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Effects of Three Different Nucleoid-Associated Proteins Encoded on IncP-7 Plasmid pCAR1 on Host *Pseudomonas putida* KT2440

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Nucleoid-associated proteins (NAPs), which fold bacterial DNA and influence gene transcription, are considered to be global transcriptional regulators of genes on both plasmids and the host chromosome. Incompatibility P-7 group plasmid pCAR1 carries genes encoding three NAPs: H-NS family protein Pmr, NdpA-like protein Pnd, and HU-like protein Phu. In this study, the effects of single or double disruption of *pmr*, *pnd*, and *phu* were assessed in host *Pseudomonas putida* KT2440. When *pmr* and *pnd* or *pmr* and *phu* were simultaneously disrupted, both the segregational stability and the structural stability of pCAR1 were markedly decreased, suggesting that Pmr, Pnd, and Phu act as plasmid-stabilizing factors in addition to their established roles in replication and partition systems. The transfer frequency of pCAR1 was significantly decreased in these double mutants. The segregational and structural instability of pCAR1 in the double mutants was recovered by complementation of *pmr*, whereas no recovery of transfer deficiency was observed. Comprehensive phenotype comparisons showed that the host metabolism of carbon compounds, which was reduced by pCAR1 carriage, was restored by disruption of the NAP gene(s). Transcriptome analyses of mutants indicated that transcription of genes for energy production, conversion, inorganic ion transport, and metabolism were commonly affected; however, how their products altered the phenotypes of mutants was not clear. The findings of this study indicated that Pmr, Pnd, and Phu act synergistically to affect pCAR1 replication, maintenance, and transfer, as well as to alter the host metabolic phenotype.

Plasmids transferred among different bacteria can confer novel phenotypes to the host, including antibiotic resistance or the ability to degrade xenobiotics (1, 2). Genes on the plasmid are regulated not only by plasmid-encoded factors but also by chromosomally encoded host factors (3). Therefore, upon transfer into a different host, regulation of genes on the plasmid may change in response to different host factors.

We examined the interaction between the plasmid and the host bacterial strain using carbazole degradation incompatibility (Inc) P-7 group conjugative plasmid pCAR1, which was originally isolated from *Pseudomonas resinovorans* CA10 (4). The whole nucleotide sequence of pCAR1 was reported previously (5, 6), and the transcriptomes of pCAR1 were shown to differ in six different *Pseudomonas* hosts (7). We also demonstrated that carriage of the plasmid affects chromosomal gene expression and the host phenotype using three different hosts: *P. putida* KT2440, *P. aeruginosa* PAO1, and *P. fluorescens* Pf0-1 (8). Moreover, comprehensive analysis of the host phenotype revealed that pCAR1 carriage reduces host fitness, swimming motility, and resistance to osmotic or pH stress and alters the primary metabolic capacity of the host cells (9). These effects on host phenotypes were considered to be dependent on various factors from both the plasmid and the host chromosome.

Nucleoid-associated proteins (NAPs) were suggested to be candidate effectors of the interaction between the plasmid and host chromosome (10). NAPs alter the shape of bacterial DNA to make it more compact and can influence global transcription (11). The best-studied NAPs are H-NS and HU proteins. H-NS was shown to repress genes acquired through horizontal gene transfer by recognizing sequences with low G+C content (12, 13). Several proteins (e.g., MvaT of *Pseudomonas* spp. or Lsr2 of *Mycobacterium* spp.) are included in the H-NS family of proteins as functional homologs due to their ability to silence the expression of xenogeneic DNA sequences, despite their almost negligible amino acid sequence homology with H-NS (13–15). In contrast, HU proteins bind preferentially to duplex DNA containing a nick or a gap, and they bend DNA in a sequence-independent manner (16, 17).

Notably, considerable numbers of plasmids from Gram-negative bacteria (7% of the 2,260 plasmids fully sequenced as of April 2010) contain at least a single NAP homolog gene (18). Previous studies on plasmid-encoded NAPs focused mainly on H-NS homologs, namely, $H\text{-}NS_{R27}$ and Sfh, in plasmid R27 and its derivatives (19, 20). The DNA binding sites of Sfh completely over-

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lapped those of chromosomally encoded H-NS, as shown by genome-wide chromatin immunoprecipitation with microarray technology (ChIP-chip) analysis (21). In contrast, H-NS_{R27}, which is 98% identical to Sfh, selectively targets horizontally acquired DNA and not core genomic DNA, whereas chromosomally encoded H-NS targets both (20). H-NS_{R27} participates in the thermoregulation of the conjugative transfer of R27 (22).

Plasmid pCAR1 carries three genes encoding the following NAPs: Pmr (plasmid-encoded MvaT-like regulator; encoded by ORF70), Pnd (plasmid-encoded NdpA-like protein; encoded by ORF93), and Phu (plasmid-encoded HU-like protein; encoded by ORF95a) (4). One of these NAPs, Pmr, binds preferentially to horizontally acquired DNA and optimizes the transcription of genes on both the plasmid and the host *P. putida* KT2440 chromosome (23). Recently, two MvaT homologs encoded on the KT2440 chromosome, TurA (PP_1366) and TurB (PP_3765), were shown to play important roles in the transcriptional regulation of Pmr (24). The present study was performed to examine the functions of the other two NAPs encoded on pCAR1, Pnd and Phu, using *P. putida* KT2440 as the model host.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in lysogeny broth (LB) (25), whereas *P. putida* KT2440(pCAR1) and its derivative strains were grown at 30°C in LB or nitrogen plus mineral medium 4 (NMM-4) (26) containing 0.1% (wt/vol) succinate or carbazole as the sole source of carbon and energy. Ampicillin (Ap; 50 μg/ml), chloramphenicol (Cm; 30 μg/ml), gentamicin (Gm; 30 μg/ml for mating experiments and 120 μg/ml for mutant preparation), kanamycin (Km; 50 μg/ml), rifampin (Rif; 25 μg/ml), and sucrose (10% [wt/vol]) were added to the selective medium. For plate cultures, the media described above were solidified with 1.6% (wt/vol) purified agar powder (Nacalai Tesque, Kyoto, Japan).

Standard DNA manipulation. Standard methods were used for the extraction of plasmid DNA, digestion of DNA with restriction endonucleases, DNA ligation, and transformation of competent *E. coli* cells (25). The primers used in this study are listed in Table 2. Total DNA was extracted from *Pseudomonas* strains by the use of hexadecyltrimethylammonium bromide, as described previously (27). Electroporation of *Pseudomonas* was performed according to the method described by Itoh et al. (28).

5′-RACE analysis. Extraction of total RNA from *P. putida* KT2440 (pCAR1) at the log growth phase was performed as described previously (9). cDNA synthesis, 5′-rapid amplification of cDNA ends (5′-RACE) PCR, and nested PCR were performed using a SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA) in accordance with the manufacturer's instructions. Nested PCR products were ligated into the EcoRV sites of plasmid pZErO-2 (Invitrogen, Carlsbad, CA), and the sequences of the DNA fragments were confirmed using primers M13-F and M13-R.

Quantitative reverse transcription (qRT)-PCR. Extraction of total RNA from *P. putida* KT2440(pCAR1) was performed as described previously (9). The primers were designed using the Primer3 program (29), and the cDNA was quantified using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) as described previously (9).

Preparation of mutants of NAP genes. The procedures for preparation of each single-gene-disruption mutant of *pmr*, *pnd*, and *phu* were performed similarly using a homologous-recombination-based gene replacement system (30). Briefly, DNA fragments containing the 5′- and 3′-flanking regions of each gene and the Gm resistance cassette, which is flanked by flippase recognition target (FRT) sites on pPS856 (31), were cloned into suicide vector pK19mobsacB (32) to yield pK19mobsacBΔpmr, pK19mobsacBΔpnd, and pK19mobsacBΔphu, respectively. *E. coli* S17-1λ*pir* cells carrying the resultant plasmids were conjugated with KT2440(pCAR1) on cellulose membrane filters (Advantec, Tokyo, Japan) (0.45-μm pore size) and incubated on LB agar plates at a donor-to-recipient ratio of 1:1 at 30°C for 15 h. Double-crossover recombinants were screened using sucrose counterselection, and then the Gm resistance cassette was removed by site-specific recombination of the FRT sites using Flp recombinase expressed by pFLP2Km (23). In our previous study, we used a *pmr* disruption mutant with a Gm resistance gene cassette without FRT sites in the *pmr* gene (23). In this study, we reconstructed KT2440(pCAR1Δ*pmr*) lacking the cassette. To prepare the double-disruption *pmr pnd* and *pmr phu* mutants, pK19mobsacBΔpnd and pK19mobsacBΔphu plasmids were transferred into KT2440(pCAR1Δ*pmr*). To prepare the double-disruption *pnd phu* mutant, the pK19mobsacBΔphu plasmid was transferred into KT2440 (pCAR1Δ*pnd*). The Gm resistance cassette was removed from the double-crossover recombinants as described above. To prepare *pmr* gene-complemented strains of KT2440(pCAR1Δ*pmr*Δ*pnd*) and KT2440 (pCAR1Δ*pmr*Δ*phu*), pK19mobsacBpmr, which contains *pmr* and a Gm resistance cassette flanked by the regions corresponding to bp 100,550 to 101,000 and bp 101,001 to 101,382 of pCAR1, was transferred into the double mutants. After successful insertion of *pmr* and the Gm resistance cassette into pCAR1, the Gm resistance cassette was removed as described above.

pCAR1 stability assay. *P. putida* KT2440(pCAR1) and its derivatives, the carbazole degradation ability of which was confirmed, were grown for 24 h in LB. Cultures were then diluted 1,000-fold with NMM-4 medium supplemented with 0.1% succinate and incubated at 30°C. After 24 h, cultures were again diluted 1,000-fold with fresh NMM-4 medium supplemented with 0.1% succinate. This procedure was repeated five times. Cultures at the first and the fifth passages were spread onto LB agar plates, and the resultant cultures were used for colony hybridization to confirm the presence of the *repA* and *carAc* genes. Probes for colony hybridization were prepared from a 1.7-kb HindIII-XbaI fragment of pCAR1 containing the *repA* gene and a 1.0-kb BglII-XhoI fragment of pCAR1 containing the *carAc* gene. Hybridization and detection was performed as described previously (9). To assess the effects of RecT overexpression on the stability of pCAR1, KT2440(pCAR1) harboring pBBad18K (33) containing the *recT* gene of pCAR1 was used in similar medium supplemented with 0.2% (wt/vol) L-arabinose to induce the expression of *recT*. To test the effects of iron ions on the stability of pCAR1, 37 or 370 μM ferric citrate was added to NMM-4 containing 0.1% succinate instead of $FeCl_3$ (originally 37 μM).

Mating experiments. *P. putida* KT2440(pCAR1) derivatives harboring a Gm resistance cassette and *P. putida* KT2440KR derivatives harboring a Km resistance cassette were used as donors and recipients, respectively. *P. putida* KT2440KR was prepared from the *P. putida* KT2440 rifampin resistance spontaneous mutant using pBSLKm^r (34). The donor and the recipient strains were precultured in 5 ml of LB liquid medium with the appropriate antibiotics for 18 h. After washing with LB liquid medium, donors and recipients were resuspended and diluted in LB liquid medium to optical densities at 600 nm (OD_{600}) of 0.2 and 2.0, respectively. Aliquots of 200 μl of each culture were mixed in 2-ml microtubes, and the lip was sealed using a gas-permeable adhesive seal (Nippon Genetics, Tokyo, Japan) with the lid open. After incubation at 30°C for 3 h, the cell suspensions were spread on selective media at the appropriate dilutions. The number of transconjugants was determined by counting colonies on selective medium 1 to 2 days after mating. The frequency of plasmid transfer was expressed as the number of transconjugants per number of donors.

PM analyses. Phenotype MicroArray (PM; Biolog, Hayward, CA) analyses were performed as described previously (9) using panels PM1 through PM4, PM9, and PM10.

Transcriptome analyses of pCAR1 and the KT2440 chromosome. Transcriptome analyses were performed with custom-made tiling arrays, which were constructed as described previously (8, 35). Total RNA extraction and cDNA syntheses in the presence of actinomycin D (Sigma-Aldrich, St. Louis, MO) were performed as described previously (9). The fragmentation and labeling of cDNA, hybridization with the tiling arrays,

TABLE 1 Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristic(s)	Source or reference(s)
Bacterial strains		
E. coli K-12 DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ^- *phoA*	Toyobo
E. coli K-12 S17-1λ*pir*	*recA thi pro hsdR*; RP4-2 integrated into the chromosome (*kan*::Tn*7 ter*::Mu) λ*pir*	44
P. putida KT2440	Naturally Cm^r	45
P. putida KT2440KR	Derivative strain of KT2440, spontaneously Rif^r, with introduced Km^r gene	This study
P. putida KT2440(pCAR1)	KT2440 harboring pCAR1	35
P. putida KT2440(pCAR1pmrHis::Gm^r)	KT2440(pCAR1pmrHis) harboring Gm^r gene cassette and FRT sites	23
P. putida KT2440(pCAR1Δ*pmr*::Gm^r)	KT2440 harboring pCAR1 carrying disrupted *pmr* gene by Gm^r gene cassette and FRT sites	This study
P. putida KT2440(pCAR1Δ*pmr*)[a]	KT2440(pCAR1) single-deletion mutant lacking *pmr* gene	This study
P. putida KT2440(pCAR1Δ*pnd*::Gm^r)	KT2440 harboring pCAR1 carrying disrupted *pnd* gene by Gm^r gene cassette and FRT sites	This study
P. putida KT2440(pCAR1Δ*pnd*)	KT2440(pCAR1) single-deletion mutant lacking *pnd* gene	This study
P. putida KT2440(pCAR1Δ*phu*::Gm^r)	KT2440 harboring pCAR1 carrying disrupted *phu* gene by Gm^r gene cassette and FRT sites	This study
P. putida KT2440(pCAR1Δ*phu*)	KT2440(pCAR1) single-deletion mutant lacking *phu* gene	This study
P. putida KT2440(pCAR1Δ*pmr*Δ*pnd*::Gm^r)	KT2440(pCAR1Δ*pmr*) in which *pnd* gene is disrupted by Gm^r gene cassette and FRT sites	This study
P. putida KT2440(pCAR1Δ*pmr*Δ*pnd*)	KT2440(pCAR1) double-deletion mutant lacking *pmr* and *pnd* genes	This study
P. putida KT2440(pCAR1Δ*pmr*Δ*phu*::Gm^r)	KT2440(pCAR1Δ*pmr*) in which *phu* gene is disrupted by Gm^r gene cassette and FRT sites	This study
P. putida KT2440(pCAR1Δ*pmr*Δ*phu*)	KT2440(pCAR1) double-deletion mutant lacking *pmr* and *phu* genes	This study
P. putida KT2440(pCAR1Δ*pnd*Δ*phu*::Gm^r)	KT2440(pCAR1Δ*pnd*) in which *phu* gene is disrupted by Gm^r gene cassette and FRT sites	This study
P. putida KT2440(pCAR1Δ*pnd*Δ*phu*)	KT2440(pCAR1) double-deletion mutant lacking *pnd* and *phu* genes	This study
P. putida KT2440(pCAR1PMGΔ*pmr*Δ*pnd*)	KT2440(pCAR1Δ*pmr*Δ*pnd*) containing Gm^r gene cassette with FRT sites and 0.57-kb *pmr* cassette inserted into 101,000–101,001 region of pCAR1	This study
P. putida KT2440(pCAR1PMΔ*pmr*Δ*pnd*)	KT2440(pCAR1Δ*pmr*Δ*pnd*) containing 0.57-kb *pmr* cassette inserted into 101,000–101,001 region of pCAR1	This study
P. putida KT2440(pCAR1PMGΔ*pmr*Δ*phu*)	KT2440(pCAR1Δ*pmr*Δ*phu*) containing Gm^r gene cassette with FRT sites and 0.57-kb *pmr* cassette inserted into 101,000–101,001 region of pCAR1	This study
P. putida KT2440(pCAR1PMΔ*pmr*Δ*phu*)	KT2440(pCAR1Δ*pmr*Δ*phu*) containing 0.57-kb *pmr* cassette inserted into 101,000–101,001 region of pCAR1	This study
Plasmids		
pBBad18K	Km^r, L-arabinose-inducible vector based on the pBBR1MCS-4 replicon	33
pBBad18K-recT	pBBad18K, BamHI-XbaI fragment containing *recT*	This study
pBSLKm^r	Mini-Tn*5*, R6K *ori*, Ap^r, Km^r	34
pFLP2Km	pFLP2, Km^r gene cassette inserted into its ScaI site	23
pK19mobsacB	Km^r, *oriT* (RP4), *sacB*, *lacZ*α, pMB1 replicon	32
pK19mobsacBΔpmr	pK19mobsacB containing 5′- and 3′-flanking regions of *pmr* and Gm^r gene cassette, which is flanked by FRT sites	This study
pK19mobsacBΔpnd	pK19mobsacB containing 5′- and 3′-flanking regions of *pnd* and Gm^r gene cassette, which is flanked by FRT sites	This study
pK19mobsacBΔphu	pK19mobsacB containing 5′- and 3′-flanking regions of *phu* and Gm^r gene cassette, which is flanked by FRT sites	This study
pK19mobsacBpmr	pK19mobsacB containing 100,550–101,000 and 101,001–101,382 regions of pCAR1, *pmr*, and Gm^r gene cassette	This study
pPS856	Ap^r, Km^r, FRT sites	31
pT7Blue T-vector	Ap^r, *lacZ*α, T7 promoter, f1 origin, pUC/M13 priming sites	Novagen
pTpmr	pT7Blue T-vector with PCR fragment amplified from total DNA of KT2440(pCAR1) with primer set pmr_qRT_F and pmr_qRT_R	35
pTpnd	pT7Blue T-vector with PCR fragment amplified from total DNA of KT2440(pCAR1) with primer set pnd_qRT_F and pnd_qRT_R	This study
pTphu	pT7Blue T-vector with PCR fragment amplified from total DNA of KT2440(pCAR1) with primer set phu_qRT_F and phu_qRT_R	This study
pTuniv16S	pT7Blue T-vector with PCR fragment amplified from total DNA of *P. resinovorans* CA10 with primer set univ16S-F and univ16S-R	35
pZErO-2	Km^r, T7 promoter, ColE1 and f1 origin, *ccdB* lethal gene, M13 priming sites	Invitrogen

[a] KT2440(pCAR1Δ*pmr*), which was used in our previous study (23), was reconstructed in this study as described in Materials and Methods.

TABLE 2 Oligonucleotide primers

Primer	Sequence (5′ → 3′)[a]	Reference
Preparation of NAP gene disruption mutants		
Gm-F	CGAATTAGCTTCAAAAGCGCTCTGA	30
Gm-R	CGAATTGGGGATCTTGAAGTTCCT	30
pmr_del_up_F	GGATCCAGGAATCACTGTTCGGCAAG	This study
pmr_del_up_R2	TCAGAGCGCTTTTGAAGCTAATTCGCTTGTCTCCTTGGTCTGGG	This study
pmr_del_down_F2	AGGAACTTCAAGATCCCCAATTCGGTTTGCGCTACCGCGGATCT	This study
pmr_del_down_R	AAGCTTGTGAGCCAAGGCTTCTTCAG	This study
pnd_del_up_F	GGATCCCGGAGTCCCAAACCGTAATA	This study
pnd_del_up_R	TCAGAGCGCTTTTGAAGCTAATTCGGAGCAAAGTCCTTGTAGTTT	This study
pnd_del_down_F	AGGAACTTCAAGATCCCCAATTCGAAAAATGCCCCGTCCATCTC	This study
pnd_del_down_R	AAGCTTGGATCAATTCCAGCCTCAAA	This study
phu_del_up_F	GGATCCTAGACGATCGACGCAAAGTG	This study
phu_del_up_R2	TCAGAGCGCTTTTGAAGCTAATTCGTCGCTTTTACTCCTTGGTTG	This study
phu_del_down_F2	AGGAACTTCAAGATCCCCAATTCGGCCCCCTCCAGCCGCCCCGA	This study
phu_del_down_R	AAGCTTGCGATGAGAAGGGCAAATAG	This study
Preparation of *pmr*-complemented strains		
Gm-F-SalI	GTCGACCGAATTAGCTTCAAAAGCGCTCTG	This study
Gm-R-PstI	CTGCAGCGAATTGGGGATCTTGAAGTTCCT	This study
ORF98_COMP_up_F	GGATCCACCTGGGTACTGGCTCATTG	This study
ORF98_COMP_up_R	CTGCAGGCCCGTTTTCCTACGCAGCT	This study
ORF99_COMP_down_F	CCCGGGCTGGTGGCACGCGTCGGCCC	This study
ORF99_COMP_down_R	AAGCTTCATATCGCATGGGATTTTCC	This study
pmr_COMP_F	CCCGGGCGCATTCTGGCCTTCCGCCG	This study
pmr_COMP_R	GTCGACCTCACAAAAAAGGCCGGGTT	This study
pCAR1 stability assay		
recT-ox-F	GGATCCAAGGAGACCTTCGGGTGCGTTTTC	This study
recT-ox-R	TCTAGACTATCACTCGCCTTGCG	This study
5′-RACE analysis		
M13-F	GTAAAACGACGGCCAGT	38
M13-R	GGAAACAGCTATGACCATG	38
pnd308	CCGGTTGAGAGGTTCGATTCTTCCAT	This study
pnd498	GGACTGCTTGTTGTTCTGCCATTCG	This study
phu157	TTGACTCGAAAGTACCAAAGCCCACGAG	This study
phu258	CTTGAAGGACTTGCCAGGGGTGAACT	This study
phu_up395	CATTACCACACTTCGGACAGAGCAATCC	This study
phu_up564	GAGGTTCCCGGAGTGGTCAATGATAATG	This study
qRT-PCR		
pmr_qRT_F	GATCCGGACTATCGAAAGCA	35
pmr_qRT_R	TTCCACTCCTTGAGCGTCTT	35
pnd_qRT_F	TGGCAGAACAACAAGCAGTC	This study
pnd_qRT_R	GTCTACACCCTCCTGGCAAC	This study
phu_qRT_F	TCGTGGGCTTTGGTACTTTC	This study
phu_qRT_R	GGTGAACTTTGGCTTGATCG	This study
univ16S-F	ACACGGTCCAGACTCCTACG	35
univ16S-R	TACTGCCCTTCCTCCCAACT	35

[a] Underlined nucleotides represent artificial restriction sites.

washing, detection, calculation of the intensities of each probe, and definition of transcriptional values for each gene were performed as described previously (8) except using 3.3 μg of cDNA. Comparisons between KT2440(pCAR1) and the mutants were performed using biologically duplicated data. We identified up- and downregulated open reading frames (ORFs) with changes of over 2-fold in all of the four data comparisons performed [between replicate 1 or 2 of KT2440(pCAR1) and replicate 1 or 2 of each mutant]. The data were visualized using the IGB software package (Affymetrix, Santa Clara, CA).

Microarray data accession number. The array data reported in this article have been deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information (NCBI) (GEO; http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession no. GSE53069.

RESULTS AND DISCUSSION

Transcription initiation start points and transcriptional profiles of *pnd* and *phu*. To identify the transcription initiation start points for *pnd* and *phu*, 5′-RACE analyses were performed with the total RNA of KT2440(pCAR1) cells at the log growth phase. When primers that anneal specifically to the internal region of *pnd* were used, the PCR products on the agarose gel electrophoresis

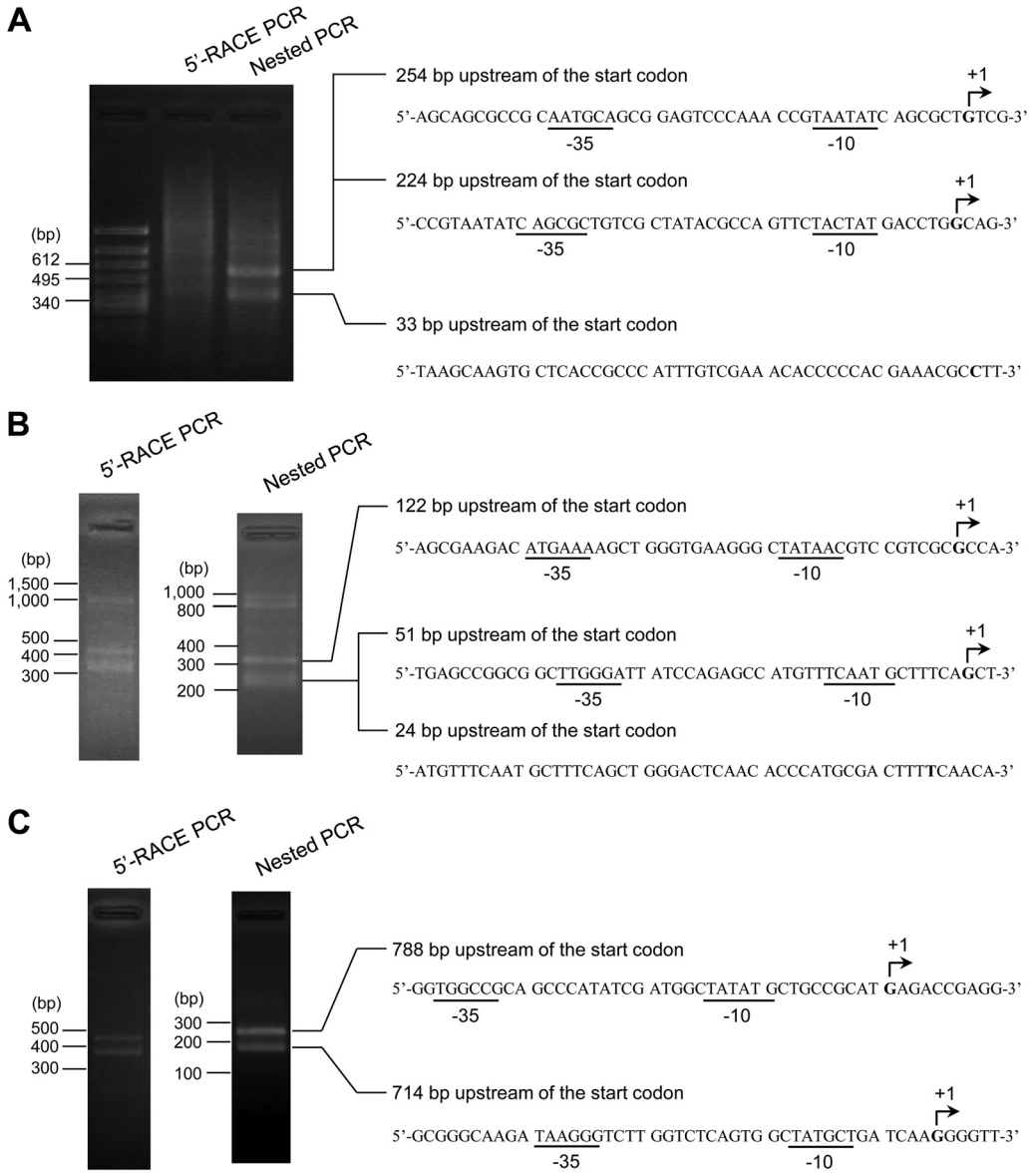

FIG 1 Identification of the transcription initiation start points of *pnd* and *phu*. 5′-RACE PCR followed by nested PCR was performed using primers designed to anneal specifically to the internal regions of *pnd* (A) and *phu* (B) and to the upstream region of *phu* (C). As templates, cDNA synthesized from the total RNA of KT2440(pCAR1) was used for 5′-RACE PCR and the 5′-RACE PCR products were used for a nested PCR. The 5′-end nucleotides of the nested PCR products are shown in bold type. The nucleotide sequences up- and downstream of the 5′-end nucleotide are also shown in the panels. The arrow indicates the transcription initiation start point (+1); the −35 and −10 hexamers are underlined.

corresponded to regions 254, 224, and 33 bp upstream of the *pnd* translational start site (Fig. 1A). Putative −10 and −35 elements for σ^{70} of pseudomonads (36) were found 254 and 224 bp upstream of the start codon, respectively, whereas no characteristic pseudomonad σ^{70} or σ^{54} binding motifs (37) were observed 33 bp upstream of the start codon. Therefore, the regions 254 and 224 bp upstream of the start codon were identified as the transcriptional start sites for *pnd*. Similarly, the transcription initiation start points of *phu* were located 788, 714, 122, and 51 bp upstream of the start codon (Fig. 1B and C). Based on the results of transcriptome analysis of KT2440(pCAR1) described below, the transcription initiation start points of *pnd* and *phu* were predicted to be ~200 and 700 bp, respectively, upstream of the translational start points (see Fig. S1 in the supplemental material), consistent with the results of 5′-RACE analyses.

qRT-PCR analyses of *pmr*, *pnd*, and *phu* along with the growth curve of KT2440(pCAR1) indicated that their transcription levels were similarly higher during the log growth phase (2 to 5 h) than in stationary phase (6 to 24 h; Fig. 2). This was consistent with the results of our previous microarray analyses (9) and the presence of putative σ^{70}-dependent promoters on *pnd* and *phu* as described above. The putative promoter of *pmr* was also predicted to be dependent on σ^{70} in our previous study (23). Whereas the levels of *pmr* and *phu* transcription were higher than those of *pnd*, the growth phase-dependent transcription profiles of *pmr*, *pnd*, and *phu* were similar to each other.

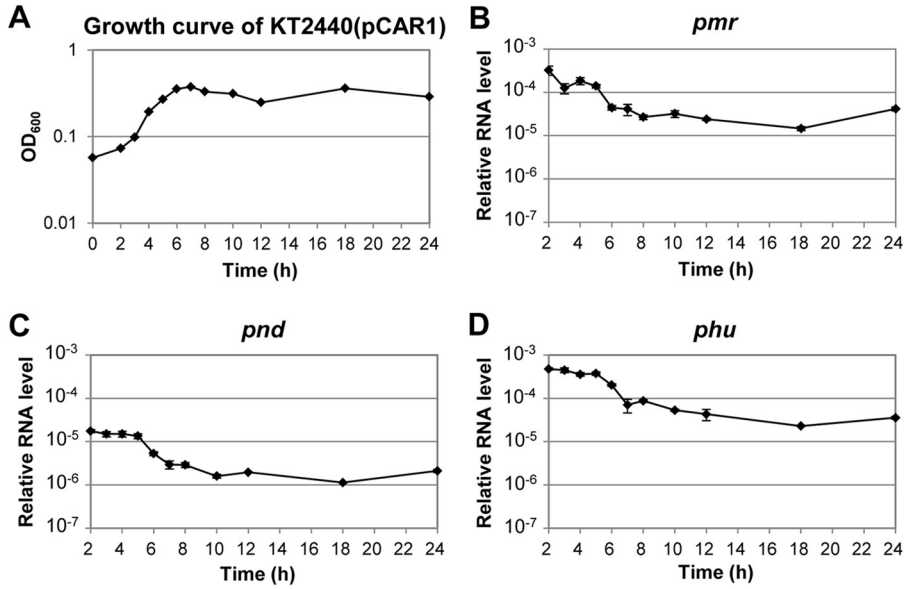

FIG 2 Transcriptional profiles of the pCAR1 genes encoding NAPs. (A) Growth curve of KT2440(pCAR1). (B to D) The mRNA levels of *pmr* (B), *pnd* (C), and *phu* (D) were measured by qRT-PCR along the growth curve. The data were normalized using the average of the 16S rRNA data as the internal standard. Means and standard deviations (error bars) of triplicate data are shown.

Effects of NAP gene disruption on host cell function. To assess the effects of NAP gene(s) disruption on *P. putida* KT2440 (pCAR1), we prepared single or double mutants of *pmr*, *pnd*, and *phu* with and without the Gm resistance gene cassette.

Stability of pCAR1. First, the stability of pCAR1 was assessed in the wild-type (WT) strain and six mutants without the Gm resistance gene cassette, which were cultured for 24 h in NMM-4 (26) supplemented with succinate as the sole source of carbon. As shown in Fig. 3, pCAR1 was maintained stably in the WT, three single mutants, and one double mutant. In contrast, some colonies from the cultures of two double mutants, KT2440(pCAR1Δ*pmr*Δ*pnd*) and KT2440(pCAR1Δ*pmr*Δ*phu*), did not contain the *repA* gene (i.e., they did not have pCAR1). In addition, many pCAR1-containing colonies of the two double mutants, particularly in the fifth passage in culture, did not have the *carAc* gene. DNA rearrangements likely due to homologous recombination between two copies of IS*Pre1* may have occurred in these strains, as described previously (38). Neither segregational instability nor structural instability of pCAR1 was detected in these two double mutants when *pmr* and its promoter region were supplied (Fig. 3). Our previous study showed that a mini-replicon of pCAR1 [containing only DNA

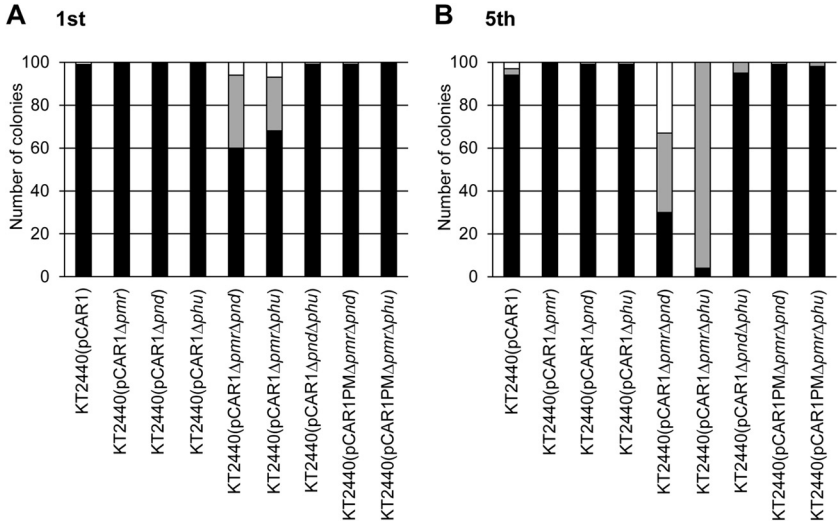

FIG 3 pCAR1 stability assay using *P. putida* KT2440(pCAR1), NAP gene disruption mutants, and *pmr*-complemented double mutants. The strains were grown on succinate as the sole source of carbon for 24 h, and then the resultant cultures were diluted into fresh media and cultured again. This dilution-incubation procedure was repeated five times, and the carriage of pCAR1 in colonies from the first (A) and fifth (B) passages in culture was examined using the *repA* and *carAc* genes. Black, gray, and white bars indicate the numbers of colonies containing intact pCAR1, pCAR1 without the *carAc* gene, and no pCAR1, respectively. Representative data from more than five independent experiments are shown.

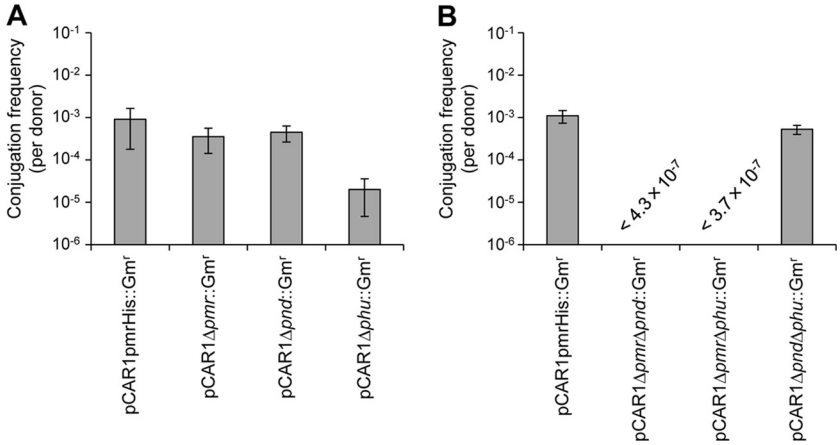

FIG 4 Conjugation frequency of NAP gene disruption plasmid pCAR1. Single mutants (A) and double mutants (B) of KT2440 harboring a Gm resistance cassette were used as the donors, and KT2440KR harboring the Km resistance cassette was the recipient. As a control, the KT2440(pCAR1pmrHis::Gm^r) strain harboring a Gm resistance cassette downstream of *pmr* was used. The donor and recipient were mixed in LB liquid medium to OD_{600} of 0.2 and 2.0, respectively, and the conjugation frequency was calculated as the number of transconjugants per number of donors. Data are expressed as means ± standard deviations of the conjugation frequencies, which were obtained in five experiments performed at the same time. Representative data from four (A) and two (B) independent experiments are shown.

regions involved in replication (*repA* and *oriV*) and partition (*parWASB*)] is not stably maintained in KT2440, whereas the full-length pCAR1 is stable, suggesting that pCAR1 encodes stabilizing factors outside the *rep* and *par* regions (39). The results of this study suggested that the stabilizing factors may be Pmr, Pnd, and Phu and also that the disruption of either a *phu* gene or a *pnd* gene with a *pmr* gene on pCAR1 could promote DNA rearrangement on pCAR1 in the host cell.

Transferability of pCAR1. To assess the effects of NAPs on the transferability of pCAR1, mating assays were performed using NAP gene disruption mutants as donors and the KT2440-derivative strain as the recipient. To select donor cells carrying each plasmid and to distinguish donors from recipients, each mutant with a Gm resistance cassette was used as a donor. As a control, we used the KT2440(pCAR1pmrHis::Gm^r) strain harboring a Gm resistance cassette downstream of the *pmr* gene (23). KT2440 (pCAR1Δ*pmr*::Gm^r) and KT2440(pCAR1Δ*pnd*::Gm^r) showed transfer frequencies similar to those seen with the control strain, whereas KT2440(pCAR1Δ*phu*::Gm^r) exhibited an about 10-fold decreased frequency (Fig. 4A). Larger differences were obtained when the double mutants were used as donors; the transfer frequencies of KT2440(pCAR1Δ*pmr*Δ*pnd*::Gm^r) and KT2440(pCAR1Δ*pmr*Δ*phu*::Gm^r) decreased markedly to below the limit of detection ($\leq 10^{-7}$ per donor cell), whereas that of KT2440(pCAR1Δ*pnd*Δ*phu*::Gm^r) was similar to the control (Fig. 4B). Notably, the transfer deficiencies of the double mutants were not recovered by complementation of *pmr* (i.e., they were below the limit of detection) ($\leq 10^{-6}$ per donor cell). As instability of pCAR1 in the double mutants was recovered by complementation of *pmr* (Fig. 3), the transfer deficiency may not have been due to plasmid replication and stability.

Taken together, these results suggest that Pmr, Pnd, and Phu are key factors in both the maintenance and the conjugation of pCAR1. As the three single mutants and KT2440 (pCAR1Δ*pnd*Δ*phu*) did not show the phenotypes described above, the pCAR1-encoded NAPs likely have synergistic functions, and Pmr may play the most important role in these processes.

Phenotypic screening of NAP gene disruption mutants. To assess the global effects of NAP gene disruption(s) in greater detail, we performed comprehensive phenotypic comparisons of the metabolic capacities and levels of stress resistance of the WT and NAP gene disruption mutants by assessing cellular respiration using Biolog Phenotype MicroArrays (PMs). This experiment was performed on the WT strain, three single mutants, and the two double mutants showing significant phenotypic differences as described above. Mutants without the Gm resistance cassette were used. The utilization of 23 [KT2440(pCAR1Δ*pmr*)], 22 [KT2440(pCAR1Δ*pnd*)], 15 [KT2440 (pCAR1Δ*phu*)], 18 [KT2440(pCAR1Δ*pmr*Δ*pnd*)], and 20 [KT2440 (pCAR1Δ*pmr*Δ*phu*)] compounds that were supplemented as the sole source of carbon, nitrogen, phosphate, or sulfur was affected by the single or double disruption of NAP genes (Table 3; see also Tables S1 to S4 in the supplemental material). Previous analyses comparing KT2440 and KT2440(pCAR1) showed that the utilization of 66 compounds was affected by pCAR1 carriage and that respiration activities for 57 of these 66 compounds had decreased (9). Notably, the utilization of intermediate compounds in the tricarboxylic acid (TCA) cycle (α-ketoglutaric acid, D,L-malic acid, succinic acid, and fumaric acid) and those several steps away from the TCA cycle (acetic acid and L-lactic acid) were affected, and the cell respiration activities for these compounds decreased (9) (see Table S1). In contrast, the disruption of NAP genes increased the cell respiration activities for these compounds, and the levels of activity for these compounds were restored to levels similar to that of pCAR1-free KT2440 (see Table S1), although a portion of double mutants lost the plasmid during the assay. No marked alterations were observed in resistance to osmotic or pH stresses by NAP gene disruption(s) (see Tables S5 and S6 in the supplemental material).

Alteration of the transcriptome by NAP gene(s) disruption. To elucidate the effects of NAP gene disruption on transcriptional networks in the host cells, transcriptome comparisons of three single mutants and two double mutants (without the Gm resistance cassette) were performed at the log growth phase using a tiling array. Among the transcribed genes in each strain, genes that showed an over-2-fold change between the WT and the mutants

TABLE 3 The numbers of compounds supplemented as the sole source of carbon, nitrogen, phosphate, or sulfur differentially metabolized between KT2440(pCAR1) and NAP gene disruptants[a]

Strain	No. of C sources (PM1 and PM2; 190 compounds)	No. of N sources (PM3; 95 compounds)	No. of P sources (PM4; 59 compounds)	No. of S sources (PM4; 35 compounds)	Total no. of sources
KT2440(pCAR1)[b]	22 (3, 19)	18 (1, 17)	22 (5, 17)	4 (0, 4)	66 (9, 57)
KT2440 (pCAR1Δ*pmr*)[c]	13 (13, 0)	4 (4, 0)	5 (2, 3)	1 (1, 0)	23 (20, 3)
KT2440 (pCAR1Δ*pnd*)[c]	10 (8, 2)	4 (4, 0)	8 (5, 3)	0	22 (17, 5)
KT2440 (pCAR1Δ*phu*)[c]	10 (9, 1)	1 (0, 1)	4 (0, 4)	0	15 (9, 6)
KT2440 (pCAR1Δ*pmr*Δ*pnd*)[c]	11 (9, 2)	1 (0, 1)	6 (0, 6)	0	18 (9, 9)
KT2440 (pCAR1Δ*pmr*Δ*phu*)[c]	10 (7, 3)	2 (0, 2)	7 (5, 2)	1 (1, 0)	20 (13, 7)

[a] Two independent analyses were performed with each strain for each compound, and cellular respiration activity was compared among the four combinations. The numbers in parentheses in columns 2 to 6 indicate the numbers of compounds with which cell respiration increased (left) or decreased (right).
[b] The results from KT2440(pCAR1) were compared with those from KT2440 (9).
[c] The results from NAP gene disruptants were compared with those from KT2440(pCAR1).

were defined as differentially transcribed genes caused by the disruption of a NAP gene(s). The ORFs of differentially transcribed genes on the KT2440 chromosome were classified into 23 groups based on their putative functions by Clusters of Orthologous Groups (COG) protein analysis.

Overview. The numbers of up- and downregulated genes on the KT2440 chromosome (with COG categories) and pCAR1 are shown in Tables 4 and 5, respectively, and the differentially transcribed genes are presented in Data Sets S1 and S2 in the supplemental material, respectively. Of the 226 genes (199 on the chromosome and 27 on pCAR1) differentially transcribed in KT2440(pCAR1Δ*pmr*), 224 were upregulated and only 2 were downregulated, suggesting that Pmr functions predominantly as a transcriptional repressor. In contrast, all 174 genes (165 on the chromosome and 9 on pCAR1) differentially transcribed in KT2440(pCAR1Δ*pnd*) were downregulated, suggesting that Pnd functions predominantly as a transcriptional activator. In KT2440 (pCAR1Δ*phu*), only 46 genes (42 on the chromosome and 4 on pCAR1) were up- or downregulated, which was less than in KT2440 (pCAR1Δ*pmr*) or KT2440(pCAR1Δ*pnd*). The upregulated genes in KT2440(pCAR1Δ*phu*) on both the chromosome and pCAR1 were also upregulated in KT2440(pCAR1Δ*pmr*), and 26 of the 33 downregulated genes on the chromosome in KT2440(pCAR1Δ*phu*) were also downregulated in KT2440(pCAR1Δ*pnd*) (see Data Sets S1 and S2 in the supplemental material), suggesting that Phu has regulons similar to the Pmr and Pnd regulons and not unique regulons. Fisher's exact test showed that the number of genes categorized in COG code C, "energy production and conversion," (19 genes) among the upregulated genes of KT2440(pCAR1Δ*pmr*) was significantly greater than that of genes categorized in COG code C (274 genes) in the whole genome in KT2440 (5,408 genes; $P<0.05$, Fisher's exact test) (Table 4), which may have been related to the increase in the respiration activity of the KT2440(pCAR1Δ*pmr*) mutant in the phenotypic scanning analysis described above. In contrast, the numbers of COG code S and uncategorized (—) genes in KT2440(pCAR1Δ*pnd*) and KT2440(pCAR1Δ*phu*) mutants were significantly greater than the number of genes in the whole genome of KT2440 ($P<0.05$, Fisher's exact test) (Table 4), indicating that disruption of *pnd* or *phu* affected the transcription of unknown genes and suggesting that these NAP genes may have unknown regulons in the host KT2440. We focused on the putative genes involved in phenotype alterations via the disruption of NAP genes (see below).

Transcriptome alterations of genes related to pCAR1 stability. As noted above, pCAR1 itself was lost in a portion of double mutants KT2440(pCAR1Δ*pmr*Δ*pnd*) and KT2440 (pCAR1Δ*pmr*Δ*phu*) (Fig. 3). The DNA regions on pCAR1 containing *carAc* were deleted in several double mutants (Fig. 3). These observations indicated that the maintenance system(s) of pCAR1 itself was disrupted or that the DNA rearrangements in the host cell were promoted. In our previous study, *repA* and *parWAB* genes of pCAR1 were shown to be involved in replication and stable maintenance of pCAR1 (40), while the *parI* (PP_3700) product, an orphan ParA family protein, is a negative host factor for the maintenance system of pCAR1 (39). The transcriptional levels of these genes were not changed in the double mutants, suggesting that the instability of pCAR1 itself in the double mutants may not have been due to a deficiency of partitions. Therefore, we attempted to find candidate genes involved in stable maintenance of pCAR1 or its DNA regions on both the chromosome and pCAR1. As the two double mutants showed similar levels of instability, commonly up- or downregulated genes in both strains were considered candidates; 49 and 10 genes on the chromosome were commonly upregulated and downregulated, respectively, in the double mutants (Table 4). Among these genes, the numbers of differentially transcribed genes classified as COG categories C ("energy production and conversion") (6 genes, upregulated) and P ("inorganic ion transport and metabolism") (3 genes, downregulated) were significantly greater than those of genes in the "C" (274 genes) and "P" (257 genes) categories among the whole genes (5,408 genes; $P<0.05$, Fisher's exact test) (Table 4). Notably, the upregulated genes in the "C" category were those encoding dehydrogenase (PP_1073, PP_4401, and PP_4667), ferredoxin (PP_1625), azurin (PP_4870), and cytochrome *c*-type protein (PP_3823), and the downregulated genes in the "P" category were putative TonB-dependent siderophore receptors (PP_3330 and PP_3340) and a copper receptor (PP_4838). Previously, we showed that pCAR1 carriage resulted in transient iron deficiency in the host and that one of the reasons for this deficiency was the constitutive expression of carbazole-degrading (Car) enzymes (8). Indeed, the deletion of *car* genes partially ameliorated the deficiency (8). In another study, DNA rearrangements were shown to occur in pCAR1 in host cells because the carriage of *car* genes would be disadvantageous to the host cells (38, 41). As pCAR1 in the double mutants also showed deletion of the *car* genes, some disadvantageous effects in the presence of pCAR1 with NAP gene deletion may be related to the DNA rearrangements of pCAR1 and instability of the plasmid. Commonly downregulated genes re-

TABLE 4 Numbers of up- and downregulated genes on KT2440 chromosome in NAP gene disruptants and their COG categories

P. putida KT2440 mutant	No. of genes in indicated COG category[a]																							Total no. of genes
	Information storage and processing					Cellular processes and signaling							Metabolism								Poorly characterized			
	J	A	K	L	B	D	V	T	M	N	U	O	C	G	E	F	H	I	P	Q	R	S	—	
Whole genome	164	2	370	200	2	38	61	232	253	120	37	165	274	200	480	87	163	178	257	79	464	420	1,162	5,408
KT2440 (pCAR1Δ*pmr*)_up	**1**[c]	0	8	6	0	1	3	10	5	6	1	6	19[b]	**1**[c]	15	3	5	6	12	3	18	16	53	198
KT2440 (pCAR1Δ*pmr*)_down	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
KT2440 (pCAR1Δ*pnd*)_up	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KT2440 (pCAR1Δ*pnd*)_down	3	0	11	4	0	1	0	6	3	4	2	5	9	5	11	0	6	4	12	2	15	23[b]	39	165
KT2440 (pCAR1Δ*phu*)_up	0	0	0	1	0	0	0	2	0	0	0	1	1	0	1	0	0	0	0	0	1	1	1	9
KT2440 (pCAR1Δ*phu*)_down	1	0	0	0	0	0	0	0	0	0	0	0	2	1	2	0	1	0	3	1	4	4	14[b]	33
KT2440(pCAR1 Δ*pmr*Δ*pnd*)_up	1	0	9	7	0	0	2	7	4	1	1	1	10	4	7	0	1	4	8	3	12	14	48[b]	144
KT2440(pCAR1 Δ*pmr*Δ*pnd*)_down	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	4[b]	0	3	4	7	21
KT2440(pCAR1 Δ*pmr*Δ*phu*)_up	1	0	4	4	0	0	1	4	2	1	0	2	8[b]	1	6	0	0	2	3	2	4	5	22	72
KT2440(pCAR1 Δ*pmr*Δ*phu*)_down	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	3[b]	0	0	2	4	12
Commonly upregulated[d]	0	0	3	3	0	0	1	2	2	1	0	1	6[b]	1	3	0	0	2	3	2	2	5	12	49
Commonly downregulated[d]	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	3[b]	0	0	2	4	10

[a] The descriptions for each COG code are as follows: J, translation, ribosomal structure, and biogenesis; A, RNA processing and modification; K, transcription; L, replication, recombination, and repair; B, chromatin structure and dynamics; D, cell cycle control, cell division; V, defense mechanisms; T, signal transduction mechanisms; M, cell wall/membrane/envelope biogenesis; N, cell motility; U, intracellular trafficking, secretion, and vesicular transport; O, posttranslational modification, protein turnover, chaperones; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism, chromosome partitioning; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, biosynthesis of secondary metabolites, transport, and catabolism; R, general function prediction only; S, function unknown; —, uncategorized.

[b] The number of ORFs categorized in the COG code in the differentially transcribed genes was significantly larger than that of ORFs categorized in the same COG code in the whole-genome ORFs ($P < 0.05$, Fisher's exact test). These cells are shown in gray.

[c] The number of ORFs categorized in the COG code in the differentially transcribed genes was significantly smaller than that of ORFs categorized in the same COG code in the whole-genome ORFs ($P < 0.05$, Fisher's exact test). These numbers are underlined and shown in boldface.

[d] "Commonly upregulated" and "Commonly downregulated" indicate the numbers of upregulated or downregulated genes in both KT2440(pCAR1Δ*pmr*Δ*pnd*) and KT2440(pCAR1Δ*pmr*Δ*phu*) mutants.

TABLE 5 Numbers of up- and downregulated genes on pCAR1 in NAP gene disruptants

Mutant	No. of upregulated genes	No. of downregulated genes	Total no. of genes
KT2440(pCAR1Δ*pmr*)	26	1	27
KT2440(pCAR1Δ*pnd*)	0	9	9
KT2440(pCAR1Δ*phu*)	3	1	4
KT2440(pCAR1Δ*pmr*Δ*pnd*)	51	2	53
KT2440(pCAR1Δ*pmr*Δ*phu*)	27	2	29

lated to the iron acquisition system suggested that pCAR1 instability and DNA rearrangements in the double mutants of NAP genes may be due to iron deficiency. Then, pCAR1 stability assays were performed under conditions of iron abundance (10-fold concentration of ferric citrate, 370 μM). However, the segregational instability and structural instability of pCAR1 did not change under these conditions (see Fig. S2 in the supplemental material). Therefore, the instability of pCAR1 itself and the DNA rearrangements in the double mutants of NAP genes may not have been caused solely by iron deficiency. In total, 27 genes on pCAR1 were commonly upregulated in KT2440(pCAR1Δ*pmr*Δ*pnd*) and KT2440(pCAR1Δ*pmr*Δ*phu*), but no genes were commonly downregulated except for *pmr* (Table 6; see also Data Set S2 in the supplemental material). Four of the 27 upregulated genes (ORF108b, ORF108c, ORF155, and *recT*) were upregulated only in the double mutants, whereas the other 23 genes were also upregulated in at least one of the three single mutants. One of these genes, *recT*, which has a translated sequence with 31% identity to RecT of *E. coli* DH10B, is a RecA-independent homologous recombination factor, and a RecT homolog of *P. syringae* was shown to promote efficient homologous recombination between genomic loci and linear DNA substrates (42). Considering our previous result (38), the elevated expression of RecT may have increased the frequency of homologous recombination between two nearly identical copies of IS*Pre1* to generate strains harboring pCAR1 without *car* operons. However, the DNA region containing *carAc* on pCAR1 was not deleted in the RecT-overexpressing strain (see Fig. S3 in the supplemental material), which suggested that upregulation of the *recT* gene did not affect the structural stability of pCAR1.

Transcriptome alterations of genes related to pCAR1 transferability. Transcriptome analyses showed that several *tra* and *trh* genes on pCAR1 were differentially transcribed in the complemented double mutants (Table 6; see also Data Set S2 in the supplemental material), which are considered to be essential for the conjugative transfer of pCAR1 (40, 43). In

TABLE 6 Commonly upregulated genes in KT2440(pCAR1Δ*pmr*Δ*pnd*) and KT2440(pCAR1Δ*pmr*Δ*phu*)

Designation or gene name	Putative function of the gene product	Regulation category[a]		
		pCAR1Δ*pmr*	pCAR1Δ*pnd*	pCAR1Δ*phu*
ORF40	Hypothetical protein	Up	NC	NC
ORF100	Hypothetical protein	Up	NC	NC
ORF101	Cobalamin biosynthesis protein	Up	NC	Up
ORF102	Cobalamin biosynthesis protein	Up	NC	NC
ORF103	Hypothetical protein	Up	NC	NC
ORF104	Hypothetical protein	Up	NC	Up
ORF105	Hypothetical protein	Up	NC	NC
ORF106	Hypothetical protein	Up	NC	NC
ORF107	Hypothetical protein	Up	NC	Up
ORF108	Hypothetical protein	Up	NC	NC
ORF108a	Hypothetical protein	Up	NC	NC
ORF108b	Hypothetical protein	NC	NC	NC
ORF108c	Hypothetical protein	NC	NC	NC
ORF109	Hypothetical protein	Up	NC	NC
ORF114	Hypothetical protein	Up	NC	NC
ORF115	Hypothetical protein	Up	NC	NC
ssb	Single-strand DNA binding protein	Up	NC	NC
recT	DNA recombination protein	NC	NC	NC
trhK	Putative transfer protein	Up	NC	NC
trhV	Putative pilus assembly protein	Up	NC	NC
trhA	Putative transfer protein	Up	NC	NC
dsbC	Putative disulfide bond isomerase	Up	NC	NC
ORF144	Hypothetical protein	Up	NC	NC
ORF145a	Hypothetical protein	Up	NC	NC
ORF145	Putative DNA primase	Up	NC	NC
ORF146	Putative DNA primase	Up	NC	NC
ORF155	Hypothetical protein	NC	NC	NC

[a] Transcription levels of genes in each single mutant in comparison with those in KT2440(pCAR1). "Up" indicates that the gene is upregulated in the mutant, and "NC" indicates that the transcription level did not change.

KT2440(pCAR1Δ*pmr*Δ*pnd*), *trhK*, *trhB*, *trhV*, *trhA*, *trhC*, *trhF*, *trhH*, and *trhG* were upregulated. Of these, *trhK*, *trhV*, and *trhA* were also upregulated in both KT2440(pCAR1Δ*pmr*) and KT2440(pCAR1Δ*pmr*Δ*phu*). Other than the *tra* and *trh* regions, ORF145 and ORF146 were upregulated commonly in KT2440(pCAR1Δ*pmr*), KT2440(pCAR1Δ*pmr*Δ*pnd*), and KT2440(pCAR1Δ*pmr*Δ*phu*). Although ORF145 and ORF146 were not essential for the transfer of pCAR1, the transfer frequency of pCAR1 decreased when these genes were disrupted (see the text in the supplemental material and Fig. S4 in the supplemental material). In the case of the other plasmid, R27, which also carries *tra* and *trh* genes and hns_{R27} (corresponding to *pmr* on pCAR1), transcription of the *tra* and *trh* genes of R27 was upregulated and the transfer frequency of R27 was derepressed when hns_{R27} was disrupted (22). The pCAR1 in the double mutants showed significantly low transfer frequencies in the mating experiments (Fig. 4B), whereas the transcriptional level of the *tra* and *trh* genes increased, perhaps due to the different media (LB or NMM-4 supplemented with succinate), growth phases (stationary or log phase), or plasmids (with or without the Gm resistance cassette in the *pnd* or *phu* gene) used in the mating experiments and transcriptome analyses. The mechanism underlying the loss of the plasmid transfer ability in the double mutants was unclear.

Conclusion. In this study, we assessed the roles of plasmid-encoded HU (Phu) and NdpA (Pnd) proteins. The function of NdpA-like proteins, which constitute a well-conserved protein family in Gram-negative bacteria, is not known regardless of their location (i.e., plasmid or chromosome). The findings of this study showed that Phu and Pnd could have alternative functions that compensate for the lack of Pmr functions in cases of *pmr* disruption. The double disruption of *pmr* (encoding H-NS family protein) and one of the other two NAP genes on pCAR1 had a significant effect on the host phenotype, especially for the stable maintenance of pCAR1. The NAPs may be stabilizing factors on the full-length pCAR1 in KT2440 which were not identified in our previous studies (39, 40). Recently, we found that these NAPs also have effects on biofilm formation of KT2440(pCAR1) (S. Lee, Y. Takahashi, H. Oura, K. Okada, H. Yamane, N. Nomura, and H. Nojiri, unpublished data). However, the molecular mechanisms by which the NAPs affected the phenotypes in the host cell remain unclear. Considering that heterologous protein-protein interactions of Pmr, Pnd, and Phu were not detected *in vitro* (see the text in the supplemental material and Fig. S5 in the supplemental material), investigation of *in vivo* DNA binding sites of these proteins will help us to understand how they regulate the phenotypes observed in this study. In addition, as the TurA and TurB chromosomally encoded H-NS family proteins have many important effects on the function of Pmr (23, 24), characterization of chromosomally encoded NdpA and HU homologs is necessary to clarify the molecular mechanisms behind the functions of Pnd and Phu. Disruption of *pnd* or *phu* did not affect transcription of the genes encoding NdpA or HU homologs on the chromosome, except for *hupA* in KT2440(pCAR1Δ*pmr*Δ*phu*) (see Data Set S1 in the supplemental material), suggesting that Pnd and Phu do not simply function to provide a molecular "backup" for their chromosomally encoded homologs. Future analyses of protein-protein interactions, DNA binding motifs, and DNA compaction using both plasmid-encoded and chromosomally encoded NAPs will clarify how these proteins function in bacterial cells, and such studies will shed light on the significance of plasmid-encoded NAPs.

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