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
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 cl 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 TAA TCC CGT CAA AAG GTT GTG TGG TGA CAA GCT TGG G and Hy2 rev 5′ CCC AAG CCT TGT GTC ACC AAT ACA CAA CCT TTT GAC GGG ATT ATA TTG AGA TCT AAA CAC CAT GTG 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 AAG TGG GTT TAG ATC ATA AGC TTG GG and Hy3 rev 5′ CCC AAG CCT TGT AAT TAA TCA ACC CAC CCT TGT 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

| Bacteria             | Pertinent features                                                                 | Origin/reference |
|----------------------|-------------------------------------------------------------------------------------|------------------|
| AG1688               | *E. coli* MC1061, F′ 128 lacF′ lacZ′::Tn5                                             | 18               |
| AG1688 (λ112OsP3)    | AG1688 containing λ112OsPs with lacZ driven by λO3P3 and a strong upstream λ operator | 19               |
| AG1688 (λXZ970)      | AG1688 containing λ112OsPs with lacZ driven by λO3P3 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 P6O6R                                    | 18               |

Plasmids

| Plasmids            | Pertinent features                                                                 | Origin/reference |
|---------------------|-------------------------------------------------------------------------------------|------------------|
| 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              | pET5c 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              | pJH391 derivative where WΦ C gene is fused to the N-terminal domain of the λ cl gene | This work        |
| pEE696              | pKK232-8 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 Smal site; Pe directs the cat gene | 17               |
| pEE923              | pJH391 derivative where WΦ C gene is fused to the N-terminal domain of the λ cl 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 TATGTTGATCGAC to GGCCAAAGT          | This work        |
| pEE920              | pET5c derivative expressing WΦ C. This work                                        | 28               |
| pEE924              | pBR322 derivative containing the T7 promoter                                       | 28               |
| pGF157              | λ cI ind1 driven by the lacUV5 promoter                                            | 18               |
| pHJ370              | Fusion between λ cl and the leucin zipper of GCN4 driven, by the lacUV5 promoter    | 18               |
| pHJ391              | First 132 nt of λ cl fused with lacZ driven by the lacUV5 promoter                  | 18               |
| pHJ622              | Fusion between λ cl and the reengineered leucin zipper of GCN4, driven by the lacUV5 promoter | 19               |
| pKH101              | Expresses only the N-terminal DNA-binding domain of λ cl                              | 18               |
| pKK232-8            | pBR322 derivative containing a promoterless cat gene                                 | 29               |
| pSS29-2             | pKK232-8 derivative containing the P2 Pc-Pe 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 SalIWΦFCor (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 Sall and BamHI, it was inserted into plasmid pJH391 cleaved with Sall 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′ GAT CCT TGA CAG TAA CTT AGT GAC TAT TCC CTT GCT CAA AAG GTT GTG TAT TGG TGA CA) and the 144-nt long fragment was inserted into the filled in Sall 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 Sall 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 GTC GAC TAT TCC CTT GCT CAA AAG GTT GTG TAT TGG TGA CA) and WPhi bot (5′ AGC TGT TCA CCA ATA CAC AAC CTT TTG ACG GGA ATA TAA GTC ACC AAT AGG TTA CTG TCA AG) and WPhi bot(5′ AGC TGT 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 GTC GAC TAT TCC CTT GCT CAA AAG TAT TGG TGA CA) and WPhiop 6ntbot (AGC TGT TCA CCA ATA CTT GTT ACG GGA ATA TAA GTC ACC AAT AGG TTA CTG TCA AG)
TTG ACG GTA AAT AGG TTA CTG TCA AG). The locations of the O1 and O2 half-sites are indicated by underlinings.

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 CGG ATG GAC TTA TAT TGG GTG ACA) and WPhiop −10bot (5’AGC TTG TCA CCA ATA GAC CGG AAT ATA AGT CAC CAA TAG GTT ACT GTC AAG). The location of the O1 and O2 half-sites are indicated by underlinings.

pEE941. Plasmid pSS32-1 was used as template for construction of an O1 library using a degenerate primer {TCGTGTTTGAGAT to ATCCGAGT} and the Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The plasmid chosen has O1 changed from ATTTAGTTTAG (K or G, A or T or C} and the Multi Site-Directed Mutagenesis Kit (Stratagene). The plasmid chosen has O1 changed from TATTGGTGAC to TATT GTGA C. The locations of the O1 and O2 half-sites are indicated by underlinings.

pEE942. Plasmid pEE905 was used as template for construction of an O1 library using a degenerate primer {GCATTGACAGTAACC KK PKP KPKK PCTAATGTTTAG (K or G, A or T or C} and the Multi Site-Directed Mutagenesis Kit (Stratagene). The plasmid chosen has O1 changed from ATTTAGTTTAG to ATCCGAGT.

pEE1020. The Wϕ C gene was amplified by PCR, using primers WϕCstartNcoI (CATGCCCATGGCAGCACTTGCGAAAAAACTGAAAGC) and WϕCendBamHI (CCGGATCCTGTAAATGT TAGTAGTAGTATACCCGTC). The Wϕ C gene was amplified by PCR, using primers WϕCstartNcoI (CATGCCCATGGCAGCACTTGCGAAAAAACTGAAAGC) and WϕCendBamHI (CCGGATCCTGTAAATGT TAGTAGTAGTATACCCGTC) and after cleavage by BamHI and NcoI it was inserted between the NcoI and BamHI 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 (OD600 = 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-32P]-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 TTC C) using 10 μg of total RNA extracted from C-1a pSS29-2, and the Pe transcript was detected with the P2 Pe primer (5’ GTC GAA GCT CGG GGG 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 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 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 MgSO4, 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 acetyltransferase (CAT) activity determination

Ten milliliter cultures were harvested at an OD600 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 [14C]-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 Ce) (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 OD600 = 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 AKTA™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 AKTA™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 P2C protein was stored in 18 mM Tris–HCl pH 7.5, 1 mM sodiumphosphate pH 7.0, 0.55 M NaCl, 4.5 mM MgCl2, 0.9 mM DTT at −20°C with 36% (v/v) glycerol.

**Circular permutation assay**

The P2C 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 S′ end labeled using [γ-32P]-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 P2C operator was incubated with 1.5 nM of purified P2C 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 P2C 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 μM/μE=cos α/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 CHC13 to the supernatant, the PFU per ml was determined on LA plates supplemented with 2.5 mM CaCl2 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 PePe 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 HindIII 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 substrates. The PCR reactions added 172 and 74 nt vector DNA to the right and left end of the WΦ C operator, or by the locations of the operator half-sites that this construct lacks the phage sequences flanking the DNA (Figure 4). This can either be explained by the fact 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 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). 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.

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.

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 GTTCTAGAT 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 32P-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–pEE946 as substrates. The PCR reactions added 172 and 74 nt vector DNA to the right and left end of the WΦ sequence.](image)
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 C8 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
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 to the C repressors bend DNA upon binding.

Figure 8a shows the results using W/C8 and its operator region. As can be seen, fragments where the operator of W/C8 is located near the ends migrates faster than the fragments where the operator had a more central location. This shows that W/C8 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_R P_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 than the negative controls pZ150 (no repressor) and pKH101.
It should be noted that the repression by both the P2 and the WΦ/C8 fusion proteins is more efficient compared to the full-length C21 CI proteins used as a positive control, indicating that the C proteins dimerize more efficiently than C21 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(λ112O3P5), 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 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 32P-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.
cooperative binding to the strong and the weak operator.

As can be seen in Figure 10, the ratio for the $\lambda$ 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. $P_e$ is turned on and $P_c$ 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 $4.5 \times 10^{-3}$ phages per bacterium and the WΦ lysogen (C-1920) produced $2.9 \times 10^{-3}$ 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
proteins were transformed into the assay strain AG1688(C21)
determination of the bending angle in the circular DNA. However, this difference may be a consequence as if P2 C induces a slightly stronger bend in the respective DNA target upon binding, and it seems to DNA. Both C repressors induce bending of its DNA, however, form higher oligomeric structures upon binding to DNA. The repressors might, as negative controls. Plasmid pHJ622 (tetrameric, engineered CI-GCN4 fusion protein) was used as positive control. Plasmid pEE694 expresses the CI-P2C fusion protein and pEE923 the 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 P2 C 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. P2 C 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 phasing 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 footprint analysis of P2 C 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 Pe should cover 75–80 nt, (from ~60 to +20), i.e. P2 C 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 differ 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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