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Bacteriocin Typing of *Burkholderia* (*Pseudomonas*) *solanacearum* Race 1 of the French West Indies and Correlation with Genomic Variation of the Pathogen

PASCAL FREY,[1,2]† JULIAN J. SMITH,[3] LAURENCE ALBAR,[2]‡ PHILIPPE PRIOR,[2] GERRY S. SADDLER,[3] DANIELE TRIGALET-DEMERY,[1] AND ANDRE TRIGALET[1]*

Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, Castanet-Tolosan Cedex, France[1]; International Mycological Institute, Egham, Surrey, United Kingdom[3]; and Laboratoire de Recherches en Productions Végétales, Institut National de la Recherche Agronomique, Pointe-à-Pitre Cedex, Guadeloupe, French West Indies[2]

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***Burkholderia solanacearum* race 1 isolates indigenous to the French West Indies were characterized by bacteriocin typing and two genomic fingerprinting methods: pulsed-field gel electrophoresis of genomic DNA digested by rare-cutting restriction endonucleases (RC-PFGE) and PCR with primers corresponding to repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and BOX elements (collectively known as rep-PCR). The survey comprised 24 reference strains and 65 isolates obtained from a field trial in Guadeloupe in 1993. Comparison of the data identified RC-PFGE as the most discriminatory method, delineating 17 pulsed-field gel profile types. rep-PCR and bacteriocin typing identified nine rep-PCR profile types and nine bacteriocin groups. Independent determination of similarity coefficients and clustering of RC-PFGE and rep-PCR data identified six groups common to both sets of data that correlated to biovar and bacteriocin groups. Further study of bacteriocin production in planta gave results consistent with in vitro bacteriocin typing. It was observed that spontaneous bacteriocin-resistant mutants exhibited a cross-resistance to other bacteriocins as identified by the typing scheme and that such mutants possessed a selective advantage for growth over isogenic nonmutants in the presence of a bacteriocin. The results are significant in the search for biological control of disease by nonpathogenic mutants of the wild-type organism.**

Burkholderia (syn. *Pseudomonas*) *solanacearum* E. F. Smith is the causal agent of bacterial wilt, one of the most devastating bacterial plant diseases in the tropical, subtropical, and warm temperate regions (24). Although the name *Burkholderia solanacearum* was recently proposed (48), the taxonomic position of this species remains unclear (16). With the advent of modern molecular DNA technology, significant advances have been made in the description of the species in recent years (5, 6). These techniques in general have complemented the previous classifications based on carbon utilization (biovars) and host association (races) and have further elucidated the probable origin of the races observed. Relatively few studies have centered on ascertaining diversity within a race, a biovar, or field populations of *B. solanacearum* (14, 15, 38).

Numerous protocols have been applied to typing bacterial populations, including multilocus enzyme electrophoresis (22, 35), bacteriocin typing (19), and genomic fingerprinting (14, 15, 27, 30). Production of bacteriocin has been reported for several genera of plant pathogenic bacteria: *Agrobacterium* (26), *Clavibacter* (10, 18), *Erwinia* (11), and *Pseudomonas* (47). Bacteriocin production was first reported for *B. solanacearum* by Okabe (32); however, few *B. solanacearum* bacteriocins have been characterized (1, 7). In recent years, improved genomic fingerprinting protocols have revealed hitherto unrealized levels of genetic diversity within populations of bacteria. Application of PCR with specific primers is a notable example. rep-PCR describes the use of primers corresponding to conserved motifs in bacterial repetitive elements referred to as enterobacterial repetitive intergenic consensus (ERIC) (25), repetitive extragenic palindromic (REP) (17), and BOX (28). PCR amplification with these primer sets has been shown to yield genomic fingerprints specific to pathovars and strains of gram-negative bacteria (8, 27, 31). Genomic fingerprinting protocols that rely on restriction endonuclease profiles of genomic DNA have also proved appropriate for similarly aimed analysis. In this context, the use of rare-cutting restriction endonuclease enzymes in which the macrofragments are resolved by pulsed-field gel electrophoresis (PFGE) yields particularly sensitive restriction profiles that are readily amenable to analysis (20, 34, 38, 40). Restriction profiling generated by frequent-cutting restriction endonucleases has also been used to type bacterial strains (14, 15), though the level of discrimination appears to be less than that obtained by rare-cutting analysis.

It is widely assumed that bacteriocins play an important role in bacterial population dynamics, with the resistance to and production of a bacteriocin(s) being advantageous traits (43). Several biocontrol studies that use nonpathogenic mutants as the biocontrol agent have aimed at utilizing the production of bacteriocin as a mechanism for enhancing the biological control (26, 46). In the case of *B. solanacearum*, this approach has met with limited success (3, 21, 44). However, recent studies

* Corresponding author. Mailing address: Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, INRA-CNRS, BP 27, 31326 Castanet-Tolosan Cedex, France. Phone: (33) 61.28.50.47. Fax: (33) 61.28.50.61. Electronic mail address: trigalet@toulouse.inra.fr.

† Present address: INRA, Unité de Recherches Ecosystèmes Forestiers, 54280 Champenoux, France.

‡ Present address: Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM), 34032 Montpellier Cedex, France.

TABLE 1. Characteristics of reference strains of *B. solanacearum* isolated from the FWI

Strain	Host	Yr of isolation	Race[a]	Biovar[a]	Bacteriocin type[b]	RC-PFGE profile[c]	rep-PCR profile[d]
Guadeloupe isolates							
GT1	Tomato	1985	1	1	4	3a	5a
GT4	Tomato	1984	1	3	1	1a	1a
GT5	Tomato	1984	1	1	8	5	4b
GA1	Eggplant	1984	1	3	1	1c	1a
GA2	Eggplant	1986	1	3	1	1a	1a
GA3	Eggplant	1984	1	3	2	1g	1a
GA4	Eggplant	1983	1	3	1	1e	1a
GA5	Eggplant	1983	1	3	1	1d	1a
GPT1	Potato	1987	1	1	5	4b	2
GP1	Pepper	1984	1	3	1	1f	1a
Martinique isolates							
MT1	Tomato	1986	1	3	1	1a	1a
MT2	Tomato	1983	1	3	1	1a	1a
MT3	Tomato	1984	1	3	1	1b	1a
MT4	Tomato	1985	1	3	1	1c	1a
MT5	Tomato	1987	1	1	5	4a	2
MA1	Eggplant	1985	1	3	1	1a	1a
MA2	Eggplant	1984	1	3	1	1a	1a
MA3	Eggplant	1985	1	3	1	1a	1a
MA4	Eggplant	1983	1	2	3	1h	1b
MPT1	Potato	1986	1	1	6	2	3
MPT2	Potato	1987	1	3	1	1d	1a
MPT3	Potato	1987	1	1	7	—[e]	4a
MP1	Pepper	1985	1	3	1	1a	1a
MB1	Ornamental banana	1987	1	3	1	1a	1a

[a] According to Prior and Steva (33).
[b] According to Table 2.
[c] According to Fig. 2.
[d] According to Fig. 4.
[e] —, not determined.

indicate that spontaneous nonpathogenic mutants of *B. solanacearum* which are impaired in exopolysaccharide production are unable to colonize the host plant (42, 45) and consequently exhibit poor biocontrol ability (41). This was addressed by the development of genetically engineered Hrp^- mutants of *B. solanacearum* that retained the ability to colonize the host. These nonpathogenic fluidal mutants were shown to confer significant biological control under pot trial conditions (13).

In this paper we present a detailed characterization of *B. solanacearum* race 1 of the French West Indies (FWI) in terms of bacteriocin production and genetic diversity. The study comprised a field population and a reference collection and was undertaken with a view to evaluating the suitability of the Hrp^- mutants as biocontrol agents against the indigenous population described.

MATERIALS AND METHODS

Reference *B. solanacearum* strains and isolation of an indigenous field population of *B. solanacearum*. *B. solanacearum* strains were routinely grown on BG medium and peptone broth (2) at 30°C. Characteristics of the reference strains of *B. solanacearum* isolated from the FWI are given in Table 1. Tomato and eggplant were used as plant baits to collect *B. solanacearum* isolates in a field experimentation plot in Guadeloupe (FWI). The field plot (20 by 38 m) was composed of 12 subplots (4 by 12 m), named A to L, planted with 4-week-old tomato plants (cv. Floradel) and eggplants (cv. Aranguez) susceptible to bacterial wilt. Plants exhibiting symptoms typical of bacterial wilt were collected, and bacterial isolations were made onto BG medium. A total of 126 *B. solanacearum* isolates were obtained. The biovar of these isolates was determined according to the ability to metabolize specific disaccharides and hexose alcohols (23). Sixty-five isolates (five or six isolates from each subplot) were randomly selected for further characterization.

Bacteriocin typing of *B. solanacearum*. Bacteriocin production and sensitivity to bacteriocins were studied as previously described (13). Bacteriocin titers were expressed in arbitrary units (AU) per milliliter corresponding to the reciprocal of the highest dilution of the culture filtrate causing a clear inhibition zone. When necessary, culture filtrates containing bacteriocins were concentrated 10-fold by lyophilization and resuspension in sterile distilled water, without loss of activity.

Heat and proteinase K treatment of bacteriocins. One-milliliter samples of culture filtrate containing bacteriocins were either heated to 100°C for 10 min or subjected to proteinase K treatment (50 μg of proteinase K ml^{-1}, 10 mM Tris [pH 8.0], 1 mM $CaCl_2$) for 2 h at 37°C. After treatment, the filtrates were tested for bacteriocin activity against an indicator strain.

Selection of bacteriocin-resistant mutants. After incubation of bacteriocin test plates for 3 to 4 days at 30°C, spontaneous bacteriocin-resistant mutants appeared in the inhibition zones. For each indicator strain, several bacteriocin-resistant mutants were picked, streaked for single-colony isolation, and then tested for bacteriocin sensitivity.

The rate of occurrence of bacteriocin-resistant mutants of GA4 was evaluated in liquid culture by sequential viable cell count of a peptone broth culture of GA4 amended with a bacteriocin preparation of GT1 at 4 AU ml^{-1}. The viable cell count was performed on BG medium and on BG medium amended with a bacteriocin preparation of GT1 at 2 AU ml^{-1}.

Production of bacteriocins in planta. Four-week-old tomato plants (cv. Supermarmande), susceptible to bacterial wilt, were root inoculated with the pathogenic strain GMI8217, a spontaneous Sm^r Rif^r derivative of strain GT1, as previously described (13). Two weeks after inoculation, 2-cm stem pieces from partially wilted plants were sampled and incubated at 10°C for 12 h in test tubes containing 4 ml of sterile distilled water to allow exudation and sedimentation of bacteria while stopping their multiplication (13). The bacteriocin titer of the bacterial suspensions exuded from the stem pieces was expressed in AU per 10^9 CFU and compared with the titer of in vitro-grown bacteria.

RC-PFGE analysis of field isolates. The method used for PFGE of genomic DNA digested by rare-cutting restriction endonucleases (RC-PFGE) was as described by Smith et al. (37). Genomic digestion was performed with endonu-

TABLE 2. Bacteriocin typing groups identified from reference strains and field isolates of *B. solanacearum* from the FWI[a]

Indicator strain	Reaction with given producing strain [group (type strain)][b]								
	1 (GA4)	2 (GA3)	3 (MA4)	4 (GT1)	5 (GTP1)	6 (MPT1)	7 (MPT3)	8 (GT5)	9 (A12)
Group 1 (GA4)	−	−	−	+	+	±	+	−	+
Group 2 (GA3)	−	−	−	−	−	−	−	−	−
Group 3 (MA4)	−	−	−	+	+	±	+	±	+
Group 4 (GT1)	−	−	−	−	+	+	−	−	−
Group 5 (GPT1)	±	−	−	−	−	−	−	−	±
Group 6 (MPT1)	+	±	±	±	±	−	−	−	−
Group 7 (MPT3)	−	−	−	−	+	±	−	+	±
Group 8 (GT5)	−	−	−	±	+	±	+	−	±
Group 9 (A12)	−	−	−	−	+	−	+	−	−

[a] Results are those of three independent tests.
[b] +, clear inhibition zone; −, no inhibition zone; ±, faint inhibition zone or variable reaction.

clease *Xba*I (Northumbria Biologicals). Duplicate analyses were undertaken. The gels were read by eye, and the presence or absence of a particular DNA fragment was converted into binary data. Similarity coefficients for all pairwise combinations were determined by Dice's coefficient (9) and clustered by the unweighted-pair-group method with arithmetic mean (UPGMA) (39).

rep-PCR analysis. The PCR protocols using REP, ERIC, and BOX primers are referred to collectively as rep-PCR. The method employed was adapted from Louws et al. (27). The primers were manufactured by Pharmacia Biotech. Sequence data are as described by Louws et al. (27). Samples for amplification were taken from overnight peptone broth cultures (10^9 CFU ml^{-1} at 27°C) that had been boiled at 100°C for 10 min. Amplification was performed on an MJ Research Inc. PTC100 programmable thermal controller in 25-μl reaction volumes containing 200 μM (each) deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, primer(s) at 10 pmol, Super Tth at 0.5 U (HT Biotechnology Ltd.), and 5 μl of boiled cells. The PCR program included an initial 7-min phase at 95°C and 30 cycles of denaturation at 94°C, annealing at 44°C (REP primers) or 53°C (ERIC and BOX primers) for 1 min, and extension at 65°C for 8 min, with a final extension at 65°C for 15 min and a final soak at 5°C. PCR mixtures were overlaid with 1 drop of mineral oil (Sigma). Products of PCR amplification were separated by agarose gel electrophoresis (2% [wt/vol] agarose [Flowgen SeaKem no. 50002], 0.5× TAE buffer [40 mM Tris, 4 mM sodium acetate, 1 mM EDTA, pH 7.9]) at 5 V cm^{-1} over 4 h, stained with ethidium bromide, and visualized under a UV transilluminator. PCR amplifications were performed in duplicate from fresh sample preparations and PCR master mix. The gels were read by eye, and bands common to both duplicate amplifications were recorded. Similarity coefficients for all pairwise combinations were determined as described above.

RESULTS

Characteristics of the bacteriocins and bacteriocin typing of reference strains and field isolates of *B. solanacearum* from the FWI. All of the *B. solanacearum* reference strains and field isolates used in this study produced substances which inhibited the growth of other *B. solanacearum* strains. No growth inhibition was detected with *B. solanacearum* culture filtrates tested against 21 bacterial strains belonging to 16 different bacterial species (data not shown). Bacteriophages were not responsible for the observed antibiosis, since serial dilutions of the culture filtrates did not result in the identification of discrete phage plaques (29). It was therefore concluded that the antagonistic substances produced by *B. solanacearum* were bacteriocins (19).

Titers of bacteriocins varied from 0.5 to 100 AU ml^{-1} according to the producer and the indicator strains. After heat or proteinase K treatment, the growth inhibition activity was abolished for all bacteriocins tested.

Bacteriocin typing of the 24 reference strains identified eight groups on the basis of their bacteriocin production and bacteriocin sensitivity profiles (Tables 1 and 2). Group 1 contained 16 strains, group 5 contained 2 strains, and the other groups contained only 1 strain each. A type strain was designated for each group (Table 2). All biovar 3 strains belonged to group 1, except strain GA3 (group 2). Biovar 1 strains belonged to bacteriocin groups 4 to 8, and the biovar 2 strain, MA4, belonged to group 3 (Table 1).

The 126 *B. solanacearum* isolates collected from diseased plants of the field plot were assigned to biovar 1 by carbohydrate utilization analysis. Sixty-five isolates (59 from tomato and 6 from eggplant) were tested for bacteriocin production and sensitivity against the eight type strains. Among the 65 isolates, three bacteriocin groups were found. Of these, two groups had been described previously by the type strains, GT1 (group 4) and GPT1 (group 5), made up of 30 and 31 isolates, respectively. Four isolates exhibited a distinct bacteriocin typing group, referred to as bacteriocin group 9 (Tables 2 and 3).

Genomic fingerprinting by rare-cutting restriction analysis and PFGE of the reference strains and field isolates of *B. solanacearum* from the FWI. RC-PFGE with the restriction endonuclease enzyme *Xba*I produced pulsed-field gel (PFG) profiles made up of DNA fragments ranging from 5 to 800 kb. The gels were read by eye in the size range 175 to 800 kb, where the profiles of typically eight to nine DNA fragments were readily amenable to interpretation. Comparison of duplicate analyses yielded identical PFG profile types, confirming their identity.

RC-PFGE analysis of the 24 reference strains identified 13 distinct PFG profile types (Table 1 and Fig. 1). No PFG profile could be obtained for reference strain MPT3. Among the 65 field isolates, five distinct PFG profile types were identified, of which three were broadly similar (Fig. 1B). The frequency with which these PFG profile types occurred varied: PFG 3a, 15 isolates; PFG 3b, 14 isolates; PFG 3c, 1 isolate; PFG 4c, 31 isolates; and PFG 6, 4 isolates (Table 3). Type strains of these PFG profile types were designated (Table 3). Comparison of the field isolate and reference strain PFG profiles identified the following profile type strains: H71 (PFG 3a), indistinguishable from GT1 (PFG 3a); A23 (PFG 3b) and D62 (PFG 3c), broadly similar to GT1 (PFG 3a); A44 (PFG 4c), broadly similar to MT5 (PFG 4a) and GPT1 (PFG 4b); and A12 (PFG 6), highly unique. These observations were consistent with the bacteriocin groupings.

Analysis of the binary data on the 23 reference strains and the 5 field PFG profile type strains and the generation of a dendrogram identified additional groupings (Fig. 2). In interpreting this analysis, it must be borne in mind that the analysis is based on the assumption that comigrating DNA fragments between PFG profile types are homologous. Hence, confident conclusions of similarity can be drawn only from comparison of broadly similar PFG profile types. At a similarity coefficient of 75%, six cluster groups are identified. Membership in these groups correlated to the bacteriocin data. The nomenclature of

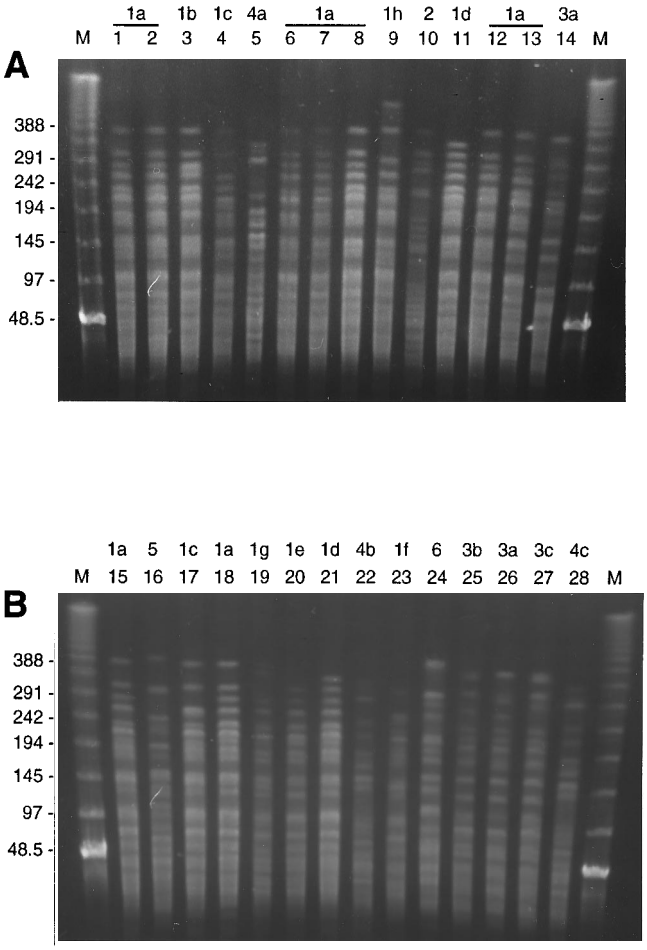

FIG. 1. *Xba*I macro-restriction fragments resolved by PFGE of *B. solanacearum* from the FWI. Lanes: (A) 1, MT1; 2, MT2; 3, MT3; 4, MT4; 5, MT5; 6, MA1; 7, MA2; 8, MA3; 9, MA4; 10, MPT1; 11, MPT2; 12, MP1; 13, MB1; 14, GT1; (B) 15, GT4; 16, GT5; 17, GA1; 18, GA2; 19, GA3; 20, GA4; 21, GA5; 22, GPT1; 23, GP1; 24, A12; 25, A23; 26, H71; 27, D62; 28, A44; M, molecular size marker (50- to 1,000-kb concatemer ladder). Sizes (in kilobases) are indicated on the left. Profile numbers are indicated above lane numbers.

TABLE 3. Characteristics of field isolates of *B. solanacearum* isolated from the FWI[a]

Isolates	Host	Bacteriocin type[b]	RC-PFGE profile[c]	rep-PCR profile[d]
H71	**Tomato**	**4**	**3a**	**5b**
E43, E73, G64, H83, I23, I41, I53, J63, J81, K21, L14, L24, L84	Tomato	4	3a	—[e]
L23	Eggplant	4	3a	—
A23	**Tomato**	**4**	**3b**	**5b**
A31, A82, B31, C24, C34, C71, D32, D43, D71, F32, G13, G22, J73	Tomato	4	3b	—
D62	**Tomato**	**4**	**3c**	**5b**
A44	**Tomato**	**5**	**4c**	**2**
B42, B53, B64, B82, C13, C53, D21, E21, E32, F51, F62, F84, G41, H21, H53, I64, I82, J21, J43, K32, K43, K52, K62, L42, L53	Tomato	5	4c	—
B62, C51, F24, H43, K12	Eggplant	5	4c	—
A12	**Tomato**	**9**	**6**	**6a**
E81, F14, G43	Tomato	9	6	—

[a] The type strain for each PFG profile is indicated in boldface type.
[b] According to Table 2.
[c] According to Fig. 2.
[d] According to Fig. 4.
[e] —, not determined.

the PFG profile types in Tables 1 and 3 is based on these groupings: group number forms the prefix, with the suffix indicating a lesser division. All biovar 3 strains shared similar PFG profiles, forming a cluster at a similarity coefficient of 57%. It was evident that the previously described biovar 2 strain, MA4, showed a marked similarity to the PFG profile type typical of the biovar 3 strains.

Genomic fingerprinting by rep-PCR of the reference strains and PFG type strains of the field isolates of *B. solanacearum* from the FWI. A parallel analysis by rep-PCR was undertaken on the reference strains and PFG profile type strains of the field isolates. ERIC, REP, and BOX primer sets were proficient at generating PCR profiles (Fig. 3). Comparison of PCR profiles after duplicate analyses identified a "core profile" with a high level of reproducibility that was readily amenable to interpretation. The data generated from ERIC, REP, and BOX primers were compiled into a single binary matrix and analyzed for similarity indices as described for PFGE data. The dendrogram generated is presented in Fig. 4. An arbitrary confidence limit reflecting the reproducibility of the data was placed at the 90% similarity coefficient. At this level of confidence, the number of discrete rep-PCR profiles identified was nine, which at a 75% similarity coefficient corresponded to six distinct cluster groups (Table 1; nomenclature as outlined for

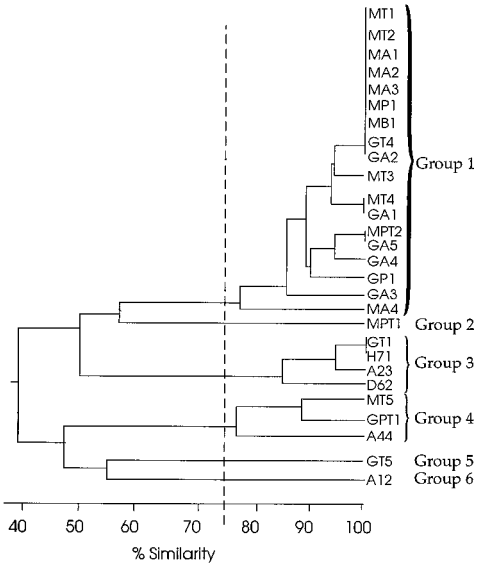

FIG. 2. Dendrogram showing relationships between *B. solanacearum* isolates from the FWI based on PFGE analysis of macro-restriction fragments of *Xba*I. Similarities were calculated by using Dice's coefficient and clustering by UPGMA.

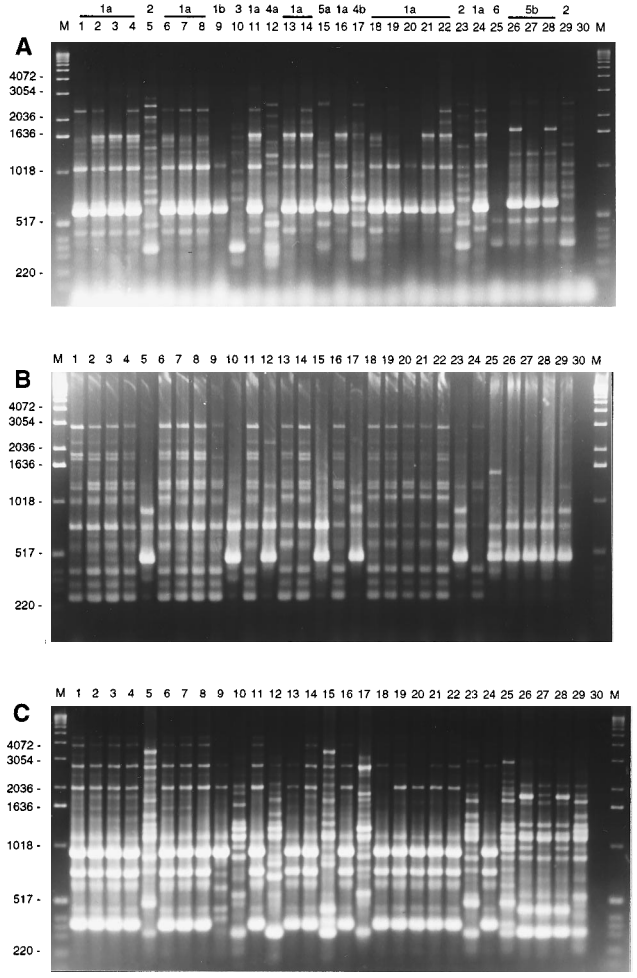

FIG. 3. rep-PCR profiles (A, ERIC; B, REP; C, BOX) of *B. solanacearum* from the FWI. Lanes: 1, MT1; 2, MT2; 3, MT3; 4, MT4; 5, MT5; 6, MA1; 7, MA2; 8, MA3; 9, MA4; 10, MPT1; 11, MPT2; 12, MPT3; 13, MP1; 14, MB1; 15, GT1; 16, GT4; 17, GT5; 18, GA1; 19, GA2; 20, GA3; 21, GA4; 22, GA5; 23, GPT1; 24, GP1; 25, A12; 26, A23; 27, H71; 28, D62; 29, A44; 30, control; M, molecular size marker (1-kb ladder). Sizes (in base pairs) are indicated on the left. Profile numbers are indicated above lane numbers.

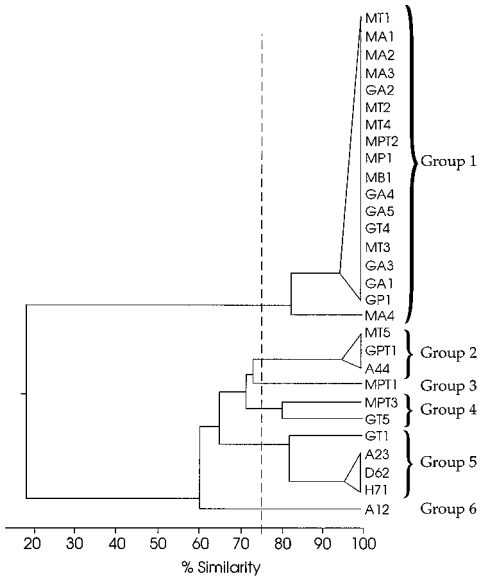

FIG. 4. Dendrogram showing relationships between *B. solanacearum* isolates from the FWI based on rep-PCR. Similarities were calculated by using Dice's coefficient and clustering by UPGMA.

PFG profile types). Membership in these rep-PCR cluster groups correlated with PFG profile and bacteriocin groupings. The principal division at a similarity coefficient of 18% corresponded to biovars 1 and 3. It was again evident that the biovar 2 strain, MA4, was very similar to the rep-PCR profile type typical of the biovar 3 strains.

Bacteriocin production in vitro and in planta. The titers of bacteriocins produced in vitro and in planta by strain GMI8217, an Sm^r Rif^r mutant of reference strain GT1, were determined with the indicator strain GA4. The bacteriocin titer of strain GMI8217 grown in peptone broth for 24 h at 30°C, determined in 20 independent tests, was 26.0 ± 5.4 AU ml^{-1}, corresponding to 5.2 ± 1.1 AU per 10^9 CFU. Bacteriocin production of the same strain exuded from tomato stem pieces, determined on 20 plants, was 5.0 ± 1.0 AU per 10^9 CFU.

Selection of bacteriocin-resistant mutants. After incubation of bacteriocin test plates for 3 to 4 days at 30°C, colonies appeared in the inhibition zones at a frequency of approximately 10^{-6} to 10^{-5}. For each indicator strain-producer strain combination, about 10 clones were selected. Bacteriocin resistance was confirmed by subsequent bacteriocin typing against the nine bacteriocin groups previously identified. It was observed that the clones that had acquired bacteriocin resistance expressed cross-resistance to all bacteriocin groups identified (Table 4).

Specific assessment of the rate of occurrence of bacteriocin-resistant mutants for reference strain GA4 in the presence of the bacteriocin of GT1 showed that bacteriocin-resistant mutants were present at time zero at a frequency of 10^{-5} (Fig. 5). Four hours after the addition of bacteriocin, the number of bacteriocin-resistant mutants began to increase, whereas the total viable cell count decreased until 15 h. Thereafter, the total viable cell count increased because of the growth of GA4 cells that were resistant to the bacteriocin.

DISCUSSION

Previous reports on bacteriocin production among *B. solanacearum* strains indicate that its occurrence is ubiquitous. In the study by Chen et al. (4), 121 of 149 strains tested produced bacteriocins active against 22 indicator strains; similarly, with 52 strains collected in Taiwan, Tsai et al. (44) demonstrated

TABLE 4. Selection of bacteriocin-resistant mutants

Strain	Bacteriocin used for selection[a]	No. of bacteriocin-resistant clones tested	No. of clones showing cross-resistance to other bacteriocins[b]
GA4	GT1	13	13
GA4	GPT1	3	3
GA4	A12	8	8
GT1	GPT1	4	4
GT1	MPT1	5	5
A12	GPT1	10	10
A12	MPT3	10	10

[a] Designation adopted for bacteriocins refers to the name of the producer strain.

[b] Mutants were tested for cross-resistance to the nine groups of bacteriocins.

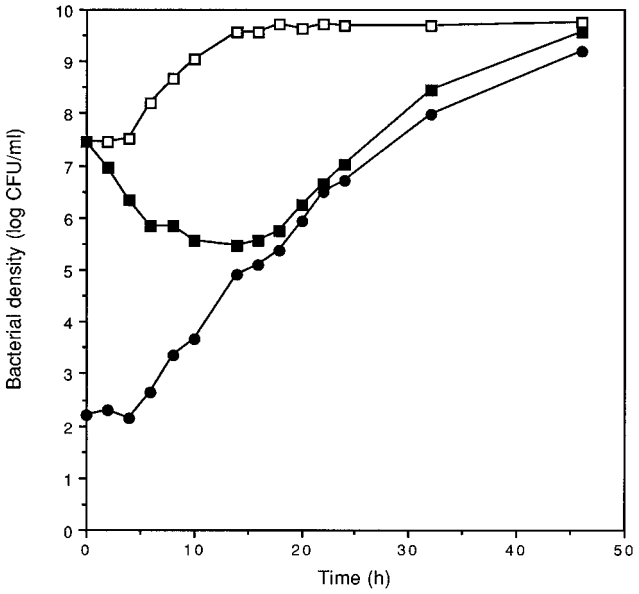

FIG. 5. Growth curve of strain GA4 in BG medium at 30°C in the presence (■) or absence (□) of a bacteriocin preparation of GT1 (4 AU ml^{-1}). The appearance of bacteriocin-resistant mutants (●) in the culture of strain GA4 in the presence of the bacteriocin was monitored by plating bacterial suspensions on BG medium containing GT1 bacteriocin (2 AU ml^{-1}). Values are the means of those from two independent experiments of three plate counts each.

that all were bacteriocinogenic against at least one of the 52 strains. In the present study, all 24 reference strains and 65 field isolates exhibited a bacteriocinogenic property; consistent with the definition of a bacteriocin (19), the antibacterial compounds under study were unable to inhibit the growth of 21 strains from 16 different bacterial species. Characterization of the bacteriocins identified them as heat labile and sensitive to proteinase K, indicating a proteinaceous nature. Arwiyanto et al. (1) showed that the bacteriocin produced by *B. solanacearum* POPS8409 was also heat sensitive.

The patterns of bacteriocin production and bacteriocin sensitivity of each strain permitted the establishment of a bacteriocin typing scheme. Similar bacteriocin schemes have been established previously for strains of *B. solanacearum* (1, 44) and other phytobacteria (19). Among the 24 reference strains and the 65 field isolates from the FWI, nine bacteriocin groups were identified.

Genomic fingerprinting identified RC-PFGE as the most discriminatory protocol employed, identifying 17 distinct profile types, with rep-PCR distinguishing 9 profile types. Independent statistical analysis of these data by Dice's coefficient and clustering by UPGMA identified six principal groupings common to both sets of data. Comparison of group membership and bacteriocin typing showed a complete correlation, strongly suggesting that the field population described was clonal. The division identified by genomic fingerprinting at similarity values of 18 and 57% by rep-PCR and RC-PFGE data, respectively, corresponded to biovars 1 and 3. The biovar 2 strain, MA4, grouped with the biovar 3 strains at similarity coefficients of 82 and 77% by rep-PCR and RC-PFGE, respectively. This level of similarity strongly indicates that MA4 has been wrongly assigned to biovar 2 and belongs to biovar 3.

It is widely recognized that PFGE of rare-cutting restriction genomic digests is a powerful method of typing bacterial strains (20, 34, 38, 40). In the study by Smith et al. (38) on *B. solanacearum* race 3 biovar 2, recognized as the most homogeneous race of *B. solanacearum*, 10 distinct PFG profile types (clonal lines) among 45 isolates obtained from diseased potatoes of Kenya in one growing season were identified. In contrast, rep-PCR has been successfully applied to typing bacterial strains principally at the pathovar level (27). The results presented here concur with the opinion that RC-PFGE is more discriminatory than rep-PCR, although both are highly appropriate to bacterial strain differentiation. In this context, the data presented also emphasize the parity of the bacteriocin typing method for epidemiological studies of this nature.

This study formed the basis of a field trial on the evaluation of nonpathogenic mutants as biocontrol agents against the indigenous population described here. To this end, the realization that the Hrp^- mutants (derivatives of GA2, GA4, and GT4; bacteriocin group 1) developed as biocontrol agents are sensitive in vitro to the bacteriocins produced by the field isolates (bacteriocin groups 4, 5, and 9) would indicate that their suitability as biocontrol agents against this population is questioned. Therefore, it would be informative to study whether antibiosis between the field population of *B. solanacearum* and the Hrp^- mutants exists. Bacteriocin-mediated antibiosis could be exerted within the rhizosphere or within the plant tissues.

Furthermore, the in planta assessment of bacteriocin production indicated that similar amounts of bacteriocins were produced in vitro and in tomato stems by strain GMI8217. Hara and Ono (21) also demonstrated that bacteriocins were produced by *B. solanacearum* in tobacco stems. A similar result was obtained with *Pseudomonas syringae* pv. syringae in bean stems (36). Logically, a nonpathogenic mutant of *B. solanacearum* with broad-spectrum bacteriocin production and insensitivity would be a more appropriate candidate for biological control.

A complementary strategy could be based on the fact that bacteriocin-resistant mutants expressed cross resistance to different bacteriocins, a phenomenon that has also been reported for *Erwinia chrysanthemi* (12). Nonpathogenic bacteriocin-resistant mutants would escape the potential antibiosis exerted by the natural bacteriocin-producing strains occurring in soil. The study of bacteriocin-resistant mutant growth on GA4 in the presence of bacteriocin GT1 indicated the selective advantage conferred by bacteriocin resistance in vitro. These observations seem to provide a basis from which data on bacteriocins produced in vitro may be discussed in relation to their natural environment and in the context of population dynamics. However, the high frequency with which spontaneous bacteriocin-resistant mutants arose may indicate that the efficacy of the biological control would break down over time.

In summary, production of bacteriocins by *B. solanacearum* isolates was shown to be ubiquitous and was highly correlated to genomic fingerprints. Collectively, these data describe a clonal population structure. In planta and in vitro studies indicate that bacteriocins are produced in infected plants and could confer a selective advantage for growth. Clearly, in view of the epidemiological implications of bacteriocin production and the need to control this devastating plant pathogen, the significance of the complexity of bacteriocin interactions and the correlation to genomic fingerprints merit greater understanding.

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