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碩 士 論 文

幽門螺旋桿菌之熱迫性蛋白 60 藉由引發調控性 T 細胞的產生而抑制週邊血液單核球之增生

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研 究 生：謝源庭

指導教授：廖光文 教授

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研 究 生：謝源庭　　Student：Yuan-Ting Hsieh

指導教授：廖光文　　Advisor：Kuang-Wen Liao

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國立交通大學生物科技學系生化工程所碩士班

摘要：

幽門螺旋桿菌是一種常見的腸胃道細菌，其特徵為可在腸道引發長期的慢性感染。目前有許多對於其毒力因子與免疫抑制上的相關研究，然而這些毒力因子並無法幫助每個亞種在體內生存。根據文獻探討，有一些物種的熱迫性蛋白在免疫上扮演著兩種截然不同的角色。例如來自 *Mycobacterium tuberculosis* 的熱迫性蛋白 60 便可引發調控性 T 細胞的增生來達到抑制免疫反應的目的。因此我們假設也許幽門螺旋桿菌的熱迫性蛋白 60 也許也有類似的功能。在我們的實驗中，我們利用週邊血液單核球以及 $CD3^+$ T 細胞做為實驗對象來研究幽門螺旋桿菌熱迫性蛋白 60 是否對於調控性 T 細胞在 $CD4^+$ T 細胞中的比例有所提昇。首先，我們先觀察該蛋白是否對細胞的增生有所影響。發現該蛋白對於週邊血液單核球的增生有嚴重的抑制。而對於週邊血液單核球的抑制乃是跟細胞生長的停滯有關。稍後我們想找出的這種抑制的現象是否跟調控性 T 細胞相關。在經過細胞表面抗原染色以及 *foxp3* 訊息 RNA 的表現程度顯示在經過幽門螺旋桿菌熱迫性蛋白 60 的刺激後，調控性 T 細胞在 $CD4^+$ T 細胞中所佔的比例會有所上升。綜合上述所得到的結論，我們發現幽門螺旋桿菌之熱迫性蛋白 60 能夠促使調控性 T 細胞的比例上升，而這樣子的上升或許可以幫助幽門螺旋桿菌逃脫於免疫系統的攻擊。

Heat Shock Protein 60 of Helicobacter pylori Suppresses The Proliferation of Peripheral Blood Mononuclear Cells by The Induction of Regulatory T Cells

Student: Yuan-Ting Hsieh Advisor: Dr. Kuan-Wen Liao

Institute of Biochemical Technology
National Chiao Tung University

Abstract

H. pylori is a common gastrointestinal bacterium that causes chronic inflammation for lifelong. Many factors were investigated to see their functions on immune suppression. However, these factors may not be strong enough to help every stain escape from immune responses. According to the literature research, some members of heat shock proteins play a dual role in immune responses. For example, *Mycobacterium tuberculosis* hsp60 (Mt hsp60) protects the rat from arthritis by inducing Treg. Thus, we hypothesized that *H. pylori* heat shock protein 60 (Hp hsp60) might also induce Treg generation to suppress almost every population of immune cells. In our study, we used PBMC and $CD3^+$ T cells as targets to investigate whether Hp hsp60 increases the percentage of Treg in $CD4^+$ T cells. At first, we demonstrated the effect of Hp hsp60 on cell proliferation which is a character for immune cell activation. We found that Hp hsp60 has a strong suppressive ability for PBMC proliferation and a slight effect on Jurkat cells, which is a T lymphoma cell line. In addition, the proliferation inhibition was caused by cell arrest. The proliferation inhibition caused by Hp hsp60 might be due to the cell arrest. Furthermore, we intended to see whether this inhibition was associated with Treg. The CD4/CD25 double staining and *foxp3* mRNA expression level showed that the Treg were increased after treated with Hp hsp60. Taken together, we found that Hp hsp60 could increase Treg cell generation and may help *H. pylori* escape from the immune system.

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時光一點一滴的流逝，轉眼間，我已經在這個實驗室待了四年。這裡充滿了我許許多多待在交大的回憶，現在回想起來，仍不禁莞爾一笑。在這些日子裡，我和許多人一起共事，一起歡樂，一起分享屬於我們自己的秘密。如今當我坐在電腦前完成我的碩士論文時，我由衷的感謝這些曾經幫助過，鼓勵過我的人。

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CONTENTS

List of Figures

List of Table

List of Appendix

List of Abbreviation

cDNA	Complementary DNA
CagA	Cytotoxin-associated gene A
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
Foxp3	Forkhead box p3
GroEL	The same as hsp60
GITR	Glucocorticoid-induced tumor-necrosis factor (TNF) receptor-related protein
Hsp	Heat shock protein
IFN-γ	Interferon -γ
Ig	Immunoglobulin
IL-1β	Interleukin - 1β
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MAPK	Microtubule-associated protein kinase
MHC	Major histocompatibility complex
NF-κB	Nuclear factor κB
NK	Natural killer cell
PAI	Pathogenicity island
PBMC	Peripheral blood lymphocyte
PML	Polymorphonuclear leucocytes
aTreg	Adaptive regulatory T cells
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper cells
TLR	Toll-like receptor
nTreg	Natural T cells
TNF-α	Tumor necrosis factor-α
Tr1	Type 1 regulatory T cells
Treg	regulatory T cells
VacA	Vacuolating cytotoxin A

Chapter 1 Introduction

1.1 *Helicobacter pylori*

1.1.1 Background

H. pylori is a very common gastrointestinal bacterium which infects various areas of stomach and duodenum. More than half population in the world were infected with this gram negative bacterium. It is a spiral-shaped bacterium discovered by Marshall and Warren in 1984 [1]. It is thought to be a microaerophile and neutralophile bacterium although stomach is an acidic environment.

H. pylori causes the chronic infection and the majority is asymptomatic. However, it is highly associated with many gastric diseases such as peptic ulcer, gastritis, duodenitis, gastric adenocarcinoma, and Mucosa-associated lymphoid tissue (MALT) lymphoma [1-4]. In Correa cancer cascade model [5], he thought that the lifelong infection causes the accumulation of DNA mutation which leads to the outcome of cancer. *H. pylori* has been categorized as a group I carcinogen by IARC (the International Agency for Research on Cancer) in 1994.

1.1.2 *H. pylori* induced immune responses

When bacteria invade the host, the immune system would be stimulated and

generate the non-specific and specific immune responses to eliminate the pathogen from the human body. When *H. pylori* infects the host, it induces both innate and adaptive immunities [6].

1.1.2.1 Innate immunity

Innate immunity is the first line to defend against the bacteria invasion. The members of innate immunity include granulocytes, macrophages, monocytes, dendritic cells, mast cells, and NK (nature killer) cells. The innate immunity is a quick and non-specific procession. Most two important functions of innate immunity are to clear the pathogen infection and participate in the initiation and subsequent direction of adaptive immune responses. Because there is a delay about one week before adaptive immunity ready, it has to control the infection in this period.

When *H. pylori* infects and colonizes at the stomach, it recruits many innate immune cells to infiltrate the area [7]. These cells include neutrophils, mast cells, macrophages, and dendritic cells. Many virulence factors of *H. pylori* involve in this recruitment. For example, HP-NAP, a 150-kD oligomeric protein, recruits neutrophils and mast cells and activates them to produce reactive oxygen intermediates or cytokines [8]. When these phagocytes infiltrate in the place, they

uptake *H. pylori* and form phagosome inside these cells. However, in previous studies, *H. pylori* seems to resistant the intracellular killing and survive in the phagosome [9].

To detect the *H. pylori* invasion, macrophages and monocytes recognize the bacterial molecules by TLRs (Toll-like receptors). After the ligand-receptor docking, TLRs would deliver a signal that induces the secretion of proinflammatory cytokines such as TNF-α (tumor necrosis factor-α), IL (interleukin) - 1β and IL-8 [10, 11]. Thus, when *H. pylori* infects host, it induces the inflammation at the infection area. *H. pylori* contains many antigenic substances including Hsp (heat shock protein), urease, and LPS. These molecules stimulate the production of inflammatory cytokines.

At first, scientists thought that since *H. pylori* is a member of Gram negative bacteria, the LPS of *H. pylori* may bind to TLR4, which is the receptor of LPS, and activate the immune responses. However, gastric epithelial cells were not sensitive to the LPS of *H. pylori* [12]. On the other hand, the neutralize antibody for TLR4 could not block the cytokine release [13]. This indicates that *H. pylori* LPS does not have the similar capability of other Gram negative bacteria LPS and the host

immunity recognizes *H. pylori* infection by other receptor (s).

In 2003, Smith MF Jr. found that TLR2 and TLR5, but not TLR4, were required for *H. pylori*-induced NF-κ B activation and chemokine expression by epithelial cells [14]. When macrophages and monocytes detect the *H. pylori* by TLR2 and TLR5, these two receptors would deliver signals to activate NK-κ B that induce the expression of proinflammatory cytokines such as IL-8. In addition to NK-κB, MAPK has also been considered to involve in the *H. pylori*-induced IL-8 expression. The proinflammatory cytokines accumulation at the infection area results in a local chemotaxis that attracts the granulocytes infiltration. These cytokines would form a positive feedback to enhance the inflammation level. However, besides the large amounts of proinflammatory cytokines, *H. pylori* also induces the secretion of TGF-β (transforming growth factor beta) which is a strong, pleiotropic regulatory cytokines [11].

1.1.2.2 Adaptive immunity

After innate immunity contacts with *H. pylori*, it helps adaptive immune system to develop responses specific for *H. pylori*. The adaptive immunity specific for *H. pylori* could be divided into two parts, cellular responses and humoral responses.

1.1.2.2.1 Cellular immunity

When innate immune cells such as dendritic cells uptake the bacterial molecule, they digest the molecule and migrate to lymph node where they present epitope to naïve T cells. After presentation, $CD4^+$ T cells would be differentiated into different phenotype of effector T cells: Th1 (T helper 1), Th2, Th17 and regulatory T cells (Treg).

The molecule presented to T cell influences the T cell differentiation outcome. The *H. pylori* antigens have the ability to result in a Th1-predominant host immune response in gastric mucosa [15]. The Th1-predominant responses were characterized by induction of IFN (interferon) -γ and IFN-γ-related genes expressions. It also induces the proinflammatory cytokines level such as IL-12, IL-18 and TNF-α. In Houghton's research, the robust mucosal Th1 response is associated with the progression to gastritis and gastric cancer [16]. This is because that inflammation cells and Th1 cells are typically responsible for clearing the intracellular pathogens. However, *H. pylori* is not one of them. These immune responses would result in gastric epithelial cell damage rather than clear the *H. pylori*. The more damage result from immune cells, the higher possibility of tumorgenesis.

1.1.2.2.2 Humoral immunity

Patients infected with *H. pylori* were confirmed to elicit a strong local and systemic antibody responses thatwere specific for *H. pylori.* The serum of patients were response to many components of *H. pylori* including membrane proteins, flagelin, urease, LPS, and HSPs. The isotype of these antibodies contain both IgG and IgA [17, 18]. In addition, there also contains the anti-*H. pylori* sIgA (secretory IgA) was also found in the gastric secretion, saliva and breast milk.

Although there are vigorous antibody responses, these antibodies seemed to have little effect to clear the bacterium from the host unless the patients were treated with combination of antibiotics. Very few researches show that the antibodies had the clearance effect on *H. pylori*. Tosi and Czinn reported that the binding of the IgG promoted the phagocytosis via PMN (polymorphonuclear leucocytes) [17]. Some reports also show that breast milk which contains high titers sIgA had the ability to protect the infects from *H. pylori* infection during infectious period [19]. However, Clyne's report did not agree with this finding because sIgA did not inhibit the *H. pylori* adhesion on the gastric cells [20]. Moreover, antibodies sometimes even worsen the patient's condition. About 20%~30% of the *H. pylori*-infected patients

develop the autoantibodies to block the gastric proton pump which contributes to the gastric damage level.

1.1.3 Factors modulating the immune response to *H. pylori* in humans

Although *H. pylori* induces various immune responses mentioned before, it still evades these attacks and causes the lifelong infection. It indicates that *H. pylori* has established the immune modulation mechanism(s) to escape from immune responses. We collected some reports about the immune modulation ability of these virulence factors in **Table 1**.

1.1.3.1 CagA

Cytotoxin-associated gene A antigen (CagA) is a 120-145kD immunodominant protein. This protein is encoded by the *cagA* gene which is localizd at *cag* PAI (cag pathogenicity island). Besides the *cagA* gene, the *cag* PAI also contains genes encode the components of type IV secretion system which injects the CagA into cells. In 2003, Umehara found that CagA plays an important role in B lymphocyte growth inhibition which may diminish the anti- *H. pylori* responses [21]. Furthermore, CagA also effects on $CD4^+$ T cell differentiation. It is associated with reduced expression of IL-4 mRNA [22]. IL-4 is a pleiotropic cytokine that promotes Th2 differentiation and

suppresses the Th1 differentiation.

1.1.3.2 VacA

VacA (vacuolating cytotoxin A) is a 90kD secret form protein that is originally thought as the toxin for gastric epithelial cells. In recent year, it turns to become a potential immune suppression toxin which aims at the adaptive immunity. Molinari showed that VacA can inhibit the processing of antigenic peptides in B cells and their presentation to human $CD4^+$ T cells by interfering with peptide loading on the newly synthesized MHC (major histocompatibility complex) II [23].

Clinical data found that PBMC (peripheral blood lymphocyte) proliferation from patients are lower than those from health donors. In 2003, VacA was found to efficiently block both *il-2* expression and IL-2Rα surface location and thus inhibits the positive feedback [24]. When PBLC (peripheral blood lymphocytes) were treated with VacA, it resulted in a reduced phosphorylation and activation of Rb (retinoblastoma) protein and then arrested the cell cycle at G1/S checkpoint [24]. In 2003, when VacA bound to an unknown receptor, it caused activation of Rac and p38 which results in inhibition of T cell proliferation and an anergic state of T cells [25, 26]. All these reports show that VacA is a powerful virulence factor for immune

suppression.

1.1.3.3 Arginase

Arginase is a highly conserved enzyme across kingdoms. It is encoded by the gene *rocF*. Arginase compares with NOS (NO synthases) for the common substrate, L-arginine. Therefore, the presence of *H. pylori* influences the synthesis of NO. NO is a critical antimicrobial agent of the innate immunity to cause intracellular killing. NO has been proved to have the toxic effect on *H. pylori* [27]. In Gobert's study, even *H. pylori* induced high level iNOS mRNA expression, the level of NO induced by *rocF*$^{-/-}$ strain is significantly lower than that induced by wild type stain [28]. Not only the innate immunity but also the adaptive immunity are influenced by arginase. It has been found that Arginase decreases the expression of the CD3ζ chain of TCR (T cell receptor) and then inhibits the TCR signal [29].

1.1.3.4 Other factors

Flagellin is a potent molecule to elicited inflammation. It is usually detected by TLR5 which delivers a signal via p38 to express IL-8. However, *H. pylori* Flagellin which encoded by *flaA* has less potent (1000 fold) than those of *Salmonella typhimurium* to induce IL-8 secretion. It may contribute to evade TLR5-mediated

detection [30].

Many bacteria adapt environment and escape from immune responses by genomic DNA rearrangement. *H. pylori* has the highest genetic recombination rate among any known bacterial species [31].

1.1.3.5 The limitation of factors mentioned above

As described before, *H. pylori* establishes many mechanisms to escape from the immune responses. However, these mechanisms and virulence factors are not present in every stain. *cag* PAI only present in about 60 % stains [32]. Despite almost 100 % of *H. pylori* carry *vacA* gene, only 50 % of stains can secrete functional protein [33]. Other factors such as Arginase also have their limitation on immune suppression. This is because it mainly effects on the innate immunity, not on the adaptive immunity. Nevertheless, the major function of innate immunity is to control the infection level. Only suppressing or weakening the killing ability of innate immunity can't guarantee that they would survive from the immune responses.

According to these limitations, it seems that not every strain of *H. pylori* is easy to survive in host. However, this is a controversy because stains without these factors

still colonize in the host. Therefore, there must have other unidentified factor to help *H. pylori* survivve. This unidentified factor should have three characters. First, it must be present in every strain. Second, the homology of this factor among stains should be very high. Finally, it has the immune suppression ability. To find out the factor, we examine the common virulence factors of *H. pylori* with these three features. These common factors includes the heat shock protein 60 (GroEL), heat shock protein 70 (DnaK), Arginase, CagA, VacA, BabA, SabA, and UreB. However, CagA is not fit the first feature. To get the homology of each factor among strains, we align the amino acid sequence from at least four stains including ATCC domestication stains and clinical stains. The alignment results are shown in **Appendix 1** and **Table 2**. According to the homology level, the factors are listed in the order: hsp60, hsp70, UreB, Arginase, SabA, BabA, VacA, and CagA. Thus, we choose hsp60 as our first candidate as the unidentified factor. However, the relationship between hsp60 and immune suppression is still unknown.

1.2 Heat shock protein

1.2.1 Background

Heat shock proteins belong to the superfamily of stress protein. They often express constitutively but can be induced and secreted by stress such as increased

temperature (ex. fever), exposed to proinflammatory factors and oxidative stress [34]. They are important for the survival of prokaryotic and eukaryotic cells. Hsps are initially thought to be chaperone whose major function is to fold newly synthesized proteins or refold the incorrectly folded proteins. However, more and more reports show that hsp have another role to be an immune response mediator.

1.2.2 The relationship between Hsps and immune responses

Hsps, initially considered as intracellular protein, has been found to be released into the extracellular environment. Because of their high conservation, both prokaryotic and eukaryotic hsps are highly antigenic. For example, when bacteria infect human body, they release their hsp at the infection area and induce proinflammatory cytokine secretion. Similarly, when host cells would contact with LPS and GroEL (another name for prokaryotic hsp60), they release their own hsps such as hsp60 and hsp70 [35]. Both hsp60 and hsp70 has been identified as danger signals. After hsp60 and hsp70 are released, they are able to activate the innate immune cells [36], such as macrophage and DC, and induces the proinflammatory cytokine expression including TNF-α and IL-6. According to these characters, hsps have powerful ability to induce inflammation.

1.2.3 Hsps play a role in immune modulation

However, hsps, including hsp60, hsp70, and hsp10, seem to not only function as an inflammation stimulator. More and more reports show that hsps might have dual role on immunity. In recent studies, scientists reported that hsps, including hsp60 and hsp70, exhibit anti-inflammation property [37]. Both hsp60 and hsp70 from *Mycobacterium tuberculosis* both have the ability to reduce the inflammation in arthiritis [38]. Besides, when rat were immunized with peptides from *M. tuberculosis* hsp70, *M. tuberculosis*-specific T cells were detected and were response to *M. tuberculosis* hsp70. However, these T cells produced IL-10 which means hsp70 from *M. tuberculosis* has the ability to suppress the immune responses, so did hsp60. However, not only microbial hsp, but also mammalian hsp has similar function. Zanin-Zhorov showed that human hsp60 enhances the function of $CD4^{+}CD25^{+}$ regulatory T cell via TLR signal pathway [39]. The immune suppression property of different species hsps are shown in **Table 3**.

1.2.4 *H. pylori* heat shock protein 60 (Hp Hsp60)

1.2.4.1 Background

As other members of hsp60, Hp hsp60 is responsible for the folding of certain protein: urease B. In addition to be an intracellular protein, it also expresses as

membrane form which is associated with adhesion [40]. In our previous preliminary result, we found that knock out the Hp hsp60 would cause death for *H. pylori*. This shows that Hp Hsp60 is an indispensable factor for *H. pylori* survival. According to the **Table 2** and **Appendix 1**, the amino acid sequence of Hp Hsp60 among different species are very conserve. Take these two finding together shows that Hp hsp60 is critical for the survival of *H. pylori*.

1.2.4.2 Hp hsp60 induced immune response

Besides the chaperone function, it also induces many proinflammatory cytokines just like its families do. Many reports confirm this character of Hp hsp60. For example, Hp hsp60 induces the IL-6 production in macrophages [41] and IL-8 in monocytes [42]. These proinflammatory cytokine secretion is associated with MAPK signal pathway. So far, the literature research shows that Hsp60 might just be an inflammation stimulator.

1.2.4.3 The immune modulation role of Hp Hsp60

According to the references have been published, it seems that Hp Hsp60 is an inflammation-stimulating factor. However, Hp Hsp60 seems to play another role in immune responses. As mentioned before, *M. tuberculosis* hsp60 has the ability to

induce the precence of Treg. Additionally, in 2003, Anna Lundgren found that there was *H. pylori*-specific Treg was present in *H. pylori* infection area [43]. Here, we collected some reference about Hsps immune modulation in **Table 1**. Thus, whether Hp Hsp60 has the similar ability like *M. tuberculosis* hsp60 to suppress the immune responses is an interesting issue.

1.3 $CD4^+CD25^+$ regulatory T cells (Treg)

1.3.1 Background

The main function of immune system is to protect host from the attack of pathogens, but result in the minimal damage to normal tissue. To achieve this goal, the negative control of the immune cell activation is very important. The first line to eliminate the self-reactive T and B cells is the negative selection in the thymus and bone marrow. However, the negative selection can't eliminate all the self-reactive lymphocyte, Thus, there had long been believed that the immune system must have peripheral mechanisms to deal with the self antigen reactive immune cells that escape from the central tolerance. In the early 1970s, Gershon found that there was a specific group of thymocytes that suppressed the immune responses. In 1995, Sakaguchi first showed that T cells express CD25, the IL-2 receptor α-chain, had the ability to tolerance the immune ability [44]. Until now, Treg can be divided into three major

populations: natural T $CD4^+CD25^+Foxp3^+$ regulatory cells (nTreg), peripherally adaptive T $CD4^+CD25^+Foxp3^+$ regulatory T cells (aTreg), and IL-10-secreting Type 1 regulatory T cells (Tr1). There are also some other kinds of Tregs, such as TGF-secreting Th3 cells, and NKT cells. In this study, we focus on the aTreg.

1.3.2 adaptive Treg (aTreg)

1.3.2.1 Differentiation of aTreg

Unlike nTreg which is mature at thymus, aTreg are induced from mature peripheral $CD4^+CD25^-$ T cells. The presence of Foxp3 (forkhead box p3) on nTreg is dependent on thymic maturation, whereas the expression of Foxp3 of aTreg can be stimulated in the presence of TGF-β and results in acquisition of suppressive function [45]. However, TGF-β alone is not fully effective in mediating Treg induction and IL-2 is an important co-stimulator. Besides, the TCR activation is also required for the expression of the Foxp3 [46]. On the other hand, other cytokines also play the regulatory role on this differentiation and maturation procession. In *in vitro* and *in vivo* study, Th1 and Th2 cytokines, such as IFN-γ and IL-4, have the antagonistic function on the peripheral induction of $Foxp3^+$ regulatory T cells [47]. It has been realized that Treg express several unique markers including extracellular and intracellular molecules. The formers include CD25, CD62L, and CD45,

glucocorticoid-induced tumor-necrosis factor (TNF) receptor-related protein (GITR), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). The latter includes the transcription factor, Foxp3.

1.3.2.2 Foxp3

Foxp3 is a transcription factor that belongs to the forkhead/winged-helix family. It constitutively expresses at high level in both nTreg and aTreg. It is not only to be a intracellular marker, but also to be a crucial transcription factor for the development and maturation of $CD4^+CD25^+$ Tregs. Both in mice and human, function mutations in Foxp3 result in the absence of Tregs which leads to severe autoimmune disorders [48], known as scurfy mice and IPEX (immunedysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome) in men. These studies indicate that Foxp3 expression is important for the differentiation and function of Treg.

1.3.2.3 Function of aTreg

Although the differentiation source and pathway is difference, the function of aTreg is very similar to nTreg. The major function of Treg is to suppress the immune responses and this suppression must be triggered by TCR activation. Interestingly, once activated, the suppression seems to be antigen-nonspecific. The mechanism of

the suppression is cell- contact dependent. This contact educates the $CD4^+$ T helper cells to become induced suppressor cells. It might mediate directly to the T effector cells by GITR and CTLA-4 [49]. On the other way, the contact suppression can also mediate indirectly. Ligation between CD80/CD86 on the DCs and CTLA-4 on Treg could modulate DC function by inducing the expression of IDO (indoleamin 2, 3-dioxygenase) which degrades tryptophan. The reduced Try concentration results in reduction of T cell activity.

Chapter 2 Strategy

Combine with the possible immune suppression function, the indispensable character, and the high conserve homology, Hp Hsp60 might be the unidentified factor which helps all the *H. pylori* strain survive in the host. In this study, we want to know the effect of Hp hsp60 on T cells, especially on the T cell differentiation toward Treg.

At first, we examined the effect of Hp hsp60 on the immune cell proliferation. Thus, we used PBMC and $CD3^+$ T cells as our targets. PBMC are composed of many kinds of immune cells from both innate and adaptive immunity. We used PBMC as our model to mimic the environment of gastric infection area. $CD3^+$ T cells involve in many areas of immunity including B cell activation, T cell differentiation, and the survive of innate immunity. Thus, we also used $CD3^+$ T cells as our investigate target. If Hp hsp60 has an inhibitory effect on the cell proliferation, we want to demostrate whether the mechanism of this inhibition is cell arrest or death. In our experiment, we confirmed it by Annexin V/PI double staining assay to detect the death signal pathway. Next, we will see whether the percentage of $CD4^+CD25^+$ T cells would be increase in $CD4^+$ T cells after the treatment of Hp hsp60 by CD4/CD25 double staining. Because the surface staining is not powerful to identify the differentiation of Treg, thus, the *foxp3* mRNA expression level would be detected by combination of reverse

transcription and real-time PCR.

Chapter 3 Material and Method

3.1 Material

3.1.1 Reagent

The following reagents and chemicals were obtained as indicated: RPMI 1640, Fetal Bovine Serum (FBS), BSA, and Tryzol from Invitrogen (Gaithersburg, MD, USA). Penicillin/ streptomycin/ amphotericin (PSA) from Biological industries (Beithaemek, Israel). Restriction enzymes from Promega (WI,USA). Kanamycin, *pfu* polymerase, DNA agarose, Tryptone and Tris bas from MDBio Inc. (Rockville, MD, USA). Ethidium bromide (EtBr), Isopropyl-beta- D-thiogalactopyranoside (IPTG), NaCl, yeast extract, agar, Tris-HCl, Triton X-100, 40% 29:1 acrylamide: Bis-acrylamide, TEMED and imidazole from Amresco (Solon, OH, USA). Ficoll-Paque^{TM} Plus from GE healthcare (Uppsala, Sweeden). Recombinant human interleukin-2 (rhuman IL-2) and rhTGF-β from Peprotech (Rocky Hill, NJ). Primers from Protech (headquartered in Taipei, Taiwan). Sephadex G-25 Medium from Amersham Bioscciences (Uppsala, Sweeden). Nitrocellulose (NC) paper from PALL(Ann Arbor, MI, USA). Developer and fixer from Kodak (Rochester, NY, USA). Isopropanol from E-ECHO (Miaoli,Taiwan). Propidium iodide (PI), RNase A, Sodium deoxycholate, APS (ammonium persulfate), and SDS (sodium dodecyl sulfate) from SIGMA-ALDRICH (Steinheim, Germany). EDTA and chloroform from TEDIA

(Fairfield, OH, USA). NaOH, H_3PO_4, KH_2PO_4, Na_2HPO_4, Tween 20, $KHCO_3$, NaN_3, and KAc from SHOWA (Saitama, Japan). KCl from Scharlau (Barcelona, Spain). Na_2HPO_4 from J. T. Baker (Phillipsburg, NJ, USA).

3.1.2 Antibody

The following antibodies were obtained as indicated: Goat anti-mouse IgG MicroBead from Miltenyi Biotec (Bergisch Gladbach, Germany). Mouse anti-human CD3 (UCHT1), mouse anti-human CD3-FITC (HIT3a), mouse anti-human CD4-FITC (RPA-T4), and mouse anti-human CD25-PE (BC96) from Biolegend (Sandiego, CA, USA). HRP- conjugated rabbit anti 6X His antibody from Novus (Littleton, CO, USA). HRP-conjugated goat anti rabbit IgG from MP Biomedicals (Aurora, OH, USA). Mouse anti-human CD3 (OKT3) was kindly provided from Dr. Steve R. Roffler (ACADEMIA SINICA, Institute of BioMedical Sciences).

3.1.3 Kit

The following kits were obtained as indicated: superscript III RT kit from Invitrogen. Human $TGF\beta_1$ ELISA kit, and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay kit from Promega. RealQ-PCR master mix kit from Ampliqon (Copenhagen, Denmark).

Coomasie Plus™ Protein Assay Reagent kit and Enhanced chemiluminescence (ECL) system from Pierce (Rockford, IL, USA). Annexin V-FITC apoptosis detection kit from Invitrogen.

3.1.4 Instrument

HisTrap™ HP column from GE healthcare. NeucleoBond ion-exchange resin from Macherey-Nagel (Düren, Germany). MACS® separation LS column from Miltenyi Biotec (Bergisch Gladbach, Germany). UV photography system from EZlab. Sunrise remote control (TECAN). ABI prism 7000 from ABI (USA). FASCan from BD (Bedford, MA, USA).

3.1.5 Others

Escherichia coli (BL21 and DH5α) from Yeastern Biotech Co. *H. pylori* genome from Department of Internal Medicine, College of Medicine, National Taiwan University. Human cDNA (complementary DNA) library were kindly provided from Dr. Chich-Sheng Lin (NCTU, Laboratory of Biomedical Engineering, Biological Science & Technology Lab). Nitrocellulose paper (NC paper) and 0.45μm syringe filter from PALL. X ray film from Midsci. Human white blood cells were obtained from Hsinchu Blood Center.

3.2 Method

3.2.1 pET-Hp Hsp60 plasmid construct

For rHp Hsp60 expression, pET-HpHsp60 was constructed (see Appendix). The vector is derived from pET-30a which carries an N-terminal His-tag and multiple cloning sites under the control of T7 promoter. The full-length of Hp Hsp60 was amplified from *H. pylori* genomic DNA by *pfu* DNA polymerase. *H. pylori* genome was isolated clinical strain (HC28) which obtained from gastric cancer patient. The full-length *H. pylori* was amplified with forward primer 5'- ATC GAA TTC ATG GCA AAA GAA ATC AAA TTT TCA - 3' and backward primer 5'- GAT CTC GAG TTA CAT CAT GCC GCC CAT G -3' by PCR (polymerase chain reaction). PCR consisted of a 94℃ denaturation step followed by 35 cycles of 45 s at 95 ℃, 45 s at 50 ℃ and 2 min at 72 ℃. After these cycles, incubate the PCR mixture at 72 ℃ 10 min for complete elongation. The PCR product was checked with 0.8 % DNA agarose gel by electrophoresis (**Figure 1A**) and the DNA length is about 1641 bps. The full gene and pET-30a vector were digested with EcoR I and Xho I at 37 ℃ for 3 hr and checked with gel electrophoresis (**Figure 1B**). The digested fragment length of vector and insert should be 5422 and 1641 bps, respectively. After ligation and transformation, we picked several single colonies from the plate and checked the

insert by screening with RE digestion (**Figure 1C**). After check, the plasmids were sequenced with primers. The DNA sequence inserted into vector has been submitted to NCBI nucleotide data base (DQ674277).

3.2.2 RE digestion

Restriction enzyme digestion was used for cloning and checking. We used 0.5 μl RE (EcoRI and XhoI) (10 U/μl) to digest 1 μg DNA in 20 μl volume at 37℃ for 3 hr. The reaction mixture consists of DNA, 10 X RE buffer H, 100 X BSA, RE, and DDW. The digested DNA fragments were checked with 0.8 % DNA agarose gel by electrophoresis. The gels were analyzed with UV after staining with EtBr for 10 min.

3.2.3 Transformation

Remove the appropriate number of competent cells tubes from the -80 ℃ freezer. DH5α was used for cloning and DNA amplification. BL21 was used for protein expression. After the cells were thawed, add 1 ng DNA into the cells, mix by gently swirling the tip. Incubate the competent cell on ice for 30 min. Heat shock the cell at 42 ℃ for 90 s. Place the cells on ice for 2 min and add 250 μl LB (10 g tryptone, 10 g NaCl, 5 g Yeast extract per liter) and incubate at 37 ℃ with shaking 225 rpm for 1 hr. Spread 100 μl mixture onto each LB agar plate (10 g tryptone, 10 g NaCl, 5 g Yeast

extract, 20 g agar per liter) containing kanamycin (30 mg/ml) and incubate at 37 ℃ for 12~16 hr.

3.2.4 Midi-preparation

After transforming the competent cell with plasmid, we picked single colony from the LB agar plate and incubate with 100 ml LB containing kanamycin (30 μg/ml) at 37 ℃ for 16 hr at 225 rpm shaking incubator. The broth was harvested by centrifuging at 8000 rpm for 15 min. The supernatant was discarded and the pellet was resuspended in 8 ml buffer S1 (50 mM Tris-HCl, 10 mM EDTA, 100 μg/ml RNase A, pH 8.0). 8 ml buffer S2 (200 mM NaOH, 1 % SDS) was added for lysis, gently mixed by inverting 6~8 times, and incubate at room temperature for 3 min. 8 ml ice-cold buffer S3 (2.8 M KAc, pH 5.1) was added for neutralizing, gently mixed by inverting 6~8 times, and incubate on ice for 2 min. Centrifuge the cell lysates at 12000 rpm for 30 min at 4℃. Wash NeucleoBond ion-exchange resin with 5 ml buffer N2 (100 mM Tris, 15 % ethanol, 900 mM KCl, 0.15 % Triton X-100, adjusted to pH 6.3 with H_3PO_4) while the lysate centrifuging. After centrifugation, apply the supernatant to the pre-rinsed resin, followed by washing with 12 ml buffer N3 (100 mM Tris, 15 % ethanol, 1.15 M KCl, adjusted to pH 6.3 with H_3PO_4) twice. Elute the column with 5 ml buffer N5 (100 mM Tris, 15 % ethanol, 1 M KCl, adjusted to pH

8.5 with H_3PO_4) and separate the eluted mixture into six fractions equally (~800µl). Precipitate DNA by adding 0.7 volume of isopropanol (about 700 µl) and kept on ice for 10 min. Centrifuge at 13000 rpm for 30 min at 4℃. The DNA pellet was washed by 1 ml ice-cold 70% ethanol. Discard the ethanol, air-dried, and dissolve with 30 µl DDW. Measure the absorbance at 260 and 280 nm to check the DNA quantity and quality.

3.2.5 rHp hsp60 Protein expression

E. coli [BL21 (DE3)] freshly transformed with pET-Hp hsp60 were grown on LB plates containing kanamycin (30 mg/ml) at 37°C. After 16 h incubation, 5 colonies grown on the LB plates were picked and inoculated into 100 ml LB medium containing kanamycin (30 mg/ml) at 37 °C for 16 hr. Refresh the culture broth in 900 ml LB medium with shaking at 37 ℃ until the value of OD600 reaches 0.6 (about 40 min). Add IPTG from a 800 mM stock to a final concentration of 1 mM and continue the incubation for 4 hr. Harvest the cells by centrifugation at 5000 rpm for 15 min at 4 ℃. Discard the supernatant and resuspend the pellet with 30 ml binding buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 40 mM imidazole, pH 7.4). Total cell lysates were sonicated with short burst of 1 sec followed by intervals 1 sec and the sonication processing was maintained for 15 min. Centrifuge the solution at 12000 rpm for 30

min at 4 ℃. Harvest the supernatant. In this experiment, we purified our proteins with HisTrap™ HP column. All the solutions used in this experiment were needed to be filtered with 0.45 μm syringe filter. To prepare the column, wash the column with 5 column volumes of DDW and equilibrate the column with 5 column volume of binding buffer at the flow rate about 1ml/min. Apply the pretreated sample and wash with wash buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 60 mM imidazole, pH 7.4) about 60 volume. Elute with elution buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 200 mM imidazole, pH 7.4) for 10 volumes. Detect which fractions contain proteins with coomasie reagent. Collect the positive fractions and use G25 column to remove the unnecessary salt from the solution and replace the buffer with PBS (Phosphate Buffered Saline, 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH_2PO_4, pH 7.4). To prepare the G25 column, we need to swell the 7 g Sephadex G-25 Medium with filtered PBS at room temperature for overnight. Fill the column with PBS. Resuspent the medium and pour the medium into the column. We poured the protein into the G25 column and eluted with PBS. Detect which fractions contain proteins with coomasie reagent and collect the fractions. Poll the fractions together and filtered with 0.22 μm syringe filter. Check the protein concentration of each part with coomassie reagent (**Figure 2**) and dilute the product to the final concentration to 1 mg/ml. The recombinant protein was checked by SDS-PAGE (sodium dodecyl sulfate

polyacrylamide gel electrophoresis) and Western blotting with anti-His conjugate HRP (**Figure 3**).

3.2.6 SDS-PAGE and Western blotting

The purified proteins were mixed with 3X protein sample dye and denature at 95 ℃ for 10 min. In our experiment, we used the 10 % acrylamide gel to analysis the protein size. Each lane was loaded with 10 μl mixture and the electrophoresis was processed with 130 Volt about 80min. the gel were stained with staining buffer for 1 hr and followed by destaining with destain buffer I for 30 min and destain buffer II overnight. For Western blotting, after electrophoresis, the gel was transferred with nitrocellulose paper (NC paper) with 200 mA for 2 hr at room temperature. After transfer, the NC paper was blocked by blocking buffer (5 % skim milk, 0.05 % tween 20 in PBS) at room temperature for 1 hr. Because the recombinant proteins contain histidine tag, we used HRP- conjugated rabbit anti 6X His antibody (1000X dilution) to recognize the recombinant protein. Wash the NC paper with PBST (0.05 % tween 20 in PBS) at room temperature on shake for 5min three times for all washing steps. The membrane was developed with ECL system in dark at room temperature for 5 min and exposed to X ray film for 10 s. Develop the film with developer until band was present and fixed the film with fixer for 1 min. Wash the film with water.

3.2.7 PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were separated from human white blood cell solution by using Ficoll-Paque^{TM} Plus. Dilute human white blood cells with equal volume of PBS. Add Ficoll-Paque Plus (6 ml) into the 15ml centrifuge tube and carefully load the diluted blood sample (8 ml) on Ficoll-Paque Plus. Centrifuge the tubes at 400 g for 40min at 18 ℃. Remove the plasma layer and collect the PBMC layer. Wash the cells with 2 volume of PBS for centrifuging at 1500 rpm for 15 min. Discard the supernatant and lyse the red blood cells by ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in DDW) at room temperature for 10 min and followed by centrifuging at 1500 rpm for 10 min. Discard the supernatant and wash the cell with 10 ml PBS. Centrifuge for another 10 min. Discard the supernatant and count the cell number. The purified PBMC were checked by flow cytometry as shown in **Figure 4A**.

3.2.8 $CD3^+$ T cell isolation

$CD3^+$ T cells were purified by magnetic bead cell sorting with mouse anti-human CD3 and Goat anti-mouse IgG MicroBead. Magnetic staining buffer (0.5 % BSA, 2 mM EDTA in PBS, pH 7.2) was the only buffer used in this experiment. Count 10^8

PBMC and suspend in 800 µl staining buffer. Stain the cells with 200 µl mouse anti human CD3 antibody to capture the $CD3^+$ T cells at 4 ℃ for 30 min. Wash the cells with 5 ml buffer twice. Resuspend the cells with 800 µl buffer and stain the cells with 200 µl Goat anti-mouse IgG MicroBead at 4 ℃ for 5 min. Wash the cells twice and resuspend in 1 ml buffer. Put the column on the separator and wash the LS column with 3 ml buffer. Load the sample into the column and wash the column with 3 ml buffer for three times. Remove the LS column from the separator and add another 5 ml buffer. Immediately flush out the cells with plunger. Centrifuge the cells at 1500 rpm for 15 min and count the cell number. The purified $CD3^+$ T cells were checked by staining with anti-human CD3 conjugated FITC and analyzed by flow cytometry. Compare with the unstrain cells, the purity of our purified $CD3^+$ T cell is over 99 % (**Figure 4B**).

3.2.9 Cell culture

Jurkat is an acute T cell leukemia cell line cultured with RPMI 1640 containing 10 % heat-inactivated FBS, 1 % PSA. The PBMC and $CD3^+$ T cells were cultured with RPMI 1640 containing 10 % heat-inactivated FBS, 1 % PSA, 100 U/ml rhuman IL-2. All the cells were incubated at 37℃, 5% CO_2 condition.

3.2.10 MTS assay

To evaluate the growth rate of Hp Hsp60-treated cells, MTS assay was used in this experiment. PBMC ($2*10^5$/well), $CD3^+$ T cells ($2*10^5$/well) and Jurkat ($2*10^4$/well) were seeded in anti-CD3 mAb-precoated (1 μg/ml, 30 μl per well at 37 ℃ for 2 hr.) 96 well and incubated with various doses of Hp Hsp60 (1 μg/ml, 5 μg/ml, and 10 μg/ml). After incubated for 48 hr (Jurkat) and 4 day (PBMC), 40 μl MTS was added into wells and incubated for another 4 hr in 37 ℃. The OD absorbance was measured at 490 nm with sunrise remote control.

3.2.11 Annexin V-FITC/PI apoptosis assay

To evaluate the survive rate of Hp Hsp60-treated cells, Annexin V-FITC/PI apoptosis detection kit was used in this experiment. PBMC ($2*10^5$/well) and $CD3^+$ T cells ($2*10^5$/well) were seeded in anti-CD3 mAb-precoated 96 well plate and incubated with various doses of Hp Hsp60 (1 μg/ml, 5 μg/ml, and 10 μg/ml) for 4 day. After 4 day, cells were washed with 1 ml PBS. Each sample was stained with 1μl Annexin V-FITC, 25 ng PI, and suspended in 20 μl staining buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2, pH 7.4) for 15 min at room temperature. Add 800 μl PBS into the mixture and analyze the sample on FACScan in FL1 and FL3 channels with dot plots with quadrant line.

3.2.12 TGF-β cytokine secretion detection

10^6/ml PBMC and $CD3^+$ T cells were seeding in one well of 24-well plate. PBMC was treated with different doses (100 pg/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml) of Hp hsp60 for 24 hr. Harvest the supernatant and stock at -80 ℃ refrigerator until the assay beginning. Before assay, we acidified 100 μl sample with 5 μl 1N HCl for 15 min at room temperature. Next, 5 μl 1N NaOH was added to neutralize the sample. The 96 well ELISA plate was coated with 1000 X coating mAb and incubate at 4℃ for overnight. Remove the coated plate from refrigerator and allow it to room temperature. Flick out the contents of the wells and slap the plate upside down on a paper towel until the well dry. Add 270 μl 1 X block buffer to each well at 37℃ for 35 min. Wash the plate with PBST five times and flick out the contents of the wells and slap the plate upside down on a paper towel until the well dry. Add 100 μl standard or sample to each well at room temperature for 2 hr. Repeat wash procedure. Add 100 μl 1000 X anti-TGF-β pAb at room temperature for2 hr. Repeat wash procedure. Add 100 μl 100 X TGF-β-HRP conjugate at room temperature for 2 hr. Repeat wash procedure. Add 100 μl 1000 X anti-TGF-β pAb at room temperature for 2 hr. Add 100 μl TMB for each well at room temperature for 15 min in the dark. Next, add 100 μl 1 N HCl to stop the reaction and measure the

absorbance at OD 490nm.

3.2.13 Cell surface marker staining

PBMC (10^6 cells/well) and $CD3^+$ T cells (10^6 cells/well) were treated with 10 μg/ml Hp Hsp60 and 2 ng/ml TGF-β for 6 days. Centrifuge the cells at 1500 rpm for 10 min. Count the cell number and take $2*10^5$ cells for this experiment. Resuspend the cells with 500 μl staining buffer (1% BSA, 0.05 % NaN_3 in PBS). Stain the cells with antibody mixture (2 μl mouse anti-human CD4-FITC and 1 μl mouse anti-human CD25-PE in 500 μl staining buffer (1 % BSA in PBS)) on ice in the dark for 30 min. For compensation, we also stained cells with 2 μl anti-human CD4-FITC and 1 μl mouse anti-human CD25-PE respectively as control. After washing the cells with 500 μl staining buffer, centrifuge the cells at 1500 rpm for 5 min. Repeat again. Analyze the cells on FACScan in FL1 and FL3 channels with dot plots with quadrant line.

3.2.14 RNA isolation

PBMC and $CD3^+$ T cells were seeded with the same condition of the surface marker staining assay. Count the cell number. Take 10^6 cells and freeze the cell pellet in -80 ℃ freezer. In this experiment, we used Trizol reagent for RNA isolation. The cell pellets were homogennizated by adding 1ml Trizol. Shake violently and incubate

at room temperature for 5 min. Add 0.2 ml chloroform and shake vigorously by hand. Incubate at room temperature for 3 min and 12000 rpm for 15 min at 4 ℃. Transfer the aqueous phase to a fresh tube, precipitate the RNA from the aqueous phase by mixing with 0.5 ml isopropanol. Incubate the sample at room temperature for 10 min. Centrifuge at 1200 rpm for 10 min and discard the supernatant. Wash the pellet with 1ml 70 % ethanol by vortex and centrifuge at 7500 rpm for 5 min. Discard the supernatant and air-dry for 15 min. Dissolve the pellet with 10 µl DEPC water and incubate at 60 ℃ for 10 min. Check the RNA quality and quantity by measuring the OD 260 and 280.

3.2.15 Reverse transcription

After quantitating the RNA level, 1 µg RNA from each sample was used for reverse transcription by using the superscript III RT kit. By following the manufacturer's recommendation, we mix the RNA with 1 µl dNTP, 1 µl random haxamer and DEPC water up to 10 µl. Incubate the sample at 65 ℃ for 5 min. Then, each sample is added with 2 µl 10X RT buffer, 4 µl 25mM $MgCl_2$, 2 µl 0.1M DTT, 1 µl RT (200 U/µl), and 1µl RNaseOUT (40 U/µl). Incubate the mixture at 25 ℃ for 10 min and followed by 50 ℃, 50 min. To terminate the reaction, incubate the mixture at 85 ℃ for 5 min and chill on ice. To remove the RNA contamination, add 1 µl RNase

H to each tube and incubate at 37 ℃ for 20 min. The cDNA was ready to use for PCR and real-time PCR. To monitor whether the reverse transcription was successful, the cDNA were examined with β-actin primers (forward: 5' TTG GGT ATG GAA TCC TGT GG 3', backward: 5' TCG TAC TCC TGC TTG CTG AT 3'). PCR consists of a 94 ℃ denaturation step followed by 35 cycles of 45 s at 95 ℃, 45 s at 50 ℃ and 2 min at 72 ℃. After these cycles, incubate the PCR mixture at 72 ℃ for 10 min to complete elongation. The PCR results were checked by agarose gel electrophoresis.

3.2.16 Real-time PCR

After checking the cDNA quality, the *foxp3* mRNA level was detected by real-time PCR performed with ABI PRISM 7000. The relative expression of *foxp3* mRNA was determined by normalizing expression of each target to *β-actin* using the primers: human *foxp3* forward primer: 5' ACT GGG GTC TTC TCC CTC AA 3', human *foxp3* backward primer: 5' CGT GGG AAG GTG CAG AGT AG 3', human *β-actin* forward primer: 5' TTG CCG ACA GGA TGC CAG AA 3', human *β-actin* backward primer: 5' GCC GACT CCA CAC GGA GTA CT 3'. The reaction mixture contained: 1 μl cDNA, 0.25 μl forward primer, 0.25 μl backward primer, 11 μl DDW, and 12.5 μl 2X realQ PCR master mix (with 10 mM $MgCl_2$, Green DNA dye). PCR consists of a 95 ℃ denaturation step for 10 min and followed by 40 cycles of 15 s at

95 ℃, 1 min at 60 ℃. The Ct number was gotten by ABI prism 7000 SDS software. The relative *foxp3* gene expression levels were calculated with the followed equation: relative foxp3 mRNA expression level =2^ - [(the Ct (the cycle of threshold) of *foxp3* of sample – the Ct of *β-actin* of sample) – (the Ct of *foxp3* of control – the Ct of *β-actin* of sample)].

3.2.17 Statistical analysis

All data are expressed as mean ± SD. Statistical analyses were done by SPSS statistics software (SPSS Inc., Chicago, IL, USA). In cell proliferation study, PBMC assay were obtained from two independent experiments, $CD3^+$ T cell and Jurkat cells assay were obtained from three independent experiments. In Annexin-V-FITC/PI study, PBMC assay were obtained from two independent experiments, $CD3^+$ T cell and Jurkat cells assay were obtained from three independent experiments. In TGF-β level detection study, PBMC and $CD3^+$ T cell assays were obtained from three independent experiments. In surface marker staining study, PBMC assay were obtained from four independent experiments and $CD3^+$ T cell assay from six independent experiments. In *foxp3* mRNA expression study, $CD3^+$ T cell assay was obtained from one independent experiment. All statistical significant was set at $p < 0.05$.

Chapter 4 Results

4.1 The effect of Hp hsp60 on immune cells.

4.1.1 Hp hsp60 inhibits the proliferation of PBMC and jurkat cells, whereas has no effect on $CD3^+$ T cells.

As mentioned before, we thought that Hp hsp60 might have the immune modulation ability. To test this, we used PBMC and $CD3^+$ T cells as our targets. PBMC were composed of many kinds of immune cells from both innate immunity, which includes monocytes, macrophages, dendritic cells, and NK cells, and adaptive immunity, such as T and B lymphocytes. Because of this feature, we used it to mimic the infection area. T cells were important immune responses. They are responsible for many immune cell activation such as APCs and B cells. Thus, we investigate $CD3^+$ T cells in our model. We also used Jurkat cells as model. Jurkat cells are acute T cell leukemia cells and are usually used for the investigation of T cell signal transduction. To monitor the proliferation of these immune cells, we cultured $2*10^5$/well (10^6/ml) of PBMC and $CD3^+$ T cells in the anti-CD3 mAb-pre-coading 96 well plate with culture medium (see 3.2.9) for 4 days. Jurkat cells ($2*10^4$/well, 10^5/ml) were cultured in a 96-well plate for 2 days. Each well was added with 40 μl MTS and incubated at 37 ℃ for with 4 hr.

The relative OD490 showed that the proliferation of PBMC was strongly inhibited by Hp hsp60 about 30% compared with the anti-CD3 activated, Hp- hsp60 untreated group, even with the TCR (T cell reception) stimulation (**Figure 5**). Different concentration of Hp hsp60 (1 μg/ml, 5 μg/ml, and 10 μg/ml) all have this inhibitory effect. In Jurkat cells, the inhibition rate only was 4% and only high dose (10 μg/ml) has effect (**Figure 7**). However, high dose Hp hsp60 seemed to have no effect on $CD3^+$ T cells (**Figure 6**).

4.1.2 Hp hsp60 inhibit the cell proliferation neither by apoptosis nor by necrosis.

Since the cell proliferation induced by TCR stimulation was inhibited by Hp hsp60, we wanted to demonstrate the mechanism of this inhibition. The cell proliferation inhibition can be divided into two major possibilities: cell cycle arrest and cell death. The cell death is also divided into two possibilities: apoptosis and necrosis. To find out the possibility, we use the Annexin V-FITC/ PI double staining assay. Annexin V is characterized by binding to the translocated PS (phospholipid phosphatidylserine) on the cell membrane which is a feature of early apoptosis. PI is a DNA intercalating agent and a fluorescent molecule. Combine with these two factors, we can determine the cell physiological stage including the apoptosis cells, necrosis cells, and alive cells. The analysis rule shows in **Figure 8**. In this experiment, we used

the same culture condition as used in MTS assay.

All the anti-CD3 activated, Hp hsp60 untreated cell group (**Figure 9, lane 2**) and the activated, Hp hsp60-treated groups (**Figure 9, lane3, 4, and 5**) exhibited no difference in the cell percentage of apoptosis, necrosis and alive cells. It means that the cell proliferation inhibition might not due to cell death, but cell cycle arrest. On the other hand, in $CD3^+$ T cell experiment, the cell percentage of apoptosis, necrosis and alive cells in the activated, untreated cell group (**Figure 10, lane2**) were no difference compared with those in the activated, Hp hsp60-treated groups (**Figure 10, lane3**). This result was fit with the result in MTS assay.

4.2 The effect of Hp hsp60 on Treg differentiation

4.2.1 Hp hsp60 increase the percentage of Treg in CD4+ T cells

Since Hp hsp60 inhibits the cell proliferation of PBMC, we wanted to know how Hp hsp60 inhibits the proliferation. As mentioned before, Treg are a powerful immune modulation cells that suppress almost all kinds of immune cells. If Hp hsp60 inhibit the cell proliferation via Terg, the number of Treg should be increase. To clarify this possibility, we cultured 10^6 cells/ml PBMC and $CD3^+$ T cells with 100 U/ml rHuman IL-2 in anti-CD3 mAb- pre-coaded 24 well plate, respectively. 10 μg/ml Hp hsp60

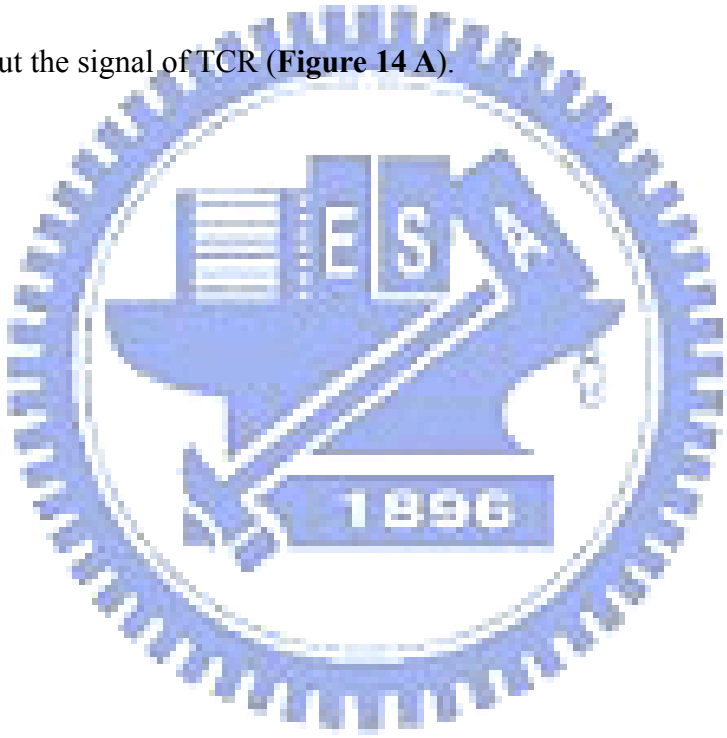
was used for this experiment. After treatment for 6 days, cell were harvested and stained with anti-human CD4-FITC and anti-human CD25-PE. After detected with cytometry by FL1 and FL2 channels, the cell percentage was analysized as shown in **Figure 11**.

In PBMC, Hp hsp60 enhanced the percentage of $CD4^+CD25^+$ T cell in $CD4^+$ T cell under the stimulation of TCR (**Figure 12**). Although the percentage of $CD4^+CD25^+$ T cell also increased in $CD4^+$ T cell without TCR stimulation, however, this is no significant that compared with the untreated group (**Figure 12**). Similar result was happened in $CD3^+$ T cells. Compared with the untreated group, the Hp hsp60 enhanced the percentage of $CD4^+CD25^+$ T cell in $CD4^+$ T cell with the help of TCR stimulation (**Figure 13B**), however, the increase percentage was not significant when cultured without the present of anti-human CD3 mAb (**Figure 13A**). Collect these finding together, we found that Hp hsp60 has the ability to increase the number of $CD4^+CD25^+$ T cell with the TCR stimulation.

4.2.2 Hp hsp60 induces the *foxp3* mRNA expression under the TCR stimulation

Although Treg express both CD4 and CD25 on the surface at the same time, however, $CD4^+CD25^+$ T cells do not equal to Treg. In this experiment, we wanted to

confirm that whether these increased $CD4^+CD25^+$ T cells were Treg. *foxp3* is a key factor for differentiation and suppression function of Treg. Therefore, we used real-time PCR to detect the mRNA expression level of the *foxp3* with the same culture condition as the experiment in 4.2.1. In **Figure 14 B**, we found that *foxp3* mRNA expression level in activated, Hp hsp60 treated $CD3^+$ T cells could be induced up to 2.5 times compared with the untreated group. However, the phenomenon was not present without the signal of TCR (**Figure 14 A**).



Chapter 5 Discussion

How does *H. pylori* inhibit the immune responses that specific to it? As mentioned in introduction, many reports suggested that virulence factors may play the role to suppress the host immune system. However, these factors all have their own defects to help every strain of *H. pylori* survive and escape from the immune response [21-25, 28, 30, 50]. Thus, we thought there are some factor else that helps *H. pylori* inhibit immune responses needs to coincide with three features: i) it presents in every stain, ii) its amino acid sequence is mostly conserved in every strain, and iii) it should exhibit immune suppressive ability. After checking the eight common virulence factors with these three features, Hp hsp60 has the highest homology and is present in every strain. On the other hand, Hp hsp60 also helps *H. pylori* to adhere and colonize in gastric mucosa [40], which means this protein has posibility to contact with immune cells. Taken these together, Hp hsp60 has the highest possibility to help *H. pylori* as long as it has the ability to suppress immune responses.

In this study, we found that after treated with Hp hsp60, the cell proliferation of PBMC was seriously inhibited and Jurkat cells were slightly inhibited. However, the cell proliferation of $CD3^+$ T cell was not affected. This proliferation inhibition might be due to the cell arrest. Besides, the $CD4^+CD25^+$ T cells percentage in Hp hsp60

treated PBMC and $CD3^+$ T cells were both increase. These increased $CD4^+CD25^+$ T cells were confirmed as Treg by detecting the induction of *foxp3* mRNA expression. Taking these finding together, we found that Hp hsp60 has the ability to help *H. pylori* escape from the immune attacks by increasing the number of Treg which inhibit the proliferation of PBMC. However, there were also some doubtful points in our study.

These controversial results between PBMC and $CD3^+$ T cells might be due to whether the cells had been activated. Because the monoclonal antibody we used in T cell isolation has the ability to activate the T cells. During the T cell purification procession, this antibody might have activated the T cells. This activation can be confirmed by the difference between cells that cultured with or without antibody. The difference in PBMC is about 40%, however, the difference in $CD3^+$ T cells only 17%. Therefore, when cells treated with Hp hsp60, PBMC was inactivated, whereas $CD3^+$ T cells had been activated. Thus, whether the cells have been activated might be the key factor that influences the inhibitory function of Hp hsp60.

In our study, we detected the increased number of Treg after treated with Hp hsp60, but where did the Treg come from? According to the literatures, $CD4^+CD25^+$ Treg are arisen from two major sources. Natural $CD4^+CD25^+$ Treg were generated in

the thymus and the proliferation of nTreg can be stimulated by antigen stimulation [51, 52]. On the contrary, the adaptive $CD4^+CD25^+$ Treg were converted from $CD4^+CD25^-$ T cells in the peripheral blood under the help of TGF-β and IL-2 [53]. If the proliferated Treg were arisen from nTreg, they needed the help of antigen presentation. Although the APCs in PBMC could give this help, however, there were no any other APCs in $CD3^+$ T cells. Thus, these enhancements might not be associated with nTreg in the $CD3^+$ T cell culture system. If the proliferated Treg were converted from $CD4^+CD25^-$ T cells, it also needed the help of TGF-β. Therefore, if Hp hsp60 could stimulate the secretion of TGF-β from PBMC and $CD3^+$ T cells, Hp hsp60 might influence the differentiation of Treg though this mechanism. In our preliminary data, we also test the TGF-β_1 level in the culture supernatant of PBMC and $CD3^+$ T cells after treated with Hp hsp60 (**Figure 9**). In this experiment, we used the ELISA kit purchased from Promega. Although the TGF-β_1 concentration were increase in a dose-dependent curve, unfortunately, we also found that this ELISA kit cross reacted with Hp hsp60 recently. The relationship among Hp hsp60, Treg, and TGF-β is still not clear. Thus, we could not provide strong enough evidence about the source of these increased Treg.

There might be some other ways to increase percentage of Treg that may help *H.*

pylori escape from immune system. The escape mechanisms might be similar to that of Mt hsp60. In human T cell differentiation progress, the negative and positive selections delete almost self-reactive T cells. However, minor self-reactive T cells would escape from these selections and play a role in immune suppression by acting as regulatory T cells. Self-hsp60-specific $CD4^+$ T cells might be activated in many ways. First, because of the high homology of amino acid sequence, the self-hsp60 specific $CD4^+$ T cells might also be activated by cross reaction after exposed with Hp hsp60 in the mucosa-associated lymphoid tissue. The crossreactive T cells might be maintained by non-professional antigen-presenting cells such as gastric epithelial cells and differentiated to regulatory T cells [54]. Second, after infected with *H. pylori*, human hsp60, which acts as a danger signal, might be released from damage cells and then activated the self-hsp60 specific $CD4^+$ T cells. On the other hand, the secreted human hsp60 might decrease the function of Hp hsp60-specific T cells by altered-peptide- ligand (APL) effect. The APL is defined as analog of immunogenic peptide in which the TCR contact sites have been manipulated. These high homology peptides still reacts with T cells but only partially activate T cells. This partial activation often not includes the cell proliferation. Taken these mechanisms, *H. pylori* can escape from the immune system easily.

Figure

Figure1

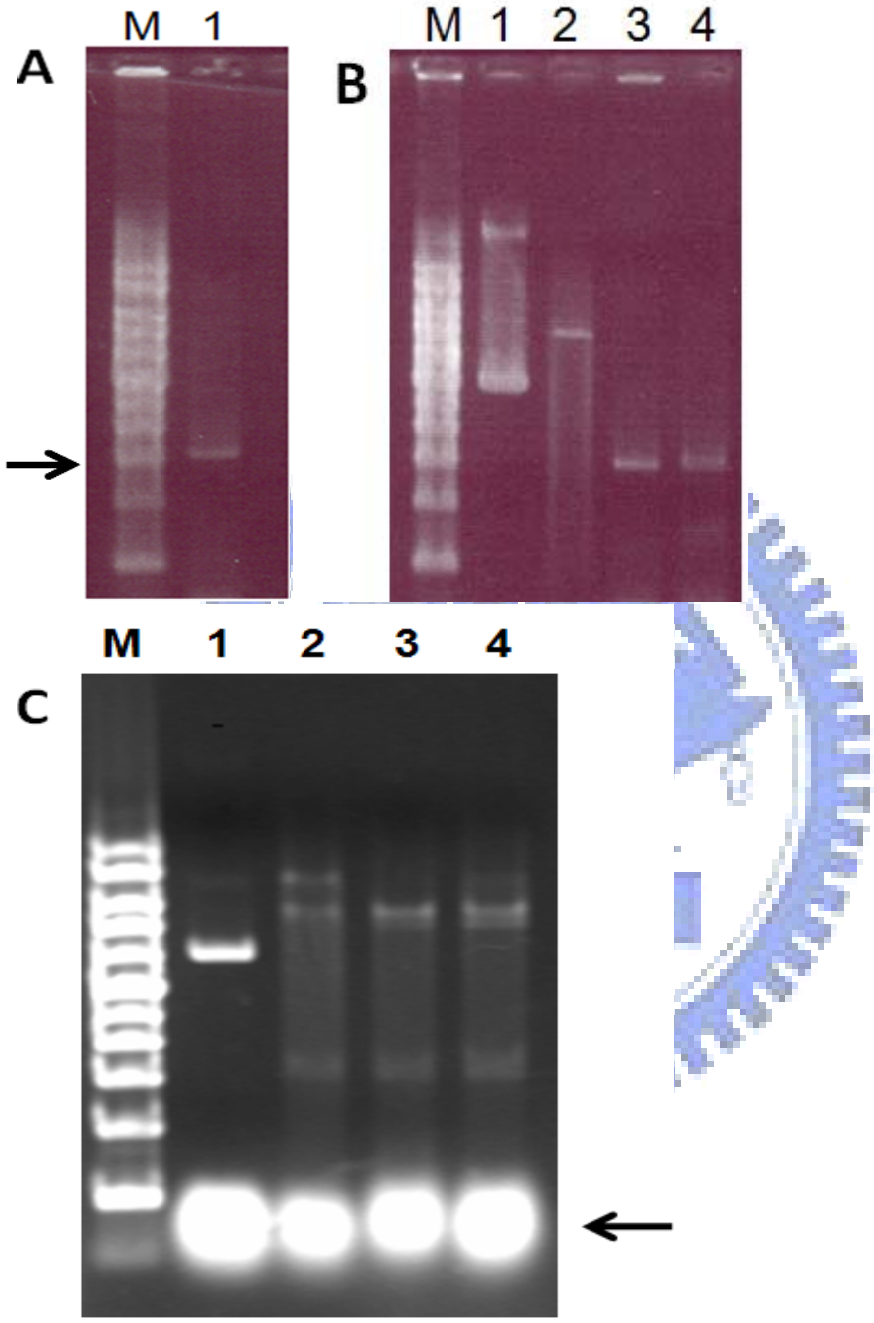
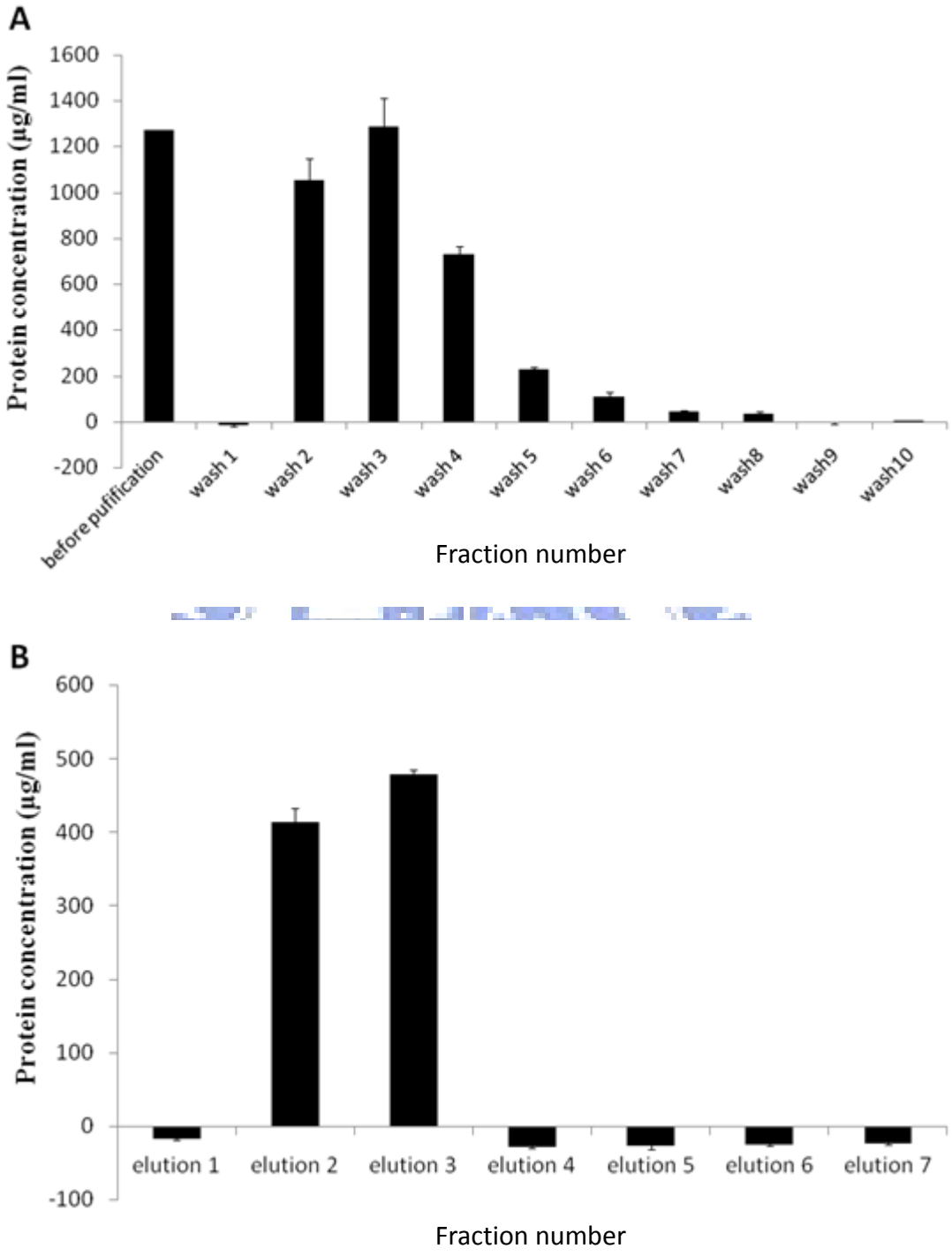


Figure1. The PCR product and the cloning RE check gel electrophoresis. All DNA were checked with 0.8% agarose gel. (A) The PCR product should be 1641bps and the marker pointed by arrow is 1.5kb. (B) The vector and insert were both digested with EcoR I and Xho I. The samples were loaded in the order: M: 1kb marker, 1: uncut vector, 2: vector + EcoR I + Xho I (5422bps), 3: uncut insert, 4: insert + EcoR I + Xho I (1641bps). (C) The plasmids isolated from ligation results were screened with RE check. The samples were loaded with the follow order: M: 1kb marker, 1: uncut plasmid, 2~4: plasmid + EcoR I + Xho I. The digested fragments should be 5422 and 1641bps. The spots pointed by arrow might be RNA contaminant.

Figure 2


A
Protein concentration (μg/ml)
1600
1400
1200
1000
800
600
400
200
0
-200
before puffication
wash 1
wash 2
wash 3
wash 4
wash 5
wash 6
wash 7
wash8
wash9
wash10
Fraction number
B
Protein concentration (μg/ml)
600
500
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elution 5
elution 6
elution 7
Fraction number

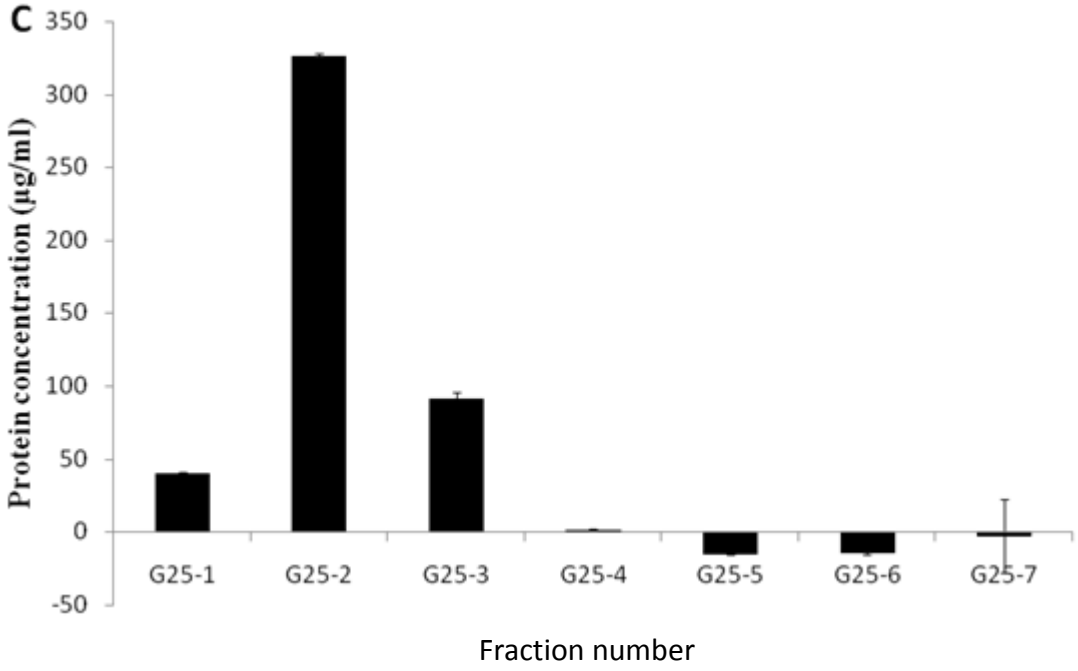


Figure 2. The protein concentration of each fraction in the protein purify procession. We collected each fraction with the 1ml volume during the purify procession which were consisted of (A) wash, (B) elution, and (C) desalting. 10 μl of sample from each fraction were reacted with 290 μl coomassie reagent at room temperature in the dark for 10 min and followed measured by OD 595nm. Each bar represented the mean value ± SD (standard deviation).

Figure 3

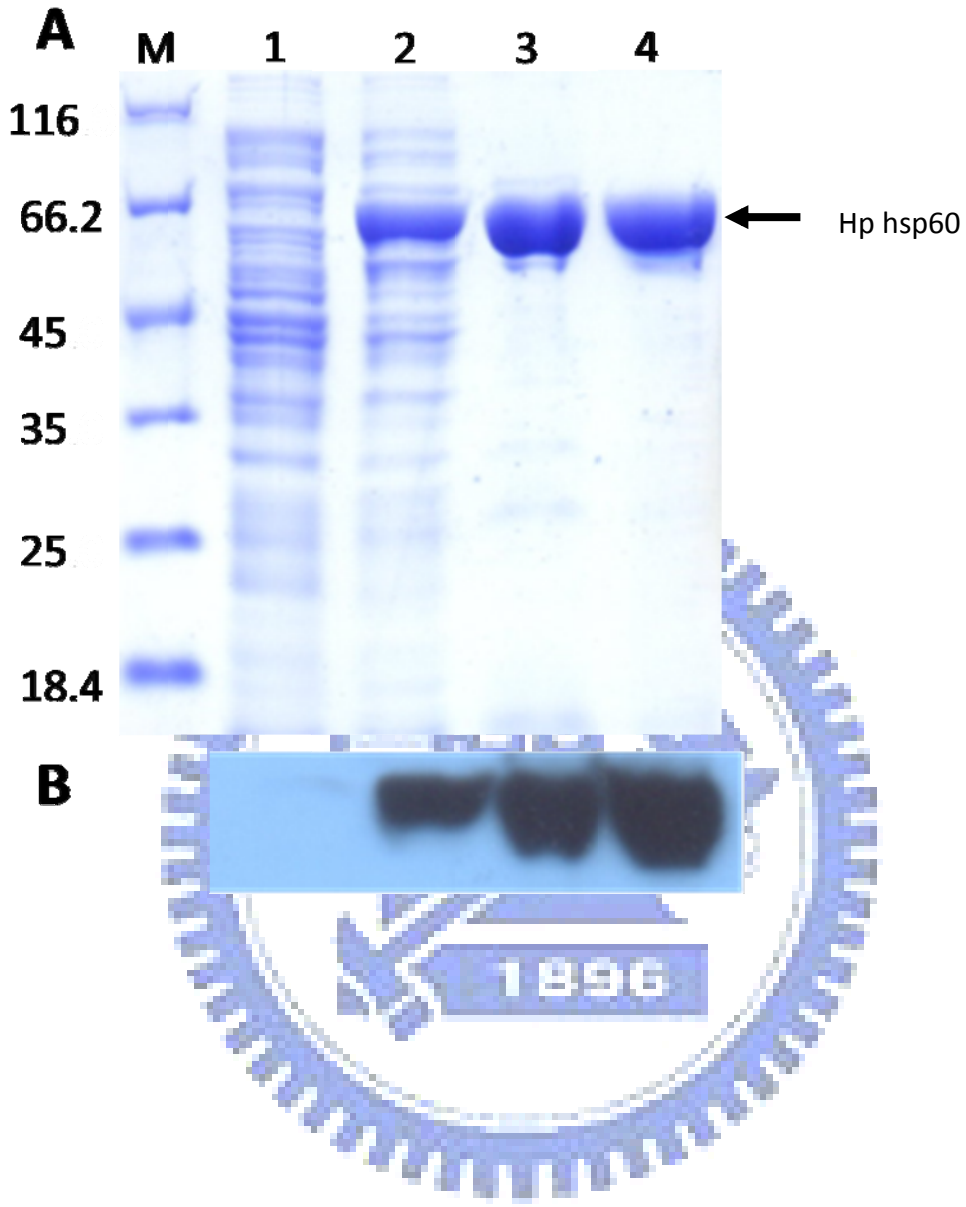


Figure 3. (A) SDS-PAGE and (B) Western blotting of rHp hsp60. 10 μl sample from each step was mixed with 5 μl sample dye and followed denatured at 95℃ 10min. the samples were loaded in 10 % gel with the order: M. protein unstained marker, 1. No induction, 2 induction before purify, 3. After purified with nickel column, 4. After desaulting with G25 column.

Figure 4

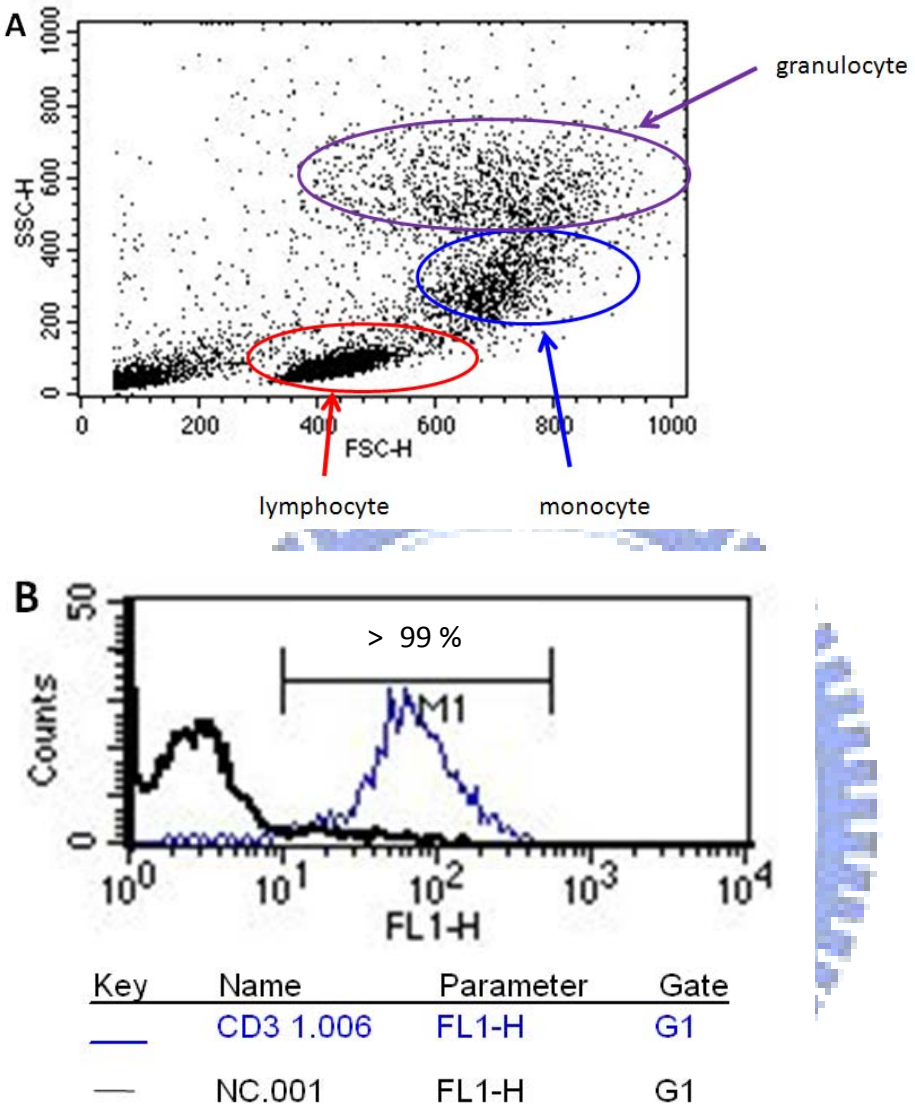


Figure 4. The PBMC and CD3$^+$ T cell purity. (A)PBMC was purified human whole blood by Fique Plus and detected by flow cytometry with FSC and SSC channels. The lymphocytes, monocytes, and granulocytes were pointed by red, blue, and purple arrows, respectively. (B) CD3$^+$ T cells were purified by magnetic bead. To test the purity, the CD3$^+$ T cells were stained with (blue line) and without (black line) anti-human CD3-FITC respectively and detected with FL1 channel. The average of the cell purity is over 99%.

Figure 5

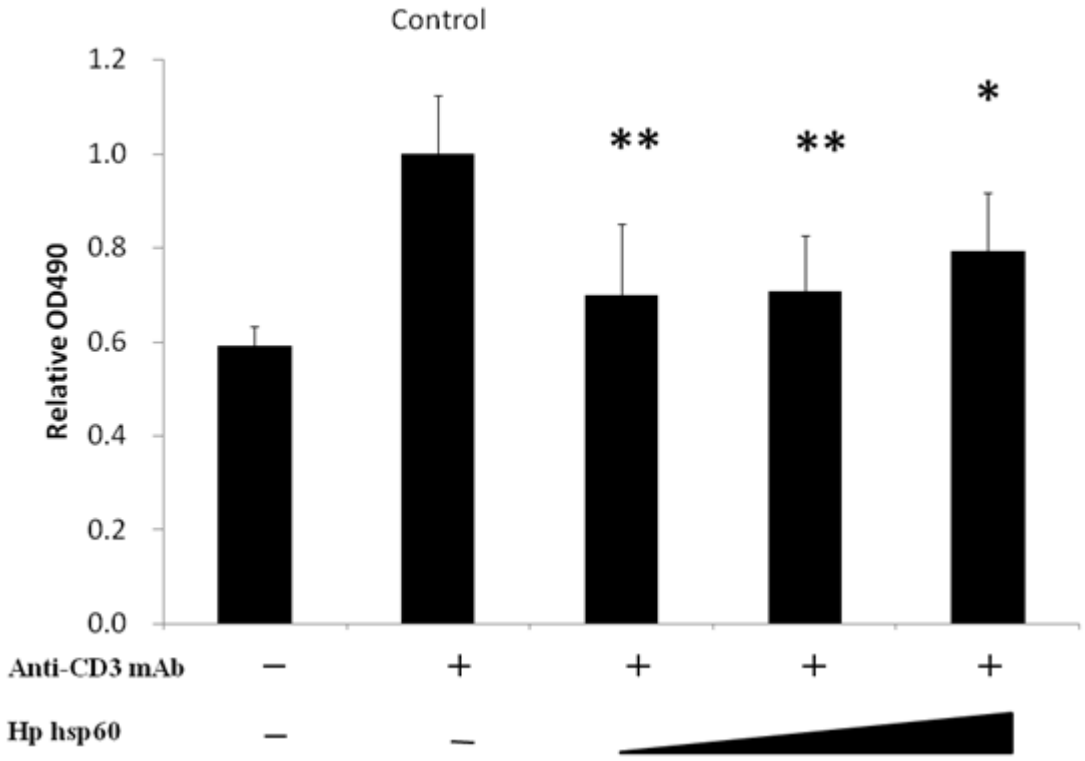


Figure 5. The effect of Hp hsp60 on proliferation of PBMC. Hp Hsp60 added to a concentration of 1, 5, 10 μg/ml (black wedges) to treat with PBMC ($2*10^5$cells/well) for 4 days. The PBMC were seeded in the anti-human CD3 mAb pre-coaded 24 well plate. The relative absorption at 490nm were calculated with the followed equation: the relative OD490 = the OD490/ the average of OD490 of the control. The value of anti-CD3 mAb unstimulated group was 0.591 ± 0.040. The value of anti-CD3 mAb stimulated but Hp hsp60 untreated group was 1.000 ± 0.123. The values of anti-CD3 mAb stimulated cell treated with different dose of Hp hsp60 were 0.697 ± 0/154, 0.705 ± 0.121, and 0.791 ± 0.125. Each bar represented the mean value ± SD from two independent experiments. (*: $P < 0.05$ indicated a significant difference compared to the control group. **: $P < 0.005$ indicated a significant difference compared to the control group.)

Figure 6

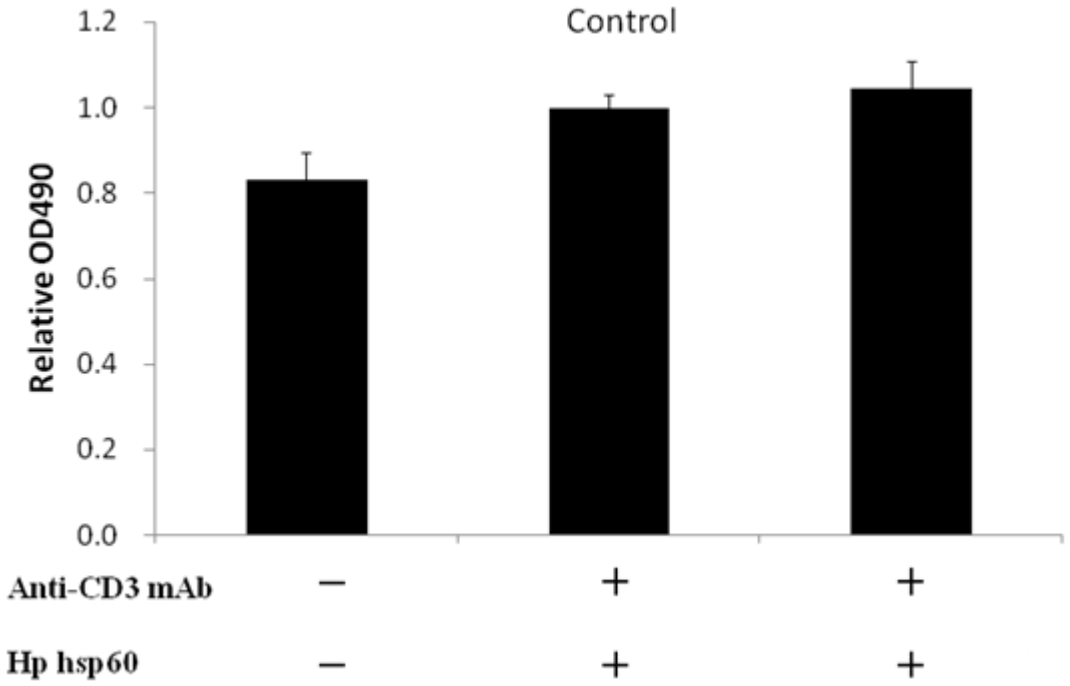


Figure 6. The effect of Hp hsp60 on proliferation of $CD3^+$ T cells. $CD3^+$ T cells ($2*10^5$ cells/well) were treated with 10 μg/ml of Hp Hsp60 for 4 days. The $CD3^+$ T cells were seeded in the anti-human CD3 mAb pre-coaded 24 well plate. The relative absorption at 490nm were calculated with the followed equation: the relative OD490 = the OD490/ the average of OD490 of the control. The value of anti-CD3 mAb unstimulated group was 0.844 ± 0.061. The value of anti-CD3 mAb treated but Hp hsp60 untreated group was 1.000 ± 0.032. The value of anti-CD3 mAb stimulated and Hp hsp60 treated group was 1.048 ± 0.062. Each bar represented the mean value ± SD from three independent experiments.

Figure 7

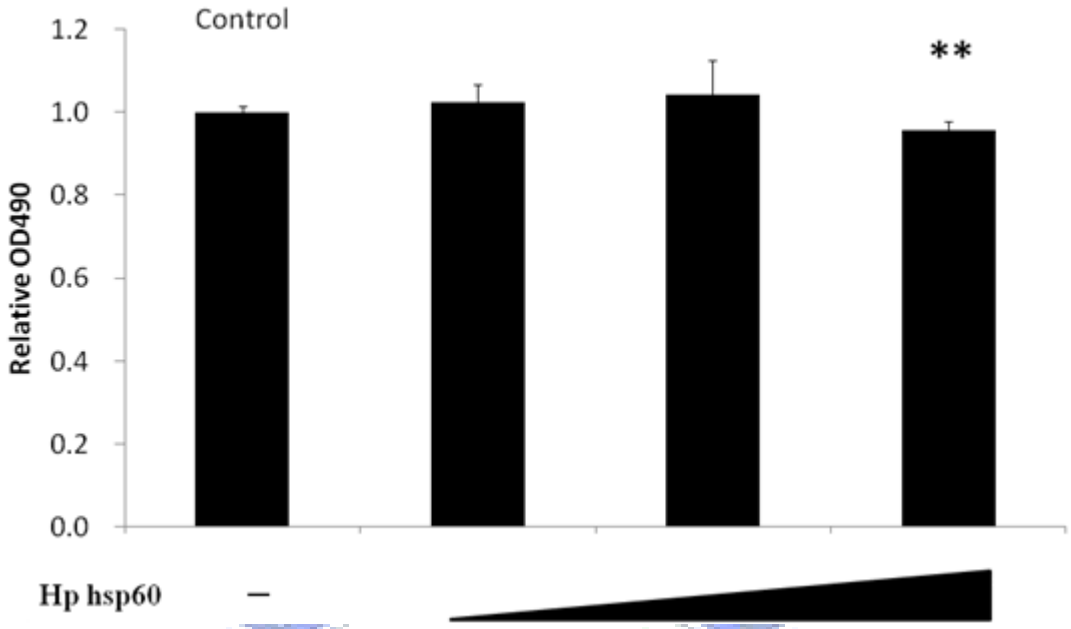


Figure 7. The effect of Hp hsp60 on proliferation of Jurkat cells. Hp Hsp60 added to a concentration of 1, 5, and 10 μg/ml (black wedges) to treat with Jurkat cells ($2*10^4$cells/well) for 2 days. The Jurkat cells were seeded in the anti-human CD3 mAb pre-coaded 24 well plate. The relative absorption at 490nm were calculated with the followed equation: the relative OD490 = the OD490/ the average of OD490 of the control. The value of the untreated group was 1.000 ± 0.017. The values of Jurkat cells treated with different dose of Hp hsp60 were 1.026 ± 0.043, 1.042 ± 0.083, and 0.958 ± 0.019, respectively. Each bar represented the mean value ± SD from three independent experiments. (**: $P < 0.005$ indicated a significant difference compared to the control group.)

Figure 8

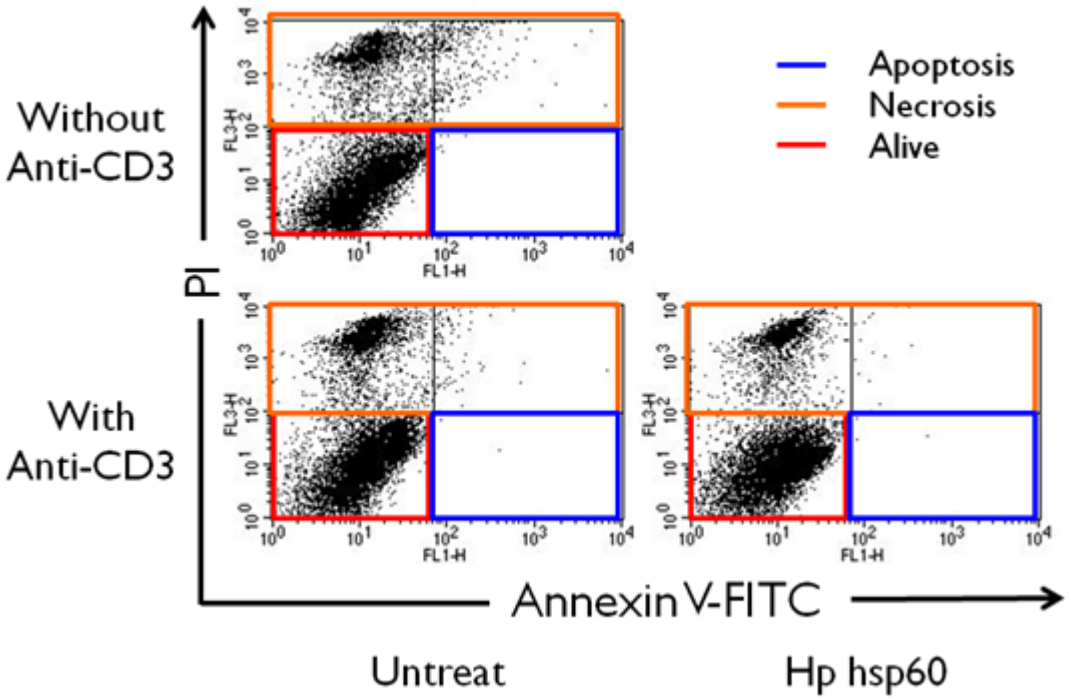


Figure 8. Different physiological stage of cells in Annex V-FITC / PI double staining assay. In our experiment, we used Annexin V-FITC /PI double staining assay to determine the physiological stage of cells. The vertical and cross axle mean the fluorescence intensity of Annexin V-FITC and PI respectively. The cells were cultured with or without anti-CD3 mAb, respectively. The anti-CD3 mAb stimulated cells also were divided into two groups that treated with or without Hp hsp60. After detected with flow cytometry by FL1 and FL3 channels, the results were presented in dot plots with quadrant line. The lower right part was defined as the apoptotic cells (the blue frame). The sum of the up left and up right part were defined as the necrosis cells (the orange frame). The lower left part was defined as the alive cells (the red frame).

Figure 9

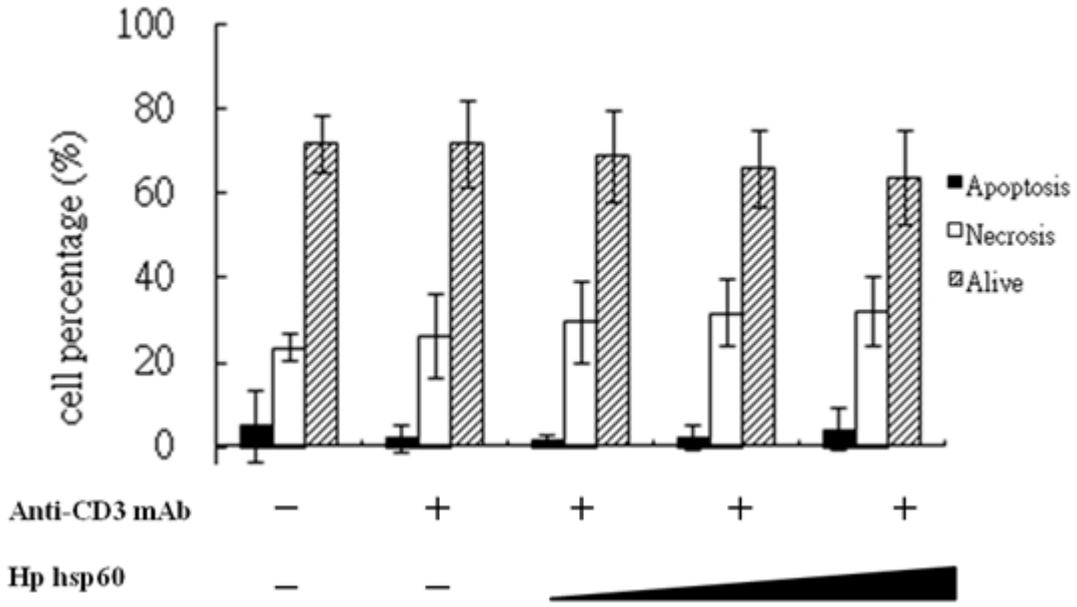


Figure 9. The effect of Hp hsp60 on PBMC cell proliferation inhibition. PBMC were cultured with the same condition of the cell proliferation experiments. After stained with Annexin V-FITC / PI, the cells were detected with flow cytometry by FL1 and FL3 and the cell percentage were calculated as shown in Figure 8. The apoptosis cell percentage of different groups were list as shown in the picture, 4.943 ± 8.497, 2.139 ± 3.189, 1.548 ± 1.364, 2.384 ± 2.926, and 4.144 ± 5.080, respectively. The necrosis cell percentage of different groups were 23.488 ± 3.190, 26.239 ± 10.004, 29.583 ± 9.620, 31.744 ± 8.053, and 32.168 ± 8.092, respectively. The alive cell percentage of different groups were 71.630 ±6.633, 71.623 ± 10.424, 68.870 ±10.904, 68.870 ± 10.904, 65.841 ± 9.082, and 63.690 ± 11.134. Each bar represents the mean value ± SD from four independent experiments.

Figure 10

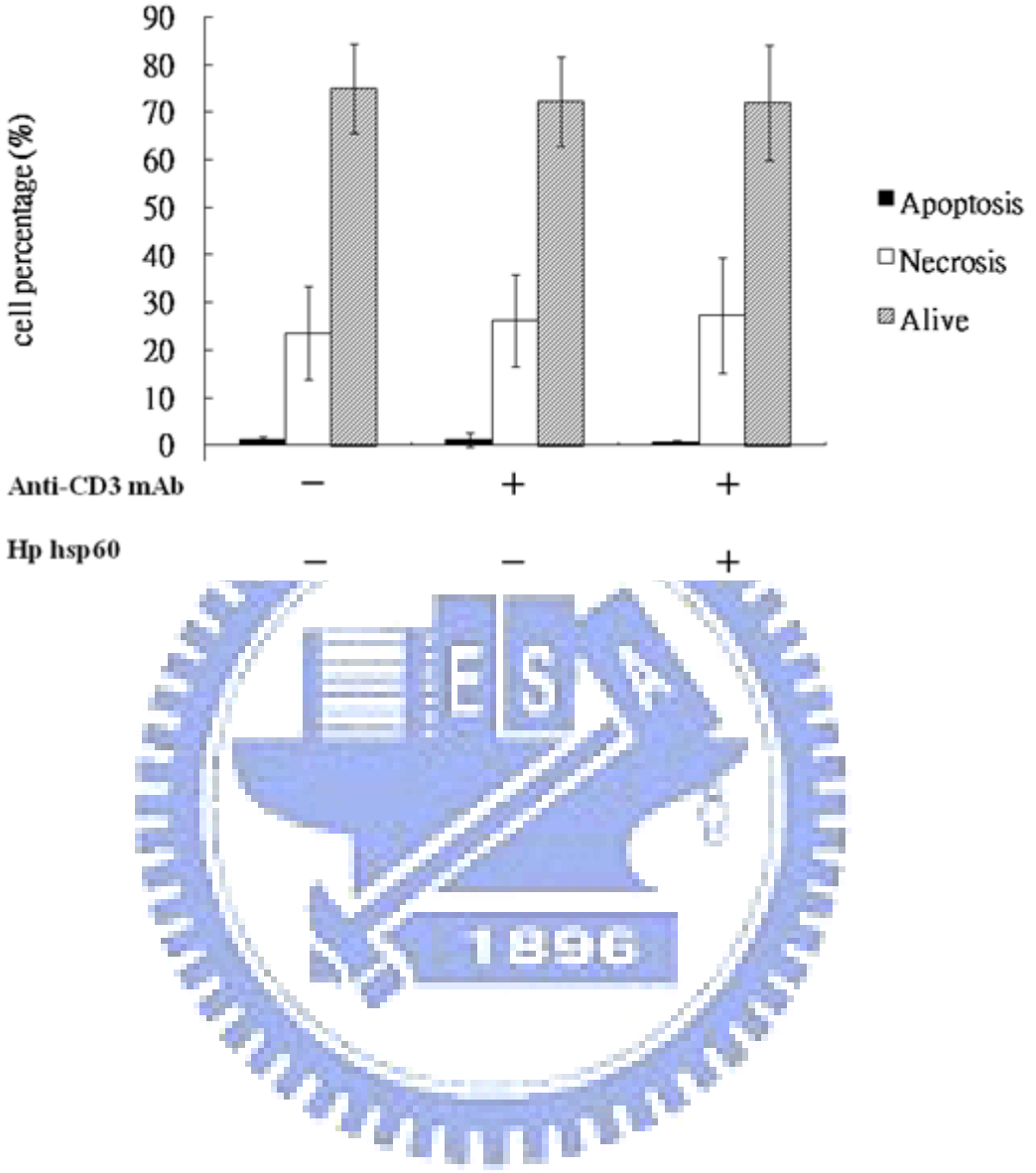


Figure 10. The effect of Hp hsp60 on CD3$^+$ T cell physiology. CD3$^+$ T cells were cultured with the same condition of the cell proliferation experiments. After stained with Annexin V-FITC / PI, the cells were detected with flow cytometry by FL1 and FL3. The cell percentages were calculated as shown in Figure 8. The apoptosis cell percentage of different groups were list as shown in the picture, 1.11 ± 0.86, 1.29 ± 1.51, and 0.55 ± 0.43, respectively. The necrosis cell percentage of different groups were 23.63 ± 9.87, 26.34 ± 9.62, and 27.36 ± 12.02, respectively. The alive cell percentage were 75.10 ± 9.46, 72.37 ± 9.37, and 72.10 ± 12.03, respectively. Each bar represents the mean value ± SD from six independent experiments.

Figure 11

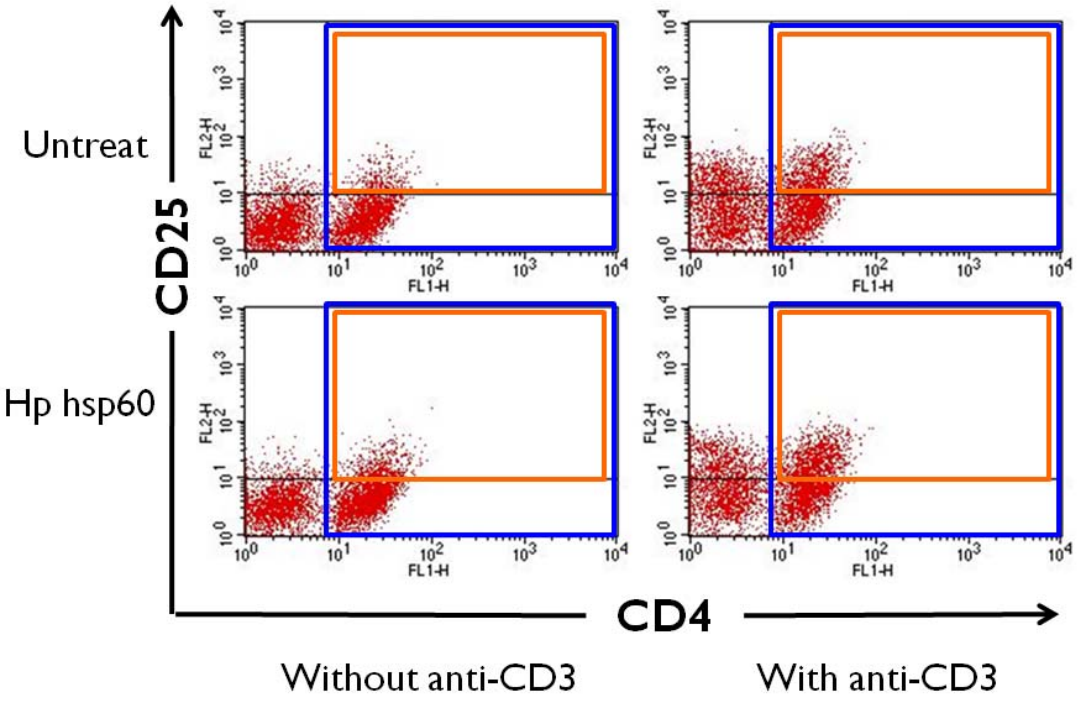


Figure 11. The $CD4^+CD25^+$ T cell percentage in $CD4^+$ T cells. In our experiment, we used anti-human CD4-FITC/ anti-human CD25-PE double staining assay to determine the $CD4^+CD25^+$ T cell percentage in $CD4^+$ T cells. The vertical and cross axle mean the fluorescence intensity of anti-human CD4-FITC and anti-human CD25-PE, respectively. The cells were cultured with or without anti-CD3 mAb, respectively. The anti-CD3 mAb stimulated or unstimulated cells also were divided into two groups that treated with or without Hp hsp60. The cells were harvested and stained with anti-human CD4-FITC and anti-human CD25-PE. The cell fluorescence was detected by FL1 and FL2 channels with compensation, the results were presented in dot plots with quadrant line. The sum of lower right and up right parts were defined as $CD4^+$ T cells (the blue frame). The up right part was defined as $CD4^+CD25^+$ T cells (the red frame). The cell percentage = the red frame/ the blue frame.

Figure 12

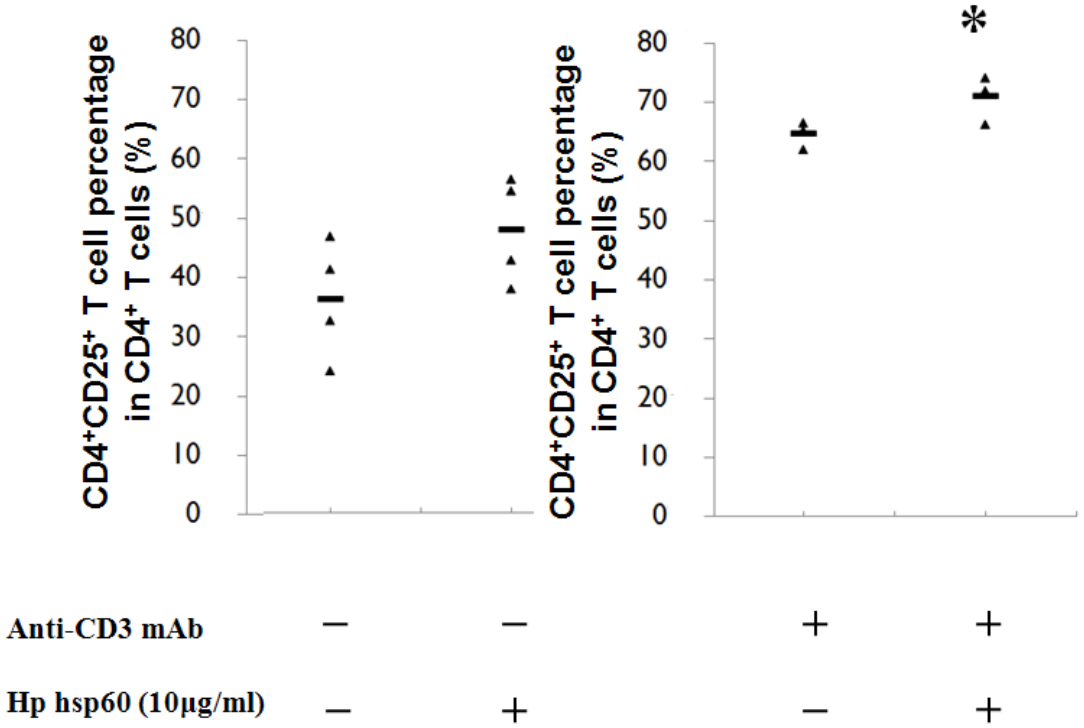
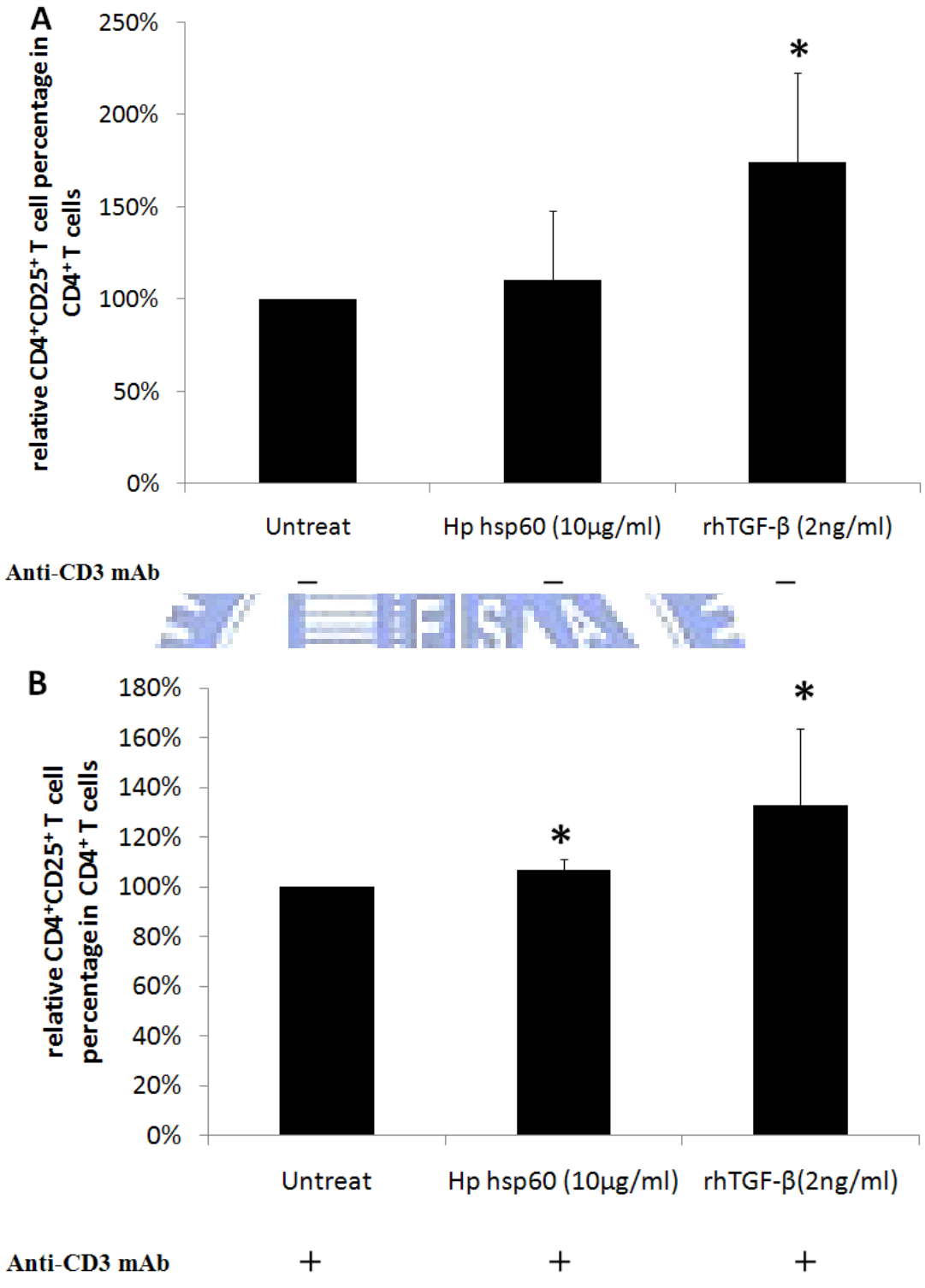


Figure 12. The effect of Hp hsp60 on $CD4^+CD25^+$ T cell population of $CD4^+$ T cells in PBMC. 10^6 PBMC were divided into two groups to culture with or without anti-CD3 mAb. These groups were treated with or without Hp hsp60 for 6 days. The equation of $CD4^+CD25^+$ T cell percentage in $CD4^+$ T cells has shown in Figure 11. The triangles mean the samples we had been test. Mean values are indicated by horizontal bars. The percentage of the anti-CD3 mAb unstimulated and Hp hsp60 untreated group was 36.26 ± 10.09 %. The percentage of the anti-CD3 mAb unstimulated but Hp hsp60 treated group was 48.21 ± 9.11 %. The percentage of the anti-CD3 mAb stimulated but Hp hsp60 untreated group was 64.69 ± 1.89 %. The percentage of the anti-CD3 mAb stimulated and Hp hsp60treated group was 70.98 ± 3.34 %. These samples were from four different donors. (*: $P < 0.05$ indicated a significant difference compared to the untreated group.)

Figure 13


A
relative CD4+CD25+ T cell percentage in CD4+ T cells
250%
200%
150%
100%
50%
0%
*
Untreat
Hp hsp60 (10μg/ml)
rhTGF-β (2ng/ml)
Anti-CD3 mAb
–
–
–
B
relative CD4+CD25+ T cell percentage in CD4+ T cells
180%
160%
140%
120%
100%
80%
60%
40%
20%
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rhTGF-β(2ng/ml)
Anti-CD3 mAb
+
+
+

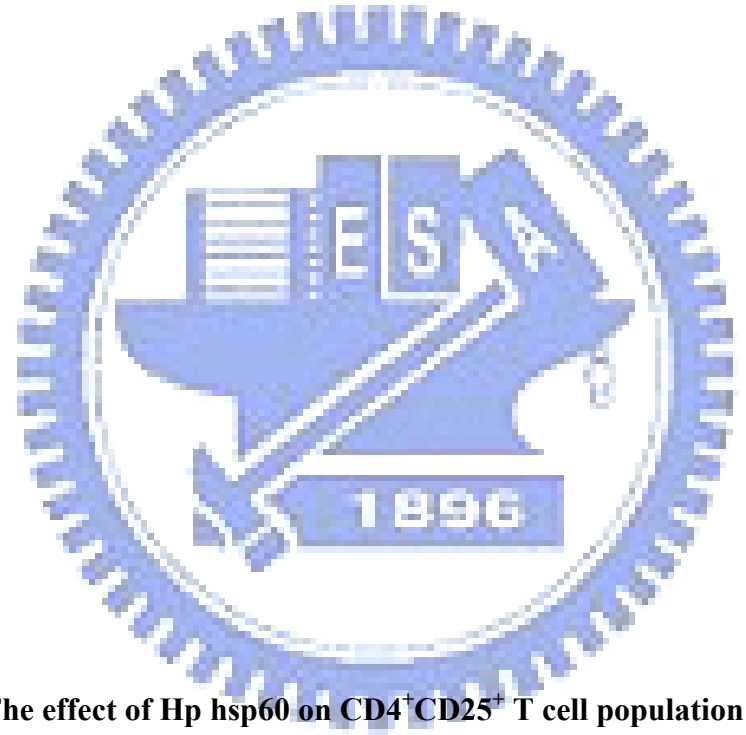


Figure 13. The effect of Hp hsp60 on $CD4^+CD25^+$ T cell population of $CD4^+$ T cells in $CD3^+$ T cells. The $CD3^+$ T cells were treated with different culture conditions as shown in the figure for 6 days. The cells were harvested and stained with anti-human CD4-FITC and anti-human CD25-PE. The fluorescence was detected by FL1 and FL2 channels with compensation. The results were presented in dot plots with quadrant lines as shown in Figure 11. To calculate the relative $CD4^+CD25^+$ T cell percentage in $CD4^+$ T cell, cell percentages were calculated with the equation: the cell percentage = the up right / (up right + lower right). In Figure 13B, the relative $CD4^+CD25^+$ T cell percentage in $CD4^+$ T cells was calculated with the equation: the relative cell percentage = sample cell percentage / control cell percentage. Each bar represented the mean value ± SD from multiple independent experiments. (*: $P < 0.05$ indicated a significant difference compared to the untreated group.)

Figure 14.

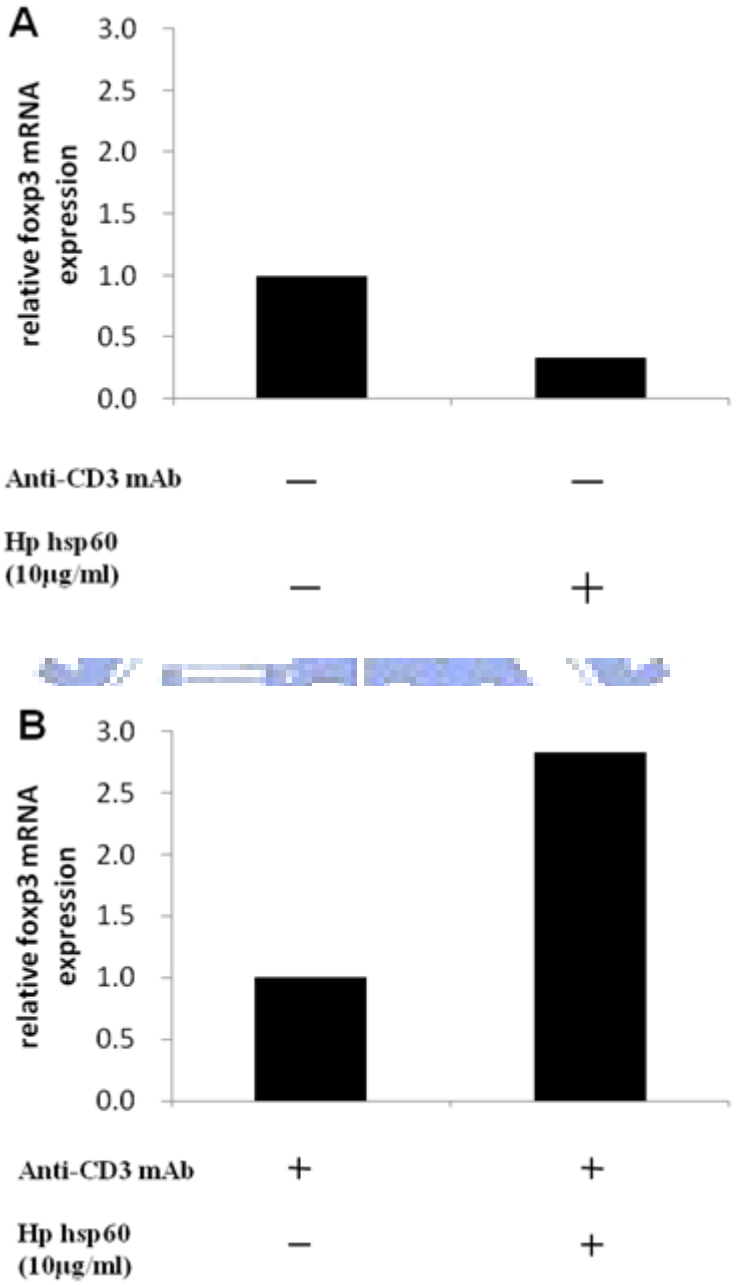
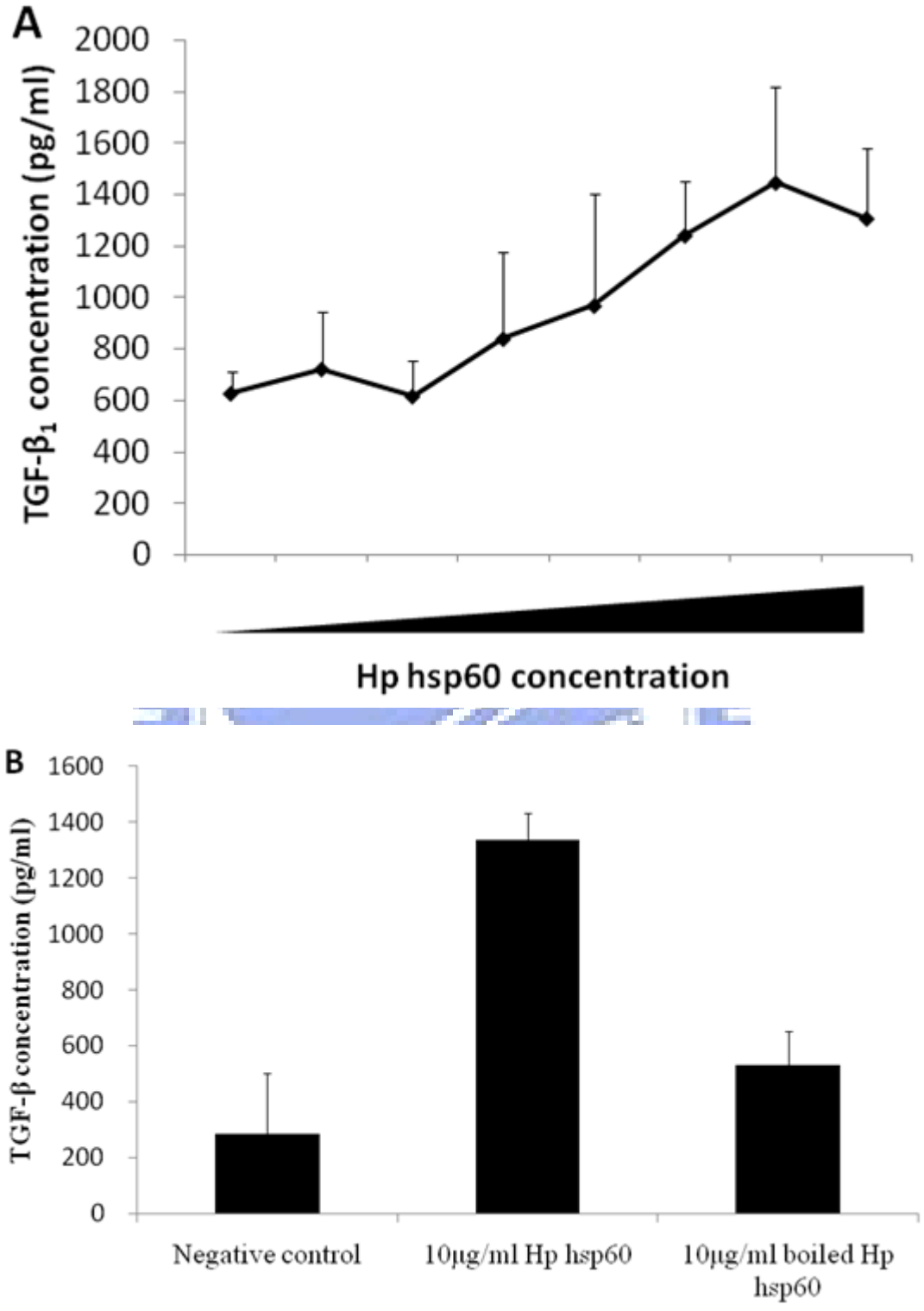


Figure 14. The *foxp3* mRNA expression in T cells. After cultured with the same condition in flow cytometry experiment, mRNA were isolated from 10^6 $CD3^+$ T cells and then reverse transcripted into cDNA. The *foxp3* mRNA expression levels were detected by real-time PCR. The *β-actin* was used as loading control. Each bar represented the mean value from one independent experiment.

Figure 15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TGF-β1 concentration (pg/ml)
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1800
1600
1400
1200
1000
800
600
400
200
0
Hp hsp60 concentration
B
TGF-β concentration (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
Negative control
10μg/ml Hp hsp60
10μg/ml boiled Hp hsp60

Figure 15. The TGF-β_1 concentration in Hp hsp60-treated PBMC and $CD3^+$ T cell supernatant. (A) 10^6/ml PBMC were treated with different doses of Hp hsp60 (100 pg/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml, 10 μg/ml, and 30 μg/ml) in 24 well plate for 24 hr. (B) 10^6 $CD3^+$ T cells were also treated with 10 μg Hp hsp60 and boiled Hp hsp60 for 24 hr. The culture supernatant were harvested and the TGF-β_1 concentration were detected by ELISA kit (Promega).

Table

Table 1

factor	Target cell	response	Reference
Arginase	Innate immune cells	Compare with NOS for the substrate: L-arginine	Gobert AP, PNAS, 2001
Flagellin	Innate immune cells	Lower immunogenic ability	Gewirtz, A.T., J Infect Dis , 2004
HP-NAP	neutrophil	Modulate the oxidative burst	Med Microbiol Immunol., Petersson C, 2006
CagA	B cells	Growth inhibition	Umehara, S., Oncogene, 2003
	T cells	Reduce IL-4 mRNA expression	Orsini, B., Infect Immun, 2003
VacA	B cells	Inhibit the processing of antigenic peptide	Molinari, M., J Exp Med, 1998
	B cells	Inhibit the presentation by interfering the peptide loading on MHC II	Molinari, M., J Exp Med, 1998
	T cells	Inhibit the IL-2 expression	Gebert, B., Science, 2003
	T cells	IL-2Rα surface location	Gebert, B., Science, 2003
	T cells	Inhibit T cell proliferation	Boncristiano, M., J Exp Med, 2003
	PBL	Reduce phosphorylation and activation of Rb and cell arrest	Gebert, B., Science, 2003

Table 1. The reference about the effects of *H. pylori* virulence factors on immune suppression. In this table, we collected the reports about the inhibition functions of Arginase, Flagellin, HP-NAP, CagA and VacA. The relationship between these factors and immune responses were investigated. NOS: Nitrous Oxide Synthase. PBL: Peripheral Blood Lymphocyte.

Table 2

Protein name	Amino acid sequence length	Identity (%)	Positive (%)
Hsp60	546	99.1	100
Hsp70	620	98.1	100
UreB	559~569	95.6	100
Arginase	322	91.0	100
SabA	98~103	78.6	97.1
BabA	737~742	76.4	96.7
VacA	1287~1310	68.9	96.6
CagA	1167~1247	62.9	93.7

Table 2. The homology of H. pylori virulence factors among different strains. These eight virulence factors are arranged in the order of the percentage of identity. Percent of identity means the percentage of the identical amino acids in the full length. Percent of positive means the percentage of the same group amino acids in the full length. The alignments were done by comparing the sequences from at least four stains. The alignment results can be seen in Appendix. The strains compared in this table were listed as follow: Hsp60: 26695, HC28 (used in our study), CCUG 178874, HPAG1, and J99. Hsp70: 26695, HPAG1, J99, Shi470. UreB: Ch-CTX1, Iran-HP031, J99, HC28, Y06, MEL-HP27. Arginase: 26695, G27, AG1, B5, B7, and J99. SabA: G1, M30, M23, G26, and M65. BabA: 92-18, 92-26, J116, CCUG 17875. VacA: ATCC 49503, J99, F26, OK111, NCTC11638, and TX30A. CagA: ATCC43526, F26, J16, J99, NCTC11638, and OK111.

Table 3

Disease model	Hsp	Species	Reference
Arthritis	Hsp10	*M. tuberculosis*	[55]
Arthritis	Hsp60	*M. tuberculosis*	[56]
Arthritis	Hsp70	*M. tuberculosis*	[57]
Arthritis	GroEL	*E. coli*	[58]
Arthritis	DnaK	*E. coli*	[58]
Type 1 diabetes	Hsp60	*M. tuberculosis*	[59]
Type 1 diabetes	Hsp60 – derived peptide	*Homo sapeins*	[60]
Atherosclerosis	Hsp60	*M. tuberculosis*	[61]
Experimental allergic encephalomyelitis	Hsp60	*M. tuberculosis*	[62]
Allergic asthma	Hsp60	*M. leprae*	[63]

Table 3. The immune suppression of different species Hsps protect the host from different disease models. These disease models were all experimental inflammatory diseases that have been proven to be rescued by the administration of Hsps. GroEL means the hsp60 of *E. coli*. DnaK means the hsp70 of *E. coli*.

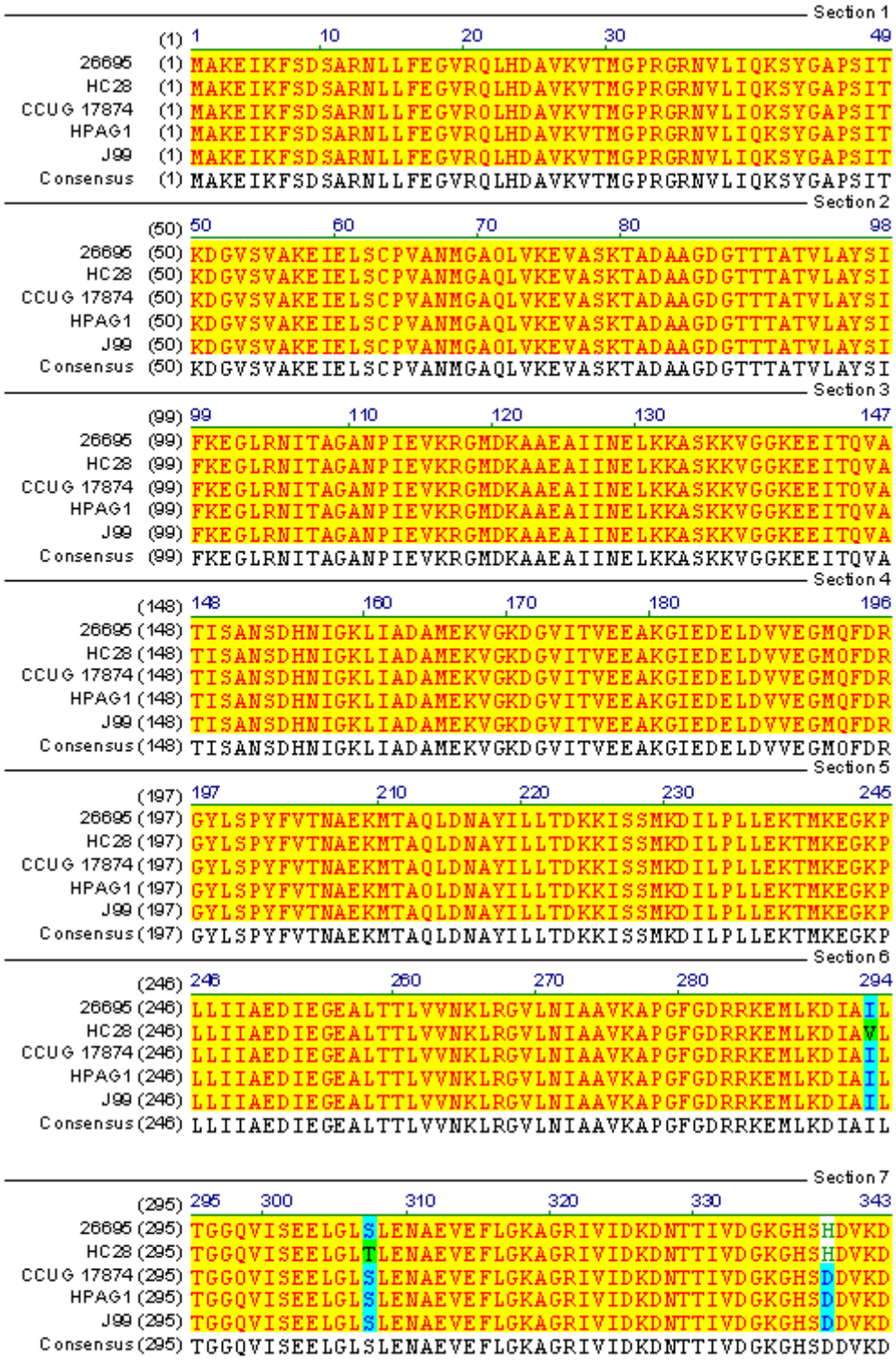
Appendix

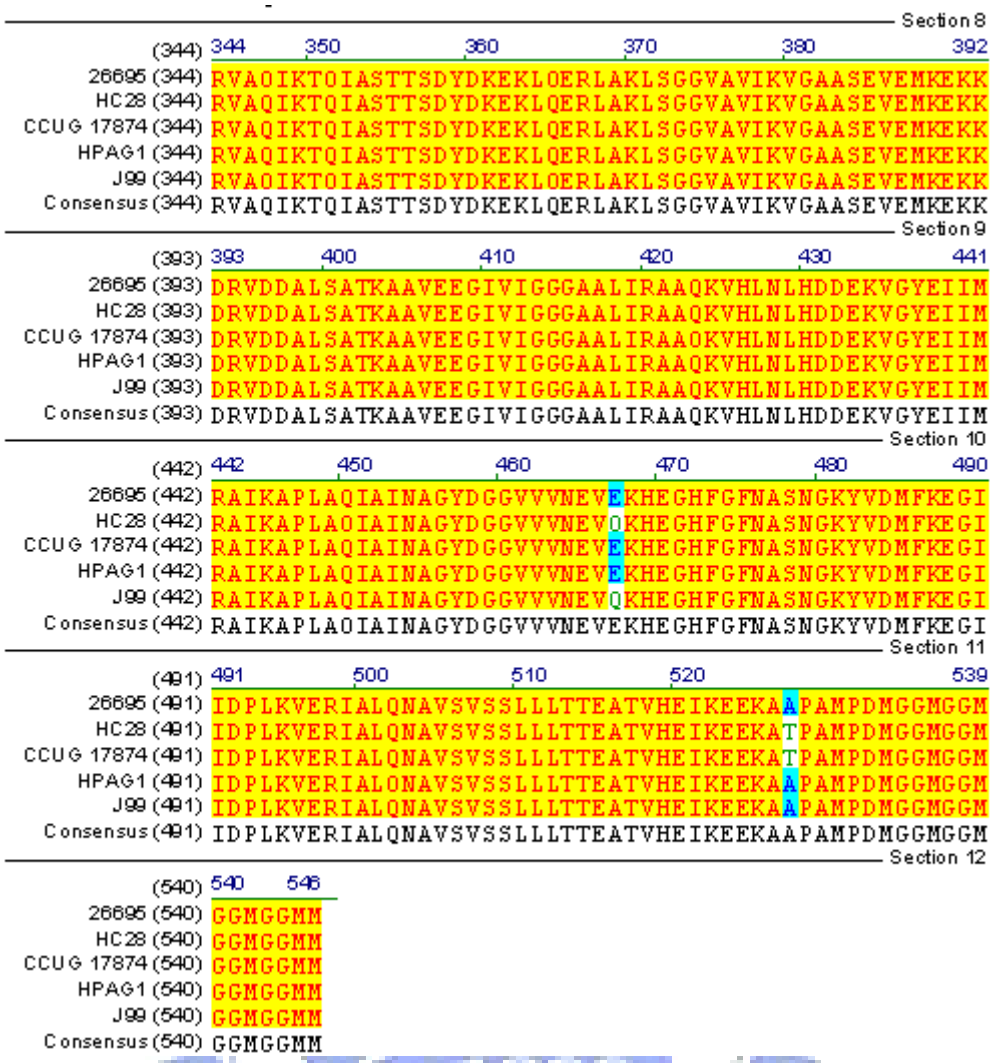
Appendix 1

1. The amino acid homology of different virulence factors in different stains.

1.1 Hsp60

1.1.1 Alignment result





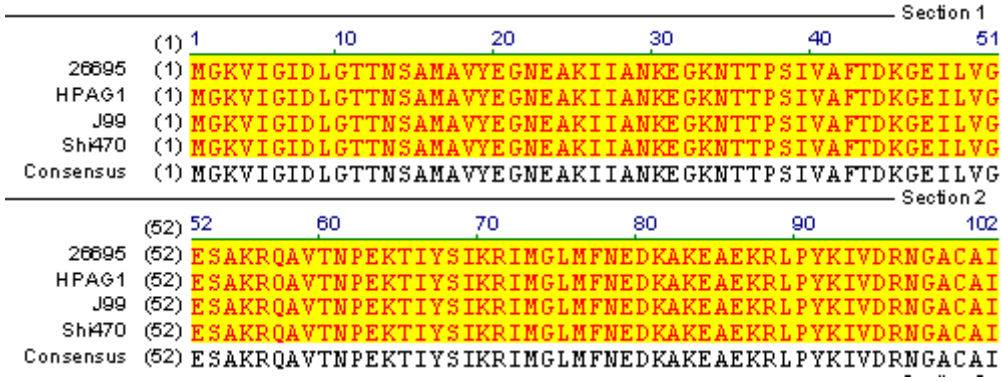
1.1.2 Strains: 26695, HC28 (used in our study), CCUG 178874, HPAG1, and J99.

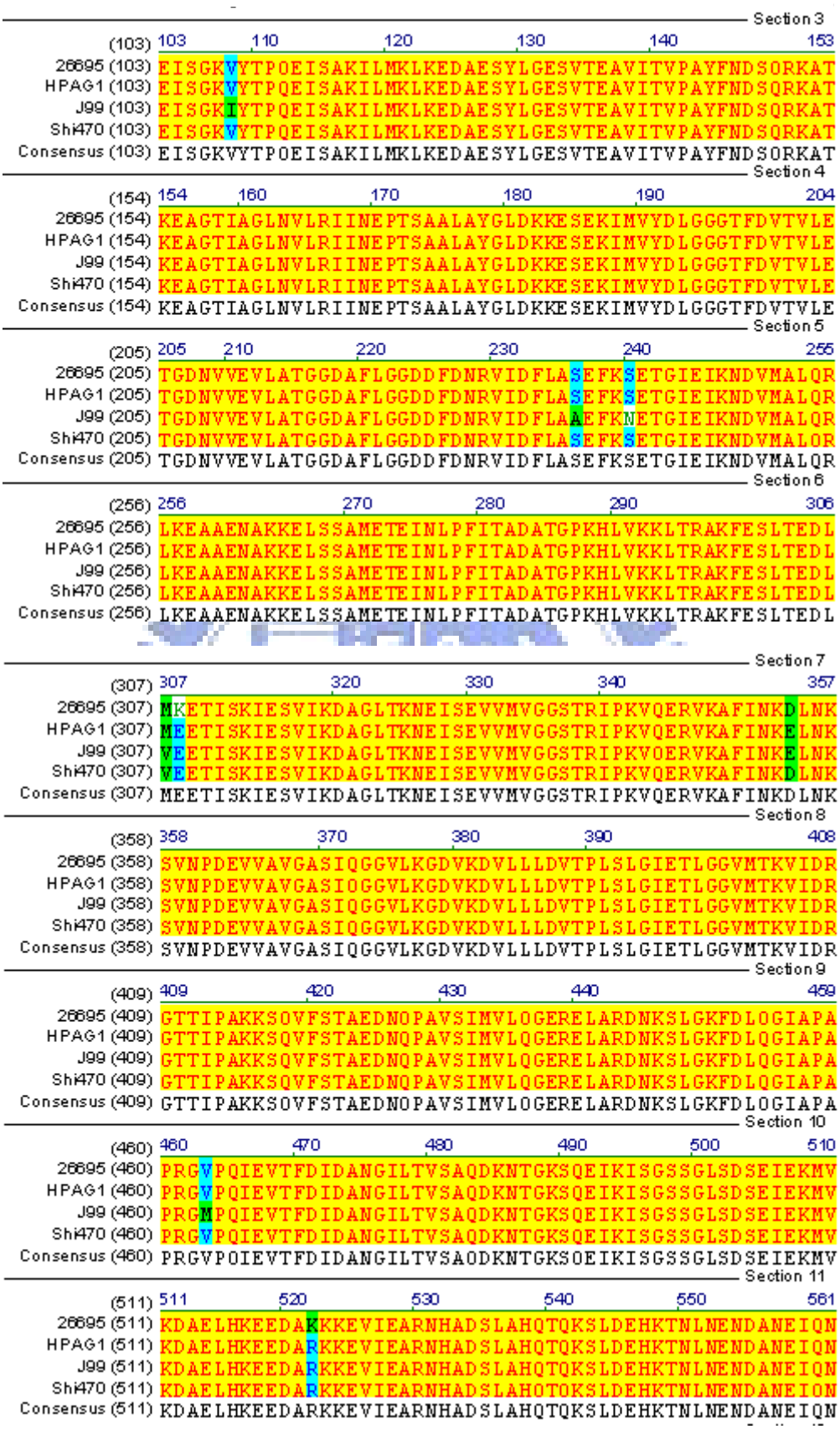
1.1.3 Identity: 99.1%

1.1.4 Positive: 100%

1.2 Hsp70

1.2.1 Alignment result

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J99 (205) TGDNVVEVLATGGDAFLGGDDFDNRVIDFLAAEFKNETGIEIKNDVMALQR
Shi470 (205) TGDNVVEVLATGGDAFLGGDDFDNRVIDFLASEFKSETGIEIKNDVMALQR
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J99 (256) LKEAAENAKKELSSAMETEINLPFITADATGPKHLVKKLTRAKFESLTEDL
Shi470 (256) LKEAAENAKKELSSAMETEINLPFITADATGPKHLVKKLTRAKFESLTEDL
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J99 (460) PRGMPQIEVTFDIDANGILTVSAQDKNTGKSQEIKISGSSGLSDSEIEKMV
Shi470 (460) PRGVPQIEVTFDIDANGILTVSAQDKNTGKSQEIKISGSSGLSDSEIEKMV
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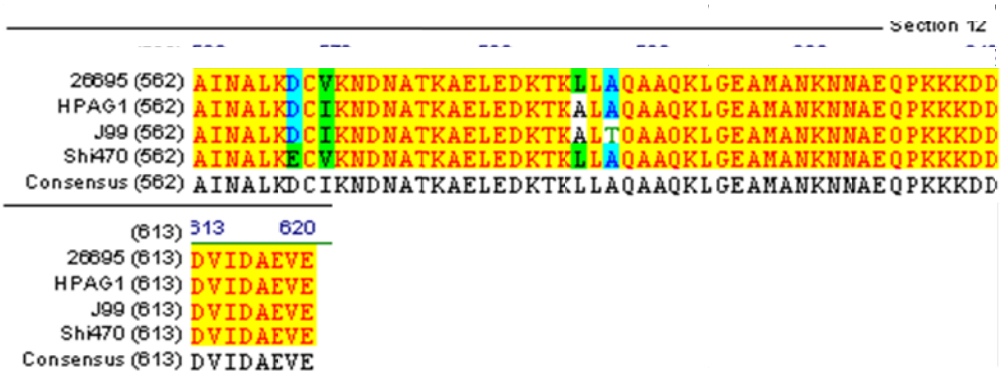


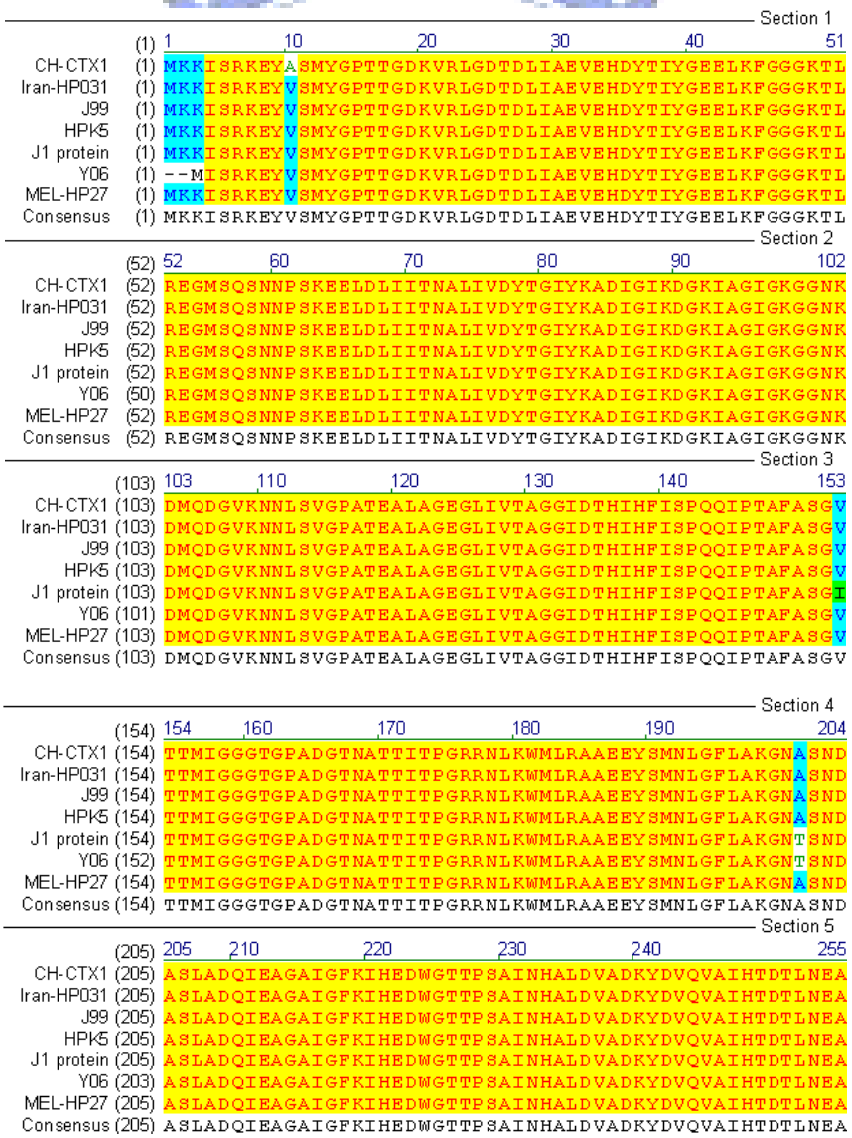
1.2.2 Strains: 26695, HPAG1, J99, Shi470

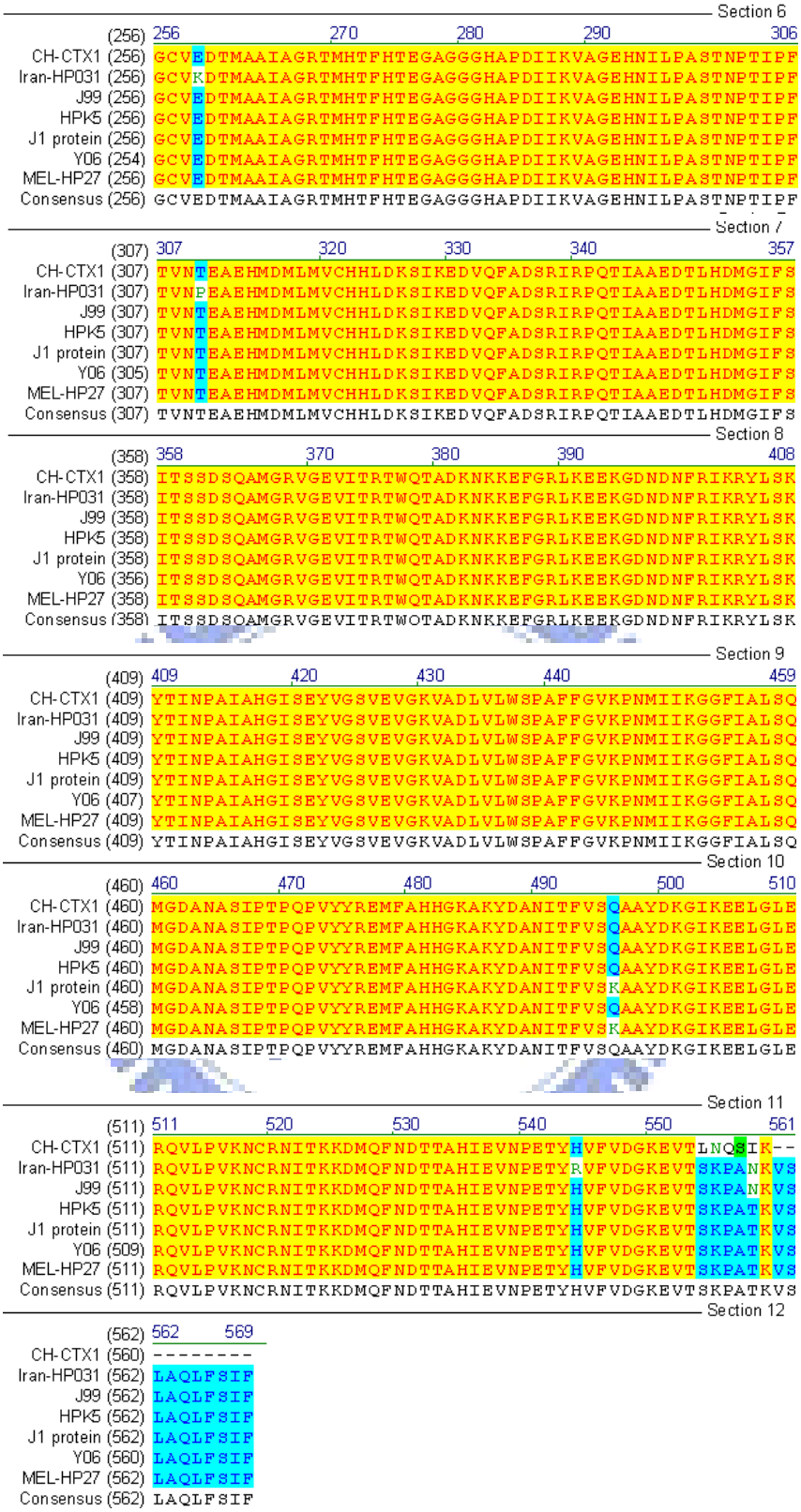
1.2.3 Identity: 98.1%

1.2.4 Positive: 100%

1.3 UreB

1.3.1 Alignment result:





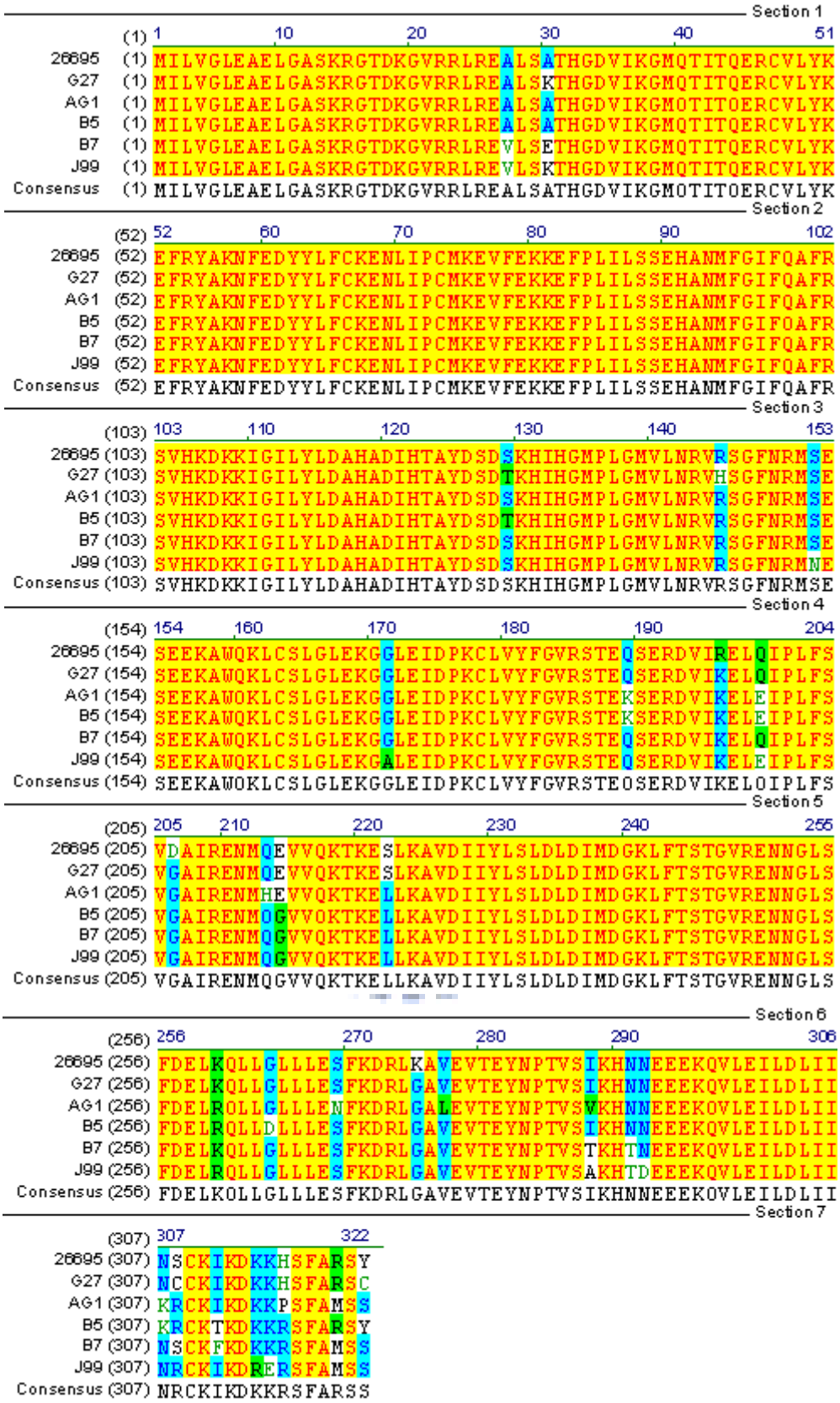
1.3.2 Strains: Ch-CTX1, Iran-HP031, J99, HC28, Y06, MEL-HP27.

1.3.3 Identity: 95.6%

1.3.4 Positive: 100%

1.4 Arginase

1.4.1 Alignment result:



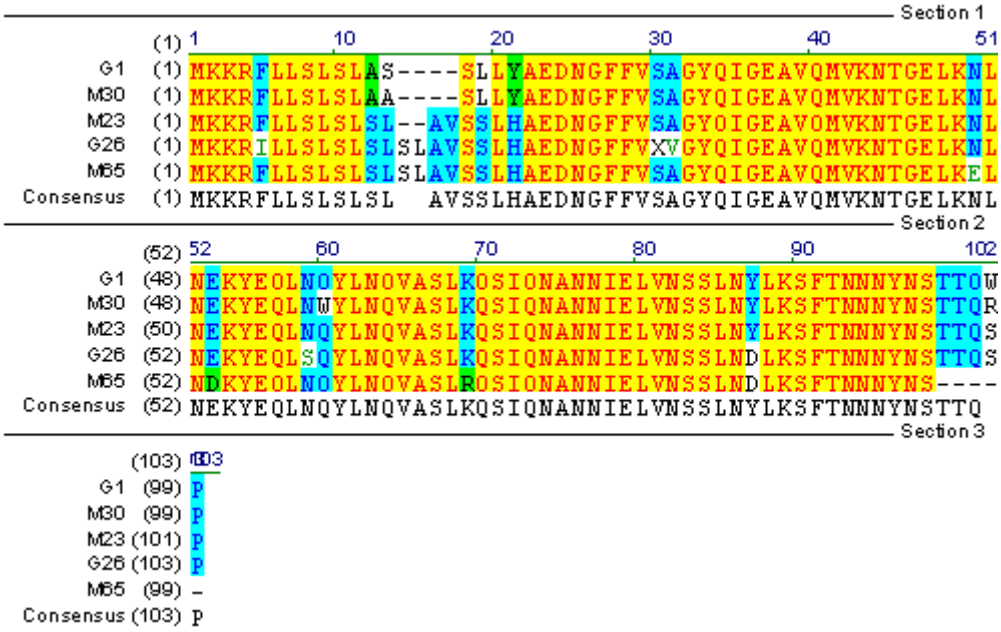
1.4.2 Strains: 26695, G27, AG1, B5,B7, and J99

1.4.3 Identity: 91%

1.4.4 Positive: 100%

1.5 SabA

1.5.1 Alignment result:



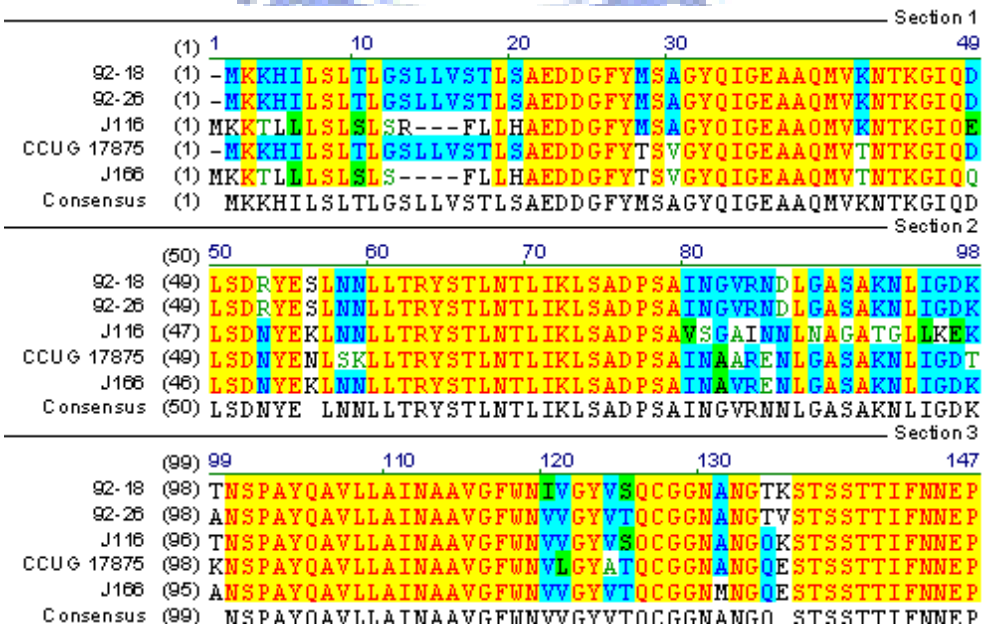
1.5.2 Strains: G1, M30, M23, G26, and M65

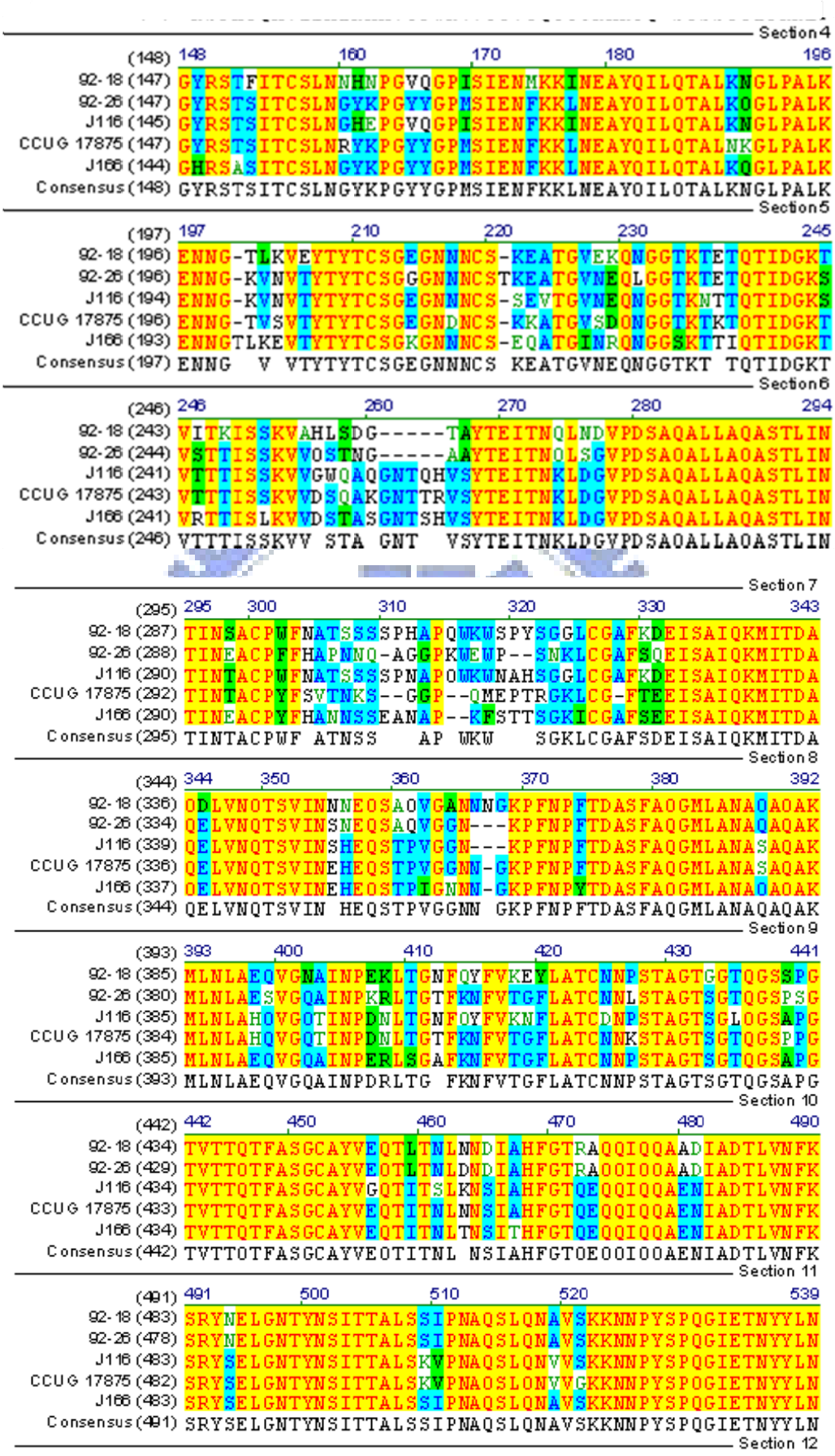
1.5.3 Identity: 78.6%

1.5.4 Positive: 97.1%

1.6 BabA

1.6.1 Alignment result:

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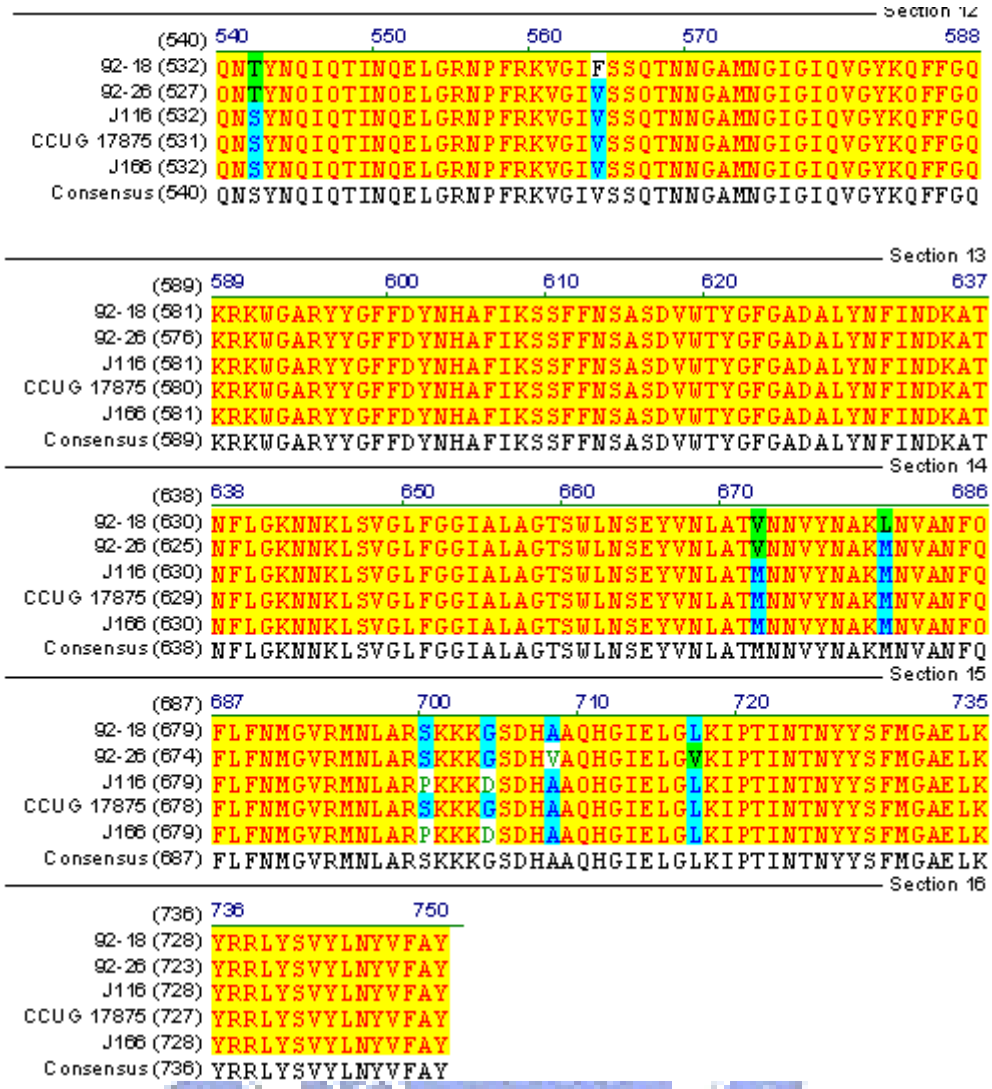


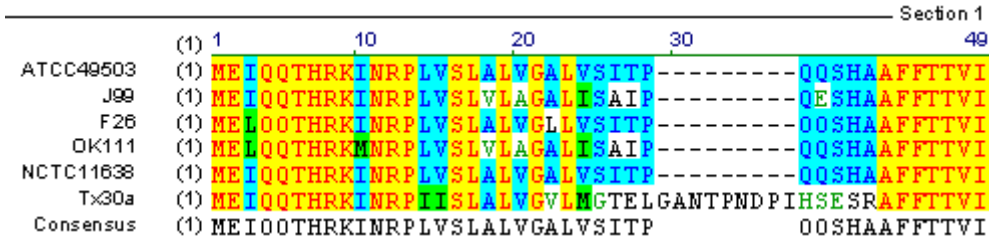
1.6.2 Strains: 92-18, 92-26, J116, CCUG 17875

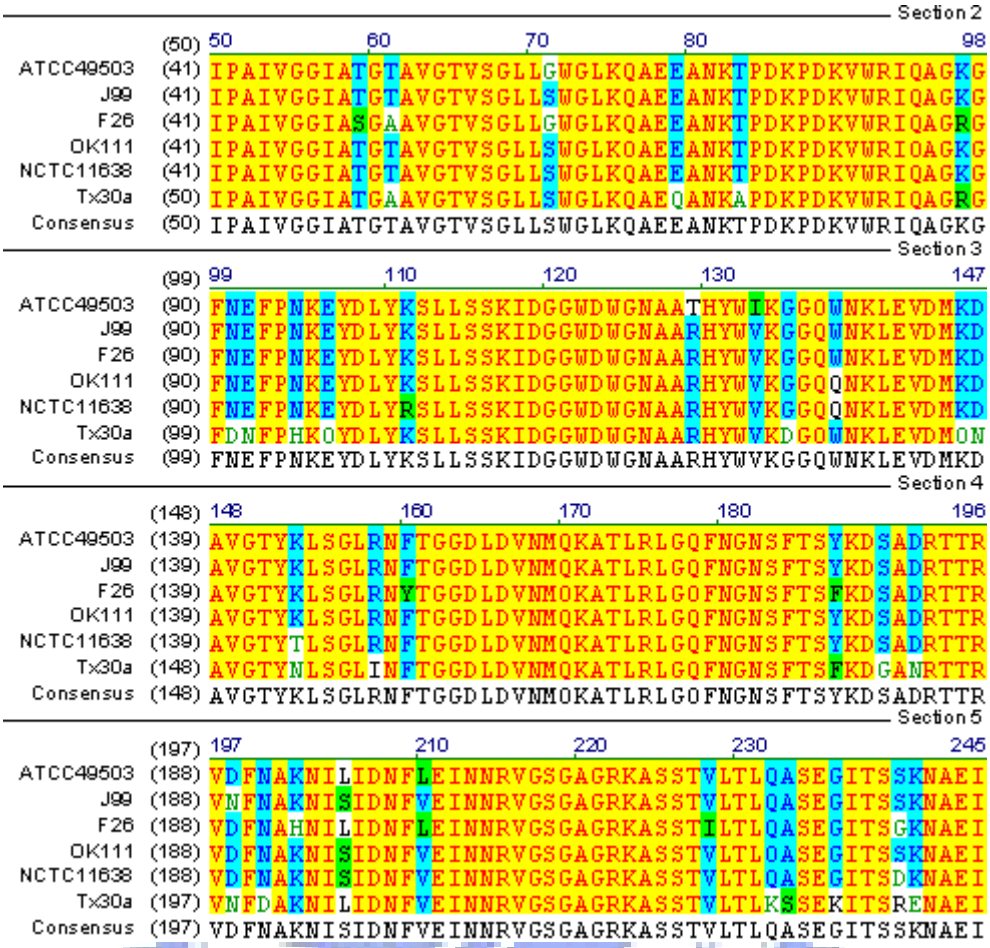
1.6.3 Identity: 76.4%

1.6.4 Positive: 96.7

1.7 VacA

1.7.1 Alignment result:

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J99 (188) VNFNAKNISIDNFVEINNRVGSGAGRKASSTVLTLQASEGITSSKNAEI
F26 (188) VDFNAHNILIDNFLEINNRVGSGAGRKASSTILTLQASEGITSGKNAEI
OK111 (188) VDFNAKNISIDNFVEINNRVGSGAGRKASSTVLTLQASEGITSSKNAEI
NCTC11638 (188) VDFNAKNISIDNFVEINNRVGSGAGRKASSTVLTLQASEGITSDKNAEI
Tx30a (197) VNFDAKNILIDNFVEINNRVGSGAGRKASSTVLTLKSSEKITSRENAEI
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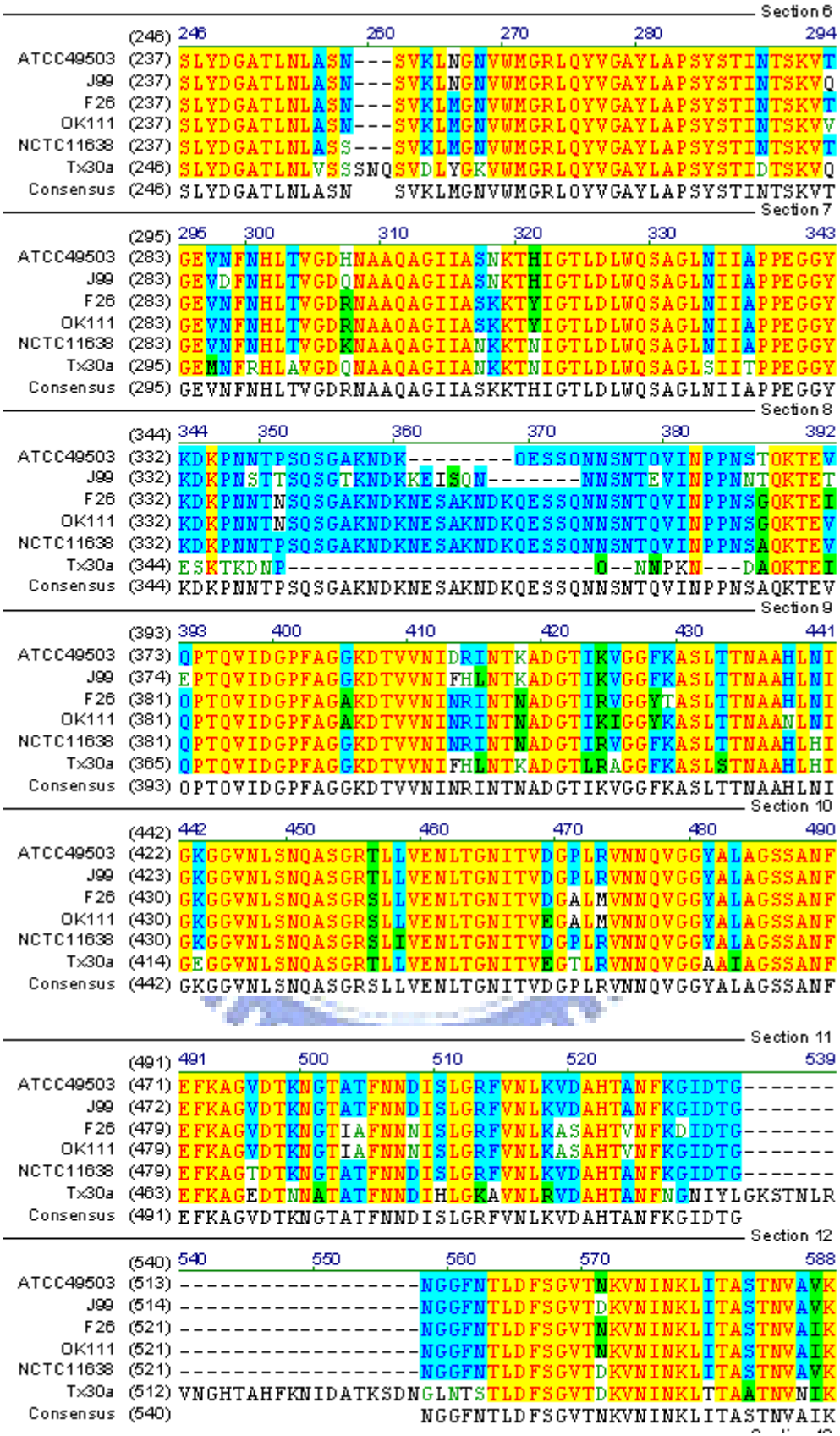
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F26 (237) SLYDGATLNLASN---SVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVT
OK111 (237) SLYDGATLNLASN---SVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVV
NCTC11638 (237) SLYDGATLNLASS---SVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVT
Tx30a (246) SLYDGATLNLVSSSNQSVDLYGKVWMGRLQYVGAYLAPSYSTIDTSKVQ
Consensus (246) SLYDGATLNLASN SVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVT
Section 7
(295) 295 300 310 320 330 343
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J99 (283) GEVDFNHLTVGDQNAAQAGIIASNKTHIGTLDLWQSAGLNIIAPPEGGY
F26 (283) GEVNFNHLTVGDRNAAQAGIIASKKTYIGTLDLWQSAGLNIIAPPEGGY
OK111 (283) GEVNFNHLTVGDRNAAQAGIIASKKTYIGTLDLWQSAGLNIIAPPEGGY
NCTC11638 (283) GEVNFNHLTVGDKNAAQAGIIANKKTNIGTLDLWQSAGLNIIAPPEGGY
Tx30a (295) GEMNFRHLAVGDQNAAQAGIIANKKTNIGTLDLWQSAGLSIITPPEGGY
Consensus (295) GEVNFNHLTVGDRNAAQAGIIASKKTHIGTLDLWQSAGLNIIAPPEGGY
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J99 (332) KDKPNSTTSQSGTKNDKKEISQN-------NNSNTEVINPPNNTQKTET
F26 (332) KDKPNNTNSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSGQKTEI
OK111 (332) KDKPNNTNSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSGQKTEV
NCTC11638 (332) KDKPNNTPSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSAQKTEV
Tx30a (344) ESKTKDNP----------Q--NNPKN---DAQKTEI
Consensus (344) KDKPNNTPSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSAQKTEV
Section 9
(393) 393 400 410 420 430 441
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F26 (381) QPTQVIDGPFAGAKDTVVNINRINTNADGTIRVGGYTASLTTNAAHLNI
OK111 (381) QPTQVIDGPFAGAKDTVVNINRINTNADGTIKIGGYKASLTTNAANLNI
NCTC11638 (381) QPTQVIDGPFAGGKDTVVNINRINTNADGTIRVGGFKASLTTNAAHLHI
Tx30a (365) QPTQVIDGPFAGGKDTVVNIFHLNTKADGTLRAGGFKASLSTNAAHLHI
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(442) 442 450 460 470 480 490
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F26 (430) GKGGVNLSNQASGRSLLVENLTGNITVDGALMVNNQVGGYALAGSSANF
OK111 (430) GKGGVNLSNQASGRSLLVENLTGNITVEGALMVNNQVGGYALAGSSANF
NCTC11638 (430) GKGGVNLSNQASGRSLIVENLTGNITVDGPLRVNNQVGGYALAGSSANF
Tx30a (414) GEGGVNLSNQASGRTLLVENLTGNITVEGTLRVNNQVGGAAIAGSSANF
Consensus (442) GKGGVNLSNQASGRSLLVENLTGNITVDGPLRVNNQVGGYALAGSSANF
Section 11
(491) 491 500 510 520 539
ATCC49503 (471) EFKAGVDTKNGTATFNNDISLGRFVNLKVDAHTANFKGIDTG-------
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F26 (479) EFKAGVDTKNGTIAFNNNISLGRFVNLKASAHTVNFKDIDTG-------
OK111 (479) EFKAGVDTKNGTIAFNNNISLGRFVNLKASAHTVNFKGIDTG-------
NCTC11638 (479) EFKAGTDTKNGTATFNNDISLGRFVNLKVDAHTANFKGIDTG-------
Tx30a (463) EFKAGEDTNNATATFNNDIHLGKAVNLRVDAHTANFNGNIYLGKSTNLR
Consensus (491) EFKAGVDTKNGTATFNNDISLGRFVNLKVDAHTANFKGIDTG
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(540) 540 550 560 570 588
ATCC49503 (513) ----------NGGFNTLDFSGVTNKVNINKLITASTNVAVK
J99 (514) ----------NGGFNTLDFSGVTDKVNINKLITASTNVAVK
F26 (521) ----------NGGFNTLDFSGVTNKVNINKLITASTNVAIK
OK111 (521) ----------NGGFNTLDFSGVTNKVNINKLITASTNVAIK
NCTC11638 (521) ----------NGGFNTLDFSGVTDKVNINKLITASTNVAVK
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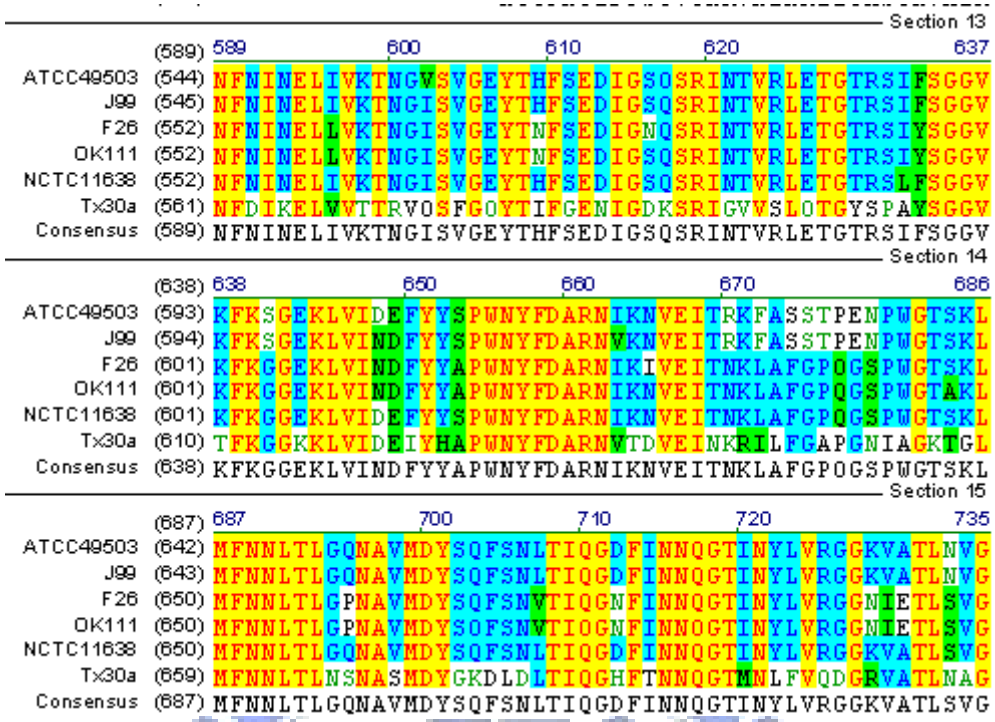
Consensus (540) NGGFNTLDFSGVTNKVNINKLITASTNVAIK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J99 (545) NFNINELIVKTNGISVGEYTHFSEDIGSQSRINTVRLETGTRSIFSGGV
F26 (552) NFNINELLVKTNGISVGEYTNFSEDIGNQSRINTVRLETGTRSIYSGGV
OK111 (552) NFNINELLVKTNGISVGEYTNFSEDIGSQSRINTVRLETGTRSIYSGGV
NCTC11638 (552) NFNINELIVKTNGISVGEYTHFSEDIGSQSRINTVRLETGTRSLFSGGV
Tx30a (561) NFDIKELWVTTRVQSFGQYTIFGENIGDKSRIGVVSLQTGYSPAYSGGV
Consensus (589) NFNINELIVKTNGISVGEYTHFSEDIGSQSRINTVRLETGTRSIFSGGV
Section 14
(638) 638 650 660 670 686
ATCC49503 (593) KFKSGEKLVIDEFYYSPWNYFDARNIKNVEITRKFASSTPENPWGTSKL
J99 (594) KFKSGEKLVINDFYYSPWNYFDARNVKNVEITRKFASSTPENPWGTSKL
F26 (601) KFKGGEKLVINDFYYAPWNYFDARNIKIVEITNKLAFGPQGSPWGTSKL
OK111 (601) KFKGGEKLVINDFYYAPWNYFDARNIKNVEITNKLAFGPQGSPWGTAKL
NCTC11638 (601) KFKGGEKLVIDEFYYSPWNYFDARNIKNVEITNKLAFGPQGSPWGTSKL
Tx30a (610) TFKGGKKLVIDEIYHAPWNYFDARNVTDVEINKRILFGAPGNIAGKTGL
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(687) 687 700 710 720 735
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J99 (643) MFNNLTLGQNAVMDYSQFSNLTIQGDFINNQGTINYLVRGGKVATLNVG
F26 (650) MFNNLTLGPNAVMDYSQFSNVTIQGNFINNQGTINYLVRGGNIETLSVG
OK111 (650) MFNNLTLGPNAVMDYSQFSNVTIQGNFINNQGTINYLVRGGNIETLSVG
NCTC11638 (650) MFNNLTLGQNAVMDYSQFSNLTIQGDFINNQGTINYLVRGGKVATLSVG
Tx30a (659) MFNNLTLNSNASMDYGKDLDLTIQGHFTNNQGTMNLFVQDGRVATLNAG
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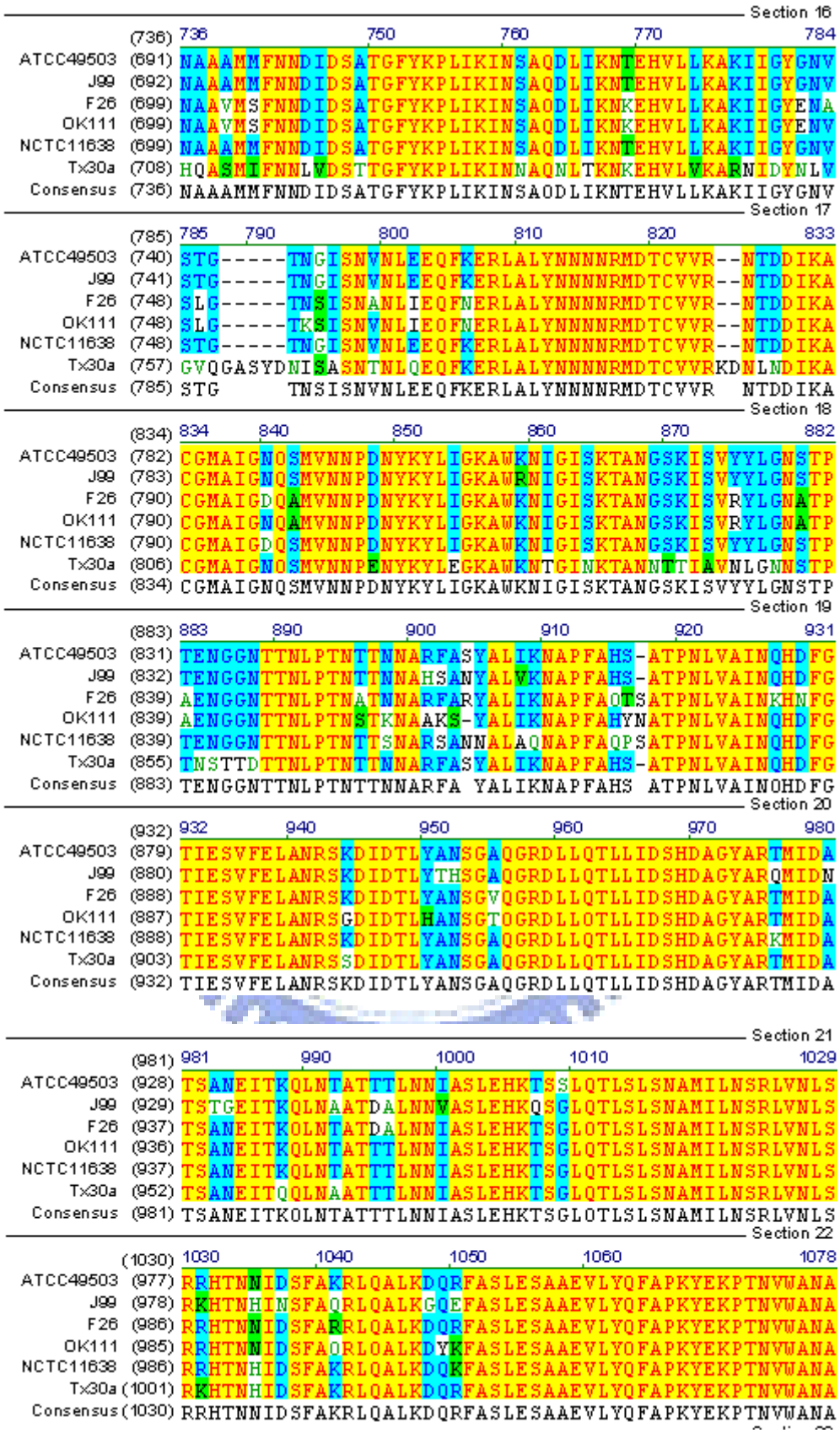
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OK111 (699) NAAVMSFNNDIDSATGFYKPLIKINSAQDLIKNKEHVLLKAKIIGYENV
NCTC11638 (699) NAAAMMFNNDIDSATGFYKPLIKINSAQDLIKNTEHVLLKAKIIGYGNV
Tx30a (708) HQASMIFNNLVDSTTGFYKPLIKINNAQNLTKNKEHVLVKARNIDYNLV
Consensus (736) NAAAMMFNNDIDSATGFYKPLIKINSAQDLIKNTEHVLLKAKIIGYGNV
Section 17
(785) 785 790 800 810 820 833
ATCC49503 (740) STG-----TNGISNVNLEEQFKERLALYNNNNRMDTCVVR--NTDDIKA
J99 (741) STG-----TNGISNVNLEEQFKERLALYNNNNRMDTCVVR--NTDDIKA
F26 (748) SLG-----TNSISNANLIEQFNERLALYNNNNRMDTCVVR--NTDDIKA
OK111 (748) SLG-----TKSISNVNLIEQFNERLALYNNNNRMDTCVVR--NTDDIKA
NCTC11638 (748) STG-----TNGISNVNLEEQFKERLALYNNNNRMDTCVVR--NTDDIKA
Tx30a (757) GVQGASYDNISASNTNLQEQFKERLALYNNNNRMDTCVVRKDNLNDIKA
Consensus (785) STG TNSISNVNLEEQFKERLALYNNNNRMDTCVVR NTDDIKA
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(834) 834 840 850 860 870 882
ATCC49503 (782) CGMAIGNQSMVNNPDNYKYLIGKAWKNIGISKTANGSKISVYYLGNSTP
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OK111 (790) CGMAIGNQAMVNNPDNYKYLIGKAWKNIGISKTANGSKISVRYLGNATP
NCTC11638 (790) CGMAIGDQSMVNNPDNYKYLIGKAWKNIGISKTANGSKISVYYLGNSTP
Tx30a (806) CGMAIGNQSMVNNPENYKYLEGKAWKNTGINKTANNTTIAVNLGNNSTP
Consensus (834) CGMAIGNQSMVNNPDNYKYLIGKAWKNIGISKTANGSKISVYYLGNSTP
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(883) 883 890 900 910 920 931
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F26 (839) AENGGNTTNLPTNATNNARFARYALIKNAPFAQTSATPNLVAINKHNFG
OK111 (839) AENGGNTTNLPTNSTKNAAKS-YALIKNAPFAHYNATPNLVAINQHDFG
NCTC11638 (839) TENGGNTTNLPTNTTSNARSANNALAQNAPFAQPSATPNLVAINQHDFG
Tx30a (855) TNSTTDTTNLPTNTTNNARFASYALIKNAPFAHS-ATPNLVAINQHDFG
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(932) 932 940 950 960 970 980
ATCC49503 (879) TIESVFELANRSKDIDTLYANSGAQGRDLLQTLLIDSHDAGYARTMIDA
J99 (880) TIESVFELANRSKDIDTLYTHSGAQGRDLLQTLLIDSHDAGYARQMIDN
F26 (888) TIESVFELANRSKDIDTLYANSGVQGRDLLQTLLIDSHDAGYARTMIDA
OK111 (887) TIESVFELANRSGDIDTLHANSGTQGRDLLQTLLIDSHDAGYARTMIDA
NCTC11638 (888) TIESVFELANRSKDIDTLYANSGAQGRDLLQTLLIDSHDAGYARKMIDA
Tx30a (903) TIESVFELANRSSDIDTLYANSGAQGRDLLQTLLIDSHDAGYARTMIDA
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(981) 981 990 1000 1010 1029
ATCC49503 (928) TSANEITKQLNTATTTLNNIASLEHKTSSLQTLSLSNAMILNSRLVNLS
J99 (929) TSTGEITKQLNAATDALNNVASLEHKQSGLQTLSLSNAMILNSRLVNLS
F26 (937) TSANEITKQLNTATDALNNIASLEHKTSGLQTLSLSNAMILNSRLVNLS
OK111 (936) TSANEITKQLNTATTTLNNIASLEHKTSGLQTLSLSNAMILNSRLVNLS
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Tx30a (952) TSANEITQQLNAATTTLNNIASLEHKTSGLQTLSLSNAMILNSRLVNLS
Consensus (981) TSANEITKQLNTATTTLNNIASLEHKTSGLQTLSLSNAMILNSRLVNLS
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ATCC49503 (977) RRHTNNIDSFAKRLQALKDQRFASLESAAEVLYQFAPKYEKPTNVWANA
J99 (978) RKHTNHINSFAQRLQALKGQEFASLESAAEVLYQFAPKYEKPTNVWANA
F26 (986) RRHTNNIDSFARRLQALKDQRFASLESAAEVLYQFAPKYEKPTNVWANA
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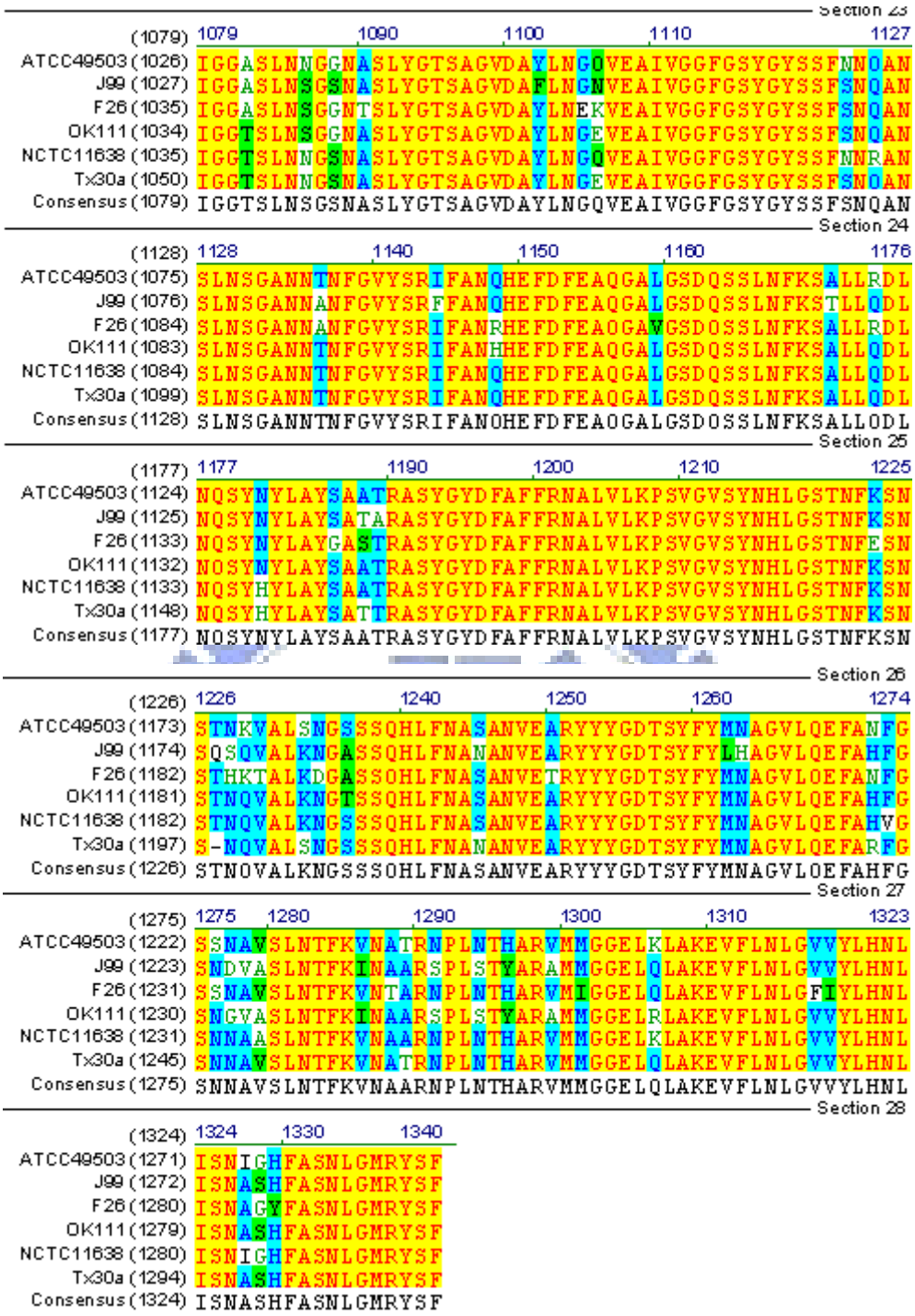


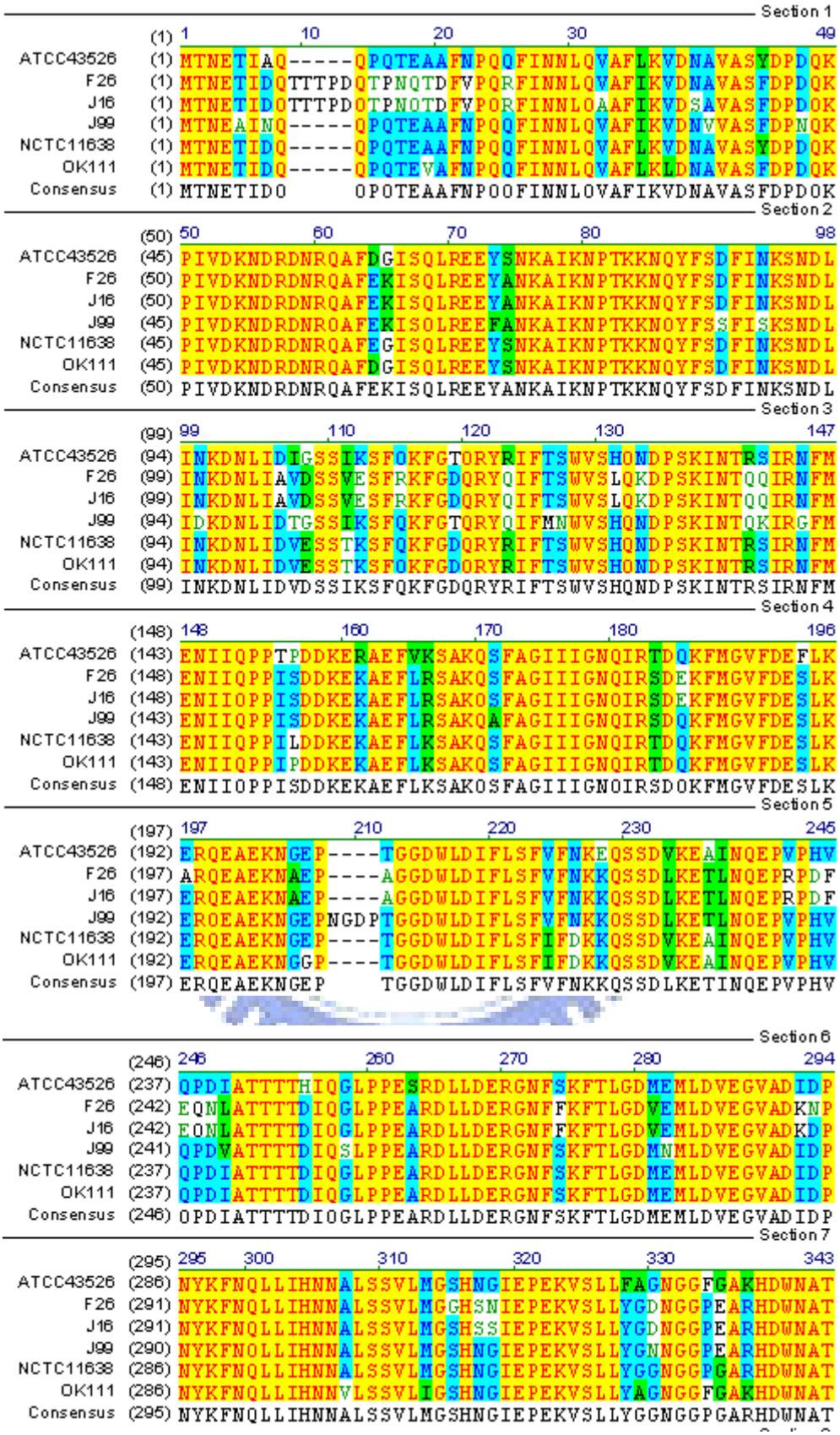
1.7.2 Strains: ATCC 49503, J99, F26, OK111, NCTC11638, and TX30A

1.7.3 Identity: 68.9%

1.7.4 Positive: 96.6%

1.8 CagA

1.8.1 Alignment result: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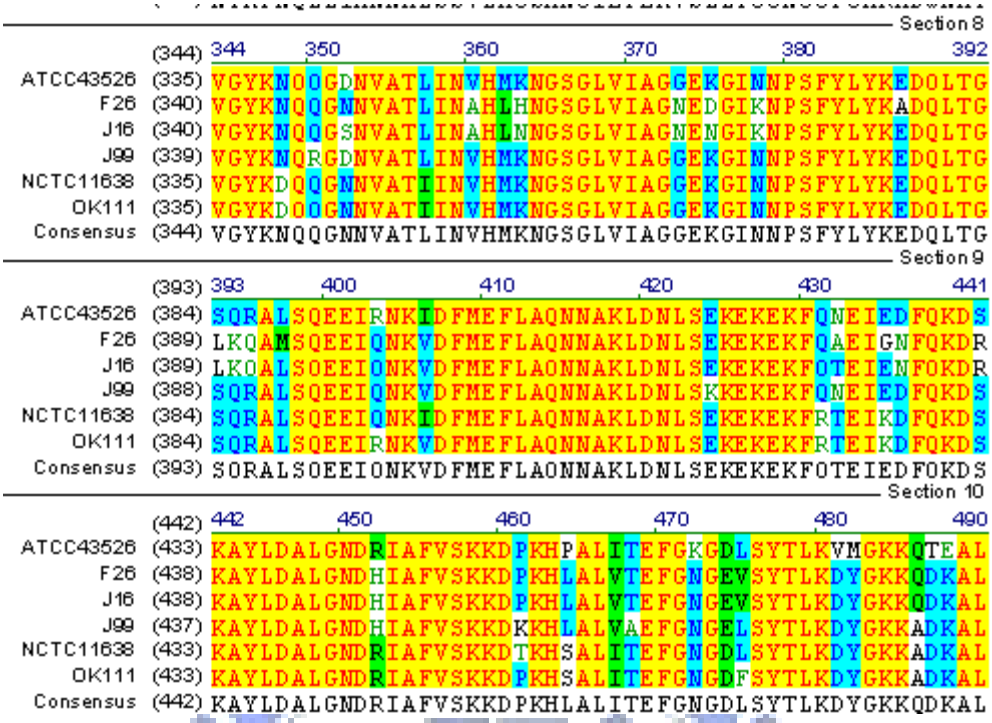
Section 2
Section 3
Section 4
Section 5
Section 6
Section 7
ATCC43526
F26
J16
J99
NCTC11638
OK111
Consensus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J16 (340) VGYKNQQGSNVATLINAHLNNGSGLVIAGNENGIKNPSFYLYKEDQLTG
J99 (339) VGYKNQRGDNVATLINVHMKNGSGLVIAGGEKGINNPSFYLYKEDQLTG
NCTC11638 (335) VGYKDQQGNNVATIINVHMKNGSGLVIAGGEKGINNPSFYLYKEDQLTG
OK111 (335) VGYKDQQGNNVATIINVHMKNGSGLVIAGGEKGINNPSFYLYKEDQLTG
Consensus (344) VGYKNQQGNNVATLINVHMKNGSGLVIAGGEKGINNPSFYLYKEDQLTG
Section 9
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ATCC43526 (384) SQRALSQEEIRNKIDFMEFLAQNNAKLDNLSEKEKEKFQNEIEDFQKDS
F26 (389) LKQAMSQEEIQNKVDFMEFLAQNNAKLDNLSEKEKEKFQAEIGNFQKDR
J16 (389) LKQALSQEEIQNKVDFMEFLAQNNAKLDNLSEKEKEKFQTEIENFQKDR
J99 (388) SQRALSQEEIQNKVDFMEFLAQNNAKLDNLSKKEKEKFQNEIEDFQKDS
NCTC11638 (384) SQRALSQEEIQNKIDFMEFLAQNNAKLDNLSEKEKEKFRTEIKDFQKDS
OK111 (384) SQRALSQEEIRNKVDFMEFLAQNNAKLDNLSEKEKEKFRTEIKDFQKDS
Consensus (393) SQRALSQEEIQNKVDFMEFLAQNNAKLDNLSEKEKEKFQTEIEDFQKDS
Section 10
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J99 (437) KAYLDALGNDHIAFVSKKDKKHLALVAEFGNGELSYTLKDYGKKADKAL
NCTC11638 (433) KAYLDALGNDRIAFVSKKDTKHSALITEFGNGDLSYTLKDYGKKADKAL
OK111 (433) KAYLDALGNDRIAFVSKKDPKHSALITEFGNGDFSYTLKDYGKKADKAL
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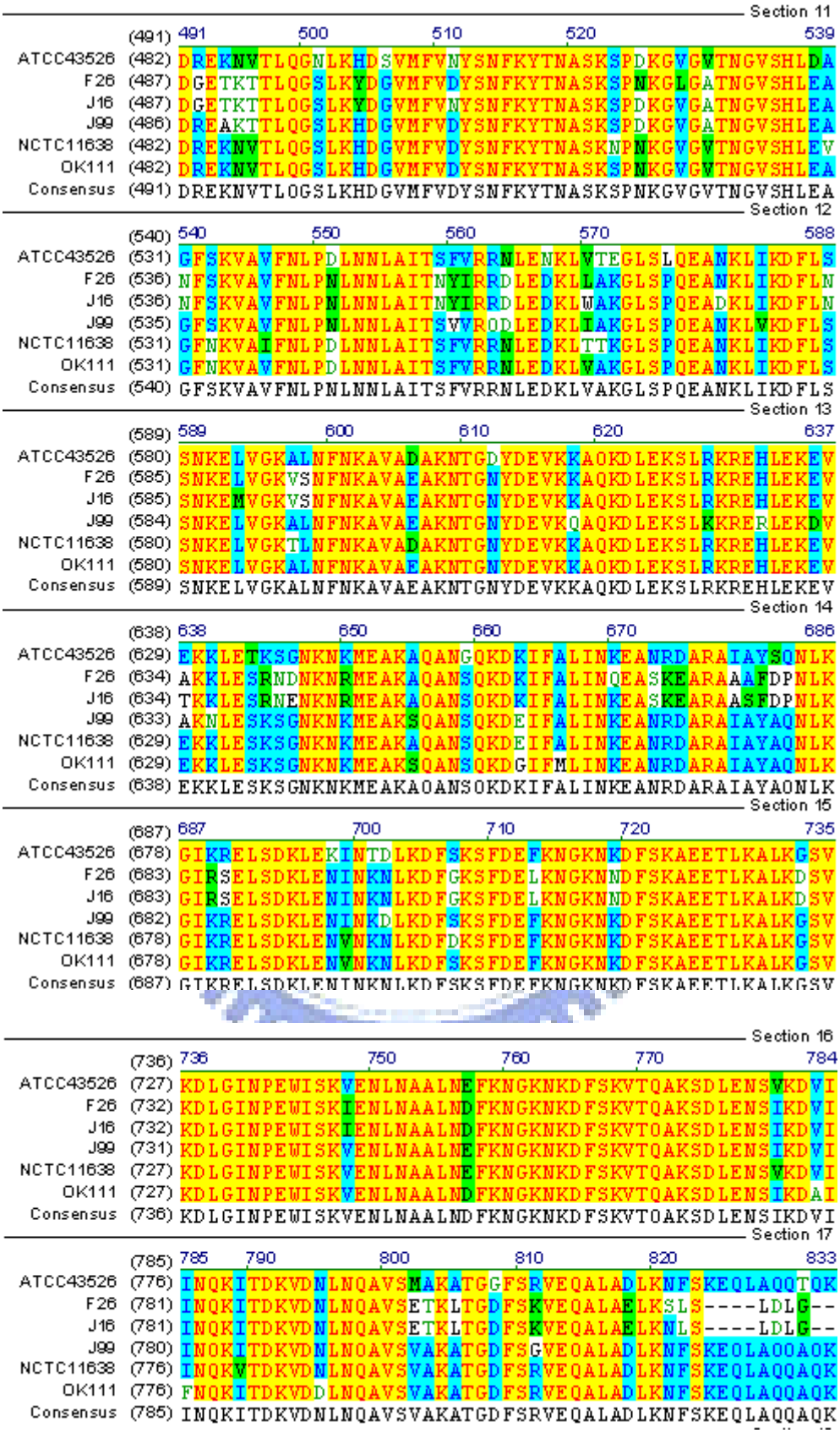
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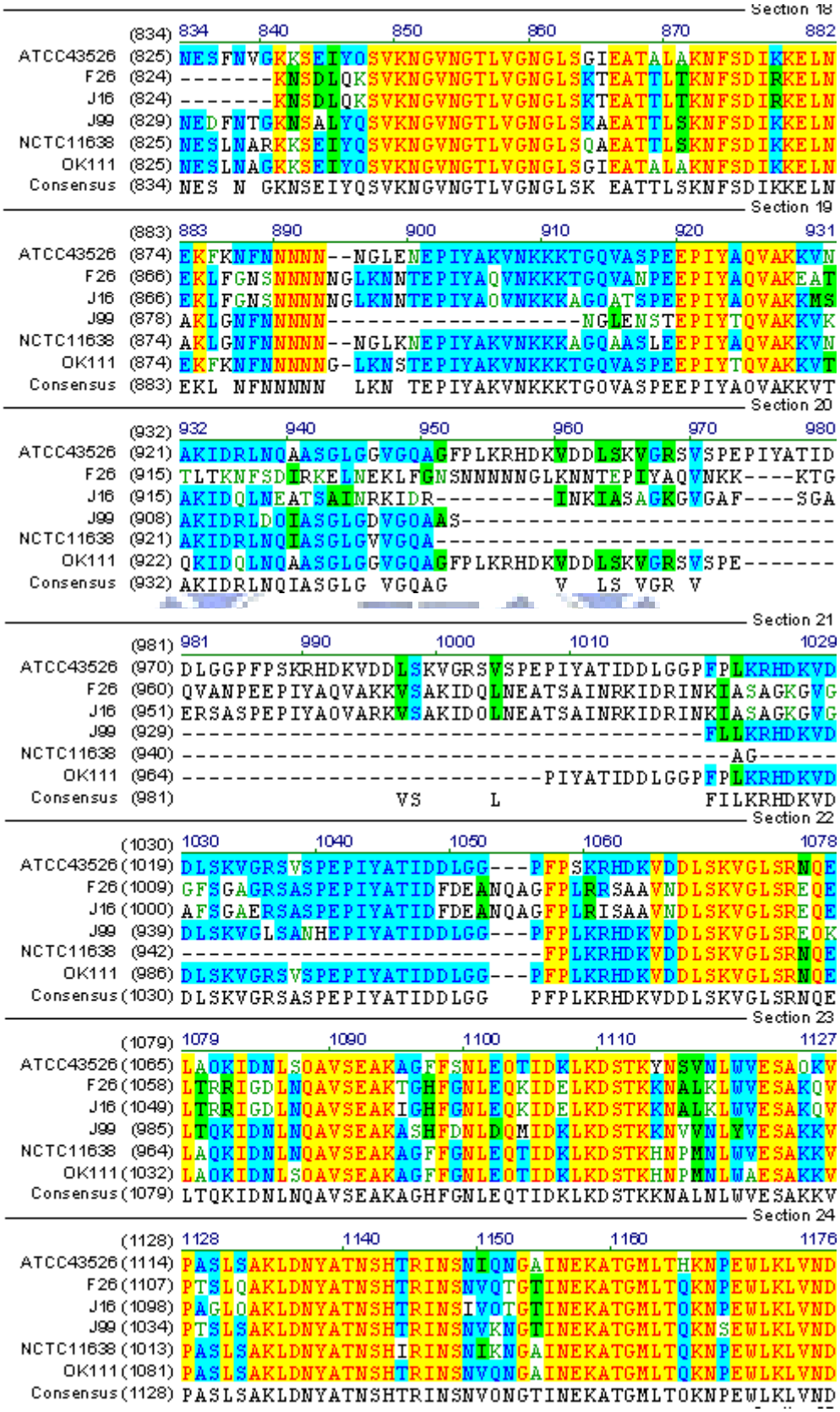
Section 13
Section 14
Section 15
Section 16
Section 17
ATCC43526
F26
J16
J99
NCTC11638
OK111
Consensus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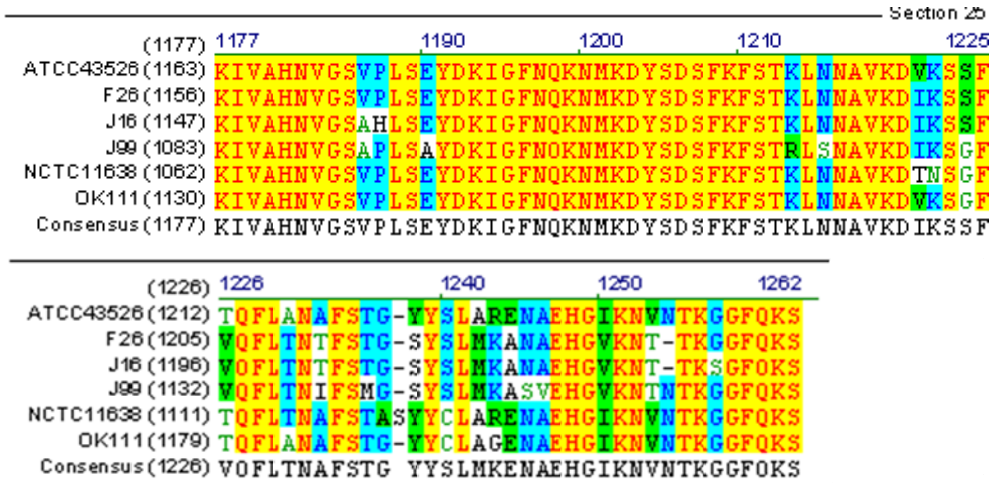
Section 22
Section 23
Section 24
ATCC43526
F26
J16
J99
NCTC11638
OK111
Consensus



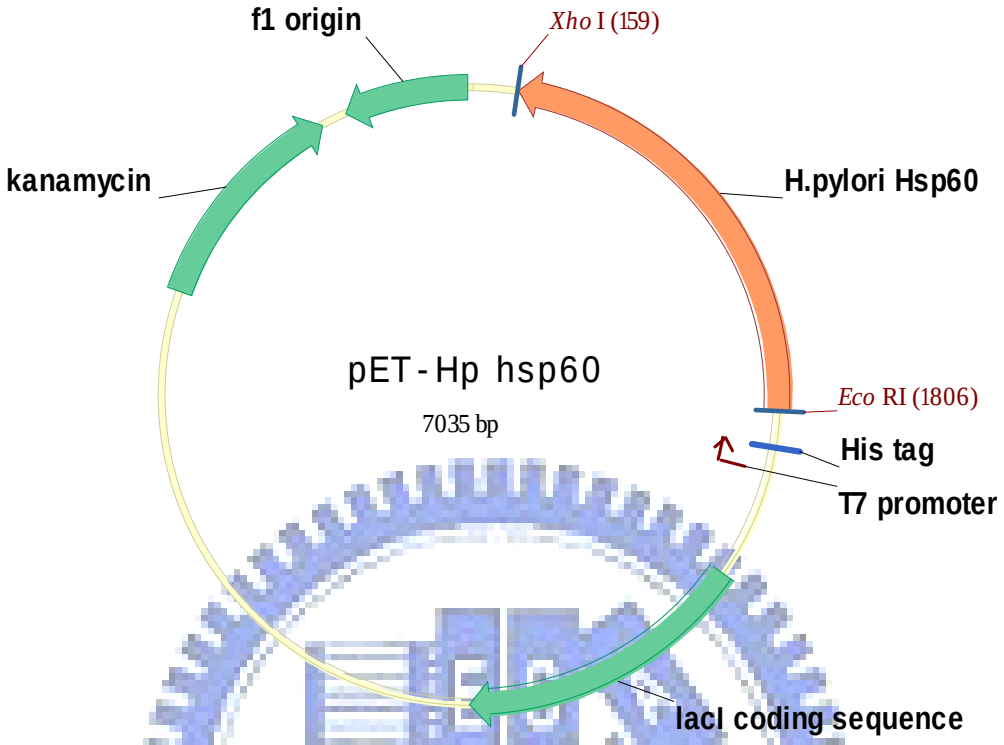
1.8.2 Strains: ATCC43526, F26, J16, J99, NCTC11638, and OK111

1.8.3 Identity: 62.9%

1.8.4 Positive: 93.7%

2. pET-Hp hsp60

2.1 Map



2.2 *H. pylori* hsp60 DNA sequence

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1381 gtggtcgtga atgaagtgca aaaacacgaa gggcattttg gttttaacgc tagcaatggc
1441 aagtatgtgg atatgtttaa agaaggcatt attgacccct taaaagtaga aaggatcgct
1501 ttacaaaatg cggtttcggt ttcaagcctg cttttaacca cagaagccac cgtgcatgaa
1561 atcaaagaag aaaaagcaac cccagcaatg cctgatatgg gtggcatggg cggtatggga
1621 ggcatgggcg gcatgatgta a

2.3 *H. pylori* hsp60 protein sequence

1 makeikfsds arnllfegvr qlhdavkvtm gprgrnvliq ksygapsitk dgvsvakeie
61 lscpvanmga qlvkevaskt adaagdgttt atvlaysifk eglrnitaga npievkrgmd
121 kaaeaiinel kkaskkvggk eeitqvatis ansdhnigkl iadamekvgk dgvitveeak
181 giedeldvve gmqfdrgyls pyfvtnaekm taqldnayil ltdkkissmk dilpllektm
241 kegkplliia ediegealtt lvvnklrgvl niaavkapgf gdrrkemlkd iavltggqvi
301 seelgltlen aeveflgkag rividkdntt ivdgkghshd vkdrvaqikt qiasttsdyd
361 keklqerlak lsggvavikv gaasevemke kkdrvddals atkaaveegi vigggaalir
421 aaqkvhlnlh ddekvgyeii mraikaplaq iainagydgg vvvnevqkhe ghfgfnasng
481 kyvdmfkegi idplkveria lqnavsvssl lltteatvhe ikeekatpam pdmggmggmg
541 gmggmm

2.4 The whole DNA sequence

1 atccggatat agttcctcct ttcagcaaaa aacccctcaa gacccgttta gaggccccaa
61 ggggttatgc tagttattgc tcagcggtgg cagcagccaa ctcagcttcc tttcgggctt
121 tgttagcagc cggatctcag tggtggtggt ggtggtgctc gagttacatc atgccgccca
181 tgcctcccat accgcccatg ccacccatat caggcattgc tggggttgct ttttcttctt
241 tgatttcatg cacggtggct tctgtggtta aaagcaggct tgaaaccgaa accgcatttt
301 gtaaagcgat cctttctact tttaaggggt caataatgcc ttctttaaac atatccacat
361 acttgccatt gctagcgtta aaaccaaaat gcccttcgtg tttttgcact tcattcacga
421 ccacaccgcc atcataaccg gcattgatag cgatttgagc taatggggct ttaatggcac
481 gcatgatgat ttcatagcct acttttttcat catcgtgtaa attcaaatgc acttttgag
541 ccgcgcgaat gagagccgca ccgccgccaa taacaatgcc ctcttcaaca gctgctttag
601 tcgcactcaa tgcgtcatca acccggtctt ttttctcttt catttccact tcactcgcag
661 cgcccacttt aatcacagcc acaccaccag agagtttggc caatctttct tgcaattttt
721 ctttgtcata atcgcttgtc gtgcttgcaa tttgggtttt gatttgcgcg actctgtctt
781 taacatcatg gctatgtcct ttgccatcta cgatcgtggt gttgtctttg tcaatcacaa

841 tccttccggc tttgcctaaa aactccactt cagcgttttc taaagtcaag cctaattctt
901 cgctaatgac ttgaccgccg gttaaaacag cgatgtcttt gagcatttct tttcttctgt
961 ccccaaagcc tggagcttta accgctgcga tattcaacac gcctcttaat ttattcacca
1021 ctagagtcgt taaagcttcg ccctcaatgt cttcagcgat gattaaaagc ggtttgccct
1081 ctttcatggt tttttctagt agcgggagaa tgtctttcat gctagagatt tttttatccg
1141 ttaaaaggat gtaagcgtta tccaattgag cggtcatttt ctcagcgttt gttacaaagt
1201 aaggggagag gtagcctcta tcaaattgca tgccctctac gacatctaat tcatcttcaa
1261 tgcccttagc ttcttcaacg gtgatcacgc cgtctttacc cacttttttcc atagcgtcag
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1381 cttctttacc acctactttt ttgctcgctt ttttaagctc attaataatg gcttcagcgg
1441 ctttatccat gcctcgtttc acttcaatag ggttagcccc agccgtgata ttcctcaagc
1501 cttctttaaa gatgctataa gcaagcacgg tcgctgtggt cgtgccatcg ccggcagcat
1561 cagcggtttt gctcgctact tctttaacga gttgagcgcc catgttagct accgggcaac
1621 ttaattcaat ctctttagcc acgctcacgc catctttggt gatgcttgga gcgccatagc
1681 ttttttggat caacacattc ctacctcttg gccccatggt tactttgaca gcgtcatgga
1741 gttgtctcac gccttcaaat aaaaggtttc ttgcactatc tgaaaatttg atttcttttg
1801 ccatgaattc ggatccgata tcagccatgg ccttgtcgtc gtcgtcggta cccagatctg
1861 ggctgtccat gtgctggcgt tcgaatttag cagcagcggt ttctttcata ccagaaccgc
1921 gtggcaccag accagaagaa tgatgatgat gatggtgcat atgtatatct ccttcttaaa
1981 gttaaacaaa attatttcta gaggggaatt gttatccgct cacaattccc ctatagtgag
2041 tcgtattaat ttcgcgggat cgagatcgat ctcgatcctc tacgccggac gcatcgtggc
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2161 ggaagatcgg gctcgccact tcgggctcat gagcgcttgt ttcggcgtgg gtatggtggc
2221 aggccccgtg gccgggggac tgttgggcgc catctccttg catgcaccat tccttgcggc
2281 ggcggtgctc aacggcctca acctactact gggctgcttc ctaatgcagg agtcgcataa
2341 gggagagcgt cgagatcccg gacaccatcg aatggcgcaa aacctttcgc ggtatggcat
2401 gatagcgccc ggaagagagt caattcaggg tggtgaatgt gaaaccagta acgttatacg
2461 atgtcgcaga gtatgccggt gtctcttatc agaccgtttc ccgcgtggtg aaccaggcca
2521 gccacgtttc tgcgaaaacg cgggaaaaag tggaagcggc gatggcggag ctgaattaca
2581 ttcccaaccg cgtggcacaa caactggcgg gcaaacagtc gttgctgatt ggcgttgcca
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2701 atcaactggg tgccagcgtg gtggtgtcga tggtagaacg aagcggcgtc gaagcctgta
2761 aagcggcggt gcacaatctt ctcgcgcaac gcgtcagtgg gctgatcatt aactatccgc
2821 tggatgacca ggatgccatt gctgtggaag ctgcctgcac taatgttccg gcgttatttc
2881 ttgatgtctc tgaccagaca cccatcaaca gtattatttt ctcccatgaa gacggtacgc
2941 gactgggcgt ggagcatctg gtcgcattgg gtcaccagca aatcgcgctg ttagcgggcc
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3061 atcaaattca gccgatagcg gaacgggaag gcgactggag tgccatgtcc ggttttcaac

3131 aaaccatgca aatgctgaat gagggcatcg ttcccactgc gatgctggtt gccaacgatc
3181 agatggcgct gggcgcaatg cgcgccatta ccgagtccgg gctgcgcgtt ggtgcggaca
3241 tctcggtagt gggatacgac gataccgaag acagctcatg ttatatcccg ccgttaacca
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6781 gatggcccac tacgtgaacc atcaccctaa tcaagttttt tggggtcgag gtgccgtaaa
6841 gcactaaatc ggaaccctaa agggagcccc cgatttagag cttgacgggg aaagccggcg
6901 aacgtggcga gaaaggaagg gaagaaagcg aaaggagcgg gcgctagggc gctggcaagt
6961 gtagcggtca cgctgcgcgt aaccaccaca cccgccgcgc ttaatgcgcc gctacagggc
7021 gcgtcccatt cgcca

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