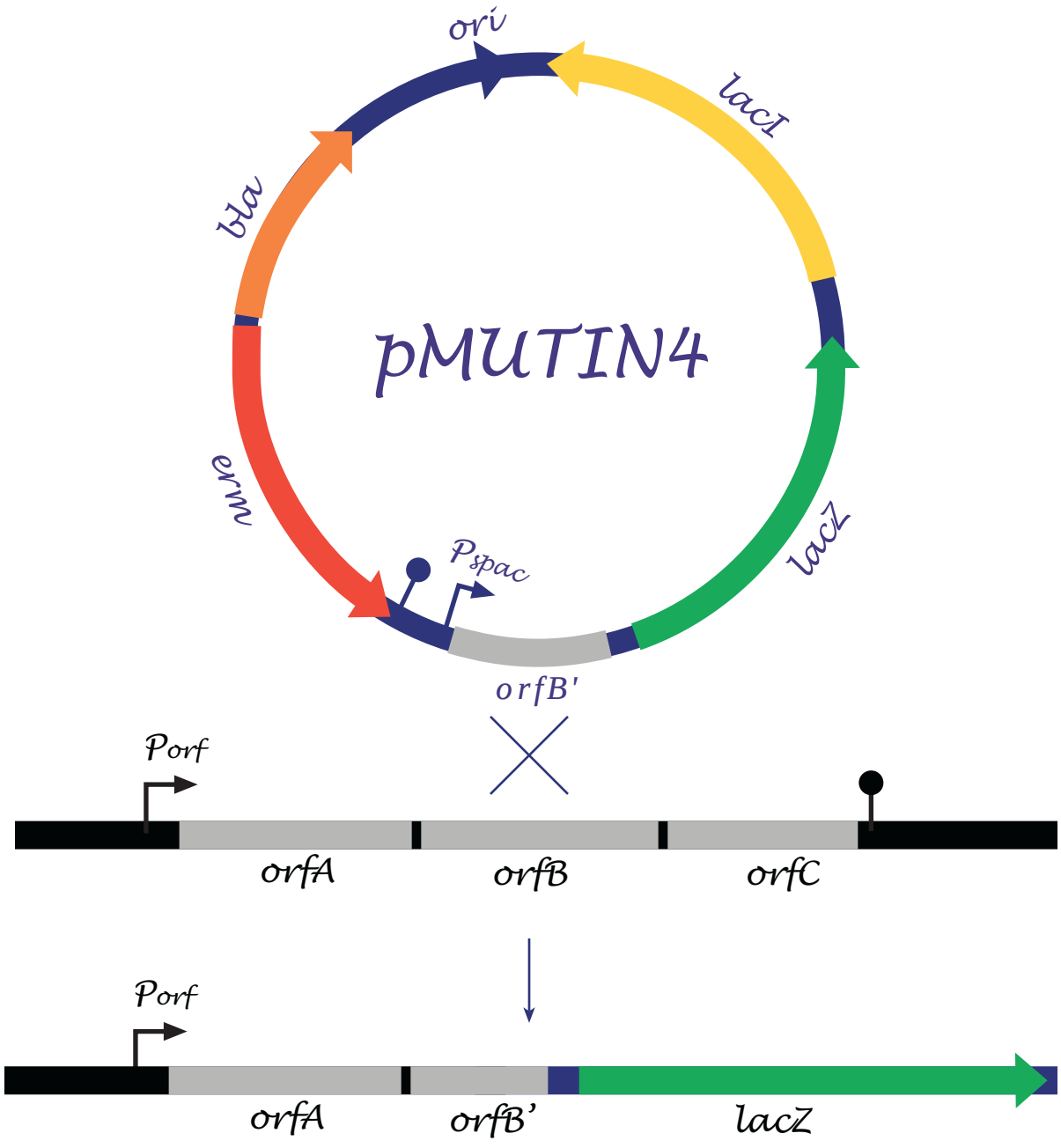
Integration Vectors

for Gram-Positive Bacteria



Bacillus Genetic Stock Center Catalog Vol. 4

Bacillus Genetic Stock Center
Catalog of Strains, Seventh Edition
Volume 4: Integration Vectors for Gram-Positive Organisms

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Disclaimer: The information in this catalog is believed to be correct. Due to the dynamic nature of the scientific process and to normal human limitations in dealing with such a large amount of data, however, some undetected errors may persist. Users bear the responsibility of verifying any important data before making a significant investment of time or other physical or financial resources.

Cover: Schematic diagram illustrating the use of the integration vector, pMUTIN4, to create a gene fusion with the *lacZ* reporter gene. An internal fragment from unknown *Bacillus subtilis* ORF, called here *orfB,* is cloned into the multiple cloning site of pMUTIN4. Upon transformation into *B. subtilis,* the plasmid can integrate into the chromosome by a single recombination event between the cloned *orfB* and the chromosomal *orfB* locus. Because pMUTIN4 cannot replicate in *Bacillus,* all erythromycin-resistant transformants will have integrated plasmids. In this example, the cloned fragment lies within an operon and does not include the promoter. Integration creates a gene fusion, placing the *lacZ* reporter gene under the control of the *orfABC* promoter. Further downstream (not shown on the diagram), the IPTG-inducible Pspac promoter now controls the distal gene of the operon, *orfC.* Because an internal fragment of *orfB* was cloned into the plasmid, chromosomal integration produces two incomplete fragments of the gene and hence a null mutation. Study of such an integration mutant could reveal much about the function of such an operon. The pMUTIN4 system should work for virtually any Gram-positive organism. See the catalog for more details on this and other useful integration vector tools!

Links: This document contains many internal hyperlinks. Clicking on a text in RED normally opens a link to another page with more information about that strain, reference, or gene. I colored a few texts for esthetic reasons—hope you don't mind! ☺

Please note: The BGSC catalog has been updated since this catalog was created. The online catalog on the BGSC website (https://bgsc.org/index.php) more accurately reflects the strains available from our collection. If you would like a full list of the E. coli strains harboring integration vectors in our collection, please email asc-microbioservice@osu.edu with your request.

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The *Bacillus* Genetic Stock Center

Who we are • What services we offer • How to order • Pricing • Important Notice

What is the *Bacillus* Genetic Stock Center?

The primary mission of the *Bacillus* Genetic Stock Center (BGSC) is to maintain genetically characterized strains, cloning vectors, and bacteriophage for the genus *Bacillus* and related organisms and to distribute these materials without prejudice to qualified scientists and educators throughout the world. Since 1978, the National Science Foundation has funded the activities of the BGSC. The Department of Biochemistry in the College of Biological Sciences at the Ohio State University provided facilities and administrative support. The Director of the BGSC, Dr. Daniel R. Zeigler, oversaw the BGSC for many years before retiring in 2021. The Bacillus Genetic Stock Center is no longer funded by the NSF and is now supported by the Infectious Disease Institute at the Ohio State University.

What kinds of cultures are available from the BGSC?

- The nomenclatural type strains for 34 species;
- 1291 mutant or plasmid bearing strains derived from *Bacillus subtilis* 168, including a large collection of genetically characterized sporulation mutants;
- 158 strains of round spore formers, comprised of 136 strains of *B. sphaericus*, 17 of *B. fusiformis*, and five of *Rumelliibacillus pycnus*;
- 191 wild type strains of *B. thuringiensis*, along with 10 cloned *B. thuringiensis* cry genes;
- 239 genetically characterized wild-type, mutant, and plasmid-bearing strains of *B. megaterium*;
- 96 lytic or lysogenic *Bacillus* bacteriophages;
- 42 wild-type and mutant strains from the thermophilic genus *Geobacillus*
- 41 wild-type, mutant, and lysogenic strains of *Bacillus licheniformis*;
- 55 other wild-type, mutant, and plasmid-bearing *B. subtilis* isolates, including 13 from *B. subtilis* subsp. *spizizenii* and 42 from other *B. subtilis* backgrounds;
- 104 wild-type strains from the *Bacillus cereus* group, also including *B. mycoides* and *B. weihenstephanensis*;
- 18 wild-type isolates from the genus *Brevibacillus*, including *B. brevis, B. borstelensis, B. centrosporus,* and *B. laterosporus*;
- 18 wild-type and mutant strains from *B. amyloliquefaciens*;
- 30 wild-type isolates from the genus *Paenibacillus*, including *P. alvei, P. dendritiformus, P. macerans, P. polymyxa, P. popilliae, P. thiaminolyticus,* and *P. vorticalis*;
- 42 isolates from 22 other related species, including *Aneurinibacillus aneurinilyticus, A. migulanus, B. atrophaeus, B. badius, B. carboniphilus, B. circulans, B. clausii, B. coagulans, B. firmus, B. lentus, B. mojavensis, 'B. natto,' B. oleronius, B. pumilus, B. shackletonii, Marinibacillus marinus, Sporosarcina ureae,* and *Virgibacillus marismortui*
- 240 *Escherichia coli* strains bearing shuttle plasmids or cloned Bacillus DNA;
- Warehoused Bacillus strain collections of Joshua Lederberg, Eugene Nester, Bernard Reilly, Patiricia Vary, Allan Yousten, Stanley Zahler, and the late Ernst W. Freese.
- Note that we **do not have—nor do we ever intend to obtain—**any strains of *Bacillus anthracis!* We simply do not have the experience, facilities, permission, or desire to deal with any serious human pathogen. Yes, we know that attenuated strains are available, but we still feel the same way. Sorry!

What you can do to help the BGSC

- *Strain contributions:* Although we have obtained a few cultures from other strain repositories, the vast majority of our holdings were contributed by individual researchers. Please take a moment to look over our collection and consider: are there strains, vectors, phage, or clones that you have developed or acquired that we do not have? Would these materials be of some potential use to others in the research community? If so, please take the time to deposit the material in the BGSC. There is no charge whatsoever to you. There is also no compensation--except for the knowledge that you have made the fruits of your labor more accessible for the benefit of others. Generally, all we would require would be a culture (or lysate) with appropriate reprints or other helpful information. Please contact us (see below) if you have any questions.

How to order cultures

There are several ways to place orders with or request information from the BGSC:

- E-mail: asc-microbioservice@osu.edu
- Internet: https://bgsc.org/index.php
- Phone: 614-292-2275

- Mail: The Ohio State University
  484 West Twelfth Avenue
  Biological Sciences Building
  556
  Columbus, OH 43210
  USA

Non-profit and for-profit users should supply a full shipping address, billing address, email address, phone number, and BGSC IDs of the requested strains. We make every effort to respond to all inquiries within one working day of receipt, together with an estimated delivery date if an order has been placed.

Pricing information—*Please note the changes!*

- *Academic, Government, and Non-Profit Users*--
  Users may purchase cultures as needed for a 55.00 USD per culture charge. If purchasing many cultures, users may pay a fee of 285.00 USD to receive up to 20 strains over the course of a year.

- *For-profit Corporate Users*--Users may purchase cultures as needed for a 179.00 USD per culture charge. If purchasing many cultures, users may pay a fee of 2850.00 USD to receive up to 50 strains over the course of a year.

- *Shipping*: Shipping and handling charges will be included in the quoted price. The shipping rate for domestic destinations is 15.00 USD and 60.00 USD for international destinations.

Important Notice

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Integration Vectors—an Introduction

A (very!) brief history of *Bacillus subtilis* genetics

Genetics is a powerful tool to unravel complex biological processes. The Gram-positive endospore-formers feature an array of fascinating processes to study, among them a primitive, but surprisingly elaborate, form of cell differentiation known as sporulation. Spizizen's announcement in 1958 that "DNA isolated from a wild-type strain can transform these bacteria to nutritional independence" (60) opened up *Bacillus subtilis* 168 as a model system for genetic analysis. We can conveniently divide the history of *B. subtilis* genetics since that time into three eras:

Classical Genetics Era (1958-1977)—During their first two decades of study, the *B. subtilis* genetics community primarily focused on developing tools for genetic exchange and combining them with biochemical approaches to analyze genetic mutants. We may perhaps pick three landmark events in the mid-1970's—the description of a gap-free circular genetic map for the *B. subtilis* chromosome (37), the publication of a landmark review of sporulation genes (49), or the convening of the first international conference dedicated solely to *Bacillus* genetics (55)—as representing the zenith of this "Classical Era."

Molecular Genetics Era (1977-1996)—The year 1977 was pivotal for the *B. subtilis* community. That year brought the first reports of *B. subtilis* genes cloned in *E. coli* (41, 57) and of the transformation of *B. subtilis* with antibiotic-resistant plasmids (14). The following year saw reports of cloning in *B. subtilis* as a host (11, 13, 19, 28). The standard genetic tricks of the *Bacillus* trade were augmented with tools produced by recombinant DNA techniques. "Reverse genetics"—purification and partial sequencing of a protein, allowing for isolation, sequencing, and genetic mapping of the gene encoding it—became common practice. The amount of DNA sequence information known for *B. subtilis* 168 began to grow exponentially. These approaches yielded key insights, among them the phosphorelay model of sporulation initiation (4), the sigma-factor cascade model of the temporal regulation of sporulation genes (40), and the two-component signaling system model of genetic response to environmental changes (23, 32).

Genomics Era (1997-?)—In the early 1990's an international group of researchers embarked on what was considered at the time an ambitious plan to sequence the entire *B. subtilis* 168 genome (34, 45). Eventually comprising twenty-five European, seven Japanese, two U.S., and one Korean laboratory, the international sequencing consortium achieved its goal in 1997 (33). The genomic sequence settled several old questions but raised many more new ones. Emphasis shifted towards analyzing the 70% of open reading frames that encode proteins of unknown function and towards elucidating the global regulatory networks that link together this organism's 4100 genes. Already, researchers are employing powerful techniques unimaginable even during the heyday of the Molecular Genetics Era—microarray technology, for example (3, 5, 22, 25, 30, 36, 46, 47, 69, 70)—to answer these questions. The two previous "eras" of *B. subtilis* genetics lasted roughly two decades each. Is it possible that within the next 15 years, researchers in the Genomics Era will succeed in identifying the function of every gene in *Bacillus subtilis* and modeling the genomic regulation as the organism responds to changes in its intra- and extracellular environments?

The integration vector: an "integral" place in this history

One key tool developed early in the Molecular Genetics Era of *B. subtilis* genetics, which continues play just as important a role in the Genomics Era, is the integration vector. Integration vectors are plasmids that feature *conditional replication* coupled with a *selectable marker.* If the plasmid is transformed into an appropriate host under conditions that select for the plasmid's presence but restrict its replication, all transformants will have integrated the plasmid into their chromosome (or some other resident DNA capable of replicating under the selective conditions). In practice, the selectable marker usually specifies antibiotic resistance. Conditional replication usually means that the plasmid has replication functions that work in *E. coli* but not in gram-positive bacteria, such as *B. subtilis.* Sometimes a temperature-sensitive replication phenotype is employed instead. Integration is targeted to a particular locus on the chromosome by including identical sequences on the plasmid. If there is a single homologous sequence, a single crossover will integrate the entire chromosome into the target locus by a Campbell-type mechanism. If there are two homologous sequences, and they are relatively close together on the chromosome, then a double crossover will result in a cassette integrating between the chromosomal targets.

Integration of a cloning vector via homologous recombination was first observed in 1978 the laboratory of Frank E. Young at Rochester University. (As an historical note, Young went on to serve as the Commissioner of the Food and Drug Administration from 1984-1989.) Duncan, Wilson, and Young (11) described cloning thymidylate synthetase (*thy*) genes from *B. subtilis* bacteriophages into *E. coli* plasmids. The plasmid carrying the β22 *thy* gene could complement *E. coli thyA* mutants but could not transform *B. subtilis* thymine auxotrophs to thymine independence. The plasmid

carrying the φ3T *thy* gene, however, could both complement *E. coli* mutants and transform *B. subtilis* Thy^- mutants to Thy^+. Analysis of these transformants showed that the entire plasmid had integrated into the chromosome by a Campbell-type mechanism, presumably by a recombination event between homologous sequences shared by the *B. subtilis* chromosome and the phage *thy* gene or its flanking sequences.

Homology-driven plasmid integration was an interesting observation, but how could it be used practically? The *B. subtilis* community was quick to come up with an answer. The Richard Losick lab at Harvard had cloned a DNA fragment from the *B. subtilis* chromosome that hybridized to a 0.4-kb mRNA species that could be isolated from early-sporulating cells (57). The problem of how to analyze the function of a gene with no known mutant alleles was a new one, requiring different approaches from the tried-and-true methods developed during the Classical Era. Bill Haldenwang, a post-doc in the Losick lab, puzzled over the problem. He recalls:

> "We had a fragment of *Bacillus* DNA cloned into pMB9 (a high tech vector for its day) and no earthly idea what the fragment actually encoded or where on the *Bacillus* chromosome it came from. I came across Duncan *et al.* in which the authors discovered that pMB9 carrying a thymidylate synthetase gene could enter the *Bacillus* chromosome. They described it as 'an additional mechanism for transformation whereby plasmids can be integrated if sufficient chromosome homology is maintained.' It seemed reasonable enough that if I could place a selectable marker in our pMB9 chimera, the cloned *Bacillus* fragment might target it to its chromosomal location and allow it to be mapped. So I cut a *cat* [chloramphenicol resistance] gene from one of Dave Dubnau's plasmids, placed it into an innocuous place in our plasmid and to our delight and surprise, it actually worked when it was transformed into *B. subtilis.*" (20)

Haldenwang *et al.* successfully used their integration vector to map the location of the "0.4 kb gene" (now *spoVG*) to a location near 5° on the *B. subtilis* genetic map (21). Other researchers were quick to appreciate the power of this technique for assigning cloned genes to a location on the chromosome. (Indeed, as of this writing, Haldenwang *et al.* has been cited 114 times, many of those citations referring to the integration vector concept.) Publications describing similar experiments followed rapidly, using integration vectors to map chromosomal fragments transcribed during stationary phase (43) or spore outgrowth (18); genes encoding alkaline protease (61, 68) and an intracellular serine protease (31); genes encoding three RNA polymerase holoenzyme subunits, alpha (63), the major vegetative sigma factor (52), and the general stress-induced sigma factor (12); structural genes for the enzymes glucose dehydrogenase (8), endo-β-1,3-1,4 glucanase (48), and phosphoribosylpyrophosphate synthetase (42); an autonomously replicating fragment from a defective prophage (2); and several ribosomal RNA operons (35). The Molecular Genetics Era was in full swing, and integrative vectors were making a key contribution.

New Uses for Integration Vectors

If integration vectors were only useful for tagging genes with a selectable marker for genetic mapping experiments, they would have fallen out of fashion with the advent of the Genomics Era—for obvious reasons. But within months after the report of Haldenwang *et al.,* researchers within the *Bacillus* genetics community seized on the integration vector concept and adapted it to their own purposes in a flurry of creativity. Many of these newer applications are pivotal in current efforts to analyze the thousands of genes with unknown function uncovered by genomic sequencing. We will briefly discuss five current uses for integration vectors in gram-positive genomics.

Construction of Knockout Mutants—In analyzing a gene of unknown function, an obvious first step is to create a knockout mutation in the gene and observe the mutant phenotype. Researchers soon realized that if they could insert a vector *into* the chromosomal locus, rather than beside it, they would disrupt its function. This approach to studying a newly cloned gene became very commonplace in the early years of the Molecular Genetics Era (12, 31, 42, 50, 53, 61, 62, 64, 66). Today, in analyzing the thousands of reading frames in a bacterial genome, a researcher can still make powerful use of the knockout mutation strategy. Figure 1 shows a basic integration vector composed of (1) *ori,* an origin of replication that functions in *E. coli* but not in gram positives; (2) *bla,* the ampicillin-resistance gene encoding β-lactamase, for maintaining the plasmid in *E. coli*; (3) *abr,* an antibiotic resistance gene selectable in gram-positives; and (4) *orfA',* an internal fragment from an open reading frame cloned from the gram-positive genome of interest. Upon transforming the gram-positive host, the plasmid integrates into the chromosomal locus of the reading frame by a single, homology-driven recombination event. Integration splits the chromosomal reading frame, *orfA,* into two incomplete parts, interrupted by the inserted plasmid sequences. The length of the chromosomal fragment required for efficient integration varies from species to species; for *B. subtilis,* a mere 75 bp of homology is sufficient to allow detectable levels of recombination between a plasmid and the chromosome (29), although in practice an insert of closer to 150 bp may be necessary to obtain useful integration frequencies (65).

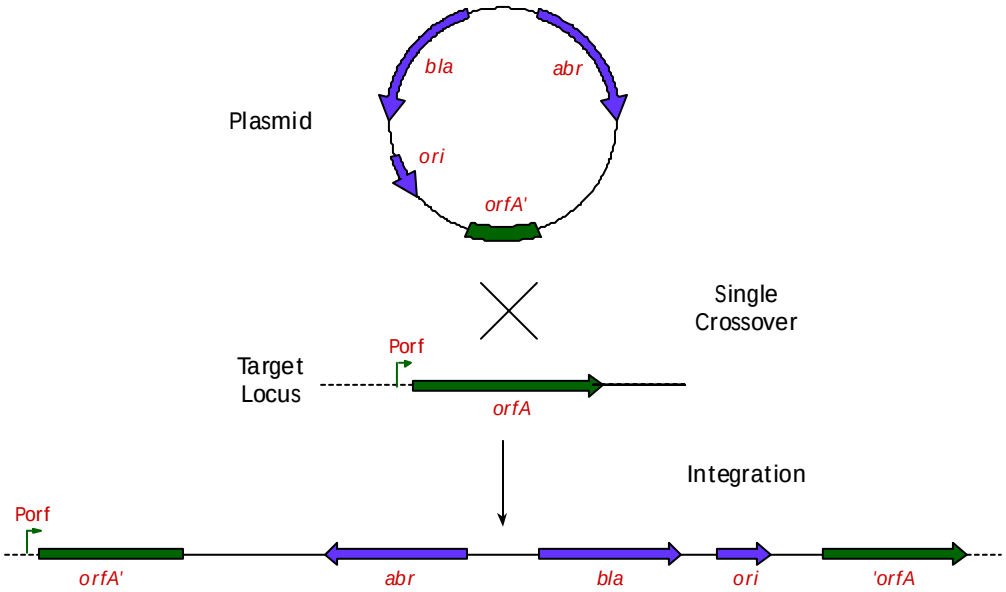

Figure 1. Use of a basic integration vector to construct a knockout mutation in a hypothetical open reading frame, *orfA*.

One important consideration in constructing knockouts is the effect on downstream genes if the disrupted ORF lies in an operon. The integration event not only disrupts the ORF but also (as Figure 1 illustrates) separates the operon's promoter from downstream genes. The integration, then, can have polar effects. To eliminate this possibility, several recent integration vectors include an inducible promoter upstream from the multiple cloning site. Integration places downstream genes under the control of this promoter, allowing the user to analyze phenotypes of the mutant with and without induction.

Finally, one interesting, although rarely used, application of gene knockouts created by integration vectors is to test which chromosome in the sporulating cell—the mother cell or forespore chromosome—is required for expression of a sporulation gene in *B. subtilis* (15, 59).

Any of the vectors listed in the **Quick Reference** table as integrating the entire plasmid (rather than a cassette) can be used to generate knockouts. Those that also include a promoter for expression of downstream sequences are indicated in the table as well.

Amplification of Chromosomal Sequences—As Figure 1 illustrates, a Campbell-type insertion into the chromosome creates direct repeats of the target fragment, flanking the inserted plasmid sequences. This arrangement makes the locus an active substrate for intra-molecular homologous recombination. If a recombination event takes place between the repeat sequences, the intervening plasmid sequences will be excised cleanly. Free plasmids will be transient; they will either re-integrate, or be lost during cell proliferation. During replication, a cell may contain two copies of any given locus. As Young has pointed out (72), if a plasmid excises from one locus and re-integrates into the other, then one of the chromosomes will contain two copies of the integrated unit. As long as selection is maintained, the daughter cell containing this duplication will grow, while the daughter inheriting the chromosome without an integrated plasmid will not. Indeed, by growing cultures with integrated plasmids in increasing concentrations of the selective antibiotic, Young was able to isolate strains with long amplifications (71). Many others have use a similar strategy to obtain up to a 50-fold amplification of integrated plasmids (1, 7, 9, 24, 51, 54).

At least two applications have been described for integration vector amplification. First, by including a gene of interest in the amplification unit, an increased gene dosage leading to overexpression of the desired product can be obtained (1). Because chromosomal amplifications are often more stable than high copy number vectors in *Bacillus* (24), integration vectors offer a potentially useful alternative for constructing genetically engineered strains. Secondly, by varying the concentration of selective antibiotic in the growth medium, it is possible to obtain a set of strains with a variety of copy numbers for a given gene. Piggot and Curtiss used this approach to study the regulation of the *spoIIA* sporulation operon in *B. subtilis* (51). Although the integration vector amplification had not been a commonly used tool in recent years, it may yet find useful application in the Genomics Era.

Chromosome walking—Plasmid integration (as in Figure 1) links a selectable marker to sequences adjacent to the cloned insert on the chromosome. If genomic DNA is digested with a restriction enzyme that does not separate the plasmid from a flanking sequence, then ligation of the fragments and transformation of the mix into *E. coli* should recover a plasmid with the flanking sequence inserted. Niaudet, Goze, and Ehrlich demonstrated the utility of integration vectors for this kind of chromosome walking (44). They recovered an *ilvA* knockout mutant from a library of *B. subtilis* clones that had been transformed with integration vectors. From classical genetic work, they knew that the *B. subtilis thyB* gene was located very near to the *ilvA* locus. By cutting chromosomal DNA from the *ilvA* integration mutant with an enzyme that did not cut the plasmid and ligating the fragments, they rescued a flanking fragment that could complement *E. coli thy*$^-$ mutants in trans and transform *B. subtilis thyB* mutants to prototrophy (44). Youngman *et al.* similarly made use of specially designed integration vectors to clone sequences adjacent to Tn*917* insertions (73). The advent of PCR technology and the availability of genomic sequences have reduced this usefulness of chromosome walking via integration vectors. It is possible, however, that in gram-positive sequencing efforts the ends of a contig could be extended using this technique.

Reporter gene fusion—By the early 1980's, the construction of *lacZ*-gene fusions was revolutionizing the field *E. coli* genetics (6). The use of β-galactosidase, for which a simple colorimetric assay was available, to tag a gene product that was difficult to quantitate was a powerful new tool for studying gene regulation. Naturally, researchers in the *B. subtilis* community were eager to adapt the technique to their model system. At the Cetus Conference on Genetics held at Stanford University (June 22-24, 1981), Donnelly and Sonenshein announced that they had created a *lacZ*-fusion with the promoter and first few codons of the *B. subtilis tms* gene. When they placed the construct on a high copy number plasmid in *B. subtilis,* copious amounts of β-galactosidase were produced (10). It had occurred to Donnelly and Sonenshein that they might be able to integrate their fusion into the *tms* locus, but their attempt was unsuccessful. Fittingly, the first reported use of an integration vector to create a gene fusion within the chromosome was in Zuber and Losick's study of the "0.4 kb gene," *spoVG* (74). Their vector allowed them to study the timing of *spoVG* expression in a natural, single-copy state and provided for an easy way to transfer the reporter gene fusion into a variety of genetic backgrounds. Reports of similar studies using integration vectors to create fusions with other genes quickly followed (16, 38, 51).

Using integration vectors to construct reporter gene fusions remains a key technology in the Genomics Era (65). A variety of reporter proteins are now available. The choice of reporter system depends on the aim of the study. For convenient *quantification of gene expression*, β-galactosidase remains a popular choice. The enzyme β-glucuronidase has also been developed as a reporter; because β-Gal and β-Glu have no cross-reactivity, it is possible to label two genes simultaneously with integration vectors (27). For *cellular localization of gene products,* the Green Fluorescent

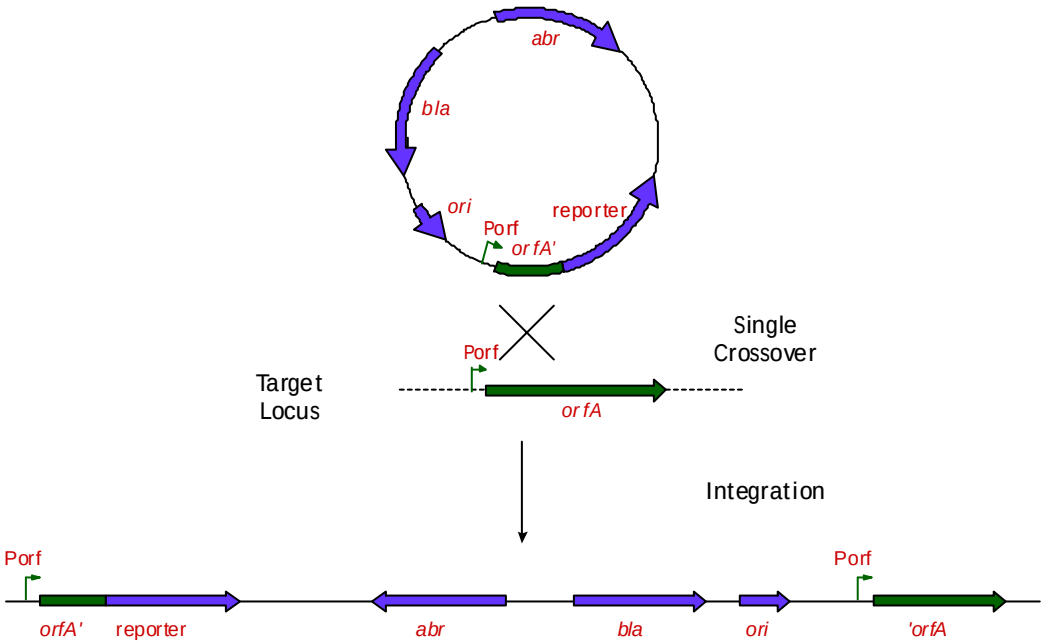

Figure 2. Use of an integration vector to construct a reporter gene fusion under the control of the promoter of a hypothetical open reading frame, *orfA*.

Integration Vectors: an Introduction (Continued)

Protein and its variants are the reporters of choice. A wide variety of integration vectors creating localization tags are now available for *B. subtilis* and other gram-positives (17, 26, 39). For *easy isolation of expressed proteins* and *detection of the proteins in immunoblots,* vectors that fuse an epitope tag—such as His_6, HA, c-Myc, or FLAG—have been developed (26, 67).

Reporter gene fusion vectors are identified in the **Quick Reference** by an entry in the "Tag" column. The specific reporter, along with where it is fused to the expressed protein, is indicated in the table.

Ectopic integration into a neutral site—Soon after it was recognized that sequence homology could direct a plasmid to integrate into a locus by a single crossover, another form of integration was detected. When a plasmid contained *two* regions of homology to the chromosome, a double crossover between the plasmid and the chromosomal loci could result in a *replacement* of the sequences between the loci with heterologous, plasmid-derived DNA (44). Shimotsu and Henner seized on this observation to develop a method of integrating gene cassettes into a defined *B. subtils* locus (58). Henner's group at Genentech had been studying the *B. subtilis trp* operon in the early 1980's. To get an accurate picture of its regulation, they needed to create a *trp-lacZ* gene fusion and study its regulation in single copy number. To accomplish this goal, they constructed the fusion, then inserted it along with a selectable marker between the "front" and "back" regions of the *B. subtilis* α-amylase gene, *amyE,* on an integration vector. Upon transformation into *B. subtilis,* the plasmid recombined with the front and back regions of *amyE.* The central region of *amyE* was replaced by a cassette containing the fusion and the selectable marker (58). Figure 3 gives a generalized picture of this type of vector. Integration into a neutral site, as opposed to the cloned locus, is known as *ectopic integration.*

Integration into a neutral site, like *amyE,* has several potential advantages. First, the integrated DNA need not be derived from the host organism at all, since homology between the insert and the chromosome is no longer a requirement. Secondly, if the insert does contain genes from the host organism, integration into a neutral site leaves the resident genes undisturbed. This arrangement allows for complementation and merodiploid analysis as well. Finally, if several genes originating from different loci or even different species are to be studied, integrating into a neutral site provides a common chromosomal environment for each of them, reducing the possibility of position effects in various chromosomal locations.

Various other sites in the chromosome other than *amyE* have been developed for ectopic integration vectors. The **Quick Reference** table indicates these vectors as integrating a *cassette* at the designated locus. A special case is the *dif* integration vector of Sciochetti and Piggot (56), which exploits the host system for resolving chromosome dimers to integrate entire plasmids into site near the host replication terminus in a RecA-independent manner.

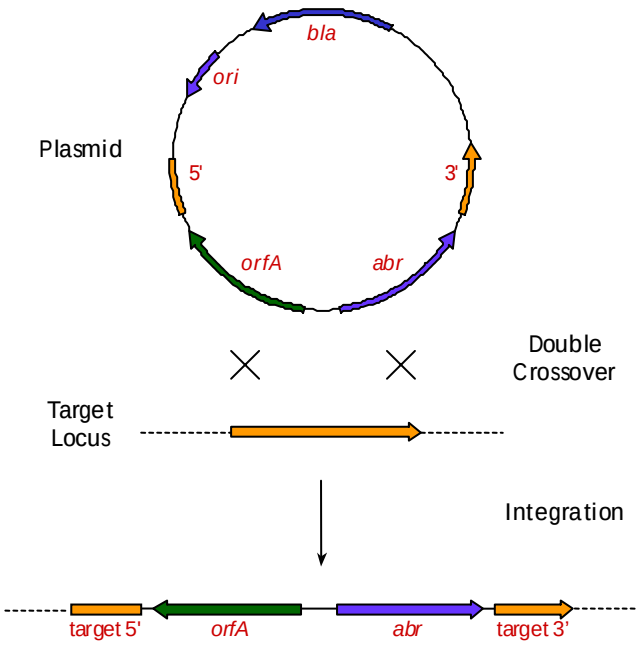

Figure 3. Use of an ectopic integration vector to insert a hypothetical open reading frame, *orfA,* into a target locus on the chromosome, such as the *B. subtilis amyE* gene.

Integration Vectors: an Introduction (Continued)

As we enter into the exciting years of the Genomic Era for *B. subtilis* and other gram-positives, integration vectors will continue to be an important tool for investigation. The Bacillus Genetic Stock Center is pleased to offer our users a collection of integration vectors that we hope will facilitate your research efforts for years to come.

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Integration Vectors—Quick Reference

Vector			Integration		Select	Upstream	Fusion		Downstream
Name	Strain	Map	Locus	Portion	with	Promoter	Tag	ORF's	Promoter
pBGSC6	ECE22	Detail	Cloned locus	Plasmid	Cm				
pCP115	ECE19	Detail	Cloned locus	Plasmid	Cm				
pMutin4	ECE139	Detail	Cloned locus	Plasmid	Em		β-Gal	C-term	Pspac
pDG364	ECE46	Detail	*B. sub amyE*	Cassette	Cm				
pMLK83	ECE103	Detail	*B. sub amyE*	Cassette	Nm		β-Glu	C-term	
pDG1661	ECE112	Detail	*B. sub amyE*	Cassette	Cm		β-Gal	C-term	
pDG1662	ECE113	Detail	*B. sub amyE*	Cassette	Cm				
pDG1728	ECE114	Detail	*B. sub amyE*	Cassette	Sp		β-Gal	C-term	
pDG1730	ECE115	Detail	*B. sub amyE*	Cassette	Sp				
pDG1663	ECE116	Detail	*B. sub thrC*	Cassette	Em		β-Gal	C-term	
pDL	ECE144	Detail	*B. sub amyE*	Cassette	Cm		BgaB	C-term	
pDK	ECE143	Detail	*B. sub amyE*	Cassette	Km		BgaB	C-term	
pDG1664	ECE117	Detail	*B. sub thrC*	Cassette	Em				
pDG1729	ECE118	Detail	*B. sub thrC*	Cassette	Sp		β-Gal	C-term	
pDG1731	ECE119	Detail	*B. sub thrC*	Cassette	Sp				
pAX01	ECE137	Detail	*B. sub lacA*	Cassette	Em	Pxyl			
pA-spac	ECE138	Detail	*B. sub lacA*	Cassette	Em	Pspac			
pPolHis1	ECE120	Detail	*B. sub rpoC*	Plasmid	Sp		His_6	C-term	
pSAS144	ECE142	Detail	*B. sub dif*	Plasmid	Cm				
pMUTIN-Flag	ECE146	Detail	Cloned locus	Plasmid	Em		FLAG	C-term	Pspac
pMUTIN-cMyc	ECE147	Detail	Cloned locus	Plasmid	Em		cMyc	C-term	Pspac
pMUTIN-HA	ECE148	Detail	Cloned locus	Plasmid	Em		HA	C-term	Pspac
pMUTIN-GFP+	ECE149	Detail	Cloned locus	Plasmid	Em		GFP+	C-term	Pspac
pMUTIN-CFP	ECE150	Detail	Cloned locus	Plasmid	Em		CFP	C-term	Pspac
pMUTIN-YFP	ECE151	Detail	Cloned locus	Plasmid	Em		YFP	C-term	Pspac
pSG1151	ECE152	Detail	Cloned locus	Plasmid	Cm		GFPmut1	C-term	
pSG1156	ECE154	Detail	Cloned locus	Plasmid	Cm		GFPuv	C-term	
pSG1186	ECE157	Detail	Cloned locus	Plasmid	Cm		CFP	C-term	
pSG1187	ECE158	Detail	Cloned locus	Plasmid	Cm		YFP	C-term	
pSG1194	ECE163	Detail	Cloned locus	Plasmid	Cm		dsRed	C-term	
pSG1164	ECE155	Detail	Cloned locus	Plasmid	Cm		GFPmut1	C-term	Pxyl
pSG1170	ECE156	Detail	Cloned locus	Plasmid	Cm		GFPuv	C-term	Pspac
pSG1154	ECE153	Detail	*B. sub amyE*	Cassette	Sp	Pxyl	GFPmut1	C-term	
pSG1192	ECE161	Detail	*B. sub amyE*	Cassette	Sp	Pxyl	CFP	C-term	
pSG1193	ECE162	Detail	*B. sub amyE*	Cassette	Sp	Pxyl	YFP	C-term	
pSG1729	ECE164	Detail	*B. sub amyE*	Cassette	Sp	Pxyl	GFPmut1	N-term	
pSG1190	ECE159	Detail	*B. sub amyE*	Cassette	Sp	Pxyl	CFP	N-term	
pSG1191	ECE160	Detail	*B. sub amyE*	Cassette	Sp	Pxyl	YFP	N-term	

Selected Methods

β-Galactosidase Assays

Permeabilization with lysozyme:

1. Measure the OD_{595} of the culture. Centrifuge 1.0 ml of culture 1 min at top speed in a microcentrifuge.
2. Discard supernatant; wash pellet in 0.5 ml of ice-cold 25 mM Tris·HCl, pH 7.4, centrifuging as before.
3. Discard supernatant; freeze pellet in dry ice/ethanol bath. The pellet can be stored at -20°C until the assay.
4. Suspend the pellet in 0.64 ml Z buffer (below). Add 0.16 ml of lysozyme stock (made up 2.5 mg/ml in Z buffer on day of use). Vortex briefly. Incubate at 37°C for 5 min.
5. Add 8 µl of 10% Triton X100. Vortex briefly. Store on ice.
6. Pre-warm the extract for 2-5 min in a 30°C water bath. Add 0.2 ml ONPG solution (4.0 mg/ml *o*-Nitrophenyl-β-D-galactoside in Z buffer). Note the time. Incubate at 30°C.
7. When the color is clearly yellow, stop the reaction by adding 0.4 ml of 1 M Na_2CO_3. Record the time.
8. Centrifuge the sample for 5 min a microcentrifuge. Read the A_{420} of the supernatant against a blank prepared from 0.8 ml Z buffer, 0.2 ml ONPG, and 0.4 ml Na_2CO_3.

Unit Definition: $(1000 \times A_{420})/(\text{reaction time (min)} \times OD_{595})$

Permeabilization with toluene:

1. Measure the OD_{595} of the culture. Centrifuge 1.0 ml of culture 1 min at top speed in a microcentrifuge.
2. Discard the supernatant and freeze the pellet at -70°C.
3. Add 1.0 ml of Z buffer to the frozen pellet. Add one drop (10 µl) toluene and vortex for 15 s.
4. Pre-warm the extract for 2-5 min in a 30°C water bath. Add 0.2 ml ONPG solution (4.0 mg/ml *o*-Nitrophenyl-β-D-galactoside in Z buffer). Note the time. Incubate at 30°C.
5. When the color is clearly yellow, stop the reaction by adding 0.5 ml 1 M Na_2CO_3. Record the time.

Unit Definition: $1000 \times (A_{420}\text{-}(1.75 \times OD_{550})/(\text{reaction time (min)} \times OD_{595})$ (Miller Units)

Assay with MUG (4-Methylumbelliferyl-β-D-galactopyranoside):

1. Centrifuge 0.5 ml of culture by a 1-min centrifugation at top speed in a microcentrifuge.
2. Suspend the cell pellet in 0.6 ml of Z buffer containing lysozyme (200 µg/ml) and Dnase I (100 µg/ml)
3. Add 0.2 ml of MUG (40 µg/ml in Z buffer). Incubate at 30°C for 40 min.
4. Stop the reaction by adding 0.4 ml 1 M Na_2CO_3.
5. Centrifuge the sample for 5 min a microcentrifuge.
6. Measure the fluorescence of the supernatant using excitation at 365 nM and emission at 450 nm.

Unit Definition: 1 unit catalyzes the production of 1 pmol of MUG per min. Calibrate the fluorometer with standard solutions of MUG.

Z-buffer:

$Na_2HPO_4 \cdot 7H_2O$	60 mM
NaH_2PO_4	40 mM
KCl	10 mM
$MgSO_4 \cdot 7H_2O$	1 mM
*β-mercaptoethanol, pH 7.0	50 mM

*add on day of use

References:

Cutting, S. M. and P. B. Vander Horn. 1990. Genetic Analysis, p. 27-74. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular Biological Methods for *Bacillus.* John Wiley, Chichester.

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Thermostable β-Galactosidase Assay

Follow the directions for the standard β-Galactosidase assay, increasing incubation temperature to 55°C.

Unit Definition: One unit hydrolyzes 1 nmol of substrate per min.

References:

Yuan, G. and S.-L. Wong. 1995. Regulation of *groE* expression in *Bacillus subtilis:* the involvement of the σ^A-like promoter and the roles of the inverted repeat sequence (CIRCE). J. Bacteriol. **177:**5427-5433.

Hirata, H., T. Fukazawa, S. Negoro, and H. Okada. 1986. Structure of a β-galactosidase gene of Bacillus stearothermophilus. J. Bacteriol. **166:**722-727.

Amylase Production Screening

To test for the production of α-amylase:

1. Inoculate transformant colonies to be tested as small patches onto a rich medium (LB or TBAB) supplemented with 1% soluble starch (Sigma Catalog No. S5651 or other similar product). Grow overnight at 30-37°C.
2. Flood the surface of the plate with 5 ml of Gram's iodine stain (a solution of 0.5% w/v iodine and 1% potassium iodide, Sigma HT90-2 or similar product). Incubate at room temperature for 1 min.
3. Decant the stain and observe plates for clear haloes around the colony. Colonies without significant zones of clearing are amylase negative, and therefore presumed to be *amy* knockouts.

References:

Cutting, S. M. and P. B. Vander Horn. 1990. Genetic Analysis, p. 27-74. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular Biological Methods for *Bacillus.* John Wiley, Chichester.

β-Glucuronidase Assay

Follow the directions for the β-Galactosidase assay of lysozyme-permeabilized cells on the previous page. Substitute *p*-nitrophenyl β-D-glucuronidide (4 mg/ml) for the ONPG solution in the protocol.

Unit Definition: Karow and Piggot (1995) expressed specific activity is as nmol *p*-nitrophenyl β-D-glucuronidide hydrolyzed per minute per mg of bacterial dry weight. Others might find it more convenient to define units in terms of OD_{595} of original culture rather than bacterial dry weight.

References:

Karow, M. L. and P. J. Piggot. 1995. Construction of *gusA* transcriptional fusion vectors for *Bacillus subtilis* and their utilization for studies of spore formation. Gene **163:**69-74.

Nicholson, W. L. and P. Setlow. 1990. Sporulation, germination and outgrowth, p. 391-429. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular Biological Methods for *Bacillus.* John Wiley, Chichester.

B. subtilis Competent Cell Preparation and Transformation

Competence development in *B. subtilis* is one of several stationary phase processes triggered by a nutritional downshift. Since the pioneering work of Anagnostopolous and Spizizen, a number of protocols for preparing competent *B. subtilis* cells have appeared. The following method, modified by Ron Yasbin from protocols developed in the Frank Young lab at Rochester, was used routinely in Stan Zahler's wonderful bacterial genetics course at Cornell. This protocol assumes that you use a spectrophotometer that accepts 16×125 mm test tubes. If your spectrophotometer, like mine, works only with cuvettes, simply increase the culture volume to 10 or 20 ml in a 250-ml Erlenmeyer flask.

1. Streak recipient strain on one-half of a Tryptose Blood Agar Base plate. Incubate overnight (18 hr) at 37°C.
2. Inoculate a few colonies into 4.5 ml of Medium A in a 16×125 mm test tube that lacks visible scratches. Mix the contents of the tube thoroughly. Read its optical density at 650 nm in the spectrophotometer. Adjust the OD_{650} to be 0.1-0.2, maintaining the volume at 4.5 ml.
3. Incubate at 37°C with vigorous aeration. Read the OD_{650} every 20 min, plotting OD_{650} against time on semi-log paper. After a brief lag, the OD should increase logarithmically—that is, they should fall on a straight line. Note the point at the culture leaves log growth—the graph points fall below the straight line. In *B. subtilis* genetics, this point is known as t_0. It should take 60-90 minutes of incubation and occur at OD_{650}=0.4-0.6.
4. Continue incubation for 90 minutes after the cessation of log growth (t_{90}). Transfer 0.05 ml of this culture into 0.45 ml of pre-warmed Medium B in a 16×125 mm test tube. Set up one tube for each transformation you intend to perform, plus an extra for a DNA-less control.
5. Incubate the diluted cultures at 37°C with vigorous aeration for 90 min. At this point, the cultures should be highly competent.
6. Add 1 µg of DNA to the competent cells and incubate at 37°C with aeration for 30 minutes.
7. Plate aliquots of the transformed cells onto selective agar.

10× Medium A base:

Yeast extract	10 g
Casamino acids	2 g
Distilled water	to 900 ml
Autoclave, then add:	
50% glucose, filter-sterilized	100 ml

10× Bacillus salts

$(NH_4)_2SO_4$	20 g
$K_2HPO_4 \cdot 3H_2O$	183 g
KH_2PO_4	60 g
Na^+citrate	10 g
$MgSO_4 \cdot 7H_2O$	2 g
Water	to 1000 ml

Medium A

Sterile water	81 ml
10× Medium A base	10 ml
10× Bacillus salts	9 ml

Medium B

Medium A	10 ml
50 mM $CaCl_2 \cdot 2H_2O$	0.1 ml
250 nM $MgCl_2 \cdot 6H_2O$	0.1 ml

References:

Yasbin, R. E., G. A. Wilson, and F. E. Young. 1975. Transformation and transfection in lysogenic strains of *Bacillus subtilis:* Evidence for selective induction of prophage in competent cells. J. Bacteriol. **121:**296-304.

Zahler, S. A. (personal communication)

Purification of His_6-tagged *Bacillus subtilis* RNA Polymerase

The plasmid pPolHis1, when integrated into the *Bacillus subtilis* chromosome, tags the RNA polymerase α subunit with a poly-histidine tail. Under the proper conditions, the entire holoenzyme can be rapidly purified using this tag.

1. Grow cells to OD_{600}= 0.8 in 2 liters of LB medium containing 50 µg spectinomycin.
2. Harvest cells by centrifugation and suspend them in 10 ml of buffer 1 (below) with 2.5 mM imidazole.
3. Disrupt the cell suspension at 4°C by two passages through a French press at 70,000 kPa.
4. Centrifuge 20 min at 5,000 rpm in a GSA rotor. Keep the supernatant.
5. Add the supernatant to 4 ml of a Ni^{++}-NTA superflow matrix (Qiagen) previously equilibrated with buffer 1 with 2.5 mM imidazole. Incubate at 4°C for 1 hr.
6. Pack the resin into a disposable column, then wash with 15 buffer 1 with 20 mM imidazole.
7. Elute the His-tagged RNA polymerase with 5 ml of buffer 1 with 300 mM imidazole.

At this stage, the RNA polymerase activity can be assayed and used in *in vitro* transcription experiments.

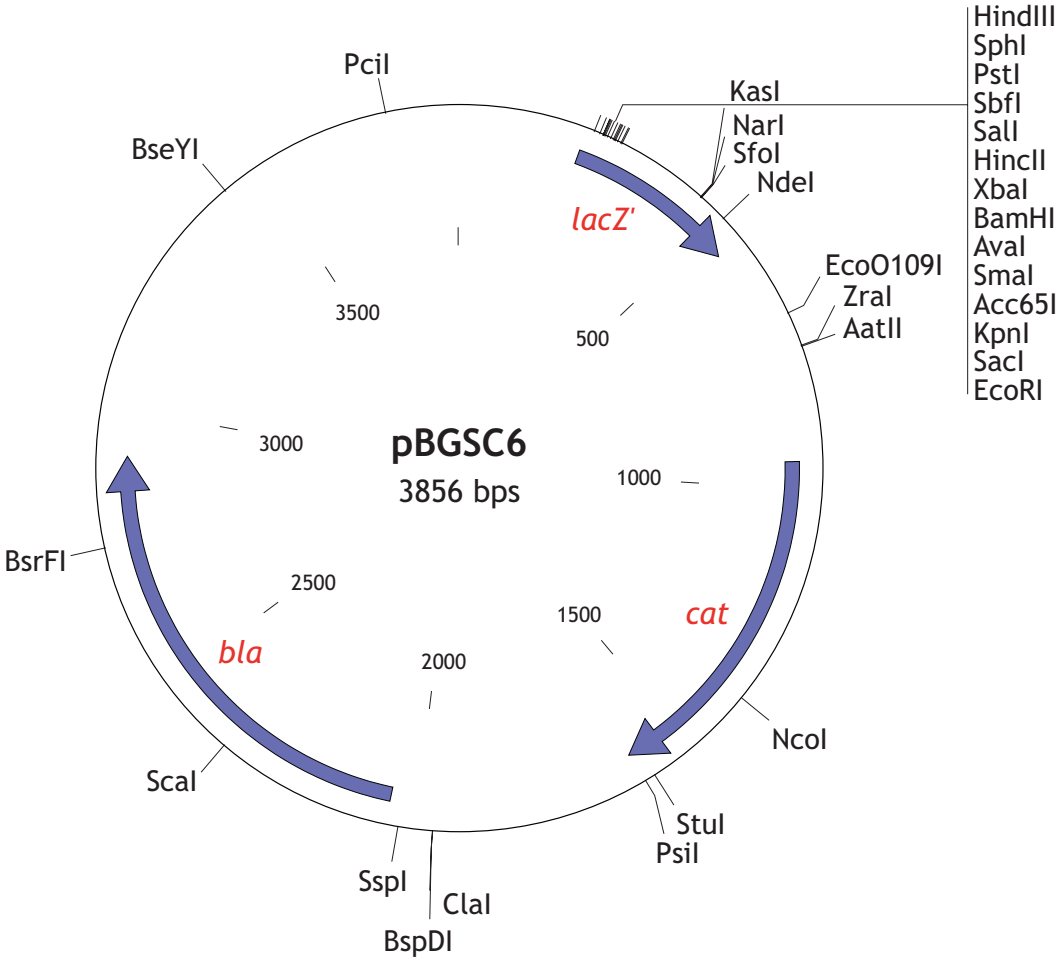
Buffer 1

Tris HCl, pH 8.0	10 mM
NaCl	0.1 M
Glycerol	5%
β-mercaptoethanol	1 mM
PMSF	1 mM

References:

Schyns, G. and C. P. Moran, Jr. (personal communication)

Integration Vector Maps



BGSC Accession: ECE22 **Orignal Code:** ECE22

Reference: D.R. Zeigler, unpublished. For an example of use, see Fajardo-Cavazos, P., *et al*. 1993. J. Bacteriol. 175:1635-1744.

Sequence: GenBank DQ483056 (Note: previous restriction map and sequence have been corrected in this release.)

Features:

lacZ'	β-lactamase α-subunit
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)

Description: Integration vector; integrates by Campbell-type recombination between cloned insert and chromosome.

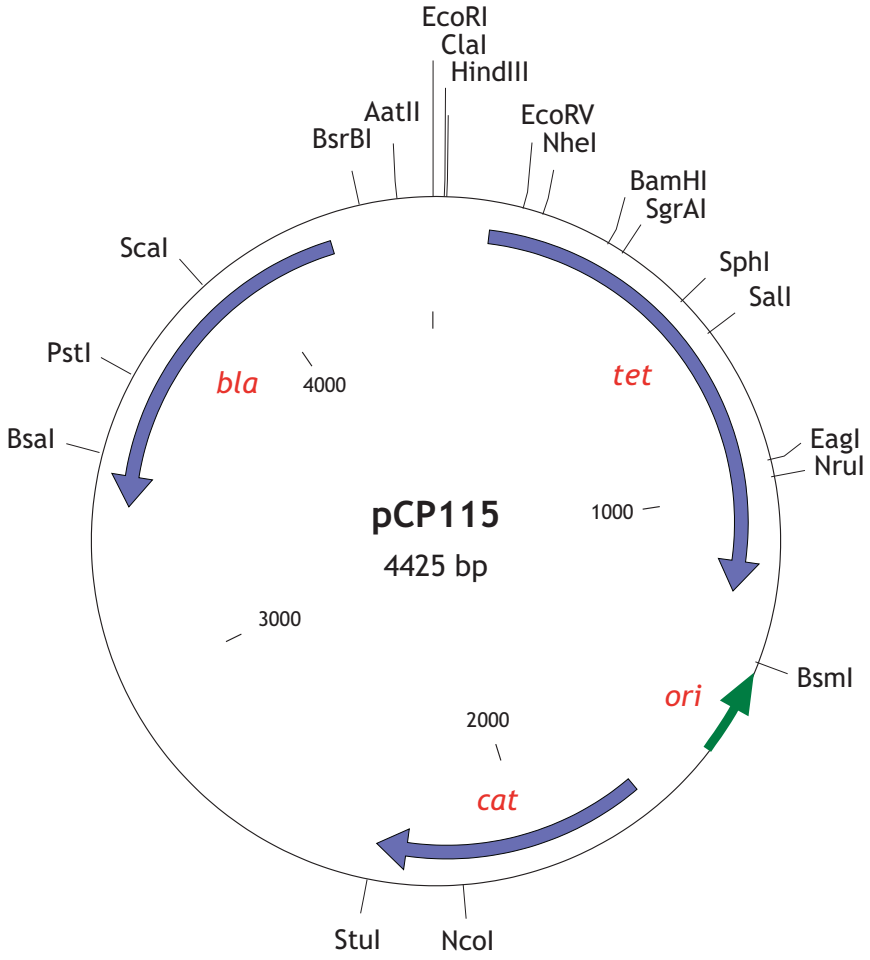
Construction: Insertion of the DraI fragment from pC194, bearing the *cat* gene, into the SspI site of pUC19.

Use: pBGSC6 is a very simple, general-purpose integration vector for Gram-positive organisms. An insert is cloned into the multiple cloning site. The blue-white system on X-gal plates or red-white on MacConkey agar detects the presence of the insert. The insert-bearing plasmid is used to transform the target organism with selection for chloramphenicol resistance. The plasmid integrates into the chormosome by homologous recombination with the cloned insert.

Recipient strains: pBGSC6 should work with any transformable bacterial species that can express *cat* but not support replication of pUC19. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE19 **Orignal Code:** BNN45(pCP115)

Reference: Price, C.W. and R.H. Doi. 1985. Mol. Gen. Genet. 201:88-95

Sequence: Not in database; not available from BGSC

Features:

tet	encodes tetracycline resistance protein; selectable in *E. coli* only (tetracycline 50 μg/ml)
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)

Description: Integration vector; integrates by Campbell-type recombination between cloned insert and chromosome.

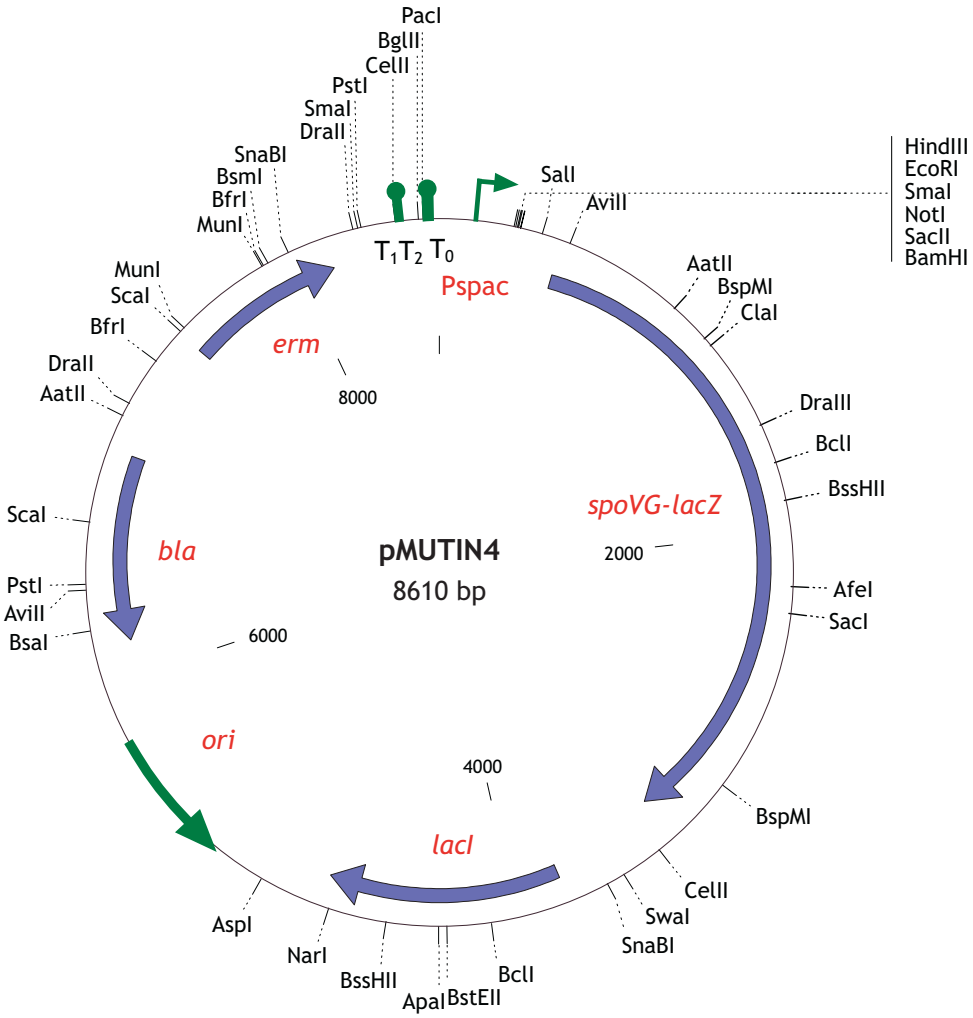
Construction: Composed of components of the vectors pBR327 (*tet, bla, ori*) and pC194 (*cat*).

Use: pCP115 is a simple, general-purpose integration vector for Gram-positive organisms. An insert is cloned into an available restriction site lying within either *bla* or *tet*. Insertional inactivation of antibiotic resistance detects the presence of the insert. The insert-bearing plasmid is used to transform the target organism with selection for chloramphenicol resistance. The plasmid integrates into the chormosome by homologous recombination with the cloned insert.

Recipient strains: pCP115 should work with any transformable bacterial species that can express *cat* but not support replication of pBR322-like plasmids. It has been used exclusively with *Bacillus subtilis,* but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation.

Integration Vector Maps (*continued*)



BGSC Accession: ECE139 **Orignal Code:** MC1061(pMUTIN4)

Reference: **Vagner, V., E. Dervyn and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097-3104.

Sequence: not available in databases; the sequence of a nearly identical sister plasmid, pMUTIN2, is available at GenBank AF072806, however.

Features:

spoVG-lacZ	*E. coli lacZ* gene, fused to the *B. subtilis* 168 *spoVG* ribosome binding site
Pspac	hybrid promoter, inducible by IPTG
lacI	encodes *lac* repressor, with modified ribosome binding site for Gram-positive expression
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.3 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)

Description: Integration vector designed for systematic inactivation of coding sequences discovered in genomic sequences.

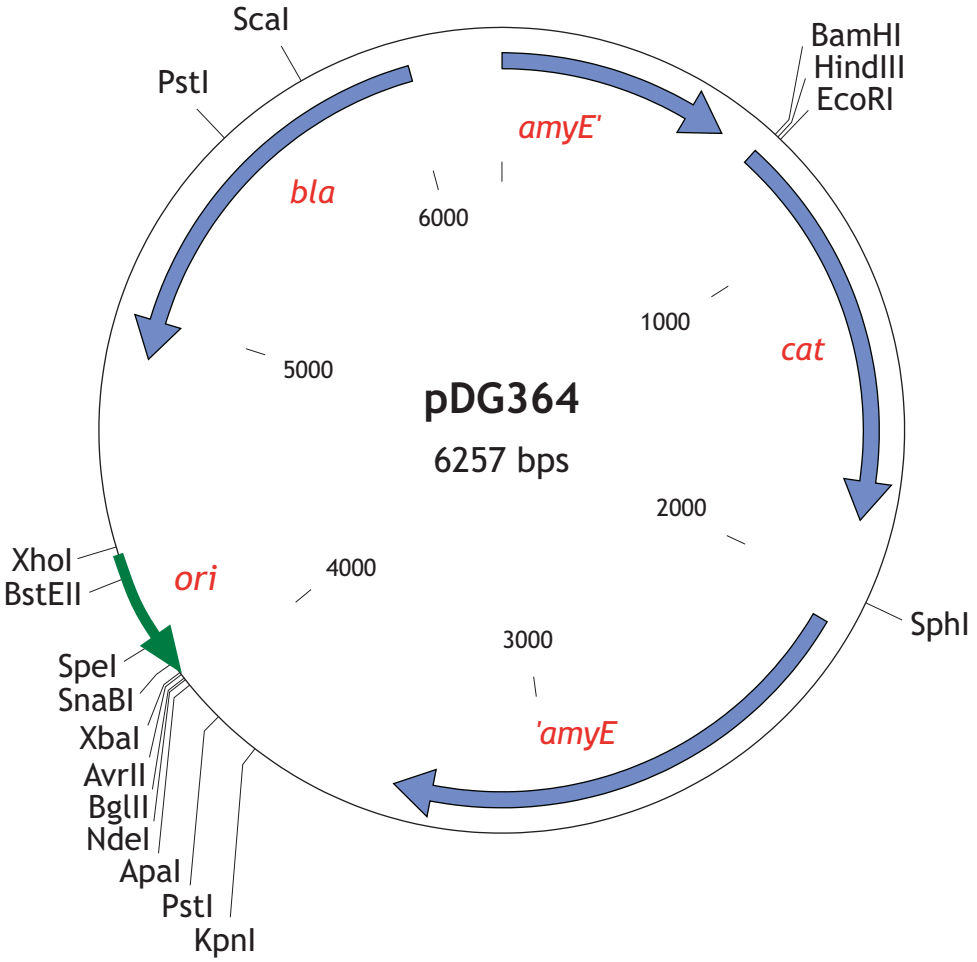
Construction: A pVE6023 *Xba*I-*Bam*HI fragment containing *erm* was joined with a pDG148 *Eco*RI fragment containing *bla*, the ColE1 origin, the *E. coli lacI* gene, and the Pspac promoter. A pJM783 BamHI-DraI fragment containing *spoVG-lacZ* was inserted into the blunted *Sph*I site of the construct. Terminators from λ and *E. coli rrnB* were amplified and inserted between *erm* and Pspac. The "oid" operator was amplified and inserted between Pspac and *spoVG-lacZ*. Finally, a multiple cloning site was inserted after pSpac.

Use: The plasmid is designed to integrate by Campbell-type insertion into the chromosomal locus of a cloned coding sequence. The net effect is to divide the coding sequence from its natural regulatory regions. The coding sequence is placed under the control, allowing for comparison of phenotypes in the presence and absence of induction. The unknown gene's promoter and regulatory regions are fused to the *spoVG-lacZ* gene, allowing for analysis of expression patterns via theβ-galactosidase reporter.

Recipient strains: Has been used in *B. subtilis,* but should perform well in any transformable Gram-positive host.

Protocols: *B. subtilis* competent cell preparation and transformation; β-galactosidase assay

Integration Vector Maps (*continued*)



BGSC Accession: ECE46 **Orignal Code:** TG1(pDG364)

Reference: Stragier, P. (unpublished); see Cutting, S. M. and P. B. Vander Horn. 1990. Genetic Analysis. In: Harwood, C. R. and S. M. Cutting, eds. Molecular Biology Methods for *Bacillus,* p. 53-54. John Wiley and Sons, Chichester.

Sequence: not available in databases; not available from BGSC.

Features:

amyE'...'amyE	5' and 3' parts of *B. subtilis* 168 α-amylase gene
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 100 μg/ml)

Description: Promotes ectopic integration into the *amyE* locus of 1A771, replacing the resident MLS resistance cassette. Integrants are Cm^R MLS^S.

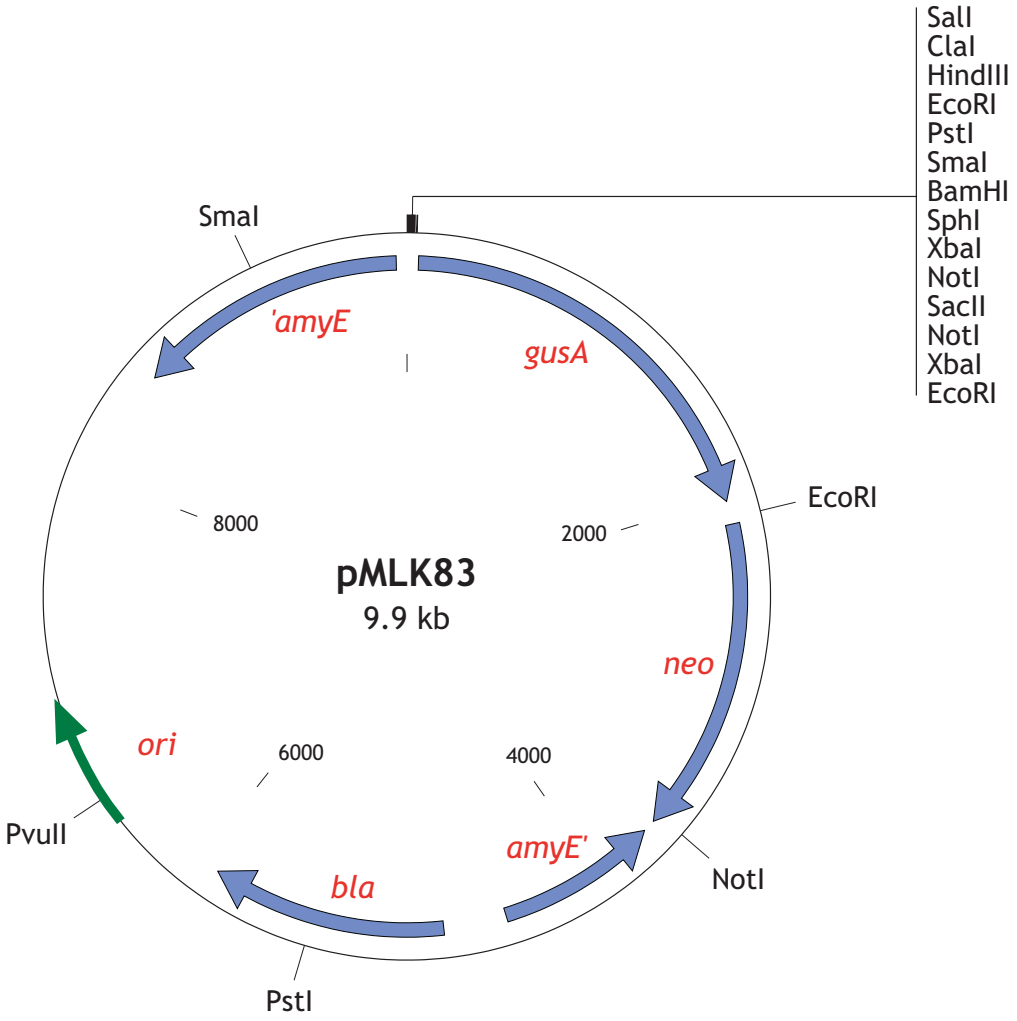
Construction: pDG268 with a 3 kb fragment containing *lacZ* deleted.

Use: The plasmid is designed to integrate a cloned insert into the *Bacillus subtilis* 168 chromosome at the *amyE* locus. The user inserts the fragment of interest into the multiple cloning site. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for chloramphenicol resistance. Transformants are screened for sensitivity to erythromycin (indicating that the resident *amyE* locus has been replaced). The second-generation vectors derived from pDG364 (such as pDG1661) make it simpler to demonstrate that a Campbell-type insertion has not occurred.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A771, with an *erm*-insertion in its chromosomal *amyE* locus, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening

Integration Vector Maps (*continued*)



BGSC Accession: ECE103 **Orignal Code:** DH5α(pMLK83)

Reference: **Karow, M. L, and P. J. Piggot**. 1994. Construction of *gusA* transcriptional fusion vectors for *Bacillus subtilis* and their utilization for studies of spore formation. Gene **163:**69-74.

Sequence: not available in databases; not available from BGSC.

Features:

amyE'...'amyE	5' and 3' parts of *B. subtilis* 168 α-amylase gene
neo	encodes ; selectable in either *E. coli* or *B. subtilis* (neomycin or kanamycin 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)
gusA	encodesβ-glucuronidase reporter

Description: Promotes ectopic integration into the *B. subtilis amyE* locus; allows for fusions with a β-glucuronidase reporter..

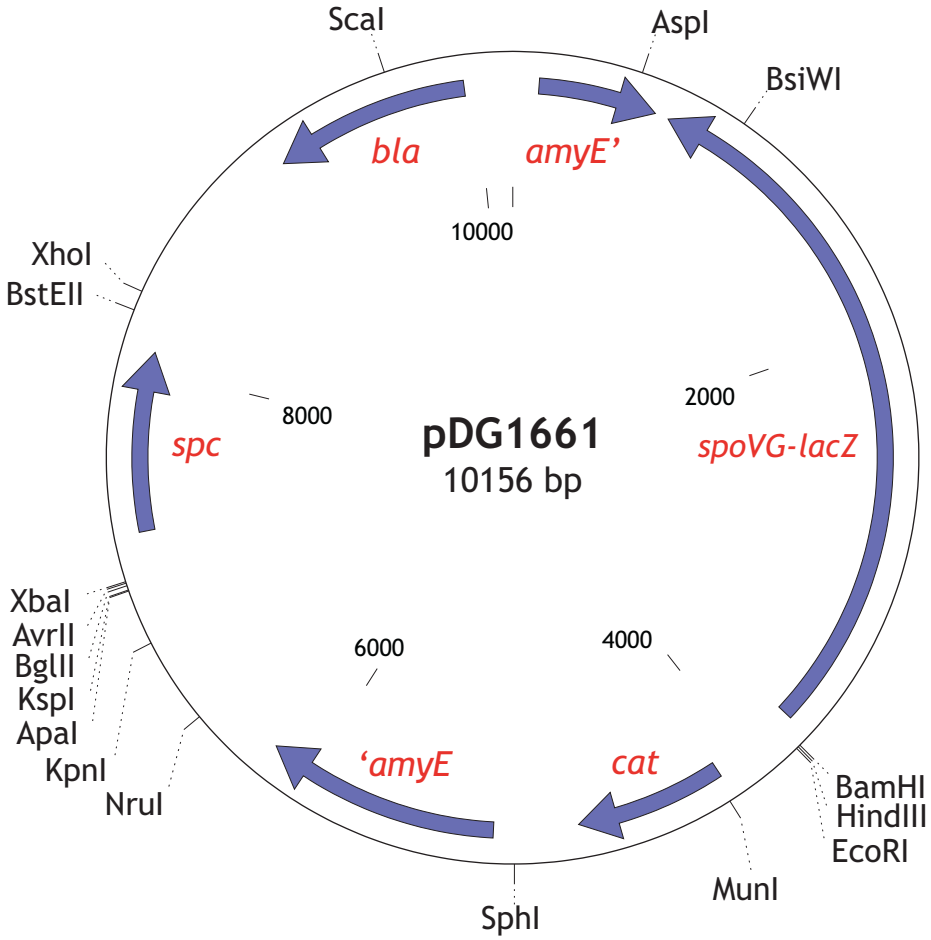
Construction: The *lacZ* gene was removed from pDH32. A *neo* cassette from pBEST501 was inserted into the SalI site of the resulting construct. A *gusA* cassette, formed by modifying the ribosome binding site of the gene in pRAJ275 (Clontech) and adding a multiple cloning site, was inserted to produce pMLK83.

Use: The plasmid is designed to create a *gusA* gene fusion and then to integrate it into the *Bacillus subtilis* 168 chromosome at the *amyE* locus. The user inserts the fragment of interest into the multiple cloning site. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for neomyhcin or kanamycin resistance. Transformants are screened for sensitivity to the resident antibiotic resistance gene in the host *amyE* locus (indicating that the host *amyE* locus has been replaced). Becauseβ-glucuronidase and β-galactosidase have non-overlapping activities, two gene fusions can be introduced into the same host and monitored separately.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A771, with an *erm*-insertion in its chromosomal *amyE* locus, and 1A772, with a *cat*-insertion, allow for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; β-glucuronidase assay; amylase production screening

Integration Vector Maps (*continued*)



BGSC Accession: ECE112 **Orignal Code:** TG1(pDG1661)

Reference: **Guérout-Fleury, A. M., N. Frandsen, P. Stragier**. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

Sequence: GenBank U46196

Features:

amyE'...'amyE 5' and 3' segments of the *B. subitlis* 168 *amyE* gene
spoVG-lacZ *E. coli lacZ* coding sequence, fused to the ribosome binding site of *B. subtilis spoVG*.
spc encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin100 μg/ml)
cat encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)

Description: Promotes ectopic integration into the *B. subtilis amyE* locus; allows for fusions with a β-galactosidase reporter..

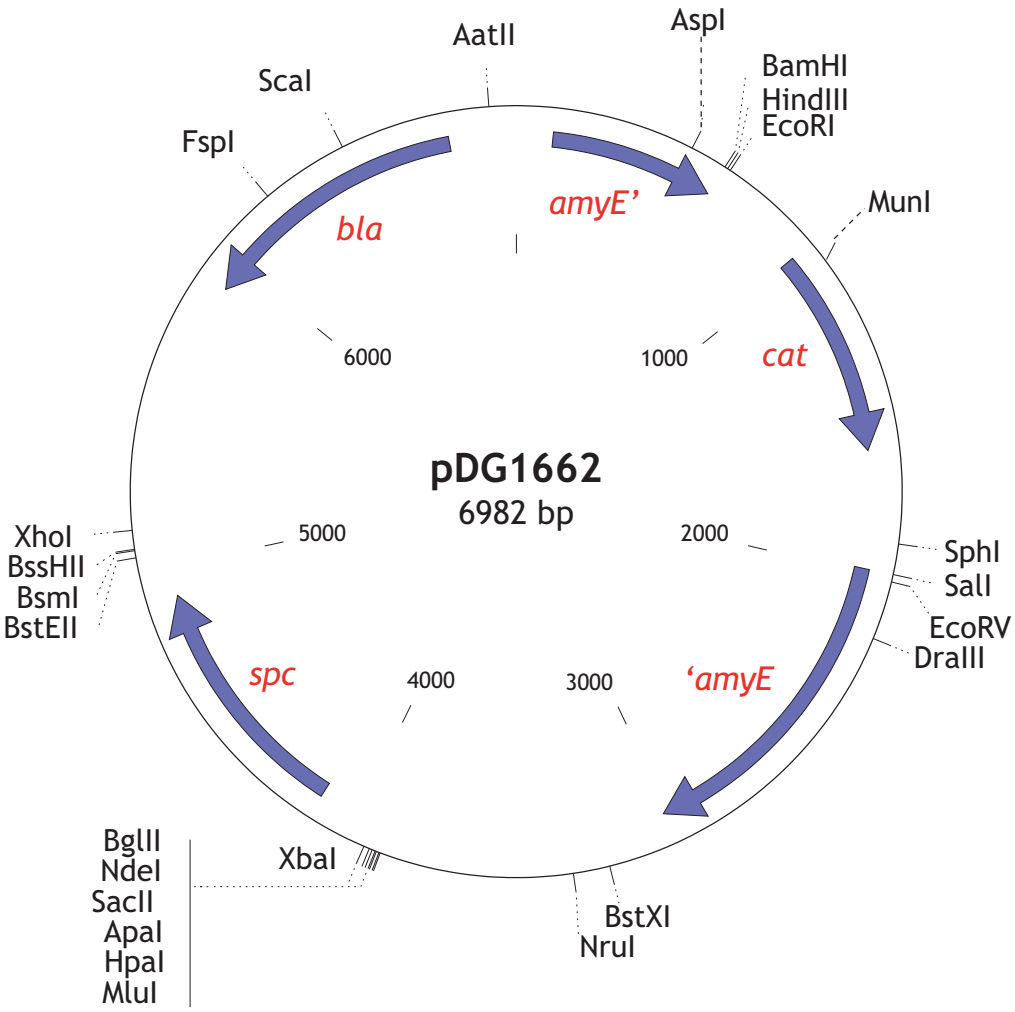
Construction: pDG268 with a spectinomycin cassette inserted into the non-integrating region of the plasmid.

Use: The plasmid is designed to integrate a *lacZ* fusion into the *Bacillus subtilis* 168 chromosome at the *amyE* locus. The user inserts the promoter-containing fragment of interest into the multiple cloning site to create a *lacZ* fusion. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for chloramphenicol resistance. Transformants are screened for sensitivity to erythromycin (indicating that the resident *amyE* locus has been replaced) and for sensitivity to spectinomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A771, with an *erm*-insertion in its chromosomal *amyE* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation; β-galactosidase assay.

Integration Vector Maps (*continued*)



BGSC Accession: ECE113 **Orignal Code:** TG1(pDG1662)

Reference: Guérout-Fleury, A-M *et al.* (1996) Gene 180:57-61

Sequence: GenBank U46197

Features:

amyE'...'amyE	5' and 3' parts of *B. subtilis* 168 α-amylase gene
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 100 μg/ml)

Description: Promotes ectopic integration into the *amyE* locus of 1A771, replacing the resident MLS resistance cassette. Integrants are Cm^R Sp^S MLS^S.

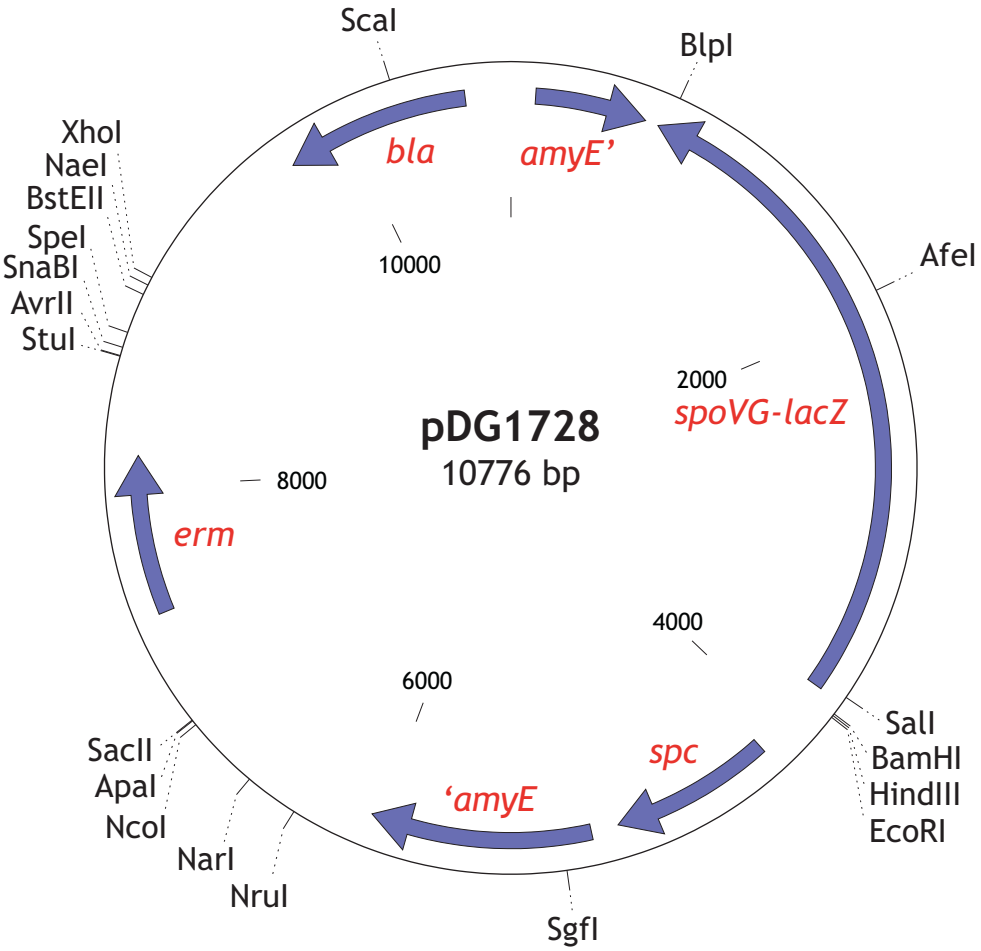
Construction: pDG268 with a spectinomycin cassette inserted into the non-integrating region of the plasmid.

Use: The plasmid is designed to integrate a cloned insert into the *Bacillus subtilis* 168 chromosome at the *amyE* locus. The user inserts the fragment of interest into the multiple cloning site. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for chloramphenicol resistance. Transformants are screened for sensitivity to erythromycin (indicating that the resident *amyE* locus has been replaced) and for sensitivity to spectinomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A771, with an *erm*-insertion in its chromosomal *amyE* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE114 **Orignal Code:** TG1(pDG1728)

Reference: **Guérout-Fleury, A. M., N. Frandsen, P. Stragier**. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

Sequence: GenBank U46198

Features:

amyE'...'amyE 5' and 3' segments of the *B. subitlis* 168 *amyE* gene
spoVG-lacZ *E. coli lacZ* coding sequence, fused to the ribosome binding site of *B. subtilis spoVG*.
spc encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin100 μg/ml)
erm encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.5 μg/ml, lincomycin 12.5 μg/ml)
bla encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)

Description: Promotes ectopic integration into the *B. subtilis amyE* locus; allows for fusions with a β-galactosidase reporter.

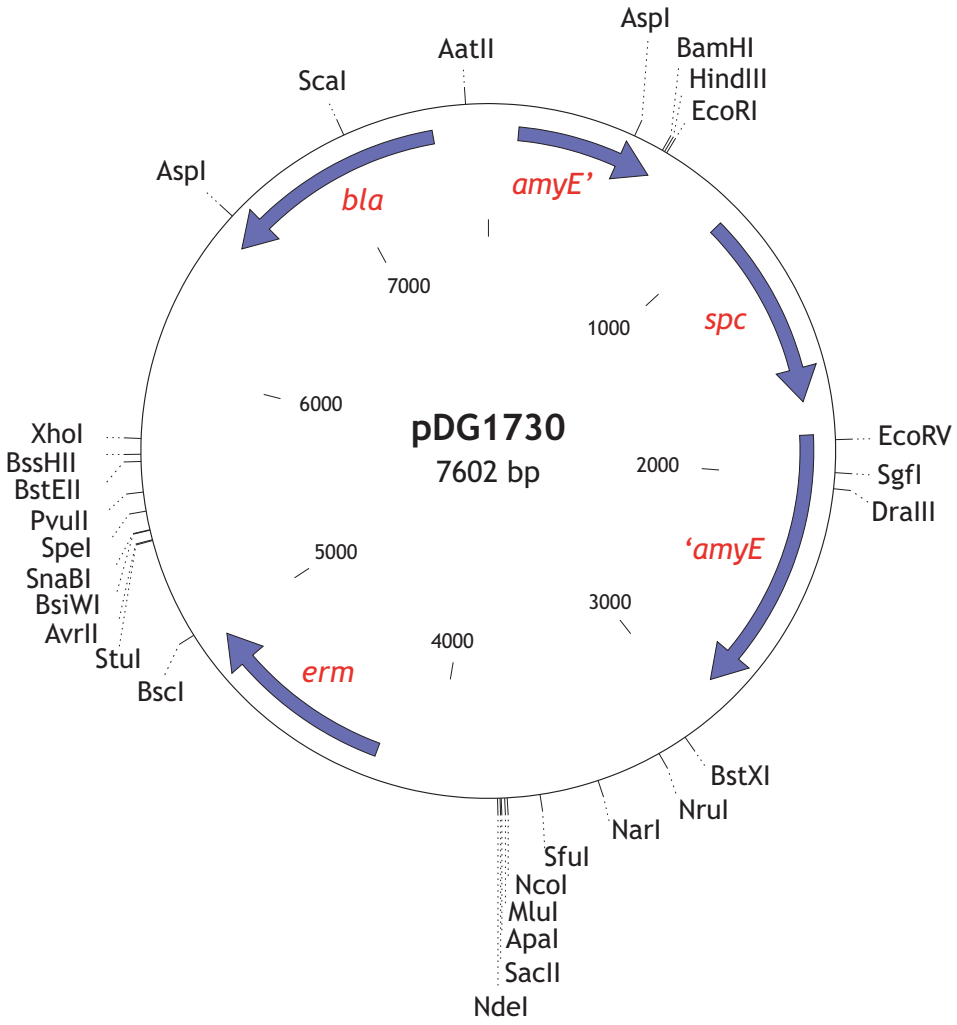
Construction: pDG1661 with a spectinomycin cassette substituted for *cat* in the integrative portion of the plasmid and *erm* substituted for *spc* in the non-integrative part.

Use: The plasmid is designed to integrate a *lacZ* fusion into the *Bacillus subtilis* 168 chromosome at the *amyE* locus. The user inserts the promoter-containing fragment of interest into the multiple cloning site to create a *lacZ* fusion. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for spectinomycin resistance. Transformants are screened for sensitivity to chloramphenicol (indicating that the resident *amyE* locus has been replaced) and for sensitivity to erythromycin-lincomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A772, witha *cat*-insertion in its chromosomal *amyE* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation; β-galactosidase assay.

Integration Vector Maps (*continued*)



BGSC Accession: ECE115 **Orignal Code:** TG1(pDG1730)

Reference: Guérout-Fleury, A-M *et al.* (1996) Gene 180:57-61

Sequence: GenBank U46199

Features:

amyE'...'amyE	5' and 3' parts of *B. subtilis* 168 α-amylase gene
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.5 μg/ml, lincomycin 12.5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 100 μg/ml)

Description: Promotes ectopic integration into the *amyE* locus of 1A772, replacing the resident Cm^R resistance cassette. Integrants are Sp^R Cm^S MLS^S.

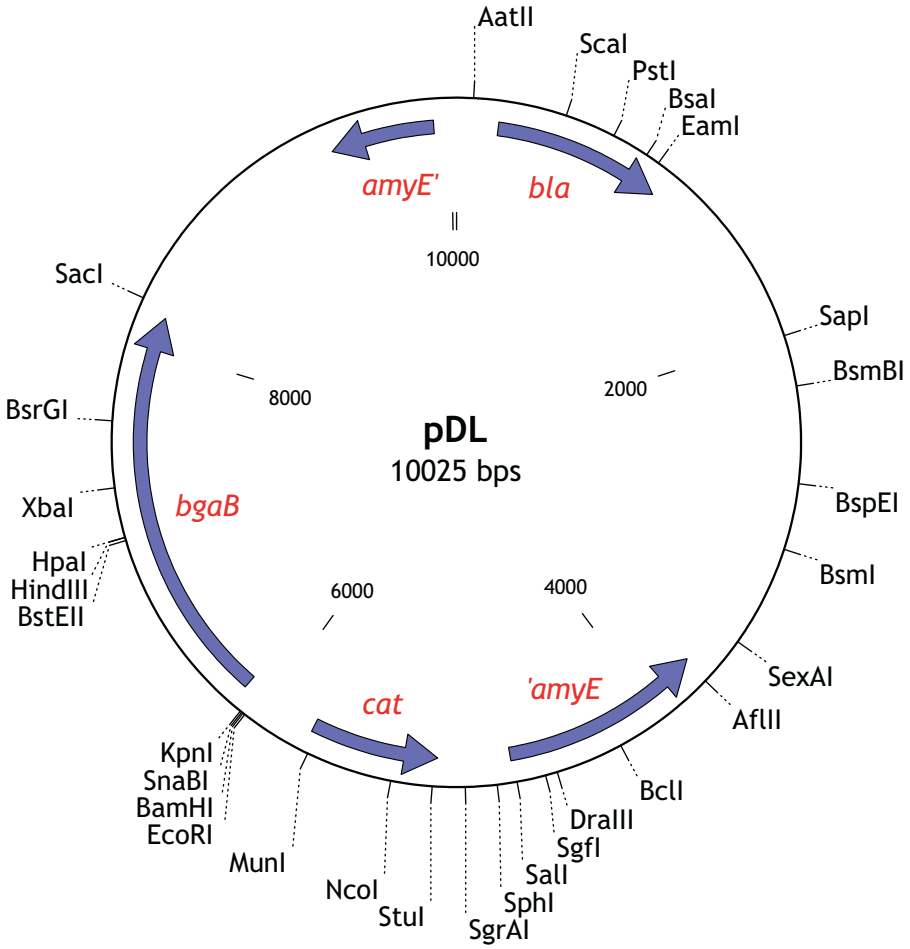
Construction: pDG268 with a spectinomycin cassette inserted into the non-integrating region of the plasmid.

Use: The plasmid is designed to integrate a cloned insert into the *Bacillus subtilis* 168 chromosome at the *amyE* locus. The user inserts the fragment of interest into the multiple cloning site. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for spectinomycin resistance. Transformants are screened for sensitivity to chloramphenicol (indicating that the resident *amyE* locus has been replaced) and for sensitivity to erythromycin-lincomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A772, witha *cat*-insertion in its chromosomal *amyE* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE144 **Orignal Code:** DH5α(pDL)

Reference: **Yuan, G. and S. L. Wong.** 1995. Regulation of *groE* Expression in *Bacillus subtilis*: the Involvement of the sA-Like Promoter and the Roles of the Inverted Repeat Sequence (CIRCE) J. Bacteriol. **177:**5427.

Sequence: Not in database; available from BGSC at http://bacillus.biosci.ohio-state.edu/sequences/pDL.htm

Features:

bgaB	encodees thermostable β-galactosidase originally isolated from *Geobacillus stearothermophilus*
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence

Description: Vector designed to fuse a sequence of choice to a thermostable reporter gene and to integrate the fusion into the *B. subtilis amyE* locus.

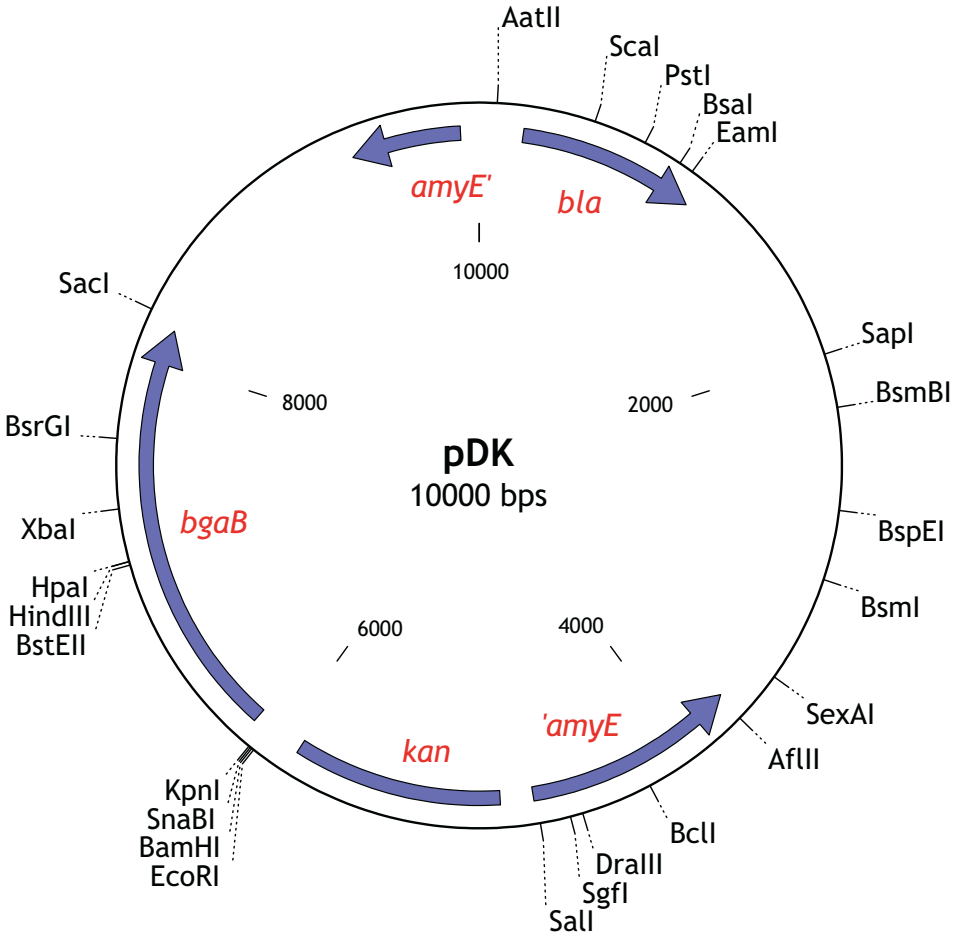
Construction: The promoterless *bgaB* coding sequence was amplified by PCR from *Geobacillus stearothermophilus* ATCC 7954, then inserted into the SnaBI-SacI backbone of pDH32/M (Kraus A., *et al*. 1994. J. Bacteriol. 176:3981-3992).

Use: A DNA fragment containing a gram-positive promoter and its regulatory region in inserted into the multiple cloning site upstream from *bgaB*. Upon transformation into *B. subtilis* 168 with selection for chloramphenicol resistance, colonies are recovered in which the resident *amyE* gene has been replaced by a cassette containing *cat* plus the *bgaB* fusion.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening; β-galactosidase assay.

Integration Vector Maps (*continued*)



BGSC Accession: ECE143 **Orignal Code:** DH5α(pDK)

Reference: **Yuan, G. and S. L. Wong.** 1995. Regulation of *groE* Expression in *Bacillus subtilis*: the Involvement of the sA-Like Promoter and the Roles of the Inverted Repeat Sequence (CIRCE) J. Bacteriol. **177:**5427.

Sequence: Not in database; not available from BGSC

Features:

bgaB	encodees thermostable β-galactosidase originally isolated from *Geobacillus stearothermophilus*
kan	encodes kanamycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (kanamycin or neomycin 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence

Description: Vector designed to fuse a sequence of choice to a thermostable reporter gene and to integrate the fusion into the *B. subtilis amyE* locus.

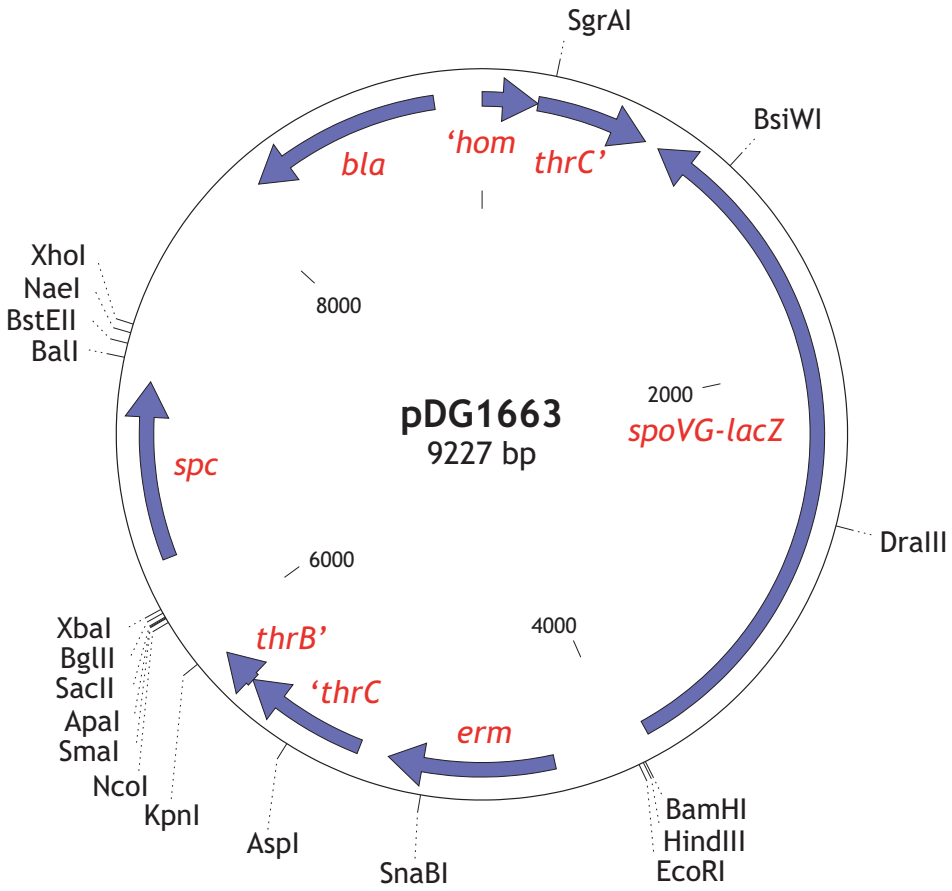
Construction: An EcoRI-SalI cassette carrying P43-*kan* was substituted for the EcoRI-SalI fragment of pDL.

Use: A DNA fragment containing a gram-positive promoter and its regulatory region in inserted into the multiple cloning site upstream from *bgaB*. Upon transformation into *B. subtilis* 168 with selection for kanamycin resistance, colonies are recovered in which the resident *amyE* gene has been replaced by a cassette containing *cat* plus the *bgaB* fusion.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening; β-galactosidase assay.

Integration Vector Maps (*continued*)



BGSC Accession: ECE116 **Orignal Code:** TG1(pDG1663)

Reference: **Guérout-Fleury, A. M., N. Frandsen, P. Stragier**. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

Sequence: GenBank U46200

Features:

thrC'...'thrC	5' and 3' segments of the *B. subitlis* 168 *thrC* gene
'hom	3' end of the *B. subtilis* 168 *hom* gene (part of the *thrC* operon)
thrB'	5' end of the *B. subtilis* 168 *thrB* gene (part of the *thrB* operon)
spoVG-lacZ	*E. coli lacZ* coding sequence, fused to the ribosome binding site of *B. subtilis spoVG*.
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin100 μg/ml)
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.5 μg/ml, lincomycin 12.5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)

Description: Promotes ectopic integration into the *B. subtilis thrC* locus; allows for fusions with a β-galactosidase reporter.

Construction: Composite of *hom-thrC* fragment from pSU11, hybrid *lacZ* fragment from pJM73, multiple cloning site from pDG268, *thrC-thrB* fragment from pSU11, multiple cloning site from pJRD184, *spc* cassette from pIC215, an origin-containing fragment from pSU11, and a *bla* fragment from pBR322

Use: The plasmid is designed to integrate a *lacZ* fusion into the *Bacillus subtilis* 168 chromosome at the *thrC* locus. The user inserts the promoter-containing fragment of interest into the multiple cloning site to create a *lacZ* fusion. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for erythromycin-lincomycin resistance. Transformants are screened for sensitivity to chloramphenicol (indicating that the resident *thrC* locus has been replaced) and for sensitivity to spectinomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A773, with a *cat*-insertion in its chromosomal *thrC* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation; β-galactosidase assay.

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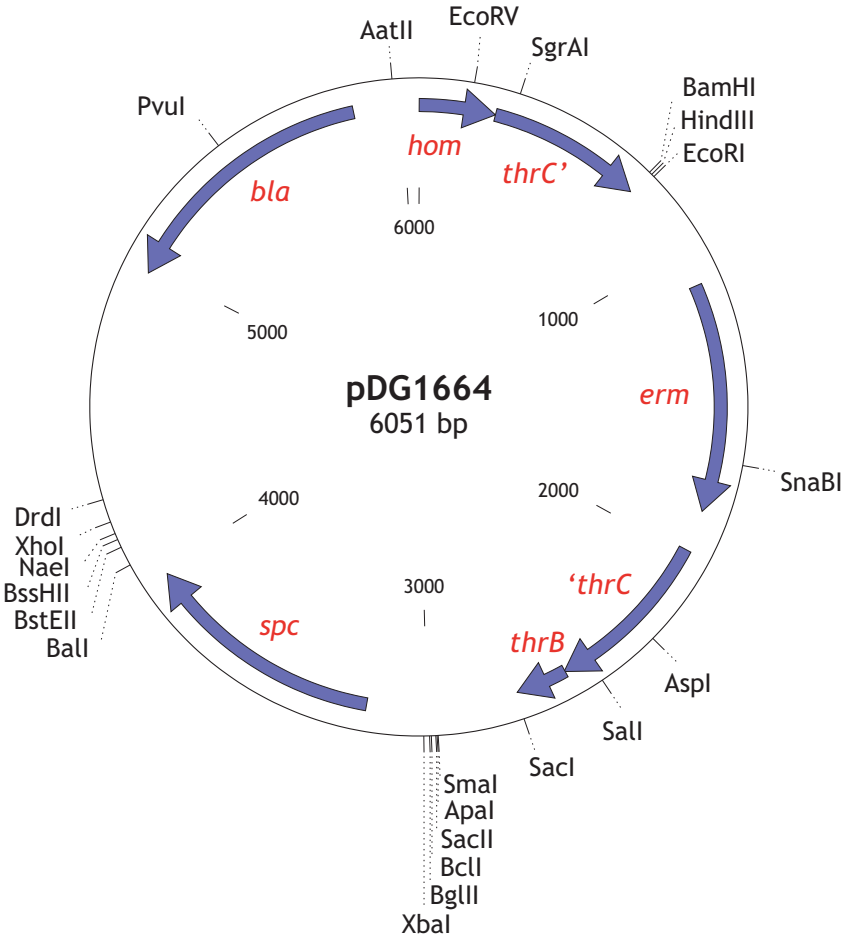
Integration Vector Maps (*continued*)



BGSC Accession: ECE117 **Orignal Code:** TG1(pDG1664)

Reference: **Guérout-Fleury, A. M., N. Frandsen, P. Stragier**. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

Sequence: GenBank U46201

Features:

thrC'...'thrC	5' and 3' segments of the *B. subitlis* 168 *thrC* gene
'*hom*	3' end of the *B. subtilis* 168 *hom* gene (part of the *thrC* operon)
thrB'	5' end of the *B. subtilis* 168 *thrB* gene (part of the *thrB* operon)
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin100 μg/ml)
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.5 μg/ml, lincomycin 12.5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)

Description: Promotes ectopic integration into the *B. subtilis thrC* locus.

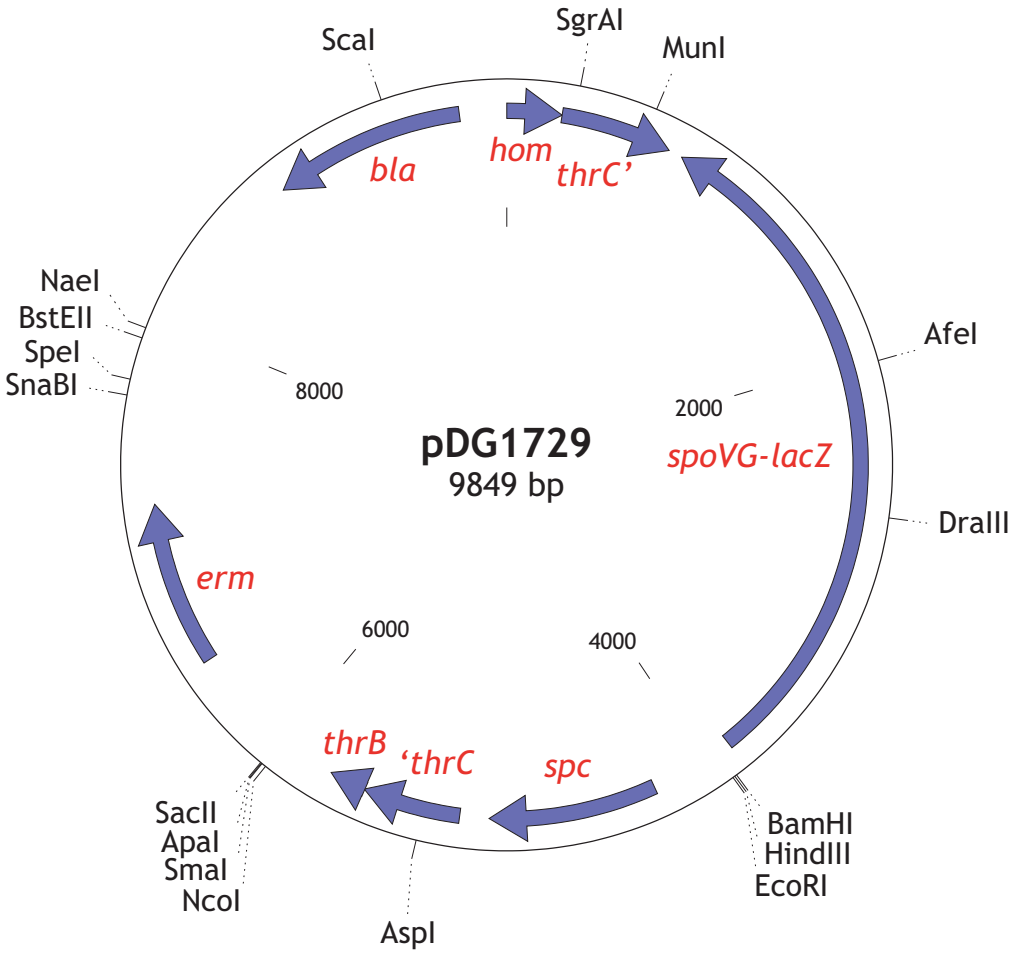
Construction: Composite of *hom-thrC* fragment from pSU11, hybrid *lacZ* fragment from pJM73, multiple cloning site from pDG268, *thrC-thrB* fragment from pSU11, multiple cloning site from pJRD184, *spc* cassette from pIC215, an origin-containing fragment from pSU11, and a *bla* fragment from pBR322.

Use: The plasmid is designed to integrate a cloned fragment into the *Bacillus subtilis* 168 chromosome at the *thrC* locus. The user inserts the promoter-containing fragment of interest into the multiple cloning site. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for erythromycin-lincomycin resistance. Transformants are screened for sensitivity to chloramphenicol (indicating that the resident *thrC* locus has been replaced) and for sensitivity to spectinomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A773, with a *cat*-insertion in its chromosomal *thrC* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation.

Integration Vector Maps (*continued*)



BGSC Accession: ECE118 **Orignal Code:** TG1(pDG1729)

Reference: **Guérout-Fleury, A. M., N. Frandsen, P. Stragier**. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

Sequence: GenBank U46202

Features:

thrC'...'thrC	5' and 3' segments of the *B. subitlis* 168 *thrC* gene
'*hom*	3' end of the *B. subtilis* 168 *hom* gene (part of the *thrC* operon)
thrB'	5' end of the *B. subtilis* 168 *thrB* gene (part of the *thrB* operon)
spoVG-lacZ	*E. coli lacZ* coding sequence, fused to the ribosome binding site of *B. subtilis spoVG*.
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin100 μg/ml)
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.5 μg/ml, lincomycin 12.5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)

Description: Promotes ectopic integration into the *B. subtilis thrC* locus; allows for fusions with a β-galactosidase reporter.

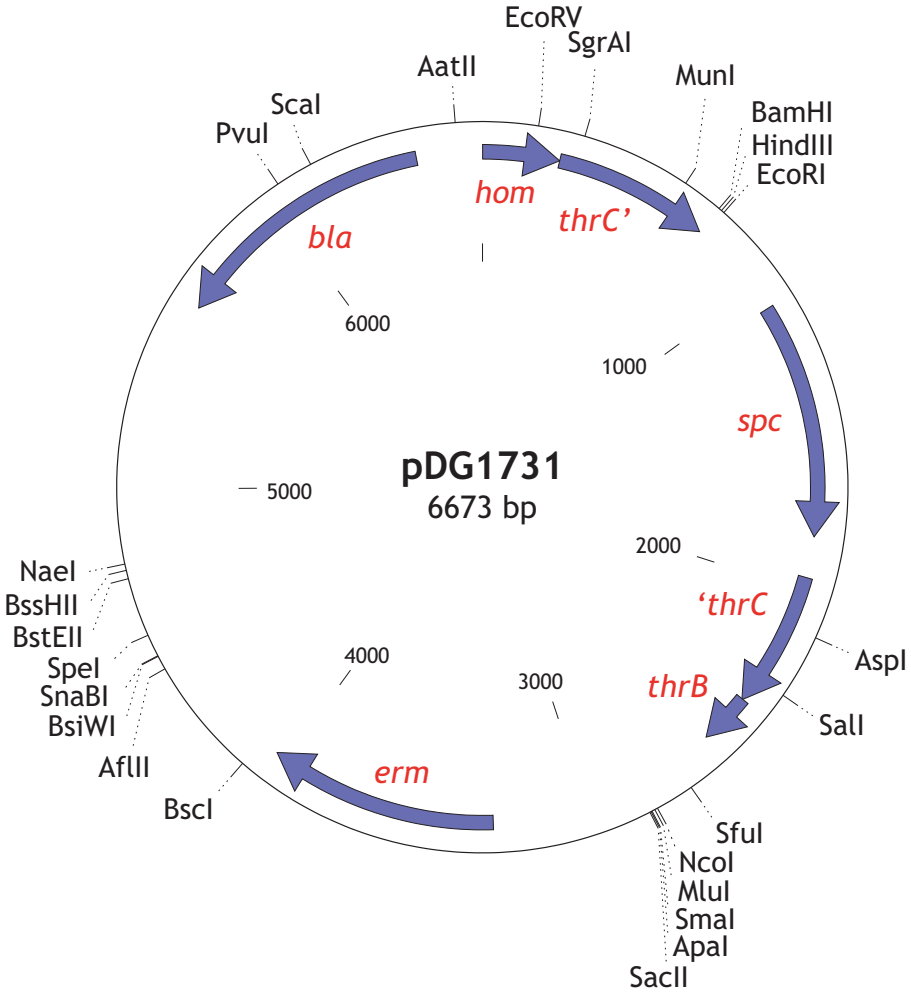
Construction: Similar to pDG1663, except that the *spc* and *erm* cassettes are switched in location.

Use: The plasmid is designed to integrate a cloned fragment into the *Bacillus subtilis* 168 chromosome at the *thrC* locus. The user inserts the promoter-containing fragment of interest into the multiple cloning site. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for spectinomycin resistance. Transformants are screened for sensitivity to chloramphenicol (indicating that the resident *thrC* locus has been replaced) and for sensitivity to erythromycin-lincomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A773, with a *cat*-insertion in its chromosomal *thrC* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation.

Integration Vector Maps (*continued*)



BGSC Accession: ECE119 **Orignal Code:** TG1(pDG1731

Reference: **Guérout-Fleury, A. M., N. Frandsen, P. Stragier**. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57-61.

Sequence: GenBank U46203

Features:

thrC'...'thrC	5' and 3' segments of the *B. subitlis* 168 *thrC* gene
'hom	3' end of the *B. subtilis* 168 *hom* gene (part of the *thrC* operon)
thrB'	5' end of the *B. subtilis* 168 *thrB* gene (part of the *thrB* operon)
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin100 μg/ml)
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.5 μg/ml, lincomycin 12.5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)

Description: Promotes ectopic integration into the *B. subtilis thrC* locus.

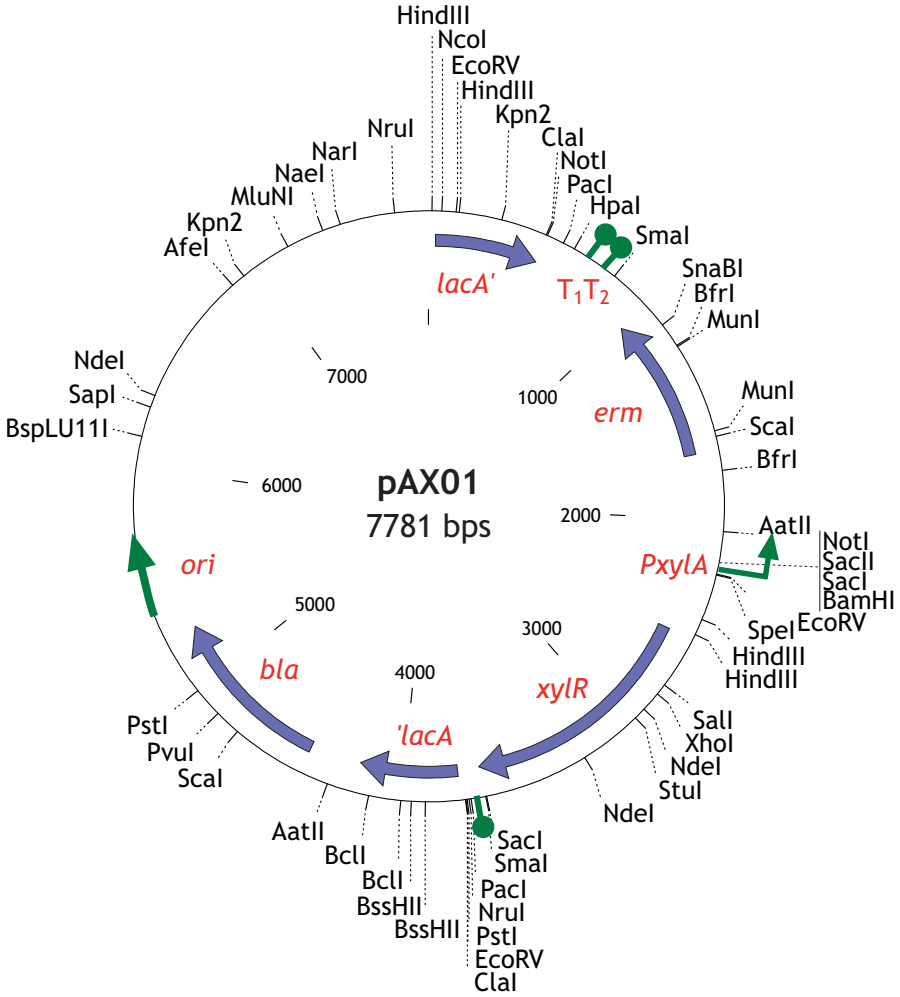
Construction: Similar to pDG1664, except that the *spc* and *erm* cassettes have been switch in their location

Use: The plasmid is designed to integrate a cloned fragment into the *Bacillus subtilis* 168 chromosome at the *thrC* locus. The user inserts the promoter-containing fragment of interest into the multiple cloning site. The plasmid is transformed into a special *B. subtilis* host (see below), with selection for spectinomycin resistance. Transformants are screened for sensitivity to chloramphenicol (indicating that the resident *thrC* locus has been replaced) and for sensitivity to erythromycin-lincomycin (indicating that a double-crossover recombination has occurred, rather than a Campbell-type insertion).

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, 1A773, with a *cat*-insertion in its chromosomal *thrC* locus, allows for rapid screening for marker replacements.

Protocols: *B. subtilis* competent cell preparation and transformation.

Integration Vector Maps (*continued*)



BGSC Accession: ECE137 **Orignal Code:** JM109(pAX01)

Reference: Härtl, B., *et al*. 2001. Development of a New Integration Site within the *Bacillus subtilis* Chromosome and Construction of Compatible Expression Cassettes. J. Bacteriol. 183:2696-2700.

Sequence: Not in database; available at http://www.genetik.uni-bayreuth.de/LSGenetik1/schumann_pax01.htm

Features:

lacA'-'lacA	end fragments of the *Bacillus subtilis* 168 chromosomal *lacA* (β-galactosidase) gene
erm	encodes adenine methylase; selectable in single copy in *B. subtilis* (erythromycin 1 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
xylR	*xyl* operon repressor from *B. subtilis*.
P_{xylA}	Promoter for the *xylA* gene; induced by xylose through the *PxylA* repressor.

Description: Ectopic integration vector; cassette integrates by double recombination between plasmid and chromosomal *lacA* sequences.

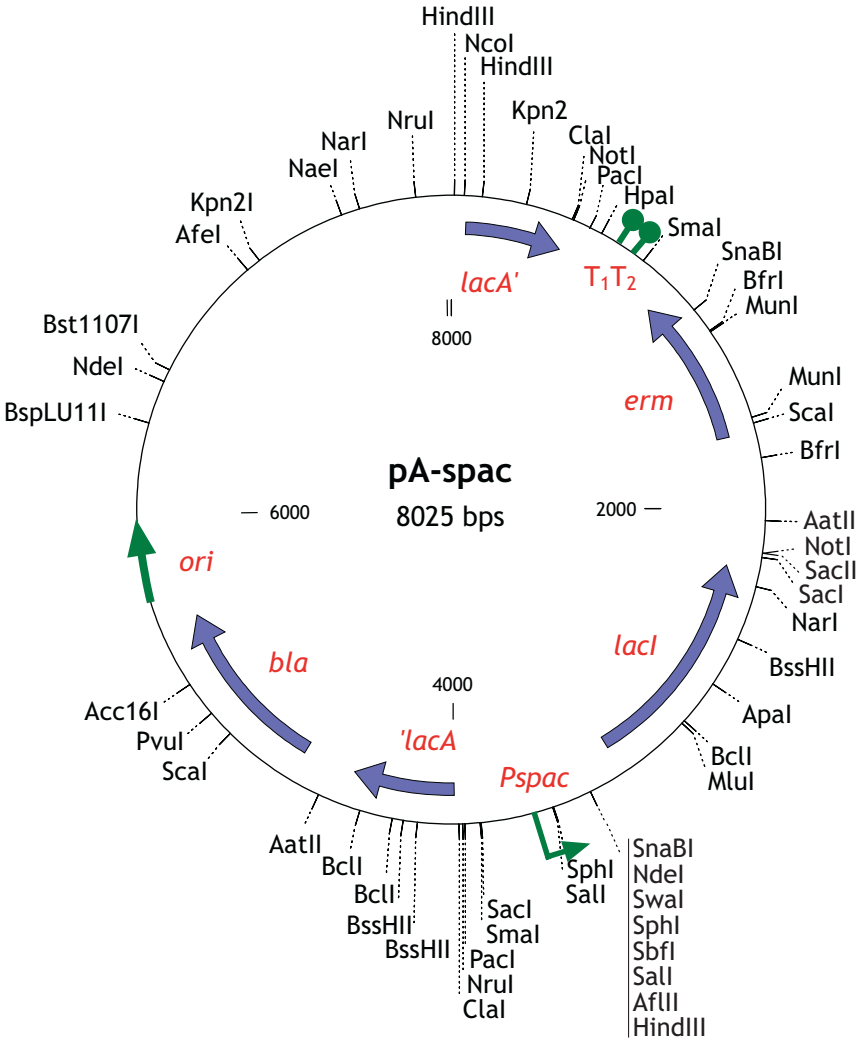
Construction: The *xylR*-P_{xylA} cassette was amplified from plasmid pX and inserted into a ColE1-*bla* backbone; the T1 and T2 transcription terminators, amplified from bacteriophage λ, and the *erm* gene, amplified from pMUTIN4, were inserted into the construct.

Use: pAX01 places a cloned gene under the control of a xylose-inducible expression system, then delivers the construct to the *B. subtilis lacA* locus on the chromosome. In practice, a gene of interest is inserted into the pAX01 multiple clonining site. The construct is used to transform the *B. subtilis* host to erythromycin resistance.

Recipient strains: pAX01 should work with any strain derived from *B. subtilis* 168. If strains IHA01 (1A785) or IHA02 (1A786) are used, the erythromycin-resistant transformats can be screened for loss of spectinomycin resistance to insure that the cassette has integrated into the *lacA* locus.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE138 **Orignal Code:** JM109(pA-spac)

Reference: **Härtl, B., et al.** 2001. Development of a New Integration Site within the *Bacillus subtilis* Chromosome and Construction of Compatible Expression Cassettes. J. Bacteriol. **183:**2696-2700.

Sequence: Not in database; available at http://www.genetik.uni-bayreuth.de/LSGenetik1/schumann_paspac.htm

Features:

lacA'-'lacA	end fragments of the *Bacillus subtilis* 168 chromosomal *lacA* (β-galactosidase) gene
erm	encodes adenine methylase; selectable in single copy in *B. subtilis* (erythromycin 1 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
lacI	*lac* operon repressor from *E. coli*, engineered for expression in Gram-positive bacteria.
P_{spac}	Hybrid promoter; regulated by the LacI repressor, inducible by IPTG.

Description: Ectopic integration vector; cassette integrates by double recombination between plasmid and chromosomal *lacA* sequences.

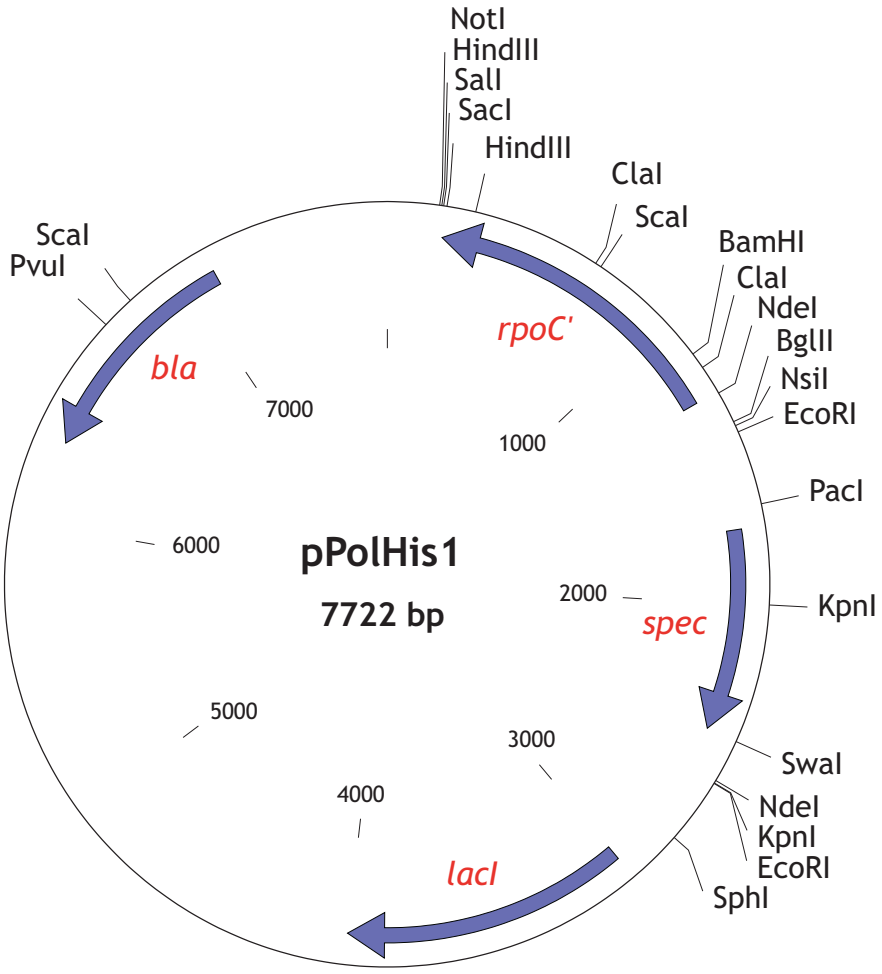
Construction: The *lacI-*P_{spac} cassette was amplified from plasmid pDR66 and inserted into SacI-linearized pAX01.

Use: pA-spac places a cloned gene under the control of an IPTG-inducible expression system, then delivers the construct to the *B. subtilis lacA* locus on the chromosome. In practice, a gene of interest is inserted into the pA-spac multiple clonining site. The construct is used to transform the *B. subtilis* host to erythromycin resistance.

Recipient strains: pA-Spac should work with any strain derived from *B. subtilis* 168. If strains IHA01 (1A785) or IHA02 (1A786) are used, the erythromycin-resistant transformats can be screened for loss of spectinomycin resistance to insure that the cassette has integrated into the *lacA* locus.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE120 **Orignal Code:** DH5α(pPolHis1)

Reference: **Wade, K. H., G. Schyns, J. A. Opdyke, and C. P. Moran, Jr.** 1999. A Region of σ^K Involved in Promoter Activation by GerE in *Bacillus subtilis*. J. Bacteriol. **181:**4365-4373.

Sequence: Not in database; available in hard copy form from BGSC.

Features:

rpoC'	the 3' end of the *B. subtilis rpoC* gene, encoding the 342-amino acid carboxy terminus of the β' subunit of RNA polymerase, fused to an in-frame coding sequence creating a C-terminal His_6 extension to the subunit.
lacI	*E. coli lacI*, encoding the Lac repressor. Not relevant for the use of this plasmid.
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 50 μg/ml)
spec	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 50 μg/ml)

Description: Integrates into the chromsomal *rpoC* locus of *B. subtilis* 168-derivatives by Campbell-type insertion, altering the gene to encode a His-tagged β' subunit for ease of RNA polymerase purification.

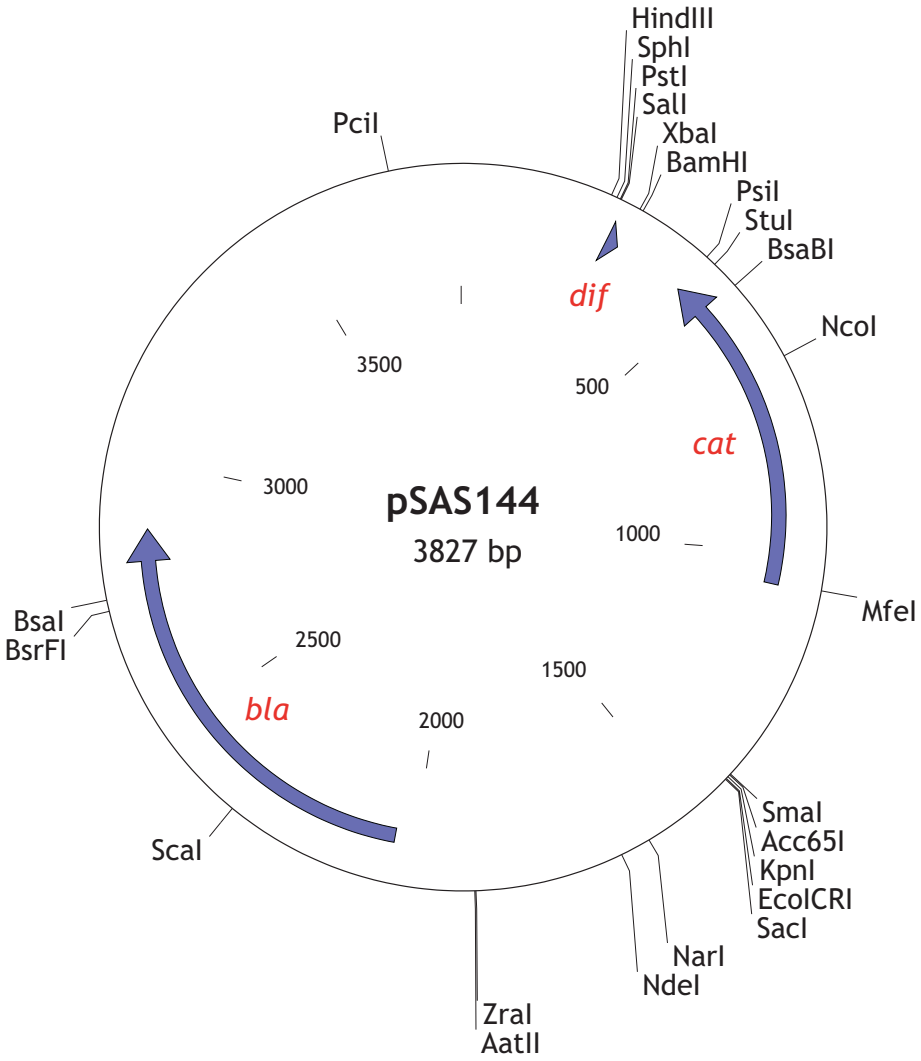
Construction: A 1.2 kb spectinomycin resistance cassette from pAH256 was inserted into the BglII site of pET21b and its orientation reversed by PstI cutting and religation. An amplified fragment containing the last 342 codons of *rpoC* was then inserted between the NdeI and SacI sites of the construct. The amplification primers created a 3' histidine tag as well.

Use: The plasmid is transformed into a competent *B. subtilis* 168 derivative of choice with selection for spectinomycin resistance. The user can then isolate RNA polymerase easily with a single step purification on a nidkel resin column.

Recipient strains: Any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient.

Protocols: *B. subtilis* competent cell preparation and transformation; purification of His-tagged RNA polymerase

Integration Vector Maps (*continued*)



BGSC Accession: ECE142 **Orignal Code:** DH5α(pSAS144)

Reference: **Sciochetti, S. A., P. J. Piggot, and G. W. Blakely.** 2001. Identification and Characterization of the *dif* Site from *Bacillus subtilis*. J. Bacteriol. **183:**1058–1068

Sequence: Not in database; available from BGSC at http://bacillus.biosci.ohio-state.edu/sequences/pSAS144.htm

Features:

dif	*Bacillus subtilis* site-specific recombination target for resolving chromosome dimers
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)

Description: Integration vector; integrates by site-specific recombination between plasmid and chromosomal *dif* sequences via the host RipX-CodV system.

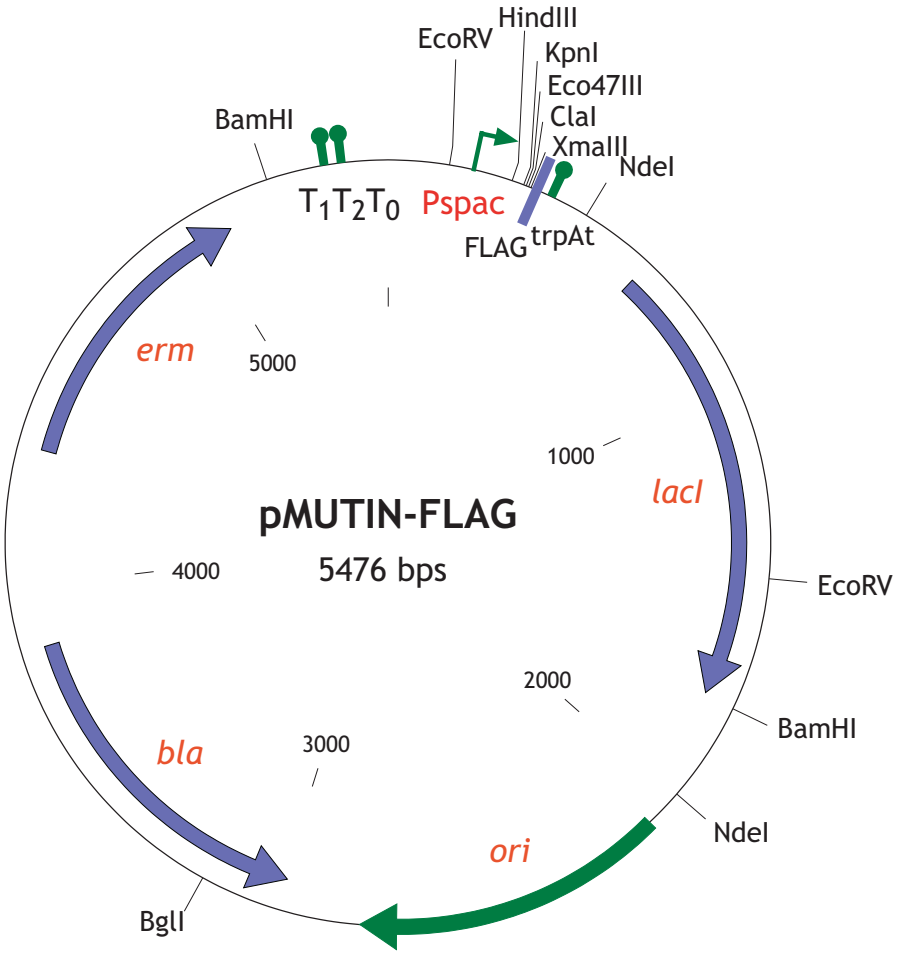
Construction: A 28 bp synthetic *dif* site was inserted between the pUC19 SalI and XbaI sites. A pDH32 NaeI-SmaI digestion fragment, containing *cat*, was inserted into the SmaI site of the resulting plasmid to produce pSAS144.

Use: pSAS144 integrates into *Bacillus subtilis* 168 at 166° of the chromosome, between bp 1941798 and 1941825 of the genomic sequence. Integration is *recA*-independent, instead making use of the host's system for resolving chromosome dimers via site-specific recombinases. Users simply insert a fragment of interest into one of the many available restriction sites. Transformation of competent *B. subtilis* with selection for chloramphenicol resistance recovers integrants.

Recipient strains: Any RipX+ CodV+ *B. subtilis* 168 derivative--even *recA* mutants--will serve as a recipient. It is not known if other Gram-positive bacteria may substitute, although it would be interesting to try.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE146 **Orignal Code:** DH5α(pMUTIN-FLAG)

Reference: **Kaltwasser, M., T. Wiegert, and W. Schumann.** 2002. Construction and Application of Epitope- and Green Fluorescent Protein-Tagging Integration Vectors for *Bacillus subtilis*. Appl. Environ. Microbiol **68:**2624-2628

Sequence: not available in databases; can be downloaded from the BGSC or from the Schumann web site at http://btbgn1.bio.uni-bayreuth.de/lsgenetik1/frames.htm.

Features:

FLAG	tagging sequence encoding the FLAG epitope
Pspac	hybrid promoter, inducible by IPTG
lacI	encodes *lac* repressor, with modified ribosome binding site for Gram-positive expression
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.3 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori	ColE1 origin of replication
trpAt, T_1T_1, T_0	transcription terminators

Description: Integration vector designed to tag genes with the artifical peptide sequence FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). Anti-FLAG antibodies are available from several commercial sources.

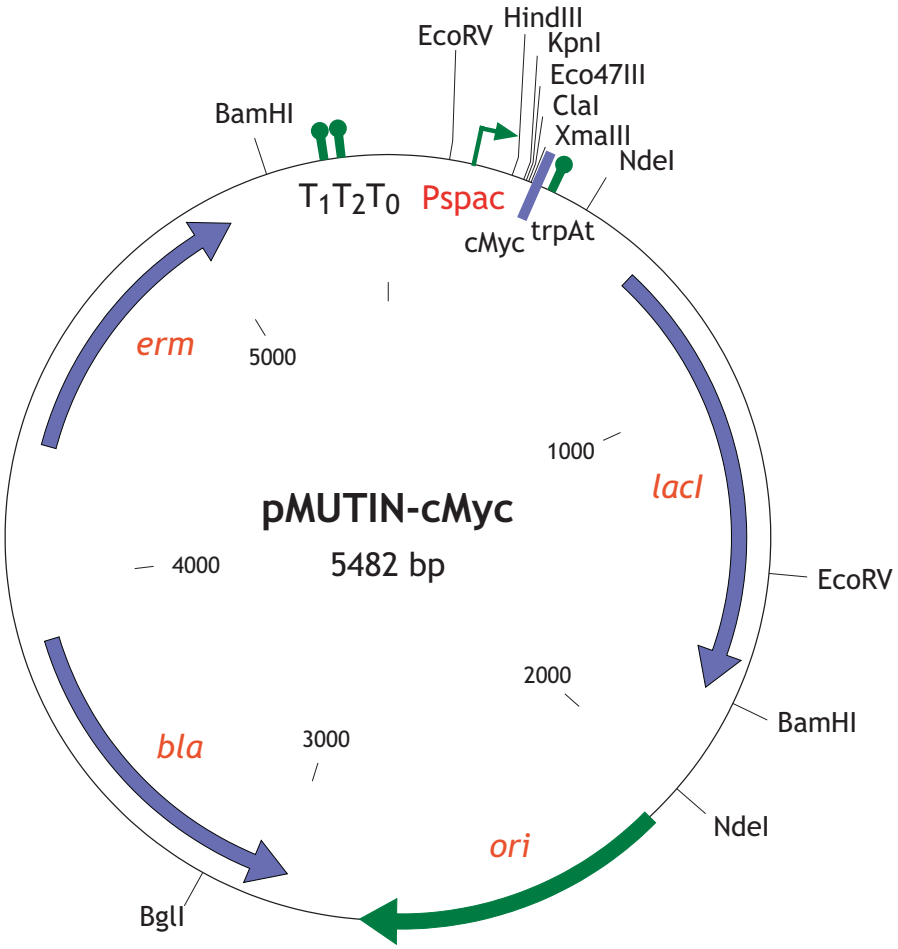
Construction: The pMUTIN2 precursor, pDE01, was altered to replace the *bgaB* reporter with a polylinker. The trpAt terminator was inserted downstream from the linker. An oligonucleotide coding for FLAG, using the optimum codons for *B. subtilis*, was then inserted between the polylinker and the terminator.

Use: A gene of interest is isolated from a gram-positive organisms. Its complete coding sequence is inserted into the polylinker in-frame with the FLAG coding sequence to produce a fusion. Upon transformation back into the gram-positive organism, the construct should integrate into the chromsome by a single recombiation event between the cloned sequence and the chromosomal locus. The transformant should produce a tagged protein from its natural promoter. If the coding sequence is present in an operon, any downstream genes will be under the control of the IPTG-inducible Pspac promoter.

Recipient strains: Has been used in *B. subtilis*, but should perform well in any transformable Gram-positive host.

Protocols: *B. subtilis* competent cell preparation and transformation; β-galactosidase assay

Integration Vector Maps (*continued*)



BGSC Accession: ECE147 **Orignal Code:** DH5α(pMUTIN-cMyc)

Reference: **Kaltwasser, M., T. Wiegert, and W. Schumann.** 2002. Construction and Application of Epitope- and Green Fluorescent Protein-Tagging Integration Vectors for *Bacillus subtilis*. Appl. Environ. Microbiol **68:**2624-2628

Sequence: not available in databases; can be downloaded from the BGSC or from the Schumann web site at http://btbgn1.bio.uni-bayreuth.de/lsgenetik1/frames.htm.

Features:

cMyc	tagging sequence encoding the cMyc epitope
Pspac	hybrid promoter, inducible by IPTG
lacI	encodes *lac* repressor, with modified ribosome binding site for Gram-positive expression
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.3 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori	ColE1 origin of replication
trpAt, T_1T_1, T_0	transcription terminators

Description: Integration vector designed to tag genes with the human peptide sequence cMyc (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu). Atni-cMyc antibodies are available from several commercial sources.

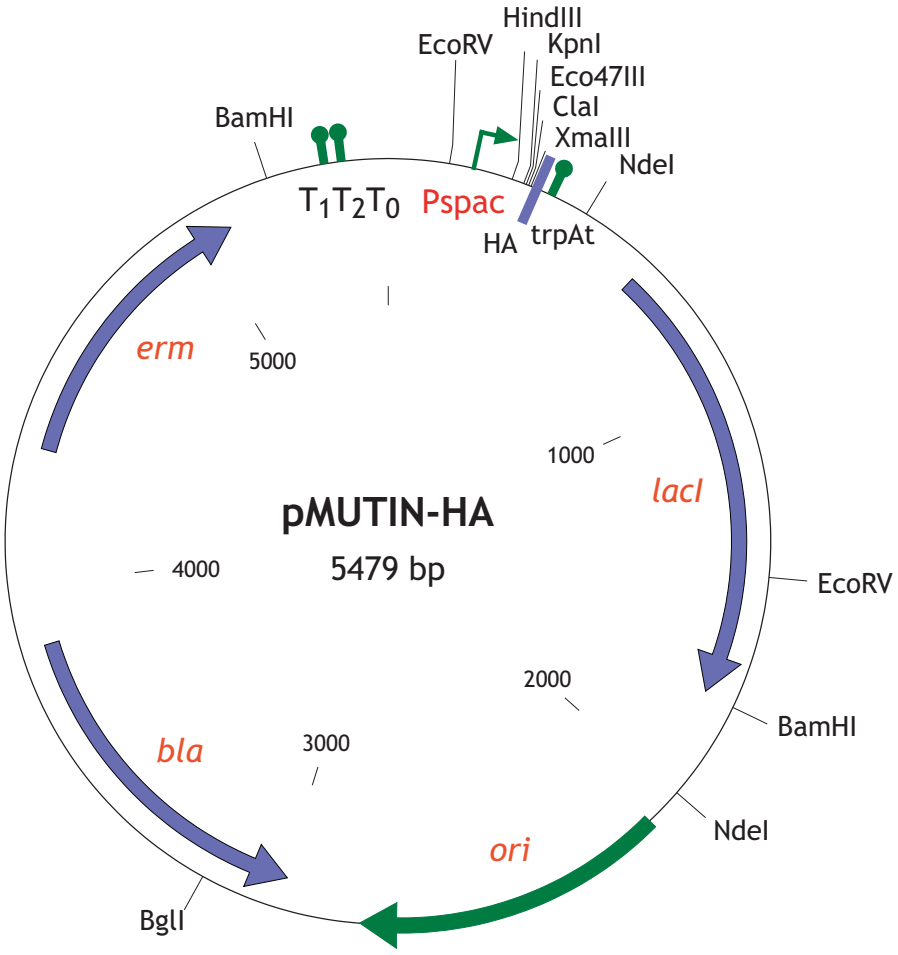
Construction: The pMUTIN2 precursor, pDE01, was altered to replace the *bgaB* reporter with a polylinker. The trpAt terminator was inserted downstream from the linker. An oligonucleotide coding for FLAG, using the optimum codons for *B. subtilis*, was then inserted between the polylinker and the terminator.

Use: A gene of interest is isolated from a gram-positive organisms. Its complete coding sequence is inserted into the polylinker in-frame with the cMyc coding sequence to produce a fusion. Upon transformation back into the gram-positive organism, the construct should integrate into the chromsome by a single recombiation event between the cloned sequence and the chromosomal locus. The transformant should produce a tagged protein from its natural promoter. If the coding sequence is present in an operon, any downstream genes will be under the control of the IPTG-inducible Pspac promoter.

Recipient strains: Has been used in *B. subtilis*, but should perform well in any transformable Gram-positive host.

Protocols: *B. subtilis* competent cell preparation and transformation; β-galactosidase assay

Integration Vector Maps (*continued*)



BGSC Accession: ECE148 **Orignal Code:** DH5α(pMUTIN-HA)

Reference: **Kaltwasser, M., T. Wiegert, and W. Schumann.** 2002. Construction and Application of Epitope- and Green Fluorescent Protein-Tagging Integration Vectors for *Bacillus subtilis*. Appl. Environ. Microbiol **68:**2624-2628

Sequence: not available in databases; can be downloaded from the BGSC or from the Schumann web site at http://btbgn1.bio.uni-bayreuth.de/lsgenetik1/frames.htm.

Features:

HA	tagging sequence encoding the HA epitope
Pspac	hybrid promoter, inducible by IPTG
lacI	encodes *lac* repressor, with modified ribosome binding site for Gram-positive expression
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.3 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori	ColE1 origin of replication
trpAt, T_1T_1, T_0	transcription terminators

Description: Integration vector designed to tag genes with the hemagglutinin peptide sequence HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala). Anti-HA antibodies are available from several commercial sources.

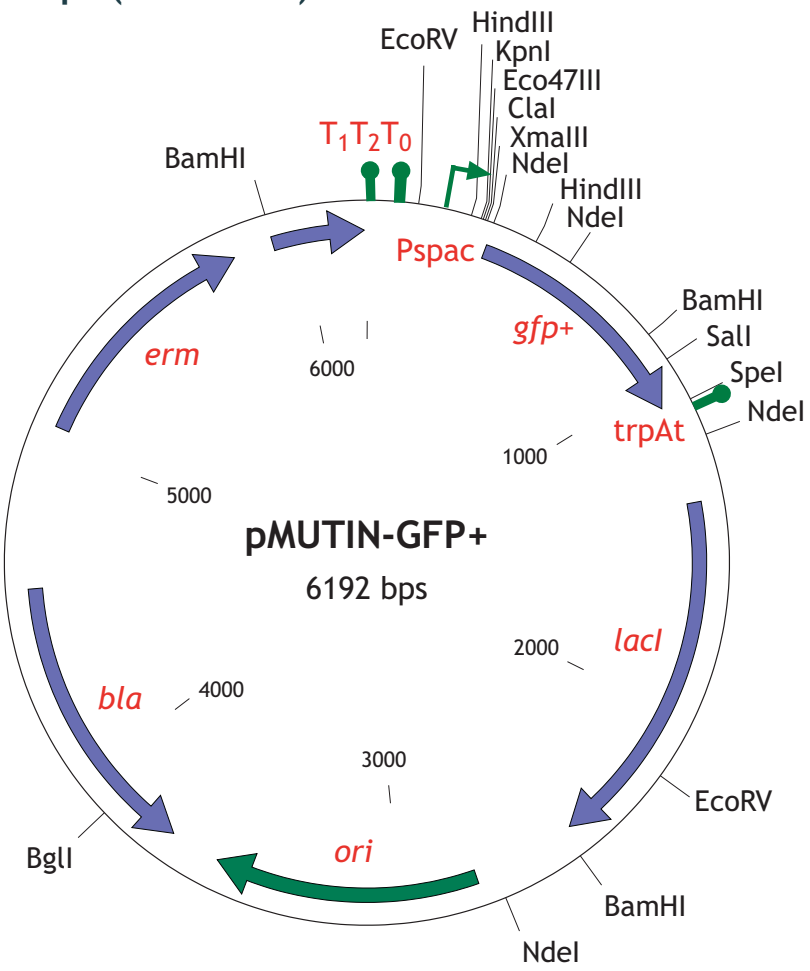
Construction: The pMUTIN2 precursor, pDE01, was altered to replace the *bgaB* reporter with a polylinker. The trpAt terminator was inserted downstream from the linker. An oligonucleotide coding for HA, using the optimum codons for *B. subtilis*, was then inserted between the polylinker and the terminator.

Use: A gene of interest is isolated from a gram-positive organisms. Its complete coding sequence is inserted into the polylinker in-frame with the HA coding sequence to produce a fusion. Upon transformation back into the gram-positive organism, the construct should integrate into the chromsome by a single recombiation event between the cloned sequence and the chromosomal locus. The transformant should produce a tagged protein from its natural promoter. If the coding sequence is present in an operon, any downstream genes will be under the control of the IPTG-inducible Pspac promoter.

Recipient strains: Has been used in *B. subtilis*, but should perform well in any transformable Gram-positive host.

Protocols: *B. subtilis* competent cell preparation and transformation; β-galactosidase assay

Integration Vector Maps (*continued*)



BGSC Accession: ECE149 **Orignal Code:** DH5α(pMUTIN-GFP+)

Reference: **Kaltwasser, M., T. Wiegert, and W. Schumann.** 2002. Construction and Application of Epitope- and Green Fluorescent Protein-Tagging Integration Vectors for *Bacillus subtilis*. Appl. Environ. Microbiol **68:**2624-2628

Sequence: not available in databases; can be downloaded from the BGSC or from the Schumann web site at http://btbgn1.bio.uni-bayreuth.de/lsgenetik1/frames.htm.

Features:

gfp+	encodes Green Fluorescent Protein variant with increased fluorescence
Pspac	hybrid promoter, inducible by IPTG
lacI	encodes *lac* repressor, with modified ribosome binding site for Gram-positive expression
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.3μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori	ColE1 origin of replication
trpAt, T_1T_1, T_0	transcription terminators

Description: Integration vector designed to fuse proteins with a high-fluorescing variant of GFP.

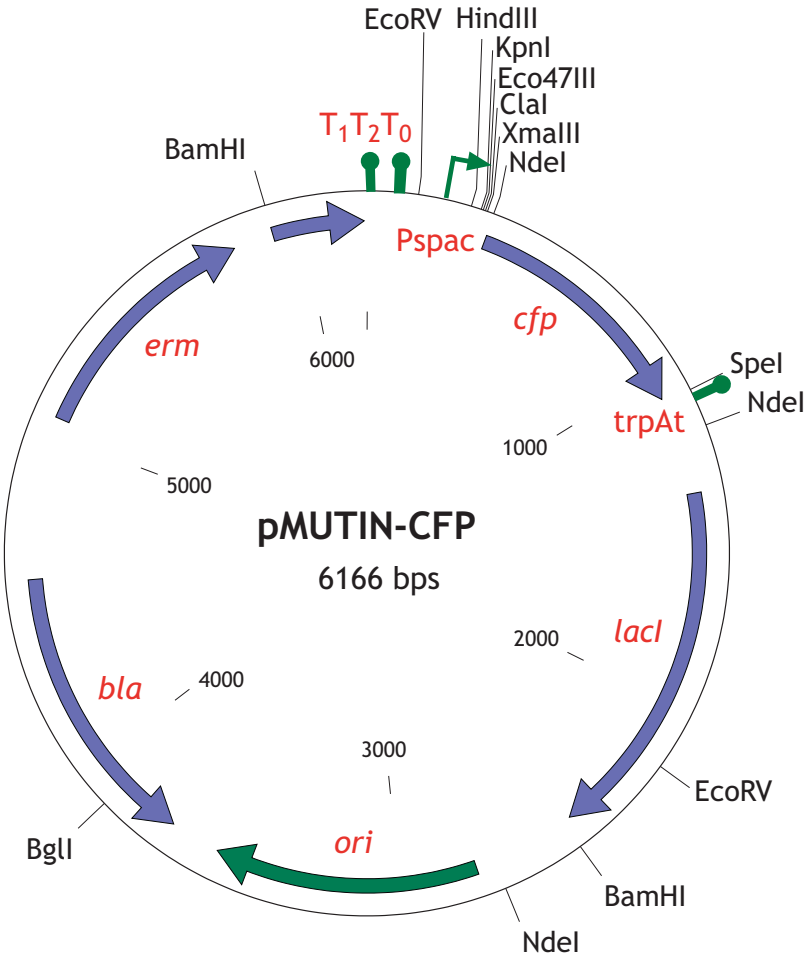
Construction: The pMUTIN2 precursor, pDE01, was altered to replace the *bgaB* reporter with a polylinker. The trpAt terminator was inserted downstream from the linker. The *gfp*+ coding sequence, amplifed by PCR from pMN402, was then inserted between the polylinker and the terminator.

Use: A gene of interest is isolated from a gram-positive organisms. Its complete coding sequence is inserted into the polylinker in-frame with the *gfp*+ coding sequence to produce a fusion. Upon transformation back into the gram-positive organism, the construct should integrate into the chromsome by a single recombiation event between the cloned sequence and the chromosomal locus. The transformant should produce a GFP fusion protein from its natural promoter. The protein can be localized in a living cell via fluorescence microscopy. If the sequence is present in an operon, any downstream genes will be under the control of the IPTG-inducible Pspac promoter.

Recipient strains: Has been used in *B. subtilis*, but should perform well in any transformable Gram-positive host.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE150 **Orignal Code:** DH5α(pMUTIN-CFP)

Reference: **Kaltwasser, M., T. Wiegert, and W. Schumann.** 2002. Construction and Application of Epitope- and Green Fluorescent Protein-Tagging Integration Vectors for *Bacillus subtilis*. Appl. Environ. Microbiol **68:**2624-2628

Sequence: not available in databases; can be downloaded from the BGSC or from the Schumann web site at http://btbgn1.bio.uni-bayreuth.de/lsgenetik1/frames.htm.

Features:

cfp	encodes Cyan Fluorescent Protein
Pspac	hybrid promoter, inducible by IPTG
lacI	encodes *lac* repressor, with modified ribosome binding site for Gram-positive expression
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.3 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori	ColE1 origin of replication
trpAt, T_1T_1, T_0	transcription terminators

Description: Integration vector designed to fuse proteins with a CFP, the cyan-fluorescing variant of GFP.

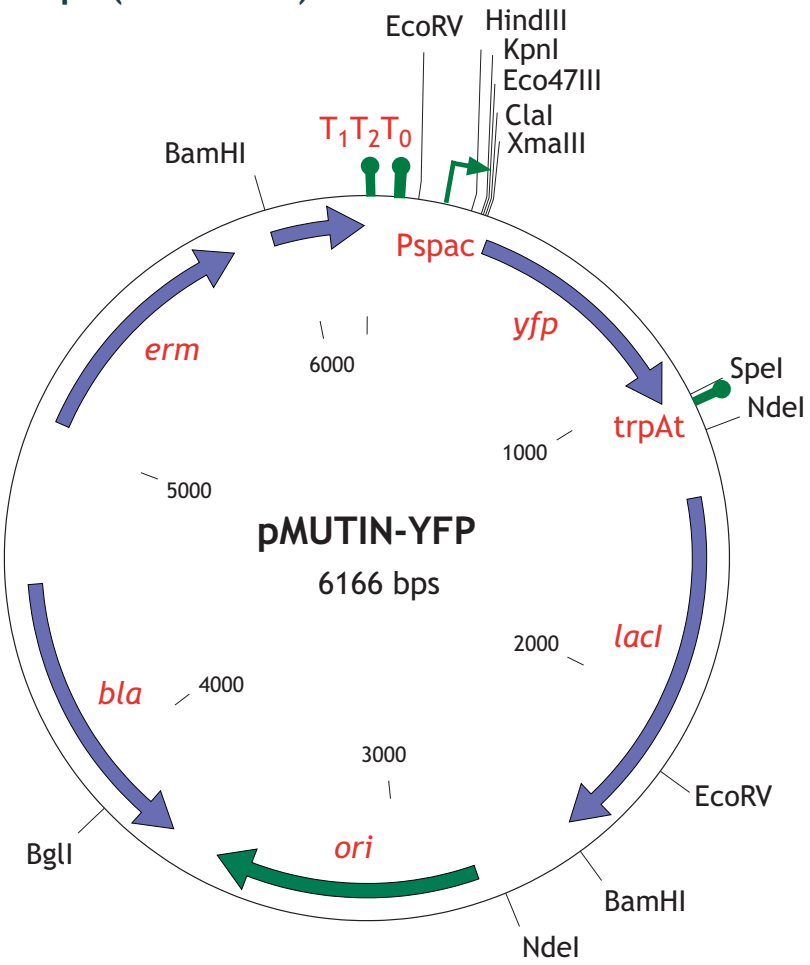
Construction: The pMUTIN2 precursor, pDE01, was altered to replace the *bgaB* reporter with a polylinker. The trpAt terminator was inserted downstream from the linker. The *cfp* coding sequence, amplified by PCR from pSG1186, was then inserted between the polylinker and the terminator.

Use: A gene of interest is isolated from a gram-positive organisms. Its complete coding sequence is inserted into the polylinker in-frame with the *cfp* coding sequence to produce a fusion. Upon transformation back into the gram-positive organism, the construct should integrate into the chromsome by a single recombiation event between the cloned sequence and the chromosomal locus. The transformant should produce a CFP fusion protein from its natural promoter. The protein can be localized in a living cell via fluorescence microscopy. If the sequence is present in an operon, any downstream genes will be under the control of the IPTG-inducible Pspac promoter.

Recipient strains: Has been used in *B. subtilis*, but should perform well in any transformable Gram-positive host.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE151 **Orignal Code:** DH5α(pMUTIN-YFP)

Reference: **Kaltwasser, M., T. Wiegert, and W. Schumann.** 2002. Construction and Application of Epitope- and Green Fluorescent Protein-Tagging Integration Vectors for *Bacillus subtilis*. Appl. Environ. Microbiol **68:**2624-2628

Sequence: not available in databases; can be downloaded from the BGSC or from the Schumann web site at http://btbgn1.bio.uni-bayreuth.de/lsgenetik1/frames.htm.

Features:

yfp	encodes Yellow Fluorescent Protein
Pspac	hybrid promoter, inducible by IPTG
lacI	encodes *lac* repressor, with modified ribosome binding site for Gram-positive expression
erm	encodes rRNA adenine N-6-methyltransferase; selectable in *B. subtilis* (erythromycin 0.3μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori	ColE1 origin of replication
trpAt, T_1T_1, T_0	transcription terminators

Description: Integration vector designed to fuse proteins with a YFP, the yellow-fluorescing variant of GFP.

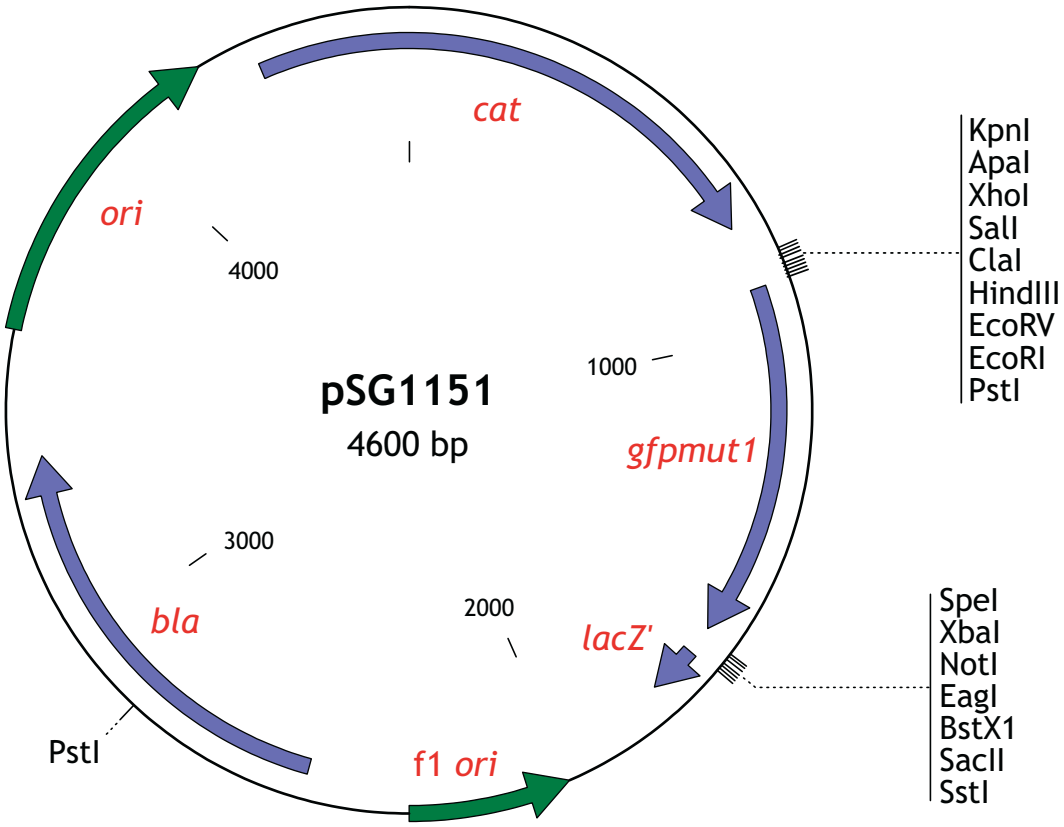
Construction: The pMUTIN2 precursor, pDE01, was altered to replace the *bgaB* reporter with a polylinker. The trpAt terminator was inserted downstream from the linker. The *yfp* coding sequence, amplified by PCR from pSG1187, was then inserted between the polylinker and the terminator.

Use: A gene of interest is isolated from a gram-positive organisms. Its complete coding sequence is inserted into the polylinker in-frame with the *yfp* coding sequence to produce a fusion. Upon transformation back into the gram-positive organism, the construct should integrate into the chromsome by a single recombiation event between the cloned sequence and the chromosomal locus. The transformant should produce a YFP fusion protein from its natural promoter. The protein can be localized in a living cell via fluorescence microscopy. If the sequence is present in an operon, any downstream genes will be under the control of the IPTG-inducible Pspac promoter.

Recipient strains: Has been used in *B. subtilis*, but should perform well in any transformable Gram-positive host.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE157 **Orignal Code:** DH5α(pSG1151)

Reference: **Feucht, A. and P. J. Lewis.** 2001. Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264:**289-297.

Sequence: Not in database; not available from BGSC

Features:

gfpmut1 encodes red-shifted, highly fluorescent variant of GFP (single excitation peak at 488 nm, emits green light maximally at 507 nm).
cat encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1 ColE1 origin of replication
ori-f1 F1 origin of replication

Description: Integration vector designed to fuse proteins with Cyan Fluorescent Protein.

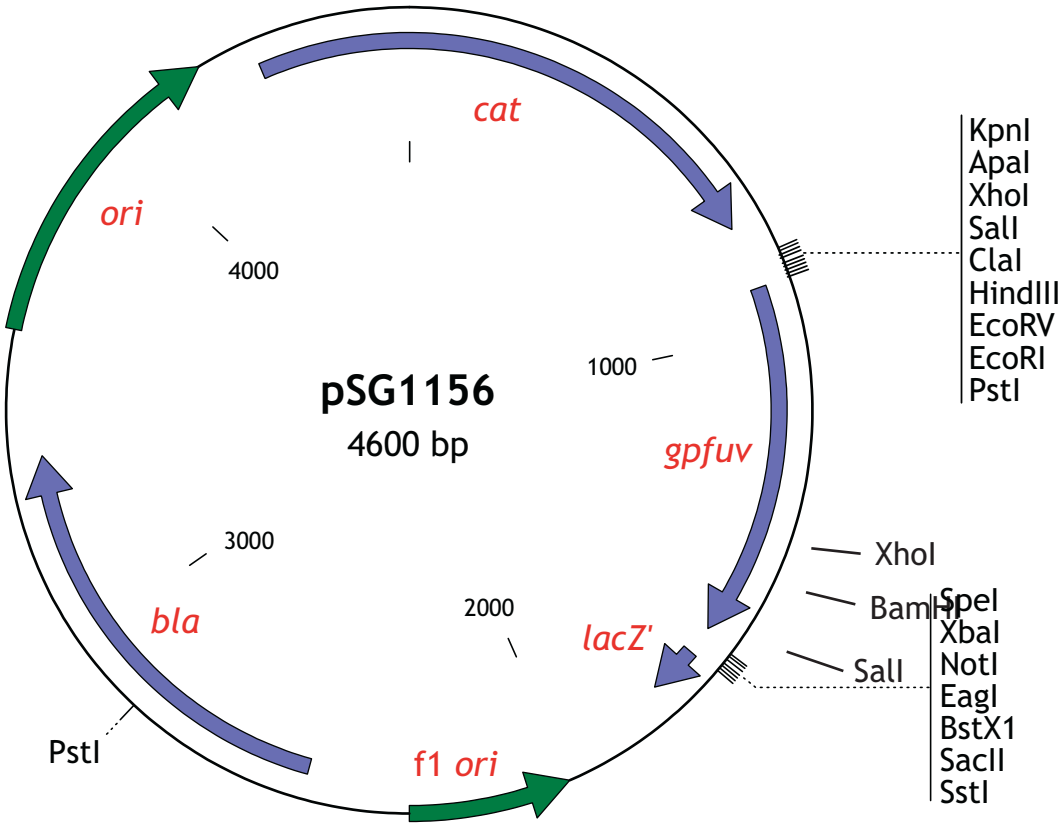
Construction: The *gfpmut1* coding sequence was amplified from pGFPmut1 (Clontech) to create BglII and EcoRI sites at the ends, then ligated to pSG1301 (as detailed by Lewis, P.J., and J. Errington. 1996. Microbiology 142:733-740) to give pSG1151.

Use: The C-terminal fragment of an ORF from a gram-positive bacterium is inserted in-frame with *gfpmut1*. Transformation back into the gram-positive with selection for chloramphenicol resistance recovers integration mutants in which the target gene is fused to *gfpmut1* and expression is driven from the natural promoter.

Recipient strains: pSG1151 should work with any transformable bacterial species that can express *cat* but not support replication of the ColE1 origin. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE152 **Orignal Code:** DH5α(pSG1156)

Reference: **Lewis, P. J. and A. L. Marston.** 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene **227:**101–109.

Sequence: Not in database; not available from BGSC

Features:

gfpuv	encodes GFP variant (single excitation peak at 395 nm, emits green light maximally at 507 nm).
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
ori-f1	F1 origin of replication

Description: Integration vector designed to fuse proteins with a high-fluorescing variant of GFP.

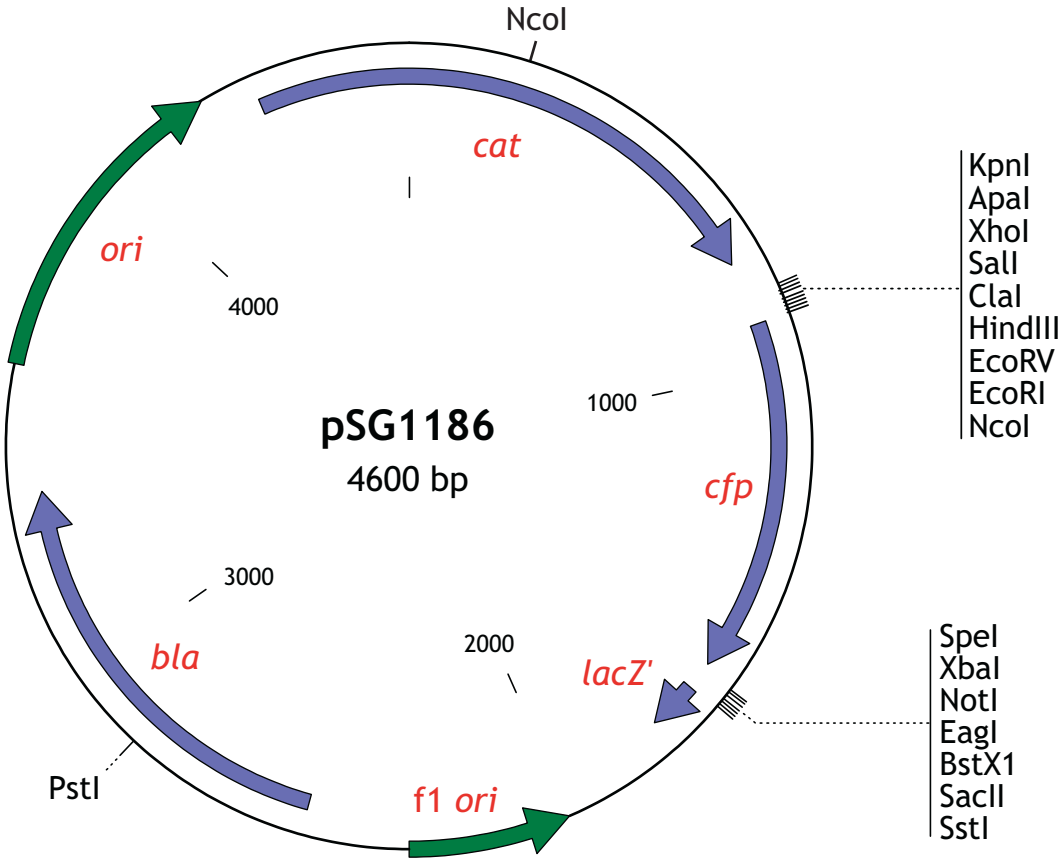
Construction: The coding sequence for *gfpuv* was PCR amplified from pGFPuv (Clontech) and inserted into pSG1301 (as detailed by Lewis, P.J., and J. Errington. 1996. Microbiology 142:733-740) to give pSG1156.

Use: The C-terminal fragment of an ORF from a gram-positive bacterium is inserted in-frame with *gfpuv*. Transformation back into the gram-positive with selection for chloramphenicol resistance recovers integration mutants in which the target gene is fused to *gfpuv* and expression is driven from the natural promoter.

Recipient strains: pSG1156 should work with any transformable bacterial species that can express *cat* but not support replication of the ColE1 origin. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE157 **Orignal Code:** DH5α(pSG1186)

Reference: **Feucht, A. and P. J. Lewis.** 2001. Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264:**289-297.

Sequence: Not in database; not available from BGSC

Features:

cfp	encodes Cyan Fluorescent Protein (single excitation peak at 434 nm, emits cyan light maximally at 477 nm).
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
ori-f1	F1 origin of replication

Description: Integration vector designed to fuse proteins with Cyan Fluorescent Protein.

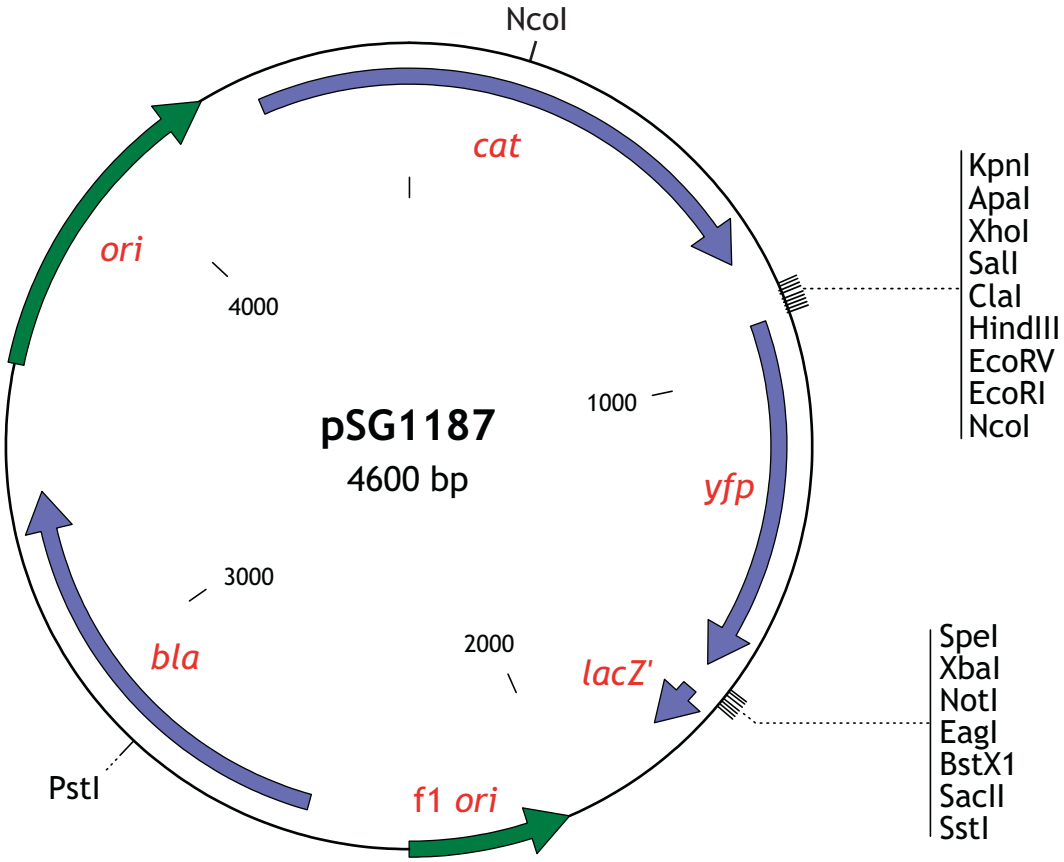
Construction: The *cfp* coding sequence was amplified from pECFP (Clontech) to create BglII and EcoRI sites at the ends, then ligated to pSG1301 (as detailed by Lewis, P.J., and J. Errington. 1996. Microbiology 142:733-740) to give pSG1186.

Use: The C-terminal fragment of an ORF from a gram-positive bacterium is inserted in-frame with *cfp*. Transformation back into the gram-positive with selection for chloramphenicol resistance recovers integration mutants in which the target gene is fused to *cfp* and expression is driven from the natural promoter.

Recipient strains: pSG1186 should work with any transformable bacterial species that can express *cat* but not support replication of the ColE1 origin. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE157 **Orignal Code:** DH5α(pSG1187)

Reference: **Feucht, A. and P. J. Lewis.** 2001. Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264:**289-297.

Sequence: Not in database; not available from BGSC

Features:

yfp	encodes Yellow Fluorescent Protein (single excitation peak at 514 nm, emits yellow light maximally at 527 nm).
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
ori-f1	F1 origin of replication

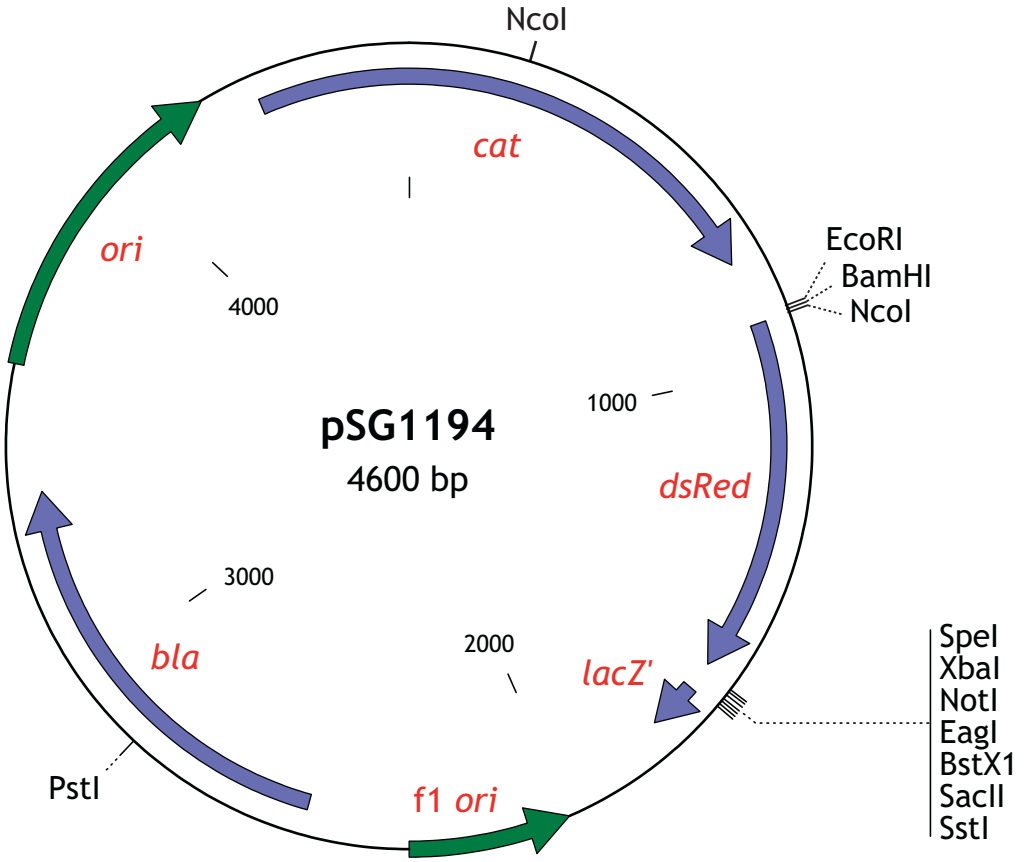
Description: Integration vector designed to fuse proteins with Yellow Fluorescent Protein.

Construction: The *yfp* coding sequence was amplified from pEYFP-C1 (Clontech) to create BglII and EcoRI sites at the ends, then ligated to pSG1301 (as detailed by Lewis, P.J., and J. Errington. 1996. Microbiology 142:733-740) to give pSG1186.

Use: The C-terminal fragment of an ORF from a gram-positive bacterium is inserted in-frame with *yfp*. Transformation back into the gram-positive with selection for chloramphenicol resistance recovers integration mutants in which the target gene is fused to *yfp* and expression is driven from the natural promoter.

Recipient strains: pSG1186 should work with any transformable bacterial species that can express *cat* but not support replication of the ColE1 origin. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation



BGSC Accession: ECE163 **Orignal Code:** DH5α(pSG1194)

Reference: **Feucht, A. and P. J. Lewis.** 2001. Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264:**289-297.

Sequence: Not in database; not available from BGSC

Features:

dsRed	encodes dsRed Fluorescent Protein (single excitation peak at 558 nm, emits red light maximally at 583 nm).
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
ori-f1	F1 origin of replication

Description: Integration vector designed to fuse proteins with dsRed Fluorescent Protein.

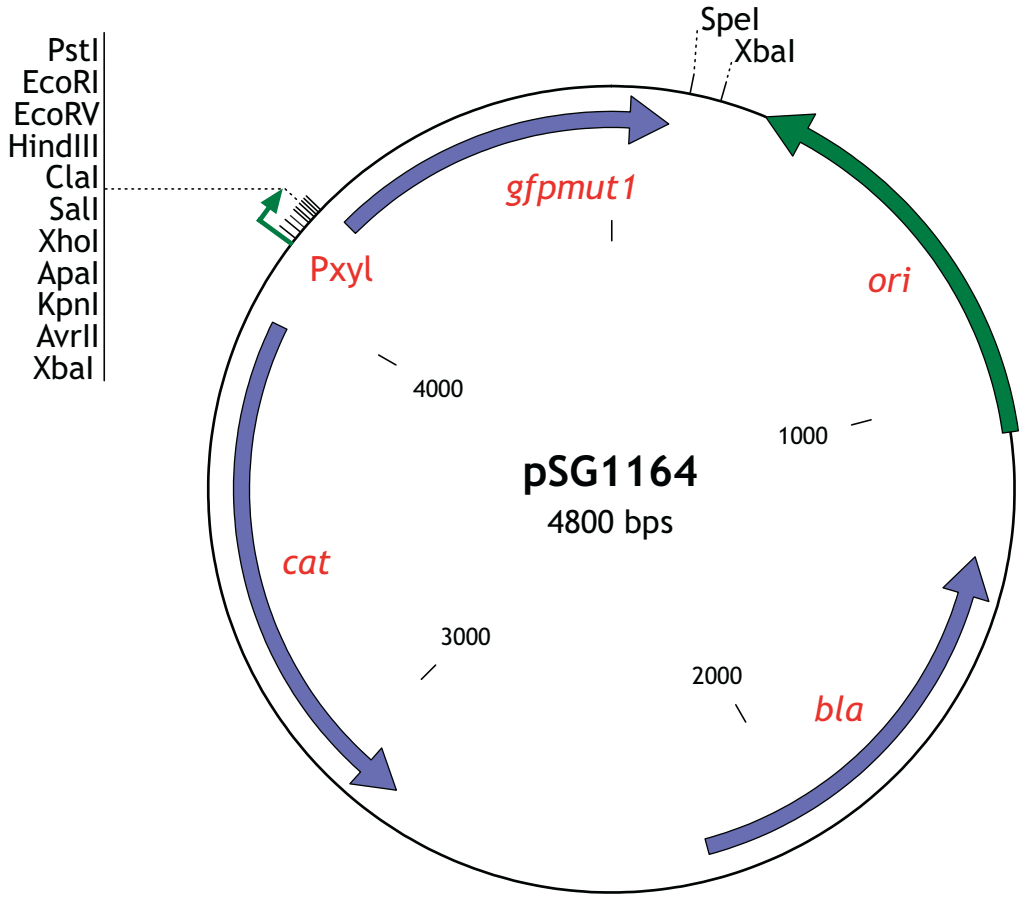
Construction: The *dsRed* coding sequence was amplified from pDsRed1-N1 (Clontech) to create BglII and EcoRI sites at the ends, then ligated to pSG1301 (as detailed by Lewis, P.J., and J. Errington. 1996. Microbiology 142:733-740) to give pSG1186.

Use: The C-terminal fragment of an ORF from a gram-positive bacterium is inserted in-frame with *dsRed*. Transformation back into the gram-positive with selection for chloramphenicol resistance recovers integration mutants in which the target gene is fused to *dsRed* and expression is driven from the natural promoter.

Recipient strains: pSG1186 should work with any transformable bacterial species that can express *cat* but not support replication of the ColE1 origin. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE155 **Orignal Code:** DH5α(pSG1164)

Reference: **Lewis, P. J. and A. L. Marston.** 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene **227:**101–109.

Sequence: Not in database; not available from BGSC

Features:

gfpmut-1	encodes highly fluorescent red-shifted GFP variant (single excitation peak at 488 nm, emits green light maximally at 507 nm).
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes b-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
Pxyl	xylose-inducible promoter

Description: Integration vector designed to fuse proteins with a high-fluorescing variant of GFP, placing any downstream genes in the operon under the control of a xylose-inducible promoter.

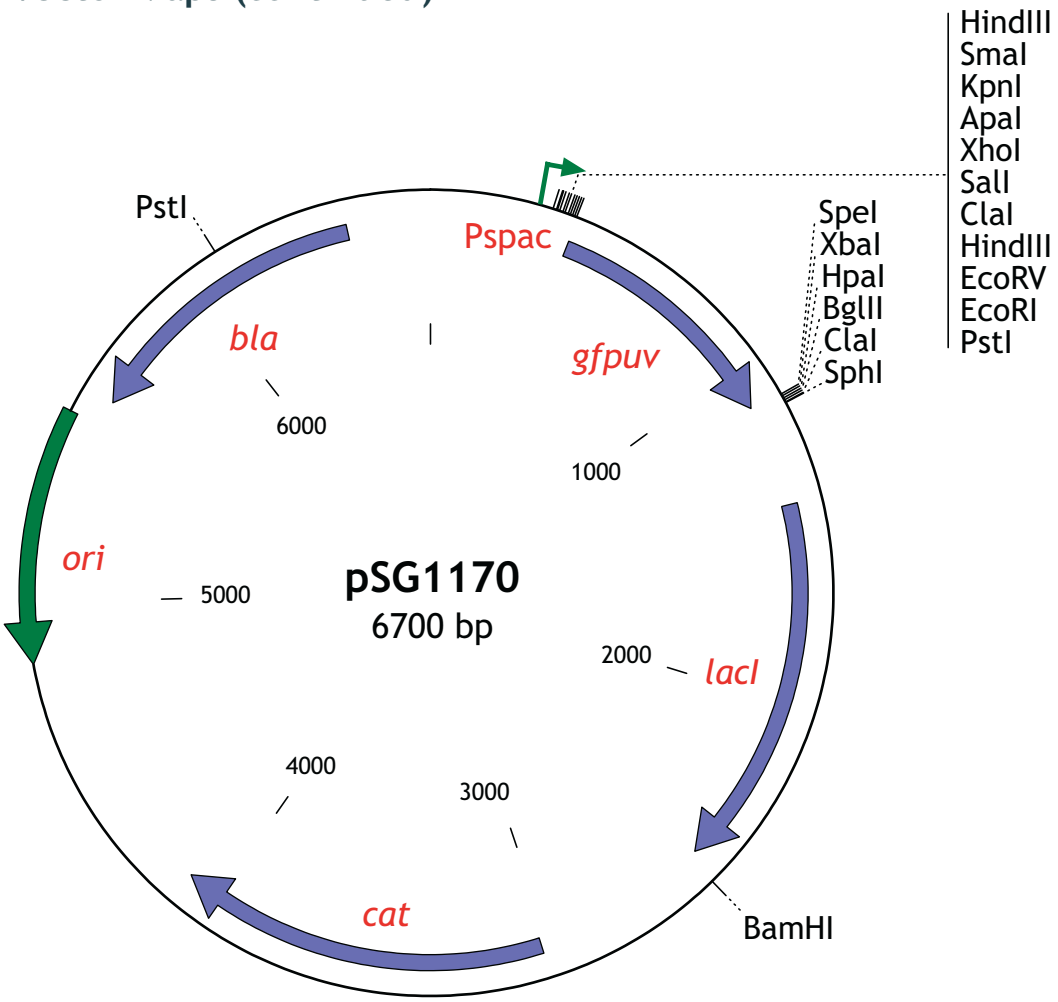
Construction: The xylose-inducible expression vector, pRD96 (Daniel, R. A., *et al*. 1998. Mol. Microbiol. 29:593–604), was modified by (1) removing several restriction sites; (2) introducing a multiple cloning site; and (3) introducing the *gfpmut-1* coding sequence.

Use: The C-terminal fragment of an ORF from a gram-positive bacterium is inserted in-frame with *gfpmut1*. Transformation back into the gram-positive with selection for chloramphenicol resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the natural promoter. If the ORF is in an operon, the downstream genes are under the control of a xylose-inducible promoter.

Recipient strains: pSG1164 should work with any transformable bacterial species that can express *cat* but not support replication of the ColE1 origin. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE156 **Orignal Code:** DH5α(pSG1170)

Reference: **Lewis, P. J. and A. L. Marston.** 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene **227:**101–109.

Sequence: Not in database; not available from BGSC

Features:

gfpuv	encodes GFP variant (single excitation peak at 395 nm, emits green light maximally at 507 nm).
cat	encodes chloramphenicol acetyl transferase; selectable in either *E. coli* or *B. subtilis* (chloramphenicol 5 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
lacI	*lac* operon repressor, engineered to be expressed in gram-positives
Pspac	IPTG-inducible promoter

Description: Integration vector designed to fuse proteins with a UV-excitable variant of GFP, placing any downstream genes in the operon under the control of an IPTG-inducible promoter.

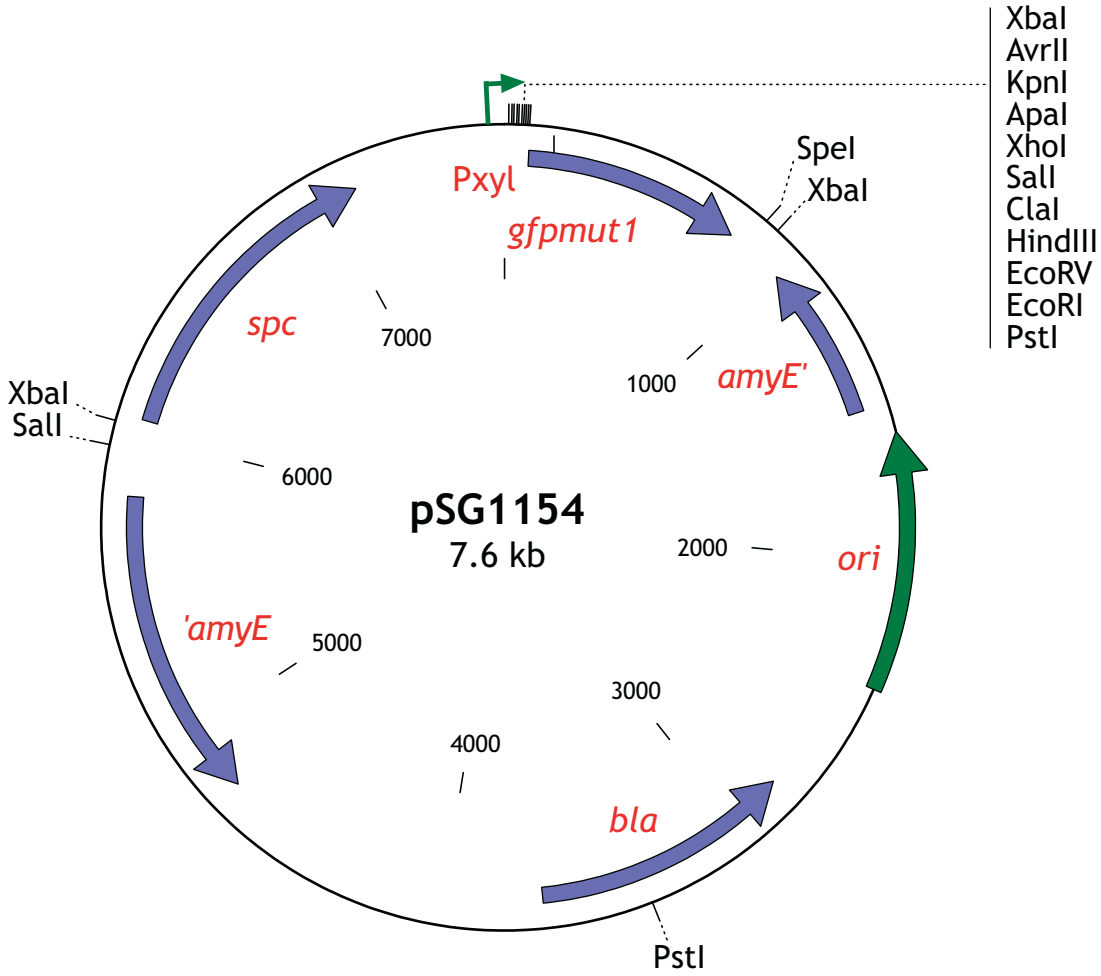
Construction: The *gfpuv*-containing *Acc*651 fragment from pSG1156 was inserted into the filled-in XmaI site of the IPTG-inducible expression vector, pDH88 (Henner, D.J. 1990. Meth. Enzymol. 185:223–228) to produce pSG1170.

Use: The C-terminal fragment of an ORF from a gram-positive bacterium is inserted in-frame with *gvpuv*. Transformation back into the gram-positive with selection for chloramphenicol resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the natural promoter. If the ORF is in an operon, the downstream genes are under the control of an IPTG-inducible promoter.

Recipient strains: pSG1170 should work with any transformable bacterial species that can express *cat* but not support replication of the ColE1 origin. It has been used primarily with *Bacillus subtilis*, but should work with other organisms equally well.

Protocols: *B. subtilis* competent cell preparation and transformation

Integration Vector Maps (*continued*)



BGSC Accession: ECE153 **Orignal Code:** DH5α(pSG1154)

Reference: **Lewis, P. J. and A. L. Marston.** 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene **227:**101–109.

Sequence: Not in database; not available from BGSC

Features:

gfmut-1	encodes highly fluorescent red-shifted GFP variant (single excitation peak at 488 nm, emits green light maximally at 507 nm).
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 50 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence
Pxyl	xylose-inducible promoter

Description: Vector designed to fuse GFP onto the C-terminus of any protein under the control of a xylose-inducible promoter and integrate the fusion into the *B. subtilis amyE* locus.

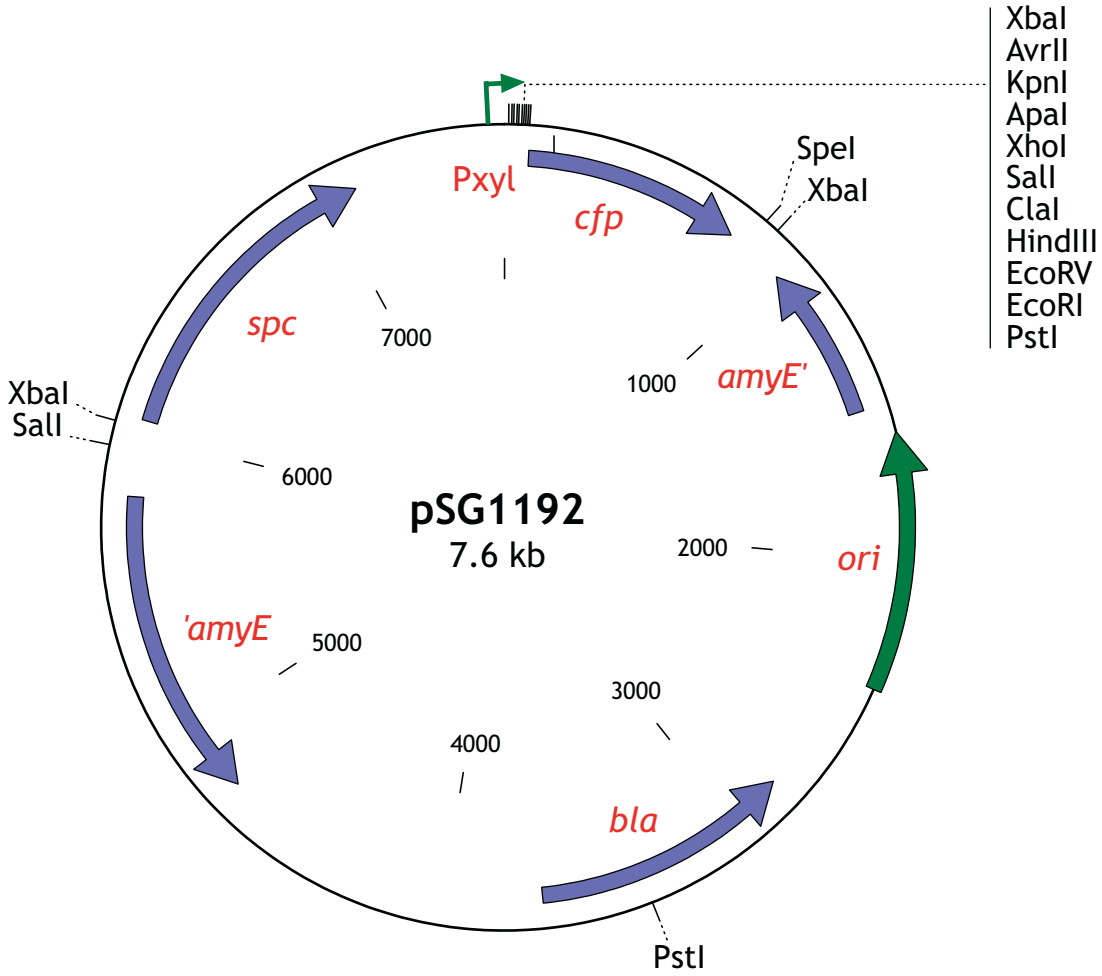
Construction: Derived from pJS2, which was in turn derived from the ectopically integrating expression vector, pMLK83. After unwanted restriction sites were removed from pJS2, a purified Pxyl-*gfpmut1* fragment from pSG1164 was inserted to produce pSG1154.

Use: The complete coding sequence of an ORF of interest is inserted upstream and in-frame with *gfpmut-1*. Transformation into *Bacillus subtilis* 168 with selection for spectinomycin resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the xylose-inducible Pxyl promoter, and the fusion (plus the spectinomycin resistance cassette) is integrated into the host chromosomal *amyE* locus.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening

Integration Vector Maps (*continued*)



BGSC Accession: ECE161 **Orignal Code:** DH5α(pSG1192)

Reference: **Lewis, P. J. and A. L. Marston.** 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene **227:**101–109.

Sequence: Not in database; not available from BGSC

Features:

cfp	encodes Cyan Fluorescent Protein (single excitation peak at 434 nm, emits cyan light maximally at 477 nm).
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 50 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence
Pxyl	xylose-inducible promoter

Description: Vector designed to fuse Cyan Fluorescent Protein onto the C-terminus of any protein under the control of a xylose-inducible promoter and integrate the fusion into the *B. subtilis amyE* locus.

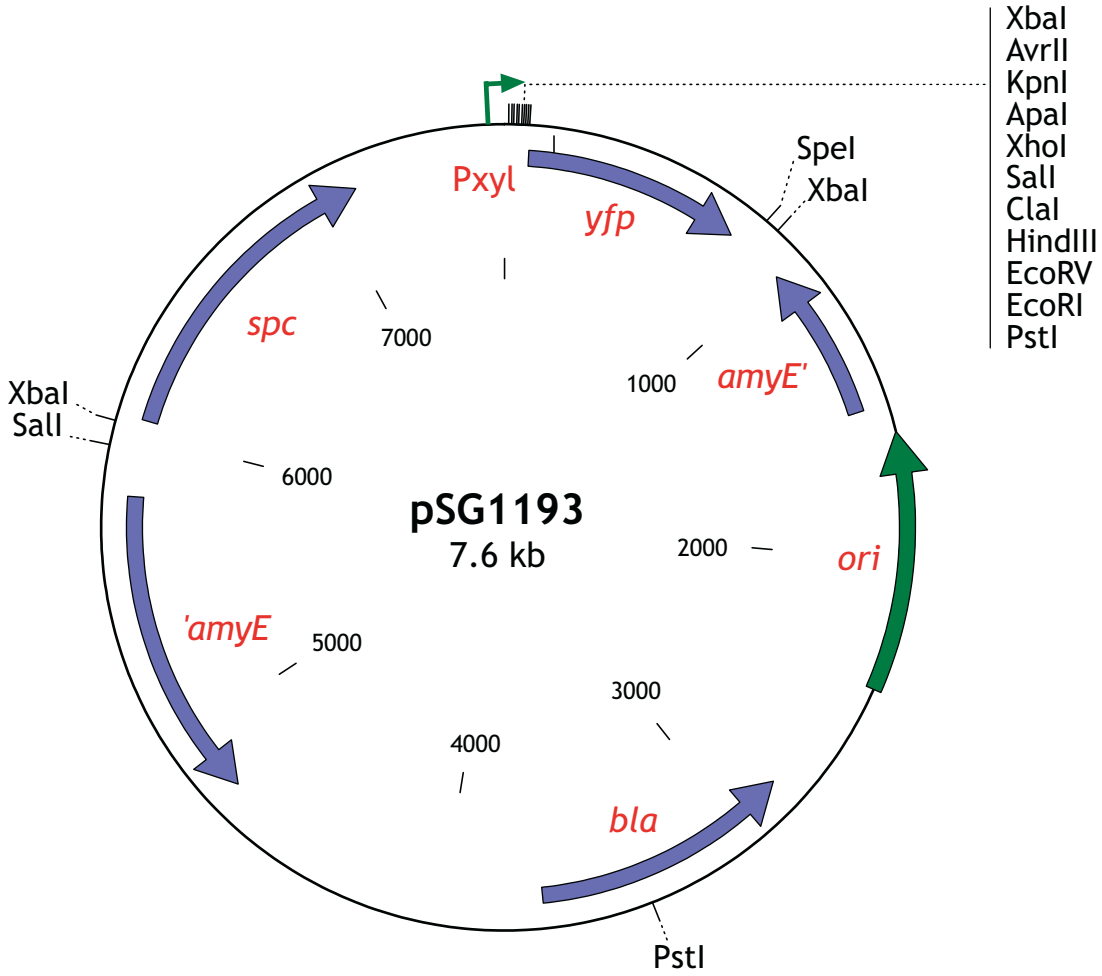
Construction: pSG1186 and pSG1154 were digested with SpeI and XhoI. The *cfp* coding sequence from pSG1186 and the vector fragment of pSG1154 were purified from the digests, then ligated together to give pSG1192.

Use: The complete coding sequence of an ORF of interest is inserted upstream and in-frame with *cfp*. Transformation into *Bacillus subtilis* 168 with selection for spectinomycin resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the xylose-inducible Pxyl promoter, and the fusion (plus the spectinomycin resistance cassette) is integrated into the host chromosomal *amyE* locus.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening

Integration Vector Maps (*continued*)



BGSC Accession: ECE153 **Orignal Code:** DH5α(pSG1193)

Reference: **Feucht, A. and P. J. Lewis.** 2001. Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264:**289-297.

Sequence: Not in database; not available from BGSC

Features:

yfp	encodes Yellow Fluorescent Protein (single excitation peak at 514 nm, emits yellow light maximally at 527 nm).
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 50 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence
Pxyl	xylose-inducible promoter

Description: Vector designed to fuse Yellow Fluorescent Protein onto the C-terminus of any protein under the control of a xylose-inducible promoter and integrate the fusion into the *B. subtilis amyE* locus.

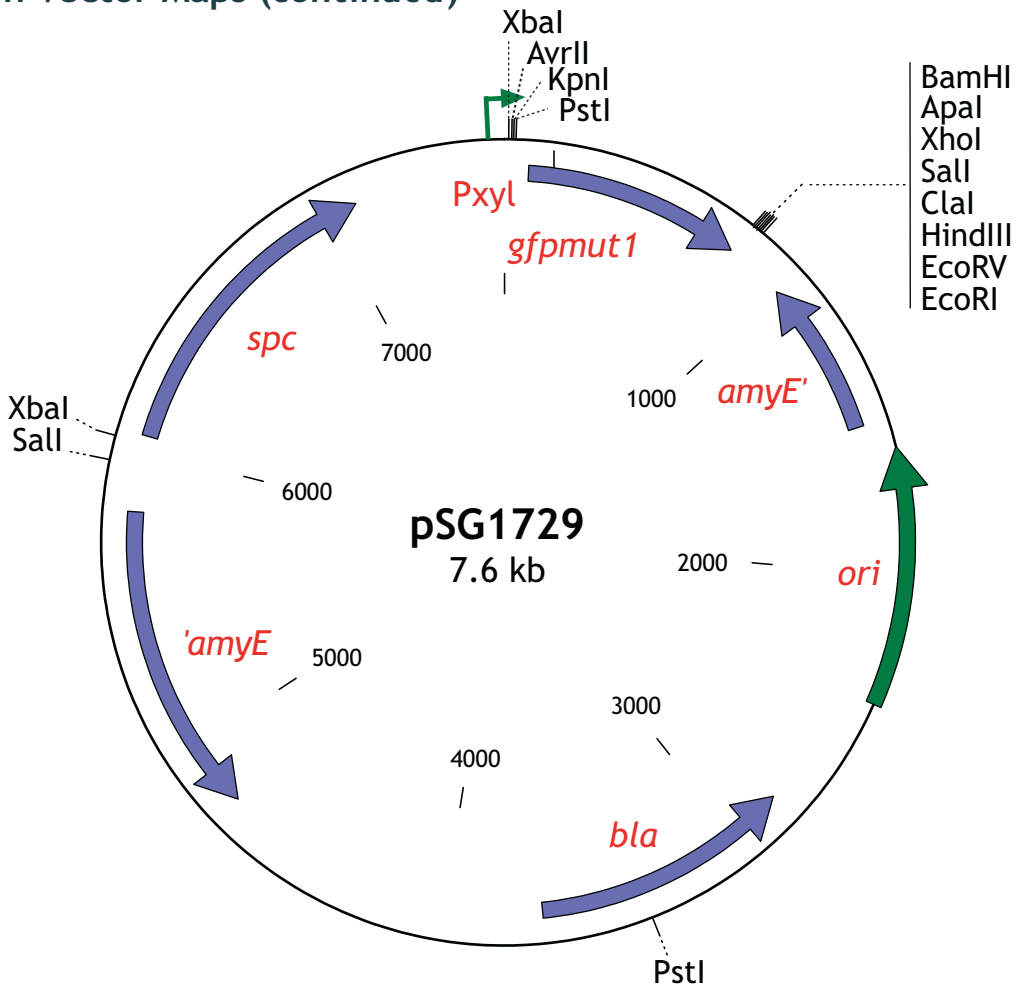
Construction: pSG1187 and pSG1154 were digested with SpeI and XhoI. The *yfp* coding sequence from pSG1187 and the vector fragment of pSG1154 were purified from the digests, then ligated together to give pSG1193.

Use: The complete coding sequence of an ORF of interest is inserted upstream and in-frame with *yfp*. Transformation into *Bacillus subtilis* 168 with selection for spectinomycin resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the xylose-inducible Pxyl promoter, and the fusion (plus the spectinomycin resistance cassette) is integrated into the host chromosomal *amyE* locus.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening

Integration Vector Maps (*continued*)



BGSC Accession: ECE164 **Orignal Code:** DH5α(pSG1729)

Reference: **Lewis, P. J. and A. L. Marston.** 1999. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene **227:**101–109.

Sequence: Not in database; not available from BGSC

Features:

gfmut-1	encodes highly fluorescent red-shifted GFP variant (single excitation peak at 488 nm, emits green light maximally at 507 nm).
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 50 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence
Pxyl	xylose-inducible promoter

Description: Vector designed to fuse GFP onto the N-terminus of any protein under the control of a xylose-inducible promoter and integrate the fusion into the *B. subtilis amyE* locus.

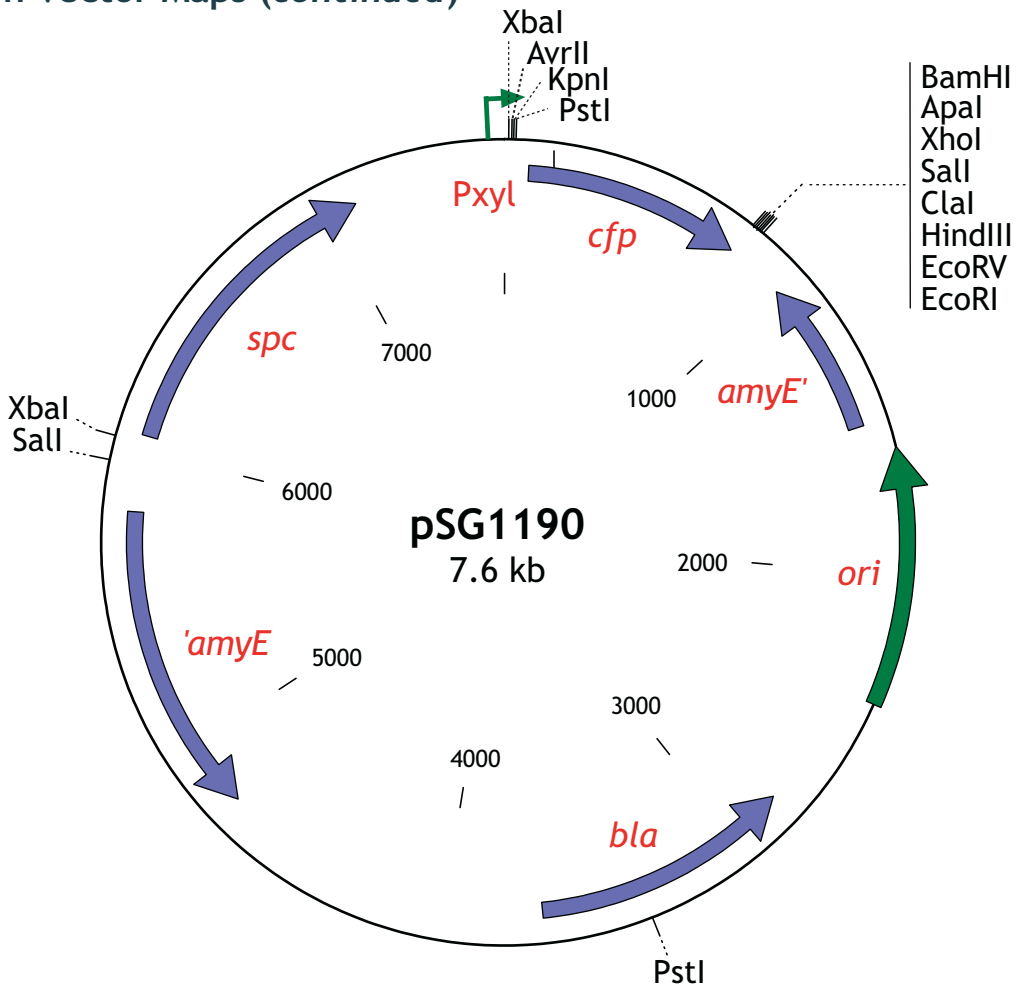
Construction: To make pSG1729, the *gfpmut1* coding sequence of pSG1154 was amplified with primers to add a new multiple cloning site with a stop codon downstream and the PCR product was inserted into the pSG1154 backbone.

Use: The complete coding sequence of an ORF of interest is inserted downstream and in-frame with *gfpmut-1*. Transformation into *Bacillus subtilis* 168 with selection for spectinomycin resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the xylose-inducible Pxyl promoter, and the fusion (plus the spectinomycin resistance cassette) is integrated into the host chromosomal *amyE* locus.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening

Integration Vector Maps (*continued*)



BGSC Accession: ECE159 **Orignal Code:** DH5α(pSG1190)

Reference: **Feucht, A. and P. J. Lewis.** 2001. Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264:**289-297.

Sequence: Not in database; not available from BGSC

Features:

cfp	encodes Cyan Fluorescent Protein (single excitation peak at 434 nm, emits cyan light maximally at 477 nm).
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 50 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence
Pxyl	xylose-inducible promoter

Description: Vector designed to fuse Cyan Fluorescent Protein onto the N-terminus of any protein under the control of a xylose-inducible promoter and integrate the fusion into the *B. subtilis amyE* locus.

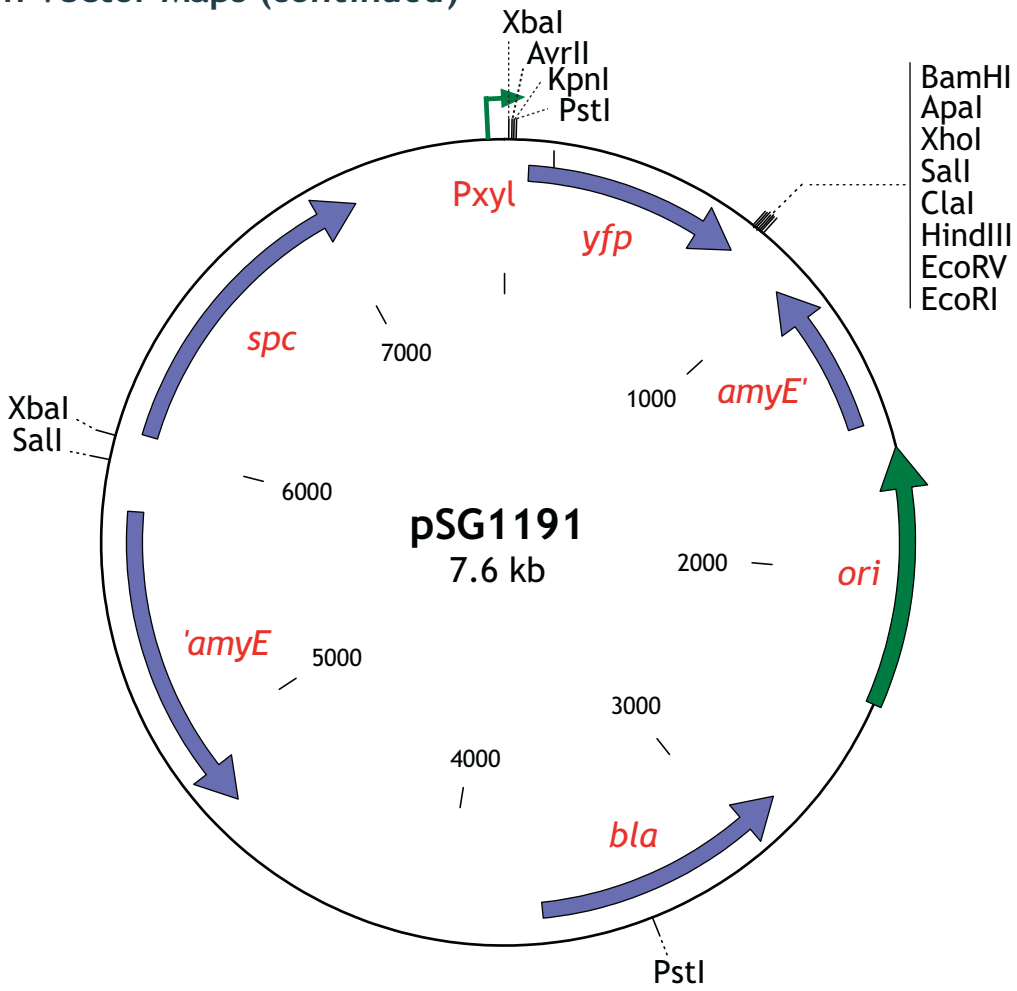
Construction: To make pSG1190, the *cfp* coding sequence of pSG1186 was amplified with primers and the PCR product was inserted into the pSG1729 backbone.

Use: The complete coding sequence of an ORF of interest is inserted downstream and in-frame with *cfp*. Transformation into *Bacillus subtilis* 168 with selection for spectinomycin resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the xylose-inducible Pxyl promoter, and the fusion (plus the spectinomycin resistance cassette) is integrated into the host chromosomal *amyE* locus.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening

Integration Vector Maps (*continued*)



BGSC Accession: ECE160 **Orignal Code:** DH5α(pSG1191)

Reference: **Feucht, A. and P. J. Lewis.** 2001. Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene **264:**289-297.

Sequence: Not in database; not available from BGSC

Features:

yfp	encodes Yellow Fluorescent Protein (single excitation peak at 514 nm, emits yellow light maximally at 527 nm).
spc	encodes spectinomycin adenyltransferase; selectable in either *E. coli* or *B. subtilis* (spectinomycin 50 μg/ml)
bla	encodes β-lactamase; selectable in *E. coli* only (ampicillin 100 μg/ml)
ori-ColE1	ColE1 origin of replication
amyE'-'amyE	5' and 3' ends, respectively, of the *B. subtilis* 168 *amyE* coding sequence
Pxyl	xylose-inducible promoter

Description: Vector designed to fuse YFP onto the N-terminus of any protein under the control of a xylose-inducible promoter and integrate the fusion into the *B. subtilis amyE* locus.

Construction: To make pSG1191, the *yfp* coding sequence of pSG1187 was amplified with primers and the PCR product was inserted into the pSG1729 backbone.

Use: The complete coding sequence of an ORF of interest is inserted downstream and in-frame with *yfp*. Transformation into *Bacillus subtilis* 168 with selection for spectinomycin resistance recovers integration mutants in which the target gene is fused to *gfp* and expression is driven from the xylose-inducible Pxyl promoter, and the fusion (plus the spectinomycin resistance cassette) is integrated into the host chromosomal *amyE* locus.

Recipient strains: Although any recombination-proficient *B. subtilis* 168 derivative will serve as a recipient, use of BGSC 1A771 or 1A772, with pre-existing antibiotic resistance cassettes in their chromosomal *amyE* loci, allows for rapid screening for marker replacements. If another host is used, the user may need to confirm that integration has been at the proper locus by screening for loss of amylase production in the transformed cell.

Protocols: *B. subtilis* competent cell preparation and transformation; amylase production screening