Recognition and Detection in Seed of the *Xanthomonas* Pathogens That Cause Cereal Leaf Streak Using rDNA Spacer Sequences and Polymerase Chain Reaction

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ABSTRACT

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Two short DNA sequences, situated in the spacer segment between the 16S and 23S rRNA genes and flanking an alanine-tRNA gene, varied among four xanthomonads. Polymerase chain reaction (PCR) primers designed to exploit this variability amplified a 139-bp fragment from strains of five leaf streak pathogens of cereals, *Xanthomonas campestris* pvs. *cerealis*, *hordei*, *secalis*, *translucens*, and *undulosa*, and five *X. campestris* pathovars pathogenic on forage grasses, *X. campestris* pvs. *arrhenatheri*, *graminis*, *phlei*, *phleipratensis*, and *poae*. These primers did not amplify DNA from 52 other bacteria, including other xanthomonads. PCR specificity was largely determined by one primer site, which is located in a 57-bp sequence present only in the rDNA spacer of DNA homology group I strains. Amplification of extracts of 2 seed lots known to be contaminated with a translucent leaf streak pathogen produced the specific 139-bp PCR fragment; 4 of 27 additional seed lots with unknown levels of leaf streak pathogens were positive for this amplified fragment. The assays proved to be fast and relatively sensitive (2×10^3 CFU/g of seed), indicating the technique might be useful for detecting pathogens in seed.

Translucent leaf streak or leaf stripe disease of cereals (6) is largely absent in Western Europe, although it is endemic in the United States, Canada, Central America, and most other countries in which the host cereals are grown. Translucent leaf streak, called black chaff when found on cereal kernels, is most destructive under warm and humid conditions (11). The quarantine list of the European Plant Protection Organization includes a group of bacterial pathogens that cause "translucent leaf stripe of cereals and related grasses" (33) that are primarily seed transmitted (37). The strains belonging to these cereal leaf streak (CLS) pathogens have been assigned to *Xanthomonas campestris* pvs. *cerealis*, *hordei*, *secalis*, *translucens*, and *undulosa*, as described by Dye et al. (12). This classification was based on the host range specializations observed with these pathogens: *X. campestris* pv. *cerealis* contains the pathogens of various cereals and the grass *Bromus inermis*; *X. campestris* pv. *hordei* contains the barley pathogens; *X. campestris* pv. *secalis* contains the rye pathogens; *X. campestris* pv. *undulosa* contains the pathogens that cause black chaff on wheat and triticale; and *X. campestris* pv. *translucens* is described as pathogenic on several cereals, including barley, wheat, rye, and triticale. However, in phytopathological practice, a strict definition of this classification is not available because the host ranges of the pathovars often overlap, and variations in the host range of the strains within a pathovar can be observed (2,3,6). There is also no clear molecular basis for this pathovar differentiation within the group of CLS bacteria (4,22,41,44), although recent studies on the genomic fingerprinting of *X. campestris* pathovars indicate that genomic sequence variation exists between CLS bacteria (3, 39). The actual pathovar assignation of the CLS strains has not been confirmed by these techniques and should be reevaluated based on both pathogenicity and genomic typing experiments (3).

Currently, CLS bacteria are routinely detected by growth on a selective medium, which takes several days (37). However, the CLS bacteria surviving in association with the seed coat often recover slowly, and plates must be incubated for up to 1 week. Subsequently, a pathogenicity test is needed to discriminate them from possible saprophytic xanthomonads and from other seed-associated bacteria with yellow colonies. Immunological typing of the CLS bacteria has been described, but cross-reaction with different phytopathogens prevents unambiguous identification (2). In addition, different serotype subgroups have been observed for the CLS bacteria depending on the antibody used (21,36).

DNA-based discrimination and unambiguous detection of the *X. campestris* pathovars associated with *Gramineae* is difficult because these organisms are closely related genetically. No data are available on selective genes or traits that can be used for specific detection of CLS bacteria. Recently, Leite et al. (24) developed a polymerase chain reaction (PCR)-mediated method of recognition of pathogenic xanthomonads, based on specific *hrp*-located sequences, showing the conservation of the *hrp* sequences among a large range of *X. campestris* pathovars. However, *hrp* genes of xanthomonads pathogenic on *Gramineae* are of a different type and are not detected with the *hrp*-specific primers described. The few examples of phytobacterial differentiation using rDNA sequences have been limited to the recognition of 16S rDNA gene sequences. DeParasis and Roth (9) proposed the use of 16S rDNA oligonucleotide sequences as hybridization probes to discriminate the phytobacterial genera *Xanthomonas*, *Pseudomonas*, and *Erwinia*. However, for application in a bacterial detection procedure,

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TABLE 1. Bacteria used in the polymerase chain reaction (PCR) specificity test with primer pair T1 and T2

Strain	Number[a]	Origin[b]	PCR[c]
Cereal translucent leaf streak pathogens			
Xanthomonas campestris pvs.			
cerealis	880, 890, 881, 887	Canada	+
	7393, 679, 7392	U.S.	+
hordei	882, 884, 8278	Canada	+
	737	India	+
	879	Japan	+
secalis	883, 7507, 877, 7445	Canada	+
	866	India	+
translucens	5261, 876, 5259, 5260, 5263	U.S.	+
	875	Canada	+
	878	Kenya	+
undulosa	885, 892, 888, 8282	Canada	+
Other xanthomonads pathogenic on *Gramineae*			
X. campestris pvs.			
arrhenatheri	727, 590, 592	Switz.	+
graminis	713, 596, 598, 615	Belgium	+
	726, 7407, 7406	Switz.	+
	595	Norway	+
phlei	716, 717, 719, 720, 723	Belgium	+
poae	728, 594	Switz.	+
phleipratensis	843	U.S.	+
holcicola	736	N.Z.	–
vasculorum	901	Mauritius	–
X. albilineans	488	Sri Lanka	–
X. axonopodis	538	Colombia	–
X. oryzae pv. *oryzae*	5047	India	–
X. oryzae pv. *oryzicola*	797	Malaysia	–
X. campestris isolated from *Bromus* grass	947	France	–
	8269	N.Z.	–
Other pathogens of cereals			
Clavibacter tritici	3728	Egypt	–
Erwinia rhapontici	2642	France	–
	2687	U.K.	–
Pseudomonas syringae pv. *atrofaciens*	5095	N.Z.	–
Other phytobacteria			
X. campestris pvs.			
alfalfae	496, 497	Sudan	–
	495	India	–
	9330	U.S.	–
begoniae	7303	N.Z	–
campestris	568, 575	U.K.	–
citri	8650	India	–
cucurbitae	7479	U.S.	–
dieffenbachiae	695	Brazil	–
glycinis	712	Sudan	–
melonis	8670, 8671	Brazil	–
phaseoli	842	...	–
pisi	847	Japan	–
poinsettiicola	849	India	–
	8676	N.Z	–
vesicatoria	667	...	–
vignicola	828	Sudan	–
X. fragariae	704	N.Z.	–
X. maltophilia	957, 10857	Blood culture	–
Agrobacterium tumefaciens	167	Belgium	–
Erwinia amylovora	1877	Denmark	–
	1969	France	–
	2024	U.K.	–
	2078	Poland	–
E. carotovora subsp. *carotovora*	311*, 328*	Belgium	–
E. carotovora subsp. *atroseptica*	2369	...	–
	2386	U.K.	–
E. stewartii	2715	U.S.	–
	2716	...	–
Pantoea spp. (*E. herbicola*)	2569, 2570	U.S.	–
	2560	...	–
	2558	India	–
Pseudomonas solanacearum	6973	Uganda	–
P. syringae pvs.			
papulans	5076	Canada	–
syringae	5141	U.K.	–

[a] When not specified, the LMG (Laboratory of Microbiology, Gent, Belgium) number for bacterial strains is given. Strains marked * have a RPZBC (Research Station for Plant Pathology, Merelbeke, Belgium) number.

[b] ... = bacterial origin not mentioned in the culture collection catalogue. Switz. = Switzerland; N.Z. = New Zealand.

[c] + = production of the single 139-bp amplification fragment from lysed bacterial cells. – = no amplification product observed on agarose gel.

short probe sequences have serious drawbacks due to low specificity. On the other hand, PCR with the 16S rDNA primer sequence gives amplified DNA fragments of different sizes, leading to recognition and detection of xanthomonads (27). Protocols based on PCR technology and 16S rDNA sequences also were developed for recognition of *P. solanacearum* (38), *Xylella fastidiosa* (15), and plant-associated mycoplasmalike organisms (1,16,29).

The close phylogenetic relationship among CLS bacteria (22,44) makes the rDNA operon a useful target for the delineation of CLS-specific sequences. The primary structure of this part of the bacterial genome is constrained and controlled by high evolutionary and functional pressures (20) and consists of strongly conserved regions interspersed with regions with different degrees of variability (20,48). Primers to the conserved sequences can be used for amplification and sequence analysis of the variable rDNA fragments. In the field of phytopathology, other reports mention the use of PCR-amplified spacer DNA between 16S and 23S rRNA genes to fingerprint and identify closely related *Agrobacterium* strains (34), some fungal species and strains (30,32,49), and intraspecific subgroups of nematodes (46). To discriminate a *X. campestris* pathovar group or cluster, specific sequences must also be searched for in rDNA regions where high sequence variability is expected. In this paper, the ribosomal 16S-23S spacer region was screened for CLS-specific oligonucleotide sequences that could be used as primer sites in PCR-based detection of CLS bacteria. PCR detection of the translucent leaf streak pathogens would be useful in cereal seed tests, in which the speed and sensitivity of the procedure are important factors.

MATERIALS AND METHODS

Bacterial strains and growth. Bacterial strains obtained from the Belgian Coordinated Collections of Microorganisms, Laboratory of Microbiology, University of Gent (LMG), and from the collection of the Research Station for Plant Pathology, Merelbeke (RPZBC), Belgium, are listed in Table 1. Bacteria were grown in rich medium containing 5 g of yeast extract, 5 g of peptone, and 10 g of glucose per liter (YPG).

Total bacterial DNA preparations. A freshly grown bacterial colony was taken from a YPG-agar plate and cultured overnight in liquid YPG medium at 28°C on a rotary shaker (200 rpm). The culture (160 µl, 10^7 to 10^8 CFU/ml) was centrifuged in a microvial (5 min at 10,000 × *g*). The supernatant was removed, and the bacterial pellet was resuspended in 60 µl of proteinase K solution (50 µg/ml in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA). After treatment at 56°C for 15 min and at 80°C for 15 min and cooling on ice, the lysis was cleared by centrifugation at 13,000 × *g* for 5 min. The supernatant (5 µl) was run on an agarose gel to estimate the DNA concentration. According to the lysis efficiency of the different bacterial strains, a 10- to 100-fold dilution of the total bacterial DNA was made for use in the PCR reaction.

Xanthomonas bacteria also lyse when exposed to high temperatures. To determine the sensitivity of the PCR in cereal seed extracts, the CLS cells were lysed by heating a small sample (<20 µl) for 7 min at 95°C. From this lysis, 3 µl was immediately used in PCR.

PCR amplifications. Bacterial 16S-23S rDNA spacer fragments were obtained for sequence analysis by amplification in PCR with the following conserved sequences used as primers: a forward 20-mer primer, 5′ AGTCGTAACAAGGTAAGCCG 3′ (C1) (derived from the *Escherichia coli* 16S rDNA, position 1493-1513 [23]), and a reverse 20-mer primer, 5′ C(T/C)(A/G)(T/C)TGCCAAGGC ATCCACC 3′ (C2) (complementary to the *E. coli* 23S rDNA sequence, position 23-43 [23]).

PCR with conserved primers C1 and C2 was performed in an Omnigene temperature cycler (HYBAID, Teddington, England). The following program was run: initial denaturation for 2 min at 95°C, followed by 29 cycles of 45 s at 95°C, 1 min at 50°C, and 2 min

at 72°C. The final extension step was for 10 min at 72°C. PCR with target primers T1 and T2 was performed in a thermal cycler PHC-3 (Techne, Cambridge, England), equipped with a heat cover, programmed for an initial denaturation at 90°C for 2 min, followed by 35 cycles of 30 s at 93°C, 45 s at 53°C, and 1 min at 68°C, and a final extension at 70°C for 10 min. All reactions had a final volume of 20 µl and contained Tris buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3), 0.2 mM of each dNTP, 60 ng of each primer, 1 unit of *Taq* polymerase (Boehringer GmbH, Mannheim, Germany), and 3 µl of sample. The PCR products were analyzed by running 5 µl of the reactions in a Tris-acetate agarose gel (2%) electrophoresis, staining with ethidium bromide, and visualizing with UV light. To determine the sensitivity of detection, the total reaction volumes were loaded on the gel.

Sequencing reactions. The PCR fragments, amplified with conserved primers C1 and C2, were purified from agarose gel by centrifugation through a HV-type filter, 0.45 µm (Millipore Corp., Bedford, MA). The fragments were directly sequenced in a dideoxy/T7 polymerase system (Deaza G/A^{T7} sequencing mixes, Pharmacia Biotech, Uppsala, Sweden) with the label α^{35}S-dATP. The products were separated on a 48% urea and 5% acrylamide wedge gel (0.2 to 0.6 mm). The sequences were read on X-ray film after 60 h of exposure. The software program Genetics Computer Group, version 7, was applied for the sequence alignments (program bestfit) and for the EMBL library searches (subdivision bacterial, program fasta) (10).

Southern hybridization. Homologous to a segment of the sequenced alanine-tRNA gene, a 37-mer (5′CCTGCTTTGCAAGC AGGGGGTCGTCGGTTCGATCCCG 3′) oligonucleotide was synthesized and 5′-end–labeled with the enzyme T4 polynucleotide kinase (Boehringer) and γ^{32}P-ATP. The DNA was blotted from the agarose gel to a Hybond nylon membrane (Amersham Life Science, Little Chalfont, England). The Southern blot hybridization was performed at 37°C for 5 h in the presence of 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 40 µg of denatured salmon sperm DNA per ml. The two washing steps were carried out at 37°C for 30 min and in 6× and 2× SSC, successively.

Cereal seed tests. Seed lots were tested: 2 United States, 4 Belgian, 15 Hungarian, and 8 Turkish. The two cereal seed lots from the United States were provided by R. L. Forster (University of Idaho, Kimberly) and contained either durum wheat (cv. Waid, lot 91 BCI) or barley (breeding line DH 85-37-01); both lots were contaminated with *X. campestris* pv. *translucens*. The four Belgian seed lots contained either wheat cvs. Castell and Estica or barley cvs. Abex and Express. The 15 Hungarian lots (provided by I. Somlyay, Pannon University, Keszthely, Hungary) contained either winter barley cvs. Eszter, Plaisant, Kompolti Kovai, GKT 0413, and GK Omega, spring barley cvs. GK Isis, Maresi, Bitrana, Cymbola, and Triangel, or wheat cvs. Marcal, Örsèg, Pinka, Rèpce, and Csörnöc. The eight wheat seed lots obtained from Turkey (provided by T. Bora, Ege University, Izmir, Turkey) contained cvs. Salihli, Ege, Ata, Cumhuriyet, Kakliç, Gönen, Izmir, or Gediz.

The bacteria associated with the cereal seeds were extracted by the method of Schaad and Forster (37) by combining 120 g of seed, 120 ml of cold aqueous saline (0.85% NaCl), and 1 drop of Tween 20 and shaking for 5 min on a rotary shaker (200 rpm) at room temperature. After 1 min without shaking, the extracts were plated and prepared for PCR. For PCR, 3 µl of the extract was put at 95°C for 7 min to lyse the bacteria and was immediately tested in PCR or stored at –80°C for later use. CLS contamination of the seed was determined by spread-plating extract dilutions on an adapted selective XTS medium (37); 1.4 mg of gentamycin per liter was used instead of 8 mg per liter. Total seed associated bacterial populations were grown on YPGA (YPG plus 1.5% Bacto [Difco Laboratories, Detroit] agar), with the addition of 50 µg of cycloheximide per ml. As a positive control on the PCR detection of CLS bacteria in the seed extracts, half of the extract volumes were artificially contaminated with 10^5 CFU of *X. campestris* pv. *translucens* (LMG 5261) per ml. To estimate the PCR detection sensitivity in cereal seeds, grains of CLS-contaminated wheat cv. Waid and of CLS-free cv. Castell were combined at 1/1, 1/10, 1/100, and 1/1,000 (wt/wt) and extracted and prepared as described above.

Pathogenicity tests. Bacterial cultures were grown in YPG for 48 h on a rotary shaker at 28°C. The cells were washed and diluted in water to about 10^7 CFU/ml. For each strain, five wheat seedlings (cv. Estica, 2 to 3 leaf stage) were inoculated by injecting the bacterial suspension into a leaf whorl using a 26-gauge needle. As a negative control, plants were injected with water. The treated plants were kept for 7 days at 25 to 28°C and high humidity in a small plastic compartment in the greenhouse.

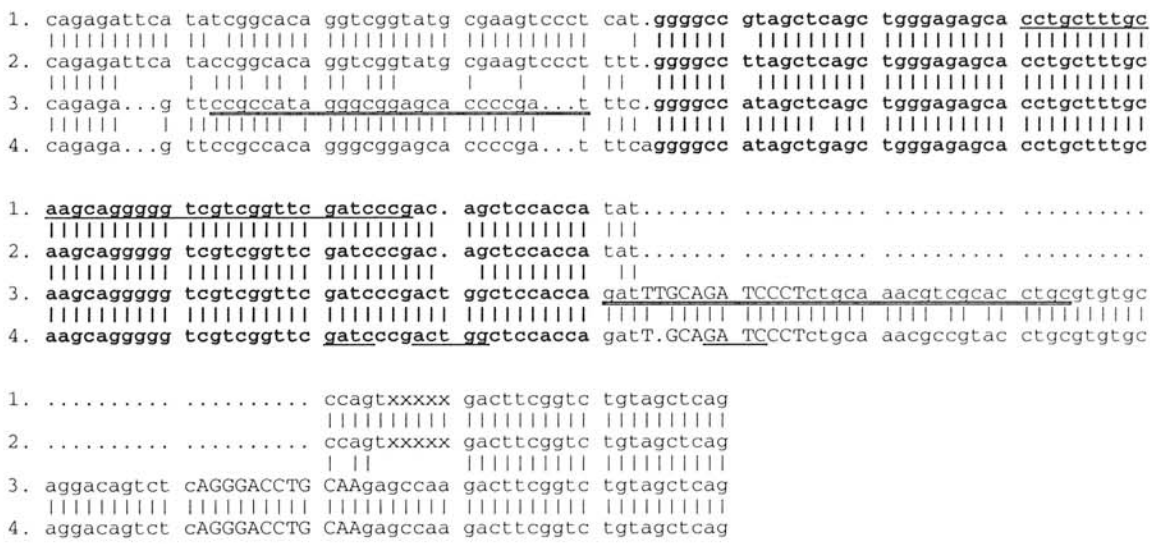

Fig. 1. Sequence comparison of the 16S-23S rDNA spacer region from row 1, *Xanthomonas campestris* pv. *alfalfae* LMG 497; row 2, *X. oryzae* pv. *oryzae* LMG 5047; row 3, *X. campestris* pv. *translucens* LMG 5261; and row 4, *X. campestris* pv. *cerealis* LMG 880. The designed polymerase chain reaction (PCR) primer sequences T1 and T2 are double underlined. The $tRNA^{Ala}$ gene sequence is in bold type. The probe sequence used in the Southern hybridization experiment, the single *Bsr*I, and the two *Sau*3AI restriction sites present in the 139-bp PCR fragment in rows 3 and 4 are single underlined. Sequence deletions are marked by dots, and undefined nucleotides are marked by x's. The two inverted repeat sequences bordering the extra 57-bp sequence in rows 3 and 4 are in capitals.

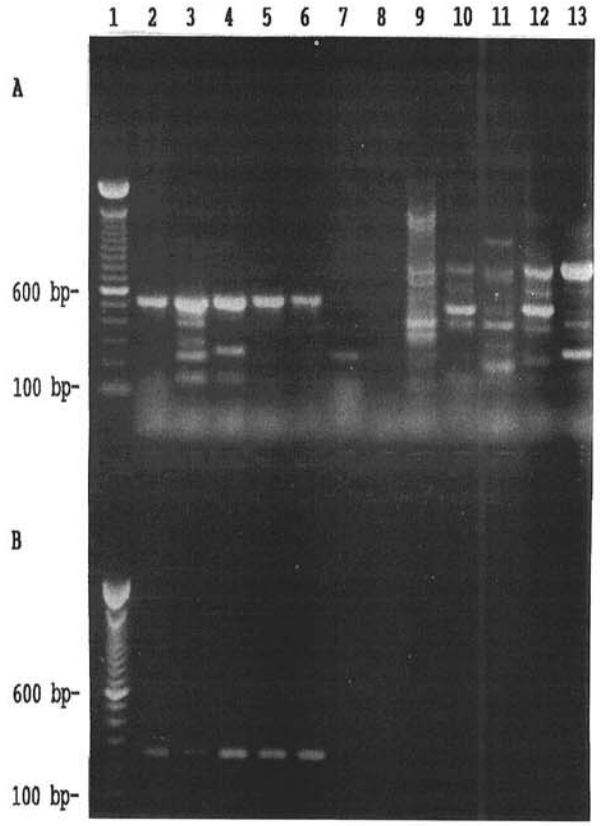

Fig. 2. Electrophoretic analysis of the fragments produced in polymerase chain reaction directed by primer pairs **A,** T1-C2 and **B,** C1-T2. Lane 1, 100-bp DNA ladder (GIBCO-BRL); lanes 2–6, *Xanthomonas campestris* pvs. *hordei* LMG 884, *cerealis* LMG 679, *undulosa* LMG 888, *translucens* LMG 5263, and *graminis* LMG 713; lane 7, *X. oryzae* pv. *oryzae* LMG 5047; lane 8, *Pantoea agglomerans* LMG 2570; lanes 9–12, *X. campestris* pvs. *holcicola* LMG 736, *vasculorum* LMG 901, *phaseoli* LMG 842, and *axonopodis* LMG 538; and lane 13, *X. campestris* LMG 947 strain isolated from *Bromus* grass.

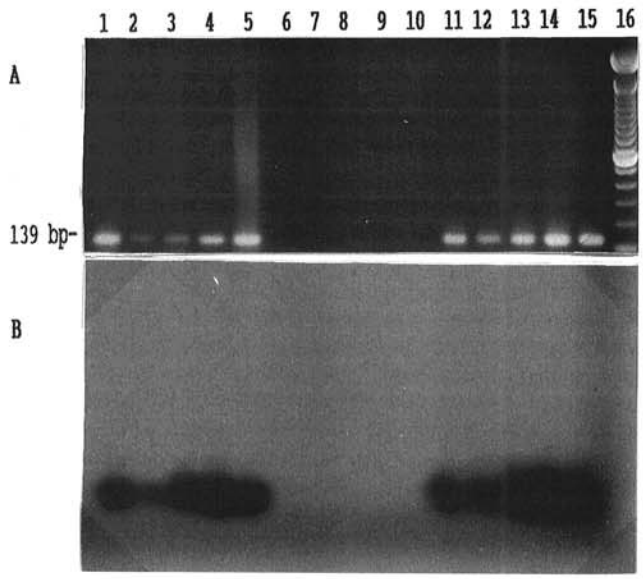

Fig. 3. A, Gel electrophoresis of the polymerase chain reaction products formed with primers T1 and T2 and **B,** Southern hybridization with the $tRNA^{Ala}$ gene probe. A hybridizing 139-bp fragment was amplified from *Xanthomonas campestris* pvs. lane 1, *translucens*; lane 2, *cerealis*; lane 3, *undulosa*; lane 4, *secalis*; lane 5, *hordei*; lane 11, *graminis*; lane 12, *poae*; lane 13, *phlei*; lane 14, *phleipratensis*; and lane 15, *arrhenatheri*. Lanes 6–10 contain the reaction products of *X. oryzae* pv. *oryzae*, *Pantoea agglomerans*, and the *X. campestris* pvs. *holcicola*, *vasculorum*, and *phaseoli*, respectively. Lane 16 has the 100-bp DNA ladder (GIBCO-BRL).

RESULTS

Sequencing data. The 16S-23S rDNA spacer regions of four taxonomically related phytobacteria were amplified by PCR, and the sequences were determined. Two CLS bacteria, *X. campestris* pvs. *translucens* and *cerealis*, another pathogen on *Gramineae*, *X. oryzae* pv. *oryzae*, and a pathogen with a totally different host range, *X. campestris* pv. *alfalfae*, were examined. Unlike many other bacteria (40), a single 16S-23S rDNA spacer fragment was observed. Within this spacer region, there was high sequence homology, except for two regions that clearly differentiated the two CLS pathovars from the two other xanthomonads (Fig. 1). The two variable regions flanked a 76-bp sequence that was conserved in the four xanthomonads. For this conserved fragment, high sequence homologies (96%) were found in the EMBL database, identifying it as an alanine-tRNA ($tRNA^{Ala}$) gene sequence. Proximal to the 5′ end of the $tRNA^{Ala}$ gene, the variable sequence was 37-bp long. Proximal to the 3′ end of the $tRNA^{Ala}$ gene a 57-bp sequence was observed in the CLS bacteria that was missing in the *X. oryzae* pv. *oryzae* and *X. campestris* pv. *alfalfae* sequences. At the borders of this extra 57-bp sequence, there were two nearly perfect inverted repeats of 12 bp.

Primer design and specificity. Two oligonucleotides, T1 and T2, homologous to the two variable regions of the *X. campestris* pv. *translucens* sequence, were synthesized. Sequence T1 was homologous to the *X. campestris* pv. *translucens* sequence upstream from the $tRNA^{Ala}$ gene and was designed as a 25-mer forward primer with a G+C content of 72% and had the sequence 5′ CCGCCATAGGGCGGAGCACCCCGAT 3′ (Fig. 1). In combination with the universal reverse primer C2, the T1 primer was not specific for the CLS bacteria; different PCR fragment patterns were produced for each xanthomonad (Fig. 2A). Only in the taxonomically more distant *Pantoea agglomerans* strain was no PCR product seen. Sequence T2 was homologous to a DNA segment downstream from the $tRNA^{Ala}$ gene, located in the extra 57-bp sequence of *X. campestris* pv. *translucens* and was constructed as a 34-mer reverse primer with a G+C content of 55.9% and had the sequence 5′ GCAGGTGCGACGTTTGCAGAGGGATCTGCAAA TC 3′. When used with the universal primer C1, the T2 primer amplified a single fragment only from CLS pathogens *X. campestris* pvs. *hordei*, *cerealis*, *undulosa*, and *translucens* and from *X. campestris* pv. *graminis*, a pathovar that causes wilt on forage grasses (Fig. 2B).

To check the specificity of the T1-T2 primer combination, 78 *Xanthomonas*, 12 *Erwinia*, 4 *Pantoea*, 1 *Agrobacterium*, 1 *Clavibacter*, and 4 *Pseudomonas* strains were used as targets. The first group represented the CLS pathogens; 28 strains belonging to *X. campestris* pvs. *cerealis*, *hordei*, *secalis*, *translucens*, and *undulosa* were tested. The second group included a range of xanthomonads pathogenic on other gramineous plants, including 21 *X. campestris* strains, 4 strains belonging to different *Xanthomonas* species, and 2 pathogenic *Xanthomonas* strains isolated from *Bromus* grass (44), viz. *X. campestris* pv. *graminis* LMG 8269 isolated in New Zealand and *X. campestris* strain LMG 947 isolated in France, without a pathovar specification. The third group consisted of four cereal pathogens outside the *Xanthomonas* genus. The fourth group had 40 other phytobacteria. With primers T1 and T2, a single 139-bp PCR fragment was produced with the lysed cells of the 28 tested CLS strains and with 19 pathogenic strains of forage grasses belonging to *X. campestris* pvs. *arrhenatheri*, *graminis*, *phlei*, *phleipratensis*, and *poae* (Table 1; Fig. 3A). Together, these 10 *X. campestris* pathovars were clearly differentiated from the 52 other phytobacterial strains tested, with which no PCR product was observed in agarose gels.

To confirm the identity of the amplified DNA fragments in the recognized *X. campestris* pathovars and to check the absence of PCR product in other strains, a hybridization experiment was performed. Since PCR primers T1 and T2 should amplify the $tRNA^{Ala}$

gene from CLS bacteria, an internal $tRNA^{Ala}$ gene probe sequence was hybridized to a Southern blot of PCR products from the 10 PCR-positive *X. campestris* pathovars and 5 related strains that gave no visual PCR amplification product (Fig. 3A). Only the visible 139-bp band on gel, produced with the 10 PCR-positive *X. campestris* pathovars, hybridized to this probe (Fig. 3B). Therefore, the same 139-bp fragment (as calculated from the sequence information) was present in all 10 pathovars and was not amplified when the other *X. campestris* pathovars were assayed.

Restriction fragment length polymorphism (RFLP) analysis of the PCR products. The 139-bp fragment amplified from the 10 *X. campestris* pathovar strains was digested with the restriction enzymes *Cfo*I, *Bsr*I, and *Sau*3AI. The fragment from all pathovars was not restricted by *Cfo*I. The same restriction pattern was produced when the 139-bp fragment from all 10 pathovars was restricted with *Sau*3AI (restriction fragments of 84, 28, and 26 bp) and *Bsr*I (restriction fragments of 91 and 41 bp; an extra 7-bp sequence was produced in this digestion but was not detected on gel). The restriction fragment lengths are in agreement with the sequence data (Fig. 1).

PCR detection in cereal seed extracts. Belgian, United States, Hungarian, and Turkish cereal seed lots were tested in PCR with primers T1 and T2 for the production of the 139-bp fragment. For the two CLS-contaminated United States lots, a single PCR fragment of the correct length was observed, whereas for the extracts of the Belgian and Hungarian seed lots no amplified DNA fragment could be detected on a gel (Fig. 4). The 139-bp fragment was produced in all artificially CLS-inoculated extracts, so the absence of the PCR signal was not due to inhibitors of PCR. In four of the eight Turkish seed lots, 139-bp fragments of different intensities were detected. The extracts of wheat cvs. Gönen and Salihli produced the most intense 139-bp band; weaker bands were detected from the extracts of cvs. Ege and Gediz.

Verification of the PCR results. All the extracts that showed a positive signal in PCR were plated on XTS medium. In United States seeds, CLS contamination of barley seed at 3.2×10^4 CFU/g and of wheat seed at 2.1×10^5 CFU/g was found. In the four positive Turkish seed extracts, CLS-type colonies were observed, but large numbers of other seed-associated bacteria prevented enumeration. From each of these four seed extracts, some CLS-type colonies were found that produced the 139-bp PCR fragment and induced leaf streak symptoms on young wheat plants after 6 to 8 days.

Detection sensitivity in seed extracts. In the Belgian wheat lot, cv. Castell, in which no CLS-contamination was detected, a seed-associated bacterial population of 4×10^5 CFU/g of seed was determined. When seed of CLS-contaminated wheat cv. Waid was titrated with seed of wheat cv. Castell, positive PCR products could be detected in seed mixtures in which the CLS population was calculated to represent 2.1×10^3 CFU/g of seed (Fig. 5).

DISCUSSION

Based on DNA sequence recognition and PCR, we found that contamination of cereal seed lots by translucent leaf streak pathogens can be determined within 6 h. The interpretation of the test results is simple and relies on the presence or absence of a single 139-bp fragment in an agarose gel. The DNA of CLS pathogens was detected at a bacterial concentration of 2×10^3 CFU/g of seed despite the presence of other seed-associated bacteria in a 200-fold excess. This level of sensitivity corresponds to the lowest degree of inoculum reportedly needed for initiation of disease in the field (37). In addition to detection in the two CLS-contaminated United States seed lots, infectious CLS contamination levels were observed in four of eight Turkish seed lots. Translucent leaf streak of cereals was recently described in Turkey (8), but its occurrence was not expected to be as frequent as was found in this research. Obviously, these eight lots are not necessarily representative of Turkish cereal production, but the high sensitivity and selectivity of the PCR detection must also be taken into account. XTS plating, which up to now was the routine detection procedure, has been inefficient in Turkish seed tests, primarily due to high concentrations of other fluorescent seed-associated bacteria that rapidly overgrow the CLS colonies. These seed bacteria probably are soilborne, because Turkish field soils are heavily populated with fluorescent pseudomonads that colonize grain during harvest when grain is left on the soil for long periods (T. Bora, *personal communication*).

In PCR, the possibility of detecting dead bacterial cells exists. However, in our experiments, the specific PCR band was not produced from dead CLS bacterial cultures and decreased in the CLS-infested United States seed tests after 14 months of seed storage (M. Maes, P. Garbeva, and O. Kamoen, *unpublished data*). This suggests that the PCR quality of the bacterial DNA is dependent on the bacterial condition; however, more research is needed on the efficiency of PCR detection in relation to the concentration of viable CLS bacteria in stored and disinfected cereal seed. The recovery of viable CLS bacteria associated with stored cereal seed is contradictorily reported to be strongly reduced after 6 months (31), to be nearly constant after 13 months (28), or to be present in excess of 63 months (18). On the other hand, the efficiency of chemical (17) or dry-heat seed treatment to control the amount of

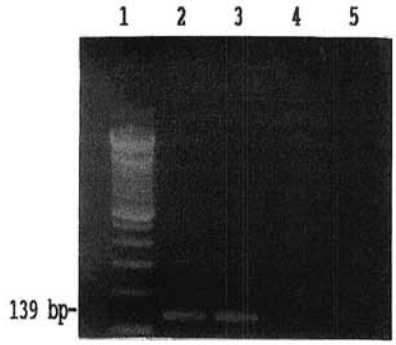

Fig. 4. Electrophoretic separation of the products formed in polymerase chain reaction with the T1-T2 primers applied to seed extracts of cereal cultivars from the United States (lane 2, barley breeding line DH 85-37-01 and lane 3, cv. Waid); from Belgium (lane 4, cv. Castell); and from Hungary (lane 5, cv. Plaisant). Lane 1 has a 100-bp DNA ladder (GIBCO-BRL). The 139-bp fragment is marked.

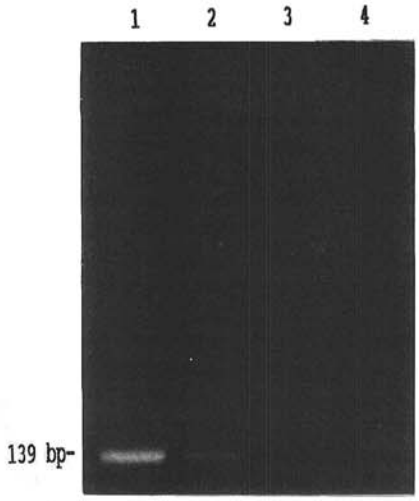

Fig. 5. Detection on gel of the polymerase chain reaction products obtained with the primers T1 and T2 and the extracts of mixed seeds. The grains of wheat cv. Waid with cereal leaf streak pathogen contamination at 2.1×10^5 CFU/g of seed and pathogen-free cv. Castell are combined in ratios of lane 1, 1/1; lane 2, 1/10; lane 3, 1/100; and lane 4, 1/1,000. The 139-bp fragment is marked.

infective CLS bacteria in infested seed has been discussed (19, 28). If a method proves useful for large and routine seed treatment, it is worthwhile to determine the risk of false positive PCR detection of dead CLS bacteria under those conditions.

In addition to the five CLS pathovars pathogenic on cereals, another five *X. campestris* pathovars pathogenic on grasses (*X. campestris* pvs. *arrhenatheri*, *phlei*, *phleipratensis*, *poae*, and *graminis*) were recognized by PCR. With these 10 pathovars, the same single 139-bp PCR fragment is produced that represents a $tRNA^{Ala}$ gene sequence. The high conservation of this gene sequence also is responsible for the lack of RFLPs in the amplified fragment from the 10 pathovars. The specificity of PCR is determined by the rDNA primers, especially by the T2 primer sequence. This primer was designed as a segment of a 57-bp sequence that is present in the same position in the rDNA spacer of the 10 *X. campestris* pathovars. The function of the extra 57-bp fragment, located downstream from a $tRNA^{Ala}$ gene, is unknown. Since two inverted repeats border this sequence, it could induce the formation of a hairpin-like structure in the unprocessed rDNA transcript. Similar hairpin structures are described in the rDNA spacers of other bacteria (5,40), and some are thought to play a role in signaling the processing of the large rDNA transcript and the maturation of functional rRNA and tRNA molecules. Nevertheless, other xanthomonads also have a $tRNA^{Ala}$ gene in the same spacer position (Fig. 1), where it is probably functional without the 57-bp sequence information.

That the 10 PCR-positive *X. campestris* pathovars have the same sequence organization in the 16S-23S rDNA spacer region suggests a close phylogenetic relatedness between these pathogens of cereals and grasses. This rDNA grouping reflects the distinct taxonomic position of this pathovar group within *Xanthomonas* (43,44). The cereal pathogens *X. campestris* pvs. *cerealis*, *hordei*, *secalis*, *translucens*, and *undulosa* and the pathogens of grasses *X. campestris* pvs. *arrhenatheri*, *phlei*, *phleipratensis*, *poae*, and *graminis* belong to DNA homology group 1, as delineated by Vauterin et al. (44). Total DNA homologies are higher than 78%, and a new *X. translucens* species is proposed for this bacterial group (43). We also found that PCR can be used to differentiate *X. translucens* bacteria from other xanthomonads, among which are the pathogens of *Gramineae*, *X. oryzae* pv. *oryzae*, *X. campestris* pvs. *vasculorum* and *holcicola*, *X. albilineans*, *X. axonopodis*, and some *Xanthomonas* isolates from *Bromus* grass with an uncertain taxonomic affiliation.

The PCR method presented is capable of detecting CLS bacteria in a seed extract without previous bacterial isolation but does not distinguish the CLS pathogens from the five other *X. campestris* pathovars with a host range restricted to forage and some ornamental grasses (13,14,25,35,47). No data are available on the survival of these grass pathogens on nonhost plants, especially on the seeds of cereals. This can pose a problem for the unambiguous PCR detection of CLS pathogens in seed. Residual populations of other xanthomonads have been described on nonhost plant species (42) and indicate that bacterial survival does not entirely depend on the availability of host plants. Until now, no specific sequences have been described that differ between the CLS bacteria and the grass pathogens that could be used for their selective detection, but recently, the repetitive extragenic palindromic (rep)-PCR method has been presented as a powerful tool for fingerprinting closely related bacterial isolates (7,45), and Louws et al. (26) reported the production of discriminative PCR patterns for different *X. campestris* pathovars, among which are one CLS strain (*X. campestris* pv. *translucens*) and two grass pathogens (*X. campestris* pvs. *graminis* and *poae*). We are currently developing a technique that combines the presented PCR detection sequences with a rep-PCR sequence that should allow the identification of a detected pathovar in a single PCR procedure by the production of a specific PCR pattern. For the moment, the rDNA-PCR method presented can be applied directly for the fast separation of harmless from potential CLS-infective seed batches. In routine seed tests, the PCR-positive seed samples can be differentiated from lots contaminated with the grass pathovars in pathogenicity tests with plate-grown *Xanthomonas* isolates.

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