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The early gene product EUO is a transcriptional repressor that selectively regulates promoters of Chlamydia late genes

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Summary

The obligate intracellular bacterium *Chlamydia* has an unusual developmental cycle in which there is conversion between two forms that are specialized for either intracellular replication or propagation of the infection to a new host cell. Expression of late chlamydial genes is upregulated during conversion from the replicating to the infectious form, but the mechanism for this temporal regulation is unknown. We found that EUO, which is expressed from an early gene, binds to two sites upstream of the late operon *omcAB*, but only the downstream site was necessary for transcriptional repression. Using gel shift and *in vitro* transcription assays we showed that EUO specifically bound and repressed promoters of *Chlamydia trachomatis* late genes, but not early or mid genes. These findings support a role for EUO as a temporal repressor that negatively regulates late chlamydial genes and prevents their premature expression. The basis of this specificity is the ability of EUO to selectively bind promoter regions of late genes, which would prevent their transcription by RNA polymerase. Thus, we propose that EUO is a master regulator that prevents the terminal differentiation of the replicating form of chlamydiae into the infectious form until sufficient rounds of replication have occurred.

Introduction

One of the defining features of the genus *Chlamydia* is a biphasic developmental cycle in which the bacterium converts between an infectious elementary body (EB) and a non-infectious, replicating reticulate body (RB) within an infected host cell (Schachter, 1988). Members of this genus include *Chlamydia trachomatis* and *Chlamydia pneumoniae*, which are major human pathogens, and *Chlamydia psittaci*, which causes an uncommon zoonotic infection transmitted by birds (Stamm and Batteiger, 2009). The chlamydial developmental cycle has three distinct stages – an early stage where an EB that has just entered the cell converts into a RB, a mid stage where the metabolically active RB divides repeatedly by binary fission, and a late stage in which RBs convert back into EBs in an asynchronous manner (AbdelRahman and Belland, 2005). Chlamydial genes are transcribed as three main temporal groups that correspond to these three stages of the developmental cycle (Shaw *et al.*, 2000; Belland *et al.*, 2003a,b). Thus, the temporal expression of chlamydial genes is linked to the progression of the developmental cycle.

The expression of the relatively small number of late genes is a critical regulatory step in determining whether an individual RB continues to divide or terminally differentiates into an EB. Many of the 26 late genes that Belland and colleagues identified as being

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upregulated at the time of EB-to-RB conversion have EB-specific functions (Belland *et al.*, 2003b). For example, *omcAB* encodes two cysteine-rich outer membrane proteins that are only found in EBs (Clarke *et al.*, 1988; Liu *et al.*, 2010). *hctA* and *hctB* encode histone-like proteins Hc1 and Hc2 respectively; these nucleoid-associated proteins bind DNA and cause chromosomal condensation, which is a characteristic feature of EBs (Barry *et al.*, 1992; Brickman *et al.*, 1993). Other late gene products include putative thioredoxin disulphide isomerases and membrane thiol proteases, which may have roles in the formation of the highly cross-linked outer membrane complex of an EB that provides protection against the extracellular environment (Newhall and Jones, 1983; Hackstadt *et al.*, 1985; Wang *et al.*, 2006; Liu *et al.*, 2010). It is likely that the expression of these late genes is tightly regulated because premature expression prior to the end of the developmental cycle would induce conversion into EBs before the multiple rounds of RB division have been completed. If this were to happen, the infectious yield of the developmental cycle, which can be several hundred to a thousand EBs, would be greatly reduced.

Late chlamydial genes have been shown to be expressed as two separate subsets that are each regulated by a different transcriptional mechanism. One subset is transcribed by the major form of chlamydial RNA polymerase, which contains the major sigma factor, σ^{66} (Engel and Ganem, 1990; Koehler *et al.*, 1990; Douglas *et al.*, 1994). As σ^{66} RNA polymerase also transcribes early and mid genes, there must be regulatory mechanisms to control expression of each temporal class of σ^{66}-dependent genes. A second subset of late genes is transcribed by a second form of RNA polymerase containing an alternative sigma factor σ^{28} instead of σ^{66} (Yu and Tan, 2003; Yu *et al.*, 2006). σ^{28}-dependent transcriptional activity has been proposed to be regulated by an anti-sigma factor RsbW that binds and sequesters σ^{28}, although this model remains controversial and unproven (Hua *et al.*, 2006).

It is not known how σ^{66}-dependent late genes are regulated so that they are not prematurely expressed until late in the developmental cycle. Transcription of σ^{66}-dependent late genes cannot be inhibited during early and mid times by an anti-sigma factor, as has been proposed for σ^{28}, without also affecting transcription of early and mid genes. Mid genes have been proposed to be upregulated by increased DNA supercoiling in midcycle, but changes in DNA supercoiling are unlikely to regulate late genes because their promoters are not supercoiling-responsive (Niehus *et al.*, 2008; Case *et al.*, 2010). Instead, σ^{66}-dependent late genes could be controlled by another major mechanism of prokaryotic gene regulation, which is the co-ordinate regulation of a group of genes by a transcription factor. For example, an activator could selectively upregulate the expression of σ^{66}-dependent late genes at late times. Alternatively, a repressor could selectively prevent transcription of these late genes during early and mid stages, but not late in the development cycle.

Chlamydia encodes remarkably few transcription factors (Akers *et al.*, 2011), but a chlamydial protein called EUO (early upstream ORF) has many of the features that would be predicted for a repressor of late genes. First, a candidate late repressor must be present starting at an early time in the intracellular infection in order to prevent premature expression of late genes. *euo* was one of the first early chlamydial genes to be identified, and *euo* transcripts and EUO protein are detectable as early as 1 h post infection (hpi) (Wichlan and Hatch, 1993; Zhang *et al.*, 1998). Second, there is evidence that EUO is a DNA-binding protein that binds near the promoter of a known late gene. Specifically, EUO contains a helix–turn–helix motif and has been shown to bind the promoter region of the late operon *omcAB*, which encodes EB-specific cysteine-rich outer membrane proteins (Zhang *et al.*, 2000). EUO shows a preference for binding A/T-rich sequences, and a 15 bp consensus DNA-binding site for EUO has been proposed based on DNA footprinting and a SELEX analysis (Zhang *et al.*, 1998; 2000). Third, EUO caused a twofold decrease in transcription of the *omcAB* promoter *in vitro* (Zhang *et al.*, 2000), suggesting that it may function as a

transcription factor. However, EUO has not been shown to bind or modulate the promoter activity of other late genes, and it is not known if EUO has specificity for late and not early and mid chlamydial promoters.

To determine if EUO is a transcription factor that regulates the expression of late genes by σ^{66} RNA polymerase, we first examined EUO binding to its operator in the context of the *omcAB* promoter. We then used *in vitro* assays to test if EUO could bind and modulate transcription of representative promoters for early, mid and late chlamydial genes. We found that EUO selectively bound promoter regions of late genes and repressed transcription, providing support for this early expressed protein as a repressor of late genes in *Chlamydia*.

Results

Examining how EUO regulates a target promoter

We first determined the location of EUO-binding sites relative to the promoter in the context of the *C. trachomatis* late operon *omcAB*. In an electrophoretic mobility shift assay (EMSA), recombinant *C. trachomatis* EUO bound to two 60 bp fragments containing different portions of the *omcAB* promoter region (Fig. 1A). EUO bound to sequences from –122 to –60 (upstream EUO-binding site) as well as from –58 to +5 (downstream EUO-binding site) relative to the transcription start site (Lambden *et al.*, 1990). For each fragment, EUO bound in a concentration-dependent manner and produced a similar pattern of two shifted species consistent with EUO binding to DNA in different molar ratios. Using Western blot analysis of the EMSA gel, we showed that antibodies against recombinant His-tagged EUO recognized both shifted bands for the downstream *omcAB* fragment, indicating that both bands represent complexes containing EUO bound to DNA (Fig. 1B). These results demonstrate that there are at least two EUO-binding sites upstream of the *omcAB* promoter, and we propose that EUO can bind to each DNA site with either a 1:1 or 2:1 stiochiometry. These findings are consistent with published reports showing that recombinant and native EUO bound to the *C. psittaci omcAB* promoter in the region from –200 to +67, and that rEUO protected the region from –53 to –14 on the top strand and –62 to –9 on the bottom strand in DNase I footprinting analysis (Zhang *et al.*, 1998).

We performed *in vitro* transcription assays to determine if this binding by EUO leads to repression of the *omcAB* promoter. rEUO decreased transcription of the *C. trachomatis omcAB* promoter (contained on a template from –122 to +5) by 75% (Fig. 1C). The same level of repression was observed when the transcription reactions were performed with either *C. trachomatis* RNA polymerase (71% repression) or *Escherichia coli* RNA polymerase (75% repression). These results show that EUO binds to and represses transcription from the *omcAB* promoter and are in agreement with results from Hatch and colleagues that were performed with *C. psittaci* EUO (Zhang *et al.*, 2000).

Using the proposed 15 bp EUO-binding site as a guide (Zhang *et al.*, 2000), we predicted that the downstream EUO-binding site overlaps the –35 element of the *C. trachomatis omcAB* promoter (Fig. 2A). To test if EUO binds to this sequence, we designed mutant templates containing nucleotide substitutions and assayed if EUO binding was altered in EMSA experiments (Fig. 2A). Substitution of three nucleotides (mutants M1 and M2) in the putative EUO-binding site had minimal effects on EUO binding (Fig. 2B). However, substitution of nine of the 15 nucleotides in our predicted EUO-binding site (M3) completely disrupted binding of EUO (Fig. 2A and B). This result indicates that these sequences are necessary for EUO binding and provides support for the presence of an EUO operator overlapping the *omcAB* promoter.

To determine if one or both of the two EUO-binding sites that we identified upstream of *omcAB* are necessary for repression, we repeated the transcription reactions with *omcAB* promoter templates containing only one EUO-binding site. To test the downstream site alone, we deleted sequences upstream of –55, which removed the upstream site (Fig. 3A). To test the upstream site alone, we used a template with the mutant downstream site (M3) from the EMSA experiment (Figs 2 and 3A). We had designed this 9 bp substitution so that it did not alter the sequence of the –35 promoter element, and we verified that promoter activity was not affected in an *in vitro* transcription assay compared to the wild-type promoter (data not shown). EUO produced a similar level of repression from a template containing both native binding sites or only the downstream site (73% vs. 67%, see Fig. 3B). However, there was less repression (22%) of an *omcAB* promoter template containing the upstream binding site alone (Fig. 3B). These results demonstrate that the downstream, and not the upstream, EUO-binding site of *omcAB* is important for repression. Furthermore, these studies provide additional experimental evidence that the operator for EUO overlaps the *omcAB* promoter. These findings are consistent with EUO functioning as a transcriptional repressor that binds its cognate operator in the vicinity of the promoter, blocking promoter recognition by RNA polymerase through steric hindrance.

EUO selectively binds and represses late promoters

To determine the role of EUO in temporal gene regulation in *Chlamydia*, we tested if it can bind and repress representative promoters for early, mid and late chlamydial genes. As an initial screen, we used competitive EMSA experiments to determine if a hundred-fold excess of unlabelled 60 bp DNA fragment containing promoter regions of representative early, mid or late genes could disrupt EUO binding to the *omcAB* promoter. Promoter fragments for the late genes *ssc2* and *ltuB*, as well as *omcAB* itself, caused a loss of bound complexes, demonstrating that these sequences were able to compete for EUO binding (Fig. 4). In contrast, promoters for early (*dnaK* and *groESL*) and mid (*cdsC*) genes did not disrupt binding between the labelled probe and EUO (Fig. 4). We observed partial competition with the promoter for the mid gene *fliF* (Fig. 4).

To directly test the ability of EUO to bind to individual chlamydial promoters, we performed EMSA experiments with promoters representing the three temporal classes of chlamydial genes. EUO bound to DNA fragments containing the promoter region (–55 to +5 relative to the start of transcription) of the late genes *ltuB*, *scc2* (Fig. 5) and *cdsU* (Table 1). For each of these late promoters, EUO produced two shifted species that were similar to those observed for each site in the *omcAB* promoter (Fig. 1A). In contrast, EUO did not significantly bind the promoters from two early genes and two mid genes (Fig. 5 and Table 1). In particular, there was no binding to the mid gene *fliF*, which was able to partially compete for EUO binding in the competitive EMSA. These results demonstrate that EUO binding is promoter-specific and suggest that EUO preferentially binds to late promoters.

We next tested if the ability of EUO to selectively bind late promoters resulted in transcriptional repression of late but not early and mid chlamydial promoters. Using *in vitro* transcription assays, we found that EUO decreased transcription from the three late promoters *ltuB*, *cdsU* and *scc2* by 46–75% compared to transcription in the absence of EUO (Fig. 6 and Table 1). In contrast, EUO did not significantly alter transcription from promoters of three early (*dnaK*, *groESL* and rRNA) and four mid genes (*cdsC*, *fliF*, *ct665* and *ompA*) (Fig. 6 and Table 1). Together, these results demonstrate that EUO selectively binds to and represses promoters of late chlamydial genes but not early and mid genes. These findings support a role for EUO as a repressor of late genes in *Chlamydia*.

We explored if EUO could regulate additional late genes by testing its ability to bind the promoter regions of five additional genes whose transcripts are upregulated at late times in

the developmental cycle (Belland *et al.*, 2003b). We based these predicted promoter regions on the transcription start sites identified in a deep sequencing study by Albrecht *et al.* (2010). In EMSA experiments, EUO bound to the predicted promoter regions of each of these five late genes, albeit with lower affinity than the *omcAB* promoter, while EUO did not bind the *groESL* promoter (Fig. 7). These five predicted late promoters and promoters of other late genes have not previously been studied. Experiments to determine if EUO also represses their transcription will first require that these predicted promoters be shown to be transcriptionally active. However, our studies provide experimental evidence that EUO regulates at least nine late chlamydial genes.

Discussion

The developmental regulation of chlamydial genes as three main temporal classes is a hallmark of the intracellular *Chlamydia* infection. Prior to this study, it was not known how late genes transcribed by the major form of chlamydial RNA polymerase, σ^{66} RNA polymerase, are regulated so that they are only expressed at the time of conversion from RBs to infectious EBs. We now present data showing that the early chlamydial protein EUO specifically represses promoters of late genes, but not early and mid genes. We also demonstrate that the basis of this specificity is the ability of EUO to selectively bind the promoter regions of late genes. EUO bound to an operator that overlaps the –35 element of the *omcAB* promoter, and the presence of this operator was necessary for EUO-mediated repression. These findings are consistent with a mechanism of repression in which RNA polymerase is selectively blocked from transcribing target late genes by steric hindrance.

We thus propose that EUO specifically regulates σ^{66}-dependent late genes because only these target genes, and not early and mid genes, contain an EUO operator in the vicinity of their promoters. EUO has been shown to preferentially bind A/T-rich sequences (Zhang *et al.*, 1998), and promoters for σ^{66}-dependent late chlamydial genes have been noted to be A/T-rich relative to mid promoters (Niehus *et al.*, 2008). In all, we show that EUO binds upstream of nine late genes. Our strongest data are for four late genes, including *omcAB*, which encodes two EB-specific cysteine-rich outer membrane proteins, and *cdsU* and *scc2*, which encode proteins involved in the type III secretion system for secreting chlamydial effectors into the inclusion membrane and the host cytosol. For these late genes, we have identified candidate EUO-binding sequences overlapping –35 or –10 promoter elements, based on the proposed 15 bp DNA-binding site for EUO (Zhang *et al.*, 2000) (Fig. 8). However, A/T-rich sequences resembling the proposed 15 bp EUO-binding site are also present in the promoter regions of early and mid promoters that were not bound by EUO in our studies. Thus with the available data, EUO target genes cannot be predicted by sequence alone, which underscores the importance of defining EUO operators with functional studies.

The operator sequence recognized by EUO appears to be a degenerate A/T-rich sequence rather than a specific DNA sequence. Hatch and colleagues performed *in vitro* binding studies and demonstrated that EUO has low DNA-binding specificity (Zhang *et al.*, 2000). In agreement, we found that three-nucleotide substitutions in the *omcAB* operator had only slight effects on EUO binding and that a more extensive 9 bp substitution was necessary to disrupt binding (Fig. 2). Prokaryotic transcription factors that are global regulators have been noted to have a lower DNA-binding specificity (Zhang *et al.*, 2000). Thus, the ability of EUO to bind to a degenerate binding site is consistent with a potential role as a global regulator whose regulon may include the 29 late genes identified in *C. trachomatis* (Belland *et al.*, 2003b).

We identified a second EUO-binding site further upstream of the promoter elements and operator of *omcAB*. This upstream EUO-binding site, which was located between –122 and

–60, was not necessary for EUO-mediated repression of the *omcAB* promoter, and its significance is not known. It is possible that this upstream binding site may compete with bona fide operators for EUO binding, or alternatively may increase the local concentration of EUO in the neighbourhood of the *omcAB* promoter. Two EUO-binding sites have also been identified in the *omcAB* promoter for *C. psittaci* (Zhang *et al.*, 1998), but it is not known if this is a feature of other EUO-regulated genes.

There is evidence to support a role for EUO as a conserved regulator of late gene expression in *Chlamydia*. EUO is encoded by all eight sequenced *Chlamydia* spp. and the protein is well conserved with 62–86% identity and 77–92% similarity (based on a BLAST search for *C. trachomatis* EUO protein). An EUO homologue is also encoded by *Chlamydia*-like organisms, including *Protochlamydia amoebophila*, *Simkania negevensis* and *Waddlia chondrophilia*, which share a common ancestor with the *Chlamydiaceae*. Where it has been examined in *C. pneumoniae* and *C. psittaci*, EUO is also an early gene that is expressed during the early and mid stages of the developmental cycle (Zhang *et al.*, 1998; Maurer *et al.*, 2007). *C. psittaci* EUO binds and represses transcription of the *omcAB* promoter (Zhang *et al.*, 2000), but its ability to selectively regulate promoters of other *C. psittaci* late genes has not been examined.

A major unresolved question is how EUO-mediated repression is relieved in order to allow transcription of its target genes at late times in the developmental cycle. The mechanism must account for asynchrony in the timing of RB-to-EB conversion, which occurs in a proportion of chlamydiae in a late inclusion while other RBs continue to divide. Thus, derepression of EUO-regulated late genes must be able to occur at the level of an individual chlamydia independently of other chlamydiae in the same inclusion. Two potential mechanisms for relieving EUO-mediated repression involve the regulation of EUO protein levels or the control of EUO activity levels by a cofactor.

Data from *C. psittaci* and *C. pneumoniae* indicate that EUO levels are downregulated at late times. In *C. psittaci*, *euo* transcript levels decreased after 4 hpi and EUO protein levels decreased by 20 hpi (Zhang *et al.*, 1998). In a *C. pneumoniae* microarray analysis, *euo* mRNA levels were high at early times but decreased after 6 hpi (Maurer *et al.*, 2007). In *C. trachomatis*, *euo* transcript levels from a microarray study did not decrease at late times (Belland *et al.*, 2003b), although the temporal pattern of EUO protein levels is not known. If EUO levels fall below a certain threshold at late times in the developmental cycle, it could lead to derepression of target genes such as *omcAB*. Potential means of downregulating EUO levels include transcriptional, post-transcriptional and translational control mechanisms.

EUO activity could instead be regulated by a cofactor such as a small molecule or a regulatory protein. A number of small molecules have been shown to serve as cofactors for chlamydial transcription factors. For example, amino acids such as tryptophan and arginine are cofactors for the repressors TrpR and ArgR respectively (Akers and Tan, 2006; Schaumburg and Tan, 2006). Similarly, Zn^{2+} is a co-repressor for the dual function YtgCYtgR repressor (CT069) (Akers *et al.*, 2011), and nucleotides have been proposed to be a cofactor for NrdR, which is a regulator of DNA synthesis (Case *et al.*, 2011). The molecular chaperone GroEL functions in an ATP-independent manner as a cofactor for the stress response regulator HrcA (Wilson *et al.*, 2005; Chen *et al.*, 2011). EUO repressor function could be dependent on a co-repressor that is present in chlamydiae during early and mid stages of the developmental cycle but depleted by late time points. Alternatively, repression by EUO could be antagonized by an inducer that prevents binding of EUO to its DNA targets when it is present at late times. However, our ability to demonstrate binding

and repression by EUO in *in vitro* studies with defined components argues against a requirement for a co-repressor.

This proposed model for the negative regulation of σ^{66}-dependent late genes stands in contrast to the positive regulation of midcycle chlamydial genes. Promoters for representative mid genes, but not late genes, have been shown to be upregulated by increased DNA super-coiling (Niehus *et al.*, 2008; Case *et al.*, 2010). As chlamydial supercoiling levels are highest in midcycle, mid genes have been proposed to be positively regulated by global DNA supercoiling (Niehus *et al.*, 2008). In contrast, EUO provides a mechanism for σ^{66} RNA polymerase to transcribe early and mid genes while selectively repressing late genes. Intriguingly, supercoiling-dependent mid promoters are disproportionally G/C-rich (Niehus *et al.*, 2008; Case *et al.*, 2010), while EUO shows a strong preference for binding to A/T-rich DNA sequences, which are typical of late promoters (Fig. 8). These differences suggest that the temporal classes of chlamydial genes are hardwired via the sequence of their promoter regions to be differentially regulated.

The proposed role of EUO in repressing late genes may be relevant to chlamydial cell culture persistence, which is an altered growth state in which RB-to-EB conversion is blocked. When *Chlamydia*-infected cells are treated with factors such as IFN-γ or depleted of specific nutrients such as tryptophan, they enter this persistent state in which large aberrant RBs continue to replicate their DNA but do not divide (Beatty *et al.*, 1993; 1994). Another feature of chlamydial persistence is that late gene expression, which is associated with RB-to-EB conversion, is downregulated (Belland *et al.*, 2003a). Interestingly, *euo* is one of the few genes that is dramatically upregulated during persistence (Belland *et al.*, 2003a). Our results predict that these high levels of EUO are the direct cause for the repression of late genes during cell culture persistence. The mechanism by which EUO expression is upregulated during chlamydial persistence remains to be determined. However, our findings suggest that by controlling late gene expression and the conversion of RBs into EBs, EUO is an important regulator of both the normal developmental cycle and chlamydial persistence.

In summary, we propose that EUO is a transcriptional repressor that prevents premature expression of σ^{66}-dependent late genes until late in the developmental cycle. As EUO-regulated target genes are involved in RB-to-EB conversion, control of their expression is likely to play an important role in determining whether a RB continues to replicate or if it differentiates into an EB. Thus, we propose that EUO is a transcriptional regulator that determines the balance between the production of chlamydial progeny and the proportion of these progeny that are infectious.

Experimental procedures

Construction of in vitro *transcription plasmids*

Promoter sequences were amplified by PCR from *C. trachomatis* serovar D UW-3/Cx genomic DNA and cloned upstream of a promoterless G-less cassette transcription template in pMT1125, as previously described (Wilson and Tan, 2002). Plasmids used in this study are listed in Table 2.

For construction of pMT1636, primers T1710 (5′AGCGAATTCTAGACGATTTGTT; EcoRI site underlined) and SP6 (5′ CGCCAAGCTATTTAGGTGACACTATAG) were used to amplify the *omcAB* promoter region from –55 to +245 by PCR using pMT1150 as a template. The DNA fragment was cloned upstream of the promoterless G-less cassette template of pMT1125 at EcoRI and BamHI sites. For construction of pMT1637, primers T180 (5′ AGCGAATTCTTTGAATCCGAGCTGTTTATTATTT; EcoRI site underlined)

and T1709 (5′ TTATAGAACAATATTACATTATAAAATAAAA**GCCG**TATCAA**GGCCC**TTTAAAA CAATCGT; nucleotide substitutions indicated in bold) were used to amplify a mutant *omcAB* promoter by PCR from pMT1150. The DNA fragment was cloned upstream of the promoterless G-less cassette template of pMT1125 at EcoRI and EcoRV sites. All constructs were verified by sequencing (Genewiz).

Purification of recombinant EUO

Recombinant EUO containing a 6×His tag at the C-terminus was expressed from pMT1181 (Elizabeth DiRusso Case, unpublished), which contains the *C. trachomatis euo* gene cloned into the IPTG-inducible expression plasmid pRSETC (Invitrogen). *E. coli* strains containing pMT1181 were grown in 1 l LB to an OD_{600} of 0.4. Cultures were induced with 1 mM IPTG for 2 h at 37°C and cells were harvested by centrifugation. The cell pellet was resuspended in buffer N [10 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 10 mM 2-mercaptoethanol] containing 20 mM imidazole. The resuspended cells were sonicated twice with a Branson digital sonifier 250D for 30 s at 22% output. The material was centrifuged and the supernatant was added to a 1 ml slurry of Qiagen Ni-NTA beads. The slurry was incubated for 1 h at 4°C and then applied to a 25 ml Poly-Prep chromatography column (Bio-Rad). The beads were washed with 500 ml of buffer N containing 20 mM imidazole. Recombinant EUO protein was eluted from the column with 2 ml of buffer N containing 250 mM imidazole. Eluted protein was then dialysed overnight against 1 l of storage buffer [10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol, 30% (v/v) glycerol]. Dialysed protein was aliquoted and stored at –70°C.

Purification of C. trachomatis *RNA polymerase*

RNA polymerase was partially purified from *C. trachomatis* LGV serovar L2 at 18 hpi by heparin-agarose chromatography as previously described (Tan and Engel, 1996). Essentially L929 mouse fibroblast cells were infected with *C. trachomatis* LGV serovar L2 EBs at an MOI (multiplicity of infection) of 3. Infected cells were harvested and resus-pended in PBS (pH 7.5). Cells were lysed by dounce homogenization and cell debris was removed by low-speed centrifugation. Chlamydiae (in the supernatant) were pelleted by high-speed centrifugation. The pellet was resuspended in lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 7.5% (v/v) glycerol, 0.6 M NaCl, 10 mM β-mercaptoethanol, 0.1% (v/v) Nonidet-40] followed by sonication three times in a Branson digital sonifier 250D for 10 s at 22% output. Cellular debris was removed by centrifugation and the supernatant was diluted with buffer I [10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 7.5% (v/v) glycerol, 10 mM β-mercaptoethanol] resulting in a final NaCl concentration of 0.25 M and incubated with heparin agarose equilibrated in buffer I containing 0.25 M NaCl for 1 h. The slurry was applied to 25 ml Poly-Prep chromatography column (Bio-Rad) and the beads were washed with 50 ml of buffer I containing 0.25 M NaCl. RNA polymerase was eluted from the column with buffer I containing 0.6 M NaCl, followed by dialysis in 1 l of storage buffer overnight. Dialysed samples were aliquoted and stored at –80°C.

Electrophoretic gel mobility shift assays (EMSAs)

Annealed 60 bp complementary primers were labelled by T4 polynucleotide kinase (New England Biolabs) with approximately 30 μCi [γ-^{32}P]-ATP (10 mCi ml^{-1}, 6000 Ci $mmol^{-1}$; MP Biomedicals). Free nucleotides were removed with a mini Quick Spin DNA column (Roche). Approximately 0.5 nM labelled DNA was incubated with rEUO over a range of concentrations from 80 to 320 nM in binding buffer [40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 70 mM KCl, 125 μM EDTA, 100 μM dithiothreitol, 7.5% glycerol, 10 ng of salmon

sperm DNA] at room temperature for 20 min. For competitive EMSAs, 50 nM of an unlabelled 60 bp DNA fragment was added to the binding reaction. Samples were loaded onto a 6% polyacrylamide EMSA gel at 150 V in 0.5× Tris-borate-EDTA (TBE) buffer (Read, 1996). After electrophoresis, the gel was dried on Whatman paper and exposed to a phosphorimager screen. The screen was scanned with a Bio-Rad Personal FX scanner.

EMSA–Western blot analysis

For detection by Western blot analysis, the preceding EMSA experiments were performed using higher DNA and protein concentrations: 50 ng (125 nM) of unlabelled 60 bp DNA fragment containing the *omcAB* promoter (–55 to +5) and 10 μM EUO. Bound DNA fragments were separated on a 6% polyacrylamide EMSA gel and then transferred to a Protran nitrocellulose membrane (Whatman). The membrane was preincubated with 5% milk, followed by incubation with anti-His antibody (1:10 000; Qiagen) overnight at 4°C. The membrane was then washed and probed with goat anti-mouse antibody (1:10 000) for 1 h at room temperature. Bands were visualized by chemiluminescence with exposure to HyBlot CL autoradiography film (Denville).

In vitro *transcription assays*

Approximately 3 nM plasmid DNA containing the transcription template was incubated with 5 μM rEUO at room temperature for 15 min. Transcription assays were initiated as described previously (Tan and Engel, 1996) using 2 μl of chlamydial RNA polymerase or 0.4 U *E. coli* RNA polymerase holoenzyme (Epicentre). However, transcription with the *ompA* promoter was performed with *C. trachomatis* RNA polymerase as this promoter is not transcribed by *E. coli* RNA polymerase (Douglas and Hatch, 1996). The transcripts were resolved by electrophoresis on an 8 M urea–6% polyacrylamide gel. The gels were then fixed, dried and exposed to a phosphorimager plate. The plate was scanned with a Bio-Rad Personal FX scanner and the amount of transcripts was quantified using Quantity One software (Bio-Rad). Repression was calculated as the percentage of transcripts in the presence/absence of EUO. For each plasmid, the transcription assays were performed as a minimum of three independent experiments, and values are reported as the mean of the repression [H11006] standard deviation.

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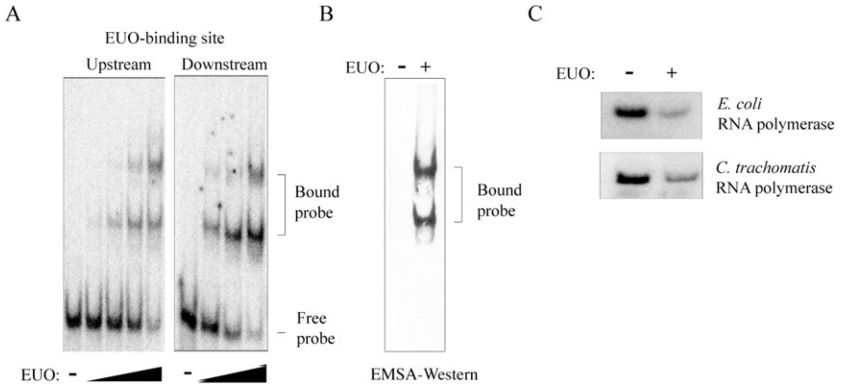

Fig. 1.

EUO binds and represses the *C. trachomatis omcAB* promoter.

A. EMSA experiments with upstream (nucleotides –122 to –60 relative to the start of transcription) or downstream regions (–58 to +5) of the *omcAB* promoter. The upstream probe was incubated with 0, 80, 160, 320 or 640 nM rEUO, while the downstream probe was incubated with 0, 80, 160 or 320 nM rEUO. Bands corresponding to the bound and free probes are indicated on the right.

B. Western blot verifying that the bound probe contains EUO. EMSA reaction for the downstream *omcAB* probe was performed in the absence or presence of 10 μM His-tagged rEUO and then transferred to nitrocellulose membrane and hybridized with anti-His antibodies. Bands corresponding to the bound DNA fragment are indicated to the right.

C. *In vitro* transcription of the *omcAB* promoter (pMT1150) by *E. coli* RNA polymerase or *C. trachomatis* RNA polymerase, as indicated, in the absence or presence of 2.5 μM EUO.

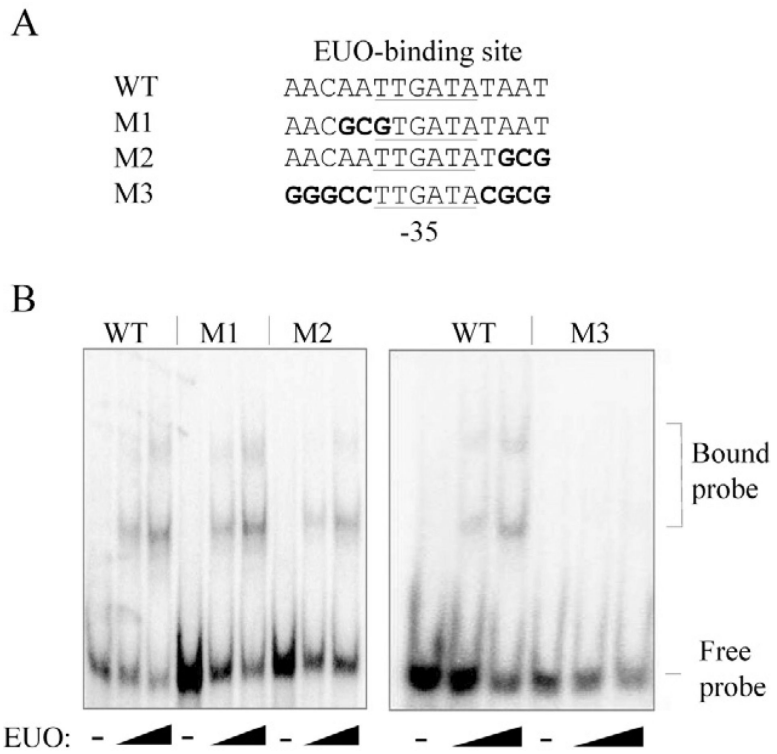

Fig. 2.

Mutational analysis to locate the downstream EUO-binding site of the *omcAB* promoter. A. DNA sequence of the wild-type (WT) EUO-binding site and mutant templates containing nucleotide substitutions (highlighted in bold). The –35 promoter element is underlined – note that the 9 bp substitution in M3 did not alter the sequence of this –35 promoter element. B. EMSA experiments with DNA probes containing these wild-type or mutant sequences. EMSA reactions were performed with 0, 80 or 160 nM rEUO. Bands corresponding to the bound and free probes are indicated on the right.

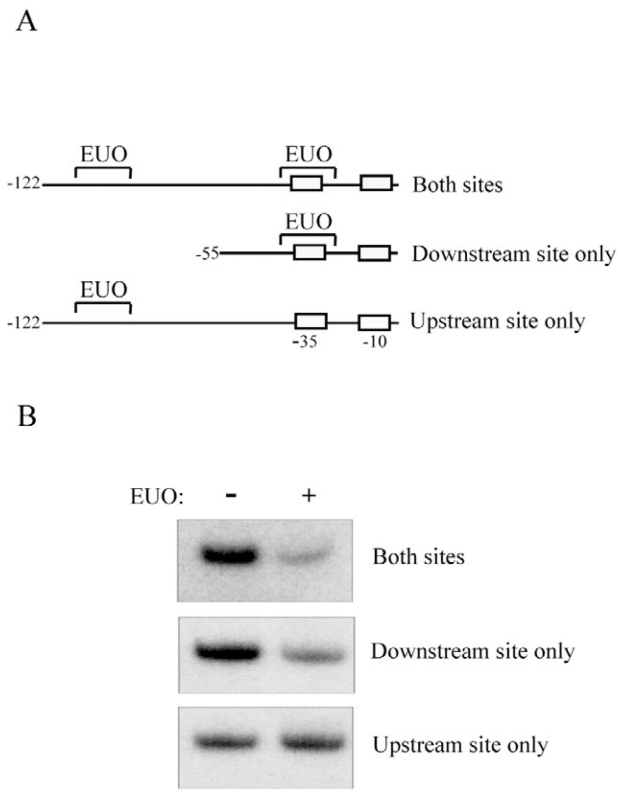

Fig. 3.

Only the downstream, and not the upstream, EUO-binding site in the *omcAB* promoter is important for repression by EUO.

A. Diagram showing transcription templates used to test the roles of the two EUO-binding sites in repression of the *omcAB* promoter. The template labelled 'Both sites' contains the two native EUO-binding sites upstream of *omcAB* in the region from +5 to –122. 'Downstream site only' is a truncation from –55 to +5 containing only the downstream EUO-binding site. 'Upstream site only' contains the *omcAB* promoter region from –122 to +5 in which the downstream EUO-binding site has been disrupted by a 9 bp substitution (M3 in Fig. 2). Positions of the EUO-binding sites, and the –10 and –35 promoter elements are shown.

B. *In vitro* transcription of these three transcription templates by *E. coli* RNA polymerase in the absence or presence of 2.5 μM *C. trachomatis* EUO.

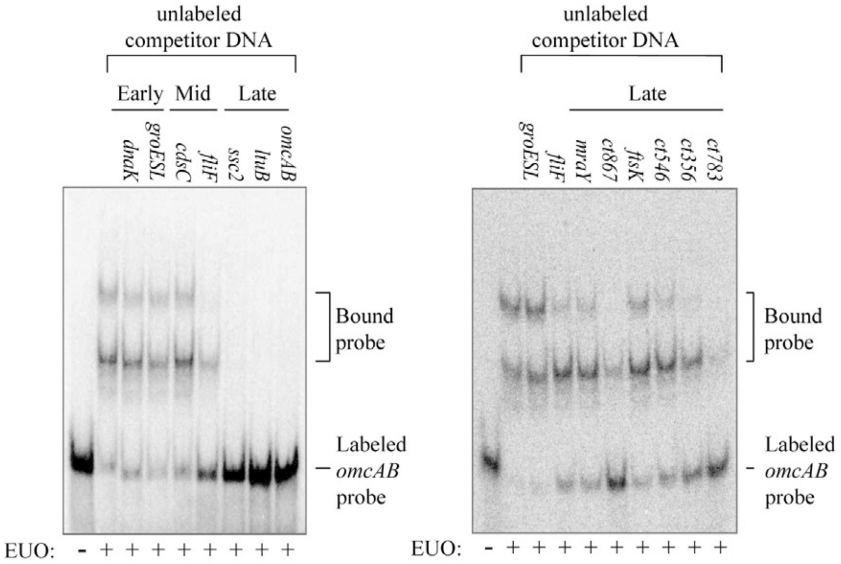

Fig. 4.

DNA fragments from late promoters, but not early or mid promoters, effectively compete for EUO binding. Competitive EMSAs were performed with the 60 bp labelled *omcAB* and 160 nM EUO in the presence of 50 nM unlabelled competitor DNA fragments (100-fold excess compared to the labelled *omcAB* probe). The promoter present on each DNA fragment is indicated for each reaction. Bands corresponding to the bound and free probes are indicated on the right.

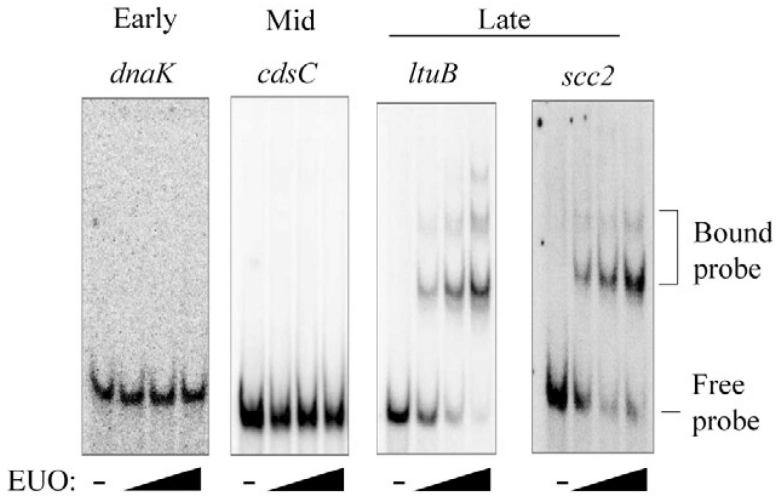

Fig. 5.

Differential binding of EUO to promoters of different temporal classes. For each promoter contained on a 60 bp DNA probe, EMSA reactions were performed in the absence of EUO or with 80, 160 or 320 nM EUO. Data for representative early (*dnaK*), mid (*cdsC*) and late promoters (*ltuB* and *scc2*) are shown. Bands corresponding to the bound and free probes are indicated on the right. Additional early, mid and late promoters were tested, and the results are shown in Table 1.

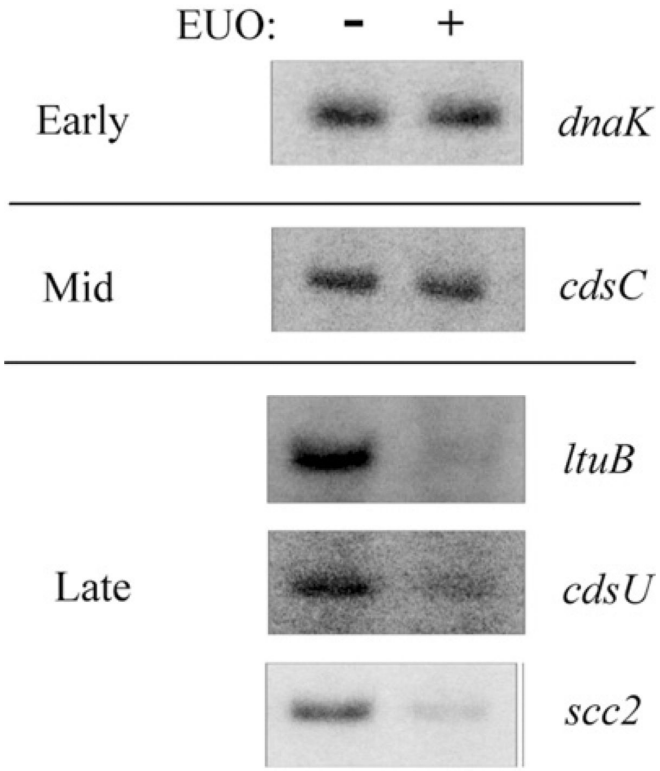

Fig. 6.

EUO represses late, but not early or mid promoters. Transcription templates containing the promoter regions of early (*dnaK*), mid (*cdsC*) and late (*ltuB*, *cdsU* and *scc2*) were transcribed with *E. coli* RNA polymerase in the absence or presence of 2.5 μM EUO. Additional early, mid and late promoters were tested, and the results are shown in Table 1.

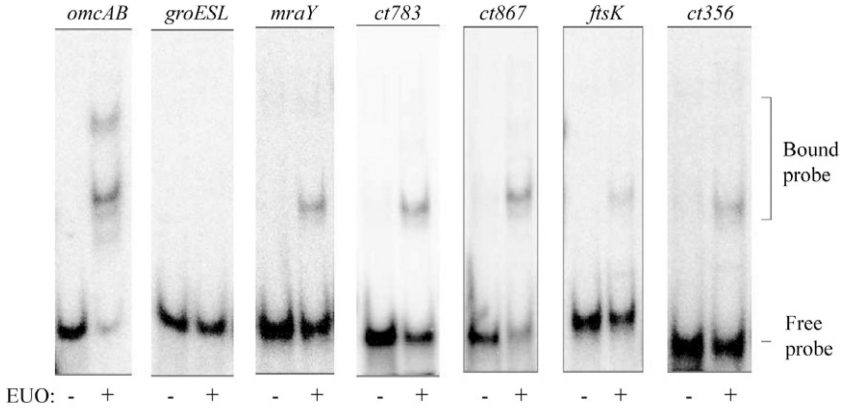

Fig. 7.

EUO binds to the predicted promoter regions of five additional late genes. For each promoter region contained on a 60 bp DNA probe, EMSA reactions were performed in the absence of EUO or with 320 nM EUO. Bands corresponding to the bound and free probes are indicated on the right.

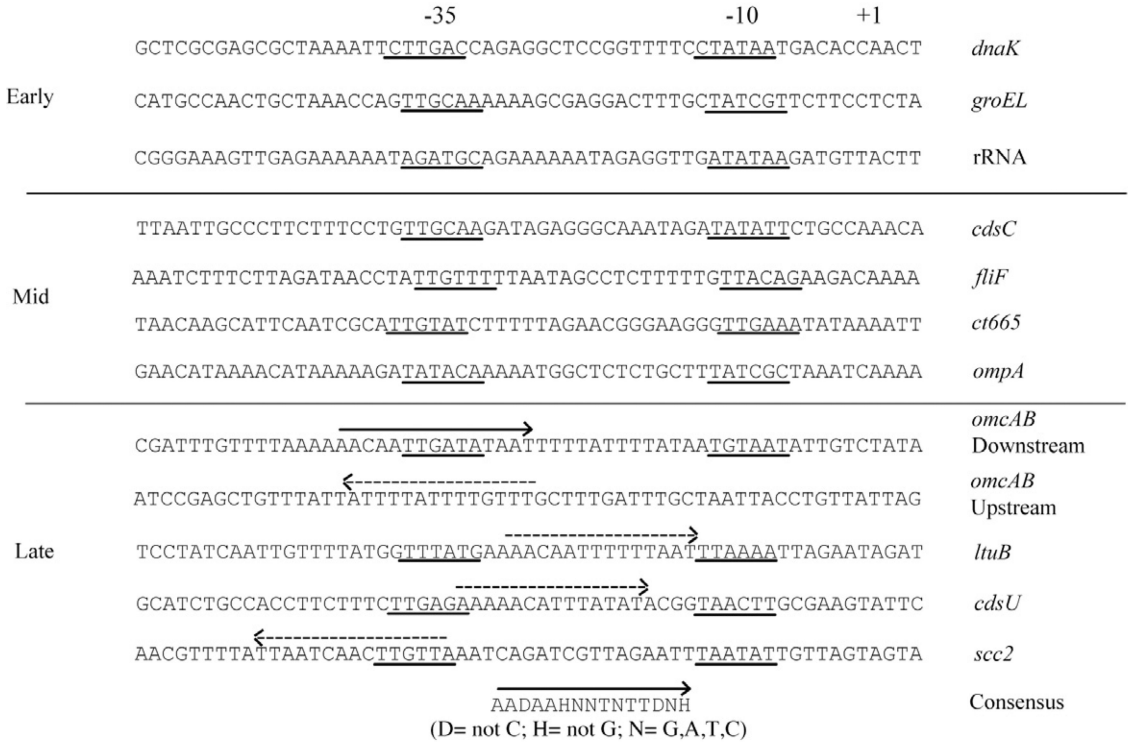

Fig. 8.

Identification of putative EUO operators in promoter regions of *C. trachomatis* genes tested in this study. Except for *omcAB* upstream, DNA sequences were aligned by the transcriptional start site (indicated by +1). The –35 and –10 promoter elements are underlined. The EUO-binding site for the downstream *omcAB* promoter region is indicated with a solid arrow above the sequence. The published consensus EUO-binding site is indicated at the bottom of the figure (Zhang *et al.*, 2000). These two reference EUO-binding sites were used to identify putative binding sites (dashed arrows) for late, but not early or mid genes, on the basis of sequence similarity. Arrows pointing from right to left represent the EUO-binding site on the complementary DNA strand. DNA sequences for the promoter regions of the late genes tested in Fig. 7 are not listed, as the –35 and –10 elements have not yet been defined.

Table 1

Binding and transcriptional repression of chlamydial promoters by EUO.

	Promoter	Binding[a]	Repression (%)[b]
Early genes	*dnaK* (*ct396*)	No	6 ± 5
	groESL (*ct110*)	No	21 ± 13
	rRNA	n.t.	7 ± 8
Mid genes	*cdsC* (*ct674*)	No	12 ± 6
	fliF (*ct719*)	No	11 ± 12
	ct665	n.t.	21 ± 11
	ompA (*ct681*)	n.t.	12 ± 10
Late genes	*omcB* (*ct443*)	Yes	60 ± 18
	ltuB (*ct080*)	Yes	69 ± 9
	ssc2 (*ct576*)	Yes	75 ± 2
	cdsU (*ct091*)	Yes	46 ± 19
	mraY (*ct757*)	Yes	n.t.
	ct783	Yes	n.t.
	ct867	Yes	n.t.
	ftsK (*ct739*)	Yes	n.t.
	ct356	Yes	n.t.

n.t., not tested.

[a]Binding was determined by EMSA using EUO protein over a range of 80–320 nM for each 60 bp labelled DNA probe containing the respective promoter.

[b]Per cent repression was calculated as the ratio of transcript levels in the presence/absence of EUO. Results are from at least three independent experiments and are reported as the mean and standard deviation for each promoter.

Table 2

List of transcription templates.

Plasmid	Promoter (nucleotides relative to transcription start site)	Reference
pMT1013	*ltuB* promoter region from –132 to +5; rRNA promoter region from –54 to +5	Schaumburg and Tan (2000)
pMT1149	*euo* promoter region from –90 to +5	Adam Wilson (unpublished)
pMT1150	*omcAB* promoter region from –122 to +5	Wilson and Tan (2002)
pMT1178	*groESL* promoter region from –137 to +5	Wilson and Tan (2004)
pMT1225	*dnaK* promoter region from –65 to +5	Adam Wilson (unpublished)
pMT1441	*cdsU* promoter region from –153 to +5	Case *et al.* (2010)
pMT1442	*scc2* promoter region from –144 to +5	Case *et al.* (2010)
pMT1513	*cdsC* promoter region from –146 to +5	Case *et al.* (2010)
pMT1515	*ct665* promoter region from –170 to +5	Case *et al.* (2010)
pMT1516	*fliF* promoter region from –154 to +5	Case *et al.* (2010)
pMT1636	*omcAB* promoter region from –55 to +5	This work
pMT1637	*omcAB* promoter region from –122 to +5 containing a 9 bp nucleotide substitution in the downstream EUO-binding site	This work