Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4964–4968, July 1987
Genetics

Transformation of *Corynebacterium diphtheriae, Corynebacterium ulcerans, Corynebacterium glutamicum,* and *Escherichia coli* with the *C. diphtheriae* plasmid pNG2

(protoplast formation/transfection/heterospecific plasmid replication/erythromycin resistance)

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Communicated by Stanley Falkow, April 13, 1987

ABSTRACT **The transfection and transformation of members of two species of pathogenic corynebacteria, *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*, is described. Protoplasts were produced by treatment with lysozyme following growth in glycine, and a medium was defined on which a significant fraction of the osmotically sensitive cells were regenerated. Transfections were carried out with DNA from corynephage 782, a member of the β family of converting phages, and transformations were performed with DNA of plasmid pNG2, a 9500-kDa plasmid that was isolated from an erythromycin-resistant strain of *C. diphtheriae* and carries the resistance gene. Strains of *Corynebacterium glutamicum* and *Escherichia coli* were also successfully transformed with pNG2 DNA. Transfection frequencies were in the range of 3–8 × 10^3 plaque-forming units/μg of phage DNA, and transformation frequencies were in the range of 0.2–150 colony-forming units/μg of plasmid DNA. Plasmid pNG2 replicated and was stably maintained in all transformants both in the presence or absence of erythromycin. Thus, it displayed the ability to replicate in strains of both Gram-positive and Gram-negative bacteria without the intervention of genetic engineering. pNG2 DNA isolated from any of the transformed strains was able to transform all parental strains. The host range of pNG2 suggests its possible utility in or as a shuttle vector for the study and manipulation of genes from corynebacterial strains of animal origin.**

The discovery of conversion to toxinogeny in *Corynebacterium diphtheriae* identified members of the β family of corynebacteriophages as carriers of the gene *tox* for diphtheria toxin. This provided a natural clone for the *tox* gene and a basis for its genetic analysis (1, 2). Since that discovery, a method has been sought that would permit broader genetic studies of *C. diphtheriae* as well as other pathogenic bacteria of this genus. However, conversion to toxinogeny and a single report of generalized transduction in *Corynebacterium renale* (3) remain the only published reports of genetic exchanges between animal pathogens of this genus.

For the past few years we have been working to develop a protocol for transformation of *C. diphtheriae* and *Corynebacterium ulcerans*. Our interest in this project was renewed with the isolation and characterization of *C. diphtheriae* plasmid pNG2 (4–6). This plasmid carries a gene for erythromycin-resistance and thus provides a defined piece of autonomously replicating and selectable DNA. In addition a number of reports of transformation in the soil organism *Corynebacterium glutamicum* and in closely related *Brevibacterium* species have been published (7, 8) demonstrating that transformation was possible in these genera. Nevertheless, major roadblocks remained, namely a method for making *C. diphtheriae* or *C. ulcerans* into protoplasts so they would be permeable to DNA and defining a medium on which osmotically-sensitive cells could be regenerated into normally growing cells. These technical problems have now been solved, and two significant findings are reported in the present study. First, we have successfully transformed selected strains of *C. diphtheriae* and *C. ulcerans* as well as *C. glutamicum* with plasmid pNG2 DNA. Second, we have shown that plasmid pNG2 can replicate autonomously not only in Gram-positive *Corynebacterium* species but also in Gram-negative *Escherichia coli*.

MATERIALS AND METHODS

Bacterial Strains and Media. *C. diphtheriae (belfanti)* 1030, *C. ulcerans* 712, *C. ulcerans* 712(782) a lysogen carrying phage 782, *C. glutamicum* ATCC 13032, and *E. coli* JM109, a restriction minus strain, were from our stock collection.

C. diphtheriae strains were cultured on heart infusion agar (Difco) supplemented with 0.2% Tween 80 (HITW agar). *C. ulcerans* and *C. glutamicum* were grown on tryptose/yeast extract plates containing 10 g of tryptose, 5 g of yeast extract, 5 g of NaCl, and 15 g of agar per liter of medium. HITW broth was also used for growing all species of *Corynebacterium* described here. *E. coli* JM109 was maintained on an M9 minimal A salts medium (9) containing 0.2% glucose, but was grown on LB broth (9) for transformation experiments or plasmid extractions.

The regeneration medium contained 10 g of tryptose, 5 g of yeast extract, 5 g of NaCl, 30 g of polyvinylpyrrolidine (M_r, 10,000), and 135 g of sodium succinate per liter of medium. The pH was 7.4 without adjustment. Agar was added to a final concentration of 1.3% (wt/vol) for plates and 0.6% for soft agar overlay medium. Fetal calf serum (GIBCO), heat inactivated at 56°C for 30 min and filter sterilized, was added to a final concentration of 10% (vol/vol) just before use in agar medium or broth. Other calf sera and one sample of equine serum were also successfully employed. Serum was added to agar medium along with antibiotics, when required, after the medium had been melted and cooled to 60°C–70°C. Soft agar was additionally supplemented with 2 mM $CaCl_2$ and 1.5% (wt/vol) maltose prior to use, held at 56°C–60°C, and overlaid in 2-ml aliquots on plates containing agar regeneration medium.

DNA Extractions and Manipulations. Corynebacterial plasmid and chromosomal DNA extractions were performed as reported (6, 10) as were gel electrophoresis, Southern blotting, nick-translation of probes using ^{32}P-labeled nucleotides, DNA·DNA hybridizations, and autoradiography (10). Hybridizations were carried out under conditions allowing 10–12% mismatch.

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Abbreviation: cfu, colony-forming unit(s).

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Buffers. The buffers used in both transfection and transformation procedures were as follows: TSMC (10 mM $MgCl_2$/20 mM $CaCl_2$/500 mM sodium succinate/50 mM Tris·HCl, pH 7.5), TES (10 mM Tris·HCl/10 mM NaCl/1 mM EDTA, pH 8) (7), and R buffer. R buffer (11) was made by combining 1.5 g of KH_2PO_4, 3.5 g of K_2HPO_4, 145 μl of $FeSO_4$·4–6 H_2O at 14 mg/ml, 5 g of casein hydrolysate, 135 g of sodium succinate in 960 ml of distilled H_2O. After autoclaving and cooling, filter-sterilized solutions of the following were added: 20 ml of 1 M $MgCl·H_2O$, 1 ml of $MnSO_4$·4–6 H_2O at 2 mg/ml, 20 ml of 50% (wt/vol) glucose, 200 μl of 1% thiamine hydrochloride, and 60 μl of biotin at 0.25 mg/ml. The buffer was stored at 4°C.

Procedure for Protoplast Formation. The procedures for both *C. ulcerans* 712 and *C. diphtheriae (belfanti)* 1030 differed only in minor details. The following describes the procedure for *C. ulcerans*. Using a freshly streaked plate as a source of inoculum, cells were grown overnight with shaking in 5 ml of HITW broth at 37°C for 13–16 hr. At this time the OD (500–570 nm) was ≈1.0. HITW broth (100 ml in a 500-ml flask) was inoculated with 0.6 ml of the overnight culture and incubated with shaking for 3.5 hr to an OD of ≈0.2. Eight milliliters of a 25% (wt/vol) solution of filter-sterilized glycine (J. T. Baker Chemical) was then added to a final concentration of 2%, and the cultures incubated for an additional 5.5 hr at which time the OD was ≈0.5. The cells were harvested by centrifugation in a GSA rotor (6500 × *g*) for 10 min, and the supernatant was discarded. The cells were resuspended in 10 ml of R buffer, distributed in 2-ml aliquots into screw-capped tubes, and refrigerated overnight at 4°C.

In the morning, 100 μl of a stock of lysozyme at 40 mg/ml of R buffer was added to each tube, the cells Vortex mixed lightly, and then incubated without shaking for 60 min at 37°C. The protoplasts were then harvested in a Sorvall angle centrifuge (4500 × *g*) for 5 min. The pellets were resuspended in 2 ml of 1× TSMC buffer with limited Vortex mixing and harvested. After discarding the supernatant, 2 ml of 1× TSMC buffer was added to each tube, the pellets were resuspended, and 1-ml samples were transferred to 5-ml screw-capped tubes. The protoplasts were centrifuged again, the supernatants were discarded, and the pellets were resuspended in 100 μl of cold 1× TSMC buffer with Vortex mixing and placed on ice. The protoplast concentration is critical since we have observed that doubling or reducing it by 50% reduces the frequency of transformation and transfection.

Transformation and Transfection. Transformation and transfection procedures were similar to those developed by Katsumata *et al.* (7). Transforming pNG2 DNA or transfecting phage 782 DNA (50 μl of DNA in TES buffer and 50 μl of 2× TSMC buffer) was added to 100 μl of the protoplast preparation. After gentle hand mixing, 1.8 ml of a 20% (wt/vol) solution (in 1× TSMC buffer) of Carbowax PEG 8000 (Fisher) was added, and the tube contents were mixed by hand again. The samples were incubated for 5 min on ice and then heat-shocked for 90 sec in a water bath at 56°C. The samples were then placed on ice for 2 min, and the preparation was harvested in the angle centrifuge (4500 × *g*, 5 min). The pellets were resuspended in no less than 4 ml of 1× TSMC, Vortex mixed, centrifuged again, resuspended in 1 ml of regeneration broth, and incubated for 1–2 hr at 35°C to allow expression of DNA. The protoplasts were then harvested as before, the supernatant was removed, and the contents of the pellet were transferred with a Pasteur pipette and then spread on regeneration medium containing erythromycin at 15 μg/ml (Sigma). Plates were incubated at 35°C. After 1 day, the plates were placed in a plastic bag to prevent drying out, and then returned to the incubator. Colonies of *C. ulcerans* appeared in 6–12 days, and those of *C. diphtheriae (belfanti)* appeared in 10–20 days.

The procedure for transfecting these organisms with corynephage 782 DNA was identical to the transformation procedure up to the point of the expression step. Transfected protoplasts were only incubated 1 hr at 35°C for expression. At that time samples were diluted 1:10 and 1:100 in regeneration broth. The diluted preparations were then harvested by centrifugation as above, and the supernatant was discarded. Each pellet was resuspended in the remaining fluid and transferred by Pasteur pipette to 2 ml of soft regeneration agar held at 56°C to which 60 μl of a freshly grown suspension of *C. diphtheriae (belfanti)* 1030 or *C. ulcerans* 712 (OD 0.6) were added as indicator. The tube contents were mixed and then poured over a regeneration plate. The overlays were allowed to harden and then exposed for 30 sec to a standardized level of UV radiation normally used for induction (12). The plates were incubated at 35°C for 2 days or until plaques were observed.

C. glutamicum was grown, and protoplasts were made and transformed with pNG2 DNA under the conditions described by Katsumata *et al.* (7) except that a heat-shock treatment of 90 sec at 56°C was used.

E. coli JM109 was made competent and subsequently transformed by the Hanahan method (13).

Photomicroscopy. Cells were grown as above for making protoplasts, harvested by centrifugation, and resuspended in R buffer. Portions of these cultures were then used to form protoplasts. Normal cells were observed by placing a drop of cell suspension on a glass microscope slide on which a thin layer of molten 1% agar in distilled H_2O had been allowed to harden for 10 min. Protoplasts were destroyed if treated in this manner and had to be observed in wet mounts. The cells were photographed under oil immersion using a phase-contrast microscope and camera combination, the Ultraphot II (Carl Zeiss, Oberkochen, Wurtt, Federal Republic of Germany). The final magnification was ×630. The film used was Plus-X pan, 35-mm black and white print film, ASA 125 (Eastman Kodak). Areas of the slides with the least amount of cell movement were photographed.

RESULTS

Protoplast Formation for *C. diphtheriae* and *C. ulcerans*. The first step in developing a protocol for transformation was to establish a method for making protoplasts and then regenerating *C. diphtheriae* and *C. ulcerans* cells. After preliminary studies, strains *C. diphtheriae (belfanti)* 1030 and *C. ulcerans* 712 were selected for intensive study since protoplasts were easier to make with those cells. In addition, both strains lacked the gene for diphtheria toxin, but could be converted to toxinogeny by members of the *tox*-bearing β family of corynephage (14), and both were sensitive to the wide host-range phage 782, a β-related non-*tox*-bearing phage (15).

Cells of the two strains were grown, made into protoplasts, and photographed. The term protoplast as used describes morphologically altered, osmotically sensitive forms that appear under our conditions of treatment. The transition of the *Corynebacterium* species from normal cells to spheres is illustrated in Fig. 1. During growth in glycine, which had been previously used to make protoplasts in a strain of *Brevibacterium* (16), the cells elongated and bulged at sites where the cell wall had apparently been damaged. When treated with lysozyme and subsequently washed in TSMC buffer, the misshapen rods were replaced by spherical forms.

The regenerability and osmotic sensitivity of the glycine-grown, lysozyme-treated preparations was examined by serial dilution in distilled H_2O and in hypertonic R buffer. After 20 min at room temperature, samples were plated on regeneration agar plates and incubated at 35°C for 2–3 days, and colonies were counted. Under the conditions of the protocol, 5–6% of the original glycine-grown population

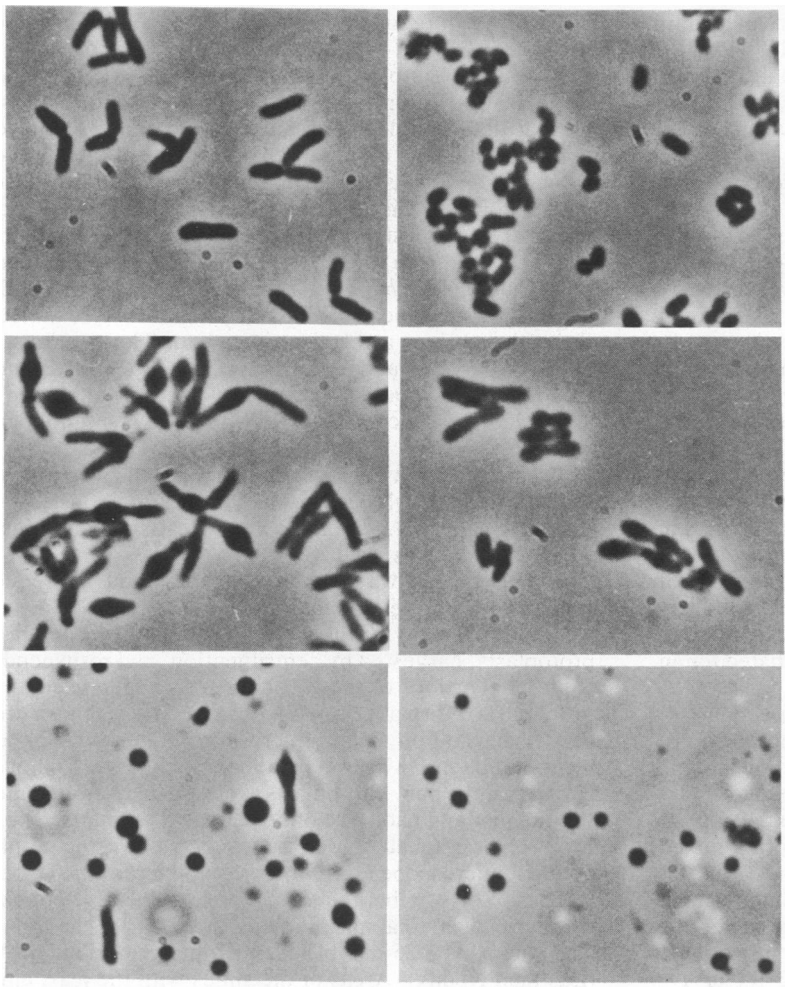

FIG. 1. Formation of *C. diphtheriae (belfanti)* 1030 and *C. ulcerans* 712 protoplasts. Two flask cultures of each organism were grown as described in the protoplast formation procedure. Glycine was added to one of the two flasks of *C. ulcerans* 712 after 3.5 hr and of *C. diphtheriae (belfanti)* 1030 after 4 hr of growth. Cells were harvested by centrifugation after a total of 9 hr of growth, resuspended in R buffer, and held at 4°C overnight. In the morning, cells of each strain from normal and glycine-grown cultures were mounted on agar slides and photographed under phase-contrast microscopy. Lysozyme was added to an aliquot of each of the glycine-grown cultures, and the cells were incubated at 37°C. The lysozyme-treated cells were washed once and then resuspended in 1× TSMC buffer. Wet mounts were made, and photographs were taken. (*Left*) *C. diphtheriae (belfanti)* 1030. (*Right*) *C. ulcerans* 712. (*A*) Cells grown without glycine. (*B*) Cells grown with glycine. (*C*) Cells grown with glycine and treated with lysozyme.

produced colonies after dilution in R buffer, and 80–90% of these regenerable cells were osmotically sensitive, i.e., did not produce colonies on dilution in distilled H_2O. If the period of incubation in lysozyme was shortened, a greater proportion of cells could be regenerated, however, as osmotic resistance increased, transformability declined.

Transfection of *C. diphtheriae* and *C. ulcerans* Protoplasts. Once regeneration had been observed, the ability of protoplast preparations to take up and express DNA was tested by transfecting with DNA from phage 782. Transfection assays are advantageous in that (*i*) they can be completed in far less time than transformation assays, (*ii*) the frequency of transfection is greater than that of transformation, being 100-fold greater in studies with soil corynebacteria (7), and (*iii*) if successful can be used to define optimal conditions for DNA uptake and expression.

Transfection was successful in both *C. diphtheriae (belfanti)* 1030 and *C. ulcerans* 712. Under the optimal conditions and using phage 782 DNA isolated following infection of *C. ulcerans* 712, the yield was 3×10^3 plaque-forming units/μg of DNA for strain 1030 and 8×10^3 for strain 712. In control platings, plaques were not formed when phage 782 DNA was added directly to the indicator systems. In optimizing the procedure, the experimental conditions that followed growth in HITW buffer with 2% (wt/vol) glycine were varied one at a time. For the 1030 strain the optimal conditions were defined sequentially as a 60-min exposure to lysozyme at 3 mg/ml, 20% (wt/vol) PEG, and a 60-sec heat-shock treatment at 56°C. These conditions were defined by varying the exposure to lysozyme in 10-min increments over a 45- to 85-min period, varying the PEG concentration in 5% (wt/vol) increments over a 10–30% range, and finally varying the heat-shock treatment in 30-sec increments over a 30- to 150-sec range. If any of the conditions was altered by more than one test increment in either direction, plaque-forming units were reduced by a factor of 2–10. Conditions for transfecting *C. ulcerans* 712 were defined in a similar manner.

To verify that phage 782 had produced the plaques in transfection experiments, presumptive lysogens of 1030 and 712 were isolated from turbid plaques. Chromosomal DNAs were extracted, digested with restriction endonuclease *Eco*RI, electrophoresed, Southern-blotted, and probed with labeled phage 782 DNA. The results (not shown) demonstrated that the lysogens had chromosomal digest patterns characteristic of these strains and that, when probed with labeled phage 782 DNA, each gave a pattern of hybridization identical with that of known 782 lysogens. The chromosomal DNA from nonlysogenic parental 1030 and 712 strains did not hybridize with the phage 782 probe.

Transformation of *Corynebacterium* spp. with Plasmid pNG2. Using the optimal conditions defined for transfection, we tested transformation of *C. diphtheriae (belfanti)* 1030 and *C. ulcerans* 712 with plasmid pNG2 DNA. The initial transformation protocol was identical to that used in transfection except that after adding pNG2 DNA and allowing a period for expression, cells were harvested and plated out on regeneration plates containing erythromycin at 15 μg/ml. Colonies were visible between 6 and 20 days after plating, and, as described below, transformation by pNG2 was verified. Once transformation was demonstrated, the range of each experimental variable was again tested, and the protocol was defined. Under these defined conditions, the frequency of transformation of strain 1030 was $\approx$0.15 colony-forming units (cfu)/μg of *E. coli*-derived pNG2 DNA, and 1.5×10^2 cfu/μg

for strain 712. *C. glutamicum* 13032 was also transformed with pNG2 DNA using the slightly modified protocol of Katsumata *et al.* (7). Transformation of *C. glutamicum* occurred at a frequency of ≈0.3 cfu/μg of *E. coli*-derived pNG2 DNA.

To verify transformation, total genomic DNAs were extracted from erythromycin-resistant isolates of each of the transformed strains. The DNAs were digested with restriction endonuclease *Eco*RI and electrophoresed. In each case (data not shown) the genomic DNA, largely chromosomal, had the same digest pattern as the parental organism, thus ruling out contaminants. In addition plasmid DNA isolated from each strain was the same size and had the same *Eco*RI digest pattern as pNG2. Finally all the plasmid *Eco*RI fragments hybridized with a pNG2 probe (data not shown). Plasmid pNG2, which originated in a strain of *C. diphtheriae*, was stably maintained during extensive passage in all transformants in the presence or absence of erythromycin.

Transformation of *E. coli* with *C. diphtheriae* Plasmid pNG2. There are three reports of plasmids from a Gram-positive organism replicating in a Gram-negative organism, the latter in all cases being *E. coli*. Two of these involve plasmids from *Staphylococcus aureus* (17, 18) and the third from *Streptococcus cremoris* (19). Since we had shown that the gene for erythromycin resistance carried by pNG2 was expressed in *E. coli* (6), we attempted to transform *E. coli* strain JM109 with pNG2. Competent cells were exposed to <1 μg of pNG2 DNA, and selection was made in the presence of erythromycin at 200 μg/ml. After a few days of incubation at 37°C, colonies appeared on LB plates. Transformation was demonstrated by showing that the chromosomal digest pattern of the erythromycin-resistant isolates matched that of the JM109 recipient and that the resistant clones contained pNG2 DNA. Again the plasmid was unaltered and stably carried through extensive passage in the absence of erythromycin. The yield of plasmid pNG2 from *E. coli* was much greater than that from any of the *Corynebacterium* species in which we estimate there are 1–2 copies per cell, and it appears from the intensity of the plasmid band in gels of *Eco*RI digests of whole cell DNA and dilutions therefrom that pNG2 was maintained in *E. coli* at a copy number exceeding 100. Because it was easier to prepare DNA from this source, much of the pNG2 DNA used in testing transformation of *C. diphtheriae (belfanti)* 1030 and *C. ulcerans* 712 originated in *E. coli* JM109.

Host Range of Plasmid pNG2 Isolated from Transformants. Plasmid pNG2 DNA was isolated from transformed strains of *C. diphtheriae (belfanti)* 1030, of *C. ulcerans* 712, of *C. glutamicum* 13032, and of *E. coli* JM109 and purified on CsCl gradients. The plasmid DNA from each strain was then used to transform the parent, which lacked plasmids, of each of the other strains. A diagram illustrating the directions of transformation and their efficiencies is shown in Fig. 2. Transformation was successful between all strains tested and in all possible directions. The frequency of transformation varied with the plasmid's strain of origin, and, as one would expect, homologous transformations were more efficient.

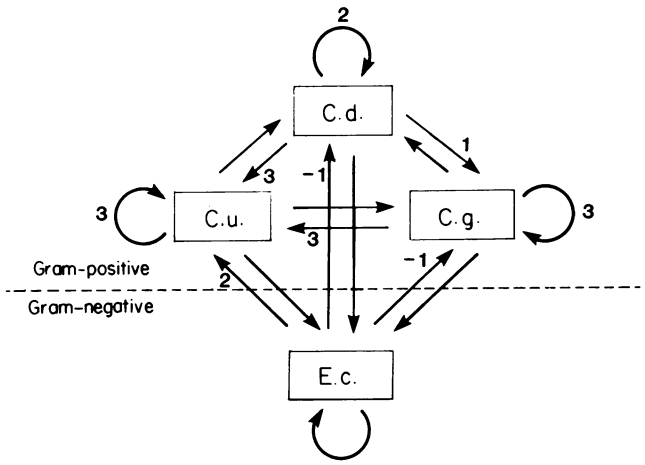

FIG. 2. Directions and efficiency of transformation of plasmid pNG2 between species of *Corynebacterium* and *E. coli*. Strain designations: C.d., *C. diphtheriae (belfanti)* 1030; C.u., *C. ulcerans* 712; C.g., *C. glutamicum* 13032, E.c., *E. coli* JM109. The arrows indicate the directions of successful transformation with plasmid pNG2 and point from the strain from which the plasmid DNA was isolated to the transformed strain. The numbers code for the frequencies of transformation which have been determined in some but not all directions: −1, 10^{-1} cfu/μg of DNA; 1, 10 cfu/μg of DNA; 2, 10^2 cfu/μg of DNA; 3, 10^3 cfu/μg of DNA.

DISCUSSION

The demonstration of transfection and transformation of animal pathogens of the genus *Corynebacterium* by *C. diphtheriae* plasmid pNG2 opens the door to a more extensive analysis of genes of interest in these organisms and a method for exchanging genetic material between them. The observation that plasmid pNG2 can also replicate autonomously and stably in *E. coli* indicates that a shuttle vector can be developed that will permit manipulation of those genes in *E. coli*. Most obviously, it may now be possible to study the regulation of diphtheria toxin synthesis by Fe^{2+} in its natural intracellular environment.

The genetic engineering of plasmids that contain two origins of replication and can replicate in both Gram-positive and Gram-negative organisms is commonplace, and shuttle vectors of this type have been described for *C. glutamicum* or *Brevibacterium* sp. and *E. coli* (20–22). However, the replication of naturally occurring plasmid pNG2 of *C. diphtheriae* in both a Gram-positive (*Corynebacterium* spp.) and Gram-negative (*E. coli*) organism is a distinct trait and is of interest from both an evolutionary as well as practical point of view. Barany *et al.* (17) reported that a biologically engineered composite of two *Staph. aureus* plasmids produced derivative plasmids in *Streptococcus pneumoniae* that replicated and expressed erythromycin-resistance in both their Gram-positive host and Gram-negative *E. coli*. In an earlier report, Goze and Ehrlich (18) had shown that an *E. coli-Staph. aureus* chimeric plasmid composed of pBR322 and pC194, respectively, could replicate in both *E. coli* and *Staph. aureus* even after the origin of replication for pBR322 had been excised. Finally Kok *et al.* (19) reported that a cloning vector engineered from a *Strep. cremoris* plasmid and two fragments from *Staph. aureus* plasmids could replicate in *E. coli* and probably did so through the replication functions of the streptococcal plasmid alone. Nevertheless, pNG2 is the only plasmid isolated in its natural state from one Gram type that has been shown to replicate in a strain of the other Gram type. Despite these laboratory demonstrations, it has yet to be shown that natural exchanges of genetic material occur between Gram-positive and Gram-negative organisms. Circumstantial evidence for such an exchange involving a gene coding for kanamycin resistance has been reported by Trieu-Cuot *et al.* (23).

The mechanism by which pNG2 is able to replicate in such unrelated hosts as *C. ulcerans* and *E. coli* is unknown. pNG2 may be a naturally evolved composite plasmid that has Gram-positive and Gram-negative origins of replication. Alternatively it may have a single origin that is compatible with the replication and partitioning mechanisms of both types of organisms. A third possibility is that pNG2 carries all of the genetic information necessary for its survival in a

variety of bacterial hosts. However, the difference between the estimated copy number of pNG2 in *C. diphtheriae* and *E. coli* indicates that there is some interdependence between the plasmid and its host. Regardless of the mechanism, the ability of pNG2 to replicate in both species qualifies it as the most obvious starting material for the development of a shuttle vector between *Corynebacterium* spp. of animal origin and *E. coli*.

Although the protocol we have developed for transformation has been successful for some strains of *C. diphtheriae* and *C. ulcerans*, it should be noted that it is not immediately applicable to every strain. Whether this reflects a need to adjust the parameters we have already examined or to identify some additional conditions has to be determined on a strain-by-strain basis. For example, protoplasts from *C. ulcerans* 712 and *C. glutamicum* 13032 could be formed using a combination of growth in either penicillin G or glycine followed by treatment with lysozyme, whereas *C. diphtheriae* C7 (unpublished observations) and *C. diphtheriae (belfanti)* 1030 only formed protoplasts if first grown in glycine. The effective concentration of glycine also varied with each strain, *C. ulcerans* requiring much less than *C. diphtheriae*. To produce protoplasts that could be successfully transformed, the glycine concentration had to be high enough to cause a slight alteration in cell morphology yet low enough to permit substantial cell growth. Levels that inhibited cell growth dramatically produced exaggeratedly misshapen cells that were resistant to the effects of lysozyme and useless for transformation. In general we have found that the ability to transform is linked to the conversion of most of the population of cells to the spherical form. This observation suggests that it is spherical cells that are transformed, though it is possible that a small fraction of the rod-shaped cells are actually involved.

While each species of *Corynebacterium* was transformed quite efficiently with homologous DNA, only *C. ulcerans* 712 was transformed easily with nonhomologous DNA. The tolerance of that strain for foreign DNA suggests that it lacks a DNA restriction system, at least one that recognizes DNA from *E. coli* strain JM109. In contrast to *C. ulcerans* 712 it appears that restriction may be a significant factor in the much studied C7 strain of *C. diphtheriae*. We have not been successful in transforming C7 with pNG2 DNA and have only succeeded in transfecting it with phage 782 DNA when the phage was grown in C7 itself. Not only was *C. ulcerans* 712 the most versatile host tested for transformation but also it is a strain that can be converted to toxinogeny, and in which diphtheria toxin synthesis is regulated by Fe^{2+} (14). Thus, it appears to be the best strain in which to pursue questions relating to the regulation of toxin synthesis.

We thank Dr. T. Philip Wong for his role in initiating the protoplast work, and the laboratory of Dr. J. Staley for its technical help with the photomicroscopy. This work was supported by Grant AI-10492 from the National Institutes of Health.

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