Published in final edited form as:
Plasmid. 2011 September ; 66(3): 180–185. doi:10.1016/j.plasmid.2011.07.007.

Vectors for ligation-independent construction of *lacZ* gene fusions and cloning of PCR products using a nicking endonuclease

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Abstract

Several ligation-independent cloning methods have been developed that offer advantages for construction of recombinant plasmids, including high efficiency while minimizing cloning artifacts. Here we report new plasmid vectors that use the nicking endonuclease Nt.*Bsp*QI to generate extended single stranded tails for direct cloning of PCR products. The vectors include pLacCOs1, a ColE1-derivative plasmid imparting resistance to ampicillin, which allows facile construction of *lacZ* translational fusions and pKanCOs1, a pSC101-derivative cloning vector that imparts resistance to kanamycin, for cloning of PCR amplicons from genomic DNA as well as from ampicillin-based plasmids. We have successfully used these plasmids to directionally clone and characterize bacterial promoters that exhibit temperature regulated expression, as well as for cloning a variety of PCR products. In all cases, constructs with the correct configurations were generated at high efficiency and with a minimal number of manipulations. The cloning vectors can also be easily modified to incorporate additional reporter genes or to express epitope-tagged gene products.

Keywords

recombinant DNA; PCR cloning; cloning vectors; *lacZ* gene fusions

1. Introduction

Gene fusions between promoters and reporter genes are a fundamental tool for the genetic analysis of bacterial gene expression and protein (Hand and Silhavy, 2000; Hughes and Maloy, 2007; Silhavy, 2000; Silhavy and Beckwith, 1985; Slauch and Silhavy, 1991). Multiple reporter genes have been described with *lacZ* (encoding-galactosidase) (Hand and Silhavy, 2000; Silhavy and Beckwith, 1985), *gfp* (green fluorescent protein) (Phillips, 2001; Southward and Surette, 2002), *lux* (luciferase) (Engelbrecht *et al.*, 1985; Stewart and Williams, 1992), and *phoA* (alkaline phosphatase) (Manoil *et al.*, 1990) being among the most widely used.

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Promoter fusions are frequently constructed using standard techniques of recombinant DNA, which includes the use of restriction endonucleases to generate DNA fragments that are joined by T4 DNA ligase. In constructing multiple gene fusions, however, this approach can be time-consuming and subject to artifacts inherent to recombinant DNA methodology. In response, a variety of ligation-independent cloning methods have been reported to improve the efficiency of constructing recombinant molecules (Aslanidis and de Jong, 1990; Huan *et al.*, 1992; Li and Evans, 1997; Quan and Tian, 2011; Sharan *et al.*, 2009; Yang *et al.*, 2010), including specialized plasmid vectors for purifying epitope tagged proteins (Bardóczy *et al.*, 2008; Berrow *et al.*, 2007; Chanda *et al.*, 2006; Curiel *et al.*, 2011; Du *et al.*, 2011). Multiple strategies have been developed, such as use of homologous or site-specific recombination *in vivo* (Sharan et al., 2009; Zhang *et al.*, 2002) or enzymatic manipulation (PCR, T4 DNA polymerase, or uracil-DNA glycoslase for example) to join DNA molecules *in vitro* (Quan and Tian, 2011; Tachibana *et al.*, 2009). More recently, the availability of nicking endonucleases that cut only a single strand of a double-stranded DNA recognition site (Chan *et al.*, 2011; Jeltsch *et al.*, 1996; Zhang *et al.*, 2010; Zheleznaya *et al.*, 2009) has been used to generate relatively long complementary single-stranded tails that can be efficiently linked in the absence of T4 DNA ligase (Vroom and Wang, 2008); Yang *et al.*, 2010; Du *et al.*, 2011), as well as to study a model DNA replication fork (Ishikawa *et al.*, 2009; Ishikawa *et al.*, 2011).

We reasoned that this approach could also be used to efficiently construct *lacZ* reporter gene fusions. To demonstrate this, we modified a plasmid carrying a promoterless *lacZY* operon (Jain, 1993) to enable direct fusion of PCR products in-frame with *lacZ*. For this, we positioned a recognition site for the nicking endonuclease Nt.*Bsp*QI immediately upstream of *lacZ*. Nt.*Bsp*QI recognition sites were also incorporated into PCR primers used to amplify specific promoter regions from bacterial genomic DNA. Treatment of linearized vector DNA and PCR products with Nt.*Bsp*QI prior to heating the molecules resulted in single-stranded regions on both plasmid and insert with sufficient homology to generate recombinant plasmids without the need for T4 DNA ligase (Fig. 1).

Here we report the use of this strategy to construct a series of *lacZ* gene fusions to characterize and compare expression of genes from *Yersinia pestis* and *Escherichia coli*. Historically, *Yersinia pestis* has been a significant human pathogen, and it remains a concern as a potential bioterrorism agent. Expression of several chromosomally-encoded *Y. pestis* genes are known to vary with temperature, which reflects its unique natural reservoirs that include both fleas and mammalian hosts (Han *et al.*, 2004; Motin *et al.*, 2004). To better understand the genetic basis for temperature-regulated gene expression in *Y. pestis*, we sought to construct several *lacZ* gene fusions. The ligation-independent cloning system described here was found to be an efficient method to generate *lacZ* reporter gene fusions to multiple bacterial promoters. The cloning system is relatively simple in that PCR products are cloned directly into the vectors without the need for expensive enzymes, recombinases, or additional oligonucleotide linkers. The success of this method further promoted us to construct a separate plasmid for direct cloning of PCR products. Further modifications to these plasmids can be made for additional applications such as creating fusions to alternative reporter genes and epitope tagging of PCR products.

2. Materials and Methods

2.1 Bacterial Strains Growth Media and Enzymes

Promoters were cloned from *E. coli* K-12 strains, including LMG194 (F^-*mcrA*, Δ[*mrr-hsdRMS-mcrBC*] Φ80*lacZ*ΔM15, Δ*lacX74*, *recA1*, *araD139*, Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL*, *endA1*, *nupG*) (obtained from Invitrogen, Inc., Carlsbad, CA), or derivatives of *Y. pestis* strain CO92 (Parkhill and et al., 2001) deleted for either the *pgm* locus (*pgm*)

(Fetherston *et al.*, 1992) or pCD1, the large virulence plasmid required for pathogenesis (Lcr^{-}) (Perry *et al.*, 1998), enabling manipulation under BSL-2 laboratory conditions. *E. coli* NEB 5-alpha (*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) was obtained from New England Biolabs (NEB) (Ipswich, MA) and used as a host for all cloning experiments. *Y. pestis* mutants were grown on Tryptic Soy Blood Agar Base #[0–9][a-zA-Z]2 (TSB) from Difco (obtained through Thermo Scientific, Waltham, MA), while *E. coli* was cultured using LB medium (Miller, 1992). When appropriate, media were supplemented with antibiotics including ampicillin (Amp, 100 g/ml), chloramphenicol (Cam, 20 g/ml), or kanamycin (Kan, 30 g/ml) (Sigma-Aldrich, St. Louis, MO).

All enzymes required for recombinant DNA, including the nicking endonuclease Nt.*Bsp*QI, restriction endonucleases and T4 DNA ligase were obtained from NEB. PCR was performed using polymerase master mixes that do not add additional bases on the 3′ end of the amplification products, including Thermo Scientific Extensor Master Mix (Thermo Scientific, Waltham, MA).

2.2. Construction of pLacCOs1 and pKanCOs1

To construct pLacCOs1 (Fig. 2), pLacZY2 (Jain, 1993) was first modified by eliminating a unique *Sap*I recognition site. The plasmid was digested with *Sap*I (an isoschizomer of *Bsp*QI) and then treated with the Quick Blunting Kit (NEB) before recircularizing the vector with T4 DNA ligase.

To introduce recognition sites for Nt.*Bsp*QI, PCR was used to amplify a Cam^{R} cassette from pKD3 (Datsenko and Wanner, 2000) using primers BspQI-KD3.S and BspQI-KD3.AS (Table 1), obtained from Integrated DNA Technologies (IDT, Coralville, IA). PCR was performed by an initial step of 2 minutes at 94°C, followed by 30 cycles of: 94°C for 30 sec., 55°C for 30 sec, 72°C for 1 minute 30 sec, and completed with a final incubation at 72°C for 10 min. The PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and colonies were selected for Cam and Kan resistance. The resulting plasmid DNA was digested with *Hin*dIII and a 1.3-kbp fragment was purified using a QIAQuick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into pLacZY2(*Sap*I^{o}) digested with the same enzyme. The resulting plasmid was then digested with *Stu*I, which deleted the Cam^{R} cassette while maintaining the flanking Nt.*Bsp*QI sites, and religated yielding pLacCOs1 (Fig. 2).

pKanCOs1 was constructed by modifying pJPK12 (Peterson and Phillips, 2008) in a similar manner. Plasmid DNA was first digested with *Eag*I and religated to eliminate a unique *Bsp*QI site. The resulting vector was then digested with *Eco*RI and ligated to the 1.3-kbp Cam^{R} cassette flanked by *Bsp*QI recognition sites originating from the pCR2.1-derivative plasmid, as just described. Finally, the resulting plasmid was digested with *Stu*I and religated to delete the Cam^{R} cassette, resulting in pKanCOs1. pKanCOs1 is compatible with other plasmids with ColE1-like replication origins, including pMB1 (pBR322 and pUC-derivative vectors) and p15A (pACYC177 and pACYC184 vectors).

2.3 PCR amplification of promoters

PCR primers designed to amplify *Y. pestis* and *E. coli* promoters are shown in Table 1. Each primer was designed to amplify a region of the bacterial chromosome containing a promoter of interest, as well as include a region of homology to pLacCOs1 and Nt.*Bsp*QI recognition sites, as summarized in Fig. 1. Genomic DNA was isolated from either *Y. pestis* CO92 (*pgm* or Lcr^{-}) or *E. coli* LMG194 using the Epicentre MasterPure Complete DNA & RNA Purification Kit (Epicentre, Madison, WI) and used as a template for PCR. PCR products were purified using the QIAQuick Gel Extraction Kit.

2.4 Promoter cloning and characterization

To prepare pLacCOs1 for cloning, plasmid DNA was digested with *Stu*I and nicked in a subsequent reaction with Nt.*Bsp*QI for 3 hours at 50°C. Purified PCR products were likewise treated with Nt.*Bsp*QI and both vector and insert were then incubated at 65°C for five minutes. DNA was immediately treated using the PCR purification/DNA concentration protocol from the Qiagen QIAExII Gel Extraction Kit. The DNA was quantified using a Nanodrop ND-1000 Spectrophotometer. The vector and insert were mixed at a 3:1 ratio in a 20 l reaction with NEB Buffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol), containing 100X bovine serum albumin (NEB) at a final concentration of 1X. The reaction was incubated at 65°C for 10 minutes and then allowed to slowly cool to room temperature. DNA was then transformed directly into NEB 5-Competent *E. coli* (NEB) and plating on LB agar containing Amp and spread with ~100 l of 10 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (Gold Biotechnology, Inc., St. Louis, MO). Blue colonies were chosen for plasmid extraction and sequencing. All *lacZ* fusions were confirmed by DNA sequencing using using LacZRev2 primer (Table 1) at Iowa State University's DNA Facility.

To study gene expression, plasmid DNA was introduced to *E. coli* K12 strains or *Y. pestis* (*pigmy* or Lucre$^-$) by electrooration (Murphy and Compellone, 2003).-galactosidase assays were performed as described (Miller, 1992). DNA sequences of pLacCOs1 and pKanCOs1 have been deposited in Genbank with accession numbers JF837312 and JF837313, respectively.

3. Results and Discussion

3.1 Development of a nicking endonuclease system for lack gene fusions

Development of a more efficient cloning system was prompted by our efforts to clone and characterize several promoters of *Y. pestis* that are differentially regulated by growth temperature. A survey of published microarray experiments revealed multiple chromosomally encoded genes whose transcription was elevated upon a shift from 30°C to 37°C, including *KatY*, YPO1996 and *psaAC*, as well as genes whose expression was reduced at 37°C, including YPO0498 and the *mal* operon (Han et al., 2004; Motin et al., 2004). Our approach to this end was to construct plasmid-borne *lacZ* translational gene fusions representing selected genes from both *Y. pestis* and *E. coli* and measure-galactosidase activity in both bacterial species.

To facilitate construction of multiple *lacZ* gene fusions, we developed a system that allowed direct cloning of PCR products, representing promoters and their flanking regions, directly in-frame with *lacZ* without the use of ligase and conventional restriction endonucleases. The strategy, as summarized in Fig 1, uses pLacCOs1 (Fig. 2) to generate a cloning vector with extended single stranded DNA overhangs that easily assemble into desired recombinant molecules *in vitro*. Since the sequence of each overhang is distinct, inserts are cloned in the proper orientation for direct in-frame fusion with the *lacZ* reporter gene. Lactose permease (*lacY*) is also expressed from this plasmid, enabling genetic selections for altered promoter activity (Shuman and Silhavy, 2004).

3.2 Construction of lack gene fusions

To construct *lacZ* gene fusions using pLacCOs1, selected promoters were PCR amplified using primer pairs with homology to the bacterial chromosome in addition to Nt.*Bsp*QI recognition sites (Table 1 and Fig. 1). Antisense primers were designed so that when inserted into pLacCOs1 a translational fusion was made between the first few amino acids of the target gene and *lacZ* (Table 1). pLacCOs1 was prepared for cloning by linearization with

*Stu*I before digestion with Nt.*Bsp*QI to generate DNA molecules with two nicks on opposite DNA strands. Both plasmid DNA and PCR products are then heated to 65°C to yield molecules with relatively long single-stranded overhangs.

To generate recombinant plasmids, both linearized vector and PCR products were purified and mixed, as described in *Materials and Methods*. The DNA mixtures were then used to directly transform chemically competent *E. coli* with selection on LB Amp plates containing X-gal. Several hundred transformants were typically obtained from each cloning reaction and 75–95% of the colonies were blue on X-gal. In each case, all of the blue colonies tested (typically 4–6) were shown by DNA sequencing to encode the predicted construct with the target promoter driving expression of a *lacZ* translational fusion.

In constructing gene fusions with this method, the PCR amplicons were typically 0.5–1.5 kbp, and all efficiently yielded gene fusions. In constructing a fusion between the *E. coli* heat shock promoter *rpoHP3* and *lacZ*, however, we designed primers that yielded only a very short (~200 bp) PCR product. In this case, cloning was successful with only a PCR clean up reaction without gel elution.

3.3 Reporter gene expression

To test the utility of the *lacZ* gene fusions, we tested each for temperature-dependent gene regulation in both *Y. pestis* and *E. coli*. While a more complete analysis of these studies will be reported elsewhere, results for *mal* operon expression is shown in Fig. 3. We observed that while expression of the *mal* genes from *Y. pestis* or *E. coli* showed no change in regulation at 37°C when expressed in *E. coli*, expression of the loci was significantly reduced at 37°C when expressed in *Y. pestis*. While the basis for temperature-dependent gene expression of the *mal* genes in *Y. pestis* is not yet clear, the plasmids constructed in this study are currently being used to identify the genetic basis for this regulation. We are especially interested in the potential role of the transcriptional activator MalT in regulating gene expression in response to growth temperature.

3.4 PCR amplicon cloning

Because of the efficiency at which *lacZ* gene fusions were made using pLacCOs1, we also constructed a vector to allow direct cloning of any PCR product.pKanCOs1 (Fig. 2) was made by modifying pJPK12, a pSC101-derivative cloning vector that replicates with elevated copy number and imparts Kan^R (Peterson and Phillips, 2008). This vector offers advantages for cloning PCR products in that the replicon is compatible with ColE1 plasmids, allowing recombinants to be immediately transformed into strains with compatible plasmids. Also, since pKanCOs1 imparts Kan^R, use of this plasmid eliminates background colonies that typically arise when cloning PCR products generated from Amp^R-based plasmid templates.

For an initial test, we PCR amplified *bla* from pUC18 and cloned the resulting Amp^R gene cassette into pKanCOs1 using the Nt.*Bsp*QI nicking endonuclease cloning system. Transformants were first selected for Kan^R and then tested for Amp^R. The cloning efficiency was similar to that of pLacCOs1 with 70–90% of the Kan^R transformants also conferring Amp^R. These efficiencies were consistently obtained with several other genes from *E. coli* and *Y. pestis*, including *malT*, with a length of over 3-kbp.

In selecting genes for cloning, including *malT*, we observed that *Bsp*QI sites were occasionally found within the desired regions. Nevertheless, the presence of these sites did not interfere with cloning efficiency using the methods described here. In all of these cases, however, the *Bsp*QI site was greater than a few hundred base pairs from the end of the PCR product. In the case where it is not possible to avoid a *Bsp*QI site near the end of the PCR

amplicon, we predict that the denaturing step to generate single stranded overlapping regions could be lowered to minimize denaturation of the PCR products. It should also be possible to build similar cloning vectors that contain recognition sites for other commercially available nicking enzymes.

3.5 Conclusions

We have further developed a ligation independent cloning method that uses nicking endonucleases for construction of recombinant plasmids, including *lacZ* gene fusions. We found the method to be a reliable approach that requires relatively few steps to progress from PCR amplification to confirmation of recombinants by DNA sequencing. Although the method was specifically developed for construction of *lacZ* translational gene fusions, pLacCOs1 can easily be modified to also construct *lacZ* transcriptional fusions. Since the cloning system also allows for directional insertion of PCR products, it is ideal for generating additional reporter gene fusions, or adding epitope tags to cloned genes. It should be also be well suited for applications that require construction of a large number of recombinant plasmids, such as promoter libraries (van Dyk *et al.*, 2001; Zaslaver *et al.*, 2008), or cloning and expressing gene products for structural analysis (Betton, 2004). As additional nicking enzymes become commercially available, new vectors can be constructed to expand the choices of enzymes used constructing recombinant plasmids.

Acknowledgments

This work was funded by grants from the Office of Naval Research (ONR-MURI N00014-06-1-1176) and NIH (NIAID R21 AI074591).

References

Aslanidis C, de Jong PJ. Ligation-independent cloning of PCR products (LIC-PCR). Nucleic Acids Res. 1990; 18:6069–6074. [PubMed: 2235490]

Bardóczy V, Géczi V, Sawasaki T, Endo Y, Mészáros T. A set of ligation-independent in vitro translation vectors for eukaryotic protein production. BMC Biotechnol. 2008; 8:32–38. [PubMed: 18371187]

Berrow NS, Alderton D, Sainsbury S, Nettleship J, Assenberg R, Rahman N, Stuart DI, Owens RJ. A versatile ligation-independent cloning method suitable for high-throughput expression screening applications. Nucleic Acids Res. 2007; 35:e45. [PubMed: 17317681]

Betton JM. High throughput cloning and expression strategies for protein production. Biochemie. 2004; 86:601–605.

Chan SH, Stoddard BL, Xu SY. Natural and engineered nicking endonucleases--from cleavage mechanism to engineering of strand-specificity. Nucleic Acids Res. 2011; 39:1–18. [PubMed: 20805246]

Chanda PK, Edris WA, Kennedy JD. A set of ligation-independent expression vectors for co-expression of proteins in *Escherichia coil*. Protein Exp Purif. 2006; 47:217–224.

Curiel JA, de las Rivas B, Mancheño JM, Muñoz R. The pURI family of expression vectors: A versatile set of ligation independent cloning plasmids for producing recombinant His-fusion proteins. Protein Exp Purif. 2011; 76:44–53.

Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coil* K-12 using PCR products. Proc Natl Acad Sci USA. 2000; 97:6640–6645. [PubMed: 10829079]

Du R, Li S, Zhang X. A modified plasmid vector pCMV–3Tag–LIC for rapid, reliable, ligation-independent cloning of polymerase chain reaction products. Anal Biochem. 2011; 408:357–359. [PubMed: 20816743]

Engelbrecht J, Simon M, Silverman M. Measuring gene expression with light. Science. 1985; 227:1345–1347. [PubMed: 2983423]

Fetherston JD, Schuetze P, Perry RD. Loss of the pigmentation phenotype in *Yersinia pests* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. Mol Microbiol. 1992; 6:2693–2704. [PubMed: 1447977]

Han Y, Zhou D, Pang X, Song Y, Zhang L, Bao J, Tong Z, Wang J, Guo Z, Zhai J, Du Z, Wang X, Zhang X, Wang J, Huang P, Yang R. Micro array analysis of temperature-induced transcriptome of *Yersinia pests*. Microbiol Immunol. 2004; 48:791–805. [PubMed: 15557737]

Hand NJ, Silhavy TJ. A practical guide to the construction and use of *lac* fusions in *Escherichia coil*. Methods Enzymol. 2000; 326:11–35. [PubMed: 11036632]

Huan RS, Serventi IM, Moss J. Rapid, reliable ligation-independent cloning of PCR products using modified plasmid vectors. Biotechniques. 1992; 13:515–518. [PubMed: 1362067]

Hughes KT, Maloy SR. Use of operon and gene fusions to study gene regulation in *Salmonella*. Methods Enzymol. 2007; 421:140–158. [PubMed: 17352921]

Ishikawa K, Handa N, Kobayashi I. Cleavage of a model DNA replication fork by a Type I restriction endonuclease. Nucleic Acids Res. 2009; 37:3531–3544. [PubMed: 19357093]

Ishikawa K, Handa N, Sears L, Raleigh EA, Kobayashi I. Cleavage of a model DNA replication fork by a methyl-specific endonuclease. Nucleic Acids Res. 2011; 39:5489–5498. [PubMed: 21441537]

Jain C. New improved *lacZ* gene fusion vectors. Gene. 1993; 133:99–102. [PubMed: 8224902]

Jeltsch A, Wenz C, Wende W, Selent U, Pingoud A. Engineering novel restriction endonucleases: principles and applications. Trends Biotechnol. 1996; 14:235–238. [PubMed: 8771796]

Li C, Evans RM. Ligation independent cloning irrespective of restriction site compatibility. Nucleic Acids Res. 1997; 25:4165–4166. [PubMed: 9321675]

Manoil C, Mekalanos JJ, Beckwith J. Alkaline phosphatase fusions: sensors of subcellular location. J Bacteriol. 1990; 172:515–518. [PubMed: 2404939]

Miller, JH. A short course in bacterial genetics, vol. Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y.: 1992.

Motin VL, Georgescu AM, Fitch JP, Gu PP, Nelson DO, Mabery SL, Garnham JB, Sokhansanj BA, Ott LL, Coleman MA, Elliott JM, Kegelmeyer LM, Wyrobek AJ, Slezak TR, Brubaker RR, Garcia E. Temporal global changes in gene expression during temperature transition in *Yersinia pests*. J Bacteriol. 2004; 186:6298–6305. [PubMed: 15342600]

Murphy KC, Compelling KG. Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. BMC Mol Biol. 2003; 4:11. [PubMed: 14672541]

Parkhill J, et al. Genome sequence of *Yersinia pests*, the causative agent of plague. Nature. 2001; 413:523–527. [PubMed: 11586360]

Perry RD, Straley SC, Fetherston JD, Rose DJ, Gregor J, Blattner FR. DNA Sequencing and Analysis of the Low-Ca2+-Response Plasmid pCD1 of *Yersinia pests* KIM5 Infect. Immun. 1998; 66:4611–4623.

Peterson J, Phillips GJ. New pSC101-derivative cloning vectors with elevated copy numbers. Plasmid. 2008; 59:193–201. [PubMed: 18295880]

Phillips GJ. Green fluorescent protein--a bright idea for the study of bacterial protein localization. FEMS Microbiol Lett. 2001; 16:9–18. [PubMed: 11682170]

Quan J, Tian J. Circular polymerase extension cloning for high-throughput cloning of complex and combinatorial DNA libraries. Nat Protoc. 2011; 6:242–251. [PubMed: 21293463]

Sharan SK, Thomason LC, Kuznetsov SG, Court DL. Recombineering: a homologous recombination-based method of genetic engineering. Nat Protoc. 2009; 4:206–223. [PubMed: 19180090]

Shuman HA, Silhavy TJ. The art and design of genetic screens: *Escherichia coli*. Nat Rev Genet. 2003; 4:419–431. [PubMed: 12776212]

Silhavy TJ. Gene fusions. J Bacteriol. 2000; 182:5935–5938. [PubMed: 11029410]

Silhavy TJ, Beckwith J. Uses of *lac* fusions for the study of biological problems. Microbiol Rev. 1985; 49:398–418. [PubMed: 3005818]

Slauch JM, Silhavy TJ. Genetic fusions as experimental tools. Methods Enzymol. 1991; 204:213–248. [PubMed: 1658562]

Southward CM, Surette MG. The dynamic microbe: green fluorescent protein brings bacteria to light. Mol Microbiol. 2002; 45:1191–1196. [PubMed: 12207688]

Stewart GSAB, Williams P. *lux* genes and the applications of bacterial bioluminescence. J Gen Microbiol. 1992; 138:1289–1300. [PubMed: 1512559]

Tachibana A, Tohiguchi K, Ueno T, Setogawa Y, Harada A, Tanabe T. Preparation of long sticky ends for universal ligation-independent cloning: Sequential T4 DNA polymerase treatments. J Biosci Bioeng. 2009; 107:668–669. [PubMed: 19447347]

van Dyk TK, Wei Y, Hanafey MK, Dolan M, Reeve MJ, Rafalski JA, Rothman-Denes LB, LaRossa RA. A genomic approach to gene fusion technology. Proc Natl Acad Sci USA. 2001; 98:2555–2560. [PubMed: 11226277]

Vroom JA, Wang CL. Modular construction of plasmids through ligation-free assembly of vector components with oligonucleotide linkers. Biotechniques. 2008; 44:924–926. [PubMed: 18533903]

Yang J, Zhang Z, Zhang XA, Luo Q. A ligation-independent cloning method using nicking DNA endonuclease. Biotechniques. 2010; 49:817–821. [PubMed: 21091446]

Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG, Alon U. A comprehensive library of fluorescent transcriptional reporters for *Escherichia coil*. Nat Methods. 2008; 3:623–628. [PubMed: 16862137]

Zhang P, Li MZ, Elledge SJ. Towards genetic genome projects: genomic library screening and gene-targeting vector construction in a single step. Nature Genet. 2002; 30:31–39. [PubMed: 11753384]

Zhang P, Too PH, Samuelson JC, Chan SH, Vincze T, Doucette S, Bäckström S, Potamousis KD, Schramm TM, Forrest D, Schwartz DC, Xu SY. Engineering *Bsp*QI nicking enzymes and application of N.*Bsp*QI in DNA labeling and production of single-strand DNA. Protein Exp Purif. 2010; 69:226–234.

Zheleznaya LA, Kachalova GS, Artyukh RI, Yunusova AK, Perevyazova TA, Matvienko NI. Nicking endonucleases. Biochemistry (Mosc). 2009; 74:1457–1466. [PubMed: 20210703]

Research Highlights

Vectors for ligation-independent construction of lacZ gene fusions and cloning of PCR products using a nicking endonuclease

YPLAS 2059/YPLAS_PLASMID-11–23

- Plasmids have been constructed for direct cloning of PCR products.
- Cloning uses a nicking endonuclease without the need for T4 DNA ligase.
- Vectors were designed to make *lacZ* gene fusions and to clone any PCR product.
- Gene fusions enabled the study gene regulation in *Yersinia pests*.

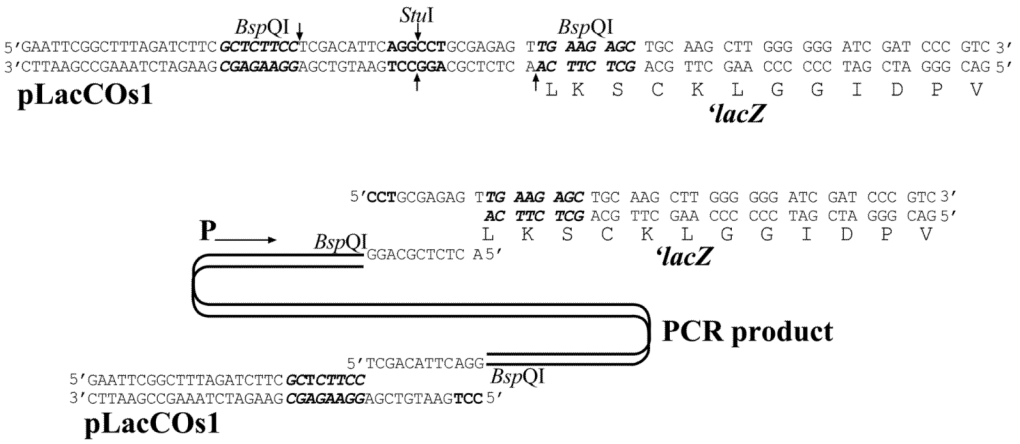

Figure 1. Strategy for construction of *lacZ* gene fusions using the nicking endonuclease Nt*Bsp*QI

Top. The site of insertion into pLacCOs1 is shown, including the *Stu*I restriction enzyme recognition site (shown in bold) for linearizing the vector and the two Nt.*Bsp*QI recognition sites (shown in bold italics). The arrows indicate the positions where the DNA strands are cut by each endonuclease. Bottom. The compatible single-stranded overhangs, generated as described in *Materials and Methods*, of both vector and PCR product are shown. The location of a promoter (P) is shown within the PCR product.

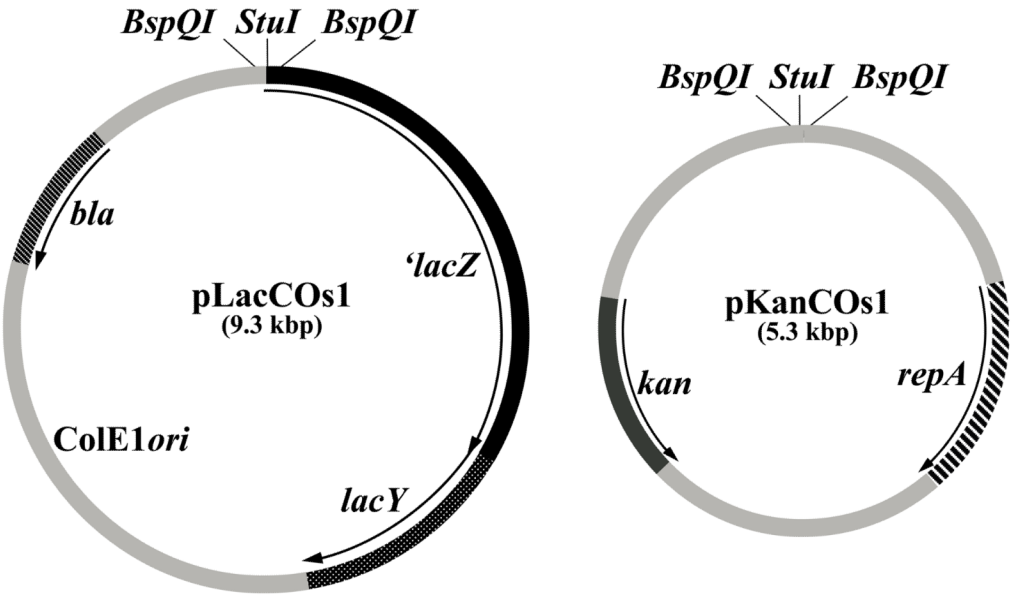

Figure 2. pLacCOs1 and pKanCOs1 plasmids

Shown are sizes and relevant features of each plasmid.pLacCOs1: ColE1*ori*, ColE1 origin of replication; *bla*, -lactamase imparting ampicillin resistance gene; '*lacZ*, -galactosidase, which requires a promoter, ribosome binding site, and initiation codon for expression; *lacY*, lactose permease, which is expressed coordinately with *lacZ*. pKanCOs1; *repA*, replication initiation protein for the medium copy number, ColE1-like compatible, pSC101-derivative plasmid (Peterson and Phillips, 2008); *kan*, kanamycin resistance. Also shown for each vector are the locations of the Nt.*Bsp*QI and *Stu*I recognition sites.

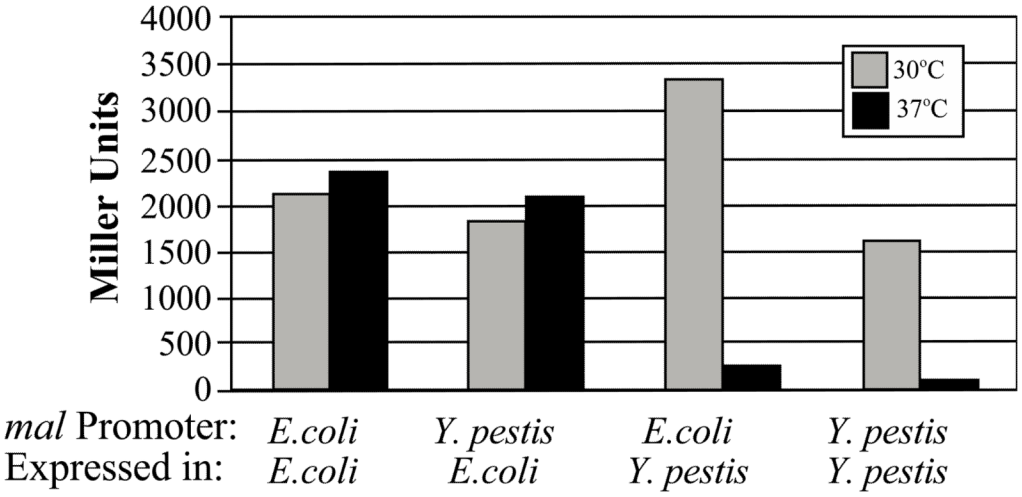

Figure 3. Temperature regulation of *mal* genes as measured by *lacZ* gene fusions expressed from pLacCOs1

Translational fusions between the *malEFG* promoter, from both *Y. pestis* and *E. coli* K-12, and *lacZ* were constructed in pLacCOs1 as described in *Materials and Methods*. Each gene fusion was expressed in both *Y. pestis* (*pigmy* or Lucre$^-$) and *E. coli* K-12 as shown. -galactosidase activity (Miller units) was measured in bacterial cultures grown at 30°C and 37°C, revealing that *mal* gene expression was temperature regulated only in *Y. pests*.

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Table 1

PCR and DNA sequencing primers used in this study.

Primer	Sequence (5′to 3′)*
BspQI-KD3.S	AAGCTTGCA**GCTCTTCA**ACTCTCGCAGGCCTCATATGAATATCCTCCTTAG
BspQI-KD3.AS	TAGATCTTC**GCTCTTCC**TCGACATTCAGGCCTTGTGTAGGCTGGAGCTGCTTCG
YpP0498-BspQI.S	TCGACATTCAGG**GGAAGAGC**CCCACGCGAAGTCATTATATAGC
YpP0498-BspQI.AS	A CTC TCG CAG G**TG AAG AGC** CTC TAG CAG CTT ATT GGC TTG C
YpP*KatY*-BspQ.S	TCGACATTCAGG**GGAAGAGC**GACAGGATTTCAGTAGGGT
YpP*KatY*-BspQ.AS	A CTC TCG CAG G**TG AAG AGC** GGC GAG AGT TAT TAG TAC GGG TAA G
YPO1996P-BspQI.S	TCGACATTCAGG**GGAAGAGC**GTTGAAATCGCCGATCAGGTATC
YPO1996P-BspQI.AS	A CTC TCG CAG G**TG AAG AGC** TGA CGG ACT TCT ACC AAT AAG CC
YpP*psaC*.BspQI.S	TCGACATTCAGG**GGAAGAGC**AATGGCAGCTATGTTCCGCCATTC
YpP*psaC*.BspQI.AS	A CTC TCG CAG G**TG AAG AGC** AGG CAC AAT AAA CGC CTG CGA TG
YpP*psaE*.BspQI.S	TCGACATTCAGG**GGAAGAGC**TTCTGTTTATGCACCGATGGCAGC
YpP*psaE*.BspQI.AS	A CTC TCG CAG G**TG AAG AGC** CGT TGG GCC ATT CAT CTT GGT TGT
YpP*malK*-BspQI.S	TCGACATTCAGG**GGAAGAGC**CGTGGTTAACGCCGAAAGAGCC
YpP*malK*-BspQI.AS	A CTC TCG CAG G**TG AAG AGC** ACT CAC CGG ATG AAT GCG GTA CAA
YpP*lsrA*-BspQI.S	TCGACATTCAGG**GGAAGAG**TGCCGCTTTCTTGCCGGTTGATCTTTC
YpP*lsrA*-BspQI.AS	A CTC TCG CAG G**TG AAG AGC** GGG TTG CAG AGT AAA GTC GAT AC
EcP*malK*-BspQI.S	TCGACATTCAGG**GGAAGAGC**TCCGGATGCTCAACGGTGACTTTA
EcP*malK*-BspQI.AS	A CTC TCG CAG G**TG AAG AGC** CAA ATG CGC CAG TTG TAG CAC TTC
EcP*rpoH*P3.-BspQI.S	TCGACATTCAGG**GGAAGAG**CTTATACTCTTTCCCTG
EcP*rpoH*P3-BspQI.AS	A CTC TCG CAG G**TG AAG AGC** CAT TCAAATCCTCTCGATATCAACGAGGTTATCATTCACTG
Yp*lsrR*-BspQI.S	TCGACATTCAGG**GGAAGAGC**GGGAACGAGATAAATACCCAGC
Yp*lsrR*-BspQI.AS	ACTCTCGCAGG**TGAAGAGC**TTCCAGGTCGAATATCACAGC
bla-BspQI.S	TCGACATTCAGG**GGAAGAGC**CATGGCAGTCTTTCGACTGAGCCTTTC
bla-BspQI.AS	ACTCTCGCAGG**TGAAGAGC**CATGGAGCTTAGTACGTTAGCCATGAGG
Yp*malT*.BspQI.S	TCGACATTCAGG**GGAAGAGC**GAAACCCAACACCGGTAAATCC
Yp*malT*.BspQI.AS	ACTCTCGCAGG**TGAAGAGC**GGATAATTTAACCAGCAGGCGGTC
malT-Ec-BspQI.S	TCGACATTCAGG**GGAAGAGC**GGTTGTGACATAGGAGTTCCAC
malT-Ec-BspQI.AS	ACTCTCGCAGG**TGAAGAGC**GAAAGAGGTGAAACGCTACCTG

Primer	Sequence (5′to 3′)*
LacZRev2	ACAAACGGCGGATTGACCGTAATG

*Nt.*Bsp*QI recognition sites are shown in bold and the *Stu*I sites are underlined. The regions of selected genes joined in-frame to *lacZ* are shown by triplet spacing within the appropriate antisense (AS) primer sequences.