Determination of the DNA-binding kinetics of three related but heteroimmune bacteriophage repressors using EMSA and SPR analysis

Petri Henriksson-Peltola, Wilhelmina Sehlén and Elisabeth Haggård-Ljungquist*

Department of Genetics, Microbiology and Toxicology, Stockholm University, S-106 91 Stockholm, Sweden

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ABSTRACT

Bacteriophages P2, P2 Hy dis and WΦ are very similar but heteroimmune *Escherichia coli* phages. The structural genes show over 96% identity, but the repressors show between 43 and 63% identities. Furthermore, the operators, which contain two directly repeated sequences, vary in sequence, length, location relative to the promoter and spacing between the direct repeats. We have compared the *in vivo* effects of the wild type and mutated operators on gene expression with the complexes formed between the repressors and their wild type or mutated operators using electrophoretic mobility shift assay (EMSA), and real-time kinetics of the protein–DNA interactions using surface plasmon resonance (SPR) analysis. Using EMSA, the repressors formed different protein–DNA complexes, and only WΦ was significantly affected by point mutations. However, SPR analysis showed a reduced association rate constant and an increased dissociation rate constant for P2 and WΦ operator mutants. The association rate constants of P2 Hy dis was too fast to be determined. The P2 Hy dis dissociation response curves were shown to be triphasic, while both P2 and WΦ C were biphasic. Thus, the kinetics of complex formation and the nature of the complexes formed differ extensively between these very closely related phages.

INTRODUCTION

The immunity repressors of the temperate 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 (1). The P2-like C proteins are small homologous proteins of ~100 aa. They recognize two direct repeats and are expressed from one promoter only. The C proteins of phage P2, P2 Hy dis and WΦ represent three different immunity classes, and so far the only C proteins where the operators have been located (2–4). The identity between the immunity C repressors is 38% (P2/P2Hy dis 63%, P2/WΦ 43% and WΦ/P2 Hy dis 44% identity at the amino acid level) (Figure 1A). The C proteins recognize direct repeats, termed half-sites, but the repeats differ in sequence, lengths, locations relative to the early promoters, and spacing (Figure 1B). P2 C binds to two direct repeats of 8 bp with a center-to-center distance of 22 bp. In P2 Hy dis, the half-sites are 8 bp with a distance of 26 bp, and the half-sites of WΦ are 10 bp long with a distance of 34 bp. In P2 and WΦ, the operators are flanking the −10 region of the Pe-promoter compared with P2 Hy dis where the half-sites are located on either side of the −35 region of the Pe-promoter.

To compare the affinities and the binding capacities of P2 C, P2 Hy dis C and WΦ C to their wild type and mutated operators, we have used one *in vivo* and two *in vitro* methods. To analyze the effects of the respective C protein on Pe *in vivo*, a plasmid system has been used where a reporter gene is under the control of the respective Pe promoter/operator region and the C protein is supplied in *cis*. Electrophoretic mobility shift assays (EMSA) have been used to analyze the complexes formed by the repressors with their respective operator and their dissociation constants. To determine the kinetics of the repressor–operator interactions in real time, we have used surface plasmon resonance (SPR) analysis.

MATERIALS AND METHODS

Biological materials

Bacterial strains and plasmids used are shown in Table 1.

Chloramphenicol acetyltransferase (CAT) activity determination

Ten milliliters cultures were harvested at an OD$_{600}$ of 0.8. The cultures were washed in 100 mM Tris–HCl, pH 7.9 and lysed by sonication in a total volume of 2 ml, and the cell extracts were cleared by centrifugation. Total protein
Figure 1. A comparison of the amino acid sequences of the C proteins and organization of the promoter-operator regions of P2, P2 Hy dis and WΦ. (A) The amino acid sequence of the C proteins and the predicted secondary structure. Amino acids conserved among all three proteins are shaded in dark gray; amino acids conserved among two proteins are shaded in light gray. (B) DNA sequences of the early promoter–operators of P2, P2 Hy dis and WΦ. The locations of the −10 and −35 regions are underlined. The transcriptional start sites are indicated by bent arrows. The operator half-sites are shaded in light gray, and the initiation codons are shaded in dark gray. The center-to-center distances between the half-sites are indicated in parenthesis. (C) The DNA sequences of the wt and mutated operators used in this work. The mutated nucleotides are shaded in gray.

Table 1. Bacterial strains and plasmids used

| Strain or plasmid | Pertinent features | Origin/reference |
|-------------------|-------------------|-----------------|
| Escherichia coli strains | ompT hsdR hsdM lon | BL21(DE3) E. coli B strain with T7 RNA polymerase under control of lac promoter |
| Plasmids | | |
| pEE675 | pKK232-8 derivative with P2 Pe-Pc region where Pe directs the cat gene |
| pEE679 | pET8c derivative with P2 C gene under the control of the T7 promoter. This plasmid has been found to contain a tandem DNA fragment downstream of the C gene from phage P4 (7870–8582) |
| pEE905 | pKK232-8 derivative with WΦ Pe-Pc region where Pe directs the cat gene |
| pEE909 | pEE905 derivative with 1 mutation in O1 site |
| pEE911 | pEE905 derivative with 1 mutation in both O1 and O2 sites |
| pEE915 | pKK232-8 derivative with P2 Hy dis C-Pe-Pc region where Pe directs the cat gene |
| pEE916 | pEE915 derivative with 1 mutation in O1 site |
| pEE917 | pEE915 derivative with 1 mutation in O2 site |
| pEE918 | pEE915 derivative with 1 mutation in both O1 and O2 sites |
| pEE922 | pET8c derivative with P2 Hy dis C gene under the control of the T7 promoter |
| pEE1020 | pET8c derivative with WΦ C gene under the control of the T7 promoter |
| pEE1021 | pEE675 derivative with 1 mutation in O1 site |
| pEE1022 | pEE675 derivative with 1 mutation in O2 site |
| pEE1023 | pEE675 derivative with 1 mutation in both O1 and O2 sites |
| pET8c | pBR322 derivative containing T7 promoter |
| pKK232-8 | pBR322 derivative containing a promoterless cat gene |
| pLysS | Plasmid expressing low level of T7 lysozyme |

*Unpublished
concentration was determined by the Bradford method using bovine serum albumin as standard (10). The supernatants were diluted and an equal amount of protein was added for the CAT determinations with \(^{14}C\)-chloramphenicol. The acetylated forms were separated by thin-layer chromatography (11). The CAT activity was determined 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) (5) containing plasmids pEE679 expressing P2 C (6), pEE922 expressing P2 Hy dis C (4), or pEE1020 expressing W\(\Phi\) C (8), were grown at 37 °C in LB or M9 minimal medium with ampicillin (100 \(\mu\)g/ml). The proteins were overexpressed and purified as described previously (8). The protein concentrations were determined by the Bradford method (10).

The purified proteins were stored in 10 mM sodium phosphate pH 7.0 supplemented 0.4 M NaCl for P2 C and P2 Hy dis C, and 0.6 M NaCl for \(W\Phi\) C, at 4 °C either with 40% (v/v) glycerol for EMSA or without glycerol for SPR analysis.

**Electrophoretic mobility shift assay (EMSA)**

The DNA fragments containing the wild-type operators (O1:O2), operators with a point mutation in one of the half-sites (O1mut: O2 ) or operators with a point mutation in both half-sites (O1mut: O2mut) (Figure 1), were expressed using primers 7 and 28R (GCATTAAGACTATCTTCTCG) and pKK240L (CTTACTAGCTTCTGTCAAA) with plasmid pEE675, pEE1021 or pEE1023 as sub-strates for the P2 operators, primers \(W\Phi\) 8R (GCTTTCCTAAAATGCTTTCG) and pKK232HindIII (GTCTTACTACAAGCTTGGCTG) using the phage specific primers and the biotinylated vector primer, biotKK232HindIII (GTCTTACTACAAGCTTGGCTG) that had a biotin molecule attached to its 5′ end. A sensor chip (SA) (Biacore) containing a streptavidin surface was activated by three consecutive injections of 1 M NaCl + 50 mM NaOH. Approximately 0.5 ng of the different operator fragments were immobilized in the respective flow cell, which corresponds to 200–300 RU for P2 C and P2 Hy dis C and for \(W\Phi\) C. The wild type or mutated operators were used to determine the affinity and the binding capacity of the C protein. The labeled and purified DNA was incubated in the same buffer. The gels were vacuum dried before the phosphor imager analysis.

The DNA fragments containing the wild-type operator (O1:O2) of each phage, as described above, were used to determine the dissociation constants (\(K_D\)) of each C repressor. The labeled and purified DNA was incubated with increasing concentrations of the protein in a BB buffer, as above, and the final DNA concentration in each reaction was 1 nM. The samples containing different concentrations of protein but the same amount DNA were loaded into the 5% non-denaturing PAA. The gel was vacuum dried. The separated radioactivity was analyzed and quantified using the phosphor imager (Fuji Film FLA-3000). Dissociation constants were determined after fitting the 1:1 binding isotherm to the experimental data by using the SIMFIT program (url:http://www.simfit.man.ac.uk).

**Real-time kinetic analyses**

Surface plasmon resonance analyses were carried out using the Biacore 3000 system (Biacore, Uppsala, Sweden). The operator containing DNA fragments described in the EMSA above were amplified by PCR using the phage specific primers and the biotinylated vector primer, biotKK232HindIII (GTCTTACTACAAGCTTGGCTG) that had a biotin molecule attached to its 5′ end. A sensor chip (SA) (Biacore) containing a streptavidin surface was activated by three consecutive injections of 1 M NaCl + 50 mM NaOH. Approximately 0.5 ng of the different operator fragments were immobilized in the respective flow cell, which corresponds to 200–300 RU for P2 C and P2 Hy dis C and for \(W\Phi\) C. The HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) was used as an immobilization buffer (Biacore). The proteins were diluted in the running buffer, which was the same as the immobilizing buffer. The protein concentrations used are given in the text. Two different flow rates were used, 30 and 2 \(\mu\)l/min and two injection cycles were performed. After each cycle, the chip was regenerated by washing with consecutive injections of 1 M NaCl and 50 mM NaOH. The SPR data was evaluated using the BIAevaluation software from (Biacore). Since the response curves were di- or triphasic the \(k_a\) and \(k_d\) values were determined separately for specified parts of the sensorgrams, and the fastest \(k_a\) and the slowest \(k_d\) values were used for the \(K_A\) and \(K_D\) determinations.

**RESULTS**

Different operator constructs have been used for each phage. The wild type or mutated operators were used to determine the affinity and the binding capacity of the respective C repressor. Two of the mutated operators contained one point mutation in either O1 or O2 and in the third both half-sites were mutated (Figure 1). The purpose of mutating the operators was to investigate how the mutations affect the affinity and the binding strength between the C repressor protein and the operator for a comparison of their in vivo effects.
The capacities of the C proteins to repress their cognate promoter in vivo using wild type and mutated operators and a plasmid reporter gene system

The effects of the operator mutants used in this work have been analyzed for WΦ C, using the cat gene as a reporter (3). Mutations in just one operator half-site did not affect the capacity of the C protein to repress the reporter gene, but when both half-sites were mutated the CAT activity was as high as the fully derepressed Pe promoter.

To investigate the in vivo effects of the operator mutations of P2 and P2 Hy dis, the C-Pe-Pe region of each phage was cloned upstream of the promoterless cat gene of plasmid pKK232-8 (9), and the capacity of the respective C protein to repress Pe was determined by measuring the CAT activity.

The mutation in either O1 or O2 half-site of the P2 operator does not abolish the repression capacity since the CAT-activity is as low as with the wild-type operator (Figure 2A, lanes 2, 3 and 5). However, mutations in both half-sites reduce the capacity of the P2 C protein to repress Pe since the CAT activity was 24% of the fully derepressed P2 promoter, i.e., in the absence of the C protein (Figure 2A, lanes 1, 4).

Since the operator of P2 Hy dis contains a natural mutation in O2 (4) (Figure 1), the repression pattern differs between P2 or WΦ Pe as seen in Figure 2B. The point mutation in O1 has no affect on the repression capacity, since the CAT activity is as low as with the wild-type operator (lanes 2 and 3), but when a point mutation is introduced in O2, the repression of the cat gene is abolished since the CAT activity is as high as the fully derepressed P2 promoter, i.e., in the absence of the C protein (Figure 2A, lanes 1, 4).

The P2, P2 Hy dis and WΦ C proteins give different band patterns in electromobility shift analysis

At the N-terminus of the repressors there are three alpha helices, and the 2nd and 3rd helix may constitute a HTH motif where the 3rd helix would be the DNA recognition helix. This assumption is supported by the fact that the C proteins differ in the 3rd helix (Figure 1A), but the 3D structure has not yet been determined. In contrast to most phage repressors the P2 C protein recognizes two direct repeats. It has been shown to form dimers, and the dimerization domain has been located to the C-terminal part of the protein (6,12).

The band shifts generated with P2 C are shown in Figure 3A. The P2 C protein gives two major band shifts, I and II (lanes 2–5). This fits with previous observations using crude extracts (6). The point mutations in O1 (lanes 7–10) or in both O1 and O2 (lanes 12–14) have no large effects on the band pattern. It should be noted that the single point mutations in O1 or O2 do not affect the capacity of C to repress the Pe promoter, while the double O1 and O2 mutation will reduce the capacity of C to repress Pe in vivo (Figure 2A).

The band pattern generated with P2 Hy dis C and its operator is similar to what has been observed using crude extracts (Figure 3B, lanes 2–5) (4). The major complex formed (II) has a very slow mobility compared to complex I formed by P2 C binding to its operator. A point mutation in O2 (lanes 6–10) or in both O1 and O2 (lanes 12–15) has no major effects on the complexes formed. In vivo it has been shown that the O2 mutation is enough to make Pe refractory to repression by P2 Hy dis C (4). Thus, the band pattern does not reflect the capacity of the C protein to repress the Pe promoter. The differences in migration of the protein–DNA complexes formed with P2 and P2 Hy dis proteins can either be due to different number of C proteins bound per operator or that the proteins cause different conformations of the DNA target.

WΦ C shows three retarded bands with the wild-type operator, I, II and III (Figure 3C, lanes 2–5). Complex III has about the same mobility as complex II obtained with P2 C (Figure 3A). A point mutation in O1 leads to a loss of the weak band I (lanes 7–10), and having a point mutation in both O1 and O2, which in vivo makes the WΦ C protein unable to repress expression of the cat reporter gene, gives the strong retarded band I, no band II and a relative decrease of radioactivity in band III (lanes 12–15). The decrease in the formation of complex III only when both half-sites are mutated, is in agreement with the in vivo data where the single point mutation does not affect the capacity of WΦ C to block the Pe promoter while the double mutant is refractory to the presence of WΦ C (2).

Estimation of the dissociation constants (K_D) of the C repressor/DNA complexes from electromobility shift analysis

Since the complexes formed between the C proteins and the respective operator varied, we were interested to determine their dissociation constants, using EMSA. Thus, a titration with a constant amount of DNA and an increasing amount of C protein was performed for each repressor-operator system, and the percent shifted DNA have been calculated from the preparations having the highest specific activities, which in the case of P2 Hy dis C corresponds to that used in Figure 3. The K_D for P2 C was estimated to 2.4 × 10^{-10} M, for P2 Hy dis C to 3.5 × 10^{-9} M and for WΦ C to 2.2 × 10^{-9} M (Figure 4).

An analysis of the interactions between the respective C protein with their wild type and mutated operators using surface plasmon resonance (SPR)

The determination of K_D-values using EMSA is dependent on the specific activity of the purified protein. Since the specific activity of our C preparations varied, we were interested to compare the values obtained by EMSA with
those generated in real time by SPR. To be able to analyze the association and dissociation by SPR we have used a Biacore 3000 system with streptavidine-coated SA chips containing four flow cells. The biotinylated 150-mers oligonucleotides were anchored to the surface of the SA chip at the respective cell. The first flow cell in all experiments contained non-specific DNA as a reference and a control in order to reduce background noise. DNA was recovered as the absolute resonance units (RU), i.e., the difference between the initial and final values of the bulk refractive index. The RU-values of the different DNA loaded flow cells are shown in Table 2.

Since the protein–DNA interactions may be affected by the flow rate, the initial kinetic studies were carried out at two flow rates, 2 and 30 μl/min, using a protein concentration of 23 pM. As can be seen in Figure 5, differences in the binding responses of the C repressors to the surfaces containing the wild-type operators are observed. P2 C shows after injection an initially fast off rate, after which a very slow dissociation rate is reached. The response is ~200 U higher after the initial fast dissociation at 2 μl/min flow rate compared to the 30 μl/min flow rate (Figure 5A). P2 Hy dis C shows a triphasic dissociation, and it has only about a 50 RU higher

![Figure 2](https://example.com/image2.png)

**Figure 2.** CAT assays showing the effects of the mutations in the direct repeats of P2 (A) and P2 Hy dis (B). In both cases, lane 1 is a control showing the strength of the Pe promoter in absence of the C repressor, and this CAT activity was set to 1. Lane 4 in B represents the operator where the O2 repeat extended with 2 nt corresponding to the O1 repeat. The relative CAT activity labeled 0, indicates that no activity could be detected in the phosphor imager analysis.
response of C protein with a slow dissociation at 2 μl/min flow rate compared to the 30 μl/min (Figure 5B). The Wφ C protein shows about a 200 RU higher response at 2 μl/min than at 30 μl/min (Figure 5C). Therefore, a 2 μl/min flow rate was used in our experiments. Saturation is not reached during the time span of the experiment for P2 C and Wφ C, which might be due to a specific as well as an unspecific DNA binding of the C proteins to the DNA. The calculated ratio of bound C monomers/DNA molecule at the end of injection is about 3 for P2 C, 2 for P2 Hy dis C and 1.5 for Wφ C.

To study the effects of the operator mutations, the SA chips were loaded with the wild-type operator (O1:O2), the operator containing one point mutation in one half-site (O1mut:O2), and the operator in which both half-sites were mutated (O1mut:O2mut). The RU-values are shown in Table 2. Since the P2 Hy dis O2 half-site already contains a natural point mutation (Figure 1) (4), a point mutation in only O1 (O1mut:O2) was used as a double mutant.

Determination of the kinetics of the interactions of the C repressor proteins and their wild type and mutated operators using the Biosensor system

The kinetic analyses were carried out with different protein concentrations. The first flow cell, containing non-specific DNA, was used as a negative control for the cells containing wild type or mutated operator sites.
Table 2. Amount of DNA template bound per flow cell

| Flow cell | DNA-template | RU |
|-----------|--------------|----|
| Fc1       | Random DNA   | 800|
| Fc2       | O1:O2 (P2)  | 300|
| Fc3       | O1mut:O2(P2)| 260|
| Fc4       | O1mut:O2mut(P2) | 250|
| Fc1       | Random DNA   | 440|
| Fc2       | O1:O1(P2 Hy dis) | 215|
| Fc3       | O1mut:O2(P2 Hy dis) | 210|
| Fc4       | O1:O2mut(P2 Hy dis) | 290|
| Fc1       | Random DNA   | 557|
| Fc2       | O1:O2 (WΦ)  | 344|
| Fc3       | O1mut:O2(WΦ) | 327|
| Fc4       | O1mut:O2mut(WΦ) | 333|

*One SA-chip per phage was used. In all three chips, the first flow cell (Fc1) was used as a reference. Other flow cells contained different operator constructions.

*The amount of bound DNA/cell is shown in RU.

The sensorgram data were analyzed with the BIA-evaluation software. The experimental data from each flow cell were used in the calculations unless otherwise stated, and evaluated for closeness of fit (chi-square) to the 1:1 Langmuir-binding model. The curves were normalized before the kinetic analysis, since there might be some conformational changes of DNA due to the binding of the C repressors. The calculations were carried out as described in Experimental Procedures.

**P2 C.** The relative responses (RU) of the binding of P2 C to its operator were found to correlate to the protein concentration (28.5–39.9 pM), i.e., the RU increased with increasing concentration. As can be seen in the sensogram (Figure 6A), the RU-values of the wild-type operator increase with 1200 RU during injection. However, the association rate of P2 C is biphasic, initially there is a very high-association rate that changes to a slower association rate, but it does not reach an equilibrium state at any concentration used. Estimations of the initial fast association rates (16–20 s) for the wild type and mutated operators indicate similar $k_a$. The range of the $k_a$ varied between $2.3 \times 10^6$ and $1.5 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ (Table 3). The slow association rate constants calculated between 80 and 240 s, were between $6.5 \times 10^5$ and $3.9 \times 10^6 \text{M}^{-1}\text{s}^{-1}$. The dissociation curve after the injection is also biphasic, i.e., after an initial fast dissociation of about half the RU, there is a steady state level with a very slow dissociation (Figure 6A). This may be interpreted in different ways. One possibility is that the initial fast dissociation rate is due to the release of non-specifically bound repressor, while the slow dissociation is the very tight complex of C with its operator. Alternatively, they represent two different forms of repressor–operator complexes where one represents a very tight binding. We believe the former is correct, and have calculated the dissociation rate constant from the steady state slow dissociation, i.e., between 260 and 510 s. The level of RU with a slow dissociation rate was reduced by 40 U at 23 pM between the wild-type operator and the O1mut:O2 operator, and the O1mut:O2mut operators showed to 20 U further reduction. The dissociation rate constant $k_d$ for the wild-type operator was $1.5 \times 10^{-3} \text{s}^{-1}$ and for the two mutated operators it was $1.8 \times 10^{-3}$ and $2.1 \times 10^{-3} \text{s}^{-1}$ (Table 3).

These rate constants show that P2 C has a higher association rate and a lower dissociation rate to the wild-type operator and to the operator where O1 was mutated compared to the O1mut:O2mut operator. This means that P2 C forms a more stable complex with its wild-type operator and the O1mut:O2 operator than with the O1mut:O2mut operator. The calculated association constant $K_A$ and the dissociation constant $K_D$ were $1.5 \times 10^9 \text{M}^{-1}$ and $6.3 \times 10^{-10} \text{M}$, respectively, for the wild-type operator compared to $2.2 \times 10^9 \text{M}^{-1}$ and $4.7 \times 10^{-10} \text{M}$ for the O1mut:O2 operator and $7.0 \times 10^8 \text{M}^{-1}$ and $1.4 \times 10^{-9} \text{M}$ for the O1mut:O2mut operator. These $K_D$ and $K_A$-values confirm that P2 C has a stronger affinity and binding to the operators which also are functional in vivo.
P2 Hy dis C. The sensorgrams using the P2 Hy dis C at different protein concentrations (22.9–39.8 pM) and the wild-type operator are shown in Figure 6B. The association rates as well as the dissociation rates seem to be triphasic at all concentrations. The values for the two fast association rates do not fit the 1:1 Langmuir model adapted for two binding sites, and the chi-square values are too high to give reliable rate constants. However, after ~120 s a very slow association rate is obtained, giving a $k_a$ between 2.5 and $3.2 \times 10^3$ M$^{-1}$ s$^{-1}$ when measured between 80 and 210 s. Unlike P2 C, also the dissociation seems to be triphasic since after a fast dissociation event the sensorgrams indicate two different dissociation rates (Figure 6B). The dissociation rate constants for the wild type and the mutated operators are therefore calculated twice, between 240 and 300 s and between 300 and 510 s after injection, respectively. The first dissociation rate constants ($k_d^1$) were $4.9 \times 10^{-2}$ s$^{-1}$ for the wild-type operator and $6.8 \times 10^{-2}$ and $7.8 \times 10^{-2}$ s$^{-1}$ for the mutated operators (Table 3). The second $k_d^2$ also shows a similar tendency for the operators, but a slightly increased dissociation rate constant was obtained for the operator containing a mutation in one half-site (Table 3). The level of RU with a slow dissociation rate at 23 pM was also slightly increased (5 U) between the wild type and the O1mut : O2 operator but the O1 : O2mut operator showed a 13 U reduction compared to the wild-type operator. The $k_d^2$ values obtained confirm that the dissociation is lower for the wild type and the O1 mutated operator, meaning that the complex formed with P2 Hy dis C and either the wild type or the O1mut : O2 operator is more stable than the complex with O1 : O2mut operator. An almost steady state was reached with all concentrations used as opposed to P2 and Wϕ C (see below) where the steady state could not be reached. This indicates that P2 Hy dis C has a lower level of non-specific binding compared to P2 and Wϕ C. This is also supported by the very fast binding seen in the EMSA analyses (Figure 4B), and by the fast association rate of P2 Hy dis C (Figure 6B). In spite of the steady state, neither the association constant $K_A$ nor the dissociation constant $K_d$ could be calculated because of some fluctuations between the concentrations after subtracting the reference from the analyte-binding sensorgrams. The lowest concentration, 22.9 pM had the highest RU. The RU increased from 25.7 to 28.8 pM, but decreased again from 28.8 to 39.8 pM (data not shown). It seems as if P2 Hy dis C could not bind efficiently to its operator at higher concentrations. Such a dose-dependent binding is not observed with P2 C.

Wϕ C. The sensorgrams obtained with Wϕ C at different protein concentrations (9.3–23.4 pM) with the wild-type operator are shown in Figure 6C. As for P2 C, the association of Wϕ C was biphasic and an equilibrium state was not reached at any protein concentration used. The initial very fast association rate constituted only ~20% of the RU obtained after 200 s. The estimated association rate constants determined between 17 and 20 s varied between $5.1 \times 10^5$ and $7.6 \times 10^6$ M$^{-1}$ s$^{-1}$. Also the dissociation was biphasic, but compared to P2 C, a much smaller fraction showed the very fast dissociation rate. The dissociation rate constant $k_d$ was calculated between 275 and 400 s and varied between $1.5 \times 10^{-4}$ and $7.7 \times 10^{-4}$ s$^{-1}$ (Table 3). As for P2 C and P2 Hy dis C, Wϕ C has the highest affinity and the strongest binding to the wild-type operator. The O1mut : O2 operator has a slightly lower affinity and $K_d$ value than the wild-type operator, but a little higher compared with the O1mut : O2mut operator (Table 3). No difference in the level of RU of the complex with a slow dissociation rate was found between the wild type and operator mutants. Thus, Wϕ C, like P2 C and P2 Hy dis C, forms a stable protein–DNA complex with the operators that are active in vivo. This complex is, however, more stable than the complexes formed with P2 C and P2 Hy dis C to their respective operator, since a smaller fraction of the bound repressor shows a fast dissociation rate and the dissociation rate constant for the remaining complex is lower.
O1:O2 (P2) 2.3
phage lambda, where the CII protein activates the PRE maintenance of the lysogenic state. This is in contrast to the same promoter during establishment as well as during the establishment of the lysogenic state, while promoter PRM controls CI expression during the lysogenic state (14). In contrast to most temperate phages, the P2-like phages are not inducible by UV light, in accordance with the fact that the P2 C protein is not stimulated by RecA to undergo self-cleavage like lambda CI.

In our electrophoretic mobility assays (EMSAs), the C proteins were shown to form different protein–DNA complexes, where the major complex formed with P2 Hy dis C has a slower migration compared to P2 C and Wϕ C. This slow migration might be caused by a different oligomeric state of P2 Hy dis C, or by a distortion of the DNA structure upon protein binding. The number of C proteins in the respective complex is not known, but the SPR analysis does not indicate a higher number of C proteins per DNA molecule for P2 Hy dis C. A genetic analysis of the P2 C and Wϕ C proteins has shown that in the absence of DNA they form dimers, but not higher oligomeric structures (8). During gel-filtration all C proteins were shown to form different protein–DNA complexes, where the major complex formed with P2 Hy dis C, or by a distortion of the DNA structure upon protein binding. The number of C proteins in the respective complex is not known, but the SPR analysis does not indicate a higher number of C proteins per DNA molecule for P2 Hy dis C. A genetic analysis of the P2 C and Wϕ C proteins has shown that in the absence of DNA they form dimers, but not higher oligomeric structures (8). During gel-filtration all C proteins elute at the same positions, indicating also that P2 Hy dis C is a dimer in solution (data not shown). Therefore, the higher mobility is more likely caused by a distortion of the DNA structure, possibly a consequence of the fact that the operator half-sites are separated by two and a half helical turns, and thus are not located on the same side of the DNA helix. A comparison of the complexes formed by P2 C and Wϕ C may indicate that they form similar complexes, but in the case of P2 there is no difference in the affinity to half-site compared to the other. Using a circular permutation assay, the P2 and Wϕ C proteins have been shown to bend their respective DNA targets upon binding, and Wϕ C as opposed to P2 C is able to bind to only one half-site (8). This may explain why three retarded bands can be detected with Wϕ C but only two with P2 C.
Surprisingly, the EMSA failed to detect any significant differences in binding of the P2 C and P2 Hy dis C proteins to the operator mutants, not even to the operators having a point mutation on both half-sites. Using in vivo plasmid reporter gene systems, and the same point mutations no significant effect on the repression of the reporter gene is found with the single mutations, but a mutation in both half-sites showed a clear reduction in repression by P2 C, and in P2 Hy dis and Wϕ repression is totally abolished (3, and this work). A possible explanation for this discrepancy between our in vivo and in vitro results is the configuration of the DNA targets. In the in vivo experiments the operator/promoter regions are in a supercoiled plasmid, but in vitro they are in the form of linear DNA fragments. Using SPR, however, point mutations in both half-sites clearly reduce the association constants and increases the dissociation constants of P2 and Wϕ C. Previous determinations of dissociation constants for P2 C has been obtained by filter-binding experiments where a constant concentration of P2 was exposed to increasing concentrations of full length linear P2 DNA (22). The $K_D$ obtained using a filter-binding assay with full-length P2 DNA covered the range $2.0 \times 10^{-10}$ to $3.3 \times 10^{-10}$ M, which is about the same as we found in our EMSA analysis ($2.4 \times 10^{-10}$ M) using a short DNA fragment containing O1 and O2. This implies that there are no additional strong binding sites for P2 C on the P2 genome. The dissociation constants measured by SPR have previously been shown to be lower compared to other methods, for example, using EMSA the lactose repressor-operator gives a $K_D$ of $4.2 \times 10^{-9}$ and using SPR the $K_D$ was $2.0 \times 10^{-10}$ (23). In this work, the $K_D$ values were found to be slightly higher using SPR in the case of P2 C and ~20-fold lower for Wϕ C.

The sensorgrams obtained in the SPR analysis for the P2 and P2 Hy dis C proteins show initially a very fast association rate, followed by a slower association rate. In the case of P2 Hy dis C the association seems triphasic and this work might be the capacities of the C proteins to oligomerize upon binding to its DNA target. The CI protein of phage lambda is known to form dimers in solution, but octamers between dimers bound to different operator regions, with a looping of the DNA in between allowing long distant effects, are known (21). Furthermore, the crystal structure of phage 186 CI has revealed a heptamers of dimers around which the DNA is wrapped (24). The formation of higher oligomers upon DNA binding has also been shown for EthR, a repressor of the TetR/CamR family, which binds to its operator as an octamer, but in solutions it seems to be a monomer or dimers but not higher oligomers (25). This could be the case for the C proteins. A possible scenario is that in the absence of DNA the C proteins only forms dimers, but upon binding to their respective DNA targets they forms tetramers or higher oligomers. Possibly, the point mutations in both half-sites prevent tetramerization, but not binding of the dimeric form of the C protein to the mutated half-sites.

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