Variation Among Strains of *Clavibacter michiganense* subsp. *nebraskense* Isolated from a Single Popcorn Field

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ABSTRACT

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The variation among 50 single-colony strains of *Clavibacter michiganense* subsp. *nebraskense* (=*Corynebacterium michiganense* subsp. *nebraskense*) was assessed on the bases of color and morphology of colonies on solid media, bacteriocin production, pathogenicity, and bacteriophage sensitivity. All strains were isolated in 1982 from popcorn residue and popcorn plants in one 46-ha field in Nebraska. Seven major groups were identified by the first three criteria. Groupings were independent of the location within the field and the type of plant material from which they were isolated. Strains in the seven groups were further subdivided by bacteriophage sensitivity, making a total of 20 subgroups that identify a minimum of that many individual strains.

Additional key words: Goss's bacterial wilt and blight of corn.

Clavibacter michiganense subsp. *nebraskense* (*C. m.* subsp. *nebraskense* [4]) (=*Corynebacterium michiganense* subsp. *nebraskense*) was first described as the causal agent of Goss's bacterial wilt and blight in 1969 (20). Since then, many strains of the pathogen have been collected from throughout Nebraska and several other states. The variation within these strains was previously found to be remarkably slight (18). In 1982, we made additions to the collection from infected fresh plant material and residue from one popcorn (*Zea mays* L.) field (15). We examined the variation within this new group of single-colony strains by the same criteria Vidaver et al (18) used and on the basis of colony color and morphology. A preliminary portion of this study was reported elsewhere (14).

MATERIALS AND METHODS

Field site and plant samples. Collections were made from one field (about 46 ha) in Chase County, Nebraska. The field was planted to popcorn in 1981 and 1982. Isolations were made from two types of material: 1) residue from the 1981 crop collected during the winter and throughout the 1982 growing season until harvest and 2) fresh plant material collected during the 1982 growing season either as whole plants early in the season or as detached leaves later in the season. Both types of material were collected from near the centers of the four quadrants of the field or from a series of plots arranged in a line across one-half of the field.

Isolation from field samples. Isolations were made as described previously (15). Briefly, residue was pulverized, suspended in broth, and shaken for 1–2 hr before the slurry was diluted serially and aliquots were plated on CNS agar (7), which is semiselective for *C. m.* subsp. *nebraskense*. Fresh plant material from four plants was combined and washed in sterile water and then homogenized in sterile 12.5 mM potassium phosphate buffer, pH 7.1. Both the wash water and the homogenates were diluted serially and aliquots plated on CNS agar.

After about 7 days, strains were transferred from the CNS plates to NBY agar (16) with sterile toothpicks. All strains were purified by streaking and selecting single colonies three times. In some instances, two colony types were apparent on the first or second streak plates. These were separated and labeled "A" and "B." Strains were stored on NBY slants at 4 C. The strains and their origins are shown in Table 1.

Pathogenicity test. A qualitative pathogenicity test was performed with each of the purified strains. The small end of a sterile flat toothpick was dipped into bacterial colonies on NBY plates. The stem of a four- to five-leaf-stage greenhouse-grown plant of the susceptible sweet corn cultivar Golden Cross Bantam was then stabbed twice with the end of the toothpick containing the bacteria. The site of inoculation was about 2 cm from the soil surface, which placed the bacteria into the whorl approximately at the growing point of the plant. Goss's bacterial wilt and blight symptoms appeared first on the leaves that were punctured by the toothpick, then spread to the entire plant and usually resulted in death of the plant.

Bacteriophage and bacteriocin typing. Bacteriophage sensitivity was evaluated by previously described methods (18). In addition to the five phages used by Vidaver et al, a sixth one, ϕXC (isolated by Y. Shirako in our laboratory), was used.

Bacteriocin production and sensitivity were evaluated as previously described (6). *C. m.* subsp. *michiganense* (4) 13-3 was used as an indicator because it differentiated strains that either made no bacteriocin, made bacteriocin CN1, or made bacteriocins CN1 and CN2. CN1 is produced on both NBY and modified Burkholder's (6) agar and causes a small zone of inhibition (5–7 mm in diameter) in a lawn of *C. m.* subsp. *michiganense* 13-3. CN2 is produced on modified Burkholder's but not NBY agar and produces a large zone of inhibition (16–20 mm in diameter) with this indicator. Production of both bacteriocins on modified Burkholder's agar is revealed by a double zone of inhibition where mutants resistant to CN2 in the outer zone occur with a higher frequency than mutants resistant to both CN1 and CN2 in the inner zone (18).

All the new strains were tested both as indicators and producers. Plates of modified Burkholder's medium (6) were spotted with 5-μl drops of cell suspensions of five or six isolates. The cell suspensions were made to about 10^8 colony-forming units (cfu) per milliliter in 12.5 mM potassium phosphate buffer, pH 7.1, with a loopful of cells from NBY agar medium. Cells were in stationary phase. After 3–4 days of incubation at 25 C, producer colonies were killed with chloroform vapor and overlaid with 2.6 ml of soft water agar (0.75%) seeded with 2×10^7 cfu of the indicator strains.

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Culture morphology and pigmentation. Strains were grown on NBY or CNS plates for 4–5 days at 25 C. The relative color of each isolate and fluidity of the growth was observed.

Polyacrylamide gel electrophoresis (PAGE) of cellular proteins. PAGE of soluble cellular proteins was done by the methods of Carlson and Vidaver (2). Gels were stained with silver (11).

RESULTS

Differences in culture morphology. On the basis of colony color and morphology, four types of strains were present. The first type was represented by the greatest number of isolates, which had orange fluidal colonies typical of those previously described (19). Strains of the second type were similar in pigment color but were not fluidal and thus appeared to be a darker orange. The nonfluidal characteristic was more noticeable in areas of heavy growth on a plate than in single colonies (Fig. 1). Strains of the third type were fluidal and yellow-pigmented. The fourth type was one strain that was fluidal but contained less orange pigment so that young

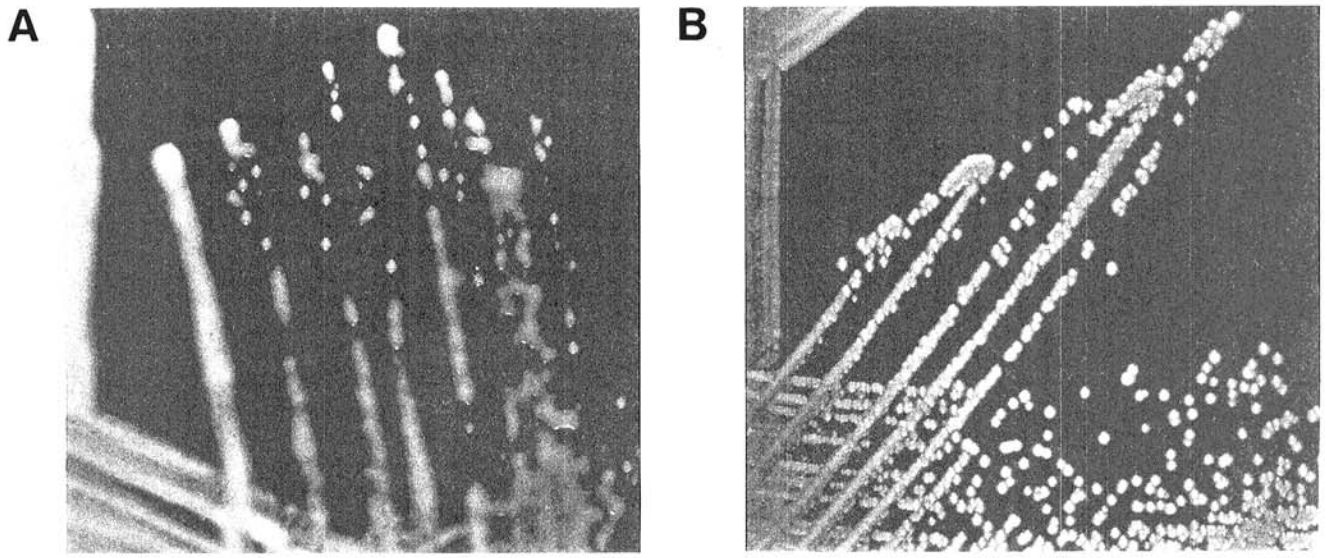

Fig. 1. Variability in culture morphology of *Clavibacter michiganense* subsp. *nebraskense*. **A,** Morphology of CN72-8A, which is fluidal and **B,** nonfluidal morphology of CN72-26B. Both plates are NBY agar and were incubed for 5 days at 25 C.

TABLE 1. Origin and pathogenicity of *Clavibacter michiganense* subsp. *nebraskense* strains collected from a single field in Chase County, Nebraska, in 1982

Isolate	Origin	Date	Pathogenicity
CN72-2	Residue from SE quadrant	14 February	+[a]
CN72-3A & B[b]	Residue from SW quadrant	14 February	+
CN72-4A & B	Residue from NE quadrant	14 February	+
CN72-5A & B	Residue from NW quadrant	14 February	+
CN72-6A & B	Residue from center of field	14 February	+
CN72-7	Residue from center of field	14 February	+
CN72-8A & B	Residue from center of field	14 February	+
CN72-10	Residue from SW quadrant	14 February	+
CN72-11A & B	Plants from plot 103	1 July	+
CN72-12A & B	Residue from SW quadrant	14 February	+
CN72-17	Plants from the plot 401	1 July	+
CN72-18A,B,BB	Plants from plot 301	1 July	+
CN72-19	Plants from plot 401	28 July	+
CN72-20	Plants from plot 205	28 July	+
CN72-21A & B	Plants from plot 205	28 July	+
CN72-22A & B	Plants from plot 205	28 July	+
CN72-23A & B	Plants from plot 205	23 August	+
CN72-24A & B	Plants from plot 205	23 August	+
CN72-25A & B	Plants from plot 303	23 August	+
CN72-26A & B	Plants from plot 303	23 August	+
CN72-28	Plants from plot 404	28 September	−
CN72-29	Plants from plot 204	28 September	−
CN72-30	Plants from plot 204	28 September	−/+[c]
CN72-31	Plants from plot 305	28 September	+
CN72-32	Plants from 303	28 September	+
CN72-33	Plants from plot 302	28 September	−/+[c]
CN72-34	Plants from plot 401	28 September	+
CN72-36	Plants from unlabeled plot	28 September	+
CN72-37	Residue from plot 402	28 September	−
CN72-38	Residue from plot 402	28 September	−
CN72-39	Residue from plot 103	28 September	+
CN72-40	Residue from plot 203	28 September	+
CN72-41	Residue from plot 203	28 September	+
CN72-42	Residue from unlabeled plot	1 December	+
CN72-43	Residue from unlabeled plot	1 December	+

[a] + = Typical Goss's bacterial wilt and blight symptoms developed; − = no disease symptoms developed.
[b] Designation of A, B, BB indicates different derivatives from an original single isolated colony.
[c] Pathogenicity lost after about 4 mo in storage.

cultures appeared white. This strain also grew more slowly than the others. The colors of the last two types grown on NBY agar were compared with color charts and corresponded to narcissus (yellow) and maise [sic] (pale orange) (10). Colony colors were slightly darker on CNS than NBY agar, but fluidity was the same. Among the strains of the first type were a few (group 6, Fig. 2) that were coryneform and could not be distinguished from any of the others on a morphological basis but did not cause Goss's bacterial wilt and blight when inoculated into corn seedlings. These, nevertheless, were included in subsequent tests.

Differences in bacteriocin production and bacteriophage sensitivity. Additional separations among strains were made by analysis of their bacteriocin production. With *C. m.* subsp. *michiganense* strain 13-3 as the indicator (6), the strains could be separated according to production of CN1, both CN1 and CN2, or neither. No strain produced only CN2.

Using the criteria of colony morphology and pigmentation, pathogenicity, and bacteriocin production, we separated the 50 strains into seven groups as illustrated in Figure 2.

When the strains were used as indicators as well as producers in assays, sensivity to CN1 was not detected, but unlike the results of Gross and Vidaver (6), some appeared to be sensitive to CN2. This sensitivity was variable from experiment to experiment and was not reliable for making additional separations among the strains. Two strains, CN72-30 and CN72-33, showed entirely different patterns of sensitivity from any others but were similar to each other. They may indicate production of a third bacteriocin by some of the other strains, but because both of these strains lost pathogenicity before these experiments were done, their identity and the importance of their behavior is questionable. Bacteriocin production and sensitivity patterns of our strains are shown in Figure 3.

Some of the patterns of phage sensitivity exhibited by our strains (Table 2) were the same as those described by Vidaver et al (18) except for phage ϕXC. Several additional patterns of sensitivity were observed, i.e., groups G–M. Phage sensitivity enabled further subdivision of the seven groups that were outlined in Figure 2 (Table 3). None of the groupings were correlated with the location or type of plant material from which the bacteria were isolated or the time they were isolated.

PAGE of cellular proteins. PAGE of cellular proteins did not reveal additional differences among strains that were not already apparent by other criteria.

DISCUSSION

The strains of *C. m.* subsp. *nebraskense* described here were chosen for study because many of them appeared slightly different from typical colonies and their identity was questioned. Consequently, the relative numbers of strains in our groups do not

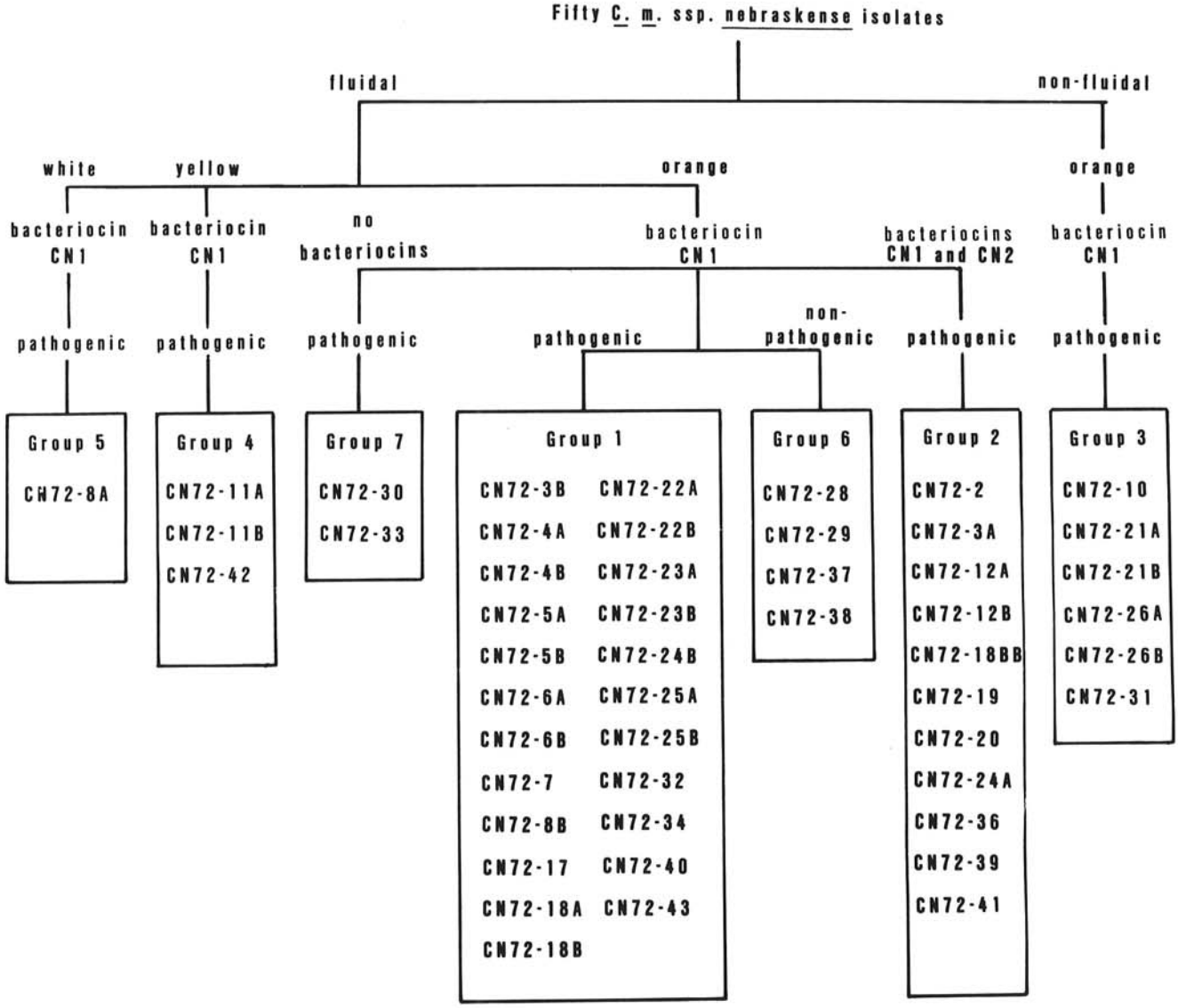

Fig. 2. Separation of *Clavibacter michiganense* subsp. *nebraskense* strains into seven groups. Separations were on the bases of colony color, fluidity, pathogenicity, and bacteriocin production, with *Clavibacter michiganense* subsp. *michiganense* 13-3 as the indicator strain.

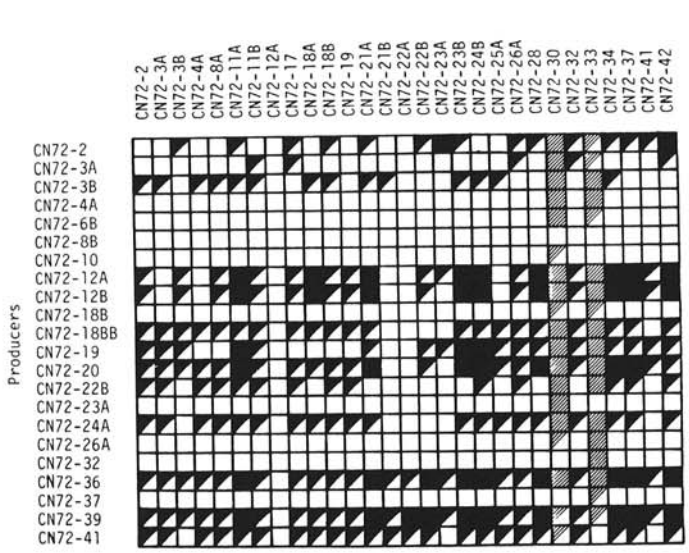

Fig. 3. Bacteriocin production and sensitivity of *Clavibacter michiganense* subsp. *nebraskense* strains. Black squares indicate a large clear zone of inhibition ranging from 10 to 15 mm around the producer strain. Shaded squares indicate a smaller inhibition zone (4–7 mm) that was sometimes turbid. Half-filled squares indicate that the results were variable, i.e., inhibition zones were present in some experiments and absent in others. Equivalence of reaction patterns as indicators were: CN72-2 = CN2-4B, CN72-5B, CN72-6A, CN72-6B, CN72-7, CN72-8B, CN72-25B, and CN72-29; CN72-3A = CN72-26B; CN72-8A = CN72-10, CN72-31, CN72-36, CN72-38, and CN72-40; CN72-12A = CN72-12B, CN72-18BB, CN72-20, CN72-24A, and CN72-39; CN72-24B = CN72-5A; CN72-42 = CN72-43. Equivalence of reaction patterns as producers were: CN72-4A = CN72-4B, CN72-5A, CN72-5B, CN72-6A, CN72-7, CN72-8A, CN72-21A, CN72-21B, CN72-25A, CN72-25B, CN72-29, and CN72-42; CN72-8B = CN72-11A, CN72-11B, CN72-17, CN72-18A, CN72-23B, CN72-30, and CN72-33; CN72-10 = CN72-24B; CN72-18B = CN72-22A, CN72-28 and CN72-31; CN72-26A = CN72-26B, and CN72-43; CN72-32 = CN72-34; CN72-37 = CN72-38, and CN72-40.

necessarily reflect their proportions in natural populations. They do illustrate the degree of variation that is possible among strains of this pathogen in a single field even in such basic characteristics as pigmentation and colony morphology. These characteristics have proven stable for each of our strains and useful in distinguishing them just as they have been for distinguishing strains of other coryneform bacteria (13). Polypeptide patterns produced by PAGE were of limited use in strain separation in contrast to species and subspecies differentiation (2,4).

Methods for determining variation among strains that have been useful in other systems, i.e., plasmid profiles (1,3,9) and serology (5,12), were assessed but not used. Plasmid isolations from *C. m.* subsp. *nebraskense* strains by previous workers showed only a single plasmid of common size was present in fewer than one-third of the strains examined (8). No plasmid was present in a random sample of 14 of our new strains (14), thus the method was not considered useful for our purposes. A serological study was not attempted because conflicting results have been produced with other corynebacteria (17), and attempts to produce a specific antiserum for *C. m.* subsp. *nebraskense* have been unsuccessful.

Many of our strains were sensitive to bacteriocins made by the other newly isolated *C. m.* subsp. *nebraskense* strains, suggesting a potential typing scheme based on bacteriocin sensitivity. However, our results were highly variable, and occasionally, we observed evidence that some strains could produce a bacteriocin inhibitor resulting in a ring of growth inside the zone of inhibition. A typing scheme based on bacteriocin sensitivity would require standardizing the procedures to give consistent results.

The differences between these results and those previously reported for *C. m.* subsp. *nebraskense* (18) may be explained in several ways. The strains investigated in the previous study were collected from infected plants over a period of 5–7 yr beginning

TABLE 2. Phage sensitivity groups of *Clavibacter michiganense* subsp. *nebraskense*

Group	Sensitivity to bacteriophage at RTD[a]					
	ϕ8	ϕ11	ϕC	ϕRH	ϕX	ϕXC
A[b]	–[c]	–	–	–	+	+
B	+	+	+	+	+	+
D	+	–	+	+	+	+
F	+	+	–	+	+	+
G	–	–	–	–	–	+
H	–	–	–	–	–	–
I	–	–	–	+	+	+
J	+	–	–	+	+	+
K	+	+	–	–	+	+
L	+	+	–	–	–	+
M	+	–	–	–	+	+

[a] RTD = routine test dilution is about 10^5 plaque-forming units/ml.
[b] Groups C and E were described by Vidaver et al (18) but are not represented in this collection of isolates.
[c] + = Clear or turbid plaque; – = no plaques.

TABLE 3. Subdivision of seven major groups of *Clavibacter michiganense* subsp. *nebraskense* isolates by phage sensitivity patterns

Group[a]	Phage type	Strain(s)
1	A	CN72-4B, CN72-6A, CN72-7, CN72-8B, CN72-17, CN72-18A, CN72-18B, CN72-22A, CN72-22B, CN72-24B, CN72-25A, CN72-32, CN72-34, CN72-40
1	B	CN72-23A, CN72-43
1	D	CN72-23B
1	F	CN72-25B
1	G	CN72-4A
1	I	CN72-3B
1	J	CN72-5A
1	K	CN72-5B
1	L	CN72-6B
2	A	CN72-2, CN72-12A, CN72-12B, CN72-18BB, CN72-19, CN72-20, CN72-29, CN72-41
2	B	CN72-24A
2	I	CN72-3A
2	M	CN72-36
4	A	CN72-11B, CN72-42
4	G	CN72-11A
6	A	CN72-29, CN72-37, CN72-38
6	B	CN72-28

[a] Strains in groups 3, 5, and 7 were not subdivided. Members of groups 3 and 5 were all of phage type A; members of group 7 were both phage type H.

with the first outbreaks of the disease. The strains we used were collected nearly 10 yr later and were from both plants and debris. The extended period of time may have been sufficient for the development of more strain variability, assuming that *C. m.* subsp. *nebraskense* was a recently evolved pathogen in 1969. The field from which these strains were isolated had been planted to dent corn or popcorn for at least four successive years before this study. An alternate explanation may lie in the methods by which the previous strains were selected and/or our use of a semiselective medium, which was not available for the original isolations. The original strains of the pathogen were characterized as having a "fluidal, orange, domed colony. . ." (19). Thus, some other colony types may not have been recognized without the semiselective medium. Regardless of its origin, recognition of the diversity within this pathogen should be useful to those working with natural populations. This study also raises questions about assumptions of homogeneity in a bacterial pathogen within a single field.

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