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MSHA-like pili of non-toxigenic *Vibrio cholerae* strains

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Abstract. **Aim.** In this study, we set out to identify the homologues of genes from the *msh*-cluster in the genomes of non-toxigenic *V. cholerae*, to perform the bioinformatics analysis of their products, as well as to study the adhesive properties of strains containing altered genes. **Materials and methods.** We analysed 17 clinical strains of non-O1/non-O139 *V. cholerae* and 2 strains of the O1 serogroup isolated from water bodies. Genes belonging to the *msh*-cluster were identified in the whole genomes using the BLASTN 2.2.29 and BioEdit 7.2.5 programs. Gene translation, comparative analysis of their nucleotide sequences and the amino acid sequences of deduced products were performed using the Vector NTI Advance 11 (Invitrogen). **Results and discusssion.** In 18 out of the 19 studied genomes we identified gene clusters responsible for production of adhesion pili (*mshH-Q*) represented by diverse alleles, the majority of which differed from the prototype genes of the *msh*-cluster in nucleotide composition but had the same localization and arrangement. Only one strain had a cluster that was close to that of the prototype. A bioinformatics analysis of their deduced products indicated that the amino acid sequence of the major MshA pilus subunit is homologous to the prototype only in a short N-terminal region (1–41) while sharing no similarities with the rest of the sequence. Nevertheless, this protein, similar to VcfA described by Kuroki H. *et al.* (2001) and designated by us as MshA-like, retained a putative pilus domain. A similar pattern was observed in the minor subunits designated as MshC-like. Other minor subunits also retained their characteristic domains. All of the strains agglutinated human erythrocytes (group O) and chicken erythrocytes, and in isolates harboring modified *mshA-like* and *mshC-like* genes the reaction was not inhibited by mannose. Since most of the studied strains were isolated from hospitalized patients, it is possible that in non-toxigenic *V. cholerae* lacking the pathogenicity island VPI, MSHA-like pili may serve as a colonization factor of the human intestine, in contrast to VPI-positive strains. The obtained information provides a basis for experimental verification of this assumption.

Key words: Vibrio cholerae, MSHA pili, bioinformatics analysis, adhesion, haemagglutination.

Conflict of interest: The authors declare no conflict of interest.

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MSHA-ПОДОБНЫЕ ПИЛИ НЕТОКСИГЕННЫХ ШТАММОВ ХОЛЕРНЫХ ВИБРИОНОВ

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Цель исследования состояла в идентификации гомологов генов *msh*-кластера в геномах нетоксигенных холерных вибрионов, биоинформационном анализе их продуктов и изучении адгезивных свойств штаммов, содержащих измененные гены. **Материалы и методы**. В работе использовано 17 клинических штаммов холерных вибрионов не O1/не O139 и 2 штамма O1 серогруппы, выделенные из водоемов. Гены *msh*-кластера идентифицировали в полных геномах с помощью программ BLASTN 2.2.29 и BioEdit 7.2.5; трансляцию генов, сравнительный анализ их нуклеотидных, аминокислотных последовательностей продуктов трансляции осуществляли с использованием пакета программ Vector NTI Advance 11 (Invitrogen). **Результаты и обсуждение.** В геномах 18 из 19 исследуемых штаммов идентифицированы кластеры генов, ответственных за продукцию пилей адгезии (*mshH-Q*), представленные разными аллелями, большинство из которых отличались по нуклеотидному составу от прототипных генов *msh*-кластера, однако имели такую же локализацию и порядок расположения. Лишь у одного штамма кластер был близок таковому у прототипа. Биоинформационный анализ продуктов их трансляции показал, что аминокислотная последовательность мажорной субъединицы пилей MshA лишь в небольшом N-концевом участке (1–41) гомологична таковой прототипа, тогда как остальная часть не имеет с ней ничего общего. Этот белок, сходный с описанным H. Kuroki *et al*. (2001) VcfA и обозначенный нами как MshA-like, тем не менее сохранил потенциальный домен пилина. Аналогичная картина наблюдалась и в минорных субъединицах, обозначенных как MshC-like. Другие минорные субъединицы также сохранили характерные для них домены. Все штаммы агглютинировали эритроциты человека I группы крови и куриные эритроциты, причем у содержащих измененные гены *mshA-like* и *mshC-like* реакция не ингибировалась маннозой. Поскольку большинство изученных штаммов выделено от госпитализированных клинических больных, не исключено, что у нетоксигенных холерных вибрионов, лишенных острова патогенности VPI, MSHA-подобные пили могут служить фактором колонизации кишечника человека, в отличие от VPI-позитивных. Полученные данные создают основу для экспериментальной проверки этого предположения.

Ключевые слова: *Vibrio cholerae*, пили MSHA, биоинформационный анализ, адгезия, гемагглютинация.

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Mannose-sensitive haemagglutinin (MSHA) pili are mainly associated with the persistence of *V. cholerae* in water bodies due to the formation of biofilms on abiotic and biotic surfaces and providing resistance towards grazing by aquatic invertebrate predators [1–3]. The role of MSHA pili in human intestinal colonisation is considered insignificant [4–6], although contradicting opinions were expressed [7, 8]. The *msh*-cluster includes 17 genes organised in two operons: *mshHIJKLMNIJ* encoding secretory proteins and *mshBACDOPQ* responsible for production of the structural components (subunits) of MSHA pili [9].

It is known that the expression of adhesion pili by toxigenic strains depends on their habitats. In the intestine the production of toxin-coregulated pili (TCP) increases, and the synthesis of MSHA is repressed; whereas the opposite process is observed in water [10]. However, the fact that most of the non-toxigenic strains lack TCP and Cep (pilin encoded by a gene of CTX or preCTX prophages) suggests that other factors – possibly, including MSHA – are responsible for their adhesive properties. As far back as in 1990s, a group of Japanese researchers [11–13] studied and described pili produced by three strains of non-O1/non-O139 *V. cholerae* serogroups (NAGs), which are similar, although not identical, to MSHA. These pili not only agglutinated chicken, human and rabbit erythrocytes but also adhered to rabbit intestinal cells. The sequences of N-terminal amino acid residues (aa) in the pilins of two strains were found to be identical and one was very similar to that of MshA. Nevertheless, all three proteins differed in molecular weights. Later on, H. Kuroki *et al.* [14] sequenced the genes of two of these three strains and designated them as *vcfA* (*V. cholerae* flexible pili). Their products consisted of 161 and 162 aa, of which only the N-terminal aa (1–41) were homologous to those of MshA. The genes themselves were flanked by homologues of *mshB* and *mshC*, as in the *msh*-cluster of O1/O139 *V. cholerae* serogroups. The PCR revealed a wide distribution of *vcfA* among NAGs. Subsequently, the authors sequenced several more structural genes flanking *vcfA* (*mshB*, *mshCDOP*) in the genome of one of these strains and found that they were 78–89 % homologous to prototypes [15]. The *mshQ* (*vcfQ*) gene was 651 bp longer than the prototype, its product (necessary for the assembly of pili) was highly homologous to the prototype protein with the exception of 270 N-terminal aa [16]. However, the entire sequence of the *msh*-cluster was not determined, only fragments of *mshBACDOPQ* operon (AB049152, AB050252, AB064660) are presented in the NCBI.

Earlier we reported the identification of *mshA*- and *mshC*-like genes in two strains of the O1 serogroup isolated from water bodies, which exhibited a high rate of biofilm formation on abiotic surfaces under experimental conditions [17]. VPI-negative clinical NAG strains which caused diseases in humans are also of great interest. In most of them PCR revealed no *mshA* genes [18]. It remains unknown due to what factors both groups can exhibit adhesive properties in different ecological niches.

In this study, we set out to identify the homologues of genes from the *msh*-cluster in the genomes of non-toxigenic *V. cholerae*, to perform the bioinformatics analysis of their products, as well as to study the adhesive properties of strains containing altered genes.

Materials and methods

In this study we analysed 17 clinical NAG strains and 2 strains of the O1 serogroup isolated from water bodies. Their origin is shown in Figure 1 in the Results and Discussion section.

Using the BLASTN 2.2.29 (http://blast.ncbi.nlm.nih.gov) and BioEdit 7.2.5 (http://www.mbio.ncsu.edu/bioedit) programs, the genes of the *msh*-cluster were identified in the whole genomes sequenced by us on the MiSeq platform (Illumina) in accordance with the attached protocol. Gene translation, comparative analysis of their nucleotide sequences and amino acid sequences of deduced products were carried out by means of the Vector NTI Advance 11 (Invitrogen). The corresponding sequences of the reference *V. cholerae* strain N16061 (AE004128) served as prototypes. In order to determine the presence and localization of active domains in proteins, as well as to identify their homologues in strains from other world regions, we performed Blastp analysis (http://blast.ncbi.nlm.nih.gov). The analysis also included products of the above-mentioned *vcfA* genes found in the NCBI [15, 16].

The haemagglutination test was carried out as described in [11]. The cells of *V. cholerae* precipitated by centrifugation from one-day-old cultures grown in Martin's broth (pH 7.2) were washed three times with saline and diluted to an initial concentration of 10^9 microbial cells per millilitre. The suspensions were titrated in a 96-well microplates in a volume of 25 µl, followed by the addition of 1 % suspension of human (blood group O) or chicken erythrocytes in the same volume. Preliminarily results were accounted after one-hour incubation at room temperature. The final results were considered after 2 hours at 4 °C. Reaction inhibition was studied by adding D-mannose at a final concentration of 1 %.

Results and discussion

As noted above, the results for 18 out of the 19 strains selected for the PCR study using primers for detection of the prototype gene for the major pilus subunit *mshA*, were negative [17, 18]. However, in their whole-genome sequences genes were revealed with 5′-terminal regions (1–122 bp) highly homologous to the prototype

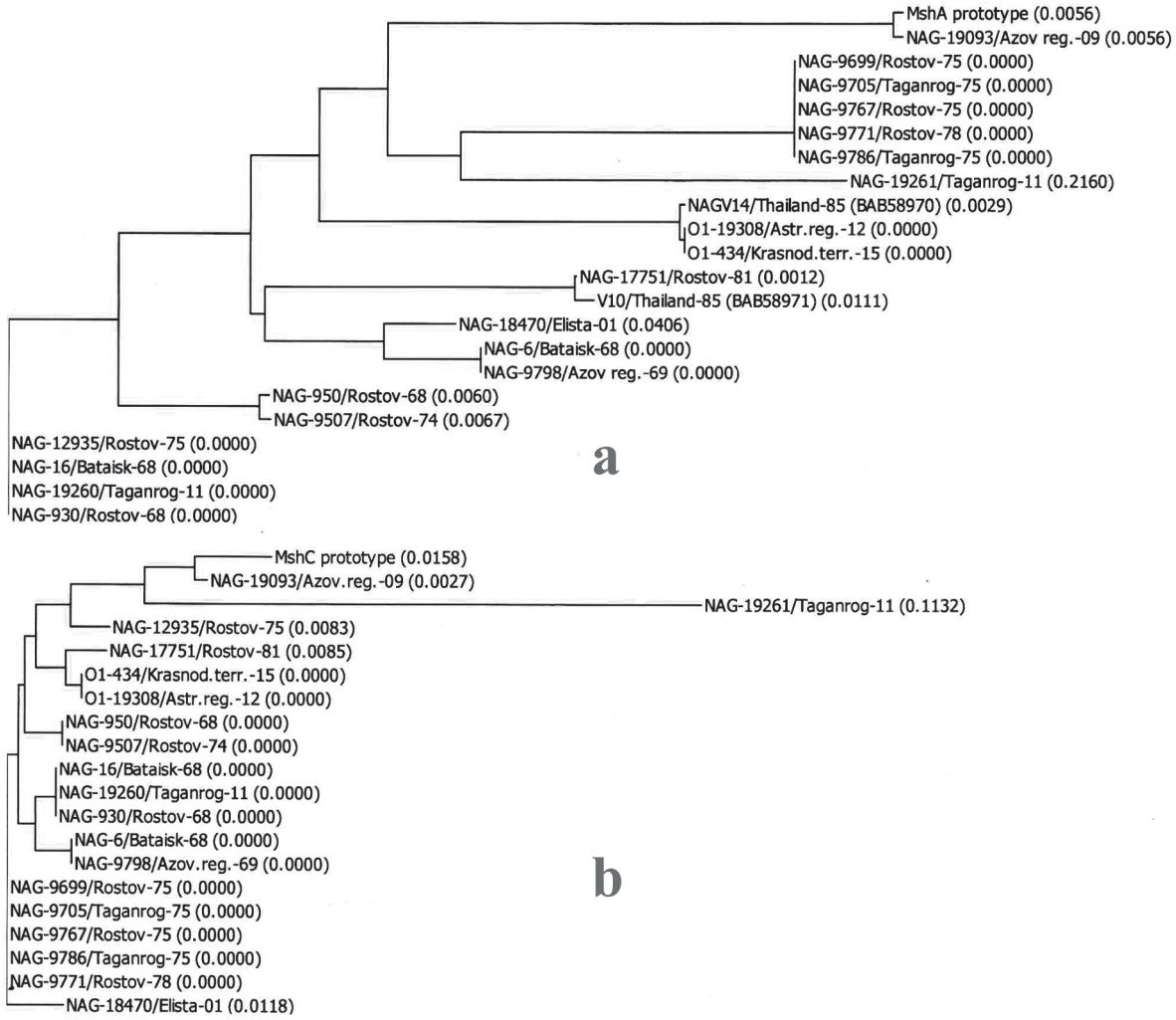

Fig. 1. Dendrograms constructed by definition of the AlignX analysis of MshA-like (*a*) and MshC-like (*b*) proteins of non-toxigenic *V. cholerae* strains

(96–100 %). The remaining sequences shared no similarities with the prototype, and the genes varied in length (from 447 to 489 bp). Only in strain 19093 (PCR positive), the *mshA* gene had 99 % identity to the prototype and the same length (537 bp). Other genes from *msh*-clusters, encoding minor pilus subunits (*mshB, C, D, O, P*) and the protein necessary for their assembly (*mshQ*), also largely differed in length and nucleotide composition. The *mshC* genes, designated as *mshC-like*, showed the most pronounced difference from the prototype (strain 19261 – 498 bp; the rest – 513 bp), with only the gene in strain 19093 having a standard length of 489 bp. However, the transcription of genes by means of Vector NTI revealed that many nucleotide substitutions were silent mutations; therefore, the amino acid sequences of their products were used for further analysis.

According to the AlignX analysis, which also included two of the above-mentioned VcfA proteins, the products of *mshA*-like genes ranging in length from 148 to 162 aa were represented by 11 variants. One of the variants (strain NAG-17751) was close to the VcfA of strain V10 (BAB58971), while another (two strains of the O1 serogroup) was similar to the VcfA of NAGV14 [14], with both being significantly different from the rest (Fig. 1, *a*). N-termini (41 aa) were completely homologous to each other, the prototype and MshA of the strain 19093, which is consistent with the data presented by the above-mentioned authors. The MshC-like (Fig. 1, *b*), MshB and MshO proteins exhibited a lower degree of variability as compared to MshD, MshP and MshQ, which manifested itself in smaller distances between dendrogram branches. The Table shows protein variants – the products of different alleles of genes belonging to operon *mshBACDOPQ* in the studied strains. Even such a restricted set of strains provides insight into the mosaic structure of this cluster. For example, in strains 6 and 9798, MshB is identical to the proteins of 16, 930, 10260, 9507, 17751, 18470, 19093, 19308 and 434; and their MshD and MshO are identical to those of 12935. Strain 12935 has the same MshA as in strains 16, 930 and 19260; whereas in strain 19093, MshO is identical to those of strains 9767, 9699, 9786 and 9705. In addition, for some of the variants presented in the Table (in particular MshB, MshA-like, MshC-like), full homologues belonging to strains from other world regions were found in the NCBI.

Variants of deduced products of *mshBACDOPQ* operon genes of *Vibrio cholerae* strains

Strains	Structural units of Msh							Truncated biogenesis genes	HA titre
	B	A	C	D	O	P	Q		
O1 prototype	**196**	**178**	**162**	**203**	**256**	**143**	**1252**		1/32
NAG 6		148	170			141	1250	*mshL*	1/64
NAG 9798		148	170			141	1250		1/32
NAG 16		156	170	202				*mshF*	1/64
NAG 930		156	170	202					1/128
NAG 19260		156	170	202					1/128
NAG 19261		161	165			141	1245		1/32
NAG 950		157	170			141	1218	*mshH*	1/64
NAG 9507		157	170		259	nd	nd	*mshH*	1/64
NAG 12935		156	170			141	nd	*mshI*	1/32
NAG 17751		162	170			141	1240		1/32
NAG 9767		162	170			140	1332		1/128
NAG 9699		162	170			140	1332		1/128
NAG 9786		162	170			140	1332		1/128
NAG 9705		162	170			140	1332		1/128
NAG 9771		162	170			140	1328		1/32
NAG 18470		148	170	202		141		*mshN*	1.32
NAG 19093						140	1328		1/64
O1 434 (18787)		161	170	202					1/64
O 19308		161	170	202					1/64
NAGV14	nd	161	nd	202		140	1328*		nd

Notes:

Protein variants in each column are marked in different colours, with identical variants sharing the same colours and non-identical variants being marked by different ones. Numerals indicate the numbers of aa in proteins differing in length from the prototypes. In the first column, the strains-representatives of different clonal complexes are highlighted [17, 18]. HA – hemagglutination, nd – not determined.

*Deduced product of the ORF revealed by us in the partial sequence of *vcfQ* (AB064660).

The same putative pilin domain as in the prototype and in exactly the same localization (4–45) was identified in all MshA-like proteins by the Blastp program, which is not surprising as it is located in the N-terminal part of the molecule. A similar situation was observed in MshC-like proteins, some of which contained the same domain PRK10574 (12–33 or 10–44), while the other part contained PRK10557 (12–97), as opposed to the prototype (6–89). Furthermore, the same domains as those of prototypes were identified in MshD and MshP, while MshB and MshO (similar to prototypes) revealed no potential domains (however, as well as in prototypes). A lectin_VcfQ domain was found in MshQ.

T. Miyazato *et al.* [16] previously reported that the *vcfQ* gene in strain NAGV14 (4410 bp) exceeds *mshQ* in length by 651 bp, with an extra fragment extending from the 5'-terminus. This fragment was characterised by a reduced GC content, which might indicate its foreign origin. However, the authors deposited an incomplete nucleotide sequence of this gene (266–4410 bp, AB064660) in the NCBI in which we found two open reading frames (ORFs) – 3660 and 3990 bp in length. The latter was similar in size to the *mshQ* of strain 19093 (3887 bp). We included the deduced product of this ORF into the AlignX analysis, which revealed 99.1 % similarity and 98.7 % identity to these proteins. They also shared the lectin_VcfQ domain.

The presence of putative domains in structural subunits does not necessarily mean that the pili assembled of them exhibit sufficient adhesive properties; earlier they were confirmed only for three strains [11–14]. Therefore, we determined the ability of the 19 studied cultures to agglutinate human (blood group O) and chicken erythrocytes. The cells of all strains agglutinated erythrocytes of both species in dilutions of up to 1/32 – 1/128 ($3.12 \cdot 10^8$–$7.8 \cdot 10^7$ microbial cells per millilitre). Additionally, the reaction was inhibited by mannose only in strain 19093 possessing genes *mshA* and *mshC*, which are close to the prototypes, with no inhibition occurring in the other strains having altered *mshA*-like and *mshC*-like genes. This is consistent with data presented by N. Nakasone *et al.* [11], who demonstrated that the pili of NAG strains were not sensitive to mannose. Figure 2 shows chicken erythrocyte agglutination by several strains.

The above-mentioned authors [11, 14–16] characterised the genes of operon *mshBACDOPQ* for one NAG strain and proposed to designate this locus as VCF. We succeeded to assemble complete sequences of *msh*-clusters for most of the studied strains, including operon *mshHIJKLMNIJ* responsible for the secretion of structural components and the assembly of pili. It was established that these clusters are localized between the same genes as in the prototype (VC0397 and VC0415) and include all their genes (*mshH-Q*), although for the most part they differ significantly from the standard ones in length and nucleotide composition. Taking into account the common structure and localization of these clusters, we considered the designation proposed by the aforementioned authors (locus VCF) to be irrational. In order to avoid confusion in the course of analysis, we propose to keep the name "*msh*-cluster" for them.

When analysing *mshHIJKLMNIJ* operons, we noticed that one of the genes in some strains was truncated – *mshH, mshI, mshL, mshN* or *mshF* (see Table), which did

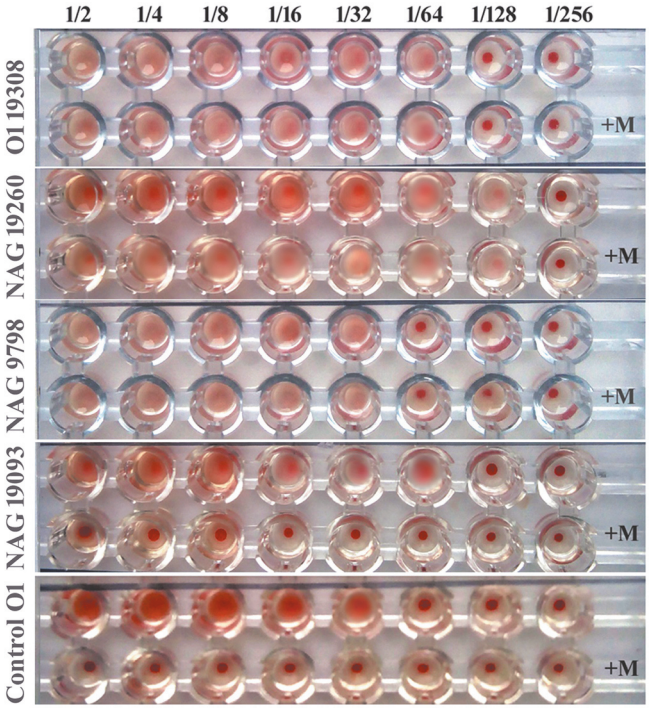

Fig. 2. Agglutination of chicken erythrocytes by the cells of *V. cholerae*. The initial concentration – 10 billion microbial cells per millilitre, + M – reaction with the addition of mannose

not affect significantly their haemagglutinating activity, although occasionally resulting in a slight decrease in titres (see Table). MshH is known not to be involved in the pilus biogenesis; however, its gene contains the promoter of the entire operon [9, 19]. We additionally studied the *mshI* promoter region against that of the reference strain [9]. In all strains, including those containing truncated *mshH*, the *mshI* promoter was 100 % homologous to the prototype. In the experiments of J.W. Marsh *et al.* [20], the disruption of the *mshL* gene resulted in the loss of the haemagglutinating activity of the mutant. However, the truncated MshL protein of strain 6 retained active domains of secretin. The role of other proteins in the pilus biogenesis remains almost unknown. No active domains were found in MshF, thus making it impossible to assess the significance of gene truncation in strain 16. The truncated MshI in strain 12935 lost its sole domain PilN found in proteins responsible for the pilus assembly, whereas the truncated MshN in strain 18470 lost the prototype domains and acquired new ones, including TadD, which is also associated with pilus assembly. Nevertheless, the above-mentioned strains agglutinated erythrocytes to a various degree. These issues require further research that was not conducted since 2009. At the moment, we can only speculate that the products of 10 genes from the biogenesis operon are probably interchangeable so that damage to one of them can be compensated by the activity of others, which allows to maintain the ability of MSHA production.

It is known that the expression of *msh*-operon occurs constitutively in water; however, it becomes repressed upon entering the intestines. The ToxT regulator capable of binding to three promoters within the locus (*mshH, mshI, mshB*) plays a key role in this process. Concurrently it activates the expression of *tcp* genes [19, 21]. Besides the repression at the transcriptional level, MSHA production is repressed at the post-translational level due to the enzymatic activity of the prepilin peptidase TcpJ which degrades the primary structural pilin of MSHA. [10]. The genes of both proteins, *toxT* and *tcpJ*, are part of the VPI. All the experimental data were obtained using VPI-positive strains, with only *tcpA* deletion mutants being constructed which preserved all the other genes of the island [4, 6]. This suggests that the initially VPI-negative strains can constitutively express MSHA also *in vivo*.

On the other hand, the repression of MSHA *in vivo* implies that these pili interact with the secretory immunoglobulin S-IgA of the host, which prevents both the penetration of vibrios through the intestinal mucus and adhesion to epithelial cells [21]. That's why these pili were referred to as 'an anticolonisation factor' [19]. S-IgA is a glycoprotein containing mannose residues that bind to MSHA; this reaction is mannose-sensitive [21]. Thus, these pili are most likely to be produced in VPI-negative strains containing the prototype *msh*-cluster; however, being bound to S-IgA, they cannot promote adherence to the intestinal mucosa. Conversely, MSHA-like pili are not sensitive to mannose, which suggests that the acquisition and preservation of their determinants in the genome may be considered as one of the ways to 'trick' the non-specific immunity of a macroorganism and ensure the pathogen adhesion. Clearly, this hypothesis requires experimental verification. Interestingly, one of our strains (17751) has a preCTX prophage and a significantly altered *tcpA* gene. We failed to find the *toxT* gene in its whole-genome sequence; however, a 94 % homologue of *tcpJ* was found. *In vitro* the strain produced MSHA-like pili, but their production or activity is probably blocked *in vivo*, with TCP or Cep (encoded by a gene of preCTX) serving as an adhesion factor. Concerning strains lacking *tcp* and *cep* genes, further studies will show whether their MSHA-like pili can serve as a colonisation factor of the human intestine.

The nucleotide sequences of *msh*-clusters from the studied strains were entered in the NCBI GenBank database under the accession numbers MN172245-MN172261, MG551945 and 551946.

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