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The use of subtractive hybridization to obtain a DNA probe specific for *Pseudomonas solanacearum* race 3

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Summary. *Pseudomonas solanacearum*, the causal agent of bacterial wilt, has been classified into three races based on host range and into five biovars based on physiological properties. Strains of race 3 belong exclusively to biovar 2 and primarily affect potatoes. Although this race is thought to have originated in the Andean highlands, it has unusual physiological properties that make it a potential threat to potatoes grown at the cooler latitudes worldwide. Consequently, there is need for a rapid and sensitive method for detection of race 3 strains. We have used subtractive hybridization to enrich for race 3-specific DNA sequences in total race 3 genomic DNA, and thereby obtained a 2 kb clone homologous to DNA from all 28 race 3 strains tested, but with only five of 90 non-race 3 strains. In addition, two larger regions of the genome, containing a minimum of 23 kb of DNA, are also specific for race 3. Deletion of this DNA did not affect virulence. This race 3-specific DNA is a potentially useful diagnostic tool for the detection of race 3 strains.

Key words: Bacterial wilt – *Pseudomonas solanacearum* – DNA probe – Potato – Subtractive hybridization

Introduction

Bacterial wilt, caused by *Pseudomonas solanacearum* E.F.Sm., is one of the most important plant diseases in tropical and subtropical areas of the world. The species has been classified into three races based on host range (Buddenhagen et al. 1962), and five biovars based on the ability of the bacterium to oxidize various sugars and sugar alcohols (Hayward 1964). Two groups of strains, included in races 1 and 3, are able to infect potatoes. Race 1 is composed of strains with a wide host range that are primarily a problem in the warm, tropical and subtropical areas of the world. Race 3, on the other hand, is composed of strains that are prevalent on potatoes grown at cooler temperatures (Buddenhagen 1985; Seneviratne 1969; Ciampi and Sequeira 1980). For example, in tropical highland areas, where cool temperatures predominate, bacterial wilt caused by race 3 can reach epiphytotic proportions (Thurston 1963; Seneviratne 1969). Thus race 3 may be a problem in climates where *P. solanacearum* is not normally found, as was demonstrated by the outbreak of bacterial wilt on potatoes in southern Sweden in 1976 (Olson 1976).

Race 3 is thought to have evolved in the Andean highlands of Colombia and Peru, where tuber-bearing *Solanum* species grow wild and were first cultivated by man. However, strains of race 3 are common in potatoes grown in Africa, Europe, Australia and Asia, and some researchers believe that this race is indigenous to these areas as well (Seneviratne 1969; Buddenhagen 1985). Recently, Cook et al. (1989) reported that DNAs from race 3 strains, regardless of geographic origin, had very similar restriction fragment length polymorphism (RFLP) patterns. Other researchers have also indicated that race 3 is a homogeneous group, based on studies of carbon source utilization (Harris 1972). The high degree of similarity among race 3 strains, therefore, supports the contention that its world-wide distribution is due to transport of infected potato tubers from South America by man.

By comparison with other strains of *P. solanacearum*, strains of race 3 have an unusually narrow host range, being limited almost entirely to potatoes. They can remain latent in potato tubers, making their detection difficult and greatly increasing the risk of disseminating the pathogen via tubers used for planting. It is not surprising, therefore, that race 3 is considered a serious threat to potato cultivation in temperate zone countries. Consequently, there is a need for a rapid and sensitive diagnostic assay to detect race 3 in potatoes being shipped from one location to another. For example, race

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1 is prevalent in the southern United States and, in certain states (i.e. Florida), it causes severe problems for potato growers. Presently, the practice of planting potatoes in early spring, when moderately low temperatures prevail, minimizes the losses due to infection by race 1. However, if race 3 were introduced, bacterial wilt could become a serious problem even early in the growing season.

Other diseases of potato, caused by unrelated pathogens, are easily mistaken for bacterial wilt. These include, the wilt diseases caused by species of *Fusarium* and *Verticillium*, blackleg caused by pathovars of *Erwinia carotovora*, and ringrot caused by *Clavibacter michiganense* subsp. *sepedonicum*. Methods for detection of *P. solanacearum* based on monoclonal or polyclonal antisera lack specificity for individual races of *P. solanacearum* (E. Barlow, unpublished data; Morton et al. 1966). Biochemical tests can discriminate accurately biovar 2 strains from other strains of *P. solanacearum*, but these tests require the isolation and purification of the bacterium followed by a 2 to 3 week assay period. DNA probes that are specific for a number of pathogens have been identified, including *Mycoplasma pneumoniae* and *M. genitalium* (Hyman et al. 1987), *Shigella* and *Escherichia coli* (Wood et al. 1986), *Plasmodium falciparum* (Barker et al. 1986), and *Clavibacter michiganense* subsp. *sepedonicum* (Johansen et al. 1989). Most of these studies involved the use of random screening of gene libraries to identify pathogen-specific clones. In the present study we have improved on these methods by the use of subtractive hybridization to enrich for DNA fragments that are specific for *P. solanacearum* race 3, and ultimately to obtain a race 3-specific DNA clone. Furthermore, since it was possible that race-specific DNA might encode information required for disease, we deleted the race-specific DNA region and assessed the effect of the deletion on virulence.

Materials and methods

Bacterial strains and plasmids. The origins and characteristics of bacterial strains and plasmids are listed in Table 1.

Media and antibiotics. P. solanacearum strains were cultured routinely at 28° C on liquid CPG medium (Hendrick and Sequeira 1984) or on TZC medium (CPG containing 1.8% agar and 0.05% 2,3,5-triphenyltetrazolium chloride; Kelman 1954). *Escherichia coli* strains were grown on LB medium (Miller 1972) at 37° C. For transduction experiments, bacteria were cultured on LB supplemented with 0.2% maltose and 10 mM $MgSO_4$. Culture media were supplemented with the following antibiotics when required: kanamycin (Km), 25 μg/ml; ampicillin (Ap), 50 μg/ml for *E. coli* or 200 μg/ml for *P. solanacearum* UW23; tetracycline (Tc), 15 μg/ml.

General DNA manipulations. Procedures for general DNA manipulations, including agarose gel electrophoresis, Southern blotting, colony blotting and plasmid DNA isolation have been described elsewhere (Ausubel et al. 1988). Chromosomal DNA was isolated from *P. solanacearum* as described by Cook et al. (1989). Southern blot hybridization and subsequent washes were completed by the method of Amasino (1986) except that the hybridization solution was modified to omit NaCl and polyethylene glycol (S. Leong, personal communication). Probe DNA was labeled with [^{32}P]dATP either by nick translation or random primer labeling according to manufacturer's instructions with kits purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and Promega Corporation (Madison, Wis.), respectively.

Subtractive hybridization. For subtractive hybridization, strain K60 DNA or strain UW23 DNA was purified by CsCl density gradient centrifugation, digested with the restriction enzyme *Eco*RI, and then extracted with phenol:chloroform (1:1) and chloroform, and precipitated with cold ethanol. The UW23 DNA (0.075 μg) was labeled with [^{32}P]dATP by the random primer method, denatured in a boiling water bath for 2 min, and combined with similarly denatured, unlabeled K60 DNA (75 μg). The combined DNAs were then allowd to reanneal at 42° C for 6 h in a 600 μl volume of hybridization buffer (Amasino 1986) containing 0.25 M dibasic sodium phosphate adjusted to pH 7.2 with phosphoric acid, 1 mM EDTA (pH 8.0), 7% sodium dodecylsulphate, and 50% formamide. This preparation was used directly as probe by adding it to Southern blots of K60 and UW23 genomic DNAs.

Construction of a genomic library of strain UW23. A cosmid library of *P. solanacearum* strain UW23 was prepared essentially as described by Maniatis et al. (1982). Total genomic DNA was isolated from strain UW23, as described by Cook et al. (1989), and purified by CsCl density gradient centrifugation. Purified DNA was partially digested with the restriction enzyme *Sau*3A, and DNA fragments were separated according to size by centrifugation in a 10–40% sucrose gradient (26000 rpm for 20 h in a Beckman SW27 rotor). DNA fragments of 25 to 35 kb in size were recovered from the fractionated sucrose gradient and ligated to *Bam*HI-digested, alkaline-phosphatase-treated pLAFR3 or pLAFR5. The concatamerized DNA was packaged into lambda phage heads with Gigapack Plus (Stratagene) in vitro packaging extracts, and then used to transduce *E. coli* DH5α cells. Transductants were selected by the acquisition of resistance to tetracycline.

Transformation and marker exchange in P. solanacearum. Transformation-competent cells were prepared from log phase CPG cultures at an O.D. of 0.4 to 0.6 at 600 nm. Bacterial cells were pelleted and washed four times by repeatedly centrifuging and resuspending them in sterile Milli-Q water. The final bacterial pellet was resuspended in 20% glycerol at 1/100th of the original volume before storing at −80° C. Competent cells (40 μl) were mixed with 0.1 to 1.0 μg plasmid DNA and transformed by electroporation in an electric field of 10 kV/cm with a

Table 1. Bacterial strains and plasmids

UW #	R	Bv	Location	Host	RG #	Source
Pseudomonas solanacearum						
UW23	3	2	Israel	potato	26	Volcani
T334	BBD		Jonggol, W. Java	banana		Green
T384	BBD		Bontosunggu, S. Sulawesi	banana		Green
T394	BBD		Wajo, S. Sulawesi	banana		Green
T440	BBD		Paniki, N. Sulawesi	banana		Green
T394A	BBD		Wajo, S. Sulawesi	banana		Green
UW21	2	1	Honduras	banana		Buddenhagen
UW36	1		Coto, Costa Rica	*Physalis*		Sequeira
UW42	2	1	Honduras	banana		Sequeira
UW210	1	1	N. Carolina, USA	tobacco		Hisada
UW263	1	1	Yurimaguas, Peru	potato		French
UW272	1	1	Turrialba, Costa Rica	potato		Sequeira
UW72	3	2	Greece	potato		Panagopoulos
UW220a	3	2	Poona, India	potato		Kelman
UW224	3	2	Embu, Kenya	potato		Harris
UW225	3	2		potato		Harris
UW257	3	2	Chicoa, Costa Rica	potato		Gonzalez
UW262	3	2	Cajamarca, Peru	potato		French
UW276	3	2	Mexico	potato		Fucikovsky
CIP180	3	2	Pangalengan, Java	potato		French
CIP181	3	2	Lambang, W. Java	potato		French
CIP265	3	2	Sumber Bromtas, E. Java	potato		French
CIP263	1	3	Jambegede, Java	peanut		French
CIP266	1	3	Cikeumeuh, Bogor, Java	potato		French
CIP264	1	4	Jambegede, Java	potato		French
0228	1	4	Queensland, Australia	ginger		Hayward
0284	1	4	Queensland, Australia	ginger		Hayward
0211	1	4	Queensland, Australia	ginger		Hayward
0249	3	2	Queensland, Australia	potato		Hayward
0223a	3	2	Wa., Australia	potato		Hayward
015a	3	2	Queensland, Australia	potato		Hayward
01058	3	2	Victoria, Australia	potato		Hayward
01059	3	2	Victoria, Australia	potato		Hayward
01018r	3	2	New S. Wales, Australia	potato		Hayward
01021a	3	2	New S. Wales, Australia	tomato		Hayward
01020as	3	2	New S. Wales, Australia	tomato		Hayward
01022	3	2	New S. Wales, Australia	potato		Hayward
01020ar	3	2	New S. Wales, Australia	tomato		Hayward
0239s	1	3	Queensland, Australia	chilli		Hayward
0190	1	3	Queensland, Australia	*X. pungens*		Hayward
0369a	1	3	New S. Wales, Australia	tomato		Hayward
001	1	3	Queensland, Australia	tomato		Hayward
0170a	1	3	Queensland, Australia	tobacco		Hayward
0171b	1	3	Queensland, Australia	*S. melongena*		Hayward
0131a	1	3	Queensland, Australia	potato		Hayward
0158	1	3	Queensland, Australia			Harris
012bs	1	3	New S. Wales, Australia	*R. rososum*		Hayward
010	1	3	Queensland, Australia	tomato		Hayward
0672	1	3	Queensland, Australia	zinnia		Hayward
01023s	1	3	New S. Wales, Australia	*S. reginae*		Hayward
01017s	1	3	Australia	*S. nigrum*		Hayward
0234	1	3	Queensland, Australia	*P. villosa*		Hayward
0256	1	4	Queensland, Australia	ginger		Hayward
0263	1	4	Queensland, Australia	ginger		Hayward
0279a	1	4	Darwin, Australia	tomato		Hayward
0224	1	4	Queensland, Australia	ginger		Hayward
003	1	4	Queensland, Australia	ginger		Hayward
0319	1	4	Queensland, Australia	*S. nigrum*		Hayward
007	1	4	Queensland, Australia	ginger		Hayward
043	1	4	Queensland, Australia	tomato		Hayward
CIP259	3	2	Munanira, Burundi	potato		French
UW39	1	1	Puerto Rico	*Zebrina*		Kelman
UW127	2	1	Peru	plantain	25	Sequeira
UW136	2	1	Costa Rica	*Heliconia*	24	Berg
021BR	3	2	Queensland, Australia	potato		Hayward

Table 1 (continued)

UW#	R	Bv	Location	Host	RG#	Source
015	3	2	Queensland, Australia	potato		Hayward
075	1	4	Queensland, Australia	ginger		Hayward
CIP77	1	1	Yurimaguas Loreto, Peru	potato		Gutarra
CIP120	1	1	La Chinaca, Peru	potato		Pinedo
CIP128	1	1	San Ramon Junin, Peru	potato		Gutarra
CIP137	1	1	San Ramon Junin, Peru	potato		de Lindo
CIP176	1	1	Yurimaguas Loreto, Peru	potato		Linde
CIP218	1	1	Divinolandia, Brazil	potato		Neto
CIP235	1	1	CNPH, Brazil	potato		EMBRAPA
CIP239	1	1	CNPH, Brazil	potato		Reifschneider
CIP267	1	1	San Ramon Junin, Peru	potato		French
CIP301	1	1	San Miguel, Peru	potato		Nydegger
CIP117	3	2	Nigeria	potato		Graham
CIP162		N2	San Ramon Junin, Peru	soil	32	Martin
CIP221	3	2	B. Paulista, Brazil	potato		Neto
CIP300	3	2	San Miguel, Peru	potato		Nydegger
CIP296		N2	Nigeria	potato		NCPPB1703
CIP61		N2	Yurimaguas Loreto, Pru	potato	29	Turkensteen
CIP172		N2	La Chincana Junin, Peru	potato	32	Martin
CIP175		N2	Yurimaguas Loreto, Peru	potato	29	Linde
CIP211		N2	Itatiba, Brazil	potato	30	Neto
CIP217		N2	San Paulo, Brazil	potato		Neto
CIP223		N2	Cristalina, Brazil	potato	32	Neto
CIP224		N2	Brasilia, Brazil	potato		Neto
CIP226		N2	Santa Juliana, Brazil	potato	33	Neto
CIP240		N2	CNPH, Brazil	potato		Reifschneider
CIP312		N2	La Chincana, Peru	eggplant	31	Aley
UW25	1	1	United States	tomato	1	Kelman
UW26	1	1	United States	tomato	2	Kelman
UW278	1	1	Mexico	tobacco	3	Fucikovsky
UW256	1	1	Costa Rica	potato	4	Gonzalez
UW275	1	1	Costa Rica	*M.p.*	5	Sequeira
UW154	1	1	Colombia	tobacco	6	Granada
UW90	1	1	Brazil	tobacco	7	Robbs
UW255	1	3	Costa Rica	pepper	8	Gonzalez
UW130	1	3	Peru	tomato	9	Sequeira
UW152	1	3	Australia	potato	10	Hayward
UW380	1	3	China	olive	11	He
UW147	1	3	Australia	tobacco	12	Hayward
UW8	1	3	Costa Rica	*E.o.*	13	Sequeira
UW119	1	3	Costa Rica	potato	14	Gonzalez
UW74	1	4	Sri Lanka	potato	15	n.a.
UW375	1	4	China	peanut	16	He
UW27	1	4	United States	tobacco	17	Kelman
UW378	1	4	China	olive	18	He
UW361	1	5	China	mulberry	19	He
UW373	1	5	China	mulberry	20	He
UW141	1	4	Australia	ginger	21	Hayward
UW151	1	4	Australia	ginger	22	Hayward
UW359	1	4	China	ginger	23	He
UW139	2	1	Costa Rica	plantain	24	Berg
UW162	2	1	Peru	plantain	25	Sequeira
UW73	3	2	Sri Lanka	potato	26	n.a.
UW80	3	2	Colombia	potato	27	Thurston
UW20	2	1	Venezuela	banana	28	Buddenhagen

Other bacteria:

G1	*Xanthomonas campestris phaseoli* var. *fuscans*	Gilbertson
B301D	*Pseudomonas syringae* pv *syringae*	Willis
61	*P. syringae* pv. *syringae*	Willis
B3A	*P. syringae* pv. *syringae*	Willis
B728A	*P. syringae* pv. *syringae*	Willis
CIAT 899	*Rhizobium* spp.	Handelsman
DH5a	*Escherichia coli*	

Table 1 (continued)

Plasmids:			Source
pK1358	*hrp* genes from UW25	Tc^r	P. Xu
pBS2338	from UW23	Ap^r	This study
p3i58	UW23 cosmid clone	Tc^r	This study
p5A89	UW23 cosmid clone	Tc^r	This study
p5C58	UW23 cosmid clone	Tc^r	This study
p3H27	UW23 cosmid clone	Tc^r	This study
pBS23D	UW23 deletion clone	Km^r, Ap^r	This study
pUC4K		Km^r, Ap^r	Pharmacia LKB Biotechnology, Inc, Pleasant Hill, CA.

R, race; Bv, biovar; RG#, RFLP group; N2, new biovar 2 (lowland strains); n.a., not available; *X. pungens, Xanthium pungens*; *S. melongena* (eggplant), *Solanum melongena; R. rososum, Rapistum rososum; S. reginae, Strelitzia reginae; S. nigrum, Solanum nigrum; P. villosa, Pultenaea villosa; M.p., Melampodium perfoliatum; E.o., Eupatorium odaratum*

capacitance of 14 μF and an external resistance of 400 Ω. Prior to plating on selective CPG medium, the electroporated cells were incubated for 4 h at 28° C in CPG broth without antibiotics.

Potential marker exchange mutants were identified by screening transformed cells for acquisition of kanamycin resistance but not of the vector-derived ampicillin resistance. Marker exchange, resulting from double homologous crossover events, was confirmed by Southern blot analysis of genomic DNA.

Virulence assays. For virulence assays, eggplant (*Solanum melongena* L. cv. Black Beauty) seeds were surface-sterilized by successivly washing with 95% ethanol (1 min), 1.6% sodium hypochlorite (3 min), and three rinses with sterile distilled water. The treated seeds were placed on sterile, water-saturated filter paper discs in petri plates, and incubated for 2 days at 28° C. Germinated seeds were placed in 17 × 150 mm culture tubes containing 10 ml Hoagland's solution in 1.5% agar. Seedlings were grown in a growth chamber at 28° C under fluorescent lighting. Ten- to fourteen-day-old seedlings were inoculated by cutting the midvein of one cotyledon with scissors dipped in bacterial suspensions. Bacterial inoculum from mid to late log phase CPG broth cultures was prepared by centrifuging and resuspending the bacterial pellet in water to an O.D. of 0.5 at 600 nm. Wild-type strains, used as positive controls, caused wilt symptoms and leaf necrosis after 6 days and complete seedling collapse after 9 days.

In an alternate assay to determine the virulence of deletion mutants and wild-type strains, the intercostal tissue of fully-expanded leaves of tobacco (*Nicotiana tabacum* cv. Bottom Special) or potato (cv. Russett Burbank) was infiltrated with bacterial inoculum, prepared as described above. Tobacco plants were grown in a growth chamber under fluorescent lighting at 28° C, and potato plants were grown in an air-conditioned greenhouse maintained at 22 to 28° C. Typically, tobacco leaves inoculated with virulent strains developed confluent necrosis of the infiltrated area within 48 h, whereas in potato leaves a similar reaction occurred within 72 to 96 h.

Results

Subtractive hybridization

When total genomic DNA from the *P. solanacearum* race 3 strain UW23 was labeled with [^{32}P]dATP and used to probe Southern blots containing *Eco*RI-digested genomic DNA from *P. solanacearum* race 1 (UW25) and race 3 (UW23), both DNAs gave equally strong hybridization (Fig. 1 A). However, when total genomic DNA from UW23 was labeled to a high specific activity by the random primer method and preincubated with a 1000-fold excess of unlabeled race 1 (UW25) DNA, there was substantial preferential hybridization to the race 3 genomic DNA (Fig. 1 B). The enrichment of probe DNA for race 3-specific sequences by subtractive hybridization was dependent on the concentration of driver DNA (race 1, UW25) and on the duration of the period allowed for subtractive hybridization. When probe DNA was incubated with decreasing concentrations of driver DNA for decreasing periods of time, preferential hybridization to race 3 DNA was reduced from that illustrated in Fig. 1 B.

Cloning of a race 3-specific DNA fragment

After subtractive hybridization, numerous race 3 (UW23) restriction fragments still hybridized to the probe DNA. In particular, a region of the Southern blot containing UW23 DNA of approximately 2 kb in size retained a high degree of hybridization (Fig. 1 B). To isolate the fragment(s) responsible for this specific hybridization, the region of an agarose gel containing fragments of *Eco*RI-digested UW23 DNA between 1.8 and 2.2 kb was excised. The DNAs were recovered from the gel, ligated to *Eco*RI-digested pBluescript, and used to transform competent *E. coli* DH5α cells. Plasmid DNA was isolated from 80 transformants and, after digestion with *Eco*RI and agarose gel electrophoresis, was found to contain insert DNAs distributed in size between 1.6 and 2.2 kb. Ten of these clones were selected because they contained different-sized insert DNAs; they were labeled by the random primer method, and used to probe

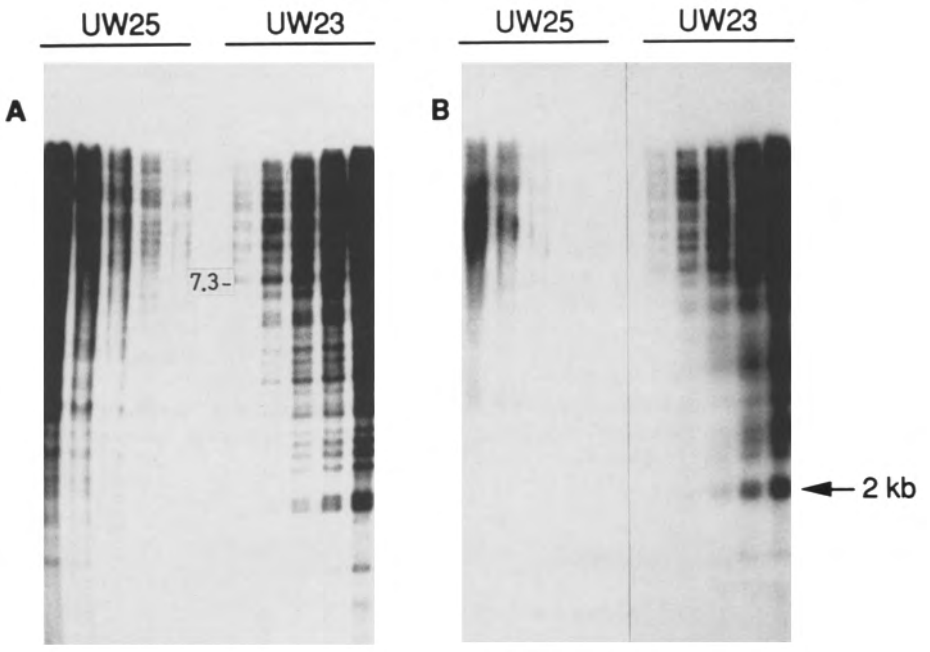

Fig. 1A and B. Southern blot of UW25 (race 1) and UW23 (race 3) genomic DNA, probed with ^{32}P-labeled UW23 genomic DNA with or without subtractive hybridization with excess UW25 DNA. UW25 and UW23 DNAs are present at comparable concentrations. Several different DNA concentrations were obtained for each strain by making a series of two-fold dilutions; the most concentrated samples were run in the outermost lanes. **A** Prior to subtractive hybridization both UW25 and UW23 DNA hybridized strongly with labeled UW23 DNA. **B** After subtractive hybridization there was a decrease in hybridization to UW25 DNA, but many UW23 DNA fragments continued to exhibit strong hybridization; of these, the region of the gel corresponding to 2 kb in size was examined further.

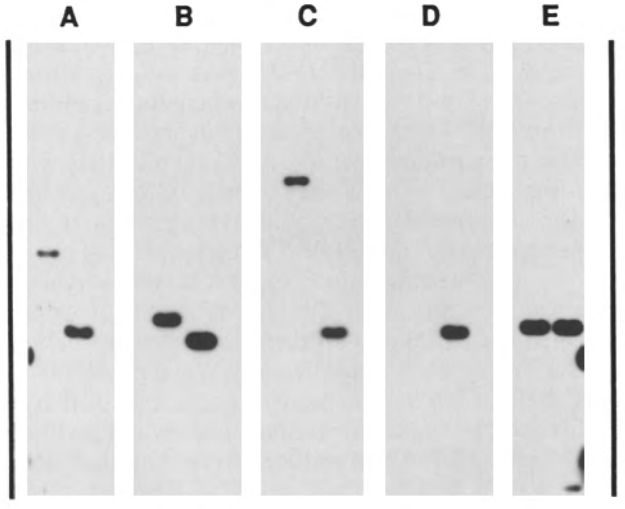

Fig. 2A–E. Screening ^{32}P-labeled UW23 (race 3) *Eco*RI clones for race 3-specific hybridization to Southern blots of UW23 and UW25 genomic DNA. Panels **A–E** represent five separate Southern blots containing equal amounts of UW25 and UW23 genomic DNA. In each blot, UW25 DNA is on the left and UW23 is on the right. The blots were probed with separate ^{32}P-labeled clones obtained from UW23. In **A**, **B**, **C** and **E**, the probes hybridized to both UW25 and UW23 genomic DNA. In panel **D**, probed with pBS2338, hybridization was detectable only to the UW23 DNA

Southern blots of race 1 (UW25) and race 3 (UW23) genomic DNAs. One clone, pBS2338, hybridized with genomic DNA from race 3 but not from race 1 (Fig. 2, lane D).

Screening strains of P. solanacearum *and other bacteria for homology to pBS2338*

To determine if the specificity of pBS2338 for strain UW23 was indicative of a broader specificity for all race 3 strains, it was used to probe a Southern blot containing *Eco*RI- and *Bam*HI-digested genomic DNAs from 61 strains of *P. solanacearum*. Among these strains were representatives of all races and biovars, including 21 race 3 strains from 14 distinct locations in nine different countries. When the blot was hybridized with the plasmid pK1358, which contains a cluster of *P. solanacearum hrp* genes identified by Boucher et al. (1988), all of the test strains showed homology to the probe DNA (Fig. 3). However, when the same blot was reprobed with nick-translated pBS2338, only the race 3 strains had detectable hybridization (Fig. 3). With all of the race 3 genomic DNAs, pBS2338 hybridized to a single, similar-sized restriction fragment.

Additional Southern blots were prepared with DNAs from 57 other strains of *P. solanacearum*, including members of all 28 RFLP groups (Cook et al. 1989), thus bringing the total number of strains of *P. solanacearum* analyzed to 118 (Table 1). Of these, the 28 that belonged to race 3 all showed homology to pBS2338. In contrast, of the remaining 90 non-race 3 strains, only five showed homology to pBS2338. There were no obvious characteristics shared in common among these five strains that might indicate a common origin: three of the five were from Peru (UW127 and UW162 from plantain, and CIP137 from potato), and one each was from Australia (UW141 from ginger) and Costa Rica (UW275 from *Melampodium perfoliatum*). Five plant-pathogenic bacteria unrelated to *P. solanacearum*, and one *Rhizobium* sp., also lacked homology with pBS2338 (Table 1).

Determining the limits of the race 3-specific DNA

To determine whether the race 3-specific DNA contained in pBS2338 was part of a larger segment of race 3-specific DNA, it was first necessary to isolate the region of the UW23 genome that contained the pBS2338 insert DNA. For this purpose, we constructed a cosmid geno-

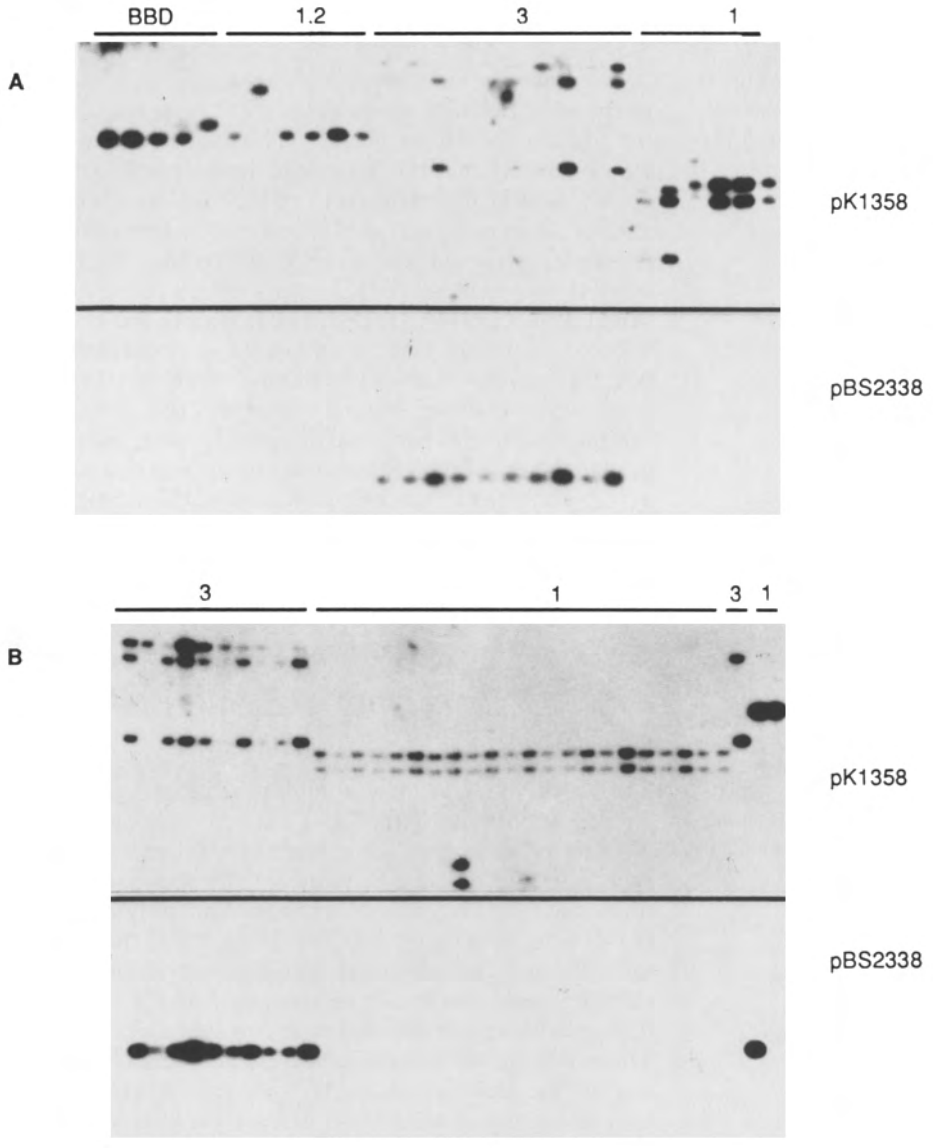

Fig. 3A and B. Southern blots of *P. solanacearum* genomic DNA from 61 different strains probed with pK1358 and pBS2338. Southern blots in panels **A** and **B** were first probed with pK1358 (upper part of **A** and **B**) and then stripped of hybridizing DNA and reprobed with pBS2338 (lower part of **A** and **B**). Strains are listed in Table 1 according to their order of appearance in the Southern blot, starting at the top left and ending at the bottom right. BBD, banana blood disease strains; 1, race 1; 2, race 2; 3, race 3

mic library of strain UW23. Preliminary analysis of plasmid DNA isolated from 18 randomly selected clones from this library indicated that all had distinct *Eco*RI restriction patterns, and that the average size of the insert DNAs was 24 kb. When colony blots of the UW23 genomic library were screened with labeled pBS2338, 12 out of 2200 colonies showed homology to the probe. Plasmid DNA was isolated from these strains and, based on a preliminary examination of *Eco*RI digestion patterns, four clones were selected for further analysis. Restriction mapping revealed that these four cosmid clones contained a series of overlapping DNA inserts spanning a total of 40 kb and encompassing the 2 kb *Eco*RI fragment of pBS2338 (Fig. 4).

The limits of the race-specific DNA were determined by isolating 12 contiguous restriction fragments from two plasmids, p3i58 and p3H27, which together span the entire 40 kb region (Fig. 4). These fragments were labeled with ^{32}P and used as probes of Southern blots containing UW23 (race 3) and UW25 (race 1) total geno-

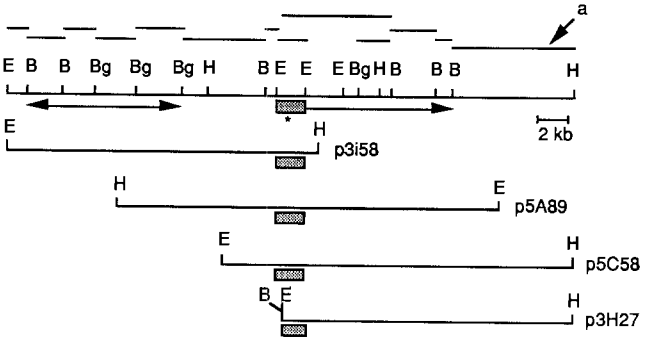

Fig. 4. The UW23 genome contains two clusters of race 3-specific DNA comprising at least 23 kb. The restriction fragments indicated at the top (*arrow* a) were used individually to probe Southern blots containing total genomic DNA from race 1 (UW25) and race 3 (UW23). Clones p3i58, p5A89 and p3H27 are cosmid clones containing race 3 DNA (Table 1). The 2 kb region cloned in pBS2338 is indicated by the *stippled box* and *asterisk*. Horizontal arrows indicate contiguous DNA fragments having homology to UW23 (race 3) and not to UW25 (race 1). E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII

mic DNAs. Two separate and continuous stretches of DNA contained within this region hybridized to UW23 DNA, but not to UW25 DNA (Fig. 4). The UW23-specific regions spanned a minimum of 23 kb of DNA, but this was interrupted by a region of approximately 6.5 kb which also had homology to UW25 DNA (race 1).

Construction of a UW23 deletion mutant

It seemed plausible that race or strain-specific DNA sequences might be required for pathogenicity. To test this hypothesis, mutants of UW23 were created by deleting most of the UW23-specific region, and then their ability to produce disease symptoms was determined. For this purpose, a deletion clone pBS23D was constructed in the pBluescript vector (Fig. 5). This clone contained the 1.6 kb *Eco*RI-*Bam*HI fragment from p3i58 and the 3.4 kb *Bam*HI fragment from p3H27, with an intervening 1.4 kb kanamycin resistance cassette from pUC4K. A single, asymmetrical *Eco*RV site within the 3.4 kb *Bam*HI fragment of p3H27 was used to determine the orientation of DNA fragments containing the deletion. When competent UW23 cells were transformed with pBS23D several Km^r Ap^s colonies were obtained. To confirm that these strains contained the deletion, a Southern blot prepared with *Eco*RI- and *Bam*HI-digested genomic DNA from these strains was probed with a ^{32}P-labeled mixture of p3i58 and p3H27. Several of these strains lacked homology to the internal 25 kb region that had been removed in the construction of pBS23D (Fig. 6), indicating that, in these strains, the wild-type region was replaced by the deletion construct.

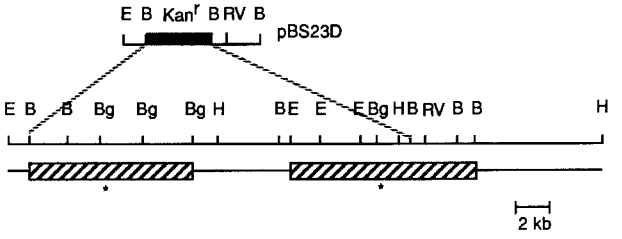

Fig. 5. Construction of a deletion clone, pBS23D, for marker exchange into UW23. Plasmid pBS23D was constructed by combining the left most *Eco*RI-*Bam*HI restriction fragment from p3H27 and the 3.4 kb *Bam*HI fragment from p3H27 (above and Fig. 4) in pBluescript. The correct orientation of the p3H27 fragment was determined using a single asymetrical *Eco*RV site contained within this fragment. The kanamycin resistance gen (kan) was obtained from *Bam*HI-digested pUC4K; it was ligated to the p3i58/p3H27 plasmid (discussed above) that had been linearized by partial digestion with *Bam*HI. E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; RV, *Eco*RV. *Hatched boxes* indicate the race 3-specific DNA illustrated in Fig. 4

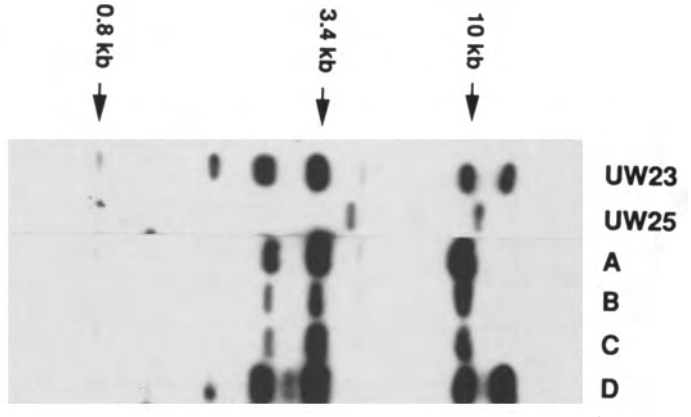

Fig. 6A–C. Southern blot analysis to confirm marker exchange of the kanamycin resistance-containing deletion of pBS23D into the UW23 genome. All DNAs were digested with *Eco*RI and *Bam*HI. The Southern blot was probed with a 1:1 mixture of ^{32}P-p3i58 and p3H27 (Fig. 4) which together contain the entire deleted region. Panels **A**, **B** and **C**, Deletion mutants; Panel **D**, This strain seems to be a cis-merodiploid of pBS23D and the wild-type region, probably the result of a single recombination event. Deletion mutants contain four restriction fragments that flank the deletion and have homology to the probe DNA (see Figs. 4 and 5); the smallest of these fragments is 0.8 kb and is difficult to visualize. Deletion mutants lack homology with all restriction fragments internal to the deletion. In the wild-type strain, UW23, there is a doublet at 3.4 kb and a triplet at the next smallest hybridizing band.

Virulence of wild-type UW23 and of UW23 deletion mutants

The wild-type UW23 was highly virulent to eggplant seedlings, both at 20 and 28° C. When deletion mutants were tested, however, some were as virulent as the wild type, but others were non-virulent. The non-virulent mutants also lacked visible extracellular polysaccharide (EPS) when grown on TZC medium, while the virulent mutants had the wild-type fluidal colony morphology. EPS deficiency could not be complemented by transformation with any of the wild-type cosmid clones in Fig. 4. There was no difference, however, between UW23 and any of the deletion mutants with regard to plant response following infiltration of bacteria into leaf tissue. Tobacco leaves that were inoculated with suspensions of either mutant or wild-type strains developed a mild chlorotic symptom after 4 to 5 days that was localized to the infiltrated tissue, as is typical of race 3. In potato leaves all of the deletion mutants, regardless of their colony morphology, caused confluent necrosis and collapse of the infiltrated area within 72 to 96 h after inoculation, reactions that were indistinguishable from those caused by the wild-type strain, UW23.

Discussion

The object of this study was to identify regions of the *P. solanacearum* genomic DNA that are associated only with race 3. Such a DNA region could be useful for the eventual development of a probe for diagnosis of race 3 strains in infested tissues. To this end, we used subtractive hybridization to enrich for race 3-specific sequences in race 3 genomic DNA. We identified a 2-kb restriction fragment from the race 3 strain, UW23, that hybridized to all 28 race 3 strains tested but to only five out of 90 non-race 3 strains. In the vicinity of this

2 kb *Eco*RI fragment we found two larger regions of DNA that also lacked homology with the race 1 strain, UW25. Even though the 2 kb fragment, cloned in pBS2338, had homology with DNA of a few strains of *P. solanacearum* other than race 3, pBS2338 is still likely to be a useful tool for detection of race 3 strains. One important factor determining the usefulness of this probe may be the frequency with which non-race 3 strains having homology to the probe occur in potato plants. Based on the present results, 95% (18 of 19) of race 1 strains from potato lack homology to pBS2338. It is not certain whether this is a fair estimate of the potential accuracy of this probe under field conditions. However, if the degree of specificity of pBS2338 for detection of race 3 is not acceptable, then other DNA fragments may exhibit the required race 3-specific homology. For example, following subtractive hybridization, Southern blot analysis indicated that the UW23 genome contained additional restriction fragments exhibiting preferential hybridization to race 3. Some of these candidate restriction fragments were found to be adjacent to the one that was cloned in pBS2338, and may themselves be suitable for diagnostic purposes.

The presence of sequences with homology to pBS2338 in a few strains other than race 3 reveals an intriguing situation: how did homology to this DNA arise in these five non-race 3 strains? Because these five strains are closely related to other *P. solnacearum* strains that lack homology to pBS2338, but only distantly related to each other and to race 3, common ancestry is an unlikely mechanism. One possible answer, however, is horizontal gene transfer. Thus, it is possible that the race 3-specific DNA of pBS2338 is part of a prophage, associated primarily with race 3, which upon induction is capable of infecting other races of *P. solanacearum*. The fact that humans have only recently moved race 3 from its origin in the Andean highlands to new locations, where it has presumably come into contact with other races, would explain the scarcity of homology for this DNA in non-race 3 strains.

Although the methods we employed were adequate to identify race 3-specific DNA fragments, there are several probable ways to improve the efficiency of subtractive hybridization. In the experiments presented here, we used UW23 (race 3) DNA that was digested with *Eco*RI prior to labeling and subtractive hybridization. Thus, many of the fragments were large and, therefore, likely to contain both race 3-specific and race 3 non-specific DNA sequences. Depending on the ratio of race 3-specific and race 3 non-specific DNA in a given restriction fragment, one might obtain inefficient removal of certain race non-specific sequences, or the unintended removal of certain race-specific sequences. Digestion with a restriction enzyme that generated smaller DNA fragments would probably circumvent this problem and increase the efficiency of subtractive hybridization.

An intriguing question with respect to race-specific DNA concerns its function. It is tempting to speculate that the unique ability of the race 3 pathogen to be virulent at high altitudes and cool temperatures, or its restricted host range, might depend on information encoded by novel DNA sequences that hybridize exclusively to race 3 strains. The fact that some deletion mutants of UW23 are still highly virulent towards eggplant seedlings at both 28 and 20° C, suggests that the deleted sequence is not essential for virulence of race 3. This has been demonstrated more recently by the ability of these deletion mutants to rapidly kill entire potato plants (data not shown). The origin of the non-virulent, EPS-deficient deletion mutants is uncertain. *P. solanacearum* strains can undergo spontaneous phenotypic shifts, in still culture, to EPS-deficient variants (Kelman and Hruschka 1973; Coplin and Cook 1990). If such a phenotypic shift occurred, this could explain the inability of wild-type cosmid clones to complement the EPS-deficient phenotype of these mutants. However, successful complementation might depend on the presence of the entire deleted region in trans, which was not possible because none of the cosmid clones we used contained the entire deleted region. Similarly, if either of the deletion endpoints bisects DNA that is required for complementation, clones would have to span the deletion endpoint in order to complement the mutant phenotype. This requirement was met only at the right border by p3H27 which contains greater than 8 kb of DNA adjacent to the deletion.

Historically, the designation "race 3" has been considered synonymous with biovar 2. When biovar 2 strains have been isolated outside of the Andean highlands it seemed likely that they had been introduced by man via infected potato tubers (Buddenhagen 1985; Cook et al. 1989). However, Martin et al. (1981) have reported the occurrence of indigenous biovar 2 strains in lowland tropical jungle areas of Peru, thus raising the possibility that race 3 may not be indigenous only the highland Andean areas. We analyzed 12 biovar 2 strains from lowland areas of Peru and Brazil, typical of those first described by Martin et al. (1981; E. French, personal communication), and found that none of these strains had homology with pBS2338. However, 28 biovar 2 strains that are known to belong to race 3 based on RFLP analysis (L. Barlow et al., unpublished data) all had homology to pBS2338. Thus, lowland biovar 2 strains are distinct from highland biovar 2 strains of race 3. This conclusion is supported further by the ability of RFLP analysis to distinguish lowland biovar 2 strains from biovar 2 strains of race 3 (L. Barlow et al., unpublished data).

The existence of *P. solanacearum* DNA with specificity for race 3 provides strong support for the idea that race 3 constitutes a separate, homogeneous group of strains. The ability to distinguish these strains by DNA hybridization should be useful for identification and detection of race 3 and, in combination with the polymerase chain reaction technology, should allow detection of latent infections. Our current efforts, therefore, are directed towards the evaluation and application of pBS2338 as a tool for rapid diagnosis of race 3 of *P. solanacearum*.

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