

## PdhR (Pyruvate Dehydrogenase Complex Regulator) Controls the Respiratory Electron Transport System in *Escherichia coli*<sup>∇</sup>

Hiroshi Ogasawara,<sup>1,2</sup> Yuji Ishida,<sup>1</sup> Kayoko Yamada,<sup>1,3</sup>  
Kaneyoshi Yamamoto,<sup>4</sup> and Akira Ishihama<sup>1,2,3\*</sup>

Department of Frontier Bioscience,<sup>1</sup> and Research Center for Micro-Nano Technology,<sup>2</sup> Hosei University, Koganei, Tokyo 184-8584, Nippon Institute for Biological Science, Ome, Tokyo 198-0024,<sup>3</sup> and Department of Advanced Bioscience, Kinki University, Nara 631-8505,<sup>4</sup> Japan

Received 11 February 2007/Accepted 10 May 2007

**The pyruvate dehydrogenase (PDH) multienzyme complex plays a key role in the metabolic interconnection between glycolysis and the citric acid cycle. Transcription of the *Escherichia coli* genes for all three components of the PDH complex in the *pdhR-aceEF-lpdA* operon is repressed by the pyruvate-sensing PdhR, a GntR family transcription regulator, and derepressed by pyruvate. After a systematic search for the regulation targets of PdhR using genomic systematic evolution of ligands by exponential enrichment (SELEX), we have identified two novel targets, *ndh*, encoding NADH dehydrogenase II, and *cyoABCDE*, encoding the cytochrome *bo*-type oxidase, both together forming the pathway of respiratory electron transport downstream from the PDH cycle. PDH generates NADH, while Ndh and CyoABCDE together transport electrons from NADH to oxygen. Using gel shift and DNase I footprinting assays, the PdhR-binding site (PdhR box) was defined, which includes a palindromic consensus sequence, ATTGGTNNNACCAAT. The binding *in vitro* of PdhR to the PdhR box decreased in the presence of pyruvate. Promoter assays *in vivo* using a two-fluorescent-protein vector also indicated that the newly identified operons are repressed by PdhR and derepressed by the addition of pyruvate. Taken together, we propose that PdhR is a master regulator for controlling the formation of not only the PDH complex but also the respiratory electron transport system.**

The pyruvate dehydrogenase (PDH) complex of *Escherichia coli* contains three components, pyruvate dehydrogenase (E1p), dehydroliipoate acyltransferase (E2p), and dihydroliipoate dehydrogenase (E3), and catalyzes the NAD-linked oxidative decarboxylation of pyruvate and the concomitant formation of acetyl coenzyme A (acetyl-CoA) (25, 34), which then reacts with oxalacetate to produce citrate in the first reaction of the citric acid (tricarboxylic acid [TCA]) cycle. The *E. coli* PDH complex is composed of 24 units of E1p (AceE [pyruvate dehydrogenase]), 24 units of E2p (AceF [dehydroliipoamide acyltransferase]), and 12 units of E3 (LpdA [dihydroliipoamide dehydrogenase]). The genes (*aceE*, *aceF*, and *lpdA*) encoding these three enzymes form a single operon, together with the *pdhR* gene, encoding a self-regulator of this operon, in the order *pdhR-aceE-aceF-lpdA*.

Transcription of the *pdh* operon is controlled by two major promoters: the upstream promoter  $P_{pdh}$  generates a *pdhR-lpdA* readthrough transcript, and the internal promoter  $P_{lpd}$  generates independent *lpdA* transcript (26). The primary *pdh* promoter  $P_{pdh}$  is negatively autoregulated by PdhR, the product of the first gene in this *pdh* operon (26). PdhR is a member of the Gnt family of transcription factors and shares sequence similarity in its N-terminal DNA-binding domain with other members (14). PdhR senses the intracellular pyruvate pool, and its activity is controlled by pyruvate. Since the PdhR-pyruvate complex is unable to bind target DNA, the *pdhR-aceEF-lpdA*

operon is derepressed when pyruvate is available (26). The secondary promoter  $P_{lpd}$  is under the control of ArcA, which is the global repressor of TCA cycle genes under anaerobic conditions (8). Besides the *pdhR* operon, the only regulation target of PdhR identified so far is the *yfiD* gene, encoding a putative formate acetyltransferase (36), that is induced at low pH or by pyruvate (3).

Since the PDH multienzyme complex is a key enzyme for the metabolic interconnection between glycolysis and the TCA cycle, PdhR could be an important regulator for the steady-state maintenance of the central metabolism for energy production in response to changes in external environmental conditions. In order to identify the whole set of target genes under the control of PdhR, we performed a systematic search *in vitro* for PdhR-binding sequences in the *E. coli* genome using the newly developed genomic systematic evolution of ligands by exponential enrichment (SELEX) method (30). In this study, we identified two targets, the *ndh* gene, encoding NADH dehydrogenase II, and the *cyoABCDE* operon, encoding the cytochrome *bo*-type oxidase (4, 5, 17), both together forming the terminal respiratory electron transport system from NADH to oxygen, which is located downstream of the PDH system in the metabolic pathway of energy production. In *E. coli*, NADH dehydrogenase II is a primary dehydrogenase that plays a major role in aerobic and nitrate respiration. Under anaerobic conditions, the expression of *ndh* is repressed by FNR (regulator of fumarate and nitrate reduction) (13, 20, 21), while the expression of *cyoABCDE* is repressed by FNR under anaerobic growth conditions (6). Except for the involvement of FNR, the overall regulation of these two operons for the terminal electron transport system remained unsolved.

\* Corresponding author. Mailing address: Department of Frontier Bioscience, Hosei University, Kajino-cho 3-7-2, Koganei, Tokyo 184-8584, Japan. Phone and fax: 81-42-387-6231. E-mail: aishiham@hosei.ac.jp.

<sup>∇</sup> Published ahead of print on 18 May 2007.

In this study, we performed a detailed analysis of the regulation of *ndh* and *cyoABCDE* promoters. Results described herein demonstrate that the *ndh* and *cyoABCDE* operons are under the direct control of PdhR. We then propose that PdhR is a master regulator of the genes involved in the main pathway of energy production, starting with the PDH system for the transfer of pyruvate, the final product of glycolysis, into the TCA cycle, followed by the Ndh-Cyo system for terminal electron transport from NADH to oxygen.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *E. coli* KP7600 (W3110 *lacI<sup>q</sup> lacZΔM15*) (30) and JD20368 (*pdhR* deletion derivative of KP7600), used for the analysis of the regulatory roles of PdhR, were gifts from T. Miki (Fukuoka Dental College). The *pdhR* disruptant JD20368 was constructed after transposon insertion and was kanamycin resistant. *E. coli* KP74201 (KP7600 *λcyoA::lacZ*) and KP74202 (JD20368 *λcyoA::lacZ*), used for the single-copy assay of the *cyoA* promoter, were constructed according to a method described previously by Simons et al. (32). In brief, pRScyoA-Cm was recombined with phage λRS45, and the recombinant phages were lysogenized into KP7600 or JD20368 to isolate KP74201 or KP74202, respectively, on an LB agar plate with chloramphenicol. Plasmid pRScyoA-Cm was constructed from plasmid pRScyoA carrying the *cyoA* promoter between EcoRI and BamHI sites on pRS551, a *lacZ* reporter assay vector, after the replacement of its Km-resistant gene with a chloramphenicol (Cm)-resistant gene.

*E. coli* BL21(DE3) [*F<sup>-</sup> ompT hsdSB(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) dcm gal* (DE3)] was used for the overproduction of PdhR. Cells were grown in LB medium at 37°C with shaking. When necessary, ampicillin was added at a final concentration of 100 μg/ml.

**Purification of His-tagged PdhR protein.** His-tagged PdhR was overexpressed in *E. coli* BL21(DE3) using expression vector pPdhR and purified by affinity chromatography as described previously (37, 38).

**SELEX search for PdhR-binding sequences.** The genetic SELEX method was performed as described previously (24, 30). A collection of 200- to 300-bp DNA fragments of the *E. coli* genome was prepared by PCR amplification using the *E. coli* DNA library plasmids as templates and a set of primers, primer-EcoRV-F (5'-CTTGGTTATGCCGGTACTGC-3') and primer-EcoRV-R (5'-GCGATGCTGTCGGAATGGAC-3'), which hybridize with vector pBR322 at EcoRV junctions. PCR products thus generated were purified by 6% polyacrylamide gel electrophoresis (PAGE).

For the genomic SELEX screening of PdhR-binding sequences, 5 pmol DNA fragments and 20 pmol His-tagged PdhR were mixed in a binding buffer (10 mM Tris-HCl [pH 7.8 at 4°C], 3 mM Mg acetate, 150 mM NaCl, 1.25 μg/ml bovine serum albumin [BSA]) and incubated for 30 min at 37°C. The mixture was applied onto a Ni-nitrilotriacetic acid column, and after washing unbound DNA with the binding buffer containing 10 mM imidazole, DNA-PdhR complexes were eluted with an elution buffer containing 200 mM imidazole. If necessary, this SELEX cycle was repeated several times. For sequencing of PdhR-bound DNA fragments, DNA fragments were dissociated, isolated from DNA-PdhR complexes by PAGE, and PCR amplified. PCR products were cloned into the pT7 Blue-T vector (Novagen) using a blunt-end cloning kit (Takara) and transformed into *E. coli* DH5α. Fluorescently labeled DNA was prepared using 2T7P-primer (5'-TAATACGACTCACTATAGGG-3'), and sequencing was performed with an ABI DNA sequencer.

**Plasmid construction.** The promoter assay vector pGRP carries two types of fluorescent protein genes, one for red fluorescent protein (RFP) under the control of reference promoter *lacUV5* and the other for green fluorescent protein (GFP) under the control of a test promoter (19, 31). The *ndh* promoter sequence upstream from the initiation codon was amplified by PCR using the genomic DNA from *E. coli* KP7600 as a template and a pair of primers, K701S (5'-ACATCTGAAGAGATCTATCATTATTACGAG-3') and K701T (5'-TCAATGGCGTATGCATCGTGACCCCTTAA-3'), while the *cyoA* promoter sequence was amplified by PCR using a pair of primers, N073S (5'-CGATGCATCATTTAACGACTCAATTCCACGG-3') and N073T (5'-GAAGATCTCGTGCTTGGTGGTTTGTGG-3'). These primers contain EcoT22I, BglII, or BamHI sites suitable for cloning into vector pGRP at the initiation codon of the GFP coding frame. The PCR products were digested with restriction enzymes and then ligated into the EcoT22I and BglII sites of pGRP. The sequences of the inserted promoter and junction with the GFP coding frame were confirmed by

sequencing. The plasmids thus constructed were named pGRK701 and pGRN073, respectively.

For the construction of plasmid pPdhR for PdhR expression, a DNA fragment corresponding to the PdhR coding region was amplified by PCR using the *E. coli* KP7600 genome DNA as a template and a pair of primers, PDHRF (5' to 3' direction) and PDHRR (3' to 5' direction), which were designed to express the full-length PdhR from the initiation codon to the termination codon. Primer PDHRF contained the BamHI recognition sequence and the His tag sequence in this order, while primer PDHRR contained the NotI recognition sequence. After digestion with BamHI and NotI, the PCR product was cloned into pET21a(+) (Novagen) at the corresponding sites.

**Measurement of the promoter activity.** Promoter strength was determined as described previously (19, 30). In brief, RFP was expressed under the control of test promoter *lacUV5*, while GFP was under the control of a test promoter. For measurement of the fluorescence intensity of RFP or GRP expressed in transformed *E. coli*, cells were grown in LB medium at 37°C for various lengths of time and harvested by centrifugation. Cells were resuspended in phosphate-buffered saline and diluted with phosphate-buffered saline to obtain approximately the same cell density (optical density at 600 nm [OD<sub>600</sub>] of 0.6) for all samples. For measurements of bulk fluorescence, aliquots of a 0.2-ml cell suspension were added to 96 0.4-ml flat-bottom wells, and the fluorescence was measured with a Wallace 1420 ARVox apparatus (Perkin-Elmer Life Sciences). GFP was measured using 485-nm excitation and 535-nm emission wavelengths, while RFP was measured using 544-nm excitation and 590-nm emission wavelengths, respectively. The fluorescence intensity of GFP by test promoter was normalized using the equation  $[X/Y]/[A/B]$ , in which X and Y indicate the fluorescence intensities of GFP (test promoter) and RFP (*lacUV5* promoter), respectively, while A and B represent the fluorescence intensities of GFP (*lacUV5* promoter) and RFP (*lacUV5* promoter), respectively. Promoter assays were repeated at least twice for confirmation.

**Gel shift assay.** Probes were generated by PCR amplification of the *ndh*, *cyoA*, *yfiD*, and *pdhR* promoter regions (approximately 500 bp from the respective initiation codon) using a pair of primers, 5' fluorescein isothiocyanate (FITC)-labeled T7-pro-primer (5'-TAATACGACTCACTATAGGG-3') and T7-R primer (5'-GGTTTCCCAGTCACACGACG-3'), the genomic SELEX plasmids containing the respective promoters as templates, and Ex *Taq* DNA polymerase. PCR products with FITC at their termini were purified by PAGE. For gel shift assays, 0.5 pmol each of the FITC-labeled probes was incubated at 37°C for 30 min with various amounts of PdhR in 12.5 μl of gel shift buffer consisting of 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 3 mM Mg acetate, and 25 mg/ml BSA. After the addition of the DNA dye solution (40% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol), the mixture was directly subjected to 6% polyacrylamide gel electrophoresis.

**DNase I footprinting assay.** A DNase I footprinting assay was carried out using the FITC-labeled DNA probes used for the gel shift assay. A total of 1 pmol each of the FITC-labeled probes was incubated at 37°C for 30 min with various amounts of PdhR in DNase I footprinting buffer consisting of 25 μl of 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 3 mM magnesium acetate, 5 mM CaCl<sub>2</sub>, and 25 μg/ml BSA. After incubation for 30 min, DNA digestion was initiated by the addition of 5 ng of DNase I (Takara). After digestion for 30 s at 25°C, the reaction was terminated by the addition of 45 μl of DNase I stop solution (20 mM EDTA, 200 mM NaCl, 1% sodium dodecyl sulfate, 250 μg/ml yeast tRNA) to the mixture. Digested products were precipitated with ethanol, dissolved in formamide dye solution, and analyzed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

## RESULTS

### Isolation of PdhR-binding sequences by genomic SELEX.

For the identification of DNA sequences that are recognized by *E. coli* PdhR, we used the genomic SELEX method (24, 30), which uses a complete library of *E. coli* genome DNA fragments, instead of synthetic oligonucleotides, with all possible sequences used with the original SELEX method (33). First, we constructed the plasmid library, each carrying a piece of the size-fractionated DNA fragment (100 to 300 bp in length) from a pool of sonicated *E. coli* W3110 genome DNA. In each SELEX experiment, the fragment mixture was regenerated after amplification of the inserted DNA fragments by PCR. From mixtures of these DNA fragments and a fourfold molar

TABLE 1. PdhR-bound DNA fragments<sup>a</sup>

Group and no. of clones	Upstream gene	SELEX fragment	Downstream gene
<b>A (SELEX fragments within spacer regions)</b>			
48	<b><i>aroP</i></b> ←	(0122011) S (0122303)	→ <b><i>pdhR</i></b>
14	<b><i>cyoA</i></b> ←	(0450896) S (0451132)	← <b><i>ampG</i></b>
3	<b><i>yfiD</i></b> ←	(2714930) S (2715311)	→ <b><i>ung</i></b>
2	<b><i>ycfD</i></b> →	(1167498) S (1167753)	→ <b><i>ndh</i></b>
<b>B (SELEX fragments on open reading frames)</b>			
2	<b><i>wcaA</i></b> ←	(2135986) S ( <i>wzc</i> (2136196)	← <b><i>wzb</i></b>
1	<b><i>ygeD</i></b> ←	(2974235) S ( <i>aas</i> (2974451)	→ <b><i>galR</i></b>
1	<b><i>degP</i></b> →	(0182534) S ( <i>cdsA</i> (0182770)	← <b><i>yaeH</i></b>
1	<b><i>ybiS</i></b> ←	(0857353) S ( <i>ybiT</i> (0857554)	← <b><i>ybiU</i></b>
1	<b><i>yhiD</i></b> →	(3964400) S ( <i>yhiE</i> (3964580)	← <b><i>yhjG</i></b>

<sup>a</sup> Using the genomic SELEX method, a total of 73 DNA fragments have been isolated as complexes with the purified PdhR protein, which were all cloned into the sequencing vector. Group A clones contained the sequences from spacer regions between the indicated neighboring genes, while group B clones carried the sequences within the indicated coding frames. The numbers on both sides of each SELEX (S) fragment indicate the boundaries in the *E. coli* genome map (27). The arrows indicate the direction of transcription of the neighboring genes. The genes shown in boldface type indicate the PdhR regulation targets.

excess of the purified His-tagged PdhR protein, the PdhR-bound DNA complexes were affinity purified. In the early period of SELEX cycles, the PdhR-bound DNA fragments gave smear bands on PAGE, as did the original genome fragment mixture. After four SELEX cycles, however, the width of the gel band decreased, indicating enrichment of specific DNA fragments with PdhR-binding activity. The SELEX DNA fragments were recovered from the gel and cloned into the pT7

Blue plasmid (Novagen) for sequencing (see Materials and Methods).

A total of 73 independent clones were isolated, sequenced, and classified into two different groups. Group A included 67 independent clones, which had unique sequences from four different spacer regions between two neighboring genes in the *E. coli* genome, i.e., 48 clones from the *aroP*-*pdhR* spacer, 14 clones from the *cyoA*-*ampG* spacer, 3 clones from the *yfiD*-*ung* spacer, and 2 clones from the *ycfD*-*ndh* spacer (Table 1). On the other hand, group B included six clones, which carried parts of five protein-coding sequences, i.e., *wzc*, *aas*, *cdsA*, *ybiT*, and *yhiE* (Table 1). Since these sequences in the group B clones are widely separated from transcription initiation sites, it is unlikely that the group B sequences play some regulatory roles in transcription initiation, but possible influence of group B sequence-bound PdhR on transcription elongation cannot be ruled out. For the identification of the target genes under the control of PdhR, a detailed analysis has been focused on the group A sequences.

Judging from the orientation of the genes included in the group A clones, we predicted that the promoters for the *aroP*, *pdhR*, *cyoA*, *yfiD*, *ung*, and/or *ndh* gene could be the regulation targets of PdhR (Table 1). A total of 48 independent SELEX clones carried the sequences, which are located within the narrow 293-bp-long region between *E. coli* genome positions 122011 and 122303 (the location is based on the revised *E. coli* K-12 genome sequence) (26), upstream of *aroP* and *pdhR* (*aroP* and *pdhR* are transcribed divergently towards opposite directions). The target of the PdhR box within this region has previously been found to be the *pdhR*-*aceEF*-*lpaA* operon (15, 26).

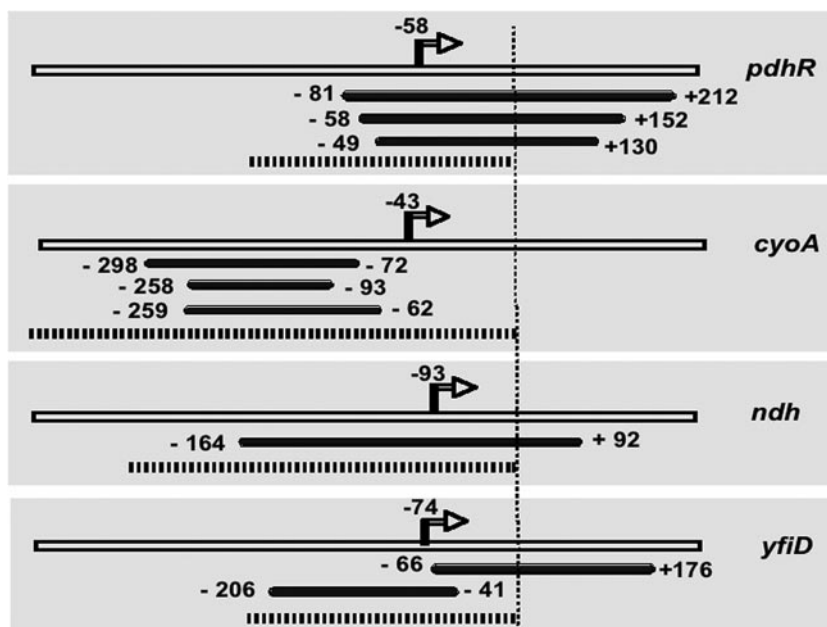


FIG. 1. PdhR-bound DNA fragments. Using genomic SELEX, PdhR-bound DNA fragments were isolated, the indicated four groups of which contained the promoter regions from the *pdhR*, *cyoA*, *ndh*, and *yfiD* genes. Open bars indicate the genomic DNA sequence, while thick bars indicate the regions of fragments isolated by SELEX. The dotted lines show the DNA fragments used for gel shift and DNase I footprinting assays. The vertical line shows the translation initiation site. Numbers on each line represent the distance (bp) from the respective initiation codon. Transcription initiation sites, shown by arrows, are from *pdhR* (25), *cyoA* (22), *ndh* (13), and *yfiD* (12).



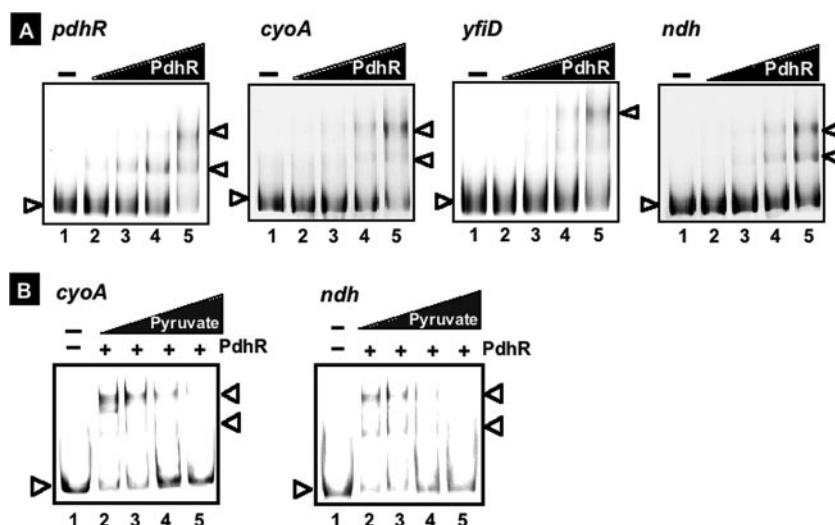


FIG. 2. Gel shift assay. (A) Fluorescently labeled DNA probes, each containing a segment of the *pdhR*, *cyoA*, *yfiD*, or *ndh* promoter (for the region of each probe, see Fig. 1), were incubated at 37°C for 30 min with the indicated amounts (0, 0.625, 1.25, 2.5, and 5 pmol) of PdhR (lanes 1 to 5) and directly subjected to PAGE. (B) Fluorescently labeled DNA probes of *cyoA* or *ndh* were incubated at 37°C for 30 min in the absence (lane 1) or presence (lanes 2 to 5) of 5 pmol of PdhR and in the absence (lane 2) or presence (lane 3, 100 mM; lane 4, 200 mM; lane 5, 500 mM) of increasing concentrations of pyruvate. Samples were subjected directly to PAGE.

Three SELEX fragments included the sequences from 382-bp-long region of the genome at positions 2714930 to 2715311, upstream of *yfiD* (putative formate acetyltransferase) and *ung* (uracil-DNA glycosylase) (Table 1). YfiD is one of the acid-inducible proteins with unidentified function. Between the two regulation targets *yfiD* and *ung*, PdhR has been indicated to be an anaerobic repressor of *yfiD* (36). The PdhR-binding sequence (cAaTGGTtttACCAATT, 12/14 match with the consensus [nonconsensus bases are in lowercase type]) within this region is similar to the consensus PdhR box.

A total of 14 group A clones carried segments within the 237-bp-long sequence upstream of the *cyoA* (cytochrome *bo*-type oxidase) gene, where PdhR binding has never been shown, although the presence of the PdhR box had been predicted (25), and two other clones included segments from the 256-bp-long sequence upstream of the *ndh* (NADH dehydrogenase II) gene (Table 1), where the binding of PdhR has never been detected. These results indicate that PdhR plays a key regulatory role in not only the formation of the PDH complex for the metabolic interconnection between glycolysis and the TCA cycle but also the synthesis of two key enzymes (Ndh and CyoA) in the terminal electron transport system.

**Identification of PdhR-binding activity for SELEX DNA fragments.** The group A SELEX clones contain promoter regions for the *pdhR-aceE-aceF* operon, the *cyoABCDE* operon, the *ndh* gene, and the *yfiD* gene (Fig. 1). To confirm the presence of a PdhR-binding site in these four promoter regions, we performed a gel shift assay. Under standard conditions, we detected the PdhR-dependent gel shift for all the promoter fragments (Fig. 2A).

Previous studies on the regulation of the *pdhR-aceE-aceF* operon indicated that pyruvate prevents PdhR binding to the promoter of this operon, as detected by gel retardation assays (26). The same was found in the gel shift assays with the *ndh* and *cyoABCDE* promoter fragments, where the formation of

PdhR-DNA complexes was reduced concomitantly with an increase in the pyruvate addition (Fig. 2B).

**Identification of PdhR-binding sequences on the *ndh* and *cyoABCDE* promoters.** The PdhR-binding sites on the newly identified *cyoABCDE* and *ndh* promoter fragments were then examined by DNase I footprinting. PdhR was found to bind to a 26-bp sequence between positions -18 and -43 upstream of the *ndh* transcriptional start position (Fig. 3) and to a 28-bp sequence between positions -158 and -185 upstream of the *cyoABCDE* transcriptional start position (Fig. 4A and B). In both PdhR-binding sequences, there is a common 15-bp ATTGGTNNNACCAAT sequence, which includes an inverted ATGGGT repeat, referred to as the PdhR box (Fig. 5). The gel shift assay indicated the formation of two kinds of PdhR complexes with these promoter fragments (Fig. 2). One possible explanation is that two molecules of PdhR bind to each half of the inverted repeat of the PdhR box. Previously, Quail et al. (26) reported that on the *pdhR-aceEF* operon promoter, PdhR binds to the sequence between positions +8 and +28 on the coding strand and positions +10 and +33 on the noncoding strand downstream from transcriptional start site (+1). This sequence also includes the inverted repeat sequence AATTGGTNNNACCAATT of the PdhR box.

The site of PdhR binding on the *ndh* promoter overlaps with its -35 region of the promoter in concert with the repression mode of PdhR. On the other hand, the PdhR-binding site on the *cyoA* promoter is far from the previously reported transcription initiation site (22). We then examined the transcription initiation site of the *cyoA* operon in the strains used. Primer extension assays indicated the presence of only a single initiation site at the same position with that previously identified (Fig. 4C).

**PdhR-dependent repression of *cyoABCDE* and *ndh* transcription in vivo.** The *pdhR-aceEF* operon is repressed by PdhR and derepressed by pyruvate, the substrate of the

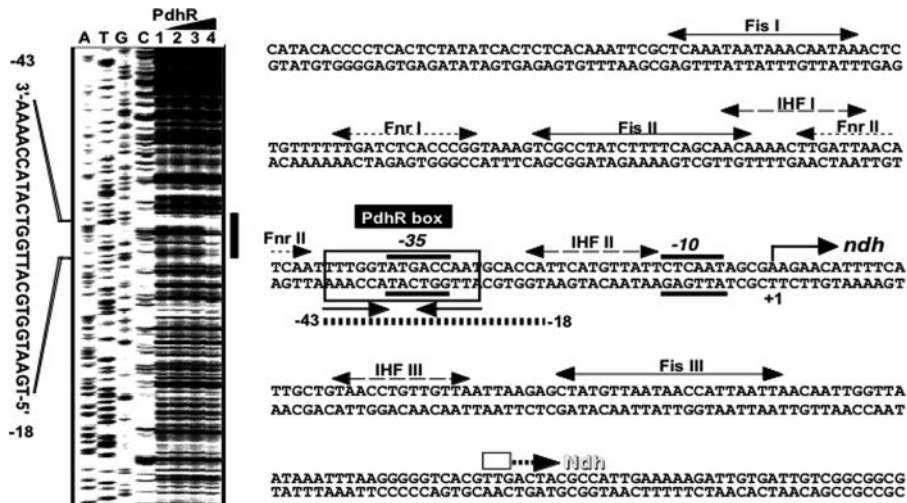


FIG. 3. DNase I footprinting of the *ndh* promoter. (Left) The fluorescently labeled *ndh* promoter (Fig. 2) was incubated with increasing amounts of purified PdhR (lane 1, 0 pmol; lane 2, 2.5 pmol; lane 3, 5 pmol; lane 4, 10 pmol) and subjected to DNase I footprinting assays. Lanes A, T, G, and C represent the sequence ladders. The black bar on the right indicates the PdhR-binding region. (Right) The PdhR-binding site on the *ndh* promoter, determined as described above, is indicated by a thick bar between positions -43 and -18. The transcription initiation site according to Green and Guest (13) is marked by an arrow. Recognition sequences for Fis, FNR, and IHF (boxed) were described previously by Jackson et al. (18), Meng et al. (21), and Green et al. (11), respectively.

PDH complex (26). To examine the mode of transcription regulation of *ndh* and *cyoABCDE* by PdhR in vivo, we employed the two-fluorescent-protein (TFP) promoter assay system (19, 31). The *cyoABCDE* and *ndh* promoters were

inserted into the TFP vector so as to adjust the *cyoA* or *ndh* initiation codon to that of GFP (see Materials and Methods), while the RFP gene on the same plasmid was expressed under the control of the reference promoter

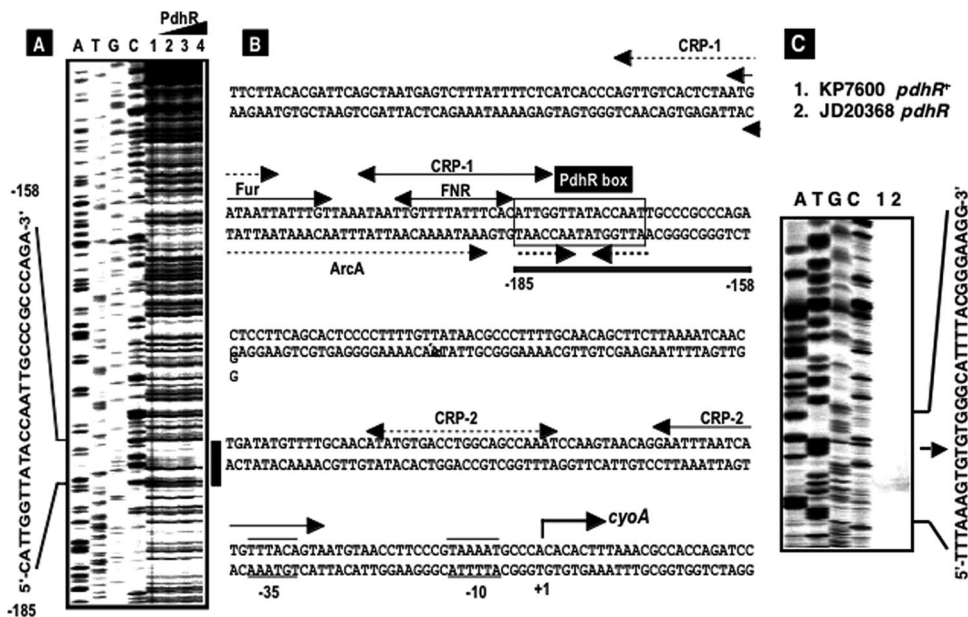


FIG. 4. DNase I footprinting of the *cyoA* promoter. (A) The fluorescently labeled *cyoA* promoter was incubated with increasing amounts of purified PdhR (lane 1, 0 pmol; lane 2, 2.5 pmol; lane 3, 5 pmol; lane 4, 10 pmol) and subjected to DNase I footprinting assays. Lanes A, T, G, and C represent sequence ladders. (B) The black bar (positions -185 to -158) on the right indicates the PdhR-binding region located upstream of the *cyoA* promoter. The nucleotide numbers represent the distance from the transcription initiation site, which was proposed previously by Minagawa et al. (22) and confirmed in this study. Recognition sequences for Fur, ArcA, and Fnr were predicted based on sequence analyses reported previously by Stojiljkovic et al. (35), Shalel-Levanon et al. (29), and Salmon et al. (28), respectively. Two CRP-binding sequences, CRP1 and CRP2, were proposed previously, but at different positions, by Minagawa et al. (22) (dotted lines) and Zheng et al. (40) (solid line). (C) Primer extension assay of the transcription initiation site of the *cyoA* operon in both wild-type KP7600 and its *pdhR* disruptant, JD20368, was performed using a fluorescently labeled probe. Data for the site identified thus agreed with those reported previously by Minagawa et al. (22).

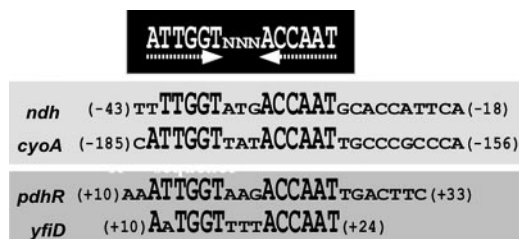


FIG. 5. Consensus sequence for the PdhR box. The PdhR-binding sites are derived from the *ndh* and *cyoA* promoters determined in this study, the previously reported *pdhR* promoter (25), and the predicted *yfiD* promoter (36). After searching the PdhR-box-like sequence along the entire *E. coli* genome, three additional targets have been identified: *ybaJ*, with the sequence (480189)ATTGGTgacActAAT(480203); *hemL* or *clcA*, with the sequence (175079)ATTNGTNNNACCAAT(175093); and *lldP*, with the sequence (3863230)ATTGGNNNNACCAAT(3863216).

*lacUV5*. The promoter activity can be accurately determined by measuring the GFP/RFP ratio.

The promoter assays were carried out with both the wild type and a mutant lacking PdhR under both aerobic and anaerobic conditions. As expected, the activities of both *ndh* and *cyoA* promoters in the wild-type strain decreased under anaerobic growth conditions (Fig. 6A and C, lanes 1 and 2). In the *pdhR* deletion strain, the activities of both promoters increased under both anaerobic and aerobic conditions (Fig. 6A and C, lanes 3 and 4), supporting the repression model of these operators by PdhR. In particular, *ndh* promoter activity increased more than fourfold in the *pdhR* mutant (Fig. 6A, lanes 3 and 4). In the wild-type strain, the addition of pyruvate markedly

increased the activity of the *ndh* promoter due to the inactivation of PdhR (Fig. 6B, lane 5). This pyruvate-induced derepression of *ndh* was significant under aerobic conditions (Fig. 6B, lane 5), where the respiratory electron transport system operates. The derepression of *ndh* was also observed with the addition of glucose (Fig. 6B, lanes 3 and 4). Under anaerobic conditions, the pyruvate- and glucose-induced derepressions were not so significant (Fig. 6B, lanes 2, 4, and 6). This is in good agreement with the previous observation that in *E. coli*, the expression of *ndh* is repressed under anaerobic growth conditions (13, 20). In contrast, the activity of the *cyoABCDE* promoter stayed unaffected even in the presence and absence of pyruvate and glucose (Fig. 6D). Under the culture conditions herein employed, i.e., shaking culture in LB medium, the activity of *cyoABCDE* must be activated even in the absence of a glucose or pyruvate addition.

The promoter assay was also carried out using a single-copy fusion on the chromosome. The *cyoA* promoter-*lacZ* fusion was inserted into both wild-type KP7600 and its *pdhR* mutant, JD20368, to generate KP74201 and KP74202, respectively, as described in Materials and Methods. In agreement with the results obtained using the multicopy promoter assay, *cyoA* promoter activity increased in the *pdhR* disruption mutant, as measured by  $\beta$ -galactosidase activity (Fig. 6E).

## DISCUSSION

In the energy production pathway of *E. coli* under aerobic conditions, pyruvate, the terminal product of glycolysis, is oxidatively decarboxylated by the PDH multienzyme complex to produce  $\text{CO}_2$  and acetyl-CoA, with the concomitant

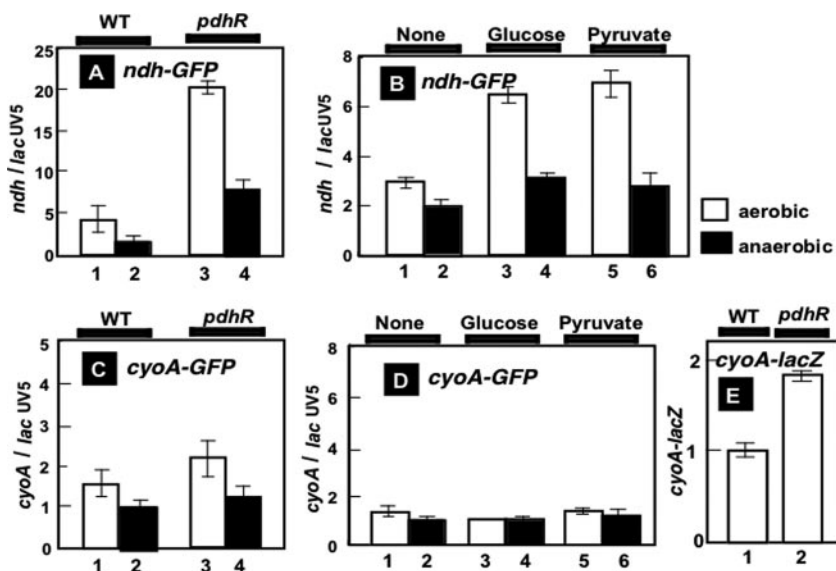


FIG. 6. Effect of PdhR on the *ndh* and *cyoABCD* promoters. (A and B) The promoter assay vectors containing the *ndh* or *cyoABCD* promoters were transformed into wild-type (KP7600) and *pdhR* deletion mutant (JD20368) cells and grown in LB medium under aerobic (open bars) and anaerobic (closed bars) conditions. Promoter activities were determined at a cell density of an  $\text{OD}_{600}$  of 0.6 and are represented as GFP/RFP ratios. (C and D) Wild-type cells and *pdhR* mutant transformants were grown in LB in the absence (lanes 1 and 2) or presence of 10 mM glucose (lanes 3 and 4) or 40 mM pyruvate (lanes 5 and 6) and under the aerobic (open bars) and anaerobic (closed bars) conditions. Promoter activities were determined at a cell density of an  $\text{OD}_{600}$  of 0.2. (E) The activity of a single-copy *cyoA* promoter-*lacZ* fusion in wild-type (KP74201) (lane 1) and *pdhR* mutant (KP74202) (lane 2) cells was determined by measuring  $\beta$ -galactosidase activity at a cell density of an  $\text{OD}_{600}$  of 0.6 under aerobic conditions.



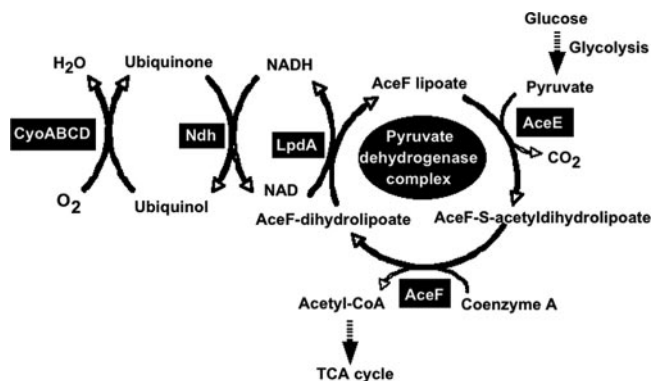


FIG. 7. Role of PdhR as a master regulator of the metabolic pathways including both NAD-linked oxidative decarboxylation of pyruvate to acetyl-CoA and respiratory electron transport of NADH to oxygen.

reduction of  $\text{NAD}^+$  (9). The electron flow into respiration follows alternative routes to oxygen via an NADH dehydrogenase coupled or uncoupled to quinone (1, 9, 16). *E. coli* is capable of synthesizing two quinones, ubiquinone-8 and menaquinone-8 (17). NADH dehydrogenase II transfers electrons to ubiquinone-8 to form ubiquinol-8 (Fig. 7). The cytochrome *bo*-type oxidase (*cyoABCDE* gene) transfers electrons from ubiquinol-8 to oxygen, exporting two protons per electron (16, 17).

The expression of the genes for metabolic energy production is influenced by the nature of the available terminal electron acceptor. For instance, the expression of *cyoA* is repressed in the absence of oxygen as the terminal electron acceptor under anaerobic conditions. The anaerobic repression of *cyoA* is known to be mediated, in part, by Fnr, as evidenced by a derepression of *cyoA'*-*lacZ* expression in an *fnr* deletion strain (6). Using the genomic SELEX system followed by *in vitro* and *in vivo* transcription studies, we found that PdhR, the regulator of the genes for the PDH complex, regulates both the *ndh* gene and the *cyoABCDE* operon (Fig. 7). The *ndh* gene product (NADH dehydrogenase II) and the *cyoABCDE* gene products (cytochrome *bo*-type oxidase) are involved in the pathway of energy production downstream of the PDH pathway. Thus, we propose that PdhR is a master regulator in the entire pathway of oxidative energy production.

In this study, we identified the PdhR-binding site (PdhR box) on the *cyoABCDE* operon (between positions  $-158$  and  $-185$  upstream of transcriptional start position) near the upstream cyclic AMP receptor protein (CRP)-binding site (Fig. 4) (two different CRP1 sites have been predicted) (22, 40). The same site was detected previously in database searches for PdhR boxes by Quail and Guest (25), who suggested that the *cyoABCDE* operon might be regulated by PdhR. *cyoABCDE* promoter activity increased in a *pdhR* deletion strain under aerobic conditions (Fig. 4C and 6B). In general, the repression of transcription initiation by transcription factors takes place when the repressors bind at or downstream of the target promoters and interfere with either the binding of the RNA polymerase or its escape from the promoter complexes. In some instances, however, the upstream-bound repressors interact with the promoter-bound RNA polymerase and prevent its migration from promoters or even induce its dissociation from

promoter complexes (37, 38). However, it should be noted that in addition to Fnr and CRP, ArcA was also proposed to associate near the PdhR site on the *cyoA* promoter (29) (Fig. 4); the mode of transcription regulation of the *cyoA* promoter must be complex. For instance, the apparent repression by PdhR could be due to an interference of activation by another factor such as CRP. The repression effect by PdhR was, however, not so strong under anaerobic conditions (Fig. 6B), implying that Fnr plays a major role in the repression of the *cyoA* gene under anaerobic conditions. However, it remains unsolved whether Fnr plays a direct role in the transcription regulation of the *cyoABCDE* operon (28).

On the other hand, NADH dehydrogenase II is a primary dehydrogenase used in *E. coli* during both aerobic and nitrate respiration. Under anaerobic conditions, the transcription factor Fnr represses *ndh* expression by binding at two sites centered at positions  $-94.5$  and  $-50.5$  (13, 21) (Fig. 3). The histone-like protein Fis binds to the three sites (centered at positions  $-123$  [Fis I],  $-72$  [Fis II], and  $+51$  [Fis III]) in the *ndh* promoter (10, 11). Using *ndh::lacZ* promoter fusions carrying 5' deletions or replacement mutations, it was shown that Fis that binds to site III functions as a repressor, while Fis that binds to sites I and II plays a role as an activator (18). Deletion of the C-terminal domain of the RNA polymerase  $\alpha$  subunit abolished Fis-mediated activation of *ndh* expression, suggesting that *ndh* has a class I Fis-activated promoter (18). In accordance with the established pattern of Fis synthesis (2), *ndh* transcription was greatest during exponential growth (18). In the absence of Fnr, *ndh* expression is activated by the amino acid response regulator (Arr) during anaerobic growth in rich medium (11). Integration host factor (IHF) was shown to bind at three sites centered at positions  $+26$  (IHF I),  $-17$  (IHF II), and  $-58$  (IHF III) in the *ndh* promoter (11). In this study, we identified the PdhR-binding site on the *ndh* promoter (between positions  $-18$  and  $-43$  upstream of the transcriptional start position), which overlaps with the  $-35$  region of the promoter (Fig. 3), and *ndh* promoter activity increased in the *pdhR* deletion strain under both aerobic and anaerobic conditions (Fig. 6A). The promoter  $-35$  and  $-10$  regions are recognized by RNA polymerase-associated  $\sigma^{70}$  and isomerized into transcription-competent open promoter complexes. The marked repression of *ndh* gene expression by PdhR might be due to the competitive inhibition of RNA polymerase binding to the promoter.

Recent genetic and biochemical analyses with an  $F_1$ -ATPase-defective mutant (enhanced glucose metabolism) indicated that the upregulation of the PDH complex by pyruvate takes place in both transcription and enzyme activities (23). Interestingly, in the same mutant, the expression level of *ndh* was significantly elevated, and *cyoA* expression also showed a tendency toward upregulation, even though the effect was not statistically significant because of the low level of activation. The  $F_1$ -ATPase-defective mutant was constructed by the transduction of a defective gene for the  $\alpha$  subunit of  $F_1$ -ATPase into an *E. coli* strain, a lipoic acid-requiring pyruvate producer (39). The pyruvate production in this  $F_1$ -ATPase-defective strain was found to be improved markedly compared with that of the wild-type strain. Taken together with our observations reported here, these results suggest that the increased level of pyruvate leads to the inactivation of the repressor PdhR, lead-

ing to the derepression of the *ndh* and *cyoA* promoters in the F<sub>1</sub>-ATPase-defective strain.

#### ACKNOWLEDGMENTS

We thank Takenori Miki (Fukuoka Dental College) for the *pdhR* disruptant and Ayako Kori (Hosei University) for technical assistance.

This work was supported by grants-in-aid (Scientific Research Priority Area 17076016; Scientific Research 18310133) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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