Cloning and Deletion Analysis of a Genomic Segment of *Bacillus subtilis* Coding for the *sdhA*, *B*, *C* (Succinate Dehydrogenase) and *gerE* (Spore Germination) Loci

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From a *Bacillus subtilis* gene bank constructed in *Escherichia coli* and based on a low copy number cloning vector we have isolated a hybrid plasmid, pSH1047, containing an 8.0 kb segment of *B. subtilis* DNA coding for the *sdhA*, *B* and *C* genes, which code for the component polypeptides of succinate dehydrogenase, and the *gerE* gene, which may code for or regulate a protease involved in producing spores which germinate normally. We report the restriction map of this segment and the analysis of deletion derivatives which allow us to correlate the physical and genetic maps of these chromosomal segments.

INTRODUCTION

Bacillus subtilis is a Gram-positive bacterium whose natural habitat is the soil. Biochemical and genetical analyses of *B. subtilis* have been facilitated by its convenient growth characteristics and because certain laboratory strains can be easily made competent for genetic transformation. One of the reasons for interest in *B. subtilis* is that it is capable of undergoing physiological specialization to produce endospores under nutrient limiting conditions. This process of sporulation, which leads to spores of high chemical and physical resistance that can subsequently germinate in a range of favourable conditions, has been studied biochemically and genetically, and the positions on the genetic map of *B. subtilis* of a large range of genetic loci (*spo* and *ger*) involved in these processes have been determined (Henner & Hoch, 1980).

There has been considerable progress recently in cloning these genes (Bonamy & Szulmajster, 1982; Dubnau *et al.*, 1981; Ferrari *et al.*, 1981; Jenkinson & Mandelstam, 1983; Mahler *et al.*, 1984; Moir, 1983). Since selection for a *Spo*⁺ or *Ger*⁺ phenotype is not as easy as selection for prototrophy in an auxotrophic mutant, one approach adopted for cloning such genes has been to select hybrid plasmids or bacteriophages carrying the wild-type allele of a nearby auxotrophic marker and subsequently to screen for the presence or absence of the relevant *spo* or *ger* locus. The *citF* gene cluster, recently redesignated *sdh* for succinate dehydrogenase, and *gerE* are one such closely linked set of loci, both of which are of considerable fundamental interest.

Membrane bound succinate dehydrogenase (SDH) is an important component of all aerobic cells. Its activity is modulated by several activators and inhibitors and SDH is a complex enzyme containing non-haem iron, acid-labile sulphur and covalently bound flavin adenine dinucleotide (FAD) (Hederstedt & Rutberg, 1981). The composition, enzymology and basis of the membrane-binding properties of *B. subtilis* SDH have been extensively studied. However, little is known about the biosynthesis and genetics of this complex although the *sdh* operon of *Escherichia coli* has recently been cloned and sequenced (Wood *et al.*, 1984). In *B. subtilis* the SDH enzyme contains equimolar amounts of three subunits: a *M*_r 65 000 flavoprotein (FP), a *M*_r

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Abbreviations: AEC, S-(2-aminoethyl)cysteine; SDH, succinate dehydrogenase.

28000 iron protein (IP) and a M_r 19000 cytochrome b_{558} (Hederstedt *et al.*, 1979; Hederstedt, 1980; Hederstedt & Rutberg, 1983). These are coded for by the *sdhB*, *C* and *A* loci, respectively (Hederstedt & Rutberg, 1981; Hederstedt *et al.*, 1982), which appear to compose an *sdh* operon (Magnusson *et al.*, 1983) that is probably expressed constitutively. Sdh mutants accumulate organic acids (Carls & Hanson, 1971) and are also defective in sporulation (Fortnagel & Freese, 1968; Rutberg & Hoch, 1970; Freese *et al.*, 1978; Freese, 1981) probably due to inadequate production of ATP (Fortnagel & Freese, 1968) and to the accumulation of inhibitory acidic intermediates (Freese *et al.*, 1974).

The role of the product of the *gerE* locus in sporulation and germination is not fully understood. The spores of *gerE36* mutants are smaller than those of wild-type strains, germinate slowly in a variety of germinants, are sensitive to lysozyme and have an incomplete spore coat (Moir, 1981). *gerE36* mutants lack a protease which seems to be required for processing a number of polypeptides normally found in the spore coat (Jenkinson & Lord, 1983). It is not clear, however, whether the protease is the product of *gerE* or whether its synthesis is regulated in some way by the *gerE* product.

Clearly, the cloning of both *sdh* and *gerE* loci would aid in the investigation of these different systems. In this paper we report the cloning of a genomic segment coding for the *sdhA*, *B* and *C* genes as well as *gerE*. The location of these genes relative to the restriction map is reported as well as a demonstration that the GerE^+ phenotype is dominant over GerE^- .

METHODS

Bacterial strains and plasmids. These are listed in Tables 1 and 2, respectively.

Bacterial growth. *Escherichia coli* strains were grown in L broth or on L agar (Kahn *et al.*, 1979) at 37 °C. Stock plates were stored at room temperature. Medium was supplemented with kanamycin (50 µg ml⁻¹), tetracycline (25 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹) or penicillin (300 µg ml⁻¹ in L agar, 150 µg ml⁻¹ in L broth) to select for appropriate antibiotic-resistance markers (Km^R , Tc^R , Cm^R and Ap^R , respectively).

Bacillus subtilis was grown at 37 °C or 42 °C using Difco Penassay Broth, Potato Glucose Yeast Extract (PGYE) agar (Dring & Gould, 1971), Tryptose-blood Agar Base (Difco) solidified with 0.75% (w/v) Difco agar and TY agar (Biswal *et al.*, 1967) as rich nutrient media. Minimal Agar (Anagnostopoulos & Spizizen, 1961) was supplemented with glucose (0.5%) and amino acids (50 µg ml⁻¹) where required. CitF^+ transformants were selected on minimal agar containing 0.5% (w/v) sodium lactate (Rutberg & Hoch, 1970). Nutrient agar containing bromocresol purple (Carls & Hanson, 1971) was also used for screening for the CitF^+ phenotype. Chloramphenicol (5 µg ml⁻¹) and *S*-(2-aminoethyl)cysteine (AEC, 100 µg ml⁻¹) were used for the selection of resistance genes, Cm^R and *aecA*, respectively. The spore germination phenotype (Ger) was scored by the tetrazolium overlay method (Trowsdale & Smith, 1975; Lafferty & Moir, 1977).

Transformation of bacteria. *E. coli* bacteria were made competent and transformed with plasmid DNA as described by Meyer *et al.* (1977) and modified by Thomas (1981). Transformation of *B. subtilis* was as described by Anagnostopoulos & Spizizen (1961).

Extraction of DNA. For plasmid DNA a slightly modified version of the method of Birnboim & Doly (1979) as described by Smith & Thomas (1983) was used. *B. subtilis* chromosomal DNA was prepared according to Marmur (1961).

Restriction digestion and ligation. Restriction endonucleases and bacteriophage T4 DNA ligase were obtained from Bethesda Research Laboratories, P and S Biochemicals or NBL Enzymes and used under the conditions recommended by the suppliers.

Gel electrophoresis. Agarose gel electrophoresis (0.7–2.0%, w/v) was done as described by Kahn *et al.* (1979) with slight modifications (Thomas, 1983).

Dephosphorylation of vector DNA. Calf intestinal alkaline phosphatase (Boehringer Mannheim) was used to remove the terminal 5' phosphates of digested vector plasmid DNA (Chaconas & van de Sande, 1980).

Size fractionation of chromosomal DNA. Chromosomal DNA of *B. subtilis* which had been partially digested with *Sau3A* was fractionated by velocity centrifugation through a sucrose density gradient to isolate fragments in the range 5–15 kb as described by Maniatis *et al.* (1978).

Characterization of spores. Spores were prepared on PGYE agar as previously described (Lafferty & Moir, 1977). Lysozyme sensitivity was tested as described by Moir (1981) but using 100 µg lysozyme ml⁻¹. Germination was measured as described by Moir (1981) and Sammons *et al.* (1981).

Southern blotting. The *Bam*HI to *Sal*I 6.6 kb fragment from the *B. subtilis* DNA cloned in pSH1047 was isolated by bulk digestion of pSH1047 DNA with *Sal*I and *Bam*HI, agarose gel electrophoresis and electroelution. It was rendered radioactive by nick translation (Rigby *et al.*, 1977) using [α -³²P]dCTP. Plasmid (1 µg) or chromosomal

Table 1. *Bacterial strains*

Strain	Description	Reference or source*
<i>E. coli</i> K12		
MV10	C600 Δ <i>trpE5</i>	D. R. Helinski
MV10Ch3/86	MV10 carrying hybrid λ prophage Ch3/86 with <i>trfA</i> and <i>trfB</i> regions of plasmid RK2	Thomas <i>et al.</i> (1981)
HB101	F ⁻ <i>hsdS20</i> (r _g m _g) <i>recA13</i> <i>ara-14</i> <i>lacY1</i> <i>proA2</i> <i>galK2</i> <i>rpsL20</i> (Sm ^R) <i>xyl-5</i> <i>mtl-1</i> <i>supE44</i> λ ⁻	D. R. Helinski
ED8654	<i>supE</i> <i>supF</i> <i>hsdR</i> ⁻ M ⁺ S ⁺ <i>met</i> <i>trpR</i>	J. R. Guest; Murray <i>et al.</i> (1977)
<i>B. subtilis</i>		
PY79	Prototroph	Youngman <i>et al.</i> (1984)
4600	<i>gerE36</i> <i>trpC2</i> <i>hisA1</i>	Moir (1983)
4700	<i>citF2</i> <i>trpC2</i>	L. Rutberg
4736	<i>ilvB2</i> <i>gerE36</i>	Moir (1981)
4709	<i>citF78</i> <i>leuC2</i>	L. Rutberg
4822	<i>citF83</i> <i>leuC2</i>	L. Rutberg
4985	<i>lys-1</i> <i>aecA</i> <i>trpC2</i> <i>sul</i>	W. Steinberg
4985.1	<i>aecA</i> <i>trpC2</i> <i>sul</i>	Present study†
1604	<i>trpC2</i>	Moir <i>et al.</i> (1979)

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† *B. subtilis* strain 4985 was transformed with chromosomal DNA from PY79, selecting prototrophs for lysine on minimal plates.

Table 2. *Plasmids*

Plasmid	Relevant properties		Size (kb)	Reference or source
pBR322	Ap ^R Tc ^R	pMB1 replicon	4.362	Bolivar <i>et al.</i> (1977)
pCT571	Km ^R Tc ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	9.5	unpublished
pSH1047	Km ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	17.5	present study
pSH1047.1	Km ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	15.95	present study
pSH1047.3	Ap ^R Tc ^R Cm ^R	pMB1 replicon	8.2	present study
pSH1047.4	Km ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	15.85	present study
pSH1047.5	Km ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	16.2	present study
pSH1047.6	Km ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	16.6	present study
PSH1047.7	Km ^R <i>oriV</i> _{RK2}	pSC101 replicon	11.9	present study
pSH1047.10	Km ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	12.5	present study
pSH810	Km ^R Cm ^R <i>oriV</i> _{RK2}	pSC101 replicon	20 (approx.)	unpublished

(10 µg) DNA digested to completion with restriction endonucleases was separated by agarose gel electrophoresis, denatured, transferred to a sheet of Biodyne A (Pall Process Filtration, Portsmouth, UK) as described by Southern (1975) and then hybridized with the radioactive probe by the method recommended by the manufacturers of the Biodyne A nylon membranes.

RESULTS

Isolation of hybrid plasmids carrying the citF region

The vector used in this study is a pSC101-derived plasmid pCT571 (Fig. 1) whose construction will be described elsewhere (S. Hasnain & C. M. Thomas, unpublished). It is 9.5 kb in size, has single restriction endonuclease sites for *Bam*HI, *Eco*RI, *Sst*II, *Xho*I, *Sma*I and *Sal*I and carries genes conferring resistance to the antibiotics kanamycin, tetracycline and chloramphenicol in *E. coli* but only to chloramphenicol in *B. subtilis*. Plasmid pCT571 normally replicates in *E. coli* with a copy number of 6–8 per chromosome. However, it also contains a mutant vegetative replication origin from plasmid RK2, *oriV*_{RK2}, allowing it to replicate at 35–40 copies per chromosome when *trfA* is supplied in *trans* (Thomas *et al.*, 1984). Plasmid pCT571 cannot replicate in *B. subtilis*: it can only be used to transform *B. subtilis* to Cm^R if it gains either a *B.*

Fig. 1. Structure of the vector pCT571 and the physical and genetic map of *B. subtilis* genomic DNA present in pSH1047 and its derivatives. Restriction endonuclease cleavage sites are indicated as: B, E, H, S, Sm, Sp, Ss, X for *Bam*HI, *Eco*RI, *Hind*III, *Sal*I, *Sma*I, *Sph*I, *Sst*II and *Xho*I, respectively. Plasmid pSH1047.1 was made by deletion of the 1.55 kb *Sal*I fragment; pSH1047.3 consists of the approximately 3.8 kb *Eco*RI fragment carrying the Cm^R marker inserted into pBR322; pSH1047.4, pSH1047.5, pSH1047.6 and pSH1047.7 were created by partial digestion of pSH1047 DNA with *Hind*III, ligation, transformation of *E. coli* and examination of the resulting plasmids for deletions of *Hind*III fragments; pSH1047.10 was made by deletion of the *Pst*I fragments within the *B. subtilis* DNA. The scale bar refers to the inserts, not the circular plasmid map.

subtilis replicon or chromosomal DNA allowing it to integrate into the chromosome by homologous recombination.

A *B. subtilis* gene bank was created by ligating dephosphorylated *Bam*HI-cleaved pCT571 to 5–15 kb chromosomal fragments produced by *Sau*3A partial digestion and fractionation on a sucrose gradient. After transformation of *E. coli* with the ligated DNA, Km^R transformants were screened for insertional inactivation of the tetracycline resistance marker and plasmid DNA was extracted. Samples of the plasmid DNA preparations were pooled in batches of approximately 30 and introduced by transformation into *E. coli* strain MV10Ch3/86, where pCT571 derivatives will have a higher copy number, in order to obtain larger quantities of plasmid DNA with less RNA and chromosomal DNA contamination. This DNA was used to screen for the presence of wild-type *citF* DNA by transformation of *B. subtilis* strain 4822 (*citF*83) and selection on lactate plates. Chromosomal DNA of *B. subtilis* PY79 was used as a positive control and as an indicator of competence, while competent cells without DNA were used as a negative control. Out of 30 pools one showed positive results and on screening the individual plasmid DNAs from this pool two identical plasmids, with an approximately 8.0 kb

Fig. 2. Integration of pSH1047 into the chromosome of a *B. subtilis gerE* strain by homologous recombination. Integration could involve monomeric or multimeric single stranded plasmid DNA molecules. If only part (*gerE**) of the wild-type *gerE* gene were present in pSH1047, then for integration to yield a functional *gerE* gene, recombination (dashed cross) must take place in the interval B, not in interval A. If the whole of the wild-type *gerE* gene were present in pSH1047 then integration would always yield one functional *gerE* gene. The different types of DNA are shown as: , *B. subtilis* DNA; , pCT571 vector DNA; , coding regions for relevant *B. subtilis* genes; ●, hypothetical site of mutation in *gerE* gene.

insertion of *B. subtilis* DNA, were identified. These plasmids (pSH1041 and pSH1047) transformed the *citF83* strain (4822) to $CitF^+$, indicating the presence of at least part of the *sdhC* gene in which the *citF83* mutation is located. Plasmid pSH1047 was used to transform *B. subtilis* strain 4709 carrying the *citF78* mutation, which lies in *sdhA* at the other end of the *sdh* cluster of genes. Many $CitF^+$ transformants were obtained, indicating that pSH1047 contains part, at least, of *sdhA*. If no deletion has occurred then pSH1047 should also contain *sdhB* which lies between the *sdhA* and *C* loci.

Plasmid pSH1047 carries the whole *gerE* locus

Previous experiments (Moir, 1981; Hederstedt *et al.*, 1982) have established that the order of loci in this region of the *B. subtilis* chromosome is *sdhA* (*citF78*) – *sdhB* (*citF2*) – *sdhC* (*citF83*) – *gerE* and that *gerE* is 90% linked by transformation to *sdhC*. It therefore seemed possible that *gerE* might be contained within the 8.0 kb chromosomal fragment. In order to determine if it was present, DNA of pSH1047 was used to transform a *gerE* strain (4736) to Cm^R and 100 transformants were screened for Ger^+ by the tetrazolium overlay method. The results showed that 99% of the transformants were Ger^+ as indicated by the red colour of the colonies.

Integration of the Cm^R marker must occur by homologous recombination between the *B. subtilis* DNA in pSH1047 and the resident genome, either via a simple Campbell-type integration or by a double cross-over with a single strand derived from a dimeric plasmid molecule (Fig. 2). In both cases the result is a duplication of the genomic segment present in

pSH1047. A cross-over can occur at a range of sites so that if only part of the *gerE* gene was present in pSH1047 then only a proportion of the Cm^R transformants would be expected to restore *gerE*⁺, while if the whole of *gerE*⁺ is present then all Cm^R transformants should have acquired an extra wild-type copy of *gerE*. Since we found that virtually all Cm^R transformants were Ger⁺ it was concluded that the whole of *gerE* is likely to be present in pSH1047.

In order to confirm that the colonies identified by the tetrazolium overlay method represent transformants that have been fully converted to Ger⁺, Cm^R transformants obtained with pSH1047 were used to prepare spores. These were found to be fully resistant to lysozyme, of wild-type size rather than small as found for *gerE* spores, and able to germinate normally in 10 mM-alanine (data not shown). Cm^R transformants obtained with a different hybrid plasmid, pSH810, which was picked at random, were found to retain *gerE* characteristics, indicating that mere integration of pCT571 into the *B. subtilis* genome does not alter sporulation and spore characteristics. Since the results show that the Cm^R transformants of the *gerE* strain, which should have become partial diploids for the *gerE* locus and therefore contain both mutant and wild-type *gerE* loci, are fully Ger⁺ phenotypically, it is clear that the GerE⁺ phenotype is fully dominant over GerE⁻.

Physical characterization of B. subtilis DNA in pSH1047

In order to generate a restriction map of the *B. subtilis* DNA in pSH1047 the following endonucleases were used separately and in combination: *EcoRI*, *SalI*, *XhoI*, *HindIII*, *BamHI*, *SstII*, *PstI*, *BglII*. There were three *PstI*, two *EcoRI*, one *SstII*, four *HindIII* and one *SalI* sites in the approximately 8.0 kb *B. subtilis* segment. Interestingly, the *Sau3A* site at one end of the insert restored the *BamHI* site near the *EcoRI* and *HindIII* sites of the vector.

To check that the cloned segment in pSH1047 was really *B. subtilis* DNA and to determine whether any significant deletions had occurred in the cloning process, Southern blotting was used to examine chromosomal DNA of *B. subtilis* PY79, from which the fragments came originally, using the *BamHI* to *SalI* fragment of pSH1047 as a radioactively labelled probe. Chromosomal DNA digested with *EcoRI*, *HindIII*, *PstI* and *EcoRI* plus *SalI* together was examined in this way, comparing the radioactive bands produced with those produced by the same digestions of pSH1047 DNA (data not shown). First, the results confirmed that the cloned DNA is homologous to PY79 DNA, ruling out the possibility that it is a totally spurious segment of DNA. Second, while we were unsuccessful in confirming the presence in the chromosomal digests of hybridizing bands corresponding to the small *EcoRI* and *HindIII* bands of pSH1047, hybridization did occur to both a *PstI* band and an *EcoRI* to *SalI* band of exactly the same sizes as the ones covering the major area coding for *sdhA*, *B* and *C* and *gerE* in pSH1047 (Fig. 1). We therefore concluded that no significant deletion has occurred in this cloned segment and that it probably represents a natural segment of the *B. subtilis* genome.

Deletion analysis of genetic loci

We used deletion derivatives of pSH1047 to locate on the *B. subtilis* genomic segment the regions coding for the subunits of the SDH complex, as defined by previously isolated mutants, and *gerE*. A series of seven deletion derivatives, as shown in Fig. 1, were utilized. Plasmids pSH1047.1, pSH1047.4, pSH1047.5, pSH1047.6 and pSH1047.10 retain the Km^R and Cm^R markers of pSH1047, whereas pSH1047.7 has lost the Cm^R. All these plasmids represent the results of digestion, partial or complete, with restriction endonucleases *SalI*, *HindIII* or *PstI*, ligation and transformation of *E. coli* MV10 selecting the appropriate antibiotic resistance markers. Plasmid pSH1047.3 was made by inserting the *EcoRI* fragment containing the Cm^R marker of pSH1047 into pBR322 at the *EcoRI* site and consequently it confers Ap^R, Tc^R and Cm^R. In order to determine the positions of the three *sdh* loci on the inserted fragment, competent *citF78* (*sdhA*), *citF2* (*sdhB*) and *citF83* (*sdhC*) mutant strains were transformed with DNA of pSH1047 and its derivatives. Mutants defective in tricarboxylic acid cycle enzymes accumulate organic acids and on medium containing the pH indicator bromocresol purple they form yellow colonies while wild-type colonies are blue (Carls & Hanson, 1971). We selected

Table 3. Results of testing pSH1047 and its derivatives for wild-type *sdh* genes

B. subtilis strains 4709, 4700 and 4822, carrying *citF* mutations, were transformed with plasmid DNA. Selection of CitF⁺ transformants on lactate plates was carried out at least twice, yielding qualitatively similar results. One set of results are shown; they represent the number of transformants per µg plasmid DNA. For screening the CitF phenotype of Cm^R transformants on bromocresol purple plates the cumulated results from several experiments are shown, the total number of Cm^R transformants being low.

Plasmid	<i>sdh</i> locus (<i>citF</i> mutation in strain used for test):				
	<i>sdhA</i> (<i>citF78</i>)		<i>sdhB</i> (<i>citF2</i>)	<i>sdhC</i> (<i>citF83</i>)	
	No. of CitF ⁺ transformants on lactate plates	No. of CitF ⁺ /no. of Cm ^R tested	No. of CitF ⁺ /no. of Cm ^R tested	No. of CitF ⁺ transformants on lactate plates	No. of CitF ⁺ /no. of Cm ^R tested
pSH1047	650	0/0	23/23	400	11/12
pSH1047.1	600	45/46	9/9	200	1/12
pSH1047.3	100	13/13	1/63	0	0/13
pSH1047.4	80	2/2	4/4	0	0/0
pSH1047.5	22	3/3	26/26	0	0/0
pSH1047.6	100	0/0	26/26	0	0/0
pSH1047.7	0	ND	ND	0	ND
pSH1047.10	0	0/1	0/1	0	0/0

ND, Not determined due to lack of the Cm^R marker in pSH1047.7.

transformants of *citF* strains both on such plates supplemented with chloramphenicol and on lactate plates (Table 3).

With the *citF78* mutant strain it was found that all pSH1047 derivatives which gave CitF⁺ transformants on lactate plates contained the *Bam*HI to *Eco*RI fragment adjacent to the Cm^R marker. Of the two plasmids which did not yield such CitF⁺ transformants, pSH1047.10 contains the *Bam*HI to *Pst*I subsegment of this fragment (Fig. 1), suggesting that the wild-type allele of *citF78* lies in the remaining *Pst*I to *Eco*RI fragment. The results of selecting Cm^R transformants on bromocresol purple plates, which allowed CitF⁺ to CitF⁻ to be scored, were not thoroughly convincing due to the generally low numbers of transformants. However, as argued above for screening for the *gerE* gene, since all Cm^R transformants with plasmid pSH1047.3 were CitF⁺ it appears likely that the *sdhA* gene lies entirely to the left (in Fig. 1) of the *Eco*RI site and may or may not extend left of the *Pst*I site. The single yellow (CitF⁻) Cm^R transformant obtained with pSH1047.10 confirms the location of at least part of *sdhA* right of the *Pst*I site. The disappointingly low number of Cm^R transformants obtained is a reflection of the poor competence of *citF* strains. However, these results are not the sole basis of our conclusions and are reported simply for completeness.

With the *citF83* mutant strain the ability of pSH1047 to give CitF⁺ transformants was abolished by deletion of the 0.9 kb *Hind*III fragment (pSH1047.6). Thus, the only derivative that gave CitF⁺ transformants was pSH1047.1, which retained this DNA fragment; the other plasmids lacked this fragment and none of them gave CitF⁺ transformants. Again, the low number of Cm^R transformants on bromocresol purple plates meant that this test was inconclusive. However, the results using lactate plates do show clearly that the *sdhC* gene must lie at least partly within the 0.9 kb *Hind*III fragment.

With the *citF2* mutant strain we were unsuccessful in getting transformation to CitF⁺ on lactate plates. However, the number of Cm^R transformants was significant for most of the plasmids and the results show that all Cm^R transformants obtained with plasmids carrying DNA left of the leftmost *Hind*III site (in Fig. 1) are CitF⁺ while with pSH1047.3, carrying DNA left of the leftmost *Eco*RI site, only one out of 63 Cm^R transformants was CitF⁺. This suggests that while pSH1047.3 may have the wild-type sequence which is mutant in *citF2* the whole of the cistron cannot be present. The *sdhB* gene may therefore flank the leftmost *Eco*RI site.

This order of *sdh* loci is consistent with the results of classical genetic mapping. It appears that they are encoded mainly, if not entirely, by the approximately 3.0 kb segment, from the leftmost *Pst*I site to the rightmost *Hind*III site.

Table 4. *Results of testing pSH1047 and its derivatives for wild-type gerE and aecA genes*

B. subtilis strains with *gerE* and *aecA* mutations were transformed to Cm^R with plasmid DNA and then tested for Ger⁺ by the tetrazolium overlay method or for AEC sensitivity (Aec⁺) as described in Methods.

Plasmid	<i>gerE</i>	<i>aecA</i>
	No. of Ger ⁺ / no. of Cm ^R tested	No. of Aec ⁺ / no. of Cm ^R tested
pSH1047	198/200	0/24
pSH1047.1	50/50	0/24
pSH1047.3	0/50	0/24
pSH1047.4	50/50	0/10
pSH1047.5	49/50	0/1
pSH1047.6	50/50	0/5
pSH1047.7	36/250*	ND
pSH1047.10	0/50	0/12

* Ger⁺/Trp⁺ determined by congression experiment (see text).

ND, Not determined due to lack of the Cm^R marker in pSH1047.7.

These pSH1047 derivatives were also tested for retention of *gerE* (Table 4). For all plasmids except pSH1047.7 Cm^R transformants were selected and tested for the Ger⁺ phenotype. Plasmid pSH1047.7 was tested by a congression experiment as follows. *B. subtilis* strain 4600 (*gerE36 trpC2 hisA1*) was transformed with *gerE36 trp⁺* DNA in the presence of pSH1047.7 DNA and transformants for the unlinked marker *trpC2* were selected. All Trp⁺ transformants will have a high probability of having been transformed with pSH1047.7 DNA due to its being present in close to saturating amounts. Since pSH1047.7 is the only potential source of *gerE⁺* DNA then only if it is *gerE⁺* should we find a significant proportion of Trp⁺ Ger⁺ double transformants. The results (Table 4) show that the only segment common to all plasmids which gave Ger⁺ transformants is the *Hind*III to *Sal*I fragment of 2.25 kb (Fig. 1). Since all Cm^R transformants obtained with pSH1047.1 and pSH1047.4 are Ger⁺ it appears that both these plasmids must contain a complete gene. Further deletion analysis and subcloning will be necessary to establish the exact location of the *gerE* locus.

The *aecA* gene, mutations in which give resistance to the lysine analogue AEC maps near to the *sdh* gene cluster but on the side distal to *gerE* (Magnusson *et al.*, 1983). *B. subtilis* strain 4985.1 (*trpC2 aecA sul*) was transformed with DNA of pSH1047 as well as DNA of all the Cm^R deletion derivatives. Cm^R transformants were obtained and then tested for their Aec phenotype. All the transformants remained resistant to AEC (Table 4), indicating either that pSH1047 does not carry the whole of *aecA* gene or that AEC resistance is dominant to AEC sensitivity.

DISCUSSION

High copy number vectors can be useful in producing large amounts of both the DNA and the products of cloned genes. However, they may accentuate problems of genetic instability if the gene being sought is itself, or is closely linked to segments which are, deleterious to the bacterial host being used, or which promote recombination. Since attempts to clone some genes of *B. subtilis* have been unsuccessful we have created a novel gene bank based on a low copy number vector, pCT571, which utilizes the pSC101 replicon (6–8 copies per chromosome equivalent) in *E. coli*. This vector cannot replicate in *B. subtilis* but does carry a Cm^R marker which is expressed in *B. subtilis*. Transformation of *B. subtilis* strains to Cm^R is dependent on insertion of *B. subtilis* chromosomal DNA into the plasmid thus allowing homologous recombination to occur. The presence of specific genetic markers can be tested either by directly transforming for an easily selected marker or by first selecting Cm^R transformants and then screening for the acquisition of a chromosomal marker. Either method depends on efficient transformation of competent bacteria – particularly if pooled hybrid plasmids are being screened. Plasmid pCT571 carries a

mutant vegetative replication origin of plasmid RK2 so that on introduction into an *E. coli* strain that provides the *trfA* gene, which is essential for replication from *oriV*_{RK2}, the copy number of pCT571 rises to 35–40 per chromosome equivalent. Over short periods at least there seem to be no problems either with host growth or with plasmid structural stability in increasing the copy number of the hybrid plasmids described in this paper. We found that the higher copy number obtained in this way allowed us to isolate, by standard rapid techniques, DNA which gave better transformation frequencies in *B. subtilis* than those obtained for DNA isolated from the low copy number strains. The reason for this may be the more favourable ratio of plasmid DNA to contaminating chromosomal DNA and RNA which may competitively inhibit transformation. However, since the stimulation of transformation to Cm^R, which depends on the Cm^R being either in a circular plasmid or flanked by direct repeats of chromosomal DNA, is greater than that for directly selectable chromosomal markers, whose integration depends only on linear homology with chromosomal DNA, it seems likely that increased copy number is having a major part of its effect by increasing the amount of dimeric plasmid molecules present since these are likely to be important in integration of Cm^R into the chromosome (Canosi *et al.*, 1978) (see Fig. 2). This explanation is consistent with the observation that DNA of pSH1041 and pSH1047, when isolated from a strain in which only the pSC101 replicon is functional, gives no visible dimeric form while if isolated from MV10Ch3/86, where the higher copy number mutant RK2 replicon is activated, it gives clearly visible quantities of dimeric and trimeric forms (S. Hasnain, unpublished observation). The rather poor transformation frequencies for Cm^R obtained in this present study probably reflect a combination of this extra constraint coupled with the difficulties of making *citF* mutant strains competent for transformation. Further analysis of the gene bank used here, including the examination of the effect of copy number on recovery and stability of different chromosomal loci, will be reported elsewhere (S. Hasnain & C. M. Thomas, unpublished).

The genomic segment in pSH1047 appears to be a true representation of the chromosomal genes rather than being a deleted or rearranged version. The arrangement of *sdh* loci and *gerE* are consistent with previously reported genetic maps and the relative distances, as far as we can judge, are in agreement with recombination frequencies between loci reported previously (Magnusson *et al.*, 1983; Moir, 1981). The results described here also suggest that at least part of the *aecA* locus is absent from pSH1047. Interestingly, in screening for this marker we observed that we needed 100 µg AEC ml⁻¹ to inhibit growth of our wild-type in contrast to 50 µg ml⁻¹ reported previously (Mattioli *et al.*, 1979) and that in the presence of 50 µg lysine ml⁻¹ the wild-type is resistant to even 400 µg AEC ml⁻¹. Thus the exact relationship between lysine and its analogue may be strain dependent.

Possession of this clone provides the opportunity to investigate the *sdh* and *gerE* loci in more detail, both at the level of the primary sequence of genes and with regard to the nature of their gene expression signals. It will be interesting to analyse the operon structure of the *sdh* genes and to determine whether *gerE* falls into the category of genes whose expression is switched on during sporulation by the alterations in RNA polymerase specificity due to sigma factor changes. Such work is currently being pursued.

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