Communication between Binding Sites Is Required for YqjI Regulation of Target Promoters within the \textit{yqjH-yqjI} Intergenic Region

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The nickel-responsive transcription factor YqjI represses its own transcription and transcription of the divergent \textit{yqjH} gene, which encodes a novel ferric siderophore reductase. The intergenic region between the two promoters is complex, with multiple sequence features that may impact YqjI-dependent regulation of its two target promoters. We utilized mutagenesis and DNase I footprinting to characterize YqjI regulation of the \textit{yqjH-yqjI} intergenic region. The results show that YqjI binding results in an extended footprint at the \textit{yqjI} promoter (site II) compared to the \textit{yqjH} promoter (site I). Mutagenesis of \textit{in vivo} gene reporter constructs revealed that the two YqjI binding sites, while separated by nearly 200 bp, appear to communicate in order to provide full YqjI-dependent regulation at the two target promoters. Thus, YqjI binding at both promoters is required for full repression of either promoter, suggesting that the two YqjI binding sites cooperate to control transcription from the divergent promoters. Furthermore, internal deletions that shorten the total length of the intergenic region disrupt the ability of YqjI to regulate the \textit{yqjH} promoter. Finally, mutagenesis of the repetitive extragenic palindromic (REP) elements within the \textit{yqjH-yqjI} intergenic region shows that these sequences are not required for YqjI regulation. These studies provide a complex picture of novel YqjI transcriptional regulation within the \textit{yqjH-yqjI} intergenic region and suggest a possible model for communication between the YqjI binding sites at each target promoter.

Metal ions play an important role in all organisms. It is estimated that about one-third of all proteins require a metal for proper function, with approximately half of (40%) all enzymes having metal bound to them as a cofactor (1). However, even essential transition metals can catalyze spurious side reactions if present in excess. Due to their dual nature, acquisition and intracellular trafficking of essential first-row transition metals, such as iron, zinc, copper, and nickel, are carefully controlled by metal homeostasis systems. Metal homeostasis systems consist of metal transporters, metal storage proteins, and metallochaperones that work in concert to maintain the proper cellular metal quota (2). Expression of metal homeostasis genes is often controlled by specialized transcription factors known as metalloregulatory proteins (3). Besides their functional DNA binding domains, metalloregulatory proteins often have an allosteric metal binding domain that is able to directly sense cellular metal changes (4). Binding or dissociation of the metal ion(s) at the metal binding domain switches the DNA binding domain to increase or decrease its affinity for target promoter(s). This metal-responsive allosteric switch coordinates expression of the genes encoding metal utilizing proteins and other metal homeostasis proteins with cellular metal levels.

In a previous study, we discovered that the predicted transcription factor YqjI from \textit{Escherichia coli} is a nickel-responsive transcriptional repressor that cross-regulates an iron homeostasis gene in response to cellular nickel levels (5). YqjI is a flavin adenine dinucleotide (FAD)-bound ferric reductase that is able to reduce ferric siderophores, such as enterobactin, presumably to release iron for \textit{in vivo} utilization (5, 6). \textit{In vivo} experiments revealed that YqjI-dependent repression of \textit{yqjI} and \textit{yqjH} is abolished under high Ni$^{2+}$ levels that may disrupt iron metabolism. This cross-regulation leads to the elevated expression of YqjH in order to compensate for disruption of iron homeostasis by nickel stress. Furthermore, the YqjI metalloregulatory protein was shown to bind Ni$^{2+}$ and Zn$^{2+}$ (5). Two highly similar palindromic sequences were identified in the 287-bp intergenic region between \textit{yqjH} and \textit{yqjI}: one located at +6 to +25 relative to the predicted transcriptional start site of \textit{yqjH} and the other located at +8 to −10 relative to the mapped transcriptional start site of \textit{yqjI} (Fig. 1). YqjI is a member of the PadR family of transcriptional regulators but contains an additional N-terminal extension that is rich in potential metal-binding residues (such as Cys, His, and Glu) (7, 8). Apo-YqjI is purified as both a monomer and in an oligomeric form. \textit{In vitro} fluorescence anisotropy assays using oligonucleotides containing the palindromic sequences showed that apo-YqjI oligomer binds these two sequences with 69 nM affinity for the \textit{yqjH} site and 48 nM affinity for the \textit{yqjI} site (5). \textit{In vivo} regulatory studies also indicate that apo-YqjI represses its own transcription to a greater degree than the transcription of \textit{yqjH}, despite the high similarity between the palindromic sequences at each promoter (5). The addition of divalent metal ions, such as Ni$^{2+}$, Fe$^{2+}$, or Zn$^{2+}$, diminishes the DNA binding affinity of YqjI for these putative target sequences \textit{in vitro}, possibly by interacting with YqjI via the N-terminal extension. Together these results suggest a model.
where transcription repression by apo-YqjI is abolished when nickel binds to YqjI, leading to increased expression from YqjI-regulated target promoters (5).

Although these studies provided a preliminary analysis of YqjI-dependent regulation, a number of important questions remain to be answered. The yqjH-yqjI intergenic region is complex. The yqjH promoter is repressed by the iron metalloregulatory protein Fur under iron-replete conditions, and this repression is relieved under iron starvation stress (5,9,10). While Fur binding to the promoter DNA has not been mapped biochemically, the predicted Fur binding sites overlap the predicted –10 RNA polymerase binding site upstream of yqjH (Fig. 1) (9,10). The yqjH-yqjI intergenic region also contains two repetitive extragenic palindromic (REP) elements, 228a and 228b, between the two promoters but slightly closer to the yqjI promoter (5,11,12). REP elements are imperfect palindromes and contain several asymmetrical features that can be used to establish a “tail-to-head” orientation (Fig. 1) (13,14). Hundreds of REP elements are found within the E. coli genome and are often organized into bacterial interspersed mosaic elements (BIMEs) that contain two or more REP elements flanked by one of several conserved sequence motifs (see Fig. S1 in the supplemental material) (13,14). The functional roles of REP elements within BIMEs are diverse and include transcriptional termination and mRNA stabilization (15,16). Some BIMEs can serve as binding sites for proteins such as integration host factor (IHF) and DNA gyrase (17–19). Since the yqjI gene is framed by pairs of REP elements located upstream (REP 228a and -b) and downstream (REP 229a and -b) of its open reading frame, the REP elements may also be remnants of transduction- or recombination-mediated horizontal gene transfer of the yqjI gene into the E. coli chromosome in the evolutionary past (20,21).

The studies described here more closely examine the mechanism of YqjI regulation in the yqjH-yqjI intergenic region and the possible role of the REP elements in that regulation. DNase I footprinting was used to confirm YqjI binding sites at the yqjH and yqjI promoters. Point mutations or deletions in the yqjH-yqjI intergenic region were generated by site-directed mutagenesis. The mutated promoters were analyzed through in vivo gene reporter constructs in order to characterize YqjI-dependent transcriptional regulation at each promoter in the yqjH-yqjI intergenic region. Using these approaches, we have discovered that the YqjI binding sites at the yqjH and yqjI promoters are not equivalent. Apo-YqjI binding sites in an extended footprint at the yqjI promoter compared to the yqjH promoter. In vitro and in vivo results also show that the two YqjI binding sites appear to communicate over a distance of 200 bp in order to provide full YqjI-dependent regulation. The REP elements per se do not appear to be important for YqjI-dependent regulation of its target promoters. However, deletion mutagenesis shows that the exact length of the intergenic region is critical for the proper regulation of the promoters. These results provide a complex picture of novel YqjI transcriptional regulation within the yqjH-yqjI intergenic region.

MATERIALS AND METHODS
Plasmid construction and promoter modification. All promoter mutants were constructed by modifying the plasmids PyqjH_pPK7035 and PyqjI_pPK7035 (5). Deletions were constructed by PCR amplification using Flu Ultra polymerase (Agilent) and primers described in Table S1 and Fig. S2 in the supplemental material. After PCR amplification, the template plasmids were removed by DpnI digestion, and the remaining PCR products were blunt-ended and religated into pPK7035 using the NEB Ultra Blunting and Ligation kits. Nucleotide substitutions were generated through site-directed mutagenesis of the PyqjH_pPK7035 and PyqjI_pPK7035 plasmids. The subsequent steps followed an established site-directed mutagenesis protocol (22). Plasmid mutations were confirmed by sequencing. Mutant plasmids were used as PCR templates to generate promoter-lacZ fusion fragments with the primer pair 5’-lacI- Kn(69bp) and Homol Recom Rev. The promoter-lacZ fusion fragments contain part of lacI, the KanR gene, and the modified yqjH or yqjI promoters fused to lacZ. The promoter-lacZ fusion fragments replace the native promoter of the lacZ gene via an in vivo recombination process described by Kang et al. to generate single-copy gene reporters (23). All fusions in genomic DNA were confirmed by DNA sequencing of colony PCR fragments generated from the recombinant colonies using primers described in Table S1 (5).

| Fig 1 | Summary of regulatory features within the yqjH-yqjI intergenic region. The nucleotide sequence numbering begins with the first nucleotide upstream of the yqjH start codon and ends with the first nucleotide prior to the yqjI start codon. Regions of the intergenic sequence protected from DNase I cleavage by apo-YqjI are indicated in black boxes (see the text and Fig. 2). Light gray boxes with dashed outlines indicate the three potential Fur binding sites proposed from bioinformatic analysis (9,10). The originally identified palindromic elements are indicated by arrows within the YqjI footprints (5). REP228a, and REP228b elements are indicated with bracketed lines. The –35 and –10 RNA polymerase binding sites are in boldface font, and the +1 transcriptional start sites are in boldface italic font. The +1 transcriptional start site for the yqjH promoter has not been experimentally determined and is only predicted. |
β-Galactosidase assays. E. coli wild-type MG1655 cells containing native or mutated yqjH-lacZ or yqjI-lacZ constructs were grown in LB with 30 μg/ml kanamycin to mid-exponential phase (optical density at 600 nm [OD600] of 0.4 to 0.6) at 37°C with shaking at 225 rpm. β-Galactosidase activity was measured using an established protocol (5).

Apo-YqjI expression and purification. The previously described expression plasmid yqjI-pET21a was expressed in the E. coli BL21(DE3) strain (5). Several changes were made to the previous YqjI expression and purification protocol to acquire better activity and higher purity. Overnight cultures were diluted 1:100 into bottles filled to the top by LB medium with 100 μg/ml ampicillin. Cells were grown anaerobically at room temperature for about 8 h to an OD600 of 0.3 to 0.4 and then induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at room temperature for 24 h. Cells were harvested by centrifugation for 10 min at 6,000 × g. Harvested cell pellets were resuspended in binding buffer (20 mM HEPES [pH 7.5], 0.2 M NaCl, 10 mM 2-mercaptoethanol [β-ME], 5% glycerol), lysed by sonication for 2 min, and cleared by centrifugation for 20 min at 16,000 × g. Cleared lysate was loaded onto a 5–ml HisTrap heparin HP column (GE Healthcare) and eluted with a linear gradient of 0.2 to 1 M NaCl. The fractions containing oligomer and monomer forms of YqjI were pooled separately for further purification. The volumes of the heparin column eluates were measured, and then they were mixed with HisTrap elution buffer (20 mM HEPES [pH 7.5], 0.5 M NaCl, 0.5 M imidazole, 5% glycerol) to provide a final imidazole concentration of 20 mM. The treated elute from the heparin column was loaded onto a 5–ml HisTrap FF column (GE Healthcare), and YqjI was eluted with a linear gradient of 20 to 500 mM imidazole. To avoid chelation of Ni2+ off the HisTrap column by apo-YqjI, β-ME was excluded in both the HisTrap binding buffer and elution buffer. The fractions containing YqjI were pooled, mixed with 10 mM β-ME, concentrated, and loaded onto a Superdex 200 gel filtration column (HiLoad 16/60; GE Healthcare). The column was run with 20 mM HEPES (pH 7.5), 10 mM β-ME, 500 mM NaCl, and 5% glycerol. YqjI oligomer and monomer were separately pooled, concentrated, and kept at −80°C. YqjI purity was estimated by SDS-PAGE to be >95%, and the YqjI concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin (BSA) (Bio-Rad) as the standard.

Preparation of DNase I footprinting probes. A two-step PCR scheme was used to optimize production of the footprinting probes (see Table S1 in the supplemental material). For the wild-type promoter analysis, a 507–bp sequence, including the 287–bp intergenic region between yqjH and yqjI along with 110 bp of flanking DNA on each end, was amplified by PCR from genomic DNA using Taq DNA polymerase (catalog no. M0273; NEB) and primers yqjH + 100_Xhol_up and yqjI + 100_BamHI_dn (see Table S1). After the first PCR, the nonlabeled 507–bp DNA sequence was generated to use as the template for PCR amplification of fluorescein-labeled fragments for DNase I footprinting. To study the full-length intergenic region containing both promoters, the primer pair [56FAM]-yqjH + 100_ up and [5HEX]-yqjI + 100_dn was utilized to amplify the whole intergenic region. The initial long template DNA was purified by a Freeze’N Squeeze DNA gel extraction column (Bio-Rad). After the second PCR (using the purified DNA fragment as the template and the primers mentioned above), fluorescence-labeled yqjH and yqjI promoter fragments were purified by a PCR purification kit (Qiagen) before being used in footprinting reactions. Fluorescein-labeled DNA concentrations were determined by measuring the absorbance at 260 nm, and the purity was determined by calculating the ratio of the absorbance at 260 nm to the absorbance at 280 nm.

DNase I footprinting. A 2 nM concentration of fluorescein-labeled DNA fragment (PyqH287, PyqI287, or the full-length yqjH-yqjI intergenic region fragment) was added to the reaction mixture containing 20 nM HEPES (pH 7.8), 50 mM NaCl, 10 mM MgCl2, 1 mM β-ME, 50 μg/ml BSA, 5 μg/ml sheared salmon sperm DNA (ssDNA), and 5% glycerol. The oligomeric form of apo-YqjI was diluted to various concentrations (0 to 400 nM) in a final reaction volume of 200 μl. After incubation at room temperature for 15 min, DNase I (NEB) (0.2 U for the full-length promoter, 0.1 U for half promoters) was added to the samples to initiate the DNA cleavage. After digestion at room temperature for 10 min, reactions were stopped by heating at 70°C for 10 min. The digested samples were purified using both a PCR purification column (Qiagen) and a subsequent gel matrix desalting step (24). Purified and digested DNA samples were analyzed by an Applied Biosystems 3730 DNA analyzer. Sequence data were first analyzed with the software GeneMapper 4.0 (Applied Biosystems). The footprinting samples were mixed with GeneScan 500 LIZ dye size standard (Applied Biosystems), and the DNA fragments were aligned using this size standard. For quantitative DNase I footprinting, the following concentrations of oligomeric apo-YqjI were used: 1, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, and 400 nM. Further details on quantitative footprinting methods and data analysis are provided in Materials and Methods in the supplemental material.

RESULTS

YqjI binding to the yqjH and yqjI promoters is not equivalent. Sequence analysis of the yqjH-yqjI intergenic region revealed a palindrome at the yqjI promoter that is quite similar to another palindrome at the yqjH promoter, with only two nucleotide differences between them (Fig. 1). Apo-YqjI binding to these putative YqjI binding sites was confirmed by DNA fluorescence anisotropy assays with labeled oligonucleotides (5). In the present study, DNase I footprinting was used to better define the apo-YqjI binding sites at each promoter in the context of the full yqjH-yqjI intergenic region (Fig. 2). Apo-YqjI binding to the yqjH promoter reduced DNase I cleavage in a region from +8 to +25 relative to the predicted +1 transcriptional start site, and we refer to this region as site I (Fig. 1 and 2). The sequence of site I mapped by DNase I footprinting overlaps most of the palindrome identified previously, indicating this region is the binding site for apo-YqjI (5).

Apo-YqjI binding to the yqjI promoter reduced DNase I cleavage from −23 to +8 relative to the previously determined transcriptional start site, and we refer to this region as site II (Fig. 1 and 2B) (25). Although the minimal palindromic sequences identified previously are quite similar, YqjI binding at the yqjI promoter resulted in an extended DNase I footprint in comparison to the footprint at the yqjH promoter (Fig. 1 and 2B) (5). Close examination of the extended footprint at the yqjI promoter revealed that site II contains two protected regions separated by a short, unprotected spacer (Fig. 2B). Further examination of the specific nucleotides protected by apo-YqjI revealed that site I closely resembles half of the extended site II footprint but does not contain the other half of site II (Fig. 2C). These results indicate that site I may contain only half of the full-length apo-YqjI operator, while site II contains the entire binding site.

Based on the footprinting results for sites I and II, we next determined if specific mutations in site I have differential effects on YqjI-dependent regulation of the promoter in vivo. The mutated yqjH promoter fragments were fused to lacZ, and the resulting constructs were used to create single-copy gene reporters on the E. coli chromosome. These constructs were inserted into the lac operon on the chromosome and do not disrupt the native yqjH-yqjI locus. Mutation of all A nucleotides to C nucleotides in the upstream portion of site I increased yqjH expression by less than 2-fold (Fig. 3). In contrast, similar A-to-C mutations in the downstream half-site of site I (which closely matches the half-site in site II) caused a 20-fold increase in yqjH expression. Combining

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the mutations that cover the entire site I region caused a 25-fold increase in \(yqjH\) expression. For comparison, in the \(\Delta yqjI\) strain lacking the YqjI repressor, \(yqjH\) expression is increased approximately 40-fold (from 12 Miller units for the wild-type strain to 480 Miller units in the mutant). Unfortunately, due to the extensive overlap of site II with the −10 element in the \(yqjI\) promoter, we were unable to construct similar mutations at site II without also disrupting RNA polymerase (RNAP) binding (Fig. 1). Nonetheless, these results suggest that site I contains only half of the full-length YqjI operator.

**Evidence for communication between sites I and II.** Quantitative DNase I footprinting was conducted on the full-length \(yqjH-yqjI\) intergenic region as well as on partial fragments that lack site I or II (Fig. 4). Quantitative footprinting of the full-length \(yqjH-yqjI\) intergenic region revealed that the dissociation constant (\(K_d\)) of apo-YqjI for both sites I and II was decreased by 3-fold relative to the \(K_d\) measured using small oligonucleotides containing the previously identified palindromes (5). YqjI had a higher affinity for site II compared to site I, consistent with site II containing the full-length operator rather than only a half-site (Fig. 4A). When apo-YqjI binding to the partial fragments was analyzed and compared to the full-length fragment, we observed that the affinity of apo-YqjI for site I was moderately decreased if site II was absent (Fig. 4A). In contrast, the affinity of apo-YqjI for site II was not
affected by the presence or absence of site I. For site II, the $K_I$ for each half-site of the full operator was calculated separately, but both half-sites showed similar $K_I$ values for all fragments analyzed (Fig. 4A). The footprinting data for site I showed a sigmoidal shape with a calculated Hill coefficient of 1.9 when the full $yqjH$-$yqjI$ intergenic region was used but a Hill coefficient of only 0.8 if site I in the $yqjH$ promoter was measured in isolation (Fig. 4B). The Hill slope above 1 indicates some type of positive cooperativity for YqjI binding to site I in the $yqjH$ promoter when site II is also present as part of the full intergenic region. The simplest hypothesis to explain this cooperativity is that apo-YqjI binding to site I at the $yqjH$ promoter is enhanced by apo-YqjI binding to the distant but higher-affinity site II at the $yqjI$ promoter.

To confirm this in vitro result, we next tested if site II at the $yqjI$ promoter is required for full in vivo repression at site I of the $yqjH$ promoter. Deletions were constructed in the YqjI operator in site II (at the distal $yqjI$ promoter) in single-copy reporters where the $yqjH$ promoter is fused to $lacZ$ in a transcriptional fusion (Fig. 5A). Surprisingly we observed that deletions within distal site II (at the divergent $yqjI$ promoter) caused partial derepression of the proximal $yqjH$-$lacZ$ promoter fusion, despite the 200-bp distance between site I and site II in the construct (Fig. 5A). Deletion of the downstream half-site from the site II palindrome resulted in $yqjH$ expression increasing approximately 17-fold compared to the wild-type gene reporter construct. Deletion of the downstream half-site and the spacer region from the site II operator further increased $yqjH$ expression to 20-fold compared to the wild-type construct. Deletion of only the upstream half-site in site II did not cause a detectable change in $yqjH$ expression. However, when the upstream half-site was deleted in combination with the spacer region or when the entire site II operator was deleted, an additive effect was observed resulting in a maximum 25-fold increase in $yqjH$ expression (Fig. 5A). For comparison, $yqjH$ expression in the $\Delta yqjI$ strain completely lacking the YqjI repressor is increased approximately 40-fold compared to that in the wild-type strain. Thus, while apo-YqjI binding to site I alone provides about 40% repression of $yqjH$, site I must somehow communicate with site II in the distal $yqjI$ promoter for full repression of the $yqjH$ promoter. The upstream half-site in site II is not absolutely required for repression of the distal $yqjH$ promoter, but it plays an additive role in combination with the rest of site II. When the promoter fusion constructs were analyzed in the $\Delta yqjI$ strain background, the distal mutations had little to no effect on $yqjH$-$lacZ$ expression, indicating the mutations specifically disrupt apo-YqjI regulation of $yqjH$ and do not cause other changes in transcription of that region (Fig. 5A).

Next we carried out the reciprocal experiment to determine if mutations in distal site I at the $yqjH$ promoter affect divergent expression from the proximal $yqjI$ promoter (Fig. 5B). Individual nucleotides within the distal site I binding site were mutated (from A to C) in single-copy $yqjI$-$lacZ$ gene reporter fusions as described in Fig. 3. Any combination of the mutations throughout site I resulted in a 10-fold increase in $yqjH$ expression, suggesting some loss of $yqjI$-dependent repression (Fig. 5B). However, $yqjH$ expression in a $\Delta yqjI$ strain lacking the YqjI repressor is increased approximately 130-fold to 4,416 ± 111 Miller units compared to the wild-type strain (34 ± 0.3 Miller units). Based on these results, apo-YqjI binding to site II alone is enough to provide 90% of the YqjI-dependent repression of $yqjH$ observed in vivo, but there is a slight increase in repression (10%) if site I is also occupied.

**The length of the intergenic region is critical for YqjI-dependent regulation of its target promoters, but the REP elements are not.** The REP elements comprise a large internal segment of the $yqjH$-$yqjI$ intergenic region, spanning 79 bp of the 216 bp between transcriptional start sites (Fig. 1). The palindromes within the REP elements could serve as binding sites for additional regulators and/or distort the local DNA structure of the intergenic region, which in turn may alter regulation of the $yqjH$ and/or $yqjI$ promoters. To test if the REP elements are required for YqjI-dependent regulation of its target promoters, we initially deleted the REP 228b sequence, which covers 34 bp of the intergenic region (Fig. 6A). The REP 228b deletion caused a dramatic 60-fold increase in the expression of the $yqjH$-$lacZ$ reporter construct. Surprisingly the REP 228b deletion had no effect on expression from the $yqjI$-$lacZ$ reporter construct (Fig. 6B). This result could imply that the REP elements are important for $yqjH$ regulation. However, since internal deletion of REP 228b shortens the spacing between the $yqjH$ and $yqjI$ promoters (from 200 bp to 166 bp), we also mutated individual nucleotides within the REP 228a and 228b elements to disrupt their palindromes without changing the length of the intergenic region. Each element was mutated individually and in concert to create single and double REP element mutant constructs (Fig. 6; see Fig. S1 in the supplemental material). Disruption of the palindromic sequences within REP 228a caused a mild 2-fold increase in $yqjH$ transcription, while similar mutations in REP 228b or combined mutations in both REP elements showed no effect on $yqjH$ expression (Fig. 6A). Similar results were obtained when the same mutations were characterized using the $yqjI$-$lacZ$ reporter construct (Fig. 6B). Together these
Quantitative DNase I footprinting for each YqjI binding site. (A) $K_d$ values were calculated based on DNase I protection upon oligomeric apo-YqjI binding at site I or site II operators when using the partial or full-length intergenic region for footprinting. Half-sites within the site II operator were characterized separately, with "down" indicating the downstream half-site and "up" indicating the upstream half-site relative to the direction of $yqjI$ transcription. (B) The sums of peak heights for select protected peaks in each site were plotted as a function of the apo-YqjI concentration for the entire apo-YqjI titration data set (filled circles). Solid lines indicate nonlinear regression fit using the sigmoidal dose-response model provided by GraphPad Prism.
results indicate that the spacing between the \( yqjH \) and \( yqjI \) promoters is critical for full repression of \( yqjH \) expression but that the specific palindromic sequences of the REP elements themselves are largely dispensable for that regulation (Fig. 6A). In contrast, regulation of the \( yqjI \) promoter is not greatly affected by either deletion or disruption of the REP elements (Fig. 6B).

**FIG 5** Effect of mutations in distal YqjI binding sites on YqjI repression of proximal promoter-\(lacZ\) fusions \textit{in vivo}. (A) Dashed gray outlines of arrows indicate deletion of the half-site(s) within the site II operator (open arrows); the spacer regions between the half-sites are shown as dashed gray lines. (B) Asterisks indicate mutations within the site I operator (with the half-sites shown as open arrows) as described in Fig. 3 and Fig. S2 in the supplemental material. All constructs were single-copy reporters in the wild-type (MG1655) or \( \Delta yqjI \) strain of \( E. coli \). Promoter activity is indicated as Miller units, and each value is the average from triplicate experiments.

**DISCUSSION**

**Binding of oligomeric apo-YqjI to its operator.** Based on DNase I footprinting and \textit{in vivo} expression studies (Fig. 2 and 3), the oligomeric, DNA-binding form of apo-YqjI binds to operators at both the \( yqjH \) and \( yqjI \) promoters. DNase I footprinting results show that site I is actually a half-site similar to the downstream half-site present in the full YqjI operator in site II (Fig. 2 and 4). Promoter mutation constructs indirectly suggest some asymmetry in the operator at site II in the \( yqjI \) promoter with the downstream palindrome being more significant for \textit{in vivo} communication between sites I and II (Fig. 5). However, we were unable to test this directly \textit{in vivo} at the \( yqjI \) promoter due to the significant overlap between site II and the –10 element of the RNAP binding site. While many transcriptional regulators bind palindromic DNA sequences symmetrically as protein dimers, there are exceptions to this trend. The bacteriophage \( \lambda \) repressor (\( \lambda C1 \)) is a well-characterized example. \( \lambda C1 \) was shown to bind asymmetrically to...
the O$_{31}$ palindromic operator, and further analysis suggested this binding mode holds for all six lac target operators (26). The asymmetric binding of lac to its operators is thought to result from the inherent asymmetry of the lac dimer itself that may cause steric clash between lac monomers when the protein tries to bind DNA symmetrically (26–28). Our results suggest that the structures of YqjI oligomer forms are inherently asymmetric.

**Communication between site I and site II operators.** In vivo expression analysis of mutated promoter constructs revealed that site II is required for full transcriptional repression of yqjH at the distant site I (Fig. 5). Conversely, full transcriptional repression of yqjH at site II also requires the distant site I, although site I makes a much weaker contribution to yqjH repression in comparison to the need for site II for yqjH repression (Fig. 5). The simplest model to explain these results is that site I and site II can communicate via bending of the yqjH-yqjI intergenic region such that apo-YqjI bound at each promoter interacts to form a promoter-bridged complex. DNA loop formation is sensitive to the distance and periodicity of DNA between sites. If a nonintegral number of helix turns are present, the torsional energy required to twist DNA to place sites on the same face of the helix can disrupt complex formation (29–31). If we assume 10.5 bp per B-DNA helical turn, the spacing between site I and site II contains an integral number of DNA helical turns: 19 helical turns (200 bp) for site I-to-site II spacing (1). This spacing would place apo-YqjI operators on the same face of the DNA helix to potentially facilitate loop formation. The severe derepression of the yqjH expression when 34 bp of REP 228b are deleted from the intergenic region (Fig. 6) is also consistent with a DNA bending model. Since it does not translate to an integral number helical turns, a 34-bp internal deletion changes the relative locations of the YqjI binding sites on the helical face of the DNA. The shortening of the intergenic region may also increase the energetic cost of bending the DNA due to increased rigidity as the distance between operators gets closer to the persistence length of DNA.

The DNA bending model is also consistent with the previous observation that only an oligomeric (possibly hexameric) form of YqjI is competent to bind target DNA in vitro (5). Formation of a higher-order complex is a likely prerequisite for apo-YqjI to stabilize a bent structure of DNA since it would allow dimer subunits that initially bind the target operators to form tetramer or larger complexes that bridge the two bent strands of the yqjH-yqjI region. lac transcriptional repression provides a well-studied parallel regulatory system for comparison. lac binds its target operators as a dimer but forms an octamer in the final bent or looped conformation of the regulatory region it controls (32–35). In the case of lac, there are four operators involved in formation of the higher-order looped complex during negative autoregulation, hence the formation of a final octamer complex of the protein (one dimer per operator). In the case of apo-YqjI, we observed an oligomeric complex with an apparent molecular weight consistent with a hexamer (5). Since there are only two distinct operators present in the yqjH-yqjI regulatory region (sites I and II), it is not clear how apo-YqjI would access each site, resulting in a hexamer complex in the final higher-order looped structure. Future structural studies on an apo-YqjI–DNA complex are necessary to resolve these questions.

In conclusion, here we define the apo-YqjI binding sites within the yqjH-yqjI intergenic region and demonstrate that apo-YqjI binding is not equivalent at each operator. Our results also suggest an inherent asymmetry in the DNA binding mode of apo-YqjI at site II, which may indicate structural asymmetry in the apo-YqjI oligomer. Finally we have shown that full in vivo repression of either promoter requires both operators and that the intergenic spacing (but not the REP elements per se) is necessary for proper repression. Future studies will test the regulatory model suggested by our results and determine if DNA looping occurs as part of YqjI regulation of its target promoters.

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