Universidade do Algarve

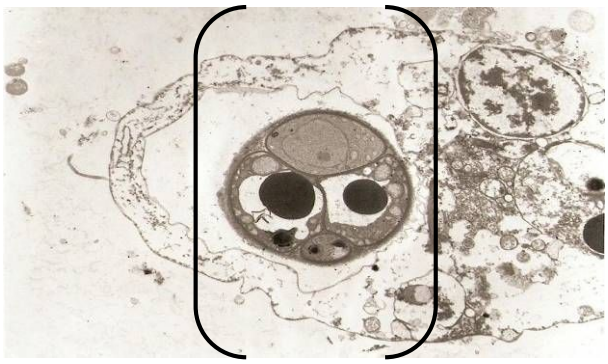
Faculdade de Ciências do Mar e do Ambiente

*

Study of *Perkinsus olseni* infection mechanisms: Identification and regulation of parasite genes differentially expressed in response to host and environmental stress

*

Thesis for obtaining the degree of doctor in Biology, speciality of Molecular Biology



Rita Margarida Teixeira Ascenso

Supervisor: Doctor Maria Leonor Quintais Cancela da Fonseca
Co-supervisor: Doutor Gerardo Vasta

Juri
President of the Juri: Doctor Rui Santos
Vogals: Doctor Idilio Jorge Matias Pereira Pinto
Dr. Domitília da Conceição Coutinha Matias
Doctor João Carlos Serafim Varela
Doctor Miguel João Baptista Gaspar
Doctor Dina Cristina Fernandes Rodrigues da Costa Simes
Doctor Maria Leonor Quintais Cancela da Fonseca

Faro
(2008)

The figure in the cover was adapted from a photography kindly provided by Professor Carlos Azevedo.

Universidade do Algarve

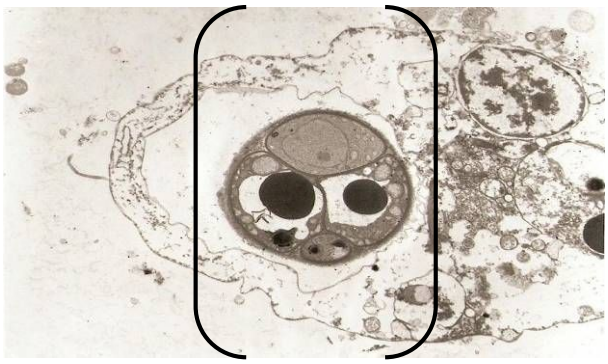
Faculdade de Ciências do Mar e do Ambiente

*

Study of *Perkinsus olseni* infection mechanisms: Identification and regulation of parasite genes differentially expressed in response to host and environmental stress

*

Thesis for obtaining the degree of doctor in Biology, speciality of Molecular Biology



Rita Margarida Teixeira Ascenso

Supervisor: Doctor Maria Leonor Quintais Cancela da Fonseca
Co-supervisor: Doutor Gerardo Vasta

Juri
President of the Juri: Doctor Rui Santos
Vogals: Doctor Idilio Jorge Matias Pereira Pinto
Dr. Domitília da Conceição Coutinha Matias
Doctor João Carlos Serafim Varela
Doctor Miguel João Baptista Gaspar
Doctor Dina Cristina Fernandes Rodrigues da Costa Simes
Doctor Maria Leonor Quintais Cancela da Fonseca

Faro
(2008)

Dedicated to
all those are part of my life, in my heart, in my memory,
by my side.

Dedicado a
todos os que fazem parte da minha vida, no meu coração, na minha memória,
a meu lado.

NAME: Rita Margarida Teixeira Ascenso

FACULTY: Faculdade de Ciências do Mar e Ambiente

SUPERVISOR: Doctor Maria Leonor Cancela, Universidade do Algarve

CO-SUPERVISOR: Doctor Gerardo Vasta, Center of Marine Biotechnology, USA

DATE: 7th November, 2008

THESIS TITLE: Study of *Perkinsus olseni* infection mechanisms: Identification and regulation of parasite genes differentially expressed in response to host and environmental stress

Abstract

Host-parasite interaction is very common in nature. *Perkinsus olseni* was described as the parasite of carpet-shell clam, *Ruditapes decussatus*, responsible for heavy mortalities that occurred in the 80ies, in the Southern coast of Portugal, Ria Formosa. The objective of this thesis was to contribute to better understand the molecular mechanisms of infection of *P. olseni* through the identification of parasite host-response genes differentially expressed in the presence of host hemolymph. From two SSH approaches, 300 new *P.olseni* ESTs were identified, from which 98 were selected for further studies. They were shown to present a pattern of response when the parasite was exposed to hemolymph of permissive and non-permissive bivalves. Two genes apparently relevant for parasite host-response were selected for further molecular characterization and gene expression studies, namely those encoding a putative ion transporter (PoNHE) and an adhesion-related protein (PoAdh). These genes showed an inversion in their normal pattern of response when the parasite was challenged with non-permissive compared to permissive bivalves and the permissive host, this one economically relevant in Southern coast of Portugal. Their levels of expression upon environmental stress conditions were also analysed. The EST most represented in the library was also objective of study. It revealed a close relation to a pyrimidine- salvage pathway protein (PoClhl). Lastly, efforts were also made towards defining a strategy to develop a methodology for efficient transfection of *P. olseni* cells.

Key-words

Parasite, *Perkinsus*, host-parasite interaction, SSH, gene expression

NOME: Rita Margarida Teixeira Ascenso
FACULDADE: Faculdade de Ciências do Mar e Ambiente
ORIENTADORA: Doutora Maria Leonor Cancela, Universidade do Algarve
CO-ORIENTADOR: Doutor Gerardo Vasta, Center of Marine Biotechnology, USA
DATA: 7 de Novembro de 2008
TÍTULO DA TESE: Estudo dos mecanismos de infecção do parasita *P. olseni*: Identificação de genes e expressão genética diferencial em resposta ao hospedeiro e condições de stress ambiental

Resumo

A interação parasita-hospedeiro é muito comum na natureza. *Perkinsus olseni* foi descrito como o parasita responsável pelas mortalidades da amêijoa-boa, *Ruditapes decussatus,* que ocorreram na costa do Sul de Portugal, Ria Formosa, nos anos 80. O objectivo desta tese foi contribuir para uma melhor compreensão dos mecanismos moleculares envolvidos no processo de infecção de *P. olseni*, através da identificação de genes diferencialmente expressos pelo parasita na presença de hemolinfa do hospedeiro. Foram utilizadas duas estratégias com base na técnica de SSH. Trezentos novos ESTs foram identificados, dos quais foram selecionados 98 para estudos posteriores. Estes apresentaram um padrão de resposta variável quando o parasita foi exposto a hemolinfa de bivalves susceptíveis e não-susceptiveis à perkinsosis. Dois genes aparentemente relevantes para a resposta do parasita ao hospedeiro foram selecionados para caraterização molecular e estudos de expressão genética. A saber, um que codifica para um possível transportador de iões (PoNHE) e outro que poderá estar relacionado com adesão celular (PoAdh). Esses genes mostraram um padrão de resposta que se inverte aquando da exposição do parasita a bivalves não-susceptiveis comparando com os susceptíveis, nomeadamente o hospedeiro, o qual tem interesse económico na costa do Sul de Portugal. Os seus níveis da expressão genética foram analisados em diferentes condições de stress ambiental. O EST mais representado na biblioteca subtractiva foi também objecto de caracterização e estudo. Este gene revelou algumas características que indicam uma possível função no metabolismo *salvage* das pirimidinas. Por fim, foram feitos esforços no sentido de desenvolver um método de transferência de ADN para o interior das células de *P. olseni*.

Palavras-chave

Parasita, *Perkinsus*, interacção parasita-hospedeiro, SSH, expressão genética

Aims of the Present Investigation

(i) To understand the molecular process involved in parasite response to host:

- Identification of parasite up-regulated genes involved in host response;
- Selection of genes with predicted functions related to host- parasite interaction;
- Investigation of parasite host-response up-regulated genes expression profile in response to commercially relevant bivalves of Ria Formosa.

(ii) To comprehend the patterns of expression of specific parasite genes in response to different types of bivalves and stressing environmental conditions:

- Molecular characterization of selected parasite genes highly expressed in response to it's' host;
- Analysis of gene expression patterns of selected parasite genes in response to permissive and non-permissive bivalves;
- Study of pattern of gene expression when parasite was exposed to simulated stressing environmental conditions.

(iii) To perform application of a parasite gene in an exogenous system:

- Molecular characterization of the most represented EST among the parasite up-regulated genes in response to its host;
- Characterization of a gene involved in organo-nitrogen compound catabolism;
- Expression of a parasite gene in an exogenous system.

(iv) To pursue the development of a methodology to transfer genes into *P. olseni* cells:

- Compilation of diverse strategies for parasite transfection;
- Testing of various methodologies for gene transfer;
- Construction of a genus specific transfection vector as a tool for further transfection assays.

Listo of Communications, Publications and Submitted sequences

Oral Communications in Meetings

R. M. T. Ascenso, M. Leonor Cancela and Gerardo R. Vasta, Study of the infection mechanisms of the parasite *Perkinsus atlanticus* when interacting with its host, the clam *Ruditapes decussatus*: Identification and regulation of *Perkinsus atlanticus* genes differentially expressed in response to host and environmental Stress, Universidade do Algarve, PhD students annual seminar, 21 February, 2005

R. M. T. Ascenso, M. Leonor Cancela and Gerardo R. Vasta, Study of the mechanism of the parasite *Perkinsus atlanticus* when interacting with its host, the clam *Ruditapes decussatus* – Molecular characterization of *Perkinsus* atlanticus response to its host *Ruditapes decussatus* - Seminários Mar e Ambiente, Universidade do Algarve, Faculdade de Ciências do Mar e Ambiente, 18-21 May, 2006

R. M. T. Ascenso, R. B. Leite, R. Afonso, M. L. Cancela, Does *P. olseni* parasite respond differently to diverse bivalve challenge?**,** WOPER Workshop for the Analysis of the Impact of *Perkinsosis* to the European Shellfish Industry, Vigo, 12-14 Sepetember 2007

Poster Presentation

R. M. T. Ascenso, R. B. Leite and M. L. Cancela, *Perkinsus atlanticus* Host-Response Expressed Genes – A FUNCTIONAL APROACH –Vila Moura, ALgarve, Biochemistry Congress, 2-4 December, 2004

R. M. T. Ascenso and M. L. Cancela, Characterisation and regulation of *Perkinsus atlanticus* genes differentially expressed in response to host and environmental stress. University of Algarve, PhD students' annual seminar, 18 June 2004

L. M. Elandalloussi, R. B. Leite, R. M. Ascenso, R. M. Afonso, and M. L. Cancela, Identification of differentially expressed genes in *Perkinsus atlanticus* exposed to pyrimethamine. Center for Marine Sciences-CCMAR, University of Algarve, Campus de Gambelas, 8005-139-Faro, Nice, France, 2004

R. M. T. Ascenso, R. Leite and M. L. Cancela, Cloning of the marine parasite suicide gene cytosine deaminase: a promising tool for negative selection. Second International Congress on Stress responses in Biology and Medicine, Tomar, 24-28September, 2005

R. M. T Ascenso and M. L. Cancela, *Perkinsus olseni* cytosine deaminase: expression vector construction for negative selection application, IX Congresso Ibérico de Parasitologia, Universidade de Coimbra, 25-28 October, 2005

R. B. Leite, L. Elandalloussi, R. Afonso, P. Rodrigues, R. Ascenso, M. L. Cancela *"An Overview Of The Metabolic Pathways In The Protozoan Parasite Perkinsus Atlanticus/olseni: Identification Of Potential Targets For Therapeutic Drugs."* IX Congresso Ibérico of Parasitologia, Coimbra, 25-28 October

R. M.T. Ascenso, R. M. Afonso, R. B. Leite and M. L. Cancela, Identification of differentially expressed target genes involved in *Perkinsus*-host interaction: comparison of two alternative SSH methods, Genomic Perspectives to Host Pathogen Interactions, CSHL meeting, Hinxton, UK, 7-10 September, 2006

R. M. T. Ascenso, R. B. Leite, R. M. Afonso and M. L. Cancela, Does *Perkinsus olseni* express pathogenic factors?, XV National Congress of Biochemistry, Aveiro, 8-10 December 2006

R. B. Leite, L. Fonseca, R. Afonso, M. Simão, R. M. T. Ascenso and M. L. Cancela, Clam Lectins: Species-specific patterns of expression upon *Perkinsus* exposure and Evolutionary considerations, MGE – final General Assembly, Faro, 13-16 May 2008

Articles in international refereed journals

R. M. T. Ascenso, R. B. Leite, R. M. Afonso and M. L. Cancela, 2007, Suppression-subtractive hybridization: A rapid and inexpensive detection methodology for up-regulated *Perkinsus olseni* genes, AJBR, 1, 24-28.

R. M. T. Ascenso and M. L. Cancela, 2007, Dissecting approaches for gene discovery – Using a mollusc parasite as example, Experimental Pathology and Health sciences, Research, Clinics, Teaching and Society, 1 (1) pp 44- 45.

Accepted for Publication in the Journal of Fish Disease: R. M. T. Ascenso, R. B. Leite, R. Afonso, M. L. Cancela, Expression pattern of Perkinsus olseni genes in response to bivalves with different susceptibility to Perkinsosis

In revision for Publication in the Experimental Parasitology: R. M. T. Ascenso, R. B. Leite, R. Afonso, M. L. Cancela, Molecular characterization of two novel *Perkinsus olseni* genes encoding putative Sodium Hydrogen Exchanger and Adhesion-related proteins.

Conferences Papers

R. M. Ascenso e M. L. Cancela, 2005, *Perkinsus olseni* cytosine deaminase: expression vector construction for negative selection application, Acta Parasitológica Portuguesa, 12 (1-2) p384.

R. M. T. Ascenso and M. L. Cancela, 2007, Dissecting approaches for gene discovery – Using a mollusc parasite as example, Experimental Pathology and Health Sciences, Research, Clinics, Teaching and Society, 1 (1) pp 44- 45.

António dos Anjos, Hamid Shahbazkia, Rita M. T. Ascenso, 2008, Automatic Macroarray Analysis - Tool and Methods, 21st International Conference on Computer Applications in Industry and Engineering, International Society for Computers and Their Applications (ISCA), March 23-25, 2006, Washington, USA

Publication in Book

Abstract "Does *Perkinsus* olseni parasite respond differently to diverse bivalve challenge? R.M.T. Ascenso, R.B. Leite, R. Afonso, M.L. Cancela at Villalba, A. (2008). Workshop for the analysis of the impact of Perkinsosis to the European Shellfish Industry. Vigo, Centro de Investigacións Mariñas, Conselleria de Pescas e Asuntos Marítimos da Xunta de Galicia; Centro Tecnológico del Mar - Fundación CETMAR.

Submissions to Nucleotide Sequence Databases

The *P. olseni* ESTs isolated from host hemolymph treated parasite, *P. olseni* forward cDNA subtractive library were submitted at DNA Data Bank of Japan (DDBJ) using Mass Submission System (MSS) with consecutive accession numbers from BB999049 to BB999146. The PoNHE and PoAdh were molecularly characterized in this thesis and were submitted to DDBJ/EMBL/GenBank nucleotide sequence databases and the nucleotide sequence data reported in this thesis will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB439284 and AB439285, respectively. PoUbq was publicized with the accession number DQ291152.

Acknowledgments

Each and every person owes itself from the background, family… that gave me life. To FAMILY I owe so many uncountable trust, faith, encouragement… not even all good word in this or any other world could describe how much you, Mum and Dad, Grandmother Maria and Grandfather José, Grandmother Agostinha, Brother, Paula and Gui contributed for me, myself and I. My lovely beautiful nice Margarida (Gui) was born… she is the purpose of frequent visits home, Alcogulhe de Cima, Maceira, where I receive encouragement all the time. I also show appreciation to my friends and colleagues from, Maceira, Leiria and Coimbra...

A person has so many aspects, from child to adult… I remember too little from childhood, but… the story does starts there.

The privilege of living in a small village, Alcogulhe de Cima and the close contact with familiar agriculture made me more sensitive to life… but the last straw that led me to Biology was the staying in Angola, a country with a huge diversity, poorly divulgated or known. At 8 years old, 15 days in family far away, in a place that looked virgin, allowed me to observe crabs, green crabs, only cooked turn out to be red!!!, fish and huge trees. I could have seen marine turtle if it were nowadays… at that time, there was an enthusiasm of observation that came up to me…

The studying trail was just starting in elementary school with a single teacher, Maria Arlete. Eugénia Cunha showed me the Natural Sciences when I was ten and I loved the classes and the subject above all. Ana Margarida, later introduce the human biology field and it was then that I decided to be a Natural Sciences and Biology teacher. By the way you never answered my question, but I found the way (thanks any way) and many other teachers, even Maths, Jorge Francisco and the actual school director, Jorge Bajouco contributed to my growth in knowledge. Next step seemed

bigger than my legs, even though I followed the Sciences closely. Earth and Life Sciences was the favourite discipline among all the others, from the just arrived new learning/ teaching system. Thanks Cândida. Biology Laboratory classes were an obligatory choice for me with Idalina Gameiro teaching. I must acknowledge also English teacher, Júlia Guarda Ribeiro, the continuation of English practice and learning with such a rigorous teaching. The high School studies were very intense and social life started to show the relevance I never felt before. Bárbara tried hard to show me, but now I recognise how helpful it would have been… later I got there with other Friends help, thanks to Sofia Pinto, Vanessa Santos, Inês Henriques and João Carvalheiro. These were the solid rocks supporting me to live away from family during graduation and they still do. From graduation in Biology I acknowledge all Professors and Researchers and Assistants in University of Coimbra who contributed with lectures, practical classes and seminars and Colleagues of those days and nights of study and… you all now what I mean. The possibility of studying in Coimbra gave me an open mind and called my attention to investigation. There was an increasing interest but the teaching took more relevance at that time. Thank you all at Escola Secundária Quinta das Flores, mainly to Isabel, the supervisor, Catarina, Vanessa and "my" students for the construction of myself as a Teacher. I still needed some development… we always fell this… don't you agree? I touch one more year at Colégio Senhor dos Milagres being thankful to all those that were there to teach and learn. Despite my move into research, I will keep feeling that each student is still mine and each of us can learn from each other.

But I had not only this interest… research became a stronger will and the first goal was helping in a novel project in Iberian Peninsula, a bivalves' hatchery. Since the project did not continue, I did: pure research in a PhD project. In this step I must acknowledge the support and enthusiastic push from you, Doctor Professor Leonor

Cancela. Like you always say, from Teaching to Research it takes a hard work… and we must be completely involved. I must THANK you. I acknowledge specially and greatly the group team (*Perkinsus* Group) that received me, heading with Ricardo Leite, Ricardo Afonso, Pedro Rodrigues, Patrícia Nunes. The order of the names is not chaos. Then followed other members that came… and went… and stayed: Joana Patrícia, Anabela, Márcio… and all *Perkinsus* fellows. Aside I thank you all from Molecular Biology Laboratory (the 2.13 people) citing this way twice the *Perkinsus* group in recognition for the friendship and shared knowledge; Carla, Marta Valente, Sandra, Catarina, Vera, Asunción, Natércia, Gavaia, Vincent, Vanesa, Nuno, António, Sara, Sofia, Brigite, Cátia, Marta, Nelson, Vânia, Daniel Tiago, Daniel, Bruno, Rui, Susana, Rita, Cindy, Michael, Joana, Paulo, Rui, João, Luis e Celine, THANKS. I must thank also the colleagues from second floor corridors, from all FCMA, namely the 2.28 and 2.51 laboratories people and other places of University of Algarve.

In scientific contexts I ACKNOWLEDGE for the third times to Doctor Professor Leonor Cancela the main foundation of knowledge and best example of research, but also to Doctor Professor Gerardo Vasta and PhD José António F. Robledo. Also thank to Doctor Professor Dina Simes, Doctor Professor Paulo Martel, Doctor Professor Ester Serrão, Doctor Vincent Laizé and Nuno Henriques for some practical and theoretical knowledge. I express gratitude to JAF Robledo and Keiko for the welcome I had in USA and my living place landlords, Lisa, Toshie and you also JAF. I am very thankful to all colleagues of COMB, namely from the laboratory Erick Odom, Theresa, Wolf, Erick , Hafiz, Ahmed, Satoshi and Nona, and also, for the second time, I thank Doctor Professor Gerardo Vasta, José António and Keiko. I show my appreciation to Nagla, Naomi and Frederico for their friendship. Far from home I felt intensely all new feelings and experiences… I thank you all the sharing of happiness, sadness and “saudade”

moments, including princess, Toshie's cat that waited for me to open my eyes every morning at 5am. JAF, you know how much you contributed for the good times I spend away from my family and my surroundings… even speaking a bit of Portuguese some times… and eating once cod, salted cod.

In Algarve, away from my hometown I started enjoying driving my car, through Ribatejo, Alentejo and Algarve. I show appreciation to those that were by my side at living places in Gambelas, Paulo Silva, Luis Gonzaga, José António, Eduardo Carrano, Adriano Benvenho, Susana Pereira, Nelson Coelho, Ana Gomes e Paula Silva, Margarida, Pedro e José António Bentes. I am thankful for the possibility of continuing some proximity with students in collaborative projects between the Center of Marine Sciences and the Centro de Ciência Viva do Algarve, like "Os cientistas vão às Escolas". A special thank to Vasco for friendship and living lessons and ride home and also Amadeu for all the support. Aside, so many other friends I met that I am thankful, Marta Valente Bernardo, Sofia Cavaco and their families … and so many others for support. Other colleagues that shared walking, riding bicycle and horse, watching movies, dancing, kayaking, kite surfing, geo-caching, eating lunch and dinner and conversing with me, I am grateful and sorry for the short time I spent doing all this things with you. Thank you all around me.

In this fraction of this thesis, I tried to show my gratitude to all that have contributed and still contribute to the development of what I am as an individual, a professional and a researcher. There would be so many others to thank and so many other circumstances to be reminded but there would be no words capable of it and I am not able to do it also in writing, but I can feel it!

The work plan depicted below is inserted into the mixed fellowship (SFRH/BD/11130/2002) funded by for Science and Technology Foundation (FCT) and was done as a collaboration between the two laboratories involved, through training and exchange of research expertise and results, supervised by Doctor Professor M. Leonor Cancela and Co-supervised by Doctor Professor Gerardo Vasta, to whom I am grateful. I also had the possibility of visiting the Plant Genetic Engineering laboratory, so I am thankful for the availability of facilities at ITQB and the knowledge of Doctor Margarida Oliveira and Sónia Negrão.

This work was supported in part by grants from FCT PERKLAM (PDCTM/M/Pmar/15308/1999), P-TARGET (POCI/CVT/57982/2004) and IsoPerk (PTDC/CVT/72083/2006) including funding from POCI 2010 and UE funds from FEDER and from DG-Pescas, Ambiperk (MARE/0092/2005/AT), and was performed as a collaborative study between the Center of Marine Sciences (CCMAR) - University of Algarve, Portugal and the Center of Marine Biotechnology (C.O.M.B.) - University of Maryland, Baltimore, U.S.A.. Some work has taken advantage of preliminary data analysis obtained from The Institute for Genomic Research through the website at http://www.tigr.org, and from The *Perkinsus marinus* Genome Sequencing Project accomplished with the support of the National Science Foundation (NSF).

I am so truly thankful to you, you all that liked me and took care of me, thanks.

Table of Contents

List of Figures

List of Tables

List of abbreviations

aa	Amino acids
ATCC	American Type Culture Collection
BLAST	Basic local alignment search tool

BLASTN	Nucleotide-nucleotide BLAST
BLASTP	Protein-protein BLAST
BLASTX	Translated query vs. protein database BLAST
BSA	Bovine serum albumin
CBB	Comassie brilliant blue
CD	Cytosine deaminase
CDD	Conserved domain database
cDNA	Complementary DNA
CDS	Coding sequence
Clhl	Chlorohydrolase-like protein
AtrzA	Atrazine chlorohydrolase
TrzA	N-ethylameline hydrolase
TrzA	Triazine hydrolase
EC	Enzyme commission
5-FC	5- fluorocytosine
5-FU	5- fluorouracil
CEIT	2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine
CAAT	2-chloro-4,6-diamino-1,3,5-*s*-triazine
cDNA	complementar deoxyribonucleotide acid
DME	Dublecco's modified eagle medium
Hams F12	Hams F12, mutrient mixture
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
FBS	Fetal bovine serum
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMBOSS	The European Molecular Biology Open Software Suite
EST	Expressed sequence tag
Et/Br	Ethidium bromide
FTM	Fluid thioglycollate medium
H_2O_2	Hydrogen peroxide
HAM	Ham's nutrient mixture F12
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl 1-thio-D-galactopyranoside
LDS	Lithium dodecyl sulphate
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-tetrazolium, inner salt
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline (20 mM phosphate buffer, 150 mM NaCl, pH 7.2)
PCR	Polymerase chain reaction
Pm	*Perkinsus marinus*
PMS	Phenazine methosulfate
Po	*Perkinsus olseni*
RACE-PCR	Rapid amplification of cDNA ends by polymerase chain reaction
SDS	Sodium dodecyl sulfate
SSH	Suppression Subtractive Library

TIGR	The Institute for Genomic Research
TNT	Transcription and translation system
U	Unit
Ubq	Polyubiquitin
UTR	Untranslated region

CHAPTER I – GENERAL INTRODUCTION

"Parasitism involves an intimate association between two different kinds of organisms – one, the host, providing food and shelter for the other, the parasite" by William Trager from John Guardiola, L. Luzzatto and William Trager (1983) Molecular Biology of Parasites, Raven Press

I-1 *Perkinsus*, a worldwide distributed genus

I-1.1 *Perkinsus* species and parasite hosts

Perkinsus (*P.*) is the genus of a protozoan parasite affecting molluscs in the five continents. Several species have been described to be responsible for disease in large number of molluscan species, primarily bivalves, which are ecologically, and often also commercially relevant. *P. marinus,* initially named *Dermocystidium marinum,* was first described as infecting the native eastern oyster *Crassostrea* (*C.*) *virginica* in Louisiana, although previous works indicated a wider distribution including the Gulf of Mexico (Mackin *et al*., 1950). According to the first species designation, the disease was called Dermo disease, in the specific case of USA. It was also named *Labyrinthomyxa marina* (Mackin and Ray, 1966). Further ultrastructure examination of zoospores led to a reclassification to *P. marinus* within the Family Perkinsidae, Class Perkinsasida, within the Apicomplexa protozoan parasites (Levine, 1978).

Another member of the genus, *P. olseni, was* described as infecting two abalone species (*Haliotis (H.) rubra* and *H. laevigata)* from Australia (Lester and Davis, 1981), and later *P. atlanticus* was identified in the clam *Ruditapes* (*R.*) *decussatus* in Southern Europe (Azevedo, 1989b). In 2002, *P. atlanticus* was synonymized to *P. olseni* (Murrell *et al*., 2002)

In 1991, a new species (*P. karlssoni*) was described in the east coast of Canada based on both morphological and epidemiological differences, where it was found to infect the introduced bay scallop *Agropecten irradians* (McGladdery *et al*., 1991). However, isolation and purification of both trophozoite and schizont stages of *P. karlssoni* (Whyte *et al*., 1993) remained limited; later, it was suggested that this was not a valid species because the prezoosporangias shape and structure and the movement and life span were not consistent with the genus *Perkinsus* (Goggin *et al*., 1996).

More recently, several additional species were described: (i) *P. qugwadi* infecting the Japanese scallop *Patinopecten yessoensis* cultured in the West coast of Canada (Blackbourn *et al*., 1998), (ii) *P. andrewsi,* found in the Atlantic coast of the USA in both *Macoma balthica* and *Mercenaria mercenaria* clams, as well as in the eastern oyster *C. virginica* (Coss *et al*., 2001a, Coss *et al*., 2001b, Kotob *et al*., 1999) and *P. chesapeaki* infecting the clam *Mya arenaria* harvested in the Chesapeake Bay (McLaughlin *et al*., 2000). In 2005, Burreson and collaborators proved that *P. andrewsi* and *P. chesapeaki* were indistinguishable and thus declared synonyms, prevailing the name *P. chesapeaki* according to the International Code of Zoological Nomenclature rules of priority (Burreson *et al*., 2005).

Recently, other *Perkinsus* species were described: *P. mediterraneous*, found in the Balearic Islands, infecting the European flat oyster *Ostrea edulis* (Casas *et al*., 2004), *P. honshuensis* infecting Manila clams, *Ruditapes* (*Tapes*, *Venerupis*) *philippinarum* (*semidecussatus*), collected in Gokasho Bay, Japan (Dungan and Reece, 2006) and lately *P. beihaiensis* (Moss *et al*., 2008). Altogether, there are seven valid species described by now: *P. marinus*, *P. olseni*, *P. qugwadi*, *P. chesapeaki*, *P. mediterraneous*, *P. honshuensis* and *P. beihaiensis*. Although the first description of each species was associated with a certain host, there has been since then an update of possible molluscs infected by *Perkinsus* species. *P. marinus* have been found not only in *C. virginica*, but also in *C. rhizophorae* (Bushek *et al*., 2002a). Oysters such as *C. gigas* and *C. ariakensis* (Calvo *et al*., 2001) and clams, *M. arenaria* and *M. balthica* (Dungan *et al*., 2007) were also susceptible to experimental infection by exposure to *P. marinus* and by mantle cavity inoculation of parasite cells, respectively.

Table I- 1 *Perkinsus* spp and hosts identified.

***Perkinsus* species**	**Type host**	**Type location**	**Other hosts**
P. marinus	*Crassostrea virginica*	East and Gulf Coasts of USA	*Crassostrea rhizophorae; C. ariakensis*
P. olseni/ Atlanticus	*Haliotis rubra, Ruditapes decusatus*	Australia, New Zeland, Korea, Japan, China, Portugal, Spain, Italy, Uruguay	*Anadara trapezia* *Austrovenus stutchburyi* *Chamus pacificus* *Haliotis laevigata* *Ruditapes philippinarum* *Pitar rostrata* *Protothaca jedoensis* *C. ariakensis* *C. hongkongensis* *Pinctata margaritifera* *P. martensii* *H. laevigata* *H. scalaris* *H. cyclobates* *Saccostrea cucullata*
P. qugwadi	*Patinopecten yessoensis*	British Columbia, Canada	
P. chesapeaki/ andrewsi	*Mya arenaria, Macoma balthica*	Chesapeake Bay, Delaware Bay, USA	*Crassostrea virginica* *Mya arenaria* *Macoma balthica* *Mercenaria mercenaria* *Tagelus plebeius* *Tridacna maxima* *Tridacna crocea*
P. mediterraneus	*Ostrea edulis*	Balearic Islands, Spain	
P. honshuensis	*R. philippinarum*	Gokasho Bay, Japan	
P. beihaiensis	*C. hongkongensis, C. Ariakensis*	Fujian to Guangxi, southern China	

(Adapted from Villalba *et al*., 2004, Villalba, 2008)

P. olseni from Australia was found to infect various molluscs in the wild, including abalones, *H. rubra, H. laevigata* (Lester and Davis, 1981), *H. scalaris, H. cyclobates*), and clams, *R. decussatus* (Da Ros and Canzonier, 1985), *R. philippinarum, Venerupis (V.) aurea, V. pullastra* (Villalba and Navas, 1988), *Tridacna (T.) crocea, T. maxima* and *T. gigas* (Goggin and Lester, 1987). Experimentally, it was found that it

could also infect oysters such as *Pinctada sugillata, P. maxima, C. rhizophorae* (Bushek *et al*., 2002a) and cockles, *Austrovenus stutchburyi, Macomona liliana, Barbatia novae-zelandiae, Katelysia rhytiphora and Anadara trapezia* (Goggin *et al*., 1989). *P. olseni* was found to infect also *Paphai undulata* (Leethochavalit *et al*., 2004) and *Pitar rostata* (Cremonte *et al*., 2005) and was identified in other molluscs, including *C. gigas* and *P. fucata martensii* (Choi and Park, 1997). *P. qugwadi* infects mainly the aberrant host *Patinopecten yessonesis* (Bower *et al*., 1998), being commonly designated as scallop protistan X (SPX), and is probably enzootic in other native scallops such as *Chlamys rubida* and *C. hastate, w*hich revealed to be resistant to infection. In contrast, *P. qugwadi* has strong pathogenic effects in British Columbia, where its infection of *Patinopecten yessonesis* is sporadically deathly causing up to 90% of losses in stocks (Bower *et al*., 1999). *P. chesapeaki,* initially described in Chesapeake Bay infecting *Mya arenaria,* is now known to also infect *M. balthica* and *M. mercenaria* (Coss *et al*., 2001a), *Tagelus Plebeius* (Bushek *et al*., 2007b) as well as *M. mercenaria, C. virginica, T. maxima* and *T. crocea*. Lately, *P. beihaiensis,* was described to parasitize the oysters *C. hongkongensis* and *C. ariakensis*, as well as other bivalves in Southern China (Moss *et al*., 2008).

The *Perkinsus* species that affects the largest number of mollusc species is *P. olseni* (Table I-1), being present in the Atlantic and Mediterranean coasts of Europe, in Southeast Asia and in South Australia, South America and New South Wales, according to recent data (Cremonte *et al*., 2005). In Ria Formosa, Southern coast of Portugal, *P. olseni* was shown to cause heavy mortalities to *R. decussatus* (Azevedo, 1989b, Ruano, 2001) and along the Iberian Atlantic coast *P. olseni* prevalence was observed in all sites sampled (Leite *et al*., 2004).

More recently, *P. atlanticus* was found to be synonymous of *P. olseni* as proposed by Murrell (Murrell *et al*., 2002)**,** and since the name *P. olseni* (Lester and Davis, 1981) has taxonomic priority, it prevailed.

I.1.2 Geographical distribution and environmental conditions of Perkinsosis

Perkinsus species affects molluscs all over the five continents, from temperate through subtropical to tropical regions of the Atlantic and Pacific oceans (Perkins, 1996). Perkinsosis may result in high mortality of some mollusc species, leading to huge economic and/or ecologic impacts. However, in some cases *Perkinsus* prevalence and intensities do not cause disease in certain host molluscs.

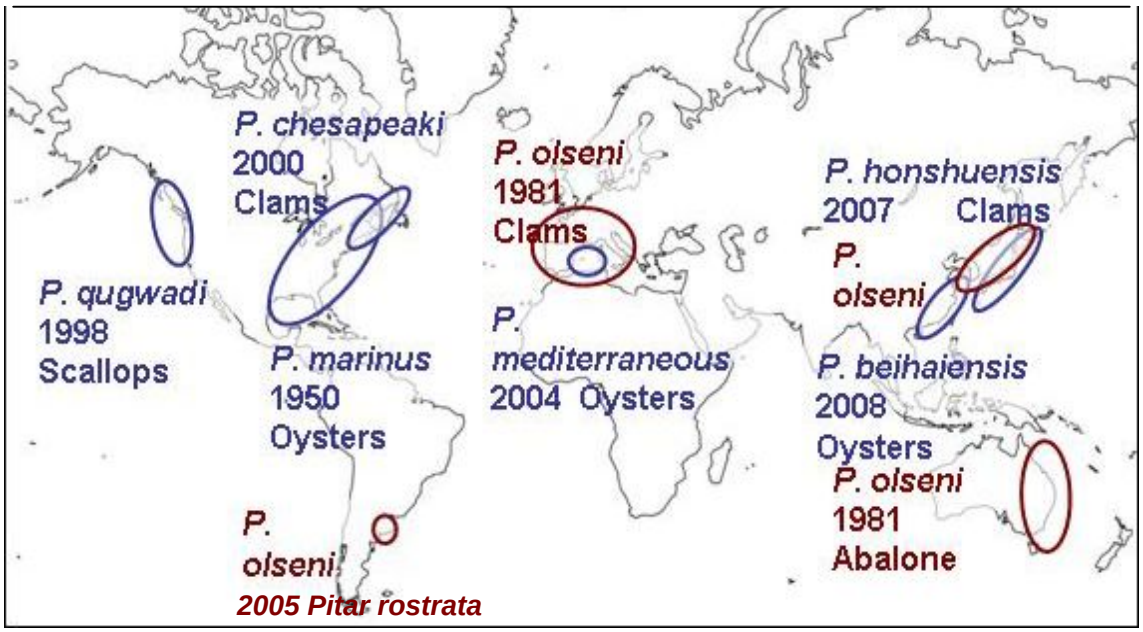


Figure I- 1 World distribution of the seven species of the genus *Perkinsus* with respective date and host as shown in the first description of the species, *P. olseni* marked in dark red and the other species in blue (figure constructed based on web world map, http://www.bristolstories.org/site_images/big_world _map.jpg).

The two species better characterized are *P. marinus* and *P. olseni*. *P. marinus* was described to be spread along the Atlantic coast of the United States, from Maine to Florida (Andrews and Ray, 1988), along the Gulf of Mexico coast to the Yucutan Peninsula (Burreson *et al*., 1994). The geographical distribution and abundance of *P.*

marinus are mainly controlled by temperature and salinity; proliferation and prevalence of this parasite increases dramatically during the summer when the temperature is over 20°C and salinity higher than 10‰ (Ragone and Burreson, 1993). Increases in sea-surface temperatures and repeated introductions over many years are probably responsible for its expansion into Delaware Bay, New Jersey and Cape Cod (Ford, 1996). Recently the northward spread of *P. marinus* associated with climate changes corroborated the Dermo disease distribution from the Gulf of Mexico to Delaware Bay and further north, into Maine (Brander, 2007). Studies on the role of temperature and salinity in the Dermo disease were pursued, namely by observing apoptosis. Results showed that higher salinity increased hemocyte apoptosis but had the opposite effect on parasites, which showed significantly higher rates of apoptosis when cultured in low salinities (17.1 ‰) at both low and high temperature tested (De Guise *et al*., 2005). Dermo disease was also associated with temperature, with *P. marinus* described to be the cause of oysters decimation at water temperatures above 25ºC (Fisher *et al*., 1992), whereas it rarely caused advanced disease in waters below 18ºC (Chu and Greene, 1989). Pollution has been also hypothesized to contribute to some aquatic epizootics (Chu and Hale, 1994) and estuarine contaminant mixtures have been shown to increase oyster susceptibility to parasitism by *P. marinus* (Bushek *et al*., 2007a).

P. olseni has been reported to infect a large number of mollusc species from Australian waters (Goggin and Lester, 1987) into the Pacific Ocean, South Korea (Choi and Park, 1997), Japan (Hamaguchi *et al*., 1998) and China (Liang *et al*., 2001). *P. olseni* affects different areas of the Atlantic and Mediterranean coasts of Portugal and Spain (Azevedo, 1989b) as well as several European countries, including France (Goggin, 1992), Italy (Da Ros and Canzonier, 1985) and Mediterranean coast of Northern Africa, Tunisia (Hili *et al*., 2007), Morocco (Belhsen *et al*., 2007) and also

South America. In Galician coast, *Perkinsus* lethality over infected *R. decussatus* was found to be much less than in Southern Portugal, thus being speculated that higher temperatures may impact on disease severity (Villalba and Casas, 2001). This was confirmed by a five year survey which revealed an annual pattern of *P. olseni* infection of *R. decussatus* clam beds influenced by temperature (Casas *et al*., 2002b). Later, a temporal dynamics study in Galicia corroborated the annual pattern of *P. olseni* infection of *R. decussatus*; lower infection intensity and prevalence in winter and higher values from spring to autumn, with 2 main annual peaks in spring and latter summer. The spring peak of infection intensity occurred when seawater temperature was around 15ºC (Villalba *et al*., 2005). Ria Formosa presents high values for water salinity and temperature and medium levels of pollution (Bebiano, 1995, Mudge *et al*., 2008), two conditions known to ease *Perkinsus* sp. infection. *P. olseni* was described as responsible for the high mortality rates in the native cultivated clam, *R. decussatus*, ranging from 50% to 80% per year, in the south of Portugal (Azevedo, 1989b). In France the prevalence of *P. olseni* remains low and does not lead to mortality in northern regions but mortality appears in the Mediterranean coast, where temperature is higher (Miossec *et al*., 2007).

Other environmental conditions have also been shown to contribute to major effects of Perkinsosis (Bushek and Allen, 1996). A 2 year survey of infection along the Iberian Atlantic and Mediterranean coasts, observed that increasing Perkinsosis intensity was associated with increasing man interference, with the higher values of infection encountered in Ria Arosa, Ria de Aveiro and Ria Formosa (Leite *et al*., 2004). In this same study, despite the lower temperature of Ria Arosa, there was a high prevalence and intensity of *P. olseni* compared to Ria Formosa. This peak of infection observed in winter 2002/2003 was associated to pollution caused by Prestige fuel oil

disaster which occurred at the end of Autumn 2002 (Leite *et al*., 2004). Thus, *Perkinsus* sp. prevalence, intensity and lethality is associated not only with higher temperatures and salinities but also to pollutants such as TBT and hydrocarbons (Anderson *et al*., 1996), which appear to intensify host mortalities.

The geographical distribution of *P. olseni* species was also correlated to different degrees of parasite pathogenicity, higher for *R. decussatus* and lower for *R. philippinarum*. In Japan, a *Perkinsus* sp. purified from clam *R. philippinarum* (Hamaguchi *et al*., 1998) showed to be intermediate between *P. olseni* from Australia, and *P. olseni/atlanticus* from Spain and Portugal, denoting their close relationship (Robledo *et al*., 1997). This facts suggested that the Manila clam or short-necked clam was its natural host, and *P. olseni* was introduced in Europe from the Pacific/Australasian/Southeast Asian region, where *R. philippinarum* is native (Cigarría *et al*., 1997). So, *P. olseni* presents a wide distribution throughout Southeast Asia to Europe (Robledo *et al*., 1997). The synonymy of *P. olseni* and *P. atlanticus* (Murrell *et al*., 2002) also supported the hypothesis that *P. olseni* was transported by movement of the clam host *R. philippinarum* from Asia to Europe (Hine and Thorne, 2000). In Europe, France, England, Spain and Italy soon introduced the exotic *R. philippinarum* because of its less susceptibility to disease (Cigarría *et al*., 1997). Despite the higher susceptibility of *R. decussatus* to Perkinsosis, in Ria Formosa, a Natural Park and clam bed situated in Southern Portugal, the cultivation of exotic introduced *R. philippinarum* is performed in very small scale, separate from native clam species.

Albeit the prevalence and intensity of *P. olseni*, *R. decussatus* produced in south Portugal constitutes 90% of Portuguese mollusc production (IPIMAR, 2003). Among several bivalve species caught, the carpet-shell clam *R. decussatus* represents by far the most important produced bivalve in Portugal, 38% (3,007 t) of total aquaculture

production (7,829t), compared to mussels (4%) and oyster (5%) (http://www.fao.org/fishery/countrysector/FI-CP_PT). Despite the decrease of production, in 2005, Portugal was the second biggest producer of carpet-shell clam in Europe (http://www.fao.org/, (Villalba, 2008). This production was deadly affected in the 80ies with heavy mortalities (Azevedo, 1989b) deeply affecting the economy of Ria Formosa producers. However, no other massive epizootic event was described since then. Perkinsosis is a wide spread and complex disease and parasite-host mollusc interactions are difficult to decipher, because they are related to environmental conditions which are in constant changing.

I.2 Taxonomic classification of *Perkinsus* genus

Perkinsus species (Levine, 1978), *Parvilucifera* spp. and *Cryptophagus* spp., constitute the phylum Perkinsozoa (Norén *et al*., 1999). According to Systema Naturae 2000, the genus *Perkinsus* belongs to Family Perkinsidae (Levine, 1978), Order Perkinsorida, Class Perkinsea, Infraphylum Protalveolata, Subphylum Dinozoa and Phylum Myzozoa (Cavalier-Smith, 1999), Infrakingdom Alveolata, Subkingdom Biciliata and Kingdom Protozoa of Eukaryota Domain. Over the years, the taxonomic placement of *Perkinsus* has been problematic, so other than morphologic studies were performed in order to clarify the phylogeny of these marine mollusc parasites. Phylogenetic analyses based on the RNA gene from the small ribosomal subunit and the actin gene indicated that the Perkinsasida may not belong to Phylum Apicomplexa (Levine, 1978), but be one of the earliest diverging groups of the lineage leading to Dinoflagellates (Goggin and Barker, 1993). Since Perkinsids share features with both Dinoflagellates and Apicomplexans, they are described as a taxon within the Alveolata group, with the phylum name of Perkinsozoa (Norén *et al*., 1999).

I.3 Parasite characterization, life-cycle and phylogeny

The two Perkinsus species of interest are the two most characterized, *P. marinus* and *P. olseni*, being included in the Aquatic Animal Health Code. *Perkinsus* sp. trophozoite was firstly characterized as a spherical spore, 2-4 µm with a very large eccentric vacuole and nucleus ordinarily oval observed in stained sections (Mackin *et al*., 1950). In 1976, Perkins updated the description of trophozoites structure as cells with a pair of centrioles in the invagination of nuclear envelope, a granular cell wall and tubulovesicular mitochondria in the cytoplasm, which mature to have the described signet-ring shape with 5-10 µm (Perkins, 1996). The life cycle of *Perkinsus* was established in two species *P. marinus* (Perkins and Menzel, 1966) and *P. olseni* (Lester and Davis, 1981). Four life stages, trophozoites, prezoosporangia, zoosporangia, and biflagellated zoospore have been identified and described. The two first phases are host tissue-associated, while the two latter are free in sea water and were defined on the basis of *in vitro* observations. Life cycle was firstly characterized as consisting of vegetative reproduction involving bipartition with alternating karyokinesis and cytokynesis. From one trophozoite, called aplanospore, by successive bipartition, multiple cells formed the sporangia, the schizont, from which each cell was released. Each cell matured to reach the signet-ring mature trophozoite shape described in 1987 by Perkins, having a large eccentric vacuole containing a vacuoplast and a peripheric nucleus with a central endosome (Perkins, 1987). These *P. marinus* trophozoite cells enlarge to about 15 to 20 µm, called prezoosporangia, and have been found in moribund oysters. Its release into water resulted in zoospore formation by bipartition. These biflagellated zoospores presented a conoid in the anterior part of the cell, rhoptries and micronemes, found in Apicomplexa, resembling the coccidian (Perkins, 1987).

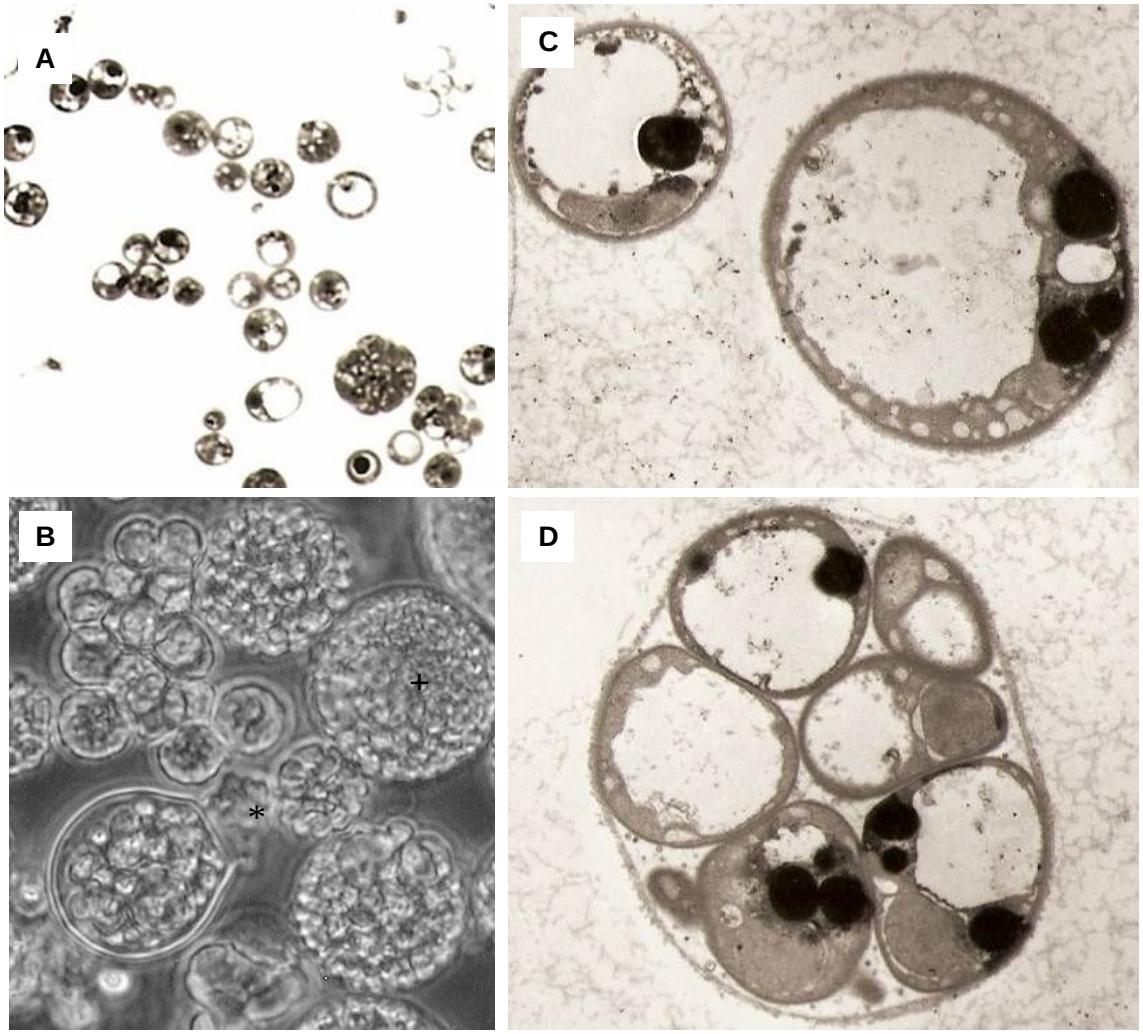


Figure I- 2 *P. olseni* life cycle stages. Optical microscopy: (A) Trophozoites and Schizonts life stages in *in vitro* cell culture (kindly provided by Professor Carlos Azevedo); (B) *In vitro* zoosporolation with Zoospores (*) and Prezoosporangia (+). Electron microscopy: Ultrastructure of Trophozoite, signet-ring (C) and Schizont (D), adapted from the electromicrographies kindly provided by Professor Carlos Azevedo.

In the early *P. olseni* description by Carlos Azevedo (1989) there were clear differences relative to *P. marinus* description concerning dimensions and structure of the zoospores and its flagella. There were no such details described by Lester (1981) for comparison with *P. olseni* (Lester and Davis, 1981) and thus the morphological characteristics were of taxonomic relevance at that time. *P. marinus* trophozoites presented the same general morphology although with 3-10 µm compared to *P. olseni* with 4.6- 16 µm (Robledo *et al*., 2002). Zoospores were described for both species as elliptic cells with two different flagella located laterally and having an apical complex

with a polar ring, rhoptries, micronemes, conoid and subpellicular microtubules (Perkins, 1987). The flagellated zoospores contained an apical organelle with similarities to the Apicomplexan leading to the conclusion that *Perkinsus* could represent an early lineage of the Apicomplexans (Levine, 1978). In 1997, Siddall described the 'conoid' structure as open along one side. This feature led to a reinterpretation of the significance of the structure for the taxonomy of the genus *Perkinsus* (Siddall *et al*., 1997) and made Perkinsids distinct from Apicomplexans (Leander and Keeling, 2003). The presence of two dissimilar flagella inserted ventrally, one with mastigonemes along one side of *P. marinus*, make this species closely related with Dinoflagellates (Perkins, 1996). Ultrastructural observation of cell division in *Perkinsus* also appeared to be Dinoflagellate-like because the nuclear envelope remains intact during mitosis, with the mitotic spindle running through continuous channels and attaching to kinetochore-like structures on the nuclear envelope (Perkins, 1996). In 1995 a second type of division, a schizogony-like division, was described for *P. marinus* (Peyre *et al*., 1995), leading to formation of schizonts. In addition to the morphological analysis, the molecular characterization of small-subunit (SSU) rRNA, actin, and α- and β-tubulin sequences allowed the definition of a key ancestral taxonomic position and defined the *Perkinsus* phylogeny in the divergence of Apicomplexans and Dinoflagellates (Saldarriaga *et al*., 2003).

The development of *in vitro* pure cultures for each of these parasite species provided an important tool to better characterize these parasites. *P. marinus* culture was successfully developed simultaneously in different laboratories (Gauthier and Vasta, 1993, Peyre *et al*., 1993) both derived from trophozoites isolated from its oyster host and later optimized (Gauthier and Vasta, 1995a, Peyre *et al*., 1995). For *P. olseni*, continuous in vitro cultures were also developed at the same time, both derived from

parasites infecting the carpet-shell clam, *R. decussatus* (Robledo *et al*., 2002, Casas *et al*., 2002a). Deposited in the American Type Cell Culture, both *P. marinus* (ATCC 50983) and *P. olseni* clonal cultures (ATCC 50984) of trophozoites are maintained independent of host and are important tools for *in vitro* studies and further characterization. Molecular characterization of *Perkinsus* spp. has been used to support *Perkinsus* species designations and to develop PCR-based assays specific to accurately assess *P. olseni* epizootics in clam populations around the world (Goggin, 1994). These tools have proven to be reliable to distinguish *Perkinsus* species from Europe, Australia, and the Atlantic coast of North America (Goggin, 1994). After *P. marinus* SSU sequence (Goggin, 1994), the ITS, SSU, and NTS rRNA sequences of a *P. olseni* isolate from Galicia, Spain (Robledo *et al*., 2000) were reported. To clarify the phylogeny of this genus the SSU rRNA, actin, α-tubulin and β-tubulin sequences were also characterized (Kuvardina *et al*., 2002). Molecular analyses of nuclear encoded genes pointed to a closer relationship of Perkinsozoa to Dinoflagellata (Reece *et al*., 1997, Saldarriaga *et al*., 2003, Saldarriaga *et al*., 2004).

In terms of molecular characterization, cDNA and genome cloning from *Perkinsus* spp. allowed the identification of several structural proteins such as actin (*P. marinus*, AAB62065; *P. olseni*, ABF61886; *P. chesapeaki*, AAX82887; *P. mediterraneus*, ABQ01414 and *P. honshuensis*, ABF61883), ribosomal proteins, *P. marinus* 40S (ABV22180) and *P. olseni* L38 (AAW55921) and proteins belonging to different metabolic pathways such as the *P. marinus* fatty acid metabolic pathway (delta 9-elongating activity protein (ABF58686), delta 5-desaturase (ABF58685), delta 8-desaturase (ABF58684) responsible for arachidonic acid biosynthesis (Venegas-Calerón *et al*., 2007) and acetyl CoA carboxylase (CAK51558) or the *P. olseni de novo* purine biosynthesis pathway (Leite *et al*., 2008, Leite *et al*., 2004), including the

phosphoribosylaninomidasole synthetase (AAT99016), phosphoribosylanino-midasole formyltransferase (AAT46683) and HIF prolyl hydrolase (ABS82069) (Leite *et al.*, 2008).

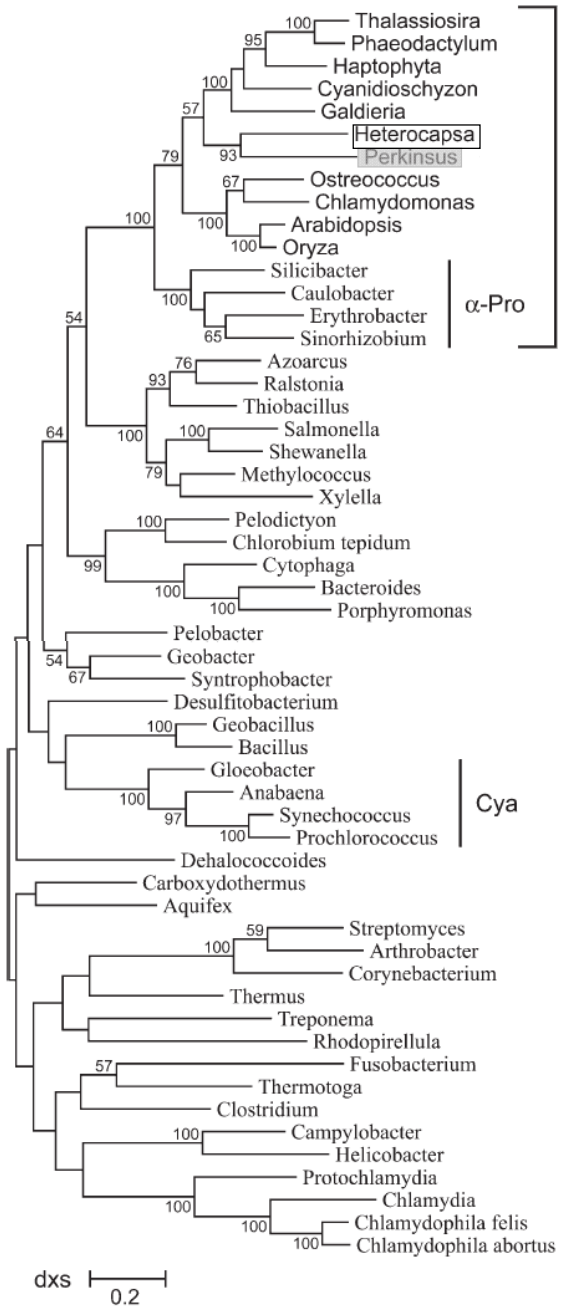


Figure I- 3 Phylogenetic analysis of *Perkinsus* based on dxs gene from MEP pathway. Unrooted ML tree constructed using Phyml with WAG substitution matrix, based on a matrix comprising 55 OTUs and 497 sites. *P. marinus* dxs ortholog marked with arrow forming a highly supported clade with the Dinoflagellate. The scale bar indicates the number of substitutions per site (adapted from Matsuzaki *et al.*, 2008).

The *P. marinus* genome project, although not yet fully sequenced nor annotated, has been a useful tool for molecular characterization studies. In May 2004 the first preliminary contigs were released from Celera Assembler assembly of 163,052 sequences with mean length of 861 nt (www.tirg.org, www.umbi.umd.edu/comb). The *P. marinus* karyotype has not been completely resolved, its size being estimated to be 28 Mb by CHEF gels, with the largest chromosomes size of 5-7 Mb and the lowest of 1-2 Mb (www.tigr.org). In *P. olseni,* nine chromosomes were identified by pulsed field gel electrophoresis (PFGE) combined with densitometry analysis. Eight distinct chromosomal DNA bands were obtained: 0.15, 1.5, 2.1, 2.9, 3.4, 4.0, 5.4 and 6.5 Mb and the whole genome size estimated in 28Mb (Teles-Grilo *et al*., 2007a). *P. olseni* karyotype revealed a chromosome organization like that of the early branches of Dinoflagellate lineage (Teles-Grilo *et al*., 2007a). Very recently the existence of a non-photosynthetic secondary cryptic plastid in *P. marinus* ensured the close relation to plastid-bearing Dinoflagellates (Figure I-3) and Apicomplexans (Matsuzaki *et al*., 2008). Teles-Grilo and collaborators identified plastids in *P. olseni* in a region of the apical end of zoospore by ultrastructural observations. This revealed also the existence of a four membrane structure that resembled the Apicomplexans more than the Dinoflagellates (Teles-Grilo *et al*., 2007a, Teles-Grilo *et al*., 2007b). The genomic, transcriptomic and proteomic of *P. olseni* and *P. marinus* are areas in rapid development, and the new data should contribute decisively to obtain a better understanding of these two *Perkinsus* species.

I.4 Host-parasite interactions

Parasitic protozoans of the genus *Perkinsus* are among the most important pathogens associated with disease outbreaks, both in cultivated and in natural mollusc

populations all around the world, and for which an increasing prevalence has been reported. In 1994, Kleinchuster and collaborators announced that the described *P. olseni* infection of the clam *R. decussatus* could be appropriate to develop a model system to study *Perkinsus* species/host interactions, because of this parasite ease of culturing through *in vitro* life cycle (Kleinschuster *et al*., 1994). The relevance of this genus was motivated by the massive mortalities described for the eastern oyster cultured in the Atlantic coast of USA (Mackin *et al*., 1950) and for the carpet-shell clams in Southern Portugal (Azevedo, 1989b), also because of the decrease in commercial value of abalones in Australia (Lester and Davis, 1981) Though, both ecological and economical impacts justified the careful characterization of these host-parasite systems.

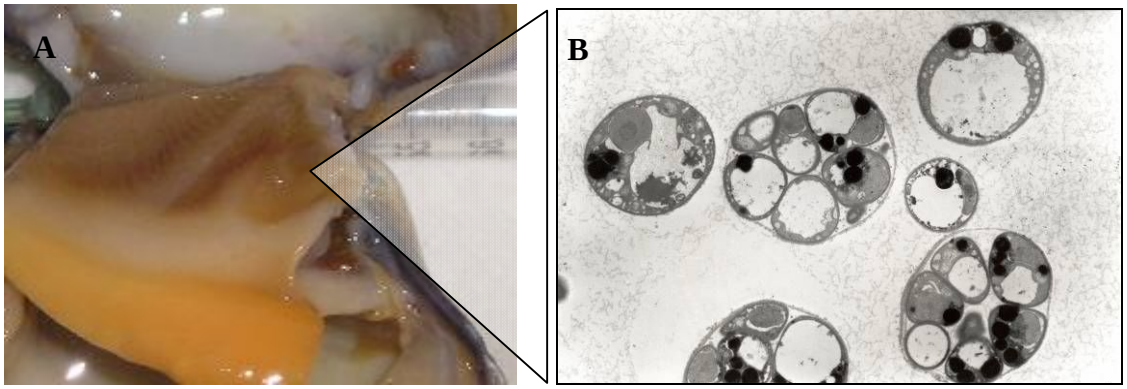


Figure I- 4 *R. decussatus* clam and *P. olseni* parasite: (A) carpet-shell clam gills macroscopic observation with white nodules of *P. olseni* pustules and (B) *P. olseni* cell culture cells observed in electron microscope (kindly provided by Professor Carlos Azevedo).

The natural interaction of this parasite with its type host was described for each species. *P. marinus* was often found intracellularly, phagocytosed by hemocytes (Mackin *et al*., 1950), being thus designated as intracellular, albeit facultative. *P. marinus* infections were described to progress rapidly in the hemolymph and other tissues because parasite cells are avidly phagocytosed by the hemocytes and are probably disseminated throughout the host via the circulation. Although hemocytes are

thought to be important microbicidal, they seem to have little ability to destroy *P. marinus*, contributing instead to spread the infection (Anderson *et al*., 1995).

P. olseni infection was observed in the foot, mantle tissues and gill of carpet-shell clams (Azevedo, 1989b) and in tissues of gill filaments in which several dense host cells surrounded the parasite cells originating structures that could reach 1 mm diameter, being macroscopically visible (Azevedo, 1989a). *P. olseni* infecting *Haliotis* spp. formed pustules in the foot and mantle, up to 8 mm, which diminished the commercial value of abalones in Australia (Goggin and Lester, 1995). At higher levels of infection, *Perkinsus* were found in all types of tissues, including adductor muscle and gonadal connective tissues (Choi *et al*., 1989). Morphological observation showed *P. olseni* cells aggregated and surrounded by lysed dense hemocytes of *R. decussatus* (Azevedo, 1989a) and *R. semidecussatus* (Sagristà *et al*., 1995). It was also found within the hemocytes and both intracellular and extracellular in hemolymph of *Saccostrea cucullata* (Hine and Thorne, 2000). This looked like the privileged way for *Perkinsus* spp. to invade the host, affecting them from mantle to gonad tissues. In the west and south coasts of Korea, clams exhibited pathologic symptoms such as digestive epithelium atrophy, hemocyte infiltration and tissue inflammation caused by *Perkinsus* heavy infection (Choi and Park, 1997). In Northern Mediterranean coast of Spain, examination of *R. philippinarum* revealed a co-infection by *P. olseni* and bacteria and/or viruses and these opportunistic pathogens were favoured by *P. olseni* infection (Montes *et al*., 2001). Many pathogenic microorganisms can enter eukaryotic host cells, like hemocytes, and use the normally hostile environment as a niche, evading the host immune response. Several studies have aimed to characterize the close interaction between the parasite and its host hemocytes/hemolymph. Microelectrode arrays and impedance techniques have been used to show the interaction between *R. decussatus*

hemocytes, the parasite *P. olseni* cells and its surroundings, identifying the cells, their adhesion, coverage, and even cell motility (Gomes *et al*., 2004).

Although much progress has been made in elucidating the immunological interactions and the molecular mechanisms involved in the process of immune evasion, relevant in human and animal parasites (Gupta, 2005), little has been done to characterize the system *P. olseni*-*R. decussatus*. The bivalve internal defence system consists of non-specific cellular and humoral components. *R. decussatus* possesses two main types of hemocytes, hyalinocytes and granulocytes, which are represented by three types, basophilic, acidophilic and mixture (López *et al*., 1997). The defensive response to *P. olseni* infection by *R. decussatus* and *R. semidecussatus* involves the redifferentiation of recruited granulocytes and the expression *de novo* of polypeptide p225, not observed when *R. decussatus* was exposed to inoculations of bacteria, algae and non-viable *P. olseni* prezoosporangia (Montes *et al*., 1997).

P. marinus is known to promote different cellular immune responses according to permissive and resistant oyster hosts. In addition, there was more phagocytosis in infected *C. virginica* than in infected *C. gigas* three days post-infection (Goedken and Guise, 2004). Another study investigating host response to parasite identified of *C. virginica* plasma protein p35, an anti-protease (Oliver *et al*., 1999). Also, analysis of oysters' *C. virginica* and *C. gigas* molecular response to *P. marinus* by suppression subtractive hybridization (SSH) has lead to the identification of 107 differentially expressed gene sequences for *C. virginica* and 69 for *C. gigas*. Of these, nineteen genes involved in immune system and cell communication, protein regulation and transcription, cell cycle, respiratory chain and cytoskeleton from *C. virginica* were characterized (Tanguy *et al*., 2004). Until now, only few studies have described the parasite molecular response when interacting with its host, identifying a *P. olseni* cell

wall protein (PWP-1) related to parasite protection (Montes *et al*., 2002) and subtilisin-like serine protease (Brown and Reece, 2003).

The host-parasite interaction and the establishment of pathogen infection depend upon the physiological status of the host and its defensive capabilities and the ability of the pathogen to avoid/evade host defences and acquire the essential nutrients for its development and growth. Food Salinity and temperature are environmental factors involved in regulating *P. marinus* infection and thus the spreading of disease among *C. virginica* populations (Hofmann *et al*., 1995). Intracellular pathogens must circumvent host defences, acquire nutrients and either kill or maintain the host cell according to their needs (Leirião *et al*., 2004). Nevertheless, the virulence factors and pathogenicity mechanisms of *Perkinsus spp.* are still poorly understood. Ford and collaborators observed that *P. marinus* parasites freshly isolated from infected *C. virginica* were much more virulent than those maintained in an *in vitro* culture (Ford *et al*., 2002). Supplementation of *P. marinus* cell culture with host plasma or tissue homogenate of eastern oyster altered both cell size and proliferation, and also increased infectivity (Earnhart *et al*., 2004). Among the several life stages of *P. marinus*, trophozoites have been shown to be the most efficient in initiating serious mortalities (Volety and Chu, 1995). It was also hypothesized the existence of a certain level of specificity in the recognition/endocytosis of *P. marinus* by its natural host species (Gauthier and Vasta, 2002).

Parasites can also represent sensitive biological indicators of various types of pollutants in both freshwater and marine ecosystems (MacKenzie *et al*., 1995) though host-parasite interaction is even more valuable. *R. decussatus* have been described as a bioindicator, in particular when mussels are not available, and several biomarkers were validated among which metallothioneins (MT), superoxide dismutase (SOD), catalase

(CAT), glutathione peroxidases (GPx), lipid peroxidation, glutathione S-transferase (GST) and acetylcholinesterase (AChE), measured in different tissues of the clam (Bebianno *et al*., 2004). Other relevant biomarkers, including ferritin, HSP70 and thioredoxin H, were analysed in parallel with metallothioneins when the clam *R. decussatus* was submitted to *P. olseni* infection. Interestingly, both ferritin and HSP70 were up-regulated when the carpet-shell clams were infected by *P. olseni* (Leite *et al*., 2007). Because the parasite determines the clam condition, analysis of the host-parasite interaction can provide relevant information, both ecologically and economically, especially in the lagoon system of Ria Formosa.

I.5 *P. olseni* affects carpet-shell clam in Ria Formosa

Ria Formosa is located in southern Portugal, extending for about 55 km of lagoon area defined by a true barrier island system, which covers a total area of 163 km^2, with 20 km^2 occupied by salty marshes and aquaculture ponds. The main water reservoir of the salty marshes and the extensive aquaculture ponds behaves like small lagoons with openings to a tidal channel (Gamito, 1997) with an average depth of 4m (Ruano, 1997). The sediment varies from sand/mud to coarse sand with water temperatures ranging from 10 to 25ºC and salinity from 33.8 to 38‰ (Ruano, 1997), being described as an important ecosystem acting as nursery and optimal habitat for bivalves, with carpet-shell clam being the most used species for culture (IPIMAR, 2003). In addition, other bivalves are economically relevant in the south coast of Portugal, including blue mussel, *Mytilus (M.) edulis*, Mediterranean mussel, *M. galloprovincialis*, Portuguese oyster, *C. angullata*, Japanese oyster, *C. gigas*, other clams such as *Venerupis (V.) pullastra* and *V. rhomboides*, cockle, *Cerastoderma edule*, and the wedge clam, *Donnax (D.) trunculus* (Ruano, 1997). In the summer of 1983,

heavy mortalities were described for the carpet-shell clams, and those substantial losses continued throughout the 80ies, when they were finally associated with *P. olseni* infection (Azevedo, 1989b).

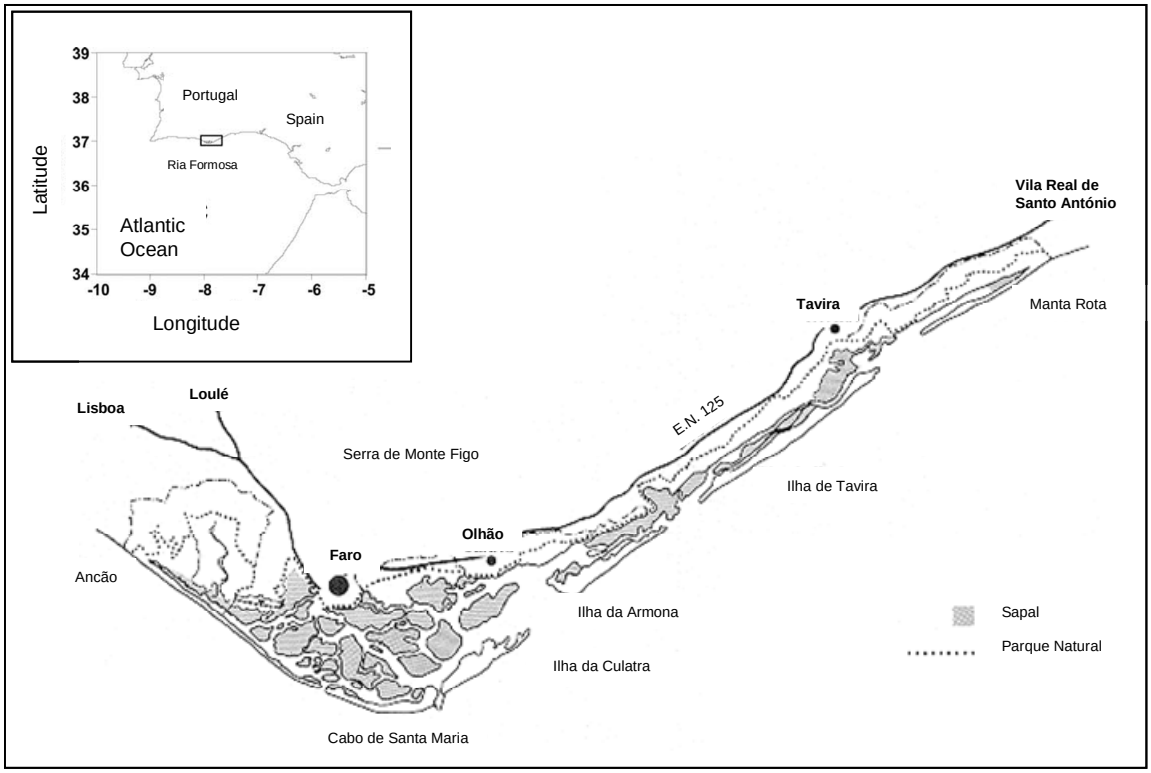


Figure I- 5 Ria Formosa Natural Park localization in South Portugal (adapted from www.olhao.web.pt/ ParqueNatural.htm).

The bivalves' collection has been important in this region ever since the Roman occupation, from 140 b.C. to 400 a.C. until th XX century, with a broad distribution in the Portuguese river estuaries and lagoon systems. Throughout time, there has been a reduction in the natural beds and in bivalves' diversity and the decline in bivalves' production observed in the XXth century can be related to overharvesting and habitat degradation (Ruano, 1997). In contrast to clam, production of oyster was more relevant in the past, during the XXth century, albeit the varied production of *R. decussatus,* as well as its economical and social importance, has always remained high thus the relevance of controlling Perkinsosis. This disease, caused by heavy infection of clams

with *P. olseni*, is an epidemic disease, and often infection intensity, as well as prevalence, is positively correlated with the density of host organisms (Da Ros and Canzonier, 1985). In the natural clam beds of Ria Formosa, farmers started preparing culture beds to seed small clams, 5-10 mm, collected from natural beds, before the development of hatcheries. These seeds grew in 1½ to 2 years to commercial size (Ruano, 1997). These clams, when transferred into closely areas, gave no problems, but the seed densities had to be maintained within a certain range, otherwise the epidemics would cause huge losses and expenses. As in aquaculture, because of overcrowding, severe parasitological problems frequently appear even in free waters, as *P. marinus*, *incerta sede* parasitic organisms of almost all commercial bivalves (Perkins, 1996). *Bonamia* spp., *Mikrocytos mackini*, and *Perkinsus* spp. transmit directly from one host to another (Berthe, 2001) requiring care in managing the host. Transfer and introduction of commercial species without a serious health control may support and spread parasitic diseases from different countries. This was a common practice when *R. philippinarum* was found out to be more resistant to plagues (Cigarría *et al*., 1997). In Portugal, other bivalves then *R. decussatus*, living in the south coast, can also be infected by *Perkinsus*, as was the case of *C. angulata* (Azevedo, 1989b), although not to the same extent nor the same virulence as *R. decussatus*. This host-parasite system remains poorly characterized for the particular case of Ria Formosa and thus constitutes an interesting research objective.

CHAPTER II – GENERAL MATERIALS AND METHODS

"Considering the complexity of host and environmental issues, study of the pathogen offer an attractive system to learn more about the biology of disease" by Karen Joy Shaw (2002) Pathogen Genomics Impact on Human Health, Humana Press

II-1 Eukaryotic and bacterial cell cultures

The clonal cell line from *P. olseni* used in this work was established from trophozoites isolated directly from the infected host *R. decussatus*, (ATCC 50984-ALG4; (Robledo *et al*., 2002) and resulted from a collaboration between the Molecular Biology Laboratory of University of Algarve and the Center of Marine Biotechnology of the University of Maryland (COMB). *P. olseni* cells were cultured in DME: Hams F12 (1:2) medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Pen/Strep) antibiotics (all Gibco, Grand Island, NY, USA), as described (Robledo *et al*., 2002). The *Perkinsus marinus* cell line TXsc (ATCC 50983) was supplied by Doctor Professor Gerardo Vasta from COMB and maintained in DME:Ham's F12 (1:2) with 5% FBS and antibiotics mix as described (Gauthier and Vasta, 1995a).The *Xenopus laevis* cell line A6 (derived from kidney epithelial cells, ATCC CCL102) was cultured at 22 °C in 60% L15 medium supplemented with 5% fetal bovine serum and 1% Pen/Strep antibiotics (all Gibco). These cells were selected as Eukaryote exogenous system because of its availability and transfectability.

E. coli DH5α competent cells (Invitrogen, Carlsbad, USA) were used for cloning, being cultured at 37ºC in Luria-Bertani (LB) medium (Sambrook *et al*., 1989). BL21 (DE3) pLysS cells (Strategene, La Jolla, CA) were used as prokaryotic system for protein expression. They were grown in LB media in the presence of antibiotic (ampicillin or chloramphenicol at 100 µg/ml). T7 polymerase was inducted by IPTG (Invitrogen).

II-2 Extraction and purification of nucleic acids

II-2.1 Total RNA extraction

Total RNA was extracted from log phase cell cultures, grown in standard medium. Since *Perkinsus* sp. cell culture presents a rosette multicellular morphology and individual throphozoites with a cell wall, there was a need to improve cell disruption. Cell lysis and homogenization were performed using a Glass/Teflon Potter Elvehjem homogenizers and the resulting lysate was passed through a 21G needle to increase cells disruption. Total RNA isolation was performed according to Chomczynski and Sachi method (Chomczynski and Sacchi, 1987) or using a commercial kit (RNeasy total RNA isolation Kit from Qiagen, Chatworth, USA), according to the manufacturer's procedure. Total RNA was quantified spectrophotometrically at A_{260nm}. Protein contamination was assessed by A_{260}/A_{280nm} ratio. RNA was used if ratio was between 1.7 and 2.0. RNA integrity was evaluated by the existence of clear and definable ribosomal RNA bands and the relative ratio of 18S to 28S, ensuring that RNA was not degraded, following a formaldehyde/agarose gel electrophoresis.

II-2.2 Poly$^+$A RNA purification

Messenger RNA (mRNA) was purified from total RNA isolated from *P. olseni* and *P. marinus* cells using the Oligotex mRNA Midi Kit (Qiagen), according to manufacturer's instructions. Quality of poly$^+$A RNA obtained was evaluated following denaturing gel electrophoresis and by amplification of the reference genes by PCR.

II-2.3 Genomic DNA isolation

DNeasy Tissue kit (Qiagen) was used for extraction of high molecular weight (HMW) genomic DNA through advanced silica-gel–membrane technology allowing a rapid and efficient purification following cell lysis through selective binding of DNA to the DNeasy membrane. *P. olseni* or *P. marinus* cells collected from a stationary growth phase were lysed using proteinase K and then loaded onto the DNeasy Mini spin column using tips with larger tip diameter to reduce DNA fragmentation. DNA was selectively bound to the DNeasy membrane and after two wash steps using brief centrifugations, the HMW DNA was eluted in TE buffer. Purified DNA was quantified by measuring A_{260nm} and purity analysed by the A_{260}/A_{280nm} ratio and electrophoresis in a low percentage agarose gel.

II-3 Molecular cloning and characterization of selected cDNAs and genes

The molecular characterization of a given gene comprises cloning and sequencing of its full length cDNA and gene, identification of its gene organization and prediction of the corresponding protein.

II-3.1 RACE-full length cDNA amplification

Rapid amplification of both 5' and 3' cDNA ends (RACE) from the same template was achieved using a Marathon cDNA library (Clontech, Palo Alto, USA), previously constructed and available at the Molecular Biology laboratory or a GeneRacer Kit (Invitrogen) constructed in the laboratory of the Centre of Marine Biotechnology. RACE amplification was selected based on our previous experience and excellent results obtained for cloning full-length cDNAs from differentially expressed ESTs (Expressed Sequence Tags) identified with the PCR Select™ cDNA Subtraction Kit (Clontech). For each 5'- and 3'-RACE reactions, 36 µl of sterile H_2O were placed in a 0.5ml tube to which were added 5µl of cDNA PCR Reaction Buffer (10x), 1 µl dNTP

Mix (10 µM ea), 1 µl Advantage 2 Polymerase Mix (50x) completing 43 µl final volume. Both 5' and 3' RACE PCR reactions were primed with an internal gene specific primer (GSP) and the Marathon Adaptor Primer (AP1). The amplification was performed in a Perkin Elmer GeneAmp System 2400 (Applied Biosystems, USA) using the following conditions: 30 sec at 94°C (denaturation step), followed by 5 cycles of 94°C for 5 sec and 72°C for 4 min, followed by 5 cycles of 94°C for 5 sec and 70°C for 4 min and finally 25 cycles of 94°C for 5 sec and 68°C for 4 min. Amplification results were analysed by gel electrophoresis using 5 µl from each PCR product along with appropriate DNA size markers, on a 1.2% agarose/EtBr gel. PCR products were gel band extracted and ligated into available cloning vectors.

II-3.2 *P. marinus* Gene Racer library construction

For *P. marinus*, the 5' and 3' UTR regions were amplified using the GeneRacer Kit (Invitrogen) methodology following the instructions. cDNAs were oligo dT-primed cDNAs that were synthesized with SuperScript II RT. This library follows the same steps and principles as described for Marathon cDNA library, after first and second cDNA strand synthesis, these were ligated to adaptors. The 5' and 3' cDNA ends of a gene were obtained using the GeneRacer primers included in the kit and GSPs designed specifically. RACE PCR products were cloned and sequenced.

II-3.3 Gene cloning using the Universal Genome Walker library

Library construction

The BD GenomeWalker™ Universal Kit (Clontech) was used because of our previous experience where it proved to be a powerful method for gene amplification from high molecular weight genomic DNA (HMW gDNA). The kit was used following

the manufacture's procedures. The first step consisted on the construction of pools of uncloned, adaptor-ligated genomic DNA fragments, which were obtained following genomic DNA (gDNA) digestions with two selected restriction enzymes: two libraries were constructed. For this, separated aliquots of 2.5 µg HMW gDNA were completely digested with either EcoRV or StuI, two restriction enzymes that leave blunt ends. Each batch of digested genomic DNA was ligated separately to the BD GenomeWalker Adaptor. Finally, 10x diluted libraries were stored at -20ºC for gene characterization by PCR-based DNA walking.

Upstream and downstream gene amplification

After construction of StuI and EcoRV libraries, two PCR amplifications per library were performed using the outer Adaptor Primer (AP1) provided in the kit and a pair of GSPs (forward and reverse, see Appendix II-1), each used separately to amplify both downstream and upstream, from the starting gene sequence, respectively. If needed, the primary PCR mixture was diluted and used as template for the nested PCR with the nested Adaptor Primer (AP2) and the same GSP or nested GSP. The resulting PCR bands of amplified DNA fragments were gel band extracted, cloned and sequenced.

II-3.4 cDNA template for amplification

The availability of a cDNA library was used for cDNA amplification purpose, but for regular amplifications for cDNA sequence confirmation, a reverse transcribed cDNA using the dT adaptor was chosen. The PCR reaction was always performed in a final volume of 25 µl, including 0.4 µM of each primer, 0.05 mM of each nucleotide and 1U Taq DNA Polymerase (Invitrogen). After an initial denaturation step (4 min at 94ºC), amplification was performed for 25 to 30 cycles (each cycle: 30 sec at 94ºC, 30

sec at oligonucleotide melting temperature, 30 sec at 72ºC) with a final elongation step at 72ºC with time adjusted according to the amplicon size. Amplification of complete ORFs, for example to prepare expression constructs, was always performed using Advantage cDNA Polymerase (Clontech) because of its proofreading properties and amplification of significantly longer fragments.

II-3.5 Genomic DNA as PCR template

To confirm gene sequences, genomic DNA was frequently used as template for PCR amplifications. These reactions were performed using 0.4 µM of each primer, 0.05 mM of each nucleotide and 2 U Taq DNA polymerase (Invitrogen). After an initial denaturation step (10 min at 95ºC), amplification was performed for 30 cycles (one cycle: 1 min at 95ºC, 1 min at 62ºC, 2 min at 72ºC) with a final elongation step of 10 min at 72ºC.

II-3.6 Ligation of DNA fragments

The cDNA fragments extracted from gel bands using GFX PCR DNA and Gel Band Purification Kit were ligated to the appropriate cloning vector, pGEM®-T Easy (Promega, Madison, USA) or pCR-II TOPO (Invitrogen), following manufacture's instructions. Taq DNA polymerase yields fragments with A (Adenine) overhangs facilitating the T/A cloning; when using Advantage cDNA Polymerase, and to improve ligation efficiency into a T/A cloning vector, 10 µl of purified PCR product was submitted to dATP addition by performing a 15 minutes PCR step with Taq DNA Polymerase (Invitrogen).

II-4 Cloning before sequencing

DH5α cells (Invitrogen) were transformed with the ligation product and then spread in agar plates together with 100 µl X-gal (20 mg/ml) and 5 µl IPTG (100 mg/ml). Individual transformants of DH5α carrying exogenous cDNA fragments (white

colonies), were grown in LB with ampicillin (100 µg/ml). Plasmid DNA was isolated as described (Sambrook *et al*., 1989) and positive clones confirmed by EcoRI digestion, before sequencing.

II-5 cDNA sequencing

Positive clones were sequenced to confirm their identity and obtain the DNA sequence for analysis. The sequencing was mainly performed at Macrogen (Macrogen, South Korea), although some sequencing was also performed at the Max Planck Institute of Berlin, within the MGE (Marine Genomics Europe) platform, and more recently at the Center of Marine Sciences (CCMar, University of Algarve, Faro) sequencing laboratory.

II-6 *In silico* sequence analysis

In order to deduce some of the characteristics that may help the molecular characterization of *Perkinsus* sp. genes and predicted the corresponding polypeptides, *in silico* analysis was performed; first sequences chromatograms were checked using available software Chromas lite 2.01 and further treatments were performed using *in silico* tools available at EXPASY (http://www.expasy.org). The online servers allowed analysis and predictions that permit construction of accurate and comprehensible representative schemes.

II-6.1 Sequence characterization

DNA sequences (either cDNA or gDNA) and predicted encoded polypeptides were, as a first step, cleaned of adaptors and vector sequences, and then submitted for homology search using the online databases, (EST, genome or proteins), namely at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). EST cDNA sequences from the cDNA forward subtractive library were submitted at BLASTx search (Basic Local Alignment Search Tool, (Altschul *et al*., 1997) to identify

homologies between the translated cDNA and sequences in the protein database. Gene sequence homologies were carried on by performing nucleotide searches both at NCBI and at TIGR, against the *P. marinus* genome sequences available (http://www.tigr.org/tdb/e2k1/pmg). Sometimes the BLASTx of genomic DNA sequences allowed the clarification of intron-exon frontiers. When possible, the predicted polypeptide sequences were compared by homology with the protein database through BLASTp search.

II-6.2 ORF prediction and cDNA structure

To predict the ORF (open reading frame), the full length cDNA was submitted to ORF Finder (http://www.ncbi.nlm.nih.gov) to predict the corresponding polypeptide. This was confirmed by comparison with other described proteins comparing with available databases (BLASTx). The final schematic drawing of the cDNA molecular structure, including 5' and 3' untranslated (UTR) regions and ORF was performed using the software for translation of a sequence for publication available through the computer system BIOINFO, developed by HKUCC (The University of Hong Kong Computer Centre), HKU-Pasteur Research Centre and Centre de Resources INFOBIOGEN (France), and skill sharing from Centre de Resources INFOBIOGEN (France), based on the BIOSUPPORT project (http://bioinfo.hku.hk).

II-6.3 Definition of gene structure

The gene sequence obtained through the previously described methods was compared with the cloned cDNA sequence in order to identify the corresponding gene structure. Finding 100% identity between these sequences allowed the identification of exons using the Align 2 sequence tool at National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov). Then the translation of a sequence for publication tool (http://bioinfo.hku.hk), previously mentioned, allowed the representation of the

gene structure with the identification of intron-exon frontiers, as well as the position, size and type of introns.

II-6.4 Promoter analysis

The promoter sequence was analysed for the presence of putative transcription factor binding sites using the MatInspector at Genomatix Server (http://www.genomatix.de).

II-6.5 Predicted polypeptide characterization

After the identification by homology search of the putative protein, diverse analyses were performed. Sometimes, the BLASTp search allows the recognition of conserved domains and polypeptide classification in the respective protein family (http://www.ncbi.nlm.nih.gov). With the vector NTI software, used as laboratory database, it was possible to infer the molecular weight, the isoelectric point and charge at pH 7. For certain polypeptides it was possible to predict the existence of possible post-translational modification (http://www.expasy.ch/tools/), namely the signal peptide (SignalP) as well as the hypothetical location of the predicted encoded proteins using ChloroP to predict chloroplast transit peptides, MITOPROT for mitochondrial targeting sequences and PATS for apicoplast targeted sequences. In the case of putative membrane proteins it was possible to estimate the presence of transmembrane helices (TMHMM) and compare it with the prediction of transmembrane regions and protein orientation (TMpred). All proteomic tools used are available at ExPASy web site (http://www.expasy.ch/tools/).

II-7 Gene expression analysis by northern blot, macroarray and Real-time qPCR

When parasite was exposed *in vitro* to various conditions the expression of particular genes of interest was assessed with cDNA macroarray technology, which can provide a rough measure of numerous genes expression profile. Results were confirmed

by using northern blotting and/or a more sensitive and more accurate gene expression measurement, the analysis by Real-time qPCR.

II-7.1 Northern blot analysis

Gene expression was initially evaluated by Northern Blot analysis (Sambrook *et al*., 1989). Five to ten µg of total RNA extracted from *P. olseni* cells to which different environmental stress simulated conditions were applied, were size fractionated by electrophoresis on a 1% agarose formaldehyde-containing gel and transferred onto a Hybond^{TM}-XL nylon membrane (Amersham Biosciences, UK) by a capillary method (Sambrook *et al*., 1989). The resulting blot was pre-hybridized at 42ºC in 10ml of ULTRAhyb buffer for 1h. The DNA probe was labelled with α^{32}P-dCTP using RediPrime^{TM} II Kit (Amersham Pharmacia Biotech) and column purified from unincorporated nucleotides with a MicroSpin^{TM} S.200 HR column (Amersham Pharmacia Biotech). The labelled probe was added to the prehybridization solution and incubated overnight with the membrane, under the same conditions described for prehybridization. Blots were then washed twice in 2xSSC (1xSSC:150mM NaCl, 15mM sodium citrate pH 7.0), 0.1% SDS at 42ºC for 5min and twice in 0.1xSSC, 0.1%SDS at 42ºC for 15min. Autoradiography was performed using Kodak BioMax MS film (Kodak, USA) at -80ºC. Two independent hybridizations were performed and at least three exposure times were developed per experiment.

II-7.2 Real-time qPCR expression quantification

Real-time qPCR was used to quantify expression of selected genes. Total RNA isolated from treated and control *P. olseni* cells were used for expression analysis using Real-time qPCR. First-strand synthesis was performed using 1µg of total RNA treated with RQ1 RNase-Free DNase (Promega), gene specific reverse oligonucleotides (Appendix II-1) and M-MLV reverse transcriptase. Real-time qPCR was performed in

an iCycler iQ system (Bio-Rad, Richmond, USA) using GSP primer sets (Appendix II-1) for the genes in study. Each reaction was prepared by adding 2 µl of a 1:10 cDNA dilution to reaction mix containing 0.5 µM of each primer and 10 µl of iQ SYBR green Supermix, in a final volume of 20 µl. After some experiments iQ SYBR green Supermix was substituted by ABsolute^{TM} Q PCR SYBR Green Flurescein mix (ABgene, Surrey, UK), but the reaction and amplification conditions were optimized to keep the same conditions. The qPCR program contained an initial cycle of 10 min at 95ºC and 60 cycles comprising an initial denaturation step at 95ºC for 30 sec, followed by annealing and extension at 68ºC for 20 sec. The fluorescence was measured at the end of each extension cycle in the FAM-490 channel. Relative levels of expression were determined by comparing results of treatments with results obtained with control cells. All experiments were performed at least twice, and with at least duplicate wells.

CHAPTER III – PARASITE HOST-RESPONSE

"During the past years, functions have been discovered for several genes that were previously of unknown function" by Karen Joy Shaw (2002) Pathogen Genomics Impact on Human Health, Humana Press

III-1 Introduction

The protozoan pathogen *Perkinsus atlanticus* (Azevedo, 1989b), recently considered synonymous of *P. olseni* (Murrell *et al*., 2002), is the causative agent of perkinsosis, a severe disease that affects the grooved carpet shell clam *Ruditapes (Tapes) decussatus* (Linnaeus, 1758) and can be emphasized under certain environmental conditions. Similarly, on the East Coast of the United States, *P. marinus*, a parasite from the same genus, is responsible for devastating the populations of oyster *C. virginica* (Burreson and Ragone Calvo, 1996) but the molecular mechanisms underlying the pathogenicity of *Perkinsus sp.* were described to be poorly understood (Anderson *et al.*, 1996). Lately several studies have been published aiming at further exploring these mechanisms. Biochemical analyses have shown the involvement of parasite-associated extra-cellular proteins (ECP), especially proteases, in the pathogenicity of *P. marinus* (Oliver *et al*., 1999), and more recently the *P. marinus* serine protease gene, correlated with virulence / pathogenicity was described (Brown and Reece, 2003), but much remains to be unveiled concerning the parasite molecular responses to its host.

P. marinus karyotype has been resolved and its genome size was estimated by CHEF gels indicating at least 28 Mb (http://www.tigr.org); this genome size value was recently confirmed for *P. olseni* (Teles-Grilo *et al*., 2007a) and the karyotype was determined (Teles-Grilo *et al*., 2007a). A preliminary sequence analysis of the *P. marinus* genome from Celera Assembler was obtained through a whole genome shotgun strategy using the strain (PmCV4CB5 2B3 D4) isolated from an infected oyster from Bennet Point (Maryland, USA) and was released in May 2004 (http://www.tigr.org/tdb/e2k1/pmg/). This release contained the first contigs derived from 163,052 sequences (mean length of 861 nt), and was not annotated. More recently,

large insert cDNA library EST were released (Robledo *et al*., unpublished) completing 31,696 EST available in the GeneBank (http://www.ncbi.nlm.nih.gov/). Nevertheless, to better comprehend the parasite answer to its host, the transcriptome must also be studied.

Several strategies are available for analysis of differential gene expression at the whole genome scale, each of them claiming specific advantages. However, and although less discussed, they also have intrinsic disadvantages. Differential display approaches (Liang *et al*., 1994) have been used for the less complex genomes but are also suitable for those showing higher levels of complexity, ranging from prokaryotes to eukaryotes, and including symbiontic interacting organisms such as *Azorhizobium caulinodans* and *Sesbania rostrata* (Lievens *et al*., 2001). Microarray techniques (Heller, 2002) have evolved lately, being a useful tool for differential gene expression and providing a large pool of information useful to better comprehend host-parasite interactions (Koltai and Weingarten-Baror, 2008). This technology is expensive and its use is frequently limited to the most standardized models, for which an annotated genome is already available. In contrast, the Suppression Subtractive Hybridization (SSH) method does not require previous knowledge of the genome but in this case highly efficient methods for gene identification are required to perform genome annotations and molecular characterization of microbiological systems not yet fully characterized, like in the unicellular mollusc parasite *P. olseni*. In SSH, subtraction and normalization are performed simultaneously (Mahalingam *et al*., 2003) thus equalizing abundance of target cDNAs in the subtracted population (Diatchenko *et al*., 1996). To decrease the number of background clones in the libraries generated by SSH, a Mirror Orientated Selection procedure (MOS) (Rebrikov *et al*., 2000) was developed although "false positive" clones are not fully eliminated due to simultaneous use of PCR amplification.

In the present work a cDNA forward subtraction library was constructed for transcriptome analysis of the parasite exposed to hemolymph from its natural host, the clam *R. decussatus*. Two different methodologies were used to uncover different non redundant contigs correspondent to parasite up-regulated genes. Gene ontology was used for characterization of the molecular function of the identified genes in order to compare results obtained by these two different SSH approaches. The results allowed identification of numerous and diverse parasite genes up-regulated in response to the presence of its host hemolymph.

III-2 Specific materials and methods

III-2.1 Differentially up-regulated genes (SSH)

Although there are several different methods, the basic theory behind subtraction hybridization is simple and a powerful technique for identification of cDNAs that refer to specific transcripts expressed in a given condition (differentially expressed). Briefly, this technique enables to compare two populations of mRNA and identify genes differentially expressed by the two populations. Tester and driver cDNAs are hybridized, and the hybrid sequences, corresponding to transcripts present in both conditions under comparison are removed due to linear amplification. Consequently, the remaining unhybridized cDNAs represent genes that were expressed in the tester, but are absent from the driver mRNA and thus were amplified exponentially. In this work, SSH was performed using the PCR Select™ cDNA Subtraction Kit (Clontech Laboratories Inc., Palo Alto, CA), following the protocol recommended by the company.

III-2.1.1 Parasite challenge

The *P. olseni* log phase cell culture, previously expanded and grown in 100 ml volumes at 28ºC, under shaking of 118 rpm for 2 days, was collected and centrifuged for 6 minutes at 2,000 rpm, to reduce medium volume and then treatments were applied. The parasite host-challenged condition was performed by incubating the parasite cells exposed to host hemolymph for six hours using transwells (Costar, Cambridge, MA), as exemplified in the scheme (Figure III-1). 100 ml *P. olseni* cell culture was submitted to the same procedure but in absence of host hemolymph.

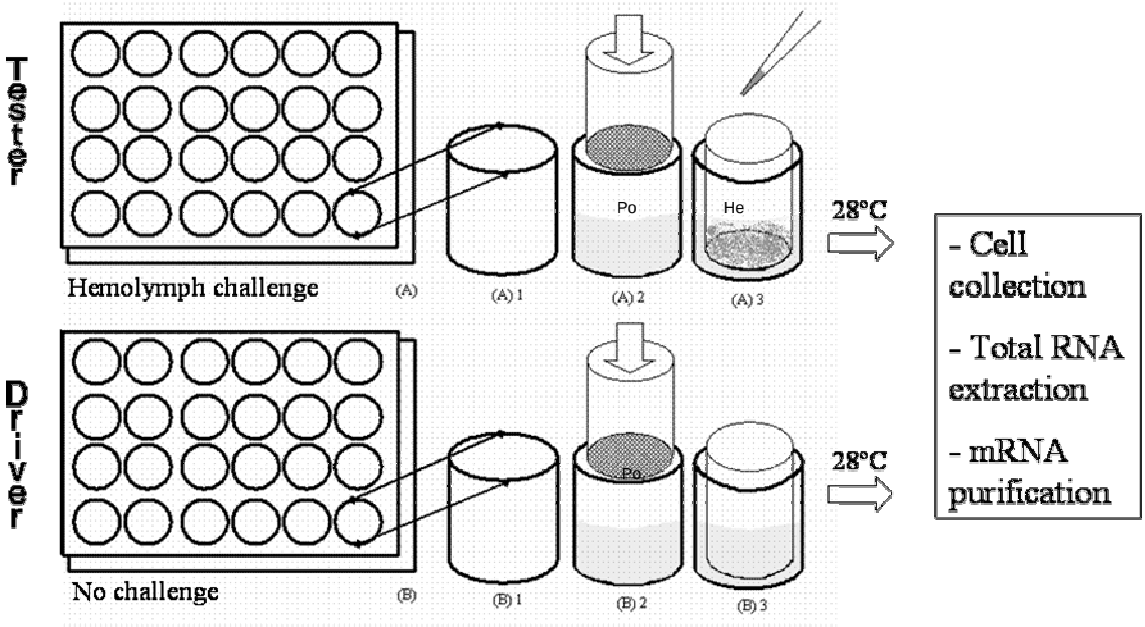


Figure III- 1 Schematic representation of *P. olseni* cells (Po) exposure to bivalve hemolymph (He) *in vitro* performed in 24-wells plates (A, B). Concentrated *P. olseni* culture was placed in each well (A2, B2) and the hemolymph applied on the 0.4 µm filter (A3).

III-2.1.2 *P. olseni* host response subtractive cDNA library construction

Clontech PCR-Select^{TM} cDNA Subtraction was the methodology used, based on selective amplification of differentially expressed sequences, which overcomes technical limitations of traditional subtraction methods (Diatchenko *et al.*, 1996). For

both cell culture conditions the respective mRNA populations were converted into cDNA: (i) the "parasite host-response" cDNA that contains differentially expressed transcripts made from mRNA of challenged *P. olseni* cell culture with host hemolymph, designated as *tester*, and (ii) the "normal" cDNA from mRNA of unchallenged cells, designated as *driver*. The final PCR mixture was enriched for differentially expressed cDNAs. In addition, differentially expressed transcripts that varied in abundance in the original mRNA sample should now be present in roughly equal proportions.

cDNA synthesis and RsaI digestion

First strand was synthesized with 1 µl cDNA synthesis primer (10 µM, Clontech) in a mixture containing 1 µg of host-challenged and normal mRNA, 1 µl of dNTP Mix (10 µM), 1 µl of AMV reverse transcriptase (20 U), 2 µl of 5× first strand buffer in a final volume of 10 µl at 42ºC for 1.5 h. Then, second strand synthesis was carried out using 10 µl first strand reaction volume, 4 µl of 20× second strand enzyme cocktail, 48.4 µl of sterile water, 1.6 µl of dNTP Mix (10 µM), 16 µl 5× second strand buffer solution for 2 h at 16ºC. Polymeric reaction was performed at 16ºC for 0.5 h after 2 µl (6 U) of T4 DNA polymerase was added into the above reaction volume. The second strand synthesis was terminated adding 4 µl of 20× EDTA/Glycogen Mix (Clontech). After was performed phenol:chloroform:isoamyl alcohol (25:24:1) phase separation and chloroform:isoamyl alcohol (24:1) second extraction, and 4 M NH_4OAc and 95 % ethanol precipitation. The double stranded cDNA pellet was washed in 80 % ethanol, air dried and dissolved in 50 µl of sterile H_2O. Before digestion, 6 µl of undigested cDNA was kept for prior agarose/EtBr gel electrophoresis analysis. The cDNA was digested with 1.5 µl of RsaI (10 U) in a final volume of 50 µl at 37ºC for 1.5 h. Enzyme digestion was terminated by adding 2.5 µl 20× EDTA/Glycogen Mix. After extraction and precipitation of digested cDNAs, the pellet was washed in 80% ethanol, air dried and dissolved in 5.5 µl of sterile water.

Adaptor ligation and hybridization

Forward subtraction represents specific cDNAs up-regulated during parasite exposure to its host. The forward subtracted cDNA was prepared as follows: 1 µl of the parasite challenged digested cDNA was diluted in 5 µl of sterile water and subdivided into two (2 µl each). Each fraction was ligated to a different adaptor (NP1 and NP2), by adding 1 µl of T4 DNA ligase (400 U/µl) and 6 µl of Master Mix (Clontech) in a final volume of 10 µl, while 2 µl of each adaptor-linked mineralizing cDNA were mixed prior to ligation, and this mixture was used as an unsubtracted cDNA control sample. Ligation to adaptors was completed at 16ºC overnight. Subtractive hybridization was performed in two rounds. First, 1.5 µl of each adaptor-linked cDNA population was separately mixed with an excess of normal cDNA in 1 µl of 4× hybridization buffer solution at 68ºC for 8 h, after denaturation for 1.5 min at 98ºC. This first hybridization round enriches for cDNA sequences specifically expressed during host-parasite interaction. Second, the two reaction products were mixed in the presence of a fivefold excess of denatured normal cDNA and left at 68°C for 16 h and afterwards 200 µl of dilution buffer was added to the subtracted product. During the second hybridization, single-stranded cDNA specific for the interaction condition bearing different adaptors formed hybrids that were subsequently amplified by two rounds of PCR. The reverse subtracted cDNA was prepared by using the same protocol but switching the parasite host-challenged and normal cDNA.

PCR amplification of subtracted products

Hybridization was followed by two rounds of PCR. The primary PCR amplifications were conducted for forward and reverse subtractions, from both conditions, using diluted subtracted products following the second hybridization and the diluted unsubtracted cDNA as control. One µl of sample was added to 24 µl PCR

master mix prepared using the reagents supplied in the kit and cycling conditions commenced as follows: 75°C for 5 min to extend the adaptors; 94°C for 25 sec; and 25 cycles at 94°C for 10 sec, 66°C for 30 sec, and 72°C for 1.5 min. Amplified products were diluted 10 fold in sterile water and 1 µl of diluted primary PCR products were added to 24 µl of secondary PCR master mix containing nested primers, NP1 and NP2R using Advantage cDNA polymerase (Clontech), to ensure specific amplification of double-stranded templates containing both adaptors. Considering the amount of amplification of the first PCR, 10 cycles for secondary PCR were performed at 94°C for 10 sec, 68°C for 30 sec and 72°C for 1.5 min. Primary and secondary PCR products were analyzed on a 2% agarose ethidium bromide gel.

III-2.1.3 Construction of the Direct forward cDNA subtractive library (Dfsl)

To target diverse up-regulated genes, *P. olseni* SSH cDNA forward subtractive library was constructed using the PCR-SELECT cDNA Subtraction Kit (Clontech). Ten µl of forward subtractive library PCR mixture was then submitted to dATP addition by performing a 15 min PCR step with DNA polymerase (Invitrogen, Carlsbad, USA) to improve T/A ligation efficiency into TOPO cloning vector (Invitrogen). The forward subtractive library, consisting of cDNA fragments ligated into pCRII-TOPO, was used to transform DH5α cells (Invitrogen) which were subsequently grown in agar plates (144 cm2) with 100 µl X-gal (20 mg/ml) and 5 µl IPTG (100 mg/ml). To perform the screening, the library was transferred to Hybond^{TM}-XLfilters (Amersham Biosciences, Piscataway, USA) by bacterial plate lifts (Sambrook *et al.*, 1989). Duplicate filters were prepared for each master plate.

III-2.1.4 Construction of the enriched forward MOS library (efMOSl)

Mirror oriented selection (MOS) technique was performed in order to enrich the library with differentially expressed genes (Rebrikov *et al.*, 2000). Briefly, PCR product

was purified using the GFX column PCR DNA clean (Amersham Biosciences) and ressuspended in TE buffer (pH 8); 5 µl of this purified product was then XmaI-digested (New England Biolabs, Ipswich, USA) to remove the NP1 adaptor and 1 µl of the 15 µl of digestion inactivated product was denatured 1.5 min. at 98ºC and then hybridized for 12 h at 68ºC. One µl of a 40x dilution of hybridization product was used for PCR amplification and 1 µl of the resulting product was inserted into the T/A cloning vector pGEM®-T Easy (Promega, Madison, USA). Individual transformants of DH5α, carrying exogenous cDNA fragments, were isolated from selected white colonies on X-gal/IPTG agar plates, as previously described, being stored arrayed in 10 plates of 96-well format. For screening, the 96-well plate MOS library clones grown in liquid LB were spotted (3 µl per spot) in duplicate membranes and processed by bacterial dot blot as described (Fonseca *et al*., 2005).

III-2.1.5 Identification of differentially up-regulated genes

Differential screening was performed by double filter hybridization. After digestion and neutralization *in situ*, each membrane was hybridized with a probe consisting on the pool of cDNAs (i.e. the products of the second PCR) obtained in each subtractive library (forward or reverse). These complex probes were carefully synthesized by PCR. Briefly, four tubes of either forward or reverse subtracted secondary PCRs were combined and purified using GFX column PCR DNA clean (Amersham Biosciences) according to manufacturer instructions, reducing the volume to 24 µl to concentrate the probes to 50 ng. Afterwards, removal of the adaptors was performed by digesting first with RsaI (10 U) and EaeI (10 U) for 2 h at 37ºC and secondly with SmaI (10 U) for 2 h at room temperature (New England Biolabs). Separation was made through electrophoresis and DNA was recovered from the gel band using Gel Band Purification KIT (Amersham Biosciences). Adaptor-free cDNAs

from forward and reverse subtractions were radiolabeled with α-^{32}P dCTP using the random prime labeling kit (Rediprime™ II, Amersham Biosciences) and purified by spin filtration (Microspin™ S.200 HK columns, Amersham Biosciences), according to manufacturer instructions. Pre-hybridization and hybridization using UltraHyb solution (Ambion, Austin, USA) were carried out at 42ºC. Membranes were then washed twice in 1x SSC, 0.1% SDS at 42ºC for 5 min, followed by two 10 min washes in a more stringent solution (0.1x SSC, 0.1% SDS) at 42ºC. Autoradiography was performed with Kodak X-Omat AR film. The impressed signals were quantified by densitometry (QuantityOne, BioRad, Richmond, USA). Naked eye analysis was also performed. DNA fragments in dots showing more than twofold difference in impressed signal strength were further processed for sequence analysis.

III-2.2 *In silico* data analysis for gene characterization

Gene ontology annotation of the efMOSl library clones was performed after removal of vector sequence and correction of obvious reading errors, using VecScreen (http://www.ncbi.nlm.nih.gov), through GOblet server (http://goblet.molgen.mpg.de). On the other hand, the Dfsl EST sequences were trimmed against the vector and adaptors using the contig program included in Vector NTI (Invitrogen), and submitted to GOblet (http://goblet.molgen.mpg.de). Sequence characterization used a format based on server instructions (Groth *et al*., 2004), defining E value $<1x10^{-1}$ as cutoff value. The information selected was the GO hits in the molecular function categories and subcategories. Homology search against NCBI protein database (BLASTx) was performed and protein hits and significance values recorded (http://www.ncbi.nlm.nih.gov). A search of each hit function was performed in the pathway maps for biological processes server of Kyoto Encyclopedia of Genes and

Genomes (KEGG, http://www.genome.jp/kegg/). A BLASTn search was also performed against *P. marinus* genome data base (http://www.tigr.org/tdb/e2k1/pmg/).

III-3 Results and Discussion

SSH analysis was used to uncover numerous and diverse parasite up-regulated genes in response to its host hemolymph, permitting a better understanding of parasite molecular host-response and supplementary information for eventual genome annotation.

II-3.1 How to characterize the protozoan mollusc parasite transcriptome?

There are reports that certain pathogen genes are specifically expressed only after host infection by the parasite (Kuboki *et al*., 2007). To identify genes differentially expressed by the protozoan parasite *P. olseni*, upon challenge with its host hemolymph, the SSH approach was chosen. For this, poly A^+ RNA was purified from *P. olseni* cells in culture and cells exposed to its host *R. decussatus* hemolymph, thus becoming the driver and tester, respectively, for forward subtracted cDNA library construction.

The parasite library construction was followed step by step: (i) the expected smear of cDNA was checked before and after RsaI digestion (Figure III-2A); (ii) the products from first and second PCR, optimized to 10 cycles, were fractioned to confirm the presence of cDNA smear (Figure III-2B). This direct approach of cDNA subtractive library was designated direct forward subtractive library (Dfsl), where the second PCR was directly cloned as described in materials and methods. In parallel, the reverse cDNA subtractive library was also constructed and each step verified as well. Because SSH has been described to originate false differentially expressed genes, the MOS methodology was used to attempt to eliminate these false positives from the SSH-generated libraries, which represents the non-differentially expressed genes (Rebrikov *et al*., 2000), originating an enriched library (Figure III-2C) called enriched (efMOSl).

Dfsl and efMOSl allowed the identification of 96 and 204 over-expressed clones, respectively.

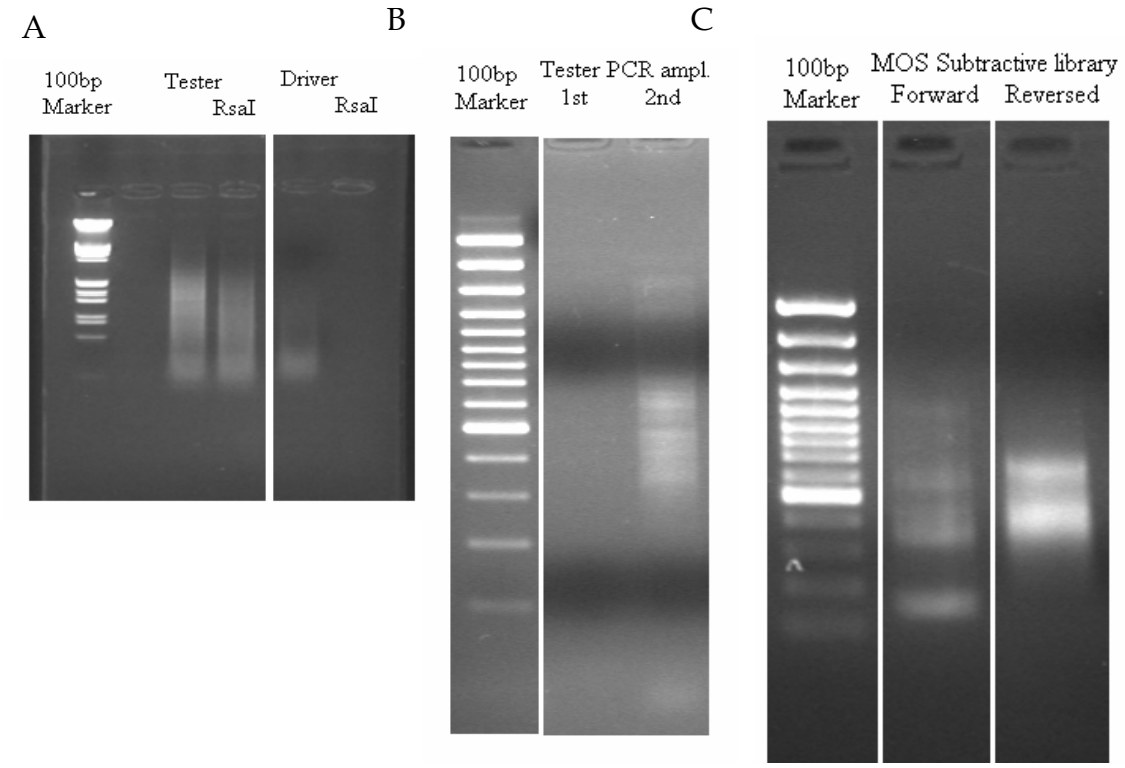


Figure III- 2 cDNA subtractive library construction: tester and driver cDNA before and after digestion with RsaI restriction enzyme (A) and PCR amplification of forward subtracted library, first and second PCR amplification results (B). MOS of the cDNA subtractive library: forward and reverse library PCR products fractionation after enrichment using MOS (C).

A similar SSH strategy was followed to test the hypothesis that African trypanosomes could have *in vivo* specific genes for adaptation to host's environment, but in the order Kinetoplastida some genes are expressed polycistronically, and regulated at post-transcriptional level. 328 clones were identified from the *in vivo* library and 160 clones from the *in vitro* SSH library were analyzed, but none of the genes showed differential expression (Kuboki *et al*., 2007).

III-3.2 Which SSH approach was more profitable?

The two different SSH-based approaches (efMOSI and DfsI) were tested in parallel to determine which more profitable and allowed identification of a bigger set of differentially expressed genes from the parasite *P. olseni*, in response to the presence of

hemolymph from its natural host, the carpet shell clam *R. decussatus* (Ascenso *et al*., 2007).

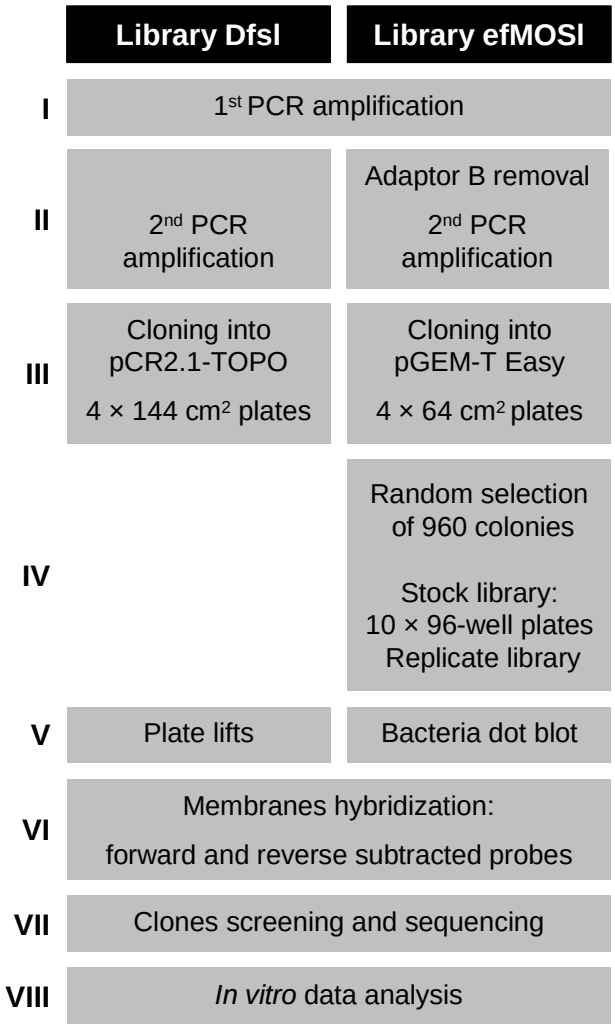


Figure III- 3 Comparison of the two methodologies followed for construction of the subtracted libraries, represented in eight stages (adapted from Diatchenko (Diatchenko *et al*., 1996) and Rebrikov (Rebrikov *et al*., 2000). Dfsl, Direct forward subtractive library and efMOSl, enriched forward Mirror Orientation Selection library.

Dfsl was found to be less labor-intense than efMOSl since step IV (Figure III-3), which involved the manipulation of 960 clones twice before spotting, was omitted. Another disadvantage noted for efMOSl was the independent dot blotting of bacterial clones into two different membranes, resulting in comparisons made between hybridizations performed in two separate blotting experiments, a procedure that may

introduce experimental and/or interpretation errors. In addition, the screening procedure was simpler for eight Dfsl membranes then for twenty efMOSl bacteria dot blots (Figure III-3). Furthermore, *in silico* analysis of the efMOSl-derived cDNA sequences required individual cleaning from pGEM®T-easy vector sequence contamination prior to storage in local database. In contrast, all Dfsl-derived cDNA sequences were trimmed together in one single bioinformatics step, much less labor intensive. These results were compiled into a short note (Ascenso *et al*., 2007) aiming at improving the detection of differentially expressed genes (Appendix III-1 and Appendix III-2) quickly and cost efficiently from non-standard models as the mollusc parasite, *P. olseni*.

III-3.3 Which SSH approach was more efficient in terms of GO diversity?

After cloning the two forward libraries, Dfsl and efMOSl, they were screened and selected for genes up-regulated at least twice.

Table III- 1 GO analysis of molecular function determined using the GOblet server.

Library	Dfsl	efMOSl
Sequenced cDNA	96	204
Goblet submitted	96	108
GO subclasses	25	6
GO molecular function hit	44	21
No GO match	52	87

Two hundred and four clones were identified, sequenced and analyzed *in silico* (Appendix III-3 and III-4) using Gene Ontology (GO) at Goblet server. The two different SSH-based approaches (efMOSI and DfsI) were compared to determine which one was more efficient and less redundant in the identification of parasite differentially expressed genes in response to the presence of hemolymph from carpet shell clam. In

the Dfsl procedure, all colonies were present in both replica filters through plate lifts and for filter hybridization (Appendix III-1). Screening analysis was also more efficient for Dfsl since 100% of the 96 clones selected resulted in useful sequences; while from the 204 clones selected from efMOSl (Appendix III-2), resulted in only 108 quality sequences (Table III.1, Ascenso *et al*., 2007).

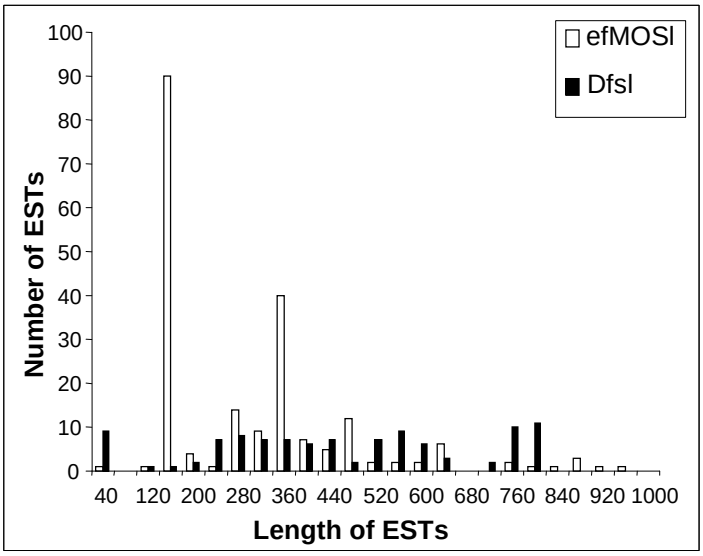


Figure III- 4 Subtractive library cDNA fragments analysed. Distribution of the cloned cDNA fragments from each subtractive library, according to the sequence length for Direct forward subtractive library (Dfsl) and enriched forward MOS subtractive library efMOSl).

According to cDNA fragments size obtained, efMOSl presented a majority of sequences ranging in size from 160 to 179nt, most corresponding to the same EST. On the other hand, the Dfsl presented a much broader size distribution, from 240 to 800nt (Figure III-4). The 96 cDNA sequences of Dfsl library were classified according to GO (e-value of 0.1 of homology at GOblet server) in 27 different subcategories of molecular functions (45.8% ESTs matched, Figure III-5). The diversity obtained was 61%. In contrast, from the 204 clones selected by efMOSl, only 108 resulted in quality sequences, allowing the identification of 6 different GO function subcategories (10.3% ESTs matched, Figure III-5). The diversity obtained in terms of GO, was 2.1x higher in

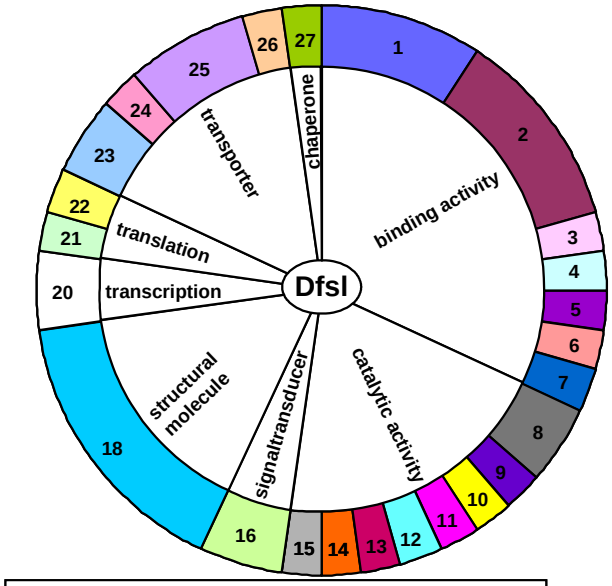
Dfsl compared to efMOSl, corresponding to 4.2x increase in number of GO categories in Dfsl compared to efMOSl.

There are few comparisons of SSH and MOS, but Lathia and collaborators compared the results obtained in cDNA libraries derived from the spinal cord with those derived from the visual cortex. This resulted in an overall representation of repeated sequence reduction from 38 in SSH libraries to 22 in MOS libraries which makes this procedure marginally useful in these complex subtractions. Although it was quite visible a reduction in the diversity obtained by Lathia and collaborators, this comparison was not specifically analysed. In that work, two libraries were compared in terms of SSH and MOS approaches; resulting in a higher number of genes identified in the library named 4-SSH than in the 4-MOS (Lathia *et al*., 2006), similarly to what was obtained for *P. olseni* host-response libraries. This shows that MOS libraries result in larger number of differentially expressed clones, but it is also more redundant in terms of identity of genes obtained, associated with the second PCR performed in MOS technique used for false positive elimination, a result that is in agreement with our data (Ascenso *et al*., 2007).

III-3.4 Which parasite genes are up-regulated in response to host?

The 300 ESTs corresponding to the subtracted transcriptome (204 from efMOSl and 96 from Dfsl) were further analysed on a Gene Ontology (GO) search engine. The most represented molecular function class in clones obtained from the Dfsl approach was binding activity (32% with 14 out of 44 different ESTs obtained). Altogether, the forty four sequences matched the following GO molecular function categories: binding, catalytic, transporter, chaperone activity, signal transducer, structural function, transcription and translation activities (Figure III-5). 14 ESTs corresponding to binding activity hits represented 7 different subclasses, indicating a higher diversity (Figure III-

5) than with efMOSI, while for the GO class of catalytic activity; the resulting positive 16 ESTs were distributed into only two sub-classes (Figure III-6).



1	DNA binding (9,1%)
2	RNA binding (11,4%)
3	transcription factor binding (2,3%)
4	transition ion binding (2,3%)
5	purine nucleotide binding (2,3%)
6	cytoskeletal protein binding (2,3%)
7	androgen binding (2,3%)
8	ATP-dependent helicase activity (4,5%)
9	carbon-carbon lyase (2,3%)
10	oxidoreductase (2,3%)
11	hydrolase on carbon-nitrogen bond (2,3%)
12	peptidase activity (2,3%)
13	forming carbon-nitrogen bonds (2,3%)
14	transferring acyl groups (2,3%)
15	transferring one-carbon group (2,3%)
16	transmembrane receptor (4,5%)
18	ribosome structural molecule (15,9%)
20	transcription factor activity (4,5%)
21	translation initiation factor (2,3%)
22	translation termination factor (2,3%)
23	amino acid transporter (4,5%)
24	electrochemical transport (2,3%)
25	electron transporter (6,8%)
26	cation transporter activity (2,3%)
27	heat-shock protein activity (2,3%)

Figure III- 5 GO results for Dfsl. The classes and subclasses are represented according to GO molecular function category of GOblet tool and the area is proportional to EST sequence number. Dfsl, Direct forward subtractive library.

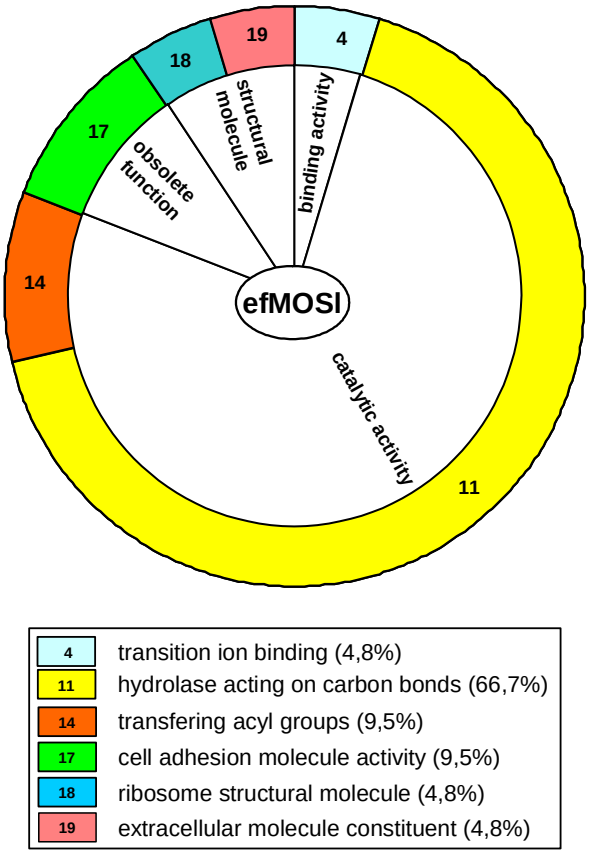


Figure III- 6 GO results for efMOSl. The classes and subclasses are represented according to the molecular function category of GOblet tool and the area is proportional to EST sequence number. efMOSl, enriched forward Mirror Orientation Selection library.

In contrast, in data resulting from efMOSl approach, only twenty one sequences were included in the GO categories of molecular functions, including binding, catalytic activities, structural and obsolete function (Figure III-6). A total of 139 sequences presented no match with GO molecular function in both libraries (Table III-1), being designated as hypothetical. A percentage of hypothetical or unknown genes have also been obtained in similar studies performed in other microorganisms as also other categories of gene function associated with host-pathogen interaction. In *Escherichia*

coli, several pathogenic genes were identified by selective capture of transcribed sequences such as putative adhesins, lipopolysaccharide core synthesis, iron-responsive, plasmid- and phage-encoded genes, are specifically expressed in response to host (Dozois *et al*., 2003). In another a study using the protozoan parasite *Trypanosoma brucei gambiense,* the SSH technique resulted in 328 clones arbitrarily picked up from the *in vivo* library and 160 from the *in vitro* library resulting in 36 and 5 differentially expressed unique genes, respectively. Based on homology to genes in the DNA database, results from both *in vivo* and *in vitro* libraries resulted in nearly half as unknown genes, the remaining belonging essentially to the categories of ribosomal protein genes, RNA binding/transcription protein genes, protease genes, other functional genes and conserved protein genes (Kuboki *et al*., 2007).

Altogether, and despite the lack of information on unicellular parasite genomes, the results obtained allow a progressive unveiling of parasites transcriptome contributing for a better understanding of host-parasite interaction. Furthermore, the homology found among genes belonging to the various parasites genomes and present in the various available databases helps to infer a molecular characterization of parasite host-response.

III-3.5 Was diversity observed also evident through BLASTx protein hits?

Not all genes could be annotated. Those genes sharing similarities to known proteins were named according to their database hits. Genes without significant peptide similarity but with EST similarity were named as 'unknown' proteins. Genes without protein or EST similarity are annotated as 'hypothetical' proteins.

The *Perkinsus* diversity was confirmed by individual BLASTx analysis, resulting in 82 different hits were obtained from Dfsl (Appendix III-5 and III-7), while 27 singular hits out of the 108 tested from efMOSl (Appendix III-6 and III-8). As for

GO, the BLASTx analysis resulted in an evident higher diversity in protein homology hits for Dfsl (3x) compared to efMOSl. Based on the BLASTx homology hit results and taking into account the organism hit, for efMOSl the majority of the clones (n=53, representing 48% of total clones) were homologous to Protists while for Dfsl 32% corresponded to Protist kingdom (Figure III-7).

Table III- 2 BLASTx analysis of *P. olseni* ESTs determined using the NCBI website.

Dfsl NCBI BLAST x results	
ESTs sequenced	96
ESTs with hit	90
ESTs with no hit	6
BLASTx hits	82
efMOSl NCBI BLAST x results	
ESTs sequenced	204
ESTs with hit	109
ESTs with no hit	95
BLASTx hits	27

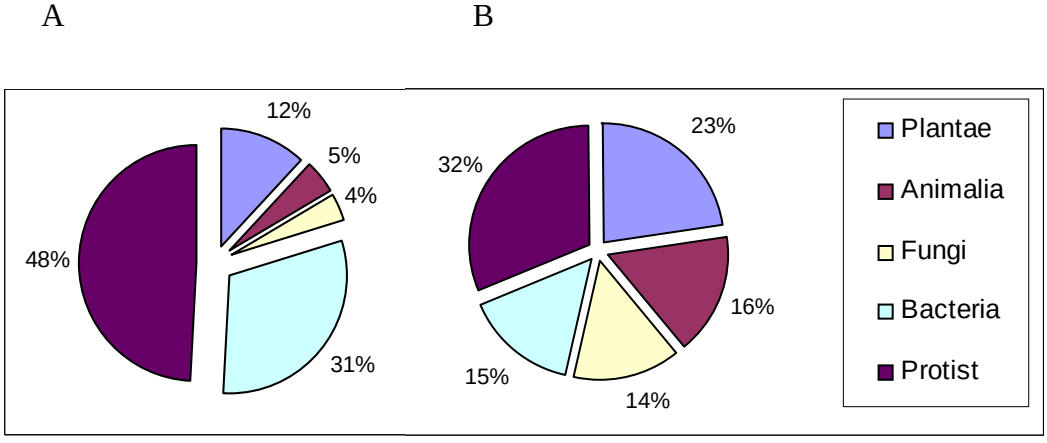


Figure III- 7 Percentage of clones from efMOSl (A) and from Dfsl (B) approaches distributed by kingdoms of the represented organisms, Plantae, Animalia, Fungi, Bacteria and Protist.

It was thus possible to infer proximity with the Protozoans, especially with parasites that are quite characterized as *Plasmodium*, *Cryptosporidium* and *Entamoeba*, which were more represented in homology results.

Then the two most representative groups of organisms with hits are the Bacteria and Plantae. Each EST was submitted to homology search against *P. marinus* genome sequencing TIGR database. But the majority of cDNA fragments obtained had no homology hit at NCBI neither at TIGR. This *in silico* analysis allowed the selection of genes of interest for further molecular characterization, in an attempt to provide further insight into our comprehension of parasite host-response.

III-4 Conclusions

In recent years, identification of pathogenic genes has been achieved by using a number of different approaches. The most commonly used methods for bacterial pathogens have included signature-tagged mutagenesis (Song *et al*. 2004) a negative-selection method that involves comparative isolation of individual specifically tagged transposon-generated mutants applied to *Salmonella typhimurium* (Hensel *et al*., 1995), to *Yersinia enterocolitica* (Darwin and Miller, 1999) and selective capture of transcribed sequences in *Escherichia coli* (Dozois *et al*., 2003). An alternative approach for studying the role of genes in physiological, morphological and biochemical processes in bacteria is to investigate changes in gene expression. For this, cDNA-amplified fragment length polymorphism (cDNA-AFLP) was firstly reported to study differential gene expression in *Erwinia carotovora* (Dellagi *et al*., 2000), but these authors already suggest that SSH could allow the isolation of previously unidentified genes.

In this case, the SSH strategy was selected for the study of the non-standard mollusc parasite *P. olseni* up-regulated genes when challenged with hemolymph from

its host, the carpet-shell clam, *R. decussatus*. Likewise, SSH was the technique chosen for studies of host parasite-response, like the detection of Eastern (*Crassostrea virginica*) and Pacific (*C. gigas*) oysters genes expressed in response to *P. marinus* (Tanguy *et al*., 2004) and identify new antimicrobial peptides in carpet-shell clam hemocytes in response to bacterial stimulation (Gestal *et al*., 2007). Two approaches to parasite subtracted transcriptome, direct SSH and an enriched library by MOS, were compared to find out which one was less labour intensive, cheaper and able to provide more genes with a higher diversity. The first approach (direct SSH or Dfsl) was found to be less labour intensive and was recommended not only because of being faster, but also because it allowed identification of a higher number of genes. The diversity obtained with Dfsl in terms of GO molecular function was at least twice that obtained with efMOSl, with the majority of the EST identified corresponding to Protist kingdom.

CHAPTER IV – MOLECULAR CHARACTERIZATION OF A CHLOROHYDROLASE-LIKE GENE

"Effective chemotherapy requires inhibition of critical molecular processes in the target pathogen without significantly impinging on those of the host. This simple principle requires that host and pathogen must be different in some aspect, usually in a metabolic or structural pathway" by Karen Joy Shaw (2002) Pathogen Genomics Impact on Human Health, Humana Press

IV-1 Introduction

Perkinsus sp. is a poorly characterized molluscan parasite. Through a cDNA subtractive library were identified those parasite genes up-regulated when challenged with host hemolymph (see Chapter III). From these, the gene represented in more copies was obtained from the efMOSl cDNA subtracted library and first identified, following BLASTx analysis as a putative cytosine deaminase (CD) related to pyrimidine salvage pathway. Later a higher homology was obtained with proteins from the chlorohydrolase family.

The chlorohydrolase was shown to be firmly linked with a major amidohydrolase protein superfamily (Seffernick *et al*., 2001). Members of the amidohydrolase protein superfamily catalyze different reactions and are generally divergent in their amino acid sequence, presenting less than 50% identity. However, these superfamily members are known to share common tri-dimensional structure and catalytic mechanisms. For example, *Pseudomonas* sp. strain NRRL B-12227 melamine deaminase was described to be 98% identical to the enzyme atrazine chlorohydrolase from the strain ADP, differing in 9 amino acids only (Seffernick *et al*., 2001) although their function is quite different, thus confirming that proteins with high identity may catalyze different reactions in different metabolic pathways.

Inference by homology is currently the most powerful computational method of function assignment and structure prediction for the protein products of newly identified genes (Holm and Sander, 1997). Homologous proteins catalyzing different reactions are being discovered at an increasing rate with functional genomics focusing attention on the interplay between molecular sequence and function. This amidohydrolase superfamily comprehend some of the enzymes responsible for nitrogen recycle, essential metabolites for DNA and nucleic acid synthesis (Seffernick *et al*., 2001),

including 30% of the steps in four intermediate metabolic pathways (Holm and Sander, 1997). Cytosine deaminase (EC 3.5.4.1) is one of the proteins responsible for the salvage pathway of pyrimidine, being related with parasitism mechanisms of nutrient scavenge. It was found in a variety of bacteria and fungi, but not in other eukaryotes. It functions in the pyrimidine salvage pathway during nutritional stress deaminating cytosine to uracil (Danielsen *et al.*, 1992). CD is also capable of catalyze the deamination of 5-fluorocytosine to form 5-fluorouracil, being very useful as negative selectable marker since CD has a very narrow range of substrates (Porter and Austin, 1993). Indeed, the enzyme prodrug CD/ 5-FC strategy was first used for selection (Nishiyama *et al*., 1985), and since then has been applied to gene cancer therapy (Huber *et al*., 1994), (Nyati *et al*., 2002), (Zhengwang *et al*., 2002), (Dabrowska *et al*., 2004), (Lee *et al*., 2004), (Goblirsch *et al*., 2006) and (Chaszczewska-Markowska *et al*., 2008) as well as for selection of parasitosis transfectants (Fox *et al*., 1999), (Duraisingh *et al*., 2002) and (Davoudi *et al*., 2003).

Besides the enzymes responsible for deamination, there are those chlorohydrolases that catalyse hydrolytic displacement of amino groups and chlorine substitutives from s-triazine ring compounds (Holm and Sander, 1997). That is the case of Atrazine chlorohydrolase (AtzA), N-ethylameline hydrolase or Triazine hydrolase (TrzA), enzymes involved in detoxification of the pesticide atrazine. Atrazine, first synthesized in Switzerland in 1955 for JR Geigy (CIBA-GEIGY Corporation), was first licensed in the US in 1959. More than 2 billion pounds of the herbicide atrazine have been applied to soils globally, and this has provided selective pressure for the evolution of new metabolisms. The clearest example to date is atrazine chlorohydrolase, an enzyme which has been shown to have evolved to allow the catabolism of atrazine.

AtzA was shown to exclusively catalyse dehalogenation of halo-substitued triazine ring compounds (Seffernick and Wackett, 2001), such as atrazine (2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine, CEIT). Although AtzA was not active with any of the pyrimidine substrates (Seffernick *et al*., 2000) it dehalogenates atrazine. CD catalyses specifically the deamination of cytosine to uracil and amonia (Danielsen *et al*., 1992). N-ethylameline hydrolase (TrzA) from *Rhodococcus corallinus* NRRL B-15444R catalyzes the deamination of unalkylated aminotriazines, such as melamine or 2-chloro-4,6-diamino-1,3,5-*s*-triazine (CAAT) but fails to catalyze either deamination or dechlorination reactions with atrazine (CEIT) (Grossenbacher *et al*., 1984).

Bivalves behave like a biological filter therefore they can accumulate and concentrate contaminants within their tissues (Gagnaire *et al*., 2004). Bivalves can concentrate pollutants in their tissue at concentration greater than the ambient seawater (El-Shenawy, 2004). In this context, it was described that atrazine affects the peroxidase activity of the hemocytes thus interfering with the bivalve defense mechanisms (Gagnaire *et al*., 2004).

The effect of drugs with different chemical properties and mode of action have been shown to contribute significantly to increase our understanding of parasite biology and physiology. Increasing information has became available on *Perkinsus* drug susceptibility, and in particular *P. olseni,* which was highly susceptible to iron deprivation as induced by iron-chelators desferrioxamine (DFO) and 2,2-bipyridyl (BIP) (Elandalloussi *et al*., 2003). Also some compounds known for their antimalarial and antiprotozoal properties showed in vitro inhibitory effect on *Perkinsus* proliferation, namely cycloheximide, pyrimethamine, DFO and BIP (Elandalloussi *et al*., 2005). Despite the lack of effect by PAHs in *P. marinus* in vitro proliferation (Bushek *et al*.,

2007a), it was noteworthy the effects that these chemicals can have on oysters, increasing haemocyte activity.

The *P. olseni* EST was identified, through *in silico* analysis, as being part of the chlorohydrolase protein family, with significant hits for TrzA, AtzA and CD, and named Chlorohydrolase-like protein (Clhl). Substrate specificity and protein functionality were studied in order to distinguish and characterize this *P. olseni* protein. Our findings suggest that either (i) another enzyme(s) is present which confers the broader substrate range in *Perkinsus* or (ii) the Clhl itself has a broader substrate range. The molecular characterization and functional analysis of this PoClhl allowed a better definition of this novel parasite protein and contribute to understand its role in host-parasite interaction.

IV-2 Specific materials and methods

IV-2.1 *P. olseni* Clhl cDNA and gene molecular characterization

A partial sequence of one clone from *P. olseni* chlorohydrolase-like protein **(**PoClhl) was obtained by screening the subtractive cDNA library (see Chapter III) and the complete cDNA was obtained by RACE-PCR using Marathon cDNA library and PoClhl GSP, PoClhl Frw1 and PoClhl Rev1 (Appendix II-1) specific primers, as well as the AP1 primer, according to previously described method (see Chapter II). The PoClhl gene was obtained through a combination of PCR using specific primers (PoClhl GW Frw1 and PoClhl GW Rev1; PoClhl gene Frw5 and PoClhl gene Rev5, Appendix II-1) and the Universal Genome Walker commercial kit, as previously described (see Chapter II). For identification of gene structure the resulting genomic sequence was aligned with the sequence of the cloned PoClhl cDNA. There was 100% identity between the common regions of both gene and cDNA sequences for PoClhl.

IV-2.2 *P. marinus* Clhl cDNA and gene molecular characterization

A PmClhl gene sequence was identified by BLASTn analysis using the PoClhl cDNA sequence against *P. marinus* genomic sequences available from its genome sequencing project (http://tigrblast.tigr.org). A high homology hit identified a preliminary genomic DNA fragment (#23200) which was used to design specific primers (PmClhl Frw1 and PmClhl Rev1, Appendix II-1) which allowed amplification of its partial cDNA. Amplification of the corresponding 5' and 3' cDNA regions was achieved following the manufacture's instructions using the GeneRacer Kit (Invitrogen) methodology and specific primers PmClhl Frw3 and PmClhl Rev3 Rev (Appendix II-1). The amplified cDNA fragments were cloned into pCR II TOPO T/A cloning vector (Invitrogen) in One Shot Top10 Competent cells (Invitrogen). To eliminate internal sequencing error, the ORF was re-sequenced using specific primers PmClhl Frw2 and PmClhl Rev2 (Appendix II-1).

The complete sequence of the PmClhl gene was obtained by searching the available *P. marinus* database with the sequence of the cloned full length PmClhl cDNA and by comparison with the PoClhl gene. The PmClhl gene sequence was later confirmed by amplification of *P. marinus* high molecular weight DNA using PmClhlg Frw1 and PmClhlg Rev1 oligonucleotides (Appendix II-1). There was 100% identity between the common regions of both gene and cDNA sequences for PmClhl.

IV-2.3 *Perkinsus* spp. sensitivity to specific Clhl substrates and inhibitors

To test the possibility of PoClhl behaving like a CD, P. olseni cells were cultured in the presence of its specific substrates, prodrug, 5-FC and the respective drug 5-FU. Cellular proliferation was also assayed in the presence of the specific CD substrate. Posterior BLASTx analysis revealed other hypothesis of protein hit above CD, AtzA, TrzA. AtzA was shown to exclusively catalyse dehalogenation of atrazine

(2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine, CEIT). TrzA catalyzes the deamination of unalkylated aminotriazines, such as melamine (Grossenbacher *et al*., 1984). CD crystal structure was described in complexes with the inhibitor 2-hydroxypyrimidine (Ko *et al*., 2003). AtzC competitive inhibitor is 5-azacytosine (Shapir *et al*., 2002) being used as AtzA and TrzA inhibitors, also.

IV-2.3.1 *P. olseni* growth curve under Clhl substrates treatments

Cells from both Perkinsus species were cultured after adding the chemical treatments, prodrug and drug, at various concentrations and incubated at 28ºC during 12 days. A similar experiment was performed using solely the vehicle used (0.2 % dimethylsulfoxide (DMSO) as control. The growth was followed by absorbance measured at 600 nm, along the 12 days of the experiment. This method tested each chemical concentration, the blank control, with no treatment and solvent control, measuring duplicates for each sampling. At the 2^{nd} and 12^{th} day 3 replicates of 100 µl were sampled and used to perform the cellular viability assay. Cell proliferation assay was performed after 72 hours exposure, adding 20 µl MTS/ PMS, allowing an incubation of 2 hrs at 28ºC, protected from light, following Cell titter 96 aqueous non-radioactive cell proliferation assay instructions (Promega). Absorbance was read at 490 nm with a microplate reader, preceded by 10 seconds agitation.

To screen other chlorohydrolases activities, all known substrates and inhibitors were used. The chemicals cytosine, 5-FC, 5-FU, CEIT, melamine, azacytosine and hydroxypirimidine were purchased from Sigma Aldrich. Azacytosine is a DNA methylation inhibitor, a competitive inhibitor of N-Ethylameline (TrzA). 2-Hydroxypirimidine was described as an inhibitor of recombinant yeast cytosine deaminase. The concentration of each chemical responsible for 50% of cell viability (EC50) was determined by nonlinear regression analysis of log dose response curves

using GraphPad Prism v.4.00 software. These values were calculated from triplicates of two independent experiments. The assays were carried out in 100µl of medium containing the different factors to be tested and evaluation of cell proliferation was determined after 72 hours, using a cell proliferation assay (Promega) according to manufacturer instructions. A linear association (R^2=0.9755) was previously found between the proliferation assay signal and cell density, measured by 600nm absorbance, confirming that the assay absorbance at 490nm was proportional to cell culture density (Elandalloussi *et al*., 2003).

IV-2.4 Characterization of PoClhl gene expression pattern

In order to characterize the pattern of parasite gene expression, *P. olseni* cell culture media was prepared using DME: Hams F12 (Gibco, Invitrogen Co) supplemented with 5 % FBS and streptomycin/ampicilin antibiotics as the control condition once is the standard medium. DME commercial medium can be found supplemented or not with glutamine. The nutrient mixture Hams F12 contains basic aminoacids and lipids including alanine, asparagine, proline, vitamine B12, putrescine, lipoic and linoleic acids as well as nucleotide precursors (proline, hypoxanthine and thymidine). The conditions prepared for *P. olseni* gene expression were thus designed to prevent interference from specific components of the culture medium which are known to be pyrimidine pathway precursors**.**

IV-2.4.1 PoClhl expression in response to host hemolymph challenge

PoClhl gene expression was observed by RT-PCR following *in vitro* exposure of *P. olseni* cells to *R. philippinarum* serum (hemolymph cell free) along time. *P. olseni* cells were grown in 250 cm^2 Erlenmeyer. Hemolymph was withdrawn from the

adductor muscle of *R philippinarum* without dissecting the clams, which were obtained from North Eingstown, USA. Only cell-free hemolymph from non infected clams was used. A number of $2x10^7$ trophozoites were resuspended in a minimum volume of 0.5 ml cell free-hemolymph to perform the parasite-host exposure. The serum was isolated by centrifugation and hemocytes were used for DNA extraction and *Perkinsus sp.* diagnose PCR assay. The serum quality was checked under the microscopy. Total RNA was isolated using the RNeasy mini Kit (Qiagen). cDNA was synthesized using SuperScript II RT (Invitrogen) and PoClhl relative gene expression was analysed by gel electrophoresis of RT-PCR products, using *P. olseni* Polyubiquitin (PoUbq, accession number DQ 291152) as reference gene.

IV-2.4.2 PoClhl expression in media depleted of pyrimidine percursors

Medium depleted of different nucleotide precursors was prepared using DME medium with and without glutamine in various proportions in the absence or presence of Hams F12 nutrient mix. Without Hams F12, nutrients as alanine, asparagine, proline, hypoxanthine, thymidine, vitamine B12, putrescine and lipoic and linoleic acids are not available, allowing to test the relevance of external nucleotides precursors (proline, hypoxanthine and thymidine) availability in parasite salvage for the ribonucleotide metabolism.

Table IV- 1 Different cell culture media prepared using DME (Gibco, Invitrogen Co), Hams F12 (Gibco, Invitrogen Co) and DME with glutamine.

A	B	C	D	E	F	G	H	Treatment
-	-	-	-	+	+	+	+	5-FC treatment
-	-	+	+	+	+	-	-	Glutamine
-	+	-	+	+	-	+	-	Hams
+	+	+	+	+	+	+	+	DME medium

The cells were cultured 3 days in the various media described, then collected for total RNA isolation for further PoClhl gene expression study by Northern blot (Chapter II), using PoUbq as reference gene.

IV-2.4.3 PoClhl expression in response to various environmental conditions

The environmental stressing conditions were previously mentioned, temperature, salinity and organic and inorganic pollutants (Table IV-1). The Northern blot was the strategy used for gene expression study, using PoUbq as reference gene.

IV-2.4.4 PoClhl expression following exposure to substrates and inhibitors

P. olseni cells were cultured in 8ml medium and incubated with chlorohydrolases substrate and inhibitors. For treatment with the prodrug 5-FC, the EC50 dose (33.8 µM) was used, as also for treatment with the drug 5-FU (EC50, 4.15 µM). The other substrates: cytosine, CEIT, melamine and inhibitors Azacytosine and 2-hydroxypirimidine; a final concentration of 100 µM was used. After 3 days, total RNA was isolated using Denhardt's solution as previously described (Chapter II). Cellular viability was checked to ensure number and quality of cells using the previously described methodology of cell proliferation assay (Promega).

IV-2.5 PoClhl *in vitro* transcription/ translation

The PoClhl ORF sequence was amplified by PCR using recommended oligonucleotides (Appendix II-1). PCR product obtained was confirmed by DNA sequence analysis to represent the correct amplified sequence prior to its use. The PCR product was then transcribed and translated *in vitro* by TNT quick coupled transcription and translation system (Promega) as described by the manufacturer, but in 25 µl final volumes. The PoClhl synthesis products were then used for functional assays. To confirm the *in vitro* protein expression, a control reaction was performed without addition of DNA template for transcription/ translation. The polyacrylamide gradient gel denaturing conditions were used to analyse the products of *in vitro* synthesis. Aliquots of 1 and 2 µl were added to LDS sample buffer (Nupage, Invitrogen) reductant

agent according to manufactures' instructions (Nupage, Invitrogen). The sample mix was heated to 90ºC for 10 min. Then the samples were electrophoresed in a commercial gel of 1 mm x 12 columns, of 4-12 % SDS-polyacrylamide (Nupage, Invitrogen) with 0.1 % de SDS (Nupage, Invitrogen). This was performed using PowerPac **(**HC, Biorad**)** under 200 V. To analyse the protein profile, the gel was fixated and coloured with the CBB solution (Comassie Brilliant Blue, R-250 0.2 % ($^{p}/_{v}$) (Biorad), 10 % ($^{p}/_{v}$) trichloroacetic acid and 10 % ($^{p}/_{v}$) sulfosalicilic acid) during 8 h, followed by de-colouration with acetic acid (7.5 % ($^{v}/_{v}$) and methanol (10 % ($^{v}/_{v}$) solution. The scanned result allowed the comparison between TNT *in vitro* PoClhl protein synthesis and control.

IV-2.6 PoClhl *in vitro* expression using a prokaryote system

P. olseni Clhl cDNA was obtained by RT-PCR amplification from reverse transcription of *P. olseni* RNA using M-MLV reverse transcriptase (Invitrogen), according to the manufacture's instructions. The amplification was performed using the universal primer and a specific primer PoClhlFrw2 (Appendix II-1). Amplification conditions were started with 5 min denaturation at 94ºC proceeding with 10 cycles of 40 sec at 94ºC, 40 sec at 64ºC and 1.5 min at 68ºC, the followed by 35 cycles of 40 sec at 94ºC and 1.5 min at 68ºC and at last the extension of 7 min at 68ºC, using Advantage cDNA Polymerase (Clontech, BD). The 1.6 Kb PCR product of the CD gene was gel band extracted after fractionation in agarose-ethidium bromide gel electrophoresis. The DNA isolated was used as template in a reamplification of the gene using primers specifically designed for expression *Po*Clhl_exp_NotIF1 and *Po*Clhl_exp_NotIR1, with NotI enzyme restriction sites (Appendix II-1). Amplification conditions were as follows: 5 min denaturation at 94ºC, 10 cycles of 30 sec at 94ºC, 2 min at 69ºC and 25

cycles of 30 sec at 94ºC, 2 min at 6 ºC and the last 5 min at 68ºC, using a proofreading polymerase (Advantage cDNA polymerase, Clontech BD). A 1.5 Kb amplicon was isolated from gel band and digested using the NotI restriction enzyme. The pET3-His2 expression vector was similarly digested in order to perform the oriented cloning.

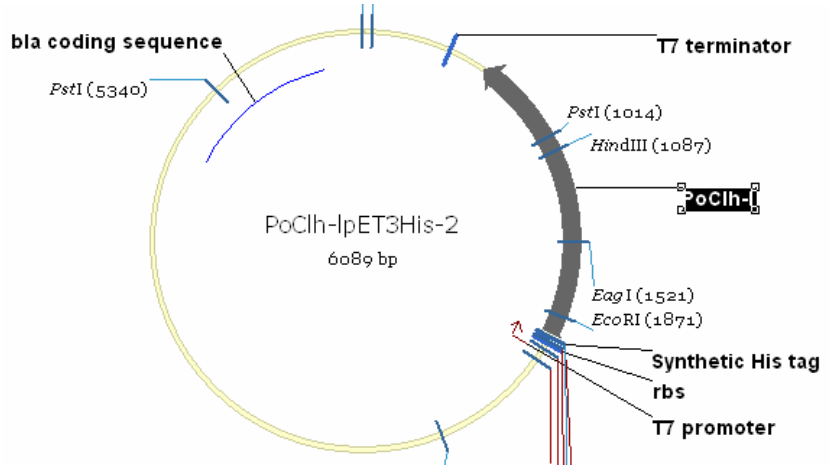


Figure IV- 1 PoClhlpET3His-2 plasmid construct for exogenous PoClhl expression in a prokaryotic system.

The resulting construct was designed PoClhlpET-3His and inserted firstly into DH5α for direction and misleading polymerase synthesis sequence confirmation, then was expressed as exogenous protein in BL21 pLys. The BL21 strain was selected for protein expression due to its deficiency in *lon* protease3 as well as the *ompT* outer membrane protease that can degrade proteins during purification. Their presence reduces basal activity of T7 RNA polymerase in the uninduced state. The strain BL21-Gold (DE3) [F^- *ompT hsdS*(rB^- mB^-) *dcm*$^+$ Tetr *gal* λ(DE3) *endA* Hte (pLysS Camr)] carries a derivative of the plasmid pACYC184 which expresses the T7 lysozyme gene at low levels. T7 lysozyme binds to T7 RNA polymerase and inhibits transcription by this enzyme. In addition to inactivation of T7 RNA polymerase transcription, T7 lysozyme has a second function involving specific cleavage of the peptidoglycan layer of the *E. coli* outer wall. Upon IPTG induction, overproduction of the T7 RNA polymerase renders low level inhibition by T7 lysozyme virtually ineffective and the protein of

interest can be over-expressed. PoClhlpET3His-2 construct was used to transform the BL21pLys by heat shock. For control of protein expression pET3His-2 vector (gently provided by Marc Ohresser) was cloned also in BL21pLys. For control of the transformation reaction, the pET3a vector was used (gently provided by Marc Ohresser). The colony growth selection was performed with chloramphenicol antibiotic and single colony growth was performed in LB broth containing 100 µg/ml of ampicilin and 50µg/ml chloramphenicol and incubated with shaking at 220–250 rpm, at 37°C, overnight. The rest of the culture was induced adding IPTG to a final concentration of 1 mM being incubated with shaking at 220–250 rpm at 37°C for 2 hours. The cultures without induction constituted the uninduced control samples.

IV-2.6.1 PoClhl protein activity in expressing colonies

A simple resting cell assay was devised to measure the enzyme activity based on a spectrophotometric assay described previously (Bouquard *et al*., 1997). A stationary phase cell culture was obtained growing the pre-inoculum in N-free medium. Cells were harvested by centrifugation and washed three times with phosphate-buffered saline solution (10mM potassium phosphate). Then, cells were resuspended in N-free medium and incubated with 5-FC, 5-FU, atrazine, melamine or cytosine as nitrogen source. 50 µl of 10mM atrazine was added and the same was performed in parallel with each substrate. Samples (500µl) were withdrawn at 5-min intervals and centrifuged immediately for 3 min in a microcentrifuge at full speed. Substrate degradation was controlled by decreasing absorbance (García-González, 2003) measured along time, using a Helios gamma spectrophotometer (Thermo Electro Corporation).

IV-2.6.2 Chlorohydrolase substrate degradation in cell extract

Cultures were grown at 37°C overnight in Luria-Bertani medium and induced with IPTG (1 mM). Induction of expression was studied taking in account the time of

induction and IPTG concentration, ranging from 20 to 1mM. Cells were centrifuged at 14,000 × *g* for 2 min at 4°C. Cell pellets were resuspended in ice-cold phosphate buffer after being twice washed. Cell suspensions were subjected to lysis by repeated freeze-thaw cycles. Lysed cell suspensions were centrifuged at 14,000 × *g* for 15 min at 4°C to obtain cell extracts. Saturated solutions of the various atrazine, melamine and cytosine compounds were prepared in 100mM phosphate buffer (pH 7.0). All stock solutions at 100mM were filtered through a 0.2 µm syringe filter and diluted to 10mM in the experimental condition. The atrazine, melamine and cytosine solutions were incubated with 50 µl of cell extracts for 16 and 48 h at 28°C. Enzymatic reactions were stopped by heating for 2 min at 95 to 100°C. Reactions were carried out in 1 ml of 10 mM phosphate buffer, pH 7.0, containing atrazine, melamine or cytosine at 37°C. Control samples without enzyme were handled in parallel with the enzyme- treated samples. Enzyme activity was measured by monitoring the decrease in absorbance of atrazine, melamine and cytosine throughout time. Readings were then taken at OD_{290} for 5-FC and OD_{255} for 5-FU (Mahan *et al*., 2004), at OD_{225} for Atrazine (García- González *et al*., 2003), at OD_{235} for Melamine (UNEP CAS Nº 108-78-1) and at OD_{275} for Cytosine (Garriga *et al*., 1992). Substrate degradation by enzyme activity was measured by decreasing in specific absorbance of each chemical along time using a Helios gamma spectrophotometer (Thermo Electro Corporation).

IV-2.6.3 PoClhl His- tag protein isolation following *in vitro* translation

Two BL21- Lys transformants with PoClhl were grown at 37°C overnight in Luria-Bertani medium and induced with IPTG (20 mM). Two experiments of protein expression were performed:, (i) induction of a 2 hours recovered culture in 1 ml with 20 mM IPTG for 2 hours at 37ºC and (ii) induction of a 10 ml culture grown overnight at room temperature. Both clones were prepared, PoClhlBL21-41 and PoClhlBL21-45 in

BL21-lys and also control BL21-Lys only, for induction and no induction in both experiments. One ml of culture was used to measure cell density by absorbance at 600 nm. The remaining cells were centrifuged at 3,000 rpm for 5 min. Cell pellets were resuspended in 100mM HEPES buffer prior to protein isolation. His-tag based protein isolation was performed using Maxwell™ 16 Polyhistidine Protein Purification Kit (Promega). The product was observed by SDS- PAGE and stained using CBB. When needed a Western blot analysis was performed using anti- His- tag antibody available.

IV-2.7 PoClhl *in vitro* expression using a eukaryote system

The epithelial cell line established from the kidney of the aquatic south African clawed toads *Xenopus laevis* (A6 cell culture, ATCC# CCL102) was the exogenous eukaryotic system selected for PoClhl expression once the transfection methodology was settled and optimized (Conceição *et al*., 2002).

IV-2.7.1 Construction of the expression vector PoClhlpcDNA3

P. olseni Clhl gene was obtained by PCR amplification from *P. olseni* cDNA library, constructed using M-MLV reverse transcriptase (Invitrogen), according to the manufacture's instructions. The amplification was performed using specific primers PoClhlFrw2 and PoClhlRev2 (Appendix II-1). Amplification conditions were those described previously at IV-2.6. The 1.6 Kb PCR product of the Clhl gene was gel band extracted after fractionation and the DNA isolated was used for reamplification of the gene using specifically designed primers for expression (PoClhl_exp_Frw1 and PoClhl_exp_Rev1, II-1), thus introducing BamHI and XhoI enzyme restriction sites. Amplification conditions were as follows: 5 min denaturation at 94 ºC, 35 cycles of 40 sec at 94ºC, 1.5 min at 68ºC and the last 5 min at 68ºC. A 1.5 kb amplicon was isolated from gel band and cloned in TOPO T/A vector. To facilitate TA vector ligation and subsequent cloning, an additional adenylation was performed with a single cycle at

72ºC for 15 min with dATP, using Taq cDNA polymerase (Clontech). The plasmid was amplified in *E. coli* DH5α, purified using plasmid miniprep Kit (Amersham Biosciences) and sequenced to check for absence of misleading polymerase synthesis. The fragment of interest was then amplified by PCR using a proofreading polymerase and digested using the BamHI and XhoI restriction enzymes, as also the pcDNA3 expression vector in order to perform the oriented subcloning.

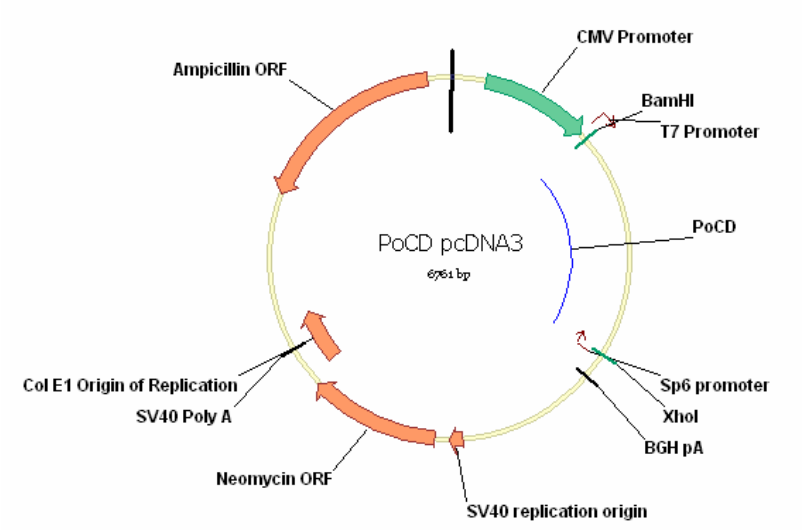


Figure IV- 2 PoClhpcDNA3 plasmid construct for exogenous PoClhl expression in eukaryotic systems.

The PoClhl ORF was subcloned into the multiple cloning site of eukaryotic expression vector pcDNA3.0 to generate PoClhlpcDNA3_BamHI/XhoI plasmid. Sequencing step was repeated to ensure the sequence quality, avoiding errors in protein expression. This plasmid was named PoClhl-pcDNA3.

IV-2.7.2 Construction of vector directing expression of PoClhl protein fused to GFP (PoClhlpEGFP)

The PoClhl ORF was cloned into the multiple cloning site of eukaryotic expression vector pEGFP-N1 to generate PoClhlfusGFP_BamHI/ XhoI plasmid. A sequencing step was repeated to ensure the sequence quality, avoiding errors in protein expression. This plasmid was named PoClhl pEGFP-N1.

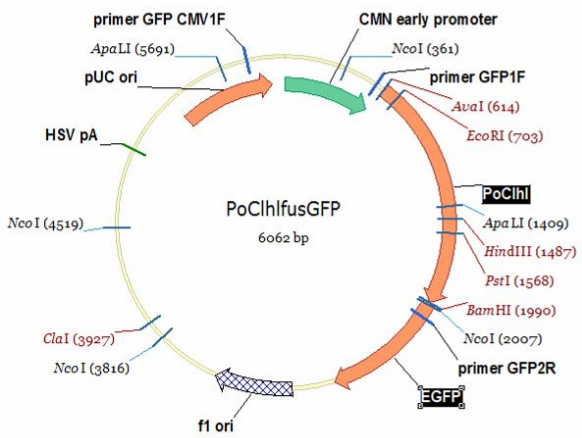


Figure IV- 3 PoClhfusGFP plasmid construct for exogenous PoClhl expression in eukaryotic systems.

IV-2.7.3 PoClhl expression using *X. laevis* A6 cell line

The *X. laevis* A6 cell line (kidney epithelium derived cells) were seeded at $3x10^5$ cells/ ml the day before, to reach at the transfection moment 80 % confluence in 6- well plates. Transient transfections were carried out in standard medium without FBS using FuGene6 (Roche Diagnostics, Indianapolis, USA). The DNA/ transfection reagent complexes were prepared in diluted medium and allowed to incubate 15 min at room temperature. PoClhlfusGFP and PoClhlpEGFP-N1 constructs were used to transfect A6 cells. pEGFP-N1 empty vector were used as control. No DNA complexes were used as transfection negative control. All experiments were repeated at least two times. The transfection efficiency was observed by fluorescence microscopy under blue light after 48 hours. Transcription was checked by RT-PCR, after 5 days following total RNA isolation and using gene specific primers and *X. laevis* actin GSP for control. For stable transfection, pEGFP-N1 plasmid confers geneticin resistance to the cells modified with this plasmid DNA. Geneticin (Gibco) dose response curve was constructed for A6 cells to determine the lethal concentration (EC_{90}= 2 mg/ml of geneticin) and selection concentration (EC_{50}=of 0.5 mg/ml) (r^2 0.9444). To check the exogenous PoClhl production and activity, the transfected A6 clones were seeded in triplicate in a 96-well

plate (cells/ well), cultured for two hours and exposed to above mentioned substrates at various concentrations (2 pM to 2 mM) for 3 to 5 days. Cell viability was determined by MTS assay using cell titter aqueous non radioactive cell proliferation kit (Promega) as previously described (Davoudi *et al*., 2003). These experiments were repeated at least two times.

IV-2.7.4 PoClhl *in vitro* protein expression and western blot confirmation

X. laevis cells transfected with PoClhlfusGFP and expressing Clhl were obtained and maintained under selection. The transformant cells were observed at 48 hours to ensure transfection and partially collected at 72 hours for RT- PCR to verify the Clhl transcription. Having enough cells, these were centrifuged at 3,000 rpm for 5 min and crude extracts were prepared for SDS- PAGE analysis, stained using CCB. Crude extracts were also prepared from *X. laevis* cells transfected with empty vector pEGFP-N1 to be used as negative control for protein expression. To confirm expression a Western blot was performed following established procedures and analysed using 1:500 anti-GFP antibody (Invitrogen) as primary antibody and anti-alkaline phosphatase as secondary antibody.

IV-3 Results and Discussion

IV-3.1 Identification of the most represented EST in the SSH library

From the 300 EST resulting from both approaches of SSH, we chose to focus on those highly represented. From Direct forward subtractive library (Dfsl) we identified 3 ESTs corresponding to a sequence sharing homology with *Pisum* sp. putative senescence associated protein (Accession number BAB33421) and 3 ESTs for another unknown sequence homologous to *Nicotiana* sp. cytochrome p450- like (Accession

number BAA10929). In the enriched forward MOS library we identified 20 ESTs corresponding to a sequence similar to *Cryptosporidium* sp. putative senescence associated protein (Accession number EAL34999) and 15 ESTs corresponding to a sequence sharing homology with the *Moorella* sp. cytosine deaminase (Accession number ZP-00331135; see Chapter III).

This later sequence was chosen because it was previously well characterized in several organisms and described to exist only in bacteria and lower eukaryotes as yeast. In addition, it was thought to be relevant for negative selection, a useful property for gene therapy. If confirmed, *P. olseni* would be the first protozoan described as possessing a CD, a relevant enzyme in the pyrimidine salvage pathway, which enables the organism to utilize exogenous pyrimidine bases and nucleosides, which are not intermediate in the *de novo* pyrimidine synthesis. Pyrimidine and purine nucleotides are fundamental to life as they are involved in nearly all biochemical processes.

Purine and pyrimidine salvage pathways are characteristic of prokaryotes and lower eukaryotes and have been successfully used in the past as target pathways for drugs used to treat microorganism-induced pathologies (Kilstrrup *et al.*, 1989). This EST was thus further characterized and the structure of its cDNA, gene and encoded protein unveiled. However, while the partial sequence was firstly identified as CD, the full cDNA and encoded protein was later considered broadly as a chlorohydrolase-like protein (PoClhl) according to the significative homology hits in BLASTx. This protein thus appeared to rather belong to the amidohydrolase protein superfamily, which comprehends some of the enzymes responsible for nitrogen recycle of essential metabolites required for DNA and nucleic acid synthesis.

IV-3.2 Molecular characterization of *Perkinsus* spp. Chlorohydrolase-like

The molecular characterization of the cDNA and gene was the first step to obtain the complete amino acid sequence of the predicted protein. The EST sequence obtained from SSH spanned 234 base pairs and was used to design specific primers to amplify the complete cDNA and allow its molecular characterization. The resulting full length PoClhl cDNA spanned 1,446 bp and encoded a polypeptide with 457 amino acid residues (Figure IV-4).

The corresponding PoClhl gene spanned 2,823 bp from putative site of transcription initiation to the site corresponding to the 3' end of the cDNA. The PmClhl cDNA sequence was also fully cloned and found to span 1,459 bp and encode a 458 amino acid residue polypeptide (Figure IV-5).

The corresponding PmClhl gene was cloned and found to span 3,381 bp. Alignment of the orthologous cDNA sequences with their corresponding genes identified an equal number of 15 exons for each gene (Figure IV-6), all showing conserved canonical splicing signals (GT/AG) but with different intron sizes and types (Table IV-2). The PmClhl gene was the largest Clhl protein gene (3,381 bp) identified so far.

The promoter was carefully analysed *in silico* and its characteristics compared to other *Perkinsus* spp. promoters. PoClhl and PmClhl had no canonical TATA box, but a T- rich region was identified (Table IV-3). A comparable T-rich region was identified in other Perkinsus gene promoters such as the PmSOD1 gene, which contains a proximal TATA (TATTTTA) 34 bases from the transcription start point, a position typical of many eukaryotic promoters (Schott *et al*., 2003b), while PmNramp has a putative TATA box at -162 position (Robledo *et al*., 2004).

```
                                                                  _ gtcttacttgttctatccttactgacatc    29
 S  S  E  I  S  T  T  S  S  G  S  G  I  T  C  I  R  A  G  W  V  I  Q  V  D  E  E  N  S     30
TCC AGC GAA ATC TCC ACC ACT TCA AGC GGC TCT GGA ATT ACC TGC ATT CGC GCT GGT TGG GTT ATC CAA GTC GAT GAA GAG AAT TCT   119

 I  P  D  G  C  V  V  W  D  N  E  S  H  R  I  L  N  V  C  P  F  S  E  L  P  A  D  V  E     60
ATT CCC GAC GGT TGT GTC GTC TGG GAC AAC GAG TCC CAT CGA ATC CTC AAC GTT TGC CCT TTC TCT GAG CTC CCC GCT GAC GTC GAA   209
                                        Intron I

 T  E  H  L  P  K  H  A  I  M  P  G  M  V  N  C  H  Q  H  T  P  M  A  P  L  R  G  Y  S     90
ACT GAG CAT CTT CCC AAG CAT GCC ATC ATG CCC GGC ATG GTC AAC TGC CAT CAA CAC ACC CCT ATG GCG CCT CTC AGA|GGT TAC TCT   299
                                                                                Intron II

 D  Q  N  L  Q  D  W  L  Q  Q  Y  V  W  P  A  E  A  K  F  L  C  S  E  Y  V  K  L  G  T    120
GAC CAG AAC TTG CAG GAC TGG CTC CAG CAG TAC GTC TGG CCT GCT GAA GCG AAG TTC CTC TGC TCG GAA TAT GTG AAG CTC GGA ACT   389

 L  S  V  Y  E  M  L  L  T  G  S  T  T  F  V  D  M  Y  Q  F  P  H  E  T  A  E  V  A  N    150
CTT AGC GTC TAT GAG ATG CTT CTC ACC GGT TCG ACT ACA TTC GTC GAT ATG TAC CAG|TTC CCC CAC GAA ACG GCC GAG GTT GCT AAC   479
                                     Intron III                          Intron IV

 A  H  I  R  C  F  N  G  E  S  V  M  D  L  G  D  G  T  I  D  K  M  I  D  D  G  A  E  Y    180
GCT CAT ATC AGG TGT TTC AAT GGC GAG TCA GTG ATG GAC CTT GGT GAC GGT ACC ATC GAC AAG ATG ATC GAC GAT GGT GCT GAA TAC   569
          Intron V

 N  N  K  E  N  R  S  E  M  V  T  P  L  N  I  A  H  A  T  Y  T  V  P  K  D  K  L  K  R    210
AAC AAT AAA GAA AAT CGC AGC GAG ATG GTG ACT CCC TTG AAC ATC GCT CAT GCT ACT TAT ACG|GTT CCC AAG GAT AAG CTG AAG AGA   659
         Intron VI                                                  Intron VII

 A  A  I  A  K  P  A  G  T  L  V  H  I  H  L  N  E  S  Q  A  E  V  D  D  Y  F  K  Q  H    240
GCG GCA ATC GCT AAG CCA GCT GGA ACT CTA GTT CAT ATT CAT CTG AAC GAG TCA CAG GCC GAG GTT GAT GAT TAC TTC AAA CAG CAT   749
                                                                  Intron VIII

 E  S  A  I  D  A  I  D  E  A  G  L  L  N  D  H  L  I  A  A  H  C  V  H  M  T  D  E  E    270
GAG TCT GCA ATT GAT GCC ATA GAC GAA GCG GGC TTG CTT AAC GAC CAC TTG ATC GCC GCC CAC TGT GTG CAC ATG ACT GAC GAG|GAG   839
                                                                                Intron XI

 A  R  F  A  K  A  G  A  S  A  V  H  C  P  R  S  N  A  K  L  A  S  G  I  A  K  V  Q  R    300
GCC CGG TTC GCG AAA GCT GGC GCA AGC GCG GTT CAT TGC CCG CGC TCT AAT GCC AAG CTT GCG AGT GGC ATA GCT AAG|GTA CAG AGG   929
                                                                                Intron X

 L  D  A  G  V  N  V  C  L  G  T  D  G  P  C  S  N  N  S  V  D  M  L  Q  E  M  Q  Y  A    330
CTG GAT GCT GGT GTT AAC GTG TGC CTT GGT ACT GAT GGC CCC TGC AGC AAC AAC TCT GTG GAT ATG CTA CAG GAG ATG CAG TAC GCG  1019

 L  L  G  K  V  A  G  P  G  M  N  P  K  A  V  N  C  Y  T  A  V  R  M  A  T  I  N  G  A    360
CTC TTA GGG AAG GTT GCA GGG CCC GGC ATG AAC CCT AAA GCC GTT AAC TGC TAC ACC GCC GTC CGT ATG GCA ACC ATA AAC GGT GCT  1109
                    Intron XI

 A  V  R  R  E  S  D  L  G  S  L  E  V  G  K  L  V  D  M  I  A  I  D  L  G  R  L  E  N    390
GCT GTC AGG CGA GAG TCG|GAC TTG GGA TCT CTT GAG GTC GGC AAG CTC GTC GAT ATG ATC GCC ATC GAC CTT GGC CGT CTG GAG AAT  1199
                     Intron XII

 P  V  Y  D  P  V  S  A  I  V  Y  T  N  Q  R  S  V  T  D  V  W  I  G  G  D  R  V  V  Q    420
CCC GTA TAC GAT CCT GTG TCA GCT ATT GTG TAC ACC AAC CAG AGA TCA GTG ACC GAC GTA TGG ATC GGA GGC GAC CGC GTC GTG CAG  1289
                                     Intron XIII

 R  E  V  L  S  M  R  K  P  S  A  E  K  I  A  H  Y  Q  K  Q  I  A  E  F  K  A  E  R  E    450
AGA GAG|GTT TTG TCG ATG CGC AAG CCT TCA GCT GAG AAG ATA GCG CAC TAC CAG AAG CAG ATA GCA GAG TTC AAG GCG GAG CGA GAG  1379
    Intron IV

 V  A  A  V  Q  A ***                                                                      457
GTT GCG GCA GTA CAA GCA TAG  tttttacttgctattttaccagcagattcggatttctcaatct                  1446
```

Figure IV- 4 Nucleotide sequences of the cDNA molecule encoding *P. olseni* chlorohydrolase-like (PoClhl). Numbering on the right side is according to the 5'end of the longest cDNA obtained (identified as number 1). The stop codon is marked with asterisks, the intronic insertion positions are indicated with a vertical line.

```
                                                                                                                ttcgtccctcccatcacaactgccgct     27

 S   S   E   V   S   T   K   S   S   T   A   P   V   T   C   I   R   A   G   W   V   V   Q   V   D   E   D   N   S      30
TCA AGC GAA GTG TCC ACG AAA TCA AGT ACG GCC CCA GTC ACA TGC ATT CGT GCT GGG TGG GTG GTC CAA GTC GAT GAG GAT AAC TCT    117

 I   P   D   G   C   V   V   W   D   N   E   S   R   R   I   L   N   V   C   P   F   S   E   L   P   A   D   F   E      60
ATT CCT GAC GGT TGT GTC GTT TGG GAC AAC GAG TCT CGC CGT ATC CTC AAC GTT TGC CCT TTT TCT GAG CTC CCG GCT GAT TTC GAG    207
                                        Intron I

 T   E   H   L   P   E   H   A   L   M   P   G   L   I   N   C   H   T   H   S   P   M   T   P   L   R   G   Y   S      90
ACT GAG CAT TTG CCC GAG CAT GCC CTC ATG CCT GGC TTG ATC AAC TGC CAT ACA CAC TCC CCG ATG ACG CCC CTT AGA|GGA TAT TCT    297
                                                                                                    Intron II

 D   Q   N   L   Q   D   W   L   Q   K   Y   V   W   P   A   E   G   K   F   V   C   P   E   F   V   K   L   G   S     120
GAT CAG AAT TTG CAG GAT TGG CTT CAG AAG TAT GTC TGG CCT GCT GAA GGG AAG TTT GTT TGT CCA GAG TTT GTA AAG CTC GGC TCG    387
                                                    Intron III

 L   G   V   Y   E   M   L   L   S   G   S   T   A   F   V   D   M   Y   M   F   P   H   S   V   A   E   V   A   N     150
CTC GGC GTG TAT GAG ATG CTC CTC TCT GGT TCT ACC GCC TTT GTG GAT ATG TAT ATG TTC CCT CAC TCA GTG GCT GAG GTA GCT AAT    477
                                        Intron IV

 A   H   I   R   C   F   N   G   E   A   V   M   D   I   G   D   G   S   I   D   Q   M   I   D   A   G   A   D   Y     180
GCA CAC ATC CGA TGC TTC AAT GGC GAA GCT GTG ATG GAT ATT GGC GAT GGC TCC ATC GAC CAG ATG ATC GAT GCT GGT GCT GAT TAT    567
                Intron V

 N   N   K   D   N   R   S   D   M   V   T   P   L   N   I   A   H   T   C   Y   T   V   P   K   D   K   L   S   R     210
AAC AAC AAG GAT AAT CGC AGC GAC ATG GTT ACC CCC CTG AAC ATT GCA CAT ACC TGC TAC ACA GTG CCG AAG GAT AAG CTC AGC AGA    657
          Intron VI                                                                 Intron VII

 S   T   I   A   E   S   A   G   T   R   V   H   V   H   L   H   E   S   Q   A   E   V   D   D   Y   L   K   Q   H     240
TCT ACC ATT GCT GAA TCA GCC GGG ACC AGA GTT CAT GTC CAT CTT CAC GAA TCA CAG GCC GAG GTT GAC GAT TAC|CTC AAG CAA CAC    747
                                                                                                Intron

 E   S   A   I   D   A   L   D   E   A   G   L   L   N   D   H   L   I   A   A   H   C   V   H   M   T   D   D   E     270
GAG TCG GCT ATT GAT GCC TTG GAC GAA GCA GGC TTA CTT AAT GAC CAT CTT ATT GCG GCC CAC TGT GTG CAC ATG ACT GAT GAC|GAG    837
                                                                                                            Intron IX

 A   R   F   A   E   A   G   A   N   A   V   H   C   P   R   S   N   A   K   L   A   S   G   I   A   K   V   Q   R     300
GCT CGG TTT GCT GAG GCG GGC GCT AAT GCT GTC CAC TGT CCA CGA TCG AAC GCC AAG CTT GCT AGT GGC ATA GCT AAG|GTA CAG AGA    927
                                                                                                Intron X

 L   D   A   G   V   N   V   C   L   G   T   D   G   P   C   C   N   N   S   M   D   M   L   Q   E   M   Q   Y   A     330
CTG GAT GCT GGT GTG AAT GTG TGT CTT GGA ACT GAC GGA CCG TGT TGC AAC AAC TCG ATG GAT ATG CTA CAG GAG ATG CAA TAT GCA   1017

 L   L   G   K   V   A   G   P   V   M   S   P   K   N   V   N   C   Y   T   A   V   R   M   A   T   I   N   G   A     360
CTA CTG GGA AAG GTT GCA GGA CCT GTT ATG AGC CCT AAG AAC GTC AAC TGT TAC ACT GCA GTT CGC ATG GCC ACT ATC AAC GGC GCT   1107
                        Intron XI

 A   V   G   R   E   A   D   L   G   S   L   E   A   G   K   L   V   D   M   I   A   I   D   L   G   R   L   E   N     390
GCT GTT GGG AGA GAG GCG|GAC CTG GGC TCA TTG GAA GCT GGT AAA CTC GTT GAT ATG ATC GCC ATC GAT CTG GGC CGT TTG GAG AAT   1197
                    Intron XII

 P   V   Y   D   P   V   S   S   I   V   Y   T   N   Q   R   S   V   T   D   V   W   I   G   A   E   R   V   V   R     420
CCC GTG TAT GAT CCT GTG TCA TCA ATT GTG TAC ACT AAT CAG AGG TCA GTG ACG GAT GTG TGG ATT GGG GCC GAG CGT GTT GTC CGA   1287
                                Intron

 R   E   V   Q   T   M   C   K   P   S   A   E   K   L   A   E   Y   Q   Q   Q   I   A   D   F   K   A   E   R   E     450
AGA GAA|GTT CAG ACC ATG TGT AAG CCA TCA GCC GAG AAG CTA GCT GAG TAC CAG CAG CAA ATA GCT GAT TTC AAG GCC GAA CGC GAG   1377
    Intron IV

 A   E   S   T   A   Q   A  ***                                                                                      459
GCT GAA TCT ACC GCA CAA GCT TAG  tttcggactgttttgtttctactgrcaattctatcagctgagcctcgtgggagga                            1459
```

Figure IV- 5 Nucleotide sequences of the cDNA molecule encoding *P. marinus* chlorohydrolase-like (PmClhl). Numbering on the right side is according to the 5'end of the longest cDNA obtained (identified as number 1). The stop codon is marked with asterisks, the intronic insertion positions are indicated with a vertical line.

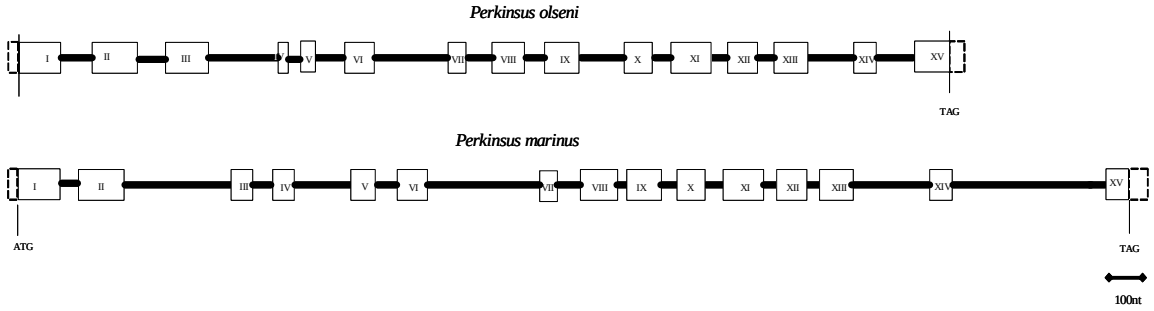


Figure IV- 6 PoClhl and PmClhl gene organization. Comparison of PmClhl and PoClhl gene structures. The exons are numbered for both species. The first nucleotide identified as number 1 for each gene corresponds to the first nucleotide of the corresponding full length cDNA.

Table IV- 2 PoClhl and PmClhl gene organization. Clhl genes exon-intron boundaries are compared. The first nucleotide identified as number 1 for each gene corresponds to the first nucleotide of the corresponding full length cDNA.

P. olseni					***P. marinus***			
Exon		**Position**	**Intron**		**Exon**	**Position**	**Intron**	
No	**Size**		**Size**	**Type**	**Size**		**Size**	**Type**
I	154	-29 - 1-125	93	1	153	1-152	48	1
II	136	219-354	81	2	136	201-336	323	2
III	130	436-565	209	0	65	660-724	55	1
IV	29	775-803	35	2	65	780-844	169	0
V	44	839-882	84	1	73	1014-1086	47	1
VI	89	967-1055	219	0	89	1134-1222	338	0
VII	53	1275-1327	75	2	53	1561-1613	72	1
VIII	98	1403-1500	56	0	112	1686-1797	31	2
IX	103	1557-1659	129	2	121	1829-1931	42	2
X	84	1787-1870	49	0	84	1981-2064	52	2
XI	121	1920-2040	49	2	121	2117-2237	44	0
XII	89	2090-2178	51	0	89	2282-2370	43	2
XIII	101	2230-2330	140	2	101	2414-2514	235	1
XIV	67	2471-2537	107	0	67	2750-2816	416	2
XV	149	2645-2792			163	3233-3395		

For *P. olseni* this T- rich motif was located at -42 and for *P. marinus* at -29 nt. In gene promoters from other chlorohydrolase family members, a Nitrogen- control motif was described (Andersen *et al.*, 1989) which appears to be present in both Po and PmClhl genes at least twice (Table IV-3). The predicted PoClhl polypeptide also presents the amidohydrolase domain (e- value 29.2 $x10^{-67}$), as expected (InterPro Scan, www.ebi.ac.uk).

Table IV-3 Putative regulatory motifs in PoClhl and PmClhl promoters. Gene promoter analysis *in silico* revealed three putative motifs (A, B and C), common to *P. olseni* and *P. marinus* species. Sequences were aligned using Clustal W and edited using WebLOGO.

Type of motif	Species	Localization	Motif name	Promoter motif
Putative NAC (ATA N9 TAT)	*P. olseni*	-105		
	P. Marinus	-146	Motif A	
	P. olseni	-52	Motif B	
	P. Marinus	-64		
T-rich sequence				
	P. olseni	-42	Motif C	
	P. Marinus	-29		

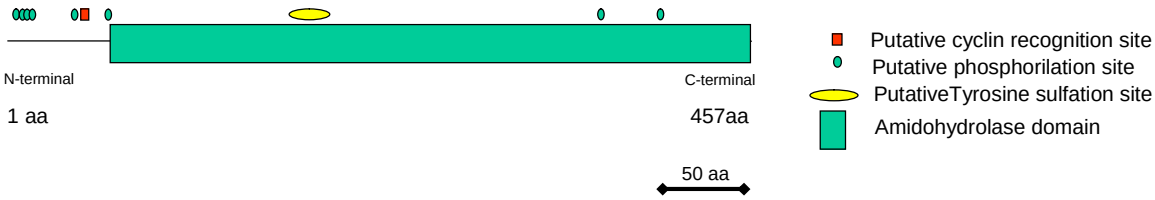


Figure IV- 7 Scheme of the predicted PoClhl polypeptide as deduced from *in silico* analysis using SignalP and Motif Scan (www.expasy.ch).

From *in silico* analysis, the PoClhl polypeptide appears as a non-secretory protein as expected, a result confirmed by SignalP-NN analysis since no signal peptide was identified. Motif Scan predicted various putative phosphorylation sites, one cyclin recognition site and one putative tyrosine sulphating site (myhits.isb-sib.ch/cgi-bin/motif_scan), as represented in figure IV-7. The comparison of the presently described polypeptides with those from amidohydrolase family demonstrated a higher similarity with the Proteobacteria, *N. oceani*, amidohydrolase (Table IV-4), described as a cytosine deaminase and related metal-dependent hydrolase according to predicted function. The *in silico* identification of PoClhl polypeptide as a member of the amidohydrolase family remains to be confirmed by other type of analysis.

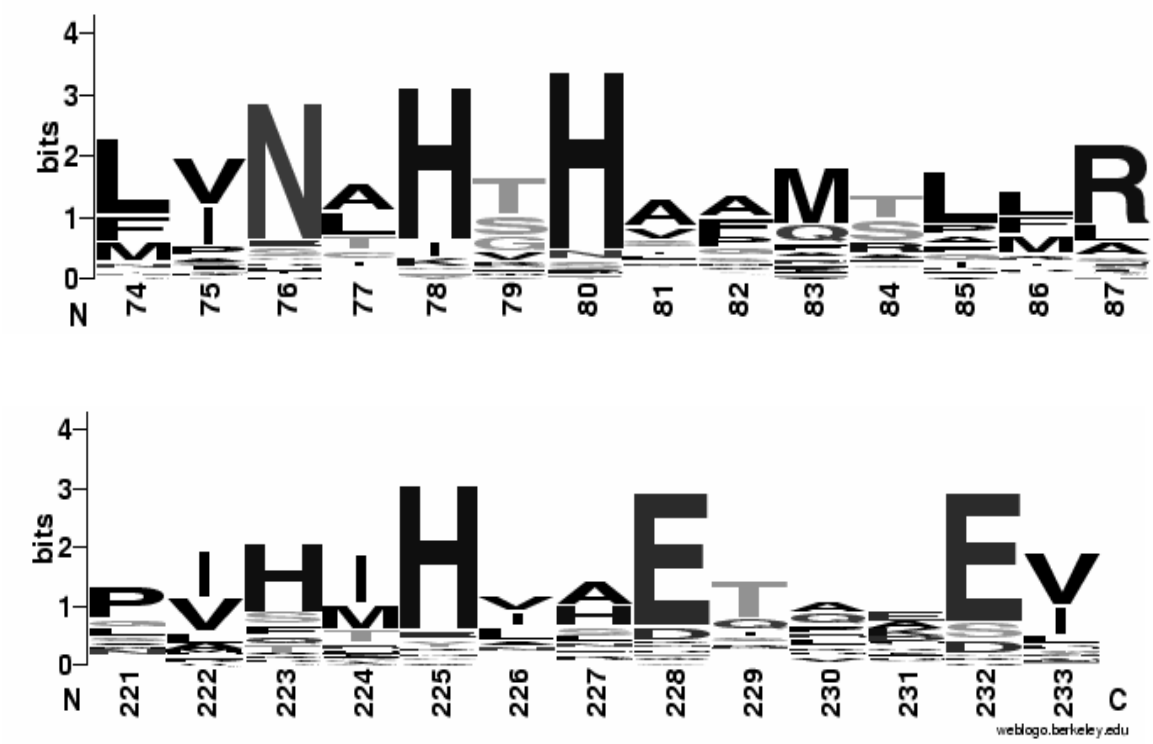


Figure IV- 8 *P. olseni* and *P. marinus* Clhl alignment with amidohydrolase proteins performed with clustalW. The schematic presentation was constructed using WebLOGO. The highlighted residues are those which are relevant for the active site and those evolutionary conserved according to height in yy axes'. Numbering of the amino acid residues is referent to PoClhl. Amidohydrolase protein sequences were obtained from NCBI data base and accession numbers are those indicated in Table IV-4.

PoClhl and PmClhl presented the metal-dependent family domain and the motif HxH, suggested to be coordinated by divalent-metals bound to specific residues. It also contains the motif HxHxxE, relevant in substrate coordination at the active site (Figure IV-8). The metal ligands, four histidines and one aspartic acid residue, are strictly conserved in the three enzyme families, and define a subtle but sharp sequence signature of a metal-dependent hydrolases superfamily (Holm and Sander, 1997). The N-terminal HxH regions proposed to contain metal-coordinating histidine residues were reasonably well conserved in all members of amidohydrolase protein family, which includes dihydroorotase, adenine deaminase, ureases, cytosine deaminases, and *s*-triazine hydrolase (Holm and Sander, 1997). For AtzA, this HxH motif was described to be related to divalent cation coordination and was, accordingly, expected to be involved in enzyme catalytic site, because the residues involved in its interaction with the substrate

are also conserved (Sadowsky *et al*., 1998). These motifs were also described as conserved for *E. coli* CD (Ireton *et al*., 2002). This metal ligand motif was found to be well conserved in *Perkinsus* sp. Clhl (Figure IV-8), thus corroborating *in silico* analysis which resolved PoClhl as part of superfamily of metal-dependent hydrolases. Among the members of amidohydrolase superfamily there are a number of metalloenzymes. From these, cytosine deaminase was identified as the most closely related to the atrazine enzymes by sequence alignments (Shapir *et al*., 2005). However, the sequence identity in pairwise comparisons among AtzA, AtzB, and AtzC was only on the order of 20% (Sadowsky *et al*., 1998). PoClhl and PmClhl were also submitted to alignment to highlight conserved residues between both sequences (Figure IV-8), revealing a maximum identity of 35% with *N. oceani* amidohydrolase (Table IV-4).

The amidohydrolase superfamily is characterized by having a conserved (αβ) 8 barrel structure (Wilson and Quiocho, 1993). Accordingly, *in silico* analysis indicates the same secondary structure for PoClhl and PmClhl. Comparison with amidohydrolase family proteins from both bacterial and eukaryotic origin showed that both *Perkinsus* Clhl proteins present conserved residues and motifs, although no conclusive proximity to one functional protein neither its corresponding evolutionary distance can be resolved. All known *s*-triazine catabolic enzymes that remove groups from an *s*-triazine ring, including AtzB, AtzC, TrzA, TriA, TrzC, and TrzN, are evolutionarily related to AtzA. Therefore, they are also members of the amidohydrolase superfamily as CD and other enzymes that potentially bind catalytically essential metals (Seffernick and Wackett, 2001). It was a surprise to find AtzA, AtzB, and AtzC placed in an ancient class of amidohydrolases (Sadowsky *et al*., 1998). However, chlorohydrolase family protein gene phylogenetic tree was not enough for meaningful in-group phylogenetic analyses.

Table IV- 4 *P. olseni* and *P. marinus* Clhl alignment with amidohydrolase proteins performed with clustalW and identity determined at Vector NTI.

Protein	Organism	NCBI accession no	% identity	% simil.
PoClh-I	*P.olseni*		100	100
PmClh-I	*P. marinus*		82	92
Amidohydrolase	*N. oceani*	ABA58753	35	68
Trza	*X. campestris*	YP_364306	32	68
Amidohydrolase	*M.flagellatus*	ZP_0056636	32	67
Amidohydrolase	*P.fluorescens*	ABA75817	31	67
Amidohydrolase	*N.multiformis*	ABB75479	33	67
Trza	*P.fluorescens*	YP_349808	31	67
Trza	*Halobacterium.sp*	AAG20370	33	67
Trza	*P.furiosus*	AAL81662	32	67
Chlorohydrolase/CD	*T.denitrificans*	AAZ96900	33	67
Trza	*P.syringae*	YP_236719	31	66
Atza	*C.tetanigi*	NP_782771	31	66
Atza	*D.desulfuricans*	YP_388304	30	66
Cytosine.deaminase	*P.aeruginosa*	ZP_0013651	31	66
Amidohydrolase	*M.burtoniigi*	ZP_0056228	29	65
Chlorohydrolase	*B.cereus*	YP_083276	30	65
Trza	*A.aeolicus*	G70352	28	65
Atza	*S.pombe*	T38111	28	64
Chlorohydrolase	*S.pneumoniae*	NP_345814	28	64
Chlorohydrolase	*M.maripaludis*	NP_988611	29	64
Trza	*O.sativa*	ABA98417	27	63
Trza	*H.marismortui*	AAV44960	22	62
Chlorohydrolase	*M.thermautotrophicus*	NP_276129	12	60
Trza	*RHOCO*	Q52725	21	58
Atza	*B.suis*	NP_700095	14	57
Chlorohydrolase	*H.pylori*	NP_207065	18	57
Atza	*P.fluorescens*	YP_346090	16	57
Atza	*P.syringae*	YP_233477	13	57
Atza	*Y.pseudotuberculosis*	YP_070485	12	56
Imidazolonepropionase	*B.bacteriovorusgi*	NP_969518	15	56
Atza	*X.campestris*	YP_363411	13	56
Trza	*B.bronchiseptica*	NP_886637	17	55
Haloalkane.dehalogenase	*LINB_PSEPA*	P51698	2	54
PcpA	*S.chlorophenolicumgi*	AAC64295	5	54
Hydrolase	*P.yoelii.yoelii*	XP_726045	1	53
Hydrolase	*C.hominis*	XP_666846	2	49
NAD.synthase	*P.chabaudi.chabaudi*	XP_745433	4	16
Glutamine-dependent synthetase	*P.yoelii.yoelii*	XP_730516	9	13

Therefore it cannot provide important insights on the phylogenetic position of *Perkinsus sp*. as a whole, on the identity of their close relatives and on specific questions of evolutionary history. In addition to the molecular characterization, functional analysis should be helpful to acquire a better description of this gene function, followed by identification of the function of the corresponding protein and improvement in the comprehension of Clhl activity.

IV-3.3 Do 5-FC prodrug and 5-FU drug affect parasite growth?

In silico analysis indicated initially a higher homology to an amidohydrolase, cytosine deaminase (CD) functionally related with a metal-dependent hydrolase.

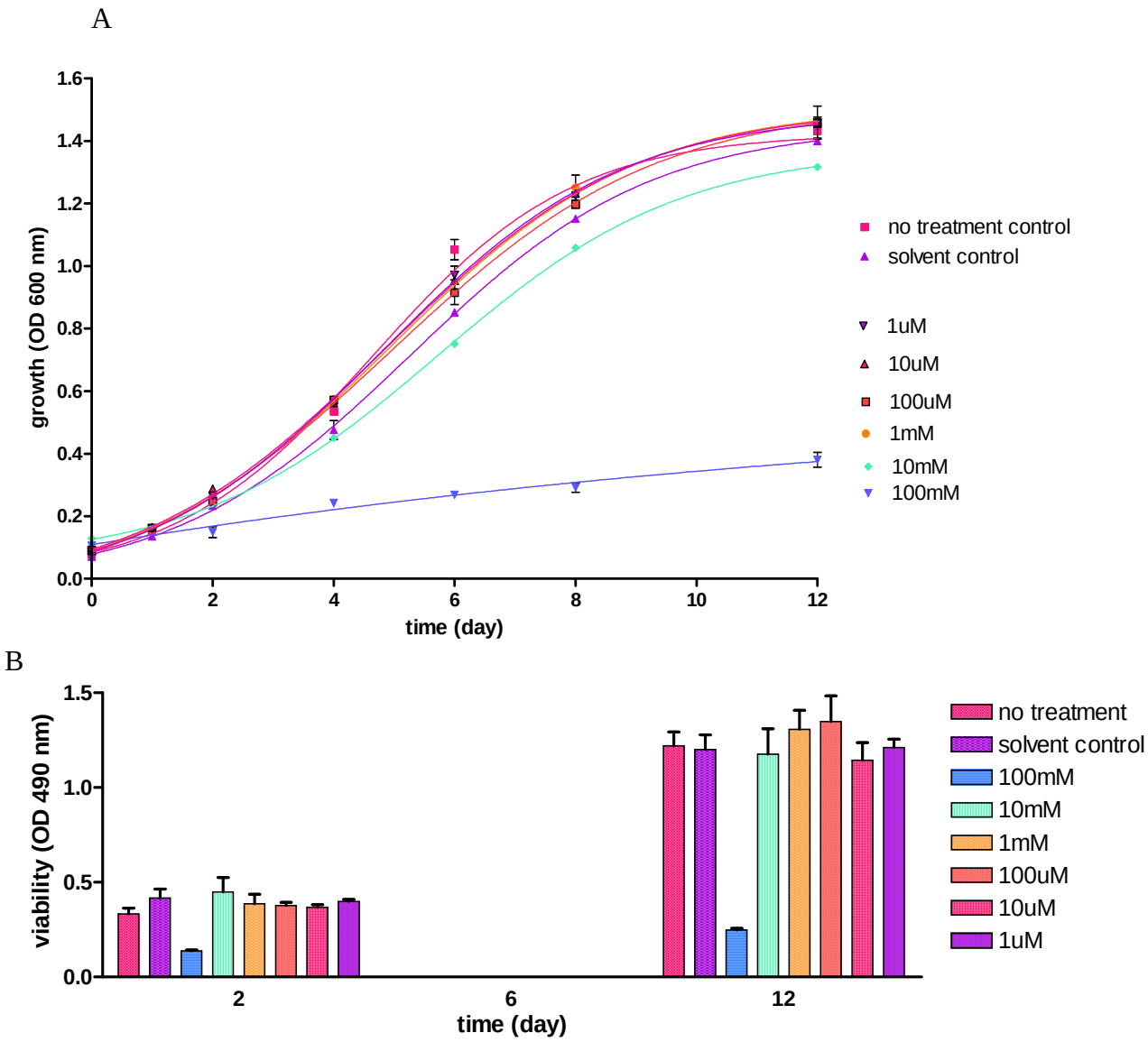
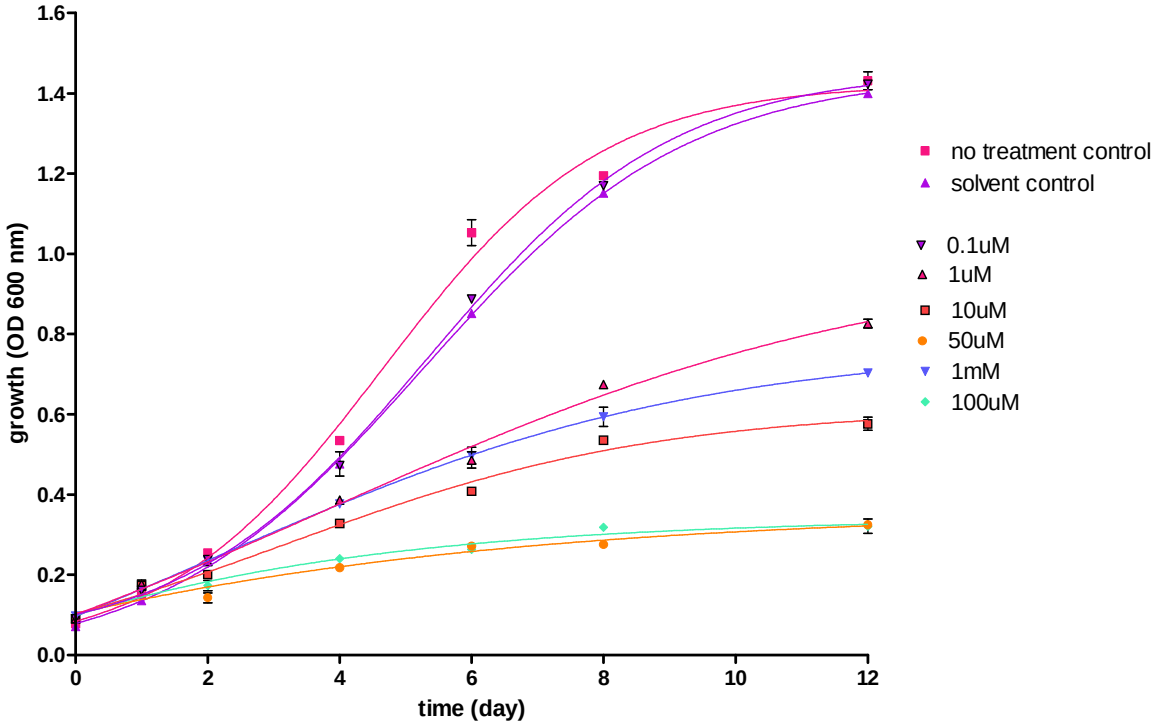


Figure IV- 9 *P. olseni* growth curve following exposure to various concentrations of the cytotoxic percursor 5-FC, during 12 days and monitored by absorbance at 600nm (A).Cell viability was checked in the 2nd and 12th day of treatment (B). Cell growth and viability under control conditions, in the absence of treatment and when treated with the vehicle solvent only.

Taking into account the specific characteristic of CD enzymes, we investigated if this putative CD was active in a similar way in *Perkinsus*. 5-FC acts as an inhibitor of both DNA and RNA synthesis through the intracytoplasmic conversion of 5-fluorocytosine to 5-fluorouracil, the cytotoxic drug.

A



B

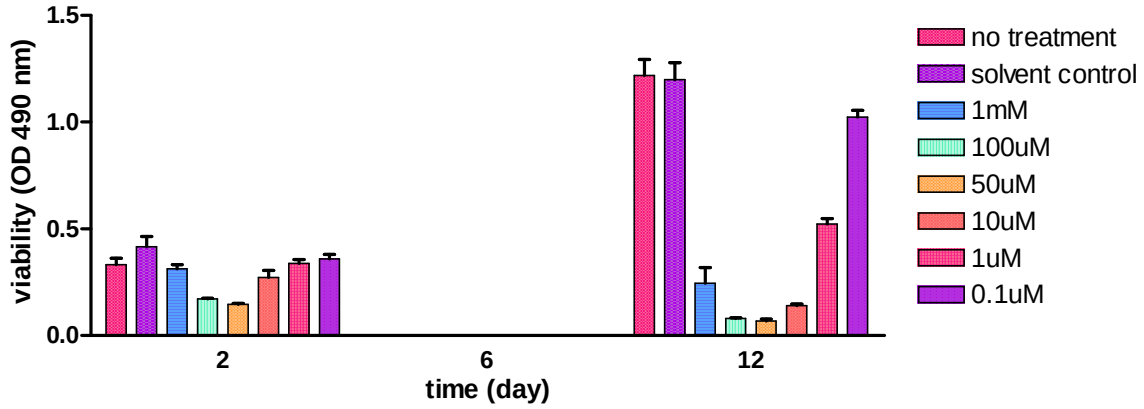


Figure IV- 10 *P. olseni* growth curve under exposure to various cytotoxic concentrations of the drug 5-FU during 12 days and monitored by Absorbance at 600nm (A).Cells viability was checked in the 2nd and 12th day (B). Cell growth and viability under control conditions, in the absence of treatment and when treated with the control solvent only.

The prodrug is a cytosine analogue that is nontoxic to cells that lack the enzyme cytosine deaminase (CD). To investigate the effect of CD specific substrates, the growth of *P. olseni* culture was observed following treatment with various concentrations of the prodrug (5-FC) and the drug (5-FU). Although *P. olseni* cell growth was apparently

affected by 5-FC at a concentration of 10mM after a 3 days exposure (Figure IV-9A), these differences were not confirmed by one-way ANOVA statistical analysis. However, *P. olseni* proliferation was heavily compromised under the higher 5-FC concentration (100mM). This fact was observed both after 2 and 12 days of treatment (Figure IV-9B) although the cytoxicity may be resultant of CD activity leading to deamination of 5-FC into the toxic product 5-FU. The statistical paired students t test analysis revealed significant difference (P= 0.0152 *). The parasite trophozoite cell culture growth was also affected by the direct cytoxicity of 5-FU at a concentration of 1mM and 10µM after 3 days exposure (Figure IV-10). In the case of *P. olseni* sensitivity to the drug 5-FU, although the means did not show significant difference (P= 0.1014), the variances appeared to be significantly different (P< 0.0001 ***).

P. olseni cell culture was obviously more sensitive to the drug 5-FU. In the 4th day of treatment the cell growth was significantly affected by the second lowest concentration, i.e. 1µM (Figure IV-10A) and cell viability was already affected in the 2nd day of treatment at concentrations between 1mM and 50 µM. In the 12th day of treatment the cells were severely affected by a range of lower concentrations, 1mM to 1 µM (Figure IV-10B) with significant differences (P= 0.0013 **) in proliferation between cells grown in the presence or absence of drug treatment. This first approach allowed the selection of the effective concentration range affecting cell growth and cell viability. Although no direct conclusion about the possibility of Clhl being a CD could be taken, it was possible to infer that this parasite possessed capability of deamination 5-FC to 5-FU, leading the prodrug to became cytotoxic.

IV-3.4 How sensitive is *P. olseni* to other chlorohydrolases substrates/inhibitors?

The proliferation of parasite cells was tested in nitrogen-free medium when the cells were submitted *in vitro* to specific enzyme substrate. In such nutrient stressing

conditions, the provided substrate was the unique nitrogen source. Knowing the substrate specificity of the hypothetical chlorohydrolases, these were tested to recognize the protein function. In contrast to cytosine, the CD specific substrate, which led to an increase in cell viability (Figure IV-11A) inducing a positive sigmoid growth- response curve (r^2=0.8378), the other substrates tested did not show any obvious utilization as nitrogen source by the parasite cells. The same time of exposure to the substrate in nitrogen-free medium, in the presence of increasing concentrations of atrazine (r^2=0.7217) and melamine (r^2=0.9521) led to a decrease in cell proliferation according to a sigmoid curve (Figure IV-11A).

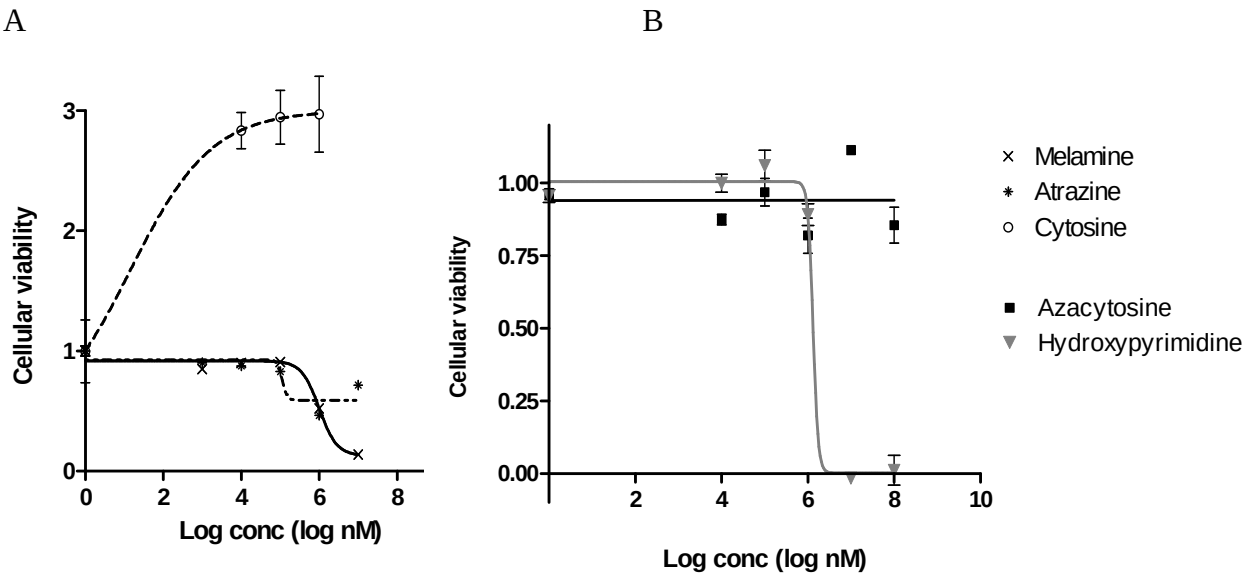


Figure IV- 11 *P. olseni* cells sensitivity to chlorohydrolases specific inhibitors (A) and specific substrates (B). The results came from 2 independent experiments with triplicates.

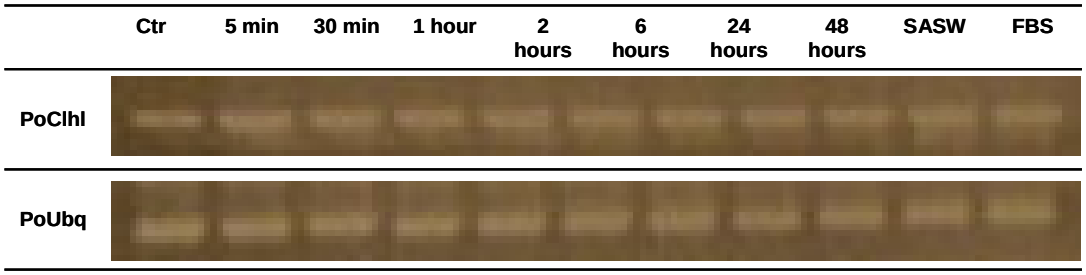
Sensitivity assays do not confirm the existence of an AtzA or a TrzA in *P. olseni* parasite. TrzA was described to catalyze the deamination of melamine (Grossenbacher *et al.*, 1984) but in our experiments no change was observed in viability or proliferation of *P. olseni* trophozoits induced by melamine. Both substrates had effects on proliferation at the higher doses tested, probably associated with toxic concentration.

From the three possible substrates tested (cytosine, melamine and atrazine), cytosine was the only one that increased cell proliferation *in vitro* (Figure IV-11A). TrzA and AtzA inhibitor, azacytosine had no effect in the range of concentrations used (deviation not significantly different from zero for P=0.9815). The cellular viability decreased with increasing concentration of hydroxypyrimidine (r^2=0.81), the CD inhibitor (Figure IV-11B). This indicates the existence of enzymes able to metabolize this single substrate as nitrogen source. By homology, *P. olseni* cells also deaminate the prodrug 5-FC into the cytotoxic agent 5-FU (Figure IV-9 and Figure IV-10). Although CD inhibitor was the only that decreased the cell proliferation, these facts could not confirm if the observed endogenous activity of CD was due to the activity of PoClhl enzyme. Altogether, our data corroborated the hypothesis of the existence of an endogenous protein in *P. olseni* responsible for the utilization of cytosine as substrate and inhibited by hydroxypyrimidine. However, the hypothesis of PoClhl being a CD could not be answered and will need further studies.

IV-3.5 Does exposure to *R. philippinarum* affects PoClhl gene expression?

Since PoClhl was the most represented EST obtained in the cDNA subtractive library constructed to identify *P. olseni* up-regulated genes in response to host hemolymph, we analysed if PoClhl gene expression was altered upon exposure of parasite trophozoites to cell- free hemolymph (serum) from the clam *R. philippinarum*, a permissive host. These clams were obtained from North Eingstown (American Mussels Harvesters, Inc) and were verified not to be infected by *Perkinsus* sps. using PCR diagnosis assay.

A



B

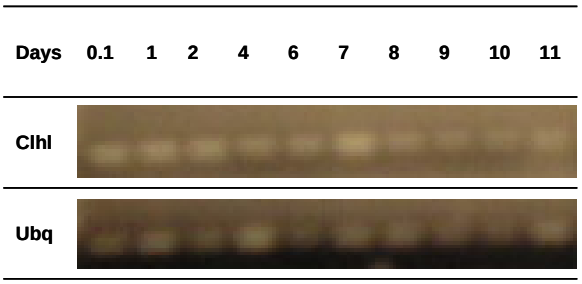


Figure IV- 12 PoClhl expression by RT-PCR. Total RNA was extracted after different times of *P. olseni* cell exposure to host serum (hemolymph cell-free): (A) exposed from 5 min to 2 days and (B) from 2 hours to 11 days. RT- PCR PoClhl gene expression was normalized to Po ubiquitin expression, used as housekeeping gene.

PoClhl gene expression was observed by RT- PCR during a 2 day challenge but no differences where observed compared to control expression of polyubiquitin gene (PoUbq) expression (Figure IV-12A). Later, the PoClhl gene expression was also analysed in response to a longer 11 day exposure, and again no change was detected in gene expression (Figure IV-12B). However, some pathogenic protozoa have the ability to *salvage* pyrimidines under nutrient stressing conditions (Marr and Müller, 1995). If Clhl was a CD there should be a relation between gene expression and the parasite exposure to host. Based on the results of specific deamination of 5-FC and increased cell proliferation by cytosine there were some evidence for the presence of an endogenous CD enzyme.

From our gene expression studies it was clear that host presence did not have any influence in PoClhl gene expression. Although the possibility of acquiring their nucleotides from their host using *salvage* pathways (Marr and Müller, 1995) is of vital importance for parasites, *in silico* evidence indicated the presence of *de novo* pyrimidine

synthesis, corroborating the fact of *Perkinsus* being a facultative parasite. Under nutrient stress conditions the parasite *P. olseni* probably can synthesize its pyrimidines using either *de novo* pathway and/ or *salvage* pathway, according to surrounding conditions. This may also explain the absence of gene regulation following host serum treatment. So PoClhl presents no visible regulation in response to host exposure. Also the fact of the parasites being maintained in just a reduced volume of cell culture medium may explain a non- stressing nutrient condition, so the *salvage* pathway would not be activated and no variations in gene expression would be observed.

IV-3.6 Is PoClhl a CD enzyme involved in the pyrimidine salvage pathway?

If PoClhl was a CD, its expression pattern would be influenced by 5-FC and 5-FU when cells were exposed to 3 different concentrations of CD specific substrate. These concentrations were tested and in all three cells survived allowing extraction of total RNA for gene expression analysis. Results showed no visible difference in PoClhl gene expression (Figure IV-13).

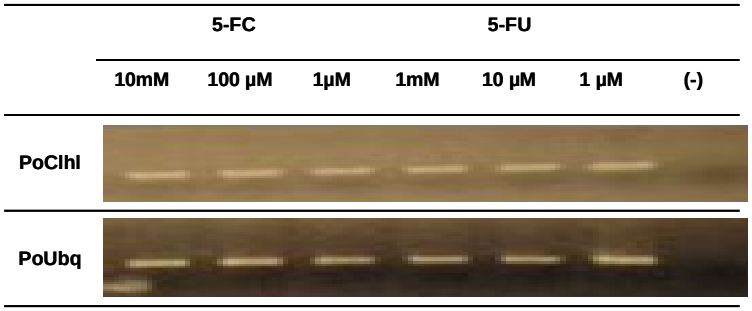


Figure IV- 13 PoClhl expression by RT-PCR. Total RNA was extracted from *P. olseni* cell culture submitted to various concentrations of the prodrug, 5-FC and to the drug, 5-FU. Changes in expression of PoClhl gene were checked and compared to those of Po ubiquitin, chosen as housekeeping gene.

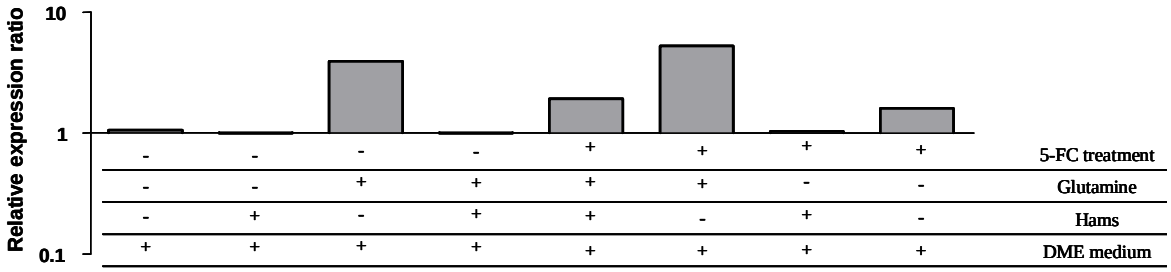


Figure IV- 14 PoClhl expression by northern analysis. Total RNA was extracted from *P. olseni* cells submitted to various treatments (Table IV-1). Five micrograms were electrophoresed, transferred to a nylon membrane, and hybridized with a 32P-labeled PoClhl cDNA and Po ubiquitin as housekeeping gene. Radioactivity signal density levels were determined to assess for relative gene expression after autoradiography, using QuantityOne (BioRad).

CD regulation is known to respond to purines, pyrimidines and nitrogen supply of the medium (Andersen *et al*., 1989). In this particular case, the cells were not under nitrogen stressing conditions and there was no visible regulation of PoClhl by either 5-FC or 5-FU arguing against a CD-like function for this protein. The hypothetical presence of CD in both *Perkinsus* species could indicate its involvement in the pyrimidine *salvage* pathway. To investigate this possibility, PoClhl gene expression was followed in the presence or absence of nucleotide precursors and glutamine, and then submitted or not to 5-FC treatment in nucleotide precursors depleted medium (Figure IV-14). Purine and pyrimidine nucleotides are fundamental for life being the monomeric units of DNA, RNA and ATP and being involved in nearly all biochemical process. Most pathogenic protozoa seem to be able to synthesize pyrimidines *de novo* but their ability to *salvage* pyrimidines is limited (Marr and Müller, 1995). Both metabolic pathways are present in *P. olseni* and *P. marinus* (Leite *et al*., unpublished). PoClhl was regulated in the artificial nutrient stress conditions tested. When 5-FC was available, this CD specific substrate increased PoClhl gene expression except in the presence of glutamine. The nutrient availability in Hams nutrient mixture masked the up- regulation observed in the presence of 5-FC as substrate (Figure IV-16).

PoClhl transcription was up-regulated in the presence of glutamine, the most important metabolite in the pyrimidine pathway, which induces CD in order to balance uracil/cytosine nitrogen bases. Nitrogen source was described to be responsible for the regulation of *codBA* operon of *Escherichia coli* (Muse *et al*., 2003) corroborating our results for PoClhl gene expression. In the absence of nutrient mixture, i.e. the Hams medium that supplements the media with amino acids such as proline and alanine, and in the presence of glutamine, there was a clear up-regulation of Clhl. In contrast, the presence of nucleotide precursors in the medium reduced gene expression to the constitutive levels (Figure IV-14). These results could be related to the fact that alanine metabolism may lead to uracil synthesis. In alanine's presence, as also for other nucleotide precursors, thymidine and hypoxanthine alternative metabolic pathways could be used to maintain nitrogen base levels in the cell, instead of using *salvage* pathway (Figure IV-15). Hypoxanthine presence in the medium decreased PoClhl expression (Figure IV-14) as was described for *E. coli codBA* expression (Muse *et al*., 2003). PoClhl was up-regulated by 5-FC in the absence of Hams, consequently, hypoxantine may explain a reduction of this gene expression in presence of 5-FC (Figure IV-14). Surprisingly, in contrast with data observed for PoClhl and for *E.coli codBA*, it was previously published that hypoxanthine increased *K. pneumoniae* gene expression and increased also the sensitivity to fluorocytosine for *K. pneumoniae* (Muse *et al*., 2003), a result difficult to explain in light of both *P. olseni* and *E. coli* available data.

Figure IV-15 illustrates a possible hypothesis to interpret the results obtained through a model diagram showing the up-regulation of Clhl in the presence of glutamine, which is determinant in two steps of pyrimidine metabolism. So, data on

PoClh gene expression may help to deduce the presence of a CD activity in the pyrimidine *salvage* pathway (Figure IV-15).

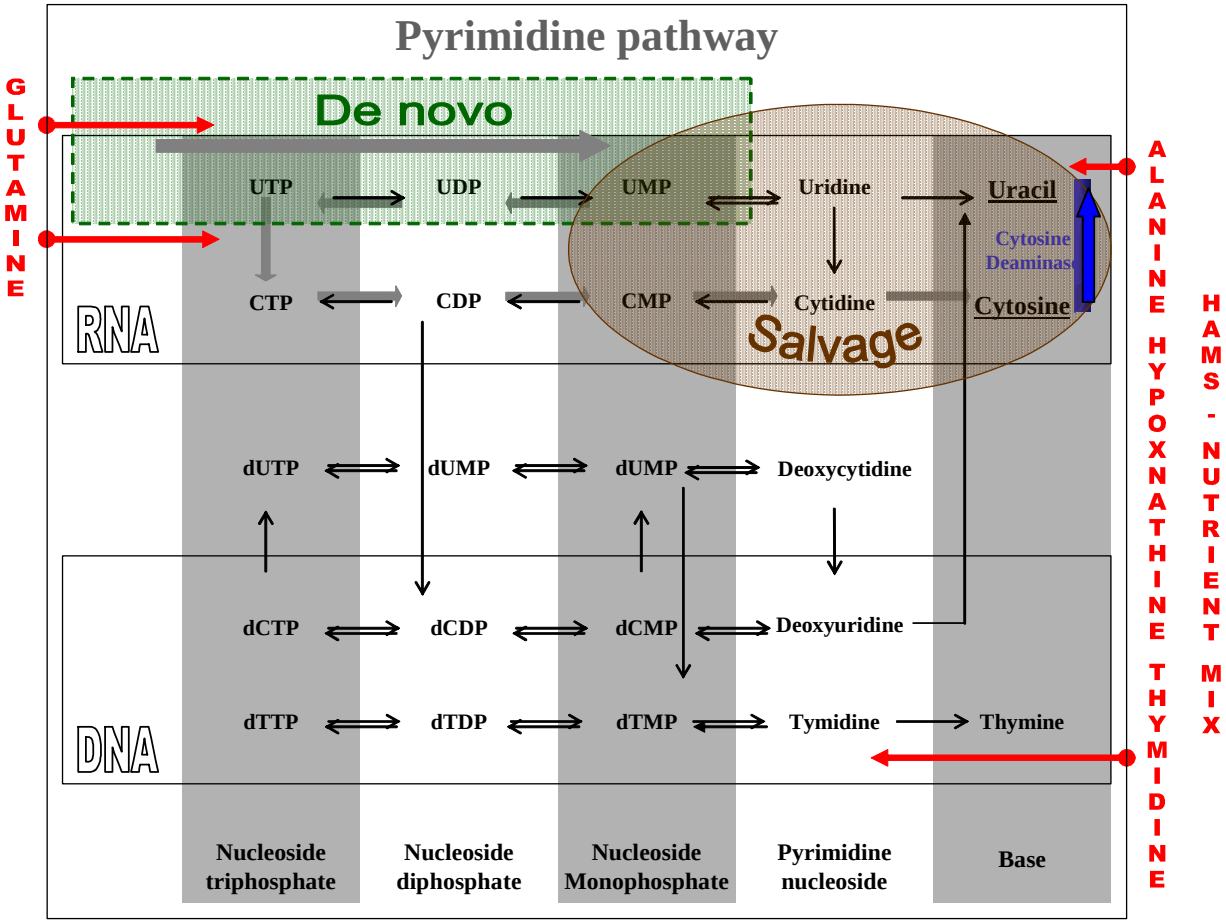


Figure IV- 15 Influence of the glutamine and Hams nutrient mixture in the pyrimidine metabolic pathway. Grey thick arrows represent the CD up-regulation in presence of glutamine and of nutrient mixture components, which can supplement the basal levels of nitrogen through other metabolic pathways. Circled and curve legend refers to the pyrimidine *salvage* pathway and *de novo* pathway is delimited by discontinuous lined box.

This model explains also the observed decrease in PoClhl gene expression when the parasite was incubated in a nitrogen supplemented medium, like Hams nutrient mixture presence. In the absence of additional nutrients and glutamine, the cell expresses basal levels of Clhl. When glutamine was added, the gene was up- regulated. This effect was less significant when nutrients were also added to the medium, suggesting they were sufficient to provide the correct amount of nucleotides for normal cell metabolism without requirement of a *salvage* pathway. Addition of an exogenous

substrate such as 5-FC led to an additional increase in Clhl expression except when nutrients were provided and glutamine was absent. According to the gene expression pattern and by comparison with the pyrimidine metabolic pathways it was possible to hypothesize that PoClhl behaved as a CD. This response could be related to the presence of Ntr transcription regulation since a putative Nitrogen-control motif, previously found in the promoter region of other chlorohydrolases, was also present in Clhl promoter region for both species (Table IV-3). A similar situation was already observed for *E. coli codBA* (Muse *et al.*, 2003). Altogether, parasite cells responded in agreement with the hypothesis that Clhl enzyme was involved in the pyrimidine *salvage* metabolism. But additional gene expression studies were performed in order to clarify if PoClhl was a CD or other member of the family of chlorohydrolase proteins.

IV-3.7 Is PoClhl endogenous expression affected by specific substrates?

PoClhl gene expression was analysed in response to the various specific substrates and inhibitors of the potential chlorohydrolase proteins. There were no significative differences in gene expression in response to any specific substrate tested (Figure IV-16), only a slight up-regulation in response to cytosine, the CD substrate, compared to not treated (basal line 1). PoClhl gene regulation was not significantly associated with either pyrimidine precursors or with the specific substrates. Only CD substrates showed a slight up-regulation effect (Figure IV-16).

After analysing all the data obtained, no clear conclusions could be taken from the gene expression analysis. In a similar study, in two bacterial strains isolated from soil, *atz* genes showed a basal expression, as observed for *P. olseni*, but in response to atrazine treatment the gene was up-regulated (Devers *et al*., 2004), a result that was was not observed for PoClhl. Since PoClhl gene expression indicated that *P. olseni*

possessed an enzyme involved in nitrogen metabolism, it probably has no association with atrazine degradation. Based on the effect of CD substrates and inhibitors on *P. olseni* cells, further studies must be performed at the protein level to ascertain whether the hypothesis of PoClhl being a CD is real.

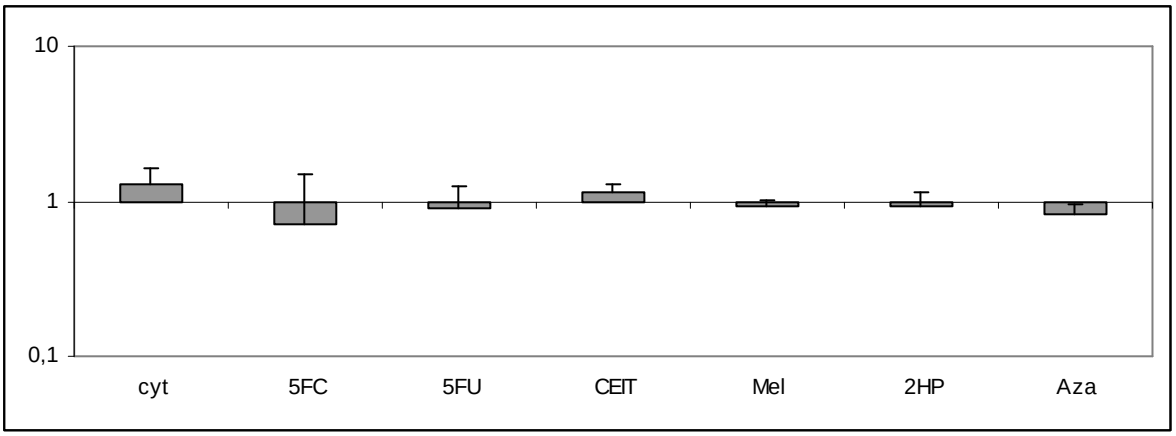


Figure IV- 16 PoClhl gene expression in response to Clhs specific substrates and inhibitors. The gene expression was analysed by Real Time pPCR, when the parasite cells were submitted to EC_{50} concentration of each chemical. PoClhl gene expression was determined and compared to expression of PoUbq, used as housekeeping gene (substrates: cyt states for cytosine, 5FC for 5- fluorocytosine, 5FU for 5-fluorouracil, CEIT for Atrazine, Mel for Melamine and inhibitors: 2HP states for 2- hydroxypyrimidine and Aza for Azacytosine).

IV-3.8 Was PoClhl *in vitro* expressed a functional protein?

The TNT® Quick Coupled Transcription/Translation Systems (Promega) was convenient for single-tube, coupled transcription/translation reactions to study *in vitro* translation. In this case and to ensure the synthesis of PoClhl protein, two reactions, experiment and control, were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and compared. The predicted size expected for PoClhl was 50kD (Vector NTI) but no protein band was visible at this MW making it impossible to ensure that it had been indeed transcribed/translated. This did not allow us to investigate if there were any differences between control and experiment (Figure IV-17). Since there is no antibody available, there was no possibility of confirmation by western blot. The functional assays were performed as an attempt to detect the activity of the protein even

though there was no visible presence of the protein in 1 and 2 µl of reaction product. For functional analysis, various concentrations of each substrate were observed under all wave lengths and the expected results of decreasing absorbance with time were not observed. So activity of the hypothetically translated PoClhl could not be confirmed.

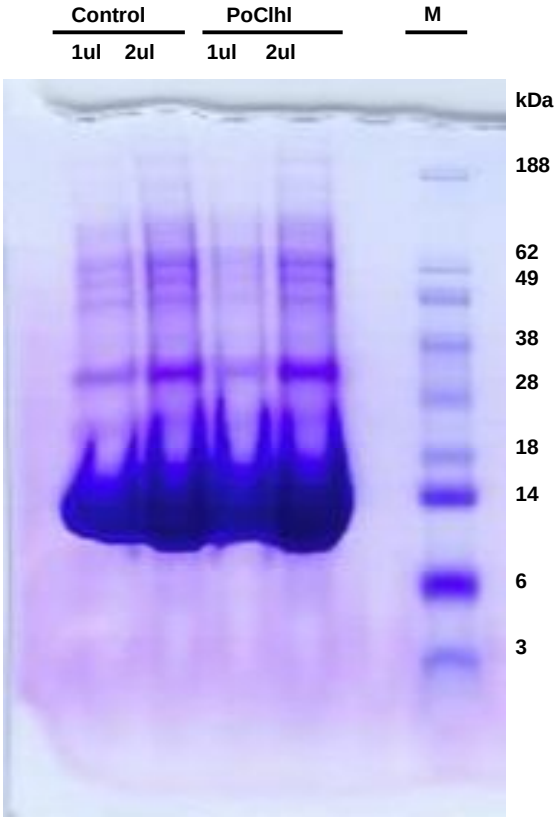


Figure IV- 17 TNT protein *in vitro* expression using the TNT quick coupled transcription and translation system (Promega) and SDS- PAGE in reductant conditions followed by CBB staining. M signs for SeeBlue protein Marker (Invitrogen).

IV-3.9 Is the PoClhl protein expressed and functional in the prokaryotic system?

PoClhl was successfully cloned in pET3His-2 vector kindly provided by Marc Ohresser and BL21 competent cells were transformed with the construct PoClhlpET3His-2. The construct PoClhlpET3His-2 was confirmed by sequence analysis after being cloned in DH5-alpha and the isolated vector was later cloned in BL21 p-Lys cells. Two clones were identified as positive, used to obtain the final

construct, and then used for PoClhl protein functional assay. They were designated PoClhlpET3His-2_BL21-41 and 45. These clones were stored in 50% glycerol at -80ºC.

First, no significant differences in the protein content were observed (P= 0.2998) for BL21 cells, either transformed with the pET3a empty vector or with PoClhlpET3His-2 (Figure IV-18A), as quantified by BCA protein assay (Pierce) and during 3 hours.

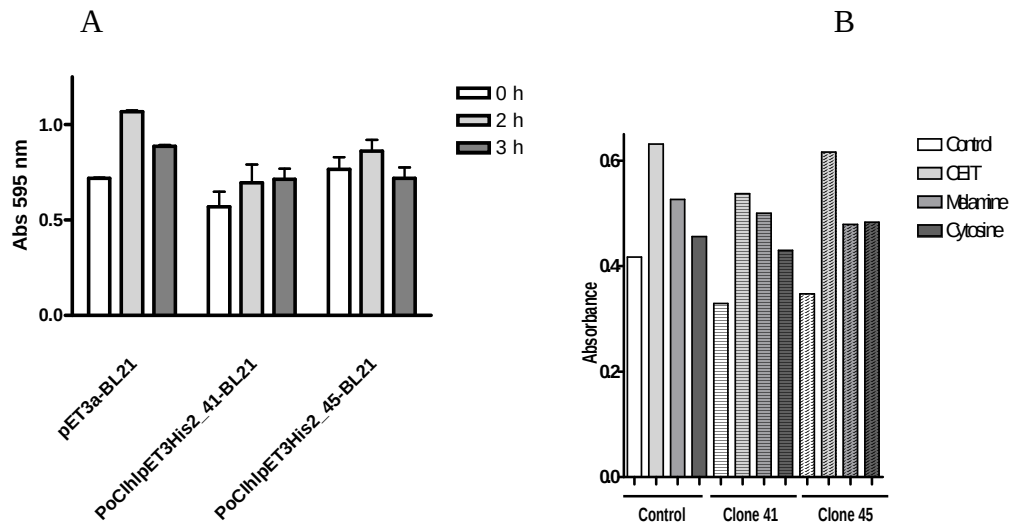


Figure IV- 18 Protein activity observation in the BL21 transformants with PoClhlpET3His-2. Quantification of protein content using BCA protein assay (Pierce) along time (A) and preliminary results of measurement of substrates specific absorbance at 39 hours to check PoClhl activity (B).

Although results showed no differences in protein expression between the control, empty vector and the two clones with the construct, preliminary functional assays were performed. The PoClhl protein activity was not visible using 1 mM of IPTG inducting the reaction of substrate degradation. This was observed by decreasing specific OD of each substrate (Figure IV-18B). The substrate degradation using the cell extracts revealed no PoClhl activity also.

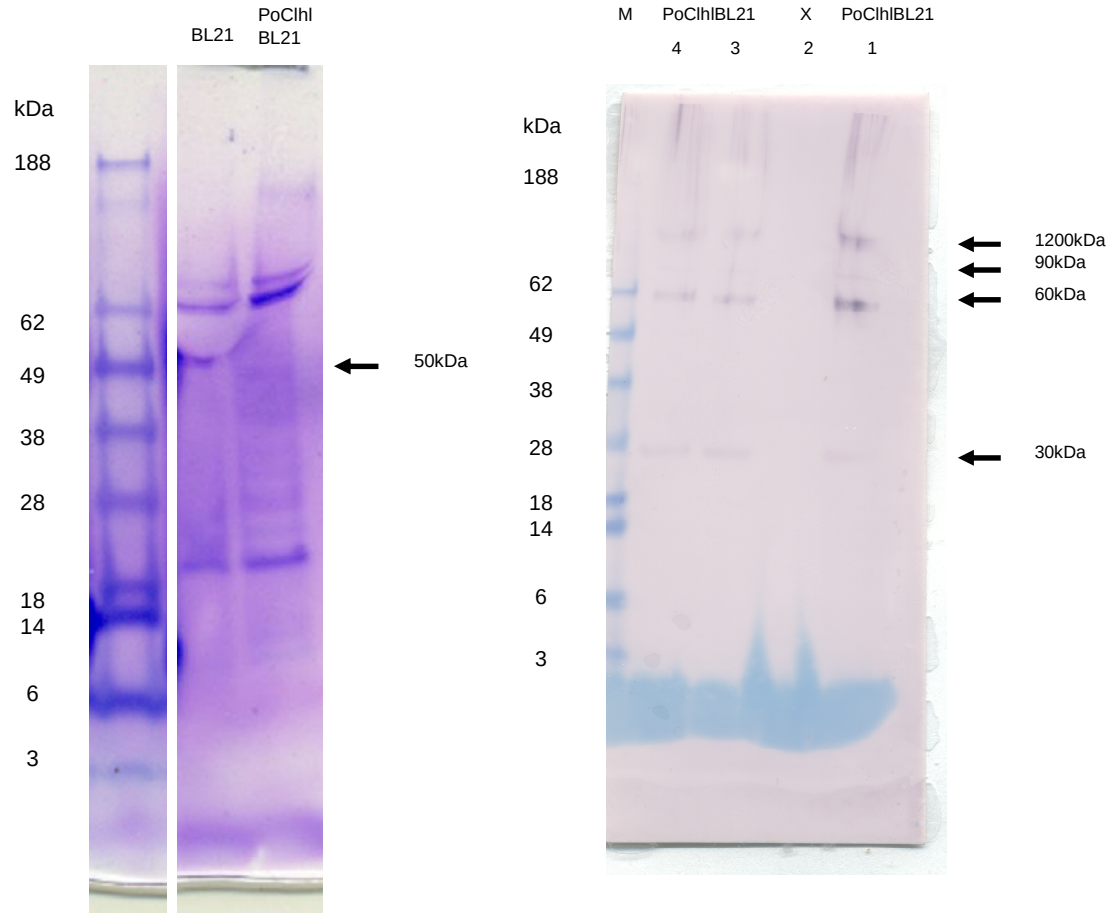


Figure IV- 19 PoClhl protein expression in prokaryotic system. PoClhlpET3His-2_41 expressed by BL21-pLys induced ON at RT by IPTG and electrophoresed in 4-12% polyacrilamide gel compared to BL21 induced (A) and PoClhlpET3His-2_41 and 45 SDS-PAGE and western blot with anti-his, SaBGP-his alfa primary antibody and anti- alkaline fosfatase secondary antibody (B).

The positive clones maintained the construct but there was no observable protein activity along time using the substrates mentioned. It were also performed resting cell assays using the two clones and the control plated in N-free medium in order to observe the growth of the clones cultured in presence or not of N-source and in presence of each substrate as the only N-source. No differential growth was observed between the various plates with different N-source substrates. The induction of protein expression in the BL21 expressing clones (PoClhlpET3His-2_BL21-41 and 45) was tested using different IPTG inductor concentration.

The induction and growth was then performed at 20mM IPTG. This was the minimum concentration that resulted in the maximum increase in protein content. The

expected 50kDa protein was isolated by his- tag MaxWell system (Promega) from BL21-pLys cells induced by 20mM IPTG, overnight (ON) at room temperature (RT) and the protein production was checked by SDS-PAGE (Figure IV-21A). PoClhl expression was confirmed using anti- his- tag antibody by western blot using crude extracts and isolated proteins from induced cell cultures grown for 2 hours at 37ºC and ON for both clones were obtained. It was observed a smaller protein of 30kDa and typical aggregation of His-tagged protein with multiples size (Figure IV-19B). In conclusion, although PoClhl was expressed in prokaryotic system, the protein function was not observed in crude extracts.

IV-3.10 How was the PClhl expression and activity in the eukaryotic exogenous system?

Clonogenic assays were performed in a eukaryotic system to express functional PoClhl and assess the sensitivity of transformants to cytosine, melamine and atrazine in nitrogen-free medium. The *X. laevis* epithelial cell line was used and previously assayed for the enzyme activity to ensure no endogenous activity in presence of the substrates in study.

X. laevis A6 cells responded in a sigmoid-dose response curve to 5-FU (0.9559) while for 5-FC the linear regression reveals that the deviation from zero is not significant (Figure IV-20). So although the prodrug was observed, no toxic effects were detected since those eukaryotic cells do not possess the CD enzyme that converts the prodrug 5-FC to the cytotoxic 5-FU. For this fact this model was used to check if the PoClhl is a CD and identify its function. The presence of PoClhl transcripts in both *Perkinsus* sp. suggested the existence, in these parasites, of a nitrogen active enzyme. This hypothesis was supported by preliminary results suggesting that the Clhl behaves

as CD when expressed in the eukaryotic exogenous system. The protein activity in transient transfected cells confirmed the utilization of cytosine as substrate as also the transformation of 5-FC into the cytotoxic drug 5-FU.

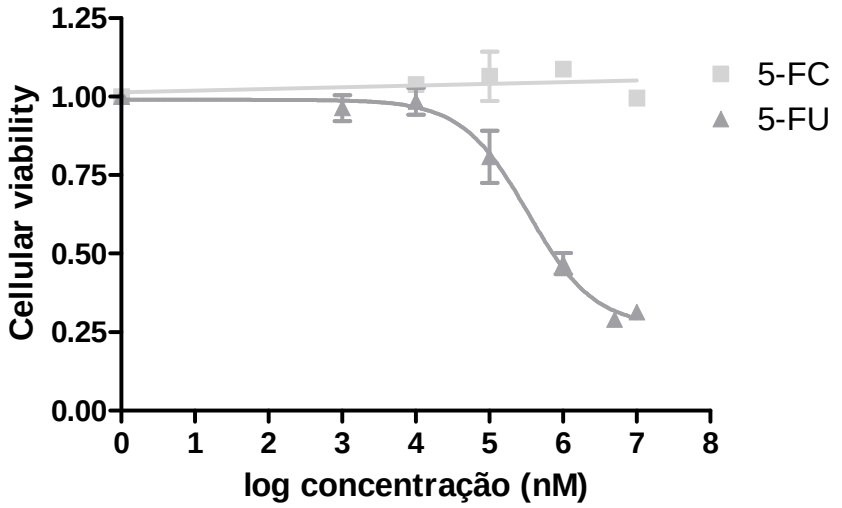


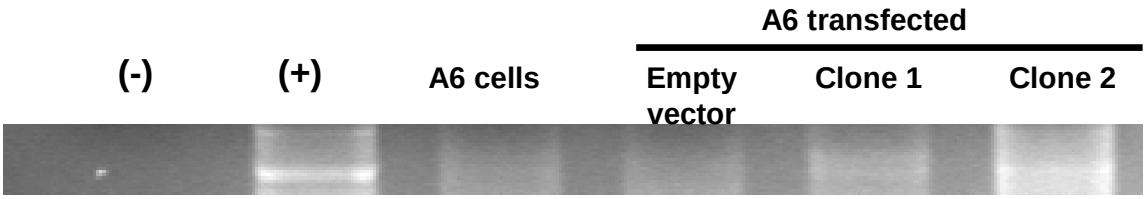
Figure IV- 20 *X. laevis* kidney epithelial derivate cells sensitivity to prodrug, 5- FC and drug, 5- FU.

To confirm these preliminary results, we needed to develop stable clones expressing PoClhl to make an activity assay. A6 cells transfected with PoClhlpcDNA3 were submitted to selection in order to try to develop clones that would have kept and integrated the construct into their genome. The positive clones were stored in liquid nitrogen and used to test for protein activity, by checking cells for their sensitivity to specific substrates and inhibitors. Clone stability was observed by RT-PCR which confirmed the presence of the construct and its transcription (Figure IV-21A). Although not statistically significant, for clone 1 there appeared to be a slight decrease in cell viability when they were submitted to 2-HP, the CD specific inhibitor (a result needing confirmation). However the substrates that appeared to increase cells proliferation were melamine and atrazine (CEIT, Figure IV-21B). No conclusive results could be taken

after a second experiment that did not corroborated the first results. So, it was not possible to infer if PoClhl was being expressed neither if PoClhl was active as a CD.

A second approach was performed cloning PoClhl fused to EGFP protein using pEGFP-N1. A6 cells were definitely transfected with this construct. There was a confirmation of protein expression in the eukaryote system by detection of the fluorescent protein (Figure IV-22A). Western blot using the cell extract showed a band of a size different from what was expected which could indicate that the expected protein was displaying a different behaviour in the gel, appearing to run at a MW less than the expected (Figure IV-22A).

A



B

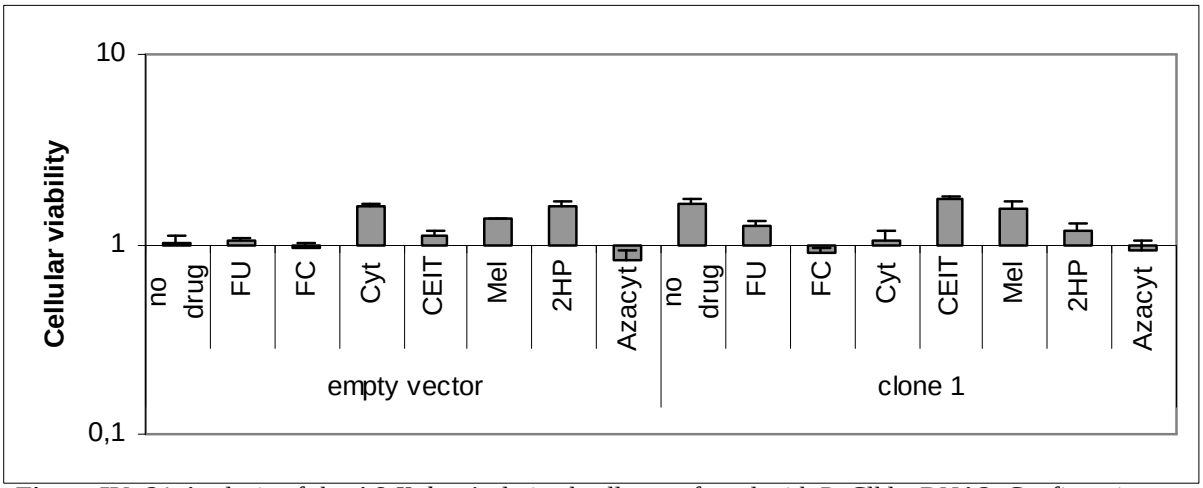


Figure IV- 21 Analysis of the A6 *X. laevis* derived cells transfected with PoClhlpcDNA3. Confirmation of the presence of the construct/ PoClhl expression (A) and analysis of the transfected cells sensitivity to PoClhl potential substrates and inhibitors (B).

A careful observation of the fluorescence in the cells revealed a differential localization of EGFP, mainly nuclear, while PoClhl-fused-EGFP was mainly

cytoplasmatic (Figure IV-22B and Figure IV-22C). Despite the various tests of functionality checking for substrates specificity, no further information was obtained in terms of PoClhl activity, which could indicate that the fusion protein was not active. One clone expressing EGFP and 6 clones expressing PoClhl-fused-EGFP were cryopreserved and further studies must be performed using different strategies in order to ascertain whether the expressed protein is functional or not. If it was proven to be a cytosine deaminase, this *P. olseni* protein could then be tested for GDEPT after calculating the degree of activation (Springer and Niculescu-Duvaz, 2000) According to its characteristics this could be a potential tool for negative selection as previously pointed out for other systems and compiled in Table IV-5.

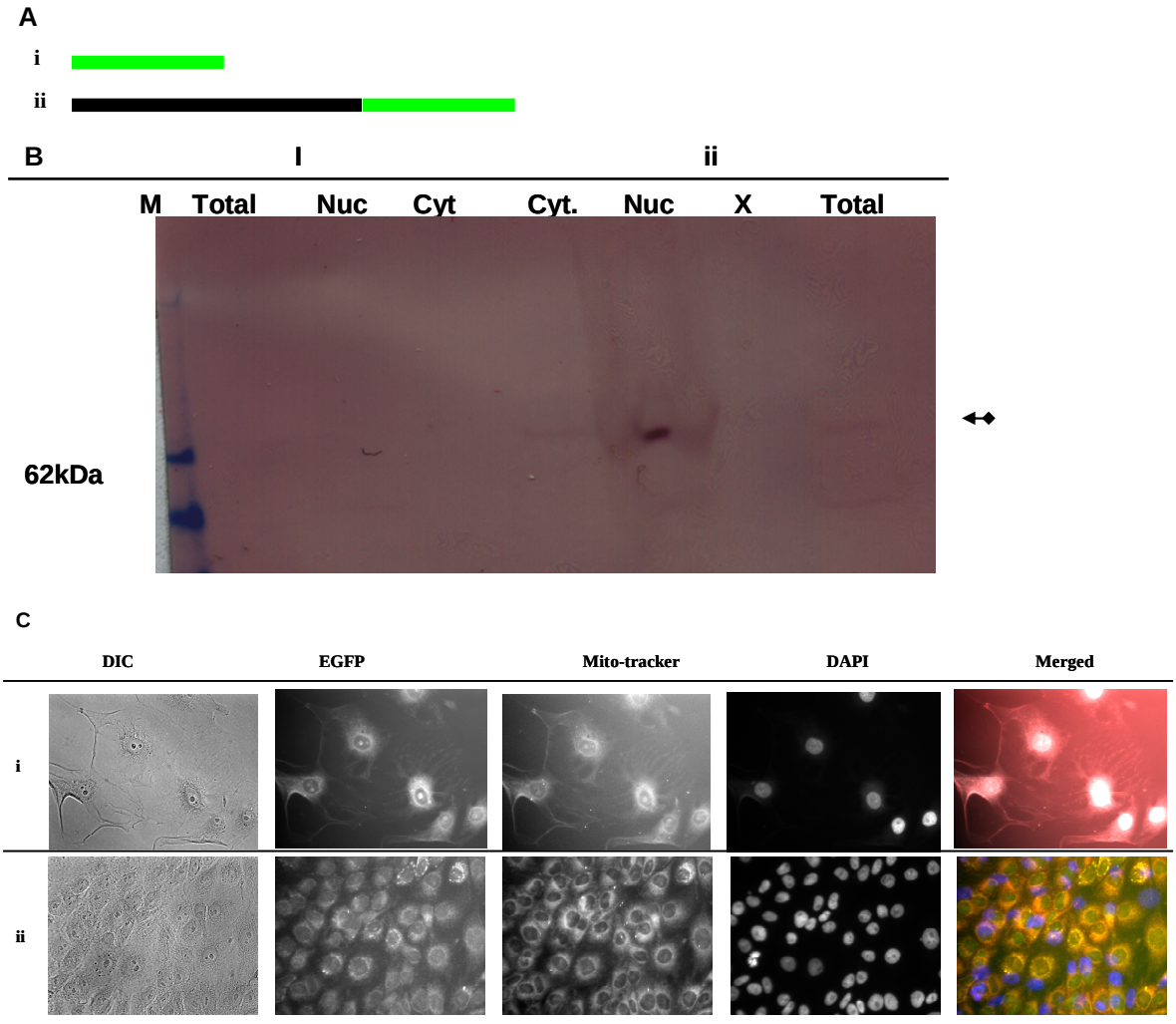


Figure IV- 22 Expression analysis of protein α by Western blot. A6 cells transfected with 0.5 µg of plasmid pEGFP-N1 (i) and PoClhlfusGFP, green bar indicates EGFP and black bar indicates protein

PoClhl (ii) EGFP- fusion protein (A) was harvested at 48 h posttransfection and assayed using anti-GFP by Western blot analysis (B). Localization of EGFP-α fusion protein in transfected A6 cells. Cells were transfected with PoClhlfusGFP plasmid with Fugene6 (Roche). A6 cell expressing EGFP- fusion protein at 36 h posttransfection: image of nucleus stained with DAPI, image of mitochondria stained with Mitotraker and merged images. The merged image represents a superimposition of green, blue and red signals where areas of fluorescence co-localization are yellow-orange. The images were viewed with a Leica 550 IW fluorescence microscope and photographed in 200× amplification.

Table IV- 5 Quantitative data on (GDEPT) systems. Comparison of various systems for GDEPT based on the data from Springer and Niculescu-Duvaz (2000).

		Potency, IC50 (uM)				
no	Enzyme/ prodrug system	Prodrug	Drug	Potential of activation[B] (fold)	Degree of activation[C] (fold)	Clinical trial
1	CA/CPT-11	1.6–8.1	SN-38: 0.003–0.011	150–3,000	7–17	1
2	XGPRT/6-TX, 6-TG	6-TX > 50; 6-TG = 0.5	Ara-MT*P* < 1Q K	K	6-TX: >20; 6-TG: 10	None
3	CPG2/CMDA, CJS278H	CMDA: 1,700–3,125; CJS278: 0.256	CMBA: 8–65;	CMDA: 26– 390; Doxorubicin: 11	CMDA: 10– 115;	None
4	Cyt-450/CP, IF, ipomeanol, 2-AA	CP, IF ~ 4,000	Doxorubicin: 0.012	K	5–60 50–100L	1
5	dCK/ara-C	0.3–0.6	J	K	2–100	None
6	SV-TK/GCV, ACV	GCV: 200–600	K	K	20–1,000	> 21
7	NR/CB1954	> 1,000	GCVTPK	> 50,000	14–10,000	None
8	PNP/6-MePdR	> 200	0.02N	25–1,000	40	None
9	TP/5¢-DFUR	17	3.7	7000	165	k
10	VZV-TK/ara-M	> 2,000	5-FUdR: 0.0023	> 2,000	55–600	None
11	CD/5-FC	26,000	5-FU: 4–23.5	100–8,000	70–1,000	2
12	PoCD/5-FC	33.884	4.149	8.16	?	None

IV-4 Conclusions

P. olseni chlorohydrolase like protein was found to be the most represented transcript from the cDNA subtractive library of parasite host- response. Both *P. olseni* and *P. marinus* presented these novel described proteins and *P. olseni* gene was up-regulated only in the presence of 5- FC as nitrogen source. PoClhl presented a basal expression in several conditions studied, namely in response to host exposure and environmental stressing conditions. PoClhl gene expression was increased in nutrient stressing conditions in the presence of CD specific substrate, while the presence of CD specific inhibitor resulted in decreased PoClhl gene expression, reinforcing the hypothesis of PoClhl being a CD. This was a potentially important result since this

protein was never described in protozoan parasites. Given the possibility that this gene could be involved in nitrogen metabolism, and some preliminary evidence suggested that PoClhl could be a CD enzyme involved in pyrimidine *salvage*, functional studies were attempted using an exogenous system. However, no observable activity was detected using the *in vitro* synthesized protein, nor in protein expressed in the BL21-pLys prokaryotic system. These functional assays were based on substrate degradation observed through the decreasing absorbance at each substrate specific wave length. Another attempt was made using a eukaryotic system, but the results were again not conclusive. Endogenously, both *Perkinsus* sp. parasite trophozoites revealed sensitivity to 5-FC, the prodrug and CD specific substrate. *P. olseni* presented an increased cell proliferation with cytosine as complementary source of nitrogen and was affected by hydroxypirimidine, a CD specific inhibitor. All this corroborated the inference of PoClhl being a CD but we could not confirm this data by functional assays and therefore further studies are required. Interestingly, since the expression of PoClhl did not appear to be affected in most of the conditions tested it could be used as a reference gene for gene expression analysis in *Perkinsus*. In subsequent work in our laboratory we have already used PoClhl as a housekeeping gene with success (Leite *et al*., 2008).

CHAPTER V – PARASITE RESPONSE TO SURROUNDINGS

"Host-parasite interaction is important to the parasite protozoa, since their survival depends on the host cells that supply the environmental and nutritional requirements" by William Trager from John Guardiola, L. Luzzatto and William Trager (1983) Molecular Biology of Parasites, Raven Press

V-1 Introduction

The protozoan pathogen *P. olseni* (Lester & Davis, 1981) is known to infect clam beds along southern Europe including French, Spanish and Portuguese coasts. However, it has been associated with episodes of massive mortalities of the carpet-shell clam *Ruditapes (Tapes) decussatus* (Linnaeus, 1758) essentially in southern Portugal, in the 80ies (Azevedo, 1989b). Available data suggests that environmental conditions, such as temperatures, salinities and pollutants, may be involved in this higher rate of mortality, but until now there is insufficient knowledge concerning the molecular response of this parasite to its host, thus precluding our understanding on how these factors can affect infection. *R. decussatus* and *R. philippinarum* are both susceptible bivalves to *P. olseni* infection but Perkinsosis does not significantly affect the wedge-shell clam *Donax trunculus* (Linnaeus, 1767), the blue mussel *Mytilus galloprovincialis* (Lamarck, 1919) or the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) and Japanese oyster, *C. gigas* (Thunberg, 1873), which also constitute important fishing resources. These bivalves have different habitats within the same coast, with *D. trunculus* found in the open, wave exposed sites, while the other two species inhabit more sheltered areas. The wedge-shell clam is known not to be infected by *Perkinsus* although it may be affected by other parasites as Rickettsia (Comps and Chagot, 1987). Similarly, *C. gigas* is not affected although on the East Coast of the United States; although, populations of a closely related species, *C. virginica,* have been devastated by another member of this parasite genus, *P. marinus*, for many years (Burreson and Ragone Calvo, 1996).

In Europe, environmental conditions seem to be related to *Perkinsus* lethality. Indeed, and in contrast with the high mortalities observed in Southern Portuguese coast, *Perkinsus* infection does not appear to cause serious mortalities in the infected beds of

R. decussatus in the Galician coast, Northwest of Spain, being speculated that the higher water temperatures in the southern regions may impact on disease severity (Villalba and Casas, 2001). On the other hand, in Asia, *Perkinsus* sp. was considered to be the cause of epizootic mortalities of *R. philippinarum* in Korea (Park *et al*., 1999), China (Liang *et al*., 2001) and Japan (Hamaguchi *et al*., 1998). However, results of short-necked clam disease surveys in Korea (Lee *et al*., 2001) indicated that caution should be applied when determining a causal relationship between *Perkinsus* sp. infections and *R. philippinarum* mortalities. Pollution has been also hypothesized to contribute to some aquatic epizootics (Chu and Hale, 1994) and estuarine contaminant mixtures have been shown to increase oyster susceptibility to parasitism by *P. marinus* (Bushek *et al*., 2002b). Molecular characterizations of the parasite in response to these various factors that affect the host-parasite interaction were not explored yet. Biochemical analyses have been conducted showing the involvement of parasite-associated extra-cellular proteins (ECP), especially proteases, in the pathogenicity of *P. marinus* (Oliver *et al*., 1999, Peyre, 1996). In *P. olseni*, we have recently identified, through a subtractive hybridization strategy, a set of parasite genes differentially expressed upon host infection (Ascenso *et al*., 2007). As a continuation of that work (Chapter III), we next evaluated their expression pattern in the presence of permissive, more or less susceptible and nonpermissive bivalves. A selected set of these parasite genes were used to construct a cDNA macroarray in order to compare their levels of expression when *Perkinsus* was challenged with hemolymph from infected and non infected *R. decussatus*. Two of them, up-regulated more than one hundred fold in response to the presence of its non-infected host, were further analysed by cloning their full length cDNAs and analysing the corresponding genes and encoded proteins. Their pattern of

expression was then analysed when *P. olseni* was challenged with hemolymph from permissive and non permissive bivalves, or subjected to environmental stresses.

V-2 Specific materials and methods

V-2.1 Construction of cDNA macroarray

A series of cDNA macroarrays were constructed using a total of 98 cDNA fragments identified from a previously constructed subtractive library (Ascenso *et al.*, 2007). To construct the macroarray, selected cDNAs cloned in pCR II TOPO vector (Invitrogen, Carlsbad, USA) and pGEM T-easy (Promega, Madison, USA) as well as control vectors, were purified, denatured, spotted onto a nylon membrane (Amersham Biosciences, UK) manually and cross-linked by a 2 hour incubation at 80ºC.

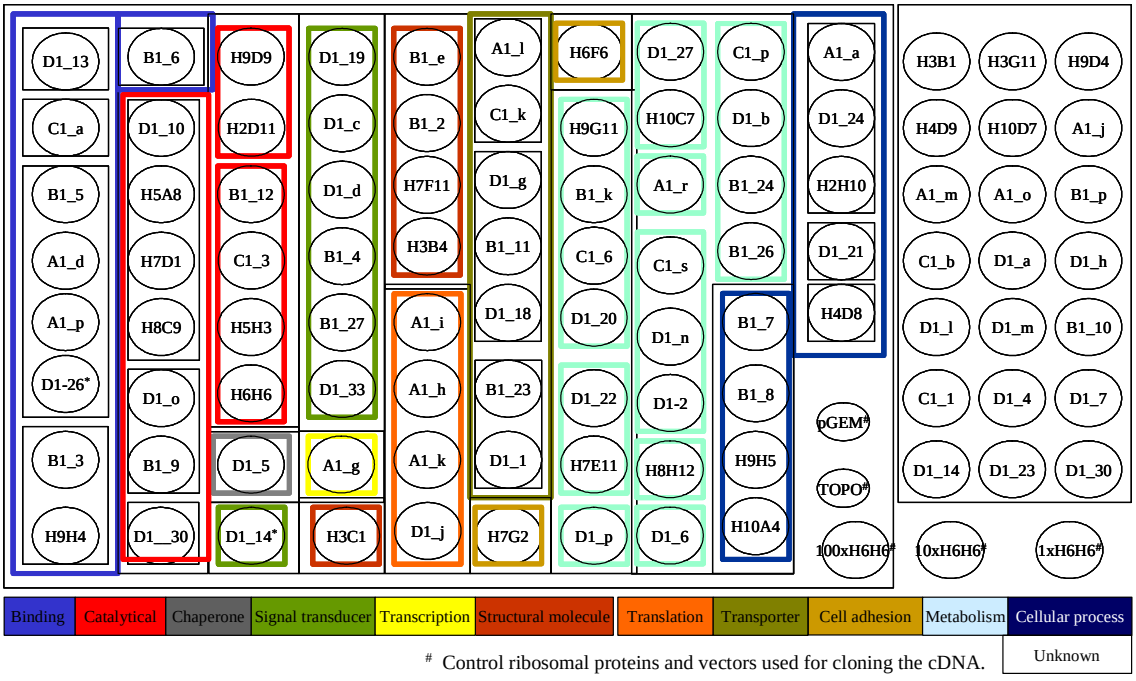


Figure V- 1 Scheme of the membrane containing the cDNA macroarray. Representation of all 98 cDNA clones included in the macroarray, organized according to molecular function according to the GO categories. The array also included both vectors used for EST cloning, reference genes (*) and three dilutions of one selected clone, as internal standard.

Four replica nylon membranes were constructed, each of them contained 98 unique cDNA clones, the two empty plasmid vectors, possible reference genes, signed with (*) and one clone spotted in three different dilutions as internal control (Figure V-1).

V-2.2 Differential gene expression in response to bivalve's hemolymph

Ten micrograms of total RNA was labelled and used as probe to hybridize the array filters. Each individual radioactive probe was obtained by reverse transcription in were normal dCTP was substituted by α^{32}P dCTP. In brief, the RNA was heated to 70°C to remove secondary structures and labelled by reverse transcription for 1.5h at 37°C in the presence of 50 µCi α ^{32}P dCTP, 0.8mM each dATP, dTTP, and dGTP, and 400U M-MLV reverse transcriptase (Invitrogen), added in two steps. cDNA synthesis reaction was stopped by treatment at 70°C for 30 min with 5 µl of 0.5M EDTA and denaturation performed with 10µl of 0.1M NaOH, and then equilibrated at room temperature for 15 min. Neutralization was performed by adding 25µl of 1M Tris–HCl. Unincorporated nucleotides were removed by filtration using a microspin column (Microspin^{TM} S.200 HK columns, Amersham Biosciences, Piscataway, USA). Purified probes were denatured for 5 min at 100°C, cooled on ice for 5 min, then added to hybridization buffer (UltraHyb, Ambion, Austin, USA) and incubated with membranes overnight at 42°C. After hybridization, membranes were washed three times in 0.1x SSC, 0.1% SDS (10min/wash) at 42°C, and then exposed to Kodak BioMax MS film (Kodak, USA) for different periods to optimize signal intensity quantification. Two independent hybridizations were performed to calculate the response of parasite genes to host hemolymph challenge. Prior to re-hybridization, membranes were stripped with three successive immersions in boiling 0.1% SDS for 15 min (Mazurais *et al.*, 2005) and probe removal confirmed by autoradiography. The impressed signals were quantified by

densitometry (QuantityOne, BioRad, Richmond, USA) and levels of gene expression were calculated relative to expression of the same housekeeping genes in control conditions.

V-2.3 Molecular characterization of genes of interest

Partial cDNA sequences of *P. olseni* genes of interest were obtained by sequencing the clones resulting from screening the forward cDNA subtractive library. Two ESTs (PoNHE and PoAdh) were identified as highly expressed (over 100 fold) in parasites upon challenge with non-infected host hemolymph and thus selected to be further characterized. Complete cDNAs were obtained through rapid amplification of cDNA ends using a Marathon cDNA library (Clontech BD, Palo Alto, USA) previously constructed in our laboratory (our unpublished data), using gene specific primers (Frw1 and Rev1, Appendix II-1). The resulting PCR products were inserted into pCR II TOPO vector (Invitrogen), cloned in DH5- alpha cells (Sambrook *et al*., 1989) and final identification achieved following DNA sequencing (Macrogen, South Korea) and homology search using tBLASTx tool (http://www.ncbi.nlm.nih.gov). Gene structure organization was achieved by amplification using two *P. olseni* Universal Genome Walker libraries (Clontech) previously constructed in our laboratory (our unpublished data). Then, using the gene specific (Frw1 and Rev1, Appendix II-1) and adaptor specific primers, selected genes were PCR amplified upstream and downstream from the known sequence, following manufacture recommendations. Resulting PCR products were cloned and sequenced, as previously described. There was 100% identity between PoNHE and PoAdh genes and the corresponding cloned cDNAs.

V-2.4 *P. olseni* clonal culture and parasite cell treatments

The clonal cell line of *P. olseni* (ATCC 50984) was cultured in DME:Hams F12 (1:2) medium supplemented with 5% Foetal Bovine Serum (FBS) according to the method of Gauthier and Vasta (Gauthier and Vasta, 1995b, Robledo *et al.*, 2002). Cultures were maintained in exponential growth phase at 28ºC for 3 days with no medium changes before treatment. *P. olseni* log phase cell cultures were expanded and grown in 80ml medium at 28ºC, for 2 days, then pelleted to reduce the volume prior to treatments. The parasite host-challenged condition was performed by incubating the parasite cells in a 12-well plate exposed to bivalve hemolymph through a 0.4 µm membrane (Transwell, Costar, Cambridge, MA), for six hours. Specifically, *P. olseni* cells were challenged with infected and non infected hemolymph from either permissive bivalves (*R. decussatus* and *R. philippinarum*) or perkinsosis-nonpermissive bivalves (*C. gigas, M. galloprovincialis* and *D. trunculus*). Reference condition was the parasite cells exposed to infected *R. decussatus*.

Table V- 1 Culture conditions used in vitro to simulate *P. olseni* environmental stress conditions.

Environmental conditions	Temperature	Salinity
Control condition	28ºC	30‰
Salinity stress conditions	28ºC	26‰
	28ºC	37‰
	28ºC	31‰
Temperature stress conditions	15ºC	30‰
	20ºC	30‰

To study the response of *Perkinsus* to environmental xenobiotics, parasite trophozoite cells were exposed to two types of pollutants, organic (PAH, PCB and TBT) and inorganic (heavy metals, cadmium, copper and zinc). Pollutants were added to 8 ml

cell cultures; each compound was dissolved in DMSO solvent at concentrations of 10ng/ml for TBT, 360ng/ml for PCB, 145ng/ml for PAH, 500ng/ml for Cd, 150ng/ml for copper and 250ng/ml for Zn. Incubation with cells was performed for 72h. The adequate concentrations to be tested were obtained following toxicity curves developed for each compound studied.

To study the response of *Perkinsus* to environmental conditions (Table V-1), cells were acclimated for a week in the conditions to be tested, e.g. temperatures (15ºC and 22ºC) and salinities (26‰ and 37‰). Then $1x10^6$ cells were transferred to a new culture vessel with 8 ml of medium and incubated for 72h in the same conditions prior to collection. Following total RNA extraction, gene expression was analyzed by Real Time qPCR.

V-2.5 Gene expression analysis by Real Time qPCR

Reverse transcription-PCR was performed to further investigate and quantify differential expression of genes of interest. Total RNA isolated from challenged and control *P. olseni* cells were used for expression analysis using Real Time qPCR. First-strand synthesis was performed after DNase treatment with RQ1 RNase-Free DNase (Promega) using 1µg of total RNA, gene specific reverse oligonucleotides and M-MLV reverse transcriptase (Invitrogen). Real-time PCR was performed in a iCycler iQ system (Bio-Rad) with master mix for SYBR Green (ABgene, Surrey, UK), using the following primer sets: (i) PoNHE Frw1/Rev2 to amplify PoNHE (accession number AB439284), (ii) PoAdh Frw2/Rev1 to amplify PoAdh (accession number AB439285) and (Chalikonda *et al.*) PoClhl Frw1/Rev1 to amplify PoClhl, the housekeeping gene used for this study. Each sample was prepared by adding 2 µl of a 1:10 cDNA dilution to reaction mix containing 0.5 µM of each primer and 10 µl of ABsolute^{TM} Q PCR SYBR

Green Flurescein mix (ABgene), in a final volume of 20 µl. The PCR program comprised an initial cycle of 10 min at 95ºC followed by 60 cycles comprising an initial denaturation step at 95ºC for 30 sec, and a step of annealing/extension at 68ºC for 20 sec. The fluorescence was measured at the end of each extension cycle in the FAM-490 channel. Relative levels of expression were determined by comparing results of treatments with data from control cells. For relative gene expression determination, all experiments were performed at least twice and in triplicate.

V-3 Results and Discussion

As part of our ongoing efforts to understand the molecular events involved in the response of parasite *P. olseni* to the presence of its dedicated host, the carpet-shell clam *R. decussatus*, 300 *Perkinsus* ESTs were previously identified (Ascenso *et al*., 2007) following screening of a forward subtractive library and classified by Gene Ontology, the majority without known counterparts in other organisms. From these, 98 were further characterized *in silico* and their expression analyzed through macroarray hybridization in response to challenge with hemolymph from permissive and nonpermissive bivalves. Among these genes highly up- regulated (e.g. more than 100 fold) in the presence of host *R. decussatus* hemolymph, two were selected due to their apparent homology to genes encoding electron transport and cell adhesion proteins, two functions possibly involved in host-parasite interaction. The two genes identified were further characterized as an attempt to provide clues suitable to unveil their function.

V-3.1 Does *P. olseni* respond differently to permissive & non-permissive bivalves?

Variations in the population density of host species lead to variations in the parasite burdens of host species; in some cases this allows pathogenic parasites to establish in populations of hosts that would otherwise be too small to sustain them, as happens with *P. olseni*, in *R. decussatus* population in Ria Formosa (Leite *et al*., 2004). In the Algarve southwest coast there are various native commercially relevant bivalves, which include the carpet-shell clam, the Pacific and Portuguese oyster, the blue mussel and the wedge-shell clam, and these are not equally infected by the parasite *P. olseni*. The blue mussel was never described as being infected while *R. decussatus* is its endemic host (Azevedo, 1989b). Therefore, in this work we investigated the molecular response of *Perkinsus* to challenge with hemolymph of these various bivalves of interest using a cDNA macroarray. A total of 98 cDNA fragments selected from the two *P. olseni* library screenings, were used for construction of the macroarray (see chapter III for details), 72 from Dfsl and 26 from efMOSl (Table V-2). To study the parasite response to various bivalves, the genes selected were those related to cell adhesion, binding, chaperone, signal transducing, transport and cellular process because they are known to be involved in host parasite interaction in other biological systems. In addition, cDNAs from genes involved in transcription, translation, structural, catalysis and basal metabolism for parasite survival were included. Furthermore, twenty one clones corresponding to hypothetical parasite genes overexpressed in the presence of hemolymph from *R. decussatus*, and showing no homology to any previously identified ESTs (Table V-2) were also selected and included in the array. Through one step radioactive hybridization of the macroarray, the relative expression of these selected parasite genes in response to various bivalves' hemolymph, was identified. After removal of the background, their

average relative gene expression was calculated and the number of genes up-regulated one hundred fold or more in response to each bivalve (*R. decussatus*, *R. philippinarum* and *D. trunculus*) hemolymph was counted and reported in Figure V-2. Fifty eight genes were up-regulated in response to *R. philippinarum* hemolymph (59.2 %). The majority of the genes presented an increased expression in response to *D. trunculus* hemolymph (84 genes, 85.7 %). Only eight did not present this high expression level (>100x), but they were still up-regulated when compared to control, the parasite exposed to hemolymph from infected *R. decussatus* (Ascenso *et al*., unpublished a).

Table V- 2 Macroarray selected gene clones related to parasite host-response classified according to GO molecular function category (Appendix V-1), using GOblet server (http://goblet.molgen.mpg.de).

Genes related to host-parasite interaction		
GO molecular Function		**Occu-rrence**
Category	**Sub-category**	
Obsolete	Cell adhesion	2
Binding	Purine nucleotide binding Dna binding Rna binding Transition metal ion binding Cytoskeletal protein binding	9
Chaperone	Chaperone	1
Signal transducer	Receptor activity Signaling molecule	7
Transporter	Aminoacid transporter Electron transporter Cation transporter	7
Cellular process	Membrane transport Cell growth and death Cellular motility Cell communication Immune system	9
Genes related to basal metabolism		
Transcription	Transcription regulator	1
Translation	Translation regulator	4
Structural molecule	Ribosome component	5
Catalytic activity	Hydrolase Lyase Ligase Transferase Oxidoreductase	13
Metabolism	Carbohydrate metabolism Energy metabolism Lipid metabolism Nucleotide metabolism Aminoacid metabolism Glycan metabolism Polyketides metabolism	19
Not classified:	hypothetical, unknown	21

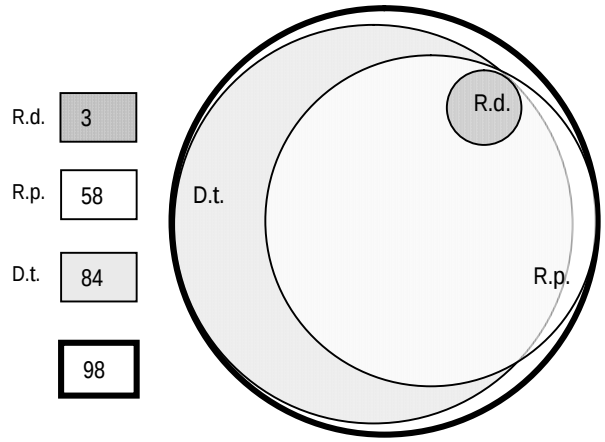


Figure V- 2 Number of parasite genes up-regulated one undread fold or more (log≥2). The genes corresponding to parasite response to each bivalve's hemolymph were counted. R.d. states for *R. decussatus*, R.p. for *R. philippinarum* and D.t. for *D. trunculus*.

Even when a parasite can develop in a large number of different hosts, it usually develops best in one or two host species and less readily in the rest. The vast majority of parasites are restricted to one or a few closely related species, and this is what is meant by host specificity. An apparent resistance, or non-susceptibility, may result from a lack of adaptation of the parasite to a new host (Langand *et al.*, 1998). It may be that, once a parasite is exposed to a bivalves' hemolymph for the first time it naturally activates a large number of gene, from basal metabolism to possible defense and invasive related genes. This may have been the case when observing the number of parasite up-regulated genes in response to hemolymph from wedge-shell clam. *R. philippinarum* hemolymph also induced a high level of response in number of genes up-regulated. These clams, known to be permissive but less susceptible to this parasite, were bought from French aquacultures and only some presented low infection levels, thus explaining the strong response of the parasite. The weaker parasite response was towards its endemic host, *R.*

decussatus, that were collected from Ria Formosa, all heavily infected, which again may explain the differences observed between the parasite response to *R. decussatus* and *R. philippinarum*, since both bivalves are permissive to the parasite infection (Ascenso *et al*., unpublished a). According to Navas and colaborators, the parasite, *P. olseni* shows a special incidence in *R. decussatus* (Navas *et al*., 1992). In infecting its permissive host, where the parasite is able to invade and infect easily, it looks like the parasite spends less energy in gene expression machinery in response to its host environment. In this case the fact of the clams being infected may have contributed as an additional factor to diminish the host defences against the parasite and facilitate the parasite invasion with fewer expenses. A non-permissive bivalve is resistant to the parasite and either the parasite does not survive or exists only at a very low level of parasitemia. This may imply an increase in parasite costs to invade and/ or defend from host defences, corresponding to *P. olseni* active response to *D. trunculus* hemolymph. From analysis of the host-parasite system *Biomphalaria glabrata*/*Echinostoma caproni*, the host non-susceptibility was genetically determined (Langand *et al*., 1998). No specific studies of parasite host-response involving permissive and non-permissive hosts are known so a clarifying comparison could not be made.

V-3.2 Which genes were highly up-regulated in response to *R. decussatus?*

Parasite gene expression showed a differential response when challenged with various bivalves but among these, our interest was focused on its dedicated host, the carpet-shell clam. Macroarray screening by differential hybridization confirmed that 84 (85.7 %) of the 98 selected genes were up-regulated at least 2x (Figure V-3).

GO classification			Accession	Relative Expression (log)		
	Category	Subcategory	nos.	R.d.	R.p.	D.t.
GENES RELATED TO HOSPARASITE INTERACTION	Binding	Nucleotide	BB999049	-	2	1
		DNA	BB999050	-	2	2
		RNA	BB999051	-	2	2
			BB999052	-	2	2
			BB999053	-	2	2
			BB999054	-	2	2
		Metal ion	BB999055	-	2	2
			BB999056	-	2	3
		Protein	BB999057	-	2	1
	Catalysis	Hydrolase	BB999058	-	2	2
			BB999059	-	2	2
			BB999060	-	2	2
			BB999061	-	2	2
		Lyase	BB999062	-	2	2
			BB999063	-	2	2
		Ligase	BB999064	-	1	2
		Transferase	BB999065	-	2	1
			BB999066	-	2	2
		Oxidoreductase	BB999067	-		2
			BB999068	-	2	2
			BB999069	-	2	2
			BB999070	-	1	1
	Chaperone	Chaperone	BB999071	-	1	1
	Signal transducer	Receptor	BB999072	-	1	2
		Signaling	BB999073	-	1	2
			BB999074	-	2	2
			BB999075	-	2	2
			BB999076	-	2	2
			BB999077	-	2	2
			BB999078	-	1	2
	Transcription	Transcription	BB999079	-	1	2
			BB999080	-	1	2
	Structure	Ribosome	BB999081	-	2	2
			BB999082	-	2	2
			BB999083	1	2	2
			BB999084	1	2	2
	Translation	Initiation	BB999085	-	2	2
			BB999086	-	1	2
			BB999087	-	1	2
		Termination	BB999088	-	1	2
	Transporter	Aminoacid	BB999089	-	1	1
			BB999090	-	1	2
		Electron	BB999091	1	3	3
			BB999092	1	2	2
			BB999093	-	2	2
		Cation	BB999094	-	1	2
			BB999095	-	1	2
	Cellular process	Transport	BB999113	-	2	2
			BB999114	1	2	2
			BB999115	1	2	2
			BB999116	-	2	2
		Motility	BB999117	-	2	2
			BB999118	-	1	2
			BB999119	-	1	2
			BB999120	-	1	1
		Growth & death	BB999121	-	1	2
			BB999122	1	2	2
			BB999123	-	2	1
		Communication	BB999124	-	1	2
		Immunity	BB999125	-	1	2
	Obsolete	Cell adhesion	BB999096	-	1	2
			BB999097	4	5	5
BASAL METABOLISM	Metabolism	Carbohydrate	BB999098	2	5	3
			BB999099	1	3	2
			BB999100	-	1	1
			BB999101	-	2	2
		Energy	BB999102	-	1	2
			BB999103	-	1	2
		Lipid	BB999104	-	1	1
			BB999105	1	2	2
			BB999106	1	2	2
		Nucleotide	BB999107	1	2	2
		Amino acid	BB999108	-	1	2
			BB999109	-	2	1
			BB999110	-	1	1
		Glycan	BB999111	-	1	2
		Polyketide	BB999112	-	1	2
Unknown	Hypothetical		BB999126	-	2	2
			BB999127	-	2	2
			BB999128	-	2	2
			BB999129	1	2	2
			BB999130	1	2	2
			BB999131	1	2	2
			BB999132	-	2	2
			BB999133	-	2	2
			BB999134	-	2	2
			BB999135	-	1	2
			BB999136	-	1	1
			BB999137	-	1	2
			BB999138	-	1	1
			BB999139	-	1	2
			BB999140	-	1	2
			BB999141	-	1	2
			BB999142	-	1	2
			BB999143	1	2	3
			BB999144	-	2	2
			BB999145	-	1	2
			BB999146	2	2	5

Figure V- 3 *P. olseni* genes relative expression in $\log_{10}$ scale based on the macroarray hybridization. Parasite gene expression, in response to challenge with hemolymph from each bivalve, was quantified based on two hybridization of the cDNA macroarray and selected autoradiography exposures. The macroarray genes were classified by Gene Ontology (GO) and grouped by function in relation with host- parasite interaction. R.d. states for *Ruditapes decussatus*, R.p. states for *R. philippinarum* and D.t. for *Donax trunculus*. The control condition was the parasite not treated.

From these, a cell adhesion-related gene showed the highest degree of overexpression (more than 100x); four others where overexpressed at least 10x, one of them appearing to be related to transport, two linked to carbohydrate metabolism and one hypothetical (Figure V-3). Two genes were selected to be further characterized, the Adhesion-related (*P. olseni* Adhesion-related protein, PoAdh, BB999097, recently AB439285) and the transporter, Na^+/H^+ antiporter (*P. olseni* Na^+/H^+ antiporter, PoNHE, BB999091, recently AB439284), since in addition to being strongly up-regulated by the parasite when challenged with *R. decussatus* hemolymph (Ascenso *et al*., unpublished b), they encode proteins which putative molecular functions may be related to host-parasite interaction based on data known for other organisms (Table V-2). The other genes appear to either be related to basal metabolism of the parasite or have unknown functions, and thus may be interesting in future work when more information about this group of parasites becomes available.

The two novel genes selected were therefore cloned and their architecture and complete structure of the encoded proteins determined. The one which is the most expressed shares no homology with known proteins present in the protein database, but based on its putative features (Gene Ontology, GO:0007155), identified through *in silico* analysis, we hypothesize that it might be involved in cell adhesion and so have named accordingly: Adhesion-related protein (Figure V-1). The PoAdh gene was up-regulated log 1000,000 times in response to hemolymph of *R. philippinarum* and to *D. trunculus* (Figure V-3), but no further functional information can be inferred at this

time. PoNHE presented 1,000 fold augment in expression in response to *R. philippinarum* hemolymph and to *D. trunculus*. Instead the PoNHE was identified and belongs to cation proton antiporter (CPA) superfamily, being a transporter.

V-3.3 What is the molecular architecture of the PoAdh and PoNHE genes?

V-3.3.1 Characterization of PoAdh, a putative adhesion-related protein

The cell adhesion-related protein encoded by the clone with accession number AB439285 (the nucleotide sequence data reported in this thesis will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the mentioned accession number) had no homology in available protein databases, but according to Goblet hit (GO:0007155, (Ascenso *et al*., 2007, Ascenso *et al*., unpublished b) it was designated as *P.olseni* (Po) Adhesion- related gene (PoAdh). PoAdh cDNA spans 1,187 bp from the longest 5' end obtained to the site of insertion of the poly A tail and includes an ORF of 1,038 bp, encoding a 346 aa polypeptide (Figure V-4). No introns were found when the cDNA was compared to the corresponding genomic DNA sequence. The predicted protein had 41kDa and presented a putative isoelectric point of 8.62 (Vector NTI, Invitrogen). *In silico* analysis revealed the presence of a tyrosine rich region spanning from 163 to 337 aa (e-value 1.2e-8, MotifScan, http://myhits.isb-sib.ch) close to its C-terminus. Eleven tyrosin residues were predicted to be phosphorilated using the *in silico* analysis (score>0.9, http://www.cbs.dtu.dk/services/NetPhos/). *In silico* SignalP (http://www.cbs.dtu.dk/-services/SignalP) suggests that PoAdh may be a secreted protein, with a predicted signal peptide of 19-21 amino acids.

In an effort to find a possible function for this region as well as a possible association with virulence and involvement in the parasite response to its host, we searched for other proteins with similar structures, in particular a C- terminal tyrosine rich region.

```
                                        -28 tgctcaagttcatattctagcttctcga     -1

 M   L   Y   A   S   L   A   A   A   L   S   F   V   A   A   A   A   A      18
ATG CTT TAC GCT TCA CTT GCC GCT GCA CTG TCG TTC GTT GCC GCC GCC GCC GCC     54

 E   A   H   C   M   A   S   Y   V   W   Q   Q   D   Y   W   T   G   K      36
GAG GCC CAC TGT ATG GCT TCC TAT GTA TGG CAA CAA GAC TAC TGG ACG GGT AAG    108

 D   S   S   Y   D   D   F   Y   K   A   L   R   K   D   H   K   Y   D      54
GAC TCG TCC TAC GAT GAC TTC TAC AAA GCC CTC CGC AAG GAC CAC AAG TAT GAT    162

 D   Y   D   C   G   D   L   Y   V   N   V   A   D   A   S   N   Q   G      72
GAT TAC GAT TGT GGT GAT CTC TAC GTC AAC GTT GCC GAT GCC TCC AAT CAG GGG    216

 H   L   A   N   P   K   G   L   A   K   F   M   E   D   Y   Y   K   H      90
CAC CTT GCT AAC CCC AAG GGA CTG GCC AAA TTC ATG GAA GAC TAC TAC AAG CAC    270

 N   S   K   G   R   V   Y   L   F   Y   A   G   G   A   K   G   Y   D     108
AAC AGC AAG GGA AGA GTG TAC CTG TTC TAC GCT GGT GGT GCC AAG GGG TAT GAT    324

 D   K   K   W   A   P   E   Y   V   K   T   Y   T   E   F   V   Y   K     126
GAC AAG AAG TGG GCA CCC GAG TAT GTG AAG ACG TAC ACG GAG TTC GTC TAC AAG    378

 Y   G   P   K   Y   H   W   G   P   I   G   I   S   F   V   D   V   E     144
TAC GGC CCC AAG TAC CAC TGG GGA CCC ATC GGT ATC AGC TTC GTT GAC GTC GAG    432

 L   D   E   H   A   W   K   D   I   F   H   H   T   D   E   M   K   H     162
CTT GAC GAG CAT GCA TGG AAG GAT ATT TTC CAT CAC ACT GAT GAG ATG AAA CAC    486

 Y   L   Y   K   H   Y   K   Y   D   V   Y   V   D   V   H   F   Y   Y     180
TAT CTT TAC AAG CAT TAT AAG TAC GAC GTT TAT GTT GAT GTG CAC TTC TAC TAC    540

 N   Y   H   Y   K   H   L   V   E   L   Y   M   K   R   A   D   H   V     198
AAC TAC CAC TAC AAG CAC CTG GTT GAA CTG TAC ATG AAG CGA GCT GAT CAC GTT    594

 S   V   A   T   Y   S   N   T   Y   E   G   L   I   K   L   Y   K   Y     216
AGT GTT GCC ACG TAC AGC AAC ACT TAC GAG GGT CTC ATC AAA CTT TAC AAG TAC    648

 F   F   K   Y   T   F   P   H   A   Y   G   K   D   Y   Y   K   Y   K     234
TTC TTC AAG TAT ACT TTC CCT CAT GCC TAC GGT AAG GAC TAC TAC AAA TAC AAG    702

 A   K   I   T   F   V   G   E   G   N   C   E   S   K   H   S   C   G     252
GCG AAG ATC ACC TTC GTT GGT GAG GGC AAC TGC GAG AGC AAG CAC TCC TGC GGG    756

 K   T   S   F   C   A   Y   Y   S   D   K   Y   D   D   P   K   G   G     270
AAG ACG TCC TTC TGT GCC TAT TAC TCT GAC AAA TAC GAT GAT CCC AAG GGC GGC    810

 I   R   Y   A   Y   D   V   F   E   K   A   D   T   Y   V   R   R   H     288
ATC CGC TAC GCT TAT GAC GTC TTC GAG AAG GCC GAC ACC TAC GTT CGC CGT CAC    864

 I   L   S   R   K   Q   Y   E   H   Y   F   E   G   Y   G   S   K   Y     306
ATC CTC TCA AGG AAG CAG TAC GAG CAT TAC TTC GAA GGT TAC GGC TCC AAG TAC    918

 G   L   N   Y   F   Y   W   V   Q   C   Y   Y   G   K   D   L   W   Y     324
GGC TTG AAC TAC TTC TAT TGG GTC CAG TGC TAT TAC GGT AAG GAC CTC TGG TAC    972

 K   A   G   L   K   H   C   R   K   Y   R   Y   Y   S   D   Q   C   R     342
AAG GCT GGC CTC AAG CAC TGT CGT AAG TAC CGT TAC TAC TCC GAC CAG TGC AGA   1026

 R   H   Y   *                                                           346
CGT CAT TAC TAA  ggacttgtctgatgctacgatgacagcgtgtgtagtctcttgtaacgaaactgta  1090

caagatatcttaccacgcgttaaatcttcacttcttcttttcaatttttgtagtagtcgattgattgcaag  1162

acgcttctcgatactcatcatccgc                                               1187
```

Figure V- 4 PoAdh cDNA and correspondent amino acid sequence. Location of primers used for PCR amplification are indicated by arrows. Stop codon is marked with an asterisk.

The association of these two characteristics was found in *E. coli* tyrosine autokinase (Wzc). Wzc_{CPS} is a tyrosine autokinase essential for the assembly of a high-molecular-weight (HMW) group 1 capsular polysaccharide in *Escherichia coli* (Wugeditsch *et al*., 2001, Paiment *et al*., 2002). Capsular polysaccharides and exopolysaccharides are important virulence determinants in many plant and animal pathogens, but the biological role of this phosphorylation of the C-terminal tyrosine residues has not been established (Paiment *et al*., 2002). This *E. coli* protein presents 7 C-terminal tyrosine residues concentrated in the last 17 amino acids. This feature is similar to what has been described for another tyrosine autokinase from the bacteria *K. pneumoniae*, responsible for capsular polysaccharide biosynthesis required for protein envelope biogenesis, and directly related to pathogenesis (Preneta *et al*., 2002). PoAdh presents a C-terminal tyrosine rich region with 31 tyrosine residues distributed in 174 amino acids in the C-terminal. The finding of similar motifs in PoAdh, together with the fact that this protein is highly overexpressed by the parasite when in contact with its host hemolymph suggests that it may also be involved in *Perkinsus* response to its host.

V-3.3.2 Characterization of PoNHE, a transporter-like protein

The protein encoded by the clone with accession number AB439284 (the nucleotide sequence data reported in this thesis will appear in the DDBJ/EMBL/ GenBank nucleotide sequence databases with the mentioned accession number) had its highest homology with a Na^+/H^+ antiporter (NHE) protein, being thus designated as a putative PoNHE (Ascenso *et al*., unpublished b). *In silico* analysis of PoNHE using available databases identified the presence of both transmembrane and ion transporter domains, indicating that this gene is likely to encode a membrane transporter.

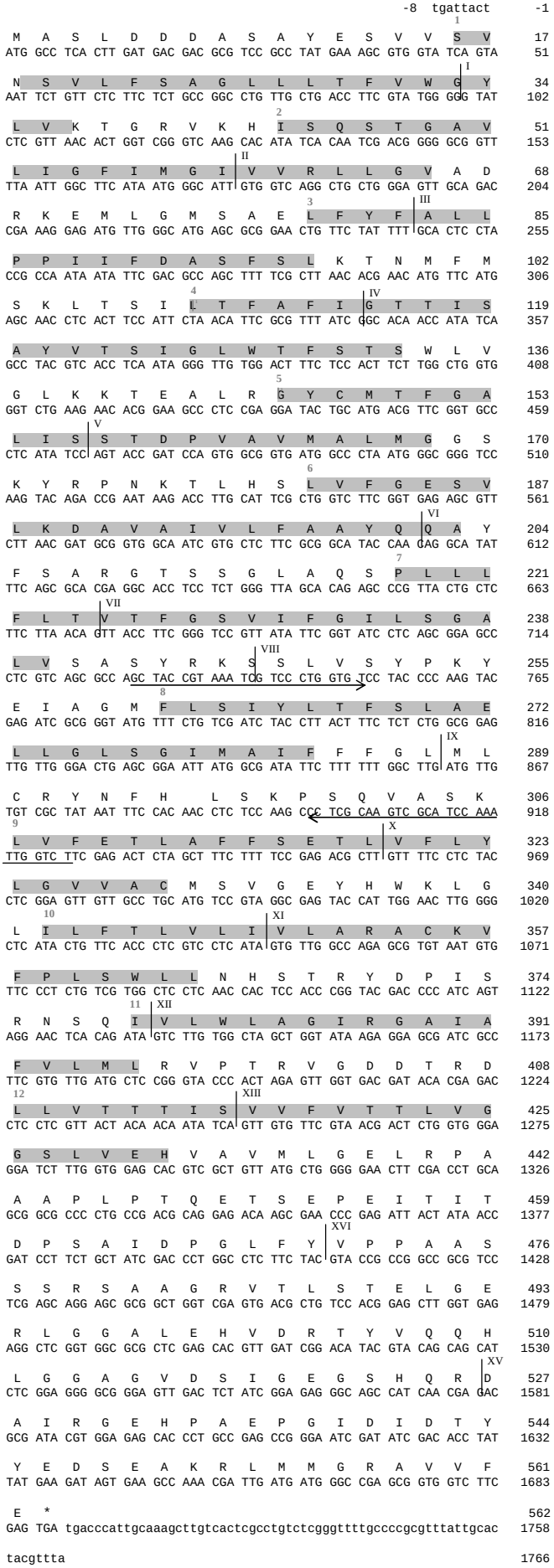

-8 tgattact -1
1
M A S L D D D A S A Y E S V V S V 17
ATG GCC TCA CTT GAT GAC GAC GCG TCC GCC TAT GAA AGC GTG GTA TCA GTA 51
I
N S V L F S A G L L L T F V W G Y 34
AAT TCT GTT CTC TTC TCT GCC GGC CTG TTG CTG ACC TTC GTA TGG GGG TAT 102
2
L V K T G R V K H I S Q S T G A V 51
CTC GTT AAC ACT GGT CGG GTC AAG CAC ATA TCA CAA TCG ACG GGG GCG GTT 153
II
L I G F I M G I V V R L L G V A D 68
TTA ATT GGC TTC ATA ATG GGC ATT GTG GTC AGG CTG CTG GGA GTT GCA GAC 204
3
III
R K E M L G M S A E L F Y F A L L 85
CGA AAG GAG ATG TTG GGC ATG AGC GCG GAA CTG TTC TAT TTT GCA CTC CTA 255
P P I I F D A S F S L K T N M F M 102
CCG CCA ATA ATA TTC GAC GCC AGC TTT TCG CTT AAC ACG AAC ATG TTC ATG 306
4
IV
S K L T S I L T F A F I G T T I S 119
AGC AAC CTC ACT TCC ATT CTA ACA TTC GCG TTT ATC GGC ACA ACC ATA TCA 357
A Y V T S I G L W T F S T S W L V 136
GCC TAC GTC ACC TCA ATA GGG TTG TGG ACT TTC TCC ACT TCT TGG CTG GTG 408
5
G L K K T E A L R G Y C M T F G A 153
GGT CTG AAG AAC ACG GAA GCC CTC CGA GGA TAC TGC ATG ACG TTC GGT GCC 459
V
L I S S T D P V A V M A L M G G S 170
CTC ATA TCC AGT ACC GAT CCA GTG GCG GTG ATG GCC CTA ATG GGC GGG TCC 510
6
K Y R P N K T L H S L V F G E S V 187
AAG TAC AGA CCG AAT AAG ACC TTG CAT TCG CTG GTC TTC GGT GAG AGC GTT 561
VI
L K D A V A I V L F A A Y Q Q A Y 204
CTT AAC GAT GCG GTG GCA ATC GTG CTC TTC GCG GCA TAC CAA CAG GCA TAT 612
7
F S A R G T S S G L A Q S P L L L 221
TTC AGC GCA CGA GGC ACC TCC TCT GGG TTA GCA CAG AGC CCG TTA CTG CTC 663
VII
F L T V T F G S V I F G I L S G A 238
TTC TTA ACA GTT ACC TTC GGG TCC GTT ATA TTC GGT ATC CTC AGC GGA GCC 714
VIII
L V S A S Y R K S S L V S Y P K Y 255
CTC GTC AGC GCC AGC TAC CGT AAA TCG TCC CTG GTG TCC TAC CCC AAG TAC 765
8
E I A G M F L S I Y L T F S L A E 272
GAG ATC GCG GGT ATG TTT CTG TCG ATC TAC CTT ACT TTC TCT CTG GCG GAG 816
IX
L L G L S G I M A I F F F G L M L 289
TTG TTG GGA CTG AGC GGA ATT ATG GCG ATA TTC TTT TTT GGC TTG ATG TTG 867
C R Y N F H L S K P S Q V A S K 306
TGT CGC TAT AAT TTC CAC AAC CTC TCC AAG CCC TCG CAA GTC GCA TCC AAA 918
9
X
L V F E T L A F F S E T L V F L Y 323
TTG GTC TTC GAG ACT CTA GCT TTC TTT TCC GAG ACG CTT GTT TTC CTC TAC 969
L G V V A C M S V G E Y H W K L G 340
CTC GGA GTT GTT GCC TGC ATG TCC GTA GGC GAG TAC CAT TGG AAC TTG GGG 1020
10
XI
L I L F T L V L I V L A R A C K V 357
CTC ATA CTG TTC ACC CTC GTC CTC ATA GTG TTG GCC AGA GCG TGT AAT GTG 1071
F P L S W L L N H S T R Y D P I S 374
TTC CCT CTG TCG TGG CTC CTC AAC CAC TCC ACC CGG TAC GAC CCC ATC AGT 1122
11
XII
R N S Q I V L W L A G I R G A I A 391
AGG AAC TCA CAG ATA GTC TTG TGG CTA GCT GGT ATA AGA GGA GCG ATC GCC 1173
F V L M L R V P T R V G D D T R D 408
TTC GTG TTG ATG CTC CGG GTA CCC ACT AGA GTT GGT GAC GAT ACA CGA GAC 1224
12
XIII
L L V T T T I S V V F V T T L V G 425
CTC CTC GTT ACT ACA ACA ATA TCA GTT GTG TTC GTA ACG ACT CTG GTG GGA 1275
G S L V E H V A V M L G E L R P A 442
GGA TCT TTG GTG GAG CAC GTC GCT GTT ATG CTG GGG GAA CTT CGA CCT GCA 1326
A A P L P T Q E T S E P E I T I T 459
GCG GCG CCC CTG CCG ACG CAG GAG ACA AGC GAA CCC GAG ATT ACT ATA ACC 1377
XVI
D P S A I D P G L F Y V P P A A S 476
GAT CCT TCT GCT ATC GAC CCT GGC CTC TTC TAC GTA CCG CCG GCC GCG TCC 1428
S S R S A A G R V T L S T E L G E 493
TCG AGC AGG AGC GCG GCT GGT CGA GTG ACG CTG TCC ACG GAG CTT GGT GAG 1479
R L G G A L E H V D R T Y V Q Q H 510
AGG CTC GGT GGC GCG CTC GAG CAC GTT GAT CGG ACA TAC GTA CAG CAG CAT 1530
XV
L G G A G V D S I G E G S H Q R D 527
CTC GGA GGG GCG GGA GTT GAC TCT ATC GGA GAG GGC AGC CAT CAA CGA GAC 1581
A I R G E H P A E P G I D I D T Y 544
GCG ATA CGT GGA GAG CAC CCT GCC GAG CCG GGA ATC GAT ATC GAC ACC TAT 1632
Y E D S E A K R L M M G R A V V F 561
TAT GAA GAT AGT GAA GCC AAA CGA TTG ATG ATG GGC CGA GCG GTG GTC TTC 1683
E *
GAG TGA tgacccattgcaaagcttgtcactcgcctgtctcgggttttgccccgcgtttattgcac 1758
tacgttta 1766

Figure V- 5 PoNHE cDNA sequence with intron insertion marked with vertical lines and numbered I to XIV; grey boxes mark TM protein domains numbered 1 to 12. Location of primers used are indicated by arrows. Stop codon is marked with an asterisk.

Table V- 3 PoNHE gene organization. PoNHE gene exon-intron boundaries, sizes of introns and exons and type of intron according to Pathy (Pathy, 1994). The nucleotide identified as +1 of the gene corresponds to the first nucleotide of the full length cDNA.

Exon			Exon/intron junction		Intron	
No.	Size	Position	5'donor	3'acceptor	Size	Type
I	170	1-170	TGGGGgtact	ttcagGTATC	43	2
II	84	214-197	GCATTgtggg	ttcagGTGGT	38	0
III	69	366-404	ATTTTgtgag	actagGCACT	150	0
IV	89	555-643	TATCGgtagt	cccagGCACA	75	1
V	126	7179-823	GTTCGgtgcc	ggtagTACAC	44	1
VI	134	889-1022	CCAACgtaaa	ttcagAGGCA	64	1
VII	69	1087-1155	AACAGgtagt	cgtagTTACC	264	1
VIII	67	1420-1486	AAATCgtgag	atcagGTCCC	55	2
IX	121	1542-1662	GCTTGgtgag	ctcagATGTT	49	0
X	95	1712-1806	CGCTTgtaag	accagGTTTT	53	0
XI	90	1860-1949	TCATAgtaag	ttcagGTGTT	115	0
XII	92	2065-2156	AGATAgtagc	ctgagGTCTT	51	0
XIII	111	2208-2318	TATCAgtact	tgcagGTTGT	71	0
XIV	163	2390-2552	TCTACgtagc	tctagGTACC	43	0
XV	169	2596-2764	ACGAGgtggg	ctcagACGCG	93	1
XVI	496	2558-3054				

Sequence analysis revealed homology to NHEs from a number of organisms, including mammals and plants. A direct BLASTn search into *P. marinus* genome sequencing project using *P. olseni* sequence allowed identification of one single gene,

suggesting the presence of a single copy of the NHE gene in *Perkinsus*, in contrast with other species where multiple copies were identified. The PoNHE gene presents 15 introns (Figure V-5), ranging from 43 to 263 bp in length, with all intron-exon frontiers are corresponding to the canonical consensus (Table V-3). Alignment of the various cDNA sequences obtained for PoNHE with the corresponding genomic sequence identified three types of transcripts (Figure V-6).

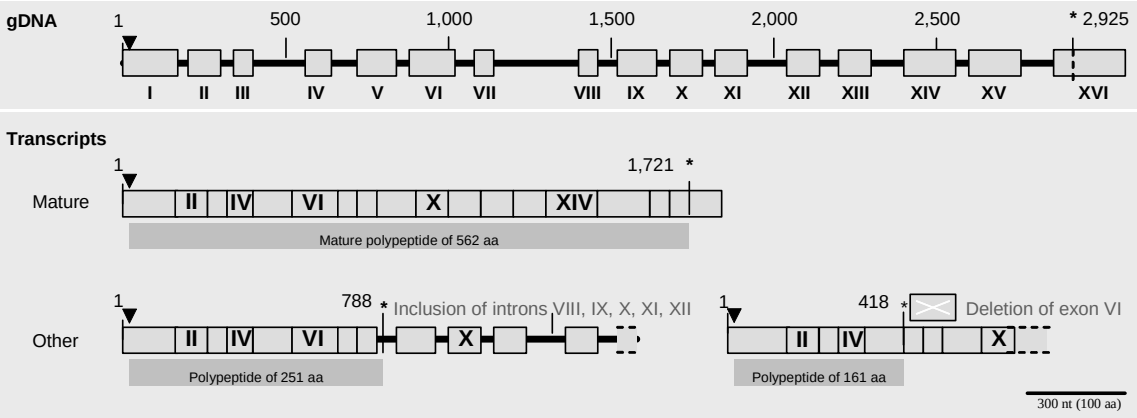


Figure V- 6 Molecular characterization of PoNHE. Scheme of genomic and transcripts sequence and predicted polypeptides size. PoNHE gene structure (black lines corresponds to intronic sequences and grey rectangles to exon sequences), the various transcripts (grey boxes) and the corresponding polypeptide size (dark grey bars) are represented. Arrowhead marks the start codon and asterisk the STOP codon.

All identified variants appear to result from different alternative splicing events. The longer transcript, considered the PoNHE mature mRNA, corresponds to the putative functional protein with an ORF of 1,689 bp and encodes a polypeptide of 562 aa (Figure V-6), considered the PoNHE mature mRNA. The two smaller transcripts can be explained by partially non-processed mRNAs, resulting in the use an intronic stop codon and the other there was an alternative splicing event, splicing the 5^{th} and 6^{th} intron with the 6^{th} exon also, altering the reading frame. These premature stop codons resulted in shorter peptides of 251 or 161 residues, respectively (Figure V-6). The PoNHE predicted polypeptide sequence hydrophobicity was analysed using Kyte-

Doolittle algorithm (Kyte and Doolittle, 1982) and twelve putative transmembrane regions (TM) were clearly identified and confirmed by TMprep (Figure V-7).

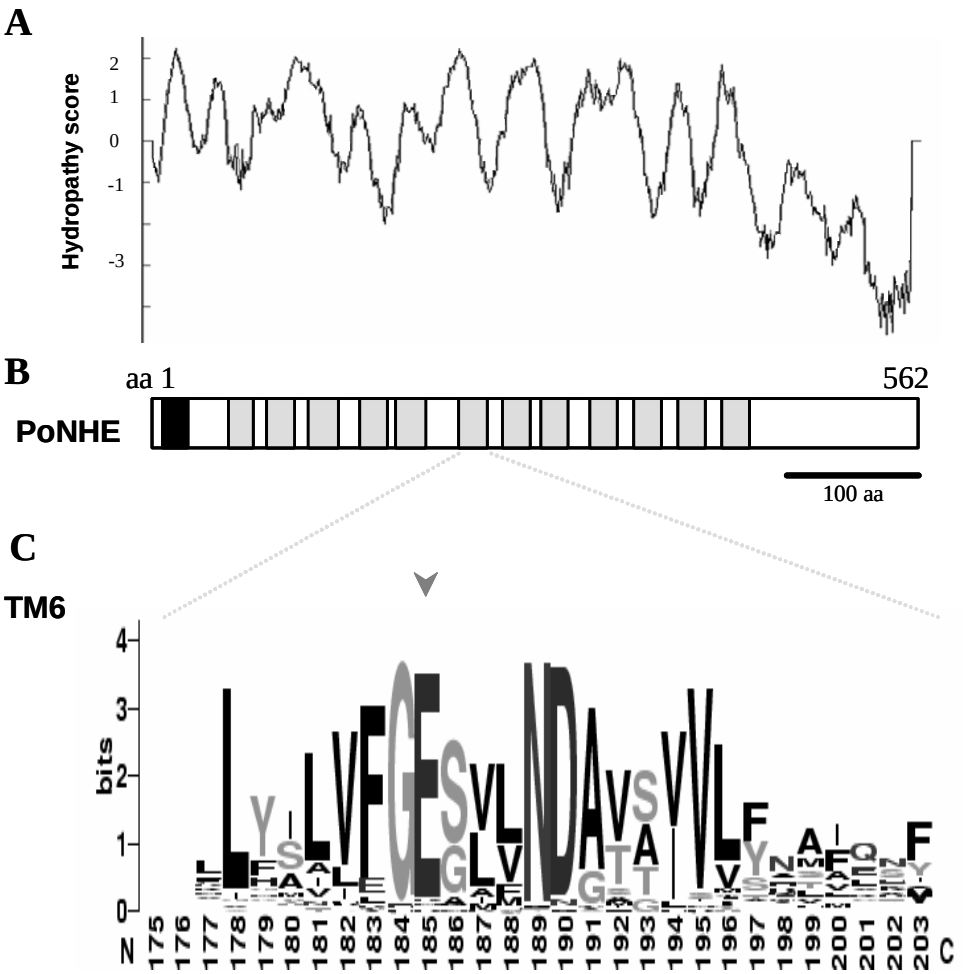


Figure V- 7 Characterization of PoNHE polypeptide. Hydropathy (y-axis) profile was calculated using TMprep server (www.ch.embnet.org/software/TMPRED_form.html) for identification of TM segments (A); in the same scale, is shown the schematic of PoNHE domains with predicted signal peptide (black box) and transmembrane domains (TM, grey boxes) of the predicted polypeptide (B); comparison of conserved residues in the 6th TM domain from PoNHE aligned with CPA1 proteins, noticing the most conserved residue, Glu (E) marked with arrowhead (C). For TM6 sequences comparison used 46 CAP1 proteins (Supplementary table 1) aligned in ClustalX, analysed using GeneDoc and scheme produced using WebLOGO.

This finding provides evidence for its identification as a NHE protein since all known NHE isoforms have a similar topology with 10-12 conserved TMs (Brett *et al.*, 2005). Analysis of the identity and similarity among the 46 CAP1 proteins indicated a higher value of 26% and 44% (identity/ similarity) of *P. olseni* with NHX4 from corn,

Z. mays. But if using only TM6 for comparison, this resulted in 59% and 77%, identity/ similarity with NHX3, 4, 5 and 6. The membrane-spanning segments shared a large identity/ similarity, with corresponding segments present in the different CAP1 proteins. It was also described that M6 and M7 were the most highly conserved (95% identity) (Orlowski and Grinstein, 1997). Alignment of PoNHE with its orthologs (46 CAP1 proteins) from mammals, yeast, protozoan and bacteria confirmed the relative position of the TM domains and high conservation of specific residues within the 3rd, 5th, 6th and 8th TM, as expected. Based on data obtained for all other known NHEs, where these residues are always conserved, namely in TM6 (Figure V-7) there is a motif highly conserved, L(nX)GE(nX)ND(nX)V, being E185 a residue conserved among all described proteins (Figure V-7) suggesting that it may be crucial for three dimensional conformation and protein function, as was described for NHE1 (Putney *et al.*, 2002). Altogether, available evidence indicates that PoNHE is indeed a NHE (Ascenso *et al.*, unpublished b).

V-3.3.3 *In silico* analysis suggests an intracellular localization for PoNHE

PoNHE was identified as an intracellular protein using GOblet server analysis, resolving a significative hit ($6e^{-12}$ e-value, GO:0016021). Although the majority of NHEs characterized have been localized in the plasma membrane, PoNHE showed characteristics for this localization within the membrane but also sequences suggesting it to be intracellular.

Therefore, to better understand the origin of this protein and provide insights into its function we explored its evolutionary relationship through a phylogenetic analysis of the transporters of the monovalent cation proton antiporter (CPA1) superfamily.

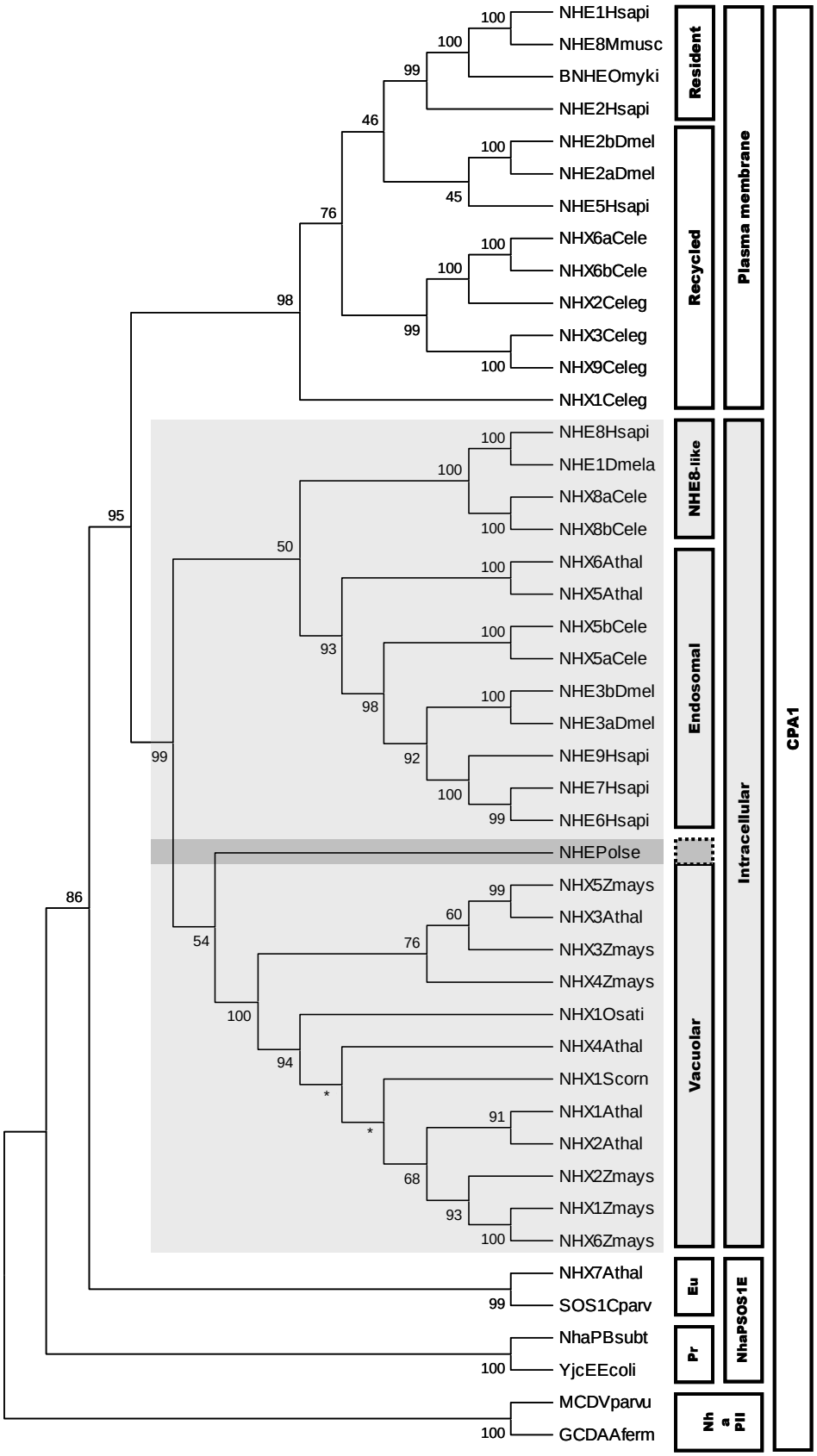


Figure V- 8 Relative classification of the PoNHE protein within the CPA1 protein family. The linearized tree shows the phylogenetic relashionships between 46 monovalent cation proton

antiporter 1 (CPA1) proteins (Supplementary table 1). Multiple alignments were performed using clustalX. Phylogenetic tree was prepared using the alignment region of full PoNHE sequence, according to neighbour- joining method using MEGA4, with 1000 replicates of bootstrapping and 45% cut-off (Bootstrap analysis showed a confidence value below 45 at the nodes indicated by the asterisk).

Based on the evolutionary relationships among members of the superfamily CPA, previously characterized in the review (Brett *et al*., 2005), we performed a study exploring the localization of PoNHE in-between the defined clades, using comparative biology to predict a putative function for this uncharacterized gene. Figure V-8 shows a phylogram constructed with PoNHE and 45 additional protein sequences (Appendix V-2) from diverse CPA1 members using ClustalX for alignment (ClustalX 1.83, (Thompson *et al*., 1997) followed by phylogeny inferring using MEGA4 (http://www.megasoftware.net, (Tamura *et al*., 2007). The complete information on accession numbers of individual genes can be found in Appendix V-2. Multiple pairwise alignments and Neighbour Joining phylogenetic tree corroborated the CAP phylogeny determined by Brett and collaborators, and provided insight towards unveiling PoNHE function. The *P. olseni* NHE was included in the CAP1 clade thus suggesting an intracellular location, with a close relationship to plant vacuolar NHEs (Figure V-8). The so called “Endossomal/TNG” or “Plant vacuolar” are part of the intracellular clade (Brett *et al*., 2005). In plants, vacuolar NHEs have been shown to be related to salt tolerance (Fukuda *et al*., 2004) and in *S. cerevisae*, Nhx1 contributes to osmotolerance after acute osmotic shock (Nass and Rao, 1999). Our results suggest that PoNHE may be responsible for adjustment to different environmental conditions, possibly responding to an osmotic shock related with the interface parasite-host. This predicted property could also be related to salt tolerance as this protozoan parasite is a facultative parasite and has a host-free life stage in marine waters, often in lagoons like Ria Formosa, where osmolarity can vary quite significantly (13 ‰ to 36.5 ‰, (Newton

and Mudge, 2003). If a similar function was maintained in *Perkinsus*, PoNHE could be involved in parasite adjustment to different environmental conditions such as the osmotic stress that may occur at the interface parasite-host upon infection (Ascenso *et al*., unpublished b).

V-3.4 How is gene expression pattern of parasite exposed to bivalves' hemolymph?

When *P. olseni* cells were exposed to hemolymph from permissive hosts not previously exposed to this parasite, there was an increase in expression of PoNHE gene suggesting an involvement of this gene in host-parasite interaction process (Figure V-9, Panel A).

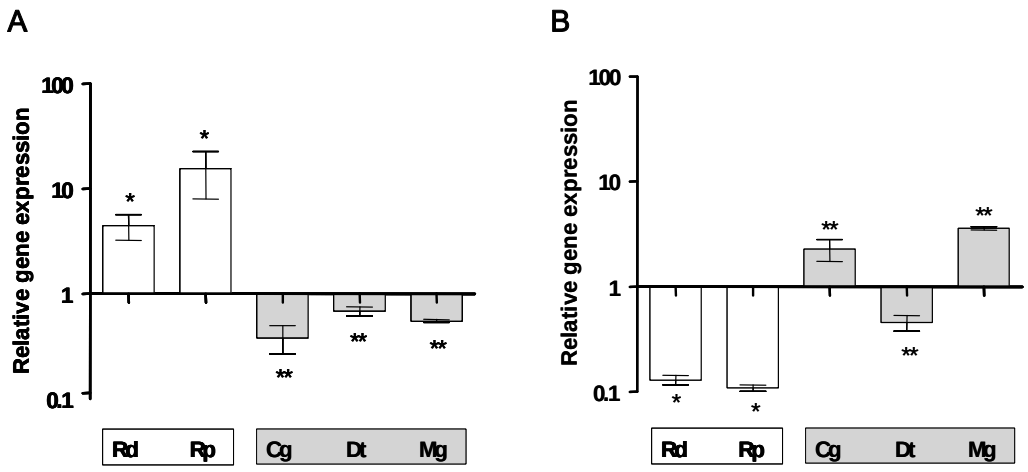


Figure V- 9 Expression of PoNHE and PoAdh genes upon exposure to different bivalves' hemolymphs. Relative levels of gene expression (in log scale) of PoNHE (Panel A) and PoAdh (Panel B) after 6 hours of exposure of *P. olseni* to bivalves' hemolymph: permissive host (white bars), the carpet-shell clam *Ruditapes decussatus* not infected (Rd), the clam *R. philippinarum* (Rp); and non permissive bivalves (grey bars): the oyster *Crassostrea gigas* (Cg), the wedge-shell clam, *Donax trunculus* (Dt) and the blue mussel, *Mytilus galloprovincialis* (Mg). All expression levels and ANOVA two ways statistical analysis are relative to results obtained for *P. olseni* exposed to infected type host hemolymph, *R. decussatus*.

Although the decreasing levels of expression are not significant, there is a clear inversion of parasite PoNHE expression when the parasite is exposed to non-permissive bivalves' hemolymph (Figure V-9, Panel A). Accordingly, levels of PoNHE mRNA

appear to be significantly higher when the parasite is in the presence of hemolymph from non-infected clams (Figure V-9, Panel A), suggesting an adjustment in preparation for infection. In contrast, a down regulation was observed upon presence of hemolymph from non permissive hosts. The correlation between NHE and infection has also been shown in *T. gondii*, which possesses two NHEs. One of them (TgNHE2) was the first NHE to be characterized as intracellular in a protozoan parasite and immunoflurescence studies showed its localization in the rhoptries, secretory organelles being involved in the invasion (Arrizabalaga *et al*., 2004, Karasov *et al*., 2005). Altogether, this suggests a possible involvement of PoNHE in the host-parasite interaction with its' overexpression possibly associated with the infection of permissive hosts (Ascenso *et al*., unpublished b).

On the other hand, the levels of PoAdh mRNA decreased 10x in the presence of hemolymph from *R decussatus* not infected and from *R. philippinarum,* when compared to levels observed in the presence of hemolymph from infected *R. decussatus* indicating that this gene is mainly and significantly expressed when infection is effective (Figure V-9, Panel B). This two parasite genes expression pattern is inverted when the parasite is exposed *in vitro* to host hemolymph. Taking in account the presence of these two genes the parasite interacts, probably with a certain level of specificity with permissive bivalves when observing the PoAdh expression pattern. PoNHE may explain the high tolerance of *P. olseni* parasite to variations at the moment of environment-host interface.

V-3.5 How is parasite gene expression altered when exposed to stress conditions?

In Ria Formosa, water temperature ranges from 12ºC in winter to 27ºC in summer and salinity from 13‰ to nearly 37‰ (Newton and Mudge, 2003) and it has

been suggested that this variation in environmental conditions may affect efficiency of parasite infection since periods of higher salinities, associated with higher temperatures, appear to favour infection. We have therefore investigated the effect of these environmental parameters on expression of PoNHE and PoAdh genes. PoNHE relative mRNA levels were up-regulated at 22ºC (Figure V-10, Panel A) while no effect was detected at 15ºC, suggesting that 22ºC, a temperature closer to average natural bivalves environmental temperature, can lead to PoNHE up-regulation when compared to standard cell culture conditions of 28ºC. Culture of the parasite at lower then normal salinities also affected expression, while no significant effect was observed at the higher salinity (37‰) suggesting that an adjustment to lower salinities is probably more stressful to the parasite, requiring adjustments to ion transport (Ascenso *et al.*, unpublished b).

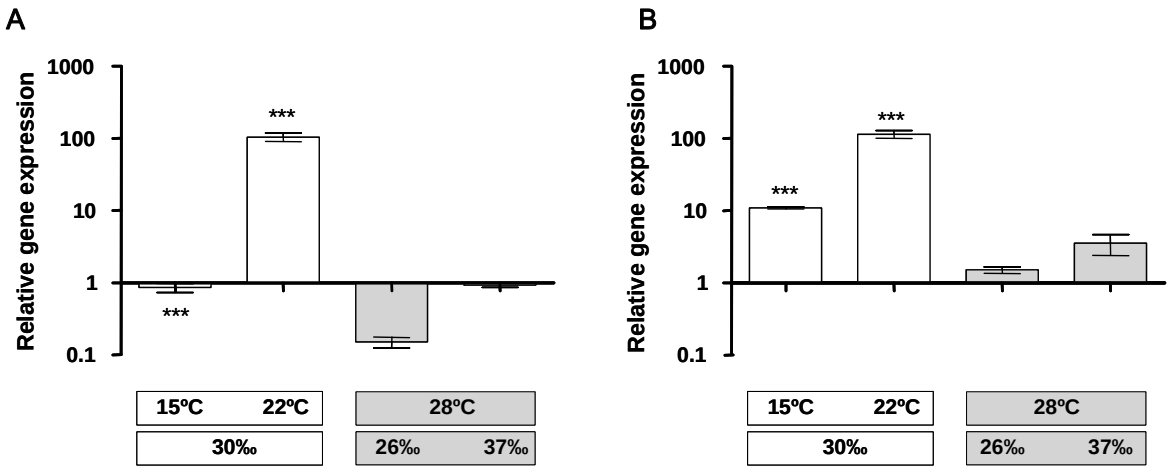


Figure V- 10 Expression of PoNHE and PoAdh genes upon exposure to different temperatures and salinities. Relative levels of gene expression (in log scale) of PoNHE (Panel A) and PoAdh (Panel B) after 3 days of exposure of *P. olseni* to treatment conditions simulating Ria Formosa environmental conditions. Growing temperatures (white boxes) corresponded to 15ºC and 22ºC and the growing salinities (grey boxes) corresponded to 26‰ and 37‰. All expression levels and ANOVA two ways statistical analysis are relative to results obtained for *P. olseni* in standard cell culture condition (28ºC and 30‰).

Indeed, less favourable conditions tend to repress parasite infectiveness as observed at 26‰ salinity. Relatively high prevalence and infection intensity observed in

summer along the Korea west and south coasts suggests that *Perkinsus* sp. has a wide range of salinity tolerance and, although this tolerance range is not well known (Park and Choi, 2001), expression of PoNHE may explain the high tolerance of *P. olseni* parasite to salinity variations. Accordingly, available data confirms that infection by *Perkinsus* is much more effective at sites of higher temperatures and salinities such as Ria Formosa (Leite *et al*., 2004), where the highest host mortality episodes are observed, then in regions of lower temperatures and salinities like the Ria de Arosa in Galicia, Spain (Villalba and Casas, 2001). The transmembrane exchange of protons for sodium ions is ubiquitous in organisms across all phyla and kingdoms, and suggests a fundamental homeostatic mechanism to control these ions. PoNHE gene expression pattern variation with salinity is in agreement with the observed evolutionary proximity to "plant vacuolar" proteins, providing further evidence towards an involvement of this protein with *P. olseni* salt tolerance, a possibility that should be the scope of future work.

In contrast, temperature appeared to be the major factor responsible for changes in relative levels of PoAdh mRNA which were already up-regulated at 15ºC and further increased (up to 100 fold) at 22ºC (Figure V-10, Panel B), when compared to levels expressed during normal *in vitro* culture conditions of 28ºC. Salinity appeared to only moderately affect expression of this gene, and particularly at (37‰) (Figure V-10, Panel B).

Temperature and salinity are prime environmental factors described to affect spatial and temporal distribution of *Perkinsus* parasitism and periods of higher salinities associated with higher temperatures appear to favour infection (Soniat, 1996, Burreson and Ragone Calvo, 1996). Low salinity areas in the upper reaches of an estuary are described to allow host population to survive even extreme *Perkinsus* epizootics

(Bushek and Allen, 1996). There were no significative effects of the pollutants studied on these genes regulation. Our results may contribute to understand the higher survival rate of similarly infected clams in Northern, cooler and less salty, versus Southern, warmer and more salty, lagoons in Portugal, during the summer months (Leite *et al*., 2004), if we hypothesize the possible relation of PoAdh and PoNHe with pathogenicity.

V-4 Conclusions

The sequences characterized in this work are newly identified in *P. olseni* parasite and this data will complement those already obtained for the host studies (Tanguy *et al*., 2004, Kang *et al*., 2006) and will provide more information for eventual parasite genome annotation. The protein PoNHE characteristics allowed to infer that it is related to the parasite host interaction process, namely in homeostasis. PoAdh molecular characterization showed the inverse expression of PoNHE in response to permissive host hemolymph at 6 hours exposure (Ascenso *et al*., unpublished b).

The molecular characteristics and gene expression patterns of PoNHE and PoAdh permit to support the hypothesis that both genes are involved in host-parasite interaction and in its adjustment to environmental conditions, in particular its tolerance to salinity and temperature variations. Although little work has been done in this area with such a model system, so little comparisons can be taken, PoNHE and PoAdh appear to be novel genes described for this species and its expression pattern revealed an involvement in parasite response to its host but with a clear interaction with environmental conditions.

This work is a first step toward *P. olseni* overall host-response expression mechanisms and should help us to understand this parasite's infection mechanisms.

CHAPTER VI – DEVELOPMENT AND OPTIMIZATION OF TRANSFECTION METHODOLOGY FOR *PERKINSUS OLSENI*

"Within the last ten years, the application of modern molecular biological approaches has provided a wealth of knowledge regarding gene structure, organization and expression in parasitic organisms." by Elisabetta Ullu and Timothy Nilsen (1995) Molecular Biology of Protozoan and Helminth parasites at J. Joseph Marr and Miklós Müller (1995) Biochemistry and Molecular Biology of Parasites, Academic Press

VI-1 Introduction

When this PhD was initiated in 2003, no transfection protocol had ever been described for *P. olseni*. However, a transfection methodology using the Nucleofector® technology and its validation for *P. marinus* was recently reported (Fernández-Robledo *et al*., 2008), although with a moderate efficiency (37.8%). This methodology was also applied to *P. olseni*, where a similar fluorescence pattern was observed but with less clear results, described by the authors as "intriguing" (Fernández-Robledo *et al*., 2008).

Little is known about the molecular biology of this mollusc parasite, but it is easily cultured *in vitro*, independently of the host cells, making it a potentially useful unicellular model for facultative parasites. Given its phylogenetic ancestry in the Alveolata group (Saldarriaga *et al*., 2003), knowledge of its molecular mechanisms of action and signalling pathways may provide important clues to better understand the protozoans parasitism. Therefore, organisms within the *Perkinsus* genus are potentially useful model systems to study interactions between the virulent form of the disease and its host. This parasite is responsible for Perkinsosis, associated with heavy carpet-shell clam mortalities (Azevedo, 1989b) and Dermo, deathly affecting the eastern oyster (Mackin *et al*., 1950), so any developments contributing to delineate new strategies to prevent or modulate growth of *Perkinsus* cells would be important. Within this line of action, the possibility of genetic manipulation could provide a tool to better understand the biology of this parasites and its interaction with their hosts. Many groups of microorganisms have evolved natural mechanisms of transformation (Redfield, 1993) but induced transfection was achieved much later with yeast, trypanosomatid parasites, *Leishmania*, *T. gondii*, and *E. histolytica.* For malarial parasite, electroporation was reported to be successful, first in *P. gallinaceum* (Goonewardene *et al*., 1993) and later

in *P. falciparum* (Wu *et al.*, 1995). In this work described in this chapter we have attempted to transfect P. olseni cells using both chemical and physical approaches.

VI-1.1 Transfection methodologies

The ability to introduce nucleic acids into cells should contribute decisively to improve our knowledge on genetic regulation and protein function in parasite cells. The process of introducing nucleic acids into cells by non-viral methods is defined as "transfection", which is distinct from "infection" or viral transduction. There are various methods of introducing foreign DNA into a cell. These methods can be broadly classified in: 1) chemical-based methods: liposome-mediated, non-liposomal lipids, dendrimers; 2) physical-based methods: electroporation, microparticle bombardment, microinjection, heat shock, magnetic assisted and 3) viral-based methods: through the use of retrovirus, adeno-associated virus and lentivirus (Müller *et al*., 2007).

The pioneering successful studies of Vaheri and Pagano led to the development of the DEAE dextran-mediated transfection technique (Pagano and Vaheri, 1965), paving the way for future experiments necessitating DNA transfer into cells cultured *in vitro*.. Another of the first transfection methods to be developed and widely used for many years is the calcium phosphate methodology, which is also one of the, cheapest (Graham and Eb, 1973). A HEPES-buffered saline solution (HeBS) containing phosphate ions combined with a calcium chloride solution can complex with exogenous DNA upon mixing and then precipitates. The resulting precipitate covers the cells, usually grown in a monolayer, which then can uptake some of the precipitate by a process not entirely understood, and with it, the exogenous DNA. The reproducibility is described as poor and dependent on the experimentalist. More recently, the original method was greatly improved by developing a simple and reproducible method to

prepare well-defined DNA-coated calcium phosphate nanoparticles, showing good transfection efficiency, with complexes allowed to be stored for weeks without loss of their transfection activity (Welzel *et al*., 2004).

Later, chemical transfection methodologies were developed, using lipid and polymer-based carrier molecules. Mixtures of lipids, and certain polymers, are capable of binding to RNA or DNA and creating liposomes, which can permeate the cellular membrane and deliver DNA inside the cell. Depending upon the cell line, lipofection has been described to be 5- to >100-fold more effective than either the calcium phosphate or the DEAE dextran transfection techniques (Felgner *et al*., 1987). A very efficient method is the inclusion of the DNA to be transfected in liposomes (Nabel *et al*., 1993), small membrane-bounded bodies that mimics the structure of a cell and can actually fuse with the cell membrane, releasing the DNA into the intracellular compartment.

Cation-lipid based transfection (Lasic and Papahadjopoulos, 1995) is typically used for eukaryotic cells, because these are more sensitive. Highly branched organic compounds, called dendrimers, have been developed for transfection of cell lines hard-to-transfect, as is the case of *P. olseni*. Another class of organic compounds, polycationic polymers such as polyethyleneimine (PEI), was developed for delivering biologically active compounds into a cell through a targeting group covalently bound to its primary amine, which can interact with the surface of the cell (Kopatz *et al*., 2004). Nowadays, numerous companies developed their own transfection methods *versus* reagents and are commercially available, such as PEI (Sigma Aldrich) or TransPEI (Eurogentec, Herstal, Belgium).

Physical transfection methods include the direct microinjection of materials by electroporation, biolistic particle delivery and heat shock. Early experiments exposing

cell membranes to strong electric fields involved diverse systems, ranging from nerve fibre to bacteria. In 1958, the Ranvier nodes of nerves were reported to involve some type of ‘‘breakdown’’; these results showed the development of strong electric field pulses which in turn caused molecular transport across a biological membrane (Neumann and Rosenheck, 1972). In electroporation, which mechanism involves ultra-short and broad-band voltage pulses that modify membrane charge, transient hydrophilic pores are created in the more fluid, lipid regions of a membrane due to both electric field interactions and thermal fluctuations, thus allowing entrance of the exogenous DNA (Weaver, 2003). Electroporation is regularly performed in a small chamber with two parallel plane electrodes that use larger volumes and induces, generally, damage to cells. The last advances eliminate many problems of conventional electroporation, since the gap size between the two electrodes is maximized and the surface area of electrode is minimized in a capillary electroporation chamber compared to the cuvette type chamber, reducing the cells damage. This is called microporation instead of electroporation (Chang *et al*., 2005) and was developed by Digital Bio (www.microporator.com).

Biolistics, or particle bombardment, involves coupling the DNA to a nanoparticle of an inert solid (commonly gold) which is then "shot" directly into the target cell's nucleus. This has been used successfully to introduce nucleic acids into plant cells, animal tissues or organs, and whole organisms that typically are not amenable to transfection. This method was originally developed to use with plant cells, where biolistics have been the most universally successful method for introduction of nucleic acids. Biolistics provides also a practical method for *in vivo* studies using whole organisms such as multicellular parasites, for which cell lines and transgenic methods are currently not available. The first delivery by particle bombardment was developed

by Sanford and co-workers (Klein *et al*., 1987, Johnston *et al*., 1988). Initial reports were restricted to transient expression of marker genes in onion, corn, soybean, wheat and rice (Klein *et al*., 1987, Klein *et al*., 1992), quickly followed by production of transgenic maize (Fromm *et al*., 1990, Gordon-Kamm *et al*., 1990). This technology was widely applied to bacteria (Klein *et al*., 1992), fungi (Armaleo *et al*., 1990), algae (Mayfield and Kindle, 1990), insects and mammals (Klein *et al*., 1992, Cheng *et al*., 1993, Luo and Saltzman, 2000).

DNA can also be introduced into cells using viruses as carriers. The technique is called viral transduction, and the cells are said to be transduced.

One factor critical to successful gene therapy was the development of efficient delivery systems. Based on how long the genetic material is expressed through transfection, the process can be designated as transient or stable transfection. In transient transfection, plasmid DNA (pDNA), messenger RNA (mRNA), short interfering RNA (siRNA) and microRNA (miRNA) can be transferred and stay present in the cell, but nucleic acids do not integrate into the host cell chromosomes. Transient transfection results in high expression levels of introduced DNA 48-96 hours post-transfection. Stable transfection is achieved by integration of exogenous DNA into chromosomal DNA thus remaining permanently expressed in the genome of the cell.

VI-1.2 Development of transfection methods for parasites

Many groups of bacteria have evolved mechanisms of natural transformation which enable them to take up free DNA fragments from their environments (Redfield, 1993). In addition, experimental transfection was achieved with yeast and trypanosomatid parasites, *Leishmania*, *T. gondii*, and *E. histolytica*. Parasite genetic manipulation, as *P. gallinaceum* transfection, was shown to be essential for

understanding the complex biology of this group of pathogens and also to study implications for both vaccine and drug development. Goonewardene and his group were the first to describe the transient expression of luciferase in *P. gallinaceum* transfected by electroporation (Goonewardene *et al*., 1993). Similar studies followed for *P. falciparum* (Wu *et al*., 1995). Almost simultaneously, the first set of targeted chromosomal integration events were reported confirming the stable transfection of *P. falciparum* and *P. berghei*, (Wu *et al*., 1996, Waters *et al*., 1997), respectively. Electroporation was usually the technique of choice for introducing DNA in *P. falciparum* within infected red blood cells, following the methods of low voltage and high capacitance (Fidock *et al*., 1998) or high voltage and low capacitance (Wu *et al*., 1995). Electroporation of noninfected red blood cells allowed spontaneous uptake of DNA by the erythrocyte-stage *P. falciparum* cells and was an alternative approach to improve this parasite transfection efficiency, instead of direct transfection of parasite cells in infected red blood cells (Deitsch *et al*., 2001). In parallel, other alternative approaches have been documented but electroporation of infected red blood cells generally remains the method of choice for introducing DNA into *P. falciparum* (Skinner-Adams *et al*., 2003).

Classic RNAi approaches were used to reduce gene expression at multiple *Leishmania* loci through improved protocols of transient and stable transfection (Robinson and Beverley, 2003). Molecular characterization of the *Leishmania* genome has been greatly facilitated by the development of stable transfection methods and expression vectors. Gene knockout coupled to stable transfection of *Leishmania* and trypanosomes requires the use of dominant selectable markers, and a number of independent markers have been used successfully (Goyard and Beverley, 2000). In *T. brucei*, equaly diploid, RNAi has proven a powerful tool for both forward and reverse

genetic analysis introduced by electroporation (Bastin *et al*., 2000). For *T. annulata* sporozoites a method to transiently transfect this cells was developed using lipid transfection agent SuperFect™ (Adamson *et al*., 2004).

The physical methods for gene transfer are described as methodologies which permit advantages and better performance compared to other transfection systems (Mehier-Humberta and Guy, 2005).

VI-1.3 Importance of the vector features for transfection

Despite advances in gene transfer technology, some cases require an ideal vector system specifically constructed. For *T. annulata* the transfection vectors were constructed using a number of *T. annulata* 5' gene flanking sequences linked to the enhanced green fluorescence protein (eGFP) reporter gene (Adamson *et al*., 2004). The selectable markers are determinant for use in genetic manipulations.

For protozoan parasites transfection, *L. mexicana* and *T. brucei*, mutant parasite contain various well-established selectable marker genes which confer resistance to: hygromycin (hyg^R), neomycin (neo^R), phleomycin (ble^R), puromycin (pac^R) and streptothricin acetyltransferase (sat). The gene encoding blasticidin S deaminase (BSD) was used as a selectable marker gene for targeted chromosomal integration in trypanosomatids (Brooks *et al*., 2000). Plasmid transfection vectors could carry other selectable markers as gene locus encoding a drug-resistant form of the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (DHFR/TS) (Goonewardene *et al*., 1993). The dhfr-ts genes, which confer resistance to pyrimethamine or WR99210, came from either *T. gondii* (tgdhfr-ts) or human sources (hdhfr) (Wu *et al*., 1996) (Fidock *et al*., 1998). Other selectable markers have also been successfully developed to be used as transfection vectors such as geneticin (G418) (Mamoun *et al*., 1998)

(Mamoun *et al*., 1999). In addition, here are several other selectable markers adjusted to each experiment.

VI-1.4 Aims of the work

Advances in transfection technology suitable to allow genetic manipulation have occurred regularly since 1990s but currently available transfection tools are essentially useful for well explored model organisms, in particular parasites of vertebrates. Progress in the development of new vectors could improve this situation and hence our ability to understand the fundamental biology of other, less known parasites such as *Perkinsus*. To overcome the lack of an efficient transfection methodology for *P. olseni*, a compilation of possible methods to use was done through an extended bibliographic search and several methods selected for testing using *P. olseni* trophozoites maintained in suspension culture. The present work aimed (a) at attempting to develop a transfection methodology for *P. olseni* by adapting and testing various pre-existing techniques, (b) to demonstrate transformation of *P. olseni* and (c) to optimize cell transfection conditions and examining some key parameters.

VI-2 Materials and Methods

VI-2.1 Transfection by chemical methodologies

VI-2.1.1 Calcium phosphate

DNA-calcium phosphate co-precipitate was used to introduce genetic material into cells, based on a previously described method (Jordan and Wurm, 2004). The cell culture was seeded with $1x10^6$ cells to allow exponential growth phase and obtain approximately 3.5 $x10^6$ cells/ml of medium after one day of culture. Then, cells were washed and incubated in FBS-free cell culture medium prior to experiment. In 96-well

plates, 50µl of cell suspension were plated per well immediately prior to transfection. For each transfection assay, 0.5 µg pEGFP plasmid was prepared in 15 µl final volume of sterile water. Five microlitters of $CaCl_2$ 1 M was added to DNA Just before transfection 20 µl of the 2x phosphate buffer (0.5 M HEPES pH 7.1 , 3.0 M NaCl 1.0 M NaPO4 pH 7.1) was added and briefly mixed with the pipette to obtain the DNA/Ca complexes. After 1 min incubation at room temperature, the 40 µl transfection cocktail was added per well. A 1:1 and 1:1.2 ratio of DNA:transfection-reagent were tested. The controls included DNA only, calcium phosphate cocktail only and no treatment at all. The plate was incubated with shaking at 80rpm for 4 h at 28ºC before changing cell medium to standard medium, DME/Hams (1:2) with 5% FBS. Transfection was analysed after 16 and 48 hours by observation of 20µl sample replicates under the microscope, excited by blue light (440-490nm).

VI-2.1.2 Cationic polymer – PEI and TransPEI

Another chemical transfection methodology was tested with the available cationic polymers, PEI and TransPEI. Using the TransPEI (Eurogentec) the PEI nitrogen/DNA phosphate ratio (N/P ratio) was calculated based on 7.5 mM of cation nitrogen of TransPEI working solution. The N/P ratio was a measure of the ionic balance of the complexes; it referred to the number of nitrogen residues of PEI per DNA phosphate. The best transfection results were described for N/P = 5 to 10, expressed in nitrogen residues and 1 µg of DNA contains 3 nmoles of anionic phosphate. PEI (Sigma Aldrich) working solution was 1/25 dilution of stock solution. Immediately following plating cells at $1x10^6$ cells/ml, transfection was performed with PEI and TransPEI in the absence of FBS and then incubated 6 hours before changing to standard medium. 500 ng pEGFP DNA was prepared in 150 mM NaCl, to which was added the transfection

reagent, incubated at room temperature and the complexes added drop wise to the cells. Various N/P ratio were assayed in 96-well plate according to the following table, in a final volume of 100 µl per well. The results were observed after 24 hours and 48 hours. In another assay in 6-well plate involving PEI as transfection reagent, the ratio selected was 1,4 in two ml cell culture.

Table VI- 1 PEI and TransPEI transfection reagent/ DNA ratio used for transfection.

Reagent	Ratio Reagent/DNA							
TransPEI*	1,5	3	5	6	10	16	20	32
PEI±	0,014	0,14	1,4	14,4	144,2	288,4	576,8	824

* Number of residues of nitrogen in PEI per DNA phosphate
± µg PEI to µg DNA

VI-2.2 Physical methodologies for transfection

Physical transfection methods in the present study include electroporation and particle bombardment.

VI-2.2.1 Electroporation

The use of high-voltage pulses to introduce DNA into cultured cells is based on the use of a short high-voltage pulse that causes the membrane potential of the cells to break down. As a result, pores are formed through which macromolecules such as DNA can enter. *P. olseni* cells were submitted to electroporation using a Gene pulser Xcell™ Electroporation system (BioRad). There are few variables defined; electroporation media is recommended to be of high resistance. For that various transfection media were tested: (a) 10 % glycerol, (b) cytomix (120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4-KH2PO4, 25 mM Hepes, 2 mM EDT and 5 mM MgCl2 at pH 7) and (c) 1 M Sorbitol. According to the cuvette sizes (2 mm and 4 mm) it was used 40 and 80 µl,

respectively. The cell culture used was at exponential growth phase with $2x10^8$ cells/ml cell density. The cells were washed twice and resuspended in the electroporation media. Increasing amount of DNA was tested, using 50 ng to 500 ng of purified plasmid DNA in DNase- free water. pEGFP_N1, pcDNA3 and pβGal_Basic were the plasmids used to analyse the introduction of foreign DNA in *P. olseni* cells allowing various methods of verification of transfection efficiency. According to the cell type and some pre- set protocols for electroporation of bacteria and yeast, resistance 200 Ω, capacitance 25 F, voltage of 1500 v, 2500 v or 3000 v and pulse length from 1 ms to 5 ms were applied. The results were checked at 48 hours observing the cells under the microscope with fluorescence emission in blue wave length range (440-490 nm). Selection of electroporated *P. olseni* cells using the geneticin resistant was performed with standard medium containing 0.72 mg/ml geneticin (G418, Gibco), and initiated 48 hours after transfection. Under continuous selection, geneticin-resistant-like cells were observed and grown to be cryopreserved. The integration and expression of target DNA in regenerated geneticin resistant cells was confirmed by RT-PCR using Geneticin resistance gene specific primers. Results were observed and analysed accordingly.

Pre-treatment to improve transfection

Treatment of cells with pronase before application of the pulses abolishes the ionic modulation of both electro-permeabilization and electrofusion and a rate of expansion of permeabilization should be obtained.

The permeabilization assay was prepared testing a cell culture at $1x10^7$ cells/ml of cell medium and various pronase concentrations, 20 mg/ml, 10 mg/ml, 5 mg/ml, 1 mg/ml, 200 µg/ml and 125 µg/ml, incubated at 37ºC and 28ºC. Other permeabilization method used was the incubation with 25 mM DTT at 37ºC and 28ºC. The cells viability

was assayed throughout time, at 5 min, 20 min, 1 hour, 2 hours and 24 hours using trypan blue coloration observed under the microscope. After determination of the minimal treatment for membrane permeabilization, this treatment was followed by different methods of transfection, previously described and repeated twice.

The transfection methods used were those based on chemical methods (calcium-phosphate and PEI) and on a physical method (electroporation). The conditions selected were those according to the less aggressive state of the cells. This experiment was repeated twice, including transfection reagent and DNA control conditions. The results were based on the observation of cells under the fluorescence microscope.

Table VI- 2 *P. olseni* transfection plan using calcium phosphate, PEI transfection and electroporation methods after *P. olseni* cells permeabilization with DTT and pronase pre-treatment.

Method	Vessel	Cell density	Volume/well	Plasmid	DNA		Reagent		
					Amount	Volume	Type	Volume	Final volume
Chemical	Three plates of 12-well	$1,4x10^7$ cells/ml	1ml	pcDNA3 and pEGFP	0,5µg	5µl pcDNA3 + 5µl pEGFP µg to 100µl of 150mM NaCl	PEI	0,105µl in 600µl of 150mM NaCl	330µl cells + 200µl complexes
					0,5µg	5µl pcDNA3 + 5µl pEGFP and 55µl sterile water	CaPO4	22,2µl 1M $CaCl_2$ 200µl 2x phosphate buffer	330µl cells + 200µl complexes
Physical					0,5µg	5µl pcDNA3 + 5µl pEGFP	1M sorbitol	1 pulse, 5ms, 2500v	330µl cells
Control					No DNA				330µl cells
					0,5µg	5µl pcDNA3 + 5µl pEGFP	Not transfection		330µl cells

VI-2.2.2 Microparticle bombardment

Biolistics, or particle bombardment, has been used successfully to introduce nucleic acids into plant cells, animal tissues or organs, and whole organisms that typically are not amenable to more traditional methods of transfection. A biolistic based transformation procedure was applied in the protozoan parasite, *P. olseni* trophozoite cells during a short stay of visiting the laboratory of Plant Genetic Engineering, hosted at ITQB/IBET. The parasite stock cell culture was prepared 4 days before at least. The stock cells from small flasks were used to inoculate an 80 ml of $1x10^6$ cells/ ml cell culture at least 2 day before transfection. Each plate for bombardment was seeded with 100 µl in the centre of $2x10^9$ and $2x10^8$ cells/ml cell density to get $2x10^8$ and $2x10^7$ cells per plate, respectively. The preparation of the microparticles started by weighting 8 mg gold particles (0.6 µm) counting 500 ng per bombardment. Those were washed firstly with 70 % ethanol (400 µl), ressuspended by vortex 3-5 min and allowed to settle down for 15 min. After that, the microparticles were collected in the bottom of the tube to wash again with 100% ethanol. Finally the particles were washed twice with sterile water, under the hood and were kept at 4ºC in 50 % glycerol, approximately 16.67 µl per mg of gold (134 µl). The microcarrier- DNA complex preparation was produced by addition of DNA to 50 µl microparticles and allowed to rest 2 min, followed by mixing. Two sets were prepared for pcDNA3 plus pEGFP_N1 and for PoClhl_pcDNA3 plus pEGFP. The control without DNA was also prepared. Then 50 µl $CaCl_2$ (2.5 M) and 20 µl spermidine (0.1 M) previously prepared and sterilized by filtration (0.22 mm) were added and incubated 10 min. To discard the supernatant the mix was vortexed for 2 min and allowed to settle for 2 min before spinning down. Then followed by a wash with 70 % ethanol (200 µl) and then with 100 % ethanol. vortexing 2 min, allow to rest and spin down. Starting with 50 µl microparticles and adding 21 µg DNA (3.3 µg pcDNA3 or

PoClhlpcDNA3 and 0.2 µg pEGFP per bombardment) the complexes were resuspended in 60 µl 100 % ethanol in order to load 8 µl of the macrocarrier with the microcarrier. Before bombardment, the macro and microcarrier holders, the rupture disks and all the equipment were pre-sterilize with 70% ethanol. The Petri dishes, paper and stopping screens were previously sterilized. The chamber was sterilized and washed with 70% ethanol.

Table VI- 3 Transfection of *P. olseni* by microparticle bombardment, distribution of assayed plates per experiment

Flying distance	Cell number (100µl)	Plasmid		Control		
		pEGFP+ pcDNA3	pEGFP+ PoClhlpcDNA3	Shoot		No shoot
				No DNA	No cells	
1 mm	$2x10^6$	1	6	9	13	15
	$2x10^7$	3	8	10		16
0.1 mm	$2x10^6$	5	2	11	14	-
	$2x10^7$	7	4	12		-

Then, having the cells settled in the bombardment area, the bombardment parameters for gap distance between the rupture disk and the microcarrier (0.1 mm and 1 mm) were adjusted as also the helium bottle pressure for 2000psi and the discharge tube for 1600 psi, so a 1350 psi pressure shoot was performed at each bombardment. The macrocarrier was loaded with 8 µl of well-homogenized complexes. Those were pipetted to the macrocarrier center homogeneously distributed let the ethanol to evaporate and place it in the macrocarrier holder. For each plate of cells was performed a single bombardment according to the table VI-3. Immediately after the bombardment the cells were incubated at 28ºC, kept on solid agar medium. Transfection result was checked at 48 h by observation of each plate in inverted fluorescence microscope under blue light (440-490 nm). Selection for geneticin resistant *P. olseni* cells of post-

bombardment was performed after 48 hours with standard medium containing 0.72 mg/ml geneticin. Under continuous selection, geneticin-resistant-like cells were observed and later inoculated in standard liquid medium with geneticin selection. The integration and expression of target DNA in regenerated geneticin resistant cells was confirmed by RT-PCR using specific primers.

VI-2.2.3 MicroPoration

Microporation (Digital Bio Technology, Suwon, Korea) was a novel electroporation technique available in a demonstration for one test, allowing us to continue to search and optimize the transfection condition for *P. olseni*. This produced high-voltage pulses in a pipette tip as electroporation space creating uniform electric field with minimal heat generation and oxide formation. The cell culture used was incubated as described above to reach exponential growth phase and transferred 24 hours before transfection to medium without antibiotics. The cells were washed twice and finally resuspended in the antibiotic-free medium. For an optimization assay in 24-well plate a final cell number of $2{,}5x10^6$ cells, were used as recommended by manufactures' protocol (Digital Bio Technology). These cells were pelleted and resuspended in Solution R, according to manufactures' protocol (Digital Bio Technology). Five hundred nanograms of purified plasmid DNA pEGFP_N1 in DNase-free water was used per well, so 12 µg were added to the cells suspension and incubated 15 min at room temperature. According to the pre- set optimization protocol for microporation, the voltage of the pulse ranged from 900 to 1700 volts in combination with 20 to 30 ms pulse repeated from one to three times. Transfection results were checked after 48 h by observation of each plate under the inverted microscope, excited by blue light (440-490 nm).

VI-3 Results and Discussion

VI-3.1 Which approach for *P. olseni* cells transfection?

To develop a transfection methodology for *P. olseni* cells, a bibliographic search was performed to compile possible applicable strategies. Other unicellular organisms' transfection methodologies were taken into account to search for techniques to be applied for *Perkinsus* transfection, namely those used for other protozoan parasites (Appendix VI-1). Among the collected information, two methods were chosen to try: transfection and biolistic.

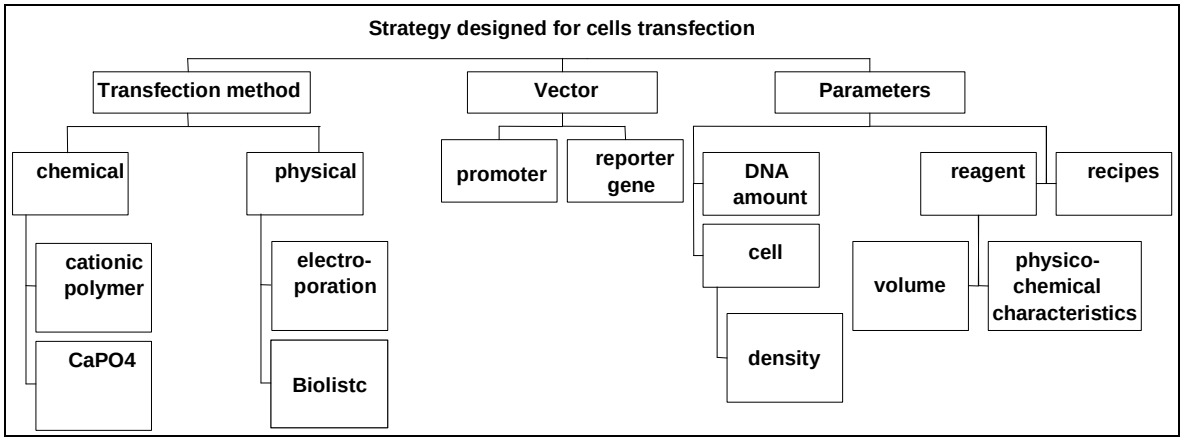


Figure VI- 1 Schematic summary of factors to take into account to develop a transfection methodology for *P. olseni* throphozoites.

For consistent results, it was important to maintain healthy proliferating cells; therefore they were seeded at $1x10^6$ cells two days before the transfection attempt in order to get trophozoites in exponential growth phase. In addition to cell conditions, the following factors had to be taken into account in order to maximize transfection efficiency: transfection reagent, its amount and/or transfection procedure, the plasmid DNA, its concentration or ratio and cell medium for their incubation during and after transfection (Figure VI-1). A comparison of some relevant characteristics between other

organisms, for which successful transfection strategies were developed and *P. olseni* parasite was performed. *P. olseni* trophozoites and schizonts are known to present a cell wall which composition has not been fully described. According to surface/volume (S/V) ratio the *Perkinsus* cells are more closely related to yeast (Table VI-4) and so we analysed the methods used to introduce DNA into yeast cells over time.

Table VI- 4 Comparison of cell characteristics to allow selection of transfection methodology to apply for *P. olseni* cells (adapted from Dumont *et al*., 2004).

Reference	Organism	Type of cell	Shape	Mean vol[a] (μm^3)	S/V ratio (μm^{-1})	Description of cell wall
Robledo, 2002	*P. olseni*	Protozoan	Spherical*	65-4188±	0,3-0,07±	
Montes, 2002	*P. olseni*	Protozoan				PWP-1 disulphide-rich
Afonso, unpublished	*P. olseni*	Protozoan				cell wall mannan protein / chitin
Dumont, 2004	*E. coli*	Bacterium gram negative	Straight rods	6	3.3	Very thin, high in lipid content
Dumont, 2004	*S. cerevisiae*	Yeast	Ellipsoidal	250	0.7	Predominantly glucan, mannan, and chitin polymers
Dumont, 2004	*C. utilis*	Yeast	Spherical	270	0.75	Primarily cellulose, mannan, and glucan polymers
Dumont, 2004	K562	Leukemia cell	Spherical	523	0.6	No cell wall

* Based on the visible shape in the developed *P. olseni* trophozoites cell culture which exhibited size heterogeneity with tetrads and clusters consisting of small cells (Robledo *et al*., 2002)

± Determined based on the described sizes for actively growing trophozoites, 4.6–16.0 µm (Robledo *et al*., 2002)

The procedures followed to attempt transfection of *P, olseni* cells were used to obtain transient transfection only, and both chemical and physical techniques were proposed. For the first one, carrier molecules as calcium phosphate and commercial cationic polymers, polyethylenamine (PEI, Sigma) and TransPEI (Eurogentec) were used. In the case of direct delivery, electroporation was performed using various

protocols based on comparison with those used for other Alveolata parasites and Yeast (Appendix VI-1). Biolistic was also tested.

VI-3.2 Selection of a reporter gene; how to confirm effective transfection?

To determine transfection efficiency, we used plasmid vectors available in the laboratory such as pEGFP_N1 (Clontech), pcDNA3 (Invitogen) and pβGal_Basic (Promega).

Vector pcDNA3 was chosen because it confers neomycin resistance (Neo^r),allowing the selection of transformants clones under geneticin (G418, Gibco) initially submitted to lethal concentration (EC90) and later by transformant growth under EC50 concentration.

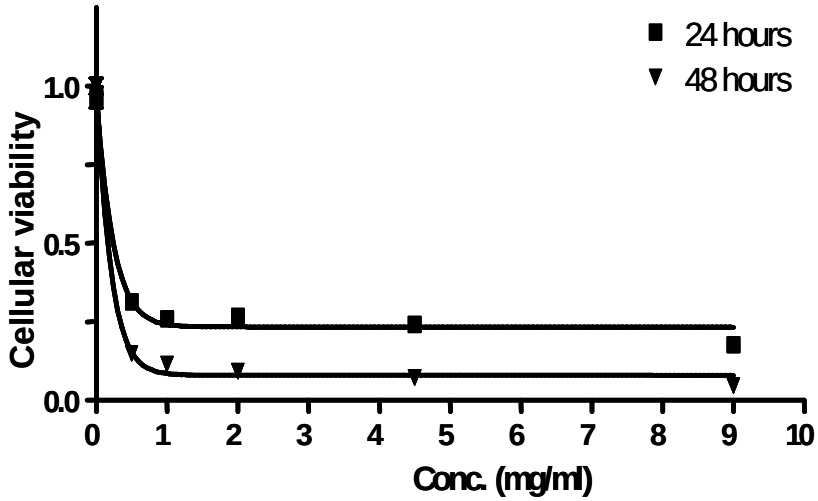


Figure VI- 2 *P. olseni* trophozoites sensitivity to geneticin (G418, Invitrogen). Cell proliferation assay was performed to assess *P. olseni* cellular viability using 100 µl *P.* olseni cells in triplicates at least. This was evaluated using the Cell Titer 96 Aqueous Non-Radioactive Cell proliferation assay (Promega) measuring absorbance at 490 nm.

Lethal (EC_{90}) and selection (EC_{50}) concentrations of geneticin were determined by *in vitro* assay to identify *P. olseni* cells sensitivity (Figure VI-2). Briefly, log-phase

cell culture was submitted to various concentrations of geneticin (0 to 9 mg/ml of active antibiotic, G418 sulphate, Invitrogen) and cell viability assay performed at 24 and 48 hours. The sigmoid dose response curve constructed allowed the determination of EC_{50}, 0.1 mg/ml and EC_{90}, 0.72 mg/ml (r^2= 0.9936). These were used as selection and lethal concentrations, respectively, during transfection experiments.

Vector pβGal_Basic contains the gene that codes for β-galactosidase, which enzyme activity direct assay resulted in activity of β-galactosidase in all conditions. The expression of β-galactosidade reporter gene allowed degradation of the exogenous substrate galactoside (o-nitrophenyl-beta-D-galactopyranoside) thus leading to the identification of a blue product in all conditions tested, but also in control conditions (Figure VI-3). Therefore, the possibility of using the gene β-galctosidase as reporter gene was discarded. Indeed, the β-galactosidase gene was predicted to be present in *P. marinus* genome by *in silico* analysis through BLASTn search at TIGR using *E. coli* sequence for this enzyme.

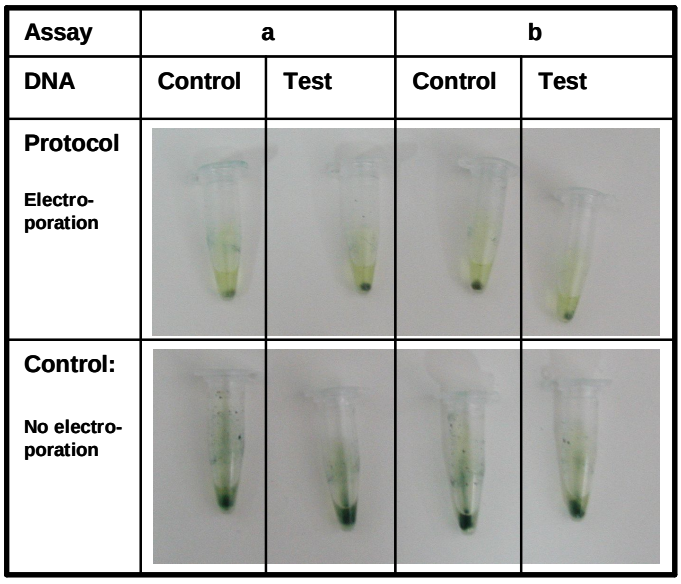


Figure VI- 3 Testing pβGAL_Basic plasmid for transfection efficiency observation. The transfection method used was the electroporation of *P. olseni* cells and β-galactosidase assay performed at 6th day after transfection (a and b refers to two independent experiments).

For pEFGFP-N1, it allowed a direct checking of the efficient entrance of exogenous DNA into *P. olseni* cells through expression of green fluorescent protein (GFP) because of its characteristic; emission of green fluorescence when excited by blue light at wave length range 440-490 nm. Enhanced GFP expression was observed at 24, 48 or 72 hours under inverted fluorescence microscope (Olympus U-RFL-T), according to the experiment design. To assay the efficiency of parasite transfection another reporter gene, selected, the GFP at pEGFP_N1. In combination, pcDNA3 was also used because it contained the Neo^r gene, thus allowing screening of the transfected cells along time; both plasmids containing neomycin-resistance cassette (Neo^r). This characteristic allowed the geneticin selection to be maintained in the transfected cells throughout time.

Various selectable markers have been successfully developed for transfection vectors but geneticin (Mamoun *et al.*, 1999) was chosen to be used in our work. There was no visible GFP expression in *P. olseni* cells submitted to transfection, so the green fluorescence emission observed could not be associated with efficient transfection. These difficulties at GFP observation were caused by a slight auto-fluorescence of the *P. olseni* cells in response to excitation with blue light (440-490 nm). However, each assay results were observed under fluorescent microscope, in parallel with the application of geneticin selection at 48h after transfection and followed, when possible, by RT-PCR assay to check for plasmid presence in the cells. Several methodologies were performed using both pEGFP-N1 and pcDNA3.1 to check effective transfection in the experiments.

VI-3.3 Are chemical methodologies efficient for *P. olseni* cells transfection?

Transfection by calcium phosphate, originally discovered by S. Bacchetti and F. L. Graham in 1973 was the first to be tested in *P. olseni* cells. In the experiments using proportions of 1:1 and 1:1.2 of DNA: calcium phosphate, no fluorescence was observed in the transfection assay in comparison to control at 16 hours or at 40 hours after transfection. The complex precipitation condition is of decisive importance for cell transfection efficiency and the parameters, pH, salt and DNA concentrations, temperature, type of cells and the time between precipitation and transfection were described as crucial (Bachetti and Graham, 1977). In these experiments like incubation temperature parameter was adjusted to *P. olseni* cell type.

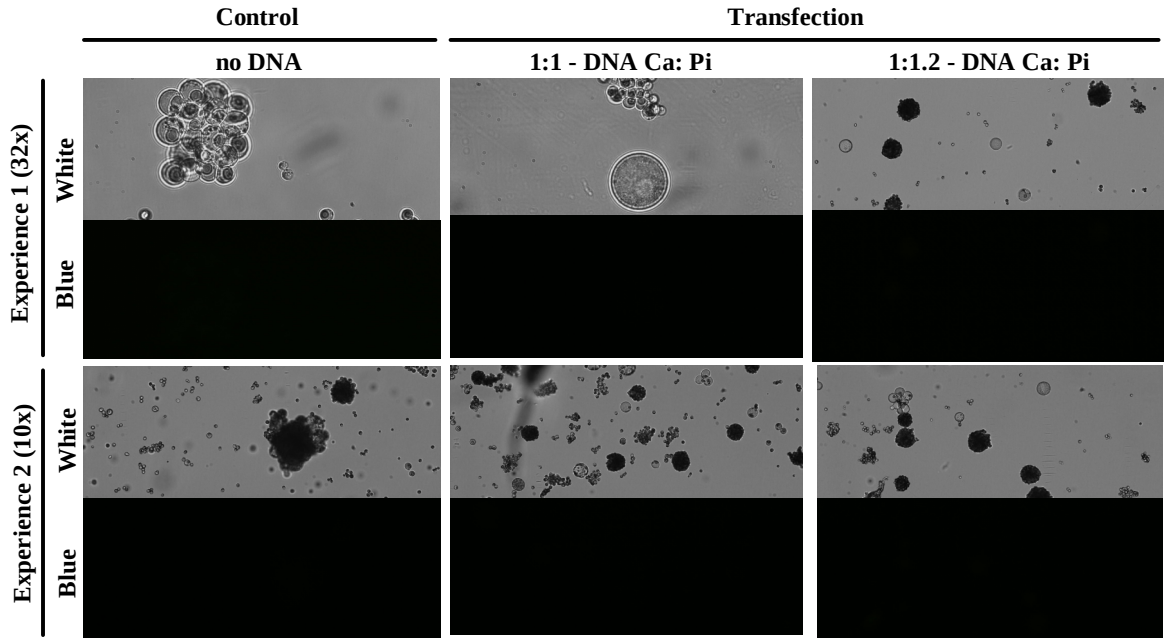


Figure VI- 4 *P. olseni* cells submitted to calcium phosphate transfection assay. The results compared two ratios between DNA and calcium phosphate; two experiments were performed in duplicated and observed at different magnification under white and blue light.

Other chemical transfection methodologies were performed; two commercial polyethylenimine polymers were tested: PEI (Sigma Aldrich) and TransPEI (Eurogentec). PEI amount was added to DNA in various proportions recommended but

any ratio of polycationic polymer reagent with DNA used to transfect the *P. olseni* cells gave no significative emission of green fluorescence, either after 24, 48 and 72 hours (Figure VI-5).

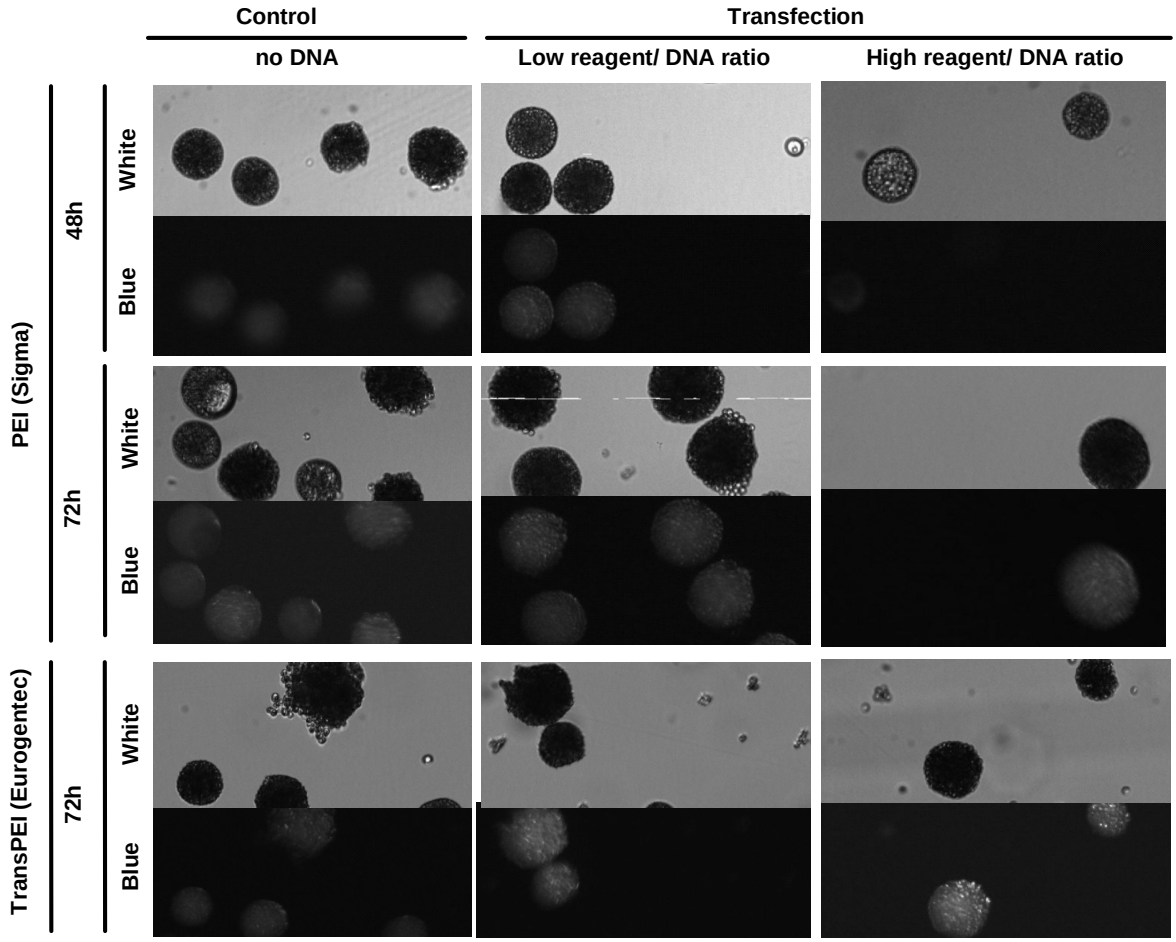


Figure VI- 5 *P. olseni* cells submitted to polycationic polymer transfection assay using two different commercial products, PEI (Sigma Aldrich) and TransPEI (Eurogentec). The results compared two ratios between DNA and transfection reagent. Two independent experiments duplicated were performed and observed at different magnification at 48 and 72hours under white and blue light.

Linear PEI was considered one of the most efficient and popular compounds for delivery of DNA into animal cells (Kopatz *et al*., 2004) being described that it is able to cross the cells membrane carrying the exogenous DNA. However, presence of a cell wall in *P. olseni* trophozoite cells (Ordas and Figueras, 1998 and Montes *et al*., 2002) may interfere in the endocytosis of chemical agent/DNA complexes.

PEI available in the laboratory was 25kDa MW but there are polyanionic macromolecules smaller, with a number of polycationic fragments in the range from about 8 to about 15, described to transfer DNA across biological barriers as a cell wall or plasma membrane (US Patent 7060498). Other chemical transfection approaches based on DEAE- dextran, polymer and lipid carrier molecules were mostly described to be highly efficient in Eukaryotic cells. These techniques were not further pursued once it was clear that the parasite trophozoites presented a cell-wall (Ordás and Figueras, 1998, Montes *et al.*, 2002). The following approach was the transfer of DNA into the cells by physical methods.

VI-3.4 Is electroporation efficient to transfect *P. olseni* cells?

Electroporation was the technique most used for transfection of parasites and other cell wall-possessing organisms (Appendix VI-1), so it was also one of the methodologies explored to transfer DNA into *P. olseni* cells. This physical method implied the application of an electric pulse to the cells between two plate electrodes using BioRad Gene Pulser. For this, the medium of cells is crucial. Different cell type medium for electroporation were screened based on information available for other parasites (Appendix VI-1), in order to get the most appropriate medium to perform the assays, for a better pulse diffusion and to increase the rate of cell survival. The cells were observed after each test in the various media and viability was checked for each assay.

FBS is one component of the cell culture standard medium that was avoided because when used for electroporation, FBS caused electric circuit diffusion due to its high salt content. Despite the removal of FBS from standard medium there were too many salts that interfered with pulsing producing arching (Figure VI-6). Glycerol was found to cause no disturbs in the electric pulse, however the cells were severely injured and no recovery was observed in the trophozoite cells after electroporation (Figure VI-

6). Cytomix was referred as a media for electroporation of *P. knowlesi* (van-der-Wel *et al*., 1997) and *E. histolytica* (Nickel and Tannich, 1994) but frequently the pulse was not constant and the system did arching when testing *P. olseni* cells. Parasite trophozoite cells showed extended cell damages (Figure VI-6) compared to results obtained with sorbitol 1M, the solution also described for yeast transfection (De-Backer *et al*., 1999). In this media, *P. olseni* cells presented the same morphology as in their standard cell culture medium (Figure VI-6) and recovered very well from electroporation. No arching problems were observed using this medium. So electroporation assays were performed in sterile 1M sorbitol medium and after, *P. olseni* cells were directly added to standard culture medium and allowed to recover prior to observation for transfection efficiency.

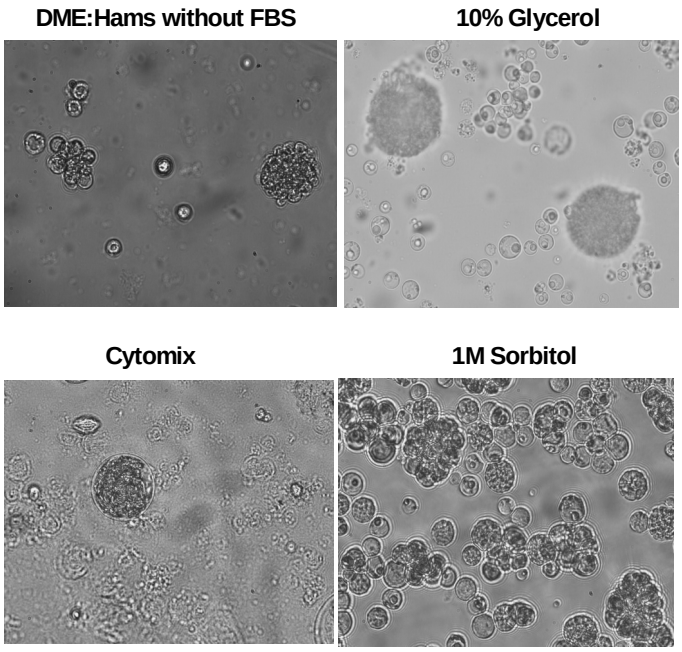


Figure VI- 6 Viability of *P. olseni* cells submitted to electroporation. Observation through optical microscopy of *P. olseni* trophozoites viability checking for changes in morphology in the various media used for electroporation.

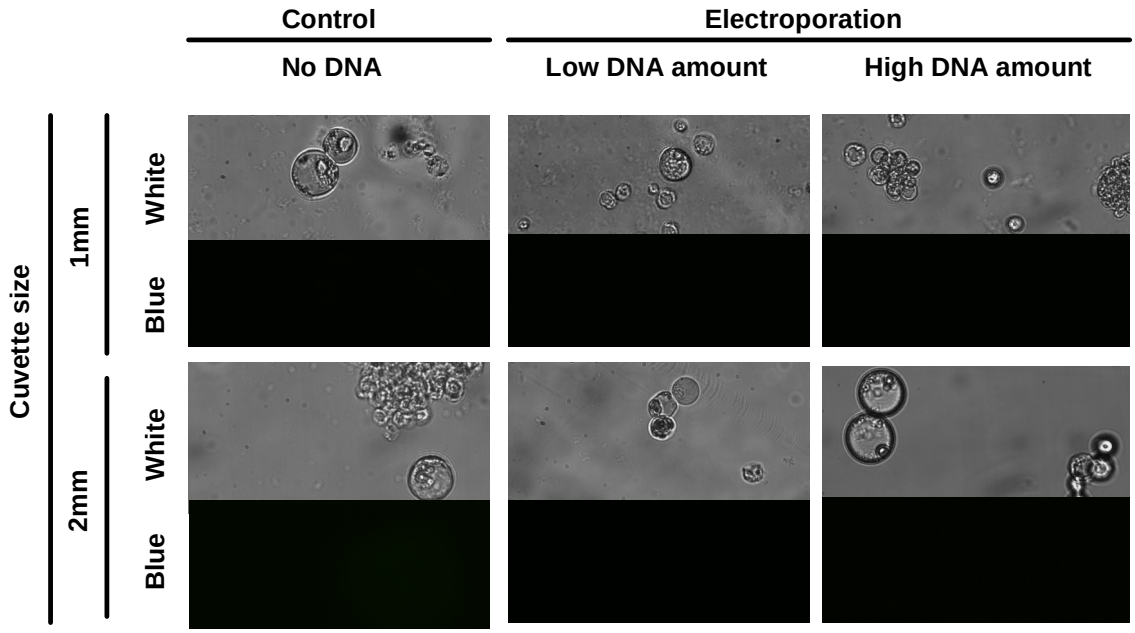


Figure VI- 7 Electroporation of *P. olseni* cells with two different amounts of purified plasmid DNA, (50 ng and 500 ng).

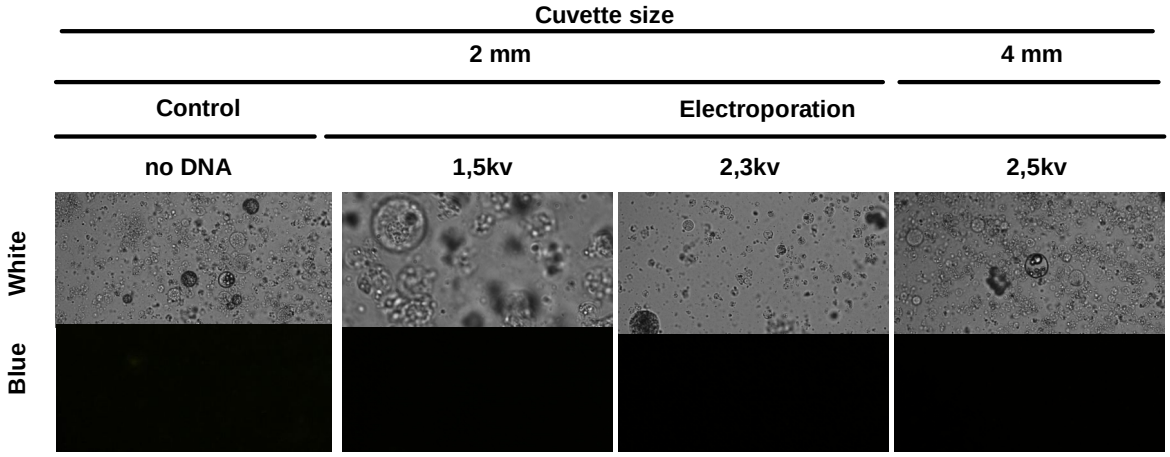


Figure VI- 8 Electroporation of *P. olseni* cells at various conditions of electro-pulse and cuvette size.

In electroporation various quantities of plasmid DNA, various cell numbers according to available cuvettes (1mm and 2mm) and a diversity of electroporation conditions were tested as described in the materials and methods. The results were observed after 24 and 48 hours but no fluorescence was observed in any condition (Figure VI-7 and Figure VI-8).

Figure VI-9 shows fluorescent schizonts six days after transfection; cels appearedl viable and the GFP fluorescence was above background. This result was obtained using one pulse of 2ms at 2.5 kv voltage, 25 μF capacitance, 200 Ω resistance, but it also resulted in arching in the moment of electroporation. This arching means the pulse was not constant and therefore no direct relation between this result and transfection efficiency or conditions tested can be taken. Following this assay, the cells were kept under selection for 12 days. During that time, fluorescence was not visible but 15 days after transfection, the cells looked viable and positive for the presence of transfection vector.

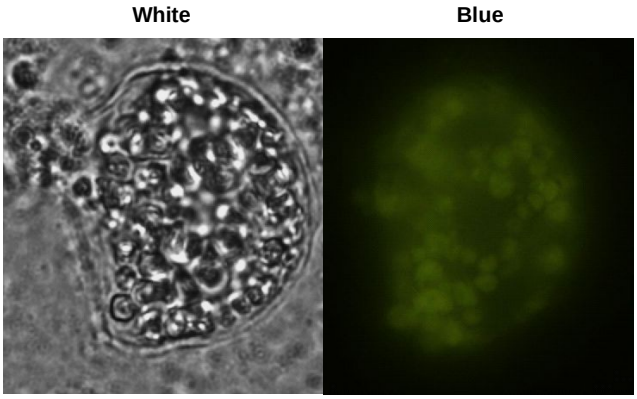


Figure VI- 9 *P. olseni* fluorescent schizont submitted to electroporation transfection assay. The electroporation assay did arching and the fluorescence was observed at 6th day after transfection using 400x magnification under fluorescence microspcope.

Although DNA transfers looked originally promising, electroporation conditions were not characterized because of arching circumstances (Figure VI-10). Furthermore, when the experiment was repeated, the same result was not obtained thus it was considered to be an artefact of the first experiment.

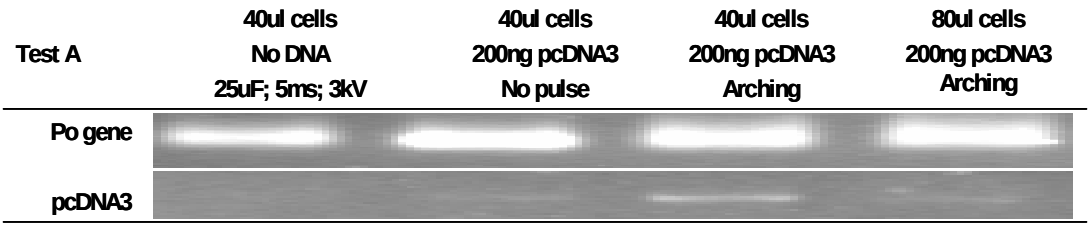


Figure VI- 10 P. *olseni* cells submitted to electroporation assay at various conditions of electro-pulse, in 2 mm cuvette. RT-PCR was performed for each assay 15 days after transfection using pcDNA3s Po gene, specific amplification primers and PoClhl as reference gene for PCR amplification.

Many described conditions were tested with no observed transfection of *P. olseni* cells. The lack of confirmed transfection or the inability of the parasite cells to express GFP, many issues remain to be studied. Having unacceptable results either with PEI and TransPEI and Calcium phosphate tests or with electroporation it were performed assays to determine the possibility to improve permeabilize of trophozoits cell wall as a pre-treatment before transfection.

VI-3.5 How to increase the probability of transfection of *P. olseni* cells?

According to literature, cell permeabilization could improve transfection effectiveness. Two approaches were selected; one based on enzymatic digestion, by pronase and other by chemical stress, dithiothreitol (DTT). Reece and her collaborators used pronase 125µg/ml to permeabilize *Perkinsus* sp. (Elston *et al*., 2004). So, for *P. olseni* trophozoites, the permeabilization was assayed at 28ºC and 37ºC using pronase at various concentrations (Figure VI-11). At 5 min incubation, 10mg/ml of pronase was cytotoxic and the permeabilization condition was defined as the concentration before the concentration for which there was a decrease in cell viability, in shorter time incubation. So, 15 min incubation of the cells at 28ºC with 5mg/ml concentration (Figure VI-11) was used from then on. Two more transfection attempts were performed using three different methods previously tested, after cells pre-treatment permeabilization with pronase or DTT. In *S. pombe* the efficiency transformation was

enhanced with a pretreatment with 25mM DTT (Suga and Hatakeyama, 2001), so this treatment was used also for *P. olseni*.

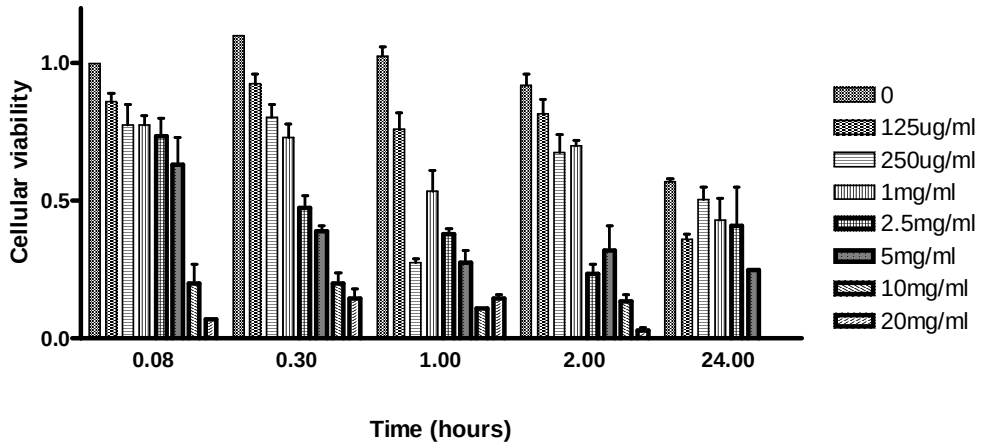


Figure VI- 11 Observation of *P. olseni* cell viability under a range of pronase concentration treatment, at 28ºC. Trypan blue was the procedure used to quantify the cellular viability and the cell counting was performed in photos taken to the cells homogeneously distributed in the heamocytometer observed at 10x magnification.

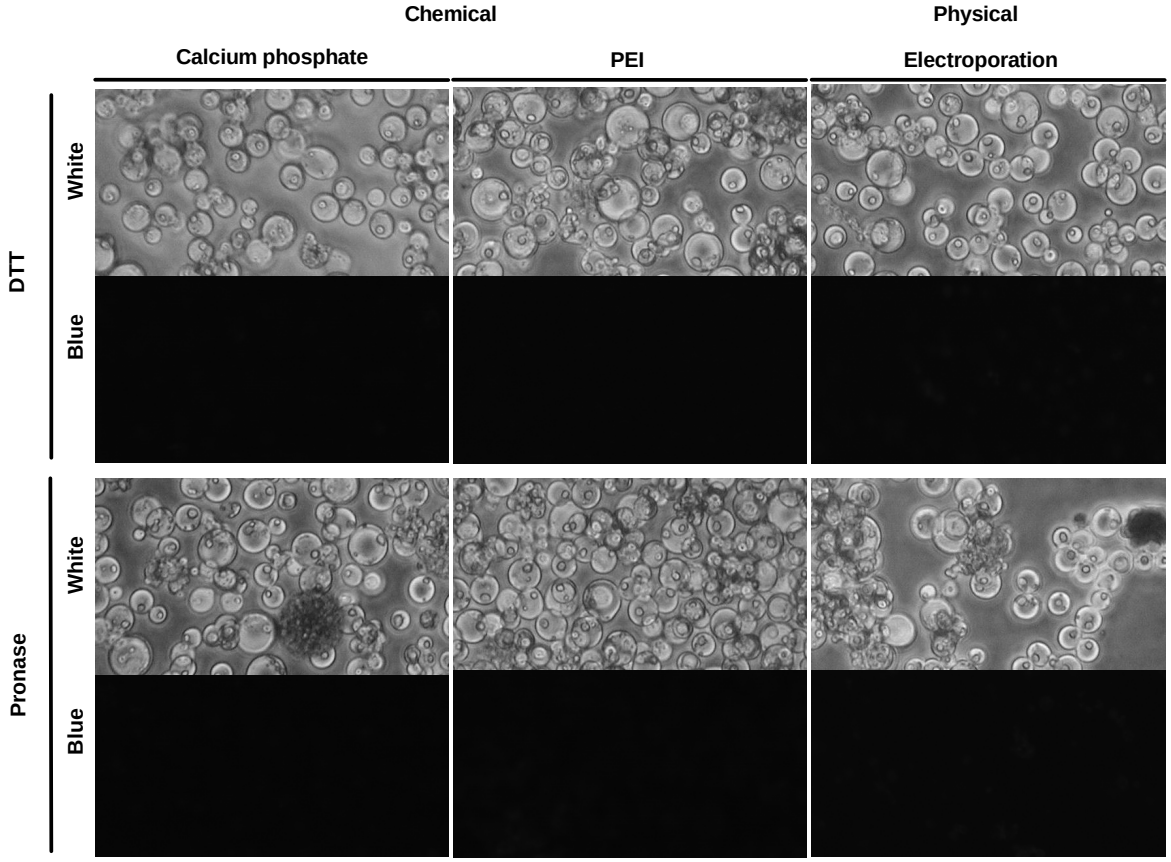


Figure VI- 12 *P. olseni* cells submitted to calcium phosphate and PEI transfection and to electroporation, after pre-treatments with either DTT or Pronase. for cells permeabilization. Results were observed at 48h after transfection using 200x magnification under the inverted fluorescence microspcope.

From all experiments performed there was no visible fluorescence associated with the diverse transfection methodologies tested (Figure VI-12), after permeabilization treatment of parasite cells. This indicates that the pre-treatments tested had no effect on the development of transfection method.

VI-3.6 Is Biolistic technique applicable to transfect *P. olseni* ?

Biolistics, or particle bombardment, has been used successfully to introduce nucleic acids into plant cells, animal tissues or organs, and whole organisms that typically are described not amenable to more traditional methods of transfection. Microparticle bombardment was the alternative to electroporation method. These experiments were performed in the laboratory of Plant Biotechnology at ITQB under Dr. Margarida Oliveira supervision. For the application of this procedure it was taken into account various conditions: pressure, DNA and gold amount per shoot (Table VI-5).

Table VI- 5 Factors influencing transformation efficiency by microparticle bombardment of *P. olseni* (based on Rasco Gaunt, 2001).

Variable factors	**Cell number per 100µl**	$2x10^7$
		$2x10^8$
	Flying distance (cm)	0.1
		1
Constant factors	**Bombardment pressure (psi)**	1,350
	Amount of gold (mg/ shoot)	0.5
	DNA amount (µg/ shoot)	3.5

The parasite trophozoite cell culture was transported and acclimated to the reference conditions and passed twice to ensure the good quality of the culture. A two day cell culture was used to seed the previously prepared plates containing solidified medium using 2% agar (W/ W). Cells were spread in the centre one third of a plate of solid agar medium 1 hour before bombardment. Immediately after the bombardment the cells were incubated at 28ºC, in solid agar medium for examination at 24 and 48 hours but no fluorescence was observed.

Table VI- 6 Procedure for microparticle bombardment of *P. olseni* cells**.**

Cell Culture	Biolistic equipment	Microcarrier	Bombardment
Day -3 1x10^6 cell culture	**Day 0** Sterilize paper, macrocarriers, nests, petri dishes forceps, pipette tips Prepare agar medium plates	**Day 0** Prepare gold Prepare the DNA Start the complexes	**Day 1** Plate 100ul cells 2x108c/ml 2x109c/ml Prepare complexes DNA-gold Sterilize all equipment Bombardment
Day -1 1x10^6 cell culture			
Selection	**Clone selection**	**Transformant**	**Store transformants**
48h after transfection Check by GFP exp Start geneticin selection	**6th week** Pick 3 replica colony (a# b# c#) Growth and selection Of transformants	**Until 6th month** No growth 36 colonies picked	**Until 6th month** No growth No transformants No transfection observed
Until 3rd week Selection 0.72mg/ml **Until 6th week** Selection 0.1mg/ml			

After 48 hours, geneticin selection was started and the master plates were maintained for 6 months to observe the growth of possible resistant colonies, corresponding to positively transfected *P. olseni* trophozoites. Although fluorescence was never observed, viable colonies of cells were transferred to liquid medium and allowed to grow under geneticin selection. The plates were maintained protected from

desiccation and contamination for transport back to EDGE laboratory. After 3-4 weeks selection, the cells were tested for presence of the transferred DNA through RT-PCR but no amplification was obtained with GFP gene specific primers. The first particle delivery was developed by Sanford and co-workers in (Klein *et al*., 1987) (Johnston *et al*., 1988). Despite the various attempts, integration and expression of target DNA in geneticin resistant *P. olseni* cells was not confirmed since no exogenous DNA could be detected in these conditions, indicating that cells were not successfully transfected.

VI-3.7 Is Microporation technique applicable to transfect *P. olseni* ?

Microporation (Digital Bio Technology, Suwon, Korea) is a recently developed electroporation technique producing high-voltage pulses in a pipette tip as electroporation space creats uniform electric field with minimal heat generation and oxide formation. Various conditions were tested, from one to three pulses of various voltage ranging from 900 to 1700 volts. After 48 hours there was no evidence of fluorescence (Figure VI-13). However, this was a single experiment because the equipment was not available for a additional trials. However, the absence of expected fluorescence may be due to ineffective introduction of exogenous DNA into the cells or the inability of the transfected cells to express EGFP. Although the advantages of this technique are obvious, i.e the fact of being less aggressive than electroporation and use a predefined transfection medium, there were no positive results for the development of a transfection methodology for *P. olseni* cells.

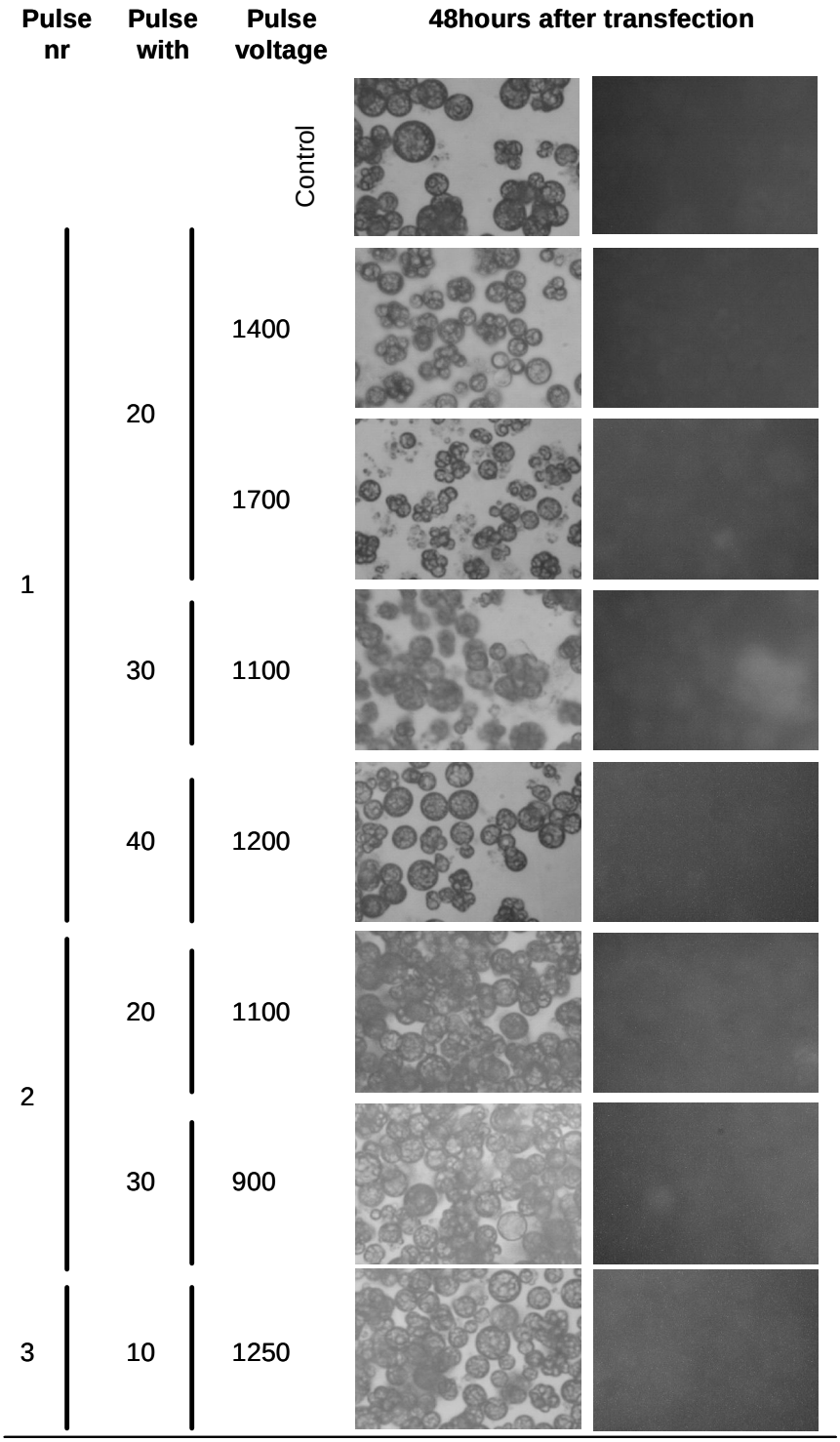


Figure VI- 13 *P. olseni* cells submitted to microporation and observed at 48h after transfection using 200x magnification under inverted fluorescence microscopy.

VI-3.8 Could a *Perkinsus* directed construct produce effective *P. olseni* transfection?

One attractive alternative involves the use of species specific constructs, transfection vectors like it was designed for *Theileria annulata* using *T. annulata* 5' gene flanking sequences linked to the enhanced green fluorescence protein (eGFP) reporter gene (Adamson *et al*., 2004). This strategy was recently followed for *P. marinus* parasite. A plasmid (pPmMOE-GFP) was engineered based on a *P. marinus* identified gene, Pm*MOE*. For *P. marinus* it was used Cell Line Optimization Nucleofector Kit in the Nucleofector II (Amaxa) following the manufactures' instructions resulting in the identification of a combination of transfection solution and program that resulted in 37.8% efficiency of transfection (Fernández-Robledo *et al*., 2008). Despite the evolutionary proximity of *P. olseni* with *P. marinus*, the results for *P. olseni* were not shown and were described as intriguing. This was the first report of a transfection methodology developed for a member of the Perkinsozoa but a successful methodology for *P. olseni* is still missing. Therefore, we are presently trying to use a similar methodology for *P. olseni*. A genus specific transfection construct was prepared using a 600nt fragment containing the 5' UTR of PmHSP70 and 600nt 3'UTR of PoHSP70 flanking the eGFP ORF of the vector pEGFP-N1. The lack of availability of electroporation apparatus was impeditive of testing this construct so far but further studies remain to be addressed in order to develop an efficient methodology for *P. olseni* cells transfection.

VI-4 Conclusions

Development of *P. olseni* transfection involved first the definition of a reporter gene so the efficiency could be followed, according to available transfection vectors:

pβGal- basic, pEGFP-N1 and pcDNA3. The assays screened the last two for regular use since galactosidase was excluded because an ortholog gene was identified *in silico* in the *P. marinus* genome.

To select a series of suitable possible methods to transfect *P. olseni* trophozoites, a bibliography search was performed. Results were compiled but were also indicative of the proximity of *P. olseni* to yeast in terms of methodology available. This was validated when the best medium for electroporation was found to be 1M sorbitol, already used for yeast transfection, and the more promising technique was electroporation, largely used for Apicomplexans transfection. Various chemical (calcium-phosphate and cationic polymers (PEI) and physical (electroporation, microporation and microparticle bombardment) methodologies were tested as possible transfection methodologies for *P.olseni*. However they all resulted in no visible transfection of *P. olseni* cells. Very recently, a transfection procedure by electroporation was finally developed for this genus, in particular for *P. marinus* (Fernández-Robledo *et al*., 2008) through the use of a genus specific construct. A similar approach was taken by the authors for *P.olseni* but was not very successful. We are in the process of attempting to optimize this technique for *P. olseni* cells in culture using a similar genus--specific plasmid (5'UTR-PmHSP70pEGFP3'UTR-PoHSP70). This tool is available for further experiments aiming at introducing exogenous DNA construct in *P. olseni* trophozoites by a transfection strategy.

CHAPTER VII – GENERAL CONCLUSIONS AND PERSPECTIVES

"*This interaction is not a game of solitaire but rather a complex game of chess, the rules of which we are only just beginning to decipher*" by Lakshmi Goyal, editor of Cell: Host and Microbe

This thesis was focused on the molecular characterization of the parasite, *P. olseni* when interacting with its local host in Ria Formosa, the carpet- shell clam, *R. decussatus*.

The study focused on the unveiling of *P. olseni* genes up-regulated when challenged with hemolymph of its host, using a SSH strategy. Being a non-standard system, little EST information was available at the beginning of this work which justified the use of SSH, a widely used approach when other, more sophisticated and highthroughput strategies such as microarrays are not available or too expensive. The SSH approach has been previously used with success to identify differently expressed genes in many other organisms, such as bacteria (Dwyer *et al*., 2004), plants (Bassani *et al*., 2004), rodents (Kirsch *et al*., 2001), birds (Wang, 2006), and humans (Liang *et al*., 2004) but in particular, to identify genes differently expressed in situations of *host-parasite* interaction, such as the clam response to *Perkinsus* infection (Tanguy *et al*., 2004, Kang *et al*., 2006), the response of clam hemocytes to bacterial stimulation (Gestal *et al*., 2007), the *Bonamia ostreae* interaction with *Ostrea edulis* (Morga *et al*., 2007) and the expression profile of genes from *Sparus aurata* brain in response to nodavirus infection (Dios *et al*., 2007). These studies have contributed to identify the genes expressed by an organism in response to a given stress (physiological, environmental, exogenous infection, etc) allowing a "glimpse" into the responsive mechanisms involved and permitting in particular a better understanding of the molecular pathways involved in host-pathogen interactions.

For *P. olseni*, two strategies were followed to come up with the various diverse genes involved in the parasite response to its host, both based on a forward subtractive library (Ascenso *et al*., 2007). Our results allowed the identification of 300 new cDNA

sequences providing a snapshot of the processes taking place in the parasite during its relationship with its host and the environmental conditions that influence host-parasite interaction. By analysing the resulting cDNA sequences with the GO program, we were able to allocate to many of the predicted protein sequences a potential function, thus contributing to better comprehend the parasite response to bivalves' hemolymph exposure (Ascenso *et al.*, unpublished a, b).

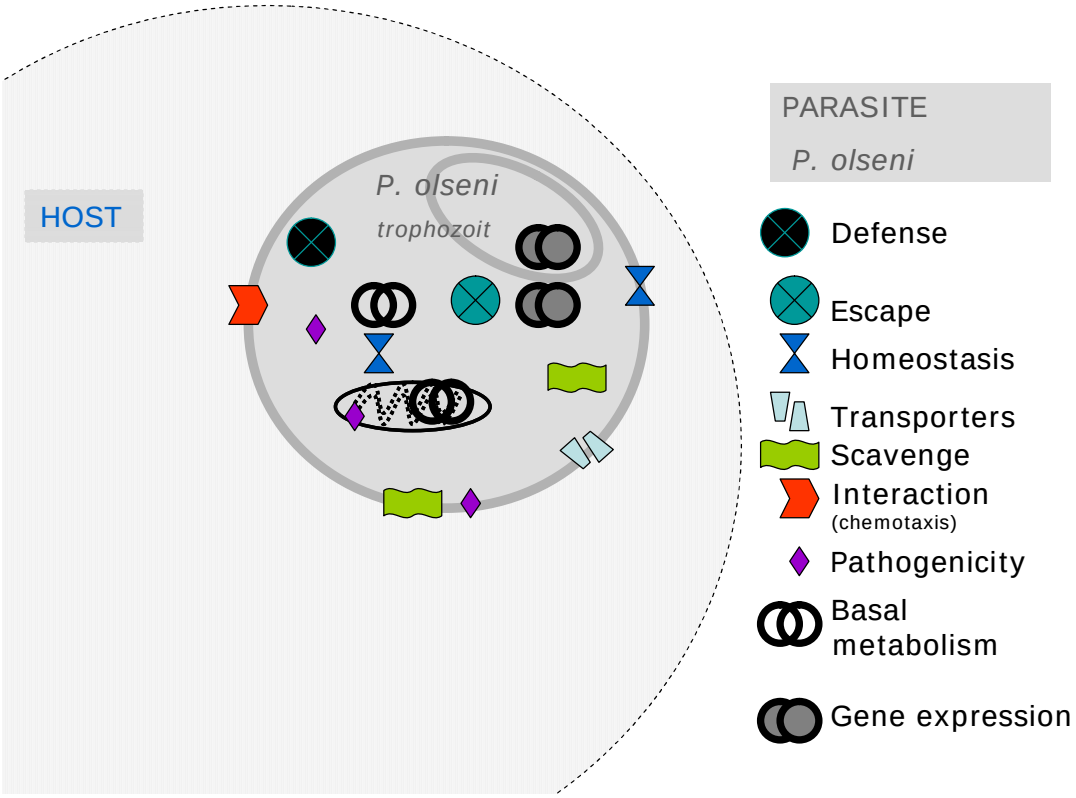


Figure VII- 1 Model proposed for *P. olseni* response to host hemolymph exposure. Compilation of the diverse parasite genes identified by SSH according to the function and possible location in the parasite trophozoite cells when exposed to host hemolymph.

In many other pathogenic microorganisms, the use of SSH strategy allowed the identification of a number of differences in gene expression including (1) *Erwinia amylovora*, the causal agent of fire blight disease in various rosaceous species, namely apple and pear, showed genomic differences between *Erwinia* strains with differing host ranges including two type III secretion systems, putative tyrosine phosphatase effectors,

membrane transporters and polyssacaride biosynthesis (Triplett *et al*., 2006); or (2) *B. ostreae* expressed actin, HSP90, cofilin and alpha tubulin genes in response to *O. edulis* hemocytes (Morga *et al*., 2007). Similarly, our results showed that *P. olseni* responded to host hemolymph by up- regulating a set of genes whose putative functions appear to/could be related to host interaction, including binding activities, catalysis, chaperone, signal transduction, transcritption and translation, transport, cellular processes and cell adhesion. Some hypothetical, not annotated genes were also identified (Ascenso *et al*., 2007), opening the way to additional studies. In figure VII-1 is proposed a cartoon showing the diversity of parasite gene expression upon host interaction: (i) in the defence mechanism it may be involved genes related to catalytic function, cell communication and signalling, (ii) for escape/evasion, catalytic genes and one related with the immune system, (iii) for homeostasis there are several genes associated with transport, sorting and degradation, (iv) for transport there are several transporter genes related, including membrane transporters. The scavenge activity of the parasite is related with some transporters and catalysis of carbohydrates, lipids, nucleotides and amino acids. Lipids are essential, the capacity of *P. marinus* to acquire and use host lipids contributes directly to its pathogenicity. The incorporation, storage and bioconversion of exogenous lipids, the *de novo* synthesis of fatty acids, were studied for *P. marinus* and provided good insights into the metabolic abilities of the protozoan parasite affecting the oyster *C. virginica*, which is able to sequester lipids from host to its survival (Chu *et al*., 2000, Soucieta *et al*., 2000, Lund *et al*., 2007, Venegas-Calerón *et al*., 2007).

The parasite *P. olseni* interactions with its host carpet- shell clam are mainly associated with signalling and transduction genes and the pathogenicity may be determined by several groups of genes previously mentioned combined with cell

communication, cell motility and cell growth and death. Basal metabolism related genes include the structural, transcription and translation associated functions annotated by GO. *P. olseni* presented the up-regulation of chaperone related genes in response to host hemolymph as was observed for *B. ostreae* in response to its host (Morga *et al*., 2007).

There are non-annotated genes which may bring up other functions relevant for host-parasite interaction that were not described previously in any other species studied until now. It has been known for some time that parasitic protozoa have capacity to evade the immune system of their host, expressing numerous proteins of self defence, as were already described for *P. marinus* (Schott *et al*., 2003a) (Schott *et al*., 2003b) (Ahmed *et al*., 2003) (Pecher, 2007). Parasites have enormous advantages in their life cycle and ability to alter themselves and their host to their own advantage. The mechanism used by the parasite to adapt to their host and their own environment in order to obtain essential nutrients for survival and growth and endure the complexity of the interactions between parasites and their hosts, is one of the intriguing aspects of parasitism that we begin to decipher in this non-model system of *P. olseni* that infects various molluscs. This work is a first step toward *P. olseni* overall host-response expression mechanisms and should help us understand the mechanisms involved in parasite infection.

Gene-for-gene interactions between pathogen and host are little known for this host-parasite interaction system but comparative genomic studies could enrich this characterization; first approach was the identification of parasite candidate genes but the analysis of their roles in host range and pathogenicity would be challenging.

For *P. olseni* it was followed the strategy of macroarray for closer analysis of parasite response to permissive, resistant and non permissive bivalves using a selection of 98 cDNA sequences from SSH results; those most expressed. SSH followed by

macroarray was used to reveal differential gene expression profiles of *S. aurata* brain infected with nodavirus (Dios *et al*., 2007). The pattern of gene expression was clarifying of the up-regulation of the identified genes and some genes pointed a differential expression in response to the various bivalves present in Ria Formosa, namely, *R. decussatus*, type host susceptible, *R. philippinarum* a permissive, less susceptible host and *D. trunculus* non-permissive to infection.

The highest up-regulated in the presence of host *R. decussatus* hemolymph and differentially expressed in response to bivalves were further studied. PoNHE, encoding a putative electron transporter and PoAdh, a putative cell adhesion associated protein were characterized because of their functions. These genes revealed a pattern of expression in response to environmental stresses, such as extreme salinities and temperatures. But there was evidence that PoNHE gene expression increased with the increasing level of infection of *R. decussatus* (Ascenso *et al*., unpublished b) corroborating the fact that parasites may experience a wide range of environments and nutrients availabilities for their episodes of growth and differentiation (Woo, 1998), leading to different gene expression patterns. The effect of environmental modulation of the virulence factors, the pathogenicity and the metabolism of *P. olseni* could be further explored using a field approach comparing virulence and metabolism of parasites purified and cultivated from infected molluscs coming from different sites.

Collaborative efforts would have a positive impact on our knowledge of the parasite host-response and could contribute to understand outbreaks and evaluate their impact on molluscs' populations. Ultimately it could contribute to improve aquaculture, namely crossing information about parasite and host separately and integrating it with host- parasite interaction studies.

The most represented gene from the forward cDNA subtractive library constructed, PoClhl was further analysed. This gene, first classified as related to catalysis of nucleotides, was thought to code for cytosine deaminase (CD), a pyrimidine *salvage* pathway enzyme. This fact was considered very valuable because this pathway has only been described to be present in bacteria, yeast and lower eukaryotes including, now, the parasite *Perkinsus*. CD activity in the parasite led to further studies to compare the system CD/5FC with other model systems, namely *E. coli* and yeast. Bacterial and fungal cytosine deaminase are commonly used in gene-direct enzyme prodrug therapy (GDEPT) based on their potential of activation (Springer and Niculescu-Duvaz, 2000). PoClhl potential of activation was calculated around eight fold, representing a high conversion of the nontoxic substrate to the toxic product.

To characterize a GDEPT system, another parameter must be taken into account, the degree of activation (Springer and Niculescu-Duvaz, 2000). Therefore, further studies should be performed to calculate the PoClhl degree of activation using a transfected derivate cell line. Tests were performed using eukaryote and prokaryote exogenous systems but no significant results could be achieved. Although the bacterial CD is the most used until these days and has been improved for better cancer treatment by gene therapy (Shieh *et al*., 2006) (Kaliberov *et al*., 2007), new models could be valuable and deserve to be investigated, namely systems distant enough in terms of metabolic pathways, to be satisfactory and effective when applied.

Secondly, the thesis has addressed the issue of development of a transfection methodology, in order to enable the transfer of DNA into the parasite cells for gene regulation studies. Although not achieved, several methods were screened, still many new techniques were not tested and lately a transfection method was described (Fernández-Robledo *et al*., 2008). The methodology used for *P. olseni* trophozoites

transfection was developed using a specific construct, and although not highly efficient, it should now serve as the basis to pursue the development/optimization of the methodology for *P. olseni* trophozoites transfection. Further optimization studies must be performed to improve the efficiency of transfection, which is required in order to use *P. olseni* as a potential model of research.

To complement studies of parasite host- response and comprehend the functions of the numerous non-annotated genes identified it would also be useful to develop gene-specific knock-downs, as well as sub and sober-expression parasite cell lines. This would lead us to a better comprehension of the molecular mechanisms involved in protozoan parasite infection.

CHAPTER VIII- REFERENCES

ADAMSON, R., LYONS, K., SHARRARD, M., KINNAIRD, J., SWAN, D., GRAHAM, S., SHIELS, B. & HALL, R. (2004) Transient transfection of Theileria annulata. *Molecular & Biochemical Parasitology,* 114**,** 53-61.

AHMED, H., SCHOTT, E. J., GAUTHIER, J. D. & VASTA, G. R. (2003) Superoxide dismutases from the oyster parasite Perkinsus marinus: purification, biochemical characterization, and development of a plate microassay for activityq. *Analytical Biochemistry,* 318**,** 132-242.

ALTSCHUL, S. F., MADDEN, T. L., SCHÄFFER, A. A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25**,** 3389-3402.

ANDERSEN, L., KILSTRUP, M. & NEUHARD, J. (1989) Pyrimidine, purine and nitrogen control of cytosine deaminase synthesis in Escherichia coli K 12. Involvement of the glnLG and purR genes in the regulation of codA expression. *Arch Microbiol,* 152**,** 115-118.

ANDERSON, R. S., BURRESON, E. M. & PAYNTER, K. T. (1995) Defense responses of hemocytes withdrawn from *Crassostrea virginica* infected wth *Perkinsus marinus*. *Journal of Invertebrate Pathology,* 66**,** 82-889.

ANDERSON, R. S., UNGER, M. A. & BURRESON, E. M. (1996) Enhancement of *Perkinsus marinus* disease progression in TBT-exposed oysters (*Crassostrea virginica*). *Marine Environmental Research,* 42**,** 177-180.

ANDREWS, J. D. & RAY, S. M. (1988) Management strategies to control the disease caused by *Perkinsus marinus*. *American Fisheries Society Special Publication,* 18**,** 257-264.

ARMALEO, D., YE, G.-N., KLEIN, T. M., SHARK, K. B., SANFORD, J. C. & JOHNSTON, S. A. (1990) Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi *Current genetics,* 17**,** 97-103.

ARRIZABALAGA, G., RUIZ, F., MORENO, S. & BOOTHROYD, J. C. (2004) Ionophore-resistant mutant of *Toxoplasma gondii* reveals involvement of a sodium/hydrogen exchanger in calcium regulation. *The Journal of Cell Biology*, 165**,** 653-662.

ASCENSO, R. M. T., LEITE, R. B., AFONSO, R. & CANCELA, M. L. (2007) Suppression-subtractive hybridization: A rapid and inexpensive detection methodology for up-regulated *Perkinsus olseni* genes. *African Journal of Biochemistry Research,* 1 (3)**,** 24-28.

ASCENSO, R. M. T., LEITE, R. B., AFONSO, R., CANCELA, M. L. (unpublished a) Expression pattern of Perkinsus olseni genes in response to bivalves with different susceptibility to Perkinsosis, *Journal of Fish Disease, *,*-**

ASCENSO, R. M. T., LEITE, R. B., AFONSO, R. & CANCELA, M. L. (unpublished b) Molecular characterization of two novel *Perkinsus olseni* genes encoding putative Sodium Hydrogen Exchanger and Adhesion-related proteins. *Experimental Parasitology, **, *-*.

AZEVEDO, C. (1989a) Fine structure of *Perkinsus atlanticus* n. sp. (Apicomplexa, Perkinsea) parasite of the clam *Ruditapes decussatus* from Portugal. *J. Parasitol.,* 75**,** 627-635.

AZEVEDO, C. (1989b) *Perkinsus atlanticus* nova espécie protoparasita da ameijoa *Ruditapes decussatus* (Mollusca, Bivalvia). *Pesca e Navegação.*

BACHETTI, S. & GRAHAM, F. L. (1977) Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex viral DNA. *Proc. Natl. Acad. Sci. USA,* 74**,** 1590-1594.

BASSANI, M., NEUMANN, P. & GEPSTEIN, S. (2004) Differential expression profiles of growth-related genes in the elongation zone of maize primary roots. *Plant Molecular Biology,* 56**,** 367-380.

BASTIN, P., ELLIS, K., KOHL, L. & GULL, K. (2000) Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system. *Journal of Cell Science,* 113**,** 3321-3328.

BEBIANNO, M. J., GÉRET, F., HOARAU, P., SERAFIM, M. A., COELHO, M. R., GNASSIA-BARELLI, M. & ROMÉO, M. (2004) Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. *Biomarkers,* 9**,** 305-330.

BEBIANO, M. J. (1995) Effects of pollutants in the Ria Formosa lagoon, Portugal. *The Science of the total environmental,* 171.

BELHSEN, O., KODDAD, S., TALBAOUI, E. & ORBI, A. (2007) The Moroccan plan of zoo sanitary surveillance of shellfish. *WOPER Workshop for the analysis of the impact to the European shellfish Industry.* CETMAR, Vigo, Spain.

BERTHE, F. (2001) Report about mollusc diseases. *IFREMER.*

BLACKBOURN, J., BOWER, S. M. & MEYER, G. R. (1998) *Perkinsus qugwadi* sp.nov. (*incertae sedis*),a pathogenic protozoan parasite of japanese scallops, *Patinopecten yessoensis*, cultured in British Columbia, Canada. *Can. J. Zool.,* 76**,** 942-953.

BOUQUARD, C., OUZZANI, J., PROMÉ, J.-C., MICHEL-BRIAND, Y. & PLÉSIAT, P. (1997) Dechlorination of Atrazine by a *Rhizobium* sp. Isolate. *Applied and Environmental Microbiology,* 63**,** 862-866.

BOWER, S. M., BLACKBOURN, J. & MEYER, G. R. (1998) Distribution, prevalence, and pathogenicity of the protozoan *Perkinsus qugwadi* in japanese scallops, *Patinopecten yessoensis*, cultured in British Columbia, Canada. *Can. J. Zool.,* 76**,** 954-959.

BOWER, S. M., BLACKBOURN, J., MEYERL, G. R. & WELCH, D. W. (1999) Effect of *Perkinsus qugwadi* on various species and strains of scallops. *Diseases of Aquatic Organisms,* 36**,** 143-1511.

BRANDER, K. M. (2007) Global fish production and climate change. *PNAS,* 104**,** 19709-19714.

BRETT, C. L., DONOWITZ, M. & RAO, R. (2005) Evolutionary origins of eukaryotic sodium/proton exchangers. *American Journal of Cell Physiology,* 288**,** C223-C239.

BROOKS, D. R., MCCULLOCH, R., COOMBS, G. H. & MOTTRAM, J. C. (2000) Stable transformation of trypanosomatids through targeted chromosomal integration of the selectable marker gene encoding blasticidin S deaminase. *FEMS Microbiology letters,* 186**,** 287-291.

BROWN, G. D. & REECE, K. S. (2003) Isolation and characterization of serine protease gene(s) from *Perkinsus*. *Diseases of Aquatic Organisms,* 57**,** 117-126.

BURRESON, E. M., ALVAREZ, R. S., MARTINEZ, V. V. & MACEDO, L. A. (1994) *Perkinsus marinus* (Apicomplexa) as a potential source of oyster *Crassostrea virginica* mortality in coastal lagoons of Tabasco, Mexico. *Dis. aquat. Org.,* 20**,** 77-82.

BURRESON, E. M. & RAGONE CALVO, L. M. (1996) Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake bay, with emphasis on data since 1985. *Journal of Shellfish Research,* 15**,** 17-34.

BURRESON, E. M., REECE, K. S. & DUNGAN, C. F. (2005) Molecular, Morphological, and Experimental Evidence Support the Synonymy of Perkinsus

chesapeaki and Perkinsus andrewsi. *The Journal of Eukaryotic Microbiology,* 52**,** 258-270.

BUSHEK, D. & ALLEN, S. K., JR. (1996) Host-parasite interactions among broadly distributed populations of the eastern oyster *Crassostrea virginica* and the protozoan *Perkinsus marinus*. *Mar. Ecol. Prog. Ser.*, 139**,** 127-141.

BUSHEK, D., FORD, S. E. & CHINTALA, M. M. (2002a) Comparison of in vitro-cultured and wild-type Perkinsus marinus. III. Fecal elimination and its role in transmission. *Disease of Aquatic Organism,* 51**,** 217-25.

BUSHEK, D., HEIDENREICH, M. & PORTER, D. (2007a) The effects of several common anthropogenic contaminants on proliferation of the parasitic oyster pathogen Perkinsus marinus. *Marine Environmental Research,* 64**,** 535-540.

BUSHEK, D., LANDAU, B., SCARPA, E., BURT, I. & DUNGAN, C. (2007b) First report of *Perkinsus chesapeaki* in Delaware Bay razor clams, *Tagelus plebeius*. *Aquaculture.* San Antonio, TX, USA.

BUSHEK, D., SCARPA, J. & LARAMORE, S. E. (2002b) Susceptibility of the Caribbean oyster Crassostrea rhizophorae to Perkinsus marinus. *Journal of Shellfish Research,* 21**,** 371-372.

CALVO, G. W., LUCKENBACH, M. W., STANDISH K. ALLEN, J. & BURRESON, E. M. (2001) A Comparative Field Study of Crassostrea ariakensis and Crassostrea virginica in Relation to Salinity in Virginia. *Journal of Shellfish Research,* 20**,** 221-229.

CASAS, S. M., GRAU, A., REECE, K. S., APAKUPAKUL, K., AZEVEDO, C. & VILLALBA, A. (2004) *Perkinsus mediterraneus* n. sp., a protistan parasite of the European flat oyster Ostrea edulis from the Balearic Islands, Mediterranean Sea. *Diseases of Aquatic Organisms,* 58**,** 231-244.

CASAS, S. M., PEYRE, J. F., REECE, K. S., AZEVEDO, C. & VILLALBA, A. (2002a) Continuous *in vitro culture* of the carpet shell clam *Tapes decussatus* protozoan parasite *Perkinsus atlanticus*. *Dis Aquat Organ,* 52**,** 217-231.

CASAS, S. M., VILLALBA, A. & REECE, K. S. (2002b) Study of Perkinsiosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). I.Identification of the aetiological agent and *in vitro* modulation of zoosporolation by temperature and salinity. *Dis Aquat Organ,* 50**,** 51-65.

CAVALIER-SMITH, T. (1999) Zooflagellate phylogeny and Systematics of Protozoa. *Biol. Bull.,* 196**,** 393-396.

CHANG, J. G., CHUNG, C., CHO, K., SHIN, Y. S., KIM, J. A. & JUNG, Y. C. (2005) Electroporator Having an Elongated Hollow Member. IN TECHNOLOGY, D. B. (Ed.).

CHASZCZEWSKA-MARKOWSKA, M., STEBELSKA, K., SIKORSKI, A., MADEJ, J., OPOLSKI, A. & UGORSKI, M. (2008) Liposomal formulation of 5-fluorocytosine in suicide gene therapy with cytosine deaminase - for colorectal cancer. *Cancer letters,* 262**,** 164-172.

CHENG, L., ZIEGELHOFFER, P. R. & YANG, N.-S. (1993) In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proc. Natl. Acad. Sci. USA,* 90**,** 4455-4459.

CHOI, K.-S. & PARK, K.-I. (1997) Report on the occurrence of *Perkinsus* sp. in the Manila clams, *Ruditapes philippinarum* in Korea. *Journal of Aquaculture,* 10**,** 227-237.

CHOI, K.-S., WILSON, E. A., LEWIS, D. H., POWELL, E. N. & RAY, S. M. (1989) The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate method. *Journal of Shellfish Research,* 8**,** 125-131.

CHOMCZYNSKI, P. & SACCHI, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem,* 162**,** 156-159.

CHU, F.-L. E. & GREENE, K. H. (1989) Effect of temperature and salinity on *in vitro* culture of the oyster pathogen, *Perkinsus marinus* (Apicomplexa, Perkinsea). *Journal of Invertebrate Pathology,* 53**,** 260-268.

CHU, F.-L. E. & HALE, R. C. (1994) Relationship between pollution and susceptibility to infectious disease in the eastern oyster, *Crassostrea virginica*. *Marine Environmental Research,* 38**,** 243-256.

CHU, F., SOUDANT, P., VOLETY, A. & HUANG, Y. (2000) Uptake and interconversion of fluorescent lipid analogs in the protozoan parasite, *Perkinsus marinus,* of the oyster, *Crassostrea virginica*. *Experimental Parasitology,* 95**,** 240-251.

CIGARRÍA, J., RODRIGUEZ, C. & FERNÁNDEZ, J. M. (1997) Impact of *Perkinsus* sp. on Manila clam *Ruditapes philippinarum* beds. *Diseases of Aquatic Organisms,* 29**,** 117-120.

COMPS, M. & CHAGOT, D. (1987) Pathologie Animale - Une parasitose nouvelle chez la Palourde *Ruditapes decussatus* L. *C. R. Acad. Sc. Paris,* 304**,** 41-44.

CONCEIÇÃO, N., HENRIQUES, N. M., OHRESSER, M. C. P., HUBLITZ, P., SCHÜLE, R. & CANCELA, M. L. (2002) Molecular cloning of the Matrix Gla Protein gene from *Xenopus laevis* Functional analysis of the promoter identifies a calcium sensitive region required for basal activity. *Eur. J. Biochem.,* 269**,** 1947-1956.

COSS, C. A., ROBLEDO, J. A. F., RUIZ, G. M. & VASTA, G. R. (2001a) Description of Perkinsus andrewsi n. sp. Isolated from the Baltic Clam (Macoma balthica) by Characterization of the Ribosomal RNA Locus, and Development of a Species-Specific PCR-Based Diagnostic Assay. *The Journal of Eukaryotic Microbiology,* 43**,** 52-61.

COSS, C. A., ROBLEDO, J. A. F. & VASTA, G. R. (2001b) Fine structure of clonally propagated *in vitro* life stages of a *Perkinsus* species isolated from Baltic Clam *Macoma balthica*. *Journal of Eukaryotic Microbiology,* 48**,** 31-51.

CREMONTE, F., BALSEIRO, P. and FIGUERAS, A. (2005) Occurrence of *Perkinsus olseni* (Protozoa: Apicomplexa) and other parasites in the venerid commercial clam *Pitar rostrata* from Uruguay, southwestern Atlantic coast. *Diseases of Aquatic Organisms* 64:85–90.

DA ROS, L. & CANZONIER, W. J. (1985) *Perkinsus*, a protistan threat to bivalve culture in the Mediterranean basin. *Bull. Eur. Ass. Fish Pathol.,* 5**,** 23.

DABROWSKA, A., SZARY, J. A., KOWALCZUK, M., SZALA, S. & UGORSKI, M. (2004) CEA-negative glioblastoma and melanoma cells are sensitive to cytosine deaminase/5-fluorocytosine therapy directed by the carcinoembryonic antigen promoter. *Acta Biochimica Polonica,* 51**,** 723-732.

DANIELSEN, S., KILSTRUP, M., BARILLA, K., JOCHIMSEN, B. & NEUHARD, J. (1992) Characterization of the *Escherichia coli* codBA operon encoding cytosine permease and cytosine deaminase. *Mol Microbiol,* 6**,** 1335-1344.

DARWIN, A. J. & MILLER, V. L. (1999) Identification of Yersinia enterocolitica genes affecting survival in an animal host using signature-tagged transposon mutagenesis. *Mol. Microbiol.,* 32**,** 51-62.

DAVOUDI, N., MAHBOUDI, F., AZIZI, M., ADELI, A. & MCMASTER, R. W. (2003) Introduction of Three Independent Selection Markers in Leishmania. *Iran. Biomed. J.,* 7**,** 13-18.

DE-BACKER, M. D., MAES, D., VANDONINCK, S., LOGGHE, M., CONTRERAS, R. & LUYTEN, W. H. M. L. (1999) Transformation of *Candida albicans* by Electroporation. *Yeast,* 15**,** 1609-1618.

DE GUISE, S., DUNGAN, C., GOEDKEN, M., MORSEY, B. & SUNILA, I. (2005) The effects of temperature and salinity on apoptosis of *Crassostrea virginica* hemocytes and *Perkinsus marinus*. *Journal of Shellfish Research,* 24**,** 177-183.

DEITSCH, K. W., DRISKILL, C. L. & WELLEMS, H. E. (2001) Transformation of malaria parasites by spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Research,* 29**,** 850–853.

DELLAGI, A., BIRCH, P. R. J., HEILBRONN, J., LYON, G. D. & TOTH, I. K. (2000) cDNA-AFLP analysis of differential gene expression in the prokaryotic plant pathogen Erwinia carotovora. *Microbiology,* 146**,** 165-171.

DEVERS, M., SOULAS, G. & MARTIN-LAURENT, F. (2004) Real-time reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil *Journal of Microbiological Methods,* 56**,** 3-15.

DIATCHENKO, L., LAU, Y.-F. C., CAMPBELL, A., CHENCHICK, A., MOQADAM, F., HUANG, B., LUKYANOV, S., LUKYANOV, K., GURSKAYA, N., SVERDLOV, E. D. & SIEBERT, P. D. (1996) Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *PNAS,* 93**,** 6025-6030.

DIOS, S., POISA-BEIRO, L., FIGUERAS, A. & NOVOA, B. (2007) Suppression subtraction hybridization (SSH) and macroarray techniques reveal differential gene expression profiles in brain of sea bream infected with nodavirus. *Mol Immunol,* 44**,** 2195-2204.

DOZOIS, C. M., DAIGLE, F. & CURTISS, R. (2003) Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic *Escherichia coli* strain. *PNAS,* 100**,** 247-252.

DUMONT, F., MARECHAL, P.-A. & GERVAIS, P. (2004) Cell Size and Water Permeability as Determining Factors for Cell Viability after Freezing at Different Cooling Rates. *Applied and Environmental Microbiology,* 70**,** 268-272.

DUNGAN, C. F. & REECE, K. S. (2006) In Vitro Propagation of Two Perkinsus spp. Parasites from Japanese Manila Clams Venerupis philippinarum and Description of Perkinsus honshuensis n. sp. . *The Journal of Eukaryotic Microbiology,* 53**,** 316-326.

DUNGAN, C. F., REECE, K. S., MOSS, J. A., STOCKES, N. A., HUDSON, K. L., HAMILTON, R. M. & BURRESON, E. M. (2007) Epidemiology evidence contradicts experimental results, and indicates consistent partitioning of hosts by two *Perkinsus* spp. that are co-endemic among sympatric chesapeake bay bivalve molluscs. *WOPER.* Vigo, Spain.

DURAISINGH, M. T., TRIGLIA, T. & COWMAN, A. F. (2002) Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. *International Journal for Parasitology,* 32**,** 81-89.

DWYER, K., LAMONICA, J. & SCHUMACHER, J. (2004) Identification of *Bacillus anthracis* specific chromosomal sequences by suppressive subtractive hybridization. *BMC Genomics,* 5**,** 1-11.

EARNHART, C. G., VOGELBEIN, M. A., BROWN, G. D., REECE, K. S. & KAATTARI, S. L. (2004) Supplementation of *Perkinsus marinus* Cultures with

Host Plasma or Tissue Homogenate Enhances Their Infectivity. *Applied and Environmental Microbiology,* 70**,** 421-431.

EL-SHENAWY, N. S. (2004) Heavy-Metal and Microbial Depuration of the Clam Ruditapes decussatus and Its Effect on Bivalve Behavior and Physiology. *Wiley periodicals, Inc. Rnviron. Toxicol.,* 19**,** 143-153.

ELANDALLOUSSI, L. M., AFONSO, R., NUNES, P. A. & CANCELA, M. L. (2003) Effect of desferrioxamine and 2,2'-bipyridyl on the proliferation of *Perkinsus atlanticus*. *Biomolecular Engeneering,* 20**,** 349-354.

ELANDALLOUSSI, L. M., LEITE, R. B., RODRIGUES, P. M., AFONSO, R., NUNES, P. A. & CANCELA, M. L. (2005) Effect of antiprotozoal drugs on the proliferation of the bivalve parasite *Perkinsus olseni*. *Aquaculture,* 243**,** 9-17.

ELSTON, R. A., DUNGAN, C. F., MEYERS, T. R. & REECE, K. S. (2004) Perkinsus sp. infection risk for Manila clams, Venerupis philippinarum on the Pacific Coast of North and Central America. *Journal of Shellfish Research,* 23**,** 101-105.

FARZAN, M., CHOE, H., VACA, L., MARTIN, K., SUN, Y., DESJARDINS, E., RUFFING, N., WU, L., WYATT, R., GERARD, N., GERARD, C. & SODROSKI, J. (1998) A Tyrosine-Rich Region in the N Terminus of CCR5 Is Important for Human Immunodeficiency Virus Type 1 Entry and Mediates an Association between gp120 and CCR5. *Journal of Virology,* 72**,** 1160-1164.

FELGNER, P. L., GADEK, T. R., HOLM, M., ROMAN, R., CHAN, H. W., WENZ, M., NORTHROP, E. P., RINGOLD, G. M. & DANIELSEN, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA,* 84**,** 7413-7417.

FERNÁNDEZ-ROBLEDO, J. A., LIN, Z. & VASTA, G. R. (2008) Transfection of the protozoan parasite Perkinsus marinus. *Molecular & Biochemical Parasitology,* 157**,** 44-53.

FIDOCK, D. A., NOMURA, T. & WELLEMS, T. E. (1998) Cycloguanil and Its Parent Compound Proguanil Demonstrate Distinct Activities against *Plasmodium falciparum* Malaria Parasites Transformed with Human Dihydrofolate Reductase. *Molecular pharmacology,* 54**,** 1140-1147.

FISHER, W. S., GAUTHIER, J. D. & WINSTEAD, J. T. (1992) Infection intensity of *Perkinsus marinus* disease in *Crassostrea virginica* (Gmelin, 1791) from the Gulf of Mexico maintained under different laboratory conditions. *Journal of Shellfish Research,* 11**,** 363-369.

FONSECA, V. G., LAGO-LESTÓN, A., LAIZÉ, V. & CANCELA, M. L. (2005) Rapid Identification of Differentially Expressed Genes by In Situ Screening of Bacteria. *Molecular Biotechnology,* 30**,** 163-165.

FORD, S. (1996) Range Extension by the oyster parasite *Perkinsus marinus* into the Notheastern United States : Response to climate Change? *Journal of Shellfish Research,* 15**,** 45-56.

FORD, S. E., CHINTALA, M. M. & BUSHEK, D. (2002) Comparison of in vitro-cultured and wild-type *Perkinsus marinus*. I. Pathogen virulence. *Dis Aquat Organ,* 51**,** 187-201.

FOX, B. A., BELPERRON, A. A. & BZIK, D. J. (1999) Stable transformation of Toxoplasma gondii based on a pyrimethamine resistant trifunctional dihydrofolate reductase-cytosine deaminase-thymidylate synthase gene that confers sensitivity to 5-fluorocytosine. *Molecular and Biochemical Parasitology,* 98**,** 93-103.

FROMM, M. E., MORRISH, F., ARMSTRONG, C., WILLIAMS, R., THOMAS, J. & KLEIN, T. M. (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. *Nature biotechnology,* 8**,** 833-839.

FUKUDA, A., NAKAMURA, A., TAGIRI, A., TANAKA, H., MIYAO, A., HIROCHIKA, H. & TANAKA, Y. (2004) Function, Intracellular Localization and the Importance in Salt Tolerance of a Vacuolar Na+/H+ Antiporter from Rice. *Plant and Cell Physiology,* 45**,** 146-159.

GAGNAIRE, B., THOMAS-GUYON, H. & RENAULT, T. (2004) In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes *Fish and Shellfish Immunology,* 16**,** 501-512.

GAMITO, S. (1997) Sustainable management of a coastal lagoonal system (Ria Formosa, Portugal): An ecological model for extensive aquaculture. *International Journal of Salt Lake Research,* 6**,** 145-173.

GAUTHIER, J. & VASTA, G. R. (1995a) In vitro culture of the eastern oyster parasite *Perkinsus marinus* optimization of the methodology. *Journal of Invertebrate Pathology,* 66**,** 156-168.

GAUTHIER, J. D. & VASTA, G. R. (1993) Continuous *in vitro* culture of the eastern oyster parasite *Perkinsus marinus*. *Journal of Invertebrate Pathology,* 62**,** 321-323.

GAUTHIER, J. D. & VASTA, G. R. (1995b) *In vitro* culture of the eastern oyster parasite *Perkinsus marinus*: optimization of the methodology. *Journal of Invertebrate Pathology,* 66**,** 156-168.

GAUTHIER, J. D. & VASTA, G. R. (2002) Effects of plasma from bivalve mollusk species on the in vitro proliferation of the protistan parasite perkinsus marinus. *Journal of Experimental Zoology***,** 221-230.

GESTAL, C., COSTA, M. M., FIGUERAS, A. & NOVOA, B. (2007) Analysis of differentially expressed genes in response to bacterial stimulation in hemocytes of the carpet-shell clam Ruditapes decussatus: identification of new antimicrobial peptides. *Gene,* 406**,** 134-143.

GOBLIRSCH, M., ZWOLAK, P., RAMNARAINE, M. L., WEIHONG PAN, LYNCH, C., ALAEI, P. & CLOHISY, D. R. (2006) Novel Cytosine Deaminase Fusion Gene Enhances the Effect of Radiation on Breast Cancer in Bone by Reducing Tumor Burden, Osteolysis, and Skeletal Fracture. *Clinical Cancer Research,* 12**,** 3168-3176.

GOEDKEN, M. & GUISE, S. D. (2004) Flow cytometry as a tool to quantify oyster defence mechanisms. *Fish & Shellfish Immunology,* 16**,** 532-559.

GOGGIN, C. L. (1992) occurrence of parasitism of the genus *Perkinsus* in France. *Bull. Eur. Ass. Fish Pathol.,* 12**,** 174.

GOGGIN, C. L. (1994) Variation in the two internal transcribed spacers and 5.8S ribossomal RNA from five isolates of the marine parasite *Perkinsus* (Protista, Apicomplexa). *Molecular and Biochemical Parasitology,* 65**,** 179-182.

GOGGIN, C. L. & BARKER, S. C. (1993) Phylogenetic position of the genus *Perkinsus* (Protista, Apicomplexa) based on small subunit ribossomal RNA. *Molecular and Biochemical Parasitology,* 60**,** 65-70.

GOGGIN, C. L. & LESTER, R. J. G. (1987) Occurrence of *Perkinsus* species (Protozoa, Apicomplexa) in bivalves from the Great Barrier Reef. *Dis. aquat. Org.,* 3**,** 113-117.

GOGGIN, C. L. & LESTER, R. J. G. (1995) *Perkinsus*, a protistan parasite of abalone in Australia: a review. *Mar. Freshwater Res.,* 46**,** 639-646.

GOGGIN, C. L., MCGLADDERY, S. E., E, S. K. W. H. Y. T. & CAWTHORN, R. J. (1996) An assessment of lesions in bay scallops Argopecten irradians attributed to Perkinsus karlssoni (Protozoa, Apicomplexa). *Diseases of Aquatic Organisms,* 24**,** 77-80.

GOGGIN, C. L., SEWELL, K. B. & LESTER, R. J. G. (1989) Cross-infection experiments with australian *Perkinsus* species. *Dis. aquat. Org.,* 7**,** 55-59.

GOMES, H. L., LEITE, R. B., AFONSO, R., STALLINGA, P. & CANCELA, M. L. (2004) A Microelectrode impedance method to measure interaction of cells. *Proceedings of IEEE,* 2**,** 1011-1013.

GOONEWARDENE, R., DAILY, J., KASLOW, D., SULLIVAN, T. J., DUFFY, P., CARTER, R., MENDIS, K. & WIRTH, D. (1993) Transfection of the malaria parasite and expression of firefly luciferase. *Proc. Natl. Acad. Sci. USA,* 90**,** 5234-5236.

GORDON-KAMM, W. J., SPENCER, T. M., MANGANO, M. L., ADAMS, T. R., DAINES, R. J., START, W. G., O'BRIEN, J. V., CHAMBERS, S. A., WHITNEY R. ADAMS, J., WILLETTS, N. G., RICE, T. B., MACKEY, C. J., KRUEGER, R. W., KAUSCH, A. P. & LEMAUX, P. G. (1990) Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants. *The Plant Cell,* 2**,** 603-618.

GOYARD, S. & BEVERLEY, S. M. (2000) Blasticidin resistance: a new independent marker for stable transfection of *Leishmania*. *Molecular and Biochemical Parasitology,* 108**,** 249-252.

GRAHAM, F. L. & EB, A. J. V. D. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology,* 52**,** 456-467.

GROSSENBACHER, H., HORN, C., COOK, A. M. & HUTTER, R. (1984) 2-Chloro-4-Amino-1,3,5-Triazine-6(5H)-One: A New Intermediate in the Biodegradation of Chlorinated s-Triazines. *Applied and Environmental Microbiology,* 48**,** 451-453.

GROTH, D., LEHRACH, H. & HENNIG, S. (2004) GOblet: a platform for Gene Ontology annotation of anonymous sequence data. *Nucleic Acids Research,* 32**,** 313-317.

GUPTA, S. (2005) Parasite immune escape: new views into host–parasite interactions. *Current Opinion in Microbiology,* 8**,** 428-433.

HAMAGUCHI, M., SUZUKI, N., USUKI, H. & ISHIOKA, H. (1998) Perkinsus protozoan infection in short-necked clam Tapes (=Ruditapes) philippinarum in Japan. *Fish Pathology,* 33**,** 473-480.

HELLER, M. (2002) DNA microarray technology: devices, systems, and applications. *Annual Reiew Biomed Eng,* 4**,** 129-153.

HENSEL, M., SHEA, J. E., GLEESON, C., JONES, M. D., DALTON, E. & HOLDEN, D. W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science,* 269**,** 400-403.

HILI, H. A. E., SALAH, C. B., AYARI, W. & AMOR, N. B. (2007) Spatial and temporal distribution of Perkinsus olseni in clams Ruditapes decussatus from Medenine costal area (Southern Tunisia). *WOPER Workshop for the analysis of the impact to the European shellfish Industry*. CETMAR, Vigo, Spain.

HINE, P. M. & THORNE, T. (2000) A survey of some parasites and diseases of several species of bivalve mollusc in northern Western Australia. *Disease of Aquatic Organism,* 40**,** 67-78.

HOFMANN, E. E., POWELL, E. N., KLINCK, J. M. & SAUNDERS, G. (1995) Modelling diseased oyster populations I. Modelling *Perkinsus marinus* infections in oysters. *Journal of Shellfish Research,* 14**,** 121-151.

HOLM, L. & SANDER, C. (1997) An Evolutionary Treasure: Unification of a Broad Set of Amidohydrolases Related to Urease. *Proteins: structure, function and genetics,* 28**,** 72-82.

HUBER, B. E., AUSTIN, E. A., RICHARDS, C. A., DAVIS, S. T. & GOOD, S. S. (1994) Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: Significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase

Proc. Natl. Acad. Sci. USA, 91**,** 8302-8306.

IPIMAR (2003) Relatório de actividades do IPIMAR. *ipimar-iniap.ipimar.pt*.

IRETON, G. C., MCDERMOTT, G., BLACK, M. E. & STODDARD, B. L. (2002) The Structure of Escherichia coli Cytosine Deaminase. *J. Mol. Biol,* 315**,** 687-697.

JOHNSTON, S., ANZIANO, P., SHARK, K., SANFORD, J. & BUTOW, R. (1988) Mitochondrial transformation in yeast by bombardment with microprojectiles. *Science,* 240**,** 1538-1548.

JORDAN, M. & WURM, F. (2004) Transfection of adherent and suspended cells by calcium phosphate *Methods,* 33**,** 136-143.

KALIBEROV, S., MARKET, J., GILLESPIE, G., KRENDELCHTCHIKOVA, V., MANNA, D. D., SELLERS, J., KALIBEROVA, L., BLACK, M. & BUCHSBAUM, D. (2007) Mutation of Escherichia coli cytosine deaminase significantly enhances molecular chemotherapy of human glioma. *Gene Therapy,* 14**,** 11111-11119.

KANG, Y.-S., KIM, Y.-M., PARK, K.-I., CHO, S. K., CHOI, K.-S. & CHO, M. (2006) Analysis of EST and lectin expressions in hemocytes of Manila clams (*Ruditapes philippinarum*) (Bivalvia: Mollusca) infected with *Perkinsus olseni*. *Development and Comparative Immunology,* 30**,** 1119-1131.

KARASOV, A. O., BOOTHROYD, J. C. & ARRIZABALAGA, G. (2005) Identification and disruption of a rhoptry-localized homologue of sodium hydrogen exchangers in *Toxoplasma gondii*. *International Journal for Parasitology,* 35**,** 285-291.

KILSTRRUP, M., MENG, L. M., NEUHARD, J. & NYGAARD, P. (1989) Genetic Evidence for a Repressor of Synthesis of Cytosine Deaminase and Purine Biosynthesis Enzymes in *Escherichia coli*. *Journal of Bacteriology,* 171**,** 21244-2127.

KIRSCH, T., WELLNER, M., LUFT, F., HALLER, H. & LIPPOLDT, A. (2001) Altered gene expression in cerebral capillaries of stroke-prone spontaneously hypertensive rats. *Brain Research,* 910**,** 106-115.

KLEIN, T. M., ARENTZEN, R., LEWIS, P. A. & FITZPATRICK-MCELLIGOTT, S. (1992) Transformation of Microbes, Plants and Animals by Particle Bombardment. *Nature biotechnology,* 10**,** 286-291.

KLEIN, T. M., WOLF, E. D., WU, R. & SANFORD, J. C. (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature Biotechnology,* 327**,** 70-73.

KLEINSCHUSTER, S. J., PERKINS, F. O., DYKSTRA, M. J. & SWINK, S. L. (1999) The *in vitro* life cycle of *Perkinsus* species (Apicomplexa, Perkinsidae) isolated from *Macoma balthica* (Linnaeus, 1758). *Journal of Shellfish Research,* 13**,** 461-465.

KO, T.-P., LIN, J.-J., HU, C.-Y., HSU, Y.-H., WANG, A. H.-J. & LIAW, S.-H. (2003) Crystal structure of yeast cytosine deaminase: Insights into Enzyme Metabolism and Evolution. *The Journal of Biological Chemistry,* 278**,** 19111-19117.

KOLTAI, H. & WEINGARTEN-BAROR, C. (2008) Specificity of DNA microarray hybridization: characterization, effectors and approaches for data correction. *Nucleic Acids Research,* 36**,** 2395-2405.

KOPATZ, I., REMY, J.-S. & BEHR, J.-P. (2004) A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *The Journal of Gene Medicine,* 6**,** 769-776.

KOTOB, S., MCLAUGHLIN, S., VANBERKUM, P. & FAISAL, M. (1999) Characterization of two Perkinsus spp. from the softshell clam, Mya arenaria using the small subunit ribosomal RNA gene. *Journal of Eukaryotic Microbiology,* 46**,** 439-444.

KUBOKI, N., KIBE, M. K., THEKISOE, O. M. M., SUGIMOTO, C. & INOUE, N. (2007) Transcriptional analyses of Trypanosoma brucei gambiense from infected mice and in vitro culture. *Journal of Protozoology research,* 17**,** 25-31.

KUVARDINA, O. N., LEANDER, B. S., ALESHIN, V. V., MYL'NIKOV, A. P., KEELING, P. J. & SIMDYANOV, T. G. (2002) The Phylogeny of Colpodellids (Alveolata) using Small Subunit rRna gene sequences sugest they are free-living sister group to Apicomplexa. *J Eukaryot Microbiol,* 49**,** 498-504.

KYTE, J. & DOOLITTLE, R. F. (1982) A Simple Method For Displaying the hydropahitic character of a protein. *Journal of Molecular Biology,* 157**,** 105-132.

LANGAND, J., JOURDANE, J., COUSTAU, C., DELAY, B. & MORAND, S. (1998) Cost of resistance, expressed as delayed maturity, detected in the host-parasite system *Biomphalaria glabratal/ Echinostoma caproni*. *Heredity,* 80**,** 320-325.

LASIC, D. & PAPAHADJOPOULOS, D. (1995) Liposomes revisited. *Science,* 267**,** 1275-1276.

LATHIA, K. B., YAN, Z. & CLAPSHAW, P. A. (2006) Spinal Cord Transcriptome Analysis Using Suppression Subtractive Hybridization and Mirror Orientation Selection. *Cellular and Molecular Neurobiology,* 26**,** 259-275.

LEANDER, B. S. & KEELING, P. J. (2003) Morphostasis in Alveolata evolution. *Trends in Ecology evolution,* 18**,** 395-402.

LEE, H., RYU, J., KIM, K.-A., LEE, K. I. S. O., LEEJA, J. Y., PARK, E.-B., PARK, J. & CHOI, S. Y. (2004) Transduction of yeast cytosine deaminase mediated by HIV-1 Tat basic domain into tumor cells induces chemosensitivity to 5-fluorocytosine. *Experimental and Molecular Madicine,* 36**,** 43-51.

LEE, M. K., CHO, B. Y., LEE, S. J., KANG, J. Y., JEONG, H. D., HUH, S. H. & HUH, M. D. (2001) Histopathological lesions of Manila clam, *Tapes philippinarum*, from Hadong and Namhae coastal areas of Korea. *Aquaculture,* 201**,** 199-209.

LEETHOCHAVALIT, S., CHALERMWAT, K., UPATHAM, E. S., KWANGSIK, C., SAWANGWONG, P. & KRUATRACHUE, M. (2004) Occurrence of Perkinsus sp. in undulated surf clams *Paphia undulata* from the Gulf of Thailand. *Diseases of Aquatic Organisms,* 60**,** 165-171.

LEIRIÃO, P., D.RODRIGUES, C., S.ALBUQUERQUE, S. & M.MOTA, M. (2004) Survival of protozoan intracellular parasites in host cells. *EMBO Reports,* 5**,** 1142–1147.

LEITE, R. B., AFONSO, R., ASCENSO, R., SIMÃO, M. & CANCELA, M. L. (2007) Effect of Perkinsus infection on expression of *Ruditapes decussatus* biomarkers

genes in response to environmental pollutants. *Marine Genomics International Congress.* Sorrento, Italy.

LEITE, R. B., AFONSO, R. & CANCELA, M. L. (2004) *Perkinsus* sp. infestation in carpet-shell clams, *Ruditapes decussatus* (L), along the Portuguese coast. Results from a 2-year survey. *Aquaculture,* 240**,** 39-53.

LEITE, R. B., BRITO, A. B. & CANCELA, M. L. (2008) An Oxygen Molecular Sensor, the HIF Prolyl 4-Hydroxylase, in the Marine Protist Perkinsus olseni Protist, 159, 355-368.

LESTER, R. J. G. & DAVIS, G. H. G. (1981) A new Perkinsus species (Apicomplexa, Perkinsea) from the abalone *Haliotis ruber*. *Journal of Invertebrate Pathology,* 37**,** 181-187.

LEVINE, N. D. (1978) Perkinsus gen. n. and other new taxa in the protozoan phylum Apicomplexa. *journal of Parasitology,* 64**,** 549.

LIANG, G., ZHANG, X. D., WANG, L. J., SHA, Y. S., ZHANG, J. C., MIAO, S. Y., ZONG, S. D., WANG, L. F. & KOIDE, S. (2004) Identification of differentially expressed genes of primary spermatocyte against round spermatid isolated from human testis using the laser capture microdissection technique. *Cell Research,* 14**,** 507-512.

LIANG, P., ZHU, W., ZHANG, X., GUO, Z., P.O'CONNELI, R., AVERBOUKH, L., WANG, F. & B.PARDEEL, A. (1994) Differential display using one-base anchored oligo-dT primers. *Nucleic Acids Research,* 22**,** 5763-5764.

LIANG, Y. B., ZHANG, X. C., WANG, L. J., YANG, B., ZHANG, Y. & CAI., C. L. (2001) Prevalence of *Perkinsus* sp. in the Manila clam *Ruditapes philippinarum* along northern coast of Yellow Sea in China. *Oceanologia et Limnologia Sinica,* 32**,** 502-511.

LIEVENS, S., GOORMACHTIG, S. & HOLSTERS, M. (2001) A critical evaluation of differential display as a tool for identify genes involved in legume modulation: looking back and loocking forward. *Nucleic Acids Research,* 29**,** 3459-3468.

LÓPEZ, C., CARBALLAL, M. J., AZEVEDO, C. & VILLALBA, A. (1997) Morphological characterization of the hemocytes of the clam, *Ruditapes decussatus* (Mollusca: Bivalvia). *J. Invert. Pathol.,* 69**,** 51-56.

LUND, E., CHU, F., SOUDANT, P. & HARVEY, E. (2007) Perkinsus marinus, a protozoan parasite of the eastern oyster, has a requirement for dietary sterols. *Comp Biochem Physiol A Mol Integr Physiol,* 146**,** 141-147.

LUO, D. & SALTZMAN, W. M. (2000) Synthetic DNA delivery systems. *Natura biotechnology,* 18**,** 33-37.

MACKENZIE, K., WILLIAMS, H. H., WILLIAMS, B., MCVICAR, A. H. & SIDDAL, R. (1995) Parasites as indicators of water quality and the potential use of helminth transmission in marine pollution studies. *Advances in parasitology,* 35**,** 86-144.

MACKIN, J. G., OWEN, H. M. & COLLIER, A. (1950) Preliminary note on the occurrence of a new protistan parasite, *Dermocystidium marinum* n. sp. in *Crassostrea virginica* (Gmelin). *Science.*

MACKIN, J. G. & RAY, S. M. (1966) The taxonomic relationships of *Dermocystidium marinum* Mackin, Owen and Collier. *Journal of Invertebrate Pathology,* 8**,** 544-545.

MAHALINGAM, R., GOMEZ-BUITRAGO, A., ECKARDT, N., SHAH, N., GUEVARA-GARCIA, A., DAY, R. R. P. & FEDOROFF, N. V. (2003) Characterizing the stress/defense transcriptome of *Arabidopsis*. *Genome Biology,* 4**,** 1-14.

MAMOUN, C. B., DAVID J. SULLIVAN, J., BANERJEE, R. & GOLDBERG, D. E. (1998) Identification and Characterization of an Unusual Double Serine/Threonine Protein Phosphatase 2C in the Malaria Parasite *Plasmodium falciparum*. *The Journal of Biological Chemestry,* 273**,** 11241-11247.

MAMOUN, C. B., GLUZMAN, I. Y., GOYARD, S., BEVERLEY, S. M. & GOLDBERG, D. E. (1999) A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA,* 96**,** 8716-8720.

MARR, J. J. & MÜLLER, M. (1995) *Biochemistry and Molecular Biology of Parasites,* London, Academic Press Harcourt Brace & Company Publishers.

MATSUZAKI, M., KUROIWA, H., KUROIWA, T., KITA, K. & NOZAKI, H. (2008) A Cryptic Algal Group Unveiled: A Plastid Biosynthesis Pathway in the Oyster Parasite *Perkinsus marinus*. *Molecular Biology and Evolution,* 25**,** 1167-1179.

MAYFIELD, S. P. & KINDLE, K. L. (1990) Stable nuclear transformation of Chlamydomonas reinhardtii by using a C. reinhardtii gene as the selectable marker. *Proc. Natl. Acad. Sci. USA,* 87**,** 2087-2091.

MAZURAIS, D., MONTFORT, J., DELALANDE, C. & GAC, F. (2005) Transcriptional analysis of testis maturation using trout cDNA macroarrays. *Gen Comp Endocrinol* 142**,** 143-154.

MCGLADDERY, S. E., CAWTHORN, R. J. & BRADFORD, B. C. (1991) *Perkinsus karlssoni* n. sp. (Apicomplexa) in bay scallops *Argopecten irradians*. *Dis. aquat. Org.,* 10**,** 127-137.

MCLAUGHLIN, S. M., TALL, B. D., SHAHEEN, A., ELSAYED, E. E. & FAISAL, M. (2000) Zoosporulation of a new Perkinsus species isolated from the gills of the softshell clam Mya arenaria. *Parasite,* 7**,** 115-122.

MEHIER-HUMBERTA, S. & GUY, R. H. (2005) Physical methods for gene transfer: Improving the kinetics of gene delivery into cells. *Advanced Drug Delivery Reviews,* 57**,** 733-753.

MIOSSEC, L., GARCIA, C., ARZUL, I., FRANÇOIS, C., JOLY, J. P., CHOLLET, B. & ROBERT, S. (2007) Results of the Perkinsosis survey in France (2004- 2005). *WOPER Workshop for the analysis of the impact to the European shellfish Industry.* CETMAR, Vigo, Spain.

MONTES, J. F., DEL RIO, J. A., DURFORT, M. & GARCIA-VALERO, J. (1997) The protozoan parasite *Perkinsus atlanticus* elicits a unique defensive response in the *Tapes semidecussatus*. *Parasitology,* 114**,** 339-349.

MONTES, J. F., DUFORT, M., LLADÓ, A. & GARCÍA-VALERO, J. (2002) Characterization and immunolocalization of a main proteinaceous component of the cell wall of the protozoan parasite *Perkinsus atlanticus*. *Parasitology,* 124**,** 477-484.

MONTES, J. F., DURFORT, M. & GARCÍA-VALERO, J. (2001) Parasitism by the protozoan *Perkinsus atlanticus* favours the development of opportunistic infections. *Diseases of Aquatic Organisms,* 46**,** 57-66.

MORGA, B., ARZUL, I., FAURY, N., GAGNAIRE, B., CHOLLET, B. & RENAULT, T. (2007) Study of interactions between flat oyster, *Ostrea edulis*, and the parasite *Bonamia ostreae* using flow cytometry and suppression subtractrive hybridization (SSH). *13th EAFP Internation Conference "Diseases of Fish and Shellfish"* Grado, Italy, European Association of Fish Pathologists.

MOSS, J., XIAO, J., DUNGAN, C. & REECE, K. (2008) Description of *Perkinsus beihaiensis* n. sp., a new *Perkinsus* sp. parasite in oysters of Southern China. *Journal of Eukaryotic Microbiology,* 55**,** 117-130.

MUDGE, S.M., ICELY, J.D., and NEWTON, A. (2008). Residence times in a hypersaline lagoon: Using salinity as a tracer. Estuarine, Coastal and Shelf Science *77*, 278-284.

MÜLLER, O. J., KATUS, H. A. & BEKEREDJIAN, R. (2007) Targeting the heart with gene therapy-optimized gene delivery methods. *Cardiovascular Research,* 73**,** 453-462.

MURRELL, A., KLEEMAN, S. N., BARKER, S. C. & LESTER, R. J. G. (2002) Synonymy of *Perkinsus olseni* Lester & Davis, 1981 and *Perkinsus atlanticus* Azevedo, 1989 and an update on the phylogenetic position of the genus *Prekinsus*. *Bulletin of the European Association of Fish Pathologists* 22**,** 258-265.

MUSE, W. B., ROSARIO, C. J. & BENDER, R. A. (2003) Nitrogen Regulation of the codBA (Cytosine Deaminase) Operon from *Escherichia coli* by the Nitrogen Assimilation Control Protein, NAC. *Journal of Bacteriology,* 185**,** 2920–2926.

NABEL, G. J., NABEL, E. G., YANG, Z.-Y., FOX, B. A., PLAUTZ, G. E., GAO, X., HUANG, L., SHU, S., GORDON, D. & CHANG, A. E. (1993) Direct gene transfer with DNA-liposome complexes in melanoma: Expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA,* 90**,** 11307-11311.

NASS, R. & RAO, R. (1999) The yeast endosomal Na+/H+ exchanger, Nhx1, confers osmotolerance following acute hypertonic shock. *Microbiology,* 145**,** 3221-3228.

NAVAS, J. I., CASTILLO, M. C., VERA, P. & RUIZ-RICO, M. (1992) Principal parasites observed in clams, *Ruditapes decussatus* (L.), *Ruditapes philippinarum* (Adams et Reeve), *Venerupis pullastra* (Montagu) and *Venerupis aureus* (Gmelin), from the Huelva coast (S.W. Spain). *Aquaculture,* 107**,** 193-199.

NEUMANN, E. & ROSENHECK, K. (1972) Permeability changes induced by electric impulses in vesicular membranes. *Journal of Membrane Biology,* 10**,** 279-290.

NEWTON, A. & MUDGE, S. M. (2003) Temperature and salinity regimes in a shallow, mesotidal lagoon, the Ria Formosa, Portugal. *Estuarine, Coastal and Shelf Science,* 56**,** 1-13.

NICKEL, R. & TANNICH, E. (1994) Transfection and transient expression of chloramphenicol acetyltransferase gene in the protozoan parasite *Entamoeba histolytica*. *PNAS,* 91**,** 7095-7098.

NISHIYAMA, T., KAWAMURA, Y., KAWAMOTO, K., MATSUMURA, H., YAMAMOTO, N., ITO, T., OHYAMA, A., KATSURAGI, T. & SAKAI, T. (1985) Antineoplastic effects in rats of 5-fluorocytosine in combination with cytosine deaminase capsules. *Cancer Research,* 45**,** 1753-1761.

NORÉN, F., MOESTRUP, Ø. & REHNSTAM-HOLM, A.-S. (1999) *Parvilucifera infectans* Norén et Moestrup gen. et sp. nov. (Perkinsozoa phylum nov.): a parasitic flagellate capable of killing toxic microalgae. *European journal of protistology,* 35**,** 233-254.

NYATI, M. K., SREEKUMAR, A., LI, S., ZHANG, M., RYNKIEWICZ, S. D., ARUL M. CHINNAIYAN, REHEMTULLA, A. & LAWRENCE, T. S. (2002) High and Selective Expression of Yeast Cytosine Deaminase under a Carcinoembryonic Antigen Promoter-Enhancer. *Cancer Research,* 62**,** 2337–2342.

OLIVER, J. L., LEWIS, T. D., FAISAL, M. & KAATTARI, S. L. (1999) Analysis of the Effects of *Perkinsus marinus* Proteases on Plasma Proteins of the Eastern

Oyster (*Crassostrea virginica*) and the Pacific Oyster (*Crassostrea gigas*). *Journal of Invertebrate Pathology,* 74**,** 173-183.

ORDÁS, M. C. & FIGUERAS, A. (1998) *In vitro* culture of *Perkinsus atlanticus*, a parasite of the carpet shell clam *Ruditapes decussatus*. *Dis. aquat. Org.,* 33**,** 129-136.

ORLOWSKI, J. & GRINSTEIN, S. (1997) Na+/H+ exchangers in mammalian cells. *The Journal of Biological Chemistry,* 272**,** 22373-22376.

PAGANO, J. S. & VAHERI, A. (1965) Enhancement of infectivity of poliovirus RNA with diethylaminoethyl-dextran (DEAE-D). *Archives of Virology,* 17**,** 456-464.

PAIMENT, A., HOCKING, J. & WHITFIELD, C. (2002) Impact of Phosphorylation of Specific Residues in the Tyrosine Autokinase, Wzc, on Its Activity in Assembly of Group 1 Capsules in *Escherichia coli*. *journal of Bacteriology,* 184**,** 6437-6447.

PARK, K.-I. & CHOI, K.-S. (2001) Spatial distribution of the protozoan parasite *Perkinsus* sp. found in the Manila clams, *Ruditapes philippinarum*, in Korea. *Aquaculture,* 203**,** 9-22.

PARK, K.-I., CHOI, K.-S. & CHOI, J.-W. (1999) Epizootiology of Perkinsus sp. found in the Manila clam, Ruditapes philippinarum in Komsoe Bay. *Journal of the Korean Fisheries Society,* 32**,** 303-309.

PATHY (1994) Introns and exons. *Curr. Opinion in Structural Biology,* 4**,** 383-392.

PECHER, W. T. (2007) Host preference of Perkinsus species: epizootiological, environmental, and molecular aspects. *Center of Marine Biotechnology*. Baltimore, University of Maryland Biotechnology Institute.

PERKINS, F. O. (1987) Protistan parasites of commercially significant marine bivalve molluscs - life cycles, ultrastructure and phylogeny. *Aquaculture,* 67**,** 240-243.

PERKINS, F. O. (1996) The structure of *Perkinsus marinus* (Mackin, Owen and Collier, 1950) Levine, 1978 with comments on taxonomy and phylogeny pf *Perkinsus* spp. *Journal of Shellfish Research,* 15**,** 67-87.

PERKINS, F. O. & MENZEL, R. W. (1966) Morphological and cultural studies of a motile stage in the life cycle of *Dermocystidium marinum*. *Proceedings of the National Shellfisheries Association.*

PEYRE, J. F. (1996) Propagation and *in vitro* studies of *Perkinsus marinus*. *Journal of Shellfish Research,* 15**,** 89-101.

PEYRE, J. F., FAISAL, M. & BURRESON, E. M. (1993) In vitro propagation of the protozoan *Perkinsus marinus*,a pathogen of the eastern oyster, *Crassostrea virginica*. *J. Euk. Microbiol.,* 40**,** 304-310.

PEYRE, J. F. L., CHU, F.-L. E. & VOGELBEIN, W. K. (1995) In vitro interaction of Perkinsus marinus merozoites with eastern and pacific oyster hemocytes. *Development and Comparative Immunology,* 19**,** 291-304.

PORTER, D. J. T. & AUSTIN, E. A. (1993) Cytosine Deaminase The Roles os Divalent Metal Ions in Catalysis. *The Journal of Biological Chemistry,* 268**,** 24005-24011.

PRENETA, R., JARRAUD, S., VINCENT, C., DOUBLET, P., B. DUCLOSA, ETIENNE, J. & COZZONE, A. J. (2002) Isolation and characterization of a protein-tyrosine kinase and a phosphotyrosine-protein phosphatase from *Klebsiella pneumoniae*. *Comparative Biochemistry and Physiology,* 131**,** 103-112.

PROMEGA Transfection guide.

PUTNEY, L. K., DENKER, S. P. & BARBER, D. L. (2002) The changing face of the Na+/H+ Exchanger, NHE1: Structure, Regulation, and Cellular Actions. *Ann. Rev. Pharmacol. Toxicol.,* 42**,** 527-552.

RAGONE, L. M. & BURRESON, E. M. (1993) Effect of salinity on the infection progression and pathogenicity of *Perkinsus marinus* in eastern oyster, *Crassostrea virginica* (Gmelin). *Journal of Shellfish Research,* 12**,** 1-7.

REBRIKOV, D. V., BRITANOVA, O. V., GURSKAYA, N. G., LUKYANOV, K. A., TARABYKIN, V. S. & LUKYANOV, S. A. (2000) Mirror orientation selection (MOS): a method for eliminating false positive clones from libraries generated by suppression subtractive hybridization. *Nucleic Acids Research,* 28**,** 1-4.

REDFIELD, R. J. (1993) Evolution of Natural Transformation: Testing the DNA Repair Hypothesis in Bacillus subtilis and Haemophilus intuenzae. *Genetics,* 133**,** 755-761.

REECE, K. S., SIDDALL, M. E., BURRESON, E. M. & GRAVES, J. E. (1997) Phylogenetic analysis of *Perkinsus* based on actin gene sequences. *J. Parasitol.,* 83**,** 417-423.

ROBINSON, K. A. & BEVERLEY, S. M. (2003) Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. *Molecular & Biochemical Parasitology,* 128**,** 217-228.

ROBLEDO, J.-A. F., COURVILLE, P., CELLIER, M. F. M. & VASTA, G. R. (2004) Gene organization and expression of the divalent cation transporter Nramp in the Protistan parasite *Perkinsus marinus*. *J. Parasitol.,* 90**,** 1004-1014.

ROBLEDO, J. A. F., COSS, C. A. & VASTA, G. R. (2000) Characterization of the ribossomal RNA locus of *Perkinsus atlanticus* and development of a Polymerase Chain Reaction-based diagnostic assay. 7 pages.

ROBLEDO, J. A. F., GARDNER, M. J., EL-SAYED, N. M. & VASTA, G. R. (****) Expressed Sequence Tags from the early Dinoflagellate *Perkinsus marinus*. *Unpublished*.

ROBLEDO, J. A. F., NUNES, P. A., CANCELA, M. L. & VASTA, G. R. (2002) Development of an In Vitro Clonal Culture and Characterization of the rRNA Gene Cluster of *Perkinsus atlanticus,* a Protistan Parasite of the Clam *Tapes decussatus*. *J Eukaryot Microbiol,* 49**,** 414-422.

ROBLEDO, J. A. F., WRIGHT, A. C., COSS, C. A., VASTA, G. R. & GOGGIN, C. L. (1997) Further studies of conserved genes from *Perkinsus* isolates. *Journal of Shellfish Research,* 16**,** 342.

RUANO, F. (1997) Fisheries and farming of important marine bivalves in Portugal. *NOOA Tech. Rep. NMFS,* 129**,** 191-200.

RUANO, F. (2001). Doenças da Amêijoa-boa (*Ruditapes decussatus*) e implicações na sua cultura no Litoral Algarvio. In IPIMAR Divulgação.

SADOWSKY, M. J., TONG, Z., SOUZA, M. D. & WACKETT, L. P. (1998) AtzC Is a New Member of the Amidohydrolase Protein Superfamily and Is Homologous to Other Atrazine-Metabolizing Enzymes. *Journal of Bacteriology,* 180**,** 152-158.

SAGRISTÀ, E., DURFORT, M. & AZEVEDO, C. (1995) *Perkinsus* sp. (Phylum Apicomplexa) in Mediterranean clam *Ruditapes semidecussatus*: ultrastructural observations of the cellular response of the host. *Aquaculture,* 132**,** 153-160.

SALDARRIAGA, J. F., MCEWAN, M. L., FAST, N. M., TAYLOR, F. J. R. & KEELING, P. J. (2003) Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellates lineage. *J. Syst. Evol. Microbiol.,* 53**,** 355-365.

SALDARRIAGA, J. F., TAYLOR, F. J. R. M., CAVALIER-SMITH, T., MENDEN-DEUER, S. & KEELING, P. J. (2004) Molecular data and the evolutionary history of dinoflagellates. *European Journal of Protistology,* 40.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989) Molecular Cloning: A Laboratory Manual. *Cold Spring Harbor Press, Cold Spring Harbor, New York, USA.*

SCHOTT, E. J., PECHER, W. T., OKAFOR, F. & VASTA, G. R. (2003a) The protistan parasite *Perkinsus marinus* is resistant to selected reactive oxygen species *Experimental Parasitology,* 103**,** 232-240.

SCHOTT, E. J., ROBLEDO, J. A. F., WRIGHT, A. C., SILVA, A. M. & VASTA, G. R. (2003b) Gene organization and homology modeling of two iron superoxide dismutases of the early branching protist Perkinsus marinus. *gene,* 309**,** 1-9.

SEFFERNICK, J. L., JOHNSON, G., SADOWSKY, M. J. & WACKETT, L. P. (2000) Substrate Specificity of Atrazine Chlorohydrolase and Atrazine-Catabolizing Bacteria. *Applied and Environmental Microbiology,* 66**,** 4247-4252.

SEFFERNICK, J. L., MERVYN L. DE SOUZA, SADOWSKY, M. J. & WACKETT, L. P. (2001) Melamine Deaminase and Atrazine Chlorohydrolase: 98 Percent Identical but Functionally Different. *Journal of Bacteriology,* 183**,** 2405-2410.

SEFFERNICK, J. L. & WACKETT, L. P. (2001) Rapid Evolution of Bacterial Catabolic Enzymes:A Case Study with Atrazine Chlorohydrolase. *Biochemistry,* 40.

SHAPIR, N., OSBORNE, J. P., JOHNSON, G., SADOWSKY, M. J. & WACKETT, L. P. (2002) Purification, Substrate Range, and Metal Center of AtzC: the N-Isopropylammelide Aminohydrolase Involved in Bacterial Atrazine Metabolism. *Journal of Bacteriology,* 184**,** 5376-5384.

SHAPIR, N., ROSENDAHL, C., JOHNSON, G., ANDREINA, M., SADOWSKY, M. J. & WACKETT, L. P. (2005) Substrate Specificity and Colorimetric Assay for Recombinant TrzN Derived from Arthrobacter aurescens TC1. *Applied and Environmental Microbiology,* 71**,** 2214-2220.

SHIEH, G.-S., SHIAU, A.-L., YO, Y.-T., LIN, P.-R., CHANG, C.-C., TZAI, T.-S. & WU, C.-L. (2006) Low-Dose Etoposide Enhances Telomerase-Dependent Adenovirus-Mediated Cytosine Deaminase Gene Therapy through Augmentation of Adenoviral Infection and Transgene Expression in a Syngeneic Bladder Tumor Model. *Cancer Research,* 66.

SIDDALL, M. E., REECE, K. S., GRAVES, J. E. & BURRESON, E. M. (1997) 'Total evidence' refutes the inclusion of *Perkinsus* species in the phyllum Apicomplexa. *Parasitology,* 115**,** 165-176.

SKINNER-ADAMS, T. S., LAWRIE, P. M., HAWTHORNE, P. L., GARDINER, D. L. & TRENHOLME, K. R. (2003) Comparison of Plasmodium falciparum transfection methods. *Malaria Journal,* 2.

SONG, J. L., HARRY, J. B., EASTMAN, R. T., OLIVER, B. G. & WHITE, T. C. (2004) The *Candida albicans* Lanosterol 14-┐ - Demethylase (ERG11) Gene Promoter Is Maximally Induced after Prolonged Growth with Antifungal Drugs. *Antimicrob Agents Chemother,* 48**,** 1136-1144.

SONIAT, T. M. (1996) Epizootiology of *Perkinsus marinus* disease of eastern oysters in the Gulf of Mexico. *Journal of Shellfish Research,* 15**,** 35-43.

SOUCIETA, J.-L., AIGLE, M., ARTIGUENAVE, F., BLANDIN, G., BOLOTIN-FUKUHARA, M., BON, E., BROTTIER, P., CASAREGOLA, S., MONTIGNY, J. D., DUJON, B., DURRENS, P., GAILLARDIN, C., LËPINGLE, A., LLORENTE, B., MALPERTUY, A., NEUVËGLISE, C.,

OZIER-KALOGËROPOULOS, O., POTIER, S., SAURIN, W., TEKAIA, F., TO¡ANO-NIOCHE, C., WËSOLOWSKI-LOUVEL, M., WINCKER, P. & WEISSENBACH, J. (2000) A set of yeast species for molecular evolution studies. *FEBS letters,* 487**,** 3-12.

SPRINGER, C. J. & NICULESCU-DUVAZ, I. (2000) Prodrug-activating systems in suicide gene therapy. *The journal of Clinical Investigation,* 105**,** 1161-1167.

SUGA, M. & HATAKEYAMA, T. (2001) High efficiency transformation of *Schizosaccharomyces pombe* pretreated with thiol compounds by electroporation. *yeast,* 18**,** 1015-1021.

TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution,* 24**,** 1596-1599.

TANGUY, A., GUO, X. & FORD, S. E. (2004) Discovery of genes expressed in response to Perkinsus marinus challenge in Eastern (*Crassostrea virginica*) and Pacific (*C. gigas*) oysters. *Gene,* 338**,** 121-131.

TELES-GRILO, M. L., DUARTE, S. M., TATO-COSTA, J., GASPAR-MAIA, A., OLIVEIRA, C., ROCHA, A. A., MARQUES, A., CORDEIRO-DA-SILVA, A. & AZEVEDO, C. (2007a) Molecular karyotype analysis of *Perkinsus atlanticus* (Phylum Perkinsozoa) by pulsed field gel electrophoresis. *European journal of protistology,* 43**,** 315-318.

TELES-GRILO, M. L., TATO-COSTA, J., DUARTE, S. M., MAIA, A., CASAL, G. & AZEVEDO, C. (2007b) Is there a plastid in *Perkinsus atlanticus* (Phylum Perkinsozoa)? *European journal of protistology,* 43**,** 163-167.

THOMPSON, J., GIBSON, T., PLEWNIAK, F., JEANMOUGIN, F. & HIGGINS, D. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research,* 25**,** 4876- 4882.

TRIPLETT, L. R., ZHAO, Y. & SUNDIN, G. W. (2006) Genetic Differences between Blight-Causing Erwinia Species with Differing Host Specificities, Identified by Suppression Subtractive Hybridization. *Appl. Environ. Microbiol.,* 72**,** 7359.7364.

VAN-DER-WEL, A. M., TOMÁS, A. M., KOCKEN, C. H. M., MALHOTRA, P., JANSE, C. J., WATERS, A. P. & THOMAS, A. W. (1997) Transfection of the Primate Malaria Parasite *Plasmodium knowlesi* using Entirely Heterologous Constructs. *J. Exp. Med.,* 185**,** 1499-1503.

VENEGAS-CALERÓN, M., BEAUDOIN, F., SAYANOVA, O. & NAPIER, J. A. (2007) Co-transcribed Genes for Long Chain Polyunsaturated Fatty Acid Biosynthesis in the Protozoon Perkinsus marinus Include a Plant-like FAE1 3-Ketoacyl Coenzyme A Synthase. *The Journal of Biological Chemistry,* 282**,** 2996-3003.

VILLALBA, A. (2008) *Workshop for the analysis of the impact of Perkinsosis to the European Shellfish Industry*, Vigo, Centro de Investigacións Mariñas, Conselleria de Pescas e Asuntos Marítimos da Xunta de Galicia; Centro Tecnológico del Mar - Fundación CETMAR.

VILLALBA, A., CASAS, S., LOPEZ, C. & CARBALLAL, M. (2005) Study of perkinsosis in the carpet shell clam Tapes decussatus in Galicia (NW Spain). II. Temporal pattern of disease dynamics and association with clam mortality. *Diseases of Aquatic Organisms,* 65**,** 257-267.

VILLALBA, A. & CASAS, S. M. (2001) Effect of perkinsosis on the energetic physiology of the clam Ruditapes decussatus. *Journal of Shellfish Research,* 20**,** 559-560.

VILLALBA, A. & NAVAS, J. I. (1988) Occurrence of *Minchinia tapetis* and a *Perkinsus*-like parasite in cultured clams, *Ruditapes decussatus* and *R. philipppinarum*, from south Atlantic coast of Spain. Preliminary results. *3rd. Internat. Colloq. Pathol. Marine Aquacult.*

VILLALBA, A., REECE, K. S., ORDÁS, M. C., CASAS, S. M. & FIGUERAS, A. (2004) Perkinsosis in molluscs: A review. *Aquat. Living Resour.,* 17**,** 411-432.

VOLETY, A. K. & CHU, F.-L. E. (1995) Supression of chemiluminescence of eastern oyster (*Crassostrea virginica*) hemocytes by the protozoan parasite *Perkinsus marinus*. *Development and Comparative Immunology,* 19**,** 135-142.

WANG, L. (2006) Polycationic water soluble copolymer and method for transferring polyanionic macromolecules across biological barriers. IN ISSUED, U. P. (Ed.) USA, Genta Salus LLC.

WATERS, A. P., DIJK, M. R. V., RAMESAR, J. & JANSE, C. J. (1997) Stable transfection of the blood stages of malarial parasites. *Annals of Tropical Medicine and Parasitology,* 91**,** 63.66.

WEAVER, J. C. (2003) Electroporation of Biological Membranes from Multicellular to Nano Scales. *IEEE Transactiions on Dielectrics and Electrical Isolation,* 10**,** 754-768.

WELZEL, T., RADTKE, MEYER-ZAIKA, W., HEUMANN, R. & EPPLE, M. (2004) Transfection of cells with custom-made calcium phosphate nanoparticles coated with DNA. *Journal of Matererials Chemistry,* 14**,** 2213-2217.

WHYTE, S. K., CAWTHORN, R. J., MACMILLAN, R. J. & DESPRES, B. (1993) Isolation and purification of developmental stages of *Perkinsus karlssoni* (Apicomplexa: Perkinsea), a parasite affecting bay scallops *Argopecten irradians*. *Diseases of Aquatic Organisms,* 15**,** 199-205.

WILSON, D. K. & QUIOCHO, F. A. (1993) A pre-transition-state mimic of an enzyme: X-ray structure of adenosine deaminase with bound 1-deazaadenosine and zinc-activated water. *Biochemistry,* 32**,** 1689-1694.

WOO, P. T. K. (1998) Protection against *Cryptobia* (*Trypanoplasma*) *salmositica* and *Salmonid Cryptobiosis Parasitology Today,* 14**,** 272-277.

WU, Y., KIRKMAN, L. A. & WELLEMS, T. E. (1996) Transformation of *Plasmodium falciparum* malaria parasites by homologous integration. *Proc. Natl. Acad. Sci. USA,* 93**,** 1130-1134.

WU, Y., SIFRI, C. D., LEI, H.-H., XIN-ZHUAN & WELLEMS, T. E. (1995) Transfection of *Plasmodium falciparum* within human red blood cells. *PNAS,* 92**,** 973-977.

WUGEDITSCH, T., PAIMENT, A., HOCKING, J., DRUMMELSMITH, J., FORRESTER, C. & WHITFIELDI, C. (2001) Phosphorylation of Wzc, a Tyrosine Autokinase, Is Essential for Assembly of Group 1 Capsular Polysaccharides in *Escherichia coli*. *The Journal of Biological Chemistry,* 276**,** 2361-2371.

ZHENGWANG, Z., LIANHUA, Y., YONGKANG, Z. & FENGDI, Z. (2002) In situ transduction of cytosine deaminase gene followed by systemic use of 5-fluorocytosine inhibits tumor growth and metastasis in orthotopic prostatae cancer mouse models. *Chinese medical journal,* 115**,** 227-231.

APPENDIX

Appendix II-1

Appendix II-1

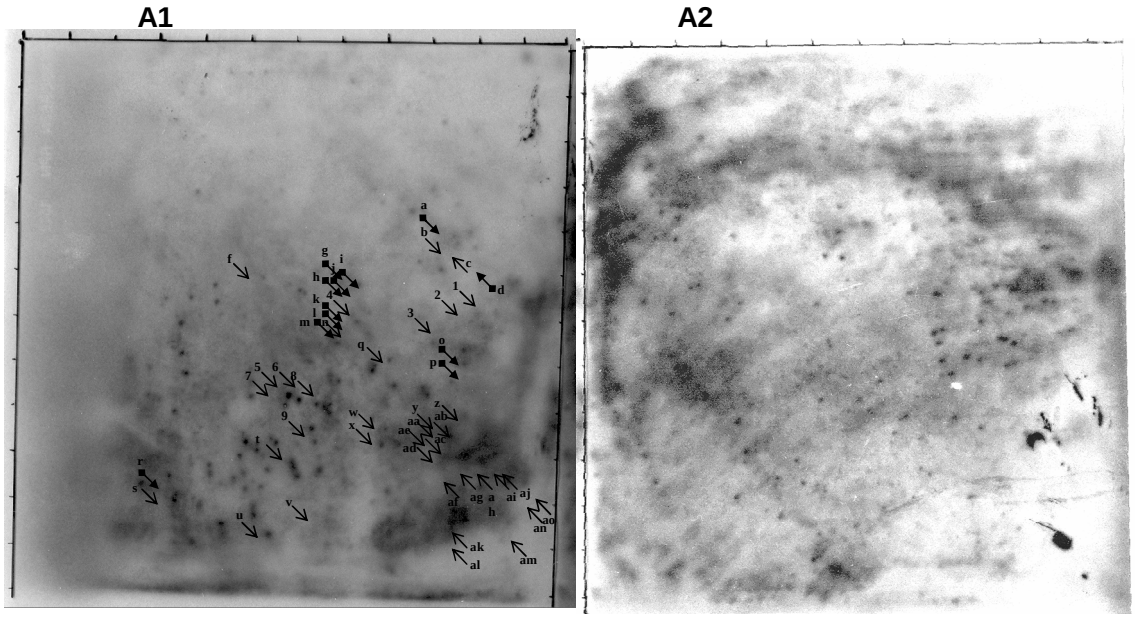
Table of oligonucleotides used in the molecular tasks performed. Oligonucleotides used for PCR amplification of *P. olseni* selected genes (GSP, Gene Specific Primers) and manufacturers' Adaptor Primers (AP). Each pair organized by species and by gene with the respective procedure of use described and the sequence.

Species	Gene		Oligonucleotide	Sequence (5'-3')
P. olseni	PoClhl	PCR/Gene expression	PoClhl Frw1	CTTCAAACAGCATGGCGAGTCTGCAATT
			PoClhl Rev1	ACATGACTGACGAGGAGATGACCCGGTT
		PCR/ Gene	PoClhlGWFrw1	AAAGCCGTTAACTGCTACACCGCCGT
			PoClhlGWRev1	ATCTCCACCACTTCAAGCGGCTCTGG
		PCR/ Full cDNA	PoClhlgeneFrw2	CATGTCCAGCGAAATCTCCACCAC
			PoClhlgeneRev2	AGATTGAGAAATCCGAATCTGCTGGTAA
		Expression in Prokaryote system	PoClhl_exp_NotI F1	CCGCGGCCGCCatcgaaggtcgtATGTCCAGCGAA ATCT
			PoClhl_exp_NotI R1	CACGCGCGGCCGCCTATGCTTGTACTGCCGC
		Expression in Eukaryote system	PoClhl_exp_Frw1	ccgctcgagatgtccagcgaaatctccacca
			PoClhl_exp_Rev1	cacgcggatccgcttgtactgccgcaacg
	PoAdh	PCR/ Gene expression	PoAdh Frw2	ACTCGGGTGCCCACTTCTTGTCATCA
			PoAdh Rev1	CTCCAATCAGGGGCACCTTGCTAACC
	PoNHE	PCR/ Gene expression	PoNHE Frw1	GCTACCGTAAATCGTCCCTGGTGTCC
			PoNHE Rev1	AGACCAATTTGGATGCGACTTGCGAG
		PCR/ cDNA/ Gene	PoNHEfullFrw2	TGATTACTATGGCCTCACTTGATGACGACG
			PoNHEfullRev2	AGTGACAAGCTTTGCAATGGGTCATCACT
	PoUbq	PCR/Gene expression	PoUb Frw	AGAACGTCAAGGCGAAAATCCAAGACAAGG
			PoUb Rev	TCACGGGGAAGACCATCACCCTCGAT
P. marinus	PmClhl	PCR/cDNA	PmClhl Frw1	GGTTACCCCCCTGAACATTGC
			PmClhl Rev1	AGATTGCTCGGTTTGCTGAGG
		PCR/ Full cDNA	PmClhl Frw3	TATGTCAAGCGAAGTGTCCACGAAA
			PmClhl Rev3	GTAGAAACAAAACGGTCCGAAACTAAGC
		PCR/ cDNA/ Gene	PmClhl Frw2	GCTATGTCAAGCGAAGTGTCC
			PmClhl Rev2	CTATGGTATCCCCTTTCACC
	SSH	Adaptor primer	AP1	GTAATACGACTCACTATAGGGC
			AP2	tgtagcgtgaagacgacagaa
		Nested AP	Nested AP1	TCGAGCGGCCGCCCGGGCAGGT
			Nested AP2R	AGCGTGGTCGCGGCCGAGGT
Manufacturers	Marathon/Genome walker	AP	AP1	CCATCCTAATACGACTCACTATAGGGC
			AP2	ACTCACTATAGGGCTCGAGCGGC

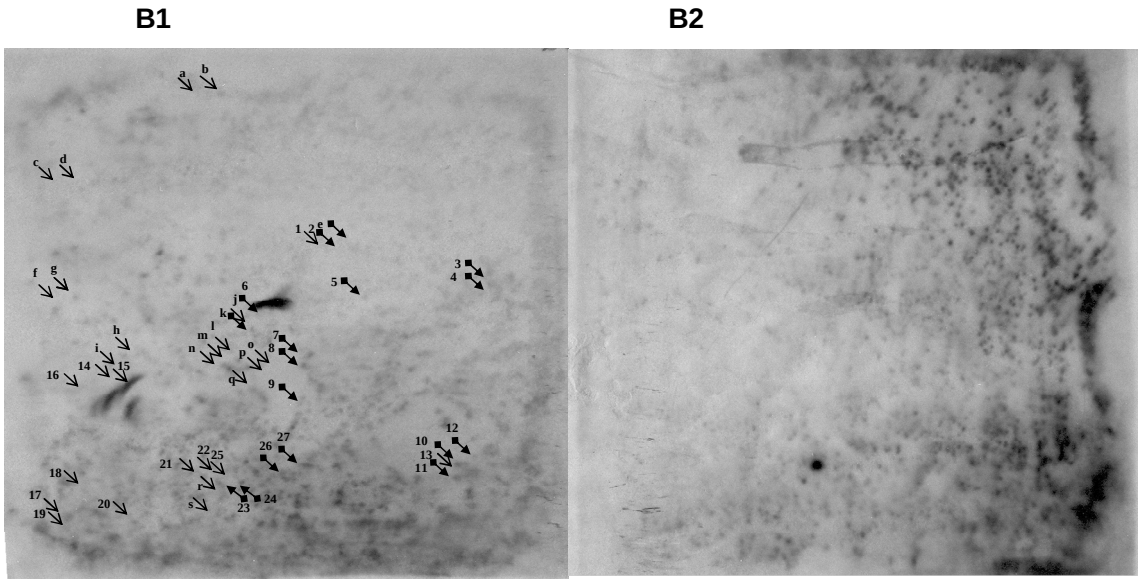
Appendix III-1

Appendix III-1

Filters resulting from the hybridization of the Dfsl library, these were analysed to identify the differentially expressed genes from.

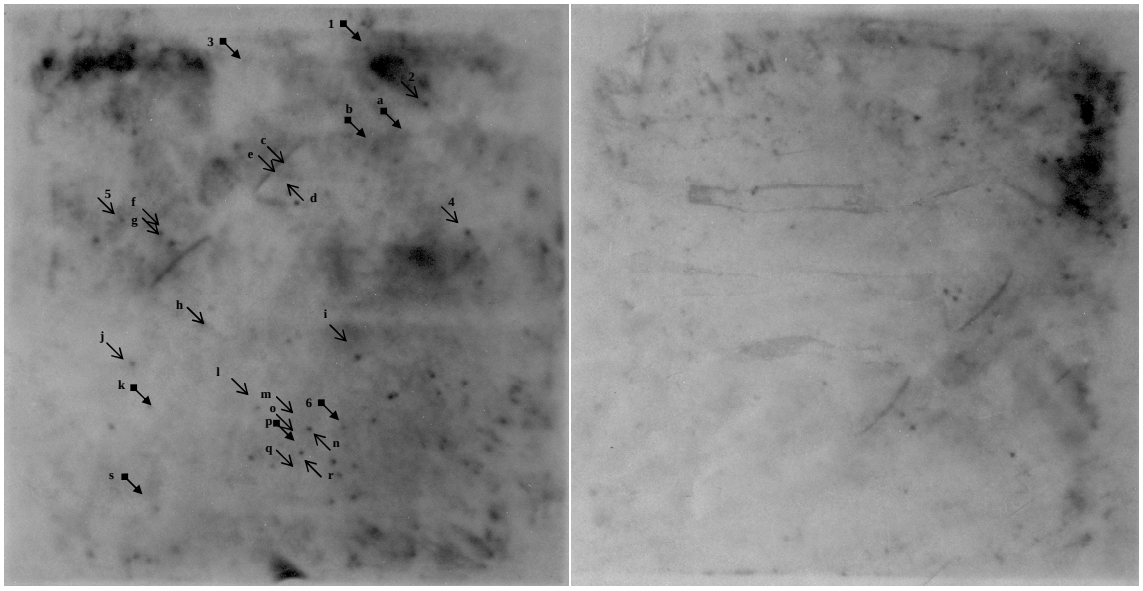


↘ Differentially expressed gene (48)
↘ More than twice up-regulated (Macroarray)



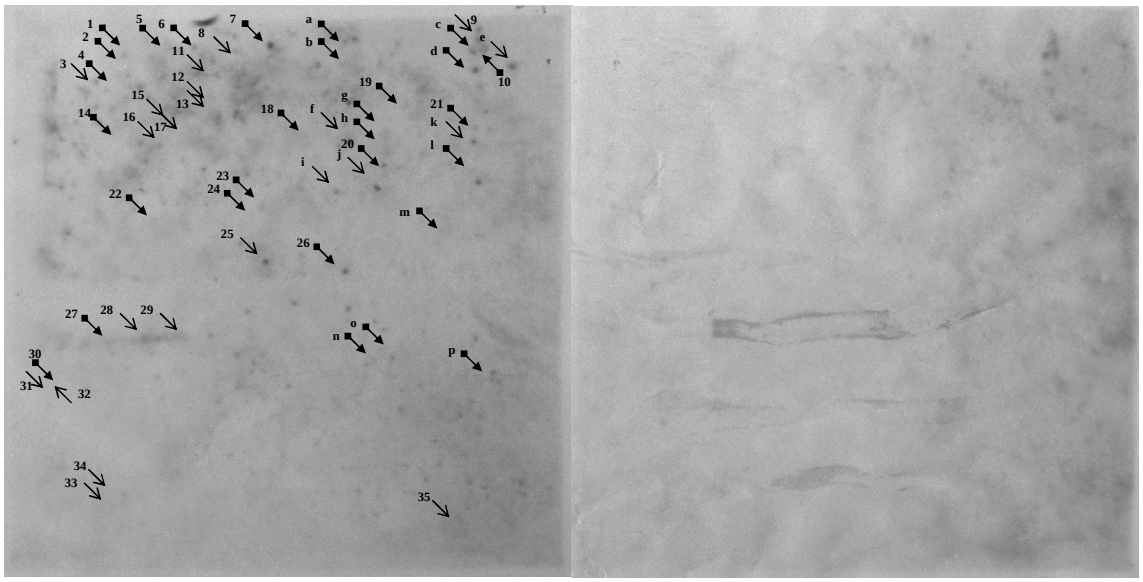
↘ Differentially expressed gene (19)
↘ More than twice up-regulated (Macroarray)

C1 **C2**



↘ Differentially expressed gene (19)
■↘ More than twice up-regulated (Macroarray)

D1 **D2**

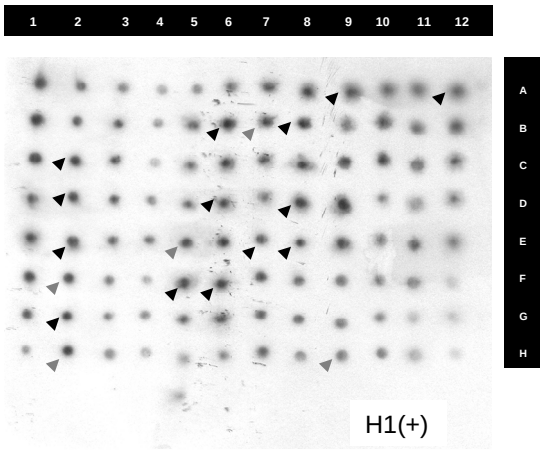


↘ Differentially expressed gene (19)
■↘ More than twice up-regulated (Macroarray)

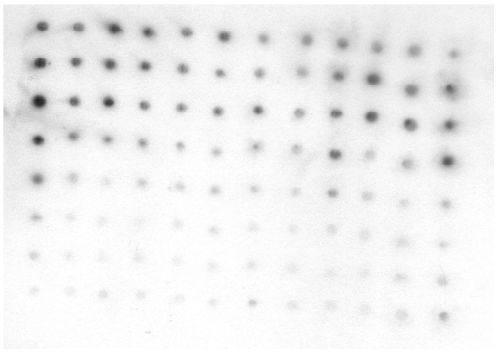
Appendix III-2

Appendix III-2

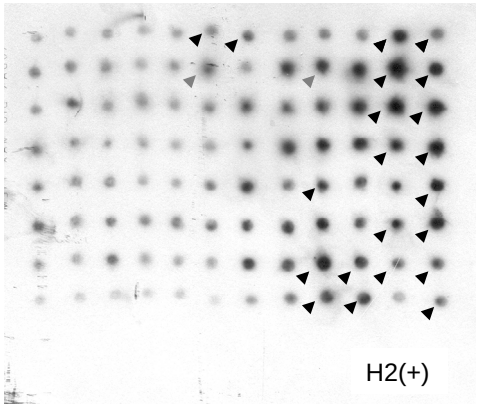
Filters resulting from the hybridization of the Dfsl library, these were analysed to identify the differentially expressed genes from.



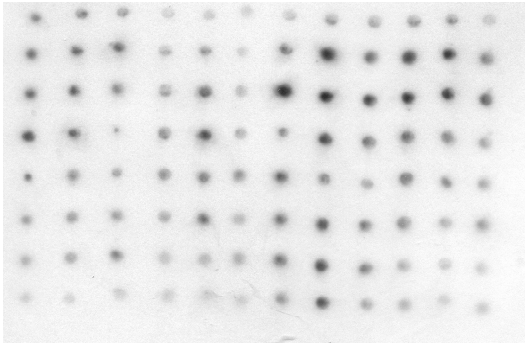
H1(+)



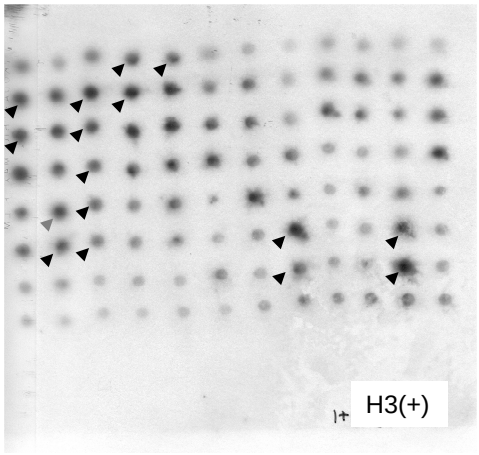
H1(-)



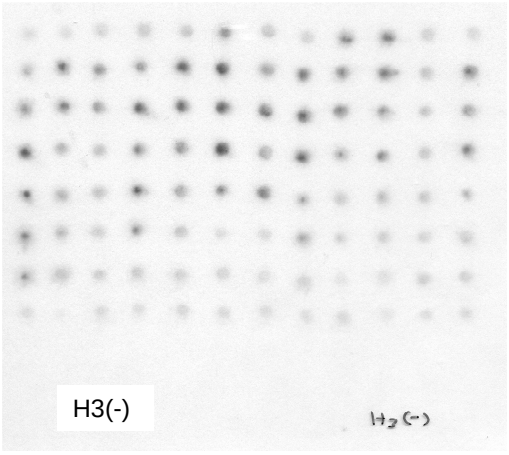
H2(+)



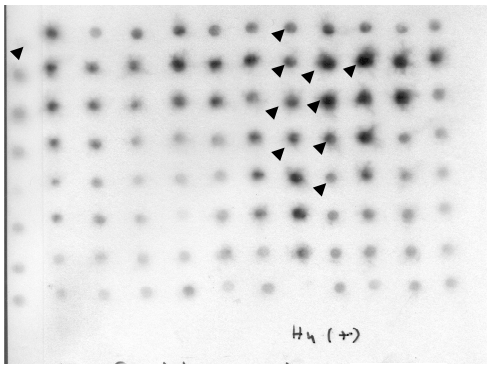
H2(-)



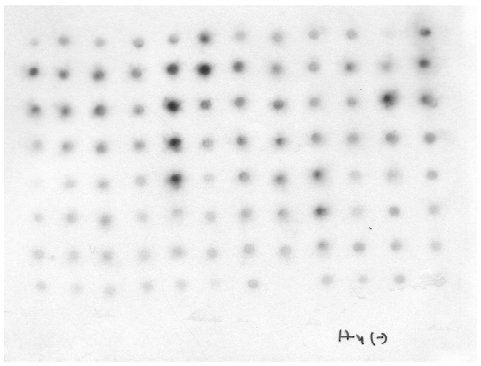
H3(+)



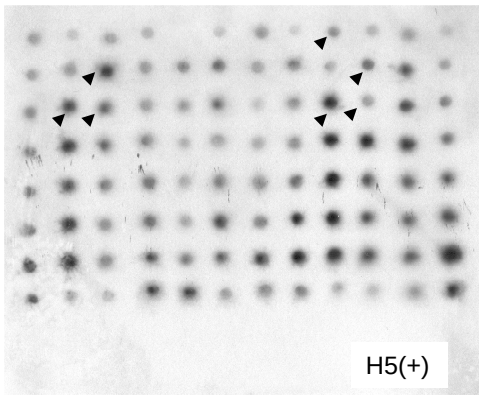
H3(-)



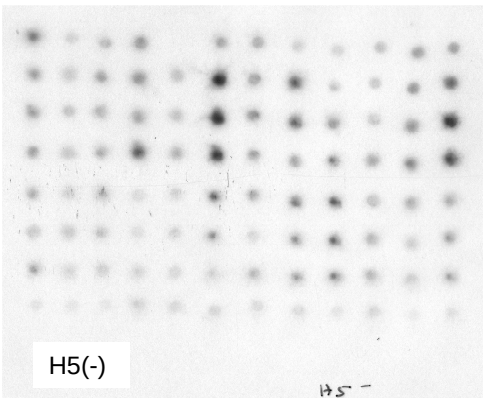
H4(+)



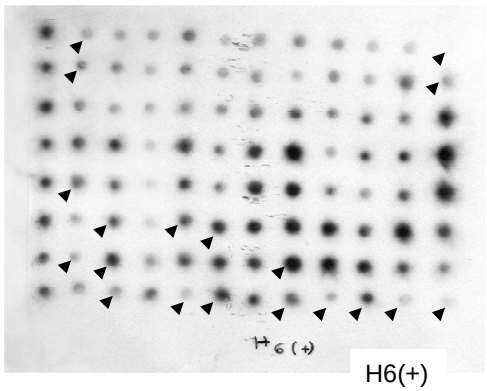
H4(-)



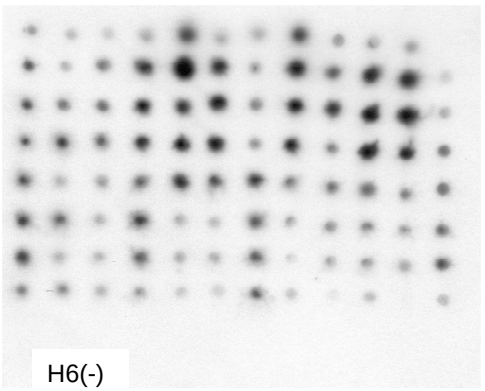
H5(+)



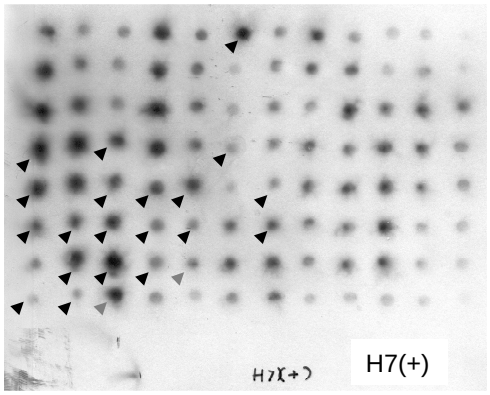
H5(-)



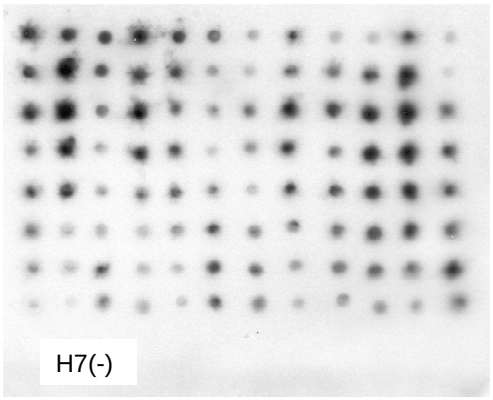
H6(+)



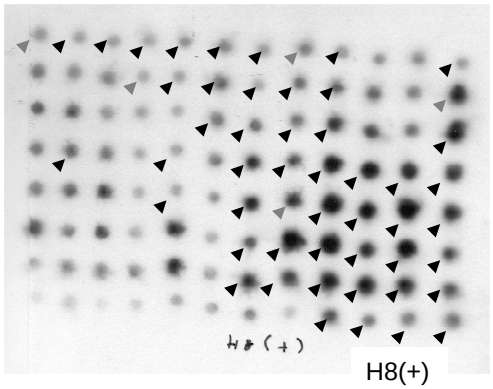
H6(-)



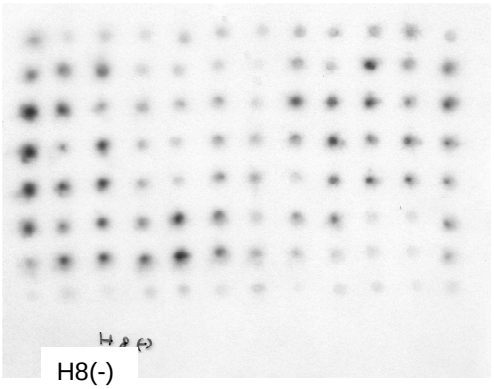
H7(+)



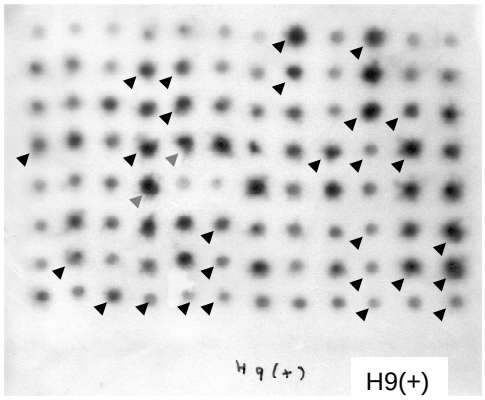
H7(-)



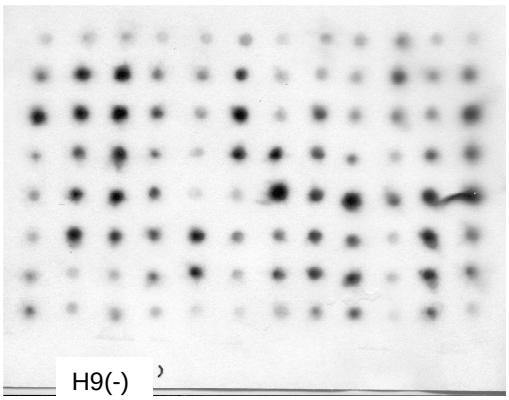
H8(+)



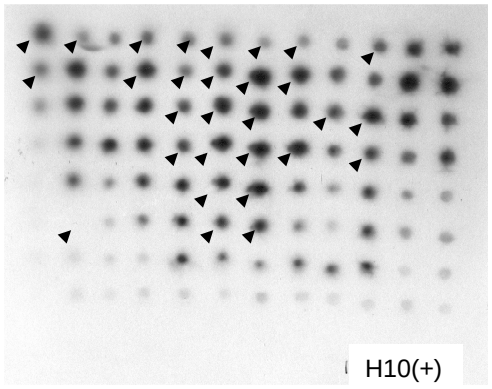
H8(-)



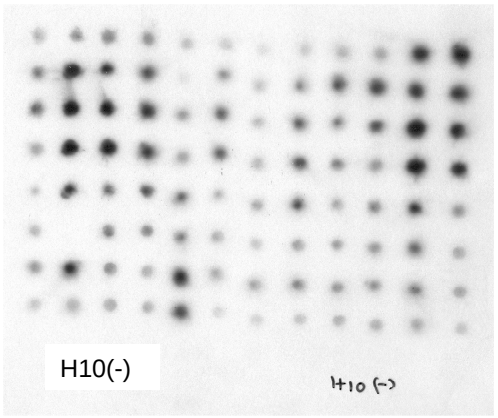
H9(+)



H9(-)



H10(+)



H10(-)

Appendix III-3

Appendix III-3

GOblet *in silico* analysis and results for Dfsl. EST designation, size and the protein hits results obtained from GO search at Goblet server are presented.

DfsI	GOblet *in silico* analysis		
clone	GO molecular function	Subcategory of GO	EST nr
C1-a	binding	Dna binding	1
A1-d		RNA binding	4
A1-p			
A1-h			
A1-q			
D1-e		transcription factor binding	1
A1-p	catalytic activity	ATA-dependent helicase activity	2
A1-q			
D1-o		carbon-carbon lyase	1
A1-r		oxidoreductase	1
D1-g	signal transducer activity		1
A1-d	structural molecule activity		5
A1-f			
B1-e			
B1-s			
A1-k			
D1-c	transcription regulator activity	DNA binding andtranscription factor activity	2
A1-g			
A1-i	translation regulatory function	translation initiation factor	1
D1-j		translation termination factor	1
A1-l	transporter activity	aminoacid transtorter	2
C1-k			
D1-g		electrochemical-potential driven transport	1
B1-3	binding	transition ion binding	1
B1-6		DNA binding	3
A1_9			
B1-3			
D1-26		RNA binding	1
D1-13		purine nucleotide binding	1
B1-6		cytoskeletal protein binding	1
D1-18		androgen binding	1
D1-10	catalytic activity	acting on carbon-nitrogen bond	1
D1-5		peptidase activity	1
D1-30		forming carbon-nitrogen bonds	1
D1-27		transferring acyl groups	1
D1-18		transferring one-carbon group	1
D1-5	chaperone activity	heat-shock protein activity	1
D1-14	signal transducer	transmembrane receptor	1
B1-1	structural molecule activity	structural constituent of ribosome	2
B1-2			
B1-3	transporter activity	electron transporter	3
B1-11			
D1-18			
B1-23		cation transporter activity	1
no hit			52
total			96

Appendix III-4

Appendix III-4

GOblet *in silico* analysis and results for efMOSl. EST designation, size and the protein hits results obtained from GO search at Goblet server are presented

efMOSI	GOblet *in silico* analysis		
clone	GO molecular function	Subcategory of GO	EST nr
H9H4	binding activity	metal ion binding activity	1
H10A2	Catalytic activity	hydrolase activity	14
H10E7			
H1D6			
H1E7			
H7D1			
H8C9			
H8D9			
H8E5			
H8F12			
H8G10			
H8G12			
H9C5			
H9F12			
H9G6			
H1G2		transferase activity	2
H2D11			
H6F6	obsolete moleclar functuion	cell adhesion melecule activity	2
H7G2			
H3B4	structural molecule activity	structural melecule of ribosome	1
H3C1		extracellular molecule consituent	1
no hit			183
total			204

Appendix III-5

Appendix III-5

NCBI *in silico* analysis and results for Dfsl. EST designation, size and the protein hits results obtained from GO search at Goblet server are presented

DfsI		NCBI database BLASTx			
clone	size	hit e-value	accession number	organism hit	protein hit
A1.a	337	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
A1.c	500	1.5	AAU44818	*Thalassiosira pseudonana*	silaffin precursor Sil2p
A1.d	300	2,00E-17	XP_416225	*Gallus gallus*	similar to NHP2-like protein 1 (High mobility group-like nuclear protein 2
A1.f	257	7,00E-12	CAI76286	*Theileria annulata*	60S ribosomal protein L36, putative
A1.g	532	2,00E-08	NP_191043	*Arabidopsis thaliana*	amino acid permease/ amino acid-polyamine transporter
A1.h	442	6,00E-45	CAI76286	*Theileria annulata*	60S ribosomal protein L12, putative
A1.i	132	1,00E-07	AAX95709	*Oryza sativa*	DEAD/DEAH box helicase, putative
A1.j	462	6,00E-13	XP_675686	*Plasmodium berghei*	hypothetical protein PB106694.00.0
A1.k	316	3,00E-29	CAD1441	*Crassostrea gigas*	ribosomal protein L19
A1.l	791	4,00E-09	EAN85393	*Trypanosoma cruzi*	transmembrane amino acid transporter, putative
A1.m	224	1.2	NP_142962	*Pyrococcus horikoshii OT3*	hypothetical protein PH1053
A1.o	790	2,00E-04	CAF98772	*Tetraodon nigroviridis*	unnamed protein product
A1.p	469	2,00E-49	NP_191790	*Arabidopsis thaliana*	ATP binding / ATP-dependent helicase/ RNA helicase/ helicase/ nucleic acid binding
A1.q	470	1.8e-49	NP_191790	*Arabidopsis thaliana*	ATP binding / ATP-dependent helicase/ RNA helicase/ helicase/ nucleic acid binding
A1.r	308	2,00E-09	ABA74211	*Pseudomonas fluorescens*	Short-chain dehydrogenase/reductase SDR
B1.e	789	4,00E-67	BAD11816	*Lentinula edodes*	putative S-phase specific ribosomal protein cyc07
B1.j	556	0.003	CAI76842	*Theileria annulata*	40S ribosomal protein S10, putative
B1.k	635	7,00E-20	AAZ09952	*Leishmania major*	d-lactate dehydrogenase-like protein
B1.m	780	8,00E-27	BAB33421	*Pisum sativum*	putative senescence-associated protein
B1.o	245	none			
B1.p	271	1,00E-14	XP_679643	*Plasmodium berghei*	hypothetical protein
B1.q	547	0.48	ABA56642	*Nitrosococcus oceani*	Formate/nitrite transporter
B1.r	338	2,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
B1.s	198	2,00E-07	CAA48558	*Aplysia californica*	40S ribosomal protein S3a (Lysine-rich protein KRP-A
C1.a	654	3,00E-10	XP_539198	*Canis familiaris*	similar to Zinc finger CCCH-type domain containing protein3

C1.b	812	1,00E-14	XP_453836	*Kluyveromyces lactis*	unnamed protein product
C1.c	796	1,00E-45	EAL34999	*Cryptosporidium hominis*	senescence-associated protein
C1.d	636	7,00E-20	AAZ09952	*Leishmania major*	d-lactate dehydrogenase-like protein
C1.k	392	2,00E-07	EAN89347	*Trypanosoma cruzi*	transporter, putative
C1.m	429	6,40E-01	XP_729354	*Plasmodium yoelii yoelii*	extragenic suppressor of the BimD6 mutation
C1.p	769	5,00E+00	BAD81020	*uncultured bacterium*	ATPase component of ABC-type transport system
C1.s	590	4.9	EAA22428	*Plasmodium yoelii yoelii*	SERA-3
D1.a	338	2,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
D1.b	771	6.6	XP_650610	*Entamoeba histolytica*	non-transporter ABC (ATP-binding cassette) protein
D1.c	259	4.4	AAZ09927	*Leishmania major strain Friedlin*	protein kinase-like protein
D1.d	388	2.5	EAA03808	*Anopheles gambiae str. PEST*	ENSANGP00000013418
D1.e	790	none			
D1.g	574	4,00E-22	BAD93487	*Rosa hybrid cultivar*	vacuolar Na+/H+ antiporter
D1.h	379	0.003		*Rattus norvegicus*	similar to Ac1147
D1.i	314	3,00E-23	EAL34999	*Cryptosporidium hominis*	senescence-associated protein
D1.j	411	1,00E-57	AAL17660	*Chlamydomonas reinhardtii*	eukaryotic release factor 1
D1.l	558	4,00E-09	AAN37152	*Plasmodium falciparum 3D7*	hypothetical protein PF14_0539
D1.m	517	3.5	EAN78069	*Trypanosoma brucei*	hypothetical protein, conserved
D1.n	262	1.5	AAO17380	*Mus musculus*	zinc metalloendopeptidase
D1.o	491	5,00E-41	EAQ63662	*Marinomonas sp*	transaldolase
D1.p	786	3	AAQ84157	*Streptomyces sp.*	Plm2-3
A1.9	651	3,00E-10	XP_539198	*Canis familiaris*	similar to Zinc finger CCCH-type domain containing protein3
B1.1	340	5,00E-19		*Ixodes pacificus*	ribosomal protein S15
B1.2	788	4,00E-66		*Lentinula edodes*	putative S-phase specific ribosomal protein cyc07
B1.3	791	4,00E-37	Q6BXH8	*Debaryomyces hansenii*	40S ribosomal protein S6
B1.4	368	1.9	NP_032506	*Mus musculus*	laminin, alpha 1
B1.5	790	5,00E-09	XP_463583	*Oryza sativa*	putative terminal ear1

B1.6	628	7,00E-16	AAP09047	*Bacillus cereus*	hypothetical Membrane Spanning Protein
B1.7	603	0.12	XP_523076	*Pan troglodytes*	tropomodulin 2 (neuronal)
B1.8	359	0.88	AAX80044	*Trypanosoma brucei*	kinesin, putative
B1.9	801	0.007	XP_826696	*Trypanosoma brucei*	sphingosine phosphate lyase-like protein
B1.10	769	8.5	AAW27075	*Schistosoma japonicum*	SJCHGC01117 protein
B1.11	404	6,00E-10	NP_990458	*Gallus gallus*	xanthine dehydrogenase
B1.12	787	7,00E-26	BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
B1.12	759	7,00E-26	BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
B1.13	592	6,00E-41	ABB32789	*Geobacter metallireducens*	Fe-S type hydro-lyases tartrate/fumarate alpha region
B1.23	574	4,00E-22	BAD93487	*Rosa hybrid cultivar*	vacuolar Na+/H+ antiporter
B1.24	463	1.2	YP_468931	*Rhizobium etli*	putative mechanosensitive ion channel protein
B1.25	579	6.2	AAA29908	*Schistosoma mansoni*	p48 eggshell protein
B1.26	791	2,00E-10	ZP_00242021	*Rubrivivax gelatinosus*	ABC-type phosphate/phosphonate transport system, permease component
B1.27	377	2,00E-05	CAG70682	*Datisca glomerata*	putative sucrose-H+ symporter
C1.1	471	3,00E-06	CAB38602	*Schizosaccharomyces pombe*	hypothetical protein SPBC405.03c
C1.2	576	9,00E-12	XP_627364	*Cryptosporidium parvum*	SSM4 like ring finger
C1.3	384	2.5	NP_176450	*Arabidopsis thaliana*	disulfide oxidoreductase/ monooxygenase
C1.6	294	2.6	AAM35598	*Xanthomonas axonopodis*	N-acetylglucosaminidase
D1.1	289	9.9	NP_956927	*Danio rerio*	potassium channel, subfamily K, member 5
D1.2	79	9,00E-50	AAW70160	*Phaeodactylum tricornutum*	condensing enzyme
D1.4	748	2.1	AAX88150	*Haemophilus influenzae*	conserved hypothetical protein
D1.5	776	6,00E-67	AAM02971	*Crypthecodinium cohnii*	BiP
D1.6	782	1.3	ABV27966	*Hahella chejuensis*	Non-ribosomal peptide synthetase modules and related protein
D1.7	325	1.5	NP_915097	*Oryza sativa*	B1099D03.20
D1.9	791	1,00E-17	XP_453836	*Kluyveromyces lactis*	unnamed protein product
D1.10	432	1,00E-35	AAQ98879	*Dictyostelium discoideum*	asparaginase
D1.12	595	0.091	AAB46781	*human*	rai, ShcC=51.9 kda Shc-related protein

D1.13	605	5,00E-62	AAP13994	*Litchi chinensis*	S-adenosylmethionine synthetase
D1.14	800	3,00E-17	EAN31411	*[Theileria parva*	hypothetical protein, conserved, RNA regognition motif
D1.15	234	none			
D1.17	382	4.4	NP_188870	*Arabidopsis thaliana*	ATP binding / ATP-dependent helicase/ helicase/ nucleic acid binding
D1.18	791	5,00E-67	ZP_00794740	*Yersinia pseudotuberculosis*	NAD-dependent aldehyde dehydrogenases
D1.19	431	1.1	XP_729354	*Plasmodium yoelii yoelii*	extragenic suppressor of the BimD6 mutation
D1.20	285	5,00E-26	ABC23006	*Rhodospirillum rubrum*	tartrate dehydrogenase
D1.21	558	0.27	YP_395662	*Lactobacillus sakei*	hypothetical membrane protein
D1.22	537	1,00E-15	NP_916683	*Oryza sativa*	putative MRP protein (ATP/GTP-binding protein)
D1.23	618	1.4	NP_900570	*Chromobacterium violaceum*	hypothetical protein CV0900 Tetratricopeptide repeat domain
D1.24	517	3.5	ZP_00768811	*Chloroflexus aurantiacus*	survival protein SurE
D1.26	257	4,00E-07	XP_667137	*Cryptosporidium hominis*	RNA binding protein
D1.27	665	0.12	EAN97318	*Trypanosoma cruzi*	diacylglycerol acyltransferase, putative
D1.28	288	3.4	XP_762299	*Ustilago maydi*	hypothetical protein UM06152.1
D1.30	345	1,00E-30	XP_624223	*Apis mellifera*	similar to CG6854-PC, isoform C
D1.32	788	8,00E-25	BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
D1.33	300	0.40	ZP00972480	*Pseudomonas aeruginosa*	GGDEF domain

Appendix III-6

Appendix III-6

NCBI *in silico* analysis and results for efMOSl. EST designation, size and the protein hits results obtained from GO search at Goblet server are presented

efMOSl		NCBI database BLASTx			
clone	size	hit e-value	accession number	organism hit	protein hit
H1A9	373	7,2	NP703691	*Plasmodium falciparum*	Hypothetic protein
H1A12	496	4,1	NP070278	*Archaeoglobus fulgidus*	pyruvate formate lyase
H1B6	376	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H1B7	#				
H1B8	176				
H1C2	126				
H1D2	176				
H1D6	279	4,00E-16	ZP_00331135	*Pisum sativum*	Cytosine deaminase and related metal-dependent hydrolases
H1D8	176				
H1E2	375	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H1E5	#				
H1E7	292	3,00E-16	ZP_00331135	*Pisum sativum*	Cytosine deaminase and related metal-dependent hydrolases
H1E8	164				
H1F2	#				
H1F5	176	3,2	NP913654	*Oryza sativa*	OSJNBa0004G10.18
H1F6	810				
H1G2	597	1,00E-18	EAA20530	*P. yoelii yoelii*	Acyl CoA synthase
H1H2	#				
H1H9	#				
H2A6	178				
H2A7	177				
H2A11	178				
H2A12	176				
H2B6	#				
H2B9	#				
H2B11	179				
H2B12	170				

H2C11	163	7,1	AAL59385	*Citrobacter freundii*	Intl
H2C12	374	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H2D11	597	7,00E-18		*cryptosporidium parvum*	Acyl CoA synthase
H2D12	176				
H2E9	177				
H2E12	176				
H2F11	177				
H2F12	176				
H2G9	352	9,00E-30	EAL34999	*cryptosporidium hominis*	putative senescence-associated protein
H2G10	176				
H2G11	164				
H2G12	565	4,00E-16		*Zea maiz*	cytochrome P450 like_TBP
H2H9	377	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H2H10	363	9,00E-30	EAL34999	*cryptosporidium hominis*	putative senescence-associated protein
H2H12	363	9,00E-30	EAL34999	*cryptosporidium hominis*	putative senescence-associated protein
H3A4	496	4,10E+00	NP070278	*Archaeoglobus fulgidus*	pyruvate formate lyase
H3A5	428	3,80E-01	EAL46739	*Entamoeba histolytica*	protein kinase, putative
H3B1	475	7,20E-01		*Plasmodium chabaudi*	Hypothetic protein
H3B3	344	3,00E-05	CAE76360	*Neurospora crassa*	Related to ethanolaminephosphotransferase
H3B4	555	8,00E-34	1909359A		ribosomal protein S19
H3C1	408				
H3C3	40				
H3D3	353	4,00E-27	BAB33421	*Pisum sativum*	putative senescence-associated protein
H3E2	#				
H3E3	496	2,1	AAH59336	*Xenopus leavis*	MGC69088
H3F2	496	2,1	AAH59336	*Xenopus leavis*	MGC69088
H3F3	362	4,00E-27	BAB33421	*Pisum sativum*	putative senescence-associated protein
H3F8	176				
H3F11	652	2,4	NP650778	*Enterococcus faecium*	Predicted phosphosugar isomerase
H3G8	362	3,00E-18	NP182246	*Arabidopsis thaliana*	long-chain fatty acid CoA ligase protein
H3G11	471	0,71	CAH82113	*Plasmodium chabaudi*	Hypothetic protein
H4A1	177	0,042	AAC46904	*Trypanosoma cruzi*	mucin like protein

H4A8	177				
H4B8	176				
H4B9	176				
H4B10	178	9,2	NP913654	*Oryza sativa*	hypothetical protein
H4C8	176				
H4C9	176				
H4D8	378	0,22	AAW29114	*Homo sapiens*	MK2 non alergic IgE long chain
H4D9	377	1,1	NP703691	*P. falciparum*	Hypothetic protein
H4E9	177				
H5A9	178				
H5B3	373	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H5B10	180	9,2	NP_913654	*Oryza sativa*	OSJNBa0004G10.18
H5C2	373	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H5C3	176				*
H5C9	176				*
H5C10	178				*
H7A6	176				
H7D1	291	3,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H7D3	175				
H7D6	363	4,00E-22	EAL34999	*Cryptosporidium hominis*	senescence-associated protein
H7E1	498	4,2	NP070278	*Archaeoglobus fulgidus*	pyruvate formate lyase
H7E3	179				
H7E4	346	2,00E-04	AAL68843	*Sorghum bicolor*	aminoalcoholphosphotransferase
H7E5	392	3,2	EAL46739	*Entamoeba histolytica*	protein kinase, putative
H7E7	179				
H7F1	179				
H7F2	651	0,71	YP_172668	*Synechococcus elongatus*	N-acetylmuramoyl-L-alanine amidase
H7F3	177				
H7F4	176				
H7F5	308	0,44	AAG00637	*Moloch horridus*	NADH dehydrogenase
H7F7	378	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase

H7G2	630	1,00E-24	BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
H7G3	378				
H7G4	630		BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
H7G5	#				
H7H1	177		BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
H7H2	176				
H7H3	#				
H8A1	#				
H8A2	176				
H8A3	178				
H8A4	855	1,00E-24	BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
H8A5	176				
H8A6	345	2,00E-04	AAL68843	*Sorghum bicolor*	aminoalcoholphosphotransferase
H8A7	428	4,2	EAL46739	*Entamoeba histolytica*	protein kinase, putative
H8A8	#				
H8A9	179				
H8A12	176				
H8B4	#				
H8B5	176				
H8B6	305	9,3	NP_907232	*Wolinella succinogenes*	Magnesium and Cobalt Transport Protein
H8B7	177				
H8B8	363				
H8B9	178				
H8B12	#				
H8C6	176				
H8C7	363	7,00E-30	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8C8	362	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8C9	291	3,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H8C12	179				
H8D2	176				
H8D5	176				

H8D7	362	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8D8	177				
H8D9	291	3,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H8D10	304	9,3	NP_907232	*Wolinella succinogenes*	Magnesium and Cobalt Transport Protein
H8D11	353	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8D12	176				
H8E5	291	3,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H8E7	303	9,3	NP_907232	*Wolinella succinogenes*	Magnesium and Cobalt Transport Protein
H8E8	#				
H8E9	364	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8E10	363	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8E11	463	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H8E12	353	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8F7	179				
H8F8	177				
H8F9	180				
H8F10	176				
H8F11	177				
H8F12	292	2,00E-13	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H8G7	176				
H8G8	362	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H8G9	179				
H8G10	291	3,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H8G11	177				
H8G12	291	3,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H8H8	530	9,00E-14	T02955	*maize*	probable cytochrome P450 monooxygenase
H8H9	177				
H8H10	402	3,20E+00	EAL46739	*Entamoeba histolytica*	protein kinase, putative
H8H11	177				
H8H12	468	0,004	AAC46906	*Trypanosoma cruzi*	mucin-like protein
H9A8	386	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein

H9A10	339	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H9B4	329	3,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H9B5	177				
H9B8	178				
H9C5	291	2,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H9C10	304	9,3	NP_907232	*Wolinella succinogenes*	Magnesium and Cobalt Transport Protein
H9C11	401	3,20E+00	EAL46739	*Entamoeba histolytica*	protein kinase, putative
H9D1	378	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H9D4	881	9,00E-23	CAH00932	*Kluyveromyces lactis*	unnamed protein product
H9D5	#				
H9D9	305	9,3	NP_907232	*Wolinella succinogenes*	Magnesium and Cobalt Transport Protein
H9D10	178				
H9D11	176				
H9E4	#				
H9F6	177				
H9F10	345	2,00E-05	CAE76360	*Neurospora crassa*	related to ethanolaminephosphotransferase
H9F12	291	1,00E-15	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H9G2	362	7,00E-30	BAB33421	*Pisum sativum*	putative senescence-associated protein
H9G6	292	1,00E-15	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H9G10	304	7,1	NP_940322	*Corynebacterium diphtheriae*	Putative membrane protein
H9G11	861	2	AAN30046	*Brucella suis*	pyruvate dehydrogenase complex, E3 component, lipoamide dehydrogenase
H9G122	353	3,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H9H3	178				
H9H4	291	3,00E-16	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H9H5	450	0,026	AAA29908	*Schistosoma mansoni*	p48 eggshell protein
H9H6	176				
H9H10	#				
H9H12	377	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H10A1	184				
H10A2	299	3,00E-16	ZP_00331135	*Pyrococcus furiosus*	Cytosine deaminase and related metal-dependent hydrolases

H10A4	480	0,013	CAD2081	*Ostertagia ostertagia*	SXC2 protein
H10A5	177				
H10A6	#				
H10A7	177				
H10A8	346				
H10A10	479	0,72	CAH82113	*Plasmodium chabaudi*	hypothetic protein
H10B1	178				
H10B4	363	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H10B5	176				
H10B6	176				
H10B7	#				
H10B8	363	1,00E-29	BAB33421	*Pisum sativum*	putative senescence-associated protein
H10C5	866	6,00E-63	XP4255663	*Gallus gallus*	similar to IF2
H10C6	765				
H10C7	911	2,2	NP072926	*Mycoplasma genitalum*	lipoprotein, putative
H10C9	177				
H10C10	176				
H10D5	658				
H10D6	634				
H10D7	476	0,72	CAH82113	*Plasmodium chabaudi*	hypothetic protein
H10D8	432	0,72	CAH82113	*Plasmodium chabaudi*	hypothetic protein
H10D10	176				
H10E6	757				
H10E7	249	1,00E-11	ZP_00331135	*Moorella thermoacetica*	Cytosine deaminase and related metal-dependent hydrolases
H10F2	#				
H10F6	#				
H10F7	376	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H6A2	#				
H6A12	369	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H6B2	176				
H6B12	176				

H6E2	373	1,9	CAD13249	*Schistosoma mansoni*	tyrosine kinase
H6F3	177				
H6F5	183				
H6F6	390	0,5	AAG00637	*Moloch horridus*	NADH dehydrogenase subunit 2
H6G2	395	3,2	EAL46739	*Entamoeba histolytica*	protein kinase, putative
H6G3	177				
H6G8	178				
H6H3	175				
H6H5	176				
H6H6	944	2,00E-37	BAA10929	*Nicotiana tabacum*	cytochrome P450 like_TBP
H6H8	502	2,00E-23	EAL34999	*Cryptosporidium hominis*	senescence-associated protein
H6H9	177				
H6H10	175				
H6H11	176				
H6H12	426	9,4	EAL36536	*Cryptosporidium hominis*	importin-beta2

Appendix III-7

Appendix III-7

Resume of NCBI data base *in silico* analysis and results for Dfsl. Number of EST and the protein hits results obtained from BLASTx search at NCBI database (http://www.ncbi.nlm.nih.gov).

Dfsl NCBI BLAST x results			
ESTs sequenced			96
ESTs with hit			90
ESTs with no hit			6
BLASTx hits			82
E-valus	**Accession numbers**	**Protein hits**	**ESTs**
1,00E-29	BAB33421	Pisum putative senescence-associated protein	3
2,00E-08	NP_191043	Arabidopsis amino acid permease/ amino acid-polyamine transporter	
1.5	AAU44818	Thalassiosira silaffin precursor Sil2p	
2,00E-17	XP_416225	Gallus similar to NHP2-like protein 1	
7,00E-12	CAI76286	Theileria 60S ribosomal protein L36, putative	
1,00E-07	AAX95709	Oryza DEAD/DEAH box helicase, putative	
6,00E-13	XP_675686	Plasmodium hypothetical protein	
3,00E-29	CAD1441	Crassostrea ribosomal protein L19	
4,00E-09	EAN85393	Trypanosoma transmembrane amino acid transporter, putative	
1.2	NP_142962	Pyrococcus hypothetical protein PH1053	
2,00E-04	CAF98772	Tetraodon unnamed protein product	
2,00E-49	NP_191790	Arabidopsis ATP binding / ATP-dependent helicase	
2,00E-09	ABA74211	Pseudomonas Short-chain dehydrogenase/reductase SDR	
4,00E-67	BAD11816	Lentinula putative S-phase specific ribosomal protein cyc07	
7,00E-20	AAZ09952	Leishmania d-lactate dehydrogenase-like protein	
8,00E-27	BAB33421	Pisum putative senescence-associated protein	
1,00E-14	XP_679643	Plasmodium hypothetical protein	
0.48	ABA56642	Nitrosococcus Formate/nitrite transporter	
2,00E-07	CAA48558	Aplysia 40S ribosomal protein S3a (Lysine-rich protein KRP-A	
3,00E-10	XP_539198	Canis similar to Zinc finger CCCH-type domain containing protein3	
1,00E-14	XP_453836	Kluyveromyces unnamed protein product	
1,00E-45	EAL34999	Cryptosporidium senescence-associated protein	
7,00E-20	AAZ09952	Leishmania d-lactate dehydrogenase-like protein	
2,00E-07	EAN89347	Trypanosoma transporter, putative	
6,40E-01	XP_729354	Plasmodium extragenic suppressor of the BimD6 mutation	
5,00E+00	BAD81020	uncultured bacterium ATPase component of ABC-type transport system	
4.9	EAA22428	Plasmodium SERA-3	

Appendix III-8

Appendix III-8

Resume of NCBI data base *in silico* analysis and results for efMOS. Number of EST and the protein hits results obtained from BLASTx search at NCBI database (http://www.ncbi.nlm.nih.gov).

efMOSI NCBI BLAST x results			
ESTs sequenced			204
ESTs with hit			109
ESTs with no hit			95
BLASTx hits			27
e-value	**Accession numbers**	**Protein hits**	**ESTs**
0,72	NP703691	*Plasmodium* Hypothetic protein	7
4,1	NP070278	*Archaeoglobus* Pyruvate formate lyase	5
2	AAN30046	*Brucella* Pyruvate dehydrogenase, lipoamide dehydrogenase	1
2,2	NP072926	*Mycoplasm* Lipoprotein, putative	3
1,9	CAD13249	*Schistosoma* Tyrosine kinase	14
3,2	EAL46739	*Entamoeba* Protein kinase	6
3,00E-16	ZP_00331135	*Moorella* Cytosine deaminase	15
1,00E-18	EAA20530	*Plasmodium* Acyl CoA synthase	2
3,2	NP913654	*Oryza* hypothetical protein	3
7,1	AAL59385	*Citobacter* IntI class1 integron integrase	1
1,00E-29	EAL34999	*Cryptostoridium* Putative senescence-associated protein	20
1,00E-24	BAA10929	*Nicotiana* Cytochrome P450 monooxygenase	7
2,00E-05	CAE76360	*Neurostora* Related to ethanolaminephosphotransferase	3
8,00E-34	1909359A	Ribosomal protein S19	1
2,4	NP650778	*Enterococcus* Predicted phosphosugar isomerase	1
3,00E-18	NP182246	*Arabidopsis* Long-chain fatty acid CoA ligase protein	1
0,004	AAC46904	*Trypanosoma* Mucin like protein	2
2,20E-01	AAW29114	*Homo* MK2 non alergic IgE long chain	1
2,00E-04	AAL68843	*Sorghum* Aminoalcoholphosphotransferase	2
2,00E-04	AAL68843	*Synechococcus* N.acetylmuramoyl-L-alanini amidase	1
0,44	AAG00637	*Moloch* NADH dehydrogenase	2
9,3	NP_907232	*Wolinella* Magnesium and cobalt transport protein	5
9,00E-23	CAH00932	*Kluyveromyces* Unnamed protein product	1
0,026	AAA29908	*Schistosoma* P48 eggshell protein	1
0,013	CAD20812	*Ostertagia* SXC2 protein	1
6,00E-63	XP4255663	*Gallus* Similar to IF2	1
9,4	EAL36536	*Cryptosporidium* Importin beta2	1
7,1	NP_940322	*Corynebacterium* putative membrane protein	1

Appendix V-1

Appendix V-1

Classification of Macroarray Sets according to *in silico* analysis, presenting the clone designation associated with the submission name and the respective accession number.

Category	Sub-category		Clone	Submission designation	AccessionNumber
binding activity	nucleotide binding	purine nucleotide binding	D1-13	ESTfrwSSHPo00002_D1_13-A1	BB999049
		DNA binding	C1-a	ESTfrwSSHPo00003_C1_a-B1	BB999050
		RNA binding	B1_5	ESTfrwSSHPo00004_B1_5-C1	BB999051
			A1-d	ESTfrwSSHPo00005_A1_d-D1	BB999052
			A1-p	ESTfrwSSHPo00006_A1_p-E1	BB999053
			D1-26*	ESTfrwSSHPo00007_D1_26-F1	BB999054
	metal ion binding activity	transition metal ion binding	B1-3	ESTfrwSSHPo00008_B1_3-G1	BB999055
		transition metal ion binding	H9H4	ESTfrwSSHPo00009_H9H4_H1	BB999056
	protein binding	cytoskeletal protein binding	B1-6	ESTfrwSSHPo00010_B1_6-A2	BB999057
catalytical activity	hydrolase activity	acting on carbon-nitrogen bond	D1-10	ESTfrwSSHPo00011_D1_10-B2	BB999058
			H5A8	ESTfrwSSHPo00012_H5A8-C2	BB999059
			H7D1	ESTfrwSSHPo00013_H7D1-D2	BB999060
			H8C9	ESTfrwSSHPo00014_H8C9-E2	BB999061
	lyase activity	carbon-carbon lyase	D1-o	ESTfrwSSHPo00015_D1_o-F2	BB999062
			B1_9	ESTfrwSSHPo00016_B1_9-G2	BB999063
	ligase activity	forming carbon-nitrogen bonds	D1-30	ESTfrwSSHPo00017_D1_30-H2	BB999064
	transferase activity	transferase activity, transferring acyl groups	H9D9	ESTfrwSSHPo00018_H9D9-A3	BB999065
			H2D11	ESTfrwSSHPo00019_H2D11-B3	BB999066
	oxidoreductase		B1_12	ESTfrwSSHPo00020_B1_12-C3	BB999067
			C1_3	ESTfrwSSHPo00021_C1_3-D3	BB999068
			H5H3	ESTfrwSSHPo00022_H5H3-E3	BB999069
			H6H6	ESTfrwSSHPo00023_H6H6-F3	BB999070
chaperone activity	chaperone activity	heat-shock protein activity	D1-5	ESTfrwSSHPo00024_D1_5-G3	BB999071
signal transducer activity	receptor activity	transmembrane receptor	D1-14*	ESTfrwSSHPo00025_D1_14-H3	BB999072

	Signaling molecule		D1_19	ESTfrwSSHPo00026_D1_19-A4	BB999073
			D1_c	ESTfrwSSHPo00027_D1_c-B4	BB999074
			D1_d	ESTfrwSSHPo00028_D1_d-C4	BB999075
			B1_4	ESTfrwSSHPo00029_D1_13	BB999076
			B1_27	ESTfrwSSHPo00030_B1_27-E4	BB999077
			D1_33	ESTfrwSSHPo00031_D1_33-F4	BB999078
transcription regulator activity	transcription regulator activity	DNA binding andtranscription factor activity	A1-g	ESTfrwSSHPo00032_A1_g-G4	BB999079
	extracellular molecule consituent	extracellular molecule consituent	H3C1	ESTfrwSSHPo00033_H3C1-H4	BB999080
structural molecule activity	structural constituent of ribosome	structural melecule of ribosome	B1-e	ESTfrwSSHPo00034_B1_e-A5	BB999081
	structural constituent of ribosome		B1-2	ESTfrwSSHPo00035_B1_2-B5	BB999082
			H7F11	ESTfrwSSHPo00036_H7F11-C5	BB999083
			H3B4	ESTfrwSSHPo00037_H3B4-D5	BB999084
translation regulatory function	translation factor activity	translation initiation factor	A1-i	ESTfrwSSHPo00038_A1_i-E5	BB999085
			A1_h	ESTfrwSSHPo00039_A1_h-F5	BB999086
			A1_k	ESTfrwSSHPo00040_A1_k-G5	BB999087
		translation termination factor	D1-j	ESTfrwSSHPo00041_D1_j-H5	BB999088
transporter activity	amine/ polyamine transporter	aminoacid transtorter	A1-l	ESTfrwSSHPo00042_A1_1-A6	BB999089
			C1-k	ESTfrwSSHPo00043_C1_k-B6	BB999090
	carrier activity	electrochemical-potential driven transport	D1-g	ESTfrwSSHPo00044_D1_g-C6	BB999091
	channel/pore class transporter activity	electron transporter	B1-11	ESTfrwSSHPo00045_B1_11-D6	BB999092
			D1-18	ESTfrwSSHPo00046_D1_18-E6	BB999093
	ion transporter activity	cation transporter activity	B1-23	ESTfrwSSHPo00047_B1_23-F6	BB999094
			D1_1	ESTfrwSSHPo00048_D1_1-G6	BB999095
obsolete molecular function	cell adhesion molecule activity	cell adhesion molecule activity	H7G2	ESTfrwSSHPo00049_H7G2-H6	BB999096
			H6F6	ESTfrwSSHPo00050_H6F6-A7	BB999097

Metabolism	Carbohydrate metabolism	Citrate cycle (TCA cycle); Pyruvate metabolism	H9G11	ESTfrwSSHPo00051_H9G11-B7	BB999098
			B1_k	ESTfrwSSHPo00052_B1_k-C7	BB999099
			C1_6	ESTfrwSSHPo00053_C1_6-D7	BB999100
			D1_20	ESTfrwSSHPo00054_D1_20-E7	BB999101
	Energy metabolism	Oxidative phosphorylation	D1_22	ESTfrwSSHPo00055_D1_22-F7	BB999102
			H7E11	ESTfrwSSHPo00056_H7E11-G7	BB999103
	Lipid metabolism	Glycerolipid metabolism	D1_p	ESTfrwSSHPo00057_D1_p-H7	BB999104
			D1_27*	ESTfrwSSHPo00058_D1_27-A8	BB999105
			H10C7	ESTfrwSSHPo00059_H10C7-B8	BB999106
	Nucleotide metabolism		A1_r	ESTfrwSSHPo00060_A1_r-C8	BB999107
	Aminoacid metabolism		C1_s	ESTfrwSSHPo00061_C1_s-B8	BB999108
			D1_n	ESTfrwSSHPo00062_D1_n-E8	BB999109
			D1_2	ESTfrwSSHPo00063_D1_2-F8	BB999110
	Glycan biosynthesis and metabolism	O-Glycan biosynthesis	H8H12	ESTfrwSSHPo00064_H8H12-G8	BB999111
	Biosynthesisof polyketides and nonribosomal peptides		D1_6	ESTfrwSSHPo00065_D1_6-H8	BB999112
Cellular process	Membrane transport		C1_p	ESTfrwSSHPo00066_C1_p-A9	BB999113
			D1_b	ESTfrwSSHPo00067_D1_b-B9	BB999114
			B1_24	ESTfrwSSHPo00068_B1_24-C9	BB999115
			B1_26	ESTfrwSSHPo00069_B1_26-D9	BB999116
	Cell motility		B1_7	ESTfrwSSHPo00070_B1_7-E9	BB999117
			B1_8	ESTfrwSSHPo00071_B1_8-F9	BB999118
			H9H5	ESTfrwSSHPo00072_H9H5-G9	BB999119
			H10A4	ESTfrwSSHPo00073_H10A4-H9	BB999120
	Cell growth and death		A1_a	ESTfrwSSHPo00074_A1_a-H10	BB999121
			D1_24	ESTfrwSSHPo00075_D1_24-B10	BB999122

			H2H10	ESTfrwSSHPo00076_H2H10-C10	BB999123
	Cell comunication		D1_21	ESTfrwSSHPo00077_D1_21-D10	BB999124
	Imune system		H4D8	ESTfrwSSHPo00078_H4D8-E10	BB999125
Unknown/ HypotheticalUnk		Unknown 1	H3B11	ESTfrwSSHPo00079_H3B11-A11	BB999126
		Unknown 2	H3G11	ESTfrwSSHPo00080_H3G11-A12	BB999127
		Unknown 3	H9D4	ESTfrwSSHPo00081_H9D4-A13	BB999128
		Unknown 4	H4D9	ESTfrwSSHPo00082_H4D9-B11	BB999129
		Unknown 5	H10D7	ESTfrwSSHPo00083_H10D7-B12	BB999130
		Unknown 6	A1_j	ESTfrwSSHPo00084_A1_j-B13	BB999131
		Unknown 7	A1_m	ESTfrwSSHPo00085_A1_m-C11	BB999132
		Unknown 8	A1_o	ESTfrwSSHPo00086_A1_o-C12	BB999133
		Unknown 9	B1_p	ESTfrwSSHPo00087_B1_p-C13	BB999134
		Unknown 10	C1_b	ESTfrwSSHPo00088_C1_b-D11	BB999135
		Unknown 11	D1_a	ESTfrwSSHPo00089_D1_a-D12	BB999136
		Unknown 12	D1_h	ESTfrwSSHPo00090_D1_h-D13	BB999137
		Unknown 13	D1_l	ESTfrwSSHPo00091_D1_l-E11	BB999138
		Unknown 14	D1_m	ESTfrwSSHPo00092_D1_m-E12	BB999139
		Unknown 15	B1_10	ESTfrwSSHPo00093_B1_10-E13	BB999140
		Unknown 16	C1_1	ESTfrwSSHPo00094_C1_1-F11	BB999141
		Unknown 17	D1_4	ESTfrwSSHPo00095_D1_4-F12	BB999142
		Unknown 18	D1_7	ESTfrwSSHPo00096_D1_7-F13	BB999143
		Unknown 19	D1_14	ESTfrwSSHPo00097_D1_14-G11	BB999144
		Unknown 20	D1_23	ESTfrwSSHPo00098_D1_23-G12	BB999145
		Unknown 21	D1_30	ESTfrwSSHPo00099_D1_30-G13	BB999146

Appendix V-2

Appendix V-2

CAP1 protein family. Classification of CAP1 proteins with respective designation and accession number.

CAP1	NhaPSOS1E	**Prokaryotic Nha**		NhaPBsubt	Bacillus subtilis ATCC9372524 (a Gram +)	NP_391222
				YjcEEcoli	Escherichia coli K12 (a Gram -)	NP_418489
		Eukaryotic SOS1		NHX7Athal	Arabidopsis thaliana (thale cress)	NP_178307
				SOS1Cparv	Cryptosporidium parvum (a parasitic protozoan)	CAD98616
	NHE	**Intracellular NHE**	**Endosomal/Trans-Golgi Network NHE**	NHX5Athal	Arabidopsis thaliana (thale cress)	NP_175839
				NHX6Athal	Arabidopsis thaliana (thale cress)	NP_178079
				NHX5aCele	Caenorhabditis briggsae (a nematode)	CAE60804
				NHX5bCele	Caenorhabditis elegans (a nematode)	NP_741891
				NHE3aBmel	Drosophila melanogaster (fruit fly)	NP_609064
				NHE3bBmel	Drosophila melanogaster (fruit fly)	NP_609064
				NHE6Hsapi	Homo sapiens (Human)	NP_006350
				NHE7Hsapi	Homo sapiens (Human)	NP_115980
				NHE9Hsapi	Homo sapiens (Human)	NP_775924
			NHE8-like NHE	NHX8aCele	Caenorhabditis elegans (a nematode)	NP_740923
				NHX8bCele	Caenorhabditis elegans (a nematode)	NP_740923
				NHE1Dmela	Drosophila melanogaster (fruit fly)	AAL39869
				NHE8Hspi	Homo sapiens (Human)	NP_056081
			Plant Vacuolar NHE	NHX1Athal	Arabidopsis thaliana (thale cress)	NP_198067
				NHX2Athal	Arabidopsis thaliana (thale cress)	NP_187154
				NHX3Athal	Arabidopsis thaliana (thale cress)	NP_187288
				NHX4Athal	Arabidopsis thaliana (thale cress)	NP_200358
				NHX1Osati	Oryza sativa, japonica cultivar group (rice)	AAQ63678
				NHX1Scorn	Suaeda maritime subsp. salsa (a sea blight)	AAP15178
				NHX1Zmays	Zea mays (corn)	AAP20428
				NHX2Zmays	Zea mays (corn)	AAP20429
				NHX3Zmays	Zea mays (corn)	AAP20430
				NHX4Zmays	Zea mays (corn)	AAP20431
				NHX5Zmays	Zea mays (corn)	AAP20432
				NHX6Zmays	Zea mays (corn)	AAP20433
		Plasma Membrane NHE	**Recycling PM NHE**	NHX1Celeg	Caenorhabditis elegans (a nematode)	NP_510622
				NHX2Celeg	Caenorhabditis elegans (a nematode)	NP_495614
				NHX3Celeg	Caenorhabditis elegans (a nematode)	NP_504943
				NHX6aCele	Caenorhabditis elegans (a nematode)	NP_494035
				NHX6aCele	Caenorhabditis elegans (a nematode)	NP_494035
				NHX9Celeg	Caenorhabditis elegans (a nematode)	NP_502260
				NHE2aDmel	Drosophila melanogaster (fruit fly)	NP_724311
				NHE2bDmel	Drosophila melanogaster (fruit fly)	NP_610088
				NHE5Hsapi	Homo sapiens (Human)	NP_004585
			Resident PM NHE	NHE1Hsapi	Homo sapiens (Human)	NP_003038
				NHE2Hsapi	Homo sapiens (Human)	NP_003039
				NH8Mmusc	Mus musculus (house mouse)	NP_683731
				βNHEOmyki	Oncorhynchus mykiss (rainbow trout)	Q01345
	NaT-DC Transporter Family (Prokaryotic)			GCDAAferm	Acidaminococcus fermentans (a Gram - coccus)	Q06700
				MCDVparvu	Veillonella parvula (a gram-negative coccus)	CAA80872

APPENDIX

Appendix VI-1

Appendix VI-1

Table of transfection methods described for some organism of interest. This was a selection of organisms and methodologies according to the objective of Chapter VI.

Technology		Vector designation	Promoter	Gene	Organism	Source
Name	**Methodology**					
PROTOZOANS						
Electroporation	Cytomix (120 mM KCl, 0.15 mM CaCl, 2 mM EGTA, 5 mM MgCl2, 10 mM K2HPO4/KH2PO4, 25 mM Hepes, pH 7.6)	p Bluescript PHRP CAT pCAT Basic DNA	lac promoter 5' hrp3 SV40 promoter	Chloramphenicol acetyltransferase	*P falciparum*	Wu *et al*., 1995
Electroporation		pBluescript pHRPCAT vector	Lac promoter Promoter regions DHFR-TS	Hrp, hsp86, CAT Pf e luciferase	P falciparum	Wu *et al*., 1996
Electroporation	Wu *et al*., 1995	PGEM 4Z CAT PCAT pCAM	Lac promoter hrp3; hrp2	DHR-TS Tg	*P. falciparum*	Crabb *et al*., 1996
Electroporation	RPMI 1640 + Glutamine (Life Technologies)	PHH-1	MMTV	DHFR selectable	*P falciparum*	Fidock, 1997
Electroporation	Cytomix	pD.D B .D. pD.D T .D. Plasmids	*P. berghei* or *P. falciparum* promoters	*dhfr-ts* from *Plasmodium* or *T. gondii*	*Plasmodium Knowlesi*	Van der Well, 1997

Electroporation		PDT-Tg23 vector	Pf Histidine-rich-protein III gene promoter	Pf DHFR	*P. falciparum*	Mamoun *et al*., 1999
Electroporation		pDB.DTm.DB./DB.AF.DB, pDB-DTm.DB./AB.AF.DB	*pb66/ama-1* and *dhfr-ts* promoters	AMA-1	*P. Knowlesi*	Kocken, 1998
Electroporation		pPICZ A vector	oxidase (AOX1) gene promoter	Circumsporozoit protein *T. gondii* dhfr/ts flanked by *P. berghei* dhfr/ts	*P. Knowlesi*	Kocken, 2002
Electroporation		Plasmid PHluc-3m	5’ flanking regions of gene encoding hrp3/ 2	Parasitophorous vacuolar membrane	*P. falciparum*	Burghaus *et al*., 2001
Electroporation	Fidock *et al*., 1997	PHLH1 and pHD22Y	Pf hrp3 promoter	DHFR	*P. falciparum*/ erythrocytes	Deitsch *et al*., 2001
Electroporation	SuperFect Transfection Reagent	pBS-GFP	*P. falciparum* histidine rich protein (HRP) III gene promoter	mutant *T. gondii* dhfr/ts or GFP	*P. falciparum*	Ben Mamoun, 2001

Comparison of transfection methods		PHH1	MMVT	DAG resistance to WR99210	*P. falciparum*	Skinner-Adams *et al*., 2003
Electroporation	Cytomix	pA5'A3' CAT and Pl5' A' CAT	UTR sequences of E. histolytica actin	CAT	*Entamoeba histolytica*	Nickel, 1994
Electroporation	Nickel, 1994	pA5'A3' NEO		NEO	*E. histolytica*	Hamann, 1995
Electroporation	cytomix supplemented	pGEM-luc pGL2 -control SV40 - luc	5' / 3' flancking region of hgl1	Luciferase reporter gene	*E.histolytica*	Purdy, 1994
Electroporation		pX63-HYG leishmanial	"PARP promoter"	LNP18	*Leishmania*	Papageorgiou 2002
Electroporation	cytomix	pXG1-GFP	*L. major* rRNA promoter	GFP	*Leishmania*	Robinson 2003
Electroporation		pXbGAL 673-73-bGAL PARP.P5%bGAL BGAOS pXbGAL PARP-P	*T. brucei* Procyclic acidic repetitive protein or procyclin promoter		*Leishmania*	Clayton 2000

Electroporation		pR-NEO	30-kilobase extrachromosomal amplified DNA	Dhfr/ts	*Leishmania major*	Kapler, 1990
Electroporation			*L. seymouri* mini-exon sequence	CAT gene	*Leptomonas seymouri*	Bellofatto and Cross, 1989
Electroporation	cytomix buffer supplemented	pSAG 1/trpB	SAG 1 surface antigene gene sequences	tryptophan synthase B gene from *E. coli*	*T. gondii*	Sibley, 1994
Electroporation		pYX122(HIS3)	yeast triose phosphate isomerase promote	Cryptosporidium mtHSP70 - GFP	*Toxoplasma*	Slapeta, 2004
Electroporation	intracellular buffer supplemented with 2mM ATP and 5mM glutathione	pDHFR-TS/M2M4	DHFR-TS promoter	CAT	*Toxoplasma gondii*	Donald, 1993
Antisense RNA vector	antisense construct of clag9			Clag gene	*P. falciparum*	Gardiner *et al*., 2000