THE ISOLATION AND IDENTIFICATION OF SOFT ROT *ERWINIA* FROM SAGUARO (*CARNEGIEA GIGANTEA*) FLOWERS

by

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TABLE OF CONTENTS

LIST OF TABLES

ABSTRACT

The objectives of this study were to determine if Erwinia carnegiana is present in saguaro flowers and to clarify the taxonomic position of the organism. A selective broth containing pectin, salts, glutamic acid, and 4 μg/ml erythromycin, and a calcium polypectate differential solid medium were employed in isolations from saguaro flowers. Eleven percent of postblooms (permanently closed flowers) were found to contain pectolytic, soft rotting, Gram negative bacteria. Only two of a total of 189 Cactobrosis larvae found free on the arms (branches) or in the flowers of the saguaro were found to contain soft rotting bacteria. E. carnegiana is very similar biochemically to the species E. carotovora, E. aroideae, and E. atroseptica. Seven of 12 isolates of E. carnegiana produced soft rot of carrot slices; all of them soft rotted tomato fruits. The organism should be placed in the genus Pectobacterium Waldee with the other soft rot Erwinia, probably in the same species.

INTRODUCTION

The saguaro or giant cactus (Carnegiea gigantea Britt and Rose) is the most obvious and best known of the Sonoran Desert plants in the Lower Sonoran Life Zone. It has come to be the symbol of the west and desert life in general, and of Arizona in particular. Its flower is the state flower of Arizona.

Possibly the first written account of a soft rotting disease of the saguaro was that of Hubbard in 1899 (11); Hubbard was probably the first to inoculate an apparently healthy plant with tissue from a rotting plant. On revisiting the site later, he reported, "I found the plant almost entirely consumed by the rot with which I had infected it. Other trunks which I had cut down about the same time without inoculation have not rotted and are still sound and green."

Lightle, Standring, and Brown (16) were the first to isolate Erwinia carnegiana Standring from rotting tissues and to verify it as the causative agent of the necrotic disease of the saguaro. They gave the following description of the disease as caused by E. carnegiana:

> The first symptom of the bacterial necrosis of Carnegiea is a small, circular, light-colored spot usually with water soaked margin, on the surface of the trunk and branches.

> Underneath the surface discoloration the soft parenchymatous tissues become water-soaked after bacterial invasion and subsequently brown to almost black. As the infection progresses the spot enlarges and assumes a purplish hue. In cases in which the infection advances rapidly the central part of the spot breaks and a brown liquid exudes. Rapid destruction of the tissues is then accompanied by "bleeding" but slower internal decay may precede exudation. The rotted tissues dry, break up into granular to lumpy pieces and fall to the ground, leaving the woody stelar strands bare.

This disease caused a 29.3% mortality in a stand of 1380 cacti in the Saguaro National Monument east of Tucson, Arizona in the 21 years from 1942 to 1961 (3). If mortality due to bacterial necrosis continues at its present rate and the problems relating to seedling establishment persist, it was predicted by Alcorn and May (3) that the stand will be wiped out by 2000 A. D. Since in the U. S. saguaros naturally exist in Arizona and, only marginally, in California, the relationship of bacterial necrosis to population decrease of this cactus is of continuing concern.

There is confusion in the literature concerning the proper taxonomic position of some members of the genus Erwinia, including E. carnegiana. Phytopathogenic bacteria may be divided into groups based on the changes they make in plant tissue. Soft-rot producing, coliform bacteria possess enzymes that break down the pectic substances in the middle lamella of the cell walls of parenchymatous tissues, thus making plant tissues soft and

slimy (9). In addition to possessing pectic enzymes, these bacteria are Gram negative, pertrichously flagellated, and facultatively anaerobic. They reduce nitrate to nitrite, and ferment many carbohydrates to acid (9). Since they are similar to enteric bacteria, they have been placed in the genus Erwinia.

The genus Erwinia, however, also contains other bacteria which do not cause "soft rot" diseases. One of these species, the type species of the genus, Erwinia amylovora (Burrill) Winslow et al., causes fire blight of pear and apple, a dry necrosis. The recognizable and important differences between the non-soft rotting members of the Erwinia, represented by E. amylovora, and the soft rotting species, E. carotovora (Jones) Holland, E. aroideae (Townsend) Holland, E. atroseptica (van Hall) Jennison, and E. chrysanthemi Burkholder et al., have been pointed out by several different authors, first by Waldee (5), later by Sutton (28), Graham (9), and Lockhart and Koenig (17). The lack of similar biochemical characteristics is so definite that these authors believe it is necessary to recognize the two groups as distinct genera by removing the pectolytic species from Erwinia to the genus Pectobacterium Waldee. Sutton (28) has suggested that these be reduced to two species of Pectobacterium, i.e., P. carotovora and P. chrysanthemi. The biochemically defined P. carotovora would, then, include E. aroideae and E. atroseptica.

However, Graham (9) recognizes only one species, P. carotovorum. This he divides into four varieties, P. carotovorum, itself, P. carotovorum var. atroseptica, P. carotovorum var. aroideae, and P. carotovorum var. chrysanthemi.

The organism responsible for the necrosis of the saguaro cactus, E. carnegiana, was originally described (16) as being a Gram positive, peritrichous, encapsulated, nonsporogenus rod. It was noted as being similar to E. carotovora in the type of disease produced, and in reactions to most media tests. However, when E. carotovora was inoculated into the saguaro no disease resulted, and when E. carnegiana was inoculated into carrots and other hosts of E. carotovora no soft rotting occurred. Apparently, because of the difference in Gram stain reaction and pathogenicity, the organism was considered a new species.

The Gram reaction was confirmed by Boyle (4) who noted, however, that old laboratory stock cultures became Gram negative. On the other hand, Burkholder found this organism to be Gram negative, with old cultures showing Gram positive granules (5). Boyle found E. carnegiana to belong to the soft rot group on the basis of physiological and cultural similarities to E. carotovora. However, it was also found to have a thermal death point of 59 C, 7 C above that published for E. carotovora, and to be sensitive

to penicillin, while E. carotovora was not. Sutton (28) and Graham (9) in their studies of the genus Erwinia examined cultures of E. carnegiana and found them not to cause pectolysis and stated therefore that it could not be a soft rotter. Indeed the literature is confused regarding E. carnegiana.

Several different possible modes of transmission of E. carnegiana have been proposed by Boyle (4): root to root transmission, infection from contaminated soil, falling of an infected plant or branch against a healthy plant, and by insects. Alcorn and May (3) substantiated the observation of Boyle (4) that proximity of healthy to infected plants could be a factor in transmission. Takacs (29) found that injuries must be present in order for infection to occur when saguaro seedling roots are either dipped in bacterial suspensions or planted on soil infested with bacteria. In the field, roots may be injured by the action of weather, rodents, or insects (4).

Boyle considered the night flying moth, Cactobrosis fernaldialis Hulst to be the most important vector concerned with the spread of bacterial necrosis. When diseased cacti were cut down, and sliced thru the middle longitudinally, many tunnels with occasional rot pockets occurring along them were revealed. E. carnegiana was isolated from the tissues surrounding the tunnels and the surface and intestine of the Cactobrosis fernaldialis

larvae responsible for the tunnels. On rearing these larvae in cages, the bacterium was isolated from the adult and the surface of the eggs. When larval descendants of those collected from the tunnels were inoculated into cacti, typical soft rot characteristic of the disease resulted.

Boyle believed that Cactobrosis larvae had one life cycle a year with the largest part spent tunneling in the cactus. She observed larvae leaving plants to pupate under debris on the ground from May through August. In laboratory studies, pupation required 28-33 days; the adult nocturnal moth laid its eggs and died within a three day period. The eggs required 30 days to hatch.

Butler and Werner (6) collected Cactobrosis adults in a black light trap in the Saguaro National Monument during 1960 and 1961. Adults first appeared in March in small numbers. They were numerous in April and May, but were most numerous in June. Small numbers were found in July, August, and September.

From our own observations larvae change color as they develop: at 2 mm they are pink, from 3 to 15 mm brown, and from 18 to 24 mm blue. Small pink larvae can enter small greenhouse reared plants anywhere, however they can only enter large cacti near the apex (the growing point) due to heavy cutinization elsewhere (4). Larvae of all sizes, however, have been found inside the flowers that

form a crown around the apex in May and June (Alcorn and Butler, unpublished 1967). Often larvae are seen eating their way out of flowers; they could enter the main body of the cactus at this time.

Boyle (4) recovered E. carnegiana from partially rotted flowers and fruits found on the ground around cacti. No larvae were found in them, but holes thought to have been made by the larvae were found. Boyle (4) and Takacs (29) caused flowers to rot by spraying them with suspensions of E. carnegiana. These findings raised the question of whether the flowers containing E. carnegiana found on the ground by Boyle were infected on the plant or after falling, since the bacterium can survive in soil (4, 29). The question arose, also, whether the Cactobrosis larvae were responsible for the infection of flowers or if possibly the reverse might be the case.

The purpose of this investigation was fourfold:

1. To determine if E. carnegiana is present in any of the flower stages--prebloom (bud), bloom (open flower), or postbloom (permanently closed flower) on the plant.
2. If the flowers are infected on the plant, then to determine what relationship exists between the presence of Cactobrosis larvae in the flowers and the presence of E. carnegiana; that is, to determine if larvae carry the bacteria to the flowers.

3. If the Cactobrosis larvae are not carrying the bacterium to the flowers, then to determine if flowers are becoming contaminated by flying insects or dust-borne bacteria.
4. To more fully biochemically characterize E. carnegiana and to compare it with some of the other species of Erwinia in hopes of clearing up some of the confusion in the literature about E. carnegiana.

MATERIALS AND METHODS

Plant Selection

Saguaro cacti in the East Division of the Saguaro National Monument and in the West Division (30 miles away on the west slopes of the Tucson Mountains) were selected for recovery experiments in 1966. In all, 110 plants were chosen that had flowers on the apices of their main stems or branches (arms) that were accessible either from the ground or from a deck on the roof of a panel truck. Three of the 110 were obviously diseased. Of the plants in the East Division ten were treated as controls; in order to keep out insects, one flowering arm was covered with three to four layers of cheesecloth and a non-systemic insecticide, ORTHO-KLOR-44 (Ortho Division, California Chemical Company, San Francisco, Calif.) was painted around the arm below the cheesecloth to form a band approximately an inch wide. Although the insecticide was applied to prevent crawling insects from visiting the flowers of the saguaro, ants and Cactobrosis larvae were subsequently seen on most arms.

Twenty-five saguaro cacti in the north cactus forest of the East Division of the Saguaro National Monument were selected for the recovery experiments in 1967. The plants were all 20 to 25 feet tall with two to five

arms except for one approximately ten feet tall with only one arm. Plants of this description were chosen at random; that is, plants were chosen that could be reached from the road by truck and had two flowering trunk and/or branch tips that could be reached when the collector stood on the top of the truck. None of the plants was obviously diseased; diseased plants were in the area but none was closer than 70 feet. On each plant one flowering apex was treated with cheesecloth and insecticide as described for 1966.

Collection Technique

Collections were made from approximately 7 AM to 10 AM. The collector's hands were washed with 2% AMPHYL (Lehn and Fink Products Corp., Bloomfield, N. J.) before moving from one plant to another, and frequently moistened with AMPHYL between flower collections from a given plant. Each flower stage removed was placed in a new, small, brown paper bag on which was recorded the plant number, whether or not the apex was covered with cheesecloth, the stage of the flower, and if there were signs of larvae being present. If a Cactobrosis larva was visible, it was placed in a sterile, screw capped vial with the aid of AMPHYL treated forceps. Any larvae found crawling free on the branch were also placed in a separate, sterile vial.

In 1966 collections were made from one uncovered flowering apex on each plant except for the ten plants that had covered apices; from these, flowers were collected from two apices, one covered and one uncovered. From each of the plants up to three blooms and three postblooms were removed from each arm. Collections were made on seven different days in the period of May 6 to June 14. Each plant was collected from only once, except for the covered and noncovered arms on the same plants, two collections were made from these. A total of 323 postblooms and 250 blooms were collected from uncovered apices; 32 postblooms and 20 blooms from covered apices.

In 1967 collections were made from one covered and one uncovered apex on each plant. On May 11, 46 covered and 45 uncovered preblooms, 8 covered and 15 uncovered blooms, and 9 covered and 15 uncovered postblooms were collected from a total of 16 plants. Not more than three of each stage were collected from each apex. On the succeeding collection dates May 16, 20, 23, and 26, only covered and uncovered postblooms were collected. No more than six of each came from each apex. Laboratory processing procedures necessitated restricting the collections to 250 postblooms or less on a given date. Because of this, and the plant to plant variation of suitable flower stages, at times material was collected from as few as 18 plants. In all, 401 covered postblooms and 405

uncovered postblooms were collected. In addition to the larvae collected on the covered and uncovered arms, 146 more larvae were collected from other plants between May 20 and June 9.

Isolation Techniques

In 1966, in the laboratory, each flower was placed in a sterile, glass, petri dish, and sliced longitudinally with a flamed scalpel. One section was placed in a test tube containing 10 ml of sterile distilled water. If a larva was found in a flower, it was placed in a separate tube. After not more than one-half hour, the tube contents were mixed on a Vortex stirrer and then one loopful was streaked for isolation on a potato dextrose peptone (PDP) agar plate. This medium contains 39 g Bacto-potato dextrose agar, 10 g Bacto-peptone, 5 g Bacto-agar per liter of distilled water; this was adjusted to pH 7.5 before autoclaving at 15 psi for 15 minutes. After 24 hr incubation at 30 C any colonies resembling E. carnegiana were picked off and streaked on a PDP slant. After 24 hr incubation the bacteria on these slants were suspended in 3 ml of sterile distilled water and up to 1/4 ml of this suspension was inoculated with a sterile, disposable syringe into a two year old saguaro seedling. After 48 hr, bacteria were reisolated from diseased seedlings by streaking a small amount of aseptically isolated, soft

rotted tissue on PDP plates. Subsequently, bacterial growth was checked for colony morphology and Gram reaction.

To facilitate the isolation of E. carnegiana from saguaro flowers a new isolation technique was developed in 1967. The ability to produce pectic enzymes does not necessarily mean an organism is a plant pathogen (19); however, soft rotting Erwinia produce pectic enzymes more rapidly and in greater variety than non-pathogens (14, 19). For this reason a selective broth was developed by modifying Nasuno and Starr's medium for pectic enzyme production by E. carotovora (22) by the addition of erythromycin, a macrolide antibiotic active against Gram positive bacteria in very low concentrations (12). Since erythromycin is a basic molecule and its activity increases fivefold for an increase of one unit of pH in the range from 6.5 to 7.5 (12), the medium was adjusted to pH 8.0. The selective medium contained 2 separately autoclaved parts. Part A was a 1% solution of Sunkist Pectin N. F. #3466. Part B was 0.24% $KHPO_4$, 0.08% Na_2HPO_4, 0.05% $MgSO_4 \cdot 7\ H_2O$, 0.3% $(NH_4)_2SO_4$, 0.3% glutamic acid. To part B was added enough 10 N NaOH to make the pH of the combination of A and B 8.0. One ml of a stock 4 mg/ml erythromycin solution was made up to a volume of 25 ml with sterile pH 8 phosphate buffer and then added to the pH 8.0 combination of parts A and B. The stock erythromycin solution was prepared by dissolving 4 mg/ml erythromycin base in a small

quantity of reagent grade methanol and then making up to volume with sterile pH 8 phosphate buffer. The solution can be kept for no more than one week at 4 C.

In order to determine the maximum concentration of erythromycin that could be used without inhibiting the growth of E. carnegiana, tubes with ten ml aliquots of the selective medium containing respectively 0, 2, 4, and 8 μg/ml erythromycin were inoculated with 0.1 ml physiological saline containing approximately 2×10^4 cells of E. carnegiana. A duplicate set of tubes was inoculated with 0.1 ml physiological saline containing approximately 2×10^4 cells of Staphylococcus aureus. After incubation for 24 hr at 30 C, tenfold serial dilutions in physiological saline were made of the broth in each tube. Tenth ml portions of each dilution were plated three times and the number of colonies developing after 24 hr at 30 C were counted. From the inoculated tubes containing 0, 2, 4, and 8 μg/ml of erythromycin, there were respectively isolated an average of $38 \pm 5 \times 10^7$, $38 \pm 6 \times 10^7$, $210 \pm 34 \times 10^6$, and $204 \pm 24 \times 10^4$ cells/ml of E. carnegiana. There were no S. aureus cells in any of the tubes containing erythromycin; in the tube containing no erythromycin there were $202 \pm 21 \times 10^4$ cells/ml. Four μg/ml thus appeared to be the highest concentration of erythromycin at this pH that can be used to inhibit Gram positive organisms and still effectively inhibit E. carnegiana.

A modification of the solid calcium polypectate gel developed by Starr (26) was used as a solid, differential medium for soft rot Erwinia. It contained 15 g sodium polypectate (Sunkist), 2.25 ml 1 N NaOH, 3 ml fresh 10% $CaCl_2 \cdot 2\ H_2O$, 0.5 ml 1.6% brom thymol blue (in 95% ethanol) and 500 ml distilled water. The ingredients were mixed in a Waring Blendor for one minute and then autoclaved in an unplugged flask for 15 minutes at 15 psi. To the 500 ml of medium 50 ml of a melted 4% agar solution was added, and the mixture poured immediately. The gel cannot be remelted; the final color is grass green with a pH of 6.5. On this medium, isolated colonies of E. carnegiana and other soft rotting Erwinia hydrolyze the pectate so that cylindrical holes are formed which extend to the bottom of the petri dish in 48 to 72 hr at 30 C; other bacteria grow on the surface only, or form more shallow depressions in the gel in the same period of time.

In 1967, the bloom and postblooms were sliced longitudinally and placed in 50 ml of selective medium. After 24 hr at 30 C 0.1 ml of the broth was removed with a sterile disposable 1 ml pipette, and spread (with a flamed bent glass rod) on the solid differential medium. Each larva found in a flower was removed and placed in a sterile tube containing selective broth and subsequently treated in the same manner as the flowers. If the larva was in the

base of the flower, the base was cut off and placed in a separate tube of selective broth.

Bacteria from the holes or liquified areas of the differential plates were streaked with a sterile loop for isolation on a comparable plate. Bacteria from isolated holes on the latter plates were streaked on PDP plates, incubated, and observed for colony appearance. Colonies with morphology in the range of variation of those of the soft rotting Erwinia were Gram stained (15) and streaked on PDP slants. These slants were used for determining pathogenicity in saguaro seedlings as was done in 1966.

Cultures

Twelve isolates of E. carnegiana were examined: three from rotting saguaros, 1-12, 106, and 64-28, isolated in 1957, 1958, and 1964 respectively (stocks from Department of Plant Pathology), and nine from saguaro flowers, 65-164, 65-145, and 65-214 isolated in 1965, and 66-185 through 66-190 isolated in 1966. In addition, three isolates each of E. aroideae, E. carotovora, and two isolates of E. atroseptica were tested (Table 1). Organisms used as controls on the physiological tests were obtained from the Department of Microbiology at the University of Arizona; they included Escherichia coli (Migula) Castelloni and Chalmers, Serratia marcescens Bizio, Proteus vulgaris Hauser, Pseudomonas aeruoginosa (Schroeter) Migula,

Table 1. Sources of isolates of Erwinia species tested

Species	Code No.	Source	Date
E. aroideae	Ea 14	Dickey, Cornell	1964
E. aroideae	Ea 6	Dickey, Cornell	1964
E. aroideae	Ea 139	Starr, U.C. at Davis	1965
E. carotovora	Ec 6	Dickey, Cornell	1964
E. carotovora	Ec 14	Dickey, Cornell	1964
E. carotovora	$Ec_1 18$	Friedman, USDA[a]	1964
E. atroseptica	E 28	Dickey, Cornell	1965
E. atroseptica	E 70	Dickey, Cornell	1964

[a]The late Dr. Friedman was at the USDA Market Pathology Lab., N.Y.C. His code no. for the isolate $Ec_1 18$ was ES4-7. The other code numbers are the numbers that the senders used.

Staphylococcus aureus Rosenbach, and Aerobacter aerogenes (Kruse) Beijerinck.

Media

Studies were done to determine whether rhamnose, dulcitol, salicin, sorbitol, sucrose, dextrose, lactose, and mannitol were fermented. The media contained 10 g Bacto-peptone, 10 g carbohydrate, and 1 ml of a 1.6% solution of Bacto-brom thymol blue (in 95% ethanol) per liter of distilled water and were sterilized by filtration through EKS2 filters having 0.1 micron pore size (Microcell Filter Co., Grosse Pointe Farms, Mich.). They were incubated one week at 30 C to check for sterility before inoculation. The results were read every other day for the first week and twice a week after that for a total of 3 weeks.

Other biochemical studies included: tests for hydrolysis of gelatin (using nutrient agar plates containing 0.4% gelatin), the use of citrate as a sole source of carbon, the production of H_2S (using 1% tryptone broth and lead acetate papers), and the reduction of nitrate to nitrite. These were done as specified in the Manual of Microbiological Methods (25). Positive controls were: S. marcescens on the nutrient gelatin, A. aerogenes for citrate utilization on Bacto-Koser citrate medium, and E.

coli for the reduction of nitrate to nitrite on Bacto-Nitrate agar.

Indole production and molitity were checked with Bacto-SIM medium with P. vulgaris as the positive check. Urease production was detected by the use of Christensen's urea agar medium and phenylalanine deaminase by Bacto-phenylalanine deaminase by Bacto-phenylalanine agar; in both cases P. vulgaris served as the positive check. The presence of oxidase was determined by Steele's (27) modification of Kovak's (13) technique using P. aeruginosa as the positive control. Procedures used for determining the oxidation of gluconate to 2 keto-gluconate were those of Graham and Dowson (10), with A. aerogenes as the positive control. The presence of pectic enzymes was detected with Starr's calcium polypectate gel (26). The production of arginine, lysine, ornithine and glutamic acid decarboxylases was checked by incorporating these amino acids into separate lots of Bacto-decarboxylase base medium. P. aeruoginosa was the positive check for arginine decarboxylase, and S. marcescens for lyxine and ornithine. P. vulgaris was the positive check for glutamic acid decarboxylase, according to the method of Moeller (20).

For the methyl red and Voges-Proskauer reactions, organisms were grown two days at 30 C in Bacto-MR-VP broth. The methyl red test was performed as specified in the

Manual of Microbiological Methods; the Voges-Proskauer test was done according to the "Standard Methods" of the APHA (1946) as given in the same manual (25). E. coli and A. aerogenes were used in both tests as controls.

Antibiotics

Sensitivity to penicillin G was assayed using the petri plate method (12) employing 1/2 inch paper disks (No. 740-E, Carl Schleicher and Schuell Co., Keene, N. Y.) on which was pipetted .09 ml of the penicillin solution. The "base" layer was 20 ml of G and R agar medium #1 and the "seed" layer was 4 ml of G and R agar medium #2 (12). Five replicate disks were tested on each organism at each concentration of penicillin.

Pathogenicity

Pathogenicity to carrots, tomato fruits, and saguaro seedlings was checked. Carrots were surface sterilized for five minutes with 1:1000 $HgCl_2$ and then rinsed three times with sterile distilled water. Four 1 cm thick slices were placed in sterile, glass petri dishes on pieces of sterile moistened filter paper. Three of the slices were inoculated with 0.05 ml of a suspension containing 5-10 x 10^9 cells per ml; the concentration of the suspension was determined spectrophotometrically (29). On the fourth slice 0.05 ml of sterile distilled water was pipetted. If the carrots were soft rotted a glass rod

could be easily pushed through the slice after 24 hr at 30 C.

Tomato fruits, two year old saguaro seedlings (1 to 1-1/2 inch) and four year old saguaro seedlings (3 to 4 inches) were surface sterilized with 2% AMPHYL 15 minutes prior to inoculation with between 1/4 and 1/2 ml of the bacterial suspension by means of a sterile disposable syringe. Three of each were inoculated; one was injected with 1/4 to 1/2 ml sterile distilled water.

RESULTS

Isolations from Flowers

In May and June of 1966, 4 of 323 uncovered postblooms contained E. carnegiana. Of 32 covered postblooms 2 contained the bacterium. The postblooms from which these isolations were made did not contain Cactobrosis larvae. E. carnegiana could not be isolated from 250 uncovered and 20 covered blooms.

The strikingly low incidence of E. carnegiana recovered from uncovered postblooms cast some doubt on the efficiency of the isolation technique used. Using the new technique an attempt was made in 1967 to determine how few cells of E. carnegiana in flowers could be detected. Since numerous attempts in 1966 had failed to allow isolation of E. carnegiana from blooms collected at 7 AM after having been open since about 10 PM the preceding evening, blooms were seeded and then recovery by the new technique attempted. Ten blooms each were inoculated with 560 $\pm$ 90 cells, 56 $\pm$ 9 cells, and 4-6 cells of E. carnegiana suspended in 0.1 ml of physiological saline. Ten blooms were left as uninoculated controls. After 4 hr the blooms were sliced longitudinally, and put in 125 ml flasks containing the selective broth. After 24 hr at 30 C, 0.1 ml of the broth was removed from each flask with a

separate, sterile disposable 1 ml pipette and spread (with a flamed bent glass rod) on the solid differential medium. Typical sunken colonies were obtained after 48 hr from all inoculated blooms. While it was not certain that the inoculated flowers did not already contain E. carnegiana, no typical sunken colonies were found on the plates spread with the broth containing the ten control blooms.

Using the new isolation technique three of the 15 uncovered postblooms collected May 11, 1967 were found to contain E. carnegiana-like bacteria (i.e., rapid pectate hydrolyzers pathogenic to saguaro seedlings). However, none of the 45 uncovered preblooms or 15 uncovered blooms, 46 covered preblooms, 8 covered blooms, or 9 covered postblooms contained the bacterium. Since the longer the time the blooms are open, the greater the chance the bacteria might multiply and make isolation easier, postblooms only were subsequently collected. Of a total of 401 postblooms from covered arms, only two were found to have E. carnegiana-like bacteria (.52%); of a total of 405 postblooms from uncovered arms 45 were found to harbor similar bacteria (11%). The percent of postblooms that contained these bacteria varied with collection date (Table 2).

Seven percent (29/401) of the covered postblooms and 4.4% (18/405) of the uncovered postblooms either contained Cactobrosis larvae or had tunnels revealing that

Table 2. Fraction of saguaro postblooms containing soft rotting bacteria.

Date	Covered[a]		Uncovered[b]	
	PB[c]	PBL[d]	PB	PBL
May 11	0/6	0/3	3/11	0/7
May 16	1/53	0/1	9/50	0/2
May 20	0/104	1/13	14/105	1/4
May 23	0/90	0/4	7/98	0/5
May 26	1/119	0/8	12/123	0/0

[a]Flowering tip covered with cheesecloth and arms banded with 44% ORTHO-KLOR-44.

[b]No cheesecloth covering; no insecticide applied.

[c]Fraction of postblooms that had no signs of larvae that contained soft rotting bacteria.

[d]Fraction of postblooms that had larvae in them or signs of larvae that contained soft rotting bacteria.

larvae had entered the base of the flower. Of these, only the larvae in these two cases were large and blue; since they were inadvertently sliced when the postblooms were cut, the whole flower and the sliced larva were cultured together. For this reason, it could not be determined whether the larvae alone had the bacteria or if the bacteria were in the upper portion of the postbloom only.

Of 20 larvae found free on the outside of the covered arms, 23 found similarly on uncovered arms, and the 146 larvae found on plants (other than the 25 from which flowers were collected), no soft rotting bacteria were recovered.

Identification of Organisms

All the isolates from flowers and cacti and the isolates of E. carotovora, E. aroideae, and E. atroseptica were found to be motile, Gram negative rods that fermented dextrose, rhamnose, salicin, sucrose, mannitol, and sorbitol without gas. Dulcitol was fermented by none of them. They all gave the same reactions on 14 additional physiological tests (Table 3). Especially noteworthy is the pectate liquefaction since this is the "hallmark" of the "soft rotters." The controls on each Gram stained (15) slide, S. aureus and E. coli, were Gram positive and Gram negative respectively. All organisms inoculated into test media as positive controls produced positive reactions.

Table 3. Biochemical reactions of Erwinia carnegiana and soft rot Erwinia.

Biochemical Test	E. carnegiana[a]	Soft Rot Erwinia[b]
Indole production	-	-
H_2S production[c]	+	+
Gelatin hydrolysis	+	+
Pectate liquefaction	+	+
Urease	-	-
Nitrate reduction	+	+
Gluconate test	-	-
Oxidase test	-	-
Growth in citrate	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Glutamic acid decarboxylase	-	-
Arginine decarboxylase	-	-
Phenylalanine deaminase	-	-

[a]Results of one replication of tests on 12 isolates of E. carnegiana.

[b]Results of one replication of tests on three isolates of E. aroideae and E. carotovora, and two of E. atroseptica (Table 1).

[c]H_2S production was detected on lead acetate papers only; none could be detected with SIM medium.

All of the organisms produced soft rot of two and four year old saguaro seedlings and of tomato fruits. However only seven of the twelve cactus isolates tested were able to produce consistent, typical soft rot of carrots (Table 4). Ten of the twelve cactus isolates produced acid during the fermentation of lactose only after two to three weeks in contrast to acid production within two days by the three species of soft rotting Erwinia (Table 4). There was great variation in the methyl red and Voges-Proskauer reactions given by the cactus isolates (Table 4).

The colonial morphology of E. carnegiana was compared with that of E. carotovora, E. aroideae, and E. atroseptica. After 24 hr incubation at 30 C on PDP agar plates the colonies of most isolates of E. carnegiana were small, convex, entire, smooth, glistening, grayish or somewhat opalescent, depending on the angle of the microscope light, and translucent becoming white and more opaque with age. Lines or "bars" could be seen in the colonies. The colonies of the other three species were less convex, with irregular edges and more pronounced "bars." The colonies of E. carnegiana isolates, however, varied somewhat in the regularity of edge and degree of convexity, and some of them approached the colonial morphology of the three other species.

The minimum inhibitory concentration of penicillin was found to be 0.5 µg/ml for S. aureus, 100 µg/ml for

Table 4. Some differences between Erwinia carnegiana and the isolates of three species of soft rot Erwinia.

Isolate No.	VP	MR	Carrot[a]	Lactose[b]
66-185	-	+	---	(+)
66-186	-	+	+++	(+)
66-187	-	-	---	(+)
66-188	+	-	++-	(+)
66-189	+	+	++-	(+)
66-190	+	-	+++	(+)
106	+	+	+++	(+)
1-12	+	+	+++	(+)
64-28	+	-	+++	(+)
65-214	-	-	---	(+)
65-164	+	-	+++	+
65-145	+	+	+++	+
Ec 6, Ec 14	-	+	+++	+
Ea 139, $Ec_1$18	+	+	+++	+
Ea 14, Ea 6	-	+	+++	+
E 28, E 70	-	+	+++	+

[a]Carrot pathogenicity test; + means soft rot of one of three slices inoculated.

[b]Lactose fermentation; + means acid production in two days; (+) means acid production delayed.

isolate 66-188 of E. carnegiana, 100 μg/ml for Ec 14, an isolate of E. carotovora, and 10 μg/ml for Ea 6, an isolate of E. aroideae.

DISCUSSION

Saguaro flowers definitely can be infected while on the plant; soft rotting bacteria can be isolated from postblooms, but not from blooms or preblooms. It seems improbable that the Cactobrosis fernaldialis larva is responsible for the presence of these bacteria in flowers since the bacterium could not be isolated from the 189 larvae found on the surface of the arms, irrespective of the treatment of the arm. Further, only 2 of the 47 postblooms with larvae were also found to harbor such bacteria. Since 11% of the uncovered postblooms were found to contain soft rotting bacteria, it is possible that instead of the Cactobrosis larvae bringing these to the flowers, the larvae might there become contaminated. Since Boyle (4) observed that larval tunnels appeared to generally originate at or near the branch apices, the larvae might carry bacteria into the body of a saguaro immediately after leaving the flowers, thus causing the distribution of rot pockets along tunnels, as described by Boyle.

Erwinia carnegiana has been recovered from soil (4, 29), therefore the possibility exists that flowers could become contaminated by wind-borne dust. The present tests did not specifically include experiments to determine whether or not dust could sift through the three to four

layers of cheesecloth. Presuming, however, that dust could penetrate to the flowers, the fact that only one-half percent of the postblooms on the cheesecloth covered arms contained the bacterium compared with eleven percent of the postblooms from uncovered arms, suggests that contamination of flowers with the bacterium is caused by other means.

A number of night and/or day flying winged animals visit the saguaro blooms for pollen and/or nectar (21). Some of these, as the bumble bee (Bombus Latreille) and Carpophilus longiventris Sharp, may nest or pupate in the soil (3, 22). Should such sites be in areas that contained a high concentration of bacteria due to the rotting of a cactus nearby, transfer of bacteria to flowers could be effected.

Many insects of the Dipterous group are known to be associated with rotting saguaros; of these, Sanatana (24) found Volucella isabellina Willston larvae to be the most common in rapidly rotting saguaros. While one or two adults were observed by him at the opening of rot pockets, more were commonly found on flowers (presumably saguaro flowers, although they were not specifically identified). Boyle (4) collected Volucella larvae from rot pockets and was able to isolate Erwinia carnegiana from their surfaces, however, she could not isolate the bacterium from laboratory reared adults. It is still possible however, that

adults might become contaminated with the bacterium while laying eggs in rot pockets and then later transmit the bacteria to flowers. Apart from the implication by Santana (23), Volucella isabellina has not yet been reported visiting saguaro flowers. Drosophila larvae also inhabit rot pockets and have been shown by Graf (7) to be possible vectors of E. carnegiana. Although small Dipterous insects were noted in the proximity of flowers, these were not identified. Reports are also not known of their visits to saguaro flowers. Thus, proof of the relationship of rot visiting insects to saguaro flower contamination yet remains.

Saguaro blooms produce as much as 4 to 5 ml of nectar; it is not known whether E. carnegiana is able to multiply in the nectar. Thomas and Ark (30) found that E. amylovora could not multiply in the flowers of apples and pears unless rain diluted the nectar because of the high osmotic pressure. Whether or not E. carnegiana can multiply in the nectar, it is logical that it would be able to multiply in the parenchymatous cells of the flower as it does in the plant. After the flower closes there is a greenhouse effect, that is, moisture is trapped inside and the heat from the sun makes a favorable situation for the growth of E. carnegiana.

Erwinia carnegiana is a soft rot coliform. The 12 isolates from the saguaro cactus and saguaro flowers were

found to possess pectic enzymes, the most definitive characteristic of the "soft rotters"; in 1967 this fact was used to facilitate in the isolation of E. carnegiana from saguaro flowers. These 12 isolates also stained Gram negative, possessed motility, reduced nitrates to nitrites, and fermented many sugars to acid, as did the soft rotting Erwinia tested. The culture that Sutton (28) examined was obtained from Dr. Brown of the University of Arizona in 1944; it is possible that in the intervening years it became contaminated with a non-pectolytic organism. Graham (9) did not state the source or age of his supposedly authentic E. carnegiana cultures.

E. carnegiana is very similar biochemically to the three species of soft rotting Erwinia tested; all test reactions were identical (Table 3) except for four (Table 4). All of the results obtained for E. carotovora, E. aroideae, and E. atroseptica agree with those obtained by Sutton (28) and Graham (9) except for the acid production in the fermentation of sorbitol. Two different lots of sorbitol from Difco and one from Eastman Organic Co. were tried with acid production resulting in all cases. Sutton mentions that others had found these organisms to produce acid from the fermentation of sorbitol and assumed that the sorbitol was contaminated with glucose, since it is used to produce sorbitol commercially. The three lots of sorbitol on which acid was produced were tested with Benedict's

qualitative reagent for reducing sugars (7); a positive reaction was obtained when a saturated solution was tested, indicating the presence of reducing sugars, probably glucose.

The isolate of E. carnegiana tested was not found to be more sensitive to penicillin than the isolates of E. carotovora and E. aroideae tested; this is in disagreement with the finding of Boyle (4). She did not state the pH or concentration of penicillin that she used or the number of isolates checked. More isolates of E. carnegiana and the other species should be tested before any conclusions can be drawn about the relative sensitivity of E. carnegiana and the other three species to penicillin.

In contrast to the finding of Lightle, Standring and Brown (16) that E. carotovora did not produce disease in the saguaro and that E. carnegiana did not produce disease in any of the hosts of E. carotovora, E. carotovora has been found to soft rot saguaro seedlings, and E. carnegiana has been found to cause disease in some hosts of E. carotovora. Alcorn (1) found E. carnegiana to soft rot tomato and squash fruits and honey dew melons. All of the isolates in this study soft rotted tomato fruits; seven of them produced consistent, typical soft rot of carrots.

Since Erwinia carnegiana is a soft rotter it should be placed in the genus Pectobacterium along with the other soft rotting species of Erwinia. Because of the few

differences that exist between it and the three other species, they probably should be considered as the same species.

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