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Leaf Freckles and Wilt of Corn Incited By *Corynebacterium nebraskense* Schuster, Hoff, Mandel, Lazar, 1972

M. L. Schuster[1]

INTRODUCTION

The new bacterial disease Leaf Freckles and Wilt (LFW) of corn (*Zea mays* L.) was first observed in 1969 on two farms in southcentral Nebraska. Since then it has spread to other areas in the state. LFW is a serious disease on farms in six Nebraska counties (Clay, Custer, Dawson, Furnas, Hall and Phelps) and has been found in isolated cases in at least 28 other counties.

In 1971 LFW was found in a seedcorn field in western Iowa, in 1973 in two Kansas counties, and in 1974 in South Dakota and Colorado.

Because of its explosive nature, several facets of the disease and the causal agent, a previously undescribed plant pathogenic bacterium, have been investigated.

The new bacterium was identified and described as *Corynebacterium nebraskense* Schuster, Hoff, Mandel, and Lazar, 1972 (18). The common name, Leaf Freckles and Wilt, was chosen because it is descriptive and characteristic of the disease symptoms. In several scientific papers terms such as Nebraska leaf freckles and wilt, bacterial leaf blight and wilt, bacterial leaf freckles and wilt and Nebraska wilt and leaf freckles, have been employed. Leaf Freckles and Wilt, finally selected as the preferred common name, was so reported in national and international scientific journals (3, 4, 5, 7, 17, 18, 19, 20, 22, 23, 24).[2]

SYMPTOMS AND SIGNS

The gross morphological symptoms (wilt and leaf blight) of the new disease are indistinguishable from Stewart's bacterial wilt and leaf blight caused by *Erwinia stewartii* (E. F. Smith) Dye, 1963. Because of this the corn disease causal agent was considered as a more virulent and orange-colored strain of *E. stewartii* that attacked field corn.

In the new corn disease light greenish-yellow stripes (occasionally reddish depending on hybrid or inbred) with wavy and irregular margins occur following the leaf veins; however, discrete watersoaked

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[2]Editor's Note: Another common name used in connection with this disease is "Goss's Bacterial Wilt and Blight."

spots (freckles) along the veins are characteristic of the new disease (Fig. 1—see color plate). These spots are dark green to blackish (Fig. 2) in appearance and may result from direct infection of old or young leaves or via the roots or stems of young plants. The spots, especially when infected leaves are turning brown, look like "freckles," hence the common name (Fig. 3—see color plate). Eventually, coalescence of stripes results in a leaf scorch reminiscent of drought effects. Ivanoff (9) indicated that *E. stewartii* may induce watersoaked spots which are transitory since they coalesce to form the characteristically linear watersoaked lesions with wavy or irregular margins.

The corn plant can be infected at any age and may wilt and die at any stage of development. Seedlings may die sooner than older plants which may or may not produce tassels and ears depending on age at infection. Late infection may not affect full development of seedlings or older plants. Wilting, stunting and typical leaf symptoms were found in corn in all development stages. Leaves of infected seedlings may stick together due to exuding organisms. In older plants this may prevent emergence of tassels and cause the stalk to bend in the form of a loop or to form a "buggy whip." A dry or watersoaked brown root and stalk rot has been found in the field depending on the moisture regime (Fig. 4). Discoloration of interior nodes and internodes, three or more above the adventitious roots, and general crown or basal rotting can occur.

A diagnostic sign of the wilt phase is the orange bacterial exudate (not as viscous as that of *E. stewartii)* discharged from vascular elements of a cross sectioned stalk of maize (Fig. 5—see color plate). Under severe infections an orange bacterial exudate can occur as pockets of pure cultures in the stem pith. A bacterial exudate frequently is found on the surface of infected leaves and usually appears colorless but can be recognized because of a sheen on the leaf surface. The pathogen is primarily a vascular parasite but invades other tissues such as parenchyma. This is vividly demonstrated by the bacterial pockets in the pith and the presence of "freckles" along leaf veins. The organism produces a toxin which precedes the bacterium by a few days. Initial symptoms due to a toxin are grayish-green stripes on leaves of young or older plants (Fig. 6). Preliminary studies by Calub, Schuster, Hoff (unpublished) have shown that a toxin is produced in culture by *C. nebraskense.* Orange bacterial ooze and orange to watersoaked "freckles" occur in the inner husks (Fig. 7—see color plate) of severely diseased ears. The pathogen has been isolated from most plant parts, including roots and stems, leaf blades and sheaths, tassels, husks and silks, cobs and kernels.

METHODS OF INOCULATION

To determine reactions of corn inbreds and hybrids and to make pathogenicity studies, different inoculation methods were compared.

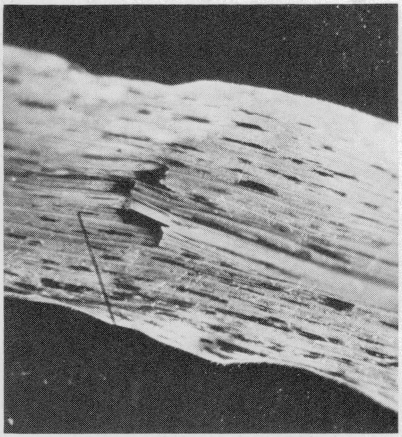

Fig. 2. The freckles can be dark green to blackish on older leaves.

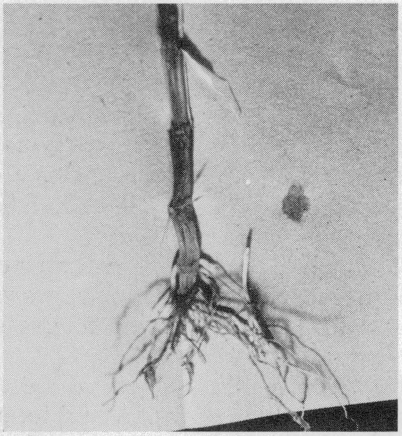

Fig. 4. Root and stalk rot induced by freckles bacterium.

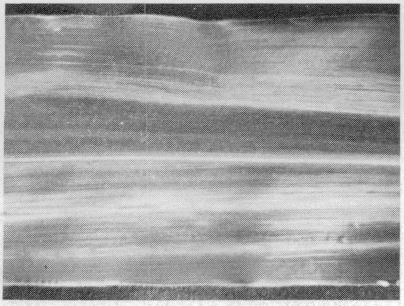

Fig. 6. Gray to greenish-yellow stripes parallel to long axis of corn leaf caused by *C. nebraskense*.

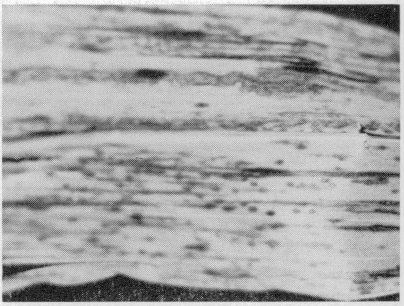

Fig. 8. Leaf infections on grain sorghum or shattercane.

Fig. 9. Source of new infection is the overwintered and infested corn stubble.

Fig. 10. Portion of field (dent) corn ear infected with *C. nebraskense* showing shrivelling of kernels which are water-soaked and orange in color.

Spraying of corn plants by general atomizing (without watersoaking) occasionally resulted in infection, especially from application of bacterial suspension into the whorl area. Watersoaking leaves by forcing inoculum into the leaf through the stomata using a paint sprayer or atomizer attached to a 15 P.S.I. air line did not result in infection. Neither the general atomizing nor the watersoaking method proved useful.

Spraying a bacterial suspension to clipped leaves or injection by hypodermic syringe into bases of seedlings one inch above soil line resulted in fairly consistent infection. In the cut-spray method older leaves appeared less susceptible than younger leaves. Based on these tests, pathogenicity tests, and evaluation of different types of corn, the cut-spray of leaves (2 X 10^9 cells/cc) and/or hypodermic injection of stem (10^6 or 10^7 cells/cc) was employed in the following experiments unless otherwise designated. Air temperature of 25°C proved favorable for disease evaluation. In comparative temperature tests 18, 25 and 28°C symptoms required 28, 3–5, and 1–3 days to develop, respectively.

HOST RANGE

Some genotypes of maize, *Zea mays* L., are susceptible to the pathogen, with inbreds and hybrids varying in disease reactions. Natural infections were found on field and sweet corn. The pathogen was also isolated from green foxtail and shattercane, common weeds in field (dent) corn in Nebraska.

Artificial inoculations have been made with a number of diverse plant species. Leaf symptoms resembling those induced in maize were obtained in teosinte (*Euchlaena mexicana* Schrad.), eastern gama grass (*Tripsacum dactyloides* L.), and green foxtail (*Setaria viridis* L.).

Shattercane, grain sorghum, sudangrass and sugarcane were found susceptible. Symptoms were a red vein discoloration and long red or small irregularly shaped lesions following the leaf veins (Fig. 8). Freckles in these hosts were perhaps masked by the red pigmentation. Infection was not obtained on Proso millet, yellow foxtail, crabgrass, barnyard grass, bromegrass, Johnson grass, oats, barley, tobacco, soybean, and cabbage. No infection resulted from inoculations of primary host plants (beans, alfalfa, tomato, wheat, petunia, potato, chrysanthemum, orchardgrass, poinsettia, and sugarbeet) of other closely related bacteria. The related bacteria in turn were not infectious for field and sweet corn.

This inoculation study indicates that a small number of plant species are susceptible to Leaf Freckles and Wilt bacterium (Table 1). It appears that non-corn host plants are relatively unimportant in the disease picture but additional studies are required along these lines. The pathogenicity studies confirm that a new disease and a new pathogenic coryneform bacterium have been discovered (18, 22, 23).

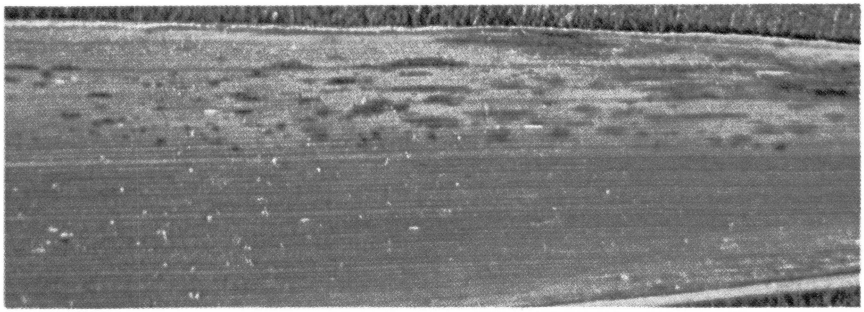

Fig. 1. Watersoaked spots (freckles) along the leaf veins of green foxtail are characteristic of the disease.

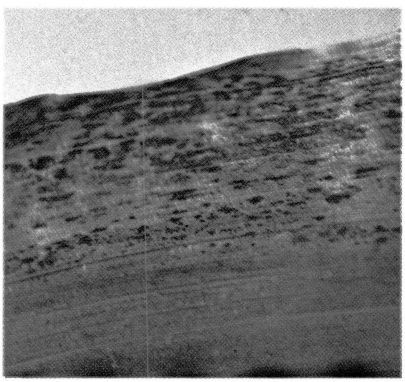

Fig. 3. The spots are more reminiscent of "freckles" on leaves turning brown.

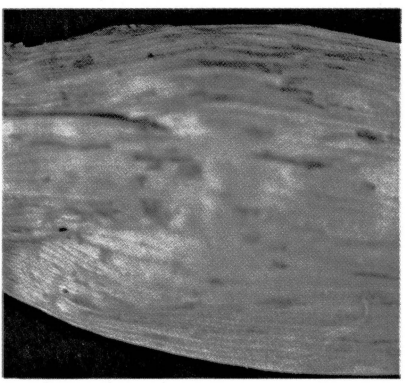

Fig. 7. Orange discolored spots (freckles) on inner husk induced by *C. nebraskense*.

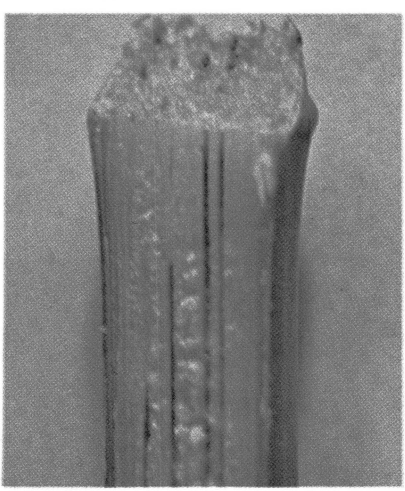

Fig. 5. Vascular bundles of corn stem infected by *C. nebraskense* are orange in color.

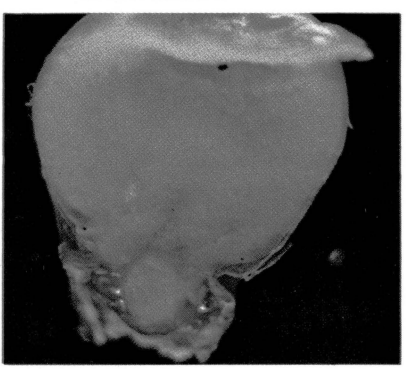

Fig. 13. Longitudinal sections of a kernel of field (dent) corn infected with *C. nebraskense*. Note watersoaked and orange discolored vascular elements of pedicel and chalazal areas; cavities are present in chalazal areas. Compare with figure 12.

Table 1. Summary of susceptibility of selected plant species to *Corynebacterium nebraskense*.

Plant species	Reaction[a]	Leaf symptoms[b]
Euchlaena mexicana (teosinte)	S	N
Zea mays (corn, maize)	S	N
Setaria viridis (green foxtail)	S	N
Tripsacum dactyloides (Eastern gama grass)	S	N
Saccharum officinarum (sugarcane)	S	A
Sorghum bicolor (shattercane)	S	A
Sorghum vulgare (grain sorghum)	S	A
Sorghum vulgare sudanense (sudangrass)	S	A
Avena sativa (oats)	R	
Bromus inermis (brome grass)	R	
Dactylis glomerata (orchardgrass)	R	
Digitaria sanguinalis (crabgrass)	R	
Echinocloa crusgalli (barnyard grass)	R	
Hordeum vulgare (barley)	R	
Panicum miliaceum (Proso millet)	R	
Setaria lutescens (yellow foxtail)	R	
Sorghum halepense (Johnson grass)	R	
Triticum aestivum (wheat)	R	
Beta vulgaris (sugarbeet)	R	
Brassica oleracea var. *gemmifera* (cabbage)	R	
Chrysanthemum morifolium (chrysanthemum)	R	
Euphorbia pulcherrima (poinsettiae)	R	
Lycopersicon esculentum (tomato)	R	
Nicotiana tabacum (petunia)	R	
Solanum tuberosum (potato)	R	

[a] S = susceptible; R = resistant.

[b] N = normal symptoms as commonly expressed in corn; A = atypical symptoms – refer to text.

SOURCES OF PRIMARY INFECTION

A possible source of infection is bacteria that have overwintered on or in corn residues (Fig. 9). Infested leaf material from fields in southcentral Nebraska was checked for the disease organism during the spring and early summer of 1970, 1971, 1972, 1973, and 1974. The "freckles" bacterium overwintered in leaf material each of the five years.

Studies were made to determine bacterium survival under more closely controlled and varied conditions. In sunken clay tile, leaves were placed on the soil surface or incorporated to a depth of 8 inches. In 1970, 1971, 1972, and 1973 survival was evident in leaves maintained on the soil surface but not at a depth of 8 inches. The same procedure was used to indirectly assess survival in field survival tests by using the corn plant as the recovery medium.

In fall 1971 a more extensive experiment on survival was set up in Lincoln, Nebraska, using different infested plant parts (leaves, stems,

cobs, ears) and a pure culture of the pathogen, each placed in individual sunken clay tiles at three different depths (0, 4, and 8 inches). Cell growth on 12 Petri plates of pure cultures (four plates of each of three bacterial isolates—293, 296, 313), was applied to soil in each of three tiles. Air dry weights (in grams) of the various plant parts in each tile series was 83 for leaves, 330 for stems, 100 for cobs, and 840 for ears. In June 1972, plant parts incorporated at depths of 4 and 8 inches were placed in wire cloth baskets. This did not interfere with contact between the soil and plant materials. One-fourth of each plant sample was recovered in June, 1972, and soaked in 500 ml of distilled water for 4 hours before inoculation with the supernatant. Five aliquot samples were recovered using a soil probe (1-inch diameter) sunk to a depth of 10 inches in tile soil with pure bacterial cultures and soaked in 500 ml distilled water for 4 hours. The water soakate from each composite sample was used for inoculating ten 10-day-old seedlings (Golden Cross Bantam) by injecting 1 ml of soakate into seedling base about one inch above the soil line (Table 2).

Seedlings were classified into four disease reaction categories: 0 = no symptoms; 1 = few lesions and freckles on first leaf; 2 = lesions and freckles on first and second leaf; 3 = lesions and freckles on three leaves and/or death or wilting seedlings. Disease ratings on individual plants were converted to a disease index or percentage rating for the group of plants based on:

$$\text{Disease Index} = \frac{\text{Category No. X 100}}{\text{Total No. plants X 3}} = \%$$

The three in the denominator represents maximum infection and 100 is used to convert to percentage. The summarization of category numbers is obtained by multiplying the number of plants in each category by their respective category numbers from 0 to 3 and adding the products. The result is the comparative infection rating for the different treatments.

Table 2 shows that different treatments differentially affected pathogen survival. Placement of plant parts in soil affected survival

Table 2. Effect of different infested corn parts and pure cultures placed at three different soil depths in sunken clay tile for 10 months on the survival of *Corynebacterium nebraskense*.

Plant part	Disease index (%)[a]		
	0″	4″	8″
Leaves	80	0	0
Stems	75	30	40
Cobs	63	5	0
Ears	64	0	0
Pure culture	0	0	0

[a] See text for description.

differentially; the bacterium in leaves did not survive for 10 months when incorporated into the soil to an 8-inch depth. This substantiates preliminary tests.

Different soil depths did not affect bacteria in stems, cobs, or ears to the same extent as bacteria in leaves. Pure cultures of the pathogen proved the poorest mechanism for survival—recovery was not realized at any placement depth.

The pathogen survives and is pathogenic in different plant parts under natural field conditions. Proof is necessary to demonstrate that under field conditions infection can result from infested corn residues. Infested corn stubble was transported to an isolated area where the disease was not found previously. The infested corn stubble was placed in each of three plots (10 ft. X 30 ft.) with comparable check plots. On every 2 sq. ft. of treated plots material from one infested corn plant was applied to the soil surface in the autumn of 1970. Three plots were left untreated.

Infested corn stubble was shown to be a source of primary infection and occurring naturally under field conditions. A standard susceptible field corn hybrid was found to have an average disease index of 33% on July 27, 1971, and 42% on September 3, 1971. In the adjacent four non-infested plots, the average percent infection on the two respective dates was 2% and 8%. The explanation for the occurrence of infection in the control plots was that in spring disking infested corn stubble was moved from infested to check plots. This spring land preparation simulated minimum tillage practiced in the severely infested counties in Nebraska. In 1971 a corn grower in Hall county deep fall plowed a 20-acre field in which LFW caused a 22% loss in yield. The following year the field was sown to soybeans, a non-susceptible crop. In 1973 and 1974 a very susceptible corn hybrid was seeded but LFW was present in only trace amounts. The results verified the assumption that under natural conditions a primary and important inoculum source is infested corn stubble.

SURVIVAL IN SOIL

In greenhouse tests, environmental factors were studied as they affect survival of *Corynebacterium nebraskense* strain No. 296. Pure cultures and infested corn leaves (ground in a Wiley mill) were maintained in air-dry (0.95% of field water-holding capacity) or moist soil (30% of field water-holding capacity) in 25.4 cm clay pots at 25°C; other treatments were sterilized and non-sterilized soil.

Twelve corn seedlings were inoculated for each treatment combination. Each replication consisted of four seedlings placed in a randomized block design. The initial concentration of bacterial cells per gram of leaves was 6.7×10^6; the concentration in the pure culture treatment that was added per ml of soil was 10^8 cells. Leaves added to 3000 ml of soil weighed 44.8 grams — equivalent to 10^8 cells/ml.

Table 3. Comparisons of different treatments on the maintenance of *Corynebacterium nebraskense* based on disease index percentages at weekly intervals.

Treatment	Week					
	0	1	2	3	4	5
NMC[a]	95[b]	0	0	0	0	0
NDC	96	2	0	0	0	0
NML	100	51	5	3	0	0
NDL	93	62	30	53	17	33
SMC	96	8	0	2	0	0
SDC	95	5	8	0	0	0
SML	90	29	2	0	0	0
SDL	100	69	64	54	10	47

[a] Letters refer to: N = nonsterile; S = sterile; M = moist; D = dry; C = pure culture; L = infested leaf material.

[b] Refers to disease index percentage; see text.

Each treatment combination involved a final concentration of 10^8 bacterial cells/ml of soil at 0 hours. The disease indexes ranged from 90% to 100% for the different treatments, indicating that the test is valid. Samplings were made at weekly intervals to determine survival up to 5 weeks (Table 3). The procedure of recovery was transfer of 100 ml of each soil combination into 50 ml of sterilized distilled water. After four hours the water soakate was used for inoculation by submerging a multineedle inoculator and then puncturing the leaves of 10-day-old seedlings. Plants were kept in 25°C dew chamber for 24 hours and incidence of disease was recorded one week later.

Differences were noted between the two opposing treatments: moisture regimes, types of inoculum source, and sterility of the soil (Table 3). Bacterium was recovered in virulent form up to five weeks, the duration of the experiment. Disease indexes were 33% in nonsterile soil and 47% in sterilized soil. Initial disease indexes for the two respective treatments were 100% and 93%.

Pure cultures were a poor inoculum source for survival under all other conditions employed. In the pure cultures the inoculum was mixed in the soil and not necessarily maintained on the soil surface alone. Under nonsterile field soil conditions the pathogen can over winter in leaves on the soil surface. Presumably the organism is in a drier environment and not exposed to antagonistic organisms which are favored by moist conditions. By the same token moist conditions are required for bacterial multiplication but the bacteria are more amenable to destruction during their active than their dormant state in the dried leaves.

Bacteria in pure culture under comparable conditions are in a different state and therefore are viable for a shorter duration even in dry soil. This might be presumptive evidence that the pathogen in dried leaves is in a state of "dormancy" whereas in pure cultures the bacterial cells are in an active state of growth and/or are not protected

by some matrix material. Under the pure culture survival is not favored by any of the other experimental conditions employed — moisture regimes or soil sterility. Possibly the bacterium in its exponential growth phase is more susceptible to killing due to desiccation or other adverse effects than in its stationary phase. Which state is more comparable to a dormant phase, as it were, would be an assumption. Incorporation of leaves in soil is comparable to non-sterile dry soil. Organic decomposition may have created a condition of antibiosis to the bacterial pathogen in nonsterile soil but not in air-dry soil. This might explain the longer survival time in leaves maintained in air-dry soil whether sterilized or not sterilized.

INFECTED KERNELS AS INOCULUM SOURCE

Even though infested corn stubble can serve as a source of primary inoculum, the role of seed in production of LFW was investigated. Detection of bacterium in the kernel can be accomplished by isolation and by histopathological procedures. In the fall of 1970 corn ears were collected from infected plants with this in mind. From 118 ears collected from commercial dent corn, 13 (11%) yielded the pathogen. The isolation method consisted of piercing (with a dissecting needle) five kernels from the base to the tip of each ear and then streaking on nutrient dextrose agar + thiamine (Vitamin B-6). This nutrient medium contained per liter of distilled water beef extract 3 g, peptone 5 g, dextrose 20 g, agar 15 g, and thiamine 100 mg. With this isolation procedure location of the bacterium in or on the seed could not be determined.

Since the orange bacterial exudate can be found on inner husks from badly infected plants, this exudate could provide a source of surface-borne contamination of kernels. Also, since the pathogen is primarily a vascular parasite, the organism could enter the seed via the vascular elements; orange discoloration was found in the vascular elements of the pedicel. Ears from infected plants were examined in more detail for the presence of the bacterium. The ears showed evidence of the pathogen in the vascular elements of cob, ear shank (Figs. 10, 11), and in the outer as well as the inner vascular bundle ring leading to the seed. Before the chalazal region became dark at maturity, removal of the infected kernels would show evidence of water-soaking, orange discoloration and bacterial exudate. Isolations from the exposed chalazal areas yielded the pathogen. The orange bacterium in heavily infected kernels could be easily seen macroscopically between the scutellum and the endosperm and in the vicinity of the embryo (Figs. 12, 13—see color plate). In fact, a cavity or cavities filled with orange bacterial ooze were noted in the chalazal region of infected seed collected in the soft dough stage. The cavity was apparently the result of the breakdown of the chalazal and adjacent cells; prior to dissolution this area was watersoaked (translucent) and then

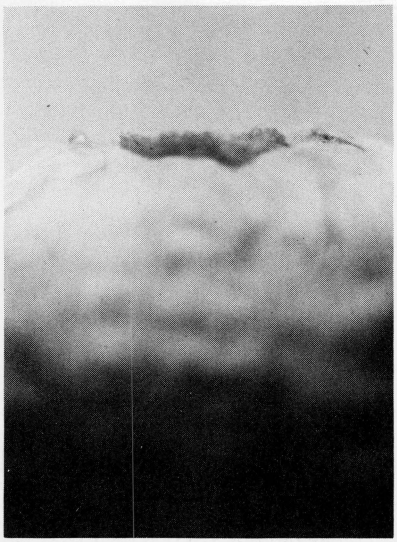

Fig. 11 Orange ooze of the bacterium at base of infected ear.

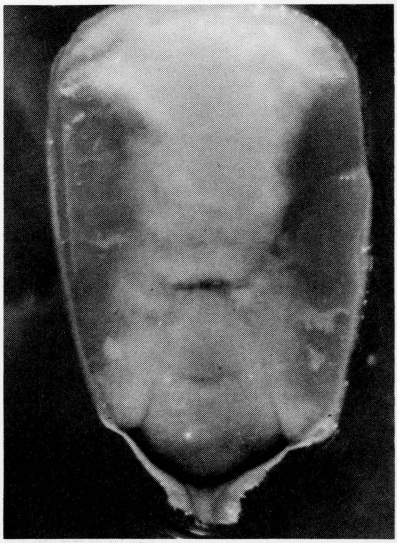

Fig. 12 Longitudinal section of non-infected kernel of field (dent) corn. Note the narrow dark chalazal areas.

filled with orange bacterial slime. The pathogen could be readily isolated from kernels 9 or 12 months old. These conditions illustrated that the bacterium was still viable and did not die before the next year's planting. Also one-year-old seed would produce just as many diseased seedlings as seed one or two month's of age. Experiments were started to ascertain the relationship between internally borne inoculum and disease occurrence.

To determine the percentage of internal seed infection, kernels were alcohol-flamed, dipped in 15% chlorox (0.9% NaOCl) for five minutes, and then rinsed in sterile distilled water. The seeds were transferred aseptically to Petri Plates, five per plate, embryo side up and pressed into the 1% nutrient dextrose agar + thiamine and incubated at 24°C for 1 week. Orange bacterial growth from the kernels was tested for pathogenicity and inducement of typical symptoms after hypodermic inoculation into bases of 10-day-old field corn seedlings. Five field corn seed lots from as many different fields in three southcentral counties in Nebraska were assessed for seed infection. Results are shown in Table 4. Of 399 ears, 31.6% carried the disease organism. Of 2,480 kernels tested (from the above ears) 20.8% were infected. Ears were selected from infected plants so the amount of infection was high.

ROLE OF INFECTED KERNELS IN TRANSMISSION OF LFW

Previous experiments demonstrated that the organism is carried

Table 4. Number and percent of ears and kernels from five lots of commercial field (dent) corn internally infected with the pathogen *C. nebraskense* nine months after harvest.

	Ears			Kernels		
Lot No.	No. non-infected	No. infected	% infected	No. non-infected	No. infected	% infected
I	45	31	40.8	314	137	30.4
II	77	27	26.0	467	97	17.2
III	120	31	20.5	754	111	12.8
IV	9	10	52.6	120	40	25.0
V	22	27	55.1	308	132	30.0
	Total 273	126	Avg. 31.6	1963	517	Avg. 20.8

internally and that isolations from the interior of the kernels were pathogenic. Experiments were started to determine the relationship between inoculum from the interior of the kernels and occurrence of the disease.

To be certain that infection came from the interior of the seed, surface-sterilized kernels were germinated and grown in autoclaved soil in a 24°C greenhouse until plants were six weeks old. Seeds used in this experiment were taken from infected ears reported in Table 4. Fifty seeds from each ear were tested. Diseased plants were checked for pathogen presence by piercing diseased leaves and streaking on nutrient agar. Orange bacterial isolates were also hypodermically inoculated into seedling bases for assessment of their pathogenic capabilities. Results are shown in Table 5.

Only 30 (1.6%) of 1,934 seedlings showed disease symptoms. Since the average percentage of seed actually infected was 48.1, the seed transmission was in reality 0.8%. Furthermore, 416 of the 2,350 seed planted did not germinate; severe infection may have precluded seed germinability. The disease may, however, be introduced into new areas and disseminated long distances; commercial corn as well as seed corn may serve in this capacity. Captan (75% at 2 oz./bu.)-treated seed had a slightly higher number of infected seedlings, 2.2%, compared to 1.4% for untreated. These seed treatment data are based on a total of 250 seeds from five different lots. The low rate of seed

Table 5. Seed transmission of LFW bacterium as evidenced by planting in autoclaved soil.

Lot no.	No. ears	No seed planted	No seed germinated	No. plants infected	% infected
I	9	450	273	2	0.7
II	11	550	508	11	2.2
III	12	600	423	5	1.2
IV	2	50	35	2	5.7
V	14	700	395	10	2.5
	Total 48	2350	1934	30	Avg. 1.6

transmission is not surprising since observations in Petri plates seedlings growing in pure cultures of the bacterium showed little or no infection.

Seed corn was also assessed for presence of the bacterium. Leftover seed corn lots planted in fields that later in the season showed severe infection were assessed for infection. In 1970 three such seed lots (100 kernels/lot) which had been treated with 75% Captan (2 oz./bu.) did not yield the bacterium upon isolation nor was the disease transmitted to seedlings. In 1971, three of 22 seed corn lots from different parts of Nebraska contained the bacterium. These data indicate a low rate of seed contamination. Precautionary measures in selection of seed from disease-free fields would be desirable.

Infected kernels play a minor role in the production of LFW even when the plants are grown in the greenhouse with other sources of infection eliminated. Why greenhouse-grown plants in sterilized soil from seed known to be infected did not have a greater percentage of disease is a moot question. Injury to host tissue might be involved in the entrance of *C. nebraskense* into the host plant.

RELATION OF INJURY TO INFECTION

General means by which bacteria might enter the host plant were studied. These were through natural and artificial wounding of leaves and roots in nutrient dextrose agar or infested soil. Entrance through stomata and water pores was also attempted.

The low percentage of seedling infection from internally infected seed might be caused by lack of injury during germination. In development of suitable inoculation procedures, it became apparent that injury of the host was a prerequisite to infection. Bacteria might enter the host through various means.

Breaks in the pericarp made by an emerging primary root during seed germination is a possible entrance route for external bacterial inoculum. To test this 100 non-infested kernels were surface-sterilized and transferred to the surface of nutrient agar in Petri dishes with embryo sides up. Before the coleorhiza emerged through the pericarp, a drop of bacterial suspension was applied to each kernel tip. After the coleorhiza emerged through the pericarp and the primary root tip appeared through the broken root sheath, bacterial growth was present on the agar and in contact with base of the kernel. As the root grew and developed secondary roots, bacterial growth increased and completely surrounded the roots and the remainder of the kernel. Three weeks after germination no symptoms were evident on leaves of any seedlings. Closer examination under the dissecting microscope revealed bacterial slime between the pericarp and the ruptured coleorhiza, and between the coleorhiza and the surface of the primary root, but there was no evidence of symptoms in any of the 50 seedlings. Roots of 50 seedlings had been punctured with a fine

sterilized needle and six days later watersoaking of upper part of seedling and the characteristic water-soaked spots appeared in the leaves of 64% of the punctured plants. These data indicate that injury is a prerequisite to infection.

To determine whether bacteria can infect plants through wounded roots in soil, greenhouse experiments were started. Non-infected seeds were germinated and six days later were divided into two seedling groups and given certain treatments before planting in sterilized soil.

Each seedling was painted with a camel's hair brush at the primary root, at the broken coleorhiza, and at the lower part of the kernel with a suspension of the new LFW bacterium. Some primary roots of one group of seedlings were punctured with a fine needle and treated with bacterial inoculum. Seedlings of the second group were treated in a similar way, except that sterilized water instead of bacterial inoculum was employed. After these treatments seedlings were transplanted into holes punched into potted soil. Ten days after transplanting, the typical water-soaked spots (freckles) appeared along the leaf veins of 21 seedlings (84%) painted with bacterial suspension and pierced with a needle. Seedlings without punctured roots showed no symptoms.

Experiments were conducted to determine the effect of root wounding and infested soil on infection. In limited pot experiments, infested corn debris did not provide infection unless roots were injured. This involved 25 plants for each treatment; 13 plants (52%) in the root wounded series and one plant in the non-wounded series became infected.

In hydroponics the effect of root wounding and bruising was studied with respect to soil infestation. Roots were cut with scissors on three successive weeks after the corn was three weeks of age. During each root cutting, inoculum consisting of equal amounts of three virulent cultures was applied to the nutrient solution in nutrient tank at a concentration of 100,000 bacterial cells/ml of solution. Of 250 root-wounded plants, 75% became infected compared to none for the plants without root wounding. Stunting, however, did occur in the noncut inoculated plants even though leaf symptoms were not evident. The average height for the inoculated plants was 42 and 62 inches for injured and not injured roots, respectively, while the non-inoculated non-injured and injured plants were 80 and 78 inches, respectively. Apparently, presence of bacteria in the vicinity of the non-injured roots provoked some damage resulting in stunting. This experiment was repeated on three occasions and comparable results were obtained in each case.

Entrance through stomata is not an uncommon phenomenon for certain bacterial species. After corn plants had attained the second, third and fourth-leaf stage, 15 were inoculated by dropping a bacteri-

al suspension on the tip of each leaf. Fifteen other plants were treated with sterile distilled water. After six weeks no infection was noted in either treatment. A comparable experiment was conducted by applying 3 ml of bacterial suspension in the funnel (whorl) formed by the uncurling leaves. The plants were kept in a humidity chamber for 2 days at 27°C, then transferred to a 24°C greenhouse. No symptoms appeared at the end of the sixth week. Comparable experiments were run substituting ground up (Wiley mill) infested leaves and placement in the whorl of plants two, three and four weeks old. Periodic sprinkling of water into the whorl area was done, but no infection resulted, indicating that infection through the whorl is not common.

LOCATION OF PATHOGEN IN HOST TISSUES

In stems of systemically infected plants the vascular bundles were filled with orange bacteria. The bacteria were also found in disrupted pith tissue of the stem apparently destroying the adjacent tissue. The vascular bundles of the cob and shank were also filled at times with orange bacteria.

In the leaves, bacteria were found in the vessels of the vascular bundles, in cavities adjacent to the vessels, in the intercellular spaces, and as a colorless exudate on the leaf surface.

In the parenchyma tissue of the infected leaf, bacterial pockets were noted adjacent to the openings in the vessel walls. These pockets were found on both sides of a bacterial-filled vessel. These pockets corresponded to the water-soaked spots or freckles. These were either semi-circular or irregular in shape and at times attained ¼ inch in length (Fig. 1).

In the seed, bacteria were located in the parenchymatous tissue of the chalazal region. Bacteria were found in vessels that are extensions of the vascular elements of the inner vascular ring of the cob. Removal of kernels from infected ears during soft dough stage exposed the orange and slimy nature of the bacterium in the abscission layer; removal of kernels of comparable age from non-infected ears exposed a white abscission layer.

Severely infected ears were usually soft, mushy and stunted; they had poorly developed kernels which were orange due to the profuse amounts of orange bacterial slime (Fig. 10). In less severely infected ears, bacteria were evident in the vicinity of the endosperm, scutellum, and embryo of the seed. Concentration of bacteria was greatest at the base (chalaza) of the kernel and decreased towards the top, indicating apical direction of movement.

Shelled kernels usually have the pedicels attached, thus providing a protective cover to the infected chalazal region. Vascular elements and tissues adjacent to the pedicels also harbored the bacterial pathogen, providing additional amounts of bacteria to the shelled kernels. Perhaps such internal infection partially explains the failure

to control seed infection by certain seed treatments such as 75% Captan (1:2,000), mercuric chloride, (1:1,000), for 10 minute dip, or by either of two formulations of tetracycline [(oxytetracycline hydrochloride) (terramycin)] or chlorotetracycline hydrochloride (aureomycin) applied at rates of 100 and 200 ppm. These treatments and the control series were preceded by dipping in 15% chlorox (0.9% Na-OCl) for 5 minutes. Before treatments all seeds were alcohol-flamed. Seventy-six percent of seed treated with mercuric chloride yielded the pathogen. Ninety percent of seed treated with tetracyclines yielded the pathogen compared to 80% for the controls. The two antibiotics, however, inhibited bacterial growth when incorporated in Bactodiscs or filter paper discs (30 mcg) and applied to the surface of a bacterial lawn.

REACTION OF CORN TYPES TO THE DISEASE

Preliminary studies were made in the greenhouse to determine disease reaction of inbreds, hybrids and other types of corn. Found susceptible were the following corn types: popcorn (KP47, La 7, Sg5, Sg16, Sg18, Sg30A, Tom Thumb 8); flint corn [(N8)4612-1, (N7)4627-1, B144601-1, (N6)4594-1, Gaspé)]; sugary corn (725C); sweet corn (Span Cross, Carmel Cross, Iochief, Golden Beauty, Golden Cross Bantam, etc., (Tables 6 and 7).

Table 6. Reaction of sweet corn entries to two isolates (#42 and #10) of the LFW bacterium. Seed planted 3/26/71; inoculated by leaf cut-spray method on 4/14/71; disease readings on 4/21/71.

Hybrid	Disease reaction[a]	
	42	10[c]
Pride of Canada	4[b]	4
Hybrid 4th of July	4	4
Hybrid Golden Beauty	4	4
Hybrid Tendergold	4	4
Hybrid Golden Bantam	4	4
Golden Bantam	4	4
Honey & Cream Hybrid	4	4
Hybrid 10-Chief	4	4
Xtra Sweet Hybrid	3	4
Country Gentleman Hybrid	4	4
Stowell's Evergreen Hybrid	4	4
Carmel Cross	4	4
Country Gentlemen	4	4
Span Cross	4	4
New Hybrid Silver Queen	3	4
Stowell's Evergreen	4	4
New Jubilee Hybrid	4	4
Early Xtra Sweet	4	4

[a] Reaction numbers refer to movement of bacteria from tip of leaf inoculated. A reading of 0 means no infection; 1 refers to movement up to 1 inch; 3 up to 3 inches; and 4 over 3 inches.

[b] Each value number refers to average of 4 plants inoculated.

[c] O.D. for 42 & 10 = 1.8 and 1.8, respectively.

Table 7. Reaction of inbred lines of agronomic corn types to LFW bacteria using two methods of inoculation.

Inbred line	Avg. disease reaction	
	Mixture[a]	Mixture[b]
Popcorn KP47	2.0	2.5
Popcorn Sg5	2.7	4.0
Popcorn La7	4.0	4.0
Popcorn Tom Thumb—H	4.0	1.7
Popcorn Sg16	4.0	3.2
Popcorn Sg30 A	4.0	3.0
Popcorn Sg18	4.0	2.8
Flint (N8)4612-1	4.0	2.8
Flint (N7)4627-1	4.0	2.0
Flint (B14)4601-1	4.0	0.5
Flint (Wf9)	3.0	2.8
Flint (N6)4594-1	2.0	3.2
Flint Gaspé	4.0	3.3
Sugary 725C	4.0	3.3

[a] See previous table for legends; mixture consisted of Max Beyl, Fagot A, Sitorious, and Dek 363 isolates at concentration of 1.3 O.D. (420 mu) applied by cut-spray method.

[b] Same as above using stem injection method.

Table 8. Reaction of sweet corn entries to the 3 isolates (50, 48, & 10) of the LFW bacterium, on 4/22/71.

Corn entry	Disease reaction[a]		
	50	48	10[c]
Co Gert III. 13	3[b]	3	3
Calumet	3	3	3
Golden Cross Bantam	4	4	4
Golden Beauty	4	4	4
Midway	4	4	4
XP348	4	4	4
Vanguard	4	4	4
Florida 104	4	4	4
XP1279	4	4	4
XP299	4	4	3
Golden Security	4	4	4
Victory Golden	4	4	4
Merit	4	4	4
Gusto	4	4	4
Deep Gold	4	4	3
Pacer	4	4	3
XP1323	4	4	3
Wintergreen	3	4	3
Golden Fancy	4	4	3
Bravo	4	4	4
XP358	4	4	4

[a] Disease reading based on distance of bacterial movement down the leaf tip as evidenced by water soaking. A reading of 0 means no infection; 1 refers to movement up to ½ inch; 2 refers to up to 1 inch; 3 up to 3 inches, and 4 refers to movement over 3 inches.

[b] Each number refers to average of 4 plants inoculated.

[c] O.D. for 50, 48 & 10 = 1.3, 1.8 & 1.5, respectively; 420 mu wave-length employed.

Evaluations of field and sweet corn hybrids, solicited by corn companies, ranged from slight to very susceptible with the majority in the moderate to the susceptible categories (Tables 8-10). Immunity was not found in any of the hybrids or inbreds. Over 200 corn inbreds tested for reaction to the new disease bacterium by Schuster, Compton and Hoff (19) and Calub, Schuster, Compton, and Gardner (5) ranged from slightly to severely susceptible. Schuster, *et al,* (19) also reported Texas male sterile (Tms) cytoplasm did not provoke the disease. This conclusion was based on the reactions of eight paired normal (N) and Texas male sterile cytoplasms. Gardner and Schuster (7) in preliminary genetic studies found that probably more than one gene locus controls LFW reaction. Refinements of inoculation techniques (4) are essential and then further investigations will be required to establish the exact genetic nature of LFW reaction.

GENETIC VARIATION OF THE PATHOGEN

Many bacterial species are a confederation of strains having some characters in common, but differing in others, including degree of virulence. Accordingly, *Corynebacterium nebraskense*, Schuster, Hoff, Mandel and Lazar, 1972 (18) comprises a population of strains (18, 19, 20, 22, 23, and Tables 6-11). The occurrence of numerous strains in a pathogenic species complicates control through breeding and maintaining disease-resistant cultivars. Variation in pathogenicity of

Table 9. Reaction of sweet corn hybrids to isolates of LFW bacterium 5/28/71.

	Isolate No.		
Hybrid No.[a]	10	34	48
GG-1	4[b]	2	3
GG-4	6	0	1
GG-20	4	0	2
GG-38	4	3	0
GG-41	10	1	1
GG-D42	3	1	0
GG-043	3	0	0
GG-48	4	0	2
GG-51	3	0.5	0
GG-52	3	–	0
GG-53	4	2	1
GG-120	4	0	0.5
GG-141	5	0	0
GG-521	–	0	–
GG-702	6	0	0
GG-914	2	2	0
GG-918	6	0	2
GG-923	4	0.5	2

[a] These are coded numbers of Green Giant Lines.

Disease reaction reading numerals refer to distance (inches) the bacteria moved down the inoculated leaf; inoculations made by cutting the tips of leaves and simultaneously spraying with the bacterial suspension (O.D. = 0.1, 420 mu setting).

isolates indicated that strains exist as evidenced by reaction of seven inbreds to three cultures (Table 11). The very susceptible inbreds, A632 and N7A, were poor differentials. The reaction of the four other inbreds indicated that pathogenic strains do exist. In another example, a certain hybrid varies in its reaction to three different isolates of the "Freckles" bacterium: 293, 296, and 298 (Fig. 14). Culture 293 was the least virulent of these three isolates.

Table 10. Reaction of dent corn inbreds (F1-1B to F17-1B) and hybrids (F18-54) inoculated with 2 LFW isolates (Bennett and Fagot) on 1/19/71.

Entry No.[a]	Average disease rating[b]		
	Bennett	Fagot	Avg.
F1-1B	3	2	2.5
F2-1B	4	3	3.5
F3-1B	2	0	1.0
F4-1B	2	1	1.5
F5-1B	2	0	0.0
F6-1B	4	1	2.5
F7-1B	2	3	2.5
F8-1B	4	1	2.5
F9-1B	3	2	2.5
F10-1B	4	0	2.0
F11-1B	3	0	1.5
F12-1B	4	1	2.5
F13-1B	3	2	2.5
F14-1B	3	4	3.5
F15-1B	3	1	2.0
F16-1B	3	2	2.5
F17-1B	2	1	1.5
F18	2	1	1.5
F19	2	1	1.5
F20	3	0	1.5
F21	4	3	3.5
F22	3	0	1.5
F23	4	2	3.0
F24	1	2	1.5
F25	4	0	2.0
F26	3	2	2.5
F27	4	1	2.5
F28	3	1	2.0
F29	4	2	3.0
F30	3	1	2.0
F31	3	3	3.0
F32	4	3	3.5
F33	3	2	2.5
F34	4	1	2.5
F35	3	2	2.5
F36	4	4	4.0
F37	4	2	3.0
F38	4	3	3.5
F39	4	1	2.5
F40	4	3	3.5

Table 10. (continued).

Entry No.[a]	Average disease rating[b]		
	Bennett	Fagot	Avg.
F41	3	1	2.0
F42	3	1	2.0
F43	1	1	1.0
F44	3	1	2.0
F45	4	1	2.5
F47	4	1	2.5
F48	3	0	1.5
F49	4	1	2.5
F50	3	0	1.5
F51	4	3	3.5
F52	4	1	2.5
F53	4	1	2.5
F54	4	1	2.5

a These are Funk's coded numbers.

b The disease readings were graded from 0 = no symptoms to 4.0 = symptom expression on leaves observed at a distance greater than 3 inches from point of inoculation using clip-spray method.

During greenhouse pathogenicity investigations, certain isolates of *C. nebraskense* subcultured on nutrient dextrose agar frequently assumed a substantial reduction or complete loss of virulence. Comparisons of isolates are meaningless if they were isolated, for example, in different years and maintained on nutrient media. The comparisons above were between cultures isolated about the same month or so. Therefore, to obtain fair representation of the pathogen present in Nebraska, our 1972, 1973 and 1974 cooperative field plots were located in three geographical locations in Nebraska to ensure exposure of the inbreds and hybrids to a variable pathogen population (3, 4, 5). To ensure presence in our testing program of a variable pathogen population in laboratory, greenhouse and field inocula-

Table 11. Variation in virulence of three isolates of *Corynebacterium nebraskense* using seven corn inbreds as differentials.

Inbred	Isolates[a]		
	10	50	53
A632	4[b]	4	3
A635	4	4	2
B14	2	1	3
B14A	3	4	1
H95	3	4	1
Oh43	2	3	1
N7A	4	4	3

a Concentration of inoculum consisted of 2×10^9 bacterial cells/ml of water.

b Disease ratings based on distance bacteria moved down the clip-sprayed leaf: 0 = no movement or visible symptoms; 1 = water-soaking of the leaves or necrosis of tissues up to one-half inch from inoculation point; 2 = above symptoms up to 1 inch from inoculated leaf tip; 3 = symptoms up to 3 inches from inoculated leaf tip; 4 = symptom expression exceeding 3 inches from inoculated leaf tip.

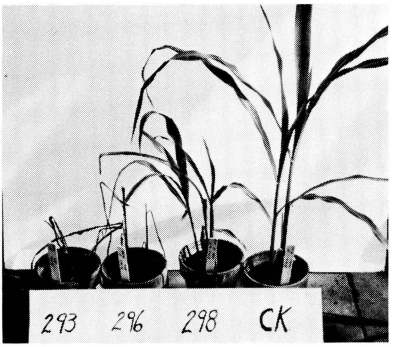

Fig. 14. Reaction of corn hybrid to three strains (293, 296, 298) of *C. nebraskense*.

Fig. 15. Differences in reaction of four corn inbreds to *C. nebraskense*.

tions, a mixture of strains of the bacterium was employed to be representative of pathogen variability (Fig. 15).

MAINTENANCE OF VIRULENCE

Because subculturing on nutrient agar frequently resulted in attenuation, it became clear that an effective storage medium or method was needed for maintaining the coryneform bacterium in pathogenic form (17, 18, 22, 23).

Several types of storage media were used in searching for an effective storage medium: mineral oil, distilled water, infested leaves, and infested seeds. The first three media were seeded or inoculated with each of five isolates. Infested seeds were collected from infested commercial field corn. Assessment of the percentage of seed infested was made before conducting longevity studies. Isolations from each storage medium were made periodically by plating on NDAT [nutrient dextrose agar + thiamine (100 mg/l)] and then inoculating the bases of 10-day-old corn seedlings (A619 × A632 hybrid) with the recovered organisms. The method of inoculation is described elsewhere (18).

Results are given in Table 12. *C. nebraskense* virulence was maintained up to 23 months, the termination of the tests, and, therefore, any storage medium used is more satisfactory than repeated subculturing on nutrient media. Distilled water might prove less satisfactory because of possible mutations after 23 months storage; this same problem may have occurred after 13 months but could have been inadvertently overlooked. In fact, to date rugose (R) colonial variants (293 R and 303 R) were only noted in isolations from distilled water (Fig. 16). Rugosity in this instance was not necessarily associated with loss of virulence. This might be due to the tendency of R colonies to change frequently to smooth (S) types.

Table 12. Survival of *Corynebacterium nebraskense* in different media after 7, 13, and 23 months storage.

Storage time (months)	Storage media			
	Mineral oil[a]	Distilled water[a]	Corn leaves[b]	Corn seeds[b]
7	5/5[c]	5/5	3/3	0/0
13	4/4	5/5	4/4	2/5[d]
23	2/5[d]	5/5	4/4	7/7

[a] Stored at 10°C.

[b] Stored at 25°C.

[c] Numerator = number of isolates virulent; denominator = total number isolates tested. With respect to seeds reference is made to seed numbers.

[d] Plates contaminated with fungi.

Preservation by lyophilization was found satisfactory for *Corynebacterium nebraskense* nomenclatural strain 296 (ATCC 27794, ICPB CN101, NCPPB 2581) and a representative strain 363 (ATCC 27795, ICPB 102) in subsequent tests. Results confirm previous findings that the pathogen can survive in various corn plant tissues in the field (17, 18). The problem of virulence loss can be overcome by maintenance in an appropriate storage medium and temperature conditions.

RESTORATION OF VIRULENCE

Different approaches were used to alter the degree of virulence and recovery of virulence of *C. nebraskense*. One method involved successive passages through a susceptible corn hybrid. Five successive passages of five attenuated isolates, Nos. 6, 10, 42, 50 and 65, through corn hybrid, A619 × A632, did not result in recovery of virulence. Perhaps the proportion of virulent to avirulent bacterial cells was too low to express virulence in the isolates upon successive passages through the susceptible host since a concentration of 10^7 cells/ml or more was required for infection. Reisolates from hosts after these passages did not appear too different insofar as colony characteristics were concerned. In fact, the bacterial isolates, which lost virulence upon subculturing on NDAT, did not differ in colony characteristics from the original virulence cultures: convex, mucoid, glistening colonies, orange in color. One culture, No. 293, was somewhat less mucoid but its colony was convex. Another corn pathogen, *Erwinia stewartii*, was altered in virulence upon successive passages through either susceptible or resistant hosts (12, 14).

Restoration of virulence in *C. nebraskense* was attempted also by growing the isolates in media containing different forms of nitrogen, ammonium nitrate, ammonium sulfate, or sodium nitrate. The four isolates of *C. nebraskense* used in this test were not affected in these experiments.

A correlation was found between *E. stewartii* virulence and its abil-

ity to utilize inorganic nitrogen (14); susceptibility increased with an increase of nitrogen, especially ammonium nitrogen, in contrast to nitrate forms. Most of the virulent strains of *E. stewartii* reduce nitrates to nitrites (14) while we found this did not occur in the tested *C. nebraskense* strains. Some of the Stewart's wilt symptoms have been attributed in part to nitrite toxicity (13).

The effect of amino acids on virulence was investigated. Amino acids were injected with a hypodermic syringe into bases 1-inch above soil line of 10-day-old corn seedlings simultaneously with each of three strains of *C. nebraskense* at concentrations of 2×10^9 cells/ml. One ml of the suspension was injected into each seedling base. Amino acids and concentrations in mg/l employed included L-alanine (300); L-asparagine (300); L-glutamine (367); L-cystine (150). Guanylic acid (150); and inositol (275) were also employed. None of these compounds visibly altered the virulence of one attenuated or two virulent isolates of *C. nebraskense*. Chesnin (UN-L soils scientist) was unable to alter symptom development of *C. nebraskense*-inoculated corn plants by application of iron chelates to the soil medium. Relation of nutrition of LFW is currently under study.

CULTURAL CHARACTERISTICS OF NEW BACTERIUM

Comparisons in cultural characteristics were made between avirulent and virulent strains of *C. nebraskense* in anticipation of determining the nature of pathogenicity and whether differences existed between attenuated and pathogenic strains (Table 13). All cultures were incubated at 25°C unless stated otherwise: virulent and avirulent were similar unless reported differently. Physiological characteristics were examined according to accepted standard methods (1, 25).

Potato dextrose agar plates (pH 7.0). In seven days growth became abundant, slimy, glistening, spreading colonies, white to cream in color; the colony edges were entire. Virulent cultures retained their virulence on PDA.

Potato dextrose agar + thiamine, 100 mg/l, (pH 7.0). The colonies were mucoid, convex, and endopigment color was orange.

Gelatin plate. A single culture streak was applied on surface of hardened medium and, following incubation, the plate was flooded with a gelatin precipitan = saturated ammonium sulfate solution. A white precipitate indicates presence of non-hydrolyzed gelatin; absence of the precipitate in the region of growth indicates gelatin hydrolysis (clear area). Plates were incubated for 5-7 days before adding ammonium sulfate solution. The width of the cleared area was measured the next day. Eight virulent and five avirulent cultures were positive.

Gelatin tube (pH 7.0). Bacteriological grade gelatin, 12%, was added to distilled water, sterilized, pipetted into deep tubes, and kept at 21°C. Variable results were obtained between virulent and aviru-

Table 13. Comparison of cultural characteristics of seven virulent (293, 296, 356, 363, 408, 411, 752) and five avirulent (6, 10, 42, 50, 65) cultures of *Corynebacterium nebraskense*.

Media	Culture No.											
	293	296	356	363	408	411	752	6	10	42	50	65
Gelatin (plate)	+[a]	+	+	+	+	+	−	+	+	+	+	+
Gelatin (tube)	−	+	+	−	−	−	−	+	+	−	+	−
H_2S	+	+	+	+	+		+	+	+	−	+	+
Indole	−	−	−	−	−		−	−	−	−	−	−
Litmus	+	+	+	+	+		+	+	+	+		
Loeffler blood serum		−					+	+			+	
Methyl red	−	−	−	−	−		−	−	−	−	−	−
Voges Proskauer	−	−	−	−	−		−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−		−	−	−	−	−	−
Temp. 37°C	−	−	−		−				−		−	−
Catalase	+	+						+				
Starch	+	+			+			+			+	+
Glycerol	+	+	+	+	+		+	+	+	+		
	A−[b]	A−	A−	A−	A−		A−	A−	A−	A−		
Lactose	+	+	+	+	+		+	+	+	+		
	A−	A−	A−	A−	A−		A−	A−	A−	A−		
Maltose	+	+	+	++	+		+	+	+	+		
	A−	A−	A−	A−	A−		A−	A−	A−	A−		
Mannitol	−	−	+	−	−		−	−	−			
			A+									
Salicin	++	+	+	+	+		+	+	+	+		
	A−	A−	A−	A−	A−		A−	A−	A−	A−		
Sorbitol	+++	+	+	+	+		+	+	+	+		
	A−	A−	A−	A−	A−		A−	A−	A−	A−		
Succinic	+++	+	+	+	+		+	+	+	+		
acid	Alk+[c]	nc[d]	nc	nc	A−		nc	nc	nc	nc		
Sucrose	+++	+	+	+	+		+	+	+	+		
	A−	A−	A−	A−	A−		A−	A−	A−	A−		

Table 13. Comparison of cultural characteristics of seven virulent (293, 296, 356, 363, 408, 411, 752) and five avirulent (6, 10, 42, 50, 65) cultures of *Corynebacterium nebraskense*.

	Culture No.											
Media	293	296	356	363	408	411	752	6	10	42	50	65
Xylose	+++	++	++	+	+		++	+	++	+	++	+
	A+	A−	A−	A−	A−		A−	A−	A−	A−	A−	A−
Arabinose	+++	+++			+++		+++	+++			+++	+++
Dextrin	++	+			+		+	+			+	+
Galactose	+++	+++			+++		+++	+++			+++	+++
Aesculin	+	+	+	+	+		+	+			+	+
Dulcitol	+++	+++	+++	+++	+++		+++	+			+	+
Inulin	++	++	++	++	+		++	++			++	++
NaCl–8%		−		−	−				−		−	−
NaCl–5%		−	+	+	+				+		+	+
Raffinose	+	++	+	+	++		+	++		+++	+++	
PDA plates[e]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
PDAT plates[f]	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Origin	LN	LN	LN	KN	LN	KN	LN	SN	KN	KN	KN	LN

[a] + = slight growth; ++ = moderate growth; +++ = excellent growth; − = no growth.

[b] A− = acid negative. A + = acid positive.

[c] Alk+ = alkaline

[d] nc = no change in pH.

[e] PDA = Potato dextrose agar, Difco, pH. 7.0.

[f] PDAT = Potato dextrose agar, Difco, + thiamine 100 mg/1, pH 7.0.

[g] L, K, S, refer to infected leaf, kernel, and stalk of dent corn, respectively; N refers to Nebraska.

lent cultures, ranging from negative to moderate amount of liquefaction.

Hydrogen sulfide. Slants of Difco-Lead Acetate agar were streaked on the surface and stabbed into the butt. Hydrogen sulfide production was indicated by surface browning or browning along the line of puncture. There was positive hydrogen sulfide production for 11 isolates tested.

Indole. Difco-Bacto-tryptone (1%), NaCl (0.5%), agar (1.5%), and water was used to prepare slants. Strips of filter paper previously dipped in a saturated solution of oxalic acid were inserted in top of tubes. The strips turn pink in presence of indole. The test was negative for 11 cultures.

Litmus milk. Difco litmus milk was used and reaction was observed on one to two day intervals for first week and weekly thereafter up to six weeks. Partial peptonization was noted for nine cultures.

Loeffler blood serum. Difco Loeffler blood serum indicated no digestion for four cultures tested.

Methyl red-Voges Proskauer. Difco MR-VP medium was used and cultures were agitated on a rotator for four days or not agitated up to 20 days. Eleven cultures were negative for methyl red and Voges-Proskauer.

Nitrate reduction. No nitrate reduction to nitrites was observed for 11 cultures tested. Cultures were grown in nitrate broth (peptone 10g, nitrite-free KNO_3, lg, distilled water, 1000ml) and tested with sulfanilic acid and naphtamine in acetic acid at the end of 8 and 13 days.

Sodium chloride-8%. Cultures were incubated in nutrient broth yeast extract for five days on a shaker and growth was assessed by spectrophotometer. Agar plates were read after 21 days. Negative growth was noted for six cultures; growth was variable in 3% and 5% NaCl.

Starch. Hydrolysis of starch was positive for seven virulent and three avirulent strains.

Catalase. Air bubbles from colonies of two avirulent and one virulent isolate resulted from application of 12% H_2O_2, indicating positive catalase activity.

Temperature–37°C. No growth of seven cultures was noted on plates of nutrient broth yeast extract after 17 days.

Basic nutrient solution for action on carbon compounds (glycerol, lactose, maltose, mannitol, salicin, sorbitol, succinic acid, sucrose, xylose, arabinose, dextrin, and galactose) consists of the following materials in grams/1 of distilled water: nicotinic acid, 0.010; NH_4Cl, 1.0; KH_2PO_4, 4.0; K_2HPO_4, 4.0; $MgSO_4.7H_2O$, 0.2; $FeSO_4$, 0.028; $ZnSO_4$, 0.003; phenol red, 0.018; and thiamine, 0.0001. The thiamine hydrochloride is filter sterilized; nicotinic acid is dissolved first and

then the other chemicals are added. The solution is then adjusted to pH 7.0.

Glycerol. Nine bacterial cultures showed very slight growth. All cultures were negative for acid.

Lactose. Nine cultures showed very slight growth. All cultures were negative for acid.

Maltose. Very slight growth was found for eight cultures. Culture No. 293 showed a moderate growth. Nine cultures were negative for acid.

Mannitol. No growth was noted for 11 cultures.

Salicin. Ten cultures showed slight growth. No acid production was noted for 10 cultures; culture 293 showed a moderate amount of growth.

Sorbitol. Slight growth and a negative for acid was observed for 10 cultures; excellent growth and a positive for acid was observed for No. 293 (virulent).

Succinic acid. Very slight growth and no pH change was noted for eight cultures. No. 293 showed excellent growth and alkaline reaction.

Sucrose. Very slight growth, negative for acid for eight cultures; excellent growth and positive for acid for No. 293.

Xylose. Slight to moderate growth and negative for acid noted for 10 cultures; No. 293 showed excellent growth and positive for acid production.

Raffinose. Positive growth for nine cultures after 14 days.

Arabinose. Excellent growth was observed for nine cultures.

Dextrin. Slight growth was observed for nine cultures.

Galactose. Excellent growth was observed for nine cultures.

Aesculin. Slight growth for six virulent and three avirulent cultures was noted.

Dulcitol. Excellent growth obtained for six virulent and three avirulent cultures.

Inulin. Moderate growth obtained for six virulent and three avirulent cultures.

NaCl-5%. No growth for one virulent culture and slight growth for three virulent and three avirulent cultures was noted.

SEROLOGY AND DNA ANALYSES OF NEW BACTERIUM

Lazar (10) studied the serological relationship of coryneform bacteria and categorized them into five groups. He included three isolates, 10, 293, and 296, of *C. nebraskense* in standard slide cross-agglutination tests with *Corynebacterium michiganense* (Nos. 1, T_1, T_2, 201, 382, 704), *C. insidiosum* No. 83, *C. poinsettiae* Nos. 845, and 9682, *C. flaccumfaciens* Nos. 706 and 712, *C. flaccumfaciens* var. *aurantiacum* No. 558, *C. betae* No. 373, *C. tritici* No. 255, *C. rathayi* No. 797, *C.*

Table 14. Cross-agglutination reaction of three isolates of *Corynebacterium nebraskense* and *C. insidiosum* No. 83 and *C. michiganense* Nos. 1, T_1, T_2, 201, 382, and 704.

Antibody of species		Antigen of species *C. nebraskense*		
		No. 10	No. 293	No. 296
C. michiganense	1	++++	++++	++++
" "	T_1	±	++	±
" "	T_2	+++	++++	++++
" "	201	++++	++++	++++
" "	382	++	++++	++++
" "	704	++++	++++	++++
C. insidiosum	83	+++	++++	++++

± = fine agglutination and evident after at least one minute.

sepedonicum Nos. 299 and 708, and *C. fascians* Nos. 156 and 469. The results of cross-agglutination tests conducted by Lazar of the three *C. nebraskense* isolates and *C. insidiosum* and *C. michiganense* are entered in Table 14. Standard serological methods used were reported previously by Lazar (10).

The three *C. nebraskense* isolates appear to belong to Lazar's Group 2 which includes *C. michiganense* and *C. insidiosum* since cross-agglutination occurred between the three *C. nebraskense* strains and each of the *C. michiganense* (6 strains) and *C. insidiosum* (one strain). The avirulent *C. nebraskense* 10 was not different from the two virulent strains Nos. 293 or 296 in cross reaction with the *C. michiganense* and *C. insidiosum* strains. The cross-agglutination reaction between avirulent and virulent *C. nebraskense* strains and any of the strains of *C. poinsettiae, C. flaccumfaciens, C. flaccumfaciens* var. *aurantiacum, C. betae, C. tritici, C. rathayi, C. sepedonicum* and *C. fascians* was negative indicating that *C. nebraskense* is not serologically related to any of them.

There are detailed descriptions of most of the phytopathogenic coryneforms, but many are based on methods no longer in general usage and are misleading in today's viewpoint. In the most recent Bergey's Manual of Determinative Bacteriology (2), the editors include only characters in which different workers are in agreement. They attempt to group species, particularly on their serological relationships, the amino acid composition of their cell walls, and their minimal nutritional requirements. Thus *C. sepedonicum* is not grouped with *C. insidiosum* and *C. michiganense* because it seems to share few nucleoprotein and polysaccharide antigens with them. Thus, in Bergey's 1974 Manual (2) Group 4 includes *C. insidiosum* and *C. michiganense*; the AB serotypes of both species are identical in agglutinogen complement (15).

SUMMARIZATION OF BIOLOGICAL CHARACTERISTICS OF NEW BACTERIUM

At this point a recapitulation of some points on variability and characteristics of the pathogen is in order. Strains of *C. nebraskense*

differed in pathogenicity based on reaction of differential hybrids. Definitive colony characteristics of this coryneform bacterium were not associated with virulent or attenuated culture types as reported for the corn pathogen *Erwinia stewartii* (9, 12, 28). Both of these bacterial species became attenuated upon repeated *in vitro* cultivation. Successive passage of five avirulent *C. nebraskense* strains through susceptible hosts did not restore virulence.

Wellhausen (28) and Lincoln (12) found changes in virulence of *E. stewartii* during host passage as well as colony morphology. They found that virulence was directly related to the ratio of virulent to avirulent bacteria. Because clear-cut differences in colony characteristics of the two types in *C. nebraskense* were not available at the time of the test it would be cumbersome and time-consuming to prove this point. *C. nebraskense* avirulent types did not recover virulence when cultured with the addition of any of three nitrogen forms (ammonium nitrate, ammonium sulfate or sodium nitrate) to the inoculum, whereas susceptibility increased with type of nitrogen used, especially ammonium nitrogen, as opposed to nitrate forms insofar as *E. stewartii* is concerned (14). *E. stewartii* reduced nitrates to nitrites while *C. nebraskense* did not.

Pathologists have commonly sought to associate nutritional and physiological factors with virulence or degrees of virulence. Employment of cyclic AMP, amino acids or nitrogen forms did not alter virulence of *C. nebraskense*. Factors or nutrients that can change avirulent to virulent cultures were not found despite use of a wide spectrum. Presumably, serology should indicate differences in cell walls but no differences could be demonstrated.

Liquefaction of gelatin was positive on plates and variable in gelatin tubes. Production of hydrogen sulfide from sulfur-containing amino acids was positive. Indole was not produced as a product of tryptophan breakdown and hydrolysis of starch was positive. Litmus milk was partially peptonized and Loeffler blood serum showed no digestion. Eight cultures (four virulent, four avirulent) were nonmotile, nonfluorescent under ultra-violet light, produced no change in arginine or dehydrogenase oxidase, were negative for the Voges-Proskauer reaction, and were positive for catalase activity. Apparently catalase does not play a significant role in the virulence of *C. nebraskense* as had been reported for *Pseudomonas phaseolicola* (16) and for *Pseudomonas solanacearum* (6) in which avirulent mutants lack functional catalase.

Variability was noted in growth on sodium chloride. Four virulent isolates and four avirulent isolates grew in nutrient broth and 3% NaCl but growth could not be measured in 5% or 8%; on NDAT the cultures were a darker orange with 3% NaCl than without the salt. In seven days, four virulent cultures grew on NDAT + 5% NaCl. At 37°C no growth by seven cultures was observed on plates on NDAT

after 77 days. Cultures grew abundantly on potato dextrose agar (PDA) with slimy, glistening, and watery growth; color was white to cream; colony edges were entire. On PDA + thiamine (100 mg/1) the colonies were mucoid, convex and the endopigment was orange in color. On potato plugs no differences were observed with growth appearing slimy orange in color.

On a basic nutrient solution the effect on growth of the following carbon compounds was negative: glycerol, lactose, maltose, mannitol and xylose; slight to excellent growth was noted on salicin, sorbitol, benzoic acid, gluconic acid, propionic acid, succinic acid, arabinose, dextrin, galactose, raffinose inulin, dulcitol, aesculin, and sucrose. Variable growth differences between virulent (six) and avirulent (three) cultures were noted in dulcitol. Culture No. 293 exhibited excellent growth and alkaline reaction in succinic acid, positive for acid in sucrose and excellent growth and acid reaction in xylose and sorbitol and moderate growth in salicin. Culture No. 293 will be checked for purity and other characteristics.

The causal organism is a new coryneform bacterium based on its pathogenic capabilities and biological properties (1, 18, 22, 23). As reported under the section "Host Range," the new bacterium does not infect plant hosts of other phytopathogenic corynebacteria. These bacteria in turn were not pathogenic for corn (Golden Cross Bantam or Nebraska 501-D). The cross inoculation tests were repeated three times but without success. Inoculations were accomplished by injecting stems of plants and clip-spraying with suspensions of 2 X 10^9 bacterial cells/ml. Each experimental unit consisted of five plants replicated four times for each experiment. Strider (27) failed to infect corn with *Corynebacterium michiganense*. Pathologists have identified new species on the basis of their host range and symptoms they induce. That a new phytopathogenic bacterium was discovered was substantiated on the basis of its biological properties (Table 13). Morphological and physiological properties were examined according to standard bacteriological methods (25).

The orange endopigment bacterium is gram-positive and coryneform. Cells from 36 hour cultures ranged in size from 0.8–2.5 u by 0.3–0.5 u under electron microscopy. The generation time was about 3 hours at 26°C. The bacteria are non-motile, pleomorphic rods without flagella. After about seven days visible colonies on nutrient dextrose agar + thiamine (NDAT) were commonly round, convex, mucoid, viscid, glistening with entire margins and attaining a diameter of 3–6 mm. Centers of the colonies were usually a darker, and margins a lighter, orange. The colonies at 25°C were usually capucine or apricot orange but were darker orange at 10°C or on NDAT + NaCl 3%. On potato dextrose agar (Difco) the pigmentation of colonies was white to cream and the consistency was more watery, resulting in coalescence of colonies.

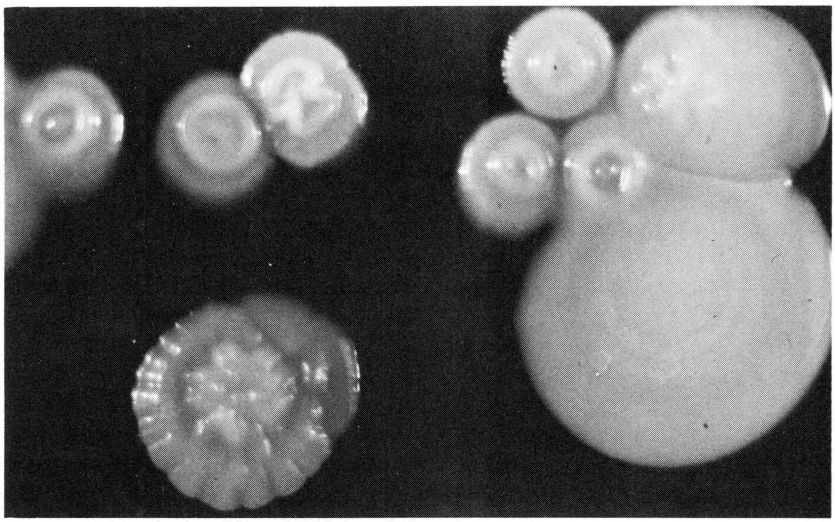

Fig. 16. Rough and smooth colonial variants of *Corynebacterium nebraskense*.

Differences between 12 isolates (seven virulent and five avirulent) on these two media (NDAT or PDA) were quite similar except culture No. 293 which was less mucoid and convex on NDAT. Thiamine appears to be one factor necessary for growth and orange endopigment production. The amount of thiamine required is considerably higher (50–100 mg/l) than for orange bean wilt bacterium, *Corynebacterium flaccumfaciens* var. *aurantiacum* Schuster and Christiansen, 1957 (21). In the corn plant *C. nebraskense* usually is orange, but the exudate on the surface of infected leaves appears colorless and on occasion yellow-orange. Technically, the LFW bacterium is orange in color but can vary depending on the nutrient medium employed. Rugose mutants (293R, 296R, 300R, 313R) which were obtained from 23 months storage in distilled water differed in that the colonies were rough (Fig. 16) with uneven margins; the orange pigmentation on NDAT was similar to the original parent cultures (293, 296, 300, 313). Maintaining rugose cultures was difficult because of their tendency to change to the original mucoid types; this mutability might explain the retention of virulence by the rugose cultures.

Four isolates (296, 311b, 313, 408) grew on nutrient broth + NaCl (3%), but growth could not be measured in 5% NaCl. On NDAT the four cultures assumed a darker orange with 3% NaCl than without the salt. In seven days, cultures 311b, 313, and 408 grew on NDAT + 5% NaCl; No. 296 did not grow on this medium. *C. insidiosum*, alfalfa wilt bacterium, grew on 5% NaCl while *C. michiganense* did not grow in 3% NaCl (1).

In a base deoxyribonucleic acid (DNA) analyses Dr. M. Mandel, University of Texas–Houston, reported in September, 1970 that each of six isolates (293, 296, 363, 5, 10, 50) of *C. nebraskense*

contained a guanine + cytosine (GC) percentage of 73.5. The moles percent guanine and cytosine was determined by a method previously described (26) except that bacterial cells were treated with lysozyme (100 mg/ml) in the presence of saline: EDTA for three hours at 37°C prior to lysis with sodium dodecyl sulfate. *E. stewartii* which incites a corn disease similar to LFW in gross morphological symptoms, has a GC% of 55 (26) indicating that the two bacterial species are not closely related. The GC% of *C. nebraskense* most closely approaches the GC% of 73 for *C. michiganense* and *C. insidiosum* (18). The consistent DNA differences may suffice to separate *C. nebraskense* from these two coryneforms. On the basis of serology, Lazar did relegate *C. nebraskense* into Group 2 which includes *C. michiganense* and *C. insidiosum.*

On the basis of DNA base composition, serology, absence of flagella, negative nitrate reduction, non-motility, temperature requirements, growth rates, opaque growth, cell shape, aesculin hydrolysis, and fermentation of glucose and salicin the LFW bacterium appears most closely related to *C. insidiosum* and *C. michiganense* (Table 15). But the LFW bacterium is distinct from these two corynebacteria on the basis of pathogenic capabilities (plant host range) as well as certain biological characteristics as illustrated by comparative characters (1, 10, Table 15). *C. nebraskense* is a generally larger coryneform than the other two species. *C. nebraskense* differs from one or the other two species on the basis of diastase production, use of NaCl, endopigment production, indogoidine production, and fermentation of galactose, maltose, xylose, lactose, mannitol, and raffinose.

C. nebraskense differs from either of the two other coryneforms on the basis of gelatin liquefaction, litmus milk peptonization, hydrogen sulfide production, starch digestion, fermentation of sucrose, and glycerol. The DNA base composition of *C. nebraskense* (73.5%) differs sufficiently from *C. insidiosum* (73%, 78.1%) and *C. michiganense* (73%; 67.3–70.7%) to be considered adequately different. On the basis of the character differences the author feels that the LFW bacterium is a new bacterium and warrants the new species binomial, *Corynebacterium nebraskense*, as first described in 1972 by Schuster, Hoff, Mandel, Lazar (18) and employed in several subsequent scientific publications.

Culture No. 296 was considered the holotype strain and several others as representative strains. Subsequent to the original description of the new species and new disease (18), requests in 1973 for cultures of the nomenclature strain No. 296 and other representative strains of *C. nebraskense* have been received from curators of international phytopathogenic bacterial repositories: American Type Culture Collection (ATCC), Baltimore, Md., International Collection of Phytopathogenic Bacteria (ICPB), Davis, California, and the National Collection of Plant Pathogenic Bacteria (NCPPB), England. The type strain No. 296 was listed by the respective bacterial curators as ATCC

Table 15. Differential characters of three closely related species of Corynebacterium.[a]

Character	*C. insidiosum*	*C. michiganense*	*C. nebraskense*
Cell size (approx) umm	0.4–0.5×0.7×1.0	0.6–0.7×0.7–1.2	0.3–0.5×0.9–2.5
Cell shape — pleomorphic	+	+	+
Motility	–	–[b]	–
Flagella	–	–	–
Growth slow	+	+	+
Growth opaque	+	+	+
Growth at 37°C	–	(+)	–
Optimum temperature	23°C	26°C	26°C
Urease	–	–	
Gelatin liquefaction	+	–	++
Litmus milk peptonization	–	–	+[c]
Hydrogen sulfide	–	–	+
Diastase on starch	±	±	+
Nitrite from nitrate	–	–	–
NaCl 5%	+	–	(+)
NaCl 3%	+	–	+
Esculin hydrolyzed	+	+	+
Medium yellow pigment	–	(+)	–
Orange pigment	–	–	+
Pink pigment	–	(–)	–
Indigoidine produced	+	–	–
Fermentation of:			
glucose	+	+	+
sucrose	+	+	–
galactose		+	+
maltose		+	–
glycerol	+	±	–
lactose		±	–
mannitol		±	–
salicin	–	–	–
raffinose	–	+	+
xylose	+[d]	–	–
Host plant	alfalfa	tomato	corn
(G+C) % – Mandel, 1972	73	73	73.5
(G+C) % – Bergey's, 1974	78.1	67.3–70.7	

[a] + All the few strains examined reported positive; ± weakly positive; – all of few strains examined reported negative; (+) most strains positive; (–) most strains negative; d strains differ.

[b] Some authors claim to have detected motility.

[c] Colonies on nutrient dextrose agar < 1 mm diameter after 3 days.

[d] Variable in degree at 7 days.

27794, ICPB-CN101, and NCPPB 2581. Several other representative strains of *C. nebraskense* were given corresponding number descriptions by the respective curators.

A portion of the key in Bergey's Manual (1) on plant pathogenic corynebacteria can be modified as follows to include the new bacterium:

II. From plant sources
  a. Non-motile

1. Nitrites not produced from nitrates
   a. Colonies cream—colored
   b. Slow liquefaction of gelatin
   c. Bluish granules in growth or ordinary media; attacks alfalfa.
      18. *C. insidiosum*
   bb. No liquefaction of gelatin
      20. *C. humiferum*
   aa. Colonies yellow—No liquefaction of gelatin; attacks tomato.
      21. *C. michiganense*
   aaa. Colonies orange—slow liquefaction of gelatin; LFW of corn
      22. *C. nebraskense*

ORIGIN OF NEW BACTERIUM

The origin of plant parasitic bacteria concerns applied microbiologists. When mechanisms by which microorganisms acquire new characteristics became more complete, investigations were started on the manner of adaptation of bacteria to a parasitic life on plants.

Such studies are confronted by polemics on inheritance and there is a question of the acquisition of inherited characteristics. The explanations were that this occurred as a result of "mutations" of some inherited substance in the bacterial cells or that the phenomena resulted from adaptation of bacteria to their natural environment (plants) by gradual acquisition of corresponding factors, first by modification of fermenting systems and then by inheritance as a response to the modified metabolic activity of the cells in the new biological environment. There is then the question of the later acquisition of phytopathogenic capabilities.

In nature there exists populations of saprophytic bacteria, which are similar to parasitic bacteria in their basic biological properties, except for the plant parasitic capacities. These bacterial populations develop directly on the plant parts or in their vicinity. It should be possible to coordinate the acquisition of phytopathogenic abilities of these bacteria with the individual bacterial populations.

It has been presumed that origin of coryneform parasites arose from populations of similar saprophytic bacteria. For example, the ancestor could be *Corynebacterium helvolum* or other corynebacteria which are widespread in nature and soil. From such ancestors bacterial pathogens of plants could arise: *C. michiganense* with a pigment similar to *C. helvolum*, or *C. sepedonicum* which loses its pigment when infecting the subterranean parts of the plant or even *C. insidiosum* for which one saprophytic var. (*C. insidiosum* var. *saprophyticum*) was described (1, 2).

Why did *C. nebraskense* make its initial appearance in southcentral Nebraska? This might be a matter of circumstances. Although the complete epidemiology of LFW is not known, it appears that *C. nebraskense* is of local origin.

If the assumption is that it arose from a soil-borne coryneform, it is possible that corn roots have been in a "bacterial soup" in the soil long enough for inoculum buildup to occur from common soil-borne corynebacteria or for another species of vascular parasite to change in a stepwise fashion in the direction of parasitism for corn.

Perhaps the LFW bacterium develops directly on the plant organs as an epiphyte of corn or as a parasite or epiphyte of common weeds, such as shattercane or foxtail. Another possible source is that the closely related alfalfa wilt bacterium, *C. insidiosum* (or its saprophytic variety) which has been present in southcentral Nebraska many years, could have mutated or had been transformed by action of pesticides or other farming practices to *C. nebraskense*. By selection pressures due to favorable environment, cultural practices (corn monoculture, minimum tillage) and genetic vulnerability of narrow corn germplasm, LFW inoculum buildup could have been so rapid as to reach sufficient proportions (epidemic) to be recognized as a problem. In this regard the question of survival of primary inoculum (initial source) of infection in the spring and/or early summer needs immediate consideration.

The primary source of LFW inoculum is infested corn stubble that overwinters on or near the soil surface (seed transmission is low but dissemination over short or long distances must be involved in its distribution pattern). In southcentral Nebraska minimum tillage and corn monoculture have been widely applied for the past decade before the sudden appearance of Leaf Freckles and Wilt. It is improbable that *C. nebraskense* appeared *de novo* and assumed epidemic proportions in a decade. A pathogen may be the product of long evolution. Because of the recent recognition of the LFW bacterium which is not confounded by many years of change due to modified farming practices, this reconstruction of its evolution appears plausible.

Numerous researches of parasitic bacteria were directed to seeking "toxic" materials or enzymes that favor maintenance on the plant or virulence of the bacteria. We have tried comparisons of virulent vs. avirulent strains with respect to their cultural characteristics but were not successful (Tables 13, 15). The only possible lead was the ability of virulent strains to use dulcitol in its metabolism. If better understanding is found of the difference between the virulent and attenuated strains, a better means of protecting cultivated plants from phytopathogenic bacteria might be forthcoming.

DISEASE CONTROL

Development of Leaf Freckles and Wilt of corn to some extent

parallels the employment of certain farming practices. Since the primary inoculum source of the new corn disease bacterium is infested corn residues and since it has a relatively narrow host range, use of cultural practices (deep plowing preferably in the fall, and/or crop rotation) should minimize the incidence of Leaf Freckles and Wilt.

Because many growers in the intensive corn growing areas are reluctant to change traditional farming practices (minimum tillage and monoculture) which have contributed to high corn yields, cooperative corn breeding and genetic studies have been initiated in anticipation of developing tolerant and adapted hybrids. This appeared feasible since greenhouse tests have demonstrated that certain inbreds are tolerant to the new organism (Fig. 15) and hybrids developed from tolerant lines also tend to be tolerant.

Infested seed corn can also provide primary inoculum and serve as a means of introducing the disease to new areas. Selection of seed corn from non-infested areas should decrease the chance of harvesting infected seed. Infested feed, corn, cobs and other plant parts could be instrumental in dissemination of the disease. Seed treatments with antibiotics, such as tetracycline formulations or fungicides, Captan or mercuric chloride, do not appear promising in controlling internal seed infections. This emphasizes the need for seed selection from non-infested fields.

Research already started involves breeding, genetic studies and the relationships of insects to the new corn disease. A cooperative breeding and genetic project was initiated in 1972 with Drs. W. A. Compton, C. O. Gardner, and A. Calub to determine the reaction of corn inbred and hybrids under conditions of natural infection in the field. Although greenhouse reactions were usually more severe, the field reactions correlated fairly well with the greenhouse data.

During the course of the cooperative breeding and genetic experiments, certain aspects of host-parasite relations were investigated. A more refined method of inoculation, the most suitable inoculum concentration, and age of plant most suitable for inbred and hybrid reaction differentiation were facets that required additional study. Calub, Schuster, Gardner, and Compton (3) inoculated corn inbreds and hybrids at four different age groups and four inoculum concentrations and found that plant age played a significant role in differentiating resistant from susceptible lines with the hypodermic syringe injection technique. Plant age did not affect reaction differences in susceptible corn materials as was evident in resistant genotypes. Infection decreased with increased plant maturity with the greatest change occuring at tasseling period. Inoculum concentration was critical principally in young plants. However, optimum concentration was difficult to determine because of variable results. In mature plants, high inoculum concentrations yielded more consistent differences. An improved inoculation procedure, which entailed use of a multineedle

inoculator, proved effective in evaluating reaction of corn to *C. nebraskense* in field and greenhouse tests (4).

Field assessments might be more meaningful since they occur under natural conditions; however, since field reactions may occur under most favorable conditions, it will be necessary to conduct these experiments over a period of years. The field tests will include experimental lines developed over a period of years by 13 North Central Experimental Stations. Some of these inbreds are used in currently grown commercial hybrids. The reactions of the inbreds to LFW will be released cooperatively to the corn industry and experimental stations for use in their programs.

In other studies, Drs. R. Staples and Z. B. Mayo, UN–L entomologists, are studying the possible relationships of certain insects to primary and secondary infections. This information may have a bearing on disease control suggestions.

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