tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*

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A PCR-based detection system was developed for *Xanthomonas albilineans*, a pathogen of sugarcane, and other related xanthomonads, using the conserved sequence of two adjacent tRNA genes and the variable length and sequence of the spacer region between them. An appropriate region was identified as follows: tRNA genes with the same anticodon from a wide variety of bacteria were aligned and the most frequent base at each position was chosen to derive primers that would anneal to the gene in either orientation. Pairs of such primers were screened against various *Xanthomonas* species and members of related genera using PCR at low to moderate annealing stringency. A subset of these pairs of tRNA consensus primers gave one or more PCR products which generally displayed interspecific length variability. The primer pair 5'–3' tRNA^{ala} and 3'–5' tRNA^{ile} showed interspecific length polymorphisms between *X. albilineans* and all other *Xanthomonas* species examined. These PCR products were cloned and sequenced from four isolates of *X. albilineans* and four isolates from different pathovars of *X. campestris*, and the spacer length variation confirmed. Specific tRNA gene primers were derived from the tRNA gene sequences. These primers yielded a PCR product of a characteristic length within most *Xanthomonas* species and pathovars tested. When a primer that projected from tRNA^{ala} into the 3' end of the variable intergenic spacer was used with a tRNA^{ile}-specific primer, PCR was a very sensitive diagnostic test for *X. albilineans*-infected sugarcane and gave no product or only a faint product with other species of bacteria. The specificity of this PCR-based detection system was further enhanced by a nested PCR reaction that took advantage of the fact that the tRNA^{ala}–tRNA^{ile} region was found to be embedded in a 16S rRNA–23S rRNA gene spacer. By amplifying the region between the 16S rRNA gene and tRNA^{ile} or between the tRNA^{ala} and the 23S rRNA gene, the subsequent nested PCR product was shown to be *X. albilineans*-specific.

Keywords: *Xanthomonas albilineans*, tRNA, polymerase chain reaction, phytopathogen, leaf scald disease, sugarcane

INTRODUCTION

Xanthomonas are Gram-negative bacteria in the *Pseudomonadaceae* family of the Gracilicutes. All *Xanthomonas* species are plant pathogens. Currently six *Xanthomonas* species are recognized: *X. albilineans*, *X. axonopodis*, *X. campestris*, *X. fragariae*, *X. oryzae* and *X. populi*. *X. campestris* has the most complex taxonomic structure, being subdivided into over 140 pathovars specific for

certain plant hosts or diseases (Vauterin *et al.*, 1990). This requires further taxonomic clarification as recent molecular evidence based on DNA homologies indicates that *X. campestris* is not a single species (Palleroni *et al.*, 1993; Hildebrand *et al.*, 1990).

X. albilineans is the causal agent of leaf scald disease in sugarcane, a systemic disease characterized by chronic and acute phenotypes (reviewed by Ricaud & Ryan, 1989). In addition, under certain environmental conditions, latent infections can become fulminant. Current methods for detection rely on isolation of pure cultures onto selective media (Davis *et al.*, 1994a) or immunoassays of tissue

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extracts (Comstock & Irey, 1992). Serotyping (Rott *et al.*, 1994) and RFLP fingerprints and electrophoretic karyotypes from PFGE (Davis *et al.*, 1994b) have revealed variation within *X. albilineans*. These methodologies, useful for detection or identification of some isolates, do not provide a sufficient combination of sensitivity, speed, reliability and high output to serve as a diagnostic test for *X. albilineans*. Such a tool would facilitate ecological studies as well as quarantine assessment.

PCR-based tools have been developed that are useful in determining genomic variation, e.g. arbitrarily primed PCR (APPCR; Welsh & McClelland, 1990; Williams *et al.*, 1990) and rep-PCR (Versalovic *et al.*, 1991). Welsh *et al.* (1992) used APPCR characters to distinguish phyletic groups within *Borrelia burgdorferi*, and rep-PCR fingerprinting has been demonstrated as a potential means of identifying *Xanthomonas* and *Pseudomonas* strains (Louws *et al.*, 1994). Leite *et al.* (1994) combined specific PCR primers designed from a *hrp* gene, followed by restriction digestion of amplified products to reveal variation among some *Xanthomonas* strains that are pathogenic on plants. The *hrp*-specific primers did not produce products in *X. albilineans* or in all pathovars of *X. campestris*.

Most tRNA genes in eubacteria are found in tandem clusters with genes separated by a short spacer region. Previously, Welsh & McClelland (1991) had designed pairs of PCR primers based on a consensus sequence for all *Bacillus* tRNAs. At low to moderate annealing stringency, these primers generate fingerprints consisting mainly or exclusively of products derived from these tandem arrays, with products generally spaced by about one tRNA gene repeat unit (Welsh & McClelland, 1991). If a number of species are compared, it is possible to identify repeats that are of different lengths due to interspecific variations in the intergenic spacer regions between tRNA genes (McClelland *et al.*, 1992; Welsh & McClelland, 1992). These authors speculated that the sequence and order of tRNA genes probably evolves very slowly compared to neutral DNA or protein coding regions, so primers are likely to match perfectly with genes in all strains of a species or even genus. However, variation in intergenic spacer length probably occurs almost as quickly as in neutral DNA. This results in variations on the length of the PCR product which may often be diagnostic for particular species or sub-species. Consensus tRNA primers have since been used to assess genetic variability and phylogenetic relationships among isolates of *Pseudomonas solanacearum* (Seal *et al.*, 1992), *Acinetobacter* (Wiedmann-Al-Ahmad *et al.*, 1994) and entomopathogenic fungi (Tigano-Milani *et al.*, 1995a, b).

When products polymorphic in length are cloned and sequenced, new specific tRNA-gene-based PCR primers can be derived. The resulting high stringency PCR products that span the intergenic length polymorphisms are diagnostic markers that define a group of species by the presence of a PCR product and identify taxonomic subsets of these organisms by virtue of the size of the PCR product (McClelland *et al.*, 1992; Welsh & McClelland, 1992). Here we have used a further refinement of this

approach based on consensus primers derived from aligning homologous tRNAs (that share the same anticodon) from all eubacteria. These primers have been used to distinguish between members of the genus *Xanthomonas* at various taxonomic levels.

METHODS

Bacterial strains, leaf samples and DNA extraction. Bacterial strains used in this study are listed in Table 1. Samples from ATCC or ICPB were received as bacterial cultures; all other samples were received as genomic DNA. Strains were grown in either Media 3 or Media 825 (as recommended by ATCC) or YDCP media for strains received from ICPB. Genomic DNA was isolated according to standard protocols (Ausubel *et al.*, 1987), and quantified on agarose gels. Alternatively, single bacterial colonies were boiled in 500 µl sterile water for 10 min, then centrifuged at 12000 r.p.m. for 5 min and the supernatant used as a template for PCR.

Leaf samples of sugarcane cultivar LCP82-89, as well as *X. albilineans* isolated from LCP82-89 grown in the field, were kindly supplied by Michael Grisham (USDA-ARS, Houma, LA, USA). Symptomatic and asymptomatic leaves were fixed in ethanol. Sectors of leaf blade (about 0.4 g wet wt) were excised and DNA extracted using a CTAB-based protocol (Murray & Thompson, 1980). DNA preparations were quantified by spectrophotometry and used as templates for PCR.

PCR primers. Primers were designed using four different criteria.

(a) Consensus tRNA primers made after aligning all *Bacillus subtilis* tRNAs (Welsh & McClelland, 1991): T5A, 5' AGTCC-GGTGCTCTAACCACCTGAG 3' and T3B, 5' AGGTCGC-GGGTTCGAATCC 3'.

(b) Each set of 21 homologous eubacterial tRNAs were aligned separately (GenBank version 11). From each of these alignments two PCR primers were derived. Details and nomenclature are given in Results. Of the 42 possible primers, six consensus anticodon-specific tRNA primers were used in this study: phenylalanine tRNA (FEU), 5' TTGGKAGAGCRWYRGA-CTGAA 3'; tryptophan tRNA (WEU), 5' TGGTAGACR-SMGGTCTCCA 3'; alanine tRNA (AEU), 5' GYTGGKAG-AGCRCCTGCTTTG 3'; initiator methionine tRNA (iMEL), 5' CCKRSGACCTWWGGGTTATG 3'; isoleucine tRNA (IEL), 5' GAACYWSYGACCTYACSTTATC 3' and proline tRNA (PEL), 5' GAACCTSCGACCCMYGGTCCCA 3'.

(c) Genus- and species-specific primers were designed using sequence alignment of AEU+IEL amplified PCR products from four isolates of *X. albilineans* and four isolates of *X. campestris* pathovars with bacterial and plant tRNAs in GenBank. These primers were named for their relative positions on tRNA^{ala} and tRNA^{ile}: Ala1, 5' TTTGCAAGCAGGGGG-TCGTC 3'; Ala2, 5' TCGTCGGTTTCGATCC CGAC 3'; Ala4, 5' CCCGACTGGCTCCACCACTG 3'; Ile1, 5' AGGGGTG-CGCTCTAACCAC 3' and Ile2, 5' CCTTATCAGGGGTGC-GCTC 3'.

(d) Primers for ribosomal RNA sequences from conserved regions at the 3' end of the 16S rRNA gene and 5' end of the 23S rRNA gene (D. Ralph & M. McClelland, CIBR, unpublished data) were: 16S uni1330, 5' GTTCCCGGGCCTTGTAACAC 3' and 23S uni322anti, 5' GGTTCCTTTTCACCTTTCCCTC 3'.

All primers used in this study were synthesized by Genosys.

PCR conditions. Pairs of consensus tRNA primers were initially screened using two *X. albilineans* (CTC193 and ATCC 29186) isolates and *X. campestris* pv. *holcicola* at two template concen-

Table 1. *Xanthomonas* and *Pseudomonas* isolates used in this study

Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; CTC, Copersucar, Piracicaba, Brasil; ICPB, International Collection of Phytopathogenic Bacteria, Berkeley, CA, USA; MSRI, Mauritius Sugar Industry Research Institute, Reduit, Mauritius; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK.

Species	Strain	Host	Origin	Supplier
<i>Xanthomonas</i>				
<i>X. albilineans</i>	CTC193	<i>Saccharum officinarum</i>	Brazil	CTC
<i>X. albilineans</i>	CTC293	<i>Saccharum officinarum</i>	Brazil	CTC
<i>X. albilineans</i>	CTC393	<i>Saccharum officinarum</i>	Brazil	CTC
<i>X. albilineans</i>	CTC493	<i>Saccharum officinarum</i>	Brazil	CTC
<i>X. albilineans</i>	MSRI3201	<i>Saccharum officinarum</i>	Mauritius	MSRI
<i>X. albilineans</i>	MSRI3284	<i>Saccharum officinarum</i>	Mauritius	MSRI
<i>X. albilineans</i>	MSRI3295	<i>Saccharum officinarum</i>	Mauritius	MSRI
<i>X. albilineans</i>	MSRI2905	<i>Saccharum officinarum</i>	Mauritius	MSRI
<i>X. albilineans</i>	XaOD1	<i>Saccharum officinarum</i>	Florida	J. Comstock, USDA-ARS, FL
<i>X. albilineans</i>	XaOD2	<i>Saccharum officinarum</i>	Florida	J. Comstock, USDA-ARS, FL
<i>X. albilineans</i>	XaCP2	<i>Saccharum officinarum</i>	Florida	J. Comstock, USDA-ARS, FL
<i>X. albilineans</i>	XaCP3	<i>Saccharum officinarum</i>	Florida	J. Comstock, USDA-ARS, FL
<i>X. albilineans</i>	XaCP4	<i>Saccharum officinarum</i>	Florida	J. Comstock, USDA-ARS, FL
<i>X. albilineans</i>	LA334	<i>Saccharum officinarum</i>	Louisiana	M. Grisham, USDA-ARS, LA
<i>X. albilineans</i> subsp. <i>paspali</i>	ATCC 29186 (NCPPB 208)	<i>Paspalum dilatatum</i>	Mauritius	ATCC
<i>X. axonopodis</i>	ATCC 19312 (NCPPB 457)	<i>Axonopus scoparius</i>	Colombia	ICPB
<i>X. campestris</i> pv. <i>badrii</i>	ATCC 11672 (NCPPB 571)	<i>Xanthium strumarium</i>	India	M. Simcox, Stratagene
<i>X. campestris</i> pv. <i>campestris</i>	B293 (ATCC 22913, NCPPB 528)	<i>Brassica oleracea</i> var. <i>gemmifera</i>	?	ICPB
<i>X. campestris</i> pv. <i>celebensis</i>	XC145 (ATCC 19045, NCPPB 1832)	<i>Musa</i> sp.	New Zealand	ICPB
<i>X. campestris</i> pv. <i>holcicola</i>	ATCC 13461	<i>Holcus</i> sp.	?	M. McClelland
<i>X. campestris</i> pv. <i>malvacearum</i>	ATCC 9924	<i>Gossypium</i>	?	M. Simcox, Stratagene
<i>X. campestris</i> pv. <i>manihotis</i>	7AS1	<i>Manihot esculenta</i>	?	M. Simcox, Stratagene
<i>X. campestris</i> pv. <i>pelargonii</i>	XP15 (ATCC 8721)	<i>Pelargonium</i> sp.	?	ICPB
<i>X. campestris</i> pv. <i>phaseoli</i>	XP200 (NCPPB 1680)	?	?	ICPB
<i>X. campestris</i> pv. <i>taraxaci</i>	XT11 (ATCC 19318, NCPPB 940)	<i>Taraxacum kok-saghyz</i>	USA	ICPB
<i>X. campestris</i> pv. <i>undulosa</i>	ATCC 35935 (NCPPB 2821)	<i>Triticum turgidum</i>	?	ICPB
<i>X. campestris</i> pv. <i>vasculorum</i>	ATCC 13901	<i>Saccharum officinarum</i>	Puerto Rico	ATCC
<i>X. fragariae</i>	Strain305	<i>Fragaria</i> sp.	?	ICPB
<i>X. oryzae</i> pv. <i>oryzae</i>	ATCC 35933 (NCPPB 3002)	<i>Oryza sativa</i>	?	ICPB
<i>X. oryzae</i> pv. <i>oryzicola</i>	XO111	<i>Oryza</i> sp.	?	ICPB
<i>X. populi</i>	XP233 (NCPPB 2961)	<i>Populus balsamifera</i>	The Netherlands	ICPB
<i>Pseudomonas</i>				
<i>P. syringae</i> pv. <i>lapse</i>	ATCC 10859	?	?	ATCC
<i>P. rubrisubalbicans</i>	ATCC 19308 (NCPPB 1027)	<i>Saccharum officinarum</i>	?	ATCC
<i>Acidovorax</i>				
<i>A. avenae</i> subsp. <i>avenae</i>	ATCC 19307 (NCPPB 920)	<i>Saccharum officinarum</i>	?	ATCC
<i>A. avenae</i> subsp. <i>avenae</i>	ATCC 12530	?	?	ATCC
<i>Stenotrophomonas maltophilia</i>	ATCC 13637	<i>Homo sapiens</i>	?	ICPB
<i>Bacillus amyloliquefaciens</i>	H	?	?	ATCC
<i>Escherichia coli</i>	RY13			M. McClelland
<i>Streptomyces achromogenes</i>	ATCC 12767			M. McClelland
<i>Streptomyces albus</i>	G			M. McClelland
<i>Fusobacterium nucleatum</i>	D			M. McClelland
<i>Rhodobacter spaeroides</i>				M. McClelland
<i>Norcardia aerocolonigenes</i>	ATCC 23870			M. McClelland
<i>Flavobacterium okeanoikoites</i>	ATCC 33414			M. McClelland
<i>Arthrobacter luteus</i>	ATCC 21606			M. McClelland

(a)

Species	Strain	5'	3'
<i>X. campestris</i> pv. <i>badrii</i>	11672	T G C A A G C A G G G G G T C G T C G G T T C G A T C C C G A C A G G C T C C A C C A	
<i>Acholeplasma laidlawii</i>	216199	- C - - - G - - - - G - - - - C - - - - C - - - - A - - - - A G - - - - - G C T A / - - - - -	
<i>Acholeplasma laidlawii</i>	38852	- C - - - G - - - - G - - - - C - - - - C - - - - A - - - - A G - - - - - G - T - / - - - - -	
<i>Bartonella bacilliformis</i>	431273	- C - - - G - - - - A - - - - T - - - - C -	
<i>Blastocystis culicis</i>	459692	- T - - - G - - - - G - - - - T - - - - - - - - - A - - - - A - - - - - - - - - T T C A C - - - -	
<i>Bacillus</i> spp.	40228	- C - - - G - - - - A - T - - C - - - - A - - - - A - - - - G - - - - - - - - - T - T C - - - -	
<i>Bacillus subtilis</i>	143424	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - - - - - C T - - - -	
<i>Coxiella burnetii</i>	475887	- T - - - G - - - - A T - - C - - - - - - - - - A - - - - G - - - - - C T - - - C T - - - -	
<i>Caulobacter crescentus</i>	144276	- T - - - T - - - - G - - - - T - - - - - - - - - T - / - - - - - G - A T - - T T - - - -	
<i>Campylobacter jejuni</i>	440400	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - A T - - C T - T T - - -	
<i>Escherichia coli</i>	147585	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - T G - - - - - C A T A - - - -	
<i>Enterococcus faecalis</i>	290644	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - C T - - - -	
<i>Enterococcus hirae</i>	148320	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - G - T / - - - - T T - - -	
<i>Kinetoplastibacterium crithidii</i>	459861	A T - - - G - - - - G - - - - T - - - - - - - - - A - - - - A - - - - - T T C A C - - - -	
<i>Lactococcus lactis</i>	44070	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - C T - - - -	
<i>Leuconostoc oenos</i>	488593	- C - - - G - - - - A - - - - T - - - - - - - - - A - - - - A - - - - - A - - - - T T T - - -	
<i>Mycoplasma capricolum</i>	216788	- C - - - G - - - - A - - - - C - - - - C - - - - A - - - - A - - - - - T T - - G - - -	
<i>Mycoplasma mycoides</i>	44468	- C - - - G - - - - A - - - - C - - - - C - - - - A - - - - A - - - - - T T - - G - - -	
<i>Neisseria meningitidis</i>	466506	- T - - - T - - - - A - - - - T - - - - C - - - - A - - - - A - - - - - T T T C - - - -	
<i>Pseudomonas cepacia</i>	453541	- C - - - G - - - - A - - - - T - - - - - - - - - / - - - - - - - - - T T T C - - - -	
<i>Pseudomonas gladioli</i>	453546	- C - - - G - - - - A - - - - T - - - - - - - - - / - - - - - - - - - T T T C - - - -	
<i>Pseudomonas mallei</i>	453548	- C - - - G - - - - A - - - - T - - - - - - - - - / - - - - - - - - - T T T C - - - -	
<i>Pseudomonas mendocina</i>	453549	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - C T T - - -	
<i>Pseudomonas mendocina</i>	453552	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - C T T - - -	
<i>Pseudomonas pickettii</i>	453553	- C - - - G - - - - A - - - - T - - - - - - - - - / - - - - - - - - - T T T C - - - -	
<i>Pseudomonas pseudoalcaligenes</i>	453554	- C - - - G - - - - A - - - - T - - - - - - - - - / - - - - - - - - - T T T C - - - -	
<i>Rhodobacter sphaeroides</i>	46449	- C - - - G - - - - A - - - - T - - - - - - - - - A - - - - A - - - - - T T T C - - - -	
<i>Staphylococcus aureus</i>	310605	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - C T - T - - -	
<i>Spiroplasma meliferum</i>	47278	- C - - - G - - - - A - - - - C - - - - C - - - - - - - - - - - - - - - T T C - G - - -	
<i>Streptococcus pneumoniae</i>	153796	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - C T - - -	
<i>Streptococcus pneumoniae</i>	466514	- C - - - G - - - - G - - - - T - - - - C - - - - A - - - - A G - - - - - C T - N - - -	
<i>Thiobacillus ferrooxidans</i>	48171	- T - - - G - - - - A - - - - T - - - - - - - - - A - - - - A - - - - - G A - G T C A - - -	
<i>Stenotrophomonas maltophilia</i>	453566	- C - - - G - - - - A - - - - T - - - - - - - - - / - - - - - - - - - G A - G T C A - - -	
<i>X. albilineans</i>	29186		- T -
<i>X. albilineans</i>	CTC193		- T -
<i>X. albilineans</i>	CTC393		- T -
<i>X. albilineans</i>	CTC493		- T -
<i>X. campestris</i> pv. <i>malvacearum</i>	9924		- T -
<i>X. campestris</i> pv. <i>manihotis</i>	7AS1		- T -
<i>X. campestris</i> pv. <i>vasculorum</i>	13901		- T -
<i>X. campestris</i> pv. <i>badrii</i>	11672	T G C A A G C A G G G G G T C G T C G G T T C G A T C C C G A C A G G C T C C A C C A	
AEU		G Y T G G K A G A G C R C C T G C Y T T G	

(b)

Species	Strain	5'	3'
<i>X. campestris</i> pv. <i>badrii</i>	116720	G G G T C T G T A G C T C A G G T G G T T A G A G C G C A C C C C T G A T	
<i>Acholeplasma laidlawii</i>	216199	- - - C - - - - - - - - - T - - - - - - - - - A - T - G - T - - - - C - A G G - - - - G A T G - - -	
<i>Acholeplasma laidlawii</i>	38852	- A C - C - - - - - - - - T - - - - - - - - - T A C - G G - C - - - - C C - G T C - - - - G T T G - - -	
<i>Bartonella bacilliformis</i>	431273	- C - / - - - - - - - - - T - - - - - - - - - - - - - - - G - G - T - - - - C - G - - - - G G A G - - -	
<i>Blastocystis culicis</i>	459692	- - - - - - - - - - - - - - - C - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T T G - - -	
<i>Bacillus subtilis</i>	143734	- - - C - - - - - - - - - C - - - - - - - - - - - - - - - G - - - - C - G - - - - G G T G - - -	
<i>Bacillus subtilis</i>	143425	- - - C - - - - - - - - - C - - - - - - - - - - - - - - - G - - - - C - G - - - - G A T G - - -	
<i>Bordetella</i> sp.	49212	- - - - - - - - - - - - - - - T C - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T T G - - -	
<i>Coxiella burnetii</i>	475887	- - - - - - - - - - - - - - - T - - - - - - - - - A - - - - - - - - - G - - - - - - - - - G G A A - - -	
<i>Escherichia coli</i>	174462	- C - - - C - - - - - - - - - T - - - - - - - - - A - C A - T - - - C - T - G T G G G - - - - G G T G - - -	
<i>Escherichia coli</i>	43163	- C - - T C A - - - - - - - - - T - - - - - - - - - A - C A - T - - - C - T - G T G G G - - - - G T T G - - -	
<i>Kinetoplastibacterium crithidii</i>	459861	- - - - - - - - - - - - - - - C - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T T G - - -	
<i>Mycoplasma</i> -like	149821	- - - C - A - - - - - - - - - T - - - - - - - - - A - - - G - - - - C - G - - - - G G T G - - -	
<i>Mycoplasma</i> sp.	435160	- - - C - - - - - - - - - T - - - - - - - - - A - - - G - - - - C - G - - - - G G T G - - -	
<i>Neisseria meningitidis</i>	466506	- - - T - - - - - - - - - C - - - - - - - - - A - - - G - T - - - - C - G G - - - - G G A G - - -	
<i>Pseudomonas aeruginosa</i>	45429	- - - - - - - - - - - - - - - T - - - - - - - - - - - - - - - G - - - - G - - - - G G C A - - -	
<i>Pseudomonas aeruginosa</i>	453538	- - - - - - - - - - - - - - - T - - - - - - - - - - - - - - - G - - - - G - - - - G G C A - - -	
<i>Pseudomonas cepacia</i>	453541	- - - - - - - - - - - - - - - T C - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T T G - - -	
<i>Pseudomonas fluorescens</i>	294377	- - - - - - - - - - - - - - - T - - - - - - - - - - - - - - - G - - - - G - - - - G G C A - - -	
<i>Pseudomonas fluorescens</i>	294378	- - - - - - - - - - - - - - - C - T - - - - - - - - - - - - - - - G - - - - G - - - - G G C A - - -	
<i>Pseudomonas gladioli</i>	453546	- - - - - - - - - - - - - - - T C - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T T G - - -	
<i>Pseudomonas mallei</i>	453548	- - - - - - - - - - - - - - - T C - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T T G - - -	
<i>Pseudomonas mendocina</i>	453549	A - - - - - - - - - - - - - - - T - - - - - - - - - - - - - - - G T / G - - - - G G C A - - -	
<i>Pseudomonas pickettii</i>	453553	- - - - - - - - - - - - - - - T - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T A G - - -	
<i>Pseudomonas pseudoalcaligenes</i>	453554	- - - - - - - - - - - - - - - T C - - - - - - - - - A - C G T - T - - - - G C G G G - - - - G T T G - - -	
<i>Rhodopseudomonas marina</i>	450362	- - - / - G - - - - C - - - - G G T G - - -	
<i>Stenotrophomonas maltophilia</i>	453566	- G - - - - G G T A - - -	
<i>X. albilineans</i>	29186		- -
<i>X. albilineans</i>	CTC193		- -
<i>X. albilineans</i>	CTC393		- -
<i>X. albilineans</i>	CTC493		- -
<i>X. campestris</i> pv. <i>malvacearum</i>	9924		- -
<i>X. campestris</i> pv. <i>manihotis</i>	7AS1		- -
<i>X. campestris</i> pv. <i>vasculorum</i>	13901		- -
<i>X. campestris</i> pv. <i>badrii</i>	116720	G G G T C T G T A G C T C A G G T G G T T A G A G C G C A C C C C T G A T	
		G A T A A G S G T Y A G G T C Y S W Y G T T C IEL	

Fig. 1. Alanine and isoleucine tRNAs sequences in GenBank (version 11) were aligned using *X. campestris* pv. *badrii* as the reference sequence. The eubacterial alignments used to generate the AEU and IEL primers are included in the left-most 21 bases of (a) and the right-most 23 bases of (b), respectively. K = G or T, R = A or G, Y = T or C, S = G or C, W = A or T. '-' represents an identical base, '/' represents a deleted base and '' represents an additional base relative to the reference sequence. The new tRNA sequences from AEU+IEL amplification products generated in this study (Fig. 2) are included at the bottom of (a) and (b) as indicated.

trations in 50 µl reactions [1.25 U AmpliTaq, 1 × Taq buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.5 µM each primer and 5 µCi (0.185 MBq) ³²P-dCTP] for 40 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min. Products were resolved on 5% denaturing polyacrylamide gels with electrophoresis for 2.5 h at 58 W constant power. The gels were dried onto Whatman 3MM paper and exposed overnight on X-Omat AR film.

Specific PCR amplifications using primer pairs developed in this study were performed as above. Some reactions also included 0.5 U PerfectMatch (Stratagene). Cycling parameters were either 40 cycles at 94 °C for 20 s and 70 °C for 20 s, 40 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s, or 30 cycles at 94 °C for 20 s and 65 °C for 20 s, as indicated in the Figure legends. Products were resolved on 3% NuSieve (FMC) agarose gels prepared in 1 × TBE with electrophoresis at 12 V cm⁻¹ for 2.5 h. The molecular mass markers (123 bp and 1 kb ladders) were purchased from Gibco BRL.

Reactions for experiments involving 16S uni1330 or 23S uni322anti were performed using 40 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min.

Reamplification, cloning and sequencing. PCR products from amplifications with AEU + IEL primers were excised from dried gels and eluted overnight at room temperature in 50 µl of sterile water, then used as a template for reamplification. The final concentration of reaction components and cycling parameters were as above except 1 and 2 µl of the eluted products were used as templates. Fifteen microlitres of each reaction was loaded onto a 2% agarose gel prepared in 0.5 × TBE and electrophoresis was performed at 12 V cm⁻¹ for 2.5 h. The remainder of each reaction product was purified by chloroform/isoamyl alcohol extraction, ethanol-precipitated and cloned.

Reamplified PCR products were blunt-end ligated into pCRScript (Stratagene) and transformed into *Escherichia coli* XL1-blue MRF' (Stratagene). Clones containing inserts were identified as white colonies from which single-stranded DNA was prepared according to standard protocols, and sequenced using a Sequenase 2.0 kit (USB).

Sequence alignments. Sequences were aligned using GeneWorks software (Intelligenetics) and compared to both bacterial and plant tRNA sequences of GenBank (version 11) (Fig. 1).

RESULTS AND DISCUSSION

Primer design

tRNA genes in bacteria occur in mixed cistrons of direct tandem repeats (e.g. Jinks-Robertson & Nomura, 1987). Here, we present a variation on the tRNA gene PCR fingerprinting strategy of Welsh & McClelland (1991). This variation uses primers derived from particular tRNA anticodon types rather than a consensus of all tRNAs. These primers were designed so that two or three bases at the 3' end are the conserved anticodon loop (underlined in primer sequence). Thus, for example, we aligned all known eubacteria alanine tRNAs, anticodon TGC, to derive a primer, AEU, oriented 5'–3' relative to transcription (the left-most 21 bases in Fig. 1a). Another primer oriented in the opposite direction and also ending with its 3' end at the anticodon was designed for tRNA^{ala} but is not shown. Fig. 1(b) shows the alignment of the

Fig. 2. Amplification products of *Xanthomonas* species using tRNA consensus primers AEU (alanine) + IEL (isoleucine) and 5 ng bacterial DNA resolved on a 5% denaturing polyacrylamide gel. Lanes: 1, 123 bp standard; 2, 1 kb standard; 3, no DNA control; 4, *Bacillus amyloliquefaciens* H; 5–8 *X. albilineans* strains (ATCC 29186, CTC193, CTC393 and CTC493, respectively); 9, *X. campestris* pv. *vasculorum* (ATCC 13901); 10, *X. campestris* pv. *malvacearum* (ATCC 9924); 11, *X. campestris* pv. *badrii* (ATCC 11672); 12, *X. campestris* pv. *holcicola* (ATCC 13461); 13, *X. campestris* pv. *manihotis* (7AS1); 14, 123 bp standard; 15, 1 kb standard.

isoleucine consensus anticodon-specific primer IEL in the 3'–5' orientation relative to transcription.

Names for these primers consist of the one letter amino acid code, 'E' for eubacteria, then 'U' or 'L' for 'upper' and 'lower' strands of the gene. U and L indicate primers that are oriented 5'–3' or 3'–5' in the consensus tRNA gene, respectively.

Consensus anticodon-specific primers derived from two particular tRNA types used in combinations (one primer oriented 5'–3' and the other 3'–5') will yield PCR products only if that particular combination of tRNAs occurs in the correct order in at least one of the tRNA gene tandem arrays. It can reasonably be speculated that once such a combination is found in one species, then it almost certainly occurs in most or all other species in the same genus (Jinks-Robertson & Nomura, 1987; Welsh & McClelland, 1992; McClelland *et al.*, 1992). When PCR products amplified from different species are then visualized side-by-side using PAGE, intergenic length polymorphisms may be revealed. These PCR products can be cloned, sequenced and specific PCR primers can be derived, as required to meet particular taxonomic criteria.

Fig. 3. Sequence of AEU + IEL PCR product from *X. albilineans*. The AEU and IEL primer sequences are boxed and the anticodon underlined. The location and orientation of primer sequences used in this study are shown.

Detection of a polymorphism among *Xanthomonas* species

tRNA consensus primers were screened in pairwise combinations, oriented so that a productive PCR amplification could take place. Primers were chosen that would hybridize to opposite strands of the tRNA gene sequences. Consensus anticodon-specific tRNA primers (see Methods) and the consensus universal tRNA primers T5A and T3B (Welsh & McClelland, 1991; Welsh *et al.*, 1992) were used in a total of 13 combinations. Some combinations revealed complex patterns. For example, for the *X. albilineans* and *X. campestris* isolates tested, primers T5A + T3B gave three prominent PCR products, each probably a multiple of tRNA genes from the same or different tRNA gene clusters (data not shown). Other primer combinations gave single or no products within the *Xanthomonas* species tested. For example, the AEU + IEL anticodon-specific primers for alanine and isoleucine tRNAs, respectively, resulted in single products of different lengths for *X. albilineans* and *X. campestris* isolates (Fig. 2) as compared to *Pseudomonas rubrisubalbicans*, *Pseudomonas syringae* pv. *lapse* or *Acidovorax avenae* subsp. *avenae* isolates which gave multiple products (data not shown). With the exception of pv. *badrii*, *X. campestris* pathovars gave products of a similar length, which were distinct from those found in *X. albilineans* isolates. Thus, the products generated with this particular pair of primers were chosen for further study because a potential *X. albilineans*-specific length polymorphism was present between an alanine and isoleucine tRNA gene, which would meet our objective of a PCR-based diagnostic test.

Clones of the AEU + IEL products were obtained in both orientations for three isolates of *X. albilineans*, one isolate of *X. albilineans* subsp. *paspali*, and one isolate each of *X. campestris* pv. *badrii*, pv. *malvacearum*, pv. *manihotis* and pv. *vasculorum*. The complete sequence of the *X. albilineans* tRNA^{ala}-tRNA^{ile} region is shown in Fig. 3, along with the primers designed to amplify subsequences of this region. All sequences were divided into three sections; alignments of all known bacterial tRNA^{ala} genes, including the one we have sequenced here, are shown in Fig. 1(a) and all tRNA^{ile} genes in Fig. 1(b). The spacers sequenced in this study are presented in Table 2.

All *X. albilineans* isolates included a spacer region of 14 bases while the spacer region in the *X. campestris* pathovars varied in length from 17 to 66 bases (Table 2). The 66 bp spacer sequence from *X. campestris* pv. *badrii* shared over 60% sequence identity with the 73 bp spacer between these genes in *Stenotrophomonas maltophilia* (S. D. Tyler, C. A. Strathdee, W. M. Johnson & K. P. Rozee, unpublished), synonym *X. maltophilia* (Palleroni & Bradbury, 1993). Alignments of tRNA^{ala} and tRNA^{ile} sequences to other bacterial sequences found in GenBank are shown in Fig. 1. Based on these alignments, additional primers, Ala1, Ala2, Ala4, Ile1 and Ile2, were designed (Fig. 3). All of these primers, except Ala4, were positioned in such a way that they had a perfect match in the tRNAs of *Xanthomonas*. Because the tRNAs are well conserved between genera, these primers also exhibited a good, but imperfect, match with the tRNAs from known *Pseudomonas* and related species. In contrast, Ala4 + Ile1 and Ala4 + Ile2 was predicted to yield an approximately 70 bp product only in *X. albilineans* because the 3' end of Ala4 lies within the spacer region, a sequence that may be unique to *X. albilineans* (Fig. 3 and Table 2).

Length polymorphisms revealed among *Xanthomonas* species

PCR using the primer pairs Ala1 + Ile2 or Ala2 + Ile2 was performed on an array of isolates including representatives of six *Xanthomonas* species, five DNA homology groups within *X. campestris* (Palleroni *et al.*, 1993; Vauterin *et al.*, 1990), two *Pseudomonas* species, one

Table 2. tRNA^{ala}-tRNA^{ile} spacer sequences from the AEU + IEL amplification products shown in Fig. 2

Species	Strain	Spacer length (bp)	Sequence (5'-3')
<i>X. campestris</i> pv. <i>badrii</i>	ATCC 11672	66	GTACGCGGAT CCTTCCGCAA GAGCCCGCAC ATCCATGTGC GCACTTTTAT GGAAGCGCAA GACTTT
<i>X. campestris</i> pv. <i>vasculorum</i>	ATCC 19301	19	TAT TGAGTGAAAA GACTTC
<i>X. campestris</i> pv. <i>malvacearum</i>	ATCC 9924	18	TT TGAGTGAAAC GACTTT
<i>X. campestris</i> pv. <i>manihotis</i>	7AS1	17	T TTGAGTGAAA CACTTT
<i>X. albilineans</i>	ATCC 29186	14	CTGAGGAC GACATT
<i>X. albilineans</i>	CTC193	14	CTGATGAC GACTTT
<i>X. albilineans</i>	CTC393	14	CTGATGAC GACTTT
<i>X. albilineans</i>	CTC493	14	CTGATGAC GACTTT

Fig. 4. Length polymorphisms produced using Ala1+Ile2 and Ala2+Ile2 primers for representative *Xanthomonas*, *Pseudomonas*, *Acidovorax* and *Stenotrophomonas* isolates. Lanes: 1, *MspI*-digested pBR322 DNA; 2, no DNA control; 3, *X. albilineans*; 4, *X. axonopodis*; 5, *X. campestris* pv. *campestris*; 6, *X. campestris* pv. *phaseoli*; 7, *X. campestris* pv. *undulosa*; 8, *X. oryzae* pv. *oryzicola*; 9, *X. campestris* pv. *pelargonii*; 10, *X. campestris* pv. *celebensis*; 11, *X. campestris* pv. *vasculorum*; 12, *X. fragariae*; 13, *S. maltophilia*; 14, *X. oryzae* pv. *oryzae*; 15, *A. avenae*; 16, *P. rubrisubalbicans*; 17, *P. syringae* pv. *lapsa*. Reactions were as described in Methods and included PerfectMatch and about 1 ng bacterial genomic DNA. The thermal profile was 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s for 40 cycles. Products were resolved on a 6% denaturing polyacrylamide gel.

Acidovorax and one *Stenotrophomonas* species. Similar patterns of PCR products were observed among isolates for both primer pairs (Fig. 4).

Length polymorphisms found in *X. albilineans* and *S. maltophilia* isolates were distinct from those of other *Xanthomonas* species. The length of the product observed in *X. campestris* pv. *undulosa* was greater than for other *X. campestris* pathovars, and was similar in size to the *S. maltophilia* product. These data suggest that *X. campestris* may be comprised of more than one species, as noted by Hildebrand *et al.* (1990) based on DNA:DNA hybridization studies, assuming that tRNA spacer length is species-specific.

A potential diagnostic test for *X. albilineans*

Sensitivity. For quarantine-testing, the sensitivity of the PCR reactions is important, especially for *X. albilineans* because there can be a latency phase in the host (Ricaud & Ryan, 1989). The sensitivity of PCR using the potentially *X. albilineans*-specific primer pair Ala4+Ile2 was determined by titration of the bacterial DNA template in the presence of an excess of plant genomic DNA. For this study, purified genomic DNA from *X. albilineans* CTC193, at 30 ng, 3 ng, 0.3 ng, 30 pg, 3 pg and 0.3 pg per reaction was mixed with 50 ng sugarcane genomic DNA. A 70 bp product was observed for all *X. albilineans* + sugarcane templates tested, while no product was observed in the sugarcane-only control reaction (data not shown). This ability to detect approximately 180 genomes (calculated assuming a genome size of 5×10^6 bp) is typical of PCR. Given that most bacteria contain more than one copy of the genome, less than 180 bacterial cells can probably be detected using this method.

Although these experiments used mixtures of purified

genomic DNA, the results indicate that PCR with these primers is much more sensitive than most immunological methods. Even the most sensitive monoclonal antibody protocols require bacteria in culture to reach sensitivities of 5000 cells (e.g., Tsai *et al.*, 1990). In infected sugarcane material, only levels of *X. albilineans* above 100000 cfu ml⁻¹ can be detected (Comstock & Irey, 1992).

Ala4+Ile2 was also useful in detecting *X. albilineans* in sugarcane tissues. DNA was extracted from mature leaves of field-grown plants inoculated with *X. albilineans* and from plants not infected by this bacterium. Templates of 150, 15, 1.5 and 0.15 ng DNA from asymptomatic leaf tissue and 180, 18, 1.8 and 0.18 ng DNA from symptomatic leaf tissue were used in PCR with Ala4+Ile2 (Fig. 5). Even after only 30 cycles of PCR, the 70 bp product was observed in the symptomatic leaf samples with template masses of 180 and 18 ng; the 1.8 ng template yielded a very faint band not visible in reproductions (Fig. 5) but was prominent at 40 cycles (data not shown). One-hundred-and-eighty nanograms of DNA represented a 5 mg sample of leaf. A 70 bp product was also observed in reactions of *X. albilineans* isolate LA334 cultured from the symptomatic leaves. The amount of *X. albilineans* in this sample has been quantified as 10% by mass and is detectable down to 1000 *X. albilineans* genomes in a 12 pg sample of total infected sugarcane DNA from 0.5 µg leaf (R. J. Honeycutt & M. McClelland, unpublished data). Incorporation of a radioactive nucleotide during PCR allowed even more sensitive detection (data not shown).

When PerfectMatch is included in the reaction, a faint product of about 250 bp is observed only in lanes that give a 70 bp product (Fig. 5). This 250 bp product requires both the Ala4 and the Ile2 primers and is not seen when PerfectMatch is omitted (data not shown). PerfectMatch is a DNA binding protein prepared from

Fig. 5. PCR amplification from the DNA of sugarcane infected with *X. albilineans* (symptomatic) and uninfected sugarcane (asymptomatic) using the primer pair Ala4 and Ile2. DNA template concentrations are as annotated. Reactions were as described in Methods and included PerfectMatch. The thermal profile was 94 °C for 20 s and 65 °C for 20 s for 30 cycles.

E. coli. We suspect the 250 bp product is a cross-over PCR event between the 70 bp *Xanthomonas* product and a tRNA gene sequence contaminant in the PerfectMatch, presumably *E. coli* DNA. Most likely the other end of this product is primed in *E. coli* DNA on the conserved Ile2 primer. Fortunately, the 250 bp product is of no consequence for this study.

Ideally, for a species-specific diagnostic test, all *X. albilineans* strains should be identical in the part of the tRNA^{ala}-spacer-tRNA^{ile} region targeted by the Ala4 primer. To screen for possible major differences between strains of this species, a single-stranded conformation polymorphism gel (SSCP, Hayashi, 1991) was used to resolve products amplified with Ala4 + Ile2 from the four *X. albilineans* (MSRI) isolates from Mauritius, isolate CTC293 from Brazil, isolate LA334 from Louisiana, the three CTC isolates from Brazil and ATCC 29186. All isolates yielded products of the same mobility (data not shown), except ATCC 29186, which differs by 2 bases in length relative to the three Brazilian isolates (Table 2). ATCC 29186 was isolated from *Paspalum dilatatum* in Mauritius and is designated subsp. *paspali*, whereas all other *X. albilineans* were isolated from sugarcane.

SSCP is sufficiently sensitive for our inference that all strains from sugarcane are probably identical or very nearly identical in this region, as desired for this diagnostic test. Nevertheless, the *X. albilineans* isolates used in this study originated either in Brazil, Mauritius, Florida or Louisiana. They do not represent the worldwide genetic diversity of *X. albilineans*, or even that found within their countries of origin, although variation that would affect Ala4 or Ile2 annealing is unlikely. Confirmation of PCR

Fig. 6. PCR amplification from the DNA of *Xanthomonas* isolates using the primer pair Ala4 and Ile2. Reactions were as described in Methods. All reactions included PerfectMatch, 25 ng genomic sugarcane DNA and 1 ng bacterial DNA. The thermal profile was 94 °C for 20 s and 70 °C for 20 s for 40 cycles. Lanes: 1, 1 kb standard; 2, sugarcane only; 3–16, *X. albilineans* strains (CTC193, CTC293, CTC393, CTC493, MSRI3201, MSRI3284, MSRI3295, MSRI2905, XaOD1, XaOD3, XaCP2, XaCP3, XaCP4 and ATCC 29186, respectively); 17, *X. axonopodis* (ATCC 19312); 18, *X. campestris* pv. *campestris* (B293); 19, *X. campestris* pv. *phaseoli* (XP200); 20, *X. campestris* pv. *undulosa* (ATCC 35935); 21, *X. oryzae* pv. *oryzicola* (XO111); 22, *X. campestris* pv. *pelargonii* (XP15); 23, *X. campestris* pv. *celebensis* (XC145); 24, *X. campestris* pv. *holcicola* (ATCC 13461); 25, *X. campestris* pv. *vasculorum* (ATCC 13901).

with our recommended primers as a routine diagnostic tool will only come as a larger number of isolates from a broader geographical range are tested. The test is to be disseminated to sugarcane research stations for this purpose.

Specificity. The specificity of Ala4 + Ile2 was tested using 15 isolates of *X. albilineans*, as well as representatives of the closest relatives of *X. albilineans* that are also pathogens of sugarcane (Bradbury, 1984): one isolate of *X. campestris* pv. *vasculorum*, one isolate of *P. rubrisubalbicans*, two isolates of *A. avenae* subsp. *avenae* (formerly *P. rubrilineans* Willems *et al.*, 1992) and one isolate of *P. syringae* pv. *lapsa*. In addition, species from most major branches of the bacterial phylogenetic tree were analysed (Table 1). All reactions included genomic DNA from sugarcane.

As shown in Fig. 6, the 70 bp product was generally amplified only from *X. albilineans* isolates; no amplification occurred in the sugarcane-only control except the primer dimer product at about 35 bp. The primer dimer, when it occurs, confirms that the PCR reaction was viable and acts as a convenient positive control for PCR in samples that do not have bacterial DNA.

Isolates of other *Xanthomonas* species and *X. campestris* pathovars (listed in Table 1) were tested and, in general, again no amplification was observed (data not shown). However, a faint product of about 70 bp was sometimes seen in samples containing *X. campestris* pv. *vasculorum* (Fig. 6, ATCC 13901) and in two *Pseudomonas* and one *Acidovorax* species (data not shown). These products did not always occur and when they did occur they were always faint. These PCR products also required the presence of many hundreds of thousands of genomes to

Fig. 7. PCR in the 16S rRNA–23S rRNA spacer region. (a) Diagram of the 16S and 23S region showing the location of tRNA^{ala} and tRNA^{ile}. The location and orientation of the primers are shown that yield products in the nested reactions in (b) and (c). (b) 16S+23S, 16S+Ile1 and Ala1+23S reactions separated on a 2% agarose gel. Reactions were done as described in Methods. The thermal profile was 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s for 40 cycles. Lanes: 1, 1 kb standard; 2, *X. albilineans* CTC193; 3, *X. campestris* pv. *vasculorum* ATCC 13901; 4, *P. rubrisubalbicans* ATCC 13908. (c) Ala4+Ile2 nested reactions using as templates 1 µl of a 1/1000 dilution of the 16S+Ile1 or Ala1+23S products shown in (b). Lanes as for (b). The reactions were done as described in the Methods and included PerfectMatch. The thermal profile was 94 °C for 20 s and 70 °C for 20 s for 40 cycles.

be detected in 40 cycles (data not shown). Nevertheless, these products could not be completely eliminated even at the highest stringency. Presumably these products derived from inefficient PCR at adjacent tRNA genes that partly but imperfectly matched the primers. While detection of those other species that are pathogenic on sugarcane is equally desirable, it would be better to have a method to distinguish faint positive results due to low levels of *X. albilineans* versus heavier infections from these other possible sources. A strategy to enhance specificity was devised using nested PCR, as explained below.

tRNA^{ala}–spacer–tRNA^{ile} is in a 16S–23S rRNA spacer region

The order of tRNA genes is generally conserved within a bacterial genus and sometimes, between related genera (e.g. Jinks-Robertson & Nomura, 1987). Among the closest relatives of the genus *Xanthomonas*, the available sequence data showed a locus in all *Pseudomonas* species examined in which the tRNA^{ile} and tRNA^{ala} occurred embedded in the 16S–23S rRNA gene spacer region, but the tRNAs occurred in the opposite order to that found in *Xanthomonas*: 16S–tRNA^{ile}–tRNA^{ala}–23S (S. D. Tyler, C. A. Strathdee, W. M. Johnson & K. P. Rozee, unpublished; GenBank accession number L28166). Indeed, the organization we observed in *Xanthomonas*, tRNA^{ala}–spacer–tRNA^{ile}, was not observed in any of the tRNA gene arrays that have been characterized in eubacteria, except *S. maltophilia* where tRNA^{ala}–spacer–tRNA^{ile} was also located between the 16S rRNA and 23S rRNA genes (S. D. Tyler, C. A. Strathdee, W. M. Johnson & K. P. Rozee, unpublished; GenBank accession numbers L28148–L28165). Given that this related genus contained the gene order 16S–tRNA^{ala}–tRNA^{ile}–23S, we suspected that the same order may occur in *Xanthomonas*. To test this possibility for *X. albilineans*, PCR and nested PCR reactions were performed as outlined in Fig. 7(a). The primary product observed using universal 16S and 23S rRNA gene primers on the genome of *X. albilineans* was about 1080 bp (Fig. 7b, lane 2). When PCR was performed using a universal 16S primer in combination with the Ile1 primer, or the Ala1 primer in combination with a universal 23S primer, the most prominent products observed were 340 bp and 815 bp, respectively, in *X. albilineans* (Fig. 7b, lanes 5 and 8). If the order of genes is 16S–tRNA^{ala}–tRNA^{ile}–23S, then these products should be amplified by PCR with the nested primers Ala4+Ile2 as was indeed the case (Fig. 7c, lanes 1 and 3). Nested PCR can be expected to be more sensitive than a single PCR reaction so it is entirely possible that 10 or fewer molecules of *X. albilineans* might be detected with this nested PCR assay in the presence of a vast excess of sugarcane DNA.

For species other than *X. albilineans*, the faint PCR products seen when using Ala4+Ile2 on total genomic DNA probably do not derive from the 16S+23S region. The order of tRNA^{ala} and tRNA^{ile} genes is inconsistent with all characterized *Pseudomonas* rRNA spacers and the sequence we have for *X. campestris* pv. *vasculorum* matches very poorly with Ala4 at the critical 3' end. Presumably,

these faint products derive from one of the approximately 100 other tRNA genes organized in cistrons throughout a typical eubacterial genome, among which two or three other copies of each of the tRNA^{ala} and tRNA^{ile} genes can be expected. Thus, one can expect that after PCR of the 16S + 23S spacer region only *X. albilineans* will present an abundant template for Ala4 + Ile2. This was indeed the case as shown for two examples in Fig. 7(c), lanes 2, 3, 5 and 6.

In summary, the length of the product from the consensus tRNA primer pair AEU + IEL distinguishes *X. albilineans* from other *Xanthomonas* species. Pairs of primers based on these adjacent tRNAs gave PCR products that showed length variation that distinguished some *Xanthomonas* species and pathovars. When one primer included a *X. albilineans*-specific portion of the tRNA^{ala}-tRNA^{ile} spacer region, a PCR product was obtained in *X. albilineans* which allowed sensitive detection of very low levels of *X. albilineans* alone, or in the presence of sugarcane genomic DNA, and in infected sugarcane. Nested PCR produced a diagnostic test highly specific for *X. albilineans*.

The strategy presented could be used to find taxon-specific length polymorphisms at other tRNA loci or in other genera of bacteria. This strategy also has the advantage over other PCR-based methods in that not only is a PCR product present only in a set of related species, but also its size can be taxonomically informative.

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NOTE ADDED IN PROOF

The specificity of the primer Ala4 and its localization in the ribosomal spacer region has now been utilized in another study for 58 strains of *X. albilineans* (Yong-Bao Pan, USDA-ARS Sugarcane Research Unit, Houma, LA, USA, personal communication).

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