A Complex Signaling Cascade Governs Pristinamycin Biosynthesis in *Streptomyces pristinaespiralis*

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Pristinamycin production in *Streptomyces pristinaespiralis* Pr11 is tightly regulated by an interplay between different repressors and activators. A γ -butyrolactone receptor gene (*spbR*), two TetR repressor genes (*papR3* and *papR5*), three SARP (*Streptomyces* antibiotic regulatory protein) genes (*papR1*, *papR2*, and *papR4*), and a response regulator gene (*papR6*) are carried on the large 210-kb pristinamycin biosynthetic gene region of *Streptomyces pristinaespiralis* Pr11. A detailed investigation of all pristinamycin regulators revealed insight into a complex signaling cascade, which is responsible for the fine-tuned regulation of pristinamycin production in *S. pristinaespiralis*.

Streptomycetes are filamentous, Gram-positive soil bacteria that are well known for their ability to produce varieties of bioactive secondary metabolites, including more than 70% of the commercially important antibiotics (1). The production of antibiotics is controlled by a vast array of physiological and nutritional conditions, communicated by extracellular and intracellular signaling molecules (2). The beginning of antibiotic biosynthesis is often coordinated with processes of morphological differentiation. The characteristic *Streptomyces* life cycle involves the formation of a feeding substrate mycelium and subsequent development of aerial hyphae, which finally septate into spores (3). Generally, antibiotic production begins as the culture enters stationary growth in liquid culture and coincides with the onset of morphological differentiation in agar-grown cultures (reviewed in reference 4). In many *Streptomyces* strains, antibiotic production is regulated by low-molecular-weight compounds, called γ -butyrolactone autoregulators (GBLs) (5, 6). GBLs are small diffusible signaling molecules that are synthesized and gradually accumulated in a growth-dependent manner, at or near the middle of the exponential phase of *Streptomyces* growth, when they trigger the onset of antibiotic biosynthesis and/or morphological differentiation at nanomolar concentrations (7). Often, the GBL signal is transmitted via a hierarchical signaling cascade including pleiotropic and pathway-specific regulators, which all together control the antibiotic production: when the GBL concentration reaches a critical level, the signal is transmitted into the cells by binding to specific cytoplasmic receptor proteins, the GBL receptors (7). GBL receptors belong to the TetR family of transcriptional regulators (8). In the absence of the corresponding ligand, the GBL receptor binds to conserved AT-rich, partially palindromic sequences (9), the so-called “ARE” sequences (autoregulatory element) (10), within the promoter regions of its target genes and thereby represses the transcription of these genes. By binding of the GBLs to their receptors, the latter undergo a conformational change and dissociate from the target DNA, allowing expression of the derepressed genes (11). Predominantly, targets of GBL receptors are transcriptional regulatory genes, such as TetR and SARP (*Streptomyces* antibiotic regulatory protein) genes. The different families of regulatory proteins together control secondary metabolite production in a complex cascade. Such cascades can consist of several levels of regulation, which can have either a pleiotropic mode of action, by affecting a broad range of morphological and physio-

logical processes, or a pathway-specific activity that affects only a single antibiotic biosynthetic pathway (4). TetR regulators prevalently have a repressive function on the transcription of their target genes and act on a higher level within the regulatory signaling cascade (12), whereas SARP-type regulators are a family of pathway-specific transcriptional activators that directly control the expression of the respective antibiotic biosynthetic gene cluster (13). The best-understood model for a targeted coordination of antibiotic biosynthesis is the A-factor regulatory cascade of *Streptomyces griseus*, which controls streptomycin biosynthesis (14). Further examples have been reported, e.g., for the regulation of the pristinamycin-related antibiotic virginiamycin of *Streptomyces virginiae* (15, 16), tylosin production in *Streptomyces fradiae* (17), lankacidin/lankamycin biosynthesis in *Streptomyces rochei* (18), or auricin biosynthesis in *Streptomyces aureofaciens* (19).

Streptomyces pristinaespiralis Pr11 produces the streptogramin-type antibiotic pristinamycin, which consists of two chemically diverse antibiotics: the cyclohexadepsipeptide pristinamycin I (PI) and the polyunsaturated macrolactone pristinamycin II (PII) (Fig. 1). PI and PII are coproduced in a 30:70 ratio (20). Each compound alone displays only a little bacteriostatic activity by binding to the 50S subunit of the bacterial ribosome and thereby blocking protein synthesis (21). In combination, the pristinamycins exhibit a strong synergistic antibacterial activity against a wide range of Gram-positive and some Gram-negative bacteria, including methicillin- and vancomycin-resistant strains (22). The genes that code for PI and PII biosynthesis, regulation, and resis-

Received 11 March 2015 Accepted 27 June 2015

Accepted manuscript posted online 17 July 2015

Citation Mast Y, Guezguez J, Handel F, Schinko E. 2015. A complex signaling cascade governs pristinamycin biosynthesis in *Streptomyces pristinaespiralis*. Appl Environ Microbiol 81:6621–6636. doi:10.1128/AEM.00728-15.

Editor: M. A. Elliott

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00728-15>.

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FIG 1 (Upper panel) HPLC spectrum of the culture extract from *S. pristinaespiralis* Pr11 wild-type strain (48-h sample). Wavelength monitoring was performed at 210 nm. PIA-specific (R_t = 8.2 min) and PIIA-specific (R_t = 10.3 min) peaks are indicated by arrows. (Lower panels) Corresponding UV-visible (UV-Vis) spectra of PIA (left) and PIIA (right) with their respective chemical structures; R (in PIA structure) represents CH_3 .

tance are organized together with a cryptic type II polyketide synthase (PKS) gene cluster (*cyp*) in a large supercluster (*prs*) (23). This gene region contains a variety of diverse regulatory genes, including a GBL receptor-like gene (*spbR*), two TetR-type regulatory genes (*papR3* and *papR5*), and four SARP-type genes (*papR1*, *papR2*, *papR4*, and *cyp1*), as well as one response regulator gene (*papR6*) (20, 23). SpbR has already been characterized as an auto-regulator receptor protein, which acts as a pleiotropic regulator and hereby influences growth, morphological differentiation and

pristinamycin biosynthesis of *S. pristinaespiralis* (10). Production of pristinamycin is induced by nanomolar concentrations of A-factor-like quorum-sensing molecules, which are secreted by the strain 3 h prior to the initiation of antibiotic production (24). However, the chemical structure of the *S. pristinaespiralis*-specific GBL receptor ligand(s) is still not elucidated. *papR1* encodes the SARP-type regulator PapR1 and itself is under transcriptional control of SpbR (10). As a *papR1* deletion mutant still produces 30% pristinamycin, it was assumed that there is another SARP-

type regulator (PapR2), which is involved in the control of pristinamycin biosynthesis (10). The function of the other *papR* genes has not been investigated before. The SARP gene *cpl1*, which is located within the *cpl* subcluster, is not involved in pristinamycin biosynthesis, as deletion of *cpl1* had no influence on pristinamycin production (23). Altogether, the multitude of different regulatory genes within the pristinamycin supercluster suggests a complex network for the regulation of the antibiotic's synthesis.

In this study, we describe the characterization of the regulatory function of PapR1 to PapR6 by mutational as well as overexpression, electrophoretic mobility shift assay (EMSA), and RT-PCR analyses. These regulators constitute a hierarchical signaling cascade governing the fine-tuned biosynthesis of pristinamycin in *S. pristinaespiralis*, which is summarized in a comprehensive regulatory model.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. For routine cloning strategies, *Escherichia coli* XL1-Blue (25) was used. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C (26) supplemented with kanamycin, apramycin, or ampicillin (50, 100, or 150 µg/ml, respectively) when appropriate. *S. pristinaespiralis* Pr11 (Aventis Pharma) was used for the generation of gene insertion mutants and overexpression strains. *Streptomyces lividans* T7 (27) was used for protein expression experiments. *Streptomyces* strains were grown at 28°C on yeast malt (YM), R5, R2YE, or mannitol soy flour (MS) solid medium for isolation of spores (28). For cultivation and harvesting of genomic DNA, *Streptomyces* strains were grown in 100 ml of S-medium (28) in 500-ml Erlenmeyer flasks (with steel springs) on an orbital shaker (180 rpm) at 28°C. Kanamycin and apramycin (50 and 100 µg/ml, respectively) were added to liquid cultures when required. For production analysis, *S. pristinaespiralis* strains were cultivated as reported previously (23).

Molecular cloning. The basic procedures for DNA manipulation were performed as described by Sambrook et al. (28) for *E. coli* and for *Streptomyces*. The primers used for PCR were obtained from MWG Biotech AG (Ebersberg, Germany) and Integrated DNA Technologies (IDT; Coralville, IA, USA) and are listed in Table 2.

Targeted disruption of pristinamycin regulatory genes. For the construction of gene insertion mutants ~1- to 1.5-kb fragments up- and downstream of the regulatory genes *papR1*, *papR2*, *papR3*, *papR4*, *papR5*, and *papR6* were amplified by PCR using *S. pristinaespiralis* Pr11 genomic DNA and the primers listed in Table 2. For amplification of the downstream fragments, primer pairs labeled m1/m2, which added an artificial EcoRV restriction site to the 5' end, were used, resulting in amplicates *papR1a* (1.1 kb), *papR2a* (1.0 kb), *papR3a* (1.0 kb), *papR4a* (1.2 kb), *papR5a* (1.3 kb), and *papR6a* (1.5 kb), respectively. For the amplification of the upstream fragments, primer pairs labeled m3/m4, which added an artificial EcoRV restriction site to the 3' end, were used, resulting in amplicates *papR1b* (1.2 kb), *papR2b* (1.3 kb), *papR3b* (1.2 kb), *papR4b* (1.1 kb), *papR5b* (1.5 kb), and *papR6b* (1.5 kb), respectively. All fragments were subcloned into the EcoRV-restricted *E. coli* vector pDRIVE, resulting in the constructs pDRIVE/*papR1a* and -*b*; pDRIVE/*papR2a* and -*b*; pDRIVE/*papR3a* and -*b*; pDRIVE/*papR4a* and -*b*; pDRIVE/*papR5a* and -*b*; and pDRIVE/*papR6a* and -*b*, respectively. All "a" fragments were isolated from the respective pDRIVE/*papRa* constructs as XbaI/BamHI fragments and ligated into the XbaI/BamHI-restricted *E. coli* vector pK18 (29), resulting in the constructs pK18/*papR1a*, pK18/*papR2a*, pK18/*papR3a*, pK18/*papR4a*, pK18/*papR5a*, and pK18/*papR6a*, respectively. The respective "b" fragments were excised as HindIII/EcoRV fragments from the respective pDRIVE/*papRb* constructs and were ligated into the HindIII/EcoRV site of the respective pK18/*papRa* plasmids, which resulted in the constructs pK18/*papR1'*, pK18/*papR2'*, pK18/*papR3'*, pK18/*papR4'*, pK18/*papR5'*, and pK18/*papR6'*, respectively. A 1.5-kb *aac(3)IV*

cassette (Apr^r) was isolated as an EcoRV/SmaI fragment from pEH13 (30) and was cloned into the EcoRV restriction site between fragments "a" and "b" of the respective pK18/*papR'* derivatives, resulting in the mutational constructs pYM11, pYM12, pYM13, pYM14, pYM15, and pYM16, in which the regulatory genes were inactivated by the insertion of the Apr^r cassette. The pYM11 to -16 plasmids were each transferred into *S. pristinaespiralis* Pr11 by protoplast transformation, followed by selection for apramycin-resistant and kanamycin-sensitive transformants, resulting in the *papR1::apr*, *papR2::apr*, *papR3::apr*, *papR4::apr*, *papR5::apr*, and *papR6::apr* mutants. Transformants were confirmed by PCR and Southern hybridization (data not shown). The Δ *papR1* Δ *papR4* double mutant was constructed on the basis of an *S. pristinaespiralis* NRRL2958 Δ *papR1'* deletion mutant, kindly provided by Sanofi-Aventis Pharma GmbH. This mutant shows the same pristinamycin production profile as the Pr11 *papR1::apr* mutant (data not shown). Plasmid pYM14 was introduced into the Δ *papR1'* mutant. Transformants were selected for apramycin-resistant and kanamycin-sensitive clones, resulting in the Δ *papR1* Δ *papR4* strain.

Construction of *papR* overexpression strains. For overexpression experiments, the genes *papR1*, *papR2*, *papR3*, *papR4*, and *papR5* were amplified by PCR using *S. pristinaespiralis* Pr11 genomic DNA and the primers listed in Table 2. The primers were designed in such a way that a BamHI restriction site at the 5' end and a HindIII restriction site at the 3' end were added to the *papR* coding sequence. This resulted in the fragments *papR1ex* (1.0 kb), *papR2ex* (1.0 kb), *papR3ex* (0.8 kb), *papR4ex* (0.9 kb), and *papR5ex* (0.7 kb), respectively. All "ex" fragments were restricted by BamHI/HindIII and subcloned into the BglIII/HindIII restriction site of the *E. coli* expression plasmid pRSETB. With this cloning procedure, each *papR* gene is fused to a His tag-encoding sequence, which is localized behind the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible T7 promoter. From the resulting constructs pRSETB/*papR1*, pRSETB/*papR2*, pRSETB/*papR3*, pRSETB/*papR4*, and pRSETB/*papR5*, respectively, the *his**papR* fragments were excised with NdeI/HindIII and ligated into the NdeI/HindIII-restricted *E. coli*/*Streptomyces* shuttle plasmid pGM190 (31), where they are under the control of the thiostrepton-inducible P_{tipA} promoter. The targeting plasmids pYM17, pYM18, pYM19, pYM20, and pYM21, respectively, were each transferred to *S. pristinaespiralis* Pr11 for pristinamycin production analyses, resulting in the strains SP*papR1*-OE, SP*papR2*-OE, SP*papR3*-OE, SP*papR4*-OE, and SP*papR5*-OE, respectively, and into *S. lividans* T7 for protein purification experiments, resulting in the strains SL*papR1*-OE, SL*papR2*-OE, SL*papR3*-OE, SL*papR4*-OE, and SL*papR5*-OE, respectively.

Fermentation and pristinamycin production analysis. For pristinamycin production analyses, the *S. pristinaespiralis* Pr11 wild-type strain, the *papR* mutant strains, and the SP*papR*-OE strains (see above) were cultivated as described previously (23). Strains harboring the pGM190 plasmid were induced for gene expression by adding 25 µg/ml thiostrepton.

Expression and purification of the His-tagged pristinamycin regulators. For protein purification, the SL*papR*-OE strains were grown in 100 ml of yeast extract-malt extract (YEME) medium with 50 µg/ml kanamycin in 500-ml Erlenmeyer flasks (with steel springs) on an orbital shaker (180 rpm) at 28°C. After 2 to 3 days, 17 ml of the YEME preculture was inoculated into 200 ml of fresh YEME medium with 25 µg/ml thiostrepton for gene expression and then incubated for a further 3 to 4 days in 1-liter Erlenmeyer flasks with steel springs on an orbital shaker (180 rpm) at 28°C. Cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C. Cell pellets were resuspended in ice-cold lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8), and then cells were disrupted by a French press (10,000 lb/in²; American Instruments) with two consecutive passages. Cell debris and the insoluble protein fraction were harvested by centrifugation at 10,000 rpm for 20 min and 4°C. The His-tagged proteins were purified from the soluble crude extract by metal chelate affinity chromatography using Ni-nitrilotriacetic acid resins according to the standard protocol by Qiagen. The collected fractions were

TABLE 1 Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
<i>E. coli</i> XL1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q ZΔM15 Tn10 (Tet ^r)]	25
<i>S. pristinaespiralis</i> Pr11	Pristinamycin-producing strain/wild type; natural isolate of <i>S. pristinaespiralis</i> ATCC 25486	Aventis Pharma
<i>papR1::apr</i> mutant	Gene interruption of <i>papR1</i> , <i>aac(3)IV</i>	This work
<i>papR2::apr</i> mutant	Gene interruption of <i>papR2</i> , <i>aac(3)IV</i>	This work
<i>papR3::apr</i> mutant	Gene interruption of <i>papR3</i> , <i>aac(3)IV</i>	This work
<i>papR4::apr</i> mutant	Gene interruption of <i>papR4</i> , <i>aac(3)IV</i>	This work
<i>papR5::apr</i> mutant	Gene interruption of <i>papR5</i> , <i>aac(3)IV</i>	This work
<i>papR6::apr</i> mutant	Gene interruption of <i>papR6</i> , <i>aac(3)IV</i>	This work
Δ <i>papR1</i> ' mutant	<i>papR1</i> deletion mutant of <i>S. pristinaespiralis</i> NRRL2958	Aventis Pharma
Δ <i>papR1</i> Δ <i>papR4</i> mutant	Gene interruption of <i>papR4</i> , <i>aac(3)IV</i> in Δ <i>papR1</i> ' mutant	This work
SPpGM190	<i>S. pristinaespiralis</i> /pGM190	This work
SPp <i>papR1</i> -OE	<i>S. pristinaespiralis</i> /pYM17	This work
SPp <i>papR2</i> -OE	<i>S. pristinaespiralis</i> /pYM18	This work
SPp <i>papR3</i> -OE	<i>S. pristinaespiralis</i> /pYM19	This work
SPp <i>papR4</i> -OE	<i>S. pristinaespiralis</i> /pYM20	This work
SPp <i>papR5</i> -OE	<i>S. pristinaespiralis</i> /pYM21	This work
<i>papR5::apr</i> /pYM21	Gene interruption of <i>papR5</i> , <i>aac(3)IV</i> , pYM21	This work
Δ <i>papR2::apr</i> /p <i>papR1</i> -OE	<i>papR2::apr</i> /pYM18	This work
Δ <i>papR2::apr</i> /p <i>papR4</i> -OE	<i>papR2::apr</i> /pYM20	This work
<i>S. lividans</i> T7	<i>tsr</i> , T7 RNA polymerase gene	27
SLpGM190	<i>S. lividans</i> /pGM190	This work
SLp <i>papR1</i> -OE	<i>S. lividans</i> /pYM17	This work
SLp <i>papR2</i> -OE	<i>S. lividans</i> /pYM18	This work
SLp <i>papR3</i> -OE	<i>S. lividans</i> /pYM19	This work
SLp <i>papR4</i> -OE	<i>S. lividans</i> /pYM20	This work
SLp <i>papR5</i> -OE	<i>S. lividans</i> /pYM21	This work
Plasmids pDRIVE	<i>lacZ'</i> complementation system, ampicillin and kanamycin resistance, multiple cloning site	Qiagen
pK18	pUC derivative, <i>aphII</i> , <i>lacZ'</i> complementation system	29
pEH13	pUC21 derivative carrying the 1.8-kb apramycin resistance cassette (Apr ^r)	30
pRSETB	<i>bla</i> , pT7, pUC derivative	Invitrogen
pGM190	<i>Streptomyces-E. coli</i> shuttle vector, <i>tsr aphII</i> , pSG5 derivative, <i>tipA</i> promoter shuttle vector	31
pUC18C	<i>bla cya</i> -T18	32
pKT25	<i>aph cya</i> -T25	32
Plasmids for <i>papR</i> mutant construction		
pYM11	pk18 derivative, <i>aphII</i> Apr ^r <i>lacZ'</i> α <i>papR1ab</i>	This work
pYM12	pk18 derivative, <i>aphII</i> Apr ^r <i>lacZ'</i> α <i>papR2ab</i>	This work
pYM13	pk18 derivative, <i>aphII</i> Apr ^r <i>lacZ'</i> α <i>papR3ab</i>	This work
pYM14	pk18 derivative, <i>aphII</i> Apr ^r <i>lacZ'</i> α <i>papR4ab</i>	This work
pYM15	pk18 derivative, <i>aphII</i> Apr ^r <i>lacZ'</i> α <i>papR5ab</i>	This work
pYM16	pk18 derivative, <i>aphII</i> Apr ^r <i>lacZ'</i> α <i>papR6ab</i>	This work
Plasmids for <i>papR</i> overexpression		
pYM17	pGM190 derivative, <i>PtipA tsr aphII his papR1</i>	This work
pYM18	pGM190 derivative, <i>PtipA tsr aphII his papR2</i>	This work
pYM19	pGM190 derivative, <i>PtipA tsr aphII his papR3</i>	This work
pYM20	pGM190 derivative, <i>PtipA tsr aphII his papR4</i>	This work
pYM21	pGM190 derivative, <i>PtipA tsr aphII his papR5</i>	This work

TABLE 2 Primers used in this study

Primer	Primer sequence (5'–3') ^a	Temp (°C)
For <i>papR</i> mutant construction		
papR1m1	GCGTAGAGGTGGTCGGTGAT	59
papR1m2	ATGATATCACGGCCGGCTGACCGG	65
papR1m3	ATGATATCATGGCGTTTCGTCTTC	55
papR1m4	TACGCCGCCGACCACGGCAT	67
papR2m1	GTATCTGCCCCGCTCCT	59
papR2m2	ATGATATCACTAGGCCCTGCCCCG	66
papR2m3	TAGATATCCATTGTCTTCCTCGCA	55
papR2m4	CGGGCACTTCTACTTCC	58
papR3m1	GGCGAGCGCGTTGTG	52
papR3m2	ATGATATCCACGCCGCTGAACCC	65
papR3m3	ATGATATCGGTACGCCCGTGGGATCG	71
papR3m4	GGGAGACGGCGTGGACATCG	62
papR4m1	ATCTATAGCAGCGCTCGATCCTGATG	61
papR4m2	ATGATATCCAGCGCTCGATCCTGATG	63
papR4m3	ATGATATCCAGGCCGCCGAGATCAG	67
papR4m4	CCGCCGTCCGTCACTGAG	57
papR5m1	CGATCTGGCCTGCATCCCGGTTTC	68
papR5m2	ATGATATCTAGCAGACCACCGCCCTGTTTC	68
papR5m3	ATGATATCACGCTCCTGCTTGAC	55
papR5m4	ATGATCTCGACCCTGAAC	44
papR6m1	AACAGGGTGTGCAGCGCGGG	72
papR6m2	ATGATATCTGCGCTGACGCCCGCA	70
papR6m3	ATGATATCTTCCGTCAATGACCCGC	61
papR6m4	TGCGCGTGCACCCCGAGACC	73
For <i>papR</i> overexpression		
papR1ex1	ATGGATCCAGACATCGACATACTCGGCGC	70
papR1ex2	ATAAGCTTTTACGCCGGCCGTGGCGGCG	73
papR2ex1	TGGATCCAAGTTCCGCATTCTCGGTCCGGTG	75
papR2ex2	TAAGCTTAGTGGCCCGAGGCCGGGTTG	75
papR3ex1	ATGGATCCCGGCACGCGGCACGCGATCC	81
papR3ex2	TAAGCTTTCAGCGGGCGTGGGCGGGGC	77
papR4ex1	ATGGATCCGACATCGATGTGCTGGGGGAG	73
papR4ex2	TAAGCTTTTACGCCGGCCCGCTCAGCCG	76
papR5ex1	ATGGATCCTGCGGTCCGCCGTCCGTCAGTG	78
papR5ex2	TAAGCTTCTATTGGGGGGTGGGGGTGC	68
For amplification of promoter regions		
propapR1fw	AGCCAGTGGCGATAAGAACGACGGCTGCCTGACCCG	77
propapR1rev	AGCCAGTGGCGATAAAGTATGTCTGATGTCCATGGCGT	73
propapR2fw	AGCCAGTGGCGATAAAGATCGGCCGGCCCGGGCGCGA	81
propapR2rev	AGCCAGTGGCGATAAAGCAGGGCGTTGGTCGCGTTC	77
propapR3fw	AGCCAGTGGCGATAAAGAGCGGCTGAGCCGGGCCCGC	81
propapR3rev	AGCCAGTGGCGATAAGTTGCCCTGGTGACCCTGG	77
propapR4fw	AGCCAGTGGCGATAAGACCCCTTACGCCCGTTCCTT	75
propapR4rev	AGCCAGTGGCGATAAAGAGCTCCCCAGCACATCGAT	75
propapR5fw	AGCCAGTGGCGATAAAGTCTGTCCCGGTTCCCGGCC	78
propapR5rev	AGCCAGTGGCGATAAAGATCGCAGCGATCCCCCTCACT	75
propapR6fw	AGCCAGTGGCGATAAGAATGTCTCCATGATCGTCCC	73
propapR6rev	AGCCAGTGGCGATAAAGACGAGGCCCTGCCGCCGTG	78
prospbRfw1	AGCCAGTGGCGATAAAGAGCGTTCTGTGCCGGCTCCGG	78
prospbRrev1	AGCCAGTGGCGATAAAGTCCCTTCAAGCGGAATGAT	72
prospbRfw2	AGCCAGTGGCGATAAAGATCATTCCTTGAAGGGA	72
prospbRrev2	AGCCAGTGGCGATAAAGTCGGCCGCCGCCACCAAAAT	76
proснаBfw	AGCCAGTGGCGATAAAGAGCCGCTGCTCGTCCGTGG	78
proснаBrev	AGCCAGTGGCGATAAAGTGTGAGGGTGGCGACGAGG	77
proснаDfw	AGCCAGTGGCGATAAGAAGGGCGGGGAACGGCTGCC	78
proснаDrev	AGCCAGTGGCGATAAGTCCGGCGGTGCGGGGGTTCT	78
proснаE3fw	AGCCAGTGGCGATAAAGCGCGCGGCTCGGCCCGGTCT	77
proснаE3rev	AGCCAGTGGCGATAAAGTCTGCGCGCGGACGCGGAG	76
proснаFfw	AGCCAGTGGCGATAAGAAGTGGGCTGGAAC	60

(Continued on following page)

TABLE 2 (Continued)

Primer	Primer sequence (5'–3') ^a	Temp (°C)
proснаFrev	AGCCAGTGGCGATAAGCGCGGTGGAACATC	60
procpp1-snaRfw	AGCCAGTGGCGATAAGGGTTCTCCGTA	74
procpp1-snaRrev	AGCCAGTGGCGATAAGGGCGCCCGAAAGTA	77
propipA-snbAfw	AGCCAGTGGCGATAAGAACGCATCCGTCAGCATCG	75
propipA-snbArev	AGCCAGTGGCGATAAGTCGCGCCGCGCCAGGACCCA	79
prosnbCfw	AGCCAGTGGCGATAAGGCAGAACCTGCTGAACAAG	60
prosnbCrev	AGCCAGTGGCGATAAGTGTGAAGACGGGACTGTG	60
propapB-papCfw	AGCCAGTGGCGATAAGACGTCCAGCCAGGTCACCGC	77
propapB-papCrev	AGCCAGTGGCGATAAGAGGGCGGCGTCCGCGGCGTC	81
prosnbRfw	AGCCAGTGGCGATAAGGGATCCCTCGCCAGGGCC	79
prosnbRrev	AGCCAGTGGCGATAAGGTTGTCGAGCAGGACGACGA	75
Cy5	AGCCAGTGGCGATAAG	60
For amplification of cDNA (RT-PCR)		
papR1int1	ACCGTGCAGACCTACATCC	62
papR1int2	TCAGTTCGGCGAGCAGTTC	62
papR2int1	GCGGGAACGTTTCTACGACCTG	66
papR2int2	TTCGAGGGAGAGGTGCTCGATG	66
papR3int1	ACCTCGGTGATCCAGGTCTG	65
papR3int2	TCCTCGCGGGCGCCAGATG	71
papR4int1	AACTGGCCGTGCAGGTTCTC	65
papR4int2	AAGGACGTGCTGGTGACCTC	65
papR5int1	AGAAGCCGGTGATCTTGC	60
papR5int2	GCACTTCCACTTCGAGAAC	60
papR6int1	GTGTCATAGGGGAGGACGAG	60
papR6int2	CTGGGAGGTGGTGGAGTG	60
spbRint1	AGATCCTGCGTCTGCTCCATCC	66
spbRint2	GGTGTTTCGACGAGGTCGGTTAC	66
snaBint1	ATCACCGCCCCGCTCCCGGC	73
snaBint2	ATCTTGGCGTGCGGTGCCTG	67
snaE3int1	CTGTCTACCTGCTGGACCT	60
snaE3int2	GAGGGGTGCAGGTAGAGGTT	60
snbAint1	GGAGGTGAAGGTGACGTGTT	60
snbAint2	AGTGTCATCTGGCGGTGCT	60
snbCint1	CCTACGTCCAGTGGCTGAC	60
snbCint2	CCTCCCTCGTAGGGGTAGC	60
hrdBfw	CCGGTCAAGGACTACCTGAA	60
hrdBv	GTGGCGTACGTGGAGAACTT	60

^a Restriction sites are underlined; Cy5 homologous base pairs are in bold.

analyzed by standard SDS-polyacrylamide gel electrophoresis (PAGE) in 12% gels (26) and Western blot analysis.

EMSA. DNA fragments (100 to 250 bp) of the upstream regions of the various genes were amplified by PCR from genomic DNA of *S. pristinaespiralis* Pr11 with the primers listed in Table 2. For Cy5 labeling, the DNA amplicates were used as the templates in a second PCR approach together with the Cy5 primer (Table 2). DNA binding reactions were performed at room temperature in 20 μ l of 100 mM HEPES (pH 7.6), 5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM dithiothreitol, 1% (wt/vol) Tween 20, 150 mM KCl, 5 mM MgCl₂, 2 ng of Cy5-labeled DNA, and 8 μ l of crude extract. After a 10-min incubation, 2 μ l loading buffer (0.25 $\times$ Tris-borate EDTA [TBE] buffer, 60% glycerol) was added, and the samples were loaded onto a 2% agarose gel. To verify the specificity of the regulator-DNA binding, an excess of unlabeled, specific, or nonspecific DNA was added to the EMSA mixture, separately. DNA bands were visualized by fluorescence imaging using a Typhoon Trio Variable Mode Imager (GE Healthcare).

Transcriptional analysis by RT-PCR experiments. *S. pristinaespiralis* Pr11 strains were grown in 100 ml of preculture medium (see above) and incubated at 28°C in 500-ml Erlenmeyer flasks (with steel springs) on an orbital shaker (180 rpm). Samples were taken after 24, 36, 48, 72, and 96 h. Cell disruption was carried out with glass beads (150 to 212 μ m; Sigma)

using a Precellys Homogenizer (6,500 rpm, once for 20 to 30 s; Peqlab). Total RNA was extracted from *S. pristinaespiralis* and used as the template for RT-PCR in accordance with the instructions from the RNeasy minikit (Qiagen). DNA that bound to the RNA purification column was digested with RNase-free DNase (Fermentas) once on the column for 30 min at 24°C and a second time for 1.5 h at 24°C before elution of the RNA. RNA concentrations and quality were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNA from 3 mg RNA was generated with random nonamer primers (Sigma), reverse transcriptase, and cofactors (Fermentas). For PCRs, primers that amplify cDNA of 200 to 300 bp from internal gene sequences were used (Table 2). PCR conditions were 98°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 40 s, and finally 72°C for 5 min. As a positive control, cDNA was amplified from the major vegetative sigma factor (*hrdB*) transcript, which is expressed constitutively. To exclude DNA contamination, negative controls were carried out by using total RNA as a template for each RT-PCR.

BTH assays. Bacterial two-hybrid (BTH) complementation assays were carried out with the nonreverting adenylate cyclase-deficient (*cya*) *E. coli* strain BTH101 (32). For the construction of recombinant plasmids used for BTH assays, the genes of interest were amplified by PCR using *Taq* polymerase (Qiagen) and the oligonucleotide pairs described in

TABLE 3 Characteristics of genes, predicted functions, and protein matches from other *Streptomyces* species

Gene	Size (bp)	No. of amino acids	pI	Predicted function	ID/SM ^a (%)	Match	Origin reference	GenBank accession no.
<i>spbR</i>	1,050	228	5.72	GBL receptor	63/77	TylP	<i>S. fradiae</i>	AAD40801
					46/64	SrrA	<i>S. rochei</i>	BAC76540
					46/64	BarA	<i>S. virginiae</i>	BAA06981
<i>papR1</i>	857	285	10.58	SARP-type regulator	72/81	TylS	<i>S. fradiae</i>	AAD40804
					71/83	SrrW	<i>S. rochei</i>	BAC76513
<i>papR2</i>	995	331	7.03	SARP-type regulator	63/72	VmsS	<i>S. virginiae</i>	BAF50715
					60/69	TylT	<i>S. fradiae</i>	AAD40805
					44/55	RedD	<i>S. lividans</i> TK24	EFD66212
<i>papR3</i>	824	275	9.92	TetR-type regulator	39/55	BarB	<i>S. virginiae</i>	BAA23612
					53/65	SrrC	<i>S. rochei</i>	BAC76532
<i>papR4</i>	902	300	10.05	SARP-type regulator	73/83	TylS	<i>S. fradiae</i>	AAD40804
					72/78	SrrY	<i>S. rochei</i>	BAC76533
<i>papR5</i>	662	220	6.08	TetR-type regulator	57/68	TylQ	<i>S. fradiae</i>	AAD40803
					56/69	SrrB	<i>S. rochei</i>	BAC76537
<i>papR6</i>	749	249	9.74	Response regulator	52/65	VmsT	<i>S. virginiae</i>	BAF50712

^a ID/SM, % identity/similarity of amino acid sequences.

Table 2. PCR products were cloned as XbaI/BamHI- or XbaI/KpnI-digested DNA fragments in frame with either the T18 or the T25 fragment of the catalytic domain of the *Bordetella pertussis* adenylate cyclase (*cyaA*) into the equally restricted pUT18C and pKT25 vectors (BACTH System kit; Euromedex). For BTH complementation assays, recombinant pKT25 and pUT18C plasmids carrying the genes of interest (Table 1) were used in various combinations to cotransform *E. coli* BTH101/pRARE2 cells. The transformants were plated onto M63-X-Gal-IPTG (where X-Gal is 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) or MacConkey medium with ampicillin (75 mg/ml), kanamycin (25 mg/ml), and chloramphenicol (25 mg/ml) supplemented with lactose. *E. coli* BTH101/pRARE2 with the empty pUT18C and pKT25 vectors was used as a negative control. *E. coli* BTH101/pRARE2 with the plasmids pUT18-*zip* and pKT25-*zip* was used as a positive control. Protein-protein interaction is observed when cells stain blue on M63-X-Gal-IPTG and red on MacConkey agar.

Phylogenetic analysis. SARP sequences from different antibiotic producers were identified with the BLAST software and aligned using the Clustal Omega software. A phylogenetic analysis was performed with the aligned sequence data using the Clustal W2 program.

RESULTS

In silico analysis of the regulatory gene products. In the course of sequence analysis of the pristinamycin gene region, several regulatory genes were identified. The respective genes were designated *spbR*, *papR1*, *papR2*, *papR3*, *papR4*, *papR5*, and *papR6* (23). The *spbR* gene is localized at the right border of the pristinamycin gene region (see Fig. S1A in the supplemental material) and encodes the autoregulator receptor SpbR, with high similarity to TylP and BarA of *S. fradiae* and *S. virginiae*, respectively (Table 3). *papR1*, *papR2*, and *papR4* code for SARP-type regulators belonging to the “small SARP” group (2). The deduced proteins PapR1 and PapR4 both show high similarity to the SARP TylS of *S. fradiae*, whereas PapR2 is similar to VmsS of *S. virginiae* and TylT of *S. fradiae* (Table 3; see also Fig. S2A in the supplemental material). Two TetR-type regulatory genes are present in the pristinamycin gene region, *papR3* and *papR5*, both of which are part of a “regulatory

island,” consisting of *papR3*-*papR4*-*papR5*, in the proximity of the cluster border (see Fig. S1A in the supplemental material). The deduced PapR3 protein is similar to BarB of *S. virginiae*, whereas the predicted PapR5 protein is more similar to TylQ of *S. fradiae* (Table 3). PapR3 and PapR5, as well as the autoregulator receptor SpbR, consist of the typical TetR-like protein structure (see Fig. S2B in the supplemental material). The gene *papR6* encodes a predicted response regulator of bacterial two-component transduction systems that shows similarity to VmsT of *S. virginiae* (Table 3). However, no cognate sensor kinase gene is present in the pristinamycin gene cluster. Thus, PapR6 represents an orphan response regulator, like AurIP in *Streptomyces aureofaciens* (activator of auricin biosynthesis) (33) or JadR1 in *Streptomyces venezuelae* (activator of jadomycin biosynthesis) (34).

Regulatory influence on *S. pristinaespiralis* morphological development. To analyze the functions of *papR1*, *papR2*, *papR3*, *papR4*, *papR5*, and *papR6*, the genes were inactivated by insertion of an apramycin resistance cassette (*Apr*^r) and the morphologies of the respective mutants, i.e., *papR1::apr*, *papR2::apr*, *papR3::apr*, *papR4::apr*, *papR5::apr*, and *papR6::apr* mutants, were analyzed on MS solid medium. An *S. pristinaespiralis* NRRL2958 *spbR* apramycin insertion mutant (*spbR25*) has been constructed before and was described to have severe defects in growth and morphological differentiation and not produce any pristinamycin (10). The *papR1::apr*, *papR2::apr*, *papR3::apr*, *papR4::apr*, and *papR6::apr* mutants grew as the wild-type strain, which forms the aerial mycelium after ~3 days and starts producing gray spores after around 7 days. The *papR5::apr* mutant failed to form any aerial mycelium or spores on solid medium (Fig. 2). This phenotype was observed on different media, such as LB, R5, R2YE, YM, or MS agar (see Fig. S3 in the supplemental material), and was restored after complementation with the native *papR5* gene (see Fig. S4A in the supplemental material). Altogether, these data show that *papR1*, *papR2*, *papR3*, *papR4*, and *papR6* do not exert any effect on

FIG 2 Morphological phenotype of *S. pristinaespiralis* wild-type strain and the *papR1::apr*, *papR2::apr*, *papR3::apr*, *papR4::apr*, *papR5::apr*, and *papR6::apr* mutants on MS solid agar after 5 days.

morphological development, whereas *papR5* strongly influences differentiation in *S. pristinaespiralis*.

Pristinamycin production of *papR* mutants and overexpression strains. To investigate the regulatory functions of *papR1*, *papR2*, *papR3*, *papR4*, *papR5*, and *papR6*, the pristinamycin production of the respective regulatory mutants (*papR1::apr*, *papR2::apr*, *papR3::apr*, *papR4::apr*, *papR5::apr*, and *papR6::apr* mutants) and that of overexpression strains were analyzed by HPLC and compared to the antibiotic production of the *S. pristinaespiralis* wild-type strain. For the overexpression experiments, additional copies of *papR1*, *papR2*, *papR3*, *papR4*, and *papR5* were each cloned into the medium-copy-number plasmid pGM190 under the control of the thiostrepton-inducible promoter P_{tipA} and then transferred into *S. pristinaespiralis*, resulting in the overexpression strains SP*papR1*-OE, SP*papR2*-OE, SP*papR3*-OE, SP*papR4*-OE, and SP*papR5*-OE, respectively. *S. pristinaespiralis* with the empty pGM190 plasmid served as a control. All strains were cultivated in pristinamycin production medium. Samples were taken at different time points (24 h, 48 h, 72 h, and 96 h) and then analyzed for pristinamycin production by high-performance liquid chromatography (HPLC).

The *papR1::apr* and *papR4::apr* mutant strains showed similar production profiles and produced less pristinamycin than the wild-type strain. The production performance was around 30% to 50% that of the wild-type strain. The deletion of *papR2* led to a complete loss of antibiotic production (Fig. 3). In contrast, the overexpression of the SARP genes *papR1* and *papR2* each led to an earlier and greater (increased by up to 100%) pristinamycin production, whereas the *papR4* overexpression did not have any significant effect on the starting time or amount of production (see Fig. S5 in the supplemental material). Altogether, these data show that PapR1, PapR2, and PapR4 act as activators of pristinamycin biosynthesis. Thereby, *papR2* is essential for pristinamycin biosynthesis, whereas *papR1* and *papR4* are not.

The deletion of the TetR-like *papR* genes led to an increase of pristinamycin production: the *papR3::apr* mutant produced up to 150% and the *papR5::apr* mutant even 300% more pristinamycin than did the wild-type strain (Fig. 3). Here, deletion of *papR5* had

a more dramatic effect on PII than on PI production. In contrast, the overexpression of *papR3* resulted in a decreased pristinamycin production, which was around 40% that of the wild-type strain (see Fig. S5 in the supplemental material). The overexpression of *papR5* in *S. pristinaespiralis* caused a complete loss of antibiotic production. This was observed when *papR5* was overexpressed in *S. pristinaespiralis* Pr11 (strain SP*papR5*-OE) (see Fig. S5 in the supplemental material) but also in the pYM21-complemented *papR5::apr* mutant strain (see Fig. S4B in the supplemental material). These data show that both PapR3 and PapR5 are repressors of pristinamycin biosynthesis but *papR5* has a more dramatic effect on production than *papR3*.

The deletion of the response regulator gene *papR6* led to a decrease of pristinamycin production, which was around 40% that of the wild-type level (Fig. 3), whereas *papR6* overexpression resulted in a slightly increased pristinamycin production (data not shown), suggesting that PapR6 is an activator of pristinamycin biosynthesis.

The pristinamycin production profiles of all mutant strains were restored, at least partially, to wild-type levels after complementation with the respective *papR* genes using the pGM190 derivatives described above (data not shown).

Identification of TetR- and SARP-binding sequences within the pristinamycin gene cluster. To identify binding sequences in the upstream regions of the pristinamycin gene cluster that are potential targets of the diverse PapR regulators, we performed bioinformatical analyses using the software PatScan (35). TetR-like regulators are known to bind to conserved partially palindromic sequence motifs, the so-called ARE sequences (10). Three ARE motifs have already been identified in the upstream region of *spbR* (*PspbR*-ARE1 and *PspbR*-ARE2) and *papR1* (at bp −19 to −42; *PpapR1*) (10). Our analysis identified two further ARE motifs, upstream of *papR4* and *papR5*, as well as another less conserved one in front of *papR2* (Fig. 4A). No ARE sequences were found in the upstream region of *papR3* and *papR6* or any of the pristinamycin biosynthetic genes. Thus, except for the promoter region of the regulatory genes *papR3* and *papR6*, all other pristinamycin regulatory promoters contain ARE binding motifs and therefore are suggested to be regulated by TetR-like proteins.

In the same manner, the cluster was screened for putative SARP binding sites. SARP regulators are known to bind to specific direct heptameric sequence motifs, of which the 3' repeat is located 8 bp from the −10 promoter element of the target gene (for example, *ActII*-ORF4, 5'-TCGAGCC/G-3'). It is suggested that two SARP monomers cooperatively bind to a direct repeat at the same face of the DNA, whereas the RNA polymerase is recruited to the opposite face of the DNA (36). Putative SARP binding motifs were detected upstream of the PI-specific genes *snbC* and *snbA-pipA* and the PII-specific genes *snbA*, *snbD*, *snbE3*, and *snbR-cpp1*, where *cpp1* is not related to pristinamycin biosynthesis, as well in front of the regulatory gene *papR1* and the predicted ABC transporter gene *snbR* (Fig. 4B). Thus, nearly all predicted pristinamycin operons (37) contain a SARP binding motif in their cognate promoter regions, except for the intergenic regions in front of *snbF* and *papB-papC*.

PapR2 is the central SARP activator of pristinamycin biosynthesis. From the pristinamycin production analysis reported above, we know that PapR2 is essential for pristinamycin biosynthesis. To identify the targets of the PapR2 regulation, we performed EMSAs with the purified HisPapR2 protein and the

FIG 3 Pristinamycin production of the *S. pristinaespiralis* wild-type strain (WT) and the *papR* mutant *papR1::apr*, *papR2::apr*, *papR3::apr*, *papR4::apr*, *papR5::apr*, *papR6::apr*, and $\Delta papR1 \Delta papR4$ mutant strains.

SARP-motif containing intergenic regions of all regulatory and structural genes (see above). In these analyses, HisPapR2 specifically bound to the upstream regions of the SARP regulator gene *papR1* (Fig. 5C), the PI structural genes *snbA*-*pipA* and *snbC*, and the PII structural genes *snbB* and *snbE3*, which all contain conserved SARP-binding motifs (Fig. 4A). To confirm an effect on transcription, reverse transcription-PCR (RT-PCR) experiments

using RNA isolated from the *papR2::apr* mutant and the wild-type strain were performed. The isolated RNA was used as the template in RT-PCRs with primers annealing to internal parts of the various genes. The transcriptional analysis showed that there is no or almost no transcription of *papR1* and the pristinamycin structural genes *snbA*, *snbC*, and *snbE3* in the *papR2::apr* mutant (Fig. 6A and B). Furthermore, transcripts of the PII structural genes *snbB*

A		
IUPAC	TNANAWACNNACYNNNCGGTTTCTTT	100%
<i>PspbR</i> (ARE1)	AAAGATACGTACCCACCGGTTTGT	96, 2%
<i>PspbR</i> (ARE2)	TAAGATACGAACCCGCCGTTTCTTT	96, 2%
<i>PpapR1</i>	TGACAAACCGACCGTGCCGTTTCTTT	96, 2%
<i>PpapR2</i>	GAAGACAATGAAGTTCCGCATTCTCG	61, 5%
<i>PpapR4</i>	TGACAAACCGTCCGTCCTGTTTCTTTA	92, 3%
<i>PpapR5</i>	TTACAAACCGCGTAAGCGGTTTGT	88, 4%
<i>prist</i> consensus	TAASAAACCGACCSWSCGGTTTCTTT	
B		
<i>Psnab</i>	CTCACACGCTATCAGTTC CCG GTGAGCG	
<i>Psnad</i>	GTCAGCCGCACGTCAGCGGCACGTCGGCG	
<i>PsnbC</i>	ATCAGGAGCGGGTTCAGCGCCCGTCAACC	
<i>Psnar-cpp1</i>	GTCAGGCATGTGTCAGTGCCGCGTCAGGC	
<i>PsnadE3</i>	CTGAGGGACCGTCACGCCCGTGCCTGCG	
<i>PpapR1</i>	GTCAGGCGGGCGGTGCGCAGGTGTGGTTG	
<i>PsnbA-pipA</i>	GTCAGGAGCCGATCAGGGCGGCGTCAGCC	
<i>PsnbR</i>	GTGTCACACCGGTGACTCCCGTGCCGTG	
<i>prist</i> consensus	GTCAGSSNNNNGTCAGSSNNNNGTCAGSS...8nt...-10	

FIG 4 (A) ARE sequences and their respective conformities in front of the genes *spbR*, *papR1*, *papR2*, *papR4*, and *papR5*. The sequences were compared to the consensus "IUPAC string" mentioned by Folcher et al. The *S. pristinaespiralis*-specific ARE consensus sequence (*prist*) is shown in the lowest row. The underlined sequences represent half sites of the palindrome, supposed to be bound by the TetR-like monomers. (B) SARP binding sequences of pristinamycin-related genes. Heptameric repeats, which are supposed to be bound by two SARP monomers and RNA polymerase, are shown in bold. The *S. pristinaespiralis*-specific SARP consensus sequence is shown in the lowest row.

and *snad* or the transporter gene *snbR* were absent in the *papR2::apr* mutant samples, whereas all these genes were transcribed in the wild-type strain (data not shown). Thus, even if not all SARP motif-containing samples shifted in our EMSAs (*snad* and *snbR*), we have supporting evidence from RT-PCR data that these genes are also regulated by PapR2. Altogether, these data indicate that PapR2 is a superior SARP-type regulator that directly activates the transcription of the SARP regulatory gene *papR1* as well as the

FIG 6 Transcriptional analysis of the *S. pristinaespiralis* wild-type and different *papR::apr* mutant strains. (A and B) A 5- μ l volume of the GeneRuler 1-kb ladder (Fermentas) was loaded onto each gel as an internal control. The first picture in a row shows the 250-bp and 500-bp bands (lower and upper bands, respectively) of the 1-kb ladder (M); the second picture in a row shows the RT-PCR sample (S). RT-PCR analysis of genes *papR1*, *papR2*, and *papR4* (A) and of genes *snbA*, *snbC*, and *snadE3* (B) in the WT, *papR1::apr*, and *papR2::apr* strains at 36 h. (C and D) RT-PCR analysis of the genes *papR1* and *papR4* in the WT and *papR5::apr* strains (C) and of the genes *papR4* and *papR5* in the WT and *papR3::apr* strains (D). *hrdB* was used as a control.

transcription of the pristinamycin structural and putative resistance gene(s).

PapR1 and PapR4 are accessory regulators. The production analyses of the *papR1::apr* and *papR4::apr* mutant showed that the two SARP regulators are dispensable for pristinamycin biosynthesis, as they still produced low levels of the antibiotics (Fig. 3). In

FIG 5 (A) EMSAs with HisPapR2 and Cy5-labeled promoter regions of the pristinamycin structural genes *snbA-pipA*, *snbC*, *snbA*, and *snadE3*. —, negative control without protein; +, addition of purified His-tagged PapR protein. (B) EMSAs with HisPapR1 and Cy5-labeled promoter regions of the pristinamycin structural genes *snbA-pipA*, *snbC*, and *snbA*. (C) EMSAs with the HisPapR2, HisPapR4, HisPapR5, and HisPapR3 and Cy5-labeled promoter regions of different *papR* genes. The specificity of the reaction was checked by the addition of 500-fold specific (S) and unspecific (U) unlabeled DNA.

DISCUSSION

Regulatory influence on the synergistic production ration.

From our regulatory studies, we know that at least seven different regulators are involved in controlling the pristinamycin biosynthesis. The question is why *S. pristinaespiralis* needs such a multitude of control elements. We speculated that this might be needed to ensure the cosynthesis of the two different streptogramin-type antibiotics in the synergistic active 30:70 ratio (PI/PII). However, whenever we inactivated or overexpressed our regulators in *S. pristinaespiralis*, we did not observe any influence on the PI/PII production ratio (except for PapR6). Thus, the synergistic production ratio is not the result of a fine-tuned regulation but rather is based on the antibiotic synthesis efficiency: The polyketide-type antibiotic PII is composed mainly of malonyl-coenzyme A (-CoA) units and three proteinogenic amino acids, whereas the peptide antibiotic PI consists of two proteinogenic and five aprotogenic amino acids (23). Overall, the PI biosynthesis may be more laborious, since all the aprotogenic amino acid precursors have to be synthesized beforehand, whereas in terms of PII synthesis, malonyl-CoA and the proteinogenic amino acids are available immediately. This assumption is supported by more-detailed HPLC analyses (data not shown) and the production curves presented in reference 37, in which PI production is seen to increase during the later growth phase.

Effector synthesis and autoregulator receptor SpbR. In a previous study, it has been shown that the autoregulator receptor SpbR is the major regulator of pristinamycin biosynthesis. It was suggested that SpbR senses an A-factor-like effector molecule (24); however, the SpbR-interactive ligand has not been characterized so far. In *S. griseus*, the GBL synthase AfsA is responsible for the biosynthesis of the A-factor signaling molecule (38). However, no putative orthologous gene is present in the pristinamycin biosynthetic gene region. Instead, at the right border of the cluster, between the autoregulator receptor gene *spbR* and the TetR-like gene *papR5*, a putative P450 monooxygenase-encoding gene, *snbU*, is localized. The predicted SnbU protein shows similarity to Orf16 of *S. fradiae* (64% identity, 75% similarity). Orf16 is a deduced cytochrome P450 and together with Orf18 (a deduced acyl-CoA oxidase) has been suggested to play a role in the synthesis of the GBL receptor (TylP)-interacting ligand in *S. fradiae* (12). Actually, no *orf18* orthologous gene is present in the pristinamycin gene cluster. However, due to the sequence homology to Orf16, we suggest that SnbU is involved in the pristinamycin effector synthesis. The respective autoregulator receptor SpbR has previously been shown to bind to the promoter region of its own gene, as well as to the *papR1* promoter (10). Our SpbR EMSA analyses additionally showed a binding to the *papR2*, *papR4*, and *papR5* promoter regions (data not shown), meaning that SpbR is an autoregulator that binds to all promoters harboring an ARE element and thereby controls the transcription of nearly all *papR* genes (except for *papR3* and *papR6*).

TetR-like regulators PapR3 and PapR5 and their putative receptor role. A protein alignment of the TetR-like proteins from *S. pristinaespiralis* and those from other antibiotic-producing streptomycetes revealed that PapR3 (275 amino acids) has a 60-amino-acid-longer N-terminal sequence residue (see Fig. S2B in the supplemental material). Within the respective gene region, a less conserved ARE motif (57.7%) was identified. Thus, it may be possible that the *papR3* gene has been annotated incorrectly and the

actual PapR3 protein is shorter (215 amino acids). However, we are quite sure that the data generated with the longer PapR3 version are authentic, for several reasons: overexpression analyses with the larger PapR3 version clearly showed a repressive function on pristinamycin biosynthesis, and EMSAs exhibited a specific binding performance property (see above); furthermore, from BTH analysis we know that the longer PapR3 protein is capable of forming functional dimers (Fig. 7), whereas the shorter version is not (see Fig. S7 in the supplemental material).

In silico analyses of the amino acid composition of the TetR-like regulators PapR5 and PapR3 revealed that the two proteins considerably differ from each other in respect to their deduced isoelectric points (*pI* values), which is a rather acidic one for PapR5 (*pI* = 6.08) and a more basic one for PapR3 (*pI* = 9.92) (Table 3). Such a difference in the *pI* values of antibiotic cluster-originated TetR-like regulators has been observed before. Here, it was stated that the basic TetR-like proteins are supposed to be “pseudo-GBL receptors,” whereas the acidic ones act as “real” GBL receptors (19, 39, 40). Given this classification, PapR3 would be expected to be the pseudo-GBL receptor whereas PapR5 should act as the real GBL receptor protein. However, previously published phylogenetic analyses suggest a pseudo-GBL receptor function for PapR5 (19, 41). At present, the receptor role of PapR3 and PapR5 is unclear and cannot be predicted from phylogenetic trees; instead, it should be resolved experimentally in future studies, e.g., by effector-dependent EMSAs. Our data showed that PapR5 has the strongest effect on pristinamycin production amount, which theoretically makes it a good candidate for a sensor protein that may detect pristinamycin and/or its intermediates as effector(s) and in a feed-forward mechanism drives the antibiotic biosynthesis in a way similar to that described for the JadR2-mediated regulation of jadomycin biosynthesis in *Streptomyces venezuelae* (42, 43).

Different SARPs exert discrete regulatory effects. As outlined above, PapR2 is the essential activator for pristinamycin biosynthesis, which controls *papR1*- and pristinamycin structural gene transcription, whereas PapR1 and PapR4 play only a subordinate role, as they are dispensable for pristinamycin production. PapR1, like PapR2, directly bound to the promoter regions of the pristinamycin structural genes. Thus, we suggest that PapR1 has an assisting function as a helper protein for PapR2. A similar “SARP helper” activity has already been proposed for TylU of *S. fradiae*, which is a nonessential, non-SARP-type regulator that together with the essential SARP TylS activates the expression of the pathway-specific activator TylR of tylosin biosynthesis (44). In EMSAs, PapR4 bound to the *papR2* promoter region, which suggests that it may have an activating function in *papR2* gene transcription. A comparable SARP-SARP system has been described for the lankamycin/lankacidin producer *S. rochei*, whereby the SARP-type regulator SrrY activates the transcription of the SARP gene *srrZ* (45). In summary, we can state that each of the pristinamycin SARPs exerts its individual role during pristinamycin biosynthesis.

Correlation between *pI* characteristics and functionalization of SARPs. Interestingly, *in silico* analyses of the amino acid composition of the SARP-type regulators PapR1, PapR2, and PapR4 also revealed striking differences in *pI* values: PapR2, the essential regulator of pristinamycin biosynthesis, has a neutral character (*pI* = 7.03), whereas PapR1 and PapR4 are very basic proteins (*pI* = 10.58 and 10.05, respectively) (Fig. 8; Table 3). To investigate if there is a relationship between the essentiality of different

FIG 8 Phylogenetic tree of different SARPs. Sources: *S. pristinaespiralis* (PapR1, CBW45751; PapR2, CBW45736; PapR4, CBW45766; Cpp1, CBW45731); *S. fradiae* (TyIS, AAD40804; TyIT, AAD40805); *S. lavendulae* (FarR3, BAG74713; FarR4, BAG74714); *S. aureofaciens* (Aur1PR2, ADM72850; Aur1PR3, ADM72849; Aur1PR4, ACK77758); *S. rochei* (SrrY, BAC76533; SrrZ, BAC76529; SrrW, BAC76513); *S. virginiae* (VmsR, BAA96296; VmsS, BAF50715); *S. griseoviridis* (SgvR3, AGN74872; SgvR2, AGN74902); *S. nanchangensis* (NanR1, AAP42853; NanR2, AAP42854); *S. coelicolor* A3(2) (RedD, AAA88556; ActII-ORF4, CAC44198); *S. peucetius* (DnrI, AAA26736); *S. clavuligerus* ATCC 27064 (CcaR, AAC32494); *S. argillaceus* (MtmR, CAK50770); *S. ambofaciens* ATCC 23877 (AlpV, CAJ87890). Phylogenetic distances are given as numbers. Essential SARP regulators are framed. Proteins with an acidic ($pI < 6.5$), neutral ($pI = 6.5$ to 7.5), or basic ($pI > 7.5$) character are indicated by boxes with vertical lines, black boxes, or boxes with diagonal lines, respectively.

SARPs, such as TyIS (activator of tylosin biosynthesis) from *S. fradiae*, SrrY from *S. rochei* (activator of lankacidin and lankamycin biosynthesis), DnrI from *Streptomyces peucetius* (activator of daunorubicin biosynthesis), RedD and ActII-Orf4 from *Streptomyces coelicolor* A3(2) (activator of undecylprodigiosin and actinorhodin biosynthesis, respectively), and Aur1PR3 from *S. aureofaciens* (activator of auricin biosynthesis) (46, 47, 48, 49, 50, 51) in antibiotic biosynthesis and different pI values, a phylogenetic analysis was performed with the known SARP-type regulators from various antibiotic-producing streptomycetes (Fig. 8). From this analysis, we found that, with a few exceptions, acidic and basic SARPs are part of discrete clades. Essential SARPs have a rather neutral or basic character. Interestingly, we observed that, except for the NanR regulators of *Streptomyces nanchangensis* (52), the SARPs from different strains are more similar to each other than the different SARPs from a specific host strain, meaning that the different SARPs from one strain most likely did not evolve from duplication events in the host strain but rather were acquired separately by horizontal gene transfer. In conclusion, the difference in protein acidity seems to be a more unitary tool that nature uses to develop functionally different regulators from the same protein family. Thus, the importance of protein acidity in the differential functionalization of regulators definitely needs further attention.

Putative interaction partners of response regulator PapR6.

Our analysis showed that nearly all predicted pristinamycin operons (37) contain a SARP binding motif in their cognate promoter regions, except *snaF* and *papB-papC*. Thus, the expression of the SARP motif-containing genes is suggested to be controlled by SARP-type regulators, whereas the *snaF* and *papB-papC* operon expression may be governed by a non-SARP-type regulator. Such a regulatory function most likely is exerted by the response regulator PapR6. This is supported by data from reference 37, which showed by EMSAs that PapR6 binds to the *snaF* promoter region. PapR6 is an orphan response regulator with no cognate sensor kinase gene in the pristinamycin cluster. A sensor kinase, Spy1, of which the encoding gene is located outside the pristinamycin gene cluster, has previously been reported to positively influence PI biosynthesis but has a negative effect on PII biosynthesis (53). The Dun et al. study (37) showed that a *papR6* deletion or overexpression had a more dramatic effect on PII production than on PI biosynthesis. Even if this is not obvious from the graph in Fig. 3, such a differential influence on PI and PII production has also been observed for several individual growth curves of *papR6* deletion and overexpression strains in the present study (data not shown). Thus, we concluded that PapR6 is an activator of PII but not PI biosynthesis. Whether Spy1 interacts with PapR6 is doubt-

FIG 9 Model of the regulation of pristinamycin biosynthesis in *Streptomyces pristinaespiralis*. SpbR ligands are indicated as black-filled diamonds. Pristinamycins and/or intermediates are shown as gray-filled circles. Regulators are represented by ellipses. The major pristinamycin regulators PapR2 and PapR5 are highlighted by thick lines. Arrows indicate transcriptional activation, and perpendicular lines represent transcriptional repression. The dashed line illustrates pristinamycin (intermediate) production.

ful, as the two regulators have opposite effects on PI and PII production.

With BTH analysis, we found that PapR6 interacts on the protein level with the TetR-like regulator PapR5 (Fig. 7). The fact that PapR6 interacts with PapR5 fits more with the data of jadomycin regulation in *S. venezuelae* and auricin regulation in *S. aureofaciens*. Here, it was hypothesized that the response regulator JadR1 forms a novel two-component system with the pseudo-GBL receptor JadR2, and together they govern jadomycin biosynthesis (53). A similar protein pair, Aur1P (response regulator)/Aur1R (TetR-like regulator), controls auricin production in *S. aureofaciens* (33). However, in these analyses the regulation has always been reported to take place on the protein-DNA level. Our data show an additional regulatory level, which occurs on the protein-protein stage and may disclose a completely novel regulatory interaction pair.

Pristinamycin regulation model. To comprehend our data obtained from the regulatory studies of the PapR regulators into a broader context, we derived a hierarchical organized regulatory model that governs pristinamycin biosynthesis in *S. pristinaespiralis* (Fig. 9): in the absence of ligands, the autoregulator receptor SpbR represses its own transcription, as well as that of *papR1*, *papR2*, *papR4*, and *papR5*. As this repression prohibits the expression of all SARPs, which are the pathway-specific activators, no (or only basal) pristinamycin biosynthesis can occur. In the presence of a critical concentration of ligands, the repressive function of SpbR is relieved and the signaling cascade switches on. PapR2, as the major activator of pristinamycin biosynthesis, together with PapR1, activates the transcription of the pristinamycin structural genes.

PapR2 expression is indirectly regulated by PapR5, as PapR5 represses the transcription of *papR4*, the product of which activates *papR2* transcription. Furthermore, PapR5 interacts on the protein level with the PII activator PapR6. The function of PapR6 in the regulatory cascade remains unclear. PapR5 represents the major control element for the pristinamycin production amount. As a GBL receptor-like protein, PapR5 may sense pristinamycin or intermediate(s) of the pathway. During the early growth phase, PapR5 directly represses the transcription of the SARP genes *papR1* and *papR4* and indirectly that of *papR2* (via PapR4), which results in a tight inhibition of pristinamycin production. At the beginning of the stationary phase, when the ligand-induced derepression of SpbR is initiated, the protein concentration of PapR5 is decreased by the function of PapR3, which inhibits the transcription of *papR4* and *papR5* and in turn allows low-level pristinamycin production. Initially, such a timely controlled drug homeostasis may be necessary for the strain to develop self-resistance and thereby prevents self-toxicity from the compound. The low-level pristinamycin synthesis finally drives the feed-forward mechanism and by inactivating the PapR5 repression leads to full pristinamycin production. Alternatively, PapR5 may act as a late repressor that allows to switch off pristinamycin production. In such a scenario, the ligand-induced derepression of SpbR allows *papR5* transcription, which leads to an accumulation of PapR5 protein in the cell. When the PapR5 concentration is high enough, it represses the SARP gene transcription, resulting in a shutdown of pristinamycin production.

Regulation in related streptogramin producers. A comparison of the two other characterized streptogramin antibiotic gene clusters—the virginiamycin cluster (*vir*) from *S. virginiae* (54) and the griseoviridin/viridogrisein (GV/VG) cluster (*sgv*) from *Streptomyces griseoviridis* (55)—revealed that the amount of pathway-specific regulatory genes, as well as their overall localization within the gene cluster, is quite conserved (see Fig. S1A in the supplemental material). However, a closer look at the abundance of different regulator types and their function, which has been partially shown for virginiamycin regulation (15, 16, 56, 57, 58) but only deduced for GV/VG regulation (55), suggests that there exist different regulatory networks in all streptogramin producers (see Fig. S1B in the supplemental material): e.g., the virginiamycin cascade has no equivalent for PapR1 and PapR5 in abundance and function. The autoregulator ligand synthesis seems to be different in all three streptogramin producers: in *S. virginiae*, BarX, BarS1, and BarS2 are involved in virginiae butanolide synthesis (58), and in *S. pristinaespiralis* a P450 monooxygenase is suggested to be involved in the biosynthesis of an A-factor-like GBL (see above), whereas in *S. griseoviridis* a *barX* homologous gene is present, in addition to two other putative GBL synthesis genes, of which the products are not homologous to BarS1 and BarS2. In conclusion, it seems that even in strains with a similar—or in the case of virginiamycin, nearly identical—gene cluster, the regulatory network governing the respective antibiotic biosynthesis is unique for every strain.

So far, there are only few reports on antibiotic signaling cascades consisting of multiple regulators. However, regulator-based strain engineering is an efficient tool, and knowledge of regulatory effects is a prerequisite for improving antibiotic production and/or activating a silent gene cluster. In this regard, our data contribute to a better understanding of antibiotic regulation processes by disclosing a comprehensive new regulatory cascade that

governs the fine-tuned biosynthesis of pristinamycin in *S. pristinaespiralis*.

ACKNOWLEDGMENTS

This study was supported by The German Center for Infection Research (DZIF) grant number TTU 09.802. J.G. acknowledges a grant from the Promotionsverbund Antibakterielle Wirkstoffe of the University of Tübingen. Y.M. was supported by scholarships funded by the Landesgraduiertenförderungsgesetz des Landes Baden-Württemberg and the DFG (Graduiertenkolleg Infektionsbiologie), as well as by a grant from the Athene-Programm für Nachwuchswissenschaftlerinnen of the University of Tübingen. Sanofi-Aventis partially financed this work.

We thank H.-P. Fiedler and Andreas Kulik (Universität Tübingen) for help in HPLC measurements and Regina Ort-Winklbauer for excellent technical assistance.

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