学校编码：10384　　分类号　　密级

学　　号：B200227006　　UDC

厦 门 大 学

博 士 学 位 论 文

养殖青石斑鱼(*Epinephlus awoara*)溃疡病细菌病原及其DNA疫苗的研究

Studies on the Pathogenic Bacterium and the DNA vaccine of Ulcer Disease in cage-cultured *Epinephelus awoara*

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专 业 名 称：海 洋 生 物 学

论文提交日期：2005 年 6 月

论文答辩时间：2005 年 7 月

学位授予单位：厦 门 大 学

学位授予日期：2005 年　月

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2005 年 **6** 月 **8** 日

目　录

主要英文缩写词表 I

摘　要 III

Abstract VII

第一章　文献综述 1

第一节　鱼类弧菌病（vibriosis）研究进展 1

1.1 鱼类的致病弧菌 1

1.2 鱼类弧菌病 9

第二节　鱼类 DNA 疫苗研究进展 16

2.1 DNA 疫苗的概况 16

2.2 DNA 疫苗的免疫机制 18

2.3 鱼用 DNA 疫苗的研究进展 25

第二章　网养青石斑溃疡病细菌病原的研究 36

第一节　细菌性病原的分离与鉴定 36

1.1 材料与方法 37

1.2 结果 39

1.3 讨论 45

第二节　哈维氏弧菌 TS-628 菌株胞外产物（ECP）蛋白酶活性的研究 48

2.1 材料与方法 50

2.2 结果 52

2.3 讨论 55

第三节　哈维氏弧菌（*Vibrio harveyi*）TS-628 菌株抗原性研究.....58
3.1 材料与方法.....59
3.2 结果.....62
3.3 讨论.....64
第三章　哈维氏弧菌 DNA 疫苗的构建及其免疫保护性研究....68
第一节　哈维氏弧菌鞭毛丝蛋白 *Fla*A 基因的克隆及真核表达载体的构建.....68
1.1 实验材料.....70
1.2 实验方法.....73
1.3 结果.....79
1.4 讨论.....87
第二节　pc*Fla*A 在青石斑中的表达及免疫保护性的初步研究.....89
2.1 实验材料.....91
2.2 实验方法.....92
2.3 结果.....98
2.4 讨论.....104
参考文献.....111
在学期间发表的论文.....127
致　　谢.....128

CONTENTS

Abbreviation ..... **I**

Chinese abstract ..... **III**

English abstract ..... **VI**

Chapter 1 Review **1**

Section 1 Research progress in vibriosis of fish **1**

1.1 The pathogenic vibrios of fish **1**

1.2 Fish vibriosis **9**

Section 2 Research progress in DNA vaccinea of fish **16**

2.1 General situation of DNA vaccine 16

2.2 Immune mechanism of DNA vaccine 18

2.3 Advances in research of DNA vaccines of fish 25

Chapter 2 Studies on the pathogenic bacteria of ulcer diseasein cultured *E. awoara* **36**

Section 1 Isolation and identification of the pathogenic bacterium **36**

1.1 Materials and methods 37

1.2 Results 39

1.3 Discussion 45

Section 2 Studies on the protease activities of the ECP of the pathogenic bacterium V. harvey strain TS-628 ..... **48**

2.1 Materials and methods 50

2.2 Results 52

2.3 Discussion 55

Section 3 Studies on the antigenicity of V. harveyi TS-628 ..... **58**

3.1 Materials and methods 59

3.2 Results 62
3.3 Discussion.....64
Chapter 3 Construction and immunoprotection of DNA vaccine of *V.harveyi* · 68
Section 1 Cloning of FlaA Gene of Polar Flagellin of V. harveyi and construction of eukaryotic expression plasmid.....68
1.1 Materials 70
1.2 Methods 73
1.3 Results 79
1.4 Discussion 87
Section 2 Studies on the expression and immunoprotection of pcFlaA in E. awoara.....89
2.1 Materials 91
2.2 Methods 92
2.3 Results.....98
2.4 Discussion 104
References 111
Publication 127
Acknowledgements.....128

主要英文缩写词表

英文词	英文全称	中文译名
ADV	Adenovirus	腺病毒
APC	Antigen-presenting cell	抗原提呈细胞
BSA	Bovine serum albumin	牛血清白蛋白
CAT	Chloramphenicol acetyltransferanse	氯霉素乙酰转移酶
CD4	An antigenic marker of helper /inducer T cells	辅助性/诱导性 T 细胞抗原标志
CD8	An antigenic marker of cytocoxic /suppressor T ells	细胞毒性/抑制性 T 细胞抗原标志
CMV	Cytomegalovirus	巨细胞病毒
CTL	Cytotoxic T lymphocyte	细胞毒性 T 细胞
ELISA	Enzyme-linked immunosorbent Assay	酶联免疫吸咐试验
ECP	Extracellular products	胞外产物
HBV	Hepatitis B virus	乙型肝炎病毒
HIV	Human immunodeficiency virus	人类免疫缺陷病毒
IFN	Interferon	干扰素
IHNV	Infectious hematopoietic necrosis virus	传染性造血器官坏死病毒
IL2	Interleukin-2	白细胞介素 2
ISS	Immunostimulatory sequences	免疫刺激序列

LPS	Lipopolysaccharide	脂多糖
Luc	Luciferase	荧光素酶
MCS	Multiplecloning site	多克隆位点
MHC	Major histocompatibility complex	主要组织相容性复合体
MTT	Thiazolyl blue	噻唑蓝
NK	Natural killer cells	自然杀伤细胞
OMP	Outer membrane protein	外膜蛋白
RSV	Respiratory syncytial virus	劳氏瘤病毒
SDS-PAGE	Sodium dodecyl sulfate-poly-acrylamide gel elextrophoresis	十二烷基磺酸纳--聚丙稀酰胺凝胶电泳
SI	Stimulation indices	刺激指数
SHRV	Snakehead rhabdovirus	蛇头棒状病毒
SV40	Simian virus	猴空泡病毒
Th	Helper T cell	辅助性 T 细胞
VHSV	Viral haemorrhagic septicemia virus	病毒性出血败血症病毒

摘　要

弧菌病是海水鱼类养殖业中造成经济损失最严重的细菌性疾病，本论文致力于养殖石斑鱼弧菌病病原菌及 DNA 免疫防治的研究，主要结果如下：

1、调查研究了 2002 年夏季厦门同安湾网箱养殖石斑鱼大面积暴发的溃疡病，对细菌病原进行分离纯化，编号为 TS-628，回归感染证实这株菌是引发同安湾网箱养殖石斑鱼溃疡病的病原菌。采用生化鉴定的方法对病原菌进行初步鉴定，同时采用 PCR 技术获得病原菌 16S rRNA 基因一段长 1121bp 的序列，该序列登录基因库（序列号为 AY747308）。结合生化鉴定结果和 16S rRNA 基因同源性比较结果，确认分离到的病原菌为哈维氏弧菌(*Vibrio harveyi*)。42 种药物的药敏实验结果表明病原菌对氯霉素、壮观霉素、磷霉素、利福平、环丙沙星等 16 种抗生素敏感，对万古霉素、青霉素 G、林可霉素、苯唑青霉素、氨苄青霉素等 16 种抗生素不敏感。

2、采用复性电泳技术，研究环境 pH、温度及培养时间对病原菌 TS-628 胞外产物(ECP)蛋白酶活性的影响，结果发现胞外产物蛋白酶最适反应 pH 在 8.5 左右,酸对 ECP 蛋白酶活性的抑制明显强于碱对它的抑制作用；最适反应温度在 20~50℃之间,20℃以下或 50℃以上蛋白酶活性则受到明显抑制，表明蛋白质酶不仅对高温敏感，对低温也很敏感；12h 培养的胞外产物蛋白酶活性最强。最适温度和 pH 条件下,主要出现 3 种蛋白酶,其中分子量为 94kDa 和分子量为 26kDa 的 2 种蛋白显示了很强的蛋白酶活性,而且能在比较极端的温度和pH值条件下保持活性,而分子量为35kDa蛋白酶只在最适反应条件下出现，并且该蛋白酶的最适反应条件与弧菌病暴发时的环境条件极为相似。由此可推测分子量 94kDa 和分子量为 26kDa 的 2 种蛋白可能是弧菌维持生存所必需，而分子量为 35kDa 的蛋白酶虽然只

出现在最适条件下,却可能在病原菌的致病过程中起重要作用。

3、分别提取病原菌 TS-628 菌株的鞭毛蛋白、外膜蛋白(OMP)和脂多糖(LPS)，应用 Western blot 技术分析检测了这几种主要表面抗原的抗原性。结果发现鞭毛蛋白主要的免疫印迹带约有 6 条，大致分子量分别为 16 kDa、20 kDa、35 kDa、38 kDa、43 kDa、52 kDa，其中 43 kDa、52 kDa 免疫印迹反应最强 ；OMP 主要的免疫印迹带约有 7 条，大致的分子量分别为 16 kDa、20 kDa、35 kDa、38 kDa、43 kDa、47 kDa、52 kDa，其中 43 kDa 免疫印迹反应最强。可见，43kDa 和 52kDa 的鞭毛蛋白以及 43kDa 的外膜蛋白具有较强的抗原特异性，可作为哈维氏弧菌疫苗理想的候选成分。而 LPS 没有检测到免疫印迹反应。

4、采用 PCR 的方法从哈维氏弧菌 TS-628 株基因组中，扩增出鞭毛丝蛋白 *Fla*A 基因。将该基因克隆到 T 载体后测序，经序列分析该基因全长 1140bp，编码 379 个氨基酸，该基因编码的多肽在 N-、C-两端的氨基酸序列更为保守，中间区域的变异较大，没有发现半胱氨酸，酪氨酸和组氨酸的含量也非常低，推测该蛋白质的平均分子量为 40.6kDa。BLAST 程序检索表明该基因与其他细菌鞭毛丝蛋白 *Fla*A 基因具有高度同源性，序列登录基因库，序列号为 AY956422。在哈维氏弧菌鞭毛丝蛋白 *Fla*A 基因末端加上一段编码 Flag 短肽的核苷酸序列后克隆到真核表达载体 pcDNA3.1(+)，酶切、PCR 及测序证实基因片段插入正确，将该重组质粒命名为 pc*Fla*A。

5、将 pc*Fla*A 以肌肉注射方式免疫青石斑(*E. awoara*)，实验组注射重组质粒 pc*Fla*A，对照组 I 注射空载体质粒 pcDNA3.1，对照组 II 注射无菌生理盐水。PCR 技术在 DNA 水平上检测质粒的转染，RT-PCR 法在 mRNA 水平上检测转染质粒在鱼肌肉中的表达，免疫组化染色技术在蛋白质水平上检测目的蛋白的表达。结果证实 pc*Fla*A 可以转染鱼类肌肉细胞并可在

其中进行表达，而且质粒在鱼体内持续表达的时间至少一个月。为检测 pc*Fla*A 质粒免疫青石斑的免疫效果，免疫后第一周到第四周分别取样品鱼的头肾淋巴细胞，在体外用抗原刺激，测定淋巴细胞的增殖反应，结果对照组 II 的刺激指数比较稳定，而且明显低于实验组和对照组 I，而实验组和对照组 I 的刺激指数有较大的波动，实验组淋巴细胞增殖的平均 SI 值略微高于对照组 I，但并不明显，表明经 pc*Fla*A 和 pcDNA3.1 空载体免疫的青石斑淋巴细胞增殖能力都有所增强，但 pc*Fla*A 并未表现出明显强于 pcDNA3.1 的免疫效应，可能是 pcDNA3.1 骨架的 CpG motif 在免疫过程中发挥了较为重要的作用。ELISA 技术重复多次实验，但没有在实验鱼的血清中检测到特异性抗体。攻毒感染试验中对照组 II 的死亡率为 100%，而实验组鱼的死亡率仅为 35.3%。经统计学检验也进一步证实，实验组鱼的抗感染能力明显高于对照组 II。对照组 I 在攻毒感染试验中虽然也表现出 26.67%的保护率，但经统计学检验与对照组 II 差异不显著，与实验组在 5%水平上差异不显著，但在 10%水平上差异显著。总之，以上实验结果表明 pc*Fla*A 可转染鱼的肌肉细胞并在其中获得表达，表达时间至少 1 个月以上，并且 pc*Fla*A 对青石斑具有一定的免疫保护作用，它的免疫保护很可能是表达的目的蛋白的抗原效应及质粒中非甲基化的 CpG 免疫刺激序列的佐剂效应两者共同作用的结果。

关键词：哈维氏弧菌（*V. harveyi*）；DNA 疫苗；青石斑鱼(*E. awoara*)

Abstract

Vibriosis is the most serious bacterial disease that economically affects marine fish culture. In this thesis, an attempt had been made to study the pathogenic bacteria of vibriosis in cultured grouper and the feasibility of DNA vaccine technology as a prophylactic treatment to prevent the vibriosis.

The results were shown as follows:

1、The characteristics of ulcer disease occurred in cage-cultured *Epinephelus awoara* in Tongan Bay of Xiamen, China during summer of 2002 were documented. The dominant bacterium, designated TS-628, was isolated from diseased grouper and an artificial challenge confirmed that TS-628 was the pathogenic bacterium. Automatic bacterial identification system and biochemical methods were used to do a primary identification to the isolate. Also a 1121bp 16S rRNA gene sequence of the isolate was amplified by PCR, which was accessed into GenBank (accession number: AY747308). According to the biochemical characteristics and comparing 16S rRNA gene homology of the isolate, the pathogenic bacterium was identified as *Vibrio harveyi*. Drug sensitivity tests showed that this pathogenic bacterium was sensitive to 16 antibacterials, especially to chloramphenicol and actinospectacin, but completely resistant to antibacterials like vancomycin, penicillin, lincomycin, and so on.

2、The SDS-gelatin-PAGE assay was used to study the effects of growing time, temperatures, and pH on the protease activities of the ECP of the pathogenic bacterium TS-628. The results showed that the optimal pH for ECP protease activity was about 8.5 and the protease activity was more easily inhibited by low pH than by high pH. The optimal temperature for the protease was 20~50℃.The significant decrease in activity observed at temperatures

below 20℃ or above 50℃ indicated that this protease was sensitive to both low and high temperatures. The highest level of the protease activity was present after 12h growing. Under the condition of optimal temperature and pH, there were three kinds of protease present: 94kDa, 26kDa and 35kDa. The 94kDa and 26kDa proteases displayed high activity and could remain active under extreme temperatures and pH, while the 35kDa protease presented only under the optimal condition. These suggested that the 94kDa and 26kDa proteases may be necessary in maintaining the vibrio survival and the 35kDa protease could play an important role in the pathogenicity of the bacteria.

3、The flagellin, outer membrane proteins (OMP), and lipopolysaccharide (LPS) of *V. harveyi* TS-628 were extracted. Western blot analysis was used to detect the antigenicity of these extractions. The results of the Western blot assay revealed 6 positive flagellin bands about 16 kDa、20 kDa、35 kDa、38 kDa、43 kDa、52 kDa，of which the 43kDa and 52 kDa bands displayed the strongest reaction. There were 7 positive OMP bands about 16 kDa、20 kDa、35 kDa、38 kDa、43 kDa、47 kDa、52 kDa , of which the 43kDa appeared the strongest reaction. However the LPS was Western blot-negative. These results indicated that the 43kDa and 52 kDa flagellin and the 43 kDa OMP could be the candidates to develope vaccines against *V.harveyi*.

4、The *Fla*A gene of *V. harveyi* TS-628 encoding the flagellin A was amplified by PCR and inserted into pMD-18 T vector. After sequencing and analyzing, the *Fla*A gene was found to contain 1140bp, which encodes 379 amino acids that contains conserved amino- and carboxy-terminal regions when compared to other flagellins, with the center region being more variable. Like previously reported flagellins, it lacked cysteine residues and had low amount of histidine and tyrosine residues. Based on DNA-deduced amino acid sequence, the predicted molecular mass of the protein was 40.6kDa. The *Fla*A

gene of *V.harveyi* has a high degree of homology with other *Fla*A genes compared by Blast homological search via GenBank and was accessed into GenBand(accession number: AY956422). The *Fla*A genes of *V. harveyi,* with a short nucleotide sequence encoding Flag tag, was cloned to eukaryotic expression vector pcDNA3.1(+). The cloning gene was correctly inserted into the vector pcDNA3.1(+) when confirmed by restriction endonuclease digestion, PCR amplification and sequencing. The recombined plasmid was designated pc*Fla*A.

5、Seventy-five fish were separated into three groups. The experimental group was immunized with pc*Fla*A. Control I was immunized with empty plasmid pcDNA3.1 and Control II was immunized with physiological saline. All the fish were immunized by intramuscular injection. PCR was performed to identify if the fish muscle cells were transfected with pc*Fla*A. The expression of pc*Fla*A was analyzed by RT-PCR at the level of mRNA and by immunohistochemistry at the level of protein. The results demonstrated that pc*Fla*A could transfect the fish muscle and keep expressing in the cells at least for one month. To evaluate cell-mediated immune response after pc*Fla*A vaccination, MTT method was used to measure the kidney cells proliferation *in vitro* of immunized fish in response to antigen stimulation. The results showed that the stimulation indices(SI) of Control II were more stable but significantly lower than the other two groups, while the stimulation indices of experimental group and Control I were variable. The SI of experimental group was a little bit higher than that of Control I, which indicated that the CpG motif in pcDNA3.1 played an important role in this DNA immunization. Antibodies were not detected in sera of vaccinated fish regardless that enzyme-linked immunosorbent assay(ELISA) was repeated several times. The results of bacteria challenge showed that the mortality of Control II was 100% while the experimental group was only 35.3%.A x^2 test also indicated that

experimental group conferred significant protection against *V. harveyi* infection when compared with Control II. Although Control I displayed 26.67% protective efficacy, it was not significantly different from Control II and was only significantly different from experimental group by 10%. In summary, pc*Fla*A could transfect the fish muscle and keep expressing in the cells at least for one month. Furthermore, pc*Fla*A showed protective efficacy in the challenge tests, which may come from the combined effects of expression protein and the unmethylated CpG immunostimulatory sequences.

Key words: *Vibrio harveyi*; DNA vaccine; *Epinephelus awoara*