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Low copy expression vectors for use in *Yersinia* sp. and related organisms

Markus W. Obrist[1] and **Virginia L. Miller**[1,2,*]

[1]Department of Genetics, University of North Carolina School of Medicine, Chapel Hill, NC 27599

[2]Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC 27599

Abstract

In *Yersinia*, the most commonly used expression vectors for genetic studies such as gene complementation do not effectively allow for both induction and repression of gene expression. Additionally, there is no expression system available that can be induced in bacteria growing *in vitro* as well as *in vivo*, e.g. in eukaryotic cell lines or in living animal models. Here, we present a series of novel inducible low copy expression vectors that are well suited for use in the *Yersinia* species. Their *tet* operator/promoter/repressor system makes them distinct from other vectors, and gene transcription in bacteria can easily be induced by addition of anhydrotetracyline (ATc) either to the growth medium, to tissue culture medium during bacterial infections of cell lines or by injection into animals infected with bacteria. Researchers can choose between two different antibiotic resistances (kanamycin or spectinomycin), between two copy numbers (5 or 12-22) as well as between two different versions for expression from either the native RBS and ATG or RBS and ATG encoded in the plasmid. The whole vector series contains the same multi-cloning site from pBluescript II KS+ that allows for easy subcloning. Moreover, these vectors are built in a modular fashion that makes it simple to adapt them for other purposes. Finally, in addition to their use in *Yersinia* they are suitable for use in many other *Enterobacteriaceae*.

Keywords

complementation vector; tet operator; tetracycline inducible; Yersinia; low copy number

Introduction

Bacterial genetics often involves complementation studies that rely on inducible promoters to allow for control of expression of the complementing gene. One way to achieve this goal is to design an inducible gene construct that is introduced on the chromosome of a given deletion mutant. As this strategy is often time consuming and sometimes hindered by a limited number of genetic tools for certain bacterial species, plasmid expression vectors have gained huge popularity. However expression vectors have their own drawbacks. They often tend to over-produce the protein of interest because of the high copy number, their

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*Corresponding author: Mailing address: Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, 804 Mary Ellen Jones Bldg, Campus Box 7290, Chapel Hill, NC 27599-7290, Phone: 919-966-9956, Fax 919-962-8103, vlmiller@med.unc.edu.

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induction system can be difficult to titrate, some promoters are not fully repressed in the absence of the inducer, and some vectors do not replicate in the species of interest in contrast to *Escherichia coli*, where most cloning is performed.

Low copy vectors often used for gene complementation in the *Yersinia* species such as pWSK129, pWKS130, pKW1, pACYC184, pRW50 or pTM100 are based on either the p15A or the pSC101 origin of replication (Chang and Cohen, 1978; Michiels and Cornelis, 1991; Wang and Kushner, 1991; Lodge et al., 1992; Wagner et al., 2008). Several of these vectors contain either the *tac* or *lac* promoter which can be repressed by LacI (Jacob and Monod, 1961; de Boer et al., 1983). As none of these vectors contain the *lac* repressor-encoding *lacI* gene, they rely on the repressor gene to be provided *in trans* e.g. on a plasmid or on the chromosome. The Yersinia enterocolitica, Yersinia pseudotuberculosis and *Yersinia pestis* chromosomes do encode the *lacI* gene but in our experience, their gene products do not efficiently repress the aforementioned promoters. Therefore, these vectors are used under derepressed conditions, and genes cloned into these vectors are constitutively transcribed. An inducible low copy expression vector that has been successfully used in *Yersinia* is pBAD33 (Guzman et al., 1995; Yamaguchi et al., 2010). The vector contains the *araBAD* promoter that can be induced by addition of arabinose to the medium. Unfortunately, this induction system produces mixed populations under subsaturating arabinose concentrations where some cells are not induced in contrast to others that are fully induced. This result was found due to unequal uptake of arabinose, which does not freely diffuse into the cells but is actively taken up by transport systems which are under arabinose induction themselves (Siegele and Hu, 1997).

Another common expression system uses the *tet* operator/promoter, which can be induced by the antibiotic tetracyline (Hillen and Berens, 1994). Anhydrotetracyline (ATc) is a tetracyline derivative that also induces the *tet* operator/promoter system. ATc does not depend on active uptake into either bacterial or eukaryotic cells, binds stronger to the *tet* repressor and is less toxic to the cells than tetracycline (Gossen and Bujard, 1993; Skerra, 1994; Lutz and Bujard, 1997). Thus, ATc can be used to induce tetracycline inducible promoters in bacteria as well as in eukaryotic cells (Gossen and Bujard, 1993). Recently, the *tet* operator/promoter system was successfully used to induce expression of a reporter construct in *Y. pestis* cells residing in the lungs of mice by injection of ATc into the animals (Lathem et al., 2007). Therefore, we decided to use the *tet* operator/promoter system to construct a series of low copy expression vectors. The vector series has a modular basis where the origin of replication and antibiotic resistance can be exchanged for individual purposes. These vectors increase the number of genetic tools for studying gene expression in the *Yersinia* species as well as in other *Enterobacteriaceae*.

2. Materials and methods

2.1 Bacterial strains and growth conditions

Bacterial strains used in this work are presented in Table 1. *Escherichia coli* strain DH5α was used for cloning purposes and grown at 37°C in LB medium (Difco; Sparks, MD). *Yersinia enterocolitica* strain JB580v was used for control experiments and grown for 15 h at 26°C in LB medium or for 48 h at 26°C on LB plates. *Yersinia pseudotuberculosis* strain IP32953 and *Yersinia pestis* strain YP6 were used for control experiments and were grown for 15 h at 26°C in filter sterilized brain heart infusion medium (BHI, Bacto; Sparks, MD) or for 48 h at 26°C on BHI plates (BHI Agar, Bacto; Sparks, MD). *Yersinia enterocolitica* strain YVM1250 was used to test expression and was grown at 26°C in LB medium. To perform β-galactosidase tests *Y. entercolitica* strain YVM1250 was grown overnight in L-broth (1% tryptone, 0.5% yeast extract; referred to hereafter as LB-0) and subcultured into L-broth containing 290mM NaCl (referred to hereafter as LB-290). We noticed that cells

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carrying the new vectors were growing more slowly and forming much smaller colonies when selected with our previously published antibiotics concentrations (Walker and Miller, 2004; 2009; Walker et al., 2010). We therefore lowered concentrations for kanamycin and spectinomycin. For plasmid selection in *E. coli*, antibiotics (all from Sigma-Aldrich Corp.; St. Louis, MO) were added at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 25 μg/ml; spectinomycin, 50 μg/ml. For selection of plasmids in the *Yersinia* species antibiotics were added at the following concentrations: chloramphenicol, 12.5 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml.

2.2 DNA isolation, manipulation and oligonucleotides

Genomic DNA was isolated from *Y. enterocolitica* strain JB580v according to standard protocols (Sambrook and Russell, 2001). Plasmid DNA was isolated from *E. coli* strains using Wizard Plus SV Miniprep (Promega; Madison, WI). DNA extraction from agarose gels was performed using Wizard SV Gel and PCR Clean-Up System (Promega). DNA manipulations were carried out according to standard procedures (Sambrook and Russell, 2001) unless otherwise stated. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (NEB; Ipswich, MA) and were used as recommended by the supplier. Cloned Pfu DNA polymerase was purchased from Agilent Technologies Inc. (Santa Clara, CA). Chemically competent *E. coli* cells were made according to the rubidium chloride method (NEB protocol) and were transformed by heat-shock. Competent *Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis* cells were prepared as described in (Walker and Miller 2004) and transformed by electroporation (BIORAD Gene Pulser II; settings: 1.8 kV, 25 Ω, 400 mA). Oligonucleotides used in this study are listed in Table 2 and were purchased from Integrated DNA Technologies (IDT; Coralville, IA). DNA sequence analysis was performed by either Eton Bioscience Inc. (San Diego, CA) or GENEWIZ (South Plainfield, NJ).

2.3 Plasmid construction

Vectors constructed in this study are presented in Results and are listed in Table 3. Plasmids used and constructed in this study are listed in Table 1. All plasmids were confirmed by restriction digestion as well as by sequencing of the critical regions. To test the newly constructed vector pMWO-005, a derivative plasmid, pMWO-038, encoding 6xHis-*ysrR* was constructed. The *ysrR* gene was PCR amplified from *Y. enterocolitica* genomic DNA using MWO-147 and MWO-072. MWO-147 adds a 6xHis-tag encoding sequence to the 5′-end of the *ysrR* gene. The PCR product was digested with *Kpn*I and *Xba*I and ligated into pMWO-005. As we had set the start codon too far from the RBS, we saw almost no expression from pMWO-038 (data not shown). Therefore, we designed a new forward primer, MWO-150, using the same restriction site. Using pMWO-038 as template, 6xHis-*ysrR* was PCR amplified with MWO-150 and MWO-072. The PCR product was digested with *Kpn*I and *Xba*I and ligated into pMWO-005 thereby creating pMWO-081. The insert was sequenced with MWO-fw and MWO-rv-1.

2.4 ATc toxicity test

Y. enterocolitica (JB580v), *Y. pseudotuberculosis* (IP32953) and *Y. pestis* (YP6) were grown overnight at 26°C. The next morning, two subcultures for each strain were inoculated to an optical density at 600 nm (OD_{600}) ≈ 0.2. To one culture of each pair of strains, anhydrotetracycline hydrochloride [ATc (Fluka Analytical; Sigma-Aldrich)] was added using the following concentrations: 1.0 or 2.0 μg/ml and additionally in the case of *Y. enterocolitica*: 0.1, 0.25, 0.5, 0.75 μg/ml. Cultures were grown at 26°C and optical densities were checked after 8 h.

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2.5 Growth curve experiment

Y. enterocolitica, Y. pseudotuberculosis and *Y. pestis* harboring either pMWO-005, pMWO-073, pMWO-074 or pMWO-075 were grown overnight at 26°C with antibiotic selection. The next morning, one subculture for each strain was subcultured to $OD_{600} \approx 0.2$ and grown at 26°C under antibiotic selection up to $OD_{600} \approx 0.4$. Then each culture was split into two subcultures and one was induced with 50 ng/ml ATc. Cells were grown for 6 h and optical densities were monitored each hour.

2.6 Plasmid stability test

Y. enterocolitica, Y. pseudotuberculosis and *Y. pestis* harboring either pMWO-005, pMWO-073, pMWO-074 or pMWO-075 were grown overnight at 26°C with antibiotic selection. The next morning, two subcultures for each strain were subcultured to $OD_{600} \approx$ 0.2. One culture of each strain pair was grown with and one without antibiotic selection. Cells were grown for 6 h. Samples were taken and serial dilutions were made and plated onto LB or BHI plates with or without antibiotics. Plates were incubated for 48 h at 26°C, colonies were counted and numbers were analyzed and compared.

2.7 Protein expression test and Western blot

pMWO-081 was transformed into YVM1250 by electroporation and transformants were grown on selective LB agar plates at 26°C for two days. Five ml LB cultures containing 20 μg/ml nalidixic acid and 100 μg/ml kanamycin were inoculated with a single colony and grown overnight at 26°C on a roller drum. These cultures were subcultured to $OD_{600} \approx 0.2$ into 2 ml fresh medium with antibiotics. Cultures were grown at 26°C on a roller drum until $OD_{600} \approx 0.4$, where they were induced with 50 ng/ml ATc and grown for 2 h under the same conditions. Initially, we also tested other concentrations but found 50 ng/ml ATc to be the optimal induction level. OD_{600} was read and 100 μl samples of cultures were harvested and pelleted. Pelleted cells were resuspended in 5x SDS-PAGE sample buffer to 1 ml per OD_{600} = 1.0. Samples of 0.10 OD were analyzed by SDS-PAGE on 16% tricine gels (Schägger and Jagow, 1987) and transferred onto nitrocellulose membranes by Western blot. His-YsrR was detected using an anti-His antibody (monoclonal mouse anti-His antibody; 0.1μg/ml; THE™ His Tag Antibody; GenScript; Piscataway, NJ) and a secondary antibody [goat-anti-mouse IgG (Fc specific)-peroxidase; 1:50,000 dilution; Sigma-Aldrich], followed by chemiluminescence detection (Amersham™ ECL™ Western Blotting Detection Reagents; GE Healthcare; Waukesha, WI).

2.8 β-galactosidase assay

Prior to each β-galactosidase test, YVM1250 was transformed with the appropriate plasmids by electroporation and colonies were grown on LB-agar plates for two days at 26°C. Saturated cultures grown overnight at 26°C on a roller drum in LB-0 were diluted into fresh LB-290 to OD_{600} of 0.2 and grown for 2 h with or without 50 ng/ml ATc using the same growth conditions. Antibiotics were added as necessary to retain plasmids. After 2 h, cultures were put immediately on ice to prevent further growth and β-galactosidase assay was performed as described before (Miller, 1992).

3. Results and discussion

3.1 Design and characteristics of new vectors

The vector series presented in this study is intended for gene complementation, low level protein expression and related purposes. We designed the vectors to have the following characteristics: (i) replicate in the *Yersinia* species (*Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis*) as well as in *E. coli*, (ii) have a low copy origin of replication, (iii) bear a

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selection marker suitable in all mentioned organisms, (iv) contain a versatile multi-cloning site, (v) have a tightly controlled inducible promoter, (vi) have vector derivatives suited for genes that require expression from the native RBS and translational start codon and (vii) are built in a modular fashion, to allow for future adaptations.

To fit these criteria we chose the pSC101 as well as the p15A origin of replication. Both origins of replication have been shown to replicate in *E. coli* as well as in *Y. enterocolitica, Y. pseudotuberculosis* and *Y. pestis* (Cozzarelli et al., 1968; Cohen et al., 1973; Pierson and Falkow, 1993; Pepe et al., 1995; Jackson et al., 2004; Heroven and Dersch, 2006; Lenz et al., 2011). For complementation studies to succeed, it is desirable not to overexpress the gene of interest. Balancing between lowest possible copy number and cloning practicalities, the pSC101 and the p15A origin of replication seemed to be an ideal fit. Plasmids bearing the pSC101 origin of replication were shown to have about 5 copies per cell (Cabello et al., 1976; Stoker et al., 1982) whereas plasmids carrying the p15A origin of replication were shown to generate between 12-22 copies per cell (Cozzarelli et al., 1968; Chang and Cohen, 1978).

With regard to natural antimicrobial susceptibility (Martins et al., 1998; Galimand et al., 2006) *Y. pseudotuberculosis* and *Y. pestis* are susceptible to most antimicrobials (Martins et al., 1998; Galimand et al., 2006), but *Y. enterocolitica* strains are often naturally resistant to some ß-lactams, whose derivatives, e.g. ampicillin or carbenicillin, are commonly used in laboratories (Stock and Wiedemann, 1999). Therefore, we chose to use kanamycin and spectinomycin resistance genes for our vectors. To be able to choose a variety of cloning strategies, we selected the multi-cloning site present in pWKS130, which originates from pBluescript II KS+ (Alting-Mees and Short, 1989; Wang and Kushner, 1991). As this multi-cloning site is present in all vectors, genes of interest can be easily subcloned from one vector to another.

Controlled induction of gene transcription is important and we therefore chose to use the tetracyline inducible promoter/operator/repressor system reviewed in (Hillen and Berens, 1994). In particular we chose to use a promoter derivative that combines the promoter of phage lambda with the operator of the *tet* operon (Tn*10*) (Lutz and Bujard, 1997). A tetracycline derivative, ATc, has been shown to diffuse freely into bacterial and eukaryotic cells without being dependent on an active uptake system (Gossen and Bujard, 1993). This allows induction of gene transcription in bacteria which reside in either eukaryotic cells or within living animals (Lathem et al., 2007). Furthermore, ATc is less toxic and binds tighter to the *tet* repressor than tetracycline (Gossen and Bujard, 1993).

As membrane protein encoding genes and some other genes often include special leader sequences, we designed some vector derivative, which contain neither the RBS nor the ATG start codon at the beginning of the multi-cloning site. In addition, we designed this vector series in a modular fashion where the antibiotic resistance as well as the origin of replication can be easily exchanged for future adaptations.

3.2 Sequencing of pWKS130

As we intended to use pWKS130 as a source for the pSC101 origin of replication as well as for the multi-cloning site, we sequenced this vector by primer walking using primers MWO-010 to MWO-036 (Table 2). When assembling the sequences, we noticed that the *f1ori-lacZ*α fragment has the opposite orientation as published (Wang and Kushner, 1991). This finding was confirmed by restriction analysis (data not shown). The sequence of pWKS130, starting with the *Bgl*II site, was deposited in GenBank (accession number JQ283951).

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3.3 Construction of pMWO-005 and pMWO-034

In a first step, the pSC101 origin of replication and the kanamycin resistance gene were combined. For this purpose, the *kanR* gene was PCR amplified from the pET24b+ vector using primers MWO-042 and MWO-043 (PCR #1, Fig. 1A). Primer MWO-042 anneals upstream of the *kanR* promoter and adds two unique restriction sites, *Mlu*I and *Nhe*I. MWO-043 anneals at the 3′-end of *kanR* and introduces a 26 bp overlapping sequence at the 3′-end of the PCR #1 product. To prevent read-through from the *kanR* gene the *rrnB* T1 transcription terminator (Brosius et al., 1981; Brosius, 1984) was amplified from *E. coli* BL21PRO genomic DNA using primers MWO-044 and MWO-045 (PCR #2, Fig. 1A). MWO-044 adds the corresponding 26 bp overlapping sequence of MWO-043 at the 5′-end of the *rrnB* T1 terminator and primer MWO-045 adds two unique restriction sites, *Sph*I and *Pvu*II, to the 3′-end of the product of PCR #2. The products of PCR #1 and #2 were then fused using the overlap PCR technique (Horton et al., 1989) with the products as templates and MWO-042 and MWO-045 as primers (PCR #3, Fig. 1A). Vector pWKS130 was digested with *Nae*I resulting in five fragments of different length, each having blunt ends. The 2008bp DNA fragment containing the pSC101 origin of replication was isolated and blunt-end ligated with PCR product #3 (Fig. 2A) creating pStep1 (Fig. 2B). The orientation of ligated fragments was determined by restriction analysis.

In a second step, the *tetR* gene was introduced into pStep1. For this purpose, three elements were combined: (i) the promoter P_{N25} from coliphage T5, which is a very strong and constitutive transcribed promoter (Deuschle et al., 1986), (ii) the *tet* repressor gene, *tetR*, (Hillen and Berens, 1994) and (iii) the *rrnB* T1 transcription terminator (Brosius et al., 1981; Brosius, 1984). On the chromosome of *E. coli* strain DH5αPRO, promoter P_{N25} and *tetR* are already combined. To avoid carrying over the restriction sites, *Eco*RI and *Xba*I, which are present at the 5′-end of *tetR*, primers MWO-047 and MWO-048 carry two point mutations that abolish these restriction sites. Additionally, these two primers overlap by 33 bp. P_{N25} was amplified using primers MWO-046 and MWO-047, and *tetR* was amplified with primers MWO-048 and MWO-049 (PCR #5), both from *E. coli* DH5αPRO genomic DNA. To prevent read-through from the *tetR* gene the *rrnB* T1 transcription terminator was amplified from *E. coli* BL21PRO genomic DNA using primers MWO-050 and MWO-051 (PCR #6). Primers MWO-049 and MWO-050 overlap by 32 bp. The products of PCR #4, #5 and #6 where fused using overlap PCR (PCR #7, Fig. 1B) with primers MWO-046 and MWO-051, which add *Sph*I and *Pvu*II restriction sites to the ends of the product. pStep1 and the product from PCR #7 were digested with *Sph*I and *Pvu*II and ligated, generating pStep2 (Fig. 2B).

The third step completed the first vector construction by adding the sequence for promoter, operator and multi-cloning site to pStep2. A single fragment containing the strong phage lambda promoter (Deuschle et al., 1986) and the operator *tet*O_2 from the *tet* operon (reviewed in (Hillen and Berens, 1994)) present in expression vector pLP-PROTet-6xHN (Lutz and Bujard, 1997) was PCR amplified using primers MWO-052 and MWO-053 (PCR #8, Fig. 1C). Also, the versatile multicloning site from pBluescript II KS+ was amplified from vector pWKS130 using primers MWO-054 and MWO-055 (PCR #9, Fig. 1C). Primers MWO-053 and MWO-054 overlap by 22 bp. The products of PCR #8 and #9 were fused by overlap PCR using primers MWO-052 and MWO-055 that add *Nhe*I and *Mlu*I restriction sites to the product (PCR #10, Fig. 1C). pStep2 and the product from PCR #10 were digested using *Nhe*I and *Mlu*I and ligated, generating pMWO-005 (Fig. 2C). The promoter, operator and multi-cloning site encoding sequence are shown in Fig. 3A. Vector pMWO-005 has already been successfully used in our lab to express autotransporter-encoding genes from *Y. pestis* (Lenz et al., 2011).

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To construct a derivative of pMWO-005 that is ideal for cloning membrane protein encoding genes, the RBS and the ATG encoding sequences were deleted. For this purpose, the promoter and operator sequences were PCR amplified from vector pMWO-005 with primers MWO-085 and MWO-086 (PCR #11, Fig. 1C). The multi-cloning site was amplified using primers MWO-087 and MWO-088 with pMWO-005 as template (PCR #12, Fig. 1C). Primers MWO-086 and MWO-088 each encode a *Kpn*I site. To prepare for combination of the required fragments, PCR product #11 was digested with *Nhe*I and *Kpn*I, PCR product #12 was digested with *Kpn*I and *Mlu*I and vector pMWO-005 was digested with *Nhe*I and *Mlu*I. Purified fragments were ligated in a three-piece ligation, generating pMWO-034 (Fig. 2C). This shortened version of promoter, operator and multi-cloning site sequence is shown in Fig. 3B.

3.4 Construction of pMWO-073 – pMWO-078

To have more options we expanded the vector series by constructing several derivatives. Making use of the modular design of vectors pMWO-005 and pMWO-034 (Fig. 2) we either alone, or together, exchanged the antibiotic resistance and the origin of replication. The gene encoding spectinomycin resistance was PCR amplified using primers MWO-139 and MWO-140 from vector p34S-Sm2 (Dennis and Zylstra, 1998). The primers add *Nhe*I and *Sph*I sites to the PCR product. Both vectors and the spectinomycin gene PCR product were digested with *Nhe*I and *Sph*I. Purified vector backbones from pMWO-005 and pMWO-034 were each ligated with the spectinomycin resistance insert, giving rise to pMWO-073 and pMWO-076. By changing the origin of replication from pSC101 to p15A it is possible to choose a different copy number, 5 vs. 12-22 respectively (Cozzarelli et al., 1968; Cabello et al., 1976; Chang and Cohen, 1978; Stoker et al., 1982). The origin of replication p15A was PCR amplified from vector pACYC184 (Chang and Cohen, 1978) using primers MWO-137 and MWO-138. *Mlu*I and *Pvu*II sites were added to the product by the primers. The vectors and the p15A PCR product were digested with *Mlu*I and *Pvu*II. Purified vector fragments without origin were ligated with the new P15A origin of replication insert, generating pMWO-074 and pMWO-077. To construct versions where both antibiotic resistance and origin of replication are exchanged, spectinomycin resistance was introduced into pMWO-074 and pMWO-077 as described above using *Nhe*I and *Sph*I. The resulting vectors were named pMWO-075 and pMWO-078 respectively. All vectors constructed in this study are summarized in Table 3 with their respective characteristics. Sequences of new expression vectors, starting with the *Nhe*I site, were deposited in GenBank and accession numbers are listed in Table 3.

3.5 Control tests with vectors

To make sure that the newly generated vectors do not negatively affect the bacterial cells they are used with, we performed control tests regarding toxicity of the inducer ATc, growth behaviour of cells and plasmid stability. Toxicity of the inducer ATc was tested by growing wild type strains of *Y. enterocolitica* (JB580v), *Y. pseudotuberculosis* (IP32953) and *Y. pestis* (YP6) with and without the presence of different concentrations of ATc as described in Materials and Methods. *Y. enterocolitica* was found to be resistant up to 0.5 μg/ml, *Y. pseudotuberculosis* up to 1.0 μg/ml and *Y. pestis* up to 2.0 μg/ml ATc, which confirmed previous findings (Lathem et al., 2007). These threshold levels of ATc are well above the working concentration of 0.05 μg/ml that we typically use for induction.

Growth behaviour with or without addition of ATc of *Yersinia* wild type strains mentioned above harboring either pMWO-005, pMWO-073, pMWO-074 or pMWO-075 was assessed as described in Materials and Methods. This vector subset includes one vector of each combinations of origin of replication and antibiotic resistance. None of the tested strain pairs showed any growth defect (results not shown).

Plasmid stability was tested as described in Materials and Methods using the same strain and vector selection mentioned above. No plasmid loss was detected with or without antibiotic selection over the tested time (6 h) (results not shown). Taken together, at the recommended ATc and antibiotic concentrations *in vitro* at 26°C, we could not detect any toxicity towards the cells, impact on growth nor plasmid instability.

3.6 Test expression of His-YsrR in Yersinia enterocolitica

To test the efficacy of the promoter-operator system present in this vector series, we cloned the gene sequence encoding the N-terminally 6xHis-tagged response regulator, YsrR, into pMWO-005 as described in Materials and Methods. The new plasmid, pMWO-081, was transformed into *Y. enterocolitica* strain YVM1250, which is a *ysrR* null mutant (Walker et al., 2010). Induction of expression of His-YsrR, a 27 kDa protein, was tested with and without 50 ng/ml inducer (ATc) and samples were analyzed using SDS-PAGE and detection with anti-His antibodies as described in Materials and Methods. His-YsrR was detected under inducing conditions but absent under repressing conditions (Fig. 4A). If any His-YsrR was expressed, it was below the detection limit of the Western blot. As a further test, we performed β-galactosidase assays for YsrR function with YVM1250. This strain has a chromosomally integrated *ysaE-lacZ* reporter fusion, which is activated by YsrR (Walker et al., 2010) as well as His-YsrR. Expression from the *ysaE* promoter is activated ~180-fold after ATc induction compared to the Δ*ysrR* mutant carrying the vector pMWO-005 (Fig. 4B). Without ATc induction, the Δ*ysrR* mutant carrying pMWO-081 typically showed 6 to 9-fold higher levels of *ysaE-lacZ* expression compared to the Δ*ysrR* mutant alone or the Δ*ysrR* mutant carrying the vector. As the readout of the β-galactosidase assay is amplified by the fact that YsrR is a transcriptional activator (Walker and Miller, 2004) and the stability of the β-galactosidase enzyme (Bachmair et al., 1986), it is likely that promoter repression by TetR is nearly complete and only a few molecules of His-YsrR are present during uninduced conditions. Therefore, this inducible vector series should be suitable for most experiments. If complete repression is required, such as for cloning of genes encoding toxic proteins, an additional *tetR* copy can be added *in trans* either on a plasmid or on the chromosome.

Conclusion

In this study, we constructed a series of versatile low copy expression vectors well-suited for use in the *Yersinia* species. The *tet* operator/promoter system makes them distinct from other vectors frequently used in *Yersinia*, and it allows for control of gene expression not only *in vitro* and potentially also *in vivo*. As the same multi-cloning site is present in all vectors, genes of interest can be easily subcloned from one vector to another. Vectors can be chosen according to antibiotic selection, copy number and or special needs for use of the native RBS and translational start site. Further, the modular design of these vectors makes them easily adapted for other purposes. And last but not least, it should be possible to use these vectors for gene expression in many different species within the *Enterobacteriaceae*.

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Highlights

- Construction of small vectors for use in *Yersinia sp.* and other *Enterobacteriaceae*
- Vectors are low-copy (pSC101 ori or p15A ori) and share pBluescript II KS+ MCS
- Use of the *tet* operator allows highly regulated control of expression
- Vectors contain either kanamycin or spectinomycin resistance

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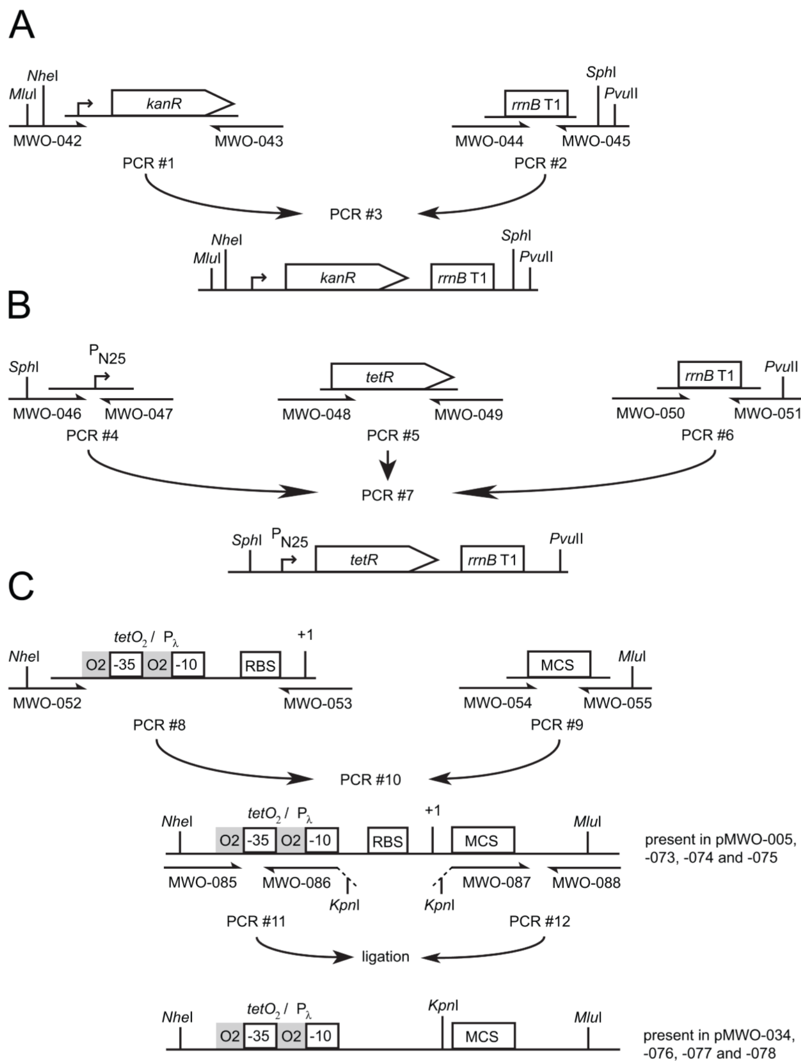

Fig. 1. PCR and cloning schemes of vector modules

(A) antibiotic resistance module. (B) *tet* repressor module. (C) promoter/operator and multi-cloning site modules.

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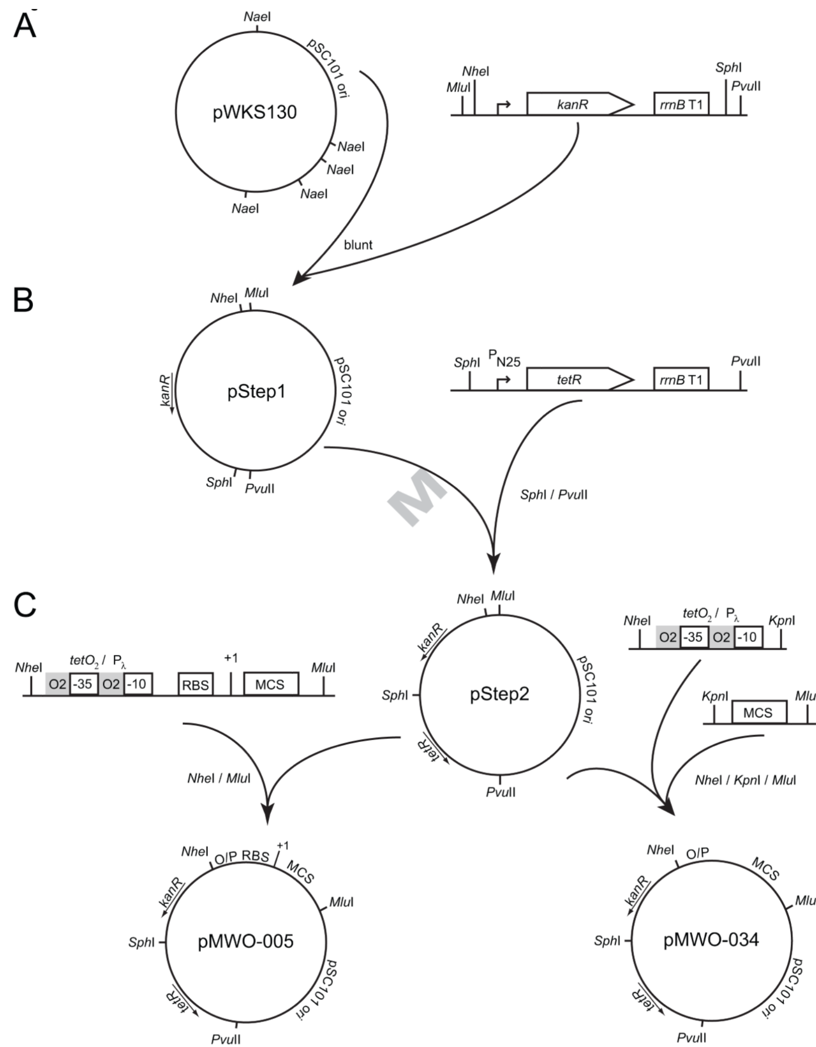

Fig. 2. Vector cloning steps

(A) Ligation of kanamycin resistance gene with pSC101 origin of replication. (B) Digestion of pStep1 and ligation of *tet* repressor module with pStep1. (C) Digestion of pStep2 and ligation with module containing promoter/operator sequence and multi-cloning site.

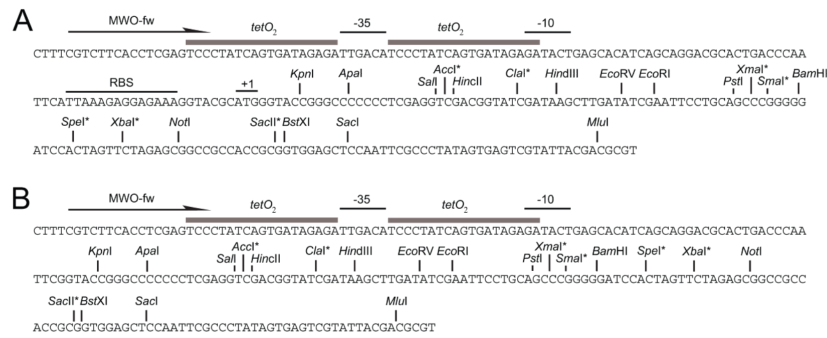

Fig. 3. Nucleotide sequence of modules containing promoter/operator and multi-cloning site

(A) Module with RBS and ATG start codon. (B) Module without RBS and ATG start codon. Restriction sites marked with an asterisk are present in some vectors but not all.

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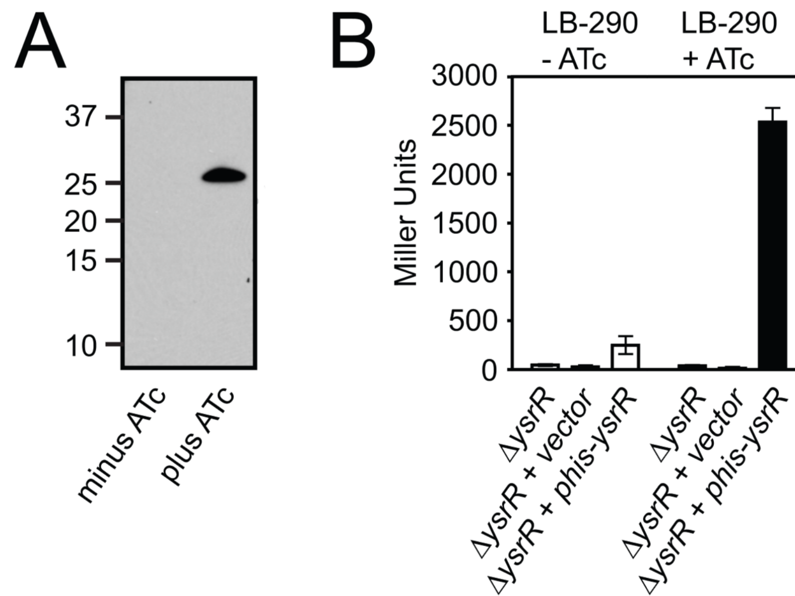

Fig. 4. Expression test of *his-ysrR*

(A) Immunoblot with anti-His antibodies against samples of *Y. enterocolitica* strain YVM1250 complemented with pMWO-081 encoding *his-ysrR* that was treated with or without the inducer ATc. Lane "minus ATc" represents a culture without induction and lane "plus ATc" shows a culture induced with 50 ng/ml ATc as described in Materials and Methods. Numbers reflect the protein standard in kDa. (B) Induction of His-YsrR expression. β-galactosidase assay with uninduced and induced (50 ng/ml ATc) cultures of *Y. enterocolitica* strain YVM1250 grown in LB-290. Δ*ysrR* refers to YVM1250; Δ*ysrR* + vector refers to YVM1250 + pMWO-005; Δ*ysrR* + p*His-ysrR* refers to YVM1250 + pMWO-081.

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Table 1

Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
E. coli		
DH5α	F^- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44 thi-1 gyrA96 relA1* γ^-	Invitrogen
BL21PRO	Source for P_{25N} & *tetR*	Clontech
Y. enterocolitica		
JB580v	8081v (r^- m^+ Nal^r) serotype O:8	(Kinder et al., 1993)
YVM1250	*ysaE*$^+$, *ysaE*::*lacZYA*, Δ*ysrR*, Cm^r	(Walker et al., 2010)
Y. pseudotuberculosis		
IP32953		(Chain et al., 2004)
Y. pestis		
YP6	CO92 pCD1$^-$	(Cathelyn et al., 2006)
Plasmids		
pET24b(+)	Source for *kanR*	Novagen
pWKS130	Source for pSC101 ori, multi-cloning site	(Wang and Kushner, 1991)
pACYC184	Source for p15A ori	(Chang and Cohen, 1978)
p34S-Sm2	Source for Sm^r	(Dennis and Zylstra, 1998)
pLP-PROTet-6xHN	Source for $P_{L\ tetO}$	Clontech
pMWO-038	His-*ysrR* in pMWO-005	this study
pMWO-081	His-*ysrR* in pMWO-005	this study

Table 2

Oligonucleotides used in this study

Name	Oligonucleotide sequence (5′ - 3′)[a]
Sequencing primers	
MWO-010	GGTTTCATGCTCCGTTAAGTC
MWO-011	GGTTCGTTCTCATGGCTCACGC
MWO-012	GTTCCCTTTAGTGAGGG
MWO-013	GCGAAGAGGCCCGCACCG
MWO-014	CCCATATAAATCAGCATCC
MWO-015	CTCCTTCATTACAGAAACGGC
MWO-016	GTTCCGTTGCGCTGCCCGG
MWO-017	GATTCTTCTCGCTTCCGGCG
MWO-018	GTCTCAGCCAATCCCTGGG
MWO-025	GGCAGTGAATGGGGGTAAATGGC
MWO-026	CTCAAAACCGCCCGCTTAACACC
MWO-027	GCGTTCCAGCTAAGGCTAAGGC
MWO-028	GCCTTTAGGGTTTTAAGGTCTG
MWO-029	GCGATTCAGGCCTGGTATGAG
MWO-030	CTTGCCATCCTATGGAACTGCCTCGG
MWO-031	GGCAATACGCACGCTTTCAGGC
MWO-032	GGAAAGCGGGCAGTGAGCGCAACG
MWO-033	GCTCTGCCAGTGTTACAACC
MWO-034	CAACAAAGCCACGTTGTGTCTC
MWO-035	CTATGCCAAGTTCTCAAGCG
MWO-036	CATGGTGAACAGCTTTGAATGCACC
Primers for construction of pMWO-005	
MWO-042	GGGGAAACGCGTATAGCTAGCGGAACGAAAACTCACGTTAAGGG
MWO-043	AAATCAAATAATGATTTTATTTTGACCATTCAAATATGTATCCGCTC
MWO-044	GTCAAAATAAAATCATTATTTGATTTCAATTTTGTCCCACTCCCCC
MWO-045	GGGGAACAGCTGATAGCATGCGGCAGCAAAACCCGTACCCTAGG
MWO-046	GGGGAAGCATGCGGCACGACAGGTTTCCCGACTGG
MWO-047	CTTTTATCTAATCT**G**GACATAT**C**AATTCGGGGCGGGATTTC
MWO-048	GCCCCGAATT**G**ATATGTC**C**AGATTAGATAAAAG
MWO-049	CTACTCAGCTA**C**CTAGA**A**TGCTTAAGACCCACTTTCAC
MWO-050	GTGGGTCTTAAGCA**T**TCTAG**G**TAGCTGAGTAGCAATTTTGTCCCACTCCCCC
MWO-051	GGGGAACAGCTGGGCAGCAAAACCCGTACCCTAGG
MWO-052	GGGGAAGCTAGCCCATTATTATCATGACATTAACC
MWO-053	CCTCGAGGGGGGGCCCGGTACCCATGCGTACCTTTCTCCTCTTTAATGAATTGGGTCAGTGCGTCC
MWO-054	GGTACCGGGCCCCCCCTCGAGGTCGACG
MWO-055	GGGGAAACGCGTCGTAATACGACTCACTATAGGGC
Primers for construction of pMWO-034	
MWO-085	CGTGAGTTTTCGTTCCGCTAGCCC
MWO-086	CCCAAGTGGTACCGAATTGGGTCAGTGCGTCC
MWO-087	GGGAAGTGGTACCGGGCCCCCCCTCGAGG
MWO-088	GGAAACGCGTCGTAATACGACTCAC
Primers for construction & sequencing of pMWO-073 to pMWO-078	

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Name	Oligonucleotide sequence (5′ - 3′)[a]
MWO-137	GGAAACGCGTGGAGTGTATACTGGCTTACTATG
MWO-138	GGGGAACAGCTGACAACTTATATCGTATGG
MWO-139	CTACTAGCTAGCGCGCTCGTTCGCCAGCCAGG
MWO-140	GGGGAAGCATGCTTATTTGCCGACTACCTTGG
MWO-142	CTATTATACAGAAAAATTTTCC
MWO-143	GGCAAAGCCGTTTTTCCATAGG
MWO-144	GCTGAGTAGCAATTTTGTCC
Primers for construction of pMWO-038 and pMWO-081	
MWO-072	GGCAAGTTCTAGATTATAGAGAAATTTCATGAGC
MWO-147	CCGAAGTGGTACCAAATGCATCACCATCACCATCACACACAAACGAAAACGCTC
MWO-150	GGGAAGTGGTACCCATCACCATCACCATCAC
General sequencing primers for vector series	
MWO-fw	CGTCTTCACCTCGAGTCC
MWO-rv-1	CAGCCTGAAACAGGCGATGCTGC
MWO-rv-2	GTATCACATATTCTGCTGACGC

[a]restriction sites are underlined; overlapping regions are double underlined; nucleotide exchanges are in bold; His-tag encoding nucleotides are underlined with dashes

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Table 3

Vectors constructed in current study

name	RBS	replicon	copies	resistance	rv-primer	size (bp)	accession number
pMWO-005	yes	pSC101	5	Kan	MWO-rv-1	4469	JQ283952
pMWO-073	yes	pSC101	5	Spec	MWO-rv-1	4517	JQ283945
pMWO-074	yes	p15A	12-22	Kan	MWO-rv-2	3354	JQ283946
pMWO-075	yes	p15A	12-22	Spec	MWO-rv-2	3402	JQ283947
pMWO-034	no	pSC101	5	Kan	MWO-rv-1	4444	JQ283944
pMWO-076	no	pSC101	5	Spec	MWO-rv-1	4492	JQ283948
pMWO-077	no	p15A	12-22	Kan	MWO-rv-2	3329	JQ283949
pMWO-078	no	p15A	12-22	Spec	MWO-rv-2	3377	JQ283950