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Native extrachromosomal deoxyribonucleic acid in Erwinia carotovora var. carotovora.

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NATIVE EXTRACHROMOSOMAL DEOXYRIBONUCLEIC ACID IN *ERWINIA CAROTOVORA* VAR. *CAROTOVORA*

A Thesis Presented

By

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INTRODUCTION

Under conditions of high humidity and high inoculum potential, soft rot disease can be a problem in the field, but it leads to even greater economic loss when it is manifested during storage of fleshy plant tissue. Soft rot bacteria, having gained access to internal plant tissue, can quickly macerate and destroy cells by the action of polysaccharide-degrading enzymes.

A clear understanding of the biochemical process resulting in soft rot is important to our search for efficient controls to curtail the loss of food material before and after harvest. Ultimately, not only the chemical reactions, but also the genetic basis for the pathogenic properties of a soft rot bacterium should be determined. A thorough knowledge of the way the bacterium is programmed for attack on plant tissue, and the signals which modulate this behavior, should allow food suppliers to alter conditions to reduce the toll of soft rot.

The present study was spurred by the recent findings regarding the mechanism for oncogenesis by the crown gall bacterium, Agrobacterium tumefaciens. Extrachromosomal plasmid deoxyribonucleic acid (DNA) has been found to include genetic information essential to the development of the crown gall disease, but not essential to the life processes of the bacterium. This discovery has allowed researchers to focus more and more closely on certain of the segments of DNA involved in this disease, and has made possible a great leap in our ability to closely scrutinize the exact genetic mechanisms for

disease development. Although it might be argued that crown gall is an extraordinary disease, it nevertheless is worthwhile to consider the possible contributions of plasmid DNA to other plant pathogen interactions, since genes for pathogenesis carried on extrachromosomal DNA may be manipulated for detailed study most advantageously. Thus, this investigation was intended to determine whether or not plasmid DNA was significant to the etiology of soft-rot disease caused by Erwinia carotovora var. carotovora.

REVIEW OF LITERATURE

Erwinia carotovora (L.R. Jones) Holland is a gram-negative rod-shaped bacterium responsible for serious damage to fleshy plant tissue in the field and during storage. The virulence of this organism is derived, at least in part, from its ability to synthesize and excrete pectin-degrading enzymes that macerate the tissue of the invaded plants, causing a soft rot. Friedman (25) reported the isolation of an ultraviolet light (UV)-induced *E. carotovora* mutant of greatly reduced virulence, and noted the concomitant reduction in the quantity of pectinolytic enzymes which the strain produced. Beraha and Garber (3) carried this line of investigation one step further, by examining not only an avirulent (AV) mutant, but also a virulent revertant mutant derived from the AV strain. Two traits were seen to reappear along with the virulence capacity in the revertant strain: phosphatidase activity and high production of polysaccharidases. These authors concluded that virulence was the result of pectin esterase, polygalacturonase, pectate lyase, cellulase and phosphatidase acting in concert. Mount, *et al.* (49) determined that electrolyte loss, tissue maceration and cell death, which typify soft rot, are attributable to the endopolygalacturonate *trans*-eliminase (EC 4.2.2.2) (PGTE) secreted by the pathogen. Proteinases and peptidases, in the absence of pectinase, did not cause cell death in this study. It is thought that pectic enzymes serve to modify plant cell walls, providing access to substrates for the

other degradative enzymes possessed by the bacterium (2). Studies have indicated (33,50) that PGTE synthesis, along with the catabolism of ∝ -lactose, L -arabinose, D-galactose, L-rhamnose, D-xylose, raffinose, D-cellobiose and glycerol, is under adenosine 3',5'-cyclic monophosphate regulation in E. carotovora. Mere possession of pectic enzymes is not sufficient for pathogenesis in many cases; such enzymes are common in saprophytes (67). Thus, the manner of genetic regulation might be the distinguishing feature between saprophytic and parasitic bacteria.

The genetics of Erwinia carotovora has not received extensive attention, and little is understood of the genetic basis for its pathogenic habit. In addition to the bacterial genome, extrachromosomal deoxyribonucleic acid (DNA) might code for traits essential to pathogenesis. This possibility has been investigated in several other systems.

Relationship between plasmid DNA and pathogenesis has been established for the crown gall bacterium, Agrobacterium tumefaciens, in its induction of non-selflimiting tumours of many woody and herbaceous plants, primarily dicots. Certain species of extrachromosomal DNA in A. tumefaciens are essential for tumorigenesis. The presence of unusually large plasmids in tumorigenic strains was demonstrated by Zaenen et al, in 1974 (66), who with Van Larebeke et al. (63) and Watson (64) showed that strains of A. tumefaciens lost their pathogenicity when cured of their plasmids by incubation at supra-optimal temperatures.

Workers have studied the "Ti" (tumour-inducing) plasmids

intensively, and have succeeded in locating areas of the plasmid genetic map (identified as particular restriction enzyme digest fragments) which appear to be transferred to the infected plant genome (10). Nucleic acid hybridization studies with tobacco tumour callus culture DNA and restriction enzyme Sma I digest fragments of pTi-B6-806 showed that fragment 3b was present in the transformed tobacco DNA. There appeared to be 18 copies of 3 b per tumour cell. Although a second fragment, 10c, was also detected in the crown gall culture cells (11), Drummond et al (20) found transcripts of only the 3b information in the crown gall culture ribonucleic acid (RNA), and no evidence of RNA copies of the 10c fragment.

Chilton et al (12) manipulated restriction enzyme Sma I and Hpa I fragments of pTi-B6-806, hybridizing the sets together and performing serial digestions, until a map of the plasmid could be reconstructed from the two sets of restriction pieces. An interesting aspect of this work is the discovery that the two Sma I restriction fragments which have appeared in DNA of cultures of tumour tissue, 10c and 3b, appear to lie side by side on the genetic map of the B6-806 Ti plasmid. This information lends credence to the possibility that a single section of a Ti plasmid genome might be incorporated into the plant cell during oncogenesis.

Researchers showed previously that additional plasmid markers for unusual amino acid derivative utilization were associated with crown gall transformation (44,28,29). Pathogenic bacteria containing a Ti plasmid may have the capability of using octopine or nopaline

as sole carbon source (53), while plant tumour cells may exhibit dehydrogenase activity, producing the octopine or nopaline which the inciting bacterial strain is able to utilize (G. Bomhoff, thesis, cited in (47). The Ti plasmid genome of a pathogenic bacterium determines what, if any, arginine derivatives will be produced by the plant after tissue transformation (4). The catabolism of octopine or nopaline by the bacterium is also mediated by plasmid genes (4). Comparison of the octopine oxidase and dehydrogenase enzymes (Bomhoff, thesis, cited in (47) and work with bacterial mutant strains deficient in catabolism of the guanido amino acids octopine or nopaline (47) suggests that there may be two separate plasmid genes for the synthesis and utilization of octopine/nopaline. The possibility of a single gene policed by a regulator which responds differently to the bacterial and plant cell environments also exists.

Another genetic marker has been found to be associated with certain of the tumorigenic Agrobacterium plasmids. Sensitivity to a bacteriocin produced by A. radiobacter strain 84, known as agrocin 84, has been correlated with plasmid possession in A. tumefaciens strain C-58 and certain other strains (22). Agrocin 84 sensitivity is lost in C-58 derivatives whose plasmids are cured by growth at 37°C. Sensitivity does not appear to be essential for tumor induction, as strains exist which are agrocin resistant, but fully oncogenic. Biological control of crown gall by establishing the agrocin-producing strain A. radiobacter 84 in the plant rhizosphere when transplanting has been very effective in some instances (48).

The tumor-inducing plasmids of A. tumefaciens are not all identical; they have evolved and diverged, but have retained the useful genetic information that allows their bacterial hosts to colonize plant tissue. Variation in size ($1.1x10^8$-$1.6x10^8$) (17, 64), bacteriocin sensitivity (48), octopine or nopaline utilization (58) and variation in gross homology as determined by hybridization studies (17) and Sma I fingerprinting (57) is common for the Ti plasmids. A. tumefaciens naturally hosts a number of large, cryptic plasmids, which may accompany the Ti plasmids (17). Recent studies have shown that the Ti plasmids coding for octopine utilization, even when isolated from distant geographic locations, are genetically very similar to one another, while nopaline plasmids are, for the most part, highly dissimilar (57).

In addition to the well-documented case for plasmid involvement in A. tumefaciens pathogenesis, there are a number of other cases demonstrating or suggesting plasmid contributions to the plant pathogenicity of bacteria. Pseudomonas syringae, causal agent of holcus spot of maize, commonly loses its virulence in agar culture, simultaneously losing its ability to produce a phytotoxic peptide, syringomycin. This observation prompted Gonzalez and Vidaver to investigate the possibility of an extrachromosomal location for the gene specifying syringomycin production (30). Acridine orange-cured isolates lost syringomycin-producing ability and provoked a hypersensitive reaction when injected into maize leaves. The cured strain was compared to the parent strain by agarose gel electrophoresis; only the parent strain possessed a plasmid of 22 megadaltons.

For Erwinia chrysanthemi strains isolated from corn with bacterial stalk rot, plasmid DNA of similar size has been isolated from samples from Hawaii, Wisconsin and Rhodesia (37). The pattern of occasional spontaneous loss of virulence in cultured E. chrysanthemi is suggestive of an association between plasmids and virulence (G. Lacy, personal communication) (36).

Agarose gel electrophoresis of Erwinia stewartii DNA has revealed numerous small plasmids, some of which appear to be held in common by many different geographically isolated strains of bacteria (David Coplin, personal communication) (14).

The apparent gene linkage for B-glucosidase, phloretin hydrolase and a bacteriocin in Erwinia herbicola suggested to Gibbons et al. (27) that the genes for these functions might be plasmid borne. Attempts were made to facilitate study by co-transfer of a native 6.4 megadalton plasmid with RP1, but low conjugation transfer percentages caused this line of investigation to be abandoned. E. herbicola is not itself a pathogen, but it is often found associated with Erwinia amylovora in fire blight infections. Interestingly, some strains of E. herbicola from human clinical isolates contain a sex factor, E, which appears to be linked to the lac^+ marker (7).

Curiale and Mills (16) found plasmid DNA of varying sizes in Pseudomonas glycinea strains from different geographic locations, but have as yet not observed any effects of plasmids on pathogenicity to soybean.

Panopoulos et al. (51) have reported the presence of naturally occurring plasmids in strains of E. amylovora, P. lachrymans, P.

savastanoi and P. tonelliana. P. savastanoi and P. tonelliana both cause galling on their host plants. Since these pathogens cause a disease symptom which is similar to crown gall, it would be particularly interesting to try to determine the phenotypic markers to be found in their plasmid DNA.

Plasmid genomes may be involved in the infection process for Rhizobium, the legume-nodulating bacterium (57). Studies of Rhizobium trifolii have shown that a loss of viomycin sensitivity in this bacterium, which is correlated with a loss of symbiotic efficiency, is enhanced by plasmid-curing treatments. Infectivity is normally lost by a small percentage of untreated clones, which suggests the possibility of extrachromosomal influence on plant-bacterium interaction.

The emerging evidence for plasmid involvement in plant pathogenesis is in accord with previous observations of aggressive traits coded for by episomal DNA in animal systems. Plasmid genes may code for pili, which may affect a bacterium's physical interaction with its host. The pili of Neisseria gonorrhoeae, for example, allow attachment to human epithelial cells, and perhaps help the pathogen to evade host defenses (55). Enterotoxin production by E. coli, leading to disease in swine and humans, is a plasmid-mediated character (59). Evidence has also been accumulated for the association of a large plasmid in Clostridium perfringens with the production of betatoxin, which causes diarrhea in domestic animals (21).

There are a number of cases of utilization of plasmids for

investigations of the stability and transferability of antibiotic resistance factors in plant pathogenic bacteria. Resistance factors originally found in Pseudomonas species, the "Group P" plasmids, have been transmitted to Agrobacterium tumefaciens (19,41), as well as to plant pathogenic pseudomonads (38,52), erwinias (13, 37), and xanthomonads (40). The study of Lai et al. (40) established that R factor survival in planta could be significantly greater than laboratory tests on artificial media might indicate. This work was also significant in achieving the first conjugational transfer with a Xanthomonas species, overcoming the thick polysaccharide slime barrier problem by growing and mating the cells on a succinate medium. Chatterjee and Starr (6) also transferred F'lac from E. coli to E. herbicola, E. amylovora and E. chrysanthemi, and noted the percentage of transfer of tetracycline resistance on the R100rd-56 plasmid from E. coli to fourteen pathogenic Erwinia species.

Conjugation systems have been established with some of the plant pathogens, utilizing sex factors from E. coli or Pseudomonas. In 1973, Chatterjee and Starr (8) achieved stable strains of E. amylovora 178R, containing F'lac (probably integrated into the chromosome), and successfully transferred arg, cys, gua, ilv, met, pro, ser and trp markers to auxotrophic recipients. Lacy and Leary (39) developed efficient chromosomal donors by transferring R6886 to Pseudomonas glycinea; Panopoulos et al. (52) and Guimaraes (thesis cited in 57) have also established conjugative transfer systems with Pseudomonas species.

Genetic mapping has been achieved with mobilization of genes by plasmids in Erwinia amylovora, E. chrysanthemi and Pseudomonas glycinea. Pugasetti and Starr (54), using integrated (presumably) F'lac, could transfer virulence to avirulent auxotrophic strains of E. amylovora via conjugation. During a mating period of fifteen minutes, both the ser marker and virulence on Barlett pear fruit were transferred. There was no direct correlation between prototrophy and virulence, and addition of the necessary amino acid to an avirulent auxotroph at time of inoculation did not serve to restore virulence. Fulbright and Leary (26) used plasmid R68 to determine linkage for ilv, his and trp markers in Pseudomonas glycinea.

With an F'lac Hfr strain of Erwinia chrysanthemi, Chatterjee and Starr (9) were able to transfer tissue maceration ability to an avirulent EMS mutant of E. chrysanthemi. The AV mutant was unable to polygalacturonic acid (PGA) as sole carbon source at pH 8, but could use PGA at pH 5.4 via hydrolase enzymes. These investigators concluded that the PGTE enzyme active at high pH values is essential for the aggressive degradation of plant tissue characteristic of virulent strains of E. chrysanthemi. As no recombinants were obtained showing segregation of excretion and synthesis of the PGTE enzymes, single gene control was indicated. Synthesis and excretion for E. carotovora 153, on the contrary, are apparently not closely coupled (Chatterjee and Starr, ms. in preparation). The mapping permitted by the donor system also showed some similarities in gene order between E. chrysanthemi and other members of the

Enterobacteriaceae, with close linkage between *leu*$^+$ and *thr*$^+$, and *his*$^+$ and *trp*$^+$ markers.

Dependable techniques for plasmid isolation are essential for studies of the genetic contribution of native plasmids and for confirmation of genetic evidence of plasmid addition or cure. Methods commonly employed for the isolation of total DNA from bacterial cells fail to protect large plasmids from shearing or to separate covalently closed circular (CCC) species from the bulk of the DNA (23). Several procedures have been developed, however, for enhancing the proportion of plasmid DNA recovered relative to the total bacterial DNA (46). These methods frequently make use of the greater resistance of CCC DNA to alkaki denaturation, or of the physical limitations on the amount of acridine dye which can intercalate within such a molecule. It is also possible to capitalize on the small size of plasmids, centrifuging preparations of detergent-lysed bacteria in order to pellet the chromosome along with the cell debris, and leave plasmids in the supernatant (32). After a preparatory step which disposes of 90% or more of the chromosomal DNA, sucrose gradients or dye-buoyant density cesium chloride centrifugation are employed to purify the plasmid DNA and isolate it from the remainder of the chromosome. CsCl gradients without added propidium iodide or ethidium bromide fail to separate CCC and chromosomal species if the GC content of the DNA forms is very similar (as is the case with F factor and R factors in *E. coli*), but it is sometimes effective to collect the lower portions of the heterogeneous DNA band and recentrifuge, or to carry out buoyant

density centrifugation after transferring the plasmid of interest to a host strain with a sufficiently different GC content (56). Alternatively, Hoechst dye has been employed in the buoyant density centrifugation of very large plasmid DNA species sheared to linear fragments in the course of isolation, magnifying the inherent differences in GC content between plasmid and chromosome (24). The greater than 200 megadalton plasmid DNA of Inc P-2 plasmids may be visualized under UV light as a discrete band set off from the chromosomal DNA by this technique. Ethidium bromide also fluoresces with long or short wave UV light, and allows observation of supercoiled DNA within a cesium chloride gradient; used at a high concentration, this dye achieves plasmid separation by binding to a greater extent to the linear chromosomal fragments, lessening their buoyant density relative to the CCC molecules whose conformational stress limits the amount of dye with which they can become associated (32).

Rapid plasmid screening methods have been developed which involve placing single colony lysates (1) or crude bulk lysates (45) directly upon agarose gels for electrophoretic separation.

The rapid screening technique of Meyers et al. (45) was used in this study to determine the natural plasmid complement of 25 isolates of Erwinia carotovora var. carotovora. The purpose of this investigation was to examine the possibility that plasmids might be relevant to the identity of E. carotovora var. carotovora as a soft-rot pathogen.

MATERIALS AND METHODS

Bacterial Strains:

Wild-type strains of E. carotovora var. carotovora were procured from various labs. See Table 1 for a complete description of names, plant source, geographic location and suppliers.

Media:

Bacteria for plasmid isolation were grown overnight to saturation in 100 ml volumes of PM (nutrient broth plus .05% glucose and .05% casein hydrolysate) on a reciprocal shaker at 125 rpm, at room temperature. When a larger mass of cells was desired, cultures were incubated for 48 hours in 200 ml of liquid medium.

CVP medium (4.5 ml 1 N NaOH, 3 ml of 10% $CaCl_2$, 15 g sodium polypectate, 1 g $NaNO_3$, 1 ml of 0.075% crystal violet and 1.5% agar per liter) (15) was used to test bacterial strains for the typical sunken colonies characteristic of E. carotovora var.carotovora.

To test for pectic enzyme production, a solid medium containing 1.0% polygalacturonic acid, disodium salt (Sunkist) and 1.5% agar was employed. After 48 hours of bacterial growth, plates were flooded with a 1% solution of hexadecyltrimethylammonium bromide (Eastman) and examined 15 minutes later for clear zones indicative of polysaccharide degradation.

Bacteriocin Screening:

To screen for bacteriocin production, bacterial smears were

Table 1. Bacterial Strains.

A. *Erwinia carotovora* var. *carotovora* strains

Strain	Plant Source	Location	Supplier
SR-11	carrot	Wisconsin	A. Kelman
SR-17	tomato	Florida	"
SR-52	broccoli	England	"
SR-169	caladium	Florida	"
SR-170	lettuce	Florida	"
SR-200	potato	The Netherlands	"
SR-218	potato	Peru	"
SR-250	potato	Arizona	"
SR-252	tomato	North Carolina	"
SR-259	cassava	Columbia	"
SR-288	potato	Scotland	"
ECC-CT#1	potato	Connecticut	G. Lacy
SR-53	carrot	Vermont	"
E-107	potato	Connecticut (?)	"
E-125	tobacco	Connecticut	"
E-126	"	"	"
E-128	"	"	"
E-129	"	"	"
E-130	"	"	"
E-131	"	"	"
E-132	"	"	"
EC-14	yellow calla	California	R.S. Dickey
EC-301	Chinese cabbage	Japan	"
EC-311	chrysanthemum	Florida	"
EC-527	onion bulb	Barbados	"

B. Marker Plasmid Strains

Background	Plasmid	Size*	Supplier
J53 *E. coli* K12F⁻ pro met	RP1	36	G. Jacoby
W3110T⁻ *E. coli* K12F⁻ *thy deoC*	R1033	45	"
J53 *E. coli* K12 F⁻	R1	60	"
J53 *E. coli* K12 F⁻	R6K	25	"
J53 *E. coli* K12 F⁻	Sa	25	"
W3110 *E. coli* K12 F⁻*lac*	Col E1	4.2	B. Levin
W3110 *E. coli* K12 F⁻*lac*	Col E3	5	B. Levin

*In megadaltons

grown on 1% nutrient agar plates for three days, then exposed to chloroform for one-half hour. A soft agar overlay of 0.1 ml of the sensitive strain in 3 mls of 0.5% agar was made to cover the potential producer colonies. After 24-48 hours, zones of inhibition of sensitive strain growth would be noted in the immediate area of an inhibitor-producing colony. To distinguish bacteriocins from lysogenic phage, a zone-causing strain was grown in liquid culture. After cells were killed by mixing with chloroform and centrifuged, the supernatant was passed through a 0.45 u Millipore filter. A series of one-half dilutions of the filtrate was dotted onto fresh soft agar overlays of sensitive bacterial strains in 0.5% agar. Points of filtrate contact were examined after the lawn developed for areas of growth inhibition. Phage inhibition was anticipated to result in isolated plaques within the spot of inhibition at some critical dilution, whereas a bacteriocin would be diluted in strength rather than reduced in number of propagative particles, as a virus (42). In addition to tests on nutrient agar, R medium (per liter: 10 g Bacto tryptone, 1 g Bacto yeast extract, 8 g NaCl and 1.2 g agar, plus 2 mM $CaCl_2$ and 5 mls of sterile 20% glucose added after autoclaving) was used to screen for plaques in bacterial lysates.

Plasmid Isolation Techniques:

Rapid Screening. Plasmids were isolated from overnight shake cultures (100 mls) of E. carotovora var. carotovora by a modification of the method of Meyers et al. (45) which included alterations by Tom Korfagen and Dan Krogstaad (personal communication) (35). Bacteria

were harvested by a 12-minute spin in a Sorvall preparative ultra-centrifuge at 13,200 g, followed by resuspension of the pellet in 150 ml of distilled water and a second 12 min., 13,200 g spin to help eliminate cell surface polysaccharides. Over 90% of the polysaccharide is present in the medium after the initial centrifugation (A.Woods, personal communication) (65).

Washed pellets from a 100 ml culture were thoroughly resuspended in 5 ml of 0.05 M Tris (tris(hydroxymethyl)aminomethane), pH 8 containing 25% sucrose (wt/wt), transferred to SS34 centrifuge tubes and placed in an ice bath. One ml of 5 mg/ml lysozyme (Sigma) in 0.25 M Tris, pH 8 was added, and the solution mixed well and incubated for 5 min. One ml of 0.25 M, pH 8 Na_2EDTA (ethylenediamine tetraacetic acid, disodium salt) was added and the mixture was swirled and incubated an additional 5 min. Two ml of 5 M NaCl were added to bring the mix to 1 M salt. While swirling the sample gently, 1 ml of 10% SDS (Sodium dodecylsulfate)(Bio-Rad) was added, and the tubes immediately inverted gently several times to achieve as rapid mixing as possible.

Following production of the viscous lysate, tubes were either first placed at $4^{o}C$ for 3 hours, or immediately spun for 1 hour at 20,200 g at $2^{o}C$. The clear or slightly cloudy supernatant, a salt-cleared lysate, (31) was retained in conical calibrated 15 ml glass centrifuge tubes, and any floating flecks of SDS removed with a Pasteur pipette. Each sample was incubated with 20 ul of ribonuclease (1 mg/ml in .05 M Na acetate, pH 5.0, pre-treated at 80^{o} for 10 minutes to destroy contaminating deoxyribonuclease) per each

ml of supernatant (RNase A, Worthington Biochemicals) and kept for 1-2 hours in a water bath at 37°C. Samples were then incubated for an additional half hour to an hour with 600 ug of pronase for each ml of supernatant. Pronase was prepared as a 20 mg/ml stock in 0.05 M Tris, pH 8 (Sigma, Protease Type IV, from Streptomyces griseus), and self-digested at 37°C for two hours prior to freezing in small vials to be thawed as needed. Sodium acetate (1.0 M), pH 5 was then added, to a concentration of 0.3 M and the samples were poured back into SS34 centrifuge tubes. Two volumes of cold (-20°C) 95% ethanol were added, and each sample inverted very carefully several times to prevent freezing of the DNA. Isolates were kept at -20°C overnight or up to an additional 48 hours, and then the DNA was harvested by centrifugation for 40 minutes at -10°C at 6,250 g. The prescribed procedure indicates a 30 minute spin at 12,100 g; the reduced force and increased time were recommended by the Sorvall Company to reduce stress on the centrifuge compressor. The ethanol-precipated DNA formed a disc-shaped white pellet, which was air-dried for about 30 min. and then resuspended by swirling in 0.4 ml TES buffer (0.05 M Tris, 0.005 M Na_2EDTA, and 0.05 M NaCl, pH 8). Crude DNA samples were stored at 4°C for up to several weeks prior to agarose gel electrophoresis.

Agarose Gel Electrophoresis. Agarose slab gels were utilized for electrophoretic separation of the DNA isolated from E. carotovora var. carotovora strains. A vertical lucite apparatus supporting glass gel plates (15.8 cm x 13.1 cm) was employed, with a lucite comb (Future Plastics, Cambridge) of 13 teeth, 0.45 cm wide with 0.55 cm

space between tracks. Plates, with 3 mm thick lucite spacers at sides and across the bottom, were first set up outside the apparatus, and 5 ml of acrylamide (0.965 g acrylamide and 0.035 g Bis-acrylamide (Eastman) in 5 ml of electrophoresis buffer, 1 x E,activated by 25 ul ammonium persulfate and 12.5 ul TEMED (N,N, N',N' Tetramethyl-ethylenediamine) (Eastman) pipetted in to form an adhesive layer to support the agarose slab. Electrophoresis buffer was made up as a stock, 10 x E, of 0.40 M Tris, 0.05 M sodium acetate, and 0.01 M Na_2EDTA, with pH adjusted to 8 with glacial acetic acid, and diluted to 1/10 (1 x E) strength just before use. After the acrylamide had hardened for 10 minutes, the bottom spacer was removed, and the plates fastened to the apparatus with G-clamps. (A 3 cm x 16 cm x 0.3 cm piece of lucite was placed horizontally along the front of the glass plates to help equalize the pressure exerted by the clamps across the face of the gel). A layer of vaseline was smeared across the vertical wall supporting the plates, to seal off the top chamber and prevent leakage of buffer behind the back plate. Agarose (Bio-Rad) at 0.7%, 1%, or 1.2% was dissolved in 50 ml of the electrophoresis buffer (1 x E) in a boiling water bath for about 10 minutes. The molten agarose was then cooled to 55°C in a water bath before pouring the gel. The 55°C agarose was poured between the plates with the comb in place using a Pasteur pipette, taking care not to create air bubbles within the slab or at the base of the comb teeth. Gels were allowed 30 minutes to harden, at room temperature. Buffer (1 x ELB) was then poured into the top chamber. After several minutes, the comb was gently and carefully jiggled up and down, and

side to side to loosen the contact between comb and gel and to allow buffer to flow onto the sample wells. When the comb appeared to be free of adhesive restraint, it was slowly pulled up away from the gel, tilting the teeth towards the top chamber. DNA samples were loaded into each well after being combined in a 4:1 ratio with dye (40% sucrose, 0.125% bromphenol blue). Preparations were initially screened with 40 ul DNA and 10 ul dye per sample well, with the amounts adjusted for future gels according to the initial results. Slab gels were run at 20-30 milliamps (ma) for 3-6 hours using a Canalco power source Model 300B. The tracking dye had run off into the lower reservoir after about 3½ hours. Difficulties with leakage behind the glass plates could be counteracted with a pump carrying drops of buffer from the lower to the upper reservoir; this was not often necessary.

After the run, spacers, plates and acrylamide were removed and the gels were soaked overnight in buffer containing 0.5 ug/ml ethidium bromide (Sigma) in a glass baking dish. Gels were sometimes destained in distilled water for a few hours before photographing. Plasmid bands were visualized by placing the gels directly on the face of a long-wave UV light source. Photographs were taken using a Kodak Wratten 2A filter on a Polaroid CU-5 camera with an 88-5 lens, using Type 665 positive/negative film. Exposures of 2-7 minutes were used, depending on plasmid band brightness. Measurements of plasmid mobility were made with a ruler on a 5 x 7 " enlargement from the negative.

Alkali denaturation plasmid isolation. Several strains were

also tested for plasmid DNA presence following an isolation by the method of Currier and Nester (18). Two grams of frozen bacterial cells were resuspended in 80 ml of TE buffer (0.05 M Tris, 0.02 M Na_2EDTA, pH 8) plus 20 ml of protease (Sigma) and incubated at 37°C until lysis was complete. The solution was somewhat cleared and extremely viscous. The preparation was then sheared slightly by slowly spinning a blade of a Virtis mixer through it for one minute, until the glutinous mass was broken down slightly into smaller clots of thick lysate. (Samples were poured back and forth to determine relative viscosity). The pH of the preparation was then raised to pH 12 by the dropwise addition of 3 M NaOH. After allowing it to stand for 10 minutes, the sample was quickly neutralized to pH 9 with 2 M Tris, pH 7, by adding approximately 3X the volume of NaOH that had been necessary to raise the pH to 12. After neutralization, samples were brought to 3% NaCl with solid NaCl. An equal volume of redistilled phenol, saturated with 3% NaCl, was then added to each sample. The preparations were stirred gently for 5 minutes, and then centrifuged at 4,080 g for 5 minutes. The supernatant was poured off carefully, taking care not to disturb the white degraded material at the interface. An equal volume of chloroform:octanol (10:1) was then added to each supernatant fraction. Samples were stirred to mix and then spun for an additional 10 minutes at 4,080 g. The supernatant was taken up through the wrong end of a 10 ml Pasteur pipette and transferred to an acid-washed beaker. Two point 2 ml 1 M $MgCl_2$ and 0.4 ml 1 M $NaPO_4$ buffer, pH 8 were mixed into the sample prior to the addition of 75 ml cold redistilled

ethanol at -20°C. The DNA was allowed to precipitate for 3 hours; if the DNA had not begun to precipitate out after 20 min. at -20°C, a bit more of the salt solutions was added. Pellets were resuspended in a minimal volume of 0.1 M Na_2EDTA, pH 8, and stored at 4°C until electrophoresis or CsCl-ethidium bromide purification.

Purification of Isolated Plasmid DNA.

CsCl-ethidium Bromide Gradients. Both the alkali denaturation and cleared lysate procedures were used to prepare crude DNA isolates for further purification in a CsCl-ethidium bromide gradient. Samples from an alkali treated preparation were diluted to 9.25 ml with distilled water; samples of dialyzed untreated supernatant from a cleared lysate or resuspended ethanol-precipated DNA from a cleared lysate were adjusted to 9.25 ml with TES buffer. Nine point two five grams of cesium chloride (Sigma) were dissolved in the 9.25 ml of DNA solution by gently inverting the mixture several times in a cellulose nitrate centirfuge tube. Ethidium bromide (0.6 ml of a 5 mg/ml stock in distilled water) was mixed with the tube contents and a layer of mineral oil added to fill the space remaining in the tube before capping. Tubes were spun for 36-72 hours in a Type 65 rotor at 20°C at 35,000 rpm in a Beckman Model L ultracentrifuge. Chromosomal and plasmid bands could be observed by illuminating the tubes with long wave UV light. After removing the set screw in the cap, plasmid bands were extracted by inserting a syringe equipped with an 18-gauge needle into the tube just beneath the fluorescing satellite band of supercoiled DNA.

The extracted DNA was then freed of ethidium bromide by

inverting the sample slowly four times with three changes of an equal volume of isopropanol saturated with 20 x SSC (3 M NaCl, 0.3 M citric acid, trisodium salt). The colorless DNA solution was then dialyzed overnight against two changes of distilled water or TES buffer to remove the cesium chloride. Samples could then be reconcentrated by evaporation under nitrogen or by precipitation with 3 volumes of ethanol before visualization of agarose gels.

Sucrose gradients. Linear-log sucrose gradients (5) with ethidium bromide at 10 ug/ml (R. Sparks, personal communication) (60) were also used to separate plasmid species from the remaining chromosomal material. Layers were poured by hand into 13.5 ml cellulose nitrate tubes. Sucrose stock was prepared at a concentration of 600 g/liter in TES buffer. Layer #1, (30% sucrose) was 1.35 ml of a solution of 11 ml sucrose, 12 ml TES and 20 ul ethidium bromide (from a 5 mg/ml aqueous stock). Layer #2 (27.5% sucrose) was 3.50 ml of a solution of 11 ml sucrose, 12 ml TES, 1.00 ml distilled water and 48 ul ethidium bromide. Layer #3 (23.22% sucrose), was 3.04 ml of 7.74 ml sucrose, 10.00 ml TES, 2.26 ml H_2O and 40 ul ethidium bromide. Layer #4 (18.22% sucrose) was 2.53 ml of a solution of 4.86 ml sucrose, 8.00 ml TES, 3.14 ml H_2O and 32 ul ethidium bromide. Layer #5 (11.8% sucrose) was 1.68 ml of a solution of 2.36 ml sucrose, 6.00 ml TES, 3.64 ml H_2O and 24 ul ethidium bromide. Layer #6 (0% sucrose) was 1.35 ml of a solution of 5.00 ml TES, 5 ml H_2O and 20 ul ethidium bromide. After equilibration at 4°C overnight, a volume of Layer #6 equivalent to the volume of DNA

sample (0.5, 0.8 or 1.0 ml) was removed, with a 200 ul Pipetman (Gilson), and the DNA gently pipetted onto the surface of the gradient. Gradients were spun at 25,000 rpm in an SW40Ti rotor for 10-24 hours. Bands could be visualized under UV light, and were removed with a Pasteur pipette from the top of the tube after the chromosomal material was drawn off. Ethidium bromide was removed with isopropanol saturated with 20 x SSC, or dialyzed away overnight. DNA was then reconcentrated by precipitating with three volumes of cold 95% ethanol at -20°C overnight, and centrifugation at -10°C for 40 min at 6,250 x g.

RESULTS

All Erwinia carotovora var. carotovora strains (Table I) used in this study gave comparable positive results in the pectic enzyme plate test. After two days growth on a solid sodium polypectate medium, the pectic material had been broken down in a zone 5 mm wide around the bacterial smears (Fig. 1). No quantitative or qualitative variation was discernible among the various strains. When inoculated onto CVP medium, all bacterial strains exhibited efficient sodium polypectate degradation and resistance to crystal violet, developing into sunken, milky-white colonies characterisitc of E. carotovora var. carotovora.

Bacteriocin screening yielded no positive results for the majority of the strains tested when E-107 and EC-14 were used as indicator strains in soft agar overlays. One isolate, however, SR-259, generated some factor which was inhibitory to the growth of not only E-107 and EC-14, but also to all the other E. carotovora strains available, as well as to the E. coli strain CSH-23. A series of half dilutions of an SR-259 culture filtrate passed through an 0.45 u Millipore filter gave no visible growth inhibition when loopfuls were placed onto a soft agar overlay of E-107, EC-14 or CSH-23. Neither was any growth inhibition noted when 0.1 ml of culture filtrate was mixed with 0.1 ml of sensitive bacterial culture and plated in soft agar. When the SR-259 liquid culture was chloroformed before 0.45 u filtration, soft agar overlays of sensitive strains with this filtrate again showed no areas of growth inhibition,

Fig. 1. Pectic enzyme production by plasmid-containing (SR-17, EC 527, E-130, E-131) and plasmidless (EC301, EC311) strains of Erwinia carotovora var. carotovora. Bacteria were grown on 1.5% agar with 1% sodium polypectate for 48 hours; plates were then flooded with 1%-hexadecyltrimethylammonium bromide to precipitate polysaccharide.

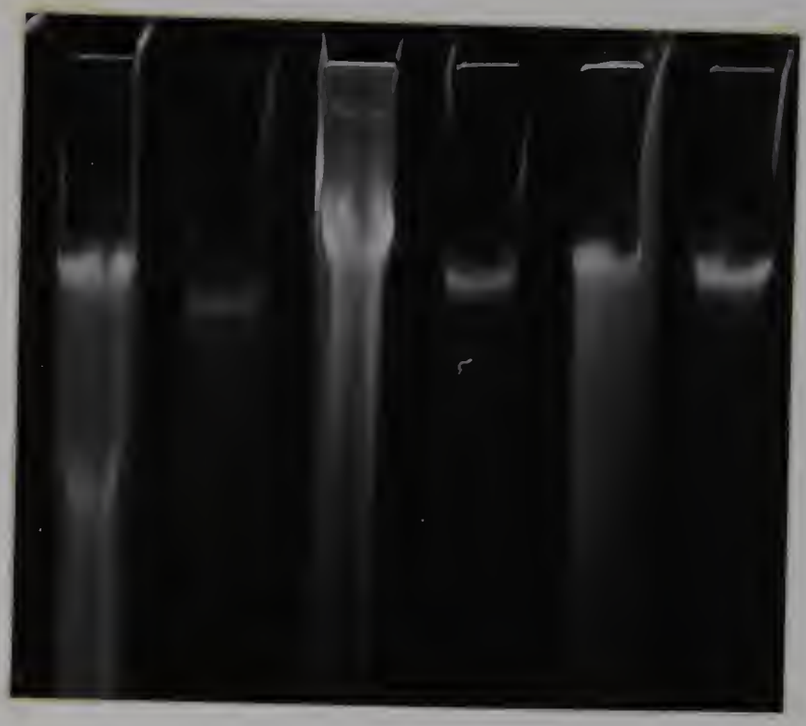


Fig. 4. Plasmid bands from E. carotovora var. carotovora. Constricted band in Track 1 showing typical pattern of isolated EC301 DNA. Track 3 and Track 6 show large E-130 and E-131 plasmid bands, respectively. Tracks 2,4 and 5 show chromosomal DNA from SR-53, EC14 and EC301. Electrophoresis was conducted at 24 milliamps for 4 hours, 35 minutes in a 1.2% agarose gel.

Fig. 3. Plasmid bands from E. carotovora var. carotovora. Track 1: SR-170; Track 2: EC527; Track 3: Col E3 from E. coli. Uppermost band in each case is chromosomal DNA. Electrophoresis was conducted at 25 milliamps for 4 hours, 37 minutes in 1% agarose.

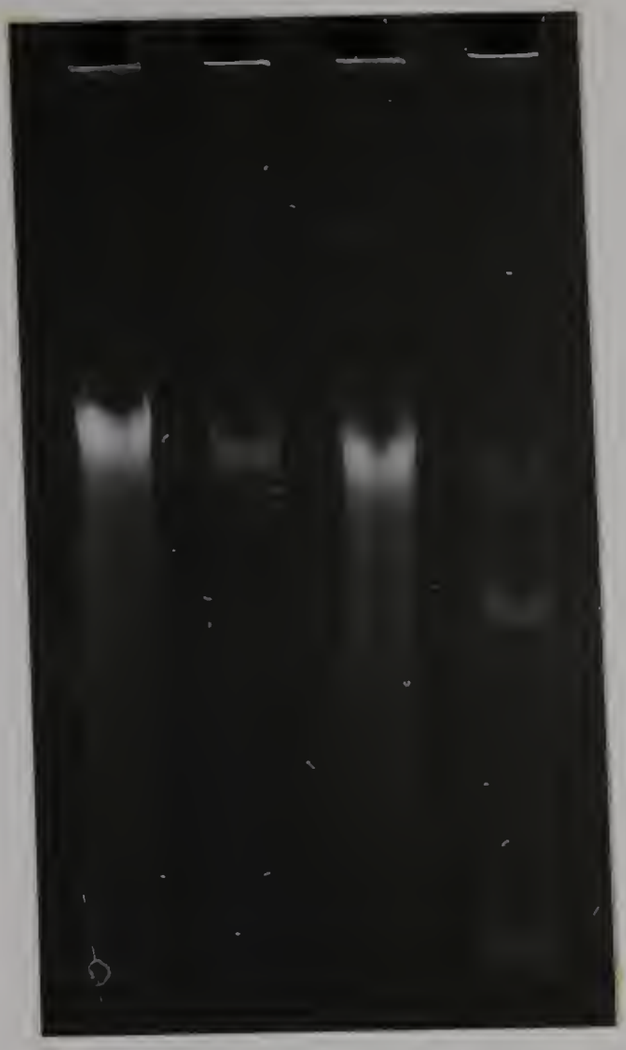


Fig. 2. Plasmid bands from E. carotovora var. carotovora. Track 1: E-130 DNA; Track 3: E-131 DNA; Track 4: EC527 DNA. Thick, diffuse bands are heterogeneous chromosomal DNA fragments. Tracks 2 and 5 show chromosomal DNA only in SR-53 and EC301, respectively. Electrophoresis was conducted at 30 milliamps for five hours in 1% agarose.

whether on nutrient agar or R medium.

The rapid screening technique utilizing agarose gels to examine pronase- and ribonuclease-treated cleared lysates disclosed plasmid bands in five of the twenty-five strains tested. Strains E-130 and E-131, isolated from tobacco hollow stalk infections in Connecticut, each contained a single, large plasmid species (Fig. 2). Strain 527, isolated from onion bulb in Barbados, displayed two prominent bands (Fig. 2) which ran ahead of the chromosomal fragments in roughly the same size category as the Col El plasmid of E. coli. Strains SR-17 and SR-170, both of which were isolated in Florida, showed apparently identical complements of extrachromosomal DNA; SR-170 lysates had six bands that appeared on agarose gels in the low molecular weight plasmid range (Fig. 3).

In addition to those strains which were shown to contain distinct plasmid DNA bands by agarose gel electrophoresis, isolates of strains SR-259 and EC-301 consistently showed abnormal "constricted" bands in the small plasmid range. Ethidium bromide concentration occurred in a spindle-shaped arrangement in the tracks of these two strains, the phenomenon always occurring at relatively the same location relative to the heterogeneous chromosome band (Figs. 4 & 5).

All other strains of E. carotovora var. carotovora which were examined in this investigation gave no evidence of extra-chromosomal DNA by the rapid screening technique. The DNA was isolated three or more times from each bacterial strain, and run on agarose gels in a diagnostically useful sample size on at least three occasions.

Fig. 7. Linear-log sucrose gradients containing 10 ug/ml ethidium bromide. Tube 1: E-130 DNA; Tube 2: EC301 DNA; Tube 3: Klebsiella sp. DNA with a large plasmid already pelleted; Tube 4: E-131 DNA. Tubes 1 and 4 show concentrated bands within the diffuse chromosomal band which are possibly a nicked form of the large plasmids present in those strains.

of concentrated ethidium bromide stain were noted to appear within the chromosomal DNA area after 15-25 hours of centrifugation in E-130 and E-131 isolates (Fig. 7). Since these bands were far too high in the tube to be in the form of compact, supercoiled DNA, if they do represent the large plasmids seen on agarose, they are most likely nicked supercoils, or open circle molecules.

Agarose gel electrophoresis of E. carotovora var. carotovora DNA on the same gel as DNA isolated from E. coli strains containing plasmids of known size provided approximate size determinations for the species observed in strains E-131, SR-170 and EC-527 (Fig. 8). The relative migration distance of the unusual SR-259 constricted band was also determined in this manner. A log-log plot of relative migration distances of known plasmids was used to estimate sizes of unknowns, assuming that the detected extra-chromosomal bands noted were in the supercoil configuration (Fig. 9). The migration distance of plasmid RP1 was arbitrarily assigned a value of 10, and the distance traveled by other plasmids given proportionate values for graphing purposes. A linear relationship between the log of the molecular weight and the relative migration distance was observed to hold true for marker E. coli-isolated plasmids of established sizes. From gel measurements, E-131 appeared to be about 55 megadaltons, EC-527 bands indicated sizes of 8.4 and 3.9 megadaltons, while SR-170 showed bands at 3.4, 4.0, 4.6, 6.7, 9.6 and 10.2 megadaltons. The SR-259 constriction is centered at a migration distance indicative of a size of 3.8 megadaltons, if indeed it is a plasmid. Bands a and d of the SR-170 set could easily

Fig. 6. Linear-log sucrose gradients containing 10 ug/ml ethidium bromide. Tube 1: SR-17 DNA; Tube 2: SR-170 DNA; and Tube 3: EC527 DNA. Two plasmid bands are apparent in each tube after 17 hours centrifugation at 25,000 rpm in an SW40Ti rotor. Uppermost band is chromosomal DNA.

Strains yielding smaller plasmids appeared to possess these abundantly, whereas E-130 and E-131, containing large plasmids, showed relatively faint plasmid bands which were not always detectable in the isolated DNA samples. The constricted bands of SR-259 and EC-301 were ordinarily, but not invariably, detected.

Cesium-chloride centrifugations following the isolation procedure of Currier and Nester (18) were carried out repeatedly on strain EC-14, the primary experimental organism for the laboratory. Although Agrobacterium and Klebsiella controls containing large plasmid species showed distinct plasmid bands beneath the wide chromosome-fragment band, no such supercoiled DNA band was ever detected in the E. carotovora strain. Neither was a plasmid band detectable in single isolation attempts with EC-311, E-130, E-131, or SR-259, or in many repetitions of EC-14 isolations, when a cleared lysate (without pronase or ribonuclease treatment) was the DNA sample to be mixed with the cesium chloride.

The ethidium bromide-sucrose linear log gradients clearly showed the two bands also observed in agarose gels for strain EC-527 plasmids (Fig. 6). SR-17 and SR-170 showed two plasmid bands after 15 hours centrifugation. As the extrachromosomal DNA moved down to the base of the tube, however, each of the early-detectable bands was resolved into two components. E-130 and E-131 preparations showed no distinct supercoiled DNA bands in the sucrose gradients in the approximately location of a control plasmid of similar size which sedimented in slightly more than 15 hours. Glowing lines

Fig. 5. Plasmid bands from E. carotovora var. carotovora. Constricted band in Track 3 showing typical pattern of isolated SR-259 DNA. Track 2 contains EC527 DNA, Track 1 contains Col E3 DNA from E. coli. Top band in all tracks is chromosomal DNA. Electrophoresis was conducted at 25 milliamps for 4 hours in a 1% agarose gel.

Fig. 8. Standard sized plasmids and E. carotovora var. carotovora isolates. Track 1: Col E1 DNA, supercoil, open circle and multimer bands in addition to E. coli chromosome. Lowest band (supercoil) is 4.2 megadaltons. Track 2: SR-17 DNA, showing no plasmids. Track 3: SR-170 DNA, with 6 plasmid bands calculated to be approximately 3.4, 4.0, 4.6, 6.7, 9.6 and 10.2 megadaltons. Track 4: EC527 DNA, with 2 plasmid bands calculated to be approximately 3.9 and 8.4 megadaltons. Track 5: Col E3 DNA, from E. coli, showing chromosomal material as well as supercoil and open circle plasmid bands. Lower band is the supercoil, 5 megadaltons in size. Track 6: SR-259 DNA, faintly showing the constricted band just slightly beyond the migration distance of the lower EC527 band. Track 7: DNA from EC311, having no plasmids. Track 8: E-130 DNA, with too little plasmid DNA present to be discernible. Track 9: E-131 DNA, with large plasmid calculated to be approximately 55 megadaltons. Track 10: E. coli containing RP1, a large 36 megadalton plasmid. Track 11: E. coli containing Sa, a 25 megadalton plasmid. Approximate sizes of cryptic E. carotovora plasmids were calculated (Fig. 9) on the assumption that all bands were in the supersoil configuration. Electrophoresis was carried out for 4 hours, 37 minutes at 25 milliamps on 1% agarose.

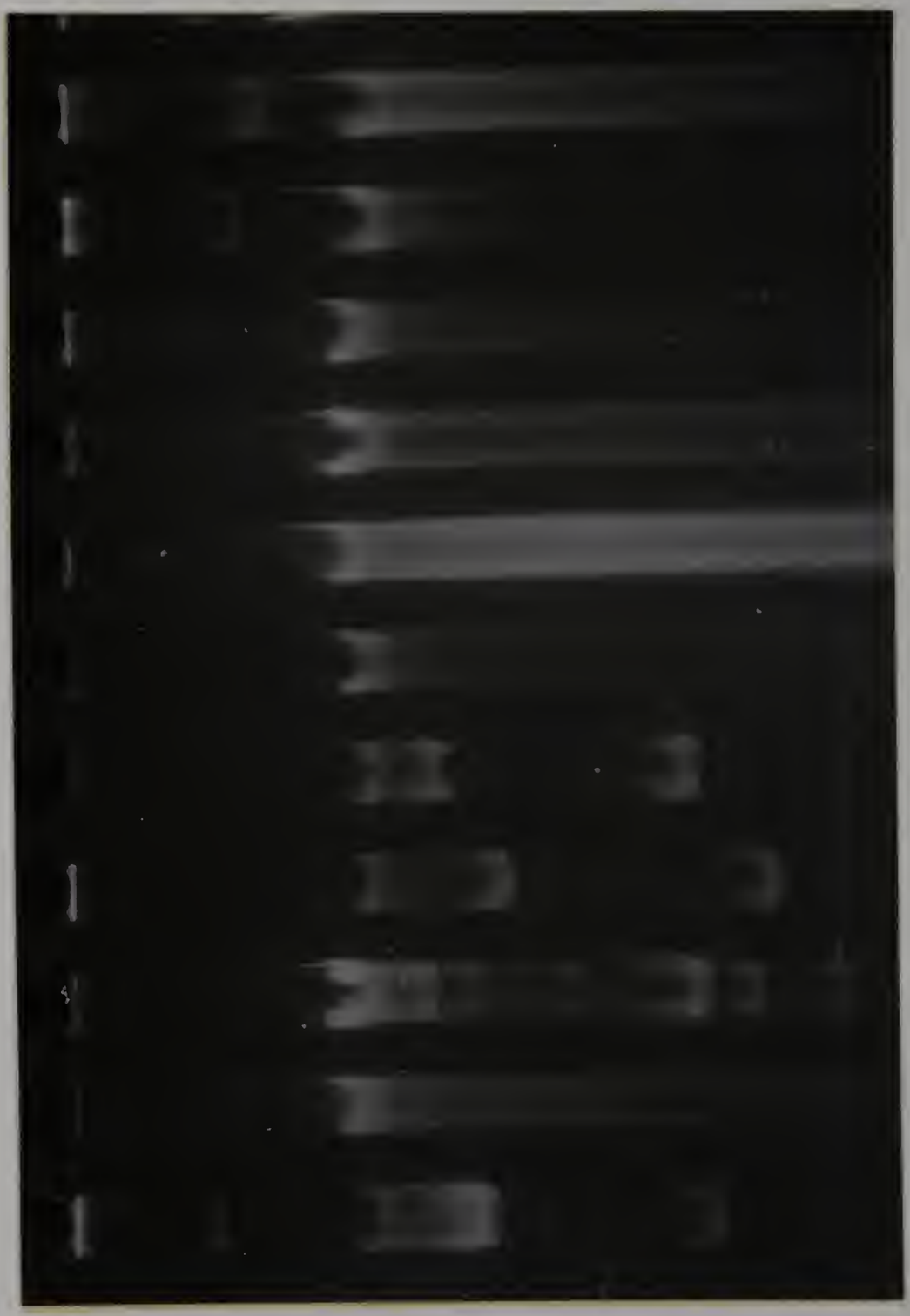
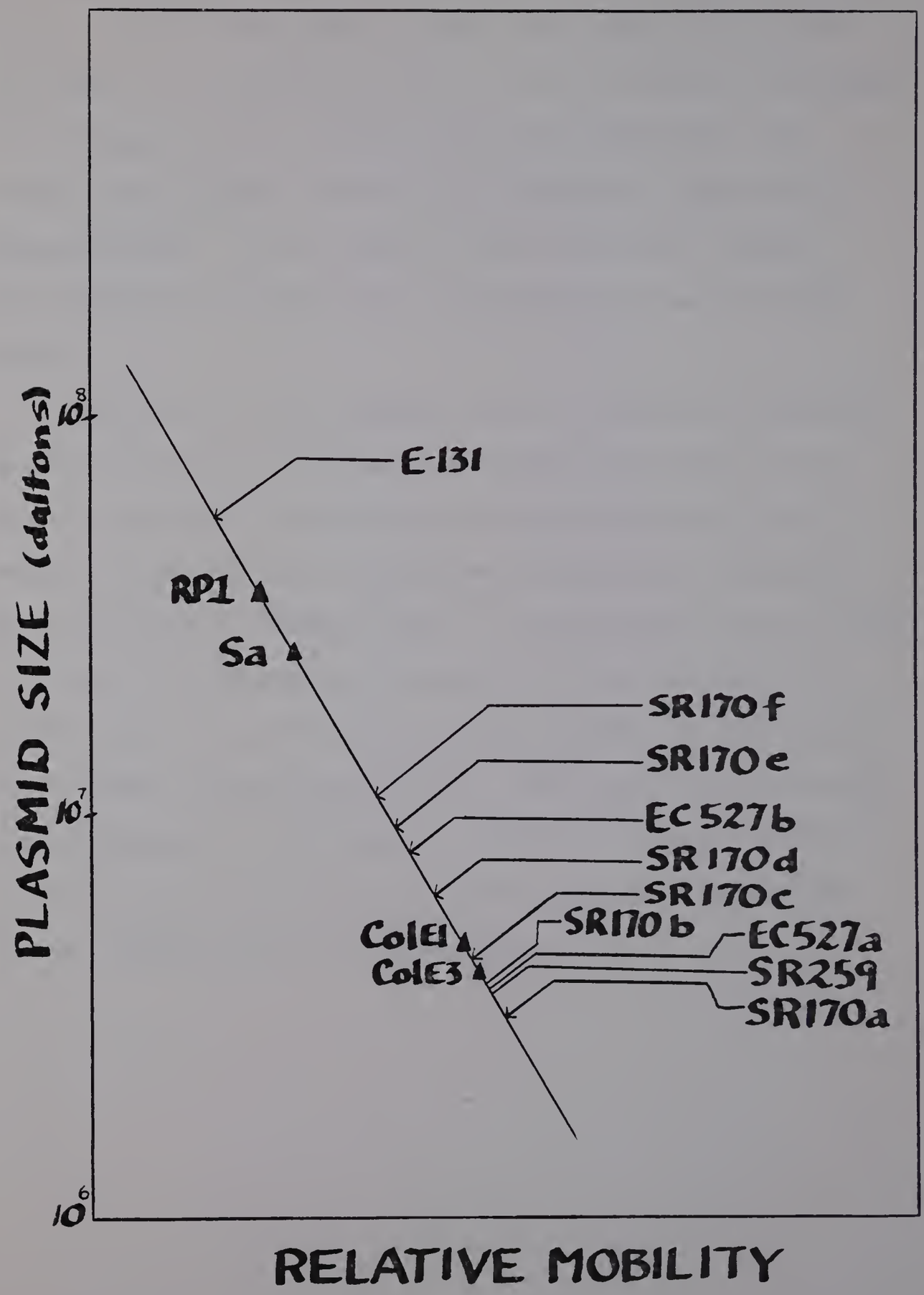


Fig. 9. Relative mobility of plasmids isolated from E. carotovora var. carotovora (arrows) as compared to marker plasmids of known size isolated from E. coli (▲). RP1 was arbitrarily assigned a mobility value of 10, and other plasmid bands were plotted accordingly.


PLASMID SIZE (daltons)
10^8
10^7
10^6
E-131
RP1
Sa
SR170f
SR170e
EC527b
SR170d
SR170c
SR170b
EC527a
ColE1
ColE3
SR259
SR170a
RELATIVE MOBILITY

be a monomer:dimer pair, according to their estimated sizes. The SR-170 gel pattern suggests that there may be two supercoil forms (lines a and d), with linear and open circle molecules migrating above each of the supercoiled forms. The EC-527 bands, in contrast, do not appear to have a monomer:dimer size relationship. On sucrose, as on agarose, the two bands consistently appear to be supercoiled DNA: on one occasion, faint open circle "shadows" were observed on an agarose gel accompanying the usual prominent bands.

The E-130 and SR-17 plasmids were not isolated in detectable amounts on the slab gels intended for sizing; hence their sizes must be interpreted from data gained from the E-131 and SR-170 strains. Figure 10 shows a preliminary screening gel containing SR-17 and SR-170 in adjacent tracks: no differences in their plasmid complement are discernible. Likewise, in the sucrose gradients shown in Fig. 5, the SR-17 and SR-170 DNA samples are indistinguishable in their sedimentation behavior. Both 1% and 1.2% agarose gels show a difference in the migration distance of the E-130 and E-131 plasmids (Figs. 2 & 4); the E-130 species appears to be somewhat larger, perhaps as high as 65 megadaltons.

Fig. 10. Plasmid bands from E. carotovora var. carotovora. Preliminary screening gel showing similarity between SR-17 (Track 1) and SR-170 (Track 2) plasmid complements. See also Fig. 6. Electrophoresis was carried out for 4 hours at 30 milliamps in a 1% agarose gel.

DISCUSSION

This study was begun with the intent of determining whether extra-chromosomal DNA was the location for any of the genetic determinants of pathogenesis for the soft-rot bacterium, Erwinia carotovora var. carotovora. After screening 25 bacterial strains to determine their plasmid complement, it became apparent that the majority of these strains, although without any plasmid DNA that could be isolated by the standard procedures used here, still were active pectic enzyme producers. We are as yet unable to understand the distinctions between the virulence of this pathogen, which appears to be strongly correlated with its pectic enzymes, and the factors which make E. carotovora an aggressive colonizer of plant tissue rather than just another saprophyte which possesses pectic enzymes. Future investigations will hopefully allow us to distinguish between the genetic determinants of pathogenicity and virulence.

The possibility of plasmid involvement in soft rot pathogenesis is certainly not entirely ruled out by this study: plasmids larger than 120 megadaltons, smaller than 1-2 megadaltons or tightly associated in folds of chromosome might have escaped detection, as might any large plasmids present primarily as relaxation complexes that would be converted to open circles when treated with protease or SDS. Repeated tests of EC-14 in cesium chloride-ethidium bromide gradients following the two isolation procedures were intended to insure that any large plasmid species that could not penetrate the

agarose gels under the stated conditions would be revealed by that alternative approach. Although a large class of possible plasmid species has been checked for, no characteristic E. carotovora var. carotovora plasmid was found.

The only patterns noted in plasmid possession in this study were related to the geographic location of the isolates. SR-17 and SR-170 appeared to contain the same complement of small plasmids; these were both isolated from vegetables in Florida, perhaps from the same field. As the host plants for these isolates were different (tomato and lettuce), plasmid presence might be interpreted as reflecting the environmental pool of plasmids available to E. carotovora var. carotovora; certain plasmid genes might perhaps be of assistance to members of the Enterobacteriaceae living under similar soil conditions, e.g. with the same Actinomycete competitors. Two of the tobacco hollow stalk isolates, E-130 and E-131, possess similarly large-sized plasmids. As the strains screened in this investigation had all been maintained in culture for several to many years, it is certainly possible that some of the plasmid species present in the original isolates may have been lost with repeated subculturing. In the case of the Connecticut tobacco hollow stalk isolates, for example, prompt examination of extra-chromosomal DNA might have disclosed large plasmids in all of the strains. Even with only two of the original seven isolates still showing large plasmids, it provokes speculation that a plant pathogenic bacterium might employ a plasmid conferring resistance to a certain toxic plant alkaloid (of which tobacco has many) that would allow it to extend its host range.

A study by McIntyre et al. (43) showed that isolates of E. carotovora var. carotovora from tobacco hollow stalk would cause the disease on tobacco and bring about a rapid necrosis of tobacco callus cultures at a low inoculum concentration. Few E. carotovora var.carotovora isolates from other plants, however, could cause pith necrosis, and none could kill callus cells as quickly or with as dilute an inoculum as the tobacco isolates. The relative degree of host specificity shown by these tobacco isolates could be a plasmid-mediated resistance to toxic compounds in the tobacco plant. Plasmids quite commonly confer resistance to poisons, such as heavy metals (62), encountered in the environment.

Much of our knowledge regarding E. carotovora var. carotovora centers about its pectic enzyme production, and its integrity as a distinct taxonomic type is subject to some doubt. Serological comparisons and gel electrophoresis of protein complements of various E. carotovora var. carotovora strains have indicated an extreme amount of variability among the different strains (A. Kelman, personal communication) (34). In view of the apparent differences in the genetic makeup of the bacterial chromosome, it is hardly surprising that the extrachromosomal DNA shows a similar variation. Perhaps these bacteria need to be subdivided according to their pathogenicity, rather than grouped according to certain metabolic capabilities.

It seems that, in order for us to achieve a better understanding of the pathogenicity of these soft rot bacteria, studies should focus upon the features which insure compatability between an E. carotovora var. carotovora strain and a particular host. The selective pressure

for being well-suited to a plant host is removed when bacteria are isolated and maintained on rich artificial media in the laboratory. Investigations of freshly isolated E. carotovora var. carotovora strains, with particular attention to similarities among strains isolated from a particular host, might lead to discoveries of important contributions of extrachromosomal DNA to the precise host-parasite interaction necessary for infection.

E. carotovora var. carotovora has been reported to lose virulence when maintained in culture for a number of years (3), but no avirulent segregants have been remarked upon. Such a segregation of the trait might be expected if plasmids were necessary to the pectic enzyme production of the bacteria. Until more sophisticated plasmid isolation techniques are developed which might uncover plasmids presently beyond the capabilities of our methodology, it is impossible to deny that difficult-to-isolate plasmids which are highly stable in agar culture might be involved in providing or regulating virulence capabilities in this pathogen. The results of this investigation, however, make it seem very likely that plasmid DNA is not essential for the virulence of E. carotovora var. carotovora. The plasmids isolated from strains EC-527, E-130, E-131, SR-17 and SR-170 remain cryptic, as no unusual traits were observed which invited further genetic study. The bacteriocin-like substance produced by the SR-259 strain lends support to the identification of the unusual constricted bands as plasmid DNA; further study of this substance will require attempts to induce its production in liquid culture with mitocmycin C, or to recover it from solid agar

media, as the inhibitory substance is apparently not produced in liquid culture. Better knowledge of this bacteriocin-like substance may provide us with some useful tools for control of soft rot diseases occurring in field or storage conditions.

SUMMARY

Plasmid deoxyribonucleic acid (DNA) was isolated from five of twenty-five strains of Erwinia carotovora var. carotovora by a salt-cleared lysate procedure. Isolate E-131 from tobacco hollow stalk showed a large plasmid of approximately 55 megadaltons by co-electrophoresis with plasmids of known size, and strain E-130 contained an even larger plasmid species. An onion isolate, EC-527, contained two plasmid bands of approximately 8.4 and 3.9 megadaltons. Florida isolates SR-170 and SR-17, from lettuce and tomato, respectively, each showed multiple small bands of extrachromosomal DNA. SR-170 bands indicated supercoil molecular weights of 3.4, 4.0, 4.6, 6.7, 9.6 and 10.2 megadaltons. Two strains, SR-259 from cassava and EC-301 from Chinese cabbage, consistently showed an unusual "constricted" plasmid band in the small molecular weight range. One of these strains, SR-259, produced a bacteriocin-like substance on solid media which was inhibitory to the growth of all other Erwinia carotovora var. carotovora strains, and to the E. coli strain CSH-23 as well. The growth inhibitor was not produced in detectable concentration in liquid culture. As all strains were strongly pectolytic but of variable plasmid content, no significance of plasmids to virulence of the pathogen was observed in this investigation.

LITERATURE CITED

1. BARNES, W.M. 1977. Plasmid detection and sizing in single colony lysates. Science 195:393-394.

2. BATEMAN, D.F. 1976. Plant cell wall hydrolysis by pathogens, p. 79-103. In J. Friend and D.R. Threlfall (eds.) Biochemical Aspects of Plant-Parasite Relationships. Academic Press, New York.

3. BERAHA, L. and E.D. GARBER. 1971. Avirulence and extracellular enzymes of Erwinia carotovora. Phytopathology Z. 70:335-344.

4. BOMHOFF, G., P.M. KLAPWIJK, H.C.M. KESTER, R.A. SCHILPEROORT, J.P. HERNALSTEENS and J. SCHELL. 1976. Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of Agrobacterium tumefaciens. Molec. Gen. Genet. 145:177-181.

5. BRAKKE, M.K. and N. VAN PELT. 1970. Linear-log sucrose gradients for estimating sedimentation coefficients of plant viruses and nucleic acids. Anal. Biochem. 38:56-64.

6. CHATTERJEE, A.K. and M.P. STARR. 1972. Genetic transfer of episomic elements among Erwinia sp. and other Enterobacteria: F'lac. J. Bacteriol. 111:169-176.

7. CHATTERJEE, A.K. and M.P. STARR. 1973. Gene transmission among strains of Erwinia amylovora. J. Bacteriol. 116:1100-1106.

8. CHATTERJEE, A.K. and M.P. STARR. 1973. Transfer of lac by the sex factor E in Erwinia strains from human clinical sources. Inf. and Imm. 8:563-572.

9. CHATTERJEE, A.K. and M.P. STARR. 1977. Donor strains of the soft-rot bacterium Erwinia chrysanthemi and conjugational transfer of the pectolytic capacity. J. Bacteriol. 132: 862-869.

10. CHILTON, M.D., M.H. DRUMMOND, D.J. MERLO, D. SCIAKY, A.L. MONTOYA, M.P. GORDON and E.W. NESTER. 1977a. Stable incorporation of plasmid DNA into higher plant cells: The molecular basis of crown gall tumorigenesis. Cell 11:263-271.

11. CHILTON, M.D., M.H. DRUMMOND, M.P. GORDON, D.J. MERLO, A.L. MONTOYA, D. SCIAKY, R. NUTTER and E.W. NESTER. 1977b. Foreign genes of plasmid origin detected in crown gall tumor. Microbiology, in press.

12. CHILTON, M.D., A.L. MONTOYA, D.J. MERLO, M.H. DRUMMOND, R. NUTTER, M.P. GORDON and E.W. NESTER. 1978. Restriction endonuclease mapping of a plasmid that confers oncogenicity upon Agrobacterium tumefaciens strain B6-806. Plasmid 1: 254-269

13. CHO, J.J., N.J. PANOPOULOS, and M.N. SCHROTH. 1975. Genetic transfer of Pseudomonas aeruginosa F factors to plant pathogenic Erwinia species. J. Bacteriol. 122:192-198.

14. COPLIN, D. Personal Communication. Department of Plant Pathology, Ohio Agricultural Research and Development Center, Wooster, Ohio.

15. CUPPELS, D., and A. KELMAN. 1974. Evaluation of selective media for isolation of soft rot bacteria from soil and plant tissue. Phytopathology 64:468-475.

16. CURIALE, M.S. and D.M. MILLS. 1977. Detection and characterization of plasmids in Pseudomonas glycinea. J. Bacteriol. 131:224-228.

17. CURRIER, T.C. and E.W. NESTER. 1976a. Evidence for diverse types of large plasmids in tumor inducing strains of Agrobacterium. J. Bacteriol. 126:157-165.

18. CURRIER, T.C. and E.W. NESTER. 1976b. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 76:431-441.

19. DATTA, N. and R.W. HEDGES. 1972. Host ranges of R factors. J. Gen. Microbiol. 70:453-460.

20. DRUMMOND, M.H., M.P. GORDON, E.W. NESTER and M.D. CHILTON. 1977. Foreign DNA of bacterial origin is transcribed in crown gall tumours. Nature (London) 269:535-536.

21. DUNCAN, C.L., E.A. ROKOS, C.M. CHRISTENSON and J.I. ROOD. 1978. Multiple plasmids in different toxigenic types of Clostridium perfringens: possible control of beta-toxin production, p. 246-248. In D. Schlessinger (ed.) Microbiology--1978. American Society for Microbiology, Washington, D.C. 449p.

22. ENGLER, G., M. HOLSTERS, M. VAN MONTAGU and J. SCHELL. 1975. Agrocin 84 sensitivity: a plasmid determined property in Agrobacterium tumefaciens. Molec. Gen. Genet. 138:345-349.

23. FALKOW, S., R.V. CITARELLA, J.A. WOHLHIETER and T. WATANABE. 1966. The molecular nature of R factors. J. Molec. Biol. 17:102-116.

24. FENNEWALD, M., W. PREVATT, R. MEYER and J. SHAPIRO. 1978. Isolation of the Inc P-2 Plasmid DNA from Pseudomonas aeruginosa. Plasmid 11:164-173.

25. FRIEDMAN, B.A. 1962. Physiological differences between a virulent and a weakly virulent radiation-induced strain of Erwinia carotovora. Phytopathology 52:328-332.

26. FULBRIGHT, D.W. and J. LEARY. 1977. Linkage analysis of Pseudomonas glycinea. Proc. Am. Phytopathol. Soc. 4: 141 (Abstr.).

27. GIBBINS, L.N., P.M. BENNETT, J.R. SAUNDERS, J. GRINSTED, and J.C. CONNOLLY. 1976. Acceptance and transfer fo R-factor RP1 by members of the "Herbicola" group of the genus Erwinia. J. Bacteriol. 128:309-316.

28. GOLDMAN, A., J. TEMPE and G. MOREL. 1968. Quelques particularities de diverses souches d'Agrobacterium tumefaciens. C.R. Acad. Sci. (Paris) 162:630-631.

29. GOLDMAN, A., D.W. THOMAS and G. MOREL. 1969. Sur la structure de la nopaline, metabolite anormale de certaines tumeurs de crown gall. C.R. Acad. Sci. (Paris) 268:852-854.

30. GONZALEZ, C.F. and A.K. VIDAVER. 1977. Syringomycin and holcus spot of maize: plasmid associated properties. Proc. Am. Phytopathol. Soc. 4:107 (Abstr).

31. GUERRY, P., D.J. LE BLANC and S. FALKOW. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064-1066.

32. HELINSKI, D.R. and D.B. CLEWELL. 1971. Circular DNA. Ann. Rev. Biochem. 40:855-898.

33. HUBBARD, J.P., J.D. WILLIAMS, R.M. NILES and M.S. MOUNT. 1978. The relation between glucose repression of endopolygalacturonate trans-eliminase and adenosine 3',5'-cyclic monophosphate levels in Erwinia carotovora. Phytopathology 68:95-99.

34. KELMAN, A. Personal Communication. Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin.

35. KORFAGEN, T. and D. KROGSTAAD. Personal Communication. Infectious Disease Unit, Massachusetts General Hospital, Boston, Massachusetts.

36. LACY, G. Personal Communication. Connecticut Agricultural Experiment Station, New Haven, Connecticut.

37. LACY, G.H. and A. KELMAN. 1977. Genetic studies with RP1 in Erwinia chrysanthemi strains pathogenic on corn. Proc. Am. Phytopathol. Soc. 4:106 (Abstr).

38. LACY, G.H. and J. LEARY. 1975. Transfer of antibiotic resistance plasmid RP1 into Pseudomonas glycinea and Pseudomonas phaseolicola in vitro and in planta. J. Gen. Microbiol. 88:49-57.

39. LACY, G.H. and J. LEARY. 1976. Plasmid mediated transmission of chromsomal genes in Pseudomonas glycinea. Genet. Res. 27: 363-368.

40. LAI, M.N., J. PANOPOULOS and S. SHAFFER. 1977. Transmission of R plasmid among Xanthomonas spp. and other plant pathogenic bacteria. Phytopathology 67:1044-1050.

41. LEVIN, R.A., S.K. FARRAND, M.D. GORDON and E.W. NESTER. 1976. Conjugation in Agrobacterium tumefaciens in the absence of plant tissue. J. Bacteriol. 127:1331-1336.

42. MAYR-HARTIG, A., A.J. HEDGES and R.C.W. BERKELEY. 1972. Methods for studying bacteriocins. P. 315-422 In J.R. Norris and D.W. Ribbons (eds). Methods in Microbiology. Academic Press, London and New York.

43. MCINTYRE, J.L., D.C. SANDS and G.S. TAYLOR. 1978. Overwintering, seed disinfestation, and pathogenicity studies of tobacco hollow stalk pathogen, Erwinia carotovora var. carotovora. Phytopathology 68:435-440.

44. MENAGE, A. and G. MOREL. 1964. Sur la presence d'octopine dans les tissus de crown-gall. C.R. Acad. Sci. (Paris) 259:4795-4796.

45. MEYERS, J.A., D. SANCHEZ, L.P. ELWELL and S. FALKOW. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.

46. MEYNELL, G.G. 1972. Bacterial plasmids. Macmillan, London. 164 p.

47. MONTOYA, A.L., M.D. CHILTON, M.P. GORDON, D. SCIAKY and E.W. NESTER. 1977. Octopine and nopaline metabolism in Agrobacterium tumefaciens and crown gall tumor cells: Role of plasmid genes. J. Bacteriol. 129:101-107.

48. MOORE, L.W. 1977. Prevention of crown gall on Prunus roots by bacterial antagonists. Phytopathology 67:139-144.

49. MOUNT, M.S., D.F. BATEMAN and H.G. BASHAM. 1970. Induction of electrolyte loss, tissue maceration, and cellular death of potato tissue by an endopolygalacturonate trans-eliminase. Phytopathology 60:924-931.

50. MOUNT, M.S., P.M. BERMAN, R.P. MORTLOCK and J.P. HUBBARD. (Ms. submitted for publication) Regulation of endo-poly-galacturonate trans-eliminase in an adenosine 3',5-cyclic monophosphate-deficient mutant of Erwinia carotovora.

51. PANOPOULOS, N.J., W.V. GUIMARAES, S.S. HUA, C.C. SABERSKY-LEHMAN, S. RESNICK, M. LAI and S.S. SHAFFER. 1978. Plasmids in phytopathogenic bacteria. p. 238-241. In D. Schlessinger (ed.) Microbiology-1978. American Soc. for Microbiology, Washington, D.C. 449p.

52. PANOPOULOS, N.J., W.V. GUIMARAES and M.N. SCHROTH. 1975. Conjugative transfer of Pseudomonas aeruginosa R factors to plant pathogenic Pseudomonas spp. Phytopathology 65:380-388.

53. PETIT, A., S. DELHAYE, J. TEMPE and G. MOREL. 1970. Recherches sur les guanidines des tissus de crown gall. Mise en evidence d'une relation biochimique specifique entre les souches d'Agrobacterium tumefaciens et les tumeurs qu'elles induisent. Physiol. Veg. 8:205-213.

54. PUGASETTI, B.K. and M.P. STARR. 1975. Conjugational transfer of genes determining plant virulence in Erwinia amylovora. J. Bacteriol. 122:485-491.

55. PUNSALANG, A.P. and W.D. SAWYER. 1973. Role of pili in the virulence of Neisseria gonorrhoeae. Infection and Immunity 8:255-263.

56. ROWND, R., R. NAKAYA and A. NAKAMURA. 1966. Molecular nature of the drug-resistance factors of the Enterobacteriaceae. J. Molec. Biol. 17:376-393.

57. SCHWINGHAMER, E.A. 1977. Genetic aspects of nodulation and dinitrogen fixation by legumes: The microsymbiont, p. 577-622. In R.W.F. Hardy and W.S. Silver (eds) A Treatise of Dinitrogen Fixation, Section III: Biology. John Wiley and Sons, New York. 675p.

58. SCIAKY, D., A.L. MONTOYA and M.D. CHILTON. 1978. Fingerprints of Agrobacterium Ti plasmids. Plasmid 1:238-253.

59. SKERMAN, F.J., S.B. FORMAL and S. FALKOW. 1972. Plasmid-associated enterotoxin production in a strain of E. coli isolated from humans. Inf. and Imm. 5:622-624.

60. SPARKS, R. Personal Communication. Connecticut Agricultural Experiment Station, New Haven, Connecticut.

61. SPARKS, R.B. and G.H. LACY. 1977. Characterization of Erwinia chrysanthemi plasmids pEC1 and pEC2. Proc. Am. Phytopathol. Soc. 4:198. (Abstr.)

62. SUMMERS, A.O., G.A. JACOBY, M.N. SWARTZ, G. MCHUGH and L. SUTTON. 1978. Metal cation and oxyanion resistances in plasmids of gram-negative bacteria. p. 128-131. In D. Schlessinger (ed.) Microbiology-1978. American Soc. for Microbiol. Washington, D.C. 449p.

63. VAN LAREBEKE, N., G. ENGLER, M. HOLSTERS, S. VAN DEN ELSACKER, I. ZAENEN, R.A. SCHILPEROORT and J. SCHELL. 1974. Large plasmid in Agrobacterium tumefaciens essential for crown gall-inducing activity. Nature (London) 252:169-170.

64. WATSON, B., T.C. CURRIER, M.P. GORDON, M.D. CHILTON, and E.W. NESTER. 1975. Plasmid required for virulence of Agrobacterium tumefaciens. J. Bacteriol. 123:255-264.

65. WOODS, A. Personal Communication. Department of Plant Pathology, Cornell University, Ithaca, New York.

66. ZAENEN, I., N. VAN LAREBEKE, H. TEUCHY, M. VAN MONTAGU and J. SCHELL. 1974. Supercoiled circular DNA in crown-gall inducing Agrobacterium strains. J. Mol. Biol. 86:109-127.

67. ZUCKER, M., L. HANKIN and D. SANDS. 1972. Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. Physiol. Pl. Path. 2:59-67.