The Fumarase Genes of *Escherichia coli*: Location of the *fumB* Gene and Discovery of a New Gene (*fumC*)

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(Received 16 May 1985)

The *fumB* gene of *Escherichia coli*, which complements the fumarase deficiency of a *fumA* mutant when present in multiple copies, has been located at 93.5 min in the *E. coli* linkage map and its product has been identified as a polypeptide of 61 kDal. Four overlapping ColE1-*fumB*⁺ plasmids representing a continuous segment of 23.3 kb of bacterial DNA have been isolated from the Clarke–Carbon *E. coli* gene bank and the location of the *fumB* gene relative to the restriction map and the adjacent *mel* operon has been defined. Hybridization studies have shown that the *fumB* gene is homologous to the *fumA* gene, which complements the *fumA1* mutation in single and multi-copy situations, and encodes an analogous 61 kDal product formerly regarded as the *E. coli* fumarase. The hybridization studies also showed that the *Bacillus subtilis* fumarase gene (*citG*) is homologous to an independent gene, *fumC* (formerly *g48*), which lies adjacent to the *fumA* gene at 35.5 min in the *E. coli* linkage map. The N-terminal sequences of the *citG* and *fumC* products exhibit a 51% identity over 88 residues. It is possible that the *fumC* and *citG* genes are fumarase structural genes of *E. coli* and *B. subtilis*, and that the *fumA* gene may encode a differentially-regulated fumarase or be a positive regulator gene which is essential for the expression of *fumC* (but not *citG*). If so, the *fumB* gene may encode a related enzyme or activator that can replace the *fumA* function when amplified.

INTRODUCTION

Fumarate hydratase or fumarase (EC 4.2.1.2) catalyses the interconversion of fumarate and L-malate. In facultative organisms such as *Escherichia coli* it functions in the citric acid cycle during aerobic respiratory metabolism, and during anaerobic glucose fermentation it participates in the reductive conversion of oxaloacetate to succinate. Consistent with this dual role, the synthesis of fumarase is only 40–60% repressed by anaerobiosis, and by glucose during aerobic growth (Gray *et al.*, 1966). Studies with a mutant lacking fumarase have shown that the enzyme is essential for aerobic growth on fumarate and acetate, whereas growth on malate, glucose or complex media is only impaired by the deficiency (Guest & Roberts, 1983). The fumarase mutation (*fumA1*) does not prevent anaerobic growth on glucose minimal medium. However, this is not surprising since an anaerobic requirement for succinate (succinyl-CoA) is only imposed when isocitrate lyase and fumarate reductase are both blocked (Creaghan & Guest, 1978). It would also appear that alternate pathways exist for the conversion of oxaloacetate to fumarate under anaerobic conditions. These involve either aspartate or malate as intermediates, and it may be significant that the enzymes of the former route, aspartate aminotransferase and aspartase, are induced anaerobically (Smith & Neidhardt, 1983; Courtright & Henning, 1970), whereas malate dehydrogenase and fumarase are repressed (Courtright & Henning, 1970; Gray *et al.*, 1966). It is also interesting that the *fumA1* mutation does not prevent anaerobic growth on glycerol plus H₂ with malate as the source of the terminal oxidant (fumarate), which suggests that an alternative route from malate to fumarate may be operating.

Fig. 1. Scale diagram showing the segments of *E. coli* and *B. subtilis* DNA cloned in λ phages that complement the fumarase deficiency of the *fumA1* mutant of *E. coli*. The positions and polarities of known genes are indicated relative to the *EcoRI* (▲) and *HindIII* (△) sites. The sizes of the gene products (kDa) and specific restriction fragments (kb) are included. The shaded regions denote the segments of DNA cloned in the M13 phages that were used as hybridization probes.

Two fumarase genes, *fumA* and *fumB*, have been isolated in recombinant λ phages by virtue of their ability to complement the fumarase deficiency of the *fumA1* mutant (Guest & Roberts, 1983). The *fumA* gene encodes a 61 kDa polypeptide which is expressed from a 6.2 kb *HindIII* fragment that also contains the 6-phosphomannose isomerase gene (*manaA*) and a gene (*g48*) specifying an unidentified 48 kDa product (Fig. 1). These genes are located at 35.5 min in the *E. coli* linkage map and the nucleotide sequences of the *fumA* and *manaA* genes and part of the unidentified gene (*g48*) have been defined (Miles & Guest, 1984*a, b*). The *fumB* gene is expressed from an 8.2 kb *HindIII* fragment (Fig. 1), which encodes polypeptides of 61, 56, 43, 33 and possibly 52 kDa, and it differs from *fumA* in being unable to complement the *fumA1* mutant except in high-copy situations such as transduction plaques or transformants with multi-copy plasmids. The *fumA1* mutant is also complemented by the *Bacillus subtilis* fumarase gene (*citG*) which has also been cloned and sequenced (Moir *et al.*, 1984; Miles & Guest, 1985). The *citG* gene encodes a 50 kDa polypeptide which has no homology with the *fumA* gene product.

The existence of two *fum* genes raises questions concerning their products and about the nature of the *fumA1* mutation. If it is assumed that *fumA* is the structural gene for the major fumarase then *fumB* could encode a minor or differentially-regulated fumarase. It could, for example, specify a fumarase that is only synthesized under anaerobic conditions or in high-copy situations where an aerobic repressor may be titrated by the *fumB* operators. It could also represent a vestigial or silent fumarase gene that is only expressed when coupled to a phage or plasmid promoter. The putative minor fumarase could resemble the unrelated *B. subtilis* fumarase (*citG* product). On the other hand, if there is only one fumarase in *E. coli*, the *fumB* gene could encode some other hydratase having a very weak fumarase activity (but sufficient to complement the *fumA1* lesion when amplified), or it could be a suppressor. The latter could have a direct effect on the *fumA1* mutation, or function indirectly by opening up an alternative metabolic route to bypass the need for fumarase.

This paper describes the properties of some ColE1-*fumB*⁺ plasmids isolated from the Clarke-Carbon plasmid gene bank (Clarke & Carbon, 1976) and the location of the *fumB* gene in the 93rd min of the *E. coli* linkage map. Evidence is presented that the *fumA* and *fumB* genes encode homologous products ($M_r = 61\,000$) which could either be fumarases, or regulatory proteins that activate the expression of a fumarase structural gene. Evidence is also presented for the existence of a new fumarase gene (*fumC*, formerly designated *g48*) that is adjacent to *fumA* and encodes a protein that is analogous to the *citG*-encoded fumarase of *B. subtilis*.

Table 1. *Strains of Escherichia coli* K12

Strain	Relevant genotype or phenotype	Source* or reference
NK-1	F ⁻ ; <i>spoT1</i>	E. Juni
EJ1535	<i>fumA1</i>	E. Juni; mutant of NK-1
JRG1331	<i>fumA1 gyrA</i>	Nal ^R derivative of EJ1535
JRG1555	<i>fumA1 gyrA recA srl::Tn10</i>	Tet ^R Rec ⁻ transductant of JRG1331
ED8641	Met ⁻ Trp ⁻ Aux ⁻ <i>supE hsdR recA56</i>	N. E. Murray
GM242	<i>dam-3 recA1</i>	M. Marinus
AB2480	<i>uvrA6 recA13</i>	P. J. Emmerson
C600	λ^S	N. E. Murray
PL2024	<i>rpsL</i> λ^S	Lambden & Guest (1976)
F500/GMS724	F500; <i>rpsL700</i> λ^S	CGSC 5505
H	Hfr (97 min); <i>thi-1</i>	W. Hayes
KL14	Hfr (67 min); <i>thi-1</i>	CGSC 4294
KL16	Hfr (62 min); <i>thi-1</i>	CGSC 4245
CH931	Hfr (97 min); <i>lysA polA1</i>	C. W. Hill; Greener & Hill (1980)
BW360	<i>polA1 zig-219::Tn10</i>	W. Wickner; Silver & Wickner (1983)
JRG1595	Hfr (67 min); <i>thi-1 polA1 zig-219::Tn10</i>	Tet ^R PolA ⁻ transductant of KL14
X478G3	F ⁻ ; <i>leu-6 proC purE Trp⁻ thi-1</i>	Herbert & Guest (1968)
AB2294	F ⁻ ; <i>proA2 trp-3 his-4 ilvD145 metE46 thi-1 malA1 rpsL8</i> or 9 λ^- λ^R	CGSC 2294
JC1552	F ⁻ ; <i>leuB6 trp-31 hisG1 argG6 metB1 malA1 rpsL104</i> λ^- λ^R	CGSC 4273
AB2495	F ⁻ ; <i>thr-1 leuB6 proA2 trp-35 hisG4 thyA20 argE3 thi-1 rpsL31</i>	CGSC 2495
RM1003	<i>purA</i>	Spencer & Guest (1973)
PA2004	<i>pyrB thr-1 leu-6 his-1</i>	Spencer & Guest (1973)
Q11	Fdp ⁻	Spencer & Guest (1973)
JRG780	<i>frdA11 trpA</i>	Cole & Guest (1980)
JRG820	<i>mel-1 gltC8</i> (P1)	Formerly Ymel/G; Spencer <i>et al.</i> (1976)
M2508	<i>melA7 metB1</i>	CGSC 4926
M1900	<i>melB4 metB1</i>	CGSC 5934
JA200	F ⁺ ; <i>thr-1 leuB6 trpE5 thi-1 recA56</i>	B. J. Bachmann

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METHODS

Bacterial strains. The strains of *E. coli* used are listed in Table 1. The routine transformation host was ED8641, but GM242 was used when *Bcl*I-susceptible DNA was required. Strain C600 was used for assaying λ phages except following zygotic induction when Str^R (PL2024) or Str^R F-prime (F500) strains were used. Phages P1kc and P1vir1 were assayed with *Shigella dysenteriae*. The map positions of the markers used in the linkage studies and the points of origin for the Hfr strains are indicated in Fig. 5. The *polA* derivative of KL14, JRG1595, was prepared by contrasducing the *polA1* mutation with a closely-linked Tn10 insertion using a P1vir1 lysate of BW360. Tet^R transductants were screened for: sensitivity to methyl methanesulphonate (PolA⁻), using L agar containing methyl methanesulphonate (0.08%, v/v); retention of Hfr activity, by cross-streak conjugation with JC1552; and P1-sensitivity.

Phages and plasmids. Several λ transducing phages containing the *fumA* and *fumB* regions of *E. coli* were used: λ G135 (*λfumA*) and λ G139-140 (*λfumB*), Δ *srI* λ 1-2 *shn* λ 3:::*fum att⁺ int⁺ imm²¹ nin5 shn* λ 6[°]; and λ G137 (*λfumA*) and λ G141 (*λfumB*), Δ *srI* λ 1-2 *shn* λ 3:::*fum* Δ (*att-red*) *imm²¹ nin5 shn* λ 6[°] (Guest & Roberts, 1983). A phage containing the *citG* region of *B. subtilis* was also used: λ G142 (*λcitG*), Δ *srI* λ 1-3:::*citG cI*857 *srI* λ 4[°] (Moir *et al.*, 1984). Other phages, λ b2c *imm²¹* and λ vir, were used in lysogen selection and for testing immunity and sensitivity.

Plasmid pGS56 is a derivative of pBR322 that contains the *fumB* region (8.2 kb *Hind*III fragment) and retains the Amp^R and Tet^R markers (Guest & Roberts, 1983). A culture containing samples of all the clones in the ColE1-*E. coli* plasmid gene bank of Clarke & Carbon (1976) was kindly provided by Dr S. T. Cole (Pasteur Institute,

Paris, France), and strains containing specific plasmids (pLC4-5, pLC17-38 and pLC25-33) were obtained from Dr B. J. Bachmann (Yale University, New Haven, Conn., USA). The host strain for the ColE1-hybrid plasmids was JA200.

Media. The rich medium for subculturing was L broth, or LG broth for citric acid cycle mutants, and BBL medium was used for λ phage assays (Guest, 1981). Media were solidified with Difco Bacto agar at 10 or 15 g l⁻¹ for plates or 6.5 g l⁻¹ for top layers. Minimal medium E (Vogel & Bonner, 1956) was used with carbon sources, glucose (11 mM), melibiose (5 mM), maltose (5 mM), glycerol (40 mM), potassium fumarate (40 mM), and other supplements, L-amino acids (30 μ g ml⁻¹), adenine (35 μ g ml⁻¹), uracil (35 μ g ml⁻¹), thymine (50 μ g ml⁻¹), vitamin B₁ (5 μ g ml⁻¹), as required for different nutritional tests and genetic selections. The antibiotic supplements were (μ g ml⁻¹): ampicillin (50-100); tetracycline (15); streptomycin (200) and nalidixic acid (10). The glycerol plus fumarate medium (GF) was used for Frd⁺ selection using an atmosphere of H₂ + CO₂ (5%, v/v) according to Lambden & Guest (1976).

Genetic methods. (a) Transformation with plasmid DNA was done by the method of Lederberg & Cohen (1974) except that the first wash with CaCl₂ was omitted.

(b) Transduction with phage P1 was done by the method of Spencer *et al.* (1976) using an m.o.i. of 2. For strain construction the m.o.i. was reduced to 0.01 and EGTA (2.5 mM) was added, before plating, to chelate Ca²⁺ ions and so limit superinfection with free phage particles.

(c) Conjugation. F⁺-mediated ColE1 transfer from a culture containing representatives of all the clones in the Clarke-Carbon gene bank to JRG1331 (F⁻; *fumA1 gyrA*) was done by mixing equal volumes (0.5 ml) of exponential cultures in a total of 2.5 ml L broth for 4 h at 37 °C. Fum⁺ Nal^R exconjugants were selected on fumarate minimal medium plus nalidixic acid (10 μ g ml⁻¹), and then purified by single colony isolation on the same medium before examination for the presence of ColE1 hybrid plasmids. Interrupted mating experiments for analysing zygotic induction and for time of entry mapping were done according to Jacob & Wollman (1956). Mating mixtures were sampled at 10 min intervals for up to 100 min, separated by vortex mixing for 1 min in L broth plus streptomycin, and dilutions were plated on appropriate media (with or without an indicator organism) to assay phage-generating merozygotes and different recombinant classes. Conjugational linkage analysis was done by the method of Herbert & Guest (1968). Donor and recipient cultures were mated for 100 to 120 min, vortexed for 1 min, and dilutions were plated immediately on a variety of selective media to assess the gradients of marker transmission, except in the case of Amp^R, where the mixtures were diluted 5-fold and incubated for 1 h at 37 °C before plating. Recombinants were purified on the selective medium before testing for inheritance of non-selective markers by replica-plating. Cross-streak tests for donor activity were done on solid media according to Lambden & Guest (1976).

Isolation and manipulation of DNA. The methods for isolating phage and plasmid DNA have been described previously, as have the methods for restriction enzyme digestion, agarose gel electrophoresis and DNA ligation (Guest *et al.*, 1983; Moir *et al.*, 1984). Small scale plasmid preparations were used for routine screening and transformation (Birnboim & Doly, 1979). Fragments of DNA were isolated either by electrophoresis in low melting point agarose (BRL) and phenol extraction according to the manufacturer's instructions, or with DEAE-cellulose paper (Dretzen *et al.*, 1981) for standard agarose.

'Maxicell' procedures. Polypeptides expressed by plasmid-encoded genes were labelled with L-[³⁵S]methionine by the 'maxicell' technique of Sancar *et al.* (1979) by using AB2480 derivatives as detailed by Shaw & Guest (1982).

DNA labelling and hybridization methods. Single-stranded M13 DNA containing segments of bacterial DNA was isolated by the method of Sanger *et al.* (1980) and labelled with [α -³²P]dATP to >10⁶ c.p.m. per μ g of DNA by synthesizing a complementary M13 strand with a hybridization probe primer according to Hu & Messing (1982). Restriction fragments from *λfum* and *λcitG* DNA (0.2 μ g) were separated in agarose gels and transferred overnight to nitrocellulose filters (Southern, 1975), or total genomic DNA samples (4 μ g) were applied as spots directly to the filters. The filters were baked at 80 °C for 2 h *in vacuo*, and then treated according to the prehybridization and hybridization procedures of Maniatis *et al.* (1982). Hybridization was done with 10⁵ to 10⁶ c.p.m. of ³²P-labelled probe per filter in 10 ml of a 6 × SSC hybridization solution (1 × SSC is 0.15 M-NaCl, 0.15 M-trisodium citrate, pH 7.0) at 65 °C for 18 h. The filters were subsequently washed with 4 × SSC (low stringency) and 0.1 × SSC (high stringency) solutions at the same temperature, and then autoradiographed with a fluorescent screen.

Materials. Restriction enzymes, T₄ DNA ligase, DNA polymerase (Klenow fragment) and hybridization probe primer were purchased from BRL, New England Biolabs, Boehringer and Gibco-PL. The radiochemicals, L-[³⁵S]methionine (0.8 Ci mol⁻¹; 30 GBq mol⁻¹) and 2'-deoxyadenosine 5'-[α -³²P]triphosphate (3 Ci mol⁻¹; 110 GBq mol⁻¹) were from Amersham.

RESULTS

Isolation of ColE1-fum⁺ plasmids

A culture containing representatives of all the clones in the Clarke-Carbon *E. coli* gene bank was screened by F⁺-mediated conjugation for complementation of the fumarase lesion of strain

Fig. 2. Restriction maps of the ColE1-*fum*⁺ plasmids, drawn to scale in a linearized form and showing the bacterial DNA (open bars) and ColE1 DNA (thick lines). The segment of bacterial DNA found in the *λfumb* phages and subcloned into plasmid pGS56 is indicated, as is the common segment (marked *fumB*) presumed to contain the *fumB* gene. The pLC plasmids, which are probably identical to three of the pGS isolates, are also indicated. All of the sites were unambiguously assigned except the *Pst*I site denoted (P). The restriction sites are abbreviated as follows: Bg, *Bgl*II; H, *Hind*III; P, *Pst*I; R, *Eco*RI; Sm, *Sma*I.

JRG1331 (*fumA1 gyrA*). *Fum*⁺ exconjugants were selected on fumarate minimal medium containing nalidixic acid (10 µg ml⁻¹) to counterselect the donors. The plasmid contents of ten independent *Fum*⁺ colonies were examined and four different types of ColE1-*fum*⁺ plasmid were detected: pGS77 (23.2 kb), pGS78 (23.0 kb), pGS79 (17.3 kb), and pGS80 (20.8 kb). Restriction analysis (Fig. 2) with combinations of five restriction endonucleases revealed the presence of four overlapping inserts, which also overlap the 8.2 kb *Hind*III fragment that had been isolated previously in several *λfumb* transducing phages and was later subcloned in the pBR322-*fumB*⁺ plasmid, pGS56 (Guest & Roberts, 1983). The corresponding segments of bacterial DNA (10.7 to 16.6 kb) were clearly derived from a 23.3 kb region of the bacterial chromosome containing the *fumB* gene, and the results indicated that *fumB* is expressed from a common 3.4 kb region (Fig. 2). It is not clear why no ColE1-*fumA*⁺ plasmids were recovered, but an exhaustive search was not made.

Identification of the *fumB* gene product

In previous studies with two *λfumb* phages (*λ*G139 and *λ*G140) and with pGS56, evidence was obtained that the 8.2 kb *Hind*III fragment directed the synthesis of polypeptides of 61, 56, 43, 33 and possibly 52 kDa (Guest & Roberts, 1983). The polarities of expression of the 61 and 43 kDa products were defined as leftward relative to the restriction map shown in Fig. 2. A restriction map of the *fumB* region of pGS56 is illustrated in Fig. 3. No sites were detected for *Cla*I, *Kpn*I, *Sal*I, *Sma*I, *Sst*I or *Xho*I. In order to define the precise location of the *fumB* gene and to identify its product, a series of pBR322 derivatives containing sub-segments of the *fumB* region were constructed (Fig. 3). Plasmids pGS93, pGS100 and pGS142 are deletion derivatives of pGS56, obtained by re-ligating the products of *Bam*HI, *Eco*RI and *Sph*I digestion, respectively. Plasmid pGS143 is likewise a *Bcl*II-*Bam*HI deletion derivative of pGS93. Plasmids pGS144 and pGS145 were obtained by recloning the 2.4 kb *Sph*I-*Bgl*II (pGS56) and 1.85 kb *Eco*RI-*Pst*I (pGS93) fragments into pBR322. In each case the reconstructed plasmids were recovered as Amp^R Tet^S or Tet^R Amp^S transformants of ED8641 and their structures were confirmed by restriction analysis. Their ability to complement the *fumA1* lesion was then tested non-selectively by first isolating Amp^R transformants of JRG1555 (or Tet^R transformants of EJ1535) and then screening for the *Fum*^{+/−} phenotype. The results are shown in Fig. 3, and it is apparent that the smallest fragment from which an active *fumB* product can be expressed is the 2.4 kb *Sph*I-*Bgl*II segment of pGS144. Deletions extending as far as the *Bcl*II site (in pGS143) or the *Pst*I site (in pGS145) inactivate the *fumB* function and so define an essential 0.85 kb segment of the *fumB* gene. This location is consistent with the results obtained with the ColE1 plasmids.

Fig. 3. Physical maps of derivatives of plasmid pBR322 containing different segments of the 8.2 kb *fumB* region. The phenotypic characteristics conferred by the plasmids are abbreviated: F⁺, Fum⁺; M⁺, Mel⁺; A^R, Amp^R; T^R, Tet^R. The bacterial DNA is denoted by open bars, the vector by thick lines, and sufficient vector restriction sites are indicated to define its relative orientation. The locations and polarities of specific genes are indicated. A scale (1 division = 1 kb) is included in the map and the restriction sites are abbreviated as follows: A, *Ava*I; B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; H, *Hind*III; Hp, *Hpa*I; P, *Pst*I; Pv, *Pvu*I; R, *Eco*RI; Sp, *Sph*I.

It is interesting that the smaller plasmids induce a more vigorous Fum⁺ phenotype in the *fumA* host. This presumably reflects the copy-dependent complementation of *fumA* by *fumB*⁺ and the inverse relationship between plasmid size and copy number. The smaller plasmids also improve the rate and extent of growth on the permissive substrate, malate, for unknown reasons.

Using the maxicell procedure to detect the polypeptides expressed by different plasmids, it was clear that one component (61 kDal) is specifically associated with the *fumB* gene (Fig. 4). This is the left orientated product that has the same mobility as the 1647 bp-encoded *fumA* gene product (Guest & Roberts, 1983; Miles & Guest, 1984a). The 56 kDal product appeared to be encoded to the left of the *fumB* gene, and no other products or meaningful deletion or fusion products were detected in these experiments.

Chromosomal location of the *fumB* gene

Because there are no *fumB* mutants, and because such mutants are unlikely to have a Fum⁻ phenotype (or any other easily predicted phenotype), the conventional methods of gene mapping were excluded. However, this difficulty was circumvented by strategies that rely on DNA homology to direct the insertion of the cloned *fumB* gene into the *fumB* region of the chromosome along with recognizable markers provided by the phage or plasmid vectors.

Mapping by zygotic induction. Zygotic induction occurs when an integrated prophage of a donor strain is transferred to the repressor-free cytoplasm of a non-immune recipient, and the approximate position of a prophage can be estimated from the onset of phage production in interrupted mating experiments (Jacob & Wollman, 1956). Using an integration-deficient λ *fumB* transducing phage [λ G141; λ *fumB* Δ (*att-red*) *imm*²¹], lysogenic derivatives of three different Hfr strains (Fig. 5) were constructed by *rec*-mediated integration in the *fumB* region of the host chromosome. Interrupted matings between KL14(λ G141) and AB2294(F⁻ λ ^R) gave a time of prophage entry corresponding to 90–99 min on the linkage map, just clockwise of *metE* (86 min) but anticlockwise of *proA* (6 min). Other matings involving H(λ G141) and KL16(λ G141) showed no sign of prophage transfer within the first 60 min, whereas a λ G140

Fig. 4. Autoradiogram of ^{35}S -labelled polypeptides expressed from cloned genes. Plasmid-containing cultures were labelled for 2 h by the maxicell procedure. The molecular masses (kDal) of the polypeptides were deduced by reference to a set of standards.

prophage (λ *fumB*, *att*⁺ *int*⁺ *red*⁺) entered at a time corresponding to the primary λ attachment site (*att* λ ; 17 min) in a control experiment with H(λ G140) as the lysogenic donor.

Mapping by conjugational linkage analysis. The replication of a ColE1-derived plasmid requires DNA polymerase I to be active in the transformed cells (Kingsbury & Helinski, 1970). Consequently a plasmid such as pGS56 (*fumB*⁺ Amp^R) will only persist in a *polA* mutant if it is integrated into the chromosome. As a result an Amp^R marker is delivered to a region of mutual homology, and can be used to locate genes that have been cloned but do not have a readily identifiable phenotype (Greener & Hill, 1980; Silver & Wickner, 1983). Two Hfr *polA* strains, CH931 (related to H) and JRG1595 (from KL14), were transformed with pGS56 and Amp^R transformants were recovered, albeit at low frequencies (~ 200 per μg of plasmid DNA). These were tested for retention of the *polA* mutation, Hfr activity, and ampicillin-resistance consistent with single rather than multiple copies of the plasmid ($< 100 \mu\text{g ml}^{-1}$ not $> 2 \text{ mg ml}^{-1}$, respectively). One derivative of each type (CH931::pGS56 and JRG1595::pGS56) was then used for conjugation with several multiply-marked recipients: X478G3, AB2294 and JC1552.

Fig. 5. Genetic map of *E. coli* K12 showing the locations of relevant markers and the points of origins of the Hfr strains.

Table 2. Transductional mapping of the integrated *fumB* plasmid (pGS56)

Recipient	Selection	Transductants per 10 ⁶ P1kc	Number tested	Amp ^R (<i>fumB</i>)	Cotransduction frequency (%)
JC1552	MetB ⁺	5	100	0	<1
AB2495	ArgE ⁺	7	100	0	<1
JRG820	Mel ⁺	90	200	196	98
JRG780	FrdA ⁺	60	200	96	48
RM1003	PurA ⁺	39	280	101	36
Q11	Fdp ⁺	8	400	24	6
PA2004	PyrB ⁺	14	300	3	1

The relative positions of the points of origin of the donor strains and the selective markers are shown in Fig. 5. In each case streptomycin-resistant exconjugants were selected after 100 or 120 min matings and the predictable gradients of marker transmission were obtained for nearly all the selected markers.

With the CH931 derivative the gradient was *proC*⁺ > *trp*⁺ > *his*⁺ > *metE*⁺, *ilvD*⁺, *leu*⁺ and Amp^R (*fumB*), the transfer of *leu*⁺ being unusually low for what should have been the earliest marker (Fig. 5). The coinherance frequencies for non-selective donor markers amongst the Amp^R (*fumB*) Str^R exconjugants were: *leu* (40%); *metE* (28%); *ilvD* (25%); *malA* (9.2%); *his*, *trp* and *proC* (<1%). These results indicate that *fumB* is closest to the *leu* marker, but the relatively high linkages with the late markers (*metE* and *ilvD*) suggest that it lies anticlockwise of *leu* and therefore close to the sex plasmid integration site. In this position the *fumB* plasmid could interfere with the transfer and integration of the early marker (*leu*), and if it is really a late marker the cotransfer with *leu* may be abnormally high due to F-prime activity in the donor culture.

With the KL14 derivative the gradient of marker transfer was *argG*⁺ > *malA*⁺ > *metB*⁺ > *leuB*⁺ and Amp^R (*fumB*), and the coinherance frequencies of non-selective markers amongst the Amp^R (*fumB*) Str^R exconjugants were: *metB*⁺ (78%), *leuB*⁺ (48%), *malA*⁺ (11%) and *argG*⁺ (4%). These results indicate that *fumB* is closer to *metB* (89 min) than to *leuB* (2 min), and a detailed analysis of non-selective marker distribution in the Leu⁺ Str^R exconjugants confirmed that the *fumB* gene (Amp^R) is located between these two markers. Furthermore, since *fumB* is not an early marker with HfrH derivatives, it probably lies between 89 and 97 min.

Transduction studies. The location of the *fumB* gene was further investigated by transduction tests in which a P1kc lysate of CH931::pGS56 was used with a variety of recipient strains containing selectable markers in the *metB* to *pyrB* (89–97 min) region (Table 2). The results show

that the *fumB* gene is extremely close to the *mel* operon at 93.4 min, and probably clockwise to *mel* because the *fumB*-*frdA* (48%) and *fumB*-*purA* (36%) linkages are somewhat larger than the corresponding *mel*-*frdA* (24%) and *mel*-*purA* (4.8%) linkages (Spencer *et al.*, 1976). The relationship between *fumB* and other markers in the 93–94 min region, *proP** (*leuV*) *melA,B* (*bymA*) *pheR** *lysU** *cadA* *dgkR** *aspA* *mopA,B* *ampC* (Backmann, 1983; VanBogelen *et al.*, 1983) was not investigated but it is likely that it is at least as close to *mel* as *cadA*.

Relationships between *fumB* and neighbouring genes

The very close proximity of the *mel* and *fumB* loci focused attention on the *mel* region, and it was found that two of the ColE1-*fumB*⁺ plasmids (pGS77 and pGS78; Fig. 2) and two of the seven pBR322 derivatives (pGS56 and pGS100; Fig. 3) complemented the *mel* lesions of three mutants: M2508 (*melA*), M1900 (*melB*) and JRG820 (*mel-1*). This locates the *mel* genes in the 4.6 kb *HindIII*-*EcoRI* segment on the left of the restriction maps in Fig. 2 and 3.

It was also realized that ColE1 plasmids from the Clarke–Carbon gene bank containing the *mel*, *lysU* and *cad* genes had been identified (Tsuchiya *et al.*, 1982; Hanatani *et al.*, 1984; VanBogelen *et al.*, 1983; Neidhardt *et al.*, 1983). Furthermore, the nucleotide sequences of the *mel* promoter, part of the proximal *melA* (α -galactosidase) gene, and the complete *melB* (melibiose carrier) gene have now been defined (Shimamoto *et al.*, 1984; Yazyu *et al.*, 1984). The relevant ColE1-*melA*⁺*B*⁺ (pLC17–38 and pLC25–33) and ColE1-*cadA*⁺ *lysU*⁺ (pLC4–5) plasmids were isolated and each resembled the ColE1-*fumB*⁺ plasmids in complementing the *fumA1* mutant. Their restriction patterns were identical to three of the previously-defined ColE1 plasmids: pGS77, pGS78 and pGS80, respectively (Fig. 2).

The positions of the *melA* and *melB* genes have been defined relative to the restriction map (Fig. 3) and their products have been identified as polypeptides of 50 (*melA*) and 31 kDal (*melB*) using the maxicell technique (Hanatani *et al.*, 1984). The estimate for the *melB* product is much lower than the value of 52 kDal derived from the nucleotide sequence, the discrepancy being attributed to the abnormally low mobility of hydrophobic proteins in SDS-polyacrylamide gels (Yazyu *et al.*, 1984). It is very probable that the 56 (or 52) and 33 kDal components detected previously with pGS56 and λ *fumB* correspond to the *melA* and *melB* products.

The *mel* and *lac* operons each possess promoter-proximal galactosidase genes (*melA*, *lacZ*), and comparable carrier protein genes (*melB*, *lacY*) that are flanked by analogous intercistronic elements, so the existence of a distal gene (*melC*) equivalent to *lacA* is anticipated (Yazyu *et al.*, 1984). This would be located between the *melB* and *fumB* genes where there is sufficient coding capacity for a polypeptide of ≤ 35 kDal (Fig. 3).

The ColE1-*cadA*⁺ *lysU*⁺ plasmid (pLC4–5) encodes lysine decarboxylase (~ 75 kDal), two forms (II and IV) of the inducible lysyl-tRNA synthetase (61 kDal; a component of the *htpR*-controlled high-temperature production regulon), and one or two unidentified polypeptides (~ 60 and 62 kDal) (VanBogelen *et al.*, 1983; Neidhardt *et al.*, 1983). The *fumB* product may correspond to any of the latter three, although it is unlikely to be *lysU* because only one ColE1-*lysU*⁺ plasmid has been found in the Clarke–Carbon gene bank, whereas at least four different ColE1-*fumB*⁺ plasmids have been isolated. However, the structural relationships between the plasmids suggest that pLC17–38 should include at least *cadA* or *lysU* (Fig. 2).

Hybridization studies

The *fumA* and *fumB* genes of *E. coli* and the *citG* gene of *B. subtilis* were examined for homology by DNA–DNA hybridization. Radioactive probes were prepared from M13 clones containing segments of the *fumA* and *citG* regions. The *fumA* probe included almost all of the *fumA* gene in a 1570 nucleotide *BclI* segment of DNA, whereas the specificity of the *citG* probe was conferred by a 2700 nucleotide *SstI* fragment containing all of the *citG* gene and about half of the *gerAI* gene (Fig. 1). In both cases radioactivity was incorporated by synthesizing the complementary strand of the single-stranded M13 vector DNA using a hybridization-probe primer (Hu & Messing, 1982). The results of hybridization at both high and low stringencies with Southern blots of *HindIII* plus *EcoRI* digests of λ *fumA*, λ *fumB* and λ *citG* DNA are shown in Fig. 6. The *fumA* probe hybridizes to the major *fumA* (3.5 kb) and *fumB* (3.6 kb) fragments, and

Fig. 6. Autoradiograph showing the hybridization of ^{32}P -labelled *fumA*- and *citG*-containing M13 DNA probes (see Fig. 1) to *Hind*III plus *Eco*RI fragments of λ *fumA* (λ G135), λ *fumB* (λ G139) and λ *citG* (λ G142) DNA. A diagram of the restriction patterns obtained by agarose gel electrophoresis and the sizes of key fragments is included: ---, λ vector DNA; —, bacterial DNA.

it detects some larger but minor partial digestion products. It also hybridizes weakly to the 2.7 kb λ *fumA* fragment, but not at all to λ *citG*. The *citG* probe hybridizes to the corresponding fragments of λ *citG*, but not to the *fumA*- or *fumB*-containing fragments. However, the *citG* probe does find homology in the 2.7 kb fragment that is adjacent to the 3.5 kb *fumA* fragment in λ *fumA* (Fig. 6), and the hybridization persists at high stringency (not shown). These observations strongly indicate that the *fumA* and *fumB* genes are homologous to each other but not to the *citG* gene. They also identify a region adjacent to the *fumA* gene that is homologous to the *citG* region but only weakly homologous to *fumA*. The nature of these homologies which could involve *citG* or *gerA1*, and *g48* or some other unidentified *E. coli* gene, will be discussed later.

The same probes were used for hybridization with total DNA extracted from bacteria from several genera: *Bacillus*, *Enterobacter*, *Escherichia*, *Proteus*, *Pseudomonas* and *Serratia*. Positive results were obtained for the *fumA* probe with all except *Bacillus* and *Proteus*, though the *Pseudomonas* reaction was very weak. In contrast the *citG* probe gave a positive reaction with all of the test organisms (data not shown).

Fig. 7. Alignment of the N-terminal sequences of the products of the putative fumarase structural genes of *E. coli* (*fumC* = *g48*) and *B. subtilis* (*citG*). Identical residues are indicated by asterisks and vertical bars denote conservative substitutions scoring ≥ 0.12 in MDM₇₈ (Schwartz & Dayhoff, 1978).

DISCUSSION

The *fumB* gene is characterized by its ability to complement the fumarase deficiency conferred by the *fumA1* mutation, but only in multi-copy situations. Many features of the *fumB* gene, including its relationship with the previously-defined *fumA* gene, have now been established as a result of studies with the cloned gene. Genetic mapping with λ *fumB* prophages and integrated *fumB* plasmids has located the *fumB* gene at 93.5 min, very close to the *mel* operon, but distinct from the *fumA* gene at 35.5 min. The identification of a series of ColE1 hybrid plasmids with overlapping inserts spanning the *melAB-fumB-cadA* region has defined the orientation of a 23.3 kb segment of bacterial DNA relative to the linkage map (left to right in Fig. 2 corresponds to clockwise). The *fumB* coding region has been located and shown to resemble the *fumA* gene in expressing a similarly-sized product (61 kDa) with the same anticlockwise polarity. Their mutual homology has been established in hybridization studies and this suggests that they are functionally-related genes which complement the *fumA1* lesion by analogous mechanisms, even though *fumB* appears less efficient. The discovery that the *B. subtilis* fumarase gene (*citG*) is homologous with a *fumA*-linked gene (*fumC*), but not with *fumA* or *fumB*, increases the complexity of the situation and raises further questions concerning the functions (structural or regulatory) of the *fum* (and *citG*) genes.

Relationship between citG and g48 (fumC). The detection of good hybridization between the *citG* probe and the restriction fragment adjacent to *fumA* focuses attention on a potential relationship between *citG* and *g48*. The unidentified gene (*g48*) encodes a polypeptide of approximately the same size as the *citG* product. Moreover an open reading frame encoding what is presumed to be the N-terminal segment of the *g48* product was defined during the sequencing of the *fumA* gene (Miles & Guest, 1984a). The N-terminal segments of the *citG* and *g48* products possess a remarkably high degree of homology (Fig. 7). Some 51% of the residues in the *B. subtilis* protein are retained in the *E. coli* sequence, and the homology increases to 73% when conservative substitutions scoring ≥ 0.12 in the MDM₇₈ mutation data matrix (Schwartz & Dayhoff, 1978) are included. The same degree of homology (51% identity) is found at the DNA level, but this is apparently not sufficient (or sufficiently extensive) to promote detectable hybridization between the *citG* probe and the corresponding 3.5 kb fragment of λ *fumA* (Fig. 6). Preliminary sequence analysis indicates that the homology with *citG* continues in the *g48* region of the 2.7 kb fragment, where hybridization is detected. Because the *citG* gene complements fumarase mutants of *B. subtilis* and because it also directs the synthesis of fumarase in *E. coli* it is very probable that *citG* is a fumarase structural gene (Moir *et al.*, 1984). It is also likely that *citG* expression in *E. coli* is unaffected by any regulatory constraints that may operate on *citG* in *B. subtilis*, or on the corresponding gene in *E. coli*. It would therefore appear that the homologous *E. coli* gene (*g48*) is a fumarase structural gene (now designated *fumC*). This means that *E. coli* probably elaborates a fumarase that is approximately the same size as those of the Gram-positive bacterium and mammalian tissue (Kobayashi & Tuboi, 1983).

The nature of the fumA1 mutation and the relationship between fumA and fumB. One explanation for the present results is to propose that the *fumA1* mutation inactivates a positive regulator or gene activator (the *fumA* product) that is essential for the expression of the fumarase structural gene (*g48* = *fumC*). The *fumA*–*fumC* intergenic region (139 bp) appears to contain a *rho*-independent terminator for *fumA*, a potential *fumC* promoter, and regions of hyphenated dyad symmetry in the promoter and the segment encoding the 5'-end of the putative *fumC* transcript (Miles & Guest, 1984a). No *fumA*–*fumC* readthrough transcript was detected, so it would appear that the genes are expressed independently. It is therefore feasible that the transcription of *fumC* could be controlled by the *fumA* product, possibly by a mechanism involving a coeffector, as is observed for several positive regulators (Raibaud & Schwartz, 1984).

According to this view the *fumC* gene must be tightly controlled because very little fumarase is synthesized by the *fumA*[−] *fumB*⁺ strain (EJ1535), and attempts to generate a Fum⁺ phenotype by constructing a pBR322 derivative containing the *fumC*⁺ gene but no other selectable marker were unsuccessful (Guest & Roberts, 1983). It also follows from the strong homology between *fumA* and *fumB*, that *fumB* should encode an analogous product which might only activate *fumC* expression when amplified. Presumably it could have a higher affinity for the regulatory region of some other gene, but would function in place of *fumA* in the same way that elevated levels of the Trp repressor protein repress the transcription of other amino acid biosynthetic systems (Bogosian & Somerville, 1983). The potential target of *fumB* regulation is unknown, but it is probably not an adjacent gene like the *fumA*–*fumC* system because the *mel* operon converges on *fumB* and the putative *melC* gene is likely to occupy most of the space between *melB* and *fumB*. Several regulatory genes are located in the critical segment of the linkage map (e.g. *bymA*, *pheR* and *dgkR*) but it is not known whether any of these are related to *fumB* (Bachmann, 1983). It is interesting that plasmid pGS54, which contains the 6.2 kb *Hind*III *fumA* fragment (Fig. 1), consistently generates four times as much fumarase as the *Eco*RI deletion-derivative (pGS57) with the smaller 3.5 kb *Hind*III–*Eco*RI *fumA* fragment (Guest & Roberts, 1983). This could mean that the proposed structural and activator genes (*fumC* and *fumA*) are both amplified with pGS54, whereas the activator alone is amplified with pGS57, and fumarase production would thus be limited to the amount that could be generated from the single copy of the *fumC* gene in the chromosome.

Confirmation of the functions proposed for the *fum* genes is now being sought by several methods including the isolation of specific *fumA* and *fumC* mutants, the construction of compatible *fumA* and *fumC* plasmids to test for complementation in the *trans* situation, the isolation of *fumC*–*lac* fusions to test the prediction that *fumA* is needed to activate *fumC* expression, and the isolation and enzymological characterization of the various gene products. There are other explanations for the observed results. For example the three *fum* genes could encode distinct structural or organizational components contributing to different fumarase activities. This would probably mean that the fumarase deficient strain (EJ1535) is a double mutant (*fumA fumC*). Some support for this comes from the weak hybridization between the *fumA* probe and the fragment containing *fumC*, which suggests that some homology may be found between *fumA* and *fumC* (rather than another gene in that fragment). Although they have not been excluded these and other such possibilities are at present considered plausible but less attractive. If the favoured proposals are substantiated then the *fumA* gene will be the first example of a regulatory gene controlling the expression of a citric acid cycle enzyme.

We are very grateful for the mutant strains provided by Elliot Juni, Barbara J. Bachmann and many others, to the Medical Research Council for a research studentship (J.S.M.), and for support from the Science and Engineering Research Council.

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