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Bordetella infections in animals and the role of type III secretion system in their virulence

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Declaration

I hereby declare that I have done this thesis entitled *Bordetella* infections in animals and the role of type III secretion system in their virulence independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In.................................

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Darya Kuzmenko

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Abstract

This thesis investigates problematics of animal infections caused by genus *Bordetella* with special emphasis on members encoding type III secretion system (T3SS). Virulence of numerous pathogenic bacteria utilizes T3SS in terms of colonization, multiplication and persistence in the host. *B. bronchiseptica* has been known as an animal pathogen, precisely as a mammalian respiratory disease causative agent, since 1910. Over the second half of the 20th century, a lot of research was done on aetiology of the disease. An integrated prevention strategy, including vaccination and a set of zootechnical measures, was proposed. However, reports suggest that the bacteria still impose adverse infectious pressure on domestic animals. The goal of literature review was to inspect the field of contention and determine the T3SS role in veterinary infections provoked by *Bordetella* species. We begin with a brief portrayal of *Bordetella* genus and genus-associated diseases. It is followed by an assessment of the T3S apparatus general design, function and the way it enables different bacteria to establish infection. Finally, we summarise our current knowledge of the T3SS in *Bordetella* representatives. Additionally, the thesis includes a practical part which is divided into two sections. The first section aimed to discover so far unknown *Bordetella* T3SS substrates. By means of mass spectrometry analysis and comparison of secretion profile of wild type and T3SS-deficient Δ*bscN* mutant bacteria, we identified 14 proteins plausibly being secreted via the T3SS of *B. bronchiseptica* D445 (MO211). They were examined for presence of homologies and matched to the relevant loci in *B. bronchiseptica* D445 (MO211) genome. In the next section, we analysed and annotated *B. bronchiseptica* D445 (MO211) T3SS locus in agreement with generic standard rational nomenclature. The most intriguing outcome of this thesis is the detection of a small protein secreted in T3S-dependent manner and encoded within the T3SS locus. The protein was found to show sequence homology to members of YscX superfamily and thus might be hypothesised to play role in the process of the injectisome assembly.

Key words: *Bordetella bronchiseptica*, type III secretion system, mass spectrometry, genome annotation

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List of the abbreviations used in the thesis

CBD – chaperone binding domain

CIRD – canine infectious respiratory disease

e.g. – exempli gratia; for example

LRT – lipid raft targeting

NC – needle complex

spp. – species

SNP – single nucleotide polymorphism

SPI – Salmonella Pathogenicity Island

T3SS – type III secretion system

URD – upper respiratory disease

URI – upper respiratory tract infection

1. Literature Review

1.1. *Bordetella* genus

1.1.1. Members of the genus

Taxonomically, genus *Bordetella* belongs to the family Alcaligenaceae, from the class β-proteobacteria of the phylum Proteobacteria, which harbours gram-negative, aerobic, asporogenous rods recovered from a variety of habitats [1]. The genus comprises nine species. Three of them, also called "classical" species, are well-known human and animal respiratory pathogens: *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*. Remaining six, *B. trematum, B. holmezii*, *B. avium*, *B. hinzii*, *B. petrii* and *B. ansorpii*, are more distantly related and less actively studied. Some of them have been isolated from the environment, whereas others have been documented to cause infections in immunocompromised humans and poultry [2-5].

One of the latest phylogenetic studies describes genus *Bordetella* being divided into three distinct clades on the basis of genome SNPs analysis (**Fig. 1**). Clade C includes *B. petrii* and *B. ansorpii*. These two species are assumed to have branched off first and thus to be the most distantly related to the others. It seems that, primarily, *B. petrii* and *B. ansorpii* might be environmental bacteria although with the potential to infect immunodeficient patients. In clade B, where *B. holmesii*, *B. hinzii*, *B. avium* and *B. trematum* belong, considerable diversity among the species is observed. On the other hand, genomes of "classical" *Bordetella* from clade A appear to be quite closely related. The latter clade was studied the most thoroughly due to notorious nature of its representatives as animal and human pathogens. Beyond doubt, *B. pertussis* deserves special attention for being the primary cause of infant death in pre-vaccination era together with current re-emergence of whooping cough in Western countries [6]. It was well-documented that the clade A species share plenty of similarities in relation to how their genomes are organized. On that basis, *B. pertussis* and *B. parapertussis* are widely believed to evolve from *B. bronchiseptica*-like ancestor. Correspondingly, experimental

data acquired from *B. bronchiseptica* could be relevant as well as reasonably applicable to other classical bordetellae [7, 8].

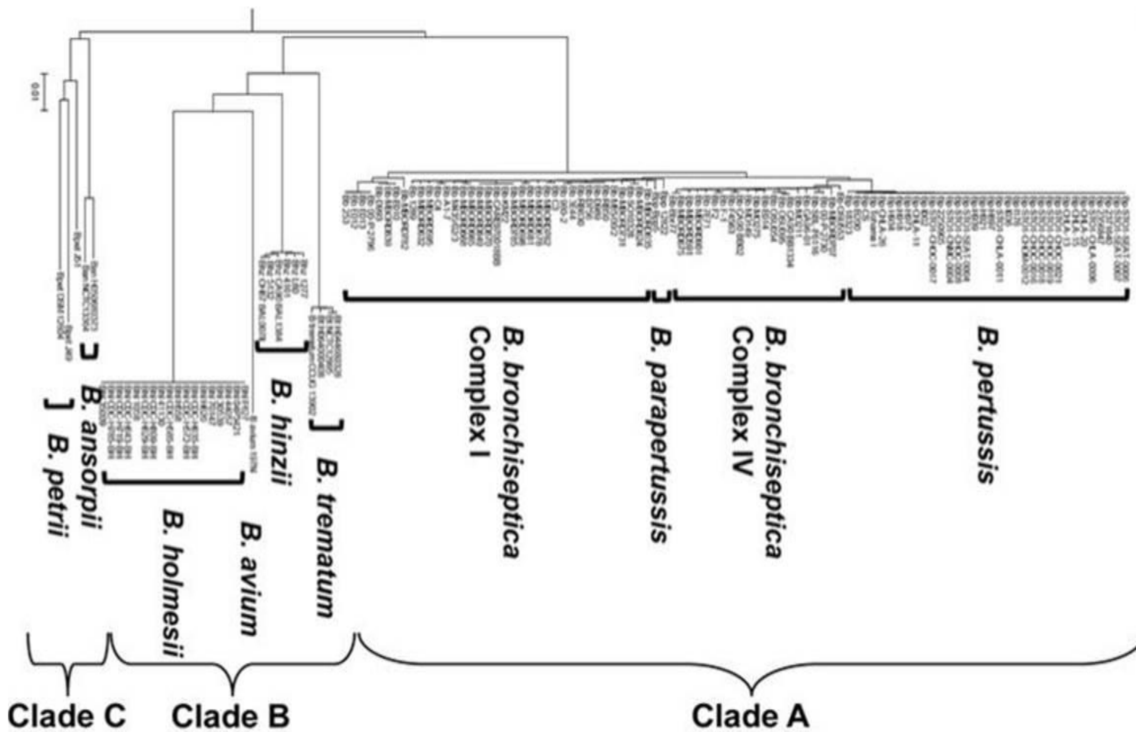

Figure 1: Phylogenetic structure of *Bordetella* genus according to a genome-wide sequence alignment based on SNPs analysis (reproduced from [7])

1.1.2. Virulence factors of classical *Bordetella* species

The extent and severity of a disease caused by individual bacterial pathogens are determined by an array of unique virulence factors produced. *Bordetella* spp. own a number of virulence determinants vital for colonisation of host respiratory tract and development of clinical disease. Virulence factors of classical bordetellae could be classified into two categories according to their biological effects. Those necessary to mediate bacterial adherence to eukaryotic cell are fimbriae, filamentous haemagglutinin and pertactin. In tandem, adenylate cyclase toxin, dermonecrotic toxin, tracheal cytotoxin, lipopolysaccharide and, finally, type III secretion system effectors empower the bacteria to escape destruction by the host immune system [9]. Exclusively,

B. pertussis possesses an additional virulence factor called pertussis toxin which is responsible for lymphocytosis of pertussis patients [10, 11]. Many of *Bordetella* spp. virulence factors are extensively studied. Most of them have a great potential as therapeutic targets and are thus used for vaccine development. A two-component signal transduction system, BvgAS, controls the expression of a large number of bacterial genes, including those responsible for establishing the infection [12]. Additionally, PlrSR regulatory system and RisA regulon were recently reported to contribute to virulence coordination in *Bordetella* [13, 14]. Environmental signals that indicate respiratory tract localization, such as temperature shift and low concentrations of niacin and sulphate ions, trigger activation of BvgA and result in bacteria switching to Bvg^+ (virulent) phase [15].

1.1.3. T3SS-possesing *Bordetella* species in animal infections

Gene clusters encoding the type III secretion system are present in the classical bordetellae as well as in *B. ansorpii*. However, they are absent from genomes of all other species. It is believed that the ancestor of the clade where *B. holmezii*, *B. avium*, *B. trematum* and *B. hinzii* belong, lost the T3SS. Nevertheless, pertinent genes were most probably present in the common *Bordetella* genus ancestor. Among classical bordetellae, a functional T3SS was demonstrated in *B. pertussis* and *B. bronchiseptica*. Functionality of the T3SS in *B. parapertussis* was never showed. Nevertheless, the sheep linage (*B. parapertussis*$_{OV}$) possesses an intact T3SS cluster [7], whereas human lineage of *B. parapertussis* (also referred as *B. parapertussis*$_{HU}$) was observed to contain two genes of the T3SS cluster in the form of pseudogenes which likely affect the T3SS function. So far *B. pertussis* and *B. parapertussis*$_{HU}$ and *B. ansorpii* have been isolated predominantly from humans [16, 17]. Henceforth, providing the chief goal of the thesis is to estimate the seriousness of *Bordetella*-caused veterinary infections and the role the type III secretion system plays in them, we will focus on diseases and syndromes caused by two species possessing the type III secretion system - *B. bronchiseptica* and *B. parapertussis*$_{OV}$.

1.1.3.1. *Bordetella bronchiseptica*

There is a broad range of respiratory diseases, varying from clinically unapparent to fatal, that are thought to be of the *B. bronchiseptica* origin. It is associated with swine atrophic rhinitis and pneumonia, canine and feline acute respiratory illness, respiratory infections of laboratory animals, including guinea pigs, rats, rabbits, ferrets and monkeys [18]. The species is known to contribute to health decline in koalas, presumably, both in captivity and in the wild [19]. *B. bronchiseptica* was demonstrated to persist in a 79-year old host for a period of two and a half years [20]. Nevertheless, the ability of *B. bronchiseptica* to cause chronic infections in humans awaits further investigation. In case of immunosuppressed patients, it may bring on severe pulmonary infections such as pneumonia [21, 22]. It has been intensively studied over the last decade due to its close evolutionary relationship to *B. pertussis*, an agent of whooping cough.

1.1.3.1.1 Infection of pigs

B. bronchiseptica was shown to cause atrophic rhinitis in pigs [23, 24]. According to World Organisation for Animal Health, atrophic rhinitis of swine is classified among list B diseases meaning it is a transmissible disease of socio-economic importance and it is significant in the international trade of animals and animal products. As the disease progresses affected swine are reported to develop turbinate atrophy, malformations of the nose and sneezing. Animals infected with *B. bronchiseptica* show signs of retarded growth rate as well as predisposition to colonisation by other pathogens such as swine influenza virus, porcine reproductive and respiratory syndrome virus, porcine respiratory coronavirus, *Haemophilus parasuis*, toxigenic *Pasteurella multocida*, and *Streptococcus suis* [18, 25, 26]. Progressive atrophic rhinitis, ultimately requiring presence of *P. multocida*, is a serious condition both in sucking and growing pigs [27]. The coryza may be so acute to the extent of haemorrhage from the nose. Severely affected young and adults have problems eating which results in considerably reduced daily gain. Thus, *B. bronchiseptica* infections may have a significant impact on economic losses in animal husbandry if not foreseen and prevented in time. Vaccination usually prevents the establishment of

infection [28] and it is widely applied in Denmark, Portugal and Brazil. However, recent research in India confirms the occurrence of *Bordetella* infection in healthy pigs as well [29].

1.1.3.1.2 Infection of dogs

Canine infectious respiratory disease (CIRD), also called kennel cough, is thought to be a complex syndrome of multifactorial aetiology. Its clinical signs such as coughing, nasal discharge and dyspnea are attributed to viral, as well as bacterial agents, which may act either in conjunction or sequentially. Canine adenovirus 2, canine parainfluenza virus and *B. bronchiseptica* are pathogens most frequently associated with the syndrome. The most severe cases of CIRD, with excessive tracheal mucus accumulation, vomiting and weight loss, are linked to the presence of *B. bronchiseptica* in affected dogs but not to canine respiratory viruses alone [18]. Despite successful vaccination [30] disease outbreaks have been documented over last decade at boarding and breeding kennels, shelters and research facilities [31]. An extensive survey has been carried out on 503 asymptomatic dogs in America. It showed a relatively high - 40.8% - prevalence of *B. bronchiseptica* in densely housed environments [32]. Recent epidemiological studies confirm steady circulation of *B. bronchiseptica* in populations of dogs in Canada and New Zealand [33, 34].

1.1.3.1.3 Infection of cats

In cats, upper respiratory tract infection (URI) often results in euthanasia of affected cats. An earlier study assessing incidence and risk factors for feline infection discovered presence of *B. bronchiseptica* in 11% of sampled individuals, with the highest rate in rescue catteries and research colonies. Regarding symptoms of the disease, only association with sneezing was statistically significant [35].

The outbreaks appear to occur especially often in shelter environment. A recent study evaluated prevalence of different pathogens known to contribute to the disease. While feline herpesvirus type 1, *Mycoplasma* spp. and *Chlamydophila felis* were the most important of them, *B. bronchiseptica*, despite its moderately increased prevalence

in some locations (up to 31.4%), was not significantly associated with URI in shelter cats. Henceforth, the species was perceived rather as opportunistic, importantly, with the potential for cross-species transmission to dogs [36].

1.1.3.1.4 Infection of rabbits

The role of *B. bronchiseptica* infection was studied in relation to upper respiratory disease (URD) of rabbits. It appears that *B. bronchiseptica* is rarely associated with the illness itself, albeit common in the respiratory tracts of healthy and diseased animals. Research conducted at a commercial rabbitry [37] reported that *B. bronchiseptica* was isolated from about 75% of weaning rabbits. In addition, it was demonstrated that only rabbits with *P. multocida* or *P. multocida* and *B. bronchiseptica* developed upper respiratory disease (URD), whereas rabbits with just *B. bronchiseptica* infection had no apparent disease. The most serious symptoms were linked again to *P. multocida* and not to *B. bronchiseptica*. Another study described respiratory pathology caused by the intranasal inoculation of *B. bronchiseptica* in rabbits [38]. Three out of the eight inoculated animals died. Post-mortem examination revealed acute rhinitis, pneumonia and pleuritic. Recently published findings suggested that in the European rabbit (*Oryctolagus cuniculus*) *B. bronchiseptica* facilitates parasitic nematode infection [39].

1.1.3.2. *Bordetella parapertussis*$_{OV}$

Two lineages of the species are distinguished – *B. parapertussis*$_{HU}$, the one infecting humans, and *B. parapertussis*$_{OV}$, which is sheep-specific.

There are only a modest number of articles describing ovine *B. parapertussis*. The bacteria were first isolated from the respiratory tract of lambs with chronic non-progressive pneumonia in New Zealand [40]. In 1988, it was shown [41] that lambs inoculated with ovine isolates of *B. parapertussis* developed degeneration of airway epithelium and alveolar epithelial cells, as well as inflammation in the lungs. Later studies reported that *B. parapertussis* primary infection increases the susceptibility of lambs and sheep to pneumonic pasteurellosis [42, 43].

Articles characterizing *B. parapertussis* infection indicate that the bacteria bring on only mild respiratory disease. Even though up to 50% of sheep might carry *B. parapertussis*, it is the coinfection with *P. haemolytica* (biotype A) which has the potential for exceeding the threshold of economic harmfulness.

1.1.4. Importance of *Bordetella* infections in animals

Conclusions regarding *B. bronchiseptica* infection severity and economic impact have been changing over time. In the early 1960s, it was common to attribute a great part of domestic animals respiratory infections to *B. bronchiseptica*. As time passed and more research on prevalence of the bacteria and aetiology of relevant diseases was conducted, the reassessment arrived [44-47]. It was repeatedly shown that *B. bronchiseptica* as a sole pathogen is not responsible for fatal cases but rather for a self-limiting, mild form of the disease. Although sudden death may occur, no specific treatment is prescribed but for quarantine and supportive care for infected individuals until clinical symptoms resolve. On the other hand, coinfections as well as secondary bacterial overgrowth occurring after a primary viral infection or following some type of respiratory tract assault, might well culminate in an acute illness [48]. The species was reported to have numerous natural and urban reservoirs [49, 50]. Altogether, according to the literature available, the most substantial effect of *B. bronchiseptica* infection could be observed in food-producing and companion animals. Thanks to the development of vaccines and persistent high vaccine coverage of the most susceptible groups of animals, the burden of *Bordetella* infection across developed countries is restrained. Assessing the extent and seriousness of associated diseases, we may conclude that neither *B. bronchiseptica* nor *B. parapertussis* are a primary cause of great economic damage connected with veterinary respiratory illnesses. Nevertheless, *B. bronchiseptica* has been repeatedly shown to enhance some pathogens, as well as facilitating invasion of others. Presence of *B. bronchiseptica* aggravates the condition of the afflicted individuals. What is more, in isolated instances, it has the potential to be transmitted to humans.

1.2. Type III secretion system and its effectors

Type III secretion system (T3SS), also known as a type III secretion injectisome, represents a sophisticated protein-export apparatus which enables bacteria to transport bacterial proteins, collectively known as effectors, directly to the cytosol of eukaryotic cells. The T3SS and its translocated effectors are central to virulence of numerous gram-negative pathogens, including such prominent examples as *Salmonella typhimurium*, a bacterium responsible for a great part of veterinary infections; *Shigella flexneri*, a notorious human pathogen, and enteropathogenic *Escherichia coli* [51, 52]. Moreover, genes for the injectisome were found almost in all plant major pathogens, comprising *Pseudomonas syringae*, *Xanthomonas* spp., *Ralstonia solanacearum* and *Erwinia* spp., along with nitrogen fixating symbionts from genus *Rhizobium*. Interestingly, the T3SS is encoded by some free-living bacteria, e.g. *Desulfovibrio vulgaris* – sulphate reducing environmental microorganism, which has been, however, occasionally linked to human disease [51, 53, 54]. Consequently, it is no wonder, the T3S system is a subject of considerable interest among those studying the field of host-pathogen interactions. Undoubtedly, pathogens aforementioned exploit additional virulence factors during the establishment of the infection. Nonetheless, the T3SS is an essential virulence determinant and it is used as a key virulence mechanism [55].

1.2.1. Type III secretion system phylogeny

According to phylogenetical analyses, the injectisome is perceived as an exaptation of the flagellar apparatus. The evidence strongly suggests their common evolutionary origin. The core proteins of flagellar apparatus and the T3SS are structurally and functionally exceptionally close. Both these nanomachines are capable of exporting proteins: filament components and effector proteins, respectively. [54, 56].

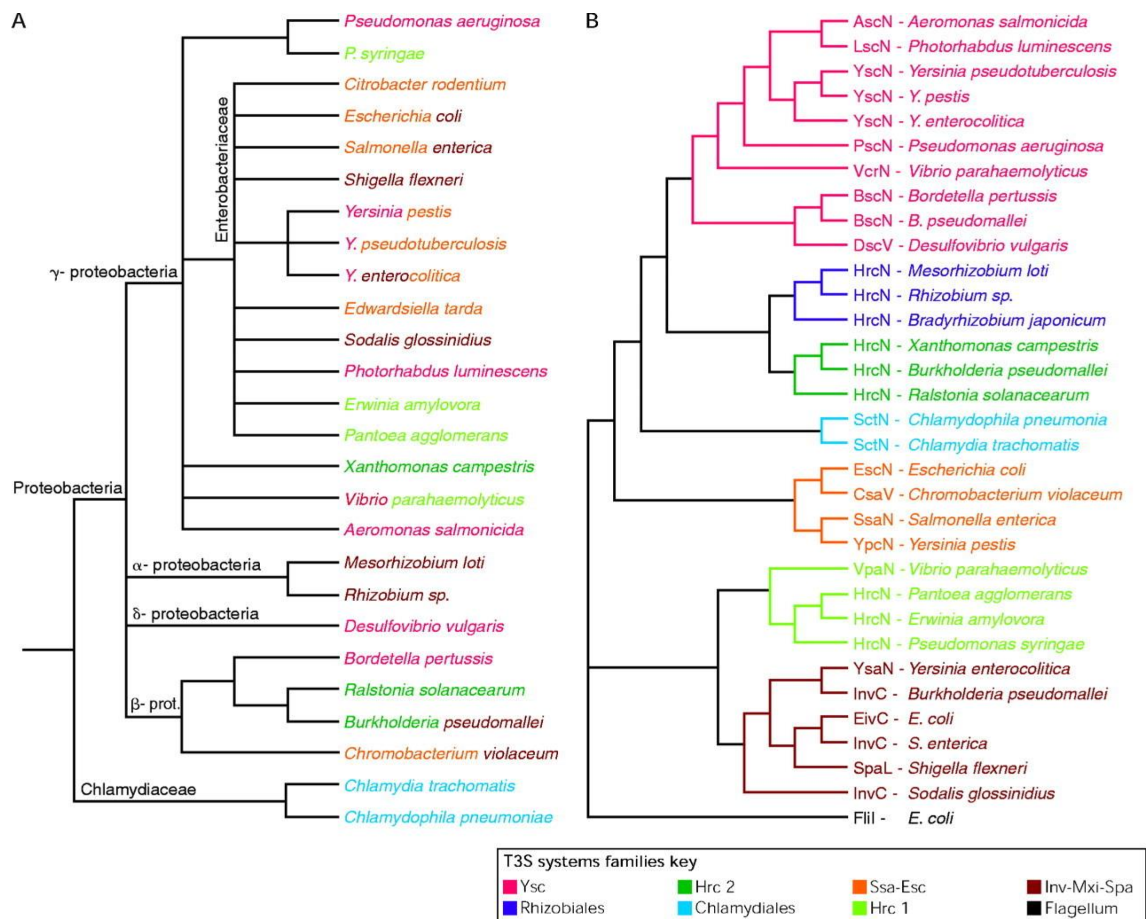

Figure 2 : Phylogenetic tree of bacteria T3S systems (reproduced from [54]) A) Phylogenetical tree of the bacteria derived from the analysis of rRNA aligned sequences. The tree reflects actual evolutionary relationship between the T3SS-possesing bacterial species. **B)** T3S systems relationship phylogram showing relatedness of the ATPases of seven families of injectisomes and FliI, the ATPase of the flagellum of *E. coli*. The phylogram demonstrates how different types of the injectisomes are interconnected, as well as illustrating which type of the T3SS a particular bacterial species possesses

The T3SSs themselves are phylogenetically divided into seven families. Although the T3SSs are encountered in fairly distantly related species of gram-negative bacteria, as evident from the **Fig.2A**, their genetic organisation, function and architecture are remarkably conserved. The T3SS genes are usually organised into a distinct cluster and frequently located on a plasmid. Some bacterial species are reported to have two different types of the T3SSs in their genomes (e.g. *Vibrio parahaemolyticus*, **Fig.2B**). These findings, indicating the T3SS gene acquisition by lateral transport, offer a possible explanation as to why *Bordetella* spp. T3SS belongs to

the Ysc family of injectisomes (**Fig.2**), together with evolutionary remote γ-proteobacteria *Yersinia* spp., *Pseudomonas aeruginosa*, *Photorhabdus luminescens* and δ-proteobacterium *Desulfovibrio vulgaris* [54].

1.2.2. T3SS structure

Current knowledge about the T3SS structure, obtained by transmission electron microscopy, high-resolution cryo-electron microscopy, Rosetta modelling, X-ray crystallography and combinations of these methods, divides the injectisome into a needle complex (NC) and associated cytosolic elements (**Fig.3**). The multi-ring structure of the NC is known to be embedded into the bacterial envelope. It consists of a base and a protruding filament with an inner rode inside. The cytosolic compounds comprise numerous proteins forming a sorting platform. Although a great host of studies have been dedicated to solving the T3SS complex structure, its interactions with cytosolic associated proteins have only recently started to be uncovered [57].

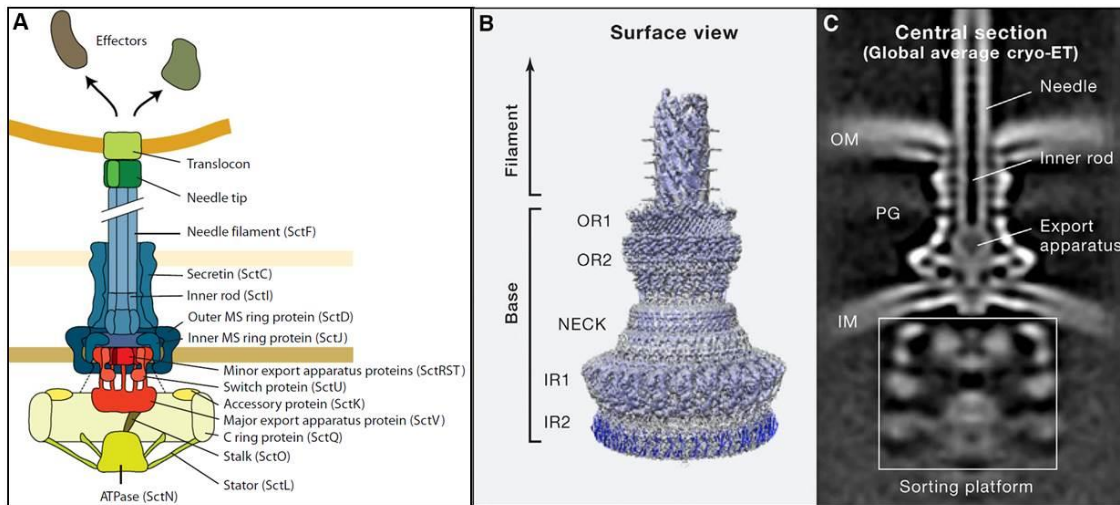

Figure 3 : Depiction of the T3SS (reproduced [57, 58]) A) Diagram of the needle complex and the associated elements employing the common nomenclature for conserved T3SS structural proteins. **B)** Structure of the T3SS needle complex. **C)** *In situ* structure of the T3SS obtained by a cryo-electron tomography imaging technique (OM – outer membrane; PG – peptidoglycan; IM – inner membrane).

Previously, a common nomenclature was proposed for the conserved T3SS apparatus proteins (**Tab.1**). The abbreviation used – Sct – stands for secretion and cellular translocation [55]. We will employ it further on in order to describe the T3SS structure and function in essence rather than in relation to the definite species of

bacteria. In case where no common name exists, an actual example of the protein from a well-studied T3SS-possesing species will be given.

General architecture of the base is represented by a cylinder composed of two pairs of rings. Two rings connected to the inner membrane (**Fig.3A** – SctJ and SctD; **Fig.3B**) are linked through a “neck”, or a “rod”, to the two rings associated with the outer membrane (**Fig.3A -** SctC). The inner rings shelter the so-called “export apparatus” consisting of five highly conserved proteins of presupposed functions (**Fig.3A** – SctR, SctS, SctT, SctU, SctV), such as to facilitate target proteins export through the inner membrane. In order to translocate proteins and to establish secretion hierarchy, the T3SS requires the presence of the sorting platform (**Fig.3A -** SctK, SctQ, SctL, SctO; **Fig.3C**) and ATPase (SctN), structurally similar to those in eukaryotic mitochondria. Both are hypothesized to dynamically associate with the NC base components [51, 58].

The filaments vary from species to species. Inside either one there is a channel of 2-3 nm, which extends from the bottom to the tip and creates a conduit for the transported proteins. Generally, the filament is represented by a hollow stiff needle, an outcome of SctF protein polymerization (**Fig.3A**). Captured images of *Shigella* and *Yersinia* needles structure show that those are tipped with a single hydrophilic protein (IpaD for *Shigella* and LcrV in *Yersinia*), serving as a scaffold for subsequent translocon pore formation in the cytoplasmic membrane. The process involves on-contact secretion of two hydrophobic proteins (YopB and YopD in *Yersinia*, IpaD and IpaB in *Shigella*), effector translocases, and is continued by their insertion into the target host plasma membrane. In some cases, structure of a protruding part differs in the way the filament is capped. The enteropathogenic *E. coli* orthologue of SctF protein – EspA – polymerises and forms an elongated superstructure that extends a bacteria shorter stiff needle. This considerable variation appears to be physiologically relevant for *E.coli* when facing the mucus layer barrier on the way to the enterocytes, its target cells. In contrast, in a pilus, consisting of pili protein HrpA, e.g. in *Erwinia amylovora*, no stiff needle structure might be present.

The actual number of the injectisomes per cell varies from one to a few of SPI-2 system in *Salmonella* [59] to about a hundred needle complexes in *Shigella* [60]. The

role the T3SS plays in the pathogenesis of bacteria depends entirely on the proteins it injects into the host cell. The function such proteins perform differs according to the pathogen needs.

Table 1 : Common nomenclature of type III secretion components and their proposed functions

Sct-common nomenclature	Proposed function
SctC	needle complex outer ring
SctD	needle complex inner ring
SctJ	needle complex inner ring
SctR	export apparatus (protein channel)
SctS	export apparatus (protein channel)
SctT	export apparatus (protein channel)
SctU	export apparatus (substrate switching)
SctV	export apparatus (proton channel)
SctK	cytoplasmic sorting platform
SctQ	cytoplasmic sorting platform
SctL	links ATPase to sorting platform
SctN	ATPase
SctO	cytoplasmic component
SctF	needle filament component
SctI	inner rod component

1.2.3. T3SS effectors

Effectors are bacterial proteins injected directly into the host cell by a contact-dependent excretion system. They switch the cell metabolism in the way favourable to bacteria that extensively use effector proteins to fight against the host immune system, establish the infection and persist. Over one hundred different bacterial effectors are known. In general, there is more than one T3SS effector protein secreted by a single bacterial species. From six to over twenty different effector proteins are usually delivered into the target cells [51]. Additionally, some of them are believed to work synergistically.

There are a number of important structural and functional commonalities present between the T3SS effectors. Although secreted proteins are highly divergent across the species [55], a recent review [61] pointed out that they are of, so-called, modular architecture. This statement basically means that they consist of well-defined distinct domains; each has its own role to play. Firstly, proteins, destined to serve as T3SS effectors, possess a strong secretion signal residing at the extreme N-terminus. Secondly, closely downstream there is a site for binding of class I chaperones – a chaperone-binding domain (CBD). It is necessary that the pre-secretory proteins maintain their partially unfolded state, and so the chaperon-effector interactions appear indispensable [62]. Finally, there are a variety of functional modules important for biological activity of effectors in the host cells.

Described functional modules could be classified into two groups: those serving for subcellular localization and those mediating subversive activity. Targeting sequences might aim the effectors for mitochondria, nucleus or plasma membrane. The subversive activity may be performed by (a) changing cell phosphorylation patterns (b) modulating cytoskeleton (c) targeting G proteins (d) directly by lipase, protease, lyase or other enzymatic activities, and so forth [61]. Examples illustrating the application of certain functional domains by some intestinal and respiratory pathogens follow on.

In *Yersinia enterocolitica*, a microorganism responsible for a number of nasty gastrointestinal syndromes in human and plague-like disease in rodents, several T3SS effectors mediate inhibition of the host inflammatory response. One of them, a tyrosine phosphatase YopH secreted to cytosol, interferes via modulating cell phosphorylation processes, (a) strategy. It has been shown to supress bacterial uptake by macrophages through specific dephosphorylation of proteins required for phagocytosis [63]. Cytotoxicity of YopE, another protein translocated by the injectisome and targeted into intracellular membrane, was proved to contribute to the inactivation of phagocytes at early stages of infection. It causes a collapse of their cytoskeleton employing its RhoGAP activity (c) [64]. YpkA effector also prevents proper phosphorylation in cells (a), as well as deterring the appropriate cytoskeleton assembly (b). It was found to contain plasma membrane targeting, kinase activity and actin binding domains [65].

Notably, around twenty five T3SS effector proteins could be found in *Shigella flexneri*, a causative agent of diarrheal disease. In contrast to *Yersinia* spp., it predominantly occupies intracellular locations of epithelial cells. The bacteria need to be transcytosed by microfold cells and then ensure their survival inside resident macrophages. *S. flexneri* triggers its uptake by changing usual cell cytoskeleton behaviour (b). The translocated effector IpaA was demonstrated to possess an actin modulatory domain and mediate localized actin depolymerisation which, in turn, causes the closure of the phagocytic cup around the bacteria. When inside the host cell, *S. flexneri* destroys the endocytic vacuole and escapes to the cytoplasm. Its intracellular motility, being actin-based, requires changes in actin-nucleation (b). While surface protein IscA ensures actin polymerization in the way favourable to the bacteria, T3SS translocated VirA functions as a microtubule-disrupting effector destabilizing host cytoskeleton[55, 61, 66].

Pseudomonas aeruginosa, a common nosocomial contaminant causing fatal lung infection in immunocompromised patients, is capable of colonizing mucosal epithelia [67]. Intensive characterization of this gram-negative bacterium T3SS yielded the identification of four proteins that are injected into the host cell. ExoS, bi-functional toxin, is targeted to intracellular membrane via its N-terminal motif. One of ExoS functions is RhoGAP-domain dependent (c). Another translocated protein, plasma membrane targeted phospholipase ExoU (d), was reported to cause rapid cell death in different eukaryotic cell types [61, 68].

All in all, it becomes apparent that many gram-negative bacteria manipulate their victim host cells using a syringe transporting mechanism. The examples previously mentioned are followed by dozens of others making the T3SS a promising pharmaceutical target [69].

1.3. *Bordetella* type III secretion system

As was indicated in the previous chapter (1.1.2), bacteria from genus *Bordetella* possess several virulence factors. The type III secretion system appears to be one of the

most intriguing of them since its role in pathogenesis was largely underestimated. The system was discovered in *Bordetella* at the end of the 20th century [70]. It was shown that the BvgAS signal transduction system, responsible for switching between virulent and non-virulent phenotypic phases, up-regulated a certain *bscN* gene. That one, in turn, was found homologous to the T3SS ATPase from *Yersinia* spp. The study basically revealed the very existence, as well as undeniable importance, of the T3SS in *Bordetella* spp. pathogenesis. Later, the whole *bsc* locus was described [71]. Until that point, none of the known *Bordetella* virulence factors had been reported to be secreted via the T3S apparatus. Yet it was anticipated from the moment of its discovery that there were bound to be numerous unidentified proteins secreted through the system [70].

1.3.1. *Bordetella* type III secretion locus and its regulation

Current knowledge of the T3SS genes clustering embraces two loci – *bsc,* encoding structural components, and *btr,* encoding regulatory proteins – and BteA effector with its chaperone BtcA genes located 2,5 Mbp "upstream" from *bsc*. All of them are controlled by the BvgAS phosphorelay. Furthermore, transcriptional data were used to divide about 30 *bsc* open reading frames (ORFs) into two distinct clusters on account of being controlled by a BtrA-BtrS regulatory node. [72].

1.3.2. *Bordetella* T3SS structure

Bordetella injectisome is most closely related to those in *Yersinia* spp. and *P. aeruginosa* [71]. With the T3SS genes being usually highly conserved, its structure is generally assumed to be similar, even though it has not been visualised yet. Nevertheless, the *Bordetella* T3SS needle filament, formed by Bsp22 protein, appears to be analogous to that aforementioned of EPEC, built of EspA protein subunits, rather than to a hollow stiff needle of *Yersinia* spp.

A recent article has outlined its probable intricate architecture (**Fig.4**) including five described and intensively studied structural proteins: BscN, Bsp22, BopB, BopD and BopN [72]. Four of them are known to be secreted into culture supernatant whereas BscN, ATPase, powers their transport. It is suggested that BopB and BopD form a translocon pore in the cytoplasmic membrane of eukaryotic cells, thus serving as T3SS translocators [73].

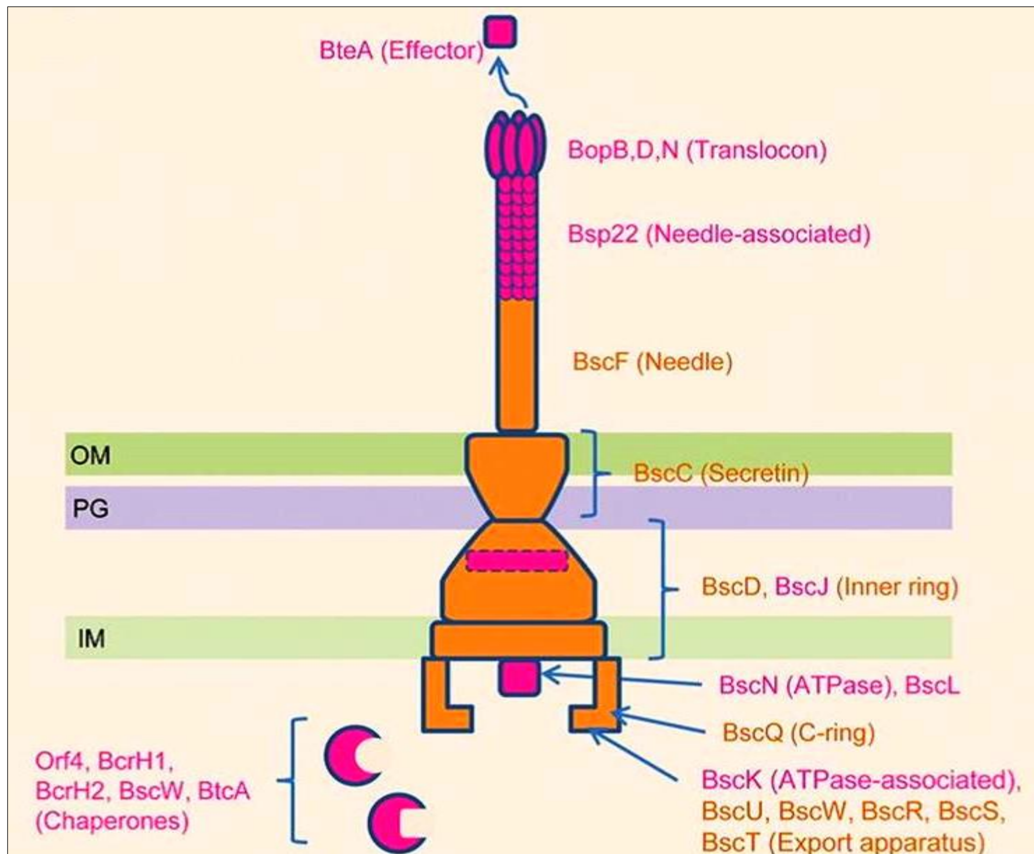

Figure 4 : Functions and locations of $T3SS_{Bsc}$ gene products (reproduced from [72]) IM – inner membrane; OM – outer membrane; PG – peptidoglycan

In turn, Bsp22 was demonstrated to interact with BopD. Bsp22 is the most abundant polypeptide in *B. bronchiseptica* culture. It has been observed to extensively multimerize and form filaments. Taking this into account, it was then proposed that the protein assembles into a long filamentous extension. Together these findings may indicate the role of Bsp22 in connecting the ND to the translocon which is similar to the earlier mentioned tip complex protein EspA from E. coli. Although no significant sequence similarity was observed between Bsp22 and EspA, their secondary structures, as well as positions within T3SS loci, are coinciding [8, 74].

1.3.3. *Bordetella* T3SS function

A number of effects on target cells *in vitro* and on respiratory tract in rodent model were attributed to the presence of an intact T3SS in *Bordetella*.

Firstly, it induces necrotic cell death [75]. Secondly, it may prevent the development of an inflammatory response by interfering with NF-κB signalling pathway, by inhibition of interferon-gamma production and by eliciting the anti-inflammatory cytokine, interleukin-10, level [76-78]. *In vivo*, the T3SS is required for bacterial persistence in the lower respiratory tract and enhances the development of nasal lesions and pneumonia in swine infection model. The system was shown to contribute to the disease severity but did not affect transmission [26] [79].

1.3.4. Effectors of *Bordetella* T3SS

1.3.4.1. BteA effector

Information about the first T3SS effector detection emerged in the ingeniously elaborated study, seven years after the identification of the T3SS in *B. bronchiseptica* [80]. As expected, the effector's gene, *bteA*, colocalized with its chaperone, *btcA*, which served as a "guide star" for the conducted genome-wide screen. BteA, the only definitely accepted *Bordetella* T3SS effector, is highly conserved among the "classical" clade. *B. parapertussis bteA* appears to be slightly more divergent although the allele is claimed to be functionally interchangeable [81]. BteA effector consists of 656 amino acid residues with molecular weight of about 69 kDa. While its structure is still being studied, previously acquired data showed a modular design which is characteristic of T3SS effectors. Two important functional domains are recognized. The BteA N-terminus, a region of 130 amino acid residues, includes CBD and lipid raft targeting (LRT) domain [81]. Its C-terminus has proved essential for BteA unusually potent cytotoxicity. Up until now, the central region has been referred as a "linker". The mystery behind its unusually potent cytotoxicity remains to be solved [82].

The protein was demonstrated to be extremely cytotoxic in a multitude of cell types such as L2, HeLa and MLE12 [81]. Being able to induce rapid non-apoptotic death, it was proposed to play a role in promoting persistent colonization of the host

respiratory tract. It was shown that *B. bronchiseptica* strain with deleted *bteA* strongly resembles the phenotype with the entirely inactivated T3SS [76, 80]. Using murine model, it was shown that BteA from *B. bronchiseptica* induced production of anti-BteA antibodies after the respiratory infection. Interestingly, none of the *B. parapertussis* tested isolates were found producing BteA protein in a culture. In addition, after *B. parapertussis* infection there were no anti-BteA antibodies [83].

In conclusion, for *B. bronchiseptica,* it appears that the T3SS and particularly its effector, BteA, may play a crucial role during the infection. As BteA was discovered relatively recently, it is not targeted by current immunization. However, the protein might be used for developing more extensive acellular vaccines.

1.3.4.2. BopN protein

Four years after the discovery of BteA protein, a study, describing BopN as another T3SS effector being translocated into the eukaryotic cell, arrived. A role in up-regulation of IL-10 by blocking nuclear translocation of NF-κBp65 was attributed to this protein [84]. Importantly, other research groups remain unconvinced by these findings and do not consider BopN being an effector [72]. In addition, recently acquired observation from crystal structure analysis confirms significant structural similarity between BopN and YopN which suggest their functional congruence (Bumba, unpublished). Initially, BopN protein was predicted to be a post-transcriptional regulator of the T3SS. The assumption was expressed on the basis of BopN homology to YopN, the *Yersinia* spp. host cell contact-dependent regulator. Unpublished experiments determined that BopN did not affect secretion of other T3SS substrates *in vitro*; however, its absence significantly decreased *B. bronchiseptica* cytotoxicity [8].

2. Aims of the Thesis

The primary goal of this thesis was to evaluate the burden caused by *Bordetella* infections in animals and to determine the role of type III secretion system (T3SS) in pathogenesis of the species by literature review. The thesis practical part was dedicated to the identification of so far unknown Bordetella T3SS effector proteins by means of proteomic analysis. The study further aimed to examine genes encoding the components of T3SS in the genome of *Bordetella bronchiseptica* D445 (MO211) by sequence similarity searching and predict their function.

3. Methods

3.1. Equipment, chemicals and media

3.1.1. Equipment

CO_2 incubator – Sanyo MCO-18 AIC, Panasonic, Japan

Digital analytical balances – Mettler Tolero AB 104-8, SRN

Dry bath – Accu Block™ Labmet International, Inc., USA

Freezer (-20 °C) – Zanussi, Italy

Freezer (-80 °C) – Jouan, France

Incubator shaker – Innova® 43, New Brunswick Scientific, Edison, USA

Laminar flow cabinet – Gelaire, Flow Laboratories, Skotland

Mass spectrometer – Thermo Orbitrap Fusion, Thermo Fisher Scientific, USA

Microcentrifuge – Biofuge pico, Heraeus Instruments, SNR

Microwave oven – Daewoo, Korea

Spectrophotometer – CO 8000 cell density meter, WPA, UK

Vortex – Minishake 1, IKA-Works, Internacional, USA

3.1.2. Chemicals

1,4-dithiotreitol (DTT) – Stratagene, USA

Acetone – VWR, France

Acetonitrile – Lachema, Neratovice

Ammonium bicarbonate – AcrosOrganics, USA

BG agar base – Difco™, France

Bovine serum albumin (BSA) - Sigma, USA

CAS – BD, France

Casein hydrolysate – Oxoid, UK

Chloroacetamide – Abcam, UK

Cyclodextrin – Zibo Qianhui Biological Technology Co., Ltd, China

Ethanol – Lachema, Brno

Glycerol – Lachema, Neratovice

L-ascorbic acid – Sigma, USA

L-cysteine hydrochloride monohydrate – Sigma, USA

L-glutathione (reduced) - Appli Chem, Germany

Monosodium glutamate – Sigma, USA

Magnesium chloride hexahydrate - Fluka, Switzerland

Nicotinic acid – Sigma, USA

Potassium chloride – Lachema, Neratovice

Potassium dihydrogen phosphate – Sigma-Aldrich, USA

Proline – Sigma, USA

Quick Start™ Bradford 1x Dye Reagent – Bio-Rad, USA

Sodium chloride – Lachema, Neratovice

Trichloroacetic acid (TCA) – Sigma, USA

Tris(hydroxymethyl)aminomethane(Tris) – Serva, SRN

Tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) – Serva, SRN

Tris-base – Serva Electrophoresis, SRN

Trypsin GOLD – Promega, USA

Urea – Merck KGaA, Germany

3.1.3. Media

Solid Bordet-Gengou (BG) medium

BG agar (Difco™)	3 % (w/v)
Glycerol	1 % (v/v)

Solid Bordet-Gengou (BG) medium supplemented with 15% sheep blood

First, solid BG medium was prepared and autoclaved (120 kPa, 20 min). Next, it was cooled down to 50 °C in the pre-heated water bath. Under sterile conditions sheep blood was added to the final concentration of 15%. The blood BG agar plates were prepared by pouring ~ 20 ml of the medium containing sheep blood to sterile Petri dishes.

Stainer-Scholte (SS) medium (pH 7)

Fraction A (10x)	
Tris-HCl	403,6 mM
Tris (base)	97,4 mM
Monosodium glutamate	572,8 mM
Proline	20,8 mM
NaCl	427,7 mM
KH_2PO_4	36,7 mM
KCl	26,8 mM
MgCl2 x 6H2O	4,9 mM
Fraction B (100x)	
L-cysteine hydrochloride monohydrate	33 mM
L-ascorbic acid	11,3 mM
L-glutathione (reduced)	32,5 mM
Nicotinic acid	3,3 mM
Casein hydrolysate	5 % (w/v)
Cyklodextrin	1 % (w/v)

The complete Stainer-Scholte defined growth medium was prepared by mixing fractions A and B. The mixture was subsequently diluted and sterilized by filtration through 0.22 μm membrane filter.

3.2. Experimental procedures

3.2.1. *Bordetella* cultivation

The bacteria, *B. bronchiseptica* strain D445 (MO211) and its Δ*bscN* mutant, were grown in pure culture on solid BG medium supplemented with 15% sheep blood at 37 °C and 5% CO_2.

For sample preparation *B. bronchiseptica* was pre-cultivated in Stainer-Scholte medium to $OD_{600} \approx 1$ and subsequently reinoculated into 50 ml of Stainer-Scholte medium to final $OD_{600} \approx 0.1$. This culture was further cultivated at 37 °C in a shaker for 12 h till $OD_{600} \approx 4$.

3.2.2. Processing of *Bordetella* cultures for mass spectrometry

The bacterial cultures grown overnight in 50 ml of Stainer-Scholte medium to $OD_{600} \approx 4$ were harvested by centrifugation (14 000 g, 20 min., 4 °C). Pellets were discarded. Supernatants containing proteins of interest were filtered through 0.22-µm membranes. In the next step, we added 9 ml of 50% TCA to 36 ml of supernatant resulting in the final TCA concentration of 10%. The samples were left to precipitate overnight at 4 °C. After that the samples were centrifuged (10 000 g, 30 min., 4 °C) and supernatant was removed. Subsequently, 5 ml of pre-cooled (-20 °C) acetone was added to each of acquired pellets. The samples were centrifuged again (10 000 g, 30 min., 4 °C). Acetone was removed, and the pellets were left to dry for 5 minutes at 37 °C. Finally, 500 µl of the solution of 8M urea in 50 mM ammonium bicarbonate ($A_{50}U_8$) was added to dissolve the dried pellets of the supernatants. Protein concentration of the samples was determined by the Bradford assay.

Equivalent amount of protein – 50 µg – from each sample was transferred to Microcon® centrifugal filters and centrifuged (14 000 g, 20 min., 25 °C). The filters were washed two times with $A_{50}U_8$ (14 000 g, 20 min., 25 °C) followed by two washes with 50 mM ammonium bicarbonate (A_{50}) alone (14 000 g, 20 min., 25 °C). Reduction of disulphide bonds in the protein samples was then carried out at 60 °C using 50 µl of 0,1M 1,4-dithiothreitol diluted in A_{50} followed by necessary alkylation of cysteine residues by adding 5,5 µl of 0,5M chloroacetamide for 30 min. After centrifugation

(14 000 g, 20 min., 25 °C), the samples were digested at 37 °C overnight with trypsin – 2 µg in 70 µl of A_{50}. The following morning, the samples were centrifuged, and remaining peptides were extracted with 60 µl of A_{50}. Next, as desalting was required, C18 spin columns were used. Firstly the columns were washed (8000 g, 1 min., 25 °C) in three steps with 50 µl of 50% MeOH, then with 100 µl of 90% water solution of acetonitrile with 0.1% trifluoroacetic acid (TFA), and finally with 100 µl of 2% water solution of acetonitrile with 0.1% TFA. After that, 130 µl of the samples were loaded on the column and centrifuged (4000 g, 1 min., 25 °C) to a new microtube. Then, 100 µl of 2% water solution of acetonitrile with 0.1% TFA was transferred to the column and centrifuged. Subsequent elution of the sample was done by adding 80 µl of 80% water solution of acetonitrile with 0.1% TFA.

Bacterial protein samples processed in such a way were dried up via vacuum concentration and resuspended in 2% water solution of acetonitrile with 0.1% TFA to the final concentration of protein equal 1 µg/µl. For mass spectrometry 3 µl of the acquired sample were used.

3.2.3. Determination of protein concentration by the Bradford assay

Coomassie Brilliant Blue G-250 dye used during the assay interacts with proteins, primarily with basic and aromatic amino acid residues, colouring the solution blue. A standard set was prepared in order to create a calibration curve. The curve was created by plotting measured absorbance values at 595 nm against standard sample concentrations of BSA protein (0; 10; 25; 50; 75; 100 µg/ml). Processed supernatants acquired from *B. bronchiseptica* D445 (MO211) cultures were diluted 20x and 40x, respectively. In the cuvette of 1 ml volume, 100 µl of the sample and 900 µl of the dye were mixed. The absorbance was measured at 595 nm in the spectrophotometer. Finally, the samples concentration was determined using the calibration curve.

4. Results

4.1. Sample preparation for mass spectrometry and analysis of the mass spectrometry data

Throughout the scientific literature describing type III secretion systems in various gram-negative bacteria, it was evident that pathogens are able to secrete a great host of proteins via their injectisome. Peculiarly, *Bordetella* representatives have been thus far documented to handle only one effector protein. Together with genome evaluation and infection experimental data, this noticeable contradiction to the prevailing tendency prompted us to find out more about *Bordetella* T3SS secreted proteins and speculate on the existence of other effectors. To investigate this phenomenon, we aimed for a widely used analytical technique – a combination of liquid chromatography and tandem mass spectrometry (LC-MS/MS). This method enables separation and identification of different chemical compounds. In our case, a compound of interest was a polypeptide.

If we presume that more effectors exist in *Bordetella,* then their detection in culture supernatant could be attainable. Importantly, for being able to distinguish proteins secreted through the T3SS from those secreted by other mechanisms, we decided to compare supernatants from *B. bronchiseptica* D445 (MO211) wild type, with a functional T3SS, and supernatants from *B. bronchiseptica* D445 (MO211) Δ*bscN*, which has a gene for the T3SS-ATPase deleted, and so the system that is incapable of secreting any effector proteins at all.

The strains *B. bronchiseptica* D445 (MO211) wild type and Δ*bscN* were cultivated as described in the chapter (3.2.1). The following day, as the cultures reached $OD_{600} \approx 4$, they were harvested, and their supernatants were processed by TCA precipitation (3.2.2). Concentration of precipitated proteins dissolved in $A_{50}U_8$ was measured by Bradford assay (3.2.3). Samples acquired after TCA precipitation were washed, reduced, alkylated, digested and desalted in several steps (3.2.2). These procedures are critical for ensuring samples compatibility for mass spectrometry.

Proteomic analysis was carried out by OMICS Proteomics laboratory (BIOCEV). Examination and processing of the data collected from two independent experiments followed. Each experiment was conducted in duplicates (**Tab.2**). The main criteria for protein selection were their presence in at least three samples from wild type supernatant, as well as their absence or significant depletion in Δ*bscN* at the same time. Notably, one more independent test will be required to prove the reproducibility of the results and provide statistical significance.

Peptides detected by LC-MS/MS are characterized by a certain intensity which, in turn, represents relative quantity of individual proteins in the analyte. Data for supernatants 1-4 wt and supernatants 1-4 dbscN, displayed in the **Tab.2**, are such intensities. In order to accomplish our goal, we focused on the proteins showing significantly different intensities in wild type compared to those in Δ*bscN*. Detection of proteins demonstrating such a distinction would suggest their secretion through the T3S apparatus. If the protein production level increased more than two times, we assumed it to be significantly upregulated in wild type compared with Δ*bscN* strain. Four proteins were found exclusively in supernatants of wild type culture and thus came as the most intriguing ones, while other ten were significantly upregulated (**Tab.3**).

Coherently, the next stage would embrace determination of their role and the reason behind upregulation. However, when we tried to identify the upregulated proteins using their ID in *B. bronchiseptica* D445 (MO211) genome (**Tab.3**), a complication arose. Since many genes in *B. bronchiseptica* D445 (MO211) genome were either given unclear names or being hypothetical ORFs, prediction of their potential role as effectors was impossible without further specification.

As effector proteins of secretion systems are known to be frequently encoded inside the pertinent locus, it was reasoned that *in silico* analysis of *B. bronchiseptica* D445 (MO211) *bsc-btr* locus might facilitate practical search for the T3SS effectors.

Table 2 : Intensity data and production level increase in wild type of proteins with significantly different concentration

Protein ID	SUP1 wt	SUP2 wt	SUP3 wt	SUP4 wt	SUP1 dbscN	SUP2 dbscN	SUP3 dbscN	SUP4 dbscN	Production level increase
KAK65919	30.36	30.66	28.18	28.86	0.00	0.00	0.00	0.00	-
KAK70248	24.11	23.89	0.00	28.64	0.00	0.00	0.00	0.00	-
KAK66808	24.12	24.21	0.00	25.56	0.00	0.00	0.00	0.00	-
KAK74435	22.81	23.11	0.00	21.88	0.00	23.03	0.00	0.00	-
KAK65929	33.51	33.87	32.20	32.41	24.63	23.15	22.96	22.55	817.87
KAK65901	38.06	37.96	34.69	34.98	26.82	27.03	26.97	26.19	816.25
KAK65893	37.86	37.87	36.07	36.04	28.71	28.02	28.14	27.01	508.06
KAK65880	33.45	33.49	30.97	30.72	23.88	23.31	22.91	0.00	443.24
KAK65945	38.81	38.48	36.90	37.11	30.35	30.83	29.68	28.97	234.09
KAK75306	37.64	37.29	36.07	35.77	28.82	28.94	30.51	29.40	154.54
KAK65923	29.01	28.18	28.18	28.25	21.82	21.05	21.32	0.00	128.53
KAK67391	22.96	21.72	0.00	25.45	20.54	21.27	0.00	0.00	5.55
KAK65952	23.76	23.14	19.46	0.00	21.57	20.91	0.00	0.00	4.61
KAK75714	24.15	24.80	22.55	23.23	21.60	21.76	0.00	0.00	4.02

(ID – identification; SUP – supernatant; wt – wild type; dbscN - Δ*bscN*)

Table 3 : Assigned names for the selected proteins (reproduced from *B. bronchiseptica* D445 (MO211) genome data – GenBank accession number: JHOJ01000131.1)

Protein ID	FASTA header for *B. bronchiseptica* MO211	Production change
KAK65919	putative N-acetyltransferase YedL OS	only present in WT
KAK70248	putative N-acetyltransferase YedL OS	only present in WT
KAK66808	putative N-acetyltransferase YedL OS	only present in WT
KAK74435	putative N-acetyltransferase YedL OS	only present in WT
KAK65929	T3S protein BopB OS	upregulated
KAK65901	T3S regulator YopN/LcrE/InvE/MxiC OS	upregulated
KAK65893	T3 secreted protein BopD OS	upregulated
KAK65880	T3 secreted protein BspR OS	upregulated
KAK65945	T3SS tip complex protein Bsp22 OS	upregulated
KAK75306	T3SS cytotoxic effector BteA OS	upregulated
KAK65923	T3S apparatus protein, YscI/HrpB, C-terminal domain protein OS	upregulated
KAK67391	ATP-dependent Clp protease, ATP-binding subunit ClpX OS	upregulated
KAK65952	T3S low calcium response chaperone LcrH/SycD OS	upregulated
KAK75714	putative N-acetyltransferase YedL OS	upregulated

(ID – identification; WT – wild type)

4.2. *In silico* analysis of *B. bronchiseptica* D445 (MO211) genome

4.2.1. *In silico* analysis of *B. bronchiseptica* D445 (MO211) *bsc-btr* locus and mass spectrometry hits found inside it

The principal objective of *in silico* analysis was to correctly annotate the T3SS locus of *Bordetella bronchiseptica* D445 (MO211). In common with the majority of other T3S systems, *Bordetella* genes encoding the injectisome are clustered together. Based on high degree of conservation between the well-annotated T3SS locus of *B. pertussis* Tohama I and the imperfectly annotated one from *B. bronchiseptica* D445 (MO211), we have attempted to provide more precise names, as well as making a basic functional prediction, for the genes of both *btr* and *bsc* loci.

Similarity search through BLAST (Basic Local Alignment Search Tool; *https://blast.ncbi.nlm.nih.gov/Blast.cgi*) was conducted using genomic sequence data of NCBI database. The FASTA format T3SS genes sequences from *B. bronchiseptica* D445 (MO211) were checked for the possibility of being significantly aligned to those from *B. pertussis* Tohama I. Once all the genes composing the T3SS locus were tested, we created an alignment of the entire loci with subsequent annotation of the ORFs from *B. bronchiseptica* D445 (MO211) (**Fig.5**). These manipulations were performed with the aid of CLC Sequence Viewer 8.0.

For the genes encoding the injectisome structural proteins a scheme was created, displaying a hypothetical appearance of *Bordetella* T3SS complex (**Fig.6**). *Yersinia* spp. needle complex and associated elements as well as its Yop-Ysc cluster were employed as a prototype for modelling the scheme. This analogy was introduced considering the fact that *Bordetella* spp. T3SS belongs to the Ysc family of injectisomes together with the one from *Yersinia* spp.

Summarized *B. bronchiseptica* MO211 T3SS locus dataset is present in **Tab.4**

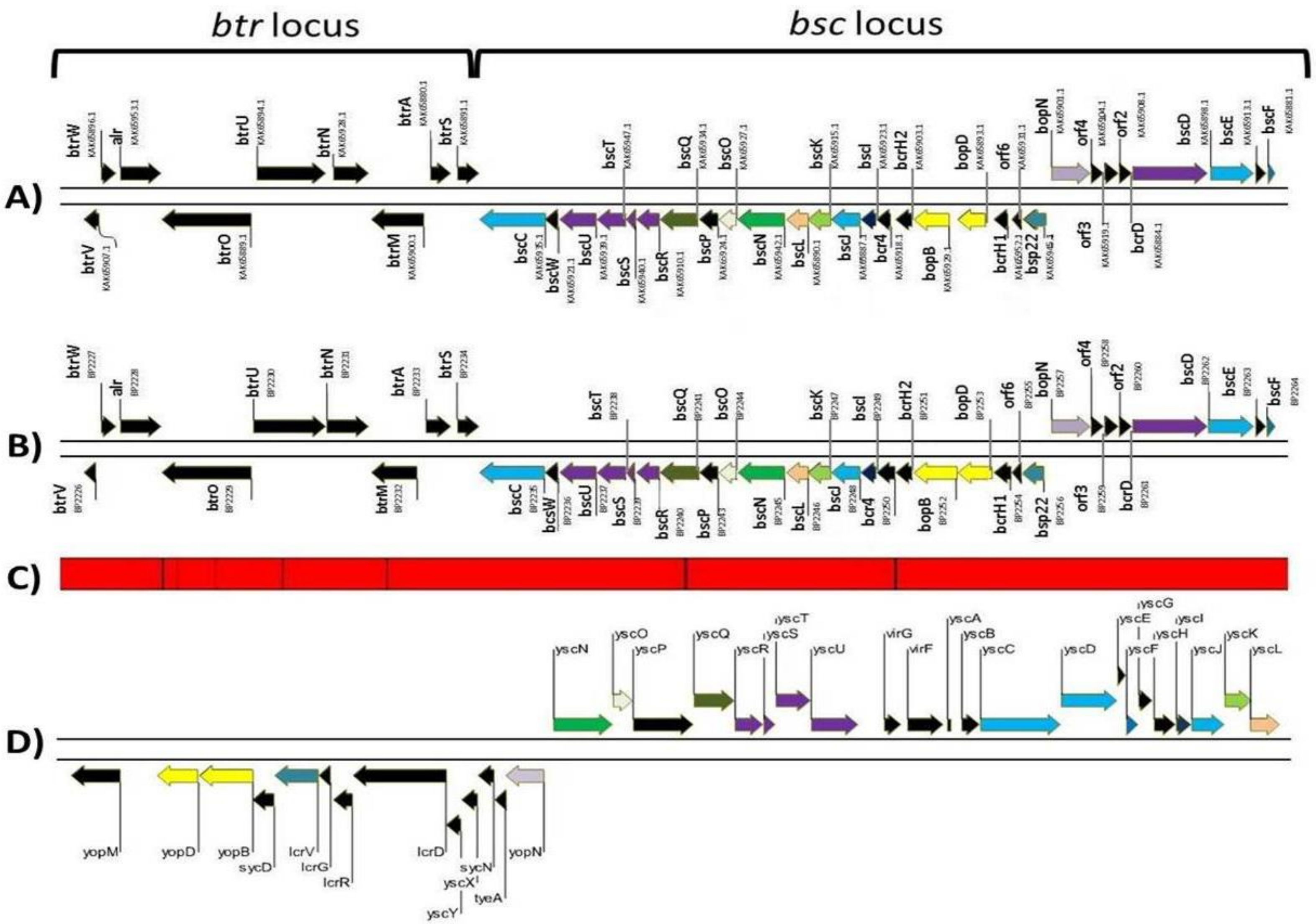

Figure 5 : Comparative visualization of T3SS loci of *Bordetella* spp. and Yop-Ysc cluster of *Y. enterocolitica* A) Organization and open reading frames of *bsc-btr* cluster in *B. bronchiseptica* MO211 (accession number: JHOJ01000131.1). Generic standard rational nomenclature name was assigned to the each ORF. Protein identification (ID) was used to further specify the genes **B)** Organization and open reading frames of *bsc-btr* cluster in *B. pertussis* Tohama I. Annotation data were reproduced from NCBI GenBank sequence database (accession number: NC_002929.2) **C)** Conservation of *bsc-btr* clusters between *B. bronchiseptica* MO211 and *B. pertussis* Tohama I: red colour in plot indicates 100% conservation; black colour in plot marks diverse regions **D)** Organization of the Yop-Ysc cluster from *Y. enterocolitica* (accession number: dbj|BA000007.2gb|AF102990). Gene arrows of **A)** and **B)** are coloured in agreement with **Fig.6**. Gene arrows colouring of **D)** is based on components homology between *Yersinia* spp. and *Bordetella* spp. T3S systems (**Tab.4**).

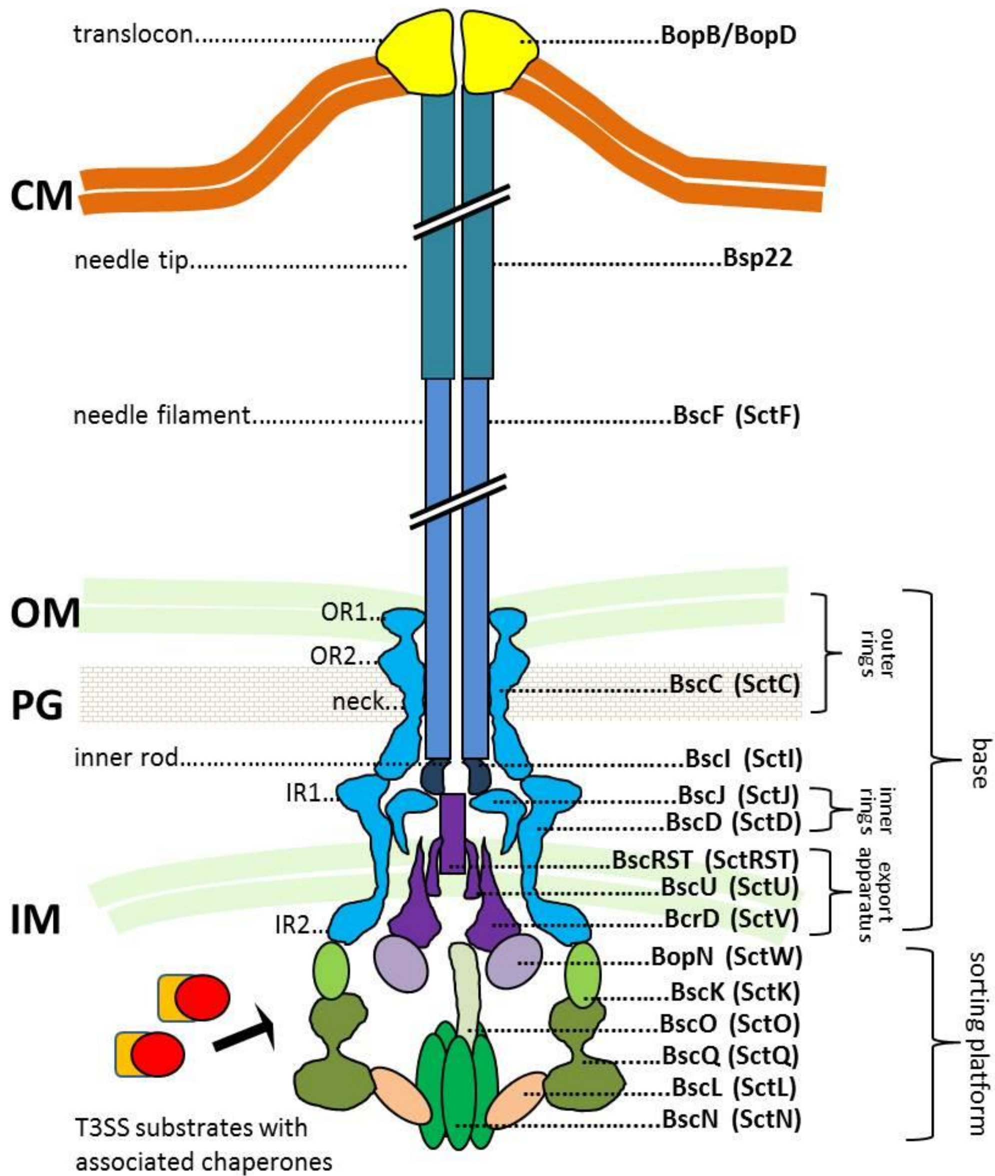

Figure 6 : Diagram of Bordetella needle complex and associated structures (based on [58, 72]) The colouring has been designated in agreement with **Fig.5A**

Table 4 : Homologies between T3SS structural proteins in *Bordetella* spp. and *Yersinia* spp. and their connection with the common T3S systems nomenclature

	B. bronchiseptica	*B. pertussis*	*Bordetella* spp.	*Yersinia* spp.	**Nomenclature**
bsc locus					
	KAK65935.1	BP2235	BscC	YscC	SctC
	KAK65921.1	BP2236	BscW		
	KAK65939.1	BP2237	BscU	YscU	SctU
	KAK65947.1	BP2238	BscT	YscT	SctT
	KAK65940.1	BP2239	BscS	YscS	SctS
	KAK65910.1	BP2240	BscR	YscR	SctR
	KAK65934.1	BP2241	BscQ	YscQ	SctQ
	KAK65924.1	BP2243	BscP	YscP	SctP
	KAK65927.1	BP2244	BscO	YscO	SctO
	KAK65942.1	BP2245	BscN	YscN	SctN
	KAK65890.1	BP2246	BscL	YscL	SctL
	KAK65915.1	BP2247	BscK	YscK	SctK
	KAK65887.1	BP2248	BscJ	YscJ	SctJ
	KAK65923.1	BP2249	BscI	YscI	SctI
	KAK65918.1	BP2250	Bcr4		
	KAK65903.1	BP2251	BcrH2	LcrH	
	KAK65929.1	BP2252	BopB	YopB	
	KAK65893.1	BP2253	BopD	YopD	
	KAK65952.1	BP2254	BcrH1	LcrH	
	KAK65931.1	BP2255			
	KAK65945.1	BP2256	Bsp22	LcrV	
	KAK65901.1	BP2257	BopN	YopN	SctW
	KAK65904.1	BP2258			
	KAK65919.1	BP2259			
	KAK65908.1	BP2260			
	KAK65884.1	BP2261	BcrD	YscV	SctV
	KAK65898.1	BP2262	BscD	YscD	SctD
	KAK65913.1	BP2263	BscE		
	KAK65881.1	BP2264	BscF	YscF	SctF
btr locus					
	KAK65907.1	BP2226	BtrV		
	KAK65896.1	BP2227	BtrW		
	KAK65953.1	BP2228	alr		
	KAK65889.1	BP2229	BtrO		
	KAK65894.1	BP2230	BtrU		
	KAK65928.1	BP2231	BtrN		
	KAK65900.1	BP2232	BtrM		
	KAK65880.1	BP2233	BtrA		
	KAK65891.1	BP2234	BtrS		

(red colour – proteins detected in MS analysis)

2.2.2 *In silico* analysis of mass spectrometry hits found outside the T3SS locus

A number of mass spectrometry hits were found outside the T3SS locus. Oddly, the name putative N-acetyltransferase YedL OS was assigned to more than one protein in the genome in *B. bronchiseptica* D445 (MO211). Furthermore, other protein headers appeared to be rather cryptic. In order to provide better understanding and reveal probable homologies, we used a bioinformatics approach. Correspondingly, a simple BLAST search was applied, which was done in the same way as described in the chapter (4.2.1). If the presence of a conserved domain was detected, we addressed Conserved Domain Database (available at www.ncbi.nlm.nih.gov/cdd/) for the assessment of similarity significance between the hits. Acquired results may serve as direction indicators for making functional predictions, as well as contemplating possible nature of the mass spectrometry hits as T3SS effectors.

Protein KAK70248.1, annotated as putative N-acetyltransferase YedL OS, is a hypothetical protein encoded by L530_4327 locus and consisting of 342 amino acid residues. BLAST similarity alignment detected presence of a putative conserved domain. Non-specific hit on the region from 42 to 340 residues for protein of an unknown function, belonging to the superfamily of nudix hydrolases, was found. Enzymes of this superfamily are known to contain a metal-binding catalytic site and be especially active during the cell cycle or periods of stress. Substrate hydrolysis, carried out by these enzymes, is of primary importance for sanitizing of the nucleotide pools and maintenance of cell viability. In an alignment to *B. pertussis*, *B. trematum* and *Achromobacter* spp., the search hit a hypothetical membrane protein.

Protein KAK66808.1, annotated as putative N-acetyltransferase YedL OS, is a hypothetical protein encoded by L530_1541 locus and consisting of 269 amino acid residues. BLAST search to the query sequence yielded a specific hit of an amino acid transport system which has been already annotated in other *Bordetella* species, including several strains of *B. bronchiseptica*. According to conserved domain architecture analysis, KAK66808.1 protein may belong to ABC (ATP-binding cassette) transporter superfamily. Members of the superfamily, showing high similarity among

the group, are responsible for transportation of organic molecules across cytoplasmic membrane.

Protein KAK74435.1, annotated as putative N-acetyltransferase YedL OS, is a small hypothetical protein encoded by L530_1943 locus and consisting of 93 amino acid residues. No conserved domains were detected and the most significant BLAST search results led to “hypothetical” or “uncharacterised” proteins of *Bordetella* spp. and *Achromobacter* spp.

Protein KAK75714.1, annotated as putative N-acetyltransferase YedL OS is a small hypothetical protein encoded by L530_3172 locus and consisting of 99 amino acid residues. No conserved domains were detected. Identical proteins were found across the *Bordetella* genus, with no proposed function.

Protein KAK67391.1, annotated as Clp protease containing ATP-binding subunit, is a 434 amino acid residues long polypeptide. Detected putative conserved domains pointed out its specific association with ClpX superfamily which comprises endopeptidases with ATP-dependent chaperone activity.

Protein KAK75306.1, annotated as T3SS cytotoxic effector BteA OS, is the only surely known *Bordetella* effector protein of the T3SS. It was more extensively described in the previous chapter (1.3.4.1)

5. Discussion

Indisputably, a T3S injectisome is of crucial importance for gram-negative bacteria. Spanning bacterial cell wall, it permits coupling of secretion and translocation of proteins into the eukaryotic cell [85]. It is vitally important to understand that, in the process of the injectisome assembly, different types of substrates, not exclusively effectors, are exported. Firstly, the T3S apparatus recognizes and exports its own distal components, as are those of inner rod (SctI), needle filament (SctF), tip complex (LcrV in *Yersinia* spp.) and early substrates (YopB and YopD in *Yersinia* spp.). Export of the effector proteins occurs only if an appropriate trigger has been introduced. Myriads of T3SS effector proteins are known with around seven to thirty being delivered into the host cell by an individual bacterial species [51]. In *Bordetella* genus, the scale is strikingly different. So far, only two effector proteins have been documented – BteA [80] and BopN [84], with the latter's nature as an effector being questionable. This contrasting difference gave us an inspiration and became our starting point in developing a strategy as to how detection of other possibly existing T3SS virulence factors might be achieved.

Currently, the scientific hunt for bacterial effectors employs *in silico* genomic and proteomic approaches, capture compound technologies and mass spectrometry analysis. In our case, considering *B. bronchiseptica* D445 (MO211) physiology, degree of genome annotation accuracy and facilities available, we chose a combination of *Bordetella* genomic engineering and mass spectrometry. It is necessary to point out that our experiments were done only twice. Subsequently, one more independent experiment is required to prove reliability of the results.

Two strains, wild type and T3SS-deficient mutant Δ*bscN*, were used in our experiments. Processing of mass spectrometry data highlighted 14 proteins which were secreted to the supernatant by wild type but not by Δ*bscN* strain (**Tab.2**). It means that transport of these proteins out of the bacterial cell is dependent on the T3SS ATPase. Although the secretion of *Bordetella* T3SS effector translocases (BopB and BopD) and a tip complex protein Bsp22 was already apparent from the FASTA headers, proteins transported via the injectisome were described by the software in an obscure way. As a

result, for other detected proteins it was still unclear whether they were distal components of the T3SS, already known effectors or yet uncharacterized polypeptides.

We resorted to bioinformatical tools presuming that names assigned to the T3SS locus of *B. bronchiseptica* D445 (MO211) would clarify our mass spectrometry results. The task turned out to be rather complex.

DNA sequencing, with its first successful attempts dated back to the 1970s, has evolved to be a powerful tool bringing about a tremendous change in our understanding of the biochemical properties of life. Nowadays, whole genome sequences for more than twenty thousand organisms are available. Scientific community faces a great host of data collected, in view of "omics" methods being rather favoured in microbiological research over last decades. However, the main issue of bioinformatics techniques is the one of quantity turning into quality in the process of genome annotation and analysis. For all that, confusion occasionally takes place resulting in misinterpretation of individual genes name, function or even existence.

Genus *Bordetella* is not an exception. Due to the recent increase in whooping cough incidence, leading research teams all over the world started taking note of other *B. pertussis* virulence determinants than those covered by the current vaccines. The T3SS of bordetellae, unusually administering secretion of only one effector protein, appears to be especially conspicuous. Notably, the vast majority of research conducted on the T3SS was held in *B. bronchiseptica* since the species offers a unique opportunity to study bacterial infection in a relevant animal model. Moreover, it is sufficiently amenable to genetic manipulation and in-culture growth. In addition, *B. bronchiseptica* D445 (MO211) belongs to complex IV of *B. bronchiseptica* strains, which is evolutionary closer to *B. pertussis* than complex I and, albeit able to infect animals, is far more often isolated from humans [7, 86]. Taking all this into account, proper annotation is imperative to further exploration. European Bioinformatics Institute currently provides reference genome annotations for 415 strains of bordetellae with more coming each day. Obviously, *B. pertussis* genome is the most thoroughly and accurately analysed while *B. bronchiseptica* D445 (MO211) T3SS genes were assigned with evidently imprecise identification.

This was the reason why we decided to perform *in silico* analysis of *B. bronchiseptica* D445 (MO211) genome.

As visible from **Fig.5**, *bsc-btr* loci from *B. pertussis* Tohama I and *B. bronchiseptica* D445 (MO211) show great similarity with more detailed description available in **Tab.4**. Although diverse regions – accented in **Fig.5C** with black vertical lines – are present, the gene order, as well as the strand transcribed, is identical. Undoubtedly, such a high degree of conservation is governed by close evolutionary relatedness of the bacteria. The organisation of *Y. enterocolitica* Yop-Ysc cluster appears to be markedly different. However, if one shades the homologous for *Yersinia* spp. and *Bordetella* spp. genes in the same colour, a discernible pattern comes out. The genes of the effector translocases YopB and YopD/BopB and BopD (yellow in **Fig.5AB** and **Fig.5D**) are clustered together with the gene of a polymerizing tip protein located right upstream – *icrV* (**Fig.5D**) in *Yersinia* spp. and *bsp22* (**Fig.5AB**) in *Bordetella* spp. Likewise, a group of genes between *yscN*-*yscU* (**Fig.5D**) exhibit the same structure as *bscN*-*bscU* (**Fig.5AB**), however, contrasting in the strand position, and consequently direction of transcription. Further, *yscJ*-*yscK*-*yscL* conjunction agrees well with *bscJ*-*bscK*-*bscL*, even though, most probably, relocation by means of chromosomal inversion occurred. Preservation of the genes reciprocal arrangement is widely described in the literature as a sign of horizontal gene acquisition [54]. Noteworthy, the previously described examples correspond to this model with considerable exactitude, and additionally suggest the T3SS genes evolution in the form of intact genetic blocks.

To look at the problem from another angle, we proposed a model of the T3SS based on works of [87], [72] and [88]. It further indicates relevancy of the asserted in **Fig.5** and **Tab.4** *Bordetella*-*Yersinia* homologies. What is more, our visualisation serves as a prototype from which the *Bordetella* T3SS genes products could be linked to a specific location and/or function in the injectisome operation.

Furthermore, the decision to analyse *bsc-btr* locus proved to be especially practical in matching the MS hits to the ORF in the T3SS locus. As could be seen in **Tab.4**, not only the translocases and the tip compound are secreted out of the bacterial cell but also other proteins encoded by *bsc-btr* locus: BscI – an inner rod component;

BopN – a post-transcriptional regulator; BcrH1 – a chaperone and BtrA – a transcriptional regulator.

No function was predicted for the protein with an identification KAK65919.1, significantly aligned to BP2259 of *B. pertussis*. The protein is supposedly 136 amino acid residues long. It is encoded by L530_1641 locus which is in the literature [72] referred as *orf3*. According to BLAST search, this protein belongs to type III secretion system YscX superfamily. The NCBI description states that members of the superfamily are required for secretion of Yops effectors and, therefore, are believed to be part of the secretion machinery (www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi). A recent study reports on evolutionary conservation among the YscX family across the species and characterises partner interactions of *ycsX* genetic equivalents [89]. The study further suggests YscX role in the physical assembly of *Yersinia* spp. T3S apparatus. Experimental work is needed to assume the same hypothesis for KAK65919.1 protein to be true.

MS hits – KAK70248.1, KAK66808.1, KAK67391.1, KAK74435.1 and KAK75714.1 – were located outside the *bsc-btr* locus. Among them, KAK70248.1 and KAK67391.1 could be viewed as the most interesting, since they were found to be homologous to the proteins with enzymatic activity (2.2.2). As was mentioned in the literature review, a great part of the known T3SS effectors are enzymes subverting the host cell metabolism [61] [55]. Proteins KAK74435.1 and KAK75714.1 are long 93 and 99 amino acid residues, respectively. If compared to the majority of known effectors of other bacteria, this length appears to be insufficient to carry out any effector function. However, a precedent of similarly small protein from *Salmonella enterica* has been recently published [90]. Importantly, BteA effector was detected, with the production increase level of 154.54, confirming validity of our approach.

While homologies are present between some of T3SS-possessing bacteria, most often the effectors are quite distinct [85]. This fact might have considerably limited the positive outcome of the BLAST search. More sophisticated versions of software were developed in order to meet T3SS effectors features and subsequently improve their *in silico* detection. Certain prediction methods of putative T3SS effectors are based on similarities of amino acid composition and residues physicochemical properties in such

domains [91, 92]. Special programs were design to target T3SS components using genomic sequences [93]. A recent study claims that it is possible to directly and accurately identify T3SS effectors by a new approach using protein N-terminal sequences [94]. Future perspectives of the thesis may include adoption of specific tools for identification of new effectors instead of using the available general annotation.

Although sequence analyses of bacterial genomes yield much invaluable information, it is still necessary to isolate mutants and apply biochemical and physiological tests to accurately characterize proteins of unknown function. After repeating the mass spectrometry analysis once more, we further plan to profoundly probe the existing protein candidates, as well as experimenting with different growth conditions to activate secretion of so far unknown *Bordetella* effectors and mimic T3SS interaction with the host cell membrane.

6. Conclusions

The thesis has proposed the evaluation of the severity of *Bordetella*-associated diseases, as well as extensively reviewing the role of the T3SS in bacterial virulence. The infectious burden was assessed in five major species of susceptible domestic animals with the conclusion that *Bordetella* spp. do not appear to be a primary cause of considerable economical losses. The T3SS virulence determinant was shown to play a crucial part in many gram-negative bacteria pathogenesis by the means of its effectors. However, definite determination of its role in *Bordetella* spp. awaits more profound and elaborate investigation.

The practical part aimed to be a functional contribution to the topic. Its first section revealed the presence of a few so far uncharacterized proteins likely secreted through the T3SS. Potentially, some of these proteins could play a role as the T3SS effectors. However, experimental procedures are required to prove or refute the hypothesis. The second part was designed to elucidate the T3SS locus nomenclature in *B. bronchiseptica* D445 (MO211) strain which was achieved by bioinformatics approach. More accurate version of the locus may serve as a tool for further research concerning the T3SS in *Bordetella*, as well as its effector proteins.

7. References

1. Austin, B., *The Family Alcaligenaceae*, in *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*, E. Rosenberg, et al., Editors. 2014, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 729-757.
2. Grespan, A., et al., *Virulence and molecular aspects of Bordetella avium isolated from cockatiel chicks (Nymphicus hollandicus) in Brazil.* Vet Microbiol, 2012. **160**(3-4): p. 530-4.
3. Majewski, L.L., et al., *Bordetella trematum sepsis with shock in a diabetic patient with rapidly developing soft tissue infection.* Diagn Microbiol Infect Dis, 2016. **86**(1): p. 112-4.
4. Moissenet, D., et al., *Septic arthritis caused by Bordetella holmesii in an adolescent with chronic haemolytic anaemia.* J Med Microbiol, 2011. **60**(Pt 11): p. 1705-7.
5. Register, K.B. and R.A. Kunkle, *Strain-specific virulence of Bordetella hinzii in poultry.* Avian Dis, 2009. **53**(1): p. 50-4.
6. Chiappini, E., et al., *Pertussis re-emergence in the post-vaccination era.* BMC infectious diseases, 2013. **13**: p. 151-151.
7. Linz, B., et al., *Acquisition and loss of virulence-associated factors during genome evolution and speciation in three clades of Bordetella species.* BMC Genomics, 2016. **17**(1): p. 767.
8. Locht, C., *Bordetella Molecular Microbiology*. 2007: Horizon Bioscience.
9. Mattoo, S. and J.D. Cherry, *Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies.* Clin Microbiol Rev, 2005. **18**(2): p. 326-82.
10. Arico, B. and R. Rappuoli, *Bordetella parapertussis and Bordetella bronchiseptica contain transcriptionally silent pertussis toxin genes.* J Bacteriol, 1987. **169**(6): p. 2847-53.
11. Graf, R., et al., *A truncated recombinant alpha subunit of Gi3 with a reduced affinity for beta gamma dimers and altered guanosine 5'-3-O-(thio)triphosphate binding.* J Biol Chem, 1992. **267**(34): p. 24307-14.
12. Bock, A. and R. Gross, *The BvgAS two-component system of Bordetella spp.: a versatile modulator of virulence gene expression.* Int J Med Microbiol, 2001. **291**(2): p. 119-30.
13. Bone, M.A., et al., *PlrSR regulatory system controls BvgAS activity and virulence in the lower respiratory tract.* Proceedings of the National Academy of Sciences, 2017. **114**(8): p. E1519-E1527.
14. Coutte, L., et al., *The multifaceted RisA regulon of Bordetella pertussis.* Scientific reports, 2016. **6**: p. 32774-32774.
15. Akerley, B.J., et al., *The bvgAS locus negatively controls motility and synthesis of flagella in Bordetella bronchiseptica.* J Bacteriol, 1992. **174**(3): p. 980-90.
16. Ko, K.S., et al., *New species of Bordetella, Bordetella ansorpii sp. nov., isolated from the purulent exudate of an epidermal cyst.* J Clin Microbiol, 2005. **43**(5): p. 2516-9.
17. Fry, N.K., et al., *The first UK isolate of 'Bordetella ansorpii' from an immunocompromised patient.* J Med Microbiol, 2007. **56**(Pt 7): p. 993-5.

18. Goodnow, R.A., *Biology of Bordetella bronchiseptica.* Microbiological Reviews, 1980. **44**(4): p. 722-738.
19. Hulse, L.S., et al., *Development and application of two multiplex real-time PCR assays for detection and speciation of bacterial pathogens in the koala.* J Vet Diagn Invest, 2018. **30**(4): p. 523-529.
20. Gueirard, P., et al., *Human Bordetella bronchiseptica infection related to contact with infected animals: persistence of bacteria in host.* J Clin Microbiol, 1995. **33**(8): p. 2002-6.
21. de la Fuente, J., et al., *Bordetella bronchiseptica pneumonia in a patient with AIDS.* Thorax, 1994. **49**(7): p. 719-20.
22. Rampelotto, R.F., et al., *Pneumonia caused by Bordetella bronchiseptica in two HIV-positive patients.* Sao Paulo Med J, 2016. **134**(3): p. 268-72.
23. Switzer, W.P., *Studies on infectious atrophic rhinitis. V. Concept that several agents may cause turbinate atrophy.* Am J Vet Res, 1956. **17**(64): p. 478-84.
24. Cross, R.F., *Bordetella bronchiseptica-induced porcine atrophic rhinitis.* J Am Vet Med Assoc, 1962. **141**: p. 1467-8.
25. Magyar, T., et al., *The pathogenesis of turbinate atrophy in pigs caused by Bordetella bronchiseptica.* Vet Microbiol, 1988. **18**(2): p. 135-46.
26. Nicholson, T.L., et al., *The Bordetella bronchiseptica type III secretion system is required for persistence and disease severity but not transmission in swine.* Infection and immunity, 2014. **82**(3): p. 1092-1103.
27. de Jong, M.F. and J.P. Nielsen, *Definition of progressive atrophic rhinitis.* Vet Rec, 1990. **126**(4): p. 93.
28. de Jong, M.F., F.T. Bouwkamp, and R.A. Oosterwoud, *A field evaluation of Nobi-Vac atrophic rhinitis vaccine.* Vet Q, 1987. **9**(1): p. 49-59.
29. Kumar, S., et al., *Occurrence of Bordetella infection in pigs in northern India.* Int J Microbiol, 2014. **2014**: p. 238575.
30. McCandlish, I.A., H. Thompson, and N.G. Wright, *Vaccination against canine bordetellosis: protection from contact challenge.* Vet Rec, 1978. **102**(22): p. 479-83.
31. Priestnall, S.L., et al., *New and emerging pathogens in canine infectious respiratory disease.* Vet Pathol, 2014. **51**(2): p. 492-504.
32. Decaro, N., et al., *Molecular surveillance of traditional and emerging pathogens associated with canine infectious respiratory disease.* Vet Microbiol, 2016. **192**: p. 21-25.
33. Ellis, J., et al., *Seroepidemiology of respiratory (group 2) canine coronavirus, canine parainfluenza virus, and Bordetella bronchiseptica infections in urban dogs in a humane shelter and in rural dogs in small communities.* Can Vet J, 2011. **52**(8): p. 861-8.
34. Sowman, H.R., N.J. Cave, and M. Dunowska, *A survey of canine respiratory pathogens in New Zealand dogs.* N Z Vet J, 2018. **66**(5): p. 236-242.
35. Binns, S.H., et al., *Prevalence and risk factors for feline Bordetella bronchiseptica infection.* Vet Rec, 1999. **144**(21): p. 575-80.
36. Bannasch, M.J. and J.E. Foley, *Epidemiologic evaluation of multiple respiratory pathogens in cats in animal shelters.* J Feline Med Surg, 2005. **7**(2): p. 109-19.
37. Deeb, B.J., et al., *Pasteurella multocida and Bordetella bronchiseptica infections in rabbits.* Journal of clinical microbiology, 1990. **28**(1): p. 70-75.

38. Glavits, R. and T. Magyar, *The pathology of experimental respiratory infection with Pasteurella multocida and Bordetella bronchiseptica in rabbits.* Acta Vet Hung, 1990. **38**(3): p. 211-5.
39. Pathak, A.K., et al., *Immuno-epidemiology of chronic bacterial and helminth co-infections: observations from the field and evidence from the laboratory.* Int J Parasitol, 2012. **42**(7): p. 647-55.
40. Cullinane, L.C., et al., *Bordetella parapertussis from lambs.* N Z Vet J, 1987. **35**(10): p. 175.
41. Chen, W., et al., *Pneumonia in lambs inoculated with Bordetella parapertussis: bronchoalveolar lavage and ultrastructural studies.* Vet Pathol, 1988. **25**(4): p. 297-303.
42. Porter, J.F., et al., *Predisposition of specific pathogen-free lambs to Pasteurella haemolytica pneumonia by Bordetella parapertussis infection.* J Comp Pathol, 1995. **112**(4): p. 381-9.
43. Brogden, K.A., H.D. Lehmkuhl, and R.C. Cutlip, *Pasteurella haemolytica complicated respiratory infections in sheep and goats.* Vet Res, 1998. **29**(3-4): p. 233-54.
44. Appel, M. and D.A. Bemis, *The canine contagious respiratory disease complex (kennel cough).* Cornell Vet, 1978. **68 Suppl 7**: p. 70-5.
45. Baskerville, A., *Pneumonia of pigs: a review.* N Z Vet J, 1981. **29**(11): p. 216-8.
46. Pedersen, K.B. and K. Barfod, *The aetiological significance of Bordetella bronchiseptica and Pasteurella multocida in atrophic rhinitis of swine.* Nord Vet Med, 1981. **33**(12): p. 513-22.
47. Rutter, J.M., *Quantitative observations on Bordetella bronchiseptica infection in atrophic rhinitis of pigs.* Vet Rec, 1981. **108**(21): p. 451-4.
48. Gamble, K.C., and M.M. Clancy, *Infectious Disease Manual*, Infectious Disease Committee, Editor. 2013, American Association of Zoo Veterinarians: Yulee, Florida. p. 1098 pp.
49. Farrington, D.O. and R.D. Jorgenson, *Prevalence of Bordetella bronchiseptica in certain central Iowa.* J Wildl Dis, 1976. **12**(4): p. 523-5.
50. Loong, S.K., et al., *Recovery of Bordetella bronchiseptica sequence type 82 and B. pseudohinzii from urban rats in Terengganu, Malaysia.* J Vet Med Sci, 2018. **80**(1): p. 77-84.
51. Cornelis, G.R., *The type III secretion injectisome.* Nat Rev Microbiol, 2006. **4**(11): p. 811-25.
52. Porwollik, S., et al., *Characterization of Salmonella enterica subspecies I genovars by use of microarrays.* J Bacteriol, 2004. **186**(17): p. 5883-98.
53. Buttner, D. and S.Y. He, *Type III protein secretion in plant pathogenic bacteria.* Plant Physiol, 2009. **150**(4): p. 1656-64.
54. Troisfontaines, P. and G.R. Cornelis, *Type III secretion: more systems than you think.* Physiology (Bethesda), 2005. **20**: p. 326-39.
55. Hueck, C.J., *Type III protein secretion systems in bacterial pathogens of animals and plants.* Microbiol Mol Biol Rev, 1998. **62**(2): p. 379-433.
56. Nguyen, L., et al., *Phylogenetic analyses of the constituents of Type III protein secretion systems.* J Mol Microbiol Biotechnol, 2000. **2**(2): p. 125-44.
57. Hu, B., et al., *In Situ Molecular Architecture of the Salmonella Type III Secretion Machine.* Cell, 2017. **168**(6): p. 1065-1074.e10.

58. Galan, J.E., et al., *Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells.* Annu Rev Microbiol, 2014. **68**: p. 415-38.
59. Chakravortty, D., et al., *Formation of a novel surface structure encoded by Salmonella Pathogenicity Island 2.* Embo j, 2005. **24**(11): p. 2043-52.
60. Blocker, A., et al., *The tripartite type III secreton of Shigella flexneri inserts IpaB and IpaC into host membranes.* J Cell Biol, 1999. **147**(3): p. 683-93.
61. Dean, P., *Functional domains and motifs of bacterial type III effector proteins and their roles in infection.* FEMS Microbiol Rev, 2011. **35**(6): p. 1100-25.
62. Page, A.L. and C. Parsot, *Chaperones of the type III secretion pathway: jacks of all trades.* Mol Microbiol, 2002. **46**(1): p. 1-11.
63. Andersson, K., et al., *YopH of Yersinia pseudotuberculosis interrupts early phosphotyrosine signalling associated with phagocytosis.* Mol Microbiol, 1996. **20**(5): p. 1057-69.
64. Aepfelbacher, M., et al., *Activity modulation of the bacterial Rho GAP YopE: an inspiration for the investigation of mammalian Rho GAPs.* Eur J Cell Biol, 2011. **90**(11): p. 951-4.
65. Galyov, E.E., et al., *A secreted protein kinase of Yersinia pseudotuberculosis is an indispensable virulence determinant.* Nature, 1993. **361**(6414): p. 730-2.
66. Schroeder, G.N. and H. Hilbi, *Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion.* Clin Microbiol Rev, 2008. **21**(1): p. 134-56.
67. Bodey, G.P., et al., *Infections caused by Pseudomonas aeruginosa.* Rev Infect Dis, 1983. **5**(2): p. 279-313.
68. Hauser, A.R., *The type III secretion system of Pseudomonas aeruginosa: infection by injection.* Nat Rev Microbiol, 2009. **7**(9): p. 654-65.
69. Lam, H., et al., *Synthetic Cyclic Peptomers as Type III Secretion System Inhibitors.* Antimicrobial agents and chemotherapy, 2017. **61**(9): p. e00060-17.
70. Yuk, M.H., E.T. Harvill, and J.F. Miller, *The BvgAS virulence control system regulates type III secretion in Bordetella bronchiseptica.* Mol Microbiol, 1998. **28**(5): p. 945-59.
71. Fauconnier, A., et al., *Characterization of the type III secretion locus of Bordetella pertussis.* Int J Med Microbiol, 2001. **290**(8): p. 693-705.
72. Ahuja, U., et al., *Differential regulation of type III secretion and virulence genes in Bordetella pertussis and Bordetella bronchiseptica by a secreted anti-sigma factor.* Proc Natl Acad Sci U S A, 2016. **113**(9): p. 2341-8.
73. Nogawa, H., et al., *The type III secreted protein BopD in Bordetella bronchiseptica is complexed with BopB for pore formation on the host plasma membrane.* J Bacteriol, 2004. **186**(12): p. 3806-13.
74. Medhekar, B., et al., *Bordetella Bsp22 forms a filamentous type III secretion system tip complex and is immunoprotective in vitro and in vivo.* Mol Microbiol, 2009. **71**(2): p. 492-504.
75. Stockbauer, K.E., A.K. Foreman-Wykert, and J.F. Miller, *Bordetella type III secretion induces caspase 1-independent necrosis.* Cell Microbiol, 2003. **5**(2): p. 123-32.
76. Yuk, M.H., et al., *Modulation of host immune responses, induction of apoptosis and inhibition of NF-kappaB activation by the Bordetella type III secretion system.* Mol Microbiol, 2000. **35**(5): p. 991-1004.

77. Legarda, D., et al., *Suppression of NF-kappaB-mediated beta-defensin gene expression in the mammalian airway by the Bordetella type III secretion system.* Cell Microbiol, 2005. **7**(4): p. 489-97.
78. Skinner, J.A., et al., *Bordetella type III secretion modulates dendritic cell migration resulting in immunosuppression and bacterial persistence.* J Immunol, 2005. **175**(7): p. 4647-52.
79. Pilione, M.R. and E.T. Harvill, *The Bordetella bronchiseptica type III secretion system inhibits gamma interferon production that is required for efficient antibody-mediated bacterial clearance.* Infect Immun, 2006. **74**(2): p. 1043-9.
80. Panina, E.M., et al., *A genome-wide screen identifies a Bordetella type III secretion effector and candidate effectors in other species.* Mol Microbiol, 2005. **58**(1): p. 267-79.
81. French, C.T., et al., *The Bordetella type III secretion system effector BteA contains a conserved N-terminal motif that guides bacterial virulence factors to lipid rafts.* Cell Microbiol, 2009. **11**(12): p. 1735-49.
82. Guttman, C., et al., *Characterization of the N-terminal domain of BteA: a Bordetella type III secreted cytotoxic effector.* PLoS One, 2013. **8**(1): p. e55650.
83. Hegerle, N., et al., *In-vitro and in-vivo analysis of the production of the Bordetella type three secretion system effector A in Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica.* Microbes Infect, 2013. **15**(5): p. 399-408.
84. Nagamatsu, K., et al., *Bordetella evades the host immune system by inducing IL-10 through a type III effector, BopN.* J Exp Med, 2009. **206**(13): p. 3073-88.
85. Coburn, B., I. Sekirov, and B.B. Finlay, *Type III secretion systems and disease.* Clin Microbiol Rev, 2007. **20**(4): p. 535-49.
86. Diavatopoulos, D.A., et al., *Bordetella pertussis, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of B. bronchiseptica.* PLoS Pathog, 2005. **1**(4): p. e45.
87. Pallen, M.J., S.A. Beatson, and C.M. Bailey, *Bioinformatics analysis of the locus for enterocyte effacement provides novel insights into type-III secretion.* BMC Microbiol, 2005. **5**: p. 9.
88. Vander Broek, C.W. and J.M. Stevens, *Type III Secretion in the Melioidosis Pathogen Burkholderia pseudomallei.* Front Cell Infect Microbiol, 2017. **7**: p. 255.
89. Gurung, J.M., et al., *Heterologous Complementation Studies With the YscX and YscY Protein Families Reveals a Specificity for Yersinia pseudotuberculosis Type III Secretion.* Front Cell Infect Microbiol, 2018. **8**: p. 80.
90. Bayer-Santos, E., et al., *The Salmonella Effector SteD Mediates MARCH8-Dependent Ubiquitination of MHC II Molecules and Inhibits T Cell Activation.* Cell Host Microbe, 2016. **20**(5): p. 584-595.
91. Arnold, R., et al., *Sequence-based prediction of type III secreted proteins.* PLoS Pathog, 2009. **5**(4): p. e1000376.
92. McDermott, J.E., et al., *Computational prediction of type III and IV secreted effectors in gram-negative bacteria.* Infect Immun, 2011. **79**(1): p. 23-32.
93. Martinez-Garcia, P.M., C. Ramos, and P. Rodriguez-Palenzuela, *T346Hunter: a novel web-based tool for the prediction of type III, type IV and type VI secretion systems in bacterial genomes.* PLoS One, 2015. **10**(4): p. e0119317.

94. Xue, L., et al., *DeepT3: deep convolutional neural networks accurately identify Gram-Negative Bacterial Type III Secreted Effectors using the N-terminal sequence.* Bioinformatics, 2018.