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Biochemistry

AMP-insensitive fructose bisphosphatase in *Escherichia coli* and its consequences

(allosteric enzyme/metabolism)

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ABSTRACT **Inhibition of fructose bisphosphatase (EC 3.1.3.11) by AMP is thought to be an important control mechanism, and, for the *Escherichia coli* enzyme, is the only control known. We here report on a mutant *E. coli* fructose bisphosphatase almost insensitive to this inhibition. The purified enzyme is normal in other respects. A strain with this enzyme instead of the wild-type enzyme grows normally in a variety of media. A strain with a very high level of the wild-type enzyme also grows normally. A strain with the very high level of mutant enzyme, however, does show growth abnormalities, but they are not clearly associated with the AMP insensitivity.**

Fructose bisphosphatase (fructose-1,6-P_2 1-phosphohydrolase, EC 3.1.3.11) is thought to be a key enzyme in regulation of gluconeogenesis (1–3). It also has an essential biosynthetic role in the growth of microorganisms on noncarbohydrate carbon sources (4, 5). The enzymes from various sources are almost all inhibited by 5′-AMP, and the eukaryotic enzyme is also inhibited by fructose-2,6-P_2. Various other processes, including phosphorylation (6), proteolysis (7, 8), and repression, may also affect the activity (5, 9).

In eukaryotic cells, evaluation of the role of AMP inhibition of fructose bisphosphatase is particularly difficult in view of the several types of control. The problem should be easier to assess in *Escherichia coli*, in which the enzyme is also AMP sensitive (10) but not clearly subject to any of the other controls. Even in *E. coli*, however, the physiological role of AMP inhibition of this reaction is not obvious. Thus, one might expect AMP concentration to be lower in growth on glycerol, when the enzyme activity is required, than on glucose, but the contrary result was found (11), and the actual levels would be expected to inhibit the enzyme. Labeling experiments bearing on the question of whether the activity functions at all during growth on glucose have been inconclusive (11, 12).

We here report on a mutant *E. coli* fructose bisphosphatase that is almost insensitive to inhibition by AMP. A strain containing this enzyme instead of the wild-type enzyme seems to grow normally, and so does a strain with a very high level of the wild-type enzyme. A strain with the high level of mutant enzyme, however, is somewhat impaired in its growth.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. All strains are isogenic to DF1001 (Hfr C *relA1 spoT pit-10 tonA22* phage T2-resistant phage λ⁻). DF656 contains a deletion of the *fbp* region [del$_1$*fbp* (ref. 13), now designated Δ*fbp*287 (B. Bachmann, personal communication)] linked to the Tn*10* insertion *zjg920*::Tn*10* (misnamed in ref. 13 as *zje*::Tn10). DF672 (*fbp-5 zjg920*::Tn*10*) has the *fbp*⁻ allele from pJS51 as a single chromosomal copy, replacing *fbp*⁺. DF672 was constructed by a variation of the allele-exchange protocol described earlier (13). Strain SY634 (*polA*) was transformed with pJS51 with selection for resistance to ampicillin (Amp^r). One Amp^r clone was grown in the absence of ampicillin, and a segregant containing AMP-insensitive fructose bisphosphatase (i.e., presumptive replacement of *fbp*⁺ of SY634 by *fbp-5*) was identified by assay. After reversion of *polA*, the region was transduced into DF1001 by employing linkage with *zjg-920*::Tn*10*; transductants were screened by enzyme assay. The resultant strain, DF672, had fructose bisphosphatase insensitive to AMP but present in an amount typical of the chromosomal enzyme. To test fidelity of the exchange, the *fbp*⁻ allele of DF672 was reisolated on a plasmid and tested for the original phenotype, as follows. DF672 was transformed with pJS24 [Δ*fbp*287 (ref. 13)] and conjugated with an F⁻ streptomycin-resistant (Str^r) strain carrying the same deletion chromosomally [DF659 (ref. 13)], selecting Amp^r Str^r exconjugants. The expectation was that in the merozygote a plasmid carrying the *fbp*⁻ allele from DF672 could be formed by recombination. Indeed, temperature-sensitive exconjugants were obtained, whose phenotype resembled the one conferred by the original *fbp-5* plasmid, pJS51, as confirmed by a further transformation.

pJS33 is a derivative of pBR322 carrying *fbp*⁺ in a 4-kilobase-pair (kbp) insert; the *fbp* gene is known to be contained in a 1.7-kbp fragment [pJS35 (ref. 13)] of this insert. pJS51 was derived by *in vivo* nitrosoguanidine mutagenesis of pJS33 as described (13). pJS52 is a derivative of pJS51 from which a 0.6-kbp *Eco*RV fragment has been deleted (Fig. 1).

pJS33 and pJS51 were converted from Amp^r to kanamycin resistance (Kan^r) by introduction of the 1.1-kbp *Pst* I fragment from pUC71K (14) into the *Pst* I site in the pBR322 portion of the two plasmids, giving pJS54 (*fbp*⁺ Kan^r) and pJS53 (*fbp-5* Kan^r), respectively.

Genetic Techniques and Media. As before (13), genetic constructions employed LB medium, and growth experiments employed minimal medium 63. Minimal medium always also contained thiamin·HCl at 1 μg/ml and the specified carbon source, usually at 0.2%. Antibiotics were ampicillin at 200 μg/ml, tetracycline at 20 μg/ml, and kanamycin at 40 μg/ml. Standard genetic and cloning techniques were used (15, 16). All incubations were aerobic.

Fructose Bisphosphatase. Assay (at 25°C) and purification were as reported for wild-type enzyme (10); 1 unit (U) hydrolyzes 1 μmol/min. The mutant enzyme was purified

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Abbreviations: Amp^r, Str^r, and Kan^r, resistance to ampicillin, streptomycin, and kanamycin, respectively; Fbp⁻, no growth on gluconeogenic compounds but normal growth on sugars; U, enzyme unit; kbp, kilobase pair(s).

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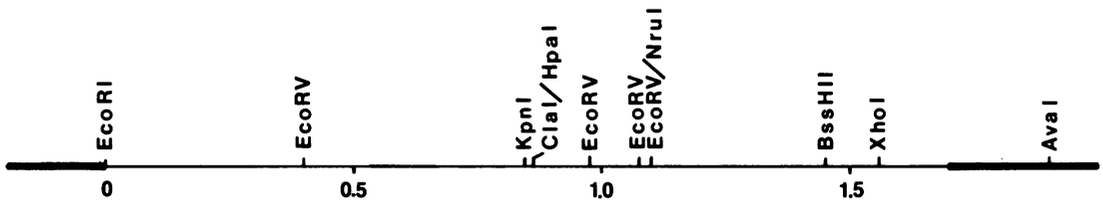

FIG. 1. Restriction map of plasmid pJS35 (*fbp*$^+$), a subclone of pJS33 (13). Numbers are kbp; the thicker line represents vector DNA. Nitrosoguanidine mutagenesis of pJS33 yielded pJS51, and the *fbp-6* mutation of pJS52 is pJS51 with the entire 0.6-kbp *Eco*RV segment (as indicated here for pJS35) deleted.

from strain DF656/pJS51 (*fbp-5*); the initial specific activity of 0.7 U/mg (as compared with 0.01 U/mg for strains with wild-type or mutant gene in a single chromosomal copy) was increased to 44 U/mg by chromatography on phosphocellulose and elution with 0.4 mM fructose-1,6-P_2; as with wild-type enzyme, a further gel filtration step on Sephacryl S-300 (Pharmacia) gave a slightly heterogeneous profile and the pooled peak fractions were used. For purposes of comparison, the previously reported preparation (10) of wild-type enzyme was used, except in studies of AMP sensitivity (Fig. 3), which employed a new preparation from strain DF656/pJS33 (*fbp*$^+$).

Metabolite Assays. The methods were described earlier (17, 18).

RESULTS

Mutant Isolation. The *fbp-5* allele, which specifies the AMP-insensitive enzyme, was obtained by mutagenesis of a plasmid, pJS33, carrying the wild-type allele *fbp*$^+$. To screen for structural gene mutants, a pool of mutagenized pJS33 was introduced into a strain deleted at *fbp* and the phenotype of temperature-sensitive growth on glycerol but not glucose was sought. Isolates of this type were indeed found to contain temperature-labile fructose bisphosphatase activity (13). An unusual isolate from that experiment, however, showed partial temperature sensitivity of growth on both glycerol and glucose, and the phenotype proved to depend on its plasmid, pJS33ts5, renamed pJS51. Extracts of strain DF656/pJS51 had about the same very high level of fructose bisphosphatase as DF656/pJS33 itself (Table 1), but the mutant activity from this allele, in crude extracts, was relatively insensitive to inhibition by AMP (Fig. 2).

Enzyme Characterization. The mutant enzyme was purified from strain DF656/pJS51. In most respects (data not shown) its properties were the same as reported for the wild-type enzyme. Thus: (*i*) The course of purification was the same. (*ii*) The final Sephacryl S-300 fraction also showed a single band on sodium dodecyl sulfate/PAGE indistinguishable in its migration from the wild-type protein (size ≈40,000 daltons). (*iii*) As for the wild-type enzyme, there was a slight shift in migration on Sephacryl S-300 in the presence of 0.1 mM AMP, indicative of some interaction with this compound. (*iv*) K_m values for fructose-1,6-P_2 were the same for wild-type and mutant enzyme (7.7 and 6.6 μM, respectively), and for both enzymes there was some reduction in activity at much higher substrate concentrations (v/V_{max} values of ≈0.7 at 1 mM fructose-1,6-P_2). (*v*) Both enzyme activities were inhibited by very high concentrations of phospho*enol*pyruvate.

However, although both purified enzymes were inhibited noncompetitively by AMP (Fig. 3), they differed substantially in their apparent K_i, slopes from Fig. 3 yielding values of 0.016 mM and 1.3 mM for wild-type and mutant enzyme, respectively. Thus, the altered sensitivity to AMP inhibition seems to be an intrinsic property of the enzyme specified by the mutant *fbp* allele of pJS51, named *fbp-5*.

Genetics of *fbp-5*. Several questions were addressed. The first concerned whether it was alteration of fructose bisphosphatase which caused the temperature-sensitive growth impairment. Since the insert of pJS33 was 4 kbp, while the enzyme should require only ≈1.1 kbp for its coding, mutation outside the gene or even in the vector itself might have accounted for the abnormal growth.

To answer this question, pJS51 was modified by introducing a 0.6-kbp deletion in its *fbp* region. The resulting plasmid, pJS52 (*fbp-6*), no longer complemented an *fbp*-deletion strain and also did not cause growth impairment (Table 1). Therefore an intact *fbp* gene was required for the impairment. The same conclusion was also supported by the fact that selection for restoration of temperature-insensitive growth gave a preponderance of Fbp$^-$ isolates (see below).

A second question was whether impaired growth might be related to the very high level of mutant fructose bisphosphatase activity in strains carrying *fbp-5* on the multicopy plasmid. For this purpose an allele exchange protocol was devised and used to construct a strain, DF672, with *fbp-5* in a single chromosomal copy instead of *fbp*$^+$ (see *Materials and Methods*). Strain DF672 contained AMP-insensitive fructose bisphosphatase activity in typical low wild-type level and did not show temperature-sensitive growth (Table 1). Thus the growth impairment seemed likely to depend on the abnormally high level of mutant enzyme in strains with plasmid pJS51 (*fbp-5*). It should be emphasized that overproduction

Table 1. Characteristics of strains

Strain (host/plasmid)	Fructose bisphosphatase: Specific activity†	Fructose bisphosphatase: AMP sensitivity‡	Growth*
DF1001 (*fbp*$^+$)/—	0.01	Yes	+
DF656 (Δ*fbp*287)/—	0.00	—	Fbp$^-$
DF656/pJS33 (*fbp*$^+$ Amp^r)	0.50	Yes	+
DF656/pJS51 (*fbp-5* Amp^r)	0.51	No	ts
DF656/pJS52 (*fbp-6* Amp^r)	0.00	—	Fbp$^-$
D672 (*fbp-5*)/—	0.01	No	+
DF656/pJS54 (*fbp*$^+$ Kan^r)	1.2	Yes	+
DF656/pJS53 (*fbp-5* Kan^r)	0.32	No	ts
DF656/pJS58 (*fbp-7* Kan^r)	≈0.5	No	+

This table also summarizes many of the results. DF1001 is the wild-type strain and DF656 has undergone a chromosomal deletion of the entire *fbp* gene. The plasmids (except pJS52) all carry the entire deleted fragment, ≈4 kbp. Plasmids pJS33 and pJS54 both carry the wild-type allele *fbp*$^+$. pJS51 and pJS53 carry the principal mutant allele *fbp-5* specifying AMP-insensitive fructose bisphosphatase; this allele is also carried by strain DF672, but in a single chromosomal copy instead of on a plasmid. Plasmid pJS52 carries *fbp-6*, an *Eco*RV fragment deletion from pJS51, and pJS58 carries *fbp-7*, obtained as temperature-insensitive reversion in DF656/pJS53.

*+, Normal growth; Fbp$^-$, no growth on gluconeogenic compounds but normal growth on sugars; ts, partially temperature-sensitive growth on several carbon sources in minimal medium.

†U/mg of protein in crude extracts.

‡See Figs. 2 and 3.

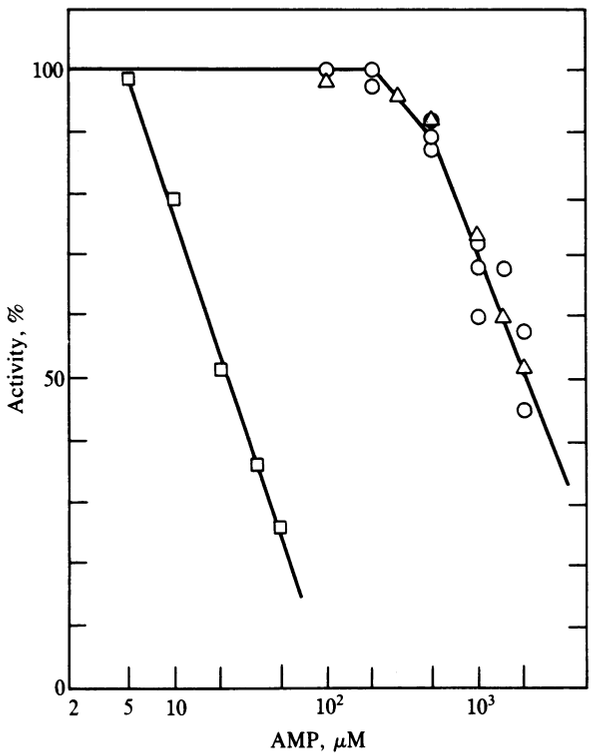

FIG. 2. AMP inhibition of fructose bisphosphatase activity in crude extracts. The standard assay mixtures contained 0.1 mM fructose-1,6-P_2 and the indicated concentrations of AMP. □, DF656/pJS33 (*fbp*$^+$); △, DF656/pJS53 (*fbp-5*); ○, DF656/pJS58 (*fbp-7*).

of wild-type fructose bisphosphatase is not deleterious (see below).

A third question concerns whether *fbp-5* was a single point mutation. A definitive answer will probably require sequencing, but we have taken the preliminary approach of selecting reversion to temperature-insensitive growth. For technical reasons, this was difficult to do with a strain carrying pJS51 (*fbp-5* Amp^r), and it was necessary to convert this plasmid to Kan^r, producing pJS53 (*fbp-5*, Kan^r; see *Materials and Methods*). About 98% of temperature-insensitive revertants of strain DF656/pJS53 (selected by their growth on glucose-6-*P* at 42°C) were Fbp$^-$ mutants, no longer complementing the host *fbp*-deletion strain for growth on glycerol, even at 30°C. Of the Fbp$^+$ temperature-insensitive isolates, several had low enzyme levels, but one carried a mutant plasmid, pJS58, that specified the same high fructose bisphosphatase level as pJS53 or pJS51. Interestingly, although growth of strain DF656/pJS58 (*fbp-7*) was normal (Table 1), its fructose bisphosphatase was still insensitive to AMP inhibition (Fig. 2). Since several interpretations are possible, we conclude only that growth impairment by pJS51 or pJS53 need not necessarily be caused by the AMP insensitivity of the mutant enzyme that they specify.

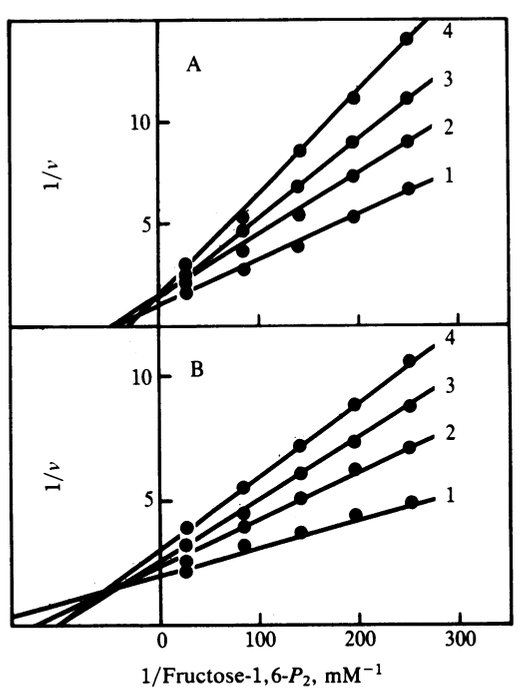

FIG. 3. AMP inhibition of purified wild-type and mutant fructose bisphosphatases. (*A*) Wild-type enzyme from DF656/pJS33 (*fbp*$^+$). The AMP concentrations were 0, 0.007, 0.014, and 0.020 mM for lines 1, 2, 3, and 4, respectively. (*B*) Mutant enzyme from DF656/pJS51 (*fbp-5*). The AMP concentrations were 0, 1.0, 1.5, and 2.0 mM for lines 1, 2, 3, and 4, respectively. Velocity is plotted in relative units.

Growth. Growth on plates of five key strains was tested on nine carbon sources at 30°, 37°, and 43°C (19). The data for glycerol, glucose, and glucose-6-*P* are shown in Table 2, and the combined results may be described as follows. (*i*) As expected, the *fbp*-deletion strain DF656 grew on none of the gluconeogenic carbon sources. Plate growth on the other carbon sources was normal. (*ii*) Strain DF672, carrying *fbp-5* in a single chromosomal copy, was indistinguishable in its growth from the isogenic wild-type strain DF1001. (*iii*) Strain DF1001/pJS33, carrying *fbp*$^+$ in multiple copies, also grew normally. (*iv*) Strain DF672/pJS51, carrying *fbp-5* in multiple copies, grew normally on all nine media at 30°C, was partially impaired at the higher temperatures on glycerol and on glucose, and showed more severe impairment on glucose-6-*P*. Its growth on glucuronate resembled that on glycerol, while its growth was normal on the weaker gluconeogenic carbon sources acetate and malate. The pattern of growth on galactose and mannose resembled that on glucose-6-*P*, while impairment on gluconate was modest.

Growth rates in liquid minimal medium on glucose and glycerol at 30°C and 37°C were also determined (Table 3). Growth of all strains was normal at 30°C, and temperature sensitivity was observed at 37°C only for the strain with the *fbp-5* plasmid, DF656/pJS53. These results are in agreement with growth observed on plates. (One exception was that the

Table 2. Growth on plates containing various carbon sources

Strain	Temp., °C	Average colony diameter, mm		
		Glycerol	Glucose	Glucose-6-*P*
DF1001 (*fbp*$^+$)	30	0.5	1.2	1.1
	37	1.4	1.9	1.9
	43	1.7	2.1	2.3
DF656 (Δ*fbp*287)	30	0.1	1.2	1.2
	37	0.1	2.0	1.9
	43	<0.1	2.2	2.3
DF1001/pJS33 (*fbp*$^+$)	30	0.5	1.0	1.0
	37	1.3	1.9	1.9
	43	1.7	2.1	2.3
DF672 (*fbp-5*)	30	0.7	1.2	1.2
	37	0.9	2.0	1.9
	43	1.5	2.1	2.1
DF672/pJS51 (*fbp-5*)	30	0.7	1.0	1.1
	37	0.9	1.4	0.9
	43	0.8	1.0	0.5

Colony size was measured after 45 hr of incubation at the indicated temperature. The concentration of carbon source was 0.2% for glycerol and glucose and 0.4% for glucose-6-*P*. Ampicillin was included at 200 μg/ml for the two strains carrying plasmids.

Table 3. Growth rates and metabolite concentrations

Strain	Growth on glucose				Growth on glycerol			
	Doubling time, min		Metabolite conc. at 37°C, mM		Doubling time, min		Metabolite conc. at 37°C, mM	
	30°C	37°C	G-6-*P*	F-1,6-P_2	30°C	37°C	G-6-*P*	F-1,6-P_2
DF1001 (*fbp*$^+$)	74	54	1.4	2.8	107	74	0.6	3.22
DF656 (Δ*fbp*287)	110	78	2.5	3.4	NG	NG	—	—
DF656/pJS54 (*fbp*$^+$)	85	66	2.0	2.5	105	69	1.5	3.1
DF656/pJS53 (*fbp-5*)	88	105	9.3	2.4	115	114	8.6	1.8
DF672 (*fbp-5*)	73	55	1.3	3.4	110	67	0.8	3.5
DF656/pJS58 (*fbp-7*)	82	67	5.6	3.6	99	72	3.9	3.2

Growth rates in minimal medium were determined at 30°C and 37°C. The metabolites glucose-6-*P* (G-6-*P*) and fructose-1,6-P_2 (F-1,6-P_2) were assessed from the perchloric acid-soluble pool of the 37°C cultures. NG, no growth.

host *fbp*-deletion strain DF656, when uncomplemented with its missing DNA, was slightly impaired in its growth on glucose at both temperatures. This result was not pursued, since the deletion is large and thus the effect may be unrelated to *fbp*.)

Metabolites. Concentrations of some key metabolites were also measured in cultures grown on glucose or glycerol at 37°C (Table 3). All the strains had normal levels of fructose-1,6-P_2 on both carbon sources. However, concentrations of glucose-6-*P* and fructose-6-*P* (not shown) were increased 5- to 10-fold on both carbon sources in strain DF656/pJS53 (*fbp-5*), situations of clear growth impairment. Increases in their concentrations were also seen in strain DF656/pJS58 (*fbp-7*), the temperature-insensitive revertant that still has AMP-insensitive enzyme. There was no indication of abnormal metabolite levels in strain DF656/pJS54 (*fbp*$^+$).

DISCUSSION

In this work we report a mutant fructose bisphosphatase with greatly decreased sensitivity to inhibition by AMP (a K_i increase of 80-fold). As mentioned, in many other respects the enzyme was normal. There was no indication of increased *in vivo* turnover (19) and, as with normal enzyme, the level was low with the gene in a single chromosomal copy and high with the gene in multiple copies. Furthermore, when the enzyme was present in high amount there were alterations in levels of hexose monophosphates consistent with excess enzyme activity. These results all argue for the AMP insensitivity being an *in vivo* characteristic of the mutant enzyme rather than an *in vitro* artefact resulting from lability to extraction.

A strain with the mutant enzyme at the normal level instead of the wild-type enzyme was not clearly different from the wild type in its growth on a variety of carbon sources. Perhaps there are conditions, as yet untested, in which the physiology of this strain is significantly altered. Perhaps some other mechanism of control of the reaction is predominant. Or, loss of control of the relatively low normal level of this activity in *E. coli* might cause little metabolic perturbation. Whatever the case, the role of AMP inhibition of fructose bisphosphatase in *E. coli* is still unclear.

We also show that a very high level of normal fructose bisphosphatase does not clearly alter growth or metabolite levels either. It seems likely that in this contrived situation (for *E. coli*) of a higher level of fructose bisphosphatase than phosphofructokinase, excessive reconversion of fructose-1,6-P_2 to fructose-6-*P* during growth on sugars—a futile cycle—is somehow prevented. Conceivably, the control involves inhibition of fructose bisphosphatase by AMP. And indeed, growth impairment was found in strains with a high level of AMP-insensitive fructose bisphosphatase. Perhaps there is analogy to bumble-bee flight muscle, with its high level of an AMP-insensitive fructose bisphosphatase and demonstrated futile cycling (20).

However, in the present case the relationship between growth impairment and AMP insensitivity of the enzyme is not known. The mutant enzyme was characterized as AMP-insensitive in studies employing 25°C as assay temperature, but growth impairment was observed only at 37°C and above. Furthermore, the temperature-insensitive revertant strain DF656/pJS58 (*fbp-7*) still had an AMP-insensitive enzyme. Thus, it is possible that characteristics other than AMP insensitivity somehow cause the temperature-sensitive growth. Resolution of the issue will likely require studies of the mutant enzymes at higher temperatures, as well as sequencing of the mutant alleles.

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