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Enhancement of edeine production in *Brevibacillus brevis* X23 via *in situ* promoter engineering

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Summary

Edeines, a group of cationic antimicrobial peptides produced by the soil bacterium Brevibacillus, have broad biological effects, such as antimicrobial, anticancer and immunosuppressive activities. However, the yield of edeines in wild-type (WT) *Brevibacillus* is extremely low, and chemical synthesis of edeines is a time-consuming process. Genetic engineering has proven to be an effective approach to produce antibiotics with high yield. In this study, the edeine biosynthetic gene cluster (*ede* BGC), which is involved in edeine production, was identified and characterized in *Brevibacillus brevis* X23. To improve edeine production in *B. brevis* X23, the *ede* BGC promoter was replaced with six different promoters, P_{mwp}, P_{spc}, P_{xylA}, $P_{shuttle\text{-}09}$, P_{grac} or P_{43}, through double-crossover homologous recombination. The new promoters significantly increased the expression of the *ede* BGC as well as edeine production by 2.9 ± 0.4 to 20.5 ± 1.2-fold and 3.6 ± 0.1to 8.7 ± 0.7-fold respectively. The highest yield of edeines (83.6 mg l^{-1}) was obtained in *B. brevis* X23 with the P_{mwp} promoter. This study provides a practical approach for producing high yields of edeines in *B. brevis*.

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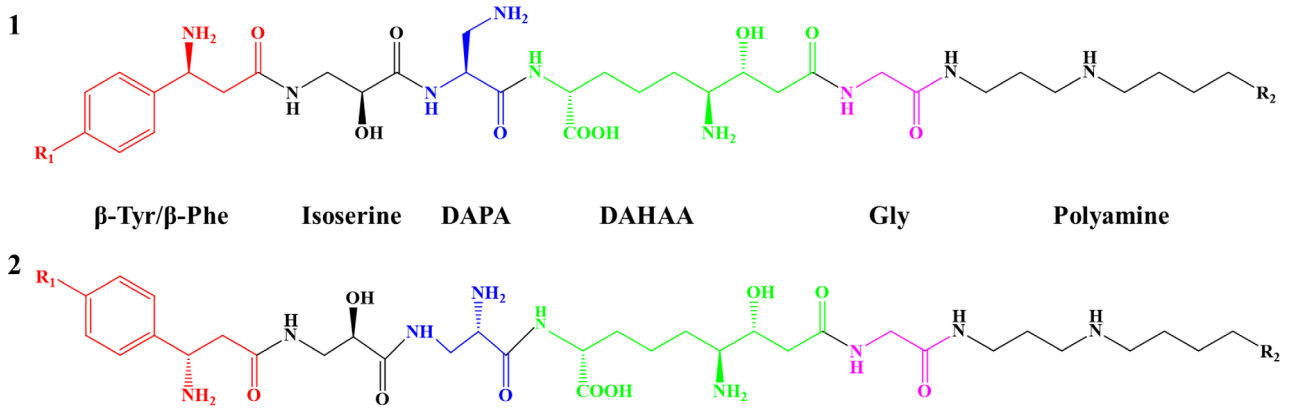
Introduction

Edeines are a group of linear pentapeptides produced by the soil bacterium Brevibacillus (Westman *et al*., 2013). Edeines A, B, D and F, which exhibit remarkable antibiotic activities, exist as two isomers, the active isomer α and the inactive isomer β (Fig. 1). Among them, edeines A_1 and B_1 have broad inhibitory activity against Gram-positive and Gram-negative bacteria, fungi, mycoplasmas and cancer cells (Hill *et al*., 1994; Czajgucki *et al*., 2006; Johnson *et al*., 2020) as well as substantial immunosuppressive activity in mice (Kierońska *et al*., 1976). Edeines inhibit DNA synthesis at low concentrations ($\leq$ 15 μg ml^{-1}) and suppress protein synthesis at high concentrations ($\geq$ 150 μg ml^{-1}) through binding to the P-site of the 30S ribosomal subunit to block t-RNA binding, contributing to their antimicrobial activities (Kurylo-Borowska and Szer, 1972; Pioletti *et al*., 2001). In addition, edeines have been widely used as transcriptional inhibitors to study ribosomal function and protein synthesis (Polikanov *et al*., 2018). However, use of edeines as drugs in human and animals is limited due to their toxicity in animal models (Czajgucki *et al*., 2007). The continuous emergence of multi-drug resistant pathogens is a great challenge to public health, which highlights the re-investigation of obsolete antibiotics such as edeines for new medical applications (Kierońska *et al*., 1976; Szaflarski *et al*., 2008).

Edeines are pentapeptides with a unique backbone, composed of a glycine, four nonproteinogenic amino acids and a C-terminal polyamine (spermidine in edeine A_1 and guanyl-spermidine in edeine B_1) (Fig. 1) (Mazerski *et al*., 1981; Czerwinski *et al*., 1983; Gumieniak *et al*., 1983). The biosynthesis of natural products has advantages over chemical synthesis in laboratories due to its low cost and high intensity (Kunga Sugumaran *et al*., 2016). *B. brevis* X23, an excellent biocontrol agent isolated by our lab (Chen *et al*., 2012), can produce



edeine	R_1	R_2
A	OH	NH_2
B	OH	$NHC(NH)NH_2$
D	H	NH_2
F	H	$NHC(NH)NH_2$

Fig. 1. Chemical structures of edeines A, B, D, and F.
Edeines have two isomers: α (1) and β (2). The α-amino group of DAPA is linked to β-serine to form the active isomer α, while the β-amino group of DAPA in the inactive isomer is linked to a β-serine. DAPA, 2,3-diaminopropionic acid; DAHAA, 2,6-diamino-7-hydroxyazaleic acid.

edeines A_1 and B_1 under propriate conditions (Lu, 2014). A previous study has demonstrated broad inhibitory activities of edeines A_1 and B_1 against a large number of plant pathogens (Lu, 2014). While the production of edeines in *B. brevis* X23 could be slightly improved through optimization of media (data not shown), the low yield of edeines produced by *B. brevis* limits their broad application in agriculture as microbicides.

Genetic engineering, such as promoter modification, deletion or overexpression of regulatory factors, feeding with key precursors and increasing BGC copy number, has significantly improved the production of antibiotics (Marahiel *et al*., 1987; Keeney *et al*., 2008; Zhang *et al*., 2016; Zhang *et al*., 2019). Promoter engineering (native promoter replacement with stronger ones) is one of the most effective ways to improve antibiotic production. The strong promoters P_{43}, P_{glv} and P_{luxS} improved the yield of sublancin in *Bacillus subtilis* 1A747 by 11.5-fold (Ji *et al*., 2015). Production of surfactin in *B. subtilis* fmbR was increased by 10.2-fold by replacing the original promoter of surfactin BGC with the inducible promoter P_{spac} (Sun *et al*., 2009).

Studies examining the biosynthetic pathway of edeines were initiated in the 1960s. Borowska *et al*. hypothesized that edeines were synthesized by non-ribosomal peptide synthetases (NRPSs; Borowska and Tatum, 1966). However, this hypothesis was not confirmed for decades. Based on genome sequencing and bioinformatics analyses, Westman *et al*. recently identified a hybrid NRPS-PKS BGC that is likely to be responsible for the biosynthesis of edeines in *B. brevis* Vm4 (Westman *et al*., 2013). The predicted edeine biosynthetic gene cluster (*ede* BGC) encodes four NRPSs and one hybrid PKS–NRPS, which is consistent with the pentapeptide structure of edeines. Additionally, the *edeQ* gene on the *ede* BGC was verified to be the self-resistance gene because its product, EdeQ, could convert edeine to inactive *N*-acetyledeine *in vitro*. However, the *ede* BGC has not been experimentally confirmed in *B. brevis* due to difficulties associated with the genetic manipulation of this organism. To confirm and characterize the *ede* BGC, we initially cloned the predicted *ede* BGC (45.1 kb) from *B. brevis* X23 and integrated it into the chromosome of *B. subtilis* via Red/ET recombination (Liu *et al*., 2016). However, edeine was not detected in the culture of the genetically modified *B. subtilis*. We recently successfully established a stable genetic manipulation system for *B. brevis* X23 and improved edeine production by disrupting the regulatory factor AbrB (Zhang *et al*., 2020).

In the present study, we confirmed the *ede* BGC of *B. brevis* X23 by gene disruption and increased the production of edeines A and B by 3.6 ± 0.1 to 8.7 ± 0.7-fold by replacing the original promoters of the *ede* operon with six stronger promoters. The highest yields of edeines A and B were obtained in *B. brevis* X23 isolated

with P_{mwp} promoter replacement. To the best of our knowledge, this is the first study to demonstrate the enhancement of edeine production in *B. brevis* via promoter engineering.

Results

Identification and characterization of ede *BGC in* B. brevis *X23*

Brevibacillus brevis X23 was initially isolated in our lab as a biocontrol agent (Table S1), which produces edeines A and B against the plant pathogen causing tobacco bacteria wilt (Lu, 2014). The genome of *B. brevis* X23 was sequenced on the PacBio sequencing platform and submitted to NCBI (accession number: NZ_CP023474.1) (Chen *et al*., 2012). Based on antiSMASH (Blin *et al*., 2017) and Big-SCAPE (Navarro-Muñoz *et al*., 2020) analyses, 12 potential secondary metabolite BGCs were identified, one of which showed high sequence similarity to the predicted *ede* BGC of *B. brevis* Vm4 reported by Westman *et al*., (2013) (Fig. 2A). The identified *ede* BGC in *B. brevis* X23 shared the same organization as that in the *B. brevis* Vm4 genome. Briefly, the *ede* BGC (45.1 kb) in *B. brevis* X23 consisted of 17 open reading frames, designated *edeA* to *edeQ* according to the homologous sequences in *B. brevis* Vm4 (Fig. 2A).

While the *ede* BGC was identified in *B. brevis* Vm4 based on bioinformatics prediction (Westman *et al*., 2013), no functional studies have yet been conducted. To confirm whether the predicted *ede* BGC was responsible for the biosynthesis of edeines, we knocked out the promoter region and the starter module *edeP* (RS18545) of the *ede* operon to generate the mutant strain *B. brevis* X23*Δede* (Fig. 2B and C). Then, both the wild-type X23 (X23-WT) and mutant strains were subjected to antimicrobial activity and comparative metabolomic analyses. The X23-WT strain exhibited considerable antimicrobial activities against *Ralstonia solanacearum* and *B. subtilis* in the plate assay. However, the X23*Δede* culture supernatant had completely lost antimicrobial ability against *R. solanacearum* (Fig. 2D-1) and *B. subtilis* (Fig. 2D-2). High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis demonstrated abolished production of edeines A and B in the crude extract of the supernatant of X23*Δede* culture compared with X23-WT (Fig. 2E and F). Taken together, these results suggested that the identified *ede* BGC was responsible for the production of antibiotic edeines A and B in *B. brevis* X23.

Construction of promoter-engineered B. brevis *X23 strains*

The present study showed that all the edeine biosynthetic genes in *B. brevis* Vm4 (*edeA-edeP*) were present on the same *ede* operon, which shared a single promoter, P_{ede} (Fig. 3). Therefore, we hypothesized that it would be possible to improve the expression of the edeine BGC by replacing the original promoter P_{ede} with stronger promoters. Thus, six strong promoters that are commonly used in *B. brevis* and *B. subtilis*, including three constitutive promoters ($P_{shuttle\text{-}09}$ (Yang *et al*., 2013), P43 (Ye *et al*., 1999) and P_{mwp} (Mizukami *et al*., 2010)) and three inducer-free promoters from inducible promoters (P_{xylA} (Kim *et al*., 1996), P_{grac} (Phan *et al*., 2006) and P_{spc} (Yansura and Henner, 1984)) generated by removing the repressor protein component, were selected to replace the original P_{ede} promoter to improve edeine production (Tables S2 and S3). By double-crossover homologous recombination via temperature-sensitive integration vectors (Fig. S1) carrying the homologous regions from upstream and downstream of the P_{ede} region of X23-WT, the original promoter P_{ede} was replaced with either one of the abovementioned six promoters (Fig. 3). The transformants were first identified by PCR (Fig. S2) and confirmed by DNA sequencing. The transformants were designated X23 (P_{ede}::P_{mwp}), X23 (P_{ede}::P_{xylA}), X23 (P_{ede}::P_{43}), X23 (P_{ede}::$P_{shuttle\text{-}09}$), X23 (P_{ede}::P_{grac}) and X23 (P_{ede}::P_{spc}).

Promoter substitutions affected the growth of B. brevis *X23*

To evaluate the effects of promoter substitution on the growth of *B. brevis*, we compared the growth curves of the X23-WT and its six promoter-engineered strains by measuring the OD_{600} of the corresponding culture broths every 12 h (Fig. 4A). No significant difference in cell density was identified between the transformants and the X23-WT strain at 12 h (exponential phase) and 24 h (stationary phase) of cultivation. However, the cell turbidity (OD_{600}) of the six promoter-engineered strains was 7.5–15.3% lower than that of the X23-WT at 36 and 48 h (decline phase) (Fig. 4B), suggesting that the promoter substitution affected both the growth and biomass of *B. brevis* X23. The results also revealed that the increased antimicrobial activity observed was not due to the biomass increment.

Promoter substitutions significantly improved the antibacterial activity of B. brevis *X23*

Given that *ede* BGC disruption abolished the antibacterial activity of *B. brevis*, we speculated that edeines were the major antibacterial substances in fermentation supernatants of X23-WT when cultured in NB media. Thus, the yield of edeines was roughly consistent with the antimicrobial activity of *B. brevis* X23. Although edeines have broad inhibitory activity against Gram-positive and

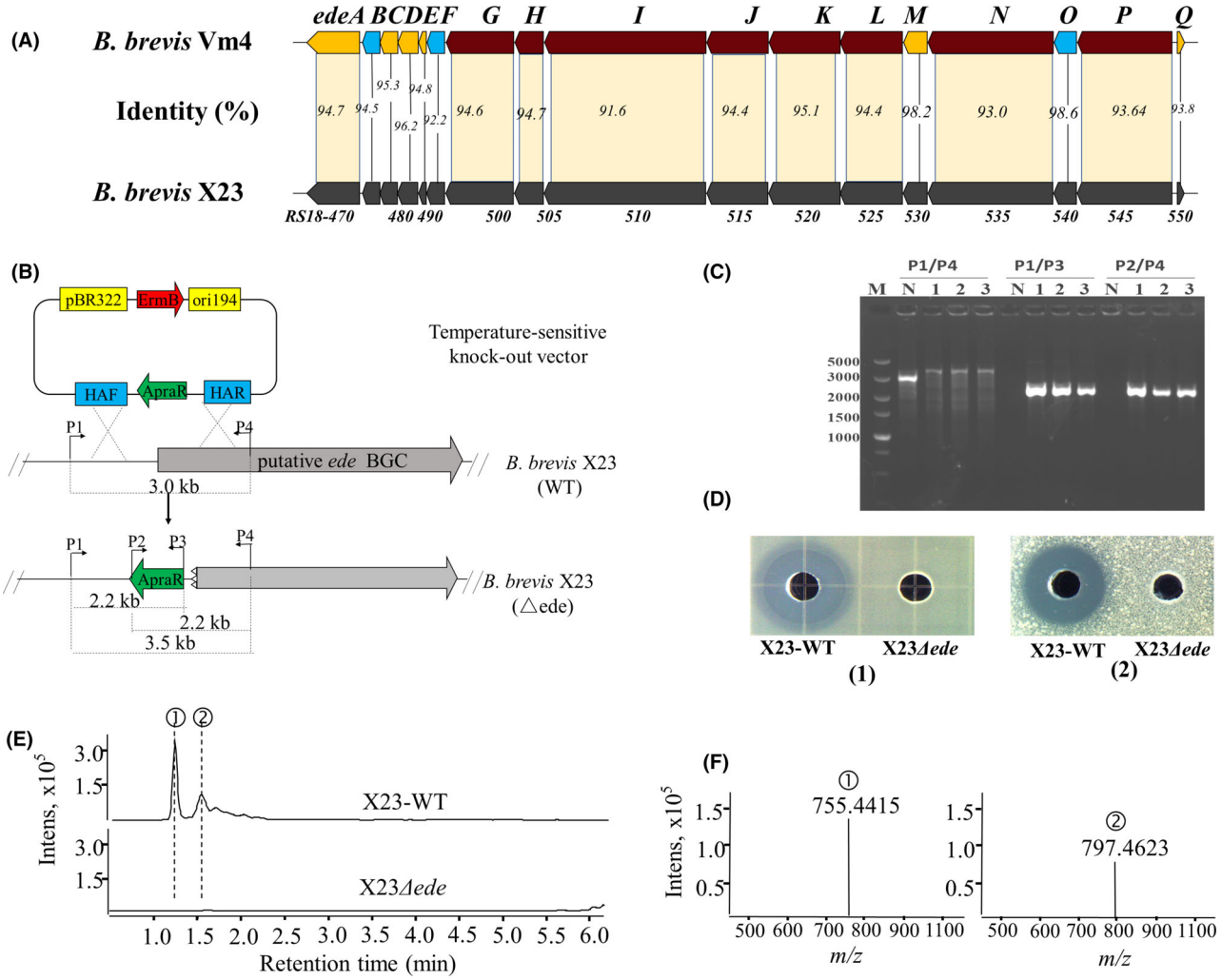


Fig. 2. Antimicrobial activity and HPLC-MS analysis of X23-WT and X23Δ*ede* strains.
A. Comparison of the *ede* BGC between *B. brevis* Vm4 (upper panel, form *edeA* to *edeQ*) and *B. brevis* X23 (lower panel, from locus RS18470 to locus RS18550). The sequences were compared by NCBI BLAST analysis. The percentage identity is displayed between homologous genes.
B. Construction and confirmation of *ede* BGC disruption in *B. brevis* X23. The temperature-sensitive knockout vector was constructed via Red/ET recombination, and then the vector was transformed into *B. brevis* X23 by electroporation. The *ede* BGC was disrupted with the apramycin resistance gene (Apr^R) through double-crossover homologous recombination. X23-WT indicates the wild type strain of *B. brevis* X23. X23Δ*ede* indicates the *ede* BGC disruption strain of *B. brevis* X23. P1 to P4 indicate the detection primer for the colony PCR to detect correct transformants.
C. Confirmation of the appropriate transformant of the *ede* BGC disruption *B. brevis* strains by PCR. M, DL 5000 marker; N, X23-WT; 1–3, transformant 1–3; P1/P4: PCR products using primer pairs P1/P4 (3.0 kb); P1/P3: PCR products using primer pairs P1/P3 (2.2 kb); P2/P4: PCR products using primer pairs P2/P4 (2.2 kb).
D. Antimicrobial activity assays of the fermentation broth of X23-WT and X23Δ*ede* strains against *B. subtilis* (1) and *R. solanacearum* (2).
E. HPLC-MS analysis (extracted ion chromatogram (EIC) 750-800), showing edeine A and B retention times at 1.2 and 1.5 min respectively (dotted lines); ①, edeine A; ②, edeine B.
F. Mass spectrogram of edeines A and B, in which the molecular ions $[M + H]^+$ were 755.4415 and 797.4623 respectively.

Gram-negative bacteria, *B. subtilis* was chosen to evaluate the antibacterial activity of all *B. brevis* strains because it exhibited the highest sensitivity to edeines based on our previous results (Lu, 2014). Therefore, to evaluate the effects of promoter substitutions on the yield of edeines, we first performed plate inhibition assay to assess the antibacterial activity of X23-WT and six promoter-engineered strains against *B. subtilis* 168 after 6, 9, 12, 24, 36 and 48-h incubations. The results showed an increased radius of the inhibition zone for all six promoter-engineered strains compared with X23-WT (Fig. 5A). After 48 h of incubation, the radii of the inhibitory zones of X23(P_{ede}::P_{mwp}), X23(P_{ede}::P_{grac}), X23(P_{ede}::$P_{shuttle-09}$), X23(P_{ede}::P_{spc}), X23(P_{ede}::P_{xylA}) and

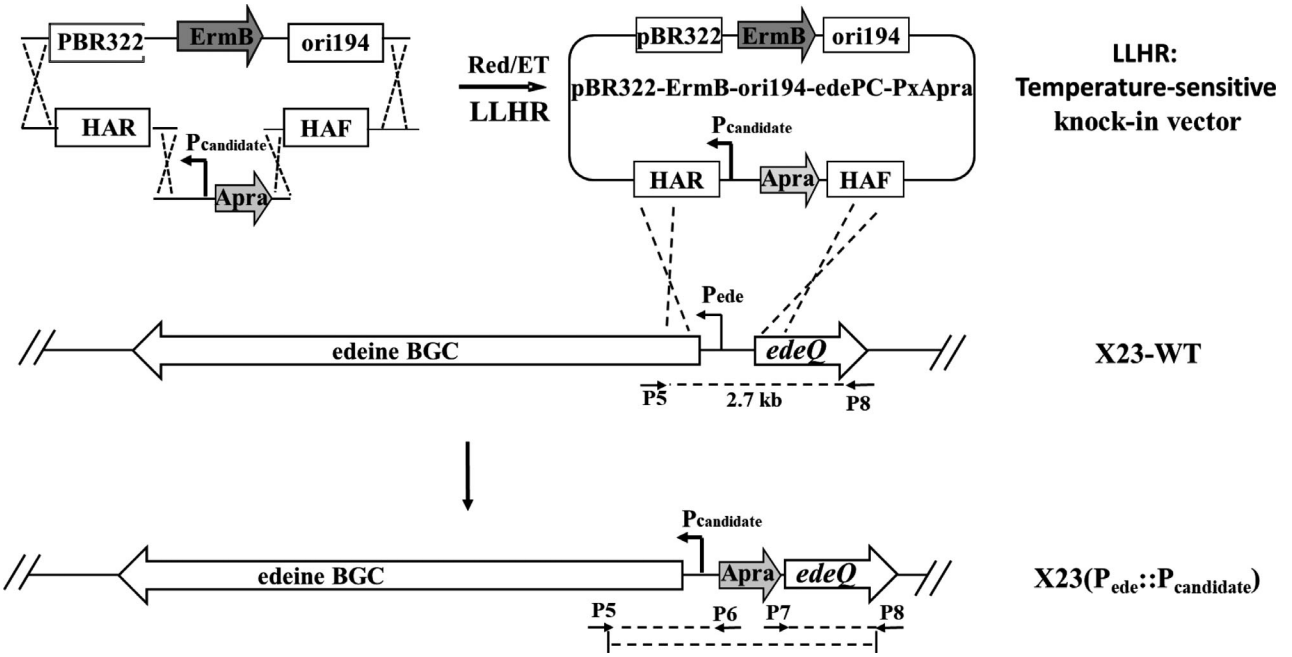


Fig. 3. Schematic diagram of promoter engineering of the *ede* BGC in X23-WT. A temperature-sensitive knock-in vector was constructed using Red/ET recombination. LLHR is the abbreviation of linear to linear homologous recombination. The homologous arms on the vector cross-exchange with homologous sequences on the genome of *B. brevis* X23. The promoter-engineered strain was generated. P_{ede} indicates the promoter of the *ede* BGC. $P_{candidate}$ indicates the six substituted promoters. X23(P_{ede}::$P_{candidate}$) is the promoter-engineered strain of *B. brevis* X23. P5 to P8 indicate the detection primer for the colony PCR to detect correct transformants.

X23(P_{ede}::P_{43}) were increased by 1.3 ± 0.2, 1.2 ± 0.2, 1.1 ± 0.2, 1.0 ± 0.1, 1.0 ± 0.2 and 0.9 ± 0.1-fold, respectively, compared with the X23-WT strain (Fig. 5B and C). Of note, there was no significant difference in the radius of inhibition zone between the promoter-engineered X23(P_{ede}::P_{mwp}) and X23(P_{ede}::P_{grac}) strains. Taken together, these results showed that promoter substitutions improved the antibacterial activity of *B. brevis* X23 against *B. subtilis* 168, and promoters P_{mwp} and P_{grac} achieved the highest antimicrobial activities among the six promoters. The results also suggested that the promoter-engineered strains produced antimicrobial substances earlier than X23-WT.

Promoter substitutions significantly increased the expression of ede *BGC*

To evaluate the effects of promoter substitutions on the expression of *ede* BGC, we assessed the transcriptional level of *edeP* gene based on quantitative real-time PCR (qRT-PCR). Compared with X23-WT, significantly increased expression of *ede* BGC was detected in the six promoter-engineered strains with *ede* promoter substitutions after 12, 24 and 36 h of culture (Fig. 6).

In the early growth stage (12 h), the level of *ede* BGC transcription in X23-WT was extremely low because the expression of secondary metabolites such as edeines was repressed during the primary metabolic period by the global regulatory systems. In contrast, *ede* BGC expression in the promoter-engineered strains was not limited by these regulatory systems, resulting in 2.9 ± 0.4 to 20.5 ± 1.2-fold higher expression of the *ede* BGC in the early stage compared with X23-WT. The highest (15.4 ± 1.9-fold compared with X23-WT) level of *ede* BGC expression after 12 h of culture was detected in X23(P_{ede}::P_{mwp}). In the middle stage (24 h), expression of the *ede* BGC in X23-WT was increased by 3.6 ± 0.8-fold compared with that at 12 h. The highest (14.6 ± 1.0-fold compared with X23-WT) expression of *ede* BGC was detected in X23(P_{ede}::P_{grac}) after 24 h of culture.

In the late period (36 h), expression of the *ede* BGC in X23-WT rapidly decreased to 0.4 ± 0.1 (compared with that at 12 h), while in the six promoter-engineered strains, it was still 3.1 ± 0.2 to 20.5 ± 1.2-fold higher than that in X23-WT. The highest (20.5 ± 1.2-fold compared with X23-WT) expression of *ede* BGC was detected in X23(P_{ede}::P_{grac}).

Taken together, these results showed that expression of the *ede* BGC was significantly increased by the promoter substitutions, and promoters P_{mwp} and P_{grac} provided the highest expression of the *ede* BGC.

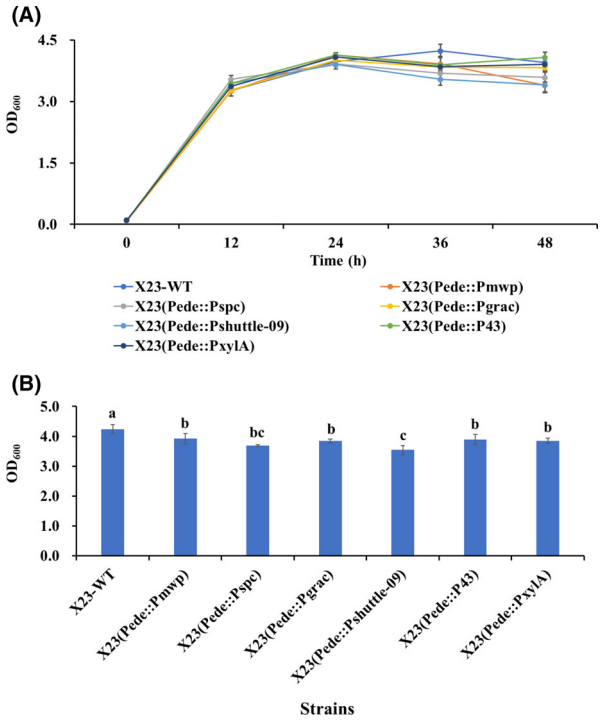


Fig. 4. Growth curves of X23-WT and promoter-engineered *B. brevis* strains.
A. Growth curves of the promoter-engineered strains and X23-WT from 0 to 48 h.
B. Significance of the growth curves at 36 h. Different letters indicate statistically significant differences ($P < 0.05$). P_{ede} indicates the promoter of *ede* BGC. X23-WT, *B. brevis* X23. X23(P_{ede}::P_{mwp}), the promoter-engineered strain in which P_{ede} was replaced by P_{mwp}. X23(P_{ede}::P_{spc}), the promoter-engineered strain in which P_{ede} was replaced by P_{spc}. X23(P_{ede}::P_{grac}), the promoter-engineered strain in which P_{ede} was replaced by P_{grac}. X23(P_{ede}::$P_{shuttle-09}$), the promoter-engineered strain in which P_{ede} was replaced by $P_{shuttle-09}$. X23(P_{ede}::P_{43}), the promoter-engineered strain in which P_{ede} was replaced by P_{43}. X23(P_{ede}::P_{xylA}), the promoter-engineered strain in which P_{ede} was replaced by P_{xylA}.

Quantitative analysis of edeine production in promoter-engineered strains by HPLC-MS analysis

While the promoter substitutions were associated with improved antibacterial activity of *B. brevis* X23, it was not clear whether the improved antibacterial activity was due to increased production of edeines. We compared the production of edeines between the X23-WT and promoter-engineered strains based on HPLC–MS analysis (Fig. 7A and B).

We purified edeines A and B from the culture broth of *B. brevis* X23 because the standard compounds are not commercially available. HPLC and high-resolution mass spectrometry (HRMS) were conducted to determine the purity of edeines A and B (Figs S3 and S4). The structure of edeine A was confirmed by nuclear magnetic resonance (NMR) spectroscopy (Figs S5 and S6). We further determined the absolute yield of edeines A and B using standard curve-based quantitative analysis (Fig. S7). As shown in Fig. 7C, the amounts of edeines A and B were significantly higher in the fermentation broth of promoter-engineered strains compared with X23-WT after 48 h of culture, which was consistent with the antimicrobial activity assays. Compared with X23-WT, the yields of edeines A and B produced by X23(P_{ede}::P_{mwp}), X23(P_{ede}::P_{grac}), X23(P_{ede}::$P_{shuttle-09}$), X23(P_{ede}::P_{spc}), X23(P_{ede}::P_{xylA}) and X23(P_{ede}::P_{43}) were increased by 8.7 ± 0.6, 8.0 ± 0.3, 4.4 ± 0.5, 7.2 ± 0.6, 7.9 ± 0.4 and 3.6 ± 0.1-fold respectively (Fig. 7C and Table S4). The highest yield of edeine A (83.4 mg l^{-1}) was detected in X23(P_{ede}::P_{mwp}) compared with the X23-WT strain (9.4 mg l^{-1}), which was consistent with the antimicrobial test and gene expression of the *ede* BGC (Table S4). In contrast, an increased yield of edeine B was only detected in X23 (P_{ede}::P_{xylA}) (0.3 mg l^{-1}) compared with the X23-WT strain (0.2 mg l^{-1}). The total yield of edeines (edeine A and B) reached 83.59 mg l^{-1} in X23(P_{ede}::P_{mwp}) (Table S4). Given that the yield of edeine B was much lower than that of edeine A and that edeine B contains an unusual guanyl-spermidine reside rather than spermidine, we speculated that the availability of the precursor guanyl-spermidine might limit edeine B production.

Discussion

Although edeines were identified over 60 years ago, the biosynthetic pathway of edeines has remained ambiguous, thus limiting improvements of edeines yield through biosynthetic pathway modification. Westman *et al.* first identified the *ede* BGC in the *B. brevis* Vm4 genome based on genomic analyses (Westman *et al.*, 2013). In this study, we confirmed the functionality of the *ede* BGC in *B. brevis* X23 through gene disruption. Identification and confirmation of the *ede* BGC allowed us to significantly improve the production of edeines through promoter engineering.

We replaced the original promoter of the *ede* BGC with stronger constitutive promoters to increase expression of the *ede* BGC and improve edeine production. Transcriptomic analyses (data not shown) and the RT-PCR assay (Fig. 6) indicated that the original promoter of *ede* BGC might be controlled by global secondary metabolic regulatory factors, and high-level expression of *ede* BGC was not reached until the mid-late stage (24 h). The three constitutive stronger promoters tested in this study, P_{43}, P_{mwp} and $P_{shuttle-09}$, mediate expression of genes that encode enzymes involved in primary metabolism and cell growth, which are active most of the time and are not controlled by secondary metabolic regulators (Ye *et al.*, 1999; Mizukami *et al.*, 2010). Therefore, the stronger constitutive promoters significantly increased the expression of *ede* BGC and the antimicrobial activity (edeine production) from the early growth stage.

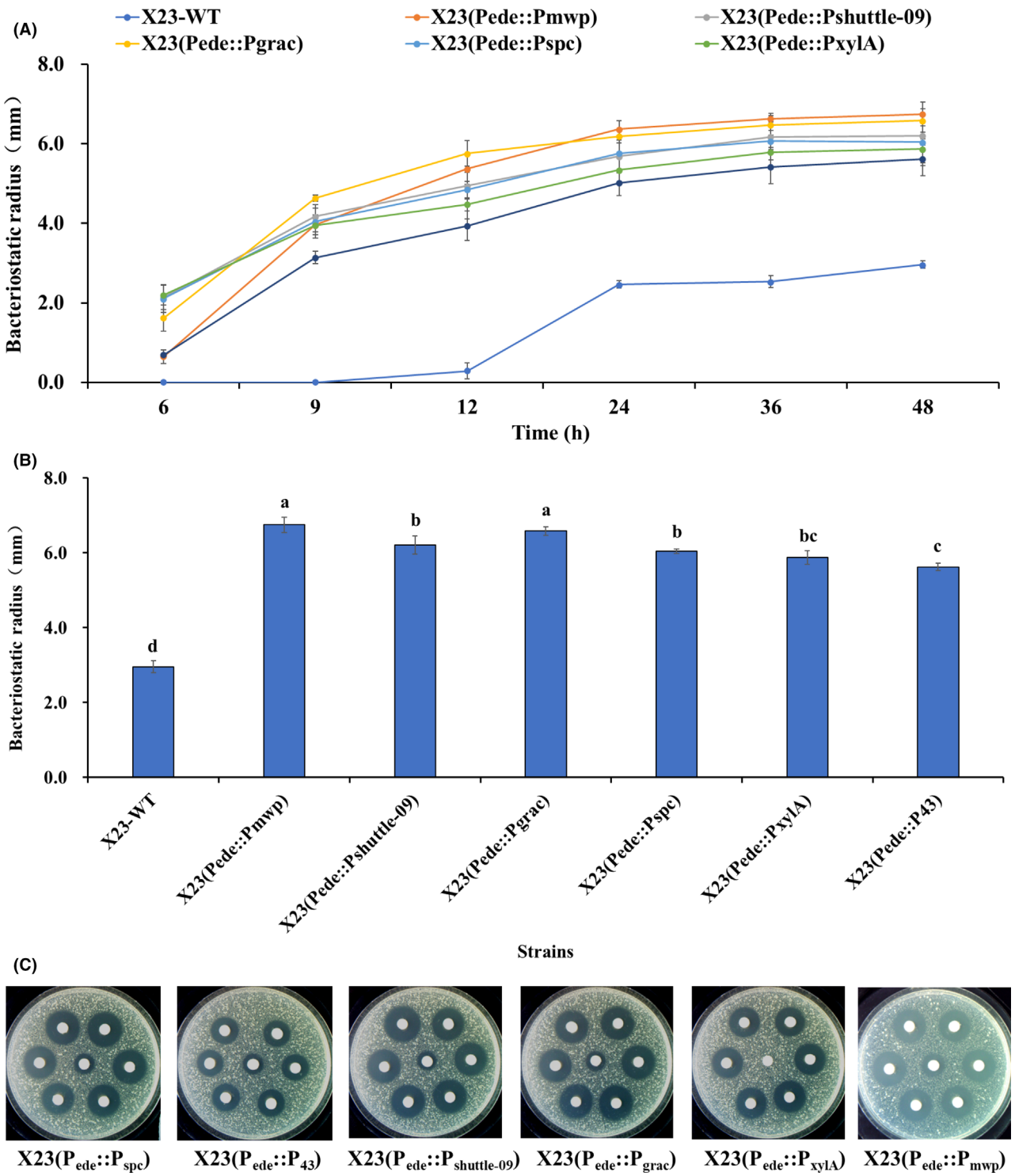


Fig. 5. Antibacterial ability of X23-WT and promoter-engineered *B. brevis* strains.
A. Bacteriostatic ability curve of the fermentation broth of X23-WT and promoter-engineered *B. brevis* strains after incubation from 6 to 48 h.
B. Significance of the differences between bacteriostatic radii at 48 h. Different letters indicate statistically significant differences ($P < 0.05$).
C. Plate antimicrobial assays of fermentation broth of the promoter-engineered strains (periphery) and X23-WT(center) against *B. subtilis* incubation for 48 h. All broths were diluted five times, and 10 μl of each dilution was added to the center of the paper disk. Experiments were performed in triplicate.

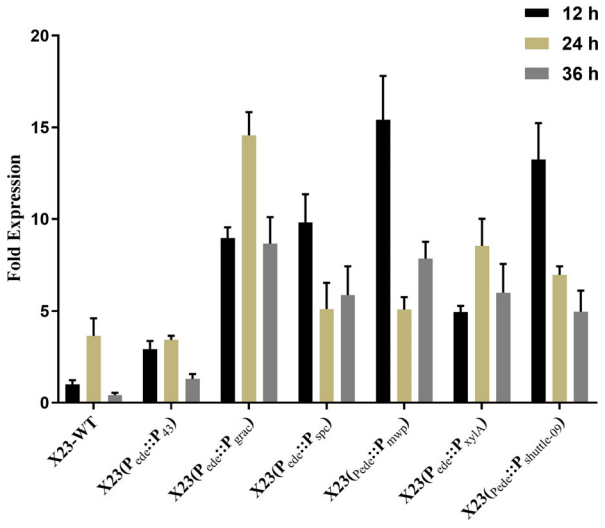


Fig. 6. Relative expression of *edeP* on the *ede* BGC from various promoter-engineered strains and X23-WT at 12, 24, and 36 h as measured by qRT-PCR. The cells of different strains were cultured in NB and incubated at 30°C for 12, 24, and 36 h. Total RNA was isolated for qRT-PCR assays. X23-WT was used as the control. The 16S rRNA was used for data normalization. Experiments were performed in triplicate.

Three chemically inducible promoters, including P_{xylA}, P_{spc} and P_{grac}, were also tested in this study. Specific stimuli (xylose for P_{xylA}; IPTG for P_{spc} and P_{grac}) are required to switch on these chemically inducible promoters (Yansura and Henner, 1984; Kim *et al*., 1996; Phan *et al*., 2006), which increases the cost of fermentation. Chemically inducible promoters are usually companied by a regulatory gene encoding a repressor protein that binds to the promoter upstream, blocking the transcription of downstream genes in the absence of inducers. Therefore, chemically inducible promoters may achieve constitutive expression when the repressor gene is disrupted. Tran *et al*. constructed an inducer-free expression plasmid with the IPTG-inducible promoter P_{grac} for *B. subtilis* by disrupting the repressor *lacI* gene (Tran *et al*., 2017). We constructed a reporter gene plasmid system in *B. brevis* X23-WT based on the green fluorescent protein (*gfp*) gene to explore whether the P_{xylA}, P_{spc} and P_{grac} promoters could be transformed into inducer-free promoters in *B. brevis*. The results showed that the promoters without repressor genes resulted in constitutive expression of the *gfp* gene in the absence of their inducers (Fig. S8). Subsequently, RT-PCR also revealed constitutive expression of the *ede* BGC in *B. brevis* promoter-engineered strains containing the P_{xylA}, P_{spc} or P_{grac} promoter throughout the growth period (Fig. 6).

Of the six promoters, the P_{mwp} promoter resulted in the most significant expression of the *ede* BGC and the highest edeine production. This impressive power of the P_{mwp} promoter in driving the expression of *ede* BGC might be explained as follows. First, the promoter of the cell wall protein gene (HWP) in *B. brevis* HPD31 has long been regarded as an extremely strong promoter in the exponential period, and it is used in the *Brevibacillus* Expression System by TaKaRa Biomedical Technology Co., Ltd (Mizukami *et al*., 2010). This expression system has been widely applied for the robust expression of a large number of genes encoding enzymes, antigens and cytokines, such as α-amylase, sphingomyelinase, xylanase, surface antigen, epidermal growth factor and interferon-γ (Mizukami *et al*., 2010). The P_{mwp} promoter was derived from a middle cell wall protein (MWP) gene, which is the homolog of HWP (78% identity in *B. brevis* X23) (Ebisu *et al*., 1990). Second, based on transcriptomic analyses of *B. brevis* X23 in the exponential growth period, the MWP gene exhibited the highest transcription among all 6291 coding genes. The expression level of the MWP gene was 18.6 $\pm$ 0.8-fold higher than that of the *ede* BGC (data not shown), suggesting that the MWP promoter is a strong promoter with the potential to improve edeine production in *B. brevis*. Indeed, the P_{mwp} promoter increased transcription of *edeP* in *B. brevis* X23 15.4 $\pm$ 1.9-fold after 12 h of incubation, which was consistent with our previous abovementioned observation. Taken together, replacing the original promoter with stronger promoters is an efficient way to improve the production of natural products in bacteria.

Interestingly, the yield of edeine B was not significantly increased or even decreased after replacing the original promoter with stronger promoters. Previously, Shimotohno *et al*. identified and purified edeine B_1 amidinohydrolase, which can transform edeine B to edeine A. They speculated that edeine B was the intermediate product of edeine A (Shimotohno *et al*., 1999). This speculation could explain the markedly reduced production of edeine B compared with edeine A or even its decrease following promoter engineering. In addition, edeine B contains an uncommon guanyl-spermidine substituted by spermidine. Thus, we speculated that low contents of the guanyl-spermidine might limit edeine B production. A number of strategies from previous studies, such as optimization of the culture media, overexpression of the precursor synthesis pathway and modification of regulatory factors (Zhang *et al*., 2016), can be applied to further improve the production of edeine A.

In this study, we successfully improved edeine A production in *B. brevis* X23 by replacing the original *ede* BGC promoter with strong promoters. Of the six strong promoters, P_{mwp} generated the highest yield of edeines (83.6 mg l^{-1}). To the best of our knowledge, this is the first report to examine the improvement of edeine production through genetic modification of the *ede* BGC. Based on these results, we are further exploring the edeine biosynthetic pathway and novel derivatives through structural modifications.

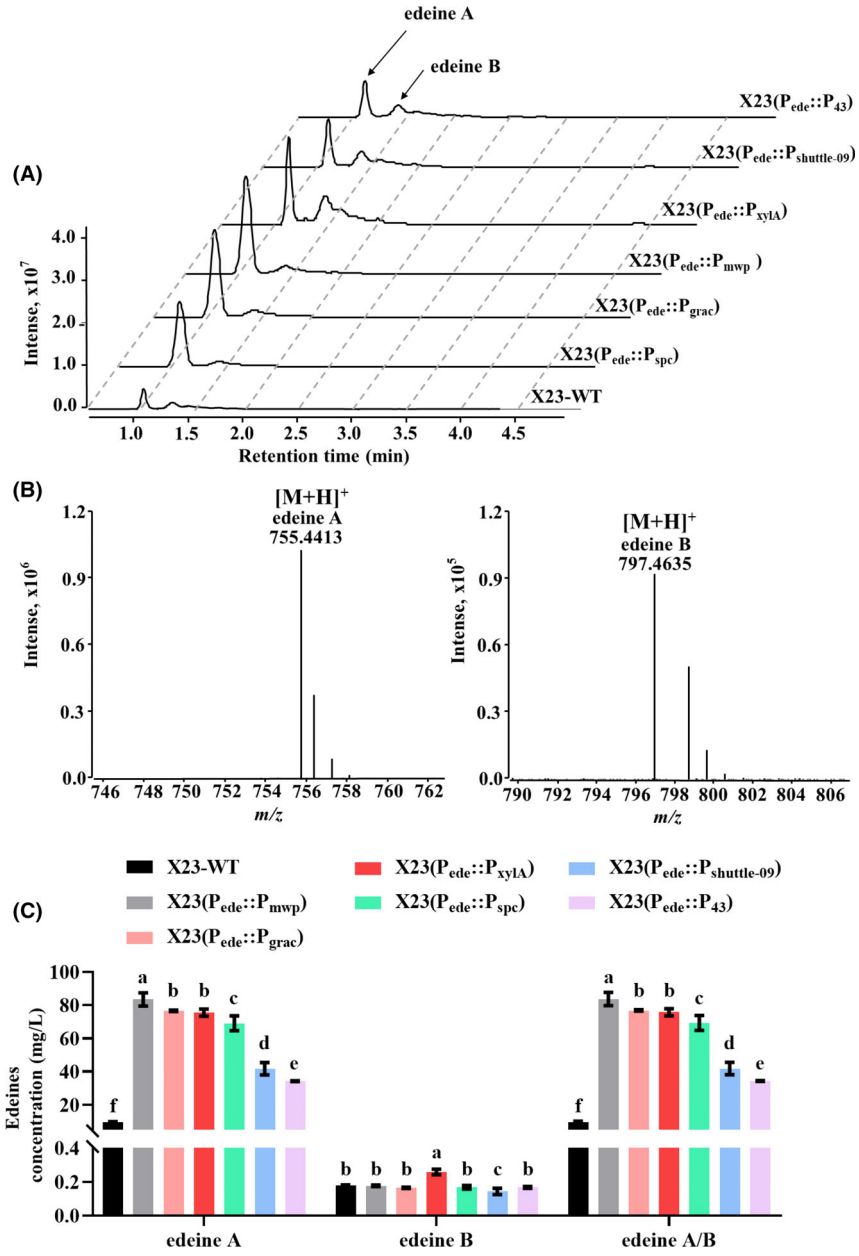


Fig. 7. Detection and analysis of edeines of the X23-WT and promoter-engineered *B. brevis* strains by HPLC-MS.
A. Comparison of edeine yield between X23-WT and promoter-engineered strains. The HPLC-MS analysis retention times of edeines A and B were 1.2 and 1.5 min respectively (indicated by arrows).
B. Mass spectrogram of edeines A and B. According to the mass spectrogram of edeines A and B, the molecular ions $[M + H]^+$ were 755.4413 and 797.4635 respectively.
C. Yield changes of edeines A and B of theX23-WT and promoter-engineered *B. brevis* strains. Data are presented as averages of three independent experiments. Error bars indicate standard deviations (SD). Different letters indicate statistically significant differences ($P < 0.05$).

Materials and methods

Bacterial strains, plasmids and promoters

All strains and plasmids used in this study are listed in Table S1. The original edeine producer *B. brevis* X23 was isolated from tobacco rhizosphere soil by our laboratory (Chen *et al.*, 2012). The primer sequences and characteristics of the six promoters used to replace the original *ede* BGC promoter are shown in Tables S2 and S3. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table S5). All restriction enzymes were purchased from New England Biolabs

(Beijing, China). Taq polymerase and DNA marker were purchased from TaKaRa Bio Inc. (Dalian, China).

The *E. coli* and *B. brevis* strains were cultured at 37°C or 30°C, respectively, in solid or liquid Luria-Bertani (LB) media (Green and Sambrook, 2012) consisting of 1% (w/v) tryptone, 0.5% yeast extract and 1% NaCl. The pH of the LB media was adjusted to 7.0. For solid media, 1.5% (w/v) agar was supplemented. For *E. coli*, apramycin (20 $\mu g\ ml^{-1}$) and ampicillin (100 $\mu g\ ml^{-1}$) were added to the media as required. Promoter-engineered *B. brevis* strains were selected on LB media containing apramycin (10 $\mu g\ ml^{-1}$) and erythromycin (1.5 $\mu g\ ml^{-1}$).

Brevibacillus brevis strain fermentation cultures consisted of Nutrient-Broth (NB) media (Green and Sambrook, 2012) containing 1% (w/v) tryptone, 0.3% beef extract, 0.5% NaCl and 1% glucose. The pH of the NB media was adjusted to 7.0.

Construction of ede *disruption and promoter-substituted mutants of* B. brevis *X23*

Genomic DNA was isolated from *B. brevis* X23 and *B. subtilis* 1A751 using an EZ-10 Spin Column Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). The primers used in this study are shown in Table S5. The upstream and downstream homologous regions were amplified from *B. brevis* X23 using PCR with primers edePC-F1/edePC-F2 and Pmwp-ede-R1/edePC-R2 respectively. The apramycin resistance gene ($Apra^R$) was amplified from the pSET152 vector using PCR with primers Apra1(edePC) and Apra2(edePmwp). The P_{mwp} promoter was amplified from *B. brevis* X23 using PCR with primers Pmwp-ede-1/Pmwp-ede-2. The P_{43} promoter was amplified from *B. subtilis* 1A751 using PCR. Other promoters, including P_{grac} (Phan *et al.*, 2006), P_{spc} (Yansura and Henner, 1984), P_{xylA} (Kim *et al.*, 1996) and P_{43} (Ye *et al.*, 1999), were amplified from the different plasmids. The $P_{shuttle\text{-}09}$ was synthesized and cloned into plasmid pUC18 by Hunan Tsingke Biocompany, Ltd. (Changsha, China) according to a previous study (Yang *et al.*, 2013).

Thereafter, the fused fragments of the corresponding promoter and $Apra^R$ were amplified using overlapping extension PCR with primers Apra1(edePC)/Pmwp-ede-2 (Yan *et al.*, 2008). The pE194 vector backbone was amplified from the temperature-sensitive plasmid pBR322-ErmB-ori194 using primers pE194-F/pE194-R. Every four fragments (i.e. the promoter-$Apra^R$, pE194 vector backbone and P_{ede} upstream and downstream homologous arms) with 45-bp overlapping sequences in both terminal regions were co-transformed into GB05dir, an *E. coli* strain LLHR-proficient recombinase (RecET, Redγ and RecA), to construct a promoter-substitution plasmid according to previous studies (Yu *et al.*, 2000; Zhang *et al.*, 2000; Muyrers *et al.*, 2001; Sharan *et al.*, 2009) (Figs S2 and S3). Promoter-engineered *B. brevis* strains were selected on LB solid media using 20 $\mu g\ ml^{-1}$ apramycin. Selected recombinant vectors were confirmed by restriction enzyme digestion and sequencing, namely pE194-edePC-P_{xylA}-Apra, pE194-edePC-Apra-P_{mwp}, pE194-edePC-Apra-$P_{shuttle\text{-}09}$, pE194-edePC-Apra-P_{grac}, pE194-edePC-Apra-P_{spc} and pE194-edePC-Apra-P_{43}.

The six promoter substitution vectors were transferred into *B. brevis* X23 by electroporation according to the previous methods (Takahashi *et al.*, 1983; Okamoto *et al.*, 1997). After double-crossover homologous recombination, the original promoter of *ede* BGC was substituted with each one of the six promoters as illustrated in Fig. 3. Recombinants were selected on LB agar containing 10 $\mu g\ ml^{-1}$ apramycin and confirmed by PCR amplification with the specific primers on the 5'(P1/P2) and 3'(P3/P4) ends of the replacement site. The PCR amplicons were sequenced to verify the inserted promoter sequence. The promoter-engineered strains were designated as X23(P_{ede}::P_{mwp}), X23(P_{ede}::P_{xylA}), X23(P_{ede}::$P_{shuttle\text{-}09}$), X23(P_{ede}::P_{grac}), X23(P_{ede}::P_{spc}) and X23(P_{ede}::P_{43}) respectively.

Assessment of bacterial growth

The growth curves of the X23-WT and six promoter-engineered *B. brevis* strains were evaluated according to a previous study (Jung *et al.*, 2012). Briefly, a single colony of each strain was cultured in 1.0 ml of liquid LB media at 30°C overnight and then transferred into 50 ml of fresh liquid fermentation media (NB) to reach an initial optical density (OD_{600}) of 0.1 and measured using a spectrophotometer T6 (Puxi, China) every 12 h for 48 h. The fermentation experiments were repeated three times, and each measurement was performed three times in parallel.

Evaluation of antimicrobial activity

Bacillus subtilis 168 was used to evaluate the antimicrobial activity of *B. brevis* X23 and the six promoter-engineered *B. brevis* strains through Kirby-Bauer disk diffusion according to a previous study (Gui *et al.*, 2019). The *B. brevis* culture in liquid NB was collected every 12–48 h. After centrifugation at 9000 *g* for 10 min at 4°C, the supernatant was passed through a filter (0.22 μm) to obtain a sterile filtrate. *B. subtilis* 168 was spread on LB agar plates after dilution to an OD_{600} of 0.1. A sterile paper disk with a diameter of 6.0 mm was placed on each plate. Each filtrate (10 μl) was added to the center of the paper disk. Sterile LB liquid media served as a negative control (Gui *et al.*, 2019). The plates were incubated for 24 h at 30°C, and the inhibition zone was measured. The antimicrobial activity was

evaluated based on comparing the radius of inhibition zone. The tests were repeated three times, with three parallel tests each time.

Evaluation of edeine production through HPLC–MS

To determine the concentration of edeine produced by the X23-WT and promoter-engineered *B. brevis* strains, edeines were extracted from bacterial culture and analyzed by HPLC–MS. Edeines were extracted according to the previous study, with some modifications (Westman *et al*., 2013).

A 100-ml culture of the X23-WT or promoter-engineered *B. brevis* strains was grown in a 500-mL flask at 30°C with rotation (180 r.p.m.) for 48 h. The supernatant was obtained by centrifugation at 9000 *g* for 10 min at 4°C (5910R, Eppendorf), combined with 5 ml of WorkBeads 100S (Bioworks) and stirred for 2 h. The resin was then filtered from the supernatant and packed into a 300/15 column. The column was washed with 200 ml of distilled water, and then edeines were eluted with 15 ml of ammonium hydroxide (0.1 M). The eluents were pooled, lyophilized and redissolved into 1 ml of distilled water to obtain the crude edeines extracts for further HPLC analysis. The HPLC system (Agilent 1260) was performed using an ODS column (Agilent XBD-C18, 2.1 $\times$ 100 mm, 5 μm) with gradient elution. The UV spectra were detected on a diode array detector with wavelengths ranging from 200 to 600 nm. HPLC parameters were as follows: solvent A, H_2O with 0.1% TFA; solvent B, methanol; gradient at a constant flow rate of 1 ml min^{-1}; 0–18 min, 10% B, 18–20 min, 10–95% B, 20–22 min, 95% B, 22–25 min, 10% B; detection by UV spectroscopy at 278 nm. HPLC–MS analyses were performed using quadrupole time-of-flight mass spectrometry (UPLC–QTOF; Agilent 6545) equipped with qualitative analysis software. Mass spectra were acquired in centroid mode ranging from 200 to 1500 *m/z* in positive ionization mode with auto MS^{2+} fragmentation.

Edeines A and B were purified according to a previous study (Westman *et al*., 2013). The purity was determined by HPLC and HPLC–MS–MS. The structure was determined by NMR spectroscopy with a Bruker Avance 600 spectrometer at 600 MHz (^{1}H) and 150 MHz (^{13}C). The standard curves of edeines A and B were established by testing 0.5, 1, 50, 100 and 500 μg ml^{-1} edeine A (EIC 755.4 $\pm$ 0.5) and B (EIC 797.4 $\pm$ 0.5). The yields of edeines A and B were determined from the peak area (extracted ion chromatogram) by reference to the standard curve.

Extraction of total RNA and quantitative real-time PCR

The mRNA level of the first gene (*edeP*) on the *ede* operon in X23-WT and the six promoter-engineered *B. brevis* strains was determined by RT-PCR. Bacterial cells cultivated for 12, 24, 36 and 48 h were collected and rinsed twice with physiological saline. Total RNA was isolated from bacterial culture using the EasyPure® RNA Kit (TransGen Biotech Co., LTD, China) according to the manufacture's protocol and reverse-transcribed into cDNA using the EasyScript® One-Step gDNA Removal and cDNA Synthesis kit (TransGen Biotech Co., LTD, China).

We conducted qRT-PCR using Fast SYBR Green Master Mix (2$\times$ Universal SYBR Green Fast For qPCR Mix, Abclonal, China) and the ABI-QS5 Real Time PCR detection system. Gene-specific primers were designed using Primer Premier 6.0 (primer sequences are listed in Table S5). The fluorescence change in SYBR Green I was monitored by the system software every cycle, and the ROX value was simultaneously used for correction. The threshold cycle (CT), melting curves and relative gene expression levels ($-\Delta^{CT}$) were automatically measured. The 16S rRNA gene was used as the internal reference gene. Real-time PCR assay was repeated three times, and each sample was assessed in triplicate.

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Conflict of interest

The authors declare no competing financial interests.

Author contributions

Q.L., L.Z., Y. W., X.B., W.C., Z.G. and D.L. designed the study; Q.L., L.Z., C.Z., T.L. and C.D. conducted the experiments; Q.L., L.Z., Z.G., Q.L., Y.T., J.D. and W.C. performed the data collection and analyses; Q.L. and L.Z. drafted the manuscript and W.C., X.B., L.D., A.L. and D.L. revised the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Table S2. Characterization of the six promoters used in this study.
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Fig. S8. Construction and functional analyses of the inducible promoters (P_{xylA}, P_{spc} and P_{grac}) with or without repressor protein using the *gfp* reporter gene system in *B. brevis* X23.

