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New Shuttle Vector-Based Expression System To Generate Polyhistidine-Tagged Fusion Proteins in *Staphylococcus aureus* and *Escherichia coli*

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Four *Staphylococcus aureus-Escherichia coli* shuttle vectors were constructed for gene expression and production of tagged fusion proteins. Vectors pBUS1-HC and pTSSCm have no promoter upstream of the multiple cloning site (MCS), and this allows study of genes under the control of their native promoters, and pBUS1-P_{cap}-HC and pTSSCm-P_{cap} contain the strong constitutive promoter of *S. aureus* type 1 capsule gene 1A (P_{cap}) upstream of a novel MCS harboring codons for the peptide tag Arg-Gly-Ser-hexa-His (*rgs-his*$_6$). All plasmids contained the backbone derived from pBUS1, including the *E. coli* origin ColE1, five copies of terminator *rrnB* T_1, and tetracycline resistance marker *tet*(L) for *S. aureus* and *E. coli*. The minimum pAMα1 replicon from pBUS1 was improved through either complementation with the single-strand origin *oriL* from pUB110 (pBUS1-HC and pBUS1-P_{cap}-HC) or substitution with a pT181-family replicon (pTSSCm and pTSSCm-P_{cap}). The new constructs displayed increased plasmid yield and segregational stability in *S. aureus*. Furthermore, pBUS1-P_{cap}-HC and pTSSCm-P_{cap} offer the potential to generate C-terminal RGS-His_6 translational fusions of cloned genes using simple molecular manipulation. BcgI-induced DNA excision followed by religation converts the TGA stop codon of the MCS into a TGC codon and links the *rgs-his*$_6$ codons to the 3′ end of the target gene. The generation of the *rgs-his*$_6$ codon-fusion, gene expression, and protein purification were demonstrated in both *S. aureus* and *E. coli* using the macrolide-lincosamide-streptogramin B resistance gene *erm*(44) inserted downstream of P_{cap}. The new His tag expression system represents a helpful tool for the direct analysis of target gene function in staphylococcal cells.

Staphylococci are commensals of human and animal skin. In some species, particularly in *Staphylococcus aureus*, community- and hospital-associated clones have acquired a multitude of virulence and antibiotic resistance mechanisms, representing a serious public health risk (1, 2). An increased understanding of the resistance and pathogenicity mechanisms of staphylococci is needed. Therefore, molecular tools for genetic analysis, including gene disruption and ectopic expression, have been developed (3). *S. aureus-Escherichia coli* shuttle vectors have been constructed using restriction fragments derived from small, natural high-copy-number *S. aureus* plasmids, such as pUB110, pT181, pE194, and pC194 (4–7). These plasmids propagate through rolling-circle replication (RCR) in staphylococci, requiring the replication initiator protein (Rep) and the double-strand origin (*dso*) to initiate leading-strand synthesis as well as the single-strand origin (*sso*) for subsequent efficient lagging-strand synthesis (8). The absence of a functional *sso* sequence in RCR plasmids has been associated with single-stranded DNA (ssDNA) accumulation, low copy numbers, and plasmid instability (9–11). While some *sso* sequences support replication only in the native host, other sequences are functional in a number of different Gram-positive bacteria (8). For example, pUB110 is a broad-host-range plasmid whose *sso* is recognized in both *Staphylococcus* and *Bacillus* (12). Related plasmids pBC16 and pAMα1 have also been isolated from *Bacillus cereus* and *Enterococcus/Streptococcus*, respectively. pAMα1 is a composite of two separable replicons that depends on the pAMα1Δ2 derivative for replication in *Enterococcus/Streptococcus* and the pAMα1Δ1 derivative, associated with pUB110/pBC16, for replication in *Bacillus subtilis* (13, 14). For simplicity, the pAMα1Δ1 replicon is referred to as the pAMα1 replicon in this and other studies. To circumvent plasmid replication via ssDNA intermediates, *S. aureus-E. coli* shuttle vectors, which replicate using a theta-mode mechanism in *Staphylococcus*, have been constructed. These constructs contain pSK1 or pI258-derived origins from natural low-copy-number *S. aureus* plasmids and might exhibit higher segregational and structural stability (15, 16). Furthermore, a series of cassette-based shuttle vectors was constructed to provide flexibility in use of Gram-positive replicons, selectable markers, and promoter regions for inducible or constitutive gene expression (16). Additionally, a number of regulated promoter systems were established for inducible gene expression from shuttle plasmids in *S. aureus*; among them are the xylose-inducible promoter P_{xyl} and the tetracycline-regulated hybrid promoter $P_{xyl\text{-}tetO}$ (17–21), the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible hybrid promoter P_{spac} (18, 20), arsenic- and cadmium-inducible promoters (16, 22), and thermally regulated promoters (23). Although an increasing number of shuttle

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S. aureus		
RN4220	NCTC8325-4 derivative, antibiotic susceptible, restriction deficient; *sau1 hsdR* (r_K^- m_K^+)	49
RN4220/pVPF5	RN4220 containing plasmid pVPF5 from *S. xylosus* VF5	43
E. coli		
DH5α	K-12 strain; *recA1 endA1 hsdR17* (r_K^- m_K^+)	Life Technologies
AG100	K-12 strain	50
AG100A	AG100 Δ*acrAB::aphA1* (Km^r) mutant; increased susceptibility to antibiotics	44
B. subtilis		
DSM4514	Derivative of strain 168 containing plasmid pUB110	DSMZ
Plasmids		
pUB110	*S. aureus* plasmid, replicates in *B. subtilis*; *aadD* (Km^r Neo^r)	51
pVPF5	*S. xylosus* pT181 family plasmid; *tet*(K)	43
pBUS1	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon, ColE1, MCS pBluescript II SK (Stratagene), (*rrnB* $T_1)_5$ *tet*(L)	24
pBUS1-P_{cap}	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon, ColE1, promoter of *S. aureus* type 1 capsule gene 1A (P_{cap}) upstream of a new MCS with joinable *rgs-his*$_6$ codons (P_{cap}-MCS-*rgs-his*$_6$), (*rrnB* $T_1)_5$ *tet*(L)	This study
pBUS1-HC	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon, *sso oriL* ColE1, MCS pBluescript II SK (Stratagene), (*rrnB* $T_1)_5$ *tet*(L)	This study
pBUS1-P_{cap}-HC	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon, *sso oriL* ColE1 P_{cap}-MCS-*rgs-his*$_6$ (*rrnB* $T_1)_5$ *tet*(L)	This study
pTSSC	*S. aureus-E. coli* shuttle vector; pT181 replicon, ColE1, MCS pBluescript II SK (Stratagene), (*rrnB* $T_1)_5$ *tet*(L)	This study
pTSSC-P_{cap}	*S. aureus-E. coli* shuttle vector; pT181 replicon, ColE1 P_{cap}-MCS-*rgs-his*$_6$ (*rrnB* $T_1)_5$ *tet*(L)	This study
pTSSCm	pTSSC with two silent mutations in the *repC* sequence to remove HindIII and XbaI sites	This study
pTSSCm-P_{cap}	pTSSC-P_{cap} with two silent mutations in the *repC* sequence to remove HindIII and XbaI sites	This study
pBJW13	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon; ColE1 P_{cap} *erm*(44) (MLS_B^r) (*rrnB* $T_1)_5$ *tet*(L)	26
pBJW13-HC	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon, *sso oriL* ColE1 P_{cap} *erm*(44) (*rrnB* $T_1)_5$ *tet*(L)	This study
pTJW13	*S. aureus-E. coli* shuttle vector; pT181 replicon, ColE1 P_{cap} *erm*(44) (*rrnB* $T_1)_5$ *tet*(L)	This study
pBJW13-RGS-His	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon, ColE1 P_{cap} *erm*(44)-*rgs-his*$_6$ (*rrnB* $T_1)_5$ *tet*(L)	This study
pBJW13-HC-RGS-His	*S. aureus-E. coli* shuttle vector; pAMα1 minimum replicon, *sso oriL* ColE1 P_{cap} *erm*(44)-*rgs-his*$_6$ (*rrnB* $T_1)_5$ *tet*(L)	This study
pTJW13-RGS-His	*S. aureus-E. coli* shuttle vector; pT181 replicon, ColE1 P_{cap} *erm*(44)-*rgs-his*$_6$ (*rrnB* $T_1)_5$ *tet*(L)	This study

vectors to express genes in *Staphylococcus* have been developed, the number remains limited compared with the versatile systems established for *E. coli*. Particularly, the use of tag fusion systems to analyze gene function is not frequently encountered in staphylococci. Shuttle vectors for the regulated coexpression of green and red fluorescent fusion proteins have been recently constructed (20). However, there are no expression vectors for the generation of polyhistidine (His) tag fusion proteins directly from *Staphylococcus* cells. The small His tag does not typically interfere with protein activity, and fusion proteins can be isolated using metal affinity matrices or can be detected using antibody-based methods. Therefore, we anticipate that the production of His tag protein fusions directly in staphylococcal cells will facilitate the analysis of the molecular interactions of this opportunistic pathogen in biochemical experiments.

Therefore, we constructed *S. aureus-E. coli* shuttle vectors for the expression of target genes and Arg-Gly-Ser-hexa-His (*rgs-his*$_6$) codon fusions. These vectors were derived from pBUS1 (24), which has frequently been used to clone genes in *S. aureus* (25–32), and comprise the Gram-positive replicon pAMα1 or pT181, the pBluescript II SK multiple cloning site (MCS) without a promoter, or a novel MCS with joinable *rgs-his*$_6$ codons downstream of the strong *S. aureus* type 1 capsule gene 1A promoter (P_{cap}) (33). The shuttle vectors were tested for plasmid stability and copy number in *S. aureus*. The macrolide-lincosamide-streptogramin B (MLS_B) resistance gene *erm*(44), cloned downstream of P_{cap} (26), was used to demonstrate the feasibility of *rgs-his*$_6$ codon fusion and the production of Erm(44)-RGS-His_6 protein in both *S. aureus* and *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in the present study are listed in Table 1. *E. coli* and *S. aureus* RN4220 strains were grown in Luria-Bertani (LB) broth with shaking at 220 rpm or on LB agar plates at 37°C. *B. subtilis* DSM4514(pUB110) was cultured in LB containing 20 μg/ml kanamycin at 30°C. Ultracompetent *E. coli* DH5α cells (34) and calcium chloride-competent *E. coli* AG100A or AG100 cells (35) were prepared and used for heat shock transformation as previously described. *S. aureus* RN4220 transformants were obtained through electroporation using the protocol of Schenk and Laddaga (36). *E. coli* and *S. aureus* transformants were selected on agar plates containing 10 μg/ml tetracycline, and this tetracycline concentration was also used to maintain the plasmids in the cells.

DNA preparation, PCR, and sequencing. Plasmid DNA and genomic DNA were isolated using peqGOLD Plasmid Miniprep Kit I and a peqGOLD bacterial DNA kit (Peqlab Biotechnologie GmbH, Erlangen, Germany), respectively. To improve lysis of *S. aureus* and *B. subtilis*, the cells were first incubated for 15 min at 37°C in solution I supplemented with 100 μg/ml lysostaphin (Sigma-Aldrich, St. Louis, MO) and 2 mg/ml lysozyme (Roche Diagnostics, Rotkreuz, Switzerland), respectively. Oligonucleotide primers were synthesized at Microsynth (Balgach, Switzerland). The primers used for plasmid construction are listed

TABLE 2 Oligonucleotide primers and probes

Function and name	Sequence (5′–3′ [modification])[a]	Target	Reference or source
Primers for plasmid construction			
capHis-pBUS1-F	*TGCAGCTAGCATATGATAAA*CCTCCTATTTTCCTTTCTTGTTTTCCATTATATATAATCCCCTGTATATTTTGCAAACTCTGGTACCACGCGTTGCGCTCAC	pBUS1	This study
capHis-pBUS1-R	*TTTATCATATGCTAGCTGCA*GGAATTCTCTGACTAGTCGACCGAAAGCTTTGCTGGATCCGCATGCTCGAGAGGTTCTCATCACCATCACCATCACTAATCTAGAGCGGCCGCCACCG	pBUS1	This study
pBUS1-oriL-F	AATGT***TGTACA***GAAAACCTCTGACACATGCAG	pBUS1, pBUS1-P_{cap}, pBJW13	This study
pBUS1-oriL-R	AGTTA***AGATCT***AGGATCAATTTTGAACTCTCTCC	pBUS1, pBUS1-P_{cap}, pBJW13	This study
oriL-BsrGI-F	TGAAT***TGTACA***CTTCCAAGTAAAGTATAACACAC	pUB110	This study
oriL-BglII-R	TTAGA***AGATCT***GCGATTGCTGAATAAAAGATACG	pUB110	This study
pBUS1-BsrGI-F	TTAGT***TGTACA***GGCCATATTGTTGTATAAGTGATG	pBUS1, pBUS1-P_{cap}, pBJW13	This study
pBlueScript-BglII-R	AGTTA***AGATCT***ATGACCAAAATCCCTTAACGTG	pBUS1, pBUS1-P_{cap}, pBJW13	This study
pT181-BglII-F	AGATA***AGATCT***ATAGAACATGCATTTATGCCGAG	pVPF5	This study
pT181-BsrGI-R	TAATA***TGTACA***CTATTTCCAAAATTTAAATTCATG	pVPF5	This study
mut-repC-F	*AAAGCT**C**CACAGAAATTC**C***AGAACAAAATATAAGAATTTG	pTSSC, pTSSC-P_{cap}	This study
mut-repC-R	*AATTTCTGTG**G**AGCTTT*CCCCATTCTTCTTCATC	pTSSC, pTSSC-P_{cap}	This study
Primers and probes for qPCR			
nuc263-F	AAAGCGATTGATGGTGATACGGTT	*nuc*	38
nuc355-R	TGCTTTGTTTCAGGTGTATCAACCA	*nuc*	38
nuc294-P	ATGTACAAAGGTCAACCAATGACATTCAGA (Cy5, BHQ2)	*nuc*	38
qPCR-tetL-F	GGCTTTCGTTCACCAAAACAGT	*tet*(L)	This study
qPCR-tetL-R	TGGTAAAGTTAAGCAAACTCATTCCA	*tet*(L)	This study
FAM-tetL-TAMRA	TTGTTTCAAGTAGCTTGAAACAGCAGGAAGCTG (FAM, TAMRA)	*tet*(L)	This study

[a] Bases binding to the template are underlined. Restriction sites used for cloning are in boldface italics. Complementary 5′ ends of cloning and mutagenesis primers are in italics. In mutagenesis primers, mismatched bases are in boldface. Cy5, cyanine fluorescein 5; BHQ2, black hole quencher 2; FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamine.

in Table 2. For analytical purposes, PCRs were performed using *Taq* DNA polymerase (Solis BioDyne, Tartu, Estonia). PCRs for plasmid construction were performed using high-fidelity DNA polymerases (such as the *Pfu* DNA polymerase [Promega, Madison, WI] or the Phusion Hot Start II High-Fidelity DNA polymerase [Thermo Scientific, Waltham, MA]) according to the manufacturers' instructions, unless otherwise specified. The PCR products were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics, Rotkreuz, Switzerland) prior to incubation with restriction endonucleases and T4 DNA ligase (ExpressLink T4 DNA Ligase [Invitrogen, Carlsbad, CA]). All plasmid constructs were generated in *E. coli* DH5α cells, examined based on restriction digestion patterns, and sequenced using an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The plasmids are listed in Table 1.

Construction of pBUS1-P_{cap}. The synthetic sequence containing both the *S. aureus* type 1 capsule gene 1A promoter P_{cap} and a new multiple cloning site with joinable *rgs-his*$_6$ codons was inserted into the plasmid pBUS1 (24) using PCR-based QuikChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). The PCR was performed with 40 ng of the DNA template pBUS1, 0.5 μM capHis-pBUS1-F primer, 0.3 μM capHis-pBUS1-R primer (Table 2), and 1U of Phusion Hot Start II High-Fidelity DNA polymerase in buffer HF (Thermo Scientific) supplemented with 5% glycerol. DNA amplification was performed for 30 cycles: 10 cycles with an annealing temperature of 55°C and an extension time of 2 min, followed by 20 cycles with an annealing temperature of 62°C and an extension time of 2 min. The PCR products were treated with the restriction enzyme DpnI and directly transformed into *E. coli* DH5α cells. The selected clones were analyzed for the correct P_{cap}-MCS-*rgs-his*$_6$ insertion sequence through colony PCR and sequencing using primers pBUS1-F2 (5′-TTTACAAGCCCAGAGCTC) and pBUS1-R (5′-CTTTGAGTGAGC TGATAC). The plasmid DNA of one correct clone, referred to as pBUS1-P_{cap}, was completely sequenced (Table 1).

Construction of pBUS1-HC, pBUS1-P_{cap}-HC, and pBJW13-HC. The *oriL* sequence was obtained from plasmid pUB110 through PCR amplification using primers oriL-BsrGI-F and oriL-BglII-R (Table 2) and introduced into vectors pBUS1, pBUS1-P_{cap}, and pBJW13 (Table 1). PCR was performed using *Pfu* polymerase and 80 ng of pUB110 template for 30 cycles, consisting of 4 initial cycles with an annealing temperature of 56°C and an extension time of 1 min, followed by 26 cycles with an annealing temperature of 60°C and an extension time of 1 min. The vector sequences were PCR amplified using 100 ng of pBUS1, pBUS1-P_{cap}, or pBJW13 as a DNA template, *Pfu* polymerase, and primers pBUS1-oriL-F and pBUS1-oriL-R (Table 2). PCRs were performed as described above but using an extension time of 11 min and an annealing temperature of 61°C. All amplicons were digested using BglII and BsrGI, whose restriction sites were incorporated into primer sequences to facilitate cloning. The vector amplicons were treated with alkaline phosphatase prior to ligation with the *oriL* fragment. The new constructs were named pBUS1-HC, pBUS1-P_{cap}-HC, and pBJW13-HC (where HC indicates high copy) (Table 1).

Construction of pTSSC, pTSSC-P_{cap}, and pTJW13. The pAMα1 minimum replicon of pBUS1, pBUS1-P_{cap}, and pBJW13 was replaced with the pT181 replicon. To this end, pBUS1, pBUS1-P_{cap}, and pBJW13 sequences were amplified as described above for vector sequences but using the primers pBUS1-BsrGI-F and pBlueScript-BglII-R (Table 2). The pT181 replicon was amplified from pVPF5 (Table 1) using 25 ng of DNA template, *Pfu* polymerase, and the primers pT181-BglII-F and pT181-BsrGI-R (Table 2) using 30 amplification cycles with an annealing temperature of 56°C and an extension time of 3 min. Restriction digestion,

dephosphorylation, and ligation were performed as described above, generating pTSSC, pTSSC-P_{cap}, and pTJW13 (Table 1).

Construction of pTSSCm and pTSSCm-P_{cap}. The plasmids pTSSCm and pTSSCm-P_{cap} (Table 1) were generated through QuikChange site-directed mutagenesis using partially overlapping primers (37). The PCRs were performed using Phusion Hot Start II High-Fidelity DNA polymerase, 5 ng of pTSSC or pTSSC-P_{cap} as a DNA template, and the primers mut-repC-F and mut-repC-R (Table 2) for 30 cycles, including 3 initial cycles with an annealing temperature of 54°C and an extension time of 3.5 min, followed by 27 cycles with an annealing temperature of 65°C and an extension time of 3.5 min. The PCR products were DpnI digested and directly transformed into DH5α cells.

Generation of *erm*(44)-*rgs-his*$_6$ fusion plasmids. A total of 500 ng of pBJW13, pBJW13-HC, or pTJW13 was digested with BcgI, and 20 μM *S*-adenosylmethionine was added to the reaction mixture according to the manufacturer's instructions (New England BioLabs, Ipswich, MA). The DNA was purified using a High Pure PCR Product Purification Kit prior to ligation and transformation into *E. coli* DH5α. The transformants were confirmed for correct 3′ *rgs-his*$_6$ codon fusion through colony PCR and sequencing using the primers pBUS1-F2 and contig11-F1 (5′-CCAACTC TTATTTTCATCC). The suffix RGS-His was appended to vector names for variants expressing *erm*(44)-*rgs-his*$_6$ (Table 1).

Growth curve measurement. Growth of cultures of RN4220 strains was monitored in LB broth every 30 min during a 16-h incubation at 37°C with 420 shakes per minute using a Varioskan Flash plate reader (Thermo Scientific) for automated measurement of the optical density at 600 nm (OD_{600}). To obtain similar initial inocula, the RN4220 strains containing plasmids grown overnight on selective agar were first adjusted to a 0.5 McFarland turbidity standard in saline and then diluted 1:100 in LB broth containing 2 or 10 μg/ml tetracycline. Experiments were performed in triplicate in 96-well microtiter plates with culture volumes of 200 μl per well. Strain RN4220 without plasmid was inoculated in selective and nonselective medium to serve as a negative and positive control, respectively.

Plasmid stability test. For segregational stability analysis, RN4220 cells containing plasmids were grown for 24 h in LB broth without selection and subcultured on LB agar with and without 10 μg/ml tetracycline, and subsequently colonies were enumerated. Briefly, a single plasmid-carrying RN4220 colony was inoculated overnight into 5 ml of LB broth containing 10 μg/ml tetracycline. The overnight culture was diluted 1:500 into fresh LB medium without selection and grown for 8 h, followed by further dilution at 1:1,000 and incubation for 16 h. Fifty microliters of the diluted bacteria (1:1,300,000 in saline), corresponding to approximately 100 CFU, was plated onto LB agar to count the colony number. For each plasmid, the segregational test was performed in triplicate.

Structural plasmid stability was assessed through the restriction analysis of plasmid DNA isolated from RN4220 cells grown under selective conditions using enzymes NotI, PvuII, and StuI in combination with SacI.

Determination of plasmid copy number using quantitative real-time PCR assay. Multiplex real-time quantitative PCR (qPCR) was performed in a 7500 Real-Time PCR system (7500 software, version 2.0.5; Applied Biosystems) using 1× TaqMan Universal PCR master mix (Applied Biosystems). TaqMan primer and probe sets were specific for the chromosomal *S. aureus* nuclease gene *nuc* (primers, nuc263-F and nuc355-R; probe, nuc294-P) (38) and for the plasmidic gene *tet*(L) (primers, qPCR-tetL-F and qPCR-tetL-R; probe, FAM-tetL-TAMRA, where FAM is 6-carboxyfluorescein and TAMRA is carboxytetramethylrhodamine) (designed using Express Software, version 2.0; Applied Biosystems) (Table 2). DNA amplification was done with 2 μl of template in 25-μl reaction volumes with 300 nM primers and 200 nM probes and using the following cycling parameters: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. For standard curve preparation, plasmid pBUS1-P_{cap}-HC isolated from DH5α and genomic DNA extracted from RN4220 strain without plasmid were used. Separate qPCR standard curves were done for *nuc* and *tet*(L) measuring 4-fold serial dilutions ranging from 20 ng (6,783,250 copies) to 76 fg (26 copies) and from 500 pg (80,926,176 copies) to 1.9 fg (309 copies), respectively. DNA copies were calculated using the following formula: DNA copies = (g of DNA) × (6.022×10^{23} copies mol^{-1})/(bp of DNA) × (665 g mol^{-1} bp^{-1}). The size of the chromosome of RN4220 (2.67 Mb) was obtained from Nair et al. (39). DNA for standard curves was prepared using peqlab kits; dsDNA concentration was measured accurately using a Quantus fluorometer and QuantiFluor dsDNA dye (Promega). Plasmid DNA was linearized using NotI prior to measurement. DNA for plasmid copy number determination was obtained by crude lysis of three RN4220 colonies exhibiting similar sizes on selective agar (10 μg/ml tetracycline) after 18 to 24 h of incubation. The colonies were lysed in 50 μl Tris-EDTA (TE) containing 100 μg/ml lysostaphin for 10 min at 37°C. After addition of 200 μl lysis buffer (100 mM Tris-HCl, pH 8.5, 240 μg/ml proteinase K, 0.05% [vol/vol] Tween 20), the samples were incubated for 30 min at 60°C and for 10 min at 95°C. For qPCR, the lysates were diluted 1:10. Threshold cycle (C_T) values of standards and samples were measured in triplicates with automatic threshold settings.

Antimicrobial susceptibility testing. MICs were determined through the broth microdilution technique using 96-well microtiter plates with serial 2-fold dilutions of erythromycin (Sigma-Aldrich), which ranged from 256 μg/ml to 0.5 μg/ml (40). The MIC was defined as the lowest concentration of erythromycin with no visible growth after a 20-h incubation in Mueller-Hinton broth at 37°C.

Expression and purification of Erm(44)-RGS-His$_6$. RN4220, AG100A, or AG100 cells carrying the empty vector or *erm*(44) expression vector were grown to an OD_{600} of approximately 1 in LB broth containing 10 μg/ml tetracycline. Forty-five milliliters of the cultures was harvested through centrifugation and washed with 1 volume of phosphate-buffered saline (PBS; 137 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO, 1.8 mM KH_2PO_4, pH 7.4). RN4220 cells were preincubated in 0.4 ml of NPI-10 buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) supplemented with 100 μg/ml lysostaphin for 10 min at 37°C to digest the cell wall under native conditions. Subsequently, 1.2 ml of cDNPI-10 buffer (8 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) was added. The following steps were performed under denaturing conditions on ice or at 4°C. The buffers were supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2% (vol/vol) Triton X-100 (except for elution buffer DNPI-250). AG100A and AG100 cells were directly resuspended in 1.6 ml of DNPI-10 buffer (6 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). RN4220, AG100, and AG100A cells were disrupted using sonication (Branson Sonifier 250; duty cycle control, constant; output control, 3; three times for 15 s each). Unbroken cells and debris were removed through centrifugation at 16,000 × *g* for 10 min. The supernatants were used as total protein extracts for Western blot analysis or protein purification using a nickel-nitrilotriacetic acid (Ni-NTA)-agarose matrix (Qiagen, Hilden, Germany). To this end, 80 μl of the Ni-NTA-agarose suspension (washed once with 1.0 ml of DNPI-10 buffer) was incubated with 1.2 ml of total protein extract for 3 h on a lab rotator. The Ni-NTA-agarose beads were washed three times with 0.8 ml of DNPI-20 buffer (6 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and collected through centrifugation at 110 × *g* for 2 min. The proteins were eluted from the Ni-NTA-agarose matrix with 160 μl of DNPI-250 buffer (6 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) for 5 min at room temperature with shaking at 850 rpm. The protein eluates were mixed with Laemmli buffer, boiled, and separated using 15% SDS-PAGE. The gels were stained with Coomassie brilliant blue R-250 (VWR International, Radnor, PA).

Western blot analysis. Total protein extracts were prepared as described above. The protein concentration was measured according to the Bradford method (Micro Assay, Sigma-Aldrich) using bovine serum albumin (BSA) as a standard. Thirty micrograms of total protein extract was separated using 15% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were probed with primary and phosphatase-labeled secondary antibodies in TBS-T buffer (20

mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% [vol/vol] Tween 20) containing 2.5% nonfat dry milk. The target proteins were detected using the chromogenic substrates NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate) as recommended (Roche Diagnostics, Rotkreuz, Switzerland). The primary antibodies included a monoclonal mouse anti-RGS-His antibody (diluted 1:1,000) (5 Prime GmbH, Hilden, Germany) and a polyclonal rabbit antiserum specific for *E. coli* ribosomal protein L20ΔN (diluted 1:5,000) (41). The secondary alkaline phosphatase-labeled antibodies included goat polyclonal anti-mouse IgG (diluted 1:5,000) (KPL, Gaithersburg, MD) and goat polyclonal anti-rabbit IgG (diluted 1:5,000) (KPL).

Nucleotide sequence accession numbers. The nucleotide sequences for pBUS1-P_{cap}, pBUS1-P_{cap}-HC, and pTSSCm-P_{cap} were deposited in the GenBank/ENA/DDBJ databases under the accession numbers LN609189, LN609190, and LN609191, respectively.

RESULTS

Construction of a plasmid expression system containing the *S. aureus* type 1 capsule gene 1A promoter and a new multiple cloning site with joinable *rgs-his*$_6$ codons. Four vectors derived from pBUS1 (24), pBUS1-HC, pBUS1-P_{cap}-HC, pTSSCm, and pTSSCm-P_{cap}, were constructed containing the ColE1 origin for replication in *E. coli*, the resistance marker *tet*(L) for plasmid selection in both *E. coli* and *S. aureus*, and a tandem array of five copies of the T1 transcriptional terminator from the *E. coli rrnB* gene [(*rrnB* $T_1)_5$], located between the *tet*(L) gene and the MCS, to prevent RNA polymerase read-through (Fig. 1). The vectors differ in their Gram-positive replicons and MCSs. Vectors pBUS1-HC and pTSSCm contain the pBluescript II SK MCS (Stratagene) and are suitable for cloning and expressing genes under the control of their own promoters (Fig. 1A). In contrast, the expression vectors pBUS1-P_{cap}-HC and pTSSCm-P_{cap} contain the strong constitutive promoter P_{cap} (33) upstream of a novel MCS encoding the RGS-His_6 epitope for the production of tagged proteins and for high-specificity immunodetection (Fig. 1B). First, promoter P_{cap} and the new MCS were introduced into plasmid pBUS1, generating pBUS1-P_{cap}. For this, synthetic DNA containing the P_{cap} sequence including the ribosomal-binding site (RBS), a novel MCS with *rgs-his*$_6$ codons and 15 unique sites for type II restriction endonucleases, and a BcgI site was designed (Fig. 1B). BcgI recognizes the CGA$(N)_6$TGC site and cleaves DNA upstream and downstream to excise a defined 32-bp fragment with 2-base 3′ overhangs. The BcgI site was placed within the MCS to facilitate the linkage of *rgs-his*$_6$ codons to cloned genes ending at the TGA stop codon of the MCS. BcgI digestion, followed by religation, was used to convert the TGA stop to a TGC codon, leading to the expression of a C-terminal His-tagged protein variant (Fig. 1B). The 160-bp P_{cap}-MCS-*rgs-his*$_6$ sequence was inserted into plasmid pBUS1 using a modified QuikChange site-directed mutagenesis (Agilent Technologies) approach to replace the pBluescript II SK MCS. The PCR primers capHis-pBUS1-F and capHis-pBUS1-R contained the 5′ and 3′ regions, respectively, of the P_{cap}-MCS-*rgs-his*$_6$ insert at the overhangs (Table 2). In addition, these primers overlapped 20 bp at the 5′ end to facilitate *in vivo* recombination.

The backbone of pBUS1-P_{cap} and the precursor pBUS1 contain the minimum pAMα1 replicon derived from the shuttle plasmid pHY300PLK (42), containing *repB* and *dso oriU* but lacking *sso* for *S. aureus* (Fig. 1B). To optimize the stability of the shuttle vectors pBUS1-P_{cap} and pBUS1, we introduced the *sso oriL* of the staphylococcal pUB110 plasmid (12) using a PCR-based strategy with primers that carry a BglII or BsrGI site. In the newly generated constructs, pBUS1-P_{cap}-HC and pBUS1-HC (HC for high copy), *sso oriL* was inserted downstream of *tet*(L) (Fig. 1), as originally situated in pBC16 and pAMα1 plasmids. In addition, we replaced the complete minimum pAMα1 replicon of pBUS1-P_{cap} and pBUS1 with the pT181 replicon (8) using a similar PCR-based strategy and the *Staphylococcus xylosus* pVPF5 plasmid (43) as a replicon source. The resulting plasmids, pTSSC-P_{cap} and pTSSC, contained restriction sites for HindIII and XbaI in the *repC* coding sequence of pT181, which are also present in the MCS. To obtain the final constructs pTSSCm-P_{cap} and pTSSCm, these sites were removed and replaced with two silent mutations using site-directed mutagenesis (Fig. 1).

Stability and copy number of the shuttle vectors. The plasmid constructs were tested for stability and copy number in *S. aureus*. Differences among the constructs were visible in the RN4220 host colony morphology. Cells harboring vectors with complete Gram-positive replicons (pBUS1-P_{cap}-HC, pTSSC-P_{cap}, and pTSSCm-P_{cap}) displayed larger colonies than RN4220 carrying pBUS1 or pBUS1-P_{cap} on agar plates containing 10 μg/ml tetracycline (Fig. 2A). Growth retardation was also monitored in liquid cultures for RN4220 cells carrying plasmid pBUS1 or pBUS1-P_{cap} (Fig. 2B). The effect was, however, less pronounced when the tetracycline concentration was reduced from 10 to 2 μg/ml (Fig. 2B). Plasmids pBUS1-HC and pTSSCm displayed similar growth kinetics and colony sizes as their P_{cap}-carrying variants (data not shown). Segregational stability was quantified by determining the fractions of cells carrying plasmids after 24 h of growth in nonselective broth. Vectors pBUS1 and pBUS1-P_{cap} were maintained in less than 1% of RN4220 cells (pBUS1, 0.9%; pBUS1-P_{cap}, 0.8%). In contrast, vectors with a complemented pAMα1 replicon were present in almost half of the cells (pBUS1-P_{cap}-HC, 46%; pBUS1-HC, 48%), and vectors containing the pT181 replicon were maintained in at least 88% of the RN4220 cell populations (pTSSC-P_{cap}, 95%; pTSSCm-P_{cap}, 99%; pTSSCm, 88%). The plasmid yield was increased after the minimum pAMα1 replicon of pBUS1-P_{cap} was complemented with *oriL* and after the minimum replicon was replaced with the pT181 replicon. Analysis of plasmid extractions from equal numbers of RN4220 cells revealed a clear plasmid profile for pBUS1-P_{cap}-HC, pTSSC-P_{cap}, and pTSSCm-P_{cap}, while no bands were visible for pBUS1 and pBUS1-P_{cap} (Fig. 2C). The plasmid yield indicated that higher copy numbers were generated with the pT181 replicon than with the pAMα1 replicon. Plasmid extractions from RN4220 cells containing pBUS1-HC or pTSSCm were similar to extractions from pBUS1-P_{cap}-HC- and pTSSC-P_{cap}-/pTSSCm-P_{cap}-containing cells, respectively (data not shown). The absolute plasmid copy number in RN4220 transformants was determined by qPCR from strains grown under tetracycline selection by comparing the copy number ratio of the plasmidic *tet*(L) gene to that of the chromosomal *nuc* gene. Standard curves were prepared for each gene using a defined DNA concentration [PCR efficiency for *nuc*, 85.9%, $R^2 = 0.999$; for *tet*(L), 86.2%, $R^2 = 0.997$]. The estimated plasmid copy number per cell was around 16 to 20 for pBUS1-HC and pBUS1-P_{cap}-HC and 21 to 30 for vectors containing the pT181 replicon (Table 3). Structural plasmid stability was confirmed for the final vectors (pBUS1-P_{cap}-HC, pBUS1-HC, pTSSCm-P_{cap}, and pTSSCm) using restriction analysis (see Fig. S1 in the supplemental material).

Analysis of RGS-His_6 codon fusion and protein expression using the MLS_B resistance gene *erm*(44). We recently cloned the

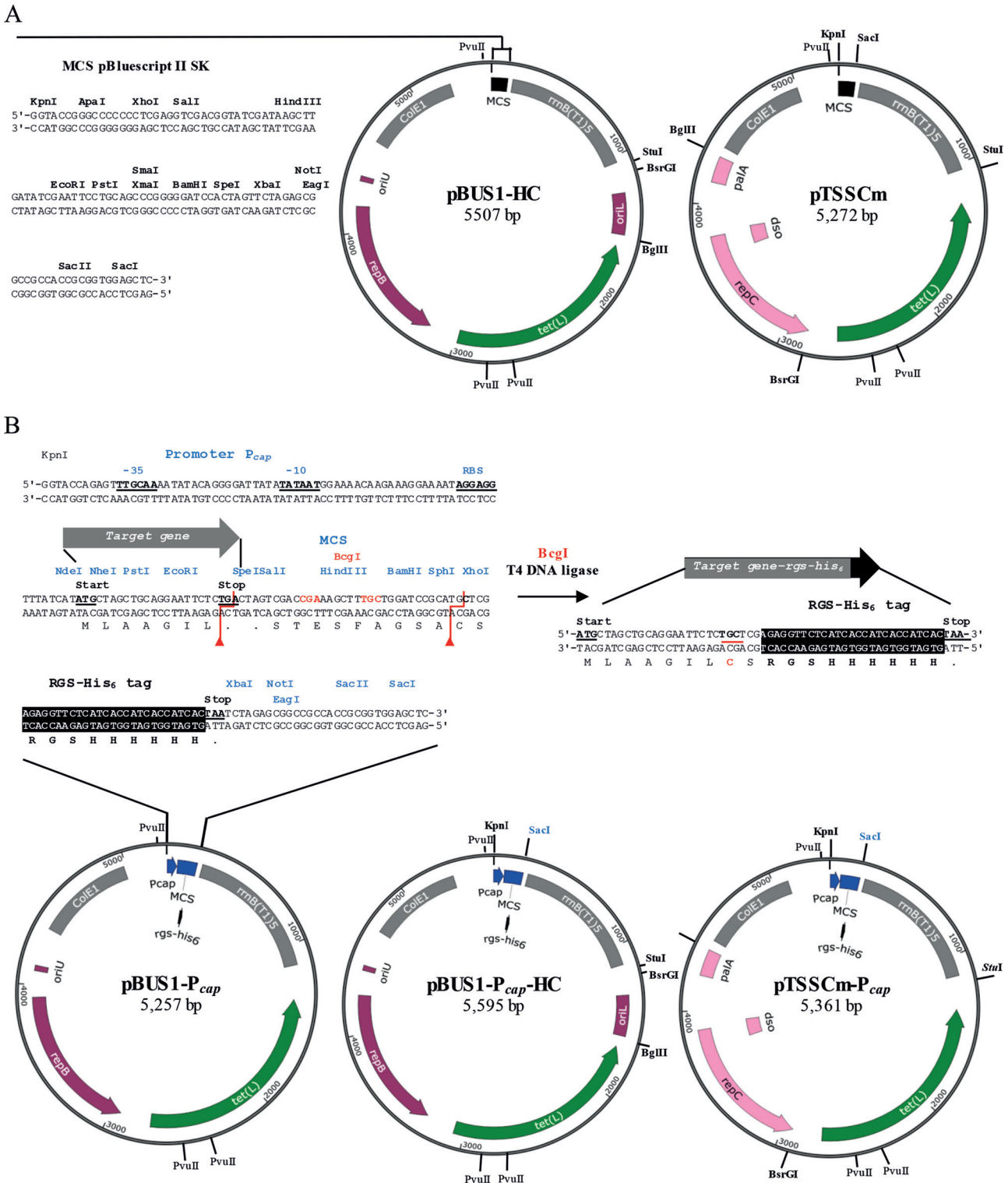

FIG 1 *S. aureus-E. coli* shuttle vectors for cloning genes with associated promoters (pBUS1-HC and pTSSCm) and for the constitutive expression of the target gene or the *rgs-his*$_6$ codon fusion from promoter P$_{cap}$ (pBUS1-P$_{cap}$, pBUS1-P$_{cap}$-HC, and pTSSCm-P$_{cap}$). The identical backbone segments in all plasmids include the *E. coli* origin ColE1 and the terminator sequence (*rrnB* T$_1$)$_5$, shown in gray, and the selectable marker for *S. aureus* and *E. coli* tet(L), in green. For propagation in Gram-positive bacteria, the plasmids contain either the pAMα1 replicon shown in violet or the pT181-family replicon in pink. The elements required for rolling-circle replication are indicated: the replication initiator protein gene (*repB* or *repC*), the double-strand origin (*oriU* or *dso*), and the single-strand origin (*oriL* or *palA*). (A) Plasmid maps of pBUS1-HC and pTSSCm containing the multiple cloning site (MCS) derived from pBluescript II SK (Stratagene) as the original pBUS1. (B) Plasmid maps of pBUS1-P$_{cap}$, pBUS1-P$_{cap}$-HC, and pTSSCm-P$_{cap}$ containing a novel MCS and the strong promoter (P$_{cap}$) and ribosomal-binding site (RBS) of *S. aureus* type 1 capsule biosynthetic gene 1A. The MCS comprises 15 unique restriction sites and codons for the peptide tag Arg-Gly-Ser-hexa-His (RGS-His$_6$), highlighted in black. The −35, −10 promoter sequences, RBS, and the start and stop codons useful for cloning are indicated in bold and underlined. Recognition and cleavage sites for the enzyme BcgI are indicated in red. Target genes inserted between the NdeI start and the TGA stop codon of the MCS (represented as a gray arrow) can be converted to a target gene-*rgs-his*$_6$ variant through BcgI digest, followed by ligation. Thereby, the TGA stop codon is mutated to TGC (red underlining), which encodes a cysteine residue. The RGS-His$_6$ tag is linked to the C-terminal end of the protein through two additional amino acids (CS). The plasmid maps were generated using SnapGene software (GSL Biotech, Chicago, IL).

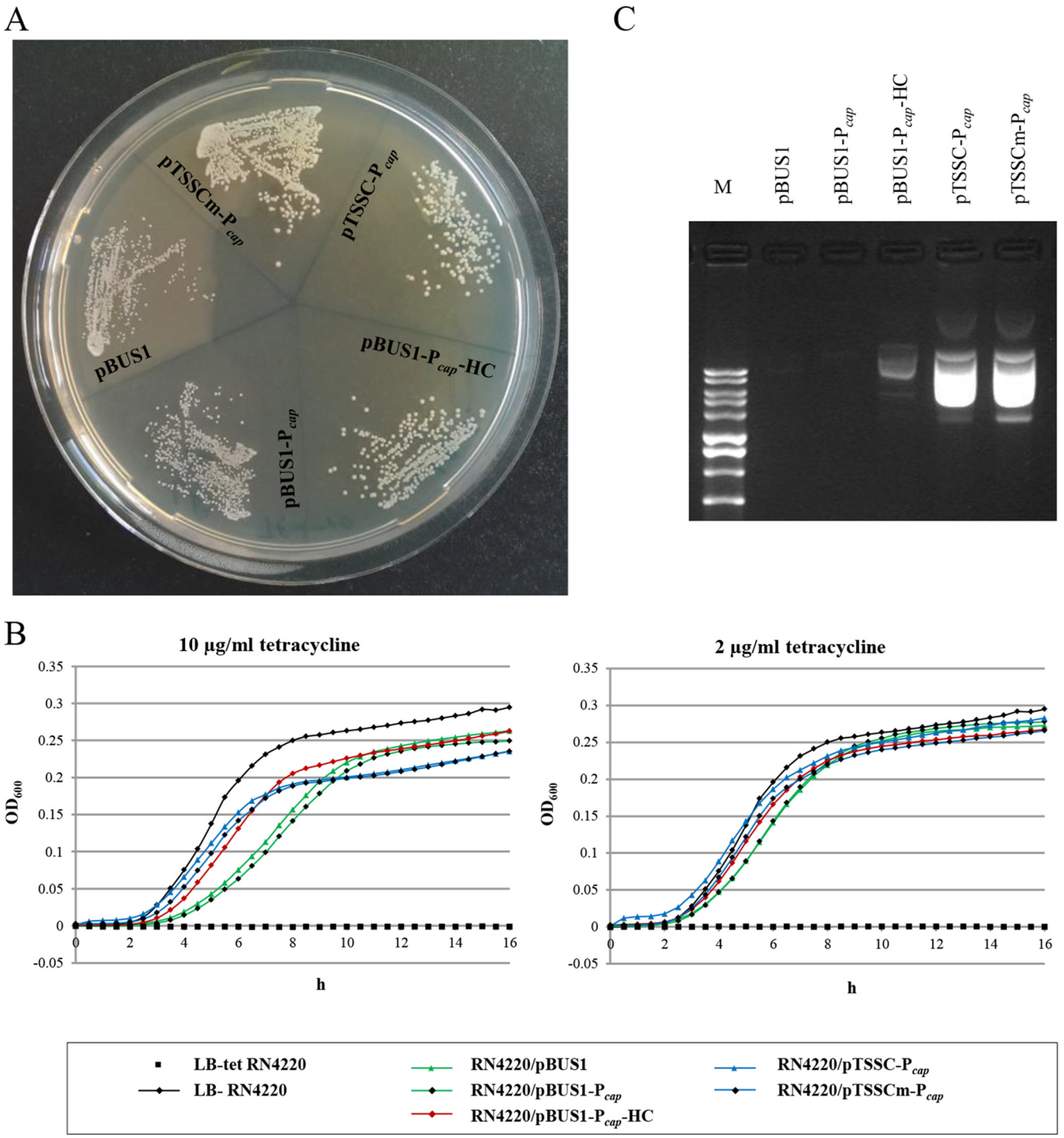

FIG 2 Analysis of cell morphology, growth curves, and plasmid profile of *S. aureus* RN4220 transformed with shuttle vector pBUS1, pBUS1-P_{cap}, pBUS1-P_{cap}-HC, pTSSC-P_{cap}, and pTSSCm-P_{cap}. (A) Colony size of RN4220 transformants after 16 h of growth on LB agar containing 10 μg/ml tetracycline. (B) Growth of RN4220 transformants in LB broth containing 10 μg/ml or 2 μg/ml tetracycline monitored through OD_{600} measurements during 16 h. Strain RN4220 without plasmid inoculated in selective (LB-tet RN4220) and nonselective (LB-RN4220) medium was used as a control. OD_{600} values are averages from triplicates. Note, OD_{600} values of 0.2 to 0.3 correspond to dense stationary-phase cultures; low values are due to measurement conditions, including short light path length. (C) Plasmid yield of the different vectors in RN4220. Plasmid DNA was isolated from similar 14-ml overnight cultures grown in selective LB broth and separated on 1% agarose gel. One-tenth of total plasmid extraction was loaded. M, 1 kb DNA ladder (Solis BioDyne).

TABLE 3 Estimated plasmid copy numbers of *S. aureus* RN4220 transformants determined by quantitative real-time PCR

Plasmid	C_T (avg ± SD)[a]		Avg no. of copies (CV [%])[b]		PCN (avg ± SD)[c]
	nuc	*tet*(L)	*nuc*	*tet*(L)	
None	21.44 ± 0.03		3.12×10^5 (1.8)		
pBUS1	21.52 ± 0.06	20.59 ± 0.11	2.97×10^5 (3.4)	3.71×10^6 (6.9)	12.48 ± 0.86
pBUS1-P_{cap}	22.04 ± 0.09	20.48 ± 0.10	2.16×10^5 (5.4)	3.98×10^6 (6.4)	18.45 ± 1.18
pBUS1-HC	21.56 ± 0.02	20.15 ± 0.03	2.91×10^5 (1.4)	4.87×10^6 (1.9)	16.74 ± 0.31
pBUS1-P_{cap}-HC	21.28 ± 0.05	19.55 ± 0.07	3.45×10^5 (3.3)	7.12×10^6 (4.2)	20.60 ± 0.86
pTSSC	21.00 ± 0.05	19.20 ± 0.02	4.12×10^5 (2.8)	8.81×10^6 (1.5)	21.41 ± 0.32
pTSSC-P_{cap}	20.77 ± 0.06	18.55 ± 0.10	4.73×10^5 (3.9)	1.32×10^7 (6.1)	27.93 ± 1.71
pTSSCm	20.98 ± 0.02	19.18 ± 0.03	4.17×10^5 (1.3)	8.91×10^6 (2.1)	21.39 ± 0.44
pTSSCm-P_{cap}	21.25 ± 0.02	19.06 ± 0.06	3.52×10^5 (1.2)	9.59×10^6 (4.0)	27.27 ± 1.06

[a] $n = 3$.
[b] CV, coefficient of variation ($n = 3$).
[c] PCN, plasmid copy number ($n = 3$).

new MLS_B resistance gene *erm*(44) into vector pBUS1-P_{cap}, generating plasmid pBJW13 (26). For that purpose, the open reading frame of *erm*(44) was inserted between the NdeI start codon and the TGA stop codon of the MCS and expressed from the strong promoter P_{cap} (Fig. 1B). Similar to vector pBUS1-P_{cap}, the plasmid stability of pBJW13 was optimized through the inclusion of the *oriL* sequence or the replacement of the minimum pAMα1 replicon with the pT181 replicon (Fig. 1B). The new *erm*(44) expression vectors were named pBJW13-HC (+*oriL*) and pTJW13 (pT181 replicon).

The conversion of *erm*(44) into *erm*(44)-*rgs-his*$_6$ through BcgI cleavage and religation was tested for plasmids pBJW13, pBJW13-HC, and pTJW13. Analysis using colony PCR showed that at least 50% of the clones carried the expected BcgI-induced deletion (Fig. 1B). Three positive clones were sequenced for each construct, and all possessed the correct *erm*(44)-*rgs-his*$_6$ fusion sequence. For these constructs, pBJW13-RGS-His, pBJW13-HC-RGS-His, and pTJW13-RGS-His, the expression of the *erm*(44)-*rgs-his*$_6$ product in *S. aureus* and *E. coli* was determined through Western blotting. Erm(44)-RGS-His_6 was detected with all three constructs in *S. aureus* RN4220 extracts (Fig. 3A). The effect of different plasmid yields was only partially apparent based on the amount of protein produced. A slight reduction in Erm(44)-RGS-His_6 synthesis was detected for RN4220 cells harboring the plasmid pBJW13-RGS-His, which contains the pAMα1 replicon without *sso* (Fig. 3A). The *S. aureus* P_{cap} promoter was functional in *E. coli*, and AG100A or AG100 cells expressed equal Erm(44)-RGS-His_6 protein levels with all constructs (Fig. 3B). The functional activity of Erm(44)-RGS-His_6 protein was examined by measuring the MIC of erythromycin for *S. aureus* and *E. coli*. Erm(44) conferred high levels macrolide resistance in RN4220, regardless of the expression vector used and whether the protein was His tagged (see Table S1 in the supplemental material). Erm(44) with or without RGS-His_6 also conferred erythromycin resistance in *E. coli* (see Table S1). As *E. coli* exhibits native resistance to macrolide antibiotics, we also transformed plasmids into the hypersensitive *E. coli* strain AG100A, an *acrAB* knockout mutant of AG100 (44). However, while vectors containing the pAMα1 replicon propagate without

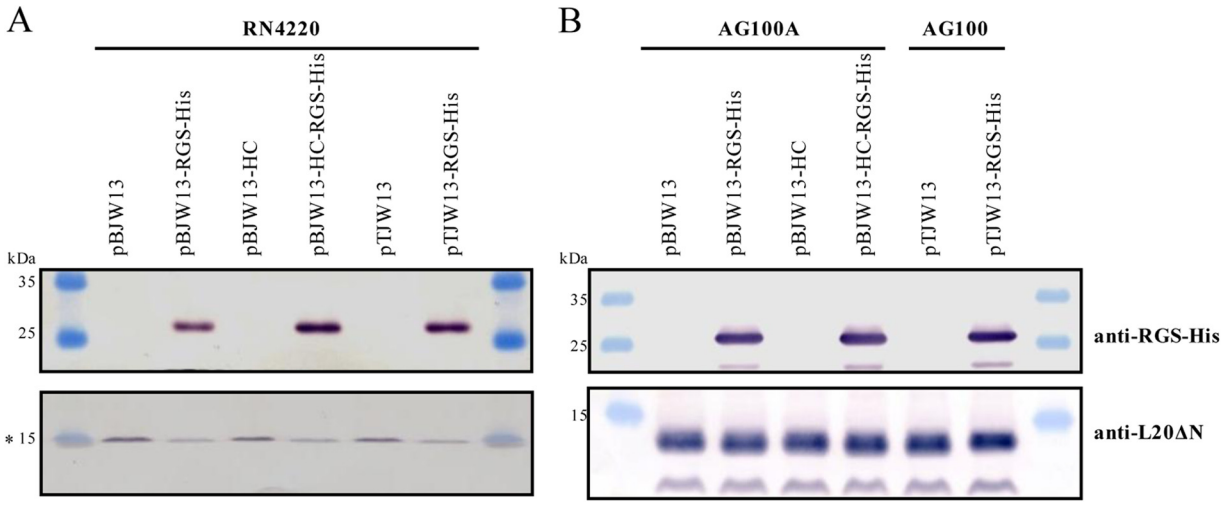

FIG 3 Analysis of Erm(44)-RGS-His_6 protein synthesis in *S. aureus* RN4220 (A) and *E. coli* AG100A or AG100 (B) cells harboring the expression vector for *erm*(44) or *erm*(44)-*rgs-his*$_6$. Thirty micrograms of total protein extract per sample was separated using 15% SDS-PAGE and analyzed through Western blotting. The upper part of the membrane was probed with anti-RGS-His antibody (5 Prime GmbH, Hilden, Germany) to detect tagged Erm(44), and the lower part of the membrane was probed with antiserum specific for the *E. coli* ribosomal protein L20ΔN (41), which served as a loading control. The asterisk at 15 kDa indicates the weak possible interaction of the antibody specific to *E. coli* L20ΔN with an *S. aureus* homolog. Prestained peqGOLD protein marker IV molecular size markers were used.

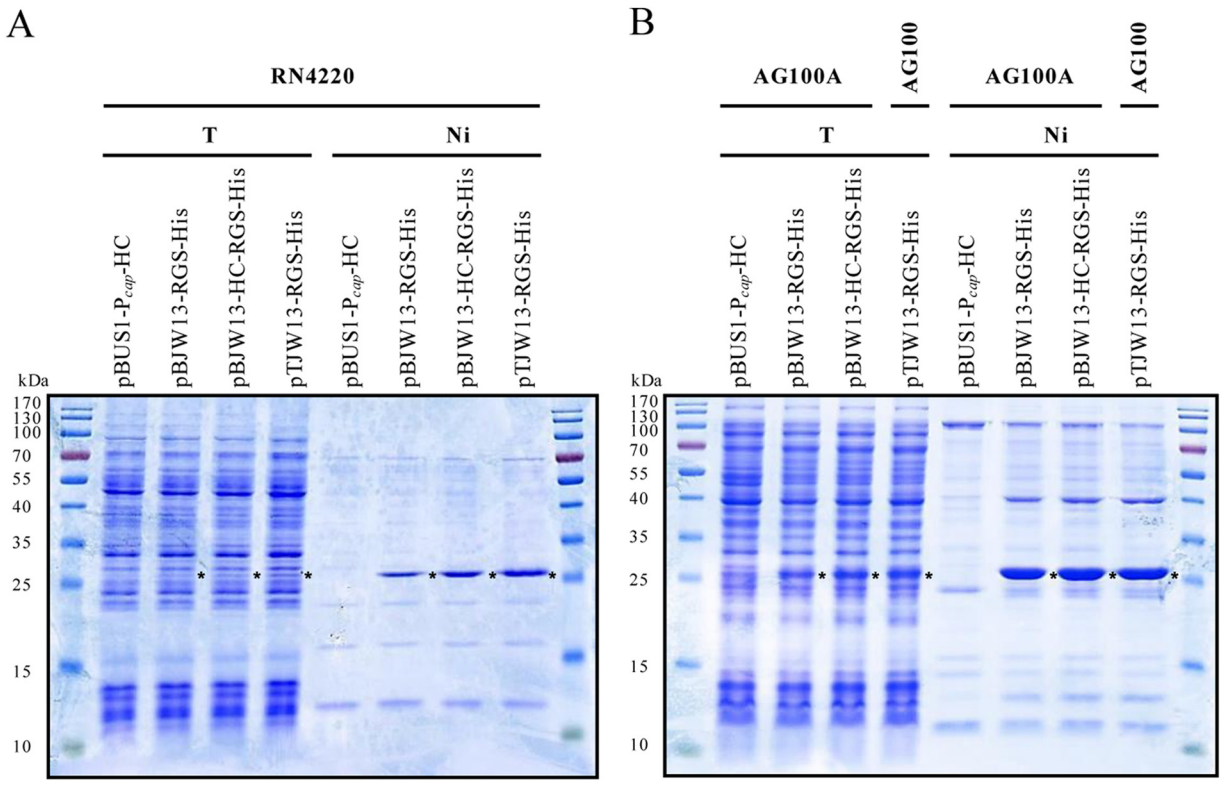

FIG 4 One-step nickel affinity purification of Erm(44)-RGS-His$_6$ from *S. aureus* RN4220 (A) and *E. coli* AG100A or AG100 (B). Cells carried one of the *erm*(44)-*rgs-his*$_6$ expression vectors (pBJW13-RGS-His, pBJW13-HC-RGS-His, and pTJW13-RGS-His) or the empty pBUS1-P$_{cap}$-HC vector as a negative control. Total protein extracts (T) or nickel affinity fractions (Ni) were separated using 15% SDS-PAGE, and the gels were stained with Coomassie brilliant blue. For RN4220, 12 μl of total protein extracts and 30 μl of nickel affinity fractions were analyzed. For *E. coli* AG100A and AG100, 10 μl of total protein extracts and 25 μl of nickel affinity fractions were used. The position of Erm(44)-RGS-His$_6$ on the gel is indicated with an asterisk. Prestained peqGOLD protein marker IV molecular size markers were used.

problems in AG100A, AG100A cells harboring shuttle vectors containing the pT181 replicon showed severe growth defects, necessitating the use of the wild-type AG100 strain for these constructs (see Table S1). To estimate protein overexpression from the gene under the control of P$_{cap}$ in *S. aureus* and *E. coli*, a simple one-step purification was performed using Ni-NTA-agarose to immobilize His-tagged Erm(44). Total protein extracts and Ni affinity fractions were analyzed using SDS-PAGE, and proteins were visualized using Coomassie staining. Although Erm(44)-RGS-His$_6$ was not clearly detected in RN4220 total protein extracts, this protein could be concentrated using Ni beads, representing the dominant band in the affinity fractions (Fig. 4A). Slightly larger amounts of Erm(44)-RGS-His$_6$ protein were detected in the fractions from RN4220 extracts carrying optimized expression vectors (pBJW13-HC-RGS-His and pTJW13-RGS-His) than in those from RN4220 extracts carrying pBJW13-RGS-His. Erm(44)-RGS-His$_6$ production was higher in *E. coli* and could be already detected in the total extract (Fig. 4B).

DISCUSSION

In Gram-positive bacteria, there are only a few tools available for protein affinity purification using small tag fusions. A pAM401-based shuttle vector has been developed for the expression of Strep-tag fusions in *Enterococcus faecalis* (45), and His$_8$ tag or Strep-tag shuttle vectors (pHT254, pHT255, and pHT253) have been developed for the expression of fusion proteins in *B. subtilis* (MoBiTec GmbH, Goettingen, Germany); however, none of these expression systems has been optimized for *Staphylococcus*.

Thus, we established a His tag expression system for *S. aureus*. The *S. aureus-E. coli* shuttle vector pBUS1 (24) was therefore optimized for better stability in *S. aureus* through either the complementation of the minimum pAMα1 replicon with the *sso oriL* or the replacement of the replicon with the pT181 replicon. The vectors were supplemented with a novel MCS region containing a His tag and the promoter P$_{cap}$ from *S. aureus* type 1 capsule biosynthetic gene 1A (33), a promoter used to regulate the strong expression of reporter genes in *S. aureus* (46, 47). Two *S. aureus-E. coli* expression vectors, pBUS1-P$_{cap}$-HC and pTSSCm-P$_{cap}$, were generated for the constitutive expression of target genes or *rgs-his*$_6$ codon fusions. These vectors offer multifunctionality as a gene of interest can be inserted under the control of P$_{cap}$ either directly as a fusion with *rgs-his*$_6$ codons or as a wild-type gene with potential to subsequently become fused with the *rgs-his*$_6$ codons through the BcgI-generated deletion of the stop codon and the linker sequence. Furthermore, these vectors are suitable for use in gene function studies in *S. aureus* and *E. coli* and for facilitating the purification of His-tagged fusion proteins from *S. aureus* or, with higher yield, from *E. coli*. In parallel, two *S. aureus-E. coli* cloning vectors were constructed, pBUS1-HC and pTSSCm, to study gene expression under the control of the native promoters.

The shuttle vectors constructed in the present study were based on Gram-positive RCR plasmids that replicate via ssDNA inter-

mediates (8). The stability of RCR-based vectors is controversial (3, 11, 15). On some occasions, vector instability might reflect incomplete replicon sequences, as observed with pBUS1. As expected for RCR plasmids without functional *sso* sequences (9–11), the vectors pBUS1 and pBUS1-P_{cap} showed low plasmid yield and severe segregational instability in *S. aureus*. However, the plasmid copy numbers did not differ substantially between RN4220 containing pAMα1 plasmids with and without *sso*. In addition, RN4220 cells containing plasmids with the minimum pAMα1 replicon displayed growth retardation compared to cells harboring plasmids with complete Gram-positive replicons in both solid and liquid cultures under tetracycline selection. These results suggest that plasmids lacking *sso* display similar levels of leading-strand synthesis but impaired completion of plasmid replication, leading to both segregational instability and replication intermediates which are lost during plasmid purification. The new constructs (pBUS1-HC, pBUS1-P_{cap}-HC, pTSSCm, and pTSSCm-P_{cap}) did not exhibit structural instability. The shuttle vectors containing the pT181 replicon supported higher plasmid copy numbers and segregational stability than the vectors carrying the complemented minimum pAMα1 replicon. However, the plasmid yield of dsDNA observed in the agarose gel for vectors containing the pT181 replicon appeared to be higher than that of vectors carrying the pAMα1 replicon, suggesting a more efficient lagging-strand synthesis. The copy numbers of the pT181 constructs from this study were in the range of 20 to 25 copies, as published for the original pT181 plasmid in *S. aureus* (48). In a previous study, an inverse correlation between the size of the recombinant RCR plasmid and copy number/segregational stability was demonstrated with pUB110, suggesting that constructs larger than 9 kb become incompatible with RCR (11). Thus, the new shuttle vectors, ranging from 5,272 bp to 5,595 bp in size, should be suitable for cloning DNA fragments up to 3 kb. Notably, shuttle vectors containing the pT181 replicon induced a severe growth defect in *E. coli* AG100A but not in the wild-type AG100 strain or in DH5α. Although AG100A transformants containing shuttle vectors with the pAMα1 replicon formed large colonies after 16 h of growth on selective agar, tiny colonies were not visible until 20 to 24 h for vectors harboring the pT181 sequence. The dysfunction induced through the pT181 replicon sequence itself or its encoded products (the RepC protein and the CopA RNAs) in the AG100A strain remained unknown and might represent a side effect of the hypersensitive phenotype of this *E. coli* strain, which has an inactivated AcrAB transporter system (44). For phenotypic analysis, such as antibiotic resistance determination in AG100A, we therefore recommend the use of the pAMα1-based shuttle vectors pBUS1-P_{cap}-HC and pBUS1-HC.

The arrangement of the BcgI restriction site within the MCS of the His tag expression vectors pBUS1-P_{cap}-HC and pTSSCm-P_{cap} facilitates the generation of a C-terminal RGS-His_6 translational fusion with a wild-type gene ending at the TGA stop codon of the MCS. The feasibility of converting a gene of interest to a 3′ *rgs-his*$_6$ fusion using a simple cut-religation method was demonstrated with the MLS_B resistance gene *erm*(44). The RGS-His_6 tag did not affect the function of Erm(44) in either *S. aureus* or *E. coli* and facilitated affinity purification and the detection of methylase protein using an RGS-His_4-specific antibody. The yields of His-tagged Erm(44) protein in *S. aureus* RN4220 were similar for both new expression vectors and were slightly higher than the yield observed with the original pBUS1-P_{cap} expression vector. Therefore, the amount of protein produced was only partially correlated with the plasmid yield of the different expression vectors. An increased amount of transcription from P_{cap} is expected (33); thus, a single plasmid copy might result in a substantial amount of transcript, whereas the amount of protein could be limited through other factors. Although, Erm(44)-RGS-His_6 was not highly overexpressed in RN4220, the yield was sufficient for Ni-NTA affinity purification. In contrast, Erm(44)-RGS-His_6 was highly expressed with the *S. aureus* promoter P_{cap} in *E. coli*. No sign of growth defect was observed for *erm*(44)-overexpressing cells, DH5α, AG100A, and AG100. The vectors presented herein might not be appropriate for the expression of genes with potential toxic effects. In such cases, the replacement of P_{cap} with an inducible promoter in *E. coli* and *S. aureus*, such as P_{spac} or $P_{xyl\text{-}tetO}$, should be considered.

In the present study, we established a plasmid-based expression system for *S. aureus*. This tool will facilitate the analysis of target gene function in biochemical experiments. For example, it could be used for the purification of fusion proteins directly from staphylococcal cells, the determination of translation initiation sites, posttranslational modifications, or even the examination of molecular interactions through the isolation of fusion protein complexes.

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