A vector for systematic gene inactivation in *Bacillus subtilis*

Valérie Vagner, Etienne Dervyn and S. Dusko Ehrlich

Author for correspondence: Valérie Vagner. Tel: +33 1 34 65 25 34. Fax: +33 1 34 65 25 21.
e-mail: vagner@biotec.jouy.inra.fr

Génétique Microbienne,
Institut National de la
Recherche Agronomique,
Domaine de Vilvert, 78352
Jouy-en-Josas cedex,
France

To study the functions of the uncharacterized open reading frames identified in the *Bacillus subtilis* genome, several vectors were constructed to perform insertional mutagenesis in the chromosome. All the pMUTIN plasmids carry a *lacZ* reporter gene and an inducible Pspac promoter, which is tightly regulated and can be induced about 1000-fold. The integration of a pMUTIN vector into the target gene has three consequences: (1) the target gene is inactivated; (2) *lacZ* becomes transcriptionally fused to the gene, allowing its expression pattern to be monitored; (3) the Pspac promoter controls the transcription of downstream genes in an IPTG-dependent fashion. This last feature is important because *B. subtilis* genes are often organized in operons. The potential polar effects generated by the integration of the vectors can be alleviated by addition of IPTG. Also, conditional mutants of essential genes can be obtained by integrating pMUTIN vectors upstream of the target gene. The vectors are currently being used for systematic inactivation of genes without known function within the *B. subtilis* European consortium. pMUTIN characteristics and the inactivation of eight genes in the *resA-serA* region of the chromosome are presented.

Keywords: integrational vector, *Bacillus subtilis*, inducible promoter, essential genes

INTRODUCTION

The last 2 years have been marked by the release of complete nucleotide sequences of several unicellular organisms. In addition to the seven bacterial genomes already available, the sequence of the *Bacillus subtilis* chromosome is now complete (Kunst *et al.*, 1997). Among the 4000 URFs (unknown reading frames; Doolittle, 1998) identified in *B. subtilis*, 26% show no significant homology to sequences in the databases, 12% show homology to unknown genes in other organisms and only about 1000 have been characterized experimentally (Kunst *et al.*, 1997). Systematic transcriptional analysis carried out on a region of the chromosome indicates that most of the newly identified genes can be expressed (Azevedo *et al.*, 1993).

The function of newly discovered genes can be studied by destruction techniques followed by the monitoring of the effect on cell fitness under different growth conditions. In some cases, sequencing and gene-disruption techniques have been combined (e.g. *Drosophila melanogaster*; Spradling *et al.*, 1995). In *Rhodobacter*

capsulatus, a defective transducing phage which packages DNA fragments has been used to replace target genes with an antibiotic-resistance marker (Kumar *et al.*, 1996). In *Saccharomyces cerevisiae*, gene disruptions have been performed *en masse* by random insertional mutations using Ty1 (Smith *et al.*, 1995). In *Escherichia coli*, a gene-replacement strategy has been used with a vector containing a temperature-sensitive origin of replication. An *in vitro*-altered sequence is introduced in the genome by recombination with the vector which is then excised at the permissive temperature. The resulting structure is an in-frame deletion without polar effects on downstream genes (Link *et al.*, 1997).

Recently, a number of laboratories have pooled their expertise to analyse the thousands of newly discovered genes, in the context of two European function analysis projects. In the case of *S. cerevisiae*, 1000 unknown genes are being analysed by a European consortium (EUROFAN), involving 144 laboratories (Oliver, 1996). A gene-by-gene replacement strategy has been chosen, which involves the insertion of a kanamycin-resistance gene flanked by PCR fragments (Oliver, 1996; Shoemaker *et al.*, 1996).

The GenBank accession number for pMUTIN2 is AF072806.

A similar project has been started in *B. subtilis*, within a network of 18 European laboratories that share a common approach to inactivate about 1200 new genes. A parallel network of 12 Japanese laboratories has adopted the same approach (N. Ogasawara, personal communication). A gene-by-gene inactivation procedure has been chosen, using the set of vectors described here. These vectors combine various elements which have been previously developed in integrational plasmids (Perego, 1993). Their main properties are: (i) an inability to replicate in *B. subtilis*, which allows insertional mutagenesis; (ii) a reporter *lacZ* gene, to facilitate the measurement of the expression of the target gene; (iii) an inducible promoter to allow controlled expression of genes downstream of, and found in the same operon as, the target gene. This last requirement has been motivated by the observation that most of the genes in the *B. subtilis* genome are organized in multicistronic units. Any insertion in an operon separates downstream genes from their promoter, which may lead to failure to express these genes. Such polar effects are not desirable when studying a unique gene function. In addition, our strategy makes it possible to create conditional mutants, where the target gene expression is regulated by the inducible promoter. The inducible Pspac promoter, developed by Yansura & Henner (1984), has been chosen for this purpose and has been modified to allow better control of the promoter activity. The use of the vectors is illustrated by the inactivation of eight genes of unknown function in the *resA-lysA* region of the *B. subtilis* chromosome.

METHODS

Strains and growth conditions. The *B. subtilis* 168 (*trpC2*) strain was provided by C. Anagnostopoulos (INRA, Jouy-en-Josas, France). The HVS320 strain is a 168 derivative (*trpC2 tyrA1 aroB2 hisH2 Cm^R*) carrying pBR322 sequences inserted in the chromosome close to the *metB* locus (Vagner & Ehrlich, 1988). The HVS597 strain is a *B. subtilis* 168 strain containing the *dnaC* gene under the control of the Pspac promoter by insertion of pMUTIN2 in front of the gene, as described below. Primers used for the amplification of the target sequence are described in Table 1.

Plasmids were prepared in *E. coli* MC1061. Luria-Bertani (LB) medium was used for cultures of *B. subtilis* and *E. coli* (Miller, 1972). Minimal medium for *B. subtilis* and transformation methods have been described previously (Anagnostopoulos & Spizizen, 1961). Antibiotics were used at the following concentrations: 0.3 µg erythromycin ml⁻¹ for *B. subtilis* and 100 µg ampicillin ml⁻¹ for *E. coli*.

DNA extraction and manipulation. Restriction enzymes were used as recommended by the supplier. Polymerase chain reactions (PCR) were carried out with *Taq* polymerase (Boehringer), according to the conditions described by the manufacturer using the following programme: 5 min at 94 °C then 30 cycles of 30 s at 94 °C, 30 s at 50 °C and then 1 min at 72 °C.

Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (Birnboim & Doly, 1979). Chromosomal DNA was extracted from *B. subtilis* as described by te Riele *et al.* (1986). Structures formed upon integration were verified by Southern blot hybridization on Hybond-N⁺ filters (Amersham), after

EcoRV chromosomal digestion. DNA probes were labelled and hybridized with the enhanced chemiluminescence direct nucleic acid labelling and detection system (Amersham).

Construction of the vectors. A *XbaI*-*BamHI* fragment containing the Em^R gene from pVE6023 (E. Maguin, personal communication) and the *EcoRI* fragment from pDG148 (Stragier *et al.*, 1988) containing the β -lactamase gene, the replication origin from ColE1, the *E. coli lacI* gene, and the Pspac promoter, were filled by the Klenow enzyme and ligated together. The *lacZ* gene flanked by a *spoVG* ribosome-binding site (RBS) was isolated from pJM783 (Perego, 1993) after a *BamHI*/*DraI* digestion. This fragment, treated by the Klenow enzyme, was cloned in the blunted *SphI* site of the previous plasmid in the same orientation as *lacI* gene. The λ terminator *t*₀ was isolated after a PCR amplification with the pSS9 vector (Scholtissek & Grosse, 1987) using primers 5' GCTCTAGACCCGGGGATCTCTGCAG 3' and 5' GCTCTAGAGCAACGTTCTTGCCATT 3' carrying a *XbaI* restriction site (shown in italics). The PCR product was cloned in the *XbaI* site of the plasmid. In the resulting pMUTIN1 vector, the λ terminator (*t*₀) stops transcription from the Em^R gene.

The pMUTIN2 vector was generated by the cloning of *t*₁*t*₂ terminators from the *rrnB* operon of *E. coli*, known to be active in *B. subtilis* (Peschke *et al.*, 1985), upstream of *λ*₀. They were isolated after a PCR amplification using primers 5' GGAAGATCTTCATCACCGAACGCGGCAGGC 3' and 5' GGAAGATCTGGTAATGACTCTCTAGCTTGAGC 3' with p602/22T2 as a matrix (P. Noirot, personal communication). After a *BglII* digestion, the amplified fragment was cloned in the *NruI* site of pMUTIN1.

A perfect palindromic sequence (5' AATTGTGAGCGCT-CACAATT 3') was put in place of the O1 *lac* operator in pMUTIN1, resulting in pMUTIN3. The palindromic operator denoted 'oid' was isolated after a mutagenized PCR reaction using primers 5' GCCCGATATCCTAACAGCACA 3' and 5' GCCGAAGCTTAATTGTGAGCGGCTCACAATTCCACACA 3'. After digestion with *HindIII* and *EcoRV* (sites in italics), the PCR product was cloned within the *EcoRV*-*HindIII* large fragment of pMUTIN1. The same experiment was carried out in pMUTIN2, resulting in pMUTIN4, which carries both the palindromic operator 'oid' and the *t*₁*t*₂ terminators.

A multicloning site *HindIII*-*EcoRI*-*NotI*-*SacII*-*BamHI* was introduced between the Pspac and the *lacZ* gene of pMUTIN2 and pMUTIN4. To do this, the oligonucleotides 5' CGCGGATCCGGCGCGCCGCCCGGGAATTCAAGCTTCGGC 3' plus the complementary strand were annealed, then digested with *HindIII* and *BamHI* (sites in italics). The resulting fragments were cloned in the pMUTIN2 and pMUTIN4 vectors after digestion with the same enzymes.

Construction of mutants. A fragment of the target genes was amplified from 168 chromosomal DNA by PCR with primers (Table 1), then extracted from polyacrylamide gels after electrophoresis. Products were digested with *HindIII* and *BamHI* in parallel with the pMUTIN1 (for insertion) or pMUTIN2 (for Pspac fusion). After heat inactivation of the enzymes, fragments and plasmid were mixed for ligation (ratio 10:1 molecules). The ligation mixtures were used to transform competent cells of *E. coli*. Recombinant plasmids were extracted and then used to transform competent cells of *B. subtilis*. Transformants were selected on LB plates containing 0.3 µg erythromycin ml⁻¹ and analysed by Southern blot hybridization to ensure that a single copy of the plasmid had integrated into the chromosome.

Enzymic assays. β -Galactosidase was assayed as previously

Table 1. Primers for construction of mutants

The primers contain around 17 bp homology with the chromosome (underline), 6 bases of a restriction site (italics) and 2 or 3 additional bases for restriction improvement (bold).

Gene	Primer (5'→3')
Interruption	
<i>ypuA</i>	GCCAAGCTTGTAACTGGGTGCTG CGGGATCCGGTTTTTCCGAGGCCT
<i>ypuC</i>	GCCAAGCTTAGAGGTGTTCTGTTAGCC CGGGATCCGAACAAAAAGATAAGCAC
<i>ypuF</i>	GCCAAGCTTAACGCTATTGGGTGGGCTTTA CGGGATCCTGCATGCTTCCTCCAGTTT
<i>ypuG</i>	GCCAAGCTTGAGCTCGACATTG CGGGATCCTTCTTCTTCCCGTTC
<i>ypuH</i>	GCCAAGCTTGAAATCGAAGAGC CGGGATCCAGATGCTTGAGAAGGC
<i>ypuI</i>	GCCAAGCTTGGGACATTGATTGTG CGGGATCCGAAAAGGCTCCTGCATCA
<i>ypuL</i>	GCCAAGCTTGCTCATGCCGGAGTTG CGGGATCCGTCACCACTTTGCG
Fusion	
<i>ypuD</i>	GCCAAGCTTGGAGGTTTAGAAATGGG CGGGATCCGAAGATGATTTTCGTTCCG
<i>ypuG,H</i>	GCCAAGCTTGAAAATGGTGAGATGATGG CGGGATCCTTCTTCTTCCCGTTC
<i>dnaC</i>	GCCAAGCTTGGAGATTTTTTAATGAGAG CGGGATCCGCGATATCAGTCAAATA

described (Msadek *et al.*, 1990). β -Galactosidase activity was expressed in Miller units (mg protein)⁻¹ (Miller, 1972). Protein concentrations were determined using the Bio-Rad Protein Assay kit.

RESULTS AND DISCUSSION

pMUTIN: an integrational vector in *B. subtilis*

The prototype vector of the pMUTINs set (Fig. 1) has the following properties. (i) It cannot replicate in *B. subtilis* but carries ColE1 replication sequences and a β -lactamase gene (Ap^R) for amplification in *E. coli*. An erythromycin-resistance gene (Em^R) provides a selectable marker in *B. subtilis*. (ii) It carries the modified *E. coli lacZ* reporter gene allowing transcriptional fusions and measurement of gene expression in *B. subtilis* (Perkins & Youngman, 1986). For this purpose, the RBS of the *spoVG* gene of *B. subtilis* and stop codons in the three possible frames are present upstream of the *lacZ* gene. (iii) It carries the Pspac inducible promoter, which contains the RNA polymerase recognition sequences of the SPO1 phage and one of the three *lac* operators designated 'O1' (Yansura & Henner, 1984). (iv) It carries the *lacI* gene which has been modified to be constitutively expressed in *B. subtilis*. Pspac is repressed by LacI and therefore can be induced by IPTG (Yansura & Henner, 1984). (v) A λ terminator of transcription, t_0 , is present at the end of the Em^R gene.

Genes can be inactivated by inserting the pMUTIN within a gene via a single crossing-over event (Fig. 2). An internal fragment of the target gene is amplified by PCR, cloned in pMUTIN, and the resulting plasmid used to transform *B. subtilis*. Upon integration, the target gene is interrupted and a transcriptional fusion is generated between its promoter and the reporter *lacZ* gene. In multicistronic organization, any gene(s) situated downstream of the target gene are placed under the control of the inducible Pspac promoter. The λt_0 terminator should prevent RNA polymerase not loaded at the Pspac to transcribe downstream genes. It should be noted that the procedure presented in Fig. 2 generates simultaneously two types of mutants: a first one null (*orf2*) via gene interruption, and a second conditional (*orf3*), via IPTG dependence. The second type can also be obtained by using for integration a plasmid carrying homology with the RBS and the 5' end of the target URF.

Activity of the Pspac promoter in the chromosome

The Pspac promoter has previously been extensively exploited for gene induction, either on multi-copy replicative plasmids (Barilla *et al.*, 1994; Chang *et al.*, 1996), or in the chromosome (Lu *et al.*, 1995; Voelker *et al.*, 1995). However, no reports exist concerning the activity of this promoter in a single copy on the chromosome, with or without LacI repression. We

Fig. 1. Map of the pMUTIN vector set. Common features of the pMUTIN vectors are presented in the figure. White boxes are for genes expressed in *B. subtilis* (*Em^R*, *lacI*, *lacZ*) and the black box is for the *Ap^R* gene expressed in *E. coli*. The black arrow represents sequences used for replication in *E. coli* (*ColE1 ori*). The broken arrow indicates the direction of transcription of the Pspac promoter. The 'Ter' element (lollipop) indicates the terminators: λ t₀ is present in all vectors; t₁t₂ are present in pMUTIN2 and pMUTIN4. 'Op' indicates the Lac operator between the Pspac promoter sequences: the 'O1' operator is present in pMUTIN1 and pMUTIN2, and the 'oid' operator is present in pMUTIN3 and pMUTIN4. Differences between the pMUTINs are set out in the table below the plasmid map.

examined this by transforming the pMUTIN1 vector into a *B. subtilis* strain (HVS320) containing pBR322 sequences integrated in the chromosome. The integration results in a fusion between the Pspac promoter and the reporter *lacZ* gene (Fig. 3a). The Pspac induction was measured at different stages of growth in the presence of 1 mM IPTG (Fig. 3b). The maximum of β -galactosidase activity during exponential growth was reached in 20 min. Pspac induction was 10-fold less efficient during the stationary phase. The Pspac activity was measured in different locations in the chromosome, showing no significant differences, which leads to the conclusion that there is no influence of the chromosomal environment on the promoter activity (data not shown). We also measured the induction of Pspac during exponential phase as a function of the IPTG concentration (Fig. 3c). A 200-fold induction was obtained with pMUTIN1, with 300 μ M IPTG in the culture. Higher concentrations of IPTG did not significantly increase the level of induction.

The β -galactosidase activity measured for pMUTIN1 without IPTG was about 10-fold higher than that measured in the strain without pMUTIN1. This residual expression can be accounted for to some extent by readthrough from external promoters located upstream of the Pspac, and/or by a low affinity of the LacI protein for the Pspac operator. Further investigations were carried out to test these two points and to obtain a better repression of the Pspac promoter.

Effects of terminators and improved operators on the promoter

The anchoring of the Lac repressor on one of its operators (O1) is directly correlated to the affinity of the protein for its operator (Straney & Crothers, 1987). It has been shown in *E. coli* that maximal repression of the *lac* promoter is due to the cooperative effect of three operators (O1, O2 and O3; Oehler *et al.*, 1994). The affinity of LacI for O1 alone is threefold lower than for all three operators, and the replacement of O1 by a perfect palindromic sequence called the 'oid' operator (Lehming *et al.*, 1987) increases repression of the *lac* promoter more than 2.5-fold. To improve the Pspac repression by LacI binding, we substituted the O1 operator present in pMUTIN1 in the vicinity of Pspac by the 'oid' operator, resulting in the pMUTIN3 vector. The putative effect of upstream transcription forks on Pspac activity was tested by adding two strong terminators (t₁t₂ from *rrnB*) close to λ t₀, resulting in the pMUTIN2 vector. The vector containing both the 'oid' sequence and the three terminators is designated pMUTIN4.

β -Galactosidase activity was measured after integration of these different vectors in the chromosome by recombination with pBR322 sequences as shown in Table 2. Without IPTG, β -galactosidase activity was reduced almost to the background level when the two terminators were present (pMUTIN2 and 4), whereas it was three- to fourfold higher when the operator alone was modified (pMUTIN3). This suggests that upstream transcription at readthrough may be responsible for the β -galactosidase activity observed for pMUTIN1 and 3. However, the twofold effect of 'oid' in pMUTIN3 on Pspac repression suggests that a better anchoring of the repressor is obtained with the 'oid' operator, which also influences Pspac activity. The threefold reduction of induction by IPTG in pMUTIN4 speaks for an additive effect of the operator and the terminators in this type of structure.

Use of pMUTIN for gene inactivation and controlled expression

A region situated between *lysA* and *resA* was chosen as an example for gene inactivation (Fig. 4). The region was previously sequenced (Sorokin *et al.*, 1993) and analysed by Northern blotting (Azevedo *et al.*, 1993). Twelve putative genes in this region have unknown functions and seven were chosen as candidates for disruption, because the other genes (namely *ypuE*, *ypuB*,

Fig. 2. Integration of pMUTIN into a target gene. Genes of the *orf1-orf3* operon are indicated as white boxes. Hatched box corresponds to the internal segment of the target gene. The vector is integrated in *orf2* by a single crossing-over event. Arrows indicate direction of transcription, broken arrows denote the promoter of the operon and *Pspac*, and the lollipop strand is for the transcription termination.

Fig. 3. (a) Structure obtained upon integration of pMUTIN into pBR322 sequences in the *B. subtilis* chromosome. The recipient strain (HVS320) contains pBR322 sequences, which are used for homologous recombination with the pMUTIN. This results in duplication of pBR322 sequences (heavy line), and a transcriptional fusion between *Pspac* and *lacZ*. (b) Induction of *Pspac* at different stages of cell growth. IPTG (1 mM) was added to cultures in rich medium at OD_{600} 0.35 (■), 0.75 (▲) or 1.3 (●). The β -galactosidase activity was measured on cells harvested at different times after addition of IPTG. (c) *Pspac* activity as a function of IPTG concentration. Cells were grown in rich medium in the presence of IPTG, and harvested at OD_{600} 0.5 before the measurement of β -galactosidase specific activity.

Table 2. β -Galactosidase activities of pMUTIN integrants of *B. subtilis*

Strain	oid	t ₁ t ₂	β -Galactosidase activity (Miller units)*	
			– IPTG	+ IPTG
HVS320†			1.3 ($\pm$ 0.7)	1.2 ($\pm$ 0.6)
+ pMUTIN 1	–	–	9 ($\pm$ 2)	1266 ($\pm$ 80)
+ pMUTIN 2	–	+	2.2 ($\pm$ 0.3)	926 ($\pm$ 126)
+ pMUTIN 3	+	–	4.5 ($\pm$ 0.35)	901 ($\pm$ 36.5)
+ pMUTIN 4	+	+	1.2 ($\pm$ 1.1)	433 ($\pm$ 17)

* β -Galactosidase activity was measured in at least two experiments; the results are shown as means $\pm$ SD.

† HVS320 cells were harvested after growth in rich medium without IPTG (– IPTG) or in the presence of 1 mM IPTG (+ IPTG) at OD₆₀₀ 0.5.

Fig. 4. Physical map of the *resA*–*lysA* region of the *B. subtilis* chromosome, from MICADO database (<http://locus.jouy.inra.fr/Micado>).

ypzC and *ypzD*) were too short to be considered (respectively 154, 201, 237 and 213 bp). In one case, *ypuD*, a small gene (345 bp), was put under Pspac control. Four of the seven genes for which disruption was attempted were disrupted by integration of pMUTIN1: *ypuA*, *C*, *F* and *I* (Fig. 4). It was not possible to obtain any transformants by integration of pMUTIN within *ypuG*, *ypuH* and *ypuL*. We observed that efficiencies of transformation varied almost linearly from 10³ to 10⁵ transformants per μ g DNA with 150–300 bp of homology (data not shown). As the length of homology was within this range for these mutants, we assume that these genes might be essential, and they were consequently put under the control of Pspac. For this purpose, pMUTIN2 was inserted in front of the RBS of the gene, preserving a functional gene after integration.

Growth curves in rich and minimal media performed with all the mutants did not reveal any difference from the wild-type strain 168 with respect to growth rate and yield (data not shown). All the strains were able to sporulate on plates. The activities of their respective promoters were tested by measuring β -galactosidase activities (Table 3). *ypuI* and *ypuG,H* are better expressed in rich than in minimal medium, whereas *ypuF* and *ypuA* are expressed better in minimal medium. Only weak activities were observed for *ypuC*, *ypuD* and *ypuL* in each medium. These results are in agreement

Table 3. β -Galactosidase activity from the natural promoters of seven genes with no known function after integration of pMUTIN2

β -Galactosidase activities are expressed in Miller units (mg protein)^{–1}. L1, L2 and L3 indicate different stages of growth: L1, OD₆₀₀ 0.2; L2, OD₆₀₀ 0.6; L3, OD₆₀₀ 2.5. M1 indicates minimal medium in exponential phase and M2 at stationary phase.

Strain	L1	L2	L3	M1	M2
<i>ypuA</i>	80	150	100	200	350
<i>ypuC</i>	2	6	12	10	10
<i>ypuD</i>	15	17	20	5	7
<i>ypuF</i>	15	27	32	50	70
<i>ypuGH</i>	600	600	150	100	75
<i>ypuI</i>	150	300	150	60	80
<i>ypuL</i>	11	31	26	15	25

with RNA analysis (Azevedo *et al.*, 1993), except that no RNA was detected for *ypuA* and *ypuG,H* at the early exponential phase and at the stationary phase in rich medium. This failure could be due either to the stability of the LacZ protein accumulated in the cells, or to the instability of the mRNAs during the stationary phase.

ypuC, for which a weak level of β -galactosidase was found, seems to be better expressed during sporulation.

We were unable to disrupt *ypuG* and *ypuH*. Insertion of pMUTIN in front of the operon was possible only when IPTG was added to the medium. This IPTG dependence during transformation indicates that the operon contains at least one essential gene.

To investigate the properties of *ypuG,H*, a further shift-down experiment was carried out, in which the behaviour of the cells was studied when IPTG was removed from the medium. As a control, *dnaC*, which encodes a helicase essential for chromosome replication, was placed under the control of the Pspac promoter by integration of pMUTIN2 in the chromosome. In both strains, cell viability started to decrease about 2 h after withdrawal of IPTG (Fig. 5). Microscope analysis at 3 h revealed the presence of filamented cells. Since RNA analysis showed an undetectable level of mRNA 10 min after IPTG removal (data not shown), we assumed that the 2 h delay before the c.f.u. decrease was due to the protein half-life in cells.

Concluding remarks

The gene inactivation vectors described in this work were constructed to study *B. subtilis* genes with unknown function. Their main advantages are that they allow the rapid construction of insertional mutants, which can be used for a number of phenotype tests, facilitating the search for the target-gene function. All the vectors also allow the polar effects of the insertion of the vector to be avoided, as when the target gene is not in a monocistronic operon, or in the last position of a multicistronic operon. Such effects could otherwise render it difficult to correlate the phenotype with the inactivation of the target gene. Furthermore, some vectors (i.e. pMUTIN2 and pMUTIN4) facilitate the construction of conditional mutants, which is important for studies of genes essential for cell survival. One of the main drawbacks of these vectors is that the single cross-over insertions require a minimum length of homology between the vector and the target gene. Although it has been previously suggested that 70 bp is sufficient for homologous recombination (Khasanov *et al.*, 1992), in our hands, at least 150 bp homology is necessary to obtain plasmid integration. It is therefore not possible to inactivate genes shorter than about 250 bp by internal insertion and, even for longer genes, the insertion cannot modify the 70 aa N-terminal domain of their products. Such a domain could have an activity that obscures the mutant phenotype. However, since it is possible to control the expression of the genes by withdrawal of the inducer, this drawback is not prohibitive, except for very short genes, for which a deletion by double crossing-over integration of a resistance cassette can be proposed.

A further development of the insertional vectors that allow systematic inactivation of individual genes can be contemplated, in response to two features of the *B. subtilis* genome. One is the possibility that an operon

Fig. 5. Viability of *dnaC* (HV5597, a, b) and *ypuG,H* (c, d) mutants. The corresponding genes were put under the control of the Pspac promoter. Cells were grown in rich medium with 1 mM IPTG, centrifuged and rinsed twice, then resuspended in LB medium with 1 mM IPTG (open symbols) or without IPTG (filled symbols). Cell growth was followed by OD₆₀₀ measurement (a, c) or by colony counting after plating on IPTG-containing medium (b, d).

contains more than one essential gene, another is that there are homologous genes (paralogues) which may have overlapping functions (Kunst *et al.*, 1997). A vector with features similar to that of the pMUTIN series, but having no homology with pMUTIN, would allow an additional gene to be inactivated, and thus study cases of two essential genes in the same operon, or two functionally related paralogues. Such a vector is presently being developed and should allow the study of most of the essential genes present in the same operon, since the average *B. subtilis* operon contains only about three genes (Kunst *et al.*, 1997). It is unlikely that an analogous systematic approach would be practical for homologous genes of overlapping function that are present in more than two copies.

In conclusion, the vectors described here have already been used to construct some 800 mutants within the network of European laboratories and about as many within a similar network in Japan (N. Ogasawara, personal communication). Generating a collection of isogenic mutants in most of the uncharacterized *B. subtilis* genes could be the final outcome of this effort.

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