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Distinct modes of promoter recognition by two iron starvation σ factors with overlapping promoter specificities

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ABSTRACT

OrbS and PvdS are extracytoplasmic function (ECF) σ factors that regulate transcription of operons required for the biosynthesis of the siderophores ornibactin and pyoverdine in the *B. cepacia* complex and *Pseudomonas* spp., respectively. Here we show that promoter recognition by OrbS requires specific tetrameric -35 and -10 element sequences that are strikingly similar to those of the consensus PvdS-dependent promoter. However, whereas *P. aeruginosa* PvdS can serve OrbS-dependent promoters, OrbS cannot utilise PvdS-dependent promoters. To identify features present at OrbS-dependent promoters that facilitate recognition by OrbS, we carried out a detailed analysis of the nucleotide sequence requirements for promoter recognition by both OrbS and PvdS. This revealed that DNA sequence features located outside of the sigma binding elements are required for efficient promoter utilisation by OrbS. In particular, the presence of an A-tract extending downstream from the -35 element at OrbS-dependent promoters was shown to be an important contributor to OrbS specificity. Our observations demonstrate that the nature of the spacer sequence can have a major impact on promoter recognition by some ECF sigma factors through modulation of the local DNA architecture.

IMPORTANCE

ECF sigma factors regulate subsets of bacterial genes in response to environmental stress signals by directing RNA polymerase to promoter sequences known as the -35 and -10 elements. In this work, we identify the -10 and -35 elements that are recognised by the ECF sigma factor OrbS. Furthermore, we demonstrate that efficient promoter utilisation by this sigma factor also requires a polyadenine tract located downstream of the -35 region. We propose that the unique

architecture of A-tract DNA imposes conformational features on the -35 element that facilitates efficient recognition by OrbS. Our results show that sequences located between the core promoter elements can make major contributions to promoter recognition by some ECF sigma factors.

KEYWORDS

Burkholderia cenocepacia, *Pseudomonas aeruginosa*, sigma factor, OrbS, PvdS, promoter recognition, gene regulation, siderophore

INTRODUCTION

Most bacterial σ factors are related to the primary (or 'housekeeping') σ factor, σ^{70}, that is responsible for directing RNA polymerase (RNAP) to the majority of promoters (1,2). σ^{70} is organised into four domains, $\sigma_{1.1}$, σ_2, σ_3 and σ_4, which are further subdivided into regions based on amino acid sequence conservation: $\sigma_{1.1}$ consists of region 1.1, σ_2 contains regions 1.2 and 2.1-2.4, and includes a long non-conserved region (NCR) that connects region 1.2 to region 2.1, σ_3 is divided into regions 3.0 and 3.1, and is connected by a long loop (region 3.2) to σ_4, which in turn is comprised of regions 4.1 and 4.2 (2,3). Region 2.4 has been implicated in recognition of the promoter -10 region while region 4.2 interacts with the -35 region (4). In addition to promoter recognition, σ^{70} is also required for initial DNA strand separation (promoter 'melting') that extends from position -11 to +2 (5,6) to form the RNAP-promoter open complex. Structural analysis indicates that regions 1.2 and 2.1-2.4 interact with the -10 region non-template strand, with region 2.3 providing most of the contacts, whereas interactions between region 2.4 and the promoter are confined to the region around position -12 (7-9). σ^{70} initiates the DNA unwinding process by 'flipping' out the adenine base located at position -11 (A_{-11}) and the thymine at position -7 (T_{-7}) of the non-template strand into complementary protein pockets in σ_2 (7,8).

The σ^{70} family can be subdivided into four groups based on their phylogenetic relatedness and function, with the housekeeping σ^{70} orthologues constituting Group 1 (1,2). The most diverse is Group 4, otherwise known as the extracytoplasmic function (ECF) σ factors (10,11). Members of this group are essentially composed of only domain 2 (lacking region 1.2 and the entire NCR) and domain 4 of σ^{70} connected by a linker sequence (2,12,13). As with all σ^{70} family members,

these σ factors recognise specific sequences located approximately 35 and 10 bp upstream from the transcription start site. ECF σ factors also tend to have more stringent promoter sequence requirements than σ^{70}, particularly at the -10 element (14-16). Moreover, in the case of the ECF σ factor, σ^{E} (RpoE), it appears that only one base (located at position -10) is flipped out of the stacked bases on the non-template strand and into a cavity in σ_2 that corresponds to the A_{-11} pocket of σ^{70} (17). Iron-starvation (IS) σ factors constitute a distinct clade within ECF σ factors that primarily regulate genes involved in iron acquisition (10,18,19). A well characterised IS σ factor is PvdS of *P. aeruginosa* which directs transcription of genes required for the biosynthesis and export of the siderophore pyoverdine, as well as additional virulence genes (19-23).

The genus *Burkholderia* includes a group of ~20 closely related species referred to as the *Burkholderia cepacia* complex (Bcc) that are notorious for causing infections in patients with cystic fibrosis and chronic granulomatous disease (24-27). As their main siderophore, members of the Bcc secrete ornibactin, which chelates exogenous ferric iron with high affinity and is then internalised by the bacterium via the TonB-dependent transporter, OrbA (28,29). Biosynthesis and transport of ornibactin by the Bcc member *B. cenocepacia* is specified by a cluster of 14 genes that is regulated by the IS σ factor OrbS in response to iron availability (30) (Fig. 1A). OrbS exhibits 40% identity to PvdS. The aim of this investigation was to conduct a detailed characterisation of the OrbS-dependent ornibactin gene promoters and to search for additional promoters that may be served by OrbS. We find that OrbS recognises very similar promoter core element sequences to those present at PvdS-dependent promoters, but that OrbS has more stringent DNA sequence requirements for optimal promoter utilisation that includes an A-tract

103 located downstream from the -35 promoter element. We also find no evidence for the existence
104 of OrbS-dependent promoters outside of the ornibactin gene cluster.

105

106

107 **RESULTS**

108 **Identification of OrbS-dependent promoters**

109 Previously, we demonstrated the existence of four iron-regulated promoters within the ornibactin
110 gene cluster but we did not confirm their precise location (30). One of these promoters, P_{orbS},
111 was observed to be located less than 71 bp upstream of the translation initiation codon of the
112 *orbS* gene and was shown to be σ^{70}-dependent (30). The remaining OrbS-dependent promoters
113 were shown to be located upstream of the *orbH*, *orbE* and *orbI* genes (Fig. 1A). To identify the
114 OrbS-dependent promoters, the transcription start sites of two of them (P_{orbH} and P_{orbI}) were
115 determined by employing the primer extension method. To confirm the location of the *orbS*
116 promoter, primer extension was also carried out using an *orbS*-specific primer.

117

118 Three *orbS*-specific cDNA products were generated that indicated mRNA 5' endpoints
119 corresponding to sites located 31, 30 and 24 bp upstream of the *orbS* translation initiation codon
120 (Fig. 1B). Given the greater abundance of the largest cDNA product, the initiation site for the
121 majority of *orbS* transcription is located 31 bp upstream of the *orbS* start codon (Fig. 1C). As the
122 second largest cDNA product is only one nucleotide shorter than the largest cDNA product,
123 transcription may also initiate at the next downstream nucleotide, but with lower efficiency. The
124 smallest product is likely to be the result of premature termination of reverse transcription due to
125 secondary structure formation or degradation of the mRNA. As the hexameric -10 element spans

positions -12 to -7 relative to the TSS (i.e. the +1 position) at the majority of σ^{70}-dependent promoters, this identifies the P_{orbS} promoter -35 and -10 elements as TTGAGA and TAAATT, respectively (Figure 1C).

The *orbH*- and *orbI*-specific primers gave rise to single cDNA products (Fig. 1B) with 3' ends corresponding to positions located at 55 and 62 bp upstream of the translation initiation codon, respectively (Fig. 1C). These products were only observed using RNA recovered from iron-starved cells. Located at positions -36 to -27 and -12 to -9 relative to both TSSs are two identical sequence motifs: CGGTAAAAAA and CGTC. Although we did not determine the TSS for P_{orbE}, a CGTC motif is located 107 bp upstream of the predicted *orbE* translation initation codon and is preceded by the TAAA component of the longer conserved motif present in the other two OrbS-dependent promoters. Notably, the TAAA and CGTC motifs are separated by 17 bp in all three cases, a distance that is typical of the spacer region separating -35 and -10 elements of σ^{70} family-dependent promoters (Fig. S1). At all three promoters the spacer region consists of a 4-5 bp A.T-rich sequence following the TAAA motif, which is in turn followed by a 10-11 bp tract consisting entirely of G.C base pairs. The three positions located immediately upstream of the TAAA -35 element also consist of G.C base pairs. Transcription initiates with a purine nucleotide at the two promoters for which the TSS was determined and is predicted to initiate with a purine nucleotide at P_{orbE}. We also observed that the TSS is located within an A+G-rich region at all three promoters (Fig. S1).

Cross-recognition of OrbS- and PvdS-dependent promoters by OrbS and PvdS

148 The sequence that is conserved at all three OrbS-dependent promoters is very similar to the
149 consensus promoter recognised by *P. aeruginosa* PvdS, i.e. TAAAT$(N)_{16}$CGT (19,20,31).
150 Therefore, we examined the possibility that OrbS and *P. aeruginosa* PvdS are functionally
151 interchangeable by using reporter fusions to measure the activities of P_{orbH} and two PvdS-
152 dependent promoters in *P. aeruginosa* and *B. cenocepacia*. The results show that the activities of
153 the PvdS-dependent *pvdF* (PA2396) and *pvdE* (PA2397) promoters (P_{pvdE} and P_{pvdF}) and the
154 OrbS-dependent P_{orbH} promoter were comparable in wild-type *P. aeruginosa* growing under iron
155 limited conditions (Fig. 2A). When cells were grown under iron replete conditions to allow for
156 repression of chromosomal *pvdS*, the activities of all three promoters were decreased. In the *pvdS*
157 mutant, expression from all three promoters was lower than observed in cells growing under iron
158 replete conditions in the presence of the σ factor. These results show that PvdS can efficiently
159 recognise an OrbS-dependent promoter, even though the promoter lacks the highly conserved
160 thymine base that is present at position 5 of the -35 element in PvdS-dependent promoters.
161

162 As expected, P_{orbH} was highly active in wild-type *B. cenocepacia* cells growing under iron-
163 limiting conditions (Fig. 2B). In contrast, P_{pvdE} was nearly 20 fold less active than P_{orbH} while
164 P_{pvdF} showed negligible activity. The P_{pvdE} promoter contains a cytidine residue following the -
165 10 element CGT motif which maximises the match to the OrbS-dependent promoter -10 region
166 (Fig. S1) and therefore may be expected to respond to OrbS and iron. Accordingly, the activity of
167 P_{pvdE} was further decreased when cells were grown in the presence of iron and it was also less
168 active in the *orbS* mutant strain. Thus, OrbS can only very inefficiently utilise a PvdS-dependent
169 promoter that contains matches to both conserved motifs present at OrbS-dependent promoters.
170 These results suggest that for efficient recognition of its target promoters, OrbS requires DNA

sequence features that reside outside the -35 and -10 sequence elements shared by OrbS-dependent promoters and P_{pvdE}.

Identification of a minimal OrbS-dependent promoter

To facilitate a more detailed analysis of the DNA sequence requirements for OrbS promoter recognition, a 'minimal' OrbS-dependent promoter was identified. *E. coli* MC1061 was transformed with plasmids pBBR2-orbS and pKAGd4 containing a series of truncated P_{orbH} derivatives, and the promoter activities were measured. The results showed that OrbS was able to serve P_{orbH} in *E. coli*, although it was much less efficient than in the native host (Table 1). In the absence of *orbS* there was no measurable activity from this promoter in *E. coli* (data not shown). Surprisingly, the longest of the truncated promoter derivatives tested, $P_{orbHds2,}$ was at least tenfold more active in *E. coli* cells expressing *orbS* than the longer P_{orbH} fragment (and was inactive in the absence of OrbS). This was also true of most of the other truncated promoter derivatives tested. The shortest DNA fragment that retained high levels of OrbS-dependent promoter activity, $P_{orbHds6}$, contained sequences extending upstream to position -37 and downstream to +5 relative to the TSS. The shortest fragment tested, $P_{orbHds1}$ (extending from -37 to -5), showed a large reduction in activity compared to $P_{orbHds6}$, suggesting that the region located between positions -5 to +6 contained an important determinant for full activity. The results were validated by performing activity measurements on some of the promoter derivatives in *B. cenocepacia* (Table 1). These results supported those obtained in *E. coli* with the exception of the longest P_{orbH} construct.

193 The activity of the minimal $P_{orbHds6}$ promoter was compared to P_{orbI} and P_{orbE} promoter
194 fragments with the same upstream and downstream endpoints relative to the TSS ($P_{orbIds1}$ and
195 $P_{orbEds1}$). The results showed that $P_{orbIds1}$ was twice as active as $P_{orbHds6}$ when measured in *B.*
196 *cenocepacia* whereas $P_{orbEds1}$ exhibited approximately 20% of the activity of $P_{orbHds6}$ (Fig. S2).
197 The weakest promoter, P_{orbE}, is distinguished by the absence of a long A-tract extending from the
198 -35 element into the spacer region in the non-template strand (Fig. S1).
199
200 **Identification of functionally important bases for promoter utilisation by OrbS**
201 To establish the DNA sequence requirements for promoter recognition by OrbS, single base pair
202 substitutions were introduced into the $P_{orbHds6}$ promoter, and their effects on promoter activity
203 were assessed. The single base pair substitutions were introduced at each position in the
204 promoter (-37 to +5) except residues -24 to -16 located within the spacer region. Substitutions at
205 -33 to -30 and -12 to -9 resulted in very strong inhibitory effects on promoter activity (Fig. 3).
206 These positions correspond to the conserved TAAA and CGTC motifs and confirm their function
207 as the -35 and -10 elements. The effect of substitutions at positions -11, -10 and -9 of the -10
208 element were particularly severe, effectively abolishing measurable promoter activity in *B.*
209 *cenocepacia*. Substitutions at some positions within the spacer region (i.e. positions -28 and -27
210 within the A-tract and also positions -15 and -14) exerted quite marked negative effects on
211 promoter activity (≥50% decrease). Interestingly, substitution of the C residue at position -25,
212 which results in extension of the A-tract by an additional base, caused a large (~70 %) increase in
213 promoter activity.
214
215 **Role of the TSS region in OrbS-dependent promoter activity**

Although the deletion analysis had identified region -4 to +5, corresponding to the A+G block, as being very important for OrbS-dependent promoter activity (Table 1), single base pair substitutions in this region resulted in more modest decreases in promoter activity or exerted no significant effect (Fig. 3). To further investigate the importance of this region, multiple base pair substitutions were introduced into this region. Promoter activity measurements in *B. cenocepacia* showed that substitution of all nine base pairs (i.e. $P_{orbHdsAGB1}$) completely abolished promoter activity (Fig. 4A). The more severe effect of the 'en bloc' substitution compared to truncation to position -5 ($P_{orbHds1}$) may be explained by the fact that replacement of the sequence downstream of position -5 by vector sequences in the latter does not result in a base change at every position. Next, non-overlapping trinucleotide substitutions were introduced that spanned the entire region. Simultaneous substitution of bases at positions -1, +1 and +2 ($P_{orbHdsAGBtri2}$) resulted in a large decrease in promoter activity (~ 90% decrease), whereas substitution of the flanking nucleotide triads exerted much less severe effects (≤30% decrease in activity) (Fig. 4A). As the bases that constitute the central triplet are also substituted in the $P_{orbHds1}$ and $P_{orbHdsAGB1}$ derivatives, the simplest explanation is that substitution of the TSS together with one or both flanking nucleotides is largely responsible for most of the decrease in promoter activity following substitution of all 9 bp of the A+G block.

Role of the spacer region in OrbS-dependent promoter activity

Located within the 17 bp spacer region of all three OrbS-dependent promoters is a 10-11 bp tract that is composed exclusively of G.C base pairs (Fig. S1). The G.C tract overlaps at nine consecutive positions within all three promoters. To explore the possible role of this region we substituted all 9 bp and measured the effect on OrbS-dependent promoter activity in *B.*

cenocepacia. Due to the fact that this manipulation rendered the promoter highly A+T rich from positions -33 to -14, we also constructed a promoter derivative in which only every alternate base in the G.C spacer block was substituted. The results showed that substitution of the entire G.C tract ($P_{orbHdsGCS1}$) caused a sharp decrease (~90%) in promoter activity, whereas less severe modification of this region ($P_{orbHdsGCS2}$) resulted in a smaller decrease (~30%) in promoter activity (Fig. 4B). The effect of spacer length on promoter activity was also investigated. Insertion of a single G.C base pair between positions -21 and -22 ($P_{orbHdsGCS+1}$) resulted in ~50% decrease in promoter activity while insertion of two base pairs ($P_{orbHdsGCS+2}$) essentially abolished promoter activity (Fig. 4B). In contrast, deletion of only a single base at position -21 ($P_{orbHdsGCS-1}$) was sufficient to almost completely inactivate the promoter.

PvdS has a less stringent promoter sequence requirement than OrbS

Although a consensus sequence for PvdS-dependent promoters in *P. aeruginosa* was established based on a bioinformatic analysis of PvdS-responsive genes (20) it has not been validated by a systematic mutagenesis analysis. As PvdS can very efficiently serve P_{orbH}, we tested its ability to utilise the P_{orbH} single base substitution mutants in *E. coli* and compared the results with those obtained for OrbS.

Overall, the ability of OrbS to serve the single substitution promoter variants in *E. coli* was qualitatively similar to the results obtained in *B. cenocepacia* (Fig. 5A). That is, substitutions at each position within the TAAA and CGTC motifs strongly impaired promoter function, with substitutions at positions -11 to -9 being most inhibitory. Expression of PvdS in *E. coli* resulted in 7 times more P_{orbH} activity than with OrbS (Fig. 5B). Although it is not clear whether this is due to higher expression of *pvdS* or more efficient assembly with the host core RNAP, the *pvdS*

gene cloned in pBBR1MCS-2 lacks its native promoter, whereas the P_{orbS} promoter is present on the plasmid expressing *orbS*. For this reason, the higher activity of the P_{orbH} promoter in the presence of PvdS may be due to more efficient incorporation of PvdS into RNAP holoenzyme in comparison to the taxonomically more distant OrbS protein. Notably, PvdS was able to efficiently serve promoters in which the fourth position of each of the two core elements recognised by OrbS had been substituted (positions -30 and -9). As with OrbS, substitution of bases at each position within the -35 element and at the first position of the -10 element exerted less severe effects than at other positions within the -10 element.

We also tested the effect of increasing and decreasing P_{orbH} spacer length on OrbS- and PvdS-dependent promoter activity in *E. coli*. Introducing mono- or dinucleotide insertions or deletions into the P_{orbH} spacer gave rise to qualitatively similar results for OrbS-dependent transcription to those observed in *B. cenocepacia* (compare Fig. 4B and Table 2). In contrast, PvdS was able to moderately tolerate a single base pair deletion in the spacer as well as a single base pair insertion (Fig. Table 2). Dinucleotide insertions or deletions in the spacer abrogated PvdS-dependent promoter activity.

Identification of a non-canonical DNA sequence element that enhances OrbS activity

To identify DNA sequence features that are required for efficient recognition of OrbS-dependent promoters in addition to the -35 and -10 core elements, we compared the effect of introducing multiple substitutions in the G.C spacer block or the downstream A+G-rich region on promoter utilisation by OrbS and PvdS, as these DNA sequence features are conserved at only some PvdS-dependent promoters (Fig. S1). The results showed that substituting every base in the G.C spacer block ($P_{orbHdsGCS1}$) exerted a significant though less detrimental effect on OrbS-dependent

transcription in *E. coli* than was the case in *B. cenocepacia* (compare Table 2 with Fig. 4B). Substitution of this region also exerted a modest inhibitory effect on promoter utilisation by PvdS. Substitution of the entire A+G tract overlapping the TSS ($P_{orbHdsAGB1}$) exerted a strong down effect on promoter utilisation by OrbS in *E. coli*, similar to what was observed in *B. cenocepacia* (compare Table 2 with Fig. 4A). Moreover, this alteration to the promoter also abrogated its utilisation by PvdS in *E. coli*.

The A-tract in the non-template strand of the -35 element of P_{orbH} and P_{orbI} extends downstream into the spacer region (to positions -26 and -27, respectively), whereas for the weakest OrbS-dependent promoter, P_{orbE}, this is not the case (Fig. S1). We also observed that extension of the tract by a single base enhanced OrbS-dependent activity at P_{orbH} in both *B. cenocepacia* and *E. coli*. Pertinently, PvdS-dependent promoter sequences do not contain this A-tract (Fig. S1). To investigate the role of this region, we introduced dinucleotide substitutions at positions -36 to -24 of $P_{orbHds6}$ and analysed their effects on OrbS-dependent promoter activity in *E. coli*. As expected, all of the dinucleotide-substituted promoters that resulted in one or two substitutions within the -35 element (TAAA) exerted a strong down effect on promoter activity (Table 3). It was notable that where both substitutions were introduced into the -35 element, the inhibitory effect on promoter activity was considerably more marked than a single nucleotide substitution. Interestingly, substitution of the AA dinucleotide at -29/-28, which resides outside of, but immediately adjacent to, the -35 element, was similarly as inhibitory as a single base pair substitution within the -35 element (compare Table 3 and Fig. 5A). Substitution of the AA dinucleotide at -28/-27 also exerted a strong inhibitory effect on promoter activity (~80% decrease). Substitution of the AA dinucleotide at -27/-26 or the AC dinucleotide at -26/-25 exerted relatively small inhibitory effects on promoter activity. Dinucleotide substitutions

311 introduced immediately upstream of the TAAA motif, exerted small or negligible inhibitory
312 effects on promoter activity. These results suggest that a conformational feature that is dependent
313 on the extended A-tract makes an important contribution to OrbS-dependent promoter utilisation.
314 To test this hypothesis, we incorporated a poly-A tract into a minimal P_{pvdE} promoter ($P_{pvdEds1}$)
315 and measured its effect on promoter utilisation by OrbS in *E. coli*. The results show that the
316 modified promoter ($P_{pvdEds7}$) was utilised nearly ten times more efficiently by OrbS than was the
317 native *pvdE* promoter, although $P_{pvdEds7}$ was still considerably less active than $P_{orbHds6}$ (Table 4).
318 In contrast, the introduction of the poly-A tract led to a small decrease in the efficiency of P_{pvdE}
319 utilisation by PvdS. PvdS was also much less sensitive than OrbS to substitution of the AA
320 dinucleotide at positions -29/-28 of P_{orbH} (compare Table 3 and Table S1).
321
322 These results prompted us to investigate the effect of all possible single base substitutions within
323 the poly-A tract on OrbS-dependent transcription. This analysis was also extended to the -35 and
324 -10 core elements, and nucleotide positions flanking each element. As expected, substitution of
325 each base pair within the experimentally determined -35 and -10 regions, by any of the other
326 three possible bases, exerted strong inhibitory effects on promoter utilisation (Fig. 6). Most
327 noteworthy was the fact that all three possible substitutions at either position of the central
328 dinucleotide of the -10 element (CGTC) abolished promoter activity. Substitutions either side of
329 the -10 element were much less inhibitory or exerted no inhibitory effect on promoter utilisation,
330 thereby clearly delimiting this element. Base pair substitutions introduced at A-tract positions
331 located downstream of the -35 TAAA motif showed a clear trend from being strongly or
332 moderately inhibitory (positions -29 and -28) to exerting little or no inhibitory effect (position -
333 26). Substitution of the base located immediately upstream from the -35 element (position -34)
334 with a C or A (but not with a T) exerted a strong inhibitory effect, although not to the extent

observed for the majority of the substitutions within the -35 element, whereas substitutions further upstream exerted little or no significant effect on promoter activity. These results confirm the importance of the poly-A tract on promoter utilisation by OrbS but also suggest a small contribution to promoter function from the G.C base pairs located immediately upstream of the TAAA motif.

DISCUSSION

We have shown that OrbS requires the tetranucleotide motifs TAAA and CGTC as the core -35 and -10 elements for target promoter utilisation. Substitutions at each position within the -10 element strongly impair promoter activity, suggesting that the σ factor makes base-specific contacts at all four positions (positions -12 to -9). Although structural analysis of the interaction of domain 2 of RpoE with its target -10 element ($_{-12}$GTCAAA$_{-7}$) has revealed base-specific interactions at positions -12 to -10 of the non-template strand (16,17,32), the occurrence of base-specific interactions at position -9 may also occur with some other ECF σ factors such as the *P. syringae* PvdS orthologue (see discussion below) and *B. subtilis* σ^X which recognises promoters with an invariant C at position 4 of the -10 element (33). The less severe effect of substituting bases within the -35 element of the OrbS target promoter is also consistent with studies carried out on other ECF σ factor-dependent promoters and supports the proposal that the -10 element contributes the main discriminatory function at such promoters (15,34).

The core elements at OrbS-dependent promoters are flanked by more loosely conserved sequence features, including G.C-rich regions located immediately upstream of the -35 element

358 and within the spacer region, and a transcription initiation region that is enriched for purine
359 residues in the non-template strand. The two strongest OrbS-dependent promoters also possess
360 an A-tract that extends from the -35 element into the spacer region. Whereas 'en bloc' nucleotide
361 substitutions of the G.C spacer and A+G TSS regions impact negatively on OrbS activity,
362 smaller modifications to the extended A-tract are sufficient to exert a strong down effect on
363 promoter utilisation by OrbS and extending it by one nucleotide stimulates OrbS-dependent
364 activity. The properties of A-tracts (specifically those that lack a TpA step) have been the subject
365 of extensive investigation (35). A-tracts as short as four consecutive base pairs, when inserted
366 into non-A.T tract DNA, can induce bending and are known to bend the DNA helix by 9^{o} into
367 the minor groove (36). A bend of ~20^{o} has been determined for an A-tract of 6 nucleotides
368 (36,37). The bending is proposed to arise from differences in tilt associated with the 5' and 3'
369 junctions flanking the A-tract and negative roll within the A-tract (36). In addition to bending, A-
370 tracts result in progressive narrowing of the minor groove and high negative propeller twist
371 (38,39). We propose that one or more of these conformational features of the A-tract is
372 instrumental in rendering the P_{orbH} and P_{orbI} -35 elements more susceptible to recognition by
373 OrbS.
374
375 The possession of an A-tract by OrbS-dependent promoters was used to inform a bioinformatic
376 search for additional putative OrbS-dependent promoters in *B. cenocepacia* and other members
377 of the Bcc which were then tested for OrbS-dependency. However, this approach did not uncover
378 additional OrbS-dependent promoters (Supplemental Text 1), leading us to conclude that the
379 three OrbS-dependent promoters located in the ornibactin gene cluster are the only
380 representatives of this promoter class in Bcc members.
381

382 The core elements present at OrbS-dependent promoters bear a strong resemblance to the
383 consensus TAAAT and CGT motifs located upstream of PvdS-dependent genes in *P. aeruginosa*
384 (20). Although these motifs have been accepted as the -35 and -10 elements for PvdS promoter
385 recognition for over a decade, a systematic analysis of DNA sequences required for PvdS-
386 dependent transcription by the *P. aeruginosa* σ factor has not been performed. Our results
387 demonstrate that the conserved CGT trinucleotide at PvdS-dependent promoters is necessary and
388 sufficient for promoter recognition by the *P. aeruginosa* σ factor. In contrast, results from a
389 single nucleotide scanning analysis suggest that for efficient promoter utilisation, the *P. syringae*
390 PvdS σ factor, like OrbS, also requires a conserved base at position 4 of the -10 element,
391 although a T is preferred rather than a C (15). As regions 2.3 and 2.4 of σ_2 make important
392 contacts with the non-template strand of the promoter -10 region, to account for the different
393 specificity of these σ factors we compared the amino acid sequences of these regions among
394 OrbS and PvdS orthologues. Interestingly, the amino acid sequence of region 2.4 is almost
395 identical in all PvdS orthologues (Fig. 7). This would suggest that region 2.3 is largely
396 responsible for discriminating between the two types of PvdS-dependent promoter, and by
397 extension, between PvdS- and OrbS-dependent promoters. In region 2.3 of the *P. syringae* σ
398 factor, the loop that connects α-helices 3 and 4 (the L3 loop) contains amino acids at three
399 positions that are different in the other PvdS orthologues (two of which are non-conservative
400 changes). It is also noteworthy that the L3 sequence of OrbS orthologues is very distinct from
401 those of the PvdS orthologues. The region of the L3 loop in which the *P. syringae* PvdS amino
402 acid substitutions occur is responsible for forming the pocket in RpoE that accommodates the
403 base at position -10 once it is flipped out of the stacked bases during open complex formation
404 (17). However, it is not clear why the L3 loop should be different in *P. syringae* PvdS when the

base at position -10 (the T base at position 3 of the -10 element) is identical at all PvdS-dependent promoters, and indeed at all OrbS-dependent promoters. Therefore, it is possible that at OrbS- and PvdS-dependent promoters it is the base at position -9 (position 4 of the -10 tetramer) that is flipped into a pocket created by the L3 loop and this is reflected in the different sequence of this region among these sigma factors .

Despite the strong conservation of bases at positions 4 and 5 of the -35 element at PvdS-dependent promoters (Fig. S1), single base pair substitutions at either position of the test promoter (positions -30/-29) impaired PvdS-dependent transcription much less significantly than at the other three conserved positions. Indeed, P_{orbH} contains an adenine at the location corresponding to position 5 of the PvdS-dependent promoter -35 element, and yet PvdS was shown to utilise this promoter as efficiently as it serves P_{pvdE}, which contains the highly conserved thymine at this position. Moreover, introduction of a consensus T base at this position in P_{orbH} did not cause an increase in PvdS-dependent promoter activity. It should be borne in mind that the more significant impairment of PvdS-dependent activity arising from substitution at position 4 (although still much less detrimental to PvdS-dependent promoter activity than the effect of substitutions at positions 1-3) may be the result of having two consecutive non-consensus bases in the -35 element, as the A base at position 5 in the P_{orbH} -35 element is also non-consensus with respect to position 5 of the PvdS-dependent promoter -35 element (i.e. substitution at position 4 results in a -35 element with the sequence TAACA compared to the TAAAT consensus for PvdS-dependent promoters).

Two lines of evidence suggest that *P. aeruginosa* PvdS can efficiently recognise target promoters lacking a consensus base at position 4 of the -35 region. First, three known PvdS-dependent

promoters contain a T residue at this position, rather than an A, although the base at position 5 is consensus in each case (Fig. S1). Second, although the consensus -35 element sequence for promoter recognition by *P. syringae* PvdS has been established as TAAAT(A/T), pertinently, base substitution at position 4 of the -35 element at such a promoter did not affect the ability of *P. syringae* PvdS to utilise the promoter (15). Therefore, we conclude that the bases at positions 4 and 5 of the PvdS-dependent promoter -35 element are not involved in base-specific interactions with PvdS but rather are likely to make important contributions to the local conformation of the -35 element and their contribution can only be observed following substitution of both of them.

Promoter utilisation by PvdS was less sensitive to the presence or absence of an A-tract adjacent to the -35 promoter element than was the case with OrbS. This suggests that although the base sequences of the -35 region at OrbS- and PvdS-dependent promoters are similar, the sigma factors may be differentially sensitive to architectural features imparted by the A-tract. The amino acid sequences of the helix-turn-helix that constitutes region 4.2 of both sigma factors, and engages with the major groove at the -35 element (40), are very similar but it is noteworthy that a proline occurs in the interhelical turn in PvdS at the position occupied by a glutamine in OrbS (Fig. 7). This may change the trajectory of the C-terminal helix relative to that in OrbS and might explain why OrbS requires a particular conformation imposed on the -35 element by the A-tract. Pertinently, it has recently been observed that some promoters recognised by the *B. subtilis* Group 4 σ factor, σ^V, have a pentameric T-tract in the non-template strand that is located immediately downstream from the core -35 element. This tract was shown to be required for optimum σ^V activity (41). Moreover, tracts of three to five T residues were observed at the

corresponding position in promoters recognised by other ECF σ factors (41). Similar to OrbS-dependent promoters, it has been proposed that these tracts change the trajectory of the DNA through introducing a bend that results in productive engagement of RNAP with the target promoter. Thus, it would appear that promoter modelling by homopolymeric A or T tracts located in the upstream spacer region are an important feature of many Group 4 σ factor-dependent promoters. To summarise, our results show that whereas OrbS recognises promoters with very similar consensus -35 and -10 promoter elements to those recognised by PvdS of *P. aeruginosa*, OrbS has more stringent requirements for optimal promoter recognition that includes a particular conformation of the -35 motif that is strongly dependent on the presence of an A-tract in the spacer region.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth media

Bacterial strains and plasmids are listed in Table 5 and Table S2, respectively. Most experiments with *B. cenocepacia* were performed using strain 715j. *B. cenocepacia* and *P. aeruginosa* were routinely cultured on M9 salts medium (42) containing 0.5% glucose and 1.5% agar. For some experiments casamino acids (BD) was included in the liquid medium at 0.1% (w/v) final concentration in addition to glucose (M9-CAA). *E. coli* MC1061 was routinely cultured on LB agar and liquid cultures were grown in LB broth. Iron replete conditions were established by inclusion of $FeCl_3$ in the medium at a final concentration of 50 μM for all bacteria/media combinations. To establish iron limiting conditions in liquid culture, 2,2'-dipyridyl was included

474 in the medium at a final concentration of 175 μM for *E. coli* strains and 100 μM (M9 medium) or
475 200 μM (LB) for *B. cenocepacia* and *P. aeruginosa* strains
476
477 For strains harbouring pKAGd4, chloramphenicol was included in the medium (*E. coli*, 25
478 μg/ml; *B. cenocepacia*, 50 μg/ml; *P. aeruginosa*, 100 μg/ml) and for strains harbouring
479 pBBR1MCS-2 derivatives, kanamycin was included (*E. coli*, 25 μg/ml; *B. cenocepacia*, 50
480 μg/ml; *P. aeruginosa*, 400 μg/ml).
481
482 **Primer extension**
483 *B. cenocepacia* 715j was grown in M9 medium under iron limited conditions and total RNA was
484 isolated using the RNaqueous Midi Kit (Ambion). Antisense primers SKorbSRev, pvdEpvdIrev
485 and orbSmbtHrev (Table S3), corresponding to sequences located 47-66, 18-38 and 39-60 bp
486 downstream of the *orbS*, *orbI*, and *orbH* translation initiation codons, respectively, were end-
487 labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase and used to prime synthesis of labelled
488 cDNA. cDNA products were then electrophoresed in a 0.4 mm thick DNA sequencing gel
489 containing 7 M urea in Tris-borate buffer and sized against DNA sequencing ladders generated
490 using the same primer and the corresponding DNA region cloned in pBluescript II KS (i.e. pBS-
491 P_{orbS}, pBS-P_{orbI} and pBS-P_{orbH}, respectively) with the Sequenase Version 2.0 kit (USB).
492 Following electrophoresis, the dried gel was imaged using a Fujifilm FLA-3000 phosphorimager.
493
494 **Plasmid constructions**
495 Oligonucleotides used for plasmid construction are listed in Table S3. pBBR2-orbS was
496 constructed by transferring a 975 bp *Bam*HI-*Hin*dIII DNA fragment from pBBR1MCS-orbS to

pBBR1MCS-2. To construct pBBR2-pvdS, a 1.875 kb *Kpn*I-*Hin*dIII fragment was removed from pUCP22-pvdS and ligated between the same sites of pBBR1MCS-2, placing the *pvdS* gene under control of the *lacZ* promoter. pKAGd4-P_{pvdE} and pKAGd4-P_{pvdF} were constructed by amplifying a 564 bp DNA fragment containing the divergently organised PvdS-dependent *pvdF* (PA2396) and *pvdE* (PA2397) promoters (P_{pvdE} and P_{pvdF}) with primers PAppvdEfor and PAppvdErev2, then cutting the amplicon with *Bam*HI and *Hin*dIII, and ligating it between the *Bam*HI-*Hin*dIII sites and *Bgl*II-*Hin*dIII sites of pKAGd4, respectively. The remaining pKAGd4 derivatives contain short (42-74 bp) DNA fragments that were assembled by annealing two complementary oligonucleotides that generate *Bam*HI- and *Hin*dIII-compatible ends and ligating the product to the corresponding sites of pKAGd4 (see Tables S2 and S3).

Transfer of DNA by conjugation

Plasmids were introduced into *B. cenocepacia* and *P. aeruginosa* by biparental conjugal transfer using the *E. coli* donor strain S17-1 as described previously (43,44).

β-galactosidase assays

All assays were performed on three independent cultures, with technical duplicates for each, growing at 37°C. *E. coli* strains were cultured in LB under iron limiting conditions. *B. cenocepacia* strains were grown in M9 medium or LB, under iron limiting or iron replete conditions, as indicated. *P. aeruginosa* was grown in M9 medium under iron limiting conditions. Assays were carried out as described previously (30).

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FIGURE LEGENDS

Figure 1. Location of OrbS-dependent promoters in the *B. cenocepacia* ornibactin gene cluster. A. Gene organisation within the ornibactin gene cluster. Genes are represented as block arrows. Genes designated with the *orb* prefix are represented by single letters that refer to the corresponding suffix (i.e. 'G' represents *orbG*). Genes correspond to QU43_RS44960 (BCAL1688)-QU43_RS45030 (BCAL1702) in J2315 and I35_RS08005 (I35_1599)-I35_RS08075 (I35_1613) in H111. Transcription start sites and the direction of transcription are indicated by bent arrows. B. Determination of transcription start sites upstream of the *orbS*, *orbH* and *orbI* genes by primer extension. Radiolabelled cDNA was generated from mRNA isolated from cells grown under iron limited conditions ('-Fe'), and (in some cases) from cells grown under iron replete conditions ('+Fe'), and electrophoresed on a DNA sequencing gel. The products of DNA sequencing reactions generated with the same primer used to generate the cDNA and a template harbouring the corresponding promoter region were run in parallel. The DNA sequence of the pertinent region is shown to the left of the DNA sequencing reactions with

bases corresponding to the -10 element shown in magenta and the transcription start site (based on the location of the cDNA product) shown in red font. C. Location of transcription start sites at the *orbS*, *orbH* and *orbI* promoters determined by primer extension. Transcription start sites determined by primer extension are indicated by bent arrows. Conserved -35 and -10 sequences are enclosed in rectangles. The more extensive region of homology shared by the -35 region of the P_{orbH} and P_{orbI} promoters is outlined by the rectangles shown by dashed lines. For reference, the translation initiation codons are shown grey highlight and the Shine-Dalgarno sequences are shown in bold font and underlined.

Figure 2. Promoter specificity of OrbS and PvdS. A. PvdS-dependent activity of an OrbS-dependent promoter in *P. aeruginosa*. β-galactosidase activities were measured in *P. aeruginosa* PAO1 and PAO1-pvdS::Gm containing transcriptional fusions of *lacZ* to the *B. cenocepacia orbH* promoter and the *P. aeruginosa pvdE* and *pvdF* promoters carried on pKAGd4. Black bars and white bars represent the activities in PAO1 grown in M9 medium under iron replete and iron starvation conditions, respectively, whereas hatched and stippled bars represent the activities in the *pvdS* mutant grown under iron replete and iron starvation conditions, respectively. B. OrbS-dependent activity of PvdS-dependent promoters in *B. cenocepacia*. β-galactosidase activities were measured in *B. cenocepacia* 715j and 715j-orbS::Tp containing transcriptional fusions of *lacZ* to the *B. cenocepacia orbH* promoter and the *P. aeruginosa pvdE* and *pvdF* promoters carried on pKAGd4. Black bars and white bars represent the activities in 715j grown under iron replete and iron starvation conditions, respectively. Hatched and stippled bars represent the activities in the *orbS* mutant grown in M9 medium under iron replete and iron starvation conditions, respectively. Activity measurements were corrected by subtraction of the background

activity in the corresponding strain containing the empty pKAGd4 vector. All assays were performed on three independent cultures, with technical duplicates for each, and bars represent the means ± standard deviation. Statistical significance was determined by performing one-tailed t-tests, **, p<0.01; ***, p<0.001; ****, p<0.0001.

Figure 3. Effect of single base substitutions on P_{orbH} activity in *B. cenocepacia*. β-galactosidase activities were measured in *B. cenocepacia* 715j cells containing pKAGd4-$P_{orbHds6}$ or variants thereof in which single nucleotide substitutions were introduced at positions -37 to -25 and -15 to +5 relative to the TSS. All substitutions were transversions to the non-base pairing nucleotide (i.e. T>G, C>A, G>T and A>C). Bacteria were grown in LB under iron limiting conditions. Activities represented by red bars are ≤15% of the wild-type activity (green bar) and correspond to substitutions in the -35 and -10 core elements. Activities have been background corrected by subtracting the activity of the same strain harbouring pKAGd4 assayed under identical conditions and then expressed relative to the activity in cells harbouring pKAGd4-$P_{orbHds6}$ (100% = 1,383.6 Miller units). All assays were performed on three cultures, with technical duplicates for each, and bars represent the means ± standard deviation. The DNA sequence of $P_{orbHds6}$ is shown below the x-axis.

Figure 4. Role of the A+G tract and G.C-rich spacer region in P_{orbH} activity. β-galactosidase activities were measured in *B. cenocepacia* 715j cells containing pKAGd4-$P_{orbHds6}$ (green bar) or variants of this plasmid (blue bars) containing nucleotide substitutions in the A+G-rich tract located at the TSS (A) and nucleotide substitutions, insertions or deletions in the G.C-rich spacer region (B). Nucleotide sequences of the promoter variants are shown in Table 2. Bacteria were

grown in LB under iron limiting conditions. Activities have been background corrected by subtracting the activity of the same strain harbouring pKAGd4 assayed under identical conditions and then expressed relative to the activity in *B. cenocepacia* harbouring pKAGd4-$P_{orbHds6}$ (100% = 1,383.6 Miller units) represented by the green bar in each case. All assays were performed on three independent cultures, with technical duplicates for each, and bars represent the means ± standard deviation. Statistical significance between the activities of the test promoters and that of $P_{orbHds6}$ was determined using a one-way ANOVA and p values of <0.0001 were obtained in each case.

Figure 5. Effect of single base substitutions on OrbS- and PvdS-dependent activity at P_{orbH} in *E. coli*. β-galactosidase activities were measured in *E. coli* MC1061 cells containing pKAGd4-$P_{orbHds6}$ or single base pair substitution variants thereof (as described in Figure 4) in combination with pBBR2-orbS (A) or pBBR2-pvdS (B). Bacteria were grown in LB under iron limiting conditions. OrbS-and PvdS-dependent activities represented by red bars are ≤15% of the wild-type activity (green bar) and correspond to promoters with substitutions in the -35 and -10 core elements. Activities have been background corrected by subtracting the activity of the same strain harbouring pKAGd4 assayed under identical conditions and then expressed relative to the activity in cells harbouring pKAGd4-$P_{orbHds6}$ and the appropriate pBBR1MCS-2 derivative (100% = 3,122 and 22,200 Miller units in the presence of OrbS and PvdS, respectively). All assays were performed on three independent cultures, with technical duplicates for each, and the data represents the mean ± standard deviation. The DNA sequence of $P_{orbHds6}$ is shown below the x-axis.

Figure 6. Effect of all possible single base substitutions at the -10 and -35 core elements and the A-tract of the P_{orbH} promoter in *E. coli*. β-galactosidase activities were measured in *E. coli* MC1061 cells containing pBBR2-orbS in combination with pKAGd4-$P_{orbHds6}$ or variants in which a single base at positions -36 to -25 (A) and -13 to -8 (B) has been substituted by the other three possible bases. Bacteria were grown in LB under iron limiting conditions. Bars representing promoter activities use a different fill depending on the base that is present at that position, as shown in the key. Activities have been background corrected by subtracting the activity of the same strain harbouring pKAGd4 and pBBR2-orbS assayed under identical conditions and then expressed relative to the activity in cells harbouring pKAGd4-$P_{orbHds6}$ and pBBR2-orbS (100% = 5,268 Miller units). A bar representing 100% activity is shown for each base position with the fill corresponding to the base present at that position in the wild-type promoter ($P_{orbHsd6}$), which is also shown below the x-axis. All assays were performed on three independent cultures, with technical duplicates for each, and the bars represent the means ± standard deviation.

Figure 7. Alignment of domains 2 and 4 of the Group 4 σ factors RpoE, OrbS and PvdS. Amino acid sequences corresponding to domains 2 (σ_2) and 4 (σ_4), along with the interdomain region, were aligned using Clustal-omega. Amino acids that are identical at the corresponding position in ≥ 50% of aligned sequences are shown in white font with black highlight while amino acids that are similar are shown in white font and shaded in grey. The locations of conserved regions 2.1-2.4 and 4.1-4.2 are based on previous assignments for RpoE (2,17) and are enclosed in coloured boxes and labelled accordingly. Amino acids corresponding to the H3-H4 interhelix loop in domain 2 (the L3 loop) are indicated by the horizontal red bar above the sequence.

Amino acids in *E. coli* RpoE that interact with the 'flipped out' base at position -10 in the RpoE-dependent promoter are shown in red font and highlighted in cyan. For reference, the RpoE residue N84 that interacts via its sidechain with the base at -12 and residues N80, I77 and A60 that interact through their sidechains with the base at position -11 are shown in red font and highlighted in magenta. Also shown similarly is Y75 that stacks against the base at -7 through its sidechain, although this interaction does not contribute to sequence specificity. Interactions of RpoE with the -10 region sequence GTCAAA (-12 to -7) are described in (17). Amino acids in the H3-H4 interhelix loop of *P. syringae* PvdS that differ from those of the other pseudomonad PvdS orthologues are highlighted in green. Also for reference, residues R171, S172, F175 and R176 in region 4.2 of *E. coli* RpoE that interact with a consensus RpoE-dependent promoter -35 element (GGAACTT, -35 to -29) are shown in red font with magenta highlighting (40). The location of the N- and C-terminal helices of the region 4.2 HTH in *E. coli* RpoE are indicated by horizontal light and dark blue bars, respectively (12). Sequences were obtained from the following strains: *E. coli* MG1655; *B. ambifaria* AMMD, *B. cenocepacia* J2315, *B. lata* 383, *B. vietnamiensis* G4, *P. aeruginosa* PAO1, *P. chlororaphis* O6, *P. entomophila* L48, *P. fluorescens* Pf0-1, *P. protegens* Pf-5, *P. putida* GB-1, *P. syringae* B728a.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Architectural similarity between OrbS- and PvdS-dependent promoters. Conserved -35 and -10 core elements are highlighted in red. G.C-rich regions located upstream of the -35 region and in the spacer region are highlighted in green and grey, respectively. A+G-rich regions overlapping the transcription start site are highlighted in cyan. Bases corresponding

802 to experimentally determined transcription start sites are underlined. Numbering of base pairs is
803 with respect to the transcription sites of the *orbH* and *orbI* promoters experimentally determined
804 in this study. PvdS-dependent promoters have been previously identified (1,2) and the
805 highlighted core elements shown here are based on DNA sequence conservation rather than a
806 functional analysis. Sequences shown, and the associated gene loci, are derived from *B.*
807 *cenocepacia* J2315 (promoter sequences from -40 to +11 are the same as in strains 715j and
808 H111) and *P. aeruginosa* PAO1. The experimentally determined transcription start sites for the
809 P_{orbH}, P_{orbI}, P_{pvdA} and P_{pvdF} promoters are underlined (1,3; this study).
810
811 1. Wilson,M.J., McMorran,B.J. and Lamont,I.L. (2001) Analysis of promoters
812 recognized by PvdS, an extracytoplasmic-function sigma factor protein from
813 *Pseudomonas aeruginosa*. *J. Bacteriol.*, **183**, 2151-2155.
814 2. Ochsner,U.A., Wilderman,P.J., Vasil,A.I. and Vasil,M.L. (2002) GeneChip®
815 expression analysis of the iron starvation response in *Pseudomonas aeruginosa*:
816 identification of novel pyoverdine biosynthesis genes. *Mol. Microbiol.*, **45**, 1277- 1287.
817 3. Leoni,L., Ciervo,A., Orsi,N. and Visca,P. (1996) Iron-regulated transcription of the
818 *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity.
819 *J. Bacteriol.*, **178**, 2299-2313.
820
821 **Figure S2. Activity of minimal OrbS-dependent promoters in *B. cenocepacia*.** β-
822 galactosidase activities were measured in *B. cenocepacia* 715j cells harbouring pKAGd4-$P_{orbHds6}$,
823 pKAGd4-$P_{orbEds1}$ or pKAGd4-$P_{orbIds1}$ following growth in LB containing chloramphenicol under
824 iron limiting conditions. Activities shown have been 'corrected' by subtracting the activity in
825 same strain containing pKAGd4 assayed under identical conditions. All assays were performed
826 on three independent cultures, with technical duplicates for each, and bars represent the means

827 ± standard deviation. Statistical significance between promoter activity values were determined
828 using a one-way ANOVA. ****, p<0.0001.
829
830 **Figure S3. Nucleotide sequence alignment of predicted OrbS-dependent promoters from**
831 **representative members of the Bcc.** A. Ornibactin operon promoters. Promoter sequences
832 which were not identified using the TAAA(A/T)A(A/T)$_2$(N)$_{13}$CGTC search string are indicated
833 by a red arrow. B. Candidate P_{fpr} promoters. P_{fpr} is located upstream of the BCAL0536 gene in
834 *B. cenocepacia* J2315 and at the corresponding location in the other members of the Bcc shown
835 here. -35 and -10 core element sequences are highlighted in blue in the consensus sequence. In A
836 and B, included species are *B. ambifaria* (AMMD and MC40-6), *B. cenocepacia* (J2315,
837 HI2424, AU1054, MC0-3 and H111), *B. lata* (383), *B. multivorans* (ATCC 17616) and *B.*
838 *vietnamiensis* (G4). Sequences from -37 to +5 relative to the TSS are shown and the consensus
839 base at each position occurs in at least 60% of the aligned sequences.
840
841 **Figure S4. Transcriptional analysis of candidate OrbS-dependent promoters.** A. Alignment
842 of *B. cenocepacia* candidate OrbS-dependent promoter sequences with P_{orbH}. Bases are
843 highlighted as described in Fig. 1D. B. OrbS-dependent activity of P_{fpr} and P_{ureA} in *E. coli*. β-
844 galactosidase assays were performed on MC1061 harbouring pKAGd4-P$_{fpr}$ and pKAGd4-P$_{ureA}$ in
845 combination with either pBBR2-orbS (+) or pBBR1MCS-2 (-) following growth in iron-limiting
846 LB medium. The $P_{orbHds6}$ promoter was included for comparison. C. Effect of OrbS and iron on
847 P_{fpr} and P_{ureA} activity in *B. cenocepacia*. β-galactosidase assays were performed on *B.*
848 *cenocepacia* 715j and 715j-orbS::Tp containing pKAGd4-P$_{ureA}$ or pKAGd4-P$_{fpr}$ following
849 growth in LB under iron replete and iron limiting conditions. The $P_{orbHds6}$ promoter was included
850 for comparison. In B and C activities are expressed in Miller units following subtraction of the

activity measured in the same strain harbouring pKAGd4 and pBBR2-orbS (for data presented in B) or pKAGd4 (for data presented in C) assayed under identical conditions. All assays were performed on three independent cultures, with technical duplicates for each, and bars represent the means ± standard deviation. Statistical significance between promoter activity values were determined using a one-way ANOVA in B and a two-way ANOVA in C. ****, $p<0.0001$; ns, not significant.

Figure S5. RT-PCR analysis of *orbI*, *fpr* and *rpoD* gene expression in *B. cenocepacia* wild-type and *orbS* strains growing under iron replete and iron limiting conditions. Template cDNA was generated by reverse transcription from mRNA isolated from (A) strains 715j (WT) and 715j-orbS::Tp (orbS::Tp) and (B) strains H111 (WT) and H111ΔorbS (ΔorbS) growing under iron replete (+Fe) and iron limiting (-Fe) conditions, and used as a template for PCR with pairs of primers specific for *orbI* (142 bp amplicon), *fpr* (340 bp amplicon) and *rpoD* (117 bp amplicon), as indicated at the top of each gel. PCRs containing genomic DNA as template (gDNA) or with no template (-) were included as controls for each cDNA template-primer pair combination. White vertical arrows highlight products corresponding to OrbS-dependent *orbI* expression, which only occurs in the wild-type strain growing under iron limiting conditions. Control PCR reactions to confirm the absence of contaminating genomic DNA in RNA samples used mRNA as template isolated from strains 715j and 715j-orbS::Tp (C) and strains H111 and H111ΔorbS (D).

Table 1. Determination of the minimal OrbS-dependent promoter

Promoter derivative	Upstream endpoint[a]	Promoter sequence[b]	Downstream endpoint[a]	β-galactosidase activity (Miller units)[c]	
				B. cenocepacia 715j	*E. coli* MC1061
P_{orbH}	-348	...GCGGCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAGCGGCCGAATCCGCCGCTTCGCCTCCTTCA...	+67	4,606 (105)	247 (17)
$P_{orbHds2}$	-40	GCGGCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAGCGGCCGAATCCGCCGCTTCGCCTCCTTCA	+34	6,141 (449)	2,873 (75)
$P_{orbHds3}$	-40	GCGGCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAGCGGCCGAATCCGCCGCTTC	+24	ND	3,903 (167)
$P_{orbHds4}$	-40	GCGGCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAGCGGCCGAAT	+14	ND	4,038 (167)
$P_{orbHds5}$	-40	GCGGCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	+5	ND	3,471 (114)
$P_{orbHds6}$	-37	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	+5	7,036 (590)	3,122 (115)
$P_{orbHds1}$	-37	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATC	-5	54 (15)	424 (13)

[a] Distances are relative to the experimentally determined transcription start site.

[b] Underlined bases correspond to the core promoter elements.

[c] Assays were performed on the indicated strains harbouring a pKAGd4 *lacZ* reporter plasmid in which the indicated promoter fragments were cloned. *E. coli* MC1061 also contained pBBR2-orbS. Bacteria were grown in LB under iron limiting conditions. Values were 'corrected' by subtracting the background activity in cells harbouring pBBR2-orbS and the 'empty' *lacZ* reporter plasmid pKAGd4. All assays were performed on three independent cultures, with technical duplicates for each, and values represent the means (± standard deviation in parentheses).

Table 2. Effect of nucleotide substitutions within the G.C spacer and the A+G block on utilisation of the P_{orbH} promoter by OrbS and PvdS in *E. coli*.

Promoter derivative[a]	Promoter sequence[b]	Promoter activity (%)[c]	
		+ OrbS	+ PvdS
$P_{orbHds6}$ (WT)	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	100.0 (5.6)	100.0 (1.1)
$P_{orbHds1}$	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATC**G**GA**TCCT**A**A**	12.9 (0.4)	31.0 (2.9)
$P_{orbHdsAGB1}$	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATC**CTCACTTCT**	1.3 (0.2)	0.8 (0.1)
$P_{orbHdsAGBtri1}$	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATC**CTC**CAGGAG	141.3 (8.6)	91.5 (4.6)
$P_{orbHdsAGBtri2}$	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGA**ACT**GAG	82.7 (2.6)	68.4 (4.5)
$P_{orbHdsAGBtri3}$	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAG**TCT**	57.9 (2.3)	85.5 (3.7)
$P_{orbHdsGCS1}$	GCGGTAAAAAAAC**TATAATTAA**AACCGTCTATCAGACAGGAG	72.0 (5.1)	62.9 (3.5)
$P_{orbHdsGCS2}$	GCGGTAAAAAAACG**A**G**A**C**T**G**A**CAACCGTCTATCAGACAGGAG	100.7 (2.0)	67.0 (0.3)
$P_{orbHdsGCS+1}$	GCGGTAAAAAAACGCG**G**CCGGCCAACCGTCTATCAGACAGGAG	59.3 (5.8)	31.0 (3.3)
$P_{orbHdsGCS+2}$	GCGGTAAAAAAACGCG**GG**CCGGCCAACCGTCTATCAGACAGGAG	4.2 (1.2)	6.2 (0.2)
$P_{orbHdsGCS-1}$	GCGGTAAAAAAACGCG**-**CGGCCAACCGTCTATCAGACAGGAG	3.3 (0.3)	39.1 (7.1)
$P_{orbHdsGCS-2}$	GCGGTAAAAAAACGCG**--**GGCCAACCGTCTATCAGACAGGAG	2.6 (2.5)	3.0 (0.5)

[a]The indicated promoters were cloned in pKAGd4 and introduced into MC1061 harbouring pBBR2-orbS or pBBR2-pvdS.
[b]All promoters extend from -37 to +5 except for $P_{orbHds1}$ which has a downstream endpoint at -5. Base substitutions and insertions are shown in bold red font. The location of deleted bases is shown by bold red dashes. Core promoter element sequences and the TSS are underlined.
[c]β-galactosidase activity measurements were performed on cells growing under iron limited conditions and activity values were adjusted by subtracting the activity in cells containing the 'empty' pKAGd4 vector together with pBBR2-orbS or pBBR2-pvdS as appropriate, and the obtained values are expressed relative to the wild-type promoter sequence ($P_{orbHds6}$). All assays were performed on three independent cultures, with technical duplicates for each, and values represent the means (± standard deviation in parentheses). 100% activity = 3,163.6 Miller units in cells containing OrbS and 21,534.7 Miller units in cells containing PvdS.
.

Table 3. Effect of dinucleotide substitutions within the -35 element and extended A tract on utilisation of the P_{orbH} promoter by OrbS in *E. coli*.

Promoter derivative[a]	Promoter sequence[b]	Promoter activity[c]	
		Miller units	% relative to control
$P_{orbHds6}$ (WT)	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	5510 (19)	100
$P_{orbHds-36a-35t}$	G**at**GTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	5448 (179)	99
$P_{orbHds-35t-34t}$	GC**tt**TAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	3587 (68)	65
$P_{orbHds-34t-33g}$	GCG**tg**AAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	719 (10)	12
$P_{orbHds-33g-32c}$	GCGG**gc**AAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	135 (5)	1
$P_{orbHds-32c-31c}$	GCGGT**cc**AAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	118 (2)	1
$P_{orbHds-31c-30c}$	GCGGTA**cc**AAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	117 (4)	1
$P_{orbHds-30c-29c}$	GCGGTAA**cc**AAACGCGCCGGCCAACCGTCTATCAGACAGGAG	619 (32)	10
$P_{orbHds-29c-28c}$	GCGGTAAA**cc**AACGCGCCGGCCAACCGTCTATCAGACAGGAG	802 (18)	13
$P_{orbHds-28c-27c}$	GCGGTAAAA**cc**ACGCGCCGGCCAACCGTCTATCAGACAGGAG	1247 (7)	22
$P_{orbHds-27c-26c}$	GCGGTAAAAA**cc**CGCGCCGGCCAACCGTCTATCAGACAGGAG	4117 (50)	74
$P_{orbHds-26c-25a}$	GCGGTAAAAAA**ca**GCGCCGGCCAACCGTCTATCAGACAGGAG	3949 (69)	71
$P_{orbHds-25a-24t}$	GCGGTAAAAAAA**at**CGCCGGCCAACCGTCTATCAGACAGGAG	5746 (99)	104

[a]The indicated promoters were cloned in pKAGd4 and introduced into MC1061 harbouring pBBR2-orbS.

[b]Bases in bold red font indicate the dinucleotide substitutions introduced into $P_{orbHds6}$. -35 and -10 core element sequences are underlined.

[c]β-galactosidase activity measurements were performed on cells growing in LB under iron limited conditions and the obtained activity values (in Miller units) were adjusted by subtracting the measured activity in cells containing pBBR2-orbS and the 'empty' pKAGd4 vector. Adjusted activities are also expressed relative to the activity of the wild-type promoter ($P_{orbHds6}$). All assays were performed on three independent cultures, with technical duplicates for each, and values represent the means (± standard deviation in parentheses).

Table 4. Effect of extending the -35 element A tract on utilisation of the *P. aeruginosa* P_{pvdE} promoter by OrbS and PvdS in *E. coli*.

Promoter derivative[a]	Promoter sequence[b]	Promoter activity (%)[c]	
		+ OrbS	+ PvdS
$P_{orbHds6}$	GCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	100.0 (3.7)	100.0 (1.1)
$P_{pvdEds1}$	**C**CG**C**TAAA**T**A**CCG**G**GCATCCTGCTT**CGTCT**G**T**C**T**G**CA**AGGAG	1.5 (0.2)	95.7 (9.6)
$P_{pvdEds7}$	**C**CG**C**TAAAAAAA**GGGCATCCTGCTT**CGTCT**G**T**C**T**G**CA**AGGAG	13.7 (0.8)	64.7 (1.6)

[a]The indicated promoters were cloned in pKAGd4 and introduced into MC1061 harbouring pBBR2-orbS or pBBR2-pvdS. $P_{orbHds6}$ and $P_{pvdEds1}$ are wild-type with respect to P_{orbH} and P_{pvdE}, respectively. $P_{pvdEds7}$ corresponds to $P_{pvdEds1}$ with an extended A-tract located downstream of the promoter -35 element.

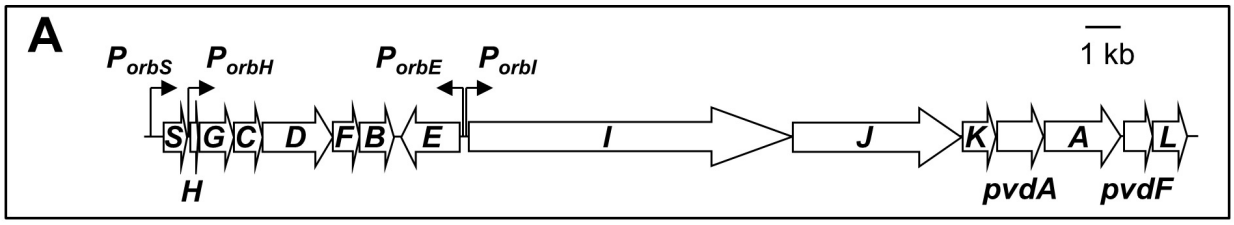
[b]Bases in bold red font deviate from the sequence of $P_{orbHds6}$. -35 and -10 core element sequences and the base that initiates transcription at P_{orbH} in the presence of OrbS are underlined.

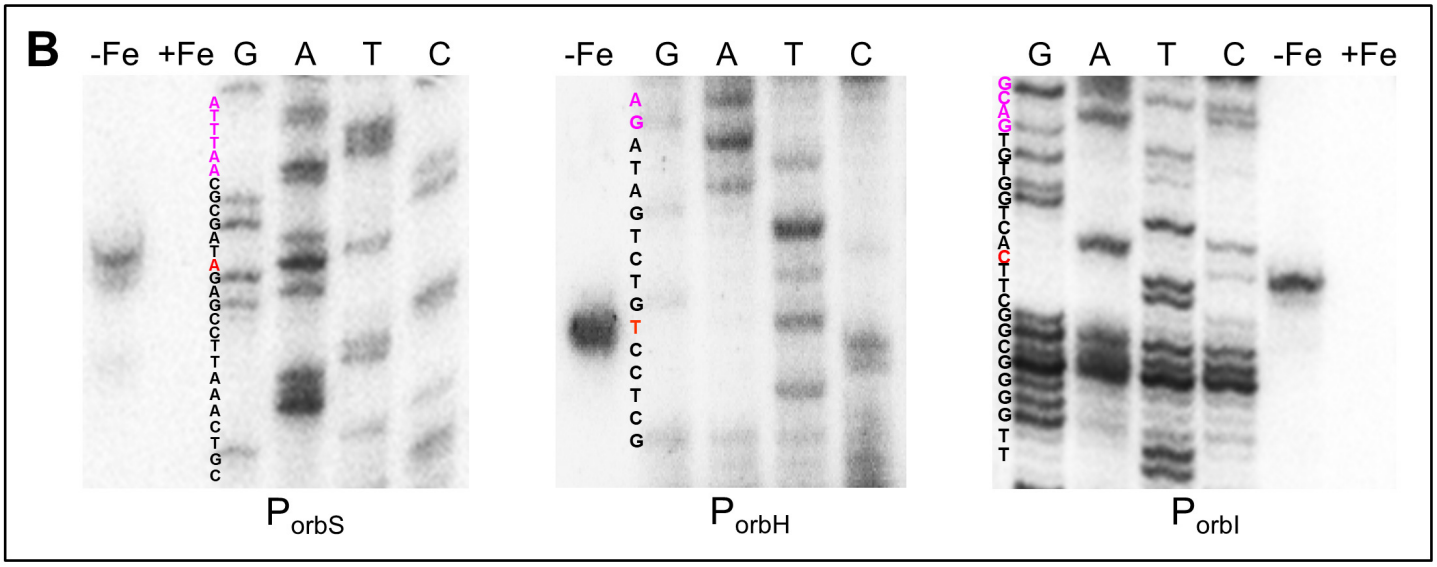
[c]β-galactosidase activity measurements were performed on cells growing in LB under iron limiting conditions and activity measurements were adjusted by subtracting the activity in cells containing the 'empty' pKAGd4 vector together with pBBR2-orbS or pBBR2-pvdS as appropriate, and the obtained values are expressed relative to the wild-type promoter sequence ($P_{orbHds6}$). All assays were performed on three independent cultures, with technical duplicates for each, and values represent the means (± standard deviation in parentheses).100% activity = 3,122 Miller units in MC1061 cells containing OrbS and 22,200 Miller units in cells containing PvdS.

973 **Table 5. Bacterial strains.**

Strain	Genotype/Description[a]	Source or reference
B. cenocepacia		
715j	CF isolate, prototroph (Orb$^+$ Pch$^+$)	(45,46)
715j-orbS::Tp	715j with *dfrB2* cassette inserted in *orbS* (Orb$^-$ Pch$^+$)	(30)
H111	CF isolate, prototroph (Orb$^+$ Pch$^+$)	(47)
H111ΔorbS	H111 containing an in-frame deletion within *orbS* (Orb$^-$ Pch$^+$)	A.B, K.A. and M.T., in preparation
P. aeruginosa		
PAO1	Wild-type, prototroph	(48,49)
PAO1-pvdS::Gm	PAO1 containing a Gm^R cassette inserted in place of a 460 bp segment of *pvdS*	(50)
E. coli		
JM83	F$^-$ *ara* Δ(*lac-proAB*) *rpsL* ϕ80d*lacZΔM15* (Sm^R)	(51)
MC1061	*hsdR araD139* Δ(*ara-leu*)7697 Δ*lacX74 galU galK rpsL* (Sm^R)	(52)
S17-1	*thi proA hsdR recA* RP4-2-*tet*::Mu-1 *kan*::Tn*7* integrant (Tp^R Sm^R)	(53)

974 [a]Gm^R, gentamicin resistant; Sm^R, streptomycin resistant; Tp^R, trimethoprim resistant; Orb, ornibactin phenotype;
975 Pch, pyochelin phenotype.





C

P orbS

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P orbH

CGGTCGCGCGCGGCGGCGGTAAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAGCGG

CCGAATCCGCCGCTTCGCCTCCTTCAACCGCCCAGCGATTTCCGATCATGACGCAAGCCCC

P orbI

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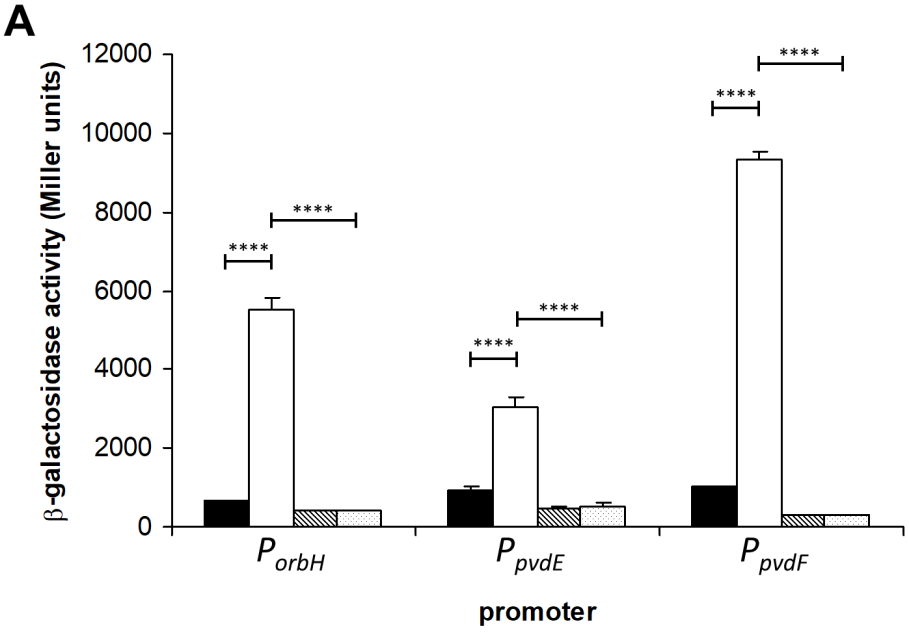
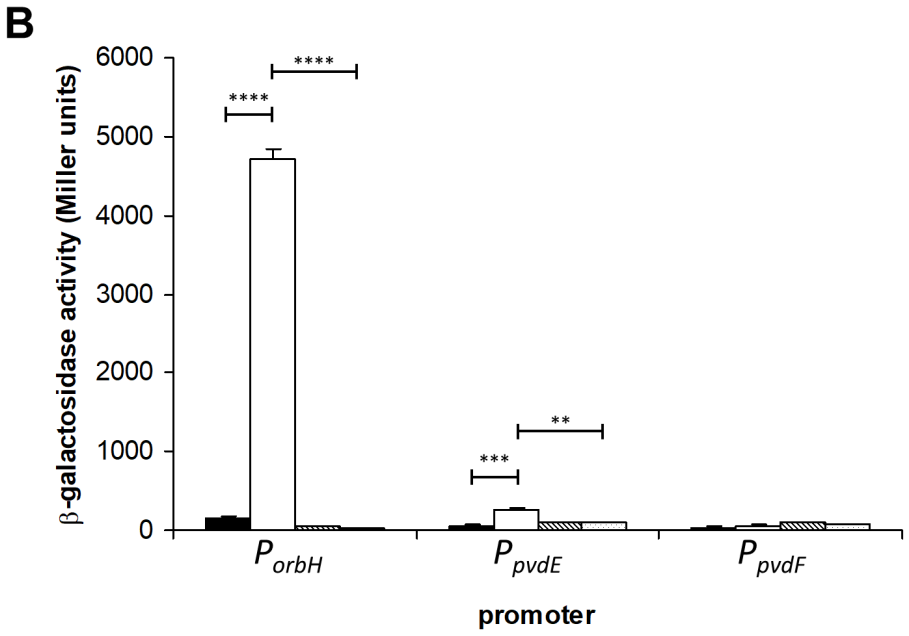

A
β-galactosidase activity (Miller units)
12000
10000
8000
6000
4000
2000
0
****
****
****
****
****
****
P_{orbH}
P_{pvdE}
P_{pvdF}
promoter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β-galactosidase activity (Miller units)
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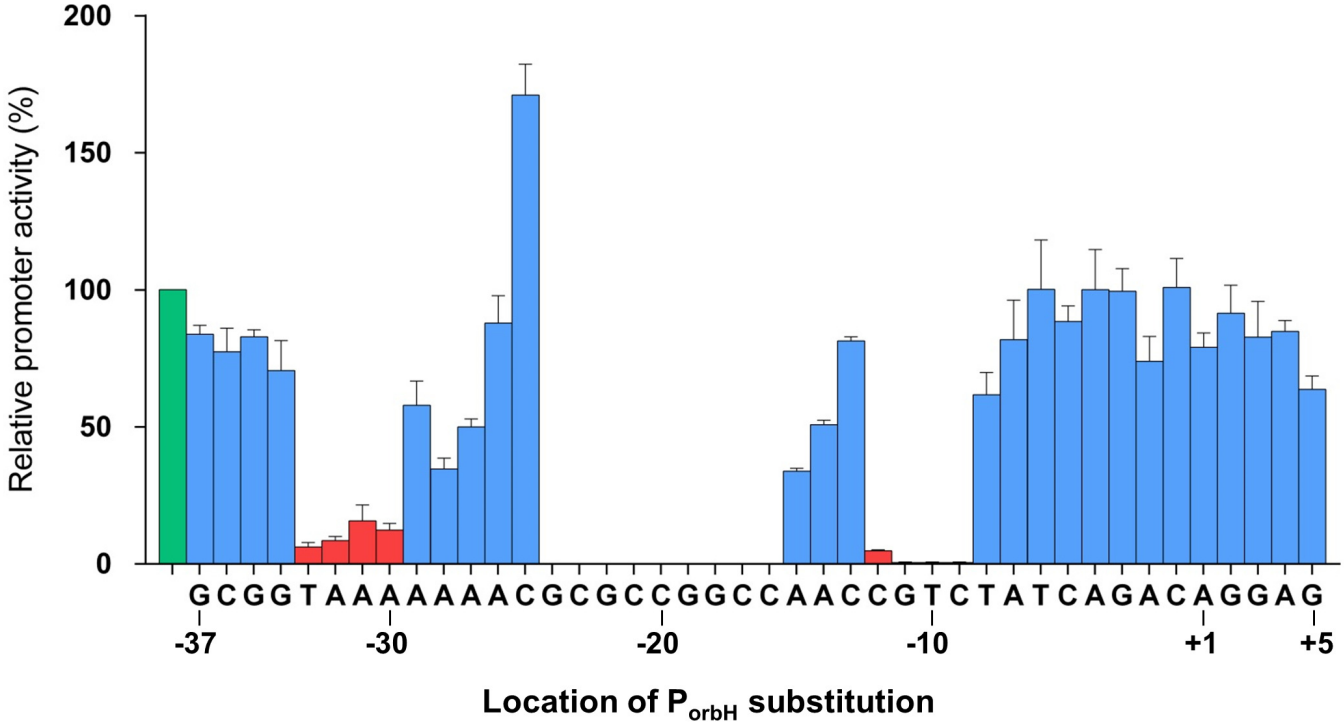
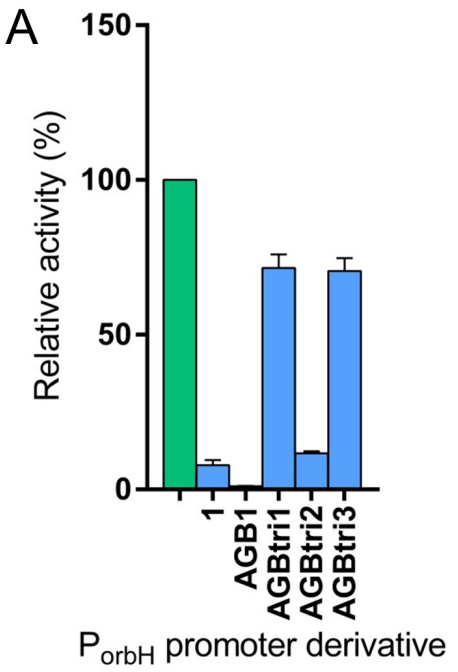
5000
4000
3000
2000
1000
0
****
****
***
**
P_{orbH}
P_{pvdE}
P_{pvdF}
promoter


200
150
100
50
0
Relative promoter activity (%)
G C G G T A A A A A A A C G C G C C G G C C A A C C G T C T A T C A G A C A G G A G
-37
-30
-20
-10
+1
+5
Location of P orbH substitution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100
50
0
Relative activity (%)
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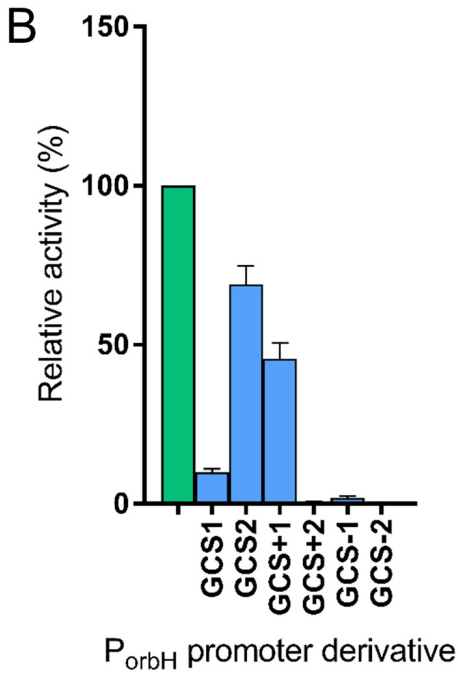
AGBtri1
AGBtri2
AGBtri3
PorbH promoter derivative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100
50
0
Relative activity (%)
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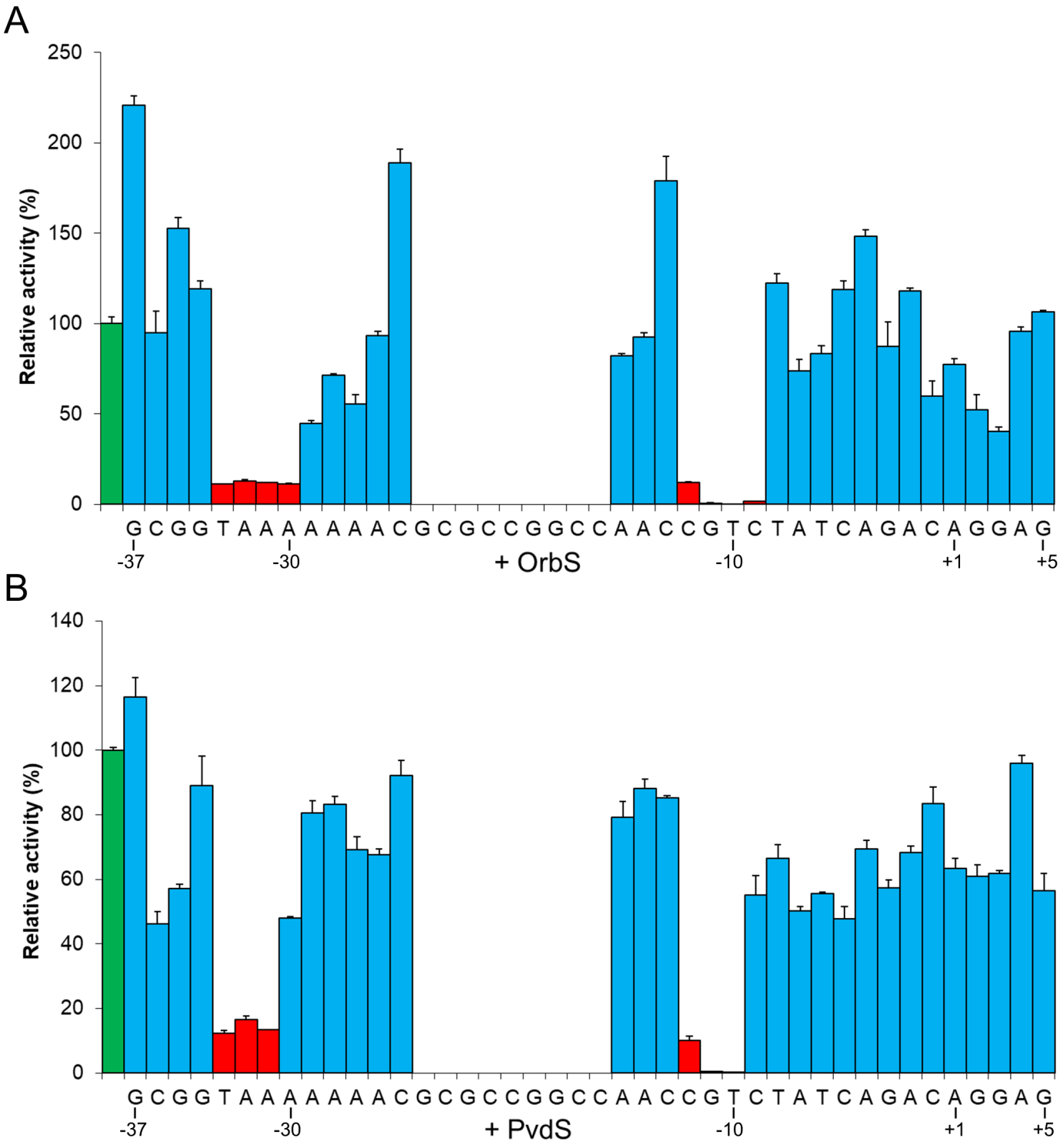
GCS2
GCS+1
GCS+2
GCS-1
GCS-2
PorbH promoter derivative


A
Relative activity (%)
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200
150
100
50
0
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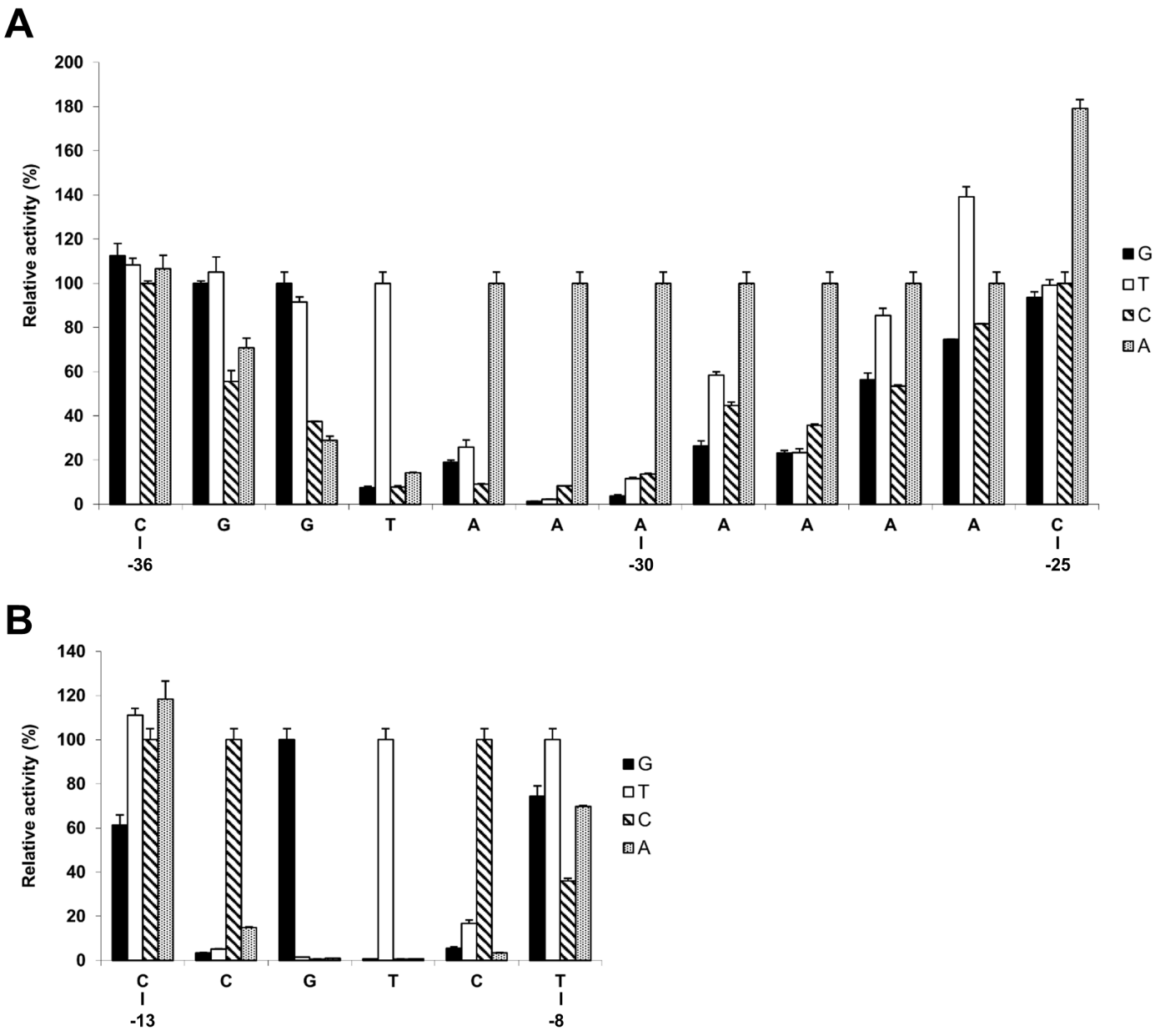
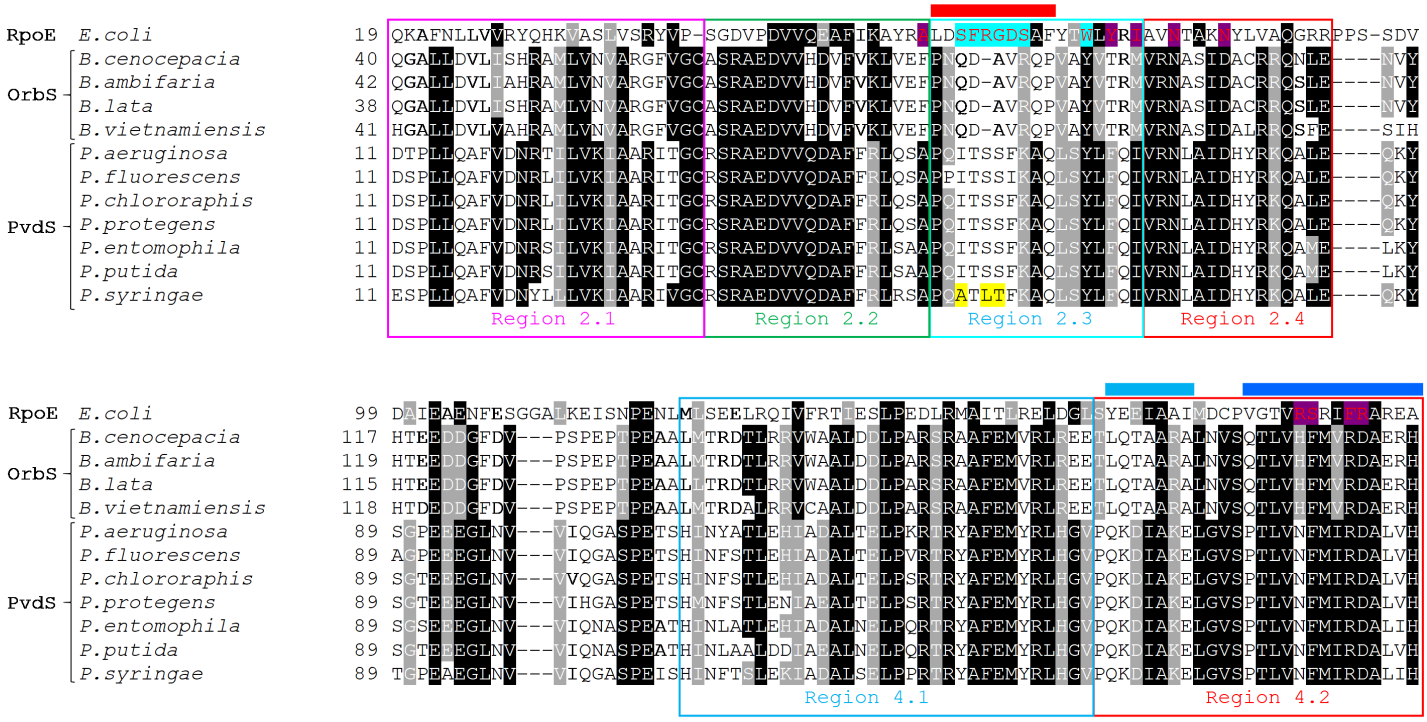
-37
-30
+ OrbS
-10
+1
+5
B
Relative activity (%)
140
120
100
80
60
40
20
0
G C G G T A A A A A A A A C G C G C C G G C C A A C C G T C T A T C A G A C A G G A G
-37
-30
+ PvdS
-10
+1
+5


A
Relative activity (%)
200
180
160
140
120
100
80
60
40
20
0
G
T
C
A
C G G T A A A A A A A C
-36
-30
-25
B
Relative activity (%)
140
120
100
80
60
40
20
0
G
T
C
A
C C G T C T
-13
-8


RpoE
E.coli
OrbS
B.cenocepacia
B.ambifaria
B.lata
B.vietnamiensis
PvdS
P.aeruginosa
P.fluorescens
P.chlororaphis
P.protegens
P.entomophila
P.putida
P.syringae
Region 2.1
Region 2.2
Region 2.3
Region 2.4
Region 4.1
Region 4.2