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The Mildiomycin Biosynthesis: Initial Steps for Sequential Generation of 5-Hydroxymethylcytidine 5′-Monophosphate and 5-Hydroxymethylcytosine in *Streptoverticillium rimofaciens* ZJU5119

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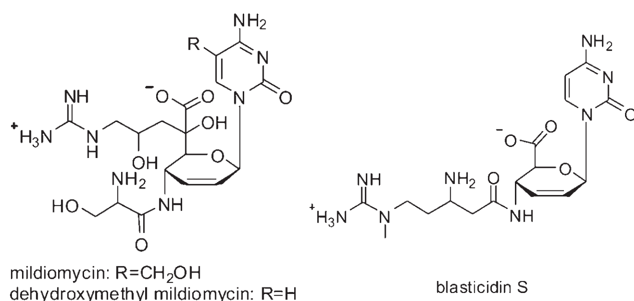
Mildiomycin (MIL) is a peptidyl nucleoside antibiotic with strong activity against powdery mildew disease of plants. We have cloned the MIL biosynthetic gene cluster in Streptoverticillum rimofaciens *ZJU5119 and shown that this organism also produces the related antifungal compound, deshydroxymethyl mildiomycin (dHM-MIL). A cosmid genomic library was screened for a putative nucleotide hydrolase gene that is related to* blsM *from the blasticidin S cluster. Six cosmids were identified that contained a 3.5 kb DNA fragment that harbors a homologue of* blsM. *The sequence of the fragment revealed two open-reading frames that are likely to function in MIL formation:* milA *is a CMP hydroxymethylase gene and* milB *is the homologue of the CMP hydrolase gene* blsM. *Insertional disruption of* milA *abolished the production of MIL but not dHM-MIL, whereas a* milB *knockout strain did not produce either of the peptidyl nucleosides. Recombinant MilA was produced in* E. coli *and shown to specifically introduce a C-5 hydroxymethyl group on CMP, but it did not accept cytosine or dCMP as a substrate. MilB was also expressed and purified from* E. coli *and shown to efficiently hydrolyze both hydroxymethyl-CMP (HMCMP) and could accept CMP as an alternative substrate. The ratio of free HMC and cytosine released by MilB was ca. 9:1 in in vitro assays, and is consistent with the higher levels of MIL compared to dHM-MIL that are produced by* Streptoverticillum rimofaciens.

Introduction

Mildiomycin (MIL) is a peptidyl nucleoside antibiotic that was originally isolated from the culture filtrate of *Streptoverticillium rimofaciens* (hereafter abbreviated as *Sv. rimofaciens*).[1,2] The compound possesses a strong inhibitory effect against powdery mildew on plants, and has been produced and sold commercially in Japan as a fungicide for agricultural and horticulture use. The mechanism of action was shown to be the inhibition of fungal protein biosynthesis.[3] The structural components of MIL include 5-hydroxymethylcytosine (HMC), which is an unusual 4-amino-pyran-3-ene moiety that bears an arginine-like side chain and a serine residue (Scheme 1).[4] There has been a great deal of interest in the family of peptidyl nucleoside antibiotics due to their diverse biological activities, including antitumor, antiviral, antibacterial, and antifungal activity.[5] The biomedical and agricultural potential of these agents, together with various unique structural features, prompted investigations into the biosynthesis of these compounds, and more recently the biosynthetic gene clusters for several peptidyl nucleosides have been reported, including those for puromycin,[6] nikkomycin,[7] streptothricin F,[8] and blasticidin S.[9]

Previous investigations of MIL biosynthesis have focused on the formation of HMC and the various means to increase the production of MIL. Among the family of peptidyl nucleoside antibiotics, the HMC moiety is only found in MIL, 5-hydroxymethylblasticidin S[10] and 5-hydroxymethylleucylblasticidin S



Scheme 1. Structures of mildiomycin, deshydroxymethyl mildiomycin and blasticidin S.

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(Sch36605 or rodaplutin).[5] 5-Hydroxymethylcytosine was first identified as a nucleic acid component of the T-even phage.[11] It was subsequently shown that hydroxymethylation took place at the nucleotide level, with deoxycytidine 5′-monophosphate (dCMP) and formaldehyde serving as substrates in the presence of tetrahydrofolate (THF) and an enzyme preparation derived from *E. coli* that was infected with T-even bacteriophages.[12] Sawada et al. used cell-free extracts of *Sv. rimofaciens* to examine MIL biosynthesis and demonstrated that cytidine 5′-monophosphate (CMP) is converted first to 5-hydroxymethylcytidine 5′-monophosphate (HMCMP) in a reaction that requires serine or formaldehyde and THF.[13] HMCMP was shown to be subsequently hydrolyzed to free HMC by a specific HMCMP N-ribotidase.[14] This group also demonstrated that the availability of free HMC might be a limiting factor in MIL biosynthesis. The addition of exogenous HMC to *Sv. rimofaciens* culture media resulted in a pronounced increase in MIL production.[15] It was also revealed that when various 5-substituted cytosines were added to MIL cultures, MIL analogues accumulated; this indicated that the enzyme involved in the incorporation of HMC into MIL exhibit some flexibility in substrate recognition.[15] The addition of ferrous ion to the culture medium also increased the yield of MIL production, and it was suggested this was due to an increased supply of amino acids precursors.[16] In iron-sufficient medium, glutamate, aspartate, serine and arginine levels in the cells were two to ten-fold greater than those that were measured in cells from iron-deficient medium, and arginine was the major amino acid that was excreted from cells in the iron-sufficient cultures.[17]

To date, there have been no published experiments that examined the incorporation of isotopically labeled arginine or related compounds into MIL. Thus, it remains to be demonstrated that the guanidine-containing segment of MIL originates from an arginine-derived α-ketoacid. However, the presence of the α-hydroxy acid, and the fact that cytosyl- or 5-hydroxymethylcytosylglucuronic acids are likely intermediates in the biosynthesis of MIL, blasticidin S, and the pentopyranines suggests a coupling of the α-keto acid of arginine with a decarboxylated nucleoside.[5,18] It also remains to be established whether hydroxylation of this guanidine side-chain precedes coupling with the nucleoside, or occurs afterwards. In this regard, mildiomycin D was identified as a minor component from the culture broth of *Sv. rimofaciens* B-98891 and differs from MIL by lacking the hydroxyl group on the arginine-like moiety.[19]

In addition to the structural resemblance of MIL and blasticidin S (Scheme 1), fermentation and biochemical studies also support related biosynthetic pathways. Similar to the findings with MIL, yields of blasticidin S increased nearly two-fold when free cytosine was added to cultures of the producing organism.[20] More recently, BlsM was characterized from the blasticidin S biosynthetic pathway as a CMP hydrolase, and was shown to be responsible for generating free cytosine from CMP,[21] analogous to the conversion of HMCMP to HMC. Based on these similarities we sought to use elements of the blasticidin S (*bls*) gene cluster to aid with the identification of the mildiomycin biosynthetic genes. We report here the cloning of the *mil* gene cluster in *Sv. rimofaciens* ZJU5119 and demonstrate in vitro that the products of *milA* and *milB* act sequentially to convert CMP to HMC and initiate mildiomycin biosynthesis.

Results

Cloning of mildiomycin biosynthetic gene cluster in *Sv. rimofaciens* ZJU5119

A nucleotide hydrolase activity in the cell-free extract of *Sv. rimofaciens* was previously shown to catalyze the specific formation of hydroxymethylcytosine (HMC) from hydroxymethylcytidine 5′-monophosphate (HMCMP).[13,14] Given the structural similarity between HMCMP and CMP, we postulated that the hydrolase that cleaves HMCMP in *Sv. rimofaciens* might share sequence similarity with BlsM, which converts CMP to cytosine in blasticidin S biosynthesis.[21] Based on the aligned sequences of BlsM and homologues, six pairs of degenerate primers were designed by using the CODE-HOP strategy (Table 2, Figure 1 A).[27] PCR products of the expected sizes that correspond to six different blocks of the *blsM* counterpart in *Sv. rimofaciens* were obtained (Figure 1 B). The patterns for both strains were found to be the same (indicated by the arrows in Figure 1 B), and implied the HMCMP hydrolase gene shared many similarities with *blsM*. The largest PCR product (Figure 1 B) was sequenced, and its deduced amino acid was found to share 62% identity and 74% positive homology with BlsM; this strongly suggests that this fragment might be part of the gene that codes for HMCMP hydrolase.

To identify the mildiomycin biosynthetic gene cluster, a genomic library was constructed in the integrative cosmid pJTU2554 (Table 1) and screened by PCR by using the primer CMPH (Table 2) with a pool-and-split PCR approach. This analysis of 20 96-well plates of clones led to six cosmids demonstrated to constitute an overlapping contig via SacI restriction map analysis. The putative HMCMP hydrolase gene was mapped to a common 3.5 kb SacI fragment by Southern hybridization (Figure 2 A). This fragment was cloned into pBluescript II SK(+) to generate pJTU1794, and sequenced. Computational analysis of the sequence by using FramePlot 3.0 predicted three putative open-reading frames.[24] BLAST analysis revealed that the product of *orf1* was 81% identical to SCO4047, a protein of unknown function that is predicted from the *S. coelicolor* A3(2) genome. The Orf2 sequence exhibited high similarity to members of the ThyA superfamily of thymidylate synthases (Pfam PF00 303) that catalyze the reductive methylation of dUMP to dTMP with concomitant conversion of 5,10-methylenetetrahydrofolate to dihydrofolate.[28] Thus, we suspected that the ThyA homologue that is encoded by *orf2* might act as a CMP hydroxymethylase and catalyze the initial step for mildiomycin biosynthesis. The deduced product of *orf3* has a conserved domain that is found in a family of nucleoside 2-deoxyribosyltransferases (Pfam PF05 014) and shares 54% identity (78/144) with BlsM. It seemed very likely that Orf3 should function as the HMCMP hydrolase required for the release of hydroxymethylcytosine from the corresponding

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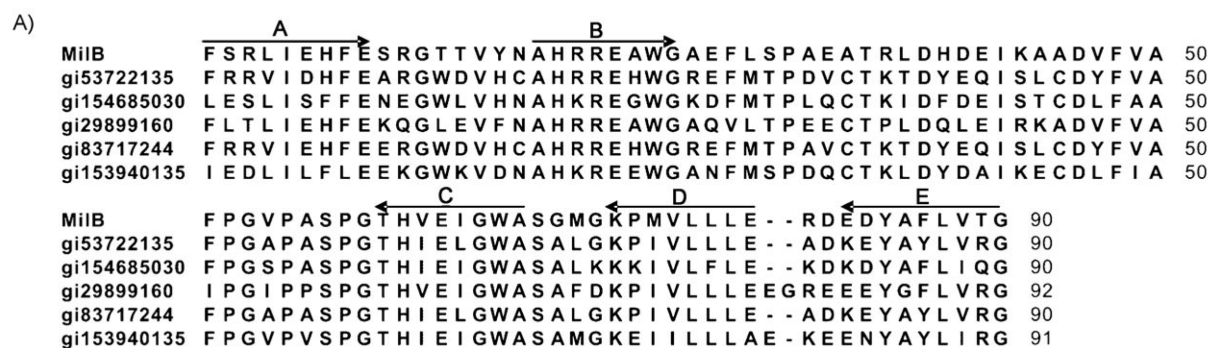
Table 1. Bacterial strains, plasmids and cosmids

Strain	Relevant properties	Source or reference
Streptoverticillium rimofaciens ZJU5119	producer of mildiomycin	this study
Streptomyces griseochromogenes	producer of blasticidin S	China General Microbiological culture collection center (CGMCG)
Rhodotorula rubra	Indicator strain of mildiomycin	[1]
Escherichia coli DH10B	F^{-} *recA lacZ* ΔM15	GIBCO BRL
Escherichia coli ET12567/pUZ8002	Strain that was used for conjugation between *E. coli* and *Streptomyces* spp. *recF*, *dam*, *dcm*, *hsdS*, Cml^{r}, Str^{r}, Tet^{r}, Km^{r}	[31]
Escherichia coli EPI300-$T1^{R}$	F- mcrA Δ(*mrr-hsd*RMS-*mcr*BC) 80d *lac*Z M15 *lac*X74 *rec*A1 *end*A1 *ara*D139 (*ara*, *leu*)7697 *gal*U *gal*K - *rps*L *nup*G *trf*A *ton*A *dhfr*	EPICENTRE Biotechnologies
Plasmid		
pJTU412	Shuttle cosmid that was derived from pHZ1358 (Sun et al., 2002), *oriT*, *ori*(ColE1), *bla*, *tsr*, *cos*, *rep*(pIJ101), *ori*(pIJ101)	Sun et al. (unpublished data)
pJTU2554	3.9-kb XbaI-XhoI fragment from pSET152 that was ligated with 5.5-kb XbaI-XhoI fragment that contained triplet COS sites from pOJ446	this study
pIJ779	pBluescript KS(+), aadA, oriT(RK2), FRT sites	[26]
14A6	pJTU2554 derived cosmid with insert from *Sv. rimonficiens* ZJU5119	this study
pJTU1794	pBluescript SK (+) with 3.5-kb SacI fragment of 1A6 that was inserted at the SacI site	this study
pJTU1796	pBluescript SK (+) with 10.6-kb Eco72I fragment of 1A6 that was inserted at the SmaI site	this study
pJTU1797	XbaI-HindIII that was digested pJTU412 with insertion of 10.6-kb XbaI-HindIII fragment from pJTU1796	this study
pJTU2952	The *milA* in the pJTU1797 substituted by *aadA* by using the PCR-targeting method	this study
pJTU2953	The *milB* in the pJTU1797 that was substituted by *aadA* by using the PCR-targeting method	this study
pJTU2955	*milA* expression vector, PCR product for *milA* has NcoI and XhoI restriction sites at its two ends, which were used for cloning into the corresponding sites of pET28a(+)	this study
pJTU2956	*milB* expression vector, PCR product for *milB* has BamHI and XhoI restriction sites at its two ends, which were used for cloning into corresponding sites of pET28a(+)	this study



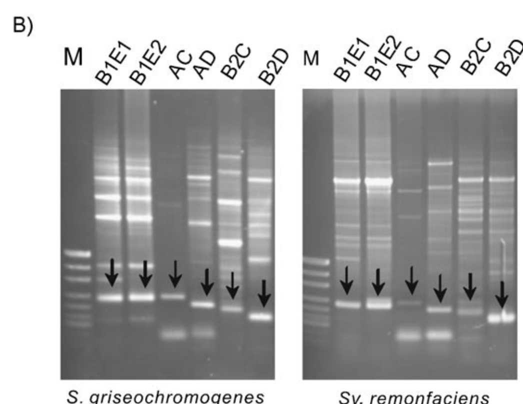


Figure 1. A) Alignment of BlsM and homologues from public databases that were used for the design of degenerate primers to clone the mildiomycin biosynthetic gene cluster. The arrows indicate the locations of the degenerate primers are shown in A, B, C, D, and E (see Table 2 for sequences). B) The patterns of PCR products of expected size from *S. griseochromogenes* and *Sv. rimofaciens* are similar. The primers that were used for each lane are shown, and the band of the expected size is indicated by the vertical arrow.

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Table 2. Primers used in this research

Primers	Forward (F)	Reverse (R)
aac(3)IV-T	CCGACTGGACCTTCCTTCT	GACACGATGCCAACACGAC
aadA-T	TCGGCAGCGACATCCTTC	CGTCATCGAGCGCCATCT
B1E1	CCCACCGGCGCGARVMNTGGGG	GGCCGCGGACCAGGWANSCRTANTC
B1E2	CCCACCGGCGCGARVMNTGGGG	CAGGCCGCGGACCARRWANSCRTA
AC	CGTTCCGGAGAGTGATCGANCAYTTYGA	TCCAGCAGCAGCACGATNGGYTTNYC
AD	CGTTCCGGAGAGTGATCGANCAYTTYGA	CCAGCCGATCTCGATGTGNGTNCCNGG
B2C	CGCCCACCGGCGNGARSMNTGGG	TCCAGCAGCAGCACGATNGGYTTNYC
B2D	CGCCCACCGGCGNGARSMNTGGG	CCAGCCGATCTCGATGTGNGTNCCNGG
CMPH	GACGGTCTACAACGCCCACC	GCCCAGCCGATCTCCACA
Ex-milA	GGCCATGGAAACCCATACGTTCGG	TCTCGAGGGAGCCGGCCGGAGAG
Ex-milB	AGGATCCGTGACCACCACCCCCAAG	TCTCGAGGCCGATGACCGTCGGCT
PT-milA	AGTCCTGTGGTACCTCACTATTACGGGCGGGCAGTG- ATGATTCCGGGGATCCGTCGACC	GGTCGGGAACGGACCGAGGTCAGGAGCCGATCGGG- GTCATGTAGGCTGGAGCTGCTTC
PT-milA-T	GTCGTGTCGCTCGGTGGTGT	GGCGGCTGAAGACGTTCTGGT
PT-milB	CCAGAGACGCGACCCCACCAGAAAGGAACAACCCC- GGTGATTCCGGGGATCCGTCGACC	CGGGCCGCCGATAAGAGGTCCATGCGCGGCCGCGC- CTCATGTAGGCTGGAGCTGCTTC
PT-milB-T	TTCGAGGCCCACCGTCAGGT	GCCGCCGATAAGAGGTCCAT

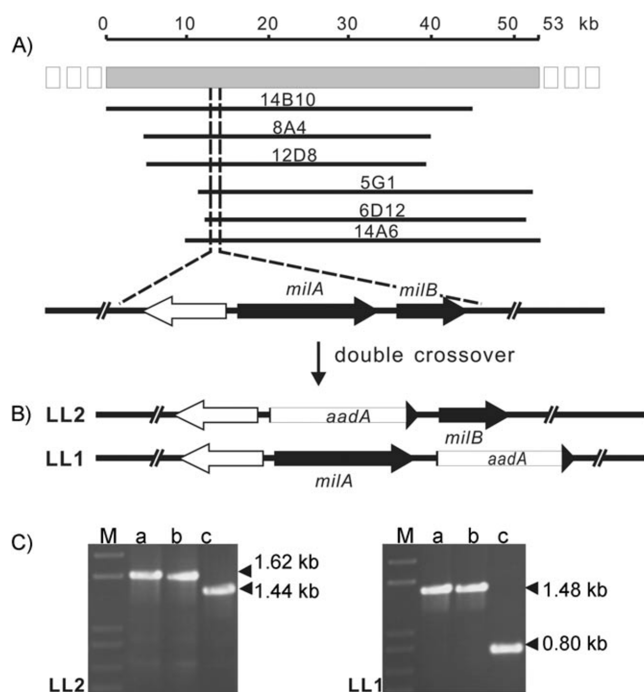

Figure 2. A) Schematic representation of six overlapping cosmids that share a common 3.5 kb SacI fragment. The boundary of the 3.5 kb fragment is indicated by the dashed lines. B) Illustration of the replacement targets gene of *milA* and *milB*. The short double slash indicates the omitted region. C) Confirmation of the mutants LL2 and LL1 by PCR. Lanes a and b show the PCR products from two independent colonies of each mutant. Lane c shows the PCR product that was obtained from the wild-type *Sv. rimofaciens* by employing the same primer set. The primer that was used for the LL2 gel was PT-milA-T, and for the LL1 gel PT-milB-T was used.

nucleotide. Based on the predicted roles of *orf2* and *orf3* in mildiomycin biosynthesis, they were renamed as *milA* and *milB*.

Identification of a new mildiomycin derivative in the extract of *Sv. rimofaciens* ZJU5119

As the HPLC chromatogram shows in the Figure 3 A, an extract of the fermentation broth of wild-type *Sv. rimofaciens* ZJU5119 showed two peaks, and peak I has the same retention time as the MIL standard. Electrospray ionization mass spectrometry (ESI-MS) analysis of peak I showed an ion at *m/z* 515, which corresponds to the protonated quasi-molecular ion of MIL, and a strong signal at *m/z* 374, which correlates to the loss of the HMC moiety. This fragmentation profile is the same as that observed for the MIL standard sample. In addition to MIL, peak II was present at a retention time of about 39 min, which had not been reported before (Figure 3 A). To determine whether it was related to mildiomycin, the peak was subjected to the ESI-MS analysis, and produced an ion at *m/z* 485, which is consistent with the protonated quasi-molecular ion of deshydroxymethyl mildiomycin (dHM-MIL). This assignment was further supported by the presence of a fragment ion at *m/z* 374 as seen with MIL.

Inactivation of *milA* abolishes the production of MIL but not dHM-MIL

To show conclusively that *milA* is required for the biosynthesis of MIL, the gene was disrupted by insertion of the *aadA* cassette, and the resulting mutant, LL2, was confirmed by PCR (Figure 2 C). In the bioassay plate (Figure 3 B), the MIL standard displayed a halo, whereas the agar patch of wild-type *Sv. rimofaciens* had an outer halo and an inner clear zone; this suggests that the halo is caused by MIL, and the clear zone is caused by other metabolites. Surprisingly, both the halo and clear zone remained in two independent LL2 strains, named LL2a and LL2b. To address this finding, mutant strain LL2 was cultured under MIL production conditions, and the metabolites were purified with the same protocol that was used for the wild-type strain, and analyzed by LC–MS (Figure 3 A). The HPLC

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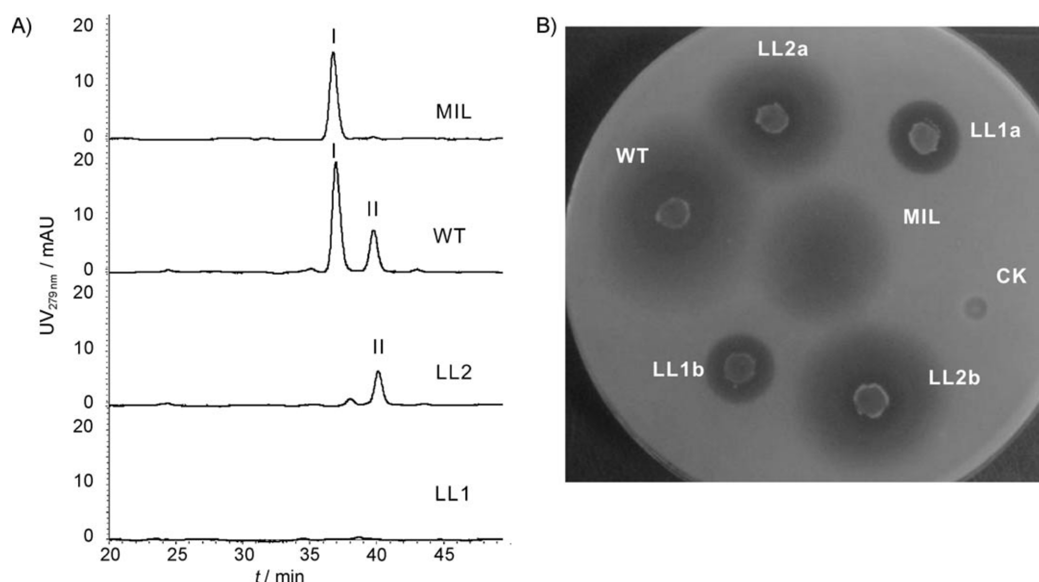

Figure 3. HPLC and bioassay comparison of the mildiomycin standard to fermentation products from wild-type *Sv. rimofaciens* and mutants. Peak I and II presents MIL and dHM-MIL, respectively; MIL: Mildiomycin standard, WT: wild-type *Sv. rimofaciens*, LL2: *Sv. rimofaciens* LL2, LL1: *Sv. rimofaciens* LL1.

chromatogram showed that peak I (MIL) disappeared but peak II (dHM-MIL) remained in the extract of LL2. Thus the outer halo around LL2 is due to the activity of dHM-MIL, which implies that dHM-MIL has antifungal activity against *Rhodotorula rubra* that is similar to that of MIL.

Inactivation of *milB* abolishes the production of both MIL and dHM-MIL

To examine if MilB is responsible for the generation of cytosine and 5-hydroxymethylcytosine, *milB* was inactivated by the insertion of the *aadA* cassette and the resultant mutant, LL1, was confirmed by PCR (Figure 2C). In the agar plate bioassay (Figure 3B), the outer halos disappeared around the two individual LL1 mutants, LL1a and LL1b, although the inner clear zone remained. For LC–MS analysis of the MIL and dHM-MIL production, LL1 was grown under MIL production conditions and the fermentation broth was processed in the same way as the wild-type sample. The HPLC chromatogram showed the disappearance of both peaks I (MIL) and II (dHM-MIL; Figure 3A) in the LL1 extract. This suggested that MilB was responsible for the hydrolysis of both CMP and HMCMP.

Analysis in vitro of the sequential action of MilA and MilB

To obtain direct evidence for the generation of free cytosine and 5-hydroxymethylcytosine by MilA and MilB, the proteins were heterologously expressed and purified from *E. coli*. The calculated molecular weights of His-tagged MilA and MilB are 38.4 and 22.7 kDa, respectively, which is consistent with those that were observed by SDS-PAGE (Figure S1). The ability of MilB to hydrolyze CMP to cytosine was confirmed. The LC–MS traces that are illustrated in Figure 4A show the time-dependent conversion of CMP to free cytosine by MilB over periods of 1 and 5 h. There is some uncatalyzed hydrolysis of CMP under the reaction conditions (top panel), but the rate of product formation is clearly increased with added MilB and supports its catalytic role.

MilA was predicted to introduce the C-5 hydroxymethyl group that is found on the cytosine moiety in MIL. To determine if hydroxymethylation occurs on free cytosine or CMP, as was previously observed in cell-free studies, both potential substrates were individually incubated with purified MilA and the appropriate cofactors. LC–MS analysis revealed product formation only when CMP was used as substrate, and nearly all of the CMP was consumed after 5 h (Figure 4). To further test the substrate specificity of MilA, dCMP, CDP, CTP, cytidine, UMP, dUMP and cytosylglucuronic acid (CGA) were evaluated. However, only CMP was consumed in the MilA reaction. The mass of peak III was consistent with that of HMCMP, and the product identity was further confirmed by one- and two-dimensional ^{1}H and ^{13}C NMR experiments (Figures S2–S4).

To further prove the roles of MilA and MilB in MIL biosynthesis, both enzymes were incubated with CMP. After 1 h a new peak dominated the chromatogram and the presence of a parent ion at *m*/*z* 142 established that it was the expected HMC (peak IV). After 5 h, all the CMP was consumed, and only HMC and a small amount of cytosine (peak II) were present. Thus, the products that are generated in vitro by MilA and MilB are consistent with the structures of the two different metabolites that are biosynthesized in *Sv. rimofaciens*. The initial steps for sequential generation of HM-CMP, HMC and cytosine in *Sv. rimofaciens* ZJU5119 are summarized in Scheme 2. Finally, maximum MilB activity was observed at pH 7.5 and the kinetic parameters for the enzyme with HMCMP as substrate were calculated to be $K_m = 1.4\times10^{-4}$ M, $k_{cat} = 3.1\times10^{-3}\,s^{-1}$ and $k_{cat}/K_m =$

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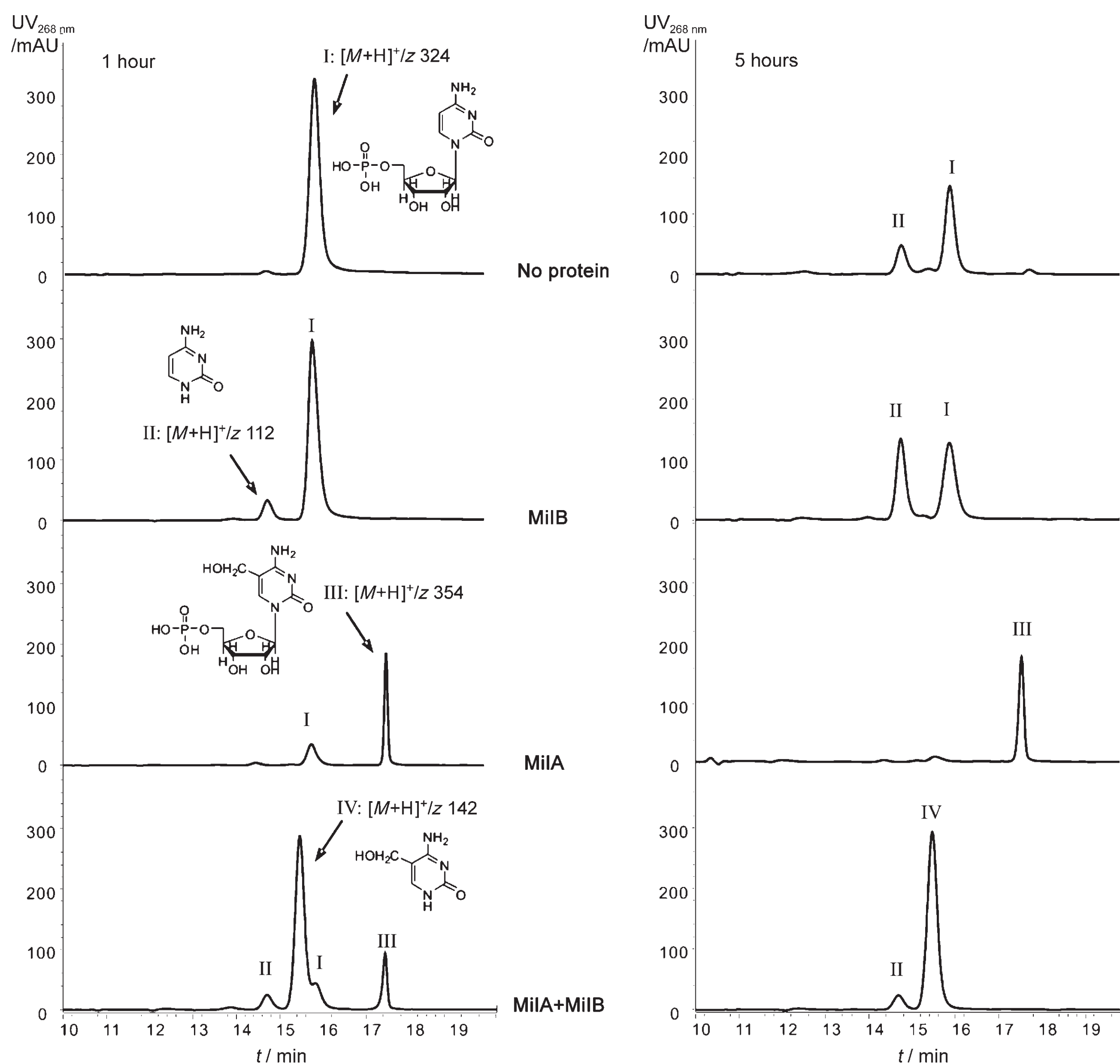

Figure 4. LC–MS analysis of products after 1 h (left) and 5 h (right) of incubation with purified MilA and/or MilB. Peaks I, II, III and IV are CMP, cytosine, HM-CMP and HMC, respectively. The chemical structure of each compound is shown.

22.1 $M^{-1}s^{-1}$ (Figure 5). The kinetic parameters for MilB with CMP as substrate were not measured due to the low level of activity.

Discussion

Previous work by using cell-free extracts of *Sv. rimofaciens* to study the biosynthesis of mildiomycin established that free 5-hydroxymethylcytosine (HMC) was a precursor to MIL and was generated from CMP by the actions of a pyrimidine hydroxymethylase and a nucleotide hydrolase.[13,14] This was consistent with the biosynthesis of the structurally related blasticidin S, which also requires free cytosine as a precursor.[21,29] The free pyrimidine was subsequently shown to originate from the hydrolysis of CMP, which is catalyzed by the nucleotide hydrolase BlsM.[21] We capitalized on the likely sequence similarity of the *Sv. rimofaciens* nucleotide hydrolase gene with the cloned *blsM* gene, and identified a fragment of the *Sv. rimofaciens* chromosome that harbored the *blsM* homologue (*milB*) as well as the gene for the CMP 5-hydroxymethylase (*milA*).

A sequence comparison of MilB and BlsM shows that the nucleotide hydrolases share 54% overall identity in 144 overlapping amino acids with the lowest similarity observed at both ends of the proteins (Figure S5). Notably, there are 22 additional amino acids at the N terminus of MilB, and the C termini share only five identical amino acids over the last 32 residues. A tempting assumption is that one or both ends of MilB dictate the substrate bias for 5-hydroxymethyl-CMP. Deletion of the N terminus and/or the replacement of the C terminus of MilB with the corresponding section of BlsM might shed light

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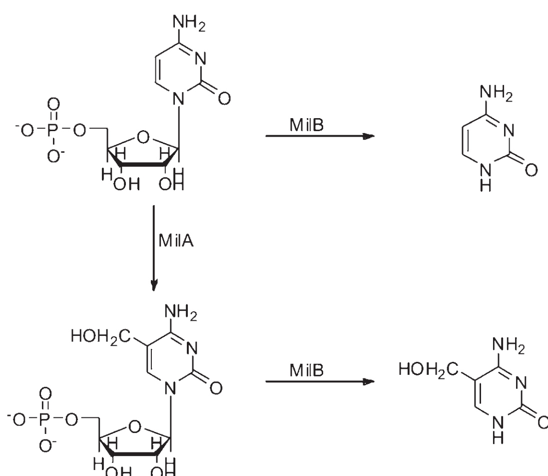

Scheme 2. Proposed steps for generation of HM-CMP, HMC, and cytosine.

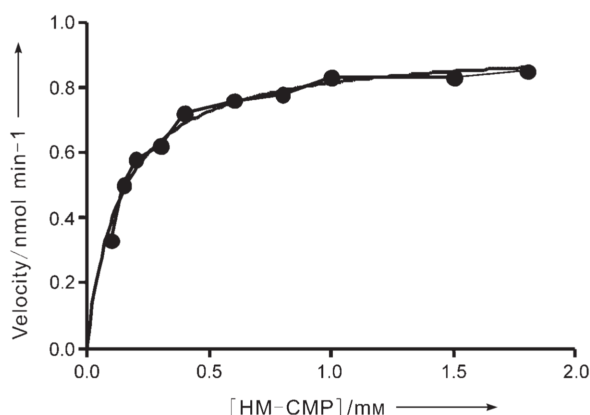

Figure 5. HM-cytosine formation by MilB with HM-CMP as substrate.

on this issue. Certainly, sequence differences throughout the highly conserved region might also play key roles in the specificity for 5-hydroxymethyl-CMP, and we are now identifying which region(s) determine the substrate preference.

It was interesting to find that a new analogue of MIL that lacks the C-5 hydroxymethyl group (dHM-MIL) was produced by wild-type *Sv. rimofaciens*. Interestingly, the ratio of MIL/dHM-MIL was about 3:1 (Figure 3 A) but when MilA and MilB are co-incubated with CMP, the proportion of HMC/cytosine is *about* 9:1 (Figure 4). The ratio in vivo for HMC/cytosine is unknown, but we confirmed that MilB accepts CMP as a poor alternative substrate, and that MilA does not convert free cytosine to HMC. It is possible that the differences in vivo and in vitro arise from subtle regulatory effects (e.g., product inhibition or other conditional differences) that have not yet been revealed by the in vitro studies. Alternatively, intermediates with a cytosine moiety might be more efficiently processed by downstream enzymes in the MIL biosynthesis pathway than those with a HMC group.

MilA was predicted by Pfam to belong to the protein family PF00303, which corresponds to thymidylate synthases (TS) and deoxycytidylate hydroxymethylases (dCMP-HMase).[30] In the presence of CH_2THF and other accessory elements, TS can convert dUMP into the dTMP that is required for DNA synthesis and likewise, dCMP-HMase can convert dCMP into hydroxymethyl-dCMP. Despite the high similarity (*E* value $=7.7\times10^{-05}$) between MilA and PF00303, it was shown here that MilA does not recognize dCMP as a substrate. It will be interesting to know which part of MilA determines the substrate specificity compared with other known PF00303 proteins.

In the blasticidin S pathway, cytosine and UDP-glucuronic acid are coupled to form cytosylglucuronic acid (CGA) by the enzyme CGA synthase (BlsD).[9,21] Sequence analysis and biochemical characterization of the corresponding hydroxymethyl-CGA synthase from *Sv. rimofaciens* should reveal more information on factors that govern substrate selection and differences in substrate processing efficiencies. However, a *blsD* homologue has not been identified, and efforts are currently underway to identify and sequence the complete mildiomycin gene cluster. This information might also reveal additional steps that are shared by the two pathways, and provide insight into various ways that these related pathways might be engineered to arrive at non-natural compounds with improved action against powdery mildew and other fungal diseases that are important in agriculture.

Experimental Section

Culture techniques and genetic manipulations: *Sv. rimofaciens* ZJU5119 (Table 1) and its derivatives were grown at 30 °C on SFM agar plates for sporulation; on R2YE for protoplast transformation or in TSB liquid medium that was supplemented with sucrose (10.3% (*w/v*)) and yeast extract (1% (*w/v*)) for growth of mycelia. Isolation of total DNA, protoplast preparation and transformations were performed according to Kieser et al.[22] Manipulations of *E. coli* strains were conducted according to Sambrook et al.[23] For selection of *Sv. remonfaciens* transformants, the concentrations of spectinomycin and thiostrepton that were used on agar were 150 μg mL^{-1} and 15 μg mL^{-1}, respectively, and in liquid medium concentrations were 50 μg $mL^{-1}$and 5 μg mL^{-1}, respectively. In vivo generation of targeted mutations in *Sv. remonfaciens* was achieved by transformation of unmethylated DNA, which was prepared from *E. coli* ET12567 (Table 1) according to Kieser et al.[22] Restriction enzymes, T4 DNA ligase, Taq polymerase and alkaline phosphatase were purchased from MBI Fermentas (Vilnius, Lithuania).

Construction of a *Sv. rimofaciens* genomic library: For the generation of a cosmid library, high-molecular-weight chromosomal DNA was prepared according to the protocol by Kieser et al., partially digested with MboI, dephosphorylated with calf intestinal alkaline phosphatase (Fermentas), and size-fractionated in low-melting-temperature agarose by using pulsed-field gel electrophoresis (PFGE).[22] The chromosome DNA fragments between 35 and 45 kb were recovered from the low-melting agarose gel that was digested by β-agarase (New England Biolabs). The recovered DNA was ligated with BamHI-digested cosmid vector pJTU2554 (Table 1) in a 1:1 molar ratio. Packaging and transfection into *E. coli* EPI300-T1 (Table 1) was done with λ-packaging mixes that were prepared according to Sambrook et al.[23] The apramycin resistant colonies were picked individually and inoculated in LB (100 μL) that was supplied with apramycin in each well of 20 96-well plates. These plates were grown at 37 °C for 20 h, and then 50% glycerol (100 μL) was added into each well for storage at −80 °C.

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PCR primers, DNA probes and Southern hybridization: The aligned DNA sequence of *blsM* and its homologues revealed five conserved regions (A, B, C, D and E; Figure 1 A) that were used to design six pairs of degenerate PCR primers (B1E1, B1E2, AC, AD, B2C and B2D; Table 2) for use in amplifying fragments of a target nucleotide hydrolase gene from *Sv. rimofaciens* and *S. griseochromogenes* (Table 1). PCR products were purified from agarose gels (0.8%) by using the DNA Gel Extraction Kit (V-gene Biotechnology Ltd.) and subsequently inserted into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing. Based on the sequence of the largest of these fragments, the specific primers CMPH (Table 2) were designed and used to screen the genomic library of *Sv. rimofaciens*.

Each of 20 96-well plates was screened by combining equal aliquots of cells from every well, and this mixture served as a template for PCR. For plates that yielded the desired PCR products, aliquots of cells from each well in a row were combined and screened by PCR. Finally, each well from the positive rows was separately screened to identify individual clones that carry the target gene. For the Southern hybridization experiments, cosmid DNA was cleaved with *Sac*I, separated on a agarose gel (0.8%), and transferred onto Hybond-N$^+$ nylon membrane (Amersham Biosciences). The probe that was produced by PCR from cosmid 1A6 with primers CMPH, was labeled with radioactive α-[^{32}P]-dCTP by using Random Priming Kit (Roche). The Southern blot hybridization was carried out overnight at 65°C followed by high stringency wash (0.1×SSC, 0.1% (*w/v*) SDS) at the same temperature, and detected with a phosphorimager (Fujifilm).

Production and analysis of mildiomycin: For bioassays, the *Streptoverticillium* strains were grown at 30°C for 5 days on SFM plates for production of MIL, agar patches were transferred to potato dextrose agar (PDA, diced potatoes 200 g, glucose 20 g and agar 15 g in 1 L tap water) that contained *Rhodotorula rubra* AS2.166 (Table 1), a fungal strain that is sensitive to MIL. Inhibition zones were observed after 20 h at 30°C. For LC–MS analysis, strains were cultured in (tryptone soya broth powder 30 g, yeast extract 10 g and sucrose 103 g in 1 L distilled water) in baffled flasks at 30°C and at 220 rpm for 6 days. The fermentation broth was harvested, adjusted to pH 5.0, and centrifuged at 12000*g* for 5 min. The supernatant was applied to Supelclean LC-SCX SPE columns (500 mg/3 mL, Supelco, Bellefonte, PA, USA), washed with water (2 mL) then 0.5% NH_4OH (2 mL). The fraction that eluted with 3% NH_4OH was filtered through a membrane (2 μm) before injection. The LC–MS analysis was done by using an Agilent 1100 LC-MSD with a C18 column (TC-C18, 250×4.6 mm, Agilent). The isocratic mobile phase was 10 mM trichloroacetic acid/MeCN (92:8, *v/v*) and the flow rate was 0.3 mL min^{-1} at room temperature. Elution was monitored with a photodiode array detector at 279 nm and electrospray ionization (ESI) MS analysis was carried out in the positive mode.

Sequence analysis: DNA sequencing was done at Shanghai Sangon Ltd. (Shanghai, China), and multiple sequence alignment was performed with BioEdit 7.0. Open-reading frames (ORFs) and ribosome binding sites (RBS) were predicted by using FramePlot 3.0.[24] Similarity comparisons of nucleotide or amino acid sequences against public databases were done by using the BLAST program on the NCBI website (http://ncbi.nlm.nih.gov/blast).[25]

Targeted disruption of *milA* and *milB*: A construct that harbored *milA* and *milB* to be used to generate a gene replacement vector was constructed by using the ReDirect technology.[26] A ca. 10.6 kb *EcoR*72I fragment that contained *milA* and *milB* was recovered from cosmid 1A6 and inserted into the *Sma*I site of pBluescript SK(+) to generate pJTU1796. Digestion with HindIII and XbaI yielded a product that was cloned into the corresponding sites of pJTU412 to give pJTU1797. This plasmid was introduced into *E. coli* BW25113/pIJ790 (Table 1) by electroporation. The *aadA* cassette was amplified by PCR from pIJ779 (Table 1) with the primers PT-milA and PT-milB (Table 2). The cassette was used to replace a 999 bp portion of *milA* in pJTU1797, to give pJTU2952, and was also used to replace a 507 bp section of *milB* in pJTU1797 to yield pJTU2953. Both pJTU2952 and pJTU2953 DNA were individually prepared from *E. coli* ET12567 and separately introduced to *Sv. rimofaciens* by protoplast transformation. Putative double-crossover strains were confirmed by PCR with the primers of PT-milA-T and PT-milB-T (Table 2).

Preparation of *milA* and *milB* expression constructs: The *milA* gene was amplified from cosmid 1A6 by PCR with the primers Ex-milA (Table 2, NcoI and XhoI sites are underlined). The *milB* gene was amplified from the same cosmid by using primers Ex-milB (Table 2, BamHI and XhoI sites are underlined). PCR was carried out with high fidelity DNA polymerase (KOD-Plus, TOYOBO) and the products were gel purified, digested with the appropriate enzymes, and cloned into the corresponding restriction sites of the pET28a (+) vector (Novagen). The resulting plasmids, named pJTU2955 and pJTU2956 were used to transform *E. coli* DH10B for sequencing. The plasmids were introduced to *E. coli* BL21 (DE3) pLysE (Novagen) for expression studies.

Expression and purification of His-tagged MilA and MilB: *E. coli* BL21 (DE3) pLysE cells that carried pJTU2955 or pJTU2956 were grown overnight in LB media that was supplemented with chloramphenicol and kanamycin (34 μg mL^{-1} and 50 μg mL^{-1}, respectively). The seed cultures (10 mL) were used to inoculate 1-L production cultures of LB with the corresponding antibiotics. The cells were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced with isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration, 1 mM). The cultures were then grown for an additional 5 h at 30°C. After centrifugation, the cells were resuspended in binding buffer (40 mL, 20 mM sodium phosphate, 20 mM imidazole, and 0.5 M NaCl, pH 7.4) and lysed by sonication in an ice bath (10×60 s at 15 W with 60 s pauses). After centrifugation (16000*g* for 45 min at 4°C), the supernatant was applied to HisTrap HP column (GE Healthcare) and purified by using an ÄKTA FPLC (GE Healthcare), by eluting with elution buffer (20 mM sodium phosphate, 500 mM imidazole, and 0.5 M NaCl, pH 7.4) in linear gradient. The purified His-tagged MilA and MilB were desalted with HiTrap Desalting column (GE healthcare) and stored in Tris–HCl buffer (50 mM, pH7.5) with glycerol (20%) at −80°C. The expression and purification of His-tagged MilA and MilB were analyzed by 12% SDS-PAGE, and protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad).

In vitro analysis of purified MilA and MilB: The assays of recombinant MilA and MilB were carried out at 37°C for 1 h and 5 h in a total volume of 100 μL that contained Tris–HCl buffer (100 mM, pH 7.5), paraformaldehyde (15 mM), 2-mercaptoethanol (50 mM), tetrahydrofolate (2 mM), CMP (1 mM), $FeSO_4$ (10 mM) and the corresponding His-tagged MilA (70 μg) and/or MilB (50 μg). The reactions were quenched by the addition of trichloroacetic acid. To test the substrate specificity of MilA, dCMP, CDP, CTP, cytosine, cytidine, UMP, dUMP (all from Sigma) and cytosylglucuronic acid (CGA) were evaluated individually at 1 mM final concentration. The product and substrate were analyzed by LC–MS as described above, but by using a mobile phase of NH_4OAc (20 mM), pH 5.5 buffer/MeCN, (98:2, *v/v*) at a flow rate of 0.3 mL min^{-1} at room temperature. The product of the MilA-catalyzed reaction was purified and confirmed by ^{1}H and ^{13}C NMR spectroscopy (400 MHz, Bruker) to be 5′-hydroxy-

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methyl-CMP. ^{1}H NMR (D_2O, 400 MHz): $\delta = 8.30$ (s, H-6), 4.62 (s, H-7), 6.02 (d, $J = 1.4$ Hz; H-1′), 4.43 (m, H-2′), 4.28 (m, H-3′), 4.16 (m, H-4′), 4.38 (m, H-5′); ^{13}C NMR (D_2O, 100 MHz): $\delta = 151.9$ (s, C-2), 161.8 (s, C-4), 108.9 (s, C-5), 145.2 (d, C-6), 59.4 (t, C-7), 92.5 (d, C-1′), 71.6 (d, C-2′), 71.9 (d, C-3′), 86.1 (d, C-4′), 77.1 (t, C-5′). A summary for the correlation is shown in Table S1.

The kinetic parameters and pH optimum for MilB were measured in Tris–HCl buffers (pH 6.8–8.8) and assays were conducted in a total volume of 100 μL that contained MilB (5 μM), HMCMP (0.1–1.8 mM), and Tris–HCl buffer (50 mM). Reactions were incubated for 30 min at 37 °C and quenched by boiling for 10 min. Precipitated protein was removed, and each reaction was analyzed by HPLC. The quantity of the substrate and product was determined by measuring and converting its peak area. Prism5 software (GraphPad Software, Inc.) was used to calculate kinetic parameters.

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[1] T. Iwasa, K. Suetomi, T. Kusaka, *J. Antibiot.* **1978**, *31*, 511–518.
[2] S. Harada, T. Kishi, *J. Antibiot.* **1978**, *31*, 519–524.
[3] E. Feduchi, M. Cosin, L. Carrasco, *J. Antibiot.* **1985**, *38*, 415–419.
[4] E. M. S. Harada, T. Kishi, *J. Am. Chem. Soc.* **1978**, *100*, 4895–4897.
[5] K. Isono, *J. Antibiot.* **1988**, *41*, 1711–1739.
[6] R. A. Lacalle, J. A. Tercero, A. Jimenez, *EMBO J.* **1992**, *11*, 785–792.
[7] C. Bormann, V. Mohrle, C. Bruntner, *J. Bacteriol.* **1996**, *178*, 1216–1218.
[8] M. A. Fernandez-Moreno, C. Vallin, F. Malpartida, *J. Bacteriol.* **1997**, *179*, 6929–6936.
[9] M. C. Cone, X. Yin, L. L. Grochowski, M. R. Parker, T. M. Zabriskie, *ChemBioChem* **2003**, *4*, 821–828.
[10] S. H. Larsen, D. M. Berry, J. W. Paschal, J. M. Gilliam, *J. Antibiot.* **1989**, *42*, 470–471.
[11] G. R. Wyatt, S. S. Cohen, *Biochem. J.* **1953**, *55*, 774–782.
[12] J. G. Flaks, S. S. Cohen, *Biochim. Biophys. Acta* **1957**, *25*, 667–678.
[13] H. Sawada, T. Suzuki, S. Akiyama, Y. Nakao, *J. Ferment. Technol.* **1985**, *63*, 17–21.
[14] H. Sawada, T. Suzuki, S. Akiyama, Y. Nakao, *J. Ferment. Technol.* **1985**, *63*, 23–27.
[15] H. Sawada, K. Katamoto, T. Suzuki, S. Akiyama, Y. Nakao, *J. Ferment. Technol.* **1984**, *62*, 537–543.
[16] K. Kishimoto, Y. S. Park, M. Okabe, S. Akiyama, *J. Antibiot.* **1996**, *49*, 770–774.
[17] K. Kishimoto, Y. S. Park, M. Okabe, S. Akiyama, *J. Antibiot.* **1997**, *50*, 206–211.
[18] "Blasticidin S and Related Peptidyl Nucleoside Antibiotics", S. J. Gould in *Biotechnology of Antibiotics*, 2nd ed. (Ed.: W. R. Strohl), Marcel Dekker, New York, **1997**, 703–731.
[19] S. Tashiro, N. Sugita, T. Iwasa, H. Sawada, *Agric. Biol. Chem.* **1984**, *48*, 881–885.
[20] S. J. Gould, J. Guo, *J. Bacteriol.* **1994**, *176*, 1282–1286.
[21] L. L. Grochowski, T. M. Zabriskie, *ChemBioChem* **2006**, *7*, 957–964.
[22] T. Kieser, M. J. Bibb, K. F. Chater, M. J. Butter, D. A. Hopwood, *Practical Streptomyces Genetics. A Laboratory Manual*, John Innes Foundation, Norwich, **2000**.
[23] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York, **1989**.
[24] J. Ishikawa, K. Hotta, *FEMS Microbiol. Lett.* **1999**, *174*, 251–253.
[25] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res.* **1997**, *25*, 3389–3402.
[26] B. Gust, G. L. Challis, K. Fowler, T. Kieser, K. F. Chater, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1541–1546.
[27] T. M. Rose, E. R. Schultz, J. G. Henikoff, S. Pietrokovski, C. M. McCallum, S. Henikoff, *Nucleic Acids Res.* **1998**, *26*, 1628–1635.
[28] P. Ross, F. O'Gara, S. Condon, *Appl. Environ. Microbiol.* **1990**, *56*, 2156–2163.
[29] H. Seto, H. Yonehara, *J. Antibiot.* **1977**, *30*, 1019–1021.
[30] R. D. Finn, J. Mistry, B. Schuster-Bockler, S. Griffiths-Jones, V. Hollich, T. Lassmann, S. Moxon, M. Marshall, A. Khanna, R. Durbin, S. R. Eddy, E. L. Sonnhammer, A. Bateman, *Nucleic Acids Res.* **2006**, *34*, D247–D257.
[31] M. S. Paget, L. Chamberlin, A. Atrih, S. J. Foster, M. J. Buttner, *J. Bacteriol.* **1999**, *181*, 204–211.

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