THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: June 26, 2009
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR	Miguel Valvano
SIGNATURE	
DEPARTMENT	Microbiology & Immunology
ADDRESS	Dental Science Building 3014
PHONE NUMBER	83996
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EMAIL	mvalvano@uwo.ca

Location of experimental work to be carried out: Building(s) SDRI Room(s) 203-208/212

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 12.0, Approvals).

FUNDING AGENCY/AGENCIES: CIHR/NSERC/Canadian Cystic Fibrosis Foundation/
GRANT TITLE(S): Mizutani Foundation for Glycoscience
(See attached pages)

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED.

Names of all personnel working under Principal Investigators supervision in this location:
Cristina Marolda, Soledad Saldias, Daniel Aubert, Xiang Ruan, Mohamad Hammad, Slade Loutet, Jenni Tolman, Kinnari Patel, Alex Skeldon, Sarah Furlong, Chelsea Clarke, Roberto Rosales

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ⊗ YES O NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)?
If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? O YES ⊗ NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
B. cepacia	⊗ Yes O No	O Yes ⊗ No	O Yes ⊗ No	1 liter	lab stock	O 1 ⊗ 2 O 3
E. coli K-12	O Yes ⊗ No	O Yes ⊗ No	O Yes ⊗ No	up to 10 liters	lab stock	⊗ 1 O 2 O 3
Salmonella	⊗ Yes O No	⊗ Yes O No	O Yes ⊗ No	100 mls	lab stock	O 1 ⊗ 2 O 3
Shigella	⊗ Yes O No	O Yes ⊗ No	O Yes ⊗ No	10 mls	lab stock	O 1 ⊗ 2 O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier. Data sheets are available for bacterial cultures.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ⊗ YES O NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture: Not applicable

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	O Yes O No		Not applicable
Rodent	O Yes O No		
Non-human primate	O Yes O No		
Other (specify)	O Yes O No		

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	Ø Yes O No	HB-1 (human bronchial)	Dr. Lukacs/McGill
Rodent	Ø Yes O No	RAW264.7 macrophages	ATCC
Non-human primate	O Yes Ø No		
Other (specify)	O Yes Ø No		

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required O 1 Ø 2 O 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? O YES Ø NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Known to Be Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		O Yes O No		O 1 O 2 O 3
Human Blood (fraction) or other Body Fluid		O Yes O No		O 1 O 2 O 3
Human Organs or Tissues (unpreserved)		O Yes O No		O 1 O 2 O 3
Human Organs or Tissues (preserved)		O Yes O No		O 1 O 2 O 3

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? Ø YES O NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? Ø YES, complete table below O NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results
E. coli K-12	recombinant vectors	E. coli	bacterial genes	See note

* Please attach a Material Data Sheet or equivalent if available.
NOTE: genes are cloned from bacteria and re-introduced into bacteria/no effect on eukaryotic cells.

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below ⊗ NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ ⊗ NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ ⊗ NO
- SV 40 Large T antigen O YES ⊗ NO
- E1A oncogene O YES ⊗ NO
- Known oncogenes O YES, please specify _____ ⊗ NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ ⊗ NO

4.5 Will virus be replication defective? Not applicable O YES O NO

4.6 Will virus be infectious to humans or animals? Not applicable O YES O NO

4.7 Will this be expected to increase the containment level required? O YES ⊗ NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES ⊗ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: Not applicable
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES ⊗ NO

5.3 How will the biological agent be administered? Not applicable

5.4 Please give the Health Care Facility where the clinical trial will be conducted: Not Applicable

5.5 Has human ethics approval been obtained? O YES, number: N/A ⊗ NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? ⊗ YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used Mice

6.3 AUS protocol # _____

6.4 Will any of the agents listed be used in live animals ⊗ YES, specify: B. cepacia O NO

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES Ø NO
- Pound source cats O YES Ø NO
- Cattle, sheep or goats O YES Ø NO
- Non-human primates O YES, please specify species _____ Ø NO
- Wild caught animals O YES, please specify species & colony # _____ Ø NO
- Birds O YES Ø NO
- Others (wild or domestic) O YES, please specify _____ Ø NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? O YES Ø NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s) N/A
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin N/A

8.4 How much of the toxin is handled at one time*? N/A

8.5 How much of the toxin is stored*? N/A

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES Ø NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. N/A

9.3 What is the origin of the insect? N/A

9.4 What is the life stage of the insect? N/A

9.5 What is your intention? O Initiate and maintain colony, give location: N/A
O "One-time" use, give location: N/A

9.6 Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

9.7 Please attach the CFIA permit. N/A

9.8 Please describe any CFIA permit conditions: N/A

_____
_____
_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O YES ⊗ NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. N/A

10.3 What is the origin of the plant? N/A

10.4 What is the form of the plant (seed, seedling, plant, tree...)? N/A

10.5 What is your intention? N/A O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO N/A
If yes, please describe: N/A

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
N/A

10.8 Is the CFIA permit attached? N/A O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions: N/A

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 ⊗ NO

11.2 Has an Import Permit been obtained from HC for human pathogens? N/A O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES N/A O NO

11.4 Has the import permit been sent to OHS? N/A O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

SIGNATURE _____ Michael Wohoon _____

13.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 Ⓧ 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?

Ⓧ YES, permit # if on-campus_____
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE ___ Michael Wohoon ___ Date: October 30, 2009

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: _____
Date: _____

Safety Officer for Institution where experiments will take place: SIGNATURE: _____
Date: _____

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: _____
Date: _____

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

See attachments: 1) List of funded proposals; 2) summary of each proposal

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

PERSONAL DATA

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Birth date and place:	August 6, 1953; Avellaneda, Buenos Aires, Argentina.
Citizenship:	Canadian

UWO CURRENT POSITION

1998-to date	Full Professor, Department of Microbiology & Immunology, University of Western Ontario, London, Ontario, Canada.
1999-to date	Full Professor (Cross-Appointment), Department of Medicine, University of Western Ontario, London, Ontario, Canada.
2002-to date	Canada Research Chair in Infectious Diseases and Microbial Pathogenesis.
2004-to date	Chair, Department of Microbiology & Immunology, University of Western Ontario, London, Ontario, Canada.

RESEARCH FUNDING (Current)

Valvano: Mechanisms of *Burkholderia cenocepacia* intracellular survival in macrophages; Canadian Cystic Fibrosis Foundation; Operating; Operating; 01/04/08-31/03/11; $260,910

Cygler, Matte & Valvano: Structure-function relationships of bacterial polysaccharide co-polymerases; Canadian Institutes of Health Research; 01/10/08-30/09/13; $ 1,190,000 (MAV operating funds $375,000).

Wright, Valvano, Davies, Burrows & Junop: Antibiotic Adjuvants: Overcoming Multi-Drug Resistance in Gram Negative Bacteria; Canadian Institutes of Health Research; Special initiative on Alternatives to antibiotics; 01/04/08-30/03/13; $ 1,393,750 (MAV operating funds $250,000).

Valvano: Lipopolysaccharide export and assembly in gram-negative bacteria; Canadian Institutes of Health Research; Operating; 01/04/07-30/03/11; $ 692,500

Valvano: Outer membrane permeability and stress responses in *Burkholderia cenocepacia*; Canadian Cystic Fibrosis Foundation; Operating; 01/04/09-31/03/12; $219,300

Valvano: Biosynthesis of core lipopolysaccharide; Natural Sciences and Engineering Research Council; Operating; 01/04/06-31/03/11; $181,250

Valvano: Functional analysis of the bacterial lipid-linked saccharide translocase Wzx; Mizutani Foundation for Glycoscience; 01/04/09-30/03/10; $63,000

Grant Title: Outer membrane permeability and stress responses in *Burkholderia cenocepacia*; Canadian Cystic Fibrosis Foundation; Operating; 01/04/09-31/03/12; $219,300

SUMMARY:

Introduction: Opportunistic, nonfermenting Gram-negative bacteria (e.g. *Acinetobacter baumannii*, *Burkholderia cepacia* complex [Bcc], *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*) are widespread in the environment and becoming an **increasing** cause of **serious** infections in humans. These bacteria mainly affect an expanding population of patients **immunocompromised** by disease, such as cystic fibrosis (CF) or by medical and surgical treatments. Most species are notable for their intrinsic multiple antibiotic resistance, leading to significant antibiotic treatment and infection control challenges. There is a **pressing need** for novel therapeutic strategies to address the emerging threat caused by these bacteria.
Burkholderia strains are fascinating **model organisms** to study the pathogenicity of nonfermenting Gram-negative bacteria. *Burkholderia* can be endosymbionts in pathogenic fungi, establish symbiotic and endophytic associations with leaf galls, roots and other tissues of plants, and also survive within free-living amoebae and phagocytes. Their adaptability to survive in the environment in contact with a broad range of hosts may be key to their pathogenicity in humans. Yet very little is known on the molecular basis for this broad adaptability.

Rationale: The urgent need to develop new therapies against *Burkholderia* will benefit from a clear understanding of the molecular basis for their intrinsic antibiotic resistances, including resistance to antimicrobial peptides (APs), which is currently lacking. ***We hypothesize that the intrinsic antibiotic resistance of B. cenocepacia, and in particular the extreme resistance to antimicrobial peptides (APs), is associated with unique properties of the bacterial cell envelope in these organisms, especially with the lipopolysaccharide (LPS)***. As nonfermenting Gram-negative bacteria share many characteristics, our discoveries may also be applicable to other opportunistic pathogens. In the previous one and half year-grant term, we have made key discoveries that strongly support the feasibility of this renewal application: **(i)** the gene cluster for 4-aminoarabinose (Ara4N) synthesis and transfer to LPS in *B. cenocepacia* is required for bacterial viability; **(ii)** a novel tool to construct unmarked gene deletions in *B. cenocepacia* representing a quantum leap in our ability to genetically manipulate this bacterium; **(iii)** the identification of an HtrA/DegP-like periplasmic protease that is essential for *in vivo* bacterial survival in a rat model of chronic lung infection; and **(iv)** the identification of alternative sigma factor RpoE that is required for osmolar and thermal stress, as well as intracellular survival in macrophages.

Aims: These exciting discoveries are the foundation for the current renewal application, which will focus on three specific aims:
1. Investigations of the mechanism of lethality associated with mutations in the *arn* gene cluster. These experiments are designed to directly demonstrate that Ara4N LPS modifications are essential for the viability of *B. cenocepacia* and elucidate the mechanism of lethality.
2. Isolation and characterization of additional *B. cenocepacia* genes and their protein products required for resistance to APs. We will perform a transposon mutagenesis screen using a *B. cenocepacia hldA* mutant to identify novel genes for resistance to APs, and will identify genes whose expression is regulated by APs as a first step toward identifying APs-responsive regulon or regulons.
3. Characterization of the function of additional HtrA proteins (as potential effectors of membrane stress) and the components of the *B. cenocepacia* RpoE regulon, which likely play key roles in maintaining the permeability barrier of the outer membrane.

Significance: We feel that the proposed research will significantly advance our current understanding of resistance to APs in *B. cenocepacia* and provide a better understanding of components of the bacterial cell envelope that are needed for its permeability barrier function and to maintain its integrity. We also expect that from these studies it will possible to develop novel inhibitory molecules targeting the Bcc outer membrane.

Grant Title: Mechanisms of *Burkholderia cenocepacia* intracellular survival in macrophages; Canadian Cystic Fibrosis Foundation; Operating; Operating; 01/04/08-31/03/11; $260,910

SUMMARY

We use *Burkholderia cenocepacia*, a member of the *B. cepacia* complex (Bcc) as a **model system** to study the pathogenicity of opportunistic bacteria. The Bcc is a group of closely related species of *Burkholderia*, which are soil-borne bacteria and once acquired from the environment, can cause devastating infections in susceptible hosts due to their extraordinary ability to persist and adapt to new niches, including host cells and tissues.

Cystic fibrosis (CF) is a recessive genetic disorder common among Caucasians. Chronic microbial colonization of the major airways and debilitating exacerbations of pulmonary infection are the main cause of morbidity and mortality in these patients. Respiratory infections by Bcc species, in particular *B. cenocepacia*, accelerate the deterioration of lung function in CF patients. These bacteria can also cause invasive infections ("cepacia syndrome") that are often lethal and can be transmitted among patients.

Our long-term goals are to discern how Bcc bacteria (especially *B. cenocepacia*) persist in the airways of CF patients, and ultimately devise strategies for the prevention of chronic infection and the deleterious effect of Bcc infection on pulmonary tissue. We have learned that Bcc isolates survive within amoebae and macrophages. Intracellular survival occurs in a modified vacuole that at least during the first few hours after infection delays acidification and the fusion with the lysosomes. We have also demonstrated that the phagolysosomal fusion delay is dramatically prolonged in *CFTR*-defective macrophages, suggesting interplay between CFTR function and *B. cenocepacia* infection. We have also discovered a bacterial type VI secretory system that appears to be critical for the secretion of effectors that modulate the host's cytoskeleton. Furthermore, Bcc bacteria survive intracellularly in phagocytes despite the presence of an oxidative burst, owing this survival advantage to the bacterial ability to produce a periplasmic superoxide dismutase, a melanin-like pigment, and to alter the assembly of the phagocyte NADP oxidase complex. These properties distinguish Bcc bacteria from other "classical" intracellular microbes and provide a new paradigm of bacteria-host cell interactions.

Our central hypothesis is that the combined effects of intracellular survival and bacterial resistance to oxidative damage in CF lung tissue contribute to enhance the persistence of B. cenocepacia in the airways. We propose to address two pivotal questions arising from our research: **(i)** How do intracellular *B. cenocepacia* bacteria interfere with the normal maturation of the phagosome? and **(ii)** How do infected macrophages respond to intracellular *B. cenocepacia*? Therefore, we propose to achieve the following specific objectives:

1) Identification of *B. cenocepacia* determinants for intracellular survival in macrophages. This involves: a) Construction of deletion mutants in genes encoding the structural components of secretion systems (e.g. types II, III, IV, and VI); b) mutagenesis of genes encoding putative effector molecules; and c) investigation of the pattern of gene expression of intracellular bacteria.
2) Characterization of macrophage responses to intracellular *B. cenocepacia*. In particular, this involves investigating the interactions of the *B. cenocepacia*-containing vacuoles (BcCVs) with the NADPH oxidase and V-ATPase complexes by a combination of biochemical and cellular biology approaches.

We will focus these studies on *B. cenocepacia* isolates from the ET12 lineage since they comprise the most prevalent Bcc complex species in Canada, and will take advantage of novel molecular tools developed in our laboratory to genetically manipulate *B. cenocepacia*. We feel that the proposed research will significantly advance our current understanding of the ability of *B. cenocepacia* to survive intracellularly. Also, methodologies developed here will be applicable to other situations such as the detailed investigations of *B. cenocepacia* interactions with respiratory epithelial cells, and other CF pathogens, like *Staphylococcus aureus*, which appear to have different fates in CFTR-defective cells and in normal cells.

Grant Title: Functional analysis of the bacterial lipid-linked saccharide translocase Wzx; Mizutani Foundation for Glycoscience; 01/04/09-30/03/10; $63,000.

Summary:
Transmembrane flipping of lipid-linked glycans is a *fundamental* biological process in *all* cell types, and is absolutely *essential* for the synthesis of glycoproteins and cell surface polysaccharides in prokaryotes and eukaryotes. However, how lipid-linked carbohydrates are translocated from one leaflet of the lipid bilayer to the other still remains unknown. It has been shown that the unassisted transbilayer movement of polyisoprenol-linked sugars in liposomes is extremely slow, suggesting the need for protein-assisted translocation. A family of bacterial and eukaryotic integral membrane proteins (referred to as Wzx and Rft1, respectively) is proposed to mediate the transbilayer movement of phosphoisoprenyl-linked glycans, but it is not understood how these proteins function as flippases. In my laboratory, we utilize the biogenesis of O antigen lipopolysaccharide (LPS) as a model system to elucidate the characteristics of the Wzx flippase and ultimately to descipher how these proteins interact with the lipid-linked glycan O antigen precursor on the cytosolic face of the membrane to mediate the translocation process.

We will take advantage of our expertise to express Wzx and the genetics systems we have developed to undertake the current studies, which will focus on: 1) Determining the membrane topology of the Wzx O antigen lipid-linked translocase; and 2) Identifying critical amino acid residues required for the function of Wzx *in vivo*.

We will accomplish these aims by examining the topology of Wzx using substituted accessibility cystein mutagenesis, which not only will provide topological information on this multi-transmembrane protein, but also will detect critical amino acid residues for function. The in vivo transmembrane flipping will be assessed using a novel bacterial protein *N*-linked glycosylation system that can add O antigen lipopolysaccharide subunits to an acceptor protein in a "clean" genetic background where downstream components for O antigen assembly (polymerase and ligase) are absent.

The experiments described in this application will provide novel information on the topology and function of the lipid-linked sugar translocase Wzx, and will advance our current understanding of the transmembrane movement of phosphoisoprenyl lipid-glycans. Proteins of the Wzx family are proposed to carry out a biochemical reaction, namely the transmembrane flipping of lipid-linked glycans, which is highly conserved in all cell types throughout the evolutionary tree. Thus, despite that our studies are heavily based on protein analyses, they impact on a central area of glycobiology.

Grant Title: Lipopolysaccharide export and assembly in gram-negative bacteria; Canadian Institutes of Health Research; Operating; 01/04/07-30/03/11; $ 692,500

SUMMARY OF RESEARCH PROPOSAL

Title: Genetics of lipopolysaccharide assembly in Gram-negative bacteria

Introduction: Lipopolysaccharide (LPS) is a unique surface molecule of Gram-negative bacteria that also plays a key role as an elicitor of innate immune responses. In addition, the O antigen moiety of LPS contributes to pathogenesis by protecting infecting bacteria from bactericidal host responses such as complement killing and phagocytosis. We investigate the biosynthesis of the LPS O antigen with the long-range goal of identifying new ways to curtail infections by interfering with the assembly of LPS. LPS is assembled at the plasma membrane followed by the transit of the molecule to the outer leaflet of the outer membrane, where it becomes surface exposed. The O antigen is synthesized as a lipid-linked saccharide intermediate. The lipid component is undecaprenyl phosphate (Und-P), a C_{55} polyisoprenol.

Rationale: Our research in the last 5-year grant period supports our central hypothesis that ***the proteins involved in O antigen synthesis interact with one another forming a multi-protein complex in the plasma membrane***. Therefore, it is critical to first determine the characteristics of each of the protein components involved in: (i) the initiation of the O antigen subunit synthesis onto Und-P, (ii) the translocation of the Und-PP-linked O antigen subunits across the plasma membrane, and (iii) the polymerization of the O polysaccharide and its ligation to the remainder of the LPS molecules. Our excellent progress in the last 5 years supports the current resubmission. We propose to use genetic and biochemical approaches for the detailed characterization of the initiation of O antigen synthesis and each of the components of the Wzy-dependent O-antigen assembly pathway.

Experimental approach: The studies proposed for the next five years will address two key areas:

1) Genetic and functional characterization of the initiation of O antigen synthesis. These studies focus on the membrane proteins WecA and WbaP, which are prototypes of the two major families of enzymes mediating the transfer of the first sugar precursor for O antigen repeating units onto the lipid acceptor. Specific experimental goals are the detailed characterization of the regions in these proteins for: **a)** recognition and binding of the nucleotide sugar substrate, and **b)** association with the Und-P lipid acceptor.

2) Characterization of the Wzy-dependent pathway protein components. These studies involve: **a)** Determining the membrane topology of the protein components of the Wzy-dependent assembly pathway; **b)** Functional analysis of the translocation of the O antigen subunit across the plasma membrane and its ligation of the lipid A core LPS moiety; **c)** Functional analysis of the O antigen polysaccharide chain regulator protein Wzz; and **d)** Identification and analysis of a putative Wzy-dependent translocation-assembly multi-protein complex.

Significance: With uninterrupted funding from MRC/CIHR since 1988 to 2006 our laboratory has gained international recognition for its contributions to elucidate the components of the Wzy-dependent pathway, and our past and present research is cutting edge in the field of bacterial glycobiology. Our proposed investigations for the next 5-year cycle will provide new knowledge on the assembly of bacterial cell surface-associated polysaccharides, and also will lay a foundation for translational medical applications derived from the genetic manipulation of LPS biosynthetic enzymes. Examples include designing new therapeutic treatments specifically targeted to key LPS biosynthetic enzymes of Gram-negative pathogens, and developing novel glycoconjugate vaccines.

Grant Title: Biosynthesis of core lipopolysaccharide; Natural Sciences and Engineering Research Council; Operating; 01/04/06-31/03/11; $181,250

3

Personal identification no. (PIN)	Family name of applicant
152576	Valvano

SUMMARY OF PROPOSAL FOR PUBLIC RELEASE (Use plain language.)

This plain language summary will be available to the public if your proposal is funded. Although it is not mandatory, you may choose to include your business telephone number and/or your e-mail address to facilitate contact with the public and the media about your research.

Business telephone no. (optional): (519) 661-3427

E-mail address (optional): mvalvano@uwo.ca

Our LONG-TERM GOALS are to elucidate the mechanism(s) of biosynthesis of lipopolysaccharides (LPS) and to utilise this information for designing novel enzyme inhibitors with antimicrobial properties. Our MODEL SYSTEM focuses mainly on the LPS molecule of Escherichia coli, but also the LPS of opportunistic bacteria such as Burkholderia cenocepacia and Pseudomonas aeruginosa. We are particularly interested in two areas of biological and biotechnological interest: (i) the biosynthesis of LPS core oligosaccharide, and (ii) the structure-function analysis of bacterial glycosyltransferases (GCTs).

(i) We will FOCUS the current research program on the identification and characterization of a novel pathway for the synthesis of the core oligosaccharide LPS precursor, ADP-L-glycero-D-manno-heptose (ADP-LDHep). This molecule is a building block required for the synthesis of the core oligosaccharide in most Gram-negative bacteria. Mutants with a heptoseless core display a dramatic increase in permeability to hydrophobic compounds and they survive very poorly in the host. Thus, targeting the enzymes involved in the ADP-LDHep biosynthesis pathway may result in the development of new antimicrobials.

(ii) In the second part of our proposal, we will continue with the detailed characterization of the four glycosyltransferases (GCTs) involved in the synthesis of the O7 antigen repeating subunit. We have described a novel analogue of the isoprenoid lipid undecaprenol phosphate, which facilitates the characterization of glycosylation products and used it to identify the function of WbbD, the first of the four GCTs for O7 antigen synthesis. We will to continue with the characterization of the remaining glysosyltransferases WbbC, WbbB, and WbbA, and initiate crystallization trails for each of these enzymes.

In terms of SIGNIFCANCE, the areas of research described in this proposal build on solid preliminary data from the PI's laboratory and his collaborators, and will contribute to further our basic knowledge on LPS biosynthesis, while at the same discover novel strategies to curtail infection.

Second Language Version of Summary (optional).

Grant Title (Cygler, Matte & Valvano): Structure-function relationships of bacterial polysaccharide co-polymerases; Canadian Institutes of Health Research; 01/10/08-30/09/13; $ 1,190,000 (MAV operating funds $375,000).

CYGLER, Mirek

Operating Grants/Subventions de fonctionnement Application/Demande 2008-03-03

Summary of Research Proposal/Résumé de la proposition de recherche

Bacteria produce a highly diverse array of cell surface polysaccharides including lipopolysaccharide (LPS) O antigens (Oag), capsules (CPS), loosely attached exopolysaccharides (EPS), enterobacterial common antigen (ECA), teichoic acids (TA), and lipoteichoic acids (LTA). Many of these macromolecules are important virulence determinants. Despite many years of study the mechanistic details of their synthesis and translocation to the cell surface are poorly understood. Several key proteins required for synthesis and assembly of cell surface polysaccharides reside in the inner membrane of Gram-negative bacteria. Synthesis of these complex polysaccharides commences in the cytosol where the oligosaccharide repeat units are assembled on an undecaprenyl phosphate anchor. Assembly and translocation of synthesis intermediates is carried out by two different mechanisms: the Wzy polymerase-dependent block polymerization pathway and ABC transporter-dependent pathway. The length of the polysaccharide chains has a modal distribution unique to different polymers and different strains. For polysaccharides synthesized by the Wzy-dependent pathway this modal length distribution requires Wzz proteins belonging to the family called polysaccharide co-polymerases (PCPs). PCPs are anchored in the inner membrane by N- and C-terminal transmembrane helices with the bulk of the polypeptide exposed to the periplasmic space, and they form oligomeric complexes.

In recent years we have made significant breakthroughs toward molecular understanding of the Wzy-dependent system. We have demonstrated by genetic reconstruction experiments of OAg biosynthesis that the assembly of OAg and ECA undecaprenyl phosphate-linked polymers depends on interactions among three membrane proteins (the Wzx OAg flippase, the OAg co-polymerase Wzz, and the OAg polymerase Wzy). We have elucidated the atomic structure of the large periplasmic region of several distantly related Wzz length chain modulators and showed that they (a) share the same protomer fold, and (b) assemble into bell-shaped oligomers of variable sizes with a large internal cavity. Finally, we have shown that WbaP, a critical membrane protein for the initiation of OAg synthesis, has a periplasmic domain that is involved in modulating OAg polysaccharide length, possibly by establishing critical interactions with Wzz or Wzy proteins.

Taken together, these data allowed us to propose a hypothesis that PCPs act as molecular scaffolds for the assembly of lipid-linked polysaccharide intermediates, which are subsequently exported to the bacterial cell surface. The purpose of this application is to determine at the molecular level how the PCPs exert control of the polysaccharide lengths and how do they interact with other proteins of the LPS assembly machinery. We propose to investigate in detail the structure-function aspects of three PCPs: WzzB, Wzz_{FepE} and WzzE. Specifically, we would like to address the following questions:

1. What is the relationship between the modal length of the polysaccharide chain and the oligomeric size of the PCP in the inner membrane?
2. Which parts of the PCPs are essential for their functional role?
3. How PCPs interact with the polysaccharide chain intermediate during its synthesis?
4. Which other proteins involved in assembly and export of the polysaccharide do PCPs interact with and how?

These recent key discoveries now provide the structural basis to begin uncovering molecular basis for the workings of this complex machinery. Combining structural, biochemical and functional data allowed us to designed novel experimental strategies to address the questions stated above. To this end we have assembled a team of internationally recognized researchers in LPS biosynthesis (Valvano, Morona), structural biology (Cygler, Matte), biophysical studies of protein-protein (Bouvier, O'Connor-McCourt) and protein-carbohydrate (Grandbois) interactions to achieve our long-range goal of elucidating the molecular interactions and mechanisms required for the assembly of complex carbohydrate polymers at the bacterial surface. The biogenesis of LPS in bacteria and lipid-linked saccharide moieties for eukaryotic N-linked protein glycosylation are remarkably similar, underscoring the general biological relevance of our research. The potential outcomes of these studies are new approaches to the treatment of bacterial pathogens.

File Number/Numéro de dossier 00034800

Grant Title (Wright, Valvano, Davies, Burrows & Junop): Antibiotic Adjuvants: Overcoming Multi-Drug Resistance in Gram Negative Bacteria; Canadian Institutes of Health Research; Special Initiative on Alternatives to antibiotics; 01/04/08-30/03/13; $ 1,393,750 (MAV operating funds $250,000).

G. Wright NET in Antibiotic Adjuvants $300,000 Research Proposal

The continuing evolution of antibiotic resistance in well known pathogenic bacteria along with the emergence of new multi-drug resistant pathogens represent a growing and significant challenge to the treatment of infectious disease. This problem impacts both the health care and agricultural sectors and spans numerous bacterial genera. The problem though is especially acute for Gram-negative pathogens, which are emerging as major clinical problems. Pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Burkholderia cepacia* infect the most vulnerable patients e.g. children with cystic fibrosis and burn victims, and these organisms are typically multi-drug resistant. Furthermore, the horizontal spread of mobile genetic elements that confer multi-drug resistance in nosocomial pathogens, is contributing to the increase of resistance to the latest drugs, for example with extended spectrum β-lactamase resistance.

The solutions to this problem will require new strategies to address the inevitable problem of antibiotic resistance using a variety of methods. The approach we are championing in this proposal is the use of **antibiotic adjuvants**. We define these as molecules that synergize with antibiotics to overcome resistance. The objectives of this NET are to use well-established antibiotics in combination with other non-antibiotic compounds to expand the impact of the antibiotics, to decrease drug dosing, and to resurrect older agents whose efficacy has diminished as a result of resistance. This proposed work builds strategically on preliminary results obtained by members of the group and as a result, the scientific leverage is substantial.

Three aims are envisioned:

1) The development of cell-based screens of antibiotic-natural compound combinations to overcome genetic and physiological antibiotic resistance.
2) The blockade of Gram-negative outer membrane biosynthesis and assembly to disrupt the cell envelope thereby enabling antibiotics to penetrate the cell.
3) Identification of potentiators of antibiotics across bacterial cell states such as biofilm growth.

In each of these aims we will pursue a common strategy that includes establishment of a suitable assay followed by screens of molecules using this assay to identify potential antibiotic adjuvants. For example, in Aim 1 we will screen cellular reporters for inhibitors of resistance enzymes (e.g. extended spectrum β-lactamases) and tracsritpion of antibiotic efflux pump genes; in Aim 2 we are focusing on inhibitors of the biosynthesis if the essential lipopolysaccharide component ADP-heptose; in Aim 3 we are seeking molecules that impair biofilm formation and quorum sensing. Unique to the Team is a collection of several thousand natural product extracts that we have already shown have great potential to discover such antibiotic adjuvants as well as over 150,000 small organic molecules available through the McMaster High Throughput Screening laboratory.

This NET brings together researchers with complimentary skill sets ranging from state of the art high throughput screening technology, to animal models of infection, to X-ray crystallography along with the necessary infrastructure to pursue this research. The NET therefore provides a unique technological environment that will result in an exceptional and engaging training environment suitable for the discovery of new therapeutic strategies in infectious disease.