Pathogenic and Genetic Diversity of *Xanthomonas translucens* pv. *undulosa* in North Dakota

Tika B. Adhikari, Suraj Gurung, Jana M. Hansen, and J. Michael Bonman

First, second, and third authors: Department of Plant Pathology, North Dakota State University, Department 7660, P.O. Box 6050, Fargo 58108; and fourth author: United States Department of Agriculture–Agricultural Research Service, Small Grains and Potato Germplasm Research Unit, Aberdeen, ID 83210.

Current address of S. Gurung: Department of Plant Pathology, University of California, Davis, c/o United States Agricultural Research Station, 1636 E. Alisal Street, Salinas, CA 93905.

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ABSTRACT

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Bacterial leaf streak (BLS), caused by *Xanthomonas translucens* pv. *undulosa*, has become more prevalent recently in North Dakota and neighboring states. From five locations in North Dakota, 226 strains of *X. translucens* pv. *undulosa* were collected and evaluated for pathogenicity and then selected strains were inoculated on a set of 12 wheat cultivars and other cereal hosts. The genetic diversity of all strains was determined using repetitive sequence-based polymerase chain reaction (rep-PCR) and insertion sequence-based (IS)-PCR. Bacterial strains were pathogenic on wheat and barley but symptom severity was greatest on wheat. Strains varied greatly in aggressiveness, and wheat cultivars also showed differ-

ential responses to several strains. The 16S ribosomal DNA sequences of the strains were identical, and distinct from those of the other *Xanthomonas* pathovars. Combined rep-PCR and IS-PCR data produced 213 haplotypes. Similar haplotypes were detected in more than one location. Although diversity was greatest ($\approx 92\%$) among individuals within a location, statistically significant ($P \leq 0.001$ or 0.05) genetic differentiation among locations was estimated, indicating geographic differentiation between pathogen populations. The results of this study provide information on the pathogen diversity in North Dakota, which will be useful to better identify and characterize resistant germplasm.

Additional keywords: linkage disequilibrium, resistance, *Triticum aestivum*, virulence.

Several pathovar names have been used to describe *Xanthomonas translucens* strains isolated from small grains (17), and earlier researchers have used *X. translucens* pv. *translucens* in a general sense for any cereal streak pathogen (9). According to the International Society for Plant Pathology list of plant-pathogenic bacteria, *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens* are two pathovars that can mostly induce leaf streak symptoms on wheat and barley (14). Strains pathogenic on both wheat and barley were considered to be *X. translucens* pv. *undulosa* while strains pathogenic only on barley were proposed as *X. translucens* pv. *translucens* (11).

Bacterial leaf streak (BLS) or black chaff (53), caused by *X. translucens* pv. *undulosa* (10), is an important disease of wheat (*Triticum aestivum* L.) worldwide. Yield losses due to BLS have been reported as high as 40% from Idaho (22). Crop debris, alternative hosts, and soil appear to be major sources of primary inoculum (8,30,53,60). The bacterium can infect (59) and infest seed (53) and, although a low transmission rate was observed, seed also was considered an important source of primary inoculum (56). The pathogen can disseminate a short distance through dew, rain, and contact between plants (8), and long-distance or intercontinental dissemination can occur through wheat germplasm exchange (35).

In recent years, BLS epidemics have increased in the Upper Midwest of the United States, particularly in Minnesota, North Dakota, and South Dakota. There are at least three possible reasons for the occurrence of BLS epidemics in this region. First, winter wheat production in this region has increased recently and fall-sown wheat could be more prone to BLS infection and, thus, serve as a potential source of inoculum for spring wheat. Secondly, there has been increased planting of wheat cultivars moderately resistant to Fusarium head blight such as ‘Alsen’ and ‘Steel-ND’ (38), which are susceptible to *X. translucens* pv. *undulosa*. Finally, humid and mild weather conditions during late growth stages of winter wheat may favor infection and dissemination of inoculum into the adjacent spring wheat fields.

Understanding of the genetic diversity of bacterial plant pathogens is a key component for successful disease resistance breeding and deployment of host plant resistance (31). *X. translucens* pv. *undulosa* is one of the pathovars of *X. translucens*, with each pathovar being distinguished based on compatibility to various cereal host plants (15,37). Among many potential cereal hosts, *X. translucens* pv. *undulosa* has been described infecting wheat, barley, oat, rye, and triticale (10,11). Pathogenic variation in 81 strains of *X. translucens* pv. *undulosa* from North and South America was analyzed by inoculating a set of 19 differential wheat cultivars (39). Although the strains were highly variable, no significant interactions between the wheat cultivars and the bacterial strains were observed (39). In the past, the main research conducted on BLS has focused on evaluating wheat germplasm for resistance to *X. translucens* pv. *undulosa* (2,18,35,40,55). Three wheat cultivars (‘Pavon’, ‘Mochis’, and ‘Angostura’) (18, 35) and several winter wheat accessions (2) were identified as potential sources of resistance to *X. translucens* pv. *undulosa*.

Corresponding author: T. B. Adhikari, E-mail address: tadhikari31@yahoo.com

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In the past decades, the genetic diversity within pathovars (e.g., *cerealis*, *hordei*, *secalis*, *translucens*, and *undulosa*) of *X. translucens* has been assessed using polyclonal and monoclonal antibodies, neutral molecular markers, membrane proteins, fatty acid profiling, and ribosomal intergenic sequences (11,12,44, 47,49,54,61). Kolliker et al. (27) found a significant effect of geographic location on genetic diversity of *X. translucens* pv. *graminis* using amplified fragment length polymorphism (AFLP) markers. Repetitive sequence-based polymerase chain reaction (rep-PCR) genomic fingerprint profiles supported by pathogenicity assays revealed that the 33 strains from ornamental asparagus and all *X. translucens* reference strains discriminated *X. translucens* into pathovars *arrhenatheri*, *graminis*, *phlei*, and *phlei-pratensis*, and enabled identification of *X. translucens* pv. *undulosa* and *X. translucens* pv. *translucens* groups (10,49). Importantly, all strains isolated from ornamental asparagus belonged to *X. translucens* pv. *undulosa* (49). Although *X. translucens* pv. *undulosa* causes extensive damage to wheat in North Dakota and neighboring states each year, little is known about the pathogenic and genetic diversity of the pathogen.

Investigating the genetic structure of plant pathogens may provide a better understanding in order to develop geographically targeted management strategies (31). Our goal was to investigate the pathogenic and genetic diversity of *X. translucens* pv. *un-*

dulosa strains associated with the recent BLS outbreaks of wheat in North Dakota and subsequently formulate strategies to manage this reemerging pathogen. The specific objectives of this study were to (i) evaluate pathogenic variation among strains of *X. translucens* pv. *undulosa* on wheat cultivars and other cereal host plants, (ii) investigate the genetic diversity of *X. translucens* pv. *undulosa* strains from five locations in North Dakota using rep-PCR and insertion sequence-based (IS)-PCR techniques, and (iii) compare the genetic relationships of selected strains of *X. translucens* pv. *undulosa* from wheat with other reference strains and pathovars of *Xanthomonas* using 16S ribosomal DNA (16S rDNA) sequence analysis.

MATERIALS AND METHODS

Collection of BLS-infected leaf samples. Field surveys were conducted in major wheat-producing locations in North Dakota during the 2008 and 2009 seasons (Fig. 1; Table 1). A random sampling strategy was used to collect severely BLS-infected flag leaf samples from each site. The sampling sites surveyed were in fields at each of five locations. Within a field, samples were collected in a W-shaped pattern. Distances between sample collections in a field varied according to the severity and distribution of BLS. Leaf samples were collected from different

Fig. 1. Map showing the five wheat-producing locations of Carrington, Casselton, Langdon, Lisbon, and Prosper in North Dakota from which 226 strains of *Xanthomonas translucens* pv. *undulosa* were collected in 2008 and 2009.

TABLE 1. Information for 226 strains of *Xanthomonas translucens* pv. *undulosa* evaluated in this study from five locations and elevations in North Dakota collected during the 2008 and 2009 seasons

Location	Coordinates, elevation ^a	Number of fields sampled	Year	Wheat type ^b	Wheat cultivar planted ^c
Carrington	47°27'N, 99°7'W, and 464 m	10	2009	HRSW	Alsen and breeding lines
Casselton	46°54'N, 97°12'W, and 282 m	8	2008 and 2009	HRSW and HRWW	Alsen, Parshall, Jerry and breeding lines
Lisbon	46°26'N, 97°41'W, and 332 m	6	2009	HRWW	Jerry and breeding lines
Langdon	48°76'N, 98°37'W, and 492 m	12	2009	HRSW and Durum	Breeding lines
Prosper	46°97'N, 97°02'W, and 273 m	14	2009	HRSW	Alsen, Granite and breeding lined

^a Information on each of the locations represented by latitude, longitude, and elevation in meters above sea level, respectively.

^b HRSW = hard red spring wheat and HRWW = hard red winter wheat.

^c Cultivars from which strains of *X. translucens* pv. *undulosa* were collected.

hard red winter wheat, hard red spring wheat, durum, and advanced breeding lines (Table 1). At least three to five leaves were collected from each cultivar or line within a site to represent a sample.

Sixteen sites were surveyed in fall 2009 in Langdon, and most of the sites showed severe symptoms of BLS (Fig. 1). Leaf samples were collected from hard red spring wheat and durum cultivars and breeding lines planted at the farm of the Langdon Research Extension Center. Distances between the sites were 0.5 to 2 km. The other four collection locations were Casselton, Carrington, Lisbon, and Prosper (Fig. 1; Table 1). In all, 8 to 12 sites in Casselton were sampled in late spring 2008 and fall 2009, respectively. Leaf samples were collected within a 3-km sampling area and most of the samples were collected from commercial hard red spring wheat 'Alsen' and 'Parshall' and hard red winter wheat 'Jerry'. Some leaf samples also were collected from promising wheat varieties and elite lines, and barley lines planted at the demonstration plots and varietal testing trials at Agronomy Seed Farm, Casselton. Ten adjacent or nearby fields were sampled from Carrington in fall 2009. These sampling sites were within 5 km. In Lisbon, six sites were sampled within 3 km in early summer 2009 and samples were mainly collected from hard red winter wheat cultivars and breeding lines. Fourteen sites were surveyed in Prosper in fall 2009. Samples were collected from the experimental farm and farmers' fields within 10 km. Leaf samples were placed in an envelope and labeled with date of collection, location, and cultivar or line and brought to the laboratory at the Department of Plant Pathology, North Dakota State University, Fargo. The leaf samples were held at 20°C until bacterial isolations were made.

Isolation and maintenance of bacteria. Bacteria were isolated from the advancing lesion associated with bacterial exudates. The leaves were cut into 2- to 4-mm segments from each sample (three samples per site), and three leaf segments were placed in 100 µl of sterile distilled water in a small test tube for 10 min, allowing bacterial ooze to disperse into water. One wire-loop of bacterial suspension of each strain was streaked on Wilbrink's medium (50) and incubated at room temperature for 5 days. To determine morphological characteristics, a single representative colony of each strain was selected and restreaked on peptone sucrose agar (PSA) plates (48) and incubated at room temperature for 3 days. In all, 226 putative strains of *X. translucens* pv. *undulosa* from wheat and 4 strains from barley from Casselton were obtained. A pure culture of each strain was stored in 30% glycerol at -80°C and these frozen bacterial cultures served as sources of bacteria for subsequent experiments.

Pathogenicity, biochemical, and physiological tests. All putative 226 strains of *X. translucens* pv. *undulosa* from Carrington ($n = 36$), Casselton ($n = 23$), Langdon ($n = 63$), Lisbon ($n = 50$), and Prosper ($n = 54$) were tested for pathogenicity on flag leaves of hard red spring wheat line 'ND495' and barley (*Hordeum vulgare* subsp. *vulgare*) 'Robust'. Three seeds were planted in a plastic cone (3.8 cm in diameter and 20 cm long) (Stuewe and Sons, Inc., Tangent, OR) filled with Fison sunshine mix number 1 (Fison Horticulture, Vancouver, BC, Canada). Nine plants (three plants/cone and three replications) were inoculated with each strain. Plants were maintained in the greenhouse and watered and fertilized as needed. The bacterial strains were revived on PSA and incubated at 28°C for 72 h. Bacterial cells were scraped with a wire-loop, and inoculum was prepared by suspending cells in 20 ml of sterile deionized water per plate. Before inoculation, inoculum was adjusted to an optical density of 0.2 at 590 nm using a spectrophotometer, which was approximately equivalent to 1×10^7 CFU/ml based on dilution plating after inoculation. For each isolate, the flag leaf was infiltrated with ≈ 100 µl of inoculum using a disposable syringe without a needle, as described previously (2,40). Leaves infiltrated with water and known reference strains (LMG 885 and LMG 886) of *X. translucens* pv. *undulosa*

served as negative and positive controls, respectively. Plants were moved into a growth chamber at 21 and 18°C day and night temperature, respectively, with a 16-h photoperiod. Disease reactions within the infiltrated areas of 0.2 to 0.4 cm² were recorded between 7 and 10 days after infiltration using a 0-to-6 scale (2,39), where 0 = no visible symptoms, 1 = chlorosis without water-soaked lesions, 2 = <10% water soaking, 3 = 10 to 30% water soaking, 4 = 31 to 70% water soaking, 5 = 71 to 100% water soaking, and 6 = water soaking extending beyond the infiltrated area (55). Disease reactions were calculated based on the average of nine infiltrated areas on flag leaves (one site on each flag leaf). Median disease scores ≤ 2 were classified as resistant and those > 2 were classified as susceptible. To characterize strains based on disease reactions on wheat cultivars and other cereal host plants, we used the term "aggressiveness", which referred to the quantitative component of pathogenicity.

Strains of *X. translucens* pv. *undulosa* used for biochemical and physiological tests were recovered from stock cultures by streaking on PSA medium. Major bacteriological characteristics (51) were determined for a set of 29 strains of *X. translucens* pv. *undulosa* from five geographical locations (Carrington, $n = 6$; Casselton, $n = 6$; Langdon, $n = 6$; Lisbon, $n = 5$, and Prosper, $n = 6$) and some strains were also tested for their pathogenic variability (Table 2). These tests were gram reaction, oxidation and fermentative metabolism, gelatin hydrolysis, oxidase reaction, catalase reaction, starch hydrolysis, and growth on 1 and 5% sodium chloride.

Pathogenic variation of *X. translucens* pv. *undulosa* strains on wheat cultivars. Twelve wheat cultivars with different levels of resistance to BLS ('Alsen', 'Dam', 'Fil', 'Magnum', 'Mochis', 'ND495', 'Pavon', plant introduction 'PI 266860', 'PI 345476', 'PI 326301', 'PI 338387', and 'PI 351315') were selected based on previous studies (2,18,35,40,55). The spring wheat breeding line 'ND495' was used as susceptible check. Because of limited seed of the wheat cultivars, we were able to evaluate a subset of 29 strains of *X. translucens* pv. *undulosa* from five locations (six strains of each location except Lisbon) in this study (Table 2). Our initial pathogenicity tests showed that these 29 strains were highly aggressive on wheat line 'ND495'. In addition, these strains were used for pathogenic variation experiments (Table 2) and were the same strains used for biochemical and physiological tests. Planting wheat cultivars, inoculum preparation, inoculation, and greenhouse conditions were the same as described previously (2). Briefly, the experiment was conducted using a randomized complete block design (RCBD) with three replications. Two separate experiments were conducted for each strain in a greenhouse. Three plants grown in each cone were considered as the experimental unit. For each strain, 18 plants (three plants/cone, three replications, and two experiments) were inoculated. The replications were regarded as random effect while wheat cultivars were considered to be a fixed effect.

Inoculum of each strain was adjusted to 1×10^7 CFU/ml as described above and infiltrated on the adaxial surface of a fully expanded flag leaf using a disposable syringe without a needle (40). Disease reactions on wheat cultivars were assessed on only one infiltrated leaf area, as described above. Median disease scores were calculated from ordinal data using a nonparametric analysis (13,52) and used to characterize aggressiveness of the strains on a set of the 12 wheat cultivars (Table 2). In our previous study (2), median disease scores of ≤ 2 (i.e., <10% water soaking and chlorosis symptoms on the flag leaf) was a reliable criterion to differentiate resistant and susceptible wheat germplasm. Thus, median disease scores of 0 to 6 were used to generate the strain differences on a set of the 12 wheat cultivars based on aggressiveness in this study.

Comparison of aggressiveness of *X. translucens* pv. *undulosa* strains on cereal hosts. To further evaluate pathogenicity of *X. translucens* pv. *undulosa* on different cereal host plants, six highly aggressive strains (BLS-CS40, BLS-CS41, BLS-CS42,

BLS-CS43, BLS-CS48, and BLS-CS49) from wheat and four strains (BLS-CS54, BLS-CS55, BLS-CS56, and BLS-CS57) from barley in Casselton, ND were selected and tested on hard red spring wheat line 'ND495', barley 'Robust', oat (*Avena sativa* L.) 'Ajay', rye (*Secale cereale*) 'Ac Remington', and triticale 'PI 634537'. In this study, strains from wheat are referred to "wheat strains" and strains from barley are designated as "barley strains". Plants were arranged in an RCBD with three replications. The experiment was repeated twice in a greenhouse as described previously (2). Each treatment consisted of three plants per replication. Each cone was considered an experimental unit, and the flag leaf of each plant was regarded as a sampling unit. Disease reactions were assessed between 7 and 10 days after infiltration, as described above.

Phenotypic data analysis. Nonparametric analysis (13,52) was performed using the PROC MIXED procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC). Median disease scores, relative treatment effects, and their confidence intervals at 95% level were estimated to compare aggressiveness of each strain on a set of 12 wheat cultivars and five cereal hosts, and determine significant interactions ($P \leq 0.001$) between bacterial strains and wheat cultivars.

DNA extraction. Genomic DNA of each strain was extracted as described previously (29) and dissolved in 1× Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). DNA quality of each sample was checked using a spectrophotometer (Nano-Drop 1000; Thermo Scientific Inc., Wilmington, DE). DNA concentration (25 ng/ml) was prepared for each strain from the stock DNA and stored at -20°C.

Selection of PCR techniques. In previous studies, both rep-PCR (3,6,33,49,57) and IS-PCR (3) techniques were documented as reliable molecular tools to investigate population biology and genetic structure of plant-pathogenic bacteria. These improved techniques for monitoring genomic diversity in large populations of bacteria not only were simpler, faster, and cheaper than the other molecular techniques but also were useful to detect spatial distribution of pathogen haplotypes in the fields and to measure a high of level diversity (3,57). During the course of this study, the fully sequenced genome of *X. translucens* pv. *undulosa* was not available. Therefore, we chose to use rep-PCR and IS-PCR techniques to evaluate the genomic variation among the 226 strains.

Standardization of PCR techniques. All reagents were purchased from the Promega Corporation (Madison, WI) and primers were synthesized by Integrated DNA Technologies Inc.

TABLE 2. Pathogenic diversity of 29 strains of *Xanthomonas translucens* pv. *undulosa* from five locations in North Dakota evaluated on a set of 12 wheat cultivars^a

Strain ^b	Hap ^c	Score ^d												Disease reaction ^e	Pathotype ^f
		Alsen	Dam	Fil	Magnum	Mochis	ND495	Pavon	PI 266860	PI 345476	PI 326301	PI 338387	PI 351315		
BLS-LB10	75	1	2	1	2	1	3	1	2	1	1	2	1	RRRRSRRRRRR	1
BLS-LB44	23	1	2	2	3	1	4	1	2	1	2	3	2	RRRSRSRRRRSR	2
BLS-LG48	70	2	1	4	5	1	3	2	3	2	2	2	4	RRSSRSRRRRS	3
BLS-CS23	184	2	2	1	5	1	4	1	3	1	2	3	1	RRRSRSRRRRSR	4
BLS-CS1	156	2	2	2	2	2	5	2	3	2	2	2	3	RRRRRSRRRRS	5
BLS-CS8	119	2	2	2	6	1	2	2	4	2	2	2	1	RRRSRRRRRRRR	6
BLS-LB5	5	2	2	3	5	1	4	1	3	1	3	4	3	RRSSRSRRSSSS	7
BLS-LB56	82	2	2	3	6	1	3	1	2	1	2	3	2	RRSSRSRRRRSR	8
BLS-P55	176	2	2	3	6	1	3	1	3	5	3	5	3	RRSSRSRRSSSS	9
BLS-P30	39	2	3	1	2	1	3	1	2	1	2	4	2	RSRRRSRRRRSR	10
BLS-CS4	25	2	3	2	2	2	4	2	2	1	1	2	1	RSRRRSRRRRRR	11
BLS-LG42	193	2	3	2	4	1	3	2	2	1	1	3	2	RSRSRSRRRRSR	12
BLS-CR31	120	3	1	1	5	2	3	1	2	1	1	2	2	SRRSRSRRRRRR	13
BLS-LG58	108	3	2	1	2	1	3	1	1	1	1	3	2	SRRRRSRRRRSR	14
BLS-LG23	158	3	2	2	2	1	4	2	2	2	1	2	2	SRRRRSRRRRRR	15
BLS-LG63	133	3	2	2	4	1	4	2	3	1	4	4	2	SRRSRSRRSSSR	16
BLS-P37	16	3	2	5	5	2	4	4	3	2	6	5	3	SRSSRSRRSSSS	17
BLS-CR10	151	3	3	1	2	2	4	3	3	2	2	2	1	SSRRRSRRRRRR	18
BLS-LG55	131	3	3	2	6	2	3	2	3	1	2	5	2	SSRSRSRRRRSR	19
BLS-CR4	13	3	3	3	5	2	3	2	2	2	4	4	2	SSSSRSRRRRSR	20
BLS-CR61	103	3	4	3	3	1	5	2	3	1	2	2	3	SSSSRSRRRRRS	21
BLS-CR48	163	3	5	5	5	2	4	2	4	6	2	5	1	SSSSRSRRRRSR	22
BLS-CR22	158	4	2	2	2	2	4	1	2	2	2	3	2	SRRRRSRRRRRR	23
BLS-LG54	189	4	2	2	6	2	4	2	4	2	5	5	3	SRRSRSRRSSSS	24
BLS-P22	42	4	2	6	4	2	5	3	4	2	5	3	3	SRSSRSRRSSSS	25
BLS-CS2	155	4	4	5	3	3	4	3	2	4	1	3	2	SSSSSSRSRRSR	26
BLS-P13	124	5	2	6	6	5	6	2	2	3	6	6	2	SRSSSSRRSSSS	27
BLS-CS40	123	5	3	2	5	3	5	2	2	4	3	3	2	SSRSSRSRRSSSR	28
BLS-P3	34	5	5	2	2	5	5	3	6	5	2	6	5	SSRSSSSRSRSS	29

^a Wheat cultivars were arranged in randomized complete block design. Two experiments were conducted in the greenhouse. In each experiment, there were three replications. Disease scores of the nine infiltrated areas on flag leaves (one site on each flag leaf) were averaged. Each plastic cone was considered as a replication and contained three plants.

^b Twenty-nine strains of *X. translucens* pv. *undulosa* isolated from wheat in Carrington, Casselton, Lisbon, Langdon, and Prosper in North Dakota were analyzed on 12 wheat cultivars. The naming for *X. translucens* pv. *undulosa* strains from North Dakota is as follows: BLS = bacterial leaf streak, which is followed by the location from where the strains were collected: CR = Carrington, CS = Casselton, LB = Lisbon, LG = Langdon, and P = Prosper.

^c Haplotypes were identified based on the combined data of insertion sequence-based polymerase chain reaction (PCR) and repetitive sequence-based PCR techniques.

^d Disease scores within the infiltrated areas on the flag leaves were visually estimated in plants between 7 and 10 days after infiltration. The size of the infiltrated areas was 0.2 to 0.4 cm² and a disease rating scale of 0 to 6 (39,55) was used, where 0 = no visible symptoms, 1 = chlorosis without water-soaked lesions, 2 = <10% water soaking, 3 = 10 to 30% water soaking, 4 = 31 to 70% water soaking, 5 = 71 to 100% water soaking, and 6 = water soaking extending beyond the infiltrated area. A nonparametric analysis (13,52) was used to calculate median disease scores from ordinal data and used to characterize aggressiveness of strains in each wheat cultivar tested. For example, median values of 1 indicate strains are least aggressive while median values of 4 or more indicate strains highly aggressive on a particular wheat cultivar.

^e R = resistant and S = susceptible. Disease reactions corresponded with the 12 wheat cultivars tested.

^f Pathotypes were classified based on disease reactions on 12 wheat cultivars.

(Coralville, IA). Initially, concentrations of all primer sets used in both rep-PCR and IS-PCR techniques were confirmed using genomic DNA of the sequenced strain BLS-CS42 (formerly BLSW16 strain from Casselton, ND) of *X. translucens* pv. *undulosa* and reference strains. Primers and water without template DNA served as negative controls. To determine consistency to confirm banding patterns and generate haplotype patterns, both rep-PCR and IS-PCR amplifications with genomic DNA from randomly selected strains from five populations were done three times. Prior to conducting actual experiments, a series of preliminary PCRs were run to confirm and standardize PCR conditions, annealing temperatures, number of cycles, quality and quantity of template DNA, and concentration of enzyme and other reagents used in the reactions. Electrophoretic mobility of fragments in each lane was checked visually and compared with identical fragments in neighboring lanes. In addition, the rep-PCR and IS-PCR fingerprint patterns (referred to as the patterns of DNA fragments) among strains of *X. translucens* pv. *undulosa* were compared visually with the 1-kb ladder used as a size marker (Invitrogen Corporation, Carlsbad, CA). To evaluate reproducibility of weak or faint bands revealed by each primer set, two separate gels were run in different times. If confirmed, only polymorphic bands were included in data analysis.

rep-PCR analysis. Primers ERIC1R and ERIC2 (for enterobacterial repetitive intergenic consensus [ERIC]-PCR) and BOXA1R (for BOX elements [BOX]-PCR) were used (16,59) for all strains. All rep-PCR cycles and conditions were as described previously (3,33), with some modifications. For ERIC primers, each 25- μ l total volume of mixture contained 4 μ l of genomic DNA (25 ng/ml), 8.6 μ l of sterile distilled water, 2.5 μ l of dimethyl sulfoxide (DMSO), 0.4 μ l of bovine serum albumin (pH 8.8), 5 μ l of Gitschier buffer, 4 μ l of 2 μ M primer mixture, and 0.5 μ l of *Taq* DNA polymerase. For the BOX primer, each 25- μ l total volume of mixture contained 4 μ l of genomic DNA (25 ng/ μ l), 13.6 μ l of sterile distilled water, 2.5 μ l of DMSO, 0.4 μ l of bovine serum albumin (pH 8.8), 2 μ l of 5 $\times$ reaction buffer stock solution (83 mM ammonium sulfate [8.0 pH], 335 mM Tris-HCl, 33.5 mM magnesium chloride, 33.5 μ M EDTA, and 150 mM β -mercaptoethanol), 2 μ l of 2 μ M primer, and 0.5 μ l of *Taq* DNA polymerase. Each rep-PCR was carried out in a PTC-100 Peltier Thermal cycler (MJ Research, Inc., Waterman, MA) programmed for 7 min at 95°C for initial denaturation; 35 cycles consisting of 1 min at 94°C, 1 min at 52°C for annealing, and a 1-min extension at 65°C; with a final extension cycle at 65°C for 8 min followed by 20 h at 4°C for incubation.

IS-PCR-analysis. In a previous study, the IS-PCR primer J3 amplified sequences from several pathovars of *Xanthomonas* (3,4). Thus, the J3 primer was selected for use in this study. All PCR cycles and conditions for the J3 primer were as described previously (4), with minor modifications. Each 25- μ l total volume of mixture contained 4 μ l of genomic DNA (25 ng/ μ l), 13.6 μ l of sterile distilled water, 2.5 μ l of DMSO, 0.4 μ l of bovine serum albumin (pH 8.8), 2 μ l of 5 $\times$ reaction buffer stock solution (83 mM ammonium sulfate [pH 8.0], 335 mM Tris-HCl, 33.5 mM magnesium chloride, 33.5 μ M EDTA, and 150 mM β -mercaptoethanol), 2 μ l of 2 μ M primer, and 0.5 μ l of *Taq* DNA polymerase. Each reaction was carried out in PTC-100 Peltier Thermal cycler (MJ Research, Inc.) programmed for 7 min at 95°C for initial denaturation; 30 cycles consisting of 1 min at 94°C, 3 min at 68°C for annealing, and a 3-min extension at 68°C; with a final extension cycle at 65°C for 15 min followed by 20 h at 4°C for incubation.

Gel electrophoresis for rep-PCR and IS-PCR products. Approximately, 15 μ l of each PCR product was separated in 1% agarose gel in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). Electrophoresis of gels was conducted for 12 h at 115 V and stained with ethidium bromide for 15 min. The stained gel was photographed

in a Fluorochem 2200 Image system, (Alpha Innotech Corp., San Leandro, CA).

Fingerprinting data analysis. To determine the genetic diversity of *X. translucens* pv. *undulosa*, the strains were grouped into five populations according to the location of the collection. These locations and strains were Carrington ($n = 36$), Casselton ($n = 23$), Langdon ($n = 63$), Lisbon ($n = 50$), and Prosper ($n = 54$). Fingerprints for each strain were first obtained individually using BOX, ERIC, and J3 primers alone and then grouped according to unique DNA banding patterns. Both rep-PCR and IS-PCR techniques were used for all strains. For each PCR technique, representative strains of each haplotype were added in the same agarose gel to confirm band positions. The combined rep-PCR and IS-PCR data were used to identify putative haplotypes and the results were repeated to confirm the proposed haplotypes. Binary data were prepared in Microsoft Office Excel (version 2007) based on 1 for presence and 0 for absence of the amplified band. The molecular sizes for DNA bands for each strain were formatted in the GENALEX 6 (46). Haplotypes were constructed for each strain. Strains with the same haplotypes were considered clones. All of the analyses were done after clone correction. The software POPGENE version 1.32 was used to estimate the total number of alleles, gene diversity (H), and genetic distance (D) for each population or location (62). Genetic diversity across locations within each location was estimated using the Nei's unbiased gene diversity (43). Multilocus linkage disequilibrium (LD) was assessed using the index of association ($\bar{r}_d$) parameter. The $\bar{r}_d$ value for each location was estimated with the program MULTILOCUS version 1.3 (5). A corrected standardized fixation index (G'_{ST}) was calculated to estimate the pairwise measures of genetic differentiation of *X. translucens* pv. *undulosa* populations using GenoDive 2.0 Beta version software (24,36). Analysis of molecular variance (AMOVA), which partitions genetic variation within and among populations or locations, was performed using the Arlequin 3.1 software (19,20). Variance components and F_{ST} values were tested by 1,000 permutations of haplotypes among strains to obtain significance levels (19).

16S rDNA sequence analysis. To determine genetic relationships among strains from wheat with the other pathovars or strains of *Xanthomonas*, 35 strains representing the *X. translucens* pv. *undulosa* population from the five locations in North Dakota were selected to represent $\approx 15\%$ of the total collection. These strains were chosen for 16S rDNA sequence analysis because they were highly aggressive and genetically highly diverse based on the results of pathogenicity tests and rep-PCR and IS-PCR techniques. *X. translucens* pathovar reference strains or DNA kindly provided by Dr. David J. Norman, University of Florida, United States and Dr. Claude Bragard, Unité de phytopathologie, Université catholique de Louvain, Louvain-la-Neuve, Belgium were included and sequenced. These strains were *X. translucens* pv. *poae* American Type Culture Collection (ATCC) 33804, *X. translucens* pv. *undulosa* LMG 8282, *Xanthomonas* sp. X1553, *X. translucens* pv. *arrhenatheri* ATCC 33803, *X. translucens* pv. *cerealis* LMG 7393, *X. translucens* pv. *translucens* 1 LMG 5263+1, *X. translucens* pv. *translucens* 2 ATCC 10771, and *X. translucens* pv. *hordei* LMG 8281.

Genomic DNA of each strain was amplified with the 27F (5'-AGAGTTTGTATCATGGCTCAG-3') and 1378R (5'-CGGTGTGTACAAGGCCCGGAACG-3') primers (25,27). Each reaction (total volume: 25 μ l) contained 13 μ l of sterile deionized water, 1 μ l of genomic DNA (25 ng/ μ l), 4 μ l of 5 $\times$ *Taq* polymerase buffer, 2 μ l of 25 mM $MgCl_2$, 0.5 μ l of 10 mM dNTP mix, 2 μ l of 4 μ M each 27F and 1378R primer, and 0.5 μ l of *Taq* polymerase (5 units/ μ l). Each PCR was carried out in a PTC-100 Peltier Thermal cycler (MJ Research, Inc.) programmed for 15 min at 95°C for initial denaturation; 30 cycles consisting of 30 s at 95°C, 60 s at 48°C, and 2 min at 72°C; followed by a final 5-min extension at 72°C. The amplified PCR products (≈ 5 μ l) were run

in 2% (wt/vol) agarose gel (Sigma-Aldrich, Inc., St. Louis) in 0.5× TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA) for PCR confirmation. After confirmation, the remaining amplified PCR products were shipped to the Molecular Cloning Laboratories (MCLAB, San Francisco) for sequencing of the 16S rDNA region in both sense and anti-sense directions.

The 16S rDNA sequences of *X. translucens* pv. *undulosa* strains derived in this study were compared with the 16S rDNA sequences of the other closely related pathovars and type strains of *Xanthomonas* spp., as described previously by Hauben et al. (23). The 16S rDNA sequences of the *Xanthomonas* spp. were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>). According to Hauben et al. (23), the *Xanthomonas* type strain EMBL accession numbers were as follows: *X. populi* LMG 5743 (X95922), *X. fragariae* LMG 708 (X95920), *X. hortorum* pv. *hederiae* LMG 733 (Y10759), *X. arboricola* pv. *juglandis* LMG 747 (Y10757), *X. cassavae* LMG 673 (Y10762), *X. codiae* LMG 8678 (Y10765), *X. bromi* LMG 947 (Y10764), *X. cucurbitae* LMG 690 (Y10760), *X. axonopodis* pv. *axonomopodis* LMG 538 (X95919), *X. oryzae* pv. *oryzae* LMG 5047 (X95921), *X. vasicola* pv. *holcicola* LMG 736 (Y10755), *X. pisi* LMG 847 (Y10758), *X. melonis* LMG 8670 (Y10756), *X. translucens* pv. *translucens* LMG 876 (X99299), *X. sacchari* LMG 471 (Y10766), and *X. albilineans* LMG 494 (X95918).

The 16S rDNA sequences of 35 strains of *X. translucens* pv. *undulosa* from North Dakota (accession numbers JN869465 to JN869509), *X. translucens* pathovar reference strains, and *Xanthomonas* type strains downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) were compiled first in FASTA format and then aligned using the ClustalX (V1.83) program. Each aligned sequence was trimmed at the same length, which was an ≈1.11-kb fragment. The trimmed sequences were realigned and the final alignment was converted to MEGA format for analysis. A neighbor-joining tree was constructed with the software MEGA4 that uses pairwise comparisons of 16S rDNA sequences of all of the tested *Xanthomonas* strains. Analysis of reliability was subjected to a bootstrap test with 1,000 replicates.

RESULTS

Pathogenicity, biochemical, and physiological tests. Generally, *X. translucens* pv. *undulosa* induced compatible reactions on both wheat and barley, whereas *X. translucens* pv. *translucens* induced compatible reactions only on barley (10,11). In this study, the induction of water-soaked lesions was considered a compatible reaction for the wheat-*X. translucens* pv. *undulosa* interaction. All 226 strains of *X. translucens* pv. *undulosa* were pathogenic on wheat line 'ND495' and barley 'Robust' (Fig. 2) and were able to develop small water-soaked, chlorotic lesions at the infiltrated sites after infiltration. Symptoms induced by the strains were similar to those produced by *X. translucens* pv. *undulosa* reference strains (LMG 885 and LMG 886). Leaves infiltrated with sterile distilled water did not produce any symptom on flag leaves of barley and wheat. In general, wheat strains were more aggressive than barley strains (Fig. 2).

All 29 strains tested formed round, bright yellow colonies on PSA and were gram negative. The strains were strict aerobes, oxidase negative, catalase positive, and did not hydrolyze starch. A few strains liquefied gelatin and grew on 1% sodium chloride but none of the tested strains grew on 5% sodium chloride (data not shown).

Pathogenic variation among strains of *X. translucens* pv. *undulosa*. Bacterial strains varied greatly in aggressiveness on the 12 wheat cultivars. All strains were highly aggressive on the susceptible wheat line 'ND495'. In contrast, ≈10% of the strains tested were aggressive on 'Mochis' wheat (Table 2). Analysis of variance from nonparametric analysis revealed significant differences among wheat cultivars ($df = 11$, F value = 116.1) and among

bacterial strains ($df = 28$, F value = 31.36) (data not shown). In addition, interactions between wheat cultivars and bacterial strains ($df = 308$, F value = 15.19) were highly significant ($P < 0.001$), suggesting that race specificity may exist in *X. translucens* pv. *undulosa*. Among 29 strains evaluated, each strain was represented by a single pathotype (Table 1). Four strains among them—BLS-CS2 (pathotype 26 and haplotype 155), BLS-P13 (pathotype 27 and haplotype 124), BLS-CS40 (pathotype 28 and haplotype 123), and BLS-P3 (pathotype 29 and haplotype 34)—were more aggressive on the wheat cultivars than the other strains tested (Table 2).

Six wheat strains induced symptoms on both wheat and barley (Table 3). Four barley strains also were aggressive on wheat but these strains were less aggressive than wheat strains (Table 3). For example, two wheat strains (BLS-CS42 and BLS-CS43) were nonpathogenic on rye and oat (median disease score = 0) whereas one wheat strain (BLS-CS40) and one barley strain (BLS-CS57) were more aggressive on triticale (median disease score = 3). The remaining strains did not cause symptoms on triticale (Table 3).

Analysis of genetic diversity (H). In total, 47 polymorphic DNA patterns were generated by ERIC (15 bands), BOX (20 bands), and J3 (12 bands) primers (Fig. 3). The combined rep-PCR and IS-PCR techniques were used for the 226 strains and resulted in 213 distinct haplotypes (Supplementary Table 1). Some haplotypes contained two strains and were detected from a single location. For examples, haplotype 67 (BLS-CR28 and BLS-CR33), haplotype 27 (BLS-CR17 and BLS-CR18), and haplotype 152 (BLS-CR5 and BLS-CR12) from Carrington; haplotype 202 (BLS-P31 and BLS-P48), haplotype 39 (BLS-P30 and BLS-P49), haplotype 100 (BLS-P9 and BLS-P11), and haplotype 172 (BLS-P15 and BLS-P26) from Prosper; haplotype 185 (BLS-LB1 and BLS-LB12) and haplotype 36 (BLS-LB24 and BLS-LB29) from Lisbon; and haplotype 164 (BLS-LG47 and BLS-LG49) from Langdon each had two strains. Similar haplotypes were found in more than one location. For examples, haplotype 206 (BLS-LG22 and BLS-P52) was found in both the Langdon and Prosper populations. Haplotype 15 (BLS-CR15 and BLS-LB38) was found in both Carrington and Lisbon. Similarly, haplotype 158 (BLS-CR22 and BLS-LG23) also was detected in Carrington and Langdon populations. In total, 13 strains appeared to be clones and were excluded from data analysis. The Prosper population had the highest H (0.24) while the Langdon population had the lowest (0.19) (Table 4). Overall, H values for five populations ranged from 0.19 to 0.24 (Table 4).

Gametic disequilibrium. LD , measured by $\bar{r}_d$ values, was 0.017 to 0.054 and differed significantly ($P \leq 0.001$) from zero (Table 4). The Carrington population had the highest $\bar{r}_d$ (0.054) while the Lisbon population had the lowest (0.017) (Table 4).

Population differentiation (G''_{ST}) and genetic distance (D). The G''_{ST} values for pairwise comparisons five populations were 0.014 to 0.218 (Table 5). The highest G''_{ST} value (0.218) was found between Carrington and Langdon populations, which also was significant ($P \leq 0.001$). However, the lowest G''_{ST} value (0.01) was observed between the Carrington and Prosper populations (Table 5). The G''_{ST} values between the Carrington and Casselton populations, Carrington and Lisbon populations, Casselton and Lisbon populations, Lisbon and Langdon populations, and Langdon and Prosper populations were significant only at $P \leq 0.05$. G''_{ST} values between the Carrington and Prosper populations, Casselton and Langdon populations, Casselton and Prosper populations, and Lisbon and Prosper populations were not significant (Table 5).

D values calculated between pairwise comparisons among five populations were 0.01 to 0.14 (Table 5). The highest D (0.14) was detected between the Lisbon and Casselton populations and Langdon and Carrington populations. In contrast, the lowest D (0.01) was observed between the Casselton and Carrington populations (Table 5). AMOVA revealed low (≈8%) genetic differen-

tiation among locations while the majority of the genetic variation ($\approx 92\%$) occurred within locations (Table 6). In addition, significant genetic differentiation ($F_{ST} = 0.095$, $P \leq 0.001$) also occurred within and among populations (Table 6).

16S rDNA sequence analysis. The mean level of sequence similarity among these strains was 99.5%. Similarly, the other seven pathovars of *X. translucens* had exactly the same sequence similarity. When the 35 strains from North Dakota were compared with the 16 pathovars of *Xanthomonas*, the mean level of sequence similarity was 99.4%. In addition, the highest nucleotide difference was observed in *X. fragariae* (LMG 708), with a 26-nucleotide difference (data not shown). The dendrogram generated from the neighbor-joining clustering method showed two clusters for all of the tested *Xanthomonas* spp. One cluster had all strains of *X. translucens* pv. *undulosa* from North Dakota and reference strains of *X. translucens* pathovars while the

other cluster contained different pathovars of *Xanthomonas* analyzed (Fig. 4). The sequence similarity within cluster 1 was between 99.5 and 100% and the nucleotide difference was 0 to 1 (data not shown). The sequence similarities within cluster 2 were 97.9 to 99.9% and the nucleotide difference was 1 to 26 (data not shown).

DISCUSSION

The pathogenic and genetic diversity of *X. translucens* pv. *undulosa* strains associated with recent BLS outbreaks in North Dakota were analyzed with pathogenicity, standard bacteriological tests, aggressive profiles on different wheat cultivars and cereal host plants, 16S rDNA sequences analysis, and rep-PCR and IS-PCR techniques. These results suggested that *X. translucens* pv. *undulosa* populations examined in this study are

Fig. 2. Frequency distribution of mean disease scores on barley and wheat cultivars for 226 strains of *Xanthomonas translucens* pv. *undulosa* from Carrington ($n = 36$), Casselton ($n = 23$), Lisbon ($n = 50$), Langdon ($n = 63$), and Prosper ($n = 54$). To evaluate aggressiveness of each strain, disease scores were recorded visually on the flag leaf of each plant between 7 and 10 days after infiltration using a 0-to-6 rating scale (39), where 0 = no visible symptoms, 1 = chlorosis without water-soaked lesions, 2 = <10% water soaking, 3 = 10 to 30% water soaking, 4 = 31 to 70% water soaking, 5 = 71 to 100% water soaking, and 6 = water soaking extending beyond the infiltrated area. Mean value ≤ 2 was resistant and > 2 was susceptible.

composed of pathologically and genetically diverse strains. In the previous studies, the genetically diverse strains within a pathovar of *Xanthomonas* were identified by several conventional and molecular techniques (6,7,11,12,28,29,33,34,45,49). Our results also indicate that *X. translucens* pv. *undulosa* strains are capable of infecting different cereal host plants such as wheat, barley, and triticale. The finding that a high level of pathogenic and genetic

variation exists in *X. translucens* pv. *undulosa* in North Dakota may provide a basis for selecting strains that are useful for identifying and characterizing new resistance genes in wheat. To our knowledge, this is the first study to demonstrate a high genetic diversity of *X. translucens* pv. *undulosa* from wheat in the United States using genotyping techniques targeting dispersed elements in the bacterial genomes.

TABLE 3. Nonparametric analysis criteria such as median disease scores, relative treatment effects (p_{ij}), and 95% confidence intervals (CI) relative treatment effects (p_{ij}) were calculated from ordinal data and used to characterize aggressiveness of 10 randomly selected strains of *Xanthomonas translucens* pv. *undulosa* on barley, oat, rye, triticale, and wheat

Host ^a	Strain ^b	Median ^c	95% CI for p_{ij} ^d
Barley ('Robust')	BLS-CS49	3 (S)	0.281, 0.619
	BLS-CS48	4 (S)	0.288, 0.731
	BLS-CS57	3 (S)	0.414, 0.688
	BLS-CS56	4 (S)	0.457, 0.730
	BLS-CS55	3 (S)	0.296, 0.574
	BLS-CS54	3 (S)	0.344, 0.584
	BLS-CS42	3 (S)	0.272, 0.506
	BLS-CS43	3 (S)	0.414, 0.688
	BLS-CS40	4 (S)	0.576, 0.847
	BLS-CS41	3 (S)	0.196, 0.473
Oat ('Ajay')	BLS-CS49	2 (R)	0.699, 0.834
	BLS-CS48	1 (R)	0.431, 0.638
	BLS-CS57	2 (R)	0.673, 0.764
	BLS-CS56	2 (R)	0.684, 0.802
	BLS-CS55	2 (R)	0.565, 0.804
	BLS-CS54	1 (R)	0.211, 0.442
	BLS-CS42	0 (R)	0.107, 0.168
	BLS-CS43	0 (R)	0.135, 0.269
	BLS-CS40	1 (R)	0.288, 0.593
	BLS-CS41	1 (R)	0.314, 0.582
Rye ('Ac Remington')	BLS-CS49	0 (R)	0.126, 0.248
	BLS-CS48	2 (R)	0.431, 0.816
	BLS-CS57	0 (R)	0.197, 0.439
	BLS-CS56	2 (R)	0.518, 0.707
	BLS-CS55	1 (R)	0.273, 0.586
	BLS-CS54	1 (R)	0.452, 0.648
	BLS-CS42	0 (R)	0.144, 0.295
	BLS-CS43	2 (R)	0.565, 0.765
	BLS-CS40	2 (R)	0.727, 0.872
	BLS-CS41	2 (R)	0.430, 0.712
Triticale ('PI 634537')	BLS-CS49	2 (R)	0.460, 0.680
	BLS-CS48	2 (R)	0.421, 0.665
	BLS-CS57	3 (S)	0.629, 0.826
	BLS-CS56	2 (R)	0.482, 0.649
	BLS-CS55	0 (R)	0.150, 0.364
	BLS-CS54	1 (R)	0.178, 0.534
	BLS-CS42	0 (R)	0.080, 0.130
	BLS-CS43	2 (R)	0.369, 0.675
	BLS-CS40	3 (S)	0.633, 0.845
	BLS-CS41	2 (R)	0.463, 0.746
Wheat ('ND495')	BLS-CS49	4 (S)	0.436, 0.834
	BLS-CS48	4 (S)	0.439, 0.740
	BLS-CS57	3 (S)	0.265, 0.507
	BLS-CS56	3 (S)	0.299, 0.566
	BLS-CS55	3 (S)	0.228, 0.499
	BLS-CS54	3 (S)	0.299, 0.566
	BLS-CS42	3 (S)	0.119, 0.391
	BLS-CS43	4 (S)	0.563, 0.762
	BLS-CS40	4 (S)	0.433, 0.701
	BLS-CS41	4 (S)	0.563, 0.762

^a Two experiments were conducted in the greenhouse. In each experiment, disease scores of the nine infiltrated areas on flag leaves (one site on each flag leaf) were averaged. Cereal host plants were arranged in randomized complete block design with three replications. Each cone was considered as replication and contained three plants.

^b Bacterial strains were collected from barley and wheat in Casselton in North Dakota were tested. These included BLS-CS49 (durum wheat), BLS-CS48 (spring wheat), BLS-CS57 (barley), BLS-CS56 (barley), BLS-CS55 (barley), BLS-CS54 (barley), BLS-CS42 (spring wheat), BLS-CS43 (spring wheat breeding line-1032), BLS-CS40 (spring wheat), and BLS-CS41 (spring wheat breeding line 313).

^c Disease scores were recorded visually on the flag leaf of each plant between 7 and 10 days after infiltration on an ordinal 0-to-6 rating scale (39), where 0 = no visible symptoms, 1 = chlorosis without water-soaked lesions, 2 = <10% water soaking, 3 = 10 to 30% water soaking, 4 = 31 to 70% water soaking, 5 = 71 to 100% water soaking, and 6 = water soaking extending beyond the infiltrated area. A nonparametric analysis (13,52) was used to calculate median disease scores from ordinal data and used to characterize aggressiveness of strains in each wheat cultivar tested. For example, median value 1 indicates strains are least aggressive while median disease score 4 refers to strains are highly aggressive on a particular wheat cultivar. Median, p_{ij} and CI values were estimated (13,52,55). Median value ≤ 2 was resistant and >2 was susceptible. Letter in parenthesis after median value represent R = resistant and S = susceptible reaction.

^d First and second values in the parentheses indicate lower limit and upper limit at 95% CI, respectively (13).

Inoculation of the 226 strains confirmed that these strains were able to infect a wheat cultivar similar to infection by *X. translucens* pv. *undulosa* reference strains (LMG 885 and LMG 886). This finding was further supported by the observation that the 10 randomly selected strains of *X. translucens* pv. *undulosa* were able to cause disease symptoms on wheat and barley but their aggressiveness varied according to the cereal host plants tested. For example, strains BLS-CS42 and BLS-CS43 were non-pathogenic on rye and oat; however, two strains, BLS-CS40 and BLS-CS47, were more aggressive on triticale. Although pathogenicity tests support the hypothesis that *X. translucens* pv. *undulosa* might infect other cereal hosts in addition to wheat and barley, the relatively few samples evaluated in this study were not adequate to draw definite conclusions as to whether or not *X. translucens* pv. *undulosa* has broad host range. Testing additional strains is necessary to fully understand the pathogenic diversity of *X. translucens* pv. *undulosa* on different host plants.

Little previous research has examined the pathogenic variability of *X. translucens* pv. *undulosa*. Although variations in aggressiveness among strains from various geographic areas differed on

TABLE 4. Genetic diversity of *Xanthomonas translucens* pv. *undulosa* populations from five locations in North Dakota revealed by the combined data on repetitive sequence-based polymerase chain reaction (PCR) with the BOX and enterobacterial repetitive intergenic consensus primers and an insertion sequence-based PCR with the J3 primer

Population	Sample size	Clone-corrected data	Gene diversity ^a	$\bar{r}_d$ ^b
Carrington	36	32	0.22	0.054**
Casselton	23	23	0.21	0.065**
Langdon	63	60	0.19	0.047**
Lisbon	50	48	0.20	0.017**
Prosper	54	50	0.24	0.028**
Total	226	213	...	...

^a Gene diversity (42,43) within locations was calculated from clone-corrected data.

^b The standardized index of association ($\bar{r}_d$) statistics were estimated for each population (5). The significance of $\bar{r}_d$ was tested with 1,000 randomizations of the data by comparing the observed value to that expected under the null hypothesis of $\bar{r}_d = 0$. The null hypothesis of multilocus linkage disequilibrium was rejected if $P < 0.001$ or 0.05; ** indicates significant at $P = 0.001$.

Fig. 3. Agarose gels showing banding patterns from representative strains of *Xanthomonas translucens* pv. *undulosa* from North Dakota generated by three separate polymerase chain reactions (PCRs) using repetitive sequence-based PCR with the BOX and enterobacterial repetitive intergenic consensus (ERIC) primers (16,33,58) and an insertion element, IS111, from *X. oryzae* pv. *oryzae* (insertion sequence-based PCR) generated with the J3 primer (3,4,28,29). The naming for *X. translucens* pv. *undulosa* strains are as follows: BLS = bacterial leaf streak, which is followed by the location from which the strains were collected: CR = Carrington, CS = Casselton, LB = Lisbon, LG = Langdon, and P = Prosper. Strains used for ERIC and BOX primers were BLS-CS40 (haplotype 123), BLS-CS42 (haplotype 47), BLS-CS45 (haplotype 45), BLS-CS47 (haplotype 205), and BLS-CS51 (haplotype 46). Strains used for the J3 primer were BLS-CR1 (haplotype 208), BLS-LB10 (haplotype 75), BLS-LB1 (haplotype 185), BLS-LG1 (haplotype 18), BLS-P1 (haplotype 207), and BLS-P2 (haplotype 214).

wheat cultivars tested, no pathogenic races were previously found in this pathogen due to lack of significant wheat cultivar–bacterial strain interactions (39). Milus and Chalkly (39) further reported that no evidence for detecting races was found due to quantitative resistance to *X. translucens* pv. *undulosa* among 19 wheat cultivars tested. In the present study, a limited number of highly aggressiveness strains ($n = 29$) were evaluated on a set of 12 wheat cultivars (2,18,35,40,55) and each strain was represented by a unique pathotype. The genetic mechanism causing this variability is unknown. In recent BLS outbreaks, winter, spring, and durum wheat cultivars presently grown in North Dakota were severely affected by BLS. We hypothesize that the susceptible wheat cultivars may provide favorable environments for primary inoculum production, which may undergo mutation and create pathogenic diversity in this pathogen.

Wheat ‘Mochis’, ‘Pavon’, ‘PI 345476’, ‘PI 326301’, ‘PI 338387’, and ‘PI 351315’ exhibited differential responses to several pathotypes of *X. translucens* pv. *undulosa* and could be good candidates for “differential cultivars”. Two wheat cultivars (‘Mochis’ and ‘Pavon’) were highly resistant to *X. translucens* pv. *undulosa* in previous studies (18,35); however, these cultivars were susceptible to several strains tested in this study. The presence of the highly aggressive pathotypes of *X. translucens* pv. *undulosa* identified in the present study raises the question of whether or not these wheat cultivars (‘Mochis’ and ‘Pavon’) could be useful sources of resistance to multiple pathotypes of *X. translucens* pv. *undulosa* in wheat breeding programs. More importantly, the finding of significant wheat cultivar–bacterial strain interactions demonstrated in this study emphasizes the need to examine cultivar–strain interaction with more wheat cultivars. Investigating pathogenic variations with respect to diverse differential wheat cultivars and including additional strains from the Upper Midwest region of the United States of America will provide a clearer picture of pathogenic specialization in *X. translucens* pv. *undulosa*.

Previous studies on membrane protein analysis, fatty acid profiling, and 16S rDNA PCR-RFLP analysis have shown a high similarity among strains or pathovars of *Xanthomonas* (44,47, 61). The rDNA genes such as 16S, 23S, and 5S are highly conserved at the genus and species levels and, thus, are believed to be useful tools for grouping bacteria at taxonomic levels (6,26). Comparison of sequence variation in 16S rDNA region among 38 strains of *X. translucens* pv. *undulosa* from five locations in North Dakota, 8 reference strains of *X. translucens* pathovars, and 16 *Xanthomonas* type strains showed >98.5% rDNA sequence identity and the maximum number of base differences of 26. In addition, partial 16S rDNA sequences were identical among

strains of *X. translucens* pv. *undulosa* studied, and our finding also supports previous studies (23,41) that this technique was less effective in grouping the *Xanthomonas* strains at the pathovar level.

Limited information describing the genetic diversity in *X. translucens* pv. *undulosa* is available compared with such information for closely related pathovars of *X. translucens*. Using AFLP markers, Kolliker et al. (27) estimated the genetic diversity in *X. translucens* pv. *graminis* and reported significant effects based on the geographic location where samples were collected. Bragard et al. (10) analyzed the 68 strains of different pathovars of *X. translucens*, including *X. translucens* pv. *undulosa*, using pathological and molecular approaches. In addition, genetic diversity of different pathovars of *X. campestris* strains using probes containing 23S rDNA or pBSF2 resulted in clustering of strains for *X. translucens* pv. *cerealis*, *X. translucens* pv. *hordei*, and *X. translucens* pv. *undulosa* into different groups (11). Rep-PCR genomic fingerprint profiles were performed to compare 33 strains pathogenic to asparagus and 61 *X. translucens* strains pathogenic to cereals and grasses (49). In addition, all 33 strains from asparagus were identified as *X. translucens* pv. *undulosa*, and induced water-soaking symptoms in susceptible wheat ‘Florida 302’ (49). In the present study, we used rep-PCR with ERIC and BOXAIR primers (16,58) and IS-PCR with J3 primer (3,4) to analyze five populations of *X. translucens* pv. *undulosa* sampled in North Dakota. These genotyping techniques revealed a high level of molecular polymorphisms among strains of *X. translucens* pv. *undulosa*. Similarly, a high genetic diversity has also been found in *Ralstonia solanacearum* in the Philippines (32) and in *X. oryzae* pv. *oryzae* in Nepal (3) and the Philippines (57) using rep-PCR and IS-PCR techniques. Although *X. translucens* pv. *undulosa* strains are highly diverse, no correlation between pathotypes and haplotypes was found.

This pathogen has been reported as seedborne (21,50). The majority of the wheat seed lots used for planting in North Dakota originated from different sources such as foundation seed, breeder seed, and wheat germplasm exchange from other states or countries without seed certification for BLS. It is likely that these seed lots could be potential sources of bacterial transmission in the field. The combined rep-PCR and IS-PCR techniques used in this study allowed us to delineate strains of *X. translucens* pv. *undulosa* from North Dakota into distinct haplotypes. We hypothesize that these haplotypes could result in association with certain geographic locations in North Dakota, and the occurrence of severe BLS outbreaks in the region could be due to the presence of the same haplotype in different locations. The presence of unique haplotypes in certain locations in North Dakota indicates

TABLE 5. Estimates of population genetic differentiation (G'_{ST}) (above diagonal) and genetic distance (D) (below diagonal) among pairs of populations of *Xanthomonas translucens* pv. *undulosa* collected in North Dakota^a

Population	Carrington	Casselton	Lisbon	Langdon	Prosper
Carrington	...	0.146*	0.092*	0.218**	0.011 ns
Casselton	0.01	...	0.106*	0.014 ns	0.048 ns
Lisbon	0.13	0.14	...	0.101*	0.030 ns
Langdon	0.14	0.04	0.11	...	0.127*
Prosper	0.07	0.08	0.07	0.06	...

^a G'_{ST} = corrected standardized fixation index (24,36); ** and * indicate significant at $P \leq 0.001$ and ≤ 0.05 , respectively; ns = nonsignificant. Nei’s unbiased D was analyzed from clone-corrected data (42,43).

TABLE 6. Analysis of molecular variance (AMOVA) among five populations of *Xanthomonas translucens* pv. *undulosa* from North Dakota

AMOVA ^a	df	Estimated variance	Variation (%)	F_{ST} ^b	P value
Among five populations	4	1.36	8.3	0.095	0.001
Individuals within population	221	5.07	91.7	...	...

^a Genetic variation was grouped among populations and individuals within five populations from Carrington, Casselton, Lisbon, Langdon, and Prosper.

^b F_{ST} = fixation index and the significance was determined by 1,000 random permutations. Clone-corrected data were used for AMOVA.

Fig. 4. Neighbor-joining trees based on 16S rDNA sequence alignment of 35 *Xanthomonas translucens* pv. *undulosa* strains from five locations (Carrington, Casselton, Lisbon, Langdon, and Prosper) in North Dakota, other reference strains of *X. translucens* pathovars, and *Xanthomonas* pathovars (23). The naming for *X. translucens* pv. *undulosa* strains from North Dakota is as follows: BLS = bacterial leaf streak, which is followed by the location of its collection: CR = Carrington, CS = Casselton, LB = Lisbon, LG = Langdon, and P = Prosper. The eight *X. translucens* pathovars reference strains were *X. translucens* pv. *poae* (ATCC 33804), *X. translucens* pv. *undulosa* (LMG 8282), *Xanthomonas* sp. (X1553), *X. translucens* pv. *arrhenatheri* (ATCC 33803), *X. translucens* pv. *cerealis* (LMG 7393), *X. translucens* pv. *translucens* 1 (LMG 5263+1), *X. translucens* pv. *translucens* 2 (ATCC 10771), and *X. translucens* pv. *hordei* (LMG 8281). The 16 *Xanthomonas* type strains were downloaded from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/BLAST>) with accession numbers in parentheses as follows: *X. populi* LMG 5743 (X95922), *X. fragariae* LMG 708 (X95920), *X. hortorum* pv. *hederarum* LMG 733 (Y10759), *X. arboricola* pv. *juglandis* LMG 747 (Y10757), *X. cassavae* LMG 673 (Y10762), *X. codiae* LMG 8678 (Y10765), *X. bromi* LMG 947 (Y10764), *X. cucurbitae* LMG 690 (Y10760), *X. axonopodis* pv. *axonopodis* LMG 538 (X95919), *X. oryzae* pv. *oryzae* LMG 5047 (X95921), *X. vasicola* pv. *holcicola* LMG 736 (Y10755), *X. pisi* LMG 847 (Y10758), *X. melonis* LMG 8670 (Y10756), *X. translucens* pv. *translucens* LMG 876 (X99299), *X. sacchari* LMG 471 (Y10766), and *X. albilineans* LMG 494 (X95918). Numbers on branches refer to bootstrap values (based on 1,000 replicates). Scale bar represents 0.002 nucleotide changes.

the possibility of introducing new strains via short-distance migration from neighboring states or via long-distance introduction from wheat seed from other states or countries. Thus, one possible reason for significant differentiation of *X. translucens* pv. *undulosa* populations in some locations also could be the development of different haplotypes propagating as primary inoculum sources from infected seed or other sources.

We observed significant *LD* for *X. translucens* pv. *undulosa* populations from five locations in North Dakota analyzed. Similar findings also were reported when the haploid fungus *Phaeosphaeria nodorum* (causal agent of Stagonospora nodorum blotch of wheat) populations from spring wheat in North Dakota were analyzed using microsatellite markers (1). The reason for the significant disequilibrium in *X. translucens* pv. *undulosa* populations is unknown. However, several factors such as migration of individuals, sampling strategies, and lumping of strains from different locations may be responsible. Although *X. translucens* pv. *undulosa* populations appeared to be highly diverse, one possible explanation for observed significant *LD* is the low level of horizontal gene transfer and recombination which probably occurs within fields.

In conclusion, the pathological and molecular tools used in this study offer a reliable approach for analyzing genetic diversity of strains of *X. translucens* pv. *undulosa* associated with the recent BLS outbreaks at a local or regional level in North Dakota. Although some winter wheat cultivars appeared to be resistant to *X. translucens* pv. *undulosa* (2), the high genetic diversity in *X. translucens* pv. *undulosa* strains found in this study could influence the durability and effectiveness of resistances in different geographic areas when widely deployed. Future studies are necessary to collect additional bacterial strains across a broader geographic scale to investigate pathogenic variability and population genetic structure, and to determine the usefulness of specific strains for identifying new sources of resistance to *X. translucens* pv. *undulosa*. A fundamental knowledge of the interaction between wheat cultivars and bacterial strains should aid in the breeding of commercially acceptable wheat cultivars with durable resistance to *X. translucens* pv. *undulosa*. The draft sequenced genome of *X. translucens* pv. *undulosa* strains is available. The development of PCR-based diagnostic markers for tracking of the pathogen in seed lots and identifying virulence factors such as the transcription activator-like effectors involved in pathogenesis are in progress.

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