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The Border Sequence of the Balhimycin Biosynthesis Gene Cluster from *Amycolatopsis balhimycina* Contains *bbr*, Encoding a StrR-Like Pathway-Specific Regulator

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Key Words
Amycolatopsis balhimycina · Antibiotics · Glycopeptide · RT-PCR

Abstract
Balhimycin, produced by the actinomycete *Amycolatopsis balhimycina* DSM5908, is a glycopeptide antibiotic highly similar to vancomycin, the antibiotic of 'last resort' used for the treatment of resistant Gram-positive pathogenic bacteria. Partial sequence of the balhimycin biosynthesis gene cluster was previously reported. In this work, cosmids which overlap the region of the characterized gene cluster were isolated and sequenced. At the 'left' end of the cluster, genes were identified which are involved in balhimycin biosynthesis, transport, resistance and regulation. The 'right' end border is defined by a putative 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase *(dahp)* gene. The proximate gene is similar to a type I polyketide synthase gene of the rifamycin producer *Amycolatopsis mediterranei* indicating that another biosynthesis gene cluster might be located directly next to the balhimycin gene cluster. The newly identified StrR-like pathway-specific regulator, Bbr, was characterized to be a DNA-binding protein and may have a role in balhimycin biosynthesis. Purified N-terminally His-tagged Bbr shows specific DNA-binding to five promoter regions within the gene cluster. By in silico analysis and by comparison of the DNA sequences binding Bbr, conserved inverted repeat sequences for the Bbr-binding site are proposed. The putative Bbr consensus sequence differs from that published for StrR.

Introduction

The vancomycin-type glycopeptides are the drugs of last resort for the treatment of severe infections caused by antibiotic-resistant Gram-positive bacteria, in particular by methicillin-resistant staphylococci and penicillin-resistant enterococci [Yao and Crandall, 1994]. Balhimycin (fig. 1b), a vancomycin-like glycopeptide antibiotic which is synthesized by *Amycolatopsis balhimycina* (formerly *Amycolatopsis mediterranei*) DSM5908 [Nadkarni et al., 1994; Wink et al., 2003], shares the same heptapeptide aglycon as vancomycin (fig. 1a), but differs in the glycosylation pattern. The activity of glycopeptide antibiotics against a wide range of Gram-positive bacteria arises

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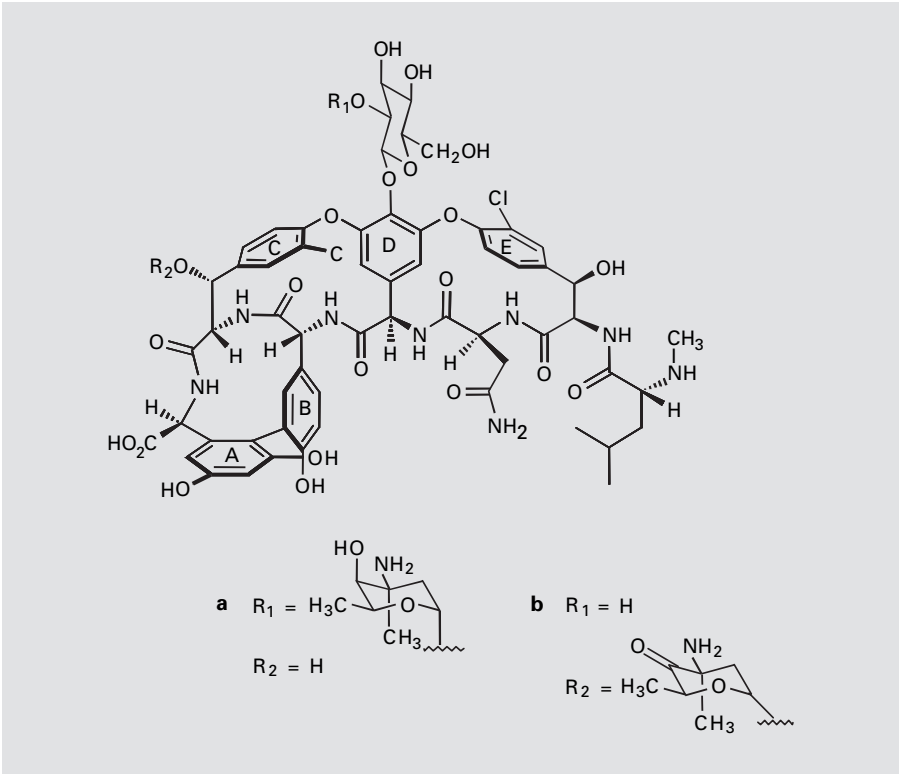

Fig. 1. Structures of vancomycin-type glycopeptide antibiotics. **a** Vancomycin (*A. orientalis* C3294). **b** Balhimycin *(A. balhimycina)*. The aromatic rings (AB, C-*O*-D, D-*O*-E) of balhimycin are assigned.

from their ability to bind to the terminal D-alanyl-D-alanine (D-Ala-D-Ala) dipeptide of bacterial cell wall precursors [Williams and Bardsley, 1999].

Recent advances in the investigation of balhimycin biosynthesis by means of genetics and biochemistry, as well as analytical chemistry of the pathway intermediates, rendered an understanding of the central steps of the glycopeptide biosynthesis (as reviewed by Stegmann et al. [2005]). Starting with the supply of the non-proteinogenic amino acid precursors, followed by the assembly of the heptapeptide scaffold, and finally by a series of modifying reactions, balhimycin displays a great structural complexity. All genes required for its biosynthesis are clustered [Pelzer et al., 1999; Recktenwald et al., 2002].

However, comparison with other glycopeptide biosynthesis gene clusters coding for chloroeremomycin [van Wageningen et al., 1998], teicoplanin [Li et al., 2004; Sosio et al., 2004], dalbavancin [Sosio et al., 2003], complestatin [Chiu et al., 2001] and A47934 [Pootoolal et al., 2002] revealed that resistance, transport and regulatory genes are still missing from the sequence of the balhimycin biosynthesis gene cluster determined so far.

In particular, the identification and characterization of regulatory genes is crucial for understanding the mechanism by which complex biosynthetic steps are coordinated and for genetically manipulating the producing strains to enhance antibiotic production.

Regulation of secondary metabolism is governed mainly by pathway-specific regulators. Many of the pathway-specific regulatory proteins that control secondary metabolism in streptomycetes belong to the SARP (*Streptomyces* antibiotic regulatory proteins) family. These transcriptional activators contain a winged helix-turn-helix (HTH) motif towards their N-termini that is also found in the OmpR family of proteins and at least some of the SARPs appear to recognize direct heptameric repeats within the promoter regions of the genes that they regulate [Wietzorrek and Bibb, 1997]. SARPs include ActII-ORF4 from *Streptomyces coelicolor* A3(2), DnrI from *Streptomyces peucetius*, which regulate the production of the polyketide antibiotics actinorhodin and daunorubicin, respectively, RedD from *S. coelicolor* A3(2), which controls synthesis of the tri-pyrolle undecylprodigiosin, and AfsR, a pleiotropic regulatory protein for antibiotic production in *S. coelicolor* A3(2) [Arias et al., 1999; Sheldon et al., 2002; Wietzorrek and Bibb, 1997].

Besides SARPs, other types of pathway-specific regulators have been identified in different *Streptomyces*

strains: such as SrmR of the spiramycin cluster in *Streptomyces ambofaciens*, which shows no significant sequence similarity to any other known regulatory protein; MmyR of the methylenomycin cluster in *S. coelicolor*, representing the first example of a negative pathway-specific regulator of antibiotic production [as reviewed by Chater and Bibb, 1997], PrpA of the phosphinothricin tripeptide cluster in *Streptomyces viridochromogenes* [Schwartz et al., 2004], BrpA and DnrN regulators for bialaphos production in *Streptomyces hygroscopicus* and for daunorubicin production in *Streptomyces peucetius,* respectively, which are similar to two-component response regulators [as reviewed by Chater and Bibb, 1997] and TylP from *Streptomyces fradiae* which negatively regulates tylosin production [Stratigopoulos et al., 2002] and is homologous to γ-butyrolactone-binding proteins.

Another type of regulator is exemplified by StrR, the pathway-specific activator protein in *Streptomyces griseus* [Retzlaff and Distler, 1995] and *Streptomyces glaucescens* [Beyer et al., 1996]. It activates the expression of streptomycin biosynthetic genes by binding to several promoter regions. Based on seven specific binding sites, a consensus sequence GTTCGActG$(N)_{11}$CagTcGAAC [Retzlaff and Distler, 1995] was deduced. Transcription of all the streptomycin biosynthesis genes apart from the resistant gene is dependent on StrR as shown by Tomono et al. [2005]. StrR has a putative HTH motif in the central region of its primary structure [Retzlaff and Distler, 1995]. Homologs of *strR* have been found in aminocoumarin, aminoglycoside and glycopeptide antibiotic biosynthetic gene clusters.

In this report, we determined further sequence of the balhimycin biosynthetic gene cluster and thereby identified besides others a regulatory gene. We characterized the StrR-like pathway-specific regulator, Bbr, in order to determine its role in balhimycin biosynthesis.

Results

Completion of the Balhimycin Biosynthesis Gene Cluster and Analysis of the Newly Identified Open Reading Frames (ORFs)

The major part of the balhimycin biosynthesis gene cluster has been identified, sequenced and characterized as shown by Pelzer et al. [1999] and Recktenwald et al. [2002]. After assigning functions to all identified genes and proposing the biosynthetic pathway for balhimycin, two questions remained open: Since no regulatory gene was found so far, how is the expression of these complex biosynthetic steps coordinated? Where are the borders of the balhimycin biosynthesis gene cluster?

In order to find adjacent cosmids overlapping with the previously identified cluster sequence on the 'left' border and to define the borders of the balhimycin biosynthesis gene cluster, an appropriate DNA probe for hybridization against available cosmid pools of the balhimycin producer was designed. To avoid cross-hybridization, primers were designed (primer Ris1 and Ris2; table 1) from a non-conserved area of the *bpsA* gene, encoding a non-ribosomal peptide synthetase (NRPS). Through Southern hybridization as well as direct polymerase chain reaction (PCR) screening of cosmid pools, we isolated two adjacent cosmids, 4.3 and 55.1 (see table 2). Numerous *Pst*I and *Eco*RI fragments of these new cosmids were cloned and sequenced. The new sequence overlaps with the upstream region of the NRPS gene *bpsA* and encodes gene products with significant homology to an ABC transporter (Tba), a prephenate dehydrogenase (Pdh), a StrR-homologous regulator (Bbr), a VanY-type carboxypepditase (VanY), a two-component system response regulator (VanR), and a two-component system sensor kinase (VanS) (see table 3 and fig. 5). Cosmid 55.1 carries only 751 bp of the *vanS* gene.

In order to define the right border of the balhimycin biosynthesis gene cluster, Cosmid 16.1 (see table 1), which has been partially sequenced previously and contains a major part of the sequenced balhimycin biosynthesis gene cluster, was further sequenced and analyzed. This resulted in the identification of a new gene *dahp* (see fig. 5), the product of which is significantly similar to a 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase from *Amycolatopsis methanolica* (table 3).

Downstream of *dahp,* a gene which encodes for a protein with 40% identity on amino acid level to a type I polyketide synthase of the rifamycin producer *A. mediterranei,* was found, indicating that this gene is not part of the balhimycin biosynthesis gene cluster.

Two Regulatory Genes Are Present in the Balhimycin Biosynthesis Gene Cluster

From the analysis described above, two regulatory genes were identified in the balhimycin biosynthesis gene cluster.

One of the regulatory proteins, VanR, consists of 225 amino acids and is significantly similar to putative two-component system response regulators with highest homology to the putative two-component system response regulator from *S. coelicolor* A3(2) (table 3) and may be involved in resistance: VanR homologs are present in resistant pathogenic bacteria [Arthur and Courvalin, 1993]

Table 1. List of primers used for this study. The names of the primers are indicated on the left. The primer sequence is 5′ to 3′

Primers used for detection of adjacent cosmids
Ris 1;CAAGGCTGAGTTCGACGACGATTCC
Ris 2;ACTGCCGTTTCTGCAACTCCGCCAG

Primers used for band shift experiments
vanS-PROM1;ACCAGGACGGACCGCTCCAG
vanS-PROM2;CGTTCCGCCGCGAGGTCTAC
vanR-PROM1;GACGGCGATGTCGTAGG
vanR-PROM2;AATCCGGCGGGTCGTC
bbr-PROM1;CTGCCGTCCGGGGAATCGCG
bbr-PROM2;CTAGCCCCCATTTGGATCTAC
pdh-PROM1;TCGCGTGACCACGGCGGAGCC
pdh-PROM2;CGTTCCTTCTTAAAACGGCTCAC
tba-PROM1;GCCGGCCGCAAAAACGATTGC
tba-PROM2;CCGGTCACCTCTCTCACACGC
bpsA-PROM1;AGGCCGACCAGATCCTCGTG
bpsA-PROM2;CGAGCTGCCGGTACGTCAGAAC
bpsC-PROM1;GGCGGTGATCGTCCAGGAAC
bpsC-PROM2;TCGAGGGCGGTGTGATAGAG
oxyA-PROM1;GAAGCGGGCCGTCAGGAGTGC
oxyA-PROM2;TGCACAAGGTCCTTTCGCCTC
oxyB-PROM1;ACTGGCCGAGATGGTGTTC
oxyB-PROM2;CGGGTCGTCATCATTCAA
oxyC-PROM1;ACCGGGGGCCCGCGACGTCCACGC
oxyC-PROM2:CATCCGCGTGGAAACTCCTTGGTC
bhaA-PROM1;GGTTCCGGTCACCTGGTAGC
bhaA-PROM2;CTCCCACCATTCCTCTGTCG
bgtfA-PROM1;CAGGTGCAGATGAAGGCGCTG
bgtfA-PROM2;TTCGCACCCTCTGTTCCGTAG
bgtfC-PROM1;AACCACACCGGGTCGGCG
bgtfC-PROM2;GCAAACGGTTCGACGTCTCCA
dvaC-PROM1;GCACCGACGGGACGACGATG
dvaC-PROM2;CGGTGGCAAGGCGGAAGAAG
orf2-PROM1;CGGTCAGTGGATCCTTTACG
orf2-PROM2;CCGGCACCGAAGGAAAGAAC
bmt-PROM1;ACGACCTGGCCGGTTGTCTC
bmt-PROM2;CCTGGATCAGGTCCCGATAG
bhp-PROM1;TTCCATACGCCGCCGAGGTGTC

Primers used for Bbr purification
bbrHIS1;GGAGATCTGCCGCAAAGTTGACGAAGTAGATCC
bbrHIS2;GGAGATCTCCGTGGTCACGCGATCATC

Primers used for band shift experiments (continued)
hmaS-PROM1;AACGGGAGCCGGCCTGGTTG
hmaS-PROM2;AGGTGAACGTGGCCGCCTCG
orf7-PROM1;TCGCCGCCGAACTCCGGAAC
orf7-PROM2;CCGCGGCAAAGGTGTGCAGC
orf7-PROM1insi;ACCTGCCGGTGGTGCTCAAG
orf7-PROM2insi;AACACCTGCCGGACGCCCTC
dvaA-PROM1;GCGGTGCCTGCCGCTATCTTG
dvaA-PROM2;ACGTCCTCGGTCCGCATGTG
dvaA-PROM1insi;TGATGGCGTCGCCGATCCTG
dvaA-PROM2insi;ACGTCCTCGGTCCGCATGTG
dpgA-PROM1;TACGCCACCTGCCTGGAG
dpgA-PROM2;GCGGGTTCGATGCTGGTC
dpgA-PROM2insi;TCGCTCCCGCCGACTTCAGG
dpgA-PROM1insi;GGGTTGCTGCCGGACTACGC
dvaD-PROM1;AGGCGTACGACATCCACTTG
dvaD-PROM2;GGGCACAGTAGACGTACTTG
dahp-PROM1;GGTCTGGACCGGGGAATAC
dahp-PROM2;GGTGACCAGGGGGACGAT

Primers used for RT-PCR experiments
bbr-RT;GTGGAACACCCTGCAGGATC
bbr-expra1;CAGGTGCTCGACGGCCTGCACCGGTTGAAG
bbr-expra2;CCCGTCCATGGAGTGCATGGTGAGCATGCG
oxyA-RT;GATGTCGAACTCGTCCTCCG
oxyA-expra1;GCTTCCTCAACCAGTACGACCCCCCGGAGC
oxyA-expra2;GAGCTGATCACCGATGCGCACGTCCTCCTG
bhp-RT;GCTTCTCCAGGTAGCCGAAG
bhp-expra1;GGTGTGGGACCTGCACCAG
bhp-expra2;GCTTCTCCAGGTAGCCGAAG
hrdB-RT;ACAGCGTCTGCAGCACCGAC
hrdB-expra1;GAGGGCAACCTCGGTCTGATCCGCGCGGTG
hrdB-expra2;AGCGTCTGCAGCACCGACTGGAGCTGGTCC
bhp-PROM2;GAAGGTCCTCCCCTGCCCGC

and a *S. coelicolor* homolog is induced by various glycopeptide antibiotics [Hutchings et al., 2006].

The second regulatory protein, Bbr, consists of 321 amino acids and shows significant similarity to putative transcriptional activators with 47% identity on an amino acid level (over a length of 289 amino acids) to StrR, the well-characterized pathway-specific regulator for streptomycin biosynthesis, in *S. glaucescens* [Beyer et al., 1996] and *S. griseus* [Distler et al., 1987]. Bbr shows the characteristic HTH motif commonly found in DNA-binding regulatory proteins. The HTH motif is located from amino acid 183 to 204 of Bbr (based on HTH prediction by the Dodd-Egan method [Dodd and Egan, 1990]).

In silico Approaches to Determine Bbr-Binding Sites within the Balhimycin Biosynthetic Gene Cluster

Based on the previously published palindromic sequence of StrR-binding site [Retzlaff and Distler, 1995] GTTCGActG$(N)_{11}$CagTcGAAC, in silico nucleotide sequence analysis was carried out to find inverted repeat sequences within the balhimycin biosynthetic gene cluster which might be targets for Bbr binding. However, an identical palindromic sequence could not be identified. Applying PatScan software, the sequence available for the balhimycin biosynthetic gene cluster was scanned in order to identify inverted repeat sequences allowing mismatches and various lengths of non-conserved spacers.

Table 2. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant features	Source or reference
Strains		
E. coli XL1-Blue	General cloning host	Bullock et al., 1987
A. balhimycina		
DSM5908	Balhimycin-producing wild type	Nadkarni et al., 1994
Plasmids		
pSP1	Gene disruption vector; Ery^r	Pelzer et al., 1997
pJOE890	Am^r	Altenbuchner et al., 1992
Cosmid 16.1	Contains part of the balhimycin biosynthetic gene cluster	Recktenwald et al., 2002
Cosmid 55.1	Contains part of the balhimycin biosynthetic gene cluster	This work
Cosmid 4.3	Contains part of the balhimycin biosynthetic gene cluster	This work

Ery^r = Erythromycin-resistant; Am^r = ampicillin-resistant.

Table 3. ORFs identified in this study and their proposed function

ORFs	Size (aa)	% aa identity	Similarities to functionally characterized proteins/ UNIPROT-Acc	Proposed function
VanS	253	66% over 253 aa	two-component system sensor kinase in *S. coelicolor* A3(2)/Q9X942	two-component system sensor kinase
VanR	225	88% over 221 aa	putative two-component system response regulator in *S. coelicolor* A3(2)/Q799B5	two-component system response regulator
VanY	206	31% over 143 aa	VanYB-carboxypeptidase in *Enterococcus faecalis* plasmid pIP834/Q9L8Y3	VanY-type carboxypeptidase
Bbr	321	47% over 289 aa	StrR, pathway-specific regulator for streptomycin biosynthesis, in *S. glaucescens*/Q54267 and *S. griseus*/Q53ID1	StrR family transcriptional regulator
Pdh	291	31% over 274 aa	cyclohexadienyl dehydrogenase (prephenate dehydrogenase) in *Zymomonas mobilis*/Q04983	prephenate dehydrogenase
Tba	651	31% over 594 aa	lipid A export ATP-binding/permease protein msbA in *Escherichia coli*/Q6ZZI9	ABC transporter ATP-binding protein
Dahp	365	60% over 348 aa	DAHP synthase in *A. methanolica*/Q6TZ04	DAHP synthase

Alignment of the inverted repeat sequences found in putative promoter regions revealed the presence of a conserved palindromic motive in the upstream regions of *bbr*, *tba*, *oxyA*, *dvaA* and *orf7* with one base pair mismatch, and in the upstream region of *dpgA* with two base pairs mismatch (fig. 2).

Overexpression of bbr *in* Escherichia coli *and Purification of the Protein by Affinity Chromatography*

To determine whether Bbr is a DNA-binding protein and whether the predicted in silico DNA-binding sites within the balhimycin biosynthetic gene cluster are true targets, Bbr was first overexpressed in *E. coli* and purified.

bbr from *A. balhimycina* was amplified by PCR with the corresponding primers (see table 1), cloned and expressed in *E. coli* under the control of the T7 promoter using the vector pRSETB. The N-terminal His_6-tagged Bbr was purified using nickel affinity chromatography. The apparent molecular weight of Bbr is about 37 kDa (calculated mass 35.324 kDa) as determined by SDS-PAGE analysis (fig. 3). Partially purified Bbr which was

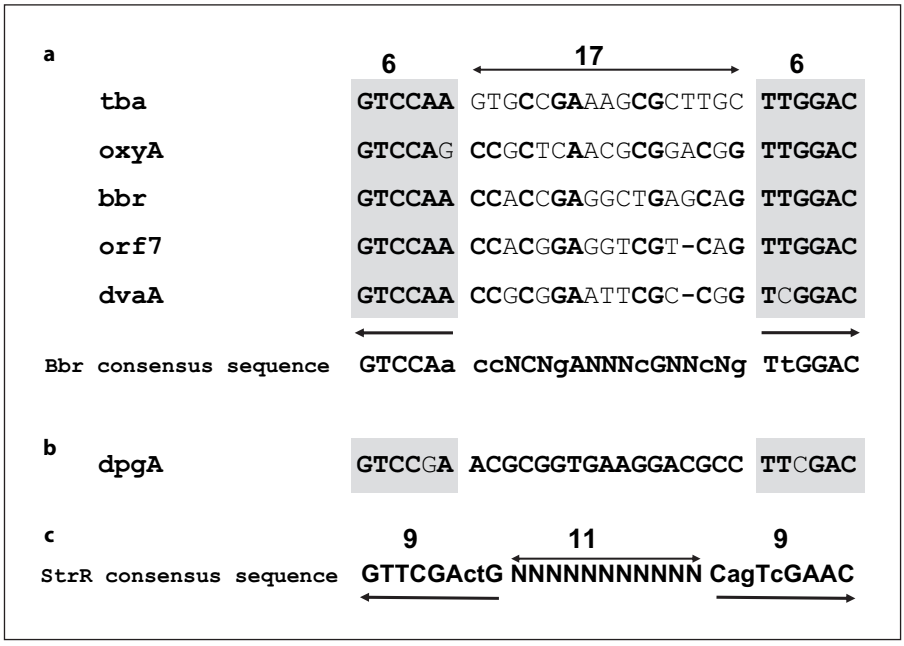

Fig. 2. Consensus sequence of potential Bbr-binding sites identified by in silico analysis. **a** CLUSTAL W (1.60) multiple sequence alignment showing positive PatScan hits. On the left are the genes where the consensus sequence indicated under **c** was identified in the promoter region. Arrows correspond to inverted repeats, double arrows to the number of spacing. Bbr binds to the promoter region of these five genes. **b** Sequence of the upstream region of *dpgA* with two mismatches. Here Bbr does not bind. **c** Consensus sequence of StrR [Retzlaff et al., 1995].

eluted with 200 and 500 mM imidazole (fig. 3, lanes 5 + 6) was used for gel retardation analysis. The protein yield of these fractions was about 0.2 mg/ml.

Bbr Has DNA-Binding Activity and Targets Several Promoter Regions of the Balhimycin Biosynthetic Gene Cluster

The purified His_6-tagged Bbr was used to determine the DNA-binding activity of Bbr by gel retardation assays. Digoxygenin (DIG)-labeled DNA fragments (100–200 bp upstream of predicted start codons) amplified via PCR from all upstream regions of coding sequences within the balhimycin biosynthetic gene cluster were used as probes.

The specificity of Bbr binding was confirmed by controls using 10- and/or 100-fold unlabeled competitor DNA of the corresponding probes as well as non-specific *Streptomyces* plasmid DNA. Unlabeled DNA fragments of the investigated putative promoter regions compete with the corresponding DIG probes for binding to His_6-tagged Bbr, whereas there is no competition in case of the non-specific *Streptomyces* DNA. In one case the protein was boiled for 10 min prior to incubation with the *bbr* probe thus denaturing Bbr and affecting its binding to the DNA probe (fig. 4a).

Analysis of gel retardation assays revealed that Bbr specifically binds to the upstream regions of the regulator gene *bbr*, the putative ABC transporter gene *tba*, the

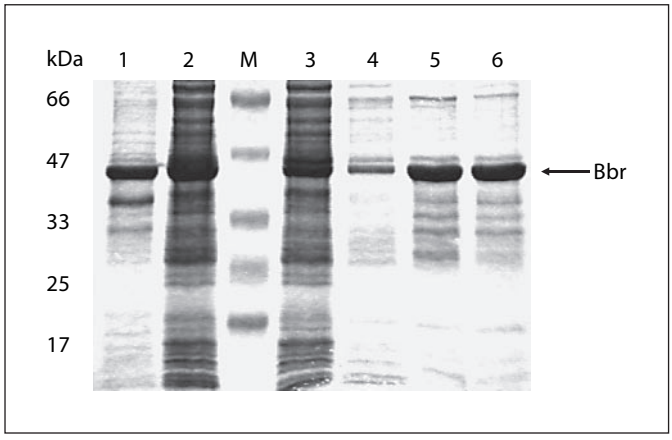

Fig. 3. Overexpression and partial purification of Bbr in *E. coli*. SDS-PAGE showing purification of N-terminal His(6)-tagged Bbr by nickel-affinity chromatography. Numbers of lanes correspond to purification steps as follows: 1: insoluble fraction of crude extract, 2: soluble fraction of crude extract, 3: flow through Ni-NTA column (Qiagen), 4: washing with buffer, 5: elution with 200 mM imidazole, 6: elution with 500 mM imidazole. M stands for protein size marker. Molecular weights are denoted on the left in kDa. Arrow corresponds to purified soluble Bbr.

P450 monooxygenase gene *oxyA*, a gene involved in the dehydrovancosamine synthesis *dvaA*, and the putative sodium-proton anti-porter gene *orf7* (fig. 4a, 5). No binding was observed to the upstream regions of the

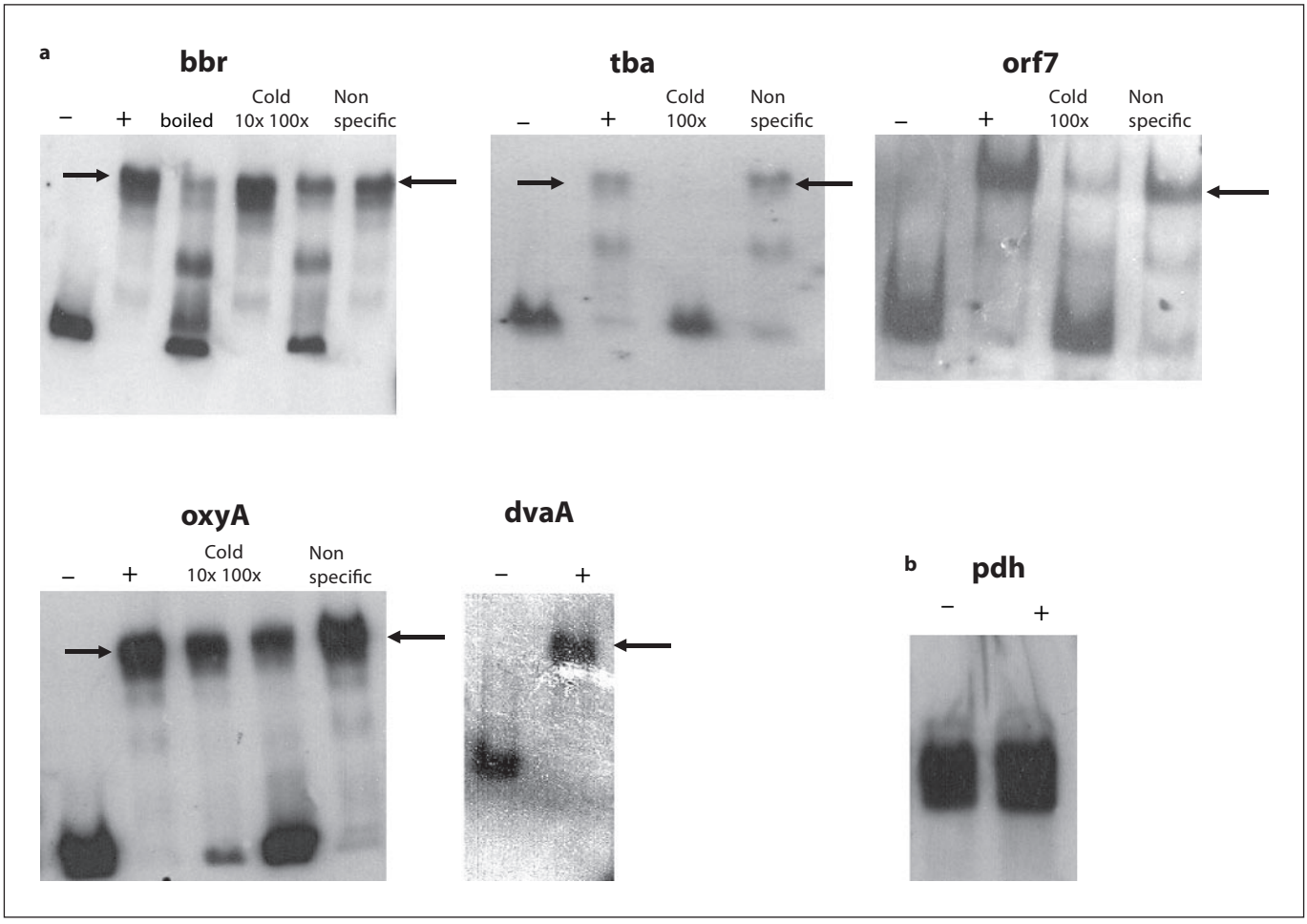

Fig. 4. Gel retardation assays with partially purified Bbr and DIG-labeled PCR fragments. **a** The investigated promoter regions are denoted on top of each assay. Lanes are marked as follows: – = DIG-labeled PCR fragment; + = DIG-labeled PCR fragment with purified His_6-Bbr; Cold = unlabeled PCR fragment corresponding to each promoter region + DIG-labeled PCR fragment + purified His-Bbr; Non specific = unlabeled non-specific *Streptomyces* DNA + DIG-labeled PCR fragment + purified His-Bbr. Arrows indicate shifts corresponding to DNA-protein complex. **b** One example of gel retardation assay *(pdh)* where no binding was observed between Bbr and the DIG-labeled DNA fragment. Rest of data for negative results is not shown. Lanes as described under **a**.

NRPS genes *bpsA* and *bpsC*, the P450 monooxygenase genes *oxyB* and *oxyC*, the halogenase gene *bhaA*, the glycosyltransferase genes *bgtfA* and *bgtfC*, a gene involved in the biosynthesis of the dehydrovancosamine sugar *dvaC*, a gene with unknown function *orf2*, the methyltransferase gene *bmt*, the hydrolase gene *bhp*, the hydroxymandelate synthase gene *hmaS*, the dihydroxyphenylglycine synthase gene *dpgA*, and the DAHP synthase gene *dahp* (only one representative example of these results is shown in figure 4b; summary of results in figure 5).

Analysis of bbr Expression in Relation to Balhimycin Production

The temporal transcription pattern of *bbr* and of some biosynthetic genes (*oxyA*, *bhp* and *bhaA*) was analyzed in correlation to antibiotic production using reverse transcription (RT)-PCR. The onset of antibiotic production was 35 h after incubation assessed by bioassay.

RNA was isolated at different time points (fig. 6) using the RNA isolation kit (Qiagen) and used to produce the corresponding cDNA. PCR was carried out using cDNA as template and the primers listed in table 1. The gene encoding the major sigma factor (*hrdB*) of *A. balhimycina*

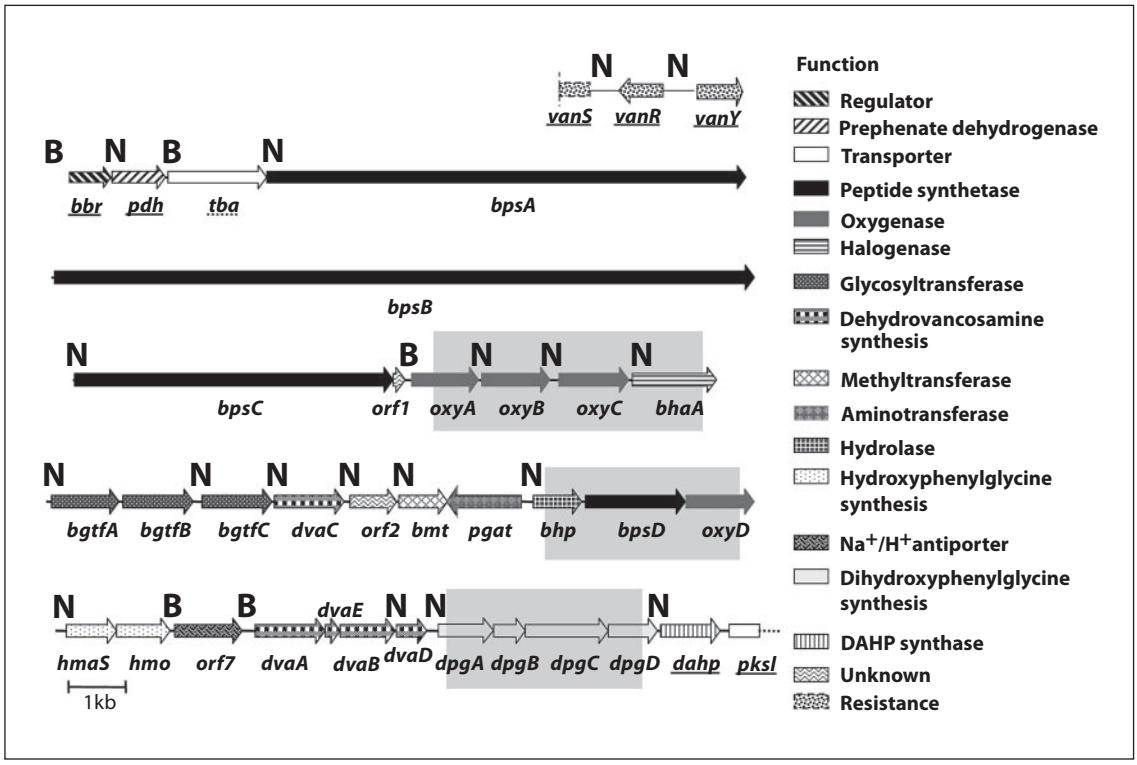

Fig. 5. Genetic organization of the balhimycin biosynthesis gene cluster and summary of Bbr binding. **a** Predicted open reading frames (ORFs) are indicated by arrows. Gene names are indicated underneath the corresponding ORFs. Functions of genes are listed on the right. Bar on the left is proportional to 1 kb. New *orfs* identified in this study are *vanS*, *vanR*, *vanY*, *bbr*, *pdh*, *tba* and *dahp* and are underlined. The *vanS* gene is uncompleted (dashed line). B denotes promoter regions where Bbr-DNA complex was observed; N denotes DNA regions where no binding of Bbr was observed; shaded squares show experimentally revealed co-transcribed genes [Pfeifer et al., 2001; Puk et al., 2004].

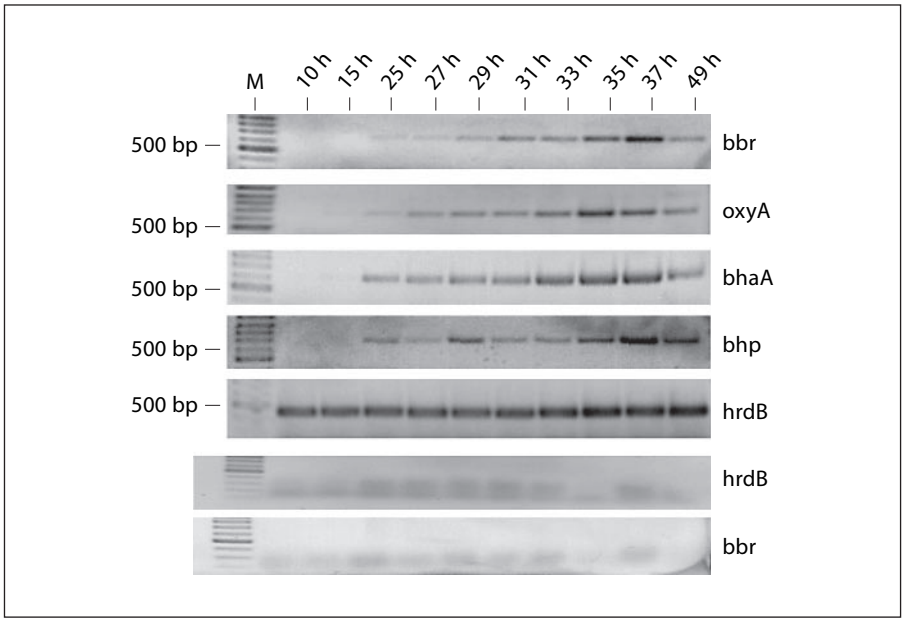

Fig. 6. Reverse transcriptase PCR experiments of *bbr* and some biosynthesis genes. The gene names are indicated on the right. *hrdB* is the major sigma factor of *A. balhimycina* WT. The amplified fragments were separated on agarose gel. M stands for DNA marker. Time points of RNA isolation are indicated on the top. A 500-bp marker is indicated on the left. Template used for each gene in brackets is indicated on the far left. As template cDNA was used.

was used as a positive control since it is constitutively expressed throughout growth [Puk, unpubl. data]. Negative controls were carried out under the same conditions using the isolated RNA as a template without reverse transcriptase. The RT-PCR experiments showed that the expression of *bbr* occurs simultaneously with that of the studied biosynthetic genes starting at least 8 h before the onset of antibiotic production (fig. 6).

Discussion

Seven New Genes Have Been Identified in the Balhimycin Biosynthetic Gene Cluster

In this work the sequence of the balhimycin biosynthetic gene cluster was extended. The newly identified genes, *tba*, *pdh*, *dahp*, *vanY*, *vanS* and two regulatory genes, *bbr* and *vanR*, are expected to be involved in the biosynthesis, resistance or regulation of balhimycin.

pdh and *dahp*, the products of which are highly similar to prephenate dehydrogenases and DAHP synthases, respectively, might represent a link between primary and secondary metabolism through the shikimate-chorismate biosynthetic pathway and are most likely needed for the supply of hydroxyphenylpyruvate, a precursor which is required for the biosynthesis of the balhimycin non-proteinogenic amino acids. Tba is probably involved in balhimycin transport, since it is highly similar to transmembrane ABC (ATP-binding cassette) transporters. VanY may be involved in resistance, since it is highly similar to D,D-carboxypeptidases which contribute to vancomycin resistance by hydrolyzing the C-terminal D-Ala residue of peptidoglycan precursors [Arthur and Courvalin, 1993]. The VanS-VanR putative two-component regulatory system identified in our study may also be involved in conferring resistance to the balhimycin producer in a manner similar to the well-established vancomycin resistance cascade in pathogenic enterococci [Arthur et al., 1992] and in the non-pathogen *S. coelicolor* [Hutchings et al., 2006].

From this study and combining previous sequence reports, a total of 66 kb sequence data was determined for the balhimycin biosynthetic gene cluster encoding 36 ORFs. One border of the balhimycin biosynthetic gene cluster has also been predicted. Sequence analysis of the right border downstream of *dahp* revealed a type I polyketide synthase of a rifamycin producer in *A. mediterranei* which could be part of another biosynthetic gene cluster located directly next to the balhimycin biosynthesis locus.

The *vanR* and *vanS* genes identified in this study are most likely to be part of the balhimycin biosynthetic gene cluster, since their homologs are located within the other glycopeptide gene clusters such as chloroeremomycin, dalbavancin, complestatin and teicoplanin gene clusters [Donadio et al., 2005]. Nevertheless, to determine the final definition of the left border of the balhimycin biosynthetic gene cluster, further sequencing of the area upstream of *vanS* is underway.

Bbr Binds Directly to the Promoter Region of the Balhimycin Biosynthesis Genes

Bbr, a StrR-like pathway-specific regulator, was identified and characterized in this study to determine a possible role in balhimycin biosynthesis. Functional studies of StrR like regulators have been limited to StrR [Beyer et al., 1996; Retzlaff and Distler, 1995], KasT from the kasugamycin producer *Streptomyces kasugaensis* [Ikeno et al., 2002] and NovG from the novobiocin producer *Streptomyces spheroides* [Eustaquio et al., 2005]. Our DNA-binding studies with Bbr provide the first characterization of a regulator of biosynthesis genes for glycopeptide antibiotics. On amino acid level, Bbr is as similar to KasT and NovG (45% amino acid identity) as it is to StrR.

The putative NovG-binding site possesses a conserved palindromic structure with the sequence GTTCAACTG$(N)_{11}$CRGTTGAAC, as determined recently by gel retardation analysis [Eustaquio et al., 2005], which differs in two positions from the StrR-binding site consensus sequence [Retzlaff and Distler, 1995]. Identical inverted repeat sequences could not be identified within the balhimycin biosynthetic gene cluster. Sequence-specific binding of Bbr to five putative promoter regions in the gene cluster allowed the deduction of a StrR-related palindromic consensus sequence, GTCCAa$(N)_{17}$TtGGAC. Even a mismatch by two bases abolishes the binding ability of Bbr experimentally as was shown for the inverted repeat sequence upstream of *dpgA* which was detected by in silico analysis.

Interestingly, the proposed palindromic sequence for Bbr-binding site is also conserved within other glycopeptide biosynthetic gene clusters [Donadio et al., 2005]. Comparison of the balhimycin biosynthetic gene cluster with the biosynthetic gene clusters of chloroeremomycin (cep), dalbavancin (dbv), A47934 (sta) and teicoplanin (tcp) revealed the presence of the suggested Bbr consensus sequence in the upstream region of the biosynthetic gene *oxyA*, the product of which is significantly homologous to P450 monooxygenases, in all five clusters. In addition, the same consensus sequence is found upstream

of the gene encoding the StrR homologous regulator in cep, upstream of the response regulatory gene in tcp, upstream of a gene unique for dalbavancin biosynthesis and upstream of a homolog to bal *orf2* from tcp [Donadio et al., 2005]. Furthermore, we have newly identified the Bbr consensus sequence upstream of the ABC transporter in sta and dbv clusters.

Operon Structures within the Balhimycin Biosynthetic Gene Cluster

Bbr binds upstream of *bbr*, *tba*, *oxyA*, *orf7* and *dvaA*. Combining these results together with our previous reports on possible operon structures within the balhimycin biosynthetic gene cluster [Pfeifer et al., 2001; Puk et al., 2004] and with sequence analysis data, the following operon structures can be suggested for the balhimycin biosynthetic gene cluster (fig. 5): the first operon comprises *bbr* and *pdh*; the second operon comprises *tba* and all three NRPS genes together with the small *orf1;* the third operon covers the region from *oxyA* to *bmt*; the fourth operon only consists of the divergent gene *pgat;* the fifth operon comprises the genes involved in the biosynthesis of *β*-hydroxytyrosine, *bhp*, *bpsD* and *oxyD* [Puk et al., 2004], as well as genes of the hydroxyphenylglycine biosynthesis, *hmaS* and *hmO*; the sixth operon contains only *orf7*, and the last operon expands from *dvaA* to *dahp*. As mentioned above, the proposal of these operon structures within the balhimycin biosynthetic gene cluster is based on either experimental data or sequence analysis revealing that the start (GTG) and stop (TGA) codons of the genes overlap with the sequence GTGA suggesting that these genes are most likely translationally coupled. All other intergenic regions were not targeted by the pathway-specific regulator Bbr. This implies that the respective genes are either co-transcribed with upstream genes, which are targeted by Bbr, or regulated via a different mechanism which has not yet been characterized.

The divergent genes at the core of the balhimycin biosynthetic gene cluster suggest that the intergenic region between *bhp* and *pgat* might contain a promoter. However, the proposed palindromic sequence for Bbr-binding site could not be identified within this intergenic region and no binding of Bbr was observed in gel retardation assays. The fact that Bbr does not bind to the promoter region of *bhp-pgat*, genes which are involved in the biosynthesis of balhimycin precursors, leads to the assumption that these genes are regulated via a different mechanism which is still elusive.

Concluding Remarks

The putative two-component regulatory system genes *vanR* and *vanS* identified in our study may be part of a resistance cascade similar to that of vancomycin [Arthur et al., 1992]. As Bbr did not bind to the promoter regions of either gene, it seems likely that resistance is controlled separately from the biosynthesis. Induction of resistance might be achieved through sensing the glycopeptide antibiotic by the histidine kinase VanS in analogy to the role of VanS in *S. coelicolor* [Hutchings et al., 2006].

In *S. griseus*, the molecular mechanism by which a γ-butyrolactone microbial hormone, A-factor, triggers streptomycin biosynthesis is well established. A transcriptional activator AdpA switches on many genes required for both morphological development and secondary metabolism. AdpA binds to the promoter region of *strR* and activates its transcription. The pathway-specific transcriptional activator StrR then induces transcription of the streptomycin biosynthetic genes by binding multiple sites in the gene cluster, thus leading to biosynthesis of streptomycin [reviewed by Horinouchi, 2002; Tomono et al., 2005]. Whether balhimycin production is triggered by a similar regulatory cascade and the transcription of *bbr* is induced by an AdpA-equivalent activator still needs to be investigated.

Ongoing studies of the complex regulation of balhimycin production will deliver a comprehensive picture of the overall antibiotic biosynthesis. This will aid in the genetic manipulation of the biosynthetic gene cluster and will help obtain new compounds to combat the sharply increasing antibiotic resistance.

Experimental Procedures

Bacterial Strains and Plasmids

The strains and plasmids used for this study are listed in table 2.

Media and Culture Conditions

E. coli strains were grown in Luria broth medium [Sambrook et al., 1989] supplemented with 100 μg of ampicillin ml^{-1} or 100 μg of apramycin ml^{-1} when necessary to maintain plasmids. *A. balhimycina* strains were grown in R5 medium [Kieser et al., 2000] at 30°C. Liquid and solid media were supplemented with 50 μg ml^{-1} of apramycin to select for strains carrying integrated antibiotic resistance genes.

DNA Preparation, Manipulations and Hybridization

The methods used for the isolation and manipulation of DNA for *E. coli* and actinomycetes were described by Sambrook et al. [1989] and Kieser et al. [2000], respectively. PCR fragments were

isolated from agarose gels with a QIAquick kit (Qiagen, Hilden, Germany). Restriction endonucleases were obtained from various suppliers and were used according to their specifications. Southern hybridization was carried out as previously described [Pelzer et al., 1999].

PCR Protocols

PCR was performed with a programmable thermal controller (MJ Research, Inc.). Each PCR mixture (100 μl) contained 100 pmol of each primer, 1.0 μg of template DNA (cosmid 16.1 or cosmid 55.1), deoxyribonucleoside 5′-triphosphates at a final concentration (each) of 20 μM (DNA polymerization mix; Pharmacia), 10× reaction buffer (Qiagen), 5× Q-solution (Qiagen), and 3.5 U of *Taq* DNA polymerase. Dimethyl sulfoxide (Stratagene) was added to the reaction mixture at a final concentration of 3% to enhance the specificity of hybridization. For amplification of the DNA fragments, the following PCR conditions were used: initial denaturation (95°C for 2 min); 25 cycles of denaturation (95°C for 30 s), annealing (52°C for 30 s), and polymerization (72°C for 30 s), and an additional polymerization step (72°C for 7 min) at the end. The primers used are listed in table 1.

Expression and Purification of His_6-Tagged Bbr

bbr from *A. balhimycina* was amplified by PCR with the primers bbrHIS1 and bbrHIS2 (table 1) that include *Bgl*II and *Hin*dIII sites, and cloned in pJOE890 *Sma*I blunt end. Then *bbr* was cloned into the expression vector pRSETB (Invitrogen) *Bam*HI/*Hin*dIII to generate an in-frame fusion with the His_6-tag (T7 promoter). His_6-tagged Bbr was expressed in *E. coli* BL21(DE3)pLysS cells and purified with nickel (II)-nitrilotriacetic acid (Ni-NTA) spin columns (Qiagen) at 4°C as described in the Ni-NTA Handbook (Qiagen). The starting point of purification was the soluble protein fraction of a 50-ml expression culture (induced with 1 mM IPTG/37°C) obtained after French Press and centrifugation of the cell lysate.

Protein Analysis

Protein concentrations were determined by the Bradford method [Bradford, 1976] using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [1970], and protein bands were stained with Coomassie Brilliant Blue G-250.

Preparation of 3′-End DIG-Labeled DNA Fragments

DNA fragments were amplified from the upstream regions of almost all genes of the balhimycin biosynthetic gene cluster. Primers used for amplification of the DNA fragments are listed in table 1; each primer pair carries the name of the corresponding gene. After purification, the 3′-end DIG-11-ddUTP labeling of the DNA fragments was carried out using DIG Gel Shift Kit, 2nd Generation (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Gel Retardation Assays

Gel retardation assays were performed using the DIG Gel Shift Kit, 2nd Generation (Roche Molecular Biochemicals) according to the manufacturer's instructions. 3 ng of DIG-labeled DNA fragment and approximately 0.2 μg of partially purified His_6 tagged Bbr, fraction eluted with 200 and 500 mM imidazole, were used for each assay. For testing the specificity of binding, competitor plasmid-DNA or corresponding unlabeled DNA fragment were added in approximately 100-fold molar excess in comparison to the labeled fragment. After 15 min of incubation, the reaction mixture was applied to a native polyacrylamide gel with 1× TBE [Sambrook et al., 1989] as running buffer. The gel was run at 80 V for about 2 h, and transferred to a positively charged Hybond-N+ nylon membrane (Amersham Biosciences) by contact blotting. Cross-linking and detection were carried out following the manufacturer's instructions.

Bioassay for Detection of Active Balhimycin

Balhimycin production was determined by bioassays using *Bacillus subtilis* ATCC6633 as test organism and cell supernatants of *Amycolatopsis* strains [Pelzer et al., 1999].

Preparation of A. balhimycina *RNA*

A. balhimycina was cultivated in 100 ml of R5 medium for 10–49 h. The cells were then harvested and shock frozen at –70°C. An aliquot was resuspended in 100 μl of P buffer [Thompson et al., 1982] containing 10 mg of lysozyme and then incubated for 7 min at 37°C. The RNA was extracted by use of an RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

RT-PCR Analysis

RNA prepared from *A. balhimycina* was treated with 3 U of RNase-free DNase I (Promega, Madison, Wisc., USA) and precipitated according to standard protocols [Sambrook et al., 1989]. The RNA concentration was photometrically determined with a Genequant fixed-wavelength photometer (Pharmacia, Freiburg, Germany). Reverse transcription (RT) reactions were performed by use of an Omniscript RT kit (Qiagen) according to the manufacturer's instructions. The primers used for RT reaction are listed in table 1. PCRs were carried out in a programmable thermal controller (MJ Research, Inc., La Jolla, Calif., USA) under the following conditions: initial denaturation (95°C for 2 min); 25 cycles of denaturation (95°C for 20 s), annealing (60°C for 30 s), and polymerization (72°C for 40 s), and finally, an additional polymerization step (72°C for 7 min). Each PCR mixture (25 μl) contained a 1-μl aliquot of RT reaction product, 100 pmol of each primer, deoxyribonucleoside 5′-triphosphates at a final concentration (each) of 20 μM (DNA polymerization mix; Pharmacia), 10× reaction buffer (Qiagen), 5× Q-solution (Qiagen), and 3.5 U of Taq DNA polymerase (Qiagen). The PCR products were analyzed by agarose gel electrophoresis (1.0%). Primers used for amplification are listed in table 1.

Computer-Assisted Sequence Analysis

The PatScan software [D'Souza et al., 1997] and the BLAST program [Altschul et al., 1990] were used for analysis of inverted repeat patterns and for homology searches in the GeneBank database, respectively.

Nucleotide Sequence Accession Number

The nucleotide sequences of the balhimycin biosynthesis genes reported in this paper are available from the EMBL data library under accession number Y16952.

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