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Sequence Analysis and Functional Expression of the Structural and Regulatory Genes for Pyruvate Dehydrogenase Complex from *Streptomyces seoulensis*

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A cluster of genes encoding the pyruvate dehydrogenase complex (PDC) of *Streptomyces seoulensis*, a Gram-positive bacterium, was cloned and sequenced. The genes of *S. seoulensis* consist of four open reading frames. The first gene, *lpd*, which encodes a lipoamide dehydrogenase, is followed by *pdhB* encoding a dihydrolipoamide acetyltransferase (E2p), *pdhR*, a regulatory gene, and *pdhA* encoding a pyruvate dehydrogenase component (E1p). E1p had an unusual homodimeric subunit, which has been known only in Gram-negative bacteria. *S. seoulensis* E2p contains two lipoyl domains like those of humans and *Streptococcus faecalis*. The *pdhR* gene appears to be clustered with the structural genes of *S. seoulensis* PDC. The PdhR-overexpressed *S. seoulensis* showed growth retardation and the decrease of E1p, indicating that PdhR regulates the function of PDC by repressing the expression of E1p. A strain of *Streptomyces lividans* overexpressing *S. seoulensis* PdhR showed a significant decrease in the level of actinorhodin, implying a regulatory role for *Streptomyces* PDC in antibiotic biosynthesis.

Key words: pyruvate dehydrogenase, dihydrolipoamide acetyltransferase, lipoamide dehydrogenase, PdhR, actinorhodin.

Pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate, producing acetyl coenzyme A (CoA) and NADH and releasing CO_2 (Berg and Kok, 1997; Patel and Rawlings, 1997). The complex consists of multiple copies of three different components, pyruvate dehydrogenase component (E1p), dihydrolipoamide acetyltransferase (E2p), and lipoamide dehydrogenase (E3). The complex is structurally similar to the 2-oxoglutarate dehydrogenase complex (OGDC) and the branched-chain 2-oxo acid dehydrogenase complex (BCDC) (Berg and Kok, 1997).

The E2p component forms the structural and functional core of the complex through its characteristic domains (Berg and Kok, 1997): one to three lipoyl domains at the N-terminal region, E1/E3 binding domain mediating noncovalent attachment of the corresponding E1p and E3 components, and a catalytic domain responsible for symmetrical aggregation at the C-terminal region. In all Gram-positive bacteria and eukaryotes studied so far, the inner core of the complex consists of 24 E2p components with a molecular arrangement of icosahedral symmetry, in which the E1p exists as multiple copies of heterotetrameric protein (Berg and Kok, 1997). In contrast, Gram-negative bacteria have a homodimeric E1p component with concomitant formation of octahedral aggregates of PDC (Berg and Kok, 1997). Recently, heterodimeric E1p components have been reported in Gram-negative bacteria, *Zymomonas mobilis* (Neveling *et al.*, 1998) and *Thiobacillus ferrooxidans* (Powles and Rawlings, 1997).

In most bacteria, the *pdh* genes encoding PDC components are organized in a single operon in a manner that the gene for E1p is followed by the gene for E2p and the *lpd* gene encoding E3. However, the *lpd* gene does not always occur as a part of the *pdh* operon, as shown in the case of *Azotobacter vinelandii* (Westphal and Kok, 1998) and *Pseudomonas aeruginosa* (Rae *et al.*, 1997).

PdhR, the negative regulator of the *pdh* genes in *Escherichia coli*, belongs to the GntR family of regulatory proteins (Fujita and Fujita, 1986) and is known to be the proximal gene of the *pdhR-aceE-aceF-lpd* operon encoding PDC (Quail *et al.*, 1994). PdhR represses the transcription from the *pdh* promoter, P_{pdh}. However, the *lpd* gene is transcribed from the independent *lpd* promoter, resulting in 1.7-kb *lpd* transcript (Quail *et al.*, 1994) that

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satisfies the E3 requirements for OGDC and the glycine cleavage system (Guest *et al.*, 1989; Steiert *et al.*, 1990).

Whereas the E3 component of *Streptomyces seoulensis* and its gene have been isolated and characterized in our previous report (Youn *et al.*, 1998), PDC of *Streptomyces* spp. has not been characterized yet. Here, we report the genes encoding the components of *S. seoulensis* PDC and the biochemical characterization of individual components. We also identified a homolog of *E. coli pdhR* in the cluster of the *S. seoulensis pdh* genes and demonstrated the effect of its overexpression on the growth of *S. seoulensis* and on the production of a polyketide pigment, actinorhodin, in *Streptomyces lividans*.

Materials and Methods

Chemicals and enzymes

Restriction endonucleases were purchased from KOSCO (Republic of Korea). T4 DNA ligase was from Promega (Madison, U.S.A.) and phosphotransacetylase was from Roche Molecular Biochemicals (Mannheim, Germany). Anti-mouse IgG antibody-alkaline phosphatase conjugate was from Jackson ImmunoResearch Laboratories (West Grove, U.S.A.). Lipoamide, 2,6-dichloroindophenol (DCIP), ampicillin, chloramphenicol, kanamycin, thiostrepton, pyruvate, α-ketobutyrate, α-ketoglutarate, α-ketoisovalerate, thiamine pyrophosphate (TPP), acetylphosphate, CoA, Brilliant blue R-250, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, bovine serum albumin, and phenylmethylsulfonylfluoride (PMSF) were from Sigma (St. Louis, U.S.A.). Dihydrolipoamide was prepared by the reduction of lipoamide with sodium borohydride as described previously (Reed *et al.*, 1958).

Bacterial strains, plasmids, and growth conditions

A variety of solid media including R2YE containing 5% sucrose (Hopwood *et al.*, 1985), modified Bennett (Hawkins *et al.*, 1989), nutrient agar (Difco), minimal media (Hopwood *et al.*, 1985) with 0.5% casamino acid as carbon source (MMC) (Hopwood *et al.*, 1985), minimal media with 0.5% glycerol as carbon source (MMGC) (Hopwood *et al.*, 1985), and minimal media with 0.5% glucose as carbon source (MMG) (Hopwood *et al.*, 1985) were used for the culture of *Streptomyces*. Minimal medium consisted of 0.2% $(NH_4)_2SO_4$, 0.06% $MgSO_4 7H_2O$, 5% polyethylene glycol 6000, 1 ml/l of minor elements solution, 150 ml/l of 0.1 M NaH_2PO_4/K_2HPO_4 buffer (pH 6.8), and 0.5% carbon source. Minor elements solution consisted of 0.1% $ZnSO_4 \cdot 7H_2O$, 0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MnCl_2 \cdot 4H_2O$, and 0.1% $CaCl_2$. Liquid media for *Streptomyces* were modified YEME (Youn *et al.*, 1995) and minimal media with 0.375% glucose and 0.125% pyruvate as carbon sources (MMGP) (Hopwood *et al.*, 1985). For *Streptomyces* containing a pIJ702-derived plasmid, thiostrepton was added to a final concentration of 50 μg/ml in solid medium or 5 μg/ml in liquid medium, respectively.

Overexpression of S. seoulensis E1p, E2p, and PdhR in E. coli

For the construction of E1p-overexpression plasmid in *E. coli*, an *Nde*I site was introduced into the *pdhA* translation start codon of *S. seoulensis* using PCR. Then an *Nde*I-*Nco*I fragment containing the *pdhA* open reading frame (ORF) was inserted into pET-32a(+). For the construction of an E2p-overexpression plasmid, 1.8-kb *Nco*I-*Not*I fragment containing *pdhB* was ligated into the *Nco*I-*Not*I fragment of pET-32a(+), giving the expression plasmid pHY201. The PdhR-overexpression plasmid was constructed using pET-3a or pET-32a(+), in which an *Nde*I site or *Nco*I site was introduced into the translation start codon of *pdhR* of *S. seoulensis* using PCR. The fidelity of all the DNA fragments generated by PCR was determined by DNA sequencing. For the expression, each plasmid was introduced into *E. coli* BL21(DE3)pLysS or AD494(DE3) pLysS. When the optical density of 0.6 at 600 nm was reached, the relevant proteins were induced by the addition of isopropyl-β-D-thiogalactoside to the cultures to a final concentration of 1 mM and incubation for additional 2.5 h.

Overexpression of S. seoulensis PdhR and E1p in S. seoulensis

For the overexpression of *phdR* in *S. seoulensis*, a 0.9-kb fragment containing *phdR* and a putative promoter region was introduced into the *Sac*I-*Sph*I sites of pIJ702, a high copy number *Streptomyces* vector, generating pHY301. For the overexpression of E1p in *S. seoulensis*, an *Nde*I-*Not*I fragment containing the whole *S. seoulensis pdhA* ORF was ligated under the *Streptomyces coelicolor* A3 (Berg and Kok, 1997) FESOD1 promoter (Chung *et al.*, 1999) in the pGEM-T easy vector. A *Sac*I-*Sph*I fragment of the resulting plasmid was introduced into pIJ702, generating pHY302. For this experiment, a DNA fragment of the FESOD1 promoter was obtained by genomic PCR using primers, one containing a *Sac*I site and the other containing an *Nde*I site.

Transformation of S. seoulensis

The pIJ702-derived plasmids were cloned in *Streptomyces lividans* and the plasmids isolated from *S. lividans* were introduced into *S. seoulensis*. Protoplast preparation and transformation were carried out according to the method described previously (Hopwood *et al.*, 1985) using R2YE plates containing 5% sucrose for the transformation of *S. seoulensis*.

Enzyme assays

Activity of E1p was measured as the reduction rate of DCIP at 600 nm using 16.1×10^3 $M^{-1} \cdot cm^{-1}$ of molar

absorption coefficient in the presence of pyruvate at 37°C. The standard reaction solution consisted of 0.5 mM pyruvate, 25 μM DCIP in 20 mM sodium phosphate buffer (pH 6.5) containing 0.1 mM TPP and 3 mM $MgSO_4$ (buffer *A*). Specific activity of E1p was defined as U/mg for $\mu mol \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$ deduced from the decrease rate of the absorbance of DCIP at 600 nm. E2p activity was measured spectrophotometrically at 240 nm by the formation of *S*-acetyldihydrolipoamide ($\varepsilon_{240}=5\times 10^3\ M^{-1} \cdot cm^{-1}$) at 37°C, as described previously (Snoep *et al.*, 1992). The reaction mixture contained 4 mM dihydrolipoamide, 10 mM acetylphosphate, 0.1 mM CoA and 2 U phosphotransacetylase in 50 mM Tris-HCl, pH 7.5. The E2p source was added and the increased absorbance at 240 nm measured.

Purification of S. seoulensis E1p

The mycelia were harvested by aspiration on filter paper in a funnel and washed with 0.85% KCl solution. The washed mycelia were suspended in buffer *A* containing 1 mM EDTA and 60 μM PMSF, and then subjected to sonication. Buffers should contain PMSF and EDTA during the disruption of mycelia and TPP and Mg^{2+} during all purification procedures. The cell debris was removed by centrifugation at 10,000×*g* for 30 min. The resulting supernatant was loaded onto a diethylaminoethyl (DEAE) Sephacel previously equilibrated with buffer *A* and washed with buffer *A*, followed by a linear concentration gradient of 0-0.3 M NaCl in buffer *A*. The fractions containing the active compound, which is eluted approximately at 0.2 M NaCl, were precipitated with solid ammonium sulfate. The proteins precipitated at 20-50% ammonium sulfate were collected and resuspended in a small volume of buffer *A*. The concentrated sample was applied to a Superose 12 column and eluted with buffer *A* containing 80 mM NaCl using the FPLC system (Amersham Pharmacia Biotech). Fractions containing E2p activity were pooled and concentrated with an Amicon YM 10 membrane. The concentrated sample was finally applied to a Resource Q column, washed with buffer *A*, and eluted with a linear concentration gradient of 0-0.3 M NaCl in buffer *A*. The active fractions were eluted around 0.15 M NaCl and immediately used for characterization.

Western hybridization analysis

To prepare antibodies against E1p, E2p, E3, and PdhR, each protein was applied to SDS-PAGE. The gel was briefly stained with Brilliant blue R-250 and completely washed with distilled water. The proteins were isolated from gels and injected into mice.

For Western blotting, SDS-PAGE was performed on a 10% polyacrylamide slab gel. The next blotting procedures were carried out according to the methods proposed previously (Towbin *et al.*, 1979).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper have been deposited in the GenBank™/EBI Data Bank under the accession number AF047034.

Results

Isolation and characterization of S. seoulensis pdh genes

In a previous report (Youn *et al.*, 1998), approximately 10-kb genomic DNA fragment was isolated from the *S. seoulensis* genomic DNA library constructed in λEMBL3 using 0.4-kb PCR-amplified fragment of the *lpd* gene as a probe. A frame computer program (Bibb *et al.*, 1984; Wright and Bibb, 1992) of the nucleotide sequence revealed that four streptomycete ORFs are present in the DNA fragment. The deduced amino acid sequences of the ORFs shared strong similarities with those of the structural genes encoding E3, E2p, E1p, and regulators of the GntR family from various sources.

The relevant characteristics of the ORFs are summarized in Table 1. The deduced amino acid sequence of the ORF shared strong similarity with those of E2 components of 2-oxo acid dehydrogenase complexes (Stephens *et al.*, 1983; Spencer *et al.*, 1984; Carlsson and Hederstedt, 1989; Hemila *et al.*, 1990; Allen and Perham, 1991; Wang *et al.*, 1993; Rae *et al.*, 1997) and exhibited 28.2-40.1% identity (39.4-58.5% similarity) to E2 components of prokaryotes and humans.

The gene product contained two repeating units of lipoyl domain [amino acid positions 5 to 76 and 137 to 208], suggesting that the ORF encodes *S. seoulensis* E2p. Up to now, only E2p components among the E2 components of three complexes, PDC, OGDC, and BCDC have

Table 1. Relevant features of the ORFs described in this paper.

ORF	RBS[a]	Start/Stop codons	Putative transcriptional terminator	Numbers of Amino acids/ deduced molecular mass	Measured molecular mass[b]	Predicted/known function
				Da	kDa	
pdhB	^{45}AAGGAG^{54}	ATG^{66}/^{1898}TAA	^{1912}GAC~GTC^{1947}	612/61,367	107	Dihydrolipoamide acetyltransferase
pdhR	^{2197}AAGGAG^{2184}	ATG^{2191}/^{2812}TGA	-	207/22,985	28	Repressor of GntR family
pdhA	^{3048}AGGAAG^{3053}	ATG^{3062}/^{577}TGA	^{5789}GAC~GTC^{5820}	903/98,727	98	Pyruvate dehydrogenase component

[a]RBS represents ribosome binding site.
[b]Molecular mass when the corresponding genes were expressed in *E. coli*.

been known to contain two or three lipoyl domains (Berg and Kok, 1997). Thus the gene was named *pdhB*. The deduced sequence was composed of 22 amino acid residues between positions 318 and 339 was highly homologous to E1/E3 binding regions of E2 components of the three complexes, which were identified based on sequence homology among the E2 components of the three complexes (Hummel *et al.*, 1988). The deduced amino acid sequence of the C-terminal region showed strong homology to those of catalytic domains of E2 components (Guest, 1987).

There was a putative translation start codon (ATG) of the third ORF at position 3,062 preceded by a putative ribosome binding site (AGGAAG) which is 13 nucleotides away from the starting codon. The ORF encodes 903 amino acids with the calculated molecular mass of 98,727 Da. The deduced amino acid sequence of the ORF was highly homologous to those of E1p from Gram-negative bacteria (Stephens *et al.*, 1983; Fleischmann *et al.*, 1995; Hein *et al.*, 1994; Hengeveld *et al.*, 1997; Inoue *et al.*, 1997; Rae *et al.*, 1997), in which its sequence identity was 46.8-51.3%. This high sequence homology suggested that the ORF encodes the E1p, and the gene was named *pdhA*. In amino acid positions 229 to 277, there is a putative TPP binding site, identified by sequence alignment among TPP-requiring enzymes (Hawkins *et al.*, 1989).

However, E1 components of PDCs of all Gram-positive bacteria studied so far possess heterotetrameric subunit composition. This is the first report that a Gram-positive bacterium contains a monomeric E1p composed of a single large subunit.

An ORF was present between *pdhA* and *pdhB* and there is a putative translation start site (ATG) at position 2,191. The ORF encodes a polypeptide of 207 amino acids with a calculated molecular mass of 22,985 Da. The putative product of the ORF in the gene cluster possesses an N-terminal helix-turn-helix motif that was found in the regulatory proteins of the GntR family (Haydon and Guest, 1991), which is similar to the deduced products of *pdhR* encoding the negative regulator of the PDC synthesis in *E. coli* (Haydon *et al.*, 1993), *ntaR* encoding a regulatory protein in *Chelatobacter heintzii* (Knobel *et al.*, 1996), *gntR* encoding the repressor of the gluconate operon of *B. subtilis* (Fujita and Fujita, 1986), and *fadR* encoding the fatty acid metabolism regulator of *E. coli* (DiRusso *et al.*, 1992). Since the ORF was clustered with the structural genes of *S. seoulensis* PDC and the putative product of the ORF is a member of GntR family of regulators, we suggest that the protein is a regulatory protein involved in the regulation of the gene expression of *S. seoulensis* PDC, and was named *pdhR*.

Overexpression of S. seoulensis E1p, E2p, and PdhR in E. coli

Recombinant proteins of E1p, E2p, and PdhR were overexpressed and analyzed by SDS-PAGE. Each plasmid resulted in the overproduction of the corresponding protein. When estimated by SDS-PAGE, the subunit molecular masses of the recombinant E1p (rE1p), recombinant E2p (rE2p), and recombinant PdhR (rPdhR) were 98, 107, and 28 kDa, respectively (Table 1). While rE1p and rPdhR accumulated in significant amounts in the inclusion body of recombinant *E. coli*, rE2p was accumulated in the cytoplasm as wholly soluble proteins.

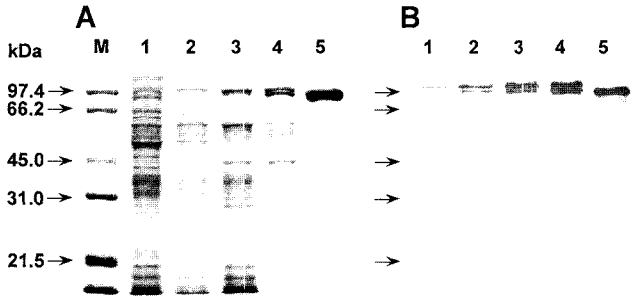

Fig. 1. Purification of E1p from E1p-overexpressed *S. seoulensis*. (A) 10% SDS-PAGE: lane M, molecular mass marker as indicated; lane 1, cell-free extract; lane 2, DEAE Sephacel fraction; lane 3, 20-50% ammonium sulfate precipitation fraction; lane 4, Superose 12 fraction; lane 5, Resource Q fraction. (B) Western hybridization analysis. The same samples as (A) except marker were subjected to SDS-PAGE and subsequent immunological analysis with anti-E1p serum.

Purification of S. seoulensis E1p

The deduced product of *pdhA* shared striking similarity to the other homodimeric E1p components. To investigate the biochemical characteristics of the *S. seoulensis* E1p, we overexpressed its gene in *S. seoulensis* (HY302 strain; transformant of the plasmid, pHY302) and purified the E1p. The strain produced approximately 10 times the amount of E1p compared to the control strain, HY702, when detected by Western hybridization analysis. Since the E1p activity in the crude extract was not measurable due to the high background of pyruvate-independent DCIP-reducing activity, we used Western hybridization analysis using anti-E1p serum as an alternative assay. Purified E1p was approximately 98% pure with specific activity 130.4 $mUmg^{-1}$ (Fig. 1). As evident in Fig. 1B, the two bands hybridized during the purification procedure with anti-serum raised against the recombinant E1p. The amount of the lower band compared with the upper band gradually increased with the procedure, suggesting that the lower band may originate from the upper band probably by protease attack. However, when the specific activity of the final preparation compared with those of the above steps, the lower band of E1p was fully active.

The subunit molecular mass of the purified E1p was measured at 93 kDa by SDS-PAGE (Fig. 1). The N-terminal amino acid sequence of the purified E1p was determined to be LMRRTL. This sequence corresponds to that of the 45th-50th residues of the deduced amino acid

sequence of *pdhA*. These results indicate that the purified E1p originated from the upper E1p band, the gene product of *pdhA*. The native molecular mass of the purified E1p was 190 kDa on Superose 12 column, indicative of homodimeric subunit composition. It has been reported that the deletion of N-terminal amino acids of *A. vinelandii* E1p had no effect on its dimeric structure (Hengeveld *et al.*, 1997). Therefore, we suggested that the purified E1p is a homodimer of *pdhA* gene product.

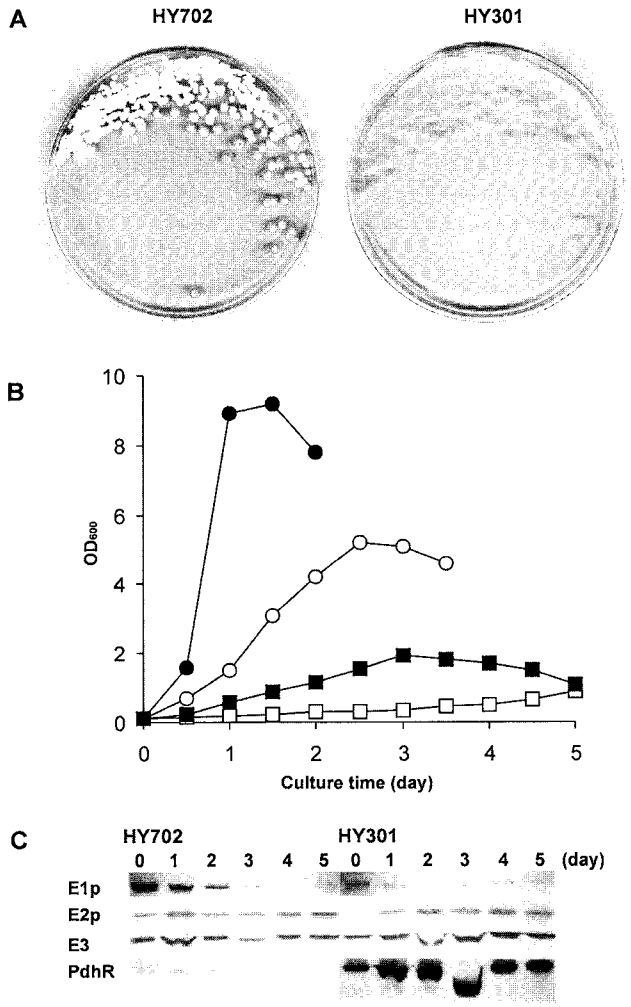

Fig. 2. Relationship between PdhR and growth of *S. seoulensis*. (A) Plate culture. The PdhR-overexpressed *S. seoulensis* strain (HY301) cultured on R2YE plates for 3 days was compared with the control strain (HY702). (B) Liquid culture. HY702 strain and HY301 strain cultured in modified YEME medium were transferred into individual liquid medium: ●, HY702 in modified YEME; ○, HY301 in modified YEME; ■, HY702 in MMPG; and □, HY301 in MMPG. To avoid possible error during spore inoculation, we adopted seed culture followed by the dilution of each cell into new media at an optical density of 0.1 at OD_{600}. (C) Immunological analysis. The cell-free extracts of MMPG-grown HY702 and HY301 as shown in (B) were analyzed by Western hybridization analysis with anti-E1p serum (E1p), anti-E2p serum (E2p), anti-E3 serum (E3), and anti-PdhR serum (PdhR). Lanes 0-5 indicate the cell-free extract of each cell collected at the time interval of 0-5 days.

Purification and molecular properties of recombinant E2p

rE2p was purified from the cell-free extract of recombinant *E. coli* through DEAE 5PW and Superdex 200 chromatography. Considering the additional N-terminal Tags, the subunit molecular mass of recombinant E2p was estimated to be about 107 kDa (data not shown), which is much higher than that of the deduced mass of 61,367 Da. To determine the native molecular mass of rE2p, the purified rE2p was again subjected to Superdex 200 gel permeation chromatography. However, the exact molecular mass could not be measured because rE2p was eluted between blue dextran (2,000 kDa) and the largest protein marker, ferritin (440 kDa), only revealing the multimeric structure of rE2p.

Effect of episomal plasmid containing pdhR on the growth of S. seoulensis

E. coli PdhR represses transcription of the *pdhR-aceEF-lpd* operon by binding to the *pdh* operator as a monomer (Quail and Guest, 1995). To assess a functional role of *S. seoulensis* PdhR in the regulation of the *pdh* genes, the *pdhR* gene was cloned into *Streptomyces* vector pIJ702 and overexpressed in *S. seoulensis*. We tested whether the overexpression of *S. seoulensis* PdhR could influence the growth on a variety of solid or liquid media. As shown in Fig. 2A, the PdhR-overexpressed strain (HY301) showed marked growth retardation in contrast to the reference strain (HY702) on solid medium, R2YE containing 5% sucrose. We also tested whether the overexpression could influence the sporulation of *S. seoulensis*. On R2YE medium containing 5% sucrose, HY301 strain entered the stage of aerial mycelium and sporulation at the sixth and seventh days, respectively, while HY702 strain entered at the fourth and fifth days, respectively. This property of growth retardation in HY301 strain over HY702 strain was also confirmed on the other surface culture media including modified Bennett, nutrient agar, MMC, MMGC, and MMG (data not shown). To confirm the effect of PdhR overexpression on *S. lividans* growth, we performed the same experiment using *S. lividans* containing the pHY301 plasmid. The strain showed the same phenotype of growth retardation as *S. seoulensis*. Surprisingly it also showed almost complete bleaching of pigment compared with the reference strain (pIJ702-transformed *S. lividans*) in a variety of solid media including R2YE containing 10% sucrose, MMGC, and MMG. The relevant pigment was identified as an antibiotic, actinorhodin in wild type *S. lividans* according to the method proposed previously (Hobbs *et al.*, 1990).

The tendency of growth retardation in HY301 strain over HY702 strain was also found in modified YEME and MMGP liquid cultures (Fig. 2B). In these cultures, we adopted seed culture (modified YEME) followed by the dilution of each cell into new medium (modified YEME

or MMGP) to avoid possible error during spore inoculation. The above results clearly indicated that PdhR exerts its adverse effect on the growth of *Streptomyces*.

PdhR negatively regulated E1p

Taking into consideration the genetic location of the *pdhR* gene, its sequence homology to the other characterized genes containing a helix-turn-helix motif, and the growth retardation phenotype in the PdhR-overexpressed strain, we postulated that PdhR can function as a negative regulator by repressing the expression of the PDC components as *E. coli* PdhR does in the *pdhR-aceEF-lpd* operon. To determine what protein is related to the phenotype of the marked growth retardation, we investigated the protein levels of the three components of PDC using Western hybridization analysis. As shown in Fig. 2C, HY301 strain expressed a reduced level of E1p with no detectable differences of the amount of E2p and E3 compared with HY702 strain. HY702 strain mostly expressed E1p in the early exponential growth stage, while HY301 expressed E1p at a basal level with no change in the amount during the growth stage. Thus, the growth retardation phenotype shown in HY301 strain could be ascribed to the reduced expression of E1p at the early exponential stage.

Discussion

We have isolated four *S. seoulensis pdh* genes arranged in a cluster. The genes for *S. seoulensis* PDC were clustered in the order of *lpd*, *pdhB*, *pdhR*, and *pdhA*. Recently, such clustering has also been identified in *S. coelicolor* (the accession number of AL096872, GenBank™/EBI Data Bank) from the *S. coelicolor* sequencing project and different from well-known PDCs (Neveling *et al.*, 1998): in most bacteria, the *pdh* genes are clustered in a single operon and organized in the order of the genes for E1p, E2p, and E3. In *A. vinelandii* and *P. aeruginosa*, the gene encoding E3 component is not positioned within the PDC operon; in *Z. mobilis* the genes for E1p and E2p are not clustered in an operon, and in *T. ferrooxidans* a single gene encoding a unique polypeptide harbors both E2p and E3 activities. Furthermore, *lpd* gene and *pdhB* gene in *S. seoulensis* seem to be under the simultaneous control of transcription since the intergenic region of the two genes exhibited no promoter-like structure when compared with the compiled *Streptomyces* promoters (Strohl, 1992). The existence of PdhR distinguishes *S. seoulensis* from many other prokaryotes that have no homolog of such a regulator although a prototype PdhR was found in *E. coli* (Quail *et al.*, 1994). In *S. seoulensis*, PdhR only represses the expression level of E1p unlike the *E. coli* PdhR. At present, although the regulation mechanism of *S. seoulensis pdh* genes is not clearly understood, the genes are thought to be composed of at least two operons, each of which is independently transcribed and regulated.

Another unusual aspect lies in that *S. seoulensis* possesses a homodimeric E1p component, a structure characteristic for Gram-negative bacteria such as *E. coli* (Saumweber *et al.*, 1981) and *A. vinelandii* (Hengeveld *et al.*, 1997). In general, the homodimer is characteristic of E1p in the Gram-negative bacteria while the heterotetrameric form is prevalent in those of the Gram-positive organisms. PDC containing such a homodimeric E1p has always been found as a supramolecular structure of octahedral symmetry (Berg and Kok, 1997). Therefore, it is supposed that the *S. seoulensis* PDC may form a structure with an octahedral symmetry. The *S. seoulensis* enzyme was very unstable against various environmental factors including a protease attack. In particular, fast removal of the N-terminal region of *S. seoulensis* E1p is very interesting because in *A. vinelandii* the N-terminal region has been suggested as a domain involved in the binding to cognate E2p (Hengeveld *et al.*, 1997; Hengeveld *et al.*, 1999). Up to now it is not clear what effect causes this truncation at a relatively specific site.

All the E2p components accommodate one to three highly flexible lipoyl domains to accomplish its essential role in carrying reducing equivalents between the active sites of PDC. *S. seoulensis* E2p has two lipoyl domains like the E2p components of human (Thekkumkara *et al.*, 1988) and *S. faecalis* (Allen and Perham, 1991). This is the second example of E2p having two lipoyl domains within Gram-positive bacteria. For its high flexibility, the molecular mass of E2 components is often overestimated on SDS-PAGE depending on the number of lipoyl domains per E2p. The apparent molecular mass of *S. seoulensis* E2p was measured to be 107 kDa, much exceeding the apparent molecular masses (83 kDa or so) of E2p components of *E. coli* and *A. vinelandii* accommodating three lipoyl domains (Guest *et al.*, 1985; Hanemaaijer *et al.*, 1988). This abnormally high apparent molecular mass of *S. seoulensis* E2p may be due to relatively long stretches of the linker polypeptides.

In aerobic organisms, PDC plays a pivotal role in energy metabolism and biosynthesis by functioning as a linker between glycolysis and the TCA cycle. The *S. seoulensis* PDC is closely related with *S. seoulensis* growth, which was demonstrated by the growth retardation with the concomitant consequence of the decreased level of E1p by the overproduced PdhR. Aside from the growth retardation phenotype, *S. lividans* strain overexpressing *S. seoulensis* PdhR showed a marked decrease of the production of a polyketide pigment, actinorhodin, suggesting a possible regulatory role of *Streptomyces* PDC in pigment production, too. The polyketide backbone of actinorhodin is composed of several acetates and branched-fatty acid units (Bao *et al.*, 1999). *Streptomyces* spp. being strictly aerobic bac-

teria, the main source of the acetate might be acetyl-CoA produced by PDC. In addition, overexpression of PdhR might regulate transcription related to actinorhodin synthesis directly or have pleiotrophic consequences leading to pigment formation. A variety of polyketide compounds is produced by *Streptomyces* such as actinorhodin in *Streptomyces coelicolor* (Matharu *et al.*, 1998) and avermectins in *S. avermitilis* (MacNeil *et al.*, 1994), which are therapeutically useful. Therefore, much remains to be learned about the functional role of *Streptomyces* PDC in pigment production as well as in central metabolism.

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