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Glycopeptide Biosynthesis in *Amycolatopsis mediterranei* DSM5908: Function of a Halogenase and a Haloperoxidase/Perhydrolase

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Summary

Glycopeptides are important clinical emergency antibiotics consisting of a glycosylated and chlorinated heptapeptide backbone. The understanding of the biosynthesis is crucial for development of new glycopeptides. With balhimycin as a model system, this work focuses on the investigation of the putative halogenase gene (*bhaA*) and the putative haloperoxidase/perhydrolase gene (*bhp*) of the balhimycin biosynthesis gene cluster. An in-frame deletion mutant in the haloperoxidase/perhydrolase gene *bhp* (OP696) did not produce balhimycin. Feeding experiments revealed that *bhp* is involved in the biosynthesis of β-hydroxytyrosine, a precursor of balhimycin. A *bhaA* in-frame deletion mutant (PH4) accumulated glycosylated but nonchlorinated balhimycin variants. The mutants indicated that only the halogenase BhaA is required for chlorination of balhimycin. Nonglycosylated and/or nonhalogenated metabolites can serve as starting points for combinatorial approaches for novel glycopeptides.

Introduction

Over the years, the glycopeptide antibiotic vancomycin has emerged as an antibiotic of last resort against multiresistant Gram-positive bacteria, as well as methicillin-resistant *Staphylococcae* (MRSA) [1]. However, the occurrence of vancomycin-resistant bacteria has been reported recently [2], and it is expected that the number of these resistances will further increase. This necessitates the search for new glycopeptide antibiotics to overcome threatening resistances. One precondition is understanding the biosynthesis of these glycopeptides in order to generate new glycopeptide antibiotics via rational combinatorial biosynthesis.

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In our attempts to study the biosynthesis of glycopeptide antibiotics and to assign the function of the respective genes [3–5], we have chosen the balhimycin biosynthesis in the actinomycete *Amycolatopsis mediterranei* DSM5908 [6] as a model system, since we are able to genetically manipulate the producer strain by applying various cloning techniques [7, 8]. The in vitro as well as the in vivo activities of balhimycin are similar to those of vancomycin, but with a slightly increased antibiotic activity toward anaerobic bacteria (for example, *Clostridiae*) [9]. The chemical structure of vancomycin-type antibiotics such as balhimycin is based on a central heptapeptide core, in which at least five residues are aromatic amino acids linked to each other to form two diaryl ether rings and one biaryl ring. The aromatic rings carry various substituents such as sugars and chlorine atoms. The aglycon of balhimycin as well as the chlorination pattern are identical with those of vancomycin and chloroeremomycin (A82846B), which are produced by different *Amycolatopsis* strains [10]. However, these glycopeptides differ in their glycosylation pattern (Figure 1), which has a strong influence on antibiotic activity [11]. The significance of glycosylation for an increased antibiotic activity has been explained by the tendency of some glycopeptides to form dimers [12, 13]. Besides glycosylation, the chlorination of the aglycon is of striking importance for antibiotic activity. Binding studies of monochlorinated and dechlorinated glycopeptide antibiotics have shown a less-effective binding of D-Ala-D-Ala peptides and analogs [14, 15]. There may be an influence on the position of the binding pocket, as well as on the dimerization of the sugar substituents [15]. Antibiotic activity tests comparing differently chlorinated glycopeptide antibiotics are in accordance with these data [11].

In order to reveal the function of the genes of the balhimycin biosynthesis gene cluster and to subsequently vary and manipulate them to obtain new glycopeptide antibiotics, we focused our interests on the characterization of the halogenation process. In nature, halogenated compounds can be found very frequently. More than 3000 different metabolites containing carbon-halogen bonds have been isolated to date [16]. The proteins first described as halogenating enzymes were the so-called haloperoxidases [17]. This was followed by the discovery of the cofactor- and metal-free haloperoxidases (perhydrolases) [18], which were later shown to belong to the α/β-hydrolase family. Structural and mechanistic investigations revealed that they were not haloperoxidases, but acted as perhydrolases [19]. Haloperoxidases and perhydrolases, which require hydrogen peroxide to catalyze halogenation reactions, lack substrate specificity and regioselectivity [18]. Further investigations demonstrated that these enzymes are not involved in the halogenation of antibiotics such as 7-chlorotetracycline [20], chloramphenicol [21], and pyrrolnitrin [22]. Hammer et al. [23] detected the genes of novel halogenating enzymes (PrnA and PrnC) by cloning the biosynthetic gene cluster of pyrrolnitrin. Biochemical

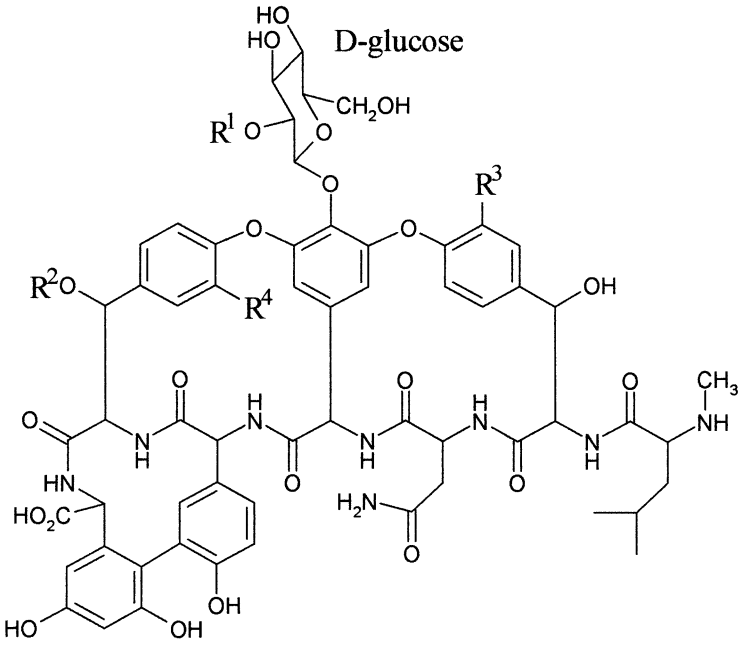
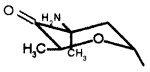
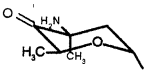
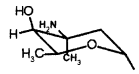
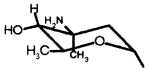
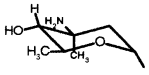


Figure 1. Structure of Different Glycopeptide Antibiotics

Structures of the glycopeptide antibiotics balhimycin (*A. mediterranei* DSM5908) and its variants PH1377 and PH1237, vancomycin (*A. orientalis* C329.4), and chloroeremomycin (*A. orientalis* A82846).

Antibiotic	R^1	R^2	R^3	R^4
Balhimycin	H	 dehydrovancosaminyl	Cl	Cl
PH1377	H	 dehydrovancosaminyl	H	H
PH1237	H	H	H	H
Vancomycin	 vancosaminyl	H	Cl	Cl
Chloroeremo-mycin	 4-*epi*-vancosaminyl	 4-*epi*-vancosaminyl	Cl	Cl

characterizations of PrnA and PrnC showed that $FADH_2$ was required for chlorinating activity [24]. Due to their substrate specificity and regioselectivity, this new class of $FADH_2$-dependent halogenases, rather than haloperoxidases or perhydrolases, was considered to be involved in halometabolite biosynthesis [18].

As reported previously, the balhimycin biosynthetic gene cluster contains a gene (*bhaA*) putatively coding for an $FADH_2$-dependent halogenase ([3] and [S.P., P.H., R.D.S., J.R., D. Heckmann, and W.W. German patent Wo 00/77182A1, December, 2000]). Here, we report that the balhimycin biosynthetic gene cluster also contains a gene (*bhp*) that putatively codes for a perhydrolase and that only the halogenase BhaA is required for the chlorination during balhimycin biosynthesis.

Results and Discussion

Features of the Proteins Encoded by *bhaA* and *bhp*

In the balhimycin biosynthetic gene cluster, the *bhaA* gene was identified downstream of the oxygenase genes *oxyA–oxyC* [3]. The similarity of the gene product to the halogenase PrnC from *Pseudomonas fluorescens* (27% identity, 42% similarity over 355 amino acids, [3]), which catalyzes the chlorination of an aromatic precur-

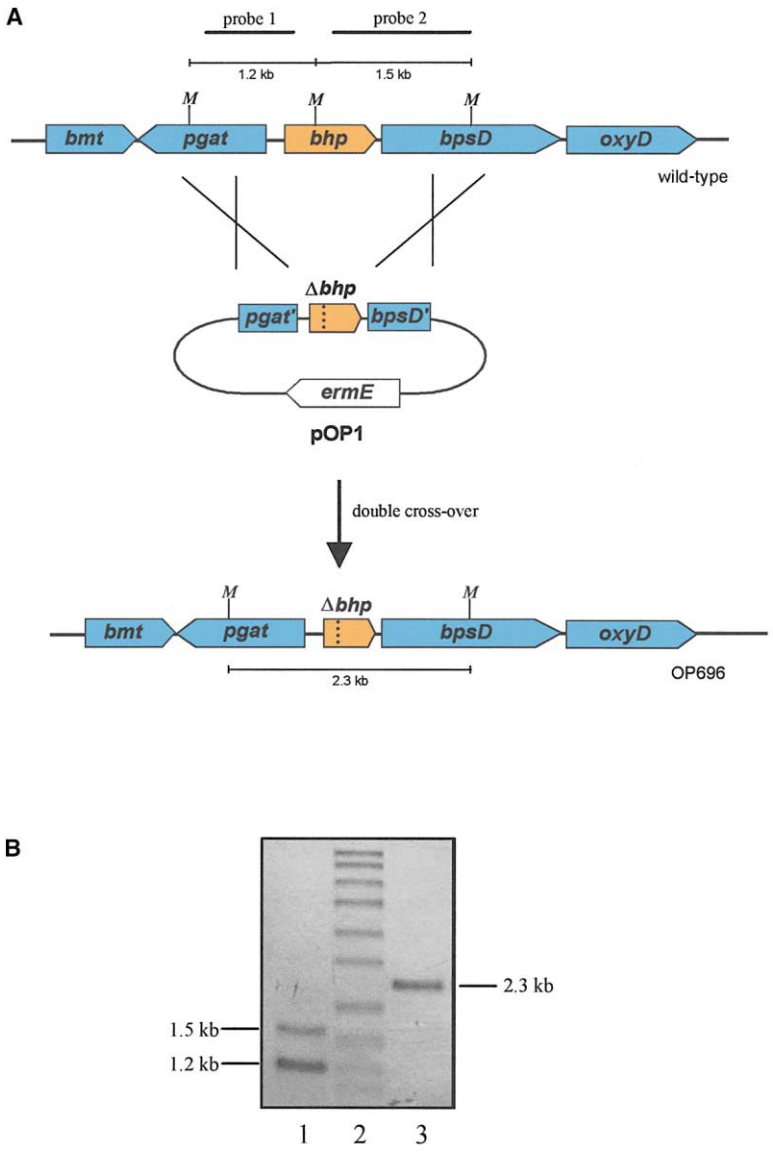


Figure 2. Mutational Analysis of the *bhp* Gene

(A) Construction of the in-frame deletion mutant OP696. MluI restriction sites are indicated (*M*). The positions of the probes used in the Southern blot analysis of OP696 are drawn above the cluster. *bmt*, *pgat*, *bpsD*, and *oxyD* are neighboring genes of *bhp*. *pgat'* and *bpsD'* are incomplete copies of the genes *pgat* and *bpsD*, respectively.

(B) Southern blot analysis of OP696 with a mix of probe 1 and probe 2. The genomic DNA of *A. mediterranei* wild-type strain (lane 1) and of OP696 (lane 3) was digested with MluI. The loss of one MluI cleavage site after the in-frame deletion of *bhp* within the chromosome of OP696 leads to the signal at 2.3 kb. Lane 2: DIG-labeled DNA Molecular Weight Marker VII (Roche).

sor of pyrrolnitrin biosynthesis [25], suggests its function to be that of an $FADH_2$-dependent halogenase. Furthermore, sequence analysis revealed an open reading frame (*bhp*), 8.5 kb downstream of *bhaA*, encoding a protein of 284 amino acids.

Bhp showed similarities to perhydrolases; for example, to BPO-A1 and BPO-A2 from *Streptomyces aureofaciens* [26] (37% and 33% similarity, respectively). By sequence comparison, the conserved serine, aspartate, and histidine residues of perhydrolases were detected in the amino acid sequence of Bhp at positions 95, 218, and 246, respectively. These residues form the catalytic triad [19] of these enzymes. The fact that it had been demonstrated that these perhydrolases are not involved in the halogenation step during biosynthesis of a number of halometabolites casts some doubt on the function of Bhp as a halogenating enzyme in balhimycin biosynthesis. To elucidate which of the two genes, *bhaA* or *bhp*, actually coded for the enzyme responsible for the incorporation of the chlorine atoms during balhimycin biosynthesis, mutational analyses were performed.

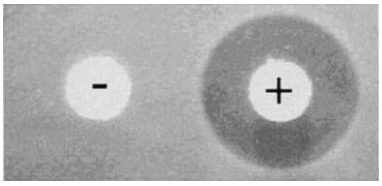


Figure 3. Bioassay of the *Amycolatopsis mediterranei* OP696 Supernatant

Bioassay of the OP696 supernatant using *B. subtilis* after incubation without (−) and with (+) supplementation of β-hydroxytyrosine.

Inactivation of the Putative Perhydrolase Gene *bhp*

To investigate whether the *bhp* gene participates in the chlorination of balhimycin or one of its biosynthesis in-

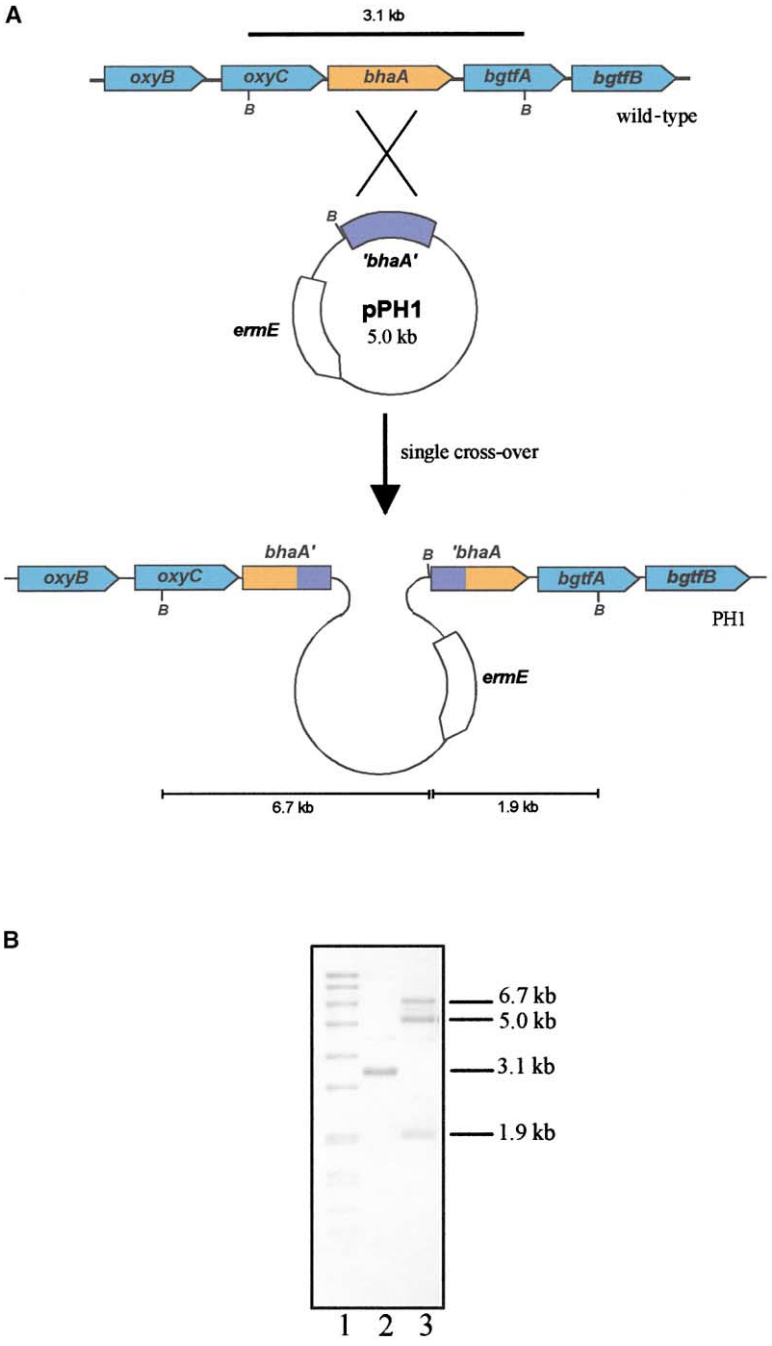


Figure 4. Analysis of the *bha* Gene by Insertional Mutagenesis

(A) Construction of the gene disruption mutant PH1. The position of the BamHI restriction sites are indicated (*B*). The location of the probe used in the Southern blot is shown. *oxyB*, *oxyC*, *bgtfA*, and *bgtfB* are neighboring genes of *bhaA*. *bhaA'*, *'bhaA*, and *'bhaA'* are incomplete copies of the gene *bhaA*.

(B) Southern hybridization of BamHI-linearized genomic DNA of the wild-type (lane 2) and of PH1 (lane 3), using the 3.1-kb BamHI fragment as a probe. The 6.7-kb and 1.9-kb signals in lane 3 indicate the integration of pPH1 into the wild-type chromosome. The additional signal at 5.0 kb most likely represents the linearized vector pPH1, indicating multiple or tandem integrations. Lane 1: DIG-labeled DNA Molecular Weight Marker VII (Roche).

termediates, an in-frame deletion mutant was constructed. The nonreplicative plasmid pOP1 (for construction, see the Experimental Procedures), carrying the in-frame deleted *bhp* gene, was used for transformation of the wild-type strain *Amycolatopsis mediterranei* by means of a modified direct transformation [8]. Via a first homologous recombination process, several erythromycin-resistant clones were generated, which contained the pOP1 plasmid integrated into the genome. One of the integration mutants was selected, and the correct integration of pOP1 by homologous recombination into the chromosomal *bhp* gene was analyzed by Southern hybridization (data not shown). In order to obtain a deletion mutant, a mutant in which a second homologous recombination event has occurred was selected for (Figure 2A). Since such events happen at a rather low frequency in *A. mediterranei*, a "stress" protocol (see the Experimental Procedures) has been developed whose application increases the probability of a second cross-over event. Following application of this "stress" protocol, 900 colonies were examined on R5 plates with and without erythromycin. Five of the tested colonies had lost the plasmid, as indicated by the lack of erythromycin resistance. A *Bacillus subtilis* bioassay showed that all of these five colonies were unable to produce balhimycin, indicating that, in all cases, the

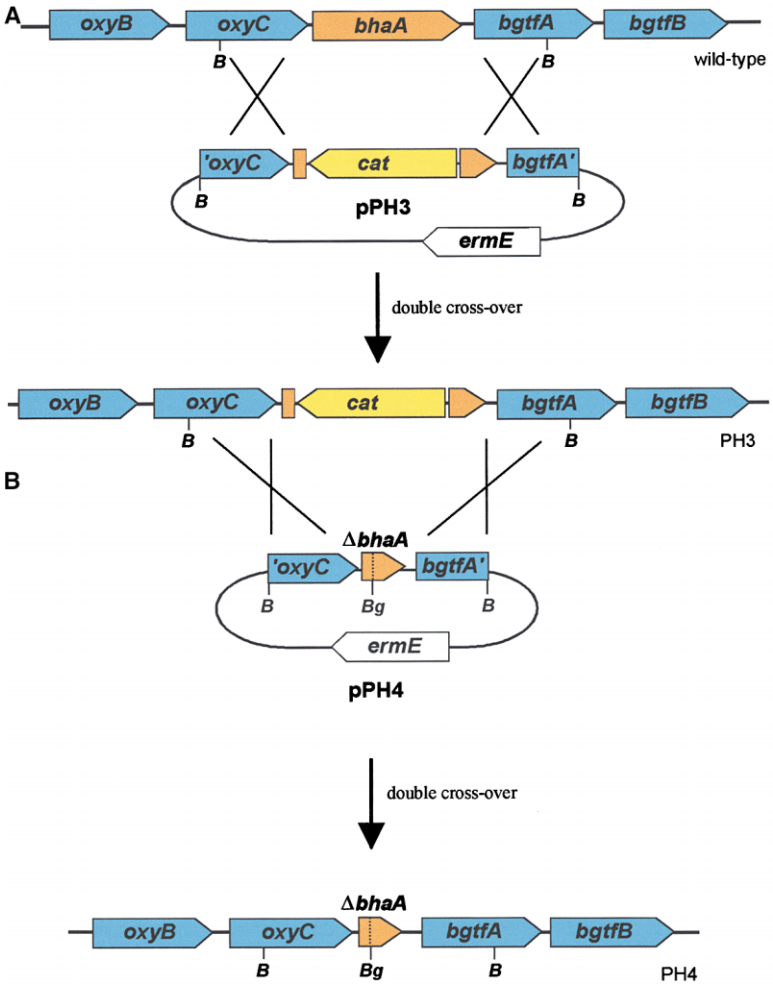


Figure 5. Analysis of the *bha* Gene by Gene Replacement and Deletion

(A) Construction of the gene replacement mutant PH3. *'oxyC* and *bgtfA'* are incomplete copies of the genes *oxyC* and *bgtfA*, respectively.

(B) Construction of the in-frame deletion mutant PH4. The relevant restriction sites are shown: B, BamHI; Bg, BglII.

second cross-over had led to the exchange of the wild-type allele against the deleted *bhp* gene (Figure 2A). The loss of balhimycin production in these strains clearly demonstrates the participation of Bhp in the biosynthesis of balhimycin. One of these balhimycin null mutants, OP696, was analyzed in detail by Southern hybridization, which confirmed the internal deletion of *bhp* (Figure 2B).

Chemical Analysis of Intermediates Isolated from OP696 Culture Filtrates

Culture filtrates of OP696 were investigated by HPLC-ESI-MS analysis. However, in accordance with the lack of antibiotic activity, neither balhimycin nor intermediates with molecular masses above 200 Da were detected in culture filtrates of OP696. The lack of balhimycin or its precursors in the culture filtrates of OP696 suggests a role of Bhp in an early step of the biosynthesis. However, since dechlorinated glycopeptide compounds have been isolated together with balhimycin [27], inactivity or lack of the chlorinating enzyme should not result in blocked balhimycin formation. Thus, the fact that *bhp* mutants are balhimycin nonproducers suggests a role of Bhp in a biosynthesis step different from halogenation. This conclusion is in accordance with the finding that these types of enzymes belong to the α/β-hydrolase family and that they are not present in the biosynthetic gene clusters of halometabolites like 7-chlorotetracycline [20], pyrrolnitrin [22], and pyoluteorin [28].

Studies of the Function of Bhp in Providing Nonproteinogenic Amino Acids by Feeding OP696 with β-Hydroxytyrosine

Downstream of *bhp*, the gene *bpsD* has been identified encoding a nonribosomal peptide synthetase domain. Feeding studies using a *bpsD* gene disruption mutant have shown that BpsD participates in the formation of β-hydroxytyrosine (J.R. et al., unpublished data), most likely by activating a tyrosine residue for the hydroxylation reaction. This assumption agrees with the results of studies on the role of the homologous protein NovH in novobiocin biosynthesis [29]. Since *bhp* and *bpsD* are cotranscribed (O.P., unpublished data), we assumed that Bhp may participate in the synthesis of β-hydroxytyrosine. To prove this, OP696 was cultivated in the presence of this amino acid. A bioassay with *B. subtilis* demonstrated that OP696 is able to produce active balhimycin after feeding β-hydroxytyrosine (Figure 3).

Since a putative thioesterase domain [30] is detectable in the amino acid sequence of Bhp (position 87–109,

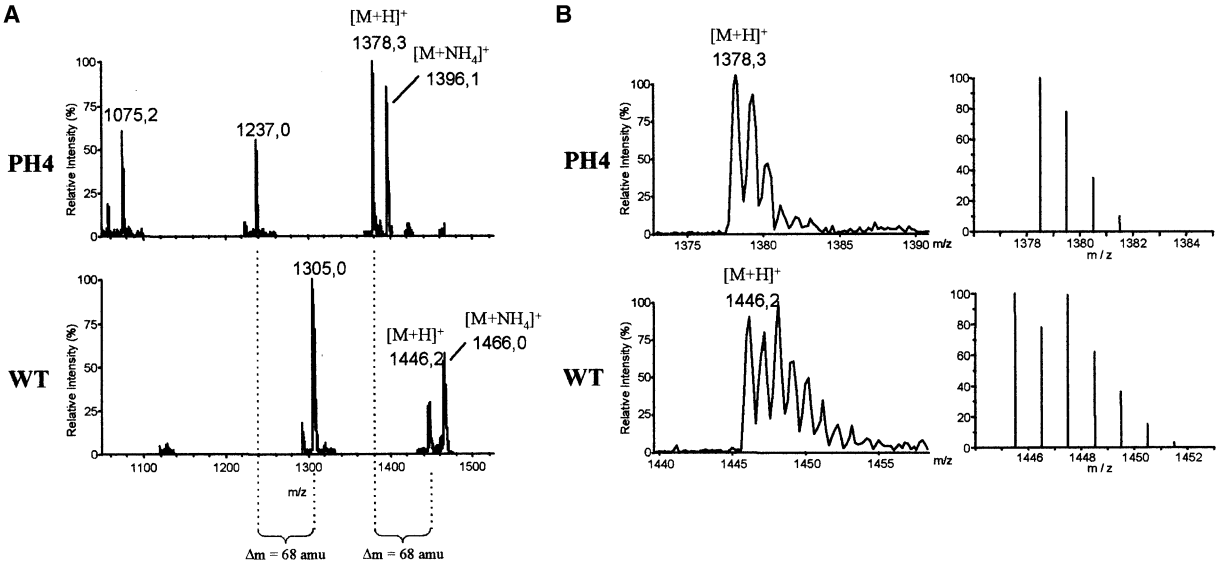


Figure 6. Chemical Characterization of Products from PH4 and Wild-Type

(A) Mass spectra of balhimycin variants produced by PH4 and the wild-type strain (WT) with a mass shift of $\Delta m = 68$ amu. The balhimycin variants indicated by the mass peaks are described in Table 1.

(B) Analysis of the isotopic pattern of balhimycin variants produced by PH4 and the wild-type strain (left). Right side: theoretically calculated isotopic patterns for nonchlorinated balhimycin (above) and for fully chlorinated balhimycin (below).

SPCRVVGTSMGSYIAQELALARP), one may speculate that Bhp catalyzes the release of the β-hydroxylated tyrosine from BpsD by thioester bond hydrolyzation. This putative enzymatic release is in contrast with the proposed reactions in novobiocin biosynthesis. In the novobiocin gene cluster [31], no gene with similarities to *bhp* was detected. For novobiocin biosynthesis, it has been postulated that the β-hydroxylated tyrosine undergoes further modifications before the product (amino-coumarin) is released from NovH by the nucleophilic attack of an *ortho*-hydroxy group [29].

Inactivation of the Halogenase Gene *bhaA* by Gene Disruption

In order to prove the halogenating function of BhaA, a *bhaA* gene disruption mutant was constructed. The wild-type strain *A. mediterranei* DSM5908 was transformed by the direct transformation method [8] with the plasmid pPH1 (for construction, see the Experimental Procedures), a nonreplicative vector carrying an internal *bhaA* gene fragment. This experiment resulted in the erythromycin-resistant transformant PH1 (Figure 4A). A Southern hybridization analysis of PH1 revealed that the plasmid pPH1 had integrated into the chromosome of the wild-type strain via homologous recombination (Figure 4B). The formation of an inhibition zone in the bioassay with *B. subtilis* demonstrated that, in contrast to the null mutant OP696, PH1 still produced antibiotically active compounds (data not shown).

Table 1. Balhimycin Variants Produced by PH4 and the Wild-Type Strain

Strain	m/z	Balhimycin Variant
PH4	1396	PH1377 (see Figure 1), ammonium-ionized
	1377	PH1377
	1237	PH1237 (see Figure 1)
	1075	Non chlorinated aglycon
Wild-type	1466	Balhimycin, ammonium-ionized
	1445	Balhimycin
	1305	Chlorinated, glucosylated peptide backbone

Chemical Characterization of the Biosynthesis Products of the Disruption Mutant PH1

To identify the balhimycin variants produced by PH1, the culture filtrate of this strain was analyzed by HPLC-ESI-MS. The mass spectrum indicated the production of the substance PH1237 (Figure 1) and its variants, all lacking the dehydrovancosamine residue, as well as both chlorine atoms (data not shown). The chlorination defect caused by the disruption of *bhaA* indicated that BhaA catalyzes the chlorination of balhimycin at both positions. The partial or complete lack of glycosylation can be attributed to polar effects on the glycosyl transferase genes downstream of *bhaA*, particularly on the *bgtfA* gene. BgtfA significantly resembles GtfA [3], which is considered to be the enzyme attaching 4-*epi*-vancosamine to the backbone of chloroeremomycin [32]. A polar effect on the *bgtfA* gene, therefore, would explain the lack of the dehydrovancosamine residue in the variants synthesized by PH1. However, from the results presented above, a function of BhaA in glycosylation cannot be excluded. Therefore, the construction of an in-frame deletion was necessary.

Inactivation of *bhaA* by an In-Frame Deletion

The construction of the in-frame deletion mutants as described for OP696 is a time-consuming procedure. The detection of the second cross-over step can require testing of a large number of colonies even after applying

Table 2. Bacterial Strains and Plasmids Used in This Study

Strain or Plasmid	Properties	Source or Reference
E. coli XL1-blue	General cloning host	[43]
A. mediterranei DSM5908	Balhimycin-producing wild-type	[6]
A. mediterranei OP696	Balhimycin non producing mutant, in-frame deletion in the *bhp* gene	This study
A. mediterranei PH1	Balhimycin non producing mutant, disruption of *bhaA* by the plasmid pPH1	This study
A. mediterranei PH4	Balhimycin non producing mutant with an in-frame deletion in the *bhaA* gene	This study
A. mediterranei PH4-2	PH4 complemented with an additional *bhaA* gene	This study
pJOE890	AP^R	[44]
pSP1	Gene disruption vector; Ery^R	[8]
pLitmus28	Ap^R	[45]
pUC18	Ap^R	[46]
pUC18ermEp1	Ap^R	[47]
pSET152	Am^R; integration system of the phage ϕC31	[48]
pIJ877	pBR322 derivative containing the *cat* gene	[33]
pUC18B3.0	pUC18 derivative containing a 3.1-kb BamHI fragment of the balhimycin biosynthetic gene cluster including the *bhaA* gene	This study
pUC18bhp	pUC18 derivative containing a 8.5-kb BglII fragment of the balhimycin biosynthetic gene cluster including the *bhp* gene	This study
pPH1	pSP1 derivative containing an internal part of the *bhaA* gene	This study
pSETbhaA	pSET152 derivative containing the *bhaA* gene under the control of the *ermE*p1-promoter	This study
pUC18hal3	pUC18 derivative containing the fragment frSP1 (see the Experimental Procedures)	This study
pUC18HalermE	pUC18ermEp1 derivative containing the fragment frSP1 (see the Experimental Procedures)	This study
pUC18BΔ	pUC18 derivative containing a part of the balhimycin biosynthetic gene cluster including the *bhaA* gene with a 969-bp in-frame deletion	This study
pPH4	pSP1 derivative containing a part of the balhimycin biosynthetic gene cluster including the *bhaA* gene with a 969-bp in-frame deletion	This study
pPH3	pSP1 derivative containing a part of the balhimycin biosynthetic gene cluster including the *bhaA* gene partly exchanged with the *cat* cassette	This study
pUC6cat	pUC18 derivative containing a part of the balhimycin biosynthetic gene cluster including the *bhaA* gene partly exchanged with the *cat* cassette	This study
pOP1	pSP1 derivative containing a part of the balhimycin biosynthetic gene cluster including the *bhp* gene with a 408-bp in-frame deletion	This study
pJOEOP1	pJOE890 derivative containing the fragment frOP1 (see the Experimental Procedures)	This study
pJOEOP2	pJOE890 derivative containing the fragment frOP2 (see the Experimental Procedures)	This study
pSPOPa	pSP1 derivative containing the fragment frOP2 (see the Experimental Procedures)	This study
pLitmus28OPa	pLitmus28 derivative containing the fragment frOP1 (see the Experimental Procedures)	This study

Am, apramycin; Ap, ampicillin; Ery, erythromycin.

the "stress" protocol. Since the recombination events that lead back to the wild-type genotype cannot be distinguished by the resistance pattern from those that result in the mutant genotype, a labor-intensive screening procedure by PCR and/or Southern hybridization is necessary. We, therefore, developed a modified strategy for the construction of the *bhaA* deletion strain. In the first step, the plasmid pPH3 (constructed via a new inverse PCR technique, see the Experimental Procedures) including *bhaA* disrupted by the chloramphenicol resistance gene *cat* [33] was used to transform *A. mediterranei*. Several chloramphenicol-resistant transformants were analyzed on erythromycin-containing media to differentiate between single cross-over (erythromycin resistant) and double cross-over (erythromycin sensitive). The mutant PH3 was chloramphenicol resistant and erythromycin sensitive, indicating a double cross-over (Figure 5A). A Southern blot experiment revealed the correct integration of the *cat* gene into the chromosomal *bhaA* gene (data not shown). To replace the *cat* cassette by an in-frame deletion, PH3 was transformed with the plasmid pPH4 that contains the mutated *bhaA* gene. After applying the stress protocol, 3 out of 250 tested colonies revealed the necessary resistance pattern (erythromycin sensitive and chloramphenicol sensitive), whereas no colony was detected (0/200) without

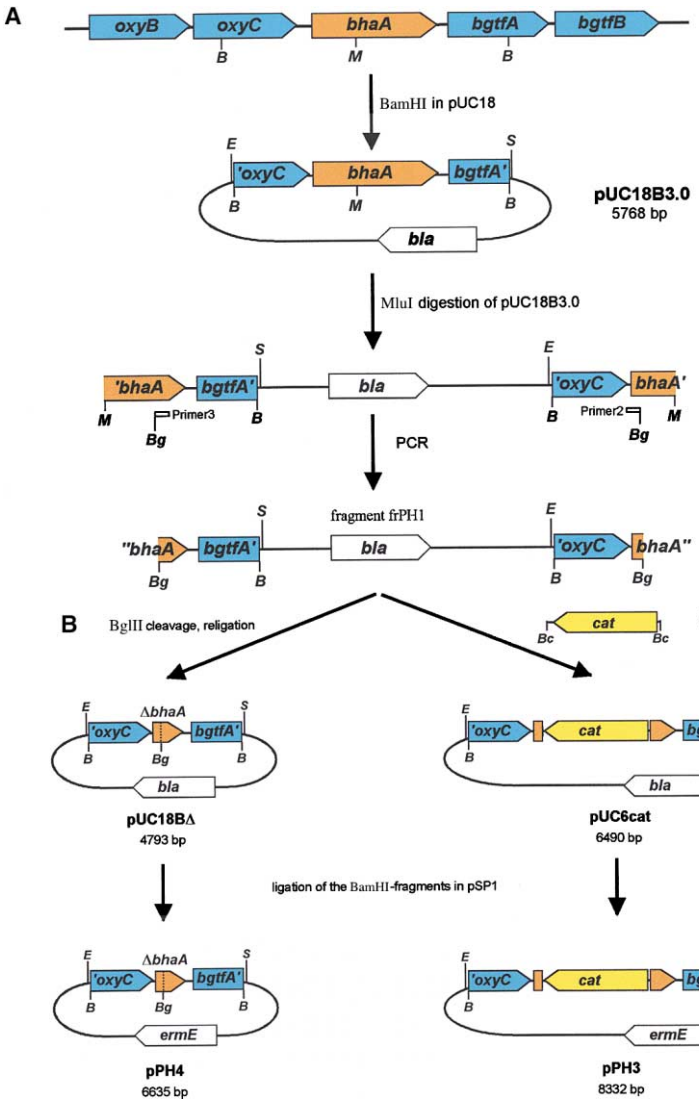


Figure 7. Construction of Plasmids Used for Mutational Analyses

(A) Amplification of the fragment frPH1. See the text for further details. *"bhaA* and *bhaA"* are incomplete copies of the gene *bhaA*.

(B and C) Construction of the plasmids (B) pPH4 and (C) pPH3. The relevant restriction sites are indicated: B, BamHI; Bg, BglII; Bc, BclI; E, EcoRI; M, MluI; S, SphI. See the text for further details.

stressing. The sensitive mutant strain PH4 was isolated, and the exchange of the *cat* cassette against the deletion by a double cross-over event (Figure 5B) was proven by Southern hybridization (data not shown). Like the disruption mutant PH1, PH4 is also able to produce antibiotically active compounds, as demonstrated by bioassays (data not shown).

Chemical Characterization of Biosynthesis Products of the Deletion Mutant PH4

For analysis of the glycosylation pattern and the chlorination degree of the balhimycin variants synthesized by PH4, samples of culture filtrates were submitted to HPLC-ESI-MS. According to the isotopic pattern, all balhimycin variants synthesized by PH4 completely lacked chlorine atoms (Figure 6B). Furthermore, characteristic mass shifts of 68 atomic mass units (amu) to lower masses in comparison with the corresponding signals from the wild-type culture broth also indicated the absence of both chlorine atoms (Figure 6A). In contrast to the gene disruption mutant PH1, the glycosylation pattern was not affected by the in-frame deletion, since completely glycosylated variants were detectable (Figure 6A; Table 1). Since both chlorine atoms are missing, *bhaA* is responsible for the halogenation at amino acids 2 and 6 of balhimycin. The occurrence of fully glycosylated balhimycins in PH4 demonstrated that BhaA is not involved in glycosylation. Furthermore, it can now be ruled out that halogenation is a precondition for subsequent biosynthesis steps. The different glycosylation degrees of the compounds synthesized by PH4 can be explained by natural variety, since differently glycosylated balhimycin variants were also found in wild-type filtrates (Figure 6A; Table 1). This is in agreement with observations of Vértesy et al. [27], who isolated up to ten different analogs of balhimycin from wild-type culture filtrates.

Complementation of the *bhaA* Gene Defect in PH4

To prove that only the in-frame deletion of *bhaA* was responsible for the loss of both chlorine atoms in the balhimycin variants produced by PH4, we complemented this mutant by integrating a copy of *bhaA* into

the chromosome. For this purpose, the integrative plasmid pSETbhaA (for construction, see the Experimental Procedures) was introduced by the direct transformation method [8]. By selection on apramycin-containing media, the complementation mutant PH4-2 was isolated. The integration of pSETbhaA into the chromosome of PH4-2 via the ϕC31 attachment site (*attb*) was demonstrated by Southern analysis (data not shown). Since *attb* lies outside of the balhimycin biosynthetic cluster (S.P., unpublished data), none of the biosynthetic genes was affected by the integration of pSETbhaA. HPLC-ESI-MS investigations of PH4-2 culture filtrates showed the existence of chlorinated balhimycin together with partially and nonchlorinated variants (data not shown). The failure of a complete restoration of chlorination could result from a positional effect. Alternatively, differences of the expression level of the *bhaA* gene in the wild-type and in PH4-2 may cause this effect, since, in PH4-2, *bhaA* is under the control of the constitutive promoter *ermE*p1 [34, 35], whereas the native *bhaA* gene is expressed from a promoter in the cluster. Comparable observations were reported, for example, on complementation experiments of a *Streptomyces clavuligerus ccaR* mutant strain [36].

The natural substrate of BhaA and, therefore, the biosynthetic step on which halogenation takes place is still unclear. Other *A. mediterranei* mutant strains, however, can give some insights into the halogenation time point. The linear heptapeptide SP-1134 [3, 4] of the oxygenase mutant SP1-1, as well as HD1, which is glycosylation deficient [3], are both chlorinated. Therefore, an early stage of chlorination prior to oxidative bridging by the oxygenases OxyA/B/C, possibly with tyrosine or β-hydroxytyrosine as the substrate, seems likely. In order to elucidate the natural substrate for the halogenase BhaA, further work is being performed via overexpression and biochemical characterization studies.

Significance

The results of this study demonstrate for the first time that a single halogenase is responsible for the incorporation of the two chlorine atoms of the glycopeptide antibiotic balhimycin, while a perhydrolase-like enzyme is required for the biosynthesis of β-hydroxytyrosine. Techniques for the construction of in-frame deletion mutants of the balhimycin-producing strain were optimized. With the help of these more-efficient techniques, glycopeptide production can be rationally manipulated with defined mutant strains. By heterologous gene expression and/or with the help of feeding experiments, a new generation of modified glycopeptides is expected to develop in the future.

Experimental Procedures

Strains and Plasmids

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

Media and Culture Conditions

Escherichia coli strains were grown in Luria broth [37], supplemented with 150 μg ml^{-1} ampicillin or 100 μg ml^{-1} apramycin when necessary to maintain plasmids. *A. mediterranei* strains were grown in R5 medium [38] at 30°C. Liquid/solid media were supplemented with 50 μg ml^{-1} erythromycin or 50 μg ml^{-1} apramycin to select for strains carrying integrated antibiotic resistance genes.

"Stress" Protocol for Increasing the Frequency of Cross-Over Events in *A. mediterranei*

After growth in 25 ml R5 medium [38] for 48 hr at 30°C, the mycelium of the *A. mediterranei* strain was harvested by centrifugation and resuspended in 12.5 ml R5. Then, fragmentation of the mycelium was induced using an ultrasound bath (Bandelin Sonorex RK100; 15 min), followed by an additional incubation for 2 hr at elevated temperature conditions (37°C) in 100 ml R5. For further fragmentation, an additional ultrasound treatment (Branson Sonifier 250; 5 mm standard-microtip; 10 s, 50% duty cycle, output control step 5) was performed. After storage on ice (10 min), 100 μl of different dilutions (10^{-1}–10^{-5}) of the cells was plated on R5 agar plates. After incubation at 30°C for 3–5 days, a few hundred colonies were obtained and used for further investigations.

Cultivation of OP696 in the Presence of β-Hydroxytyrosine

OP696 (Table 2) was incubated under standard conditions in 10 ml R5 medium [38], containing dissolved β-hydroxytyrosine that had been synthesized according to Bolhofer [39] at a concentration of 0.8 mg ml^{-1}. The supernatant was harvested after 50 hr, and 20 μl was used to determine the production of balhimycin in a bioassay with *B. subtilis*.

Preparation, Manipulation, and Sequencing of DNA

Methods for isolation and manipulation of DNA were as described by Sambrook et al. [37] and Hopwood et al. [38]. Plasmid isolation for sequencing was performed with the High Pure Plasmid Purification Kit (Roche). PCR fragments were isolated from agarose gels with QIAquick (Qiagen). Restriction endonucleases were obtained from various suppliers and were used according to their specifications. For sequencing reactions, the ALFexpress AutoRead kit (Amersham Pharmacia Biotech AB) or the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech AB) were used, according to the suppliers' instructions. Sequencing data were generated by using the ALFexpress DNA sequencer (Amersham Pharmacia Biotech AB). Alignment of sequence contigs and examination for open reading frames (ORFs) were performed by applying gap4 and nip4 [40]. BLAST [41] and FASTA [42] were used for homology searches.

PCR Protocols for Amplification of the Fragments frOP1, frOP2, frSP1, frPH1, and frPH2

PCR was performed on a Robo Cycler Gradient 40 thermocycler from Stratagene with the Expand High Fidelity PCR System (Roche). The PCR mixture (100 μl) contained 100 pmol of each primer, 1.0 μg template DNA (pUC18bhaA or pUC18bhp), deoxyribonucleoside 5′-triphosphates at a final concentration of 200 μM each (DNA Polymerization Mix; Pharmacia), 10× reaction buffer, 1.5 mM $MgCl_2$, and 3.5 U Taq DNA polymerase. Dimethyl sulfoxide at a final concentration of 3% was added to the reaction mixture to enhance the specificity of hybridization. For amplification of the fragments, the following PCR procedure was used: initial denaturation (94°C; 3 min) before the addition of the polymerase; 30 cycles of denaturation (94°C; 1 min), annealing (60°C; 1 min), and polymerization (72°C; 2 min); and an additional polymerization step (72°C; 10 min) at the end. The sequences of the primers were as follows: amplification of the fragment frOP1: prOP1-1, 5′-GGGCATGCCGGTCTTGGCGAAGGAGC CG-3′, prOP1-2, 5′-ACAGATCTCGGCGCCCCGGTGCCGGTCAG-3′; amplification of the fragment frOP2: prOP2-1, 5′-AAAGATCTGC CGACTGGCTCGACCTGTTC-3′, prOP2-2, 5′-GATCTAGACGCG GCCGGCGGCCCAGCGCTT-3′; amplification of the fragment frSP1: BhaA2RBS, 5′-AAGGATCCTCGGCAATTTGACACTCGAC-3′, BhaAC, 5′-TTTCTAGAGGTTGTTCCGCAGGTCCCGGCC-3′; amplification of the fragment frPH1: Primer2, 5′-CTCACAGATCTGATA CCGCGGGAAA-3′, Primer3, 5′-CTACCAGATCTACGTGAACGA GGAG-3′; amplification of the fragment frPH2: Primer4, 5′-AAGCGC GGCGGCACGTTCCGCTGGG-3′, Primer5, 5′-CACTGCGCGCGGCG ATGCGGTCGGC-3′.

Southern Hybridization

Digests of genomic *Amycolatopsis* DNA were separated in 1% agarose gels in Tris-acetate buffer and transferred to Hybond-N Nylon membranes (Amersham Pharmacia Biotech AB). For labeling of DNA

probes and hybridization, the nonradioactive DIG DNA Labeling and Detection Kit from Roche was used at high stringency (0.1% SDS, 0.1× SSC, 68°C). Oligonucleotides were labeled using the DIG Oligonucleotide Tailing Kit (Roche) and detected by the chemiluminescent reaction. As a size standard, the DIG-labeled DNA Molecular Weight Marker VII (Roche) with the following fragment lengths (in base pairs) was used: 81; 359; 492; 710; 718; 992; 1,164; 1,482; 1,515; 1,882; 1,953; 2,799; 3,639; 4,899; 6,106; 7,427; and 8,576.

Construction of the Plasmids pOP1, pPH1, pPH3, pPH4, and pSETbhaA

Plasmids were constructed for the internal deletion of the perhydrolase gene *bhp* (pOP1), for disruption of the halogenase gene *bhaA* (pPH1), for the internal deletion of *bhaA* (pPH3, pPH4), and for complementation of the mutant strain *A. mediterranei* PH4 (pSETbhaA). The relevant regions of the plasmid constructs were verified by sequencing.

pOP1

The 1,116-bp fragment frOP1 including the sequence encoding 32 amino acids of the N terminus of the perhydrolase gene *bhp* (855 bp) and the 1,286-bp fragment frOP2 including the sequence encoding 114 amino acids of the C terminus of *bhp* were ligated into the EcoRV site of the vector pJOE890, respectively, resulting in the plasmids pJOEOP1 and pJOEOP2. frOP2 was then ligated as an XbaI fragment into the single XbaI site of the vector pSP1, resulting in the plasmid pSPOPa. frOP1 was ligated as a BamHI fragment into the BamHI site of the vector pLitmus28, resulting in the plasmid pLitmus28OPa. frOP1 was then ligated as a BglII fragment into the single BglII site of the plasmid pSPOPa, leading to the plasmid pOP1, containing a partly deleted *bhp* gene.

pPH1

The 1,035-bp fragment frPH2 containing an internal part of the *bhaA* gene (base pair position 220–1,255) was ligated as a blunt-end fragment into the vector pUC18 and then integrated into the vector pSP1 as an EcoRI/SphI fragment, resulting in the gene disruption plasmid pPH1.

pPH3

For the construction of pPH3, a new inverse PCR strategy was used. First, the plasmid pUC18B3.0 (Table 2) was linearized at the single MluI cleavage site, localized within the *bhaA* gene 497 bp downstream of the ATG start codon. The linearized plasmid was then used as a template in a reverse PCR reaction with Primer2 and Primer3 binding 133 bp downstream of the *bhaA* start codon on the sense strand and 379 bp upstream of the *bhaA* stop codon on the antisense strand. This resulted in the fragment frPH1 (Figure 7A). Each primer contained a BglII cleavage site at the 5′ end. BglII cleavage and ligation of the 6.6-kb-sized frPH1 with the *cat* gene [33], obtained as a BclI fragment from the plasmid pIJ877 (Table 2), led to the plasmid pUC6cat. Finally, the EcoRI/SphI fragment of pUC6cat, which contains the disrupted *bhaA* gene, was inserted into pSP1 to obtain pPH3 (Figure 7C).

pPH4

The fragment frPH1 (Figure 7A, also see the construction of pPH3) was digested with BglII and religated to form the plasmid pUC18BΔ. The EcoRI/SphI fragment of pUC18BΔ, containing the deleted *bhaA* gene (969-bp deletion), was then inserted into pSP1, resulting in the plasmid pPH4 (Figure 7B).

PSETbhaA

To construct the expression plasmid pSETbhaA, the 1,565-bp fragment frSP1 containing the *bhaA* gene and its natural ribosome binding site (AGAGG) was cloned into the SmaI site of the vector pUC18 (Table 2), resulting in the plasmid pUC18hal3. For the expression of *bhaA* in *A. mediterranei*, the constitutive promoter *ermE*p1 [34, 35] was used. To place *ermE*p1 upstream of *bhaA*, pUC18hal3 was used to clone frSP1 as a BamHI/XbaI fragment into the single BamHI/XbaI site of the vector pUC18ermEp1 (Table 2), resulting in the plasmid pUC18HalermE. Finally, the *ermE*p1-*bhaA* fragment was cloned by EcoRI/XbaI digestion into the EcoRI/XbaI site of the vector pSET152 to obtain the *bhaA* expression plasmid pSETbhaA.

Determination of Balhimycin Biosynthesis

Balhimycin production was determined by bioassays using *Bacillus subtilis* ATCC6633 as a test organism and cell-free supernatants of *Amycolatopsis* strains grown on R5 medium [38].

HPLC-ESI-MS

Investigations of the balhimycin variants in the culture broths were performed via HPLC-ESI-MS analysis. Culture broths were prepared by centrifugation and filtration to obtain particle-free samples (sample injection volume: 100 μl), which were introduced via a double syringe pump (Applied Biosystems, Model 140A) to the HPLC-MS. Separations were performed on a Nucleosil C-18 column (2 × 100 mm, 5 μm) (Grom) with a flow rate of 200 μl min^{-1}. The UV detector (UVIS 204, Linear) was connected in series with the mass spectrometer (λ = 214 nm). The following gradient was used: t = 0 min: 5% B; t = 1 min: 17% B; t = 15 min: 20% B; t = 17 min: 100% B (solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile).

Mass spectra were recorded on a Perkin Elmer API III TAGA SCIEX Triple Quadrupole mass spectrometer (Thornhill), equipped with an electrospray ion source (ESI). The LC mobile phase flow rate was reduced by means of a splitter down to 70 μl min^{-1}, before introduction into the ES interface. The orifice voltage was set to 80V in positive ion mode.

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Accession Numbers

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