Characterization of the Fumarase Gene of *Bacillus subtilis* 168 Cloned and Expressed in *Escherichia coli* K12

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The fumarase (*citG*) gene of *Bacillus subtilis* 168 has been identified in a collection of λ phages carrying *EcoRI*-generated fragments of *B. subtilis* DNA. Regions of the cloned DNA have been subcloned into plasmid vectors, and the ability of prophages and multicopy plasmids to complement *Escherichia coli* and *B. subtilis* fumarase mutations has been examined. Two *EcoRI* fragments of 1.5 and 5.1 kb are both required for fumarase expression in *E. coli* and *B. subtilis*. The level of fumarase activity from a single copy of the *B. subtilis citG* gene expressed in *E. coli* is approximately one-tenth of that from the normal *E. coli* gene; the level is increased by expression from a pBR322-derived multicopy plasmid. The *citG* gene has been located within the cloned DNA by transposon mutagenesis and by expression studies, which have also identified a polypeptide of M_r 49000 as the product of the *citG* gene. The properties of a truncated derivative of this polypeptide have indicated the direction of transcription of the *citG* gene.

INTRODUCTION

The *citG* gene of *Bacillus subtilis* 168 encoding the tricarboxylic acid cycle enzyme fumarase (EC 4.2.1.2), is located at 295° on the genetic map (Rutberg & Hoch, 1970; Henner & Hoch, 1980). Lambda phages (λ *citG*) permitting weak complementation of an *Escherichia coli* fumarase-deficient mutant have been isolated from a library of *B. subtilis* DNA cloned in λ gtWES (Moir, 1983). The segments of cloned DNA were found to contain two *EcoRI* fragments of 1.5 and 5.1 kb, which encode a functional *citG* gene, at least part of a spore germination locus (*gerA*; Moir, 1983), and a genetically defined site of rearrangement in *trpE26* derivatives of *B. subtilis* 168 (Sammons & Anagnostopoulos, 1982). It was also shown that the *citG4* mutation of *B. subtilis* could be repaired by transformation with the 5.1 kb fragment purified from agarose gels (Moir, 1983). This paper describes experiments which identify the location of the fumarase gene in the cloned DNA and characterize its expression, transcription polarity and gene product.

METHODS

Bacterial strains. The derivatives of *E. coli* K12 and of *B. subtilis* 168 that have been used are listed in Table 1. Strain EJ1535, previously described as 1535 (Guest & Roberts, 1983) and BH2919 (Moir, 1983), is a fumarase-deficient derivative of NK-1.

Bacteriophages. Fumarase transducing phages (λ *citG*I and II) were selected from a gene library provided by Dr J. A. Hoch (Moir, 1983). Other λ *citG* phages, λ G142, λ G143 and λ G144, were isolated in an analogous manner from the same library using λ Pam3 as helper (this work). All of the λ *citG* phages retained the vector λ cI857 marker and only λ G143 and λ G144 were *supF*-dependent (i.e. carried at least the λ Sam7 mutation). Helper phages were essential for transduction by the suppressor-independent phages. Other phages were: λ imm⁴³⁴, a heteroimmune phage that was used for dilysogen construction; λ G134, a λ fumA phage carrying the *E. coli* fumarase gene (Guest & Roberts, 1983), used for comparative purposes; and λ G78N (λ nadC *aroP* *genA*; Guest & Stephens, 1980), which provided a λ cI857 control in post-infection labelling studies.

Table 1. *Bacterial strains*

Strain	Genotype	Origin*/reference
<i>E. coli</i>		
NK-1	<i>spoT</i> , parent of EJ1535	E. Juni
EJ1535	<i>fumA1 spoT</i>	E. Juni
ED8654	<i>supE supF hsdR⁻ M⁺ S⁺ met trpR</i>	Murray <i>et al.</i> (1977)
RB308	<i>F⁺ leu thr thyA lacY dra</i>	R. S. Buxton
AB2480	<i>pro thi uvrA6 recA8</i>	P. J. Emmerson
S159 (λ ind)	<i>gal uvrA rpsL sup^c (λimm⁺ ind)</i>	N. E. Murray
<i>B. subtilis</i>		
JH404	<i>citG4 trpC2</i>	J. Hoch
AM096	<i>citG4 gerA1 trpC2</i>	This laboratory
AM099	<i>citG4 gerA1 recE4</i>	This laboratory

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Plasmids. Plasmids constructed in this study are described in Results and illustrated in Fig. 2. Plasmid pHV33 (Primrose & Ehrlich, 1981) is a hybrid of pBR322 and a staphylococcal plasmid, pC194, that is capable of replication in *E. coli* and *B. subtilis*. A derivative of pBR322 carrying the *E. coli* fumarase (*fumA*) gene, pGS54 (Guest & Roberts, 1983), was used in comparative studies.

Media. The rich medium for subculturing *E. coli* and for phage propagation was L broth, or LG broth if used for citric acid cycle mutants (Guest, 1981). A peptone medium (Guest, 1981) was used for phage assays and for selective purposes. Media were solidified with agar (Difco) at 10 or 15 g l⁻¹ for plates and 6.5 g l⁻¹ for top layers. Strains of *B. subtilis* were routinely grown in Difco Penassay Broth and colonies of *citG* mutants were produced on TBAB agar containing glucose and MnCl₂ (Rutberg & Hoch, 1970). *Cit⁺* strains of *B. subtilis* were selected on minimal agar (Anagnostopoulos & Spizizen, 1961) containing 0.5% sodium DL-lactate as sole carbon source. Accumulation of acids, a characteristic of *Cit⁻* strains, was scored on PA agar (Carls & Hanson, 1971).

Antibiotics used for selection after transformation of *E. coli* by plasmid DNA were ampicillin (at 50 µg ml⁻¹) or tetracycline (at 15 µg ml⁻¹ or 5 µg ml⁻¹; see Results) in L or LG agar. Selection of *B. subtilis* transformants carrying plasmids was on L agar containing 3 µg chloramphenicol ml⁻¹.

Transformation. Transformation of competent cells of *B. subtilis* was performed as previously described (Warburg & Moir, 1981). Transformation of *E. coli* was by the method of Lederberg & Cohen (1974).

Conjugation. The pBR322-derived plasmid pAAM6 was mobilized by and with the F factor of RB308(pAAM6) by conjugation according to the procedure of Guyer (1978).

DNA isolation procedures. Bacteriophage DNA isolation has already been described (Moir, 1983; Spencer & Guest, 1982). Plasmid DNA from *E. coli* was purified according to Spencer & Guest (1982). pHV33 DNA was prepared from *B. subtilis* by the method of Niaudet & Ehrlich (1979). Small scale plasmid preparations (Birnboim & Doly, 1979) were used routinely for plasmid screening and transformation.

DNA restriction and ligation. Restriction enzymes *EcoRI*, *HindIII* and *Safl*, and T4 DNA ligase, were purchased from BRL and used under the reaction conditions recommended by the suppliers. DNA fragments were separated by agarose gel electrophoresis and their size estimated as previously described (Spencer & Guest, 1982). Low melting point agarose was obtained from BRL, and phenol extraction of DNA fragments from this agarose was according to the manufacturer's recommended protocol. Calf intestinal phosphatase (Boehringer) was purified by the method of Efstratiadis *et al.* (1977) before use according to the manufacturer's recommended procedure.

Enzymology. Exponential cultures (250 ml in 2 litre flasks) were grown with shaking in L broth (with ampicillin, 50 µg ml⁻¹, where appropriate) for 4 h at 30 °C (dilysogens) or 37 °C (all other strains) using inocula (5 ml) grown for 16 h in the same medium. Extracts were prepared and assayed for fumarase (EC 4.2.1.2) by measuring the conversion of L-malate to fumarate according to Guest & Roberts (1983) and Hill & Bradshaw (1969). Specific activities are expressed as µmol substrate transformed (mg protein in the high speed supernatant)⁻¹ h⁻¹ at 25 °C.

Analysis of polypeptides formed in UV-irradiated hosts. Polypeptides synthesized after infecting UV-irradiated maltose-grown aerobic cultures of strain S159 (λ ind) with transducing phages were labelled with L-[³⁵S]methionine as previously described (Guest *et al.*, 1981). Polypeptides expressed from plasmid-borne genes were labelled by the maxicell procedure of Sancar *et al.* (1979) by using glucose-grown cultures of strain AB2480 transformed with the relevant plasmids. Radioactive polypeptides were analysed by SDS-polyacrylamide gel electrophoresis as previously described (Guest & Roberts, 1983).

Fig. 1. Restriction patterns of λ *citG* DNA relative to λ^+ and λ gtWES. λ B and a restriction map of the corresponding segment of the *B. subtilis* chromosome. Phage DNA is represented by single lines (drawn to a different scale) and the arrowheads mark the phage left arms. The relative positions of the *Eco*RI ($\blacktriangledown$) and *Hind*III ($\triangle$) sites, and the sizes of some of the fragments (in kb) are indicated.

RESULTS

Properties of λ *citG* phages

Five λ *citG* transducing phages have been detected by their ability to complement the fumarase lesion of a *fumA* mutant of *E. coli* with the formation of lysogen-filled transduction plaques (see Methods). The gross physiological properties of the phages indicated that they were the products of recombination between the original hybrid phages in the gene bank and the helper phages used to supply integration and other functions needed to facilitate transduction. Restriction maps obtained by digesting the phage DNAs with *Eco*RI and *Hind*III, separately and in combination, are shown in Fig. 1. The segments of *B. subtilis* DNA cloned in these phages were clearly identified as a series of overlapping fragments (6.65 to 10.7 kb) all containing at least two specific *Eco*RI subfragments of 1.55 and 5.1 kb. It was concluded that the *B. subtilis* fumarase gene (*citG*) is very probably expressed from the 6.65 kb segment that is common to all of the λ *citG* phages. Since the gene bank was originally constructed from a size-selected fraction (10 to 13 kb) of a partial *Eco*RI digest of *B. subtilis* DNA, it is likely that the map derived from the cloned fragments corresponds to a continuous 12.4 kb segment of the *B. subtilis* chromosome, rather than a reassorted set of *Eco*RI sub-fragments.

Subcloning of DNA into plasmid vectors

To define the location of genes in the cloned segment, DNA fragments from λ *citG*I and II were subcloned into pHV33, all the plasmid constructions being carried out in *E. coli*.

Plasmids pAAM3 and pAAM4 (Fig. 2) were constructed by ligation of the complete *Eco*RI digest of λ *citG*I DNA with *Eco*RI-digested pHV33 DNA, selecting transformants of ED8654 on L agar containing 15 μ g tetracycline ml⁻¹. Plasmids pAAM3, containing the 5.1 kb fragment, and pAAM4, carrying the 1.55 kb fragment, were both identified in a screening of 17 such transformants. Strains carrying pAAM3 grew more slowly on L agar + 15 μ g tetracycline ml⁻¹ than those carrying pHV33 or pAAM4, and subsequently tetracycline was used at 5 μ g ml⁻¹ for selective purposes.

Fig. 2. Physical maps of plasmids containing segments of the *citG* region of *B. subtilis* transferred from *λcitG* to pHV33. Bacterial DNA is denoted by the open bars and vector DNA by stippled bars (pBR322 segment) and hatched bars (pC194 segment). The scale drawings show linear representations of the circular plasmids, their overall sizes (in kb), and the targets for *EcoRI* (E), *HindIII* (H), *PstI* (P) and *SalI* (S). The approximate positions and polarities of the genes encoding antibiotic resistance are indicated: *Cam^R* (*cat*); *Tet^R* (*ter*) and *Amp^R* (*bla*).

In an attempt to obtain plasmids carrying a functional fumarase gene, plasmid DNA from a pool of approximately 600 transformants was used to transform the fumarase mutant EJ1535, selecting transformants on LG + 15 µg tetracycline ml⁻¹. Some 1400 colonies were replica plated to peptone medium containing 15 µg tetracycline ml⁻¹, and three that grew more strongly were screened for plasmid content. Two carried pHV33, and were probably chromosomal revertants to *fum⁺*, while the third contained a plasmid carrying 1.55 and 5.1 kb fragments; this plasmid was designated pAAM2 (Fig. 2). A second plasmid of identical constitution was obtained from a parallel experiment.

DNA fragments of 7 to 8 kb derived from a partial *EcoRI* digest of *λcitGII* were purified by excision of appropriately sized fragments after electrophoresis in a low melting point agarose gel. These were ligated with *EcoRI*-digested and phosphatase-treated pHV33 DNA. Transformants of ED8654 resistant to 5 µg tetracycline ml⁻¹ were screened and a plasmid carrying 1.55, 5.1 and 0.9 kb fragments was retained and designated pAAM6 (Fig. 2).

A deletion derivative of pAAM2, plasmid pGS96, was constructed by religating pAAM2 after *SalI* digestion. This plasmid pGS96 retains the *bla* gene and replication functions of pBR322, the 1.55 kb cloned fragment and the proximal part of the 5.1 kb cloned fragment as far as the single *SalI* site in the cloned DNA (Fig. 2). The rest of the 5.1 kb fragment, all of pC194, and part of the pBR322 *tet* gene were lost.

Complementation and recombination studies in *B. subtilis*

Plasmids pAAM3 and pAAM4 (Fig. 2) were transformed into competent cells of the recombination-proficient strain AM096; only pAAM3 could repair the *citG4* mutation by recombination. A second genetic marker, *gerA1⁺*, was 37% cotransformed with *citG4⁺*; this linkage is similar to that observed using *λcitG* phages as donors but is lower than that observed using chromosomal DNA (Moir & Smith, 1983). Complementation of a fumarase defect was tested by transforming pAAM2 and pAAM3 into competent cells of AM099, a *recE4 citG4 gerA1* strain, selecting chloramphenicol resistance. Transformants were scored for their ability to accumulate acids when grown on PA indicator agar containing chloramphenicol. Strain AM099 carrying pAAM3 formed the characteristic asporogenic and acid-excreting colonies of the Cit⁻ parent; transformants carrying pAAM2 formed mainly Cit⁺ sporogenic colonies, although some Cit⁻ segregants were visible. So, the recombination studies indicate that *citG4⁺* is carried on the 5.1 kb fragment, and the complementation studies show that the 1.55 kb

Fig. 3. Restriction map of the *B. subtilis* DNA cloned in pGS96 (see Fig. 2). The targets for *Eco*RI (E), *Hind*III (H), *Cla*I (C), *Pst*I (P) and *Sa*I (S) are indicated. The positions of Tn1000 insertions studied are indicated by small vertical arrows; those drawn below the map inactivate *citG*, whereas the one drawn above the map does not. The minimum extent of *citG* defined by the Tn1000 insertions is cross-hatched, and the probable extent of *citG* based on the size of the polypeptide product is indicated by the dotted line extensions. The polarity of transcription is indicated by the large arrow.

fragment is also required for expression of a functional fumarase activity in *B. subtilis*. It is therefore concluded that the *citG* gene, or at least the *citG* transcriptional unit, spans the *Eco*RI target at the junction of 1.55 and 5.1 kb fragments.

Complementation studies in *E. coli*

The plasmids pAAM2, pAAM6 and pGS96 complemented the *E. coli fumA1* mutant, whereas pAAM3 and pAAM4 did not. This confirms that DNA flanking the critical *Eco*RI target contained in pAAM2, pAAM6 and pGS96 is essential for complementing the fumarase lesion.

Mapping of the *citG* gene by transposon mutagenesis

Plasmids such as pBR322 and pHV33 are mobilized by F at a low frequency, the cotransfer usually resulting in their acquiring $\gamma\delta$ (Tn1000) from F (Guyer, 1978; De Lencastre *et al.*, 1983). Following its introduction into the F⁺ strain RB308 by transformation, pAAM6 was transferred to EJ1535 in a conjugation experiment. Amp^R exconjugants containing pAAM6 were obtained at a frequency of 2×10^{-3} per donor cell by plating on minimal glucose agar containing 100 μ g ampicillin ml⁻¹. Of 177 such exconjugants, screened by replica plating to LG plus tetracycline or chloramphenicol and to peptone agar containing ampicillin, 16 had lost tetracycline resistance, none had lost chloramphenicol resistance, but 33 were now Fum⁻. Plasmids isolated from a number of these Fum⁻ strains were mapped by restriction analysis using *Eco*RI and *Sa*I, and the map of Tn1000 (Guyer, 1978). The estimated positions of the Tn1000 inserts are shown in Fig. 3. The results indicate that the *citG* gene must extend at least 0.05 kb into the 1.55 kb fragment and at least 0.8 kb into the 5.1 kb fragment. Four Tn1000 inserts which did not inactivate *citG* were mapped on the cloned DNA. They were located within the 5.1 kb *Eco*RI fragment at 2.3 kb, 2.5–2.6 kb, 2.7 kb and 2.9 kb from its left-hand end as drawn in Fig. 1. Analysis of a sufficient number of inserts to provide narrower maximum limits was not carried out.

Expression of *B. subtilis*-derived fumarase in *E. coli*

Enzymological studies. The fumarase activities of derivatives of the *E. coli fumA* mutant carrying the *B. subtilis citG* gene in phage and plasmid vectors were compared with the parental strain, and with analogous derivatives carrying the *E. coli fumA* gene in λ G134 and pGS54 (Guest & Roberts, 1983).

The dilyso-gen EJ1535(λ imm⁴³⁴, λ G142) contains a single copy of the *citG* gene whereas EJ1535(pAAM2) and EJ1535(pGS96) contain multiple copies. The results, shown in Table 2, indicate that the fumarase activity expressed from a single copy of the *citG* gene is quite significant, but is still 8- to 10-fold lower than that from a single copy of the *fumA* gene (either in the *fumA*⁺ parent or in the mutant carrying a *fumA*⁺ prophage). Expression from pAAM2 and its deletion derivative pGS96 was increased 7-fold and 10-fold respectively over that from a single *citG*⁺ prophage copy. The activities expressed from multiple copies of the heterologous *citG* gene are equivalent to those from single copies of the homologous *fumA* gene. The fumarase

Table 2. *Fumarase activities of lysogens and plasmid-containing strains*

Strain	Relevant genotype	Fumarase specific activity*
NK-1	<i>fumA</i> ⁺	85
EJ1535	<i>fumA1</i>	<1
EJ1535(λ imm ⁴³⁴)	<i>fumA1</i> (λ)	<1
EJ1535(λ imm ⁴³⁴ , λ G142)	<i>fumA1</i> (λ , λ citG ⁺)	7
EJ1535(λ G134)	<i>fumA1</i> (λ fumA ⁺)	58
EJ1535(pBR322)	<i>fumA1</i>	<1
EJ1535(pAAM2)	<i>fumA1</i> (citG ⁺)	46
EJ1535(pGS96)	<i>fumA1</i> (citG ⁺)	65
EJ1535(pGS54)	<i>fumA1</i> (fumA ⁺)	308

* Fumarase was assayed as the conversion of malate to fumarate and specific activities are quoted as μ mol fumarate produced (mg protein)⁻¹ h⁻¹.

activities for *fumA*⁺ strains in Table 2 are higher than those previously reported (Guest & Roberts, 1983) and reflect the use of mid-exponential rather than early stationary phase cells.

Identification of the *citG* product. The post-infection labelling and 'maxicell' procedures were used to detect proteins expressed from phage- and plasmid-cloned genes and to identify the *citG* gene product. By using a lysogen as the host in post-infection labelling studies, incorporation of labelled methionine was limited to a small number of λ proteins and to the products of bacterial genes that had been cloned with their own promoters. An autoradiogram of ³⁵S-labelled polypeptides expressed from three λ citG phages and a control phage (λ G78N) revealed only one product (*M*_r 49000) that is common to all λ citG tracks but not found in the controls (Fig. 4a). Other products expressed only from λ G144 were visible at *M*_r 67000, 25000 and 24000 and they are presumably encoded by the additional 4.05 kb segment of λ G144 (see Fig. 1).

The maxicell procedure was applied to strains containing plasmids that express the *citG* gene (pAAM2 and pGS96), a non-complementing plasmid (pAAM3) and vector controls (pBR322 and pHV33). These confirmed that a polypeptide of *M*_r 49000 is expressed from the *citG* region (Fig. 4b). With pAAM3, this polypeptide was replaced by one of similar intensity at *M*_r 42000, potentially a truncated derivative of the *M*_r 49000 product (Fig. 4b).

The identification of the *M*_r 49000 polypeptide as fumarase is strengthened by the properties of pAAM3, which would be expected from complementation, recombination and Tn1000 mutagenesis studies to carry part but not all of the *citG* gene. Furthermore, the replacement of the *M*_r 49000 polypeptide by one of *M*_r 42000 (whose coding requirement would be about 1.2 kb) means that the transcription of *citG* should be initiated within the 5.1 kb *Eco*RI fragment and continue outwards into the 1.55 kb fragment, and that the *citG* coding region could extend about 1.2 kb into the 5.1 kb fragment, as shown in Fig. 3. Labelled polypeptides corresponding to β -lactamase and chloramphenicol acetyltransferase, at *M*_r 30000 and 26000, respectively, are also indicated in Fig. 4(b). A number of other specific polypeptides were labelled to a lesser degree in the maxicells derived from strains carrying pAAM2, pGS96 and pAAM3 (Fig. 4b). However, their significance and origin is as yet uncertain.

DISCUSSION

The fumarase gene (*citG*) of *B. subtilis* has been located within a 12.4 kb segment of DNA, by a combination of biochemical and genetic methods. The coding region has been correlated with a gene product of *M*_r 49000 which corresponds to about 1.4 kb of DNA, and the direction of transcription has been established. The location of the *citG* promoter has not been defined but the transcriptional unit is not likely to extend beyond the *Sal*I site upstream of the coding region (Fig. 3), because deletion from this site (to form pGS96) and Tn1000 insertion close to it (Fig. 3) do not affect *citG* expression. The nearest genetic marker yet identified, *gerA11*, maps on the *citG* proximal side of a *Cla*I site (Fig. 3) 1.8 kb from the *Eco*RI target in *citG* (I.M.F., unpublished data); this mutation does not inactivate fumarase and defines a rather narrower

Fig. 4. Autoradiograms of ^{35}S -labelled polypeptides expressed from phage and plasmid genes. (a) Cultures of a UV-irradiated λimm^A lysogen were labelled for 30 min following infection with λcitG ($\lambda\text{G142-4}$) and control (λG78N) phages. (b) Plasmid-containing strains were labelled for 2 h using the 'maxicell' procedure. Labelled polypeptides were analysed by electrophoresis in SDS-polyacrylamide gels (10%) and the estimated sizes ($M_r \times 10^{-3}$) of some of the products are indicated. Control cells without phages or plasmids were used for the left-hand tracks in both (a) and (b). The polypeptides corresponding to the *B. subtilis* fumarase (*citG*) and a truncated derivative (*citG* $_{\Delta}$) are indicated. The products of the vector antibiotic resistance genes, and those expressed from *E. coli* genes of the control ($\lambda\text{nadC aroP genA}$) phage, are shown in parentheses.

limit for the *citG* unit. The truncated fumarase gene expressed from the 5.1 kb fragment lacks at most the coding capacity for approximately 7000 daltons of the polypeptide, depending on the extent of the short fusion fragment contributed by extension of the reading frame into the pBR322 sequence. This suggests that the *citG* gene extends for only a few hundred base pairs to the left of the *EcoRI* target (Fig. 3). This conclusion is consistent with the results from the transposon mutagenesis and is further supported by the properties of a plasmid (pAAM32; I. M. F., unpublished) constructed by insertion of pC194 into the sole *HindIII* target of pGS96. This plasmid can no longer complement *fumA*, although a polypeptide indistinguishable in size from the wild-type fumarase is expressed in the maxicell systems. The *citG* gene therefore extends through the *HindIII* site, but terminates soon after it. Alterations at the extreme C-terminal portion of the protein appear to critically affect the fumarase activity.

The data presented in this paper provide a basis for current studies aimed at sequencing the fumarase gene for comparison with the analogous *E. coli* gene. The *B. subtilis* fumarase is considerably smaller than that of *E. coli* (M_r 61 000; Guest & Roberts, 1983; Miles & Guest, 1984) but about the same size as the mammalian enzymes (M_r 48 500; Beeckmans & Kanarek, 1977). Differences in size between some tricarboxylic acid cycle enzymes have been noted for Gram-positive and Gram-negative organisms, and the former tend to resemble their mammalian counterparts (Weitzman, 1981). The expression of *B. subtilis* fumarase in *E. coli* permits only weak complementation, both at the level of growth rate on selective media and in terms of enzyme activity. This may reflect the rate of transcription and translation of the *B. subtilis* gene in *E. coli*. Nevertheless, the fumarase protein can be labelled efficiently (Fig. 4) and it may be that the heterologous protein functions less well or is less stable in *E. coli*. Even when expressed from a multicopy plasmid (Table 2) the activity attained is lower than that from a single copy of the *E. coli* chromosomal gene.

In addition to *citG*, the cloned DNA is known to include at least part of a spore germination locus, *gerA*. Definitive studies of the *gerA* coding regions are being undertaken. Transposon mutagenesis and complementation studies (A. R. Zuberi, I. M. Feavers and A. Moir, unpublished) have identified at least two intact *gerA* complementation groups and part of a third within the 5.1 kb fragment; part of one of these complementation groups is present in pGS96. So far, it is not possible to specifically correlate any of these *gerA* genes with minor labelled polypeptide species expressed in *E. coli* maxicells.

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REFERENCES

- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology* **81**, 741-746.
- BEECKMANS, S. & KANAREK, L. (1977). A new purification procedure for fumarase based on affinity chromatography. *European Journal of Biochemistry* **78**, 437-444.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1524.
- CARLS, R. A. & HANSON, R. S. (1971). Isolation and characterisation of tricarboxylic acid cycle mutants of *Bacillus subtilis*. *Journal of Bacteriology* **106**, 848-855.
- DE LENCATRE, H., CHAK, K. F. & PIGGOT, P. J. (1983). Use of the *Escherichia coli* transposon Tn1000 ($\gamma\delta$) to generate mutations in *Bacillus subtilis* DNA. *Journal of General Microbiology* **129**, 3203-3210.
- EFSTRATIADIS, A., VOURNAKIS, J. N., DONIS-KELLER, H., CHACONAS, G., DOUGALL, D. K. & KAFATOS, F. C. (1977). End labelling of enzymatically de-capped mRNA. *Nucleic Acids Research* **4**, 4165-4174.
- GUEST, J. R. (1981). Partial replacement of succinate dehydrogenase function by phage- and plasmid-specified fumarate reductase in *Escherichia coli*. *Journal of General Microbiology* **122**, 171-179.
- GUEST, J. R. & ROBERTS, R. E. (1983). Cloning, mapping and expression of the fumarase gene of *Escherichia coli* K12. *Journal of Bacteriology* **153**, 588-596.
- GUEST, J. R. & STEPHENS, P. E. (1980). Molecular cloning of the pyruvate dehydrogenase complex genes of *Escherichia coli* K12. *Journal of General Microbiology* **121**, 277-292.
- GUEST, J. R., COLE, S. T. & JEYASEELAN, K. (1981). Organization and expression of the pyruvate dehydrogenase complex of *Escherichia coli*. *Journal of General Microbiology* **127**, 65-79.
- GUYER, M. S. (1978). The $\gamma\delta$ sequence of F is an insertion sequence. *Journal of Molecular Biology* **126**, 347-365.
- HENNER, D. J. & HOCH, J. A. (1980). The *Bacillus subtilis* chromosome. *Microbiological Reviews* **44**, 57-82.
- HILL, R. L. & BRADSHAW, R. A. (1969). Fumarase. *Methods in Enzymology* **13**, 91-99.
- LEDERBERG, E. M. & COHEN, S. N. (1974). Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. *Journal of Bacteriology* **119**, 1072-1074.
- MILES, J. S. & GUEST, J. R. (1984). Complete nucleotide sequence of the fumarase gene, *fumA*, of *Escherichia coli*. *Nucleic Acids Research* **12**, 3631-3642.
- MOIR, A. (1983). The isolation of λ transducing phages carrying the *citG* and *gerA* genes of *Bacillus subtilis*. *Journal of General Microbiology* **129**, 303-310.
- MOIR, A. & SMITH, D. A. (1983). The genetics of spore germination in *Bacillus subtilis*. In *Fundamental and Applied Aspects of Spores*, pp. 89-100. Edited by G. J. Dring, D. J. Ellar & G. W. Gould. London: Academic Press.

- MURRAY, N. E., BRAMMAR, W. J. & MURRAY, K. (1977). Lambdoid phages that simplify the recovery of *in vitro* recombinants. *Molecular and General Genetics* **150**, 53–61.
- NIAUDET, B. & EHRLICH, S. D. (1979). *In vitro* genetic labelling of *Bacillus subtilis* cryptic plasmid pHV400. *Plasmid* **2**, 48–58.
- PRIMROSE, S. B. & EHRLICH, S. D. (1981). Isolation of plasmid deletion mutants and study of their instability. *Plasmid* **6**, 193–201.
- RUTBERG, B. & HOCH, J. A. (1970). Citric acid cycle: gene–enzyme relationships in *Bacillus subtilis*. *Journal of Bacteriology* **104**, 826–833.
- SAMMONS, R. L. & ANAGNOSTOPOULOS, C. (1982). Identification of a cloned DNA segment at a junction of chromosome regions involved in rearrangements in the *trpE26* strains of *Bacillus subtilis*. *FEMS Microbiology Letters* **15**, 265–268.
- SANCAR, A. A., HAÇK, A. M. & RUPP, W. D. (1979). Simple method of identification of plasmid-coded proteins. *Journal of Bacteriology* **137**, 692–693.
- SPENCER, M. E. & GUEST, J. R. (1982). Molecular cloning of four tricarboxylic acid cycle genes of *Escherichia coli*. *Journal of Bacteriology* **151**, 542–552.
- WARBURG, R. J. & MOIR, A. (1981). Properties of a mutant of *Bacillus subtilis* 168 in which spore germination is blocked at a late stage. *Journal of General Microbiology* **124**, 243–253.
- WEITZMAN, P. J. D. (1981). Unity and diversity in some bacterial citric acid-cycle enzymes. *Advances in Microbial Physiology* **22**, 185–244.