Biolistic transformation of prokaryotes: factors that affect biolistic transformation of very small cells

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Five bacterial species were transformed using particle gun-technology. No pretreatment of cells was necessary. Physical conditions (helium pressure, target cell distance and gap distance) and biological conditions (cell growth phase, osmoticum concentration, and cell density) were optimized for biolistic transformation of *Escherichia coli* and these conditions were then used to successfully transform *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Erwinia stewartii* and *Pseudomonas syringae* pv. *syringae*. Transformation rates for *E. coli* were 10^4 per plate per 0.8 µg DNA. Although transformation rates for the other species were low ($<10^2$ per plate per 0.8 µg DNA), successful transformation without optimization for each species tested suggests wide utility of biolistic transformation of prokaryotes. *E. coli* has proven to be a useful model system to determine the effects of relative humidity, particle size and particle coating on efficiency of biolistic transformation.

Introduction

The uptake of DNA occurs naturally in some bacterial species (Cohen *et al.*, 1972), is induced in some species by treatment with divalent cations (Hanahan, 1983) and occurs in bacterial protoplasts treated with polyethylene glycol (Klebe *et al.*, 1983). Also, transformation has been demonstrated in some Gram-negative bacteria and in protoplasts of Gram-positive bacteria using the electroporation method (Bonnassie *et al.*, 1990; Calvin & Hanawalt, 1988; Fiedler & Wirth, 1988). In this paper we describe a widely applicable transformation method which requires no pretreatment of cells but directly bombards bacterial cells spread on the surface of selective medium with DNA-coated tungsten particles.

Biolistic transformation has proven a useful tool in transformation of plant and animal cells (Daniell *et al.*, 1990; Sanford, 1988, 1990*a, b*; Williams *et al.*, 1991; Yang *et al.*, 1990). Recently our laboratory has demonstrated biolistic transformation in the prokaryote *Bacillus megaterium* (Shark *et al.*, 1990). This Gram-positive species was chosen for our initial studies of prokaryotes because of its large cell size (1.5×5.0 µm) and the relative difficulty of transforming it by other methods.

Our objective in this study was to extend biolistic technology to other bacterial species, to prove the broad applicability of this method and to better understand the factors affecting biolistic transformation of very small cells. We have therefore optimized transformation conditions for *Escherichia coli*, and have used these conditions to transform four other Gram-negative bacterial species. *E. coli* has now become useful as a model system in our lab for rapidly elucidating factors affecting biolistic transformation in general. Using the *E. coli* system we have determined the effect of relative humidity on biolistic transformation, the particle size most effective in small cell systems and we have optimized M5 tungsten particle coating.

Methods

Bacterial strains and plasmid DNA. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids pKRS101, pUC118 and pR89 were isolated from *E. coli* DH5αF' strains. pLAFR3 was isolated from *E. coli* HB101(pLAFR3). Strains were incubated with aeration (150 r.p.m.) for 16 h in Luria-Bertani (LB) broth (Maniatis *et al.*, 1982) supplemented with 100 µg ampicillin (Ap) ml⁻¹ for pKRS101 and pUC118, 15 µg tetracycline (Tc) ml⁻¹ for pLAFR3 and 50 µg kanamycin (Kn) ml⁻¹ for pR89. Plasmid DNA was isolated by the 'boiling lysis' method (Maniatis *et al.*, 1982) and was purified by CsCl density gradient ultracentrifugation (Garger *et al.*, 1983). Purified DNA was resuspended in TE buffer (1 mM-Tris, pH 7.8, 0.1 mM-EDTA) and the concentration was determined spectrophotometrically.

Abbreviations: Ap, ampicillin; Kn, kanamycin; RH, relative humidity; Tc, tetracycline; TDO medium, tryptophan drop-out medium.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Genotype or phenotype	Reference or source
<i>Escherichia coli</i> JA221	$\Delta trpE5 leu^- hsdR^- recA^-$	Miozzari & Yanofsky (1978)
<i>Erwinia amylovora</i> E4001A		Norelli <i>et al.</i> (1988)
<i>Erwinia stewartii</i> DC283	NaI ^r	Coplin <i>et al.</i> (1986)
<i>Pseudomonas syringae</i> pv. <i>syringae</i> B86-7		Legard (1991)
<i>Agrobacterium tumefaciens</i> LBA4404		Hoekema <i>et al.</i> (1983)
pKRS101	Ap ^r , <i>trpE</i> , 4.5 kb	Spindler <i>et al.</i> (1984)
pUC118	Ap ^r	Vieira & Messing (1987)
pLAFR3	Tc ^r , 22 kb	Staskawicz <i>et al.</i> (1987)
pKP201A	Ap ^r , 5.9 kb	D. Coplin (see above)
pR89	Kn ^r , 13 kb	Z. R. Liu (our laboratory)
pUB110	Kn ^r , 4.5 kb	Gryczan <i>et al.</i> (1978)

Confirmation of transformation. To confirm transformation, plasmid DNA from *E. coli*, *Erwinia amylovora*, and *P. syringae* pv. *syringae* was isolated using the 'mini-boiling prep' method (Maniatis *et al.*, 1982). Plasmid DNA was isolated from *A. tumefaciens* using a modified alkaline lysis mini-prep method (Holsters *et al.*, 1978) and from *Erwinia stewartii* using the alkaline lysis method described by Maniatis *et al.* (1982). The DNA was digested following manufacturer's directions (Gibco BRL) and visualized by agarose gel electrophoresis.

Preparation of cells and DNA for bombardment. *E. coli* JA221 was grown in LB broth for 15 h at 37 °C with aeration (250 r.p.m.). The culture (100 ml) was centrifuged for 20 min at 3500 r.p.m. at room temperature. The bacterial pellet was resuspended in 7 ml sterilized distilled water and the cell density was determined spectrophotometrically. On each plate 2×10^9 c.f.u. were spread on the surface of the agar-solidified selective medium and the surface was dried before bombardment.

P. syringae pv. *syringae* B86-7 and *Erwinia stewartii* DC283 were grown in LB broth, and *Erwinia amylovora* E4001A was grown in Kado 523 broth (Kado & Heskett, 1970) for 15 h at 25 °C with aeration (250 r.p.m.). The cultures (100 ml) were centrifuged for 10 min at 8000 r.p.m. at 4 °C. The bacterial pellet was resuspended in 5 ml of sterile medium. Cell density was determined spectrophotometrically. An overnight culture of *Agrobacterium tumefaciens* LBA4404 was used to inoculate 50 ml fresh LB medium and then the inoculated culture was incubated at 25 °C with aeration (250 r.p.m.). Growth of the culture was monitored spectrophotometrically until optical density at 550 nm equalled 0.5 (exponential growth). The bacteria were pelleted and resuspended as described above.

M5 tungsten particles (Sylvania, GTE Products Corp) were coated with plasmid DNA as previously described (Shark *et al.*, 1990), except where noted. In a microcentrifuge tube we added 50 µl of a tungsten slurry (3.6 mg tungsten), 5 µg DNA, and then 50 µl 2.5 M-CaCl₂ and 20 µl 0.1 M-spermidine. The tube was gently vortexed between the addition of each component and the tube was mixed for 10 min after all components had been added. The coated tungsten was washed with 70% ethanol and resuspended in absolute ethanol. Each plate was bombarded with approximately 600 µg tungsten, coated with 0.8 µg plasmid DNA, except where noted.

Selection of transformants and controls. *E. coli* JA221 transformants were selected on tryptophan drop-out medium [TDO: M9 salts, 0.2% Casamino acids, 2.0 mM-MgSO₄, 0.1 mM-CaCl₂, 0.2% glucose, 1.5% (w/v) agar] plus 0.6 M-sorbitol, except where noted. When Ap selection was used, to allow cells time to recover from bombardment before

selection, cells were bombarded on medium without Ap and, after a time, transferred to selective medium. First, cells were spread on a thin layer (7 ml) of LB medium plus 0.6 M-sorbitol that had been poured over the surface of a sterile piece of wet filter paper. Cells were slowly dried on the agar surface and then bombarded. After bombardment, the filter paper and agar ('pagar') were transferred to the surface of 21 ml LB agar medium plus 133.3 µg Ap ml⁻¹. The final Ap concentration after diffusion through the 'pagar' was 100 µg ml⁻¹.

The 'background' transformation rate of *E. coli* was determined. DNA alone or DNA-coated tungsten particles were mixed with cells and then the plate was exposed to vacuum alone, or vacuum with a helium burst, or bombarded with naked tungsten particles (Table 2). In addition, cells (alone) were subjected to vacuum alone or vacuum with a helium burst, bombardment with naked tungsten or bombardment with DNA-coated tungsten particles.

Optimization of particle size and coating. To determine optimum particle size, 2×10^9 *E. coli* cells per plate were bombarded with M5 and M10 tungsten particles (Sylvania) and gold particles (Dupont) which were coated with pKRS101 DNA (in each case using the coating method as described for the M5 particles).

To optimize M5 particle coating, we first varied DNA concentration keeping all other components constant. We loaded 600 µg coated tungsten per membrane and bombarded six replicate plates of *E. coli* JA221 per treatment. The mean number of transformants per treatment was quantified after 24 h incubation. The experiment was repeated four times. Secondly, M5 particles were precoated with different concentrations of pUB110 DNA (non-selected) before coating with different concentrations of pKRS101 DNA. Treatments with precoated tungsten were compared to the standard treatment. Six replicates per treatment were used and the experiment was repeated three times. Under these conditions, the optimal amount of coated tungsten per bombardment was then determined. We loaded 600, 900 and 1200 µg coated tungsten onto flying disks [circular membranes made of Kapton (Dupont) which are 2.54 cm in diameter and 0.06 mm thick] before the target cells were bombarded. Eight replicates per treatment were used.

Particle accelerator and optimization of physical and environmental parameters. A helium-driven biolistic device and the flying disk configuration of particle delivery (Sanford *et al.*, 1991a; Shark *et al.*, 1990) was used throughout this study. DNA-coated tungsten particles resuspended in 100% ethanol were dried down on the surface of a flying disk (described above). The variables tested were: helium pressure [700, 1000 and 1300 p.s.i. (1 p.s.i. = 6.895 kPa)]; gap distance - i.e. the distance between the helium source and flying disk (0.6, 1.0 and

Table 2. Production of *E. coli* JA221 transformants by particle bombardment of various control treatments using plasmid pKRS101

Cells (2×10^9 per plate) were spread on TDO medium supplemented with 0.6 M-sorbitol. In some treatments (+) 0.8 µg pKRS101 DNA and/or pKRS101 DNA-coated tungsten particles were spread on the medium surface with the target cells. All treatments were subjected to a vacuum in the bombardment chamber. Some treatments (+) were subjected to a helium burst alone and others to a helium bombardment with naked tungsten or DNA-coated tungsten. The results are the mean values of six replicates.

	Plate surface		Bombardment			Mean no. of transformants	SE
	DNA	Tungsten	Helium	Naked tungsten	DNA-coated tungsten		
Standard protocol	—	—	+	—	+	8344	968.7
Controls	+	+	—	—	—	0	0
	+	+	+	—	—	0.33	0.33
	+	—	+	+	—	0.167	0.167
	—	—	+	+	—	0	0
	—	—	—	—	—	0	0
	+	—	+	—	—	0.33	0.33

1.4 cm); and target distance – i.e. the distance between the particle launch site and target cells (6.0, 9.2 and 12.3 cm). Five replicates per treatment were used and the experiment was repeated three times. Also, the effect of a helium flushing of the vacuum chamber prior to drawing a vacuum was tested. From five to ten replicates per treatment were used and the experiment was repeated seven times.

To reduce the mortality of cells at the centre of bombarded plates, we compared transformation rates of cells bombarded at 2400 p.s.i. helium with and without a baffle mechanism (Sanford *et al.*, 1991*b*) to protect the cells from the acoustic shock and helium blast. The baffle is installed above the particle launch site and acts like the silencer of a gun by channelling the shock waves laterally (J. A. Russell, M. K. Roy & J. C. Sanford, unpublished results). Five plates per treatment were bombarded and the experiment was repeated three times.

We studied the effect of relative humidity (RH) on the 'dry-down' of DNA-coated particles onto flying disks. Five RH chambers containing saturated salt solutions were prepared using glass petri dishes. The salt solutions used were K_2SO_4 , $(NH_4)_2SO_4$, $Mg(NO_3)_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, and $LiCl$, which at 24 °C produced RHs of 96.9, 80.3, 53.8, 33.2 and 12.0%, respectively. The flying disks were loaded with 3 µl tungsten-DNA suspended in 100% ethanol, and were placed within the RH chambers and incubated within the chambers for 2 h before bombardment. *E. coli* cells (1×10^9) were spread on each TDO plus 0.2 M-sorbitol plates and bombarded. Five plates per treatment were bombarded and the experiment was repeated three times. Also, five flying disks were incubated in the 96.9% RH chamber for 5, 10 and 20 min, and 1 h, and then the flying disks were transferred to a 12% RH chamber for the balance of 2 h and used to bombard *E. coli* cells. These treatments were compared to incubation at 12% RH for 2 h and incubation at 12% RH for 1 h followed by incubation at 96.9% RH for 1 h. Plates were spread with 2×10^9 cells of *E. coli*, bombarded on TDO medium plus 0.6 M-sorbitol, and were incubated at 37 °C for 24 h before the number of transformants was quantified.

Optimization of biological parameters. The biological parameters tested were cell growth phase, concentration of osmoticum in the bombardment medium and cell density on the bombardment plate. Cells were grown for 6, 15 and 24 h, and then equal numbers of cells were spread on TDO medium plus 0.6 M-sorbitol. Ten replicates per treatment were used and the experiment was repeated three times. Next, TDO medium containing various concentrations of sorbitol (0.3,

0.5, 0.6 and 0.7 M) or mannitol (0.4, 0.5, 0.6 and 0.75 M) in the bombardment medium were compared to transformation rates on medium with no extra osmotic agent added. Also, the effect of cell density on transformation rates was studied; 7.5×10^8 , 1.0×10^9 , 2.0×10^9 and 3.0×10^9 c.f.u. per plate from 15 h LB broth cultures were spread on bombardment medium. Ten replicates per treatment were used.

Transformation of other bacterial species. Particle accelerator conditions were optimized for *E. coli* and then these optimum conditions were used to transform *Erwinia amylovora*, *Erwinia stewartii*, *P. syringae* pv. *syringae* and *A. tumefaciens*. Bacteria were grown in liquid culture overnight, centrifuged, the bacterial pellet resuspended in water and the cell density determined spectrophotometrically. On each plate 2×10^9 c.f.u. were spread. The cells were bombarded at 1000 p.s.i., 6 cm particle flight distance and 1 cm gap distance with DNA-coated M5 tungsten particles. The M5 tungsten particles were coated using the standard protocol (Shark *et al.*, 1990). *Erwinia amylovora*, *P. syringae* pv. *syringae* and *A. tumefaciens* were spread on LB medium containing either Ap, Tc or Kn to select for transformation with pUC118, pLAFR3 or pR89, respectively. *Erwinia stewartii* was spread on LB medium containing either Tc or Ap to select for transformation with pLAFR3 or pKP201A, respectively.

The bombardment media used for *Erwinia amylovora* and *Erwinia stewartii* for selection of transformants were LB medium plus 0.05 M-sorbitol and 100 µg Ap ml⁻¹, and LB medium plus 0.5 M-sorbitol and 20 µg Tc (pLAFR3) or Ap (pKP201A) per ml, respectively. The bombardment media for *P. syringae* pv. *syringae* and *A. tumefaciens* were LB medium plus 0.5 M-sorbitol and 15 µg Tc ml⁻¹ and LB medium plus 0.5 M-sorbitol and 50 µg Kn ml⁻¹, respectively.

Results

Transformation of diverse bacterial species

In addition to *E. coli*, four different Gram-negative bacterial species were transformed with the biolistic process (Table 3). An initial experiment for each species tested the effect of a range of different concentrations of

Table 3. Relative transformation rates and osmoticum concentrations for different bacterial species using conditions optimized for biolistic transformation of *E. coli* JA221

Strain	Plasmid	Sorbitol concn (M)	Approx. no. of transformants per plate*
<i>E. coli</i> JA221	pKRS101	0.60	10 ⁴
<i>Erwinia stewartii</i> DC283	pLAFR3	0.50	10 ¹
	pKP201A	0.50	10 ¹
	pUC118	0.05	10 ²
<i>Erwinia amylovora</i> E4001A	pR89	0.50	10 ¹
<i>P. syringae</i> pv. <i>syringae</i> B86-7	pLAFR3	0.50	10 ²

* Tungsten coated with 0.8 µg DNA per plate.

Fig. 1. Agarose gel electrophoresis of plasmid DNA isolated from wild-type and transformants of *E. coli* JA221. Plasmid DNA was isolated from strain JA221 (lane 2) and three *trp*⁺, Ap^r transformants of JA221 (lanes 3-6). λ HindIII markers (lane 1) and CsCl purified pKRS101 (lanes 7 and 8) were included as controls. DNA in lanes 4-6 and 8 were restricted with HindIII for 4 h before electrophoresis (12 h, 20 V).

Fig. 2. Size distribution of M5 and M10 tungsten particles and approximate size distribution of 1 µm gold (Au) particles (Dupont).

osmotic agents within the selective media. *P. syringae* pv. *syringae* and *A. tumefaciens* had difficulty growing at 0.6 M-sorbitol or higher, and the selection of *Erwinia amylovora* transformants broke down at 0.6 M-sorbitol. However, in all cases transformation rates were higher on medium with osmoticum compared to medium with no osmoticum.

Transformation of ten putative *E. coli* transformants was confirmed by growth on selective medium (TDO, LB plus Ap) and visualization of plasmid DNA by agarose gel electrophoresis (Fig. 1). All putative transformants which were tested contained pKRS101. Biolistic transformation of *Erwinia amylovora*, *Erwinia stewartii*, *P. syringae* pv. *syringae* and *A. tumefaciens* was confirmed in each case by growth on selective medium and visualization of plasmids as described above. All putative transformants tested contained their respective plasmids.

Controls consisted of DNA-coated tungsten mixed with cells and subjected to vacuum only. There were no transformants due to spontaneous uptake of DNA on such control plates in any of the experiments, with the exception of early *E. coli* experiments. When *E. coli* cells in the presence of DNA-coated tungsten or DNA were simply subjected to a helium blast, a small number (<5 per plate) of transformants was produced (Table 2).

Particle parameters

Particle size, number of microprojectiles, and DNA concentration per coating event were the major variables that affected biolistic transformation of *E. coli*. To determine which particle size gave the highest transformation rates, we compared the number of transformants produced by M5, M10 and gold particles. M5 particles are characterized by a mean diameter of 0.771 µm, a median of 0.362 µm, and a mode in the range of 0.1-0.2 µm (Fig. 2). M10 particles are characterized by a

Table 4. *Effect of DNA quantity on biolistic transformation of E. coli JA221 with plasmid pKRS101*
Quantities of tungsten, spermidine and CaCl_2 remained constant between treatments.

DNA per bombardment (μg)	Mean no. of transformants per plate*			
	Expt 1	Expt 2	Expt 3	Expt 4
1.2	588 a	69 c	—	—
0.8	852 a	63 c	3579 a	2195 a
0.6	230 a	238 b	1200 c	—
0.4	148 a	304 b	2172 a	616 b
0.2	2527 a	1122 a	3164 a	3465 a
0.08	—	—	546 b	—

* Means within a column followed by the same letter are not significantly different ($P = 0.05$) from each other according to Student's *t*-test.

mean diameter of $1.07 \mu\text{m}$, a median of $0.64 \mu\text{m}$, and a mode in the range of $0.5\text{--}0.6 \mu\text{m}$. The gold particles are characterized by a tight bimodal distribution of particles, with peaks of 1.0 and $0.2 \mu\text{m}$. The M5, M10 and gold particles produced a mean number of transformants per plate of 3488 (SE = 490), 992 (241) and 142 (47), respectively. M5 particles which contain the largest quantity of particles $0\text{--}0.2 \mu\text{m}$ in size, gave significantly more transformants ($P = 0.001$) than cells bombarded with the larger M10 or gold particles. The M10 particles gave significantly more transformants per plate than gold particles ($P = 0.006$). These data are representative of repeated experiments.

We observed that there was variation in the number of transformants per plate between individual coating events (i.e. between microcentrifuge tubes) and we therefore treated each DNA precipitation as a block in our experimental design. To further reduce the amount of variation between coating events and increase transformation efficiency we examined the relationship between DNA quantity and tungsten. When the amount of DNA-coated tungsten per bombardment was increased from the standard quantity of 600 to 900 and 1200 μg per bombardment, the mean number of transformants increased from 216 to 433 and 1693, respectively. There was a significant difference in the mean number of transformants between 600 and 1200 μg per bombardment ($P = 0.001$) and between 900 and 1200 ($P = 0.002$) but not between 600 and 900 ($P = 0.17$).

The amount of DNA per coating event affected transformation efficiency. For applications involving larger cells and larger particles, $0.8 \mu\text{g}$ DNA per bombardment has proven optimal. In three of four experiments, $0.2 \mu\text{g}$ DNA per bombardment produced consistently more transformants than our standard quantity of $0.8 \mu\text{g}$ DNA, although this difference was only significant ($P = 0.05$) in experiment two because of

the large variation within treatments (Table 4). Increasing DNA quantity ($1.2 \mu\text{g}$ per bombardment) did not significantly improve transformation over use of the standard quantity. For some reason, DNA quantities of 0.6 and $0.4 \mu\text{g}$ per bombardment consistently produced fewer transformants than either 0.8 and $0.2 \mu\text{g}$ DNA.

When tungsten was pre-coated with a small quantity ($0.48 \mu\text{g}$ per coating event = quantity for six bombardments) of non-selected DNA (pUB110) and then coated with pKRS101 DNA ($4.8 \mu\text{g}$ per coating event) transformation rates increased (745, SE = 178) over use of tungsten coated with only pKRS101 ($4.8 \mu\text{g}$ per coating event) (525, SE = 228). This result was consistent in three experiments although the difference was not significant in the individual experiments. When the amount of pre-coating DNA was increased from 0.48 to $1.2 \mu\text{g}$ per coating event, transformation rates decreased (275, SE = 146).

Relative humidity (RH) affected transformation of *E. coli* (Fig. 3). When the ethanol-suspended DNA-coated tungsten was loaded and dried on flying disks at five different RH values, the transformation rates varied. Higher transformation rates were obtained when loaded flying disks were stored at the lower RHs tested (Fig. 4). The log of the mean number of transformants was regressed against percentage RH. This yielded the equation $Y = 3.43 + 0.0037X - 0.000489X^2$, in which Y equals log of the mean number of transformants and X equals percentage RH. The mean number of transformants decreased rapidly as the time at high RH (96.9%) during dry-down of the loaded membrane increased (Fig. 5). When the mean number of transformants was regressed against time at 96% RH there was an exponential decay in the number of transformants. This yielded the equation $Y = 125.7228X^{-1.3309}$, in which Y equals the mean number of transformants and X equals the time at 96.9% RH.

Fig. 3. The effect of RH surrounding the loaded flying disks during dry-down on subsequent transformation of *E. coli* JA221. Flying disks loaded with DNA-coated tungsten were stored in RH chambers for 2 h before bombardment. Cells (2×10^9 per plate) were spread on TDO plus 0.6 M-sorbitol medium, bombarded, incubated for 24 h at 37 °C and then the mean number of transformants per treatment was quantified. Five plates per treatment were bombarded.

Fig. 4. Effect of RH on transformation of *E. coli* JA221. Loaded flying disks were stored in RH chambers for 2 h before bombardment. Each point represents the mean of five replicates. Regression analysis was used to develop an equation that describes the relationship between percentage RH and number of transformants (see Results): $Y = 3.43 + 0.0037X - 0.000489X^2$, $R^2 = 0.99$, where R is the correlation coefficient.

Fig. 5. Effect of high (96.9%) RH on transformation of *E. coli* JA221. Loaded flying disks were stored at 96.9% RH for various times and then transferred to a 12% RH chamber for the balance of 2 h before bombardment. Each point represents the mean of five replicates. Regression analysis was used to develop an equation that describes the relationship between time at high RH and number of transformants (see Results): $Y = 125.7228X^{-1.3309}$, $R^2 = 0.90$.

Gun parameters

The optimal helium pressure tested for biolistic transformation of *E. coli* was 1000 p.s.i. (Table 5). Transforma-

tion rates consistently decreased at 700 and 1300 p.s.i. compared to 1000 p.s.i. The shorter particle flight distance (6 cm) from launch site to target cells gave higher transformation rates than longer distances (9.2

Table 5. *Effect of helium pressure, particle flight distance and gap distance on transformation of E. coli JA221*

Treatment*			Mean no. of transformants per plate	SE
Pressure (p.s.i.)	Distance† (cm)	Gap‡ (cm)		
700 ab	6	1.0	1118	382
1000 a	6 a	1.0 a	1671	441
1300 b	6	1.0	622	218
1000	9.2 b	1.0	540	163
1000	12.3 a	1.0	1054	148
1000	6	0.6 b	174	52
1000	6	1.4 a	1034	433

* Treatments in bold print, within a column, which are followed by the same letter are not significantly different from one another ($P = 0.10$).

† Distance between particle launch site and target cells.

‡ Distance between helium source and flying disk.

Table 6. *Effect of helium flushing of the vacuum chamber prior to bombardment on transformation of E. coli JA221*

Cell (2×10^9 per plate) were bombarded at 1000 p.s.i. helium, 1 cm gap distance, and 6 cm target cell distance. Mean values of 5–10 replicates are given.

Experiment	Mean no. of transformants per plate (SE)		Significance (P)
	Flush	No flush	
1	2142 (507)	380 (134)	0.007
2	3788 (971)	228 (108)	0.005
3	3512 (867)	781 (249)	0.012
4	866 (251)	451 (70)	0.152
5	1485 (721)	1311 (252)	0.825
6	1835 (843)	492 (133)	0.606
7	6492 (2828)	1630 (225)	0.093

and 12.3 cm). A gap distance of 1 cm produced more transformants than 0.6 and 1.4 cm.

Helium flush of the vacuum chamber prior to bombardment significantly increased transformation in *E. coli* ($P = 0.10$) in four of seven experiments where this was tested (Table 6). Helium flush consistently produced higher transformation rates whenever tested.

Use of the baffle to protect cells from acoustic shock and helium gas blast at higher pressures decreased the area but did not eliminate the mortality of cells at the centre of the bombarded plates. Use of the baffle (mean = 3056 transformants per plate, SE = 914) significantly increased transformation rates ($P = 0.05$) at very high pressures, compared to such treatment without baffle (442 transformants per plate, SE = 270).

Biological parameters

In *E. coli*, more transformants were produced on medium containing 0.6 M-sorbitol (1315 transformants per plate, SE = 505) than medium containing no such osmoticum (664, SE = 206; $P = 0.26$). More transformants were produced on bombardment medium containing 0.6 M-sorbitol (see above) rather than an equal concentration of mannitol (366, SE = 92; $P = 0.102$). Also, the greatest number of transformants were produced on bombardment medium containing 0.6 M-sorbitol. A concentration of 0.7 M-sorbitol (132, SE = 54) reduced the mean number of transformants compared to 0.6 M-sorbitol ($P = 0.079$). These data are representative of three experiments.

Equal numbers of cells per plate from exponential (6 h), late exponential (15 h) and stationary (24 h) cultures produced 1540, 1716 and 1697 cells per plate, respectively. There was no significant difference with respect to the mean number of transformants per plate between cell ages tested ($P = 0.05$).

Transformation rates increased as cell density increased from 7.5×10^8 to 3×10^9 cells per plate (Table 7). Although 3×10^9 *E. coli* cells per plate gave higher transformation rates, 2×10^9 c.f.u. per plate produced transformed colonies which were more discrete and easier to quantify.

Discussion

Efficient transformation of *E. coli* is not new, but a simple standardized method for transformation of essentially any bacterial species would be novel and useful. Electroporation comes close to this and has

Table 7. Effect of cell density on transformation of *E. coli* JA221

Cells per plate	Mean no. of transformants per plate (SE)				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
7.5×10^8	2395 (777)	56.2 (23)	—	—	—
1.0×10^9	1951 (597)	114.8 (105)	188 (117)	249 (210)	294 (154)
2.0×10^9	1579 (374)	133.4 (39)	650 (374)	315 (88)	1440 (111)
3.0×10^9	4851 (1584)	722.0 (374)	—	—	—

enabled transformation of some previously impossible or hard to transform species. However, complex pretreatment of cells is sometimes required with electroporation, and optimization of conditions for each bacterial species can be time consuming, whilst transformation using large plasmids can be impossible. Using the biolistic process and conditions optimized for *E. coli*, we were able to readily transform four additional bacterial species with little additional investment of time. It has been possible to introduce DNA into *A. tumefaciens*, *Erwinia amylovora* and *P. syringae* pv. *syringae* by transformation via freeze thaw (Holsters *et al.*, 1978), treatment with divalent cations (Bauer & Beer, 1983) and conjugation using the triparental mating system (Ditta *et al.*, 1980), respectively. However, *Erwinia stewartii* has been impossible to transform with standard *E. coli* methods. Low transformation rates can be achieved by electroporation (D. Coplin, personal communication). In this study, transformation rates were not extremely high (<200 transformants per plate per 0.8 µg DNA) for these bacterial species, but the straight-forward, successful transformation of each additional species we have tested suggests real utility for biolistic transformation of bacteria.

Small amounts of CaCl₂ from the tungsten coating solution initially resulted in some background transformation (50 transformants per plate) in our early *E. coli* experiments. Such background transformation could be prevented by washing the coated tungsten particles in 70% ethanol before resuspending them in 100% ethanol. Additionally, the impact of the helium shock on the cell surface which was in contact with either DNA or DNA-coated particles produced a low background rate of transformation (<5 transformants per plate). Apparently, the helium blast affected the cell membrane or injured the cells in such a way that a few cells were transformed. The standard control which we included in all experiments consisted of the appropriate amounts of tungsten coated with DNA and cells mixed together, spread on the plates, and then subjected to vacuum only.

Because M5 particles yielded the most transformants, it appears that very small particles (0.1–0.2 µm) are most

effective in *E. coli* transformation by particle bombardment. It might be argued that the increased transformation with M5 was due to more individual particles hitting target cells, because the smaller M5 particles contain more individual particles per gram of tungsten. However, two additional lines of evidence suggest that it is the very small particles which are abundant in M5 tungsten that are required to transform bacteria. We have found that bacterial cells uniquely require much shorter flight distances and helium flushing of the chamber to achieve optimal results. The need for a shorter particle flight distance is consistent with the idea that the small particles are responsible for biolistic transformation of *E. coli*, since a smaller particle would lose velocity faster than a larger particle. Flushing the vacuum chamber with helium prior to drawing a vacuum leaves helium as the residual gas. Since helium is a light gas there is less drag on the particles than if air was the residual gas. Interestingly, helium flushing does not increase transformation rates of NT1 tobacco cells, which are transformed with 1 µm tungsten particles (M10) (Sanford *et al.*, 1991b).

E. coli is an ideal model system because it is easy to handle and the turnover time for experiments is rapid. Thus, we can rapidly determine the effect that changes in the gun design, and environmental and physical factors have on transformation efficiency. For example, using *E. coli* we were able to determine that low relative humidity during dry-down of coated particles onto the flying disk was critical for achieving high transformation rates. Also the effect of particle size, DNA quantity per coating event, quantity of coated tungsten per bombardment and of precoating tungsten particles on transformation efficiency could be most readily determined using *E. coli*.

Our laboratory and others, have experienced decreased rates of transformation during the summer. One factor that might have been affected by such environmental changes would be the effect of RH during dry-down of DNA-coated tungsten on the flying disk surface. We observed that particles would not dry on the flying disks as rapidly at higher RHs (>30%) typical of the summer months, apparently due to the hygroscopic

nature of 100% ethanol. The experiments reported here prove that RH reduces rates of transformation. It is not just the completeness of drying of the coated particles which affects transformation, since particles stored for as little as 5 min at 97% RH and then transferred to 12% RH for 110 min prior to bombardment produced four times less transformants per plate than those stored at 12% RH for 120 min. Rather, particles dried down on the flying disks at high RH tend to clump together while those dried at low RH remained dispersed on the flying disk. This aggregation appears to be irreversible, hence dried down particles should at no point prior to bombardment be allowed to absorb water.

We believe a portion of DNA is irreversibly bound to the tungsten particles and is not useful for transformation, while the remaining DNA comes off the particles inside the target cells. We reasoned that precoating tungsten with a small quantity of non-selected DNA prior to coating with the marker plasmid, would improve transformation rates because the more loosely bound marker DNA might be more available inside the cells after penetration. Such loosely bound DNA might be more effective for transformation of a monolayer of bacterial cells, but might not be effective for plant transformation where penetration of multiple cell-layers is required. However, the addition of too much DNA had been noted to cause clumping of the particles (Klein *et al.*, 1988). We found that the relationship between quantity of DNA versus number of transformants per plate is not linear but appears bimodal. Perhaps 0.2 µg DNA per bombardment is sufficient to coat the smaller particles optimally.

When *E. coli* cells were bombarded using higher pressures (2400 p.s.i. helium), colonies grew up in a ring, surrounding a 'zone of death'. This phenomenon can be due to tungsten toxicity, or to cell injury caused by acoustic shock, helium gas blast, or a high particle/cell ratio concentrated at the centre of the plate (Russell *et al.*, 1992). Our experiments with a baffle system reduced the size of the 'zone of death' and the mean number of transformants in the baffle treatments was higher than without the baffle, which suggests that the 'zone of death' in *E. coli* is largely due to the acoustic shock and helium blast. Although higher pressures and use of a baffle system are not necessary to produce high transformation rates in *E. coli*, higher velocities (and hence higher pressures and baffles) may be necessary for biolistic transformation in other microbial systems.

The 'pagar' cell-handling system was developed to conveniently allow newly transformed *E. coli* cells injured from particle bombardment sufficient time to recover and time to express the antibiotic resistance before being challenged with the antibiotic. In *E. coli*, the pagar system was not necessary, and direct selection with

Ap included in the bombardment medium could be used. The best use of the pagar system is for species in which a high osmoticum is necessary for transformation but not cell growth, or where cells need time to express a gene prior to selection. Cells could be spread on a pagar that had a high osmoticum concentration, then subsequently could be transferred to selective medium without osmoticum, thereby gently reducing osmoticum concentration.

Biolistic transformation of a Gram-positive bacterium, *Bacillus megaterium* was previously shown (Shark *et al.*, 1990) and now biolistic transformation of five different Gram-negative bacteria has been demonstrated. Conditions optimized for *E. coli* transformation were used to transform *Erwinia amylovora*, *Erwinia stewartii*, *A. tumefaciens* and *P. syringae* pv. *syringae*. If higher transformation rates are required in a new species, only a few simple experiments are required for optimizing transformation. A simple series of optimization experiments are described by Sanford *et al.* (1991b) and involve optimization of osmoticum concentration, gun parameters, growth phase and cell density.

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