Appl Microbiol Biotechnol (2004) 64: 382–386
DOI 10.1007/s00253-003-1502-5

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Gene replacement method for determining conditions in which *Bacillus subtilis* genes are essential or dispensable for cell viability

Received: 1 August 2003 / Revised: 21 October 2003 / Accepted: 21 October 2003 / Published online: 9 December 2003

Abstract We describe a method for determining conditions in which *Bacillus subtilis* genes are essential or dispensable for cell viability. This method utilizes a chloramphenicol-resistant plasmid containing a temperature-sensitive (ts) replication origin. In this method, the target gene is first cloned into the ts vector and the recombinant plasmid is used to transform wild-type *B. subtilis*. The second step involves transformation of the resulting strain with a linear DNA fragment containing a second antibiotic resistance marker (*tet*) that disrupts the gene of interest. Selection for tetracycline resistance forces a double crossover between the chromosomal and fragment-borne copies of the gene, thereby replacing the wild-type gene in the chromosome with the disrupted allele. Cells survive even if the gene is essential by virtue of the complementing plasmid. Transformants are then grown at the non-permissive temperature for plasmid replication under various growth conditions. Isolation of chloramphenicol-sensitive colonies indicates that the gene is dispensable, whereas the inability to isolate chloramphenicol-sensitive colonies indicates that the gene is essential. The general utility of this method is demonstrated by allowing disruption of *mtrA* and *trpE* under conditions that render each gene non-essential, but not under growth conditions in which each gene is essential.

Introduction

Gene disruption is commonly employed to determine if a gene is essential for cell viability (Hamilton et al. 1989; Ingham et al. 1999). *Bacillus subtilis* is naturally competent for the uptake of autonomously replicating plasmids and linear DNA fragments (Dubnau 1993). A common strategy for gene disruption in *B. subtilis* is to subclone an antibiotic resistance cassette in the middle of the cloned gene of interest using *Escherichia coli* as the host. The recombinant plasmid is linearized at a restriction site unique to vector sequences and subsequently used to transform *B. subtilis* by selecting for the antibiotic resistance marker used to disrupt the cloned gene. If drug-resistant colonies are not obtained it is generally assumed that the gene is essential (Ingham et al. 1999). Other methods first involve formation of a cointegrate containing wild-type and mutant copies of the gene under investigation. Resolution of the cointegrate and subsequent screening for gene replacement is then carried out (Illing et al. 1990; Biswas et al. 1993; Smith and Youngman 1992). Another method eliminates the cloning steps entirely (Fabret et al. 2002). However, these methods do not allow the investigator to readily determine if the gene of interest is essential only under particular growth conditions. We have developed a method to identify conditions in which *B. subtilis* genes are essential or dispensable for cell viability. The general utility of this method is exemplified by our ability to disrupt *mtrA* and *trpE* under conditions that render each gene non-essential, but not under growth conditions in which each gene is essential.

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Materials and methods

Bacterial strains and plasmids

Plasmid constructions utilized *E. coli* DH5α as the host strain. Prototrophic *B. subtilis* W168 (*rpoB18*) was used as the wild-type strain for these studies. Strains PLBS32 and PLBS367 were generated through the course of these studies and contain *mtrA::tet* or *trpE::tet* chromosomal alleles, respectively. The *E. coli-B. subtilis* shuttle vector pKSV7 was described previously (Fig. 1) (Smith and Youngman 1992). Plasmid pKSV7 contains a *ColE1* origin and an ampicillin resistance gene (*bla*) to allow replication and selection in *E. coli*. A polylinker and blue-white screening facilitates gene cloning. pKSV7 also contains the *cat* gene, which confers resistance to chloramphenicol in *B. subtilis*, as well as a *B.*

subtilis temperature-sensitive (ts) origin of replication. The permissive and non-permissive temperatures for replication in *B. subtilis* are 30°C and 42°C, respectively. Plasmids pTZmtrAB and pSI45 contain the entire *mtrAB* operon (Gollnick et al. 1990). pPB13 was constructed by subcloning a 0.8 kb *Eco*RI-*Bcl*I fragment containing the *mtr* promoter and the coding region of *mtrA* from pSI45 into the *Eco*RI-*Bam*HI sites in the pKSV7 polylinker. Plasmid pPB11 contains an engineered *Bgl*II site in the middle of *mtrA*. pPB14 was constructed by subcloning a 1.6 kb *Hae*III-*Acc*I fragment containing the tetracycline resistance (*tet*) gene from pHY300PLK (Ishiwa and Shibahara 1985) into the *Bgl*II site of pPB11, resulting in disruption of *mtrA*. The DNA fragments were made blunt-ended with the Klenow fragment of DNA polymerase I prior to ligation.

Plasmid pHD15 was described previously (Du and Babitzke 1998). Plasmid pYH35 was made by subcloning the 2.4 kb *Eco*RI-*Bam*HI fragment containing the *trp* operon promoter, leader and the *trpE* gene from pHD15 into the pKSV7 polylinker. The plasmid pHY36 containing a disrupted *trpE* gene was constructed as follows. A 1.6 kb PCR product containing the *tet* gene from pHY300PLK was phosphorylated with T4 polynucleotide kinase and subcloned into the pHD15 *Xho*I site that had been made blunt-ended with mung bean nuclease. Ampicillin (100 μg/ml) and/or tetracycline (10 μg/ml) were used for plasmid selection in *E. coli*. Chloramphenicol (5 μg/ml) and/or tetracycline (12.5 μg/ml) were used for selection in *B. subtilis*. Glycine (50 μg/ml), methionine (50 μg/ml), adenosine (50 μg/ml), thymidine (50 μg/ml), pantothenic acid (3 μg/ml) or tryptophan (50 μg/ml) were added to minimal medium as appropriate.

General gene disruption method and selection procedure

The gene disruption method that we developed makes use of an *E. coli*-*B. subtilis* shuttle vector (pKSV7) that contains a ts *B. subtilis* origin of replication (Fig. 1) (Smith and Youngman 1992). The general strategy of this method is outlined in Fig. 2. The wild-type gene of interest is first subcloned into the pKSV7 polylinker using ampicillin resistance for selection in *E. coli*. The recombinant plasmid is then used to transform *B. subtilis* at the permissive temperature for plasmid replication (30°C) by selecting for chloramphenicol resistance. The second step involves transformation of a linear DNA fragment carrying the gene of interest that has been disrupted by insertion of a second antibiotic resistance gene. In our case we used tetracycline resistance (*tet*), although other antibiotic markers could be used. Selection for tetracycline resistance is carried out at 30°C to maintain the ts plasmid carrying the wild-type gene of interest. Tetracycline-resistant colonies can occur in one of two ways; disruption of the chromosomal copy of the gene via a double crossover (desired outcome) or disruption of a plasmid-encoded copy of the gene (undesired outcome). These two alternative outcomes are readily distinguished genetically or by PRC analysis of plasmid DNA (see below). Importantly, replacement of the chromosomal copy of the gene with the disrupted allele can occur even if the gene of interest is essential for cell viability by virtue of the complementing ts plasmid. Individual transformants are then grown at 30°C in liquid culture in the presence of both antibiotics and subsequently plated at 42°C in the presence of tetracycline only. Surviving colonies are then screened for loss of the antibiotic resistance associated with the ts plasmid (chloramphenicol). Isolation of tetracycline-resistant, chloramphenicol-sensitive colonies indicates that the gene of interest is not essential for cell viability under that particular growth condition. Proper gene disruption is then confirmed by PCR or Southern blotting. If it is not possible to isolate tetracycline-resistant, chloramphenicol-sensitive colonies the gene of interest is essential for viability under the growth condition used.

If the replacement of a plasmid-encoded copy of the gene occurs (undesired outcome occurs in step 2—see above), the colonies that survive at 42°C will be resistant to both antibiotics due to a single crossover between the disrupted gene on the plasmid and the wild-type chromosomal gene. If the desired outcome from step 2 is obtained, a single crossover between the plasmid-encoded wild-type gene and the disrupted chromosomal gene can still occur when the cells are plated at 42°C. In each of these cases, the cells will be resistant to both antibiotics and will contain wild-type and disrupted copies of the gene (cointegrate). If it is not possible to isolate colonies that are only resistant to tetracycline the gene is essential for viability under the growth conditions used. This method will allow rapid testing of a wide variety of growth conditions to determine if the gene of interest is essential only under a particular set of conditions.

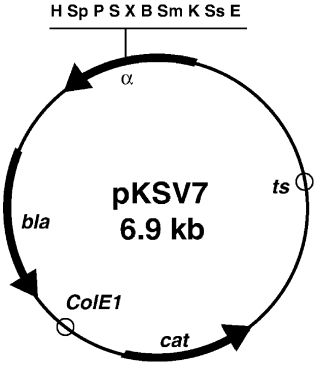

Fig. 1 Relevant features of plasmid pKSV7. *Arrows* Location and orientation of relevant plasmid-encoded genes, *cat* chloramphenicol resistance gene for selection in *Bacillus subtilis*, *bla* ampicillin resistance gene for selection in *Escherichia coli*, *α lac* alpha-complementing gene fragment, *ColE1* origin of replication for *E. coli*, *ts* temperature-sensitive origin of replication for *B. subtilis*, *H Hin*dIII, *Sp Sph*I, *P Pst*I, *S Sal*I, *X Xba*I, *B Bam*HI, *Sm Sma*I, *K Kpn*I, *Ss Sst*I, *E Eco*RI

Results

Disruption of *mtrA*

mtrA encodes GTP cyclohydrolase I, an enzyme essential for folic acid biosynthesis (Babitzke et al. 1992). Folic acid, in turn, is involved in the biosynthesis of glycine, methionine, adenosine, thymidine and pantothenic acid. Thus, *mtrA* should be essential for viability in the absence of these compounds in the growth medium but not in their presence. *B. subtilis* strain W168 was transformed with plasmid pPB13 (*mtrA* in ts plasmid) and plated at 30°C on LB in the presence of chloramphenicol. The resulting strain was subsequently transformed with *Hin*dIII-linearized pPB14 (*mtrA::tet*) by plating on LB plus tetracycline and chloramphenicol at 30°C. Subsequent screening on minimal plates indicated that the transformants survived at 30°C in the absence of the five compounds listed above; 14 of these transformants were grown overnight at 30°C in LB containing tetracycline and chloramphenicol. The next day, dilutions of each culture were plated at 42°C on minimal medium containing tetracycline in the absence or presence of the five compounds. The resulting colonies were screened for the loss of chloramphenicol resistance originating from the ts plasmid.

When cells were plated in the presence of the five compounds at 42°C, 11 of the 14 transformants gave rise to a mixture of chloramphenicol-sensitive and -resistant colonies. We subsequently found that the chloramphenicol-

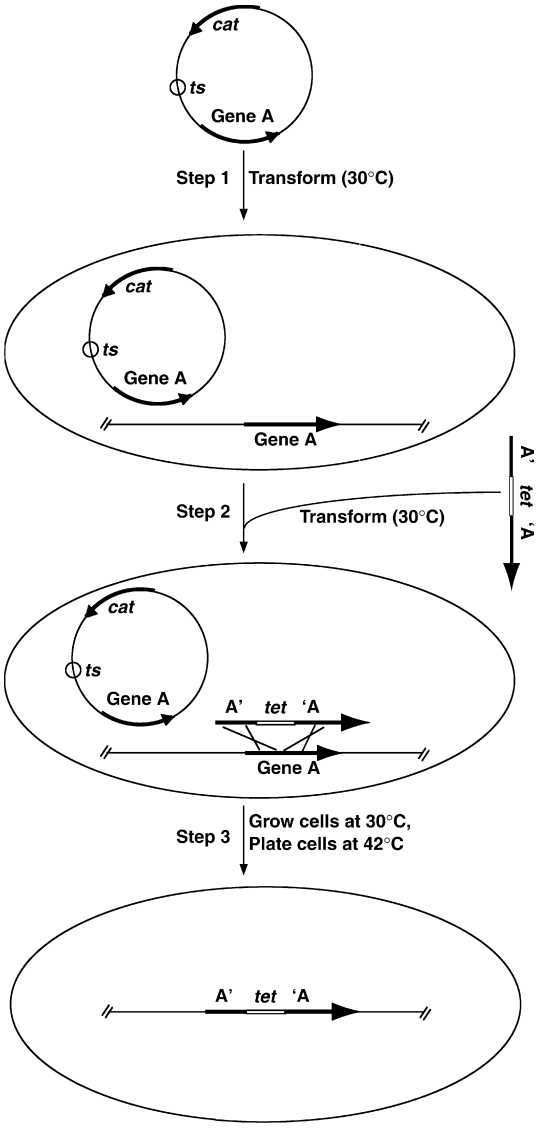

Fig. 2 Outline of the gene replacement method. The ts plasmid carrying the gene of interest (*Gene A*) is transformed into a wild-type strain. Growth is maintained at 30°C (*Step 1*). A linear DNA fragment containing disrupted Gene A is transformed into the strain containing the ts plasmid. Growth is maintained at 30°C. A double crossover results in chromosomal gene disruption (*Step 2*). Individual isolates are then grown at 30°C and subsequently plated at 42°C, the non-permissive temperature for ts plasmid replication. Individual colonies are then screened for the presence or absence of chloramphenicol resistance (*Step 3*). Disruption of the chromosomal gene of interest is then confirmed by PCR

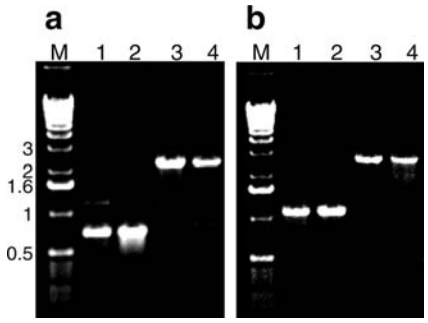

Fig. 3 Agarose gels of PCR products from the templates indicated demonstrating that the chromosomal copies of *mtrA* (**a**) and *trpE* (**b**) were disrupted. **a** Lanes: *M* Molecular size markers (kb), *1* W168 chromosomal DNA (wild type), *2* pPB13 (wild-type *mtrA*), *3* PLBS32 chromosomal DNA (*mtrA::tet*), *4* pPB14 (*mtrA::tet*). **b** Lanes: *M* Molecular size markers (kb), *1* W168 chromosomal DNA (wild type), *2* pYH35 (wild-type *trpE*), *3* PLBS367 chromosomal DNA (*trpE::tet*), *4* pYH36 (*trpE::tet*). The slower mobility of the PCR products in lanes *3* and *4* in **a** and **b** is indicative of a 1.6 kb *tet* gene insertion

col-sensitive colonies required the five compounds for growth in minimal medium, suggesting that the chromosomal *mtrA* gene had been disrupted and that the ts plasmid was lost. Interestingly, we also found that the *mtrA* null strains were unable to survive in LB unless additional thymidine was added. This finding explains why a previous attempt to disrupt *mtrA* using LB agar was not successful (Gollnick et al. 1990). Proper gene disruption of several isolates was confirmed by PCR amplification of chromosomal DNA, an example of which is shown in Fig. 3a. Disruption of *mtrA* with the *tet* gene was also confirmed by Southern blotting (data not shown). In contrast, all of the chloramphenicol-resistant colonies survived in the absence of the five compounds. Thus, in these instances it is apparent that the linearized plasmid carrying the *mtrA::tet* allele originally recombined with the wild-type chromosomal *mtrA* gene, resulting in gene disruption. When the cells were subsequently plated at 42°C, the ts plasmid apparently recombined with the disrupted gene in the chromosome by a single crossover, thereby generating a cointegrant containing both wild-type and disrupted copies of *mtrA*.

Of the original 14 transformants, only 3 gave rise to chloramphenicol resistant colonies at 42°C. PCR analysis indicated that in this case the linearized plasmid recombined with wild-type *mtrA* carried on the ts plasmid, thereby disrupting a plasmid copy of the gene (data not shown). When the cells were subsequently plated at 42°C, the *mtrA::tet* allele on the ts plasmid apparently recombined with the wild-type chromosomal *mtrA* gene by a single crossover, thereby generating a cointegrant containing both wild-type and disrupted copies of *mtrA*.

When cells derived from the original 14 transformants were plated at 42°C on minimal medium in the absence of the five compounds, we only obtained colonies that were resistant to both tetracycline and chloramphenicol. It is apparent that these cells consisted of chromosomal cointegrates containing both wild-type and disrupted copies of *mtrA*, indicating that *mtrA* is essential in the absence of the five compounds. Thus, this method allowed us to disrupt *mtrA* under conditions in which this gene is dispensable (presence of the five compounds) but not when it was essential for survival (absence of the five compounds).

Disruption of *trpE*

We carried out similar experiments with *trpE* to demonstrate the general utility of this method. Because *trpE* is required for tryptophan biosynthesis, this gene is essential for cell viability in the absence of tryptophan and dispensable in its presence (Babitzke and Gollnick 2001). *B. subtilis* strain W168 was transformed with PYH35 (*trpE* in ts plasmid) and plated at 30°C on LB in the presence of chloramphenicol. The resulting strain was subsequently transformed with *Pst*I-linearized pYH36 (*trpE::tet*) by plating on LB at 30°C in the presence of tetracycline and chloramphenicol. All of the transformants were found to survive at 30°C in the absence of tryptophan. Six of these transformants were grown overnight at 30°C by shaking in minimal-acid casein hydrolysate (ACH) medium (tryptophan-free) containing tetracycline and chloramphenicol. The next day, dilutions of each culture were plated at 42°C on minimal-ACH plates containing tetracycline in the absence and presence of tryptophan. The resulting colonies were screened for the loss of chloramphenicol resistance originating from the ts plasmid.

When cells were plated at 42°C in the presence of tryptophan, four of the original six transformants gave rise to a mixture of chloramphenicol-sensitive and -resistant colonies. We subsequently found that the chloramphenicol-sensitive colonies required tryptophan for growth, indicating that the chromosomal *trpE* gene had been disrupted and that the ts plasmid was lost. Proper gene disruption of several isolates was confirmed by PCR amplification of chromosomal DNA, an example of which is shown in Fig. 3b. All of the chloramphenicol-resistant colonies that were tested were tryptophan prototrophs. Thus, it is apparent that the linearized plasmid carrying the *trpE::tet* allele originally recombined with the wild-type chromosomal *trpE* gene resulting in gene disruption. When the cells were subsequently plated at 42°C, the ts plasmid recombined with the disrupted gene in the chromosome by a single crossover, thereby generating a cointegrant containing both wild-type and disrupted copies of *trpE*.

Two of the original six transformants grew rapidly at 30°C, resulting in large colonies on plates containing tetracycline. These two transformants only gave rise to colonies that were resistant to both tetracycline and chloramphenicol at 42°C. PCR analysis indicated that in this case the linearized plasmid recombined with wild-type *trpE* carried on the ts plasmid, thereby disrupting a plasmid copy of the gene (data not shown). We presume that the fast growing phenotype at 30°C was due to the presence of the *tet* gene on the multi-copy plasmid. When the cells were subsequently plated at 42°C, the *trpE::tet* allele on the ts plasmid recombined with the wild-type *trpE* gene in the chromosome by a single crossover, thereby generating a cointegrant containing both wild-type and disrupted copies of *trpE*.

As expected, when cells derived from the original six transformants were plated in the absence of tryptophan at 42°C, we only observed tryptophan prototrophs that were resistant to both tetracycline and chloramphenicol. Not surprisingly, these results indicate that *trpE* is essential in the absence of tryptophan. Thus, this method allowed us to disrupt *trpE* under conditions in which this gene is dispensable (tryptophan in the medium) but not when it was essential for survival (no tryptophan in the medium).

Discussion

We have developed a general method to identify conditions in which *B. subtilis* genes are essential or dispensable for cell viability. This conditional gene disruption method should be applicable to essentially any *B. subtilis* gene that can be cloned into the ts plasmid unless insertional inactivation of the gene leads to lethal polar effects. A promoter to drive expression of the gene carried on the ts plasmid is required and this should be taken into account when cloning the gene of interest. Once an appropriate strain contains the gene of interest cloned into the ts plasmid, and the chromosomal copy of the gene is disrupted, a wide variety of growth conditions can be tested to identify conditions in which the gene is dispensable or essential for viability. Our ability to disrupt two biosynthetic genes in the presence of appropriate nutrients that render each gene non-essential, but not in the absence of these nutrients, demonstrates the general utility of this technique.

The conditional gene disruption method described here could also be used to determine whether a gene is essential only in particular genetic backgrounds. For instance, many gene products carry out redundant functions. As a consequence, a mutation in only one of the genes does not lead to inviability. However, the use of the ts plasmid system could be exploited to determine whether mutant gene combinations lead to lethality. For example, if two genes encode similar functions, each gene could be disrupted individually by the method described here or by another gene replacement method (Illing et al. 1990; Smith and Youngman 1992; Biswas et al. 1993; Ingham et al. 1999; Fabret et al. 2002). If the single mutants are viable, the ts plasmid system could then be used to determine if the double mutant is lethal.

Acknowledgements We thank Phil Youngman for plasmid pKSV7 and Alexander Yakhnin for critical reading of the manuscript. This work was supported by Grant GM52840 from the National Institutes of Health. The experiments in this study comply with the current laws of the United States.

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