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Cloning and Expression of the *Escherichia coli* K-12 *sad* Gene

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The *Escherichia coli* K-12 *sad* gene, which encodes an NAD-dependent succinic semialdehyde dehydrogenase, was cloned into a high-copy-number vector. Minicells carrying a *sad*$^+$ plasmid produced a 55,000-dalton peptide, the probable *sad* gene product.

Escherichia coli strains have two succinic semialdehyde dehydrogenases (EC 1.2.1.16), one which is NADP dependent and is encoded by the *gabD* locus (min 57.6) and one which is NAD dependent and is encoded by the *sad* gene at min 34 in the terminus region of the chromosome (8, 21; for a review, see reference 5). In *E. coli* B and C, which are able to utilize 4-hydroxyphenylacetate as a sole carbon source, the NAD-dependent dehydrogenase is required to oxidize succinic semialdehyde, a product of catabolism of 4-hydroxyphenylacetate, to succinate. It is unclear why *E. coli* K-12 strains possess an NAD-dependent succinic semialdehyde dehydrogenase, since they are incapable of utilizing 4-hydroxyphenylacetate. The *sad*-encoded protein is not required for growth of *E. coli* K-12 in rich or minimal media, since strains with *sad* deletions have normal growth rates and cell morphology (13). Perhaps *sad* is a vestigial gene that was retained when K-12 strains lost their ability to use 4-hydroxyphenylacetate (5). Its retention may offer a selective advantage, since succinic semialdehyde is toxic to *E. coli* (7, 21).

We became interested in the *sad* gene because of its location in the terminus region of the chromosome (approximately min 28 to 35), which has few known genetic markers. Cloned fragments are used to determine the progress of DNA replication forks through this region and to define the endpoints of terminus region deletions (13). Analyses of some deletion mutants were incomplete because a portion of the terminus region, which included *sad*, had not been cloned. We report here the cloning of the *sad* gene of *E. coli* K-12 and expression of the gene in minicells.

The bacterial strains used in this study are listed in Table 1 and were grown on either LB (complex) or M9 (minimal) medium (18). We recently isolated *E. coli* strains with operon fusions in the terminus region of the chromosome. One of these strains, JH492, had an insertion of λ*plac*Mu53 (4) at approximately min 33.7 as determined by bacteriophage P1 transduction and hybridization with cosmid pBS12 (Fig. 1; data not shown; 1). This strain was *lac*$^+$ due to an operon fusion between the *lac* genes of the phage and a strong bacterial promoter in the terminus region of the chromosome. Because DNA on the clockwise side of this λ*plac*Mu insertion had not been cloned, we used the *lac*$^+$ genes of the prophage as a genetic marker for cloning nearby chromosomal DNA, including the *sad* gene. Cosmids which contained chromosomal DNA from strain JH492 were constructed as previously described (14); briefly, DNA was extracted from strain JH492, partially digested with *Sau*3A restriction enzyme, and ligated with cosmid vector pHC79 that was digested with *Bam*HI. Recombinant plasmids were packaged into bacteriophage λ particles (11), strain DH5 (Δ*lac*) was infected with the recombinant lysate, and Ap^r *lac*$^+$ clones, including strain JH613, were identified on MacConkey lactose medium (Difco Laboratories) containing 50 μg of ampicillin per ml.

Cosmids were also tested for their ability to complement *sad* mutants, which, unlike wild-type strains, cannot grow on medium with 2.5 mM succinic semialdehyde (21). One cosmid, pMSU613, complemented *E. coli* K-12 *sad* deletion mutants (e.g., strain JH475) as well as *E. coli* C *sad* point mutants (strains CT103 and CT101; 21), allowing them to grow on medium with 2.5 mM succinic semialdehyde. This cosmid was chosen for further analysis and subcloning (16), which is summarized in Fig. 1. Smaller *sad*$^+$ plasmids were derived by subcloning a large, >20-kilobase-pair (kb) *Pst*I fragment into pBR322 to construct pMSU645. Plasmids pMSU613 and pBR322 were digested with restriction enzyme *Pst*I, mixed, and ligated with T4 DNA ligase. Restriction enzymes and T4 DNA ligase were used as directed by manufacturers. Ligated DNA was transformed into strain DH5 (6), and tetracycline-resistant (Tc^r) transformants were selected on LB medium containing 20 μg of tetracycline per ml. pMSU645 was purified from an Ap^s clone and subjected to further restriction analysis, and a comparison of its restriction sites with the known restriction map of the terminus region (3) allowed alignment of plasmid fragments with chromosomal fragments. Plasmids pMSU687, pMSU 685, and pMSU690 were constructed by digesting pMSU645 either partially or completely with *Eco*RI, ligating fragments into the *Eco*RI site of pBR325 (2), and transforming strain DH5. Recombinant plasmids were purified from Tc^r chloramphenicol-sensitive transformants and were further analyzed by restriction mapping and hybridization to confirm their identities. Although pMSU687, which carried approximately 4.4 kb of chromosomal DNA, complemented *sad* mutations, pMSU685 and pMSU690 were unable to do so. Thus, it was likely that the *sad* gene spanned the *Eco*RI site at kb 370.

Since *E. coli* has two succinic semialdehyde dehydrogenases, we hybridized pMSU687 with chromosomal DNA from a wild-type strain and a *sad* deletion mutant to confirm that we had cloned the terminus region gene encoding the NAD-dependent dehydrogenase. As expected, chromosomal DNA from an *E. coli* K-12 wild-type strain had two *Eco*RI fragments of 2.9 and 1.8 kb that hybridized with pMSU687, whereas the terminus deletion strain did not have homologous DNA fragments, indicating that the cloned DNA was indeed from the terminus region (data not shown).

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TABLE 1. *E. coli* strains

Strain	Description	Source
MC4100	F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301*	M. Casadaban via E. Bremer (4)
JH492	MC4100 (λ*plac*Mu53); prophage at ca. kb 355 (Fig. 1)	This work
DH5	F⁻ *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA86 relA1* φ80d *lacZ* ΔM15 λ⁻	Bethesda Research Laboratories
JH613	DH5(pMSU613); pMSU613 is a cosmid with pHC79 (14) and kb 355 to 372 from the terminus region of *E. coli* K-12	This work
M2508	Hfr PO2A *relA1 spoT1 metB1 melA7*	R. Schmitt via B. Bachmann[a]
JH475	M2508 Δ5 (min 28.7 to 35.8) *zdg-232*::Tn*10*	Laboratory collection
DHS410	F⁻ *azi-8*? *tonA2*? *minA1 minB2 rpsL135 xyl-7 mtl-2 thi*-1 λ⁻	J. N. Reeve via B. Bachmann
PK2038	F⁻ *ptsG* terminus region deletion kb 360 to 455	P. Kuempel (13)
JC10240	Hfr PO45 *thr-300 recA56 srl-300*::Tn*10 relA1 ilv-318 spoT1 thi*-1 *rpsE2300* λ⁻	A. J. Clark via B. Bachmann
JH748	F⁻ *ilv his-29 arg pro thyA deoB* or *deoC trpA9605 tsx lac trpR* Δ5 (min 28.7 to 35.8) Sad⁺ (*gab*)	This work
CT101	*E. coli* C F⁻ *hpcR sad*-1	R. A. Skinner (21)
CT103	*E. coli* C F⁻ *hpcR sad*-3	R. A. Skinner (21)

[a] *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

When *sad* deletion strains were plated on 2.5 mM succinic semialdehyde medium, several Sad⁺ colonies always grew. Thus, although the *sad* gene appeared to encode the major dehydrogenase that eliminated extraneously added succinic semialdehyde, compensatory mutations allowed *sad* deletion mutants to grow on 2.5 mM succinic semialdehyde. We thought it likely that these secondary mutations were in one of the *gab* genes, since mutations in the *gabC* regulatory locus can result in a six- to sevenfold increase in the succinic semialdehyde dehydrogenase encoded by *gabD* (9, 10). To determine if the secondary mutations were near the *gab* locus (min 57.6), bacteriophage P1 transduction (18) was used to transduce *srl-300*::Tn*10* (min 58.3) from donor strain JC10240 into strain JH748 (a Sad⁺ pseudorevertant). Of 58 Tc^r transductants, 40 became Sad⁻, which indicated that the secondary mutation in strain JH748 was closely linked to the Tc^r marker at min 58 and was most likely in the *gab* locus, at either *gabC* or *gabD*. This was consistent with the mapping data of Metzer et al., who found that *gab* and *srl* are 31% cotransducible (17).

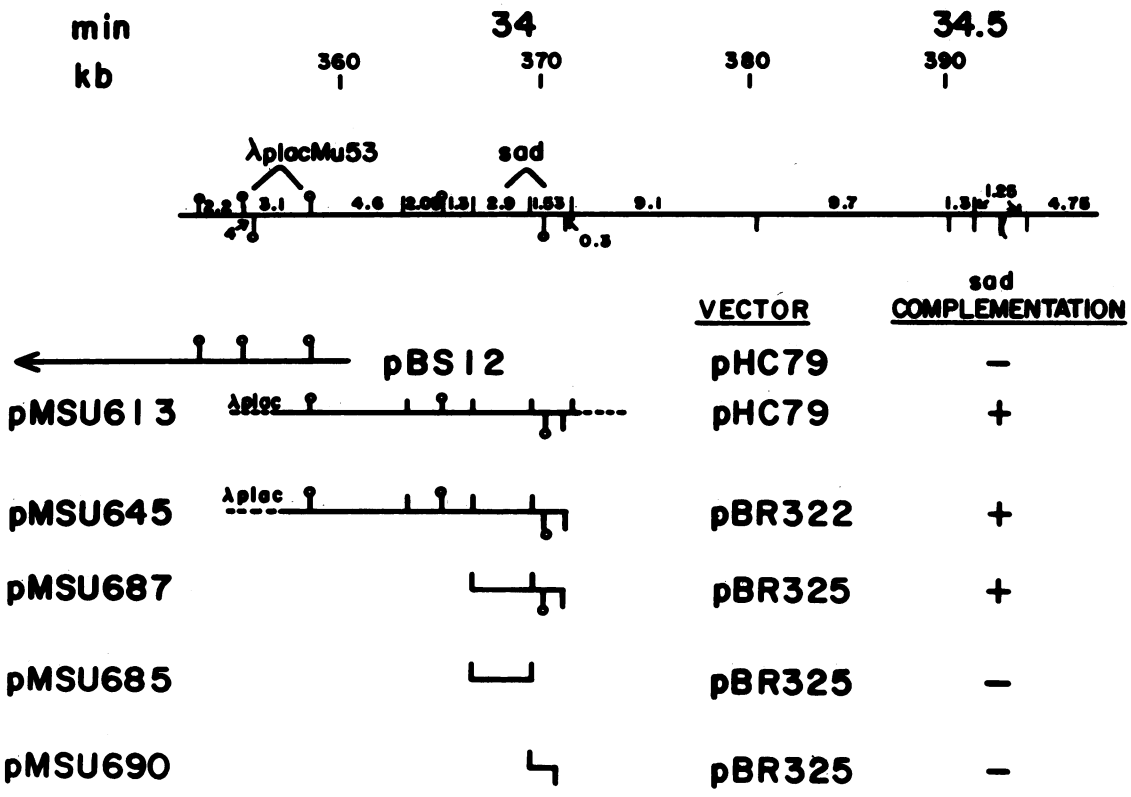

FIG. 1. Maps of the terminus region of *E. coli* K-12 near the *sad* gene and of recombinant plasmids that carried DNA from this region. The restriction map of the chromosome was constructed by Bouché (3), except for the addition of a *Bam*HI site at kb 371. Plasmid pBS12 was also constructed by Bouché and his co-workers (1). Fragment sizes are in kilobases. Symbols: ⊤, *Pst*I sites; ⊥, *Eco*RI sites; ┻, *Hin*dIII sites; ┳, *Bam*HI sites.

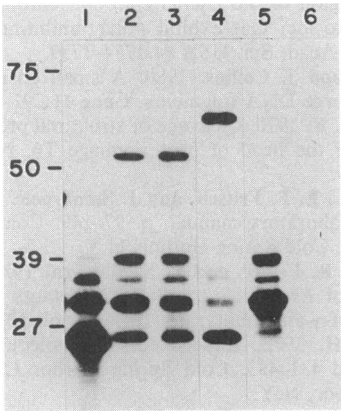

FIG. 2. Plasmid expression in minicells. Minicells were labeled with [^{35}S]methionine, lysed, and electrophoresed in a sodium dodecyl sulfate (12%)-polyacrylamide gel. The gel was treated with En^{3}Hance, and X-ray film was exposed for 4 h. Between 75,000 and 150,000 cpm was added to each well. Lanes: 1, pBR325; 2 and 3, pMSU687; 4, pMSU690; 5, pMSU685; 6, no plasmid. Minicells in lanes 2, 4, and 5 were grown and labeled in medium with 2.5 mM succinic semialdehyde. Molecular mass standards, in kilodaltons, are indicated on the left.

To identify the protein product of the *sad* gene, recombinant plasmids were transformed into strain DHS410, minicells were prepared (12), and plasmid-encoding proteins were labeled with [^{35}S]methionine (19) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2; 15). Because succinic semialdehyde induces the synthesis of NAD-dependent succinic semialdehyde dehydrogenase (7, 8), minicells containing pMSU687 were both grown and labeled in medium containing 2.5 mM succinic semialdehyde (Fig. 2, lane 2) or grown and labeled without the inducer (lane 3). In either condition, in minicells containing pMSU687 three proteins were synthesized that were not produced in minicells carrying pBR325 (Fig. 2, lane 6); these proteins had apparent molecular masses of 55, 39, and 25 kilodaltons (kDa). The 39-kDa protein was almost the same size as a 39-kDa vector-encoded protein; lighter exposures of the autoradiogram in Fig. 2 showed two 39-kDa proteins in lanes 2, 3, and 5. The 25-kDa protein encoded by pMSU687 had approximately the same molecular mass as the chloramphenicol resistance protein encoded by pBR325. In another, unrelated minicell experiment, a pBR325 derivative that had an *Eco*RI fragment inserted in the chloramphenicol resistance gene (*cam*) did not encode any 25-kDa proteins, which demonstrated that pBR325 encodes only one 25-kDa protein, the *cam* gene product (data not shown). Since the cloned *Eco*RI fragment of pMSU687 was inserted into the middle of the *cam* gene, the 25-kDa protein encoded by this plasmid cannot be the chloramphenicol resistance protein.

Minicells with pMSU690 synthesized a 25-kDa protein but not the 39-kDa protein (Fig. 2, lane 4), whereas pMSU685 synthesized a 39-kDa protein but not the 25-kDa protein (lane 5). Since neither plasmid complemented *sad* mutations, the simplest interpretation of these data is that neither of these proteins is the *sad* gene product. This interpretation assumes that the 25-kDa protein encoded by pMSU687 and seen in lanes 2 and 3 is the same 25-kDa protein encoded by pMSU690 and that the 39-kDa protein encoded by pMSU687 is the same as that encoded by pMSU685. Because the cloned *Eco*RI fragment of pMSU687, which is also present in pMSU690, picked up 0.5 kb from pBR322 during the cloning process, the gene encoding the 25-kDa protein could be a fused gene containing pBR322 and terminus region DNA.

The 55-kDa protein, which was the only apparent protein encoded by pMSU687 but not encoded by either pMSU685 or pMSU690, is probably the *sad* gene product, although other explanations for these results are possible. For example, it is possible that the Sad$^+$ phenotype requires both the 25- and the 39-kDa proteins and that the 55-kDa protein is irrelevant for growth on succinic semialdehyde. However, Donnelly and Cooper (8) purified NAD-dependent succinic semialdehyde dehydrogenase to near homogeneity and determined that the native molecular mass of the protein was 97 kDa, whereas the subunit size was 55 kDa, a size consistent with the simplest interpretation of our data.

Other plasmid-encoded proteins had molecular masses ranging from 26 to 39 kDa. The tetracycline resistance protein is 37 kDa, and the β-lactamase proteins encoded by the *bla* gene of pBR325 are 28 and 31 kDa (20); these latter two proteins appeared as one band in most lanes of Fig. 2. In addition to these proteins and the 25-kDa *cam*-encoded protein, pBR325 encoded an unidentified 39-kDa protein. This protein was apparently encoded by pMSU687 and pMSU685, but was not encoded by pMSU690. We have no explanation for the disappearance of this vector-encoded protein in pMSU690 minicells; perhaps it was made in smaller quantities, as were other vector-encoded proteins. Why minicells carrying pMSU690 had reduced expression of the vector genes is also unknown. Expression of the large (63-kDa) protein in these minicells may somehow inhibit expression of most of the other plasmid genes. The 63-kDa protein has not been identified but could be a fusion protein. The faint 26-kDa protein encoded by pMSU685 also remains unidentified but could be a truncated protein or a fusion protein.

Donnelly and Cooper reported that in *E. coli* B and K-12 the *sad* gene product is induced by succinic semialdehyde, and they demonstrated a greater-than-10-fold increase in activity when cells were exposed to the inducer (7, 8). Although the amount of the 55-kDa protein did not increase 10-fold in minicells that were exposed to succinic semialdehyde, this discrepancy in results could be due to several differences in our experiments. For example, we used 2.5 mM succinic semialdehyde to induce synthesis of the *sad*-encoded protein, whereas Donnelly and Cooper used a higher concentration (5.0 mM). Also, plasmid copy number or differences in minicell and whole-cell metabolism probably affect regulation of the *sad* gene.

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