Identification of the binding site of the σ^{54} hetero-oligomeric FleQ/FleT activator in the flagellar promoters of *Rhodobacter sphaeroides*

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Expression of the flagellar genes in *Rhodobacter sphaeroides* is dependent on one of the four sigma-54 factors present in this bacterium and on the enhancer binding proteins (EBPs) FleQ and FleT. These proteins, in contrast to other well-characterized EBPs, carry out activation as a hetero-oligomeric complex. To further characterize the molecular properties of this complex we mapped the binding sites or upstream activation sequences (UASs) of six different flagellar promoters. In most cases the UASs were identified at approximately 100 bp upstream from the promoter. However, the activity of the divergent promoters *flhAp-flgAp*, which are separated by only 53 bp, is mainly dependent on a UAS located approximately 200 bp downstream from each promoter. Interestingly, a significant amount of activation mediated by the upstream or contralateral UAS was also detected, suggesting that the architecture of this region is important for the correct regulation of these promoters. Sequence analysis of the regions carrying the potential FleQ/FleT binding sites revealed a conserved motif. *In vivo* footprinting experiments with the *motAp* promoter allowed us to identify a protected region that overlaps with this motif. These results allow us to propose a consensus sequence that represents the binding site of the FleQ/FleT activating complex.

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INTRODUCTION

Promoter recognition in bacteria is mediated by the RNA polymerase core (E) associated with a sigma factor. There are two families of sigma factors, the σ^{70} family and that of σ^{54} . These two families differ not only in their sequence but also in the mechanism that brings about transcriptional initiation. Whereas $E\sigma^{70}$ is capable of forming open complex by itself, $E\sigma^{54}$ requires an activator protein that, through ATP hydrolysis, allows open complex formation (for reviews, see Buck *et al.*, 2000; Wigneshweraraj *et al.*, 2005). Activator proteins of $E\sigma^{54}$ usually bind approximately 100 bp upstream of the promoter sequence, and a DNA loop favours the interaction of the activator with the RNA polymerase holoenzyme bound to the promoter (Reitzer & Magasanik, 1986; Ninfa *et al.*, 1987; Buck *et al.*, 1986; Hoover *et al.*, 1990; Su *et al.*, 1990; Wedel *et al.*,

1990). The mechanism that allows open complex formation is still unknown, but the current model suggests that the activator protein remodels the nucleoprotein complex formed by $E\sigma^{54}$ and DNA in order to initiate transcription (Guo *et al.*, 2000; Cannon *et al.*, 2000; Burrows *et al.*, 2004; Rappas *et al.*, 2007). The DNA region to which the activator proteins bind is known as the upstream activation sequence or UAS (Buck *et al.*, 1986; Morett *et al.*, 1988). It has been shown for the *glnA* promoter that this region can be moved more than 1000 bp without losing its ability to stimulate transcription (Ninfa *et al.*, 1987). Based on this property, the UASs are also known as enhancers, and the activator proteins as enhancer binding proteins, or EBPs.

The σ^{54} -dependent promoters show a highly conserved consensus sequence, and their main characteristics are the dinucleotides GG and GC located in the –24 and –12 boxes, respectively (Beynon *et al.*, 1983; Merrick, 1993; Barrios *et al.*, 1999).

Proteins that belong to the EBP family of transcriptional activators usually show three domains: a variable N terminus, a very well-conserved central domain, and a helix–turn–helix (HTH) motif at the C-terminal domain

Abbreviations: DMS, dimethyl sulfate; EBP, enhancer binding protein; HTH, helix–turn–helix; IHF, integration host factor; UAS, upstream activation sequence.

Two supplementary tables, listing oligonucleotides used in this work and relevant sequences for motif finding, are available with the online version of this paper.

(Studholme & Dixon, 2003; Morett & Segovia, 1993). The N-terminal domain controls the ability of the EBP to form hexameric or heptameric oligomers in response to a particular stimulus (Lee *et al.*, 1994; Chen *et al.*, 2008; Lee *et al.*, 2003; Doucleff *et al.*, 2005a; De Carlo *et al.*, 2006). The EBPs can be classified by the type of domain that is present in the N-terminal region (Studholme & Dixon, 2003). However, some EBPs do not have an N-terminal domain; in consequence, these proteins are constitutively active, and the control of this kind of proteins is usually achieved through binding of another protein (Jovanovic *et al.*, 1996; Preston *et al.*, 1998; Elderkin *et al.*, 2005; Hankamer *et al.*, 2004). The central domain is responsible for ATP hydrolysis, oligomerization and transcriptional activation (for reviews, see Wigneshweraraj *et al.*, 2005; Schumacher *et al.*, 2006; Rappas *et al.*, 2007). This domain is present in all members of the AAA+ family of ATPases. These proteins, when active, form ring shaped oligomers and exert their activity through the ATP-dependent remodelling of macromolecules (Neuwald *et al.*, 1999; Schumacher *et al.*, 2006; Wyman *et al.*, 1997; Rippe *et al.*, 1998; Lee *et al.*, 2003; Rappas *et al.*, 2005; Sallai & Tucker, 2005). Seven conserved regions have been identified in the central domain of the EBPs (C1–C7) (Morett & Segovia, 1993; Zhang *et al.*, 2002). Within these regions a distinctive signature of the EBPs is the GAFTGA sequence that is located in the C3 region; experimental evidence suggests that this sequence interacts with σ^{54} during ATP hydrolysis (Chaney *et al.*, 2001; De Carlo *et al.*, 2006). For some activators such as DctD, NifA and PspF, it has been shown that, in the absence of the N- and C-terminal domains, the central region by itself is sufficient to activate transcription (Xu *et al.*, 2004).

The C-terminal domain allows specific binding of the activator to a particular UAS (Doucleff *et al.*, 2005b), limiting the activity of the EBP to a few promoters in the chromosome; this fact allows the σ^{54} factor to be involved in the transcription of different non-related pathways in the same bacterium. Functional EBPs without an HTH motif have been described in *Helicobacter pylori*, *Chlamydia trachomatis* and *Rhodobacter sphaeroides* (Brahmachary *et al.*, 2004; Poggio *et al.*, 2005; Beck *et al.*, 2007). In the first two cases, only one EBP is present in the genome of these micro-organisms, and it has been proposed that these EBPs promote open complex formation from solution (i.e. without DNA binding) (Brahmachary *et al.*, 2004). In contrast, *R. sphaeroides* has four σ^{54} factors and five well-conserved EBPs (Poggio *et al.*, 2002, 2005). It has previously been shown that the four σ^{54} proteins present in *R. sphaeroides* are not functionally interchangeable. RpoN1 along with NifA specifically recognizes and allows transcription of the genes required for nitrogen fixation, while RpoN2 along with FleQ and FleT regulates expression of a subset of the flagellar genes (flagellar class III) required to form the flagellar structures known as the hook and basal body. The functions of RpoN3 and RpoN4

are still unknown, but several lines of evidence suggest that neither of them is able to trigger the expression of the promoters recognized by RpoN1 or RpoN2 (Poggio *et al.*, 2002, 2006). The *R. sphaeroides* flagellar EBPs FleQ and the HTH-lacking FleT protein are both required for the expression of the flagellar genes. By itself, FleQ is only capable of activating transcription of the single class II operon, which contains the *fleT* gene and six other flagellar genes. Even though FleT lacks the characteristic HTH motif in the C-terminal region, this protein forms a complex with FleQ which is required to transcribe the flagellar class III operons (Poggio *et al.*, 2005). These operons encode the majority of the flagellar proteins required for the formation of the flagellum.

The only other hetero-oligomeric EBP so far described is the HrpR/HrpS complex from *Pseudomonas syringae* (Grimm *et al.*, 1995; Hutcheson *et al.*, 2001). In this case both proteins have an HTH motif, and both are required for full expression of the genes of the pathogenic type III protein export system (Hutcheson *et al.*, 2001).

In this work, we characterize the UAS regions that are recognized by the FleQ/FleT complex. This study includes six out of the seven promoters known to be dependent upon FleQ/FleT. From deletion mapping, *in vivo* footprinting and *in silico* analysis, we propose a sequence motif that represents the binding site of this activating complex.

METHODS

Plasmids, bacterial strains and growth conditions. Plasmids and bacterial strains used in this work are listed in Table 1. *R. sphaeroides* cells were grown in Siström's minimal medium (Siström, 1962) at 30 °C, in the dark with shaking at 200 r.p.m. *Escherichia coli* was grown in LB medium at 37 °C. When required, antibiotics were added at the following concentrations: for *R. sphaeroides*, gentamicin (5 µg ml⁻¹), kanamycin (25 µg ml⁻¹) and spectinomycin (50 µg ml⁻¹); for *E. coli*, gentamicin (30 µg ml⁻¹), kanamycin (50 µg ml⁻¹), spectinomycin (100 µg ml⁻¹) and ampicillin (100 µg ml⁻¹).

Oligonucleotides. The oligonucleotides used in this work are listed in Supplementary Table S1.

Recombinant DNA techniques. Standard methods were used to obtain chromosomal or plasmid DNA (Ausubel *et al.*, 1987). To construct the plasmids used in this work, chromosomal or plasmid DNA was amplified with the appropriate oligonucleotides (Supplementary Table S1) using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) according to the recommendations of the manufacturer. All the PCR products were sequenced after cloning. Standard methods were used for transformation, ligation and other related techniques.

Conjugation. Plasmid DNA was mobilized into *R. sphaeroides* by conjugation according to published procedures (Davis *et al.*, 1988).

Deletion mapping. The DNA fragments carrying the promoter sequence along with the adjacent sequence were obtained by PCR using the appropriate oligonucleotide (Supplementary Table S1). The boundaries of each construct were considered to be from the conserved C that is part of the GC dinucleotide within the σ^{54} consensus promoter. The DNA fragments were cloned in pTZ19R

Table 1. Strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Reference or source
<i>R. sphaeroides</i> strains		
WS8	Wild-type strain	Socket et al. (1990)
SP13	<i>fleQΔ::kan</i>	Poggio et al. (2005)
<i>E. coli</i> strains		
TOP10	Cloning strain	Invitrogen
S17	RP4-2 (Tc::Mu) (Kn::Tn7)	Simon et al. (1983)
Plasmids		
pRK415	Vector for cloning, Tc ^R	Keen et al. (1988)
pBBMCS53	Transcriptional ' <i>uidA</i> fusion vector	Girard et al. (2000)
pBflgBp	pBBMCS53 carrying <i>flgBp</i> from −378 to +225	Poggio et al. (2005)
pBflgGp	pBBMCS53 carrying <i>flgGp</i> from −347 to +274	Poggio et al. (2005)
pRKfliOp	pRK415 carrying <i>fliOp</i> from −224 to +179 fused to ' <i>uidA</i>	Poggio et al. (2002)
pBmotAp	pBBMCS carrying <i>motAp</i> from −422 to +297	Poggio et al. (2005)
pBflgAp	pBBMCS carrying <i>flgAp</i> from −288 to +270	Poggio et al. (2005)
pBflhAp	pBBMCS carrying <i>flhAp</i> from −349 to +209	Poggio et al. (2005)

(Pharmacia) and after sequencing they were cloned in the proper orientation into pBBMCS53, to create the corresponding transcriptional fusion. The fragments corresponding to the *fliOp* promoter were cloned in pRK415. In these constructs, the '*uidA* reporter gene had previously been fused to the promoter fragment in pTZ19R, and finally the fusion was transferred to pRK415 in the orientation opposite to that of the plasmid *lac* promoter.

β -Glucuronidase activity. β -Glucuronidase was determined from sonicated cell-free extracts using 4-methyl-umbelliferyl- β -D-glucuronide as substrate, and following a published protocol (Jefferson et al., 1986). 4-Methyl-umbelliferone (Sigma) was used as a standard. In this work, specific activities are expressed as nmol 4-methyl-umbelliferone formed min^{−1} (mg protein)^{−1}.

In vivo footprinting. These experiments followed a published protocol (Borowiec & Gralla, 1986), with slight modifications. Briefly, *R. sphaeroides* cultures carrying the indicated plasmid were grown to OD₆₀₀ 0.5 with shaking. Dimethyl sulfate (DMS) was added at a final concentration of 10 mM and incubation was continued for 5 min. After this time, the culture was rapidly chilled to 4 °C and cells were collected by centrifugation. The plasmid was isolated with a Plasmid Mini Kit (Qiagen), precipitated with ethanol and suspended in 1 M piperidine. After incubation at 90 °C for 30 min, the cleaved DNA was desalted by chromatography on Sephadex G-50. The oligonucleotides were 5'-labelled with T4 polynucleotide kinase and [γ -³²P]ATP [3000 Ci (111 000 Bq) mmol^{−1}]. These primers were used for an extension reaction of the cleaved products using 5 μg DNA and *Taq* DNA polymerase in a final volume of 20 μl. The reaction was carried out for 15 cycles and stopped by adding 5 μl of loading buffer containing 8 M urea (Ausubel et al., 1987). A 5 μl sample from this reaction was analysed on sequencing gels. The primers used to carry out these experiments are indicated with asterisks in Supplementary Table S1.

Computational search of motifs. The sequences in Supplementary Table S2 were used as input for the MEME algorithm (Bailey & Elkan, 1994). The best motif was transformed into a degenerated recognition sequence and together with the consensus σ^{54} degenerated promoter sequence were used as a motif for the DNA-pattern of the Regulatory Sequence Analysis tools algorithm. The used motif was G[CA][TC][CG]G[AT][GC][AG]N[GC]N[GT][CG]N[70,300]GGCAN{6}TTGC[AC], and the whole genome of *R. sphaeroides* was searched.

RESULTS

Deletion analysis of the regulatory regions of promoters dependent on FleT/FleQ

The flagellar promoters dependent on FleQ/FleT have been previously identified by an *in silico* analysis and confirmed by experimental evidence, and they are: *fliKp*, *fliOp*, *flgBp*, *flgGp*, *motAp*, and the divergently transcribed *flhAp* and *flgAp* promoters (Poggio et al., 2000, 2005). In this work, we identify the binding region of the FleQ/FleT complex for six of these promoters.

As reported previously (Poggio et al., 2005), DNA fragments that included approximately 300 bp upstream and downstream of each flagellar σ^{54} promoter were cloned upstream of the '*uidA* reporter gene and proved to be sufficient for β -glucuronidase expression in a FleQ/FleT-dependent fashion [Fig. 1, first construct of panels (a–d), and line 1 of Figs 2 and 3; see Methods for details].

Sequential deletions of the upstream regions of *fliOp*, *flgBp*, *flgGp* and *motAp* allowed us to determine that a region of between 50 and 100 bp is essential for the activity of these promoters Fig. 1(a, b, c and d). No activity was observed when these constructs were introduced into either SP13 (*fleQΔ::kan*) or SP12 (*fleTΔ::aadA*) mutant strains (data not shown), indicating that no spurious promoters were generated in the different constructs. From the results shown in Fig. 1 it can be inferred that the binding site of the FleQ/FleT complex must be located in a 97 bp fragment between positions −222 and −125 upstream of the GC dinucleotide for *flgBp* (compare Fig. 1a, lines 2 and 3); for *flgGp* in an 81 bp fragment between positions −180 and −99 bp (compare Fig. 1b, lines 2 and 3); for *motAp* the binding site must be in a fragment of 50 bp between positions −217 and −167 bp (compare Fig. 1c, lines 3 and 4); and for *fliOp* in a 64 bp fragment between positions −175 and −111 bp (compare Fig. 1d, lines 2 and 3). The

Fig. 1. Deletion analysis of the regulatory region of the flagellar class III σ^{54} -dependent promoters. Promoters (striped boxes) were cloned along with their regulatory region upstream of the reporter gene *'uidA* (grey box). The scale at the top of each panel indicates the length in base pairs. The numbers to the left of each construct show the distance between the 5' end and the C of the conserved GC dinucleotide of the -12 box. Activities are expressed as percentages of the β -glucuronidase activity of the largest construct. The β -glucuronidase activities, expressed as nmol 4-methyl-umbelliferone formed min^{-1} (mg protein^{-1}), of the reference constructs were 261 905 for *flgBp*, 1 200 820 for *flgGp*, 1 961 538 for *motAp*, and 135 423 for *fliOp*. For each construct, the activity was measured at least three times independently, and the SD was less than 15 %.

sequences of the fragments identified as containing an enhancer region can be found in Supplementary Table S2.

Localization of the UAS of the divergent promoters *flhAp*-*flgAp*

The divergent promoters *flhAp* and *flgAp* are separated by only 53 bp from the conserved GG dinucleotide of each

promoter. Given the reported location of previously characterized UASs, we envisioned that the UAS for *flhAp* and *flgAp* could be contiguous to the promoter, as has been reported for FleQ in *Pseudomonas aeruginosa* (Jyot *et al.*, 2002), or at the canonical 100 bp distance from each promoter, overlapping the coding sequence of the *flhA* and *flgA* operons. To test these possibilities a deletion analysis for each promoter region was carried out, as shown in Figs 2 and 3. Initially, a DNA fragment extending from -349 to +209 from *flhAp* was cloned upstream of the reporter gene *'uidA*. The first deletion removed 202 bp from the 5' end of the original construct, causing a reduction of approximately 30 % in the activity of the reporter gene (Fig. 2, compare lines 1 and 2). Surprisingly, all of the subsequent deletions of the upstream region that removed 264, 299 or 331 bp did not cause any further decrement in the observed activity (Fig. 2, lines 3, 4 and 5). It should be noted that the last deletion left only 18 bp upstream of the conserved GC dinucleotide. For the following deletions, fragments from the 3' end were removed using the original construct as a starting point. As shown in Fig. 2, the activity level did not change when the first 60 bp were removed (compare lines 1 and 6). However, an additional deletion of 69 bp reduced the β -glucuronidase activity to 53 %, and when 57 bp were additionally deleted, 73 % of the original activity was lost (Fig. 2, lines 7 and 8). To determine whether the remaining activity was dependent on the presence of the region upstream of the promoter, 279 bp were removed from the 5' end of the last construct. The resulting plasmid yielded only 6 % of the activity of the full promoter (Fig. 2, line 9). As shown in Fig. 2, lines 3 and 4, deletion of this region by itself reduced the activity of *flhAp* by only 30 %. Together, these results suggest that approximately 30 % of the observed activity of this promoter depends on the sequences located upstream of *flhAp*, whereas 65–70 % of the activity depends on the sequences located downstream.

A similar situation was found for the *flgAp* promoter, for which a reduction of no more than 35 % in the β -glucuronidase activity was observed when 60, 130, 198 or 237 bp were deleted from the 5' end of an initial fragment that contained 288 and 270 bp upstream and downstream of the promoter, respectively (Fig. 3, lines 2–5). In contrast, a deletion that removed 53 bp from the 3' end caused a reduction of approximately 60 % (Fig. 3, line 6). Further deletions of the 3'-end did not abolish the activity of the promoter completely, since approximately 20 % residual activity could still be detected, even when only 23 bp were left downstream from the promoter (Fig. 3, line 8). This remaining activity was lost when 189 bp from the 5' end of the shorter 3' end deletion were removed (Fig. 3, line 9).

It should be noted that for *flgAp*, the promoter activity was reduced 57 % when 53 bp were deleted from the 3' end of the original DNA fragment; an additional reduction (~20 %) was observed if the next 78 bp were removed (Fig. 3, compare lines 6 and 7). A similar pattern was observed for *flhAp* (Fig. 2, compare lines 7 and 8). These

Fig. 2. Deletion analysis of the regulatory region of *flhAp*. The σ^{54} -dependent promoter *flhAp* was cloned along with its regulatory region upstream of the reporter gene '*uidA*' (grey box). The map at the top of the figure shows the general arrangement of the region, including the complete *flhA* operon, and some of the genes in the *flgA* operon. The numbers to the left of each construct show the distance between the 5' end and the C of the conserved GC dinucleotide of the -12 box of the *flhA* promoter. Striped boxes indicate the positions of the *flhA* and *flgA* promoters. Hypothetical genes belonging to the *flgA* operon are indicated by their locus tag number. Activities are expressed as percentages of the β -glucuronidase activity determined for the largest construct. The β -glucuronidase activity of this construct was 16 905 nmol 4-methyl-umbelliferone formed min^{-1} (mg protein) $^{-1}$. For each construct, the activity was determined at least three times independently, and the SD was less than 10 %.

Fig. 3. Deletion analysis of the regulatory region of *flgAp*. The σ^{54} -dependent promoter *flgAp* was cloned along with its regulatory region upstream of the reporter gene '*uidA*' (grey box). The map at the top of the figure shows the general arrangement of the region, including the complete *flgA* operon and some of the genes in the *flhA* operon. The numbers to the left of each construct show the distance between the 5' end and the C of the conserved GC dinucleotide of the -12 box of the *flgA* promoter. Striped boxes indicate the positions of the *flgA* and *flhA* promoters. Hypothetical genes belonging to the *flgA* operon are indicated by their locus tag number. Activities are expressed as percentages of the β -glucuronidase activity determined for the largest construct. The β -glucuronidase activity for this construct was 20 970 nmol 4-methyl-umbelliferone formed min^{-1} (mg protein) $^{-1}$. For each construct, the activity was determined at least three times independently, and the SD was less than 12 %.

results suggest that other binding sites of minor affinity are located in these regions that could be considered as an extended UAS. In agreement with this notion, it has been shown that several EBP binding sites are present in the regulatory region of some σ^{54} -dependent promoters; these multiple sites are required for efficient oligomerization of the EBP and in consequence for full promoter activity (for examples, see Atkinson *et al.*, 2002; Tucker *et al.*, 2006; D'Autreaux *et al.*, 2008).

In summary, our results indicate that approximately 30 % of the activity observed for the *flhAp* and *flgAp* promoters depends on the UAS located upstream, while 65–70 % of the activity depends on the UAS located downstream. Fig. 4 summarizes the position of the upstream and downstream UASs identified for these promoters. As can be observed, the main UAS region for each promoter is located downstream of the consensus promoter sequence and overlaps with the region responsible for 30 % of the activity of the contralateral promoter, suggesting that the same UAS is able to activate both promoters.

To further characterize the activation dependency of the *flhA* and *flgA* promoters on the contralateral UAS, we tested whether the 30 % activation given by these sites was affected when they are displaced to the opposite faces of the DNA. For this, we inserted 5 or 10 bp in the spacing region located between *flhAp* and *flgAp*. Since the main UASs of both promoters are located downstream, these insertions should not affect the activation of these promoters by their specific UASs; however, the 5 bp insertion should place each promoter and its downstream UAS on opposite faces of the DNA helix, disfavoured activation by the contralateral UAS. Finally, the insertion of 10 bp should restore the original topology of this region, and the activity of the *flhA* and *flgA* promoters should return to wild-type levels.

Fig. 4. Schematic representation of the FleQ/FleT binding sites in the *flhA*-*flgA* region. In the centre of the figure, the positions of the *flhAp* and *flgAp* promoters are shown as well as the translational start site for the first gene of each operon. Above and below this map the regulatory regions of the *flgAp* and *flhAp* promoters are indicated as follows: upstream enhancers (white boxes), downstream enhancers (black boxes) and extended downstream enhancer regions (black and grey boxes). The numbers indicate the position of each regulatory element with respect to the C of the conserved GC dinucleotide of the -12 box, defined as $+1$.

The DNA fragments carrying *flhAp*, *flhAp* + 5 bp and *flhA* + 10 bp extend from -349 to $+209$ bp from the conserved dinucleotide GC of *flhAp*. The DNA fragments carrying *flgAp*, *flgAp* + 5 bp and *flgAp* + 10 bp extend from -288 to $+270$ from the corresponding GC dinucleotide of *flgAp*. All these fragments were cloned upstream of the reporter gene *'uidA* and introduced into WS8 cells. A reduction in the amount of β -glucuronidase produced by *flhAp* + 5 bp and *flgAp* + 5 bp was observed (Table 2). In contrast, an activity level similar to that of the wild-type promoters was detected for the constructs carrying a 10 bp insertion (Table 2). These results suggest that the activation mediated by the contralateral UAS is sensitive to the relative position of the promoters on the DNA helix.

In vivo footprinting experiments

Given that previous attempts to determine the binding site of FleQ/FleT in the flagellar class III promoters *in vitro* were unsuccessful (Poggio *et al.*, 2005), we decided to carry out *in vivo* footprinting experiments. Plasmids carrying the complete *flgGp*, *motAp*, *fliOp* and *flgBp* promoters (line 1 of Fig. 1b, c, d and a, respectively) were introduced into wild-type WS8 and SP13 strains. SP13 carries a deletion of the *fleQ* gene and as a consequence the expression of *fleT* is practically abolished; therefore, no flagellar EBPs are present in this strain (Poggio *et al.*, 2005). Cultures of the strains carrying these plasmids were subject to a 5 min treatment with DMS, and the plasmids were then purified and cleaved. The methylation pattern was obtained with 15 cycles of primer extension. No protection could be observed from the plasmids containing the *fliOp* and *flgBp* regions. A slight protection at -182 bp upstream of the conserved dinucleotide GC of the *flgGp* promoter was detected in some experiments, but this protection was not always present and no other unambiguous protection was observed for this promoter (data not shown). In contrast, a clear protection was observed in the pattern of cleaved products corresponding to the *motAp* promoter region (Fig. 5). In this case, a comparison of the methylation

Table 2. β -Glucuronidase activities promoted by the wild-type and mutant versions of *flhAp* and *flgAp*

Plasmid	β -Glucuronidase activity (%)*
pBflhAp	100
pBflgAp	100
pBflhAp + 5	41
pBflgA + 5	30
pBflhA + 10	89
pBflgA + 10	95

*Activities are expressed as percentages of the β -glucuronidase activity measured for the construct of the respective promoter without any additional nucleotides. The absolute values of these are indicated in the legends of Figs 2 and 3 for pBflhAp and pBflgAp, respectively.

Fig. 5. *In vivo* DMS footprinting assay of *motAp*. The *in vivo* methylation pattern of the *motAp* regulatory region was obtained for WS8, SP13 and SP12 strains carrying pBmotAp, as described in Methods. Protected and hyperreactive positions in the WS8 strain are indicated by ○ and ●, respectively. Protected positions in the SP12 strain are indicated by asterisks. The sequence used as the reference for the top strand (left side of the figure) corresponds to the *flgG* regulatory region obtained with the *flgG* +274 primer. The sequence used as reference for the bottom strand (right side of the figure) corresponds to the *motA* regulatory region obtained with the *motA*10 –243 primer. In the lower part of the figure, the sequence of the protected region is shown (with the same symbols as above) together with the sequence corresponding to the motif identified by MEME analysis (underlined sequence, see below).

patterns of the plasmids isolated from the wild-type and the SP13 strain revealed several differences. In the protection corresponding to the top strand a diminished reactivity at positions –163, –164, –169 and –176, and an evident overreactivity of the bases at –160, –161 and –174, were observed. In the complementary strand a single overreactive base could be detected at position –151.

To further corroborate that the changes in the methylation pattern of *motAp* were due to the binding of the FleT/FleQ complex, we carried out the same experiments using a DMS-modified plasmid obtained from a *fleT* deletion mutant strain (SP12). As shown in Fig. 5, when the protection pattern of the plasmid obtained from the WS8 strain was compared with that of the plasmid obtained from SP12, the same DMS reactivity changes were detected to those observed when the WS8 protection pattern was compared with that of the SP13 strain. This suggested that the protection pattern observed in the WS8 strain is due to the binding of the FleQ/FleT complex. Interestingly, positions –160, –161 and –174 appeared to be protected positions when the protection pattern of the plasmid obtained from SP12 was compared with that obtained from the plasmids purified from the SP13 strain, indicating that FleQ can bind by itself to this region when FleT is not present. Further supporting this result, position –177 was protected in the bottom strand of the SP12 strain.

***In silico* analysis of the FleQ/FleT binding sites**

To identify the binding sequence of the FleQ/FleT activator we carried out a MEME analysis (Bailey & Elkan, 1994) of the regions required for activation of the flagellar

promoters identified in this work. This analysis allowed us to identify a set of putative binding sites, from which the sequence logo (Crooks *et al.*, 2004; Schneider & Stephens, 1990) shown in Fig. 6 was generated. The fact that the *motAp* region that corresponds to this motif partially overlaps with the protected bases in the footprinting experiments (see Fig. 5) supports the functionality of this motif. The motif is present in five of the six UASs with a good probability value (see legend of Fig. 6); however, it was not possible to identify it in the putative UAS region of *flhAp*, presumably due to a higher degeneracy of the binding site in this particular UAS. This motif, together with the σ^{54} promoter consensus sequence, was used to search the *R. sphaeroides* chromosome using the algorithm DNA-pattern of the Regulatory Sequence Analysis tools (RSA) (Thomas-Chollier *et al.*, 2008). Besides the promoters used to generate the FleQ/FleT consensus binding

Fig. 6. Sequence logo of the binding site of FleQ/FleT. The sequences identified by the MEME program as FleQ/FleT binding sites present in the regions characterized in the deletion analysis were converted into a sequence logo using the WebLogo server at <http://weblogo.berkeley.edu/>. The *P* values of the identified sequences are: *flgB*, 1.2×10^{-09} ; *fliO*, 8.23×10^{-09} ; *flgG*, 4.33×10^{-08} ; *motA*, 1.7×10^{-06} ; and *flgA*, 5.24×10^{-06} .

sequence, from this analysis eight other regions were identified; even though they cannot be completely discarded, we considered five of them to be false positives, either because the promoter was too far from the translation start site or because the first gene downstream of the putative promoter was not the first gene of the operon. The remaining three regions were the *fliKp* promoter, which has been previously shown to be dependent on FleQ/FleT (Poggio *et al.*, 2000), the promoter region of RSP2773 (a putative peptidase of the M15-3 family) and the promoter region of RSP0038. Since the hypothetical gene RSP0038 is located in the same chromosomal region as the promoters analysed in this work, we decided to test whether this gene was part of the flagellar hierarchy. For this, a 585 bp DNA fragment containing the predicted RSP0038 σ^{54} -dependent promoter and 469 bp upstream of the conserved GC dinucleotide of the -12 box (the predicted enhancer is located 207 bp upstream of the promoter) was cloned upstream of the promoterless *'uidA* gene. Activity levels of the β -glucuronidase enzyme produced from this construct were dependent on the presence of FleQ/FleT (data not shown). This result further supports the validity of the predicted binding site and allows us to include RSP0038 in the class III of the flagellar hierarchy of *R. sphaeroides*.

DISCUSSION

Activators of $E\sigma^{54}$ normally work in oligomeric complexes of a single protein. Until now, the HrpR/HrpS (Grimm *et al.*, 1995; Hutcheson *et al.*, 2001) and FleQ/FleT complexes are the only cases in which a hetero-oligomer is required for transcriptional activations. Besides the scarce knowledge available on this kind of σ^{54} activators, the identification of the binding site of the FleQ/FleT complex is of particular interest to understand the switch in affinity from type II to type III flagellar promoters shown by FleQ once it is in complex with the HTH-less σ^{54} activator FleT (Poggio *et al.*, 2005).

The deletion mapping carried out in this work indicates that the UAS regions of six out of the seven FleQ/FleT-dependent promoters identified so far in the genome of *R. sphaeroides* are found at sites distal to the promoter sequence. These UASs are located from 100 to 250 bp upstream or downstream of the conserved GC dinucleotide. Therefore, activation mediated by the hetero-oligomeric FleQ/FleT complex must occur using similar mechanisms to those of other well-characterized EBP proteins. In contrast, it has been reported that FleQ from *P. aeruginosa* is able to activate transcription from sites that are adjacent to the promoter sequence (Jyot *et al.*, 2002). Since FleQ from *P. aeruginosa* is highly similar to FleQ from *R. sphaeroides*, it appeared possible that these proteins shared this particular property. From our study, we conclude that this is not the case, and that at least in this regard the FleQ/FleT complex is similar to other members of the EBP family.

Previous *in vitro* attempts to determine the binding site of the FleQ/FleT complex to the class III flagellar promoters have been unsuccessful. In this work, using an *in vivo* approach, we were able to identify a DNA region that was protected from DMS modification, indicating that FleQ/FleT binds to this region. In accordance with the deletion mapping experiments, this protected region is within the minimal fragment required to activate the *motAp* promoter.

Surprisingly, when the protection pattern of the *motAp* enhancer region was tested in a *fleT* mutant strain, changes in the reactivity of the same positions detected in the WS8 strain were observed, and in most cases these changes were a switch from over-reactivity in the WS8 to protection in the SP12 strain. At the same time, the protection of some positions observed in the WS8 were lost in the SP12 strain. This indicates that although FleQ seems to be able to bind by itself to this region, this binding is different from that of the FleQ/FleT complex and does not promote transcriptional activation, since, as has been shown previously, the *motAp* promoter is not active in the SP12 strain (Poggio *et al.*, 2005). Further work will be required to elucidate how the binding of FleT to FleQ changes its DNA binding properties and how these changes are related to the transcriptional activation of the flagellar class III promoters.

Since *in vitro* evidence showing that FleQ/FleT binds to the enhancer region of the flagellar promoters is still lacking, the possibility remains that *in vivo* the FleQ/FleT complex activates the expression of another EBP that is the real activator of the flagellar class III promoters. However, it has been shown that *R. sphaeroides* has only five EBPs that show a well-conserved GAFTGA signature, which is required to productively interact with RpoN. The role of three of them has already been reported, i.e. FleQ, FleT and NifA (the regulator of the nitrogen fixation genes). The two remaining EBP proteins are encoded by the RSP1590 and RSP2800 genes; mutants of these genes do not show any motility defect (our unpublished results).

To identify the relevant bases for binding of the FleQ/FleT complex, a bioinformatic approach was taken. The regions identified in the deletion analysis of the *flgGp*, *flgBp*, *fliOp*, *flgAp* and *motAp* promoters were used as input for the MEME algorithm. The motif obtained from this analysis overlaps with the DMS-protected region observed in the *motAp* promoter. To further test the validity of this motif, we searched the complete *R. sphaeroides* genome for its presence in the vicinity of a σ^{54} promoter sequence. This strategy allowed the identification of all the previously characterized class III flagellar promoters (with the exception of the weak *flhAp*) and of the new FleQ/FleT-dependent gene RSP0038. These results together suggest that this motif represents the binding site of the FleQ/FleT flagellar activator. Since the search in the complete genome for the proposed motif did not identify the class II flagellar promoters that are dependent on FleQ alone (i.e. *fleTp*), it may be inferred that the FleQ recognition site must differ substantially from the one recognized by the FleQ/FleT

complex. Further characterization of the class II and class III enhancer regions is necessary to understand the specificity switch of the FleQ EBP mediated by the interaction with FleT.

In *R. sphaeroides*, the intercistronic region between the divergently transcribed promoters *flhAp* and *flgAp* is only 53 bp long; from this fact we hypothesized that the FleQ/FleT complex could promote activation when bound to sites lying close to the promoter, or from sites located inside the coding region of the gene located upstream. Instead, activation of these promoters depends on a region located 150–200 bp downstream from the promoter sequence. Interestingly we observed that the UAS located upstream was responsible for 25–30 % of the full activity level detected for both promoters. Our current model to explain how activation in these promoters occurs involves a biased random contact of the FleQ/FleT complex bound to the enhancer regions with $E\sigma^{54}$ bound to either promoter. The molecular bias mechanism could involve a preferential bending conformation of the DNA and/or induced bending by some other protein (e.g. integration host factor; IHF). The physiological relevance of this 30 % activation remains to be tested in the chromosomal context. Nevertheless, since the sigma factor FlhA (required for the expression of the flagellar class IV operons) is encoded in the *flgA* operon and its anti-sigma factor, FlgM, is encoded in the *flhA* operon, the shared activity of these UASs could be required to fine-tune the expression of these two operons. The release of FlhA from the inhibitory action of FlgM is one of the most important check-points during flagella formation, and it occurs when FlgM is exported out of the cell once the hook-basal body structure is completed. Hence, it is tempting to speculate that cross-regulation mediated by these UASs helps keep these two proteins in equilibrium.

So far, only two divergently transcribed σ^{54} -dependent promoters have been characterized (Charlton *et al.*, 1993; Lutz *et al.*, 1990; Hopper *et al.*, 1994). In *Klebsiella pneumoniae*, it has been reported that the IHF protein avoids activation of the *nifH* promoter by NifA when it is bound to the UAS responsible for activating the divergently transcribed promoter *nifH*. In this case, both UASs are located upstream from the promoter sequences *nifH* and *nifH* (Charlton *et al.*, 1993). In the case of the *hyc* and *hyp* operons of *E. coli*, the distance between the dinucleotide GC of the promoter sequences *hycAp* and *hypAp* is only 120 bp. This region contains an IHF binding site and the UAS that allows activation of *hycAp*, whereas the UAS responsible for the activation of *hypAp* is found between *hycA* and the following gene, *hycB* (~700 bp upstream of *hypAp*). It has been shown that *hycAp* decreases its expression in a strain deficient in IHF, whereas *hypAp* increases its activity. Therefore, it has been suggested that transcription from the *hycAp* promoter negatively affects activation of *hypAp* (Hopper *et al.*, 1994). These examples show that expression from divergent σ^{54} promoters can be affected reciprocally, although other factors such as IHF

can modify the final output. So far, the role of IHF and other similar proteins in the control of σ^{54} -dependent transcription in *R. sphaeroides* remains to be evaluated.

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