

Original Study Article

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Genetic diversity of mutations affecting the hemolytic activity of *Bordetella pertussis* bacteria during *in vitro* cultivation

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Abstract

Introduction. The two-component BvgAS system regulates the transcription of the pathogen's virulence genes and plays a key role in the pathogenesis of whooping cough, an anthroponotic infectious disease. Currently, the factors affecting the BvgAS system of *Bordetella pertussis* bacteria in the human organism have not been studied in practice. It is known that disruption of the *bvgAS* operon structure leads to phase modulations and changes in virulence. IS-elements intergration into the *bvgAS* operon and other virulence genes of *B. pertussis* bacteria is important mechanism in regulating their expression, potentially leading to the long-term persistence of this pathogen in the human body.

Aim. Identification and description of spontaneous IS-elements insertions in *B. pertussis* into *bvgAS*, *cya* and *fhaB* virulence genes responsible for hemolytic activity during *in vitro* bacterial cultivation.

Materials and methods. *B. pertussis* strains from the collection of the N. F. Gamaleya National Research Center for Epidemiology and Microbiology were used: virulent *B. pertussis* 475 strain and its isogenic attenuated strain *B. pertussis* 4MKS; virulent *B. pertussis* strain Tohama I and its avirulent mutant *B. pertussis* 347. Bacteria were cultivated on casein-charcoal agar with blood addition. The formation of haemolysis zones was assessed visually. PCR, real-time PCR and sequencing were used for the genetic characterization of the obtained insertion mutants.

Results. *B. pertussis* mutants containing insertions of IS-elements in *fhaB* and *bvgAS* genes with impaired hemolytic activity (Hly⁻ phenotype) have been isolated *in vitro*, as well as *B. pertussis* mutants that retained hemolytic activity (Hly⁺ phenotype), containing IS-elements in a previously undescribed orientation in the *bvgAS* gene. The frequency of insertional mutant formation depended on conditions of cultivation and bacteria genotype.

Conclusion. Arguments are made for the hypotheses about the IS-elements involvement in *B. pertussis* bacteria transition to a state of reduced virulence, which provides the possibility of long-term persistence of this pathogen in the human body.

Keywords: *Bordetella pertussis*, IS-elements, regulation of transcription, virulence, persistence, hemolytic activity, Hly⁻ phenotype

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Оригинальное исследование

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Генетическое разнообразие мутаций, влияющих на гемолитическую активность бактерий *Bordetella pertussis* при культивировании *in vitro*

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Аннотация

Введение. Ключевую роль в патогенезе коклюша, антропонозного инфекционного заболевания, играет двухкомпонентная система BvgAS, регулирующая транскрипцию генов вирулентности возбудителя. Факторы, оказывающие воздействие на работу системы BvgAS бактерий *Bordetella pertussis* в организме человека, практически не изучены. Известно, что нарушение структуры оперона *bvgAS* приводит к фазовым переходам и изменению вирулентности. Перемещение IS-элементов в оперон *bvgAS* и другие гены вирулентности возбудителя коклюша является важным механизмом в регуляции их экспрессии, потенциально приводящим к длительной персистенции бактерий в организме человека.

Цель работы — поиск и характеристика спонтанных инсерционных мутантов бактерий *B. pertussis* по генам вирулентности *bvgAS*, *суа* и *fhaB*, ответственным за гемолитическую активность при культивировании бактерий *in vitro*.

Материалы и методы. Использованы штаммы *B. pertussis* из коллекции НИЦЭМ им. Н.Ф. Гамалеи: вирулентный штамм *B. pertussis* 475 и изогенный ему аттенуированный штамм *B. pertussis* 4MKS, вирулентный штамм *B. pertussis* Tohama I и его авирулентный мутант *B. pertussis* 347. Бактерии культивировали на казеиново-угольном агаре с добавлением крови. Формирование зон гемолиза оценивали визуально. Для молекулярно-генетической характеристики полученных инсерционных мутантов использовали методы ПЦР, ПЦР-РВ, секвенирования.

Результаты. *In vitro* выделены мутанты *B. pertussis* с нарушенной гемолитической активностью (фенотип Hly⁻) с инсерциями IS-элементов в генах *fhaB* и *bvgAS*, а также мутанты, сохранившие гемолитическую активность (фенотип Hly⁺), содержащие IS-элементы в ранее не описанной ориентации в гене *bvgAS*. Частота образования инсерционных мутантов зависела от условий культивирования и генотипа бактерий.

Заключение. Приведены аргументы, свидетельствующие о возможной роли IS-элементов в переходе бактерий *B. pertussis* в состояние пониженной вирулентности, что обеспечивает длительную персистенцию возбудителя коклюша в организме человека.

Ключевые слова: *Bordetella pertussis*, IS-элементы, регуляция транскрипции, вирулентность, персистенция, гемолитическая активность, Hly-фенотип

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

The Gram-negative bacterium *Bordetella pertussis* causes an acute contagious infectious disease in humans called whooping cough. The genus *Bordetella* is traditionally divided into "classical" species — *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* — and "new" species — *B. avium*, *B. petrii*, *B. holmesii*, *B. hinzii*, *B. trematum* and *B. ansorpii*. In recent years, 3 more species have been described: *B. bronchialis*, *B. flabilis* and *B. sputigena* [1].

Among the virulence factors of the whooping cough pathogen, two main groups are distinguished: toxins and adhesins. Toxins include pertussis toxin, adenylate cyclase toxin (ACT), tracheal cytotoxin, dermonecrotic toxin and lipopolysaccharide endotoxin. Adhesins include filamentous hemagglutinin, agglutinogens, or fimbriae 2 and 3, the outer membrane protein pertactin, the BrkA protein and other components of the *Bordetella* type III secretion system. It is believed that the hemolysin in the bacterium *B. pertussis* is ACT [2], however, there is currently data on the influence of the FhaB protein on erythrocyte hemolysis *in vitro* when interacting with ACT [3].

An important feature of the genomes of bacteria of the genus *Bordetella* is the presence of repeated insertion sequences — IS elements — in the chromosome [4]. The number and diversity of IS elements differ among different members of the genus. The maximum number of IS481 and IS1002 is registered in the *B. pertussis* chromosome [4]. It is believed that IS elements were involved in the evolution of bacteria of the genus *Bordetella* from a common ancestor, *B. bronchiseptica* [5].

The two-component system BvgAS, consisting of the histidine kinase BvgS and the transcription regulator protein BvgA, plays a special role in the pathogenesis of whooping cough. The BvgAS system acts as a key regulator, controlling the transcription process of genes responsible for the virulence of the bacterium *B. pertussis* [6–8]. Furthermore, the BvgAS system regulates many intracellular processes, including *B. pertussis* metabolism and microbe-host interaction [8]. The level of BvgA~P production and phosphorylation determines the level of transcription of Bvg-dependent genes. The level of BvgA~P phosphorylation is determined by the activity and quantity of the BvgS phosphokinase,

which depends on a number of factors, including culture conditions. Depending on the functioning of the BvgAS system, *B. pertussis* bacteria can be in a virulent state — Phase I (Bvg⁺), an avirulent state — Phase IV (Bvg⁻), or an intermediate phase Bvgⁱ, each of which contributes to the pathogenesis of whooping cough, the persistence of bacteria in the human body, and their transmission to a new host.

Despite the lack of information about factors affecting the BvgAS system's function in a living organism, certain conditions that alter the virulence of *B. pertussis* bacteria have been described when cultured *in vitro* on solid media [6–8]. Thus, lowering the cultivation temperature to 27°C, adding 50 mM magnesium sulfate or nicotinic acid to the nutrient medium promotes the bacteria's transition to an avirulent phase.

Changes in the phase state of *B. pertussis* bacteria can occur as a result of disruption of the bvgAS virulence operon structure. Two types of induced mutations that cause changes in the phenotype of *B. pertussis* are described. The first mutants without hemolytic activity (lacking zones of hemolysis on blood agar plates, Hly⁻ mutants) were selected by culturing the laboratory virulent strain *B. pertussis* Tohama I in the presence of erythromycin [9]. The main phenotypic characteristic of Hly⁻ mutants was the absence of zones of hemolysis around individual colonies grown on Bordet–Gengou medium supplemented with sheep blood. The frequency of Hly⁻ mutant detection was characterized by the authors as 10⁻⁵–10⁻⁶. In 1989, a study by S. Stibitz et al. was published, in which the region of the *B. pertussis* Tohama I mutant responsible for hemolytic activity was mapped and sequenced. It has been shown that Hly⁻ mutants contained a *frameshift* (f.s.) mutation associated with the acquisition of a cytosine in a sequence of 6 cytosines in the *bvgS* gene [10]. Another type of *B. pertussis* mutant in the Tohama I strain was isolated by S. Stibitz in 1998 [11]. Mutants with the bvgAS operon were found to be viable under selective conditions characterized by the overproduction of the mutant BvgA protein cloned within a plasmid. The author characterized 15 *B. pertussis* insertion mutants that survived under the specified conditions, and corresponding sequences were identified in 7 of them. Five mutants contained IS481, and 2 mutants contained IS1002 at the *bvgAS* operon *ctag* site [12]. The research has shown that *B. pertussis* mutants similar to those obtained in *in vitro* experiments have been found in late-stage whooping cough convalescents, in asymptomatic carriers in contact with whooping cough patients [13], and in experimental whooping cough in Old World monkeys at a late stage of the infectious process [14].

In recent decades, various types of knockout and regulatory mutants for the *fhaB*, *kata*, *prn* and *brkA* virulence genes of *B. pertussis*, containing IS element insertions, have been isolated from patients with whooping cough. The accumulation of such *B. pertus-*

sis mutants in the population is thought to be linked to the widespread use of acellular pertussis vaccines in several countries [15–18].

We have proposed a hypothesis that IS elements are involved in the regulation of the expression of virulence genes and housekeeping genes of the whooping cough pathogen, which is one of the mechanisms for the long-term persistence of *B. pertussis* bacteria and the formation of persistence in the human body, and which determines the circulation and maintenance of anthroponotic infection foci.

The aim of this study was to identify and characterize spontaneous insertion mutants of *B. pertussis* bacteria in the virulence genes *bvgAS*, *cya* and *fhaB*, responsible for the Hly⁻ phenotype, which are formed during *in vitro* bacterial cultivation.

Materials and methods

The study used *B. pertussis* strains from the Gamaleya Research Institute of Epidemiology and Microbiology collection: virulent *B. pertussis* 475 and its isogenic attenuated *B. pertussis* 4MKS Str^RNal^RCm^R [19]; virulent *B. pertussis* laboratory strain Tohama I and its avirulent mutants *B. pertussis* 347 *bvgAS::Tn5* Str^RKm^R.

B. pertussis bacteria were cultured on casein-charcoal agar (Medgamal) supplemented with 15% sheep blood at 35°C for 24–36 hours for culture growth and for 72–96 hours for the formation of colony-forming units (CFU). The number of colonies, their size, and shape were counted and characterized visually. The absence of extraneous microflora was monitored using light microscopy after Gram staining.

DNA from *B. pertussis* bacteria was extracted using the Sorb-B DNA extraction kit (AmpliSens). For purification of amplification products, a DNA extraction and purification kit from an agarose gel (Eurogen) was used.

For real-time polymerase chain reaction (qPCR), a CFX96 Touch thermocycler (Bio-Rad) was used, and for PCR, a device from the company Tercik was used.

The nucleotide sequence of the amplification products was determined using a 3130 Genetic Analyzer (Applied Biosystems/Hitachi).

Results

Method for registering the integration of IS elements into the bvgAS operon

Among the described mutations in the virulence genes of the whooping cough pathogen, insertion mutations — the integration of IS elements into the specific site of the *B. pertussis* *bvgAS* operon — deserve special attention, according to our hypothesis. In previous studies, we described a method and test systems for detecting mutations in the *bvgAS* operon, which contains IS elements 481 and 1002 integrated in a specific, at that

time the only known, orientation, which we conditionally designated as orientation (1) [13].

To identify *B. pertussis* mutants containing IS elements 481 and 1002 integrations in a previously undescribed opposite orientation relative to the known orientation (1), we have modified a previously developed real-time PCR system. The new orientation is provisionally designated as orientation (2).

Integration of IS elements 481 and 1002 in orientation (2) — IS481 (2) and IS1002 (2), the positions of the primers and probes are schematically represented in

Fig. 1. Table 1 presents the nucleotide sequences of the probes and primers, including those flanking the specific NctagN sites located within the structure of the *cya* and *fhaB* genes.

The temperature-time profile for real-time PCR and PCR to detect IS481 and IS1002 integrations in orientation (2) and at the NctagN site of the *bvgAS* operon and *cya* and *fhaB* genes is presented in **Table 2**.

The specificity of the selected primers and probes was confirmed by determining the nucleotide sequence of the corresponding amplification products. The num-

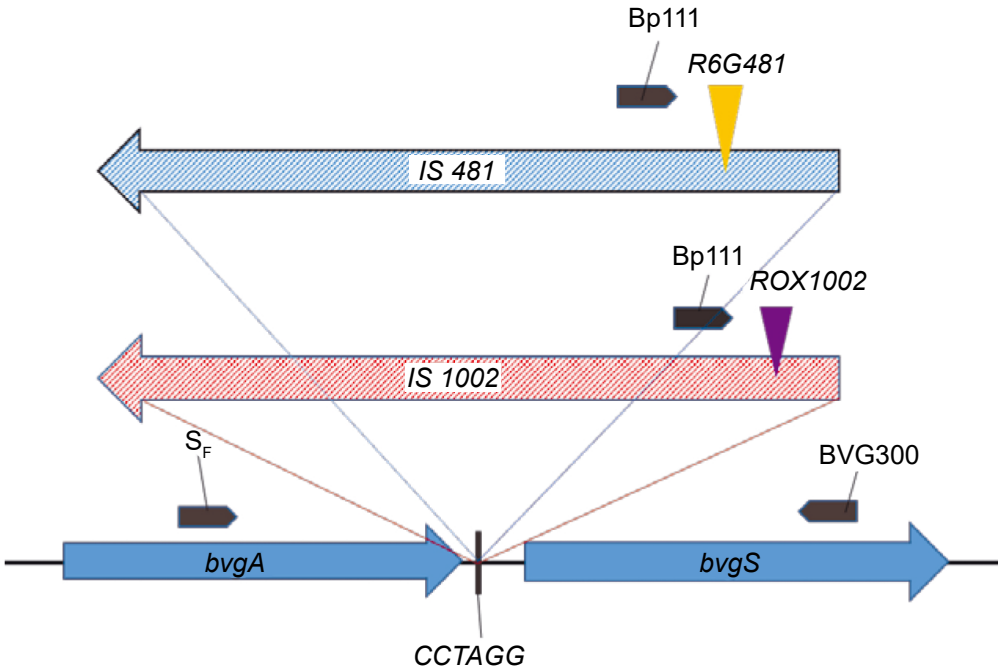


Fig. 1. Structure of the *B. pertussis* chromosome region containing the integration of IS 481 (2) or IS 1002 (2) at the *cctagg* site of the *bvgAS* operon.

The shaded arrows indicate the position of the integrated element IS 481 (blue arrow) or IS 1002 (red arrow) within the *bvgAS* operon in the *cctagg* site (blue solid arrows). The position of the primers used for amplification is indicated by dark pentagons. Colored triangles indicate the position of the DNA probes used in the real-time PCR hybridization reaction.

Table 1. Nucleotide sequences of primers and probes used to detect integrations of IS elements 481 and 1002 in orientation (2) — IS481 (2) and IS1002 (2) — into the *bvgAS*, *cya* and *fhaB* genes

Primer/probe	Nucleotide sequence
qPCR	
Bp111	GGTCAATCGGGCATGCTTATGG
BVG300	ACGTCGAACGGAGGAATGTC
R6G481, probe	(R6G)TCGCCGACCCCCAGTTCACTCAAG(BHQ1)
Rox1002, probe	(ROX)ACCACGCCATCGCAACTCAGGGCA(BHQ2)
PCR <i>bvgAS</i> ::IS; PCR <i>cya</i> ::IS; PCR <i>fhaB</i> ::IS	
CyaF	CCATGTCGAGCTGGCCCGTG
CyaR	GGCCACTTCTCGACCGTGCC
FhaB1-F	GGCTGAGCCGTTTCGACCTT
FhaB1-R	CACGGTCGTTTCAGCGCAACA
S _F	GTCGCTGGTGGAAGTATAG

Table 2. Temperature-time profiles of real-time PCR and PCR for detecting IS element integrations in the NctagN site of the *bvgAS*, *cya* and *fhaB* operons

Target	Праймеры/зонд	Temperature, °C	Time, s	Number of cycles
qPCR <i>bvgAS</i> ::IS481 (2)	Bp111-BVG300/R6G481	95	300	1
		95	20	} 40
		60	50	
qPCR <i>bvgAS</i> ::IS1002 (2)	Bp111-BVG300/Rox1002	95	300	1
		95	20	} 40
		60	50	
PCR <i>bvgAS</i> ::IS	S _F -BVG300	95	300	1
		95	20	} 40
		63	30	
PCR <i>cya</i> ::IS	CyaF-CyaR	72	30	} 40
		95	300	
		95	20	} 40
PCR <i>fhaB</i> ::IS	FhaB1-F-FhaB1-R	67	30	
		72	30	} 40
		64	30	

ber of genome equivalents (GE) of *B. pertussis* DNA and IS 481 (1) and IS 1002 (1) integrations were determined using the real-time PCR test systems (BpIS-Q) and real-time PCR BpIS-INT1 [13], respectively. The number of IS 481 (2) and IS 1002 (2) integrations was estimated based on the values of the reaction threshold cycle. The corresponding set of reagents, analogous to the set for quantifying IS element integrations in orientation (1), is designated as BpIS-INT2. The frequency of integrations was calculated as before using the ratio N_{IS}/N_{GE} , where N_{IS} is the number of integrations and N_{GE} is the number of GE in 5 μ L of the DNA solution being studied.

Phenotype of B. pertussis bacteria 4MKS, 475, Tohama I, and Tohama 347 and molecular genetic characterization of their populations

Within the framework of this study, populations of the laboratory strain *B. pertussis* Tohama I and its Bvg⁻ mutant *B. pertussis* Tohama 347, as well as virulent bacteria *B. pertussis* 475 and isogenic attenuated bacteria *B. pertussis* 4MKS, were analyzed to identify insertion mutants in the *bvgAS*, *cya* and *fhaA* virulence genes, which are responsible for the Hly⁻ phenotype that develops when *B. pertussis* bacteria are cultured *in vitro*.

B. pertussis are slow-growing, fastidious bacteria. For the phenotypic differentiation of the phase states of *B. pertussis* bacteria when cultured on solid media with blood added, the following characteristics of the colonies can be used: size, shape, and the presence of hemolysis zones. Bvg⁺ colonies are convex, smooth, shiny, small (1–2 mm), and form distinct zones of hemolysis; Bvg⁻ colonies are flat, rough, larger (2.0–2.5 mm), and do not form zones of hemolysis.

Colonies of *B. pertussis* bacteria grown on casein-charcoal agar (CCA) blood agar were analyzed for the listed parameters. *B. pertussis* culture from an ampoule was streaked onto a Petri dish containing CCA agar with added blood (primary culture), and after incubation at 35°C for 24–36 hours, it was re-streaked in a dense line onto the same Petri dish (first passage). After checking for microbiological purity using light microscopy, another subculture was performed (2nd passage). The 3rd passage culture was used to seed the ampoule culture into individual colonies by the titration method (ampoule culture analysis). Simultaneously, individual colonies from the second passage, grown on CCA medium with blood, were analyzed for morphology and the presence of hemolysis zones. Three CFUs of each strain meeting the criteria for virulent *B. pertussis* bacteria were streaked onto fresh blood agar plates, DNA was extracted from the resulting culture, and the bacteria were simultaneously serially diluted to form CFUs on the blood agar medium. Colony morphology and the presence of hemolysis zones were analyzed (culture analysis from individual colonies). This study characterized 500–1000 CFU of each strain of virulent and attenuated *B. pertussis* bacteria, grown from ampoule cultures and individual colonies.

Phenotypic and molecular biological analysis was performed on *B. pertussis* bacteria dried at different times in different laboratories. The vast majority of *B. pertussis* 4MKS and *B. pertussis* 475 ampoule cultures from the 3rd passage contained bacteria in the virulent Bvg⁺ phase; IS element integration into the *bvgAS* operon was detected by real-time PCR at a low frequency — less than 10⁻⁴ per bacterial cell. In the ampoule culture spreads, all the colonies that grew had a Bvg⁺ phenotype.

In certain cases, in *B. pertussis* 4MKS bacteria from the 3rd passage of an ampoule culture dried more than 4 years ago, the total frequency of insertion mutants in both orientations was $(2-5) \times 10^{-2}$ (Table 3). During sieving, approximately 2–3% of Hly⁺-CFU were recorded. The colonies were flat and rough in shape and 2.0–2.5 mm in size, while Hly⁺ colonies were 1–2 mm.

Similar results were obtained in a study of ampoule cultures of *B. pertussis* Tohama I. The frequency of IS481 (1) integrations into the *bvgAS* operon, detected by real-time PCR, ranged from 10^{-4} per bacterium to several percent. No integrations of other IS elements were found in the *B. pertussis* Tohama I bacteria. The maximum number of non-hemolytic CFU, reaching 95% of the total number of colonies, was identified when bacteria dried on April 13, 1974 (30 years ago) were streaked. The frequency of IS element integrations into the *bvgAS* operon was 4×10^{-2} per bacterium (Table 3).

B. pertussis Tohama 347 *bvgAS::Tn5* strain was expected not to form zones of hemolysis, and the size and shape of the colonies corresponded to the Bvg⁺ phenotype. The frequency of integrations in all *B. pertussis* Tohama 347 cultures analyzed by real-time PCR was less than 10^{-6} per bacterium.

A similar study was conducted with cultures grown from individual Hly⁺ colonies of *B. pertussis* 475, *B. pertussis* 4MKS, and *B. pertussis* Tohama I. When each strain of bacteria was streaked from individual colonies onto CCA medium with added blood from 10^3 CFU, no colonies that did not form zones of hemolysis were observed. The size and shape of the colonies corresponded to the Bvg⁺ phenotype described above. The frequency of IS element integration into the *bvgAS* operon did not exceed 10^{-4} – 10^{-5} per bacterium.

Colonies of Hly⁺ strains of *B. pertussis* 4MKS and *B. pertussis* Tohama I were selectively subjected to further analysis. 46 colonies of each strain were transferred to Petri dishes containing CCA with blood and antibiotics to test their antibiotic resistance and hemolytic ability. As a result, all individual *B. pertussis* 4MKS colonies were resistant to streptomycin, nalidixic acid, and chloramphenicol, while *B. pertussis* Tohama I bacteria were sensitive to all antibiotics tested. The identified

antibiotic resistance is fully consistent with the characteristics of the parent strains *B. pertussis* 4MKS and *B. pertussis* Tohama I. Colonies of both strains maintained a pronounced Hly⁺ phenotype in the replicates.

In PCR with SF-BVG300 primers on DNA extracted from the 3rd passage of ampoule cultures and the CFU of the tested strains, no large fragments were detected, despite the presence of IS element integration in some bacteria within the population. The absence of amplification products with the insert is due to the significantly lower efficiency of amplifying large fragments compared to small fragments without an insert.

Thus, in the cultures of virulent and attenuated *B. pertussis* strains analyzed, grown from ampoule cultures, Hly⁺ mutants lacking hemolytic activity and containing IS element integrations in the *bvgAS* operon are detected with varying frequency. Cultures grown from single bacterial colonies are homogeneous and contain insertion mutants at a frequency of less than 10^{-4} per bacterium. Bacterial populations grown from individual colonies contain non-hemolytic bacteria colonies of the Hly⁺ phenotype in insufficient numbers for phenotypic analysis.

Molecular genetic characterization of Hly⁺ mutants of *B. pertussis* 4MKS and Tohama I

DNA was extracted from 40 replicas of each strain of *B. pertussis* 4MKS and *B. pertussis* Tohama I, with an Hly⁺ phenotype. The obtained samples were analyzed using PCR with the primers SF-BVG300, FhaB1-F-FhaB1-R, and CyaF-CyaR.

The size of the amplification products from the SF-BVG300 primer-investigated Hly⁺ mutants of *B. pertussis* 4MKS was approximately 1300 bp in 25% (10 clones) and approximately 300 bp in 75% (30 clones).

Table 4 presents the results of the PCR product analysis of some Hly⁺ clones of *B. pertussis* 4MKS with primers SF-BVG300, FhaB1-F-FhaB1-R, and CyaF-CyaR.

Integration into the *cya* gene was not detected in any of the clones, whereas integration into the *fhaB* gene was recorded in 26 out of 30 Hly⁺ mutants of *B. pertussis* 4MKS that did not contain IS element integration

Table 3. The proportion of Hly⁺ colonies and the registration of IS element integration events in the *bvgAS* operon of *B. pertussis* 475, *B. pertussis* 4MKS and *B. pertussis* Tohama I strains when cultured on CCA blood agar

Strain	Ampoule culture		Culture from CFU	
	Hly ⁺ , %	<i>bvgAS</i> ::IS*	Hly ⁺ , %	<i>bvgAS</i> ::IS*
<i>B. pertussis</i> 475	N/A	5×10^{-5}	N/A	3×10^{-5}
<i>B. pertussis</i> 4MKS	2	$(2-5) \times 10^{-2}$	N/A	2×10^{-4}
<i>B. pertussis</i> Tohama I	95	4×10^{-2}	N/A	5×10^{-4}
<i>B. pertussis</i> Tohama 347	100	$\leq 10^{-6}$	100	$\leq 10^{-6}$

Note. *The total integration frequency for all IS elements is given. DNA extracted from the 3rd passage of the ampoule culture or colonies was used for PCR; N/A — not found.

in the *bvgAS* operon. For 3 Hly mutants, the nucleotide sequence of the amplification products of the *fhaB* gene fragment, 1300 bp in size, has been determined. In all cases, IS481 (1) was found integrated at a specific site, *fhaB*.

Clone #33, which did not contain integrations in the *bvgAS* or *fhaB* genes, was found to have a 2-nucleotide deletion near the *cctagc* site in the *bvgS* gene (Fig. 2). In 3 Hly clones (No. 32-2, 15-2, and 17-2), no IS element integrations or other nucleotide sequence disruptions were detected in the analyzed amplicons.

Out of 10 insertion Hly⁻BvgAS mutants with a specific nucleotide sequence, 5 contain an IS481 insertion (2), and 5 mutants contain an IS1002 insertion (1). Fig. 3 shows a fragment of the sequence containing IS481 (2) at a specific site in the *bvgAS* operon.

Among the analyzed Hly clones, no integrations of IS1002 (2) and IS481 (1) were detected, whereas the corresponding integrations are recorded in the population when analyzed by real-time PCR.

The PCR products of 40 analyzed Hly⁻ mutants of *B. pertussis* Tohama I, using primer pairs SF-BVG300, FhaB1-F-FhaB1-R, and CyaF-CyaR, had a size close to the calculated one — approximately 300 bp, 388 bp, and 261 bp, respectively. Nucleotide sequencing of 3 amplicons: *bvgAS*, *cya* and *fhaB* confirmed the absence of IS element integrations in them and did not reveal any structural alterations compared to the native sequence.

Given the presence of IS element integrations in the *bvgAS* operon in 4% of the *B. pertussis* Tohama I bacterial population and their absence in the DNA of bacteria with the Hly⁻ phenotype, we searched for IS element integrations in the *bvgAS* operon in bacteria with the Hly⁺ phenotype. PCR with SF-BVG300 primers on DNA extracted from 12 CFU with the Hly⁺ phenotype revealed products approximately 1300 bp in size in 5 (41.7%) CFU, presumably containing an IS element insertion at the analyzed site. qPCR analysis confirmed that all of them contain an IS481 (1) integra-

a

GGAAGTATAGACCTCGCCAAACGCAACAATCTCGCCTAGCGCCGCGCATGCCCCGCCCGCACCG
CCTGTACCCCGCAGTCTGATCTGCCTGGCTCAGGCGCTATTGGCATGGGCTTTGCTGCGATGGGC
GCCCCGCGCA

b

GGAAGTATAGACCTCGCCAAACGCAACAATCTCGCCTAGCGCCGCGCATGCCCCGCCCGCACCG
CCTGTACCCCGCAGTCTGATCTGCCTGGCTCAGGCGCTATTGGCATG

Fig. 2. Sequence fragment of the wild-type *bvgAS* operon of *B. pertussis* (a) and the Hly mutant *B. pertussis* No. 33 (b).

Nucleotide sequences of the *bvgAS* operon are shown in capital letters; ATG is the methionine codon of the *bvgAS* gene; **tg** is a deletion; CCTAGC is a specific integration site for IS elements.

Table 4. Results of PCR analysis of DNA from *B. pertussis* 4MKS clones with the Hly⁻ phenotype (selectively)

Clone number on strain templates	Genes, mutation types		
	<i>bvgAS</i> ::IS	<i>fhaB</i> ::IS	<i>cya</i> ::IS
2	IS481 (2)*	N/A*	N/A
3	IS481 (2)*	N/A	N/A
49	IS481 (2)*	N/A	N/A
48	IS481 (2)*	N/A	N/A
22	IS481 (2)*	N/A	N/A
9	IS1002 (1)*	N/A	N/A
10	IS1002 (1)*	N/A	N/A
32	IS1002 (1) *	N/A	N/A
34	IS1002 (1) *	N/A	N/A
43	IS1002 (1) *	N/A	N/A
31	N/A*	IS481 (1)*	N/A
33	ΔTG*	HO	N/A
3-2	N/A*	IS481 (1) *	N/A
32-2	N/A*	N/A*	N/A
15-2	N/A*	N/A	N/A
17-2	N/A*	N/A	N/A
28-2	N/A	IS481 (1) *	N/A

Note. *The structure has been confirmed by sequencing. N/A — integration was not found by PCR.

a

*atttccagtttctcaaatccggttcggatgaaccatgcatacaacctattgaatctTcacaCCTAGCGCCGCGCATGCCCCG
CCCCGCACCGCCTGTACCCCCGCAGTCTGATCTGCCTGGCTCAGGCGCTATTGGCATG*

b

*tgaaccatgcatacaacctattgaatctTcacacctaacgagcagcgacttctccgcgccacgcggaagaacgcccggac
aggccgcggttaccacatggtatccggcggtggtgcccgcgcccgcgcatccggccatcgccgccccaccacgga
ggaccgcacatg ttgagcaac*

Fig. 3. Sequence fragments of the *bvgAS* operon of *B. pertussis* 4MKS Hly⁻ mutant, clone 49 (a), and an insertion mutant of *B. pertussis* with IS481 integrated into a degenerate *cctaac* site located upstream the start of transcription of the *bteA* gene (b) [16].

The sequence of the *bvgAS* operon is indicated in capital letters, and the specific integration site CCTAGC is underlined; the sequence of the *bteA* gene is italicized, and the specifically degenerate integration site cctaac is underlined; the methionine start codons of the proteins VvgS and BteA are in bold; the 3' end sequence of IS481(1) is indicated in lowercase letters; and the presumed transcription start is indicated by a bold capital letter T.

tion into the *bvgAS* operon. Sequencing of the 1300 bp PCR products of SF-BVG300 confirmed the presence of the IS481 insertion in 3 of them (1) and the preservation of the native sequence around the integration site.

Thus, the emergence of *B. pertussis* bacteria with the Hly⁻ phenotype when cultured on solid media is not only due to previously known frameshift mutations in *bvgS*, deletions and insertions in the *bvgA* gene, integration of IS481 (2) and IS1002 (1) into the intergenic space of the *bvgAS* operon, or deletions in the *bvgS* gene, which were found in this study, but also to newly identified insertions of IS elements into the *fhaB* gene. The insertion of IS481 (1) into the *bvgAS* operon of *B. pertussis* Tohama I does not lead to the formation of a pronounced Hly⁻ phenotype in the bacteria. PCR analysis of virulence gene fragments *bvgAS*, *cya* and *fhaB*, containing specific NctagcN sites, did not reveal any visible structural changes in the analyzed gene regions in Hly⁻ mutants of *B. pertussis* Tohama I.

Discussion

A previous study conducted by us showed that a population of virulent *B. pertussis* bacteria cultured on solid CCA medium is not homogeneous; it contained a certain proportion of mutants characterized by IS element insertions at a specific site in the *bvgAS* operon. Such mutants were not only described by us, but were also obtained and characterized under selective conditions by S. Stibitz et al. In our studies, insertion mutants of BvgAS were found in convalescents and contacts of whooping cough patients, and in laboratory animals in the late stages of whooping cough infection. The qPCR test system we developed earlier, used in the experiments, allowed for the detection of IS1002 and IS481 integration events in only one orientation, which we designated as (1). We hypothesized that IS elements are capable of moving and integrating into a specific site within the *bvgAS* operon in reverse orientation (2). Therefore, this study aims to develop a method for identifying integration events of IS elements into the *bvgAS* operon in orientation (2) and to register the corresponding insertion mutants of *B. pertussis* when

cultured *in vitro*.

The same primers and probes complementary to the IS element sequences were used for PCR and the registration of amplification products resulting from the integration of IS elements in both orientations, but in pairs with primer SF for orientation (1) and with BVG300 for orientation (2) (Fig. 1). To estimate the number of integrations in the orientation (2) in this study, the values of the reaction threshold cycle were used instead of calibration curves, as in previous experiments, which slightly reduces the accuracy of the integration quantification.

In independent experiments, when screening bacteria of all strains grown from CFU, except for *B. pertussis* Tohama 347, the frequency of integration events detected by real-time PCR did not differ significantly from what we had previously determined and was 10⁻⁴–10⁻⁵ per bacterium.

In this study, we analyzed *B. pertussis* 4MKS and *B. pertussis* 475 cultures dried at different times in our laboratory, as well as *B. pertussis* 475 cultures obtained at different times from various sources, including the L.A. Tarasevich State Research Institute of Standardization and Control of Medical Biological Preparations collection. IS element integrations were identified in all ampoule cultures in both orientations (1) and (2). During the analysis of 16 preparations, IS1002 integration into the *bvgAS* operon was absent in 7 of them, either in one or both of the orientations studied. IS481 integration was present in the *bvgAS* operon in orientation (1) in 75% of cases and in orientation (2) in 25% of cases. Integration of IS 481 was not detected in only one ampoule culture of *B. pertussis* strain 475. In 25% of cases, IS1002 (1) integration was not detected, and in 3 (16%) samples, neither IS1002 (1) nor IS1002 (2) integrations were detected. Thus, the frequency of IS element detection in the *bvgAS* operon of two isogenic strains is reduced in the sequences IS481 (1), IS481 (2), IS1002 (1) and IS1002 (2). The reliability and significance of the identified patterns for other *B. pertussis* strains remain to be determined in subsequent studies.

Among the analyzed Hly⁻ clones of *B. pertussis*

4MKS ampoule cultures, no integrations of IS1002 (2) and IS481 (1) were detected (Table 4), while they are identified by real-time PCR in the overall population of *B. pertussis* 4MKS. Presumably, such insertion mutants are among the bacteria with the Hly⁺ phenotype. Analysis of 10 colonies forming zones of hemolysis did not reveal any changes in the size of the corresponding amplicon, indicating a low percentage of possible insertion mutants in the Hly⁺ clone population. The results of qPCR analysis of the bacterial population, including the studied Hly⁻ and Hly⁺ clones, support this conclusion: the frequency of detection of IS1002 (2) and IS481 (1) integrations is 10 times lower than that of IS1002 (1) and IS481 (2), which are detected at a frequency of approximately 10⁻² per bacterium. However, finding integrants without phenotypic markers at the expected frequency of less than 10⁻³ per bacterium appears difficult. For this reason, Hly⁺ clones with possible IS1002 (2) and IS481 (1) integrations were excluded from this study.

In the *B. pertussis* Tohama I bacterial population, only IS481 (1) integration was detected in the *bvgAS* operon, and no IS1002 integrations were found. When streaking the test cultures grown from CFU on blood agar, no colonies with the Hly⁻ phenotype were detected, which is consistent with the frequency of integration events identified by real-time PCR. The fact that IS481 (1) is predominantly integrated into a specific site within the *bvgAS* operon of *B. pertussis* Tohama I bacteria, as well as the different integration frequencies of IS481 and IS1002 in isogenic variants of *B. pertussis* strain 475, requires further study and may be related to the specific movement characteristics of IS elements in different strains.

The frequency of IS481 integration in *B. pertussis* Tohama 347 bacteria cultures grown from ampoules and colonies is approximately 100 times lower (10⁻⁶–10⁻⁷ per bacterium) than in isogenic virulent *B. pertussis* Tohama I bacteria. This fact was noted by us in previous studies [20, 21]. Analysis of the *B. pertussis* Tohama 347 DNA amplification product with SF-BVG300 primers showed that the Tn5 insertion in the *bvgAS* operon is located outside the analyzed fragment of the virulence operon and is likely unable to prevent the movement of IS elements into the analyzed site. This circumstance indicates the dependence of transposition frequency on the integrity of the *bvgAS* operon. It should be noted that we were unable to register a dependence of the IS element transposition frequency in the *bvgAS* operon on modulating conditions when culturing *B. pertussis* Tohama I, 4MKS, and 475 bacteria in the presence of MgSO₄ and at reduced temperature. On the one hand, this observation does not support the dependence of transposition on modulating conditions, and on the other hand, considering the identified decrease in integration frequency in *B. pertussis* Tohama 347, it can be suggested that the BvgA and BvgS proteins are not

directly involved in the regulation of transposition. The empirical result obtained requires further study.

The high frequency of *B. pertussis* insertion mutants we found in certain ampoule cultures suggests a strong dependence of the transposition frequency on the bacterial culture conditions, including, likely, within the host organism. This circumstance may be responsible for the instability in obtaining frameshift mutants and their revertants, as noted by A. Weiss et al. [9].

Thus, the presented results show that our proposed real-time PCR test systems allow for the detection of IS element integration events in both orientations within the *bvgAS* operon. The frequency of their transposition depends on the genotype, including the integrity of the virulence operon, and the conditions under which the bacteria are cultured.

Analysis of the structure of the virulence gene fragments *bvgAS*, *cya* and *fhaB* in Hly⁻ mutants showed that they contain the integration of IS481 (2) or IS1002 (1) at a specific cctagc site in the *bvgAS* operon (*B. pertussis* 4MKS), or IS481 (1) at a similar site in *fhaB* (*B. pertussis* Tohama I). Four Hly mutants were also found whose chromosomes do not have insertions in the virulence gene fragments tested. A 2-nucleotide deletion was found in the *bvgS* gene of one of them, which disrupts the expression of the BvgS phosphokinase involved in the regulation of a large number of pertussis pathogen genes, including all virulence genes. In 3 out of 4 Hly mutants, the methods used did not reveal any abnormalities in the structure of the *bvgAS* or *fhaB* genes. There were no integrations into the *cya* gene in any of the analyzed clones. It is likely that these 3 mutants contain the IS element insertions in the *bvgA* gene characterized by S. Stibitz, or other mutations, such as frameshift mutations in the *bvgS* gene, which were not identified using the methods employed.

The presence of an IS element integrated into the intergenic space of the *bvgAS* operon, blocking the transcription of the *bvgS* gene, appears to be a perfectly expected reason for the formation of the Hly⁻ mutant phenotype of *B. pertussis* 4MKS. However, there are several examples of IS481 integration into specific sites located upstream of the *brkA*, *kat* and *bvgS* genes, at least, not accompanied by the termination of their expression [16, 18, 20, 22]. In one study, not only was the hypothesis formulated that transcription from a promoter located at the end of IS481 regulates the expression of the *brkA* gene product, but the start of the corresponding transcript was also determined (Fig. 2). The promoter identified by the authors is located at the same end of IS481 (2) in the genome of clone 49 of *B. pertussis* 4MKS, but it apparently does not provide a sufficient level of *bvgS* gene transcription, resulting in the formation of the Hly⁻ phenotype of clone 49 (Fig. 2). It can be assumed that the disruption of the *bvgS* gene transcription in mutants containing the IS1002 insertion (1) occurs by the same mechanism or as a result of its

complete cessation. The lack of experimental data on the presence of promoters within the IS1002 structure currently prevents a definitive conclusion. Apparently, in this way, the IS481 insertion ensures the transcription of the *bvgS* gene and the differential expression of virulence genes in selected S. Stibitz mutants, manifested by the preservation but significant reduction in the expression of the *ptx* genes and a less pronounced reduction in *fha* [20]. An interesting case of transcription regulation is discussed by A. D'Halluin et al. [22]. Regulation of *fim2* gene transcription/translation is shown as a result of antisense RNA synthesis from the IS481 internal promoter integrated upstream of the *fim2* gene. It is suggested that the described changes in the regulation of *B. pertussis* virulence genes, particularly the *bvgAS* operon, may be one of the "triggering" mechanisms for the formation of *B. pertussis* persistence in the body of its only host — humans. Or they can increase the viability of bacteria outside the body, facilitating the transmission of infection to a new susceptible organism.

In all Hly⁻ mutants containing an insertion in the *fhaB* gene, IS481 integration was detected (1). Because the integrations are located within the coding sequence of the *fhaB* gene, they disrupt its transcription and translation, regardless of the direction of the IS element integration. While the link between mutations in the *bvgAS* operon and the Hly⁻ phenotype appears obvious, the effect of a knockout mutation in the *fhaB* gene on *B. pertussis* hemolytic activity requires further investigation. According to current understanding, the FhaB protein is not directly related to the hemolysis reaction of blood erythrocytes. This function is attributed to ACT, specifically its C-terminal region. However, recent data suggest that the FhaB protein interacts with ACT. Most likely, the hemolytic activity of ACT *in vitro* is realized after interaction with filamentous hemagglutinin on the surface of the bacterial cell and delivery of ACT into the eukaryotic cell [3].

The absence of the described *bvgAS::IS481* or *bvgAS::IS1002* insertion mutants among the bacteria isolated from whooping cough patients is noteworthy, while clinical isolates containing IS element insertions in the *fhaB*, *prn* genes and those involved in the regulation of *kata* and *brkA* have been described in the literature [16, 18, 20, 22]. In the experiments of this study, such mutant bacteria *bvgAS::IS481* (1) were detected in populations of *B. pertussis* bacteria persisting in the bodies of whooping cough convalescents and asymptomatic carriers. It can be assumed that this state of the whooping cough pathogen is optimal for persistence in the human body and survival in the external environment, and upon entering a susceptible organism, precise excision of the IS element (or inversion) occurs, the bacteria regain their virulence, the organism is infected, and the disease develops. A similar role is assigned to bacteria in a state of reduced virulence by

M.R. Farman et al., who studied the transcription profiles of a large group of genes in *B. pertussis* bacteria inside macrophages [23]. Our demonstration of the existence (accumulation) of Bvg⁻ mutants of *B. pertussis* at late stages of infection suggests a regulatory role for IS element integrations in the formation of persistent bacteria in the human body and possibly their involvement in bacterial transmission to a new host. It can be expected that the precise excision of an IS element from a virulence operon restores its structure and the ability of the bacteria to cause disease. The mechanisms and conditions that typically cause rare events of precise excision remain unclear. It is also unclear at what stage of pathogen persistence or transmission it occurs. The ability of the IS element to be precisely excised was previously demonstrated by us in the *Escherichia coli* model [21, 24].

The results of the analysis of Hly⁻ mutants of *B. pertussis* Tohama I, which we registered in approximately 95% of the analyzed individual colonies grown from one of the series of lyophilized cultures from the Gamaleya Research Institute of Epidemiology and Microbiology Museum, appear unexpected. Among 40 Hly⁻ colonies, no *B. pertussis* mutants containing integrations in the specific NctagN sequence in the *bvgAS*, *cya* and *fhaB* genes were found. The mutations responsible for the genotype formation of the Hly⁻ clones of *B. pertussis* Tohama I isolated by us remain unidentified. It is quite likely that they, like the 3 unidentified mutations in Hly⁻ clones of *B. pertussis* 4MKS, belong to the class of reading frame shift mutations in the *bvgS* gene, insertions in the specific site of *bvgA*, or contain other unidentified disruptions in the structure of the *bvgAS* operon, the *fhaB* gene or the *cya* gene.

The absence of integrations at the *cctagg* site of the *bvgAS* operon analyzed in Hly⁻ colonies against the background of their reliable detection by PCR in the population before its dispersion (up to 4% IS481 integrations (1)) allowed us to suggest that integrations at the corresponding site may be present in the genome of some bacteria that have retained hemolytic activity (with the Hly⁺ phenotype). Indeed, among the Hly⁺ phenotype bacteria, 41.7% of insertion mutants containing IS481 (1) in the *bvgAS* operon were found, while the native sequence of the operon around the integration site was preserved. Considering the analysis above and the orientation of IS481, it can be expected that an effective promoter, which ensures the transcription of the *bvgS* gene and the Hly⁺ phenotype in *B. pertussis* insertion mutants, is located at the end of the IS481 element opposite to that described by H. Han et al. [18] and in a study by S. Stibitz, who showed a differential decrease in the expression of the *fhaB* and *ptx* genes and less pronounced zones of hemolysis in *B. pertussis* insertion mutant bacterial colonies [12].

For a more detailed characterization of the *bvgAS::IS481*(1) Hly⁺ phenotype mutant, we have

planned a comparative study of the toxic activity of pertussis toxin and dermonecrotic toxin, agglutination titers, and electron microscopic analysis of the morphology and structure of *B. pertussis* Tohama I and its mutant genotype, as well as a bioinformatics analysis of the IS481 sequence aimed at identifying and comparing the putative promoters located at the ends of the IS elements.

Thus, during *in vitro* cultivation of *B. pertussis* bacteria, IS481 integrations (1) are more frequently detected in the *bvgAS* operon, which we previously identified in the genomes of *B. pertussis* bacteria isolated from whooping cough convalescents and experimental animals. Previous data on the analysis of the integration events of IS elements in one orientation (1) largely reflect the accumulation dynamics of all *bvgAS::IS* insertion mutants in *B. pertussis* bacteria.

Conclusion

The data obtained allow for several significant conclusions to be drawn regarding the characteristics of *B. pertussis* IS elements:

- IS elements are capable of moving between specific sites on the *B. pertussis* chromosome, causing gene inactivation or changes in their transcriptional regulation when the bacteria are cultured *in vitro*;
- the frequency of IS element movement and the types of spontaneous mutations they induce depend on the genotype and culture conditions of *B. pertussis* bacteria;
- factors influencing the frequency of IS element movement and the formation of spontaneous, insertion, or other mutations induced by IS elements require further study;
- during the cultivation and storage of *B. pertussis* bacteria, conditions may arise that induce the formation of a population heterogeneous in the structure of *fhaB*, *bvgAS* and likely other bacterial virulence genes;
- the movement of IS elements to a specific site in the intergenic space of the *bvgAS* operon leads to changes in the regulation of virulence genes and other *bvg*-dependent genes, possibly ensuring the long-term persistence of *B. pertussis* bacteria in the host organism.

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