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Construction of an *Escherichia coli* K-12 *ada* Deletion by Gene Replacement in a *recD* Strain Reveals a Second Methyltransferase That Repairs Alkylated DNA

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We constructed an *ada* deletion by gene replacement in a *recD1014* strain of *Escherichia coli*. Characterization of a Δ*ada-25 recD*$^+$ strain revealed the presence of a second DNA methyltransferase activity in *E. coli* K-12 which transfers a methyl group from methylated DNA to a protein with a molecular weight of 18,000 to 20,000.

Upon exposure of *Escherichia coli* cells to low levels of methylating or ethylating agents, a set of genes is induced that protects the bacteria against the mutagenic and lethal effects of much higher doses of these alkylating agents (11). This inducible system of protection, termed the adaptive response to alkylating agents, is positively regulated by the product of the *ada* gene. The Ada protein also possesses two distinct DNA repair activities in two protein domains. One activity is a methyltransferase that repairs the mutagenic DNA lesions O^6-methylguanine or O^4-methylthymine by transferring the methyl groups to Cys-321 in the carboxyl-terminal domain of Ada (3, 4). The second activity is a methyltransferase that transfers methyl groups from one of the two stereoisomers of DNA methylphosphotriesters to the Cys-69 residue in the amino-terminal domain of Ada (8, 17; B. Sedgwick, P. Robins, N. Totty, and T. Lindahl, in press). These methyl transfers to cysteines are irreversible and, hence, are suicide activities (3, 4). The inducing signal for the adaptive response is the self-methylation of Ada at the Cys-69 residue, which converts the Ada protein into an efficient transcriptional activator of the *ada alkB* operon and the *alkA* and *aidB* genes (10, 15, 16). The *ada* gene has been cloned and sequenced (4, 7, 12), and the intact 39-kilodalton (kDa) gene product has been purified (8, 9). The O^6-methylguanine-DNA methyltransferase was originally isolated as a 19-kDa protein (3) resulting from rapid cleavage of the 39-kDa Ada protein in cell extracts (14). The possible in vivo role of this cleavage remains to be determined.

Recently, we discovered that strains with our *ada* transposon insertion mutation, *ada-10* (7), still have DNA methyltransferase activity. Strains carrying the *ada-10* mutation are extremely sensitive to the mutagenic and toxic effects of alkylating agents and are unable to induce *ada* or *alkA* transcription (7). Nevertheless, we detected the transfer of ^{3}H-labeled methyl groups from a [^{3}H]methylated DNA substrate to an 18- to 20-kDa protein in cell extracts from an *ada-10* strain. This methyltransferase activity was evidently specific for O-methylated bases, since no methylated protein was detected in these assays when cell extracts from the *ada-10* strain were incubated with a DNA substrate that contained methylphosphotriesters but no O^6-methylguanine or O^4-methylthymine residues. The observation of methyltransferase activity in these strains is consistent with the results of Green et al. (5), who reported that, to generate mutations at a specific site in a plasmid where an O^6-methylguanine residue had been inserted, the *ada-10* host strain had to be previously exposed to low levels of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Such a treatment is expected to deplete the remaining methyltransferase activity in the cell.

To determine whether the methyltransferase activity in the *ada-10* strains was due to an active 19-kDa Ada fragment or to a different methyltransferase activity in the cells, we constructed an allele with the *ada* gene replaced by a fragment encoding chloramphenicol resistance (Fig. 1). Only the *ada* promoter and the first 77 base pairs of the protein-coding sequence of the *ada* gene remained in pGW3502. The first 189 base pairs of the protein-coding sequence of *alkB* were also deleted.

To move the *ada* deletion into the chromosome by homologous recombination, we modified an approach that we have previously described (18). Our original method involved transforming a *recB21 recC22 sbcB15* strain (JC7623) with a fragment of linear DNA containing a selectable marker flanked by homologous DNA. The *recB recC* mutations inactivate exonuclease V, preventing it from degrading the linear DNA, while the *sbcB* mutation restores recombination proficiency to the *recB recC* strain. However, *recB21 recC22 sbcB15* mutants grow poorly, segregating a significant fraction of nonviable cells compared with growth of the wild-type strain (1). Recently, Chaudhury and Smith (2) reported that a *recD1014* mutant has a wild-type level of recombination proficiency but lacks detectable exonuclease V activity. We reasoned that this *recD* strain (V355) might prove to be a better host for transformation with linear DNA, since it is also fully viable.

The 4.3-kilobase (kb) fragment of pGW3502 containing the *ada* deletion flanked by approximately 1 kb of chromosomal DNA on either side was isolated and used to transform competent *recD* cells. These cells were selected for Cm^r. We obtained two independent Cm^r Ap^s colonies. The Cm^r in these strains proved to be approximately 25% cotransducible with *gyrA* by P1 transduction, as was expected for the *ada* locus (7). Cm^r transductants of a *recD*$^+$ *ada*$^+$ strain (AB1157) were examined by a patch mutagenesis assay (7) and were all found to be Ada$^-$. Chromosomal DNA blots (13) of both the *recD1014* Cm^r and the *recD*$^+$ Cm^r strains

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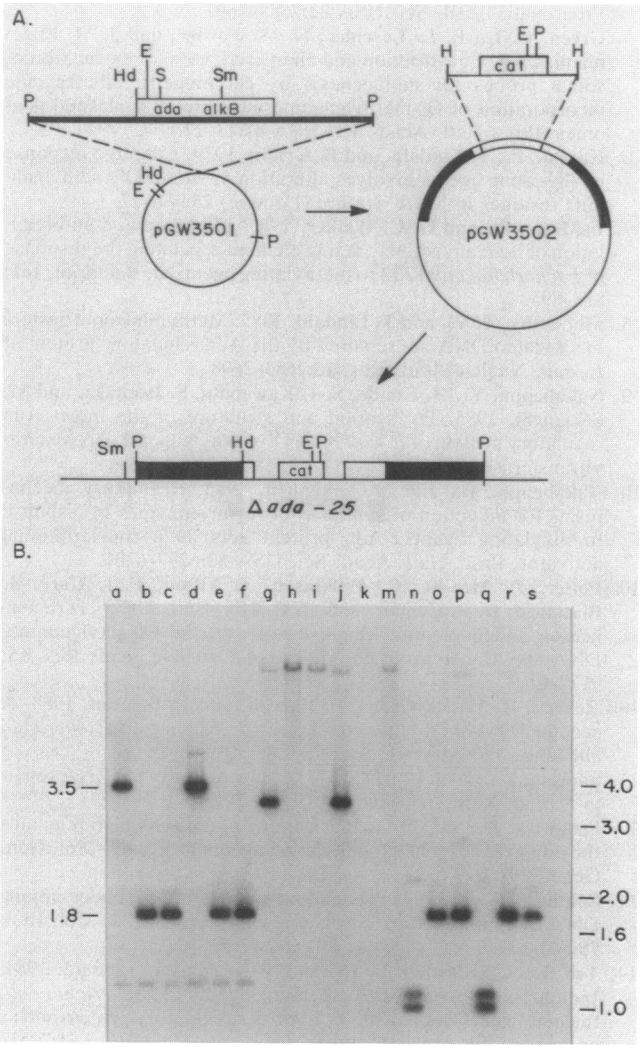

FIG. 1. Construction of an *ada* deletion. (A) pGW3501 has a 3.5-kb fragment containing the *ada alkB* operon inserted at the *Sal*I site in pBR322. We cloned a blunted 1.4-kb *Hae*II fragment containing the *cat* gene, derived from pACYC184, into the blunted *Eco*RI-*Sma*I sites of the *ada alkB* operon to generate pGW3502. pGW3502 was digested with *Bam*HI, *Pvu*I and *Nru*I, and 1.8-μg amounts of the 4.5-kb fragment containing the *ada* deletion were used to transform a *recD1014* strain (V355). Restriction sites: P, *Pvu*II; Hd, *Hin*dIII; S, *Sal*I; E, *Eco*RI; H, *Hae*II; and Sm, *Sma*I. (B) Chromosomal DNA blot of *ada*$^+$ and Δ*ada-25* strains. A total of 3 μg of DNA was loaded in each lane. The DNA blot was probed with a 2.1-kb *Sma*I-*Sma*I fragment. Lanes: a, g, and n, AB1157; b, c, h, i, o, and p, AB1157 Δ*ada-25*; d, j, and q, V355; e, f, k, m, r, and s, V355 Δ*ada-25*. The two independently isolated *ada* deletions (compare lanes b, h, and o with lanes c, i, and p, and lanes e, k, and r with lanes f, m, and s) appear to be identical. The DNA was digested with *Pvu*II (lanes a to f), *Sal*I (lanes g to m), or *Eco*RI and *Sma*I (lanes n to s). Base pairs (in thousands) are indicated on the left and right of the lanes.

confirmed that these cells contained the desired *ada* deletion (Fig. 1). We refer to the *ada* deletion mutation as Δ*ada-25*. Parallel experiments with a *recB21 recC22 sbcB15* strain did not yield any colonies containing a chromosomal *ada* deletion.

We assayed the *recD*$^+$ Δ*ada-25* strain (GW7101) and the *ada*$^+$ strain (AB1157) for DNA methyltransferase activity with ^{3}H-labeled methylated *Micrococcus luteus* DNA as a

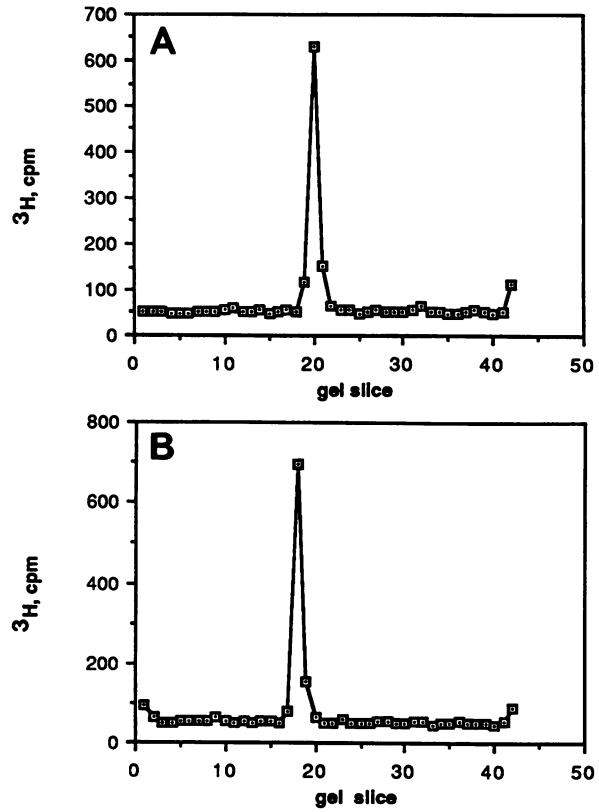

FIG. 2. Methyltransferase activity in *ada*$^+$ (AB1157) (A) and Δ*ada-25* (GW7101) (B) strains. Cell extract (1.2 mg of protein) was mixed with 10 μg of [^{3}H]methylnitrosurea (MNU) (Amersham) (2.9 Ci/mmol) -treated *M. luteus* DNA (180 cpm/μg of DNA) for 10 min at 37°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The radioactivity of 2-mm gel slices that had been incubated with Fluorosol (National Diagnostics) for 12 h at 55°C was measured in a scintillation counter. To measure phosphotriester activity, cell extract was incubated with 18 μg of [^{3}H]MNU (2.9 Ci/mmol) -treated poly(dT) hybridized to poly(dA) (140 cpm/μg of DNA) (data not shown). The alkylated *M. luteus* DNA (6) and poly(dT-dA) DNA (8) were prepared as described elsewhere. Molecular weight markers (Bio-Rad Laboratories): gel slices 17 and 18, lysozyme (14,400); gel slices 24 and 25, soybean trypsin inhibitor (21,500); gel slices 28 and 29, carbonic anhydrase (31,000); gel slice 33, ovalbumin (45,000).

substrate. Both strains possess a methyltransferase activity that transfers a methyl group to a protein with a molecular weight of 18,000 to 20,000 (Fig. 2). Interestingly, the uninduced *ada*$^+$ and Δ*ada-25* strains exhibited comparable amounts of this methylated 18- to 20-kDa protein and no 39-kDa methylated protein. These results demonstrate that there is a second DNA repair methyltransferase activity in *E. coli* that is distinct from that of the Ada protein. Moreover, this second activity is constitutively expressed and is not simply induced or selected for in strains with *ada* mutations. The lack of a detectable amount of a methylated 39-kDa protein in assays with 1.2 mg of cell extract protein from an uninduced *ada*$^+$ strain suggests that the second DNA methyltransferase accounts for most of the methyltransferase activity observed in the uninduced *ada*$^+$ *E. coli* cells. Consistent with the above observations, we found no detectable phosphotriester-DNA methyltransferase activity when we analyzed 1.2 mg of cell extract protein from a Δ*ada-25* strain or from an uninduced *ada*$^+$ strain. The above

results, together with the observations of Green et al. (5), suggest that the second methyltransferase repairs O^6-methylguanine DNA residues but not methylphosphotriesters. The spectrum of DNA lesions repaired by this novel methyltransferase remains to be determined.

We did not detect any increase in DNA methyltransferase activity in a Δ*ada-25* strain after exposure of the cells to MNNG (1, 10, or 100 ng/ml for 2 h) (data not shown). In contrast, a methylated 39-kDa protein was highly induced in an *ada*$^+$ strain after incubation of the cells with MNNG (100 ng/ml) for 2 h (data not shown). We also found that the Δ*ada-25* mutant was as sensitive to mutagenesis and killing by MNNG as the *ada-10* mutant (7) and that there was no difference in the frequency of spontaneous mutations compared with that in the parent *ada*$^+$ strain. Moreover, the Δ*ada-25* mutation had no obvious effect on cell viability and could be transduced into various genetic backgrounds.

Recently, two other groups have independently identified a second methyltransferase in *E. coli*. Potter et al. (10a) cloned a gene from *E. coli* which encoded a methyltransferase protein with 49% homology to the carboxyl-terminal domain of Ada. Rebeck et al. (10b) observed a residual DNA methyltransferase activity for O^6-methylguanine in DNA in a mutant (BS23) with a spontaneous deletion of the *ada alkB* region.

Although the Ada protein is not essential for viability in *E. coli*, the presence of a second methyltransferase suggests that a DNA methyltransferase activity could be required for some cellular function(s). The constitutively expressed second methyltransferase could protect the cell from the normal low levels of alkylation damage produced by endogenous or external sources, while the *ada* gene would respond to a large amount of alkylation damage.

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