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Differential and Cross-Transcriptional Control of Duplicated Genes Encoding Alternative Sigma Factors in *Streptomyces ambofaciens*

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The duplicated *hasR* and *hasL* genes of *Streptomyces ambofaciens* encode alternative sigma factors (named σ^{Br} and σ^{Bl}) belonging to the σ^{B} general stress response family in *Bacillus subtilis*. The duplication appears to be the result of a recent event that occurred specifically in *S. ambofaciens*. The two genes are 98% identical, and their deduced protein products exhibit 97% identity at the amino acid level. In contrast with the coding sequences, their genetic environments and their transcriptional control are strongly divergent. While *hasL* is monocistronic, *hasR* is arranged in a polycistronic unit with two upstream open reading frames, *arsR* and *prsR*, that encode putative anti-anti- σ and anti- σ factors, respectively. Transcription of each *has* gene is initiated from two promoters. In each case, one promoter was shown to be developmentally controlled and to be similar to those recognized by the *B. subtilis* general stress response sigma factor σ^{B} . Expression from this type of promoter for each of the *has* genes dramatically increases during the course of growth in liquid or on solid media and following oxidative and osmotic stresses. Reverse transcription-PCR measurements indicate that *hasR* is 100 times more strongly expressed than *hasL* from the σ^{B} -like promoter. Transcription from the second promoter of each gene (located upstream of *arsR* in the case of the *hasR* locus) appears to be constitutive and weak. Quantitative transcriptional analysis in single and double *has* mutant strains revealed that σ^{Br} and σ^{Bl} direct their own transcription as well as that of their duplicates. Only a slight sensitivity in response to oxidative conditions could be assigned to either single or double mutants, revealing the probable redundancy of the σ factors implied in stress response in *Streptomyces*.

Gene duplication is a key mechanism for genome evolution. In contrast to eukaryotes, duplication of genes in prokaryotes is considerably less well documented. However, according to the work of Ikeda et al. (20), 35% of the 7,574 *Streptomyces avermitilis* open reading frames (ORFs) cluster into 721 paralogous families, with membership ranging from 2 to 91 genes per family. The membrane-spanning components of the ATP binding cassette (ABC) transporters, the two-component transcriptional regulator systems, and the σ factors are the most prominent examples of such gene families (20). The *Streptomyces coelicolor* A3(2) (4) and *S. avermitilis* (20) genomes each contain 65 and 60 σ -factor-encoding genes, respectively, many more than the 7 reported in *Escherichia coli* (5), 16 in *Bacillus subtilis* (23), and 13 in *Mycobacterium tuberculosis* (9), the genus most closely related to *Streptomyces*. According to the classic duplication-degeneration-complementation model (14), gene duplication can lead to the overproduction of a gene product. The conservation of duplicated genes on the evolutionary scale might also involve subfunctionalization of both members of the

pair. Each duplicate can undergo complementary degenerative mutations involving reduction or specialization of its expression. The combined action of both loci would be necessary to ensure the ancestral function. In *Streptomyces*, the large-scale duplication phenomenon might correlate with a complex life cycle associated with a high level of differentiation at both the morphological (formation of aerial mycelium and sporulation) and biochemical (production of numerous secondary metabolites) levels.

Among the 65 sigma factor genes in *S. coelicolor*, nine, homologous to the *B. subtilis* stress response factor σ^{B} , were found to group into a subfamily with the deduced proteins exhibiting 38 to 73% identity (4) (*sigF*/SCO4035, *sigG*/SCO7341, *sigH*/SCO5243, *sigI*/SCO3068, *sigJ*/SCO0600, *sigK*/SCO6520, *sigL*/SCO7278, *sigM*/SCO7314, and *sigN*/SCO4034; *sig* gene names are according to the Kelemen nomenclature; G. Kelemen, personal communication). A similar situation was revealed in *S. avermitilis* (20), showing that the σ^{B} subfamily resulted from duplication events that probably arose in the *S. coelicolor*-*S. avermitilis* common ancestor.

One member of this family, called *sigB* (8) or *sigJ* (40) in *S. coelicolor* and SAV741 in *S. avermitilis*, was found in two copies in *Streptomyces ambofaciens* (13). The two copies were named *has* for homologous to alternative sigma factors and are 98% identical at the nucleotide level (13). Such a high level of identity between two duplicated genes is rare in a *Streptomyces* species. For example, the *glgBI* and *glgBII* genes of *S. coelicolor* exhibit only 73% nucleotidic identity (*glgBI* is involved in glycogen metabolism during vegetative growth, while *glgBII* participates in the accumulation of glycogen in spores and is

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Property(-ies) ^a	Reference
Strains		
<i>S. ambofaciens</i>		
ATCC 23877	wt	31
ETH11317	wt	19
DSM40697	wt	19
NSAR1, NSAR2, NSAR3	<i>hasR</i> replaced by <i>aac(3)IV/oriT</i> cassette	This study
NSAL1, NSAL2, NSAL3	<i>hasL</i> replaced by <i>aac(3)IV/oriT</i> cassette	This study
NSARL1, NSARL3	<i>hasR</i> and <i>hasL</i> replaced by <i>aac(3)IV/oriT</i> and <i>aadA/oriT</i> cassettes, respectively	This study
NSALR2, NSALR7	<i>hasL</i> and <i>hasR</i> replaced by <i>aac(3)IV/oriT</i> and <i>aadA/oriT</i> cassettes, respectively	This study
NSAR2-s1, NSAR2-s2	NSAR2 with pSETΩ integrated into the chromosomal <i>attB</i> site of ϕC31	This study
NSAR2-s _R 1, NSAR2-s _R 2	NSAR2 with pSETΩ _{s_R} integrated into the chromosomal <i>attB</i> site of ϕC31	This study
<i>E. coli</i>		
DH5α	General cloning strain	34
ET12567	Used for intergeneric conjugation	27
BW25113	Used for PCR-targeting mutagenesis	10
Plasmids and cosmids		
Supercos1	<i>bla neo</i>	11
14C4	Recombinant Supercos1 containing <i>hasL</i> locus	13
25E1	Recombinant Supercos1 containing <i>hasR</i> locus	13
pIJ790	<i>gam bet exo</i>	15
pIJ773	<i>oriT aac(3)IV</i>	15
pIJ778	<i>oriT aadA</i>	15
pSETΩ	<i>oriT attP int aadA</i>	28
pSETΩ _{s_R}	pSETΩ + <i>hasR</i> cloned into the EcoRI site	This study

^a *gam*, Gam (host's exonuclease V inhibitor) gene; *bet*, Bet (single-stranded-DNA binding protein) gene; *exo*, exonuclease gene; *aadA*, spectinomycin and streptomycin resistance gene; *aac(3)IV*, apramycin resistance gene; *neo*, kanamycin resistance gene; *bla*, ampicillin resistance gene; *int*, integrase gene of ϕC31; *oriT*, origin of transfer.

expressed only in late phases of differentiation) (6). Clustering of the *S. coelicolor* ORFs by the Basic Local Alignment Search Tool protein clustering program (excluding mobile genetic element-derived products; BLASTCLUST [2]) indeed revealed only 10 gene pairs encoding products which share the same high level of amino acid identity as the products of the *S. ambofaciens* *has* genes (data not shown). None of the 10 pairs are predicted to encode σ factors.

Taking into account the phylogenetic relationships among *S. avermitilis*, *S. coelicolor*, and *S. ambofaciens*, the last two being most closely related, the duplication of the *has* genes appears to be a recent event and therefore constitutes a useful model to study the fate of duplicated genes. With this in mind, we investigated the role of the *has* genes in *S. ambofaciens* using transcriptional and mutational analyses.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Strains and plasmids used are described in Table 1. All *Streptomyces* culture conditions, media, and selective conditions are as previously described (22). Growth curves were carried out in Hickey-Tresuer (HT) liquid medium. Exponential, transition, and stationary phases were defined as being after 9, 16, and 24 h of growth, respectively. Stress conditions were applied as follows: cells were grown to mid-exponential phase in liquid HT medium and exposed to the stressing agent for 30 min (10 mM H₂O₂, 500 mM NaCl, 34% sucrose, 0.7 M ethanol, or 30% cumene hydroperoxide). High-temperature shocks consisted of a shift from 30 to 42°C for 20 min. Oxidative stress experiments were also performed on solid medium by the disk paper method as described previously (29), with H₂O₂ concentrations ranging from 0.1 to 10 mM. Luria-Bertani and SOB liquid media were used for growing *E. coli*, and Luria-Bertani medium was used for growing *B. subtilis* (34). UV treatments were carried out on spores, spread on HT medium, by irradiation (0 to 400 J/m²) from GTE Sylvania UV lamps (254 nm, 64 mW/cm²). After irradiation, plates were incubated at 30°C in the dark to avoid photoreactivation.

Nucleic acid manipulations. Standard or pulsed-field gel electrophoresis DNA preparations from *S. ambofaciens* were made as described earlier (24, 25). Cosmid and plasmid DNAs were extracted from *E. coli* by the alkaline method (34). All restriction enzymes, PCR reagents, and other molecular biology reagents were purchased from New England Biolabs or Roche Diagnostics.

Digoxigenin-DNA labeling (digoxigenin dUTP), hybridization, washings, and detection were performed according to the recommendations of the manufacturer (Roche). Light emission was acquired with a FluorS MultiImager (Bio-Rad Laboratories). DNA sequencing was performed using the dye terminator cycle sequencing kit on an ABI Prism 310 system and analyzed with the Sequencing Analysis software (Applied Biosystems). All sequence similarity searches were performed in the nonredundant (NR) database by using the BLAST programs (2) at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). Total RNA was isolated from HT liquid or solid (growth on cellophane) medium-grown cultures of *S. ambofaciens* with the TRI reagent (Sigma), followed by a chloroform extraction. High-resolution S1 nuclease mapping experiments were performed according to the method of Kieser et al. (22) with probes consisting of PCR products (primer sequences and localization are as described in Table 2 and Fig. 1). Reverse transcription-PCR (RT-PCR) experiments were carried out according to the method of Pang et al. (30).

Real-time PCR. Real-time PCRs were carried out on an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories), and the data were analyzed using the software provided by the supplier. Sequences of primers and localizations are indicated in Table 2 and Fig. 1. Assays were performed using 5 μl of cDNA, 12.5 μl of Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 3.3 μl of SYBR Green I (10,000× dilution; Sigma), and 10 pmol of each primer in a final volume of 25 μl. Thermal cycle conditions were as follows: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of a maximum of 30 s at 95°C and 1 min at 60°C (annealing and extension) and then 80 steps of 10 s with temperature increasing by 0.5°C for each step from 55 to 94°C (determination of the melting curve). For each run, a standard dilution of the cDNA was used to check the relative efficiency of primers. A negative control (distilled water) was included in each real-time PCR assay. Each experiment was performed in duplicate. The *hrdB* gene was used as an internal control to quantify the relative expression of the target genes. The expression levels of the downstream promoters (*hasRp1* and *hasLp1*) corresponded to the total gene expression (quantified with Rqu1-Rqu2 and Lqu1-Lqu2, Fig. 1 and Table 2) deduced from those of the upstream

TABLE 2. Primers used in this work

Usage	Primer	Nucleotide sequence ^a (5'-3')	Use
Construction of mutant strains	HasR1	GCCGTGAGCGGGGCCGATCGACTGGGAGGGAACATC ATGTGTAGCCTGGACCTCCTTC	Replacement of <i>hasR</i>
	HasR2	ACCGCCGCGGGAAGGACAGCCGCGCCTGAGCGGCG CTCAATTCCGGGATCCGTCGACC	Replacement of <i>hasR</i>
	HasL1	GTGACGAGCAGGCAGTTCGAACAGGGAGGGCCCATC ATGTGTAGGCTGCAGCTGCTTC	Replacement of <i>hasL</i>
	HasL2	GCCGCCCGGTGTGCCACGGTGAGGGGCCCTCGCC TCAATTCCGGGATCCGTCGACC	Replacement of <i>hasL</i>
Complementation	CoR1	TATGAATTCTGTGACGGTGTGCGGATG	Complementation of <i>hasR</i> mutant
	CoR2	AATGAATTCGCGGGCGGTGCGAGGATGA	Complementation of <i>hasR</i> mutant
Real-time RT-PCR	R _{qu} 1	CCGATGAGGCGCGGCCGT	Gene expression of <i>hasRp1</i> and <i>hasRp2</i>
	R _{qu} 2	GATGTTCCCTCCAGTCGATCGG	Gene expression of <i>hasRp1</i> and <i>hasRp2</i>
	R _{qu} 3	CCGACGAGACTGCGCTTAC	Gene expression of <i>hasRp2</i>
	R _{qu} 4	GGCGAGACAGACCGAGAAAA	Gene expression of <i>hasRp2</i>
	L _{qu} 1	CTGCTGACGAGCAGGCAG	Gene expression of <i>hasLp1</i> and <i>hasLp2</i>
	L _{qu} 2	GATGGGCCCTCCCTGTT	Gene expression of <i>hasLp1</i> and <i>hasLp2</i>
	L _{qu} 3	CCCGAGGCCGGGGTATTG	Gene expression of <i>hasLp2</i>
	HrdB-R	CGCGAGCCCATCTCGCTGC	Gene expression of <i>hrdB</i>
	HrdB-F	CGTCGAGGGTCTTCGGCTG	Gene expression of <i>hrdB</i>
Probes for S1 mapping	R5	GTCATCCACGGTGGTGGCAGCGGCA	Probes for <i>hasR</i> (P _{R1} , P _{R3})
	G	GTCCTTGGGCGCCACCTTGGACGGG	Probe for <i>hasR</i> (P _{R3})
	E	GGCATGCCCCGTGGTGGTGTGCTCGG	Probes for <i>hasR</i> and <i>hasL</i> (P _L , P _{R1})
	L5	CACGCACAGCGTGTACGTGGTGTCC	Probe for <i>hasL</i> (P _L)
	arsR1	CGGTGTCAACGTAGACGGA	Probe for <i>hasR</i> (P _{R2})
	arsR2	TCTCACTGCCCCGATAC	Probe for <i>hasR</i> (P _{R2})

^a The sequence in boldface corresponds to *hasR* or *hasL* locus sequences (including the start and stop codons, which are underlined), while the sequence in lightface matches the end of the disruption cassette.

promoters (*hasRp2* quantified with R_{qu}3-R_{qu}4 and *hasLp2* quantified with L_{qu}3-L_{qu}2 [Fig. 1 and Table 2]).

PCR-targeted mutagenesis. Gene disruptions were achieved using the PCR-targeting system developed by Gust et al. (15). In order to replace an 840-bp sequence corresponding to the *hasR* coding region (included in the cosmid 25E1), two primers, HasR1 and HasR2 (Table 2 and Fig. 1), were used to amplify the cassettes *aac3(IV)/oriT* and *aadA/oriT* from plasmids pIJ773 and pIJ778, respectively (15). Similarly, the HasL1 and HasL2 primers (Table 2 and Fig. 1) were used to replace the 840-bp *hasL* sequence in cosmid 14C4. Allelic exchanges were confirmed by Southern blotting and PCR analysis.

Complementation. The *hasR* gene (promoter and coding sequence) was amplified using the CoR1-CoR2 primer set (Table 2 and Fig. 1) and cloned into the conjugative and integrative vector pSETΩ (28) to give pSETΩ_R (Table 1). The integrity of the insert was checked by sequencing. The recombinant plasmid was then introduced into the single mutant strains by conjugal transfer. Conjugation and screening of *S. ambofaciens* exconjugants were carried out according to the method of Kieser et al. (22), except that HT containing MgCl₂ (10 mM) was used instead of soy flour mannitol (SFM) MgCl₂.

BLASTCLUST. Analysis with use of the Basic Local Alignment Search Tool protein clustering program (BLASTCLUST) (2) was performed on the complete set of *S. coelicolor* ORFs excluding transposases (<http://www.sanger.ac.uk>) using the following parameters: minimum 95% identity and 100% length coverage.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the *hasR* (4,429-nucleotide [nt]) and *hasL* (3,659-nt) loci were deposited in the database under accession numbers AF050150 and AF050151, respectively.

RESULTS AND DISCUSSION

Characterization of the *S. ambofaciens* DSM40697 *hasR* and *hasL* duplicated genes. Each of the duplicated *has* genes is located on a different arm of the linear chromosome, *hasR* at about 850 kb from the right chromosomal end and *hasL* at 450 kb from the left end. The *has* genes constitute a substrate for homologous recombination and were discovered as a result of their implication in DNA rearrangements (13). The homology between the two *has* loci starts 4 nt upstream of the start codon

and stops 8 nt before the stop codon (Fig. 1). The nucleotide sequences of the duplicated *has* ORFs are 98% identical over 846 bp.

The putative proteins, σ^{BR} and σ^{BL} , differ by only 8 amino acids over 281 residues (sequencing of the chromosomal loci resulted in the identification of four additional divergent amino acid positions in addition to those in the initial report [13]) and share 100% identity in regions 2-4 and 4-2, which are involved in the recognition of the -10 and -35 regions of promoters (26). Homologues of σ^{BR} and σ^{BL} are listed in Table 3. The σ^{BR} and σ^{BL} factors exhibit the highest similarity with σ^{B} of *S. coelicolor* (8), also named SigJ by Viollier et al. (40). The genes controlled by σ^{B} are reported to play a role in osmoprotection and in the erection of aerial mycelium (8).

The orthologues of the *has* genes are named *sigB* (*sigJ*) in *S. coelicolor* (94 and 93% amino acid identity with σ^{BR} and σ^{BL} , respectively) and SAV741 for *S. avermitilis* (80% amino acid identity with σ^{BR} and σ^{BL}). In each organism, they belong to a gene family consisting of nine members: *sigF*, *sigG*, *sigH*, *sigI*, *sigJ* (*sigB*), *sigK*, *sigL*, *sigM*, and *sigN* in *S. coelicolor* and SAV4185, SAV7400, SAV3013, SAV3492, SAV741, SAV1087, SAV1873, SAV1151, and SAV4186 in *S. avermitilis*.

In both *S. coelicolor* and *S. avermitilis*, the *has* gene orthologue is not duplicated. Indeed, the closest homologues to σ^{B} (SigJ) in *S. coelicolor* and SAV741 in *S. avermitilis* within their respective genomes are the sigma factors SigL and SAV115, respectively. The two pairs share only 73 and 70% identity, while σ^{BR} and σ^{BL} of *S. ambofaciens* DSM40697 are 97% identical to each other at the amino acid level.

Furthermore, the *has* genes were also found to be duplicated in two other isolates of *S. ambofaciens* (ATCC 23877 and

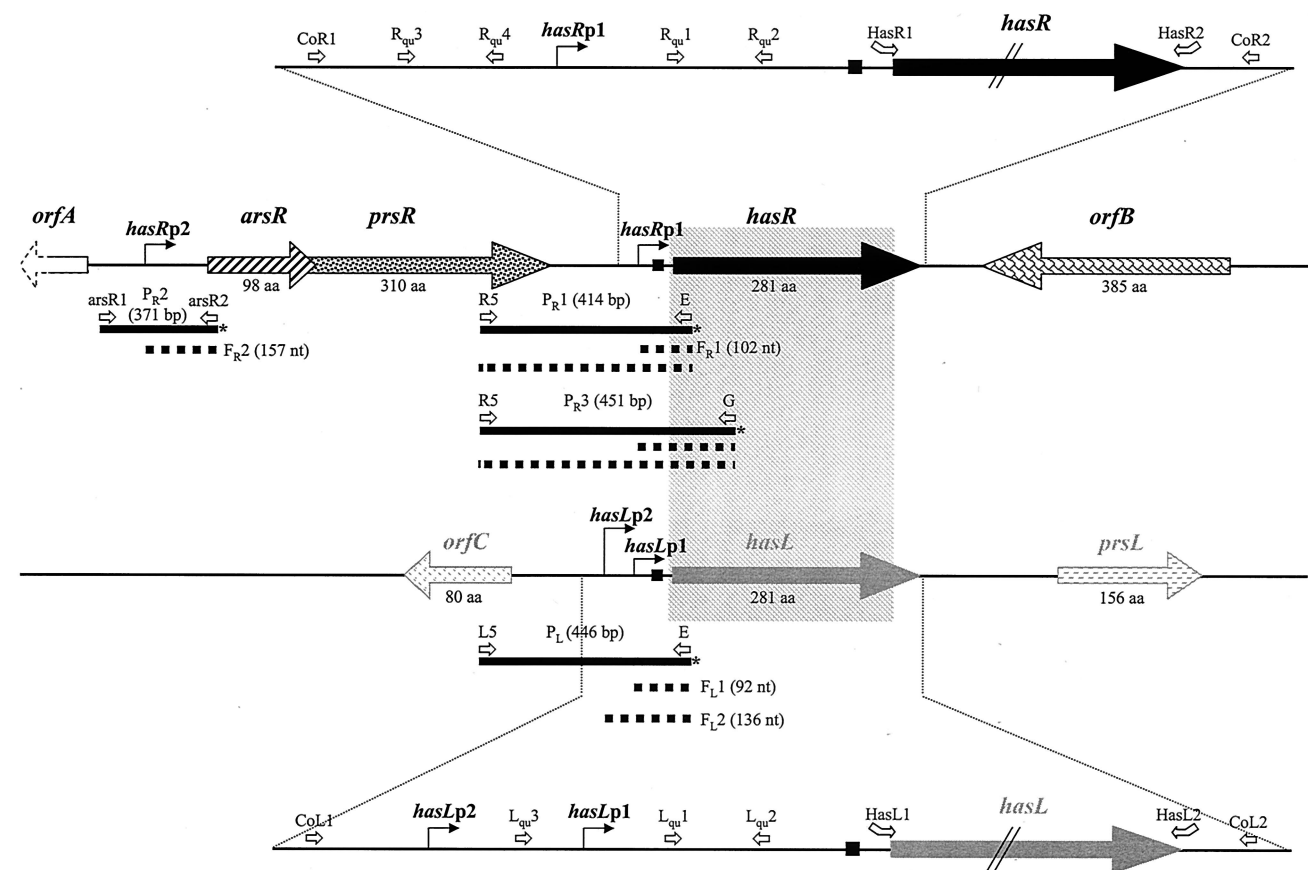


FIG. 1. Genetic organization of the *S. ambofaciens* DSM49697 *hasR* and *hasL* loci. The gray rectangle represents the homologous area between the two *has* loci. The ribosome binding sites are symbolized by the small black squares. *orfA* is incompletely sequenced (dashed arrow). S1 probes (P_R1 , P_R2 , P_R3 , and P_L) are presented as black bars with an asterisk symbolizing the radiolabeled end. The dotted thick lines represent the protected fragments obtained with each S1 probe. The different primers used in real time RT-PCR experiments, for construction of S1 probes, or for the PCR-targeted mutagenesis are symbolized by the open arrows (primer sequences are in Table 2). aa, amino acids.

ETH11317). For each strain, the nucleotidic identity between the two *has* genes was 97% (data not shown).

Since *S. avermitilis* is a species more phylogenetically distant from *S. coelicolor* than is *S. ambofaciens* (<http://avermitilis.lsc.kitasato-u.ac.jp/>), these data suggest that the duplication of the *has* genes is a recent event which has presumably occurred following divergence from the last common ancestor of the *S. coelicolor* and *S. ambofaciens* species.

As shown in Fig. 1, the sequenced region of the *hasR* locus (4,429 nt) contains five ORFs named *orfA*, *arsR*, *prsR*, *hasR*, and *orfB*. Notably, the two ORFs upstream of *hasR*, *arsR* and *prsR*, are predicted to encode homologues of anti-anti- σ and anti- σ factor proteins, respectively. Thus, *arsR* is 91% similar to the anti-anti- σ^B encoded by *rsbB* of *S. coelicolor* (8), and *prsR* exhibits 89% similarity to anti- σ^B encoded by *rsbA* of *S. coelicolor* (8).

The genetic organization of the *hasL* locus is identical to that of *sigB* (*sigJ*) in *S. coelicolor* (Fig. 2A). The initiation codon of *prsR* overlaps the termination codon of *arsR* (as in *S. coelicolor*), suggestive of coregulation, and this was subsequently demonstrated (see below). Interestingly, the conserved *sigB* (*sigJ*) locus is found on the left chromosomal arm of *S. coelicolor* 640 kb from the end (4), while the *hasR* locus is located 850 kb from the end of the right chromosomal arm in *S. am-*

bofaciens. Thus, the divergence of the two species was probably accompanied by a large terminal rearrangement.

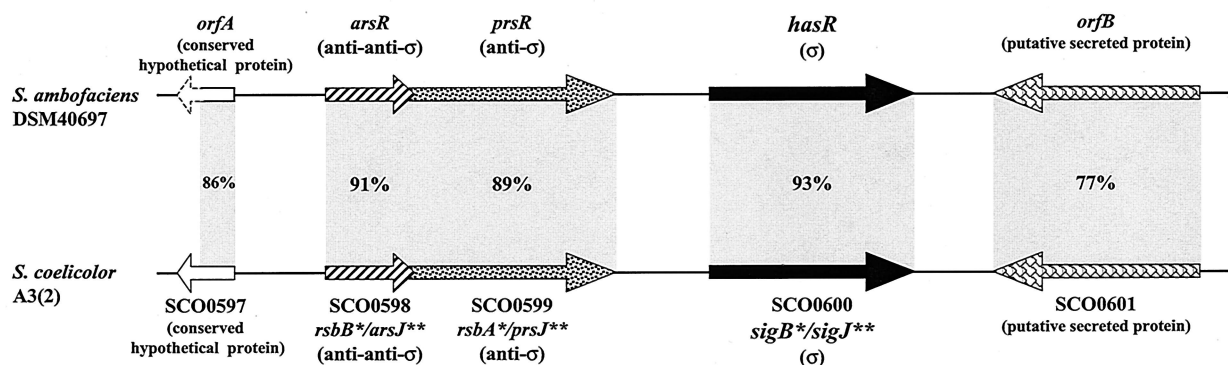
The genetic organization of the *hasL* locus is significantly different from that of *hasR* and of *sigB* (*sigJ*) in *S. coelicolor* (Fig. 2B). The product of *hasL* shares 71% amino acid identity with SigL (SCO7278) of *S. coelicolor* (one of the nine σ factors of the family previously described), but no homology could be found between the two loci when the upstream regions were compared. Immediately downstream of *hasL*, *prsL* encodes a

TABLE 3. Homologues of *S. ambofaciens* σ^{BR} and σ^{BL}

Protein (reference)	% Similarity (% identity of regions 2-4 and 4-2, respectively)	
	<i>S. ambofaciens</i> DSM40697 σ^{BR}	<i>S. ambofaciens</i> DSM40697 σ^{BL}
<i>S. ambofaciens</i> DSM40697 σ^{BL}	97 (100, 100)	
<i>S. coelicolor</i> A3(2) σ^B (SigJ) (8)	94 (100, 100)	93 (100, 100)
<i>S. avermitilis</i> SAV741 (20)	80 (100, 78)	80 (100, 78)
<i>S. coelicolor</i> A3(2) SigL (SCO7278 ^a) (4)	73 (95.5, 74)	71 (95.5, 74)
<i>Streptomyces setonii</i> CrtS (21)	64 (100, 62.5)	64 (100, 62.5)
<i>S. coelicolor</i> A3(2) SigF (32)	46 (73, 62.5)	48 (73, 62.5)
<i>B. subtilis</i> σ^B (39)	34 (73, 50)	33 (73, 50)

^a SCO7278 is named SigL according to the nomenclature of G. Kelemen.

A.



B.

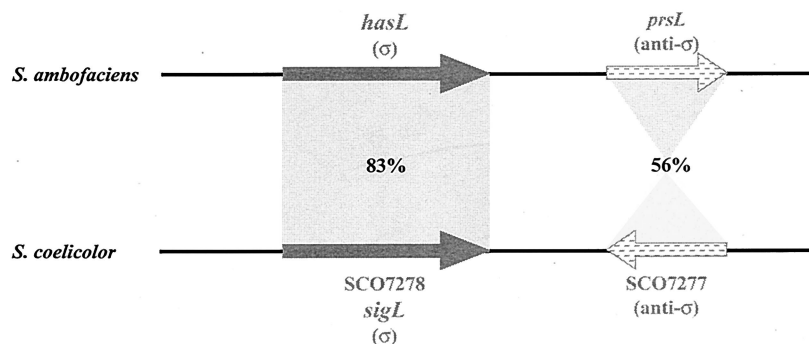


FIG. 2. Comparison of the organization of the *has* loci in *S. ambofaciens* DSM40697 and *S. coelicolor* A3(2). The percentages correspond to the similarities between the deduced products of the genes. The 86% similarity observed between the products of *orfA* and *ScF55-21c* applies to the first 104 amino acids of the proteins only, since it was incompletely sequenced (dashed arrow) in *S. ambofaciens*. *, according to the work of Cho et al. (8); **, according to the work of Viollier et al. (40) or G. Kelemen (personal communication).

putative anti- σ protein showing only 56% similarity with the product of SCO7277, located downstream from *sigL*. Furthermore, the two ORFs lie in the opposite orientation compared to their upstream σ factor-encoding genes.

Mapping of the TSPs. For *hasR*, S1 nuclease mapping experiments using the P_{R1} probe (Fig. 1), consisting of 414 bp overlapping the intergenic region between *hasR* and *prsR*, revealed two protected fragments. The shortest one, F_{R1} (102 nt), revealed a transcriptional start point (TSP) located 53 bp upstream of the *hasR* start codon (Fig. 3A), while the longer one (414 bp) could correspond either to probe-probe reannealing or to a readthrough transcript originating from the *arsR* promoter region (i.e., from *hasRp2* [data not shown]). RT-PCR experiments with the primers used for the construction of the P_{R1} probe confirmed the existence of a second promoter (data not shown). A second TSP (*hasRp2*) was indeed found 114 nt upstream of the *arsR* start codon with the use of the probe P_{R2} (fragment F_{R2} of 157 nt; Fig. 1 and 3A). These data are consistent with the chromosomal organization of the *hasR* locus, which suggests that *hasR* is part of a three-gene operon, *arsR-prsR-hasR*, although transcription from a promoter located between *hasRp1* and *hasRp2* (Fig. 2A) cannot be excluded. However, in *S. coelicolor*, a single operon including the homologous system consisting of the *rsbB*, *rsbA*, and *sigB* genes was also reported (8).

For *hasL*, two protected fragments of 92 (F_{L1}) and 136

(F_{L2}) nt were detected using the P_L probe (446 bp; Fig. 1 and 3A). They correspond to transcripts initiated from two TSPs, *hasLp1* and *hasLp2*, located 43 and 87 bp, respectively, upstream of the start codon.

Interestingly, two boxes matching the consensus sequence of promoters recognized by the *B. subtilis* general stress response σ^B factor (39) and by σ^B of *S. coelicolor* (8) were identified upstream of both *hasRp1* and *hasLp1* (Fig. 4A). A putative promoter sequence for the principal σ factor, σ^{HrdB} of *S. coelicolor* (36), was identified upstream from the TSP *hasLp2* (Fig. 4B). Only a degenerate σ^B promoter consensus was found upstream from *hasRp2* (Fig. 4A). Interestingly, two sequences that are almost identical to the promoters recognized by the sigma factor WhiG were identified upstream of *hasR* (Fig. 4C). One of these putative promoters (*hasRpWhiG1*) overlaps the *hasRp1* promoter. However, no transcription start site could be associated with these promoters with the use of either the P_{R1} or the P_{R3} probe under the experimental conditions used (Fig. 1). However, WhiG is known to be involved in regulating the early stages of sporulation in *S. coelicolor* (7), and the samples analyzed were from liquid-grown cells that do not undergo morphological differentiation. However, analysis of RNA samples prepared from surface-differentiated mycelium also did not reveal the expected transcript. The same sequence is also present upstream of *sigB* and overlapping the *sigBp1* promoter in *S. coelicolor* (Fig. 4C). Further experiments might confirm

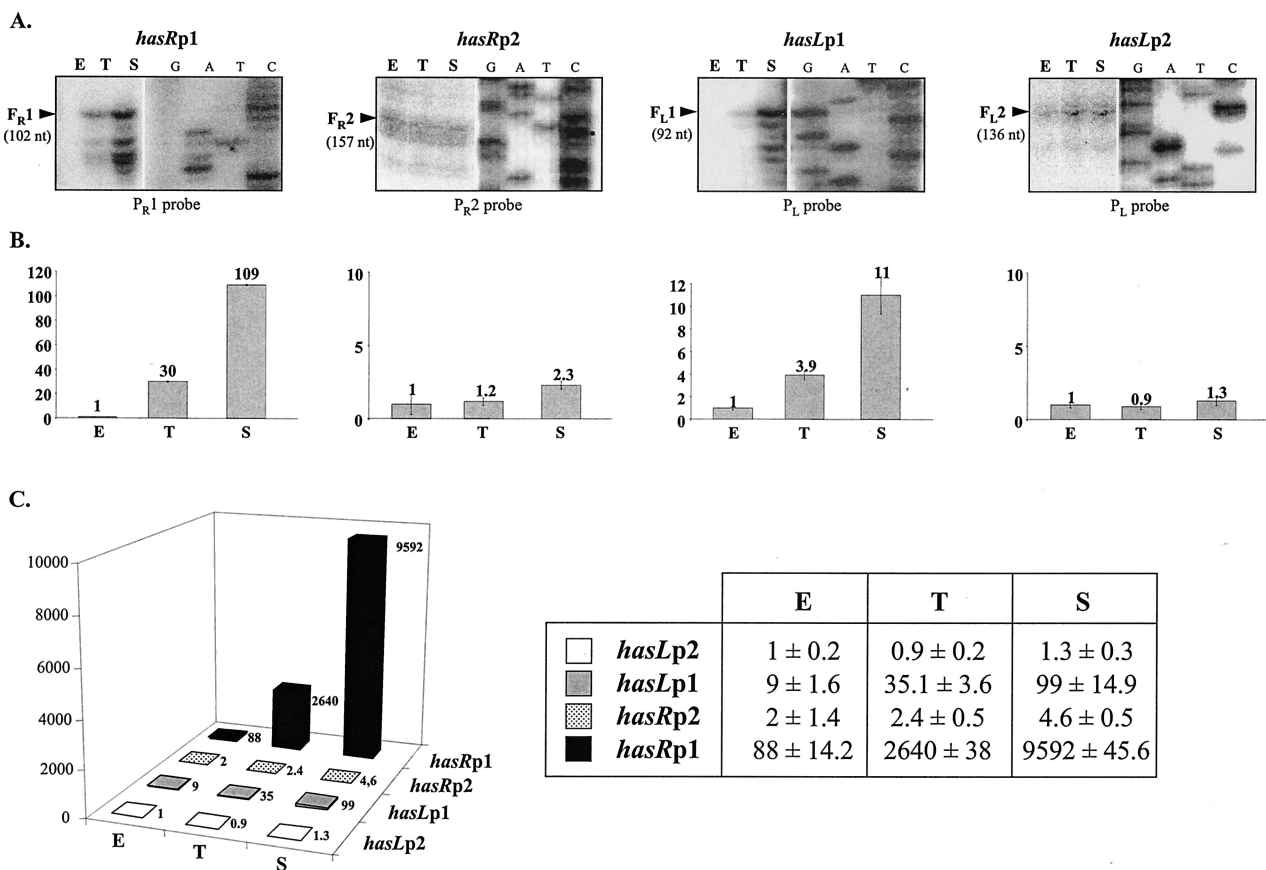


FIG. 3. Identification and quantification of the four *has* transcripts. (A) Identification of the transcription start sites of *hasR* (left) and *hasL* (right) genes by S1 nuclease mapping and determination of the level of transcription of the *has* genes during growth in liquid culture. Total RNAs were prepared from the wt strain at the exponential (E), transition (T), and stationary (S) phases of growth in liquid HT medium and analyzed using the S1 probes P_{R1} , P_{R2} , and P_L . Only the part of the autoradiographic film corresponding to each protected fragment (F_{R1} , F_{R2} , F_{L1} , and F_{L2}) is shown, alongside a panel showing the DNA sequence ladder. The experiments were performed using three independent RNA samples for each growth phase, and representative results are shown. (B) Quantification of transcription from the two *hasR* (left) and *hasL* (right) genes during growth in liquid HT medium with the use of real-time RT-PCR (see Materials and Methods). *hrdB* was used as an internal reference, as it is expressed at a constant level throughout growth. The level of expression of each transcript in the exponential phase was arbitrarily fixed at 1. These experiments were performed using the same three independent RNA samples as detailed for panel A above. (C) Comparison of the levels of expression of each *has* transcript during growth in liquid HT medium with the use of real-time RT-PCR. The level of expression of the least expressed transcript—corresponding to the *hasLp2*-initiated transcript in the exponential-phase sample—was arbitrarily fixed at 1.

the activation of these potential promoters under some specific conditions.

Developmental control of the *has* gene transcription. S1 nuclease mapping showed that, while the levels of *hasRp2* and *hasLp2* transcripts were rather constant, transcription from *hasRp1* and *hasLp1* increased significantly in transition and stationary phase (Fig. 3A). This induction was confirmed and quantified by real-time RT-PCR experiments (Fig. 3B). The most remarkable increase was observed in stationary phase, where abundance of the *hasRp1* and *hasLp1* transcripts was 109- and 11-fold higher than in the exponential-phase samples, respectively. When the levels of expression of the *has* genes are compared, it appears that *hasR* is expressed more highly than is *hasL*, with a maximum difference of 100-fold occurring between the *hasRp1* and *hasLp1* promoters in stationary phase (Fig. 3C). Analogous data were obtained using RNAs prepared from surface-grown mycelium (data not shown). The net levels of transcription of the *has* genes during growth of the wild-type (wt) strain are reported in Fig. 5 and are incorpo-

rated in a global hypothesis presented in Fig. 6. For clarity, the *hasRp2* and *hasLp2* promoters responsible for basal expression of the *has* genes are not considered in this model.

Positive autoregulation and antagonist effects. The *has* genes encode σ^B -like sigma factors, and the *hasRp1* and *hasLp1* promoters are homologous to those recognized by *B. subtilis* σ^B . We therefore tested the ability of both the σ^{BR} and σ^{BL} factors to regulate expression from their own, and each other's, promoters. For this purpose, single knockout mutant strains were first constructed by replacing either *hasR* or *hasL* coding sequence with a cassette containing an apramycin resistance gene, as described in Materials and Methods (Table 1). A double mutant strain was derived from the single mutants by replacing the remaining intact *has* gene with a cassette harboring the spectinomycin resistance gene. All the gene replacements were confirmed by Southern hybridization and PCR (see Materials and Methods), and the absence of large chromosomal rearrangements was assessed using pulsed-field gel electrophoresis (data not shown). All further experiments were

		-35	-10	+1
A.	consensus	anGnnTnn	GGGtA	
	<i>Pctc</i>	AGGT TAA	GGGTA	-N ₁₁ - A
	<i>sigBp1</i>	AGGT GTG	GGGTA	-N ₇ - G
	<i>hasRp1</i>	ATGC ATGA	GGGTA	-N ₁₁ - G
	<i>hasLp1</i>	TTGC ATAC	GGGTA	-N ₉ - T
	<i>hasRp2</i>	GGGC GTAC	GGGTA	-N ₁₀ - C
		-35	-10	+1
B.	consensus	TTGACR	TAgRRRT	
	<i>hasLp2</i>	TGGAGG	TATGAA	-N ₇ - C
		-35	-10	
C.	consensus	TAAA	GCCGATAA _{/T}	
	<i>PwhiH</i>	TCAA	GCCGACAA	
	<i>PwhiI</i>	TAAA	GCCGAAGA	
	<i>PTH4</i>	TGGA	GCCGAAGT	
	<i>PTH270</i>	TACG	CCCAGTGA	
	<i>hasRpWhiG1</i>	TAAG	GCCGATGA	
	<i>hasRpWhiG2</i>	TGAG	GCCGATCG	
	<i>sigBpWhiG</i>	TAGG	GCCGACAA	

FIG. 4. Promoter sequences. (A) Alignment of the *hasRp1*, *hasLp1*, and *hasRp2* promoters with the previously reported σ^B -type promoters *Pctc* of *B. subtilis* (33) and *sigBp1* of *S. coelicolor* (8). The consensus is according to the work of Cho et al. (8). (B) Alignment of the *hasLp2* promoter sequence with the consensus recognized by the principal sigma factor σ^{HrdB} in *S. coelicolor* (36). (C) Alignment showing putative WhiG-type promoters identified upstream of the *hasR* coding region and upstream of *sigB* (*sigI*) in *S. coelicolor*. *PwhiH* and *PwhiI* are the promoter sequences of the *whiH* and *whiI* genes known to be regulated by WhiG in *S. coelicolor* (1). *PTH4* and *PTH270* are two sequences identified as targets of WhiG in *S. coelicolor* (38). The consensus is according to the work of Tan and Chater (38). The nucleotides in boldface are those in common with the promoter consensus sequence. The two nucleotides underlined (**TA**) in the -10 region of the *hasRp1* (or *sigBp1*) promoter and in the -35 region of the *hasRpWhiG1* (or *sigBpWhiG*) promoter are shared by the two putative promoter sequences.

carried out using three representative mutants of each type (Table 1). Transcripts initiated from *hasRp1* and *hasLp1* in the single and double mutant strains during exponential-, transition-, and stationary-phase growth in liquid HT medium were quantified (see Materials and Methods), and the results are presented in Fig. 5.

In the Δ *hasR* mutant, *hasRp1* transcript abundance in the stationary-phase sample was 30-fold lower than that of the parent strain at the same time point and 1.9-fold lower in transition phase (Fig. 5A). These data indicate that σ^{Br} positively activates the transcription of its own gene from *hasRp1* at these growth stages, as illustrated in Fig. 6. This situation is common for stress response sigma factors. For example, in *S. coelicolor*, *sigB* (promoter P1) and *sigH* (promoter P2) expression show an autoregulatory activity in response to an osmotic

stress (8, 35). The same phenomenon was reported for σ^B of *B. subtilis* and of *Listeria monocytogenes* (3, 16). Surprisingly, in the exponential-phase samples, the level of transcription measured from *hasRp1* was 2.9-fold greater in the mutant than in the wt strain (Fig. 5A), suggesting that σ^{Br} might exert an antagonistic effect on the transcription of its own gene during vegetative growth (Fig. 6A). This antagonistic effect might result from competition of two (or more) σ factors for the same promoter sequence or for the RNA polymerase core enzyme as described for *E. coli* between σ^S and σ^{70} (12, 17) and for σ^X and σ^W in *B. subtilis* (18).

Transcription initiated from *hasLp1* in the Δ *hasL* mutant exhibited a slightly different pattern of transcription from that observed for *hasRp1* in Δ *hasR* (Fig. 5). Transcription from *hasLp1* was only 1.8-fold lower in the mutant during stationary phase, while a significant antagonistic effect on transcription was observed in both transition- and exponential-phase samples, which showed a 3.6- and 20-fold increase, respectively.

To confirm that the observed altered transcriptional levels are specifically the result of a deficiency in σ^{BrL} , a wt copy of *hasR* under the control of its own promoter was introduced into the NSAR or NSAL [*hasR* or *hasL* replaced by *aac(3)IV/oriT* cassette, respectively, in *S. ambofaciens* DSM40697] strain, respectively, with the integrative vector pSET Ω (28) (see Materials and Methods). Transcription was nearly fully restored in both the complemented strains: e.g., the transcription levels from *hasLp1* and *hasRp1* were 74 and 87% restored, respectively, in a complemented Δ *hasR* strain in stationary phase (Fig. 5).

Cross-regulation of *has* gene transcription. Similar net positive or negative influences on the levels of transcription were noticed when cross-regulation between the two *has* systems was surveyed (Fig. 5). In the Δ *hasL* mutant, a slight but significant decrease in *hasRp1* transcription was observed in all three growth phases: transcription was reduced by 1.4-fold in exponential phase and by 1.2- and 2.6-fold in the transition- and stationary-phase samples, respectively. Reciprocally, in the Δ *hasR* mutant strain, the transcription level from *hasLp1* decreased by 19-fold in the stationary phase of growth (Fig. 5B) and by twofold in transition phase. These data revealed a positive activation of *hasRp1* transcription by σ^{BrL} and reciprocally, and perhaps more strongly, of σ^{Br} on *hasLp1* transcription as illustrated in Fig. 6. Surprisingly however, a 2.4-fold increase in the level of *hasLp1* transcription was observed in exponential growth phase in the Δ *hasR* mutant.

In the Δ *hasR* Δ *hasL* double mutant strains, a variable effect on the level of transcription from *hasRp1* and *hasLp1* was observed (Fig. 5), strongly suggesting that additional σ factors recognize the *hasRp1* and *hasLp1* promoter sequences (noted as σ^X in Fig. 6). It is particularly interesting to note the additional effects of the absence of σ^{Br} and σ^{BrL} in the double mutant Δ *hasR* Δ *hasL* compared to the single mutant strains (Fig. 5). For example, when a net transcriptional induction was observed in both single mutant strains compared to the wt, this induction was even higher in the double mutant strain (e.g., *hasLp1* transcription in the exponential-phase samples). Furthermore, when antagonistic effects were observed in the single mutant strains, the net level of transcription in the double mutant was averaged (e.g., *hasRp1* and *hasLp1* transcription in exponential- and transition-phase samples, respectively). Fi-

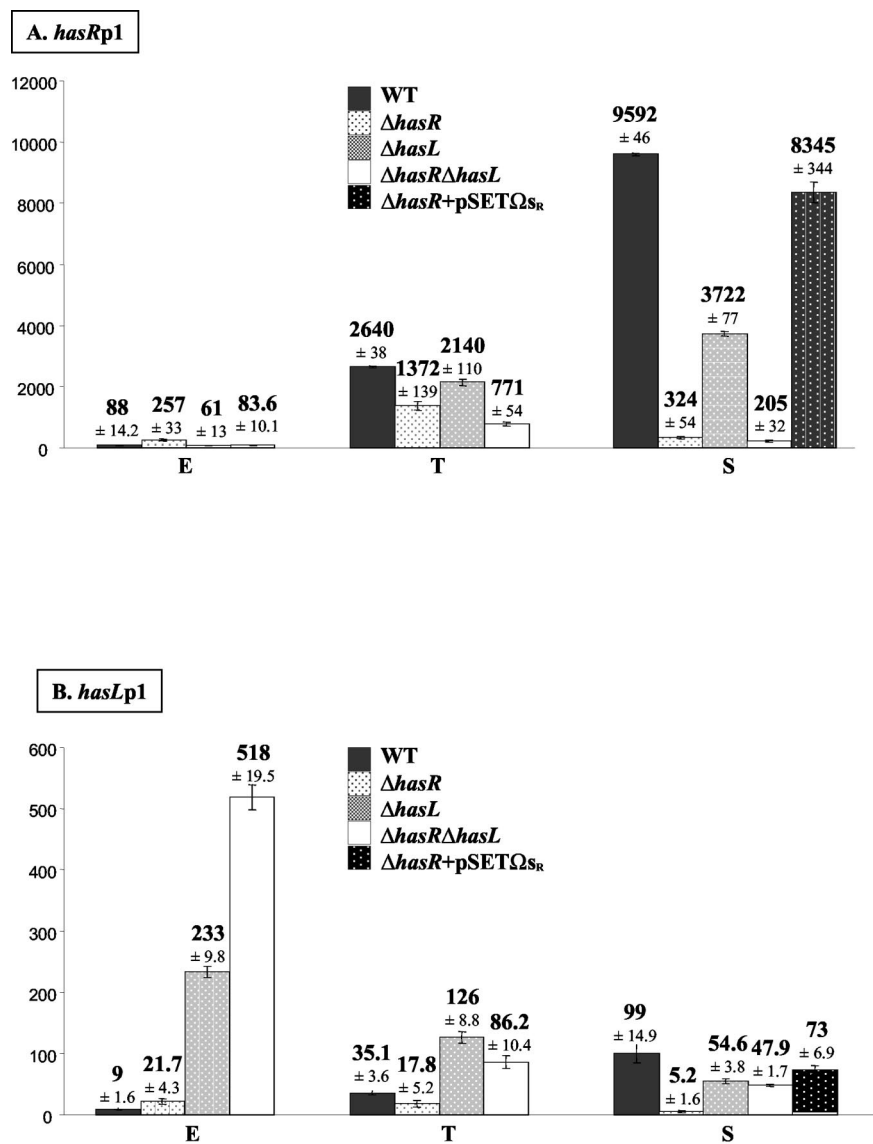


FIG. 5. Quantification of the transcription levels from *hasRp1* (A) and *hasLp1* (B) during growth and the effect of mutations in *hasR* and *hasL* genes. The genetic contexts are named $\Delta hasR$, $\Delta hasL$, or $\Delta hasR\Delta hasL$ and result from the analysis of three independent strains of each type (Table 1). The complementation experiments correspond to the $\Delta hasR+pSET\Omega_{sR}$ data and result from the analysis of two independent strains (Table 1). For each strain, three independent RNA samples have been analyzed by real-time RT-PCR. The values are relative to the level of expression from *hasLp2* in exponential phase, arbitrarily fixed at 1.

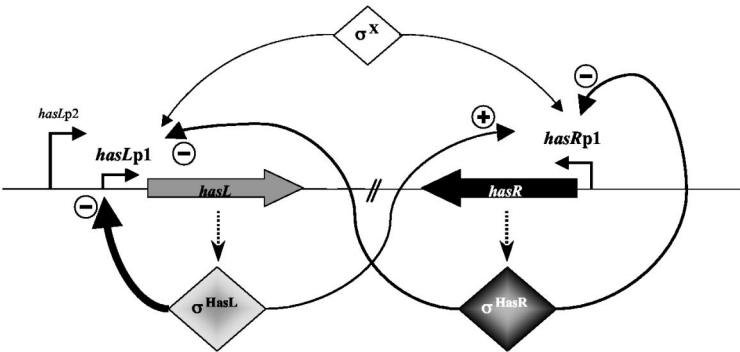
nally, when the deficiency of each σ factor was accompanied by a reduction in transcription, this decrease was greater in the double mutant (e.g., *hasRp1* transcription in transition and stationary phase and *hasLp1* in stationary phase).

Note that the regulation model can reach a higher degree of complexity if the posttranscriptional regulation is overlaid (anti-anti- σ and anti- σ factors) as reported for many stress response σ factors (41).

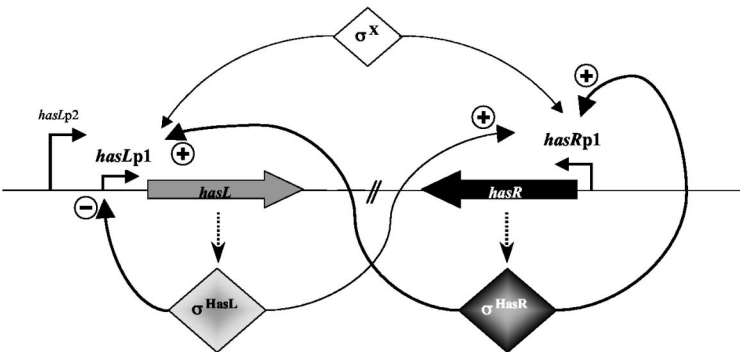
The *has* genes belong to a network involved in stress response. The single and double mutant strains were compared to the wt when grown on both rich (HT, nutrient agar [NA], SFM, R2, and R2-yeast extract) and minimal (minimal medium mannitol [MMM] and minimal medium glucose [MMG]) media. No difference in growth rate or differentiation was

detected, however, nor was any difference observed when strains were grown either at high temperature (37°C instead of the 30°C classically used) on solid medium (HT, SFM, MMM, or MMG) or in acid or alkaline conditions (pH 5.0 [acid] or pH 9.0 [alkaline]). The mutants showed no significant sensitivity to UV radiation exposure (0 to 400 J/m²), and no impairment of growth could be detected on exposure to osmotic stress conditions in liquid or solid medium. These results are significantly different from those reported by Cho et al. (8), where the *sigB* mutant in *S. coelicolor* was shown to be deficient both in aerial mycelium formation (on R2-yeast extract and NA) and in its response to osmotic stress. In addition, mutation of another member of the σ^B family, SigH, also led to slightly different conclusions. While Viollier et al. (40) could not distinguish the

A. Exponential phase



B. Transition phase



C. Stationary phase

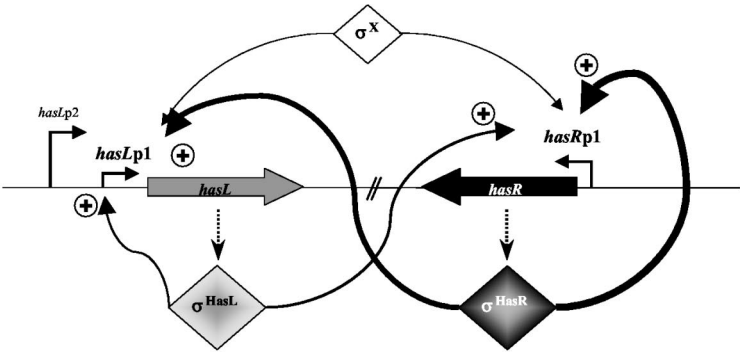


FIG. 6. Model for the control of expression of the *has* genes during growth in liquid culture. The three growth phases are depicted: exponential (A), transition (B), and stationary (C). The thickness of the lines symbolizes the level of control as deduced from real-time RT-PCR quantification (Fig. 5). + and – indicate positive or negative effects on promoter transcription, respectively, of the factors σ^{BRL} as deduced from expression analysis in the mutant strains (Fig. 5).

TABLE 4. Induction of the *has* gene transcription levels under stress conditions

Promoter and strain	Transcription level (IF ^a) under stress condition:		
	H ₂ O ₂	NaCl	Sucrose ^b
<i>hasRp1</i>			
wt	17	25	22.5
Δ <i>hasR</i>	14	18	ND
Δ <i>hasL</i>	13.2	28.4	ND
Δ <i>hasR</i> Δ <i>hasL</i>	16.5	19.1	23.9
<i>hasLp1</i>			
wt	7.5	21.1	23
Δ <i>hasR</i>	9	19.3	ND
Δ <i>hasL</i>	7.1	18	ND
Δ <i>hasR</i> Δ <i>hasL</i>	6.9	18.4	18.2

^a IFs correspond to the ratio of the transcription level following stress to the transcription level without stress.

^b ND, not determined.

phenotypes of a Δ *sigH* mutant from those of the wt strain in response to osmotic stress, Sevcikova et al. (35) reported that *sigH* is required for correct morphological differentiation and growth under conditions of high osmolarity.

Finally, a slight but reproducible sensitivity to oxidative stress conditions distinguished the *has* mutant strains (single or double) from the wt strain. The most significant difference was observed using the paper disk method with a concentration of H₂O₂ of 10 mM, where the diameter of the zone of growth inhibition around the disks was 11.2% (\pm 5.6%) higher for the double mutant strain than for the wt strain (the wt level varying by 2.8%). Furthermore, the deletion of the *prsR* gene (which encodes an anti- σ factor) seemed to confer a weak resistance to oxidative stress (data not shown). These data are consistent with the involvement of σ^{BRL} in the stress response, since the anti- σ factor may titrate the σ^{BRL} factors and prevent the induction of target genes involved in the stress response. This hypothesis is supported by the report of the probable post-translational control of three related σ factors (σ^{H} , σ^{L} , and σ^{F}) by the same anti- σ factor, RshA, in *Streptomyces griseus* (37).

The transcription levels of the *has* genes under stress conditions were measured by real-time PCR and expressed as induction factors (IF; transcription level following stress/transcription level without stress; see Materials and Methods) (Table 4). In the wt strain, transcription from *hasRp1* and to a lesser extent from *hasLp1* was increased following exposure to oxidative stress with 10 mM H₂O₂ (IF, 17 and 7.5, respectively) as well as to osmotic stress with 500 mM NaCl (IF, 25 and 21, respectively) or with 34% sucrose (IF, 22.5 and 23, respectively). In the same experimental conditions, no significant induction was observed from *hasRp2* and *hasLp2* TSPs (IF ranging between 1 and 2.5). No increase in transcription was observed after ethanol (4%), cumene hydroperoxide (30%), or heat shock (42°C) treatments for any of the four promoters.

It appears that the IFs from *hasRp1* and *hasLp1* are not significantly altered in the single and double mutants compared to the wt (Table 4). Indeed, the IFs following oxidative stress (H₂O₂, 10 mM) ranged between 13.2 and 16.5 for *hasRp1* and between 6.9 and 9 for *hasLp1* in the double (or single) mutant strains (versus 17 and 7.5, respectively, in the wt strain).

The induction response following osmotic stress (data not shown) was also retained (500 mM NaCl and 34% sucrose).

These data suggest that the induction of transcription from the *hasRp1* and *hasLp1* promoters under stress conditions results at least in part from the action of alternate σ factors (noted as σ^{X} in Fig. 6). This also provides an explanation for the absence of a marked phenotype in response to stress in the wt and mutant strains while the *has* transcription is induced under the same conditions. This is consistent with the residual transcriptional activity observed from *hasRp1* and *hasLp1* in the double mutant strain Δ *hasR* Δ *hasL* (Fig. 5). These data are also consistent with those reported by Viollier et al. (40) suggesting the existence of shared promoter recognition specificity within the stress response σ factors and notably among SigH, SigI, and SigJ (orthologue of σ^{BR} of *S. ambifaciens*) implied in the osmotic stress response in *S. coelicolor*. The stress responses might therefore rely on the activity of several specific σ factors, particularly for the response to osmotic stress. Hence, a sensory system that can coordinate the activity of multiple paralogous σ factors would be implied in the expression of genes belonging to the same stress regulon (40).

In conclusion, the conservation of the duplicated *has* genes in *S. ambifaciens* might illustrate the subfunctionalization phenomenon described in the duplication-degeneration-complementation model (14). The two σ^{BRL} factors are 97% identical at the amino acid level (and 100% in the domains involved in the -35 and -10 box recognition) and probably recognize the same regulatory signals and consequently coregulate the same genes. However, the transcriptional control revealed during this study clearly shows that the two *has* copies are differently expressed, suggesting the specialization of the two *has* genes. The redundancy of the σ^{B} -like σ factors in *Streptomyces* might increase the complexity of the response to stress. Further, the duplication of the *sigB* (*sigJ*) gene in *S. ambifaciens* would correspond to a recent event of this evolutionary process and might allow the fine-tuning of the response.

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