The Isolation of λ Transducing Phages Carrying the *citG* and *gerA* Genes of *Bacillus subtilis*

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λ gtWES derivatives carrying the *citG* (fumarase) gene of *Bacillus subtilis* have been detected by complementation of an *Escherichia coli* fumarase mutation in lysogen-filled plaques. The *gerA* gene, mutations in which affect the germination response of spores to L-alanine, is also present on the two *citG* transducing phages described. The cloned regions in these two phages include *EcoRI*-generated fragments of 5.0 kb and 1.4 kb; the former fragment carries *citG*⁴⁺.

INTRODUCTION

The cloning of *Bacillus subtilis* genes directly in *B. subtilis* using plasmids (Tanaka & Sakaguchi, 1978; Rapoport *et al.*, 1979) and bacteriophages ρ 11 (Kawamura *et al.*, 1979) and ϕ 105 (Iijima *et al.*, 1980) have met with some success but this has been limited by the complex mechanism of plasmid transformation in *B. subtilis* (Gryczan *et al.*, 1980) and the large number of restriction targets in the phage vectors.

An alternative approach has been to seek expression of *B. subtilis* genes in *Escherichia coli*. For example, *B. subtilis* *leu* genes cloned in *E. coli* plasmids have been detected by complementation of an *E. coli* *leuB* mutation (Mahler & Halvorson, 1977; Nagahari & Sakaguchi, 1978) and *rib* genes by complementation of *E. coli* riboflavin mutations (Rabinovich *et al.*, 1978). λ phages carrying *B. subtilis* *leu* and *pyr* genes have been selected as lysogenic transductants of appropriate *E. coli* mutants. However, the level of detection of such lysogens was similar to that of reversion of the *E. coli* mutations and complementation of other auxotrophic markers was not obtained (Chi *et al.*, 1978).

More recently, libraries of *B. subtilis* DNA in λ vectors have been constructed and shown by transformation of *B. subtilis* to contain many genes (Ferrari *et al.*, 1981). This paper describes the isolation, from one of these libraries, of derivatives carrying the fumarase (*citG*) gene of *B. subtilis*; these were detected by the weak complementation of an *E. coli* fumarase defect in lysogen-filled plaques. For the particular library used, a helper phage was necessary to permit plaque formation and lysogeny of the *E. coli* host.

The *gerA* gene, which encodes a spore component essential for germination in L-alanine, and which is located close to *citG* (Moir *et al.*, 1979; Sammons *et al.*, 1981), cannot be selected directly, either by complementation of *E. coli* or transformation of *B. subtilis*. The strategy of seeking clones carrying the *citG* gene has permitted the subsequent detection of the *gerA* gene in the cloned material.

METHODS

Bacterial strains. These are listed in Table 1.

Phage strains. A library was used which was generated by ligation of a 10–13 kb fraction of the products of partial *EcoRI* digestion of *B. subtilis* 168 chromosomal DNA into the purified arms of the vector λ gtWES. λ B (Leder *et al.*, 1977). The library was constructed by Dr E. Ferrari and Dr J. A. Hoch; before being used in complementation experiments this library was cycled once on ED8654 to permit methylation by the *E. coli* K modification system. λ imm⁴³⁴ *Ram54am60* (Moir & Brammar, 1976) was used as a helper phage.

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Table 1. *Bacterial strains*

Strain	Genotype/phenotype	Origin/reference
<i>E. coli</i>		
ED8654	<i>supE supF hsdR⁻M⁺S⁺ met trpR</i>	Murray <i>et al.</i> (1977)
BH2919	Fumarase-deficient	E. Juni
Ymel	<i>supF</i>	W. J. Brammar
W1485E	<i>supE</i>	W. J. Brammar
W3350	Suppressor-free	W. J. Brammar
<i>B. subtilis</i>		
1604	<i>trpC2</i>	Moir <i>et al.</i> (1979)
JH404	<i>citG4 trpC2</i>	Rutberg & Hoch (1970)
4971	<i>gerA1 metB5</i>	Sammons (1980)
4972	<i>gerA1 thr5 trpC2</i>	Moir <i>et al.</i> (1979)
BD111	<i>cysB thr5 trpC2</i>	D. Dubnau

Media. L-broth, L-agar and BBL trypticase agar were used for *E. coli* and λ phage growth and λ phage assay as previously described (Moir & Brammar, 1976). Suspension and dilution of λ phages was done in phage buffer (Murray *et al.*, 1973). Minimal agar (Anagnostopoulos & Spizizen, 1961) was used with 0.5% glucose or 0.5% sodium DL-lactate as the carbon source, and with necessary amino acid supplements at 50 $\mu\text{g ml}^{-1}$. Penassay Broth (Difco) was the liquid growth medium for routine growth of *B. subtilis* strains, while colonies of *citG* mutants were prepared on TBAB agar with MnCl_2 and glucose (Rutberg & Hoch, 1970).

λ phage techniques. Preparation of plating cells, phage propagation, purification and reisolation from single plaques, phage stock preparation and assay have all been described (Murray *et al.*, 1973). Phages were tested for the presence of suppressible mutations by spotting suspensions (at about 10^5 ml^{-1}) on lawns of Ymel, W1485E and W3350, and tested for immunity on lawns of ED8654(λ) and ED8654(λimm^{434}). Complementation by plaque suspensions was determined by spotting on a lawn of BH2919 seeded with $10^6 \lambda\text{imm}^{434} R^-$ phages.

Complementation of an *E. coli* mutant by mixed infection. One volume each of library at $10^9 \text{ p.f.u. ml}^{-1}$ (or partly purified mixtures at a lower concentration) and helper at $2 \times 10^9 \text{ p.f.u. ml}^{-1}$ were mixed and one volume of fresh plating cells of BH2919 at 10^9 ml^{-1} added. After 15 min adsorption at 30 °C or 37 °C, samples were diluted and plated with 0.1 ml BH2919 plating cells on BBL trypticase agar (Guest, 1981). After incubation at 30 °C for 24 h the total number of plaques was determined, while continued incubation for a further 4–6 d was necessary to enable complementing plaques to be distinguished from the others.

Transformation and transduction. Transformation of *B. subtilis* was as previously described (Warburg & Moir, 1981). *Cit*⁺ transformants were selected on minimal agar with lactate as sole carbon source. Transduction by phage PBS1 was carried out by the method of Jamet & Anagnostopoulos (1969). The *ger* genotype of *B. subtilis* strains was scored by the tetrazolium overlay method (Trowsdale & Smith, 1975; Lafferty & Moir, 1977).

DNA preparation. Large scale phage lysates of ED8654 were prepared on L-agar in 25 × 25 cm trays, harvested in phage buffer, concentrated by precipitation with 10% (w/v) polyethylene glycol 6000 (Yamamoto *et al.*, 1970), and resuspended in phage buffer. This suspension was treated with RNAase and DNAase (10 $\mu\text{g ml}^{-1}$ each for 2 h) and phages recovered by addition of CsCl to 41.5% (w/w) (Kaiser & Hogness, 1960) and equilibrium centrifugation for 36 h at 65000 *g*. DNA was purified by phenol extraction (Murray *et al.*, 1977).

Analysis of DNA restriction fragments. Phage DNA samples were incubated with endonucleases *EcoRI*, *BamHI* and *HindIII*, supplied by BRL, under the reaction conditions recommended by the suppliers. Standards of λ^+ DNA digested by *BamHI*, *EcoRI*, or by both *EcoRI* and *HindIII*, were used to identify and estimate the size of fragments generated from λBScitG phage DNA.

Gel electrophoresis. Electrophoresis in a BRL vertical gel apparatus (Uniscience, Cambridge, U.K.) was carried out using 0.75% agarose in Tris/borate buffer with ethidium bromide at 0.5 $\mu\text{g ml}^{-1}$ (Greene *et al.*, 1974) for 2–3 h at 10 V cm^{-1} . Gels were photographed using Polaroid Type 52 film under illumination from below by a UV light source (Chromatovue Transilluminator, Model C63). Gel separation of fragments for transformation was carried out at 4 °C using BRL low melting point agarose in Tris/acetate buffer without ethidium bromide. Side strips from the gel were stained in ethidium bromide (0.5 $\mu\text{g ml}^{-1}$ for 30 min) and the position of fragments marked; corresponding regions from the unstained gel were excised, heated to 65 °C for 20 min and 20 μl added to 0.5 ml competent cells at 37 °C for transformation.

RESULTS

Complementation by λ phage of a host defect is often most readily detected by the increased growth of lysogens in a plaque. The λgtWES library of *B. subtilis* DNA fragments was

constructed in a disabled vector which does not favour this mode of growth. It carries the *cI857* mutation, so that lysogens are only formed at low temperature; moreover, it only grows in *supF* hosts and lacks *att* and *int*, both necessary for efficient lysogenization (Leder *et al.*, 1977). These limitations can be overcome by coinfection with a helper phage, which can complement the defects in genes *W*, *E* and *S* essential for lytic growth and which can integrate efficiently into the chromosome, providing the homology for *rec*-promoted insertion of the λ gtWES genome into the prophage state. The helper used was of a different immunity to that of the library so that lysogens forming in plaques generated from mixedly-infected cells would be immune to both types of phage and would thus be lysogenic for both. The nonsense mutations in the helper phage ensured that only mixedly-infected cells would give plaques, thus minimizing the background of non-transducing plaques. Complementation by the *B. subtilis* *citG* gene of a fumarase-deficient strain of *E. coli* was sought using this helper technique.

Cells coinfecting with both phages were plated on an *E. coli* fumarase-deficient strain on BBL trypticase agar. On this medium the host grows poorly, forming a faint lawn of cells. The growth, though slow, is sufficient to permit formation of λ phage plaques.

Products of a mixed infection with library and helper phages

The fumarase-deficient strain BH2919 was infected with a mixture of λ gtWES and λ imm⁴³⁴ *R*⁻ phages as described in Methods. Phage plaques equivalent to about 12% of the total cell number in the original mixed infection developed on a thinly-growing lawn. Neither input phage could separately form plaques on this suppressor-free strain at this frequency, and thus those formed represent infectious centres, i.e. each plaque contained phages generated during multiple cycles of infection by the products of lysis of an original single cell into which two complementing genomes had been introduced. During these sequential mixed infections, recombination between the phage genomes will also have occurred.

Isolation of complementing phages

On continued incubation at 30 °C occasional clusters of colonies growing more densely than the background of surviving cells were observed on plates carrying confluent plaques. Eighteen such areas were seen on a plate with an estimated 3×10^4 plaques; at a 10-fold higher concentration of plaques, only two or three small clusters were visible. Phages were isolated from these clusters by plating with helper phage on BH2919; typically 5–10% of the plaques at this stage contained lysogens which grew better than the background (see Fig. 1). Such plaques, apparently resulting from complementation, were obtained from six of nine clusters examined. Two complementing plaques, from separate original clusters, were picked into phage buffer and the suspensions plated on ED8654; in each case, approximately equal proportions of small and normal λ -sized plaques appeared. Three well-separated plaques of each morphology type were picked. Phages from the small plaques were *imm* ^{λ} , formed plaques on suppressor-free strains, but gave complementing dense centred plaques on BH2919 only if cells were coinfecting with helper phage. Complementation could also be observed as dense growth at the junction of single plaques of these phages with plaques of λ^+ and λ imm⁴³⁴. The larger plaques contained *imm*⁴³⁴ phages which formed plaques on suppressor-free strains but did not complement BH2919 even in the presence of helper phage. One *imm* ^{λ} , suppressor-independent, fumarase-complementing plaque from each isolate was used to produce high titre lysates of phages designated λ BScitGI and λ BScitGII.

Transformation of a B. subtilis fumarase mutant

λ BScitGI and λ BScitGII both transformed *B. subtilis* JH404 to *citG*⁺ (Table 2). Intact λ phage particles can act as donors in transformation, even in the presence of DNAase (Ferrari *et al.*, 1981), although the mechanism is not understood. The efficiency of transformation was 250–500-fold higher, on a DNA weight basis (2×10^{13} p.f.u. contain approximately 1 mg DNA), than for chromosomal DNA. This reflects at least in part the relative enrichment of the region in

Fig. 1. Complementation of an *E. coli* fumarase defect by λ phages carrying the *B. subtilis* *citG* gene. The photograph shows the surface of an agar plate which represents a stage in the purification of λ BS*citG*II. The plaques were generated in a lawn of BH2919 by cells coinfecting with λ *imm*⁴³⁴ *R*⁻ and a partially purified suspension of the λ *cit* transducing phage. Examples of complementing plaques (A) and non-complementing plaques (B) are arrowed.

Table 2. Transformation of *citG4* by λ BS*citG* phages

JH404 (*citG4 trpC2*) was transformed to Cit⁺ with preparations of whole phage as donor; the competence of the recipient was reflected by the transformation efficiency using control 1604 (*trpC2*) chromosomal DNA.

Strain	Donor Amount of DNA or phage	No. of Cit ⁺ colonies ml ⁻¹	
		Experiment:	
		1	2
None		2	2
(a) 1604	0.03 μ g	6×10^3	ND
(b) 1604	0.14 μ g	ND	4×10^3
(c) λ^+	10^9 p.f.u.	3	ND
(d) λ BS <i>citG</i> I	1.5×10^6 p.f.u.	ND	1.4×10^3
(e) λ BS <i>citG</i> II	1.9×10^6 p.f.u.	ND	7.8×10^2
(f) λ BS <i>citG</i> II	10^6 p.f.u.	8×10^3	ND

ND, Not determined.

hybrid phages. Others have reported rather lower enrichments (Ferrari *et al.*, 1981) and the above results may also reflect some difference in the transforming efficiency of the chromosomal DNA and phage preparation. Alternatively, a reduced efficiency of plating of the phages may have led to underestimation of the total phage number. Wild-type λ phage and non-complementing λ *imm*⁴³⁴ phages formed from mixed infection plaques described above did not transform JH404.

Phage PBS1 lysates of a Cit⁺ transformant from each of crosses (d) and (e) in Table 2 were used to transduce BD111 (*cysB thr trpC2*) to Cys⁺. In both cases 100 Cys⁺ transductants were all Cit⁺ and the linkage of *thr5* and *cysB3* was normal, at about 45%. Thus in both cases the original Cit⁺ transformants carried the *citG*⁺ allele rather than *citG4*. The gene carried by the λ BS*citG* phage is therefore likely to be *citG*⁺ rather than some unlinked suppressor of the *citG4* mutation.

Table 3. Congression of *gerA*⁺ from λBScitG phages during transformation

Strain 4971 (*gerA1 metB5*) was transformed to Met⁺ with DNA from either 1604 (*trpC2*) or 4972 (*gerA1 thr5 trpC2*) in the presence of λ transducing phages.

Donor	No. of Met ⁺ transformants	Proportion of Met ⁺ that were Ger ⁺
Experiment 1		
None	4	
1604 DNA (0.04 µg)	5 × 10 ³	0/48
1604 DNA + λBScitGI 1.2 × 10 ¹⁰ p.f.u.	82	4/48
1604 DNA + λBScitGII 4 × 10 ⁹ p.f.u.	800	12/48
Experiment 2		
None	0	
4972 DNA (0.05 µg)	7.1 × 10 ³	0/192
4972 DNA + λBScitGI 5 × 10 ⁹ p.f.u.	423	42/200
4972 DNA + λBScitGII 6 × 10 ⁹ p.f.u.	345	18/200

Identification of the *gerA* gene in hybrid phages

Mutations of *B. subtilis* affecting the ability of spores to germinate in L-alanine and related germinants map in a cluster and are 70–90% linked to *citG4* in phage SPP1 transduction (Moir *et al.*, 1979; Sammons *et al.*, 1981). This locus, designated *gerA*, may contain one or more genes whose products are important for the initial response to germinants by the spore (Sammons, 1980). The germination phenotype of *gerA* and *ger*⁺ strains can be distinguished by a plate test involving the reduction of a tetrazolium dye (Trowsdale & Smith, 1975; Lafferty & Moir, 1977).

The ability of phages λBScitGI and λBScitGII to donate *ger*⁺ to a *gerA* strain in transformation was tested by seeking congression of the *ger*⁺ marker with a selectable, unlinked marker, *metB*. Strain 4971 (*gerA1 metB5*) was transformed to Met⁺ with a non-congressing concentration of *B. subtilis* chromosomal DNA in the presence of a high concentration of λBScitGI or II (Table 3), and the Ger phenotype of the Met⁺ transformants determined. The presence of either phage resulted in a depression of the total number of Met⁺ transformants (presumably as a result of competition between DNAs for uptake) and in the frequent appearance of *ger*⁺ amongst the transformants. The formal possibility that the phage was in some way causing the congression of *ger*⁺ from strain 1604 chromosomal DNA was eliminated by repeating the experiment using chromosomal DNA from strain 4972 (*gerA1 thr5 trpC2*) so that the only possible source of *ger*⁺ was from the donor λ phages. Essentially identical results were obtained (Table 3, experiment 2), despite some variation in the frequency of congression of *gerA*⁺. Experiment 1 was carried out using crude phage suspensions from polyethylene glycol 6000 precipitates, while in experiment 2 phages purified on CsCl gradients were used. When the selection for Met⁺ was carried out in the presence of tryptophan, none of 48 transformants from each cross carried a *trp*[−] allele; thus neither could the chromosomal DNA added cause congression at a significant level, nor was the inclusion of λ phage in the cross having a mutagenic effect. The experiments in Table 3 provide strong evidence that λBScitGI and λBScitGII phages both carry *gerA1*⁺ in addition to *citG4*⁺. While the complete *citG* gene must be present to provide the fumarase complementation, we have as yet no evidence of whether the *gerA* gene is intact in the cloned DNA. The *gerA1* mutation is, however, amongst the least-closely linked of the *gerA* mutations to *citG4* (Sammons *et al.*, 1981).

Analysis of the DNA inserts in *citG* phages

DNA from λBScitGI and II was digested with *Bam*HI and with *Eco*RI, and the fragments separated by agarose gel electrophoresis. Since genetic studies had shown that the phages were suppressor-independent recombinants between a λgtWES derivative and λimm⁴³⁴ R[−] it was not surprising that their physical maps reflected this recombination. Figure 2 shows the restriction map of λ⁺ and the two phages. The *Bam*HI digestion pattern revealed that both λ*cit* phages contain 7.3 kb and 6.8 kb fragments (K and L) and therefore have replaced the *nin* deletion of the original λgtWES with DNA from the helper. They also contain the 5.5 kb fragment (G) from

Fig. 2. Restriction map of λ^+ and $\lambda citI$ phage DNAs. $\lambda BScitGI$ and II are abbreviated to $\lambda citI$ and $\lambda citII$ respectively. Solid arrows indicate *EcoRI* cleavage sites, dotted arrows *BamHI* cleavage sites, and crosses mutations to *EcoRI* resistance. The numbers are fragment sizes in kb, and the letters denote individual fragments.

the left arm of λ . The large fragment represents the remaining segment of λ including the cloned DNA; the inserts in both phages thus lack the *BamHI* recognition sequence. *EcoRI* digests of $\lambda BScitGI$ reveal a 13.5 kb fragment corresponding to the sum of fragments D and E of λ ; the mutation in *lgtWES* to *EcoRI* resistance at the junction of these fragments is retained in this phage but is replaced in $\lambda BScitGII$ from which fragments D and E are obtained separately.

After assignment of λ fragments predicted from the known restriction map, fragments of approximately 5.0 kb and 1.4 kb remain for $\lambda BScitGI$, while these and two smaller fragments (of approximately 0.9 kb and 0.8 kb) were observed in $\lambda BScitGII$. The 5.0 kb fragment is of very similar size to λ fragment B, but if present, this λ fragment would carry a *BamHI* target which cannot be accommodated in the observed pattern of *BamHI* digestion. Transformation of JH404 to *Cit*⁺ with the *EcoRI* fragments extracted from agarose gels revealed that the 5.0 kb fragment from both phages carried the *citG4*⁺ allele.

DISCUSSION

λ phages carrying *B. subtilis* DNA which complemented an *E. coli* fumarase defect and transformed a *B. subtilis* fumarase-deficient (*citG*) strain were isolated. Although the identity of the cloned gene product with the *B. subtilis* fumarase enzyme has not been established, the genetic data suggest very strongly that the λ phages identified by complementation do carry the *citG* gene. The presence in the phages of DNA carrying the closely-linked *gerA* gene provides further evidence of the nature of the cloned region.

The complementation of an *E. coli* defect in a lysogen-filled plaque has in the past proved useful for the identification of *E. coli* genes cloned in λ (Borck *et al.*, 1976; Guest, 1981), including the fumarase gene (J. Guest, personal communication). It can equally be applied to the detection of foreign genes whose functions are selectable in *E. coli* mutants, provided that the genes are expressed and that the foreign product is sufficiently stable and active in the *E. coli* cell to provide a significant growth advantage under selective conditions. However, genetic evidence is necessary to confirm the identity of the cloned DNA, as in some cases complementation of an *E. coli* defect may be the result of the acquisition of a gene whose product has a different primary function from that of the mutated *E. coli* gene (Guest, 1981).

Using the same library and helper phages, and by selecting plaques on a minimal agar (Franklin, 1971) we have recently also identified phages complementing *leuB*, *ilvC* and *hisA* and *B. E. coli* mutations (A. Moir & D. A. Walton, unpublished results). This general approach has already been adopted for the cloning of auxotrophic genes of Gram-positive bacteria [J. Windass, quoted by Brammar (1977)].

The identification of λ transducing phages using selective or semi-selective medium facilitates the detection of phages which form a very small proportion of a mixture. This situation may arise in a library of λ hybrids, particularly if maintained as a mixture in which some phages may decrease in frequency on storage and/or repeated propagation of the lysate. The plaque complementation also provides a convenient means of selectively purifying the phage and permits a clear distinction between transducing and non-transducing plaques.

The particular library used in this work required the presence of a helper phage for effective plaque formation; the opportunities for recombination in mixedly-infected cells resulted in the frequent isolation of phages which had lost nonsense mutations and had increased in size by replacement of the *nin5* deletion (Fig. 2). Conditions may have favoured the growth of more efficient plaque-forming recombinants between helper and the hybrid phage clone originally present in the library. The introduction of *EcoRI* restriction targets into the vector portion of the hybrid is an inconvenient consequence of this recombination.

The regulation of the expression of the *B. subtilis* fumarase gene from the phages in *E. coli* has not yet been examined, although the observations of complementation based on the increased growth of lysogens in plaques would suggest that it can be expressed independently of λ vegetative promoters, probably from a promoter in the cloned DNA. Whether initiation of transcription occurs at the natural *B. subtilis* promoter for the *citG* gene remains to be established. It has been shown in the case of *B. subtilis leu* and *pyr* fragments (Chi *et al.*, 1978), the *Bacillus licheniformis* β -lactamase gene (Brammar *et al.*, 1980), and SPP1 genes (Amann & Reeve, 1981), all cloned in lambdoid phages and expressed in *E. coli*, that the expression is independent of phage repressor and thus is probably initiated in the cloned material. Promoters recognized by the major vegetative form of *B. subtilis* RNA polymerase appear to share considerable homology with those of *E. coli* (Lee *et al.*, 1980) and it is therefore possible that the natural *B. subtilis* promoter can be recognized with some efficiency by *E. coli* RNA polymerase.

The phages λ BS*citGI* and II carry regions of the *B. subtilis* chromosome, including the *citG* and *gerA* genes, which can now be analysed in detail to permit a closer study of the DNA, its products and their regulation during phases of the growth and differentiation of *B. subtilis*, and it is hoped that such studies will provide, *inter alia*, useful insights on the nature, role and regulation of a spore germination protein.

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