Transformation of *Xanthomonas campestris* pathovar *campestris* with Plasmid DNA

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Procedures for the introduction of plasmid DNA into Gram-negative bacteria have been adapted and optimized to permit transformation of the plant pathogen *Xanthomonas campestris* pathovar *campestris* with the cloning vector pKT230 and other broad-host-range plasmids. The technique involves CaCl_2 -induced competence and heat shock and is similar to that routinely used for *Escherichia coli*. Wild-type *X. c. campestris* strains appear to restrict incoming unmodified DNA, so that plasmid DNA for transformation must be prepared from *X. c. campestris* (into which it has previously been introduced by conjugation). To overcome this disadvantage a restriction-deficient mutant has been isolated.

INTRODUCTION

The introduction of plasmid DNA into bacterial cells by transformation is one of the most important elements of recombinant DNA technology, and optimized procedures for efficient transformation of a number of species have been devised (Saunders *et al.*, 1984). However, considerable variation is observed in the ability of different species or strains to be transformed, and the conditions required for hitherto unstudied organisms can only be established by trial and error.

The molecular genetics of plant pathogenic bacteria has been of increasing interest in recent years (Panopoulos & Peet, 1985; Chatterjee & Vidaver, 1986), and recombinant DNA procedures have been applied in order to identify and characterize genes involved in pathogenicity. Transformation has been described for only a small number of pathogen species (Chatterjee & Vidaver, 1986) and the absence of suitable procedures means that cloned genes can only be transferred into pathogens by conjugation. Not only is this more time-consuming but it also prevents the construction of non-transmissible disabled vectors, rendering biological containment of recombinant plasmids more difficult.

In this laboratory *Xanthomonas campestris* pathovar *campestris* (hereafter *X. c. campestris*), the causal agent of black rot of crucifers (Williams, 1980), has been used as a model for molecular genetic studies of pathogenicity (Daniels *et al.*, 1984*a, b*; Turner *et al.*, 1985; Osbourn *et al.*, 1987). Gabriel (1984) reported transformation of *X. c. malvacearum* with an indigenous plasmid, but earlier reports of transformation with chromosomal DNA in the genus are difficult to interpret (Corey & Starr, 1957*a, b*; Yamasaki *et al.*, 1966).

In this paper we describe a simple procedure for transformation of *X. c. campestris* with broad-host-range plasmid DNA, similar to procedures used routinely for *Escherichia coli*. An endogenous restriction system limits transformation with unmodified DNA and, in order to avoid this problem, a restriction-deficient mutant has been isolated.

METHODS

Bacterial strains and culture conditions. *X. c. campestris* 8004, a rifampicin-resistant derivative of strain NCPPB 1145 (Turner *et al.*, 1984), was used for most experiments; strains BM57R [a spontaneous rifampicin-

resistant mutant of NRRL B-1459 (Jeanes *et al.*, 1961)] and *X. c. translucens* XT02 (Sawczyk, 1986) were used as noted. Bacteria were cultured at 30 °C or 32 °C in NYGB medium (Turner *et al.*, 1984). *E. coli* ED8767 (Murray *et al.*, 1977) was cultured at 37 °C in NYGB or L broth (Miller, 1972).

Plasmid DNA preparation. Plasmids were isolated from *E. coli* or *X. c. campestris* strains using the alkaline lysis procedure of Birnboim & Doly (1979) and were purified by CsCl/ethidium bromide buoyant density centrifugation. Crude plasmid preparations from transformants were digested with *Eco*RI and electrophoresed in 0.8% (w/v) agarose gels (Maniatis *et al.*, 1982).

Transformation procedure. A sample (2.5 ml) of an overnight culture of *X. c. campestris* was added to 50 ml pre-warmed NYGB in a 250 ml Erlenmeyer flask and the flask was incubated in an orbital shaker (30 °C, 200 r.p.m.). Growth was monitored by measuring the optical density at 600 nm using a Unicam SP1700 spectrophotometer. When the OD₆₀₀ reached a value of 0.4, corresponding to a cell density of 8×10^8 c.f.u. ml⁻¹, the culture was rapidly chilled in ice and the bacteria were harvested by centrifugation at 4 °C in a Sorvall RC 5B centrifuge (8000 g, 5 min). The cell pellet was washed successively at 4 °C with 0.1 M-MgCl₂ and 0.1 M-CaCl₂ and finally resuspended in 2 ml 0.1 M-CaCl₂. The suspension was kept in ice for at least 30 min before use. DNA samples in either 10 or 100 µl 10 mM-Tris/HCl, pH 8.0, containing 1 mM-Na₂EDTA, were dispensed in chilled sterile microcentrifuge tubes and 200 µl portions of the competent cell suspension were added, rapidly mixed, and the tubes returned to the ice bath for 45 min, before transfer to a water bath (37 °C, 5 min) and cooling to room temperature. The suspensions were added to 25 ml universal bottles containing 1 ml NYGB supplemented with either rifampicin (for *X. c. campestris*) or spectinomycin (for *X. c. translucens*) and shaken (30 °C, 5 h) to permit expression of genes on the transformed plasmids, before portions were plated on NYG agar containing antibiotics appropriate to the strain and the transforming plasmid. Antibiotics were used at the following final concentrations (µg ml⁻¹): chloramphenicol, 25; kanamycin, 25; rifampicin, 50; spectinomycin, 50; streptomycin, 50; tetracycline, 5. Colonies were examined after incubation for 2 d at 32 °C.

Storage of competent cells. A procedure essentially as described by Morrison (1977) was used. Bacteria were harvested by centrifugation, washed successively with 0.1 M-MgCl₂ and 10 mM-CaCl₂, suspended in 60 mM-CaCl₂ containing 15% (v/v) glycerol, rapidly frozen in acetone/solid CO₂, and stored at -70 °C.

RESULTS AND DISCUSSION

The IncQ plasmid pKT230 (Bagdasarian *et al.*, 1981) encoding resistance to kanamycin and streptomycin was used for most experiments. We have found that this plasmid can be stably maintained in *X. c. campestris* after introduction by conjugation from *E. coli* using the helper plasmid pRK2073 (Leong *et al.*, 1982), and moreover the plasmid has a moderate size (11.9 kb) rendering it suitable for transformation. The efficiency of transformation decreases with increasing plasmid size, and the commonly used broad-host-range IncP plasmids, which can also be transferred to *X. c. campestris* (Turner *et al.*, 1984), are much larger than the IncQ family.

Initial attempts to transform *X. c. campestris* with pKT230 DNA purified from *E. coli* were unsuccessful, using the CaCl₂ competence-inducing procedure described above or alternative procedures incorporating RbCl solution (Bagdasarian & Timmis, 1981) or freezing and thawing (Holsters *et al.*, 1978). We suspected that the failure was due to a restriction system in *X. c. campestris* causing degradation of the unmodified incoming DNA. Although there are no reports of restriction endonucleases produced by *X. c. campestris* many other members of the genus are known to produce these enzymes (Kessler & Höltke, 1986). We therefore introduced pKT230 into *X. c. campestris* by conjugation using methods described by Daniels *et al.* (1984b) and Turner *et al.* (1984). Since conjugational transfer of plasmids proceeds via single-stranded forms the DNA is less susceptible to degradation before modification than when taken up in the double-stranded form as in transformation. Plasmid DNA, presumably modified to give protection from the putative restriction endonuclease, was purified from an *X. c. campestris* pKT230 transconjugant strain and was successfully used to transform *X. c. campestris* 8004 using the three methods mentioned above. Since the CaCl₂ method was simpler and gave more transformants than either the RbCl or the freeze-thaw methods, the latter were abandoned. The CaCl₂ procedure was optimized to derive the conditions described in Methods. The effects on transformation frequency of some deviations from the procedure are shown in Table 1.

The stage of growth at which bacteria are harvested affects transformation efficiency (Saunders *et al.*, 1984). For a constant DNA concentration (1 µg per sample) the greatest number of transformants, about 400, was obtained when *X. c. campestris* cultures were harvested at an

Table 1. *Effect of some variations in the procedure on the efficiency of transformation of X. c. campestris with pKT230 DNA*

Portions (200 μ l) of a cell suspension (about 10^9 c.f.u. per portion) were transformed with 1 μ g modified pKT230 DNA, using the procedure described in Methods, with variations as indicated. A yield of 100% obtained under optimum standard conditions corresponded to 850 transformants per sample.

Condition	Yield of transformants compared with standard conditions (%)
Temperature of heat shock ($^{\circ}$ C, 5 min):	
0	0 (i.e. <0.1)
30	69
37	100
42	50
50	0
Duration of heat shock (min, 37 $^{\circ}$ C):	
0	0
0.5	28
2	69
5	100
10	54
Concn of CaCl ₂ used for suspending bacteria (mM):	
0 (100 mM-MgCl ₂ substituted)	0
10	1
20	3
40	6
60	35
80	92
100	100
150	34

OD₆₀₀ value of 0.4. However the number of transformants per cell declined steadily throughout growth. In a typical experiment bacteria were harvested for transformation at OD₆₀₀ values of 0.02, 0.1, 0.37, 0.7 and 1.0 and yielded, respectively, 900, 460, 290, 39 and 3 transformants per 10^9 cells.

The total number of transformants rose steadily as the DNA concentration increased, up to the highest level tested (10 μ g per sample), but the yield of transformants per μ g DNA declined when amounts greater than 0.1 μ g were used.

Since *X. c. campestris* grows more slowly than *E. coli* we anticipated that a longer period of non-selective growth might be necessary after transformation to allow expression of antibiotic resistance genes, prior to plating on selective media. The number of resistant colonies which developed increased as the expression time was increased from 1 to 20 h. However, the cell concentration also increased during this period and it was found that the number of transformants per cell reached a maximum value after 5 h and thereafter remained approximately constant. A 5 h incubation period was therefore used routinely to give the maximum yield of transformants while reducing the chance of isolating siblings. Similar numbers of transformants were recovered when samples were plated on media containing either streptomycin, kanamycin or both antibiotics. Of a random sample of 50 streptomycin-resistant colonies, 88% were also resistant to kanamycin, and of a similar sample of kanamycin-resistant colonies, 100% were also resistant to streptomycin. The 12% streptomycin-resistant, kanamycin-sensitive colonies were presumably spontaneous streptomycin-resistant mutants, which occur in *X. c. campestris* cultures at a frequency of about 10^{-9} ; spontaneous kanamycin-resistant mutants have never been isolated (i.e. frequency $<10^{-11}$, M. J. Daniels, unpublished).

The presence of pKT230 in the transformants was verified by agarose gel electrophoresis of

*Eco*RI-digested cleared lysates, which revealed a single DNA band with the electrophoretic mobility of authentic cleaved pKT230 DNA, and the lysates could be used to transform *E. coli* to resistance to streptomycin and kanamycin.

All optimization experiments were done at least twice and within experiments all sampling was in duplicate or triplicate. The yield of transformants between experiments varied by a factor of about ten, from 10^2 to 10^3 per μg DNA, but replicate transformations of the same batch of cells gave numbers of transformants varying by no more than 1–2%. The procedure was successfully used to introduce several other broad-host-range plasmids into *X. c. campestris*, using cleared lysates from strains into which the plasmids had previously been introduced by conjugation. The plasmids were pKT210 (11.9 kb, Bagdasarian *et al.*, 1981), pIJ3109 (14.2 kb, Osbourn *et al.*, 1987), pLAFR3 (22 kb, B. J. Staskawicz, unpublished) and pPH1JI (54.7 kb, Hirsch & Beringer, 1984), and transformants were selected with streptomycin plus chloramphenicol, streptomycin, tetracycline, and chloramphenicol, respectively. It was not possible to estimate the relative transformation efficiencies of the several plasmids because the DNA concentrations in the crude lysates were unknown. No transformants were obtained with either linear or single-stranded pKT230 DNA.

X. c. campestris BM57R could be transformed with modified pKT230 DNA with an efficiency comparable to that of strain 8004, but *X. c. translucens* XT02 could not, suggesting that this pathovar has a different restriction system which can cleave DNA modified by passage through *X. c. campestris*.

It is convenient to be able to store competent cells in a frozen state for future use. Addition of sterile glycerol (final concentration 5–20%, v/v) to competent cells prepared by the standard method and storage at -70°C was successful, but best results were obtained with the method of Morrison (1977), which allowed storage for at least two months with no loss of transforming efficiency (using pKT230) compared with freshly prepared cells. Protocols 1, 2 and 3 of Hanahan (1985) were unsatisfactory for *X. c. campestris*; although frozen cells retained viability, the transformation efficiency was only about 1% of that obtained with comparable cells prepared by the method of Morrison (1977).

Although the ability to transfer plasmids between *X. c. campestris* strains by a one-step transformation procedure results in a useful saving of time (3–4 d against 5–6 d) compared with the standard two-stage procedure involving transformation of *E. coli* and subsequent conjugational transfer to *X. c. campestris* (Daniels *et al.*, 1984b), the versatility of the method would be greatly increased if the restriction barrier preventing transformation with unmodified DNA could be overcome. In the course of a number of experiments comparing pKT230 DNA samples prepared from either *X. c. campestris* or *E. coli*, no *X. c. campestris* transformants were obtained with DNA from the latter, implying that the transformation efficiency differs by a factor of 10^3 – 10^4 . *X. c. campestris* 8004 was mutagenized with ethyl methanesulphonate as described by Miller (1972) and competent cells were prepared from a subcultured pool of survivors. One-tenth of the suspension was treated with modified pKT230 DNA (purified from *X. c. campestris*) and yielded the expected number of transformants; the remainder was exposed to unmodified DNA purified from *E. coli* and yielded a single colony resistant to streptomycin and kanamycin. In order to verify that the strain was transformable with unmodified pKT230 DNA it was necessary to isolate a derivative which had lost the plasmid. This was achieved by shaking a subculture at 37°C for 5 h and then plating suitable dilutions non-selectively at 32°C to give single colonies, 200 of which were tested for sensitivity to kanamycin and streptomycin. *X. c. campestris* is unable to grow at 37°C , although viability is maintained for at least 20 h, and we suspected that the stressed condition of the cells might promote plasmid loss. About 30% of the colonies were sensitive to both antibiotics and were assumed to have lost the plasmid. One plasmid-free clone, designated 8004R, was taken for further study and was found to be transformable with equal efficiencies using either modified or unmodified pKT230 and pKT210 DNA. We believe that 8004R is a restriction-deficient mutant, and should be a useful working strain for future transformation experiments. The mutant retains pathogenicity to turnip seedlings, tested by the method of Daniels *et al.* (1984a).

Although the transformation procedure which we have described is unlikely to be efficient

enough to use in experiments requiring mass transfer into *X. c. campestris* strains of genomic libraries containing many thousands of clones (Daniels *et al.*, 1984b), it should considerably expedite the construction of strains carrying specific plasmids.

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REFERENCES

- BAGDASARIAN, M. & TIMMIS, K. N. (1981). Host: vector systems for gene cloning in *Pseudomonas*. In *Current Topics in Microbiology and Immunology*, pp. 47-67. Edited by P. H. Hofschneider & W. Goebel. Berlin & Heidelberg: Springer Verlag.
- BAGDASARIAN, M., LURZ, R., RÜCKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M., FREY, J. & TIMMIS, K. N. (1981). Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number RSF1010-derived vectors and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237-247.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1523.
- CHATTERJEE, A. K. & VIDAVER, A. K. (1986). Genetics of pathogenicity factors: application to phytopathogenic bacteria. *Advances in Plant Pathology* **4**, 1-218.
- COREY, R. R. & STARR, M. P. (1957a). Genetic transformation of colony type in *Xanthomonas phaseoli*. *Journal of Bacteriology* **74**, 141-145.
- COREY, R. R. & STARR, M. P. (1957b). Genetic transformation of streptomycin resistance in *Xanthomonas phaseoli*. *Journal of Bacteriology* **74**, 146-150.
- DANIELS, M. J., BARBER, C. E., TURNER, P. C., CLEARY, W. G. & SAWCZYC, M. K. (1984a). Isolation of mutants of *Xanthomonas campestris* pv. *campestris* showing altered pathogenicity. *Journal of General Microbiology* **130**, 2447-2455.
- DANIELS, M. J., BARBER, C. E., TURNER, P. C., SAWCZYC, M. K., BYRDE, R. J. W. & FIELDING, A. H. (1984b). Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pathovar *campestris* using the broad host range cosmid pLAFR1. *EMBO Journal* **3**, 3323-3328.
- GABRIEL, D. W. (1984). Plasmid transformation of *Xanthomonas campestris* pv. *malvacearum*. *Phytopathology* **74**, 837.
- HANAHAN, D. (1985). Techniques for transformation of *Escherichia coli*. In *DNA Cloning*, vol. 1, pp. 109-135. Edited by D. M. Glover. Oxford: IRL Press.
- HIRSCH, P. R. & BERINGER, J. B. (1984). A physical map of pPH1J1 and pJB4J1. *Plasmid* **12**, 139-141.
- HOLSTERS, M., DE WAELE, D., DEPICKER, A., MESSENS, E., VAN MONTAGU, M. & SCHELL, J. (1978). Transfection and transformation of *Agrobacterium tumefaciens*. *Molecular and General Genetics* **163**, 181-187.
- JEANES, A., PITTSLEY, J. E. & SENTI, F. R. (1961). Polysaccharide B-1459: a new hydrocolloid polyelectrolyte produced from glucose by bacterial fermentation. *Journal of Applied Polymer Science* **5**, 519-526.
- KESSLER, C. & HÖLTKE, H.-J. (1986). Specificity of restriction endonucleases and methylases - a review (edition 2). *Gene* **47**, 1-153.
- LEONG, S. A., DITTA, G. S. & HELINSKI, D. R. (1982). Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for δ -aminolevulinic acid synthetase from *Rhizobium meliloti*. *Journal of Biological Chemistry* **257**, 8724-8730.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MILLER, J. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MORRISON, D. A. (1977). Transformation in *Escherichia coli*: cryogenic preservation of competent cells. *Journal of Bacteriology* **132**, 349-351.
- MURRAY, N. E., BRAMMER, W. J. & MURRAY, K. (1977). Lambdoid phages that simplify the recovery of *in vitro* recombinants. *Molecular and General Genetics* **150**, 53-61.
- OSBOURN, A. E., BARBER, C. E. & DANIELS, M. J. (1987). Identification of plant-induced genes of the bacterial pathogen *Xanthomonas campestris* pathovar *campestris* using a promoter-probe plasmid. *EMBO Journal* **6**, 23-28.
- PANOPOULOS, N. J. & PEET, R. C. (1985). The molecular genetics of plant pathogenic bacteria and their plasmids. *Annual Review of Phytopathology* **23**, 381-419.
- SAUNDERS, J. R., DOCHERTY, A. & HUMPHREYS, G. O. (1984). Transformation of bacteria by plasmid DNA. *Methods in Microbiology* **17**, 61-95.
- SAWCZYC, M. K. (1986). *Pathogenicity determinants of Xanthomonas campestris - a comparative study*. PhD thesis, University of East Anglia, Norwich.
- TURNER, P., BARBER, C. & DANIELS, M. (1984). Behaviour of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pv. *campestris*. *Molecular and General Genetics* **195**, 101-107.
- TURNER, P., BARBER, C. & DANIELS, M. (1985). Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. *Molecular and General Genetics* **199**, 338-343.
- WILLIAMS, P. H. (1980). Black rot: a continuing threat to world crucifers. *Plant Disease* **64**, 736-742.
- YAMASAKI, Y., MURATA, N. & SUWA, T. (1966). Restoration of lost virulence of leucine-requiring mutants of *Xanthomonas oryzae* by DNA-mediated transformation. *Proceedings of the Japan Academy* **42**, 946-949.