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A Facile Method for Site-specific Gene Integration in *Lysobacter enzymogenes* for Yield Improvement of the Anti-MRSA Antibiotics WAP-8294A and the Antifungal Antibiotic HSAF

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Abstract

Lysobacter is a genus of Gram -negative gliding bacteria that are emerged as novel biocontrol agents and new sources of bioactive natural products. The bacteria are naturally resistant to many antibiotics commonly used in transformant selection, which has hampered the genetic manipulations. Here, we described a facile method for quick -and-easy identification of the target transformants from a large population of the wild type and non-target transformants. The method is based on a distinct yellow-to-black color change as a visual selection marker for site-specific integration of the gene of interest. Through transposon random mutagenesis, we identified a black-colored strain from the yellow-colored *L. enzymogenes*. The black strain was resulted from a disruption of *hmgA*, a gene required for tyrosine /phenylalanine metabolism. The disruption of *hmgA* led to accumulation of dark brown pigments. As proof of principle, we constructed a series of expression vectors for a regulator gene found within the WAP-8294A biosynthetic gene cluster. The yield of WAP-8294A in the black strains increased by 2 fold compared to the wild type. Interestingly, the yield of another antibiotic (HSAF) increased up to 7 fold in the black strains. WAP-8294A is a family of potent anti-MRSA antibiotics and is currently in clinical studies, and HSAF is an antifungal compound with distinct structural features and a novel mode of action. This work represents the first successful metabolic engineering in *Lysobacter*. The development of this facile method opens a way toward manipulating antibiotic production in the largely unexplored sources.

Keywords

Lysobacter; anti-MRSA antibiotics; WAP -8294A; nonribosomal peptides; metabolic engineering; site-specific gene integration

The genus *Lysobacter* is one of the most ubiquitous environmental microorganisms, belonging to the Xanthomonadaceae family within the gamma proteobacteria(1 -3). These Gram-negative gliding bacteria are drawing increased attention as novel biocontrol agents against pathogens of crop plants. For example, *L. enzymogenes* exhibits potent antifungal activity against a broad spectrum of pathogens, including *Magnaporthe poae* that causes Kentucky bluegrass summer patch, *Pythium ultimum* that causes sugar beet damping-off,

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Puccinia graminis that causes cereal crop rusts, *Uromyces appendiculatus* that causes bean plant rust, *Bipolaris sorokiniana* that causes turf grass leaf spots, and *Rhizoctonia solani* that causes brown patch on a wide range of plants (4–6). Our previous studies showed that *L. enzymogenes* produces a broad spectrum antifungal compound, HSAF (dihydromaltophilin), which is the critical factor of *L. enzymogenes* as a biocontrol agent against plant fungal diseases (7–10). In addition to the broad activities against fungal diseases, *Lysobacter* is emerging as a new source of bioactive natural products (11, 12). Several species of the genus are known to produce potent antibiotics, including the cyclic peptide lysobactin (13–15) and tripropeptins (16, 17), the cephem-type β -lactam antibiotic cephabacins (18–20), and the cyclic lipodepsi peptides WAP-8294A (21–24). Among them, the WAP-8294A family is particularly interesting because they exhibit a very potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA), and one member of the family, WAP-8294A2, is now in clinical studies (25). However, WAP-8294A compounds are produced by *Lysobacter* only under certain conditions with a very low yield (24). This is a bottleneck for structure-activity relationship studies and clinical tests.

As a relatively underexplored group of microorganisms, *Lysobacter* do not have many well developed genetic tools for gene manipulation. These bacteria are naturally resistant to most of the antibiotics commonly used in genetic selections, including ampicillin, kanamycin, streptomycin, tetracycline, and rifampin. This feature renders most of the selection marker genes useless when screening for *Lysobacter* transformants. Currently, gentamicin and chloramphenicol are the few antibiotics that can be used in *Lysobacter* transformant selection. Furthermore, no autonomous vector has been identified for *Lysobacter*, and chromosomal integration through homologous recombination is the only way for genetic engineering in these bacteria. A limiting step of this process is the initial selection of the putative transformants from a large number of colonies. Thus a facile method for quick-and-easy identifying the putative transformants is highly desirable in order to explore the potential of these prolific producers of antibiotics.

Here, we describe such a facile method for transformant identification. The method is based on a “yellow-to-black” color change for a visual selection of the putative transformants. We demonstrated the utility of this method through a site-specific integration of plasmid constructs that would express ORF8, a putative regulator gene found in the WAP-8294A gene cluster (24). ORF8 was predicted to encode a TonB-dependent receptor, which was also found in the HSAF gene cluster (26). Our results showed that the yield of WAP-8294A and HSAF in the ORF8-expressing strains increased 2 and 7 fold, respectively, compared to the wild type strain. This result not only demonstrated the usefulness of this new genetic tool, but also confirmed the positive regulatory role of the TonB-dependent receptor in the production of antibiotics in *Lysobacter*. The work also represents the first successful metabolic engineering of antibiotics in a *Lysobacter* strain.

RESULTS AND DISCUSSION

Identification and Characterization of the “Black Mutant” Generated by Transposon Random Mutagenesis

One of the characteristic features of *L. enzymogenes* OH11 is the yellowish appearance. Through transposon random mutagenesis (27, 28), we obtained a black (dark brown) mutant of the wild type strain. The subsequent sequencing of the transposon insertion site showed that the transposon was inserted into the *hmgA* gene, which is predicted to encode homogentisate 1, 2-dioxygenase. This dioxygenase catalyzes the oxidative cleavage of the aromatic ring of tyrosine/phenylalanine, which is a key step in aromatic amino acid degradation pathway (29, 30) (Figure 1). The disruption of *hmgA* gene blocks the oxidative cleavage reaction that leads to the accumulation of homogentisate. Further oxidations of

homogentisate produce ochronotic pigments and render the black color in the organisms (29, 30).

To verify this *hmgA* gene is responsible for the yellow-to-black color switch, we amplified a 633-bp fragment with in *hmgA* and cloned it into pJQ200-SK(31), which generated the construct pHmgA. This construct was then introduced into *L. enzymogenes* OH11 by conjugation. The colonies in black color were picked up for PCR verification. The results showed that all the black colonies resulted from a disruption of the *hmgA* gene through homologous recombination between the pHmgA and the chromosomal copy of *hmgA* gene (Figure 1). As a control, the introduction of pJQ200-SK vector alone only produced yellow colonies. The results confirmed that *hmgA* is responsible for the color change. The black colonies did not show any obvious difference from the wild type in growth rate (approximately 1.2–1.4 of OD_{600nm} in 24 hours) and other phenotypes. Thus, this pHmgA vector could be used for site-specific gene integration in *L. enzymogenes*, to facilitate a visual screening of putative transformants.

Construction of Broadly Adaptable Site-specific Gene Integration Vectors

To demonstrate the utility of this quick-and-easy screening method, we constructed a series of vectors that are expected to express the target gene in *Lysobacter*. To do this, we first need to put a promoter into the pHmgA vector. Our previous study had indicated that the 5'-untranslated region of the key HSAF biosynthetic gene, encoding a polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS), could be a good candidate(26). RT-PCR results showed that the PKS/NRPS gene was highly transcribed in the first three days of culture when the wild type strain was grown in 1/10 TSB medium, and the change of the transcription levels was consistent with the trend of HSAF production in the cultures. The bioinformatics analysis of the 5'-untranslated region suggested that this region may contain a putative promoter and a putative ribosomal binding site(Shine-Dalgarno sequence)(32). We subsequently amplified the 538-bp fragment and placed it behind *hmgA* in pHmgA vector, to generate pHmgA -P1 (Figure 2). This vector contains the natural SD sequence, AATGGAAAAAGAAA. In addition, we also generated a series of derivative vectors, with a modified SD sequence. pHmgA-P2 contains a shorter SD sequence, AATGGAAAAAG; pHmgA-P3 contains an even shorter SD sequence, AATGGAAA; pHmgA-P4 contains the putative SD sequence present in the 5'-untranslated region of ORF8, in addition to the promoter sequence of pHmgA -P1 (Figure 2). After sequencing the four vectors to confirm their identity, we individually transferred them into the wild type *L. enzymogenes* OH11. On the selection plates, approximately fifty percent of single colonies showed the black color. The phenotype is consistent with the expectation, because the se vectors, pHmgA -P1/P2/P3/P4, contain two homologous fragments, the *hmgA* fragment and the HSAF promoter fragment. Only the mutants resulted from the homologous recombination at the *hmgA* fragment would give the black color phenotype. The result showed all of the four vectors could be used for color screening.

Production of the Anti-MRSA WAP-8294A2 in *Lysobacter enzymogenes* Expressing a TonB-dependent Receptor

pHmgA -P1/P2/P3/P4 were constructed to express any target gene when it is placed under the control of the promoter sequence. To test the utility of the vectors and the color-based screening method, we placed the ORF8 gene of the WAP biosynthetic gene cluster behind the promoter in these vectors. ORF8 was predicted to encode a TonB-dependent receptor that positively regulates the production of WAP-8294A compounds (24). The gene was inserted behind the promoter of the pHmgA -P vectors to form pHmgA-P1-WO8, pHmgA-P2-WO8, pHmgA-P3-WO8, and pHmgA-P4-WO8, respectively (Figure 3). The vectors were introduced individually into the wild type through conjugation. Because there are three

homologous fragments in each of the constructs, the vectors could be integrated into three potential sites. Mutant strain M1 (one black colony from approximately 60 yellow colonies) was obtained from the *hmgA* site integration of pHmgA-P1-WO8, strain M2 (one black colony from approximately 120 yellow colonies) from pHmgA -P2-WO8, strain M3 (two black colonies from approximately 60 yellow colonies) from pHmgA-P3-WO8, and strain M4 (one black colony from approximately 60 yellow colonies) from pHmgA-P4-WO8.

These black strains were analyzed for the production of WAP-8294A2. As a control, WAP-8294A2 produced in the wild type was first analyzed by HPLC. This compound had a retention time of 14.9 min (Figure 3). The strain containing HmgA-alone disruption (MH) produced a similar amount of WAP-8294A2 as the wild type. This indicates that the gene integration into the *hmgA* site does not impact the production of this antibiotic. The strain (M1) containing ORF8 under the control of the natural HSAF promoter, pHmgA -P1-WO8, produced 1.9-fold more WAP-8294A2 than the wild type (Figure 3). The strains (M2, M3, and M4) containing ORF8 under the control of a modified promoter also produced a slightly higher yield (1.2, 1.1, and 1.5 fold, respectively) than the wild type. The identity of WAP-8294A2 was confirmed by mass spectrometry and comparison with the standard compound. The results suggested that, among the tested, the natural HSAF promoter probably has the best effect on ORF8 gene expression in these *Lysobacter* strains and any modification, a shorter or extra SD sequence, would lead to an adversary effect.

WAP-8294A Yield in Strains Expressing pHmgA-P1-WO8 with a Putative Terminator

Since the strain containing ORF8 under the control of the natural HSAF promoter gave the best yield of WAP-8294A2, further optimization was based on this construct, pHmgA-P1-WO8. The DNA sequence at the end of a transcription unit could potentially contain controlling elements, such as a terminator, that are important for an optimized transcription. We subsequently generated two pHmgA-P1-WO8 derivative constructs, pHmgA-P1-WO8-T1150 and pHmgA-P1-WO8-T591, which contain a 1150-bp and 591-bp fragment, respectively, originated from the 3'-untranslated region of the HSAF biosynthetic genes. Each of the fragments was inserted immediately behind the ORF8 gene (Figure 4). The black colored strains resulted from the integration of the constructs, M5 (one black colony from approximately 80 yellow colonies) from pHmgA-P1-WO8-T1150 and M6 (three black colonies from approximately 80 yellow colonies) from pHmgA-P1-WO8-T591, were analyzed for WAP-8294A2 production. Strain M5 produced 1.9 times more than the wild type, while strain M6 produced 2.0 times more than the wild type (Figure 4).

HSAF Production in the ORF8-expressing Strains

A TonB-dependent receptor is also present in the HSAF biosynthesis gene cluster (26). To test whether the expression of the TonB-dependent receptor of the WAP biosynthetic gene cluster could also affect the production of HSAF, we analyzed the yield of HSAF in the ORF8-expressing strains. The wild type produced HSAF and its analog alteramide A (33), with the retention time of 21.5 and 21.8 min, respectively (Figure 5). Interestingly, the HmgA-alone disruption strain MH produced 2-fold more HSAF than the wild type. The ORF8-expressing strain pHmgA-P1-WO8 (M1) produced a significantly higher amount of HSAF, increased 6.1-fold, than the wild type. The ORF8-expressing strains with a modified SD sequence (M2, M3, and M4) had 3.1, 1.7, and 2.6-fold more HSAF. Adding the 1150-bp putative terminator to the ORF8-expressing cassette resulted in an improved HSAF production, increased 7-fold in M5, but adding the 591-bp terminator (M6) did not have the same effect. The yield of HSAF in strain M6 was 5.2-fold of that in the wild type.

Summary and Concluding Remarks

The yellow-to-black color change provides a simple screening method for putative transformants. Recently, a color change-based method was also used to integrate genes into the fungus *Aspergillus nidulans*, which led to the discovery of cryptic polyketide metabolites (34). The pigment accumulation in *Lysobacter* did not have any obvious adversary effect on the organism. This is evident as the black colored strains showed the similar optical density as the yellow strains in the cultures and the engineered strains produced an increased amount of WAP-8294A2 and HSAF as expected. Thus, the *hmgA* locus provides a new means for site-specific gene integration in *Lysobacter*. Because of the naturally occurred resistance to many common antibiotics used in genetic selections, there are very few selection markers that can be used in genetic manipulation in *Lysobacter*. Our method is a visual screening that does not rely on an antibiotic selection marker gene. The color change in the colonies is very clear and distinct, which enables an easy identification of the target transformants even when the majority of the colonies are yellow (the wild type or transformants resulted from a random insertion of the construct). We demonstrated the utility of this method by constructing a series of vectors that express a regulator gene for the biosynthesis of WAP-8294A, a family of potent anti-MRSA antibiotics that are in clinical studies. In this proof of principle work, we were able to increase the yield of WAP-8294A2 by 2 times in strains that contain the regulator gene under the control of a promoter found in *Lysobacter*. In addition, we serendipitously found that the expression of the WAP TonB-dependent receptor led to a significant yield increase of another antibiotic, HSAF, which is produced by the same organism. One of the strains produced up to 7-fold more HSAF than the wild type. This finding suggests that the TonB-dependent regulatory mechanism may crosstalk between the WAP and the HSAF biosynthetic systems, as both gene clusters contain such a regulatory gene (24, 26).

Although species in *Lysobacter* exhibit a great potential as new sources of bioactive compounds, many of the compounds are produced only under very special conditions and often in a very low yield. The development of this facile screening method and the construction of the expression vectors open a way to ward solving these problems. These vectors contain varied promoters and multiple cloning sites, which facilitates further manipulations, such as the replacement with new promoters as well as new target genes.

METHODS

Bacterial Strains, Plasmids and General DNA Manipulations

Escherichia coli DH5 α strain was used as the host for general DNA propagations. *E. coli* S17-1 was used as the strain for conjugation. *L. enzymogenes* and other bacterial strains were grown in Luria Bertani (LB) broth or 1/10 strength tryptic soy broth (1/10 TSB, Sigma). Genomic DNA of *L. enzymogenes* was prepared as previously described (5). Plasmid mini-prep and DNA gel extraction were performed using kits from Qiagen. PCR primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). All other manipulations were carried out according to standard methods.

Construction of *hmgA* Site-specific Integration Vectors and Screening for *hmgA* Mutant

To construct the plasmid vectors for *hmgA* site-specific integration, a 633-bp internal fragment was amplified, using the primer pairs HmgA-up and HmgA-down (Table 1), from the genomic DNA of the wild type *L. enzymogenes* OH11. The fragment was digested with *SacI*/*SpeI* and cloned into the site of the conjugation vector pJQ200SK to produce pHmgA. This construct was transferred into *E. coli* S17-1, which was mated with *L. enzymogenes* OH11 for conjugal transfer of pHmgA. The black colonies grown on LB plates containing gentamicin (20 μ g/ml) were picked up and inoculated into liquid cultures containing

gentamicin. Genomic DNA was prepared from each of the black-colored cultures, and diagnostic PCR were performed to verify the mutants that resulted from homologous recombination.

Construction of Promoter-containing pHmgA-P Vectors

The promoter of the PKS/NRPS gene in HSAF biosynthetic gene cluster was amplified from the genomic DNA of the wild type strain. Three pairs of primers were used to generate three versions of promoter differing at the SD sequence. Primers p1-up and p1-down, p2-up and p2-down, and p3-up and p3-down (Table 1), were used to amplify the promoters to produce pHmgA -P1, pHmgA-P2, and pHmgA-P3, respectively. The PCR fragments were digested with *SpeI/Bam* HI and inserted into the same sites of vector pHmgA. This placed the promoter right behind the *hmgA* gene in pHmgA vector. The constructs were validated by DNA sequencing and transferred into *L. enzymogenes* OH11 by conjugation. The black colored colonies were selected and verified by diagnostic PCR.

Construction of WAP-ORF8 Expression Vectors

The coding region of WAP -ORF8 was amplified, using primers WO8-up and WO8-down (Table 1), from the genomic DNA of the wild type strain. The PCR product was digested with *Bam*HI/*Xho*I and cloned into the same sites of the vectors pHmgA -P1, pHmgA-P2, and pHmgA-P3, respectively, to generate pHmgA-P1-WO8, pHmgA-P2-WO8, and pHmgA-P3-WO8. The PCR product amplified by primers WO8 -p4-up and WO8-p4-down was used to construct pHmgA-P4-WO8, which was generated by ligating the PCR fragment with the *Bam* HI/*Xho*I digested vector pHmgA -P1. In each the constructs, the coding region of ORF8 was placed right behind the respective promoter. To add the putative terminator into the expression constructs, primers T591-up and T591-down and primers T1150 -up and T1150-down were used to amplify a 591-bp and 1150-bp fragments, respectively, from the genomic DNA. The fragments were digested with *Xho*I/*Apa*I and cloned into the same sites of pHmgA -P1-WO8, to produce pHmgA-P1-WO8-T591 and pHmgA-P1-WO8-T1150. The constructs were validated and transferred into *L. enzymogenes* OH11 by conjugation. The black colored colonies were selected and verified by diagnostic PCR.

Extraction and Analysis of WAP-8294A

Various strains of *L. enzymogenes* OH11 were grown in 1/10 strength TSB medium for 24 hours, and an aliquot of 2 ml was transferred to a 250 ml sterile flask containing 50 ml of fermentation medium (2.5% glucose, 2% soybean, 0.4% soybean oil, 0.25% NaCl, 0.5% CaCO₃, pH 7.2). The culture was incubated at 28°C for 3 days with shaking at 200 rpm. To extract WAP compounds, the 50 ml culture broth was centrifuged, and the pH of the supernatant was adjusted to 2.5 with 1N HCl. The treated supernatant was then extracted with n-butanol/ethyl acetate (1/1, vol) containing 0.05% TFA. The organic phase was dried using a Rotavapor, and the residues were re-dissolved in 2 ml methanol containing 0.05% TFA. A 50 µl aliquot of each extracts was analyzed by HPLC (Agilent, 1220 Infinity LC) using a reversed-phase column (Cosmosil, 4.6 ID × 250 mm). Water/0.05% TFA (solvent A) and acetonitrile/methanol (1/1, vol)/0.05% TFA (solvent B) were used as the mobile phases with a flow rate of 1.0 ml/min. HPLC program was as follows: 57% B in A in the first 5 min, 57–100% Bin 5–32 min, 100% B in 32–40 min, back to 57% B at 41 min and maintained to 48 min. The metabolites were detected at 280 nm on a UV detector. LCQ-MS was used to verify the mass of the peak of WAP-8294A2 and analogs

Extraction and Analysis of HSAF

Various strains of *L. enzymogenes* OH11 were grown in 1/10 strength TSB medium for 24 hours, and an aliquot of 2 ml was transferred to a 250 ml sterile flask containing 50ml of

fresh 1/10 strength TSB medium. The culture was incubated at 28°C for 3 days with shaking at 200 rpm. To extract HSAF, the 50 ml culture broth was centrifuged, and the pH of supernatant was adjusted to 2.5 with 1N HCl. The treated supernatant was then extracted with n-butanol/ethyl acetate (1/1, vol) containing 0.05% TFA. The organic phase was dried using a Rotavapor, and the residues were re-dissolved in 2 ml methanol containing 0.05% TFA. A 50 µl aliquot of each extracts was analyzed by HPLC (Agilent, 1220 Infinity LC) using a reversed-phase column (phenomenex, 150 ×4.6 mm, 3µm). Water/0.025% TFA (solvent A) and acetonitrile/0.025% TFA (solvent B) were used as the mobile phases with a flow rate of 1.0 ml/min. HPLC program was as follows: 5–25% B in A in the first 0–10 min, increased to 80% B at 25 min, to 100% at 26 min, and back to 5% B at 30 min. The metabolites were detected at 280 nm on a UV detector. LCQ-MS was used to verify the mass of the peak of HSAF and analogs.

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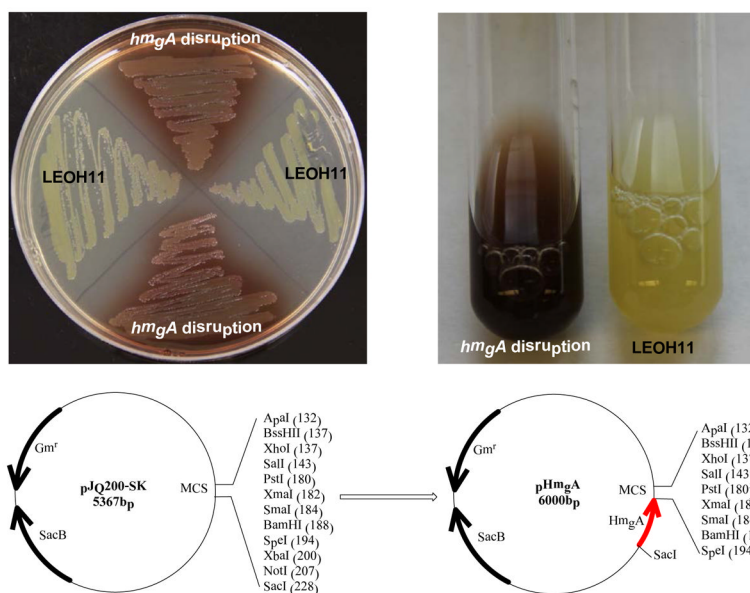


Figure 1. “Yellow-to-black” color change of *Lysobacter enzymogenes* OH11. The solid and liquid cultures of the wild type strain (LEOH11) and the *hmgA* integrated strains are shown at top, and the map of the plasmid construct containing the 633-bp fragment within *hmgA* that is cloned into pJQ200-SK is shown at bottom.

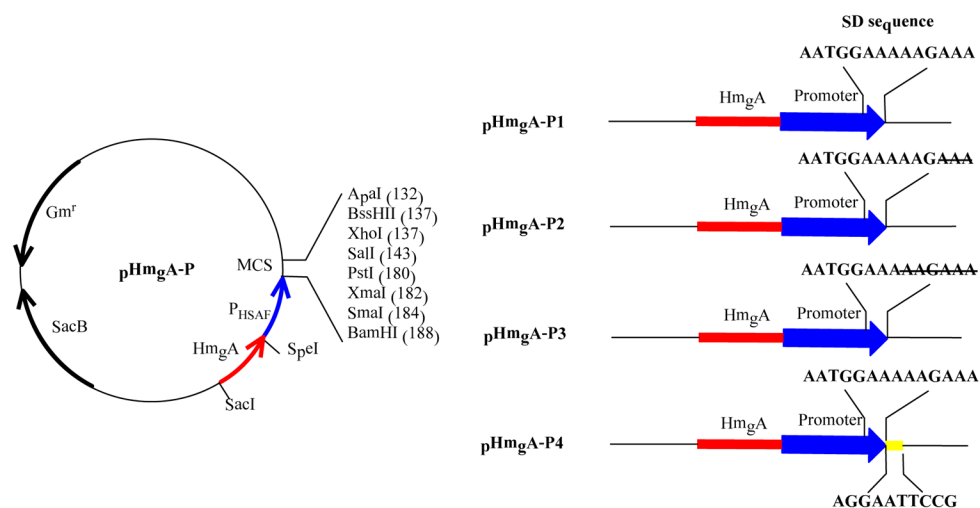
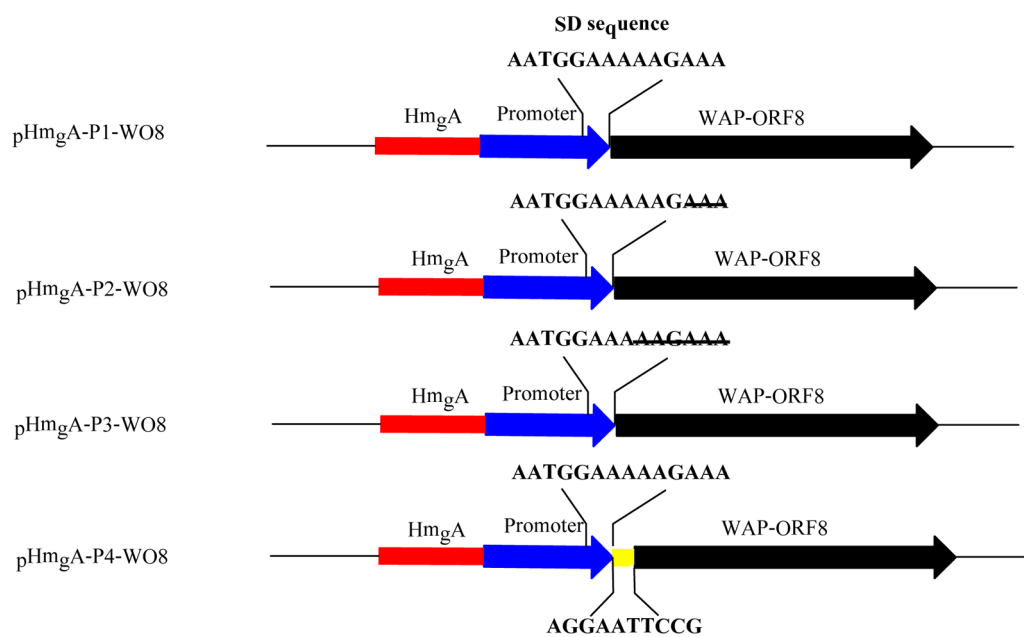
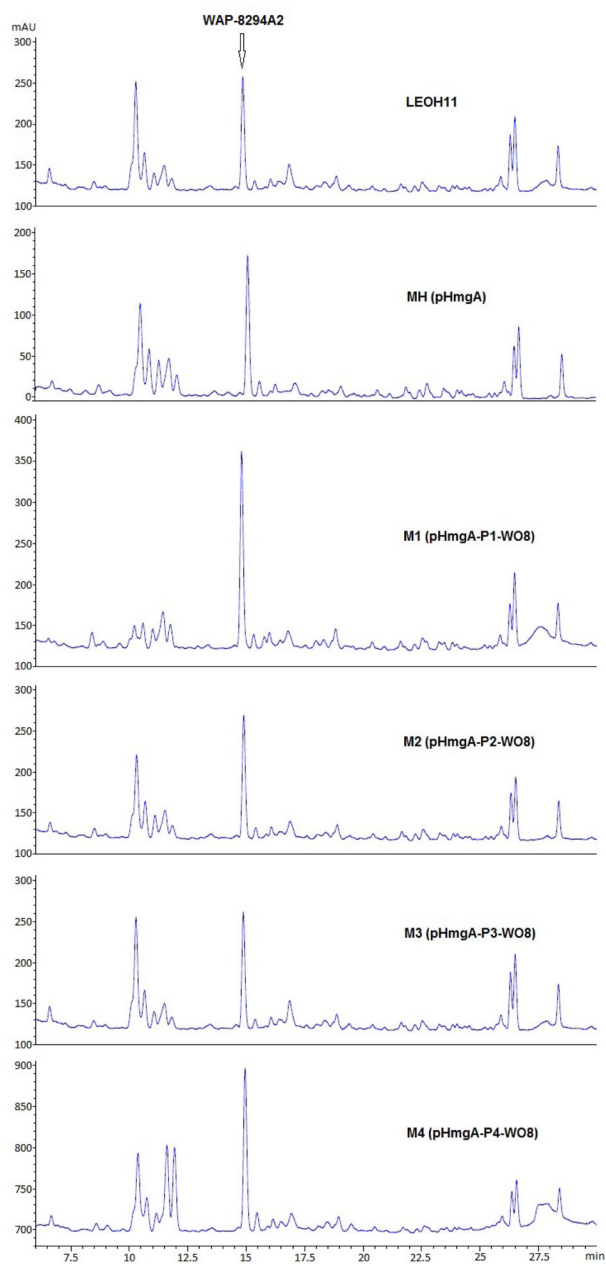


Figure 2. Construction of broadly adaptable vectors for site-specific gene integration in *Lysobacter*. The 538-bp promoter (P_{HSAF}), from the highly expressed HSAF biosynthetic gene cluster (26), was placed immediately behind *hmgA* in pHmgA vector, to generate pHmgA -P1. This vector contains the natural SD sequence, AATGGAAAAAGAAA. Three pHmgA-P1 derived vectors, with a modified SD sequence, are also shown. Note that pHmgA-P4 contains the putative SD sequence of ORF8(24), in addition to the promoter sequence of pHmgA -P1.





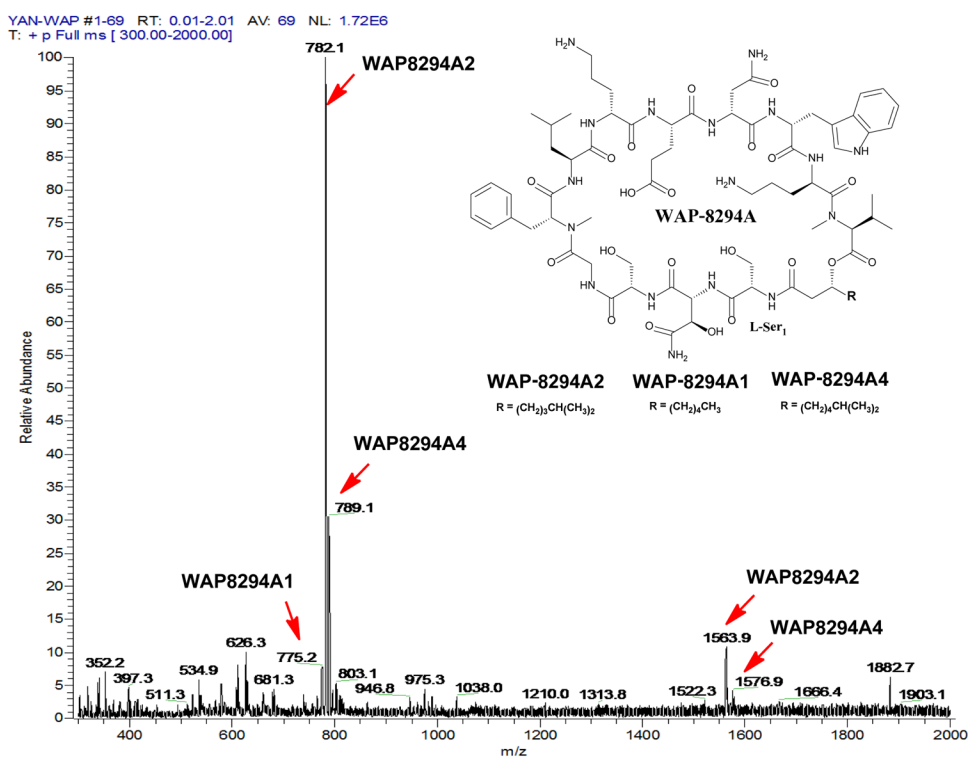


Figure 3.

Construction of ORF8 expression vectors and integration of the constructs into the color-switching *hmgA* site of *Lysobacter enzymogenes* OH11 to improve the yield of the anti-MRSA antibiotics WAP-8294A. ORF8, the TonB-dependent receptor gene in the WAP-8294A biosynthetic gene cluster (24), was placed behind the promoter of vectors pHmgA-P1/P2/P3/P4, to form pHmgA-P1-WO8 (strain M1), pHmgA-P2-WO8 (strain M2), pHmgA-P3-WO8 (strain M3), and pHmgA-P4-WO8 (strain M4), respectively. Black colonies (strain M1-M4) resulted from each of the constructs were selected, verified, cultured, and analyzed by HPLC and MS for WAP-8294A production. The wild type (LEOH11) and strain MH (pHmgA-alone integration) were used as controls.

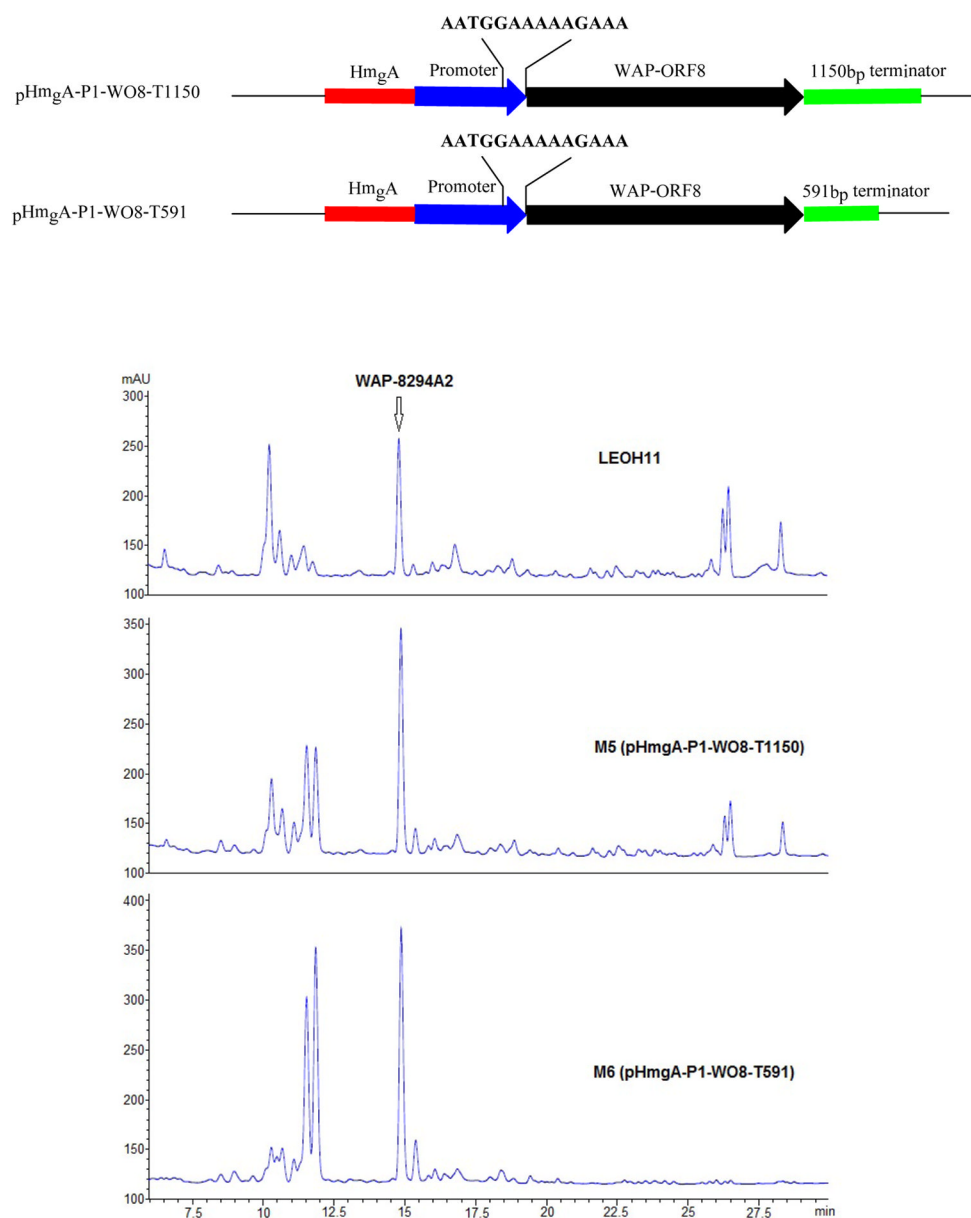
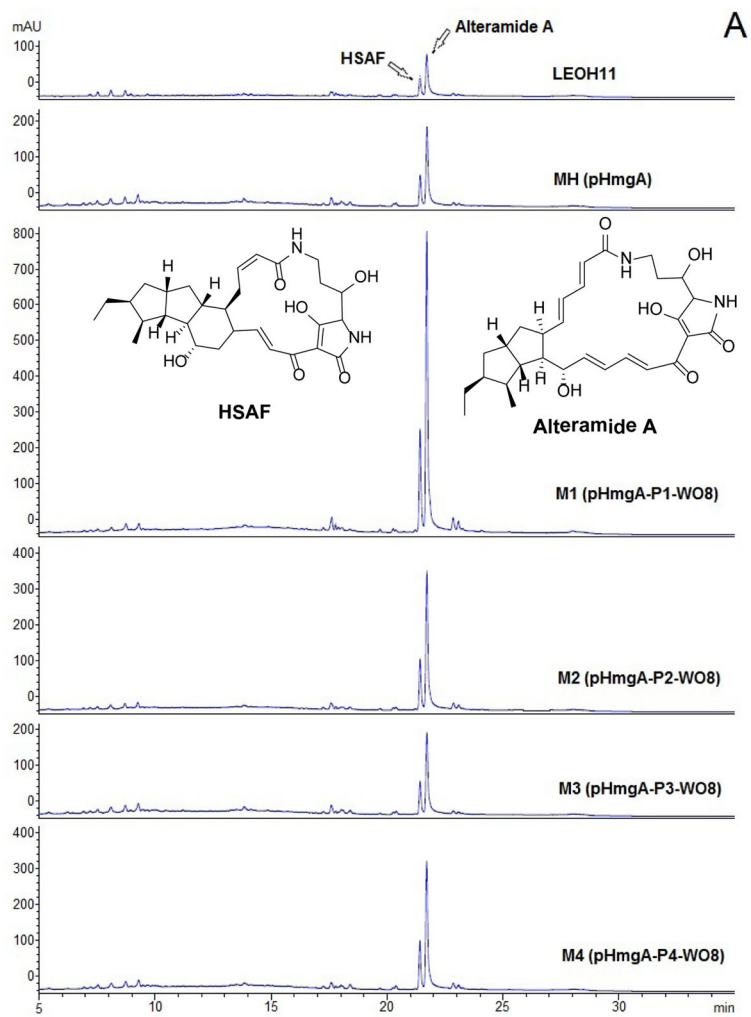


Figure 4. Optimization of ORF8 expression in *Lysobacter enzymogenes* OH11 to increase the production of WAP-8294A. pHmgA-P1-WO8-T1150 (strain M5) and pHmgA-P1-WO8-T591 (strain M6) were derived from pHmgA-P1-WO8 by inserting a 1150-bp and 591-bp fragment, respectively, of the 3'-untranslated region of the HSAF biosynthetic genes behind the ORF8 gene. The black colored strains resulted from the integration of the constructs into the *hmgA* site were analyzed for WAP-8294A production using HPLC.



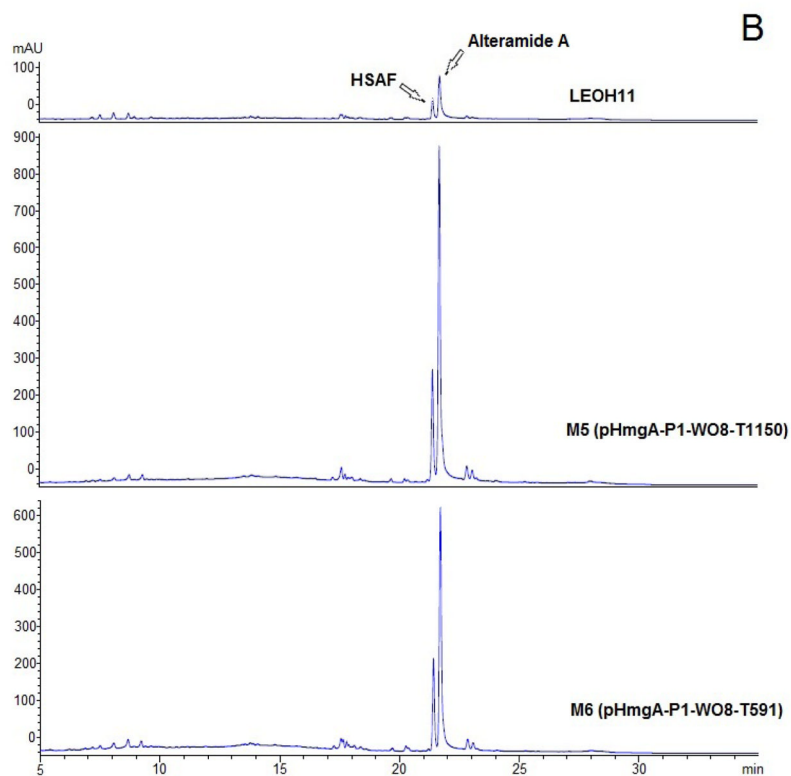


Figure 5. Production of HSAF and its analog, alteramide A, in the ORF8 expression strains M1–M4 (A) and M5–M6 (B). The wild type (LEOH11) and strain MH (pHmgA-alone integration) were used as controls.

Table 1

The sequences of primers used in this study

Primer	Sequence
HmgA-up	<u>CGAGCTCA</u> ACGACTTCGGCGACGGC
HmgA-down	GGACTAGT <u>GATCGACGGGTCGGGATG</u>
p1-up	<u>CGGGATCC</u> TTTTTCCATTATCTTTCAG
p1-down	GGACTAGTCTGGGTGACTCTGCATGGA
p2-up	<u>CGGGATCC</u> TTTTTCCATTATCTTTCAGA
p2-down	GGACTAGTCTGGGTGACTCTGCATGGA
p3-up	<u>CGGGATCC</u> TTTCCATTATCTTTCAGAAAATG
p3-down	GGACTAGTCTGGGTGACTCTGCATGGA
WO8-up	<u>CGGGATCC</u> ATGAAGCAAACGCGCGC
WO8-down	CCGCTCGAGTCAGAACTTGTAGCGCACCCC
WO8-p4-up	<u>CGGGATCC</u> AGGAATTCCGATGAAGCAAACGCGC
WO8-P4-down	CCGCTCGAGTCAGAACTTGTAGCGCACCCC
T591-up	CCGCTCGAGTCAGAACTTGTAGCGCACCCC
T591-down	CGGACC <u>GGGCC</u> CTCGCCCTTCTCAGGTCG
T1150-up	CCGCTCGAG <u>GCTGCGGAGACGAGTCACCA</u>
T1150-down	CGGACC <u>GGGCC</u> ACCCCGAGCCTTGAGCGA
verify-up	TTCTCGCCGTTGCCCCAG
verify-down	CTTCCATGCAGAGTCACCCA