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Extensive overproduction of the AdhE protein by *rng* mutations depends on mutations in the *cra* gene or in the Cra-box of the *adhE* promoter

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Abstract

Escherichia coli RNase G encoded by the *rng* gene is involved in degradation of *adhE* mRNA. Overproduction of the AdhE protein by *rng* mutants was found to depend on the genetic background of strains derived from DC272 (*adhC81*) or MC1061. We found that DC272 carried a point mutation in the Cra-binding site of the *adhE* promoter. The Cra protein encoded by the *cra* gene is known to act as a repressor of *adhE*. P1-phage-mediated transduction and *lacZ* fusion analysis with the mutant *adhE* promoter confirmed that this mutation is responsible for overproduction. On the other hand, Southern hybridization revealed that MC1061 had a 0.85-kb deletion of the *cra* gene. Overproduction of AdhE in the MC1061 background was reversed to the wild-type levels by introduction of a plasmid carrying the *cra*$^+$ gene. These results indicated that expression of the *adhE* gene was regulated transcriptionally by Cra and posttranscriptionally by RNase G.

Keywords: *adhE*; Alcohol dehydrogenase; *cra*; Cra-box; RNase G; *rng*

Escherichia coli RNase G, which is encoded by the *rng* gene at 72 min on the *E. coli* genetic map [1], was found as an endoribonuclease involved in the processing of the 5′-end of 16S rRNA precursor. Mutant strains with an *rng::cat* insertion accumulate a 16.3S precursor form that has a mature 3′-end and a 5′-end with an extra 66 nucleotides [2,3]. The endoribonucleolytic activity of RNase G was confirmed by Tock et al. [4] by using highly purified protein and synthetic oligo-ribonucleotide substrates in vitro [for a review, 5].

We also reported that RNase G is specifically involved in the degradation of the mRNA of the *adhE* gene, encoding a fermentative alcohol dehydrogenase (AdhE). The *rng::cat* mutant strains overproduced AdhE protein due to the increased stability of *adhE* mRNA [6]. Interestingly, marked overproduction of AdhE depended on the genetic background, that is, MC1061 with *rng::cat* overproduced AdhE but W3110 or CSH26 did not. Recently, we also found that the DC430 mutant strain, which was originally isolated as an AdhE overproducer [7], carried a novel *rng* mutation, named *rng430*, which causes substitution of Gly-341 with Ser of RNase G [8]. The mutant RNase G (Rng430) is defective in degradation of *adhE* mRNA but proficient in processing of 16S rRNA precursor. Marked overproduction of AdhE by DC430 was also dependent on another regulatory mutation, *adhC81* [9], the nature of which was unknown. In this study, we analyzed these genetic factors, which interact with the *rng* mutations to cause extensive overproduction of AdhE.

Materials and methods

Bacterial strains and media. Bacterial strains used are listed in Table 1. Cells were grown at 30 °C in L broth containing 1% bactopeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose (pH 7.0). Appropriate antibiotics were added for culturing the cells carrying plasmids.

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Table 1
Bacterial strains

Name	Genotype	References
GW10	The same as W3110 but *zce-726::*Tn*10*	[17]
GW11	The same as W3110 but *zce-726::*Tn*10 rng::cat*	[17]
CSH26	F$^-$ *ara* Δ(*lac-pro*) *thi*	[11]
GC11	The same as CSH26 but *rng::cat*	[6]
MC1061	*araD*139 Δ(*araABC-leu*)7697 Δ(*lac*)*X*74 *galU galK hsdR rpsL150 thi*	[18]
GM11	The same as MC1061 but *rng::cat*	[6]
DC271	*mel fadR*	[9]
DC272	The same as DC271 but *adhC81*	[9]
DC430	The same as DC272 but *rng430* (*adhR430*)	[7]
BW7622	HfrKL96: *trpB114::Tn10 relA1 spoT1 thi-1*	NIG[a]

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Ability to grow on ethanol as a sole carbon source was checked on M9 medium plates containing 2% ethanol as a carbon source and supplemented with 10 μg/ml tryptophan.

Analysis of cellular proteins. Cells suspended in sodium phosphate buffer (50 mM, pH 7.0) were disrupted by sonication. After removing unbroken cells, cell lysates were separated by SDS–PAGE and gels were stained with Coomassie brilliant blue.

Sequencing of the adhE region of adhC81 mutant strains. PCR was carried out using chromosomal DNA from DC271, DC272, DC430, or MC1061 and a set of primers, 5′-GATGTGGCGAAGTTAACATG ATGG-3′ and 5′-GGCGGAAGAGCTCTTCCACTGGGGCGG-3′, in which *Hpa*I and *Sac*I sites (underlined) were introduced for cloning, respectively, and which amplify a 4.4-kb DNA fragment containing the *adhE* gene and its upstream region. Amplified DNA fragments were digested with *Hpa*I and *Sac*I and cloned into the *Sma* I-*Sac*I site of the plasmid pMW119 (Nippon Gene, Tokyo, Japan). Three independently isolated clones of each construct were sequenced by the dideoxy chain termination method [10] using an automated fluorescence DNA sequencer (Long-Read Tower, Amersham Bioscience, NJ, USA) and Thermo Sequenase Dye Terminator (Cy5.5 and Cy5) sequencing kit (Amersham Bioscience).

Construction of adhE–lacZ fusion gene. A 1.3-kb upstream region of the *adhE* gene containing the *adhC81* mutant promoter sequence, the 5′-untranslated region and the first 27 bp of the coding region was amplified by PCR using chromosomal DNA of DC272 and a set of primers, 5′-GATGTGGCGAAGTTAACATGATGG-3′ and 5′-GCT CTACGAGTGCGTTAACTTACGCGAC-3′, in which *Hpa*I sites (underlined) were introduced for cloning. Amplified DNA was digested with *Hpa*I and joined in-frame with the *lacZ* gene at the nineth codon of *adhE* and the sixth codon of *lacZ* on a mini-F plasmid carrying ampicillin-resistance gene. The resultant plasmid, named pALF272, and pAFL1 containing the wild-type *adhE* promoter [6] were introduced into *rng* mutant strains and their parent strains. Expression of the *adhE–lacZ* fusion was examined by SDS–PAGE or by measuring β-galactosidase activity using the method of Miller [11].

Cloning of the cra gene and Southern hybridization. PCR was carried out using chromosomal DNA from GW10 and MC1061, and a set of primers, 5′-GCGCTTGGATCCTAATGAATTTAACC-3′ and 5′-CATATTGATGTCGGATCCCGTACTC-3′, in which *Bam*HI sites (underlined) were introduced for cloning, and which amplify a 1.1-kb DNA fragment containing the whole *cra* gene. Amplified DNA from GW10 was digested with *Bam*HI and cloned on the *Bam*HI site of pMW218 (Nippon Gene, Tokyo, Japan). The resulting plasmid pCRA1 was used for complementation analysis. Amplified DNA from GW10 was also used as a DNA probe for Southern hybridization. Chromosomal DNA from GW10, MC1061, DC271, and DC272 was digested with *Fsp*I or *Hae*II and separated by electrophoresis on a 0.7% agarose gel. Separated DNA was transferred onto positively charged nylon membranes (Hybond-N$^+$, Amersham Bioscience) by the capillary method. Southern hybridization was carried out using the Gene Images Kit (Amersham Bioscience).

Results

The factors involved in overproduction of AdhE by rng mutations are trans-acting in MC1061 but cis-acting in DC272

Marked overproduction of AdhE by *rng* mutations depended on the genetic background of strains MC1061 or DC272 (*adhC81*) [6,8]. In our previous paper, we reported that the unidentified factor involved in overproduction of AdhE in the MC1061 background was trans-acting [6]. To examine whether the factor in DC272 was trans-acting or cis-acting, we used an *adhE–lacZ* fusion plasmid pALF1 [6] that carries a 1.3-kb upstream region of the *adhE* gene containing the promoter sequence, the 5′-untranslated region, and the first 27 bp of the coding region of *adhE* joined in-frame to the *lacZ* gene. pALF1 was introduced into MC1061 and its *rng* derivative GM11 [6] and DC272 (*adhC81*) and its *rng* derivative DC430 [7–9]. As shown in Fig. 1, lanes 1 and 2, the AdhE–LacZ fusion protein encoded on the plasmid was overproduced in GM11, the *rng* derivative of MC1061, as was the intact AdhE protein encoded by the chromosomal *adhE* gene. On the other hand, as shown in Fig. 1, lanes 4 and 5, the AdhE–LacZ fusion protein was only slightly overproduced in DC430 in contrast to the marked overproduction of the AdhE protein. These results indicated that the factor carried by DC272 and its derivative DC430 was cis-acting, whereas the factor carried by MC1061 was trans-acting.

A mutation in the Cra-box of the adhE promoter region in DC272

Analysis using the *adhE–lacZ* fusion suggested that derivatives of DC272, such as DC430, have a mutation in the *adhE* promoter region. To clarify this possibility, the sequence of the *adhE* region of DC272 and DC430

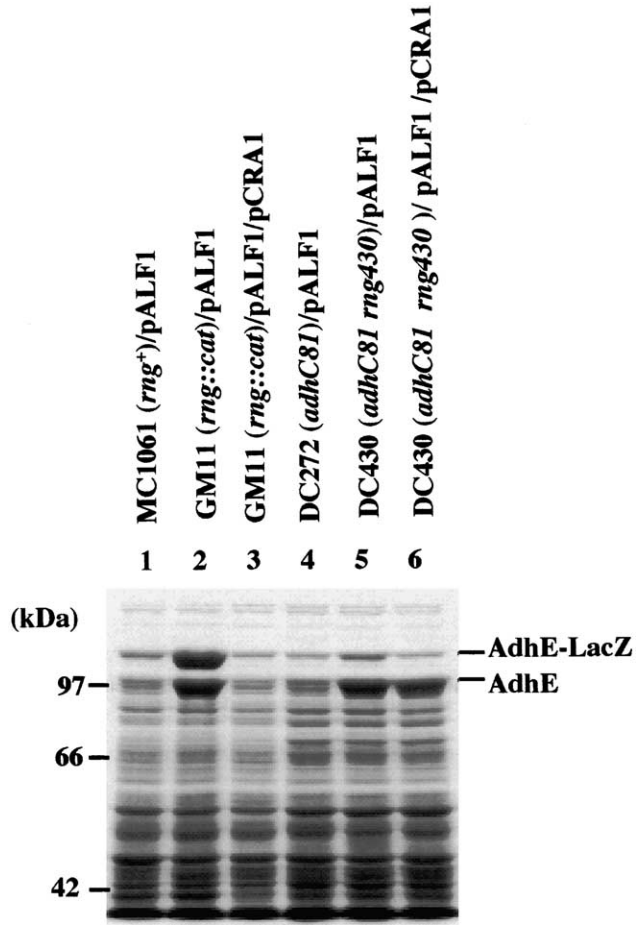

Fig. 1. A factor involved in overproduction of AdhE by *rng* mutation was trans-acting in MC1061 and cis-acting in DC272. Cellular proteins were analyzed on 7.5% SDS–PAGE as described in Materials and methods. Lane 1: MC1061 (*rng*$^+$), lane 2: GM11 (*rng::cat*), lane 3: GM11/pAFL1 (*rng::cat*/*adhE–lacZ*), lane 4: DC272 (*adhC81*), lane 5: DC430 (*adhC81 rng430*), and lane 6: DC430/pAFL1 (*adhC81 rng430*/*adhE–lacZ*). A Coomassie brilliant blue-stained gel is shown. Numbers shown at the left of the gel are molecular weights (×kDa).

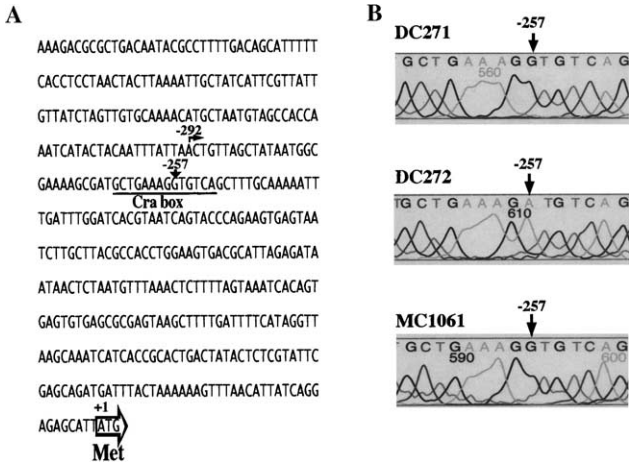

Fig. 2. Determination of the site of the *adhC81* mutation. (A) Nucleotide sequence of the upstream promoter region of the *adhE* gene. The Cra protein-binding sequence (Cra-box) is underlined. A boxed arrow indicates the ATG initiation codon of the *adhE* gene and a downward arrow, the site of the *adhC81* mutation. The possible transcription start site is −292. (B) Sequencing charts of the Cra-box regions of DC271 (*adhC*$^+$), DC272 (*adhC81*), and MC1061 (*adhC*$^+$). Arrows indicate a position of the base substituted in DC272.

was compared with that of the ancestral strain DC271. As a result, a G → A transition at position −257 bp upstream from the A of the ATG initiation codon of *adhE* was found in DC272 (Fig. 2B) as well as DC430 (data not shown). We also found in DC272 two substitutions in the coding region of the *adhE* gene (G → A at +799 bp and G → A at +1702 bp), which were previously shown to allow aerobic activity of the AdhE protein, that is, the ability to use ethanol as a sole carbon source under aerobic conditions, named *adhE** [5]. No other mutations were found in DC272 (data not shown). The *adhE* region of MC1061 had a wild-type sequence (Fig. 2B). We suspected, because of the reason mentioned below, that the upstream mutation, known as *adhC81*, was responsible for overproduction of AdhE and the coding region alterations, *adhE**, were responsible for aerobic activity of AdhE.

The *adhC81* mutation, a G → A transition at −257, is located in the Cra protein-binding site (Fig. 2A) [8]. The *cra* gene is a transcriptional regulator of numerous genes involved in carbon and energy metabolism [12,13] and acts as a repressor of *adhE* [14]. It is likely that the expression of *adhE* is de-repressed by the *adhC81* mutation due to the affinity of Cra to the Cra-box being decreased by the G → A substitution at −257.

To confirm this, P1-phage-mediated transduction of the *adhE* region was carried out using the closely linked *trpB* gene. DC430 was infected with P1-phage grown on *trpB::Tn10* strain BW7622 and tetracycline-resistant (Tc^r) transductants were selected. The transductants were checked for the amount of AdhE produced and the use of ethanol as a sole carbon source under aerobic conditions. Of 86 Tc^r transductants, 43 still overproduced AdhE as highly as the original DC430 strain and were able to grow on ethanol as a sole carbon source under aerobic conditions. The other 43 transductants no longer overproduced AdhE. Of these, 39 were unable to use ethanol and only 4 transductants still kept the ability to use ethanol. This result clearly indicated that DC430 has two mutations in the *adhE* region, very close to each other. One is responsible for overproduction of AdhE, that is, *adhC81*, and the other allows use of ethanol under aerobic conditions, that is, *adhE**. The order was *trpB::Tn10–adhC81–adhE**.

Then, to examine whether the *adhC81* mutation alone was sufficient to allow the *rng*-dependent overproduction of AdhE, a fusion of the *lacZ* gene to the promoter region with the *adhC81* mutation was constructed. As expected, the AdhE–LacZ fusion protein was overpro-

duced from pALF272 even in a *cra*$^+$ background (CSH26), where the AdhE protein encoded by the chromosomal *adhE* gene with the wild-type promoter was not overproduced (Fig. 3A, lane 8). In a Δ*cra* background (MC1061; as shown below, MC1061 has a deletion of the *cra* gene), both the wild-type promoter (pALF1) and the *adhC81* mutant promoter (pALF272) overproduced AdhE–LacZ (Fig. 3A, lanes 3 and 4). These were also confirmed by measuring β-galactosidase activity (Fig. 3B).

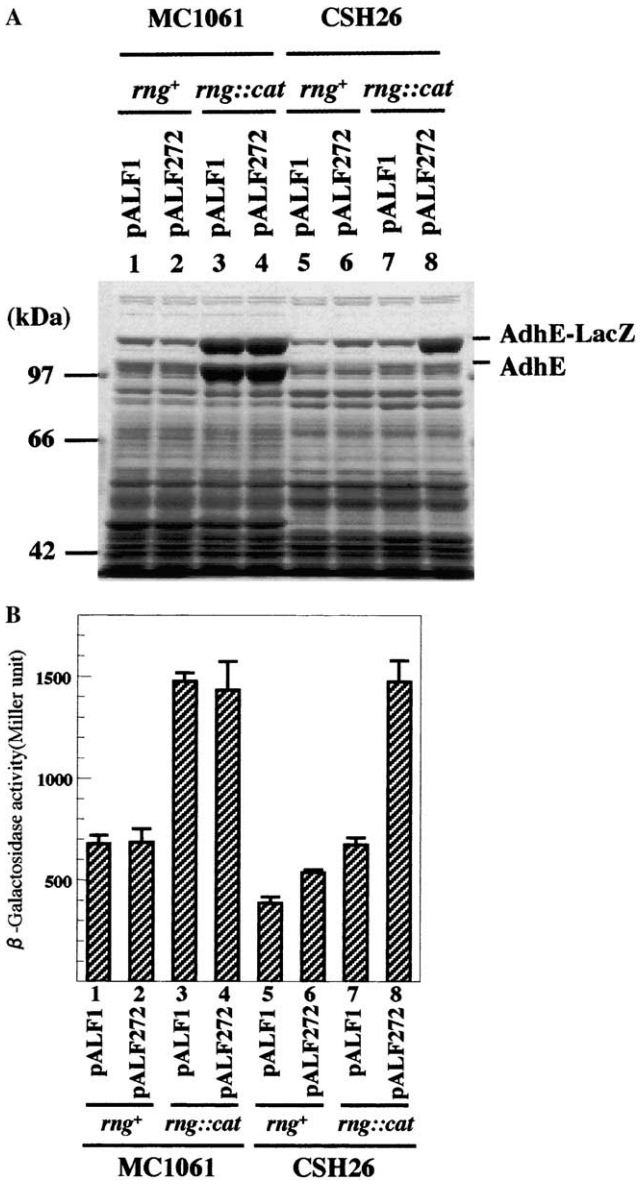

Fig. 3. Expression of AdhE–LacZ fusion protein from the wild-type and *adhC81* mutant promoters. (A) Cellular proteins were analyzed on 7.5% SDS–PAGE as described in Materials and methods. Lane 1: MC1061/pAFL1 (Δ*cra rng*$^+$/*adhE–lacZ*), lane 2: GM11/pAFL1 (Δ*cra rng::cat*/*adhE–lacZ*), lane 3: MC1061/pAFL272 (Δ*cra rng*$^+$/*adhC81–adhE–lacZ*), lane 4: GM11/pAFL272 (Δ*cra rng::cat*/*adhC81–adhE–lacZ*), lane 5: CSH26/pAFL1 (*cra*$^+$ *rng*$^+$/*adhE–lacZ*), lane 6: GC11/pAFL1 (*cra*$^+$ *rng::cat*/*adhC81–adhE–lacZ*), lane 7: CSH26/pAFL272 (*cra*$^+$ *rng*$^+$/*adhC81–adhE–lacZ*), and lane 8: GC11/pAFL272 (*cra*$^+$ *rng::cat*/*adhC81–adhE–lacZ*). A Coomassie brilliant blue stained gel is shown. Numbers shown at the left of the gel are molecular weights (×kDa). (B) Expression of the AdhE–LacZ fusion protein was examined by measuring β-galactosidase activity. Means ± SD ($n = 3$) are indicated.

A deletion of the cra gene in MC1061

The finding that the *adhC81* mutation was located in the Cra-box of the *adhE* promoter region in strain DC272 prompt us to test whether strain MC1061 had a mutation in the *cra* gene itself. The *cra* region was PCR-amplified from MC1061 and GW10 by using a set of primers that amplify a 1.15-kb DNA fragment containing the *cra* gene. As shown in Fig. 4B, no DNA fragment was amplified from MC1061 in contrast to GW10. It appeared that the primer binding site(s) were missing in MC1061. We then performed Southern hybridization using the DNA fragment amplified from

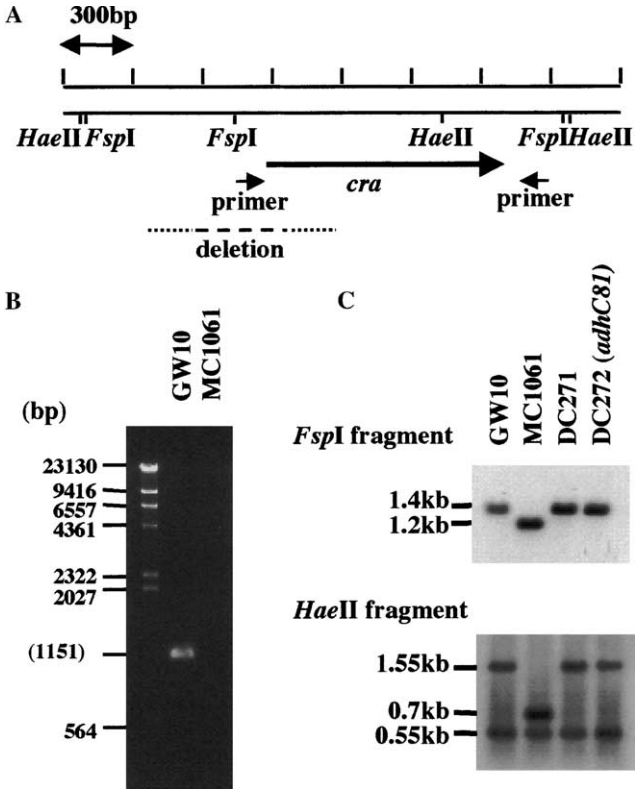

Fig. 4. A deletion of the *cra* gene in MC1061. (A) A physical map of the *cra* gene region. Small arrows indicate the set of promoters used for PCR, large arrow; coding region of the *cra* gene, dashed line; supposed deleted region in MC1061. (B) PCR products of GW10 (lane 1) and MC1061 (lane 2) were analyzed by 0.7% agarose gel electrophoresis. Numbers shown at the left of the gel are DNA length (×bp). (C) Chromosomal DNA was analyzed by Southern hybridization using a DNA probe containing the *cra* gene as described in Materials and methods. Lane 1: GW10, lane 2: MC1061, lane 3: DC271, and lane 4: DC272. Upper panel: *Fsp*I digestion; lower panel: *Hae*II digestion.

GW10 as a probe. The results indicated that about 0.85 kb of the *cra* gene encompassing the promoter and the N-terminal region was deleted in MC1061 (Fig. 4C).

To confirm that this deletion is responsible for overproduction of AdhE by *rng* mutations, a plasmid carrying the wild-type allele of the *cra* gene, pCRA1, was introduced into GM11 (Δ*cra rng*) containing the *adhE–lacZ* fusion plasmid. As expected, overproduction of the AdhE–LacZ fusion protein as well as the AdhE protein were reversed to the wild-type levels (Fig. 1, lane 3). On the other hand, when the *cra* plasmid was introduced into DC430, which carries the *adhC81* mutation on the chromosome, the AdhE protein expressed from the chromosomal *adhE* gene was still overproduced (Fig. 1, lane 6). This result again confirmed that repression of the *adhE* gene by Cra was canceled by the *adhC81* mutation.

Discussion

In this study, we analyzed other genetic factors required for the extensive overproduction of AdhE by *rng* mutations. Previously, marked overproduction of AdhE was observed only in very restricted genetic backgrounds, such as strain MC1061 and the *adhC81* mutant stain DC272 [14,17]. It was revealed that DC272 strain carried a G → A transition in the Cra-box of the *adhE* promoter, which is the basis for the *adhC81* mutation. This mutation caused de-repression of the *adhE* gene under aerobic conditions probably because of decreased affinity of the Cra protein to the mutant Cra-box. On the other hand, strain MC1061 had a deletion of the *cra* gene itself, which also caused de-repression of the *adhE* gene. In wild type cells, transcription of the *adhE* gene is strictly repressed under aerobic conditions and the effect of *rng* mutations is negligible because RNase G acts on transcribed mRNA molecules. In the presence of the *adhC81* mutation or deletion of the *cra* gene, the basal level of *adhE* mRNA is increased and the effect of *rng* mutations now becomes notable.

There have been some discrepancies in the analysis of strain DC272, which was originally isolated as a mutant that could grow on ethanol as a sole carbon source under aerobic conditions [9,15,16]. Membrillo-Hernández et al. [16] reported two substitutions in the *adhE* coding region but no base change in the promoter region was found. Holland-Staley et al. [15] also found the same two substitutions in the coding region and suggested the presence of a promoter mutation, although the mutation site was not determined. Expression of alcohol dehydrogenase activity is strictly regulated under aerobic conditions at both transcriptional and posttranslational levels. DC272 produced increased levels of alcohol dehydrogenese *activity* under both aerobic and anaerobic conditions. When the wild-type AdhE protein is produced from an expression vector under aerobic conditions, it is inactive [15]. Considering all these data, the DC272 mutant strain should have two characteristics: (1) de-repression of *adhE* transcription under aerobic conditions and (2) synthesis of *active* AdhE protein, even under aerobic conditions. Mutations in the coding region would explain the latter phenotype but seem unlikely to explain the former one. In this study, P1-phage-mediated transduction and DNA sequencing clearly revealed that DC272 has a mutation in the promoter region in addition to the two substitutions in the coding region. Moreover, *lacZ* fusion analysis indicated that this mutation, *adhC81*, is responsible for increased expression of the *adhE* gene. Now, phenotypes of mutant strain DC272 are fully explained by our findings. Application of the regulatory mechanism of the *adhE* gene expression found in the present work will be useful for industrial production of heterologous gene products in *E. coli*.

MC1061 and its derivatives have been widely used for gene expression analysis. Careful reconsideration is required to assign contribution of the *cra* mutation on the results done by using these strains, especially for the works in the field of carbon and energy metabolism.

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