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Differential Effects of Lithium Chloride on In Vitro Growth of *Clavibacter michiganense* subsp. *nebraskense* Depending upon Inoculum Source†

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The bacterium *Clavibacter michiganense* subsp. *nebraskense* (*Corynebacterium michiganense* subsp. *nebraskense*) was grown in broth cultures and inoculated into corn plants. The plating efficiency of cells from broth cultures was essentially the same on nutrient broth-yeast extract and the semiselective medium for this bacterium, CNS. However, when cells were isolated from Goss bacterial wilt- and blight-infected corn, very few were recovered on CNS compared with the amount recovered on nutrient broth-yeast extract agar. When lithium chloride was omitted from the CNS, recoveries from infected corn were nearly the same as on nutrient broth-yeast extract agar. No other ingredient of CNS was inhibitory, nor did substitution of other salts for lithium chloride cause equal inhibition. The amount of inhibition was proportional to lithium chloride concentration. The inhibition by lithium chloride occurred with several strains of the bacterium isolated from one corn cultivar and with one of the strains recovered from three different cultivars of infected corn.

Cells of plant-pathogenic bacteria grown in plants might be expected to differ in certain physiological characteristics from those grown in broth, just as do cells grown in different formulations of broth; however, such differences have never been described. In this paper, we demonstrate such a difference between plant-grown and broth-grown cells of *Clavibacter michiganense* subsp. *nebraskense* (3) (*Corynebacterium michiganense* subsp. *nebraskense*), a corn pathogen.

Recently, while isolating this organism from inoculated corn plants, we observed that when homogenates were spread onto nutrient broth-yeast extract (NBY) (9) and onto the semiselective medium CNS (4), the NBY plates had many more colonies than did the same dilutions plated onto CNS. This was not the case when broth-grown cells were plated on both media. Gross and Vidaver (4) formulated CNS by adding nalidixic acid, polymyxin B sulfate, cycloheximide, the fungicide Bravo (now marketed as Daconil 2787 by Diamond Shamrock Corp.), and LiCl to NBY. We have determined which of the ingredients of CNS inhibits growth of *C. michiganense* subsp. *nebraskense* cells isolated from plant tissues.

(Parts of these results were reported at the 6th International Conference on Plant Pathogenic Bacteria, College Park, Md. 1985.)

All strains of *C. michiganense* subsp. *nebraskense* used in this study were originally isolated from infected corn grown in Nebraska. Cells were recovered from broth cultures of strain CN72-2 grown in NBY or NMCF (22 mM $Na_2HPO_4 \cdot 7H_2O$, 22 mM KH_2PO_4, 28 mM NH_4Cl, 0.1% [wt/vol] Casamino Acids, 0.01% [wt/vol] yeast extract, 56 mM glucose, 1.0 mM $MgSO_4$, 0.6 mM L-glutamine, 0.8 μM nicotinic acid, 0.03 mM thiamine hydrochloride, 4.1 μM biotin, 0.07 mM L-methionine). The first seven ingredients were sterilized by autoclaving. The last five were each filter sterilized separately.

Growth in broth was measured with a Bausch & Lomb Spectronic 20 spectrophotometer at 640 nm. Turbidity measurements were compared with a standard curve of cell concentrations. Solidified culture media were NBY, CNS, and CNS, with certain ingredients omitted or substituted as indicated below. Media were solidified with 1.3% agar (Sigma Chemical Co. or Difco Laboratories; the source of agar did not affect the results). Each bacterial suspension was spread onto duplicate or triplicate plates. The plating efficiency of organisms grown in either NBY or NMCF broth was nearly the same on NBY and both formulations of CNS agar (Table 1).

C. michiganense subsp. *nebraskense* was recovered from corn plants grown in 10.2-cm clay pots in a soil mix of loam, sand, and peat. Corn cultivars were hybrid A619XA632, a dent corn considered highly susceptible to Goss bacterial wilt and blight; hybrid Mo17XB73, a dent corn that is considered resistant to the disease; and Golden Cross Bantam, a susceptible sweet corn. The dent corn hybrids were grown in a growth chamber with 12-h days at 32°C with 200 to 250 microeinsteins m^{-2} s^{-1} from fluorescent lights and 12-h nights at 25°C. Golden Cross Bantam was grown in the greenhouse. Two-week-old corn plants were inoculated with *C. michiganense* subsp. *nebraskense* grown in NBY broth to late log or early stationary phase. Cell concentrations were adjusted to 10^5 to 10^6 cells ml^{-1} by diluting into 12.5 mM potassium phosphate buffer (KPi buffer; pH 7.1). Inoculations were made with a Gilman Pipetman micropipet fitted with an 18-gauge hypodermic needle. A 10-μl sample of the cell suspension was injected into the stem about 2.5 cm above the soil level. This was into the whorl and resulted in wounding many leaves before they emerged. Disease symptoms first appeared on wounded leaves 2 to 3 days after inoculation. Plants were sampled 4 to 6 days after inoculation when internal bacterial concentrations were near their maxima (data not shown). Three to five plants were harvested by slicing the stem at the soil level with a razor blade. The plants were collectively weighed and then homogenized with a Waring blender in 100 ml of KPi buffer. Samples of homogenates were withdrawn, diluted serially in the same

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TABLE 1. Recovery of *C. michiganense* subsp. *nebraskense* CN72-2 from corn tissue and broth cultures on different media[a]

Source of cells[c]	Recovery with the following medium (CFU ml^{-1} or CFU g^{-1})[b]		
	NBY	CNS without LiCl	CNS with LiCl
Broth			
NBY	6.6×10^8	1.0×10^9	6.6×10^8
NMCF	1.7×10^9	6.0×10^8	1.0×10^9
Corn plants			
A619XA632	1.2×10^9	9.3×10^8	2.3×10^7
Mol7XB73	2.3×10^8	2.1×10^8	1.6×10^6
Golden Cross Bantam	1.1×10^9	7.7×10^8	3.8×10^7

[a] Results are from one representative experiment.
[b] Cell concentrations are expressed as CFU per milliliter when recovered from broth and as CFU per gram when recovered from plants.
[c] Cells were harvested from broth during mid to late log phase. Corn plants were sampled 4 to 6 days after inoculation when bacterial concentrations were near their maxima.

buffer, and spread into agar plates. Colonies were counted 4 to 7 days later.

In contrast to recoveries observed with broth-grown cells, most *C. michiganense* subsp. *nebraskense* cells grown in corn tissue were unable to grow on CNS regardless of the cultivar or growing conditions (Table 1). Recoveries on CNS of eight different isolates, CN72-2, CN72-8A, CN72-11A, CN72-18A, CN72-21B, CN72-26A, CN72-31, and CN72-42, from greenhouse-grown Golden Cross Bantam corn were similar to each other and averaged 3.0% (standard deviation, 1.4) of the recoveries on NBY. Recoveries of individual strains varied by several percent from experiment to experiment.

Omission of certain ingredients from CNS showed that LiCl had the strongest inhibitory effect against cells of CN72-2 reisolated from corn (Table 2). When CNS was formulated, LiCl was included for the purpose of inhibiting gram-negative bacteria (5) without affecting the growth of gram-positive bacteria such as *C. michiganense* subsp. *nebraskense*. Our results were contradictory for these effects when *C. michiganense* subsp. *nebraskense* was obtained from plants. Inhibition of the organism on CNS was largely eliminated by omitting LiCl, and whatever inhibition remained was probably due to the polymyxin B sulfate and nalidixic acid.

CNS with LiCl concentrations of 10, 7.5, 5.0, 2.5, 1, and 0 g $liter^{-1}$ supported the growth of 5.9×10^7, 1.1×10^8, 2.9×10^8, 6.3×10^8, 1.1×10^9, and 1.2×10^9 CFU $plant^{-1}$, respectively; 1.7×10^9 CFU $plant^{-1}$ was recovered on NBY. These results are from one representative experiment with greenhouse-grown Golden Cross Bantam corn. When 10 g of LiCl $liter^{-1}$ was added to solidified NBY, the inhibition was similar to that observed with CNS (Table 2). Identical results were obtained with three different lots of LiCl from Sigma Chemical Co. and one from Fisher Chemicals. Substitution of equimolar amounts of KCl for LiCl did not duplicate the inhibition of LiCl, indicating that the inhibition was not caused by salt concentration or the anion. Equimolar concentrations of NaCl resulted in some inhibition, but less than with LiCl (Table 2). When an equimolar amount of Li^+ as Li_2CO_3 (8.9 g $liter^{-1}$) or $LiC_2H_3O_2 \cdot 2H_2O$ (25 g $liter^{-1}$) was substituted for LiCl, no growth occurred (data not shown), suggesting that these salts were even more toxic than LiCl.

The effects of LiCl on natural populations of *C. michiganense* subsp. *nebraskense* was tested by using pieces of corn stalks collected in the spring from a field where Goss bacterial wilt and blight symptoms were observed during the previous growing season. A 50-g portion was pulverized in a Waring blender and suspended in 500 ml of half-strength NBY broth without glucose. The suspension was incubated on a gyratory shaker for 1 h, after which samples were removed, diluted in KPi buffer, and spread onto agar plates. Dent corn leaves were collected from a naturally infected field in late August. Fresh leaves showing typical Goss wilt and blight symptoms were homogenized in KPi buffer with a Waring blender. Samples of the homogenate were removed, diluted in KPi buffer, and plated. Colonies of *C. michiganense* subsp. *nebraskense*, identified by their characteristic morphology and pigmentation (10), and other bacterial colonies were counted after 7 days at 25°C.

Recovery of *C. michiganense* subsp. *nebraskense* from naturally infected corn residue and fresh leaves was also lower on CNS than on CNS without LiCl (data not shown). It was not possible to obtain counts from NBY plates because fast-growing bacteria and fungi from the plant material obscured or prevented the growth of *C. michiganense* subsp. *nebraskense* colonies. These results suggest that previous work involving quantitative field recovery of *C. michiganense* subsp. *nebraskense* on CNS likely underestimated the populations, and they bring into question the suitability of CNS as a semiselective medium for field recovery of *C. michiganense* subsp. *nebraskense*. We found that for isolations made from corn residue and fresh leaf material, CNS without LiCl sustained growth of no more miscellaneous unidentified bacteria than did CNS with LiCl. We recommend that in those cases where NBY cannot be used because of the presence of contaminating bacteria and fungi, CNS should be made by omitting LiCl, with the realization that populations of *C. michiganense* subsp. *nebraskense* are probably still slightly higher than the counts would indicate.

There are very few studies in which the physiology of phytopathogenic bacteria from infected plant tissue is compared with that of broth-grown cells. Similarities have been reported for some extracellular products, such as polysac-

TABLE 2. Recovery of *C. michiganense* subsp. *nebraskense* CN72-2 from inoculated corn tissue on NBY and modified CNS[a]

Expt no.	Culture medium[b]	Alteration of medium[b]	Recovery (CFU/ml)
1	NBY	None	8.5×10^7
	CNS	None	3.0×10^6
	CNS	−LiCl	6.0×10^7
	CNS	+NaCl, −LiCl	4.7×10^7
2	NBY	None	1.0×10^8
	NBY	+LiCl	1.8×10^7
3	NBY	None	8.6×10^7
	CNS	None	7.7×10^6
	CNS	−Polymyxin B sulfate	1.1×10^7
	CNS	−Nalidixic acid	1.1×10^7
	CNS	−Cycloheximide	6.2×10^6
	CNS	−Bravo	6.6×10^6
	CNS	−LiCl	9.5×10^7
	CNS	+KCl, −LiCl	8.1×10^7
	CNS	+NaCl, −LiCl	3.3×10^7

[a] Cells were inoculated into and recovered from greenhouse-grown Golden Cross Bantam corn as described in the text.
[b] Concentrations of LiCl, polymyxin B sulfate, nalidixic acid, cycloheximide, and Bravo are as described by Gross and Vidaver (4). NaCl and KCl were present at 14 and 18 g $liter^{-1}$, respectively. + indicates addition; − indicates omission.

charides (1), toxins (6), bacteriocins (8), and plant hormones (7), which are synthesized in broth culture and are also detected in infected plant tissues. Also, some surface components such as serological determinants (2) are similar on broth-grown cells and cells in infected plant tissue. Ours is the first report of a physiological difference between plant-grown and broth-grown cells of a bacterial plant pathogen.

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