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Heterogeneity of Diphtheria Toxin Gene, *tox*, and Its Regulatory Element, *dtxR*, in *Corynebacterium diphtheriae* Strains Causing Epidemic Diphtheria in Russia and Ukraine

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Diphtheria toxin (*tox*) and its regulatory element (*dtxR*) from 72 *Corynebacterium diphtheriae* strains isolated in Russia and Ukraine before and during the current diphtheria epidemic were studied by PCR-single-strand conformation polymorphism analysis (PCR-SSCP). Twelve sets of primers were constructed (eight for *tox* and four for *dtxR*), and three regions within *tox* and all four regions of *dtxR* showed significant variations in the number and/or sizes of the amplicons. Two to four different SSCP patterns were identified in each of the variable regions; subsequently, *tox* and *dtxR* could be classified into 6 and 12 different types, respectively. The great majority of epidemic strains from both Russia and Ukraine had *tox* types 3 and 4, and only in a single preepidemic strain isolated in Russia were all eight *tox* regions identical to those of *C. diphtheriae* Park-Williams No. 8 (*tox* type 1). Epidemic strains from Ukraine can easily be identified by *dtxR* type 5, while the majority of the Russian epidemic strains have *dtxR* of types 2 and 8. No differences in the *tox* regions between mitis and gravis biotype strains were observed. However, *dtxR* types 2, 5, and 8 were identified only in the gravis biotype, and *dtxR* type 1 was characteristic for the mitis biotype strains. PCR-SSCP is a simple and rapid method for the identification of variable *tox* and *dtxR* regions that allows for the clear association of *tox* and *dtxR* types with strains of distinct temporal and/or geographic origins.

Since 1990, epidemic diphtheria has reemerged in the newly independent states of the former Soviet Union. In 1994, 47,802 cases and 1,746 deaths from diphtheria were reported (4), and the incidence of diphtheria was still increasing in 1995. In 1994, at least 20 imported cases of diphtheria were reported in the neighboring and western European countries, demonstrating the potential for further spread of this epidemic (5). The reasons for the resurgence of the diphtheria epidemic in the newly independent states are not fully understood. Low levels of vaccine coverage in infants and children, the waning of vaccine-induced immunity in adults, and increased movements of the population are considered to be the most important factors (8). However, factors other than immune status, such as the effects of environment on the causative organism and potential variations in the virulence factors, must also be taken into consideration. In the present study we focused on the *tox* gene that encodes for diphtheria toxin, the primary virulence factor of *Corynebacterium diphtheriae*. Fragment A of the toxin contains the ADP-ribosyltransferase activity; fragment B contains the receptor binding and membrane-associating domains. The structural gene, *tox*, is carried by a family of corynebacteriophages, including the well-characterized β phage, and is under control of a chromosomal iron-dependent repressor, *dtxR*, and a promoter-operator region. To investigate the heterogeneity of *tox* and *dtxR* among different strains of *C. diphtheriae* isolated in Russia and Ukraine during the current diphtheria epidemic, we amplified regions of *tox* and *dtxR* and then used single-strand conformation polymorphism (SSCP) to identify variable regions. Double-stranded amplified DNA is denatured to single-stranded DNA and is then electrophoresed in a nondenaturing polyacrylamide gel. The single-stranded DNA migrates not only according to its length but also according to its secondary structure, determined by the nucleotide sequence. SSCP is reportedly capable of detecting 99% of point mutations in DNA molecules of 100 to 300 bp in length and 89% of mutations in molecules of 300 to 450 bp in length (10, 11, 13, 15, 17). Significant diversity among *C. diphtheriae* organisms has been recently demonstrated by ribotyping and multilocus enzyme electrophoresis, indicating that particular ribotypes and enzyme types are associated with the occurrence and spread of the current diphtheria epidemic (6, 21). Variations within *tox* and *dtxR* could have significant effects on the choice of primers used in diagnostic PCR assays aimed at the detection of these genes as a culture confirmation method or for direct detection of organisms from clinical specimens. These variations are also potential molecular subtyping markers for the surveillance of the spread of particular strains within a local community, country, and worldwide.

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TABLE 1. Origin and designations of *C. diphtheriae* strains from Russia and Ukraine[a]

Strain no.	Origin	Year	Biotype[b]	SSCP type	
				tox	*dtxR*
711	Russia	1985	M	1	11
721	Russia	1986	G	2	6
713, 724	Russia	1985–1987	M	3	1
483, 486, G4170, G4179	Russia	1990–1993	M	3	1
G4172, G4174, G4175, G4183, G4186, G4190, G4197	Russia	1991–1993	G	3	2
719	Russia	1986	G	3	4
922, 923, 924, 925, 927	Ukraine	1994	G	3	5
G4169	Russia	1990	G	3	5
496	Russia	1993	M	3	7
G4185, G4189	Russia	1992–1993	G	3	8
G4177	Russia	1993	G	3	10
926	Ukraine	1994	G	3	10
714, 716	Russia	1985–1986	M	4	1
G4168	Russia	1990	M	4	1
918	Ukraine	1993	M	4	1
749, 752, G4204	Russia	1987–1989	G	4	2
484, 485, 490, 855, G4205, G4208	Russia	1990–1993	G	4	2
880, 881, 882, 883, 884, 909, 910, 911, 912, 913, 914, 915	Ukraine	1993	G	4	5
919, 920, 921	Ukraine	1994	G	4	5
856, G4199, G4207, G4211	Russia	1991–1993	G	4	8
722	Russia	1986	G	4	11
726	Russia	1987	G	5	11
489	Russia	1993	G	5	11
765	Russia	1990	M	5	11
718	Russia	1986	M	6	4
753	Russia	1988	G	NT[c]	1
756, 723	Russia	1989	M	NT	1
916, 917	Ukraine	1993	M	NT	9
857, G4209	Russia	1990–1991	G	NT	11
748	Russia	1987	M	NT	11

[a] A total of 72 strains were tested.
[b] M, biotype mitis; G, biotype gravis.
[c] NT, nontoxigenic.

MATERIALS AND METHODS

Strains. Seventy-nine *C. diphtheriae* strains were included in the study; 48 strains were from Russia, 24 strains were from Ukraine, and 7 were control and reference strains (Table 1). *C. diphtheriae* Park-Williams No. 8 (PW8), ATCC 13812, isolated in New York in 1898 and used worldwide in the production of diphtheria toxoid, was used as a control strain for PCR-SSCP. *C. diphtheriae* NCTC 10648 and NCTC 10356 were used as PCR-positive and -negative controls, respectively. Four reference *C. diphtheriae* strains were provided by the Central Public Health Laboratory, London, United Kingdom: strain 1084, which was nontoxigenic, and strains 1085, 1086, and 1087, which were toxigenic, as defined by the Elek assay (7). All strains were stored in 5% defibrinated sheep blood at −70°C.

Nucleic acid extraction. Bacterial growth from an overnight culture on blood agar plates (tryptic soy agar II with 5% sheep blood; Becton Dickinson and Company, Cockeysville, Md.) at 37°C was used. Bacterial genomic DNA from all of the strains was obtained with an automatic nucleic acid extractor (ABI model 340A; Perkin-Elmer Corporation, Norwalk, Conn.) according to the manufacturer's instructions. DNA preparations were dissolved in 200 μl of sterile water and were stored at −20°C.

PCR. Eight overlapping primer sets were designed to cover the sequence of *tox* and its promoter-operator region. Oligonucleotide primers were synthesized on a DNA synthesizer (ABI model 380B; Perkin-Elmer). For the *dtxR* gene, four sets of primer were constructed. The nucleotide sequences and positions of the primers are provided in Table 2 and Fig. 1.

PCR amplification was performed in a Perkin-Elmer Cetus DNA Thermal Cycler 480 instrument, as follows: 0.5 to 2 μl of DNA solution, 10 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$), 1 μl of 100 μM (each) appropriate primer, 4 μl of deoxynucleoside triphosphate mixture (2.5 mM each), and 0.25 U of *Taq* DNA polymerase (Perkin-Elmer) were added to a 0.5-ml centrifuge tube. Sterile Milli-Q water was added up to 50 μl, and this PCR mixture was then overlaid with a drop of mineral oil. The mixture was initially denatured at 95°C for 2 min; this was followed by 35 amplification cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, ending with a final 10-min extension at 72°C. To verify the amplification, 6 μl of the amplified product was electrophoresed on a 1.2% SeaKem GTG agarose gel (FMC Corporation, Philadelphia, Pa.) for 1 h at 150 V. The gels were stained with ethidium bromide, and the amplicons were visualized on a UV transilluminator.

SSCP. Two microliters of amplified product was mixed with 18 μl of sequencing sample buffer (5 mM EDTA, 0.05% [wt/vol] bromphenol blue, and 0.05% [wt/vol] xylene cyanole in formamide). This mixture was heated to 95°C for 5 min and was then quickly placed on ice before the entire 20 μl was loaded onto the gel. A 12% polyacrylamide Tris-borate-EDTA gel (7.3 cm by 10.2 cm by 0.5 mm; 19:1 acrylamide to bisacrylamide) containing 10% glycerol was used for analysis with the Mini-PROTEAN II electrophoresis system (Bio-Rad Laboratories, Richmond, Calif.). Electrophoresis was carried out at 4°C for 6 h at 200 V of constant voltage. After electrophoresis, amplicons were detected by the silver staining method described by Bassam et al. (1).

Elek assay. All isolates were assayed by the Elek test, which was performed as described earlier (7).

RESULTS

All 79 *C. diphtheriae* strains were PCR positive with all four sets of *dtxR* primers, and 69 strains were PCR positive with all eight sets of *tox* primers. These 69 strains were also positive when they were assayed by the Elek immunodiffusion assay.

A single SSCP pattern identical to that of strain PW8 was detected within five of eight analyzed *tox* regions (regions 1, 2, 4, 5, and 7). Only one strain, *C. diphtheriae* 718, showed a different SSCP pattern (pattern 2) in *tox* region 3 of the A subunit of diphtheria toxin (Fig. 2). Amplification of two *tox* regions of the B subunit of the diphtheria toxin gene with primer sets 6 and 8 revealed several patterns different from that of PW8. Three patterns were detected for *tox* region 6 (Fig. 2), with pattern 2 being the predominant one. Three SSCP patterns were also observed in *tox* region 8; the great majority of strains were characterized by pattern 3 (Fig. 3A). On the basis of the number of different patterns observed for *tox* regions 3, 6, and 8, the *tox* genes could be classified into six

TABLE 2. Primers used for amplification of *C. diphtheriae tox* and *dtxR* genes

Name	Position[a]	Nucleotide sequence (5′ to 3′)[b]
Dipht 1F	(−130)–(−110)	TTGCTAGTGAAGCTTAGCTAG
Dipht 1R	148–169	GAATGGAATCTACATAACCAGG
Dipht 2F	118–138	GAAAACTTTTCTTCGTACCAC
Dipht 2R	349–369	ATTATCCACTTTTAGTGCGAG
Dipht 3F	316–336	GTCAAAGTGACGTATCCAGGA
Dipht 3R	565–586	CACGGGTTTCAAAATTAATCTC
Dipht 4F	535–555	GAACAGGCGAAAGCGTTAAGC
Dipht 4R	817–837	TGCCGTTTGATGAAATTCTTC
Dipht 5F	765–785	GAGCGAAAGTCCCAATAAAAC
Dipht 5R	1013–1032	ACCGTCTGCAATGCCCATTA
Dipht 6F	991–1011	ATACTTCCTGGTATCGGTAGC
Dipht 6R	1267–1287	CGAATCTTCAACAGTGTTCCA
Dipht 7F	1237–1257	CAATTTCTTCATGACGGGTAT
Dipht 7R	1504–1525	GCACACCATTACCAACATAAAC
Dipht 8F	1462–1472	GCTATAGACGGTGATGTAACT
Dipht 8R	1731–1751	TCTACCTGTGCATACTATAGC
DtxR 1F	(−123)–(−103)	GGGACTACAACGCAACAAGAA
DtxR 1R	116–135	CAACGGTTTGGCTAACTGTA
DtxR 2F	91–110	CTGAGCGTCTGGAACAATCT
DtxR 2R	353–334	TTTCACGAGCCTGCGTTCAA
DtxR 3F	309–318	TGGGAACACGTTATGAGTGA
DtxR 3R	570–550	ATCCAACACGGATGTCAGCAT
DtxR 4F	526–545	CGGATCAGTTTACACAGCTC
DtxR 4R	703–683	ATCTAATTTGCCGCCTTTAGT

[a] Nucleotide positions start with the first nucleotide of the initiation codon of *tox* and *dtxR*. Parentheses indicate positions upstream from open reading frames.
[b] Primers were synthesized by using the sequences published previously (2, 9, 12).

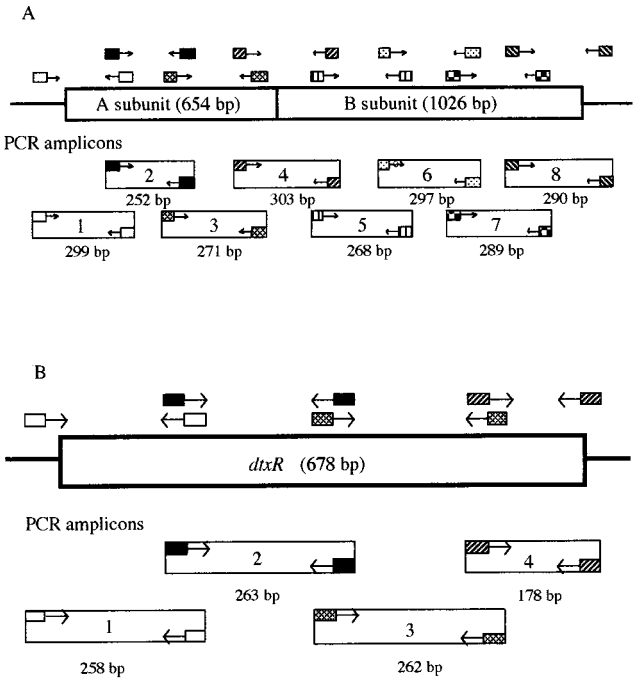

FIG. 1. Locations of PCR primers for *tox* (A) and *dtxR* (B) genes.

distinct SSCP types (Table 3). Only a single strain isolated in Russia in 1985 had *tox* type 1, which was the same as that of strain PW8. Also, a single strain (*C. diphtheriae* 721) isolated in Russia in 1986 was of *tox* type 2, which differs from *tox* type 1 in only one of eight analyzed regions. All but two epidemic toxigenic strains (*C. diphtheriae* 489 and 765) had *tox* type 3 (26 strains) or 4 (33 strains) (Table 3). Both of these *tox* types exhibited pattern 3 in region 8. Strains of both the mitis and the gravis biotypes were identified to possess toxin of *tox* types 3 and 4.

More than one SSCP pattern was observed in all four *dtxR* regions; two, four, three, and four different SSCP patterns were found in *dtxR* regions 1, 2, 3, and 4, respectively (Fig. 4). In the amino-terminal half of the gene (region 1 and 2), 52 of 78 strains had SSCP patterns identical to those of strain PW8. Contrary to that, in the carboxyl-terminal part (regions 3 and 4), only 18 of 78 strains had SSCP patterns identical to the respective patterns of PW8. On the basis of the number of different patterns observed for the *dtxR* region, 12 distinct SSCP types could be identified (Table 4). *dtxR* type 1, identical to that of PW8 in all four analyzed regions, was observed in 13 strains. Twelve of these 13 strains were of the mitis biotype, and all of them were isolated either in the preepidemic period (6 strains) or at the beginning of the epidemic (6 strains). Similarly, eight strains with *dtxR* type 11 date to the preepidemic period or the beginning of the epidemic. Twenty of 24 strains from Ukraine had *dtxR* type 5, characterized by pattern 2 in region 3 and pattern 3 in region 4. This particular combination of patterns was seen in only 1 of 48 Russian isolates (isolate G4169). Among the 31 Russian epidemic strains, significantly more heterogeneity was seen in the *dtxR* gene. The predominant *dtxR* types were type 2 (13 strains) and type 8 (6 strains). Unlike for the *tox* types, in which no significant differences were observed between mitis and gravis biotype strains, particular *dtxR* types could be clearly correlated with either gravis or mitis biotype strains (Fig. 3 A and B; Table 1). *dtxR* types 2, 5, and 8 were seen only in strains of the gravis biotype; of 53 gravis biotype strains included in the study, 43 had *dtxR* of types 2, 5, and 8. Furthermore, 40 of those 43 strains were isolated since the beginning of the epidemic. Mitis biotype strains are clearly associated with *dtxR* type 1, which is the *dtxR* type seen in strain PW8. However, mitis biotype strains from both preepidemic (7 strains) and epidemic (5 strains) periods had *dtxR* of this type. The remaining seven strains of the mitis biotype had *dtxR* types 4, 7, 9, and 11. Some of them (*dtxR* types 4 and 11) contained both gravis and mitis biotype strains. All of the PCR-SSCP patterns observed for both *tox* and *dtxR* genes were reproducible, and each strain was assayed with all 12 sets of primers at least twice.

DISCUSSION

Extensive studies have established that the *tox* gene itself is carried by a family of corynebacteriophages but that the regulation of *tox* gene expression is mediated through the chromosomally located *dtxR* (2). When DtxR becomes activated by divalent heavy metal ions, it binds to the *tox* operator and blocks transcription. Naturally occurring variants of *tox* or *dtxR* genes could be associated with increased or decreased levels of toxin production. However, point mutations that do not necessarily result in amino acid substitutions could also be expected to occur and could potentially be used for molecular subtyping, as reported earlier for cholera toxin (16). For this purpose, the PCR-SSCP assay was used to produce amplicons by using 12 overlapping sets of primers that cover the regions of the *tox* and *dtxR* genes (Fig. 1) (2, 9). Strain PW8, which was first isolated in New York in 1898, served as a control in the study because it has been used worldwide for the production of diphtheria toxoid over the past several decades. The SSCP data for the *tox* gene from 72 epidemic and preepidemic strains from Russia and Ukraine revealed significant variations in the numbers and sizes of the amplicons, resulting in several distinct *tox* types (Table 3). This heterogeneity of the *tox* gene observed in strains causing the current diphtheria epidemic compared with the *tox* gene of PW8 could point toward the possibility that the immune response obtained by vaccination with toxoid obtained from PW8 may not provide adequate protection in persons who encounter these strains with different *tox* genes.

Within *tox*, the levels of heterogeneity in its A and B subunits were quite different. Of four sets of primers that amplified regions of the A subunit in all strains analyzed, three amplified regions (regions 1, 2, and 4) were identical to those of strain PW8. A single strain had a pattern different from that of PW8 in region 3 (*C. diphtheriae* 718). The A subunit, which

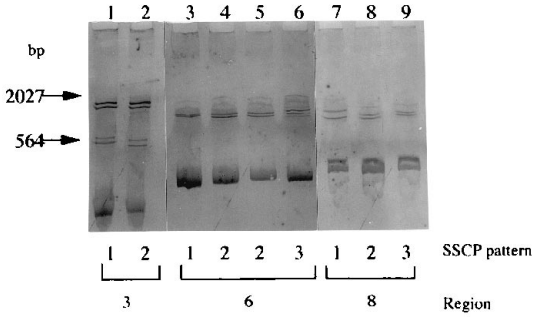

FIG. 2. PCR-SSCP analysis of *C. diphtheriae tox* gene. Lanes 1, 3, and 7, *C. diphtheriae* PW8; lane 2, *C. diphtheriae* 718; lane 4, *C. diphtheriae* 484; lane 5, *C. diphtheriae* 714; lanes 6 and 8, *C. diphtheriae* 489; lane 9, *C. diphtheriae* 483. Bacteriophage lambda DNA digested with *Hin*dIII was used as a marker; the sizes of the standards are given on the left.

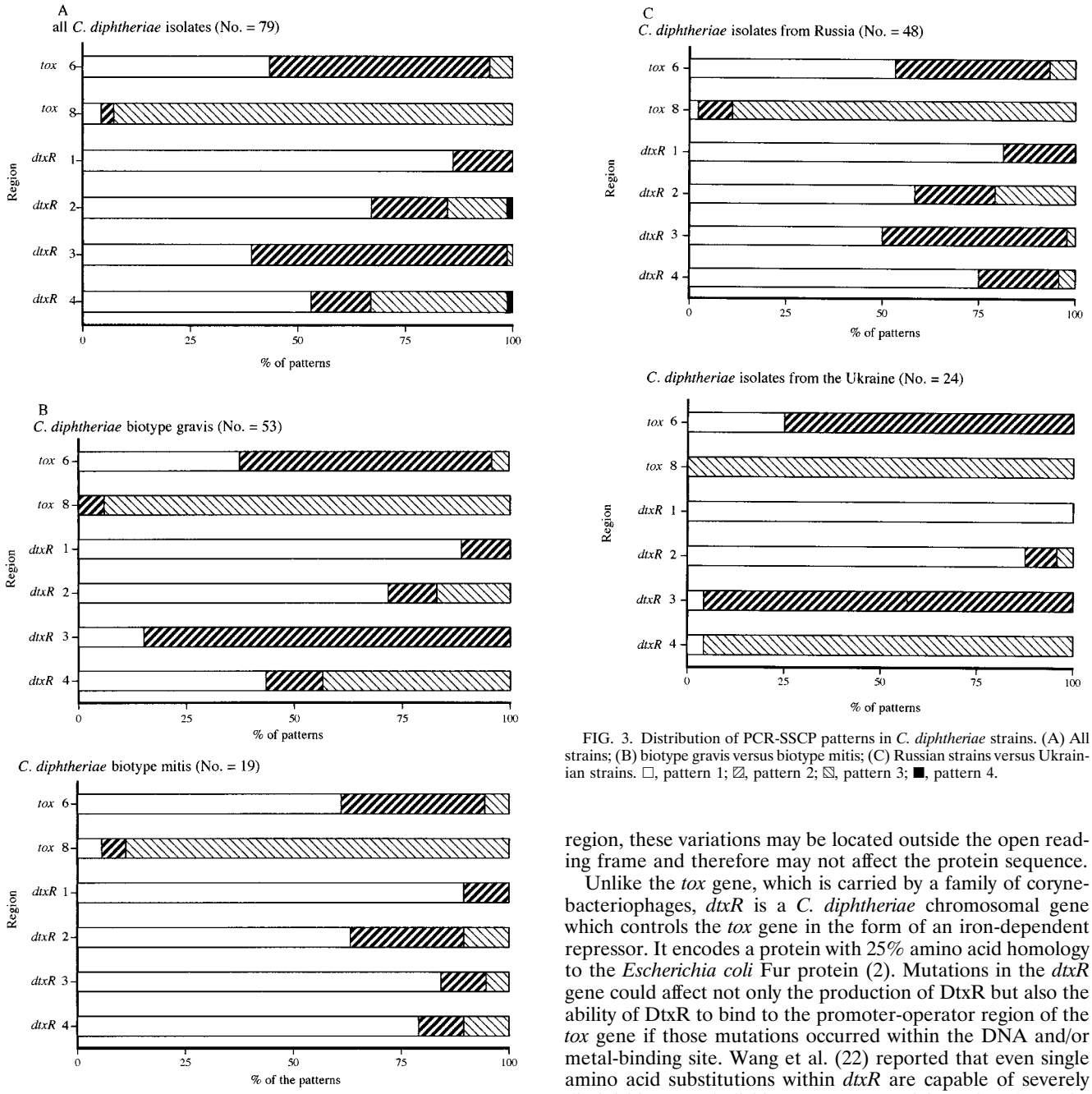

FIG. 3. Distribution of PCR-SSCP patterns in *C. diphtheriae* strains. (A) All strains; (B) biotype gravis versus biotype mitis; (C) Russian strains versus Ukrainian strains. □, pattern 1; ▨, pattern 2; ▧, pattern 3; ■, pattern 4.

is the enzymatically active subunit, appears to be very highly conserved; this observation supports the use of diagnostic PCRs that detect regions of this subunit as a tool for the rapid detection of toxigenic *C. diphtheriae* strains (14, 18). Recently, Mikhailovich et al. (14) reported an excellent correlation of a PCR that detects the 248-bp region of the A subunit with the standard Elek immunodiffusion assay that detects diphtheria toxin. The B subunit appears to be much more variable, particularly in the 3′ end region coding for the receptor binding domain, where several distinct patterns were observed. Interestingly, 64 of 65 toxigenic strains from Russia and Ukraine had SSCP patterns different from that of PW8 in region 8 (Fig. 3C). However, since *tox* region 8 contains the 3′ noncoding region, these variations may be located outside the open reading frame and therefore may not affect the protein sequence.

Unlike the *tox* gene, which is carried by a family of corynebacteriophages, *dtxR* is a *C. diphtheriae* chromosomal gene which controls the *tox* gene in the form of an iron-dependent repressor. It encodes a protein with 25% amino acid homology to the *Escherichia coli* Fur protein (2). Mutations in the *dtxR* gene could affect not only the production of DtxR but also the ability of DtxR to bind to the promoter-operator region of the *tox* gene if those mutations occurred within the DNA and/or metal-binding site. Wang et al. (22) reported that even single amino acid substitutions within *dtxR* are capable of severely diminishing or abolishing repressor activity. Therefore, the heterogeneity of *dtxR* in *C. diphtheriae* strains from Russia and Ukraine must be analyzed in light of the fact that the regulation of the *tox* gene is repressed by DtxR in the presence of iron and that the absence of DtxR could be expected to result in the increased production of the diphtheria toxin. In each of the amplified regions of *dtxR* several SSCP patterns were identified. The amino-terminal half of the gene, regions 1 and 2, appeared to be more conserved than the carboxy-terminal half (regions 3 and 4); 52 of 78 strains had an SSCP pattern that was the same as that of PW8. Previous reports are in agreement with these results. Boyd et al. (3) reported that the *dtxR* gene from the wild-type *C. diphtheriae* 1030 differed by 6 amino acid residues, all of which were located in the carboxyl-terminal half of *dtxR*, from the *dtxR* genes from *C. diphtheriae* C7 and PW8 (3). Wang et al. (22) reported that the single amino acid sub-

TABLE 3. *tox* SSCP patterns and types detected in toxigenic *C. diphtheriae* strains[a]

tox SSCP type	SSCP patterns in *tox*			No. of strains with the indicated origin			
	Region 3	Region 6	Region 8	Russia	Ukraine	Reference and control	Total
1	1	1	1	1	0	2	3
2	1	1	2	1	0	1	2
3	1	1	3	19	6	1	26
4	1	2	3	17	16	0	33
5	1	3	2	3	0	1	4
6	2	1	3	1	0	0	1
Total				42	22	5	69

[a] A total of 69 strains were tested.

TABLE 4. *dtxR* SSCP patterns and types detected in *C. diphtheriae* strains[a]

dtxR SSCP type	SSCP patterns in *dtxR*				No. of strains with the indicated origin			
	Region 1	Region 2	Region 3	Region 4	Russia	Ukraine	Reference and control	Total
1	1	1	1	1	12	1	3	16
2	1	1	2	1	16	0	0	16
3	1	2	1	1	0	0	1	1
4	1	3	1	1	2	0	0	2
5	1	1	2	3	1	20	0	21
6	1	3	1	2	1	0	0	1
7	1	2	3	1	1	0	0	1
8	1	3	2	1	6	0	0	6
9	1	2	2	3	0	2	0	2
10	1	3	2	3	1	1	0	2
11	2	2	1	2	8	0	2	10
12	2	4	1	4	0	0	1	1
Total					48	24	7	79

[a] A total of 79 strains were tested.

stitutions, when located in the amino-terminal half of *dtxR*, can significantly reduce or even abolish the repressor activity, indicating that this part of the gene, which has two main functions, is fundamental for the functioning of *dtxR*. One function is that of the iron-binding domain and another is that of the DNA-binding domain. The metal ion-binding domain of *dtxR* is located between residues 70 and 106 (22). The His residue at position 79 (His-79), His-98, Cys-102, and His-106 form a putative metal ion-binding pocket (20), and Cys-102 is considered to be positioned in the metal ion activation site and to play an essential role in the action of DtxR (19). The DNA-binding domain is located between residues 28 and 52 as the helix-turn-helix motif, which is similar to several other well-characterized DNA-binding motifs (22). The carboxyl-terminal half of *dtxR* (regions 3 and 4) seems to be much more variable, because three and four patterns were observed for regions 3 and 4, respectively. Only 18 strains had patterns in these two regions identical to that of PW8. Boyd et al. (2) found that the region from Val-174 to His-201 bears some homology to the helix-turn-helix motif for DNA-binding proteins (2). In our study, most of the strains tested (60 of 78 strains) showed SSCP patterns different from those of PW8 in regions 3 and 4; therefore, the DNA-binding ability of DtxR may have been changed, thus affecting the production of diphtheria toxin.

These observations become even more striking when the geographic and/or the temporal origins of the *C. diphtheriae* assayed strains are taken into consideration. Of 18 strains that had regions 3 and 4 identical to those of PW8, 3 were World Health Organization reference strains and 11 were strains isolated in Russia before the epidemic began; only 4 strains, 3 from Russia and 1 from Ukraine were isolated during the epidemic period. However, all three of them date back to the first 2 years of the epidemic. *C. diphtheriae* strains from Ukraine appear to be quite homogeneous as far as *dtxR* is concerned; all but one strain had identical regions 1, 3, and 4 (Fig. 3C). The only difference among these strains was observed in *dtxR* region 2, where the majority of strains (20 strains) had pattern 1, which is identical to that of strain PW8, and only 1 and 2 strains had patterns 3 and 2, respectively. Furthermore, all of the Ukrainian strains had pattern 3 in region 4. This particular combination of patterns in regions 3 and 4 (defined as *dtxR* type 5) appears to be the specific molecular marker for the Ukrainian strains. Only 1 of 48 Russian isolates (isolate G4169) had this *dtxR* type, and it originated in Penza, a city located in the southwestern part of Russia, toward the Ukrainian border. None of the reference or control strains, including strain PW8, had this particular *dtxR* type.

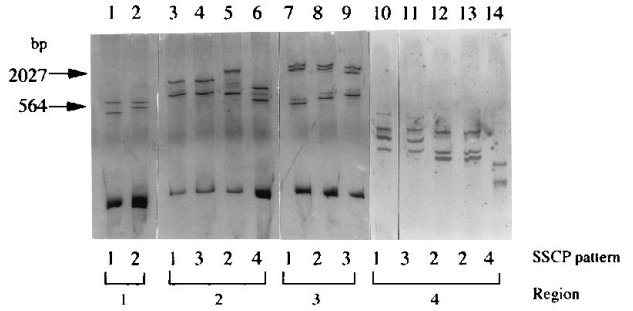

FIG. 4. PCR-SSCP analysis of *C. diphtheriae dtxR* gene. Lanes 1, 3, 7, and 10, *C. diphtheriae* PW8; lanes 2, 5, and 12, *C. diphtheriae* 489; lane 4, *C. diphtheriae* 719; lanes 6 and 14, *C. diphtheriae* NCTC 10356; lane 8, *C. diphtheriae* 484; lane 9, *C. diphtheriae* 496; lane 11, *C. diphtheriae* 916; lane 13, *C. diphtheriae* 711. Bacteriophage lambda DNA digested with *Hin*dIII was used as a marker; the sizes of the standards are given on the left.

In addition, region 8 of the B subunit of the toxin differed significantly among strains from Russia and Ukraine. All Ukrainian strains and the majority of the Russian strains (37 of 42) had pattern 3. However, in region 6, 16 of 22 Ukrainian strains had pattern 2, while 22 of 42 Russian strains had pattern 1, which is identical to that of strain PW8 (Fig. 3B). DNA sequencing of these regions is under way, and preliminary data indicate that each SSCP pattern could be associated with a particular point mutation, which supports the hypothesis that SSCP patterns have potential as molecular subtyping markers.

The diversity of the SSCP patterns identified in the present study is in striking contrast to the limited differentiation level of the traditional biotyping method. Biotyping, which distinguishes four biotypes, biotypes gravis, mitis, belfanti; and intermedius, provides a very low level of discrimination, especially given the fact that one biotype is usually predominant in a particular epidemic or endemic. Also, differences in the severity of clinical features are generally not associated with a particular biotype. A significant correlation between the patterns typical for gravis and mitis biotype strains was observed in the *dtxR* gene, especially in its carboxyl-terminal half.

Ribotyping has already proven to be a useful tool for the

molecular typing of *C. diphtheriae* strains. Recent studies showed that ribotypes G1 and G4 (both of the gravis biotype) are significantly associated with the current diphtheria epidemic in the newly independent states of the former Soviet Union (6, 21). The preepidemic period is, however, characterized by the presence of numerous other ribotypes, mainly of the mitis biotype. Ribotyping data were available for 56 strains from Russia and Ukraine; 38 strains of biotype gravis, 18 strains of biotype mitis, and 12 different ribotypes were observed (21). Regardless of ribotype, 49 of 50 toxigenic strains had pattern 3 in region 8 (3′ end of the B subunit gene), confirming once again that the carboxyl terminus of the B subunit is very conserved in both preepidemic and epidemic strains, even though this pattern is different from that of strain PW8. Region 6 of the B subunit appears to be somewhat more variable, because in only 22 of 50 toxigenic strains was this region identical to that of PW8; it was different from that of PW8 in 31 strains. When combined with ribotyping, SSCP data indicate that ribotypes G1 and G4, which are considered to be highly characteristic of the strains involved in the current Russian epidemic, account for the majority of those 31 strains. Particularly, pattern 2 in region 6 is highly characteristic of these strains and could be used as a molecular marker to identify and trace the epidemic strains as they spread through the newly independent states and further. All G1 and G4 ribotype strains had identical regions 1, 2, 3, 4, 5, 7, and 8 of the *tox* gene. The only region that showed some variation was region 6; 12 strains had a region 6 pattern identical to that of PW8, and 23 strains had pattern 2 (Fig. 2). By contrast, strains of seven different ribotypes, as seen among biotype mitis strains, were isolated in Russia, frequently before the epidemic and only sporadically during the epidemic. In spite of such a variety of ribotypes among mitis biotype strains, region 6 in two-thirds of these strains is identical to that in PW8, while two-thirds of epidemic ribotype G1 and G4 strains differ from PW8 in region 6.

A significant discrepancy in *dtxR* region 3 between epidemic strains and strains of other ribotypes was also observed. All but 1 of 35 epidemic strains had pattern 2, whereas 2 of 18 nonepidemic strains had this pattern. This pattern, like pattern 2 of region 6, appears to be highly characteristic of the epidemic strains and could also be used as a molecular marker.

In the present study, PCR-SSCP was standardized to allow for the rapid and reproducible analysis of *tox* and *dtxR*. The total time needed to perform this assay was less than 10 h. Analysis of the *tox* and *dtxR* genes identified significant variations between individual strains isolated in Russia and Ukraine and associated these variations with their geographic and/or temporal (epidemic or preepidemic) origins as well as with their corresponding biotypes and ribotypes. Most of these variations were observed in the *dtxR* gene, which is plausible since *dtxR* is a *C. diphtheriae*-determined gene that regulates the expression of *tox*, which is carried by a family of corynebacteriophages. Studies to correlate variations within the *dtxR* gene and the quantitative production of diphtheria toxin with the severity of the clinical presentation of patients with diphtheria are under way and may allow for a deeper understanding of this regulatory mechanism and of an association of the clinical features with the level of toxin produced by an individual strain. Subsequently, those data may allow for better prediction of the clinical course and the development of cardiologic and neurologic complications, which could have a further impact in that an adequate dosage of diphtheria antitoxin could be provided as treatment. In addition, DNA sequencing of *dtxR*, particularly the carboxyl-terminal half, may allow for the identification of point mutations that could be used as precise molecular markers of *C. diphtheriae* strains.

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