that is thought to be a degradation or modification product of the antibiotic (34). Interestingly, the chimerical formation of the *alp* cluster that presumably occurred through multiple horizontal gene transfer events is still distinguishable (34). Indeed, one part, which includes the minimal PKS genes responsible for assembling the polyketide chain and coding for a β -ketoacyl synthase (*alpA*), a chain length factor (*alpB*), and an acyl carrier protein (*alpC*), shows strong synteny with a part of the kinamycin cluster of *Streptomyces murayamaensis* (accession number AY228175). Consistent with this, preliminary structural determination has shown that alpomycin is related to kinamycin C and D (L. Song and G. Challis, personal communication). A second part is identical to that found in the mithramycin gene cluster carried by the pSLA2-L plasmid of *Streptomyces rochei* (28) or the chromosome of *Streptomyces argillaceus* (24). Finally, a third region that includes most of the *alp* regulatory genes is highly conserved with the regulatory module of the type I PKS tylosin cluster in *Streptomyces fradiae* (3, 34).

Within the five regulatory genes that form the *alp* regulatory subcluster, *alpT*, *alpU*, and *alpV* are predicted by comparison with database sequences to encode proteins from the *Streptomyces* antibiotic regulatory protein (SARP) family (49). Among them, *alpV* was previously shown to be essential for the production of both the orange pigment and alpomycin (1). It has been postulated that the AlpV protein occupies the critical position in the *alp* regulatory cascade, leading to the activation of transcription of the biosynthetic genes, although direct binding to promoter regions within the cluster has not yet been demonstrated. The two other deduced regulatory genes, *alpW* and *alpZ*, encode proteins from the TetR transcriptional regulator family (37). The AlpW protein shows sequence similarity to proteins acting as transcriptional repressors, such as TylQ from *S. fradiae*, which is involved in tylosin production (41), and BarB from *Streptomyces virginiae*, which regulates virginiamycin (26). The deduced product of *alpZ* shares homology with γ -butyrolactone autoregulator receptors that act as transcriptional factors to regulate antibiotic production and sometimes morphological differentiation in streptomycetes (for reviews, see references 17 and 44).

Several γ -butyrolactone receptors have been identified in various *Streptomyces* spp. and also in one non-*Streptomyces* actinomycete. (9). Typically, the autoregulator receptor binds as a homodimer to conserved DNA sequences (exemplified by the BarA responsive elements) within the promoter region of target genes to repress their transcription (20). A binding consensus sequence for autoregulator receptors has more recently been defined as autoregulatory elements (ARE) (13). Upon binding of their cognate ligand, namely, the γ -butyrolactones, the DNA-binding ability of the receptor is abolished, which in turn allows expression of the target gene(s). The γ -butyrolactone receptors most likely occupy a high regulatory level within the pathway-specific cascade by globally repressing the synthesis of antibiotics until proper physiological conditions are

reached (i.e., when a threshold concentration of γ -butyrolactone is attained).

Several previous studies characterizing signaling systems involving these receptors and their specific γ -butyrolactone interactive ligand(s) have been reported. For example, the model A-factor-ArpA system that controls the activation of the AdpA regulon (that includes a number of genes required for secondary metabolism and differentiation) in *Streptomyces griseus* (for review, see reference 17), the SCB1-ScbR system that exerts influence on actinorhodin and undecylprodigiosin production in *S. coelicolor* and directly controls the expression of a pathway-specific transcriptional regulator from the cryptic type I polyketide biosynthetic gene cluster (35, 45, 46), the virginiae butanolides-BarA system of *S. virginiae* (29), the IM-2-FarA system of *Streptomyces lavendulae* (21), and the recently published TylP system and its cognate unknown ligand controlling tylosin synthesis in *S. fradiae* (6).

In this study we address the role of AlpZ, a putative γ -butyrolactone receptor, in the *alp* regulatory cascade. We report AlpZ target promoters within the biosynthetic cluster and investigate whether activation of the *alp* cluster is influenced by γ -butyrolactone-like molecules produced in *S. ambofaciens*. The putative role of AlpZ as a sensor protein is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains, plasmids, and cosmids used in this study are listed in Table 1. *Streptomyces* strains were cultivated on or in R2, SFM, HT, or SMMS medium (19) and manipulated as described previously (34). Pigment and antibiotic production were assessed on/in R2 medium as described previously (1, 34). *Escherichia coli* strains were cultivated in Luria-Bertani (LB) and SOB liquid medium (39).

Nucleic acids and protein manipulation. Isolation, cloning, and manipulation of DNA were carried out as previously described for *Streptomyces* (23, 34) and for *E. coli* (39). Southern analyses were performed as described elsewhere (1). Total RNAs were isolated as described previously (19) from 2-ml samples of *S. ambofaciens* cultures grown in R2 liquid medium. Crude protein extracts or purified proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% resolving polyacrylamide gel). Following electrophoresis, proteins were visualized by Coomassie brilliant blue staining.

PCR and RT-PCR. Amplification of DNA fragments by PCR was performed with *Taq* DNA polymerase (NEB) or high-fidelity DNA polymerase Phusion (Finnzymes), according to the manufacturer's instructions. The method used for reverse transcription-PCR (RT-PCR) analysis was as previously described (1, 34). The cDNAs were obtained after reverse transcription of 4 μ g of DNase I-treated total RNA with SuperScript III reverse transcriptase (Invitrogen) and high-GC-content random hexamer primers (Oligo Spiking; Eurogentec). Primer pairs hrdB-F/hrdB-R, KSI-F/KSI-R, KSII-F/KSII-R, *alpV*-1/*alpV*-2, *alpT*-1/*alpT*-2, *alpU*-1/*alpU*-2 and *alpW*-1/*alpW*-2 (Table 2) were used to amplify cDNAs for an *hrdB*-like gene, *alpA*, *alpR*, *alpV*, *alpT*, *alpU*, and *alpW*, respectively. Two different sets of primers were used for transcriptional analyses of *alpZ*: *alpZ*-1/*alpZ*-2 was used in analysis of the wild type, and *alpZscar*-1/*alpZscar*-2 (amplification of the 81-bp scar; see below) was used in a double *alpZ* deletion mutant (the $\Delta\Delta$ *alpZ* strain).

Construction of *S. ambofaciens* mutant strains. (i) In-frame deletion of *alpZ*. The Redirect system (15) was used to make an in-frame deletion of the two copies of *alpZ* in *S. ambofaciens* ATCC 23877 as described in previous work for mutating other genes (1, 34).

(a) Replacement of *alpZ* by the selectable *aac(3)IV-oriT* cassette in cosmid F19. The primer pair *alpZ*-rep1/*alpZ*-rep2 (Table 2) was used to amplify the *aac(3)IV-oriT* cassette from pIJ773 (15). The replacement of *alpZ* coding sequence (only the start and stop codons were retained in the mutant) by the amplified cassette was mediated by the recombination λ RED system harbored by pIJ790 in *E. coli* BW25113 (15). The targeted F19 cosmid was designated F19 Δ *alpZ::aac(3)IV-oriT* (Table 1). Gene replacement was confirmed by Southern blotting and PCR analysis using the flanking primers CZ1 (124 nucleotides upstream from the start codon of *alpZ*) and CZ2 (149 nucleotides downstream

TABLE 1. Bacterial strains, cosmids, and plasmids used in this work

Strain, cosmid, or plasmid	Relevant properties ^a	Reference or source
Strains		
<i>S. ambofaciens</i>		
ATCC 23877	Wild type	36
ΔΔ <i>alpZ</i> (1 and 2) strain	<i>alpZ</i> loci on two chromosomal arms replaced by a scar	This study
DM1	<i>alpIABCD</i> on two chromosomal arms replaced by the <i>aac(3)IV-oriT</i> cassette	34
ΔΔ <i>alpID</i> strain	<i>alpIABCD</i> biosynthetic genes on two chromosomal arms replaced by a scar	This study
<i>S. coelicolor</i> M145	Strain used for γ-butyrolactone SCB1 extraction and antibiotic induction assay	19, 47
<i>E. coli</i>		
DH5α	General cloning strain	16
ET12567	Nonmethylating strain used for conjugation with <i>Streptomyces</i>	25
BW25113	Strain used for PCR-targeted mutagenesis	12
BL21(DE3)	Strain used for heterologous protein expression	Novagen
<i>B. subtilis</i> ATCC 6633	Strain used as indicator in bioassays	
Cosmids or plasmids		
F19	Cosmid from the genomic library of <i>S. ambofaciens</i> ATCC 23877; <i>bla neo</i>	23
F19Δ <i>alpZ</i> :: <i>aac(3)IV-oriT</i>	<i>alpZ</i> replaced by <i>aac(3)IV-oriT</i> cassette in F19; <i>bla neo</i>	This study
F19Δ <i>alpZ</i> ::scar	<i>alpZ</i> replaced by a 81-bp scar in F19; <i>bla neo</i>	This study
F19:: <i>aadA-oriT-ΔalpZ</i> ::scar	<i>alpZ</i> replaced by a 81-bp scar; <i>neo</i> gene replaced by <i>aadA-oriT</i> cassette; <i>bla oriT aac(3)IV</i>	This study
pIJ773	<i>oriT aac(3)IV</i>	15
pIJ778	<i>oriT aadA</i>	15
pIJ790	<i>gam bet exo cat</i>	15
pUZ8002	Mobilization plasmid; <i>neo</i>	33
BT340	FLP recombination plasmid; <i>flp bla cat repA101</i>	12
pGEM-Teasy	PCR cloning vector; <i>bla</i>	Promega
pGEM- <i>alpZ</i> 1	<i>alpZ</i> plus promoter region; <i>bla</i>	This study
pGEM- <i>alpZ</i> 2	<i>alpZ(G341A)</i> plus promoter region; <i>bla</i>	This study
pGEM- <i>alpZ</i> exp1	<i>alpZ, bla</i>	This study
pGEM- <i>alpZ</i> exp2	<i>alpZ(G341A) bla</i>	This study
pSET152	<i>oriT attP int aac(3)IV</i>	5
pSET152- <i>alpZ</i> 1	<i>oriT attP int aac(3)IV alpZ</i>	This study
pSET152- <i>alpZ</i> 2	<i>oriT attP int aac(3)IV alpZ(G341A)</i>	This study
pIB139	<i>oriT attP int aac(3)IV ermEp*</i>	50
pIB139- <i>alpZ</i>	<i>oriT attP int aac(3)IV ermEp* alpZ</i>	This study
pIJ8600	<i>oriT attP int aac(3)IV tipAp</i>	43
pIJ8600- <i>alpZ</i>	<i>oriT attP int aac(3)IV tipAp alpZ</i>	This study
pET-12a	<i>Bla</i> ; T7 promoter	Novagen
pET- <i>alpZ</i>	<i>bla alpZ</i>	This study
pET- <i>alpZ</i> (G341A)	<i>bla alpZ(G341A)</i>	This study
pSBET	<i>neo</i> ; tRNA ^{Arg} (AGA/AGG)	40

^a *bla*, ampicillin resistance gene; *neo*, kanamycin resistance gene; *aac(3)IV*, apramycin resistance gene; *oriT*, origin of transfer; *aadA*, spectinomycin and streptomycin resistance gene; *gam*, inhibitor of the host exonuclease V; *bet*, single-stranded DNA binding protein; *exo*, exonuclease promoting recombination along with *bet*; *cat*, chloramphenicol resistance gene; *flp*, F1p recombinase gene; *repA101*, thermosensitive replication origin; *attP*, plasmid ΦC31 attachment site; *int*, integrase gene.

the stop codon of *alpZ*), giving a product of 984 bp in the wild type and 1,658 bp after replacement with the *aac(3)IV-oriT* cassette.

(b) **In-frame deletion of *alpZ* in the F19 cosmid mediated by F1p recombinase.** Excision of the *aac(3)IV-oriT* cassette in F19Δ*alpZ*::*aac(3)IV-oriT* through the recombination between the two F1p recognition target sites flanking the cassette, leaving an 81-bp scar, was carried out as described previously (1, 15). The deletion of *alpZ* in the generated F19Δ*alpZ*::scar was confirmed by restriction and PCR analysis using primers CZ1 and CZ2 (354-bp expected fragment).

(c) **Construction of F19::*aadA-oriT-ΔalpZ*::scar.** To render the F19Δ*alpZ*::scar cosmid proficient for conjugation, the *neo* resistance gene of the cosmid part was replaced by an *aadA-oriT* cassette as described previously (1), yielding F19::*aadA-oriT-ΔalpZ*::scar.

(d) **In-frame deletion of *alpZ* in *S. ambofaciens*.** F19Δ*alpZ*::*aac(3)IV-oriT* was first introduced in *S. ambofaciens* by intergeneric conjugation via the *E. coli* donor ET12567 containing pUZ8002, and exconjugants resistant to apramycin and sensitive to kanamycin (Apra^r and Kan^s, respectively) were retained. F19::*aadA-oriT-ΔalpZ*::scar was then introduced in the Apra^r and Kan^s strains, and exconjugants resistant to spectinomycin were selected. Subsequent screening for the loss of both spectinomycin and apramycin resistance indicating the allelic exchange of the resistance marker with the unmarked, in-frame deletion was carried out. Replacement of *alpZ* in both chromosomal arms was obtained by natural homogenization between the TIRs of the chromosome during fermentation of *S. ambofaciens* Spec^c and Apra^s exconjugants. Double *alpZ* deletion

mutants (named ΔΔ*alpZ*) were confirmed by Southern blotting, PCR with CZ1/CZ2 primers (Table 2), and pulsed-field gel electrophoresis analysis.

(ii) **In-frame deletion of *alpIABCD*.** Using the DM1 strain in which *alpIABCD* biosynthetic genes are replaced by an *aac(3)IV-oriT* cassette (34), the same strategy as described above was used to remove the selectable cassette, leaving an 81-bp scar and yielding a ΔΔ*alpID* strain.

Complementation of the *alpZ* mutant. Using F19 cosmid as a template and a high-fidelity enzyme, a PCR product spanning from 528 bp upstream of the *alpZ* start codon to 15 bp downstream of the stop codon of *alpZ* was obtained with primers *alpZ*-compl-F/*alpZ*-compl-R (Table 2). After terminal dATP addition, the PCR product was first ligated with pGEM-Teasy vector (Promega). After transformation of *E. coli* DH5α with the ligation mixture, two white *E. coli* DH5α independent clones were confirmed by endonuclease restrictions to contain pGEM-Teasy including the insert. The insert part of the newly generated vectors from the two clones was further verified by sequencing to confirm the integrity of *alpZ*. The insert of one of them presented the wild-type sequence, and this vector was named pGEM-*alpZ*1, while in the other one, a single nucleotide substitution (G341 to A with respect to the first nucleotide of the start codon) was detected in the *alpZ* DNA coding sequence, resulting in a single amino acid change (Gly114 to Asp) in AlpZ. This construct, designated pGEM-*alpZ*2, was later used to analyze the effect of the mutation. The gel-purified XbaI/EcoRI restriction fragments of pGEM-*alpZ*1 and pGEM-*alpZ*2 which include *alpZ* were ligated

TABLE 2. Oligonucleotides used in this work

Primer function and name	Nucleotide sequence (5'-3') ^a
Deletion of <i>alpZ</i> and control of gene replacement	
<i>alpZrep1</i>	GGCACCGGCCGGCGTGCACCGCGGGGTGTTGTTCC <u>TCA</u> TGTTAGGCTGGAGCTGCTTC
<i>alpZrep2</i>	TGCCCGTTCCTGAGGCAAAGACCGTCGGGAGGTCCCGT <u>GATTC</u> CGGGGATCCGTCGACC
CZ1	TGTGAGAAGTACGTGCATGC
CZ2	ACAGGAAGCGGGCAGAC
Complementation, overexpression, or heterologous expression	
<i>alpZcompl-F</i>	ATTGAATTC GTGTCACCTCGGTGATTGC
<i>alpZcompl-R</i>	ATTCTAGAGCGGGT GTTGTTCC <u>TCA</u>
ExpZ1	AAACATATGGCTCGGCAGGAACGAGCG
ExpZ2	AAAGGATCCTCAGGCGGTGGCCGCGGC
RT-PCR	
<i>hrdB-F</i>	CGCGGCATGCTCTTCCT
<i>hrdB-R</i>	AGGTGGCGTACGTGGAGAAC
KSI-F	CCACGACGAACCCGAGTG
KSI-R	GTAGGCGTTGGAGCGGGT
KSII-F	CATCACCCAGTTCGCCCT
KSII-R	CCCAGGTGTTTCCGAGT
<i>alpV-1</i>	GAAGTGCTGGGGACGCTG
<i>alpV-2</i>	TGGTCGGCGTTGAGGG
<i>alpT-1</i>	GAGCAGAGCCGCTGGT
<i>alpT-2</i>	CCAGGTCGGTGAGTTCGG
<i>alpU-1</i>	CAGATTCACGACGAGCGG
<i>alpU-2</i>	CCACAACCTCGTCCACCAGG
<i>alpW-1</i>	TGATGGTCTGCAGGACGC
<i>alpW-2</i>	GCCTCGCTCACCTGATC
<i>alpZ-1</i>	AGGAACTGCGTGCGGAGT
<i>alpZ-2</i>	CAGCCACGCACTCTCGG
<i>alpZscar-1</i>	AGACCGTCGGGAGGTCCCGT
<i>alpZscar-2</i>	TGCACCGCGGGGTGTTGTT
Generation of gel shift probes	
AREU-1	GCTGCACTGTCCCGGCC
AREU-2	GCCCCGGTATTCCTCAGC
AREXW-1	GCCGTCGCCTTCTCACTCG
AREXW-2	GGCTGATTCATGCGCGTACC
AREV-1	GCCGTATCCGCCGATCT
AREV-2	CCTGACGCGGTTTGGGAG
AREZ-1	GTTGAATCCGCACGCCCG
AREZ-2	ACAGCGCCCCCTTGGTGA
d-AREXW-1	CGGTTGTTATGCTGGGG
d-AREXW-2	TCCGTGCGGTTTTTAGTG
d-AREV-1	GTGCTGTTTTTTATCAGG
d-AREV-2	AGTCGGTTTGTCAATGTG
d-AREZ-1	TCCCTGGTTTTGTTTTGG
d-AREZ-2	GTCCGTATTTTCGCAAGC
ZAREZ1	ACCCCAAACAAAACCAGGGAGTCCGTATTTTCGCAAGCC
ZAREZ1	GGCTTGCGAAAATACGGACTCCCTGGTTTTGTTTTGGGGT
ZAREV1	CCCTGATAAAAAAACAGCACAGTCGGTTTGTCAATGTGAG
ZAREV2	CTCACATTGACAAAACCGACTGTGCTGTTTTTTATCAGGG
ZAREXW1	CCCCAGCATAACGAACCGTCCGTGCGGTTTTTAGTGGGGG
ZAREXW2	CCCCACTAAAAACCGCACGGACGGTTCTGTTATGCTGGGG
ZAREU1	GGGGGGTGTGAGAACGGGGTGTCTGGTGTGTTGCGCCCGGA
ZAREU2	TCCGGCGCAAACACCAGAACACCCCGTTCTCACACCCCC

^a For the primers *alpZrep1* and *alpZrep2*, the sequences in boldface type correspond to the sequences immediately downstream and upstream of *alpZ* that include the stop and start codons (underlined). For the primers *alpZcompl-F*, *alpZcompl-R*, ExpZ1, and ExpZ2, the sequences in boldface type correspond, respectively, to the EcoRI, XbaI, NdeI, and BamHI sites used for cloning.

into pSET152 vector (5) previously digested with the same enzymes, yielding pSET152-*alpZ1* and pSET152-*alpZ2*, respectively. These integrative shuttle vectors were then introduced into the *S. ambifaciens alpZ* deletion strain by means of intergeneric conjugal transfer. For comparison, pSET152 was introduced into the wild-type and *alpZ* deletion strains.

Overexpression of *alpZ*. (i) Overexpression of *alpZ* using the thiostrepton

inducible promoter *tipAp*. Using cosmid F19 as a template, the *alpZ* coding sequence was amplified by PCR with primers ExpZ1/ExpZ2 (Table 2), which include an NdeI and BamHI site, respectively. The PCR product (729 bp) was first cloned into pGEM-Teasy (Promega), yielding pGEM-*alpZexp1*. This vector was digested with NdeI and BamHI enzymes, and the fragment corresponding to the *alpZ* coding sequence (716 bp) was gel purified and ligated

into pIJ8600 (43), previously digested with the same enzymes, yielding pIJ8600-*alpZ*.

(ii) **Overexpression using the strong, constitutive promoter *ermEp**.** Using cosmid F19 as template, the *alpZ* coding sequence was amplified by PCR with primers ExpZ1/*alpZ*-compl-R (Table 2), which includes an NdeI and XbaI site in their respective sequences. The PCR product (743 bp) was first cloned into pGEM-Teasy (Promega), yielding pGEM-*alpZ*exp2. This vector was digested with NdeI and XbaI enzymes, and the fragment corresponding to the *alpZ* coding sequence (731 bp) was gel purified and cloned into pIB139 (50) in which a typical *Streptomyces* ribosomal binding site sequence (AAAGGAGG) was previously inserted between the BamHI and NdeI sites of the MCS region, yielding pIB139-*alpZ*. The conjugative and integrative vectors pIJ8600-*alpZ* and pIB139-*alpZ* were checked for the integrity of the *alpZ* sequence and then introduced by conjugation into wild-type *S. ambifaciens*.

Antibiotic production assays and reverse-phase HPLC. Bioassays were carried out as previously described with *Bacillus subtilis* ATCC 6633 as an indicator strain using plugs from *S. ambifaciens* cultures grown in R2 agar or supernatant from cultures grown R2 liquid medium (34). High performance liquid chromatography (HPLC) for the detection of alpomycin was performed as previously described (34) with a Lichrosphere RP18 column (150- by 2-mm inner diameter; 5- μ m particle size [Merck]).

Heterologous expression and purification of recombinant and mutant AlpZ. The *alpZ* coding sequence was isolated from pIJ8600-*alpZ* (Table 1) after a NdeI/BamHI digest and inserted into the expression vector pET-12a (Novagen) digested with the same enzymes, resulting in pET-*alpZ* (Table 1). The mutated *alpZ* coding sequence [*alpZ*(G341A)] was amplified using high-fidelity DNA polymerase from pGEM-*alpZ*2 (see above and Table 1) with the primer pair ExpZ1/ExpZ2 (Table 2). After terminal dATP addition, the PCR product was ligated with pGEM-Teasy (Promega) and checked by sequencing. After an NdeI/BamHI digest, the *alpZ*(G341A) fragment was inserted into pET-12a digested with the same enzymes, yielding pET-*alpZ*(G341A) (Table 1). The *alpZ* coding sequence contains rare translated arginine codons (AGA and AGG) in *E. coli*. For heterologous expression, competent *E. coli* BL21(DE3) cells (Novagen) were electroporated with pET-*alpZ* or pET-*alpZ*(G341A) along with the pSBET vector (40) that contains an arginine tRNA-encoding gene able to efficiently recognize AGA and AGG codons. *E. coli* BL21(DE3)/pSBET/pET-*alpZ* [or with pET-*alpZ*(G341A)] was grown in LB medium containing ampicillin (50 μ g/ml) and kanamycin (50 μ g/ml) at 37°C and 250 rpm until an optical density at 600 nm of \sim 0.6 was reached. IPTG (isopropyl- β -D-thiogalactopyranoside; 0.1 mM) was added for induction. After a further 4-h incubation at 37°C, cells were collected, resuspended in TE buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8) containing NaCl 200 mM, and then disrupted by sonication. After centrifugation (30 min at 16,000 rpm at 4°C), aliquots of crude protein extract containing AlpZ and AlpZ (G114D) were kept for use in gel retardation experiments. The soluble protein extract fraction containing AlpZ was precipitated with 40% (wt/vol) ammonium sulfate. The pellet containing the partially purified recombinant AlpZ was resuspended in TE buffer-NaCl and applied to a gel filtration column, Ultrolgel AcA44 (Biosepra), previously equilibrated with the same buffer. Fractions containing AlpZ were combined and filtered through a YM10 membrane (cutoff of 10 kDa; Millipore) to remove salts. The residual extract was diluted in TE buffer, and proteins were separated on a DEAE Sepharose column (Pharmacia Biotech). Fractions corresponding to purified AlpZ were pooled and reconcentrated using a YM10 membrane. SDS-PAGE confirmed the purity of a predominantly single band with an apparent migration at 26 kDa.

Gel retardation assays. Labeling of the probes (PCR products or oligonucleotides), gel retardation assays, and chemiluminescence detection were carried out as described in the DIG Gel Shift kit, 2nd generation, following the manufacturer's instructions (Roche). Primer pairs AREU-1/AREU-2, AREXW-1/AREXW-2, AREV-1/AREV-2, and AREZ-1/AREZ-2 were used to amplify DNA probes *alpUp* (296 bp), *alpXWp* (219 bp), *alpVp* (386 bp), and *alpZp* (392 bp), respectively (Table 2; see also Fig. 4B). Primer pairs d-AREV-1/AREV-2, d-AREV-2/AREV-1, d-AREZ-1/AREZ-2, d-AREZ-2/AREZ-1, d-AREXW-1/AREXW-2, d-AREXW-2/AREXW-1, d-AREXW-1/AREXW-2 were used in PCR amplification to obtain d-AREV1 (185 bp), d-AREV2 (201 bp), d-AREZ1 (213 bp), d-AREZ2 (180 bp), d-AREXW1 (172 bp), and d-AREXW2 (47 bp) probes, respectively (Table 2). The *alpU*-ARE, *alpV*-ARE, *alpXW*-ARE, and *alpZ*-ARE probes (40 bp including the ARE sites) were generated by annealing the complementary synthetic deoxyoligonucleotides, ZAREU1 and ZAREU2, ZAREV1 and ZAREV2, ZAREXW1 and ZAREXW2, and ZAREZ1 and ZAREZ2, respectively (Table 2). Probes (20 fmol) end labeled with 3' digoxigenin (DIG)-11-ddUTP were incubated at 30°C for 15 min with purified AlpZ protein (\sim 8 ng) or crude protein extract in binding buffer containing poly(dI-dC), according to the manufacturer's protocols (Roche). When necessary, *S. ambifaciens* extract (1 μ l) or natural or

synthetic γ -butyrolactones was added directly after the 15-min incubation period, and incubation was continued at 30°C for a further 10 min. Reaction mixtures were analyzed by native PAGE using 5% or 8% acrylamide buffered and run in 0.5 \times TBE (Tris-borate-EDTA) buffer (39). DNA was then transferred onto a positively charged nylon membrane (Amersham Hybond-N⁺) by electroblotting (400 mA for 30 min in 0.5 \times TBE buffer) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Light emission was recorded with a Fluor-S MultiImager (Bio-Rad).

Extraction and physical treatments of AlpZ ligand from *S. ambifaciens* culture supernatants. To assess the production timing of AlpZ ligand, *S. ambifaciens* (wild type or the $\Delta\Delta$ *alpID* strain) was grown in 50 ml of R2 medium into 250-ml flasks and incubated at 30°C on a 250-rpm orbital shaker. During the time course, 2-ml samples of supernatant were harvested, extracted twice with 2 ml of ethyl acetate, dried on a rotary evaporator, then redissolved in 20 μ l of methanol, and stored at -20° C. For extraction of ligand quantities sufficient for supplementation experiments, 50 ml of R2 liquid culture of *S. ambifaciens* (wild type or the $\Delta\Delta$ *alpID* strain) was grown until late transition phase-early stationary phase, and culture supernatants (20-ml samples) were extracted twice with one volume of ethyl acetate. The extract was dried, resuspended in 100 μ l of methanol, and stored at -20° C. Any heat (30 min at 100°C), alkali (pH 12 for 10 min, with and without bringing back the pH to the initial level), acid (pH 1.2 for 30 min, with and without bringing back the pH to the initial level), or protease (37°C for 1 h with proteinase K [2 mg/ml; Eurobio], or proteases [2 mg/ml; Sigma] from *S. griseus*) treatments were performed prior to ethyl acetate extraction as described elsewhere (47). Control extracts were made in parallel using noninoculated R2 medium that was treated in the same manner. Extraction of γ -butyrolactones from *S. coelicolor* M145 with ethyl acetate was as described elsewhere (47).

Antibiotic induction assays. Solvent extracts (1 μ l) (see above) were applied directly on confluent lawns of *S. ambifaciens* or *S. coelicolor* spores spread on R2 or SMMS plates, respectively. Plates were incubated for 20 h to 40 h at 30°C.

RESULTS

Precocious production of orange pigment and alpomycin by *alpZ* disruption. To unravel the role of the potential γ -butyrolactone receptor protein encoded by *alpZ* in the regulation of the biosynthesis of alpomycin and orange pigment, the two copies of *alpZ* (one in each *alp* cluster present in each TIR) were deleted as described in Materials and Methods. The genomic DNA isolated from two independent mutants was verified by PCR and Southern analysis for the presence of the mutation in both TIRs and by pulsed-field gel electrophoresis to rule out any large genomic rearrangements (data not shown). The resulting strains lacking *alpZ*, designated the $\Delta\Delta$ *alpZ1* and $\Delta\Delta$ *alpZ2* strains, showed growth and morphological characteristics identical to those of the parent strain on SFM, HT, R2YE and R2 plates (data not shown) or in R2 liquid medium (Fig. 1D). The production of both the orange pigment and the antibacterial activity previously associated with the *alp* cluster was explored in more detail according to the appearance of these compounds during growth in/on R2 medium, a suitable medium for their production (34). Plugs from agar plates or supernatant from liquid-grown cultures of the mutant and parent strains were assessed during a time course for their ability to inhibit the growth of *B. subtilis*. The two mutant strains behaved identically, and only the $\Delta\Delta$ *alpZ1* strain is depicted in the figures. As shown in Fig. 1A, orange pigment production was readily detected in the mutant strain after 19 h of growth on agar plates, while the wild-type strain commenced production only after 36 h. In addition, plugs taken at 17 to 19 h from agar-grown mutant cultures exhibited inhibitory effects on the growth of *B. subtilis* whereas the same inhibitory effect was not observed for the parent strain until 35 to 36 h of growth (Fig. 1B). To ensure that the absence of *alpZ* in the $\Delta\Delta$ *alpZ* strains was responsible for the observed pre-

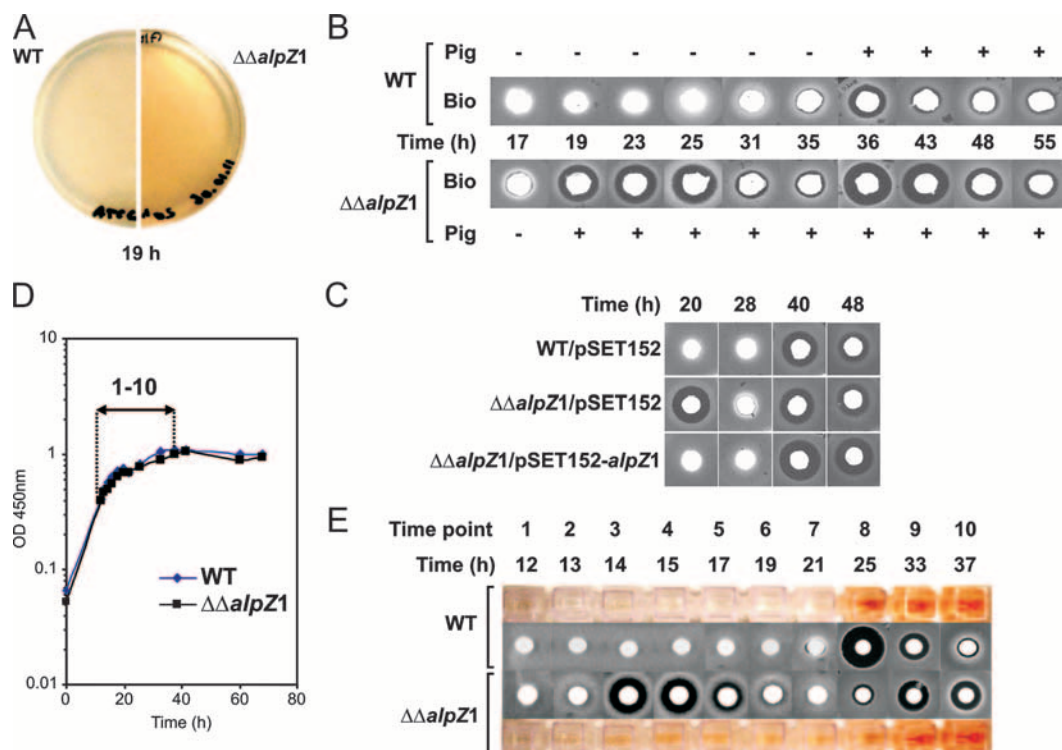


FIG. 1. Precocious production of orange pigment and antibiotic by the *alpZ* deletion strain. (A) Pigment production on R2 agar for the wild-type and *alpZ* knockout ($\Delta\Delta alpZ1$) strains after 19 h of growth at 30°C. (B) Antibiotic activity of wild type and the $\Delta\Delta alpZ1$ strain against *B. subtilis* ATCC 6633 (Bio) and orange pigment production (Pig) was followed through a time course on R2 agar; +, pigmented; -, not pigmented. Agar plugs of mycelia taken at various times were placed on an LB plate spread with *B. subtilis* spores. The antibiotic activity is visualized by the absence of *B. subtilis* growth corresponding to the dark halo surrounding the plug. (C) The pSET152 derivative pSET152-*alpZ1* was used to complement the *alpZ* mutant. Antibiotic activity (see above) of the complemented strain ($\Delta\Delta alpZ1/pSET152-alpZ1$) was compared to that of the control wild type (WT/pSET152) and *alpZ* mutant strains ($\Delta\Delta alpZ1/pSET152$) carrying the empty pSET152 vector. (D) Growth curves of the wild-type and the $\Delta\Delta alpZ1$ strains in R2 liquid cultures. At time points 1 to 10 (indicated by the arrow), total RNAs were isolated for use in the transcriptional studies shown in Fig. 3. (E) The antibiotic activity and orange pigment production at these ten time points are shown. The orange pigment was directly visualized in the spectrophotometer cuvettes. WT, wild type.

cious production of both orange pigment and alpomycin, complementation experiments were carried out by reintroducing a copy of *alpZ* under the control of its own promoter using plasmid pSET152-*alpZ1* (see Materials and Methods). As controls, both the parent and the $\Delta\Delta alpZ$ strains were transformed with the empty pSET152 vector. Compared to the wild-type strain harboring pSET152, the $\Delta\Delta alpZ$ strains bearing the vector alone precociously synthesized pigment (data not shown) and antibiotic (Fig. 1C), in a manner similar to that of $\Delta\Delta alpZ$ strains. The complemented $\Delta\Delta alpZ$ strain containing pSET152-*alpZ1* showed a production of orange pigment (data not shown) and bioactivity (Fig. 1C) over a time course comparable to that of the wild-type strain containing the pSET152 vector, confirming the involvement of *alpZ* in the regulation of orange pigment and alpomycin production. The precocious production of the antibiotic and pigment in the $\Delta\Delta alpZ$ mutant strains was also confirmed during growth in R2 liquid cultures (Fig. 1D). Whereas production of both compounds in the *S. ambofaciens* wild-type strain occurred in the late transition phase or early stationary phase (at 21 h for the antibiotic and by 25 h at the latest for the pigment), that of the mutant strains occurred significantly earlier during the mid-exponential phase (i.e., at 14 h) (Fig. 1E). Intriguingly, a second round of anti-

bacterial activity production was detected in the $\Delta\Delta alpZ$ strains after 36 h on agar-grown cultures (Fig. 1B) and around 25 h in liquid cultures (Fig. 1E). The presence of alpomycin in these early and late antibiotic production periods was confirmed by HPLC analyses (data not shown). The decrease in detectable antibacterial activity between 31 to 35 h in agar (Fig. 1B) and 19 to 21 h in liquid-grown cultures (Fig. 1E) presumably results from both a stop in the production of alpomycin (which is consistent with the reduction in transcript level of the biosynthetic gene, *alpA* (see below and also Fig. 3A), and a concomitant degradation or modification of precociously produced alpomycin (e.g., in orange pigment).

Effect of AlpZ overexpression on orange pigment and alpomycin production. To confirm the role of AlpZ as a repressor, AlpZ was overexpressed in *S. ambofaciens* by inserting the *alpZ* coding sequence under the control of either the strong, constitutive *ermEp** promoter or the thiostrepton-inducible *tipAp* promoter using the conjugative and integrative pIB139 (50) and pIJ8600 (43) vectors, respectively. As a control, *S. ambofaciens* wild-type was conjugated with empty pIB139 and pIJ8600 vectors. Using a final concentration of 50 ng/ml of thiostrepton to induce expression of *alpZ* in the wild-type strain harboring pIJ8600-*alpZ*, the production of pigment was

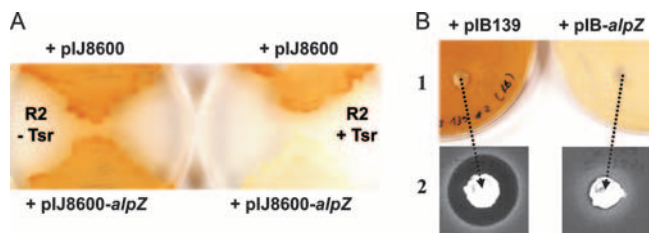


FIG. 2. Effect of the *alpZ* overexpression on pigment and antibiotic production. (A) The wild-type strain harboring pIJ8600 (control) or pIJ8600-*alpZ* was streaked on R2 agar in the absence (-Tsr) or presence of 25 μ g/ml thioestrepton (+Tsr) to induce *alpZ* expression. Plates were photographed from below after 4 days of growth at 30°C. (B) The wild-type strain harboring pIB139 (control) or pIB139-*alpZ* (constitutive expression of *alpZ*) was streaked on R2 agar. Plates were photographed from below after 3 days of growth at 30°C (panel 1). Antibiotic production was tested on *B. subtilis* with agar plugs of mycelia taken from the above plates (panel 2).

delayed by 2 days. However, when higher concentrations of inducer were employed (e.g., 25 μ g/ml of thioestrepton), the production of pigment was completely blocked (even after 2 weeks of growth) compared to the control strain (Fig. 2A). Strains overproducing AlpZ in a constitutive manner (by means of pIB139-*alpZ*) exhibited a 2-day delay in the production of both antibiotic and diffusible orange pigment compared to the control strain (Fig. 2B). Taken together, these results corroborate the role of AlpZ as a key repressor of the *alp* cluster.

Transcriptional analyses in the *alpZ* deletion strain. The role of *alpZ* as a repressor of pigment and alpomycin production was reinforced by comparative transcriptional analyses between wild-type and *alpZ* mutant strains. The presence of

transcripts for the β -ketoacyl synthase gene, *alpA*, and the five regulatory genes *alpT*, *alpU*, *alpV*, *alpW*, and *alpZ* was investigated by RT-PCR in the parent and *alpZ*-deleted strains grown in R2 liquid medium cultures. Total RNAs were isolated from mycelium samples taken from the same time points for which antibiotic production was assessed in the experiment shown in Fig. 1D and E. The transcript of the major and essential sigma factor gene, *hrdB*, which is expressed at almost constant level during the growth, was used as an internal control (7, 38) (Fig. 3A). In negative controls containing DNA polymerase but lacking reverse transcriptase, amplified products were not detected (data not shown). In the wild-type strain, the transcription of the biosynthetic *alpA* gene was shown to be induced during the late transition phase (i.e., at 19 h), following induction of the essential activator gene *alpV* (at 17 h) and prior to the onset of alpomycin production (at 21 h) (Fig. 3B). In the strain lacking *alpZ*, transcripts of both *alpA* and *alpV* were readily detectable at 12 h during the mid-exponential phase (Fig. 3A). This is in full agreement with the observed precocious production of pigment and alpomycin in the $\Delta\Delta alpZ$ strain after 14 h of growth (Fig. 1D and E). Moreover, the second round of bioactivity production observed in the $\Delta\Delta alpZ$ strain after 25 h could be correlated with the resumption of *alpV* and *alpA* expression. Expression of the other regulatory genes, *alpT*, *alpU*, *alpZ*, and *alpW*, was also induced significantly earlier (at 12 h) in the *alpZ* mutant, suggesting that the product of *alpZ* may have multiple targets and may also regulate its own expression.

In contrast to the four other *alp* regulatory genes, transcripts of *alpZ* were readily detectable even at early time points in the wild-type strain before the onset of alpomycin and orange pigment. This is consistent with the idea that the product of

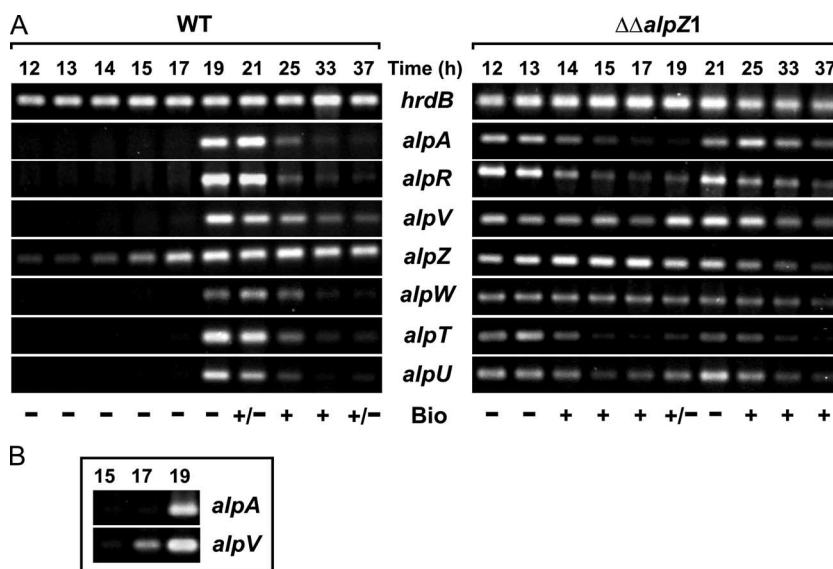


FIG. 3. Comparative transcriptional analyses of *alp* genes by RT-PCR. (A) Transcripts of *hrdB* (major and essential sigma factor gene; internal control), *alpA* (β -ketoacyl synthase gene involved in both orange pigment and alpomycin biosynthesis), *alpR* (the second *alp*-located β -ketoacyl synthase gene not involved in orange pigment and alpomycin biosynthesis), *alpV* (essential SARP activator gene), *alpZ* (repressor gene), *alpW* (deduced repressor gene), *alpT* (SARP gene), and *alpU* (SARP gene) from wild type (WT) and *alpZ* mutant ($\Delta\Delta alpZ1$) strains were analyzed by RT-PCR with 25 cycles of PCR on cDNA generated from RNA isolated from late exponential phase to early stationary phase (time points 1 to 10) (Fig. 1D). Assessed bioactivity (Bio) against *B. subtilis* at time points 1 to 10 (Fig. 1E) are indicated. +, detected; -, not detected. (B) To detect low-level transcript of *alpV* in the wild type at 17 h, 28 cycles of PCR were applied.

alpZ is needed during the early stages of growth to repress the production of alpomycin.

Another β -ketoacyl synthase present within the *alp* cluster and encoded by *alpR* has previously been shown to be dispensable for the synthesis of either the orange pigment or alpomycin (34). However, analysis of *alpR* expression by RT-PCR showed a pattern of transcription similar to that of *alpA* in both the wild-type and the $\Delta\Delta alpZ$ mutant backgrounds (Fig. 3A). It thus seems that expression of *alpR* (and presumably that of its operonic partner *alpQ*, encoding a chain length factor subunit) is efficiently regulated by AlpZ, perhaps through regulation of AlpV. The AlpZ regulon may thus include another product whose structure and role have yet to be properly defined.

DNA-binding by recombinant AlpZ. Candidate ARE-like sequences to which γ -butyrolactone receptor proteins typically bind (13) are present in the promoter regions of *alpZ*, *alpV*, and *alpU* (1). A similar ARE-like sequence was also detected in the promoter region of the putative *alpX*-*alpW* operon (Fig. 4A). In sum, the possible AlpZ binding sites are therefore located 60 bp upstream of the *alpZ* coding sequence, 90 bp upstream of *alpV*, 40 bp upstream of *alpX*/*alpW*, and 205 bp upstream of *alpU*, which is found divergent from *alpT* (Fig. 4B). To determine whether the γ -butyrolactone receptor-like protein AlpZ truly targets these promoter regions, gel mobility shift assays were performed using DNA fragments encompassing the ARE-like sequences (ranging from 219 bp to 392 bp and designated *alpZp*, *alpVp*, *alpXWp*, and *alpUp*) in the presence of purified recombinant AlpZ protein (Fig. 4B) (see Materials and Methods). Shifts were clearly observed with the probes *alpVp*, *alpZp*, and *alpXWp* but never with *alpUp*, demonstrating that AlpZ directly binds to its own promoter region and that of *alpV* and *alpXW* but was unable to bind to the promoter region of *alpU* (Fig. 4C). The ARE-like sequence upstream of *alpU* is, however, the least conserved compared to the consensus sequence. Competition with unlabeled probe DNA showed a release of the shift, verifying the specificity of the observed AlpZ interaction with the promoter regions of *alpV*, *alpZ*, and *alpXW* (Fig. 4C). In addition, use of labeled DNA fragments containing only one-half of the predicted *alpVp*, *alpZp*, or *alpXWp* ARE sequences in the gel shift assays never resulted in a shift, indicating that binding of AlpZ to these promoter regions depends upon the presence of the complete ARE motifs (data not shown). To confirm this hypothesis, shorter probes (40-bp deoxyoligonucleotides) bearing one or the other of the four *alp*-ARE sequences (26 bp) were used in further mobility shift assays. Formation of an AlpZ-DNA complex was observed for the *alpZ*-ARE, *alpV*-ARE, and *alpXW*-ARE probes but not with *alpU*-ARE even when increased concentrations of purified AlpZ were used (Fig. 4D). Altogether, these results demonstrate that AlpZ binds to a specific DNA sequence that resembles the ARE motifs recognized by typical γ -butyrolactone receptors.

Influence of solvent-extractable material on DNA-binding activity of AlpZ. In the context of AlpZ belonging to the autoregulator receptor family, the derepression of the alpomycin production might be alleviated by γ -butyrolactone-type molecules. Several members of the autoregulator receptor family have been shown to respond to small signaling molecules (the γ -butyrolactones) that are extractable from the

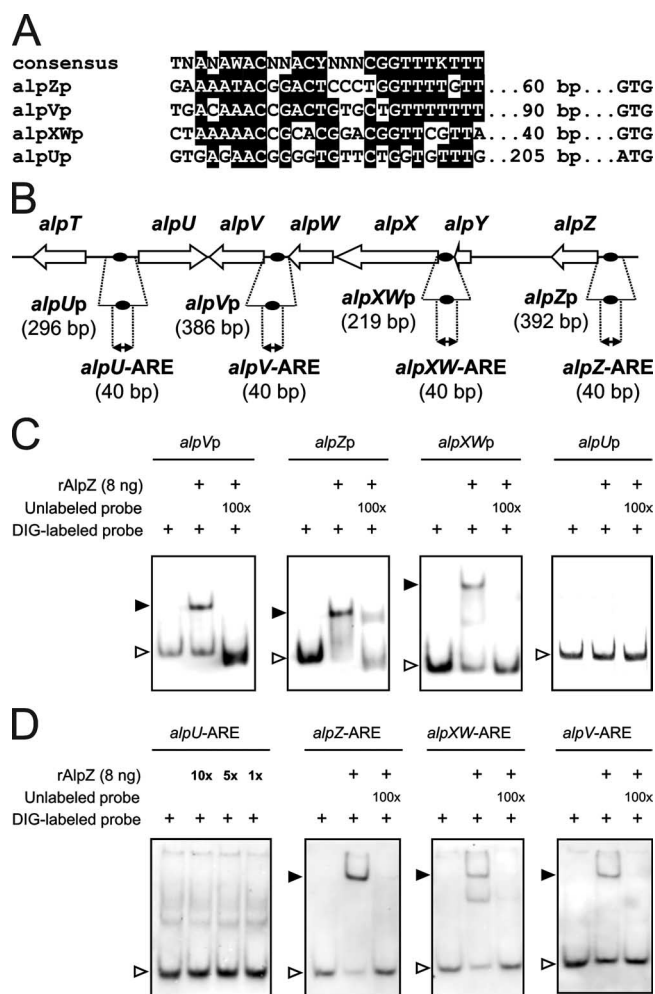


FIG. 4. DNA-binding activity of AlpZ. (A) Alignment of the putative AlpZ binding sequences. The consensus corresponds to the ARE consensus defined in Folcher et al. (13). The distance between the ARE sites and the start codon of the downstream gene is indicated. The letters W, Y, and K stand for A or T, C or T, and G or T, respectively. (B) Probes used in gel shift assays. The genetic organization of the *alp* regulatory subcluster is shown. Filled ovals symbolize the location of the ARE sequences. Sizes (bp) and names of the probes are indicated. (C) Gel shift assay using PCR probes including the respective *alp*-ARE motives. DIG-labeled probes (~ 20 fmol) were incubated in the presence (+) or absence of purified recombinant AlpZ (rAlpZ; ~ 8 ng). In competition experiments, excess of unlabeled probes ($100\times$, ~ 2 pmol) was added in the reaction mixture. (D) Gel retardation assay using synthetic short *alp*-ARE probes. DIG-labeled probes (~ 20 fmol) were incubated in the presence (+) or absence of purified recombinant rAlpZ (~ 8 ng). In competition experiments, excess of unlabeled probes ($100\times$, ~ 2 pmol) was added to the reaction mixture. In the assay using the *alpU*-ARE probe, increasing amounts of rAlpZ were tested: $1\times$, 8 ng; $5\times$, 40 ng; $10\times$, 80 ng. Open and filled triangles denote the migration of the probe alone and of probe-rAlpZ complex, respectively.

aqueous fermentation broth by the use of organic solvents. In addition, these molecules were able in gel retardation experiments to disrupt binding of the respective γ -butyrolactone receptor from their cognate DNA targets.

To determine whether similar signaling molecules were produced by *S. ambifaciens*, samples of supernatant from R2

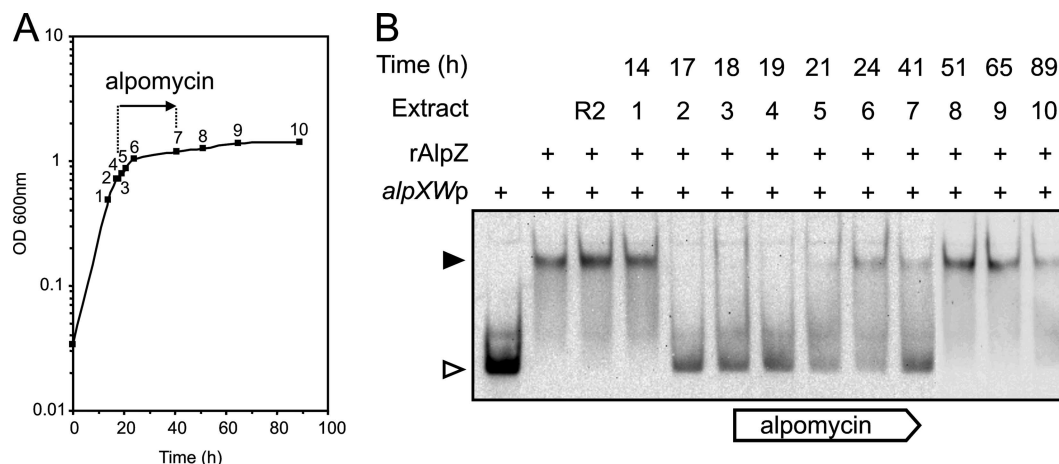


FIG. 5. Production of autoregulator molecules by *S. ambofaciens*. (A) Growth curve of wild-type *S. ambofaciens* in R2 medium. Growth was followed via optical density (OD) measurement at 600 nm. Production of alpomycin, tested with the standard bioassay (see Materials and Methods), is shown by the arrow. Time points 1 to 10 at which a supernatant sample was extracted with ethyl acetate are indicated by black squares. (B) Gel shift assay to address the presence of AlpZ-disrupting material in *S. ambofaciens* extracts. The DIG-labeled probe *alpXWp* (20 fmol) was incubated with 8 ng of recombinant AlpZ (rAlpZ) before addition of extracted material from time points 1 to 10 (as described in panel A). R2 denotes the (negative) control (noninoculated R2 medium extracted under the same conditions). Time points at which alpomycin was detected by bioassay are depicted by the arrows. Open and filled triangles denote the migration of the probe alone and of probe-rAlpZ complex, respectively.

liquid-grown cultures taken over a time course (Fig. 5A) were extracted with ethyl acetate, and the processed extracts were assayed by means of gel retardation experiments for their ability to disrupt DNA-bound AlpZ from the probe *alpXWp* (Fig. 5B). Loss of the formation of a shifted probe complex indicated the presence of a factor capable of disrupting the AlpZ-DNA interaction. This activity was detectable in cultures from early transition phase (at 17 h), 2 h before the onset of alpomycin production, up to about 24 h into stationary phase. Under these culture conditions, the absence of detectable amounts of the disrupting activity in late-stationary phase coincided with the cessation of alpomycin production. Control extracts from uninoculated R2 medium showed no activity in the assay. Extracts that disrupted the binding of AlpZ to the probe *alpXWp* also disrupted binding of AlpZ with the probes *alpZp* and *alpVp* and with the short probes *alpZ-ARE*, *alpV-ARE*, and *alpXW-ARE* (data not shown). AlpZ-DNA complex formation was also disrupted using ethyl acetate extracts obtained from the *S. ambofaciens* mutant strain $\Delta\Delta\text{alpID}$ (Table 1 and Materials and Methods) (data not shown). This strain lacks the minimal PKS (*alpABC*) involved in the synthesis of the core polyketide compound, thus indicating that the active factor is not derived from the alpomycin-biosynthetic pathway.

Influence of solvent-extractable material on pigment and antibiotic production. In *S. coelicolor* A(3)2, the γ -butyrolactone SCB1 induces the production of the two pigmented antibiotics, undecylprodigiosin and actinorhodin, when applied to a confluent lawn of growing mycelia (47). Based on this assay, a drop of the *S. ambofaciens* extract that showed disrupting activity of the AlpZ-DNA complex was spotted onto a lawn of wild-type *S. ambofaciens*. No orange halo was visible, but an inhibition zone was observed around the spot (data not shown). To avoid this inhibitory effect, perhaps due to the presence of alpomycin, an extract from the alpomycin-nonproducing $\Delta\Delta\text{alpID}$ strain was utilized. In this case, a pink/orange-

pigmented halo was now visible around the spot after 20 h of growth (Fig. 6). To test whether the observed precocious pigment production also correlated with precocious induction of antibiotic activity, agar plugs from the pigmented halo region were taken and analyzed in a bioassay against *B. subtilis* (Fig. 6). Compared to plugs taken from around the plate spotted with solvent extract from R2 medium, plugs in the halo showed an enhanced bioactivity, suggesting that material in the extract was also able to induce the production of antibiotic by the wild-type strain. The same experiment was also performed using a lawn of the $\Delta\Delta\text{alpID}$ strain instead of the wild-type strain. No halo around the extract spot of the $\Delta\Delta\text{alpID}$ strain

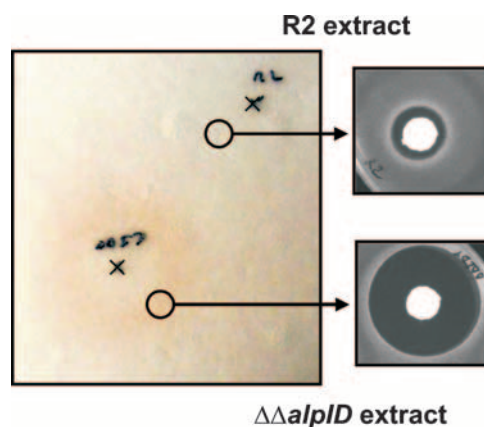


FIG. 6. Induction of orange pigment and antibiotic production by the AlpZ-interactive ligand(s). Extracts (1 μ l) from supernatant culture of the *S. ambofaciens* mutant $\Delta\Delta\text{alpID}$ strain, which lacks the biosynthetic genes responsible for alpomycin synthesis (34), and from noninoculated R2 medium (negative control) were applied (at the X sign) on a confluent lawn of *S. ambofaciens* wild-type spores. After 20 h of incubation at 30°C, an agar plug of mycelia (symbolized by the open circle) taken in the vicinity of the dropped extracts was tested for the production of bioactivity against *B. subtilis*.

was observed, demonstrating that the pink/orange halo and bioactivity detected above are derived from the *alp* cluster. To verify that the precocious bioactivity observed around the spot was not due to extracted and reconcentrated antibiotic compounds (e.g., spiramycin, which is known to be produced by *S. ambofaciens*), an equivalent amount of the $\Delta\Delta alpID$ strain extract was dropped on a noninoculated R2 plate. The plug taken in the vicinity of the “control” spot did not reveal any detectable bioactivity (data not shown). The production of alpomycin in *S. ambofaciens* is therefore stimulated by solvent-extractable material from *S. ambofaciens* cultures, presumably the same that disrupted the DNA-binding activity of AlpZ. This material was also tested for induction of the two pigmented antibiotics on an *S. coelicolor* M145 lawn without success. Conversely, an *S. coelicolor* M145 extract did not induce orange pigment and antibiotic production in *S. ambofaciens*, a result consistent with the inability of SCB1 to disrupt AlpZ from its DNA target in gel retardation assays (data not shown).

Effect of a single amino acid substitution on ligand binding ability by AlpZ. When vectors for complementation experiments of the *alpZ* mutant (the $\Delta\Delta alpZ$ strain) were constructed, one of them, pSET152-*alpZ2*, contained a single base mutation (G341A) in the *alpZ* coding sequence, resulting in the nonconservative substitution of the glycine (Gly) in position 114 (with respect to the start codon) to an aspartate (Asp). Surprisingly, after introduction of the pSET152-*alpZ2* vector into the $\Delta\Delta alpZ$ and wild-type strains, orange pigment and alpomycin production was abolished, even after prolonged incubation (Fig. 7A). According to the molecular mechanism model of γ -butyrolactone autoregulator receptors based on site-directed mutagenesis analyses of ArpA (32, 42) and crystallization studies of CprB (30), Gly114 of AlpZ would be located in the regulatory domain of the protein, i.e., the domain that interacts with the ligand. It is therefore possible that the loss of pigment and alpomycin production results from a constant repression of the *alp* cluster by the mutated AlpZ [AlpZ(G114D)] independently from the presence of disrupting ligand. To test this hypothesis, recombinant AlpZ(G114D) was used in gel retardation experiments in the presence of isolated AlpZ-disrupting ligand(s). Amounts of the respective recombinant AlpZ and AlpZ(G114D) proteins in the crude extracts used were estimated by SDS-PAGE (data not shown). Gel retardation assays were carried out with limiting amounts of both recombinant proteins to assess their ability to be displaced from their DNA target sequence by the *S. ambofaciens* disrupting material. AlpZ(G114D) was still able to bind to the tested cognate recognition sites of the probes *alpXWp* (Fig. 7B) and *alpVp* (data not shown), revealing that the mutation did not affect the DNA-binding activity of the mutated regulator. However, when solvent-extractable material that contains the AlpZ ligand was incubated with the DNA probe-AlpZ(G114D) complexes, the loss of the shift, observed in the case of the native AlpZ, was never observed (Fig. 7B). This result strongly suggests that the substitution of Gly114 to Asp rendered AlpZ(G114D) insensitive to its ligand (although the exact mechanism remains to be determined). Consequently, AlpZ(G114D) could continuously repress the target genes (particularly *alpV*, encoding an essential activator of the *alp* cluster), despite the presence of disrupting ligand. This readily

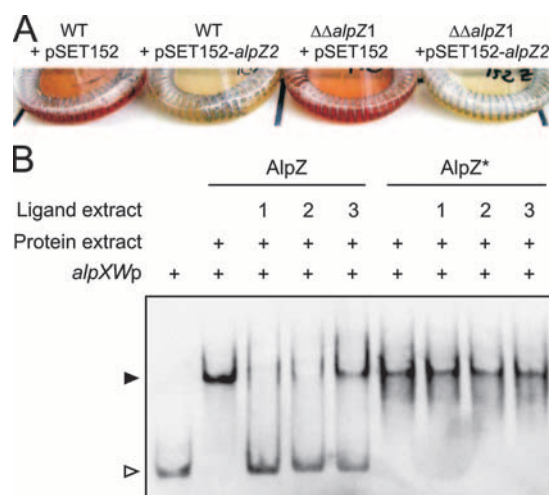


FIG. 7. Effect of a single amino acid substitution (Gly114 to Asp) in AlpZ. (A) Orange pigment production in R2 liquid cultures of the wild type and the $\Delta\Delta alpZ1$ strains carrying pSET152 (5) or pSET152-*alpZ2* (Table 2) which contains the mutated sequence of *alpZ*. Fermentation flasks were photographed after 5 days of growth at 30°C under 250 rpm constant shaking. (B) Gel retardation assay comparing the DNA-binding activity and sensitivity to the AlpZ-interactive ligand of the native AlpZ and the mutated AlpZ(G114D) (AlpZ*). The DIG-labeled probe *alpXWp* (see Fig. 4) was incubated with a limiting amount of AlpZ and AlpZ(G114D) (i.e., the minimal quantity of protein required to fully retard the probe) before addition (+) of ligand extract. Lane 1, standard extract; lane 2, 10-fold dilution; lane 3, 20-fold dilution. Open and filled triangles denote the migration of the probe alone and of probe-recombinant AlpZ complex, respectively. WT, wild type.

explains the lack of detectable orange pigment and alpomycin antibiotic in the *S. ambofaciens* strains expressing AlpZ (G114D).

Furthermore, the observed in vitro and in vivo activity of the mutant AlpZ protein is fully consistent with the proposal that AlpZ acts as a repressor of the *alp* cluster and that the presence of the ligand is essential for the activation of alpomycin and pigment production.

Properties of the AlpZ-interactive ligand(s). In order to assess the nature of the AlpZ-interactive ligand, we examined the ability of several known natural and synthetic γ -butyrolactone molecules belonging to the three defined subclasses of γ -butyrolactones to prevent shifting of DNA probes by AlpZ. The γ -butyrolactones tested included A-factor from *S. griseus*, virginiae butanolides (and derivatives) from *S. virginiae*, SCB1 from *S. coelicolor*, and IM-2 of *S. lavendulae* (kindly provided by T. Nihira and S. Kitani). None of them was able to significantly disrupt DNA-bound AlpZ (data not shown), suggesting either a strong specificity of AlpZ for its cognate ligand or a molecule with a significantly different structure.

Members of the γ -butyrolactone family have previously been shown to be resistant to proteases and to heat and acidic treatments but sensitive to treatment with alkali to pH 12. The instability in alkali is believed to result from opening of the lactone ring, rendering the molecules inactive (14, 47). In this study, the active compound in the *S. ambofaciens* cultures was surprisingly found to be resistant to all treatments, including alkali (Fig. 8). The solvent extract from noninoculated R2 medium that received the same treatments failed to disrupt

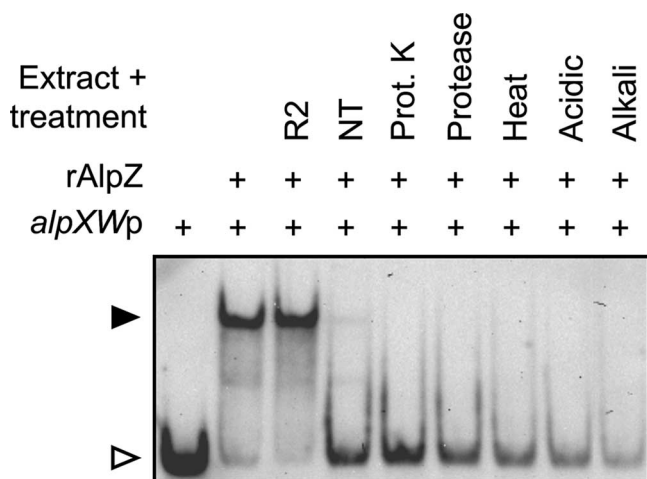


FIG. 8. AlpZ-interactive ligand properties. Gel shift experiments using the DIG-labeled probe *alpXWp* (20 fmol) and recombinant AlpZ (rAlpZ; 8 ng) were carried out with ligand extracts exposed to various treatments. NT, not treated; prot K, proteinase K; protease, proteases from *S. griseus*; heat, 100°C for 30 min; acidic, pH 1.2 for 30 min; alkali, pH 12 for 10 min. R2 corresponds to extract from noninoculated R2 medium.

binding of AlpZ from DNA probes (data not shown). In addition, a control was done with extract from the *S. coelicolor* M145 strain treated in the same way. After the alkali treatment, the *S. coelicolor* M145 extract failed to induce pigmented antibiotic production in the producing strain compared to the untreated extract, proving that the alkali treatment efficiently inactivated γ -butyrolactone molecules (data not shown). It therefore appears that the AlpZ-interacting ligand(s) present in *S. ambofaciens* is structurally different from typical γ -butyrolactones and lacks the lactone ring. This could readily explain the absence of the complementation effect by the various investigated γ -butyrolactone molecules.

DISCUSSION

A model for the regulatory cascade leading to alpomycin production. The regulation module of the *alp* cluster consists of five regulatory genes, encoding SARPs (*alpV*, *alpT*, and *alpU*) and proteins from the TetR receptor family (*alpZ* and *alpW*). Of these, AlpV has previously been shown to be an essential activator for the synthesis of alpomycin and orange pigment (1). The present study indicates that AlpZ is a transcriptional repressor that prevents alpomycin and pigment production during the early stages of growth. AlpZ was shown to bind both within its own promoter region and to the promoter regions of *alpV* and *alpXW*. In addition, expression of *alpZ*, *alpV*, and *alpW* is accelerated to the early stages of growth in the *alpZ* deletion strain. Taken together, these results indicate that AlpZ directly represses its own transcription and that of *alpV* and *alpW*. On the other hand, AlpZ does not seem to directly repress the transcription of the two other SARP genes, *alpU* and *alpT*, since gel retardation experiments failed to detect binding of AlpZ in their divergent promoter regions. AlpZ does, however, influence expression of these two genes since their transcription occurs significantly earlier in growth in

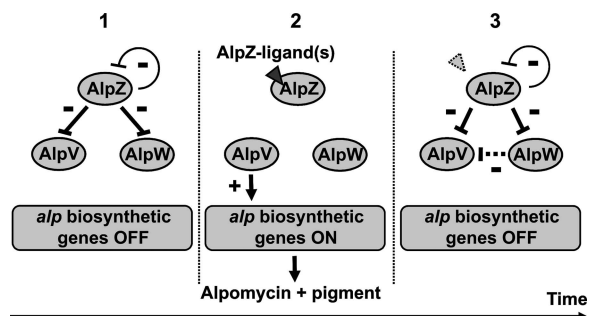


FIG. 9. Regulation model of the *alp* regulatory cascade. In the early stages of growth (panel 1), the product of *alpZ* represses the transcription of both *alpV* and *alpW*. The absence of AlpV activator prevents expression of the *alp* biosynthetic genes. When AlpZ-interactive ligands are produced (panel 2), AlpZ is dissociated from its DNA targets, which correspond to specific sequences in the promoter region of *alpV* and *alpW*, allowing transcription of the latter genes. In turn, AlpV triggers the expression of the biosynthetic genes leading to the production of alpomycin and orange pigment. Derepression of *alpW* transcription could result in accumulation of the autoregulator repressor AlpW that has been shown in preliminary gel shift assays to bind within the promoter region of *alpV*. Thus, AlpW could help to block *alpV* transcription in the late stages of growth, thereby stopping production of alpomycin and orange pigment (panel 3). Dilution or degradation of AlpZ ligands may also restore the original repression state by AlpZ.

the absence of AlpZ. The regulation of expression of these two genes most probably involves at least one intermediate regulator that is itself regulated by AlpZ. In addition, extractable material(s) produced during early transition phase by *S. ambofaciens* cultures was shown to dissociate AlpZ from its DNA promoter targets and to trigger the onset of both orange pigment and alpomycin production. This is consistent with a model (Fig. 9) in which AlpZ is located at the top of the regulatory cascade, negatively regulating *alpV* during the early stages of growth. Upon binding of its interactive ligand, which is produced in transition phase, AlpZ is displaced from the promoter region of *alpV*, leading to derepression of the gene. In turn, AlpV activates transcription of the biosynthetic genes (*alpABC*), leading to the synthesis of the polyketide compounds. Dilution or degradation of the ligand(s) or accumulation of AlpZ repressor may then explain the transient production of alpomycin. However, preliminary experiments showed that recombinant AlpW was also able to bind the promoter region of *alpV* (data not shown). Moreover, overexpression of *alpW* blocked alpomycin and pigment production in both the parent and the $\Delta\Delta\text{alpZ}$ mutant strains (data not shown), suggesting that the product of *alpW*, which hence seems to act as a repressor, is hierarchically positioned under AlpZ. The transient production of alpomycin could thus also be explained by the derepression of *alpW* transcription by AlpZ when the interacting ligand is produced, leading to the accumulation of AlpW. In turn, AlpW would block expression of *alpV*. Consequently, expression of the structural genes that are under control of AlpV would be abolished, thereby preventing synthesis of the polyketide compounds.

Interestingly, this regulatory cascade also affects expression of other *alp*-located structural genes: *alpR* (that is predicted to encode a β -ketoacyl synthase subunit) and its operonic partner

alpQ (that would encode a chain length factor subunit), which form two-thirds of a minimal PKS that is most highly homologous to the minimal PKS subunits involved in mithramycin synthesis (34). Although the product of *alpR* is not involved in the synthesis of either the diffusible pigment or alpomycin (34), transcriptional analyses in the $\Delta\Delta\text{alpZ}$ strain revealed that expression of *alpR* was affected in a manner similar to that of *alpA*. The *alpRQ* operon is thus actively coregulated with *alpABC*. If *alpR* is not involved in the synthesis of compounds associated so far with the *alp* cluster, its active regulation leads to the supposition that another compound could be produced by AlpR and AlpQ (presumptively together with AlpC, the acyl carrier protein of the minimal PKS involved in alpomycin and orange pigment synthesis). Studies of mutants lacking *alpA* and *alpB* but possessing *alpC* along with *alpR* and *alpQ* are under investigation and will possibly allow us to identify another polyketide compound synthesized by the *alp* cluster.

Nature of AlpZ by comparison with CprB crystal structure and analysis of the effect of Gly114 replacement by Asp in AlpZ(G114D). Study of AlpZ(G114D), which was still able to bind DNA but was insensitive to its cognate ligand, strengthened the role of AlpZ as a repressor. On the basis of the crystal structure analysis of the related repressor CprB of *S. coelicolor* (30), the overall tertiary structure of AlpZ(G114D) was not modified compared to AlpZ (analysis using the DeepView program of the Swiss-PdbViewer; <http://www.expasy.org/spdbv/>). CprB was shown to be composed of two domains, a DNA-binding domain (residues 1 to 52) and a regulatory domain (residues 77 to 215). According to the amino acid sequence alignment between available γ -butyrolactone receptor-like proteins (including CprB) and AlpZ, Gly114 is located in the regulatory domain and, more precisely, between helices $\alpha 6$ and $\alpha 7$ (that are involved in the ligand binding pocket formation) in a weakly conserved region among these related proteins. Nevertheless, hydrophobic residues (which are mostly not well conserved) within the ligand binding pocket seem to play an important role in the recognition of the ligand in terms of specificity. The mutation may thus impair either the recognition or the proper binding of the ligand by AlpZ(G114D). As the shift of helix $\alpha 6$ upon ligand binding is predicted to induce the relocation of helix $\alpha 4$ from the DNA-binding domain (30), it is also conceivable that the substitution may affect the normal conformational change leading to the relocation of the DNA-binding domain and subsequently the dissociation of the repressor from DNA. In both hypotheses, the nonconservative mutation of Gly114 (hydrophobic) to Asp (polar) renders impossible the release of AlpZ(G114D) to its DNA targets, which, in turn, permanently blocks alpomycin production.

A new type of small signaling molecule produced by *S. ambofaciens*. Since the material extracted from *S. ambofaciens* before and during alpomycin production was able to induce antibiotic production in young wild-type *S. ambofaciens* mycelia and to disrupt DNA-binding ability of AlpZ, a γ -butyrolactone autoregulator receptor homolog, it seemed evident, a priori, that this material could belong to the γ -butyrolactone group of small signaling molecules that are widespread in streptomycetes. However, in this study, several different natural and synthetic γ -butyrolactone molecules belonging to the three described classes (according to the length and branching of the C-2 acyl side chain and stereochemistry at C-6) were

tested in gel shift assays for their ability to release AlpZ from its DNA target, but none of them could significantly dissociate DNA-bound AlpZ. This may indicate a strict specificity of the AlpZ-ligand(s) for AlpZ but could also reflect a molecular structure that is distinct from that of the other butyrolactones. Indeed, typical γ -butyrolactones lose activity following alkali treatment, but the *S. ambofaciens* extractable material was insensitive to this treatment, suggesting that the *alp* autoinducer lacks the lactone group characteristic of the γ -butyrolactones (although we cannot totally exclude the possibility that a small but sufficient amount of active material can survive this treatment and interact with AlpZ). To our knowledge, our results support the first demonstration that a member of the so-called γ -butyrolactone autoregulator receptor family could interact with molecules different from γ -butyrolactones. This finding should extend our overview on the diversity of autoregulatory systems. Further studies aimed at purifying and characterizing the AlpZ ligand(s) are currently under way.

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