Indigenous Plasmids from Phytopathogenic *Corynebacterium* Species

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(Received 2 February 1979)

Efficient and reproducible cell lysis and the isolation of plasmid DNA from nine phytopathogenic *Corynebacterium* species is described. One to three plasmids, ranging from 23 to 77 megadaltons, were recovered from each strain, as detected by comparative agarose gel electrophoresis. *Corynebacterium michiganense* showed considerable plasmid diversity. Other species, such as *C. nebraskense* and *C. insidiosum*, did not. Bacteriocin production, colony morphology, pigmentation and virulence were not correlated with the presence of plasmids. Sedimentation through neutral and alkaline sucrose gradients and electrophoretic separation on agarose gels gave similar values for plasmid molecular weights. Log-linear alkaline sucrose gradients proved useful for molecular weight determinations and also for the isolation of purified plasmids on a preparative scale.

INTRODUCTION

The phytopathogenic *Corynebacterium* species are a diverse physiological, morphological and pathological group of Gram-positive bacteria that cause agronomically important diseases (Starr *et al.*, 1975; Vidaver & Starr, 1979). Under natural conditions, *Corynebacterium* species are generally host-specific and cause diseases of distinct symptomatology (Lelliott, 1966). Serology (Lazar, 1968), phage sensitivity (Echandi & Sun, 1973) and bacteriocin production (Gross & Vidaver, 1979) have all been used to differentiate species and strains of phytopathogenic corynebacteria. The ubiquity of bacteriocin production by 7 out of 12 phytopathogenic *Corynebacterium* species (Gross & Vidaver, 1979) and the general association of bacteriocin production by Gram-positive bacteria with plasmid DNA (Tagg *et al.*, 1976) suggested a similar relationship may exist in corynebacteria. Indeed, strains of streptococci, clostridia and bacilli that lose plasmids often lose bacteriocin production (Tagg *et al.*, 1976). However, there have been no reports of plasmids in *Corynebacterium* species including those of animal, plant or saprophytic origin.

Currier & Nester (1976) reported a procedure for isolating both small and large [> 100 megadalton (Mdal)] plasmids from Gram-negative bacteria. Plasmid enrichment involved denaturing sheared chromosomal DNA with alkali and selectively removing denatured DNA by salt precipitation in the presence of phenol. Chassy *et al.* (1976) used this method of plasmid isolation and developed a lysis procedure suitable for Gram-positive lactobacilli. Plasmid size determinations may be made by sedimentation through alkaline or neutral sucrose gradients (Nuti *et al.*, 1977; Shipley & Olsen, 1975). However, Meyers *et al.* (1976) found agarose gel electrophoresis to be a rapid and economical means of surveying molecular weights of plasmid DNA. All of the above techniques were adapted and used in this study.

We report here a survey of strains from several phytopathogenic *Corynebacterium* species for the presence of plasmids. While the presence of plasmids and bacteriocin production were not associated, the number and molecular weight of plasmids detected revealed differences in DNA content in otherwise indistinguishable strains.

METHODS

Bacterial strains. *Corynebacterium* species and strain designations along with their plant host of origin are listed in Table 1. *Escherichia coli* strain J53(RP1) was provided by R. Olsen (University of Michigan, Ann Arbor, Mich., U.S.A.) and strains J53(RSF1030) and J53(R1drd19) by S. Falkow (University of Washington, Seattle, Wash., U.S.A.).

Cultivation of bacteria. Strains of corynebacteria were maintained on a nutrient broth/yeast extract (NBY) agar medium (Vidaver, 1967) as previously described (Gross & Vidaver, 1979). *Escherichia coli* strains were maintained on NBY agar containing either 25 µg tetracycline ml⁻¹ [J53(RP1)], 25 µg neomycin ml⁻¹ [J53(R1drd19)] or 200 µg carbenicillin ml⁻¹ [J53(RSF1030)]. All strains were routinely grown in NBY broth – *E. coli* at 37 °C and *Corynebacterium* strains at 25 °C. Samples were radioisotopically labelled in medium N (Vidaver, 1967) containing 1% (w/v) yeast extract (Difco).

Bacteriocin production and assay conditions were as described by Gross & Vidaver (1979).

Labelling procedures. Exponential phase cultures of corynebacteria grown in supplemented medium N broth were subcultured to 5 × 10⁷ bacteria ml⁻¹ in medium containing 5 µCi [³H]thymidine ml⁻¹ (52 Ci mmol⁻¹; ICN Chemical and Radioisotope Division, Irvine, Calif., U.S.A.). *Escherichia coli* was grown using the same medium and procedures as described for *Corynebacterium*, except that 1 µCi [¹⁴C]-thymine ml⁻¹ (65 mCi mmol⁻¹; ICN Chemical and Radioisotope Division) was added in the presence of 250 µg 2-deoxyadenosine ml⁻¹.

Cell lysis and plasmid isolation. Bacteria were grown in 30 ml medium to between 2 × 10⁸ and 6 × 10⁸ bacteria ml⁻¹. *Corynebacterium* cells were lysed by the method of Chassy *et al.* (1976), and *E. coli* by the method of Currier & Nester (1976). All plasmids were isolated by the Currier & Nester (1976) procedure, as modified by Gross *et al.* (1979). Cells lysed with Sarkosyl NL30 (final concn 1% (v/v); ICN Pharmaceuticals, Plainview, N.Y., U.S.A.) were then sheared in 4 ml amounts and denatured with alkali. Denatured DNA was removed and plasmid DNA was concentrated as described by Gross *et al.* (1979).

Agarose gel electrophoresis. Ethanol-precipitated DNA, pelleted by centrifuging at 12100 g for 20 min, was electrophoresed in 0.7% (w/v) agarose (Sea Kem, Marine Colloids, Rockland, Me., U.S.A.) according to the method of Meyers *et al.* (1976). After resuspension of the plasmid pellet in 200 µl of a dye solution (Gonzales & Vidaver, 1979), 5 to 30 µl of the sample was applied per well and electrophoresed for 3.5 h with 40 V (approx. 5 V cm⁻¹) supplied by a constant voltage power source; the dimensions of the gel were the same as described previously (Gross *et al.*, 1979). Gels were stained with ethidium bromide (1 µg ml⁻¹ in distilled water) for 1 h and visualized according to Hansen & Olsen (1978). The average molecular mass was determined according to Meyers *et al.* (1976) by comparing the relative mobilities of *Corynebacterium* plasmids and plasmids RSF1030 [5.5 Mdal (Meyers *et al.*, 1976)], RP1 [39 Mdal (Burkardt *et al.*, 1978)] and R1 [62 Mdal (Meyers *et al.*, 1976)].

Dye-buoyant density equilibrium centrifugation. CsCl/ethidium bromide buoyant density centrifugation of [³H]DNA was done as described by Gross *et al.* (1979).

Sucrose gradient centrifugation. Plasmids to be centrifuged in neutral or alkaline sucrose gradients were first banded in CsCl/ethidium bromide density gradients as above and then recovered by syringe (20-gauge needle). Ethidium bromide was extracted with propan-2-ol (saturated with CsCl); the CsCl was removed by dialysis against TES buffer (0.02 M-Tris, 0.005 M-EDTA, 0.5 M-NaCl; pH 7.4). After dialysis, ¹⁴C-labelled RP1 was mixed with each of the ³H-labelled *Corynebacterium* plasmids at approximately a 1:3 ratio of radioactivity. The plasmid mixture was brought to 0.3 M-sodium acetate (final concn), carrier transfer RNA (Sigma) was added to 30 µg ml⁻¹ (final concn) and 2 vol. cold (–20 °C) 95% ethanol was added to precipitate the DNA. The precipitated DNA was recovered as above and suspended in 200 µl TES buffer. Samples were layered on to a 5 to 20% neutral sucrose gradient containing TES buffer and the gradients were centrifuged at 39000 rev. min⁻¹ at 20 °C in a Spinco SW50.1 rotor (Shipley & Olsen, 1975). The plasmids from *C. nebraskense* 298 and *C. poinsettiae* no. 13 were centrifuged for 80 min, and that from *C. michiganense* 156-2 for 60 min. Gradients were fractionated (125 µl per fraction) from the top using an ISCO model 640 density gradient fractionator (Instrumentation Specialties Co., Lincoln, Nebr., U.S.A.). Triton X-100/toluene/Liquifluor scintillant (Van Etten *et al.*, 1973) was then added to each fraction and ³H and ¹⁴C activity were measured. Sedimentation velocity and molecular weights of the *Corynebacterium* plasmids were calculated according to Studier (1965) and Clowes (1972) using [¹⁴C]RP1 as a standard. Sedimentation coefficients for open circular (OC) and covalently closed circular (CCC) RP1 DNA were as reported by Grinstead *et al.* (1972).

Log-linear alkaline sucrose gradients, run on a Spinco SW41 rotor (Brakke & Van Pelt, 1970), contained 0.3 M-NaOH, 0.05 M-NaCl and 0.05 M-EDTA. The ³H-labelled *Corynebacterium* plasmid(s) were mixed with ¹⁴C-labelled plasmids RSF1030 and RP1. These samples (500 µl) were applied to the SW41 alkaline gradient and centrifuged at 37000 rev. min⁻¹ for 150 min at 6 °C. Gradients were fractionated (250 µl per fraction) and

Table 1. *Bacterial strains surveyed*

<i>Corynebacterium</i> species	Strain no.	Plant host of origin	Bacteriocin(s) produced*
<i>C. betae</i>	CB102A	<i>Beta vulgaris</i>	Betacin CB102A
	CB101		None
<i>C. fascians</i>	6D21, CF101, CF107		None
<i>C. flaccumfaciens</i>	CV2, CV6	Unknown	Flaccumfacin CV2-A & CV2-B
	CV3	<i>Phaseolus vulgaris</i>	Flaccumfacin CV5-A & CV5-B
	CV7		Flaccumfacin CV7-A & CV7-B
	Neb no. 21		Flaccumfacin CV7-B
	CV4		Flaccumfacin CV4
	2AdK		Flaccumfacin 2AdK
<i>C. insidiosum</i>	CV1, CV9-SBR, ATCC 6887		None
	P9, 226, 239,		Insidiocin P9
	P2	<i>Medicago sativa</i>	Insidiocin P9 & P2
	M1B		Insidiocin P9 & M1B
<i>C. iranica</i>	NCPPB 2253		Iranicin 2253
<i>C. michiganense</i>	156-1, 140	<i>Triticum aestivum</i>	Michiganencin 13-3
	156-2	<i>Lycopersicon esculentum</i>	Michiganencin 13-3 & 156-2
	14-4, CM no. 1		Michiganencin 14-4
	15-2		Michiganencin 15-2
	1379		Michiganencin 1379
<i>C. nebraskense</i>	CN74-1, CN44-1, CN37-2,	<i>Zea mays</i>	Nebraskencin CN1
	CNC-1, CN68-1, CN18-5		
	CN18-6, CN9-1, 173		Nebraskencin CN1 & CN2
	CN76-1, CNSD-1, CN62-1		
	CN76-2, CN50-2, CN48-1, CN72-1,		
	298, 716, K293E, NTA, 311, 313		
<i>C. oortii</i>	CO101	<i>Tulipa</i> spp.	Oorticin CO101
<i>C. poinsettiae</i>	no. 1, no. 13	<i>Euphorbia pulcherrima</i>	None
<i>C. rathayi</i>	CR101	<i>Dactylis glomerata</i>	None
<i>C. sepedonicum</i>	CSea, CSmt, no. 30	<i>Solanum tuberosum</i>	None
<i>C. tritici</i>	CT101, CT102,		
	NCPPB 1857	<i>Triticum aestivum</i>	None

* Bacteriocins were biologically differentiated (Gross & Vidaver, 1978) and named after the producing species.

Fig. 1. Agarose gel electrophoresis of plasmids from phytopathogenic *Corynebacterium* species and reference plasmids from *E. coli*. Reference plasmids are: A, RSF1030 (5.5 Mdal); B, RP1 (39 Mdal); C, R1drd19 (62 Mdal). Plasmid profiles of *Corynebacterium* strains are: D, *C. nebraskense* 298 (34 Mdal); E, *C. insidiosum* M1B (53 and 28 Mdal); F, *C. michiganense* CM no. 1 (42 and 25 Mdal); G, *C. sepedonicum* no. 30 (33 Mdal); H, *C. poinsettiae* no. 13 (44 Mdal); I, *C. oortii* CO101 (68 and 46 Mdal); J, *C. fascians* CF107 (77 Mdal); K, *C. tritici* CT102 (35 Mdal). Chromosomal fragments are seen as a common band.

^3H and ^{14}C activity were measured as described for neutral sucrose gradients. The molecular weights of ^3H -labelled plasmids from corynebacteria were determined by their sedimentation rates relative to ^{14}C -labelled RSF1030 and RP1 plasmids in the log-linear gradients.

Plasmid isolated from *C. poinsettiae* no. 13 by the Currier & Nester (1976) procedure, as modified above, was directly applied to Spinco SW41 log-linear alkaline sucrose gradients and centrifuged as above. During fractionation the A_{254} was monitored on an ISCO model UA-4 absorbance monitor (Instrumentation Specialties Co.) and then ^3H was measured.

RESULTS

Cell lysis and plasmid isolation

The procedure of Chassy *et al.* (1976) gave clear lysates of all *Corynebacterium* species except *C. tritici*, *C. iranicum*, *C. rathayi* and *C. fascians*. However, these latter species lysed sufficiently to permit recovery of low levels of plasmid from all strains of the species except *C. iranicum* 2253, in which no plasmid was detected. Lysis was not improved by exposure to penicillin G (tested at 50 or 200 $\mu\text{g ml}^{-1}$) for 3 h prior to cell harvest (unpublished results).

The modified Currier & Nester (1976) procedure enabled the isolation of plasmids from nine species of *Corynebacterium* (Table 2), reducing the amount of chromosomal DNA to about the same as that of plasmid DNA (Figs 1 and 2). This procedure was superior to other methods tested. Plasmids from *C. nebraskense* 298 and *C. michiganense* 156-2 were not isolated by the cleared lysate procedure (Clewett & Helinski, 1969), and low, variable amounts of plasmid were isolated from these strains using the procedure of Guerry *et al.* (1973).

Plasmid characterization

Incorporation of [^3H]thymidine into DNA varied between strains and species. For example, *C. nebraskense* 298 incorporated less than half the amount of ^3H incorporated by

Fig. 2. Caesium chloride/ethidium bromide density gradient profiles of [^3H]DNA of *C. nebraskense* 298 (a) and *C. michiganense* 156-2 (b). After reaching equilibrium the gradient was fractionated from the bottom. For both strains fraction 24 contained the plasmid DNA peak and fraction 31 or 32 contained the chromosomal DNA peak.

Table 2. *Plasmids of phytopathogenic Corynebacterium species*

<i>Corynebacterium</i> species*	Total no. of strains surveyed	Strains containing plasmid(s)	$10^{-6} \times$ Calculated mol. wt†
<i>C. insidiosum</i>	5	239	23(± 2.0), 28(± 2.0), 53(± 4.9)
		P2, P9, 226, M1B	28(± 2.0), 53(± 4.9)
<i>C. michiganense</i>	7	1379	24(± 2.6), 32(± 2.2)
		CM no. 1	25(± 3.3), 42(± 5.0)
		15-2	31(± 2.6)
		14-4	38(± 4.7)
		156-1, 156-2, 140	52(± 2.4)
<i>C. sepedonicum</i>	3	CSmt, no. 30	33(± 3.0)
<i>C. nebraskense</i>	22	298, 173, NTA, CN48-1, CN68-1	34(± 1.3)
<i>C. tritici</i>	3	CT101, CT102, NCPPB 1857	35(± 2.1)
<i>C. poinsettiae</i>	2	no. 1, no. 13	44(± 3.4)
<i>C. oortii</i>	1	CO101	46(± 2.8), 68(± 4.2)
<i>C. rathayi</i>	1	CR101	47(± 4.3)
<i>C. fascians</i>	3	6D21, CF101, CF107	77(± 6.3)

* *Corynebacterium betae* (2 strains), *C. flaccumfaciens* (10 strains) and *C. iranicum* (1 strain) did not contain plasmids.

† Determined by electrophoretic separation on agarose gels as described in the text. All values represent averages of four to eight replications for each plasmid-containing strain. Numbers in parentheses are the standard deviation of the molecular weight determined for each plasmid.

Fig. 3. Similarity of plasmid size and number within *C. insidiosum* (a) and diversity in *C. michiganense* (b). Before agarose gel electrophoresis, plasmids were banded on CsCl/ethidium bromide density gradients.

(a) *C. insidiosum* strains (left to right) are: M1B, P2, 239, 226 and P9. All strains had a 53 and 28 Mdal plasmid; strain 239 also had a 23 Mdal plasmid. The lowest band seen for P9 consists of residual chromosomal DNA fragments.

(b) *C. michiganense* strains (left to right) are: 140 (52 Mdal), 14-4 (38 Mdal), 15-2 (31 Mdal), CM no. 1 (42 and 25 Mdal) and 1379 (32 and 24 Mdal).

an equal number of cells of *C. michiganense* 156-2. However, [^3H]thymidine served as a good source of radioactive precursor for all species, as determined by the activity in plasmid peaks from a 15 ml culture (labelled as in Methods) analysed by CsCl/ethidium bromide gradient centrifugation (Fig. 2).

Strains from nine phytopathogenic *Corynebacterium* species had one to three plasmids ranging from 23 to 77 Mdal (Table 2, Fig. 1). All *C. fascians* strains contained the largest plasmid, whereas *C. insidiosum* 239 had the smallest. Only strain 239 had three different plasmids, although a number of species and strains contained two plasmids. All strains within some species (e.g. *C. michiganense* and *C. insidiosum*) contained at least one plasmid; only five of 22 *C. nebraskense* strains contained plasmids. Bacteriocin production did not correlate with the presence or absence of plasmids (Table 1). For example, *C. nebraskense* strains with and without plasmids produced nebraskencin CN2, while strains 173 and CN68-1 had a plasmid but did not produce this bacteriocin. *Corynebacterium iranicum* 2253 that apparently lacked a plasmid produced iranycin 2253. Some species, such as *C. insidiosum*, appeared to be relatively homogeneous in the number and size of plasmids, whereas others, such as *C. michiganense*, contained diverse plasmids (Fig. 3). All five *C. insidiosum* strains had two plasmids that appeared to be common (Fig. 3a), whereas five strains of *C. michiganense* had plasmids differing in size and number (Fig. 3b).

Covalently closed circular (CCC) DNA is shown in neutral and alkaline sucrose gradients (Figs 4 and 5). Plasmid RP1 was of a similar size to the plasmids from *C. nebraskense* 298, *C. poinsettiae* no. 13 and *C. michiganense* 156-2. Grinsted *et al.* (1972) determined sedimentation coefficients of 43S and 62S for open circular (OC) and CCC RP1 DNA, respectively. Although the plasmid from *C. nebraskense* 298 appeared to be smaller than RP1 by its relative mobility on agarose gels, in neutral gradients it had sedimentation coefficients of 43S₀₀ and 60S₀₀ (Fig. 4) corresponding to a size similar to RP1 (Table 3). The plasmids from

Fig. 4. Sedimentation of plasmids in a 5 to 20% neutral sucrose gradient. ^3H -Labelled plasmids (●) from *C. nebraskense* 298 (a), *C. poinsettiae* no. 13 (b) and *C. michiganense* 156-2 (c) were co-sedimented with [^{14}C]RP1 (○) for 80 min (a, b) or 60 min (c) at 39000 rev. min $^{-1}$ (Spinco SW50.1). Samples were fractionated (125 μl per fraction) from the top of the gradient. Sedimentation coefficients, calculated using $S_{\text{OC}} = 43\text{S}$ and $S_{\text{CCC}} = 62\text{S}$ for RP1, are: *C. nebraskense* 298, fraction 18 = $S_{\text{OC}} = 43\text{S}$, fraction 24 = $S_{\text{CCC}} = 60\text{S}$; *C. poinsettiae* no. 13, fraction 18 = $S_{\text{OC}} = 46\text{S}$, fraction 26 = $S_{\text{CCC}} = 65\text{S}$; *C. michiganense* 156-2, fraction 15 = $S_{\text{OC}} = 50\text{S}$, fraction 23 = $S_{\text{CCC}} = 71\text{S}$. Calculated molecular weights are listed in Table 3.

Table 3. Comparison of plasmid molecular weights determined by different methods

Corynebacterium species	$10^{-6} \times \text{Molecular weight}$			
	Gel electrophoresis*	Neutral gradient†		Alkaline gradient‡
		OC	CCC	
<i>C. nebraskense</i> 298	34	40	37	39
<i>C. poinsettiae</i> no. 13	44	47	47	47
<i>C. michiganense</i> 156-2	52	61	63	56
<i>C. oortii</i> CO101	46, 68	ND	ND	47, 73

ND, Not determined.

* Calculated from relative electrophoretic migrations in 0.7% agarose gels: reference plasmids were 5.5, 39 and 62 Mdal.

† Determined from sedimentation coefficients using the S_{OC} and S_{CCC} of RP1 (43S and 62S, respectively).

‡ Determined from relative sedimentation of RP1 (39 Mdal) and RSF1030 (5.5 Mdal) in log-linear alkaline sucrose gradients.

strains *C. poinsettiae* no. 13 and *C. michiganense* 156-2 also had sedimentation coefficients indicative of a larger size than originally calculated from agarose gels (Table 3). In alkaline log-linear sucrose gradients the CCC DNA from *Corynebacterium* strains sedimented at a rate similar to RP1. In a strain containing two plasmids, *C. oortii* CO101, the larger plasmid (estimated at 73 Mdal) sedimented more rapidly than RSF1030 and RP1. Molecular weights for plasmids from four species (Table 3), calculated from sedimentation profiles in alkaline sucrose gradients (Fig. 5), were similar to those calculated by either gel electrophoresis or by sedimentation in neutral sucrose gradients. These results are strong evidence that the bulk of plasmid DNA from corynebacteria is CCC.

Fig. 5. Alkaline sucrose gradient profiles of ^3H -labelled plasmids (●) from *C. nebraskense* 298 (a), *C. michiganense* 156-2 (b), *C. poinsettiae* no. 13 (c) and *C. oortii* CO101 (d) co-sedimented with reference ^{14}C -labelled plasmids (○) RSF1030 (fraction 15) and RP1 [fraction 28 (with *C. nebraskense* 298) and fraction 29 (with the other three *Corynebacterium* strains)]. Single plasmid peaks were seen for *C. nebraskense* 298 (fraction 28), *C. michiganense* 156-2 (fraction 33), and *C. poinsettiae* no. 13 (fraction 31); two plasmid peaks (fractions 31 and 36) were seen for *C. oortii* CO101. Samples were fractionated (250 μl per fraction) from the top of the gradient. Radioactivity in fractions 2 to 13 represents residual linear DNA and open circular plasmid DNA. In all cases less than 10% of ^{14}C was counted in the ^3H window. Plasmid molecular weights (Table 3) were determined by comparative sedimentation to RSF1030 and RP1 through these log-linear gradients centrifuged for 150 min in a Spinco SW41 rotor.

Figure 6 shows a sedimentation profile of a plasmid preparation from *C. poinsettiae* no. 13 through an alkaline log-linear sucrose gradient. In contrast to the gradients shown in Fig. 5, plasmids had not previously been banded on CsCl /ethidium bromide gradients. Such gradients are useful for the preparative separation of plasmids from chromosomal DNA in sufficient quantity to allow detection by measurement of A_{254} . The purified plasmid was obtained in large quantity (A_{254} approx. 0.4) from a 5 ml culture grown to about 5×10^8 bacteria ml^{-1} .

Fig. 6. Correspondence of peaks of absorbance (—) and ^3H -labelled plasmid (●) from *C. poinsettiae* no. 13 in a log-linear alkaline sucrose gradient. The plasmid was isolated as described in the text and then centrifuged without prior banding on CsCl/ethidium bromide gradients. Samples were centrifuged at 37000 rev. min $^{-1}$ for 150 min in a Spinco SW41 rotor. Samples (250 μl per fraction) were collected from the top of the tube and monitored at 254 nm. The plasmid peak was in fraction 35. Residual linear DNA and open circular plasmid DNA is visible at the top of the alkaline gradient.

DISCUSSION

Plasmids of moderate size (approximately 23 to 77 Mdal) were isolated from strains representing nine species of phytopathogenic corynebacteria. The size and number of plasmids in plasmid-containing strains of *C. nebraskense* and *C. insidiosum* were limited; strains having apparently identical plasmids, however, often varied in bacteriocin production, colony morphology, pigmentation and virulence. In contrast, seven different plasmids were distributed among seven *C. michiganense* strains. Some species, such as *C. flaccumfaciens*, had no detectable plasmids. Thus, there are considerable differences in plasmid size and number among the phytopathogenic corynebacteria.

The function of the *Corynebacterium* plasmids is unknown but seems unrelated to antibiotic resistance, production of bacteriocins or virulence (Gross & Vidaver, unpublished results). Hydrocarbon breakdown is an important plasmid borne-trait in many *Pseudomonas* species (Gunsalus *et al.*, 1975). Some *Corynebacterium* species also degrade hydrocarbons (Veldkamp, 1970) and this ability may be plasmid-determined. Similarly, plasmids in *Agrobacterium tumefaciens* transform plant cells to form crown gall on a number of plant hosts (Marx, 1979); *C. fascians* also forms galls, and again, this function may be plasmid-borne. The common presence of plasmids in *Corynebacterium* suggests an important function in their survival, possibly in relation to pathological effects.

The procedures for cell lysis and plasmid isolation were adaptable and convenient for all phytopathogenic *Corynebacterium* strains. Despite poor lysis by all strains of *C. tritici*, *C. iranicum*, *C. rathayi* and *C. fascians*, plasmids were nevertheless isolated in sufficient quantity to be readily visible on agarose gels.

Plasmid copy number could be approximated by determining the ratio of plasmid DNA to total DNA from CsCl/ethidium bromide gradients (calculated from radioactivity in the cleared lysate subsamples before phenol treatment). Assuming a genome size of 2×10^9 daltons (Bak *et al.*, 1970) it was estimated that there were approximately 1 to 2 copies per cell of each plasmid for strains *C. nebraskense* 298, *C. poinsettiae* no. 13, *C. michiganense* 156-2 and *C. oortii* CO101.

Plasmid size and number were readily determined by electrophoretic separation in agarose gels, as they have been for other Gram-negative and Gram-positive bacteria. Meyers *et al.* (1976) estimated less than 10% error in determining plasmid size; linearity of plasmid

migration in 0.7% agarose gels has since been reported for plasmids up to 140 Mdal (Hansen & Olsen, 1978). In this study, a linear standard curve for plasmids RSF1030, RP1 and R1 enabled rapid and accurate determination of plasmid size. OC DNA migrates considerably more slowly than CCC DNA and was not observed if plasmids were freshly prepared and properly stored (Meyers *et al.*, 1976). The estimated plasmid size in strains from different *Corynebacterium* species using neutral and alkaline sucrose gradient centrifugation was similar to those calculated by electrophoresis, suggesting that the DNA was in the CCC configuration. In addition, satellite DNA from CsCl/ethidium bromide gradients was of identical mobility to plasmids from the crude preparations; these results also were indicative of CCC DNA (Hansen & Olsen, 1978). A small over-estimation in size was calculated from the neutral sucrose gradients, resulting in part from a larger calculated mass (40 Mdal) and sedimentation coefficient for RP1 in neutral sucrose gradients (Grinsted *et al.*, 1972) relative to the mass now reported for RP1 (39 Mdal; Burkardt *et al.*, 1978).

Alkaline sucrose gradients have been used primarily as a method of isolating plasmids (Clowes, 1972) and only sparingly to determine plasmid size (Nuti *et al.*, 1977). But by using log-linear alkaline sucrose gradients, as reported here, direct estimations of molecular weight could be obtained by comparison with the sedimentation of internal reference plasmids. Such gradients gave reproducible, sharp plasmid bands and molecular weights that agreed with those estimated from agarose gels. More significantly, alkaline sucrose gradients are an efficient and reliable means of isolating plasmid DNA. Efficiency was shown by the recovery of plasmids from crude plasmid preparations. Reliability was evident from the reproducible and predictable banding and relative positions of the plasmids as seen, for example, by the physical separation of the two plasmids of *C. oortii* CO101. If plasmids were sufficiently divergent in size, single purified plasmid species could be obtained preparatively.

We thank Dr C. F. Gonzalez for consultations and assistance on plasmid analyses. The technical help of Mark Widger is gratefully acknowledged. Published as Paper no. 5736, Journal Series, Nebraska Agricultural Experiment Station. Part of this work was supported by United States Department of Agriculture-regional research funds for project NC-135.

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