

Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
BIOLOGIA CELLULARE E MOLECOLARE

Ciclo XXXI

Settore Concorsuale: SSC 05/E2

Settore Scientifico Disciplinare: SSD BIO/11

TITOLO TESI

**Atypical LONELY GUY protein in *Bordetella pertussis*
synthetizes a cytokinin-like compound negatively
related to oxidative stress**

Presentata da: *Filippo Moramarco*

Coordinatore Dottorato

Prof. Giovanni Capranico

Supervisore

Prof. Vincenzo Scarlato

Co-Supervisore

Prof. Enrico Balducci

Esame finale anno 2019

Table of Contents

ABBREVIATIONS AND ACRONYMS	4
1. INTRODUCTION	6
1.1. Pertussis Disease	7
1.1.1. Infection and transmission	7
1.1.2. Clinical course	8
1.2. <i>Bordetella Pertussis</i>	9
1.2.1. The two-component regulatory system BvgAS	10
1.2.2. Virulence factors	11
1.3. LONELY GUY and cytokinins	13
2. AIM OF WORK	17
3. MATERIALS AND METHODS	20
3.1. Bioinformatics analysis	21
3.2. Bacterial strains and growth conditions	21
3.3. Generation of <i>E. coli</i> expressing recombinant BP1253 protein and purification procedure	21
3.4. Size-exclusion chromatographic analysis	22
3.5. Multi-angle light scattering	23
3.6. Generation of the bp1253 knockout strain	23
3.7. High-throughput purification of bp1253 mutants	24
3.8. SDS-page and western blot	24
3.9. Phosphoribohydrolase activity assay	25
3.10. Generation of mouse immune sera	25
3.11. Surface plasmon resonance experiments (Biacore)	26
3.12. Identification and characterization of cytokinins by LC-MS/MS	26
3.13. Phenotypic characterization	27
3.14. Statistical analysis	27
3.15. Protein assay	28

4. RESULTS	29
4.1. Production and purification of recombinant BP1253	30
4.2. In silico-based identification of BP1253 as a member of the LONELY GUY family	32
4.3. BP1253 binds nucleotides	34
4.4. BP1253 has phosphoribohydrolase activity	38
4.5. BpLOG synthesizes 6-O-Methylguanine	45
4.6. BpLOG is prevalently expressed in clinical isolates	48
4.7. BpLOG is negatively related to oxidative stress	49
5. DISCUSSION	52
6. BIBLIOGRAPHY	59

ABBREVIATIONS AND ACRONYMS

5-FU	5-fluorouracil
ACT	Adenylate cyclase toxin
AMP	Adenosine 5'-monophosphate
aP	Acellular pertussis
<i>B.</i>	<i>Bordetella</i>
<i>Bp</i>	<i>Bordetella pertussis</i>
cAMP	cyclic adenosine 5'-monophosphate
CHASE	Cyclase/histidine kinases-associated sensing extracellular
DNT	Dermonecrotic toxin
FHA	Filamentous haemagglutinin
FhaC	Filamentous haemagglutinin transporter protein
FIM	Fimbriae
HAP	6-N-hydroxylaminopurine
HK	Histidine kinase
Hpt	Histidine phosphotransferase
Kd	Dissociation constant
LDC	Lysine decarboxylase
LOG	LONELY GUY
LOS	Lipooligosaccharide
<i>M.</i>	<i>Mycobacterium</i>
MALS	Multi-angle light scattering
MCD	Mature C terminus distal
NO	Nitric oxide
NOS	Reactive nitrogen species
<i>P.</i>	<i>Pseudomonas</i>
pHBA	<i>para</i> -hydroxybenzaldehyde
PLP	Pyridoxal 5'-phosphate
PRN	Pertactin
PT	Pertussis toxin
Rec	Receiver
RTX	Repeats in toxin

RMS	Root Mean Squared
ROS	Reactive oxygen species
SEC	Size-exclusion chromatographic
SPR	Surface Plasmon resonance
StIR	Stimulated Innate Resistance
TCT	Tracheal cytotoxin
VFT	Venus flytrap
wP	Whole-cell pertussis

1. INTRODUCTION

1.1 PERTUSSIS DISEASE

Pertussis is a highly contagious infection of the human respiratory tract. The disease is commonly known as “whooping cough”, because of the characteristic inspiratory sound that infected people emit after cough fits. In fact, the violent and uncontrollable cough that distinguishes this disease often makes hard to breathe, leading ill people to deep breaths, which ring out as a “whooping” sound. Pertussis disease is a significant cause of morbidity and mortality in infants worldwide and despite high vaccination coverage, it continues to be a public health concern (World Health Organization 2015).

The causative agent of whooping cough is the human pathogen *Bordetella (B.) pertussis (Bp)*, a Gram-negative bacterium isolated and described for the first time by Jules Bordet and Octave Gengou in 1906 (Lynfield and Schaffner 2014, Kuchar, Karlikowska-Skwarnik et al. 2016). Pertussis disease is endemic in all countries and affects people of all ages, but it could be particularly intense with a fatal clinical course for young infants, especially of prevaccination age (Paddock, Sanden et al. 2008).

1.1.1. INFECTION AND TRANSMISSION

The site of the infection is the respiratory tract (Mallory and Hornor 1912). Indeed Pertussis colonization begins with the adherence of *B. pertussis* solely to the tufts of ciliated epithelial cells of the upper respiratory tract: nasopharynx and trachea (Tuomanen and Hendley 1983) (Fig. 1). Following this attachment *B. pertussis* starts to exert its virulence producing several factors such as adhesins, immune-modulators and toxins which damage the cilia and cause airways to swell. The combined action of all these virulence factors prevents rapid clearance of the bacteria and allows them to replicate and to disseminate to the lower areas of the respiratory tract (de Gouw, Diavatopoulos et al. 2011, Zlamy 2016).

Bordetella pertussis is transmitted from infected to susceptible individuals via aerosolized respiratory droplets released through coughing and sneezing (Warfel, Beren et al. 2012, Trainor, Nicholson et al. 2015). Whooping cough is highly contagious at the first stages of the disease and could be transmitted for three weeks or more without therapeutic intervention. In particular, vaccinated adults and adolescent, who do not manifest infection symptoms but host the pathogen, represent the major source of

transmission of bacteria to unvaccinated young infants (World Health Organization 2015). It has been demonstrated that acellular pertussis (aP) vaccines protect against the developments of pertussis symptoms but not against colonization and transmission (Warfel, Zimmerman et al. 2014).

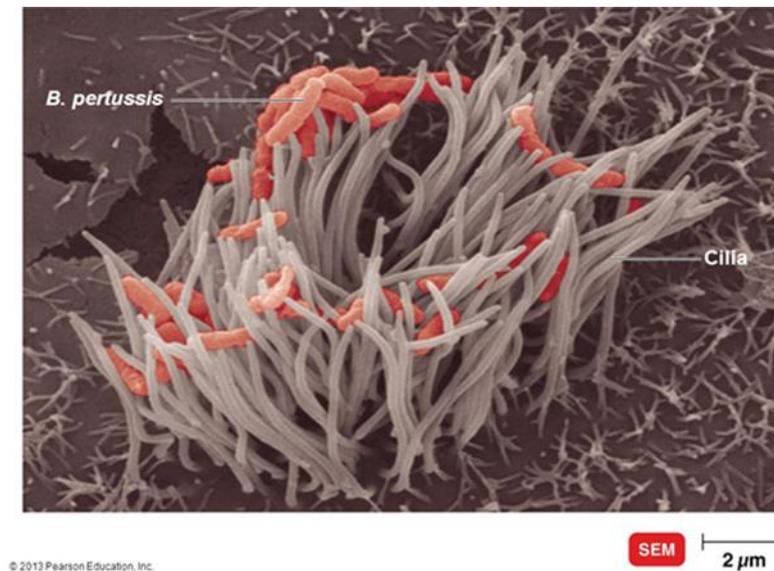


Figure 1. *Bordetella pertussis* colonization. Image at SEM of *Bordetella Pertussis* while adheres exclusively to cilia of epithelial cells in the upper respiratory tract during infection. From <https://slideplayer.com/slide/4651572/>

1.1.2 CLINICAL COURSE

Pertussis clinical course may vary depending on various factors: age, presence of transplacentally acquired antibodies and previous infection or vaccination (McGregor, Ogle et al. 1986, Elliott, McIntyre et al. 2004, Kowalzik, Barbosa et al. 2007). Classical pertussis, seen mainly in unimmunized children, has an average of incubation period of 7 to 10 days and it could last from 6 to 12 weeks. The illness is characterized by three stages: **catarrhal**, **paroxysmal** and **convalescent** phase. The **catarrhal** phase lasts from 1 to 2 weeks and presents symptoms similar to those of many upper respiratory infections, like nasal congestion, lacrimation, rhinorrhea, tolerant cough and in few cases fever. In fact, it is not immediate to diagnose whooping cough at this stage. When the cough gets worse; pertussis progresses to the **paroxysmal** phase. Characteristic of this stage is the paroxysm episodes that increase in incidence and severity. They are

repetitive series of forceful coughs during a single expiration, followed by forced inspiration that produces the distinctive whoop. The post-tussive vomiting is usually a consequence of the paroxysms (Melvin, Scheller et al. 2014). The paroxysmal stage may last from 2 to 6 weeks and represents a very critical phase for infected infants less than 6 months. Unfortunately they do not have the strength to have a whoop and therefore, fall into a state of apnea resulting in bradycardia, cyanosis and unresponsiveness (Christie and Baltimore 1989). Only in the last phase, the **convalescent** stage, the adaptive immunity begins to execute the bacterial clearance. However the cessation of symptoms, paroxysms, whooping and vomiting, occurs gradually in a period of time ranging from 1 to 12 weeks (Melvin, Scheller et al. 2014, Nieves and Heininger 2016).

In immunized or partially immune children, adolescents and adults, the clinical manifestations are much milder and could include coryza and cough, but without whoop (Nieves and Heininger 2016). In infants, less than a year old, the clinical course could evolve in a much more severe scenario. The bacteria can disseminate in lungs and induce bronchiolitis necrosis, intra-alveolar hemorrhage and fibrinous edema (Paddock, Sanden et al. 2008). In severe cases an excessive lymphocytosis could trigger a pulmonary hypertension, respiratory failure and death (Paddock, Sanden et al. 2008).

1.2 BORDETELLA PERTUSSIS

B. pertussis, belonging to the genus *Bordetella*, is an encapsulated Gram-negative, human-specific aerobic coccobacillus equipped with fimbriae. It is non-motile and non-sporulating with a size approximately of 0.5 x 1 µm.

The survival of the *B. pertussis* in the host depends on its ability to produce virulence factors able to reshape the immune system to its advantage (Higgs, Higgins et al. 2012). The main virulence factors are: pertussis toxin (PT), adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), lipooligosaccharide (LOS), filamentous haemagglutinin (FHA), pertactin (PRN) and type 2 and 3 fimbriae (FIM). Pertussis pathogenesis is not yet entirely understood, but it is demonstrated that FHA, PRN and FIM promote the attachment to the epithelial cells, whilst, PT, TCT and ACT contribute to elude host immunity and to harm the epithelium (Higgs, Higgins et al. 2012). The expression of all these and other factors is under the control of *BvgAS* locus, which encodes a two-

component regulatory system (Weiss, Hewlett et al. 1983). This system, named BvgAS, perceives environmental changes and activates the expression of hundreds of genes (Cummings, Bootsma et al. 2006, Nicholson 2007).

1.2.1 THE TWO-COMPONENT REGULATORY SYSTEM BvgAS

BvgS is a 135-kDa sensor kinase composed of several domains. At N-terminal there are two periplasmic venus flytrap (VFT) domains followed by a transmembrane region, a cytoplasmic PAS domain, a histidine kinase (HK) domain, a receiver domain (Rec) and a last histidine phosphotransferase (Hpt) domain. BvgA is a 23-kDa response regulator protein containing a Rec domain at amino terminus and a DNA-binding helix-turn-helix domain at carboxyl terminus (Decker, James et al. 2012). Specific chemical stimuli, not yet identified, activate BvgS which uses ATP to phosphorylate a histidine residue in the HK domain, triggering the enzymatic cascade (Uhl and Miller 1994). The phosphoryl group is relocated from domain to domain until BvgA Rec domain. Following this phosphorylation BvgA dimerizes and binds to specific DNA sequences to positively or negatively regulate transcription (Boucher, Murakami et al. 1997, Boucher, Maris et al. 2003). The genes regulated by BvgAS belong to four classes and their transcriptional regulation corresponds to three phenotypic phases: Bvg⁺, Bvgⁱ and Bvg⁻ (Fig.1). Bvg⁺ occurs when BvgAS is fully active. Class 1 genes (like toxins PT and ACT) and class 2 genes (like FHA and FIM), known also as virulence-activated genes (*vag*-genes), are maximally expressed in this phase. Phosphorylated BvgA activates further the repressor protein BvgR that represses the expression of virulence-repressed genes (*vrg*-genes) or class 4 genes. Bvg⁺ phase is necessary and sufficient to cause infection *in vivo* (Cotter and Miller 1994). Bvgⁱ, intermediate phase, occurs when BvgAS is partly active and the class 2 and class 3 genes are maximally expressed. Until now only *bipA* that encodes for an outer membrane protein, has been characterized as class3 genes. It is supposed that Bvgⁱ phenotype may be involved in the transmission of the bacteria. Bvg⁻ phase, instead, occurs when BvgAS is inactive and it is constituted by the highest expression of class 4 genes. The inactivation of BvgAS results under nutrient-limiting condition, therefore Bvg⁻ phase may be essential for bacteria survival in the external environment (Porter, Parton et al. 1991).

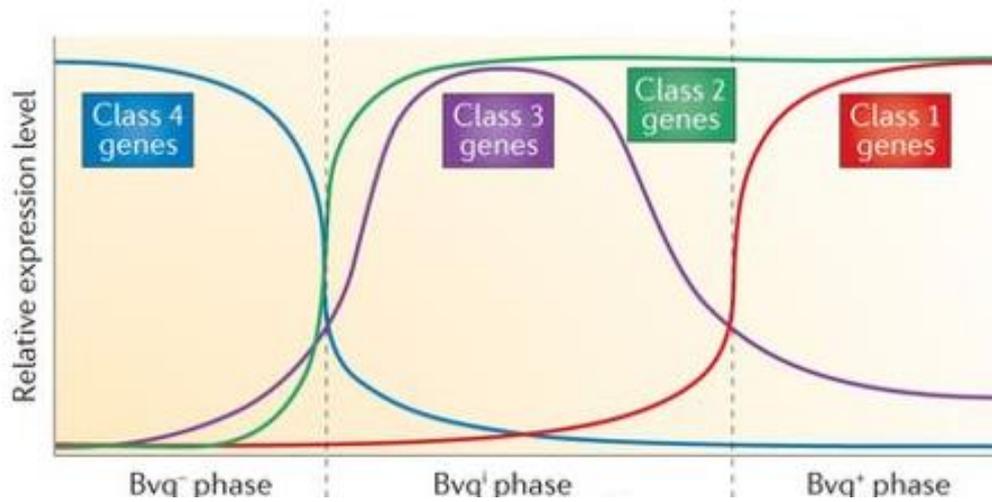


Figure 2. Schematic representation of the effects of BvgAS activation on the various classes of genes in the three phenotypic phases. The Bvg⁻ phase is characterized by maximal expression of class 4 genes and occurs when BvgAS is inactive. When instead the BvgAS is partially active, the Bvg⁺ phase begins with the highest expression of class 2 and class 3 genes. With the fully activation of the two-component regulatory system, Bvg⁺ phase starts and BvgAS induces the maxima expression of class 1 and class 2 genes. Adapted from (Melvin, Scheller et al. 2014).

1.2.2 VIRULENCE FACTORS

B. pertussis pathogenesis is the result of the action of several virulence factors that interact with host cells and coordinately alter their function. The most known *B. pertussis* virulence factors expressed during the virulent Bvg⁺ phase are proteins that can be classified as adhesins and toxins. The first category encloses surface proteins mainly involved in the adhesion of the bacteria starting the colonization of upper respiratory tract. The second one includes released proteins primarily implicated in impairing the epithelium and in evading host immunity. Filamentous haemagglutinin, pertactin and the fimbriae are the leading adhesins. FHA represents the dominant adhesin and is synthesized as pre-pro-protein of ~370 kDa (FhaB) to be then processed by peptidases to the mature form of ~250 kDa. During this process it is translocated through cytoplasmic membrane by Sec translocation system and across the outer membrane by filamentous haemagglutinin transporter protein (FhaC) (Mazar and Cotter 2007). Mature FHA has a rod shape with its functionally essential C terminus (MCD) distal to the surface (Mazar and Cotter 2006). It was reported that FHA could act as an immune suppressor in the first steps of colonization (Inatsuka, Julio et al. 2005,

Villarino Romero, Osicka et al. 2014). PRN is a protein of 69 kDa and belongs to the classical autotransporter family of outer membrane proteins (Henderson, Navarro-Garcia et al. 2004). *In vivo* PRN contributes to the adherence of the *B. pertussis* to epithelium (Edwards, Groathouse et al. 2005). It was also demonstrated its involvement in the resistance to neutrophil-mediated clearance (Inatsuka, Xu et al. 2010). The Fimbriae are filamentous appendages on the cell surface also known as type I pili. They are at the basis of *Bordetella* serotyping and the major serological types are FIM2 and FIM3. As for FHA, FIM favors the bacterial adherence to ciliated respiratory epithelium (Guevara, Zhang et al. 2016) and acts as suppressor of airways inflammation (Vandebriel, Hellwig et al. 2003). Regarding the second category, the most relevant members are pertussis toxin, adenylate cyclase toxin and dermonecrotic toxin (DNT). PT is a 105 kDa ADP-ribosylating AB₅-type toxin (Stein, Boodhoo et al. 1994) composed of A catalytic subunit and five B membrane-binding or transport subunits, which compose the B pentamer. The complex is assembled in the periplasm and is released in the extracellular space by type IV secretion system (Kotob, Hausman et al. 1995). PT enters in the host cells through a receptor-mediated endocytosis following a backward transport route up to endoplasmic reticulum from which the subunit A exits (el Baya, Bruckener et al. 1999). Once in the cytoplasm, the A subunit catalyzes the ADP-ribosylation of a cysteine residue of heterotrimeric G proteins α -subunits blocking the activities of enzymes regulated by G proteins. In particular it prevents G proteins to inhibit the adenylate cyclase activity with the consequent increase of cyclic AMP (cAMP). Overall the ADP- ribosylation induces a primary suppression of inflammatory cytokine production and an inhibition of recruiting immune cells (Graf, Codina et al. 1992, Loch, Coutte et al. 2011). ACT belongs to RTX (repeats in toxin) toxin family and is secreted by type I secretion system (Glaser, Danchin et al. 1988, Carbonetti 2010). It consists of C-terminal domain, which contains the RTX repeats and of N-terminal domain, which possesses calmodulin-dependent adenylate cyclase activity. ACT through its C-terminal binds with high affinity to complement receptor 3 situated on neutrophils, macrophages and dendritic cells surfaces. Upon binding the RTX forms a cation-selective pore in plasma membrane inducing variation in ion permeability and the calmodulin-dependent adenylate cyclase domain moves inside the host cells catalyzing the conversion of ATP in cyclic AMP (Glaser, Elmaoglou-Lazaridou et al. 1989, Ladant, Michelson et al. 1989, Sakamoto, Bellalou et al. 1992, Guermonprez, Khelef et al. 2001, El-Azami-El-Idrissi, Bauche et al. 2003). Increased level of cAMP

inhibits phagocytosis and oxidative burst in neutrophils (Confer and Eaton 1982) and prevents the complement-dependent phagocytosis by macrophages (Kamanova, Kofronova et al. 2008). DNT is a cytoplasmic toxin with a size of 160 kDa able to induce necrosis (Cowell, Hewlett et al. 1979). It possesses glutaminase activity and can activate Rho GTPases (Schmidt, Goehring et al. 1999). In the toxin category is also included the tracheal toxin, although not regulated by BvgAS system. TCT is a disaccharide-tetrapeptide monomer of peptidoglycan (Cookson, Tyler et al. 1989) that impairs the respiratory epithelium inducing the production of Interleukin 1 and nitric oxide (Heiss, Moser et al. 1993).

1.3 LONELY GUY AND CYTOKININS

LONELY GUY (LOG) proteins are key enzymes in plants for the production of phytohormones named cytokinins. The gene encoding LOG was identified in a rice mutant in an attempt to screen for defects in shoot meristem activities (Kurakawa, Ueda et al. 2007). Since the flowers of these mutants often contained only one stamen but no pistil, for analogy the protein was named LONELY GUY. It is involved directly in the synthesis of cytokinins converting inactive cytokinins nucleotides to the biological active form by its phosphoribohydrolase activity (Kurakawa, Ueda et al. 2007). The active form of cytokinins is predominantly N⁶-substituted adenine derivatives. When the modified adenine is conjugated with sugar, such as in nucleosides and nucleotides, shows a minor or completely absent biological function in activating cytokinins receptors (Bajguz and Piotrowska 2009). LOGs hydrolyze the N-glycosidic linkage between the base and the ribose, leading to the formation of free active cytokinins. In plants these phytohormones are involved in many important physiological processes, such as growth and branching (Werner, Motyka et al. 2001), chloroplast development (Cortleven and Schmulling 2015), delay of leaf senescence (Gan and Amasino 1995) and activation of plant defense responses (Bari and Jones 2009).

Over time the study of LOG proteins was limited, because they have been erroneously annotated as lysine decarboxylase enzymes (LDC) due to the presence of a highly conserved PGGxGTxxE motif (Kukimoto-Niino, Murayama et al. 2004, Jeon, Allard et al. 2006). Recently, some LOG-like proteins from non-plant organism, annotated as LDCs, have been identified to have phosphoribohydrolase activity and to produce

cytokinins (Dzurova, Forneris et al. 2015, Samanovic, Tu et al. 2015, Seo, Kim et al. 2016). Indeed many plant-interacting microbes and fungi produce mis-annotated LOG proteins able to synthesize cytokinins (Hinsch, Vrabka et al. 2015, Radhika, Ueda et al. 2015). These plant-like hormones are recognized by the plants and lead to a variation of resource allocation and to a morphological reconfiguration of the plant tissue that result in benefits for the pathogen. In fact the uncontrolled cytokinin signaling with the consequent delay of leaf senescence and creation of new organs such as galls and nodules, provides pathogens a continuous nutriment and protection against biotic and abiotic agents (Giron, Frago et al. 2013). Nevertheless a possible role of LOGs and their product cytokinins in a microbial cell-interaction has yet to be elucidated. Surprisingly cytokinin-activating homologous of plant LOG have been recently also identified in human pathogens *Mycobacterium (M.) tuberculosis* (Samanovic, Tu et al. 2015) and *Pseudomonas aeruginosa* PAO1 (Seo and Kim 2018). Interestingly, RV1205, the gene encoding *M. tuberculosis* LOG, was detected in an attempt to understand why the mycobacterial resistance to nitric oxide (NO) is linked to proteasome. Through a genetic screen in a proteasome-deficient strain, Rv1205 product was identified as proteasome regulated substrate and its accumulation in proteolysis mutants was responsible for NO sensitivity. In the absence of proteasome, which regulates and keeps LOG protein production to low levels, the amount of the cytokinins as well as of their breakdown products increase. In particular the cytokinin *p*-topolin degradation product *para*-hydroxybenzaldehyde (pHBA) resulted to be lethal in proteolysis mutant of *M. tuberculosis* in presence of NO (Samanovic, Tu et al. 2015). Despite this, the physiological role of LOGs in mammalian pathogens and why they synthesize cytokinins are completely unknown so far. Few studies suggest that cytokinins may have suppressive effects on both innate and humoral immune responses (Lappas 2015) or may induce cellular differentiation or apoptosis in immune cells (Casati, Ottria et al. 2011). Mammalian-pathogens, therefore, are likely able to reshape surrounding environment releasing plant-like cytokinins as modulator of the immune system. The specific effects that these metabolites may exert on immune cells depend on concentrations (Ciaglia, Pisanti et al. 2014). Another hypothesis is that LOGs may be involved in a signaling pathway at the inter-bacterial interface. However, a putative role of LOG protein during an infection is suggested by identification of a LOG homolog through a specific screen of genes expressed in a frog model of infection with *M. marinum* (Ramakrishnan, Federspiel et al. 2000).

The LOG domain is highly conserved among all kingdoms and this leads scientists to investigate new interesting research areas. Structural and molecular studies have proposed that the two highly conserved amino acids Glu and Arg, are the residues involved in the catalysis (Kukimoto-Niino, Murayama et al. 2004), the PGGxGTxxE motif holds the AMP moiety of substrate nucleotides (Seo, Kim et al. 2016) and that the active site needs two LOG domains for its formation (Seo, Kim et al. 2016). Notwithstanding these overall aspects, a phylogenetic analysis of LOG proteins from various organisms showed differences in the oligomeric state, in the amino acids of the binding site and variation at the PGGxGTxxE motif (Fig.2 A, B) (Seo and Kim 2017). According to this analysis it is possible to classify LOGs into at least two clusters based on the oligomeric state, the dimeric LOGs as type-I LOGs and the hexameric LOGs as type-II LOGs. Type-I and type-II LOGs may be in turn subdivided into two sub-groups, type-Ia and type-Ib, type-IIa and type-IIb respectively (Seo and Kim 2017). Type-II LOGs implement a hexameric form through an additional α -helix (Kukimoto-Niino, Murayama et al. 2004, Seo and Kim 2017). The existence of two structural clusters further divided and variations in amino acids of the binding site suggest the presence of different LOG-like proteins able to produce various cytokinin classes.

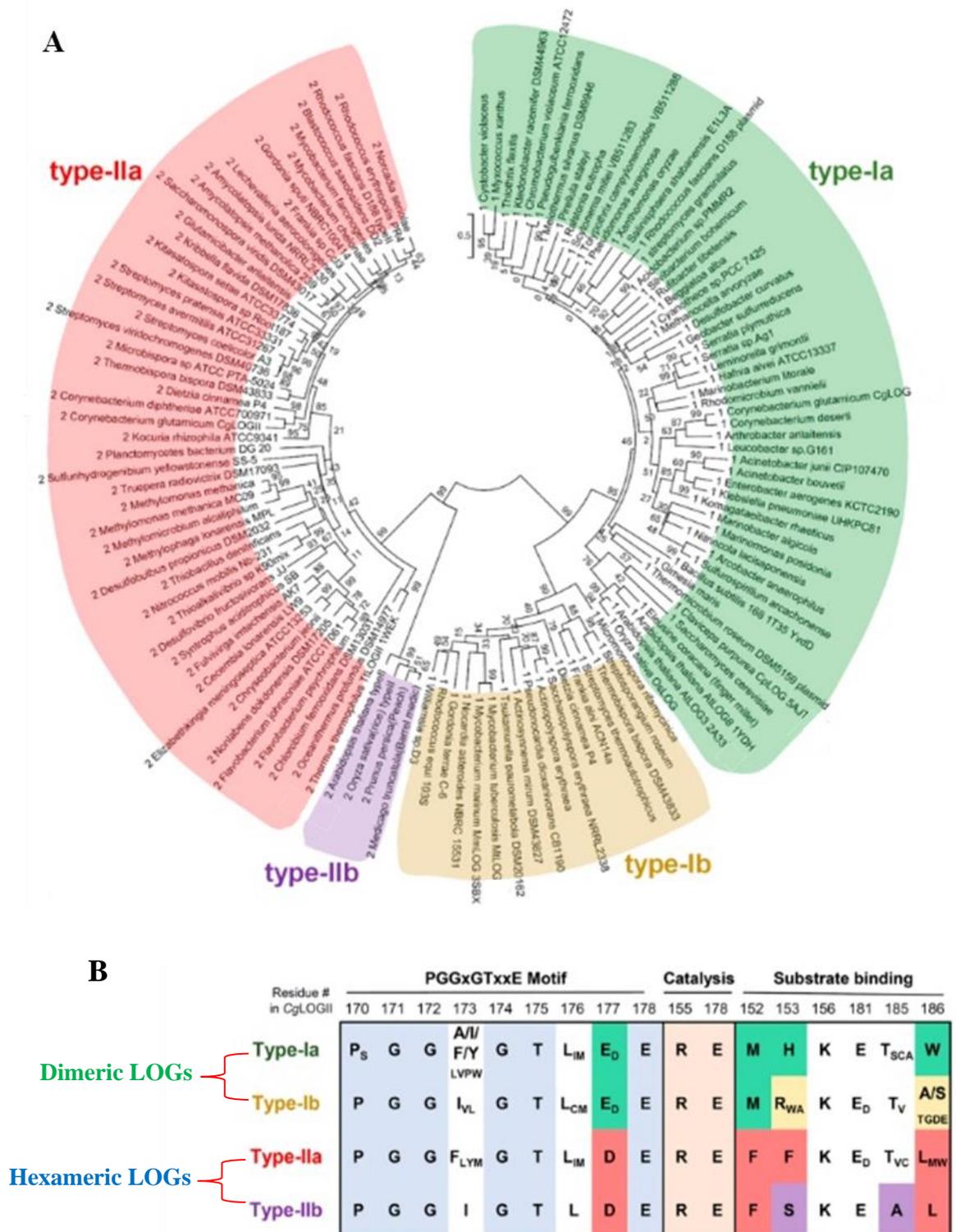


Figure 3. Phylogenetic analysis and sequences alignment from various subtypes of LOG proteins in comparison. (A) Unrooted maximum likelihood phylogenetic tree of LOGs represented in a circular model. The four subgroups are schematized and marked with different colors: green for type-Ia and yellow for type-Ib, red for type-IIa and violet for type-IIb. Bootstrap values are shown at each node. (B) Amino acids sequence alignment of key residues of the four subgroup of LOG proteins involved in the PGGxGTxxE motif, catalysis and substrate binding. The oligomeric state of each LOG type is shown. Adapted from (Seo and Kim 2017).

2. AIM OF WORK

In order to defeat and prevent Pertussis disease, in the late 1940s was introduced the whole-cell pertussis (wP) vaccine, consisting of suspensions of killed *B. pertussis* organisms (Kuchar, Karlikowska-Skwarnik et al. 2016). The introduction of the vaccine led to a rapid reduction in both the incidence and the number of deaths caused by the infection. Because of its reactogenicity for the high number of endotoxins and comprehensive concerns about its safety, in the late of 1990s it was replaced with acellular pertussis (aP) vaccine in many developed countries (Kuchar, Karlikowska-Skwarnik et al. 2016). aP is based by one to five purified antigens: PT, FHA, PRN and FIM 2/3. Unfortunately, notwithstanding a high vaccine coverage all over the world, an increase in pertussis cases has been recorded in adolescent and adults, concerning the public health about whooping cough resurgence. Although difficult to contextualize, several causes have been hypothesized, like waning immunity due to the lasting immunity induced by aP shorter than wP (Mooi, Van Der Maas et al. 2014), epidemiological changes in the circulating strains (Bart, Zeddeman et al. 2014, Bart, van der Heide et al. 2015) and the different both humoral and cell-mediated immunological response triggered by aP compared to the immunity generated by wP and natural infection (Higgs, Higgins et al. 2012). Furthermore, the difficulty of diagnosis in the first phase of the disease, leads to a loss of effectiveness of antibiotic treatment and, in severe cases, the patients may also not respond to the therapies due to breathing difficulties (Paddock, Sanden et al. 2008). The continuous increase of pertussis cases in all age groups is seriously worrying the public health, above all for the associated risk of transmission to incompletely or unimmunized infants. To solve this concern, the scientific community is operating on several fronts. On one hand it is trying to improve the current vaccine in order to have an acceptable safety, prevent transmission and provide a longer lasting immunity; on the other hand is trying to develop new therapeutic agents and targeted treatment strategies to reverse the disease at the time of diagnosis. However, to obtain all of these, it is necessary and indispensable to discover new features related to *Bordetella* pathogenesis and microbiology. On the basis of the observations above described, this PhD project aims to improve the knowledge of the physiology and pathology of *Bordetella*, which can help to improve the quality of vaccine and to develop alternative therapeutic interventions. Specific topics studied in this work were the following:

- Identification and characterization of the biochemical function of new selected proteins, potentially involved in *Bordetella* pathogenesis;
- Identification of factors playing a key role in the complex communication circuit between the host and the pathogen.

Concerning the aims, we focus our attention on protein pertussis BP1253, a putative exported protein with an unknown function, classified as possible lysine decarboxylase.

3. MATERIALS AND METHODS

3.1 BIOINFORMATICS ANALYSIS

The BLAST algorithm was used to investigate the sequence homologies between BP1253 and LOGs, while *Cluster omega* to realize amino acid sequence alignment with specific type-I and type-II LOGs. The structural analysis were performed through PDB Viewer, either to develop the modeling of BP1253 that to determinate the coordinates of atoms in the overlapping. To realize the figures, *ESPrpt* 3.0 software was used to represent the sequence alignment, instead, the graphic software PyMOL to visualize the overlaps. Regarding the phylogenetic tree, it was generated with MAFFT and figured through the software tool Archaeopteryx.js.

3.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

The following *B. pertussis* strains were used in this study: Tohama I-derivative BP536 (Weiss and Falkow 1983 153, 304-9), the clinical isolates B3629, B3621. Bacteria were stored at -80 °C and recovered by plating on Bordet-Gengou (BG) agar plates, supplemented with 15% (v/v) sheep blood, for 3 days at 37°C. Bacteria were then inoculated at initial 600 nm optical density (OD₆₀₀) of 0.05–0.1 in Stainer-Scholte medium supplemented with 0.4% (w/v) L-cysteine monohydrochloride, 0.1% (w/v) FeSO₄, 0.2% (w/v) ascorbic acid, 0.04% (w/v) nicotinic acid, 1% (w/v) reduced glutathione. Cultures were grown in rotary shakers at 37 °C.

3.3 GENERATION OF E. COLI EXPRESSING RECOMBINANT BP1253 PROTEIN AND PURIFICATION PROCEDURE

The synthetic *gene1253* from *Bordetella pertussis* was synthesized and assembled by GeneArt (Thermofisher) starting from synthetic oligonucleotides and/or PCR products. The fragment was inserted into pET24b(+)_D455 as a fusion construct with a carboxyl-terminal 6x histidine tag separated from the last amino-acid of the protein by a linker of 2 amino-acid residues. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The sequence congruence verified by sequencing of the final construct within the insertion sites was 100%. *Escherichia coli*

strain BL21 (DE3) cells (New England Biolabs) were transformed with the above construct and used for protein expression. Cells were grown using BioSilta medium (Enpresso B Animal-free growth systems), at 30°C for 8 h with gentle shaking (160 rpm). The expression of *Bp1253* gene was induced to an A_{600} of 0.5–0.6 by the addition of 1mM isopropyl β -D-1-thiogalactopyranoside (IPTG) 24 h at 17°C. Cells were harvested by centrifugation and re-suspended in lysis buffer containing 50 mM NaH_2PO_4 , 300 mM NaCl, pH 7.4 and protease inhibitor EDTA free (Boheringer Mannheim) at a ratio of 10 ml of lysis buffer per 1 g of bacterial pellet. After lysis, performed via sonication (Qsonica Q700), cell lysates were clarified by centrifugation at 15000 x g for 50 min at 4°C, and the supernatant, containing the expressed protein, filtered using a 0.22 μm membrane filters (EMD Millipore filters) before starting the first chromatography step. BP1253 was purified by a Co^{2+} -affinity chromatography (5mL HiTrap TALON crude, GE Healthcare) at room temperature (RT, 25°C) using an AKTA purifier 100 system (GE Healthcare). The column was equilibrated with buffer A (50 mM Tris pH 8, 300 mM NaCl). After loading the crude extract, the column was washed with 10 bed volumes of buffer A. Bound proteins were eluted with buffer A containing 500 mM imidazole. The content of lipopolysaccharide (LPS) on the purified protein was checked using the Endosafe nexgen-PTS system (Charles River). When the content of LPS was out of the range it was removed using the EndoTrap Red columns (Hyglos). The purity of the protein was checked using 4–12% SDS–PAGE gradient gels in MES buffer, after the identification of the fractions containing BP1253, samples were pooled and stored at -20°C for subsequent analysis.

3.4 SIZE-EXCLUSION CHROMATOGRAPHIC ANALYSIS

The investigation of BP1253 oligomerization was performed using an analytical size-exclusion chromatography. The chromatographic step was performed using a BEH200 column 4.6x300mm (Waters) at a Flow of 0.4 ml/min with a buffer containing 10 mM NaH_2PO_4 and 400 mM $(\text{NH}_4)_2\text{SO}_4$ pH 6.0. Protein samples of 15 μl 0.62 mg/ml and 3 mg/ml were analyzed. The molecular weights of the different forms of BP1253 were calculated based on the calibration curve standard proteins.

3.5 MULTI-ANGLE LIGHT SCATTERING

Multi-Angle Light Scattering (MALS) analysis was performed in an HELEOS (WYATT Technology) in combination with a SEC separation. SEC was carried out with Sepax Zenix SEC-300 3 mm 7.8 x 300 mm at a flow of 0.5 ml/min with PBS as running buffer. The BP1253 for the analysis was diluted in PBS buffer at a final concentration of 0.6 mg/ml. Results were analyzed using the ASTRA software 3.1.

3.6 GENERATION OF THE BP1253 KNOCKOUT STRAIN

The deletion strain for the genes BP1251-1252-1253 of the *B. pertussis* BP536 Tohama I-derivative was constructed as follows. The 5' and 3' extremities of the locus were amplified by PCR using *Bp* chromosome as template and the oligonucleotides FlankingUP-Fw ccgGAATTCCGAAAACCGTAGCGGTCGAA and FlankingUP-rev ggaGGATCCGGACCGATGTCGGCCAATTT, FlankingDOWN-Fw ggaGGATCCCGCGTCTATGTCGACCACG and FlankingDOWN-rev cccAAGCTTCGAACTGCACCTGACCATCC as primers, respectively.

The amplicons were then successively introduced as EcoRI-BamHI and BamHI-HindIII fragments into pUC19, together with a BamHI-BamHI fragment encoding the kanamycin resistance cassette for selection. The resulting EcoRI-HindIII fragment was then purified and introduced into the EcoRI-HindIII sites of pSORTP1, a mobilizable suicide plasmid used for conjugation between *Bp* and *E. coli*. Conjugation was performed on BG-blood agar plates containing 10 mg/ml MgCl₂ for 5 hours, and co-integrates were selected on BG-blood agar plates containing 10 µg/ml gentamicin and 20 µg/ml nalidixic acid to prevent growth of the *E. coli* donor. Single crossing overs determine the insertion of the pSORTP1 vector in the chromosome and confer gentamicin resistance and streptomycin sensitivity. Double crossing overs were selected by a successive step on BH-blood plates containing 25 µg/ml of kanamycin and 400 µg/ml of streptomycin. After 3 to 4 days growth on selective media, isolated kanamycin and streptomycin-resistant colonies were analyzed by PCR to confirm the deletion.

3.7 HIGH-THROUGHPUT PURIFICATION OF BP1253 MUTANTS

Single point conservative and not conservative mutations were inserted into the Bp1253 gene to generate six different mutants. Mutated genes were synthesized and assembled by GeneArt (Thermofisher) starting from synthetic oligonucleotides and/or PCR products using the same plasmid and procedure as above described. The mutants generated were R120A, R120K, K121A, K121R, E143A and E143D. Purification of mutants was performed under vacuum conditions in a 96-well Vacuum plate. Cells were lysed using B-PER (Sigma) and applied on a His Multitrap HP 50ml NiSepharose High Performance 96 wells previously washed with water and equilibration buffer (300 mM NaCl, 50 mM NaH₂PO₄, pH 8). After loading the samples, the plate was washed with 80 vol. of washing buffer (300 mM NaCl, 50 mM NaH₂PO₄, 20 mM imidazole, pH 8) at room temperature. The His-fusion proteins were eluted in two steps by addition of 6 vol. of elution buffer (300 mM NaCl, 50 mM NaH₂PO₄, 500 mM imidazole, pH 8) twice. All the elutions related to the same protein were subsequently pooled. All purification steps were executed applying a vacuum not exceeding the maximum pressure of 5 mmHg.

3.8 SDS-PAGE AND WESTERN BLOT

SDS-PAGE and western blot (WB) analyses were performed to monitor expression, purity and identity of BP1253 during purification. Samples mixed with 1X NuPage LDS loading buffer and 1X NuPage Sample reducing agent (Life Technologies), were heated at 70°C for 10 minutes before loading 20 µl of the mixture onto a 4-12% gradient NuPAGE Bis-Tris gel (Life Technologies). SeeBlue Plus Prestained Standard markers were run onto each gel, which was stained with Comassie Blue to visualize the proteins. Separated proteins were electro-transferred onto nitrocellulose membranes with iBlot 2 Dry Blotting System (Life Technologies). The membranes were blocked for 60 min with 0.1% Tween 20 and 10 % milk in phosphate buffer solution (PBS), incubated for 1 hour with specific mouse polyclonal α-BP1253 antibodies (1:500 dilution) in 0.1% Tween-20 and 3% milk buffer in PBS. After three washes with 0.1% Tween-20 in PBS (T-PBS) the membrane was incubated with a secondary rabbit α-mouse horseradish peroxidase conjugated antibody (Jakson Immune Research Laboratory, 1:1000

dilution), for 30 min at room temperature. Bound antibodies were visualized, after washings membranes thrice with T-PBS, with ECL immunoblotting detection system (Amersham) according to the manufacturer's instructions. The supernatants of the clinical isolates were previously TCA precipitated before western blot analysis. Briefly, the supernatants were incubated 10 min at 4°C with 20% TCA and then centrifuged at 14000 x g for 5 min. The pellets were washed twice with 200 µl of cold acetone to remove TCA. After washings, pellets were dried in heat block for 10 min to dry off acetone.

3.9 PHOSPHORIBOHYDROLASE ACTIVITY ASSAY

Phosphoribohydrolase activity was assessed by detecting the nitrogenous base ring compounds after separation with a thin layer chromatography (TLC) as described (Seo, Kim et al. 2016 6, 31390). Briefly, enzyme reaction was carried out in a mixture containing, in a final volume of 20 µl, 40 mM Tris-HCl (pH 8), 20 mM Nucleotide Mono Phosphate (NMP) as substrate and the amount of purified recombinant protein as specified in figure legends. After incubation at 30°C, samples (5 µl) were denatured at 95°C for 2 min and then 1 µl dotted on PEI-Cellulose-F-plastic TLC sheet (Merck Millipore). Since the solubility of guanine is poor in water and to enhance its visibility on TLC, the inactivation was performed with 1 M NaOH (v/v). The nitrogenous base ring was separated from the phosphoribosyl moiety using a TLC method with a mobile phase containing 1 M sodium chloride except for pyrimidine rings where the mobile phase was acetone/water 30/70 (v/v) with the addition of 0.2 mol/l NaCl. After the run, the sheet was completely dried and the separated dots visualized by UV lamp (264 nm).

3.10 GENERATION OF MOUSE IMMUNE SERA

BALB/c mice (10 females/group, 6-week old) (Charles River Laboratories International Inc., Wilmington, MA) received three intraperitoneal immunizations, with a 4-week interval, with aluminum hydroxide (2 mg/ml) adjuvanted recombinant BP1253-His (10 µg per dose) at one fifth of a human dose. Sera were collected before immunization and 2 weeks after the third immunization. Control mice immunized with adjuvant only were included in the experiments. All the experiments involving animal were performed in

compliance with the Italian law with the approval of the local Animal Welfare Body (AWB 2014/06) followed by authorization of Italian Ministry of Health.

3.11 SURFACE PLASMON RESONANCE EXPERIMENTS (BIACORE)

SPR experiments were performed using a Biacore T200 instrument (GE Healthcare). The recombinant BP1253 protein was amine-coupled at 20 µg/mL in 50 mM NaOAc at pH 4.5 to the surface of a CM5 sensor chip (GE-Healthcare), activated with 50 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) / 50 mM N-hydroxysuccinimide (NHS). Typically, response levels of 8000-9000 RU were achieved. After priming the system with HBS-EP+ buffer (10 mM HEPES; 150 mM NaCl; 3 mM EDTA; 0.05% Surfactant P20; pH 7.4), affinity data were generated under steady state conditions, injecting ligand nucleotides dissolved in HBS-EP+ at 30 µl/s and 25°C for 60 s, then allowing to dissociate for another 60 s. Blank-subtracted sensorgrams were analyzed using the Biacore T200 Evaluation Software v. 3.0. Response values were evaluated 4 seconds before the end of each injection and averaged over a window of 5 sec.

3.12 IDENTIFICATION AND CHARACTERIZATION OF CYTOKININS BY LC-MS/MS

For the analysis of the cytokinins, the supernatant was obtained spinning down 40 ml bacterial growth broth at 9000 x g for 20 min. filtered and treated as described (Dobrev, Hoyerova et al. 2017 1569, 31-39). Briefly, the supernatant was cleared by molecules with MW higher than 3kDa using a protein concentrator 3K (Amicon) following vendor's instructions. Subsequently the samples were concentrated up to 1 ml volume through a Speed Vac and loaded on a solid phase extraction (SPE) column (MCX matrix, Oasis). Before loading, samples were previously acidified to pH 3 with formic acid. The column was then washed with 0.5 mL SPE load solvent (1 M formic acid), followed by 1 mL water. The samples of interest were eluted with 0.5 mL of freshly prepared solvent 2 (0.35 M ammonium hydroxide in 70 % methanol, to 70 mL methanol were added 2.5 mL of 25% ammonium hydroxide filled to 100 mL with distilled water) and the flow-through collected into new 2 mL microcentrifuge tube was

dried in SpeedVac at 10 mBar and 40 °C. The dried fractions were stored at -20 °C until LC-MS analysis. At the time of starting the analysis in LC-MS, the dried samples were dissolved into 100 µL 5 % methanol in water and centrifuged at 20000 × g, 4 °C for 20 min. The supernatant was transferred into an autosampler vial and used for the analysis without further modification. LC-MS/MS analysis was performed using an LTQ-Orbitrap XL coupled with an Ultimate 3000 HPLC system equipped with a reverse phase column Luna C18 (2) 100mm x 2mm, 3 µm, 100 Å. The mobile phase A and B used for the analysis of the sample were 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. The gradient started with 100% of A, maintained for 3 minutes and the phase B increased up to 20% in 12 minutes. Then after two minutes the phase B reached the 100% and it was hold constant for two minutes. The flow rate was set to 200 µl/min., UV at 268 nm, while a positive ion mode with electrospray was used as mass spectrometric detection. The ESI ion source spray voltage was set to 5 kV, the capillary temperature was 300 °C, tube lens and capillary voltage were 110 and 35 V respectively. The sheat and auxiliary gas (N₂) were set to 20 and 5 a.u., respectively. The mass spectra and the MS/MS spectra were recorded using the data dependent scan mode at resolution of 30000, while the fragmentation mass spectra were recorded in low resolution with collision energy 25 a.u.

3.13 PHENOTYPIC CHARACTERIZATION

Bacteria stored with glycerol were inoculated at initial 600 nm optical density (OD₆₀₀) of 0.05–0.1 in Stainer-Scholte medium supplemented with 0.4% (w/v) L-cysteine monohydrochloride, 0.1% (w/v) FeSO₄, 0.2% (w/v) ascorbic acid, 0.04% (w/v) nicotinic acid, 1% (w/v) and reduced glutathione. Cultures were grown in rotary shakers at 37 °C for 24 h. After that we tested the bacterial susceptibility to oxidative stress by treating cultures with 0.1 and 0.3 M H₂O₂ (Sigma) for 30 minutes as specified in Figure legend. The viability of the *Bp* strains was measured after treatment.

3.14 STATISTICAL ANALYSIS

Data are presented as the means ± SD and the Kruskal-Wallis test was used to analyze significance of data. Values of P ≤ 0.05 were considered and referred to as significant.

3.15 PROTEIN ASSAY

Protein concentration was measured with the bicinchoninic acid (BCA) protein assay (Smith, Krohn et al. 1985 150, 76-85) (Thermo Scientific-Pierce, Rockford, IL, USA), using BSA (2 mg/ml) as a standard, equal amounts of proteins were resolved by SDS-PAGE.

4. RESULTS

4.1 PRODUCTION AND PURIFICATION OF RECOMBINANT BP1253

In order to obtain the *Bp* protein BP1253, we transformed BL21 (DE3) competent *E. coli* cells with known concentrations of pET24b(+)_D455 plasmid containing the corresponding synthetic gene. BP1253 was expressed as His-tagged recombinant protein after IPTG induction and purified by a single chromatographic step on an immobilized metal (Co²⁺) affinity chromatography. SDS-PAGE analysis gave a single pure band (Fig. 4A) and western immunoblot (Fig. 4B) confirmed the identity of the purified protein, instead the MS analysis of BP1253-His, resulted in an experimental mass of 24872.53 Da (Fig 4C). Due to overexpression in the bacterial system, not all the methionine residues of the recombinant protein have been removed from the proteins after translation, leading to a second peak corresponding to 25003.64 Da in intact mass measurement.

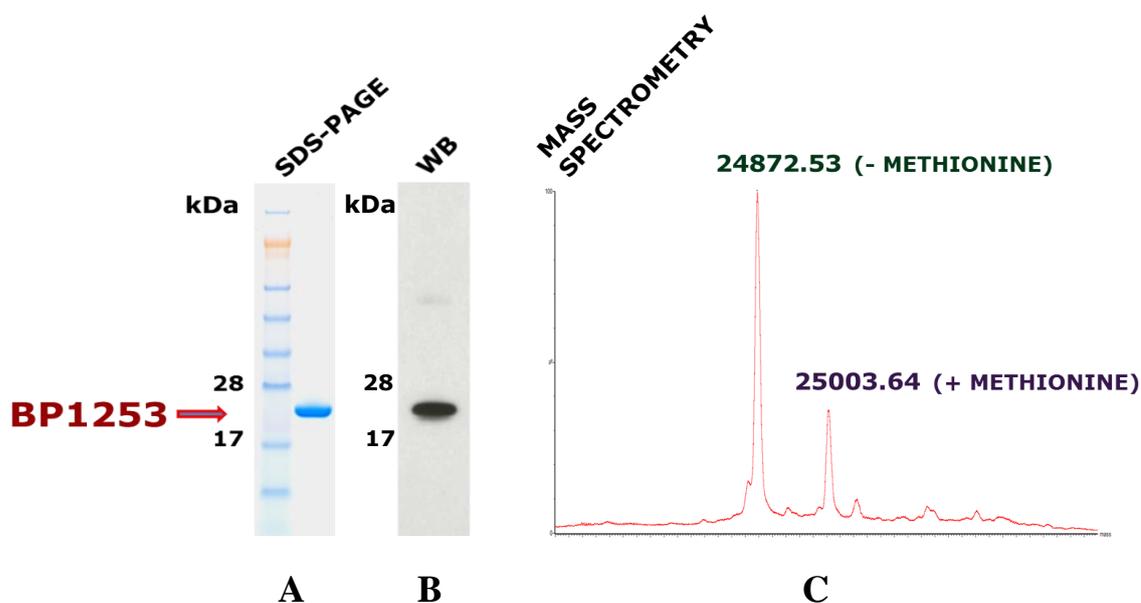


Figure 4. Expression, purification and BP1253 identification. BP1253 was cloned in *E. Coli* BL21 (DE3) and purified after IPTG induction through HiTrap TALON crude. (A) The eluate, 2 μ g, was evaluated by SDS-PAGE in 4-12% gradient NuPAGE Bis-Tris gel stained with Coomassie Blue. Molecular mass markers (kDa) are indicated on the left (B) The identity of the purified protein was further confirmed by immunoblot. The experiment was performed with 25 ng of BP1253 and with mouse polyclonal α -BP1253 (1:500 dilution) as described in Material and methods. Molecular weights of proteins markers are shown on the left. (C) Intact mass measurement revealed a BP1253-His molecular weight of 24872.53 Da. The weight of 25003.64 Da refers to BP1253-His proteins with unremoved methionine.

To characterize the functional unit and the oligomeric state of BP1253, we performed a size exclusion chromatography (SEC) (Fig. 5). The SEC gave a main peak with a retention time of 7.20 minutes. Using the molecular weights and the retention times of the protein standards, through a graphic interpolation we calculated that this peak refers to a molecule of about 50 kDa. The analysis revealed also a second, but very small peak approximately of about 100 kDa. To exclude the hypothesis that the oligomeric state of the protein depended on the concentration, we performed SEC analysis with two different concentrations of BP1253, 0.6 mg/ml and 3 mg/ml respectively. The two chromatograms acquired did not display any differences to each other suggesting that the dimeric form is the functional unit of BP1253 (data not shown).

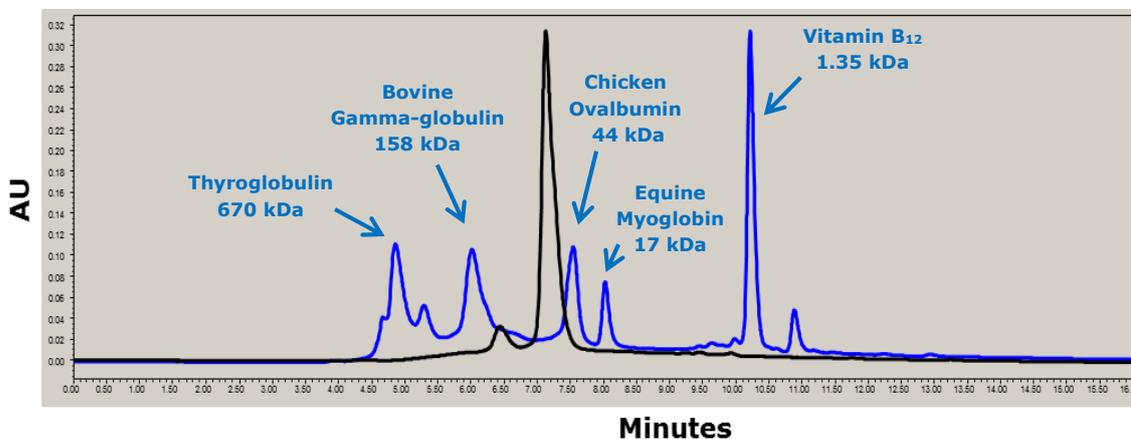


Figure 5. BP1253 is a dimer in size-exclusion chromatography. BP1253 was eluted as dimeric form with a molecular weight of 50 kDa. The analysis was carried out with concentrations of 0.6 mg/ml and 3 mg/ml of protein. The peak corresponding to BP1253 is marked in black, while the proteins standards with their own molecular weights are highlighted in blue. The figure refers to 3 mg/ml protein concentration.

To consolidate the results obtained in SEC, we performed a multi-angle light scattering (MALS) study (Fig. 6). This analysis showed further the presence of a protein with a molecular weight of 49.32 ($\pm 5\%$) kDa with a polydispersity of 1.010 ($\pm 7.7\%$). MALS definitively confirmed that the oligomeric state of BP1253 is a dimer.

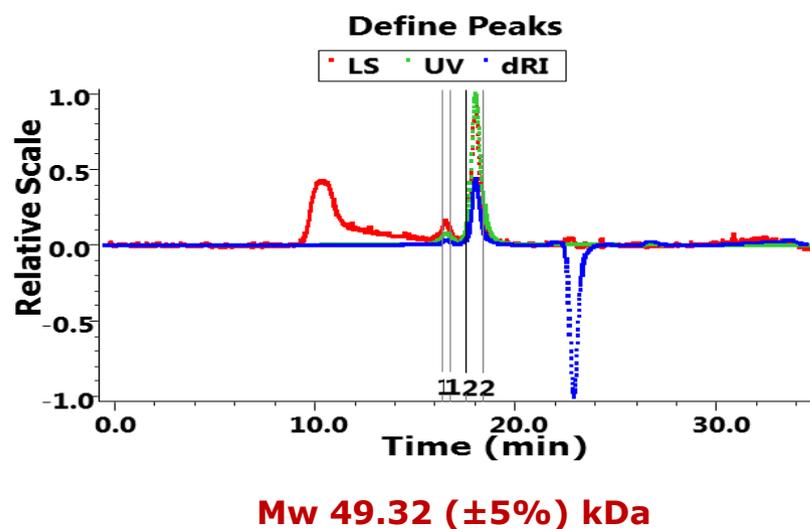


Figure 6. Multi-Angle Light Scattering confirms the dimeric state of BP1253. MALS analysis confirmed the dimer as the oligomeric state of BP1253. The study was performed in combination with SEC using a final protein concentration of 0.6 mg/ml. The data were analyzed with ASTRA software 3.1.

4.2 IN SILICO-BASED IDENTIFICATION OF BP1253 AS A MEMBER OF THE LONELY GUY FAMILY

Recently, in the literature it has emerged that many proteins from different organisms of various kingdoms, like *Plantae*, *Animalia*, *Bacteria* and *Fungi*, are mis-annotated as lysine decarboxylase because the presence of PGGxGTxxE motif in their sequence (Naseem, Bencurova et al. 2018). A more accurate analysis classifies them as members of the LOG protein family. This mis-annotated classification included also BP1253 considered a possible LDC so far. Through bioinformatics analysis and following sequence alignments we observed that BP1253 shares with LOG proteins not only the highly conserved motif PGGxGTxxE, but also the catalytic core complying with the peculiar distance among the catalytic Arg and Glu residues (Fig. 7).

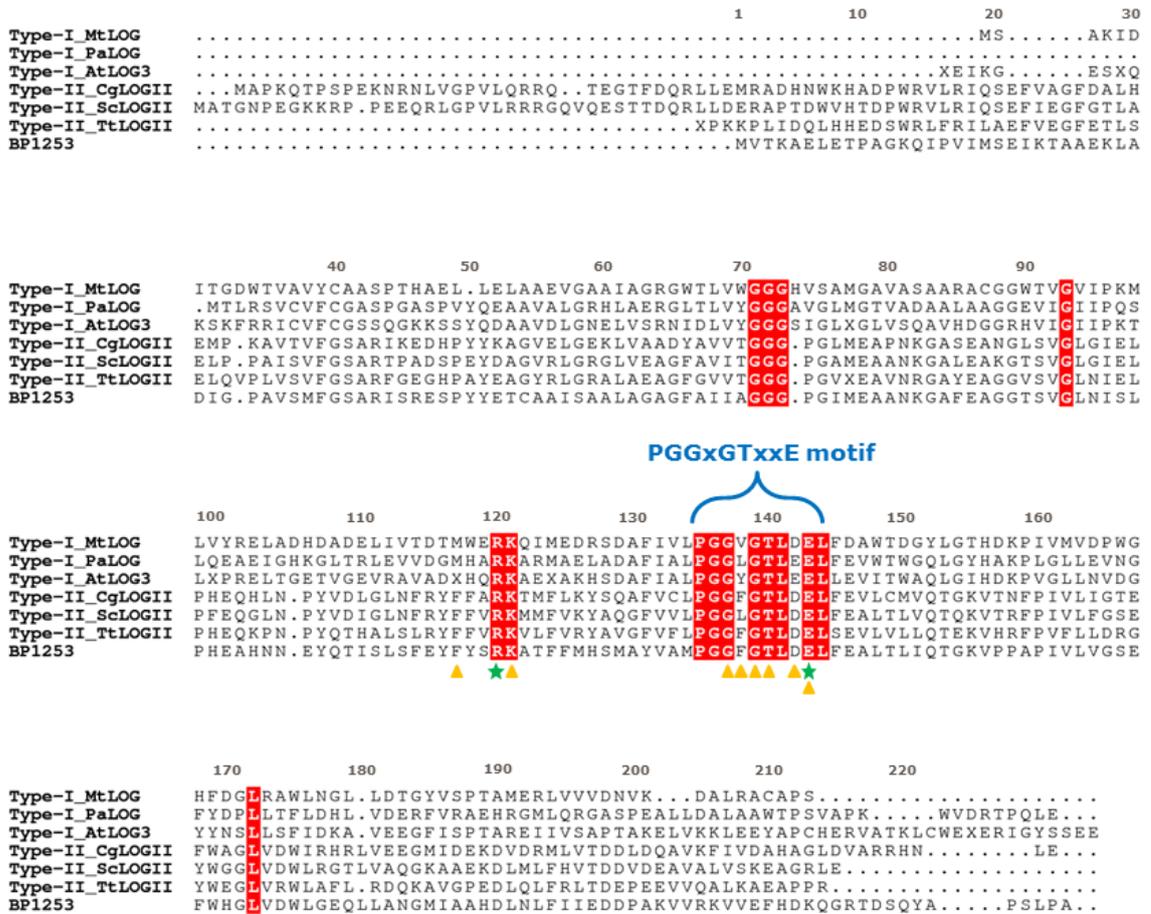


Figure 7. Sequence alignment between LOG proteins from various subtypes and BP1253. Primary structures alignment among LOG proteins type-I and type-II and BP1253 was generated by *Cluster omega* and drawn with *ESPrpt* 3.0 software. The residues involved in the catalysis are indicated with green-colored stars while, the amino acids that accommodate AMP in the catalytic site are indicated by orange-colored triangles. The PGGxGTxxE motif is specified by a blue bracket. The amino acids shared by all the LOG proteins are highlighted in red. MtLOG, PaLOG and AtLOG, are abbreviations of type-I LOG proteins from *M. tuberculosis*, *P. aeruginosa* and *A. Thaliana*. CgLOG, ScLOG and TtLOG are abbreviated name of type-II LOGs from *C. glutamicum*, *S. coelicor* and *T. thermophiles*.

We also noticed from sequence alignment, that BP1253 presents a higher sequence homology towards the LOGs type-II than the LOGs type-I. As described, the cluster type-II of LOG proteins assembles phosphoribohydrolase enzymes with a hexameric form, but we demonstrated (Fig. 5, 6) that the oligomeric state of BP1253 is dimeric. A phylogenetic tree between BP1253 and proteins described in literature to possess phosphoribohydrolase activity and classified as type-I or type-II, indicates how our protein could represent a further sub-group of LOG type-II with a dimeric form (Fig. 8).

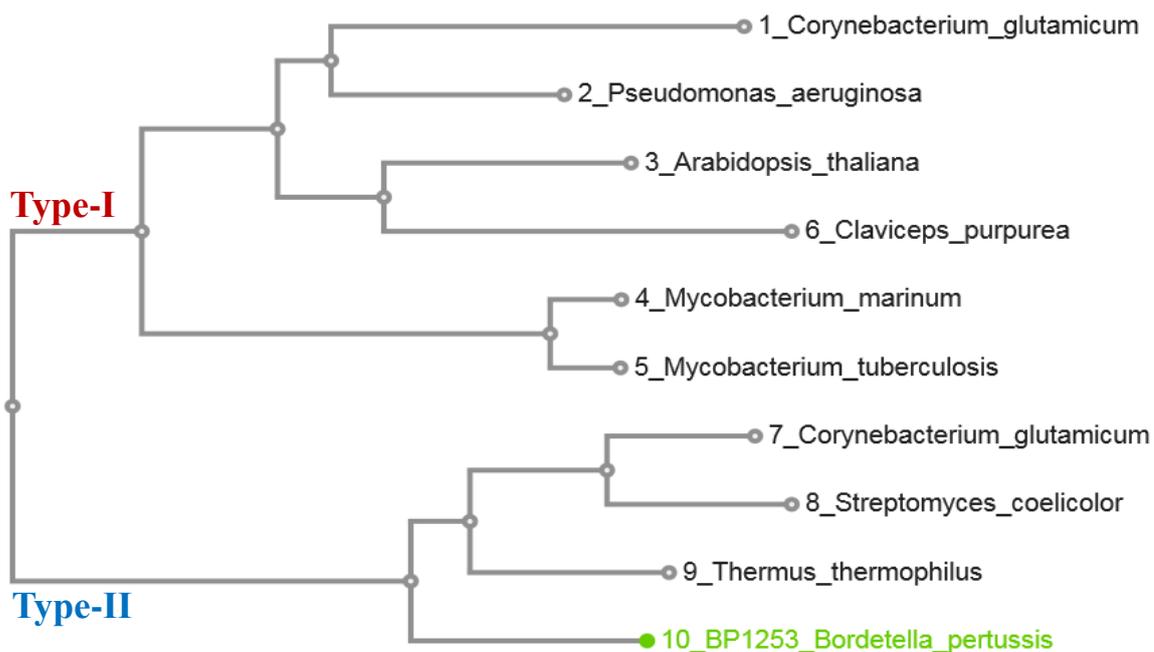


Figure 8. Likelihood phylogenetic tree between described LOGs and *Bordetella pertussis* BP1253. The phylogenetic tree was realized with MAFFT and visualized through the software tool Archaeopteryx.js. BP1253 is highlighted in green. The proteins are divided in two branches; one represents type-I LOGs while the other one the type-II LOGs. The dimeric BP1253 is grouped with Type-II LOGs characterized by having a hexameric form.

4.3 *BP1253 BINDS NUCLEOTIDES*

LOG proteins bind and cleave preferentially adenosine 5'-monophosphate (AMP). To verify whether BP1253 is able to bind AMP like the known LONELY GUY proteins, we at first resorted to a theoretical approach overlapping a modeling of BP1253 with a monomer of a *Mycobacterium marinum* protein classified in the maximum likelihood phylogenetic tree (Seo and Kim 2017) (Fig. 9) as type-I LOG protein with dimeric form (MmLOG). Characteristic of this *M. marinum* protein with PDB ID 3SBX is that it was crystallized in complex with AMP (Baugh, Phan et al. 2015). The modeling was based on a monomer of *Thermus Thermophilus* protein TT1465 (TtLOG), PDB ID 1WEK, with which BP1253 shares the 48% of homology. TT1465 is annotated in the phylogenetic tree as LOG type-II. Usually two related structures have a Root Mean

Squared (RMS) value between 0 – 2Å. In this case the overlap produced a RMS value of 1.21 Å and revealed a great similarity between the 3SBX and BP1253 binding pockets.

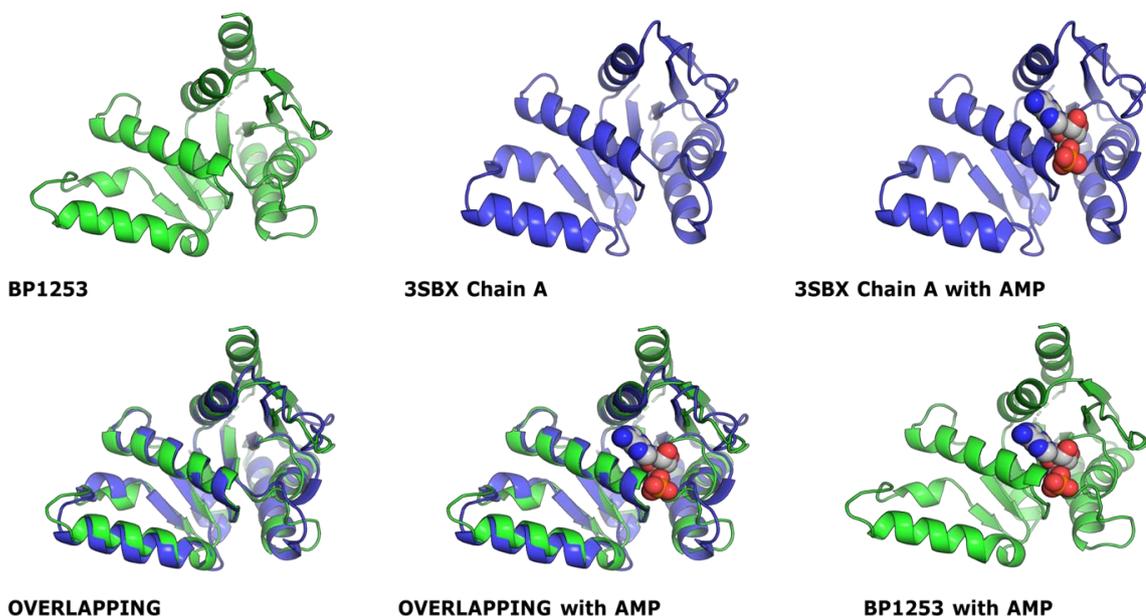


Figure 9. Overlapping between monomer structures of MmLOG and a modeling of BP1253. The prediction data obtained from the overlapping gave a RMS value of 1.21 Å and showed the analogy of the two binding sites. The modeling was created through PDB Viewer on the base of 48% homology among BP1253 and TtLOG sequences. The overlapping was realized with PDB Viewer and the figures of single monomers and of overlaps were created via the graphic software PyMOL.

To validate definitively the capability of BP1253 to bind AMP, we performed surface plasmon resonance (SPR) studies. Recombinant BP1253-His was immobilized on the surface of a golden chip CM5 and a ranging concentration from 0.78 to 400 μM of AMP was applied to measure the dissociation constant (kd). Analysis by Biacore indicated that BP1253 binds AMP with a kd of 5.7 μM (Fig. 10) suggesting a high affinity for this compound.

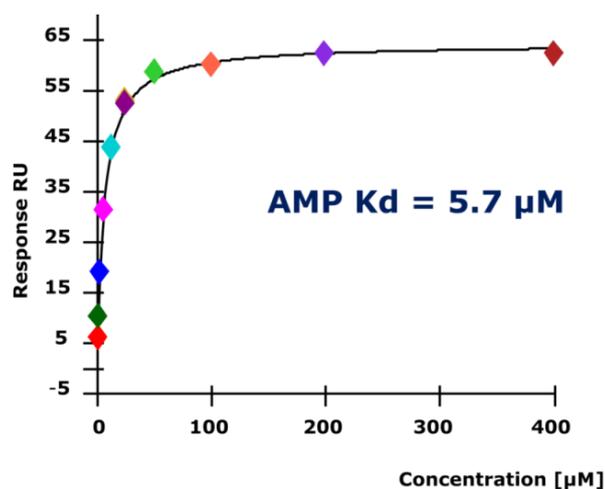


Figure 10. Surface plasmon resonance study with AMP. Biacore analysis displays that BP1253 has the ability to bind AMP with a dissociation constant of 5.7 μM . BP1253-His was immobilized via amine coupling procedure on Sensor chip CM5. Concentrations from 0.78 to 400 μM were used to measure the k_d . The results shown are representative of three independent experiments giving identical results.

To investigate if this protein is able to bind other monophosphate nucleotides, both purines and pyrimidines such as GMP, IMP, CMP, TMP and UMP were analyzed. Results shown in Fig. 11 indicate that both GMP and IMP are able to bind BP1253 with k_d of 38 μM and 71.8 μM respectively; a lower affinity was obtained with TMP and UMP (Table I).

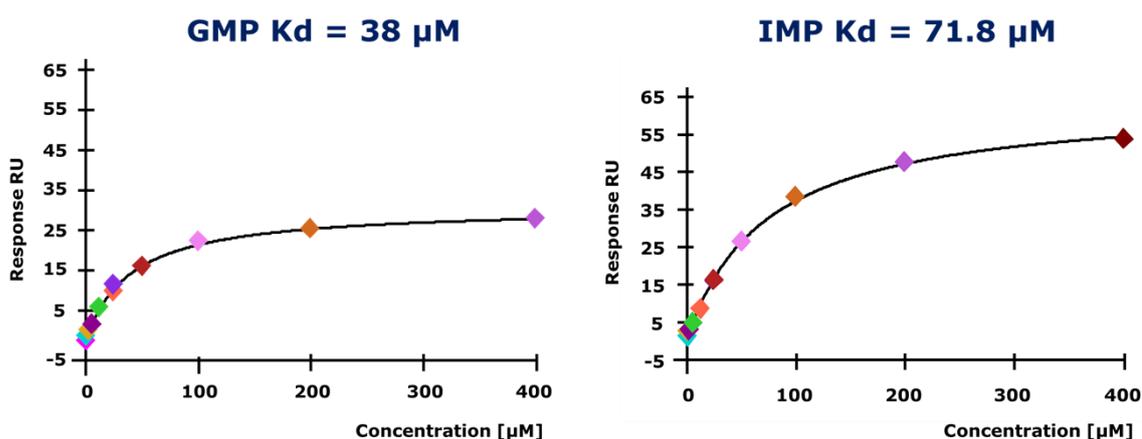


Figure 11. Biacore analysis of GMP and IMP. SPR studies between BP1253 and various monophosphate nucleotides revealed the BP1253 capability to bind GMP and IMP with good affinity. For GMP and IMP was used a ranging concentration from 0.78 to 400 μM . Identical results were obtained by independent experiments.

Nucleotides	Dissociation constant (kd)
AMP	5.7 μ M
GMP	38 μ M
IMP	71.8 μ M
TMP	2.4 mM
UMP	6.1 mM
CMP	ND

Table I. Purines and pyrimidines analyzed in surface plasmon resonance studies. List of monophosphate nucleotides evaluated by Biacore on BP1253 and the related kd.

The binding experiment was then extended to other nucleotide metabolites (Tab. II). Among them NAD, NADH and NADP revealed a kd of 0.3 μ M for NAD, 0.4 μ M for NADH and 4.4 μ M for NADP (Fig. 12). For the other substrates a very high kd or no binding was recorded. This suggests a binding capacity also for these metabolites.

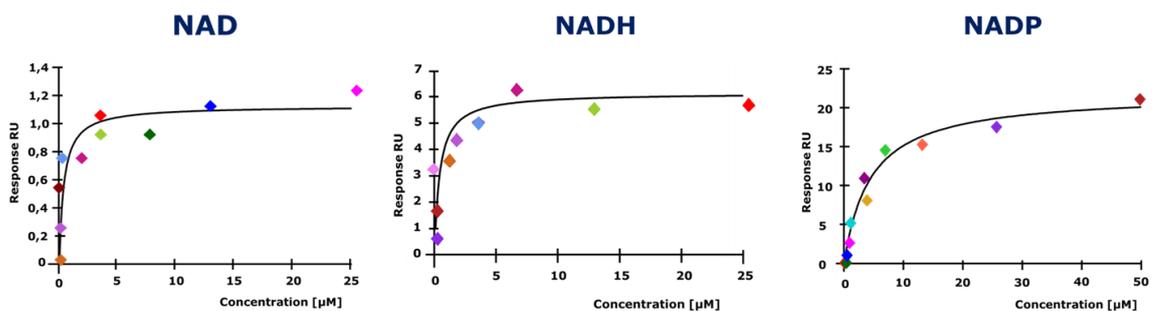


Figure 12. Biacore analysis of nucleotide metabolites. SPR investigations performed on nucleotide substrates NAD, NADH and NADP using Sensor chip CM5 and concentration of substrates from 0.097 to 50 μ M.

Nucleotide metabolites

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
NADP	Nicotinamide adenine dinucleotide phosphate
α -NAD	α -Nicotinamide adenine dinucleotide
ϵ -AMP	ϵ -Adenosine monophosphate
ADPR	Adenosine diphosphoribose
ATP	Adenosine triphosphate
NGD	Nicotinamide guanine dinucleotide
GTP	Guanosine triphosphate

Table II. List of nucleotide metabolites investigated in SPR studies.

4.4 *BP1253 HAS PHOSPHORIBOHYDROLASE ACTIVITY*

LONELY GUY catalytic activity consists in the hydrolysis of the N-glycosidic linkage between the N⁶-modified adenine and ribose 5'-monophosphate, leading to the formation of free and active cytokinins. Shown in Fig. 13 is the reaction catalyzed by phosphoribohydrolases.

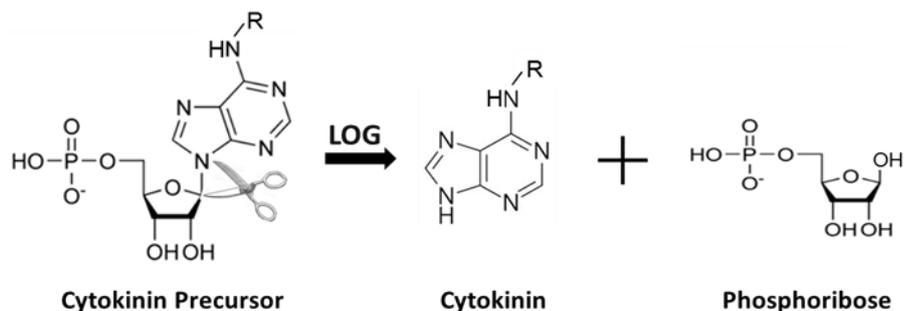


Figure 13. Schematic representation of phosphoribohydrolase catalyzed reaction.

Sequence alignments with LOG proteins showed that BP1253 has a high amino acid sequence identity with type-II LOGs and that shares with all of them the PGGxGTxxE motif and the catalytic residues. Additionally, the SPR analysis revealed the capability of the pertussis protein to bind AMP. These results led us to hypothesize that BP1253 could be a LOG protein. To examine the biochemical activity of BP1253, we performed phosphoribohydrolase activity assay. As substrate we used AMP, described to be the preferred substrate of LOG (Samanovic, Tu et al. 2015, Seo, Kim et al. 2016, Seo and Kim 2017, Seo and Kim 2018, Seo and Kim 2018). AMP and the product of the reaction adenine run at different height for their different hydrophilicity on TLC and are visible as spots under UV light at 264 nm. In dose-response experiment AMP was incubated at 30°C with different concentration of the protein and the reaction resulted BP1253 dose-dependent as shown in Fig 14 A. In the time course experiment, the substrate was incubated with 24 μ M of enzyme and the reaction stopped at different time points. It resulted that the catalytic activity increased over the time (Fig.14 B). As negative control for the experiments was used the heat inactivated protein (Fig.14 C).

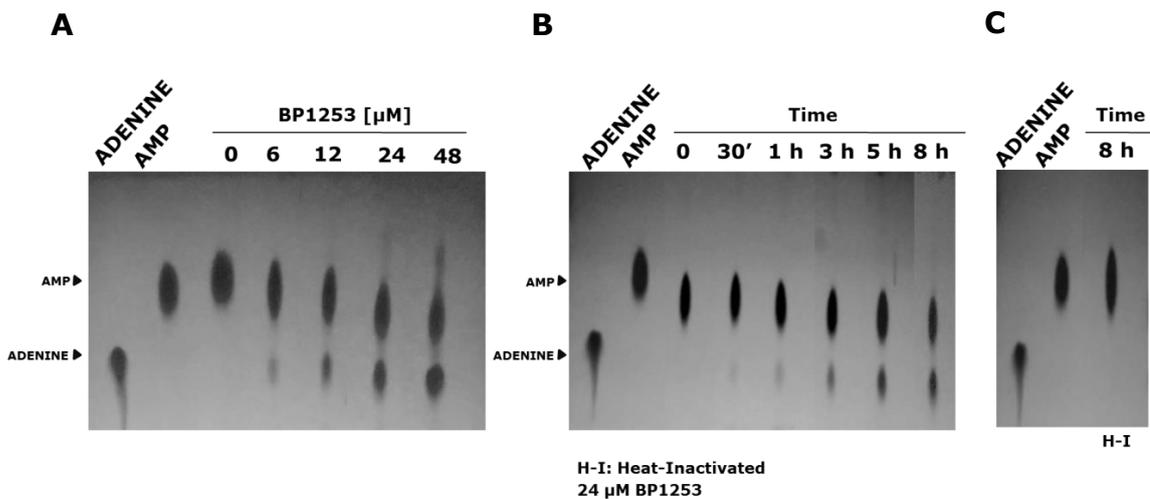


Figure 14. Dose-response and time-course experiments reveal phosphoribohydrolase activity in BP1253. The experiments were performed at 30°C in the presence of 20 mM AMP as substrate. After heat inactivation of the protein at 95°C for 2 min, the products were separated by TLC in 1 M NaCl using Cellulose F plastic sheets. The dots were evidenced under UV light at 264 nm. **(A)** Dose-response experiments carried out after 6 hours of incubation. The BP1253 concentrations used were 6, 12, 24 and 48 μ M. **(B)** Time course assay carried out with 24 μ M of enzyme at different incubation time. **(C)** TLC with the heat-inactivated protein as negative control performed after 8 hours of incubation.

Since the SPR study for GMP resulted in a dissociation constant of 38 μM comparable to that of AMP, in order to investigate if BP1253 is able to cleave the N-glycosidic linkage in GMP, we decided to carry out the enzymatic assay using GMP as substrate (Fig. 15).

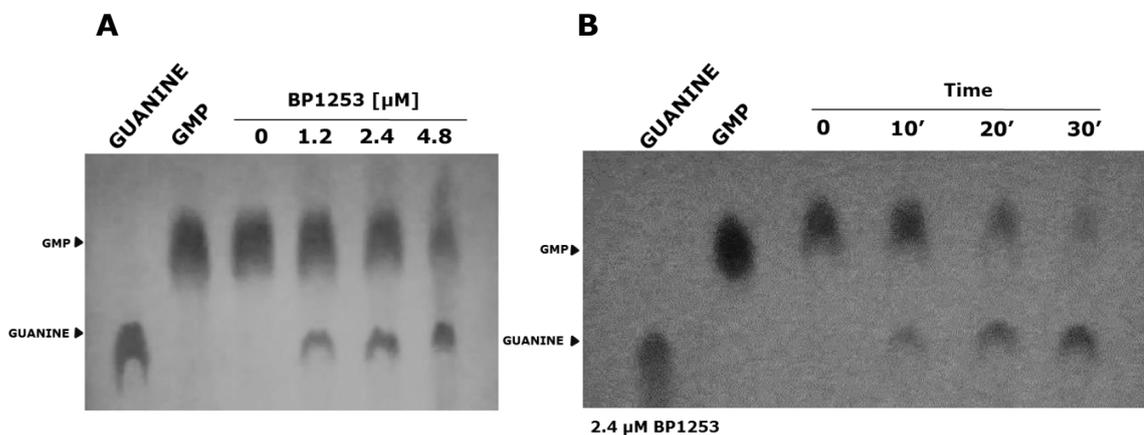


Figure 15. GMP is cleaved by BP1253. The assays were performed at 30°C in the presence of 20 mM GMP. To improve the solubility of GMP, the inactivation was performed with 1 M NaOH (v/v) and then the products of reaction separated by TLC in 1 M NaCl using Cellulose F plastic sheets. The dots were visible under UV light at 264 nm. (A) Dose-response experiments carried out in 20 minutes. 1.2, 2.4 and 4.8 μM were the enzymatic concentrations used for the assay (B) Time course experiment performed with 2.4 μM of BP1253 at different incubation time.

As clearly shown in Fig. 15, BP1253 recognizes GMP as substrate showing phosphoribohydrolase activity. Of note in the assays with GMP we used a 10-fold lower concentration of enzyme compared to that one used for the AMP. The substrate was almost completely hydrolyzed after 30 minutes of incubation indicating GMP a substrate more efficient compared to AMP for BP1253. For a more complete characterization of BP1253 we examined the protein activity with several ribonucleotides and deoxyribonucleotides (Tab. III). Among them a high phosphoribohydrolase activity was recorded also for CMP (Fig. 16). This partially explains the high k_d measured by biacore for CMP, the substrate is so fast cleaved that appears as no binding was present.

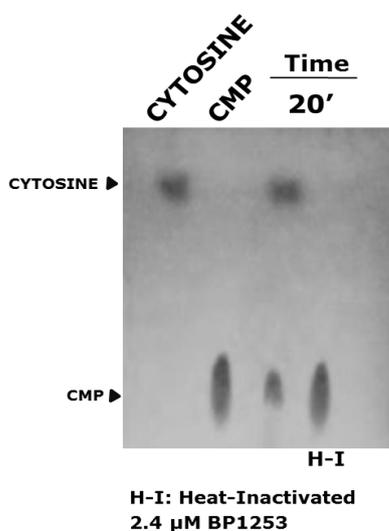


Figure 16. BP1253 cleaves CMP. The assay was carried out at 30°C in the presence of 20 mM CMP for 20 minutes. The inactivation was performed at 95°C for 2 min. To separate the products by TLC on Cellulose F plastic sheets the mobile phase was acetone/water 30/70 (v/v) with the addition of 0.2 mol/l NaCl. The dots were visible under UV light at 264 nm.

Nucleotides	20 minutes	24 hours
AMP	-	+
GMP	++	++
IMP	+	++
CMP	++	++
TMP	-	+
UMP	-	-
dAMP	-	-
dGMP	-	+
dCMP	+	++
dTMP	-	+
dUMP	+	++

Table III. Ribonucleotides and deoxyribonucleotides tested with BP1253 in phosphoribohydrolase assay. The different nucleotide substrates were incubated with 2.4 μM of BP1253 for 20 minutes and 24 hours at 30°C. The reaction was blocked with 1 M NaOH for those compounds having guanine as nitrogen base, whilst by heat at 95°C for 2 minutes for the others. The products were separated by TLC on Cellulose F plastic sheets. We used as mobile phase 1 M NaCl for purine separation while, the mobile phase for pyrimidines was acetone/water 30/70 (v/v) with the addition of 0.2 mol/l NaCl. The dots were visualized under UV light at 264 nm. We registered different activities from BP1253 depending on the incubated substrates. We indicate with - those reactions for which no phosphoribohydrolase activity was recorded, as + we specify reactions with a minimal activity, as ++ we identify high catalytic activity.

As previously described BP1253 contains the typical amino acids of LOG catalytic core. Therefore, to probe if the R120, E143 residues and K121 probably involved in substrate binding, may have a role in enzymatic activity, we generated by site-specific mutagenesis a panel of recombinant BP1253 mutant proteins. In these single point mutants R120, E143 and K121 were changed first with Ala and then with the conservative amino acid: Lys, Asp and Arg respectively. Among the amino acids implicated in the binding, we chose the K121 residue for its closeness to the catalytic amino acid R120. The mutants were expressed as His-tagged recombinant proteins in BL21 competent *E. coli* and purified by two step of high-throughput immobilized metal (Ni^{2+}) affinity chromatography. SDS-PAGE analysis and western immunoblotting confirmed the identity of the purified single point mutant proteins (Fig 17).

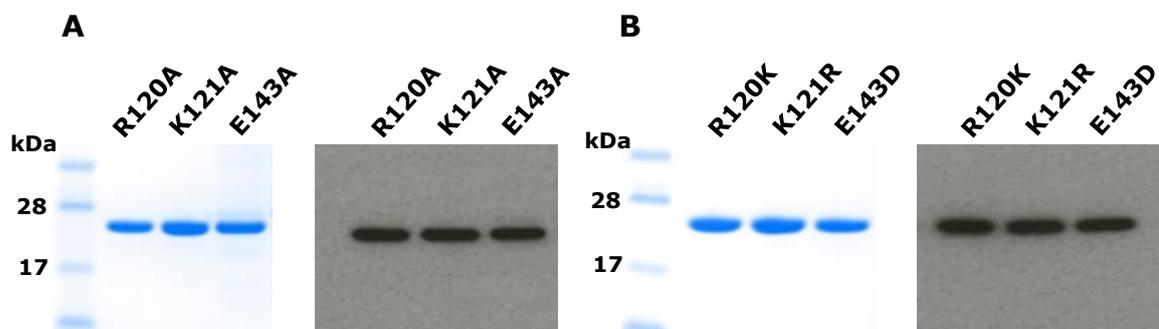
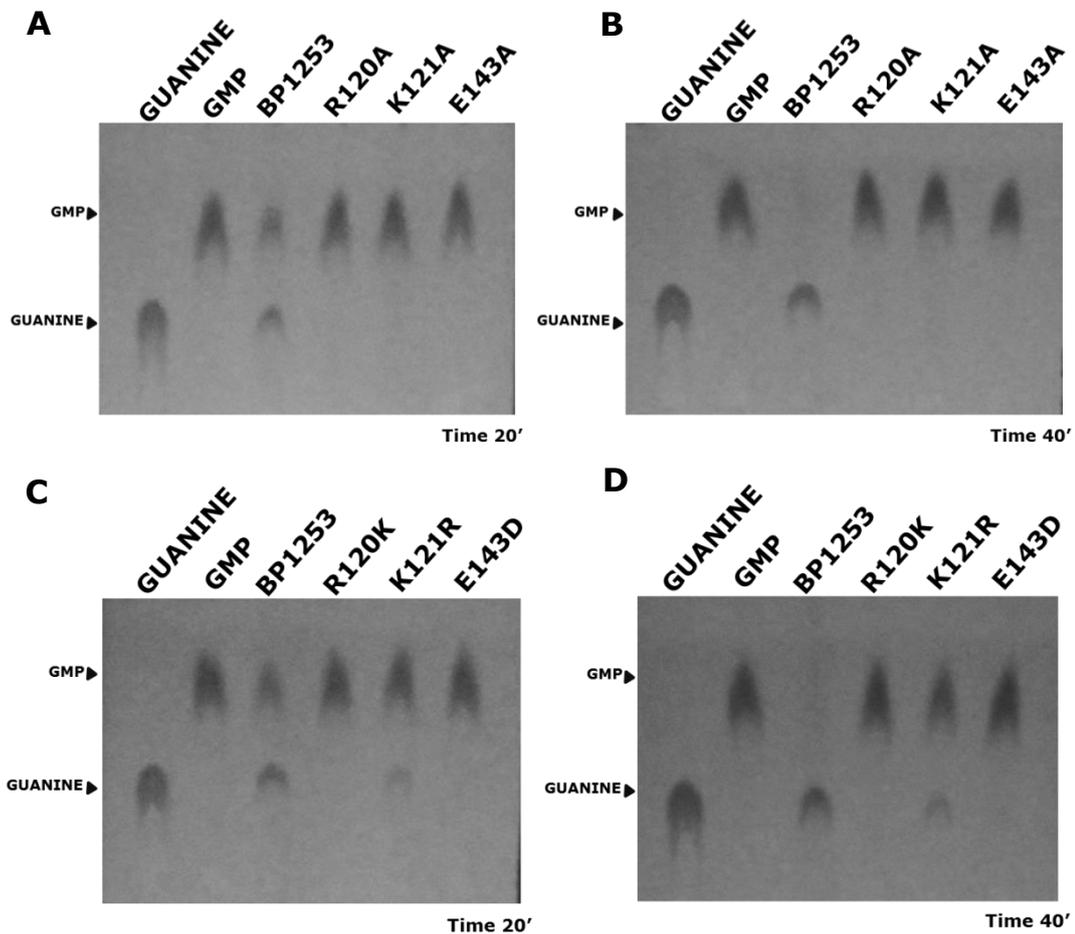


Figure 17. Purification profile of BP1253 mutants. BP1253 variants with specific site mutations with non-conservative and conservative amino acids were cloned in *E. Coli* BL21 (DE3) and purified in high-throughput through a His Multitrap HP 96-well Vacuum plate after IPTG induction. (A) SDS-PAGE (2 μg of proteins) and Immunoblotting (20 ng of proteins) performed on R120A, K121A and E143A. Mouse polyclonal α -BP1253 (1:500) was used as the primary antibody. Molecular weights of proteins markers are shown on the left. (B) SDS-PAGE (2 μg of proteins) and Immunoblotting (20 ng of proteins) carried out on R120K, K121R and E143D. Molecular mass markers (kDa) are indicated on the left and the mouse polyclonal α -BP1253 (1:500) was used for the WB analysis.

After purification, we tested the R120A, K121A and E143A mutant proteins with the substrates GMP (Fig. 18 A, B panel) and CMP (Fig. 18 E, F panel) in phosphoribohydrolase assays at 20 and 40 minutes. The non-conservative amino acid substitutions resulted in a complete loss of the enzymatic activity. When we tested conservative, R120K and E143D mutants resulted in a total absence of activity, while

the replacement of K with R at the residue 121 produced a minimal hydrolysis with GMP and CMP as substrates though not comparable to wild type (Fig. 18 C, D, G, H). These results confirm that Arg 120 and Glu 143 are the catalytic residues implicated in the phosphoribohydrolase activity and that the positive charge of the K at 121 residue is essential for the catalysis. AMP was assayed with conservative mutants for a time of 6 and 18 hours (Fig. 18 I, J). Also in these cases, R120K and E143D replacements caused the loss of the enzymatic activity and, K121R a hydrolysis almost undetectable on TLC. The results from the mutagenesis are in agreement with the recently described LOG proteins and this confirms that BP1253 is a member of the LOG family and, henceforth, we will refer to it as BpLOG.



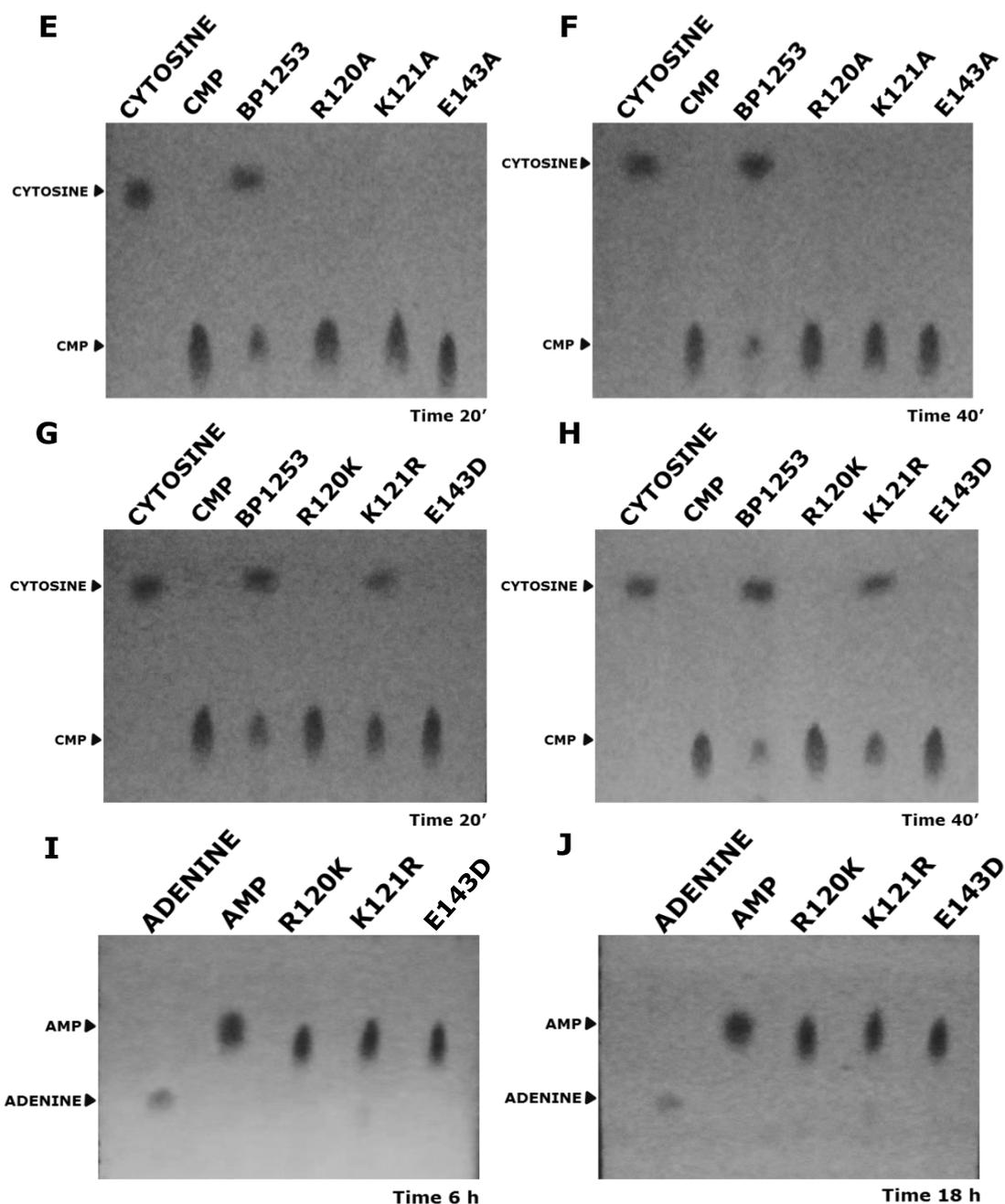


Figure 18. Phosphoribohydrolase activity assays with recombinant BP1253 mutant proteins. The experiments were all performed at 30°C in the presence of 20 mM of substrate. The incubation time was 20 and 40 minutes for GMP and CMP, and 6 and 18 hours for AMP. The reaction was blocked by heat inactivation for enzymes incubated with CMP and AMP and, with 1 M NaOH (v/v) for the proteins incubated with GMP. The products were separated by TLC on Cellulose F plastic sheets and the mobile phase was NaCl for purine and acetone/water 30/70 (v/v) with the addition of 0.2 mol/l NaCl for CMP. The dots were evidenced under UV light at 264 nm. As positive control we used wild type BP1253. (A) Non-conservative mutations incubated with GMP for 20 minutes and (B) for 40 minutes. (C) Conservative substitutions assayed with GMP for 20 minutes and (D) 40 minutes. (E) Alanine substitutions tested with CMP for 20 minutes and (F) for 40 minutes. (G) Conservative mutants assessed with CMP for 20 minutes and (H) 40 minutes. (I) Conservative replacements incubated with AMP for 6 hours and (J) 18 hours. For positive control in AMP experiments, refer to the figure number 14.

4.5 *BpLOG* SYNTHESIZES 6-O-METHYLGUANINE

BpLOG being a member of the LOG family and in analogy with its homologous proteins should be able to produce cytokinins. Therefore, we decided to try to identify the physiological product of *BpLOG*. For this purpose, we generated from Tohama I-derivative BP536 a *Bordetella* strain deleted of the operon in which is located *Bp1253* gene in order to compare the production of cytokinins between the wild type strain and the knockout. (*Bp1253* gene is located inside an operon along with other two genes, *Bp1251* and *Bp1252* that encode for a putative toxin and putative exported protein respectively. However, currently, the real function of both proteins is unknown). The attempt of this investigation is to identify the produced cytokinins by the two strains, to separate them in reverse phase through HPLC and to visualize them as peaks via UV detector exploiting the intrinsic capability of these compounds to adsorb UV light. In plants the cytokinins are hormones, therefore, they are produced in small quantities and released in the extracellular space. For this reason, we focused our attention first on supernatant. The knockout and the wild type strains were grown in Stainer-Scholte medium at 37°C until 48 hours, after that the supernatants were filtered, cleared by proteins and molecules with molecular weight higher than cytokinins as described in Methods (Dobrev, Hoyerova et al. 2017). At the end of the purification procedure, the dried samples, re-suspended in 100 µL 5 % methanol were run in HPLC and analyzed in MS. The two chromatographic spectra related to Tohama I strain supernatant and to $\Delta 1253$ supernatant arising from UV detector were identical, but we observed that at retention time of 5 minutes in wild type spectrum there was a small peak completely absent in $\Delta 1253$ (Fig. 19). This peak was isolated and analyzed in MS. It turned out that this peak corresponded to a compound with molecular weight of 165 Da, 166 Da in the mass chromatogram because of electrospray with a positive ion mode used as mass spectrometric detection, and with an apparent molecular formula of $C_6H_7N_5O$. In data banks the compound with this molecular formula that fitted better with our study was the 6-O-Methylguanin. We tested it on HPLC at the same conditions used for supernatants samples and noted that the retention time of 6-O-Methylguanin was very close to retention time of *BpLOG* physiological product (Fig. 19)

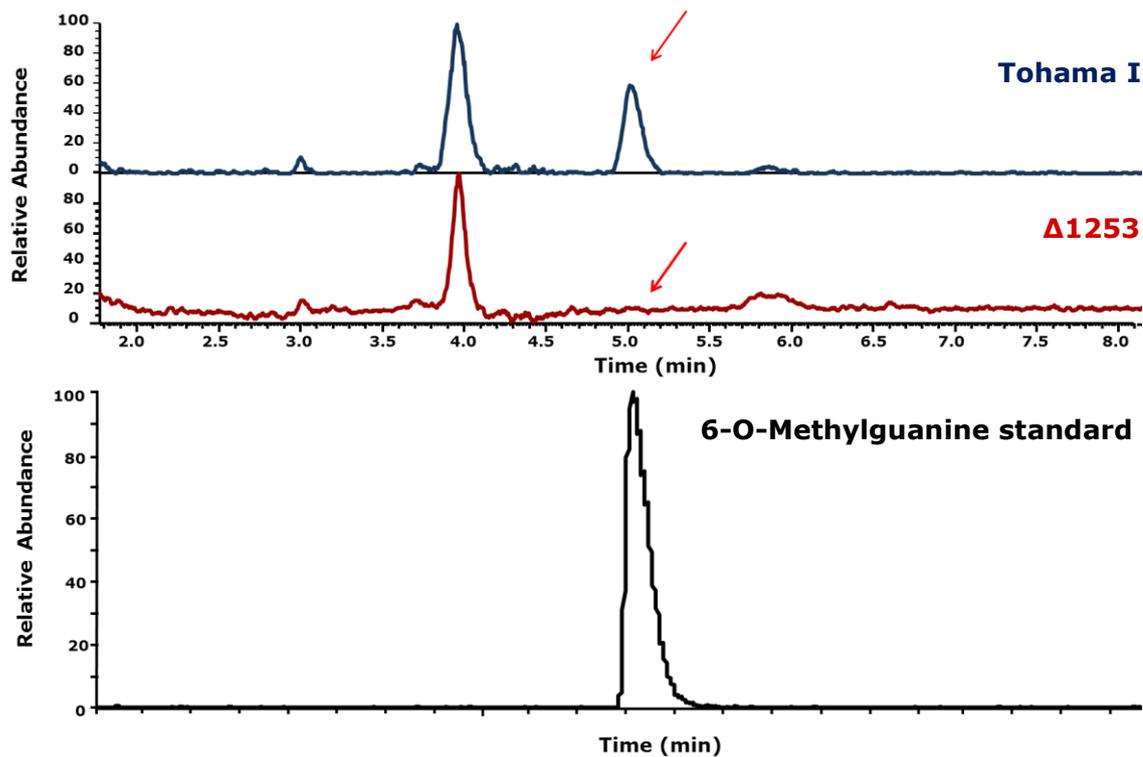


Figure 19. LC-MS analysis of BpLOG physiological product. LC investigations revealed at 268 nm the presence of a peak in Tohama I growth medium utterly absent in the medium of knockout strain. This peak isolated and analyzed in mass spectrometry in a positive ion mode with electrospray resulted in a compound of 166 Da with a putative molecular formula similar to that of 6-O-Methylguanine. The standard was run on HPLC and the retention time was similar to physiological product. The purification of cytokinins was performed by solid phase extraction with a MCX matrix after that the growth medium was filtered with a membrane 3K, the volumes reduced at 4 ml and then acidified with acid formic. The purified cytokinins were dried in SpeedVac, dissolved into 100 μ l 5 % methanol in water and centrifuged. The obtained samples were then analyzed in LC-MS and compared. The experiments were tested in triplicate.

To confirm that BpLOG is able to produce 6-O-Methylguanine, we directly applied a MS/MS approach to the isolated physiological product and to 6-O-Methylguanine standard in order to compare the two fragmentation mass spectra. We used collision energy of 25 a.u for both and observed that the two recorded fragmentation mass profiles corresponded with each other. This comparison gave the definitive proof that BpLOG synthesizes the 6-O-Methylguanine as physiological product (Fig. 20), in agreement with the ability of BpLOG to efficiently bind and cleave the GMP substrate.

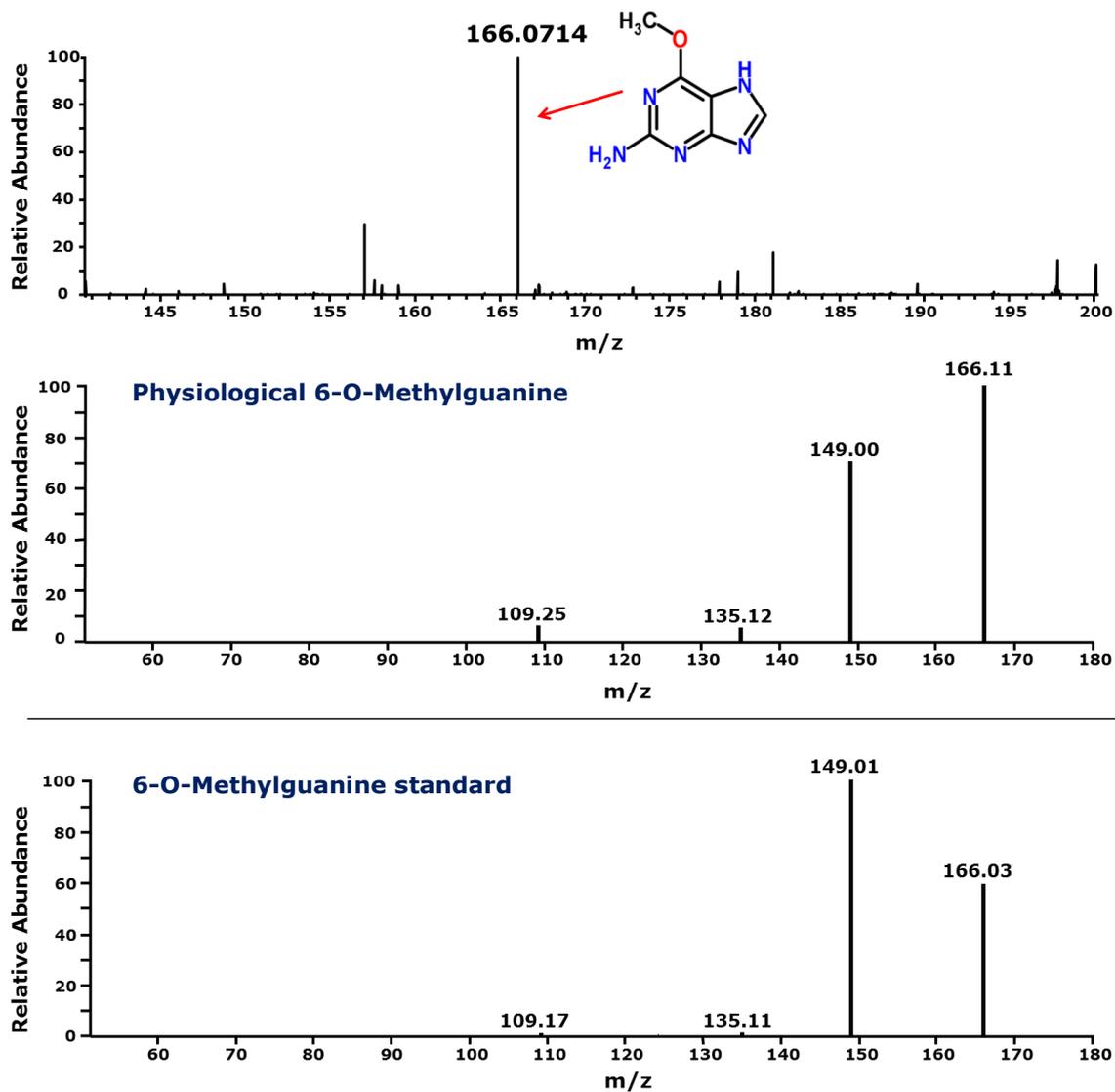


Figure 20. LC-MS/MS analysis of 6-O-Methylguanine. The MS/MS study on the physiological product and on 6-O-methylguanine confirmed the identity of the product synthesized by BpLOG. The collision energy used for the fragmentation was of 25 a.u.

Noteworthy is that we obtained the same results also for bacterial lysates and that the product 6-O-Methylguanine was found in clinical isolate strains supernatant B3629 and B3621 as well (Bart, van der Heide et al. 2015) (data not shown).

4.6 BpLOG IS PREVALENTLY EXPRESSED IN CLINICAL ISOLATES

To verify the expression of BpLOG in *B. pertussis*, we performed a Western Immunoblot analysis on total extracts and on supernatants of different strains. These strains were the lab strain Tohama I and two epidemic strains, B3621 and B3629, isolated in France in 2008 and in 2009 with the characteristic of not expressing the vaccine component pertactin and to have a novel allele for the promoter ptxP3 that lets *B. pertussis* to produce more pertussis toxin (Bart, van der Heide et al. 2015). The $\Delta 1253$ was used as negative control and the purified protein as positive control. The strains were grown for 24 hours and then lysed, whilst the proteins secreted in supernatants were TCA precipitated. The WB analysis evidenced that BpLOG is expressed in total extracts of bacteria and that is more expressed in clinical isolates compared to Tohama I (Fig. 21).

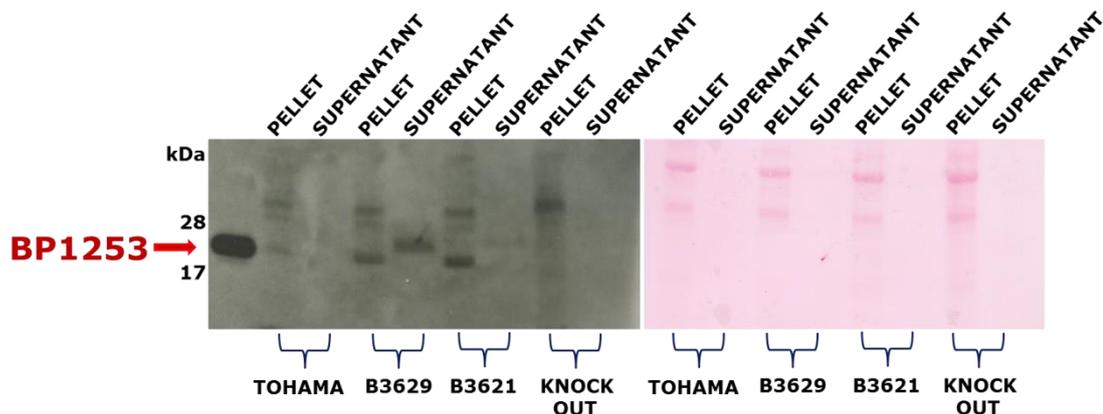


Figure 21. BpLOG is prevalently expressed in clinical isolates. In Western Immunoblot was compared the expression of BpLOG between Tohama I, clinical isolates B3629 and B3621, and $\Delta 1253$ strains. Bacterial lysates and supernatants were analyzed. The pellet of 1 OD of each strain was dissolved in 100 μ l of loading buffer with reducing agent and heated at 70°C. From these, 30 μ l were loaded onto the gel. The corresponding supernatants, after TCA precipitation, were dissolved in 20 μ l of loading buffer with reducing agent, heat inactivated and loaded onto the gel. It resulted out that BpLOG is present in total extracts and that is prevalently expressed in epidemic strains. The experiment was performed with mouse polyclonal α -BP1253 (1:500). 25 ng purified protein was the positive control. The panel on the left stained Red Ponceau was presented as control of equal loading of the proteins. Different experiments gave identical results.

4.7 BpLOG IS NEGATIVELY RELATED TO OXIDATIVE STRESS

In *Mycobacterium tuberculosis* has been recently described that an excess of cytokinins breakdown products could represent a source of toxicity for the bacterium, especially an accumulation of *p*-topolin degradation product pHBA that in the presence of Nitric Oxide (NO) could induce death of the mycobacterium itself. For this reason the MtLOG half-life is highly regulated by the proteasome that controls the level of MtLOG (Naseem, Sarukhanyan et al. 2015, Samanovic and Darwin 2015, Samanovic, Tu et al. 2015, Zhu and Javid 2015). Since BpLOG is a homologous of MtLOG and since we know that it produces 6-O-Methylguanine, we tested in the same oxidative stress conditions the Tohama I strain and Δ 1253 to estimate if even the presence of 6-O-Methylguanine can induce differences of survival between the two strains and make the wild type more sensitive. After a 24-hour growth we incubated the cultures for 30 minutes with 100 mM and 300 mM of hydrogen peroxide (H₂O₂) and following the treatment the bacteria were plated and their viability was measured (Fig. 22). From this oxidative stress condition emerged that Δ 1253 strain showed a greater resistance to hydrogen peroxide than the wild type. Indeed, Tohama I bacteria treated with 100 mM of hydrogen peroxide presented a percentage of viability of 58.8 % instead knockout strain exhibited a survival rate of 73.11 %. The same trend was observed also for the samples exposed at 300 mM of hydrogen peroxide. In these extreme conditions the wild type and Δ 1253 showed a viability of 18.34 % and 35.84 % respectively. In both treatments was measured a difference in the survival capability at oxidative stress induced by H₂O₂ in favor of Δ 1253 of 15-17 % (Fig 22).

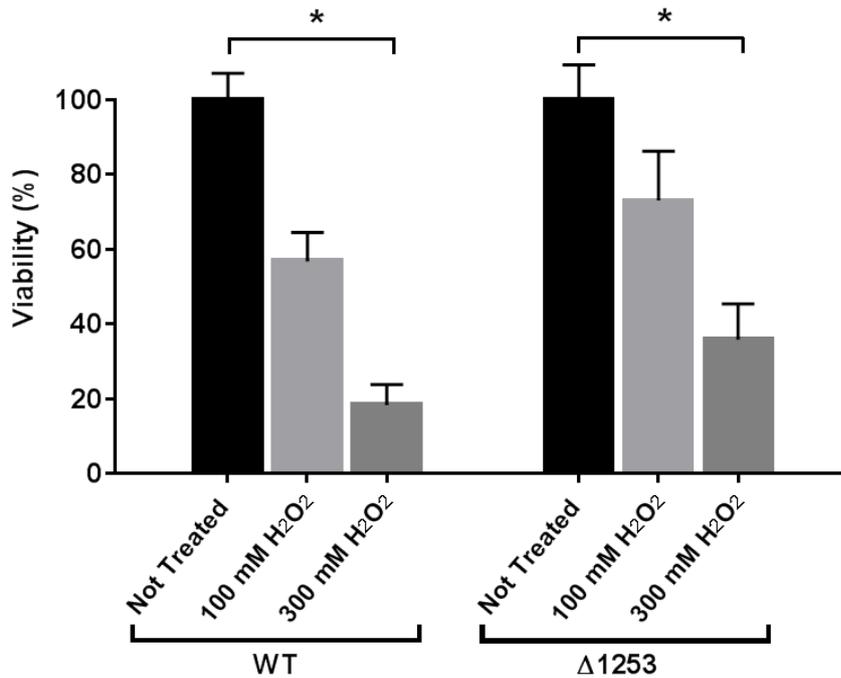


Figure 22. Tohama I and Δ1253 treated with H₂O₂. The Δ1253 strain presented a greater resistance to hydrogen peroxide. The Tohama I and Δ1253 strains after a 24 hours growth in Steiner-Scholte medium were treated for 30 minutes with 100mM and 300 mM of H₂O₂. Before treatment the corresponding OD was measured. At the end of the treatment a dilution of 10⁹ bacteria were plated on Bordet-Gengou agar plates and incubated at 37°C for three days. Finally, the number of colonies was counted. All samples exposed to H₂O₂ were compared to not treated samples with Kruskal-Wallis test. Samples incubated with 300 mM were statistically significantly with p value of 0.02. The others were not statistically significantly but we recorded a continuous decrease in vitality with increasing H₂O₂ concentration. The values presented represent the means ± SD of three independent experiments.

To verify that this recorded toxicity is due to the presence of 6-O-Methylguanine with H₂O₂, we incubated the two strains of interest either with 6-O-Methylguanine 10 μM alone or in combination with 100 mM H₂O₂ (Fig. 23). In the first case we observed a survival rate comparable to the not treated bacteria in both strains, on the contrary, in bacteria treated with H₂O₂ and 6-O-Methylguanine together, we noticed a considerable reduction of viability. In the wild type the survival percentage dropped to 19.8 %, in Δ1253 to 34.9 %. These percentages resulted much lower than that obtained incubating the bacteria only with H₂O₂ (Fig. 24). From this control experiment we confirmed that 6-O-Methylguanine alone is not toxic for *B. pertussis*, but in combination with hydrogen peroxide reduces the bacteria viability.

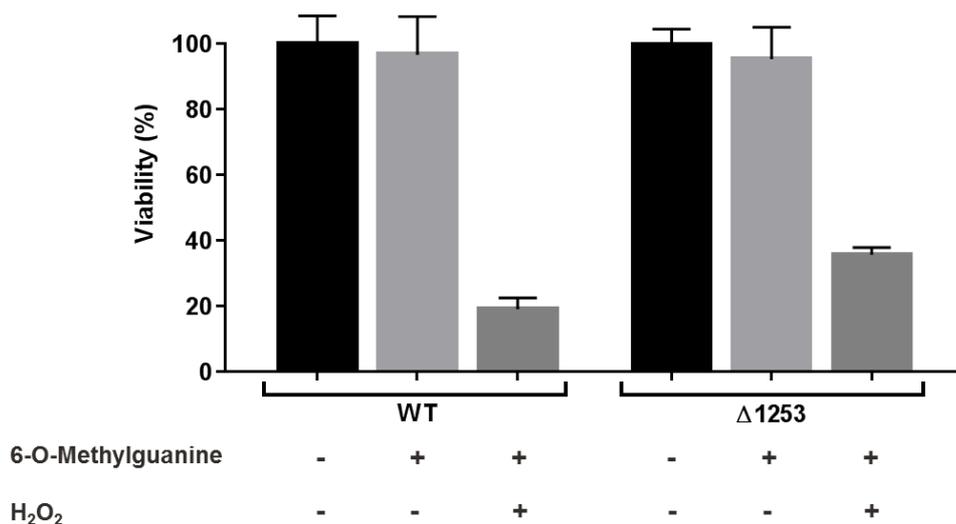


Figure 23. Tohama I and Δ1253 treated with 6-O-Methylguanaine and H₂O₂. 6-O-Methylguanaine in presence of hydrogen peroxide results to be toxic. The bacteria were grown for 24 hours, after that the OD was measured and a group of strains were incubated for 30 minutes with 6-O-Methylguanaine 10 μM and another group with H₂O₂ 100 mM and 6-O-Methylguanaine 10 μM. After treatment a dilution of 10⁹ bacteria were plated on BG agar plates and incubated at 37°C for three days for colony counts. The percentage of samples exposed to only 6-O-Methylguanaine were based on not treated bacteria, the ratio of the groups treated either with 6-O-Methylguanaine that with H₂O₂ was calculated on the base of colony number of groups treated only with 6-O-Methylguanaine. The values presented represent the means ± SD of three independent experiments.

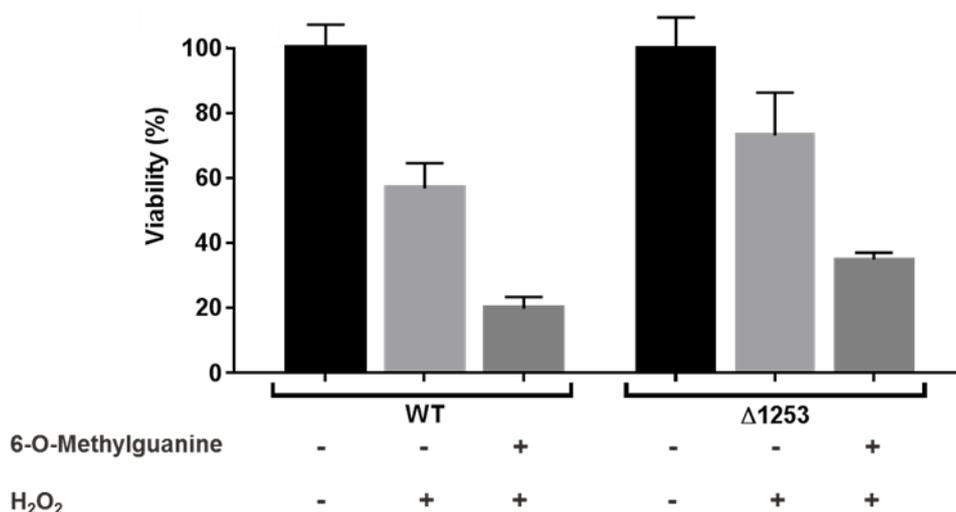


Figure 24. Effects of 6-O-Methylguanaine with H₂O₂. Viability comparison between non treated bacteria, samples exposed to 100 mM of hydrogen peroxide and samples treated with 6-O-Methylguanaine 10 μM and H₂O₂ 100 mM. In Tohama I strain 10 μM of 6-O-Methylguanaine reduces the viability of bacteria treated only with H₂O₂ of 37%, in Δ253 strains the adding of 10 μM of 6-O-Methylguanaine at 100 mM H₂O₂ decreases the vitality of 38.2 %. The values presented represent the means ± SD of three independent experiments.

5. DISCUSSION

Nowadays increasingly cases of Whooping cough infections are reported in all age groups with the consequent increase in mortality among infants. This resurgence of pertussis infection is seriously concerning the public health. As described previously, causes may be correlated to aP vaccines that induce a short last immunity not able to prevent colonization and transmission. In addition, the lack of herd immunity and the vaccine selection that leading to diffusion of *Bordetella* strains could exert a key role. Therefore, is necessary to increase our knowledge on *B. pertussis* microbiology in order to improve or find new strategies to existing ones. In this study we focused our attention on a protein of *B. pertussis* with unknown function so far. This protein named BP1253 and referred by us as BpLOG, was previously classified as lysine decarboxylase since it contains a PGGxGTxxE motif present also in the LOG family. LOG proteins have a phosphoribohydrolase activity, which consists in the cleavage of the N-glycosidic linkage between modified bases in position 6, prevalently adenines, and the ribose 5'-monophosphate moiety. Through this activity, LOG proteins synthetize and release cytokinins, phytohormones implicated in the control of many aspects of plants growth, defense and development. *In silico* analyses and *in vitro* experiments have demonstrated that BpLOG is a member of the LOG family. It shares the PGGxGTxxE motif and the catalytic amino acids Arg and Glu, it is able to bind AMP with a kd of 5.7 μ M and exerts phosphoribohydrolase activity towards AMP. Of interest was to discover that BpLOG can also bind other nucleotides like GMP, IMP and dinucleotides such as NAD, NADH and NADP. In addition, we found that surprisingly BpLOG has a higher phosphoribohydrolase activity towards GMP and CMP than AMP, though the dissociation constant for CMP was not detectable. This apparent discrepancy could be due to a strong instability of the substrate protein complex that results in a rapid release of the reaction products and in undetectable kd. A lower stability of the GMP-BpLOG complex compared to the AMP-BpLOG complex could also be the reason why GMP shows a higher kd than AMP. Currently, LOGs proteins are divided in two clusters based on their oligomeric state and each cluster is in turn further classified in two groups based on the amino acids present in the binding site. *In silico* analyses have shown that BpLOG has a primary structure similar to hexameric type-II LOGs. Based on the sequence homology, BpLOG would result a type-II LOG, but our studies have shown that the oligomeric state of BpLOG is dimeric. Moreover, the amino acid residues of the binding site are not represented in any subgroup. This latter, could explain the capability of this protein to bind and hydrolyze more effectively alternative

substrates like GMP and CMP. In the light of these differences we hypothesize that BpLOG could represent a further sub-group of type-II LOG or be the first characterized LOG of a new interposed group between the type-I and type-II LOG.

The peculiarities that distinguish BpLOG and make it unique are found also in the production of the physiological cytokinins. Generally, the cytokinins are adenine-derivatives modified at N⁶, but BpLOG produces the 6-O-Methylguanine, a guanine-derivative with a methyl-group at O⁶. Though the compounds known to possess cytokinin activity are adenine derivatives, synthesized O⁶-substituted guanine and hypoxanthine derivatives, have been described to act as cytokinins and induce cell division in plants (Hashizume, Sakai et al. 1976). Although the synthesis of the 6-O-Methylguanine as cytokinin could surprise, it sounds perfectly in agreement with the higher phosphoribohydrolase activity of BpLOG against GMP. Since BpLOG is able to produce a cytokinin-like compound different from the classical cytokinins and has substrate flexibility towards other nucleotides, we suppose that BpLOG is likely able to synthesize also other non-canonical cytokinins based on guanine and even on cytosine, compounds not identified with the methodology used in this study.

The presence of classical cytokinins was investigated and the isopentenyl-adenine and the kinetin were found in the bacterial lysates of *B. pertussis* both in wild type Tohama I and in Δ 1253 (data not shown) revealing no differences between the two strains. The production of modified adenine cytokinins is likely due to the presence of BP0547 classified as lysine decarboxylase, but which shares with LOGs the PGGxGTxxE motif and the catalytic amino acids. Sequence alignments with LOGs revealed that BP0547 is more similar to type-I LOG and has an identity of 54% with the characterized type-I *P. aeruginosa* LOG (Seo and Kim 2018) and greater homology than BpLOG with MtLOG, described to synthesize adenine-derivate cytokinins (Samanovic, Tu et al. 2015). In addition, PSORT-B prediction for BP0547 resulted in a cytoplasmic localization, while the TMHMM 2.0 server predicted for BP1253 a possible transmembrane helix at amino acids 36-58. Accordingly BpLOG could be most likely located in periplasmic space or on the external membrane. The different cellular localization of the two proteins could explain the presence of isopentenyl-adenine and kinetin in bacterial lysates, while 6-O-Methylguanine is released in the extracellular space. We have not found adenine-derivate cytokinins in the supernatant of wild type *Bp*, which could be explained by the low amount secreted, though we cannot exclude that BpLOG could be involved in the

biochemical pathway of canonical cytokinins. However, BpLOG is certainly the responsible of the synthesis of 6-O-Methylguanine.

The capability of BpLOG to recognize and metabolize several substrates could be further explained if we consider this protein as an enzyme equipped with a dual function. In *Saccharomyces cerevisiae* the overexpression of the analogous *LOG1* (*YJL055W*) gene conferred resistance to nucleotide analogues chemotherapeutic drugs. The LOG protein encoded by this gene is able to detoxify the purine analogue 6-N-hydroxylaminopurine (HAP) and the pyrimidine analogue 5-fluorouracil (5-FU) (Carlsson, Hu et al. 2018). Based on the detoxification properties of this LOG, we may hypothesize that BpLOG, besides producing and releasing 6-O-Methylguanine in the extracellular milieu, could also protect *B. pertussis* by degrading non-canonical nucleotides that could come from external environment and interfere with the normal development of the bacterium.

In plant, cytokinins are hormones that act locally or distant from the cell at very low concentrations (Dolezal, Popa et al. 2007). They modulate plant metabolism and morphogenesis in response to environmental factors such as light, water and nutrients (Hirose, Takei et al. 2008). At high concentrations, instead, cytokinins induce the plants to mount defenses against the attack of pathogens (Naseem, Wolfling et al. 2014). In plants, hence, the cytokinins are molecules involved in cellular communication triggering transcriptional changes to adapt the organism to environmental stress. Likely, under environmental stimuli, the 6-O-Methylguanine is released from *B. pertussis* as means of inter-bacteria communication to trigger the expression of specific genes and let the bacterium to adapt to these stimuli. Recently, the existence of a cytokinin signaling has been demonstrated in *M. tuberculosis* (Samanovic, Hsu et al. 2018). Through this signaling, isopentenyl-adenine, one of the most abundant cytokinin in *M. tuberculosis*, is able to regulate the expression of some genes like the *Rv0077c* and *Rv0078*. The first one encodes for an oxidoreductase, while the second one for its TetR-like regulator. The regulation of *Rv0077c* resulted in a loss of acid-fast staining of *M. tuberculosis*, generally associated with changes in bacterial envelope and virulence (Samanovic, Hsu et al. 2018). The cytokinin receptor in mammalian-pathogen has not been identified yet, but it is interesting to note that the cytokinin receptors in plants, fungi and phytopathogenic bacteria are structurally and functionally very similar to bacterial receptors associated to virulence, like the two-component regulatory system BvgAS (Steklov, Lomin et al. 2013, Wang, Cheng et al. 2017, Kabbara, Schmulling et

al. 2018). In plants the several classes of cytokinins are sensed via a two-component signaling phosphorelays, but the cyclase/histidine kinases-associated sensing extracellular (CHASE) domain, that binds cytokinins and triggers the signaling cascade, has not homologues proteins in *Bordetella* species.

Some studies have demonstrated that different cytokinins promote cellular differentiation or apoptosis in immune cells (Casati, Ottria et al. 2011), affect the production of cytokines (Ciaglia, Pisanti et al. 2014) and reduce inflammatory response (Lappas 2015, Santoro, Ciaglia et al. 2018). This latter suggested us that *B. pertussis* may secrete along with toxins also cytokinins, including 6-O-Methylguanine, to reshape the host immune system to its advantage though further investigation is necessary to confirm that 6-O-Methylguanine is released to modulate immune system synergistically with virulence factors.

In this thesis we have reported that the cytokinin-like 6-O-Methylguanine is negatively related to oxidative stress. Indeed, in the absence of 6-O-Methylguanine, the knockout $\Delta 1253$ strain treated with different concentrations of hydrogen peroxide, resulted more resistant than wild type Tohama I strain in the same conditions. After the addition of 6-O-Methylguanine to the growth medium during the treatment, $\Delta 1253$ strain reached survival rates comparable to those of wild type. Therefore, the 6-O-Methylguanine or its breakdown products could react with H_2O_2 producing a compound toxic for the bacterium. Similar results were recently obtained in *M. tuberculosis* (Samanovic, Tu et al. 2015), the non-proteasome regulation of MtLOG made the human-pathogen sensitive to nitric oxide (NO). Indeed, the overexpression of MtLOG led to an accumulation of cytokinin degradation products that interacting with NO produced toxic aldehydes. These results led us to speculate that adenine or guanine derivate molecules can react with reactive nitrogen and oxygen species (NOS, ROS) for their physical and chemical properties in oxidative stress conditions. It is interesting to note that to promote the bacterial clearance one of innate immune response during *Bordetella* infection is precisely the release of NOS and ROS in airways, as in the case of the Stimulated Innate Resistance (StIR) based on ROS production (Zurita, Moreno et al. 2013). Therefore, the host could exploit the interaction of ROS with 6-O-Methylguanine to promote the clearance of *B. pertussis*. However, since we found a greater expression of BpLOG in the clinical isolates strains compared to laboratory strain, we think that this protein is involved in infection. Our experiments on the effects of 6-O-Methylguanine in combination with H_2O_2 were all carried out *in vitro* and we do not

know what could happen *in vivo*. In alternative the release of 6-O-Methylguanine could be toxic for epithelial cells helping the colonization of *B. pertussis*. In agreement with this hypothesis, kinetin, which possesses antioxidant proprieties, was found in the *Bp* lysates (Brizzolari, Marinello et al. 2016, Othman, Naseem et al. 2016, Ahanger, Alyemeni et al. 2018, Honig, Plihalova et al. 2018). *B. pertussis* may produce the cytokinin kinetin to protect itself against oxidative stress while the release of 6-O-Methylguanine in combination with ROS can favor bacteria colonization.

All these features make BpLOG a novel exciting protein virtually involved in a whole unknown pathway in the mammalian-pathogen microbiology. This could open new branches in the research of *B. pertussis* treatments. Indeed, the importance of BpLOG in the medical field is increased if we consider that BpLOG homologous proteins are absent in humans. Accordingly, BpLOG could become a new pharmaceutical target in novel therapeutic strategies and represent an interesting research object in the study of *B. pertussis* biology.

Funding

This work was sponsored by Novartis Vaccines; in March 2015 the Novartis non-influenza Vaccines business was acquired by the GSK group of companies. The sponsor was involved in all stages of the study conduct and analysis.

Conflict of Interest statement: Filippo Moramarco participated in a post graduate studentship program at GSK.

Affiliations of supervisors: GSK and University.

6. BIBLIOGRAPHY

1. Ahanger, M. A., M. N. Alyemeni, L. Wijaya, S. A. Alamri, P. Alam, M. Ashraf and P. Ahmad (2018). "Potential of exogenously sourced kinetin in protecting *Solanum lycopersicum* from NaCl-induced oxidative stress through up-regulation of the antioxidant system, ascorbate-glutathione cycle and glyoxalase system." PLoS One **13**(9): e0202175.
2. Bajguz, A. and A. Piotrowska (2009). "Conjugates of auxin and cytokinin." Phytochemistry **70**(8): 957-969.
3. Bari, R. and J. D. Jones (2009). "Role of plant hormones in plant defence responses." Plant Mol Biol **69**(4): 473-488.
4. Bart, M. J., H. G. van der Heide, A. Zeddeman, K. Heuvelman, M. van Gent and F. R. Mooi (2015). "Complete Genome Sequences of 11 *Bordetella pertussis* Strains Representing the Pandemic ptxP3 Lineage." Genome Announc **3**(6).
5. Bart, M. J., A. Zeddeman, H. G. van der Heide, K. Heuvelman, M. van Gent and F. R. Mooi (2014). "Complete Genome Sequences of *Bordetella pertussis* Isolates B1917 and B1920, Representing Two Predominant Global Lineages." Genome Announc **2**(6).
6. Baugh, L., I. Phan, D. W. Begley, M. C. Clifton, B. Armour, D. M. Dranow, B. M. Taylor, M. M. Muruthi, J. Abendroth, J. W. Fairman, D. Fox, 3rd, S. H. Dieterich, B. L. Staker, A. S. Gardberg, R. Choi, S. N. Hewitt, A. J. Napuli, J. Myers, L. K. Barrett, Y. Zhang, M. Ferrell, E. Mundt, K. Thompkins, N. Tran, S. Lyons-Abbott, A. Abramov, A. Sekar, D. Serbzhinskiy, D. Lorimer, G. W. Buchko, R. Stacy, L. J. Stewart, T. E. Edwards, W. C. Van Voorhis and P. J. Myler (2015). "Increasing the structural coverage of tuberculosis drug targets." Tuberculosis (Edinb) **95**(2): 142-148.
7. Boucher, P. E., A. E. Maris, M. S. Yang and S. Stibitz (2003). "The response regulator BvgA and RNA polymerase alpha subunit C-terminal domain bind simultaneously to different faces of the same segment of promoter DNA." Mol Cell **11**(1): 163-173.
8. Boucher, P. E., K. Murakami, A. Ishihama and S. Stibitz (1997). "Nature of DNA binding and RNA polymerase interaction of the *Bordetella pertussis* BvgA transcriptional activator at the *fha* promoter." J Bacteriol **179**(5): 1755-1763.
9. Brizzolari, A., C. Marinello, M. Carini, E. Santaniello and P. A. Biondi (2016). "Evaluation of the antioxidant activity and capacity of some natural N6-substituted adenine derivatives (cytokinins) by fluorimetric and

- spectrophotometric assays." J Chromatogr B Analyt Technol Biomed Life Sci **1019**: 164-168.
10. Carbonetti, N. H. (2010). "Pertussis toxin and adenylate cyclase toxin: key virulence factors of *Bordetella pertussis* and cell biology tools." Future Microbiol **5**(3): 455-469.
 11. Carlsson, M., G. Z. Hu and H. Ronne (2018). "Gene dosage effects in yeast support broader roles for the LOG1, HAM1 and DUT1 genes in detoxification of nucleotide analogues." PLoS One **13**(5): e0196840.
 12. Casati, S., R. Ottria, E. Baldoli, E. Lopez, J. A. Maier and P. Ciuffreda (2011). "Effects of cytokinins, cytokinin ribosides and their analogs on the viability of normal and neoplastic human cells." Anticancer Res **31**(10): 3401-3406.
 13. Christie, C. D. and R. S. Baltimore (1989). "Pertussis in neonates." Am J Dis Child **143**(10): 1199-1202.
 14. Ciaglia, E., S. Pisanti, P. Picardi, C. Laezza, S. Sosa, A. Tubaro, M. Vitale, P. Gazzero, A. M. Malfitano and M. Bifulco (2014). "N6-isopentenyladenosine affects cytotoxic activity and cytokines production by IL-2 activated NK cells and exerts topical anti-inflammatory activity in mice." Pharmacol Res **89**: 1-10.
 15. Confer, D. L. and J. W. Eaton (1982). "Phagocyte impotence caused by an invasive bacterial adenylate cyclase." Science **217**(4563): 948-950.
 16. Cookson, B. T., A. N. Tyler and W. E. Goldman (1989). "Primary structure of the peptidoglycan-derived tracheal cytotoxin of *Bordetella pertussis*." Biochemistry **28**(4): 1744-1749.
 17. Cortleven, A. and T. Schmulling (2015). "Regulation of chloroplast development and function by cytokinin." J Exp Bot **66**(16): 4999-5013.
 18. Cotter, P. A. and J. F. Miller (1994). "BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model." Infect Immun **62**(8): 3381-3390.
 19. Cowell, J. L., E. L. Hewlett and C. R. Manclark (1979). "Intracellular localization of the dermonecrotic toxin of *Bordetella pertussis*." Infect Immun **25**(3): 896-901.
 20. Cummings, C. A., H. J. Bootsma, D. A. Relman and J. F. Miller (2006). "Species- and strain-specific control of a complex, flexible regulon by *Bordetella BvgAS*." J Bacteriol **188**(5): 1775-1785.

21. de Gouw, D., D. A. Diavatopoulos, H. J. Bootsma, P. W. Hermans and F. R. Mooi (2011). "Pertussis: a matter of immune modulation." FEMS Microbiol Rev **35**(3): 441-474.
22. Decker, K. B., T. D. James, S. Stibitz and D. M. Hinton (2012). "The Bordetella pertussis model of exquisite gene control by the global transcription factor BvgA." Microbiology **158**(Pt 7): 1665-1676.
23. Dobrev, P. I., K. Hoyerova and J. Petrasek (2017). "Analytical Determination of Auxins and Cytokinins." Methods Mol Biol **1569**: 31-39.
24. Dolezal, K., I. Popa, E. Hauserova, L. Spichal, K. Chakrabarty, O. Novak, V. Krystof, J. Voller, J. Holub and M. Strnad (2007). "Preparation, biological activity and endogenous occurrence of N6-benzyladenosines." Bioorg Med Chem **15**(11): 3737-3747.
25. Dzurova, L., F. Forneris, S. Savino, P. Galuszka, J. Vrabka and I. Frebort (2015). "The three-dimensional structure of "Lonely Guy" from *Claviceps purpurea* provides insights into the phosphoribohydrolase function of Rossmann fold-containing lysine decarboxylase-like proteins." Proteins **83**(8): 1539-1546.
26. Edwards, J. A., N. A. Groathouse and S. Boitano (2005). "Bordetella bronchiseptica adherence to cilia is mediated by multiple adhesin factors and blocked by surfactant protein A." Infect Immun **73**(6): 3618-3626.
27. El-Azami-El-Idrissi, M., C. Bauche, J. Loucka, R. Osicka, P. Sebo, D. Ladant and C. Leclerc (2003). "Interaction of Bordetella pertussis adenylate cyclase with CD11b/CD18: Role of toxin acylation and identification of the main integrin interaction domain." J Biol Chem **278**(40): 38514-38521.
28. el Baya, A., K. Bruckener and M. A. Schmidt (1999). "Nonrestricted differential intoxication of cells by pertussis toxin." Infect Immun **67**(1): 433-435.
29. Elliott, E., P. McIntyre, G. Ridley, A. Morris, J. Massie, J. McEniery and G. Knight (2004). "National study of infants hospitalized with pertussis in the acellular vaccine era." Pediatr Infect Dis J **23**(3): 246-252.
30. Gan, S. and R. M. Amasino (1995). "Inhibition of leaf senescence by autoregulated production of cytokinin." Science **270**(5244): 1986-1988.
31. Giron, D., E. Frago, G. Glevarec, C. M. J. Pieterse and M. Dicke (2013). "Cytokinins as key regulators in plant–microbe–insect interactions: connecting plant growth and defence." Functional Ecology **27**(3): 599-609.

32. Glaser, P., A. Danchin, D. Ladant, O. Barzu and A. Ullmann (1988). "Bordetella pertussis adenylate cyclase: the gene and the protein." Tokai J Exp Clin Med **13 Suppl**: 239-252.
33. Glaser, P., A. Elmaoglou-Lazaridou, E. Krin, D. Ladant, O. Barzu and A. Danchin (1989). "Identification of residues essential for catalysis and binding of calmodulin in Bordetella pertussis adenylate cyclase by site-directed mutagenesis." Embo j **8**(3): 967-972.
34. Graf, R., J. Codina and L. Birnbaumer (1992). "Peptide inhibitors of ADP-ribosylation by pertussis toxin are substrates with affinities comparable to those of the trimeric GTP-binding proteins." Mol Pharmacol **42**(5): 760-764.
35. Guermonprez, P., N. Khelef, E. Blouin, P. Rieu, P. Ricciardi-Castagnoli, N. Guiso, D. Ladant and C. Leclerc (2001). "The adenylate cyclase toxin of Bordetella pertussis binds to target cells via the alpha(M)beta(2) integrin (CD11b/CD18)." J Exp Med **193**(9): 1035-1044.
36. Guevara, C., C. Zhang, J. A. Gaddy, J. Iqbal, J. Guerra, D. P. Greenberg, M. D. Decker, N. Carbonetti, T. D. Starner, P. B. McCray, Jr., F. R. Mooi and O. G. Gomez-Duarte (2016). "Highly differentiated human airway epithelial cells: a model to study host cell-parasite interactions in pertussis." Infect Dis (Lond) **48**(3): 177-188.
37. Hashizume, T., S. Sakai, T. Sugiyama and S. Matsubara (1976). "Cytokinin activity of O6-substituted guanine and hypoxanthine derivatives." Phytochemistry **15**(12): 1813-1815.
38. Heiss, L. N., S. A. Moser, E. R. Unanue and W. E. Goldman (1993). "Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis." Infect Immun **61**(8): 3123-3128.
39. Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez and D. Ala'Aldeen (2004). "Type V protein secretion pathway: the autotransporter story." Microbiol Mol Biol Rev **68**(4): 692-744.
40. Higgs, R., S. C. Higgins, P. J. Ross and K. H. Mills (2012). "Immunity to the respiratory pathogen Bordetella pertussis." Mucosal Immunol **5**(5): 485-500.
41. Hinsch, J., J. Vrabka, B. Oeser, O. Novak, P. Galuszka and P. Tudzynski (2015). "De novo biosynthesis of cytokinins in the biotrophic fungus Claviceps purpurea." Environ Microbiol **17**(8): 2935-2951.

42. Hirose, N., K. Takei, T. Kuroha, T. Kamada-Nobusada, H. Hayashi and H. Sakakibara (2008). "Regulation of cytokinin biosynthesis, compartmentalization and translocation." J Exp Bot **59**(1): 75-83.
43. Honig, M., L. Plihalova, L. Spichal, J. Gruz, A. Kadlecova, J. Voller, A. R. Svobodova, J. Vostalova, J. Ulrichova, K. Dolezal and M. Strnad (2018). "New cytokinin derivatives possess UVA and UVB photoprotective effect on human skin cells and prevent oxidative stress." Eur J Med Chem **150**: 946-957.
44. Inatsuka, C. S., S. M. Julio and P. A. Cotter (2005). "Bordetella filamentous hemagglutinin plays a critical role in immunomodulation, suggesting a mechanism for host specificity." Proc Natl Acad Sci U S A **102**(51): 18578-18583.
45. Inatsuka, C. S., Q. Xu, I. Vujkovic-Cvijin, S. Wong, S. Stibitz, J. F. Miller and P. A. Cotter (2010). "Pertactin is required for Bordetella species to resist neutrophil-mediated clearance." Infect Immun **78**(7): 2901-2909.
46. Jeon, W. B., S. T. Allard, C. A. Bingman, E. Bitto, B. W. Han, G. E. Wesenberg and G. N. Phillips, Jr. (2006). "X-ray crystal structures of the conserved hypothetical proteins from Arabidopsis thaliana gene loci At5g11950 and AT2g37210." Proteins **65**(4): 1051-1054.
47. Kabbara, S., T. Schmulling and N. Papon (2018). "CHASEing Cytokinin Receptors in Plants, Bacteria, Fungi, and Beyond." Trends Plant Sci **23**(3): 179-181.
48. Kamanova, J., O. Kofronova, J. Masin, H. Genth, J. Vojtova, I. Linhartova, O. Benada, I. Just and P. Sebo (2008). "Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling." J Immunol **181**(8): 5587-5597.
49. Kotob, S. I., S. Z. Hausman and D. L. Burns (1995). "Localization of the promoter for the ptl genes of Bordetella pertussis, which encode proteins essential for secretion of pertussis toxin." Infect Immun **63**(8): 3227-3230.
50. Kowalzik, F., A. P. Barbosa, V. R. Fernandes, P. R. Carvalho, M. L. Avila-Aguero, D. Y. Goh, A. Goh, J. G. de Miguel, F. Moraga, J. Roca, M. Campins, M. Huang, J. Quian, N. Riley, D. Beck and T. Verstraeten (2007). "Prospective multinational study of pertussis infection in hospitalized infants and their household contacts." Pediatr Infect Dis J **26**(3): 238-242.

51. Kuchar, E., M. Karlikowska-Skwarnik, S. Han and A. Nitsch-Osuch (2016). "Pertussis: History of the Disease and Current Prevention Failure." Adv Exp Med Biol **934**: 77-82.
52. Kukimoto-Niino, M., K. Murayama, M. Kato-Murayama, M. Idaka, Y. Bessho, A. Tatsuguchi, R. Ushikoshi-Nakayama, T. Terada, S. Kuramitsu, M. Shirouzu and S. Yokoyama (2004). "Crystal structures of possible lysine decarboxylases from *Thermus thermophilus* HB8." Protein Sci **13**(11): 3038-3042.
53. Kurakawa, T., N. Ueda, M. Maekawa, K. Kobayashi, M. Kojima, Y. Nagato, H. Sakakibara and J. Kyojuka (2007). "Direct control of shoot meristem activity by a cytokinin-activating enzyme." Nature **445**(7128): 652-655.
54. Ladant, D., S. Michelson, R. Sarfati, A. M. Gilles, R. Predeleanu and O. Barzu (1989). "Characterization of the calmodulin-binding and of the catalytic domains of *Bordetella pertussis* adenylate cyclase." J Biol Chem **264**(7): 4015-4020.
55. Lappas, C. M. (2015). "The plant hormone zeatin riboside inhibits T lymphocyte activity via adenosine A2A receptor activation." Cell Mol Immunol **12**(1): 107-112.
56. Locht, C., L. Coutte and N. Mielcarek (2011). "The ins and outs of pertussis toxin." Febs j **278**(23): 4668-4682.
57. Lynfield, R. and W. Schaffner (2014). "Can we conquer coqueluche?" J Infect Dis **209 Suppl 1**: S1-3.
58. Mallory, F. B. and A. A. Hornor (1912). "Pertussis: The histological Lesion in the Respiratory Tract." J Med Res **27**(2): 115-124.113.
59. Mattoo, S. and J. D. Cherry (2005). "Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies." Clin Microbiol Rev **18**(2): 326-382.
60. Mazar, J. and P. A. Cotter (2006). "Topology and maturation of filamentous haemagglutinin suggest a new model for two-partner secretion." Mol Microbiol **62**(3): 641-654.
61. Mazar, J. and P. A. Cotter (2007). "New insight into the molecular mechanisms of two-partner secretion." Trends Microbiol **15**(11): 508-515.
62. McGregor, J., J. W. Ogle and G. Curry-Kane (1986). "Perinatal pertussis." Obstet Gynecol **68**(4): 582-586.

63. Melvin, J. A., E. V. Scheller, J. F. Miller and P. A. Cotter (2014). "Bordetella pertussis pathogenesis: current and future challenges." Nat Rev Microbiol **12**(4): 274-288.
64. Mooi, F. R., N. A. Van Der Maas and H. E. De Melker (2014). "Pertussis resurgence: waning immunity and pathogen adaptation - two sides of the same coin." Epidemiol Infect **142**(4): 685-694.
65. Naseem, M., E. Bencurova and T. Dandekar (2018). "The Cytokinin-Activating LOG-Family Proteins Are Not Lysine Decarboxylases." Trends Biochem Sci **43**(4): 232-236.
66. Naseem, M., E. Sarukhanyan and T. Dandekar (2015). "LONELY-GUY Knocks Every Door: Crosskingdom Microbial Pathogenesis." Trends Plant Sci **20**(12): 781-783.
67. Naseem, M., M. Wolfling and T. Dandekar (2014). "Cytokinins for immunity beyond growth, galls and green islands." Trends Plant Sci **19**(8): 481-484.
68. Nicholson, T. L. (2007). "Construction and validation of a first-generation Bordetella bronchiseptica long-oligonucleotide microarray by transcriptional profiling the Bvg regulon." BMC Genomics **8**: 220.
69. Nieves, D. J. and U. Heininger (2016). "Bordetella pertussis." Microbiol Spectr **4**(3).
70. Othman, E. M., M. Naseem, E. Awad, T. Dandekar and H. Stopper (2016). "The Plant Hormone Cytokinin Confers Protection against Oxidative Stress in Mammalian Cells." PLoS One **11**(12): e0168386.
71. Paddock, C. D., G. N. Sanden, J. D. Cherry, A. A. Gal, C. Langston, K. M. Tatti, K. H. Wu, C. S. Goldsmith, P. W. Greer, J. L. Montague, M. T. Eliason, R. C. Holman, J. Guarner, W. J. Shieh and S. R. Zaki (2008). "Pathology and pathogenesis of fatal Bordetella pertussis infection in infants." Clin Infect Dis **47**(3): 328-338.
72. Porter, J. F., R. Parton and A. C. Wardlaw (1991). "Growth and survival of Bordetella bronchiseptica in natural waters and in buffered saline without added nutrients." Appl Environ Microbiol **57**(4): 1202-1206.
73. Radhika, V., N. Ueda, Y. Tsuboi, M. Kojima, J. Kikuchi, T. Kudo and H. Sakakibara (2015). "Methylated Cytokinins from the Phytopathogen Rhodococcus fascians Mimic Plant Hormone Activity." Plant Physiol **169**(2): 1118-1126.

74. Ramakrishnan, L., N. A. Federspiel and S. Falkow (2000). "Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family." Science **288**(5470): 1436-1439.
75. Sakamoto, H., J. Bellalou, P. Sebo and D. Ladant (1992). "Bordetella pertussis adenylate cyclase toxin. Structural and functional independence of the catalytic and hemolytic activities." J Biol Chem **267**(19): 13598-13602.
76. Samanovic, M. I. and K. H. Darwin (2015). "Cytokinins beyond plants: synthesis by Mycobacterium tuberculosis." Microb Cell **2**(5): 168-170.
77. Samanovic, M. I., H. C. Hsu, M. B. Jones, V. Jones, M. R. McNeil, S. H. Becker, A. T. Jordan, M. Strnad, C. Xu, M. Jackson, H. Li and K. H. Darwin (2018). "Cytokinin Signaling in Mycobacterium tuberculosis." MBio **9**(3).
78. Samanovic, M. I., S. Tu, O. Novak, L. M. Iyer, F. E. McAllister, L. Aravind, S. P. Gygi, S. R. Hubbard, M. Strnad and K. H. Darwin (2015). "Proteasomal control of cytokinin synthesis protects Mycobacterium tuberculosis against nitric oxide." Mol Cell **57**(6): 984-994.
79. Santoro, A., E. Ciaglia, V. Nicolin, A. Pescatore, L. Prota, M. Capunzo, M. V. Ursini, S. L. Nori and M. Bifulco (2018). "The isoprenoid end product N6-isopentenyladenosine reduces inflammatory response through the inhibition of the NFkappaB and STAT3 pathways in cystic fibrosis cells." Inflamm Res **67**(4): 315-326.
80. Schmidt, G., U. M. Goehring, J. Schirmer, M. Lerm and K. Aktories (1999). "Identification of the C-terminal part of Bordetella dermonecrotic toxin as a transglutaminase for rho GTPases." J Biol Chem **274**(45): 31875-31881.
81. Seo, H. and K. J. Kim (2017). "Structural basis for a novel type of cytokinin-activating protein." Sci Rep **7**: 45985.
82. Seo, H. and K. J. Kim (2018). "Structural and biochemical characterization of the type-II LOG protein from Streptomyces coelicolor A3." Biochem Biophys Res Commun **499**(3): 577-583.
83. Seo, H. and K. J. Kim (2018). "Structural insight into molecular mechanism of cytokinin activating protein from Pseudomonas aeruginosa PAO1." Environ Microbiol.
84. Seo, H., S. Kim, H. Y. Sagong, H. F. Son, K. S. Jin, I. K. Kim and K. J. Kim (2016). "Structural basis for cytokinin production by LOG from Corynebacterium glutamicum." Sci Rep **6**: 31390.

85. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk (1985). "Measurement of protein using bicinchoninic acid." Anal Biochem **150**(1): 76-85.
86. Stein, P. E., A. Boodhoo, G. D. Armstrong, S. A. Cockle, M. H. Klein and R. J. Read (1994). "The crystal structure of pertussis toxin." Structure **2**(1): 45-57.
87. Steklov, M. Y., S. N. Lomin, D. I. Osolodkin and G. A. Romanov (2013). "Structural basis for cytokinin receptor signaling: an evolutionary approach." Plant Cell Rep **32**(6): 781-793.
88. Trainor, E. A., T. L. Nicholson and T. J. Merkel (2015). "Bordetella pertussis transmission." Pathog Dis **73**(8): ftv068.
89. Tuomanen, E. I. and J. O. Hendley (1983). "Adherence of Bordetella pertussis to human respiratory epithelial cells." J Infect Dis **148**(1): 125-130.
90. Uhl, M. A. and J. F. Miller (1994). "Autophosphorylation and phosphotransfer in the Bordetella pertussis BvgAS signal transduction cascade." Proc Natl Acad Sci U S A **91**(3): 1163-1167.
91. Vandebriel, R. J., S. M. Hellwig, J. P. Vermeulen, J. H. Hoekman, J. A. Dormans, P. J. Roholl and F. R. Mooi (2003). "Association of Bordetella pertussis with host immune cells in the mouse lung." Microb Pathog **35**(1): 19-29.
92. Villarino Romero, R., R. Osicka and P. Sebo (2014). "Filamentous hemagglutinin of Bordetella pertussis: a key adhesin with immunomodulatory properties?" Future Microbiol **9**(12): 1339-1360.
93. Wang, F. F., S. T. Cheng, Y. Wu, B. Z. Ren and W. Qian (2017). "A Bacterial Receptor PcrK Senses the Plant Hormone Cytokinin to Promote Adaptation to Oxidative Stress." Cell Rep **21**(10): 2940-2951.
94. Warfel, J. M., J. Beren, V. K. Kelly, G. Lee and T. J. Merkel (2012). "Nonhuman primate model of pertussis." Infect Immun **80**(4): 1530-1536.
95. Warfel, J. M., L. I. Zimmerman and T. J. Merkel (2014). "Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model." Proc Natl Acad Sci U S A **111**(2): 787-792.
96. Weiss, A. A. and S. Falkow (1983). "Transposon insertion and subsequent donor formation promoted by Tn501 in Bordetella pertussis." J Bacteriol **153**(1): 304-309.

97. Weiss, A. A., E. L. Hewlett, G. A. Myers and S. Falkow (1983). "Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*." Infect Immun **42**(1): 33-41.
98. Werner, T., V. Motyka, M. Strnad and T. Schmulling (2001). "Regulation of plant growth by cytokinin." Proc Natl Acad Sci U S A **98**(18): 10487-10492.
99. World Health Organization (2015). "Pertussis vaccines: WHO position paper - September 2015." Wkly Epidemiol Rec **90**(35): 433-458.
100. Zhu, J. H. and B. Javid (2015). "Tuberculosis: hey there, lonely guy!" Mol Cell **57**(6): 951-952.
101. Zlamy, M. (2016). "Rediscovering Pertussis." Front Pediatr **4**: 52.
102. Zurita, E., G. Moreno, A. Errea, M. Ormazabal, M. Rumbo and D. Hozbor (2013). "The stimulated innate resistance event in *Bordetella pertussis* infection is dependent on reactive oxygen species production." Infect Immun **81**(7): 2371-2378.