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

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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) (σ^F^/SCO4035, σ^G^/SCO7341, σ^H^/SCO5243, σ^I^/SCO3068, σ^J^/SCO6520, σ^L^/SCO7278, σ^M^/SCO7314, and σ^N^/SCO4034; σ 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) and 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
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) 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.

Nuclear 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.

**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 conditions are as previously described (22). Growth curves were carried out in 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.

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Property(ies)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>General cloning strain</td>
<td>34</td>
</tr>
<tr>
<td>ET12567</td>
<td>Used for intergeneric conjugation</td>
<td>27</td>
</tr>
<tr>
<td>BW25113</td>
<td>Used for PCR-targeting mutagenesis</td>
<td>10</td>
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<tr>
<td><strong>Plasmids and cosmids</strong></td>
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<td></td>
</tr>
<tr>
<td>Superco1</td>
<td>bla neo</td>
<td>11</td>
</tr>
<tr>
<td>14C4</td>
<td>Recombinant Superco1 containing hasL locus</td>
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<tr>
<td>25E1</td>
<td>Recombinant Superco1 containing hasR locus</td>
<td>13</td>
</tr>
<tr>
<td>pIJ790</td>
<td>oriT aac(3)IV</td>
<td>15</td>
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<td>pIJ773</td>
<td>oriT aadA</td>
<td>15</td>
</tr>
<tr>
<td>pSET1I</td>
<td>oriT attP int aadA</td>
<td>28</td>
</tr>
<tr>
<td>pSET1I&lt;sub&gt;R&lt;/sub&gt;</td>
<td>pSET1I + hasR cloned into the EcoRI site</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Gam, Gam (host’s exonuclease V inhibitor) gene; bet, Bet (single-stranded-DNA binding protein) gene; eso, 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 6C31; oriT, origin of transfer.

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 (hasLp1 and hasRp1) corresponded to the total gene expression (quantified with Rqu1-Rqu2, Fig. 1 and Table 2) deduced from those of the upstream...
promoters (hasRp2 quantified with Rqu3-Rqu4 and hasLp2 quantified with Lqu3-Lqu2 [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 (including the stop codon and mutations, which are underlined), while the sequence in lightface matches the end of the disruption cassette.

**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 pAO1 vector (22), except that M9 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 AF061050 and AF061051, 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, σ⁹₆ and σ⁹₆, 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 σ⁹₆ and σ⁹₆ are listed in Table 3. The σ⁹₆ and σ⁹₆ factors exhibit the highest similarity with σ₉₆ of S. coelicolor (8), also named SigI by Viollier et al. (40). The genes controlled by σ⁹₆ are reported to play a role in osmoregulation 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 σ⁹₆ and σ⁹₆, respectively) and SAV741 in S. avermitilis (80% amino acid identity with σ⁹₆ and σ⁹₆). In each organism, they belong to a gene family consisting of nine members: sigF, sigG, sigH, sigI (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 σ⁹₆ (SigI) in S. coelicolor and SAV741 in S. avermitilis within their respective genomes are the sigma factors SigL and SAV1151, respectively. The two pairs share only 73 and 70% identity, while σ⁹₆ and σ⁹₆ 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...
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.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 encoded by rsbB of S. coelicolor (8), and prsR exhibits 89% similarity to anti-σ encoded by rsbA of S. coelicolor (8).

The genetic organization of the hasR 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. ambofaciens. 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>
<thead>
<tr>
<th>Protein (reference)</th>
<th>% Similarity (% identity of regions 2-4 and 4-2, respectively)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. ambofaciens DSM40697 αRc</td>
<td>97 (100, 100)</td>
</tr>
<tr>
<td>S. coelicolor A3(2) αRc (SigJ) (8)</td>
<td>94 (100, 100)</td>
</tr>
<tr>
<td>S. avermitilis SAV741 (20)</td>
<td>80 (100, 78)</td>
</tr>
<tr>
<td>S. coelicolor A3(2) SigL (SCO7278) (4)</td>
<td>73 (95.5, 74)</td>
</tr>
<tr>
<td>Streptomyces setonii CrtS (21)</td>
<td>64 (100, 62.5)</td>
</tr>
<tr>
<td>S. coelicolor A3(2) SigF (32)</td>
<td>46 (73, 62.5)</td>
</tr>
<tr>
<td>B. subtilis αRc (39)</td>
<td>34 (73, 50)</td>
</tr>
</tbody>
</table>

a SCO7278 is named SigL according to the nomenclature of G. Kelemen.
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, FR1 (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 renanneling 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 PR1 probe confirmed the existence of a second promoter (data not shown). A second promoter (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. 3A), while the longer (414 bp) could correspond either to probe-probe renanneling 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_R2 probe confirmed the existence of a second promoter (data not shown). A second promoter (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. 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 σH factor (39) and by σH of S. coelicolor (8) were identified upstream of both hasRp1 and hasLp1 (Fig. 3A). A putative promoter sequence for the principal σ factor, σB of S. coelicolor (36), was identified upstream from the TSP hasLp2 (Fig. 4B). Only a degenerate σH 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
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 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 incorporated 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 $\sigma^N$-like sigma factors, and the hasRp1 and hasLp1 promoters are homologous to those recognized by *B. subtilis* $\sigma^N$. We therefore tested the ability of both the $\sigma^N$- and $\sigma^B$- 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 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.

![FIG. 3. Identification and quantification of the four has transcripts.](http://jb.asm.org/Downloaded from)
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 ΔhasR 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 $\sigma^B$ 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 $\sigma^H$ 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 $\sigma^H$ 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) $\sigma$ factors for the same promoter sequence or for the RNA polymerase core enzyme as described for E. coli between $\sigma^5$ and $\sigma^{70}$ (12, 17) and for $\sigma^X$ and $\sigma^W$ in B. subtilis (18).

Transcription initiated from hasLp1 in the ΔhasL mutant exhibited a slightly different pattern of transcription from that observed for hasRp1 in ΔhasR (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 $\sigma^{has}$, 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/orfT cassette, respectively, in S. ambofaciens DSM40697] strain, respectively, with the integrative vector pSET (28) (see Materials and Methods). Transcription was nearly fully restored in both the complemented strains: e.g., the transcription levels from hasRp1 and hasRp1 were 74 and 87% restored, respectively, in a complemented ΔhasR 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 ΔhasL 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 ΔhasR 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 $\sigma^{has}$ and reciprocally, and perhaps more strongly, of $\sigma^{has}$ 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 ΔhasR mutant.

In the ΔhasR ΔhasL double mutant strains, a variable effect on the level of transcription from hasRp1 and hasLp1 was observed (Fig. 5), strongly suggesting that additional $\sigma$ factors recognize the hasRp1 and hasLp1 promoter sequences (noted as $\sigma^X$ in Fig. 6). It is particularly interesting to note the additional effects of the absence of $\sigma^{has}$ and $\sigma^{ht}$ in the double mutant ΔhasR ΔhasL 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-
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., hasR p1 transcription in transition and stationary phase and hasL p1 in stationary phase).

Note that the regulation model can reach a higher degree of complexity if the posttranscriptional regulation is overlaid (anti-anti- and anti- factors) as reported for many stress response 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 σH family, SigH, also led to slightly different conclusions. While Viollier et al. (40) could not distinguish the

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 ΔhasR, ΔhasL, or ΔhasR ΔhasL, and result from the analysis of three independent strains of each type (Table 1). The complementation experiments correspond to the ΔhasR+pSETΩs 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.
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^{Has} as deduced from expression analysis in the mutant strains (Fig. 5).
TABLE 4. Induction of the has gene transcription levels under stress conditions

<table>
<thead>
<tr>
<th>Promoter and strain</th>
<th>Transcription level (IF) under stress condition:</th>
<th>H_2O_2</th>
<th>NaCl</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>hasRp1</td>
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<td>ΔhasR</td>
<td>14</td>
<td>18</td>
<td>ND</td>
<td></td>
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<tr>
<td>ΔhasL</td>
<td>13.2</td>
<td>28.4</td>
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<td></td>
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<tr>
<td>ΔhasR ΔhasL</td>
<td>16.5</td>
<td>19.1</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td>hasLp1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>7.5</td>
<td>21.1</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>ΔhasR</td>
<td>9</td>
<td>19.3</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ΔhasL</td>
<td>7.1</td>
<td>18</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ΔhasR ΔhasL</td>
<td>6.9</td>
<td>18.4</td>
<td>18.2</td>
<td></td>
</tr>
</tbody>
</table>

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 ΔsigH mutant from those of the wt strain in response to osmotic stress. Sekikawa 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 H_2O_2 of 10 mM, where the diameter of the zone of growth inhibition around the disks was 11.2% (±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-sigma factor) seemed to confer a weak resistance to oxidative stress (data not shown). These data are consistent with the involvement of α^has in the stress response, since the anti-sigma factor may titrate the α^has 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^P) by the same anti-sigma 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 H_2O_2 (IF, 17 and 7.5, respectively) as well as to osmotic stress with 500 mM NaCl (IF, 22.5 and 23, 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 (H_2O_2, 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 α^ 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 ΔhasR ΔhasL (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 α^has of S. ambofaciens) 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. ambofaciens might illustrate the subfunctionalization phenomenon described in the duplication-degeneration-complementation model (14). The two α^has 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 BR/L factors and notably among SigH, SigI, and SigJ (orthologue of α^has of S. ambofaciens) implied in the osmotic stress response in S. coelicolor might increase the complexity of the response to stress. Further, the duplication of the sigB (sigT) gene in S. ambofaciens would correspond to a recent event of this evolutionary process and might allow the fine-tuning of the response.

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