ISSN 1022-7954, Russian Journal of Genetics, 2013, Vol. 49, No. 1, pp. 110–125. © Pleiades Publishing, Inc., 2013.
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Systems of Genes and Proteins Affecting Mycobacteria Virulence and Their Homologs Participation in Conjugation of *Mycobacterium smegmatis*[1]

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Received April 1, 2012

Abstract—This review describes and summarizes the data of ESX secretory system peculiarities characteristic of mainly mycobacteria. This system is involved in the secretion of small proteins of the WXG100 family. Some of these proteins represent virulence factors of *Mycobacterium tuberculosis* and other pathogenic mycobacteria. The role of these proteins in pathogenesis apparently consists of protecting mycobacteria from lysis in the macrophages that absorb them; the cytolysis of macrophages; and, hence, mycobacterium output into the surrounding tissue. A number of proteins that make up this secretory system are homologs of proteins involved in the conjugation process in saprophytic *Mycobacterium smegmatis*.

DOI: 10.1134/S1022795413010092

INTRODUCTION

The mycobacteria family is comprised of many species, including both saprophytic and pathogenic [1]. The pathogenic species consist predominantly of a group of subspecies that form the mycobacterium tuberculosis complex (MTC), including *M. tuberculosis, M. africanum, M. bovis, M. caprae, M. pinnipedi, M. microti,* and *M. canettii*. Despite the small differences in genomes of these mycobacteria and their occurrence within only one species, their pathogenicity and, hence, their role as causative agents in tuberculosis (mainly, pulmonary tuberculosis) is rather different [2–4]. *M. tuberculosis* (MT) subspecies plays a predominant role, since it causes 2–3 million deaths per year all over the world. About one-third of the Earth's population is infected with tuberculosis bacteria known to be in latent state in the organism for decades that have not caused any disease due to the existence of a delicate restrained balance between the pathogenic bacteria and host organism [5]. It would take only one catastrophe to disturb this balance toward bacteria. Species and subspecies that consist of an *M. avium* complex are also pathogenic to humans. Leprosy caused by pathogenic bacteria *M. leprae* has been known since biblical times; fortunately, this disease became exotic, at least in developed countries. Other mycobacteria assumed to be dangerous to humans are known, including *M. marinum, M. ulcerans, M. kansasii,* etc. Finally, the saprophytic *M. smegmatis* species is widely used in empirical analysis as a fast-growing, harmless model object for studying mycobacterium genetics and metabolism and for the primary search for antituberculosis drugs [1].

During the long history of human coexistence (even with our hominid ancestors), the MT subspecies and other pathogenic mycobacteria gained a number of adaptations (called as virulence factors) that made it possible to attack multiple mechanisms of the host defense system. The virulence stage can be assessed via the infection of laboratory animals (in recent years, animals with distinct genetic lineages were used) and the observation of the duration of their lives and pathologoanatomic picture show us the character of lesion in lungs and other organs. Techniques based on cell culture infection, i.e., macrophages, are widespread (the fate of bacteria inside of the cells and the cell's fate must be observed). Moreover, the ability of mycobacteria to cause (or suppress) an inflammatory response based on the production of various interleukins and other compounds as a response to the infection of animals or cell cultures might be also measured. The degree of virulence and ability to cause the production interleukins related to the development of immunity are known to coincide within the known range. Virulence factors are very diverse. A number of evidence demonstrating an important role of lipid

[1] This issue is devoted to an outstanding Russian naturalist, Nikolai Konstantinovich Kol'tsov. N.K. Kol'tsov did not work with pathogenic microorganisms, including tuberculosis infection. Research in this field was brightly conducted by his older coeval, R. Kokh, and later by A. Calmette and K. Guerin. However, the ideas of N.K. Kol'tsov, including giant self-transcribed molecules, i.e., inherited information carriers, are the basis of the contemporary conception of genes and genomes. We were witnesses of and participants in a multiple studies of genomic structure decoding in multiple microorganisms, including such scourges of humanity as *M. tuberculosis*. Several aspects of bacteria genetics and physiology have been summarized in the present review.

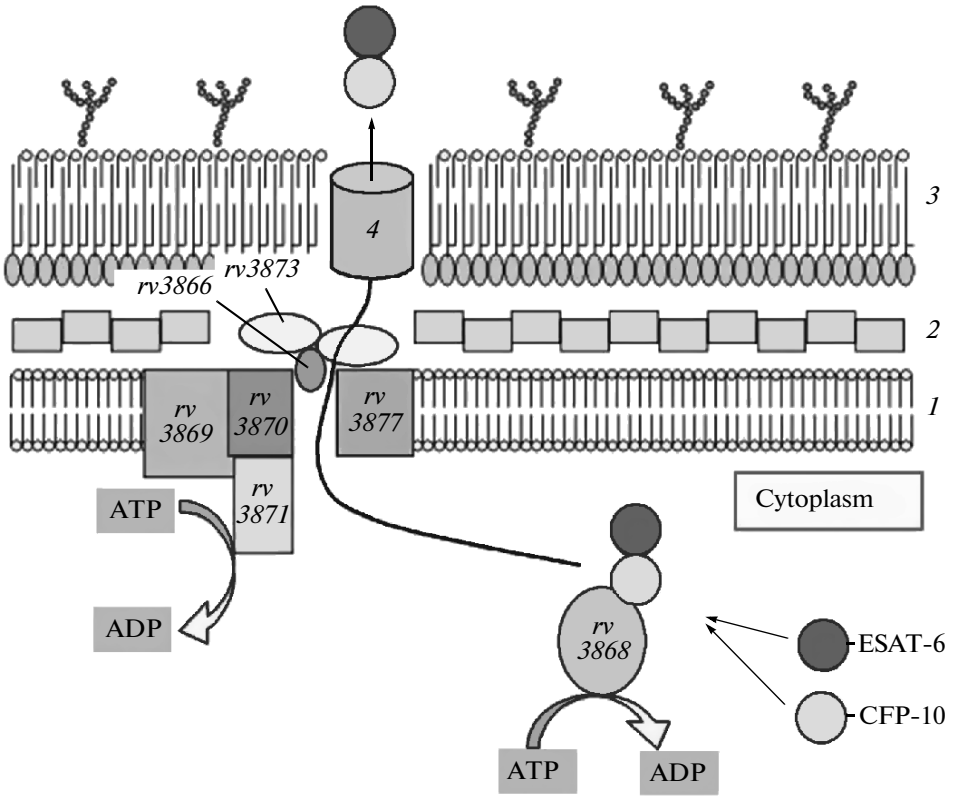

Fig. 1. Role of several gene products of ESX-1 cluster in translocation and secretion of ESAT-6 and CFP-10 proteins (according to [37], with modifications). *1*, cytoplasmatic membrane; *2*, peptide-glycan layer; *3*, polymer layer of arabinogalactan connected with a layer of branched mycolic acids; *4*, suggested pore in all outer cell layers.

complexes of mycobacterium cellular wall in tuberculosis pathogenesis has existed for a long time. Other virulence factors include proteins [6]. These factors might be tried as targets for new antituberculosis drugs.

The present review describes the properties of a distinct family of small secretory proteins predominantly characteristic for mycobacteria and the cluster structure of the encoding genes. Some of these proteins are undoubtedly involved in the virulence. As a result of the study of the secretion of these proteins, it was found that the components of their secretory apparatus were involved in the conjugation of saprophytic *M. smegmatis* bacteria.

1. ESX SECRETION SYSTEM: DEFINITION AND TERMINOLOGY

Bacteria continually enrich media with the products of their vital activity. This enrichment occurs due to both cell damage with autolysis and the active process of secretion from nondamaged bacterial cells. Secretion is accompanied by the extraction of various substances, including proteins into the media. Secretion mechanisms have been thoroughly studied in multiple studies (e.g., reviews [7, 8]). To date, at least six types of secretion are known. Over the last 10–15 years, one additional type of secretion (VII), also called an ESX system [9–12], was established and described in mycobacteria (the latter name is mainly used in the present review; however, it is sometimes also called an Snm system, i.e., secretion in mycobacteria) [13, 14]. The role of this system is limited to the secretion of the WXG100 family of proteins; corresponding proteins are small (about 5–10 kDa) and lack the N-terminal signal peptide (this peptide is assumed to be involved in the origin of proteins in other secretion systems through the surface cell layers). The extraction of these proteins was considered to occur through pores that perforate the thick, complex cellular wall of mycobacteria (see layer structure in Fig. 1). ESAT-6 protein of *M. tuberculosis* is a paradigm of these proteins (its properties are described below in detail), and it is assumed to be the major protein factor of the virulence of tuberculosis bacteria that is of interest. Genes that encode similar proteins usually comprise particular clusters with products involved in the corresponding secretory machine. *M. tuberculosis* possesses five of these clusters (ESX1-5). Moreover, there are six separate groups, including genes that also probably encode proteins similar to ESAT-6 [9].

The ESX-1 cluster has been studied more thoroughly in a number of mycobacteria. Hence, the review should be effectively started from the descrip-

tion of the genes that comprise this cluster and their products.

2. ESX-1 CLUSTER

The first publication that describes the properties of one of the key proteins of the ESX secretion system in mycobacteria was published in 1995 and investigated the properties of proteins present in liquid nutrition media at the early stages of *M. tuberculosis* growth (7 days) [15]. The media contained a protein with a low molecular weight (6 kDa, 95 amino acid residues) that lacked the N-terminal sequence. Together with the H37Rv strain, this protein was produced by the laboratory H37Ra strain; however, in a smaller quantity. Moreover, it was also produced by one of the clinical MT isolates; *M. africanum*; *M. cansasii* species pathogenic to humans; and, finally, by the *M. marinum* species pathogenic for haematocryal. Notably, the corresponding protein has not been detected in the case of BCG lineage growth. The protein had the ability to cause a cell memory response, i.e., the production of γ-interferon in murine T-lymphocytes previously treated with tuberculosis bacteria from the H37Rv strain. The gene that encodes the protein has been cloned. This protein was called ESAT-6 (early secretory antigen target 6 kDa); the name of the gene is the same; however, other aliases are known, i.e., *esx* and *esxA*.

Another low-molecular protein secreted into the media was described 3 years later [16] and had a molecular weight of 10 kDa. The gene that encodes this protein is located directly in front of the gene that encodes the ESAT-6 protein and together they possess a common operon structure. This protein was called CFP-10 (culture filtrate protein 10 kDa). Along with ESAT-6, its functions are described below. The primary name of the gene that encodes this protein was *lhp* (L45 homologous protein), since it was similar to the L45 protein in *M. leprae*. Sometimes, this gene is called *esxB*.

The following study of the secretory ESX system in mycobacteria was influenced by two almost simultaneous observations. First, the data of the complete genome sequencing of *M. tuberculosis* H37Rv strain have been published [17]. Although the sequencing of the distinct genomic regions was performed earlier, and the aforementioned study gave complete data of the structural peculiarities of the *M. tuberculosis* genome. This investigation was followed by analogous studies of the complete genome sequencing of a number of the other mycobacteria species and strains and made it possible to identify (in primary studies via *in silico* analysis) the regions in the *M. tuberculosis* genome responsible for the secretion of small ESAT-6 similar proteins that possess virulent and immunogenic properties [9, 18]. The nomenclature has been also modified; genes were renamed according to their position on the circle chromosome of H37Rv strain. For instance, the gene that encodes ESAT-6 protein was renamed *rv3875*, while the gene that encodes CFP-10 protein was renamed *rv3874*. Second, studies that demonstrate the direct relation between some genomic modifications characteristic of the BCG vaccine strain and peculiarities of the ESX secretion system have been published. In order to avoid replication in the text below, it is necessary to note the main stages in the design of the BCG vaccine strain and the peculiarities of its lineages (i.e. substrains).

The BCG (Bacillus Calmette-Guerin) strain is known to be detected as a result of the consequent subculture of *M. bovis* on a dense potato-glycerin media with the addition of bovine bile at the Institute Pasteur in Paris. Subcultivation was carried out over 11 years (1908–1919) without any selection of the distinct colonies toward their morphology and properties. A total of 230 passages were performed. Subcultivation was accompanied by the systematic infection of laboratory animals in order to test whether attenuation had taken place (virulence reduction). Since 1920, this strain has been recommended for human vaccination and gradually spread all over the world. Various lineages were isolated (Brazil, Russian, Japanese, etc.), since each country (even several Institutes of one country) used its own forces for living vaccines maintained via multiple passages (see review [19]). Much later (in the 1950s–1960s), BCG lyophilization became available, which diminished the number of passages; however, at that time, various BCG lineages accumulated a distinct number of randomly occurring genomic modifications (deletions, duplications, nucleotide substitutions, etc.; [20]). Since the 1920s, about 3 billion individuals have been vaccinated. The efficacy of vaccinations was not absolute; the level of defense against various clinical forms of tuberculosis varied in the range of 14–80% in different countries; the efficacy depended on the age of the vaccinated individual, probably ethnicity, and even the BCG lineage (due to genetic polymorphism as a result of passages). Thus, early BCG lineages (including Russian) obtained from the Institute Pasteur in the mid-1920s demonstrated stronger immunogenicity compared to later" vaccines in the mid-1930s. The BCG vaccine has not completely lost its virulence, i.e., it causes lymphadenitis and other complications. However, despite multiple attempts to replace it, it is still widely used in many countries all over the world.

The main difference between the BCG genome and *M. bovis* and *M. tuberculosis*, which is responsible for the attenuation, remained unknown for more than 70 years. A study [20] was only published in 1996 based on subtractive hybridization between the genomes of various BCG lineages and *M. bovis*, laboratory strains H37Rv and H37Ra, and several clinical *M. tuberculosis* isolates. Three regions of difference (RDs) were detected, including RD1 (chromosomal fragment 9.5 kB in size), which was absent in all BCG lineages but was present in *M. bovis* and in all tested *M. tuber-*

culosis strains. This fragment contained genes that encode ESAT-6 and CFP-10 proteins and several other genes. This fragment was cloned in the integrative shuttle plasmid and inserted into the BCG genome. The data of the absence of the RD1 fragment in various BCG lineages were later confirmed via the microarray technique [21, 22].

The cited studies have drawn the attention of researchers investigating mycobacteria, who initiated a number of new experiments. First of all, the following evidence of the involvement of the ESAT-6 encoding gene and the complete RD1 fragment with neighboring genes into MT virulence was revealed.

The deletion of the chromosomal region corresponding to RD1 and the inactivation of some genes located in this region were performed in *M. bovis* and *M. tuberculosis* H37Rv by gene-engineering methods. These strains (including H37Rv), with deletions revealed in vitro, mainly lost their virulence and might be used as vaccine strains, together with BCG in animal studies. The reverse procedure was also conducted, i.e., the knockout insert (knock-in), and the RD1 fragment was inserted into the BCG genome. This insert partly restored the virulence of BCG and enhanced the ability of the immune response of interleukin synthesis in T-lymphocytes of infected animals [23–28]. Thus, the association of genes located in the RD1 region with virulence in *M. bovis* and *M. tuberculosis* has been comprehensively testified.

Several studies conducted primarily *in silico* (based on data of complete MT sequencing and proteomic analysis) and, later, empirically were devoted to the study of the structure of the RD1 fragment and neighboring genomic regions (i.e., the gene cluster of the ESX-1 system), the functions of these cluster genes, the regulation of their expression, and the pathways of the secretion of ESAT-6 and CFP-10 proteins from the cell [9, 18, 29–31, and review 32]. The ESX-1 cluster is located near the starting point of replication on the *M. tuberculosis* chromosome on the left side (Fig. 2); it is comprised of 20 genes (*rv3864–rv3883*). The borders of this cluster exceed the RD1 fragment, especially on the left (Fig. 3). *rv3874* and *rv3875* genes, which represent the cotranscribed and cotranslated structure of the operon are located in the middle of the cluster. According to the search conducted by our group in the NCBI database, the nucleotide sequence of these two genes in 44 clinical *M. tuberculosis* isolates was completely identical to the H37Rv strain. CFP-10 and ESAT-6 proteins are encoded by the genes of this operon, respectively. One part of the genes that neighbor the operon is involved in either the regulation of expression or secretion of both proteins. However, the function of several genes of this cluster remains unknown.

Molecules of ESAT-6 and CFP-10 proteins are known to have the ability to form various complexes with each other, mainly heterodimers in a ratio of 1 : 1 or even tetramer (Fig. 1). Evidently, these complexes

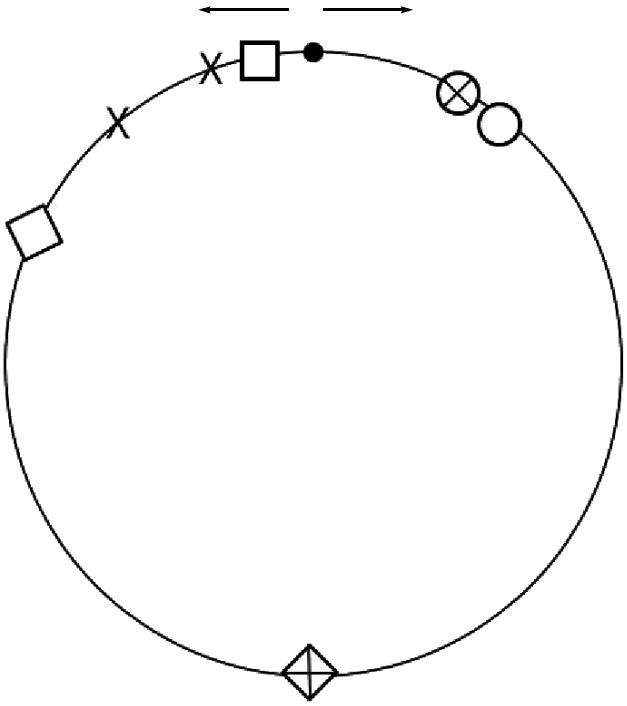

Fig. 2. Location of gene clusters of ESX-system on *M. tuberculosis* H37Rv chromosome. ⟵ ● ⟶ is starting point of chromosome replication and direction of replication branches; □ is ESX-1 (*rv3864–rv3883c*) and ESX-2 (*rv3884c–rv3895c*) clusters; × is localization of ESP system genes (*rv3614c–rv3616c* and *rv3849*); ⊗ is localization of *phoP* gene (*rv0757*); ○ is ESX-3 cluster (*rv0282–rv0292*); ◇ is ESX-4 cluster (*rv3444c–rv3450c*); ⊕ is ESX-5 cluster (*rv1782–rv1798*).

are even formed in the cell cytoplasm prior the passing through its surface layer, i.e., prior to secretion [33–38]. The heterodimeric state of these proteins promotes their resistance to denaturizing and proteases actions [39]. CFP-10 protein most likely plays the role of a chaperone protein in maintaining the configuration of the main virulence of the ESAT-6 protein.

As mentioned above, many genes of the ESX-1 cluster were involved in the regulation of the expression of *rv3874* and *rv3875* genes, in particular in the mechanism of secretion of their products. The genes that encode PE35 and PPE68 proteins (*rv3872* and *rv3873*, respectively) are located to the left of these middle genes. PE and PPE proteins represent a broad group of mycobacteria proteins; they are acid glycerin-rich proteins encoded by genes forming repeats [17]. The product of *rv3872* is closely related to the regulation of ESAT-6 and CFP-10 proteins synthesis, while the function of the *rv3873* gene remains unknown [30]. Four genes (*rv3868–3871*) are located on the left side. Their inactivation results in the inhibition of the secretion of ESAT-6 and CFP-10 proteins (gene expression was observed, however, no proteins were detected in the culture media). The phenotype of these mutants corresponds to that of the BCG strain.

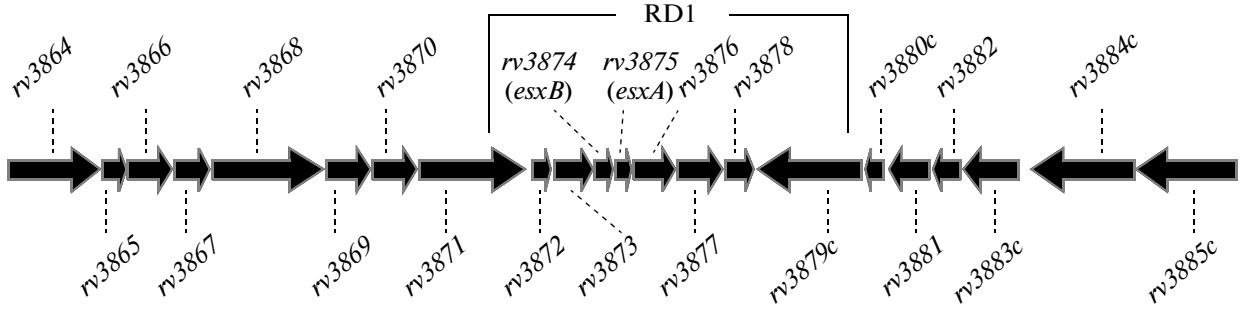

Fig. 3. Genes cluster of ESX-1 system (according to [31], with modifications). Black arrows correspond to cluster genes (*rv3864–rv3883c*), while their direction corresponds to the direction of transcription. Frame at the top corresponds to borders of RD1 fragment.

The products of *v3868* and *rv3871* genes possess ATPase activity. The proteins encoded by almost all genes from the left part of the cluster (*rv3866*, *rv3869*, *rv3870* and probably *rv3871*) and *rv3877* gene located on the right flank (Fig. 3) form a complex that is connected with the cytoplasmatic membrane and involved in the translocation of ESAT-6 and CFP-10 toward the outer cell layers (translocon) [30]. ATPases provide the energy for translocation. There are contradictory data on the protein encoded by the *rv3876* gene [29, 30]. One of the schemes that represent the role of some of these proteins in the mechanisms of secretion and translocation is shown in Fig. 1.

With respect to other products of the ESX-1 gene cluster, the proteins encoded by *rv3864* and *rv3865* genes (extremely left cluster genes) possess some influence on virulence, probably via the stabilization of the ESAT-6–CFP-10 complex. The *rv3883c* gene, which encodes mycosin-1 (one of subtilisin-like serine proteases), is located in the far right position in the cluster [40]; *rv3880c, rv3881,* and *rv3882* genes are located before it. According to the data revealed as a result of studying the homologous gene cluster in *M. smegmatis*, the *rv3882* gene, together with the *rv3883c*, is included in the operon structure of mycosin synthesis (see below). The *rv3880c* and *rv3881* genes are probably related to this operon. The inactivation of all four genes located on the far right flank of the cluster (as *rv3867* gene) demonstrated no effect on the synthesis and secretion of the ESAT-6–CFP-10 complex [29, 30, 32]. The structure of the hypothetical pore, i.e., a channel that provides the secretion of both proteins into the media, also remains unknown (Fig. 1).

The proposed protein functions of the ESX-1 cluster are shown in the table.

3. REGULATION OF ESX-1 CLUSTER FUNCTIONS BY GENES LOCATED BEYOND THE CLUSTER

The regulation system of the components of the ESX-1 cluster also involves another group of genes located in another chromosomal region of *M. tuberculosis* (Fig. 2), i.e., in an operon that consists of *rv3614c, rv3615c*, and *rv3616c* genes and apparently located *rv3849* gene (ESP system). The final product of this operon is EspA protein (ESX-1 secretion-associated protein A), which consists of a secretory protein with a size of 40 kDa that corresponds to the *rv3616c* gene [14, 41]. The protein was detected in a comparative proteomic analysis of mutants characterized by disturbances in the protein secretion of the ESX-1 system. The inactivation of the *esp* operon resulted in the cessation of ESAT-6 and CFP-10; in turn, the EspA protein was not secreted by many mutants in the ESX-1 gene cluster. The possible mechanism of the interaction of both systems was described in the following way.

The product of the *rv3849* gene activates the transcription of its operon, which caused an increased intracellular concentration of the EspA protein. In turn, this protein activated the total ESX-1 secretion system (molecular mechanisms of activation are unknown). Activation also resulted in the output of the *rv3849* gene product through the cell. The excess of this product (EspA protein), which somehow interacts with the surface cell structures, inhibited the subsequent transcription of the *rv3849* gene and the complete *esp* operon, which caused a decrease in the concentration of EspA secreted protein and the inhibition of the activity of the entire ESX-1 secretion apparatus (i.e., the negative regulation system [42]). It might be suggested that the secretion of the EspA protein occurred via the same mechanisms as known for ESAT-6 and CFP-10 secretion.

Finally, ESAT-6 and CFP-10 secretion is probably controlled by the pleiotropic positive transcription regulator PhoP. The H37Ra laboratory strain originated from *M. tuberculosis* isolate and characterized by weakened virulence was isolated in the 1930s (its origin is related to multiple passages on media with modified pH). As was observed significantly later, this strain was characterized by the strongly diminished secretion of ESX-1 protein cluster [43]. The search for differences between H37Rv and H37Ra genomes performed in the same study revealed the *s219l* mutation in *phoP* gene, which results in the substitution of serine

Genes of ESX-1 cluster in *M. tuberculosis* and corresponding proteins

Gene	Protein characteristics	Protein functions
rv3864	N/A	Stabilization of CFP-10 and ESAT-6 protein complex (?)
rv3865	N/A	Same
rv3866	Protein of ABC-transporters family	Translocation and secretion of CFP-10 and ESAT-6 protein complex
rv3867	N/A	N/A
rv3868	Protein with ATPase activity	Translocation and secretion of CFP-10 and ESAT-6 protein complex
rv3869	Transmembrane protein	Same
rv3870	Transmembrane protein	"
rv3871	Protein with ATPase activity	"
rv3872	Protein of PE family	Regulation of *rv3874* and *rv3875* genes expression
rv3873	Protein of PPE family	N/A
rv3874 (lhp; esxB)	Secretory CFP-10 protein of WXG100 family	Virulence factor (chaperone of ESAT-6 protein(?))
rv3875 (esx; esxA)	Secretory ESAT-6 protein of WXG100 family	Virulence factor responsible for membranes damage via phage lysis, cytolysis of macrophages, and release from mycobacteria
rv3876	Protein with ATPase activity	N/A
rv3877	Transmembrane protein	N/A
rv3878	N/A	N/A
rv3879	N/A	N/A
rv3880c	N/A	Regulation of mycosin-1 synthesis and secretion
rv3881	N/A	Same
rv3882c	Transmembrane protein	"
rv3883c	Subtilisin-like serine protease (mycosin-1)	Indirect involvement of secretion of ESX-1 cluster products (?)

Note: N/A means not available. The table was based on findings reported in [9, 18, 29, 30, 31, 40, 73].

for leucine in one of the DNA-binding domain regions of the PhoP protein. In H37Ra cells, the insertion of a cosmid with the correct *phoP* gene resulted in the partial recurrence of virulence in this strain. Similar findings that report the role of this mutation in the loss of virulence in H37Ra were also shown in another study [44]. It was assumed that the *phoP* gene (*rv0757*) regulated the functions of the ESX-1 gene cluster via the ESP system [43]. If this suggestion is true, the properties of both widespread strains with diminished virulence that belong to the tuberculosis complex, i.e., BCG and H37Ra, were observed due to disturbances in the secretion of the same ESAT-6 and CFP-10 proteins. In in the case of BCG, this is related to the deletion of the RD1 region, while for H37Ra, it is caused by disturbances in the functions of the same region due to a mutation in the regulatory *phoP* gene. It should be noted that the previous studies demonstrated the relationship between the presence of a mutation in the PHO gene system and decreased virulence [45–48]. One of these strains was even suggested as a living vaccine, since it was more attenuated than BCG [47]. However, these studies assumed that decreased virulence was caused by modifications in lipid synthesis, which occurred due to the mutations in the PHO system with a pleiotropic representation. Moreover, differences between H37Ra and H37Rv genomes included not only the *S219l* mutation, multiple modifications were detected [43]; however, their relation to H37Ra attenuation was indefinitely established.

4. MECHANISM OF INVOLVEMENT OF ESAT-6 AND CFP-10 PROTEINS IN VIRULENCE OF TUBERCULOSIS BACTERIA

Accordingly, multiple studies have shown that the complex of secreted ESAT-6 and CFP-10 proteins is definitely one of the major factors in the virulence of tuberculosis bacteria. Several parallel investigations aimed at detecting the distinct mechanisms of bacteria virulence that involve these proteins. For this purpose, murine and human macrophages were infected by virulent strains and strains with some mutations in ESX-1 system genes and the infected cells were observed. In the case of virulent strains, bacteria were included in macrophage phagosomes at the first stage, which was followed by the fusion of macrophages phagosomes with vesicle-like cell structures, i.e., lysosomes, and the formation of phagolysosomes (the lysosome con-

tent has a low pH with hydrolytic enzymes able to lyse and damage bacteria). One part of mycobacteria included in the phagolysosomes was lysed by the antigen output, which resulted in the immune response. The remaining living bacteria damaged the phagolysosome membrane and were released into the cytoplasm with the following reproduction. Membrane damage was preceded by the dissociation of the ESAT-6–CFP-10 complex into proteins in the acid phagolysosome content. In turn, the cellular surface of macrophages was damaged, i.e., cytolysis was followed by the release of bacteria into the intercellular area and the infection of other cells. Macrophages containing mycobacteria were also characterized by self-lysis due to cellular apoptosis induced by the ESAT-6 protein. Another observation was detected in the case of the infection of macrophages by bacteria of the BCG strain or by mutants unable to synthesize and secrete ESAT-6–CFP-10 proteins. Mycobacteria started to reproduce in macrophages, paused, and finally lysed, which resulted in the release of various antigens [48, 49–52]. Most likely, the aforementioned virulence proteins play a significant role at the first stages of tuberculosis pathogenesis, i.e., in the propagation of bacteria from primarily infected cells toward the cells of surrounding tissue. Mutations that prevent secretion in the ESX-1 system disrupt the chain of these processes.

The studies of the virulence effect of corresponding mutants have also been conducted on animals. An infection of the murine GKO lineage that is unable to produce γ-interferon and, hence, is supersensitive to tuberculosis infection was studied. The infection of these mice by virulent *M. tuberculosis* strains was accompanied by the necrosis of pulmonary tissue, resulting in the rapid death of the animals. In the case of infection by bacteria of the BCG strain or mutants with deletion in the RD1 region, favorable granulomatous pneumonia occurred. However, the complementation of mutations via plasmid with a cloned inserted RD1 fragment resulted in the mutants' ability to cause lung necrosis [53].

The study of the properties of ESAT-6 and CFP-10 also yielded a practical result, since it made it possible to design new diagnostic tests to replace the Mantoux test. The Mantoux test is known to consist of a provoked local inflammatory reaction on intracutaneous tuberculin infection based on an allergic response to repeated contact with the antigens of tuberculosis bacteria that comprise tuberculin. The primary contact includes tuberculosis infection, which even occurred in latent form. The positive Mantoux reaction, i.e., the formation of an inflammatory plaque papule in the region of the tuberculin injection, is evidence that infection occurred by tuberculosis bacteria (i.e., in sensibilization), even in clinically healthy individuals (usually in children and youths massively tested). However, the specificity of this reaction is rather low due to the presence of the large number of various antigens in tuberculin. Hence, in order to show that the organism's sensibilization occurred directly due to tuberculosis bacteria, the desirable substitution of tuberculin for a distinct protein, i.e., antigen only present in MT (and absent in many mycobacteria, especially due to the massive vaccination by BCG strain), was of great urgency. For this purpose, many countries developed tests based on the injection of ESAT-6 and CFP-10 synthetic proteins [54]. A Diaskintest preparation was constructed in Russia that consists of hybrid protein isolated from *E. coli* cells after their transfection by plasmids with the cloned *M. tuberculosis* gene that encodes ESAT-6 and CFP-10 [54, 55]. The application of an intracutaneous probe with this preparation allowed to exclude false-positive reactions in the Mantoux test related to vaccination by BCG and to specify the observation results of individuals with a latent form of tuberculosis infection (especially in risk groups with family contact with patients in open form; see monography [54, 55]).

Other diagnostic systems based on a splash of γ-interferon and the production of other cytokines by T-lymphocytes in individuals sensibilized in a preceding tuberculosis infection (as a response to contact with ESAT-6 protein). For this purpose, blood was collected from the patient, followed by the isolation of lymphocyte fractions, the treatment of lymphocytes by corresponding antigen protein, and the detection of the number of induced γ-interferons via immune-enzymatic analysis [56–59]. However, these tests are more complex and expensive compared to skin probes.

It should be mentioned that every test based on the use of synthetic or gene-engineering proteins of the ESX-1 system yield a positive reaction, even in patients with leprosy and patients with other mycobacteriosis. However, this problem mainly occurs in countries close to the equator [60, 61].

5. ESX-2–5 CLUSTERS

Together with ESX-1 cluster, the MT genome is comprised of four similar clusters located in different chromosomal regions. The ESX-2 cluster corresponds to *rv3884c–r3895c* genes and is located to the right of the ESX-1 cluster. The ESX-3 cluster corresponds to *rv0282–0292* genes, the ESX-4 cluster corresponds to the *rv3444c–3450c* genes, and the ESX-5 cluster corresponds to the *rv1782–1798* genes (Fig. 2). Despite the difference in the number of genes (less than in ESX-1 cluster), they are characterized by common properties, i.e., tandem gene repeats similar to the genes that encode ESAT-6 and CFP-10 proteins (i.e., WXG100 family proteins) represent nuclear clusters; this tandem is neighbored by genes that encode PE—PPE proteins (except for the ESX-4 cluster). According to *in silico* analysis, the proteins encoded by some genes surrounding the nuclear clusters are very similar to those observed in the ESX-1 cluster [9, 31, 62, 63, and review 13]. ESX-3 and ESX-5 systems are full-

fledged secretory systems; while the products of the ESX-2 and ESX-4 systems still have not been detected outside of bacteria cells. The proteins that correspond to tandem *rv0287–rv0288* genes (ESX-3 system) might form complexes with each other, though not with the similar ESAT-6–CFP-10 proteins of the ESX-1 system [34]. The protein expression of the ESX-3 system is controlled by the zinc regulon (similar to the observation of ESX-1 system under the control of the pho-regulon system [64].

The most prominent difference between all four systems from ESX-1 is their inability to complement mutations in both genes that encode the ESAT-6 and CFP-10 proteins due to insufficient homology. Moreover, the products of the corresponding tandem pairs of ESX-2–5 genes probably do not represent the virulence factors. However, ESX-5 system plays a distinct role in the pathogenesis of the disease caused by *M. marinum* (see below). A relatively old review [10] described the following points as problematic. What was the function of ESAT-6 protein family in ESX-2–5 systems? Was there some involvement of these proteins in virulence? What was the reason for their inability to substitute the functions of the ESAT-6 protein of the ESX-1 system disturbed by mutations? Did the secretion occur via its apparatus or via the apparatus of the ESX-1 system? The review was published in 2004; however, all of these questions remain unknown.[2]

The existence of a small group of genes in the MT genome, including genes that encode the ESAT-6 and CFP-10 proteins (except for ESX-1–5 clusters), was described above [9]. To date, there is no data that describe the level of functionality of these groups and their relation to the functions of ESX-1–5 clusters. Genes of all ESX systems were suggested to possess a single gene cluster as a precursor of the initial forms of mycobacteria, followed by expansion due to duplications and translocations of these genes within genome. The schemes of this evolution were constructed in the mycobacteria genome [10, 62].

6. STRUCTURAL PECULIARITIES OF GENOME REGIONS COMPRISING GENE CLUSTERS OF ESX-1 SYSTEM IN OTHER MYCOBACTERIA

Various methods of analyzing the structure of the bacterial genome yielded the ability to study the peculiarities of the structure of the ESX-1 system genes in mycobacteria, both of which comprise MTC subspecies and other species (pathogenic and saprophytic). The findings are described below.

M. microti

As was mentioned above, *M. microti* represents one of the subspecies that comprise MTC with a genome that is almost identical in size to H37Rv. This bacteria was isolated from voles (*Microtus agrestis*) and caused a tuberculosis-like state in them. Moreover, in some cases, *M. microti* was isolated from infected cattle, llamas, and badgers. This species was considered to be harmless for humans (it was even used as a living anti-tuberculosis vaccine in England and Czechoslovakia). However, later, several diseases were described, followed by the isolation of *M. microti*, which led this species to be considered conditionally pathogenic [65].

A comparative analysis of the genome of a typical *M. microti* strain isolated from voles (the genome was represented by an artificial-chromosome library) and the H37Rv genome demonstrated several differences, in particular in deletions; one of the deletions in the *M. microti* genome nearly corresponded to the region of the ESX-1 gene cluster in *M. tuberculosis* H37Rv (the region including 14 kb was deleted, starting from the *rv3864* gene to the *rv3876* gene). This deletion partially coincides with the deletion of the RD1 region in BCG (accompanied by the deletion of the gene that encodes the ESAT-6 and CFP-10 proteins). The corresponding deletion was called $RD1^{mic}$ in *M. microti* analogous to that observed in BCG [30, 65].

The study [65] tested whether the $RD1^{mic}$ region was observed in other *M. microti* strains isolated from the patients. This deletion occurred to be characteristic of all studied isolates; however, these isolates might differ between each other regarding to other peculiarities of genome structure. The constant $RD1^{mic}$ deletion in the genomes of *M. microti* isolates allowed it to be suggested that the reduced virulence in this subspecies was related to the stoppage in protein synthesis encoded by the *rv3875–rv3876* genes as in the bacteria of the BCG strain. The insertion of the RD1 fragment in the *M. microti* chromosome increased its virulence, as was observed in BCG [26]. To some extent, *M. microti* represents a natural example of BCG. However, this is incomplete identity, since *M. microti* differed from the primarily BCG subspecies, i.e., *M. bovis*, by several other peculiarities [30].

Mycobacteria of M. avium Complex

This complex includes some species (*M. avium, M. paratuberculosis*, etc.) and subspecies pathogenic to birds and human. The symptoms of this disease are similar to those characteristic of pulmonary tuberculosis; the cases of infection with mycobacteria of this complex are rather frequent in immunodeficient individuals; however, the frequency of this mycobacteriosis is ten times lower than that caused by MTC [66].

[2] After the present review has been sent to the Editor, the study [Bottai D., Luca M., Majlesi L., et al., Disruption of the ESX-5 System of *Mycobacterium tuberculosis* Causes the Loss of PPE Protein Secretion, Reduction in Cell-Wall Integrity, and Strong Attenuation, // *Mol. Microbiol.*, 2012, V. 83, No. 6, pp. 1195–2209] was published that demonstrates that mutations in some genes of ESX-5 system in *M. tuberculosis* significantly reduce the virulence in the experiments with macrophages and immune-deficient mice.

The *M. avium* genome is larger than MTC genomes (4829781 bp; [67]). The *M. avium* genome lacks the ESX-1 region (probably due to the extensive deletion of the genomic region starting from the *rv3861* gene ortholog to the *rv3883* gene ortholog; followed by the insertion of a fragment about 3.1 kb in size with the hydrolase gene and a gene that encodes one of the sigma factors, which is foreign to this genomic region [9]). Accordingly, together with *M. microti* and BCG, *M. avium* was characterized by the deletion of a chromosomal fragment that contains the same genes. Most likely, when the lungs are affected, to some extent, this deletion will result in another histological pattern compared to the case of classic tuberculosis. The remaining four ESX regions were observed in the *M. avium* genome.

M. leprae

M. leprae is known to be a pathogenic agent of leprosy, a severe disease that is currently spread predominantly in Africa and South America. The *M. leprae* genome is the smallest one among mycobacteria (3268203 bp [67]). Reduction in the genome size and pseudogene abundance is most likely related to the reproductive evolution (*M. leprae* is strictly intracellular human parasite unable to grow on artificial nutrition media). The reduction of the multiple genomic regions also involved ESX system clusters. Only two clusters, ESX-1 and ESX-3 possess full-fledged genes se that functions in *M. leprae*. The genes of the remaining systems were either deleted or modified by point mutations to the point of unrecognizability [9].

M. marinum

M. marinum is an independent mycobacterium species with unique properties that differ from those of the subspecies that comprise MTC; its genome is 6636827 bp and its growth is possible below the temperature range of 25–35°C. *M. marinum* causes infections in haematocryal fish and frogs; occasionally, it affects human skin in a way clinically similar to skin tuberculosis and is treated by antituberculosis antibiotics. A favorite model for studying the pathogenesis of the disease caused by this bacteria is embryos of aquarium zebrafish (*Pseudotropheus zebra*); a suspension of bacteria is injected into the caudal vein of the embryo, followed by the observation of the histological pattern, i.e., granuloma formation, etc. [67 and review 68].

Although the size of the *M. marinum* genome is 1.5 times larger than that of *M. tuberculosis*, about 3000 of their genes are orthologous. It is also related to the genes located in MTC bacteria in the RD1 region and its neighboring regions. This region is called extRD1 (extended RD1) in *M. marinum* [69]. Its borders are wider than RD1 and almost coincide with the borders of the ESX-1 gene cluster in *M. tuberculosis*. Tandem genes that encode the ESAT-6 and CFP-10 proteins in *M. tuberculosis* are especially similar in both mycobacteria (97 and 91% of homology, respectively) [70]. A number of mutations in *M. marinum*, in particular mutations in the extRD1 region, were revealed via transposon mutagenesis. These mutations prevailed in the cytolysis of infected macrophages, disturbed the bacterial transfer from infected cells to neighboring cells, and significantly decreased the virulence in fish (including the absence of granuloma formation). The possibility of the complementation of damaged genes of the extRD1 region by orthologs in tuberculosis bacteria has been demonstrated [69, 70].

Together with the ESX-1 system, *M. marinum* possesses another system of ESX-secretion, i.e., ESX-2–5 observed in bacteria of the MTC complex. It was even characterized by the presence of an additional system (ESX-6) that consists of a small gene cluster that included duplicated fragments of the ESX-1 cluster [18]. The ESX-5 system has been well studied in *M. marinum*; it is involved in the export of PPE41 protein. This protein is involved in the suppression of various cytokines synthesis in macrophages, which is responsible for the survival and reproduction of bacteria inside of cells [71, 72].

M. smegmatis

M. smegmatis is a fast-growing nonpathogenic bacteria. *M. smegmatis* strains were detected on the skin of human genitals (in the waxlike lubrication of the prepution saccus, smegma), on the skin of axillary crease and in environment. As was mentioned above, this microorganism became a favorable laboratory model in molecular genetics for studying the properties of mycobacterium due to its safety; simplicity of cultivation; and, at the same time, the significant genomic similarity to MTC bacteria.

The *M. smegmatis* genome is one of the largest among mycobacteria (about 7 million bp, which is two times longer than in MT). Despite this observation, the genomes of both species possess about 3000 gene orthologs with a high level of identity. An analysis performed *in silico* revealed that the *M. smegmatis* genome contained the ESX-1 and ESX-3 gene clusters [9]. The ESX-1 system gene cluster is located near the starting point of chromosomal replication as in MT; however, it is on the right side. A detailed description of the structure of the ESX-1 cluster and the functions of some of its genes in *M. smegmatis* were described in the study [73] on the empirical level. In both species, differences between the clusters were small; *M. smegmatis* was characterized by the presence of three transposase genes absent in MT after the *rv3879* gene and by an additional insert to the right of the *rv3881* gene. Finally, the structure of the *rv3876* gene was slightly modified. The function of the *rv3883c* gene in *M. smegmatis* in MT was predicted by a previous study [40] and confirmed experimentally; the encoding mycosin-1 enzyme (subtilisin-like serine protease).

The *rv3882* and *rv3883c* genes were observed to represent a single operon structure in *M. smegmatis*; most likely, the *rv3880c* and *rv3881* genes located to the left also comprise this structure.

Interesting findings were revealed via a comparative analysis of the genes that encode ESAT-6 and CFP-10 proteins in both species. These genes were cloned from the MT genome that followed by their insertion in the genome of the *M. smegmatis* strain with the deletion of orthologous *rv3874* and *rv3875* genes and the secretion of tuberculosis proteins from the cells of the saprophytic strain was obtained (there were doubts whether it became virulent, and the article lacks any data on the necessary experiments for this test). The possibility of complementation between some other cluster gene-orthologs of both species was also testified.

The question arises regarding the functions of a complex of connected genes that regulate secretion in saprophytic *M. smegmatis* bacteria are assumed to be responsible for the virulent properties in a number of pathogenic mycobacteria. Some authors demonstrated that the functions of some proteins of the ESX-1 gene cluster were associated with the conjugated genes transfer in *M. smegmatis*. Hence, the corresponding studies will be summarized below.

7. CONJUGATION IN *M. Smegmatis*

The main conjugation parameters in *M. smegmatis* were described in the studies of Japanese research groups undertaken as early as in the 1970s [74–76] and were later followed by the first series of respective studies conducted by the K. Derbyshire group in the United States [77]. Japanese research groups have studied independently isolated *M. smegmatis* strains, including Rabinovitschi (Rab), Jucho, and PMS; while American researchers studied the fast-growing crossing partner strains MKD6 and MKD8. The strains were cultivated separately on the dense media or filters for 2 days at 30°C and washed; washouts were mixed pairwise and seeded on plates containing dense selective media. Much later, it was reported [78] that conjugation might occur in the case of the growth of long-lasting combined cells in liquid media (without aeration); though not in the plankton part of the culture, but in the air part of the biolayer formed on the surface.

The crossed pairs were selected in order to select colonies of supposed conjugates for markers of antibiotic resistance or for the ability to grow in the absence of amino acid (if auxotrophic strains were used as partners). The combined incubation of the Rab strain and the Jucho and PMS strains (studies cited above) in turn, as well as MKD6 and MKD8 strains [77], on plates (for over 18 h) resulted in the growth of colonies that possessed the hybrid genotype. The transfer of genetic material was only unidirectional, from the donor Rab strain to the recipient strains, i.e., Jucho and PMS (or from MKD6 to MKD8, respectively). The frequency of recombinant colonies was 3×10^{-5} to 5×10^{-4} (recalculated to donor cells), which exceeded the frequency of the corresponding spontaneous mutants by hundreds of times. It was tested whether the transfer of markers was caused due to transformation or transduction (in experiments with DNase addition into the cell mixture or substitution of suspension of donor strain cells to non-cellular filtrate). The results were negative. Moreover, the possibility of the transfer of autonomous plasmids was tested. For this purpose, the donor cells were predominantly transfected by mycobacterial pYUB415 and pLP261 plasmids, which contain genes of hygromycin resistance. It was reported that plasmids were nontransferred with crossing. These experiments also show the inability of hybrid colonies to form due to the simple confluence of cells (not due to the conjugative transfer of chromosomal DNA).

Accordingly, the process of chromosomal markers' transfer resembled conjugation; however, radical differences were observed compared to the classic *E. coli* system (conjugation determined by the insertion of gender plasmid F factor in the donor chromosome). First, the donor strain did not initially contain plasmid or any extrachromosomal element; thus, the treatment of initially with acridine, which eliminates the F factor in *E. coli* did not cause the donor's capacity for genetic material transfer. Second, one part of the transconjugants obtained the capacity for the subsequent transfer of genes into recipient cells, i.e., they became secondary donors. Finally, there was an inability to construct a unified chromosomal map of the donor based on the suggested polarity of marker transfer (although, some marker groups were linked); the impression was given that a transfer might begin from several chromosomal points (and even start simultaneously), rather than from one point. Based on this suggestion, the study [77] tried to detect the maximal size of the transferred chromosomal fragment. For this purpose, the transfer of the IS1096 element was used; this element was present in the donor chromosome in 16 repeats distributed rather uniformly within the chromosome and in one copy in a recipient chromosome. According to the results of Southern blot hybridization with labeled DNA in the IS element, the chromosome of some conjugants comprised even nine copies of IS1096, which corresponded to the transfer of a fragment of more than 350 kb. However, a different interpretation could not be excluded, i.e., the simultaneous transfer of two or more smaller regions that start from various chromosomal points.

The proposition of the approximately equal possibility of the transfer of any region of donor chromosome was confirmed in experiments with transposon inserts (the mariner::km^r transposon was used) [78]. Twenty donor clones were obtained, each of which possessed one insert in a distinct position, but they completely covered the in total. Moreover, all clones

contained a hig^r marker at the same chromosomal position. Subsequent crossing enabled the determination of the frequency of the transfer of single markers, i.e., km^r, hig^r and km^rhig^r, which are characteristic of the donor clone. In turn, the frequency of this marker transfer and the frequency of the combined transfer of km^r and hig^r was independent of the km^r localization and was approximately the same in all donors. Accordingly, each region in the donor chromosome was transferred with an approximately equal probability compared to the polar transfer of genes of the chromosome of *E. coli* Hfr strains.

Attempts to determine whether the chromosome of *M. smegmatis* donor strains possessed any resemblance to the oriT region, which is the starting point of single-stranded DNA replication in the case of *E. coli* conjugation, and is known for their leading role in the entering recipient cell [79–81] were conducted. The common experimental scheme might be described as follows. The recipients carried the sm^r marker (resistance to streptomycin). The library of donor strain fragments was constructed via the cloning of the shuttle pMD30 plasmid derivatives containing the km^r gene. Plasmids that contain these fragments were transfected in the recipient cells via electroporation. The set of transformants that possess the plasmid with the selective km^r marker and the inserted fragment of some region of the donor chromosome were obtained. It was suggested that some transformants would possess the cloned chromosomal region that functionally corresponds to oriT; thus, this plasmid demonstrated the ability to mobilize and were transferred independently of the chromosome in conjugation. Actually, a number of transformant-clones was revealed that were characterized by the formation of sm^rkm^r transconjugants as a result of the following crossing with sm^r recipients. Thus, plasmids that included distinct fragments of the donor-strain chromosome became mobilized. A comparative analysis of the overlapping sequenced regions of chromosomal DNA inserted into mobilized plasmids made it possible to detect some common sequence and restrict it to 5 kb. These regions that mobilize plasmid transfer were called bom regions (basis of mobility). If a bom region was subsequently inserted into the initial pMD30 plasmid, it obtained the capacity for conjugative mobilization. Most likely, the transfer of chromosomal fragments during conjugation also started from bom regions.

Actually, the function of bom regions was similar to that of oriT in *E. coli*. However, there was no other similarity. *M. smegmatis* chromosome possessed more than 24 bom regions compared to oriT; they were significantly longer than oriT (several kb compared to the small oriT 200 bp in size).

A detailed description of the mechanisms of interaction and insertion of the donor chromosome with bom regions into the recipient genome was not summarized in the present review; the corresponding hypothesis were reported in studies [78–81]. However, experiments that involve insertion mutagenesis revealed surprising data that serve as evidence of the direct relation of the mechanisms of the conjugation and secretion of ESAT-6 proteins in *M. smegmatis* [82, 83].

About 5300 clones that contain insertions in various chromosomal regions were isolated in the recipient strain [83]; 49 of them demonstrated significantly reduced conjugation frequency. Forty percent of inserts were observed in the ESX-1 region in these clones; the remaining inserts were located in other chromosomal regions (in particular, five insertions neighbored ESX-1 from the left side, closer to the starting point of replication). 20000 clones that contain transposon insertions were isolated from the donor strain [82]. Only 20 inserts influenced the conjugation frequency, but 14 of them were located in the ESX-1 region, distinctly in *M. smegmatis* genes homologous to the following MT genes: *rv3868*, *3869*, *3871*, *3872*, *3873*, *3874* (i.e. in genes that encode CFP-10 protein), *3879*, *3881c* (some genes contained two insertions). Moreover, only clones that contain inserts in genes located on the right flank of the region and were observed in *M. smegmatis* were revealed (see above). Complementation to the phenotype modifications caused by inserts that correspond to tuberculosis genes cloned in plasmids was observed. However, the main puzzle included that insertions of the ESX-1 region in the same genes in the recipient and donor resulted in differences in transconjugants release: they decreased conjugation frequency in recipient as was mentioned above and rapidly increased it in donor (sometimes, thousands of times with the formation of superdonors from respective strains). The action of these insertions was present *in trans*, since the simultaneous presence of donor cells of the initial genotype in the conjugation mixture reduced the superdonor properties of mutants [82]. The explanation for the diametrically opposite influence of mutations in the ESX-1 region on the functioning of donor and recipient cells remains unsatisfactory, while one of the suggestions proposed that the secretion product of proteins of the ESX-1 region (probably ESAT-6) played a role in the negative regulator of conjugation by hiding donor cells and inhibiting their contact (until the beginning of the distinct phase of culture growth) with recipient cells [82, 83]. As was mentioned above, long-lasting (compared to *E. coli*) contact of partner cells was necessary for conjugation in *M. smegmatis*.

The implementation of this complex process as conjugation in *M. smegmatis* is undoubtedly influenced by the significant number of genes located in both the ESX-1 region and other chromosomal regions. Some of these genes have been already studied [78, 83, 84]. However, the involvement of small, ESAT-6-like proteins in the conjucation of the secretion system conducted by a cluster of ESX-1 genes can be confidently proposed even now; these genes are homologous in a number of virulent mycobacterium and in *M. smegmatis* (secretion systems of VII type, see

above). Most likely, the existing secretion systems present in bacterial cells,ay be adapted due to comparatively small modifications to the performance of various functions, such as the horizontal transfer of genetic material and the overcoming of defense mechanisms in the host organism. A similar situation was described previously in a number of microorganisms with type-IV secretion (see review [85, 86]). In the case of system IV, even the existence of specific structures responsible for both DNA transfer and the injection of toxin proteins into the microorganism cell was reported. These structures are unknown in mycobacteria; it might only be suggested that the secretion proteins type VII provide the close contact between conjugating cells and DNA transfer through the formed pore-channel between them. Moreover, conjugation was only observed in *M. smegmatis* compared to other mycobacteria. In summary, the coincidence of functions and substitution characteristic of the same morphological structure or organ is a common biological observation. Examples might be found in various living organisms. Thus, the progressive movement is conducted via the flagellum, projecting the cell in the majority of bacteria, whereas in spirochetes, contractive axial fibrils (similar to flagellum in the structure and content but residing inside the cell) are responsible for this function. A corresponding example from the comparative anatomy of vertebrates is that the wing transformation in the organ resemble penguin fins.

CONCLUSIONS

Tuberculosis causative agents can coexist with the host organism for many years, even ad vitam and, compared to other pathogenic bacteria, have been shown to be a part of microbiota. This is probably the cause of the diversity of virulence factors in MT and their adaptation to various stages of coexistence. The present review described the properties of small, secreted proteins characterized by mycobacterial defense from lysis in macrophages and their release into the surrounding tissue as a main function. However, there are a number of other factors responsible for reproduction, or even for the long-term existence of MT after infection, in particular, lipids and some proteins of the cell wall [6], including catalase-peroxidase [87–90], some protein kinases [91], and toxin–antitoxin systems [39]. An attempt to identify the genes that affect the number of viable bacteria of the H37Rv strain in spleen tissue for 1 month after mouse infection (this parameter was assumed to be a conditional measure of virulence) was undertaken in the study [91]. The suspension of the spleen tissue was inoculated on nutrition media after different lengths of time following infection and the number of grown colonies was calculated. Strains from the giant collection (about 200 000) of clones obtained via blind transposon mutagenesis were used for the infection. Clones containing insertions associated with reduced bacterial viability, which were predominantly demonstrated in vitro without animal infection, were discarded. Genes containing mutations that result in a decreased number of bacteria in passages were assumed to be involved in MT virulence. A total of 194 of these clones have been detected, 107 of which contain insertions in genes with the predicted functions. These functions were related to various sides of bacterial viability (lipids and carbohydrates metabolism, protein secretion, aminoacid metabolism and transport, etc.; the clones containing insertions in the ESX-1 gene cluster and, in particular, genes of the ESP system). Functions of 107 genes containing insertions proposed to affect virulence remained unidentified. Interestingly, the curves corresponding to the slope and rise in the number of vital mycobacterium in the spleen differed based on the time that passed after infection in various mutants. Most likely, different gene groups with various functions were expressed in virulent mycobacteria at different stages of host infection [92, 93] and different virulence factors were involved.

During recent years, tuberculosis infection caused by strains characterized by increased virulence, e.g., by strains of the so-called Beijing genotype [3, 4], have attracted increasing attention. It is possible that one of the mechanisms of the formation of a strain with increased virulence might include the presence of a mutation in the ESX-system, which modifies the intensity of the secretion of virulence proteins.

The majority of studies that involve pathogenic mycobacteria are directly or indirectly related to medicinal needs, e.g., disease diagnostics; vaccine development; and, in particular, the design of drugs with new mechanisms of action directed at new biotargets. Since the 1940s, antituberculosis preparations have included a number of antibiotics characterized by bacteriostatic or bactericidal action that demonstrated good results in tuberculosis treatment [87, 89, 94]. However, usually, after some periods of application, antibiotics become ineffective due to the formation and accumulation of drug resistant mutants. The schemes of treatment based on the the alternation of various combinations of existing preparations demonstrated incomplete success due to the formation of mutants with multiple drug resistance. The search for the new antituberculosis preparations remains of vital importance. This goal is also complicated by the absence of any free place in MT, since the action of antituberculosis preparations in summary affects various sides of bacterial metabolism [88, 89, 94]. Hence, a new conceptual approach to pharmaceutical tuberculosis therapy is urgent, including the search for pharmaceutical preparations with new biotargets of action; we assume that, in particular, factors of bacterial virulence might serve as these biotargets, e.g., the synthesis and/or secretion of ESAT-6 and CFP-10 proteins. The application of these preparations might result in reduced MT virulence and availability of

mycobacteria for digestion in macrophages (in an ideal case, it results in at least the phenotypical transformation of virulent mycobacteria in the BCG-like strain). Similar suggestions have already been made for the therapy of diseases caused by bacteria with secretion systems II, III, and IV [95].

Certainly, simultaneous application of antibiotics is presumed. The study of conjugation mechanisms in *M. smegmatis* demonstrated that conjugation system involved genes that encode proteins homologous to the proteins of ESX-1-system (in particular, the protein with a high level of resemblance (72%) toward ESAT-6 protein of *M. tuberculosis* [74]). This makes it possible to design a simple test-system based on the conjugation system of *M. smegmatis* for selecting substances that inhibit the synthesis and secretion of ESAT-6 protein. These substances can be detected with respect to their ability to suppress conjugation in an innocuous, fast-growing *M. smegmatis* microorganism unaffected by inoculation conditions. The obtained inhibitors of conjugation might be subjected to testing as inhibitors of the ESX secretion system in *M. tuberculosis*. It is feasible to select from among these inhibitors for antituberculosis preparations.

ACKNOWLEDGMENTS

We are grateful to A.N. Kuz'mina and I.K. Lubimova for their help in searching the literature and preparing the manuscript.

This study was supported by the Russian Foundation for Basic Research (project no. OFI-M 11-04-12026 "Development of New Test System for Screening Antituberculosis Preparations on *Mycobacterium smegmatis* As Model Object").

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