Distinct Modes of Promoter Recognition by Two Iron Starvation σ Factors with Overlapping Promoter Specificities

Kirsty Agnoli,^{a*} Sayali S. Haldipurkar,^a Yingzhi Tang,^{a*} Aaron T. Butt,^a Mark S. Thomas^a

^aDepartment of Infection, Immunity and Cardiovascular Disease, Faculty of Medicine, Dentistry and Health, University of Sheffield, Sheffield, United Kingdom

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 *Burkholderia 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 *Pseudomonas aeruginosa* PvdS can serve OrbS-dependent promoters, OrbS cannot utilize 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 the sigma binding elements are required for efficient promoter utilization 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 σ factors through modulation of the local DNA architecture.

IMPORTANCE ECF σ 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 recognized by the ECF σ factor OrbS. Furthermore, we demonstrate that efficient promoter utilization by this σ 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 σ factors.

KEYWORDS *Burkholderia cenocepacia*, OrbS, *Pseudomonas aeruginosa*, PvdS, gene regulation, promoter recognition, siderophores, sigma factors

Most bacterial σ factors are related to the primary (or “housekeeping”) σ factor, σ^{70} , which is responsible for directing RNA polymerase (RNAP) to the majority of promoters (1, 2). σ^{70} is organized 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 to 2.4 and includes a long nonconserved region (NCR) that connects region 1.2 to region 2.1, and 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

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Address correspondence to Mark S. Thomas, m.s.thomas@shef.ac.uk.

* Present address: Kirsty Agnoli, Department of Microbiology, Institute of Plant Biology, University of Zürich, Zürich, Switzerland; Yingzhi Tang, Cambridge Systems Biology Centre and Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom.

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the RNAP-promoter open complex. Structural analysis indicates that regions 1.2 and 2.1 to 2.4 interact with the -10 region of the nontemplate strand (positions -12 to -7), 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 nontemplate 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 recognize specific sequences located approximately 35 and 10 bp upstream from the transcription start site (TSS). 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 nontemplate 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-characterized IS σ factor is PvdS of *Pseudomonas 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 internalized by the bacterium via the TonB-dependent transporter OrbA (28, 29). Biosynthesis and transport of ornibactin by the Bcc member *B. cenocepacia* are 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 characterization of the OrbS-dependent ornibactin gene promoters and to search for additional promoters that may be served by OrbS. We found that OrbS recognizes promoter core element sequences very similar to those present at PvdS-dependent promoters but that OrbS has more stringent DNA sequence requirements for optimal promoter utilization, including an A-tract located downstream from the -35 promoter element. We also found no evidence for the existence of OrbS-dependent promoters outside the ornibactin gene cluster.

RESULTS

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

Three *orbS*-specific cDNA products were generated that indicated mRNA 5' endpoints corresponding to sites located 31, 30, and 24 bp upstream of the *orbS* translation initiation codon (Fig. 1B). Given the greater abundance of the longest cDNA product, the initiation site for the majority of *orbS* transcription is located 31 bp upstream of the *orbS* start codon (Fig. 1C). As the second longest cDNA product is only one nucleotide

FIG 1 Location of OrbS-dependent promoters in the *B. cenocepacia* ornibactin gene cluster. (A) Gene organization 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 (e.g., “G” represents *orbG*). Genes correspond to QU43_RS44960 (BCAL1688) to QU43_RS45030 (BCAL1702) in the J2315 strain and I35_RS08005 (I35_1599) to 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. Radiolabeled 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 harboring 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. (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 rectangles formed by dashed lines. For reference, the translation initiation codons are shown in gray and the Shine-Dalgarno sequences are in bold font and underlined.

shorter than the longest cDNA product, transcription may also initiate at the next downstream nucleotide but with lower efficiency. The shortest product is likely to be the result of premature termination of reverse transcription due to 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 (Fig. 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 observed only 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: CGGTAAAAA 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 initiation 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 (see Fig. S1 in the supplemental material). At all three promoters the spacer region consists of a 4- to 5-bp A-T-rich sequence following the TAAA motif, which is, in turn, followed by a 10- to 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.

The sequence that is conserved at all three OrbS-dependent promoters is very similar to the consensus promoter recognized by *P. aeruginosa* PvdS, i.e., TAAATN₁₆CGT (19, 20, 31). Therefore, we examined the possibility that OrbS and *P. aeruginosa* PvdS are functionally interchangeable by using reporter fusions to measure the activities of P_{orbH} and two PvdS-dependent promoters in *P. aeruginosa* and *B. cenocepacia*. The results show that the activities of the PvdS-dependent *pvdF* (PA2396) and *pvdE* (PA2397) promoters (P_{pvdE} and P_{pvdF}) and the OrbS-dependent P_{orbH} promoter were comparable in wild-type *P. aeruginosa* grown under iron-limited conditions (Fig. 2A). When cells were grown under iron-replete conditions to allow for repression of chromosomal *pvdS*, the activities of all three promoters were decreased. In the *pvdS* mutant, expression from all three promoters was lower than observed in cells grown under iron-replete conditions in the presence of the σ factor. These results show that PvdS can efficiently recognize an OrbS-dependent promoter, even though the promoter lacks the highly conserved thymine base that is present at position 5 of the -35 element in PvdS-dependent promoters.

As expected, P_{orbH} was highly active in wild-type *B. cenocepacia* cells grown under iron-limiting conditions (Fig. 2B). In contrast, P_{pvdE} was nearly 20-fold less active than P_{orbH} , while P_{pvdF} showed negligible activity. The P_{pvdE} promoter contains a cytidine residue following the -10 element CGT motif which maximizes the match to the OrbS-dependent promoter -10 region (Fig. S1) and therefore may be expected to respond to OrbS and iron. Accordingly, the activity of P_{pvdE} was further decreased when cells were grown in the presence of iron, and the promoter was also less active in the *orbS* mutant strain. Thus, OrbS can only very inefficiently utilize a PvdS-dependent promoter that contains matches to both conserved motifs present at OrbS-dependent promoters. These results suggest that for efficient recognition of its target promoters, OrbS requires DNA sequence features that reside outside the core -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. *Escherichia coli* MC1061 was trans-

FIG 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 data represent the means $\pm$ SDs. Statistical significance was determined by performing one-tailed *t* tests. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

formed 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 10-fold 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

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	
<i>P_{OrbH}</i>	−348	...GCGGCGTTAAAAAACGCGCGCGGCCAACCGTCTATATCAGACAGGAGCGGCCGGAATCCGCGCTTCGCCCTCTTCA...	+67	<i>B. cenocepacia</i> 715j	<i>E. coli</i> MC1061
<i>P_{OrbHds2}</i>	−40	GCGGCGTTAAAAAACGCGCGCGGCCAACCGTCTATATCAGACAGGAGCGGCCGGAATCCGCGCTTCGCCCTCTTCA	+34	4,606 (105)	247 (17)
<i>P_{OrbHds3}</i>	−40	GCGGCGTTAAAAAACGCGCGCGGCCAACCGTCTATATCAGACAGGAGCGGCCGGAATCCGCGCTTCGCCCTCTTCA	+24	6,141 (449)	2,873 (75)
<i>P_{OrbHds4}</i>	−40	GCGGCGTTAAAAAACGCGCGCGGCCAACCGTCTATATCAGACAGGAGCGGCCGGAAT	+14	ND	3,903 (167)
<i>P_{OrbHds5}</i>	−40	GCGGCGTTAAAAAACGCGCGCGGCCAACCGTCTATATCAGACAGGAG	+5	ND	4,038 (167)
<i>P_{OrbHds6}</i>	−37	GCGGTTAAAAAACGCGCGCGGCCAACCGTCTATATCAGACAGGAG	+5	7,036 (590)	3,471 (114)
<i>P_{OrbHds1}</i>	−37	GCGGTTAAAAAACGCGCGCGGCCAACCGTCTATATC	−5	54 (15)	3,122 (115)
					424 (13)

^aDistances are relative to the experimentally determined transcription start site.

^bUnderlined bases correspond to the core promoter elements.

^cAssays were performed on the indicated strains harboring a pKAGd4 *lacZ* reporter plasmid in which the indicated promoter fragments were cloned. *E. coli* MC1061 also contained pBBR2-orbS. *B. cenocepacia* was grown in M9 salts medium, whereas *E. coli* was grown in LB medium, in both cases under iron-limiting conditions. Values were corrected by subtracting the background activity in cells harboring pBBR2-orbS and the empty *lacZ* reporter plasmid pKAGd4. All assays were performed on three independent cultures, with technical duplicates for each, and values shown are the means (with standard deviations in parentheses). ND, not determined.

FIG 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 medium under iron-limiting conditions. Activities represented by red bars are $\leq 15\%$ 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 harboring pKAGd4 assayed under identical conditions and then expressed relative to the activity in cells harboring pKAGd4- $P_{orbHds6}$ ($100\% = 1,383.6$ Miller units). All assays were performed on three cultures, with technical duplicates for each, and data represent the means $\pm$ SDs. The DNA sequence of $P_{orbHds6}$ is shown below the x axis.

located between positions -5 and $+6$ 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 with *E. coli*, with the exception of the longest P_{orbH} construct, which was much more active in *B. cenocepacia*.

The activity of the minimal $P_{orbHds6}$ promoter was compared to those of P_{orbI} and P_{orbE} promoter fragments with the same upstream and downstream endpoints relative to the TSS (P_{orbI} and P_{orbE}). The results showed that P_{orbI} was twice as active as $P_{orbHds6}$ when measured in *B. cenocepacia*, whereas P_{orbE} exhibited approximately 20% of the activity of $P_{orbHds6}$ (Fig. S2). The weakest promoter, P_{orbE} , is distinguished by the absence of a long A-tract extending from the -35 element into the spacer region in the nontemplate strand (Fig. S1).

Identification of functionally important bases for promoter utilization by OrbS.

To establish the DNA sequence requirements for promoter recognition by OrbS, single base pair substitutions were introduced into the $P_{orbHds6}$ promoter, and their effects on promoter activity were assessed. The single base pair substitutions were introduced at each position in the promoter (-37 to $+5$) except residues -24 to -16 located within the spacer region. Substitutions at -33 to -30 and -12 to -9 resulted in very strong inhibitory effects on promoter activity (Fig. 3). These positions correspond to the conserved TAAA and CGTC motifs and confirm their function as the -35 and -10 elements. The effects of substitutions at positions -11 , -10 , and -9 of the -10 element were particularly severe, effectively abolishing measurable promoter activity in *B. cenocepacia*. Substitutions at some positions within the spacer region (i.e., positions -28 and -27 within the A-tract and positions -15 and -14) exerted quite marked negative effects on promoter activity ($\geq 50\%$ decrease). Interestingly, substitution of the C residue at position -25 , which results in extension of the A-tract by an additional base, caused a large ($\sim 70\%$) increase in promoter activity.

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

FIG 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 medium under iron-limiting conditions. Activities have been background corrected by subtracting the activity of the same strain harboring pKAGd4 assayed under identical conditions and then expressed relative to the activity in *B. cenocepacia* harboring 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 data represent the means $\pm$ SDs. Statistical significance between the activities of the test promoters and that of $P_{orbHds6}$ was determined using a one-way analysis of variance (ANOVA), and P values of <0.0001 were obtained in each case.

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 replacement of all nine base pairs (i.e., $P_{orbHdsAGB1}$) completely abolished promoter activity (Fig. 4A). The more severe

effect of the “en bloc” replacement 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, nonoverlapping trinucleotide substitutions that spanned the entire region were introduced. Simultaneous substitution of bases at positions -1 , $+1$, and $+2$ ($P_{orbHdsAGBtri2}$) resulted in a large decrease in promoter activity ($\sim 90\%$ decrease), whereas replacement of the flanking nucleotide triads exerted much less severe effects ($\leq 30\%$ decrease in activity) (Fig. 4A). As the bases that constitute the central triplet are also replaced in the $P_{orbHds1}$ and $P_{orbHdsAGB1}$ derivatives, the simplest explanation is that replacement 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- or 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 replaced all nine base pairs 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 position -33 to -14 , we also constructed a promoter derivative in which only every alternate base in the G-C spacer block was replaced. The results showed that substitution of the entire G-C tract ($P_{orbHdsGCS1}$) caused a sharp decrease ($\sim 90\%$) in promoter activity, whereas less severe modification of this region ($P_{orbHdsGCS2}$) resulted in a smaller decrease ($\sim 30\%$) 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 an $\sim 50\%$ 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_{orbHr} we tested its ability to utilize 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 for *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. Production 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 than 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 recognized by OrbS had been replaced (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 results for OrbS-dependent transcription qualitatively similar to those obtained with *B. cenocepacia* (compare Fig. 4B and Table 2). In contrast, PvdS was able to moderately tolerate a single base pair

FIG 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 the legend to Fig. 4) in combination with pBBR2-*orbS* (A) or pBBR2-*pvdS* (B). Bacteria were grown in LB medium under iron-limiting conditions. OrbS- and PvdS-dependent activities represented by red bars are $\leq 15\%$ 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 harboring pKAGd4 assayed under identical conditions and then expressed relative to the activity in cells harboring 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 data represent the means $\pm$ SDs. The DNA sequence of $P_{orbHds6}$ is shown below the x axis.

deletion in the spacer as well as a single base pair insertion (Table 2). Dinucleotide insertions or deletions in the spacer abrogated PvdS-dependent promoter activity.

Identification of a noncanonical 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 utilization 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 utilization by PvdS. Substitution of the entire A+G tract overlapping the TSS ($P_{orbHdsAGB1}$) exerted a strong negative effect on promoter utilization by OrbS in *E. coli*, similar to what was observed

TABLE 2 Effects of nucleotide substitutions within the G-C spacer and the A+G block on utilization 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)	GCGGTAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	100.0 (5.6)	100.0 (1.1)
$P_{orbHds1}$	GCGGTAAAAAACGCGCCGGCCAACCGTCTATC GGATCCTAA	12.9 (0.4)	31.0 (2.9)
$P_{orbHdsAGB1}$	GCGGTAAAAAACGCGCCGGCCAACCGTCTATC CTCACTTCT	1.3 (0.2)	0.8 (0.1)
$P_{orbHdsAGBtri1}$	GCGGTAAAAAACGCGCCGGCCAACCGTCTATC CTCCAGGAG	141.3 (8.6)	91.5 (4.6)
$P_{orbHdsAGBtri2}$	GCGGTAAAAAACGCGCCGGCCAACCGTCTATCAG ACTGAG	82.7 (2.6)	68.4 (4.5)
$P_{orbHdsAGBtri3}$	GCGGTAAAAAACGCGCCGGCCAACCGTCTATCAGACAG TCT	57.9 (2.3)	85.5 (3.7)
$P_{orbHdsGCS1}$	GCGGTAAAAAAC TATAATTAAA ACCGTCTATCAGACAGGAG	72.0 (5.1)	62.9 (3.5)
$P_{orbHdsGCS2}$	GCGGTAAAAAAC GAGACTGACA ACCGTCTATCAGACAGGAG	100.7 (2.0)	67.0 (0.3)
$P_{orbHdsGCS+1}$	GCGGTAAAAAACGCG GCCGGCCA ACCGTCTATCAGACAGGAG	59.3 (5.8)	31.0 (3.3)
$P_{orbHdsGCS+2}$	GCGGTAAAAAACGCG GGCCGGCCA ACCGTCTATCAGACAGGAG	4.2 (1.2)	6.2 (0.2)
$P_{orbHdsGCS-1}$	GCGGTAAAAAACGCG- CGGCCAACCGTCTATCAGACAGGAG	3.3 (0.3)	39.1 (7.1)
$P_{orbHdsGCS-2}$	GCGGTAAAAAACGCG- GGCCAACCGTCTATCAGACAGGAG	2.6 (2.5)	3.0 (0.5)

^aThe indicated promoters were cloned in pKAGd4 and introduced into MC1061 harboring pBBR2-*orbS* or pBBR2-*pvdS*. WT, wild type.

^bAll 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. The locations of deleted bases are shown by bold dashes. Core promoter element sequences and the TSS are underlined.

^c β -Galactosidase activity measurements were performed on cells growing in LB 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; 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 are the means (with standard deviations in parentheses). An activity of 100% = 3,163.6 Miller units in cells containing OrbS and 21,534.7 Miller units in cells containing PvdS.

in *B. cenocepacia* (compare Table 2 with Fig. 4A). Moreover, this alteration to the promoter also abrogated its utilization by PvdS in *E. coli*.

The A-tract in the nontemplate 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_{orbEr} , 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 analyzed 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

TABLE 3 Effects of dinucleotide substitutions within the -35 element and extended A-tract on utilization 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)	GCGGTAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	5,510 (19)	100
$P_{orbHds-36a-35t}$	GatGTAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	5,448 (179)	99
$P_{orbHds-35t-34t}$	GCttTAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	3,587 (68)	65
$P_{orbHds-34t-33g}$	GCGtgAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	719 (10)	12
$P_{orbHds-33g-32c}$	GCGGgcAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	135 (5)	1
$P_{orbHds-32c-31c}$	GCGGTccAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	118 (2)	1
$P_{orbHds-31c-30c}$	GCGGTAccAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	117 (4)	1
$P_{orbHds-30c-29c}$	GCGGTAAccAAAAAACGCGCCGGCCAACCGTCTATCAGACAGGAG	619 (32)	10
$P_{orbHds-29c-28c}$	GCGGTAAAaccAACGCGCCGGCCAACCGTCTATCAGACAGGAG	802 (18)	13
$P_{orbHds-28c-27c}$	GCGGTAAAAaccAACGCGCCGGCCAACCGTCTATCAGACAGGAG	1,247 (7)	22
$P_{orbHds-27c-26c}$	GCGGTAAAAaccCGCGCCGGCCAACCGTCTATCAGACAGGAG	4,117 (50)	74
$P_{orbHds-26c-25a}$	GCGGTAAAAaccAGCGCCGGCCAACCGTCTATCAGACAGGAG	3,949 (69)	71
$P_{orbHds-25a-24t}$	GCGGTAAAAaaatCGCGCCGGCCAACCGTCTATCAGACAGGAG	5,746 (99)	104

^aThe indicated promoters were cloned in pKAGd4 and introduced into MC1061 harboring pBBR2-*orbS*.

^bBases in lowercase are 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 shown are the means (with standard deviations in parentheses).

TABLE 4 Effects of extending the -35 element A tract on utilization 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
$P_{orbHds6}$	CGGTAATAAAAAACGCGCCGCCAACCGTCTATCAGACAGGAG	100.0 (3.7)	100.0 (1.1)
$P_{pvdEds1}$	CCGCTAAATACCGGGCATCCTGCTTCGTCTGTCTGCAAGGAG	1.5 (0.2)	95.7 (9.6)
$P_{pvdEds7}$	CCGCTAAATAAAAGGGCATCCTGCTTCGTCTGTCTGCAAGGAG	13.7 (0.8)	64.7 (1.6)

^aThe indicated promoters were cloned into pKAGd4 and introduced into MC1061 harboring 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.

^bBases in bold deviate from the sequence of $P_{orbHds6}$. -35 and -10 core element sequences and the base where transcription initiates 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; 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 are the means (with standard deviations in parentheses). An activity level of 100% = 3,122 Miller units in MC1061 cells containing OrbS and 22,200 Miller units in cells containing PvdS.

a single nucleotide substitution. Interestingly, substitution of the AA dinucleotide at $-29/-28$, which resides outside, 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 ($\sim 80\%$ 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 introduced immediately upstream of the TAAA motif exerted small or negligible inhibitory effects on promoter activity. These results suggest that a conformational feature that is dependent on the extended A-tract makes an important contribution to OrbS-dependent promoter utilization. To test this hypothesis, we incorporated a poly(A) tract into a minimal P_{pvdE} promoter ($P_{pvdEds1}$) and measured its effect on promoter utilization by OrbS in *E. coli*. The results show that the modified promoter ($P_{pvdEds7}$) was utilized nearly 10 times more efficiently by OrbS than was the native *pvdE* promoter, although $P_{pvdEds7}$ was still considerably less active than $P_{orbHds6}$ (Table 4). In contrast, the introduction of the poly(A) tract led to a small decrease in the efficiency of P_{pvdE} utilization by PvdS. PvdS was also much less sensitive than OrbS to substitution of the AA dinucleotide at positions $-29/-28$ of P_{orbH} (compare Table 3 and Table S1).

These results prompted us to investigate the effect of all possible single base substitutions within the poly(A) tract on OrbS-dependent transcription. This analysis was also extended to the -35 and -10 core elements and nucleotide positions flanking each element. As expected, substitution of each base pair within the experimentally determined -35 and -10 regions, by any of the other three possible bases, exerted strong inhibitory effects on promoter utilization (Fig. 6). Most noteworthy was the fact that all three possible substitutions at either position of the central dinucleotide of the -10 element (CGTC) abolished promoter activity. Substitutions on either side of the -10 element were much less inhibitory or exerted no inhibitory effect on promoter utilization, thereby clearly delimiting this element. Base pair substitutions introduced at A-tract positions located downstream of the -35 TAAA motif showed a clear trend from being strongly or moderately inhibitory (positions -29 and -28) to exerting little or no inhibitory effect (position -26). Substitution of the base located immediately upstream from the -35 element (position -34) 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 utilization 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 utilization. Substitutions at each

FIG 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 replaced by the other three possible bases. Bacteria were grown in LB medium 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 harboring pKAGd4 and pBBR2-orbS assayed under identical conditions and then expressed relative to the activity in cells harboring 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 ($P_{orbHsdg1}$), which is also shown below the x axis. All assays were performed on three independent cultures, with technical duplicates for each, and data represent the means $\pm$ SDs.

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 nontemplate strand (16, 17, 32), base-specific interactions at position -9 may also occur with some other ECF σ factors, such as the *Pseudomonas syringae* PvdS orthologue (see below) and *Bacillus subtilis* σ^X , which recognizes promoters with an invariant C at position 4 of the -10 element (33). The less severe effect of replacing 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 and within the spacer region, as well as a transcription initiation region that is enriched for purine residues in the nontemplate strand. The two strongest OrbS-dependent promoters also possess an A-tract that extends from the -35 element

into the spacer region. Whereas en bloc nucleotide substitutions of the G-C spacer and A+G TSS regions negatively impact OrbS activity, smaller modifications to the extended A-tract are sufficient to exert a strong down effect on promoter utilization by OrbS and extending it by one nucleotide stimulates OrbS-dependent activity. The properties of A-tracts (specifically those that lack a TpA step) have been the subject of extensive investigation (35). A-tracts as short as four consecutive base pairs, when inserted into non-A-T tract DNA, can induce bending and are known to bend the DNA helix by 9° into the minor groove (36). A bend of ~20° has been determined for an A-tract of 6 nucleotides (36, 37). The bending is proposed to arise from differences in tilt associated with the 5' and 3' junctions flanking the A-tract and negative roll within the A-tract (36). In addition to bending, A-tracts result in progressive narrowing of the minor groove and high negative propeller twist (38, 39). We propose that one or more of these conformational features of the A-tract are instrumental in rendering the P_{orbH} and P_{orbl} -35 elements more susceptible to recognition by OrbS.

The possession of an A-tract by OrbS-dependent promoters was used to inform a bioinformatic search for additional putative OrbS-dependent promoters in *B. cenocepacia* and other members of the Bcc which were then tested for OrbS dependency. However, this approach did not uncover additional OrbS-dependent promoters (see Text S1 in the supplemental material), leading us to conclude that the three OrbS-dependent promoters located in the ornibactin gene cluster are the only representatives of this promoter class in Bcc members.

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

FIG 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 $\geq 50\%$ of aligned sequences are shown with black highlighting, while amino acids that are similar are shaded in gray. The locations of conserved regions 2.1 to 2.4 and 4.1 and 4.2 are based on previous assignments for RpoE (2, 17) and are enclosed in colored boxes and labeled accordingly. Amino acids corresponding to the H3-H4 interhelix loop in domain 2 (the L3 loop) are indicated by the 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 highlighted in cyan. For reference, the RpoE residue N84, which interacts via its side chain with the base at -12 , and residues N80, I77, and A60, which interact through their side chains with the base at position -11 , are highlighted in magenta. Also shown similarly is Y75, which stacks against the base at -7 through its side chain, although this interaction does not contribute to sequence specificity. Interactions of RpoE with the -10 region sequence GTCAA (-12 to -7) are described in reference 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 yellow. Also for reference, residues R171, S172, F175, and R176 in region 4.2 of *E. coli* RpoE, which interact with a consensus RpoE-dependent promoter -35 element (GGAAGCTT; -35 to -29), are shown with magenta highlighting (40). The locations of the N- and C-terminal helices of the region 4.2 HTH in *E. coli* RpoE are indicated by light and dark blue bars, respectively (12). Sequences were obtained from the following strains: *E. coli* MG1655, *Burkholderia ambifaria* AMMD, *B. cenocepacia* J2315, *Burkholderia lata* 383, *Burkholderia vietnamiensis* G4, *P. aeruginosa* PAO1, *P. chlororaphis* O6, *Pseudomonas entomophila* L48, *Pseudomonas fluorescens* Pf0-1, *Pseudomonas protegens* Pf-5, *Pseudomonas putida* GB-1, and *P. syringae* B728a.

contains an adenine at the location corresponding to position 5 of the PvdS-dependent promoter -35 element, and yet PvdS was shown to utilize 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 to 3) may be the result of having two consecutive nonconsensus bases in the -35 element, as the A base at position 5 in the P_{orbH} -35 element is also nonconsensus 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 TAAAT, compared to the TAAAT consensus for PvdS-dependent promoters).

Two lines of evidence suggest that *P. aeruginosa* PvdS can efficiently recognize 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 utilize the

TABLE 5 Bacterial strains used in this study

Strain	Genotype or description ^a	Reference(s) or source
<i>B. cenocepacia</i>		
715j	CF isolate, prototroph (Orb ⁺ Pch ⁺)	45, 46
715j-orbS::Tp	715j with <i>dfrB2</i> cassette inserted in <i>orbS</i> (Orb [−] Pch ⁺)	30
H111	CF isolate, prototroph (Orb ⁺ Pch ⁺)	47
H111ΔorbS	H111 containing an in-frame deletion within <i>orbS</i> (Orb [−] Pch ⁺)	A. T. Butt, K. Agnoli, and M. S. Thomas, unpublished data
<i>P. aeruginosa</i>		
PAO1	Wild type, prototroph	48, 49
PAO1-pvdS::Gm	PAO1 containing a Gm ^r cassette inserted in place of a 460-bp segment of <i>pvdS</i>	50
<i>E. coli</i>		
JM83	F [−] <i>ara</i> Δ(<i>lac-proAB</i>) <i>rpsL</i> φ80 <i>dlacZ</i> ΔM15 (Sm ^r)	51
MC1061	<i>hsdR araD139</i> Δ(<i>ara-leu</i>)7697 Δ <i>lacX74 galU galK rpsL</i> (Sm ^r)	52
S17-1	<i>thi proA hsdR recA</i> RP4-2-tet::Mu-1 <i>kan::Tn7</i> integrant (Tp ^r Sm ^r)	53

^aGm^r, gentamicin resistant; Sm^r, streptomycin resistant; Tp^r, trimethoprim resistant; Orb, ornibactin phenotype; Pch, pyochelin phenotype.

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 these contributions can be observed only following substitution of both bases.

Promoter utilization 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 σ factors may be differentially sensitive to architectural features imparted by the A-tract. The amino acid sequences of the helix-turn-helix (HTH) that constitutes region 4.2 of both σ 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 recognized by the *B. subtilis* group 4 σ factor, σ^V , have a pentameric T-tract in the nontemplate 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 recognized by other ECF σ factors (41). Similar to the case with 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 modeling 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 summarize, our results show that whereas OrbS recognizes promoters with consensus −35 and −10 promoter elements very similar to those recognized by PvdS of *P. aeruginosa*, OrbS has more stringent requirements for optimal promoter recognition, including 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, and growth media. Bacterial strains and plasmids used in this study are listed in Table 5 and Table S2 in the supplemental material, 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 a 0.1% (wt/vol) 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 FeCl₃ in the medium at a final concentration of

50 μ M for all bacterium-medium combinations. To establish iron-limiting conditions in liquid culture, 2,2'-dipyridyl was included in the medium at final concentrations of 175 μ M for *E. coli* strains and 100 μ M (M9 medium) or 200 μ M (LB) for *B. cenocepacia* and *P. aeruginosa* strains.

For strains harboring pKAGd4 derivatives, chloramphenicol was included in the medium (*E. coli*, 25 μ g/ml; *B. cenocepacia*, 50 μ g/ml; and *P. aeruginosa*, 100 μ g/ml), and for strains harboring pBBR1MCS-2 derivatives, kanamycin was included (*E. coli*, 25 μ g/ml; *B. cenocepacia*, 50 μ g/ml; and *P. aeruginosa*, 400 μ g/ml).

Primer extension. *B. cenocepacia* 715j was grown in M9 medium under iron-limited conditions, and total RNA was isolated using the RNeasy Midi kit (Ambion). Antisense primers SKorBSRev, pvdEpvdRev, and orbSmbtHrev (Table S3), corresponding to sequences located 47 to 66, 18 to 38, and 39 to 60 bp downstream of the *orbS*, *orl*, and *orbH* translation initiation codons, respectively, were end labeled with [γ - 32 P]ATP using T4 polynucleotide kinase and used to prime synthesis of labeled cDNA. cDNA products were then electrophoresed in a 0.4-mm-thick DNA sequencing gel containing 7 M urea in Tris-borate buffer and sized against DNA sequencing ladders generated using the same primer and the corresponding DNA region cloned in pBluescript II KS (i.e., pBS-P_{orbS}, pBS-P_{orl}, and pBS-P_{orbH}, respectively) with the Sequenase version 2.0 kit (USB). Following electrophoresis, the dried gel was imaged using a Fujifilm FLA-3000 phosphorimager.

Plasmid constructions. Oligonucleotides used for plasmid construction are listed in Table S3. pBBR2-orbS was constructed by transferring a 975-bp BamHI-HindIII DNA fragment from pBBR1MCS-orbS to pBBR1MCS-2. To construct pBBR2-pvdS, a 1.875-kb KpnI-HindIII fragment was removed from pUCP22-pvdS and ligated between the same sites of pBBR1MCS-2, placing the *pvdS* gene under the control of the *lacZ* promoter. pKAGd4-P_{pvdE} and pKAGd4-P_{pvdF} were constructed by amplifying a 564-bp DNA fragment containing the divergently organized PvdS-dependent *pvdF* (PA2396) and *pvdE* (PA2397) promoters (*P_{pvdE}* and *P_{pvdF}*) with primers PAppvdEfor and PAppvdErev2, then cutting the amplicon with BamHI and HindIII, and ligating it between the BamHI-HindIII sites and BglII-HindIII sites of pKAGd4, respectively. The remaining pKAGd4 derivatives contain short (42- to 74-bp) DNA fragments that were assembled by annealing two complementary oligonucleotides that generate BamHI- and HindIII-compatible ends and ligating the product to the corresponding sites of pKAGd4 (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 or iron-replete conditions, as indicated. Assays were carried out as described previously (30).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00507-18>.

SUPPLEMENTAL FILE 1, PDF file, 2.2 MB.

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