



IDENTIFICATION OF SECRETION SYSTEM AND PHYLOGENETIC ANALYSIS OF MYCOBACTERIUM ON THE BASIS SS GENES

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ABSTRACT

Pathogenic bacteria depend on secretory pathways for the performance of virulence functions and one of the most important bacteria is mycobacterium. Recently, a novel type of secretory pathway, type VII secretion systems (T7SSs), has been characterized in mycobacteria. The chromosomes of *Mycobacterium tuberculosis* and *Mycobacterium bovis* encode five T7SS's (ESX-1 to ESX-5). The best characterized of them, ESX-1, is involved in host-pathogen interactions and its deletion is one of the main causes of *M. bovis* BCG attenuation. Type 7 secretion system is reported to be found in mycobacterial species. This study is mainly conducted to identify and detect the presence of secretion system gene clusters in Mycobacterial species and to check the closeness and relativity among different species on the basis of secretory pathway present in them.

KEYWORDS: T7SS's (ESX-1 to ESX-5), *M. bovis* BCG.

INTRODUCTION

Many pathogenic bacteria rely on specialized protein secretion pathways to secrete effector proteins that are important for virulence (Finlay and Falkow, 1997; Thanassi and Hultgren, 2000; Lee and Schneewind, 2001). An important group of pathogens that have only recently been shown to secrete virulence factors are the mycobacteria (DiGiuseppe Champion and Cox, 2007). This genus includes the fish pathogen *Mycobacterium marinum* and *Mycobacterium tuberculosis*, which is, with an estimate of 2 million deaths per year, the most deadly bacterial pathogen worldwide. Secreted proteins are among the most important molecules involved in host-pathogen interaction of *Mycobacterium tuberculosis*, the etiological agent of human tuberculosis (TB). ESX-1 of *M. tuberculosis* and related tubercle bacilli is the ESX/T7S system that has probably attracted the most attention in recent years. Located in the region of difference 1 (RD1), ESX-1 is deleted in the vaccine strain *Mycobacterium bovis* BCG and other attenuated members of the *M. tuberculosis* complex like the vole bacillus *Mycobacterium microti*, suggesting that secretion of ESAT-6 is involved in virulence of tubercle bacilli. The ESX-1/T7S system effector proteins involved in host-pathogen interaction, although several features related to pathogenicity were found to be associated with ESX-1, the primary biological function of this system in *M. tuberculosis* and other ESX-1 harboring bacteria still remains unclear. The first T7S system to be discovered was the ESX-1 system in

members of the *M. tuberculosis* complex. This system was shown to be responsible for the secretion of two small proteins that were among the major culture filtrate proteins. These two secreted proteins are now known as EsxA and EsxB, but are perhaps better known by their initially designated names, ESAT-6 and CFP-10, respectively. The ESX-1 system quickly became one of the major topics in tuberculosis research when it was discovered that ESX-1 is a major virulence factor and that the absence of this system is responsible for the attenuation of the live-vaccine strain *Mycobacterium bovis* BCG. During prolonged *in vitro* growth this attenuated strain incurred the so-called RD1 deletion, which removes a major part of the ESX-1 locus. The role of the ESX-1 system in virulence is diverse and complex and has recently been reviewed elsewhere. This study mainly focuses on the identification of secretory pathways present in Mycobacterial species actively playing their part in virulence and translocation of substrates across the cytosolic membrane. To check the closeness and association among the different species of Mycobacterium a phylogenetic analysis is being conducted in this study.

KEGG Analysis

Kyoto encyclopedia of genes and genomes (KEGG) is database used to investigate the secretion system and interactive pathways for T7SS in *Mycobacterium*. The KEGG, the Kyoto Encyclopedia of Genes and Genomes, was initiated by the Japanese human genome programme

in 1995. According to the developers they consider KEGG to be a "computer representation" of the biological systems. The KEGG database can be utilized for modelling and simulation, browsing and retrieval of data. It is a part of the systems biology approach.

Analysis of all the genome sequences of mycobacterium available at Kyoto encyclopedia of genes and genomes (KEGG) database is conducted and crosschecking of all the species to investigate whether there is presence of any other secretory pathway or not. It is also used to check that which proteins of T7SS are involved in secretory pathway of Mycobacterium.

MATERIALS AND METHODS

BlastN by using BIOEDIT

- 16s rRNA sequences of mycobacterium were downloaded from NCBI.
 - After downloading the sequences were aligned by using BIOEDIT tool.
 - After the completion of alignment they were saved in FASTA format.
 - Local blast was performed with BIOEDIT
1. Several modes of hand alignment

2. Automated ClustalW alignment
3. Automated Blast searches (local and WWW)

Identification & detection of SS genes clusters in MYCOBACTERIUM

Representatives of the secretion system genes and core components recently defined by Boyer and co-workers were used as baits to identify SS loci by sequential BLASTN, BLASTP and TBLASTX searches using all publicly available sequences from 195 *Mycobacterium* genome sequencing projects (completed or in progress). The data analysed included both chromosome and plasmid sequences. Overall, in our results we determined that type 7 secretion system was widely distributed in Mycobacterium as shown in Table 1. No presence of any other type of secretion system was detected in Mycobacterium. Research on type VII secretion system has recently become a large and competitive research topic that is tightly linked to studies of host-pathogen interactions of pathogenic mycobacteria. Insights into this matter is of utmost importance for the improvement of current prevention strategies, diagnostics and therapy of tuberculosis as well as for a better understanding of the virulence mechanisms employed.

Table NO. 1: Table above shows the presence of T7SS components in different species of *Mycobacterium*.

Genome Name	T7SS clusters
Mycobacterium abscessus	3
Mycobacterium abscessus 3A-0119-R	3
Mycobacterium abscessus 3A-0122-R	3
Mycobacterium abscessus 3A-0122-S	3
Mycobacterium abscessus 3A-0731	3
Mycobacterium abscessus 3A-0810-R	3
Mycobacterium abscessus 3A-0930-R	3
Mycobacterium abscessus 3A-0930-S	3
Mycobacterium abscessus 47J26	3
Mycobacterium abscessus 4S-0116-R	2
Mycobacterium abscessus 4S-0116-S	2
Mycobacterium abscessus 4S-0206	2
Mycobacterium abscessus 4S-0303	2
Mycobacterium abscessus 4S-0726-RA	2
Mycobacterium abscessus 4S-0726-RB	2
Mycobacterium abscessus 5S-0304	5
Mycobacterium abscessus 5S-0421	5
Mycobacterium abscessus 5S-0422	4
Mycobacterium abscessus 5S-0708	4
Mycobacterium abscessus 5S-0817	5
Mycobacterium abscessus 5S-0921	4
Mycobacterium abscessus 5S-1212	4
Mycobacterium abscessus 5S-1215	5
Mycobacterium abscessus 6G-0125-R	3
Mycobacterium abscessus 6G-0125-S	3
Mycobacterium abscessus 6G-0212	3
Mycobacterium abscessus 6G-0728-R	3
Mycobacterium abscessus 6G-0728-S	3
Mycobacterium abscessus 6G-1108	3
Mycobacterium abscessus M115	3
Mycobacterium abscessus M139	2

Mycobacterium abscessus M148	5
Mycobacterium abscessus M152	2
Mycobacterium abscessus M154	2
Mycobacterium abscessus M156	3
Mycobacterium abscessus M159	3
Mycobacterium abscessus M172	3
Mycobacterium abscessus M24	3
Mycobacterium abscessus M93	3
Mycobacterium abscessus M94	3
Mycobacterium abscessus subsp. bolletii BD	3
Mycobacterium africanum GM041182	11
Mycobacterium avium	
Mycobacterium avium 104	10
Mycobacterium avium subsp. avium ATCC 25291	10
Mycobacterium avium subsp. avium DT 78	9
Mycobacterium avium subsp. avium Env 77	9
Mycobacterium avium subsp. paratuberculosis 1281	9
Mycobacterium avium subsp. paratuberculosis 4B	10
Mycobacterium avium subsp. paratuberculosis ATCC 19698	9
Mycobacterium avium subsp. paratuberculosis CLIJ361	9
Mycobacterium avium subsp. paratuberculosis CLIJ623	9
Mycobacterium avium subsp. paratuberculosis CLIJ644	10
Mycobacterium avium subsp. paratuberculosis DT 3	1
Mycobacterium avium subsp. paratuberculosis Env 210	10
Mycobacterium avium subsp. paratuberculosis JQ5	12
Mycobacterium avium subsp. paratuberculosis JQ6	10
Mycobacterium avium subsp. paratuberculosis JTC 1285	9
Mycobacterium avium subsp. paratuberculosis K-10	10
Mycobacterium avium subsp. paratuberculosis Pt139	9
Mycobacterium avium subsp. paratuberculosis Pt144	10
Mycobacterium avium subsp. paratuberculosis Pt145	10
Mycobacterium avium subsp. paratuberculosis Pt146	9
Mycobacterium avium subsp. paratuberculosis Pt154	9
Mycobacterium avium subsp. paratuberculosis Pt155	9
Mycobacterium avium subsp. paratuberculosis Pt164	9
Mycobacterium avium subsp. paratuberculosis S397	10
Mycobacterium avium subsp. paratuberculosis S5	10
Mycobacterium bovis AF2122/97	10
Mycobacterium bovis BCG str. ATCC 35733	10
Mycobacterium bovis BCG str. ATCC 35740	10
Mycobacterium bovis BCG str. ATCC 35743	10
Mycobacterium bovis BCG str. China	10
Mycobacterium bovis BCG str. Mexico	10
Mycobacterium bovis BCG str. Pasteur 1173P2	10
Mycobacterium bovis BCG str. Tokyo 172	10
Mycobacterium canettii CIPT 140010059	10
Mycobacterium canettii CIPT 140060008	10
Mycobacterium canettii CIPT 140070008	10
Mycobacterium canettii CIPT 140070010	10
Mycobacterium canettii CIPT 140070017	10
Mycobacterium chubuense NBB4	1
Mycobacterium colombiense CECT 3035	10
Mycobacterium fortuitum subsp. fortuitum DSM 46621	10
Mycobacterium gilvum PYR-GCK	4
Mycobacterium gilvum Spyr1	4
Mycobacterium hassiacum DSM 44199	3
Mycobacterium indicus pranii MTCC 9506	10
Mycobacterium intracellulare ATCC 13950	10

Mycobacterium intracellulare M.i.198	11
Mycobacterium intracellulare MOTT-02	10
Mycobacterium intracellulare MOTT-64	10
Mycobacterium kansasii ATCC 12478	10
Mycobacterium leprae Br4923	7
Mycobacterium leprae TN	7
Mycobacterium liflandii 128FXT	11
Mycobacterium mageritense JR2009	3
Mycobacterium marinum M	9
Mycobacterium massiliense 1S-151-0930	3
Mycobacterium massiliense 1S-152-0914	3
Mycobacterium massiliense 1S-153-0915	3
Mycobacterium massiliense 1S-154-0310	3
Mycobacterium massiliense 2B-0107	4
Mycobacterium massiliense 2B-0307	3
Mycobacterium massiliense 2B-0626	3
Mycobacterium massiliense 2B-0912-R	3
Mycobacterium massiliense 2B-0912-S	3
Mycobacterium massiliense 2B-1231	3
Mycobacterium massiliense CCUG 48898 = JCM 15300	3
Mycobacterium massiliense M18	4
Mycobacterium massiliense str. GO 06	3
Mycobacterium parascrofulaceum ATCC BAA-614	4
Mycobacterium phlei RIVM601174	3
Mycobacterium rhodesiae JS60	5
Mycobacterium rhodesiae NBB3	3
Mycobacterium smegmatis JS623	1
Mycobacterium smegmatis str. MC2 155	4
Mycobacterium sp. JDM601	9
Mycobacterium sp. JLS	4
Mycobacterium sp. KMS	4
Mycobacterium sp. MCS	4
Mycobacterium sp. MOTT36Y	10
Mycobacterium sp. VKM Ac-1815D	3
Mycobacterium thermoresistibile ATCC 19527	4
Mycobacterium tuberculosis	11
Mycobacterium tuberculosis '98-R604 INH-RIF-EM'	11
Mycobacterium tuberculosis 02_1987	1
Mycobacterium tuberculosis 210	10
Mycobacterium tuberculosis 94_M4241A	12
Mycobacterium tuberculosis BTB05-552	10
Mycobacterium tuberculosis BTB05-559	10
Mycobacterium tuberculosis C	10
Mycobacterium tuberculosis CCDC5079	11
Mycobacterium tuberculosis CCDC5180	10
Mycobacterium tuberculosis CDC1551	11
Mycobacterium tuberculosis CDC1551A	11
Mycobacterium tuberculosis CPHL_A	10
Mycobacterium tuberculosis CTRI-2	10
Mycobacterium tuberculosis CTRI-4	10
Mycobacterium tuberculosis EAS054	12
Mycobacterium tuberculosis GM 1503	11
Mycobacterium tuberculosis H37Ra	10
Mycobacterium tuberculosis H37Ra [WGS]	12
Mycobacterium tuberculosis H37Rv	11
Mycobacterium tuberculosis H37Rv (Broad)	11
Mycobacterium tuberculosis H37RvAE	10
Mycobacterium tuberculosis H37RvCO	11

Mycobacterium tuberculosis H37RvHA	10
Mycobacterium tuberculosis H37RvJO	11
Mycobacterium tuberculosis H37RvLP	10
Mycobacterium tuberculosis H37RvMA	10
Mycobacterium tuberculosis HN878	10
Mycobacterium tuberculosis K85	
Mycobacterium tuberculosis KZN 1435	10
Mycobacterium tuberculosis KZN 4207	10
Mycobacterium tuberculosis KZN 4207 (Broad)	10
Mycobacterium tuberculosis KZN 605	10
Mycobacterium tuberculosis KZN R506	10
Mycobacterium tuberculosis KZN V2475	10
Mycobacterium tuberculosis NA-A0008	12
Mycobacterium tuberculosis NA-A0009	11
Mycobacterium tuberculosis NCGM2209	10
Mycobacterium tuberculosis OSDD071	10
Mycobacterium tuberculosis OSDD504	10
Mycobacterium tuberculosis OSDD518	10
Mycobacterium tuberculosis F11	1
Mycobacterium tuberculosis RGTB327	14
Mycobacterium tuberculosis RGTB423	12
Mycobacterium tuberculosis S96-129	10
Mycobacterium tuberculosis SUMu001	10
Mycobacterium tuberculosis SUMu002	10
Mycobacterium tuberculosis SUMu003	10
Mycobacterium tuberculosis SUMu004	10
Mycobacterium tuberculosis SUMu005	11
Mycobacterium tuberculosis SUMu006	10
Mycobacterium tuberculosis SUMu007	10
Mycobacterium tuberculosis SUMu008	10
Mycobacterium tuberculosis SUMu009	11
Mycobacterium tuberculosis SUMu010	9
Mycobacterium tuberculosis SUMu011	11
Mycobacterium tuberculosis SUMu012	11
Mycobacterium tuberculosis T17	10
Mycobacterium tuberculosis T46	10
Mycobacterium tuberculosis T85	10
Mycobacterium tuberculosis T92	10
Mycobacterium tuberculosis UM 1072388579	10
Mycobacterium tuberculosis UT205	11
Mycobacterium tuberculosis W-148	10
Mycobacterium tuberculosis X122	10
Mycobacterium tuberculosis str. Erdman = ATCC 35801	11
Mycobacterium tuberculosis str. Haarlem	11
Mycobacterium tusciae JS617	3
Mycobacterium ulcerans Agy99	0
Mycobacterium vaccae ATCC 25954	5
Mycobacterium vanbaalenii PYR-1	4
Mycobacterium xenopi RIVM700367	10

B173		f_s	10
	A	B	
167	Mycobacterium tuberculosis OSDD504		10
168	Mycobacterium tuberculosis OSDD518		10
169	Mycobacterium tuberculosis F11		1
170	Mycobacterium tuberculosis RGTB327		14
171	Mycobacterium tuberculosis RGTB423		12
172	Mycobacterium tuberculosis S96-129		10
173	Mycobacterium tuberculosis SUMu001		10
174	Mycobacterium tuberculosis SUMu002		10

The snapshot above shows the two highlighted species of *Mycobacterium*. The one which is highlighted yellow named *Mycobacterium Tuberculosis F11* has the least number of T7SS components present in it. Therefore it causes less pathogenicity and virulence whereas on the other hand the 2nd species of *Mycobacterium* highlighted in red color named *Mycobacterium Tuberculosis RGTB327* with the highest number of T7SS components present in it.

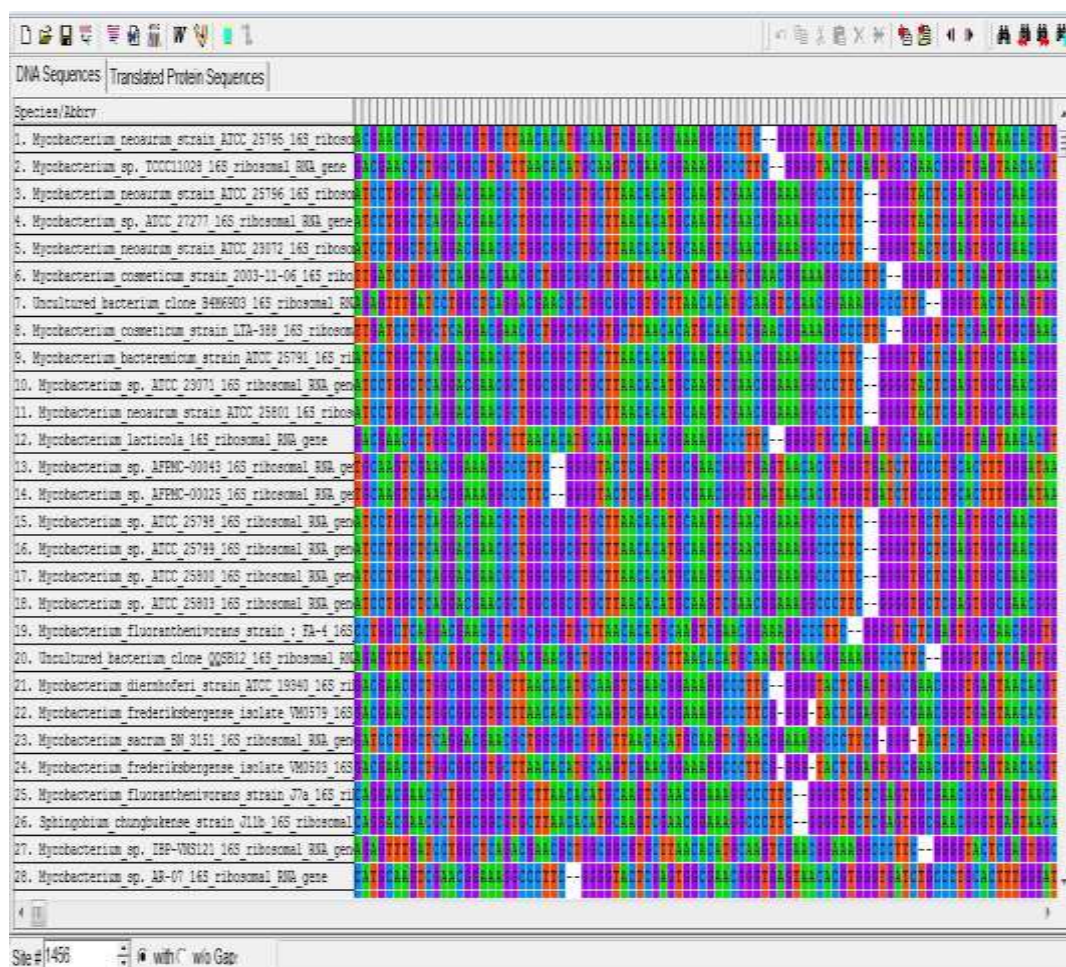
RESULTS AND DISCUSSION

Phylogenetic analysis of T7SS components and 16SrRNA

Phylogenetic analysis is conducted using the biological software Molecular Evolutionary Genetic Analysis which is abbreviated as (MEGA) and we used the 5th version of this software based on the approach described by Bingle et al. (2008). In case of T7SS loci amino acids sequences were aligned using ClustalW (Thompson et al., 1994). Similarly, to prove the ancestry of the T7SS and to understand their evolutionary pattern, 16S rRNA sequences of some selected species corresponding to species used by Bingle et al. (2008) were downloaded from RNA database **NCBI**. After that 16S phylogenetic tree of the selected species of *Mycobacterium* was constructed using MEGA version 5. (Thompson et al.,

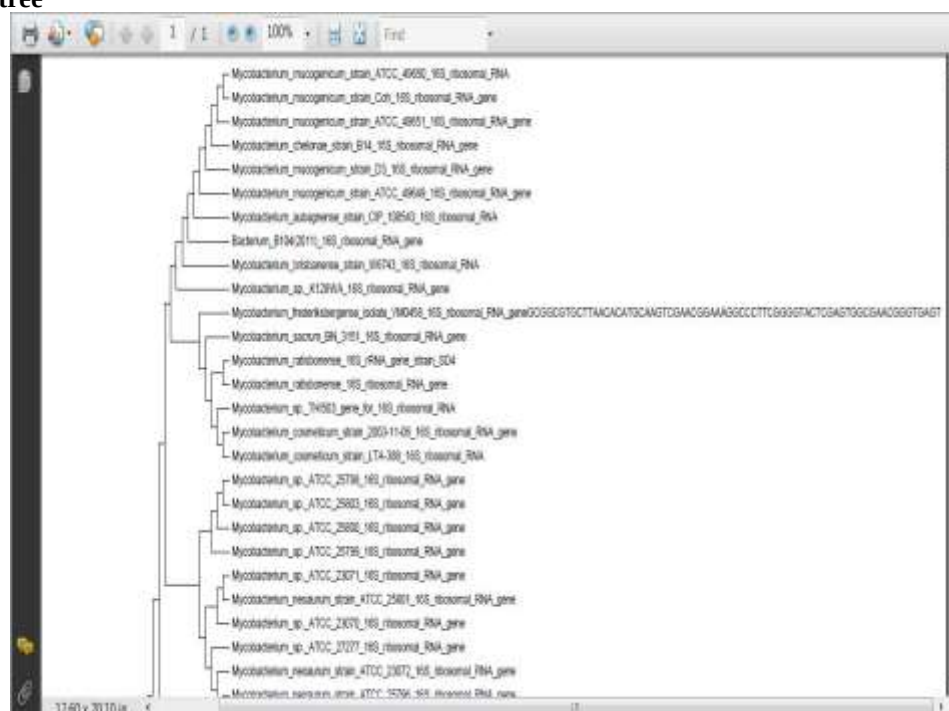
1994). The steps followed for phylogenetic study were as under:

- 16s rRNA sequences of some of the selected species of mycobacterium were downloaded from NCBI (**NATIONAL CENTER FOR BIOINFORMATICS**).
- Then the sequences were aligned by using ClustalW (Thompson et al., 1994) with default parameters.
- Pairwise alignment and multiple sequence alignment were carried out.
- After the alignment is done phylogenetic tree is being constructed.



- The snapshot shown above shows the sequences with multiple sequence alignment and pairwise alignment by using MEGA version 5 by ClustalW.
- It is the final sequence alignment after removing gaps and spaces within the sequences.

Phylogenetic tree



The above snapshot is of phylogenetic tree being derived from the sequences of mycobacterial species. The tree shows closeness and relativity among the different species of mycobacterium on the basis of secretory pathway similarities and evolutionary relationship among different species.

- First of all feature tables were downloaded from **Patho Systems Resource Integration Center (Patric)**.
- These feature tables contains the genomic data of all the species of mycobacterium. After downloading, the feature tables have been arranged according to the requirement and the undesired information was deleted from these tables.
- Then T7SS components were detected in all species of Mycobacterium.
- The species in which there is more T7SS genes present in cluster form cause more virulence and pathogenicity as compared to those who have less number of T7SS present in them shows less virulence and pathogenicity.
- The tree above shown in results show the phylogenetic analysis.
- The tree is formed on the basis of clusters of secretory components present in them.
- The species which are closely related on the basis of secretory genes are clustered together in the tree.
- They have same number of secretory genes present in them and those which are situated far in the phylogenetic tree have different number of secretory proteins in them.
- The secretory proteins present in bacteria helps them in survival within the environment also helps in adaptation within different environment.
- The secretion system also assists in adherence, virulence and pathogenicity.
- As we know that the secretion system functions mainly in pathogenicity and virulence.
- After knowing the genes encoding T7SS we can alter them in laboratory and can manipulate the information within these genes and we can use this information in pharmaceutical and in drug designing for the benefit of mankind.

REFERENCES

1. Abdallah A, Gey van Pittius N, Champion P, Cox J, Luirink J, Vandenbroucke-Grauls C, Appelmek B, Bitter W: Type VII secretion-mycobacteria show the way. *Nat Rev Microbiol*, 2007; 5: 883-891. Very complete and comprehensive overview of the ESX-related features in a large range of actinobacteria.
2. Brodin P, Rosenkrands I, Andersen P, Cole ST, Brosch R ESAT-6 proteins: Protective antigens and virulence factors? *Trends Microbiol*, 2004; 12: 500-508.
3. Coros A, Callahan B, Battaglioli E, Derbyshire KM: The specialized secretory apparatus ESX-1 is essential for DNA transfer in *Mycobacterium smegmatis*. *Mol Microbiol*, 2008; 69: 794-808
4. DiGiuseppe Champion, P.A., and Cox, J.S. Protein secretion systems in Mycobacteria. *Cell Microbiol*, 2007; 9: 1376- 1384.
5. Finlay, B.B. and Falkow, S. Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev*, 1997; 61: 136-169.
6. Guinn KM, Hickey MJ, Mathur SK, Zakel KL, Grotzke JE, et al. Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*. *Mol Microbiol*, 2004; 51: 359-370.
7. Gey van Pittius NC, Sampson SL, Lee H, Kim Y, van Helden PD, Warren RM: Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (*esx*) gene cluster regions. *BMC Evol Biol*, 2006; 6: 95.
8. This paper nicely shows evidence that PE and PPE proteins have coevolved and have preceded the PE-PGRS and PPE-MPTR proteins.
9. Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, et al. The primary mechanism of attenuation of *Bacillus Calmette-Guerin* is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc Natl Acad Sci USA*, 2003; 100: 12420-12425.
10. Lee, V.T. and Schneewind, O. Protein secretion and the pathogenesis of bacterial infections. *Genes Dev*, 2001; 15: 1725-1752.
11. Pallen MJ: The ESAT-6/WXG100 superfamily—and a new Gram-positive secretion system? *Trends Microbiol*, 2002; 10: 209-212.
12. R. Simeone, D. Bottai, R. Brosch ESX/type VII secretion systems and their role in host-pathogen interaction *Curr. Opin. Microbiol.*, 2009; 12: 4-10.
13. Stanley SA, Raghavan S, Hwang WW, Cox JS Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. *Proc Natl Acad Sci USA*, 2003; 100: 13001-13006.
14. Thanassi, D.G. and Hultgren, S.J. Multiple pathways allow protein secretion across the bacterial outer membrane. *Curr Opin Cell Biol*, 2000; 12: 420-430.
15. Way SS, Wilson CB: The *Mycobacterium tuberculosis* ESAT-6 homologue in *Listeria monocytogenes* is dispensable for growth in vitro and in vivo. *Infect Immun*, 2005; 73: 6151-6153.