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New high-cloning-efficiency vectors for complementation studies and recombinant protein overproduction in *Escherichia coli* and *Salmonella enterica*

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Abstract

Galloway et al. recently described a method to alter vectors to include Type IIS restriction enzymes for high efficiency cloning. Utilizing this method, the multiple cloning sites of complementation and overexpression vectors commonly used in our laboratory were altered to contain recognition sequences of the Type IIS restriction enzyme, *Bsp*QI. Use of this enzyme increased the rate of cloning success to >97% efficiency. L(+)-Arabinose-inducible complementation vectors and overexpression vectors encoding *N*-terminal recombinant tobacco etch virus protease (rTEV)-cleavable H_6-tags were altered to contain *Bsp*QI sites that allowed for cloning into all vectors using identical primer overhangs. Additionally, a vector used for directing the synthesis of proteins with a *C*-terminal, rTEV-cleavable H_6-tag was engineered to contain *Bsp*QI sites, albeit with different over-hangs from that of the previously mentioned vectors. Here we apply a method used to engineer cloning vectors to contain *Bsp*QI sites and the use of each vector in either *in vivo* complementation studies or *in vitro* protein purifications.

Keywords

Type IIS restriction cloning; High-efficiency cloning vectors; Complementation vectors; Overexpression vectors; *Escherichia coli*; *S. enterica*

1. Introduction

Increasing cloning efficiency is desirable for the rapid completion of experiments where complementation of function or protein overproduction is the objective. Engler et al. developed a method termed Golden Gate cloning; in which a Type IIS restriction enzyme (*Bsa*I) is utilized for high efficiency cloning into expression constructs (Engler et al., 2008). Additionally, Galloway et al. (Galloway et al., 2013) reported an approach for the introduction of *Bsp*QI sites into a vector's multiple-cloning site (MCS). Type IIS restriction enzymes (*e.g.*, *Bsp*QI, *Bsa*I, BpiI) have been used in cloning methods for the construction of gene expression reporters (Oster and Phillips, 2011), recombinant protein expression (Engler et al., 2008; Galloway et al., 2013), assembly of multiple DNA fragments (Engler et al., 2009) or gene fusions and promoter shuffling (Engler and Marillonnet, 2014).

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Type IIS restriction enzymes cleave at least one strand of DNA located outside of the recognition sequence, with *Bsp*QI cleaving both strands of DNA (Roberts et al., 2003). Type IIS restriction enzymes act as dimers, with one domain binding to a 4–7 base pair recognition sequence and another domain interacting with a cleavage site 1–20 nucleotides away from the recognition site (Pingoud et al., 2005; Szybalski et al., 1991). Having a separate cut site from the recognition sequence is valuable for vector design because the cleavage site can be engineered to have its own unique overhangs. An enzyme, such as *Bsp*QI, has a recognition sequence [GCTCTTC (1/4)] where the first number in parentheses corresponds to the position of cleavage on the coding strand and the second number to the cleavage site on the complementary strand (Roberts et al., 2003). This creates a three base pair overhang, resulting in 81 different possible overhangs (Fig. 1).

The method described by Galloway et al. simplifies cloning efforts because digestion and ligation occur in the same reaction mixture, substantially reducing the time needed to clone genes of interest (Galloway et al., 2013). Additionally, because Type IIS restriction enzymes cut outside of their recognition sequence, ligated insert-vector products no longer contain the recognition site, and any empty vector remaining in the reaction mixture can be linearized by addition of the Type IIS restriction enzyme being used, substantially reducing false-positive background (Engler et al., 2008).

In this study, we used the method described by Galloway et al. to introduce *Bsp*QI sites into the MCSs of plasmids used for arabinose-inducible expression of genes of interest, or for the overproduction of proteins. Here, we modified the MCSs of three complementation vectors (pBAD24, pBAD30, and pBAD33-SD1 (Guzman et al., 1995a)). Specifically, each MCS was modified to contain two *Bsp*QI sites. We also modified two overexpression vectors to contain *Bsp*QI sites matching those present in the complementation vectors. The resulting vectors directed the synthesis of proteins of interest fused to an *N*-terminal, re-combinant tobacco etch virus (rTEV)-cleavable hexahistidine (H_6-tag (pTEV5, (Rocco et al., 2008)) or an *N*-terminal, rTEV-cleavable H_6, maltose binding protein tag (pTEV6, (Rocco et al., 2008)). Conveniently, cloning into all of the above-mentioned vectors was done using the same primer overhangs. Lastly, an overexpression vector with a pTEV backbone was also altered to contain *Bsp*QI sites. In this case, however, the protein whose synthesis was directed by this vector was fused to a *C*-terminal, rTEV-cleavable H_6-tag. Cloning into all of the *Bsp*QI-containing vectors was ≥97% efficient. The main goal of this work is to provide investigators with information regarding the specific aforementioned vectors.

2. Materials and methods

2.1. Bacterial strains, culture media, chemicals, and sequencing methods

All strains used in this study are listed in Table S1. *Escherichia coli C*41 (λDE3) (Miroux and Walker, 1996) and DH5α (New England Biolabs) strains were grown at 37 °C in lysogeny broth (LB, Difco). Strains used for growth analysis were derivatives of *Salmonella enterica* sv Typhimurium LT2 and grown at 37 °C in nutrient broth (NB, Difco) containing NaCl (85 mM), or no-carbon essential (NCE) minimal medium (Berkowitz et al., 1968) supplemented with sodium acetate (10 mM), *L*-methionine (0.5 mM), $MgSO_4$ (1 mM), and trace minerals (Atlas, 1995). Antibiotics were used at the following concentrations:

ampicillin, 100 μg mL^{-1}; chloramphenicol, 20 μg mL^{-1}. All chemicals were purchased from Fischer unless noted otherwise; chloramphenicol, L(+)-arabinose (Sigma-Aldrich); ethylenediaminetetra-acetic acid (EDTA, VWR); and isopropylβ-D-1-thiogalactopyranoside (IPTG, IBI Scientific). All restriction enzymes were purchased from Thermo Scientific™ with the exception of *Bsp*QI (New England Biolabs). DNA sequencing was performed using Big Dye® Terminator v3.1 protocols (Applied Biosystems). DNA sequencing was performed at the Georgia Genomics Facility.

2.2. Construction of vectors containing BspQI sites

Native *Bsp*QI sites (GCTCTTC) in the backbone of pBAD24, pBAD30, pBAD33-SD1, pTEV5, and pTEV6 were eliminated *via* site-directed mutagenesis and new *Bsp*QI sites were introduced into the MCS as described previously (Galloway et al., 2013). For a detailed procedure, see Supplemental Information or reference 2. Newly engineered vectors are listed in Table S3 and are hereafter referred to as pTEV16 (derived from pTEV5), pTEV17 (derived from pTEV6), and pCV1 (for *C*omplementation *V*ector, derived from pBAD24), pCV2 (derived from pBAD30), and pCV3 (derived pBAD33-SD1).

2.3. Site-directed mutagenesis of pTEV16 and pTEV17

To reduce the number of primers and to facilitate cloning into over-expression and complementation vectors, DNA containing the cut site (pTEV16: AGC, pTEV17: ACC) was mutated to the pCV cut site (*i.e.*, TTC) by site-directed mutagenesis. Polymerase chain reaction was performed using *Pfu* Ultra II DNA polymerase using primers listed in Table S2. Modifications included an anneal time of 60 s, an extension temperature of 68 °C, and an extension time of 2.5 min kb^{-1}. DNA changes were confirmed by sequencing.

2.4. Construction of C-terminal rTEV cleavable H_6-tag BspQI vector

The pTEV18 vector was amplified with *Pfu* Ultra II DNA polymerase using primers outside of the *N*-terminal H_6-tag and MCS (Table S2). PCR product was purified using Wizard® SV DNA Clean-Up System (Promega). Linear blunt-end fragments were ligated using the Fast-Link-DNA Ligation Kit (Epicentre), and transformed into DH5α chemically competent cells (Maniatis et al., 1982). Cells were plated on LB + ampicillin, incubated overnight at 37 °C, and 10 individual Ap^R colonies were used to inoculate fresh LB + ampicillin medium. Plasmids were isolated from each overnight culture using the Wizard® Plus SV Miniprep DNA Purification System (Promega). The presence of *Bsp*QI sites in the MCS was determined by cutting with *Bsp*QI and an enzyme outside of the MCS (*e.g.*, *Sca*I). The resulting MCS was sequenced to confirm the presence of the *Bsp*QI site and *C*-terminal H_6 rTEV cleavable tag.

2.5. Cloning of SeAcs into newly designed BspQI vectors

To illustrate the efficiency of the new vectors, the *S. enterica acs*$^+$ gene encoding acetyl-CoA synthetase was cloned. For this purpose, primers were designed with *Bsp*QI sites on the 5′-ends such that when amplicons were cut, a three base pair overhang was complementary to overhangs in the corresponding digested vector (Table S2). The *acs*$^+$ gene was PCR amplified from *S. enterica* LT2 genomic DNA using *Pfu* Ultra II DNA polymerase. PCR

products corresponding to the correct size were verified *via* gel electrophoresis and extracted as described above. Gene products were cloned into pCV1, pCV2, pCV3 pTEV16, pTEV17, pTEV18, pTEV19 and pTEV20 as described elsewhere (Galloway et al., 2013). For a more detailed procedure, refer to supplemental materials or reference 2. A sample of the ligation reaction (1 μL) was used to transform *E. coli* DH5α competent cells (Maniatis et al., 1982), and cells were plated on LB containing the appropriate antibiotic. The presence of inserts in the cloning vectors was confirmed by colony PCR with Go Taq® Green Master Mix (Promega) using primers that annealed to the plasmid outside of the MCS (Table S2). PCR products were analyzed on a 1% agarose gel with Tris/Borate/EDTA buffer for 40 min at 115 V. Cells harboring plasmids containing the expected inserts were grown overnight in LB supplemented with the appropriate antibiotic, and plasmids were isolated as described above. The presence of *acs*$^+$ in each plasmid was confirmed by DNA sequencing.

2.6. Growth studies

Complementation vectors carrying the *acs*$^+$ allele or empty vector were electroporated into *acs*$^+$ and Δ*acs* strains (O'Toole et al., 1993) (Table S1). Starter cultures of each strain were grown overnight at 37 °C with shaking in NB containing the appropriate antibiotic. Fresh minimal medium (198 μl) containing sodium acetate (10 mM), appropriate antibiotics, and L(+)arabinose (250 μM) was dispensed into each well of a 96-well microtiter dish. Each well was inoculated with 2 μl (1% v/v) of the aforementioned overnight cultures. Arabinose was included in the medium to induce expression of *acs*$^+$ from the P_{araBAD} promoter. Growth was monitored at 630 nm with shaking at 37 °C for 24 h (BioTek ELx808-1 Ultra microplate reader). Three technical and three biological replicates were analyzed; a representative growth curve is shown. Data were analyzed using Prism v6 software (GraphPad) and error bars represent standard deviation.

2.7. Purification of SeAcs from pTEV16-20

Plasmids encoding H_6-*Se*Acs (pACS65 and pACS67), H_6-MBP-tagged *Se*Acs (pACS66 and pACS68), and *Se*Acs-H_6 (pACS69) were electroporated using a protocol described elsewhere (Seidman et al., 1997) into *Escherichia coli* strain *C*41 (λDE3) (Miroux and Walker, 1996) Δ*pat* (strain JE9314). Cultures of cells containing plasmids were grown to stationary phase (OD_{650} ~ 1.3) and sub-cultured (1:100 v/v) into 1 L of LB + ampicillin. Cultures were grown shaking at 37 °C to an OD_{650} of 0.5, after which ectopic gene expression was induced with IPTG (0.5 mM). Cultures were grown overnight at 25 °C, cells were harvested by centrifugation at 6000 ×*g* for 15 min at 4 °C, and cell pellets were stored at −80 °C until used Acs proteins were purified from cell pellets as described (Hentchel and Escalante-Semerena, 2015). The amount of Acs in fractions was quantified on a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) using the molecular weight (72.15 kDa) and extinction coefficient (138,770 M^{-1} cm^{-1}; ExPASy ProtParam) of the protein. Percent purity was calculated using ImageQuant™ TL.

3. Results

3.1. Cloning efficiencies of newly designed vectors

The *S. enterica acs*$^+$ gene was cloned into each vector (Materials and methods Section 2.6) to test complementation (pCV1–3) and overexpression (pTEV16-20) using primers listed in Table S2. For each vector, 20 colonies were screened using colony PCR and cloning efficiencies were calculated (Table 1). A 2.2-kb band was indicative of a positive result, as shown in Fig. S2. The cloning efficiency varied between 95 and 100%. Information on troubleshooting cloning issues can be found in Table 3 of the Conclusions section.

3.2. Description of complementation vectors (pCVs)

Plasmids pCV1, pCV2, and pCV3 contained the promoter region of the *E. coli araBAD* operon (P_{araBAD}) for the purpose of inducing expression of genes of interest by the addition of L(+)-arabinose (Cronan, 2006; Guzman et al., 1995a). All vectors contained an *rrnB* terminator, and either a *bla*$^+$ gene for ampicillin resistance (pCV1, pCV2) or a *cat*$^+$ gene for chloramphenicol resistance (pCV3) (Guzman et al., 1995b). Plasmid pCV1 contained a pBR322 origin of replication, while pCV2 and pCV3 contain a p15A origin of replication, making pCV1 compatible *in vivo* with either pCV2 or pCV3. All plasmids contained M13 intergenic regions for ssDNA packaging into phage capsids. The MCS and plasmid maps of each complementation vector are shown in Fig. 2. Noteworthy is the presence of the Shine Dalgarno (SD) sequence in plasmids pCV1 and pCV3. Plasmid pCV2 did not contain a SD sequence, allowing for the cloning of a gene and its native ribosome-binding site. A stop codon was added after the second *Bsp*QI site and when combined with a gene's native stop codon, the error of read through was reduced. *Nhe*I, *Eco*RI and *Hin*dIII restriction sites surrounded the *Bsp*QI sites for verification of inserts by restriction analysis. It was necessary to add *Bsp*QI recognition and cut sites to both forward and reverse primers. This allowed for cutting of amplified gene products so they could anneal to the corresponding digested overhangs for each vector. In the case of pCV plasmids, the primer overhangs were identical for all three vectors. A simplified list of overhangs to be added to primers for each vector can be found in Table 2.

3.3. Use of complementation vectors for growth behavior analysis

S. enterica Δ*acs* strains grow poorly on 10 mM acetate as the sole source of carbon and energy, and such growth defect can be corrected by the ectopic expression of the *acs*$^+$ allele (Chan et al., 2011). We used the above-mentioned phenotype of *S. enterica acs* strains to verify the functionality of the *acs*$^+$ allele in the newly described complementation vectors as described in the Materials and Methods Section 2.7. Plasmids pCV1, pCV2, or pCV3 encoding *S.e. acs*$^+$ (pACS62-pACS64) were induced with L(+)-arabinose (250 μM), a concentration of inducer that effectively compensated for the absence of the Acs protein in a *S. enterica* Δ*acs* strain (Fig. 3). This result indicated that introduction of *Bsp*QI sites into the MCS did not disrupt the intended function of these plasmids.

3.4. Overexpression vectors containing rTEV protease cleaving sites

All of the TEV vectors described herein contained the *E. coli lacI*$^+$ allele, whose protein product acts as a repressor to the T_7 promoter of the plasmids. Addition of IPTG had the dual effect of relieving LacI repression and inducing transcription of genome-encoded T_7 RNA polymerase. All vectors included a *bla*$^+$ gene for the synthesis of β-lactamase, which provided resistance to ampicillin, an f1 origin for ssDNA packaging into phage capsids, and a pBR322 origin of replication (Bolivar, 1978; Soberon et al., 1980). The plasmid maps and MCSs of plasmids pTEV16-20 are shown in Fig. 4.

3.4.1. Plasmids pTEV16, pTEV18—Genes expressed from plasmids pTEV16 and pTEV18 resulted in proteins with an *N*-terminal, rTEV-cleavable H_6-tag. Plasmid pTEV18 was constructed by mutagenizing the *Nhe*I site of plasmid pTEV16 to GCTTTC, rendering this site inactive, but allowed for the design of one set of primers that could be used for cloning into the complementation and overexpression vectors. Tag removal resulted in proteins with three additional residues (*i.e.*, Gly-Ala-Ser for pTEV16; or Gly-Ala-Phe for pTEV18) on the *N*-terminus. A stop codon was engineered immediately after the *Bsp*QI sites to reduce read through when combined with the stop codon of the gene of interest. Plasmids pTEV16 and pTEV18 also contained multiple restriction sites in the MCS for alternative cloning methods or verification *via* restriction analysis. An *Xba*I site in plasmid pTEV18 is present upstream of the H_6-tag, if needed for insert verification by restriction analysis.

3.4.2. Plasmids pTEV17, pTEV19—Genes expressed from plasmids pTEV17 and pTEV19 directed the synthesis of proteins with an *N*-terminal rTEV-cleavable H_6-tag fused to a maltose binding protein (MBP). The MBP-tag was included with the goal of increasing protein solubility. Plasmid pTEV19 was constructed by mutagenizing the *Kpn*I site of pTEV17 to GGTTTC, rendering this site inactive but allowing for the design of primers that could be used with complementation and overexpression vectors in this study. A stop codon was also engineered in these vectors to reduce read through. Tag removal resulted in proteins with two additional residues (*i.e.*, Gly-Thr for pTEV17; or Gly-Phe for pTEV19). Additional restriction sites different from those found in plasmids pTEV16 and pTEV18 were present in plasmids pTEV17 and pTEV19 for alternative cloning options or verification *via* restriction analysis. An *Xba*I site in pTEV19 lies upstream of the H_6-tag, if needed for insert verification by restriction analysis.

3.4.3. Vectors engineered for the synthesis of C-terminally tagged proteins—Plasmid pTEV20 was also engineered for the overproduction of proteins. However, pTEV20 differed from the aforementioned overexpression vectors by the location of the rTEV-cleavage site and the primer overhangs. Proteins overproduced in cells harboring plasmid pTEV20 have a *C*-terminal H_6-tag that can be removed by rTEV protease. Plasmid pTEV20 was constructed by amplifying plasmid pTEV18 outside of the H_6-tag and MCS; consequently pTEV20 maintained the backbone features of plasmids pTEV16 and pTEV18. Differences in the MCS of pTEV20 can be seen in Fig. 4. Designed primer overhangs also differed for this vector due to the fact that there is no stop codon engineered immediately downstream of the cloned gene. When designing primers for pTEV20, it was necessary to

remove the stop codon of the native gene to ensure read through of the rTEV cleavage site and the H_6-tag. After cleavage, proteins obtained from pTEV20 retained six additional amino acids (*i.e.*, Glu-Asn-Leu-Tyr-Phe-Gln) at the *C*-terminus. A simplified list of overhangs to add to primers for each TEV-vector can be seen in Table 2.

3.5. Isolation of Salmonella enterica acetyl-CoA synthetase (Acs) from pTEV16-20

To assess the efficacy of the overexpression vectors, *S.e. acs*$^+$ was cloned into pTEV16-20, overexpressed in *E. coli* strain *C*41 (ΔDE3), and proteins were purified as described in the Materials and Methods Section 2.8. Purity of protein purified from cells overexpressing *acs*$^+$ from pTEV16, pTEV17, pTEV18, pTEV19, and pTEV20 was assessed and final protein products are shown in Fig. 5. Yields of *Se*Acs protein purified from overexpression from each vector were 8.35 mg L^{-1} (pTEV16), 0.9 mg L^{-1} (pTEV17), 8.55 mg L^{-1} (pTEV18), 0.9 mg L^{-1} (pTEV19), 5.32 mg L^{-1} (pTEV20).

4. Conclusions

Here we describe modified cloning vectors containing *Bsp*QI restriction sites that allow for rapid and efficient cloning of genes of interest. Common issues that may arise during cloning may be resolved by solutions provided in Table 3. Using these vectors paired with a previously described cloning method (Galloway et al., 2013), the number of cells carrying empty vectors is greatly reduced. We also have designed the complementation vectors (pCV1–3) and *N*-terminal rTEV cleavable H_6-tag vectors (pTEV18–19) to carry the same primer overhangs so that only one primer set is needed for *in vivo* and *in vitro* studies. Because MBP is a 42-kDA protein, yields of the cleaved protein once fused to MBP were lower. For this reason, it may be necessary to increase expression volumes to obtain similar yields compared to the pTEV16/18 vectors. It is important to note that when purifying proteins encoded by plasmids pTEV17 or pTEV19, some MBP contamination may occur, which if necessary may be removed using an MBPTrap HP column (GE Healthcare Life Sciences). Additionally, an *N*-terminal tag may be problematic for some proteins due to folding issues or interference with the protein's activity. For this reason we have provided a *C*-terminal rTEV-cleavable H_6-tag vector (pTEV20). Due to the nature of the rTEV cleavage site, recombinant proteins purified and cleaved from the cells harboring this vector will contain additional amino acids compared to the *N*-terminally tagged vectors. Overall, we have established a set of vectors that should facilitate complementation studies or protein production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Guzman LM, et al. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol. 1995b; 177:4121–4130. [PubMed: 7608087]

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plasmid.2016.05.001.

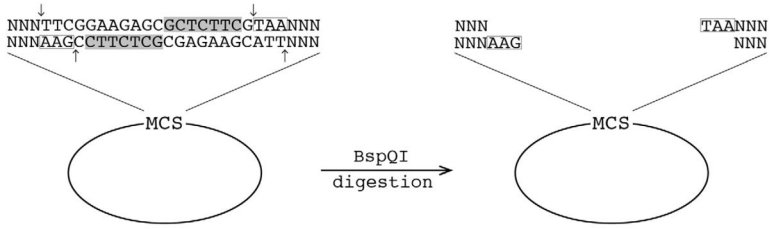

Fig. 1.

Visual representation of BspQI vector digestion. *Bsp*QI binds to the recognition site [GCTCTTC (1/4), highlighted in grey] and will cut both strands at indicated arrows. This creates a three base pair overhang. The cut site does not interfere with recognition and can be custom designed. Shown is an example of vectors constructed in this study (pCV1–3, pTEV18, 19). For a more detailed figure, see supplemental.

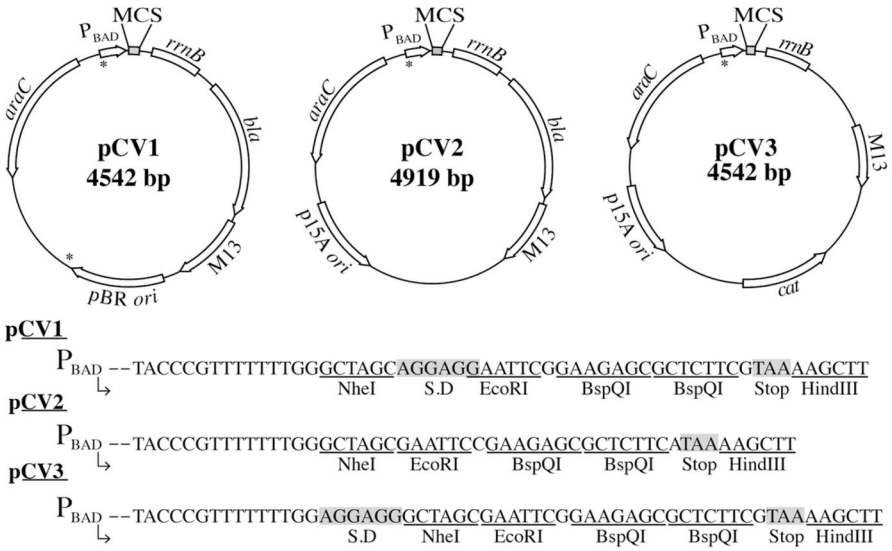

Fig. 2.

Plasmid maps and MCSs of complementation vectors. Plasmid maps show notable genetic features. Plasmid MCS sequences of each vector are shown between *Nhe*I and *Hin*dIII restriction sites. S.D. in MCSs represents Shine-Dalgarno (ribosome-binding site) sequence. Asterisks identify location of *Bsp*QI sites in original vectors that were mutated from GCTCTTC to WCTWTTC, as explained in Materials and methods Section 2.3.

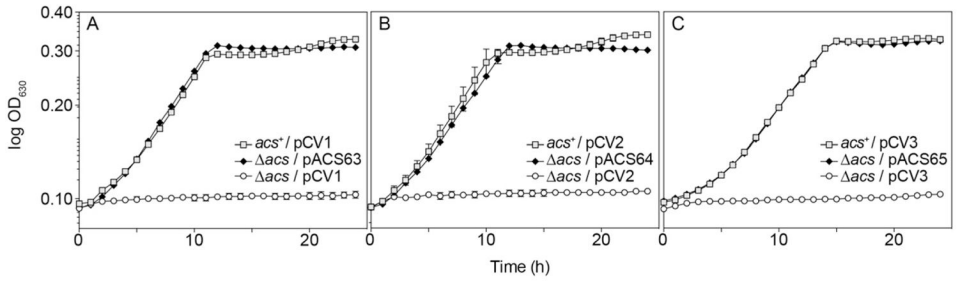

Fig. 3.

Growth analysis and complementation of *S. enterica acs phenotype. S.e. acs*$^+$ was cloned into pCV1-pCV3 and transformed into a *acs* strain to assess the effectiveness of the vectors. Cells were grown in NCE medium with acetate (10 mM) and transcription of *acs*$^+$ in each vector was induced with L(+)-arabinose (250 μM). Growth curves were obtained using a microplate reader (BioTek Instruments).

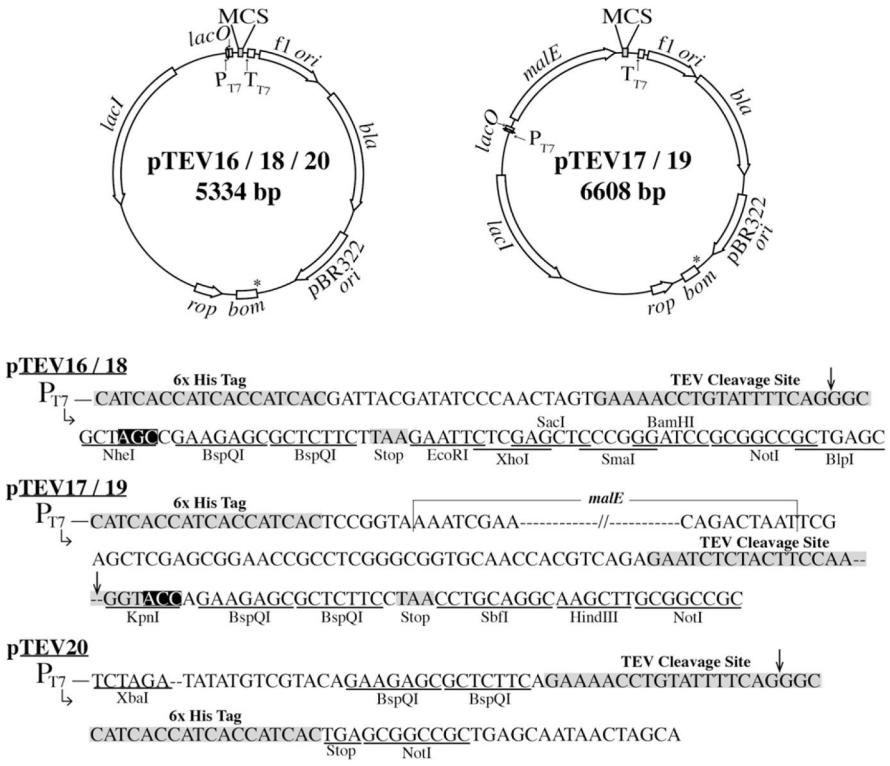

Fig. 4.

Plasmid maps and MCSs of overexpression vectors. Plasmid maps show notable genetic features. Plasmid MCS sequences of each vector are shown. Nucleotides inside black boxes of pTEV16 were changed to TTC resulting in pTEV18 and nucleotides in black boxes in pTEV17 were changed to TTC resulting in pTEV19. Therefore pTEV18 lacks an *Nhe*I site and pTEV19 lacks a *Kpn*I site. An *Xba*I site lies upstream of the H_6-tag. Plasmid pTEV20 was constructed by amplifying plasmid pTEV18 outside of the H_6-tag and *Not*I restriction site, resulting in an rTEV-cleavable *C*-terminal H_6-tag. Arrows mark the rTEV cleavage site. Asterisks identify location of *Bsp*QI sites in original vectors that were mutated from GCTCTTC to WCTWTTC, as explained in Materials and methods Section 2.3.

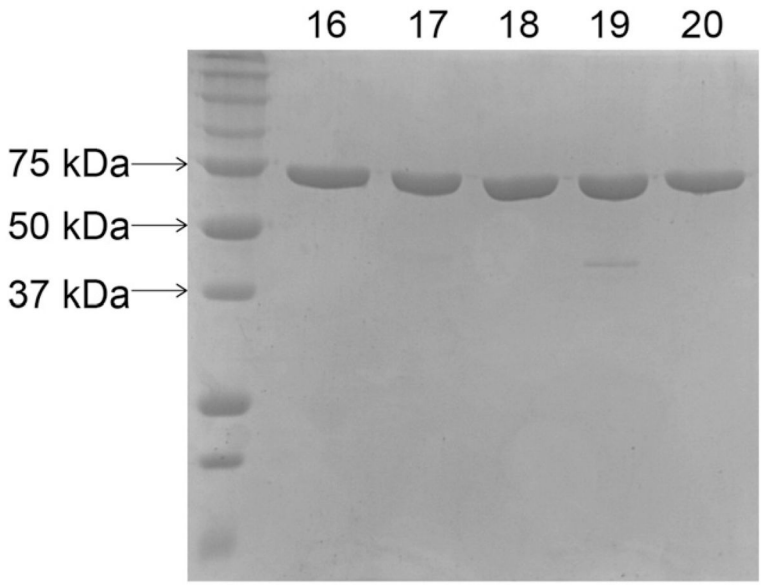

Fig. 5. SDS-PAGE gel of purified Acs

A 12.5% SDS-PAGE gel shows molecular weight standards (BioRad Precision Plus Protein™, left) and Acs (72 kDa) purified from cells harboring pACS65 (pTEV16), pACS66 (pTEV17), pACS67 (pTEV18), pACS68 (pTEV19), and pACS69 (pTEV20). Numbers above each well correspond to protein purified from cells harboring respective pTEV vectors (*e.g.*, well labeled 16 corresponds to pTEV16).

Table 1

Cloning efficiencies of newly designed vectors.

Vector	GenBank accession #	No. of positive clones (20 screened)[a]	Efficiency (%)[b]
pCV1	KU974153	19	95
pCV2	KU974154	20	100
pCV3	KU974155	19	95
pTEV16	KU974156	20	100
pTEV17	KU974157	20	100
pTEV18	KU974158	18	90
pTEV19	KU974159	19	95
pTEV20	KU974160	20	100

A positive result was indicated by a 2.2 kbband as shown in Fig. S2.

[a]Colony PCR was used to screen 20 colonies for each vector.

[b]Cloning efficiencies were calculated as described under Materials & methods Section 2.6.

Table 2

Designing primers for vectors used in this study.

Plasmid	Sequence to add to 5′ of primer
pTEV16 forward primer	5′NNGCTCTTCNAGC
pTEV17 forward primer	5′NNGCTCTTCNACC
pCV1, pCV2, pCV3, pTEV18, pTEV19 forward primer	5′NNGCTCTTCNTTC
pCV1, pCV2, pCV3, pTEV16, pTEV17, pTEV18, pTEV19 reverse primer	5′NNGCTCTTCNTAA
pTEV20 forward primer	5′NNGCTCTTCNTAC
pTEV20 reverse primer	5′NNGCTCTTCNTTC

Table 3

Troubleshooting.

Problem	Potential explanations or solutions	
Cloning yielding all empty vector	**1**	Potential primer dimers cloning into vectors, gel extract PCR reaction
	2	Check activity of *Bsp*QI
Cloning yielding incorrect insert sizes	**1**	Verify competent cells are DH5α and not contaminant
	2	Contaminants in PCR reaction, gel extract correct band size
Cloning yielding zero colonies	**1**	Confirm primer overhangs correspond correctly to vector overhangs
	2	Check for *Bsp*QI sites in gene, if present eliminate second *Bsp*QI digestion step, then inactivate ligase at 70 °C before transformation
	3	Re-PCR amplify insert to rule out mutations in overhangs
	4	Verify activity of ligase or stability of ATP