DECODING THE REGULATORY MECHANISMS OF TWO QUORUM SENSING PROTEINS, CepR AND CepR2, OF *Burkholderia cenocepacia*

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DECODING THE REGULATORY MECHANISMS OF TWO QUORUM SENSING PROTEINS, CepR AND CepR2, OF *Burkholderia cenocepacia*

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Burkholderia cenocepacia is an opportunistic pathogen of humans that encodes two LuxR-type acylhomoserine (AHL) synthases and three LuxR-type AHL receptors. Of these, *cepI* and *cepR* are tightly linked and form a cognate synthase/receptor pair, as do *cciI* and *cciR*. In contrast, CepR2 is unlinked from the other four genes, and lacks a genetically linked cognate AHL synthase gene. In the first study, a CepR-binding site (*cep* box) was systematically altered to identify nucleotides essential for CepR activity *in vivo* and CepR binding *in vitro.* The consensus *cep* box determined from these experiments was used to screen the genome and identify CepR-regulated genes containing this site. Four new regulated promoters were found to be induced by OHL and required the *cep* box for induction and CepR binding. In the second study, the regulatory mechanism of CepR2 at two divergently transcribed genes predicted to direct the synthesis of secondary metabolites was investigated. These *cepR2*-linked genes were induced by OHL and required CepR2, indicating CepR2 acts as a repressor at these promoters and is antagonized by OHL. A *lacZ* reporter fused to the divergent promoters was used to confirm these hypotheses. Promoter resections and DNase I footprinting assays revealed a single *cepR2* binding site located in the intergenic region upstream of both promoters and was required for CepR2-dependent regulation. An AraC homolog, CepS, encoded adjacent to *cepR2*, was found to be essential for expression of both promoters, regardless of the CepR2 status or OHL concentration. CepS therefore acts downstream of CepR2 and CepR2 appears to function as a CepS antiactivator.

BIOGRAPHICAL SKETCH

Gina Ryan was born number six of eight children in a small suburb of Detroit, Michigan. At an early age, she migrated with her boisterous family to the desert of northern New Mexico, where she was raised in small towns along the dusty San Juan River. The expansive skies and rich landscape fostered a deep curiosity about her surroundings and instilled in her a restless longing to understand the mysterious world around her. With her heart set on exploring the world, Gina enlisted in the Air Force and served as a Korean linguist. She was stationed in the great state of Texas.

A nomad at heart, Gina pursued her academic interests at several community colleges as she travelled across the country. In 2005, she returned to the desert and enrolled in the Biology program at the University of New Mexico. Gina was fortunate to be extended an opportunity to join the lab of Dr. Cristine Takacs-Vesbach. In the small heated basement space, she encountered a microbe isolated from the frozen waters of Antarctica that would open a gateway to the invisible underworld and, hereafter, enthrall her attention. Bacteria.

In 2007, Gina received her B.S. degree. She was accepted into the doctorate program in Microbiology at Cornell University, where she began her work studying bacterial communication systems under the guidance of Dr. Stephen Winans.

For my mother, Janice,
who always believed.

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When I made the decision to pursue my doctorate eleven years ago, I failed to appreciate the sacrifice and commitment I was asking of my family and friends as well as the enormous contribution of so many others that would be required to complete this journey. First and foremost, I wish to acknowledge the enormous support of my family (the entire tribe), whose steadfast belief in me and continued encouragement - along with the occasional kick- kept me focused and on course. To all of my friends, particularly the Ithacan-derived, for their patience and understanding, especially when plans were spontaneously and repeatedly changed because my cells were suddenly ready to be tinker with. I am especially grateful to Kara Garner-Taylor, whose friendship has been a constant source of strength and support and to whom I most assuredly owe my sanity.

I would like to thank the members of my Ph.D. committee, Drs. John Helmann (Molecular Biology) and Michael Stanhope (Genomics) for their guidance and support. Most especially, I am indebted to my advisor, Dr. Stephen Winans, from whom I have learned the finer points of research. Door ever open, he was always eager to discuss new ideas and troubleshoot finicky experiments.

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1.1.1. History and taxonomy of the *Burkholderia* genus

The history of the *Burkholderia* genus is complex and still evolving, akin to its members, that are notorious for their metabolic diversity and genetic plasticity. First described by Walter Burkholder (Cornell Plant Pathology) in the 1940s, isolates recovered from decaying onion bulbs were identified as the causative agent of soft rot in onions and later found to share 16S rRNA sequence similarity with *Pseudomonads* (Burkholder, 1950). Following the restructuring of the *Pseudomonas* genus based on rRNA-DNA hybridization and rRNA gene sequencing, seven species (*P. cepacia, P. solanacearum, P. pickettii, P. gladioli, P. mallei, P. pseudomallei,* and *P. caryophylli*), were transferred to the newly named *Burkholderia* genus (Yabuuchi *et al*., 1992).

A group of human opportunistic pathogens previously known as 'eugonic oxidisers group 1', was shown to represent the same species, *B. cepacia* (Coenye & Vandamme, 2003). Through the use of polyphasic techniques, most *B. cepacia* strains were demonstrated to actually represent a complex of closely related species that could be separated into phenotypically similar genomic groups, collectively known as the *B. cepacia* complex (Bcc) (Fig. 1.1). The Bcc is comprised of species that share a high similarity at the 16S rRNA level (> 97%) and only moderate DNA-DNA hybridization (30-60%). Given the limitations of 16s rRNA sequence to resolve below the species

level, alternative molecular genotyping approaches with objective and greater resolving power were developed to delineate the relationships between the closely related Bcc species (Vandamme & Dawyndt, Martens *et al.*, 2008). Multilocus sequence analysis (MLSA), which compares the divergence of seven housekeeping genes located on the first and second chromosome, recA gene sequence, and whole-genome sequence analysis have been successful in classifying novel species within this complex. The recent establishment of two taxon K strains, *B. contaminans* and *B. lata,* as distinct species, has expanded the current structure of the Bcc group to 17 validly described species (Vanlaere *et al*., 2009). As whole-genome sequence for additional representative and candidate strains becomes available, the taxonomy of this diverse group is expected to continue to evolve (Vanlaere et al., 2009).

1.1.2. Ecological diversity of the *Burkholderia* genus

This diverse genus is currently comprised of 70 species that are ubiquitous in nature and have been isolated from a wide array of ecological niches ranging from water and soils to the respiratory tracts in humans (see http://www.bacterio.cict.fr/). In some cases, *Burkholderia* bacteria can form opposing interactions depending on the host colonized, functioning as either a pathogen or a symbiont (Fig 1.1). The majority of *Burkholderia* species are non-pathogenic soil bacteria and several form beneficial interactions with plants, including nitrogen fixation and nodulation in legumes by *B. caribensis, B. kururienis, B. nodosa,* and *B. tuberum* and biocontrol of soil-borne plant pathogens. Some species have been introduced into agricultural crop soils as biocontrol agents (Coenye & Vandamme, 2003, Bontemps *et al.*, 2010, Gyaneshwar *et al.*, 2011).

Several members of this genus have been identified as important pathogens in cystic fibrosis (CF) patients, causing chronic and severe lung infections (Mahenthiralingam *et al*., 2005). The intrinsic antibiotic resistance of *Burkholderia* species severely limit therapeutic options and can lead to persistent colonization. In some cases, the infection progresses to a condition described as '*cepacia* syndrome,' which is marked by a progressive and rapid deterioration of lung function and is associated with increased mortality rates. While all Bcc species have been isolated from CF sputum, *B. cenocepacia* and *B. multivorans* strains predominate (McDowell *et al*., 2004). The best characterized is the virulent *B. cenocepacia* J2315 strain that represents the Edinburgh/Toronto type 12 (ET12) intercontinental epidemic strain (Mahenthiralingam *et al*., 2002).

Some *Burkholderia* species can form both deleterious and beneficial interactions depending on the host it has colonized. In the lungs of CF patients, new strains of B. vietnamensis continue to emerge. Sequence analysis have identified several environmental isolates that are identical to CF outbreak strains, showing this globally distributed soil bacterium may be well adapted to human infection (McDowell et al., 2004). In contrast, *B. vietnamensis* isolates have been recovered from the rhizosphere of rice species and shown to function in nitrogen fixation for the plant (Chen *et al*., 2003).

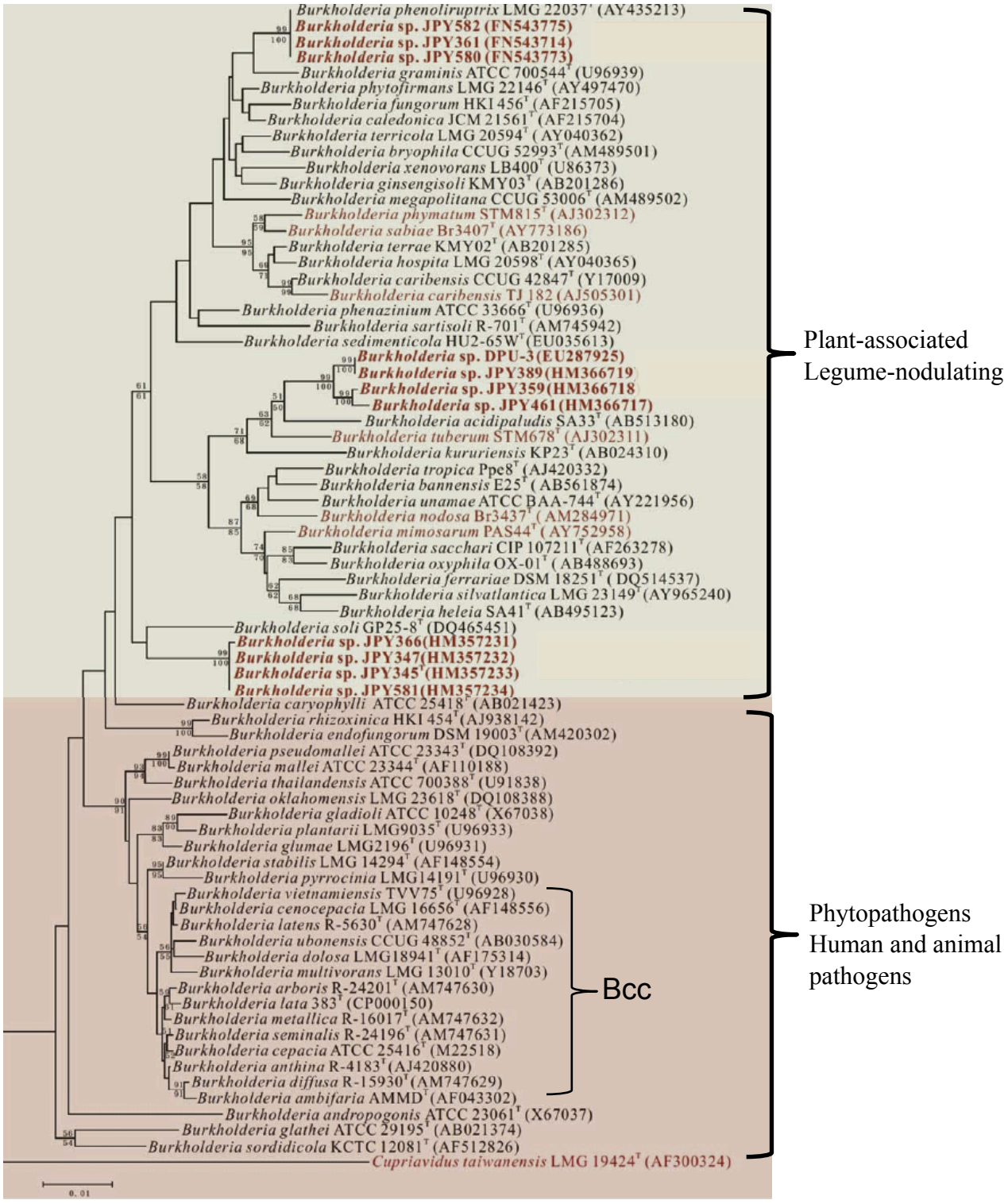

Figure 1.1. 16S rRNA gene phylogeny of known *Burkholderia* species, including proposed new *Burkholderia* species. The genus can be divided into two groups; group A contains most of the plant-associated species and all currently known legume nodulators within the genus. Group B contains all known phytopathogens and species that may cause disease in humans and animals. Candidate species are in red. Modified from (Gyaneshwar et al., 2011).

1.2. Genomics of the *B. cepacia* complex

The importance of *Burkholderia cepacia* complex species as human pathogens has prompted the sequencing of numerous genomes within this genus. Nine sequencing projects have been completed, including four *B. cenocepacia* strains J2315, AU1054, HI2424, and MC0-3, all of which were isolated from CF patients (available at the Sanger Institute; http://sanger.ac.uk/ Projects/Microbes/). Additionally, several environmental strains have been completed recently, including *B. vietnamensis* str. G4, *B. multivorans* ATCC 17616, and two *B. cepacia* strains, 383 and AMMD (all can be found at http://www.jgi.doe.gov/JGI_microbial/html/index/ html). Presently, the genome sequences of 18 strains from 10 Bcc species are publically available (http://pathema.jcvi.org/cgibin/*Burkholderia*/PathemaHomePage. cgi).

Burkholderia species possess some of the largest, most complex genomes, ranging in size from 6.2 to 9.73 Mbp. They are characterized by a high G+C content (~67%), multireplicon structure, numerous gene duplications, insertion sequences, and mobile elements that contribute to the high frequency of genetic rearrangement characteristic of species within this group. The genomes of four isolates of *B. cenocepacia* have been sequenced in their entirety while additional genomes are currently being sequenced (NCBI, 2011). All four sequenced genomes have three circular chromosomes that vary in size between 0.88 and 3.9 MB in length,. Chromosome 3 of strain J2315 was recently found to be curable (Agnoli *et al.*, 2012), and should therefore be considered a plasmid. Strains J2315 and HI2424 also have one plasmid, 93 KB and 165 KB in length respectively. The remarkable ability to adapt to diverse ecological

habitats is attributed to the metabolic diversity and genetic plasticity of its genome (Holden *et al*., 2004). Analysis of sequenced Bcc genomes estimates that more than 10% of sequence has been acquired through horizontal gene transfer. Comparative analysis of four *B. cenocepacia* strains shows significant differences in genomic content and highlights the extent of genetic plasticity even at the species level. The sequenced genome of J2315 reveals it contains approximately 21% unique DNA in comparison with the other *B. cenocepacia* strains, including a 0.93 kb plasmid found only in J2315 and HI2424, 14 genomic islands and numerous mobile genetic elements (Holden *et al*., 2009). In addition to the vast chromosomal modifications by foreign DNA, there is evidence that genes are transferred between Bcc species. Some of these genomic islands unique to the ET12 lineage share similarity with islands in other Bcc species and promote survival and pathogenesis in the CF lung ((Mahenthiralingam *et al.*, 2001, Baldwin *et al.*, 2004).

Bcc strains exhibit a high degree of antibiotic drug resistance, with some strains utilizing penicillin G as a carbon source (Beckman & Lessie, 1979). This extensive drug resistance severely limits infection control strategies and is therefore considered as virulence factors of this pathogen. *Burkholderia* species possess multiple mechanisms for antibiotic resistance including efflux pumps, degradative enzymes, low cell membrane permeability, and antibiotic target modification. The genome of J2315 encodes numerous efflux pump systems belonging to six families associated with drug resistance, at least four β-lactamases, and evidence of a non-synonymous substitution in the dihydrofolate reductase (*dfrA*) gene targeted by trimethoprim, all of which contribute to an incredible

intrinsic resistance to a wide range of clinically-relevant drugs (Holden et al., 2009).

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1.3. AHL-quorum sensing systems in Proteobacteria

1.3.1. Quorum sensing systems

Bacteria in the environment exist within complex communities that rely on various signaling mechanisms to perceive their surroundings and efficiently modulate gene expression for rapid adaptation to ever changing conditions. Quorum sensing (QS) is one type of communication system that enables bacteria to coordinate the synthesis of genes in a density dependent manner in response to an endogenously produced signal molecule, *N*-acyl-homoserine lactone (AHL), also described as an autoinducer. This simple AHL-driven system, first described for the regulation of bioluminescence genes in Vibrio fischeri, is composed of a signal synthase, LuxI, and a transcription regulator, LuxR (Hastings & Nealson, 1977). At low cell density, basal level expression of LuxI results in a minimal production of *N*-oxohexanoyl-homoserine lactone (OHHL) that accumulates extracellularly. Dependent upon cell density, external diffusion constraints, and conditions affecting signal stability, the OHHL concentration reaches a critical threshold and diffuses back across the cytoplasmic membrane. The autoinducer is bound by newly synthesized LuxR protein and stable LuxR-OHHL complexes accumulate. Active LuxR-OHHL complexes recognize a 20-bp dyad sequence in the promoter region, designated the *lux* box, and up-regulate gene expression of the *lux* operon (Boyer & Wisniewski-Dyé, 2009).

AHL synthases direct the synthesis of *N*-acylhomoserine lactones from an acyl-ACP and *S*-adenosylmethionine. AHLs vary in fatty acid chain length (4-18 carbons) and modifications at the third carbon (either unsubstituted or substituted with hydroxyl, oxo groups, or acyl chain desaturation) (Eberhard *et al*., 1981). This variation in the fatty acid chain is believed to provide specificity in recognition between the LuxR homolog and its cognate AHL (Nasser & Reverchon, 2007).

1.3.2. Phylogenetic distribution and evolution of quorum sensing systems

AHL-based QS systems are unique to the Proteobacteria phylum. Analysis of 265 proteobacterial genomes identified genes for QS systems in 68 species. Within these bacteria, QS has been described to regulate diverse functions that play a role in symbiosis and pathogenesis such as bioluminescence, nodulation, motility, biofilm formation and production of virulence factors (Whitehead *et al.*, 2001, Case *et al.*, 2008).

Phylogenetic analysis of LuxI/R family members show they are subdivided into two groups with one group restricted to the γ-proteobacteria (family B), and the other more widely distributed across the α, β, and γ classes (family A) (Lerat & Moran, 2004, Case et al., 2008). While both groups share similar functions, the divergence of aligned amino acid sequences suggests they are not paralogous but arose through speciation events. Neighbor-joining tree reconstructions of the LuxI and LuxR homologs within their respective groups reveal the two trees are globally congruent, indicating co-evolution of the AHL synthase and regulator (Fig 1.2) (Lerat & Moran, 2004). Furthermore, the agreement between the SSU rRNA and the LuxI/R trees indicates an

ancient evolution of the QS mechanism within these bacteria (Lerat & Moran, 2004, Case et al., 2008).

In many lineages, the genes for the inducer and regulator are genetically linked and retain pairwise functional relationships. This is not always the case, as in A. tumefaciens, whose *traR* and *traI* genes are separated by more than 60 kb on the octopine-type plasmid (Fuqua & Winans, 1994). In cases where lateral transfer events lead to the movement of QS genes across bacterial species (Boucher *et al.*, 2003, Lerat & Moran, 2004), this contiguous arrangement may ensure that the QS system remains intact, thereby retaining regulatory ability that can be harnessed by the recipient cell.

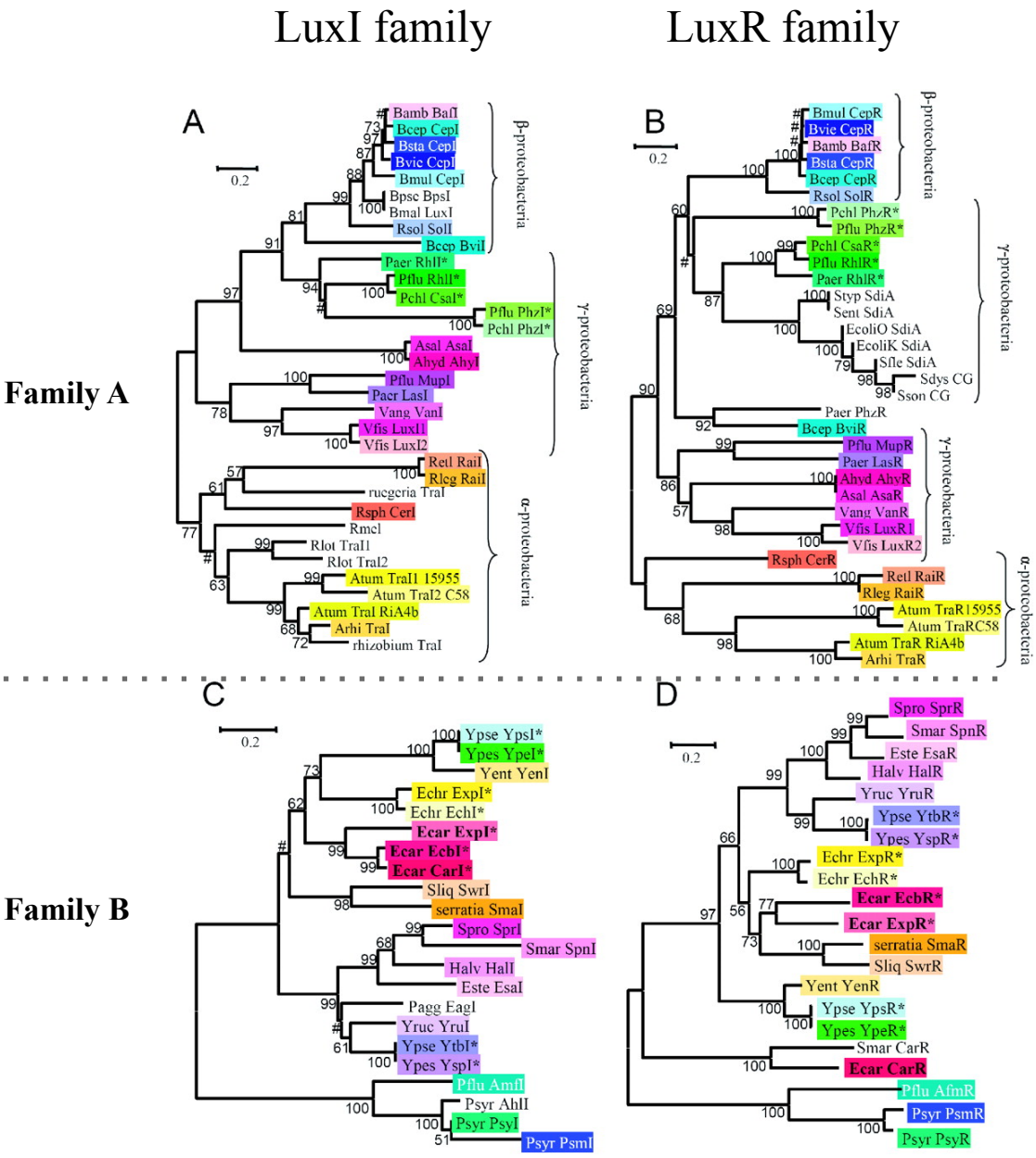

Figure 1.2. (A) Tree based on the protein alignment of the LuxI members of family A. (B) Tree based on the protein alignment of the LuxR members of family A. (C) Tree based on the protein alignment of the LuxI members of family B. (D) Tree based on the protein alignment of the LuxR members of family B. Species abbreviations as in table 1. Different phylogenetic methods yielded the same topology, and we present trees obtained using NJ and γ correction. Asterisks (*) indicate paralogous copies. The number sign (#) indicates nodes with bootstrap values less than 50%. Modified from (Lerat & Moran, 2004).

1.3.3. LuxR-type solo regulators

Of the 68 proteobacterial genomes encoding a complete QS system, 45 harbor genes encoding LuxR homologs that exceed the number of genes for predicted AHL synthases. While the cognate signal for these additional regulators is not immediately clear, it is very plausible that they can perceive the AHL signal produced by the host synthase. In cells that possess at least one complete AHL QS system, these additional LuxR homologs, also described as LuxR solos, may be integrated with the native QS regulatory network and function to finely modulate AHL signaling within the cell. QscR, in Pseudomonas aeruginosa, closely interacts with the two native QS systems, LasI/R and RhlI/R, to coordinate temporal regulation of a number of virulence factors, including production of rhamnolipids, elastases, and biofilm formation (Lequette *et al*., 2006). In some organisms, the *luxR*-type gene is not accompanied by an AHL synthase gene. These QS regulators are predicted to perceive AHL signals produced other bacteria. SdiA, encoded by *Escherichia coli* and *Salmonella enterica* sv. Typhimurium, has been shown to respond to several exogenous AHL species (Ahmer, 2004, Yao *et al.*, 2006, Janssens *et al.*, 2007).

1. 4. Quorum Sensing in *B. cepacia* complex species

1.4.1. Quorum sensing genes in Bcc species

Of the BCC genomes that have been analyzed, members of the *B. cepacia* complex encode genes for the CepI/R QS system. The genes for CepI and CepR proteins are highly conserved in Bcc species, indicating that this system is important for the successful persistence in the diverse habitats of the Bcc species (Table 1.1). CepI and CepR show the highest similarity (64% and 67%, respectively) to the SolIR quorum

sensing genes in *Ralstonia solanacearum*. CepI in *B. cenocepacia* K56-2 produces two AHL signals, *N*-octanoyl-L-homoserine lactone (Coenye *et al.*) and *N*-hexanoyl-L-homoserine lactone (HHL) in a 10:1 ratio (Huber *et al*., 2001). Expression of the *cepI* gene is controlled from a 20-bp *cep* box that partially overlaps the -35 region of the promoter. The product of the divergently transcribed gene, CepR, positively regulates *cepI* expression at this site upon perception of OHL at high cell density (Fig 1.3) (Weingart *et al*., 2005).

Some members of the complex also carry genes for an additional QS system. *B. vietnamensis* expresses a BviR protein which regulates gene expression in response to decanoyl-homoserine lactone (DHL) and 3-oxodecanoyl-homoserine lactone (ODHL) that are produced by the AHL synthase, BviI (Conway & Greenberg, 2002). Several *B. cenocepacia* strains harbor a second system, CciI/R, encoded on a pathogenicity island that was likely acquired through horizontal gene transfer (Baldwin et al., 2004). CciR responds to HHL produced by CciI and is reported to negatively regulate several genes within the CepR regulon, thereby acting to partially antagonize CepR-dependent function (Fig. 1.3) (O'Grady *et al*., 2009).

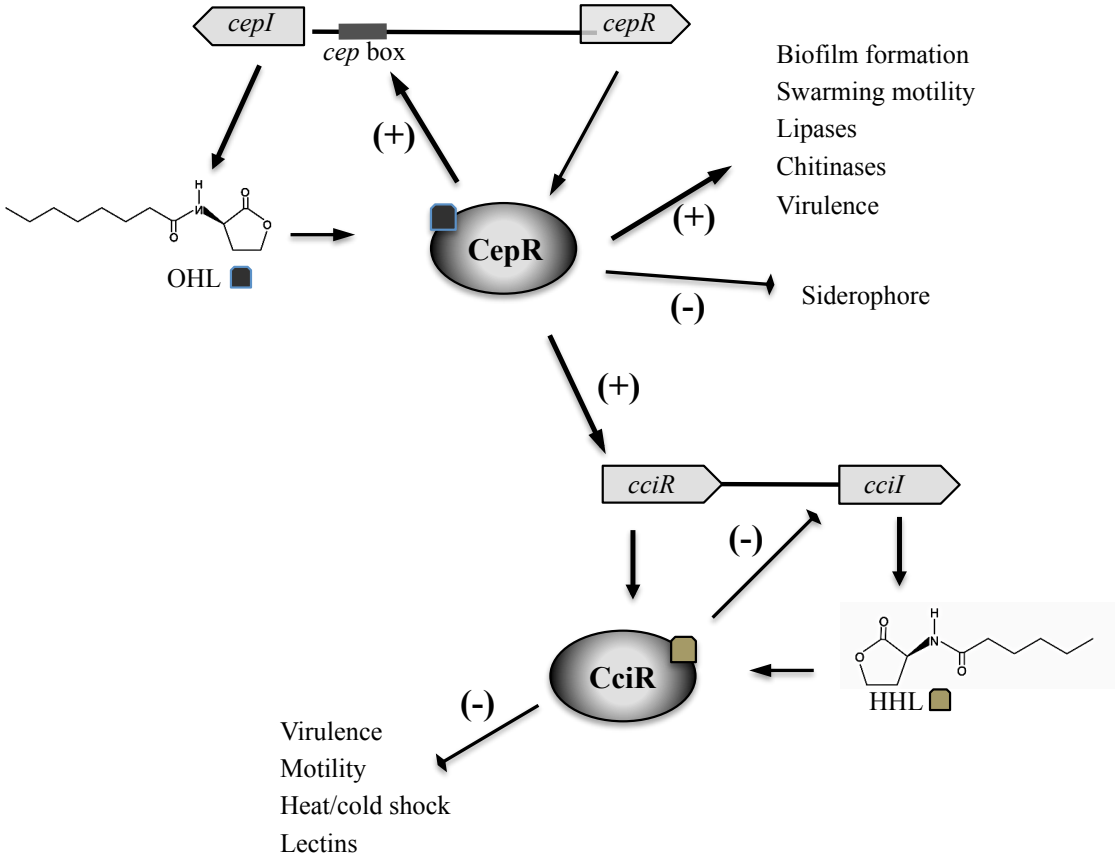

Figure 1.3. CepI/CepR quorum sensing system in *Burkholderia cenocepacia*. CepI directs the synthesis of OHL which is then bound by the transcriptional regulator, CepR, to regulate expression of genes at high cell density. CepR-OHL induce or repress target genes through DNA binding at the *cep* box. CciI produces HHL, which is recognized by CciR. CciR-HHL negatively regulates several genes, including *cciI* expression which initiates negative feedback regulation of the CciIR regulon (Eberl, 2006; O'Grady *et al.*, 2009).

Many of the bacterial genomes within the *Burkholderia* genus contain at least one additional LuxR homolog. The animal pathogens, *B. mallei* and *B. pseudomallei*, both possess at least two LuxR homologues in addition to multiple complete QS circuits. These regulators exhibit relaxed AHL specificity and recognize several endogenous signal molecules (Case et al., 2008). *B. cenocepacia* strains encode a single LuxR solo, CepR2, that has weak similarity to CepR (38% identity). CepR2 has been found to negatively regulate genes encoding nearby divergent non-ribosomal peptide synthase operons, as well as several virulence factors within the CepR regulon (Malott *et al.*, 2009, O'Grady et al., 2009).

1.4.2. Regulation of genes by CepIR and CciIR in *B. cenocepacia*

The CepI/R and CciI/R systems regulate the production of proteases, chitinases, and lipases, swimming and swarming motility, biofilm formation and maturation, as well as components of efflux pumps, lectins, pili and type II, III and IV secretion systems (Lewenza *et al.*, 1999, Huber et al., 2001, Lewenza & Sokol, 2001, Aguilar *et al.*, 2003, Eberl, 2006).

CepR is a global regulator and functions primarily as an activator of gene expression, including the transcription of *cepI* and *cciIR* operons (Fig 1.3). CciR has been shown to reciprocally regulate many CepR-dependent genes, thus providing a regulatory feedback of QS-dependent expression (Malott *et al*., 2005). The LuxR solo, CepR2, has been shown to negatively regulate several genes within the CepR regulon, including an efflux system and protease (Malott et al., 2009). Interestingly, CepR2 was demonstrated to activate a *luxI* promoter in an AHL independent manner, suggesting a novel

mechanism may exist for this LuxR-type regulator (Malott et al., 2009).

To date, several studies have revealed many targets of the CepI/R regulon. However, most of these are likely regulated indirectly by CepR. Deciphering the CepR binding site was deemed crucial for identifying new genes directly under the control of the regulator and is the focus of Chapter 2.

1.4.3. Quorum sensing-mediated pathogenesis in *B. cenocepacia*

Pulmonary infections with *B. cenocepacia* strains typically occur in the later stages of CF and follow *P. aeruginosa* colonization (Coenye & Vandamme, 2003). Recovery of OHL, the principle autoinducer produced in *B. cenocepacia*, from CF lung tissue and sputum samples, established the use of quorum sensing in *B. cenocepacia* pathogenesis in CF pulmonary infections (Eberl, 2006). CepI and CepR mutant strains demonstrated reduced virulence in rat and mouse agar bead infection models of chronic lung infection (Sokol *et al*., 2003). The functional significance of this system is further underscored by the fact that the QS circuit and activity remains intact, while mutations accumulate in genes involved in late stage infections including motility, iron acquisition, and O-antigen biosynthesis (McKeon *et al*., 2011). Two genes involved in oxidative stress response, *katC* and *sodB*, were found to be down-regulated in *cepR* mutants compared to wild type and are predicted to play a role in intracellular survival within macrophages and respiratory epithelial cells (O'Grady et al., 2009). However, this would require a quorum is attained and the lipid-soluble autoinducer is permitted to reach sufficient concentrations within the host cell.

Table 1.1 QS genes, signals, and regulated phenotypes in the *B. cepacia* complex

Bcc species	Designation	QS phenotypes and genes controlled	Amino acid identity		Presence of *ccilR* QS system	Presence of *bviIR* QS system	*N*-acyl HSL	References
			CepI	CepR				
B. cepacia	Genomovar I	onion maceration, protease activity	100	100	No	No	C_6-, C_7-, C_8-HSL	(Aguilar et al., 2003; Gotschlich et al., 2001)
B. multivorans	Genomovar II	n.d.	78	94	No	Yes	C_6-, C_7-, C_8-HSL	Gotschlich et al., 2001)
B. cenocepacia	Genomovar III	protease activity, chitinase activity, swarming motility, siderophore production, biofilm development, lipase activity	98	97	Yes	No	C_6-, C_7-, C_8-HSL	Gotschlich et al., 2001)
B. stabilis	Genomovar IV	n.d.	97	95	No	No	C_6-, C_8-HSL	Gotschlich et al., 2001)
B. vietnamensis	Genomovar V	n.d.	91-97	96-97	No	No	C_6-, C_7-, C_8-HSL	Gotschlich et al., 2001)
							C_{12}HSLs; 3OC_{10}HSL	(Conway and Greenberg, 2002)
B. dolsa	Genomovar VI	n.d.	92	93	No	No	C_6-, C_7-, C_8-HSL	Gotschlich et al., 2001)
B. ambifaria	Genomovar VII	n.d.	96	93	No	No	C_6-, C_7-, C_8-HSL	Gotschlich et al., 2001)(Conway and Greenberg, 2002)
B. pyrrocinia	Genomovar VIII	n.d.	n.d.	n.d.	No	No	n.d.	(Coenye and Vandamme, 2003)
B. anthina	Genomovar IX	n.d.	n.d.	n.d.	No	No	n.d.	Coenye and Vandamme, 2003)

Amended from Venturi et al. (2004)

1.5. Mechanism of LuxR-type regulators

1.5.1. Classification of LuxR homologues

The LuxR family proteins feature two domains, with the NTD serving to recognize the AHL and the CTD recognizing nucleotides within the DNA binding site (Whitehead et al., 2001, Pappas *et al.*, 2004). Amino acid alignments show a low sequence similarity overall (~25%); however, several residues making critical contacts in the NTD and CTD are highly conserved (Vannini, *et al*., 2002).

Proteins within the LuxR family are grouped into four classes based on interactions with AHL and their multimeric properties (Fig. 1.4) (Stevens *et al*., 2011). Classes I-III proteins are transformed into an active state upon perception of their cognate signal also described as the holo-form state. Class I regulators, such as TraR from *Agrobacterium tumefaciens*, binds its cognate signal at a buried hydrophobic pocket that serves as a structural scaffold during peptide synthesis. Apo-TraR is unable to dimerize and is targeted for rapid proteolysis by Clp and Lon proteases (Zhu & Winans, 1999). CepR binds OHL during protein synthesis and is similarly required for proper folding and transcription activation (Weingart et al., 2005, Wei *et al.*, 2011). LuxR is a representative of Class II regulators that requires AHL for protein folding, but unlike Class I proteins, AHL binding is reversible and OOHL can be diluted away from the binding domain without loss of protein stability. While, nanomolar OHHL concentrations are inhibitory to LuxR, the phenotype can be rescued with additional inputs of the autoinducer signal (Urbanowski *et al*., 2004). Class III regulators, such as QscR in *P. aeruginosa*, are stable in the absence of AHL but require binding of the signal for dimerization and transcriptional activation (Yang *et al*., 2009).

In contrast, Class IV regulators, such as EsaR from *Pantoea stewartii*, are active in the absence of their signal, to bind as dimers to operator sites within the DNA (Fig. 1.4) (Minogue *et al*., 2005). Binding the AHL molecule decreases affinity of the LuxR protein for the DNA and antagonizes its regulatory function (Patankar & González, 2009, Subramoni & Venturi, 2009). Only a few members have been characterized, including YenR and EsaR, and are reported to repress to their target genes at low cell density when AHL levels are minimal (Castang *et al.*, 2006, Tsai & Winans, 2011). Differences in structural elements differentiate γ-proteobacteria class IV proteins from their holo-active counterparts. Amino acid sequence analysis of members of this class reveals an extended linker region between the NTD and an extended C-terminus.

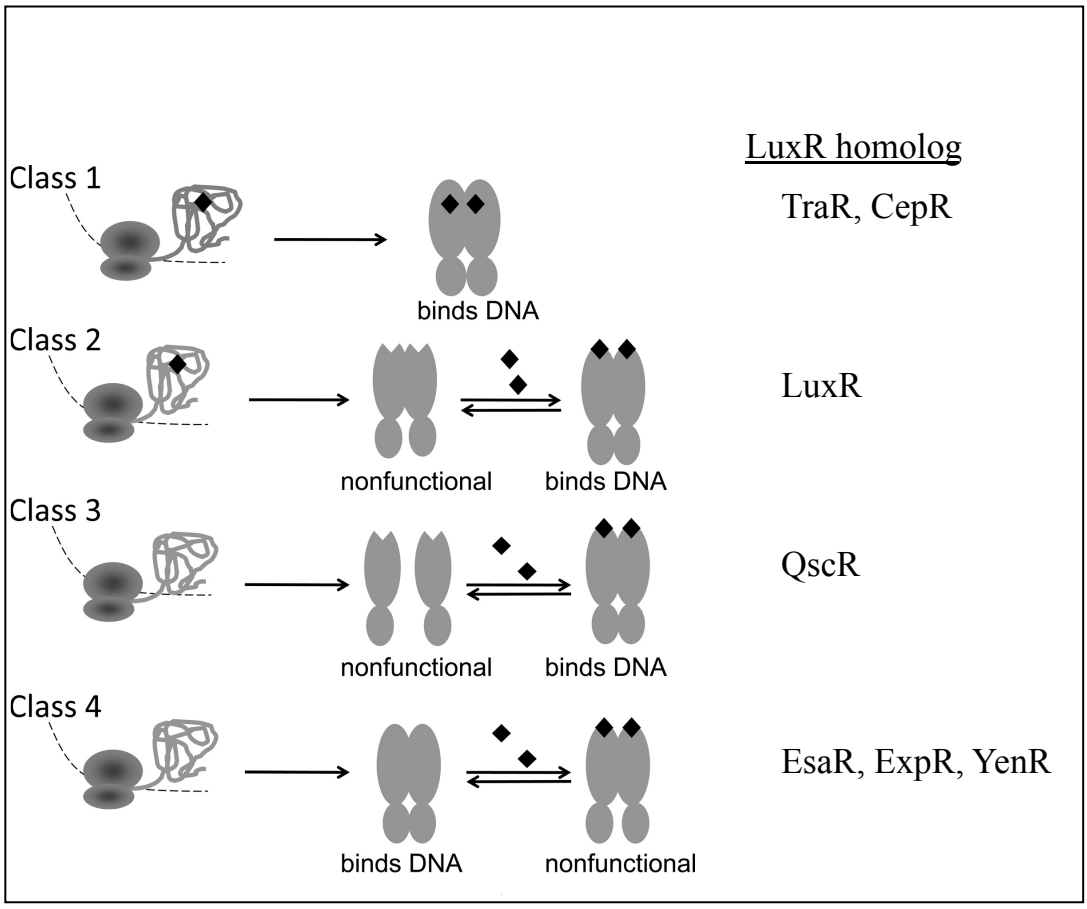

Figure 1.4 Classification of LuxR homologues. The cognate AHL for a given LuxR homologue is represented by a black diamond. White and grey diamonds represent non-cognate AHLs. Adapted from (Stevens et al., 2011).

With a significant number of LuxR homologs characterized over the past few decades, phylogenetic analysis of these genes has begun to yield insights beyond sequence relatedness and enable predictions about possible regulatory mechanisms for newly described QS regulators. LuxR-type proteins whose activity is stimulated upon perception of their cognate AHL (class I-III), are widely distributed across the Proteobacteria (α, β, γ) (Fig. 1.2b), yet cluster into a distinct group, described as Family A in this text. In contrast, apo-active QS regulators, whose activity is antagonized upon binding their AHL (class IV), cluster separately from their counterparts in Family B (Fig. 1.2d). Until recently, this intriguing group of apo-active regulators has been described only in organisms within the γ-proteobacteria. However, as the search for novel LuxR homologs continues, facilitated by the increasing number of sequenced genomes, examples of apo-active regulators are emerging in other Proteobacterial classes. VjbR, a LuxR-type protein in the α-proteobacterial organism, *Brucella melitensis*, is reported to regulate genes for flagella motility and type IV secretion at low cell density and its activity is antagonized through binding its cognate AHL (Delrue *et al.*, 2005). Another homolog in the β-proteobacterium, *B. cenocepacia*, CepR2, regulates secondary metabolite genes in the absence of OHL and is the focus of Chapter 3. While the correlation between phylogeny and biological function has been well established, it remains to be seen whether this relationship will extend to regulatory mechanistic function, as well.

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Chapter 2: [1]Saturation mutagenesis of a CepR binding site as a means to identify new quorum-regulated promoters in *Burkholderia cenocepacia*

2.1 Abstract

Burkholderia cenocepacia is an opportunistic pathogen of humans that encodes two genes that resemble the acyl-homoserine lactone synthase gene *luxI* of *Vibrio fischeri* and three genes that resemble the acyl-homoserine lactone receptor gene *luxR*. Of these, CepI synthesizes octanoyl-homoserine lactone (OHL), while CepR is an OHL-dependent transcription factor. In the current study we developed a strategy to identify genes that are directly regulated by CepR. A CepR binding site (*cep* box) was systematically altered upstream of a target promoter to identify nucleotides that are essential for CepR activity *in vivo* and for CepR binding *in vitro*. Thirty-four self-complementary oligonucleotides containing altered *cep* boxes were constructed, and binding affinity was measured for each. These experiments allowed us to identify a consensus CepR binding site. **I show that purified CepR induces a bend in a DNA fragment containing this consensus sequence**. Several hundred similar sequences were identified, some of which were adjacent to probable promoters. Dr. Ana Lidia Flores-Mireles fused these 13 promoters to a reporter gene with and without intact *cep* boxes. This allowed her to identify four new regulated promoters that were induced by OHL, and that required a *cep* box for induction. **In collaboration with Dr. Flores-Mireles, we show that expression of all four CepR and OHL-dependent promoters could be reconstituted in *Escherichia coli.* I then confirmed purified CepR-OHL complexes bound to each of these sites in**

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electrophoretic mobility shift assays. Furthermore, I found that seven promoters containing non-symmetric putative *cep* boxes, identified by Chambers *et al*., 2006 as direct targets of CepR, were not bound by the purified regulator.

2.2 Introduction

The genus *Burkholderia* encompasses a fascinating collection of diverse β-proteobacteria (Coenye and Vandamme, 2003). This genus includes over 50 species, some of which are potentially useful in bioremediation, while other members are capable of forming nitrogen-fixing root nodules with legumes (Chen *et al*., 2003; Bontemps *et al*., 2010). Some members protect host plants against fungal pathogens, while others are themselves pathogenic against plants, animals and humans (Coenye and Vandamme, 2003; Jones and Webb, 2003). Seventeen pathogenic species are members of the *Burkholderia cepacia* complex, or BCC (Vandamme *et al*., 1997; Vanlaere *et al*., 2008; 2009), two of which are described by the Center for Disease Control as category B select agents (Godoy *et al*., 2003).

Burkholderia cenocepacia, previously known as *B. cepacia* genomovar III (Vandamme *et al*., 2003), is recognized as an opportunistic pathogen of humans and is a particular threat to cystic fibrosis (CF) patients (Vandamme *et al*., 1997; Mahenthiralingam *et al*., 2005). Colonization of the CF lung by *B. cenocepacia* (Vandamme *et al*., 2003) tends to occur in patients already infected with *Pseudomonas aeruginosa*, another opportunistic pathogen of the CF lung (Vandamme *et al*., 1997; Jones and Webb, 2003). An infection caused by both organisms can result in serious clinical complications. *B. cenocepacia* strains are resistant to most antibiotics, making

them virtually impossible to eradicate (Nzula *et al*., 2002). Infections with *B. cenocepacia* may have variable clinical outcomes ranging from asymptomatic carriage to a sudden fatal deterioration in lung function (Mahenthiralingam *et al*., 2005).

Four strains of *B. cenocepacia* have been sequenced in their entirety, one of which is described in a publication (Holden *et al*., 2009). The Joint Genome Institute is currently sequencing nine additional strains (http://www.jgi.doe.gov/genome-projects/). All four sequenced isolates have three circular chromosomes that vary in size between 3.9 and 0.88 MB in length. The third chromosome has been found to be a curable and is considered a plasmid (Agnoli *et al.*, 2012). Strains J2315 and HI2424 also have one plasmid, 93 KB and 165 KB in length respectively.

Many or possibly all *Burkholderia* spp. encode at least one regulatory system that resembles the LuxR and LuxI proteins of *Vibrio fischeri*, where LuxI synthesizes an acyl-homoserine lactone (AHL)-type pheromone, also called an autoinducer, and LuxR is an AHL-dependent transcriptional regulator (Eberhard *et al*., 1981; Engebrecht and Silverman, 1984; Choi and Greenberg, 1992). Regulatory systems of this family are found in countless proteobacteria, where they are thought to allow individual bacteria to coordinate their physiology with their siblings. Collectively, these systems regulate diverse processes, including pathogenesis, biofilm formation, bacterial conjugation and the production of antibiotics and other secondary metabolites (Whitehead *et al*., 2001). In general, target genes are transcribed preferentially at population densities high enough to favor AHL accumulation (Eberhard *et al*., 1991), a phenomenon referred to as quorum sensing (Fuqua *et al*., 1994). *Burkholderia thailandiensis* has three such systems, one of which is implicated in cell aggregation, while another is required for antibiotic

production (Chandler *et al*., 2009; Duerkop *et al*., 2009). A plant growth promoting isolate of *Burkholderia ambifaria* uses quorum sensing to regulate the production of the anti-fungal compound pyrrolnitrin (Schmidt *et al*., 2009).

LuxR-type proteins have two domains, an N-terminal pheromone binding domain and a C-terminal DNA binding domain (Pappas *et al*., 2004). Purified LuxR, TraR of *Agrobacterium tumefaciens* and LasR of *P. aeruginosa*, when complexed with their respective AHLs, bind with high specificity to recognition sequences (referred to as *lux*, *tra* or *las* boxes, respectively) that are found at target promoters (Zhu and Winans, 1999; Schuster *et al*., 2004; Urbanowski *et al*., 2004). LasR is also able to bind to sequences that have no obvious resemblance to canonical *las* boxes. A few members of this family bind DNA only in the absence of AHLs (Cui *et al*., 2005; Fineran *et al*., 2005; Minogue *et al*., 2005; Castang *et al*., 2006; Sjoblom *et al*., 2006).

Burkholderia cenocepacia J2315 encodes three LuxR homologues and two LuxI homologues (Lewenza *et al*., 1999; Malott *et al*., 2005; 2009). Among these, CepR and CepI appear to be well conserved within the BCC (Venturi *et al*., 2004). CepI synthesizes primarily octanoyl-homoserine lactone (OHL), and lower levels of hexanoyl-homoserine lactone (Lewenza *et al*., 1999; Gotschlich *et al*., 2001; Huber *et al*., 2001; Aguilar *et al*., 2003a). Null mutations in *cepI* or *cepR* increased the production of the siderophore ornibactin, and decreased the production of secreted lipases and metalloproteases ZmpA and ZmpB (Lewenza *et al*., 1999; Lewenza and Sokol, 2001; Sokol *et al*., 2003; Kooi *et al*., 2006). CepI and CepR are also required for swarming motility and biofilm formation (Huber *et al*., 2001) and for pathogenicity in several animal models (Kothe *et al*., 2003; Sokol *et al*., 2003). *B. cenocepacia* also expresses the

CciI and CciR proteins, which are encoded on a genomic island called *cci* (cenocepacia island), that is associated with epidemic strains (Malott *et al*., 2005). The CepIR and CciIR systems extensively interact, in that CciR negatively regulates *cepI*, while CepR is required for expression of the *cciIR* operon (Malott *et al*., 2005). Transcriptional profiling studies indicate that CepR and CciR regulate many of the same genes, but do so in opposite ways (O'Grady *et al*., 2009). *B. cenocepacia* also encodes an orphan LuxR homologue called CepR2, which represses a cluster of genes that may direct production of an antibiotic or other secondary metabolite (Malott *et al*., 2009).

In addition to transcriptional profiling several other approaches have been used to identify genes whose expression is influenced by CepR and/or OHL. In one study, the proteome of a wild-type *B. cenocepacia* was compared with that of a *cepR* mutant. Fifty-five proteins were found to be differentially expressed in the two strains, approximately 10% of all detected proteins (Riedel *et al*., 2003). In a second study, fragments of a *B. cepacia* strain were cloned into a promoter trap plasmid and introduced into an *E. coli* strain that expressed CepR (Aguilar *et al*., 2003b). Twenty-eight promoter fragments were identified as being induced by OHL, and in all cases, induction required CepR. In a third study, a library of *B. cenocepacia* DNA fragments were introduced into a plasmid containing a promoterless *luxCDABE* operon (Subsin *et al.,* 2007). That study identified 58 OHL- inducible promoters and 31 OHL-repressible promoters. Regulation of nine of these genes required CepR, while the others were not tested. Seven OHL-inducible genes were identified by screening a library of *lacZ* fusions (Weingart *et al*., 2005). Induction of all of these genes required CepR. Purified CepR-OHL complexes bound with high affinity and specificity to specific DNA sequences at two target promoters (Weingart *et*

al., 2005). These binding sites contained a 16 nucleotide imperfect dyad symmetry and were centered approximately 44 nucleotides upstream of the transcription start sites. These two sites are to date the only experimentally confirmed CepR binding sites. Most of the studies described above do not distinguish whether a target promoter is controlled by CepR directly or indirectly. CepR could regulate a promoter indirectly, for example, by directly regulating an unknown regulatory gene whose product directly regulates that promoter. Alternatively, a CepR mutation might perturb cellular physiology in such a way that various promoters are affected by secondary effects.

To date, the most comprehensive study attempting to define the optimal CepR binding site was done by Chambers, Sokol and colleagues (Chambers *et al*., 2006), who approached this question with an impressive combination of genetics and bioinformatics. Mutagenesis of the known CepR binding site within the *cepI* promoter completely abolished induction (Chambers *et al*., 2006). The promoters of six genes known to be induced by OHL were used to formulate a consensus CepR binding motif (Chambers *et al*., 2006). This information was used to test eight additional candidate promoters, six of which were CepR- regulated. Ultimately, 10 inducible promoters were used to refine the consensus sequence, and 57 possible CepR binding sites were identified upstream of various genes.

The consensus motif identified in the Chambers study included the sequence CTG-N10-CAG, which has dyad symmetry. However, several other bases in the consensus did not preserve this symmetry, and some of those non-symmetric bases were said to be highly conserved (Chambers *et al*., 2006). The partial dyad symmetry suggests that CepR binds DNA as dimer and that the two DNA binding domains have rotational symmetry.

Although we have no proof of this, structural studies of a related protein support this idea (Vannini *et al*., 2002; Zhang *et al.,* 2002). Several other LuxR-type proteins are thought to decode dyad symmetrical sequences (Whitehead *et al*., 2001; White and Winans, 2007; Antunes *et al*., 2008). In the present study, we tested the 10 putative CepR binding sites described above for the ability to bind purified CepR-OHL complexes. We also systematically resected and mutated a known CepR binding site, and use the resulting information to identify four new promoters that are regulated directly by CepR. All four promoters are regulated by CepR *in vivo*, require their binding sites for regulation, and bind with high affinity to CepR-OHL *in vitro.*

2.3 Results

Note that only partial results correlating to the work in which I contributed are presented below.

2.3.1. Binding of CepR to 10 known or putative CepR binding sites

As described above, another group identified 10 putative CepR binding sites that lay upstream of CepR-regulated promoters (Chambers *et al*., 2006). However, these DNA sequences were not tested for CepR binding, and were not shown to be required for CepR-dependent gene expression *in vivo*. As CepR-OHL complexes have been shown to bind DNA fragments in electrophoretic mobility shift assays (EMSA) and DNase footprinting (Weingart *et al*., 2005), I therefore assayed CepR binding to these 10 sequences. The 10 sites include the two that we had previously tested, one in the *cepI* promoter and the other in the *aidA* promoter. Synthetic double-stranded oligonucleotides 38 base pairs long were radiolabelled, combined with purified CepR-OHL complexes in

the presence of a 10 000-fold excess (mass/mass) of calf thymus DNA, and size fractionated using native gel electrophoresis. CepR bound to the 38-mer containing the *cepI* binding site with an affinity of approximately 65 nM, in reasonable agreement with previous estimates (Fig. 2.1). It also bound to the fragment containing the *aidA cep* box, although significantly more weakly, also as expected (Weingart *et al*., 2005). To our surprise, of the eight other DNA fragments tested, only one was detectably shifted, even using very high concentrations of CepR (Fig. 2.1). The shifted fragment was that of the *phuR* promoter, which appeared to form two shifted complexes. I also tested the consensus sequence that was derived in the Chambers study (Chambers *et al*., 2006). Because this consensus was only 18 nucleotides in length, we used flanking sequences that were derived from the *cepI cep* box. This consensus sequence was bound by CepR with relatively high affinity (Kd = 210 nM, Fig. 2.1).

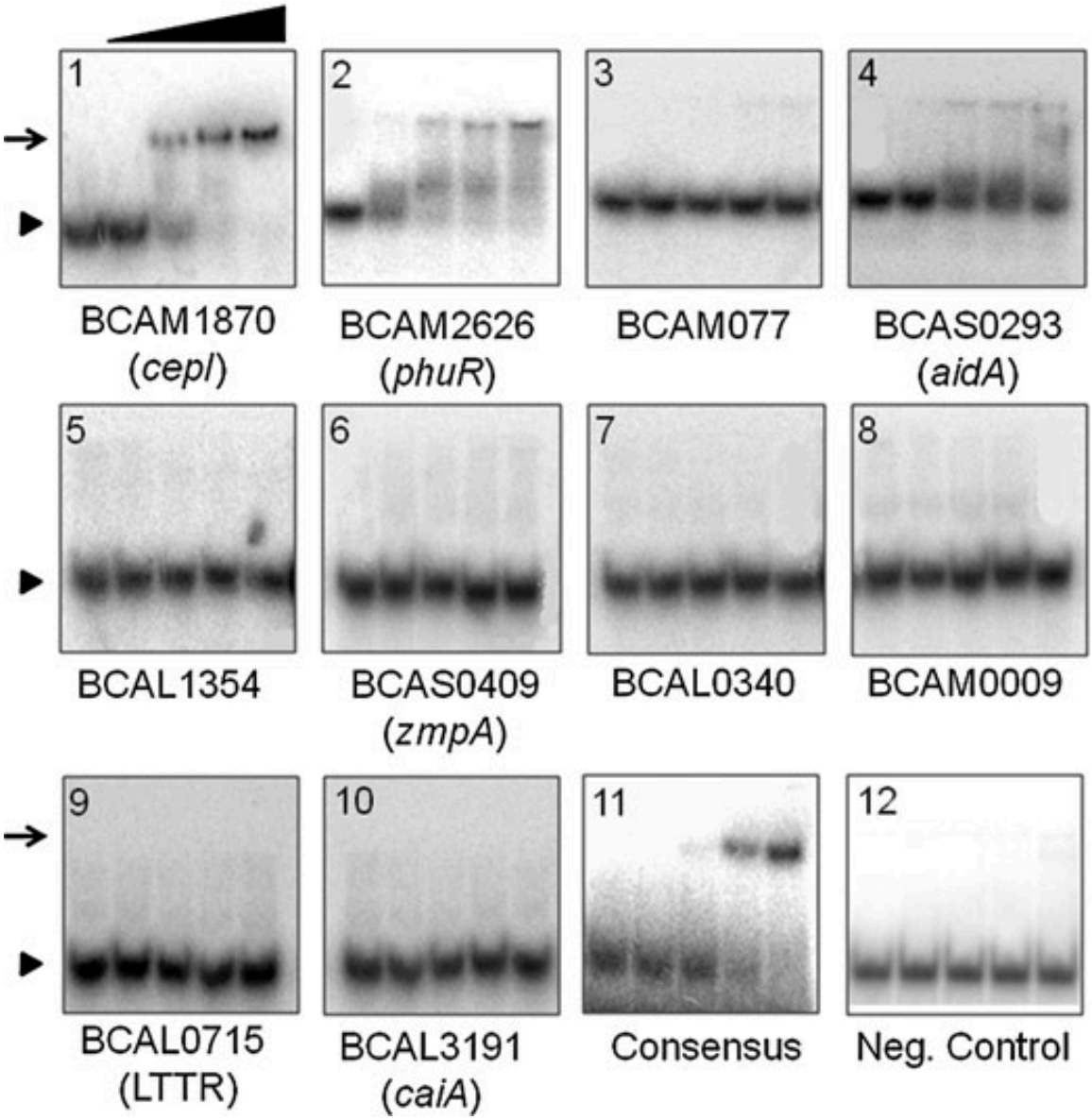

Fig. 2.1. Electrophoretic mobility shift assays of putative CepR binding sites. Synthetic double-stranded DNA fragments 38 nucleotides long were end-labelled, combined with purified CepR-OHL complexes, and size-fractionated using native PAGE. In panel 1 and 11, CepR-OHL was added at the following concentrations (lanes 1–5): 0 nM, 21 nM, 65 nM, 210 nM and 650 nM. In all other panels, CepR-OHL was added at the following concentrations (lane 1–5): 0 nM, 650 nM, 2060 nM, 6500 nM and 20 600 nM. Free DNA is indicated with a filled triangle and shifted complexes are indicated using an arrow. **Work done by Ryan.**

2.3.2. Contribution of each base to binding affinity in vitro

As a working hypothesis, it was assumed that the CepR DNA binding domain probably has twofold rotational symmetry and that the DNA binding sites should therefore have dyad symmetry. Identifying a fully symmetric binding site would greatly facilitate mutagenesis studies, as we could then construct duplex DNA molecules each containing two copies of the same oligonucleotide. We therefore set out to identify a high-affinity CepR binding site that was perfectly symmetric.

This work was conducted by Dr. Esther Costa, who constructed two DNA fragments, designated L-L′ and R′-R, which are fully symmetrical, and designed using the left half and right half of the wild-type *cep* box of the *cepI* promoter respectively (Fig. 2.2). Fragment L-L′ bound CepR with threefold higher affinity than the wild-type sequence, and appeared to form well-focused complexes rather than smears. This finding provides further evidence that CepR binds DNA as a rotationally symmetric dimer. In contrast, fragment R′-R bound CepR weakly. This indicates that sequences within the left (promoter-distal) half-site of the wild-type *cep* box contribute more to the overall binding affinity than do the sequences within the right (promoter-proximal) half-site. Fragment L-L′ was there- fore used as a starting point for systematic mutagenesis.

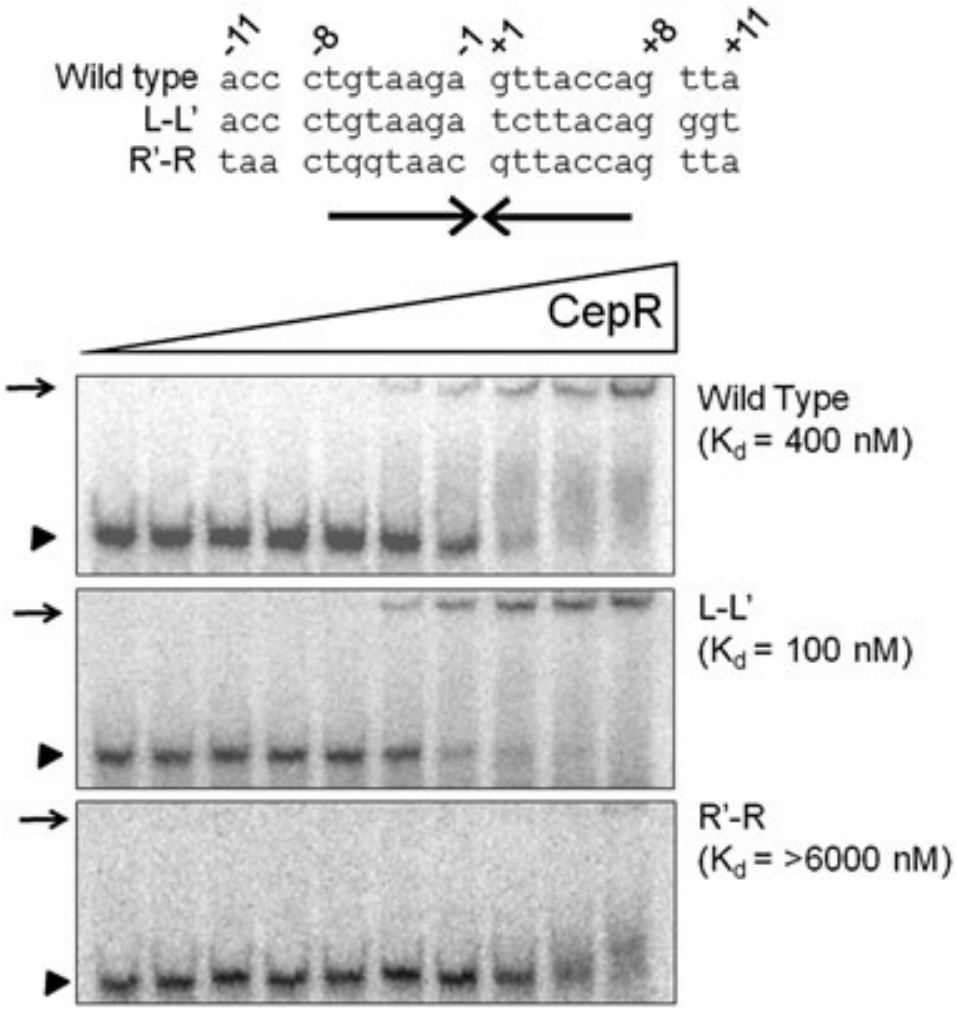

Fig. 2.2. CepR binding affinity for fully symmetrical CepR binding sites. The sequence designated L-L′ is composed of sequences of the native *cep* box of the *cepI* promoter from positions -11 to -1 followed by the inverse complement of these sequences. Fragment R′-R is similar but contains sequences of the native *cep* box from positions +1 to +11, and their inverse complements at positions -11 to -1. DNA fragments were radiolabelled and combined with CepR-OHL in the following concentrations (lanes 1–10) 0 nM, 0.6 nM, 1.8 nM, 6 nM, 18 nM, 60 nM, 180 nM, 580 nM, 1830 nM and 5780 nM. Complexes were size-fractionated by native PAGE. Kd values were calculated by determining the amount of CepR-OHL required to shift half of the DNA fragments. **Word was done by Costa.**

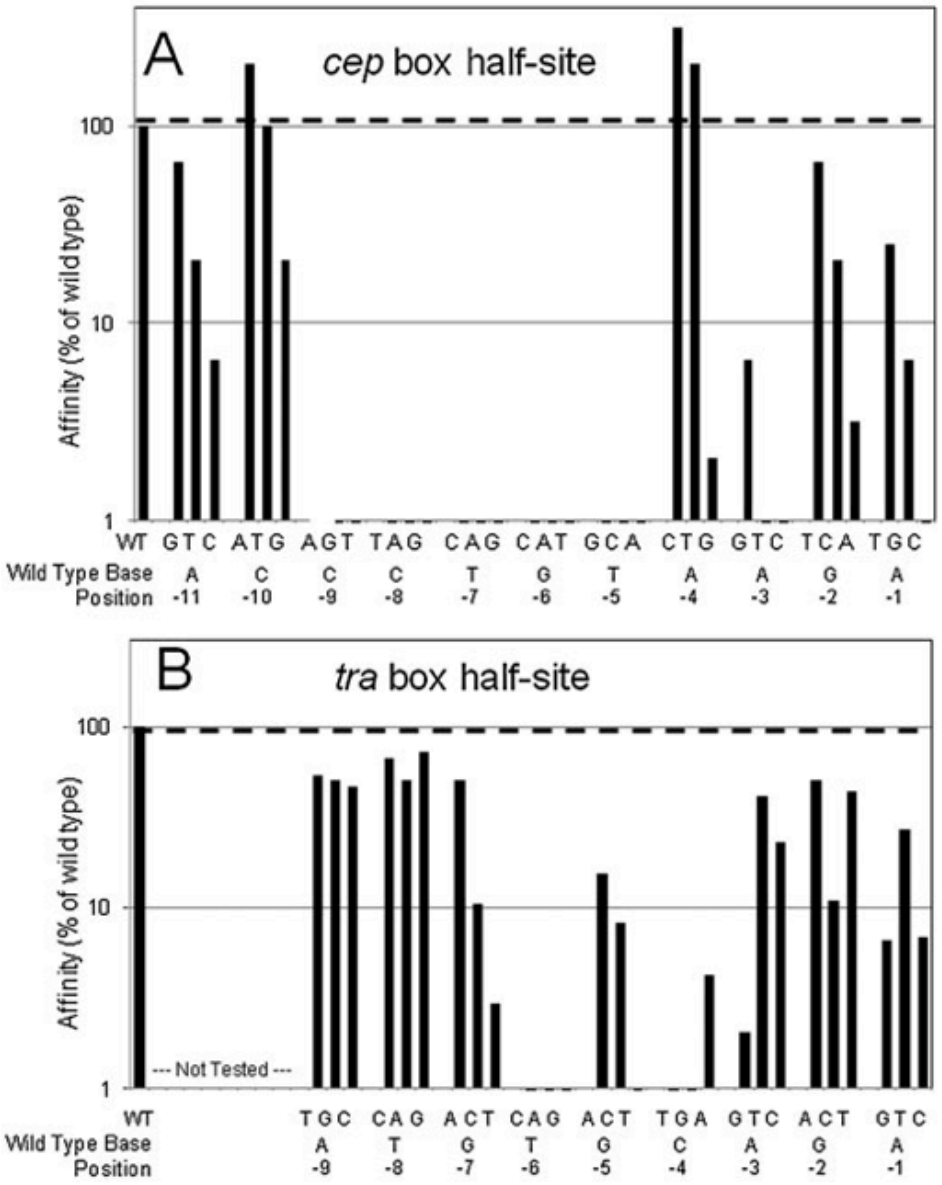

Fig. 2.3. Summary of binding affinities of *cep* boxes having the indicated alterations. Only the left half-site is shown. Wild-type binding affinity is indicated using a dashed horizontal line.
A. Affinity of wild type and mutant *cep* boxes for CepR-OHL. **Work done by Wei.** B. Affinity of wild type and mutant *tra* boxes for TraR of *A. tumefaciens* is shown for comparison (White and Winans, 2007).

2.3.3. CepR causes a DNA bend at its binding site

As described above, the critical bases required for CepR binding extend from nucleotide -9 to -5 (and +5 to +9 on the opposite half-site, see Fig. 2.3A). This is in some ways quite different from the sequences decoded by TraR of *A. tumefaciens* (White and Winans, 2007), where nucleotides -6 to -4 (and +4 to +6) were essential (Fig. 2.3B). Structural studies showed hydrogen bonding between TraR and these bases (Vannini *et al*., 2002; Zhang *et al*., 2002). Therefore, the critical bases detected by CepR are located considerably further from the dyad axis than those bases detected by TraR. Binding each of the half-sites of the *cep* box might require that the DNA recognition helices of CepR be further apart from each other than their counterparts in TraR. Alternatively or additionally, CepR might impart a higher angle DNA bend to a *cep* box than does TraR to a *tra* box, in effect bringing the two half-sites of DNA closer together. TraR induces a 30° bend in this DNA sequence (Vannini *et al*., 2002; Zhang *et al*., 2002; Pappas and Winans, 2003), and this model predicts that CepR might impart a greater bend.

To determine whether CepR causes a DNA bend at its binding site, I introduced a DNA sequence containing a consensus *cep* box into plasmid pBEND3, a plasmid that facilitates the study of intrinsic or protein-induced DNA bending (Zwieb and Brown, 1990). This plasmid allows the creation of a set of DNA fragments that are the same length and circularly permuted (Fig. 2.4.C). The *cep* box will therefore lie near one end of some fragments and closer to the center of other fragments. If CepR causes a DNA bend, then complexes having this bend near the center of the fragment will migrate in electrophoretic gels more slowly than complexes whose bend is closer to one end of the fragment (Zwieb and Brown, 1990). The resulting plasmid was digested individually with three different restriction endonucleases, added sufficient CepR to shift these fragments

and size-fractionated these complexes by native PAGE (Fig. 2.4). For comparison, a similar analysis was done using the TraR binding site and purified TraR.

The mobility of the EcoRV-generated fragment was considerably lower than fragments generated by the other two enzymes (Fig. 2.4A). This is diagnostic of a DNA bend. An identical analysis using TraR and *tra* box DNA showed a somewhat similar result (Fig. 2.4B). However, the differences in mobility were less pronounced, indicating a lower angle DNA bend. Using the equation of Thompson and Landy (Thompson and Landy, 1988), I estimate the bend angle to be approximately 45° for CepR and 30° for TraR. Structural studies of TraR-DNA complexes agree with this estimate (Vannini *et al.,* 2002; Zhang *et al.,* 2002). These data indicate that CepR causes a higher angle DNA bend than does TraR, confirming our predictions.

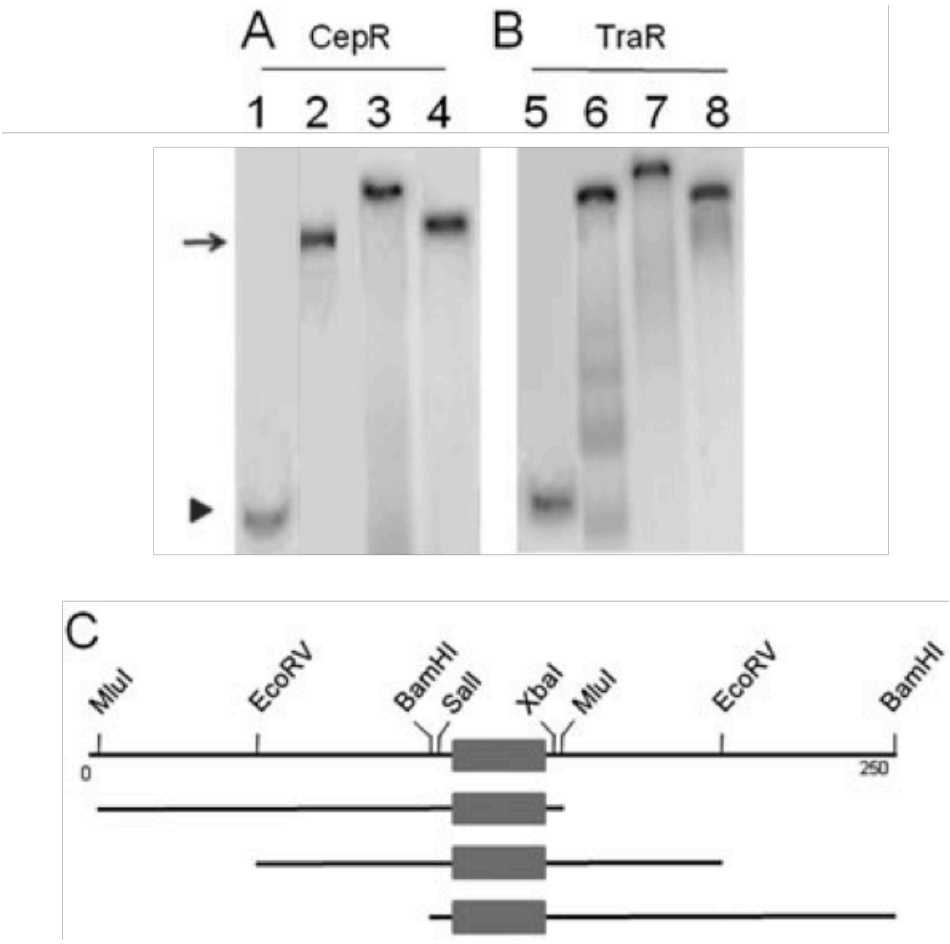

Fig. 2.4. Assays for a CepR-directed DNA bend. A. The optimal CepR binding site, as determined in Fig. 5 (ACCCTGTCAGATCTCACAGGGT) was introduced into plasmid pBEND3, and the resulting plasmid was digested individually with restriction endonucleases BamHI (lane 1, 2), EcoRV (lane 3) or MluI (lane 4), combined with CepR-OHL (lanes 2–4), and size-fractionated by native PAGE. B. A similar plasmid was constructed containing the consensus *tra* box, cut with BamHI (lane 5, 6) EcoRV (lane 7) or MluI (lane 8). Fragments were combined with TraR-OOHL (lanes 6–8), and size-fractionated by native PAGE. C. A map of the multiple cloning site of the pBEND3 derivatives containing a *cep* box or a *tra* box. **Work done by Ryan.**

2.3.4. Identification of new CepR-regulated promoters

The enoLOGOS web server (Workman *et al*., 2005) was used to obtain a pictorial representation of the most favored bases in a canonical *cep* box. The dissociation

constants were used as input, and the few mutant sequences that were not detectably bound by CepR were arbitrarily assigned a dissociation constant of 1 mM, which is eightfold weaker than the weakest detected binding. The resulting *cep* box logo is shown in Fig. 2.5. The EnoLOGOS web server also converted these dissociation constants into a log-likelihood matrix. This matrix was used to obtain a similarity score between the *cep* box logo and every 22 nucleotide sequence in the *B. cenocepacia* genome. This was done by Dr. David Schneider (USDA) using the MOODS algorithm (Korhonen *et al*., 2009), which provided a list of 237 possible *cep* boxes and scored their similarity to the consensus sequence.

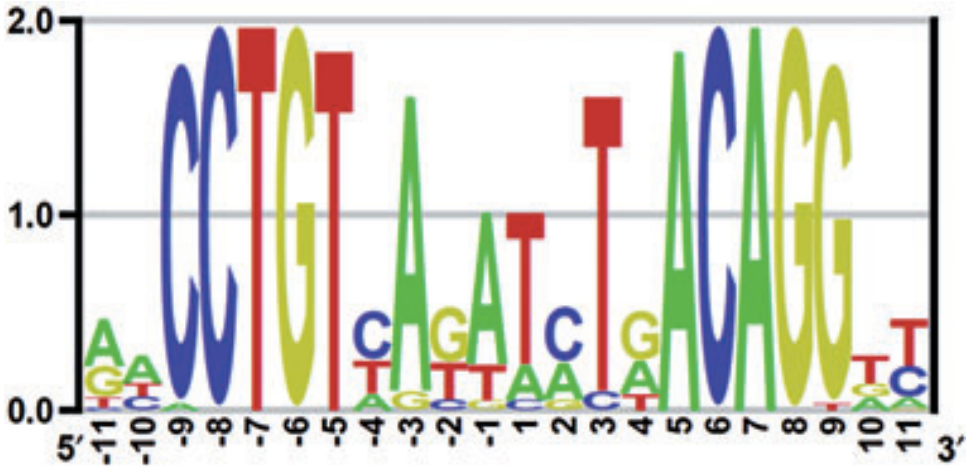

Fig. 2.5. A *cep* box logo, derived from the experimental dissociation constants for mutant *cep* boxes and enoLOGOS.

Many of the DNA sequences that resemble the *cep* box logo lie far from any predicted promoter. However, 142 of these sites lie within 300 nucleotides upstream of a predicted translation start site. The expression of most of these genes is not affected by a CepR mutation (O'Grady *et al*., 2009). However, 43 genes that have possible nearby *cep* boxes are differentially expressed at least twofold by a mutation in *cepR*. Of these 43

genes, 13 were chosen for further analysis (Table 2.1). These 13 were chosen for a variety of reasons. Two were *cepI* and *aidA*, which served as positive controls. We also chose the *cepR* promoter, which was said to be autorepressed in one study (Lewenza and Sokol, 2001) but not in another (O'Grady *et al*., 2009). BCAM0188 encodes CepR2, which if induced would show a quorum cascade. Two other genes are adjacent to BCAM0188. BCAM1869 was chosen because it lies adjacent to and divergent from *cepR*. BCAM1413a and BCAM1414 were chosen because they encode three AidA homologues. The promoter of pBCA055 was found to be induced in an earlier study from our lab (Weingart *et al*., 2005).

To determine whether these genes were indeed regulated by CepR, Dr. Flores-Mireles fused each of the 13 putative promoters to *lacZ* on a multicopy plasmid. Each fragment extended upstream just far enough to include the putative CepR binding site plus 6–8 extra nucleotides. Thirteen similar fragments were constructed that included only the downstream half of the predicted binding sites. All 26 plasmids were introduced into the *cepI* mutant strain K56-2I and tested for induction of β-galactosidase in the absence of OHL or in the presence of two OHL concentrations, one that causes approximately half-maximal induction of *cepI* and *aidA* (Weingart *et al*., 2005), and one that causes maximal expression.

Of the 13 plasmids containing full CepR binding sites, 12 were predicted to be induced by OHL, as one (*cepR*) was predicted to be repressed. Of these twelve, seven were induced at least fourfold by 1 mM OHL (Table 2.1). Of these seven, induction of six was abolished or severely reduced in isogenic plasmids containing only half of the putative *cep* box (Table 2.1). The exception was the BCAM0186 promoter, which was

strongly induced even with only half of its *cep* box. This gene lies close to BCAM0188 (*cepR2*), and may be regulated by the CepR2 protein (Malott *et al*., 2009). Of the six promoters whose OHL induction required a *cep* box, two were previously characterized (*cepI* and *aidA*), while four were new [BCAL0510, BCAM1869, BCAS0156 and pBCA055-4 (*bqiCD*)]. Three of these four genes were previously shown to be induced by CepR, and to be unaffected by mutations in CciR or CepR2 (Malott *et al*., 2009; O'Grady *et al*., 2009), although those studies did not show whether CepR acted directly or indirectly. The fourth is BCAS0156, which was not previously reported to be induced.

The four newly identified CepR-regulated promoters are divided among the four replicons. BCAL0510 lies on the largest chromosome and its product resembles a group of hypothetical proteins (data not shown). BCAM1869, lies on chromosome 2 and is adjacent to and divergent from *cepR*. The *cep* box lies 114 nucleotide upstream of the BCAM1869 translation start site. Induction was reduced, although not abolished, by removing the promoter-distal half of the *cep* box. BCAM1869 and *cepR* are adjacent in many species of *Burkholderia*, providing suggestive evidence that their proteins are in some way functionally linked. A protein homologous to BCAM1869 was recently shown to play a role in transcription regulation (Mattiuzzo *et al*., 2010). The *cepR* gene was previously identified as being autorepressed in one study (Lewenza and Sokol, 2001), although in another study it was found to be unregulated by CepR (O'Grady *et al*., 2009). Our data support the latter study (Table 2.1). BCAS0156, which lies on chromosome 3, resembles a family of b-lactamases and other penicillin-binding proteins (pfam00144). pBCA055 (*bqiC*) and pBCA054 (*bqiD*) lie on the 93 KB plasmid and appear to be expressed as an operon. pBCA055 is a multidomain protein whose central domain

resembles GGDEF proteins (COG2199) and whose C-terminal domain resembles EAL proteins (pfam00563), and may therefore synthesize and or degrade c-di-GMP (Romling and Amikam, 2006). pBCA054 has a C-terminal domain that resembles the DNA binding domains of LuxR proteins (pfam00196), suggesting that it may be a transcription factor.

The fact that these six promoters required OHL and a putative CepR binding site for induction suggested that CepR might directly activate them. To provide additional evidence, I determined whether CepR and OHL could activate these promoters in a heterologous host lacking any other LuxR-type protein. I introduced into *E. coli* strain MC4100 plasmids containing each of the six inducible promoters as well as plasmids containing the same promoters but containing only part of the CepR binding site. These strains also contained a second plasmid expressing CepR or a vector control. Strains containing CepR were cultured in the presence or absence of OHL and assayed for β-galactosidase. All six strains containing CepR and a full *cep* box showed strong induction of β-galactosidase by OHL (Table 2.2). Strains whose plasmids lacked the full *cep* boxes were either uninduced or weakly induced by OHL, as expected. Strains lacking CepR were not induced by OHL, also as expected. I conclude that for each promoter, induction by OHL requires CepR and a CepR binding site. The reconstitution of CepR-dependent induction of these promoters in *E. coli* provides further evidence that each is directly regulated by CepR.

Table 2.1 Induction of *B. cenocepacia* genes by OHL. **Work done by Flores-Mireles.**

Gene	Sequence	Score	cep box	No OHL	OHL (1nM)	Ratio	OHL (1uM)	Ratio
BCAL0510	CGCCCGCCAGAATTGACAGGCC	6.338	full	38 ± 2	469 ± 117	3.5	747 ± 73	5.4
			half	351 ± 27	167 ± 40	0.5	303 ± 16	0.9
BCAM0186	ACCCTGTGATTTGATGCCGGTC	9.398	full	43 ± 7	97 ± 6	2.3	77 ± 67	17.9
			half	63 ± 6	69 ± 3	1.1	762 ± 34	12.1
BCAM0188 (*cepR2*)	ATCCTGTTCAAAAGGACAGTTT	-1.875	full	543 ± 134	743 ± 141	1.4	145 ± 105	2.6
			half	669 ± 23	876 ± 27	1.3	1678 ± 223	2.5
BCAM0189 (*cnaR*)	ATCCTGTTCAAAAGGACAGTTT	-1.875	full	180 ± 17	150 ± 17	0.8	360 ± 16	2
			half	170 ± 5	123 ± 9	0.7	329 ± 40	1.9
BCAM1413 a (*aidC*)	TACCTGTCAGGTTTGATGGGGG	6.26	full	134 ± 6	145 ± 6	1.1	408 ± 26	3.1
			half	173 ± 16	201 ± 8	1.1	217 ± 29	1.2
BCAM1414	TACCTGTCAGGTTTGATGGGGG	6.26	full	80 ± 23	83 ± 8	1.03	175 ± 52	2.1
			half	101 ± 8	93 ± 9	0.9	114 ± 10	1.1
BCAM1868 (*cepR*)	ACGCTGTCATACTTGTCAGGTT	-8.188	full	205 ± 6	193 ± 4	0.94	224 ± 16	1.1
			half	nd	nd	nd	nd	nd
BCAM186	ACGCTGTCATACTTGTCAGGTT	-8.188	full	179 ± 13	1598 ± 193	8.9	3536 ± 125	19.7
			half	121 ± 12	153 ± 3	1.3	609 ± 45	5
BCAM1870 (*cepI*)	ACCCTGTAAGAGTTACCAGTTA	-5.885	full	60 ± 3	655 ± 25	10.9	2489 ± 162	41.5
			half	40 ± 16	39 ± 3	0.9	133 ± 42	3.3
BCAS0155-0153	ATACTGTTAAAACCGGCAGGTT	-9.521	full	67 ± 4	72 ± 6	1.1	176 ± 44	2.6
			Half	87 ± 7	61 ± 4	0.7	114 ± 10	1.3
BCAS0156	ATACTGTTAAAACCGGCAGGTT	-9.521	full	110 ± 22	518 ± 64	4.7	1399 ± 144	12.7
			half	101 ± 7	84 ± 6	0.8	116 ± 14	1.1
BCAS0293 (aidA)	AAGCTGTAAAAGTAAACAGGTC	-1.315	full	109 ± 9	416 ± 18	3.8	1648 ± 61	15.1
			half	118 ± 8	108 ± 7	0.9	174 ± 32	1.6
pBCA055-054 (*bqiCD*)	CCACTGTCAAATCTACGAGGGC	2.799	full	149 ± 49	775 ± 193	5.2	3126 ± 1052	20.9
			half	167 ± 6	167 ± 1	1	272 ± 106	1.6

Table 2.2 Activation of CepR-regulated promoters in the heterologous host *E. coli*. **Work done by Ryan.**

Gene	*cep* box	+ CepR		Ratio	- CepR	Ratio
		No OHL	(1 µM)		(1 µM)	
BCAM1870 (*cepI*)	full	4 ± 0.2	491 ± 6.2	128	4 ± 0.7	120
	half	4 ± 0.2	12 ± 2.7	3	4 ± 1.1	3
BCAS0293 (*aidA*)	full	4 ± 0.2	176 ± 17.7	44	4 ± 0.7	43
	half	4 ± 0.1	8 ± 1	2	3 ± 0.3	3
BCAL0510	full	4 ± 0.1	49 ± 3.3	13	4 ± 0	14
	half	4 ± 0.1	11 ± 0.4	3	5 ± 0.9	2
BCAM1869 (*cepJ*)	full	3 ± 0.1	211 ± 6.9	65	4 ± 0.3	52
	half	4 ± 0.2	29 ± 0.7	7	4 ± 0.1	8
pBCA055-054 (*bqiCD)*	full	4 ± 0.1	159 ± 13.4	44	3 ± 0.2	48
	half	4 ± 0.1	4 ± 0.5	1	3 ± 0	1
BCAS0156	full	4 ± 0.2	41 ± 1.3	10	3 ± 0.4	12
	half	4 ± 0.1	3 ± 0.1	1	3 ± 0.2	1
pYWN302.1		4 ± 0.3	3 ± 0.1	1	4 ± 0.2	1

I used EMSA to test whether purified CepR could bind these sites *in vitro*. Synthetic duplex oligonucleotides 26 base pairs in length were combined with highly purified CepR-OHL complexes and size-fractionated by native PAGE (Fig. 2.6). All of the six inducible promoters were shifted, indicating that they can be bound by CepR. These data are therefore consistent with the hypothesis that these four genes are directly regulated by CepR-OHL complexes.

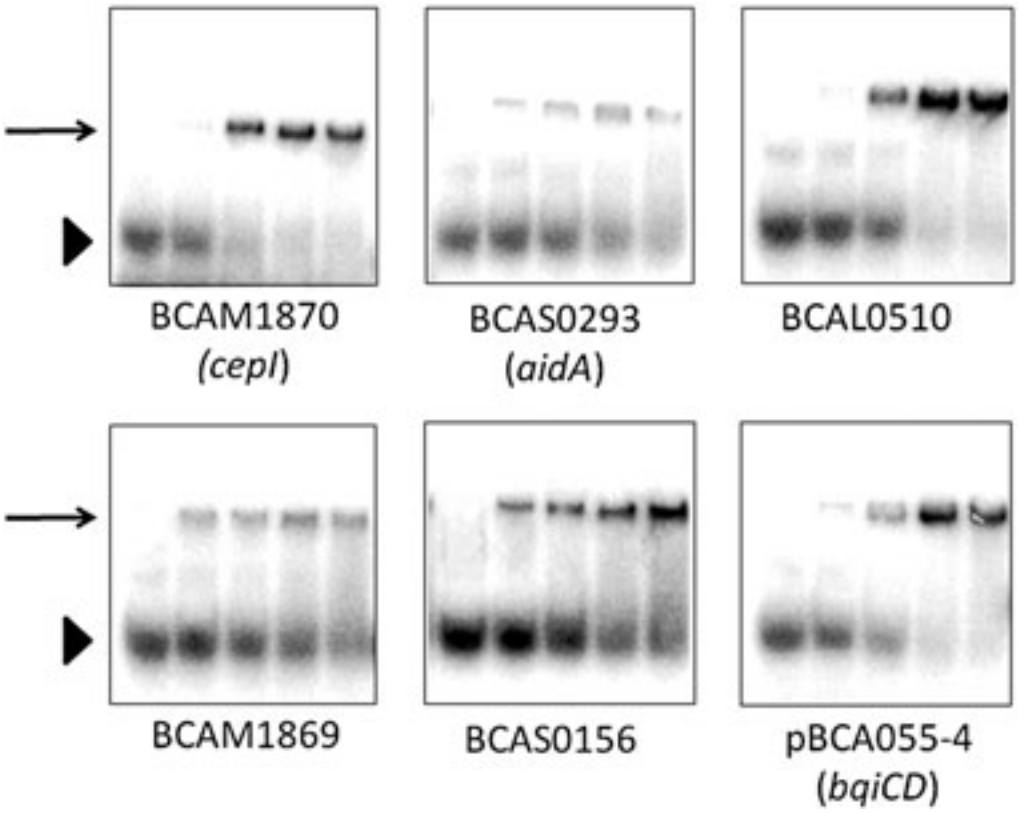

Fig. 2.6. CepR binding affinity for all known *cep* boxes. Duplex DNA molecules 26 nucleotides in length containing the sequences shown in Fig. 10 were radiolabelled, combined with purified CepR-OHL, and the resulting complexes were size-fractionated by native PAGE. For the *cepI* cep box (upper left panel), CepR-OHL was added at the following concentrations (lanes 1–5): 0 nM,
21 nM, 65 nM, 210 nM and 650 nM. In all other panels, CepR-OHL was added at the following concentrations (lane 1–5): 0 nM, 650 nM, 2060 nM, 6500 nM and 20 600 nM. **Work done by Ryan.**

2.4 Discussion

Members of our laboratory have previously used genetic, biochemical and structural approaches to study interactions between another LuxR-type protein, TraR, and its DNA binding site. TraR binds DNA as a dimer, and the two DNA binding domains of each dimer have twofold rotational symmetry. The DNA binding site also has twofold rotational symmetry, and complexes between the TraR-CTD and DNA also have

rotational symmetry. This type of symmetry is found in the binding sites of other LuxR-type proteins, and of a great number of other DNA binding proteins. The imperfect dyad symmetry of two CepR binding sites leads us to believe that CepR would follow a similar pattern. It was therefore interesting that the consensus sequence previously described had several highly conserved asymmetric bases (Chambers *et al*., 2006). Unfortunately, only three of the ten putative bindings sites was detectably bound by purified CepR-OHL. Our studies suggest that a core CTG-N10-CAG is critical for binding. Loss of one of these six bases may be tolerated, but loss of any two or more probably is not. Of the 10 DNA sequences compiled in the Chambers study, three had all six bases of this consensus and were bound by CepR-OHL, while seven sequences lacked between one and four of these bases, and were not bound. The consensus sequence identified previously (Chambers *et al*., 2006) also has all six of these bases, and was bound. In the Chambers study, there seemed to be an implicit assumption that all CepR-inducible genes would be induced directly by CepR. In light of the present data, it seems more likely that induction of some genes could occur indirectly.

In order to study specifically how CepR decodes its binding site, we felt it was important to move to an *in vitro* system using purified components. The finding that 26 nucleotides are needed for full binding affinity was somewhat surprising, as this sequence would extend 2.5 helical turns, or 1.25 turns per half-site. It is far from clear why such a long sequence would be required. CepR does not strongly discriminate specific sequences at positions -11, -10,+10 or +11 (Fig. 2.3), suggesting that the need for bases far removed from the dyad center may not be sequence-specific.

We also tested a set of 22 nucleotide, perfectly symmetric sequences based upon

the left half-site of the *cepI cep* box. Mutation of any of the bases from -9 to -5 (and the corresponding bases at +5 to +9) either abolished or strongly impaired binding affinity. From these data, we conclude that the core CepR binding site could be amended from the 16 nucleotide sequence CTG-N10-CAG to the 18 nucleotide sequence CCTGT-N8-ACAGG. We had previously discounted the bases at positions -9, -5, +5 and +9 as they are not part of the dyad symmetry of the two *cep* boxes previously aligned (Weingart *et al*., 2005). However, these bases are somewhat conserved in a new set of CepR binding sites.

The effects of mutations within the central spacer (-4 to +4) had more variable effects on binding affinity. Mutations from A to C or T at position -4 (and from T to G or A at position +4) enhanced affinity, indicating that the original L-L′ sequence was not optimal at this position. Many of the newly identified *cep* boxes contain the bases C or T at position -4 and a G or A at position +4. We denote the *cep* box residues from -4 to +4 as the central spacer, and predict that there are no sequence-specific protein–DNA contacts in this region, as was shown for TraR (Vannini *et al*., 2002; Zhang *et al*., 2002). It is well established that non-contacted bases can have large effects on protein affinity, generally via effects on the helical pitch or on the flexibility of the DNA, or by imparting a sequence-directed DNA bend (Sarai and Kono, 2005). This phenomenon is sometimes referred to as "indirect readout", while sequence decoding by direct protein-DNA interactions is called "direct readout".

The identification of the bases essential for CepR binding facilitated a search for new genes that could be regulated directly by CepR. Of the thirteen promoters that were tested, six were significantly induced by OHL and required the putative *cep* box for this

induction. These six CepR-regulated genes are distributed across all three chromosomes and the 92 KB plasmid. BCAS0293 (*aidA*) was reported previously to be OHL- regulated (Aguilar *et al*., 2003a; Riedel *et al*., 2003; Weingart *et al*., 2005; Chambers *et al*., 2006). This gene is in a two-gene operon, and the downstream gene, *aidB,* is homologous to *aidA*. This homology extends to three additional genes that we designate *aidC, aidD* and *aidE* (BCAM1413a, BCAM1412, and BCAM1414, respectively, see Fig. S3), none of which was induced more than 2–3 fold by OHL (Table 2.1). BCAM1413a and BCAM1414 are divergent and flank a *cep* box, although the score of this site is weak (Table 2.1). The roles of these proteins are unknown, although AidA was previously reported to play a role in the slow killing of the nematode *Caenorhabditis elegans* (Huber *et al.,* 2004). All five proteins are members of the PixA protein family (pfam12306).

In this study, we also examined the regulation of gene BCAM0186. This gene was unusual in that it was strongly induced by OHL and had a possible *cep* box, yet induction was *cep* box-independent. In hindsight, this should not have been surprising, as the similarity between this putative *cep* box and the consensus is rather weak, and the *cep* box lies almost 600 nucleotides upstream of the BCAM0186 translation start site. Its expression was reported to be inhibited by the product of the nearby BCAM0188 (*cepR2*) (Malott *et al*., 2009). We hypothesize that BCAM0186 may be directly repressed by CepR2, and that repression might be blocked by OHL.

2.5 Experimental procedures

Note that only the procedures for the experiments I performed are included below. For the complete experimental procudures for this study, see the published manuscript[1].

Bacterial strains and growth conditions

Strains used in this study are described in Table 2.3. As needed, *B. cenocepacia* was cultured in 100 μg ml^{-1} of trimethoprim or 300 μg ml^{-1} of tetracycline. *E. coli* strains were cultured with 15 μg ml^{-1} of tetracycline, 100 μg ml^{-1} of streptomycin or 100 μg ml^{-1} of ampicillin. Plasmid pYWN302 was constructed by digesting pKP302 (Pappas and Winans, 2003) with NsiI, and inserting an NsiI fragment containing the *tet* gene of pBBR-MCS3.

Measurement of DNA bending by CepR

Synthetic oligonucleotides containing a consensus *cep* box or *tra* box are described in Table 2.4. They were allowed to self-anneal by heating to 95°C for 5 min and then gradually cooled to room temperature, then digested simultaneously with XbaI and SalI, and introduced into plasmid pBEND3 (Zwieb and Brown, 1990) after digestion with the same enzymes. The resulting plasmids, pGR110 (containing a *cep* box) and pGR111 (containing a *tra* box), were digested individually with BamHI, EcoRV or MluI to permute the position of the box with respect to the DNA termini, radiolabelled using T4 polynucleotide kinase (New England Biolabs) and [gamma-P32]-ATP and purified using Centri-Spin columns (Princeton Separations). Binding reactions (10 ml) contained 800 ng DNA and clarified lysates containing overexpressed CepR-OHL or TraR-OOHL in a buffer containing 10 mM Tris- HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 60 mM potassium glutamate, 30 mg ml^{-1} calf thymus DNA, 20 mg ml^{-1} BSA, and 10% glycerol, and were incubated for 30 min at 4°C, size-fractionated using a 14% polyacrylamide gel in TAE buffer.

Identification of new CepR-regulated promoters

The enoLOGOS (Workman *et al*., 2005) web server was used to construct a log-likelihood matrix from a set of EMSA- derived Kd values for set of 22 nucleotide sequences shown in Fig. 6. The Kd value for the reference sequence and each of its variants were converted to association constants which were entered into the program. EnoLOGOS returned a log-likelihood matrix, calibrated for a GC content of 67%, and scaled by a factor of -1 to conform to the usual sign convention for binding energies. DNA sequences that resemble the *cep* box logo were identified using the log-likelihood matrix and the MOODS algorithm (Korhonen *et al*., 2009). This algorithm created a set of over eight million 22 nucleotide sequences derived from the *B. cenocepacia* genome, each overlapping its nearest neighbors by 21 nucleotides. Each of these sequences was compared with the canonical *cep* box using the log-likelihood matrix. The optimal and least optimal sequences received scores of approximately -39 and 160, respectively, while the two confirmed CepR binding sites, upstream of the *cepI* and *aidA* promoters, received scores of -5.9, and -1.3 respectively.

Promoters containing suspected CepR binding sites were PCR amplified using oligonucleotides shown in Table 2.4. For each promoter, a fragment containing a complete CepR binding site was amplified, as well as a similar fragment containing only the promoter-proximal half of the site. These PCR fragments contained a KpnI site at the promoter-distal end, and a PstI site at the promoter-proximal end. The PCR fragments were purified by using QIAquick Gel Extraction Kits (Qiagen) and digested with KpnI and PstI (New England Biolabs). The digested fragments were cloned into the promoter

probe plasmid pYWN302, generating transcriptional fusions between each promoter and *lacZ*. The resulting plasmids were introduced into *B. cenocepacia* strain K56-I2 or *E. coli* strain MC4100 by electroporation. For assays of β-galactosidase, *B. cenocepacia* and *E. coli* strains were cultured in LB medium supplemented with either 300 mg of tetracycline or 100 ug ml^{-1} kanamycin and 12 ug ml^{-1}tetracycline, respectively, overnight at 37°C. Each culture was diluted 1:100 into LB medium containing the indicated concentrations of OHL, and incubated with aeration at 37°C to an OD600 of 0.4, and assayed for β-galactosidase specific activity (Miller, 1972). Experiments were performed in triplicate with three different isolates of each strain.

Table 2.3 Strains used in this study

Strains and Plasmids	**Relevant features**	**Reference**
Strains		
K56-2	B. cenocepacia wild type	Lewenza et al. 1999
K56-I2	B. cenocepacia K56 cepI::TpR	Lewenza et al. 1999
DH5α	E. coli α-complementation	Stratagene
MC4100	F- araD139 Δ(argF-lac)U169 rpsL150 relA1 deoC1 rbsR fthD5301 fruA25 λ-	Ferenci et al., 2009
Plasmids		
pBEND3	plasmid to facilitate measuring bent DNA	Zwieb *et al*., 1990
pYW302	Derivative of pKP302 expressing Tc^R	This study
pGR110	pBEND3 containing *cep* box, using GR151, GR152	This study
pGR111	pBEND3 containing *tra* box, using GR157, GR158	This study
pCW106	pRSETA derivative containing *cepR*	Weingart *et al*., 2005
pSRKKm	Broad host range expression vector, Km^R	Khan *et al*., 2008
pAFM113	pSRKKm derivative containing *cepR*	This study

pAFM323	BCAM1870 (*cep*I) full cep box cloned into pYWN302-1	This study
pAFM318	BCAM1870 (*cep*I) half cep box cloned into pYWN302-1	This study
pAFM266	BCAM1413a (aidC) full cep box cloned into pYWN302-1	This study
pAFM267	BCAM1413a (aidC) half cep box cloned into pYWN302-1	This study
pAFM263	BCAL510 full cep box cloned into pYWN302-1	This study
pAFM264	BCAL510 half cep box cloned into pYWN302-1	This study
pAFM271	BCAM1869 (cepJ) full cep box cloned into pYWN302-1	This study
pAFM272	BCAM1869 (cepJ) half cep box cloned into pYWN302-1	This study
pAFM314	BCAM0156 full cep box cloned into pYWN302-1	This study
pAFM315	BCAM0156 half cep box cloned into pYWN302-1	This study

Table 2.4. Oligonucleotides used in this study

Primers for cloning *cep* and *tra* box permutated variants

GR151	GCTTCTAGAACCCTGTAAGAGTTACCAGT	*cep* box forward, pairs with GR152
GR152	GCTGTCGACTGTAACTGGTAACTCTTACA	*cep* box reverse, pairs with GR151
GR157	CTAGACCGTATGTGCAGATCTGCACATGA	*tra* box forward, pairs with GR158
GR158	TCGACAATCATGTGCAGATCTGCACATAC	*tra* box reverse, pairs with GR157

Oligonucleotides for EMSA hybridization duplexes of *cep* boxes identified by Wei *et al.*, 2011

GR159	ACTCGATTGATTCCGATTTTAA	BCAS0293 (aidA) forward
GR160	TGCGGAGGCGACGGCTAA	BCAS0293 (aidA) reverse
GR161	GGTTTATCGCGACTTTTGC	BCAL0510 forward
GR162	GCCTCGGATGGCACAA	BCAL0510 reverse
GR165	TGGTAGGCATCCTGCCAG	BCAM1869 forward
GR166	AAGGGTGAAGTCATGCG	BCAM1869 reverse
GR167	TCTTTTTCATCGATTTTCGACG	BCAS0156 forward
GR168	AACGAGGCCGCCGTCAT	BCAS0156 reverse
GR169	TGATCAAGAAACCGTTACCACGT	BCAM1870 forward
GR170	CTGTGCTTTTGTATGCGTGCATT	BCAM1870 reverse
GR171	GAACGAGGCCGCCGTCAT	BCAS0155 forward

GR172	TCTTTTTCATCGATTTTCGACGC	BCAS0155 reverse
GR173	ACTGCGACAATTGGTCTTCTAT	pBCA055-4 (BqiC) forward
GR174	ACGAGATGTTTCTTCGGAAAGGA	pBCA055-4 (BqiC) reverse
GR265	CGCCGTCACCCTGTAAGAGTTACC	BCAM1870 (cepI) gel shift forward

Oligonucleotides for EMSA hybridization duplexes of *cep* boxes identified by Wei *et al.*, 2011

GR266	CTCATTTACACTGTTAAAGTTGTC	BCAM2626 (phuR) forward
GR267	AACATCAAAATTGACAAAGTTATC	BCAM077 forward
GR268	ATCATGGAAGCTGTAAAAGTAAAC	BCAS0293 (*aidA*) forward
GR269	TATTACCTTTCGGCAATAGTTGCC	BCAL1354 forward
GR270	GACACGTCTTGTTTAAAAGTCATC	BCAS0409 (*zmpA*) forward
GR271	GTCGTGCAACCAGTAAAACTTGCG	BCALL0340 forward
GR272	GGATTCCGTTCGCTTAGAGTTGTT	BCAM0009 forward
GR273	ATCCAGTGTCAAGTCAGACTTGAC	BCAL0715 forward
GR274	TCGTCGATGGTTGAAAGTGTCATC	BCAL3191 (*caiA*) forward
GR275	CGCCGTCACCCTGTAAAAGTTACC	Consensus (*cep* box) forward
GR276	AGGAGCCTGTAACTGGTAACTCTT	BCAM1870 (*cepI*) reverse
GR277	TGTGAAAGGCAACTGACAACTTTA	BCAM2626 (phuR) reverse
GR278	TTAAAGTCATAACTGATAACTTTG	BCAM077 reverse
GR279	CATTTTCCCGACCTGTTTACTTTT	BCAS0293 (*aidA*) reverse
GR280	GCTCAATCGAAACAGGCAACTATT	BCAL1354 reverse
GR281	GGAATGCATCAAGTGATGACTTTT	BCAS0409 (*zmpA*) reverse
GR282	TTTCGACCGGAATGCGCAAGTTTT	BCALL0340 reverse
GR283	AATTCGAAATATCGAACAACTCTA	BCAM0009 reverse
GR284	CGCCTTTACAAGCTGTCAAGTCTG	BCAL0715 reverse
GR285	CCAGTGTAGCACCGGATGACACTT	BCAL3191 (*caiA*) reverse
GR286	AGGAGCCTGTAACTGGTAACTTTT	Consensus (*cep* box) reverse

Oligonucleotides for cloning *cep* box fusions

ALFM323-Y	GGGGTACCCACCCTGTAAGAGTTACCAG	BCAM1870 (*cep*I) full cep box fusion, pairs with ALFM319-Y
ALFM318-Y	GGGGTACCTTACCAGTTACAGGCTCCTCG	BCAM1870 (*cep*I) half cep box fusion, pairs with ALFM319-Y
ALFM319-Y	AACTGCAGTTTCGCGCGAACACGTAGA	BCAM1870 (*cep*I) cep box fusion pairs with ALFM318-Y and ALFM323-Y

ALFM320-Y	GGGGTACCCCGACCTGTTTACTTTTACAG	BCAS0293 (*aid*A) full cep box fusion pairs with ALFM321-Y
ALFM321-Y	GGGGTACCCTTTTACAGCTTCCATGACC	BCAS0293 (*aid*A) half cep box fusion pairs with ALFM321-Y
ALFM322-Y	AACTGCAGGTCGCCGACCTGCGCCTTCAG	BCAS0293 (*aid*A) cep box fusion pairs with ALFM320-Y or ALFM321Y
YWP263	GGGGTACCCTGACGCGGCCTGTCAATTC	BCAL0510 full cep box fusion ,pairs with YWP265
YWP264	GGGGTACCTCTGGCGGGCGCGGCGCGCA	BCAL0510 half cep box fusion, pairs with YWP265
YWP265	AACTGCAGAGCGAGCGGGCAGGAGCGGA	BCAL0510 cep box fusion, pairs with YWP263 or 264
YWP271	GGGGTACCCAACGTCACGCTGTCATACT	BCAM1869 full cep box fusion, pairs with YWP273
YWP272	GGGGTACCCTTGTCAGGTTTCAGTACCC	BCAM1869 half cep box fusion, pairs with YWP273
YWP273	AACTGCAGGAGTGCGGCCAGTGCGCCTT	BCAM1869 box fusion, pairs with YWP271 or 272
ALFM298-Y	GGGGTACCAAAACCTGCCGGTTTTAACAG	BCAS0155 full cep box fusion, pairs with ALFM301-Y.
ALFM299-Y	GGGGTACCTTTTAACAGTATCGAATCCGG	BCAS0155 half cep box fusion, pairs with ALFM301-Y
ALFM301-Y	AACTGCAGCCGACGCGGCCACGGTTTCG	BCAS0155 box fusion, pairs with ALFM298-Y or ALFM299-Y
ALFM314-Y	GGGGTACCATACTGTTAAAACCGGCAGT	BCAS0156 box fusion, pairs with ALFM317-Y
ALFM315-Y	GGGGTACCCCGGCAGGTTTTCCCGGAAA	BCAS0156 half box fusion, pairs with ALFM317-Y
ALFM317-Y	AACTGCAGGACGATGCCGATCGCCATGCC	BCAS0156 box fusion, pairs with ALFM314-Y or ALFM315-Y

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CHAPTER 3: A LUXR-TYPE REPRESSOR OF *Burkholderia cenocepacia* INHIBITS TARGET PROMOTERS VIA ANTIACTIVATION AND IS INHIBITED BY A COGNATE ACYLHOMOSERINE LACTONE

3.1. Summary

Burkholderia cenocepacia is an opportunistic human pathogen that encodes two LuxI-type acylhomoserine (AHL) synthases and three LuxR-type AHL receptors. Of these, *cepI* and *cepR* are tightly linked and form a cognate synthase/receptor pair, as do *cciI* and *cciR*. In contrast, CepR2 is unlinked from the other four genes, and lacks a genetically linked cognate AHL synthase gene. Another group showed that a *cepR2* mutant overexpressed a cluster of linked genes that appear to direct the production of a secondary metabolite and provided data suggesting that CepR2 did not detect any AHL (Malott *et al.*, 2009). We found that these same genes were upregulated by octanoylhomoserine lactone (OHL), which is synthesized by CepI. Taken together, these data suggest that several *cepR2*-linked promoters were repressed by CepR2 and that CepR2 was antagonized by OHL. Fusions of two divergent promoters to *lacZ* were used to confirm these hypotheses, and promoter resections and DNase I footprinting assays revealed a single CepR2 binding site between the two promoters. Surprisingly, the CepR2 binding site lies well upstream of both promoters, suggesting an unusual mode of repression. Adjacent to the *cepR2* gene is a gene that we designate *cepS*, which encodes an AraC-type transcription factor. CepS is essential for expression of both promoters, regardless of the CepR2 status or OHL concentration. CepS therefore acts downstream of CepR2, and CepR2 appears to function as a CepS antiactivator.

3.2. Introduction

Although the genus *Burkholderia* was recognized only 20 years ago (Yabuuchi *et al.*, 1992, Anon., 1992), it now encompasses over 50 species (Vanlaere *et al.*, 2009) that occupy extremely diverse ecological niches. Most have rather large genomes and a considerable degree of metabolic versatility. Some species are potentially useful in bioremediation of anthropogenic toxic chemicals (Chen *et al.*, 2003). Other members are capable of forming nitrogen-fixing root nodules with legumes and have a full complement of *nod* genes that were formerly thought to be limited to *Rhizobium* spp. and their allies (Bontemps *et al.*, 2010). Some members protect host plants against fungal pathogens (Parke & Gurian-Sherman, 2001). Other species are pathogenic against plants, animals, and humans. The type strain *B. cepacia* (originally *Pseudomonas cepacia*) is a pathogen of onions (Burkholder, 1950). *B. mallei* causes glanders in equines, while *B. pseudomallei* causes melioidosis in a variety of animals. Both can also be transmitted to humans, and are select agents of concern as possible bioweapons (Godoy *et al.*, 2003). *B. mallei* was used during World War I in an effort to incapacitate draft animals (Wheelis, 1998).

Seventeen pathogenic species, including *B. mallei, B. pseudomallei, B. cenocepacia, B. cepacia,* and *B. vietnamiensis*, are members of the *Burkholderia cepacia* complex, or BCC (Vandamme *et al.*, 1997, Vanlaere et al., 2009, Vanlaere *et al.*, 2008). Among these, *B. cenocepacia* is recognized as an opportunistic pathogen of humans and is a particular threat to cystic fibrosis (CF) patients (Mahenthiralingam *et al.*, 2005, Vandamme et al., 1997). Colonization of the CF lung by *B. cenocepacia* (Vandamme *et*

al., 2003) tends to occur in patients already infected with *Pseudomonas aeruginosa*, another opportunistic pathogen of the CF lung (Jones & Webb, 2003, Vandamme et al., 1997). *B. cenocepacia* strains are resistant to most antibiotics, making them virtually impossible to eradicate (Nzula *et al.*, 2002). Infections with *B. cenocepacia* may have variable clinical outcomes ranging from asymptomatic carriage to a sudden fatal deterioration in lung function (Mahenthiralingam et al., 2005).

The genomes of four isolates of *B. cenocepacia* have been sequenced in their entirety while additional genomes are currently being sequenced (NCBI, 2011). All four fully sequenced genomes have three circular chromosomes that vary in size between 3.9 and 0.88 MB in length, and two have a plasmid. Chromosome 3 of strain J2315 was recently found to be curable (Agnoli *et al.*, 2012), and should therefore be considered a plasmid rather than a chromosome.

Many or possibly all *Burkholderia* spp. encode at least one regulatory system that resembles the LuxR and LuxI proteins of *Vibrio fischeri*, where LuxI synthesizes an acylhomoserine lactone (AHL)-type pheromone, also called an autoinducer, and LuxR is an AHL-dependent transcriptional regulator (Choi & Greenberg, 1992, Eberhard *et al.*, 1981, Engebrecht & Silverman, 1984). Regulatory systems of this family are found in countless proteobacteria, where they are thought to allow bacteria to estimate their population size and for individual bacteria to coordinate their physiology with their siblings. Collectively, these systems regulate diverse processes, including pathogenesis, biofilm formation, bacterial conjugation, and the production of antibiotics and other

secondary metabolites (Whitehead *et al.*, 2001). In general, target genes are transcribed preferentially at population densities high enough to favor AHL accumulation (Eberhard *et al.*, 1991), a phenomenon sometimes referred to as quorum sensing (Fuqua *et al.*, 1994).

LuxR-type proteins have two domains, an N-terminal pheromone binding domain and a C-terminal DNA binding domain (Pappas *et al.*, 2004). Purified LuxR, TraR of *Agrobacterium tumefaciens*, LasR of *Pseudomonas aeruginosa*, and CepR of *B. cenocepacia*, when complexed with their respective AHLs, bind with high specificity to recognition sequences (referred to as *lux*, *tra, las,* or *cep* boxes, respectively) that are found near target promoters (Schuster *et al.*, 2004, Urbanowski *et al.*, 2004, Zhu & Winans, 1999). LasR is also able to bind to sequences that have no obvious resemblance to canonical *las* boxes (Schuster et al., 2004).

A few members of this family are *antagonized* by their cognate autoinducers, and bind DNA only in their *absence* (Tsai & Winans, 2010). Most of these are closely related to each other and include EsaR of *Pantoea stewartii*, ExpR of *Pectobacterium caratovorum* (formerly *Erwinia caratovora*), and YenR of *Yersinia enterocolitica* (Castang *et al.*, 2006, Cui *et al.*, 2005, Fineran *et al.*, 2005, Minogue *et al.*, 2005, Sjoblom *et al.*, 2006, Tsai & Winans, 2011). At least one LuxR-type protein that is not closely related to EsaR, ExpR, or YenR is also antagonized by its cognate AHL. This is the VjbR protein of *Brucella melitensis*, which functions as an apoprotein to activate

expression of a Type IV secretion system, and is inhibited by dodecanoyl-HSL (Delrue *et al.*, 2005).

B. cenocepacia J2315 encodes three LuxR homologs and two LuxI homologs (Lewenza *et al.*, 1999, Malott *et al.*, 2005, Malott et al., 2009). Among these, CepR and CepI are well conserved within the BCC (Venturi *et al.*, 2004). CepI synthesizes primarily octanoylhomoserine lactone (OHL), and lower levels of hexanoylhomoserine lactone (HHL) (Aguilar *et al.*, 2003, Gotschlich *et al.*, 2001, Huber *et al.*, 2001, Lewenza et al., 1999). Null mutations in *cepI* or *cepR* increased the production of the siderophore ornibactin, and decreased the production of secreted lipases and metalloproteases ZmpA and ZmpB (Kooi *et al.*, 2006, Lewenza et al., 1999, Lewenza & Sokol, 2001, Sokol *et al.*, 2003). CepI and CepR are also required for swarming motility and biofilm formation (Huber et al., 2001) and for pathogenicity in several animal models (Kothe *et al.*, 2003, Sokol et al., 2003). *B. cenocepacia* J2315 also encodes CciI and CciR, which are found on a genomic island called *cci* (*cenocepacia* island), that is found only in a subset of *B. cenocepacia* strains (Malott et al., 2005). The CepIR and CciIR systems extensively interact, in that CciR negatively regulates *cepI*, while CepR is required for expression of the *cciIR* operon (Malott et al., 2005). Transcriptional profiling studies indicate that CepR and CciR regulate many of the same genes, but do so in opposite ways (O'Grady *et al.*, 2009).

B. cenocepacia also encodes a third LuxR-type transcription factor, CepR2, whose gene is not linked to any apparent AHL synthase gene. The *cepR2* gene was

reported to be negatively regulated by the CepR2 protein and by CciR (Malott et al., 2009). A *cepR2* mutation increased the expression of 64 genes and decreased the expression of 127 others (Malott et al., 2009). These included genes involved in virulence, chemotaxis, heat shock, and signal transduction. Differential expression was strongest in a group of genes that are closely linked to *cepR2*, including *cepR2* itself, an adjacent gene *bcam0189*, which encodes an AraC type protein (that we designate CepS), a two gene operon (*bcam0191-0190*), a divergent five-gene operon (*bcam0192-0196*), and a nearby four gene operon (*bcam0199-0202*). Bcam0190-0196 are predicted to direct the synthesis of a secondary metabolite, while Bcam0199-0202 are predicted to direct the efflux of a small molecule. All of these genes were expressed more strongly in the mutant than in wild type, indicating that CepR2 inhibits their expression. CepR2 was fully functional in the absence of any AHL. In a heterologous system, the ability of CepR2 to activate a *lux* operon was not affected by the addition of any AHL. It was concluded that CepR2 functions independently of AHLs and does not detect them (Malott et al., 2009).

Members of our laboratory are interested in the genetic and biochemical properties of several LuxR-type proteins, including CepR. To further those studies, we used oligonucleotide microarrays to identify genes that are differentially expressed by exogenous OHL, and were surprised to find that several genes that are induced by OHL were previously found to be repressed by CepR2 (Malott et al., 2009). Taking the two findings together, this would suggest that OHL antagonizes CepR2 activity, though this model was difficult to reconcile with the report that CepR2 was unaffected by any AHL

(Malott et al., 2009). We therefore measured the expression of two CepR2-linked promoters in the presence or absence of CepR2 and OHL, both in *B. cenocepacia* and in *E. coli*, and studied the role of CepS (formerly Bcam0189), a possible regulatory protein, in the expression of the same promoters. We also tested the ability of CepR2 to bind OHL *in vivo* and to fold into a soluble, protease-resistant form in the presence or absence of OHL.

3.3. Results

In previous studies, we identified a set of genes that are directly regulated by CepR (Wei *et al.*, 2011). In an effort to identify additional members of this regulon, we cultured the *cepI* mutant strain CLW101 in the presence and absence of 1 μM OHL, and screened for differential gene expression using oligonucleotide microarrays. This strain contains a P*cepI-lacZ* fusion that was created by an insertion of Tn5*lac* in *cepI* (Weingart *et al.*, 2005), which allows us to test for induction. The microarray included probes complementary to *E. coli lacZ* mRNA. Cultures containing OHL expressed 100-200 fold more β-galactosidase than identical cultures lacking OHL (Table 3.1). OHL caused a 3.2-3.5 fold increase in *lacZ* mRNA abundance as measured by the microarrays (Table 3.1). These data indicate that the microarrays reflected expression of this gene but show a compressed induction ratio. Another CepR-regulated operon composed of *aidA* and *aidB*, was also strongly induced by OHL (Table 3.1).

In this transcriptional profiling experiment, we also detected OHL-inducible expression of a number of additional genes (Table 3.1), including several that are closely linked to *cepR2*. Interestingly, all of the OHL-inducible genes linked to *cepR2* were previously found to be expressed more strongly in a *cepR2* mutant than in a wild type strain (Malott et al., 2009). The two studies taken together could suggest that apo-CepR2 represses these genes, and that its ability to repress them is somehow antagonized by OHL. These genes are expressed in six apparent operons (Fig. 3.1), including *cepR2* and another possible regulatory gene *cepS* (both of which are monocistronic). The operon containing *bcam0184-0186* and the divergent *bcam0187* were induced rather weakly compared to the others and were not pursued in the present study. We focus first on the promoters of the *bcam0191-0190* operon and of the divergent the *bcam0192-0196* operon. Later, we will describe the regulation of *cepR2* and *cepS*.

Table 3.1. Transcriptional profiles of cells cultured in the presence or absence of 1 μM OHL.[a]

		OHL Induction Ratio (S.D.)				
Gene		Trial 1	Trial 2	Average	*cepR2* vs. WT (Malott et al., 2009)	Alternate Name, Comments, References
lacZ		3.51 (0.81)	3.24 (0.32)	3.38	n.a.[b]	*cepI-lacZ* reporter
bcal0510		7.61 (1.58)	6.41 (0.46)	7.01	n.d.[c]	CepR-regulated (Wei et al., 2011)
bcal0831		1.66 (0.20)	2.88 (0.81)	2.27	n.d.	
bcal0833		0.87 (0.15)	1.29 (0.28)	1.08	n.d.	*phbB*
bcal2118		1.55 (0.24)	3.56 (0.93)	2.55	n.d.	
bcal3178		1.49 (0.41)	3.09 (0.54)	2.29	n.d.	
bcam0030		2.63 (1.41)	5.36 (2.27)	3.99	n.d.	
bcam0031		2.57 (1.21)	4.48 (3.18)	3.52	n.d.	
bcam0184		**1.34 (0.28)**	**4.06 (3.23)**	**2.70**	**n.d.**	**Lectin**
bcam0185		**1.43 (0.45)**	**11.8 (1.96)**	**6.59**	**n.d.**	**Lectin**
bcam0186		**3.12 (0.40)**	**1.81 (0.52)**	**2.46**	**2.9 (0.8)**	***bclA* (lectin)**
bcam0187		**0.99 (0.22)**	**3.24 (0.32)**	**2.12**	**n.d.**	
bcam0188		**2.45 (0.99)**	**2.62 (0.69)**	**2.53**	**21.1 (3.4)**	***cepR2***
bcam0189		**1.03 (0.26)**	**2.15 (0.46)**	**1.59**	**188 (46)**	***cepS***
bcam0190		**2.00 (0.81)**	**3.25 (0.54)**	**2.62**	**11.6 (0.9)**	**Aminotransferase Class III**
bcam0191		**1.96 (0.37)**	**4.11 (1.24)**	**3.04**	**9.5 (3.0)**	**Non-ribosomal peptide synthase**
bcam0192		**4.92 (1.24)**	**4.63 (2.79)**	**4.78**	**113 (27)**	**Conserved hypothetical**
bcam0193		**3.08 (1.28)**	**6.15 (1.73)**	**4.62**	**171 (74)**	**Conserved hypothetical**
bcam0194		**7.46 (1.69)**	**5.18 (1.70)**	**6.32**	**151 (48)**	**Conserved hypothetical**
bcam0195		**3.41 (1.76)**	**6.60 (1.33)**	**5.00**	**58.3 (62)**	**Non-ribosomal peptide synthase**
bcam0196		**7.08 (3.02)**	**3.50 (3.19)**	**5.29**	**80.5 (31)**	**Conserved hypothetical**
bcam0393		2.45 (0.89)	3.34 (1.43)	2.90	n.d.	
bcam0634		3.59 (0.08)	2.64 (0.15)	3.12	n.d.	
bcam1413a		3.58 (1.60)	3.50 (3.19)	3.54	n.d.	*aidC* (Wei et al., 2011)
bcam1742		1.86 (0.65)	2.60 (1.10)	2.23	n.d.	
bcam1869		1.86 (0.65)	3.26 (1.23)	2.56	n.d.	CepR-regulated (Wei et al., 2011)
bcam2307		2.96 (0.60)	7.09 (3.23)	5.02	-1.7 ± (0.7)	*zmpB*
bcam2308		2.00 (0.57)	2.31 (0.85)	2.15	n.d.	
bcas0153		2.34 (0.42)	1.78 (0.21)	2.06	n.d.	
bcas0292		14.9 (11.8)	39.4 (41.2)	27.2	n.d.	*aidB*, CepR-regulated (Wei et al., 2011)
bcas0293		293 (299)	198 (156)	245	1.7 ± (0.2)	*aidA*, CepR-regulated (Wei et al., 2011)
bcas0409		2.31 (0.60)	2.64 (0.67)	2.47	1.6 ± (0.3)	*zmpA*
All Genes		1.00 (1.26)	1.00 (1.37)	1.00		

a. Strain CLW101 contains a chromosomal P*cepI-lacZ* fusion (Weingart et al., 2005). In Trial 1 the culture lacking OHL expressed 2.7 Miller units of β-galactosidase, while the culture containing

OHL expressed 290 units. In Trial 2, the culture lacking OHL expressed 1.5 units, while the culture containing OHL expressed 195 units.

b. Not applicable

c. Not determined

3.3.1. Regulation of *bcam0191* and *bcam0192* by CepR2 and CepS

In order to study the *bcam0191* and *bcam0192* promoters more closely, we fused each to *lacZ* on a low copy plasmid. Plasmid pGR130 contains the *bcam0191* promoter on a 493 nucleotide fragment (Fig. 3.1), while plasmid pGR136 contains the *bcam0192* promoter on a 527 nucleotide fragment. Expression of these fusions was tested in strains containing or lacking *cepR2* or *cepS*, and in the presence or absence of exogenous OHL. All strains lacked *cepI*, and so they did not synthesize OHL.

A *cepR2*$^+$ strain expressing the *bcam0191-lacZ* fusion (pGR130) expressed 35 units of β-galactosidase in the absence of OHL (Table 3.2). This fusion was induced approximately 11-fold by OHL, in reasonable agreement with the transcriptional profiling experiments described above. This fusion was also expressed 11-fold more strongly in a strain lacking CepR2 than in a CepR2$^+$ strain, in agreement with the data of the Malott study (Malott et al., 2009). Addition of OHL did not stimulate expression in the strain lacking CepR2 (Table 3.2). These data are consistent with the hypothesis that the *bcam0191* promoter is repressed by CepR2 and that repression is somehow antagonized by OHL.

As described above, the *cepS* gene is adjacent to *cepR2*, and encodes a possible transcription factor of the AraC family. The genetic linkage of *cepS* to *cepR2*, *bcam0191*, and *bcam0192* suggested a possible role in their regulation. We therefore deleted *cepS* and tested for the expression of the *bcam0191-lacZ* fusion in this mutant. Loss of *cepS* caused a severe decrease in expression of this promoter, both in the presence and absence of OHL (Table 3.2). The lack of stimulation by OHL in a *cepS* mutant indicates that when CepR2 is inactive and CepS is absent, expression is very low. In other words CepS is epistatic to CepR2.

Similar results were obtained from the divergent *bcam0192* promoter (Table 3.2). In a strain expressing CepR2, the fusion in pGR136 was expressed 11-fold more strongly in the presence of OHL than in its absence. In a strain lacking CepR2, the fusion was expressed 12-fold more strongly than in the presence of apo-CepR2 (Table 3.2) and was unaffected by OHL. The *cepS* mutant expressed this promoter at low levels that were unaffected by OHL. Evidently, the *bcam0192* promoter is repressed by apo-CepR2 and activated by CepS, similar to the *bcam0191* promoter.

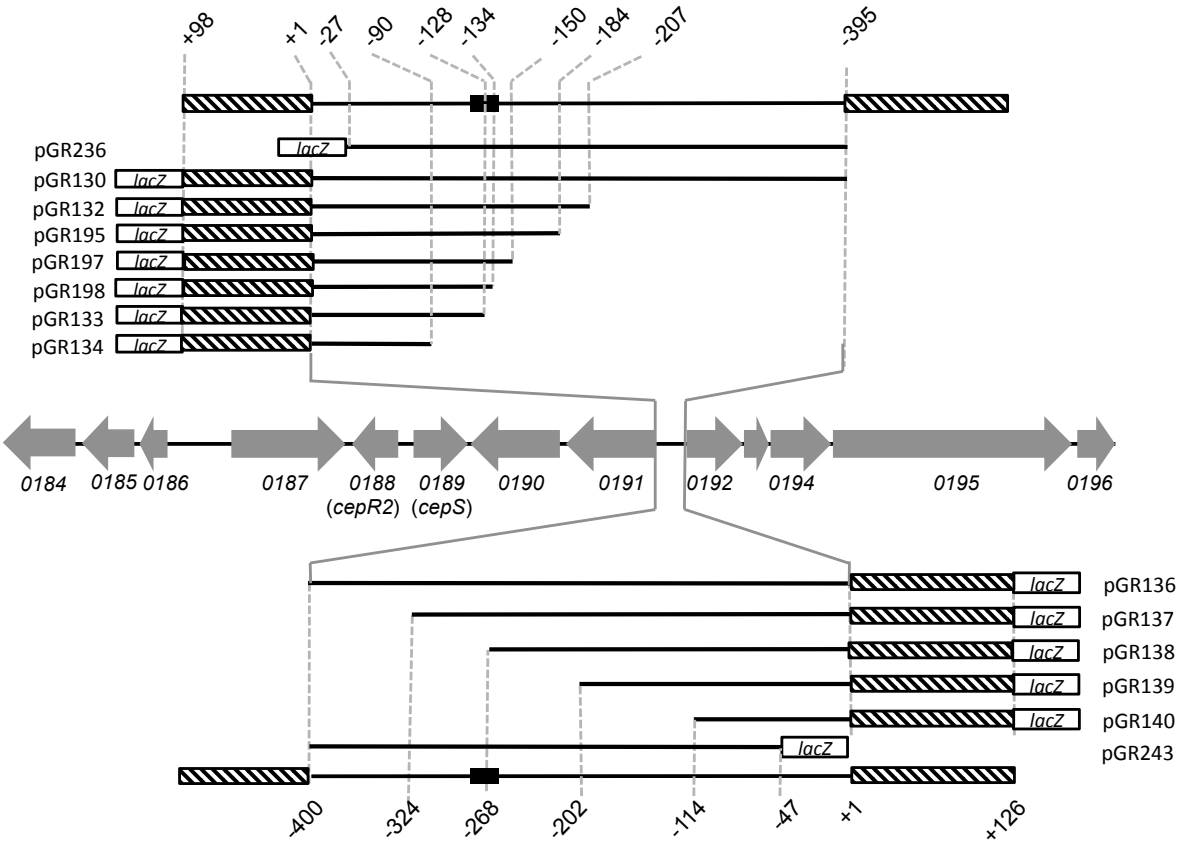

Fig. 3.1. Resections of the *bcam0191* and *bcam0192* promoters. Chromosomal organization of OHL-inducible genes is indicated using gray arrows. Genes are named in accordance with the genome sequence of strain J2315 (Holden *et al.*, 2009). Promoter-proximal portions of the *bcam0191* and *bcam0192* genes are indicated using hatched boxes. Endpoints of each resection are calculated with respect to the translation start site of regulated gene. The solid black box represents the CepR2 binding site. A promoterless β-galactosidase gene is denoted using a white box labelled "*lacZ"*.

Table 3.2. Regulation of the promoters of *bcam0191* and *bcam0192* by CepR2, CepS, and OHL.

Fusion[a]	Chromosomal genotype[b]	Plasmid genotype	OHL (uM)	β-Galactosidase	Normalized Values[c]
bcam0191	WT	none	0	35 ± 8	(1)
	WT	none	1	377 ± 30	10.8
	GR141 (*cepR2*⁻)	pSRKKm	0	383 ± 24	10.9
	GR141 (*cepR2*⁻)	pSRKKm	1	397 ± 32	11.3
	GR141 (*cepR2*⁻)	pGR192 (*cepR2*)	0	33 ± 4	0.95
	GR141 (*cepR2*⁻)	pGR192 (*cepR2*)	1	387 ± 21	11.1
	GR145 (*cepS*⁻)	pSRKGm	0	5 ± 3	0.14
	GR145 (*cepS*⁻)	pSRKGm	1	3 ± 2	0.1
	GR145 (*cepS*⁻)	pGR193 (*cepS*)	0	41 ± 8	1.2
	GR145 (*cepS*⁻)	pGR193 (*cepS*)	1	412 ± 32	11.8
bcam0192	WT	none	0	32 ± 3	(1)
	WT	none	1	362 ± 15	11.3
	GR141 (*cepR2*⁻)	pSRKKm	0	376 ± 31	11.8
	GR141 (*cepR2*⁻)	pSRKKm	1	368 ± 25	11.5
	GR141 (*cepR2*⁻)	pGR192 (*cepR2*)	0	36 ± 1	1.1
	GR141 (*cepR2*⁻)	pGR192 (*cepR2*)	1	373 ± 24	11.7
	GR145 (*cepS*⁻)	pSRKGm	0	3 ± 1	0.1
	GR145 (*cepS*⁻)	pSRKGm	1	4 ± 2	0.13
	GR145 (*cepS*⁻)	pGR193 (*cepS*)	0	43 ± 1	1.3
	GR145 (*cepS*⁻)	pGR193 (*cepS*)	1	396 ± 36	12.4

a: A *bcam0191-lacZ* transcriptional fusion was provided by pGR130, while a *bcam0192-lacZ* fusion was provided using pGR136. The vector for both plasmids, pYW302, expressed only 1-2 units of β -galactosidase activity.

b: All strains are derived from K56-I2, which carries an insertion mutation in *cepI*.

c: β-galactosidase activity is normalized to that of the wild type strain carrying the indicated plasmid and cultured in the absence of OHL.

3.3.2. Reconstitution of regulated expression in a heterologous host.

We sought to determine whether CepR2 and CepS regulate the *bcam0191* and *bcam0192* promoters directly, and therefore attempted to reconstitute regulated expression in *E. coli*. Plasmid pGR130 was introduced into derivatives of MC4100 containing plasmids that express CepR2 and/or CepS. In a strain expressing neither CepR2 nor CepS, the *bcam0191-lacZ* fusion expressed approximately 120 units of β-galactosidase and was not significantly affected by OHL (Table 3.3). The fusion was repressed approximately 4-fold by CepR2. Surprisingly, OHL had little or no effect on

CepR2-mediated repression. CepS enhanced expression of the fusion about 2.5 fold in the presence or absence of OHL. When both proteins were provided in the absence of OHL, expression fell to the same levels as with CepR2 alone (Table 3.3). However, when OHL was provided, expression increased to the same levels as with CepS alone. Very similar data were obtained using *E. coli* strains expressing the *bcam0192-lacZ* fusion (Table 3.3). The ability of CepR2 and CepS to regulate expression of these promoters in *E. coli* indicates that they both are likely to act directly upon them.

Table 3.3. Regulated expression of the *bcam0191* and *bcam0192* promoters in *E. coli*[a].

Fusion[b]	Plasmids expressing *B. cenocepacia* genes	OHL (uM)	β-Galactosidase Activity	Normalized Value[c]
bcam0191	none	0	121 ± 11	(1)
	None	1	137 ± 15	1.13
	pGR192 (*cepR2*)	0	29 ± 8	0.24
	pGR192 (*cepR2*)	1	35 ± 8	0.29
	pGR276 (*cepS*)	0	310 ± 21	2.6
	pGR276 (*cepS*)	1	305 ± 17	2.5
	pGR192 (*cepR2*), pGR276 (*cepS*)	0	35 ± 8	0.29
	pGR192 (*cepR2*), pGR276 (*cepS*)	1	307 ± 30	2.5
bcam0192	None	0	101 ± 11	(1)
	None	1	93	0.92
	pGR192 (*cepR2*)	0	23 ± 4	0.23
	pGR192 (*cepR2*)	1	32 ± 3	0.32
	pGR276 (*cepS*)	0	300 ± 11	3.0
	pGR276 (*cepS*)	1	344 ± 23	3.4
	pGR192 (*cepR2*), pGR276 (*cepS*)	0	32 ± 3	0.32
	pGR192 (*cepR2*), pGR276 (*cepS*)	1	293 ± 30	2.9

a: All strains were derived from MC4100.

b: A *bcam0191-lacZ* transcriptional fusion was provided by pGR130, while a *bcam0192-lacZ* fusion was provided using pGR136.

c: β-galactosidase activity is normalized to that of the wild type strain carrying the indicated plasmid and cultured in the absence of OHL.

3.3.3. Localization of DNA sequences required for regulated gene expression of *bcam0191* and *bcam0192.*

The intergenic region between *bcam0191* and *bcam0192* start codons is 396 nucleotides in length, and contains a strongly AT-rich region characteristic of many bacterial promoters. In order to identify the essential sequences required for regulated expression of *bcam0191*, we made several resections of this promoter from its 5' end (Fig. 3.1) and fused the remaining sequences to *lacZ*. Plasmids pGR132, pGR195, pGR133, and pGR134 resemble pGR130, but contain 207, 184, 128, and 90 nucleotides upstream of the *bcam0191* translation start site, respectively. Plasmid pGR236 contains sequences from nucleotides -395 to -26 (Fig. 3.1). The fusion in pGR132 was expressed at 3-fold higher levels than that of pGR130 in the absence of OHL, while the two fusions were expressed at similar levels in the presence of OHL (Table 3.4). Both fusions were expressed at equally high levels in the absence of CepR2 and at equally low levels in a *cepS* mutant. Similar data were obtained using pGR195 and pGR236. Together, sequences required for OHL-responsive expression are limited to nucleotides -184 to -27.

The fusion of pGR133 was expressed at equally high levels in the presence or absence of OHL (Table 3.4), and was not affected by a CepR2 mutation (Table 3.4). It was expressed at very low levels in a *cepS* mutant. These data indicate that pGR133 lacks some sequence required for repression by CepR2. Plasmid pGR195 contains all such sequences and is 59 nucleotides longer than pGR133 at the 5' end.

Table 3.4. Regulation of resected *bcam0191* promoters by CepR2, CepS, and OHL[a].

Plasmid	Fragment	Genotype	OHL (uM)	β-Galacto-sidase	Normalized Value[b]
pGR130	-395 – +98	WT	0	35 ± 8	(1)
		WT	1	377 ± 30	10.8
		GR141 (*cepR2*⁻)	0	381 ± 30	10.9
		GR141 (*cepR2*⁻)	1	365 ± 17	10.4
		GR145 (*cepS*⁻)	0	7 ± 1	0.2
		GR145 (*cepS*⁻)	1	2 ± 0	0.01
pGR132	-207 – +98	WT	0	94 ± 27	(1)
		WT	1	361 ± 34	3.8
		GR141 (*cepR2*⁻)	0	327 ± 28	3.5
		GR141 (*cepR2*⁻)	1	349 ± 31	3.7
		GR145 (*cepS*⁻)	0	9 ± 1	0.1
		GR145 (*cepS*⁻)	1	7 ± 1	0.07
pGR195	-184 – +98	WT	0	74 ± 13	(1)
		WT	1	361 ± 35	4.9
		GR141 (*cepR2*⁻)	0	326 ± 28	4.4
		GR141 (*cepR2*⁻)	1	389 ± 31	5.3
		GR145 (*cepS*⁻)	0	n.d.	n.d.
		GR145 (*cepS*⁻)	1	n.d.	n.d.
pGR133	-128 – +98	WT	0	339 ± 9	(1)
		WT	1	361 ± 20	1.1
		GR141 (*cepR2*⁻)	0	339 ± 29	1.0
		GR141 (*cepR2*⁻)	1	360 ± 11	1.1
		GR145 (*cepS*⁻)	0	1 ± 1	0.003
		GR145 (*cepS*⁻)	1	1 ± 1	0.002
pGR134	-90 – +98	WT	0	10 ± 2	(1)
		WT	1	6 ± 4	1.0
		GR141 (*cepR2*⁻)	0	9 ± 4	1.0
		GR141 (*cepR2*⁻)	1	10 ± 3	1.0
		GR145 (*cepS*⁻)	0	2 ± 1	0.2
		GR145 (*cepS*⁻)	1	2 ± 1	0.2
pGR236	-395 – -27	WT	0	45 ± 6	(1)
		WT	1	248 ± 26	5.5
		GR141 (*cepR2*⁻)	0	248 ± 19	5.5
		GR141 (*cepR2*⁻)	1	226 ± 20	5.0
		GR145 (*cepS*⁻)	0	3.3 ± 1	0.07
		GR145 (*cepS*⁻)	1	2.5 ± 1	0.06

a: Derivatives of strain K56-I2 containing the indicated *cepR2* or *cepS* mutations and the indicated plasmids were grown to an optical density of approximately 0.4 in the presence or absence of 1 μM OHL, and assayed for β-galactosidase activity. Data are the averages of three biological replicates, with standard deviations indicated.

b: β-galactosidase activity is normalized to that of the wild type strain carrying the indicated plasmid and cultured in the absence of OHL.

The fusion of pGR134 was expressed at low levels in all backgrounds and was not responsive to OHL. This plasmid therefore lacks sequences required for promoter expression, either the promoter itself or the CepS binding site. Plasmid pGR133 contains all sequences required of CepS-dependent expression and is 38 nucleotides longer (Fig. 3.1).

We noticed an imperfect dyad symmetrical DNA sequence (GACAGCCCGATTTGCGGATGTC, symmetrical bases are underlined) present in all CepR2-repressed plasmids and absent or partially absent in all CepR2-nonresponsive ones. To learn more about the role of this sequence in regulation, we constructed two additional plasmids (pGR197 and pGR198, Fig. 3.1 and Fig. 3.2). The first plasmid contains this sequence plus 13 additional promoter-distal bases, while the second plasmid lacks five bases near the promoter-distal end of this sequence. Plasmid pGR197 was induced by OHL in an *E. coli* strain expressing CepR2, while pGR198 was not affected (Fig. 3.2). We used site-directed mutagenesis to alter small groups of nucleotides within this dyad symmetry. Plasmids pGR259, pGR260, and pGR261 have 3- or 4-nucleotide mutations in the upstream half of this sequence. All three mutations significantly reduced induction by OHL (Fig. 3.2), providing additional evidence that this dyad is essential for CepR2 activity. We will demonstrate that this site is bound by CepR2 *in vitro* (see below).

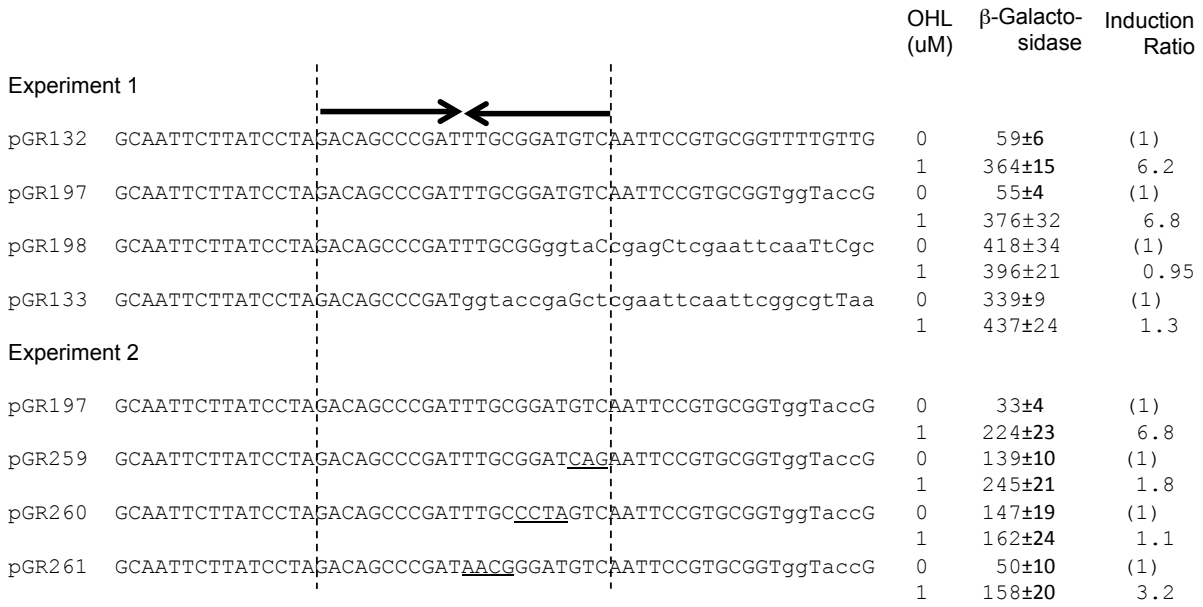

Fig. 3.2. Resections and alterations of the CepR2 binding site. The dyad symmetrical CepR2 binding site is indicated using inverted arrows. All sequences shown were part of a *bcam0191-lacZ* fusion. *B. cenocepacia* sequences are capitalized while vector sequences are shown in lower case. Vector sequences that fortuitously match the original DNA sequence are capitalized. Site-directed mutations of the CepR2 binding site are underlined. β-galactosidase activity was determined for cells cultured in the presence or absence of OHL.

Similar experiments were carried out to identify cis-acting sites necessary for regulated expression of the divergent gene *bcam0192*. Four plasmids, pGR137, pGR138, pGR139, and pGR140 were constructed that resemble pGR136 but have 324, 268, 202, or 114 nucleotides of upstream DNA, respectively (Fig. 3.1). Plasmid pGR243 resembles pGR136 but contains sequences from nucleotides -400 to -47 (Fig. 3.1). Significantly, pGR137 contains all of the dyad symmetry described above and 21 additional nucleotides, while pGR138 lacks half of the dyad, and pGR139 and pGR140 lack all of it. Plasmid pGR137 resembled pGR136 in that it was derepressed by OHL and by a *cepR2* mutation, and was expressed at very low levels in a *cepS* mutant (Table 3.5).

Table 3.5. Regulation of the promoter of *bcam0192* by CepR2, CepS, and OHL[a].

Plasmid	Fragment	Genotype	OHL (uM)	β-Galacto-sidase	Normalized Value[b]
pGR136	-400 – +126	WT	0	32 ± 3	(1)
		WT	1	367± 30	11.5
		GR141 (*cepR2*$^-$)	0	376 ± 31	11.8
		GR141 (*cepR2*$^-$)	1	368 ± 25	1.0
		GR145 (*cepS*$^-$)	0	3 ± 1	0.1
		GR145 (*cepS*$^-$)	1	4 ± 2	0.01
pGR137	-324 – +126	WT	0	78 ± 10	(1)
		WT	1	357 ± 15	4.8
		GR141 (*cepR2*$^-$)	0	356 ± 23	4.6
		GR141 (*cepR2*$^-$)	1	374 ± 34	1.0
		GR145 (*cepS*$^-$)	0	7 ± 3	0.9
		GR145 (*cepS*$^-$)	1	5 ± 2	0.01
pGR138	-268 – +126	WT	0	217 ± 23	(1)
		WT	1	263 ± 15	1.2
		GR141 (*cepR2*$^-$)	0	374 ± 18	1.7
		GR141 (*cepR2*$^-$)	1	382 ± 23	1.7
		GR145 (*cepS*$^-$)	0	4 ± 2	0.02
		GR145 (*cepS*$^-$)	1	7 ± 3	0.03
pGR139	-202 – +126	WT	0	370 ± 21	(1)
		WT	1	375 ± 23	1.0
		GR141 (*cepR2*$^-$)	0	364 ± 26	1.0
		GR141 (*cepR2*$^-$)	1	384 ± 43	1.0
		GR145 (*cepS*$^-$)	0	12.5 ± 4	0.03
		GR145 (*cepS*$^-$)	1	18.7 ± 9	0.05
pGR140	-114 – +126	WT	0	5 ± 7	(1)
		WT	1	4 ± 9	0.8
		GR141 (*cepR2*$^-$)	0	8 ± 6	1.6
		GR141 (*cepR2*$^-$)	1	5 ± 3	1.0
		GR145 (*cepS*$^-$)	0	3 ± 2	0.7
		GR145 (*cepS*$^-$)	1	3 ± 1	0.7
pGR243	-400 – -46	WT	0	75 ± 14	(1)
		WT	1	332 ± 32	4.4
		GR141 (*cepR2*$^-$)	0	392 ± 32	5.2
		GR141 (*cepR2*$^-$)	1	421 ± 28	5.6
		GR145 (*cepS*$^-$)	0	2.4 ± 1.2	0.03
		GR145 (*cepS*$^-$)	1	5.6 ± 1.2	0.07

a: Derivatives of strain K56-I2 containing the indicated *cepR2* or *cepS* mutations and the indicated plasmids were grown to an optical density of approximately 0.4 in the presence or absence of 1 μM OHL, and assayed for β-galactosidase activity. Data are the averages of three biological replicates, with standard deviations indicated.

b: β-galactosidase activity is normalized to that of the wild type strain carrying the indicated plasmid and cultured in the absence of OHL.

In contrast, the fusions in pGR138 and pGR139 were expressed at high levels and not significantly affected by CepR2 or OHL. They were expressed at low levels in a

cepS mutant. Plasmid pGR140 expressed its fusion at very low levels under all conditions. Expression of the fusion of pGR243 was similar to wild type, indicating that all sequences required for regulation lie upstream of nucleotide -47. These data suggest that the dyad symmetry is required for regulation of the *bcam0192* promoter, just as it was for the divergent *bcam0191* promoter. In both cases, the repressor binding site appears to lie well upstream of the regulated promoters.

3.3.4. Regulation of the *cepR2* and *cepS* promoters

The microarray data described above shows that OHL may cause induction of *cepR2* and *cepS*, though the effect is very slight. In contrast, microarray data of Malott and colleagues indicate that both these genes are expressed far more strongly in a *cepR2* mutant than in a *cepR2*$^{+}$ strain (Malott et al., 2009). Although these data do not directly contradict ours, the two datasets are nonetheless somewhat difficult to reconcile.

In order to study the expression of the *cepR2* and *cepS* genes further, we constructed two plasmids in which each promoter is fused to *lacZ*. These plasmids were introduced into strains lacking one or the other of these genes, and cultured in the presence or absence of OHL. Both fusions gave similar results. In the strain containing *cepR2* and *cepS*, expression was increased about 2-fold by OHL (Table 3.6). Perhaps surprisingly, this slight increase also was detected in a *cepR2* mutant, indicating that CepR2 is not required. The *cepS* mutation caused a mild decrease in expression of both promoters, but did not affect the very slight stimulation by OHL. These data tend to

support our microarray data. In the Discussion, we will describe a possible explanation for the data of the Malott study.

Table 3.6. Regulation of the *cepR2 and cepS* promoters.

Fusion[a]	Fragment[b]	Chromosomal	OHL (uM)	β-Galactosidase	Induction
P*cepR2-lacZ*	-195 - +170	WT	0	237 ± 8	(1)
		WT	1	480 ± 30	2.0
		GR141 (*cepR2*$^-$)	0	247 ± 23	1.0
		GR141 (*cepR2*$^-$)	1	446 ± 26	1.9
		GR145 (*cepS*$^-$)	0	116 ±13	0.5
		GR145 (*cepS*$^-$)	1	126 ± 12	0.5
P*cepS-lacZ*	-198 - +127	WT	0	264 ± 23	(1)
		WT	1	468 ± 35	1.8
		GR141 (*cepR2*$^-$)	0	237 ± 17	0.9
		GR141 (*cepR2*$^-$)	1	482 ± 32	1.8
		GR145 (*cepS*$^-$)	0	172 ± 14	0.7
		GR145 (*cepS*$^-$)	1	155 ± 21	0.6

a: The *cepR2-lacZ* fusion was carried by plasmid pGR141, while the *cepS-lacZ* fusion was carried by pGR146.
b: Coordinates of the two fusions are calculated relative to the *cepR2* and *cepS* translation start sites, respectively.
c: All strains are derivatives of K56-I2, and therefore have mutations in *cepI*.

3.3.5. Specificity of CepR2 for AHL-type pheromones

Throughout this study, we have used strains that have null mutations in *cepI*, and have been providing exogenous OHL where indicated. These strains still have *cciI*, and therefore presumably synthesize hexanoyl-HSL (HHL), and smaller amounts of similar pheromones. The fact that OHL influences CepR2 indicates that CciI-synthesized AHLs do not activate this fusion, at least not fully. However, they could in principle play some role in CepR2 function. To address this question, we assayed the expression of a *bcam0191-lacZ* fusion in the presence of different AHL-type pheromones, with acyl

groups that vary in length and substitution. Among these, OHL was the most effective at derepressing the fusion (Fig. 3.3). The only other pheromone that showed significant activity was 3-oxooctanoyl-HSL (OOHL). Decanoyl-HSL (DHL) showed a trace of activity when provided at high concentrations, while five other AHL pheromones (hexanoyl-HSL, 3-oxo-hexanoyl-HSL, 3-oxo-decanoyl, dodecanoyl-HSL, and 3-oxo-dodecanoyl-HSL) were inactive (data not shown). We conclude that endogenous levels of pheromones synthesized by CciI did not detectably impact CepR2 activity.

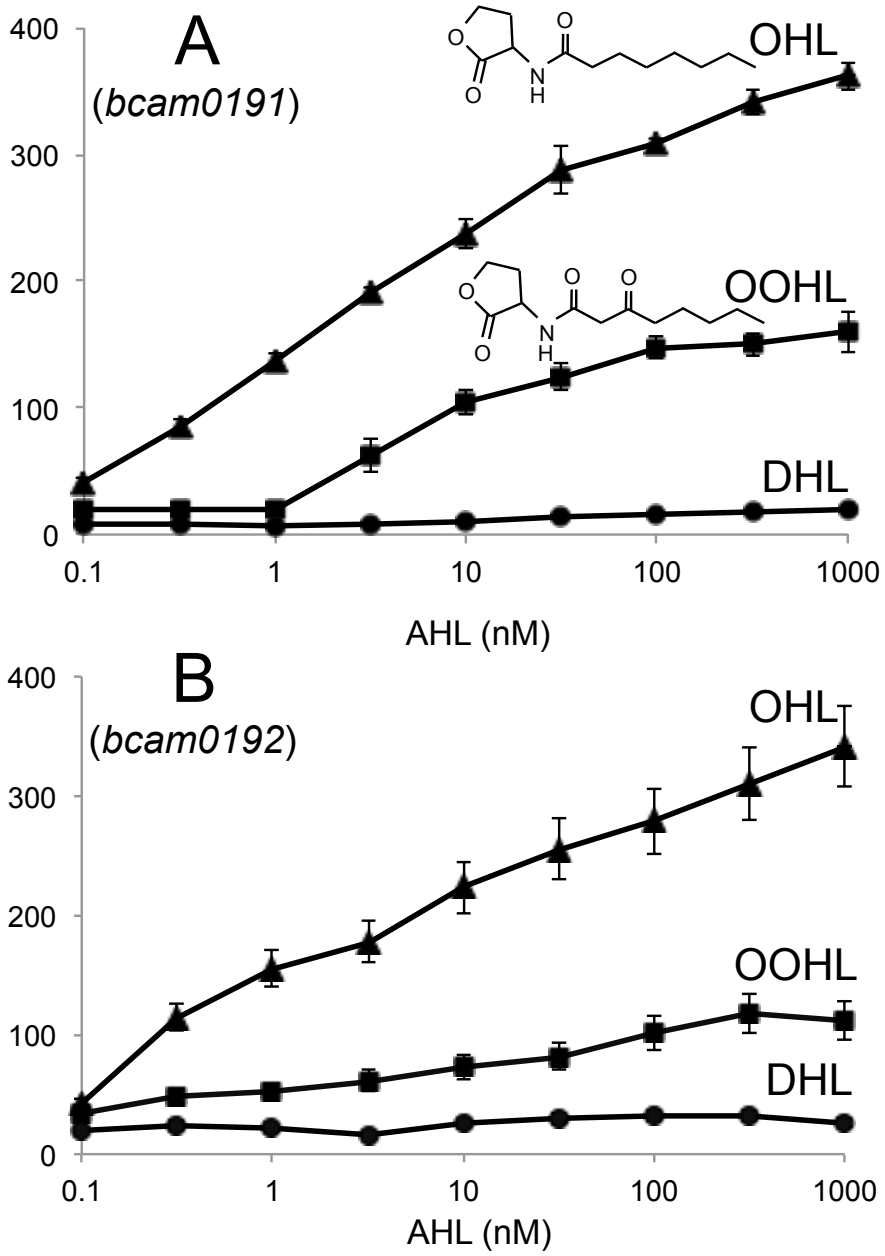

Fig. 3.3. Detection of heterologous AHLs by CepR2. Strains K56-I2(pGR130) and K56-I2(pGR136) were used to test the induction of the *bcam0191* (A) and *bcam0192* promoters (B), respectively. β-galactosidase activity of cultures amended with selected concentrations of OHL (triangles), 3-oxooctanoyl-HLS (OOHL, squares), and decanoyl-HSL (DHL, circles) were measured at mid-log growth. The values shown are the mean standard deviation (error bars) from triplicate experiments. Five other AHLs (hexanoyl-HSL, 3-oxo-hexanoyl-HSL, 3-oxo-decanoyl-HSL, dodecanoyl-HSL, and 3-oxo-dodecanoyl-HSL) did not detectably induce expression of the fusion (data not shown).

3.3.6. Ability of cells expressing CepR2 to sequester AHLs

The hypothesis that CepR2 is antagonized by OHL and OOHL predicts that it should be able to bind these AHLs stably and preferentially. To test this, we overexpressed CepR2 using the T7 promoter in *E. coli* in the presence of each of eight different AHLs, then washed the cells of each culture to remove unbound or weakly bound AHLs, and bioassayed for CepR2-bound AHLs. Of the eight AHLs tested, OHL was detected at the highest levels, followed by OOHL and ODHL (3-oxodecanoyl-HSL) (Fig. 3.4). Trace amounts of HHL and OHHL (3-oxohexanoyl-HSL) were bound, while DHL, dDHL and OdDHL (dodecanoyl-HSL and 3-oxododecanoyl-HSL) were not detectably sequestered. These data agree fairly well with the preference for OHL *in vivo* as described above, except that DHL was more active then ODHL in the former assay, while ODHL was sequestered more effectively than DHL. It appears that ODHL can bind CepR2 without altering its DNA binding properties as profoundly as other AHLs.

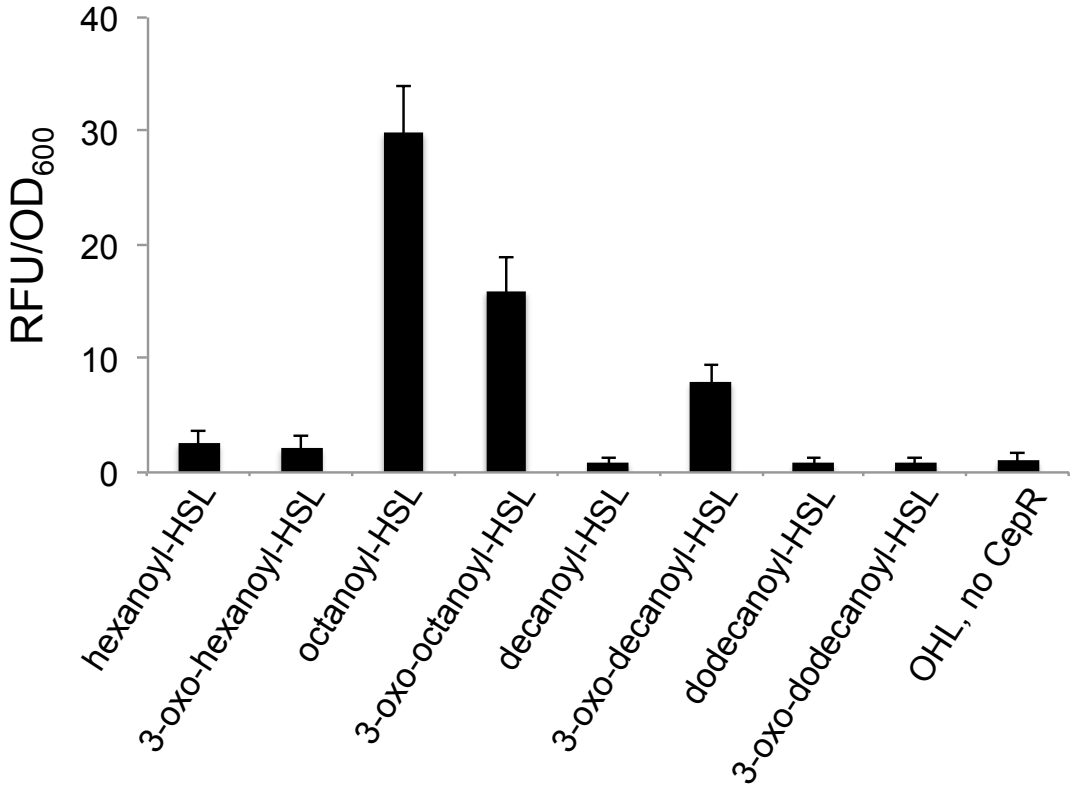

Fig. 3.4. Ability of CepR2 overproduced in *E. coli* to sequester eight different AHLs. *E. coli* strain BL21(DE3)(pGR107) was incubated in medium containing 10 nM of the indicated AHL. Bound AHLs were extracted and bioassayed (Zhu et al., 1998). The bioassay strain was calibrated using each AHL. The values shown are the mean and standard deviations (error bars) and triplicate experiments.

3.3.7. AHL-independent folding of CepR2.

Several LuxR-type transcription factors that require AHLs for activity fail to fold into a soluble, protease resistant form in the absence of AHLs (Zhu & Winans, 1999, Zhu & Winans, 2001, Urbanowski et al., 2004, Schuster et al., 2004, Weingart et al., 2005). In contrast, several LuxR-type proteins that are antagonized by cognate AHLs fold into

soluble, protease resistant forms in the absence of their cognate pheromones (Tsai & Winans, 2011, Minogue *et al.*, 2002, Castang et al., 2006). Solubility of some LuxR-type proteins is also enhanced by artificial overexpression of the chaperone GroESL (Chai & Winans, 2009, Choi & Greenberg, 1992). We assayed the accumulation of soluble CepR2 in the presence and absence of OHL, and in strains that express normal or elevated levels of GroESL. CepR2 was detected in a soluble form only when GroESL was overproduced (Fig. 3.5). The yield of soluble CepR2 may have been enhanced somewhat by OHL, but it was significantly soluble in the absence of OHL. CepR2 therefore resembles at least three other LuxR-type proteins that function as apo-proteins in that none requires its ligand for folding into a soluble form.

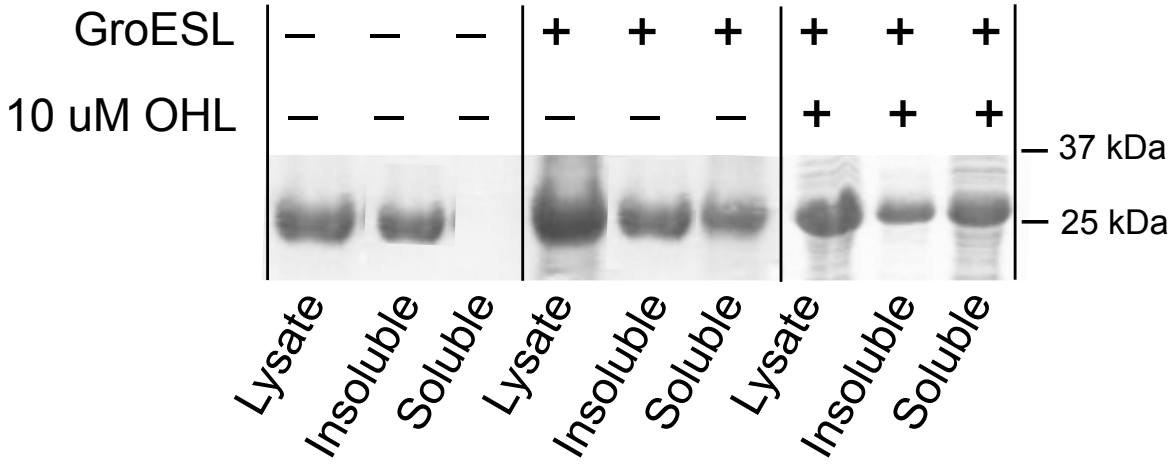

Fig. 3.5. Ability of CepR2 to fold into a soluble form requires GroESL but does not require OHL. *E. coli* strain BL21(DE3)(pGR107) containing or lacking pT7-GroESL was cultured in medium containing or lacking 1 μM OHL, lysed, and clarified extracts were size-fractionated by SDS-PAGE and stained using Coomassie Brilliant Blue.

3.3.8. Electrophoretic mobility shift assays with CepR2.

Data described above suggested that CepR2 binds to a dyad symmetrical DNA sequence in the intergenic region between *bcam0191* and *bcam0192*. We sought to obtain biochemical support for this hypothesis by carrying out electrophoretic mobility shift assays (EMSA) using radiolabelled DNA fragments containing this sequence. Clarified supernatants containing apo-CepR2 shifted a DNA fragment containing 83 nucleotides of DNA that contains this sequence (Fig. 3.6, Fragment 2) under conditions including a 10,000-fold excess non-specific competitor DNA. The extract containing apo-CepR2 did not shift two fragments containing nearby sequences (Fragments 1 and 3). We also tested two fragments identical to Fragment 2 (denoted Fragments 4 and 5) that contained either a 3-nucleotide or 4-nucleotide alterations in the dyad sequence (Fig. 3.6, bottom panel). Binding affinity was greatly weakened with these mutant DNA fragments, although residual binding was detectable.

The data described above using fusions indicates that CepR2 is antagonized by OHL and suggests that its ability to bind DNA might be antagonized by OHL. To test this, we set up binding reactions using Fragment 2, apo-CepR2, and a range of OHL concentrations. As predicted, OHL inhibited DNA binding by CepR2 (Fig. 3.6, right panel).

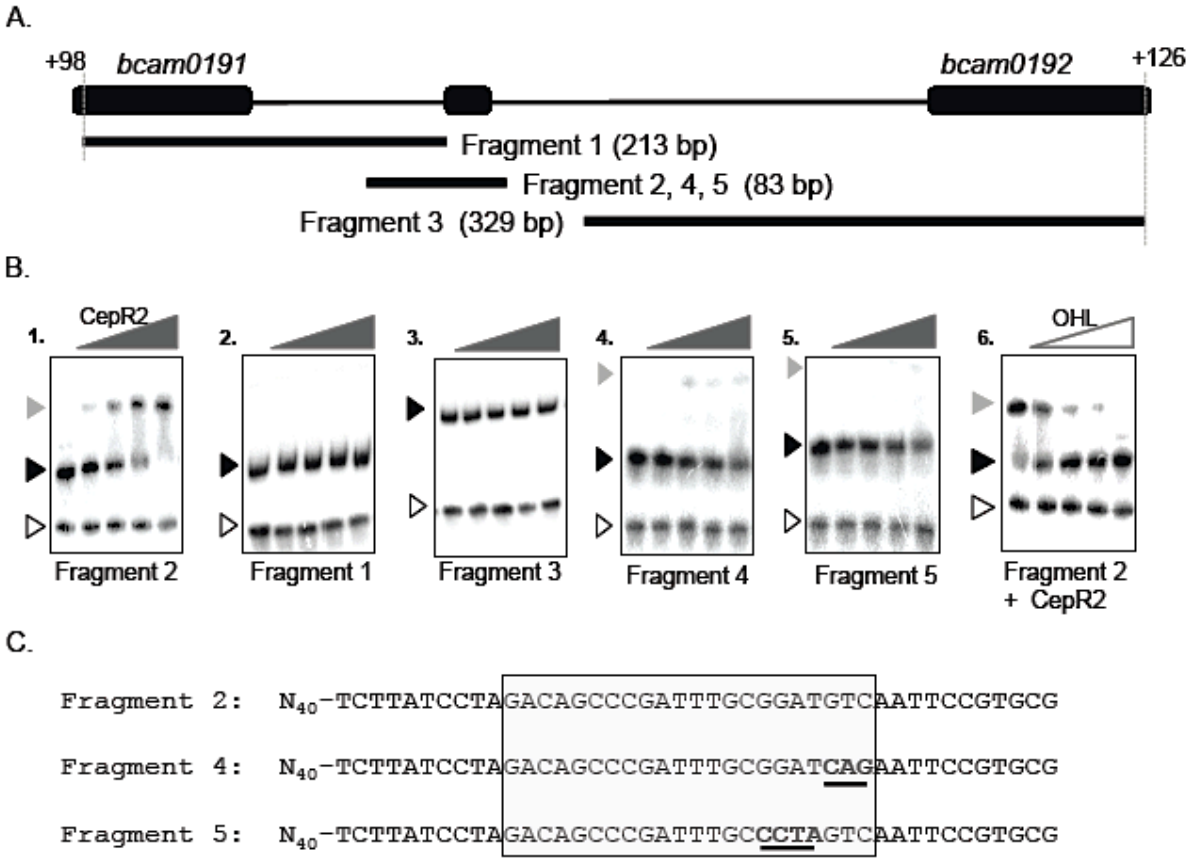

Fig. 3.6. Electrophoretic mobility shift assays of fragments containing the CepR2 binding site. A: location and size of DNA fragments used in Part B. B. Clarified supernatants containing CepR2 were used for all binding reactions. A 65-bp PCR amplified *lacZ* DNA fragment was used as a negative control (open arrowhead). Free DNA is indicated using a black arrowhead, while CepR2-DNA complexes are indicated using a grey arrowhead. CepR2 supernatants were diluted 3.16-fold in reactions with DNA fragments in the absence of OHL (gels 1-5). In gel 6, binding reactions containing CepR2 and Fragment 2 were amended with OHL to final concentrations of 0 μM, 0.032 μM, 0.1 μM, 0.315 μM, and 1.0 μM. C. Sequence of fragments containing the wild type CepR2 binding site (Fragment 2) or near-identical fragments having the indicated sequence alterations (Fragments 4 and 5). The dyad symmetrical CepR2 binding site is boxed, and altered sequences are underlined.

Earlier in this study we provided evidence that CepR2 does not autoregulate, nor does it regulate *cepS*. Supporting these conclusions, CepR2 did not detectably shift a DNA fragment containing the *cepR2-cepS* intergenic region.

3.3.9. DNase I footprinting of the CepR2 binding site.

In order to further localize the CepR2 binding site, we carried out DNase I footprinting experiments using fluorescently end-labeled DNA fragments containing this sequence. Clarified supernatants containing apo-CepR2 protected a region of approximately 20 nucleotides that contains this dyad symmetry (Fig. 3.7). On the basis of promoter resections, point mutations, EMSA, and DNase I footprinting, we conclude that CepR2 binds specifically to this dyad DNA sequence.

3.3.10. Identification of the transcription start sites of *bcam0191* and *bcam0192*.

In an effort to identify possible transcription start sites for the two promoters, we isolated total mRNA from strain K56-I2 cultured in the presence or absence of OHL and hybridized it with a 5' fluorescently labeled oligonucleotide complementary to *bcam0191* mRNA, and in a separate reaction, did the same experiment using an oligonucleotide complementary to *bcam0192* mRNA. These oligonucleotides were used as primers for DNA synthesis by reverse transcriptase, and resulting cDNA transcripts were size-fractionated by automated capillary electrophoresis.

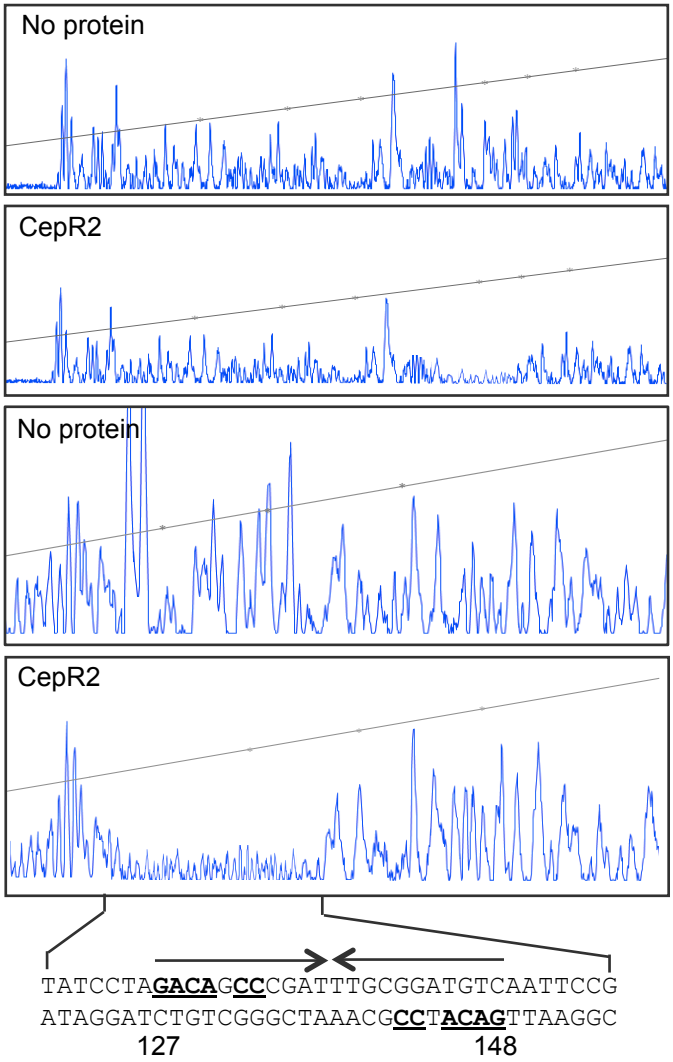

Fig. 3.7. DNase I protection of the CepR2 binding site by CepR2. A fluorescently end-labeled DNA fragment was combined with a clarified extract containing CepR2 (second and fourth panel) or an extract lacking CepR2 (first and third panel), partially digested with DNase I, and size fractionated by automated capillary electrophoresis. The bottom two panels are enlargements of the left third of the top two panels. The DNA sequence of the protected region is shown at the bottom. The CepR2 binding site is indicated using inverted arrows, and symmetrical nucleotides are underlined. Nucleotides are numbered with respect to the 5'- end of fluorescently-labeled amplicon.

Using the former primer, the major reverse transcripts were 61, 62, and 63 nucleotides in length, corresponding to apparent start sites lying 54, 55, and 56 nucleotides upstream of the *bcam0191* translation start site (Fig. 3.8). Upstream of these sites are sequences that resemble the -10 and -35 motifs of proteobacterial vegetative promoters. The promoter motif and apparent starts sites are ATGAAAN$_{17}$TATTTTTTATTAAA, where single underlined sequences resemble consensus promoters, and the double underlines indicate the three apparent transcription start sites. Plasmid pGR134 contains this putative promoter with no additional upstream sequences. It expresses this promoter at very low levels, suggesting that it may lack a binding site for CepS. The CepR2 binding site is centered 75 nucleotides upstream of this putative transcription start site.

Using the fluorescent primer that hybridizes to *bcam0192* mRNA, we detected several reverse transcripts ranging in size from 40 to 53 nucleotides (Fig. 3.8). These correspond to apparent transcription start sites between and 112 and 126 nucleotides upstream of the *bcam0192* translation start site. Upstream of these apparent start sites is the sequence TTGAATN$_{19}$TATTTAGCATCGACGCCTGAAA, where single underlined sequences resemble consensus promoters, and the double underlines indicate the apparent transcription start sites. The positioning of a promoter motif with respect to the three candidate start sites suggests that the middle candidate, a G residue, may represent the true transcription start site. The CepR2 binding site is centered 150 nucleotides upstream from this putative transcription start site.

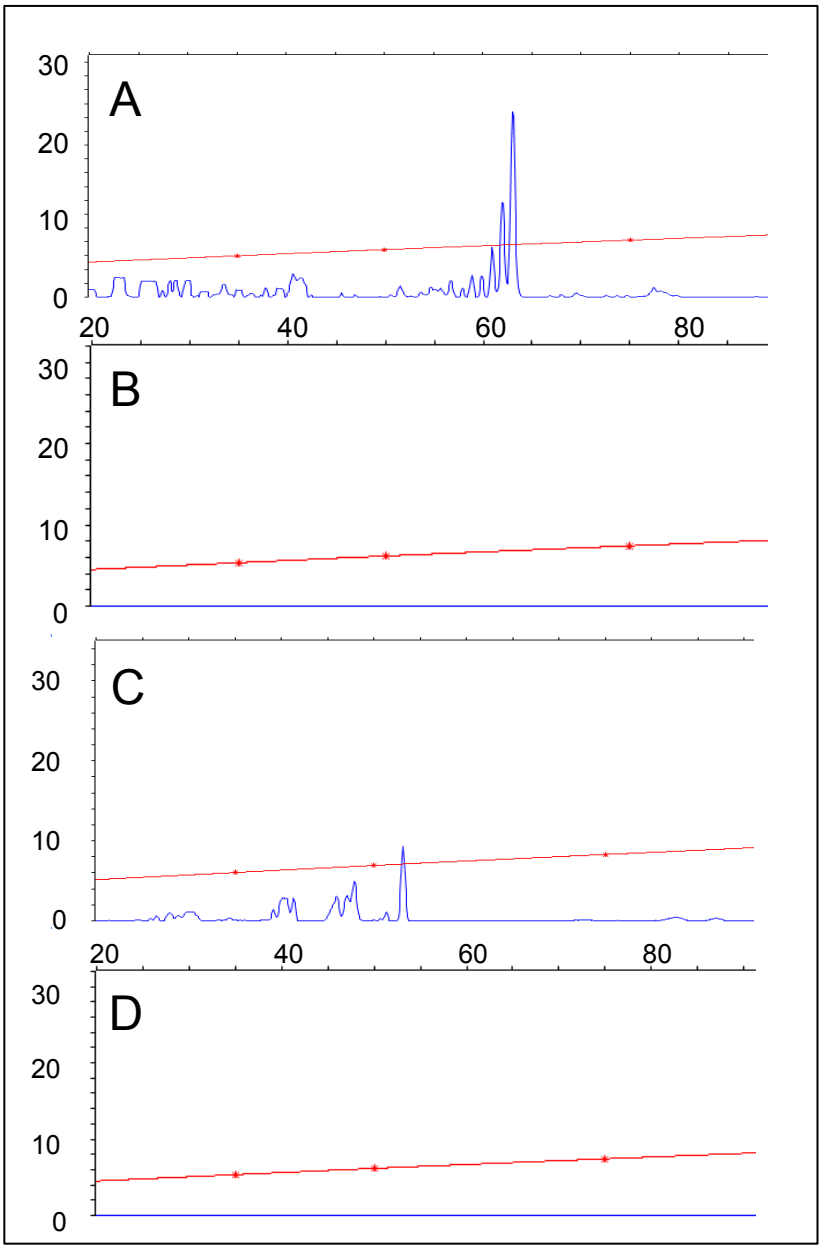

Fig. 8. Localization of the *bcam0191* and *bcam0192* promoters by primer extension of mRNA. Total RNA was purified from strain K56-I2 cultured in the absence (B and D) or presence (A and C) of 1 μM OHL. Oligonucleotides GR458 and GR459 were used to prime reverse transcription of *bcam0191* and *bcam0192* mRNA, respectively, and the resulting cDNA fragments were size-fractionated by automated capillary electrophoresis. Sizes are relative to the 5' ends of the two fluorescently labeled primers.

3.4. Discussion

CepR2 is active only as an apo-protein

This study was initiated while trying to reconcile microarray data of our lab with that of another group. Malott and colleagues showed that a strain lacking CepR2 overexpressed a number of genes tightly linked to *cepR2* (Malott et al., 2009), while we had found that OHL stimulated the expression of an overlapping set of genes. The hypothesis that CepR2 was a repressor whose activity was blocked by a cognate pheromone seemed worth exploring, as most LuxR-type proteins require a cognate AHL for activity. The only known exceptions are the VjbR protein of *Brucella melitensis* and a clade of proteins that resemble EsaR of *P. stewartii* (Tsai & Winans, 2010, Delrue et al., 2005).

Our data confirm that CepR2 is antagonized by OHL, making it similar to VjbR and to members of the EsaR clade. EsaR-type proteins are relatively closely related, and may therefore have evolved from a common ancestor that had similar properties. However, EsaR-type proteins are not closely related to CepR2 or to VjbR. CepR2 and VjbR are also only distantly related. These data suggest that the ability of these proteins to function only as apo-proteins may have evolved at least three times independently. It seems quite plausible that more LuxR-type proteins will turn out to be AHL-inhibited rather then AHL-stimulated.

The study of Malott and colleagues provided data that CepR2, when expressed in *E. coli*, activated the *luxI* promoter in the absence of any AHL (Malott et al., 2009).

Activation was not affected by addition of ten different AHLs, including OHL. It was concluded that CepR2 does not detect AHLs. The ability of CepR2 to function in the absence of AHLs agrees well with our findings. The lack of inhibition by AHLs is also reminiscent of some of the data in the current study. CepR2, when expressed in *E. coli*, repressed both the *bcam0191* and *bcam0192* promoters whether or not OHL was provided (Table 3.3). OHL-responsiveness was restored only when CepS was co-expressed. In both studies, CepR2 was expressed by fusing the *cepR2* gene to the P*lac* promoter. We believe that in both studies, CepR2 may inadvertently have been overexpressed. If so, perhaps this overexpression may overcome the inhibitory activity of OHL. One could imagine that CepR2 binds DNA only as a dimer, that OHL weakens dimerization, and the overexpression of CepR2 may shift the equilibrium toward dimers, such that enough dimers exist to populate the binding site and repress transcription (in our study) or activate transcription (in the Malott study).

CepR2 acts as a repressor

Although EsaR-type members of the LuxR family are sometimes referred to as repressors, at least some of them can act as both repressors and activators, depending largely on the position of their binding sites relative to the target promoter. For example EsaR is both an autorepressor and an activator of a divergent gene, *esaS* (Schu *et al.*, 2011). YenR also is an activator of a small RNA gene (Tsai & Winans, 2011). In the present study, CepR2 was demonstrated to act as a repressor. However, it is plausible that it could also activate one or more other promoters in this organism. The fact that CepR2 can activate the *luxI* promoter of *V. fischeri* in a system reconstituted in *E. coli*

provides further evidence that it could act as an activator in *B. cenocepacia*. Malott and colleagues reported that the *cepR2* mutation caused decreased expression of 127 genes, though the effects were generally modest (Malott et al., 2009).

Efforts to reconstitute regulated expression of these promoters in *E. coli* were met with some success and a few surprises. CepR2 inhibited expression of both promoters, and CepS activated both, just as they did in *B. cenocepacia,* strongly suggesting that they act directly. The expression of both promoters in the absence of these proteins was far higher in *E. coli* than in *B. cenocepacia*, probably due at least in part to a ColE1 replication origin in the reporter plasmid that functions to produce high copy number in *E. coli* but which is inactive in *B. cenocepacia.* It was initially surprising that OHL did not seem to block CepR2 repression, though these results were rationalized as due to CepR2 overproduction. The fact that OHL-responsiveness was restored by CepS could be due to synergistic effects of OHL and CepS.

Regulation of cepR2 *and* cepS.

In the present study, we found that the divergent *cepR2* and *cepS* genes were very slightly up-regulated by OHL in transcriptional profiling experiments. Fusions between these promoters and *lacZ* confirmed these results, and showed curiously, that the effect was CepR2-independent. One possibility is that this regulation is mediated by CepR, although the induction is so slight as to be of unknown significance. Both promoters were also downregulated by a mutation in *cepS*, though once again, the effect was subtle.

In another study, a mutation in *cepR2* was described as causing a large increase in the expression of *cepR2* and of *cepS* (Malott et al., 2009) . We believe that the apparent discrepancy between those data and ours could be due to cis-acting effects of the *cepR2* mutation used in the Malott study. In that study, the *cepR2* null mutation was constructed using a trimethoprim resistance cassette inserted near the 5' end of *cepR2*. Significantly, this cassette has two divergent promoters (DeShazer & Woods, 1996). We believe that transcription from one promoter continued into *cepR2* while transcription from the other promoter continued into *cepS*. If so, the mutant would express both genes at higher levels than the wild type, exactly as reported. However, the implication that this enhanced expression occurred that the native promoters of the two genes would have to be re-evaluated. If we are right that the *cepR2* mutation caused increased expression of *cepS*, the increased accumulation of CepS protein could have implications for the expression of all CepS-dependent promoters described in the Malott study.

Identity of a secondary metabolite

The functions of the regulated genes remain a matter for speculation. Analysis of these protein sequences suggests a role in synthesizing a secondary metabolite. The N-terminal half of Bcam0195 is predicted to bind ATP and leucine, while the C-terminal half contains a phosphopantatheine binding site and a reductase domain. *bcam0191* is a condensation domain while Bcam0190 is an aminotransferase. Based on these homologies, one could hypothesize that this pathway could convert a yet unknown ketone into an amine, condense it to leucine, and then reduce the dipeptide into a terminal aldehyde. Further chemistry probably could occur on the reactive aldehyde (Michael

Burkart, personal communication). We are currently collaborating in an attempt to purify such a metabolite and identify its structure.

Opposing roles for CepR2 and CepS.

The two CepR2-repressed promoters that we examined are unusual in that the repressor binding site appears to lie upstream of the regulated promoters. In the case of *bcam0191*, the binding site is centered 75 nucleotides upstream of the transcription start site. In the case of *bcam0192*, the binding site is centered 150 nucleotides upstream. Promoter resections confirm that the CepR2 binding site lies well upstream of both promoters. This is atypical of most repressible promoters, as repressor binding sites generally lie within the target promoter or directly downstream (Perez-Rueda *et al.*, 1998). We believe that this unusual promoter geometry can be explained only in the context of CepS, a positive regulator of both promoters. Data obtained from promoter resections can be used to predict the region of the CepS binding site. We have several 5' resections that are blind to CepR2 yet are still CepS-dependent, indicating that CepS must bind downstream of CepR2. This binding site must lie fully within plasmid pGR133, but may be fully or partly absent in pGR134. These plasmids contain 75 and 37 nucleotides of DNA upstream of the *bcam0191* transcription start site, respectively. These data strongly suggest that CepS binds DNA between the promoter and the CepR2 binding site.

A *cepS* mutant expressed both promoters at very low levels irrespective of OHL status. This indicates that when CepS is absent, CepR2 has no effect on expression of these promoters. In other words, CepS appears to work downstream of CepR2, and

CepR2 appears to act by inhibiting CepS activity. One possibility is that CepR2 binding sterically blocks CepS binding, and that OHL, by blocking CepR2 activity, allows CepS to bind and activate the two promoters. If so, there must be two CepS binding sites, as plasmid pGR133 and pGR138, which share no *B. cenocepacia* DNA, have two different CepS-dependent promoters and therefore two different CepS binding sites. Interestingly, when CepR2 and CepS function were reconstituted in *E. coli*, CepR2 was able to decrease expression even in the absence of CepS, while this was not true in *B. cenocepacia*. It seems possible therefore that CepR2 may regulate these promoters in two ways, one dependent on CepS, and one that is independent.

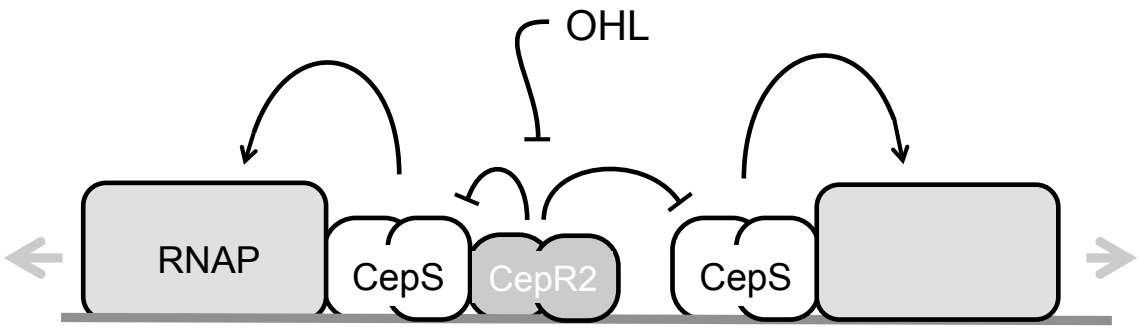

Fig. 3.9. A model of proposed activities of CepR2 and CepS. In this model, apo-CepR2 binds to a single site between the *bcam0191* and *bcam0192* promoters. Bound CepR2 inhibits the stimulatory activity of CepS, which binds between CepR2 and the two target promoters. At high-cell density, OHL accumulates and releases CepR2 from the DNA, permitting CepS to activate both promoters. CepR2 thus functions as an antiactivator of CepS.

The interactions of the CepR2 repressor and the CepS activator are somewhat reminiscent of the CytR repressor and CAP activator of *E. coli*, which function antagonistically at several promoters (Shin *et al.*, 2001, Tretyachenko-Ladokhina *et al.*, 2006, Valentin-Hansen *et al.*, 1996). CytR binds to a site centered 70 nucleotides upstream of the *deoP2* promoter, flanked by two binding sites for CAP, one centered at -40.5 and the other at -93.5. Binding of CytR does not dislodge CAP, but may block the proper positioning of the C-terminal domain of the alpha subunit of RNA polymerase (RNAP). By analogy, apo-CepR2 could act by blocking the interactions between CepS and RNAP (Fig. 3.9). Equally plausibly, CepR2 might simply block the binding of CepS to a site near these promoters.

3.5. Experimental Procedures.

Strains, oligonucleotides, and growth conditions.

Bacterial strains and plasmids used in this study are listed in Tables 3.7 and 3.8. Oligonucleotide primers (IDT, Coralville, Iowa) used for PCR amplification and DNA-mutagenesis are listed in Table 3.9. *Burkholderia cenocepacia* and *Escherichia coli* were cultured at 37°C in Luria-Bertani (LB) medium and *Agrobacterium tumefaciens* was cultured at 28°C in AT minimal medium. Antibiotics were added where described at the following concentrations: 100 μg ml^{-1} ampicillin, 100 μg ml^{-1} kanamycin, 35 μg ml^{-1} chloramphenicol, and 12 μg ml^{-1} tetracycline for *E. coli*; 300 μg ml^{-1} tetracycline, 700 μg ml^{-1} kanamycin, 400 μg ml^{-1} gentamicin for *B. cenocepacia*; 100 μg ml^{-1} spectinomycin and 35 μg ml^{-1} tetracycline for *A. tumefaciens*. Media was supplemented with 500 μM isopropyl β-d-galactopyranoside (IPTG) where indicated.

Transcriptional activity of *bcam0191* and *bcam0192* promoters.

Recombinant DNA techniques were performed using standard methods (Sambrook & Russell, 2001). The intergenic region containing promoter and regulatory elements for each divergent promoter was resected by PCR amplification. For each resection, the amplicon was cloned into the promoter-less transcriptional *lacZ* reporter plasmid pYWN302 at KpnI and XbaI sites creating a transcriptional reporter fusion. Reporter fusion plasmids were transformed into *B. cenocepacia* or *E. coli* strain MC4100 by electroporation (Cangelosi *et al.*, 1991). To assay promoter activity, overnight cultures were diluted to 1:100 into LB medium and grown at 37°C to an OD_{600} of 0.35 with the appropriate antibiotics and 1 μM OHL. Cultures aliquots (150 μL) were transferred to

the wells of opaque microtitre plates containing 4 μl of a 1.5 mg/ml solution of 4-methylumbelliferyl-β-D-galactopyranoside (MUG) dissolved in DMSO. β-galactosidase specific activities were measured using a Biotek Synergy HT microplate fluorescence reader. Experiments were performed in triplicate with three different isolates of each strain.

To construct a plasmid expressing a regulated P*lac-cepR2* fusion, the *cepR2* gene was cloned into the pSRKKm broad-host range vector (Khan *et al.*, 2008) to create pGR192. This promoter is regulated by $LacI^q$ encoded on the plasmid and is induced with IPTG. pSRKGm was used to construct plasmid pGR193 which expresses an IPTG-inducible *cepS* gene. Constitutive expression of CepS was obtained by cloning the *cepS* gene into plasmid pSW208 to create pGR276.

Mutations of the putative *cepR2* box were made by PCR amplification using oligonucleotide primers containing mutagenic oligonucleotides. The PCR fragments were cloned into the transcriptional reporter vector pYWN302 that had been digested with KpnI and XbaI.

Construction of deletion mutations in *cepR2* and *cepS*.

To create an internal deletion *cepR2* mutant, oligonucleotides GR329 and GR330 were used to PCR amplify a 741-nucleotide fragment upstream of *cepR2*, while oligonucleotides GR331 and GR332 were used to PCR amplify a 737-nucleotide fragment downstream of *cepR2*. These fragments were digested with EcoRI, ligated, and PCR amplified using oligonucleotides GR329 and GR332, creating a 1.5 kb fragment

with a 633 nucleotide deletion of *cepR2* (nucleotides 21-652 of the *cepR2* reading frame). This fragment was digested using HindIII and XbaI and ligated into pEX18Tet-pheS (Barrett *et al.*, 2008), and introduced into strain SM10(*λpir*) by transformation, creating pGR178. This plasmid was introduced into *B. cenocepacia* K56-I2 by conjugation. Tetracycline-resistant single-crossover recombinant mutants were screened by PCR for correct integration of the plasmid and double crossover recombinants were selected using M9 agar supplemented with 0.1% p-chlorophenylalanine (Sigma-Aldrich) (Barrett et al., 2008). The resulting colonies were screened by PCR amplification for the 633 nucleotide *cepR2* deletion and verified by DNA sequencing (Cornell Biotechnology Resource Center). The resulting *cepR2* deletion was designated GR141.

A similar strategy was used to delete *cepS*. Oligonucleotides GR345 and GR346 were used to amplify a 474-nucleotide fragment upstream of *cepS*, while GR347 and GR348 were used to PCR amplify a 492-nucleotide fragment downstream of *cepS*. These fragments were digested with SpeI, and PCR amplified using oligonucleotides GR345 and GR348, yielding a 0.95 kb fragment that contains a 0.9 kb deletion of *cepS*. This fragment was digested using BamHI and EcoRI and ligated into pEX18Tet-*pheS*, to create pGR182. The *cepS* deletion was crossed into the genomic DNA of strain K56-I2 as described above, creating strain GR145.

AHL detection by CepR2.

To measure CepR2 AHL ligand specificity, strains K56-I2(pGR130) and K56-I2(pGR136), was cultured at 37°C to mid-log phase (OD_{600} 0.4) in 2 ml LB medium supplemented with tetracycline and AHLs at concentrations ranging from 1 pM to 1 μM.

Promoter activity was determined by measuring β-galactosidase activity as described above. Experiments were performed in triplicate.

Overexpression of CepR2.

To overexpress CepR2 in *E. coli*, the *cepR2* gene was PCR amplified using oligonucleotides GR295 and GR288 and inserted into pRSETa (Invitrogen) after digesting both with NdeI and XhoI, creating pGR107. *E. coli* strain BL21 (DE3) (Novagen) harboring plasmids pGR107 and pT7-*groESL* (which expresses the chaperone GroESL) were grown in LB medium supplemented with 0.4% glucose, 400 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol at 37°C. At an OD_{600} of 0.4, cultures were cooled to 28°C and 10 μM OHL was added as indicated. Protein expression was induced using 0.5 mM IPTG and growth was continued for three additional hours at 28°C. Cells were harvested and resuspended in TEDG buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 20% glycerol) supplemented with 200 mM NaCl. Cells were disrupted using a French press (three passages, 10,000 psi) and the lysate was clarified by ultracentrifugation (50,000 r.p.m., 30 min, 4°C). Protein fractions were analyzed on SDS-PAGE gels stained with Coomassie blue.

AHL sequestration assays.

E. coli strain BL21(DE3)(pGR107) was used to test for the sequestration of AHLs. Cells were cultured at 18°C in 10 ml LB medium supplemented with 100 μg ml^{-1} ampicillin. When the OD_{600} reached 0.4, IPTG was added to a final concentration of 0.5 mM, and AHLs were added at a final concentration of 10 μM. When the cultures reached an OD_{600} of 0.7 (approximately 4 hours), they were harvested, washed twice with LB, then washed three times with TE buffer (10 mM Tris (pH 8), 0.5 mM EDTA) and resuspended

in lysis buffer (200 mM Tris (pH 8), 400 mM EDTA, 0.7 mM sucrose). Cell-associated autoinducers were extracted twice with ethyl acetate:acetonitrile (99.5:0.5 v/v) (HPLC grade, Fisher). Organic phase extracts were pooled and dried under nitrogen gas. Pellets were resuspended in 10 μl ethyl acetate and added to cultures inoculated with the biosensor strain *A. tumefaciens* WCF47(pCF218)(pCF372), which detects a wide range of AHLs (Zhu *et al.*, 1998). The detection of each AHL was calibrated using known concentrations of each AHL. Cultures were grown for 12 h at 28° and assayed for β-galactosidase specific activity. Experiments were performed in triplicate.

Electrophoretic mobility shift assays.

For all EMSA reactions, a clarified supernatant from BL21(DE3)(pGR107)(pT7-*groESL*) was dialyzed against EMSA buffer (50 mM Tris-HCl pH 7.0, 2 mM EDTA, 2 mM DTT, 60 μM potassium acetate, 39 μM potassium glutamate, 20% glycerol). DNA fragments were PCR amplified using oligonucleotides described in Table 3.9 and end-labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP (Perkin Elmer). Binding reactions contained 2.5 pM of DNA and varying concentrations of CepR2 protein in a 15 μl total volume containing EMSA buffer, 20 μg ml-1 of calf thymus DNA, and 20 μg ml-1 of BSA. Reactions were incubated at room temperature for 30 minutes, and complexes were size-fractionated at 4°C using 10% polyacrylamide gels (Dgel Sciences) containing 20 mM Tris-acetate pH 8.5, and 1 mM EDTA (0.5 x TAE). Gels were analyzed using a Storm B840 Phosphorimager (Molecular Dynamics). All reactions were performed in triplicate.

DNase I protection assay

A fluorescently labeled 84-bp fragment was PCR amplified using primers GR280 and GR458 (Table 3.9). Binding reactions contained ~ 200 ng DNA and a clarified

supernatant of strain BL21(DE3)(pGR107)(pT7-*groESL*) (10 mg ml^{-1} total protein) or BSA (for control) diluted in 20 μl EMSA buffer and incubated at room temperature for 30 minutes. $MgCl_2$ (2.5 mM), $CaCl_2$ (0.5 mM) and 0.1 units of DNase I (Ambion) were added to the reaction and allowed to incubate at room temperature for 2.5 minutes. The reaction was stopped by addition of 0.75 μl stop solution (20 mM EDTA (pH 8.0), 200 mM NaCl, 1% SDS). DNA was purified with the Qiagen PCR kit and eluted in 20 μl water. DNA fragments were analyzed using an Applied BioSystems 3730xl DNA Analyzer (Cornell University Life Sciences Core Laboratories Center).

Primer extension assays

Strain K56-I2 was cultured to mid-log phase in LB with or without 1 μM OHL at 37°C. DNA-free mRNA preparations were isolated from 2 ml cell culture aliquots using Qiagen RNeasy Plus Mini kit. Residual DNA in mRNA extracts was degraded using Turbo DNA-free kit (Applied Biosystems) and mRNA was purified by isopropanol precipitation. cDNA transcripts containing *bcam0191* and *bcam0192* transcriptional start sites were obtained with the Superscript III RT kit (Invitrogen) using GR458 or GR459 fluorescently labeled primers, respectively. cDNA transcripts were purified (Qiagen PCR purification kit) and DNA fragment analysis was performed as above.

All reactions were performed in triplicate.

Transcriptional profiling.

Whole genome microarray slides containing 3-5 different probes for each gene of the *B. cenocepacia* genome were purchased from Agilent (AMADID #016249). Bacterial strains were cultured to exponential phase in AT minimal medium and subjected to RNA extraction as described previously (Cho & Winans, 2005). Preparation of fluorescent

cDNA was performed following a published procedure (Hegde *et al.*, 2000). Hybridization and washing of slides was performed according to the manufacturer's protocol. Fluorescence intensity was analyzed using a GenePix 400B scanner (Axon). Induction ratios were calculated after normalization with locally weighted linear regression (lowess) analysis. Experiments were performed in duplicate, with independent bacterial culturing, RNA preparation, cDNA probe synthesis, dye coupling and hybridizations. The Cy3 and Cy5 dyes were swapped in the two trials.

Table 3.7. Bacterial strains used in this study.

Strain	Description	Source and/or
B. cenocepacia		
K56-I2	*B. cenocepaci*a K56-2 *cepI*::TpR	(Lewenza *et al.*,
GR141	K56-I2, Δ*cepR2*, *cepI*::TpR	This study
GR145	K56-I2, Δ*cepS*, *cepI*::TpR	This study
E. coli		
DH5α	*F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+)*	Stratagene
BL21(DE3)	P*lac*-gene 1 of bacteriophage T7; pTet-TVMV protease, Km^R	(Studier *et al.*,
MC4100	F- a*raD139* Δ(*argF-lac*)U169 *rpsL150 relA1 deoC1 rbsR fthD5301*	(Ferenci *et al.*,
SM10(λ*pir)*	*λpir,* RP4 *tra* regulon, host for *pir*-dependent plasmids; Kan^R	(Donnenberg &
EPMax10B	F−*mcrA* Δ(*mrr-hsdRMS-mcrBC*) 80*dlacZ*ΔM15 Δ*lac*X74 *deoR recA1*	(Barrett *et al.*,
Agrobacterium tumefaciens		
WCF47(pCF218)	R10 Δ*traI;* P*tetR-traR*, P*traI-lacZ* fusion; Tet^R, Sp^R	(Fuqua & Winans,

Table 3.8. Plasmids used in this study.

Plasmid	Description	Source and/or reference
pRSETa	T7 promoter cloning vector, ColE1 ori, Ap^R	Invitrogen
pT7-*groESL*	PT7-*groESL*, ColE1; Cm^R	(Yasukawa *et al.*, 1995)
pYWN302	Broad host range, promoterless transcriptional *lacZYA* reporter, Tet^R	(Wei *et al.*, 2011)
pSRKGm	pBBR1MCS-2-derived broad-host range expression vector containing *lac* promoter; Gm	(Khan *et al.*, 2008)
pSRKKm	pBBR1MCS-2-derived broad-host range expression vector containing *lac* promoter; Kan^R	(Khan et al., 2008)

pEX18Tet-*pheS*	Suicide plasmid for gene replacement based on *pheS* and Tet^R	(Barrett et al., 2008)
pSW208	pACYC184-derived plasmid containing *lac* promoter; Cm^R	Lab collection
pGR107	*cepR2* cloned into *NdeI* and *XhoI* sites of pRSETa using oligonucleotides GR295 and GR288.	This study
pGR130	PCR fragment using oligonucleotides GR283 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion.	This study
pGR132	PCR fragment using oligonucleotides GR280 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion.	This study
pGR133	PCR fragment using oligonucleotides GR281 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion.	This study
pGR134	PCR fragment using oligonucleotides GR279 and GR284 cloned into pYWN302 KpnI and XbaI sites; *bcam0191-lacZ* fusion	This study
pGR136	PCR fragment using oligonucleotides GR196 and GR191 cloned into pYWN302 at KpnI and XbaI sites; *bcam0192-lacZ* fusion.	This study
pGR137	PCR fragment using oligonucleotides GR195 and GR191 cloned into pYWN302 at KpnI and XbaI sites; *bcam0192-lacZ* fusion	This study
pGR138	PCR fragment using oligonucleotides GR194 and GR191 cloned into pYWN302 at KpnI and XbaI sites; *bcam0192-lacZ* fusion	This study
pGR139	PCR fragment using oligonucleotides GR193 and GR191 cloned into pYWN302 at KpnI and XbaI sites; *bcam0192-lacZ* fusion	This study
pGR140	PCR fragment using oligonucleotides GR192 and GR191 cloned into pYWN302 at KpnI and XbaI sites; *bcam0192-lacZ* fusion.	This study
pGR141	PCR fragment using oligonucleotides GR197 and GR202 cloned into pYWN302 at KpnI and XbaI sites; *cepR2-lacZ* fusion.	This study
pGR146	PCR fragment using oligonucleotides GR203 and GR208 cloned into pYWN302 at KpnI and XbaI sites; *cepS-lacZ* fusion.	This study
pGR178	PCR fragment made using GR329, GR330, GR331, and GR332, cloned into pEX18Tet-*pheS*; *cepR2* deletion with flanking DNA	This study
pGR182	PCR fragment made using GR345, GR346, GR347, and GR348 cloned into pEX18Tet-*pheS*; *cepS* deletion with flanking DNA.	This study
pGR192	pSRKKm derivative containing *cepR2* using GR288 and GR295.	This study
pGR193	pSRKGm derivative containing *cepS* using GR322 and GR323	This study
pGR195	PCR fragment using oligonucleotides GR292 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion	This study
pGR197	PCR fragment using oligonucleotides GR293 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion	This study
pGR198	PCR fragment using oligonucleotides GR294 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion	This study
pGR236	PCR fragment using oligonucleotides GR275 and GR283 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion	This study
pGR243	PCR fragment using oligonucleotides GR196 and GR380 cloned into pYWN302 at KpnI and XbaI sites; *bcam0192-lacZ* fusion	This study

pGR259	PCR fragment using oligonucleotides GR392 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion with altered CepR2 binding site	This study
pGR260	PCR fragment using oligonucleotides GR393 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion with altered CepR2 binding site	This study
pGR261	PCR fragment using oligonucleotides GR394 and GR284 cloned into pYWN302 at KpnI and XbaI sites; *bcam0191-lacZ* fusion with altered CepR2 binding site	This study
pGR276	pSW208 derivative containing *cepS* using GR323 and GR417	This study

Table 3.9. Oligonucleotides used in this study.

Name	Sequence	Comments
GR191	ATTTCTAGACGAATACGTGCCATTCCATG	For PCR amplifying *bcam0192* promoters in pGR136, pGR137, pGR138, pGR139, and pGR140 and Fragment 3.
GR192	ATTGGTACCCTGAAATTGCTGC	For PCR amplifying of *bcam0192* promoter in pGR140.
GR193	ATTGGTACCTGGATGGATGAGGAGTCTG	For PCR amplifying Fragment 3 and pGR139
GR194	ATTGGTACCTTGCGGATGTCAATTCC	For PCR amplifying of *bcam0192* promoter in pGR138.
GR195	ATTGGTACCTCGCATCGTGCATTTC	For PCR amplifying of *bcam0192* promoter in pGR137.
GR196	ATTGGTACCTCACGTCGTTTCTCCTG	For PCR amplifying *bcam0192* promoter in pGR136 and pGR243
GR197	ATTTCTAGACGACGATCTGCATGTCG	For PCR amplifying *cepR2* promoter in pGR141
GR202	ATTGGTACCGTCGATCAGTCCTGATAC	For PCR amplifying *cepR2* promoter in pGR141
GR203	ATTGGTACCCATACTCGTGAGCAAGC	For PCR amplifying *cepS* promoter in pGR146
GR208	ATTTCTAGAATCGGTGATCCTCG	For PCR amplifying *cepS* promoter in pGR146
GR275	GCTTCTAGAGGTGGATGAATTAAATGC	For PCR amplifying *bcam0191* promoter in pGR236
GR279	ATTGGTACCATGAAATGCACGATGCG	For PCR amplifying *bcam0191* promoter in pGR134
GR280	ATTGGTACCCCTCATCCATCCATCAA	For PCR amplifying *bcam0191* promoter in pGR132
GR281	ATTGGTACCATCGGGCTGTCTAGGATAAG	For PCR amplifying *bcam0191* promoter in pGR133
GR283	ATTGGTACCGGACTCTCCTAGTAATGTCC	For PCR amplifying *bcam0191* promoter in pGR130, pGR236.
GR284	ATTTCTAGAGCGTAGATATGCGTCGAC	For PCR amplifying *bcam0191* promoters in pGR130, pGR132, pGR133, pGR134, pGR195, pGR197, pGR198, pGR259, pG260, pGR261 and Fragment 1.
GR288	ATTCTCGAGTTTTTCACGTCACGG	For amplifying *cepR2* for cloning into pGR107 and pGR192
GR292	ATTGGTACCTACTGGAAAGGCTTTGACGCA	For PCR amplifying *bcam0191* promoter in pGR195
GR293	ATTGGTACCCGCACGGAATTGACATC	For PCR amplifying *bcam0191* promoter in

		pGR197
GR294	ATTGGTACCCCGCAAATCGGGCTGTC	For PCR amplifying *bcam0191* promoter in pGR198
GR295	GGAATTCCATATGGACCTGACAATACTG	For amplifying of *cepR2* for cloning into pGR192 and pGR107
GR301	CCTCTTCGCTATTACGCCAGC	For PCR amplifying of *lacZ* fragment for negative control for EMSA experiments
GR302	CGTTACCCAACTTAATCG	For PCR amplifying of *lacZ* fragment for negative control for EMSA experiments
GR322	GGAATTCCATATGACCAGCGTTCAAGACG	For PCR amplifying *cepS* for cloning into pGR193
GR323	CCCAAGCTTGATCGTGCATGCGATC	For PCR amplifying *cepS* for cloning into pGR193 and pGR276.
GR324	GATCGTGCATGCGATC	For sequence verification of *cepR2* deletion
GR328	GCATGCGTCTTGTTCATCGC	For sequence verification of *cepS* deletion
GR329	ACTAAGCTTCTGCAGTCGCTCGCACAGCTT	With GR330, GR331, and GR332, for construction of *cepR2* deletion fragment cloned into pGR178.
GR330	ATTGAATTCTTGCAGTATTGTCAGGTCCATC	With GR329, GR331, and GR332, for construction of *cepR2* deletion fragment cloned into pGR178.
GR331	ATTGAATTCTCGACGCGATGAACAAGAC	With GR329, GR330, and GR332, for construction of *cepR2* deletion fragment cloned into pGR178.
GR332	ACTTCTAGAGCACGTACGATTCGATCATTCGC	With GR329, GR330, and GR331, for construction of *cepR2* deletion fragment cloned into pGR178.
GR345	ATGGATCCGAACGCATTCCATACGACC	With GR346, GR347, and GR348, for construction of *cepS* deletion fragment cloned into pGR182.
GR346	GCACTAGTGATCAGTCCTGATACGAAACCG	With GR345, GR347, and GR348, for construction of *cepS* deletion fragment cloned into pGR182..
GR347	GCACTAGTGATCGCATGCACGATCCG	With GR345, GR346, and GR348, for construction of *cepS* deletion fragment cloned into pGR182..
GR348	CGGAATTCGACATCATGTGCTTGGC	With GR345, GR346, and GR347, for construction of *cepS* deletion fragment cloned into pGR182..
GR351	ATTTCTAGACATATTCGCATCGTGCATTTC	For PCR amplifying Fragments 2, 4, and 5
GR365	ATTTCTAGAGGATAAGAATTGCGATTCATC	For PCR amplifying Fragment 1
GR380	ATTTCTAGATTCCGGCGCCGGAAACCGTTT	For PCR amplification of *bcam0192* promoter in pGR243
GR391	ATGGTACCCGCACGGAATTGACATCCGCAA ATCGGGCTGTC	For PCR amplifying Fragment 2
GR392	ATGGTACCCGCACGGAATTCTGATCCGCAA ATCGGGCTGTC	For PCR amplification of bcam0191 promoter in pGR259 and Fragment 4
GR393	ATGGTACCCGCACGGAATTGACTAGGGCAAA TCGGGCTGTC	For PCR amplification of bcam0191 promoter in pGR260 and Fragment 5
GR394	ATGGTACCCGCACGGAATTGACATCCCGTT ATCGGGCTGTC	For PCR amplification of bcam0191 promoter in pGR261
GR417	ATGAATTCAGGAGGCGATAGATGACCAGCG TTCAAGA	For PCR amplifying *cepS* and cloning into pGR276
GR458	6-FAM-ATCGGTCACGTCGTTTCTCC	For mapping *bcam0191* promoter and DNA footprinting analysis
GR459	6-FAM-AATTGCCTGCATGCCGT	For mapping *bcam0192* promoter

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Chapter 4: Conclusions and a brief comparison of CepR and CepR2

4.1. Decoding of the respective binding sites by CepR and CepR2

In Chapter 2, the consensus binding sequence for CepR was identified and the contributions of each nucleotide to binding affinity and specificity were determined. The 18-bp consensus sequence was defined by a five-nucleotide palindrome that flanks a variable four-nucleotide spacer around the dyad axis. Each half-box contributed five nucleotides required for CepR specificity and mutations to any nucleotide resulted in defective phenotype (Fig. 2.3). In contrast, nucleotides within the spacer region were better tolerated by CepR and did not greatly alter DNA binding recognition. Furthermore, new gene promoters directly regulated by CepR contained *cep* box motifs with strong agreement to the consensus sequence. This suggests that the highly conserved symmetric bases are predicted to be involved in CepR-DNA interactions. The features of the *cep* box are similar to other well-defined LuxR-type binding sites that each contains conserved dyad nucleotides separated by a non-symmetrical spacer region (Fig. 4.1). The individual regions appear to contribute to different interactions made by the protein at its respective binding site. TraR makes sequence-specific contacts with six bases within the conserved region of each half-site of the *tra* box, while sequence-independent contacts are made at nucleotides within the central spacer (Zhang *et al*., 2002).

A look at the binding site for the second *B. cenocepacia* LuxR-type regulator, CepR2, reveals the sequence is defined by similar features and contains four palindromic nucleotides flanking seven non-symmetrical nucleotides. Binding affinity and promoter

activity for this binding site also required sequence-specific recognition by CepR2, though mutations of the palindromic nucleotides were more defective than those made at positions with imperfect symmetry (Fig. 3.2). Additional mutations are needed to further identify all the bases critical for sequence-specific interaction within the *cepR2* box. Given the similarity in binding site characteristics and phenotypes for mutations at nucleotides within the dyad, it is predicted that residues within CepR and CepR2 C-terminal domain (CTD) would make similar contacts at critical bases within their respective DNA binding sites. Scanning alanine mutagenesis would permit the analysis of important amino acid residues involved in direct contact of both regulators with their respective binding sites.

LuxR-type binding	half box sequence
tra box (consensus)	**ATGTGC**AGA
lux box (consensus)	**CTG**TAGGAT
cep box (consensus)	**CCCTGT**AAGA
cepR2 box ($P_{bcam0191}$)	**GACA**GCCCGAT

Figure 4.1. Alignment of the left half of the binding sites for LuxR, TraR, CepR, and CepR2, respectively. Palindromic nucleotides are bolded.

It was hypothesized that the width of the *cep* box central spacer, whose spacer region is two nucleotides larger than that of the *tra* box, would result in a higher angle DNA bend when bound by CepR compared to TraR at the *tra* box. Indeed, CepR was found to induce a 45° bend at this site. There appears to be a correlation between the width of the central spacer and the magnitude of the DNA bend conferred by the LuxR-type protein. It is interesting to consider the implications of this for CepR2 at its binding

site, given that the *cepR2* box spans more than 2-helical turns. It is possible that CepR2 will cause a sharper bend. Alternatively, CepR2-*cepR2* box interaction may be constrained by a particular CepR2 conformation. That is, the absence of OHL may impart a flexibility in the CTD that can accommodate the span of the 22-nt binding site not possessed in the OHL-bound CepR2 structure. These two hypotheses can be tested by determining the DNA angle bend and shed insight into the CepR2 interaction with its binding site. If the former is true and apo-CepR2 contorts the DNA around its structure, the DNA bend angle would be expected to be greater than that observed in CepR at its binding site. If the latter is true and CepR2 affinity to the *cepR2* box is determined by the OHL-induced structural conformation, than the bend angle would be expected to be less than 45°.

4.2. Effect of OHL on CepR2 expression and function

In Chapter 3, CepR2 was shown to act as a repressor and interact with its DNA binding site in the absence of its cognate signal, OHL. This requires the protein to stably accumulate at low cell density when OHL levels are low. Accumulation of soluble CepR2 was found to be independent of OHL but required co-expression of the chaperone, GroESL, supporting this assertion. As discussed above, CepR2 recognized its DNA site through sequence-specific interactions; however, it is unclear how binding of the autoinducer signal alters this affinity for this site.

Comparison of amino acid alignments highlight structural differences between the apo-active regulators in the γ-proteobacteria and those from other classes, and include an extended linker region between the AHL-binding domain and an extended C-terminus

(Fig. 4.2). The importance of this linker region on translating the signal to the CTD is not understood. EsaR protein stability and multimeric state of both full-length and an EsaR-NTD subunit were recently reported to be unaffected by autoinducer binding (Schu et al., 2011). It is postulated that binding of AHL may be translated to the CTD and result in DNA-binding prohibitive conformational changes. This leads to interesting questions about the role of autoinducer binding on the dimerization of individual domains. However, CepR2 does not possess this linker and therefore, it is unclear whether the insights gained from the EsaR experiments are relevant to the mechanism of CepR2 regulation. Preliminary experiments, including western immunoblots and pulse-chase, are needed to address questions about the effect of OHL binding on CepR2 stability and accumulation in the cell. The role of dimerization on DNA recognition also needs to be addressed. It would be interesting to determine if CepR2-CTD subunits are functional independent of the NTD. If that is the case, the CTD domain could then be probed for mutations that inhibited functional DNA binding.

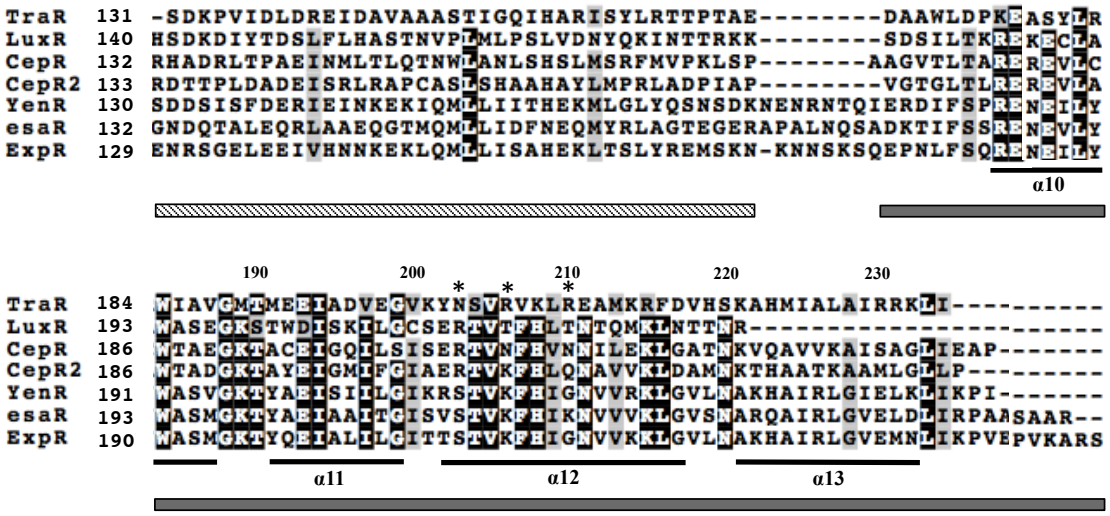

Figure 4.2. Protein alignment of LuxR-type C-terminal domain sequences using the solved TraR model as the template. TraR, LuxR, CepR, CepR2, YenR, EsaR, and ExpR are involved in quorum sensing in *A. tumefaciens*, *E. carotovora, V. fischeri, B. cenocepacia, Y. enterocolitica,* and *P. stewartii,* respectively. Identical and conserved residues are shaded in black and grey, respectively. Position of TraR residues are numbered above the sequence. Bases directly interacting with TraR residues are denoted by an asterix. Predicted alpha helices are denoted by black lines under the alignment. Protein domains are shown as boxes below the sequence: N-terminal domain, hatched box; C-terminal domain, black box.

Regulation of gene expression for divergent NRPS promoters was found to be dependent on CepR2 and CepS activity. Negative regulation is mediated by CepR2 in the absence of OHL. The site of this repression, *cepR2* box, is located distal to both promoters and predicted CepS activation sites. CepS is required for positive regulation and is predicted to bind DNA at separate sites located between the *cepR2* box and the respective promoter elements for each gene. CepS-mediated activation is independent of both OHL and CepR2, indicating that CepS is epistatic to CepR2. Apo-CepR2 is predicted to act as an anti-activator of CepS. Apo-CepR2 may act by obstructing access of CepS to the DNA or may bind adjacent to CepS and alter CepS activity through protein-protein interactions. Preliminary studies, using DNase I protection assay and additional promoter resections, will be useful in determining the DNA binding site of CepS.