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Fructose Bisphosphatase of *Escherichia coli*: Cloning of the Structural Gene (*fbp*) and Preparation of a Chromosomal Deletion

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The *fbp* locus at 96 min on the *Escherichia coli* chromosome governs fructose bisphosphatase (fructose-1,6-P_2 1-phosphatase). We have cloned and subcloned *fbp* on vector pBR322 to obtain strains with high levels of the enzyme. In vivo mutagenesis of the clone was used to show that *fbp* is the structural gene. The gene was deleted on the plasmid in vitro, and the chromosomal wild-type locus was replaced with this deletion by a method involving stabilization of a heterozygous intermediate resulting from plasmid integration, followed by segregation of the wild-type gene.

The fructose bisphosphatase reaction converts fructose-1,6-P_2 to fructose-6-P and P_i and is necessary for the growth of *Escherichia coli* on substances such as glycerol, succinate, and acetate. Deficiency mutants fail to grow on these carbon sources but grow on most sugars (14). Their genetic locus at 96 min has been known as *fdp* (13), but in accord with current usage it will be renamed *fbp*. The enzyme is present in low activity, and only a limited purification was achieved in earlier work (16).

For a new study of the reaction, we have now cloned *fbp*, obtaining strains with very high levels of the enzyme. We also show *fbp* to be the structural gene and have used a new technique to obtain a chromosomal deletion mutation.

MATERIALS AND METHODS

Strains. Strains are *E. coli* K-12 and are listed in Table 1. Plasmid pBR322 (5) was from M. Syvanen.

Chemicals. All chemicals were obtained commercially and used as supplied.

Media. The following growth media were used: LB; MacConkey with 2% glycerol; and M63 minimal salts with either 0.2% glucose or 0.4% each of glycerol and succinate, as described by Miller (26). Superbroth was 3.2% tryptone (Difco)–2% yeast extract (Difco)–0.25% NaCl–1% 3-[*N*-morpholino]propanesulfonic acid (pH 7.0). When required, amino acids were included at 25 μg/ml each, and antibiotics were included at the following concentrations: tetracycline, 20 μg/ml; ampicillin, 200 μg/ml; and nalidixic acid, 25 μg/ml. Growth was at 37°C unless indicated otherwise.

Genetic techniques. Standard genetic techniques were employed (26). Transformation with plasmid DNA employed $CaCl_2$ treatment (24) as described by Daldal (10).

Recombinant DNA techniques. Restriction endonuclease buffers were: (high salt) 100 mM NaCl, 6 mM $MgCl_2$, 6 mM Tris (pH 7.6), and 2 mM dithiothreitol; (low salt) 6 mM KCl, 10 mM $MgCl_2$, 10 mM Tris (pH 7.6), and 10 mM dithiothreitol. Rapid plasmid DNA isolation from 5-ml cultures employed the lysozyme-EDTA-Triton X-100 lysis treatment, followed by phenol extraction and ethanol precipitation (11). For large-scale preparation of DNA, cultures in superbroth (absorbance at 600 nm of 1.5 to 2.0) were treated overnight with chloramphenicol (200 μg/ml), lysed as above, and further purified by cesium chloride-ethidium bromide equilibrium centrifugation. Agarose gel electrophoresis was in Tris-acetate-EDTA (TAE) buffer (25).

Southern blotting. Plasmid DNA was nick translated with *E. coli* DNA polymerase I by using [α-^{32}P]dCTP according to the instructions of the supplier (Bethesda Research Laboratories). Other techniques were as described in Maniatis et al. (25).

Enzyme assays and preparation of antiserum. Cells were grown to stationary phase in LB medium supplemented with ampicillin as required. Extracts were prepared (15) and assayed for fructose bisphosphatase by the method of Babul and Guixé (1). Rabbit antiserum was obtained as follows: for the first injection, 400 μg of purified fructose bisphosphatase (1) was first denatured by heating for 5 min at 90°C in the presence of 3% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol and then was mixed with Freund complete adjuvant; the second injection, 4 weeks later, was of 200 μg of enzyme (without adjuvant), and the antiserum was obtained 1 week later.

Radioactive labeling, immune precipitation, and electrophoresis. Cultures of 5 ml in logarithmic growth in minimal medium containing glucose and ampicillin were labeled with [^{35}S]methionine (10 μCi/ml) for 5 min. Sodium azide was added to 3 mM, the cells were quickly chilled, washed, and resuspended in 0.25 ml of 50 mM Tris (pH 8.0)–5 mM $MgCl_2$–1 mM EDTA–7 mM β-mercaptoethanol, and, after addition of SDS (to 1%) and β-mercaptoethanol (to 5%), they were boiled for 4 min. Fractions of ca. 1/20 per gel lane were used for immune precipitation. The antiserum was preincubated with a 10-fold excess of unlabeled extract of strain DF658(pBR322), prepared in the same way. Antibody-antigen complexes were recovered with heat-fixed, formalinized *Staphylococcus* cells (20) as described by Ito et al. (19). SDS-polyacrylamide gel electrophoresis was done according to Laemmli (21) but with slabs (14 by 25 by 0.15 cm), and it employed 4% stacking and 12.5% separating gels. Staining and fixing were done as previously described (8). Gels were prepared for autofluorography by the method of Chamberlain (6).

Nitrosoguanidine mutagenesis of plasmid DNA. A 250-ml culture in LB medium plus ampicillin was grown to an absorbance at 600 nm of 0.6, washed twice with 100 ml of citrate buffer (26), and resuspended in 100 ml of the same buffer. Nitrosoguanidine was added to 25 μg/ml, and after gentle agitation for 30 min at 37°C, the cells were washed twice with 150 ml of phosphate buffer (26), resuspended in 250 ml of superbroth, and incubated on a gyrotory shaker at

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TABLE 1. *E. coli* strains[a]

Strain	Genotype	Source (reference)
DF1000	HfrC *relA1 spoT pit-10 tonA22* $T2^r$	Is strain K10[b]
DF961	F^- *endA* hsr_k^- hsm_k^+ *pro thi recA56*	(10)
DF964	(DF1000) Δ_{15}(*rha-pfkA*) *pfkB1 zwfL2*	(10)
DF1001	(DF1000) λ^-	This laboratory
DF1100	HfrH *fbp-1 lacI22* λ^- *rel-1 thi-1*	Is strain Q11 (14)
DF666	(DF1100) *recA56* Tet^s	—[c]
SY634	F^- *ara* Δ(*lac-pro*)XIII *argE*(Am) *nalA* Rif^r *thi polA34*	M. Syvanen (28)
JC411	F^- *metB leu-6 his-4 argG6 lacY1* or *Z4* λ^- *malA1 xyl-7 mtl-2 gal-6 str-104 sup-59*	(2)
DF667	(SY634) Δ_1*fbp*	This work
DF657	(DF1001) Δ_1*fbp* Tet^s	—[d]
DF658	(DF1001) Δ_1*fbp recA1* Tet^s	—[d,e]
DF659	(JC411) Δ_1*fbp* Tet^s	—[d]

[a] Gene designations are as described in Bachmann (3), except that *fdp* has been changed to *fbp*. Tet^s indicates that Tn*10* has been removed by the selection procedure of Bochner et al. (4) as modified (23).

[b] Markers are according to B. Bachmann, Coli Genetic Stock Center, Yale University.

[c] *recA56* from NK5306 (N. Kleckner), introduced by cotransduction with *srl*::Tn*10*.

[d] Δ_1*fbp* from DF667, by cotransduction with *zje*::Tn*10* from DF602 (7).

[e] *recA1* from CK1700 (C. Kumomoto), introduced into DF657 by cotransduction with *srl*::Tn*10* and Tet^s selection repeated.

30°C. After 1 h of incubation, chloramphenicol was added to 200 μg/ml, and the incubation was continued for 16 h. Plasmid DNA was prepared by the usual rapid isolation technique, but it was scaled up for the 250-ml culture volume. Mutagenized DNA was transformed at concentrations within the linear portion of the dose-response curve (micrograms of DNA versus number of transformants).

Temperature inactivation of fructose bisphosphatase activity. Crude extracts were diluted to the same activity (0.1 U/ml) and supplemented with bovine serum albumin to 10 mg/ml (9). Portions of 0.4 ml were placed for 2 min in a water bath at the desired temperature, chilled in ice water, and 0.1-ml portions (0.01 U of original activity) were assayed immediately.

RESULTS

Cloning and subcloning *fbp*. The *fbp* gene was obtained from a DNA pool of *Eco*RI fragments from strain DF964 (*fbp*$^+$) cloned into pBR322 (10). Strain DF666 (*fbp-1 recA*) was transformed with selection for resistance to ampicillin, and complementation of the *fbp* mutation was screened by replica plating onto minimal medium containing glycerol and succinate as the sole carbon sources. All apparent Fbp^+ clones contained a plasmid with a ca. 20-kilobase pair (kbp) *Eco*RI insert. One such plasmid, pFD12, was chosen for further study. Its Fbp^+ phenotype was 100% linked to ampicillin resistance, both by retransformation and by segregation. Furthermore, strains carrying pFD12 or its subclones had greatly elevated levels of fructose bisphosphatase: e.g., specific activities of >0.5, as compared with values of ca. 0.01 in wild-type strains and 0.001 in *fbp* mutants (Table 2).

Screening of the Fbp phenotype could also be done on MacConkey-glycerol indicator plates, with Fbp^- strains forming pale colonies and Fbp^+ strains (either wild-type or complemented mutants) forming red colonies. The use of such plates, supplemented with ampicillin, allowed the immediate identification of Fbp^+ and Fbp^- subclones. The restriction map of pFD12 and the subcloning procedure are shown in Fig. 1. First, the putative *fbp* gene was localized by deletion analysis to the right-hand side of the map. An 8.8-kbp *Sal*I-*Bam*HI fragment subcloned into pBR322, pJS23, still complemented, whereas removal of a 4-kbp *Sac*II fragment from this insert gave pJS24, which did not. Subcloning of the same *Sac*II fragment into the *Hae*II site at 1646 base pairs of pBR322 gave pJS25, which did complement.

The positive complementation by pJS25 and high enzyme level conferred by it (Table 2) showed that its 4-kbp *Sac*II insert contains the whole gene and, accordingly, that the deletion plasmid pJS24 lacks the whole gene. Strains carrying pJS25, however, grew somewhat irregularly (data not shown), and rearrangements in the plasmid were occasionally observed. Assuming that these properties might somehow depend on the location of the insert in the tetracycline resistance determinant, the *Sal*I-*Hin*dIII fragment was removed from pJS25, giving pJS33, which was apparently stable. Deletion of a *Mlu*I-*Eco*RI fragment from pJS33 gave pJS35, which still complemented, thus localizing the gene to a 1.7-kbp *Mlu*I-*Sac*II fragment.

Construction of a chromosomal deletion. The *fbp* mutant strains reported to date all revert at low frequency. To obtain a deletion strain, we decided to incorporate the *Sac*II deletion, constructed in vitro in pJS24, into the chromosome. This was done relying on the fact that the origin of replication of a multicopy plasmid such as pBR322 is not active in a DNA polymerase mutant (31). If pBR322 contains chromosomal sequences, then transformation into a *polA* strain gives, by single crossover, integration of the plasmid into the chromosome (12, 17, 27). Then, as was recently reported (18), subsequent segregation allows substitution of chromosomal sequences by the corresponding ones originally on the plasmid. In the protocol developed in the present work, integration of the plasmid carrying the mutant gene was accomplished by Hfr transfer. (It is known that plasmid-chromosome recombination does occur in a *polA*$^+$ strain [22]. The integrands are not stable, but they are capable of Hfr transfer [30].)

Accordingly, we transformed the deletion plasmid pJS24 into HfrC strain DF1000 and conjugated it with an F^- *polA* strain, SY634. Exconjugants from the mating were obtained at a frequency of ca. 10^{-5}, with ampicillin selection; nalidixic acid was used to counterselect the donor. Only about 10% grew upon restreaking to the same medium; this

TABLE 2. Fructose bisphosphatase activity

Strain	Fructose bisphosphatase activity (U/mg of protein)
JC411 (*fbp*$^+$)	0.004
DF659 [Δ_1*fbp*]	<0.001
DF659(pFD12 *fbp*$^+$)	0.777
DF659(pJS23 *fbp*$^+$)	0.415
DF659(pJS24[Δ_1*fbp*])	<0.001
DF659(pJS25 *fbp*$^+$)	0.667
DF1001 (*fbp*$^+$)	0.020
DF657 [Δ_1*fbp*]	<0.001
DF658 [Δ_1*fbp*]	<0.001
DF658(pBR322)	<0.001
DF658(pJS24[Δ_1*fbp*])	<0.001
DF658(pJS33 *fbp*$^+$)	0.550
DF658(pJS33ts1)	0.025
DF658(pJS33ts2)	0.033
DF658(pJS35 *fbp*$^+$)	0.459

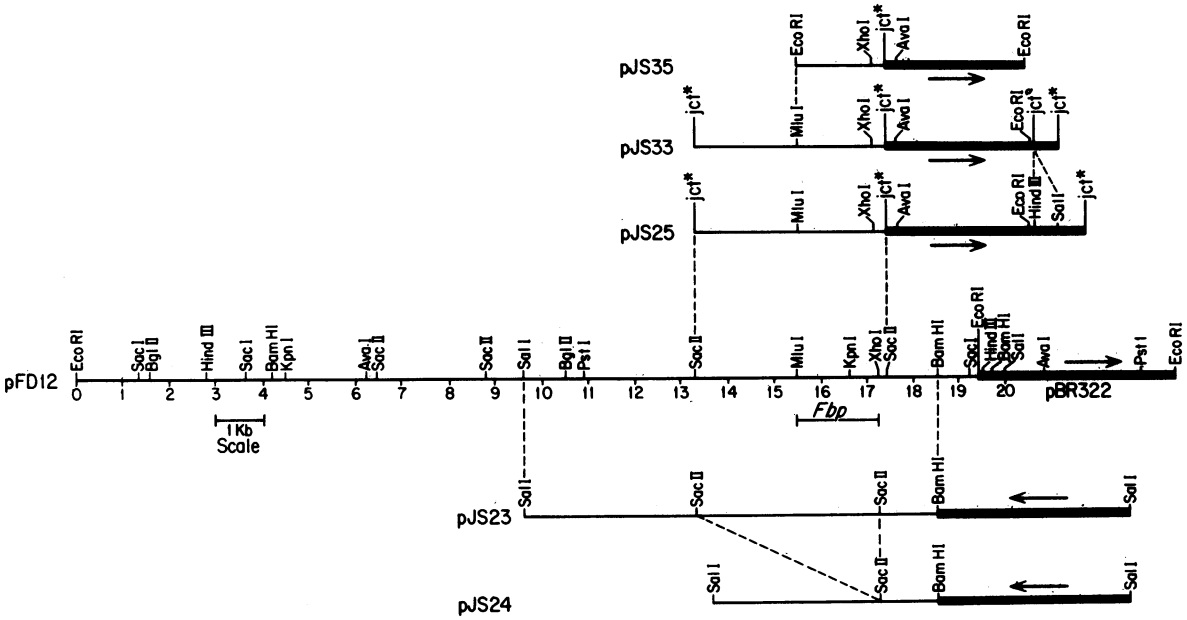

FIG. 1. Partial restriction map and subcloning procedure of pFD12. The *fbp* gene was localized to a 1.7-kbp *Mlu*I-*Sac*II fragment as indicated by a bar under pFD12, the original *Eco*RI clone. All subcloning employed pBR322 as the vector; the heavy arrow indicates its orientation. pJS23 is a *Sal*I-*Bam*HI subclone. pJS24 is a *Sac*II dropout from pJS23. pJS25 is a *Sac*II subclone into the *Hae*II site at 1646 base pairs of pBR322, produced by partial digestion (*Hae*II gives a 3′ four-base overhang, the terminal two bases of which are complementary to the 3′ two-base overhang produced by *Sac*II); neither the *Sac*II or the *Hae*II sites can be regenerated at the junctions (indicated as jct*). pJS33 is a *Sal*I-*Hin*dIII dropout from pJS25; neither the *Sal*I nor the *Hin*dIII sites were regenerated at the junction (indicated as jct*). pJS35 is an *Eco*RI-*Mlu*I dropout from pJS33 produced by filling in and blunt-end ligation; the *Eco*RI site was regenerated at the junction.

may be due to the β-lactamase produced by the donor cells. After several purifications, one isolate was grown for 30 to 40 divisions in the absence of ampicillin. Amp^s colonies were then present at a frequency of 10% and, of these, about 10% were Fbp$^-$. In such a strain, DF667, the Fbp$^-$ phenotype did not revert ($<10^{-10}$), as compared with the *fbp-1* mutation of DF1100, which reverted at ca. 10^{-6}.

Southern analysis (Fig. 2) employed pJS23 (*fbp*$^+$) made radioactive by nick translation. Its insert contains no *Bam*HI site, but it does have a single *Ava*I (*Xho*I) site within the *Sac*II fragment. Probing *Bam*HI digests of chromosomal DNA revealed a single fragment in the wild-type strain SY634 of the expected size, ca. 15 kbp, whereas the putative *fbp* deletion strain, DF667, showed a single fragment of ca. 11 kbp. Probing an *Ava*I chromosomal digest of the wild-type strain revealed a single fragment of ca. 11 kbp (i.e., coordinates of 6 to 17 kbp in pFD12; Fig. 1) which was absent in the mutant and replaced by one of ca. 18 kbp. The appearance of the larger fragment accords with the loss of the *Ava*I site. (A second *Ava*I fragment was expected in the wild type from coordinate 18 rightwards. This fragment is probably the same size as the other, for if the 18-kbp fusion fragment contains the 4-kbp deletion, then the "missing" fragment would be 18 + 4 − 11, i.e., 11 kbp.) Also, *Sac*II-*Bgl*II double digests showed that the *Sac*II fragment, coordinates of 13.4 to 17.4 kbp, was missing in strain DF667 (data not presented). These results are all consistent with the new chromosomal mutation being the 4-kbp *Sac*II deletion.

Cloned gene is *fbp*. The fact that chromosomal deletion of a region carried on the original Fbp$^+$ clone (pFD12) gave a Fbp$^-$ phenotype did not establish that the cloned gene was *fbp* itself rather than a suppressor. The new chromosomal deletion was therefore mapped by using the marker *zje*::Tn*10*, which is cotransducible with *fbp* at a frequency of a few percent (7). Since phage P1 grows poorly on *polA* strains, PolA$^+$ reversion was first obtained in strain DF667 by selection for resistance to methyl methane sulfonate. Tet^r

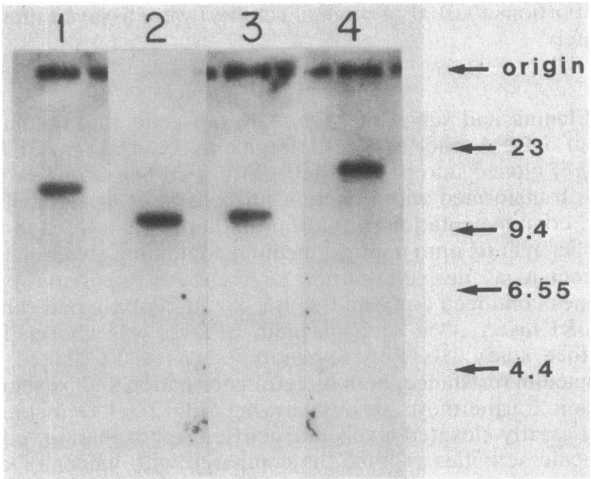

FIG. 2. Southern blot analysis of the chromosomal deletion mutation. DNA from strain DF667 (deletion) and the parental strain SY634 (wild type) was analyzed with *Bam*HI and *Ava*I. The probe was pJS23. *Bam*HI digests: lane 1, SY634; lane 2, DF667. *Ava*I digests: lane 3, SY634; lane 4, DF667. The deleted fragment contains one *Ava*I site and no *Bam*HI sites. Molecular weight markers indicated on right-hand side were λ DNA cut with *Hin*dIII.

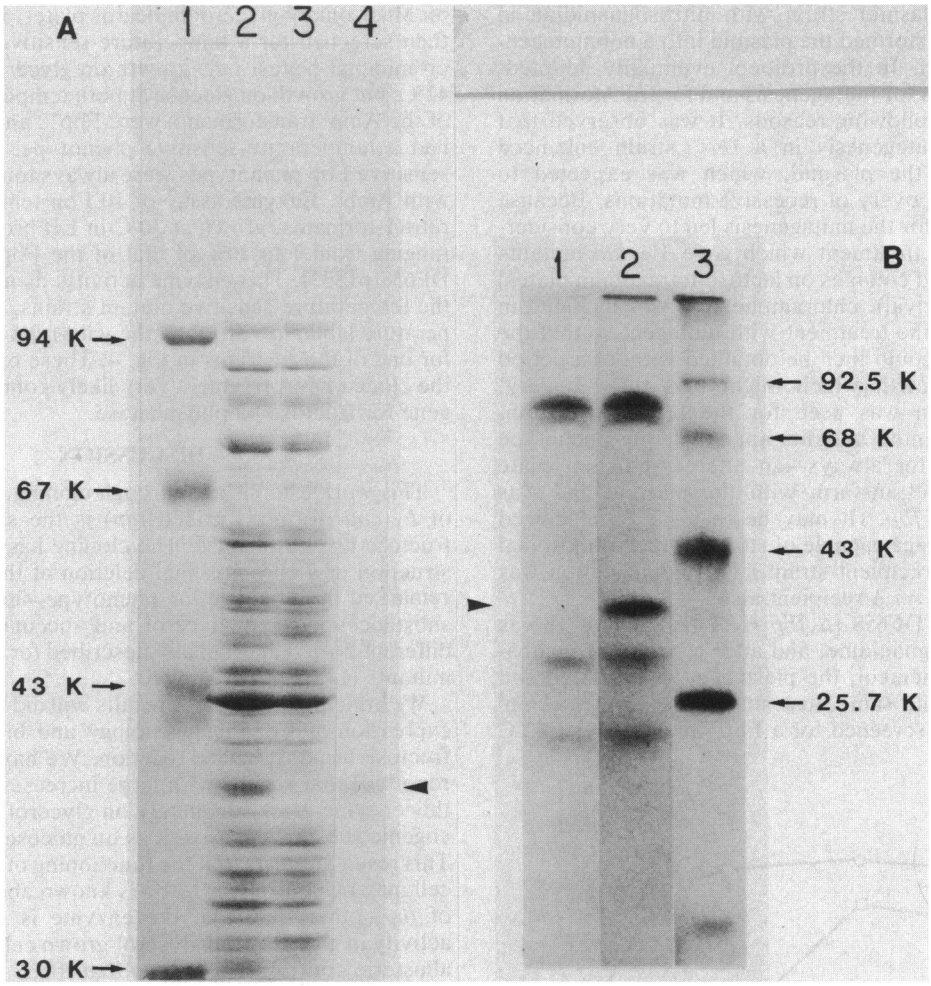

FIG. 3. Productions of fructose bisphosphatase protein from multicopy plasmids. (A) Total cellular proteins from strains DF961/pFD12 (lane 2) and DF961 (lane 3) were analyzed by SDS-polyacrylamide gel electrophoresis. Staining was with Coomassie blue. Molecular weight markers (lane 1) were from Pharmacia: phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000. Purified *E. coli* fructose bisphosphatase (lane 4, position indicated by arrow) was from Babul and Guixé (1). (B) Precipitation of [^{35}S]methionine-labeled cell extracts with antibodies to *E. coli* fructose bisphosphatase. Lane 1, DF658(pBR322). Lane 2, DF658(pJS33 *fbp*$^+$). Lane 3, ^{14}C-labeled molecular weight markers (Bethesda Research Laboratories); phosphorylase *b*, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700. The left-hand arrow indicates the migration of fructose bisphosphatase, according to Fig. 3A.

was then introduced by P1 transduction from a *zje*::Tn*10* strain, and an Fbp$^-$Tet^r transductant was identified. Phage P1 was then used to transduce Tet^r out of that strain into *fbp*$^+$ strains, such as JC411 or DF1001; in such crosses there was ca. 10% cotransduction of Fbp$^-$. The Fbp$^-$ transductants (e.g., DF657-659; Table 2) did not revert to Fbp$^+$ and did not contain fructose bisphosphatase activity. When used as recipients, strains carrying the transduced deletion gave Fbp$^+$ transformants with Fbp$^+$ plasmids pFD12, pJS23, pJS25, pJS33, and pJS35, and the transformants contained 30 to 50 times the wild-type level of enzyme. The deletion plasmid, pJS24, did not complement the new deletion strains or confer fructose bisphosphatase activity on them (Table 2).

These results make it very likely that the cloned gene is *fbp* and that strain DF667 and the transductants made from it carry a chromosomal deletion, Δ_1*fbp*.

***fbp* is the structural gene.** The elevated fructose bisphosphatase activity in strains containing Fbp$^+$ plasmids was accompanied by a similar increase in fructose bisphosphatase protein. Figure 3A shows crude extracts of strains DF961(pFD12) (*fbp*$^+$) and DF961 after SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue; a strong band comigrating with purified fructose bisphosphatase appeared in the extract of the former strain (lane 2). That this new band indeed represents the enzyme was confirmed by precipitation of [^{35}S]methionine-labeled extracts of an analogous pair of strains [DF658(pBR322) and DF658(pJS33 *fbp*$^+$)] with rabbit antiserum raised against the purified enzyme (Fig. 3B).

Although the appearance of high-level enzyme activity and antigen in a strain with a multicopy plasmid for *fbp* would fit with *fbp* being the structural gene for the enzyme, the results might equally accord with *fbp* being a positive regulatory gene. Therefore, to determine whether *fbp* is the structural gene, we sought plasmid-linked mutations affecting in vitro characteristics of the enzyme. Since obtaining such mutations was likely to require fairly massive random mutagenesis of a relatively large piece of DNA, we treated

an *fbp*$^+$-carrying plasmid strain with nitrosoguanidine in vivo and then retransformed the plasmid into a nonmutagenized recipient strain. In the protocol eventually adopted, both the strains used for mutagenesis and for transformation were *recA* for the following reasons. It was observed that nitrosoguanidine mutagenesis in a *rec*$^+$ strain enhanced multimerization of the plasmid, which was expected to depress eventual recovery of recessive mutations. Because use of a *recA* strain for the mutagenesis led to very considerable killing (e.g., a treatment which gave 1% *lac* mutants among survivors [red colonies on lactose-tetrazolium plates] gave only 10^{-4} survival), chloramphenicol was included in an incubation after the treatment with mutagen, so that the mutagenized DNA could then be obtained without a period of prolonged outgrowth which might bias the recovery. When a *rec*$^+$ strain was used for the retransformation, although many potential mutants appeared, the phenotype that was screened for always—an altered indicator plate reaction—did not retransform with the plasmid and was unlikely to involve *fbp*. (It may be that the mutagenized DNA sustained damage capable of stimulating chromosomal mutagenesis in the recipient strain.) This complication was avoided by use of a *recA* recipient also.

Therefore, strain DF658 [Δ_1*fbp recA* (pJS33 *fbp*$^+$)] was treated with nitrosoguanidine, and after a period of incubation with chloramphenicol, the plasmid was reisolated and used to transform the same host strain (see above). Amp^r transformants were screened for a Fbp$^-$ phenotype at 42°C on MacConkey-glycerol indicator plates. Pale colonies were then screened for a temperature-sensitive Fbp$^-$ phenotype on minimal plates, i.e., growth on glycerol at 30°C and not 42°C, but growth on glucose at both temperatures. About 1% of the Amp^r transformants were Fbp$^-$, and of these ca. 10% had a temperature-sensitive phenotype. The temperature-sensitive Fbp phenotypes were always found to cotransform with Amp^r. Enzyme assay of 10 Fbp temperature-sensitive retransformants, grown at 30°C in LB broth, gave activities ranging from 1 to 10% of that of the Fbp$^+$ parental strain, DF658(pJS33). This enzyme activity, as measured in two of the temperature-sensitive mutant strains, was far more temperature labile in vitro than the wild-type activity, as shown for one of those strains in Fig. 4. These results indicate that the cloned DNA fragment very likely contains the structural gene for fructose biophosphatase.

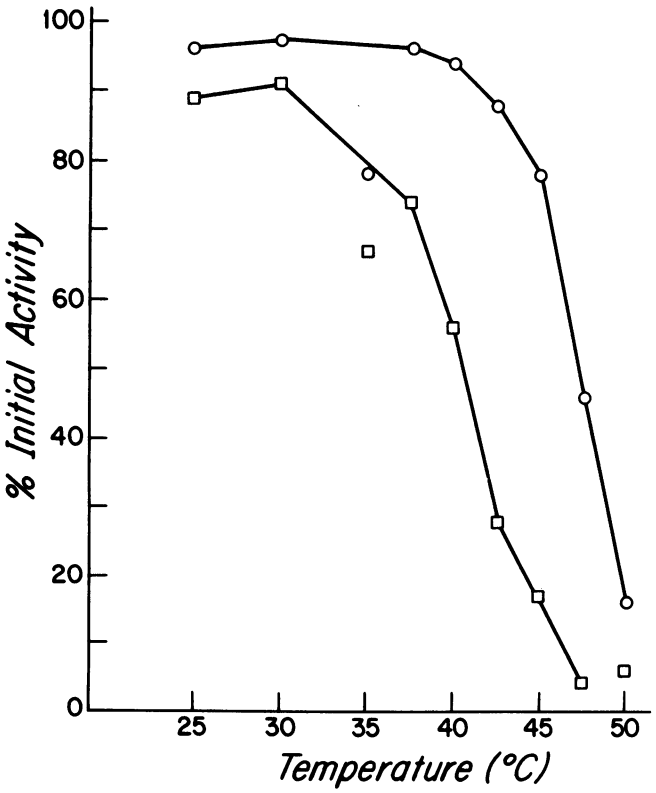

FIG. 4. Thermolability of mutant fructose bisphosphatase (see the text). Extracts were placed for 2 min in a water bath at the indicated temperatures, chilled, and assayed at 25°C. Activities are expressed as percents of unheated controls. DF658(pJS33), open circles; DF658(pJS33ts2), open squares.

DISCUSSION

This work establishes, through cloning, that the *fbp* locus of *E. coli* (formerly called *fdp*) is the structural gene for fructose bisphosphatase. The cloning has also allowed construction of a chromosomal deletion of the gene. It may be remarked that the deletion phenotype—inability to grow on substances such as glycerol and succinate—is not clearly different from that originally described for presumptive point mutants (14; data not shown).

We anticipate that these results will aid new studies of *fbp* expression and of the physiology and biochemistry of the fructose bisphosphatase reaction. We have observed (to be reported) that strains with large increases in the amount of this enzyme grow adequately on glycerol and other gluconeogenic substances, as well as on glucose and other sugars. This result suggests that the functioning of the reaction in the cell may be controlled. Little is known about the expression of *fbp*, other than that the enzyme is present in similar activity in glucose- and glycerol-grown cells. With respect to allosteric control, it is known that the *E. coli* enzyme, like many other fructose bisphosphatases (29), is very sensitive to inhibition by 5'-AMP (16). Using a strain carrying pFD12, the original clone, Babul and Guixé have now reported the first adequate purification, with a new characterization, of the *E. coli* enzyme (1).

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