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EXPERIMENTAL ARTICLES

Genetic Diversity in Pseudomonads Associated with Cereal Cultures Infected with Basal Bacteriosis

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Abstract—The genetic properties of 45 pseudomonad strains isolated from cereal cultures exhibiting symptoms of basal bacteriosis have been investigated. Considerable genetic diversity has been demonstrated using DNA fingerprints obtained by amplification with REP, ERIC, and BOX primers. Restriction analysis of the 16S–23S internal transcribed spacer (ITS1) allowed the strains to be subdivided into two major groups. In a phylogenetic tree, the ITS1s of these groups fell into two clusters, which also included the ITS1 of *Pseudomonas syringae* ("Syringae" cluster) and the ITS1 of *P. fluorescens, P. tolaasii, P. reactans, P. gingeri*, and *P. agarici* ("Fluorescens" cluster) from the GenBank database. Comparison of the ITS1 divergence levels within the "Fluorescens" cluster suggests expediency of treating *P. tolaasii, P. reactans*, various *P. fluorescens* groups, and, possibly, *P. gingeri* and *P. agarici* as subspecies of one genospecies. The intragenomic heterogeneity of ITS1s was observed in some of the pseudomonad strains studied. The results of amplification with specific primers and subsequent sequencing of the amplificate suggest the possibility of the presence of a functionally active *syrB* gene involved in syringomycin biosynthesis in the strains studied.

Key words: phytopathogens, *Pseudomonas*, genetic diversity, ITS1.

Basal bacteriosis is the most common disease in cereals and significantly decreases harvests. In every Russian region investigated in this respect, the symptoms of basal bacteriosis have been found in wheat, barley, and rye, initiating studies of the biochemical properties and virulence of the casual agent [1]. Modern standards of investigation imply study of the genomic features of the casual agent in addition to its traditional phenotypic and biochemical characterization. For the characterization and identification of pathogenic pseudomonads, several approaches have been recently used: DNA typing with the use of a polymerase chain reaction (PCR) [2]; restriction analysis of rRNA operons [3]; and determination of the nucleotide sequences of various DNA regions, such as the ITS1 region of the ribosomal operons, *hrpL* and *hrpS* genes, *gyrB* gene, and *rpoD* gene [4–6]. The pseudomonads attacking cereal cultures in Russia have been little studied, and data on their genetic characteristics are practically nonexistent. The goal of the present study was therefore to characterize the genetic diversity of pseudomonads isolated from infected plants in different regions of Russia. For this purpose, we used methods of genomic fingerprinting based on PCR amplification of repeated DNA elements with REP, ERIC, and BOX primers [7] and ITS1 amplification with subsequent restriction analysis and sequencing.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were obtained from the collection of the Russian Research Institute for Phytopathology. All the strains had been isolated from various cereal cultures exhibiting symptoms of basal bacteriosis (see table).

DNA isolation and PCR amplification. DNA was isolated from bacterial cultures according to the procedure described in [8]. DNA fingerprints were obtained by amplification of repeated DNA elements with REP, ERIC, and BOX primers [7]. The amplification was performed in the standard reaction mixture recommended by the DNA polymerase manufacturer (Fermentas, Lithuania), which had an increased $MgCl_2$ content (4 mM). ITS1 amplification was performed with the primers 5'-AGC CGT AGG GGA ACC TGC GG-3' and 5'-TGA CTG CCA AGG CAT CCA CC-3', which are complementary to the 3'-proximal part of the 16S rRNA gene and to the 5'-proximal part of the 23S rRNA gene [3]. The fragment of the *syrB* gene, which participates in syringomycin biosynthesis, was amplified with the primers 5'-CTT TCC GTG GTC TTG ATG AGG-3' and 5'-TCG ATT TTG CCG TGA TGA GTC-3' [9].

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Characterization of *Pseudomonas* strains according to the results of restriction analysis and *syrB* gene fragment amplification

Restriction group	Strain	*SyrB*	*Hae*III	*Taq*I	*Msp*I	*Sau*96I	*Bst*KTI	*Mse*I
I-1	R-1026	++	A	A	A	D	H	E
	R-1/9	++	A	A	A	D	H	E
I-2	W-7	++	A	A	A	D	H	F
	W-9	++	A	A	A	D	H	F
	B-908	++	A	A	A	D	H	F
	B-92-2	++	A	A	A	D	H	F
	W-920	++	A	A	A	D	H	F
	W-913	++	A	A	A	D	H	F
	R-218	++	A	A	A	D	H	F
	W-914	++	A	A	A	D	H	F
	W-918	++	A	A	A	D	H	F
	W-194-3	+	A	A	A	D	H	F
II-1	W-905	+	B	B	–	B	B	C'
	B-69/1	+	B	B	–	B	B	C'
II-2	R-915	+	B	B	–	B	B	C
	R-936	+	B	B	–	B	B	C
	R-216-7	+	B	B	–	B	B	C
II-3	B-t21	+	B	B	B	A	C	B'
II-4	W-237-6	+	B	B	–	A	C	D
	R-93-4	+	B	B	–	A	C	D
II-5	W-238-4	+	B	B	–	A	E	D
	W-27-10	+	B	B	–	A	E	D
II-6	W-Sn-7	+	B	B	–	E	C	D'
II-7	W-20-7	+	B	B	–	E	I	D
	W-217-4	+	B	B	–	E	I	D
II-8	B-224-5	+	B	B	–	E	A	B
II-9	W-11/2	+	B	B	–	E	A	A
	W-912	+	B	B	–	E	A	A
	R-200	+	B	B	–	E	A	A
	W-930	+	B	B	–	E	A	A
	R-72	+	B	B	–	E	A	A
	W-922	+	B	B	–	E	A	A
	R-1/2	+	B	B	–	E	A	A
II-10	B-t5	+	B	B	B	E	I	A'
	S-10	+	B	B	B	E	I	A'
II-11	B-t8	+	B	B	–	E	I	A'
II-12	B-909	+	B	B	–	E	D	B
	147-15	+	B	B	–	E	D	B
II-13	R-85	+	B	B	–	E	D	D
	W-6005	+	B	B	–	E	D	D
II-14	R-56	+	B	B	–	E	E	D
	R-88	+	B	B	–	E	E	D
	R-102	+	B	B	–	E	E	D
II-15	W-901	+	B	B	–	E	G	E
II-16	B-t6	+	B	B	C	C	F	A

Note: In the strain names, the first letter, as a rule, signifies the host plant; thus, W is wheat, B is barley, and R is rye. Maize and oats are the hosts for strains S-10 and 147-15, respectively. The letters in the table indicate restriction group type; the dash signifies the absence of a restriction site. Intense and weaker *syrB* amplifications are designated as "++" and "+," respectively.

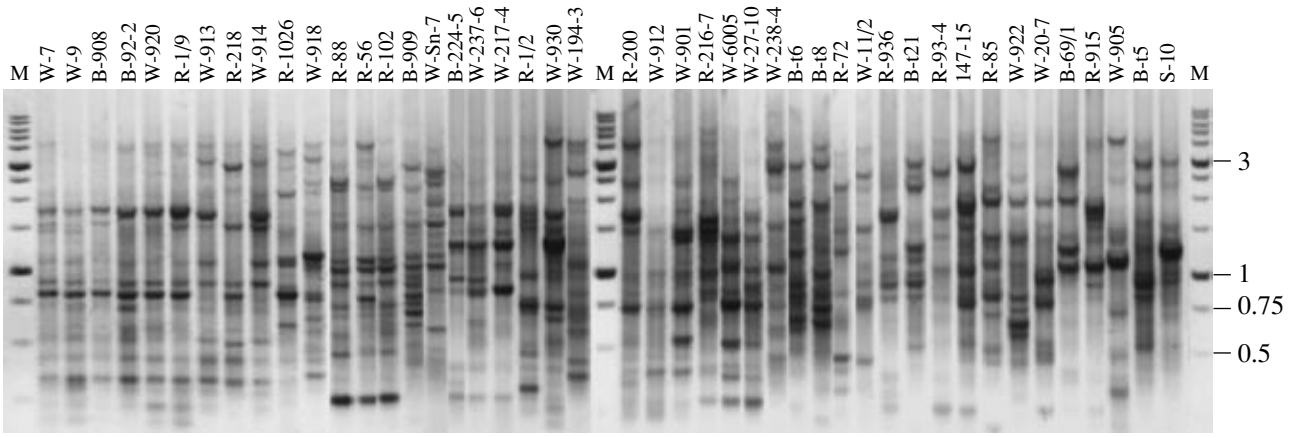

Fig. 1. DNA fingerprints of pseudomonads obtained after amplification with BOX primers. The upper line contains the strain names. M is a length marker. The numerals on the right are fragment lengths in kb.

Restriction analysis and nucleotide sequence determination. For restriction analysis of the ITS1 sequences, *Hae*III, *Taq*I, *Msp*I, *Sau*96 I, *Bst*KT I, and *Mse*I endonucleases were used (Fermentas, Lithuania). The amplified products were precipitated with ethanol and dissolved in 20 µl water; 3 µl of this solution was used for digestion with each endonuclease (2.5 U) under the conditions recommended by the manufacturer. The fragment length was estimated by comparison with a marker possessing a 100-bp step after separation in 2% agarose gel. ITS1 nucleotide sequences were determined either by direct sequencing of the amplified products or, in cases where the ITS1 sequences in individual operons were different, by cloning and subsequent sequencing of the individual ITS1, as described in [10]. The *syrB* gene fragments were sequenced in both directions after cloning in *E. coli* NM 522 cells, which was performed with the use of the pBluescript KS^+ vector, and the sequence data were deposited with the GenBank database under accession numbers AY764296 and AY764297 for strains R-1/9 and W-11/2, respectively. The accession numbers of the ITS1 sequences are shown in Fig. 4.

Phylogenetic reconstruction. The nucleotide sequences most similar to the sequences determined in the present study were retrieved from the GenBank database using BLAST software. For the construction of phylogenetic trees, MrBayes 3.0 software [11] and the GTR + I + G model of nucleotide substitutions were used. To find the optimal tree, 1000000 generations were performed, every tenth tree was saved, and the first 9000 trees (before reaching the stationary state) were disregarded. In the consensus tree built according to the 50% majority rule, *P. chlororaphis* (AJ279240) was used as an outgroup.

RESULTS

Genomic DNA fingerprinting with REP, ERIC, and BOX primers. The genetic diversity of the pseudomonads was estimated by genomic fingerprints obtained with REP, ERIC, and BOX primers specific to different repeated sequences of the bacterial genome. DNA amplification with these primers and subsequent electrophoresis in agarose gel resulted in reproducible banding patterns. The number of bands varied from 5 to 18 for different primers, and the size of the corresponding fragments was 200 to 4000 bp. The genomic DNA fingerprints obtained with the BOX primers are presented in Fig. 1. The use of the BOX primers revealed substantial polymorphism among the strains. The fingerprints obtained with the REP and ERIC primers (data not shown) consisted of a greater number of bands than those obtained with the BOX primers; however, the number of bands common to all of the fingerprints obtained was small, precluding the use of these data for the determination of genetic relatedness among the

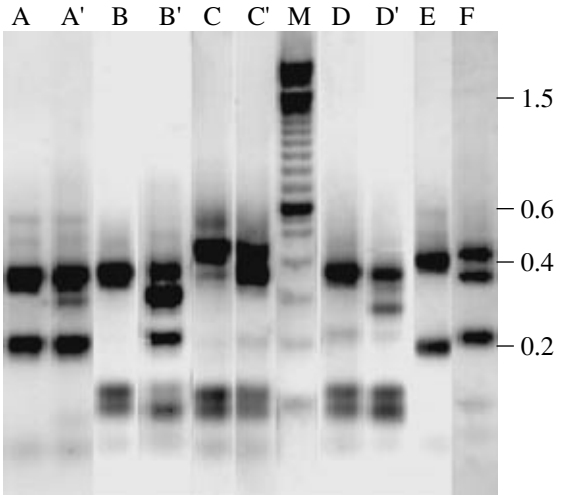

Fig. 2. Types of restriction patterns obtained after digestion of amplified pseudomonad ITS1s with *Mse* I endonuclease. M is a length marker. The numerals on the right are fragment lengths in kb.

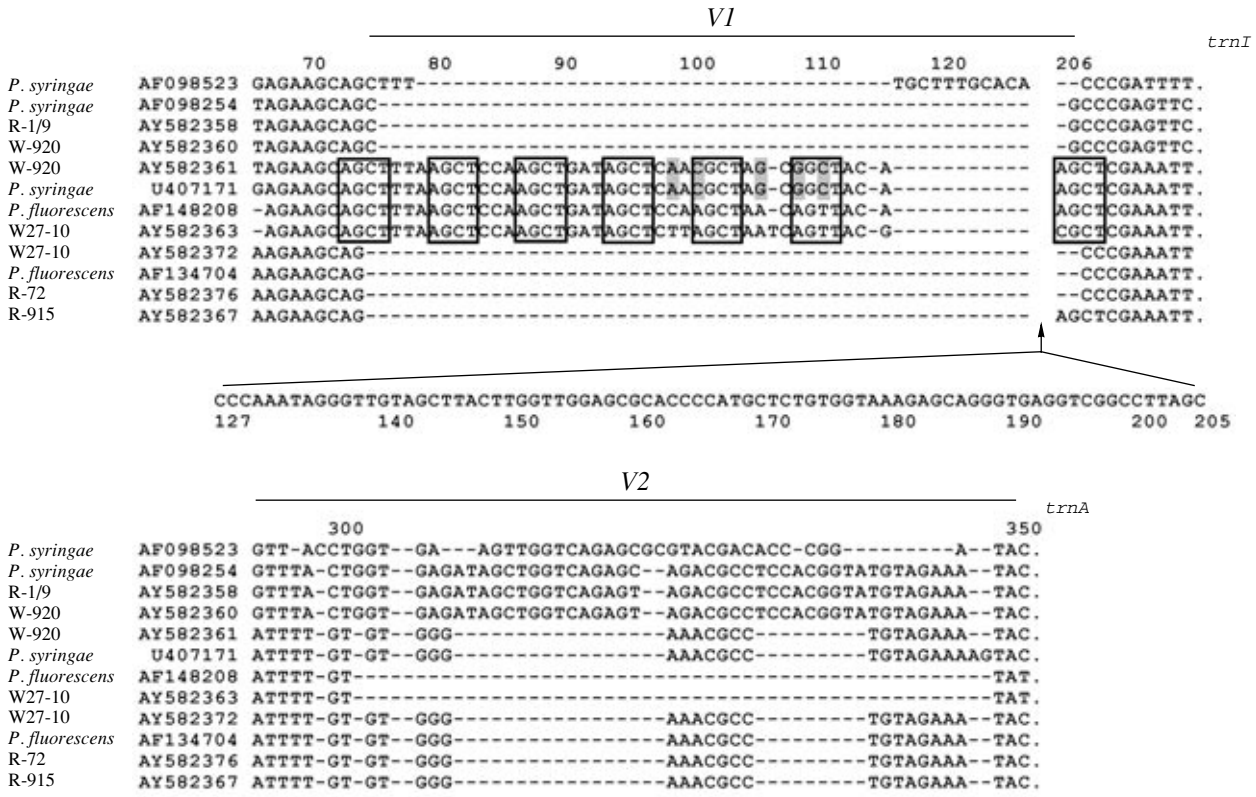

Fig. 3. Alignment fragments of pseudomonad ITS1s. V1 and V2 are variable regions. A 80-bp insertion occurring in strain R-915 is shown separately, and its position is indicated. Repeated tetranucleotide motifs are framed. The nucleotides marking *P. syringae* are shaded.

strains by cluster analysis. We succeeded only in visual subdivision of the strains into groups with similar genotypes. Some of the strains had unique BOX patterns from which bands shared with the other strains were absent.

Restriction analysis of ITS1s was performed for 45 pseudomonad strains. For most of the strains, the ITS1 amplification product was represented in the gel by a single band of ca. 550 bp. In some of the strains, the product was represented by two bands that differed in length by ca. 80 bp, indicating the presence of ITS1s of different lengths in the ribosomal operons of these strains.

Digestion of the amplified ITS1s by restriction endonucleases *Hae*III, *Taq*I, *Msp*I, *Sau*96 I, *Bst*KT I, and *Mse*I revealed a substantial degree of strain polymorphism. Based on the restriction analysis with *Hae*III, *Taq*I, and *Msp*I restriction endonucleases, all the strains were subdivided in two groups: 12 strains were assigned to group I, and 33 strains, to group II (see table).

Comparative analysis of the *Sau*96 I, *Bst*KT I, and *Mse*I restriction patterns allowed the subdivision of group II into 16 subgroups, each including one to seven strains. Restriction analysis with the *Mse*I endonuclease proved to be the most informative: it revealed ten different restriction patterns (A, A', B, B', C, C', D, D', E, and F) for the 45 strains studied (Fig. 2).

Comparison of the lengths of the initial PCR products with the total length of the restriction fragments indicated that all the types of restriction patterns, except A, B, and E, resulted from the simultaneous presence of at least two ITS1 types. In order to ascertain that these complex patterns were not caused by heterogeneity of the cell populations of the strains, we performed control experiments in which DNAs were extracted from at least three colonies of a strain and the ITS1 restriction patterns obtained upon DNA amplification were compared. The identity of the three replicate restriction patterns indicated the absence of heterogeneity among the cell population.

Intragenomic ITS1 heterogeneity was confirmed by cloning the amplified ITS1s from strains in which the presence of different ITS1 variants was expected, with subsequent *Mse*I restriction and sequencing of the individual ITS1s from individual operons.

Analysis of ITS1 nucleotide sequences. ITS1 sequences were determined for ten strains: W-11/2, W-912, W-27-10, W-238-4, W-194-3, R-72, R-915, W-920, R-1/9, and B-t5. For five strains, belonging to restriction groups I and II, in which intragenomic ITS1 polymorphism was expected, cloning and sequencing

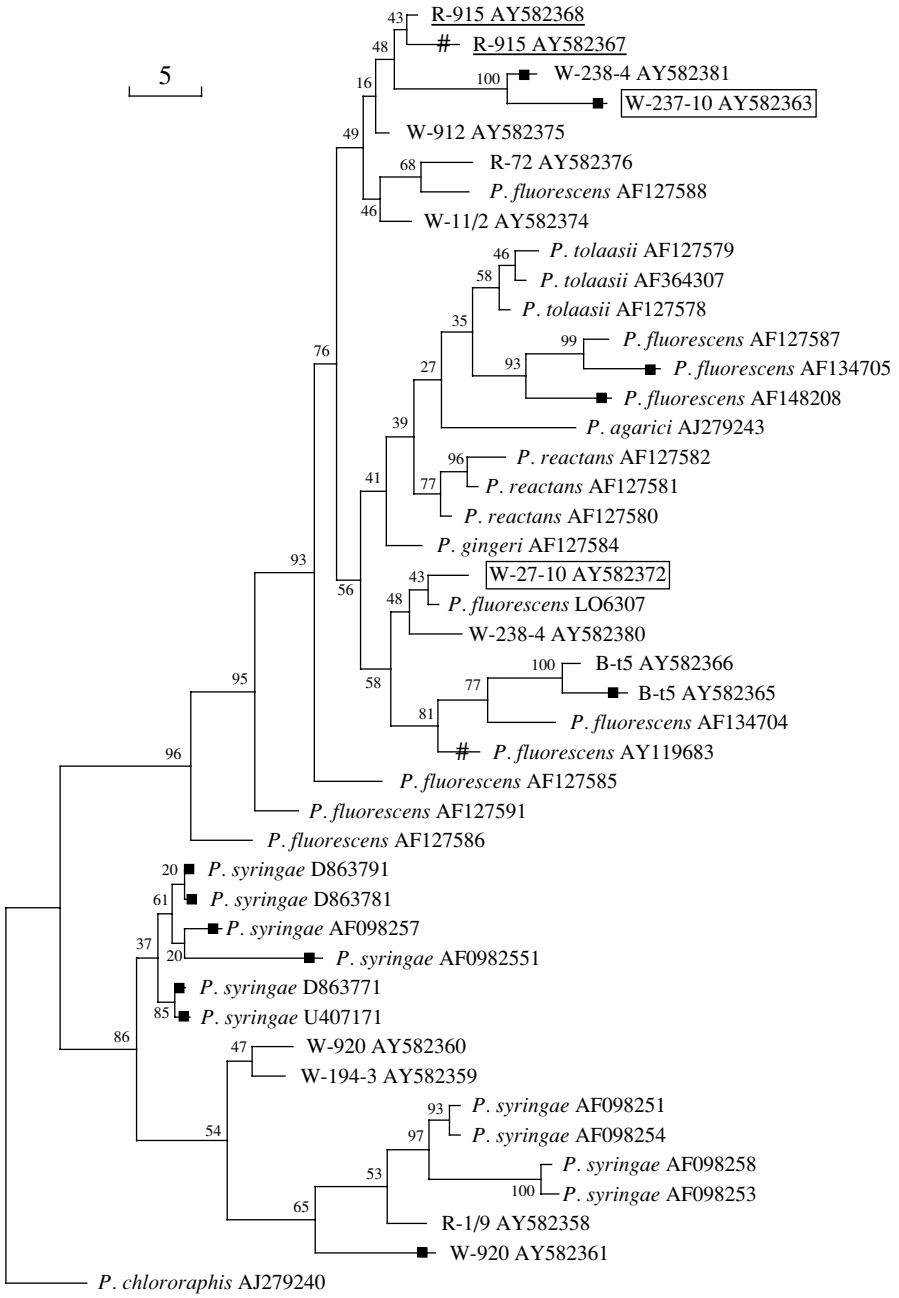

Fig. 4. Phylogenetic tree constructed based on 44 ITS1 sequences from 39 pseudomonad strains using MrBayes 3.0 software. The branch lengths are proportional to the expected number of nucleotide substitutions per site. Pairs of different ITS1 operons from the same strains are in bold, bold italic, underline, bold italic underline, or frame. IST1s that contain 80-bp and 41- to 43-bp insertions are marked with grids and squares, respectively.

of ITS1s from individual operons was performed. The ITS1 sequences of the analyzed strains were aligned with the closely related sequences retrieved from GenBank. The final alignment included ITS1 sequences of 44 ribosomal operons from 39 *Pseudomonas* strains. The borders of the ITS1 and coding regions were determined by comparison with the GenBank sequences. The ITS1 length varied from 503 to 594 bp in most of the strains, being smaller in *P. agarici* (466 bp) and *P. chlororaphis* (456 bp). All of the ITS1s included genes of isoleucine and alanine tRNA.

Two main clusters can be discerned in the phylogenetic tree constructed from the ITS1 nucleotide sequences. Since these clusters include the *P. syringae* and *P. fluorescens* sequences obtained from GenBank, they were designated as the "Syringae" and "Fluorescens" clusters (Fig. 4).

In the pseudomonad ITS1 sequences, apart from conservative regions, variable regions could be discerned. The first variable region, V1, preceded the $tRNA^{Ile}$ gene sequence; the second, V2, was located between $tRNA^{Ile}$ and $tRNA^{Ala}$ genes; and the third, V3, adjoined the 3' end of the $tRNA^{Ala}$ gene (Fig. 3). The inter- and intragenomic differences in the ITS1 sequences were revealed mainly in these regions and were represented by two main types of variations.

The first type of variation consists in a change in the ITS1 length due to an insertion in V1. For instance, strains R-915 and *P. fluorescens* (AY119683) have an 80-bp insert in this region. This insert has a high similarity to the major part of the following $tRNA^{Ile}$ gene and possibly originated from its duplication. In strain R-915, the 80-bp insert is the only difference between the two ITS1s present in this strain.

The second type of variation was not related to changes in ITS1 length; rather, it consisted in the correlated occurrence of a 41- to 43-bp insert in V1 (Fig. 3) and deletions in the other variable regions. An example of deletions is presented in Fig. 3. The 41- to 43-bp insert occurs in strains W-238-4, W-27-10, B-t5, and W-920, as well as in certain strains from GenBank (Fig. 4). This insert is a sevenfold repeated AGCT motif separated by nucleotide triplets. The first 24 nucleotides of this insert are identical in the strains belonging to the "Syringae" and "Fluorescens" clusters. The other part of the insert contains variable nucleotides that mark strains belonging to different clusters.

In the phylogenetic tree (Fig. 4), the "Syringae" clade includes *P. syringae* ITS1 sequences obtained from GenBank and four ITS1 sequences belonging to the three strains of restriction group I. The "Fluorescens" clade includes ITS1 sequences belonging to the strains of restriction group II and the GenBank ITS1 sequences of *P. fluorescens, P. tolaasii, P. reactans, P. gingeri*, and *P. agarici.* The ITS1 sequences of restriction group II strains determined in the present study cluster in the phylogenetic tree with some of the *P. fluorescens* ITS1 sequences from GenBank. In some cases, the ITS1s of different operons of the same strain cluster together (as in the case of strains B-t5 and R-915), while, for other strains, they may be remote from each other and closely related to the ITS1s of other strains (as with strains W-920, W-27-10, and W-238-4).

The *syrB* gene. Amplification with primers specific to the *syrB* gene, which is involved in syringomycin biosynthesis, yielded a 756-bp product for all the strains studied. Its amount, however, varied from strain to strain, being much higher for the 12 strains of restriction group I than for the other 33 strains. The amplification products obtained for strains R-1/9 (restriction group I) and W-11/2 (restriction group II) were cloned and sequenced. The analysis of the nucleotide sequences of the *syrB* gene fragments of these strains revealed, in strain W-11/2, a G to A substitution in the region complementary to the 3' end of one of the primers, which was possibly the reason for the weak amplification observed for the strains of restriction group II. The homologies of the *syrB* gene fragment sequences of strains R-1/9 and W-11/2 both to each other and to the *P.syringae* pv. *syringae* (U25130) sequence were 95.0, 96.5, and 93.5% respectively.

DISCUSSION

The results of our investigation demonstrate a high degree of genetic variability among the Russian strains of pseudomonads obtained from affected tissues of cereal cultures exhibiting symptoms of basal bacteriosis. Analysis of the restriction products of ITS1 and of the nucleotide sequences of these regions enabled us to subdivide the 45 strains studied into two genetic groups.

Group I consisted of 12 strains with very similar REP, ERIC, and BOX fingerprints; these strains had only two types of ITS1 restriction patterns (table). The restriction patterns obtained after digesting amplified ITS1s with *Hae*III, *Taq*I, and *Msp*I endonucleases were similar to the patterns previously found in various pathovars of *P. syringae* [3]. In the phylogenetic tree built using ITS1 sequences, all the investigated strains of restriction group I (R-1/9, W-920, and W-194-3) belonged to the "Syringae" cluster and were evidently related to *P. syringae*.

The strains of restriction group II exhibited higher genetic diversity, both in their DNA fingerprints and in the results of ITS1 digestion with *Bst*KT I, *Sau*96 I, and *Mse*I restriction endonucleases. They were subdivided in 16 subgroups based on the similarity of their restriction patterns. This subdivision, however, may be imprecise due to the difficulty of discrimination between fragments of similar lengths. In the phylogenetic tree, seven of the strains from restriction group II (W-11/2, W-912, W-27-10, W-238-4, R-72, R-915, and B-t5) were located on various branches of the "Fluorescens" cluster, grouping with the GenBank *P. fluorescens* strains. These data lead us to the conclusion that the pseudomonad strains of restriction group II are related to various strains of *P. fluorescens*.

The results of amplification of the *syrB* gene fragment also support subdivision of the strains studied into two groups. The 12 strains exhibiting intense amplification of the *syrB* gene fragment evidently belong to the species *P. syringae*, in which the presence of this gene has previously been reported [12, 13]. Amplification of a 756-bp fragment in restriction group II strain W-11/2 probably indicates the presence of the *syrB* gene in the genomes of this and other strains supposedly belonging to *P. fluorescens.* The fact that the amplification of this fragment was weak may be due to a nucleotide substitution in the region of primer annealing or to the structural features of the region flanking the fragment.

The high degree of homology between the *syrB* gene fragment of strain W-11/2 and GenBank *P. syringae* sequence U25130 also indicates the presence of a functioning syringomycin biosynthesis gene in strain W-11/2. It has previously been shown that the capacity for synthesis of toxic lipodepsipeptides is not confined to *P. syringae* but is also inherent in *P. fuscovaginae* [14]. Our data indicate that an even wider range of pseudomonads is probably capable of synthesizing this toxin.

The extent of the divergence of ITS1 sequences within the "Syringae" and "Fluorescens" clades were similar (Fig. 4). The strains of *P. tolaasii, P. reactans, P. agarici*, and *P. gingeri* presented in the tree exhibited a relatively small divergence in their ITS1 sequences. The *P. tolaasii* and *P. reactans* strains form compact monophyletic groups deep within the "Fluorescens" cluster, which agrees with the results of an earlier performed analysis of *gyrB* and *rpoD* gene sequences of pseudomonads [6]. This result suggests the possibility of treating *P. tolaasii, P. reactans, P. agarici, P. gingeri*, and various groups of *P. fluorescens* as subspecies of a single genospecies.

Cloning and subsequent sequencing of individual ITS1s have demonstrated the presence of ITS1 copies differing in length or nucleotide sequence in the DNA of certain pseudomonad strains. Intragenomic heterogeneity of ribosomal operons is widespread among bacteria [15] and hampers phylogenetic reconstructions [16, 17]. We have demonstrated this phenomenon in various pseudomonad strains and discussed it in detail in a previous paper [10].

Among the 45 pseudomonad strains associated with basal bacteriosis that we investigated in the present study, 12 were found to belong to the species *P. syringae* and the rest proved to be related to *P. fluorescens*. Strains B-t5, W-27-10, and W-238-4 form a compact subcluster within the "Fluorescens" clade in the phylogenetic tree, and this subcluster should be treated as a group of the same rank as *P. tolaasii* and *P. reactans.*

The classification of the "Fluorescens" strains performed in this study based on their genomic characteristics coincides only in part with the classification based on biochemical characteristics. This fact is not surprising, since even a single mutation can lead to enzymatic dysfunction and thus seriously affect metabolism.

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