An enhanced GFP reporter system to monitor gene expression in *Borrelia burgdorferi*

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*Borrelia burgdorferi* regulates genes in response to a number of environmental signals such as temperature and pH. A green fluorescent protein (GFP) reporter system using the *ospC*, *ospA* and *flaB* promoters from *B. burgdorferi* B31 was introduced into infectious clonal isolates of strains B31 and N40 to monitor and compare gene expression in response to pH and temperature in vitro. GFP could be assayed by epifluorescence microscopy, immunoblotting or spectrofluorometry and was an accurate reporter of target gene expression. It was determined that only 179 bp 5’ of *ospC* was sufficient to regulate the reporter *gfp* in vitro in response to pH and temperature in *B. burgdorferi* B31. The loss of linear plasmid (lp) 25, lp28-1, Ip36 and Ip56 had no effect on the ability of *B. burgdorferi* B31 to regulate *ospC* in response to pH or temperature. The amount of OspC in N40 transformants was unaffected by changes in pH or temperature of the culture medium. This suggests that regulation of gene expression in response to pH and temperature may vary between these two *B. burgdorferi* strains.

The ability to monitor gene expression in *B. burgdorferi* at different stages of the infectious cycle is essential in identifying proteins that are involved in this process. Earlier, a chloramphenicol acetyltransferase (CAT) reporter system was used to monitor *flaB, ospC* and *ospAB* expression in *B. burgdorferi* (Sohaskey et al., 1997, 1999). However, in this system cat expression was transitory due to plasmid instability and not adequate to observe progressive gene expression over long periods of time in vivo or in vitro. A useful technique that has been applied as a quantitative reporter system for gene expression during in vivo and in vitro growth in numerous bacterial systems is the transcriptional fusion of promoter regions to the gene encoding green fluorescent protein (GFP). Many derivatives (mutants) of wild-type GFP now exist, each having its own unique properties and advantages. Moreover within the last year, shuttle vector constructs expressing the yellow fluorescent protein (YFP), the cyan fluorescent protein (CFP) and two GFP derivatives (Eggers et al., 2002; Sartakova et al., 2000) (all driven by the constitutive promoter *flaB*) have been transformed into and expressed in *B. burgdorferi*. The efficacy of these GFP derivatives to monitor differential gene expression has not been assessed.

We have developed a reporter system in the shuttle vector pBSV2, employing the ‘cycle 3’ mutant GFP allele, to examine gene expression in *B. burgdorferi*. The cycle 3 GFP
derivative (c3 GFP) has the desirable properties of being highly soluble and exhibiting a greater than 40-fold increase in fluorescence relative to wild-type GFP, while maintaining excitation/emission maxima (395/507 nm) similar to that of wild-type GFP (Crameri et al., 1996). The promoter regions controlling the expression of ospC, ospA and flaB were transcriptionally fused to c3 GFP and introduced into electroproporation-competent, infectious *B. burgdorferi* cells, where fluorescence and c3 GFP expression were monitored in response to changes in culture pH and temperature.

**METHODS**

**Bacterial strains and growth conditions.** Low-passage (<3 passages), infectious *B. burgdorferi* B31 clone A3 (Elias et al., 2002) and intermediate passage, infectious clone N40 (passage 32) (Barthold et al., 1993, 1988; Thomas et al., 2001) were grown to mid-exponential phase (5 × 10^6 cells ml^-1) in BSK II in 5% CO2 at 35°C and were prepared for electroporation as described by Samuels (1995). *B. burgdorferi* transformants were plated into semi-solid BSK plating medium with 200 µg kanamycin ml^-1 as described by Bono et al. (2000). For *gfp* expression studies, spirochaetes were inoculated from thawed freezer stocks into a 5 ml BSK II starter culture supplemented with kanamycin. From the starter culture the cells were inoculated to a final concentration of 10^7 cells ml^-1 into BSK-H medium (lot 21K-8408) (Sigma) or into HEPES-buffered BSK-H adjusted to pH 7-0 or 8-0 (Carroll et al., 1999) and incubated at either 35 or 23°C. The growth rate of N40 and B31 is slow at 23°C and cells generally took 1 month to reach the desired cell density. Spirochaetes were harvested by centrifugation (8000 g; 10 min; 4°C) when a cell density of 5 × 10^7 cells ml^-1 was reached. Enumeration of spirochaetes in culture was performed by dark-field microscopy using a Petroff–Hauser counting chamber. All *gfp* expression studies were carried out under kanamycin selection. Transformation-competent *Escherichia coli* TOP10 cells were obtained from Invitrogen. *E. coli* transformants were selected for by growth in Luria broth (LB) supplemented with either 100 µg ampicillin ml^-1 or 40 µg kanamycin ml^-1.

**Plasmids and cloning.** Plasmids used in this study were purified from *E. coli* using the Qiagen Maxi plasmid purification kit (Qiagen). The promoters for ospC, ospA and flaB were amplified by PCR with target specific primers using the GeneAmp kit (Perkin-Elmer) with *B. burgdorferi* B31 genomic DNA as the template. Primers used to amplify the flaB promoter were proFlaBS (5′-TGTCTGTTGCTGTGCGGCGCGAGAGAG-3′) and proFlaB3′ (5′-GATTGATAATCATATGCTATTCTCATCAG-3′), to amplify the *ospA* promoter proA15.1 (5′-AATGCCAAACTGGGACT-3′) and proA15.4 (5′-TCTAATATATTCTCCTTTT-3′) were used, and to amplify the *ospC* promoter proOspC1 (5′-GCATATTGGCTTTGCTTATGCG-3′) and proOspC4 (5′-TAATGATGATATCTTATTCAATTAGGTG-3′) were used. PCR products were cloned into the vector pCDNA3.1/CT-GFP-TOPO from Invitrogen and transformed into chemically competent *E. coli* TOP10 (Invitrogen). The cloned region 5′ of *ospA* included a 203 bp DNA fragment, consisting of the RNA polymerase-binding site, the ribosome-binding site (RBS) and the first codon of the structural gene to produce pJAC*E*(ospA-*gfp*). Likewise, the *ospC* promoter was amplified and cloned encompassing a 194 bp DNA fragment, consisting of the RNA polymerase-binding site, ribosome-binding site and the first five codons of the structural gene to produce pJAC*E*(ospC-*gfp*). The *flaB* promoter was amplified and cloned encompassing a 367 bp DNA fragment, consisting of the RNA polymerase-binding site, ribosome-binding site and the first four codons of the structural gene to produce pJAC*E*(flaB-*gfp*).

Promoter-*gfp* fusion transformants were screened by PCR, plasmids containing promoter-*gfp* fusions were isolated and DNA inserts were sequenced at least twice using the GFP reverse primer (5′-GGGTAAGCCTTTCCATGATAGC-3′) to verify that the promoters were correct and in-frame. Sequencing reactions were performed by adding 3-0 µl Big Dye Ready Reaction mix and 1-5 µl sequencing buffer (400 mM Tris/HCl pH 9-0; 10 mM MgCl2) to 22 pmol primer and 750 ng plasmid DNA. The reaction volume was brought up to 15 µl with distilled water and PCR was carried out with 45 cycles of 95°C for 10 s, 50°C for 5 s and 60°C for 4 min. Samples were cleaned with Centrerpin spin columns (Millipore) and sequencing was performed on an ABI 3700 sequencer. The promoter-*gfp* fusions were then excised by double digestion of pJAC*E*(ospC-*gfp*), pJAC*E*(ospA-*gfp*) or pJAC*E*(flaB-*gfp*) with KpnI and SphI, and ligated into the shuttle vector pBSV2 digested similarly to generate pBSV*E*(ospC-*gfp*), pBSV*E*(ospA-*gfp*) and pBSV*E*(flaB-*gfp*) (Fig. 1). These shuttle vector promoter-*gfp* constructs were transformed into chemically competent *E. coli* TOP10 and clones were again screened by PCR and/or fluorescence. A *gfp* construct lacking a promoter (pBSV-*gfp*) was created by ligation of an XbaI–SphI fragment from pJAC*E*(ospC-*gfp*) into pBSV2 and used as a control (Fig. 1). Shuttle vector promoter-*gfp* fusion constructs and the control were electro- porated into electroporation-competent N40 or B31 clone A3. The cells were allowed to recover for 1 day in 2 ml BSK II at 35°C without selection and then the electroporation was plated on semi-solid BSK plating medium supplemented with 200 µg kanamycin ml^-1. After 12–14 days at 35°C, transformants were individually picked and screened by PCR and fluorescence. All clones were stored frozen at −80°C.

**Electrophoresis and immunoblotting.** *B. burgdorferi* controls and transformants were grown to mid-exponential phase, centrifuged, rinsed in HN buffer (10 mM HEPES, 50 mM NaCl, pH 8.0), centrifuged again and solubilized in 1× Laemmli sample buffer (Bio-Rad). Approximately 2 × 10^6 cells were loaded per lane and proteins were separated by SDS-PAGE with SE600 gel apparatus (Hoefer Scientific). Proteins were visualized by staining with the Silver Stain Plus kit (Bio-Rad) or prepared for immunoblotting. Molecular mass standards were purchased from Bio-Rad.

For Western blot analysis, the proteins were electrophoretically transferred to nitrocellulose (0-45 : MTrans-Blot Transfer Medium; Bio-Rad) as described by Towbin et al. (1979) with a Bio-Rad Trans Blot Cell (30 V, 12 h, 4°C). After transfer, the proteins were visualized with Amido Black (0.1%, w/v, Amido Black dye in 1-0% v/v, acetic acid) and the standards were marked. The nitrocellulose membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline (150 mM NaCl in 10 mM Tris/HCl, pH 8.0) with the addition of 0.1% (v/v) Tween 20 (TBS-T20) (3 h, 24°C) and immunoblots were probed with anti-B. burgdorferi B31 polyclonal hyper immune serum (Carroll & Gherardini, 1996) or anti-GFP immune serum (Invitrogen) diluted 1:3000 and 1:5000, respectively, in TBS-T20 (1 h, 24°C). Immunoblots were washed twice in 100–200 ml TBS-T20 for 10 min. Horseradish-peroxidase-conjugated goat anti-rabbit (Sigma) was diluted 1:5000 in TBS-T20 and applied to the blot (45 min, 24°C), followed by three washes with 100–200 ml TBS-T20. Bound antibodies were visualized with the Enhanced Chemiluminescence kit (Amersham Life Sciences) in accordance with the manufacturer’s specifications. Integrated density values of immunoreactive bands were assessed using an Alphalmerager 2000 digital imaging system (Alpha Innotech). All immunoblots were performed independently, at least twice.

**Determination of plasmid content in B31 A3 GFP clones.** Genomic DNA from the clones of spirochaetes was obtained with the Wizard Genomic DNA Purification kit (Promega). The plasmid content of each clone was assessed by PCR with primers specific for target genes that reside on the known plasmids from the sequenced...
B31 genome (Fraser et al., 1997). The 29 different PCR primer pairs, set-up and conditions were identical to those of Elias et al. (2002). Briefly, 50 ng genomic DNA served as the template in a total reaction volume of 20 μl per primer set, with reaction conditions consisting of a 5 min, 94 °C denaturation step followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 7 min. PCR products were separated on a 1–2% (w/v) agarose gel and visualized with ethidium bromide by UV transillumination.

Analysis of GFP expression by spectrofluorometer. Ten millilitre cultures of spirochaetes harbouring different promoter-gfp fusion constructs were grown under various environmental conditions to mid-exponential phase (5×10⁷ cells ml⁻¹), harvested by centrifugation, rinsed in ice-cold HN buffer and centrifuged again. Cell pellets were resuspended in ice-cold HN buffer at a concentration of 1×10⁹ cells ml⁻¹ and 100 μl was assayed for fluorescence with a SpectraMax Gemini XS spectrofluorometer (Molecular Devices). Spectrofluorometer analysis and settings were as follows: 96-well plates were analysed by the ‘Well Scan’ setting with a total of 19 reads per well, excitation was at a wavelength of 395 nm, detection of emission was at a wavelength of 507 nm and data were recorded and plotted in relative fluorescence units (RFU). All spectrofluorometer experiments were performed independently, at least twice.

Photography of B. burgdorferi expressing gfp. Fluorescent spirochaetes were centrifuged, rinsed in HN Buffer, suspended in HN Buffer and visualized and photographed by epifluorescence microscopy using a Nikon Eclipse E800 fitted with a V-2A filter block and an FDX 35 mm Nikon camera. Camera settings were as follows: DX setting of 1600 with exposure time of 2 s. The 35 mm film used was Kodak Ektachrome P1600 designed for push processing.

RESULTS

Construction and characterization of promoter-gfp fusion constructs

We amplified by PCR the regions 5′ to ospA, ospC and flaB of strain B31 and cloned them into pcDNA3.1/CT-GFP-TOPO to produce transcriptional fusions of these promoters to gfp (Fig. 1). These three promoter-gfp constructs were transformed into E. coli, resulting in numerous fluorescent clones from all constructs. Sequence analysis of random clones confirmed that all fluorescent E. coli contained plasmids with Borrelia promoters in the same orientation as gfp, whereas non-fluorescent clones contained Borrelia promoters in the opposite orientation. This suggested that E. coli recognized the ospA, ospC and flaB promoters. The promoter-gfp transcriptional fusions were excised, directionally cloned into the B. burgdorferi shuttle vector pBSV2 (Fig. 1) and transformed into E. coli. All shuttle vector constructs conferred a fluorescent phenotype in E. coli (not shown). A promoterless gfp construct (pBSV-gfp) was used as a negative control and did not fluoresce in any E. coli transformants (data not shown).

Expression of gfp in B. burgdorferi B31 and N40

pBSVΦ(flaBp-gfp), pBSVΦ(ospCp-gfp), pBSVΦ(ospAp-gfp) and the promoterless control (pBSV-gfp) were transformed into infectious B. burgdorferi B31 or N40 by electroporation (refer to Table 1 for clone designations and descriptions).
We obtained 7–300 colonies per transformation by this method (data not shown). Seven to 20 colonies (depending on the number of transformants) from each electroporation were picked, screened by PCR and analysed for gfp expression and the ability to regulate gene expression in response to pH. All B. burgdorferi clones harbouring promoter-gfp fusions were clearly visible by epifluorescence microscopy either when picked directly from an agar plate or after growth in BSK II or BSK-H medium. No fluorescence was observed by epifluorescence microscopy in any of the promoterless gfp construct transformants (not shown).

Transcriptional fusions of the ospA and flaB promoters to gfp (refer to Table 1 for descriptions) resulted in uniform fluorescence in B31. Likewise, pBSVΦ(flaBp-gfp) transformed into N40 conferred a similar phenotype to that of B31 (Fig. 2). Fusion of the ospC promoter to gfp resulted in heterogeneous fluorescence in B31, yet we routinely observed approximately 90% of the cells by fluorescence microscopy (Fig. 2 and data not shown). Fusion of the ospC promoter to gfp in N40 produced uniformly fluorescent spirochaetes with similar intensities (Fig. 2).

Plasmid profile of B. burgdorferi B31 expressing gfp

Plasmid loss is one potential factor in the loss of gene expression and/or virulence when B. burgdorferi is passaged in vitro (McDowell et al., 2001; Purser & Norris, 2000; Schwan et al., 1988; Xu et al., 1996). We determined the plasmid profiles of B31 transformants chosen for study. Elias et al. (2002) found that B. burgdorferi clone A3 lacked circular plasmid (cp)9 but contained all remaining 21 plasmids. In addition to the absence of cp9, our results indicated that B31-CGFP lacked linear plasmid (lp) 25, lp28-1 and lp36, B31-FGFP lacked lp25, lp28-1 and lp56, and B31-AGFP lacked lp25 alone (Table 1). It would be informative to determine the plasmid content of the B. burgdorferi N40 transformants as well, but we currently lack the tools to quickly and definitively establish a complete picture of the N40 plasmid profile.

Analysis of the GFP reporter in B. burgdorferi strains N40 and B31 under varying pH

We subjected B31 and N40 transformants harbouring the gfp constructs to changes in the pH of the medium. All B31 transformants chosen for further characterization in this study displayed the expected alteration in protein profile when cells were cultured at pH 7–8 relative to pH 8–9 (Fig. 3), but surprisingly the B31 clone A3 transformants did not down-regulate OspC at pH 8–9 as strongly as we have observed with a previous non-clonal B31 culture (Carroll et al., 1999). We have discovered that the original parental B31 clone A3 does not respond to changes in pH and temperature as robustly as other B31 isolates for reasons that are not entirely clear. By densitometry of immunoreactive bands from Western blots, the amount of OspC...
observed in all B31 transformants analysed averaged 4.2-fold higher in those grown in BSK-H at pH 7.0 compared to cells grown at pH 8.0 (Fig. 3 and data not shown). When these same cell lysates were probed with antiserum against c3 GFP, the only observable significant difference in the amount of c3 GFP was in samples from B31 clone A3 transformed with pBSVΦ(ospCp-gfp) (B31-CGFP). B31-CGFP produced 5.8-fold more c3 GFP when grown at pH 7.0 than at pH 8.0 (Fig. 3), suggesting that the amount of c3 GFP produced under these conditions was a direct reflection of the amount of OspC produced.

Interestingly, no change in the amount of OspC or c3 GFP was detected in cell lysates of N40 transformed with pBSVΦ(ospCp-gfp) grown at pH 7.0 or 8.0 (data not shown). In addition, when the parental N40 clone was grown in BSK-H at pH 7.0 and 8.0, we were unable to detect any change in the amount of OspC (data not shown). This suggested that in the N40 clone from which these transformants were derived that ospC is either regulated in a different manner than in B31 or the regulation of ospC in this N40 strain is altered resulting in constitutive expression in vitro.

**Fig. 2.** Fluorescence of spirochaetes transformed with *gfp* constructs. *B. burgdorferi* B31 and N40 *gfp* construct transformants (as labelled) were grown in BSK-H medium at pH 7.6 and 35 °C, rinsed and resuspended in HN Buffer, and then visualized and photographed using a Nikon E800 epifluorescence microscope at the same magnification.
We measured gfp expression in transformants with a spectrofluorometer to obtain a more sensitive and quantitative measurement of the alteration of gene expression in response to environmental pH (Fig. 4). Live spirochaetes were assayed for their intensity of fluorescence under varying pH, which is a reflection of the amount of c3 GFP present in the cells. The amount of fluorescence in cultures grown at pH 7.0 compared to pH 8.0 varied significantly in B31-CGFP, but not in the other B31 or N40 transformants. In B31-CGFP, a 3.5-fold increase in the amount of fluorescence was observed (1342 RFU at pH 7.0 compared to 380 RFU at pH 8.0), similar to the increase determined by densitometry of immunoreactive c3 GFP and OspC in cell lysates from B31-CGFP grown under identical pH conditions (Fig. 3). Spectrofluorometric analysis demonstrated that spirochaetes harbouring pBSVW (flaBp-gfp) were slightly more fluorescent at pH 8.0 relative to pH 7.0, with a mean increase of only 1.15-fold in B31 and 1.06-fold in N40 (Fig. 4). When B31 (not shown) or B31 transformed with the promoterless gfp construct (Fig. 4) was analysed by spectrofluorometer, no significant fluorescence was detected (4.66 RFU and 4.02 RFU, respectively).

**Fig. 3.** Immunoblot analysis of cell lysates from *B. burgdorferi* B31 GFP-expressing transformants at pH 7.0 and 35 °C versus pH 8.0 and 35 °C. Equivalent numbers of cells of each transformant were loaded per lane, separated by SDS-PAGE, transferred to nitrocellulose and probed with antiserum against either *B. burgdorferi* B31 (a) or GFP (b). Promoter-gfp fusion transformants are indicated above the lanes and the locations of FlaB, OspA, OspC and GFP are indicated. Relative molecular masses in kDa are indicated to the left of each immunoblot.

Analysis of GFP by temperature shift in *B. burgdorferi* B31 and N40

OspC synthesis (Schwan *et al.*, 1995) and *ospC* expression (Yang *et al.*, 2000) decrease when spirochaetes are shifted from 35 to 23 °C in medium, which correlates with the temperature variations observed during the infectious cycle (Schwan *et al.*, 1995). This response is enhanced when spirochaetes are shifted from pH 7.6 at 35 °C to pH 8.0 at 23 °C (Yang *et al.*, 2000). To determine if c3 GFP expression from these promoters was influenced by a shift in pH and temperature, c3 GFP-expressing transformants were grown in BSK-H at pH 7.0/35 °C and inoculated in BSK-H at pH 8.0 and grown at 23 °C. When spirochaetes were shifted to pH 8.0 and 23 °C, the *ospC* promoter fused to gfp did not confer fluorescence in B31, but the *ospC* promoter fused to gfp in N40 and the flaB promoter fused to gfp in B31 did impart fluorescence (not shown). By immunoblot of cell lysates of spirochaetes shifted to and grown at pH 8.0 and 23 °C, we could not detect OspC or c3 GFP in B31-CGFP, but could detect c3 GFP in B31-FGFP (Fig. 5). OspC and c3 GFP were readily detectable by immunoblot cell lysates of N40-CGFP grown at pH 8.0/23 °C (Fig. 5),

![Graph](image-url)
reaffirming that ospC regulation in vitro between these B31 and N40 clonal isolates differs.

DISCUSSION

Researchers are just beginning to develop the molecular tools necessary to decipher gene regulation and adaptation in B. burgdorferi. This pathogen has been shown to differentially express several genes, some alleged to play a role in pathogenesis, in response to environmental cues in vitro and in vivo (Babb et al., 2001; Carroll et al., 1999, 2000, 2001; Cassatt et al., 1998; Fikrig et al., 1998; Indest et al., 1997; Ramamoorthy & Philipp, 1998; Ramamoorthy & Scholl-Meeker, 2001; Schwan et al., 1995; Stevenson et al., 1995; Yang et al., 2000). To better understand and study gene regulation in B. burgdorferi, we have developed a reporter system using a promoter-gfp transcriptional fusion carried on the shuttle vector pBSV2 (Stewart et al., 2001) and introduced it into two electroporation-competent, clonal, infectious B. burgdorferi sensu stricto strains (B31 and N40) (Fig. 2). Unfortunately the resultant B31 isolates presented here are likely to be non-infectious due to the loss of the virulence-associated plasmids lp25 and/or lp28-1 (McDowell et al., 2001; Purser & Norris, 2000).

All three promoter constructs proved to be functional in E. coli, B31 and N40, making it feasible to screen for gfp construct transformants merely by fluorescence. This can be attributed to the gfp used in these studies (c3 GFP), which was developed by DNA shuffling to have an improved

Fig. 4. Spectrofluorometric analysis of fluorescence from B. burgdorferi B31 and N40 gfp construct transformants at pH 7-0 and 35 °C, and at pH 8-0 and 35 °C. RFU values are indicated on the left. Transformants and pH are as indicated. Data are the means of two independent experiments with bars indicating the range.

Fig. 5. Immunoblot analysis of cell lysates of B. burgdorferi B31 and N40 gfp construct transformants when shifted from pH 7-0 and 35 °C to pH 8-0 and 23 °C. Equivalent numbers of solubilized cells from pH 8-0 and 23 °C were loaded per lane and proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-GFP (a) and then probed with polyclonal serum against OspC (b). Locations of OspC and GFP are indicated. Relative molecular masses in kDa are indicated to the left of each panel.
solubility with a greater than 40-fold increase in fluorescence over that of wild-type GFP (Cramer et al., 1996). While other derivatives of the GFP allele have been introduced into *B. burgdorferi*, their utility as a practical reporter to monitor gene expression was not determined. Sartakova et al. (2000) introduced the enhanced GFP allele from Clontech transcribed from the *flaB* promoter into a high-passage (presumably avirulent) *B. burgdorferi B31*, but this allele was weakly expressed and only fluorescent after aeration of the culture. Likewise, Eggers et al. (2002) recently introduced a cp32-based shuttle vector containing the *gfpmut1* (GFPmut1), *yfp* and *cfp* under the control of the constitutive *flaB* promoter into a clone of *B. burgdorferi 297* (297-c155) and into high-passage *B. burgdorferi* B31. Their *gfpmut1* construct was more fluorescent in *B. burgdorferi* than that of Sartakova et al. (2000) and did not require aeration.

The c3 GFP allele used in our study may have several advantages over the allele used by Eggers et al. (2002). Although a direct comparison of the c3 GFP and the GFPmut1 alleles has not been made, a comparison of the fluorescence of both relative to the GFPmut2 allele (Cormack et al., 1996; Cramer et al., 1996) suggests that c3 GFP is 18-fold more fluorescent than GFPmut1 in *E. coli*. Thus, the use of c3 GFP may allow for the detection of lower levels of transcript from promoters of interest in *B. burgdorferi*. Furthermore, c3 GFP has been shown to fold more efficiently at higher temperatures (Patterson et al., 1997; Tsien, 1998; Yokoe & Meyer, 1996) than other GFP derivatives, yielding less lag time between expression and fluorescence (Albano et al., 1996). This indicates changes in gene expression can be more accurately measured in real time with c3 GFP.

The validity of gene expression data gathered by using GFP has gained attention due to concerns with cytoplasmic accumulation of GFP, a remarkably stable protein. A recent report by Lu et al. (2002) demonstrates that the obstacle associated with c3 GFP accumulation can be overcome and changes in gene expression can be accurately estimated in near real time by measuring the rate of change in c3 GFP fluorescence over time. We did not design our initial studies to monitor the kinetics of gene expression, but results suggest that accumulation of c3 GFP was not a concern due to comparable changes in OspC synthesis, GFP synthesis and fluorescence in B31 transformed with pBSVΦ(ospCp-gfp) in response to pH.

We were careful to perform all GFP experiments in the same lot number of fresh BSK-H to ensure accuracy and reproducibility. Our results indicate that 179 bp 5′ of the first codon of *ospC* are sufficient for regulation in response to temperature and pH in strain B31 A3. This observation narrows down the functional promoter/operator region of *ospC* to within the 179 bp reported here from the over 500 bp used by Sohaskey et al. (1997) in their CAT assay.

Results with N40 were quite different from those observed with B31. The discrepancy in *ospC* regulation in response to pH or temperature between clonal isolates of strains B31 and N40 was surprising. The cloned *ospC* operator/promoter from B31 fused to *gfp* shares greater than 96% identity with that of N40 (not shown), yet when the B31 *ospC* promoter-gfp construct was transformed into N40 it was no longer regulated by environmental signals. Clonal variation in spirochaete populations is not uncommon (Ohnishi et al., 2001; Schwan et al., 1995) and our data suggest that the lack of regulation is not due to the cloned promoter from B31, but is likely to be the result of an uncharacterized variation in the N40 strain used in these studies.

There does appear to be a difference in the RFU values between B31 and N40 *gfp* construct transformants. B31 *gfp* construct transformants gave higher RFU values per cell than did the comparable N40 transformants when grown at pH 7.0 (Fig. 4). This might reflect differences in promoter strengths, proper c3 GFP folding, protein turnover or differences in cell volumes between strains. Moreover, we observed no difference in the levels of c3 GFP in B31 transformed with pBSVΦ(ospAp-gfp) grown at pH 7.0 or 8.0 (Figs 3 and 5). These observations correlate with our previous results with a non-clonal *B. burgdorferi* B31, in which we detected no change in the amount of OspA or in the amount of *ospA* transcript at pH 7.0 relative to pH 8.0 (Carroll et al., 1999, 2000). Our results differ somewhat from those of Yang et al. (2000) who reported that in strains B31 and 297 they detected a decrease in *ospA* transcript and in the amount of OspA in spirochaetes grown at pH 6.8 relative to pH 8.0, albeit with some inconsistency. We did not grow B31-AGFP at pH 6.8 because our earlier studies concluded that the amount of OspA was not significantly altered with growth of B31 at pH 6.0, 7.0 or 8.0 (Carroll et al., 1999). Differences could exist between lab strains, clonal isolates or the way the experiments were performed that might account for this discrepancy in *ospA* response to pH.

We have developed and implemented a GFP reporter system in *B. burgdorferi* for monitoring gene regulation in response to pH and temperature. The studies presented here using promoter-gfp fusion constructs demonstrate and validate the utility of our GFP reporter system in *B. burgdorferi*. With the caveat that our data were derived from individual *B. burgdorferi* clones, our results suggest that clones of *B. burgdorferi* strain B31 and N40 may differ in their mechanisms of *ospC* regulation in response to environmental signals in *B. burgdorferi*. The regulation of *ospC* expression by other genospecies that cause Lyme disease in response to pH and temperature should be performed using similar methods. This functional, easily assayable reporter system could be used in a multitude of scenarios, such as monitoring target gene expression in ticks or mammals, tracking spirochaetes in tissues and as a rapid way to screen transformants. Monitoring gene expression during the infectious cycle will provide valuable insight into how the spirochaete responds to its environment and makes the
changes necessary for adaptation and infection. Moreover, this reporter system could play a central role in identifying and characterizing regulatory elements in B. burgdorferi.

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