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A Dual-Replicon Shuttle Vector System for Heterologous Gene Expression in a Broad Range of Gram-Positive and Gram-Negative Bacteria

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Abstract

Origin of replication (*ori* in theta-replicating plasmids or *dso* in rolling circle replicating plasmids) initiates plasmid replication in a broad range of bacteria. These two kinds of plasmids were both identified in *Streptococcus*, a genus composed of both human commensal bacteria and pathogens with the ability to cause severe community-acquired infections, including meningitides, septicemia, and respiratory tract diseases. Given the important roles of *Streptococcus* in the exchange of genetic elements with other symbiotic microbes, the genotypes and phenotypes of both *Streptococcus* spp. and other symbiotic species could be changed during colonization of the host. Therefore, an improved plasmid system is required to study the functional, complicated, and changeable genomes of *Streptococcus*. In this study, a dual-replicon shuttle vector system named pDRE was constructed to achieve heterologous gene expression. The vector system contained theta replicon for *Escherichia coli*. The origin of rolling circle replicon was synthesized according to pMV158 in Gram-positive bacteria. By measuring the products of inserted genes at multiple cloning sites, the ability of this vector system in the replication and expression of heterologous genes was assessed in four *Streptococcus* and three other Gram-positive bacteria: *Bacillus subtilis, Lactococcus lactis*, and *Staphylococcus aureus*. The results showed that the newly constructed vector could simultaneously replicate and express heterologous genes in a broad range of Gram-positive and Gram-negative bacteria, thus providing a potentially powerful genetic tool for further functional analysis.

Introduction

As one of the most widely distributed bacteria in the natural environment and human community, *Streptococcus* currently contains >100 recognized species [29]. Numerous *Streptococcus* species, such as *S. pneumoniae, S. pyogenes, S. agalactiae*, and *S. suis*, were considered as important human pathogens and could cause a broad range of diseases, such as pharyngitis, septicemia, pneumonia, meningitis, and toxic streptococcal syndrome [2, 33]. Notably, the re-emergence of *S. pyogenes* has been considered as a public health problem [33]. Other non-pathogenic *Streptococcus*, such as *S. thermophilus, S. oralis*, and *S. mutans*, have also been frequently isolated from human epidermis and cavities as commensal bacteria [20]. These non-pathogenic *Streptococcus* act as genetic donors for the phenotypic changes of pathogenic acceptors [12, 14]. However, limited by the technologies and efficient molecular tools available for the classification, conservation, and culture of *Streptococcus*, the functional analysis of its major genes associated with pathogenic mechanism, antimicrobial resistance, serotype, and other phenotypes is still insufficient [7, 10, 24]. Therefore, a powerful genetic tool with the ability to easily express certain genes could accelerate the study of functional genomics in these species.

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The most characterized genetic tool in the functional analysis of bacteria is plasmid, which was used to investigate the expression of native and heterologous genes in different microorganisms during functional genomics studies. In Gram-negative bacteria, thousands of plasmids were developed in the model species *Escherichia coli*, such as pEX1/2/3, pET-5a, and pKK223-3 [9]. The common structure of these plasmids contained a typical origin of replication, which initiated the theta-type replicons not only in *E. coli* but also in most Gram-negative bacteria [5]. In contrast, rolling circle replicating (RCR) plasmids are abundant in Gram-positive bacteria, although they have also been reported in many Gram-negative microorganisms and in archaea. *Streptococcus*, as a Gram-positive genus, mainly employed the RCR mechanism for the replication of circular plasmids [13]. A few non-integrative RCR plasmid vectors have been designed for this genus. They were derived from pVA380-1 (*S. orals*) or pMV158 (*S. agalactiae*) [15, 25]. In addition, several theta-replicated plasmids have also been isolated from Gram-positive bacteria, while a series of plasmid vectors have been designed for streptococcal species based on theta replicons [4].

To obtain an efficient plasmid with the ability to replicate and express heterologous genes in *Streptococcus*, a dual-replicon shuttle vector system was designed and synthesized in this study by combining the theta and RCR replicons. This vector system could be well maintained in both Gram-positive and Gram-negative bacteria, and could successfully express heterologous genes. This system may be widely used in the functional genetic analysis of *Streptococcus* and other Gram-positive bacteria.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

Fourteen bacterial strains and three plasmids were used in this study (Table 1). Gram-positive strains, including *Streptococcus, Bacillus subtilis, Lactococcus lactis*, and *Staphylococcus aureus*, were cultured on Columbia Blood Agar Base (Oxoid, Basingstoke, UK) supplemented with 8% sheep blood or a brain heart infusion (BHI) medium (Oxoid Ltd., Hampshire, UK). *E. coli* strain TOP10 (Invitrogen, Paisley, UK) was cultured on a Luria–Bertani (LB) medium. This study was approved by the local Ethics Committee of Beijing Ditan Hospital, Capital Medical University. No Human Participants or Animals were involved in this study.

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Characteristics	Origin
E. coli TOP10	F–*mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ (*ara leu*) 697 *galU galK rpsL* (StrR) *endA1 nupG*	Invitrogen
B. cereus ATCC11778	Wild type, erm^S chl^S	ATCC
L. lactis ACCC11092	Wild type, erm^S chl^S	ACCC
S. agalactiae ATCC12386	Wild type, erm^S chl^S	ATCC
S. agalactiae ATCC12403	Wild type, erm^S chl^S	ATCC
S. agalactiae ATCC12813	Wild type, erm^S chl^S	ATCC
S. agalactiae ATCC27956	Wild type, erm^S chl^S	ATCC
S. agalactiae ATCCBAA-611	Wild type, erm^S chl^S	ATCC
S. dysgalactiae ATCC35666	Wild type, erm^S chl^S	ATCC
S. pyogenes ATCC12344	Wild type, erm^S chl^S	ATCC
S. pyogenes ATCC12384	Wild type, erm^S chl^S	ATCC
S. pyogenes ATCC19615	Wild type, erm^S chl^S	ATCC
S. thermophilus ATCC 14485	Wild type, erm^S chl^S	ATCC
S. aureus ATCC 6538P	Wild type, erm^S chl^S	ATCC
pDRe	PHCAmpR carrying RCR replicon, maltose regulation elements	This study
pDRe (*erm/egfp*)	pDRe carrying erythromycin resistance gene and enhanced green fluorescent protein gene	This study
pSMART-LCKanR	Kan^R	Lucigen
pJ444-01	Amp^R	Addgene
pHCAmpR	Amp^R	This study
pMG36e	Erm^R	Addgene
pUC57-*egfp*	Amp^R with *egfp* gene	Lab collection

ACCC agricultural culture collection of China, *ATCC* American type culture collection

DNA Extraction and PCR Amplification

The bacterial genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A PCR strategy was implemented to detect the target gene in the plasmid or the transformed bacteria, and to amplify the functional elements of the plasmid using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Beverly, MA, USA) (Table S1).

Construction of Plasmids Using a Gibson Protocol

Two DNA segments were synthesized based on the sequence of pLS1ROM plasmid (accession number: JN381945.1) [22]. Gene synthesis method was applied by synthesizing reverse complementary short DNA oligos using an automatic DNA synthesizer firstly, then short double-strand DNA fragments were obtained by anneal and joined by an one-step thermocycled in vitro recombination system [1]. The large segment was assembled according to Gibson Assembly method [8]. Another DNA segment derived from pSMART-LCKan was amplified and linearized using PCR method.

A total of 100 ng of linear DNA fragments were mixed into a 20μL reaction system and assembled using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). The mixture was transformed into *E. coli* TOP10 competent cells via a chemical transformation method. Positive strains were screened using a LB medium containing 50 μg/ml ampicillin. The plasmid was purified with a GenElute Plasmid Miniprep Kit (Sigma, St Louis, USA) and validated using Sanger sequencing on an ABI 3730 machine (Applied Biosystems, Carlsbad, CA, USA). The sequence has been submitted to the GenBank database (accession number: MG799394). The primers designed for pDRE sequencing are listed in Table S2.

Cloning of Target Genes into the Constructed Vector

Two genes, the erythromycin resistance gene (*erm*) from plasmid pMG36e and the enhanced green fluorescent protein gene (*egfp*) from plasmid pUC57-*egfp* (Table 1), were amplified and cloned into the constructed vector to validate its function as an expression tool (Table S1). The two genes were inserted at BsaI restriction sites using Golden Gate Assembly Mix (New England Biolabs) and at *Hin*dIII/*Eco*RI restriction sites using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs), respectively. The plasmid was verified by restriction enzyme digestion.

Transformation of Gram-Positive Bacteria with the Vector Containing *erm*/*egfp*

Competent Gram-positive bacterial cells were exposed to a single electrical pulse generated by a Gene-Pulser (Bio-Rad Laboratories, Richmond, CA, USA). The electric field strength of the pulse was set at 12 kV/mm. The transformed cells were plated on Columbia Blood Agar Base supplemented with 8% sheep blood and subsequently screened using erythromycin. The concentration of erythromycin was 2 μg/mL for *L. lactis, Streptococcus* and *S. aureus*, and 20 μg/mL for *B. subtilis*.

Validation of the Expression of the Vector Containing *erm*/*egfp*

After transforming the target bacteria with pDRE containing *erm*/*egfp*, the expression of *erm* and *egfp* in the bacteria was detected to confirm the presence of the plasmid. Real-time PCR of *erm* was performed to measure the relative amount of the plasmid in transformed colonies [19]. The *16S rDNA* was used as the internal reference, and the relative DNA contents of the vector in the hosts were normalized as per chromosomic equivalent via multiplying the relative quantity by the copy number of *16S rDNA* in different bacterial genomes [31]. A similar real-time PCR protocol was used to detect the expression of target genes. The cDNA was obtained using an One-Step reverse-transcription-PCR kit (Qiagen), and 2×EvaGreenFast qPCR Master Mix (Biorise, Beijing, China) was used to amplify *erm* and *egfp*. The 16S rRNA was used as the internal reference. All samples were run on a Roche LightCycler 480 Instrument (Roche Diagnostics, Indianapolis, IN, USA). The green fluorescence was detected using a fluorescence microscope (Zeiss, Oberkochen, Germany). Fluorescence emission was recorded at 480 nm. Primers designed for plasmid validation are listed in Table S3. The $2^{-\Delta\Delta C_T}$ method was used to calculate the relative expression of the target genes (*erm*/*egfp*), the amplification efficiency of the *erm* and *16s rDNA* were tested [19], and the amplification curve is shown in Fig. S1.

Results and Discussion

Reconstruction of *E. coli* Replicable Plasmid with Ampicillin Resistance

Due to the increased penicillin resistance of Gram-positive bacteria in clinic application and natural environments [16, 30], pSMART-LCKan was modified to pHCAmp in this study to adapt to the resistant profiles in Gram-positive bacteria. According to its Manual (http://www.lucigen.com/docs/manuals/MA004-CloneSmart-HCLC-Blunt-Cloni

ng.pdf), pSMART-LCKan is a 1968 bps plasmid with the low copy number pBR322 origin of replication (*ori*). During the modification of this plasmid, the *ori, cat* promoter, bidirectional terminator (*tonB*), and another two terminators (*T3Te* and *T7Te*) were retained (Fig. 1a). In this step, the *rop* gene was removed to maintain the high copy number of the plasmid in *E. coli*. Using the primers ligated with two adaptors, a 1152 bp sequence was amplified for the subsequent ligation. Meanwhile, *ampR* gene was amplified from plasmid pJ444-01 with the same adaptors (Fig. 1b). After the two DNA fragments were assembled and transformed into *E. coli* TOP10, ampicillin-resistant colonies were screened out to extract the plasmid, which was named pHCAmp (Fig. 1c, d). This plasmid modification provides an efficient choice of kanamycin resistance to screen transformed strains in the future when limited antimicrobial susceptibility is exhibited by penicillin resistant Gram-positive bacteria [32].

Construction of Plasmid pDRE

Theta and RCR are two major replication mechanisms for circular plasmids. pSMART-LCkan harbors a ColE1 replicon, which belongs to theta class B and requires DNA polymerase I to initiate replication. During theta replication, the DNA duplex is opened by the transcription of a long (~600 bp) pre-primer RNA II, which is then extended by DNA Pol I to initiate the leading-strand synthesis. Subsequently, DNA Pol III is loaded on the complex, and maintains the leading-strand synthesis and initiates the lagging-strand synthesis [17]. Nevertheless, in many Gram-positive bacteria, another replication mechanism named RCR is accessible for circular plasmids [6, 21]. The two-step replication proceeds as follows: (i) Leading-strand replication is initiated at the strand-specific double-strand origin (*dso*) until it generates a single-stranded plasmid DNA. (ii) Lagging-strand synthesis is started at the single-strand origin (*sso*) [26].

Therefore, to extend the host range of the constructed plasmid, a new plasmid, which was named pDRE and contained the above two replication origins, was constructed in this study. First, pHCAmp was linearized using the PCR method. Second, two DNA fragments, FA and FB, were synthesized based on the core elements, including *ssoA, dso, copG, repB, tet* promoter, *tet* terminator, maltose operator, and Pm promoter [26] (Figs. 2a, S2), of the pLS1ROM plasmid [22]. The sequences of these elements were the same as those in pLS1ROM. In this step, two short intergenic fragments were removed according to the comparison with pMV158 (accession number: NC_010096.1), so as to reduce the size of the plasmid (Fig. S2). FA contained

Fig. 1 Construction of pHCAmp. **a** The sequence from *cat* to T7 terminator in pSMART-LCKan was linearized by PCR amplification using primers P1 and P2 (Table S1). The terminators (*T7Te, T3Te*, and *tonB*), *cat* promoter, and *ori* in pSMART-LCKan were retained to allow the replication and regulation of pHCAmp in *E. coli*. **b** Terminators were represented by gray ellipses, while *cat, ori, rop*, and *kanR* were represented by gray arrows. The *ampR* obtained from plasmid pJ444-01 was represented by a pink arrow. Primers with overlapping sequences (the two blocks shown in red and blue) between pSMART-LCKan and *ampR* gene were designed and named as P3 and P4 (Table S1). **c** The above two fragments were assembled and transformed into *E. coli* TOP10, while the positive strains were screened out on LB plates containing ampicillin (AMP). **d** The extracted plasmid was sequenced and annotated. (Color figure online)
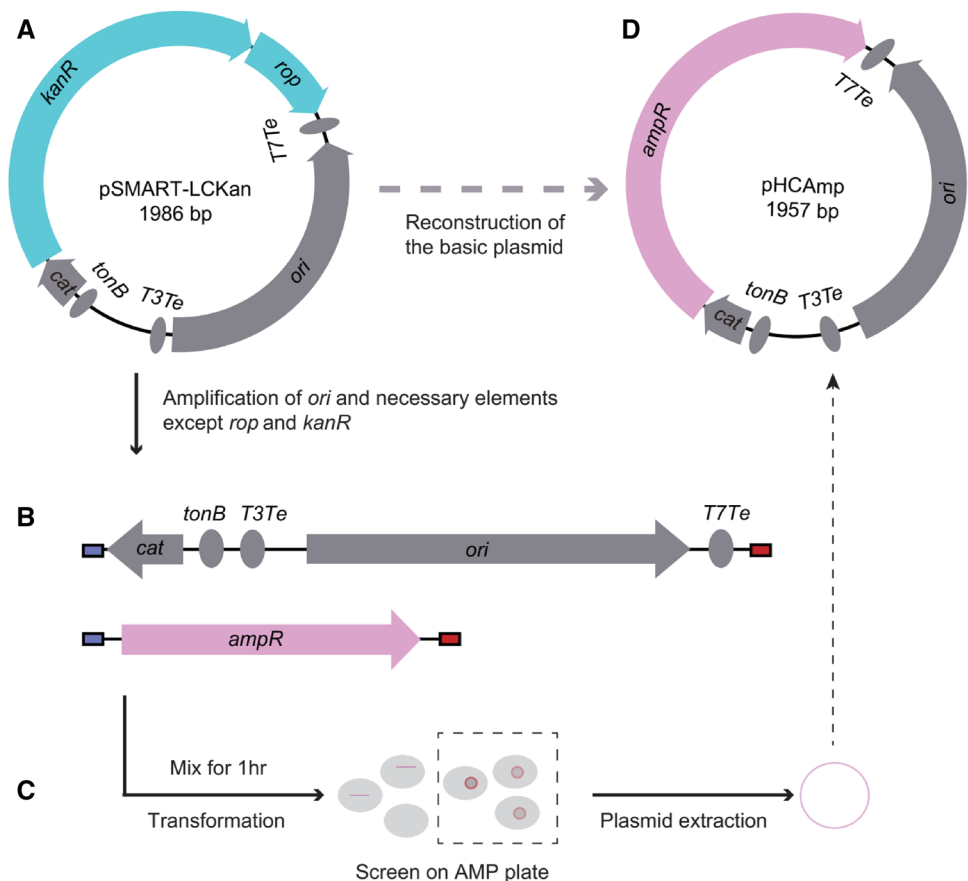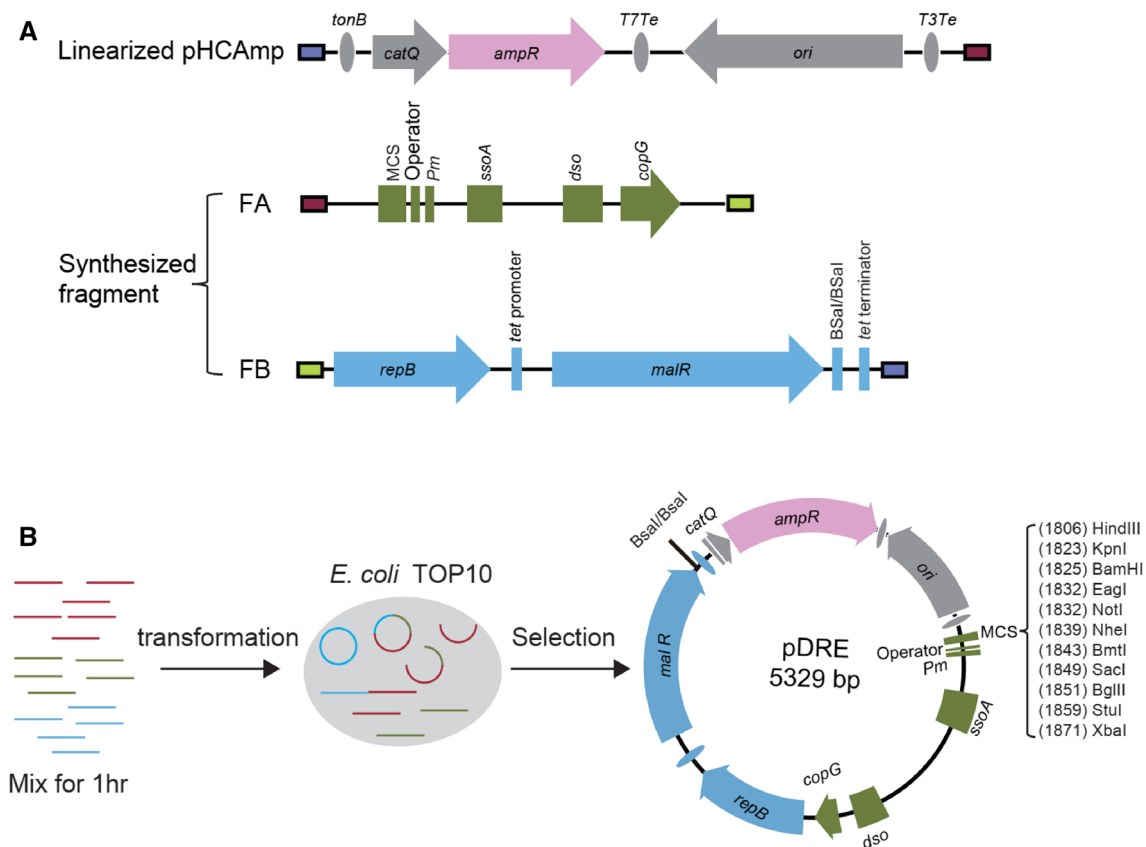
Fig. 2 Construction of pDRE. **a** Three fragments (linearized pHCAmp, FA and FB) were used to assemble pDRE. Overlapping sequences flanking the fragments were presented by blocks in blue, red, and green. FA contained *dso, ssoA*, and *copG* for RCR replication, *Pm* promoter, *operator*, and MCS. FB included *repB, tet* pro- moter, *malR*, and *tet* terminator. **b** The final plasmid, pDRE, was assembled using the Gibson method. The circular sketch map was shown on the right, with the locus of MCS marked. (Color figure online)

dso, ssoA, copG, a Pm promoter (*Pm*), a maltose operator, and multiple cloning sites (MCS) different from those in pLS1ROM (Fig. 2a). Among these elements, *dso* and *ssoA* were of RCR origin. The gene *copG* encoded a transcrip- tional regulator, which could regulate the copy number of the plasmid by limiting the availability of the replica- tion protein RepB [26]. The MCS was designed to include frequently used restricted sites for the insertion of exoge- nous genes (Fig. 2b). The *malR* gene encoded the maltose operon regulator, while the *Pm* promoter was assigned to the upstream of MCS to regulate the expression of inserted exogenous genes. FB contained *repB*, the *malR* gene with *tet* promoter and terminator, and two BsaI restriction sites. The *repB* encoded a replication initiator protein, and *malR* gene was designed to regulate the expression of cloned gene in MCS. Finally, these fragments (linearized pHCAmp, FA and FB) with overlapping sequences (symbolized by different colors in Fig. 2a) were assembled using the Gibson assem- bly method. The mixture was transformed into *E. coli* strain TOP10, and the positive strain was successfully screened out on LB plates containing penicillin. After extraction, the

plasmid was sequenced and annotated, and named pDRE (Fig. 2b). Fig. S2 showed the similarities between the syn- thesized sequences of FA/FB and their original sequences in pLS1ROM and pMV158.

The Genetic Characters of pDRE

This constructed shuttle vector, pDRE, was a 5329 bp cir- cular plasmid containing two individual replication origins that could be used as a shuttle vector in *Streptococcus* and some of the Gram-negative bacteria. RCR plasmid pMV158 uses *repB* and *dso* to initiate its replication in *Streptococcus* [11], while *ori* plays the same role in the theta replication of plasmids. Two types of restriction sites, MCS and BSaI/BSaI, were also included in pDRE to make it easy to operate. The *malR* and *operator*, and the *Pm* promoter, were introduced into pDRE to control the expression of inserted genes. MalR, which belongs to the LacI–GalR family of transcriptional repressors, spe- cifically binds to *operator*. In the absence of maltose, the complex of MalR–*operator* acts as a repressor of inserted

genes [23]. Thus, the concentration of maltose in the culture medium determines the expression level of inserted genes [22].

By combining these elements, the plasmid pDRE has the potential to act as a shuttle vector in both Gram-positive and Gram-negative bacteria. The expression level of the genes carried by the plasmid could be altered using different culture conditions (Table 2).

E. coli Expression of Heterologous Genes Carried by pDRE

To validate that pDRE has the ability to express heterologous genes in *E. coli*, two genes were inserted into pDRE, *egfp* at *Hin*dIII/*Eco*RI and *erm* at BsaI/BsaI restriction sites, to generate a plasmid termed pDRE (*erm*/*egfp*), which was then transformed into *E. coli* TOP10 (Figs. 3a, S3). The *erm* gene was cloned in BsaI/BsaI restriction sites with its own promoter, while *egfp* was cloned in the MCS under the control of Pm promoter. The *E. coli* cells carrying pDRE (*erm*/*egfp*) were cultured on LB medium containing 0.2 mg/ml erythromycin. The cultured cells were then washed and diluted (1:10) in LB medium containing maltose (3 mM). The cultures were grown to $OD600 = 0.5$ and harvested by centrifugation. Subsequently, the expression of inserted genes was successfully detected at DNA and RNA levels (Fig. 3b, c). In the next step, the phenotype of the transformed strain was identified, and both erythromycin resistance and green fluorescence signal could be observed at 510 nm in transformed cells (Fig. 3d). These data showed that the inserted genes were expressed in *E. coli*.

## Expression of Heterologous Genes in *Streptococcus* Using pDRE

To validate that pDRE has the ability to express heterologous genes in *Streptococcus*, ten *Streptococcus* strains were chosen: *S. agalactiae* (five strains), *S. dysgalactiae* (ATCC 35666), *S. pyogenes* (three strains), and *S. thermophilus* (ATCC 14485). Subsequently, the plasmid pDRE (*erm*/*egfp*) used in *E. coli* above was used to transform the *Streptococcus* strains, and transformed cells were cultured on BHI medium containing erythromycin (2 μg/ml) and maltose (3 mM). The positive strains were isolated by verifying the presence of *erm* gene in bacterial total DNA (Fig. S4). The presence of *erm* DNA and RNA was detected in all strains. The relative DNA quantities of *erm* were 2.23–9.76, indicating the different copy number of the plasmid in these strains. When compared with 16S rRNA, the relative level of *erm* expression was 0.002–0.28 (Fig. 4). The highest level of DNA abundance was observed in *S. dysgalactiae*, followed by *S. agalactiae* and *S. pyogenes*, whereas the highest level of expression was observed in *S. pyogenes*, followed by *S. agalactiae*. Green fluorescence could be observed in all strains. The above results confirmed that pDRE could be used as a tool to achieve gene expression in a broad range of streptococcal species.

In *Streptococcus*, shuttle vectors based on two major types of replicons have been designed for functional studies. One type of replicons involves RCR replication, such as those derived from pVA380-1 of *S. oralis* origin [15,

Table 2 Characteristics of pDRE

pDRe element	Location (start..end)	Feature
tonB	49..80	*tonB* bidirection terminator
catQ	81..183	*cat* gene universal promoter
ampR	184..1044	AMP resistant gene
T7Te	1068..1095	T7 universal terminator
ori	1107..1694	Replication origin in *E. coli*
T3Te	1716..1745	T3 universal terminator
MCS	1819..1862	Multiple cloning site
malO	1888..1903	Maltose operon
Pm	1912..1940	Maltose promoter
ssoA	2140..2349	The origin of lagging strand
dso	2927..3096	The origin of double strand
copG	3181..3318	Transcriptional repressor involved in the copy number control of pMV158
repB	3379..4011	Replication initiator gene of pMV158
tet promoter	4109..4136	Universal promoter
malR	4262..5248	Transcriptional repressor involved in the regulation of the maltose operon in *Streptococcus pneumoniae*

Fig. 3 Validation of the expression of inserted genes (*egfp*/*erm*) in *E. coli*. **a** Circular sketch map of constructed pDRE (*erm*/*egfp*) plasmid. The *egfp* gene (light green arrow) was inserted between *Hin*dIII and *Eco*RI sites, and *erm* (dark purple arrow) was inserted at the BSaI/BSaI sites of pDRE. Subsequently, the constructed plasmid was transformed into *E. coli* TOP10. **b** Detection of *egfp* and *erm* genes in transformed *E. coli* by PCR using the primers in Table S3. The *erm* gene was detectable in the transformed strains, thus validated its presence in the strains transformed by pDRE (*erm*/*egfp*). Both *erm* and *egfp* genes were absent in the original strains (data not shown). **c** The relative RNA expressions of *egfp* and *erm* were calculated by RT-qPCR using 16S rRNA as the internal reference (mean±SD, *n*=3). The sizes of amplicons of *egfp* and *erm* were 447 bps and 508 bps, respectively. **d** Green fluorescence was successfully detected. (Color figure online)
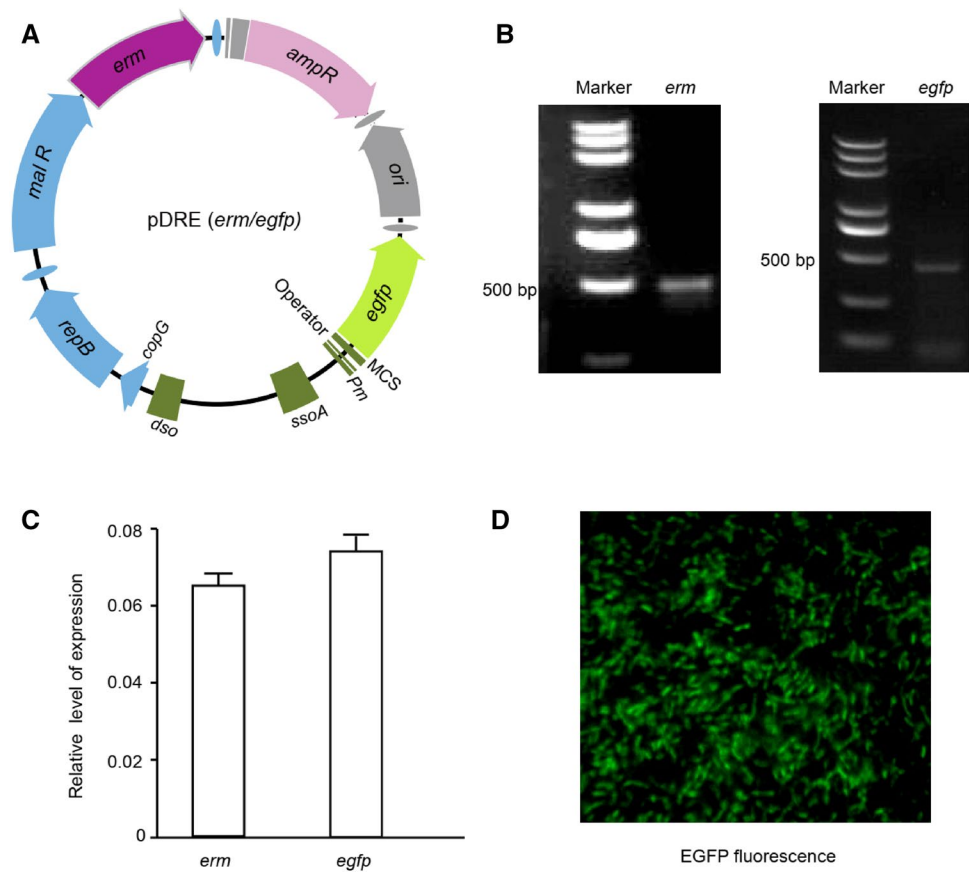
22]. These plasmids can replicate in a broad range of Gram-positive bacterial hosts but can rarely be expressed in Gram-negative bacteria [26]. Given their incompatibility with RCR plasmids already present in the hosts, plasmid vectors using theta replication have been developed, such as those derived from pAMβ1 of *Enterococcus faecalis* origin, pFW213 from *S. parasanguinis*, and its derivative, pCG1 [3, 4]. These plasmids have been widely used in Gram-negative bacteria [27, 28]. Considering that RCR replication is frequent in Gram-positive bacteria [13], we constructed the plasmid pDRE contains both types of replicons, theta replicon from *E. coli* and RCR replicon synthesized based on the sequence of pLS1ROM replicon. It should be mentioned that plasmid pMV158 is a RCR plasmid carrying two different *ssos, ssoU* and *ssoA*, for the initiation of lagging-strand synthesis [18]. Compared with *ssoU, ssoA* is more *Streptococcus*-specific [26]. To achieve better expression in *Streptococcus, ssoA* was chosen in this study to initiate the replication of the lagging strand. The expression of both inserted genes, *erm* and *egfp*, showed that pDRE could be expressed in *E. coli* and most *Streptococcus*.

Validation of pDRE Expression in Other Gram-Positive Genera

The replication of pDRE and the expression of cloned genes were also observed in multiple Gram-positive species, such as *B. subtilis, L. lactis*, and *S. aureus*. Compared with *16S rDNA*, the relative quantities of target gene *erm* cloned in the BsaI/BsaI restriction sites were 6.37, 2.44, and 3.62 in *B. subtilis, L. lactis*, and *S. aureus*, respectively, whereas the relative expression levels of *erm* were 2.21, 0.07, and 0.29. These results showed that pDRE may achieve similar levels of expression in *Lactococcus* and *Staphylococcus*, while the expression of pDRE was higher in *Bacillus* (Fig. 5).

Validation of the Function of the Maltose Inducible Promoter in pDRE

Four representative isolates harboring pDRE (*erm*/*egfp*) were selected to analysis the relative expression of cloned *egfp* gene under the control of the maltose inducible promoter. *E. coli* TOP10, *S. agalactiae* (ATCC 12513), *L. lactis*

Fig. 4 The expression of inserted genes in pDRE in *Streptococcus*. Plasmid pDRE (*erm*/*egfp*) was transformed into ten strains: *S. agalactiae* (five strains), *S. dysgalactiae* (one strain), *S. pyogenes* (three strains) and *S. thermophilus* (one strain), while positive colonies were selected on the basis of erythromycin resistance; the plasmid could be extracted from the strains above. The relative DNA content of *erm* (top) and the relative mRNA expression level of *erm* (bottom) were calculated, using *16S rDNA* and 16S rRNA as the internal reference, respectively (mean±SD, $n=3$). The results of negative controls with untransformed cells were at the left of those of the transformed cells, respectively
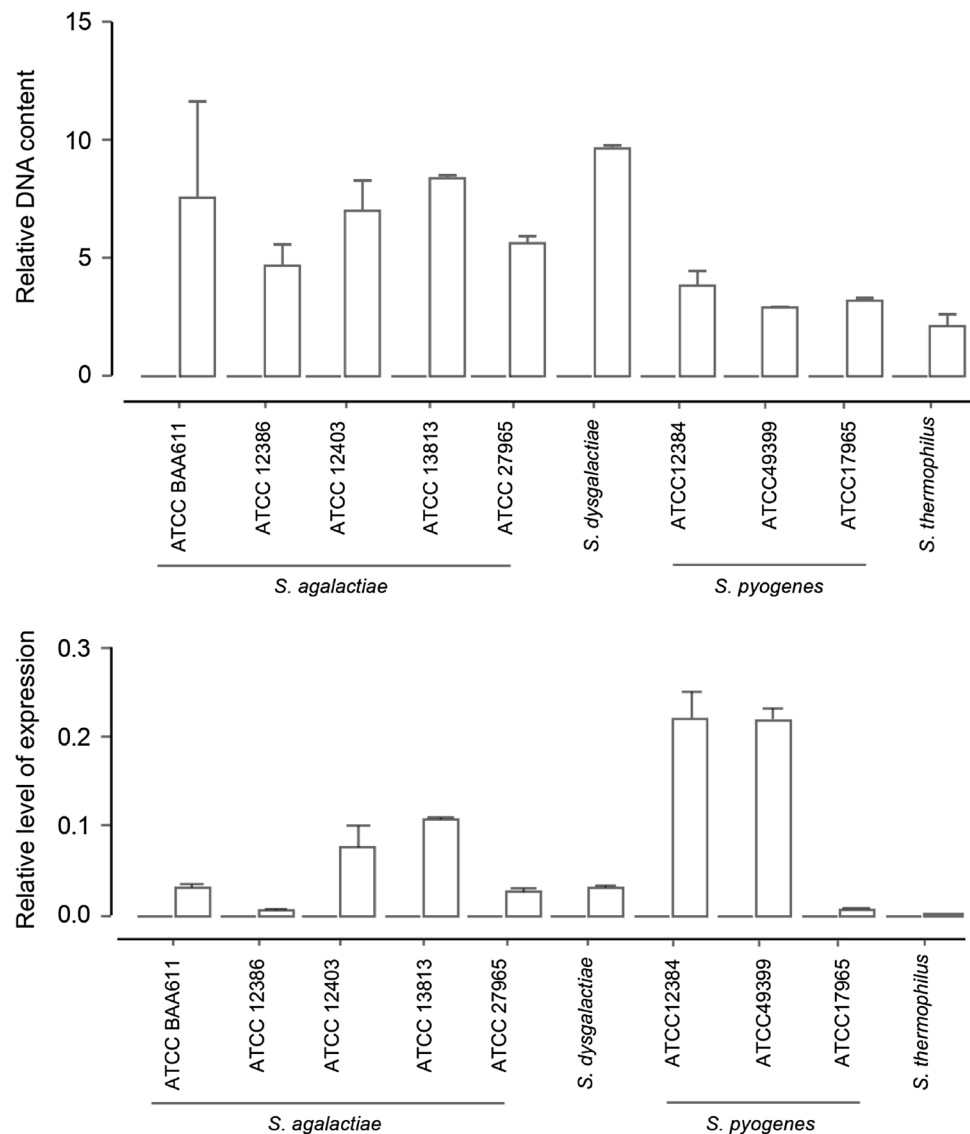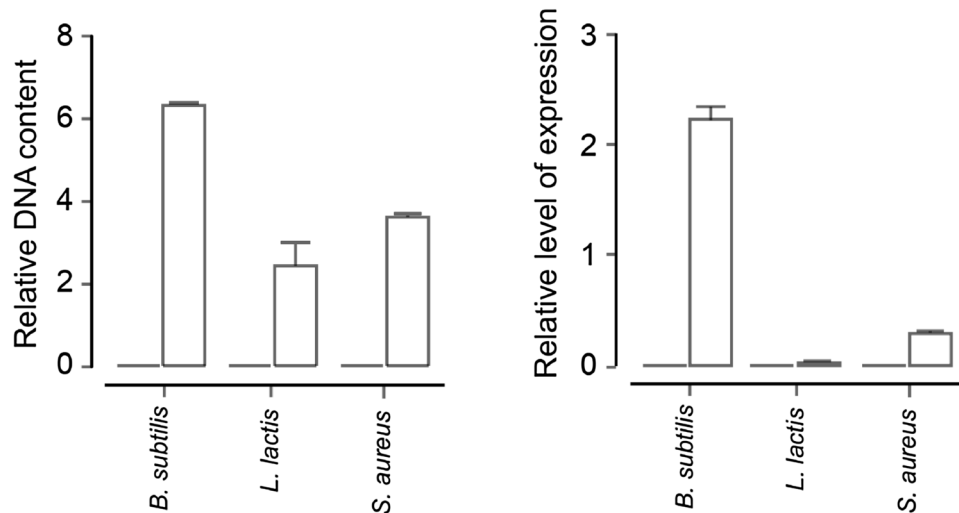
(ACCC 11902), and *S. aureus* (ATCC 6538P) were cultured in BHI medium containing erythromycin (0.2 mg/ml for *E. coli* TOP10 and 2 µg/ml for the other three strains). The cultures were grown to OD600 = 0.5 and harvested by centrifugation, then washed and diluted (1:10) in BHI medium containing maltose at final concentrations of 0.3, 3, and 30 mM. After the cultures were grown to the same OD, the mRNA levels of *egfp* were measured and the relative mRNA expression values were compared with those of *erm* gene (Fig. S5). Then the green fluorescence signal of the induced isolates were observed and the fluorescent intensity were detected. In contrast, the fluorescence were extremely low (0.18–1.8) in the four isolates when no maltose was added (Fig. 6).

Plasmid-based genetic tools with the ability to express both native and heterologous genes in different microorganisms can play key roles in both basic functional research and specific application such as vaccine development [34]. In most Gram-positive bacteria, one of the difficulties encountered in genetic functional analysis is the lack of an efficient overexpression system in the host. Once a shuttle vector with the ability to replicate in both Gram-negative and Gram-positive bacteria has been constructed, various exogenous genes can be cloned into the plasmid and transformed into *E. coli* with high quantitative efficiency. Subsequently, the plasmid can be introduced into an appropriate bacterial host to analyze the functions of target genes. The expression and replication of pDRE in *B. subtilis* and *S. aureus* indicated that this plasmid could be widely used in Gram-positive bacteria.

In conclusion, a shuttle vector system, pDRE, was constructed in this study for the heterologous gene expression in *Streptococcus* and other bacteria. The genes (*erm* and *egfp*) carried by pDRE could be expressed in seven

Fig. 5 The inserted genes in pDRE could be expressed in other Gram-positive strains. pDRE (*erm/egfp*) was transformed into *Bacillus subtilis* (ATCC 6051), *Lactococcus lactis* (ACCC 11902), and *Staphylococcus aureus* (ATCC 6538P). The positive strains were obtained by erythromycin selection. The relative DNA content of *erm* (left) and relative mRNA expression of *erm* (right) were calculated using *16S rDNA* and 16S rRNA as the internal reference, respectively (mean±SD, $n=3$). The results of negative controls with untransformed cells were at the left of those of the transformed cells, respectively
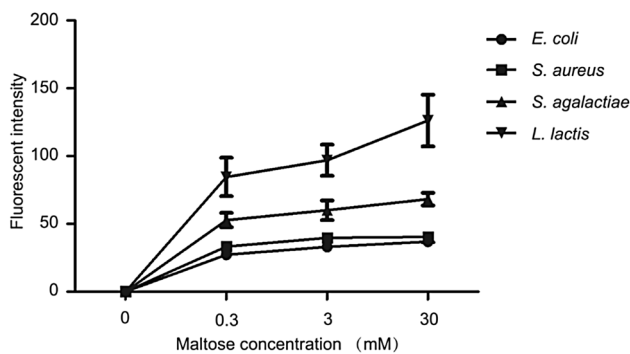
Fig. 6 *egfp* gene expression could be induced by adding maltose in transformed isolates. Detection of fluorescence in *E. coli* TOP10, *S. agalactiae* (ATCC 12513), *Lactococcus lactis* (ACCC 11902), and *Staphylococcus aureus* (ATCC 6538P) harboring pDRE (*erm/egfp*) after culturing in BHI medium containing different concentrations of maltose

Gram-positive species, including four *Streptococcus* spp. and three other species. Furthermore, the maltose inducible promoter could regulate the expression of cloned gene in transformed isolates, suggesting that this shuttle vector could become a useful genetic tool for genetic and functional studies in *Streptococcus* and other Gram-positive bacteria.

Author Contributions HZ and PD conceived the project. HZ, CC, PD, and MH designed the study. MH, JG, ML, YZ, DJ, and CS performed the experiments. HZ, CC, PD, and MH wrote the paper. All authors have discussed the results, commented on the manuscript, and have given final approval for the submitted version.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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