DETECTION OF DIFFUSIBLE CHEMICAL SIGNALS BY TWO SPECIES OF PROTEOBACTERIA

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DETECTION OF DIFFUSIBLE CHEMICAL SIGNALS BY TWO SPECIES OF PROTEOBACTERIA

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This dissertation focuses on how two different species of *Proteobacteria*, *Yersinia enterocolitica* and *Agrobacterium tumefaciens*, detect chemical signals that are synthesized by the same species or by other organisms.

Y. enterocolitica encodes proteins YenR and YenI, that resemble the LuxR and LuxI proteins of *Vibrio fischeri.* YenI synthesizes mainly 3-oxohexanoylhomoserine lactone (OHHL), while YenR is thought to be an OHHL-dependent transcriptional activator. Chromatin immunoprecipitation assays suggested that YenR might bind to a region upstream of the *yenR* gene. This was confirmed using purified YenR in electrophoretic mobility shift assays. Bound YenR activated a gene divergent from *yenR*, designated *yenS*, and did so only in the absence of OHHL. The *yenS* gene encodes two overlapping non-translated RNAs that share the same promoter. In cells expressing *yenI*, the *yenS* promoter was expressed preferentially at low cell densities.

A bioinformatic analysis showed that a portion of YenS was complementary to the ribosome binding site and start codon of the *yenI* mRNA. The activity of *yenI-lacZ* translational fusions was inhibited by YenS. Earlier studies of the YenR/YenI system suggested a role in swimming and swarming motility. A *yenI* mutant was strongly motile on semisolid agar, while the wild

type strain, the *yenR* mutant, and the *yenS* mutant were nonmotile. A *yenI*, *yenR* double mutant and a *yenI, yenS* double mutant were nonmotile, suggesting a direct positive role for YenR and YenS.

A separate study focused on the OccR protein, which is encoded by the tumor inducing (Ti) plasmid of *A. tumefaciens*. OccR activates an operon that directs the uptake and catabolism of a tumor-released nutrient called octopine. In the absence of octopine, OccR binds to its operator and causes a high angle DNA bend. In contrast, OccR-octopine complexes bind DNA and cause a low angle DNA bend. Eight positive control mutants were identified by random mutagenesis. Of these, six mutants showed the same high angle bend as wild type in the absence of octopine, but retained this bend in the presence of octopine. These data support the idea that relaxation of the DNA bend by the inducing ligand is critical for transcriptional activation.

BIOGRAPHICAL SKETCH

Ching-Sung Tsai was born in Taipei, the Capital of Taiwan. In the second year of high school, he decided to follow his father's path to become an engineer. He entered the Department of Environmental Engineering, National Cheng-Kung University. After graduation, he got a scholarship from National Taiwan University and finished his master's degree in the Graduate Institute of Environmental Engineering. He then applied for the Ph. D. program of Cornell University and joined the Department of Microbiology. He rotated in Dr. Eugene Madsen's lab and Dr. John Helmann's lab. Attracted by the new field of quorum sensing in *Yersinia enterocolitica*, he rotated in and joined Dr. Stephen Winans' lab.

To my parents, Nydia, and my sisters.

改蘇軾　六月二十日夜渡海 以自況

參橫斗轉欲三更，苦雨終風也解晴。

雲散月明誰點綴，天容海色本澄清。

空餘魯叟乘桴意，粗識軒轅奏樂聲。

九載拓荒吾不恨，茲遊奇絕冠平生。

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CHAPTER ONE
INTRODUCTION

[1]1.1 LuxR type Quorum Sensing Regulators that Are Detached from Common Scents

Bacteria are 'scentient' beings. Collectively, they synthesize and detect a rich variety of diffusible chemical signals that impact diverse behaviours, including bioluminescence, horizontal DNA transfer, biofilm formation, pathogenesis, and the production of antimicrobials and other secondary metabolites [1]. New signals, signalling systems, and signal-controlled behaviours are continually being discovered. Chemical communication within populations of bacteria enable them to estimate their population density, a process sometimes referred to as quorum sensing. The ability of bacterial populations to coordinate their behaviour might reflect the difficulty of an individual bacterium to impact its environment. Bacteria give new poignancy to the aphorism 'To be is to be perceived' [2].

Two general classes of diffusible signals, or pheromones, have been described in bacteria. Communication among Gram-positive bacteria often involves oligopeptides that are detected by membrane-spanning kinases [3] or by cytoplasmic receptors [4, 5]. In contrast, signalling among Proteobacteria often involves N-acyl-homoserine lactones (AHLs), which have identical polar head groups and a variety of hydrophobic acyl groups that differ in length, oxidation, and desaturation. AHL signal molecules are often referred to as autoinducers. A number of other bacterial pheromones have also been described, including coumaryl homoserine lactone [6], γ-butyrolactones [7],

[1] Section 1.1 is modified from a published manuscript, with permission from the co-author and the publisher. Tsai, C.S. and Winans, S. C. (2010) LuxR-type quorum-sensing regulators that are detached from common scents. *Molecular Microbiology* 77:1072-1082.

unsaturated fatty acids [8-10], a fatty acid methyl ester [11], a quinolone [12], and a substituted alkane [13]. One signal, denoted autoinducer-2, is synthesized by many species of bacteria, and might therefore serve as an intergeneric signal, though this conclusion remains somewhat controversial [13].

This review focuses on members of the LuxR family of transcription factors. Virtually all known members of this family are controlled by AHLs, which are synthesized by LuxI-type AHL synthases. Most LuxR-type receptors require AHLs for function and in at least some cases, AHLs are required for protein folding and protease resistance [14, 15]. This review focuses primarily on several atypical members of this family that are active only in the absence of cognate AHLs and whose activity they inhibit. We will briefly highlight recent biochemical and structural advances of three proteins that require AHLs for activity, LuxR, LasR, and TraR. We will then review in greater depth the genetic and biochemical literature on LuxR-type proteins that function only as apoproteins.

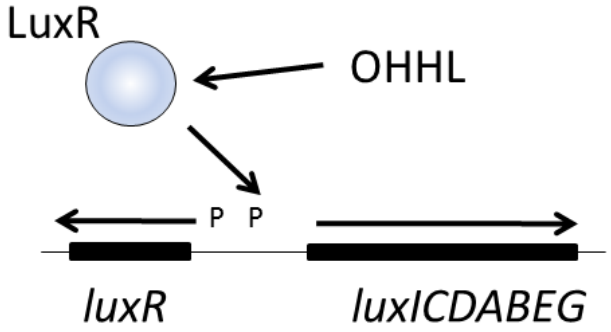
LuxR, LasR, and TraR: three representative AHL-dependent transcription factors

LuxR and LuxI are encoded by the bioluminescent bacterium *Vibrio fischeri* [16, 17]. LuxI synthesizes N-3-oxohexanoyl-l-homoserine lactone (OHHL) [18] while LuxR is an OHHL sensor and an OHHL-dependent transcriptional activator of the luciferase operon [16, 17, 19]. As a population of *V. fischeri* cells grows in density, the concentration of external OHHL increases. When the concentration of this signal reaches the nanomolar range, its passive efflux

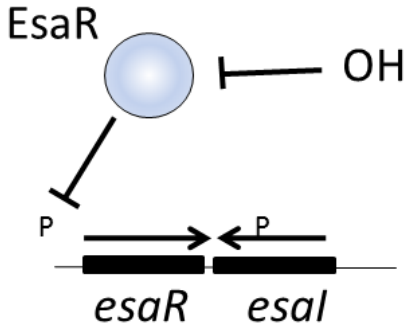
from the cells becomes balanced by an influx, so that it can interact with LuxR. LuxR-OHHL complexes bind the promoter of the *luxICDABEG* operon and activate its transcription [20] (Fig. 1.1).

LuxR has two domains, an N-terminal domain that binds pheromone, and a C-terminal domain that binds DNA [21, 22]. A strain producing the N-terminal domain can sequester exogenous OHHL [22]. Such a fragment can also block the activity of full length LuxR by forming inactive heterodimers [23]. Taken together, these studies suggest that AHLs stimulate dimerization of the N-terminal domains. A LuxR fragment containing only the C-terminal domain is constitutively active and unaffected by AHLs [21]. This indicates that the C-terminal domain contains all the sites essential for LuxR-DNA and LuxR-RNA polymerase interactions. Three amino acids clustered in the C-terminal domain of LuxR are required for positive control of transcription, and are presumed to make direct contacts with RNA polymerase [24, 25]. Experiments using RNA polymerase mutants suggest that LuxR contacts the alpha and sigma subunits [26, 27, 28].

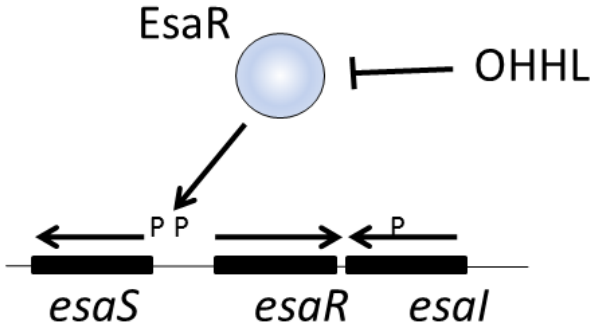
Figure. 1.1. Gene induction at high cell density can be achieved via a diffusible signal that activates a transcription activator (top panel, for example LuxR, LasR, and TraR) or that inhibits a repressor (middle panel, for example, EasR, ExpR1, ExpR2, SmaR). Gene activation at low cell density can be achieved via a signal that activates a repressor (not shown) or by a signal that inhibits an activator (bottom panel, EasR family proteins).



OHHL activates an activator: expression at high cell density



OHHL inactivates a repressor: expression at high cell density



OHHL inactivates an activator: expression at low cell density

LuxR binds to a DNA binding site called a *lux* box, that is 20 nucleotides in length and centred 42.5 nucleotides upstream of the transcription start site [20, 29]. This sequence has imperfect dyad symmetry, suggesting that the DNA binding domains are multimeric and have a corresponding two-fold rotational symmetry. Bases located 6-8 nucleotides away from the dyad axis were essential for wild type activity, while other bases were less critical [30].

LuxR was purified in a soluble, active form after high-level production of the protein in the presence of OHHL [20], and bound to lux box DNA, stimulating the functional binding of RNA polymerase to an adjacent promotor. An important property of LuxR that distinguishes it from LasR and TraR (see below) is that binding of OHHL is reversible [20]. When LuxR was diluted to sub-micromolar concentrations for gel shift assays, it bound to lux box DNA only when additional OHHL was provided. LuxR bound OHHL non-cooperatively with a dissociation constant of 100 nM [20].

The LasR protein of *Pseudomonas aeruginosa* is a central component of a regulatory web that controls the expression of hundreds of genes, some of which play direct roles in disease. This organism produces the cognate AHL synthase LasI, which synthesizes 3-oxo-dodecanoyl-homoserine lactone (OdDHL), as well as another quorum sensing receptor, RhlR, and its cognate AHL synthase, RhlI (which synthesizes butanoyl-homoserine lactone (BHL), and the so-called orphan receptor QscR, which also detects OdDHL [31-34]. The regulons of these three receptors are partially overlapping. The LasR/LasI system stimulates production of the RhlI/RhlR system, causing the two *P. aeruginosa* quorum-sensing circuits to initiate sequentially. Genes that

are controlled by LasR or RhlR have been identified by Tn5*lac* mutagenesis and by transcriptional profiling [35-38].

LasR was purified in a soluble form by producing it in the presence of OdDHL as described above for LuxR [39]. LasR is dimeric in solution, and binds one molecule OdDHL per subunit. Unlike LuxR, LasR does not detectably release its AHL. It bound to six LasR-dependent promoters in EMSA experiments and by DNase I footprinting [39]. It bound some promoters non-cooperatively, causing a DNase I footprint of approximately 30 nucleotides, and bound other promoters cooperatively, causing longer footprints The former promoters were presumably bound by one dimer, while the latter were bound by two. Chromatin immunoprecipitation experiments revealed additional target genes including some encoding transcription factors and others encoding secreted proteins and secretion machinery [40].

Two groups have described the crystal structure of the LasR N-terminal domain [41, 42]. In the first study, the protein was stabilized and folded during synthesis by adding OdDHL. This fragment was dimeric and each subunit consisted of an alpha-beta-alpha sandwich with the AHL lying between the beta sheet and three alpha helices. OdDHL was entirely buried within the protein, with no contact to bulk solvent. The homoserine lactone moiety and the 1-oxo group made hydrogen bonds with a number of conserved amino acids, while the acyl chain made van der Waals contacts with hydrophobic residues. The 12-carbon acyl moiety of the AHL forms a compact S-shape, which is essential for it to be fully buried within the protein. The second study

crystalized LasR with OdDHL or with each of three different triphenyl mimic compounds [42]. Despite the lack of structural similarity between these compounds and AHLs, they aided in the folding and stabilization of LasR, and occupied the AHL binding site.

The TraR and TraI proteins are encoded by Ti plasmids of *Agrobacterium tumefaciens*. TraI synthesizes primarily 3-oxo-octanoyl homoserine lactone (OOHL), while TraR is an OOHL-dependent activator of genes required for vegetative replication and conjugative transfer of the Ti plasmid [43-48]. OOHL is essential for the folding of TraR in vivo, and for resistance to cellular proteases, as the half-life of TraR is increased over 20-fold by OOHL [14, 15]. The protein binds to sequences called *tra* boxes that have dyad symmetry and are approximately 43 or 63 nucleotides upstream of the various transcription start sites [14, 47]. TraR binds these sites as a dimer and without cooperativity. Residues on both the N-terminal and C-terminal domains of TraR are essential for positive control and probably make direct contacts with RNA polymerase [49-51].

The structure of full-length TraR-OOHL-DNA complexes was solved by X-ray crystallography [52, 53]. TraR is dimeric and contains one molecule of OOHL per subunit. OOHL is fully embedded within the N-terminal domain of the protein, just as OdDHL is embedded within LasR. The C-terminal domain of TraR contains a bundle of four helices per subunit that binds to half of a tra box [52, 53]. The N-terminal and C-terminal domains of TraR are connected by a flexible twelve amino acid linker that, if fully extended, would allow the

N-terminal domains and C-terminal domains to be separated by over 40 angstroms.

There are several surprising structural differences between LasR and TraR. First, although the 3-oxo groups of both AHLs make water-mediated hydrogen bonds to their respective receptor proteins, these 3-oxo groups and their bound water molecules are highly displaced in TraR relative to LasR (Fig. 1.2A and 1. 2B). In TraR, the water is hydrogen-bonded by Thr129, while in LasR the water is bonded by Arg61, located on the opposite face of the binding pocket. The remainder of the acyl moiety is also oriented quite differently in LasR compared to TraR (Fig.1.2C and 1.2D). Another surprising difference between LasR and TraR is in their subunit interfaces. The entire N-terminal domains are rotated by 90 degrees in TraR relative to those in LasR. Both proteins have long helices at the C-terminal end of these domains. In TraR, these helices are almost parallel, forming a coiled coil that is the primary dimerization determinant. In contrast, the corresponding helices of LasR are perpendicular and make fewer contributions to dimerization. In TraR, the C-terminal residues of the two NTDs are 10 angstroms apart, while their counterparts in LasR are separated by 30 angstroms.

The structure of a third LuxR homolog was reported in 2006, when the ligand-binding domain of SdiA of *E. coli* was solved using NMR [54]. The solubility of the SdiA fragment was enhanced by providing an AHL (octanoyl-HSL) during protein synthesis, as was done for LasR and TraR. The resolution of this structure was insufficient to identify protein-AHL contacts.

This fragment was reported to be monomeric in solution, possibly due to the harsh conditions that were required to maintain solubility.

EsaR of Pantoea stewartii *is inhibited by its cognate AHL*

As described above, a small number of LuxR homologues appear to function only as apoproteins, in that they are fully active in the absence of any AHL, and their activity is blocked by cognate AHLs. Among the best characterized of these systems is the EsaR/EsaI system of *Pantoea stewartii* (formerly *Erwinia stewartii*), a pathogen of maize. In 1995, von Bodman and Farrand reported that *P. stewartii* produced OHHL, the same AHL as made by *V. fischeri*, and isolated the *esaI* and *esaR* genes from a cosmid library [55] The two genes are transcribed convergently and their reading frames overlap by 21 nucleotides at their 3' ends (Fig. 1.3). EsaR represses transcription of its own gene, but does not affect expression of *esaI*. Disruption of *esaI* caused a sharp decrease in exopolysaccharide accumulation, and production was restored by adding OHHL [55]. In a subsequent study, it was reported that EsaR mutants overproduce the same exopolysaccharide [56], indicating that null mutations in *esaR* and *esaI* have opposite phenotypes. Apparently, whatever EsaR does to regulate EPS was antagonized by OHHL. An *esaR, esaI* double mutant had the same phenotype as an *esaR* mutant.

Figure. 1.2. Structural comparisons of LasR and TraR N-terminal domains. A and B. The conformation of OdDHL bound to LasR (A) and OOHL bound to TraR (B). The homoserine lactone group and residue W60 (LasR) and W57 (TraR) are aligned for reference. The 3-oxo group of OdDHL makes a water-mediated hydrogen bond to residue R61 of LasR, while that of OOHL makes a water mediated hydrogen bond to residue T129 of TraR. The remainders of the two acyl chains are located in different parts of the two proteins.

C and D. Dimerization of the two N-terminal domains of LasR (C) and of TraR (D). The left subunits of each dimer are aligned for reference. The C-terminal helices of the two proteins are in red, and are oriented approximately 90^{O} apart in TraR relative to LasR (black arrows).

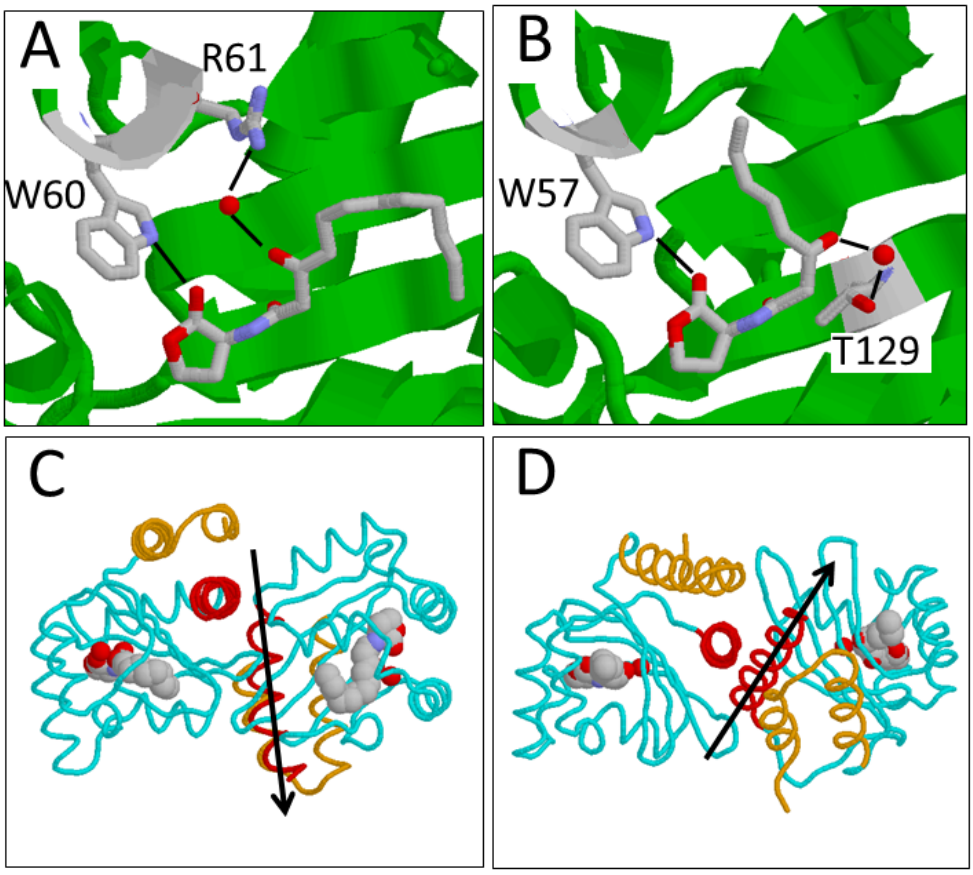
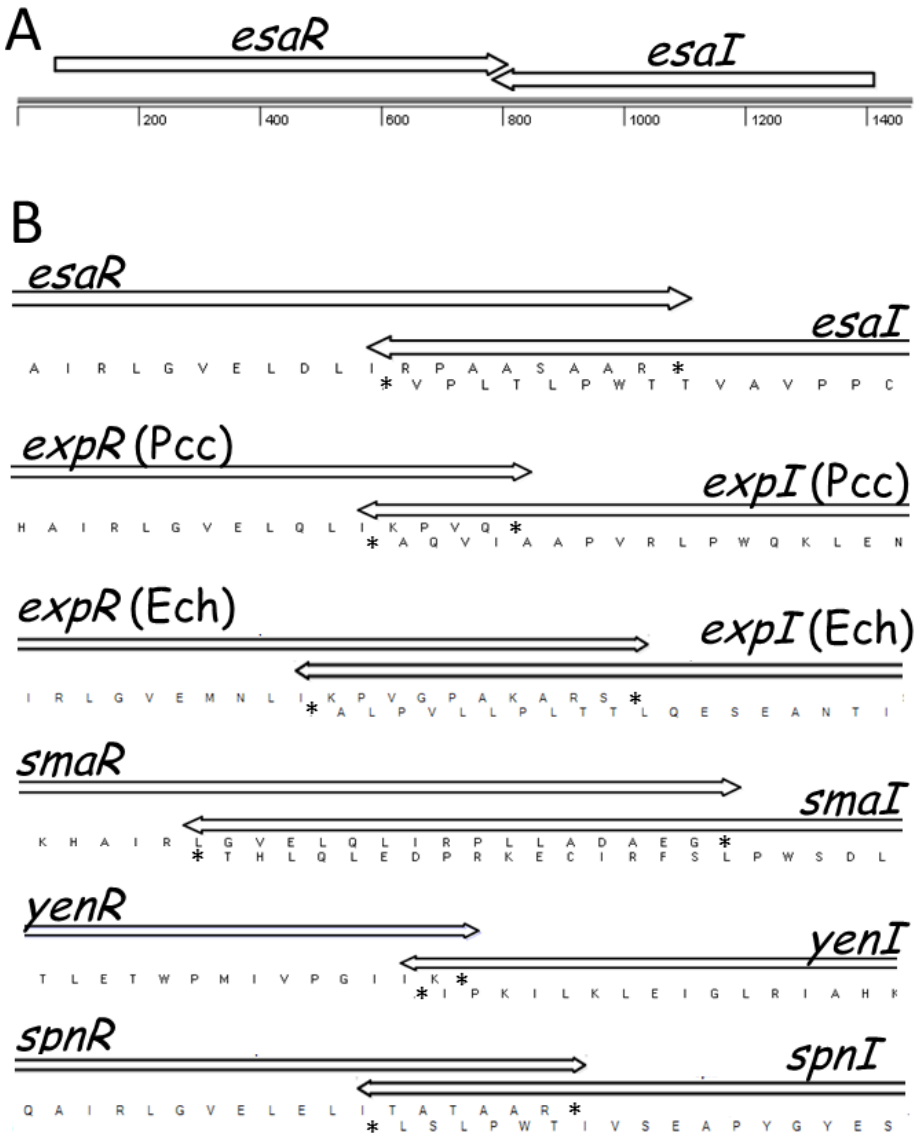

A
R61
W60
B
W57
T129
C
D

Figure 1. 3. The *esaR* and *esaI* genes are expressed convergently (A) and overlap by eight codons (B). The *expR1* and *expI* genes, the *smaR* and *smaI* genes, and the *yenR* and *yenI* genes have similar orientations and overlaps (B). This convergent and overlapping arrangement of all four gene pairs suggests that the expression of one member of a gene pair might be antagonized by the expression of the other member, either via RNA polymerase collisions, or by hybridization of the two complementary mRNAs.


A
esaR
esaI
200
400
600
800
1000
1200
1400
B
esaR
esaI
A I R L G V E L D L I R P A A S A A R *
* V P L T L P W T T V A V P P C
expR (Pcc)
expI (Pcc)
H A I R L G V E L Q L I K P V Q *
* A Q V I A A P V R L P W Q K L E N
expR (Ech)
expI (Ech)
I R L G V E M N L I K P V G P A K A R S *
* A L P V L L P L T T L Q E S E A N T I
smaR
smaI
K H A I R L G V E L Q L I R P L L A D A E G *
* T H L Q L E D P R K E C I R F S L P W S D L
yenR
yenI
T L E T W P M I V P G I I K *
* I P K I L K L E I G L R I A H K
spnR
spnI
Q A I R L G V E L E L I T A T A A R *
* L S L P W T I V S E A P Y G Y E S

It was later discovered that apo-EsaR directly represses the promoter of *rcsA*, a gene required for exopolysaccharide synthesis [57]. The fact that EsaR repressed *rcsA* and *esaR* was unusual among LuxR proteins, which generally activate transcription. However, many transcription factors can activate some promoters while repressing others, often depending on whether they bind upstream of the target promoter, or within or downstream of the promoter. Both LuxR and TraR have been converted to repressors simply by moving their binding sites, even though they are not known to repress any natural promoters [58, 59]. The fact that EsaR was a repressor could therefore be explained easily. In contrast, the fact that OHHL antagonized EsaR function was completely unprecedented, and in stark contrast to the properties of LuxR, LasR, TraR, and most related proteins. This finding shows that EsaR can fold and bind DNA in the absence of any AHL. It is not clear yet whether fully folded, active apo-EsaR can bind its AHL and if so, whether binding is reversible, as it is for LuxR, or irreversible, as it is for LasR and TraR.

EsaR negative autoregulation was reconstituted in *E. coli,* where OHHL again blocked repression [60], providing more evidence for a direct interaction between EsaR and the *esaR* promoter. As might be predicted, EsaR does not require any AHL to remain soluble. Purified EsaR bound to the *esaR* and *rcsA* promoters fragments in gel shift assays [57, 60]. At the *esaR* promoter, EsaR bound to a DNA sequence that contained an imperfect rotational symmetry. Strangely, the binding site in the *rcsA* promoter showed little or no symmetry and little or no similarity to the EsaR binding site in the *esaR* promoter. EsaR bound the *esaR* promoter as a dimer and without

cooperativity [60]. Surprisingly, OHHL did not have any effect on binding affinity in gel shift assays, though it did inhibit EsaR binding to its binding site in assays using surface plasmon resonance [60]. OHHL also altered intrinsic fluorescence of EsaR tryptophan residues and increased EsaR thermostability, providing additional evidence for a direct interaction [60].

As described above, EsaR was first characterized as a repressor. However data published in 2003 showed that EsaR can also serve as a transcriptional activator, albeit in an artificially reconstituted system. EsaR was produced in an *E. coli* strain that also expressed the *luxICDABEG* operon of *V. fischeri*. EsaR activated transcription of this operon, but only in the absence of OHHL [61]. The related protein ExpR of *Pectobacterium carotovorum* (see below) had similar properties in this assay. Activation of the *lux* operon was further enhanced by replacing the LuxR binding site with an EsaR binding site. Although this system was artificial, it proved that EsaR can in principle recruit RNA polymerase to a promoter, which would seem highly unlikely unless it had evolved to do so. A native promoter of *P. stewartii* was recently discovered that is activated by EsaR [62]. This promoter expresses a small, noncoding RNA *esaS* that is divergent from the *esaR* gene. OHHL blocked the activation of this gene by EsaR. An EsaR binding site located between *esaR* and *esaS* is required for repression of the former gene and is thought to be required for activation of the latter. This binding site is centred 60 nucleotides upstream of a possible transcription start site. Therefore, as described above, EsaR can activate or repress gene expression, depending largely on the position of the binding site, but in all cases, OHHL antagonizes EsaR function.

The exoenzyme regulators ExpR of Pectobacterium carotovorum and Erwinia chrysanthemi *are inhibited by OHHL and OOHL.*

*P. carotovorum (*formerly *Erwinia carotovora)* and *E. chrysanthemi* are plant pathogens that macerate plant tissues by releasing enzymes capable of degrading pectate, cellulose, and protein. Expression of the corresponding genes is controlled by several master regulatory proteins. Mutations in the gene for one such regulator, ExpI, abolished expression of all such genes [50]. ExpI is related to members of the LuxI family. This gene is linked to *expR*, and the two are expressed convergently, and overlap at their 3' ends [63], reminiscent of *esaR* and *esaI* (Fig. 1.3). Null mutations in *expR* caused a modest increase in plant tissue maceration and pectate lyase activity, while over-production of ExpR caused a decrease [63].

Three groups identified a second possible copy of *expR* in the genome sequence of a related bacterium, and used this sequence to identify the orthologous gene in *P. carotovorum* [64-66]. In one study, mutants lacking just one of these two receptors still produced exoenzymes at slightly elevated levels. Expression of target genes increased when AHL was added. A strain lacking both receptor proteins produced exoenzymes at high constitutive levels [66]. This suggests that there are two receptor proteins with overlapping functions, and that both are able to repress transcription of exoenzyme genes. The newly identified receptor (ExpR2) detected a broader variety of AHLs than ExpR1. In a different study using a different strain of *P. carotovorum*, this second receptor (referred to as VirR) was solely responsible for exoenzyme production, in that a *virR* mutation fully restored exoenzyme production in an

expI mutant. An *expR*, *virR*, *expI* triple mutant was a phenocopy of the *virR*, *expI* double mutant, suggesting that ExpR does not play any role in regulating these genes. Surprisingly, exoenzyme production in a *virR*, *expI* mutant was still induced at high cell density, rather than constitutive. This observation was unexplained.

E. chrysanthemi has orthologous *expI* and *expR* genes, once again transcribed convergently and overlapping at their 3' ends [67]. Mutation of *expI* abolished production of two AHLs, but did not affect the production of a third one, suggesting the existence of at least one more AHL synthase gene. Mutations of *expI* and *expR* had little effect on pectate lyase synthesis, which remained quorum-regulated. This provided additional evidence for a second quorum sensing system. Despite this genetic evidence that ExpR does not (at least solely) regulate pectate lyase genes, the protein was tested for binding to five different *pel* gene promoters. Apo-ExpR caused a gel shift at all five promoters. For the one promoter tested, OHHL blocked DNA binding in gel shift assays. DNase I protection assays were performed using three of these promoters. In the absence of OHHL, footprints were detected at two promoters. Inexplicably (in light of the gel shift data), addition of OHHL caused far longer and more distinct footprints at these promoters.

In a subsequent study, apo-ExpR was shown to autorepress its synthesis, while OHHL almost fully blocked autorepression [68]. Purified apo-ExpR bound the *expR* promoter without cooperativity, forming two high-affinity complexes (containing one or two ExpR dimers, respectively) and four additional slow migrating, low affinity complexes, possibly due to protein

aggregation. OHHL largely disrupted these complexes. ExpR protected a region extending from -40 to +6 of the *expR* promoter from DNase I digestion. This 46 nucleotide region is sufficiently long to account for the two complexes observed in gel shift assays. Similar to the pectate lyase promoters described above, protection was more pronounced in the presence of OHHL than in its absence. This finding is extremely difficult to reconcile with the gel shift data. RNA polymerase bound an overlapping but longer region, probably displacing ExpR, although the authors of that study concluded that both proteins bound simultaneously. Apo-ExpR blocked open complex formation at the expR promoter, while OHHL restored open complex formation. Similar results were obtained using runoff transcription assays [68].

Although ExpR of these species represses most known target genes, it can also activate at least one gene, *rsmA* (orthologous to *csrA* of E. coli), whose product binds mRNAs and accelerates their degradation [65, 69]. Even here, ExpR functions only as an apoprotein, as OHHL blocks activation *in vivo* and disrupts ExpR-DNA complexes *in vitro*. Both copies of ExpR are able to activate this promoter [65].

SmaR and SmaI act antagonistically to regulate antibiotic biosynthesis

SmaR of *Serratia* sp. 39006 is yet another example of a LuxR-type protein whose activity is blocked by the cognate AHL. The *smaR* gene and the cognate *smaI* gene are expressed convergently (Fig. 1.3). SmaI synthesizes predominantly butanoyl-HSL (BHL) and smaller amounts of hexanoyl-HSL (HHL). A *smaI* mutation abolishes the synthesis of the antibiotic carbapenem, the pigment prodigiosin, and several hydrolytic enzymes [70], while a *smaR*

smaI double mutant restores their production [71]. Apo-SmaR is thought to bind to the promoters of the *carR* gene and the *car* (carbapenem) biosynthetic operon and repress their expression, and binding is blocked by BHL or HHL [71, 72]. CarR is also a LuxR homolog and directly activates the car operon. CarR is rather closely related to members of the EsaR family (Fig. 1.4), which might suggest that its activity could be blocked by cognate AHLs. CarR is often referred to as "AHL-independent", as it was able to activate the *car* operon of *P. carotovorum* in a strain lacking AHLs [73]. CarR is essential for transcription of the *Serratia car* operon and functions perfectly well in an AHL-defective strain. It still seems possible that CarR could be antagonized by AHLs, although it functions in strains that produce BHL. The question is complicated by some rather intricate regulatory circuits. It would be interesting to express CarR constitutively in a SmaI SmaR double mutant of *Serratia* and measure the expression of the *carR* or *carA* promoters in the presence and absense of exogenous BHL. What makes this question even more fascinating is that a close relative of CarR clearly requires AHLs for activity. The CarR protein of *P. carotovorum* requires OHHL (synthesized by ExpI) to activate that organism's *car* operon [74]. The AHL binding domains of the two CarR proteins are 57% identical.

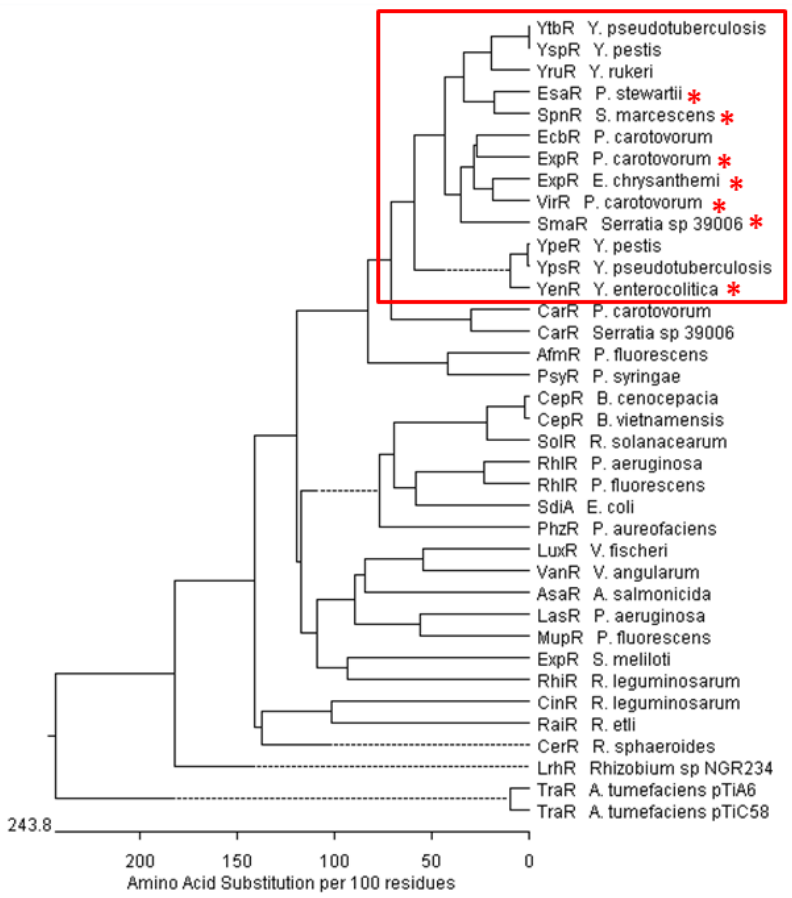


Figure 1. 4. Phylogeny of representative members of the LuxR family, constructed using the Megalign program (DNAStar). Proteins that known to be antagonized by their cognate AHLs are indictated with an asterisk. Known or suspected members of the EsaR clade are enclosed within a box.

Serratia marcescens strain SS-1 is similar to *Serratia* sp. 39006 in that its SpnI/SpnR system controls production of prodigiosin, endonuclease, and a surfactant that affects motility (this strain does not synthesize carbapenem) [75]. SpnR is thought to directly repress target promoters, while the AHL synthesized by SpnI antagonizes SpnR. SpnI and SpnR are not closely related to their counterparts in *Serratia sp.* 39006 (SmaI and SmaR), and instead are more closely related to EsaR and EsaI of *P. stewartii*. SpnI (like EsaI) synthesizes primarily OHHL, while SpnR is inhibited primarily by the same signal. SpnR activates the *spnR* promoter, providing positive autoregulation. Surprisingly, it was reported that SpnR does so more effectively in the presence of OHHL than in its absence. It is not terribly surprising that SpnR could activate some promoters and repress others, as several EsaR-type proteins can do both (see above). However, the finding that OHHL would block SpnR function at one promoter and enhance it at another is unprecedented and counterintuitive. No explanation was offered [75]. The spnR/I genes are located on mobile genetic element, and SpnR represses transcription of the Tn3-type transposase of this element [76]. The idea that a quorum of bacteria would stimulate transposition provides an interesting example of Lamarkian evolution.

Yersinia *spp*. *and* Pseudomonas syringae

At least two other gamma-proteobacteria encode proteins that fall into the EsaR clade. *Yersinia enterocolitica* encodes YenR and YenI as well as one orphan LuxR homolog, while *Y. pestis* and *Y. pseudotuberculosis* (which have extremely similar chromosomes and differ largely in their plasmid content) each encode two LuxR/LuxI systems. The former organism encodes YpeRI and

YspRI, while the latter encodes YpsRI and YtbRI. YenRI, YpeRI, and YpsRI are quite similar and are presumed to be orthologous (Fig. 1.4) while YtbRI and YspRI are also orthologous. All five pairs of genes are transcribed convergently and overlap at their 3' ends. Our works indicate that YenR and YenI are similar to other members of the EsaR clade. Apo-YenR activates transcription of a non-coding RNA gene, *yenS*, which is divergent from *yenR*. Activation is blocked by OHHL. Purified apo-YenR binds to two sites between the two genes, and binding is reversed by addition of OHHL (Chapter 2). Given that YpeR and YpsR are extremely similar in sequence to YenR, it is plausible that they have similar properties. The published literature on YpsRI and YtbRI is difficult to interpret, as the two systems may have redundant or overlapping functions and so the phenotypes of single mutants may be masked. It would be interesting to delete all four genes, and individually express YpsR and YtbR from constitutive promoters in the presence or absence of cognate AHLs, measuring expression of a target gene such as the small RNA homologous to *yenS*.

Many or perhaps all isolates of the plant pathogen *Pseudomonas syringae* encode members of the EsaR clade. The protein pairs are designated PsyRI in some strains and AhlRI in other strains. In all cases, the pairs of proteins are transcribed convergently and overlap at their 3' ends. The AHL synthases of this group synthesize predominantly OHHL. Although a number of papers have appeared that describe these proteins, no published study shows whether or not the receptor proteins are antagonized by their cognate AHLs.

Members of this group form a monophyletic clade

A phylogenetic dendrogram of LuxR homologs shows that all the proteins whose activities are blocked by AHLs (indicated using an asterisk) form a single clade (Fig. 1.4). The closest relative that is known to require AHLs for function is CarR of *E. carotovora* [74].

Members of the EsaR clade have several similarities in addition to their being antagonized by cognate AHLs. First, all confirmed members are found only within the Enterobacterialaes, which are themselves within the gamma-proteobacteriales. Second, all but one of these proteins preferentially bind OHHL. The exception is SmaR of *P. carotovorum*, which preferentially binds BHL. Third, while most LuxR-type proteins activate expression of their cognate AHL synthase genes, members of the EsaR subfamily generally neither activate nor repress these genes. The activation of AHL synthase genes is thought to play a role in the tendency of these systems to remain stably in one of the two possible states in the face of subthreshold variations in AHL concentrations, a phenomenon referred to as hysteresis. EsaR-type proteins might have other mechanisms to achieve this (see below).

A fourth similarity between these proteins is they can function as repressors (though some can also activate certain promoters), while most LuxR type proteins are thought to act solely as activators. Target genes that are repressed by a member of the EsaR subfamily would be induced at high population density, just as they are in more conventional quorum sensing systems, as repression is blocked by AHLs, whose concentration increases at high cell densities (Fig. 1.1, middle panel). On the other hand, genes that are

activated by such proteins would be expressed preferentially at low cell densities, where quorum signals are limited (Fig. 1.1, bottom panel).

A fifth similarity between members of this family is that they are all encoded by genes that are adjacent to their cognate AHL synthase genes, and each pair of genes are convergently transcribed (Fig. 1.3). In every case, the coding sequences of these gene pairs overlap by a few nucleotides at their 3' ends. Although the translation overlap is short, the transcription overlap would certainly be longer, possibly encompassing the entire length of the two genes. These genes lack any obvious transcriptional stop signals.

The convergent arrangement of these gene pairs suggests that expression of one gene might antagonize expression of the other. First, RNA polymerases transcribing the two genes could collide, possibly causing one to be dislodged and the other to continue transcription. Similarly, elongating RNA polymerases might dislodge convergently oriented RNA polymerases bound at the opposite promoter. This mechanism of transcriptional dominance, called 'sitting duck' inhibition, has been reported previously [77]. Third, the mRNAs of cognate receptor and synthase genes could form RNA-RNA duplexes. Such duplexes might inhibit the translation of one or both RNAs, or might enhance their degradation, or both [78]. If all duplexed molecules are degraded, then only the more abundant mRNA will persist. Such a contest between the two genes or their mRNAs could contribute to hysteresis. If under some conditions the promoter of a synthase gene were more active than that of a receptor gene, these mechanisms could accentuate the imbalance, locking the system in the high cell density state. Conversely, if

the receptor promoter were more active than the synthase promoter, these mechanisms would exacerbate the imbalance, locking the cells in the low cell density state. Given the antagonism between these receptor proteins and their cognate pheromones, it seems striking that the expression of the two convergent genes might also be antagonistic.

How are EsaR-type proteins inactivated by their cognate AHL pheromones? Mechanistic explanations for inhibition by AHLs are only beginning to emerge. The ability of several EsaR-like proteins to bind DNA is blocked by AHLs [68] [57, 60, 65]. The most obvious way for AHLs to block DNA binding would be to inhibit dimerization of the NTDs, which in turn would inhibit dimerization of the CTDs by cooperative effects. Alternatively, AHLs might cause the two NTDs of a dimer to bind their respective CTDs in such a way that prevents DNA binding. It seems far less likely that a conformational change caused by AHL binding could be propagated through each subunit to its DNA binding domain, especially in light of the long, flexible linkers between the two domains. The predicted linkers of EsaR-type proteins are even longer than those of other LuxR-type proteins (Fig. 1.5), and probably impart some degree of functional autonomy to the CTDs. It would be extremely useful to determine experimentally whether AHLs can inhibit NTD dimerization. In vivo, AHLs could also alter the rate of proteolysis of these proteins, analogous to the decrease in proteolysis of TraR caused by OOHL [14, 15].

It is tempting to attempt to model the structure of the EsaR NTD, using the crystal structures of TraR and LasR, and to look for differences that could explain the antagonistic effects of AHLs. All members of the EsaR clade

contain all the residues that are predicted to make hydrogen bonds with the homoserine lactone group of the pheromone (Fig. 1.5). Unfortunately, proteins in the EsaR subfamily are only distantly related to LasR and to TraR, and even the latter two proteins have surprising structural differences (see above). Therefore, it would be exceedingly difficult to make any meaningful predictions about the structures of these proteins. This fascinating group of proteins is therefore ripe for further genetic, biochemical, and structural studies.

1.2 Virulence factors of *Yersinia enterocolitica* and their impact on host immunity

Yersinia enterocolitica is a human pathogen that induces food-borne acute or chronic gastrointestinal diseases, with swine often implicated as the natural reservoir and source of infection. The gastrointestinal syndromes caused by Y. enterocolitica vary from acute enteritis to mesenteric lymphadenitis [79]. Upon entering into human intestine, the bacteria first penetrate the M-cells, then proliferate in Peyer's patches (PPs), and later distribute into liver and spleen through mesenteric lymph nodes (MLNs) to cause systemic immune responses. [80].

The uptake by the M-cells is mainly related to invasin, a chromosomally encoded 92 kDa outer membrane protein. An invasin mutant lost the ability to invade eukaryotic cells [81, 82]. Invasin binds specifically to beta1 integrin, which was retained on a column containing immobilized invasin [83].

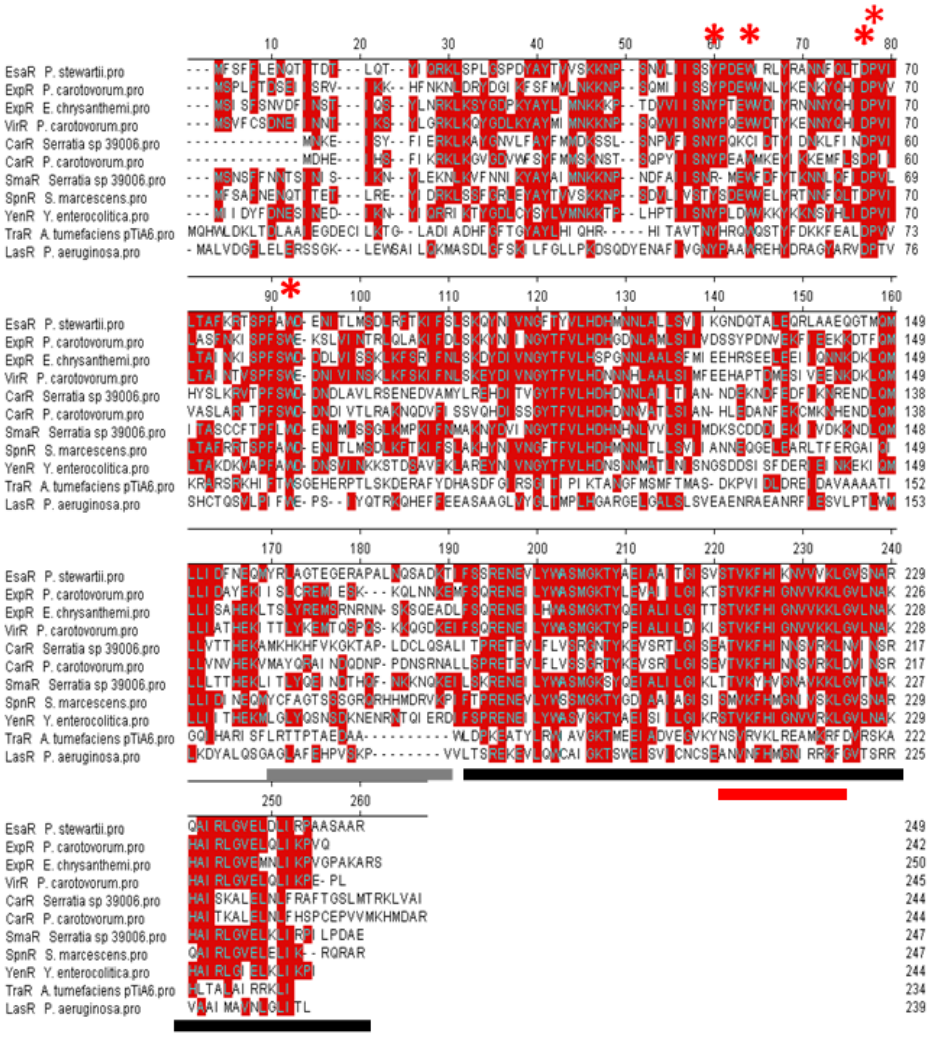


Figure 1. 5. Alignment of LasR and TraR, whose structures have been solved by X-ray crystallography, with confirmed members of the EsaR clade. The C-terminal DNA binding domain is indicated using a black bar, while the interdomain linker is indicated with a grey bar. LasR residues that make hydrogen bonds with AHLs are marked using a asterisk.

Colonization of the Peyer's patches requires a second adhesin called YadA, which is encoded on the virulence plasmid. YadA is linked to many biological functions and its homologs in *Y. pseudotuberculosis* and *Y. pestis* play obscure roles in pathogenesis. In *Y. enterocolitica*, *yadA* mutants showed no differences in invasion of Peyer's Patches when compared to wild type, but failed to persist there. YadA was also involved in blocking the innate immune system of the host, such as defensin and serum complement clearance [84, 85].

Once penetrating the M cell, invasion causes the accumulation of polymorphonuclear leukocytes (PMNs) and macrophages inside the subepithelial dome (SED). Upon contacting the phagocytic cells, invasin may trigger cell surface receptors such as β-integrin to activate guanine nucleotide exchange factors GEFs through the Cas, Fak or Fyb protein complexes [86]. GEFs later activate Rho GTPases to initiate stages of phagocytosis including the activation of kinases, actin polymerization, a shift in phospholipid metabplism, and increase rates of membrane assembly [86].

However, *Yersinia spp*. can counteract phagocytosis,. The 70 Kb virulence plasmid of *Yersinia spp.* helps the bacterium to counteract these innate immune defenses, mostly through the production of a Type 3 translocation apparatus that injects effector proteins called Yops (*Yersinia* outer proteins) directly into the host cell. *Y. enterocolitica* secretes at least 6 effectors: YopE, YopH, YopO (designated YpkA in *Y. pseudotuberculosis*), YopT, YopP(YopJ in *Y. pseudotuberculosis*),and YopM. Four of these effectors, YopH, YopE, YopO, and YopT block the phagocytosis process.

A protein tyrosine phosphatase, YopH was discovered due to its catalytic motif shared with many of its eukaryotic counterparts [87]. YopH targets proteins such as Cas, Fyb and Pyk that are required for phagocytosis. The dephosphorylation of Cas, Fyb or Pyk disrupts a signaling cascade that would have stimulated the assembly of actin, which is required for phagocytosis [86].

As mentioned above, Rho GTPases are central to the phagocytosis process. Interestingly, YopE, YopO, and YopT all act to target the host Rho GTPase. GTP-bound Rho GTPases such as RhoA, Rac-1 and Cdc42 are in their active form to interact with downstream effectors. If the gamma phosphoryl group of GTP is hydrolyzed, creating GDP, the GTPases are inactive. Moreover, the function of the Rho GTPases requires membrane association, and this is achieved by the prenylation of a Cysteine residue in the C terminal ends of these proteins. YopE is a GTPase-activating protein, facilitating the hydrolysis of the GTP molecule bound to the Rho family proteins. Experiments showed that YopE specifically targets Rac-1 or RhoA. YopT, on the other hand, is a cysteine protease dissociating the Rho GTPases from the membrane by cleaving the C terminus. Both activities decrease the activities of Rho GTPases.

YopO, the final Yop that disrupts phagocytosis, has several activities. The N-terminal domain of this protein resembles eukaryotic serine/threonine kinases, followed by a domain predicted to bind Rho GTPases, and then a C-terminal actin binding domain. The detailed mechanism used by YopO to interact with Rho GTPases is still unknown, although the uptake of a mutant

YopO by macrophages or neutrophils occurred at higher rates than a wild type, suggesting that YopO inhibits uptake [86].

The activation of Rho GTPases family proteins also induce the MAP kinase (MKK) and NF-κB (IKK) pathways, causing the expression of cytokines or triggering apoptosis of macrophages. The MKK and IKK pathways can also be turned on by the TLR4 receptor [86]. The production of cytokines could stimulate T cells or NK cells to produce gamma interferon (IFN-γ), which in turn further stimulate macrophages to engulf the pathogens [88]. However, these self defenses are also blocked by Yersiniae. YopP (YopJ in *Y. pseudotuberculosis*) was reported to inhibit the MKK and IKK pathways by acetylating the important serine and threonine residues of MKK and IKK kinases. This acetylation prevented further phosphorylation events [89] and hence stopped the activation of cytokines.

The last effector, YopM, seems has not been thoroughly studied, although one study showed that it binds to two kinases: ribosomal S6 protein kinase 1 and 2. The roles of binding these proteins is not understood [90] .

Yops also block adaptive immune responses, as T cell responses are essential for the removal of *Yersinia* after infection [91, 92]. Erfurth and colleagues [93] showed that *Yersinia enterocolitica* can neutralize the host immune responses by blocking the antigen processing and presentation processes in dendritic cells, mainly through YopP. Furthermore, YopP can induce the apoptosis of dendritic cells. *Y. pseudotuberculosis* YopH was also found to reduce IL-2 production of T cells, which is required for T cell

proliferation, by inhibiting the tyrosine phsphorylation of the T cell receptor (TCR) complex [94]. This results need to be verified by using *Y. enterocolitica* strains. In summary, *Y. enterocolitica* uses several approaches to block or neutralize the host innate and adaptive immune responses.

1.3. Quorum sensing systems in *Yersinia* spp.

As described in Section 1.1, *Y. pseudotuberculosis and Y. pestis* both encode two complete LuxI-LuxR type systems, while *Y. enterocolitica* encodes one complete system and an orphan receptor. All six receptors (YenR and YetR of *Y. enterocolitica*, YpeR and YspR of *Y. pestis*, and YtbR and YpsR of *Y. pseudotuberculosis*) are all grouped into the EsaR protein family. Of these, YpeR is orthologous to YtbR, while YspR is orthologous to YpsR. YtbR and YpsR of *Y. pseudotuberculosis* are 37.5 % identical in their N-terminal domains and 57.6 % identical in their C-terminal domains.

It is not known whether the two complete systems found in *Y. pseudotuberculosis* and *Y. pestis* might have overlapping functions, either by detecting each other's AHLs or by binding to each other's promoters. A strain expressing only YpsI synthesized primarily 3-oxo-C6-HSL, with small amounts of C6-HSL and 3-oxo-C7-HSL, while a strain expressing only YtbI produced primarily 3-oxo-C8-HSL, 3-oxo-C6-HSL, and 3-oxo-C7-HSL. Both strains made trace amounts of many other AHLs [95]. Therefore, the AHLs produced by the two synthases are partly overlapping and partly unique. Clearly, YpsR can detect at least one AHL made by YtbI, while it is not clear whether the converse is true. It is impossible to know whether these AHL receptors can

coregulate common promoters without first identifying such promoters. The DNA recognition helices of YpsR and YtbR are identical at 11 of 15 residues.

One study of *Y. pseudotuberculosis* focused on the question of a transcriptional cascade, in which one system might control the expression of the other [96]. Essentially, *ypsR, ytbR, ypsI,* and *ytbI* promoters were each fused to a reporter in a suicide plasmid (that integrates at a neutral site), and each fusion was introduced into a wild type strain, and into strains having null mutations in each of the same genes. The experimental design did not allow for the possibility of functional redundancy in any of these proteins. The findings are summarized in Fig. 1.6. The authors observed that mutations in *ypsI* and *ypsR* had similar phenotypes, as did mutations in *ytbI* and *ytbR*. These findings suggest that the two AHL receptors require AHLs to function, which is very difficult to reconcile with my findings about YenR and YenI (Chapter 2) and about other members of the EsaR subfamily.

Also as described above, many members of the EsaR subfamily function only as apoproteins. In Chapter 3, I show that YenR has similar properties. No other AHL receptor in *Yersinia* spp has been studied at this level. Such studies would be greatly facilitated by indentifying direct target promoters, but so far, none has been identified, except for one promoter that is activated by YenR (see Chapter 2).

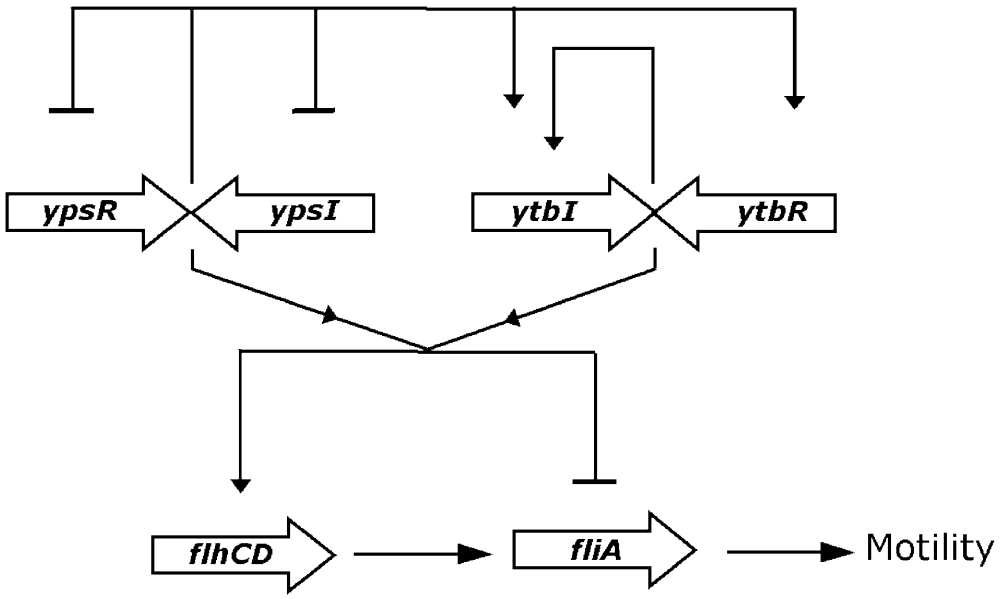


Figure. 1.6 Summary of interactions between YpsR, YpsI, YtbI, and YtbR. Adapted from reference [96].

.A small number of papers have appeared describing a possible link between quorum and motility in *Y. enterocolitica* and in *Y. pseudotuberculosis* [96-98]. In one of these studies, a *yenI* null mutant showed delayed swimming motility and a loss of swarming motility [98]. Somewhat surprisingly, the addition of extracellular autoinducer did not restore the wild type phenotype. It seems plausible that the antibiotic resistance gene used to disrupt *yenI* could have blocked expression of the adjacent and convergent *yenR* gene. In a second study, using a different serotype of *Y. enterocolitica*, a *yenI* mutation had small defect in motility [99].

A separate study focused on motility and aggregation in *Y. pseudotuberculosis.* A *ypsI* mutant resembled the wild type, as neither

aggregated in broth culture and neither released FleC into the supernatant. In contrast, a *ypsR* mutant showed a strong tendency to aggregate and released high amounts of FleC [100]. The fact that the *ypsI* and *ypsR* mutants had opposite phenotypes suggests that YpsR functions as an apoprotein. However, mutations in YpsR or YpsI were both hypermotile on semisolid agar [96]. This is difficult to reconcile with their opposite aggregation phenotypes.

Mutations in *ypsR, ypsI, ytbR* or *ytbI* caused decreased expression in the *flhDC* operon and increased expression of *fliA [96]*. Surprisingly, exogenous autoinducers did not suppress the defect of a *ypsI* or *ytbI* mutant. This could be interpreted to mean that the mutations in *ypsI* and *ytbI* has inadvertently blocked expression of the cognate receptor genes. Also, in all these experiments, the possibility that these systems may have overlapping functions seems to be overlooked.

In summary, the literature on quorum sensing in *Yersinia* spp tends to focus on the organismal level rather than the mechanistic level. Roles in motility have been investigated, but no target promoters have been described, and the conclusions are clouded by possible overlapping functions of these systems.

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[2]CHAPTER TWO

A QUORUM-HINDERED TRANSCRIPTION FACTOR FROM *YERSINIA ENTEROCOLITICA* ACTIVATES EXPRESSION OF AN UNTRANSLATED RNA AT LOW POPULATION DENSITIES

[2] Chapter 2 is a manuscript prepared for submission, with the permission from the co-author, Ching-Sung Tsai and Stephen C. Winans. A quorum-hindered transcription factor from Yersinia enterocolitica activates expression of an untranslated RNA at low cell densities.

2.1 Abstract

The YenR and YenI proteins of *Yersinia enterocolitica* resemble the quorum sensing proteins LuxR and LuxI of *Vibrio fischeri.* Chromatin immunoprecipitation experiments suggested that YenR can bind to DNA sequences upstream of the *yenR* promoter. Purified apo-YenR bound noncooperatively to two 20-nucleotide sites that lie upstream of *yenR*, and which are centered 25 nucleotides apart. Binding occurred in the absence of 3-oxohexanoylhomoserine lactone (OHHL), a pheromone made by YenI, and YenR was largely released from the DNA by addition of this pheromone. Apo-YenR activated a gene, designated *yenS*, that lies adjacent to and divergent from *yenR*. This gene was expressed preferentially at low cell density and expression was inhibited by endogenous or exogenous OHHL. *yenS* encodes two overlapping RNAs that are 105 and 165 nucleotides in length. They share the same 5' end but have different 3' ends. Sequence inspection and translational fusions indicate that these RNA molecules are not translated.

2.2 Introduction

In recent years, it has become clear that many groups of bacteria can release and detect diffusible chemical signals and can use these signals to coordinate a wide variety of behaviors [1-4]. In some pathogenic bacteria, these signals alter the expression of proteins required for pathogenicity [5-9]. Proteobacteria generally use acylhomoserine lactones (AHLs), which are normally synthesized by proteins that resemble LuxI of *Vibrio fischeri*, and are detected by receptor proteins that are similar to the *V. fischeri* LuxR transcription factor [10-12].

Most LuxR-type proteins function only in the presence of their cognate AHL [12]. Structural studies of three members of this family have shown that AHLs bind deeply within these receptors and contribute to the overall hydrophobicity of the protein core [13-16]. One such receptor, TraR of *Agrobacterium tumefaciens*, requires its cognate AHL as a scaffolding for its folding. In the absence of this signal, TraR is rapidly degraded by the cellular proteases Clp and Lon [17, 18].

A small number of LuxR homologs are active only in the *absence* of their cognate AHLs. The EsaR protein of *Pantoea stewartii* is a repressor of a gene required for exopolysaccharide biosynthesis, and also autorepresses its own synthesis [19-21]. EsaR can also activate the *esaS* promoter [22] and the heterologous *luxI* promoter [23]. In all four cases, EsaR is active as an apoprotein and its activities are blocked by the cognate AHL. ExpR of *Pectobacterium* spp. and SmaR of *Serratia* sp. have similar properties [24-27]. EsaR, ExpR and SmaR are part of a monophyletic clade within the larger family

of LuxR-type proteins [28], suggesting that the ability of these proteins to function only as apoproteins may have evolved just once.

Yersinia enterocolitica is a gamma-proteobacterium that colonizes the small intestine and can cause gastrointestinal distress and can also cause septicemia in immunocompromised patients [29]. The YenI protein synthesizes primarily 3-oxohexanoylhomoserine lactone (OHHL) and lesser amounts of hexanoylhomoserine lactone (HHL) [30, 31], which were presumed to regulate the activity of YenR. YenI and YenR orthologues are found in other species of *Yersinia*, including *Y. pestis*, the causative agent of Bubonic plague. YenR closely resembles the EsaR, ExpR, and SmaR proteins described above (46%, 50%, and 40% identical, respectively). This similarity suggested to us that YenR might function only as an apoprotein. The current study was initiated in an effort to identify promoters that are regulated directly by YenR, and led to the unexpected discovery that YenR activates expression of a small non-translated RNA. As we had predicted, YenR functioned only in the absence of AHLs, and therefore activated transcription of this target gene preferentially at low population densities.

2.3 Materials and Methods

Strains, Plasmids, and Reagents.

Bacterial strains, chromosomal mutations, plasmids and oligonucleotides used in this study are described in Tables 2.1, 2.2, 2.3 and 2.4. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Antibiotics and Protein A-Sepharose beads were purchased from Sigma. Restriction enzymes, DNase I, T4 DNA kinase, and terminal DNA transferase were

purchased from New England Biolabs. PCR fragment purification kits and RNeasy kits were purchased from Qiagen. Radionucleotides were purchased from Perkin Elmer. Trizol reagent and Superscript III reverse transcriptase were purchased from Invitrogen. Polyclonal rabbit Anti-YenR antiserum was prepared by the Cornell Center for Research Animal Resources. 3-oxohexanoylhomoserine lactone (OHHL) and hexanoylhomoserine lactone (HHL) were generously provided by A. Eberhard (Cornell University), and were chemically synthesized using published procedures [32].

Chromosomal genes were disrupted, singly and in combination, by a two-step Campbell-type integration and excision of a suicide plasmid carrying deletion alleles of the target genes. DNA fragments (approximately 500 nucleotides in length) upstream and downstream of the target genes were constructed by PCR amplification, ligated, and then cloned into plasmid pKNG101, a suicide plasmid carrying an R6K origin and a *sacB* counter-selectable gene. The resulting plasmids were introduced into *E. coli* SM10 λ-pir by transformation, and then introduced into *Y. enterocolitica* JB580 or its derivatives by conjugation. Transconjugants containing the suicide plasmid integrated into the chromosome were selected using streptomycin and nalidixic acid. Excision of the suicide plasmid was subsequently selected by plating cells on medium containing 10% sucrose and nalidixic acid. Sucrose-resistant clones were characterized using PCR amplification to confirm the deletion.

Table 2.1. Strains used in this study

Strain	Genotype	Source
JB580	*Y. enterocolitica 8081v,* restriction defective.	[33]
DH5α	*E. coli* K12 F $^-$ *endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG lacZΔM15 hsdR17*	Stratagene
SM10/λ pir	*tra* regulon of RK2, R6K *pir,* host for *pir*-dependent plasmids	[34]
BL21/DE3	*E. coli* B F $^-$, *ompT*, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)	Promega
S17-1/ λpir	*tra* regulon of RK2, R6K *pir*, host for *pir*-dependent plasmids	[35]
YWY003	JB580 *yenI102*	Thus study
CST201	JB580 *yenRI101*	This study
CST206	JB580 *yenI101*	This study
CST214	JB580 *yenRI101*, *lacZ101*	This study
CST216	JB580 *yenI101*, *lacZ101*	This study
CST217	JB580 *yenRS101, lacZ101*	This study
CST225	CST217 *yenI'-lacZ102*	This study

Table 2.2. Chromosomal mutations constructed in this study

Allele	Description	Oligonucleotides for PCR amplification of upstream DNA fragment	Oligonucleotides for PCR amplification of downstream DNA fragment
yenI101	deletion of codons 1-187 of *yenI*	o3001 and o3002	o3003 and o3004
yenRI101	deletion removing all of *yenR-yenI* coding sequences	o3005 and o3006	o3001 and o3002
yenRS101	deletion of *yenR* and the intergenic region between *yenR* and *yfrE*	o3007 and o3008	o3009 and o3010
yenI102	Cm^R cassette in the ScaI site of *yenI*	o3011 and o3012	N. A.
yenI'-lacZ102	chromosomal *yenI-lacZ* fusion with a truncated *yenI in cis.*	o132 and o133	N. A.

Table 2.3. Plasmids used in this study.

Plasmid	Description	Source
pAJD905	pRE112 with an *araHG-lacZY-araBA* insert	[36]
pAJD990	pSR47S with *ΔlacZ* insert	[36])
pRSETA	Expression vector with PT7 promoter	Invitrogen
pBBR1MCS3	Broad host range cloning vector, Tc^R	[33]
pKP302	Promoterless lacZ transcriptional fusion, Sp^R	[37]
pRA300-302	Promoterless lacZ translational fusions in three different frames, Sp^R	[38]
pPZP100	Cloning vector, Cm^R	[39]
pKNG101	Pir-dependent suicide plasmid, *sacB*.	[40]
pRU1098	*gfp3.1*(LAA), proteolytically unstable GFP	[41]
pRA504	PT7-*yenR* in pRSETA	This study
pYenR	P*lac-yenR* in pBBR1MCS3	This study
pCST2053	A pRA300 derivative containing the fragment amplified with primers o045 and o130 to detect possible translational activity in the *yenS* region	This study
pCST2054	A pRA301 derivative containing the fragment amplified with primers o045 and o130 to detect possible translational activity in the yenS region	This study
pCST2055	A pRA302 derivative containing the fragment amplified with primers o045 and o130 to detect possible translational activity in the yenS region	This study
pCST2056	A pKP302 derivative containing the fragment amplified with primers o045 and o130 to measure P*yenS* region transcriptional activity to compare with the translational activities obtained from the above three translational fusions	This study
pCST2082	A pPZP100 derivative containing the fragment amplified with primers YenR2EcoRI and o010, containing the first 100 bp of YenR gene and the whole intergenic region	This study
pCST2099	A pPZP100 derivative containing the fragment amplified with primers o029 and o068	This study
pCST2137	A pKP302 derivative containing the PCR fragment obtained with primers o097 and o323 to identify the functions of *yen* box I and *yen* box II	This study

Table 2.3. (Continued).

Plasmid	Description	Source
pCST2233	A promoterless transcriptional *gfpmut3.1*(LAA) cloned from pRU1098 as a PstI-EcoRI fragment into pPZP100	This study
pCST2395	A pAJD905 derivative containing the PCR fragment obtained with primers o708 and o712, which contains *yenS*-lacZ and intact *yen* BoxI and *yen* Box II.	This study
pCST2396	A pAJD905 derivative containing the PCR fragment obtained with primers o709 and o712, which contains *yenS*-lacZ and starts with a truncated *yen* Box I and intact *yen* Box II	This study
pCST2397	A pAJD905 derivative containing the PCR fragment obtained with primers o710 and o712, which contains *yenS*-lacZ and starts with intact *yen* Box II	This study
pCST2398	A pAJD905 derivative containing the PCR fragment obtained with primers o711 and o712, which contains *yenS*-lacZ and starts with a truncated *yen* Box II	This study
pCST2399	A pAJD905 derivative containing the PCR fragment obtained with primers o713 and o717, which contains a *yenR*-lacZ with both boxes	This study
pCST2406	A pCST2233 derivative containing a PCR fragment obtained with primers o410 and o097, using pCST2137 as a template. The PCR fragment contains the promoter region of *yenS* with two intact *yen* Boxes.	This study
pCST2410	A pAJD905 derivative containing the PCR fragment obtained with primers o723 and o712, which contains *yenS*-lacZ with an intact *yen* BoxI and a 2-bp mutated *yen* Box II.	This study
pCST2411	A pAJD905 derivative containing the PCR fragment obtained with primers o724 and o712, which contains *yenS*-lacZ with a 2-bp mutated *yen* Box II.	This study

Table 2. 4. Oligonucleotides used in this study

Name	DNA sequences (restriction sites are in upper case)
pYenR F	gcTCTAGAggtctaggttttctc
pYenR R	gcgGAGCTCgtacatcaggttgaac
YenR2EcoRI	gcGAATTCcgaagatcaccatagg
YenR1KpnI	ggGGTACCcagactaactttcttaa
YenR4EcoRI	ggGAATTCaaatgcggtataacata
o045	gtGAATTCaactagacctaaggctag
o048	ggGAATTCtagctattattatacccc
o010	ggGGTACCccctgttgtggtccaaataat
o130	ttGGTACCgtatgcgttgaggagggg
o009	ttGGTACCaccagggaaacattataac
o024	ccGAATTCcagaattagattttaatat
o029	ccGGTACCataaataaaatactgagagt
o068	ggTCTAGAcgtttttatttcgtctgtct
o132	ccGAATTCattagcaagagatatggtg
o133	gcGGTACCtggttcaacctgatgtac
o097	ccGGTACCaacagtttaattaacata
o308	gaaaacctagaccaaagtatagtttagataactagacctaaggctagct
o309	agctagccttaggtctagttatctaaactatactttggtctaggttttc
o323	ggGAATTCacctagaccaaagtatagtt
o268	6XFAM-acttggtgaatctgaagtgccctg
o276	HEX-caatagtgcaacgaattgcactgggtt
o410	gaAAGCTTCTGCAGgtactgaattaacgccgaa
o451	gaagtgccctggtgttgg
o673	ggtgaaacttgtacttggt
o698	gtttaattaacataggggtat
o699	gatctttctcagaaagaga

Table 2. 4. (Continued)

Name	DNA sequences (restriction sites are in upper case)
o708	cacGGTACCTCTAGAaagagaaaacctagaccaa
o709	ttcGGTACCTCTAGAagtatagtttagataacta
o710	tttGGTACCTCTAGAagataactagacctaaggc
o711	ctcGGTACCTCTAGAaaggctagctattattata
o712	gagGGATCCCAATTGgcaaaaacagtttaattaa
o713	tctGGTACCTCTAGAtaataatagctagcctt
o717	gagGGATCCCAATTGtattctgcgttgaatatag
o723	gaTCTAGAaagagaaaacctagaccaaagtatagtttagataaGAagacctaagg
o724	gagTCTAGAagataaGAagacctaaggctagctat
o3001	ggGGATCCaccaatagccttaccgcta
o3002	ggGGTACCacaacatcgtacacgatct
o3003	ccGGTACCataaataaaatactgagagt
o3004	ccTCTAGAtaatattgttaatggctata
o3005	gtTCTAGAcacgacaacgcgagtccat
o3006	ggGGTACCaataggccaattctactct
o3007	ccGGTACCaggaaactgggtgttttaa
o3008	ggGGATCCtatgaaatacccaaccatg
o3009	ttGGTACCatcgcgcggataatcaccgc
o3010	ggTCTAGAcgtttttatttcgtctgtct
o3011	gcGGATCCgctcggaatatcatcagctgc
o3012	gcTCTAGActccaatggaagcgacgacagta

Integration of lacZ fusions into the ara locus

Suicide plasmids with various *lacZ* fusions were transformed into S17-1/ λpir and selected on LB agar plates with chloramphenicol. The resulting strains were used as conjugative donors in overnight matings on LB agar with recipient strains CST214 and CST216. Transconjugants were selected on LB plates containing both chloramphenicol and nalidixic acid. Single colonies were streaked onto LB agar with nalidixic acid and 10% sucrose. Candidate colonies were confirmed using PCR reactions and by screening for chloramphenicol sensitivity.

Purification of YenR

YenR was overexpressed by constructing plasmid pRA504, which contains a PT7-*yenR* fusion. An overnight culture of BL21(DE3)(pRA504) was diluted 100 fold into LB broth with appropriate antibiotics. At an O.D. (600 nm) of 0.5, IPTG was added (1 mM final concentration) and the cells were cultured for 4 additional hours. The cells were then collected and disrupted using a French press. The lysate was clarified by high speed centrifugation and applied to a heparin sepharose matrix that had been equilibrated with SEDG buffer (50 mM sodium phosphate, 0.1 mM EDTA, 5% glycerol and 1 mM DTT). YenR was eluted with the same buffer containing a gradient of NaCl. Fractions containing YenR were pooled and dialyzed against the SEDG buffer with 100 mM NaCl and 50% glycerol and stored at -80^{O} C.

Chromatin Immunoprecipitation (ChIP).

Chromatin immunoprecipitation assays were performed using a published procedure [42]. Strain YWY003 was cultured in LB medium to an O.D. of 0.3, at

which time formaldehyde was added (1% v/v) to crosslink DNA binding proteins to DNA. The crosslinking reaction was terminated by the addition of excess glycine. Cells were disrupted and DNA was sheared by sonication. YenR-DNA complexes were precipitated using anti-YenR antiserum and protein A-Sepharose beads.

Precipitates were heat-treated to reverse the cross links, and DNA fragments were ligated to uni-directional linkers (oJW102 and oJW103) containing an EcoRI restriction site [43]. The ligated fragments were then amplified by PCR using oligonucleotides that bind to the linkers, digested with EcoRI, and cloned into plasmid pBluescript II SK(+). Plasmids containing inserts were submitted for automated DNA sequencing.

Electrophoretic Mobility Shift Assays.

Electrophoretic mobility shift assays were conducted using double-stranded synthetic oligonucleotides that were treated with γ-P32-ATP and T4 polynucleotide kinase. Purified YenR and 0.12 pmol of labeled DNA were combined at room temperature (RT) in a buffer containing 10 mM Tris·HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 60 mM potassium glutamate, 30 µg/ml calf thymus DNA, 20 µg/ml BSA, and 10% glycerol, and incubated at RT for 30 min. Complexes were size-fractionated using 5 or 15% polyacrylamide gels containing TAE buffer (40 mM Tris·acetate/2 mM EDTA, pH 8.5). Gels were examined using a Storm B840 PhosphorImager (Molecular Dynamics). ImageJ software [44] was used to calculate the ratio of bound to free DNA. In some experiments, autoinducers in the indicated concentrations were added 30 min after YenR-DNA binding reactions were initiated, then the reactions

were incubated for 30 min more and characterized by EMSA.

DNase I Footprinting Assays.

DNA primer o262, which contains a 5'-6XFAM fluorescent dye, and o263 were used to PCR amplify a DNA fragment containing *yen* boxes I and II. Binding of YenR was carried out as described above. Protein-DNA complexes were digested using DNase I (NEB, diluted to 0.2 U/μL) for 40 s. The reactions were then stopped by addition of SDS and EDTA. DNA was further purified using Qiaquick purification kits (Qiagen), and submitted to the Cornell Life Sciences Core Laboratories Center for analysis using an Applied BioSystems 3730xl DNA Analyzer.

RNA extraction, Northern blotting, primer extension, and RACE assays.

Cells were cultured in LB medium and collected at an O.D. of 0.3. A solution containing ethanol and phenol was added to kill the bacteria and preserve RNAs. Cells were centrifuged and frozen at -80^{O} C. RNA was extracted using either Trizol reagent (Invitrogen) or a Qiagen RNeasy kit. The total RNA was quantified by absorbance at 260 nm and RNA integrity by agarose gel electrophoresis. RNA was size-fractionated by electrophoresis in 8% polyacrylamide gels containing 8M urea and 1 x TBE, and then blotted electrophoretically onto an Amershan Hybond-XL membrane (GE Healthcare) in 1 x TBE buffer. The membranes were probed with 5'-radiolabeled oligonucleotides o451, o673, o698 and o699.

Primer extension reactions were performed using an oligonucleotide containing a 6XFAM 5' end (o268) and Superscript III reverse transcriptase. The reactions were stopped by heating and RNA was subjected to alkaline hydrolysis. Fluorescently labeled cDNA was purified and analyzed using an Applied BioSystems 3730xl DNA Analyzer.

5' RACE reactions were carried out by synthesizing cDNA using a primer specific for each gene. Terminal DNA transferase and dCTP were added to produce homopolymers at the 3' ends of each cDNA. A poly-dG primer and a second gene specific primer (YenR1EcoR1) were used to PCR amplify these cDNA fragments. The resulting fragments were cloned into pBluescript SK(+) and submitted for automated DNA sequencing.

3' RACE reactions were carried out using RNA treated with CIP (Calf Intestinal Alkaline Phosphatase, NEB). CIP was then removed by phenol/chloroform extraction. RNA adapter E1 (5'Phos-UUCACUGUUCUUAGCGGCCGCAUGCUC -3' InvdT) was denatured and chilled on ice. The CIP treated RNA, E1 adapter and T4 RNA ligase were then combined overnight at 16^{O} C. RNA ligase was then removed by phenol extraction. Primer extension with a DNA oligonucleotide complementary to the E1 adapter was performed using Superscript III reverse transcriptase (Invitrogen). The resulting cDNA was amplified using primer o024 (which is complementary to the cDNA) and an oligonucleotide complementary to E1 adapter. The PCR product was cloned into pBluescript SK(+) and sequenced.

Analysis of yenS gene expression by flow cytometry

Strains CST201(pCST2406), CST206(pCST2406), and JB580(pCST2406) were cultured in LB medium overnight, then washed and diluted 10^8-fold into AB medium containing 1% LB. Cells were collected at intervals and analyzed for GFP-mediated fluorescence using a Coulter Epics XL-MCL flow cytometer. Data were collected for 5000 cells in each sample, and analyzed using WinMDI software.

2.4 Results

Immunoprecipitation of YenR-DNA complexes.

The primary goal of this study was to identify YenR-regulated promoters. As described above, the closest YenR homologs include EsaR, ExpR and SmaR, and evidence had been published indicating or suggesting that these proteins bind DNA only as apoproteins. We therefore hypothesized that YenR might have similar properties (this hypothesis was later confirmed, see below). Accordingly, we performed chromatin immunoprecipitation experiments in a strain expressing YenR and lacking YenI. The *yenI* null mutation effectively locked this quorum sensing system into a low-cell-density state regardless of the actual cell density.

We cultured strain YW003 (a *yenI* derivative of *Y. enterocolitica* JB580) to an OD_{600} of 0.3, cross-linked all DNA bound proteins to DNA using formaldehyde, and immunoprecipitated DNA-YenR complexes with an anti-YenR antiserum. The precipitated DNA was PCR amplified, cloned into a plasmid vector, and 28 clones were sequenced (Table 2.5). One such clone consisted of an intergenic region extending from 132 nucleotides upstream of

the *yenR* translation start site to 335 nucleotides upstream. The present study focuses on this intergenic region.

We performed a reconstruction experiment, in which immuno-precipitated chromatin was PCR amplified using primers that amplify a region upstream of *yenR*. This DNA was found primarily in the precipitated fraction in YenR$^+$ strain (Fig. 2.1A, lane 7), while only trace amounts were detected in a YenR$^-$ strain (lane 5). This fragment was also not detected in mock immunoprecipitations in which the antiserum had been omitted (Fig. 2.1A, lanes 4 and 6).

To demonstrate a direct interaction between YenR and this DNA sequence, we performed electrophoretic mobility shift assays (EMSA) using a radiolabeled DNA fragment and purified apo-YenR. YenR formed two major complexes with this DNA fragment (Fig. 2.1B), suggesting that it may bind to two sites (confirmed below), and lower amounts of a third complex that migrated slightly faster than complex 2. Detection of this third complex was variable and was not pursued.

Table 2.5. DNA fragments immunoprecipitated using anti-YenR antiserium

ChIP-1-1	4283923-4283618, within ATP-binding subunit of ABC-type permease
ChIP-2-1 ChIP-12-1	3453968-3453709, Overlapping with *pstS*, upstream of *brnQ*
ChIP-2-2 ChIP-12-2	2951792-2951434, Overlapping with *cbiL,* upstream of *cbiM* (B12 biosynthesis)
ChIP-3-1	1095045-1095242, within YE0972, a hypothetical protein
ChIP-4-1	3225093-3224921, within the two-component sensor kinase KdpD
ChIP-4-2	1629671-1630031, within *myfA,* (adhesin)
ChIP-7-1	2990324-2990076 within YE2756, a hypothetical protein
ChIP-8-1	1798006-1797802, upstream of *yenR*, downstream of YE1598 (‘*yfrE*, a pseudogene)
ChIP-8-2	3279434-3279652, upstream of *lipA* (lipoic acid synthase)
ChIP-9-1	2326667-2326353, upstream of *ryhB* RNA, within *sapA* (periplasmic subunit of an ABC-type peptide transporter)
ChIP-9-2	2517947-2518153, within YE2313, a putative phage tail protein
ChIP-10-1	4220897-4220685, within *malG*, a subunit of an ABC-type maltose permease

Table 2.5. (Continued)

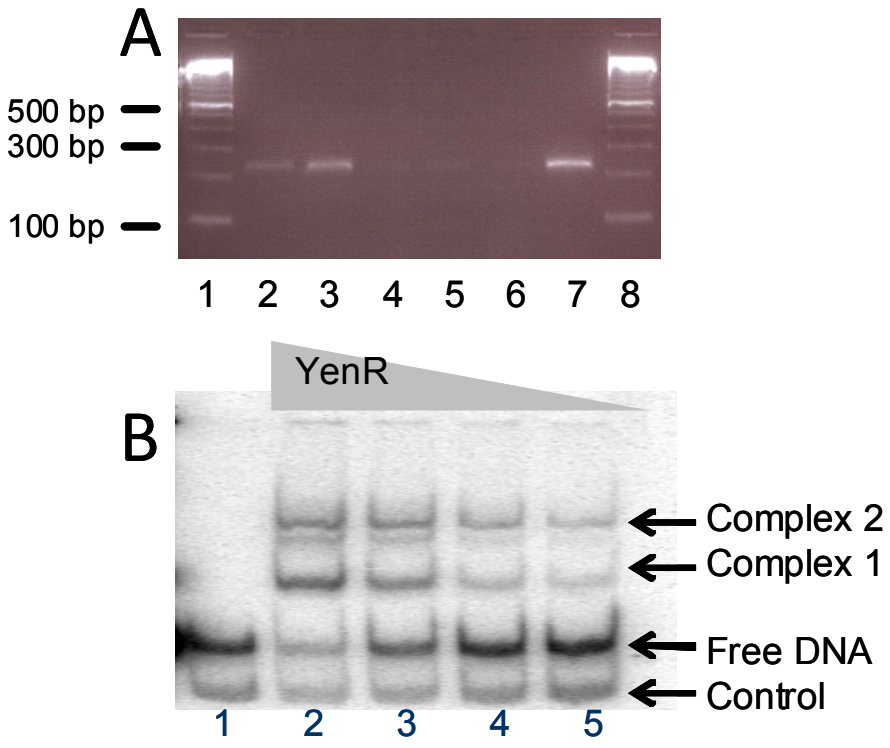
ChIP-14-1	3469155-3469596, Upstream of YE3190, a hypothetical protein
ChIP-15-1	4104720-4104911, Upstream of YE3762, a hypothetical protein
ChIP-15-2	16S ribosomal RNA
ChIP-15-3	4421861-4422069, Upstream of YE4055, a hypothetical protein
ChIP-15-4	4529730-4530071, Downstream of *fdhD,* upstream of *sodA*
ChIP-16-1	2679801-2679406, Between YE2487 and YE2486, a ferric iron reductase involved in ferric hydroximate transport
ChIP-17-1	76902-76662, Within kdtX, a glycosyl transferase (lipopolysaccharide core biosynthesis)
ChIP-18-1	4591102-4591583, within YE4189, a predicted hydrolase, within YE4190, a putative amino acid permease
ChIP-19-1 Chip-20-1	4451502-4451216, within bcsA, a cellulose synthase subunit
ChIP-19-2 ChIP-20-2	4471619-4471393, within uhpB, a sensory histidine kinase gene
ChIP-19-3 ChIP-20-3	plasmid pYVe8081, 5328-5198, upstream of sycT, 45860-45763, within YEP0064

Figure. 2.1. Interactions between apo-YenR and DNA upstream of the *yenR* promoter.

(A) Chromatin immunoprecipitation of YenR-DNA complexes.

Lanes 1 and 8: 100 bp ladder. Lane 2 and 3: PCR amplified input DNA from strain CST201 (*yenR*$^-$, *yenI*$^-$) and from CST206 (*yenR*$^+$, *yenI*$^-$), respectively. Lane 4 and 6: Mock chromatin immunoprecipitation from CST201 and CST206, respectively, in the absence of anti-YenR antiserum. Lane 5 and 7: immunoprecipitated and amplified DNA from strains CST201 and CST206. PCR amplifications were carried out using oligonucleotides YenR4EcoRI and YenR1KpnI, the PCR product size is 213 bp.

(B) Electrophoretic mobility shift assays of YenR and a 311 bp DNA fragment upstream of *yenR*. Complex 1 and complex 2 are YenR-DNA complexes, while control DNA is a 250 nucleotide fragment containing the *occR* promoter of *A. tumefaciens*. The highest protein concentration is 250 nM and was serially diluted in 4-fold increments.


A
500 bp
300 bp
100 bp
1 2 3 4 5 6 7 8
B
YenR
Complex 2
Complex 1
Free DNA
Control
1 2 3 4 5

YenR binds to two sites upstream of yenR.

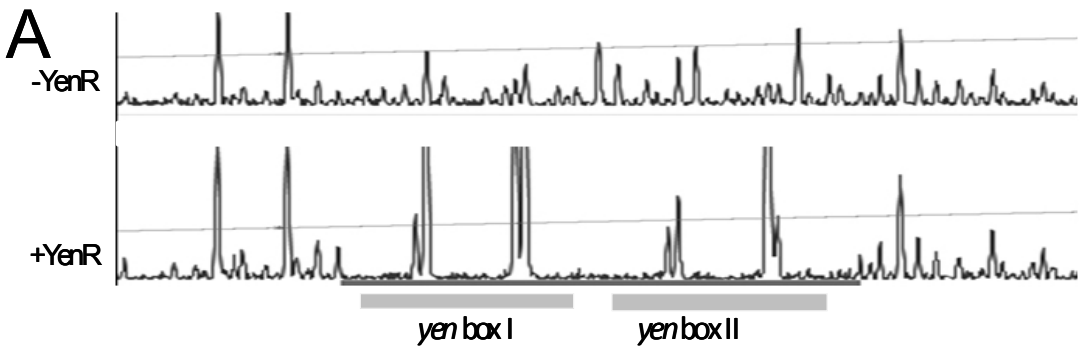
To localize the YenR binding sites, we conducted DNase I protection assays using a fluorescently labeled DNA fragment and purified apo-YenR. YenR strongly altered the protection patterns of a 55 nucleotide region that lies 123 to 178 nucleotides upstream of the *yenR* translation start site (Fig. 2.2A). Four hypersensitive sites were identified within this region, similar to those found in footprints of the TraR and CepR proteins [17, 45]. These non-protected sites suggest that YenR binds to one face of the DNA and does not protect the opposite face. Similar conclusions were reached with the TraR protein [17], and were later confirmed by X-ray crystallography [14, 16].

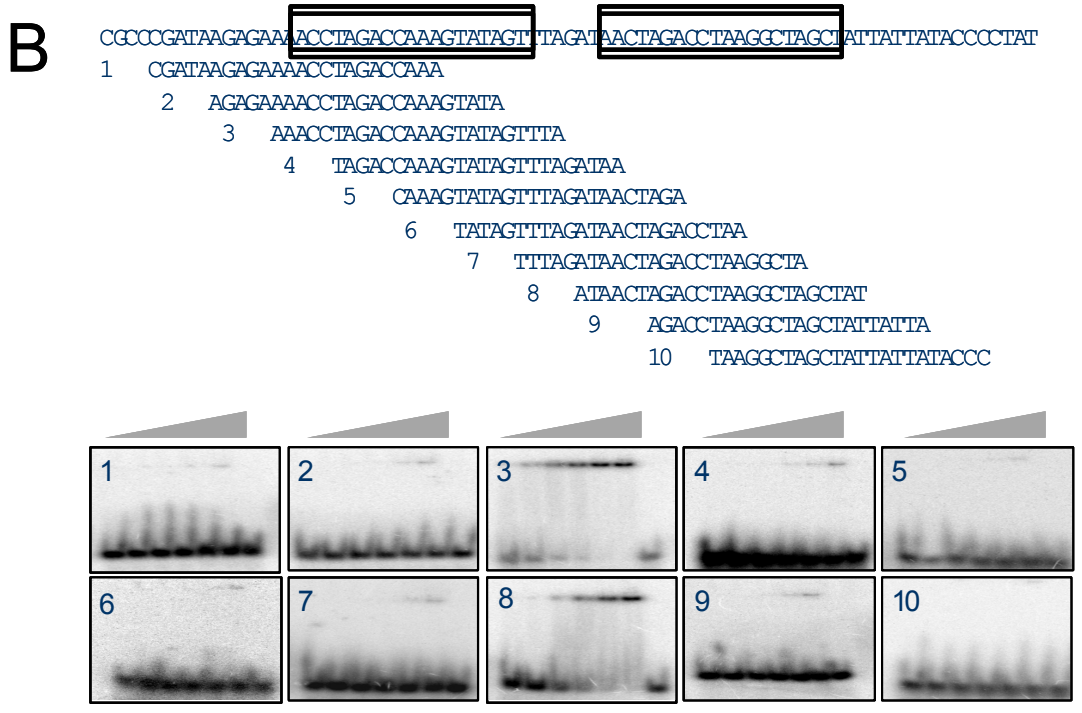
We noticed two 20-nucleotide sequences within the footprinted region that show imperfect dyad symmetry and that are similar to each other in sequence. To determine whether these sequences served as YenR binding sites, we synthesized ten duplex oligonucleotides, each 24 nucleotides in length that tiled the footprinted region (Fig. 2.2B). Of these ten fragments, fragment 3 fully contains the left sequence, while fragment 8 fully contains the right sequence. These two fragments bound YenR with high affinity (40 nM and 150 nM, respectively), while the remaining eight fragments bound very weakly or not at all (Fig. 2.2B). These sites were designated *yen* box I and *yen* box II.

Figure. 2. 2. Identification of the sites bound by YenR.

(A) DNase I footprinting of the region upstream of *yenR*. A DNA fragment extending from 82 nucleotides downstream of the *yenR* translation start site to 327 nucleotides upstream was synthesized by PCR amplification using oligonucleotides o262 and o263, the former of which contains the fluorophore 6XFAM. This fragment was subjected to DNase I digestion in the absence (top) or presence (bottom) of purified YenR. Fragments were analyzed by automated capillary gel electrophoresis.

(B) Ten duplex DNA fragments (1-10) were radiolabeled, combined with purified apo-YenR, and tested for binding using EMSA (lower panels). YenR was added at the following concentrations in each EMSA assay: 5.44 nM, 16.3 nM, 48.9 nM, 146 nM, 440 nM, 1322 nM, 0 nM.


A
-YenR
+YenR
yen box I
yen box II


B
CGCCCGATAAGAGAAAACCTAGACCAAAGTATAGTTTAGATAACTAGACCTAAGGCTAGCTATTATTATACCCCTAT
1 CGATAAGAGAAAACCTAGACCAAA
2 AGAGAAAACCTAGACCAAAGTATA
3 AAACCTAGACCAAAGTATAGTTTA
4 TAGACCAAAGTATAGTTTAGATAA
5 CAAAGTATAGTTTAGATAACTAGA
6 TATAGTTTAGATAACTAGACCTAA
7 TTTAGATAACTAGACCTAAGGCTA
8 ATAACTAGACCTAAGGCTAGCTAT
9 AGACCTAAGGCTAGCTATTATTA
10 TAAGGCTAGCTATTATTATACCC
1
2
3
4
5
6
7
8
9
10

Several other LuxR-type transcription factors bind to sites that show dyad symmetry [14, 16, 19, 24, 45-47]. To further characterize these putative binding sites, we constructed a total of four artificial fragments that were perfectly symmetric. The first replaced the right half of *yen* box I with the inverse complement of the left half (Fig. 2.3, Box I L-L'). The second replaced the left half of this site with the inverse complement of the right half (Fig. 2.3A, Box I R'-R). The remaining two artificial sequences were similar but using *yen* box II as the template sequence (Box II, L-L', and Box II, R'-R). The two wild type and four symmetric sequences were tested for YenR affinity. All six fragments bound YenR with high affinity. Interestingly, all three sequences based upon Box I showed similar affinities, while all three sequences based upon Box II also bound with similar affinities that were slightly lower than the first three. We have aligned the four half-sites and derived a consensus (Fig. 2.3B). Of the four synthetic binding sites, sequence 5 (box II L-L') most closely resembles the consensus, yet is not bound with higher affinity than the other sites. More work will need to be done to identify the optimal YenR binding site. In a preliminary search of the *Y. enterocolitica* genome, we find other predicted YenR binding sites that lie near promoter regions (data not shown).

Figure. 2. 3. YenR binding to artificial fragments having perfect rotational symmetry.

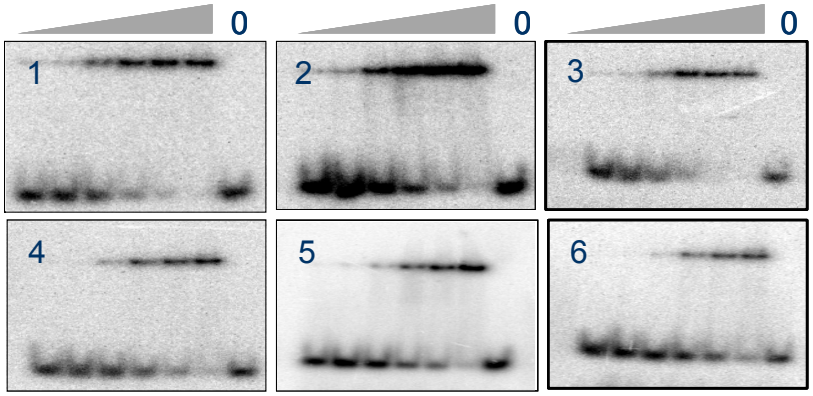
(A) Sequences 1 and 4 are wild type for *yen* box I and *yen box II*, respectively, and are denoted as L-R, for left and right half-sites. *yen* box I L-L' (line 2) contains the left half of *yen* box I followed by the inverse complement of that sequence, and therefore is completely symmetric. *yen* box II L-L' (line 5) is the same but using *yen* box II sequences. *yen* box I R'-R (line 3) and *yen* box II R'-R (line 6) contain right half-sites of *yen* box I and II, respectively, and the inverse complements of those sequences, and are also fully symmetric. Each fragment was tested for binding using the following apo-YenR concentrations: 5.44 nM, 16.3 nM, 48.9 nM, 146 nM, 440 nM, 1322 nM, 0 nM. The K_d for YenR to binding to fragments 1-3 is approximately 50 nM, while the affinity for fragments 4-6 is approximately 150 nM.

(B) The left halves of *yen* box I and II and the inverse complement of the right halves of these sites are aligned, and a consensus sequence identified. In the consensus sequence, a capital letter indicates that all four bases are identical, while a lower case letter indicates that three of the four are identical, and a lower case “n” indicates a lack of consensus at that position.

A

1 AAAC CTAGACCA AAGTATAG TTTA *yen* box I L-R
2 AAAC CTAGACCA TGGTCTAG GTTT *yen* box I L-L′
3 TAAA CTATACTT AAGTATAG TTTA *yen* box I R′-R

4 ATAA CTAGACCT AAGGCTAG CTAT *yen* box II L-R
5 ATAA CTAGACCT AGGTCTAG TTAT *yen* box II L-L′
6 ATAG CTAGCCTT AAGGCTAG CTAT *yen* box II R′-R



B

A A A C C T A G A C C A box I L
T A A A C T A T A C T T box I R
A T A A C T A G A C C T box II L
A T A G C T A G C C T T box II R

0 0 0 1 0 0 0 3 0 0 0 0 G
3 2 4 2 0 0 4 0 3 0 0 1 A
1 2 0 0 0 4 0 1 0 0 2 3 T
0 0 0 1 4 0 0 0 1 4 2 0 C

a n A n C T A g a C n t Consensus half site

The two *yen* boxes are centered 25 nucleotides apart, or 2.5 helical turns. In the DNase I footprint, there were no sensitive sites between the two binding sites, indicating that the two dimers must lie very close together, and may contact one another. It therefore seemed plausible that YenR might bind *yen* box I and *yen* box II cooperatively. This was tested using EMSA with the fragment containing both sites. The fragment was shifted by YenR, forming two complexes (Fig. 2.4A). The dissociation constant (K_d) was about 25 nM. Binding did not show detectable cooperativity, as the Hill coefficient was approximately 1 (Fig. 2.4B). This indicates that YenR bound to one site does not affect the binding affinity at the other site.

Binding of YenR to DNA is altered by addition of OHHL.

As described above, YenR binding was detected *in vivo* and *in vitro* in the absence of AHLs. We tested whether OHHL could influence binding of purified YenR to DNA. Complexes were made using apo-YenR and a DNA fragment containing two *yen* boxes (the same fragment as used in Fig. 2.4A). OHHL was then added at a range of concentrations and incubated for 30 min prior to size-fractionation by EMSA. In the absence of OHHL (Fig. 2.4C, lane 2) most of the DNA was in complex 2, while far smaller amounts of DNA was in complex 1 or unbound. As OHHL was titrated in, both complexes became less abundant (Fig. 2.4C), and increasing amounts of the DNA became unbound. In addition, some DNA molecules migrated more slowly, or formed aggregates that failed to enter the gel. Apparently, OHHL caused some complexes to dissociate, and caused other complexes to aggregate. In either case, OHHL disrupted soluble YenR-DNA complexes. Similar results were obtained by adding OHHL to YenR prior to addition of DNA (data not shown).

Figure. 2.4. YenR activity *in vitro* and *in vivo* in the presence and absence of AHLs.

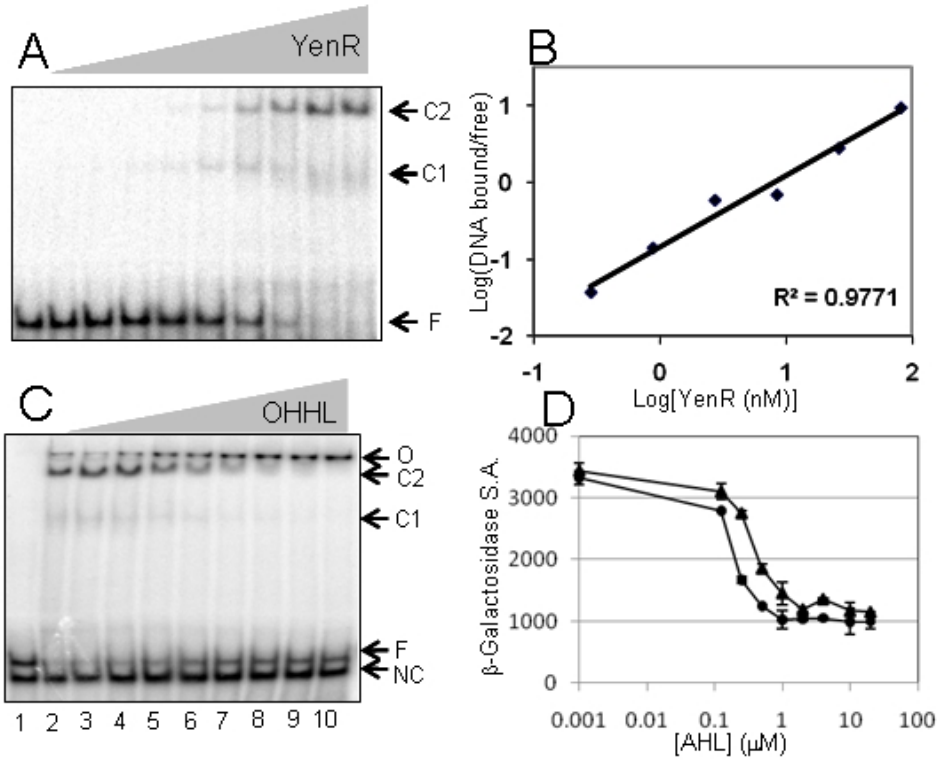
(A) Gel mobility shift assays using a DNA fragment containing both *yen* boxes (made by PCR using oligonucleotides o308 and o309. The protein concentrations for each lane is: 0 nM 0.09 nM, 0.28 nM, 0.87 nM, 2.7 nM, 8.4 nM, 26 nM, 81 nM, 250 nM, and 774 nM.

(B) Hill plot of the EMSA data shown in part A. The slope is approximately 1, indicating that binding is noncooperative.

(C) Disruption of YenR-DNA complexes by OHHL.

Complexes were formed between apo-YenR and a DNA fragment containing both binding sites (the same fragment as in part A). OHHL was then added at various concentrations, incubated for 30 m, and analyzed by EMSA. OHHL caused some complexes to dissociate so that the DNA fragment migrated as free DNA, while the remaining complexes migrated more slowly than in the absence of OOHL, and some did not enter the gel, suggesting that the complexes had aggregated. The highest OHHL concentration was 3 uM and was serially diluted in 2-fold increments.

(D) Inhibition of *yenS* expression *in vivo* by AHLs. β-galactosidase activities of the *yenS-lacZ* transcriptional fusion in strain CST216 (*yenI* $^{-}$ *yenR* $^{+}$ *lacZ* $^{-}$) containing plasmid pCST2395 cultured in the presence of the indicated concentrations of OHHL (triangles) or HHL (circles). All assays were done in triplicate in AB defined medium [41].


A
YenR
C2
C1
F
B
Log(DNA bound/free)
1
0
-1
-2
-1
0
1
2
Log[YenR (nM)]
R² = 0.9771
C
OHHL
O
C2
C1
F
NC
1 2 3 4 5 6 7 8 9 10
D
β-Galactosidase S.A.
4000
3000
2000
1000
0
0.001 0.01 0.1 1 10 100
[AHL] (μM)

YenR does not significantly autoregulate

The finding that YenR binds to a site close to the *yenR* promoter suggested that this protein might regulate one or more promoters in this region. We constructed a single-copy *yenR-lacZ* fusion (in pCST2399) containing 248 nucleotides of *yenR* extending from 179 nucleotides upstream of the translation start site to 69 nucleotides downstream. This fusion was introduced into the *ara* chromosomal locus by double homologous recombination [36], using strains CST216 (*yenR*$^+$, *yenI*$^-$, *lacZ*$^-$) or CST214 (*yenR*$^-$, *yenI*$^-$, *lacZ*$^-$). Synthesis of β-galactosidase by this fusion was not affected by the *yenR* or *yenI* status of the cell or by addition of OHHL (Table 2.6). YenR therefore does not regulate its own synthesis.

Identification of two conserved DNA sequences upstream of yenR.

In an effort to determine whether the *yen* boxes were conserved across *Yersinia* spp., we aligned these regions using BLASTN. As expected, these two sites were conserved (Fig. 2.5). To our surprise, we found that the conservation extended far further upstream. The two *yen* boxes lie at the *yenR*-proximal end of a 250-nucleotide conserved region (Fig. 2.6A, grey boxes and Fig. 2.5). Upstream of this sequence is a 210-nucleotide nonconserved region, followed by a second conserved region of approximately 160 nucleotides (Fig. 2.5). The nonconserved regions suggest that in the absence of genetic selection, genetic drift between these species has been extensive. This in turn indicates that the observed conservation is probably attributable to some conserved and adaptive function. In this study, we focus on the 250 nucleotide conserved region.

Table 2. 6. The role of YenR, OHHL, and *yen* boxes in the expression of *yenS* and *yenR.*

Integrated Plasmid	Fusion	*yen* box I	*yen* box II	CST216	CST216	CST214	CST214
				yenR^{+}, *yenI*$^{-}$, *lacZ*$^{-}$	1 µM OHHL	*yenR*$^{-}$, *yenI*$^{-}$, *lacZ*$^{-}$	1 µM OHHL
pCST2399	*yenR-lacZ*	+	+	385 ± 70[1]	317 ± 35	343 ± 50	373 ± 37
pCST2395	*yenS-lacZ*	+	+	1895 ± 65	618 ± 25	75 ± 11	50 ± 8
pCST2396	*yenS-lacZ*	half	+	964 ± 159	195 ± 30	88 ± 8	64 ±16
pCST2397	*yenS-lacZ*	-	+	942 ± 59	30 ± 5	68 ± 6	40 ± 12
pCST2398	*yenS-lacZ*	-	half	44 ± 12	48 ±12	55 ±21	35 ± 4
pCST2410	*yenS-lacZ*	+	altered[2]	1008 ±112	169 ± 35	55 ± 3	23 ± 2
pCST2411	*yenS-lacZ*	-	altered	60 ± 3	48 ± 2	60 ± 8	31 ± 11

1: β-galactosidase specific activity. Data shown in the average of triplicate experiments with standard deviations as shown. All assays were done using AB defined medium (Cangelosi *et al.*, 1991).

2: *yen* box II was altered from AA**CT**AGACCTAAGGCTACGT to AA**GA**AGACCTAAGGCTACGT.

Figure. 2.5. BLASTN alignments of *yenS* and putative orthologs in other *Yersinia* spp. The two *yen* boxes and the *yenS* promoter are indicted in open squares and the transcription terminator is indicated with inverted arrows. A second conserved region of unknown function is shown on the third page of the figure.

Multiple Alignments of the region upstream of YenR (page 1/3)

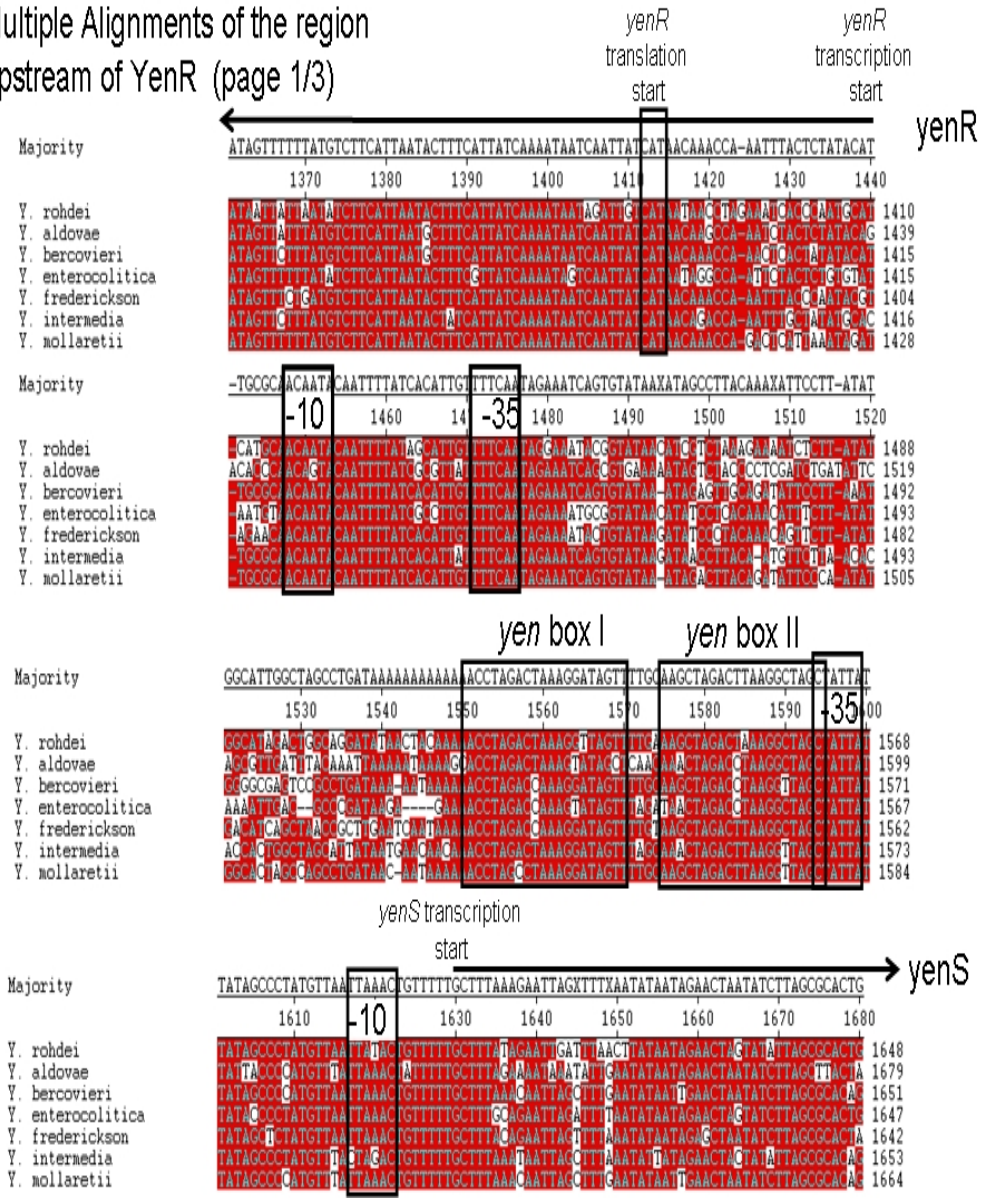


Figure 2.5. (Continued)

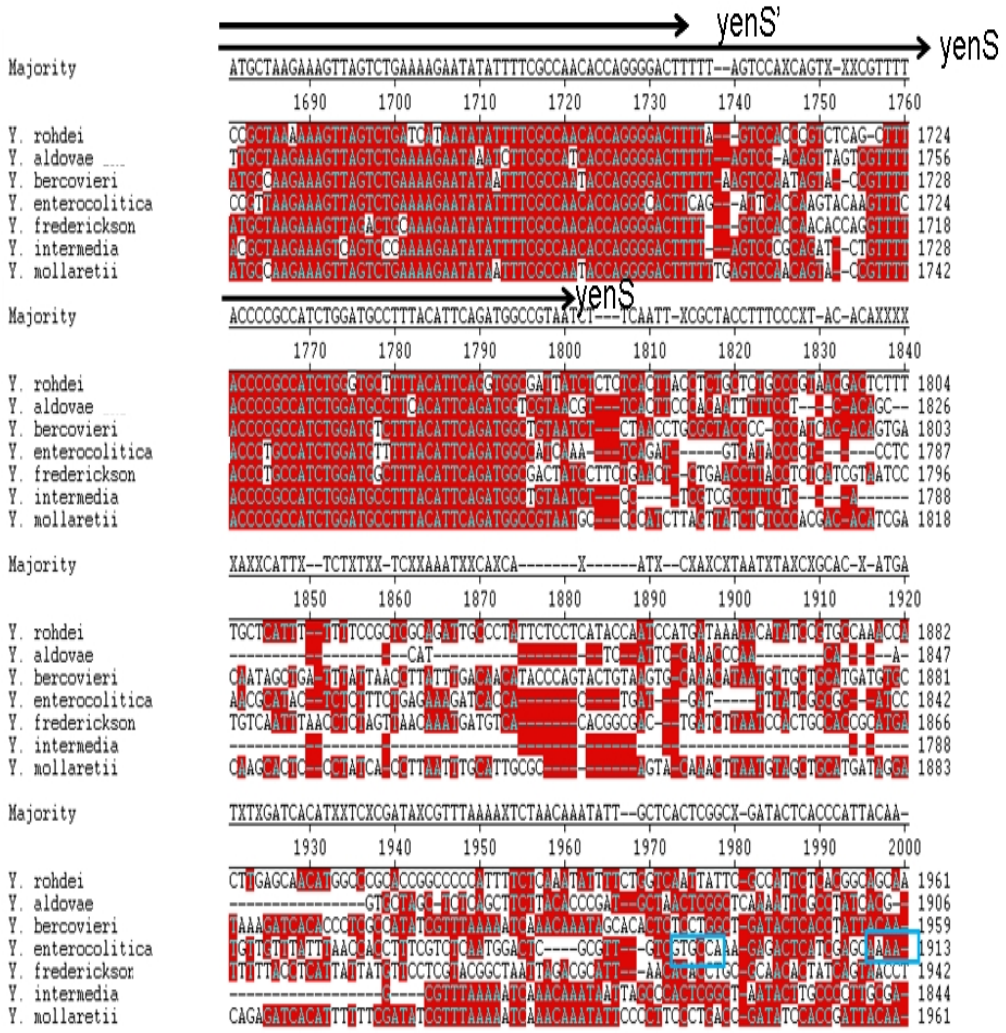
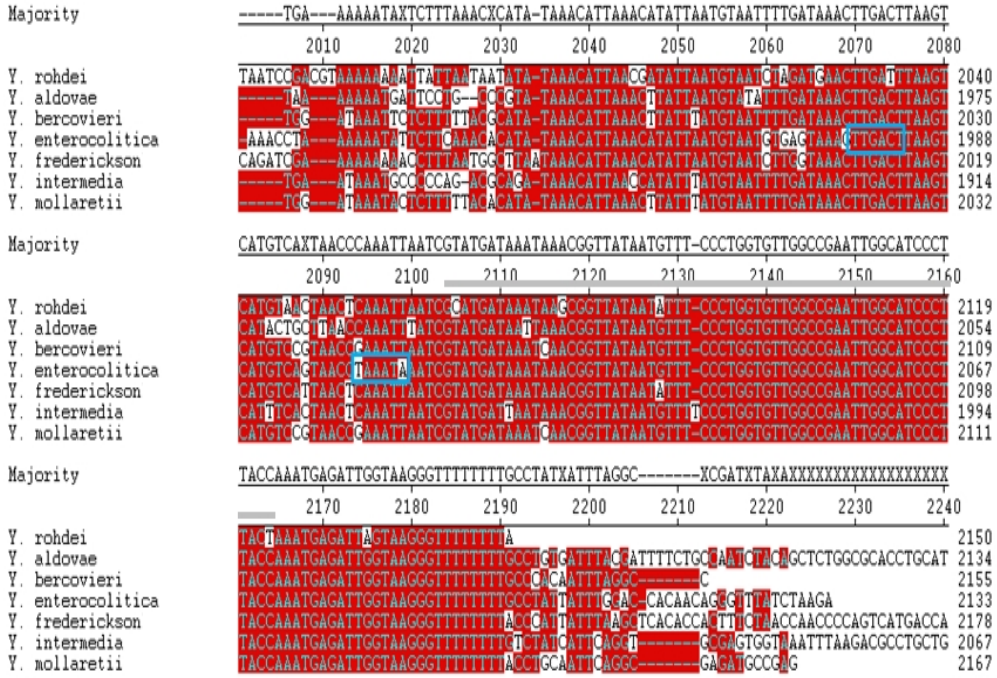


Figure 2.5. (Continued)



Apo-YenR activates a gene divergent from yenR.

To determine whether the 250-nucleotide region is transcribed, we constructed a transcriptional fusion between it and *lacZ*, oriented so as to measure transcription divergent from *yenR* (pCST2395, Table 2.6). This fusion was integrated into the chromosome at the *ara* locus, using strains CST216 (*yenR*$^+$, *yenI*$^-$, *lacZ*$^-$), and CST214 (*yenR*$^-$, *yenI*$^-$, *lacZ*$^-$). The resulting two strains were cultured in the presence or absence of 1 μM OHHL. In the absence of OHHL, the *yenR*$^+$ strain synthesized about 25-fold more β-galactosidase than the *yenR*$^-$ strain. Addition of OHHL reduced expression about 3-fold in the *yenR*$^+$ strain, but had little or no effect on the *yenR* mutant. Evidently, apo-YenR activated expression of a gene, designated *yenS*, while OHHL antagonized YenR. We used the same fusion to measure the inhibitory effects of varying amounts of OHHL and HHL. Both AHLs inhibited expression of this fusion (Fig. 2.4D). OHHL seemed to inhibit YenR activity at slightly lower concentrations than HHL.

yen boxes I and II act cooperatively to activate yenS.

To determine whether *yen* boxes I and II are required for induction of *yenS*, we compared six suicide plasmids that contained or lacked these sites (Fig. 2.6). These plasmids were integrated into the *ara* locus by double recombination, using CST216 (*yenR*$^+$, *yenI*$^-$, *lacZ*$^-$) and CST214 *(yenR*$^-$, *yenI*$^-$, *lacZ*$^-$*)*. Four of these plasmids make step-wise resections of the two *yen* boxes, while two contain point mutations in *yen* box II (from AA**CT**AGACCTAAGGCTACGT to AA**GA**AGACCTAAGGCTACGT). All six plasmids had similar levels of transcription in the absence of YenR. In this strain, the addition of OHHL caused a slight decrease in expression. The significance of this is unknown,

but might be attributable to a second LuxR homolog encoded by this strain.

In the strain synthesizing YenR but not YenI, several fusions were expressed at elevated levels (Table 2.6). As described above, the fusion containing *yen* box I and *yen* box II (pCST2395) was induced about 25-fold. The fusion containing half of *yen* box I and all of *yen* box II (pCST2396) was induced about 11-fold, while the fusion that contained just *yen* box II (pCST2397) was induced about 14-fold. Apparently, YenR can activate this promoter by binding to just *yen* box II. The fusion containing only half of *yen* box II (pCST2398) was not affected by YenR (Table 2. 6).

Of the two fusions with point mutations in *yen* box II, one lacked *yen* box I, and was not induced by YenR (Table 2.6, fusion pCST2411). The other of these two fusions contained *yen* box I (pCST2410); this fusion was inducible. These data indicate that YenR can activate *yenS* by binding to just *yen* box I. Conversely, as seen above, YenR can also activate the *yenS* promoter by binding to just *yen* box II.

All fusions that were induced by YenR were also inhibited by OHHL. However, in some cases, inhibition was rather mild, while in other cases it was more severe. For example, in the fusion containing both *yen* boxes (pCST2395), OHHL reduced expression only about 3-fold (Table 2. 6). In the strain lacking half of *yen* box I (pCST2396), *yenS* expression was inhibited about 5-fold by OHHL, while the fusion containing *yen* box I and an altered *yen* box II (pCST2410) was inhibited about 6-fold. In contrast, induction of the fusion containing just *yen* box II (pCST2397) was completely blocked by OHHL.

These data suggest either that some apo-YenR persists even in the presence of OHHL or that YenR-OHHL complexes retain residual activity. Either way, this residual YenR activity is most effective in the presence of two *yen* boxes (pCST2395), works slightly in the presence of one wild type *yen* box and a second truncated or altered *yen* box (pCST2396 and pCST2410), and does not work at all with just one *yen* box (pCST2397).

Expression of yenS *is enhanced at low cell population density*

All the *in vivo* experiments described above were done using a *yenI* mutant, which locks the system into a low population density state. Addition of exogenous AHLs mimicked conditions of high cell density. Our findings predict that in a YenI-proficient strain, *yenS* would be expressed preferentially at low cell density. To test this, we introduced a plasmid containing a P*yenS-gfp* fusion (pCST2406) into strain JB580 (wild type), CST201 (*yenR*$^-$ *yenI*$^-$), and CST206 (*yenR*$^+$, *yenI*$^-$). These three strains were diluted 10^8-fold and cultured to stationary phase. As predicted, strain CST201 expressed the fusion at low levels during all stages of growth, while CST206 expressed the fusion at high levels (Fig. 2.7). In contrast, the wild type strain expressed the fusion at moderately high levels at low population densities, and at lower levels as the culture density increased (Fig. 2.7).

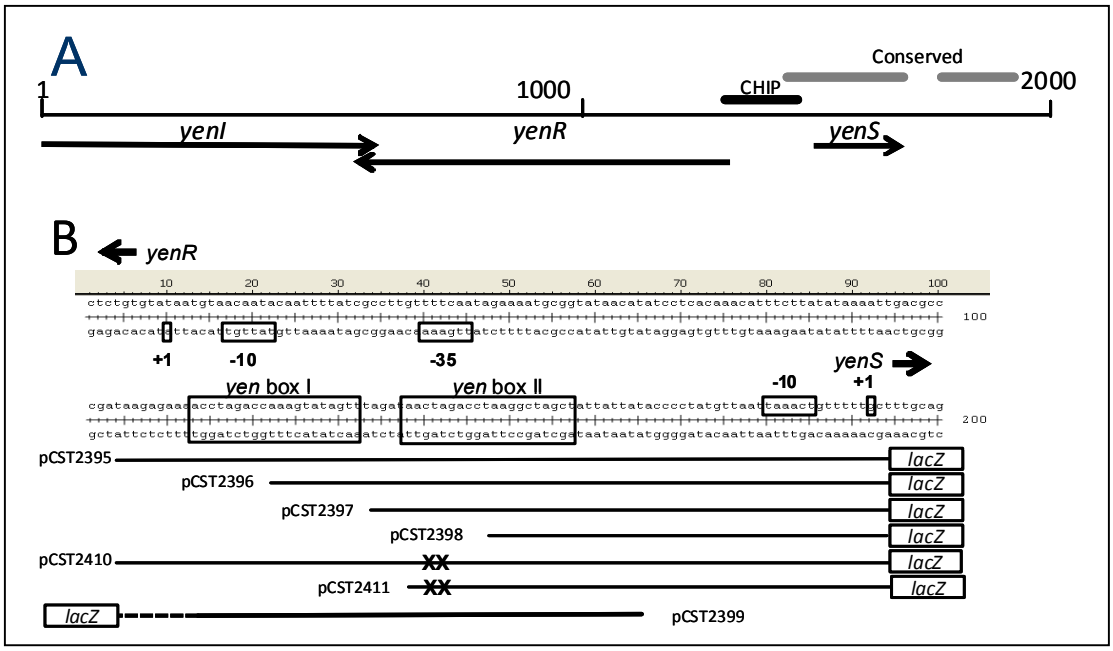


Figure. 2. 6. Genetic map of *yenI*, *yenR,* and *yenS*.

(A) The DNA fragment recovered by chromatin immunoprecipitation is indicated (CHIP). The two regions conserved in five *Yersinia* spp. is indicated with two grey bars.

(B) A *yenR-lacZ* (pCST2399) and six *yenS-lacZ* fusions containing progressive resections or other alterations of *yen* box I and *yen* box II. The transcriptional start sites and promoter motifs of both genes are indicated using open squares.

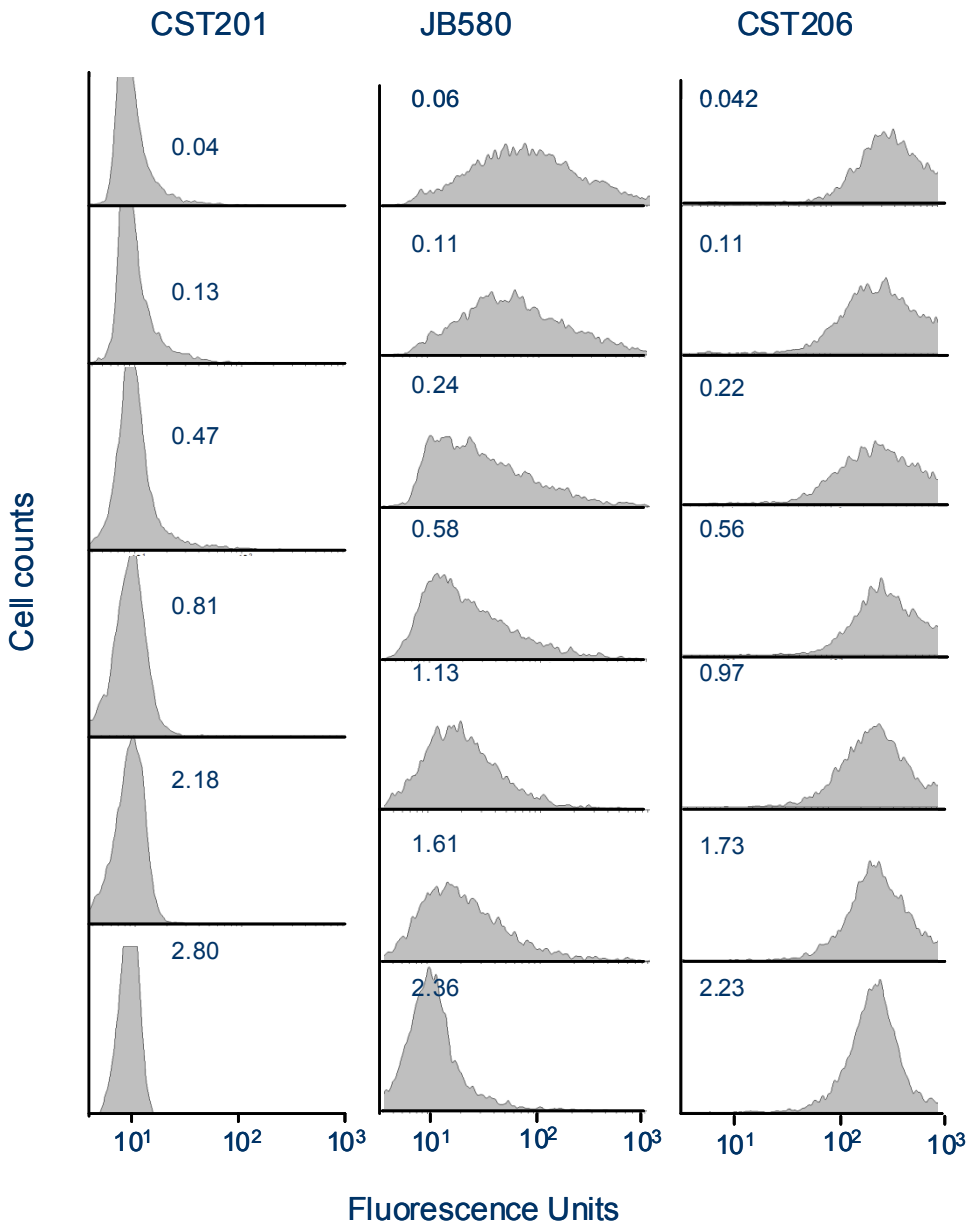


Figure. 2.7. Preferential expression of *yenS* at low cell population densities. Strains containing a plasmid borne P*yenS* gfpmut3.1 (LAA) fusion was diluted to 10 cells per ml, and cultured to stationary phase. At the indicated optical densities, samples were withdrawn and 5000 cells were assayed for the distribution of fluorescence.

yenS *encodes two transcripts with identical 5' ends and differing 3' ends*

Northern blotting experiments were conducted to determine the size of the *yenS* transcript (Fig. 2.8). We used four different oligonucleotide probes in order to give an approximate location of the upstream and downstream ends of this transcript. RNA was prepared from two strains, CST206 (*yenR*$^+$, *yenI*$^-$) and CST201 (*yenR*$^-$, y*enI*$^-$). The former strain was cultured in the presence and absence of OHHL. Using probe o451 (second panel in Fig .2. 8), two RNA species were detected, approximately 100 and 170 nucleotides in length. They were detected in the *yenI* mutant cultured in the absence of OHHL (lane 1), but not from the same strain cultured in the presence of OHHL (lane 2) or in the *yenR*$^-$ *yenI*$^-$ double mutant (lane 3). Probe o673 hybridized to the larger RNA but not the shorter one (Fig 2. 8, third panel), while probes o698 and o699 did not hybridize to either RNA (Fig. 2.8, first and last panel). These data indicate that *yenS* lies fully between probes o698 and o699, and that it encodes two RNA molecules that differ at their 3' ends. The longer transcript was designated YenS while the shorter one was designated YenS'.

The *yenS* and *yenR* transcription start sites were localized using primer extension and 5' RACE assays (data not shown). The *yenS* start site lies 44.5 nucleotides downstream of the center of *yen* box II and 69.5 nucleotides downstream of *yen* box I (Fig. 2.6B). In contrast, the two *yen* boxes are centered over 110 nucleotides upstream of the *yenR* start site. This may help to explain why YenR had no effect on *yenR* expression.

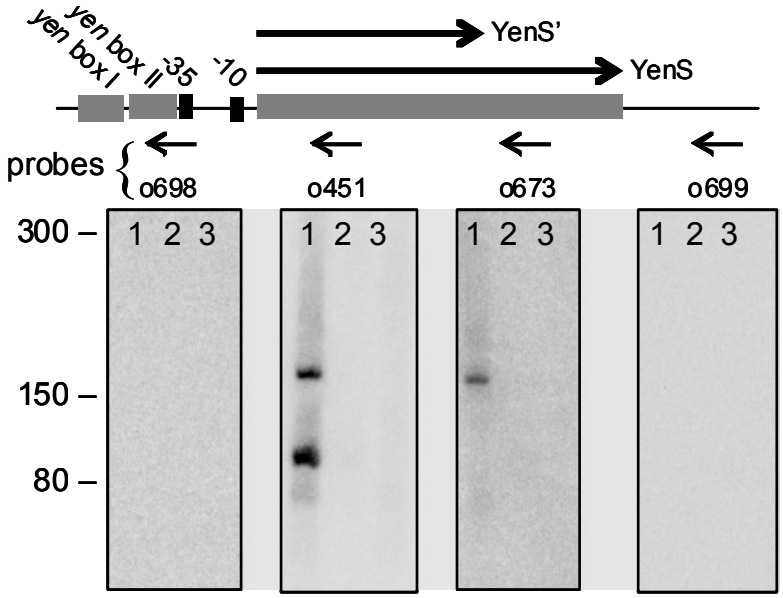


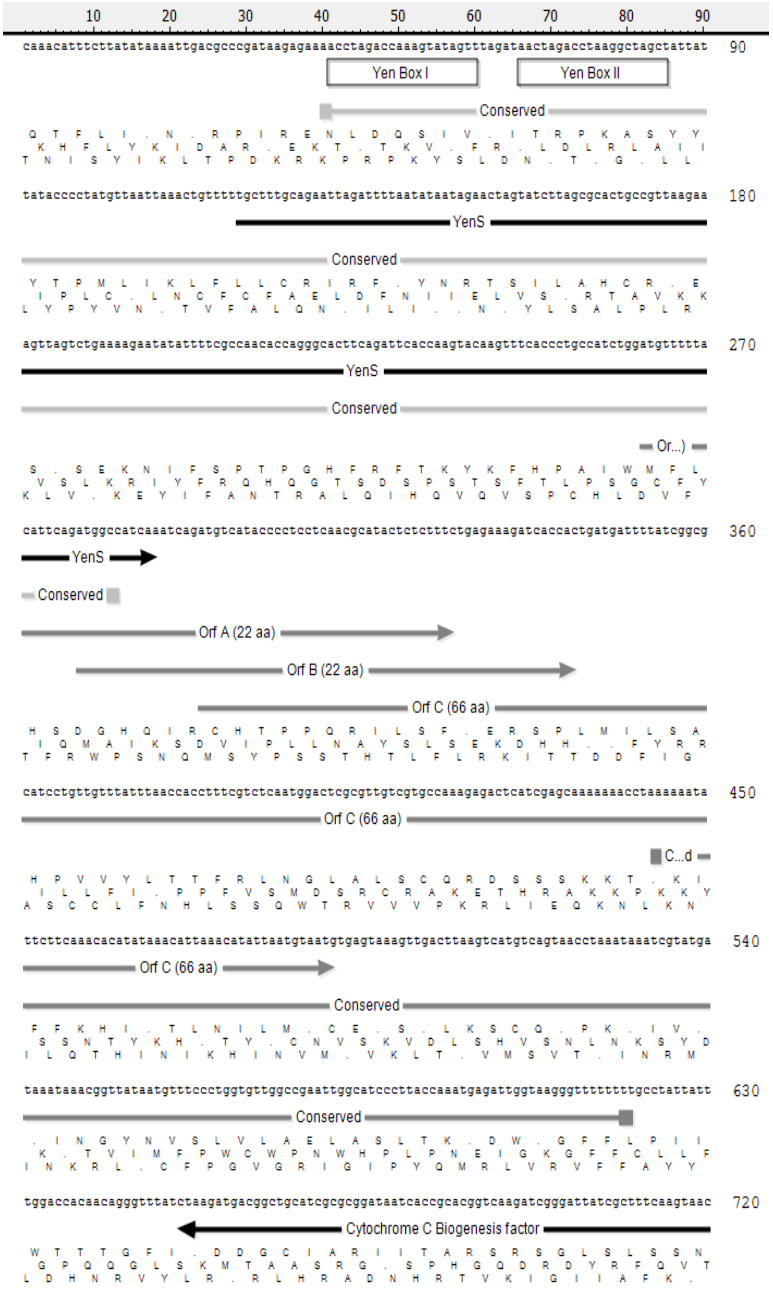
Figure. 2.8. Identification of two RNA molecules encoded by *yenS.* Northern hybridizations of YenS transcripts using oligonucleotide o698 (panel 1), o451 (panel 2), o673 (panel 3), and o699 (panel 4). In each panel, lane 1: CST206 (*yenR*$^+$, *yenI*$^-$), lane 2: CST206 cultured in the presence of 100 nM OHHL, lane 3: CST201 (*yenR*$^-$, *yenI*$^-$). Bacteria were cultured using LB medium

The prediction that YenS and YenS' share a single 5' end but have two different 3' ends was tested by using 3' RACE assays. As predicted, two different 3' ends were identified. One was located 105 nucleotides downstream from the start site, while the other was located 168 nucleotides downstream (Fig. 2.5). YenS and YenS' transcripts are therefore 168 and 105 nucleotides long, respectively, in close agreement with the Northern assays described above.

yenS *encodes a nontranslated RNA.*

Analysis of the *yenS* gene indicates that it has no open reading frames beginning with ATG, GTG, or TTG (Fig. 2.9). Near the 3' end of *yenS*, there are three short overlapping reading frames beginning with ATG (Fig. 2.9). Two of these are extremely short (22 codons each), while the third is somewhat longer (66 codons). For three reasons, it seemed highly unlikely that any of these ORFs was translated. First, all three extend beyond the downstream end of the *yenS* transcript. Second, none of these ORFs has an apparent ribosome binding site. Third, none is conserved among different species of *Yersinia*. We nevertheless determined whether any of these ORFs is translated, by constructing plasmid-based *lacZ* fusions, one transcriptional and three translational (plasmids pCST2053-2056) and introducing them into a *yenR*$^{+}$*, yenI*$^{-}$ strain and a *yenR*$^{-}$*, yenI*$^{-}$ *strain*. Each fragment contained the *yenS* promoter and both *yen* boxes. None of the translational fusions was significantly expressed in either strain, while the transcriptional fusion was expressed at readily detectable levels in the strain expressing YenR (data not shown). We conclude that the *yenS* transcript is not translated.

Figure. 2.9. Conceptual translation of *yenS*. The black arrows on the second and third lines denotes the *yenS* transcript. The two gray bars denote sequences that are conserved among five *Yersinia* spp. (*Y. entercolitica, Y. frederiksenii, Y. bercovieri, Y. intermedia,* and *Y. mollaretii,* see Fig. 2.5). The black arrow on the bottom line denotes a convergent open reading frame. OrfA, -B, and -C represent the only possible translatable sequences. We note that all three are very short, lack ribosome binding sites, are not conserved among *Yersinia* spp., and are not detectably translated.


10 20 30 40 50 60 70 80 90
caaacatttcttatataaaattgacgcccgataagagaaaacctagaccaaagtatagtttagataactagacctaaggctagctattat 90
Yen Box I
Yen Box II
Conserved
tataccctatgttaattaaactgtttttgctttgcagaattagattttaatataatagaactagtatcttagcgcactgccgttaagaa 180
YenS
Conserved
agttagtctgaaaagaatatattttcgccaacaccagggcacttcagattcaccaagtacaagtttcaccctgccatctggatgttttta 270
YenS
Conserved
Or...)
cattcagatggccatcaaatcagatgtcataccctcctcaacgcatactctctttctgagaaagatcaccactgatgattttatcggcg 360
YenS
Conserved
Orf A (22 aa)
Orf B (22 aa)
Orf C (66 aa)
catcctgttgtttatttaaccacctttcgtctcaatggactcgcgttgtcgtgccaaagagactcatcgagcaaaaaaacctaaaaaata 450
Orf C (66 aa)
C...d
ttcttcaaacacatataaacattaaacatattaatgtaatgtgagtaaagttgacttaagtcatgtcagtaacctaaataaatcgtatga 540
Orf C (66 aa)
Conserved
taaataaacggttataatgtttccctggtgttggccgaattggcatcccttaccaaatgagattggtaagggttttttttgccctattatt 630
Conserved
tggaccacaacagggtttatctaagatgacggctgcatcgcgcggataatcaccgcacggtcaagatcgggattatcgctttcaagtaac 720
Cytochrome C Biogenesis factor

2.5 Discussion

This study was initiated by a search for promoters that are regulated by YenR, and ended with the unexpected finding that YenR activates expression of a non-translated RNA. Our work does not tell us whether YenR can directly regulate additional promoters. The ChIP experiments yielded other potential YenR binding fragments (Table 2. 5), although at least some of these fragments appear to lack promoters and could be false positives. A preliminary search for other YenR binding sites yield several candidates (data not shown). It is plausible that YenR may regulate *yenS* and also regulate additional genes indirectly, through its influence on YenS accumulation.

As this dissertation was being prepared, a report appeared that *Pantoea stewartii* expresses a gene divergent from its *esaR* gene (a *luxR* homologue) whose expression is stimulated by EsaR [22]. Expression of this gene, *esaS,* in *E. coli* required EsaR, though this requirement was not confirmed in *P. stewartii*. Expression of *esaS* in both organisms was inhibited by OHHL. Sequence inspection suggested that *esaS* was not translated, though the sequence of this region has not been reported. The *esaS* promoter was not identified, and the role of EsaR binding sites was not investigated. The *esaS* transcript was approximately 100 nucleotides. There is no apparent sequence conservation between *esaS* and *yenS*. However, it is tempting to speculate that these genes may have some functional similarity, and it will be interesting to learn more about their similarities and differences.

It is far from clear why YenR binds to two sites in the *yenR-yenS* intergenic region rather than one. The distance between *yen* box I and the *yenS*

transcription start site is similar to that of class I activators, while the position of *yen* box II is similar to that of class II promoters. Class I activators are generally thought to interact with the C-terminal domain of the alpha subunit of RNA polymerase, while Class II activators are thought to interact with the alpha-CTD, alpha-NTD, and/or sigma subunits. YenR was able to activate *yenS* from either site. Most characterized LuxR homologues bind DNA non-cooperatively as dimers (Urbanowski *et al.*, 2004; Weingart *et al.*, 2005; Zhu and Winans, 1999, 2001) [17, 18, 45, 47], and usually bind approximately 42 nucleotides upstream of their transcription start sites. One LuxR protein (LasR) can bind some promoters cooperatively, forming a probable dimer of dimers [46]. No member of the family other than YenR is known to bind two adjacent sites non-cooperatively.

As described above, YenR bound to target DNA only in the absence of its cognate autoinducers, OHHL and HHL. Only a few other LuxR homologs have similar properties, including EsaR, ExpR, and SmaR (Castang *et al.*, 2006; Fineran *et al.*, 2005; Minogue *et al.*, 2005) [20, 24, 26], all of which are close relatives of YenR (Fig. 1.4). Virtually all other members of this family require AHLs for activity and for DNA binding [12]. AHLs are also required for at least some members of this family to fold into mature, soluble forms [17, 18, 45-47]. AHLs are completely buried within TraR, LasR, and SdiA and contribute to the hydrophobic core of this protein [13-16]. The fact that YenR and EsaR function only as apoproteins indicates that they must not need AHLs for folding. Moreover, these proteins must have a binding site for AHLs that is accessible in the fully folded protein. It will therefore be extremely interesting to use biochemical and structural approaches to learn how OHHL perturbs the

properties of YenR or its close relatives.

We have used the algorithm TargetRNA [48] to search for such mRNAs. Remarkably, this program predicted that YenS might interact with the mRNA of YenI. We will pursue this interaction in Chapter 3.

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[3]CHAPTER THREE
BIOLOGICAL FUNCTIONS OF SMALL RNA YENS

[3] Chapter 3 is a manuscript prepared for submission, with the permission from the co-author, Ching-Sung Tsai and Stephen C. Winans. Biological functions of small RNA YenS.

3.1 Abstract

In this chapter, two functions of YenS are studied. First, a bioinformatic analysis revealed a sequence complementarity between YenS and YenI mRNA, suggesting that they could form RNA-RNA hybrids that might inhibit AHL production. Expression of *yenI-lacZ* translational fusions was decreased by YenS expression. S1 nuclease protection assays and Q-PCR assays confirmed that *yenI* mRNA levels were decreased by YenS. This inhibition required the part of *yenI* that is complementary to YenS. As might be predicted, a *yenS* mutant also accumulated more 3-oxohexanoylhomoserine lactone (OHHL) than a wild type strain. Other experiments suggested a possible role for YenS in swimming motility. I tested motility in semisolid agar of the wild type strain, a *yenI* mutant, a *yenR* mutant, a *yenS* mutant, a *yenI, yenR* double mutant, and a *yenI*, *yenS* double mutant. Of these six strains, the *yenI* mutant was vigorously motile, while the other five were nonmotile. The fact that the motility of a *yenI* mutation was suppressed by second mutation in *yenR* or *yenS* suggests a direct, positive role for YenS in the expression of proteins that are required for motility.

3.2 Introduction

In recent years, more and more bacterial small RNAs have been discovered that regulate the expression of diverse genes. Small RNA regulators are involved in the processes of iron uptake, sugar synthesis and breakdown, the maturation of biofilms and the induction or repression of virulence genes [1-4].

Small RNAs generally function by hybridizing to mRNAs, and altering the secondary structure of these mRNA molecules. This can impact either the continued synthesis of the mRNA, or its translatability, or both. In the simplest case, a small RNA can hybridize with the ribosome binding site and start codon, blocking the binding of ribosomes. Alternatively, a small RNA could hybridize to a different part of the mRNA, causing a previously occluded ribosome binding site to become exposed. Small RNA can also alter the stability of a rho-independent transcription termination site, thereby altering mRNA synthesis. Small RNAs can also alter the processing and stability of mRNAs. In many cases, small RNAs are less stable than a regulatory protein, and their accumulation therefore requires continuous synthesis. This can be a major advantage if changing circumstances necessitate an end to this sort of regulation.

Several quorum sensing systems involve the use of small noncoding RNAs [6-9]. In *Vibrio harveyi*, quorum sensing signals HAI-1, AI-2, CAI-1 were detected by two component receptors, LuxN, LuxP/Q and CqsS respectively. At low population densities, these signal sensors act as kinases and the signal is transferred through the phosphorelay protein LuxU to the NtrC-type transcription factor LuxO. LuxO~P activates several σ^{54} dependent

promoters, each of which lies upstream of 5 small RNA genes, designated qrr1-qrr-5 [7]. These RNAs hybridize to the 5' UTR of the mRNA of LuxR and inhibit its translation [7, 9, 10]. LuxR protein activates transcription of the bioluminescence operon and represses genes required for biofilm formation and for pathogenesis. At high population densities, these pheromones convert their receptors to phosphatases, which leads to dephosphorylation of LuxO, decreased abundance of Qrr1-5, increased accumulation of LuxR, and to increased bioluminescence. The quorum sensing system in *V. cholerae* is very similar to that of *V. Harveyi*, except that it lacks the HAI-1 system and has four *qrr* genes rather than five. The LuxR ortholog of *V. cholerae* is designated HapR.

In *V. cholerae*, a two component system composed of VarS and VarA, regulates the *csrBCD* operon, which encodes three small RNAs, CsrB, CsrC, and CsrD. These RNAs attenuate a global RNA binding regulator CsrA, originally discovered in *E. coli* as a regulator of carbon storage. CsrA increases the activity of LuxO, although the mechanism is unknown [11]. In the opportunistic pathogen *Pseudomonas aeruginosa*, the GacS-GacA two-component system (orthologous to VarS-VarA) uses the CsrABCD system to inhibit the accumulation of RhlI [12].

In Chapter 2, I showed that apo-YenR activates transcription of *yenS,* which encodes two overlapping small RNAs. In this chapter, I describe two possible roles of YenS in the physiology of the organism. First, a sequence complementarity between YenS and YenI suggested that they could hybridize, causing inhibition of AHL production. Second, several studies have indicated

that quorum sensing may play a role in the motility of *Yersinia* spp. By testing the motility of strains with mutations in *yenR, yenI,* and *yenS*, I provide evidence that YenS may play a direct role in the expression of one or more proteins required for motility.

3.3 Material and Methods

Strains,and Plasmids used in this study

Bacterial strains, chromosomal mutations, plasmids and oligonucleotides used in this study are described in Tables 3.1, 3.2, 3.3 and 3.4

RNA extraction, northern blotting, primer extension, S1 nuclease assay, quantitative PCR, and RACE assays.

Cells were cultured in AB or LB medium and collected at an O.D. of 0.3. A solution containing ethanol and phenol was added to kill the bacteria and preserve RNAs. Cells were centrifuged and frozen at -80 ^{O}C. RNA was extracted using either Trizol reagent (Invitrogen) or a Qiagen RNeasy kit. The total RNA was quantified by absorbance at 260 nm and RNA integrity was checked by agarose gel electrophoresis.

S1 nuclease assays were carried out as described previously [13], primers used are listed in Table 3.4. Quantitative reverse transcriptase PCR analysis was carried out using cDNA obtained with iScript cDNA synthesis kit (Biorad). Oligonucleotide primers and iTaq SYBR Green Supermix with ROX (Biorad) were added to cDNA samples and the mixture was amplified and analyzed by an ABI 7300 Real-time PCR system.

Bioassays for extracellular AHLs.

Strains producing AHLs were diluted 5,000 fold into fresh defined AB growth medium supplemented with 2.5% LB. At intervals, 1 ml samples were removed and stored at -80^{O} C. At the end of the growth interval, all samples were thawed, centrifuged to remove bacterial cells, and 10 µl of each supernatant was added to early-log phase cultures of the *A. tumefaciens* AHL bioassay strain WCF47(pCF218)(pCF372) [14]. Cultures of this strain were assayed for β-galactosidase specific activity after overnight culturing.

Motility on semisolid medium

Motility of wild type and mutant strains of *Y. enterocolitica* was tested using growth medium containin 1% tryptone, 10 mM glucose and 0.3% agar. A volume of 2 µl of log phase cultures was spotted at the center of each plate without penetrating the agar surface. The plates were incubated at 27^{O}C for 12 hours and photographed.

Table 3.1. Strains used in this study

Strain	Genotype	Source
JB580	*Y. enterocolitica 8081v,* restriction defective.	Lab Collection
DH5α	*E. coli* K12 F $^-$ *endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG lacZΔM15 hsdR17*	Stratagene
SM10/λ pir	*tra* regulon of RK2, R6K *pir,* host for *pir*-dependent plasmids	Lab Collection
BL21/DE3	*E. coli* B F $^-$, *ompT*, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)	Promega
S17-1/ λpir	*tra* regulon of RK2, R6K *pir*, host for *pir*-dependent plasmids	Lab Collection
CST201	JB580 *yenRI101*	This study
CST206	JB580 *yenI101*	This study
HGB001	JB580 *yenS101*	This study
HGB002	CST206 *yenS101*	This study
CST213	JB580, *lacZ101*	This study
CST214	JB580 *yenRI101*, *lacZ101*	This study
CST215	JB580 *yenR101*, *lacZ101*	This study
CST216	JB580 *yenI101*, *lacZ101*	This study
CST217	JB580 *yenRS101, lacZ101*	This study
CST224	CST217 *yenI'-lacZ101*	This study

Table 3.1. (Continued)

Strain	Genotype	Source
CST225	CST217 *yenI'-lacZ102*	This study
CST226	CST213 *yenI'-lacZ101*	This study
CST232	HGB001 *yenI'-lacZ101*	This study
CST234	CST215 *yenI'-lacZ101*	This study

Table 3.2. Chromosomal mutations constructed in this study

Allele	Description	Oligonucleotides for PCR amplification of upstream DNA fragment	Oligonucleotides for PCR amplification of downstream DNA fragment
yenI101	deletion of codons 1-1 87 of *yenI*	o3001 and o3002	o3003 and o3004
yenR101	deletion of *yenR* codons 1-221	o3005 and 3006	o3007 and o3008
yenRI101	deletion removing all of *yenR-yenI* coding sequences	o3005 and o3006	o3001 and o3002
yenRS101	deletion of *yenR* and the intergenic region between *yenR* and *yfrE*	o3007 and o3008	o3009 and o3010
yenS101	deletion of *yenS*	o145 and o005	o146 and o147
yenI102	Cm^R cassette in the ScaI site of *yenI*	o3011 and o3012	N. A.
yenI'-lacZ101	chromosomal *yenI-lacZ* fusion with an intact *yenI in cis.*	o143 and o133	N. A.
yenI'-lacZ102	chromosomal *yenI-lacZ* fusion with a truncated *yenI in cis.*	o132 and o133	N. A.
lacZ 101	[15]	N. A.	N. A.

Table 3.3. Plasmids used in this study

Plasmid	Description	Source
pCST2099	A pPZP100 derivative containing the fragment amplified with primers o029 and o068	This study
pCST2123	A pVIK112 derivative containing the PCR fragment obtained with primers o302 and o303 to insert a lacZ reporter gene in the upstream of the YenS targeting site on YenI mRNA, transcriptional fusion	This study
pCST2124	A pVIK112 derivative containing the PCR fragment obtained with primers o302 and o304 to insert a lacZ reporter gene in the upstream of the YenS targeting site on YenI mRNA, transcriptional fusion	This study
pCST2132	A pVIK107 derivative containing the PCR fragment obtained with primers o302 and o307 to insert a lacZ reporter gene in the middle of the YenS targeting site, translational fusion in frame with *yenI*	This study
pCST2127	A pVIK112 derivative containing the PCR fragment obtained with primers o302 and o307 to insert a lacZ reporter gene in the middle of the YenS targeting site, transcriptional fusion in frame with *yenI*	This study
pCST2142	A pVIK112 derivative containing the PCR fragment obtained with primers o302 and o328 to insert a lacZ reporter gene in the middle of the YenS targeting site on YenI mRNA, transcriptional fusion	This study
pCST2143	A pVIK112 derivative containing the PCR fragment obtained with primers o302 and o329 to insert a lacZ reporter gene in the downstream of the YenS targeting site on YenI mRNA, transcriptional fusion	
pCST2144	A pVIK107 derivative containing the PCR fragment obtained with primers o302 and o328 to insert a lacZ reporter gene in the middle of the YenS targeting site, translational fusion in frame with *yenI*	This study
pCST2145	A pVIK107 derivative containing the PCR fragment obtained with primers o302 and o329 to insert a lacZ reporter gene in the downstream of the YenS targeting site, translational fusion in frame with *yenI*.	This study

Table 3.4. Oligonucleotides used in this study

Name	DNA sequences (restriction sites are in upper case)
o005	ccGGTACCcatagggtataataata
o029	ccGGTACCataaataaaatactgagagt
o068	ggTCTAGAcgtttttatttcgtctgtct
o132	ccGAATTCattagcaagagatatggtg
o133	gcGGTACCtggttcaacctgatgtac
o143	ggGAATTCggtccacgtcagcatcctt
o145	ccTCTAGAatattctcgggctaactt
o146	ccGGTACCtctctttctgagaaagat
o147	ggGGATCCgctggcaaaagatccttt
o097	ccGGTACCaacagtttaattaacata
o302	ggGAATTCagcgcatagccatttttta
o303	ggGGTACCgatctctatctatgagtg
o304	ggGGTACCttaaaacaacatcgtaca
o307	ggGGTACCtaacataataaaaaccaaa
o323	ggGAATTCacctagaccaaagtatagtt
o328	ggGGTACCttttaacataataaaaacca
o329	ggGGTACCattaaaatttacgttaaaga
o3003	ccGGTACCataaataaaatactgagagt
o3004	ccTCTAGAtaatattgttaatggctata
o3005	gtTCTAGAcacgacaacgcgagtccat
o3006	ggGGTACCaataggccaattctactct
o3007	ccGGTACCaggaaactgggtgttttaa
o3008	ggGGATCCtatgaaatacccaaccatg
o3009	ttGGTACCatcgcgcggataatcaccgc
o3010	ggTCTAGAcgtttttatttcgtctgtct
o3011	gcGGATCCgctcggaatatcatcagctgc
o3012	gcTCTAGActccaatggaagcgacgacagta
Q-PCR-yenI-lacZF	Acctctagaagaagcttgggatccgt
Q-PCR-yenI-lacZR	Tgctgcaaggcgattaagttgggt
Q-PCR-23S-F	Atggcattgcccggtagctaaattcgga
Q-PCR-23S-R	Tcaatgcatcgctgcacttacacaccca
S1-Nuclease-YenI	Gttatttttctaagtgagaaaatctcgtctaacttcctttggcc
S1-Nuclease-23S rRNA	Gacgcttttcgcagattagcacgcccttcatcgcctctgagacgg

3.4 Results

Identification of Possible YenS Targets using the Target RNA algorithm

Many small, nontranslated RNAs regulate gene expression by binding to complementary mRNAs, often blocking their translation and/or altering their stability to cellular RNases [16]. I used the algorithm TargetRNA [17] to try to predict possible mRNAs that might interact with YenS. Surprisingly, the *yenI* gene appeared at the top of a ranked list of possible targets (Table 3.5). In a similar search using the putative YenS ortholog of *Y. pestis*, the gene corresponding to *yenI* received also received the highest score (Table 3.6). The predicted alignment between the YenS and YenI RNAs is shown in Fig. 3. 1A.

Inhibition of YenI expression by YenS

To determine whether YenS alters the expression of YenI, I constructed a *yenI-lacZ* translational fusion in strains containing or lacking *yenS*. Expression of the fusion was lower in a strain expressing YenS than in one lacking YenS (Table 3.7). Inhibition of the fusion by YenS required YenR, as a *yenR* mutant expressed the fusion as strongly as a *yenS* mutant. This was expected, as *yenS* expression requires YenR. S1 nuclease protection assays and Q-PCR assays also showed that *yenI* mRNA is less abundant in strains containing YenR and YenS than in any single or double mutants (Fig 3.1B, Table 3.7, right column).

Table 3.5. mRNAs of *Y. enterocolitica* that are predicted to hybridize with YenS

```
TargetRNA PROGRAM PARAMETERS

Species:                Yersinia_enterocolitica
Remove terminator:              no
Before Start Codon:             -10
After Start Codon:              30
Hybridization Seed:             11
G:U Pairs in Seed:              yes
Single Target:          -
P-value:                0.01
Thermodynamic:                  no
Orthologs:              -

Score: -71 yenI   (YE1600)      N-acylhomoserine lactone synthase

14      AGA-UUUUAAUAUAAUAGAA32
        ||| |||||:|||||||:||
25      UCUCAAAAUUGUAUUAUUUU6

Score: -68 YE2751       Putative periplasmic binding protein

143     AUGUUUUUACAUUCAG     158
        |:||||||||||:||||
25      UGCAAAAAUGUGAGUC     10

Score: -68 yrbC   (YE3749)Hypothetical protein

140 UGGAUGUUUUUACAUUCAGAUGGCC  164
    |::|:||||  ||||||||||: ||
15  AUUUGCAAAUUUGUAAGUCUAUGGG  -10

Score: -64 YE1243       Putative eamino acid permease

128     CACCCUGC-CAUCUGGAUGUUUU     149
        ||| |||| || |||:||:||||
30      GUGAGACGCGUUGACUUAUAAAA     8

Score: -63 YE1177       Putative conjugal transfer protein

75      AGAAUAUAUUUUCGC      89
        ||||:|||||||||:|
21      UCUUGUAUAAAAGUG      7
```

Table 3.6. mRNAs of *Y. pestis* that are predicted to hybridize with the *Y. pestis* ortholog of YenS

argetRNA PROGRAM PARAMETERS

Species:	*Yersinia_pestis*_KIM
Remove terminator:	no
Before Start Codon:	-10
After Start Codon:	30
Hybridization Seed:	11
G:U Pairs in Seed:	yes
Single Target:	-
P-value:	0.01
Thermodynamic:	no
Orthologs:	-

Score: -76 ypeI (y1733) Homoserine lactone synthase

```
26    UAAGUGCUU--AAUAUAAUAGAA-CUAAUAUUU 55
      |||:|:|||  ||:|||||||:|| |:|| ||||
28    AUUUAUGAAAAUUGUAUUAUUUUUGGUU-UAAA -4
```

Score: -72 y1347 Hypothetical protein

```
15    GCUUUAAAGAAUAAGUG-CUUAAUAUA       40
      ||| ||||:||||||:: ||:||| ||
18    CGAUAUUUUUUAUUCGUUGAGUUACAU       -9
```

Score: -70 y2228 Hypothetical protein

```
60    UUAUUGUUGCUGAAAAAGUUAGUCUGCUGUGA-AUAUA  96
      || |||||||::|||| |  ||   | ||||| |||||
30    AAAAACAACGGUUUUUCC--UC---CAACACUGUAUAU  -3
```

Score: -68 rfaH (y0460) Transcriptional activator

```
35    AAUAUAAUAGA-ACUAAUAUUUUA   57
      ||||:||||:| ||:|| ||||:|
25    UUAUGUUAUUUAUGGUUCUAAAGU   2
```

Score: -67 y3246 Hypothetical protein

```
84    UGCUGUGAAUAU---AUUUU 100
      ||||||||||||   |||||
24    ACGACACUUAUAAAAUAAAA  5
```

Score: -65 usg (y1598) Hypothetical protein

```
39    UAAUAGAACUAAUAUUUUAGC      59
      ||||||| | ||||||::|||
30    AUUAUCUCG-UUAUAAGGUCG      11
```

Score: -65 atpC (y4134) ATP synthase subunit epsilon

```
166   ACAUCCAGAUGGU  178
      |||||||||||||
29    UGUAGGUCUACCA  17
```

Score: -64 livH (y0423) Branched-chain amino acid ABC transport system

```
67    UGCUGAAAAAGUUAGU-CUGCUGUGAAUAUAUUUUAGCC 104
      ||| ||||| :| |:  ||||| ||| :|||:::||:||
29    ACGUCUUUUAUAUUUUUGACGAGACU-GUAUGGGAUUGG -9
```

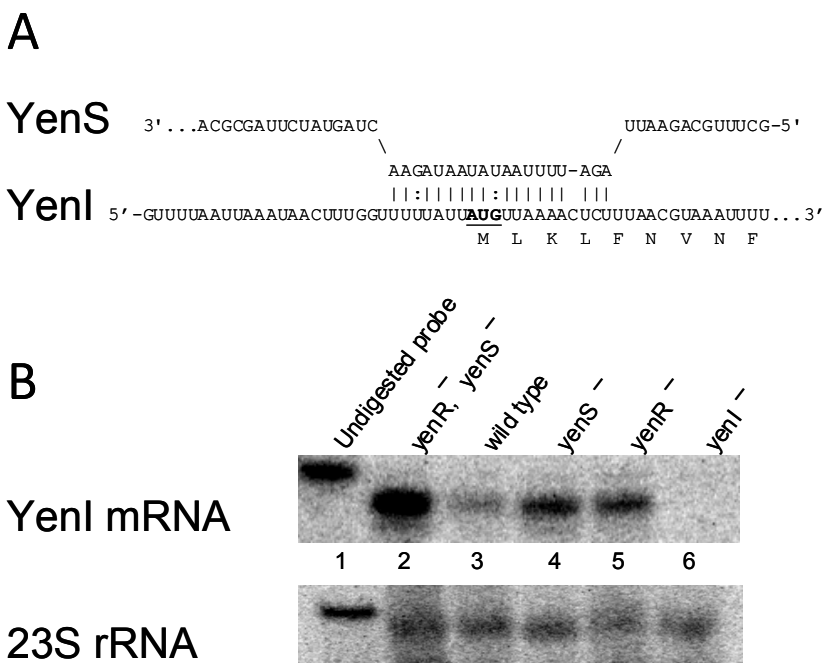


Figure. 3.1. YenS targets *yenI* mRNA.

(A) Proposed duplex formation between YenS RNA and YenI mRNA, as predicted by the TargetRNA algorithm. The YenI start codon is underlined. (B) Decrease in the abundance of YenI mRNA by YenS. In each panel, lane 1 is the radioactively radiolabeled DNA probe without nuclease digestion. The remaining lanes show the abundance of oligonucleotides that were resistant to nuclease S1 due to hybridization with YenI mRNA (upper panel) or 23S rRNA (lower panel); (2) CST224 (*yenR yenS* double mutant); (3) CST226 (wild type); (4) CST232 (*yenS* mutant); (5) CST234 (*yenR* mutant); (6) CST206, (*yenI* deletion mutant).

Table 3.7. Negative regulation of the expression of a *yenI-lacZ* translational fusion[a] by YenS.

Strain	Genotype	β-gal Activity[b]	Relative Expression	YenI-lacZ mRNA[c]
CST226	wild type	52 (6)	(1)	(1)
CST224	*yenR*⁻*yenS*⁻	573 (77)	11.0	7.7 (1.7)
CST232	*yenS*⁻	545 (17)	10.5	6 (2.3)
CST234	*yenR*⁻	418 (101)	8.0	6.8 (2.1)
CST224 (pPZP100)	*yenR*⁻*yenS*⁻ (vector)	731 (41)	14.1	nd[d]
CST224 (pCST2099)	*yenR*⁻*yenS*⁻ (*yenR yenS*)	131 (36)	2.5	nd

a: all strains contained pCST2058, which encodes a *yenI-lacZ* translational fusion.

b: Miller units [18].

c: Relative YenI abundance was calculated from the $2^{-\Delta\Delta CT}$ method by using 23S rRNA as a reference gene and CST226 as a calibrator [19]. The PCR primers used are listed in Table S5.

d: n.d. - not determined

The finding that YenS inhibits the expression of YenI predicts that YenS should also inhibit the production of extracellular AHLs. To test this, stationary phase cultures of a wild type strain and a *yenS* mutant were diluted 5,000 fold into fresh medium and cultured to midlog phase. At intervals, samples were removed and bioassayed for AHLs. As predicted, the wild type strain produced only low levels of AHL until it had reached an OD(600) of approximately 0.1, while the *yenS* mutant produced readily detectable levels of AHLs at far lower optical densities (Fig. 3.2).

The hypothesis that YenS binds YenI mRNA near the latter's translation start site predicts that inhibition of a *yenI-lacZ* fusion by YenS would require this site. I constructed a series of transcriptional *yenI-lacZ* fusions that contain or lack this region. Fusions lacking the putative YenS binding site (Table 3. 8, plasmids pCST2123-2124) were not affected by YenS, while expression of fusions containing all or part of the YenS binding site (plasmids pCST2127, pCST2142, and pCST2143) was decreased by YenS. I conclude that inhibition requires part of the region of YenI predicted to bind YenS, although evidently the entire binding site is not essential.

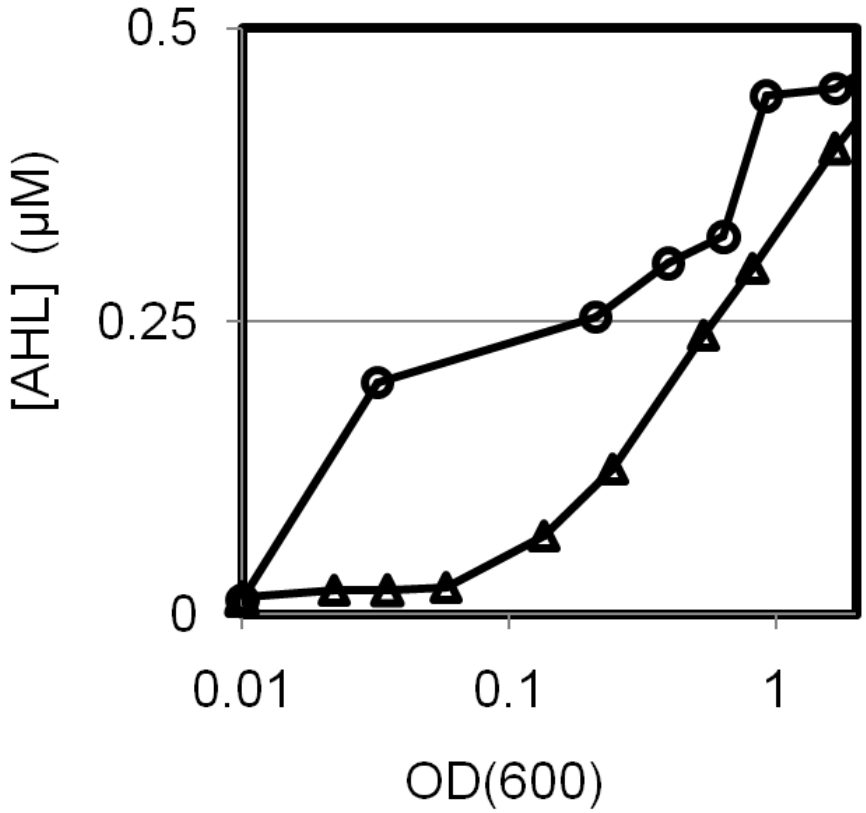


Figure. 3.2. Inhibition of AHL synthesis by YenS. A wild type strain (triangles) and a *yenS* deletion mutant (circles) were diluted 5,000 fold into fresh growth medium and cultured to midlog phase. At intervals, samples were removed and bioassayed for extracelluar AHLs. A standard dose-response curve was constructed using synthetic OHHL.

Table 3.8. Activity of *yenI-lacZ* fusions containing or lacking the putative YenS targeting site in strains containing or lacking YenS.

	Sequences in each fusion[a]	CST213 (*yenR+yenS+*)	CST230 (*yenR+ yenS-*)	CST217 (*yenR-yenS-*)
Transcriptional fusions		β-galactosidase activity[b]		
pCST2123	-412 to -41	27	20	21
pCST2124	-412 to -22	203	220	213
pCST2127	-412 to +6	58	219	176
pCST2142	-412 to +9	48	95	104
pCST2143	-412 to +30	55	91	119
Translational fusions		β-Galactosidase activity		
pCST2132	-412 to +6	60	321	321
pCST2144	-412 to +9	3	21	25
pCST2145	-412 to +30	3	16	17

a: sequence numbering is relative to the first base of the *yenI* translation start codon.

b: Miller units [18]. The data are representative of three experiments.

Mutations in yenR *or* yenS *suppress hypermotility caused by a* yenI *mutation.* A report from another lab showed that a mutation in *yenI* affected swimming and swarming motility of *Y. enterocolitica* 90/54 [20]. I tested mutants in *yenI, yenR* and *yenS* and various double mutants for similar defects, using semisolid complex medium containing 0.3% agar. Under the conditions I used, the wild type strain showed virtually no detectable motility (Fig. 3. 3), which is unlike strain 90/54 [20]. A mutation in *yenR* also was not detectably motile under these conditions. Surprisingly, a mutation in *yenI* was strongly hypermotile. This phenotype was highly reproducible and was opposite that reported previously, presumably due to differences in strains or experimental design. I also tested a *yenI, yenR* double mutant and a *yenI, yenS* double mutant. Neither strain was motile, indicating that the motility of the *yenI* mutation required YenR and YenS. Given that the lack of OHHL is predicted to activate YenR, which is predicted to activate *yenS*, I propose that YenS RNA may act upon one or more target mRNAs to stimulate motility.

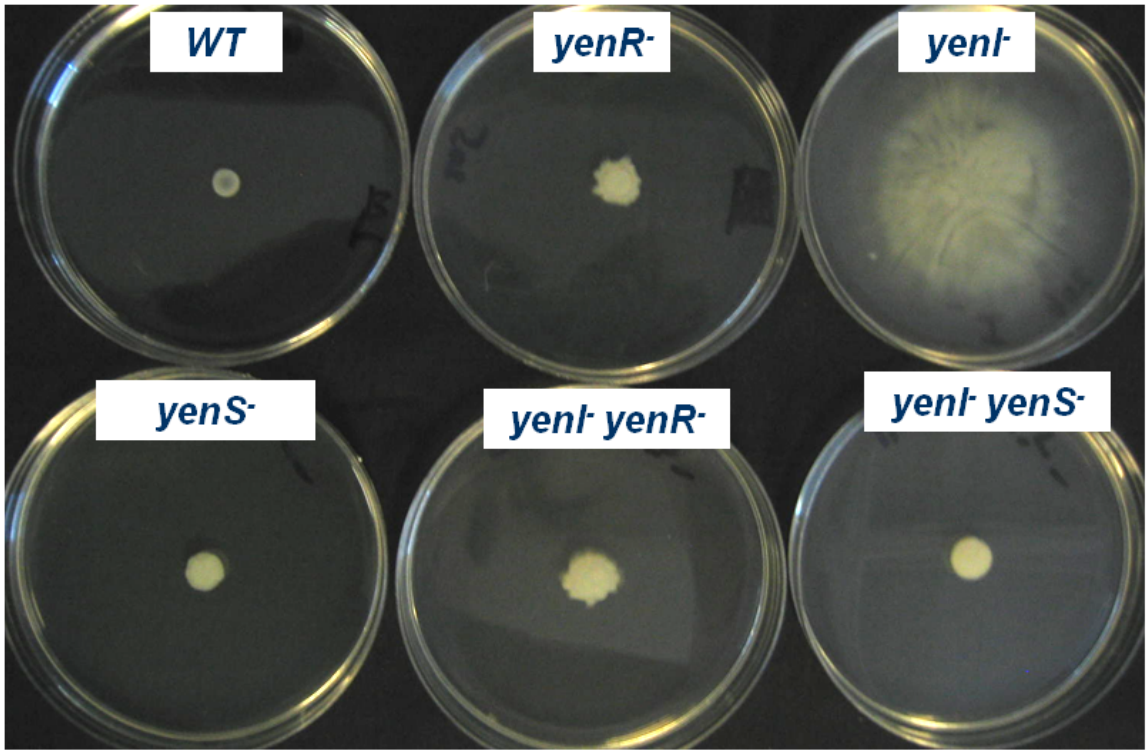


Figure. 3. 3. Motility of wild type and mutant strains of *Y. enterocolitica*. The strains used were JB580 (WT), CST206 (*yenR*), CST206 (*yenI*), HGB001 (*yenS*), CST201 (*yenI, yenR*), and HGB002 (*yenI, yenS*).

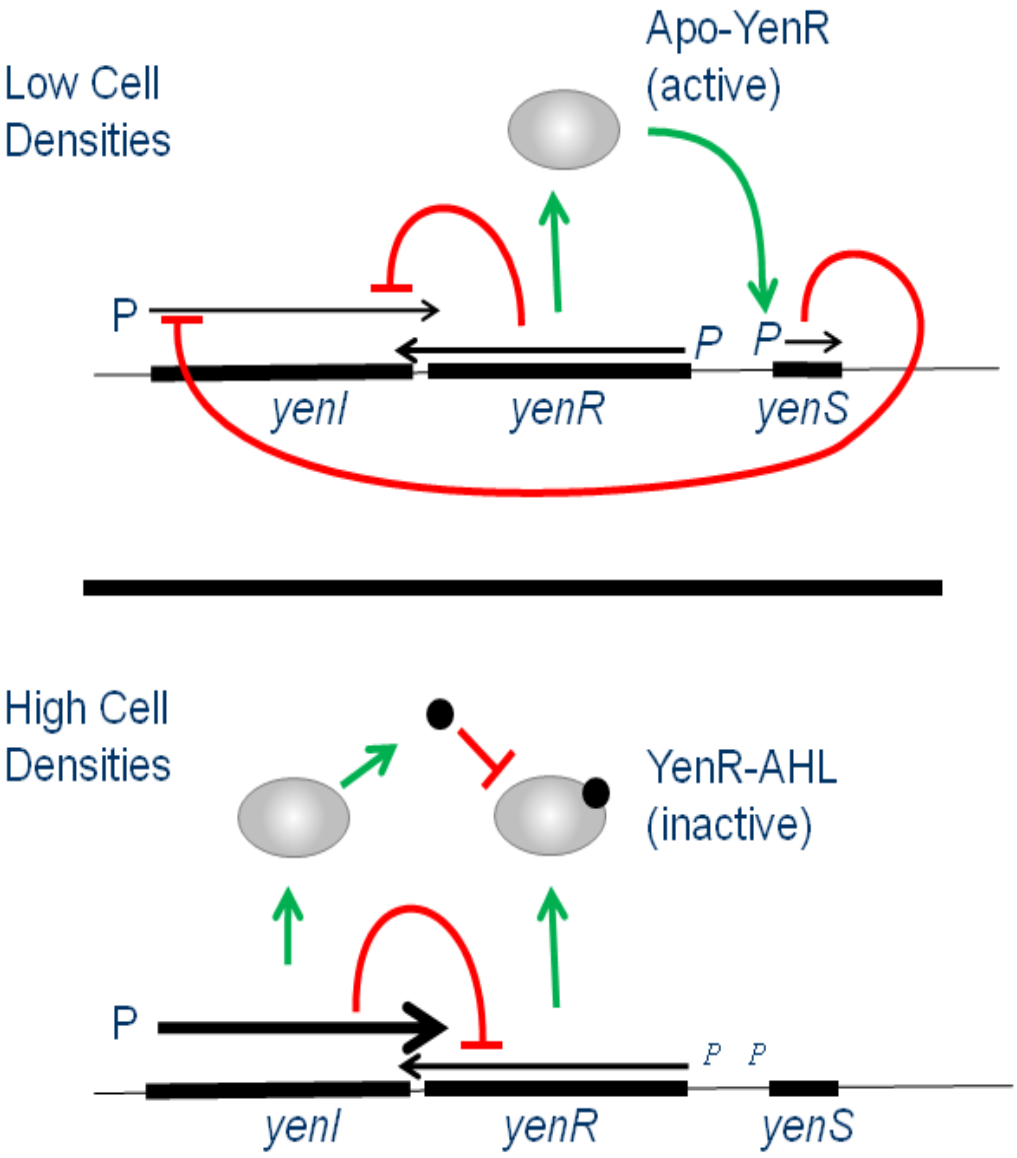
3.5. Discussion

This study was undertaken to try to discover a role for the YenS RNA in some aspect of the physiology of the organism. I used the algorithm TargetRNA to look for mRNA molecules that were complementary to YenS and was surprised to find that the top scoring mRNA was that of *yenI*. I went on to find that YenS downregulates the synthesis and translation of the YenI mRNA. I also studied the role of the *yenI, yenR, and yenS* genes in motility, and obtained evidence that YenS may directly regulate one or more functions required for motility.

My finding that YenI is regulated by YenS, which is itself regulated by YenR, whose activity is controlled by OOHL, which is made by YenI, may create an interesting positive autoregulatory loop. Many LuxR-LuxI systems exhibit hysteresis, meaning that their responses to quorum-sensing stimuli are conditioned by their recent history [21]. Simply put, these systems, when in the inactive state tend to stay inactive despite environmental perturbations, and vice versa. This is a result of positive regulation of the AHL synthase gene by the AHL receptor protein. The YenR-YenI system could also exhibit hysteresis, but by a completely different mechanism. There are at least two factors that may contribute to hysteresis, the first of which involves YenS. At low cell densities, apo-YenR accumulates and activates the *yenS* promoter (Fig. 3. 4). YenS inhibits production of AHLs, and this inhibition ensures continued accumulation of active apo-YenR. At high cell densities, inactive YenR-AHL complexes accumulate, which cannot produce YenS. This stimulates AHL production, which ensures that YenR remains inactive. In essence, apo-YenR makes an inhibitor of an inhibitor of itself, while YenR-AHL complexes cannot do so (Fig. 3. 4).

The second way that this system may exhibit hysteresis is more speculative, but highly plausible. The *yenR* and *yenI* genes are convergent and overlap by one codon, indicating that their mRNAs must overlap by at least 10-20 nucleotides and possibly much more. All the members of the EsaR/I subfamily are also encoded by convergent, overlapping genes [22]. The YenR and YenI mRNAs may therefore hybridize, which could destabilize either or both (Fig. 3. 4). If so, then at low cell densities, apo-YenR would, through YenS, decrease levels of YenI mRNA. This potential excess of YenR mRNA could stoichiometrically degrade both species of mRNA, until only YenR mRNA remained. The lack of YenI mRNA would ensure continued accumulation of apo-YenR. At high cell densities, YenR-AHL complexes would fail to activate the *yenS* promoter, causing an increase in YenI mRNA. The potential excess of YenI mRNA could cause stoichiometric degradation of YenR mRNA, further decreasing the production of YenR.

Figure. 3.4. A model describing the regulatory circuitry of YenI, YenR, and YenS. According to this model, at low population densities, OHL is expected to accumulate very poorly, causing apo-YenR to accumulate and activate transcription of *yenS*. The YenS RNA is proposed to bind to YenI mRNA, blocking its transcription and translation. The inhibition of YenI production will inhibit the production of OHHL. This ensures that YenR will remain in the unliganded, active form. At high population densities, sufficient OHHL accumulates to form complexes with YenR, thereby inactivating it. This leads to decreased expression of YenS, and that decrease causes an increase in YenI production, and an increase in OHHL accumulation, which in turn helps to ensure that YenR will remain inactive. Additionally, the YenI and YenR mRNAs may antagonize each other's transcription or accumulation, either via RNA polymerase collision or by mRNA-mRNA duplexes. In either case, the more abundant mRNA could ensure the destruction of the less abundant mRNA. These regulatory features could ensure hysteresis, that is, the tendency for the system to remain stable in one state or the other state even in the face of sub-threshold changes in environmental conditions.


Low Cell Densities
Apo-YenR (active)
P
P
P
yenI
yenR
yenS
High Cell Densities
YenR-AHL (inactive)
P
P
P
yenI
yenR
yenS

In an effort to identify physiological properties that are regulated by YenR or YenS, I undertook assays of motility using semisolid nutrient agar. The wild type strain was not motile on this medium, while the *yenI* mutant was strongly motile, covering virtually the entire petri dish within 12 hours (Fig. 3. 3). This phenotype was fully suppressed by mutations in YenR or YenS.

My model for quorum sensing shows a regulatory cycle, in which OHHL impacts activity of YenR, which impacts production of YenS, which impacts the translation of YenI, which impacts the abundance of OHHL. A mutation in *yenI* breaks this cycle, and allows me to ask which of these components is most likely to act upon motility directly. In principle, the lack of OHHL caused by a *yenI* mutation could alter the activity of an AHL receptor other than YenR. *Y. enterocolitica* encodes a second LuxR-type protein, YE1026 [23], and phylogenetic analysis suggests that it could well be a receptor for OHHL. However, a *yenI, yenR* double mutant was defective in motility, arguing against a role for a second AHL receptor. YenR is known to directly regulate just one gene, *yenS*, but could have additional direct targets, including one or more that are involved in motility. However, a *yenI, yenS* mutant was defective in motility. Although it remains conceivable that YenR could directly regulate motility genes, these data argue that YenS has a direct role in regulating motility, possibly via posttranscriptional effects on protein synthesis.

Although the *yenI* mutant constructed in this study was hypermotile, a *yenI* constructed in a different lab had a very different phenotype [20]. This could be due to differences in strain background, mutant construction, or experimental design. The parent strain used in my studies, JB580, was

derived from strain 8081v, an O:8 serotype, while the previous study was done using strain 90/54, an O:9 serotype. My *yenI* mutation was made by creating an unmarked deletion. In contrast, the *yenI* mutant of the other study was made using a Kn^R cassette. This cassette could cause the synthesis of a *yenR* antisense RNA. If so, then the *yenI* mutation would block the expression of both *yenI* and *yenR*. The reported lack of motility in that study could be due to a block in *yenR* expression. In my study, a *yenI*, *yenR* double mutant was nonmotile. The authors of the earlier study claimed to be unable to construct a *yenR* null mutation [20], while I had no trouble in doing so, possibly due to a difference in strain backgrounds. Both studies used 0.3% agar with 1% tryptone, but I also added 10 mM glucose, while the medium in previous study contained no glucose but contained 0.5% NaCl.

I have so far identified just one direct target of YenR and just one target for YenS. However, I strongly suspect that additional genes are controlled by this system. Any such genes could be regulated directly by YenR or by YenS, and could themselves regulate still other genes. YenR could act either as a repressor or as an activator, depending largely on the position of the binding site with respect to the target promoter [24]. I have carried out a preliminary bioinformatic search for YenR-regulated promoters (data not shown), and will study several such promoters in future studies. YenS could also regulate other genes, probably by inhibiting the accumulation or translation of an mRNA, though it could have the opposite effect, for example, by inactivating an inhibitory site of an mRNA. In principle, YenS targets should be identifiable by their complementarity. TargetRNA has identified several other candidate genes (Tables. 3.5, 3.6). Transcriptional profiling or proteome analysis could

provide other approaches for finding members of this regulon.

As this dissertation was being prepared, a report appeared that *Pantoea stewartii* expresses a non-translated gene divergent from its *esaR* gene whose expression was stimulated by EsaR [25]. Expression of this gene, *esaS,* in a heterologous host (*E. coli*) was activated by apo-EsaR and inhibited by OHHL. There is no apparent sequence conservation between *yenS* and *esaS* and there is no obvious complementarity between EsaS and EsaI. However, it is tempting to speculate that these genes may have some functional similarity, and it will be interesting to learn more about their similarities and differences.

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[4]CHAPTER FOUR

SIX MUTANT OCCR PROTEINS THAT ARE DEFECTIVE IN POSITIVE CONTROL HOLD OPERATOR DNA AT A FULLY OR PARTIALLY LOCKED HIGH ANGLE BEND

[4] Chapter 4 is a manuscript prepared for submission, with the permission from the co-author, Ching-Sung Tsai and Stephen C. Winans. Six mutant OccR Proteins that are defective in positive control hold operator DNA at a fully or partially locked high angle bend.

4.1. Abstract

OccR is a LysR-type transcriptional regulator of *Agrobacterium tumefaciens* that positively regulates the octopine catabolism operon of the Ti plasmid and is also an autorepressor. Positive control of the *occ* genes occurs in response to octopine, a nutrient released from crown gall tumors. OccR binds to a site upstream of the *occQ* promoter in the presence and absence of octopine. Octopine causes prebound OccR to undergo a conformational change at the DNA binding site that causes changes in footprint length and DNA bending. In order to determine the roles of these conformational changes in transcriptional activation, we isolated eight OccR mutants that are defective in activation of the *occQ* promoter but still able to repress the divergent *occR* promoter. These mutations spanned virtually the entire length of the protein. Six mutant proteins, in the presence or absence of octopine, displayed a high angle DNA bend that resembled the octopine-free, inactive conformation of wild type OccR.

4.2. Introduction

LysR type regulators (LTTR) comprise the largest DNA binding transcriptional regulators known in most proteobacteria [2]. Most are involved in the regulation of metabolic functions such as amino acid synthesis, or in catabolism, these proteins contain a highly conserved N-terminal DNA binding domain and a less conserved C-terminal ligand recognition domain.

The binding of most transcription factors to promoter DNA is controlled by environmental stimuli. LTTRs are highly unusual in that they are able to bind to their DNA recognition sites in the presence or absence of their ligands. With a few exceptions, LTTRs regulate transcription by undergoing conformational changes that have little affect on binding, but do alter their ability to recruit RNA polymerase (RNAP). Without the ligand, LysR proteins bind to a region of DNA that spans five helical turns, with one dimer centered at nucleotide -62 and the other centered at -32. In this conformation, LTTRs cause a high-angle DNA bend. Upon ligand incorporation, the dimer centered at -32 shifts to nucleotide -42, a position where many transcriptional regulators occupy to recruit RNA polymerase. This change of conformation also frees the promoter for RNA polymerase binding, and relaxes the DNA bend [3-8]. Genes encoding LTTRs are often transcribed divergently from the target promoter, and are negatively autoregulated. Both protein conformations are equally able to carry out autorepression. A small number of LTTRs have been shown to bind as a dimer in the absence of ligand, centered about approximately -62, and as tetramers in their presence, bound at -62 and -42 [9-11].

Agrobacterium tumefaciens is well known for its ability to perform horizontal gene transfer across biological kingdoms. After infection of higher plants, the pathogen injects a fragment of DNA called T-DNA into the plant cytoplasm and also injects a number of proteins that ensure its targeting to the plant cell nucleus and its integration into nuclear DNA. The transferred DNA directs transformed cells to produce a set of compounds called opines [12], that can serve as nutrients. Opines cause the induction of cognate uptake and catabolic proteins in the bacterium, and this required dedicated detection systems. The OccR protein is encoded by the tumor-inducing (Ti) plasmid, and is responsible for detecting one such opine, called octopine. OccR binds DNA in the presence or absence of octopine, but binding of octopine causes the protein to activate a nearby promoter of a 14-gene operon that encodes two ABC-type permeases, four opine catabolic genes, and *traR*, which encodes a well known quorum sensing regulator [13].

Previous studies in the Winans laboratory have shown that purified OccR binds to the *occQ* promoter in the presence or absence of octopine [1, 4, 14, 43]. The absence of octopine causes the protein-DNA complex to form a high angle bend, while the binding of octopine relaxes this bend [4]. The protection of the promoter region was also shortened by one helical turn by octopine, from a region spanning -80 to -28 to a shorter region spanning nucleotides -80 to -38. OccR binds at a tetramer in both cases [14]. Gel shift assays also showed that the upstream 20 nucleotides is crucial for binding affinity, while the downstream 30 nucleotides have minor effect on binding, but is essential for bending [1].

In this study, I mutagenized *occR* to test the idea that ligand responsive DNA bending is essential for wild type OccR function. I describe the genetic and biochemical properties of OccR point mutants that have lost the ability to activate transcription of the *occQ* promoter, but which still retain high-affinity binding to the *occ* operator. I then tested these mutants for the ability to cause a high angle DNA bend in the absence of octopine and to be able to relax this bend in the presence of octopine. On the basis of the effects of these mutations I discuss the role of the conformational change that causes ligand-responsive DNA bending in transcriptional activation.

4.3. Experimental Procedures

Mutagenesis of OccR

Strains and plasmids used in this study are listed in Table 4.1. To introduce point mutations into a cloned copy of the *occR* gene, plasmid pSS102 was introduced into the *E. coli* strain XL1-Red (Stratagene). Thirty transformants were isolated and each was cultured in LB medium for approximately 40 generations. At the end of this interval, plasmid DNA was isolated from each of the 30 cultures and introduced into *A. tumefaciens* strain KYC1211(pSS101) by electroporation. KYC1211 carries a Tn5gus insertion in the *occQ* gene that creates an *occQ-gus* fusion, while pSS101 carries an *occR-lacZ* fusion. Electroporated cells were plated on solid AT medium containing 100 µg/ml spectinomycin and 100 µg/ml gentamycin, 1 µM octopine, and 40 µM X-Glu. Strains that were defective for activation of the *occQ* gene were then streaked on the similar medium containing X-Gal in place of X-Glu. Since this strain contains an *occR-lacZ* fusion, clones containing OccR alleles unable to repress this promoter will form blue colonies on this medium. About 1000 colonies

were screened for the formation of white colonies, and eight independent clones were obtained.

Derivatives of pSS102 thought to contain positive control mutations were introduced into *E. coli* strain DH5α for automated DNA sequencing, and reintroduced into KYC1211(pSS101) to ensure that the observed phenotypes were attributable to the pSS102 plasmid. Quantitative assays of β-glucurondase and β-galactosidase expression in response to varying octopine concentrations were performed using these reconstructed strains.

Protein overexpression and crude extract preparation

Crude cell lysates were prepared with BL21/DE3(pSW213) containing mutant *occR* derivatives cloned into pRA304, a T7 promoter controlled OccR expression plasmid. The function of pSW213 is to overexpress the LacI repressor [15], which decreases the basal level expression of the the T7 RNA polymerase gene. Cells were grown in 300 ml LB at 37°C containing 1 mg/ml ampicillin and 10 μg/ml tetracycline to an OD_{600} of 0.6. After treated with 1 mM IPTG, cells are incubated for 6 more hours at 28°C. Cells were resuspended in 1 ml of TEDG buffer (50 mM Tris·HCl, pH 7.9, 0.5 mM EDTA, 1 mM DTT, 5% glycerol) plus 0.15 M NaCl, and disrupted by a French pressure cell (20,000 psi). Clarified extract was obtained after ultracentrifugation (150,000 × g for 10 min at 4°C).

Table 4. 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Reference
BL21/DE3	*E.coli* B P*lac*-gene 1 of bacteriophage T7	[16]
CST101	*A. tumefaciens* KYC1211 (pSS101)(pSS102)	This study
KYC1211	*A. tumefaciens* R10 (*OccQ*::*Tn5-gusA7*)(*occR*::pKY135) Km^R, Sm^R	[17]
pBBR1MCS5	Cloning vector	[18]
pSW213	IncP cloning vector, *lacZα, lacI*^{q},Tc^R	[15]
pBEND3	Derivative of pBluescript for assays of DNA bending, Ap^R	[19]
pRSETA	T7 promoter cloning vector, Ap^R	Invitrogen
pSS101	P*occR-lacZ* cloned into broad host range plasmid pRA301, Sp^R	This study
pSS102	*occR* cloned into pBBR1MCS5 Gm^R	This study
pRA304	*occR* cloned into pRSETA	[14]
pLW132	*occQ-occR* intergenic region cloned into pBEND3	[1]

* Only vectors, host strains, and plasmids containing wild type *occR* genes are shown. Construction of plasmid derivatives containing mutant *occR* genes is described in detail in the Materials and Methods.

DNA bending assays

For DNA bending assays with mutant proteins, pLW132 [1] was digested with *Bam*HI and end-labeled using γ-^{32}P-dATP(NEN) and the T4 polynucleotide kinase (NEB). Clarified extract and 0.12 pmol of labeled DNA were combined at room temperature (RT) in a buffer containing 10 mM Tris·HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 60 mM potassium glutamate, 30 μg/ml calf thymus DNA, 20 μg/ml BSA, and 10% glycerol, and incubated at RT for 30 min After that, samples were size-fractionated using 6% polyacrylamide gels in 1X TAE buffer (20 mM Tris-acetate, 1 mM EDTA, pH 8.5) at 4^{o}C. Octopine was added in both the gel and the running buffer at indicated concentrations. Gels were examined using a Storm B840 PhosphorImager (Molecular Dynamics).

β-Glucuronidase and β-Galactosidase assays

To quantify the activities of the mutant *occR* alleles on the *occQ* and *occR* promoters, derivatives of strain KYC1211(pSS101) containing the mutant *occR* derivatives of pSS102 were cultured overnight at 28°C with appropriate antibiotics. Cultures were diluted 100-fold into fresh AB minimal glucose medium without antibiotics and containing octopine at the indicated concentrations. β-glucuronidase and β-galactosidase specific activities [20] were measured when the cell density reached log phase.

4.4. Results

Isolation of positive control occR mutants

We have previously demonstrated that wild type OccR causes a high angle DNA bend in its operator in the absence of octopine and a lower angle DNA bend in the presence of octopine [4] . We have also described the isolation of

a large number of OccR constitutive mutants able to activate the *occQ* promoter in the absence of octopine [21] and show that these mutant proteins are predisposed to form a low angle DNA either in the absence of octopine or in the presence of very low octopine concentrations. Here we asked the converse question: Are there OccR mutants that are able to bind the operator but which fail to activate the *occQ* promoter locked in an inactive conformation even in the presence of octopine? To address this question, we used an *E. coli* mutator strain to introduce point mutations into a cloned copy of *occR*. Independently mutagenized pools of this plasmid were introduced into an *A. tumefaciens* strain that contains an *occQ-gus* fusion and an *occR-lacZ* fusion. To facilitate recovery of *occR* mutant genes, we inactivated the Ti plasmid copy of this gene. We screened for mutants that were unable to activate the former fusion but which retained the ability to repress the second fusion. Although in principle these two screens could have been done simultaneously, we found that in practice it was preferable to do them sequentially.

To make sure that the desired phenotypes were attributable to the plasmid carrying the *occR* gene, this plasmid was isolated from each of the candidates, introduced into an *E. coli* strain, and then reintroduced into KYC1211(pSS101) by electroporation. All the resulting strains showed the same positive control defect, indicating that the original constitutive phenotype was due to a point mutation in cloned *occR* gene. Eight clones having the PC phenotype and isolated from independent pools of mutagenized plasmids were retained for further study.

Mutations span the length of the protein

To determine the location and nature of the mutations, the plasmid containing the putative *occR* mutation was isolated and the *occR* gene was sequenced. Each of the eight mutant *occR* genes had a single transition or transversion mutation. In no case did two independent mutations have the same amino acid alteration, suggesting that many more positive control mutations remain to be isolated.

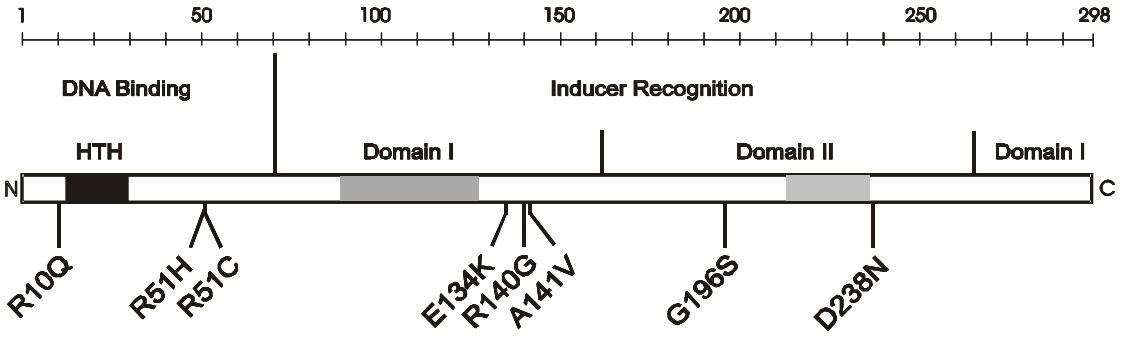

Figure. 4.1. The OccR Positive Control span the length of the protein. The DNA binding domain is colored in black box and labeled as HTH. Domain I and Domain II of the recognition domains are shown as grey boxes.

Significantly, these mutations did not appear to cluster in any particular part of OccR primary sequence, but rather spanned much of the protein (Fig. 4.1). We modeled these mutations on the crystal structure of the CbnR protein (Fig. 4.3). Three mutations are predicted to lie on the DNA binding domain. Two mutations affect the same residue, Arginine 51. This residue lies in a loop between two β-strands and is highly exposed to solvent. It would

therefore be extremely well positioned to interact with other proteins such as RNA polymerase. The other mutation in this domain, R10Q, lies in the amino terminal α-helix of the protein. This residue contacts the linker helix, and the mutation might therefore block the transmission of the conformational change between the domains.

The remaining five positive control mutations that were isolated lie within the regulatory domain, which is proposed to act as a pair of jaws that lie open in the absence of ligand to which close down upon the ligand. One mutation, G196S, lies deep at the back of the jaws in a position that is probably close to the ligand binding site. This mutation might therefore conceivably affect ligand recognition. Two other mutations, E134K and D238N, are localized on opposing surfaces of the front edge of the proposed jaws. They might therefore impede the stable shutting of the jaws, causing the mutant protein to favor an open conformation even in the presence of ligand.

The remaining two mutants are R140G and A141V. Because the two subunits of the CbnR dimer show a pronounced asymmetry, these residues lie in different environments on the two subunits. In one subunit, these residues contact the linker helix, and the mutations might therefore affect the transfer of information between the regulatory domain and the DNA binding domain. In the other subunit of the dimer, these residues line the surface of a large channel within the complex.

Quantitation of occQ-gus and occR-lacZ activities

Strains containing each *occR* mutant were tested quantitatively for expression

in the *occQ* and *occR* genes. All mutants were strongly defective in expression of the *occQ* promoter, though one mutant, A141V, showed a higher level of residual induction than did the others (Table 4.2) . Meanwhile, all mutants also repressed the *occR-lacZ* fusion, indicating that they must retain the ability to bind to operator DNA (Table 4.3).

DNA bending by OccR positive control mutants

OccR PC mutants were cloned into pRA304, under control of the strong T7 promoter, and crude extracts prepared from the resulting strains were used in DNA bending assays, as described previously [21]. For unknown reasons, mutants E134K and R51C did not accumulate in this strain and was therefore excluded from the *in vitro* analysis. The OccR binding site was placed into plasmid pBend3 [19], which is designed to measure DNA bending, and the mobilities of the resulting mutant OccR-DNA complexes were observed on 6% polyacrylamide gels. OccR-DNA complexes formed with high angle bend will move slowly in these gels, while OccR mutants complex with DNA in a low angle bend move faster since the complex is more linear. We hypothesized that PC OccR mutants would have a high-angle DNA bend even in the presence of octopine or might require higher octopine concentrations than wild-type OccR for relaxation of the DNA bend.

Table 4. 2. Activation of the *occQ* promoter by OccR positive control mutants. [a]

Amino Acid Mutation	Codon Mutation	*occQ-gus* Expression at Indicated Octopine Concentrations			
		0 μM	1 μM	10 μM	100 μM
w.t. OccR	None	2.6	218	249	250
no OccR		2.9	2.7	4.0	4.7
R10Q	CGG→ C**A**G	2.1	3.8	5.2	6.4
R51H	CGT→ C**A**T	3.0	9.9	9.8	10
R51C	CGT→ **T**GT	3.5	7.5	13.7	16.5
E134K	GAA→ **A**AA	3.2	3.6	5.6	5.7
R140G	AGG→ **G**GG	2.3	14.6	15	18
A141V	GCC→G**T**C	2.8	35.6	46.3	52.2
G196S	GGC→**A**GC	2.4	9.7	16.2	15.6
D238N	GAT→**A**AT	2.4	3.3	4.5	4.1

a β-glucuronidase activities were measured for *A. tumefaciens* strain CST101 containing derivatives of pSS102 containing the indicated *occR* mutations. Strains were cultured overnight in the absence or presence of the indicated concentrations of octopine and assayed for β-glucuronidase specific activity [22].

Table 4. 3. Repression of the *occR* promoter by OccR positive control mutants. [a]

Allele	*occR-lacZ* Expression Without Octopine	Fold Repression	*occR-lacZ* Expression With 1 μM Octopine	Fold Repression
W. T.	15	9	9	14
none	140	(1)	134	(1)
R10Q	16	9	20	7
R51H	17	8	17	8
R51C	18	8	21	6
E134K	15	9	14	10
R140G	12	11	13	10
A141V	15	9	15	9
G196S	15	9	13	10
D238N	14	10	16	8

a β-galactosidase activities were measured for *A. tumefaciens* strain CT101 containing derivatives of pSS102 containing the indicated *occR* mutations. Strains were cultured overnight in the absence or presence of the indicated concentrations of octopine and assayed for β-galactosidase specific activity [20].

As seen in Fig. 4.2, in the absence of octopine, wild type OccR migrates at the same rate as all the other mutants, forming high bend protein-DNA complexes. As the concentration of octopine is increased, wild type OccR relaxed the DNA bend and migrates more quickly (compare complex in lanes 1 to the molecular weight standards). The other mutants also migrate more quickly in the presence of octopine than in its absence. However, this difference is far more subtle than for wild type protein. We conclude that all of these mutants are defective to one degree or another in octopine-response conformation changes.

In all assays described above, we detected a range of gel mobilities under different conditions, and in all cases we detected only single bands. This could be interpreted to mean that complexes can take many different static conformations, each with a different bend angle. However, we strongly prefer the alternative hypothesis that OccR has only a small number of possible conformations (probably two), and that intermediate migration rates are due to a dynamic equilibrium between these conformations during electrophoresis.

We performed a control experiment to ensure that the mobility shifts that we had detected were due to changes in DNA bending rather than to differences in the number of bound protein subunits. Gel retardation assays were repeated with operator DNA containing the bend center near the end of the fragment rather than at the middle of the fragment. All the mutant OccR-DNA complexes migrated at the same rate in the presence or absence of octopine (data not shown). Therefore, the mutations altered the conformation of bound protein rather than the number of bound OccR monomers.

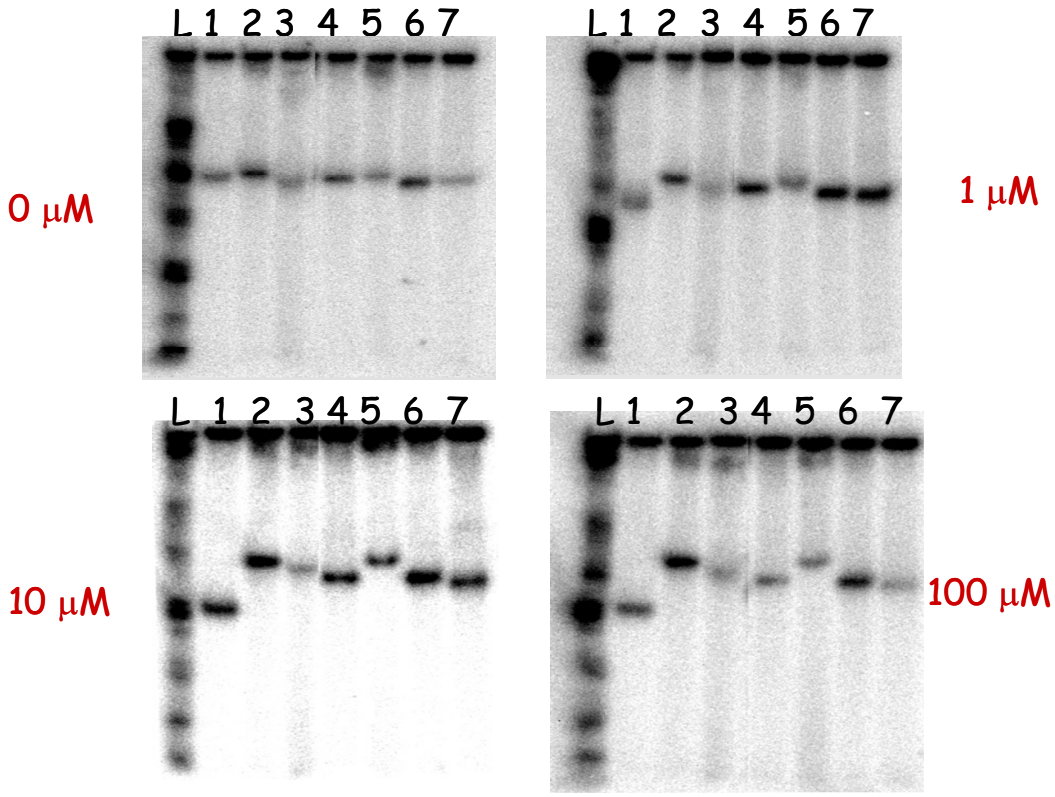

Figure. 4.2. Bending assay of positive control mutants with different concentrations of octopine. Lane 1: WT, Lane 2: R10Q, Lane 3: R51H, Lane 4: R140G, Lane 5: A141V, Lane 6: G196S, Lane 7: D238N.

4.5. Discussion

In this study, we describe the isolation and biochemical properties of positive control mutants of OccR that were fully able to bind operator DNA but which were unable to activate transcription. The autorepression of the divergent *occR* gene provided a convenient screen to DNA binding proficiency. We had predicted that two classes of mutants would be found. One class would be unable to undergo octopine-induced conformational changes. The other class would be fully able to undergo these changes, but would be unable to productively interact with RNA polymerase. These two classes could be distinguished by testing for octopine-responsive DNA bending.

Positive control mutants have been identified in several other LysR-type proteins. In NahR of *Pseudomonas putida*, mutants that are defective in activation but still retain DNA binding ability were found to be distributed in the full length of the protein [23]. The P35S allele was the only one mutant found in the DNA binding domain, although it has only 30% of the DNA binding ability of the wild type. All the other mutants, on the other hand, are in the ligand recognition domain. Bending assays was not performed, and hence it is difficult to further explore their functions. CysB of *E. coli* was more thoroughly studied. In an initial random mutation trial, a possible RNA polymerase contacting residue Y27 was identified by bending assay [24]. Also, four "inducer response" mutations in the region of residue 160 to 247 were also located (M160I, T196I, A244V and A247E). A subsequent study focused on the DNA binding region of CysB. Mutations of residues Y27, T28, S29 and Q30 decreased activation but did not alter a ligand-responsive DNA bend [25].

A set of alanine scanning mutants of the "265 determinant" of the alpha subunit of RNA polymerase was examined for defects in interactions with CysB [25].

We have previously described OccR point mutants that activate the *occQ* promoter constitutively [21]. As might be predicted, the large majority of these proteins were affected in octopine-responsive DNA bending, and maintained their active conformation (judged by their short DNase I footprint and low angle DNA bend) even in the absence of octopine. In some cases octopine was required for this conformation but less of it was required than for wild type. A few mutants appeared wild type in conformation, and their consititutive properties must have other causes. In a separate study, we created a series of mutant OccR binding sites that were designed to lock OccR into one conformation or other other conformation. Mutant operators were identified that locked OccR into a conformation resembling the inactive form (long footprint and high DNA bend) while other operators were described that locked OccR into the opposite conformation. Somewhat unexpectedly, mutations that locked OccR latter conformation did not cause constitutive activity, indicating that octopine must have additional affects on OccR conformation that allow it to form optimal protein-protein contacts with RNA polymerase. Yet it is clear from the present study as well as previous studies with constitutive mutants that a low-angle DNA bend is strongly associated with transcription activation.

Several members of this family have been studied at the structural level. These include the C-terminal sensory domains of CysB of *E. coli*, OxyR of *E.*

coli and of *Mycobacterium tuberculosis* [26, 27]. Full length structures are available for CbnR of *Ralstonia eutropha* [28], TsaR of *Comamonas testosterone*, BenM and CatM of *Acinetobacter* sp. [29, 30]. TsaR has been solved in dimeric form in the presence and absence of the inducing ligand para-toluensulfonate (TSA) [31]. CbnR has been solved in both dimeric and tetrameric forms, though only in the absence of ligand [28].

Structures of full-length proteins show a highly conserved N-termninal DNA binding domain (57 residues in length) composed of three helices followed by two beta strands. These domains contain a winged helix-turn-helix DNA binding motif. These are joined to the less conserved C-terminal ligand-binding domain by a 27-residue helix, called the linker helix. The two linker helices of a protein dimer are adjacent to each other in an antiparallel conformation, and form a coiled-coil that is the major dimerization determinant. Studies of CatR [32], TrpI [33], and OxyR [34] suggest that LysR proteins contact the C-terminal domain of the α subunit of RNA polymerase and act to increase polymerase recruitment. Positive control mutants have been isolated in several LysR-type proteins, and these mutations generally cluster within the amino terminal DNA binding domain, suggesting that this domain may contact RNAP [24, 35].

The regulatory domains of CbnR, OxyR, and CysB, closely resemble each other, and both have a pronounced structural similarity to periplasmic binding proteins (PBPs) [36]. These proteins are also structurally similar to another family of DNA binding proteins, including the Lac repressor of *E. coli*. The regulatory domains of CbnR, OxyR, and CysB are composed of two

subdomains, designated regulatory domains I and II. Domain I is composed of two amino acid sequences, corresponding to CbnR residues 91-161 and 265-294, while domain II is composed of one contiguous sequence corresponding to residues 164-259 (Fig. 4.3). The cavity formed between domains I and II is the likely ligand binding site for LysR proteins, as is observed in the family of PBPs.

Recent structural studies of TsaR has shed more light on the ligand binding site [31]. TsaR crystals were soaked overnight in a solution containing 250 mM of *para*-toluensulfonate, the natural ligand of this regulator. The protein did not show any global conformational change. The each dimer contained no fewer than eleven ligand molecules, many of which were located on the exterior of the protein, and unlikely to reflect the *in vivo* ligand binding site. One ligand molecule per protein monomer was located deep in the cleft of the ligand binding domain, between Domains I and II, and was judged to occupy the *in vivo* site. Similarly, the CysB crystal structure included a molecule of sulfate buried at a similar position, that was thought to be the binding site for the natural ligand (*O*-acetyl-L-serine). Cys199 of OxyR also lies in a similar position. As described above, the ligand-binding domains of LysR-type proteins resemble the periplasmic substrate binding proteins of ABC-type uptake systems, whose ligands are also buried in the cleft between these proteins' two domains. Octopine might therefore bind OccR at a similar site. It is interesting that only three of the positive control mutants (E134K, G196S and D238N) have mutations predicted to lie within this cleft.

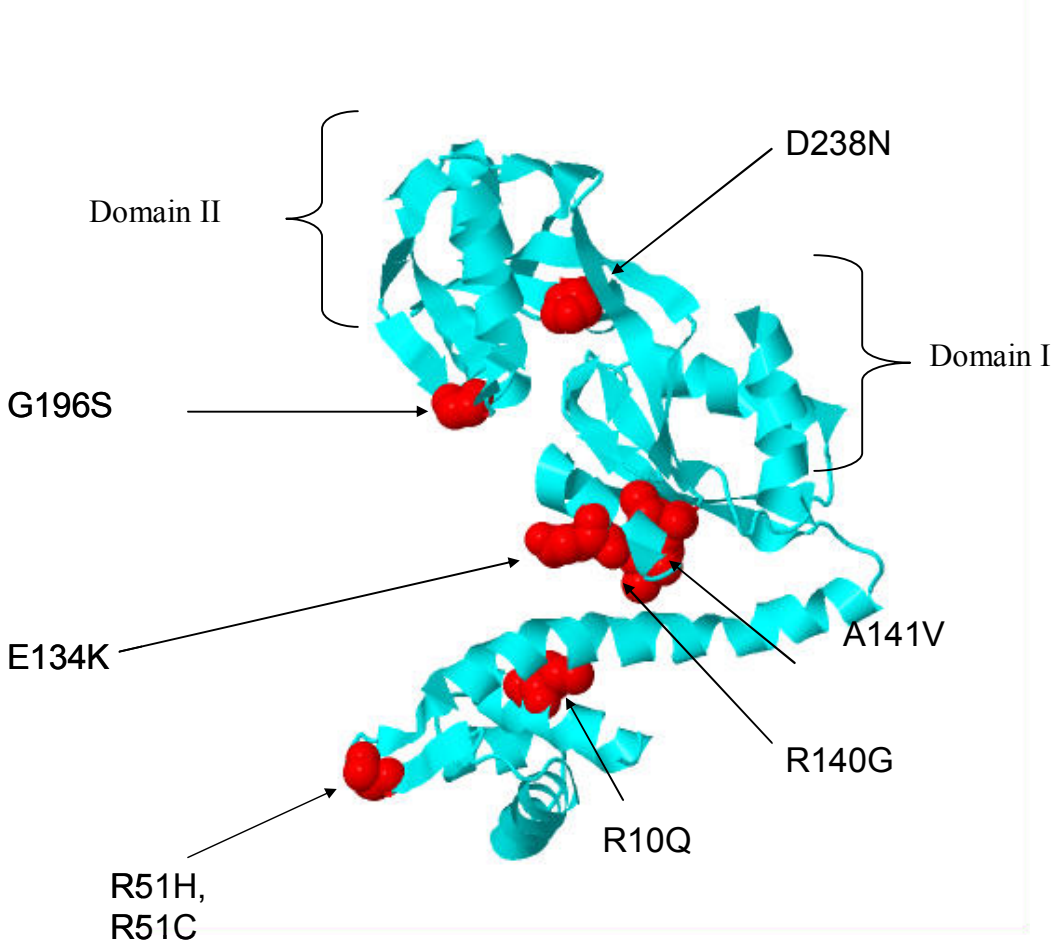

Figure. 4.3. Location of Recognition Domains I and II and the location of positive control OccR mutations, modeled using the secondary structure model of CbnR are also shown (see below).

Residues that line the inducer binding cavity (IBC) between regulatory domains I and II are likely to participate directly in ligand binding. The inducer response related regions have been revealed, they are residues 95-173 and residues 196-206 [24, 37-42]. Recently, an additional region, residues 227-255, has been proposed to be important in CysB [24] and OxyR [40]. These conclusions were supported by structural studies of TsaR.

The structure of CbnR (obtained in the absence of inducing ligand) is presumed to represent the inactive form [28]. The four DNA binding domains of the CbnR tetramer are arranged on a single face of the complex (Fig. 4.4A). In the figure, the N-terminal DNA binding domains are in green, except for the probable recognition helices, which are in yellow. These helices are highly exposed to solvent, and lie perpendicular to the DNA axis, as would be expected of recognition helices. They are arranged in an approximately antiparallel conformation, as would be expected given the dyad symmetry of the recognition sequence. Their positions suggest that they would cause a high angle DNA bend in any operator that contacts all four helices simultaneously. For comparison, I have included a cartoon of OccR-DNA complexes drawn in 1995 (Fig. 4.4B).

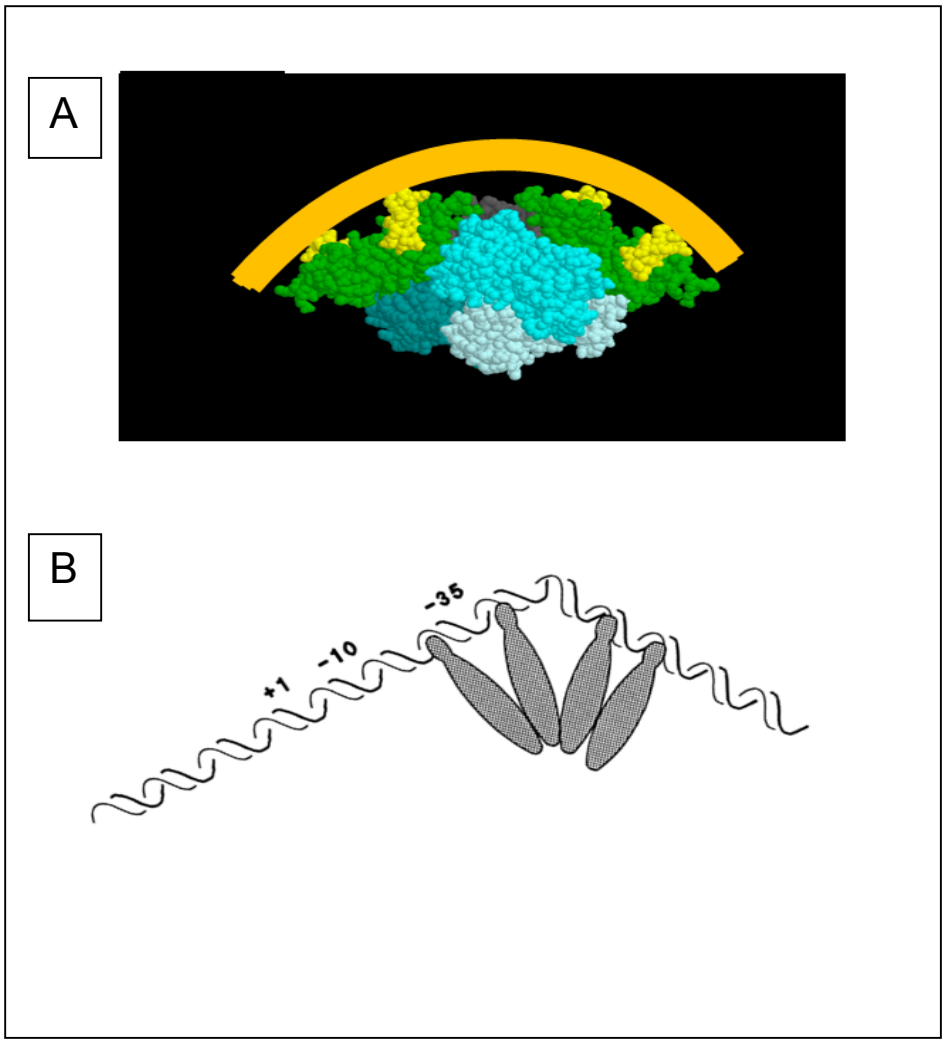

Figure 4.4. Part A. X-ray crystal structure of CbnR. The four DNA binding regions are shown in green, except for recognition helices, which are shown in yellow. The ligand binding domains are shown in three shades of cyan and in dark grey. DNA is modeled using an orange arch. Part B. A cartoon of an OccR-DNA complex, adapted from reference [1].

The spacing of the putative recognition helices of CbnR suggests that helices of domains D' and C' could occupy consecutive DNA major grooves, as could the helices of domains A' and B' (Fig. 4.5A) [28]. However, helices of domains C' and A' are positioned approximately two helical turns apart. This suggests that the four domains could span five helical turns of DNA, but would not contact the central helix, in close agreement with our data for OccR [1, 43]. We have previously developed a model to describe how octopine alters the conformation of OccR [1, 43]. In this model, octopine causes two of the four DNA binding domains to shift by one helical turn, such that the four domains bind to four *consecutive* major grooves. The structure of CbnR strongly supports our model describing how OccR binds DNA in the absence of inducer.

The overall structure of the CbnR tetramer is noteworthy. DNA binding domains D' and C' form a rotationally symmetric dimer, as do domains A' and B'. However, ligand binding domain D'' does not contact C'', nor does A'' contact B''. Instead, ligand binding domains D'' and A'' form a rotationally symmetric dimer, as to ligand binding domains B'' and C''. Fig. 4.6A shows a dimer composed of DNA binding domains A' and B'. In this figure, one can see extremely short linkers joining these DNA binding domains to their respective ligand binding domains A'' and B''. It would seem quite impossible for the ligand-binding domains A'' and B'' to dimerize. In Fig. 4.6B, the ligand-binding domains B'' and C'' form a dimer. The structure of this dimer precludes dimerization of DNA binding domains B' and C'. However, OccR is a dimer in solution and these dimers dimerize upon DNA binding [14]. We conclude that OccR dimers must have the conformations shown in Fig 4.6A (with only

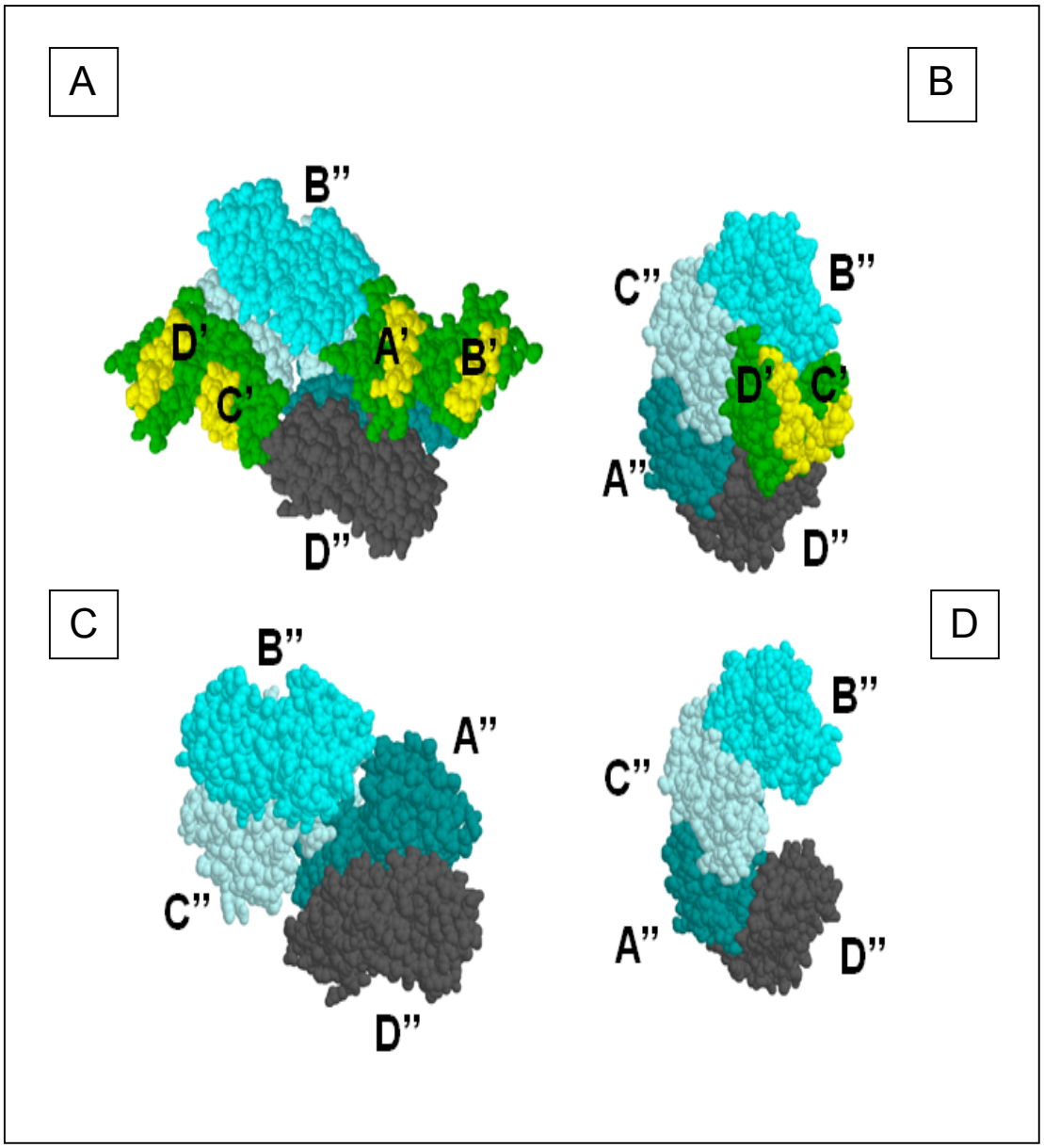

Figure. 4.5. Part A and B. Structure of full-length CbnR, colored as shown in Fig. 4.4. Part B is rotated 90 deg with respect to part A. Part C and D. CbnR tetramer showing residues 89-292, revealing the C-shape made by the four ligand-binding domains.

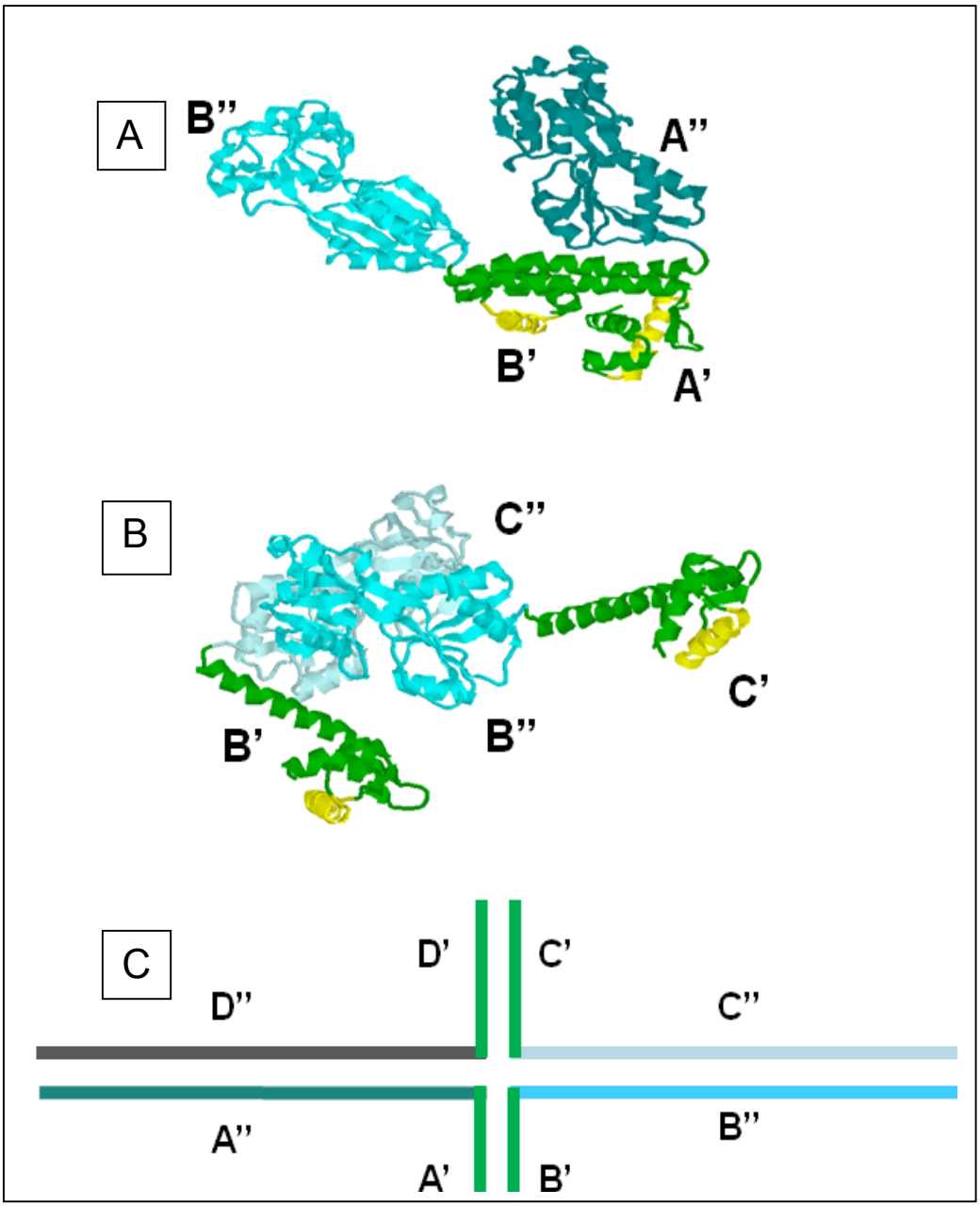

Figure. 4.6. Part A. Subunits A and B, dimerized via their DNA binding domains and linker helix. The two ligand binding domains are unable to dimerize. Part B. Subunits B and C, dimerized via their ligand-binding domains. The two DNA binding domains are unable to dimerize. Part C. Subunit interfaces in the CbnR tetramer.

the DNA binding domains dimerized) or Fig. 4.6B (with only the ligand binding domains dimerized). It is structurally impossible for both domains to participate simultaneously in dimerization. The multimeric structure of a CbnR tetramer is diagramed in Fig. 4.6C. The structure of dimeric TsaR supports these conclusions [31].

The structure of CbnR provides a possible mechanism for this conformational change. We believe that inducer may cause two changes in the protein. In the structure of CbnR, DNA binding domains C' and A' are held apart by the four ligand binding domains (A'', B'', C'' and D''), which form a C-shape (Fig. 4.5C, 4.5D) [28]. It would seem impossible for DNA binding domain C' to contact A' unless this steric hindrance is relieved. This can only happen if the C-shaped ligand-binding domains were to open. We believe therefore that octopine causes the C-shape formed by the four ligand-binding domains to open, allowing all four DNA binding domains to contact, and to bind four consecutive major grooves of operator DNA.

Second, inducer binding in the cleft between domains I and II may cause these domains to draw together. This would occur simultaneously in all four subunits of the complex. This would exert a force upon the two linker helices subunits B' and C' to move rightward and the corresponding helices of subunits A' and D' to move leftward. This would cause the complex to contact from five non-consecutive helical turns to four consecutive turns, with the downstream dimer repositioned to a site centered at approximately -42.5 nucleotides upstream of the transcription start site.

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