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A comparison of the DNA binding and bending capacities and the oligomeric states of the immunity repressors of heteroimmune coliphages P2 and WΦ

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ABSTRACT

Bacteriophages P2 and WΦ are heteroimmune members of the P2-like family of temperate *Escherichia coli* phages. Temperate phages can grow lytically or form lysogeny after infection. A transcriptional switch that contains two convergent promoters, Pe and Pc, and two repressors regulate what life mode to enter. The immunity repressor C is the first gene of the lysogenic operon, and it blocks the early Pe promoter. In this work, some characteristics of the C proteins of P2 and WΦ are compared. An *in vivo* genetic analysis shows that WΦ C, like P2 C, has a strong dimerization activity in the absence of its DNA target. Both C proteins recognize two directly repeated sequences, termed half-sites and a strong bending is induced in the respective DNA target upon binding. P2 C is unable to bind to one half-site as opposed to WΦ, but both half-sites are required for repression of WΦ Pe. A reduction from three to two helical turns between the centers of the half-sites in WΦ has no significant effect on the capacity to repress Pe. However, the protein–DNA complexes formed differ, as determined by electrophoretic mobility shift experiments. A difference in spontaneous phage production is observed in isogenic lysogens.

INTRODUCTION

The original classification of the temperate P2-like phages was based on common characteristics such as serological relatedness, host-range and non-inducibility by UV light and serologically unrelatedness to phage lambda (1). Since then, several phages or prophages have been found in various γ-proteobacteria that based on similarities of genome organization and gene content have been classified as P2-like, for a recent review see (2). P2-like phages are common in *Escherichia coli*, and ~30% of the strains in the *E. coli* reference collection ECOR (3) contain P2-like prophages (4). An analysis of the structural genes of 18 isolated P2-like coliphages has shown that they have at least 96% identity to those of P2, and therefore might be considered as different isolates of P2 (5). However, a sequence analysis of the immunity repressors of 19 P2-like prophages in the ECOR collection and 13 P2-like coliphages identified seven different immunity classes (6).

Temperate phages have after infection a choice either to grow lytically or form lysogens. A developmental, or transcriptional switch determines which pathway the phage will enter. The transcriptional switches of the P2-like phages have some common characteristics, i.e. two converging promoters and two repressors that recognize different operators. Thus, they differ from the well characterized transcriptional switch of phage λ where the repressors recognize the same three operators containing a 17-nt long inverted repeat, although with different affinities (7). The immunity repressors are termed C or CI, and the lytic repressors are termed Cox or Apl. The immunity repressors of the P2-like phages can be divided into two types depending on size, sequence similarity and control; the 186-like CI proteins and the P2-like C proteins. The CI protein of phage 186 is 196 aa long and consists of two domains. The N-terminal domain contains the DNA-binding motif and the C-terminal domain contains the oligomerization interface (8). The CI repressor recognizes three inverted repeats of two types in the early promoter region (9,10). In addition, there are distantly located operators and cooperative binding between the operators in the early promoter region and the distal operators has been demonstrated (11,12). The structure of the C-terminal domain of 186 CI has been

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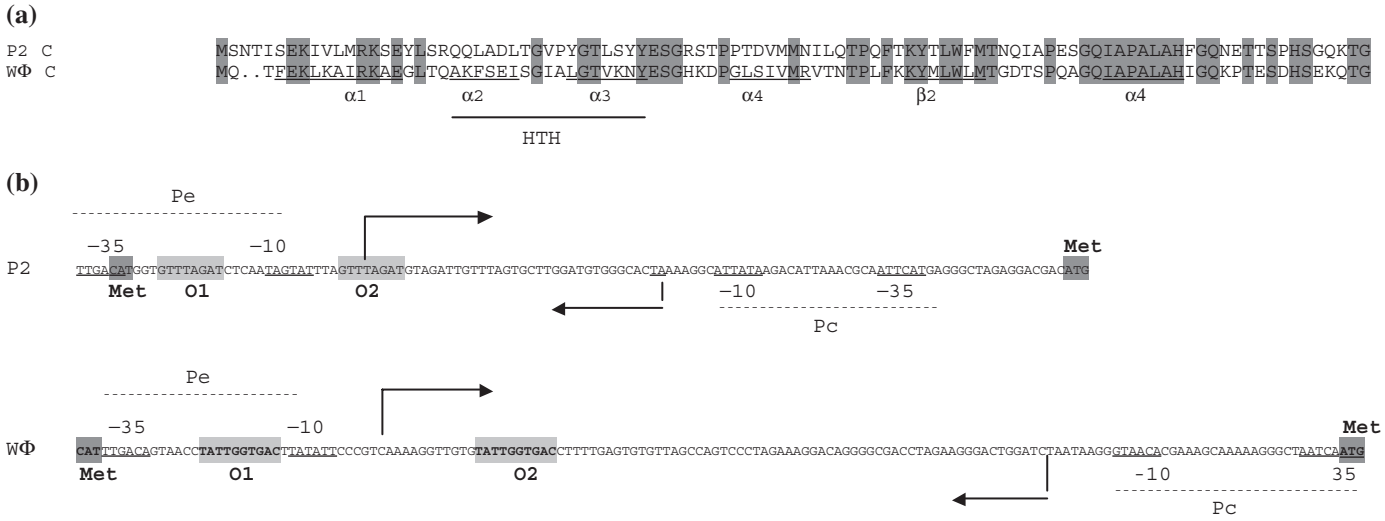

Figure 1. A comparison of the amino acid sequences and the transcriptional switch regions of phages P2 and WΦ. **(a)** Amino acid sequences and predicted secondary structures of the C proteins. Common amino acids are shaded. The predicted α helices and the β sheet are underlined, and the α helices believed to constitute the HTH DNA-binding motif are indicated. **(b)** DNA sequence of the transcriptional switches, where the C operators and the start codons of the respective operon are shaded. The −10 and −35 regions of the respective promoter are underlined and the transcriptional start sites are indicated by the bent arrows.

determined, and it has been shown to form a heptamer of dimers that are believed to wrap the operator DNA around the outside of the large 14-mer (13). Like phage λ the *cI* gene of phage 186 has two promoters, one for establishment of lysogeny and another for its maintenance. The promoter for establishment of lysogeny is activated by the phage-encoded CII protein (14,15).

The P2-like C proteins are small homologous proteins ~100 aa long. They recognize directly repeated sequences in the early promoter region and are expressed from one promoter only, thus no CII like protein has been identified. The C proteins of phage P2 and Wϕ represent two different immunity groups, where the operators have been located (16,17). The identity between the C repressors is 43% at the amino acid level (Figure 1a). The C proteins recognize a pair of direct repeats, but the repeats differ in sequence, length, spacing and location relative to the early promoter (Figure 1b).

To gain further insight into the transcriptional switches of the P2-like phages we have made comparative analyses of the C proteins of the heteroimmune *E. coli* phages P2 and WΦ.

MATERIALS AND METHODS

Biological materials

Bacterial strains and plasmids used are listed in Table 1

Plasmid constructions

Plasmid constructions were performed according to standard procedures (31). Oligonucleotides used were obtained from DNA Technology, Aarhus C, Denmark or Thermo Electron Corp, Erlangen, Germany. All constructs were confirmed by DNA sequencing (Macrogen Inc., Seoul, Korea or on an ALFexpress II, using the ThermoSequenase Kit, GE Healthcare, Piscataway, NJ, USA).

pEE865. Pairs of oligonucleotides, containing a hybrid of P2 O1 and WΦ O2 (Hy2 for 5′ CGG GAT CCT TGA CAT GGT GTT TAG ATC TCA ATA GTA TTA GTT TAG ATG TAG ATT GTT TAG TGC TTG GAT GTG GGC ACT AAA AGG CAT TAT AAG ACA TTA AAC GCA ATT CAT GAG GGC TAG AGG ACG ACA TG and Hy2 rev 5′ CCC AAG CTT GTC ACC AAT ACA CAA CCT TTT GAC GGG ATT ATA TTG AGA TCT AAA CAC CAT GTC AAG GAT CCC G) were hybridized. The recognition sequence for BamHI was included in one end of the oligonucleotide and a HindIII site at the other end. After digestion with the two restriction enzymes, the fragment was inserted between the BamHI and HindIII sites of pKK232-4. The locations of the P2 O1 and WΦ O2 in the cloned fragment are indicated by under linings. The center-to-center distance is 37 nt. In this construct, P2 O1 has the wt distance to the −10 region, and the wt distance between the −10 region and WΦ O2.

pEE866. Pairs of oligonucleotides containing WΦ O1 and P2 O2 (Hy3 for 5′ CGG GAT CCT TGA CAG TAA CCT ATT GGT GAC TTA TAA TTT ACC GTC AAA AGG TGG GTT TAG ATA AGC TTG GG and Hy3 rev 5′ CCC AAG CTT ATC TAA ACC CAC CTT TTG ACG GTA AAT TAT AAG TCA CCA ATA GGT TAC TGT CAA GGA TCC CG) were hybridized. The recognition sequence for BamHI was included in one end of the oligonucleotide and a HindIII site at the other end. After digestion with the two restriction enzymes, the fragment was inserted between the BamHI and HindIII sites of pKK232-4. The locations of the WΦ O1 and P2 O2 in the cloned fragment are indicated by under linings. The center-to-center distance is 33 nt. In this construct, WΦ O1 has the wt distance to the −10 region,

Table 1. Bacterial strains

	Pertinent features	Origin/reference
Bacteria		
AG1688	*E. coli* MC1061, F′ 128 *lacI*^{q} *lacZ*::Tn5	18
AG1688 ($\lambda 112O_SP_S$)	AG1688 containing λ112OsPs with *lacZ* driven by λO_SP_S and a strong upstream λ operator	19
AG1688 (λXZ970)	AG1688 containing λ112OsPs with *lacZ* driven by λO_SP_S and a 434 upstream promoter	19
C-1a	Prototrophic *E. coli* C strain	20
C-117	C-1a lysogenized with P2	21
C-1920	C-1a lysogenized with Wϕ	22
C-1757	Auxotrophic, *supD str*	23
JH372	AG1688 containing λ202 with *lacZ* driven by P_RO_R	18
Plasmids		
pACYC 177	p15 derivative used for the cloning	24
pBend2	pBR322 derivative containing tandemly repeated restriction enzyme cleavage cassettes	25
pEE675	pKK232-8 derivative containing P2 C-Pe-Pc region where Pe directs the *cat* gene	26
pEE679	pET8c derivative expressing P2 C. This plasmid has been found to contain a tandem DNA fragment downstream of the C gene from phage P4 (7870–8582).	27
pEE695	pJH derivative where P2 c is fused to the N-terminal domain of λ *cI*	27
pEE865	pKK-232-8 derivative containing Hy2 operator-promoter region controlling the *cat* gene	This work
pEE866	pKK-232-8 derivative containing Hy3 operator-promoter region controlling the *cat* gene	This work
pEE900	pET16b derivative containing Wϕ *C* gene	17
pEE902	pACYC 177 derivative containing Wϕ *C* gene	17
pEE905	pKK232-8 derivative with Wϕ Pe-Pc region inserted into the SmaI site; Pe directs the *cat* gene	17
pEE923	pJH391 derivative where Wϕ C gene is fused to the N-terminal domain of the λ *cI* gene	This work
pEE924	pBend2 derivative containing the P2 C operator region	This work
pEE925	pBend2 derivative containing the WΦ C operator region	This work
pEE938	pKK232-8 derivative where the Wϕ Pe promoter-operator region controls the *cat* gene	This work
pEE939	pEE938 derivative containing a 6 nt deletion between the transcriptional start site and O2	This work
pEE940	pEE938 derivative containing a 10 nt deletion between the transcriptional start site and O2	This work
pEE941	pSS32-1 derivative where O1 has been changed from GTTAGAT to ATCCGAGT	This work
pEE942	pEE905 derivative where O1 has been changed from TATTGGTGAC to CGCCAACAGT	This work
pEE1020	pET8c derivative expressing WΦ C	This work
pET8c	pBR322 derivative containing the T7 promoter	28
pGF157	λ *cI ind1* driven by the *lacUV5* promoter	18
pJH370	Fusion between λ *cI* and the leucin zipper of GCN4 driven, by the *lacUV5* promoter	18
pJH391	First 132 nt of λ *cI* fused with *lacZ* driven by the *lacUV5* promoter	18
pJH622	Fusion between λ *cI* and the reengineered leucin zipper of GCN4, driven by the *lacUV5* promoter	19
pKH101	Expresses only the N-terminal DNA-binding domain of λ *cI*	18
pKK232-8	pBR322 derivative containing a promoterless *cat* gene	29
pSS29-2	pKK232-8 derivative containing the P2 PcPe region where Pc controls the *cat* gene	30
pSS32-1	pKK232-8 derivative where P2 Pe controls the *cat* gene	30
pSS39-6	pKK232-8 derivative where P2 Pc controls the *cat* gene	30
pZ150	pBR322 derivative with an M13 origin	19

and P2 O2 is placed at the normal center-to-center distance of the WΦ operators.

pEE923. The WΦ *C* gene was amplified by PCR using primers SalIWΦCfor (5′ CTA GAC GCG TCG ACG ATG CAG ACA TTC GAA AAA CTG AAA GC) and BamHIWΦCrev (5′ CGC GGA TCC GGT AAC AAA TGG TGA AAA TG) and after cleavage with SalI and BamHI, it was inserted into plasmid pJH391 cleaved with SalI and BamHI.

pEE924. The P2 operator region was amplified by PCR using primers 76.9R (5′ CTG ATT TTC GCA TTA AGA CTA TC) and 77.4aL (5′ CCT CAT GAA TTG CGT TTA ATG TC), and the 139-nt long fragment was inserted into the filled in SalI site of pBend2. In the cloned fragment the operators are flanked on the left side by 49 nt and on the right side by 67 nt of P2 sequences.

pEE925. The WΦ operator was amplified by PCR using primers WΦ8R (5′ GCT TTC CTA ATC GCT TTC AG) and WΦ8.5L (5′ CTT CTA GGT CGC CCC TGT C), and the 144-nt long fragment was inserted into the filled in SalI site of pBend2. In the cloned fragment, the operators are flanked on the left and right side by 50 nt of WΦ sequences.

pEE938. The WΦ wild-type Pe promoter/operator region was inserted between the BamHI and HindIII sites of pKK232-8 by hybridizing the primers WPhi wtop (5′ GAT CCT TGA CAG TAA CCT ATT GGT GAC TTA TAT TCC CGT CAA AAG GTT GTG TAT TGG TGA CA) and WPhi bot (5′ AGC TTG TCA CCA ATA CAC AAC CTT TTG ACG GGA ATA TAA GTC ACC AAT AGG TTA CTG TCA AG). The locations of the O1 and O2 half-sites are indicated by under linings.

pEE939. The WΦ Pe promoter/operator region containing a 6 nt deletion between the transcriptional start site and O2 was inserted between the BamHI and HindIII sites of pKK232-8 by hybridizing the primers WPhiop −6nttop (5′ GAT CCT TGA CAG TAA CCT ATT GGT GAC TTA TAT TCC CGT CAA AAG TAT TGG TGA CA) and WPhiop −6ntbot (AGC TTG TCA CCA ATA CTT

TTG ACG GGA ATA TAA GTC ACC AAT AGG TTA CTG TCA AG). The locations of the O1 and O2 half-sites are indicated by under linings.

pEE940. The WΦ Pe promoter/operator region containing a 10 nt deletion between the transcriptional start site and O2 was inserted between the BamHI and HindIII sites of pKK232-8 by hybridizing the primers WPhiop −10top (GAT CCT TGA CAG TAA CCT ATT GGT GAC TTA TAT TCC CGT CTA TTG GTG ACA) and WPhiop −10bot (5′AGC TTG TCA CCA ATA GAC GGG AAT ATA AGT CAC CAA TAG GTT ACT GTC AAG). The location of the O1 and O2 half-sites are indicated by under linings.

pEE941. Plasmid pSS32-1 was used as template for construction of an O1 library using a degenerate primer {TCGTGTTTGACATGGTKPPPKKKPCTCAATAGT ATTTAGTTTAG (K = G or A, P = T or C)} and the Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The plasmid chosen has O1 changed from GTTTAGAT to ATCCGAGT.

pEE942. Plasmid pEE905 was used as template for construction of an O1 library using a degenerate primer {GCATTTGACAGTAACCPKPPKKPKKPTTATATT CCCGTCAAAAGG (K = G or A, P = T or C)} and the Multi Site-Directed Mutagenesis Kit (Stratagene). The plasmid chosen has O1 changed from TATTGGTGAC to CGCCAACAGT.

pEE1020. The WΦ *C* gene was amplified by PCR, using primers WϕCstartNcoI (CATGCCATGGCCATGCAG ACATTCGAAAAACTGAAAGC) and WϕCendBamHI (CGGGATCCTGTAATGTTTATAGAGTGTTAACC) and after cleavage by BamHI and NcoI it was inserted between the NcoI and BamH1 sites of pET8c, which places the *C* gene under the control of the T7-promoter.

Primer extension

RNA was prepared from 4.5 ml culture of *E. coli* C-1a pSS29-2 or 9 ml culture of *E. coli* C-1a pSS39-6 ($OD_{600} = 0.6$) using the Rneasy minikit (Qiagen, Valencia, CA, USA). The preparations were ethanol precipitated to increase the purity and the concentration. The purity of the extracted RNA was determined spectroscopically, an A260/280 ratio greater than 1.7 being regarded as acceptable. Primers were 5′ labeled with [γ-^{32}P]-ATP (GE Healthcare) and purified using a microSpin S-50HR column (GE Healthcare). The extension reactions were carried out with AMV reverse transcriptase and the Promega Primer Extension system, Madison, WI, USA. The Pe transcript was detected with the P2 Pe primer (5′ CGC ATC AGT CAT GAG TGT TAC TTG CTT GCT CAT GTC GTC C) using 10 μg of total RNA extracted from C-1a pSS29-2, and the Pc transcript was detected with the P2 Pc primer (5′ GTC GAA GCT CGG CGG ATT TGT CCT ACT CAA GCT TG) using ∼35 μg of total RNA extracted from C-1a pSS 39-6. Extended products were separated on a 5% polyacrylamide gel with 7 M urea in 1× TBE (50 mM Tris, 100 mM boric acid, 5 mM EDTA). The gels were dried prior to autoradiography.

In vivo dimerization/oligomerization assay

An *in vivo* plasmid reporter system for the analysis of dimerization has been developed by Hu *et al.* (18), and modified to also discriminate between dimerization and the formation of higher order structures (19). The reporter system utilizes the λ CI DNA-binding domain, which by itself is unable to repress a reporter gene, but when fused to a protein forming dimers or higher oligomeric forms the reporter gene will be repressed.

The ability of the λ CI-C fusion proteins to repress the reporter gene constructs was determined by measuring the β-galactosidase activity in cells grown in A medium supplemented with 0.4% glucose, 1 mM $MgSO_4$, 1% casamino acids and 1 μg vitamin B1 per ml according to (31), but the β-galactosidase activity was measured at 405 nm instead of 420 nm.

Chloramphenicol acetyltransferease (CAT) activity determination

Ten milliliter cultures were harvested at an OD_{600} of 0.8. The cultures were washed in 100 mM Tris–HCl, pH 7.9 and lyzed by sonication in a total volume of 2 ml, and the cell extracts were cleared by centrifugation. Total protein concentration was determined using serum albumin as standard (32). The supernatants were diluted and an equal amount of protein was added for the CAT determinations with [^{14}C]-chloramphenicol, unless otherwise stated. The acetylated forms were separated by thin-layer chromatography (33), and the CAT activity was calculated after phosphor image analysis (Fuji Film FLA-3000) as the amount of acetylated chloramphenicol divided by the total amount of chloramphenicol.

Protein purification

Escherichia coli strain BL21(DE3) (28), containing plasmids pEE679 (expressing P2 C) (27), or pEE1020 (expressing Wϕ C) were grown at 37°C in LB or M9 minimal medium with ampicillin (100 μg/ml). The expression of the respective C protein was induced at $OD_{600} = 0.6$ by adding IPTG to a final concentration of 0.2 mM, since the *C* genes were under the control of the T7 promoter and the T7 polymerase under the control of the *lac* promoter. The cells were harvested by centrifugation, 20 min 5500 r.p.m. at 4°C, and resuspended in 10 mM sodium phosphate pH 7.0. The cells were lyzed by sonication followed by centrifugation, 13 000 r.p.m. at 4°C. The supernatants were filtered before a two-step purification was carried out in ÄKTA^{TM}FPLC-system. In the first purification step, a DEAE (a weak anion exchanger) was used after equilibration with 10 mM sodium phosphate pH 7.0, which was also used as running buffer. The proteins were eluted with the flow-through, since the sample pH was just below the Ip of the proteins. This step was followed by affinity chromatography using a HiTrap Heparin HP column. It was equilibrated with 10 mM sodium phosphate, which was also used as a running buffer. Before loading the sample, the pH was

adjusted to 6.8. The protein was captured in the column and eluted using a salt gradient (10 mM sodium phosphate and 1 M NaCl). The proteins were recovered at 40% NaCl concentration. After each purification step, the samples were analyzed on 10–15% sodium dodecyl sulphate (SDS) polyacrylamide gels in a Phast-system and stained with Coomassie brilliant blue. All chromatography columns and protein gels were obtained from GE Healthcare, as well as ÄKTA^{TM}FPLC and Phast systems. The protein concentrations were determined by the Bradford method as described above. The specific activity of different protein preparations varied, therefore the amount of protein added in different experiment varies.

The purified WΦ C protein was stored in 10 mM sodiumphosphate pH 7.0, 0.6 M NaCl at 4°C with 20% (v/v) glycerol. The P2 C protein was stored in 18 mM Tris–HCl pH 7.5, 1 mM sodiumphosphate pH 7.0, 0.55 M NaCl, 4.5 mM $MgCl_2$, 0.9 mM DTT at −20°C with 36% (v/v) glycerol.

Circular permutation assay

The P2 C and WΦ C operators were cloned in the pBend2 vector (25), generating plasmids pEE924 and pEE925, respectively. Plasmid pBend2 contains a tandemly repeated polylinker with unique cloning sites in between. The region containing either the P2 C or the WΦ C operator and the restriction sites from the pBend2 plasmid was amplified by PCR using primers pBR322 EcoRI cw (5′ GTA TCA CGA GGC CCT T) and pBR HindIII ccw (5′ TAA CTG TGA TAA ACT ACC GC). The PCR fragments were purified using QIAquick PCR Purification Kit (Qiagen) and cleaved with pertinent restriction enzymes (MluI, ClaI, XhoI, EcoRV, SmaI and BamHI), thereby generating fragments identical in size but with the respective operator at different positions relative to the ends. The cleaved fragments were purified by gel extraction from a 2% agarose gel using QIAEX II Gel Extraction Kit (Qiagen), dephosphorylated with Shrimp alkaline phosphatase (Fermentas, Hanover, MD, USA) and 5′ end labeled using [γ-^{32}P]-ATP (GE Healthcare) and T4 polynucleotide kinase (Fermentas). The end-labeled fragments were purified using MicroSpin G-25 columns (GE Healthcare), and incubated with the respective C repressor. Here 1.2 nM of DNA containing the P2 C operator was incubated with 1.5 nM of purified P2 C repressor, and 1.2 nM of DNA containing the WΦ C operator was incubated with 220 nM of purified WΦ C repressor at 30°C for 20 min in a final volume of 20 µl including 4 µl buffer 5× BB (60 mM HEPES–NaOH pH 7.7, 60% glycerol, 20 mM Tris–HCl pH 7.9, 300 mM KCl, 5 mM EDTA, 0.05 µg/µl poly dI/dC, 0.3 µg/µl BSA and 5 mM DTT). WΦ C was diluted in A+ buffer (0.3 M potassium phosphate, pH 7.5, 3 mM EDTA), supplemented with 0.5 M KCl and the volume used in the reaction was 4.2 µl, thus the reactions were supplemented with buffer A+ accordingly. The P2 C protein was diluted in 20 mM Tris–HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA, 1 mM DTT and 40% (v/v) glycerol. The protein volume used in the reactions was 2.5 µl, thus the reactions were supplemented with the buffer mentioned above accordingly. The reactions were incubated at 30°C for 20 min and loaded onto a non-denaturing 5 or 8% polyacrylamide gel and submitted to electrophoresis at 20 mA in 0.5× TBE for ∼2 (5%) or 3.5 h (8%) with running water for cooling in a Protean II xi Cell (Bio-Rad, Hercules, CA, USA). The gels were dried prior to analysis using phosphor imager. The bending centers and the bending angles were calculated by applying the experimental data from the 8% polyacrylamide gel electrophoresis to the formula $\mu M/\mu E = \cos \alpha/2$, where µM is the complex with the lowest mobility, µE is the complex with the highest mobility and α is the bending angle (34).

Electrophoretic mobility shift assay (EMSA)

To analyze the capacity of the C proteins to bind to different operator/promoter constructs, pertinent plasmids were used for PCR amplifications. The DNA fragments used are described in the figure legends. The PCR-generated DNA fragments were labeled, purified and incubated with the C proteins and run on a 5% non-denaturing polyacrylamide gel as described in the circular permutation assay. Also, 100 ng DNA template was labeled and after the purification by using MicroSpin G-25 columns, the final DNA concentration in the reaction was calculated to be 1 nM unless otherwise stated.

Spontaneous phage production from P2 and WΦ lysogens

To compare the spontaneous phage production of a P2- and a WΦ lysogen, colony-forming units (CFU) and plaque-forming units (PFU) were determined from cultures of C-117 (P2 lysogen) and C-1920 (WΦ lysogen). The cultures were grown overnight in 30°C in LB with 0.2 M potassium phosphate (pH 6.8) to avoid read-sorption. The CFU per ml was determined by plating on LA plates. After removal of bacteria by centrifugation and addition of a few drops of $CHCl_3$ to the supernatant, the PFU per ml was determined on LA plates supplemented with 2.5 mM $CaCl_2$ with C-1757 bacteria as the indicator. The titers of free phages and bacteria were determined from seven isolated colonies.

RESULTS

The C repressor proteins of P2 and WΦ are similar, but the operators differ in spacing between the two repressor-binding sites

The sequences of the immunity repressor genes and their operators have been determined (16,17,35). As can be seen in Figure 1a, the repressors are small slightly basic proteins with 43% identity. The predicted secondary structures contain a potential HTH motif in the N-terminus. The spacing between the half-sites in the direct repeat constituting the repressor binding sites varies between P2 and WΦ (Figure 1b). In P2, the center-to-center distance between the half-sites is two helical turns (22 nt), and in WΦ approximately three helical turns (34 nt). The transcriptional start sites of the promoters of the early operon (Pe) and the lysogenic operon (Pc) of WΦ have been determined previously (17), while those of P2

are not known. To locate the Pe and Pc of P2, primer extensions were performed. To locate the P2 Pe promoter, total RNA was extracted from plasmid pSS29-2 that contains the P2 PcPe region cloned into the *cat* reporter plasmid pKK232-8. A primer located 125 nt downstream of the presumed −10 region was used to detect the start site of the Pe transcript. A major extended cDNA band corresponding to ~118 nt in length was found, and this fits well with the presumed start site (Figure 2a). To locate the Pc promoter, a *cat* reporter gene vector where Pc directs the *cat* gene, pSS39-6, was used. For the weaker Pc promoter to be detectable, the opposing Pe promoter had to be inactivated and in this construct the −35 region of Pe has been deleted. Total RNA was extracted and a primer located 155 nt downstream of the start codon of the *cox* gene was used for the extension reaction. The resulting cDNA was a weak band of ~105 nt (Figure 2b).

The spacing between the operator half-sites in the Pe region of WΦ has no significant effect on the capacity of the WΦ C protein to block the expression of a reporter gene

Since the spacing between the *C* operator half-sites varies between P2 and WΦ, it is of interest to determine the effects of changing the distance with a half or a full helical turn. In the case of P2, this cannot be done without affecting the Pe promoter and the transcriptional start site. In WΦ it is possible to change the distance between the transcriptional start site and O2 without affecting the promoter (Figure 1b). Therefore, the wild-type WΦ operator/promoter region and two constructs removing 6 or 10 nt, respectively, upstream of the O2 half–site were inserted in front of a promoter-less *cat* gene (plasmids pEE938–pEE940). The capacity of the WΦ C protein to repress the wild type and the modified operators were analyzed by determination of the CAT activity after transformation into a WΦ lysogenic strain. As can be seen in Figure 3, there is no significant difference in the level of CAT expression using the wild type and the modified

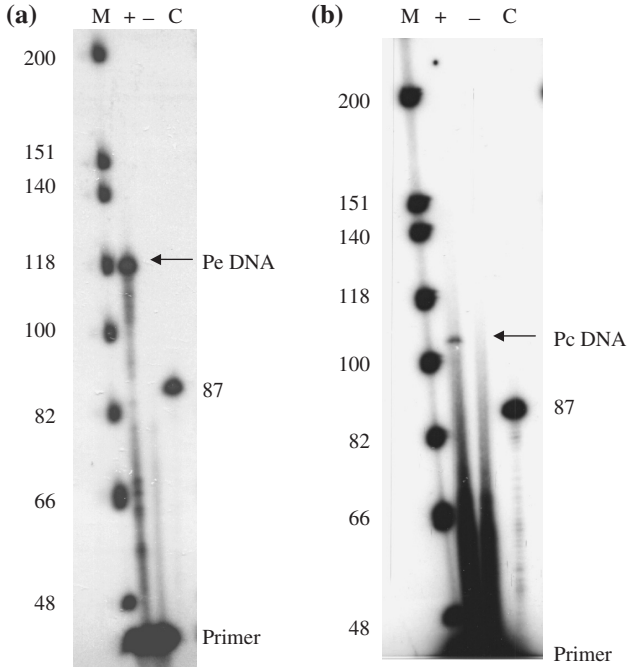

Figure 2. Primer extension experiments with P2 Pe (**a**) and P2 Pc (**b**) promoters. Lane M, ΦX174 HinfI DNA marker (fragment sizes indicated on the side). Lane+ contains the cDNA product made from Pe (a) or Pc (b). Lane−, primer extension reaction with no RNA added. Lane C, control cDNA of 87 nucleotides. Locations of the primers are indicated.

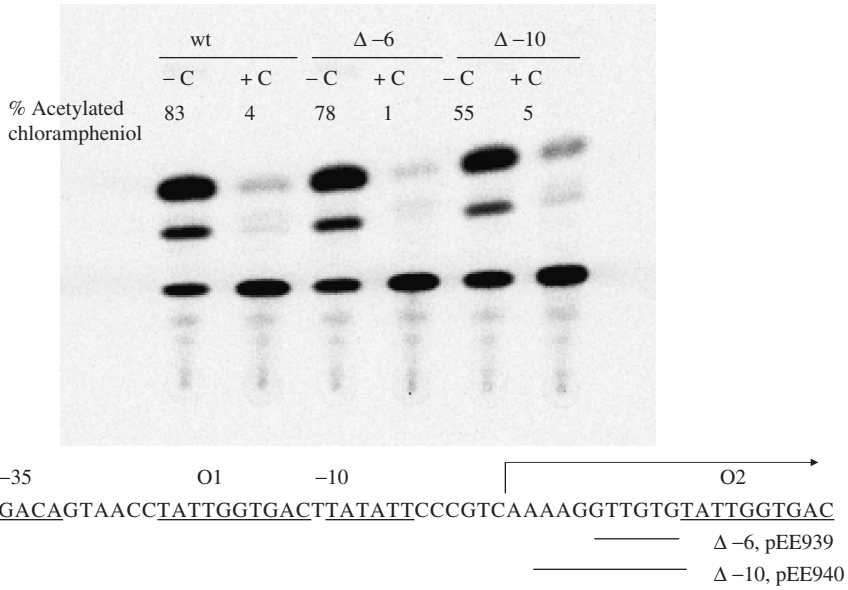

Figure 3. CAT assays of WΦ Pe where the distance between the operator half-sites is changed. The phosphoimage of the CAT analysis using the wt WΦ Pe promoter/operator region (pEE938), or the promoter regions containing a 6 nt deletion (pEE939) or a 10 nt deletion (pEE940) upstream of the O2 half-site. An equal concentration of cell extract was used in each CAT assay. The sequence of the Pe promoter/operator region, where the locations of the 6 and 10 nt deletions are indicated is shown below the CAT assay. The −10 and −35 regions and the operator half-sites are underlined, and the bent arrow indicates the transcriptional start site.

operators, and the level is only 1–9% of the activity obtained with the respective fully derepressed Pe promoter, i.e. in the absence of C. Thus, the spacing between the two operator sites of WΦ can be reduced by a half or a full helical turn without affecting the capacity of C to repress the promoter.

The distance between the operator half-sites in the WΦ C early promoter region affects the complexes formed upon C binding

P2 C has been shown to generate two retarded bands in the EMSA, compared to WΦ C that generated three retarded bands (36). The distance between the half-sites of P2 is two helical turns, and that of WΦ three helical turns. Thus, we were interested in analyzing the complexes formed between WΦ C and its operator when the center-to-center distance between the half-sites was changed to 2 and 2.5 helical turns, respectively. Using PCR fragments from the same constructs as in the reporter system above in an EMSA with the WΦ C protein (plasmids pEE938–940), only two shifted bands are evident with wt DNA (Figure 4). This can either be explained by the fact that this construct lacks the phage sequences flanking the operator, or by the locations of the operator half-sites relative to the end of the fragment, since WΦ C bends its DNA target, see below. Furthermore, the slow migrating band is not obtained when the distance between the half-sites of the operator was changed by a half or one helical turn (Figure 4). Thus, it seems as if the *in vitro* conditions used in the EMSA, using linear DNA as a target, will not allow binding of WΦ C to both half-sites simultaneously if the distance between the half-sites is decreased.

The complexes formed between WΦ C and its operator is affected by the nucleotides flanking the promoter/operator region

To clarify the possible effect of the sequences flanking the operator half-sites, we wanted to compare the promoter strength and repression capacity of the WΦ C protein in the construct containing WΦ sequences (pEE905) or vector sequences (pEE938) flanking the promoter/operator region using the *cat* gene as a reporter. To our surprise the level of CAT expression in the absence of repressor was ~100-fold higher in cells containing plasmid pEE938 compared to pEE905, but the level of repression was about the same (90%). Thus, the flanking phage sequences do not seem to have an effect on the capacity of C to repress Pe *in vivo*.

To compare the effects of the flanking nucleotides on the complexes formed, we amplified the promoter/operator regions of plasmid pEE905 and pEE938 in such a way that the fragments generated were of almost the same length and the location of the operator half-sites are at about the same position on the fragments (Figure 5a) and performed an EMSA with increasing C concentrations (Figure 5b). As can be seen, the flanking regions do affect the complexes formed. With WΦ flanking sequences three bands are obtained, two major fragments and one weak fragment. With vector sequences flanking the promoter/operator only two fragments are consistently generated, and surprisingly even though the fragments have about the same length in both constructs they migrate differently. It also seems as if the protein concentration required to detect binding is slightly higher in the absence of flanking phage DNA. However, the dissociation constants calculated from titrations, using a constant amount of DNA and an increasing amount of C, followed by a quantification of shifted DNA in the EMSA and a linear regression analysis of the generated curves, were the same.

WΦ C, as opposed to P2 C, is able to bind to one operator half-site *in vitro*, but *in vivo* repression requires both half-sites

To investigate the importance of the half-sites for C repressors binding to the operators the capacities of C proteins to repress and bind to early promoters containing multiple mutations in O1 were analyzed.

Plasmid pEE941, a derivative of pSS32-1 that has the P2 Pe promoter/operator region directing the *cat* gene, but where the O1 half-site was mutated from GTTTAGAT to ATCCGAGT was shown to express the *cat* gene to the same extent in the presence or absence of C (data not shown). Thus, the multiple mutations in P2 O1

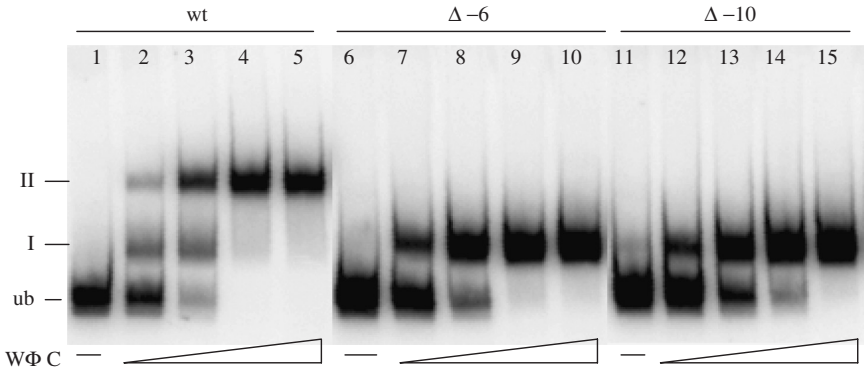

Figure 4. EMSA showing the interactions between WΦ C to its Pe operator where the distance between the operator half-sites is changed. An increasing amount of WΦ C protein (0.18–1.8 nM) was added to a ^{32}P-labeled DNA fragment containing wild-type operator or an operator with a 6 or 10 nt deletion upstream of the O2 half-site, see Figure 3. The labeled fragments were obtained by PCR using plasmids pEE938–pEE940 as substrates. The PCR reactions added 172 and 74 nt vector DNA to the right and left end of the WΦ sequence.

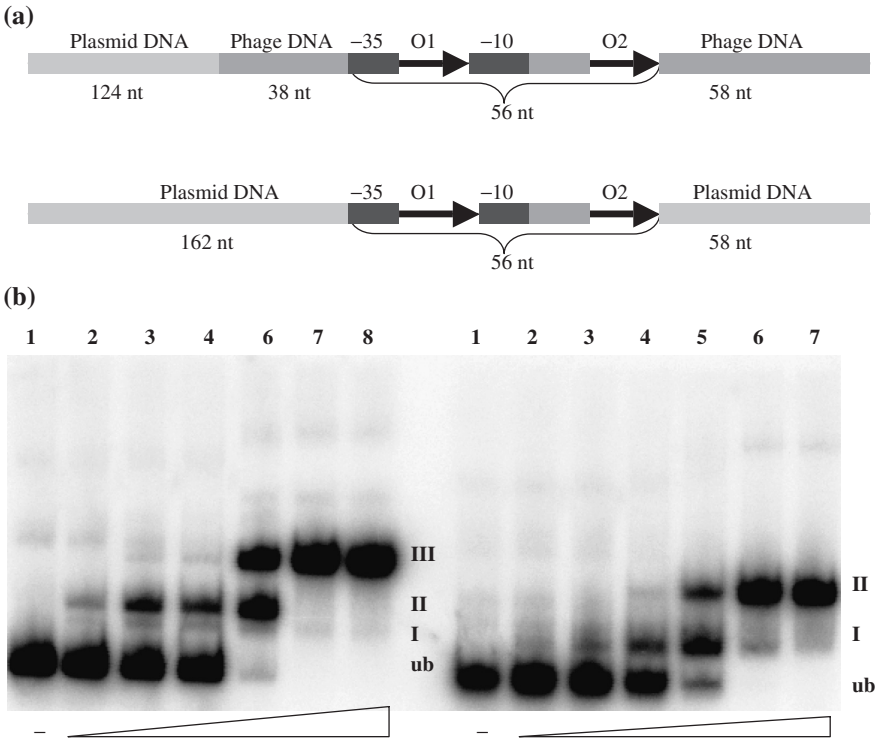

Figure 5. EMSA showing the interaction between WΦ C and its operator/promoter flanked by phage DNA (left) or vector DNA (right). An increasing amount of C protein (0.22–8.8 nM,) was added to the ^{32}P-labeled DNA fragments. A schematic drawing of the fragments used are shown above the gel. The labeled fragments were generated by PCR using plasmids pEE905 and pEE938 as substrates.

abolished the capacity of P2 to repress the early promoter *in vivo*. As can be seen in the EMSA in Figure 6a, P2 C was also unable to bind to the early promoter/operator region amplified from plasmid pEE941 at concentrations where it bound efficiently to the wild-type fragment amplified from plasmid pSS32-1.

Plasmid pEE942, a derivative of plasmid pEE905 that has the WΦ Pe promoter/operator region directing the *cat* gene, but the O1 half-site was mutated from TATTGGTGAC to CGCCAACAGT was also unable to repress the *cat* gene *in vivo*. However, as can be seen in Figure 6b, the WΦ C protein binds as efficiently to the PCR fragment generated from plasmid pEE942 as to the wild-type fragment generated from plasmid pEE905, but only one retarded fragment (II) is obtained compared to the three fragments obtained with the wild-type fragment. Thus, WΦ C as opposed to P2 C is able to bind to only one half-site.

Since the nucleotides flanking the WΦ promoter/operator regions affect the fragments generated in gel-shift analysis, see above, two synthetic hybrid Pe promoter/operator regions, one containing the O1 half-site of P2 and the O2 half-site of WΦ, and the other O1 of WΦ and O2 of P2 (Figure 7b and c) were constructed and inserted upstream of a promoterless *cat* gene in pKK232-8, generating plasmid pEE865 and pEE866. Providing the P2 C or WΦ C repressor *in trans* showed that neither repressor alone was able to repress the synthetic Pe promoters *in vivo* (data not shown). However, in an electrophoretic mobility shift analysis, WΦ C was found to have the ability to bind both constructs containing only one half-site, either O1 or O2 (Figure 7b and c), but instead of the three retarded bands obtained with the wild-type operator/promoter region with flanking phage DNA (Figure 7a), or the two retarded bands obtained with the wt operator/promoter region without the flanking phage DNA (Figure 5b) only one retarded band is evident. This in contrast to P2 C, where the synthetic promoter/operator containing only O2 shows no binding and the construct containing only O1 a weak binding. This indicates that WΦ C can bind to the respective half-site independently under *in vitro* conditions using a linear DNA substrate. Furthermore, the fact that the synthetic Pe promoters are not repressed either by P2 or WΦ C, indicates that both half-sites are required for repression *in vivo*.

The DNA-bending capacity of WΦ C and P2 C

DNA-binding proteins often cause conformational changes when they form protein–DNA complexes. Since the spacing between the half-sites of the operators differ in P2 and WΦ, binding of the two C repressors might affect DNA topology differently. To investigate the bending capacity of the two C repressors, the respective early promoter/operator region was cloned into the pBend2 vector, a pBR322 derivative containing a directly repeated polylinker region (25). The pertinent region was amplified from the vector by PCR, and the amplified fragment was cleaved with restriction enzymes generating fragments of the same size, but the location of the operator relative to the ends of the fragments varied. After incubation with

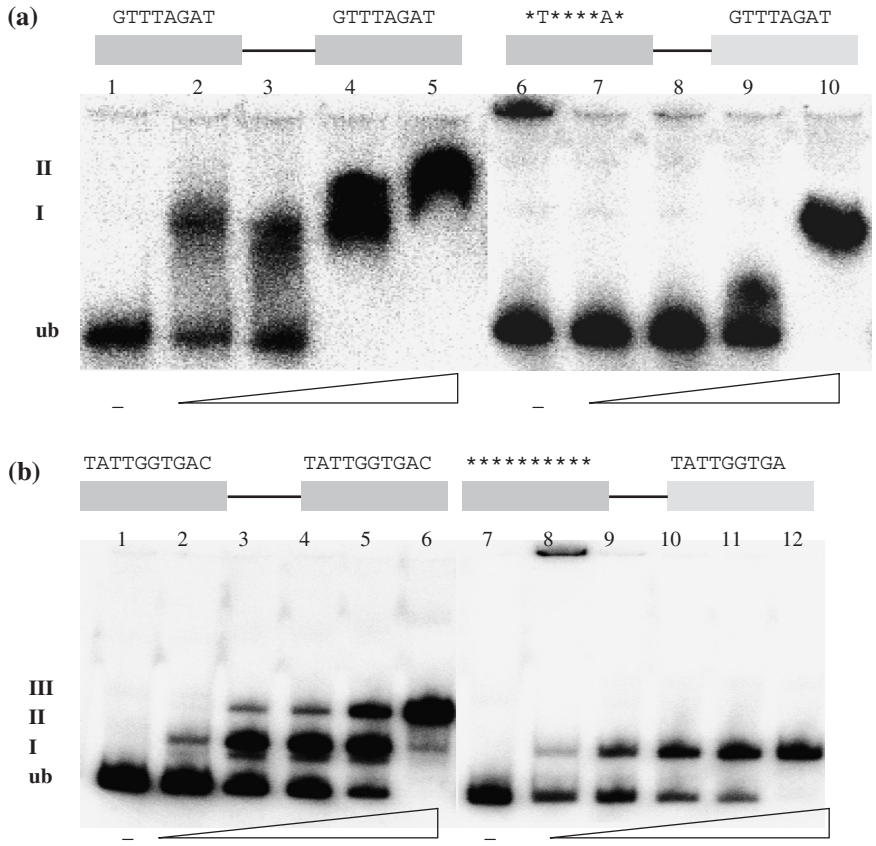

Figure 6. EMSA showing the interactions between P2 C (**a**) and WΦ C (**b**) to its wild-type promoter/operator regions (left panels) and to the same region where O1 contains multiple mutations (right panels). The sequences of the half-sites are indicated, and the stars indicate the mutated nucleotide. The ^{32}P labeled fragments were generated by PCR using plasmids pSS32-1 and pEE941 for P2 and pEE905 and pEE942 for WΦ as substrates. The protein concentrations for P2 C varied between 3.2 and 101.2 nM and for WΦ C 0.22 and 8.8 nM.

the respective C protein, the relative mobility of each fragment was analyzed on a non-denaturing polyacrylamide gel. Depending on the location of the operator, the fragments would migrate differently of the C repressors bend DNA upon binding.

Figure 8a shows the results using WΦ C and its operator region. As can be seen, fragments where the operator of WΦ is located near the ends migrates faster than the fragments where the operator had a more central location. This shows that WΦ C bends the DNA substrate. The bending center seems to be located between the O1 and O2 half-sites, which was analyzed by plotting the relative mobility values against the location of the operator (Figure 8a). The bending angle was calculated to ~60° (34).

As can be seen in Figure 8b, P2 C also bends its DNA target and the bending angle was estimated to ~90°. As in WΦ, the bending center is located between the O1 and O2 half-sites. In this experiment, conditions were selected so that only one shifted band was obtained with P2 C.

WΦ C, like P2 C, forms dimers, but not oligomers *in vivo*

A possible explanation for the different bands in our EMSA for P2 C compared to WΦ C is the native state of the repressors. P2 C has been shown to form dimers but the capacity to form higher oligomers in the absence of DNA is very weak (27). Since the oligomeric state of WΦ C had not been studied, the dimerization and oligomerization efficiency of WΦ C was analyzed and compared to that of P2 C using an *in vivo* plasmid system based on the interaction between λ CI repressor and its right operator O_R (18,19). The N-terminal domain of λ CI contains the DNA-binding motif, but efficient operator binding requires dimerization. The dimerization domain is located in the C-terminal domain of λ CI. By fusing a protein able to dimerize to the N-terminal domain of CI, high-affinity binding to the λ operator can be restored. The WΦ *C* gene was fused to the N-terminal domain of λ *cI*, which was under the control of the *lacUV5* promoter generating plasmid pEE923. The construct was transformed into the reporter strain JH372. To quantify dimerization, a *lacZ* reporter gene, controlled by the right λ operator/promoter (O_RP_R-*lacZ*), has been inserted into the chromosome of the reporter strain. The capacity of the CI-WΦC fusion protein to bind to λ O_R and repress P_R was quantified by measuring the β-galactosidase activity. As can be seen in Figure 9, the β-galactosidase activity is as low for the λ CI-WΦC fusion protein as for the λ CI-P2C fusion protein and at least 8-fold lower that the negative controls pZ150 (no repressor) and pKH101

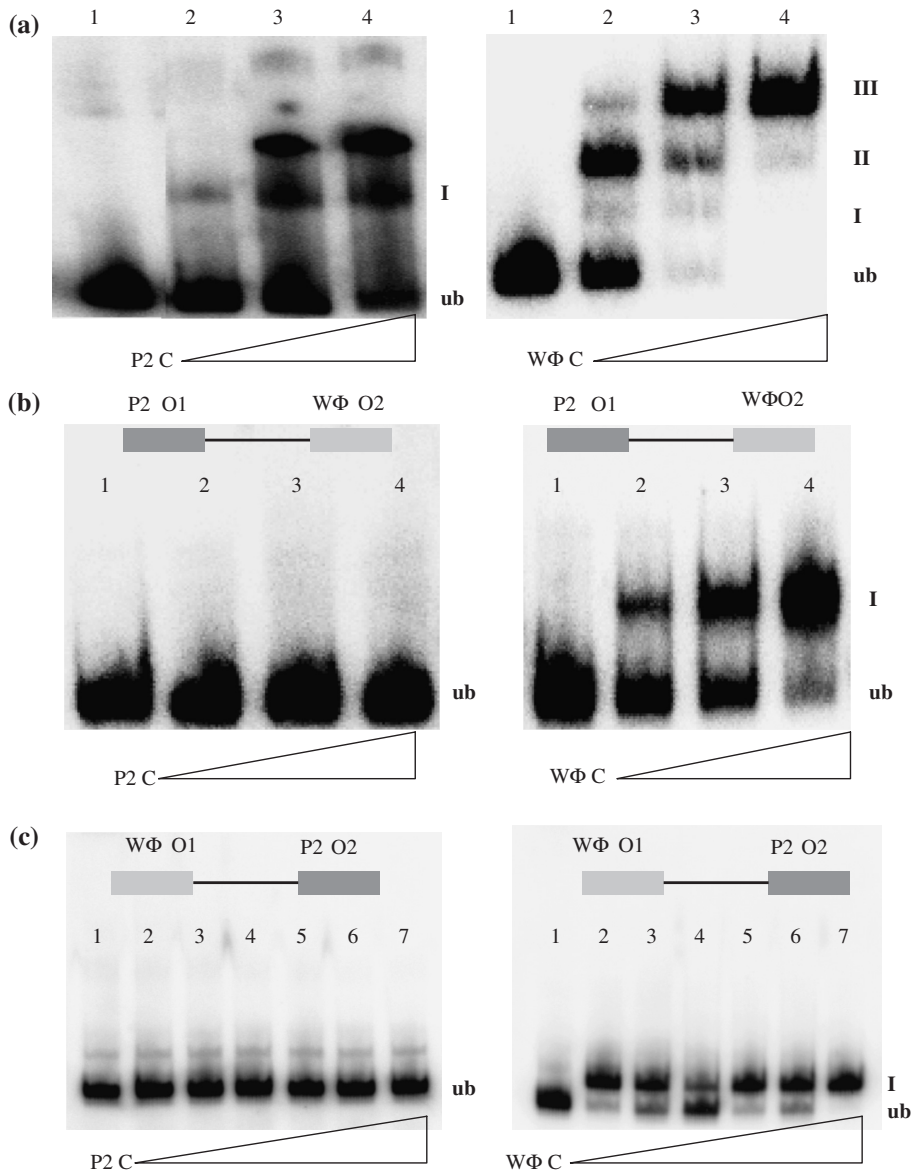

Figure 7. EMSA showing the interactions between P2 C and WΦ C to their wild-type promoter/operator regions (**a**) and two synthetic hybrid promoter/operator containing P2 O1 and WΦ O2 (**b**) and WΦ O1 and P2 O2 (**c**). Schematic drawings of the hybrid promoter/operators are shown above the respective EMSA. The ^{32}P-labeled fragments of the synthetic hybrid operator/promoters were generated by PCR using plasmids pEE865 and pEE866. The wild-type operator fragments were generated by PCR from plasmids pEE675 and pEE905 (see Figure 6). Plasmid pEE675 contains 40 nt upstream of O1 and 91 nt downstream of O2, and the PCR reaction adds 72 nt vector DNA to the right end. The first lane with each operator is without C protein. The next three lanes contain an increasing amount of the respective C protein. The shifted fragments with the respective C protein are indicated on the right side. The protein concentrations for P2 C varied between 3.2 and 101.2 nM and for WΦ C 0.22 and 8.8 nM.

(contains only the N-terminus of λ CI). It should be noted that the repression by both the P2 and the WΦ fusion proteins is more efficient compared to the full-length λ CI proteins used as a positive control, indicating that the C proteins dimerize more efficiently than λ CI.

This assay will not discriminate between dimerization and the formation of higher oligomeric forms. We therefore analyzed the capacity of WΦ to form tetramers or to bind cooperatively in an *in vivo* reporter assay system where the promoter of the *lacZ* gene is transcribed from a synthetic promoter controlled by a strong distal λ operator and a weak proximal λ operator (19). This promoter is repressed only if the λ CI repressor dimer binding to the strong upstream operator is able to recruit and stabilize another dimer at the weak operator. Plasmid pEE923 expressing the λ CI-WΦ C fusion protein was transformed into strain AG1688(λ112$O_S P_S$), where *lacZ* expression is controlled by the synthetic promoter–operator region, and into strain AG1688(λXZ970), where the promoter of the *lacZ* gene is under the control of only the weak λ operator to measure the background repression caused by binding of the dimer to the weak proximal operator. The ratio between the β-galactosidase activities between the two strains will reflect the capacity of

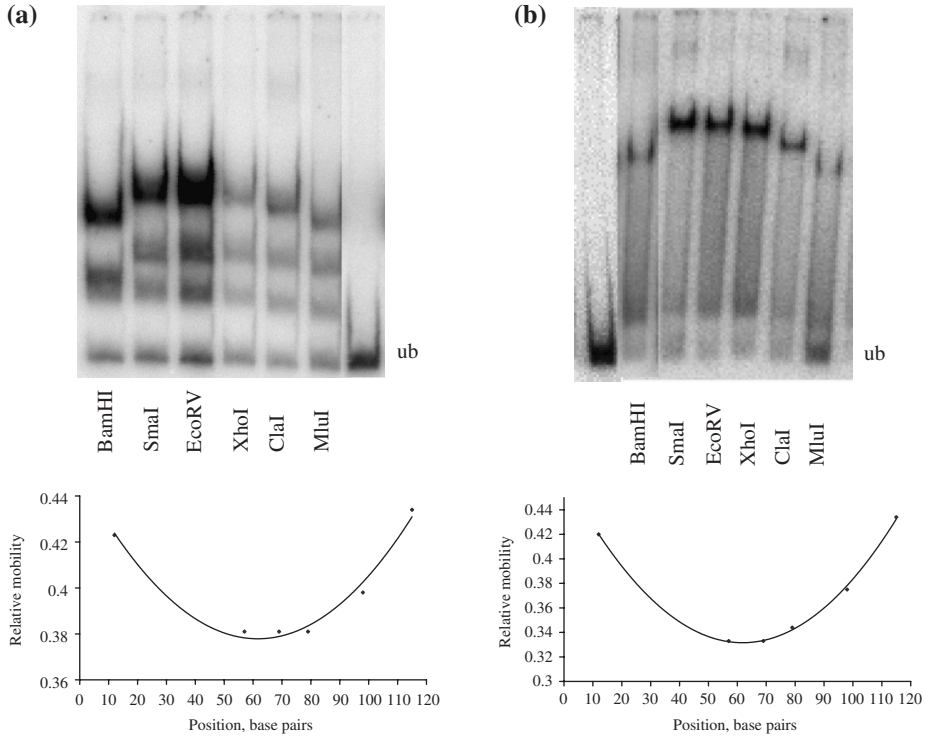

Figure 8. Circular permutation assay with WΦ C and P2 C. (**a**) The WΦ C repressor protein was incubated with the labeled restriction fragments indicated below each lane, and run on a 5% non-denaturing gel. The relative mobility of the DNA–C complexes was plotted against the location of the C operator within the fragment. (**b**) The P2 C repressor protein was incubated and analyzed as WΦ C.

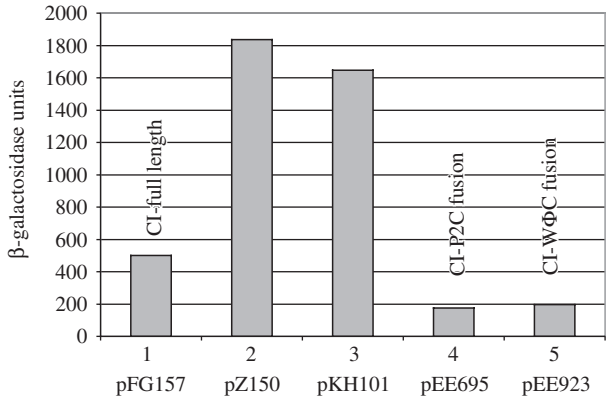

Figure 9. *In vivo* dimerization assay. Each panel shows the average of three independent β-galactosidase measurements in cell extracts of strain JH372 containing plasmids expressing different CI fusion proteins. Plasmid pFG157 (wild-type λ CI) is a positive control, pZ150 (empty vector) and pKH101 (N-terminal domain of CI) as negative controls. Plasmid EE695 expresses the λ CI-P2 C fusion protein and pEE923 the λ CI-WΦ C fusion protein. β-Galactosidase units are shown on the *Y*-axis.

cooperative binding to the strong and the weak operator. As can be seen in Figure 10, the ratio for the λ CI- WΦ C fusion of is 0.9, and that of P2 is 1.5, while the positive control pJH622 (expressing a modified CI-GCN4 fusion protein that forms tetramers) gives a ratio of 7.0, and the negative controls pJH370 (expressing the CI-GCN4 fusion protein that only forms dimers) and pZ150 (expressing no fusion protein) gives ratios around 1. This implies that under these conditions the WΦ C protein, like the P2 C protein, is unable to bind cooperatively or form higher oligomeric structures in the absence of its DNA target.

Spontaneous phage production from P2 and WΦ lysogens

Spontaneous phage production and release of free phages in a growing lysogen is a consequence of a reduction of the repressor protein below a threshold level, leading to a turn of the transcriptional switch, i.e. Pe is turned on and Pc becomes repressed by Cox. Thus, the DNA-binding properties of the repressor proteins C and Cox, their stabilities and regulation will affect the level of spontaneous phage induction. To compare the level of free phages released by P2 and WΦ lysogens, bacteria lysogenic for the respective phage were grown and the titers of free phages and bacteria were determined in overnight cultures. The P2 lysogen (C-117) had a spontaneous phage production of 4.5×10^{-4} phages per bacterium and the WΦ lysogen (C-1920) produced 2.9×10^{-3} phages per bacterium, i.e. the WΦ lysogen has a 6.4-fold higher frequency of spontaneous phage production than the P2 lysogen.

DISCUSSION

In this work, we have shown that WΦ C, like P2 C, has a strong dimerization activity *in vivo*, but in solution there are no indications of higher oligomeric forms in the

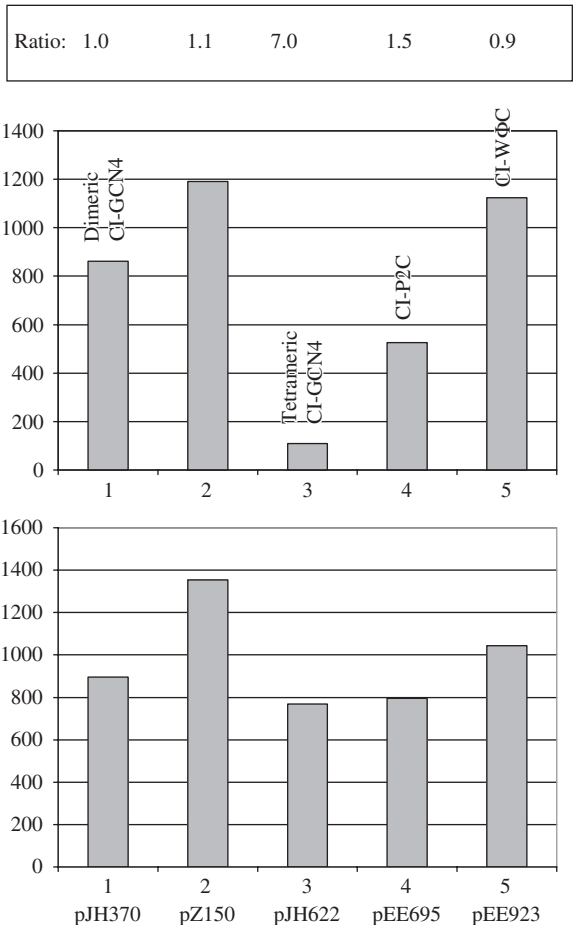

Figure 10. *In vivo* oligomerization assay. Plasmids expressing the fusion proteins were transformed into the assay strain AG1688(λ112OsPs) (top) or the control strain AG1688(λXZ970) (bottom), and the β-galactosidase activity was determined. Plasmid pJH370 (dimeric CI-GCN4 fusion protein) and pZ150 (empty vector) were used as negative controls. Plasmid pJH622 (tetrameric, engineered CI-GCN4 fusion protein) was used as positive control. Plasmid pEE694 expresses the λ CI-P2C fusion protein and pEE923 the λ CI-WΦ C fusion protein. Each panel represents the average of three independent enzyme measurements. The ratios of the enzyme activities in the two strains are shown above the panels.

plasmid reporter system used. The repressors might, however, form higher oligomeric structures upon binding to DNA. Both C repressors induce bending of its respective DNA target upon binding, and it seems as if P2C induces a slightly stronger bend in the DNA. However, this difference may be a consequence of the locations of the operator half-sites, or within the fluctuations caused by the limitations of the determination of the bending angle in the circular permutation analysis.

The distance between the operator half-sites in P2 and WΦ differs, but as shown here, a reduction of the distance between the half-sites in WΦ from 3 to 2.5 or 2 turns has no effect of the capacity of WΦ C to repress the early promoter *in vivo*. However, the complexes formed in our electrophoretic mobility shift analysis differ, only the fast migrating complex is formed when the distance is decreased. Possibly this fast migrating band correspond to binding to only one half-site, and that the substrate needs to be supercoiled for binding to both half-sites when the distance between the half-sites is reduced. This possibility is supported by the fact that in constructs containing only one half-site (O1), where O2 has been mutated or in the hybrid promoter/operator constructs, only one retarded protein–DNA complex is formed and the *in vivo* assays show that C cannot regulate the constructs that lacks the other half-site.

The electrophoretic mobility shift analysis performed in this work indicate that P2 and WΦ C have different requirements for DNA binding and that the protein–DNA complexes formed differ. P2C is unable to bind to one half-site as opposed to WΦ C. Furthermore, WΦ C gives three retarded fragments to its wild-type operator/promoter region, indicating an ability to bind to the half-sites independently. However, our analysis shows that the phage sequences upstream of the −35 region and downstream of O2 affects both the promoter activity and the repressor–DNA complexes formed. Replacing the phage flanking sequences with vector DNA leads to an almost 100-fold increase in the *in vivo* expression system using the *cat* reporter gene in the absence of the repressor, indicating an interference of some phage sequences or an enhancement of the vector DNA. This is an interesting observation that has not been studied further in this work, since the capacity of the WΦ C to repress both constructs is as efficient, indicating that the flanking sequences do not affect the capacity of C to bind and repress its operator *in vivo*. However, the repressor–DNA complexes formed in the electrophoretic mobility shift analysis differ. Instead of the three fragments generated by the wild-type operator/promoter region, only two fragments are generated when the phage flanking regions are replaced by vector sequences. A possible explanation could be that secondary binding sites are present in the flanking sequences. However, since both the construct containing multiple mutations in O1 with flanking phage DNA and the hybrid operator/promoter constructs that contain only O1 or O2 generate only one shifted band in our EMSAs secondary binding sites seems very unlikely. Our results clearly points to the difficulties in interpretation of the biological significance of complexes formed *in vitro* using EMSA, and the importance of complementary *in vivo* analysis.

Localization of the start sites of the P2 Pe and Pc transcripts confirm that the transcripts overlap by ∼35 nt. A foot-print analysis of P2C shows that the C protein covers not only the O1 and O2 region, but also ∼12 nt downstream of O2, i.e. to ∼+16 of Pe (37). The protected region is interrupted by at least three enhanced cleavage sites, giving four short protected sites spaced by enhanced cleavage two of them correspond to O1 and O2, one to the spacer region between the half-sites, and the fourth is located downstream of O2. One way of interpreting this is that four C protein molecules bind to this region. Since RNA polymerase binding to Pc should cover 75–80 nt, (from −60 to +20), i.e. P2C and the polymerase should be very close which can explain the autoregulation of C expression on Pc expression. In WΦ, however,

the Pe and Pc promoters are further apart from each other and the transcripts overlap by 82 nt, and O2 is located 60 nt from the start site of Pc. Since it is not known if WΦ C regulates its own Pc promoter, further studies are required before any conclusions can be drawn about the consequences of the difference in distance between the Pe and Pc promoters of the two phages. The observed difference in spontaneous phage production from strains carrying either P2 or WΦ prophage might reflect differences in the components of the transcriptional switch, such as relative strengths of the Pe and Pc promoters and the kinetics of the interactions between the C and the Cox repressors and their respective operator. The kinetics of the interactions between the C proteins to their respective operator has been studied (36), but not the interactions of the Cox proteins with their operators. Other phage or cellular factors could also affect the burst size.

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REFERENCES

1. Bertani,L.E. and Six,E.W. (1988) In: Calendar,R. (ed.), *The Bacteriophages*, Plenum Publishing Corporation, New York, Vol. 2, pp. 73–143.
2. Nilsson,A.S. and Haggård-Ljungquist,E. (2006) In: Calendar,R. (ed.), *The Bacteriophages*, *2nd edn*. Oxford University Press, Oxford, pp. 365–390.
3. Ochman,H. and Selander,R.K. (1984) Standard reference strains of *Escherichia coli* from natural populations. *J. Bacteriol.*, **157**, 690–693.
4. Nilsson,A.S., Karlsson,J.L. and Haggård-Ljungquist,E. (2004) Site-specific recombination links the evolution of P2-like coliphages and pathogenic enterobacteria. *Mol. Biol. Evol.*, **21**, 1–13.
5. Nilsson,A.S. and Haggård-Ljungquist,E. (2001) Detection of homologous recombination among bacteriophage P2 relatives. *Mol. Phyl. Evol.*, **21**, 259–269.
6. Karlsson,J.L., Cardoso-Palacios,C., Nilsson,A.S. and Haggård-Ljungquist,E. (2006) Evolution of immunity and host chromosome integration site of P2-like coliphages. *J. Bacteriol.*, **188**, 3923–3935.
7. Ptashne,M. (2002) *A Genetic Switch. Phage λ and Higher Organisms*, *2nd edn*. Cell Press and Blackwell Scientific Publications, Cambridge, MA.
8. Dodd,I.B. and Egan,J.B. (1996) DNA binding by the coliphage 186 repressor protein CI. *J. Biol. Chem.*, **271**, 11532–11540.
9. Lamont,I., Richardson,H., Carter,R.R. and Egan,J.B. (1993) Genes for the establishment and maintenance of lysogeny by the temperate coliphage 186. *J. Bacteriol.*, **175**, 5286–5288.
10. Shearwin,K.E., Dodd,I.B. and Egan,J.B. (2002) The helix-turn-helix motif of the coliphage 186 immunity repressor binds to two distinct recognition sequences. *J. Biol. Chem.*, **277**, 3186–3194.
11. Dodd,I.B. and Egan,J.B. (2002) Action at a distance in CI repressor regulation of the bacteriophage 186 genetic switch. *Mol. Microbiol.*, **45**, 697–710.
12. Dodd,I.B., Shearwin,K.E., Perkins,A.J., Burr,T., Hochschild,A. and Egan,J.B. (2004) Cooperativity in long-range gene regulation by the lambda CI repressor. *Genes Dev.*, **18**, 344–354.
13. Pinkett,H.W., Shearwin,K.E., Stayrook,S., Dodd,I.B., Burr,T., Hochschild,A., Egan,J.B. and Lewis,M. (2006) The structural bases of cooperative regulation at an alternative genetic switch. *Mol. Cell*, **21**, 605–615.
14. Shearwin,K.E. and Egan,J.B. (2000) Establishment of lysogeny in bacteriophage 186. DNA binding and transcriptional activation by the CII protein. *J. Biol. Chem.*, **275**, 29113–29122.
15. Neufing,P.J., Shearwing,K.E. and Egan,J.B. (2001) Establishing lysogenic transcription in the temperate coliphage 186. *J. Bacteriol.*, **183**, 2376–2379.
16. Ljungquist,E., Kockum,K. and Bertani,L.E. (1984) DNA sequences of the repressor gene and operator region of bacteriophage P2. *Proc. Natl Acad. Sci. USA*, **81**, 3988–3992.
17. Liu,T. and Haggård-Ljungquist,E. (1999) The transcriptional switch of bacteriophage WΦ, a P2-related but heterimmune coliphage. *J. Virol.*, **73**, 9816–9826.
18. Hu,J., O'Shea,E.K., Kim,P.S. and Sauer,R.T. (1990) Sequence requirements for coiled-coils: analysis with repressor-GCN4 leucin zipper fusions. *Science*, **250**, 1400–1403.
19. Zeng,X. and Hu,J.C. (1997) Detection of tetramerization domains *in vivo* by cooperative DNA binding to tandem lambda operator sites. *Gene*, **185**, 245–249.
20. Sasaki,I. and Bertani,G. (1965) Growth abnormalities in Hfr derivatives of *Escherichia coli* strain C. *J. Gen. Virol.*, **40**, 365–376.
21. Bertani,L.E. (1968) Abortive induction of bacteriophage P2. *Virology*, **36**, 87–103.
22. Kerszman,G., Glover,S.W. and Aronovitch,J. (1967) The restriction of bacteriophage lambda in *Escherichia coli* strain W. *J. Gen. Virol.*, **1**, 333–347.
23. Sunshine,M.G. and Kelly,B. (1971) Extent of host deletions associated with bacteriophage P2-mediated eduction. *J. Bacteriol.*, **108**, 695–704.
24. Chang,A.C.Y. and Cohen,S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.*, **134**, 1141–1156.
25. Kim,J., Zwieb,C., Wu,C. and Adhya,S. (1989) Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. *Gene*, **85**, 15–23.
26. Liu,T., Renberg,S.K. and Haggård-Ljungquist,E. (1997) Derepression of prophage P2 by satellite phage P4: Cloning of the P4 ε gene and identification of its product. *J. Virol.*, **71**, 4502–4508.
27. Liu,T., Renberg,S.K. and Haggård-Ljungquist,E. (1998) The E protein of phage P4 acts as an anti-repressor by binding to the C protein of helper phage P2. *Mol. Microbiol.*, **30**, 1041–1050.
28. Studier,F.W, Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) Use of T7 RNA polymerase to direct selective high-level expression of cloned genes. *Methods Enzymol.*, **185**, 60–89.
29. Brosius,J. (1984) Plasmid vectors for selection of promoters. *Gene*, **27**, 151–160.
30. Saha,S., Haggård-Ljungquist,E. and Nordström,K. (1987) The Cox protein of bacteriophage P2 inhibits the formation of the repressor and autoregulates the early operon. *EMBO J.*, **6**, 3191–3199.
31. Sambrook,J. and Russel,D.W. (2001) *Molecular Cloning: A Laboratory Manual*, *3rd edn*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
32. Bradford,M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–265.

33. Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell Biol.*, **2**, 1044–1051.
34. Thompson,J.F. and Landy,A. (1988) Empirical estimation of protein-induced DNA bending angles: applications to lambda site-specific recombination complexes. *Nucleic Acids Res.*, **16**, 9689–9705.
35. Renberg-Eriksson,S.K., Ahlgren-Berg,A., DeGrooth,J. and Haggård-Ljungquist,E. (2001) Characterization of the developmental switch region of bacteriophage P2 Hy *dis*. *Virology*, **290**, 199–210.
36. Henriksson-Peltola,P., Sehlén,W. and Haggård-Ljungquist,E. (2007) Determination of the DNA binding kinetics of three related but heteroimmune baceriophage repressors using EMSA and SPR analysis. *Nucleic Acids Res.*, in press.
37. Saha,E., Lundqvist,B. and Haggård-Ljungquist,E. (1987) Autoregulation of bacteriophage P2 repressor. *EMBO J.*, **6**, 809–814.