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# Investigation of halogenation during the biosynthesis of ramoplanin in *Actinoplanes* sp. ATCC33076

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**Abstract** Ramoplanin and enduracidin are lipopeptide antibiotics effective against Gram-positive pathogens, which share close similarity in structure and biosynthetic pathway. Both compounds have chlorine atoms attached to 4-hydroxyphenylglycine (Hpg) but with different chlorinating sites and levels. Here, to probe the factor affecting the site and level of halogenation, gene inactivation and heterologous expression were carried out in *Actinoplanes* sp. ATCC33076 by homologous recombination. Metabolite analysis confirmed that *ram20* encodes the only halogenase in ramoplanin biosynthetic pathway, and enduracidin halogenase End30 could heterologously complement the *ram20*-deficient mutant. Additionally, the mannosyltransferase-deficient mutant produces a dichlorinated ramoplanin aglycone with the halogenation site at $Hpg^{13}$. This study has refined our understanding of how halogenation occurs in ramoplanin biosynthetic pathway, and lays the foundation for further exploitation of ramoplanin and enduracidin halogenase in combinatorial biosynthesis.

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## Introduction

Ramoplanin A2 (**1**) is a potent antimicrobial agent produced by *Actinoplanes* sp. ATCC33076 and is active against a wide range of Gram-positive bacteria, including vancomycin-resistant *Enterococcus* sp. (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-intermediate resistant *Clostridium difficile* (Rolston et al. 1996; Finegold et al. 2004; Pelaez et al. 2005). Structurally, ramoplanin is a macrocycle consisting of 17 amino acids and has a single chlorine atom attached to the 4-hydroxyphenylglycine $(Hpg)^{17}$ (Fig. 1). Ramoplanin acts as an inhibitor of peptidoglycan formation, thus disrupting the biosynthesis of bacterial cell wall and eventually causes cell death (Lo et al. 2000; Hu et al. 2003). Due to the novel antimicrobial mechanism, no clinical resistance to ramoplanin was reported since the discovery of ramoplanin. Ramoplanin is now in phase III clinical trials for *C. difficile*-associated disease (CDAD) in gastrointestinal (GI) tract (Paknikar and Narayana 2012). The biosynthetic gene cluster has been isolated and reported, and the assembly of the peptide core as well as the biosynthesis pathway of the non-proteingenic amino acid were elucidated (McCafferty et al. 2002; Hoertz et al. 2012). Among the gene clusters, *ram20* was predicted to encode the only halogenase responsible for introducing the single chlorine atom onto the $Hpg^{17}$.

Enduracidin (**5**) is a lipodepsipeptide antibiotic closely related to ramoplanin (Fig. 1). It shares similarity in structure and mechanism of action with ramoplanin. Enduracidin contains six Hpg residues at the same position as its counterpart and has one chlorinated Hpg. Previous study (Yin et al. 2010)

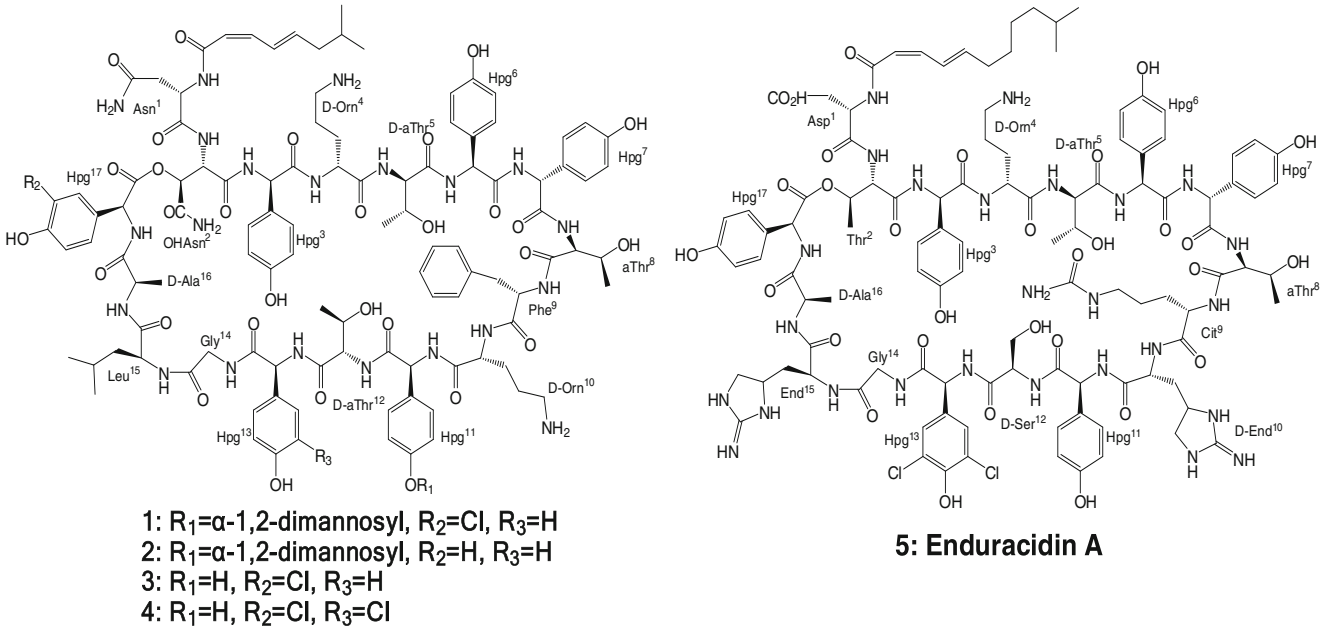



**Fig. 1** Structures of ramoplanins and enduracidin A

demonstrated that *end30* encodes the only halogenase in enduracidin biosynthetic pathway and is responsible for dichlorinating the $Hpg^{13}$.

Halogenation is a frequent modification in the biosynthesis of natural products. To date, over 4000 halogenated natural products have been reported (Gribble 2010). Halogen atoms may profoundly influence the activity of these compounds (Harris et al. 1985; Bister et al. 2003; Eustáquio et al. 2003). The halogenation strategies in natural product biosynthesis were well reviewed before (Neumann et al. 2008; Butler and Sandy 2009; Wagner et al. 2009). $FADH_2$-dependent halogenase is frequently found in nonribosomal peptide synthetase (NRPS)/PKS biosynthetic clusters and is responsible for halogenating bacterially derived secondary metabolites including ramoplanin and enduracidin. Characterization of the mechanism of chlorination, including timing and substrate specificity, is critical for utilizing halogenase to expand the diversification of these biologically important compounds.

In this work, we inactivated the *ram20* in the ramoplanin-producing strain *Actinoplanes* sp. ATCC33076 and subsequent complementary was carried out as well as the heterologous expression of enduracidin halogenase in ramoplanin-producing strain and its derivatives to assess the involvement of the halogenase in the ramoplanin biosynthesis pathway.

## Materials and methods

### Bacterial strains, plasmids, and reagents

Bacterial strains and plasmids used in this study are described in Table 1. Plasmid-bearing cultures were supplemented with appropriate antibiotics. Biochemicals, chemicals, media, restriction enzymes, and other molecular biological reagents were from commercial sources.

### DNA isolation, manipulations, and sequencing

General DNA isolation and manipulation were performed according to the standard methods (Kieser et al. 2000; Sambrook and Russell 2001). PCR reactions were performed with either Taq or PrimeSTAR HS DNA polymerase (Takara) using genomic DNA as templates. Primer synthesis and DNA sequencing were performed by Invitrogen, Shanghai, China.

### Construction of the ram20 inactivation mutant

The *ram20* in-frame deleted plasmid pCJS3003 was constructed by cloning two homologous exchange arms corresponding to the 5′ and 3′ regions flanking *ram20* into the shuttle vector pKC1139. The 1.5-kb 5′ fragment was amplified with primers 5′-AAAAAGCTTCGGCTACC TGATGGAC-3′ and 5′-AAATCTAGAGAGTTCG AGCAGCGCTAC-3′ using ATCC33076 genomic DNA as a template. The purified PCR product was then digested with *Hin*dIII and *Xba*I and then inserted into appropriately digested pSP72 to yield plasmid pCJS3001. The 1.4-kb 3′ flanking DNA fragment was amplified using primers 5′-A AATCTAGACCGGATCTGGCCGTAGTC-3′ and 5′-A AAGATATCTGCGGGTCATCGTGGT-3′ and cloned into the *Xba*I and *Eco*RV sites of pSP72 to generate pCJS3002. The identity of the fragments was verified by sequencing. The 1.5-kb *Hin*dIII/*Xba*I fragment and 1.5-kb *Xba*I/*Eco*RV fragment were then excised from pCJS3001 and pCJS3002,

**Table 1** Strains and plasmids used in this work

| Strains or plasmids | Relevant features | Source or reference |
|---|---|---|
| Strains | | |
| *Actinoplanes* | | |
| ATCC33076 | Wild type; ramoplanin producer | This Laboratory |
| *A.CJS3001* | ATCC33076 derivative with the in-frame deleted *ram20* gene | This work |
| *A.CJS3002* | *A.CJS3001* derivative, harboring the *ram20* gene under the control of *PermE** | This work |
| *A.CJS3003* | *A.CJS3001* derivative, harboring the *end30* gene under the control of *PermE** | This work |
| *A.CJS3004* | ATCC33076 derivative, harboring the *end30* gene under the control of *PermE** | This work |
| *A.CJS1001* | ATCC33076 derivative, with the in-frame deleted *ram29* gene | (Chen et al. 2013) |
| *E. coli* | | |
| DH5α | General cloning host | Invitrogen |
| ET12567 | Methylation deficient; donor strain for conjugation between *E. coli* and *Actinoplanes* | (MacNeil et al. 1992) |
| Plasmids | | |
| pSP72 | *E. coli* general cloning vector, $Amp^R$ | Promega |
| pKC1139 | *E. coli-Streptomyces* shuttle vector; $rep^{ts}$; *OriT*, $Am^R$ | (Bierman et al. 1992) |
| pSET152 | *E. coli-Streptomyces* shuttle vector; site-specific integration; *OriT*, $Am^R$ | (Bierman et al. 1992) |
| pCJS3001 | 1.5-kb PCR fragment containing the upstream of *ram20* in pSP72 | This work |
| pCJS3002 | 1.4-kb PCR fragment containing the downstream of *ram20* in pSP72 | This work |
| pCJS3003 | 1.5-kb left branch and 1.4-kb right branch of *ram20* in pKC1139 | This work |
| pCJS3004 | 1.5-kb PCR fragment encoding the intact *ram20* gene in pSP72 | This work |
| pCJS3005 | 2.0-kb PCR fragment containing the *PermE**-controlled *ram20* gene in pSET152 | This work |
| pCJS3006 | 1.5-kb PCR fragment encoding the intact *end30* gene in pSP72 | This work |
| pCJS3007 | 2.0-kb PCR fragment containing the *PermE**-controlled *end30* gene in pSET152 | This work |

respectively, and inserted into the pKC1139 prepared by digestion with *Hin*dIII and *Eco*RV to construct the in-frame inactivated plasmid pCJS3003. The resultant plasmid pCJS3003 was then introduced into ATCC33076 from *Escherichia coli* ET12567 by intergeneric conjugation according to the standard procedure (Kieser et al. 2000). One of the $Am^R$ transconjugants was streaked on the mannitol soya flour (MS) agar (Kieser et al. 2000) containing 50 μg/ml Am at 37 °C to induce integration of the plasmid to the chromosome. One colony in which integration had taken place was further cultured at 30 °C and 250 rpm for 2 days in 3-ml liquid TSB medium for five rounds in the absence of apramycin to induce the second crossover. The resulting apramycin-sensitive colonies were further screened by PCR with primers 5′-CCAAGGCCGGGTTCATGCGCAAG-3′ and 5′-GCCGGAGGCGATGCCGCCGACCAACTC-3′, and the genotype was confirmed by sequencing.

### Self-complementation of the ram20-deficient mutant

The DNA sequence encoding *ram20* was amplified from the genomic DNA of ATCC33076 using primers 5′-AAAGGATCCGTGGCTGCTCAACCGGAAG-3′ and 5′-AAATCTAGATCAGTCGGCGGCGACCCACG-3′. The gel-purified PCR product was then digested and cloned into pSP72 to give the plasmid pCJS3004. The identity of PCR product was confirmed by DNA sequencing. Together with the 0.5-kb *Eco*RI/*Bam*HI digested *PermE** fragment, the 1.5-kb *Bam*HI/*Xba*I *ram20* fragment was inserted into the integrative vector pSET152 prepared by *Eco*RI and *Xba*I to afford the self-complemented plasmid pCJS3005. This plasmid was then introduced into *A.CJS3001* by intergeneric conjugation from *E. coli* ET12567. The genotype of the resulting Am-resistant mutants was further confirmed by PCR using the primers 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ (M13-47) and 5′-GAGCGGATAACAATTTCACACAGG-3′ (RV-M), yielding the *ram20* self-complemented strain *A.CJS3002*.

### Heterologous expression of enduracidin halogenase end30 in ATCC33076 and its derivatives

The enduracidin halogenase *end30* was PCR amplified with primers 5′-AAAGGATCCGTGCCGGGAGGCCGAATGAG-3′ and 5′-AAATCTAGATCAGGACGGATGCGGCTCC-3′ from the genomic DNA of ATCC21013, and the product was gel purified and cloned into pSP72 to give the plasmid pCJS3006. Error-free *end30* was confirmed by DNA sequencing and then excised from

pCJS3006 by *Bam*HI/*Xba*I double digestion and inserted into the integrative vector pSET152 prepared by *Eco*RI and *Xba*I together with the *PermE** promoter fragment to yield the heterologously expressed *end30* plasmid pCJS3007. The resultant plasmid pCJS3007 was introduced either into the *ram20*-deficient mutant strains or the wild-type strain ATCC33076 by intergenic conjugation to construct enduracidin halogenase into heterologously complemented strains or heterologously expressed strains, respectively. The genotype of the resultant mutants was confirmed by PCR using the primers RV-M and M13-47.

### Assay of end30 transcription by reverse transcription PCR

Total RNA of ATCC33076 and its derivatives was extracted from mycelia grown in fermentation medium for 96 h using TRIzol reagent (Invitrogen). DNase I treatment and cDNA preparation were carried out using the Takara PrimeScript RT reagent kit according to the manufacturer's instruction.

One NRPS gene *ram13* involved in the biosynthesis of ramoplanin was taken as an internal control, and the 279-bp internal fragment was amplified using primers 5′-ATGCTCCGGTTCACG-3′ and 5′-GTAGGCGCTCTGGTT-3′. For evaluation of the transcriptional level of introduced *end30*, a 262-bp internal fragment was amplified using primers 5′-AGCACGGACTTCGACCTCTC-3′ and 5′-CCCAGAACATGCTGTCGGAC-3′.

### Production and preparation of ramoplanin and its derivatives

*Actinoplanes* strains were grown on 28 °C on agar plates consisting of 0.3 % beef extract, 1 % tryptone, 2.4 % corn starch, 0.1 % glucose, and 2.2 % agar, pH 7.0. Apramycin was used at a final concentration of 50 μg/ml for selection. For fermentation, the mycelium from agar plate was inoculated into seed medium (0.3 % beef extract, 0.5 % tryptone, 0.5 % yeast extract, 3 % oat meal, 0.4 % $CaCO_3$, pH 7.0), cultured at 28 °C and 250 rpm for 3 days, transferred (1:10 *v*/*v*) into production medium (5 % soluble starch, 2 % glucose, 3 % soya flour, 1 % $CaCO_3$, and 0.5 % leucine, pH 7.0), and incubated at 25 °C and 250 rpm for 6 days. The fermentation broth was adjusted to pH 3.0 with HCl and centrifuged to remove the supernatant. Then, the mycelia were extracted with an equal volume of acetone for 3 h. The suspension was then centrifuged, and the supernatant was collected and concentrated under vacuum to yield the crude extracts for further analysis.

### HPLC analysis of ramoplanin and its derivatives

HPLC was performed using a Thermo Syncronis $C_{18}$ column (5 μm, 250×4.6 mm) on an Agilent 1200 HPLC system. For ramoplanin analysis, an isocratic elution system consisting of 35 % $CH_3CN$ and 65 % $H_2O$ (0.4 % ammonium formate) was applied, at a flow rate of 0.8 ml/min, with 231 nm monitored.

Liquid chromatography-mass spectrometry (LC-MS) analyses were performed on a Thermo Finnigan LCQ Fleet system equipped with an Agilent 1100 HPLC system and under the control of Xcalibur 2.0.6, with the same conditions mentioned above for HPLC, and the positive mode electrospray ionization was used for MS detection.

MS/MS analyses were performed on ACQUITY UPLC and Q-TOF MS Premier by Instrumental Analysis Center of Shanghai Jiaotong University. An MS/MS scan was carried out by selecting the five most intense ions as the parent ion for analysis with the collision energy of 30 eV. The data were collect and processed using the MassLynx Software.

### Evaluation of antibacterial activity

Evaluation of the antibacterial activity of ramoplanin and its derivatives against *Bacillus subtilis* was determined using a disk diffusion method. Ramoplanin standards and analogues were dissolved in 50 % MeOH at a concentration of 100 μg/ml, and 100 μl of each solution was loaded onto the filter paper disk. After incubating the plates at 37 °C for 16 h, the zones of inhibition were observed and compared and the plates were photographed.

## Results

### Generation of the ram20 in-frame inactivated mutant

The biosynthetic gene cluster of ramoplanin has already been reported, and the *ram20* was annotated as the single halogenase. It was predicted to be responsible for the introduction of the chlorine atom to the $Hpg^{17}$ residue. In order to experimentally assess the in vivo function of *ram20*, a *ram20* in-frame deleted mutant was constructed by double-crossover homologous recombination and designated as *A.CJS3001*. A 192-bp fragment in the middle of *ram20* was chosen to be deleted to inactivate the Ram20 while avoiding its effects on the transcription of downstream genes. The genotype of the resultant *ram20* in-frame inactivated mutants was confirmed by PCR and sequencing (Fig. 2b). After 6-day fermentation, metabolites produced by *A.CJS3001* were extracted from the mycelia and analyzed on LC-MS. LC-MS analysis revealed that, comparing with the parent strain ATCC33076, the mutant strain *A.CJS3001* produced no ramoplanin A2 (**1**, *m*/*z* ($[M+2H]^{2+}$)=1278) but accumulated deschloro-

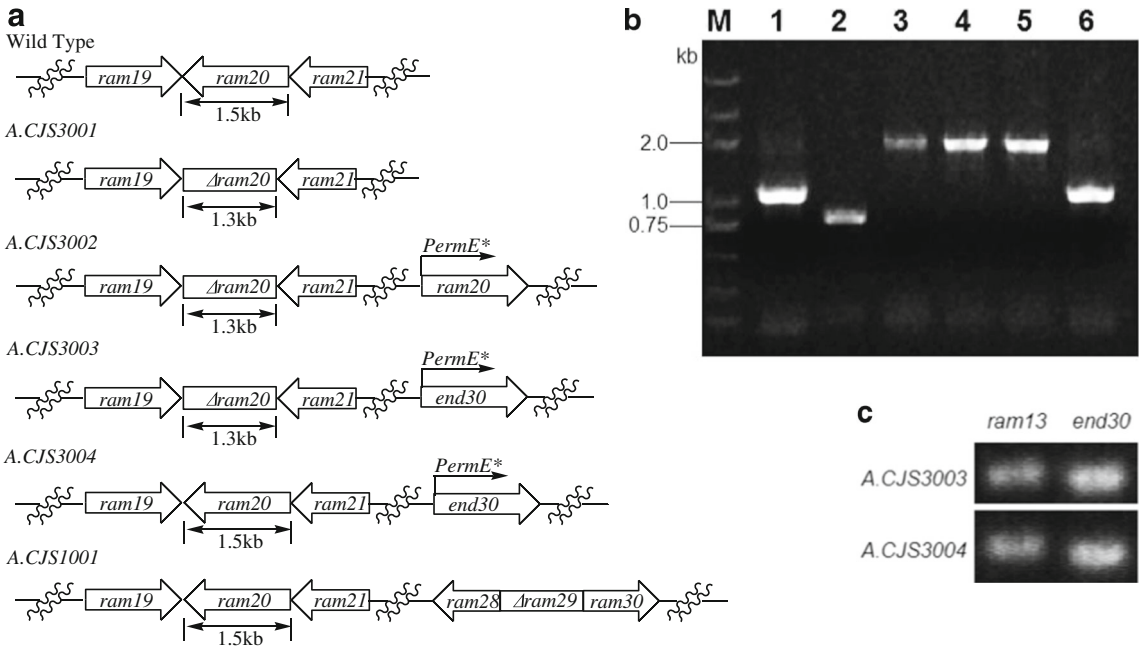



**Fig. 2** Genotypes of all recombinant strains and transcription of the *end30* gene in ATCC33076 and *A.CJ3001*. **a** Genotypes of ATCC33076 and all mutants in this work. **b** PCR analysis using 5′-CT GGAGCCGCGGGAGTTC-3′ and 5′-CACATGCTGTGGACGGAG- 3′ as primers [1.0 kb (*lane 1*) for wild type, 0.8 kb (*lane 2*) for *A.CJS3001*, and 1.0 kb (*lane 6*) for *A.CJS1001*] while using the primers M13-47 and RV-M [2.0 kb (*lane 3*) for *A.CJS3002*, 2.0 kb (*lane 4*) for *A.CJS3003*, and 2.0 kb (lane 5) for *A.CJS3004*]. All PCR products were sequenced to confirm each mutation. **c** RT-PCR analysis of the transcription level of the NRPS *ram13* gene and the heterologously expressed *end30* gene in *A.CJS3003* and *A.CJS3004*. In both heterologously expressed strains, enduracidin halogenase was transcribed in a comparable level

ramoplanin A2 (**2**, *m*/*z* $[M+2H]^{2+}$)=1260 (Figs. 3b and 4a), indicating that the Ram20-halogenated ramoplanin.

## Self-complementation of the ram20 in-frame deleted mutant

To further confirm that the *ram20* is the only halogenase gene in the ramoplanin biosynthesis pathway, a *ram20* self-complemented strain was constructed. A 1.5-kb DNA fragment coding the intact *ram20* was amplified by PCR from the genomic DNA of ATCC33076 and then inserted downstream of *PermE** in pSET152 to yield the heterologously expressed plasmid pCJS3005. pCJS3005 was then introduced into the *ram20* in-frame deleted mutant *A.CJS3001*, and the transconjugants were screened on the basis of apramycin resistance to yield the *ram20* self-complemented strain *A.CJS3002*. PCR analysis of the genomic DNAs from the recombinant strains confirmed that they have the designed genotypes (Fig. 2b). The complemented strain was fermented, and the metabolite was analyzed by LC-MS. The results showed that the self-complemented strain produces a metabolite with *m*/*z*=1278.1 (Fig. 3c), which is consistent with the molecular formula $C_{119}H_{154}ClN_{21}O_{40}$ for ramoplanin A2. Taken together, these results confirm that the *ram20* encodes the only halogenase in the ramoplanin biosynthesis pathway.

## Heterologous complementation of the ram20 deletion mutant with the enduracidin halogenase end30

Enduracidin shares great similarity in structure with ramoplanin, and enduracidin halogenase showed 62 % amino acid identity with Ram20; thus, it may be able to halogenate the macrocycle of ramoplanin. To validate this hypothesis, enduracidin halogenase was heterologously expressed in *A.CJS3001*. For heterologous expression of enduracidin halogenase *end30*, a 1.5-kb DNA fragment encodes the intact *end30* that was amplified by PCR from the genomic DNA of ATCC21013 and then inserted downstream of *PermE** in pSET152 to give the heterologously expressed plasmid pCJS3007. The heterologously complemented mutant *A.CJS3003* was obtained by introducing pCJS3007 into *A.CJS3002*, and PCR analysis confirmed the genotype of the mutant.

Compared with the parent strain, HPLC and LC-MS analysis of the metabolite reveal *m*/*z* ($[M+2H]^{2+}$)=1278.1 which is consistent with ramoplanin A2 (Fig. 3d). This indicates the heterologously expressed *end30* could halogenate the ramoplanin macrocycle. Since there are six Hpg

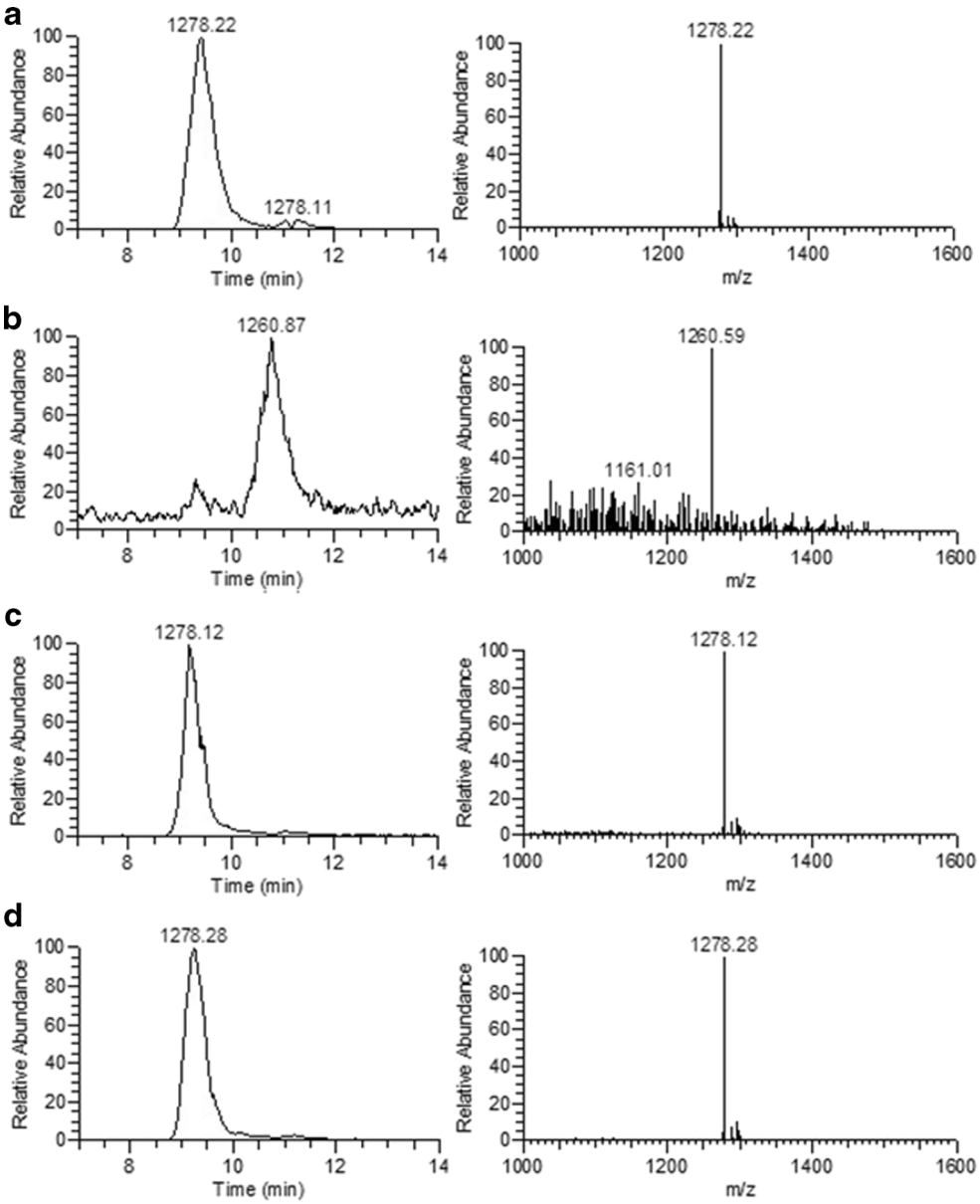



**Fig. 3** LC-ESI-MS analysis of the metabolites produced by **a** ATCC33076, **b** *A.CJS3001*, **c** *A.CJS3002*, and **d** *A.CJS3003*. The *left panel* shows the base peak corresponding to the compounds, while the *right panel* indicates the spectrum. The spectra showed doubly charged ions for wild-type ramoplanin A2 (*m*/*z* 1278), deschloro-ramoplanin A2 (**2**, *m*/*z* 1261), monochloro-ramoplanin A2 produced by *A.CJS3002* (*m*/*z* 1278), and monochloro-ramoplanin A2 produced by *A.CJS3003* (*m*/*z* 1278)

residues in the ramoplanin macrocycle that could be halogenated, Yin et al. (2010) demonstrated that the End-orf30 di-halogenates the $Hpg^{13}$ of enduracidin in the enduracidin biosynthesis pathway and it is necessary to identify the site on which the chlorine atom was introduced by the heterologously expressed halogenase. Ramoplanins were subjected to the MS/MS analysis. In the spectra of ramoplanin A2 MS/MS analysis, a series of ions are observed for fragment peptide $Gly^{14}$-Cl-$Hpg^{17}$ (*m*/*z* 443.9), D-$aThr^{12}$-Cl-$Hpg^{17}$ (*m*/*z* 694.2), and D-$Orn^{10}$-Cl-$Hpg^{17}$ (*m*/*z* 957.4) (Fig. 5a). Analysis of the MS/MS spectra for the peptide produced by *A.CJS3003* revealed ions at *m*/*z* 443.9 and 694.2, corresponding to the addition of a single chlorine atom to $Hpg^{17}$, which suggests that the enduracidin halogenase could be capable of heterologously complementing the Ram20-deficient mutant.

## Heterologous expressing End-orf30 in ATCC33076 and metabolite analysis

To further investigate the involvement of the enduracidin halogenase in the ramoplanin biosynthesis pathway, we also introduced the plasmid pCJS3007 to the wild-type ramoplanin-producing strain ATCC33076. The integration of the *end30* into the chromosome of ATCC33076 was confirmed by PCR (Fig. 2b). The resulting strain was designated *A.CJS3004*. The mutant was fermented, and the metabolites were subjected to HPLC and LC-MS analysis, but no new

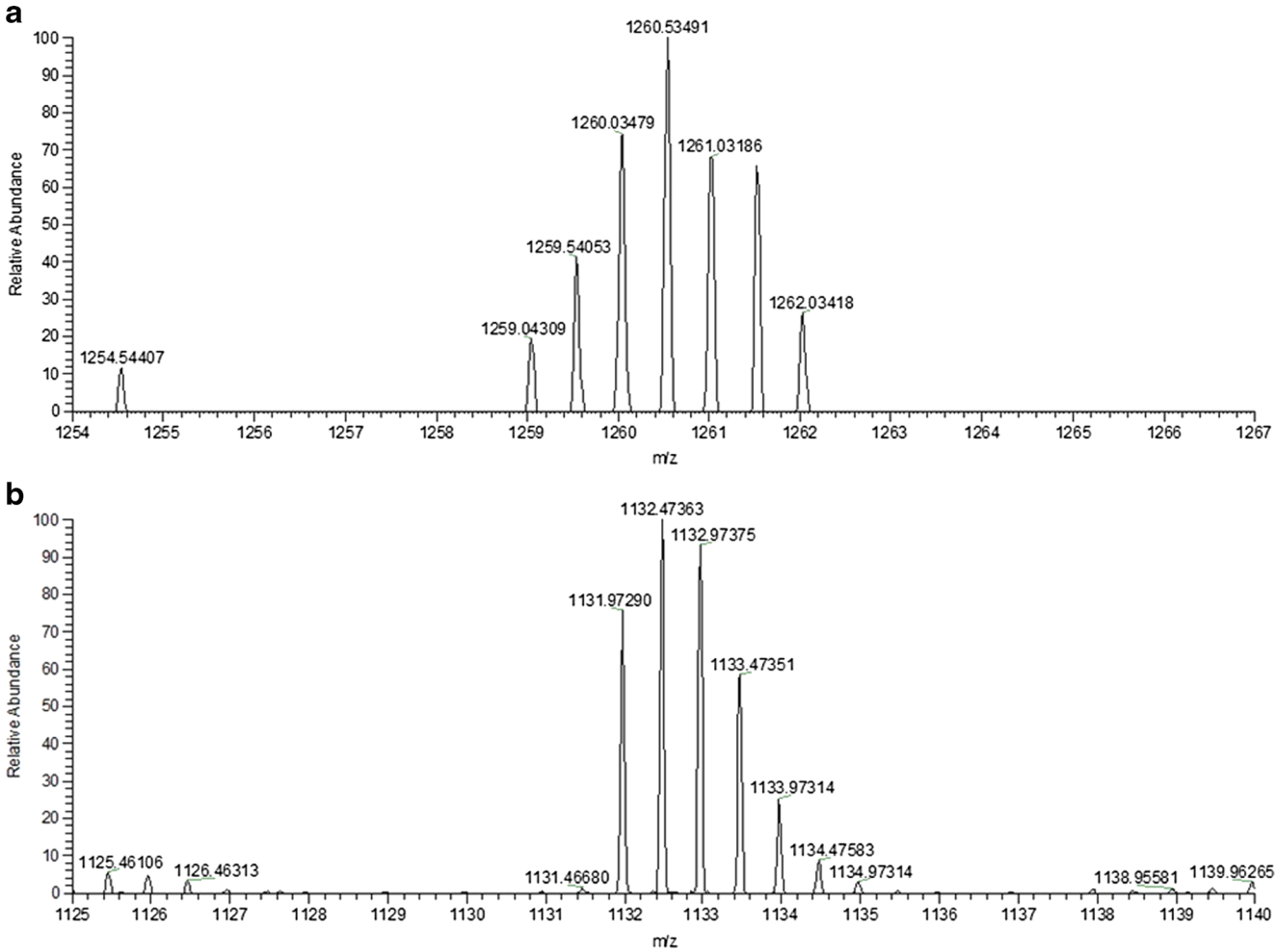



**Fig. 4** TOF-MS analysis of ramoplanins from the fermentation broth of **a** *ram20* in-frame deleted mutant *A.CJS3001* and **b** *ram29* in-frame deleted mutant *A.CJS1001*. Doubly charged molecular ions at *m/z* 1261 (**2**) and *m/z* 1132 (**4**), consistent with the proposed deschloro-ramoplanin A2 and dichloro-ramoplanin A2 aglycone, respectively

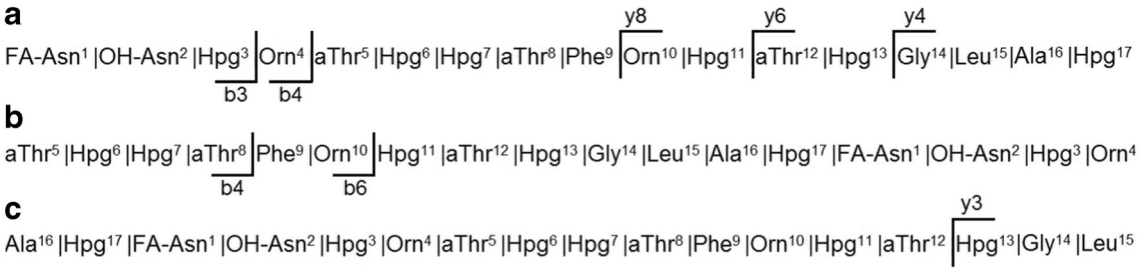
signal was detected. Furthermore, the transcription of the heterologously introduced *end30* was comparative with that of the *A.CJS3003* (Fig. 2c).

### Biological evaluation of the deschloro-ramoplanin A2

The in vitro evaluation of the deschloro-ramoplanin A2 produced from *A.CJS3001* against *B. subtilis* was conducted. The deschloro-ramoplanin A2 retained activity against *B. subtilis*, and the inhibition zone showed no measurable difference compared to the intact ramoplanin A2 (Fig. 6).

## Discussion

Halogenation is a common modification on natural products including peptide antibiotics such as ramoplanin and enduracidin. The halogen atoms may contribute to the bioactivity profoundly, and thus, many efforts were laid on manipulating halogenase for creating unnatural-natural products with altered halogenation patterns through combinatorial biosynthesis. Ramoplanin shares close similarity in both structure and biosynthetic pathway with enduracidin. However, the chlorinating site and level occurred in these two compounds were different. The factors that determine the halogenating site and level are still unknown. Generally, there may be three time points that halogenation could take place during the biosynthesis of natural products according to the substrate selectivity. Halogenation could occur on the building blocks which were utilized in subsequential procedure (Keller et al. 2000; Yeh et al. 2005), and halogenation could take place during the peptide elongation stage (Thomas et al. 2002; Dorrestein et al. 2005). Examples of late-stage halogenation are emerging (Foulston and Bibb 2010; Zeng and Zhan 2010; Wu et al. 2011). In this study, we investigated the halogenation during the biosynthesis of




| | Ramoplanin A2 | | Monochloro peptide | Dichloro ramoplanin A2 aglycone |
|---|---|---|---|---|
| Fragment | calculated | observed | observed | observed |
| y8: D-Orn$^{10}$-Cl-Hpg$^{17}$ | 956.39 | 956.49 | 956.35 | 956.36 |
| y6: D-aThr$^{12}$-Cl-Hpg$^{17}$ | 693.26 | 693.24 | 693.25 | - |
| y4: Gly$^{14}$-Cl-Hpg$^{17}$ | 443.16 | 443.11 | 443.07 | 443.17 |
| y3: Cl-Hpg$^{13}$-Leu$^{15}$ | 354.12=320.16+Cl | - | - | 354.15 |
| b4: D-aThr$^{5}$-D-aThr$^{8}$ | 501.19 | 501.07 | 501.08 | 501.19 |
| b6: D-aThr$^{5}$-D-Orn$^{10}$ | 762.34 | 762.27 | 762.29 | 762.34 |
| b4:FA-Asn$^{1}$-D-Orn$^{4}$ | 644.26 | 644.10 | 644.20 | 644.26 |
| b3:FA-Asn$^{1}$-Hpg$^{3}$ | 495.21 | 495.07 | 495.22 | 495.21 |

**Fig. 5** Localization of the chlorine atom on monochlorinated ramoplanin A2 produced by *A.CJS3003* and dichloro-ramoplanin A2 aglycone produced by *A.CJS1001* by tandem MS analysis. **a** The fragment profile with the first fragment at OH-Asn$^2$ and Hpg$^{17}$. **b** The fragment profile with the first fragment occurring at D-Orn$^4$ and D-aThr$^5$. **c** The fragment profile with the first fragment at the Leu$^{15}$ and D-Ala$^{16}$

ramoplanin through genetic manipulation of ramoplanin and enduracidin halogenase in ATCC33076.

We first conducted the bio-informatics analysis on the NRPS genes. Structurally, there are six Hpg residues in the peptide backbone and the substrate specificity of the adenylation domains in the biosynthetic gene cluster corresponding to each Hpg is identical (DAYHLGLLCK). In another word, these adenylation domains would recognize and then incorporate building blocks indiscriminately, but the eventual product only had the monochlorinated Hpg$^{17}$. Thus, this result illustrates that the free Hpg may be the substrate of the Ram20, but in the biosynthesis of ramoplanin, the Cl-Hpg may not be recognized and incorporated by NRPS, which is in accordance with studies on balhimycin halogenation (Puk et al. 2004). It can be speculated that the halogenation occurs during the peptide elongation or after the peptide backbone was assembled.

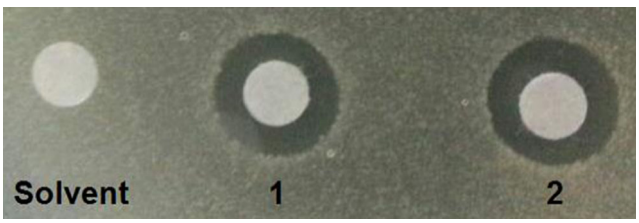



**Fig. 6** The zones of inhibition produced by the ramoplanin A2 standards and purified deschloro-ramoplanin A2. *1* Ramoplanin A2, *2* purified deschloro-ramoplanin A2. Pictures were taken after 16-h incubation at 37 °C

Then, gene manipulation was conducted to probe the function of *ram20*, the only apparent halogenase gene. The in-frame deletion of *ram20* from ATCC33076 was shown to accumulate deschloro-ramoplanins, and the self-complemented mutant restored the capacity of producing ramoplanin. These results experimentally confirmed that *ram20* encodes the only halogenase in the ramoplanin biosynthetic pathway which is in agreement with previous reports (McCafferty et al. 2002).

Enduracidin (Fig. 1) is a potent antimicrobial agent which shares great similarity in structure with ramoplanin, and *end30* is the only halogenase gene responsible for the dichlorination at the Hpg$^{13}$ (Yin et al. 2010). End-orf30 showed 62 % amino acid identity with Ram20; thus, it may be able to halogenate the macrocycle of ramoplanin. To test this hypothesis, a heterologously complemented mutant which overexpresses enduracidin halogenase in the Ram20-deficient strain was constructed. Analysis of the metabolite produced by the heterologously complemented strain revealed a monochlorinated ramoplanin species, and the chlorine atom introduced by enduracidin halogenase was located at the Hpg$^{17}$. This finding is interesting since End30 is the only halogenase in enduracidin biosynthesis pathway and is responsible for the introduction of two chlorine atoms onto the Hpg$^{13}$, but when introduced into the *ram20*-deficient strain, it preferentially

monochlorinating Hpg[17] over Hpg[13]. Due to the amino acid sequence similarity between Ram20 and End30, this finding indicates that the nonribosomal peptide (NRP) sequence may determine the halogenating site and level.

To further investigate the involvement of enduracidin halogenase in the biosynthetic pathway of ramoplanin, *end30* was introduced into ATCC33076. However, analysis of the metabolites produced by the mutant revealed no doubly or triply chlorinated species, although the transcription of the introduced enduracidin halogenase was confirmed by reverse transcription PCR (RT-PCR) (Fig. 2c). As previously reported, End30 was responsible for dichlorination of the Hpg[13] residue of enduracidin (Yin et al. 2010), and in our work, it could monochlorinate the Hpg[17] residue of ramoplanin when introduced into the halogenase-deficient strains, but when introduced into the wild-type ATCC33076, it could not halogenate the counterpart in the ramoplanin structure. This regioselectivity may be due to the difference in structure and peptide sequence between ramoplanin and enduracidin. Structurally, ramoplanin has an α-1,2-dimannosyl moiety appended to Hpg[11] but absent in enduracidin. The steric bulk of the dimannosyl moiety may pose a hindrance that could avoid the recognition of the halogenase to the substrate. In our previous study, a mannosyltransferase-inactivated strain was constructed to produce ramoplanin aglycone (Chen et al. 2013). However, in this work, further analysis revealed a dichlorinated specie of ramoplanin aglycone (**4**, Fig. 4b, *m*/*z* 1132) in the fermentation broth with a low yield. Inspection of the MS/MS spectra of this dichlorinated aglycone revealed that b ions (Fig. 5a, b) for fragment D-aThr[5]-D-aThr[8] (*m*/*z* 501.5), D-aThr[5]-D-Orn[10] (*m*/*z* 762.8), fatty acid (FA)-Asn[1]-D-Hpg[3] (*m*/*z* 495.5), and FA-Asn[1]-D-Orn[4] (*m*/*z* 644.7) and y ions (Fig. 5a) for Gly[14]-Cl-Hpg[17] (*m*/*z* 443.9) and aThr[5]-Hpg[11] (*m*/*z* 911.4) were still present, thus excluding Hpg[3], Hpg[6], Hpg[7], Hpg[11], and Hpg[17] as chlorination sites. In addition, a new ion at *m*/*z* 354 could be observed, corresponding to the addition of a single chlorine atom attached to Hpg[13] (y3 Cl-Hpg[13]-Leu[15]) (Fig. 5c). This finding could be a support to the hypothesis that the steric effects of the sugar moiety may affect the halogenation and the mannosylation takes place after the macrocycle is synthesized. Taken together, we are prone to presume that the halogenation in the ramoplanin biosynthesis occurs simultaneously or after the mannosylation, and the assembled macrocycle is taken as a substrate. Halogenation of phenolic moieties was also reported to occur in the late stage of biosynthesis of natural products (Wagner et al. 2009). However, even in the absence of the dimannosyl moiety, the yield of the dichloro-ramoplanin A2 was low, which may indicate that the local sequence of the NRP is the main determinant of the halogenating site and it may be very interesting to create a ramoplanin hybrid with the 11–17-amino acid and flanking residues substituted with the counterpart of the enduracidin to further evaluate the hypothesis that the halogenating site and level were dictated by halogenase and NRP sequence. Moreover, we demonstrated that the compatibility of related halogenase and the knowledge gained from this study could be valuable in future works in developing bioactive unnaturally natural products, including improved ramoplanin and enduracidin derivatives via combinatorial biosynthesis.

**Compliance with ethical standards**

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**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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