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ColE1 Hybrid Plasmids for *Escherichia coli* Genes of Glycolysis and the Hexose Monophosphate Shunt

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The Clarke-Carbon clone bank carrying ColE1-*Escherichia coli* DNA has been screened by conjugation for complementation of glycolysis and hexose monophosphate shunt mutations. Plasmids were identified for phosphofructokinase (*pfkA*), triose phosphate isomerase (*tpi*), phosphoglucose isomerase (*pgi*), glucose-6-phosphate dehydrogenase (*zwf*), gluconate-6-phosphate dehydrogenase (*gnd*), enolase (*eno*), phosphoglycerate kinase (*pgk*), and fructose-1,6-P_2 aldolase (*fda*). Enzyme levels for the plasmid-carried gene ranged, for the various plasmids, from 4- to 25-fold the normal level.

Clarke and Carbon constructed a hybrid plasmid bank (about 2,000 clones) of ColE1-*Escherichia coli* DNA in an *E. coli* F^+ *recA* host (5). Clones could be identified by F^+-mediated conjugation and complementation in appropriate recipients. We report herein identification of plasmids for several of the genes of glycolysis and the hexose monophosphate shunt.

MATERIALS AND METHODS

Strains. Strains are listed in Table 1.

Clone bank. Handling of the clone bank generally followed the protocol of Clarke and Carbon (5). For screening the clone bank by complementation, the clones were first replicated to a permissive rich medium, such as LBC (17), and after several hours of incubation replicated to selective minimal plates (M63; 6) spread with about 10^8 cells of recipient strain. Counterselection was usually with streptomycin-HCl (100 μg/ml); omission of the growth factors of the donor strains also sufficed. Apparently successful complementations were usually confirmed by individual matings in liquid cultures. In many cases the complemented recipients were also tested by conjugation with a second recipient.

Enzyme assays. Enzyme assays were done on crude extracts prepared from cells generally grown aerobically to stationary phase in rich medium (M63 + 1% tryptone [Difco] + 0.4% yeast extract [Difco]), supplemented as specified. (The experiments with *pgk* and *eno* used M63 + 0.1% glycerol + 0.4% malate + 0.25% Casamino Acids, since these mutants do not grow in the rich medium.) Assays were as described: phosphofructokinase (10), triose phosphate isomerase (19), phosphoglucose isomerase (11), glucose-6-P dehydrogenase (4), gluconate-6-P dehydrogenase (11), enolase (19), phosphoglycerate kinase (19), and fructose diphosphate aldolase (22).

Plasmid numbering. Plasmid numbering follows reference 5: i.e., pLC16-4 means the plasmid carried by clone 4 of plate 16 (fourth from the left in first row) of the original clone collection in strain JA200 obtained from L. Clarke and J. Carbon.

RESULTS AND DISCUSSION

***pfkA* and *tpi*.** Phosphofructokinase mutants (*pfkA*) do not grow on mannitol (12). To identify *pfkA* plasmids, strain DF456 (*pfkA*::Mu) was used as recipient on appropriately supplemented minimal plates with mannitol; counterselection of the donor was by omission of its growth factors. Complementation was found with plasmids pLC16-4 and pLC30-43. Table 2 gives the specific activities in the mutant background (lines 10 and 11) and in the original background (lines 8 and 9). pLC16-4 typically increased the phosphofructokinase level to about 20-fold that of the wild type (Table 2, lines 1-3); the factor for pLC30-43 was much less. The former plasmid was used for most experiments. It gave a high enzyme level in several other *pfkA* mutant backgrounds (lines 12-16). The high activity was precipitated by antiserum to Pfk-1 (the main phosphofructokinase of *E. coli*, specified by *pfkA* [25]). Strain JA200/pLC16-4 has been used as a convenient starting material for purification of this enzyme (E. Stellwagen, personal communication). DNA from this strain efficiently transformed (2×10^4 transformants per μg of DNA) strain JC10201 *pfkA*::Mu to *pfk*$^+$. The plasmid DNA, amplified by incubation with chloramphenicol, has also been used to program cell-free protein synthesis; about 70% of the

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TABLE 1. *Strains used*

Strain	Genotype	Reference/ Source
CS520	HfrC *glyVsu58 metB trpA58*	5
DF88	F⁻ *edd-1 galK his pfkA2 pfkB1 pfkB2 pyrD rpsL tonA22*	26
DF214	F⁻ *pgi*::Mu Δ(*eda-edd-zwf*) *his rpsL*	27
DF452	Like DF88 but *his*⁺ *recA1*	Conjugation[a]
DF456	Like JC10201 but *his*⁺ *recA1*	Conjugation[b]
DF542	Like DF88 but *bgl*⁺ *dnaA46*(Ts)/pLC16-4	P1 transduction[c]
DF543	Like DF542 but *dnaA*⁺	
DF575	F⁻ *eno-2 rpsL*	Conjugation[d]
DF576	F⁻ *pgk-1 rpsL*	Conjugation[e]
DF586	F⁻ *pgi*::Mu Δ(*eda-edd-zwf*) *recA1 rpsL*	Conjugation[f]
ET2036	F⁻ Δ(*gal-bio-uvrB*) φ80h Δ(*rha-pfkA-tpi*)	G. Pahel
ET2039	F⁻ Δ(*gal-bio-uvrB*) φ80h Δ(*rha-pfk*)	G. Pahel
JA200	F⁺ *lacY leu recA thr* Δ*trpE5*	5
JC10201	F⁻ *pfkA*::Mu in L148	L. Csonka
JF401	F *ara bgl*⁺ *dnaA46*(Ts) *lacX74 nalA thi*	J. Felton
KL16-99	Hfr *recA1*	18
L148	F⁻ *argG galK galT galC*ᶜ *galA gutC*ᶜ *his lacY mal mt1A metB rpsL su*⁺	J. Lengler
RW226	F⁻ Δ(*edd-zwf*) Δ(*gnd-his*) *lac recA1 rpsL trp*	R. E. Wolf, Jr.

[a] Conjugation, KL16-99 × DF88, His⁺ selection.
[b] Conjugation, KL16-99 × JC10201, His⁺ selection.
[c] P1 transduction, JF401 × DF88/pLC16-4, Bgl⁺(Ts).
[d] Conjugation, DF261 [HfrC *eno-2* (J. D. Hillman)] × AT713 (24).
[e] Conjugation, DF263 [HfrC *pgk-1* (J. D. Hillman)] × AT713 (24).
[f] Conjugation, KL16-99 × DF214, His⁺ selection.

TABLE 2. *Plasmids carrying pfkA and tpi genes*[a]

Strain	Enzyme activity (μmol/min per mg of protein in crude extract)	
	Phosphofructokinase	Triose-P isomerase
1. L148	0.52	ND[b]
2. JA200	0.89	ND
3. CS520	0.68	ND
4. DF456 *pfkA*::Mu	0.06	ND
5. DF452 *pkfA2*	0.02	ND
6. ET2036 Δ(*pfkA-tpi*)	0.02	0.00
7. ET2039 Δ(*pfkA*)	0.02	3.95
8. JA200/pLC16-4	13.3	ND
9. JA200/pLC30-43	1.80	ND
10. DF456 *pfkA*::Mu/pLC16-4	13.1	ND
11. DF456 *pfkA*::Mu/pLC30-43	2.65	ND
12. DF452 *pfkA2*/pLC16-4	9.3	ND
13. ET2036 Δ(*pfkA-tpi*)/pLC16-4	18.3	39.6
14. DF542 *dnaA*(Ts) *pfkA2*/pLC16-4	17.1, 17.7[c]	37.3[c]
15. DF543 *dnaA*⁺ *pfkA2*/pLC16-4	8.9, 8.3[d]	22.1[d]
16. DF542 *dnaA*(Ts) *pfkA2*/pLC16-4	31.7[e]	ND

[a] Growth was in rich medium with fructose, aerobic (except line 16).
[b] ND, Not done.
[c] Average of seven other transductants like DF542 *dnaA*(Ts); growth at 30°C.
[d] Average of three other transductants like DF543 *dnaA*⁺; growth at 30°C.
[e] Anaerobic growth.

DNA-dependent synthesis was Pfk-1, as detected by assay, radioactivity, and antibody (25).

The *pfkA* gene is known (1) to be closely linked to the gene for another enzyme of glycolysis, triose phosphate isomerase (*tpi*). Strains carrying pLC16-4 had very high levels of triose phosphate isomerase (Table 2, cf. lines 13–15 with line 7), and this plasmid complemented a *pfkA-tpi* deletion (lines 6 and 13).

The role of the host gene *dnaA* in ColE1 replication is uncertain: Goebel (13) found no ColE1 replication in a *dnaA*(Ts) strain at nonpermissive temperature, but Collins et al. (7) reported the opposite result, in which case one might expect to amplify plasmid DNA and its products at the nonpermissive temperature. We constructed isogenic strains carrying pLC16-4 in *dnaA46*(Ts) and *dnaA*⁺ backgrounds. There was a small (about twofold) effect of the *dnaA* mutation at the permissive temperature (Table 2, cf. lines 15 and 14). After incubation at temperatures (37 or 42°C) nonpermissive for *dnaA46*(Ts), phosphofructokinase levels were never higher than at 30°C; they were usually substantially lower with leakage of the enzyme, and there was also some leakage (about 3%) even at 30°C in the *dnaA*(Ts) strain. The slightly higher level of phosphofructokinase in strain DF542 *dnaA*(Ts) than in strain DF543 *dnaA*⁺ is probably related to the *dnaA* mutation, since it was shown by several independent transductants (lines 14 and 15), and a further transduction of *dnaA* from strain DF542 gave the effect in seven of eight temperature-sensitive transductants. Its significance is uncertain, and we feel these results support the experiments of Goebel, in that *dnaA*⁺ is needed for ColE1 (and ColE1 hybrid) replication.

Figure 1 shows acrylamide gel patterns of polypeptides from crude extracts from strains with and without pLC16-4. Pfk-1 is a minor protein in wild-type *E. coli*, representing about 0.1% of the total, and not clearly detectable in a gel of total cellular proteins. Strains with pLC16-

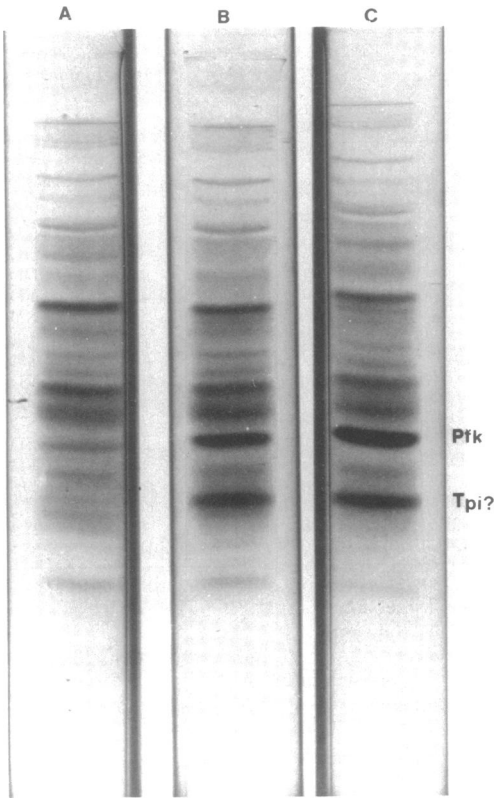

FIG. 1. *Polyacrylamide gel (7%) electrophoresis (8) of crude extracts. (A) DF88 pfkA2; (B) DF543 pfkA2 dnaA⁺/pLC16-4; (C) DE542 pfkA2 dnaA(Ts)/pLC16-4. A 40-μg amount of protein was used for each gel, and staining was with Coomassie brilliant blue. Cultures were grown in minimal medium with fructose, anaerobically, since it is known that there is somewhat more Pfk-1 in anaerobic cells (21; Table 2).*

4 show two new major proteins. The upper band is at the position of Pfk-1. The lower one has not been identified; it might be triose phosphate isomerase.

There also is a minor *E. coli* phosphofructokinase isozyme, Pfk-2. Its activity is a small percentage of the total activity in a wild-type strain (i.e., the residual level in *pfkA* mutants; Table 2, lines 4–7) but a substantial fraction of the total activity in a strain carrying the *pfkB1* mutation (at min 38, far from *pfkA*), which seems to increase the amount of Pfk-2 but not change its characteristics (2). Since the structural gene for Pfk-2 is close to the *pfkB1* mutation (unpublished data), one might expect that a hybrid plasmid for the *pfkB* gene would also have been identified by the original complementation experiments. No such plasmid was found either in the original selection (*pfkA pfkB*⁺ background), or in a selection using a strain (DF88) also lacking any Pfk-2.

pgi. Glucose-6-P can be used either via the phosphoglucose isomerase (*pgi*) reaction or by glucose-6-P dehydrogenase (*zwf*); mutants lacking both enzymes are glucose nonutilizers, but strains with either one can grow on glucose (12). Thus, selection for complementation of strain DF214 *pgi*::Mu Δ(*zwf*) should identify plasmids carrying *pgi* or *zwf* (the two genes are not linked). One plasmid was identified as almost certainly carrying *pgi*: pLC37-5 (Table 3, lines 5–7). It conferred a much higher than normal level of phosphoglucose isomerase to the recipient cells and did not alter their level of glucose-6-P dehydrogenase. DF214/pLC37-5 transferred the ability to grow on glucose and high phosphoglucose isomerase activity by conjugation to strain DF586 (line 7).

***gnd* and *zwf*.** *E. coli* forms gluconate-6-P from gluconate; gluconate-6-P is metabolized either by the inducible Entner-Doudoroff pathway (first enzyme gluconate-6-P dehydrase [*edd*]) or by gluconate-6-P dehydrogenase (*gnd*) and the pentose-phosphate pathway; double mutants (*gnd edd*) do not grow on gluconate, whereas single mutants do (12). With recipient RW226 *gnd* Δ(*edd-eda-zwf*) and selection on gluconate minimal medium, three plasmids gave complementation. One of these, pLC33-30, apparently carries *gnd* (Table 3, lines 8 and 9). Typical enzyme levels were six times or more that of the wild type. *gnd*⁺ was found to transfer by conjugation from RW226/pLC33-30 to another *edd gnd* recipient (DF710; 20). Strain

TABLE 3. *Plasmids carrying pgi, gnd, and zwf genes*[a]

Strain	Enzyme activity (μmol/min per mg of protein in crude extract)		
	Phosphoglucose isomerase (*pgi*)	Glucose-6-P dehydrogenase (*zwf*)	Gluconate-6-P dehydrogenase (*gnd*)
1. JA200	2.86	0.10	0.13
2. CS520	1.67	0.12	0.09
3. DF214 *pgi zwf*	0.00	0.00	ND[b]
4. RW226 *edd gnd*	ND	0.00	0.00
5. DF214/pLC37-5	11.4	0.00	ND
6. JA200/pLC37-5	3.9	0.25	ND
7. DF586/pLC37-5	9.2	ND	ND
8. RW226/pLC33-30	ND	0.00	1.76
9. JA200/pLC33-30	ND	0.10	0.98
10. RW226/pLC3-33	ND	1.84	0.00
11. RW226/pLC37-44	ND	0.00	0.00

[a] Growth was in rich medium.
[b] ND, Not done.

RW226/pLC33-30 has been used for purification of the gluconate-6-P dehydrogenase enzyme (manuscript in preparation).

The two other strains (Table 3, lines 10 and 11) found by complementation did not have gluconate-6-P dehydrogenase and, thus, presumably carry *edd*$^+$. *edd* is in the closely linked cluster *eda-edd-zwf* (9). pLC33-30 evidently carries *zwf*$^+$, conferring at least 10-fold the normal level of glucose-6-P dehydrogenase (line 10). pLC37-44 does not carry *zwf*$^+$ (line 11) but carries *eda*$^+$, for it was also identified in a conjugation with selection for glucuronate complementation in strain DF214.

***eno, pgk,* and *fda*.** Enolase (*eno*) and phosphoglycerate kinase (*pgk*) mutants grow neither on sugars nor on gluconeogenic substances (14, 15). Plasmids were screened by complementation with strain DF575 (*eno*) on minimal medium with glucose and streptomycin. Two plasmids (pLC10-47 and pLC11-8) were identified as carrying the *eno* gene; they raised the enolase level to about eightfold the normal level (Table 4, lines 1–6).

Likewise, two plasmids likely to carry the *pgk* gene (pLC15-31 and pLC33-5) were identified by complementation with strain DF576 (*pgk*); they increased phosphoglycerate kinase to about fivefold the normal level (Table 4, lines 8–11).

New transductional mapping (unpublished data) places the gene (*fda*) for another enzyme of glycolysis, fructose-1,6-P_2 aldolase, very close to the *pgk* gene. Both *pgk* plasmids caused (Table 4, lines 7–11) substantially higher than normal levels of aldolase activity (with the exception of pLC15-31 in JA200) and thus probably carry *fda*$^+$ as well as *pgk*$^+$.

General comments. Table 5 lists the plasmids and the approximate map location of their *E. coli* segments. In general, proof that a particular gene is carried on a plasmid is incomplete. However, other explanations for apparent complementation, such as suppression or reversion, are unlikely in view of the high enzyme levels also found in wild-type background, the demonstration of further transfer from the complemented strain, and the several cases where linked markers also show higher enzyme levels. pLC16-4 is one plasmid which has been directly shown to code for the enzyme in question (phosphofructokinase; 25).

Most genes of Table 5 were initially assigned to plasmids by complementation. As noted by Clarke and Carbon, the *E. coli* DNA in the plasmids is of average size, 8.4×10^6 daltons (5), so most plasmids should carry other bacterial genes, which may be identifiable by known linkage (*fda* was an example). It should also be noted that the screenings we have used were not efficient; repeat matings did not always give the same positive results. Thus, several genes for which we have thus far failed to find plasmids (*gap, fdp,* and *pfkB*) are not necessarily absent from the Clarke-Carbon collection. There also were several cases of apparent complementation which we do not report here, since enzyme levels were not high and reversion or recombination may have occurred.

The actual increases in gene product (from the present limited data) ranged from 4- to 25-fold above wild-type levels. It is not known whether these values reflect different plasmid copy numbers. The question of whether the genes on the plasmids are under normal control

TABLE 4. *Plasmids carrying the eno, pgk, and fda genes*[a]

Strain	Enzyme activity (μmol/min per mg of protein in crude extract)		
	Enolase (*eno*)	Phosphoglycerate kinase (*pgk*)	Aldolase (*fda*)
1. JA200	0.47	2.04	0.09
2. DF575 (*eno*)	0.01	2.01	ND[b]
3. DF575/pLC10-47	2.58	1.61	0.10
4. JA200/pLC10-47	3.85	1.66	0.06
5. DF575/pLC11-8	3.45	1.80	ND
6. JA200/pLC11-8	4.27	1.84	ND
7. DF576 (*pgk*)	0.38	0.01	0.23
8. DF576/pLC15-31	0.34	9.02	1.25
9. JA200/pLC15-31	0.28	7.19	0.13
10. DF576/pLC33-5	0.27	10.10	1.54
11. JA200/pLC33-5	0.31	10.26	0.73

[a] Growth was in minimal medium supplemented with 0.4% malate, 0.1% glycerol, 0.25% Casamino Acids, and 25 μg of tryptophan per ml.

[b] ND, Not done.

TABLE 5. *Summary of results*[a]

Gene	Map position (min)[b]	Plasmid
pfkA$^+$ *tpi*$^+$	86.5	pLC16-4, (pLC30-43[c])
pgi$^+$	90	pLC37-5
(*edd*$^+$) *zwf*$^+$	41	pLC3-33
(*edd*$^+$ *eda*$^+$)	41	pLC37-44
gnd$^+$	44.5	pLC33-30
eno$^+$	59	pLC10-47, pLC11-8
pgk$^+$ *fda*$^+$	62.5	pLC15-31, pLC33-5

[a] Genes assigned to plasmids on basis of complementation and enzyme assay. (Genes in parentheses assigned by complementation only.)

[b] From reference 3 (*pgk* [16]). The presently assigned position of *fda* (23) is slightly different.

[c] pLC30-43 was not tested for *tpi* complementation.

has not been studied extensively. Most of the enzymes in question are synthesized constitutively. For the single case where several growth conditions have been used (*pfkA* on pLC16-4) there was no indication of abnormal control, and the in vitro work showed that an active promoter is located very close to the structural gene (25).

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