

CHARACTERIZATION OF THE GENOTYPE AND THE PHENOTYPE OF NONTOXIGENIC STRAINS OF *CORYNEBACTERIUM DIPHTHERIAE* SUBSP. *LAUSANNENSE* ISOLATED IN RUSSIAN RESIDENTS

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In 2018, a few sequencing studies were published revealing the existence of two monophyletic clusters within the *C. diphtheriae* species, meaning that this species can be divided into two subspecies: *C. diphtheriae* subsp. *diphtheriae* and *C. diphtheriae* subsp. *lausannense*. The objective of our study was to describe the genotype and the phenotype of 2 nontoxigenic *C. diphtheriae* strains isolated in Russia in 2017–2018, which were classified by us as *C. diphtheriae* subsp. *lausannense* based on the aggregated data yielded by a variety of techniques, including microbiological and molecular genetic techniques, as well as a bioinformatic search for subspecies-specific genes in the publicly available genomes of *C. diphtheriae*. The isolated strains had morphological and biochemical characteristics of *C. diphtheriae*. The strains were assigned to the MLST type ST199 included in the clonal complex associated with subsp. *lausannense*. PCR revealed that both analyzed strains of *C. diphtheriae* subsp. *lausannense* carried the *ptsI* gene encoding phosphoenolpyruvate-protein phosphotransferase and did not carry the *narG* gene encoding the synthesis of nitrate reductase subunits, whereas the strains of *C. diphtheriae* subsp. *diphtheriae* had the *narG* gene and did not have *ptsI*. We experimentally proved the ability of *lausannense* strains to ferment N-acetylglucosamine. Our findings expand the knowledge of the biological diversity of *C. diphtheriae* and indicate the need for estimating the spread of these microorganisms in Russia, as well as their pathogenic potential.

Keywords: diphtheria, nontoxigenic *Corynebacterium diphtheriae*, *Corynebacterium diphtheriae* subsp. *lausannense*, multilocus sequence typing, phylogenetic analysis

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Compliance with ethical standards: the study was approved by the Ethics Committee of G. N. Gabrichevsky Research Institute for Epidemiology and Microbiology. Informed consent was obtained from all participants.

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ХАРАКТЕРИСТИКА ГЕНОТИПА И ФЕНОТИПА НЕТОКСИГЕННЫХ ШТАММОВ *CORYNEBACTERIUM DIPHTHERIAE* SUBSP. *LAUSANNENSE*, ВЫДЕЛЕННЫХ НА ТЕРРИТОРИИ РОССИИ

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В 2018 г. на основании полногеномных данных появились публикации о наличии двух монофилетических кластеров внутри вида *C. diphtheriae*, что позволяет дифференцировать этот вид на два подвида: *C. diphtheriae* subsp. *diphtheriae* и *C. diphtheriae* subsp. *lausannense*. Целью работы было описать генотип и фенотип двух нетоксигенных штаммов *C. diphtheriae*, выделенных в 2017–2018 гг. с профилактической целью, которые на основании совокупности результатов множества методов могут быть отнесены к *C. diphtheriae* subsp. *lausannense*. В исследовании использовали микробиологические и молекулярно-генетические методы, а также биоинформатический поиск подвид-специфичных генов в публично доступных геномах *C. diphtheriae*. Выделенные штаммы имели характерные для *C. diphtheriae* морфо-культуральные свойства и биохимическую характеристику. В MLST штаммы принадлежали к сиквенс-типу ST199, входящему в клональный комплекс, ассоциированный с subsp. *lausannense*. С использованием ПЦР были показаны наличие *ptsI* (гена, кодирующего фосфоенолпируват-белок фосфотрансферазы) и отсутствие *narG* (гена, кодирующего синтез субъединиц нитратредуктазы) у двух исследуемых штаммов *C. diphtheriae* subsp. *lausannense* и противоположная картина — у штаммов *C. diphtheriae* subsp. *diphtheriae*. Была экспериментально подтверждена способность выделенных штаммов подвида *lausannense* ображивать N-ацетилглюкозамин. Полученные результаты расширяют представления о биологическом разнообразии вида *C. diphtheriae* и свидетельствуют о необходимости дальнейших исследований по оценке распространенности этих микроорганизмов и изучению их патогенного потенциала.

Ключевые слова: дифтерия, нетоксигенные *Corynebacterium diphtheriae*, *Corynebacterium diphtheriae* subsp. *lausannense*, мультилокусное секвенирование, филогенетический анализ

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Соблюдение этических стандартов: исследование одобрено этическим комитетом Московского научно-исследовательского института эпидемиологии и микробиологии имени Г. Н. Габричевского. Все пациентки подписали добровольное информированное согласие на участие в исследовании.

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Diphtheria is caused by toxigenic strains of *Corynebacterium diphtheriae* harboring integrated bacteriophage DNA containing the toxin gene. The infection spreads from person to person via airborne droplets and develops into classic pharyngeal or nasal diphtheria.

Over the past century, mass immunization programs have dramatically cut down the incidence of diphtheria [1]. In Russia, the incidence rate of the disease has stabilized due to good vaccination coverage ($\geq 95\%$) [2]. In 2017, no incident cases of diphtheria and only 2 asymptomatic carriers were reported in Russia. In 2018, 4 incident cases of the disease and 3 carriers were reported, whereas in the first 9 months of 2019, there were 3 new cases of diphtheria and 2 carriers [3]. In the past few years, there have been no reports of the secondary cases or lethal infection. Most clinical forms of diphtheria are mild localized forms.

Today, diphtheria is a rare disease; therefore, it can pose a diagnostic difficulty to the clinician. This, as well as the existence of latent carriers, who act as a reservoir for the infection, and the fact that the epidemic process unfolds in the vaccinated population, still renders diphtheria a clinically important problem [3].

Recently, infections caused by nontoxigenic *C. diphtheriae* strains have been on the rise. They manifest atypically as pharyngitis, respiratory tract infections, endocarditis, osteomyelitis, septic arthritis or skin infections [4–8].

Historically, *C. diphtheriae* were classified into 4 biotypes based on their biochemical phenotypes: gravis, mitis, intermedius and belfanti [9, 10]. Representatives of the same biovar, though, can be genetically distant [11, 12]. This is why genomics does not support the use of biovars as a reliable classification tool for *C. diphtheriae* [13]. Besides, there is no correlation between the biovar and pathogenicity [14]. Multilocus sequence typing (MLST) based on the determination of allelic profiles of 7 housekeeping genes has made it possible to cluster the entire diversity of *C. diphtheriae* strains into 2 evolutionary lineages: lineage-1 (the majority of the strains) and lineage-2 (only strains of the belfanti biotype) [14].

In 2018, a paper was published describing 3 nontoxigenic strains of *C. diphtheriae* [15]. One of them had been isolated from a Swiss patient with tracheobronchitis and multiple lesions on the distal trachea and the mainstem bronchi; the 2 other strains had been isolated from nasal swabs in the UK and India. Genome comparison that used publicly available *C. diphtheriae* genomes demonstrated that average nucleotide identity between the isolated strains and the NCTC 11397 *C. diphtheriae* reference genome was lower (95.24 to 95.39%) than between the reference genome and other previously published *C. diphtheriae* genomes ($> 98.15\%$). Phylogeny reconstruction based on whole genome sequencing data confirmed the existence of two monophyletic clusters of *C. diphtheriae* corresponding to lineage-1 and lineage-2. Consequently, it was proposed to classify *C. diphtheriae* into two subspecies: *C. diphtheriae* subsp. *diphtheriae* and *C. diphtheriae* subsp. *lausannense*.

The aim of this study was to characterize the genotype and the phenotype of nontoxigenic *C. diphtheriae* strains isolated in 2017–2018 that can be identified as *C. diphtheriae* subsp. *lausannense* based on the aggregated data yielded by a variety of different methods.

METHODS

In the experimental part of the study, we analyzed 2 nontoxigenic *C. diphtheriae* strains isolated at the bacteriological laboratory

of Khabarovsk Regional Psychiatric Hospital in 2017–2018, the control toxigenic strain of *C. diphtheriae* (gravis biotype, accession number 665) from the State collection of pathogenic microorganisms (SCPM-Obolensk), freshly isolated toxigenic strains of *C. diphtheriae* (gravis biotype, strain numbers 66-19, 98-19 and mitis biotype, strain numbers 55-19, 56-19), nontoxigenic strains of *C. diphtheriae* (gravis biotype, strain numbers 57-19, 67-19 and mitis biotype, strain numbers 60-19, 91-19) that had been delivered to the Reference Center for the Surveillance of Measles, Rubella, Mumps, Pertussis, and Diphtheria (G.N. Gabrichevsky Research Institute for Epidemiology and Microbiology) from different Russian regions.

The analysis of *C. diphtheriae* strains was conducted following the guidelines № 4.2.3065-13 for laboratory diagnostics of diphtheria. The isolates were plated onto tellurite blood agar (2% fishmeal hydrolysate agar base, State Research Center for Applied Microbiology & Biotechnology; Obolensk; Russia) supplemented with 7% bovine blood (Leitran; Russia) and potassium tellurite (State Research Center for Applied Microbiology & Biotechnology; Obolensk, Russia) and kept in a temperature-controlled chamber at 37 °C for 24–48 hours. Grown colonies of *C. diphtheriae* were evaluated for their morphological, toxigenic and biochemical properties. The toxigenicity of *C. diphtheriae* strains was evaluated in a precipitation test using a Corynetoxagar medium (State Research Center for Applied Microbiology & Biotechnology; Obolensk, Russia) supplemented with 20% bovine serum (Leitran; Russia) and filter discs soaked in diphtheria antitoxin (Diagnostic Systems; Nizhny Novgorod, Russia). Each antitoxin-impregnated disc contained 5 ± 1 IU of diphtheria antitoxin (as suggested by the guidelines 4.2.3065-13). Biochemical properties of the cultures were determined from their cysteinase, urease, saccharolytic and nitrate reductase activity using the media prepared at our laboratory and a commercial DS-DIPH-CORYNE kit (Diagnostic Systems; Nizhny Novgorod, Russia).

To evaluate the ability of the analyzed strains to ferment N-acetylglucosamine, a phenol red broth was *ex tempore* supplemented with N-acetylglucosamine (Sigma-Adlrlich; USA). Then, a loop full of overnight *C. diphtheriae* cultures grown on serum agar was added to 3 ml of the solution. The cultures were incubated at 37 °C for 24–48 h. Fermentation was evaluated based on the change in the color of the solution. Two toxigenic and two nontoxigenic gravis strains, as well as two toxigenic and two nontoxigenic mitis strains, were used as controls.

The sample of the analyzed published genomic sequences comprised 204 *C. diphtheriae* genomes representing diphtheriae and *lausannense* subspecies deposited in the NCBI Refseq database, 3 genomes of *C. diphtheriae* subsp. *lausannense* from the NCBI Genbank and one genome of *Corynebacterium ulcerans* BR-AD22, which served as an outgroup for phylogenetic reconstruction. In total, 208 genomes were included in the analyzed dataset.

Coding sequences retrieved from the genome annotations in the corresponding databases were clustered into ortholog groups using OrthoMCL [16] with standard settings (inflation index of 1.5; protein sequence similarity threshold of 50%; e-value of 10⁻⁵). For phylogeny reconstruction, we used the groups of orthologs that were made up of the genes present in every genome in the amount of 1 copy. Nucleotide sequences were aligned in MUSCLE software [17] and then concatenated. Phylogeny reconstruction was performed following the Maximum Likelihood algorithm implemented in FastTree software [18] using the GTR+CAT model. MLST types of the published sequences were predicted based on the

data retrieved from PubMLST. Clonal clusters were formed in Phyloviz 2 using the goeBURST algorithm at the SLV level [19].

Total DNA was isolated from overnight *C. diphtheriae* cultures grown on fishmeal hydrolysate agar (State Research Center for Applied Microbiology & Biotechnology; Obolensk, Russia) supplemented with 10% bovine serum (Leitran; Moscow) using a standard boiling extraction method with subsequent centrifugation.

Detection of *tox* gene fragments in nontoxicogenic *C. diphtheriae* strains was performed in accordance with the protocol described in [20]. The PCR reaction mix contained 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 μM of forward and reverse primers, 200 mM of each dNTP, and 1 unit of Taq polymerase (Thermo Fisher Scientific; USA). DNA of the control toxigenic *C. diphtheriae* strain (gravis biovar, accession number 665) was used as a positive amplification control. MLST types of *C. diphtheriae* strains were determined following the international protocol [14]; fragments of 7 housekeeping genes were Sanger-sequenced, including *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, and *rpoB*. Sequencing was carried out by Evrogen JSC (Moscow). Allele identification was done using the PubMLST database.

To identify *dtxR* fragments in the sequences of *C. diphtheriae* strains, PCR was carried out with one pair of primers for the entire region of the *dtxR* gene: GGGACTACAACGCAACAAGAA and TCATCTAATTTGCGCCGCTTTA as described in [20, 21]. The following primers were used for subspecies-specific PCR: *ptsl_F*: ACTTTCCGAACCTGCCATCC and *ptsl_R*:GTGTACTCCTTCGTCTGCTC; *narG_F*: CTGACCACTGGGGCGAGG and *narG_R*: GAGTTGTCATAACGCCACTG.

RESULTS

The *C. diphtheriae* strains B-8759 and B-8760 had been isolated from the pharynx of two patients (26 and 77 years) admitted to 2 different units of a psychiatric hospital; the patients had undergone a standard preadmission test for diphtheria (see Paragraph 3.4. of the Guidelines 3.1.3018-12 on the epidemiological surveillance of diphtheria infection). The isolated strains were identified from their morphological, toxigenic and biochemical properties as recommended by the Guidelines 4.2.3065-13 on the laboratory diagnostics of diphtheria. On tellurite blood agar, the grown colonies appeared grayish-black, fuzzy, crumbly, with slightly irregular margins and a raised center. Toxigenicity of the grown colonies was determined using discs impregnated with diphtheria antitoxin (the Feldman method). After 24 and 48 hours of incubation, the

isolated cultures still had not produced specific precipitation lines, unlike the control toxigenic *C. diphtheriae* strain (gravis biotype, accession number 665), suggesting the absence of toxigenicity. PCR performed to detect the fragment of the *tox* gene demonstrated that the analyzed strains did not carry the diphtheria toxin gene.

Biochemical properties of the cultures were determined from their cysteinase, urease, saccharolytic and nitrate reductase activity. The cultures exhibited cysteinase activity and formed a brown halo following inoculation into the Pisu medium; the cultures fermented glucose, maltose, fructose and galactose, did not ferment saccharose and starch, and exhibited no urease or nitrate reductase activity (Table 1). The tests allowed us to provisionally assign the analyzed *C. diphtheriae* strains to the belfanti biotype typically seen in *lausannense* subspecies.

In the next step, we analyzed the previously published genomes of *C. diphtheriae*, which was necessary to verify that the studied species can be distinctly divided into subspecies and to conduct a search for species-specific protein-encoding genes.

The constructed phylogenetic tree (Fig. 1) confirmed the results previously obtained on a smaller sample indicating that representatives of *C. diphtheriae* constituted two clades corresponding to the subspecies *diphtheriae* and *lausannense*. The tree also showed that the representatives of these subspecies belonged to non-overlapping groups of sequence types. The goeBURST clustering analysis of MLST types described in PubMLST (Fig. 2) revealed that all representatives of the *lausannense* subspecies whose genomes had been previously sequenced belonged to the sequence types ST106, ST360, or ST409, and to one previously undescribed type that differed from ST359 in just one allele. All these sequence types formed one clonal complex.

It could be hypothesized that other sequence types (such as ST35, ST37, ST69, or ST81) constituting the same clonal complex also belong to the *lausannense* subspecies. An additional argument in favor of our hypothesis is that almost all isolates representing the sequence types from this clonal complex have been described in the PubMLST database as representing the belfanti biotype typical to the *lausannense* subspecies.

The analysis of ortholog groups revealed the existence of loci specific to *C. diphtheriae* subspecies. For example, all strains of the *lausannense* subspecies had a region (presumably, an operon) harboring genes of the phosphotransferase system, for which N-acetylglucosamine is a hypothesized substrate. The following primers were selected for the gene coding

Table 1. Characteristics of the analyzed *C. diphtheriae* cultures

Characteristic	<i>C. diphtheriae</i> strains		
	Strain 665 (control)	Strain B-8759	Strain B-8760
Glucose fermentation	+	+	+
Saccharose fermentation	-	-	-
Maltose fermentation	+	+	+
Fructose fermentation	+	+	+
Galactose fermentation	+	+	+
Starch fermentation	+	-	-
Urease	-	-	-
Nitrate reductase	+	-	-
Cysteinase test	+	+	+
Toxigenicity (the Feldman method)	+	-	-
Presence of the <i>tox</i> gene	+	-	-

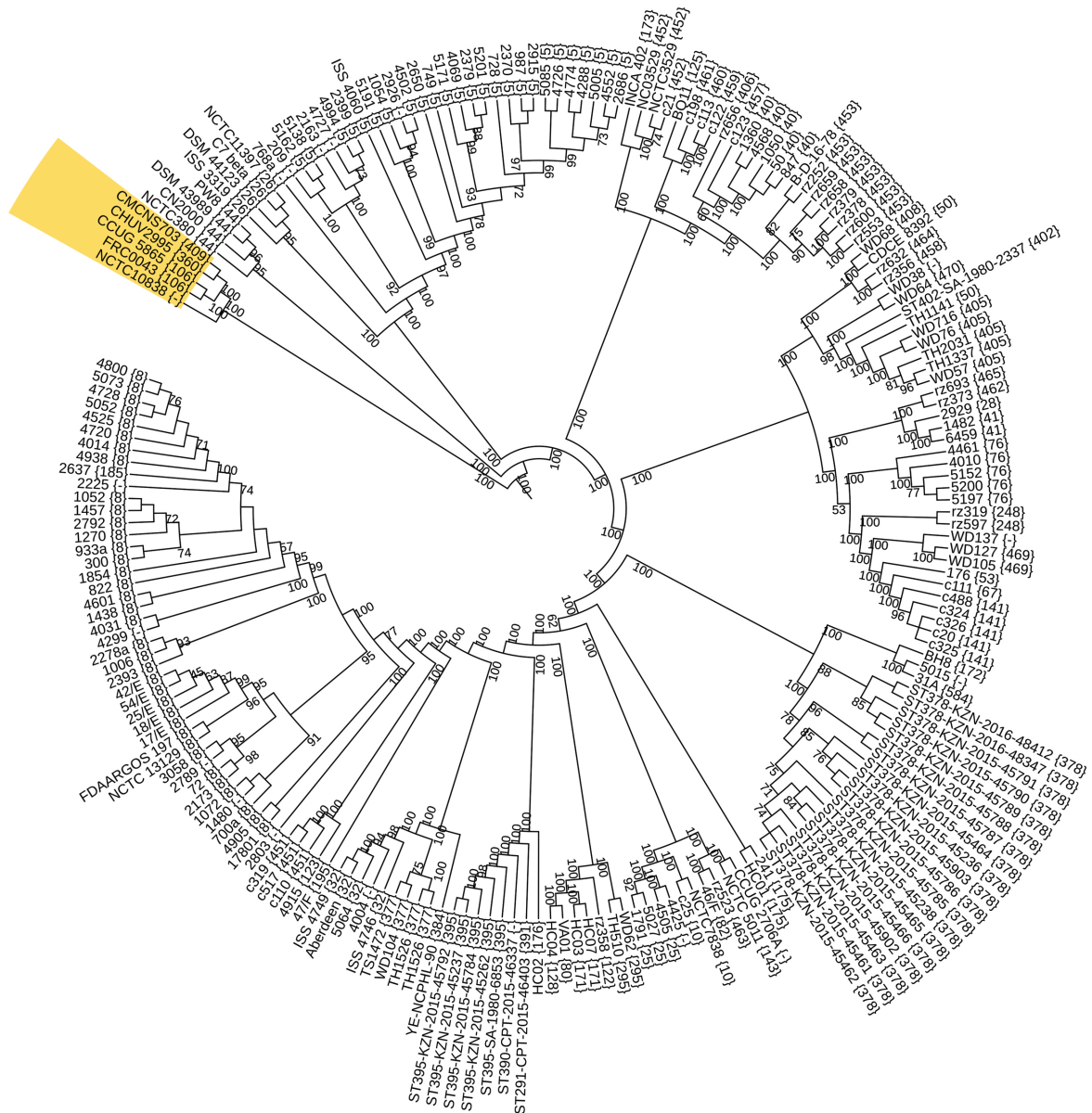


Fig. 1. A phylogenetic tree for *C. diphtheriae* strains with publicly available sequenced genomes. The length of the branches and the genome of *C. ulcerans* BR-AD22 used for rooting are not shown in the figure. The numbers on the branches represent bootstrap values. Strains of *C. diphtheriae* subsp. *lausannense* are shown in gray. The numbers in brackets refer to the predicted sequence types and follow the PubMLST nomenclature (dash marks represent yet undescribed sequence types)

for phosphoenolpyruvate-protein phosphotransferase: *ptsI*_F: ACTTTCCGAACCTGCCATCC and *ptsI*_R: GTGTACTCCTTCGTCTGCTC (the expected product length was 489 bp). At the same time, a locus encoding the synthesis of nitrate reductase subunits was detected only in the genomes of the *diphtheriae* subspecies. The following primers were selected for the gene encoding its α -subunit (the gene was present in the sequences of 201 out of 202 strains representing this subspecies in the analyzed sample): *narG*_F: CTGACCACTGGGGCGAGG and *narG*_R: GAGTTGTCAACGCCACTG (the expected product length was 691 bp).

PCR with primers for the amplification of *ptsI* and *narG* fragments (Fig. 3) showed that the samples containing DNA of B-8759 and B-8760 strains carried the *ptsI* gene and did not carry the *narG* gene, whereas "classic" *C. diphtheriae* strains had the *narG* gene and did not have *ptsI*. There were no samples that carried either both of these genomic loci or none of them.

These findings and the results of biochemical identification allowed us to conclude that the analyzed *C. diphtheriae*

strains belonged to *C. diphtheriae* subsp. *lausannense*. The conclusion was corroborated by the fact that the isolated strains represented the sequence type ST199 included in the clonal complex presumably typical to the representatives of this subspecies (Fig. 2). Another piece of evidence confirming our conclusion was the sequence of the *dtxR* gene that coincided with the sequences found in the genomes of *lausannense* subspecies.

Considering that strains of *C. diphtheriae* subsp. *lausannense* carried the gene coding for phosphoenolpyruvate-protein phosphotransferase, which is part of the phosphotransferase system for N-acetylglucosamine, we conducted a few experiments to investigate the phenotypic manifestations of this gene. The experiments showed that unlike *C. diphtheriae* subsp. *diphtheriae*, both analyzed strains, which we classified as *C. diphtheriae* subsp. *lausannense*, fermented N-acetylglucosamine (Fig. 4).

The analyzed strains, which we classified as *C. diphtheriae* subsp. *lausannense*, were deposited in the State collection of pathogenic microorganisms (SCPM-Obolensk).

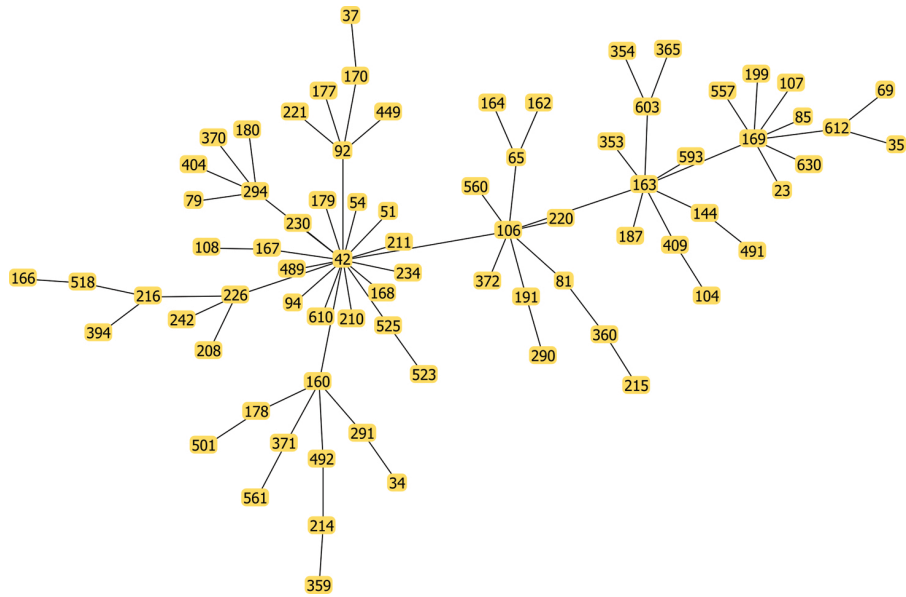


Fig. 2. The clonal complex reconstructed from PubMLST data. The complex comprises *lausannense* strains with publicly available sequenced genomes

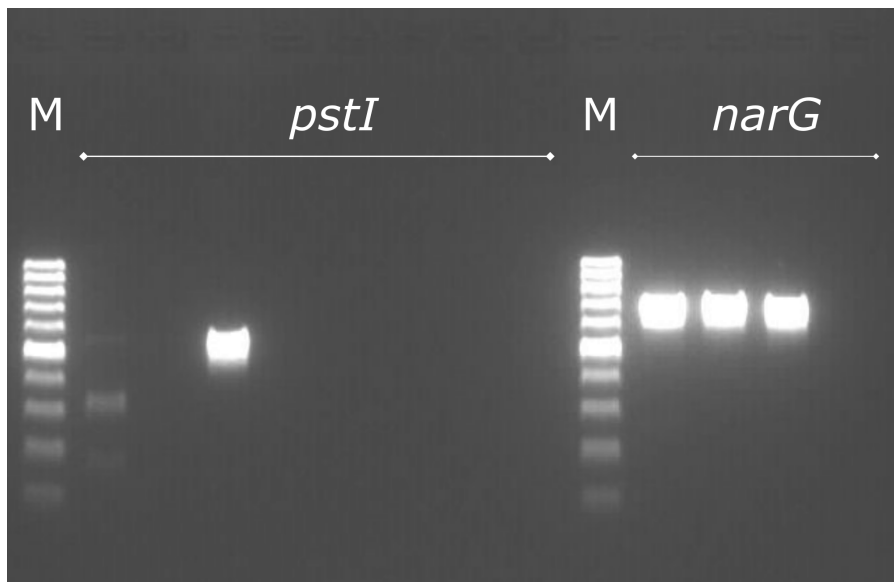


Fig. 3. Gel electrophoresis of PCR products with the following primers: *ptsI*_F — *ptsI*_R and *narG*_F — *narG*_R. M is a GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific; USA) (example)

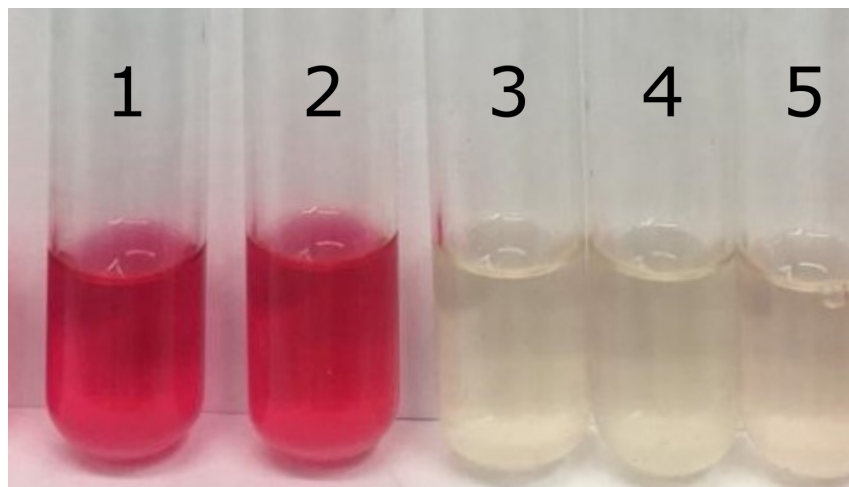


Fig. 4. Saccharolytic activity of *C. diphtheriae* subsp. *lausannense* strains: the ability to ferment N-acetylglucosamine. The crimson color of the medium means the test results are positive. 1, 2 are isolated strains of *C. diphtheriae* subsp. *lausannense*; 3 is a strain of gravis *C. diphtheriae* subsp. *diphtheriae*; 4 is a strain of mitis *C. diphtheriae* subsp. *diphtheriae*; 5 — negative control

DISCUSSION

We were able to identify the two analyzed *C. diphtheriae* strains isolated from the samples of Russian residents as nontoxicogenic representatives of subsp. *lausannense*. Our findings along with the reports of foreign researchers [14, 22] suggest that these strains are ubiquitous. They belong to the sequence type ST199, which is part of the lineage-2 cluster typical to the *lausannense* subspecies, and carry the sequence of the *dtxR* gene characteristic of *lausannense* representatives. The analysis of ortholog groups established the existence of loci specific to the subspecies of *C. diphtheriae*: the region containing the genes of the N-acetylglucosamine-phosphotransferase system (specific to the *lausannense* subspecies) and the region encoding the synthesis of nitrate

reductase subunits (specific to the *diphtheriae* subspecies). Our findings are consistent with the results of earlier genomic studies of the *lausannense* subspecies [15] and the studies of the biochemical properties of the belfanti biotype [9, 10]. Primers designed for these genes and the subsequent PCR allowed us to classify the two analyzed strains as *C. diphtheriae* subsp. *lausannense*.

CONCLUSIONS

We have identified the 2 analyzed strains collected on the territory of Russia as nontoxicogenic strains of *C. diphtheriae* subsp. *lausannense*. Our findings expand the knowledge of the biological diversity of *C. diphtheriae* and indicate the need for estimating the spread of these microorganisms

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