Colonization and Movement of Green Fluorescent Protein-Labeled *Clavibacter nebraskensis* in Maize

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Abstract

Clavibacter nebraskensis causes Goss's bacterial wilt and leaf blight, a major disease of maize. Infected crop residue is the primary inoculum source and infection can occur via wounds or natural openings, such as stomata or hydathodes. The use of resistant hybrids is the primary control method for Goss's wilt. In this study, colonization and movement patterns of *C. nebraskensis* during infection were examined using green fluorescent protein (GFP)-labeled bacterial strains. We successfully introduced a plasmid to *C. nebraskensis* via electroporation, which resulted in GFP accumulation. Fluorescence microscopy revealed that in the absence of wounding, bacteria colonize leaf tissue via entry through the hydathodes when guttation droplets are present. Stomatal penetration was not observed under natural conditions. Bacteria initially colonize the xylem and subsequently the

mesophyll, which creates the freckles that are characteristic of the disease. Bacteria infiltrated into the mesophyll did not cause disease symptoms, could not enter the vasculature, and did not spread from the initial inoculation point. Bacteria were observed exuding through stomata onto the leaf surface, resulting in the characteristic sheen of diseased leaves. Resistant maize lines exhibited decreased bacterial spread in the vasculature and the mesophyll. These tools to examine *C. nebraskensis* movement offer opportunities and new insights into the pathogenesis process and can form the basis for improved Goss's wilt management through host resistance.

Keywords: cereals, cultivar/resistance, disease management, field crops, grains, prokaryotes

Goss's bacterial wilt and leaf blight (GW), one of the most damaging bacterial diseases of maize (Mueller et al. 2016), is caused by the nonmotile gram-positive bacterium *Clavibacter nebraskensis* comb. nov. (Li et al. 2018; Vidaver and Mandel 1974). *C. nebraskensis* is a vascular pathogen that causes leaf blight and in some cases vascular wilt. Wilt symptoms typically only occur with early infection of susceptible genotypes (Calub et al. 1974a). Leaf blight, the more common symptom, is characterized by long tan to gray oval-shaped necrotic lesions with wavy margins and small, dark, water-soaked spots, referred to as freckles, that are visible on the edges of enlarging lesions (Schuster 1975). Yield losses vary depending on the susceptibility of the maize variety and the timing of infection, but ears may not be formed during severe infections of susceptible varieties (Cooper et al. 2019; Pataky et al. 1988). In inoculated trials, yield losses for some susceptible hybrids exceeded 40% (Carson and Wicks 1991; Pataky et al. 1988). Plant age has a significant effect on resistance, with younger plants more susceptible than older plants (Calub et al. 1974b).

Control of GW relies heavily on the use of host resistance, and resistance is polygenic (Cooper et al. 2018; Singh et al. 2016; Treat and Tracy 1990). There are no practical chemical controls for GW (Mehl et al. 2015). Some cultural practices, such as crop rotation and conventional tillage, can decrease the quantity of disease inoculum in the environment, but the most effective way to mitigate the effects of GW is to plant resistant maize hybrids (Jackson et al. 2007). No lines of maize have been shown to be completely resistant; rather, resistance to GW is quantitative and conferred by multiple small-effect quantitative trait loci (QTLs) with additive effects (Cooper et al. 2019; Singh et al. 2016; Treat and Tracy 1990). GW resistance for leaf blight has been shown to be associated with the common rust

resistance gene cluster at *rp1*, with hypersensitive responses observed for certain *rp1* haplotypes (Hu et al. 2018).

Resistance to xylem-colonizing pathogenic bacteria is rare, and relatively few defense mechanisms are known (Bae et al. 2015). One way plants defend themselves is to form vascular occlusions, including tyloses, as well as pectin-rich gels (Braun 1982; Clérivet et al. 2000; Mace 1978; Rioux et al. 1998; Stevenson et al. 2004; Sun et al. 2008). Vascular wall coatings and swellings are also employed by the plant to halt pathogen spread (Rahman et al. 1999; Robb et al. 1989, 1991). Restriction of pathogen growth in the vasculature has been suggested as a mechanism of resistance to *C. nebraskensis*. Early studies indicated strong positive correlations among disease severity when populations were evaluated for multiple vascular pathogens (Pataky 1985). The strong correlations were hypothesized to be related to morphological differences between genotypes or increased vascular occlusions (Ford and Mikel 1984). Resistant maize plants deposit a fibrillar matrix material in the xylem to restrict the spread of *C. nebraskensis* (Mbofung et al. 2016). Vascular resistance to Stewart's wilt of maize, caused by *Pantoea stewartii*, also a xylem-colonizing bacterial pathogen, is associated with the accumulation of electron dense materials (EDMs) in the vasculature that restrict the spread and movement of the bacterium (Braun 1982; Doblas-Ibáñez et al. 2019). Insights into how *C. nebraskensis* interacts with its host could lead to novel disease management strategies.

There are several proposed routes of bacterial entry into the plant. The pathogen enters plant tissues via wounds, such as hail damage and sand blasting (Schuster 1975). It was demonstrated that *C. nebraskensis* could enter the plant via natural openings in high humidity conditions; stomata and trichomes were the proposed entry routes (Mallowa et al. 2016). Stomata are pores found in the epidermis that facilitate gas exchange and can open and close, whereas hydathodes superficially resemble stomata but have distinctly different functions and structures that make them potential entry points for vascular pathogens. The primary purpose of hydathodes is to exude excess water via guttation. Hydathodes are found at the tips and edges of leaves, have water pores that are always open, and have direct access to the ends of vascular bundles (Dieffenbach et al. 1980). When transpiration increases as a result of changes in lighting, temperature, and humidity, guttation droplets are drawn back into the plant, which draws in any pathogens that may have been splashed into the droplet (Carlton et al. 1998). This is an entry route for several pathogens, including *C. michiganensis* infecting tomatoes and

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary videos are published online.

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Xanthomonas campestris pv. *campestris* infecting cauliflower (Carlton et al. 1998; Mew et al. 1984; Sharabani et al. 2013). In tomatoes the hydathodes were shown to be a significant secondary infection point in greenhouse outbreaks of *C. michiganensis* (Sharabani et al. 2013).

Green fluorescent protein (GFP) is a widely used bioreporter that can be used to track the growth and spread of several bacterial pathogens (Chalupowicz et al. 2012; Herrera et al. 2008; Koutsoudis et al. 2006). GFP tagging allows for the rapid imaging of live *C. nebraskensis* cells in planta, including prior to lesion formation, and for the detailed characterization of the plant-pathogen interaction without the need for chemical fixatives or tissue clearing (Han et al. 2008; Newman et al. 2003). A robust transformation system for *Clavibacter nebraskensis* will improve our understanding of gram-positive bacterial pathogenesis strategies and host resistance strategies.

Our overall goal was to develop GFP-labeled *C. nebraskensis* strains for tracking the spread of bacterial cells in planta, gain a better understanding of the pathogenesis process, and examine the effect of host genotype on pathogen spread. Experiments were designed to examine how *C. nebraskensis* enters the plant and how it colonizes tissue. We hypothesized that *C. nebraskensis* was able to enter through the hydathodes and tested this hypothesis by examining bacterial colonization following spray inoculations. To assess how bacteria spread within the host, plants were inoculated using different inoculation techniques and fluorescence was monitored. Overall, this study provides insights into how *C. nebraskensis* enters and infects maize and has implications for understanding how *Clavibacter* species enter and colonize their hosts. This study could transform our understanding of how to screen for host resistance and which factors to select for to develop resistant lines, and it could also provide insights into how to transform other gram-positive plant pathogens.

Materials and Methods

Strains. Five wild-type strains of *C. nebraskensis* were used for transformation. The isolates used were the pathogenic strains 16cmn, 4d, 44b, and B41133 and the nonpathogenic strain NP16cmn (Table 1). Strains 16cmn and NP16cmn were both collected in Illinois, while the other isolates were collected in Nebraska. NP16cmn is a mutant of 16cmn that we discovered in greenhouse inoculated plants during an experiment to reisolate 16cmn. After isolation and reinoculation with bacteria from plants inoculated with 16cmn, some plants failed to develop symptoms. We refer to this isolate as NP16cmn. NP16cmn colonies are morphologically identical to 16cmn colonies on nutrient broth yeast (NBY) agar (Vidaver 1967). All of the strains used were confirmed to be *C. nebraskensis* using species-specific primers developed by McNally et al. (2016). The *Escherichia coli* strain JM109 was used to maintain and replicate the plasmid pK2-22 (Chalupowicz et al. 2012). Growth media and laboratory conditions as described by (Chalupowicz et al. 2012; Kirchner et al. 2001) were used. Briefly, NBY was used to culture *C. nebraskensis* and nutrient agar was used to culture *E. coli*, except as described below during the transformation process.

Transformation. We used the pK2-22 plasmid to transiently express GFP in *C. nebraskensis*, as it has been successfully used in other *Clavibacter* species (Chalupowicz et al. 2012; Tancos et al. 2018). The pK2-22 plasmid contains a (enhanced) GFP gene that does not integrate into the bacterial genome and a gene for neomycin resistance (Chalupowicz et al. 2012). We obtained the plasmid from Dr. Christine Smart (Cornell University).

Plasmid containing *E. coli* JM109 cells was grown in lysogeny broth medium containing 50 µg/ml of neomycin (Bertani 1951). The plasmid was extracted using Omega Bio-tek E.Z.N.A. Plasmid Mini Kit I and the plasmid DNA concentration was adjusted to 20 ng/µl. The *C. nebraskensis* precultures for each of the wild-type strains were grown in 250 ml of NBY broth at 25°C overnight on a shaker set to 120 rpm. An aliquot of each preculture was diluted in NBY broth to an OD₅₈₀ of 0.3 and a volume of 218 ml. The cultures were then incubated under the same conditions until an OD₅₈₀ of 0.6 was reached (approximately 2 h). Then, 32 ml of 20% (wt/vol)

glycine was added to a final concentration of 2.5% (wt/vol) to increase the transformation efficiency, and the cultures were incubated for 1 h (Kirchner et al. 2001). The cells were harvested by centrifugation at 8,000 × g for 10 min at 4°C. The supernatant was removed, and the pellet was washed with 40 ml of ice-cold distilled water. The centrifugation and washing steps were repeated twice with 20 ml of 4°C distilled water. Subsequently, the cells were centrifuged and washed twice with 20 ml of 10% (vol/vol) glycerol in water. The cells were resuspended in 1 ml of 15% (vol/vol) glycerol in water after the final centrifugation. The electrocompetent cells were either used immediately for electroporation or stored at -80°C for future use.

A total of 100 µl of electrocompetent *C. nebraskensis* cells was mixed with 3 µl of plasmid DNA (60 ng total) in a chilled 0.2-cm electroporation cuvette. The cells were electroporated at 2.5 kV for a duration of 5 ms. Immediately after electroporation, the cells were mixed with 0.4 ml of sorbitol broth (SB) medium (Kirchner et al. 2001), transferred to a 1.5-ml sterile disposable tube, and incubated with shaking (140 rpm) for 3 h at 25°C. Finally, the cells were streaked on SB agar plates containing neomycin (50 µg/ml) (Chalupowicz et al. 2012; Kirchner et al. 2001). Four days later, fluorescent colonies were selected using a dark/light gel imager and a Zeiss Axio Imager 2 compound epifluorescent microscope. The presence of the plasmid in the transformed bacteria was also confirmed via PCR amplification of the GFP coding region. DNA was extracted from bacterial samples prior to running PCR. Plasmid DNA was used as a positive control and DNA from wild-type *C. nebraskensis* as a negative control. We used the forward primer 5'-TTGAACCACTAGT CAGTACTG-3' and the reverse primer 5'-ACGGGCACTAGT AGTGAG-3' (Chalupowicz et al. 2012). The reaction volume was 15 µl. It consisted of 1.5 µl of 10× DreamTaq Green Buffer, 0.3 µl of 10 mM dNTP mix, 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer, 0.6 µl of template DNA, 5 U/µl of DreamTaq DNA polymerase, and 11.325 µl of nuclease-free water. For PCR, the samples were incubated at 5 min at 95°C, followed by 40 cycles of 45 s at 94°C, 1 min at 60°C, and 1 min at 72°C, and a final elongation step of 10 min at 72°C. PCR was carried out on an Eppendorf Mastercycler nexus thermocycler. The amplified DNA was analyzed on an electrophoresis 1% agarose gel.

Plant materials. Three maize genotypes were used for this study, including Oh7B, B73, and NC344. These lines represent the spectrum of resistance and included a highly susceptible (Oh7B), moderately resistant (B73), and resistant (NC344) line. These lines were selected because they have been used as parents for biparental mapping populations and showed strong phenotypic differentiation (Cooper et al. 2018; Qiu et al. 2020). Seed for these lines was obtained from the Germplasm Resources Information Network.

Inoculum and host plant preparation. To generate inoculum, bacteria were grown on NBY agar plates for at least 3 to 4 days at room temperature (approximately 18°C). The bacteria were then scraped off with a sterile loop and diluted in an aqueous 0.1 M of NaCl solution to the desired concentration (Pataky 1985). The maize lines Oh7b, B73, and NC344 were planted at the Plant Care Facility in Urbana, Illinois, in 1-gallon pots filled with general purpose potting mix that had equal parts peat, perlite, and soil. The greenhouse day temperature was 22.5 to 24.5°C and the night temperature was 16.5 to 18.5°C with a 12-h/12-h light/dark cycle. Plants were at the V3 to V4 growth stage (Abendroth et al. 2011) when inoculated for all methods described below.

Virulence comparison among strains. The relative virulence of the wild-type isolates and GFP-expressing strains was determined by inoculating moderately susceptible B73 plants using the cut method (Mbofung et al. 2016). Cuts that spanned three major veins were made using a scalpel on either side of the midrib in the middle of the third leaf. A piece of cheesecloth dipped in inoculum (OD₆₀₀ = 0.05) was then swabbed onto the wound (Mbofung et al. 2016). For each GFP-labeled strain, we selected a random CFU that exhibited strong fluorescence in culture. A completely random design (CRD) with three replicates was used to test the virulence of the transformed and wild-type strains. Seed of the moderately susceptible line B73 was planted in the greenhouse with day temperatures of 22.5 to

24.5°C and night temperatures of 16.5 to 18.5°C. A total of 24 plants were grown. Each replicate was a whole plant with a single inoculated leaf. Plants were inoculated with one of the wild-type [16cmn, 4d, 44b, or NP16cmn] or transformed strains [16cmnGFP(17), 4dGFP(14), 44bGFP(28), or NPGFP(11)]. Plants were inoculated at the V4 growth stage (Abendroth et al. 2011) using the cut method. Disease severity was determined using a visual rating of percent diseased leaf area and proximal lesion length. Plants were rated at 3, 7, and 11 days post-inoculation (dpi). The area under the disease progress curve (AUDPC) was calculated using the agricolae package in R statistical software version 3.5.2 (De Mendiburu 2014; R Core Team 2018).

Our goal was to evaluate differences in virulence among wild-type strains and to determine whether transformation with pK2-22 affected virulence. Because we were examining differences in virulence among strains, the nonpathogenic strain was not included in the statistical analysis. The data were analyzed to test whether there were significant differences in virulence among wild-type strains and whether there were significant differences between wild-type and transformed strains. To examine differences in virulence among wild-type strains, an analysis of variance was conducted with bacterial strain as a fixed factor and included only disease severity data from plants inoculated with wild-type strains using R statistical software. Wilcoxon tests were used to determine whether there were differences between transformed and wild-type strains using the ggpubr package in R.

Stability comparison among transformed strains. The relative stability of GFP expression for 16cmnGFP(17), 4dGFP(14), 44bGFP(28), and NP16cmnGFP(11) was determined by reisolating bacteria from cut-inoculated B73 plants. Plants for the stability assay were grown and inoculated using the same methods as the virulence assay. The experiment was designed as a CRD with three replicates per strain at each of the three time points. Data were collected for a total of 36 B73 plants inoculated with one of the four following strains: 16cmnGFP(17), 4dGFP(14), 44bGFP(28), or NP16cmnGFP(11). Leaf punches were collected from plants at the leading edge of the lesion at 3, 7, and 11 dpi. A total of 12 plants were sampled at each time point and every plant was sampled only once. Leaf punches were homogenized in 1 ml of 0.1 M NaCl. The homogenate was diluted by a factor of 1,000 and 20 µl was streaked on NBY agar plates, with three plates per leaf punch. A total of 20 colonies were selected at random from each plate and then viewed under a dark light. The number of fluorescent colonies was counted and divided by 20 to determine the percentage of fluorescent colonies. The data were analyzed in R statistical software version 3.5.2 (R Core Team 2018) using the ggpubr package. Wilcoxon tests were performed that compared stability at the different time points.

Assessing modes of bacterial entry. Spray-inoculated and infiltration-inoculated plants were used to study bacterial entry. The spray inoculation method simulated bacteria being splashed onto unwounded leaves. The infiltration inoculation simulated bacteria that had entered the mesophyll through stomata.

Spray inoculation method. In the evening, well-watered plants were placed in a humidity chamber, where they remained overnight.

No humidifier was used. In the morning, the plants were sprayed with a bacterial suspension adjusted to an OD₆₀₀ of 0.4 (10⁸ cells/ml) until runoff and were returned to the humidity chamber. Only strain 4dGFP(14) was used in the spray inoculations. After 12 h in the humidity chamber, plants were placed on greenhouse benches at ambient humidity (Mallowa et al. 2016).

The spray inoculations were conducted on a total of 50 plants. Plants were inoculated in batches of no greater than five plants at a time over the course of 2 months. Of the 50 plants, two were B73, four were NC344, and 44 were Oh7b. Disease was observed in all three lines. Oh7b was used more extensively because it was a susceptible line that developed more disease. The plants were assessed qualitatively for disease severity. Spray inoculations were imaged using fluorescence microscopy between 5 and 7 dpi.

Infiltration inoculation method. Plants were infiltrated using a 12-ml NORM-JECT disposable syringe (Henke Sass Wolf, Tuttlingen, Germany) with a 4-mm-diameter opening and the needle removed. The inoculum had an OD₆₀₀ concentration of 0.05. Inoculum was added until a water-soaked region approximately 1 cm from the inoculation point was visible, which was approximately 5 µl. Care was taken not to damage the leaf tissue or vasculature. Both the second and third fully expanded leaves of each plant were inoculated. A total of 15 plants, including five of each genotype (Oh7b, B73, and NC344), were inoculated with 4dGFP(14). Plants were observed daily for symptom development. Leaves were imaged at 8 dpi, because this was adequate time for disease to develop but the symptomology did not increase after this time point.

Assessing bacterial colonization and movement and host plant resistance. Two different inoculation techniques requiring wounding were used to study the colonization and movement as well as host resistance of *C. nebraskensis*. The stem inoculation simulated the symptoms of the vascular wilt phase by introducing the bacteria directly into a puncture wound made in the stem tissue. The cut method simulated the leaf blight phase of the disease when bacteria enter the leaf tissues via wounding.

Stab inoculation method. A stab wound was made 1 cm above the soil surface using a dissecting needle, and 20 µl of inoculum with an OD₆₀₀ of 0.4 was pipetted into the wound. The plants were then observed for 15 days for signs of wilting. Three replications with one plant each of Oh7b, B73, and NC344 were inoculated with 16cmn using this method in an initial experiment. Later three plants each of Oh7b and NC344 were inoculated with 16cmnGFP(17) and used for microscopy. Qualitative assessments of severity were conducted based on visual observations of wilting severity at 14 dpi. Stem cross-sections were taken at 15 dpi and used for microscopy. No statistical comparisons were made between genotypes or isolates.

Cut inoculation method. Cut inoculations were conducted as described previously (Mbofung et al. 2016). All three maize genotypes were used with 50 Oh7b, 99 B73, and 50 NC344 plants inoculated. The strains 16cmnGFP(17) and 4dGFP(14) were used. Quantitative assessments of disease severity were made by measuring the lesion length, freckle area, freckle number, and freckle size.

Table 1. Strains used in this study

Species	Strain name	Pathogenic ^a	Plasmid	Antibiotic resistance	Fluorescence	Origin/source
<i>Clavibacter nebraskensis</i>	16cmn	Yes	No	No	No	Illinois (Cooper et al. 2018)
<i>C. nebraskensis</i>	16cmnGFP(17)	Yes	pk2-22	Neomycin	Yes	Transformation of 16cmn
<i>C. nebraskensis</i>	4d	Yes	No	No	No	Nebraska (Tamra Jackson-Ziems)
<i>C. nebraskensis</i>	4dGFP(14)	Yes	pk2-22	Neomycin	Yes	Transformation of 4d
<i>C. nebraskensis</i>	44b	Yes	No	No	No	Nebraska (Tamra Jackson-Ziems)
<i>C. nebraskensis</i>	44bGFP(28)	Yes	pk2-22	Neomycin	Yes	Transformation of 44b
<i>C. nebraskensis</i>	B41133	Yes	No	No	No	Nebraska (USDA-NRRL) ^b
<i>C. nebraskensis</i>	B41133GFP(34)	Yes	pk2-22	Neomycin	Yes	Transformation of B41133
<i>C. nebraskensis</i>	NP16cmn	No	No	No	No	Illinois (this study)
<i>C. nebraskensis</i>	NP16cmnGFP(11)	No	pk2-22	Neomycin	Yes	NP16cmn
<i>Escherichia coli</i>	JM109	NA	pk2-22	Neomycin	Yes	

^a NA = Not applicable.

^b USDA-NRRL = U.S. Department of Agriculture Northern Regional Research Laboratory.

Plants inoculated with this method were typically imaged between 5 and 7 dpi.

Image analysis. ImageJ software version 1.52k was used to quantify the number of freckles, the area of freckles, and the total leaf area

in images of three infected NC344 and three infected Oh7b leaves that were backlit to highlight the freckles. Using these data, the freckles per square centimeter, average freckle size, and the percentage of leaf area covered in freckles were calculated. For each of the traits quantified, Wilcoxon tests were conducted to compare the quantified traits between Oh7b and NC344.

Microscopy. More than 102 Oh7b and 62 NC344 plants were grown and inoculated for microscopy. We imaged at least three leaves of each genotype for the spray, cut, and infiltration inoculation methods. Stem cross-sections of three stab-inoculated plants of each genotype were imaged. Samples were imaged using microscopes at the Carl R. Woese Institute for Genomic Biology located at the University of Illinois Urbana-Champaign. To image whole leaves, a Zeiss Axio Zoom.V16 microscope with a Zeiss AxioCam HRm high-resolution monochrome camera was used, and the tile scan function was employed. Leaves were held flat by clear glass microscope slides. Color images were taken via RGB filters. Fluorescence images and color reflected light images were taken. The stitching function in ZEN Blue image analysis software was used to stitch tiles together. To image individual bacteria within leaf tissue, a Zeiss CLSM 880 confocal laser scanning microscope with a 40× water objective, GAsP detector, and an argon laser excitation of 488 nm was used. The peak emission wavelength was 537 nm with the detector set to wavelengths between 492 and 583 nm. Small pieces of leaf tissue were placed between coverslips held together with double-sided tape. Fluorescence images and pseudo-brightfield images were taken. Strains 4dGFP(14) and 16cmnGFP(17) were used for imaging experiments because they were relatively more virulent and stable compared with the other strains. Compound epifluorescent microscope images were taken with a Zeiss Axio Imager 2 microscope. The light source was a 120-V HXP metal halide arc lamp. Small pieces of leaf tissue were observed under a coverslip. Water was added to observe streaming. Both fluorescent images and differential interference contrast images were taken. ImageJ was also used to adjust the brightness and contrast of the microscope images. Cropping, resizing, and lettering necessary to make the figures was completed using Inkscape vector graphics software version 0.92.3.

Fig. 1. Strain comparison. **A**, Virulence. The plot represents area under the disease progress curve (AUDPC) values calculated from percent diseased leaf area data for transformed and wild-type strains. **B**, Virulence. The plot represents AUDPC values calculated from basipetal lesion length data for transformed and wild-type strains. **C**, Stability. Plants were inoculated with transformed strains at 3, 7, and 11 days after inoculation. The plot represents the percentage of bacterial colonies fluorescing after isolation from leaf tissue. Maize line B73 was used for these experiments. GFP = green fluorescent protein. Numbers above plots indicate *P*-values.

Results

Generation and assessment of transformed strains. The plasmid pK2-22 was successfully electroporated into all five *C. nebrascensis* strains tested, with at least one transformed colony recovered for all strains. All colonies that exhibited resistance to neomycin were fluorescent. The presence of the plasmid in the transformed bacteria was confirmed by PCR amplification of the GFP coding region (*data not shown*).

To test whether the plasmid affected virulence, we compared the virulence of the transformed and wild-type strains. There were no significant differences in virulence on cut-inoculated B73 plants between pK2-22 transformants and wild-type strains for diseased leaf area (Fig. 1A) or lesion length (Fig. 1B). The four pathogenic wild-type strains did not have significantly different AUDPC values for diseased leaf area ($P = 0.20$) or lesion length ($P = 0.78$), indicating that the strains were equivalent in terms of symptom severity despite different geographical origins. The nonpathogenic strain did not cause disease and thus the data for this strain were not included in the comparisons of virulence.

After confirming the virulence of the transformed strains, we tested the relative stability of GFP expression in the transformed isolates 3, 7, and 11 dpi. The final time point tested was 11 days, because it takes 11 days for an inoculated V3 or V4 leaf to become necrotic from an infection. We found that there were no significant differences in stability across all time points for most strains (Fig. 1C). However, 16cmnGFP(17) was significantly less stable at 11 dpi compared with 3 dpi (Fig. 1C). Strains 4dGFP(14) and 16cmnGFP(17) were selected for further in planta colonization imaging because of their high levels of virulence. Because of the relative instability of 16cmnGFP(17), samples containing this strain were imaged between 3 and 7 dpi. All symptomatic tissues exhibited bacterial fluorescence; however, it is probable that pK22-2-positive and pK22-2-negative

C. nebraskensis cells mix homogenously in the fluid-filled spaces of infected leaves.

Bacterial entry. *C. nebraskensis* is capable of causing foliar infections without mechanical damage (Mallowa et al. 2016). Our goal was to examine how the bacterium enters the leaf in the absence of mechanical damage. Spray inoculations were conducted under high humidity conditions conducive to guttation, and infection was characterized by bacterial growth originating from the tips and edges of the leaves (Fig. 2A). The vein closest to the leaf edge and the hydathodes near that vein were usually the first parts of the leaf to show fluorescence (Fig. 2B and C). In our experiments, bacteria were not observed colonizing or entering stomata during spray inoculations of healthy leaf tissue; instead, only bacteria entering from the leaf edges were observed. This suggests that the hydathodes are the likely entry point in the absence of wounding.

To determine whether bacteria could penetrate the vasculature from the mesophyll and cause disease, infiltration inoculations were conducted. We reasoned that if *C. nebraskensis* was able to cause disease symptoms when infiltrated into the leaf mesophyll, then it is possible that the stomata could serve as an infection route. However, when plants were inoculated using infiltrations, normal disease symptoms did not develop, and we only observed slight chlorosis around the inoculation site (Fig. 3B). Fluorescence microscopy revealed that bacteria were present in the mesophyll around the site of infiltration, but not in the veins (Fig. 3). This indicates that bacteria move unidirectionally from the xylem to the mesophyll, but not the mesophyll to the xylem.

Colonization and movement. After entry to the leaf through wounds caused by mechanical damage or through hydathodes, bacterial cells enter the xylem. Bacteria were observed being carried by the xylem fluid and putatively forming aggregates along xylem walls (Supplementary Video S1). In the cut inoculation experiments, bacteria spread both acropetal and basipetal from wound sites, indicating that

the pathogen can spread bidirectionally through the xylem vessels (Fig. 4A, B, C, and D). Cross-sections of stab-inoculated stems revealed that multiple vascular bundles were colonized (Fig. 4E). At the leading edge of the bacterial colonization in leaf veins, banding patterns could sometimes be observed (Fig. 4F). Leaf cross-sections showed that the bacteria were present in both the metaxylem and protoxylem (Fig. 4G and H).

After *C. nebraskensis* colonizes the xylem, it spreads to surrounding tissues, potentially through the degradation of xylem walls (Schuster 1975). *C. nebraskensis* likely degrades the xylem walls, allowing it to leak into and colonize the surrounding tissue (Supplementary Video S1). Using maize lines spanning the spectrum of resistant to susceptible, fluorescently labeled strains revealed that bacterial colonization of the mesophyll led to the formation of freckles, the characteristic symptom of the leaf blight phase of the

Fig. 3. Bacterial entry via infiltration. **A**, Infiltration of resistant line (fluorescent). Maize line NC344 (resistant) V3 leaf infiltrated with 4dGFP(14) fluorescence image was taken 8 days postinoculation with a fluorescence stereo microscope. **B**, Infiltration of resistant line (color). Color image under white light of the same leaf as A.

Fig. 2. Bacterial entry via hydathodes. **A**, Spray inoculation of susceptible line. Maize line Oh7b (susceptible) V3 leaf spray inoculated with 4dGFP(14). The leaf was viewed 11 days postinoculation (dpi) with a fluorescence stereo microscope. **B**, Bacteria 7 dpi beneath the leaf tip hydathode of an Oh7b leaf that had been spray inoculated with 4dGFP(14). **C**, Spray inoculated leaf with fluorescence in the vein along the leaf edge and water pores. wp = water pore and mjv = major vein.

Fig. 4. Bacterial colonization and movement. **A**, Cut inoculation of susceptible line (fluorescent). Maize line Oh7b (susceptible) V3 leaf inoculated with 4dGFP(14) using cut inoculation. The leaf is 5 days postinoculation (dpi) and viewed with a fluorescence stereo microscope. **B**, Cut inoculation of susceptible line (color). Color image under white light of the same leaf as **A**. **C**, Cut inoculation of resistant line (fluorescent). Maize line NC344 (resistant) V3 leaf inoculated with 4dGFP(14) using cut inoculation. The leaf is 5 dpi and viewed with a fluorescence stereo microscope. **D**, Cut inoculation of resistant line (color). Color image under white light of the same leaf as **C**. **E**, Stem inoculation of susceptible line. Cross-section of maize line Oh7b stem (susceptible) at the V3 growth stage. The plant was stab inoculated with 16cmnGFP(17) and was exhibiting systemic wilt symptoms. **F**, Banding pattern exhibited at the leading edge of infection on maize line Oh7b (susceptible) at 5 dpi on a V7 leaf with strain 16cmnGFP(17). **G**, Cross-section of a major vein in a leaf showing the presence of bacteria in the xylem strain 16cmnGFP(17) at 5 dpi. **H**, Cross-section of a major vein in a healthy uninfected Oh7b leaf. **I**, Bacteria exiting the minor veins and entering the substomatal space. **J**, Bacteria [strain 16cmnGFP(17)] in major and minor veins. **K**, Bacteria colonizing the mesophyll near an infected major vein. **L and M**, Confocal microscopy of bacteria in the apoplast near a stoma. **N**, Differential interference contrast image of bacterial exudate on the surface of the Oh7b leaf. **O**, Fluorescent image of bacterial exudate on the surface of an Oh7b leaf. **P**, Side view of bacterial exudate on the surface of an Oh7b leaf. mjv = major vein, px = protoxylem, pxl = protoxylem lacuna, mx = metaxylem, mnv = minor vein, st = stoma, bc = bacterial cell, mc = mesophyll cell, and be = bacterial exudate.

disease. The freckles appeared to have been cleared of many of the light-absorbing pigments usually present, giving them a translucent appearance when lit from behind. The freckles were typically found adjacent to infected major and minor veins (Fig. 4A, B, I, J, and K). The substomatal chambers appeared the brightest and were more heavily colonized than other regions of the mesophyll (Fig. 4K). While some cells in the mesophyll appeared to be shriveled, none appeared to have bacteria inside of them (Fig. 4L and M).

After establishing how the bacteria colonized tissue, we examined how they exited the plant. There were two observed exit routes from the leaves. Bacterial cells streamed readily from wounds in infected leaves 5 to 7 dpi in NC344 and Oh7b in the presence of water (Supplementary Video S2). Second, bacterial ooze was observed exuding through stomata from infected leaf tissue (Fig. 4N, O, and P).

Host plant resistance. We hypothesized that there would be quantitative differences in the pathogenesis process between resistant and susceptible maize plants. In order to test this hypothesis, we conducted cut inoculation experiments with resistant (NC344) and susceptible (Oh7b) lines and conducted image analysis of infected leaves to quantify the size and quantity of freckles. Both lines had similar patterns of lesion expansion via the xylem and freckle formation, although all aspects of the process were greatly restricted in the resistant line. The resistant variety, NC344, exhibited a longer incubation period before symptoms appeared and decreased spread of the

bacteria in the vasculature at 5 dpi (Fig. 4C and D). The resistant line had significantly smaller and less numerous freckles (Fig. 5).

Discussion

Here we report the first documented transformation of *C. nebraskensis* to express GFP. These methods will be useful for studying the virulence and pathogenicity of *C. nebraskensis*, the mechanisms of resistance to *C. nebraskensis*, and how *C. nebraskensis* interacts with its alternate hosts. This is the second bacterial species in the genus *Clavibacter* that has been transformed to express GFP using the plasmid pk2-22 (Chalupowicz et al. 2012, 2017; Tancos et al. 2018). It is likely that this plasmid and transformation protocol could be used to transform other important bacterial pathogens in the genus *Clavibacter*. Using the GFP strain, we found that the initial colonization steps and symptoms differ based on inoculation method, which could have important implications in identifying resistant lines and breeding for resistance.

GW disease occurs in the field in the absence of hail events or other mechanical damage, and *C. nebraskensis* can infect the plant via natural openings under high humidity conditions (Mallowa et al. 2016). Previous observations showed *C. nebraskensis* in and around stomatal openings; therefore, we tested whether stomata were the main infection route. The bacteria seemed unable to enter the vasculature unless a vein was damaged in the infiltration region. This

Fig. 5. Freckle image analysis. **A**, Images of same V4 leaf at 5 days postinoculation. (Top) Original backlit leaf image. (Middle) Using ImageJ to highlight the freckles in red to quantify freckle area and number. (Bottom) Using ImageJ to highlight entire leaf to quantify total leaf area. **B**, Differentiation between resistant and susceptible lines. Box plots of the average freckle size, number of freckles per square centimeter, and percentage of leaf area covered in freckles. Numbers above box plots indicate *P*-values.

could explain why virulent strains of *C. nebraskensis* have been found living as endophytes isolated from healthy leaf tissue and how the pathogen spreads undetected through fields (Ahmad et al. 2015; Eggenberger et al. 2016). Even though we did not observe stomatal entry, it may be possible for *C. nebraskensis* to enter stomata.

Based on our findings related to stomatal entry, we hypothesized that the likely entry point for bacteria was the hydathode water pores. The pattern of lesion formation on the edges of leaves where guttation droplets are found was like that of rice infected by *X. oryzae* pv. *oryzae*, which enters the vasculature via hydathodes but not stomata (Mew et al. 1984). Furthermore, *C. michiganensis* can enter tomato vasculature via hydathode water pores when guttation droplets are present (Carlton et al. 1998). In many plant species, hydathode water pores appear very similar superficially to stomata but have distinctly different functions and structures that make them ideal entry points for a nonmotile bacterial pathogen like *C. nebraskensis*. Unlike stomata, hydathode water pores are always open, have direct access to the ends of xylem vessels, and are found at the tips and edges of leaves (Dieffenbach et al. 1980; Kawamura et al. 2010; Singh and Singh 2013). Bacterial movement seems to be unidirectional from the vasculature to the mesophyll for *C. nebraskensis* in maize. The bacteria move from the vasculature to the mesophyll but not vice versa unless veins are damaged or if the bacteria enter through the hydathodes.

We examined bacterial colonization in maize. The leading edge of bacteria in the vasculature had a distinctive banding pattern (Fig. 4F), consistent with colonization patterns of *P. stewartii* and indicating common vascular bacterial colonization strategies across species (Koutsoudis et al. 2006). The freckles are typically found adjacent to infected major and minor veins, consistent with Schuster (1975), where bacteria were found in cavities adjacent to the vessels and in intercellular spaces. The brightness of the substomatal space is likely attributable to the area beneath the stomata being less densely packed with cells, giving the bacteria more space to fill. The microscopic images of bacterial exudate coming out of stomata within freckles suggest that *C. nebraskensis* is able to exit via the stomata.

We propose the following disease cycle for GW. The bacteria overwinter in crop residue and serve as a source of inoculum for the following growing season (Jackson et al. 2007). The bacteria then need to be introduced to the plant. One possibility is that a warm rain event splashes the bacteria onto the leaves of growing plants where some bacterial cells enter through the stomata and remain as endophytes. Other bacterial cells might be splashed into a guttation droplet that the plant draws back into the xylem when conditions get drier. If hail or other mechanical damage occurs, the bacteria enter the vasculature directly through those wounds. Once the bacteria are in the vasculature, they grow and spread in the xylem vessels, moving both acropetally and basipetally, as suggested by our data and that of Mbofung et al. (2016). The most likely scenario is that bacteria then damage the walls of xylem vessels, which causes xylem sap filled with bacteria to leak into the surrounding mesophyll, as is the case with other vascular-dwelling bacteria (Bogs et al. 1998; Gao et al. 2016). Our observations suggest that the bacteria continue to grow in the apoplast, ultimately leading to the cleared translucent appearance of the freckles. The bacteria then exit the leaf through the stomata or wounds and are spread by wind and rain, leading to secondary infection of other leaves and plants. Alternately, if infected leaves are wounded during a storm, bacteria stream from these sites in the presence of water.

The ability of the bacteria to enter the leaf via the hydathodes could be relevant to developing resistant maize lines. Symptoms like those observed with spray inoculations have been observed in the field, but the frequency of nonwounding transmission is unknown. It is highly likely that at least some spread of the disease is attributable to bacterial entry through the hydathodes. Previous QTL mapping studies have relied on causing infection solely by inoculating wounded plants (Cooper et al. 2018, 2019; Singh et al. 2016), and this inoculation method overlooks any potential for resistance at the hydathodes. In cauliflower, immunity to *X. campestris* pv. *campestris* is attributable to pathogen-associated molecular pattern-triggered immunity in the epithem, which offers a direct connection between the leaf surface and xylem vessels (Cerutti et al. 2017). In maize,

hydathode-based resistance is possible, but little is known about the anatomy and distribution of hydathodes, how guttation relates to pathogenesis, or whether there are active resistance mechanisms preventing bacterial entry through the hydathodes.

We tested several inoculation methods, and our data offer insights into the utility of different methods for germplasm screening. Typically, maize germplasm is evaluated in the field for resistance to *C. nebraskensis* using inoculation techniques that wound the plant and are evaluated using visual estimates of diseased leaf area (Cooper et al. 2018, 2019; Pataky 1985; Singh et al. 2016; Treat and Tracy 1990). The cut method combined with collecting data for incubation period and lesion length was the most consistent inoculation method for generating quantitative data associated with leaf blight symptoms. Using images of cut-inoculated leaves, we calculated the percentage of leaf area covered by freckles, average freckle size, and the number of freckles per square centimeter. All exhibited highly significant differences between resistant and susceptible maize lines, indicating that they may be useful phenotypes for characterizing resistance (Fig. 5).

Several resistance mechanisms are implicated for the resistant maize line NC344. We observed decreased colonization of the mesophyll and specifically smaller and less numerous freckles compared with the susceptible line. Given the importance of bacterial spread from xylem into mesophyll for freckle development, our findings suggest that bacteria are less able to spread from the xylem to surrounding tissues in resistant maize varieties. Furthermore, xylem vessels may be more resistant to bacterial degradation and defenses are likely being deployed to limit mesophyll proliferation in the resistant line. The pathogen exhibited decreased vascular spread in the xylem of the resistant line. These resistance mechanisms are consistent with resistance to *C. michiganensis* in some wild tomato species, where the bacteria are impaired in their ability to spread through the vascular system and macerate stem tissue (Peritore-Galve et al. 2020). Resistance to *R. solanacearum* in tomato includes the restriction of bacterial spread in the xylem and movement out of the xylem. Our results suggest that the pathogen is less able to spread in the resistant line, potentially because of a defense response such as a fibrillary matrix in the xylem, as has been observed in resistant hybrids (Mbofung et al. 2016). Our findings are also consistent with other studies that report vascular resistance in maize to other pathogens (Braun 1982; Doblas-Ibáñez et al. 2019). Two different kinds of EDMs have been observed in maize in response to *P. stewartii*, including a gel-like fibrillary material in the vascular lumen and a denser substance that was found blocking the pit membranes (Doblas-Ibáñez et al. 2019). A similar mechanism likely underlies resistance to GW, as these same maize lines also had increased resistance to *C. nebraskensis* infection (Doblas-Ibáñez et al. 2019).

In conclusion, we transformed *C. nebraskensis* to express GFP without a significant change in virulence. The instability as a result of plasmid loss was minimal and not a concern for the timescales required to study bacterial colonization in foliar tissues. Using the GFP strains, we tracked the entry, spread, and movement of the pathogen and showed that *C. nebraskensis* enters unwounded leaf tissue via hydathode water pores. The freckles were heavily colonized regions of the mesophyll, and *C. nebraskensis* can exit the leaf through the stomata located within freckles. *C. nebraskensis* infiltrated into the mesophyll was unable to cause disease without entering the vasculature. These findings provide critical insights into this disease and infection by a gram-positive plant pathogen, and they will be useful in characterizing host resistance mechanisms in future studies.

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