Transformation in Restriction-deficient *Salmonella typhimurium* LT2

By SIAO PING TSAI, RALPH J. HARTIN AND JUN-ICHI RYU*

Department of Microbiology, School of Medicine, Loma Linda University, Loma Linda,
CA 92350, USA

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Stable restriction-deficient, modification-proficient *galE* (JR501) and *F'**galE*⁺ (JR502) strains of *Salmonella typhimurium* were constructed and the effects of restriction on transformation by plasmid pBR322 were tested. Several factors which affect transformation efficiency were systematically examined to determine optimum transformation conditions and a simplified method is presented.

INTRODUCTION

Plasmid transformation is an established, routine step in gene cloning. Artificial transformation methods have been developed to induce competence in *Escherichia coli* (Mandel & Higa, 1970; Cohen *et al.*, 1972) and other bacteria (Saunders & Saunders, 1987). These methods have been improved, either by using mutants such as *hsdR* (Maniatis *et al.*, 1982; Matsushima *et al.*, 1987; Haima *et al.*, 1987), *recBC* and *sbcAB* (Oishi & Irbe, 1977) in chromosomal transformation, or by introducing various physiological treatments (Oishi & Irbe, 1977; Hanahan, 1983). Physiological conditions for optimum transformation efficiency vary, however, with the bacterial strain (Norgard *et al.*, 1978; Dagert & Ehrlich, 1979; Kushner, 1978) and type of DNA used for transformation (Oishi & Irbe, 1977).

Salmonella typhimurium, like *E. coli*, does not undergo genetic transformation naturally. Lederberg & Cohen (1974), however, reported successful transformation of a *gal* deletion mutant of *S. typhimurium* using the CaCl₂ method developed for *E. coli*. Bursztyn *et al.* (1975) also successfully applied this CaCl₂ method for P22 transfection of various rough mutants of *S. typhimurium*. Consequently, *gal* deletion and *galE* mutants have been favoured as recipients for transformation and transfection. More recently, MacLachlan & Sanderson (1985) modified the method of Lederberg & Cohen (1974) for transformation and examined plasmid transformation in many rough strains of *S. typhimurium*. Although *galE* and *rfaF* mutants both showed relatively high transformation efficiencies, *galE* strains have been recommended since these mutants can readily be converted to a P22-sensitive Gal⁺ phenotype.

S. typhimurium possess *hsdSA*, *hsdSB*, and *hsdLT* (sometimes called *hsdL*) restriction-modification genes (Colson & Van Pel, 1974; Sanderson & Hurley, 1987). Many plasmids originating from strains other than *S. typhimurium* undergo severe restriction when transformed into *S. typhimurium*. Bullas & Ryu (1983) developed a mutant (LB5000) and a *galE* derivative (LB5010) of *S. typhimurium* which are r⁻m⁺ for all three restriction-modification systems. These mutants proved to be good recipients when used in conjugation experiments with *E. coli* donors (Bullas & Ryu, 1983), as well as being readily transformed (Mizuno *et al.*, 1986). Plasmids isolated from these modification-proficient strains are fully modified and can be used to transform other *S. typhimurium* strains without encountering problems of restriction. Thus, these mutants are ideal mediators for genetic manipulation in *S. typhimurium* (Sanderson & Stocker, 1987). During the course of this study the modification phenotype of the *hsdSA* genes in both LB5000 and LB5010 was found to be unstable. A semiquantitative modification test (Bullas *et al.*, 1980) using P3 phage showed that phages propagated on strains LB5000 and LB5010 were

not modified for *hsdSA*, and thus were restricted by wild-type *S. typhimurium*. Hence, the modification phenotype has changed from m_{SA}^+ to m_{SA}^- . Bacteria distributed to other laboratories also demonstrated this same unstable modification phenotype (B. Stocker, personal communication). The purpose of this project was to construct a stable r^-m^+ strain of *S. typhimurium* and investigate the effects of restriction on plasmid transformation. In addition, several physiological conditions were examined in detail in order to simplify transformation methods in this strain.

METHODS

Bacterial and phage strains. All the bacterial strains used in this study are *S. typhimurium* LT2 derivatives (Table 1). Strain LT2 is the wild-type strain. Strains 4274 and 4419 are derived from 4247 and are thus isogenic. Phages P1, L, P22, and P3 were identical to those described by Bullas & Colson (1975), and were propagated as indicated by those authors. Phage P22HTint (Davis *et al.*, 1980) was also used in some of the detailed mapping experiments.

Bacterial culture conditions. LB medium (Davis *et al.*, 1980) containing 1% (w/v) D-glucose (L-broth) and SOB medium (Hanahan, 1983) were used for liquid culture. Agar (1.5%, w/v) and antibiotics (ampicillin, 50 $\mu\text{g ml}^{-1}$, or tetracycline, 20 $\mu\text{g ml}^{-1}$) were added to L-broth to provide selective agar media.

Plasmid and plasmid isolation. The plasmid primarily used in this study was pBR322 (Bethesda Research Laboratories) which was isolated from *E. coli* RR1 ($r_B^- m_B^-$, Maniatis *et al.*, 1982). In one experiment, pBR322 was isolated and purified from transformed *S. typhimurium* JR501 using the method described in Davis *et al.* (1986). Other plasmids were extracted from *E. coli* K (m_K^+) strains using the same method.

Original transformation procedure. Several transformation methods described for enteric bacteria (Hanahan, 1983; MacLachlan & Sanderson, 1985; Merrick *et al.*, 1987; Lederberg & Cohen, 1974) were evaluated before the following protocol, based on the methods of Cohen *et al.* (1972) and Bursztyn *et al.* (1975), was developed. An overnight culture grown in L-broth was diluted 1/20 in SOB medium and incubated at 37 °C with aeration until an OD_{510} of 0.6 was reached. Then 50 ml of the culture was chilled, and the cells were harvested by centrifugation and dispersed in 50 ml cold (4 °C) 0.1 M-MgCl₂ (magnesium wash). The cell pellet was then resuspended in 25 ml 0.1 M-CaCl₂ and kept on ice for 20 min (calcium pre-incubation), followed by centrifugation in the cold and resuspension in 5 ml cold 0.1 M-CaCl₂. Small volumes (0.2 ml) of the competent cell suspension were placed in prechilled glass test tubes (10 cm $\times$ 13 mm) or 1.5 ml plastic micro-centrifuge tubes containing 10 ng (1 mg ml⁻¹) plasmid DNA. The DNA-cell mixture was chilled on ice for another 20 min before being placed in a 42 °C water bath for 2 min. L-broth (0.8 ml, ambient temperature) was then added to the DNA-cell mixture and the culture was incubated at 37 °C for 1 h (post-incubation) without aeration. Finally 100 μl volumes were spread on selective agar containing ampicillin (50 $\mu\text{g ml}^{-1}$) unless otherwise stated, and the seeded agar was incubated at 37 °C overnight. A fraction of the post-incubated culture was diluted as necessary and spread on L-agar (in triplicate) to obtain viable cell counts. Means of the number of transformants per plate were obtained from at least two plates for calculation of transformation efficiency (transformants per μg DNA; Hanahan, 1987). Under the conditions described, there was a positive, linear relationship between the number of ampicillin-resistant transformants ml⁻¹ and the concentration of pBR322 DNA used per 0.2 ml of competent cells, up to a concentration of 1 μg DNA. All experiments in this study were repeated at least three times and the data presented are representative of the results.

Restriction and modification test. The semi-quantitative drop test described by Bullas *et al.* (1980) was used to characterize the *hsdSA*, *hsdSB* and *hsdLT* phenotypes of each *S. typhimurium* strain used. Efficiencies of plating of phages L, P3 and P22, each with proper modification, were obtained in order to determine the restriction and modification phenotypes of *hsdSA*, *hsdSB* and *hsdLT*, respectively. Phage plates were incubated overnight at 30 °C, because of the temperature-sensitive restriction phenotype of *hsdSA* and *hsdSB* genes (Colson & Van Pel, 1974).

Table 1. *S. typhimurium* strains

Strain	Relevant phenotype and genotype	Reference
LT2	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>gal</i> ⁺	Sanderson & Stocker (1987)
LB5000	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>gal</i> ⁺	Bullas & Ryu (1983)
4247	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>gal</i> ⁺	Bullas <i>et al.</i> (1980)
4274	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>gal</i> ⁺	Bullas & Colson (1975)
4419	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>gal</i> ⁺	Bullas <i>et al.</i> (1980)
SL3684	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>galE</i>	MacLachlan & Sanderson (1985)
JR501	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>galE</i>	This study
JR502	$r_{LT}^- m_{LT}^- r_{SA}^+ m_{SA}^+ r_{SB}^- m_{SB}^-$ <i>galE</i> <i>F'gal</i> ⁺	This study

* Phenotype has been changed to m_{SA}^- . See text for details.

RESULTS AND DISCUSSION

Construction of a stable r^-m^+ strain

Two strategies were tried before a new stable r^-m^+ strain of *S. typhimurium* was constructed. The initial approach was simply to replace the unstable *hsdSA* genes of LB5000 with the stable *hsdSA* genes of the parental strain, 4419. This was accomplished by P1 transduction of the 4419 *hsdSA* genes ($r_{SA}^- m_{SA}^+$) into LB5000 using Tn5 (*zji-1072::Tn5*, Fig. 1) as the selection marker. The modification phenotype of the *hsdSA* genes of the resultant strain was also unstable and soon changed to m_{SA}^- . The reason for the instability is not known. However, LB5000 may have acquired an additional chromosomal mutation outside the *hsd* genes when the *hsdSB121* mutation was introduced by nitrosoguanidine mutagenesis (Bullas & Ryu, 1983). In the second attempt to construct a stable r^-m^+ strain, the *hsdSB121* ($r_{SB}^- m_{SB}^+$) mutation of LB5000 was transferred by P1 transduction to a *galE* derivative of the parental strain, 4419, which already possessed the r_{SA}^- and r_{LT}^- phenotypes. In order to move the *hsdSB121* mutation into the same strain, Tn10 insertions close to *deo* genes, and hence close to *hsdSB* (Sanderson & Hurley, 1987), were selected from a random pool of Tn10 chromosomal insertions (obtained from G. Ames, University of California – Berkeley, USA). The insertions were mapped using both P22 and P1 transduction (Fig. 1). One of the Tn10 insertions *zji-4021::Tn10*, was used to transfer *hsdSB121* of LB5000 into strain 4419. The resultant strain was designated JR500. To examine the restriction–modification phenotype of JR500, plasmid $F'gal^+$ was introduced from strain 4529. Many Tn10 insertions isolated in this region, however, showed a lower percentage of linkage with markers close to the *hsd* genes than expected from the published genetic map (Sanderson & Hurley, 1987). Therefore, it is possible that this region contains more genetic material than previously thought. A tetracycline-sensitive derivative was subsequently isolated from JR500 $F'gal^+$ on Bochner selective agar (Bochner *et al.*, 1980) and was designated JR502. A derivative of JR502 which had lost $F'gal^+$ spontaneously was found and was designated JR501.

Effect of restriction and galE mutation

Several *hsd* mutants and *galE* mutants of *S. typhimurium* were used to investigate both the extent of the restriction of pBR322 DNA during transformation and the improvement in transformation efficiency due to *galE* mutations (Table 2). With strain 4247, which is restriction-proficient for all three systems, no transformants were obtained when up to 1 μ g DNA was used. Mutant strain 4419 had a transformation efficiency equal to strain 4274. This may simply be due to the absence of an SA recognition site on pBR322. Strain SL3684, a *galE* mutant, had a greater transformation efficiency than the wild-type, as reported earlier (MacLachlan & Sanderson, 1985), but one which was significantly lower than those of the restriction-deficient mutants. The combination of restriction-deficient and *galE* mutations (JR501) resulted in the highest transformation efficiencies. These results demonstrate that restriction is a major barrier to the introduction of unmodified plasmid into *S. typhimurium* by transformation. Although the recognition sites for *hsdSA* and *hsdLT* are not known, two *hsdSB* sites have been reported on pBR322 (Nagaraja *et al.*, 1985), which is consistent with our results.

Plasmid pBR322 was then isolated from JR501. This plasmid isolate transformed wild-type LT2 without being restricted, although the same plasmid isolate transformed SL3684 with 10^2 higher efficiency, probably due to the *galE* character. Culture temperature (30, 37 and 43 °C tested) of JR501 containing pBR322 did not affect transformation of LT2 by modified plasmid DNA, despite the fact that the *hsdSB* modification system is reported to be temperature sensitive (Colson & Van Pel, 1974). Except for JR501 having a consistently higher transformation efficiency than JR502, differences in the response of these strains to treatments in the transformation protocol were negligible. The following experiments were done to maximize transformation efficiency in strains JR501 and JR502 and to simplify the transformation protocol.

Factors affecting transformation efficiency

It has been reported that in certain strains of *E. coli*, competence is dependent upon the bacterial growth stage (Norgard *et al.*, 1978). Development of competence in strain JR502 also

Fig. 1. Genetic map of the 98–99 minute region of *S. typhimurium*. Cotransduction frequencies are given in percentages and experimental data (in parentheses) from this study. The figure is not drawn to scale. *P22 transduction, this study; †P1 transduction, this study; ‡P22 transduction, Sanderson & Hurley (1987); §P1 transduction, Kozdroj & Klopotoski (1979).

Table 2. *Effect of restriction and galE mutation on transformation efficiency of S. typhimurium strains*

Cells were harvested at OD₅₁₀ 0.2.

Strain	Restriction phenotype			<i>gal</i>	Amp ^r transformation efficiency*
	SA	SB	LT		
JR501	—	—	—	—	$1.4 \times 10^6 \pm 44\%$
JR502	—	—	—	+	$2.6 \times 10^5 \pm 22\%$
SL3684	+	+	+	—	$8.8 \times 10^1 \pm 64\%$
4419	—	+	—	+	$2.2 \times 10^2 \pm 64\%$
4274	+	+	—	+	$2.4 \times 10^2 \pm 58\%$
4247	+	+	+	+	0

* The results are means of at least three determinations, $\pm$ SD.

demonstrated a dependency on growth stage. Optimum transformation efficiency was observed at an OD₅₁₀ of 0.2 (about 1×10^8 cells ml⁻¹) after approximately 1 h of growth. The difference in transformation efficiencies between the optimum point of the growth cycle and the stationary stage was more than two orders of magnitude.

Mg²⁺ ions stimulate transformation in many *E. coli* strains (Hanahan, 1987). However, the presence of Mg²⁺ in either the medium or the cell washing buffer, or in both, failed to improve transformation in JR502. In fact, MgCl₂ (100 mM) incorporated into the wash reduced the transformation efficiency by approximately 15–70%. The primary difference between SOB and L-broth medium is the supplemental Mg²⁺, in the form of MgCl₂ and MgSO₄, contained in SOB medium. Differences in the transformation efficiencies of cells grown in these media are thus negligible.

Among the cations tested (Ca²⁺, Mn²⁺, Sr²⁺, Ba²⁺, Mg²⁺, Na⁺ and Rb⁺), Ca²⁺ gave the highest transformation efficiency in the 100–200 mM range. More than 300 mM-Ca²⁺ decreased the viable cell count. The Ca²⁺ treatment was most effective in improving transformation efficiency at the cell–DNA mixture (calcium pre-incubation) stage in our original transformation protocol.

After treatment of cells with Ca²⁺ to promote competence, cell–DNA mixtures were heat-shocked for various times at 42 °C, and the transformation efficiencies were calculated (Fig. 2).

Fig. 2

Fig. 2. Effects of heat shock at 42 °C in transformation efficiency of JR501. Cells were treated with CaCl₂ and mixed with pBR322 as described in Methods. Samples (in glass test tubes) were placed in a 42 °C water bath for various times.

Fig. 3

Fig. 3. (a) Expression of ampicillin (●) and tetracycline (○) resistance, and (b) viable cell count of strain JR501. After the heat-shock treatment, cells were suspended in L-broth, and antibiotic-resistant transformants and viable cells were counted at various times.

Optimum transformation efficiency was obtained with a 15–30 s heat shock. Longer heat-shock treatments significantly reduced transformation efficiency. Similar results were obtained whether glass test tubes or plastic microcentrifuge tubes were used for this treatment. Heat shock at 37 °C resulted in a similar curve, but transformation efficiency was slightly lower than at 42 °C.

To determine the optimum time for post-incubation of JR501 with pBR322, heat-shocked cells were incubated in L-broth for up to 2 h. Representative transformation efficiencies are shown in Fig. 3(a). Substantial numbers of ampicillin- and tetracycline-resistant transformants were recovered without post-incubation. When ampicillin resistance was used as the selective marker, no increase in the number of transformants was observed after 1 h post-incubation, although a slight increase of transformants (15%) was observed in some experiments after 15–30 min post-incubation. Fewer transformants were obtained when tetracycline resistance was used as the selective marker, unless post-incubation times were more than 1 h. Thus, post-incubation contributed to an increase in the number of tetracycline-resistant transformants. Increases in transformation efficiency after 1 h, however, appeared to be due to onset of cell division, since viable cells began to increase after this time (Fig. 3b).

Simplified transformation method

Those treatments which had a negligible effect on transformation efficiency have been deleted from the original protocol to simplify the method. Accordingly, L-broth is recommended for both overnight and exponential-phase bacterial cultures. The magnesium wash, and calcium pre-incubation steps have been deleted. The heat-shock time has been reduced from 2 min to 30 s, and incubation for expression of ampicillin resistance has been eliminated. The simplified transformation protocol is described below.

Grow 10 ml *S. typhimurium* at 37 °C in L-broth with shaking to OD₅₁₀ 0.2 (1 × 10⁸ cells ml⁻¹). Centrifuge cells at 5000 r.p.m. for 10 min at 4 °C, and resuspend them in 0.4 ml cold (4 °C) 0.1 M-CaCl₂. Dispense 0.2 ml of competent cells and mix with plasmid DNA (10–100 ng). Chill the mixture for 20 min at 4 °C. Heat-shock the mixture of cells and DNA for 30 s in a 42 °C water

bath. Add 0.8 ml L-broth (incubate the mixture for 30 min if tetracycline is used as a selection marker) and plate 0.1 ml cells on L-agar containing antibiotics. Incubate at 37 °C overnight.

When this shortened methodology was used with JR501, reasonable transformation efficiencies (10^5 – 10^6) were consistently obtained using pBR322 and other plasmids such as pGEM™-3Z (Promega), pBluescript™ (Stratagene), both with and without chromosomal inserts, and having a range of total DNA from 2.8 to 20 kb.

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