Chitobiase, A New Reporter Enzyme

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D.Y. Kalabat, J.M. Froelich, T.K. Phuong, R.A. Forsyth, V.G. Newman and J.W. Zyskind
San Diego State University, San Diego, CA, USA

ABSTRACT

N,N′-*diacetylchitobiase (chitobiase) from the marine organism* Vibrio harveyi *is a highly stable reporter enzyme for gene fusions. This enzyme hydrolyzes the disaccharide chitobiose to N-acetyl glucosamine. The advantages of the reporter gene encoding chitobiase (chb) are:* (i) *that chitobiase and N-acetyl-b-D-glucosaminidase activities are missing in* E. coli *strains,* (ii) *chitobiase can be monitored using blue/white colony indicator plates and* (iii) *convenient substrates for this enzyme are commercially available. The use of chitobiase as a reporter enzyme is generally applicable to the study of gene expression in those bacteria that do not contain* N-*acetyl-b-D-glucosaminidases. We constructed plasmid vectors containing a multiple cloning site for producing in-frame fusions to chitobiase, the* attP *of l phage for movement into the bacterial chromosome for single-copy analysis, the gene encoding chloramphenicol acetyltransferase (*cat*), the pACYC184 origin of replication and the* rrnBt1t2 *terminator region upstream of the* chb *gene to prevent read-through from other promoters. In-frame fusions between the* dnaA *gene and* chb *were moved to the chromosome by site-specific recombination with the chromosomal* attB *site. These single-copy fusions were assayed for chitobiase to examine the effects of a deletion in the* dnaA *regulatory region.*

INTRODUCTION

The most popular reporter enzyme in bacteria is β-galactosidase (β-gal), but because it is present in bacteria such as *Escherichia coli*, deletions of the *lacZ* gene must be constructed before its use. We chose to develop *N,N*′-diacetylchitobiase (chitobiase) (β-*N*-acetyl-D-glucosaminidase, EC 3.2.1. 30) as a reporter enzyme because its activity and that of the less specific *N*-acetyl-β-D-glucosaminidase, are missing in *E. coli* strains. Cellular chitobiase activity can be measured quantitatively and monitored using blue/white colony indicator plates. Substrates for this enzyme, chitobiose, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (PNAG) (Sigma Chemical, St. Louis, MO, USA) and 5-bromo-4-chloro-3-indolyl-*N*-acetyl-β-D-glucosaminide (X-Gluc) (Sigma Chemical) are commercially available.

Chitobiase is one of two enzymes that hydrolyze chitin, an abundant insoluble polysaccharide, to its monomeric unit, *N*-acetylglucosamine (GlcNac). In *E. coli* cells harboring a plasmid carrying the gene encoding chitobiase (*chb*) from the marine bacterium, *Vibrio harveyi*, the enzyme is associated with the outer membrane (11,17). Chitobiase activity is located in the cytoplasm in cells containing a fusion between the pUC19 α-peptide of β-gal and the carboxy-terminus of chitobiase (11). The vectors we describe here contain this fusion (11), which is deleted for the signal sequence of chitobiase.

Reporter-enzyme activity originating from high-copy number plasmids can vary due to differences in copy number or to titration of regulator proteins. For this reason, fusions often are integrated into the chromosome for single-copy analysis of promoter activity using lambda vectors. Because the size of lambda vectors makes them inconvenient for cloning, and in vitro packaging of lambda vectors is time-consuming, a simplified λ site-specific recombination system was developed (7). Two new vectors described here allow the use of this λ site-specific recombination system for single-copy analysis. In addition to the λ *attP* site, the vectors contain a region of pUC19 (18) including the *lac* promoter, a multiple cloning site and the amino-terminal sequence of the α peptide of β-gal that is fused in-frame to the carboxy-terminal end of chitobiase, deleted for its signal sequence. Using appropriate restriction enzymes, the *lac* promoter in these plasmids can be replaced with other promoters to create in-frame fusions with the chitobiase gene.

To test *chb* as a reporter gene, we replaced the *lac* promoter with the *dnaA* operon regulatory region (10,12) and created in-frame fusions between an amino-terminal fragment of DnaA and chitobiase. The chitobiase activities of these fusions matched the β-gal levels obtained with similar fusions between the *dnaA* regulatory region and the *lacZ* reporter gene.

MATERIALS AND METHODS

Construction of Vectors

Polymerase chain reaction (PCR) products were frequently cloned first at the *Sma*I or *Eco*RV restriction sites of plasmid pBluescript® II (pKSII+; Strat-

agene, La Jolla, CA, USA). The nucleotide sequence of all PCR products was determined by the Microchemical Core Facility (San Diego State University, San Diego, CA, USA). Unmethylated plasmid DNA, isolated from an *E. coli dam*$^-$ strain when cutting with the *Bcl*I enzyme, was required.

pDYK9 was constructed by *(i)* ligating a *Sph*I-*Kpn*I PCR product containing the *dnaA* promoter region into pRSG196 (11), also cut with *Sph*I and *Kpn*I. Plasmid pAC17 (5) served as a template with primers 5′-GCA CAT GCA TGC TGG TCA TTA AAT TTT CC-3′ and 5′-CGG GGT ACC AAC TCA TCC TGC AAT CG-3′, producing a PCR product 374-bp long that contains 353-bp from the *dnaA* promoter region (bases 583–935, numbering according to Reference 10). The forward primer (*Sph*I primer II) contained an *Sph*I site, and the reverse primer (*Kpn*I primer) contained a *Kpn*I site for cloning. This created an in-frame fusion between the amino-terminal 17 amino acids (aa) of DnaA and the carboxy-terminal end of chitobiase, deleted for the amino-terminal 22 aa including the signal peptide. *(ii)* The 3270-bp *Dra*I-*Hin*dIII (partial digest) fragment containing the *dnaA-chb* fusion was ligated to the pACYC184 (4) 2555-bp *Hin*cII-*Hin*dIII fragment carrying chloramphenicol-resistance (Cm^r) and the P15A origin. *(iii)* A *Not*I site was introduced at the *Acc*I site after digestion with *Acc*I, treatment with Mung bean nuclease and ligation to phosphorylated *Not*I linkers (NEBL). *(iv)* A *Not*I site was introduced at the *Ase*I site after digestion with *Ase*I, treatment with Mung bean nuclease and ligation to NEBL. *(v)* An *Xba*I-*Sph*I PCR product containing the *rrnBt1t2* terminator was ligated into this plasmid cut with the same enzymes creating pDYK7. *E. coli* chromosomal DNA served as a template with primers 5′-CTA GTC TAG ATG CCG AAC TCA GAA GTG A-3′ and 5′-GCA CAT GCA TGC GGG GGA TGG CTT GTA GAT-3′ to produce a PCR product 357-bp long that contains bases 6534–6869 from the *rrnB* operon (numbering according to Reference 3) and includes the complex transcription-termination region of this operon (15). The forward primer contained an *Xba*I site, and the reverse primer contained an *Sph*I site. *(vi)* A *Bcl*I-*Sma*I PCR product containing the λ *attP* site was ligated into pDYK7, cut with *Tth111*I, treated with Mung bean nuclease and then digested with *Bcl*I. Plasmid pHN894 (9) served as a template with the forward primer 5′-CAT GAT CAT GCG ACA GGT TTG ATG A-3′ and the reverse primer 5′-GGG GGC GCC TAC CTT TCA CGA G-3′, producing a PCR product 466-bp long that contains the λ *attP* site. The 466-bp PCR product containing the λ *attP* site was first cloned into the *Sma*I site of the pKSII+ plasmid to produce pDYK8. The forward primer contains a *Bcl*I site, and the reverse primer contains G's at the 5′ end to recreate a *Sma*I site when cloning the PCR fragment into a *Sma*I site. This PCR product includes bases -211 to +241 from the center of the *attP* core and the sequence required for optimum λ *attP* site integration (14). The orientation of *attP* is such that when the fusion is integrated at *attB*, the transcription direction of *dnaAp1* and *p2* promoters is the same as replication fork movement mimicking the orientation at the wild-type (WT) *dnaA* promoters.

pDYK11 was constructed by ligating an *Sph*I-*Kpn*I PCR product containing the *rpmH-dnaA* promoter region into pDYK9 also digested with *Sph*I and *Kpn*I. Plasmid pAC17 (5) served as a template with the primers 5′-CAT GCA TGC ATG AAA CGA TGG ACA CC-3′ and 5′-CGG GGT ACC AAC TCA TCC TGC AAT CG-3′ to produce a PCR product 616-bp long that contains 598 bp from the *rpmH-dnaA* regulatory region (bases 338–935, numbering according to Reference 10). The forward primer (*Sph*I primer I) contained an *Sph*I site, and the reverse primer (*Kpn*I primer) contained a *Kpn*I site for cloning.

pJMF3 was constructed by first ligating an *Ase*I linker (5′-CATTAATG-CATG-3′ self-hybridized) into the *Sph*I site of pDYK11. The pUC19 (18) *Ase*I-*Kpn*I fragment containing the *lacPO*-polylinker region was ligated to this plasmid after digestion with *Ase*I and *Kpn*I. The resulting in-frame fusion between the amino-terminal 21 aa of the pUC19 *lacZ*α peptide (18) and the carboxy-terminal end of chitobiase, deleted for the amino-terminal 22 aa is identical to the protein fusion in pRSG196 (11).

pJMF4 was constructed by *(i)* ligating the *Bam*HI-*Eco*RV fragment of pDYK8 containing the λ *attP* site into pDYK7 that had been digested with *Tth111*I, treated with Mung bean nuclease and then digested with *Bcl*I, to create pTKP9. This reversed the orientation of the *attP* site relative to the *attP* site in pDYK9. *(ii)* The pDYK11 *Bsp*MII-*Kpn*I fragment containing the *rpmH-dnaA* promoter region was ligated into pTKP9, digested with the same enzymes, to create pTKP11. This reversed the orientation of the *attP* site relative to the *attP* site in pDYK11. *(iii)* An *Ase*I linker (5′-CATTAATGCATG-3′ self-hybridized) was ligated into the *Sph*I site of pTKP11 to create pJMF2. The pUC19 (18) *Ase*I-*Kpn*I fragment containing the *lacPO*-polylinker region was ligated to pJMF2, cut with *Ase*I and *Kpn*I to create pJMF4.

Strains

The *E. coli* strains used in this study are described in Table 1.

Site-Specific Recombination

To move the chitobiase fusions in pDYK9 and pDYK11 to the *attB* site in the chromosome, *Not*I fragments from these plasmids were self-ligated and transformed (19) or electroporated into strain WM2269 (DH5α containing pLDR8) (7). Plasmid pLDR8 (7) expresses integrase from the λ P_R promoter and contains the λ cI_{857} repressor gene, a kanamycin-resistance gene and a temperature-sensitive origin of replication. The transformed or electroporated cells were incubated at 42°C with shaking for 30 min then moved to 37°C for 1 h, followed by selection on LB agar plates containing chloramphenicol (25 µg/mL) at 42°C. Transformants were screened for loss of kanamycin-resistance and therefore loss of pLDR8.

Bacteriophage P1 Transduction

Transduction with P1 bacteriophage (19) was used to construct strains and to confirm the chromosomal location of the *dnaA-chb* fusions. Co-transduction of Cm^r (carried by the fusion) and *galK* (linked to *attB*) indicated that Cm^r and

Table 1. *E. coli* K-12 Strains

Strain	Genotype/Phenotype	Source/Reference
RB220	λRB1(*dnaA-lacZ* fusion) *his* Δ(*lac*)×74 *proA rspL supF81*(Ts) *thi tsx trp*(Am)	A. Wright (2)
TP220	RB220 *fis*::767(Km^r)	(8)
MG1655	λ^- F^-	*E. coli* Genetic Stock Center
RJ1799	MG1655 *fis*::985 (Spe^r/Str^r)	R. Johnson (1)
DYK11W	MG1655 *rpmH-dnaA-chb* fusion at *attB*	This work
DYK11F	RJ1799 *rpmH-dnaA-chb* fusion at *attB*	This work (*rpmH-dnaA-chb* fusion transferred by P1-mediated transduction from DYK11W to RJ1799)
DYK9W	MG1655 *dnaA-chb* fusion at *attB*	This work
DYK9F	RJ1799 *dnaA-chb* fusion at *attB*	This work (*dnaA-chb* fusion transferred by P1-mediated transduction from DYK9W to RJ1799)
DH5α	F^- Φ80α *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 hsdR17 deoR supE44 thi-1 recA1 gyrA96 relA1*	Our laboratory
WM2269	DH5α containing pLDR8	W. Messer (7)

galK are linked on the chromosomes of strains DYK9W, DYK9F, DYK11W and DYK11F.

Chitobiase Assays

Because chitobiase activity is located in the cytoplasm, when its signal peptide is replaced by fusion with another peptide, assays are performed on toluene-treated cells (12) and washed once with M9 salts (12). Chitobiase activity is determined with PNAG as the substrate (11,16) with the following modifications. The assays contain toluenized cells in chitobiase buffer (10 mM Tris-HCl, pH 8.0 and 0.5 M NaCl) and 666 μM PNAG. NaCl is included because chitobiase has approximately 80% full activity in the absence of salt, with maximal activity occurring between 0.25 and 0.6 M NaCl. Toluenized cells (0.772 mL) are preincubated at 28°C, and the reaction is started with the addition of 0.228 mL PNAG (1 mg/mL). After incubation at 28°C, the reaction is stopped by the addition of 1 mL of 1 M Tris-base. The release of *p*-nitrophenol is measured at 400 nm and turbidity at 550 nm. *p*-Nitrophenol release is measured immediately at 400 nm with a molar absorptivity of 18×10^3 L/mol/cm. Units are calculated after subtracting the light-scattering factor [$1.5 \times$ optical density $(OD)_{550}$] from OD_{400} of the sample. The normalizing factor of 1.5 was determined previously by measuring the light-scattering ratio of bacteria at OD_{400} and OD_{550}. One unit of chitobiase activity is the amount of enzyme that catalyzes the formation of 1 pmol/min of *p*-nitrophenol at 28°C. For comparison to Miller units of β-gal (13), the units are normalized to 1 mL of culture at $OD_{450} = 1$.

RESULTS

Plasmid Vectors Containing the *lac* Promoter-Chitobiase Fusion and the λ *attP* Site

Plasmids pJMF3 and pJMF4 (Figure 1) contain the *lacPO* promoter with the first 21 aa of *lacZ*α (from pUC19) fused in-frame to the *chb* gene. These plasmids also contain the λ phage *attP* recombination site in different orientations, the gene encoding chloramphenicol acetyltransferase (*cat*) and a ribosomal terminator, *rrnBt1t2*, inserted upstream of the *lac-chb* fusion to prevent read-through from other promoters. The chitobiase activity associated with these plasmids (Table 2) is high in the absence of isopropyl-thio-β-D-galactoside (IPTG) because of titration of *lac* repressor expressed from a single-copy chromosomal gene. Induction by IPTG is approximately 10-fold (Table 2).

The *lac* promoter can be replaced with another promoter, and a fusion protein created with chitobiase, by cutting with *Sph*I or *Ase*I and either *Sal*I,

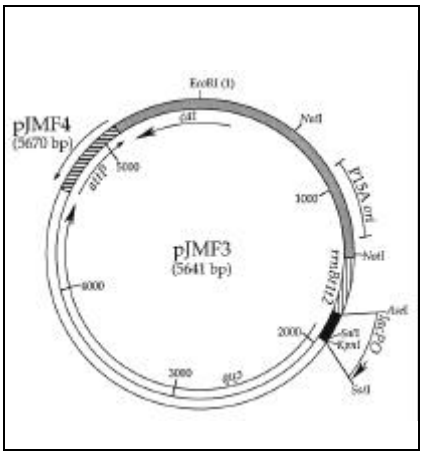

Figure 1. Plasmids pJMF3 and pJMF4 containing *attP* in 2 different orientations and the *lac* promoter with the first 21 aa of *lacZ*α (from pUC19) fused in-frame to the *chb* gene. Construction of these plasmids is described in Materials and Methods. Sequence of fusion region is shown in Figure 2. Restriction sites shown are found once in the plasmid sequences except for *Not*I, which has 2 sites flanking the P15A origin.

Table 2. Chitobiase Activity of *lacZ-chb* Fusion[a]

Plasmid	Chitobiase[b] (U[c]) -IPTG	Chitobiase[b] (U[c]) +IPTG (1 mM)
pJMF3 in DH5α	668 ± 45	9320 ± 347
pJMF4 in DH5α	788 ± 44	7188 ± 477

[a]Overnight cultures were diluted 1:1000 into 50 mL prewarmed LB and grown to OD_{450} = 0.1. 1 mM IPTG was added to half of the culture, and growth continued to OD_{450} = 0.3.
[b]Triplicate samples were assayed. Mean chitobiase activities are given with standard deviations.
[c]One unit of chitobiase activity is 1 pmol of *p*-nitrophenol/min at 28°C. Units given for 1 mL of culture at OD_{450} = 1.

*Kpn*I or *Sst*I (Figure 2). Fusions created with these vectors can be moved to the chromosome by site-specific recombination at the λ *attB* site to permit single-copy analysis of the activity of the promoter. The protocol (7) involves two components, *(i)* a circular DNA containing the λ attachment site, *attP*, the promoter-*chb* gene fusion and the *cat* gene and *(ii)* a helper plasmid, pLDR8 (7), which contains the *int* gene under the control of the temperature-sensitive repressor, *cI857* and a temperature-sensitive origin of replication. The plasmid is digested with *Not*I to remove the P15A origin, and the fragment containing the chitobiase fusion is self-ligated before transformation into cells containing pLDR8. Integration occurs by site-specific recombination between *attP* and *attB* (17.4 min on the *E. coli* chromosome) (Figure 3).

Use of the Chitobiase Reporter Enzyme to Study *dnaA* Gene Regulation

Two plasmids, pDYK9 and pDYK11, were constructed to assess the regulation of the *dnaA* gene using chitobiase as a reporter enzyme. These plasmids differ by the absence of the *rpmH* regulatory region in pDYK9 (Figure 4). These fusions were moved from the plasmid to the chromosomal *attB* site for single-copy analysis as described above. After transformation of strain WM2269 with the ligated DNA, integration occurred by site-specific recombination between the *attP* and the *attB* sites. The orientation of *attP* in pDYK9 and pDYK11 is such that when the fusion is integrated at *attB*, the transcription direction of *dnaAp1* and *dnaAp2* promoters is the same as move-

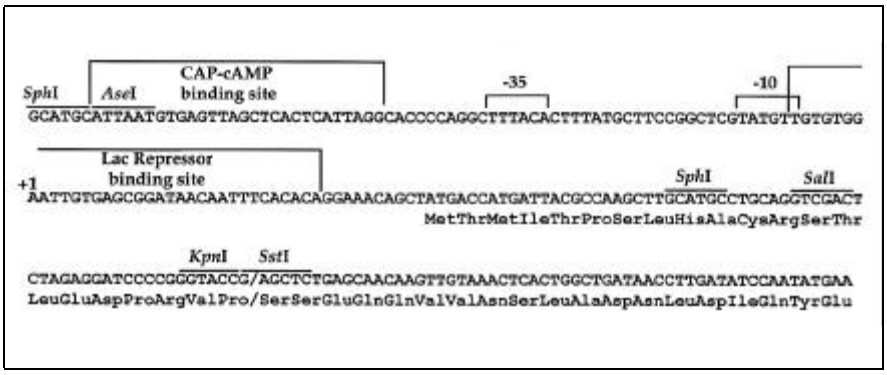

Figure 2. Sequence of the *lac* promoter and the chitobiase fusion found in pJMF3 and pJMF4 (see Figure 1). Fusion between *lacZ*α (from pUC19) and *chb* (16) is indicated by (/); start of transcription is indicated by (+1). Sequence and binding sites in the lac promoter regulatory region are found in Reference 6. Restriction enzyme sites shown are found once in the plasmid sequences except for *Sph*I, which has 2 sites; these different restriction sites can be used to replace the *lac* promoter with another promoter together with part of a coding region to produce an in-frame fusion with *chb*.

ment of the replication fork. This orientation is the same as at the *dnaA* WT location. The genetic location of the fusions was confirmed in the Cm^r transformants by demonstrating cotransduction of Cm^r and *galK*.

The fusions created in strain WM2269 were moved by P1 transduction to MG1655, creating strain DYK9W with pDYK9 and strain DYK11W with pDYK11. Deletion of the *rpmH* promoters had very little effect (1.4-fold) on chitobiase activity (Table 3, compare lines 1 and 3).

Fis protein binds to a site in the *dnaAp2* promoter that covers the -35 sequence (Figure 5) and appears to be a repressor of DnaA expression. A fusion protein with β-gal activity that is expressed from the *rpmH-dnaA* regulatory region has increased β-gal activity (1.9-fold) in a *fis*$^-$ mutant when compared to Fis WT cells (Reference 8; data shown in Table 3). Similarly, the absence of Fis leads to a greater than 2-fold increase in chitobiase activity of the DnaA-chitobiase fusion protein for the DYK9F and DYK11F strains, comparable in extent to that observed with the *dnaA-lacZ* fusion strain, TP220 (Table 3).

Table 3. Chitobiase and β-Gal Activities of *dnaA-chb* and *dnaA-lacZ* Fusions in WT and *fis* Mutant Backgrounds

Strains	Chitobiase[a] (U[b])	β-Gal[c] (Miller U[d])
DYK11W *fis*$^+$	30.0 ± 2.1	
DYK11F *fis*::985	80.0 ± 1.2	
DYK9W *fis*$^+$	44.0 ± 2.1	
DYK9F *fis*::985	96.0 ± 3.5	
RB220 *fis*$^+$		59.8 ± 7.9
TP220 *fis*::767		115.2 ± 7.2

[a]Triplicate samples were assayed during exponential growth. Mean chitobiase activities are given with standard deviation.
[b]One unit of chitobiase activity is 1 pmol of *p*-nitrophenol per min at 28°C. Units given for 1 mL of culture at OD_{450} = 1.
[c]Data from Reference 8.
[d]Unit defined in Reference 13.

DISCUSSION

Chitobiase is a useful reporter enzyme because many species of bacteria, including *E. coli*, do not synthesize this enzyme. To test whether chitobiase could be used as a reporter enzyme in other bacteria or eukaryotic cells, add PNAG to cells and look for the appearance of yellow color. Also, chitobiase is useful as a reporter enzyme because no mutations or deletions need to be created in bacterial host strains that lack this enzyme. To identify regulators of the expression of a promoter, *chb* reporter fusions can be moved using P1 bacteriophage transduction to strains deleted for suspected regulatory genes. Such

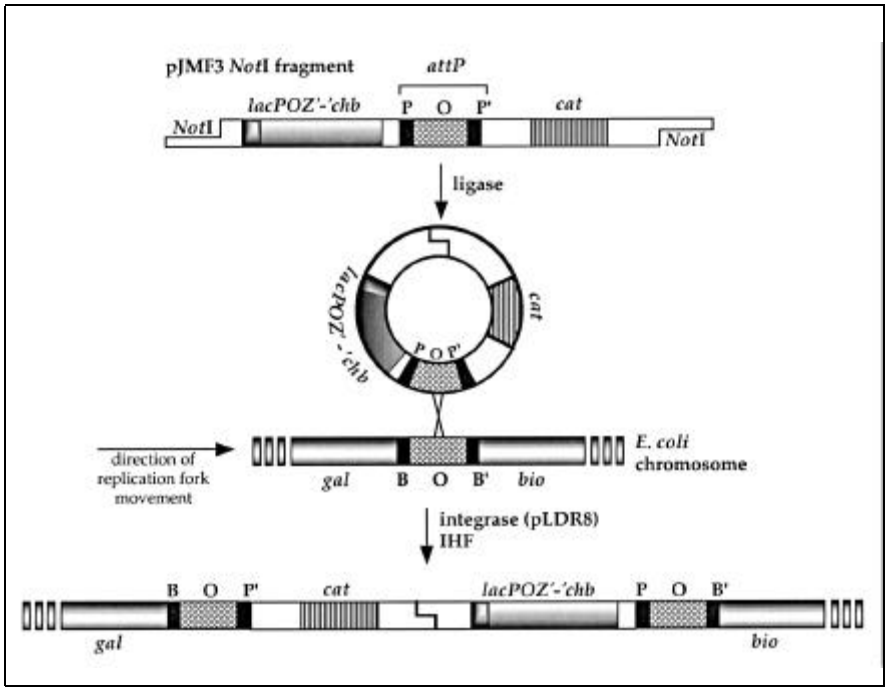

Figure 3. Integration of *chb* fusions into the chromosome by site-specific recombination between *attB* and *attP*. The steps involved are described in Materials and Methods and Reference 7.

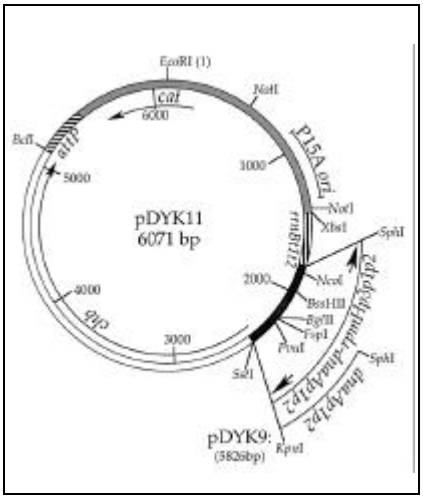

Figure 4. Plasmids pDYK9 and pDYK11 containing *dnaA-chb* fusions. pDYK9 is deleted for the *rpmH* regulatory region. The orientation of *attP* in pDYK9 and pDYK11 is the same as that of pJMF3 (see Figure 1). After integration at *attB* of the larger *Not*I fragment, the *dnaA* promoters are oriented to transcribe in the same direction as the replication fork.

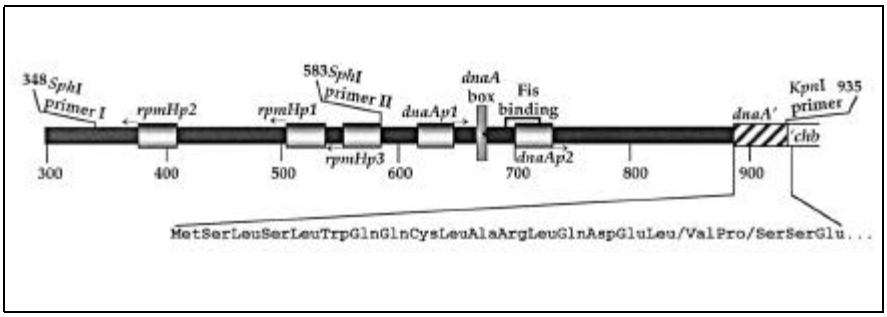

Figure 5. The *rpmH-dnaA* regulatory region and *dnaA*-chitobiase fusion. The *dnaA* box and promoters are shaded, and the coding region of the *dnaA* gene is striped. The fusion contains two aa (between the backslashes) from pUC19. The region cloned into pDYK9 is between *Sph*I primer II and *Kpn*I primer, and the region cloned into pDYK11 is between *Sph*I primer I and *Kpn*I primer. The numbers above the primers refer to the nucleotide in the sequence amplified. See References 8, 10 and 12 for sequence numbering and locations of promoters and protein-binding sites.

strain construction is greatly facilitated when no other mutations are required. In the case of the *lacZ* reporter gene, more steps would be necessary to construct the appropriate isogenic strains.

The P15A origin of replication on these vectors is compatible with all ColE1- and pMB1-derived origins. This permits one to introduce a putative regulatory protein by a compatible plasmid and to assay its effect on the promoter driving the *chb* fusion.

Chitobiase activity can be readily measured with commercially available substrates. Chitobiase-producing colonies are blue on plates that contain X-Gluc, and colonies that do not produce chitobiase are white. Quantitative measurements of chitobiase activity can be performed with either PNAG or chitobiose as the substrate (11,16), thereby measuring the level of promoter activity.

In our constructs, all upstream transcriptional activity is prevented from entering the *chb* reporter gene. The plasmid vectors, pJMF3 and pJMF4, contain the *rrnbt1t2* terminator upstream of the promoter fusion, which prevents read-through from chromosomal promoters near the insertion site. Only chitobiase activity originating from the promoters of interest is expressed.

The *attP* site in these vectors allows integration at the chromosomal *attB* in a specific orientation, depending on the vector used. With these vectors, any chitobiase fusion involving an essential gene can be moved to the chromosome, thus permitting single-copy analysis with a chromosomal orientation similar to the WT gene.

ACKNOWLEDGMENTS

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REFERENCES

1. **Ball, C.A., R. Osuna, K.C. Ferguson and R.C. Johnson.** 1992. Dramatic changes in Fis levels upon nutrient upshift in *Escherichia coli*. J. Bacteriol. *174*:8043-8056.
2. **Braun, R.E., K. O'Day and A. Wright.** 1985. Autoregulation of the DNA replication gene *dnaA* in *E. coli* K-12. Cell *40*:159-169.
3. **Brosius, J., T.J. Dull, D.D. Sleeter and H.F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. *148*:107-127
4. **Chang, A.C.Y. and S. Cohen.** 1987. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. *134*:1141-1156.
5. **Chiaramello, A.E. and J.W. Zyskind.** 1992. Coupling of DNA replication to growth rate in *Escherichia coli*: a possible role for guanosine tetraphosphate. J. Bacteriol. *172*:2013-2019.
6. **Dickson, R.C., J. Abelson, W.M. Barnes and W.S. Reznikoff.** 1975. Genetic regulation: the Lac control region. Science *187*:27-32.
7. **Diederich, L.L., J. Rasmussen and W. Messer.** 1992. New cloning vectors for integration into the λ attachment site *attB* of *Escherichia coli* chromosome. Plasmid *28*:14-24.
8. **Froelich, J.M., T.P. Phuong and J.W. Zyskind.** 1996. Fis binding in the *dnaA* operon promoter region. J. Bacteriol. *178*:6006-6012.
9. **Goodman, S.D., S.C. Nicholson and H.A. Nash.** 1992. Deformation of DNA during site-specific recombination of bacteriophage lambda: replacement of IHF protein by HU protein or sequence-directed bends. Proc. Natl. Acad. Sci. USA *89*:11910-11914.
10. **Hansen, F.G., E.B. Hansen and T. Atlung.** 1982. The nucleotide sequence of the *dnaA* gene promoter and of the adjacent *rpmH* gene, coding for the ribosomal protein L34, of *Escherichia coli*. EMBO J. *1*:1043-1048.
11. **Jannatipour, M., R.W. Soto-Gil, L.C. Childers and J.W. Zyskind.** 1995. Translocation of *Vibrio harveyi N,N*ℓ-diacetylchitobiase to the outer membrane of *Escherichia coli*. J. Bacteriol. *169*:3785-3791.
12. **Messer, W. and C.W. Weigel.** 1996. Initiation of chromosome replication, p. 1579-1601. *In* F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology. ASM Press, Washington, DC.
13. **Miller, J.H.** 1992. A Short Course in Bacterial Genetics. CSH Laboratory Press, Cold Spring Harbor, NY.
14. **Nagaraja, R. and R.A. Weisberg.** 1990. Specificity determinants in the attachment sites of bacteriophages HK022 and λ. J. Bacteriol. *172*:6540-6550.
15. **Orosz, A., I. Boros and P. Venetianer.** 1991. Analysis of the complex transcription termination region of the *Escherichia coli rrnB* gene. Eur. J. Biochem. *201*:653-659
16. **Soto-Gil, R.W., L.C. Childers, W.H. Huisman, A.S. Dahms, M. Jannatipour, F. Hedjran and J.W. Zyskind.** 1988. *N,N*ℓ-Diacetylchitobiase of *Vibrio harveyi*. Methods Enzymol. *161*:524-529.
17. **Soto-Gil, R.W. and J.W. Zyskind.** 1989. *N,N*ℓ-Diacetylchitobiase of *Vibrio harveyi* primary structure, processing, and evolutionary relationships. J. Biol Chem. *264*:14778-14782.
18. **Yanisch-Perron, C., J. Vieira and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene *33*:103-119.
19. **Zyskind, J.W. and S.I. Bernstein.** 1992. Recombinant DNA Laboratory Manual. Academic Press, San Diego.

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Address correspondence to:

Judith W. Zyskind
Biology Department
San Diego State University
San Diego, CA 92182-4614, USA
Internet: jzyskind@sunstroke.sdsu.edu