APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

2. DATE SUBMITTED	Applicant Identifier
3. DATE RECEIVED BY STATE	**State Application Identifier**

1. * TYPE OF SUBMISSION

☐ Pre-application ☒ Application ☐ Changed/Corrected Application

4. Federal Identifier

5. APPLICANT INFORMATION

* Organizational DUNS: 073130411

* Legal Name: Massachusetts General Hospital (The General Hospital Corp)

Department: Research Management Division:

* Street1: 101 Huntington Avenue

Street2: Suite 300

* City: Boston County: USA

* State: MA: Massachusetts Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 02199

Person to be contacted on matters involving this application

Prefix: * First Name: Caren Middle Name:

* Last Name: Briggs Suffix:

* Phone Number: 617-954-9302 Fax Number: 617-954-9850

Email: cbriggs1@partners.org

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN):* 1042697983A1

7. * TYPE OF APPLICANT: M: Nonprofit with 501C3 IRS Status (Other than Institution of Higher Education)

Other (Specify):

Small Business Organization Type ☐ Women Owned ☐ Socially and Economically Disadvantaged

8. * TYPE OF APPLICATION:

☒ New ☐ Resubmission

☐ Renewal ☐ Continuation ☐ Revision

If Revision, mark appropriate box(es).

☐ A. Increase Award ☐ B. Decrease Award ☐ C. Increase Duration ☐ D. Decrease Duration

☐ E. Other (specify):

* Is this application being submitted to other agencies? Yes ☐ No ☒ What other Agencies?

9. * NAME OF FEDERAL AGENCY: National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER: 93.859

TITLE: Biomedical Research and Research Training

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:

Metasystem of model organisms to study emergence of host-microbe maladaptations

12. * AREAS AFFECTED BY PROJECT *(cities, counties, states, etc.)*

N/A

13. PROPOSED PROJECT:

* Start Date: 04/01/2010 * Ending Date: 03/31/2015

14. CONGRESSIONAL DISTRICTS OF:

a. * Applicant: MA-009 b. * Project: MA-009

15. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. * First Name: Suresh Middle Name:

* Last Name: Gopalan Suffix: Ph.D.

Position/Title: Assistant in Molecular Biology

* Organization Name: Massachusetts General Hospital (The General Hospital Corp)

Department: Molecular Biology Division: Genetics

* Street1: 185 Cambridge Street

Street2:

* City: Boston County: USA

* State: MA: Massachusetts Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 02114

* Phone Number: 617-643-3323 Fax Number: 617-643-3050

* Email: gopalan@molbio.mgh.harvard.edu

OMB Number: 4040-0001
Expiration Date: 04/30/2008

424 R&R and PHS-398 Specific Table Of Contents

	Page Numbers
SF 424 R&R Face Page	1
Table of Contents	3
Performance Sites	4
Research & Related Other Project Information	5
Project Summary/Abstract (Description)	6
Public Health Relevance Statement (Narrative attachment)	7
Facilities & Other Resources	8
Equipment	9
Research & Related Senior/Key Person	10
Biographical Sketches for each listed Senior/Key Person	12
Research & Related Budget - Year 1	15
Research & Related Budget - Year 2	18
Research & Related Budget - Year 3	21
Research & Related Budget - Year 4	24
Research & Related Budget - Year 5	27
Budget Justification	30
Research & Related Budget - Cumulative Budget	32
PHS 398 Specific Cover Page Supplement	33
PHS 398 Specific Research Plan	35
Specific Aims	36
Background & Significance	37
Preliminary Studies/Progress	41
Research Design & Methods	50
Vertebrate Animals	62
Select Agent Research	63
Bibliography & References Cited	64
Letters of Support	71
Resource Sharing Plan	76
PHS 398 Checklist	77

Close Form | Print Page | About

RESEARCH & RELATED Other Project Information

1. * Are Human Subjects Involved? ☐ Yes ☒ No

1.a If YES to Human Subjects

Is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

Exemption Number: ☐1 ☐2 ☐3 ☐4 ☐5 ☐6

Human Subject Assurance Number:

2. * Are Vertebrate Animals Used? ☒ Yes ☐ No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ☒ Yes ☐ No

IACUC Approval Date:

Animal Welfare Assurance Number A3596-01

3. * Is proprietary/privileged information included in the application? ☐ Yes ☒ No

4.a. * Does this project have an actual or potential impact on the environment? ☐ Yes ☒ No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ☐ Yes ☐ No

4.d. If yes, please explain:

5.a. * Does this project involve activities outside the U.S. or partnership with International Collaborators? ☐ Yes ☒ No

5.b. If yes, identify countries:

5.c. Optional Explanation:

6. * Project Summary/Abstract 1235-ResearchSummary.pdf | Add Attachment | Delete Attachment | View Attachment

7. * Project Narrative 1239-ProjectNarrative.pdf | Add Attachment | Delete Attachment | View Attachment

8. Bibliography & References Cited 1238-Bibliography.pdf | Add Attachment | Delete Attachment | View Attachment

9. Facilities & Other Resources 1236-FacilitiesSG.pdf | Add Attachment | Delete Attachment | View Attachment

10. Equipment 1237-Equipment.pdf | Add Attachment | Delete Attachment | View Attachment

11. Other Attachments Add Attachments | Delete Attachments | View Attachments ☐

OMB Number: 4040-0001
Expiration Date: 04/30/2008

PROJECT SUMMARY

This proposal presents a novel approach for rapid study of emergence of new host-microbe maladaptations.

An unintended consequence of global industrialization and associated societal rearrangements is new interactions of microbes and potential hosts (especially mammals and plants), providing an opportunity for the rapid emergence of host-microbe adaptation and eventual establishment of new microbe-related diseases. Another scenario is when commonly innocuous microbes interact with a potential host in a condition favoring adaptation. It is hypothesized that such adaptations occur initially through host and microbe system status change (adaptive biochemical and signaling state changes). At least some environment induced diseases, zoonotic diseases and nosocomial infections likely arose this way and continue to do so in this manner. Such system status changes characteristic of different kinds of adaptations and causal components and networks can be inferred through integration of system-wide data on molecular components and other phenotypic data.

The lessons on how this proposed arm of emergence of new disease are hard to study in the relevant environments. Further, it could take too long to make inferences from natural occurrences to be of relevance. In addition, even when the signaling modules or biological components identified as possible candidates it would be difficult to validate. Here, a model metasystem has been established using seedlings of the model host *Arabidopsis thaliana* interacting with different well studied microbes representing different modes of interactions, with many unique properties that make it suitable for rapid study of such maladaptations. The well studied microbes include human opportunistic pathogen (*Pseudomonas aeruginosa*), agricultural pathogen (*Pseudomonas syringae*) and commonly considered innocuous microbes (the gram negative microbe *Escherichia coli* and the gram positive microbe *Bacillus subtilis*) all of which can cause host damage under these conditions. The use of a model host and ubiquitously used microbes should provide an excellent framework to build and test these new theories on. Other advantages of this model include amenability to high throughput automation assisted perturbation screens and real time study of signaling cross talk. The system will be used to develop a knowledge framework to infer changes in system status using biological phenotypic changes, system-wide monitoring of cellular components during progression of such adaptive interactions, and data integration approaches. Such an understanding will help identify emergence of host-pathogen adaptations that have negative impact on mankind and avoid unfavorable conditions that favor them.

PROJECT NARRATIVE

Emergence of new host-microbe maladaptations (including environment induced adaptations, zoonotic diseases and nosocomial infections) are difficult to identify and study in natural settings. A metasystem consisting of a model host (seedlings of *Arabidopsis thaliana*) interacting with different well studied microbes (including human opportunistic pathogens, agricultural pathogens and innocuous microbes) causing host damage in a particular environment has been established to study such adaptations. This proposal proposes and aims to test the hypothesis that initial stages of such adaptations occur through changes in system status (biochemical and signaling state changes) of host and microbe, and can be predicted by studying different modes of interactions represented in this model.

Resources:

Research facilities organization: The Department of Molecular Biology is located in the newly constructed (2005) Richard B. Simches Research Center on the Massachusetts General Hospital main campus. The part of the Department where I am currently housed (Ausubel lab) is located on Simches 7, which also houses the laboratories of Gary Ruvkun and Joshua Kaplan, both of whom work with *C. elegans*. The Department occupies ~ 44,000 total square feet of laboratory benches, desks, office space and shared facilities. The laboratory of Jen Sheen, working on different aspects of plant biology and signaling in plants is located across the hallway and share plant related resources mentioned below.

From a broader perspective, the Simches Research Building is designed around thematic centers that house many laboratories at MGH at the forefront of modern biological research. Thus in addition to Molecular Biology, the Simches building has multi-investigator groups studying systems biology, human genetics, stem cells, chemical genetics, genomics, and computational and integrative biology.

Simches 7 has a full complement of shared general and specific laboratory facilities including instrument rooms, cold rooms, microscope rooms, a walk-in plant growth facility, a chemical storage room, a media preparation facility, a glass washing and sterilization facility, a tissue culture facility, electrophoresis and gel rooms, a dark booth and a specialty dark room as well as laboratory supply storage space. Floor secretarial offices, the offices of the Department's Faculty members, conference rooms (with advanced audio/visual equipment), and "tea rooms" equipped with full kitchen and meeting facilities, are also located on Simches 7.

Plant and worm resources: A number of plant walk-in growth facilities and reach-in chambers are available to conduct the *Arabidopsis* and *Nicotiana* based experiments proposed, coordinated by a Greenhouse manager who also maintains the greenhouse (in the Their building that the department used to occupy), which is part of the available resources. Since three labs work with *C. elegans*, there is an extensive maintenance of common resources (e.g., regularly re-stamped RNAi library for rapid cherry picking and testing phenotypes) often coordinated on a rotating basis by postdoctoral members and students, in addition to a variety of worm mutants and reporter strains and constructs that are regularly used. This should be useful in testing contributions of any matched modules in host and pathogen proposed in Aim 3, when the microbe/pathogen is infectious in this model host. In addition, there are monthly host-pathogen meetings with presentations from one of over ten research laboratories, working on different aspects of relevant host or microbial biology, in and around MGH.

Equipment: Almost all equipment in the Department of Molecular Biology is shared. This includes most modern equipment needed for state-of-the-art cell and molecular biology research. In addition to the equipment and facilities in the Department of Molecular Biology, MGH has a robotic instrumentation facility, an oligonucleotide synthesis facility, and a DNA sequencing facility and more recently added a Solexa sequencing machine to the collection of available resources to be available to through the core. Additional services to all departmental researchers needing peptide synthesis, protein sequencing, and/or amino acid analysis are also available. In addition, many of the data collection for system-wide analyses proposed can be carried out at the core facilities available through the Harvard University and the Partners Network, usually through prior arrangement and for a charge.

Some equipment available in the Department includes: 2 Beckman L8 ultracentrifuges, 3 Beckman J2-21 high speed centrifuges, a Beckman TL-100 ultracentrifuge, a Beckman J6 low speed centrifuge, a Beckman Biomek robot, 5 -80@C freezers, a regular scintillation counter and a Perkin Elmer luminometer/scintillation counter for high-throughput reads in 96 well plates, 6 thermo-cyclers for PCR reactions, thermal cyclers for real-time quantitative PCR, a Beckman DU-640 spectrophotometer, an FPLC, an HPLC, a GLC, a capillary zone electrophoresis system, a Perkin Elmer fluorescence spectrometer, a Molecular Dynamics Phosphor Imager, a hybridization oven, fraction collectors, bacterial shakers, bacterial and cell culture incubators, inverted and epi fluorescence Zeiss microscopes, a Leica confocal microscope, ultramicrotome, lyophilizer, gel drier, table-top microcentrifuges, electrophoresis equipment, and all the miscellaneous small equipment required for carrying out standard molecular biology experiments.

Computer and Bioinformatic resources: The Department of Molecular Biology also has extensive computer facilities with web and database servers and a dedicated computer services core staff who also provide regular computer and network maintenance and consultation, as well as a new Bioinformatics Core Facility consisting of three full-time bioinformaticians. Each member of a laboratory has their own Macintosh G4 notebook or PC computer. In addition, many Macintosh G4, Linux, and PC computers are available at common workstations dispersed throughout the Department.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Suresh Gopalan, Ph.D.	Assistant in Molecular Biology
eRA COMMONS USER NAME GOPALAN	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Birla Institute of Technology and Science, Pilani, India (Dual degree scheme)	B.E. (Hons)	1988	Mechanical Engineering
Birla Institute of Technology and Science, Pilani, India (Dual degree scheme)	M.Sc. (Hons)	1988	Biosciences
Center for Biotechnology, Anna University, Madras, India	Ph.D.	1993	Biotechnology

A. PROFESSIONAL EXPERIENCE

1987	Summer internship, Cancer Institute, Madras, India. **Generation of monoclonal antibodies against the estrogen receptors from the human breast cancer cell line 247D.**
1987	Masters thesis, Birla Institute of Technology and Science, India **Leukocyte culture for scoring environmental mutagens.**
1988	Internship Program part of Bachelors degree, Hindustan Aeronautics Limited, India. **Analysis of Low Aspect Ratio Wings**
1988 – 1993	Ph. D thesis, Anna University, India (Advisor: Prof. Kunthala Jayaraman) **Recombinant DNA approaches and process optimization strategies for enhanced production of the mosquito larvicidal proteins of *Bacillus sphaericus* 1593M**
1990	Academic scientist (technology transfer), Hindustan Antibiotics Limited, India. **Large scale cultivation of *B. sphaericus* biopesticide in 27 kL reactors.**
1991	Exchange Scientist, Swiss Federal Institute of Technology, Zurich **Development of a defined medium for the study of growth, sporulation and toxin synthesis in *B. sphaericus*.**
1993 - 1995	Postdoctoral Scholar, Dept. Plant Pathology, University of Kentucky, Lexington, KY **Plant responses to bacterial hypersensitive response elicitors.** (S. Y. He Lab)
1995 - 1998	Postdoctoral Research Associate, MSU-DOE-Plant Research Laboratory, East Lansing, MI **Plant perception and signaling during gene-for-gene mediated cell death response (HR) and resistance initiated by bacterial pathogens**(S. Y. He Lab)
1999	Research Associate (Instructor), Molecular Genetics and Cell Biology Department, University of Chicago, Chicago, IL.
1999 - 2001	Postdoctoral Research Associate, Institute of Biological Chemistry, Washington State University, Pullman, WA **Molecular genetic analysis of plant-virus interactions** (J. Carrington Lab)
Nov'01 - Jan '02	Scientist at Genome Therapeutics Corporation, Waltham, MA (through Aerotek) **Mechanism of action of antimicrobials identified by screening chemical libraries and development of 'ready-to-high throughput' cell based assays for the study of biochemical processes**
Feb'02 – Aug'02	Independent Investigator **Detect SNPs (*in silico*), organize and analyze SNPs in Arabidopsis and compare to other plants and organisms.**
Sep'02–Sep' 06	Independent Investigator & Consultant: Massachusetts General Hospital & Harvard University, MA. (L. Rahme Lab) Collaborator and Visiting Scientist, MGH & Harvard University (Fred Ausubel Lab)

Genomic analysis of pathogen (*P. aeruginosa*) and host (mouse and Arabidopsis) processes.
Explore newer methods to analyze and utilize large-scale gene expression datasets

Oct'06-Mar'08 Visiting Scientist,
Mar'08-current Assistant in Molecular Biology, Department of Molecular Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA. (Fred Ausubel Lab)
Models and systems to study recalcitrant and emerging infectious diseases

HONORS AND FELLOWSHIPS

1987 ***Best lecture of the year***, IDEATION, Birla Institute of Technology and Science, India. "Cancer therapy and Monoclonal Antibodies".
1988 - 1993 Junior and Senior Research Fellowship, Council for Scientific and Industrial Research, and Department of Science and Technology (SERC), India.
1993 – 1994 Post Doctoral Fellowship, Research and Graduate Studies, University of Kentucky, Lexington.
1997 Young Researcher's bursary to participate and present talk entitled "Reversal of the plant hypersensitive response by the growth regulator auxin" in the "Plant Workshop: Leaves". La-Colle-sur-Soup, France.

OTHERS

Ad hoc reviewer: The Plant Cell, USDA, BBSRC

SELECTED INVITED PRESENTATIONS

1) Development of a defined medium by chemostat pulse and shift technique for the cultivation of the biopesticide *B. sphaericus* 1593M. Swiss Federal Institute of Technology (ETH), Honngerberg, Zurich, Switzerland. 1991.
2) Plant genes induced during the hypersensitive response to incompatible bacterial pathogens. International Laboratory for Tropical Agricultural Biotechnology/The Scripps Research Institute, California. 1995.
3) Genes galore: how do we get to their functions? Seminar at Michigan State University, 1996.
4) *Pseudomonas syringae* pv. *glycinea* AvrB elicits a genotype specific hypersensitive necrosis in transgenic *Arabidopsis thaliana* plants: dependence on RPM1 and a possible second target gene. 7th International Conference on Arabidopsis Research. Norwich, England. 1996.
5) Reversal of the plant hypersensitive response by the growth regulator auxin. The Plant Workshop: Leaves. La-Colle-sur-Soup, France. 1997.
6) *Pseudomonas syringae* avirulence protein AvrB initiates a genotype-specific hypersensitive resistance response inside the plant cell. UNESCO/DBT/SBPL sponsored workshop on Cells and Molecules in Biotechnology. Madras, India. 1997.
7) Identification of putative signaling components in *Pseudomonas syringae* AvrB-mediated plant disease resistance. 9th International Conference on Arabidopsis Research, Madison, Wisconsin. 1998.
8) Genetic Analysis of Plant Susceptibility to Tobacco Etch Virus. New England Arabidopsis Meeting, 2001.

B. PUBLICATIONS AND PATENT

1. Rajendra. K.B. and Suresh. G. (1989) Comments on the Lawrence equation for low aspect ratio wings. J. Aircraft, 26:883-885.
2. Rajamohan. F., Suresh. G. and Jayaraman. K. (1992) Enhanced expression of a mosquito larvicidal gene(s) from *B. sphaericus* 1593M in *E. coli*. Curr. Sci., 63:205-208.
3. Suresh. G., Radhika. C.G. and Jayaraman. K. (1992) Enhanced expression of a second mosquito larvicidal gene of *B. sphaericus* 1593M in *E. coli*. Biotech. Lett., 14:567-572.
4. Meenakshisundaram. S., Suresh. G (Gopalan)., Fernando. RK., Jenny. K., Sachidanandham. R., and Jayaraman. K. (1997) Metabolic response of *Bacillus sphaericus* 1593M for dual-substrate limitation in continuous and total-cell-retention cultures. Appl. Microbiol. Biotechnol. 47:554-559.

5. Collmer. A., Alfano. J. R., Bauer. D. W., Preston. G. M., Loniello. A. O., Conlin. A., Ham. J. H., Huang. H-C., Gopalan. S. and He. S. Y (1996) Secreted proteins, secretion pathways, and the plant pathogenicity of *Erwinia chrysanthemi* and *Pseudomonas syringae*. In: Biology of Plant-Microbe Interactions. Eds. G. Stacey., B. Mullin., P. M. Greshoff. International Society for Molecular Plant Microbe Interactions. St. Paul. 159-164.
6. Strobel. N. E., Ji. C, Gopalan. S, J. A. Kuc. and He. S. Y. (1996) Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ protein. Plant J. 9:413-439.
7. Gopalan. S. and He. S. Y. (1996) Bacterial genes involved in the elicitation of hypersensitive response and pathogenesis. Plant Disease 80:604-609.
8. Gopalan. S., Wei. W. and He. S. Y. (1996) *hrp* gene-dependent induction of *hin1*: A plant gene activated rapidly by harpins and an *avr* gene-mediated signal. Plant J. 10:591-600.
9. Gopalan. S., Bauer, D. W., Alfano, J. R., Loniello, A. O., He. S. Y. and Collmer, A. (1996) Expression of *Pseudomonas syringae* AvrB in plant cells alleviates its dependence on the Hrp secretion system in eliciting genotype-specific hypersensitive cell death. Plant Cell 8:1095-1105.
10. Gopalan. S. and He. S. Y (1998) Disease resistance: beyond the resistance genes. Trends in Plant Sci. 3:207-208.
11. Yuan. J., Wei. W., Gopalan. S., Hu. W., Jin. Q., Plovanich-Jones. A., Muncie. L. and He. S. Y. (2000) *hrp* genes of *Pseudomonas syringae*. Page 1-20 in Plant-Microbe Interactions, Stacey. G. and Keen. N, eds. APS Press. St. Paul, Minnesota.
12. Dormann, P., Gopalan, S., He, S. Y., Benning, C. (2000) A gene family in *Arabidopsis thaliana* with sequence similarity to *NDR1* and *HIN1*. Plant Physiol. Biochem. 38: 789-796.
13. Gopalan. S (2004) ResurfP: a response surface aided parametric test for identifying differentials in GeneChip based oligonucleotide array experiments. Genome Biol. 5: P14. (Deposited Research)
14. Padfield. K. E., Astrakas. L. G., Zhang. Q., Gopalan. S., Mindrinos. M. N., Dai. G., Tompkins. R. G., Rahme. L. G and Tzika. A. A. (2005) Burn Injury Causes Mitochondrial Dysfunction In Skeletal Muscle. Proc. Natl Acad. Sci. USA. 102: 5368-5373.
15. Déziel. E., Gopalan. S., Tampakaki. A., Lépine. F., Padfield. K., Saucier. M., Xiao. G., and Rahme. L.G. (2005) The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of N-acyl-L-homoserine lactones. Mol. Microbiol. 55:998-1014.
16. Astrakas. L. G., Goljer. I., Yasuhara. S., Padfield. K. E., Zhang. Q., Gopalan. S., Mindrinos. M. N., Dai. G., Yu. Y-M., Martyn. J. A., Tompkins. R. G., Rahme. L. G., Tzika. A. A. (2005) Proton NMR spectroscopy shows lipids accumulate in skeletal muscle in response to burn trauma induced apoptosis. FASEB. J. 19:1431-1440. 16. Padfield. K. E., Zhang. Q., Gopalan. S., Mindrinos. M. N., Tzika. A. A., Tompkins. R. G., Rahme. L. G. (2006) Local and Distal Burn Injury Alter Immuno-inflammatory Gene Expression in Skeletal Muscle. J. Trauma 61:280-292.
17. Shang. Y., Li. X., Cui. H., He. P., Thilmony. R., Chintamanani. S., Zwiesler-Vollick. J., Gopalan. S., Tang. X., Zhou. J.M. (2006) RAR1, a central player in plant immunity, is targeted by *Pseudomonas syringae* effector AvrB. Proc. Natl Acad. Sci. USA. 103:19200-19205.
18. Gopalan. S (2007) A multidirectional non-cell autonomous control and a genetic interaction restricting tobacco etch virus susceptibility in Arabidopsis. PloS ONE 2:e985.
19. Denoux. C., Galletti. R., Mammarella, N., Gopalan, S., Werck, D., De Lorezo. G., Ferrari. S., Ausubel. F.M., Dewdney. J. (2007) Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings (Molecular Plant, accepted)
20. Gopalan. S. (2008) Reversal of an immunity associated plant cell death program by the growth regulator auxin. BMC Research Notes 2008, 1:126.
Gopalan. S (2007) Reversal of an immunity associated plant cell death program by the growth regulator auxin. Nature Precedings http://dx.doi.org/10.1038/npre.2007.1347.1 (Deposited Research)
21. Gopalan, S., and Ausubel, F. M. (2008) A metasystem of framework model organisms to study emergence of new host-microbe adaptations. PLoS ONE 3:e3891.
22. Methods and systems for high confidence utilization of datasets. **US Patent 7,480,593**. (2009)

C. RESEARCH SUPPORT: None in the last three years.

2. SPECIFIC AIMS

Global industrialization and societal rearrangements brings a unique mix of organisms together. This new intermingling of microbes and eukaryotic species (especially of mammalian and plant kingdoms) not previously in contact has a huge potential to bring about new diseases and afflictions to human kind. These can be direct, or indirect - by affecting agricultural and other essential resources. This, understandably, is even more so in environments and societies with inadequate hygiene – where even some commonly innocuous interactions or in some cases typically beneficial associations can turn detrimental. Migration of humans and goods between different communities, and natural migration (e.g., seasonal migration of birds, microbe carrying vectors) leads to the spread of newly adapted and emerging disease-causing microbes to other developed communities. The latter communities would be as vulnerable as less developed and resource depleted societies for different reasons - such as lack of previous exposure, or sudden exposure to an adapted microbe, now which can be classified a pathogen or an opportunistic pathogen. Another common situation where such maladaptations can occur is during microbial interactions with vulnerable (e.g., immune compromised) hosts.

Here it is hypothesized that new diseases can emerge rapidly through transient host-microbe adaptations involving changes in system status (biochemical and signaling state changes) under appropriate environments and continued ability to interact. At least some zoonotic diseases and nosocomial infections likely arose, and continue to do so, before the establishment as a new microbe related disease. The sequencing of genomes of humans, other eukaryotes, and numerous microbes, technologies that enable measurement of specific biomolecular/molecular components of a cell or an organism, and the integration of these data with other bodies of knowledge allows us to predict key components and states leading to such adaptations.

It is difficult to study this proposed mechanism of disease emergence in the relevant environments. Further, it could take too long to make inferences from natural occurrences to be of relevance for the development of rapid ways of identification and prevention of evolution of new host-pathogen adaptations that could lead to this kind of phenomena. In addition, it is difficult to evaluate the host and microbe signaling modules or biological components identified as possible candidates. ***A model metasystem has been established using seedlings of the model host Arabidopsis interacting with different well studied microbes with many unique properties that make it suitable for rapid study of such maladaptations (Gopalan and Ausubel, 2008).*** By studying this model host, some model mammalian and agricultural pathogens, and commonly studied innocuous microbes interacting with the model host under similar conditions, one should be able to make inferences of broader consequences viz., detecting early adaptations of direct significance to human beings and their environment and possibly even make inferences on the extent such new adaptation has progressed. The use of a model host and ubiquitously used microbes should provide an excellent framework to build and test these new theories on. Other advantages of this model are its amenability for use in high throughput automation assisted perturbation screens and real time study of signaling cross talk.

The specific aims of this proposal to test these hypotheses are:

1) Gain additional biological insight via the study of progression of host damage and changes (cellular and physiological) in the host and microbes in the Arabidopsis model metasystem that represent different strategies of interaction/adaptation.

2) **2A.** Generate data on this metasystem using different technologies at a system-wide-scale (of both the host and the microbe) to aid in the inference of key indicator components of system status. **2B.** Infer system status and causal components using the data generated and the knowledge of the host-microbe interactions using integrative biological and computational tools, and

3) Prove the validity of the hypotheses generation work flow by removal and addition of specific component(s) and pathways to the host and/or microbe based on the hypotheses generated.

3. BACKGROUND AND SIGNIFICANCE

As mentioned earlier, an unintended consequence of industrialization and associated societal rearrangements is increased opportunities for artificial mingling of host and microbes previously not in contact. This new intermingling of host and microbe combinations has a huge potential to evolve as microbe associated diseases capable of causing damage to human and agricultural resources. This is even more so in societies with inadequate hygiene and poor resources to tackle them. Another situation is the interaction of commonly innocuous and even beneficial microbes interacting in an environment that alters host and pathogen local environments such that they now become detrimental. Global industrialization also has facilitated rapid migration of people and goods between different societies, thus migration of the transiently adapted microbes. Other societies can be vulnerable to some of these newly and (often transiently) adapted microbes due to lack of previous exposure. Continued availability of a niche for these microbes can evolve into opportunistic or well adapted serious diseases. Some environment-induced diseases of the nature mentioned above, zoonotic diseases, and nosocomial infections, likely evolved and continue to do so in this manner. In these scenarios, the initial stages of adaptation likely involve system status changes (defined as biochemical and signaling state changes) in host and microbe. Continued adaptation will likely lead to rapid acquisition of addition virulence related traits and antibiotic resistance through lateral genetic transfer that is very common in the microbial world (Finlay and Falkow, 1997).

Host-microbe interactions represent an intricate balance between many aspects that control host immunity/susceptibility and the microbe's genetic content (broadly termed pathogenicity determinants) and its adaptability to survive as well as to manipulate/overcome host defenses. The typical broadly classified outcomes are (i) thwarted interaction where either the pathogen does not have appropriate metabolic or pathogenic arsenal to survive in a particular host or cannot overcome the initial host innate immune defense responses, respectively, (ii) some kind of symbiotic interaction where either the host and the microbe establishes a productive interaction (e.g., rhizhobium-plant interactions, gut microbiome etc.) or creates a niche where both have sufficient control over the other to prevent any damage of consequence (e.g., tolerance – see Schneider and Ayres, 2008), and (iii) establish a maladaptation where host immunity is ineffective or actively subverted by the pathogenic arsenal and sometimes host processes are utilized to favor microbial growth causing significant host damage. The latter is a typical disease situation. This aspect of microbial pathogenesis from the host damage perspective is reviewed in Pirofski and Casadevall (2008). All three modes are actively and extensively studied in exquisite detail using specialized systems and numerous mechanisms specific to different classes of interactions have been identified.

A major affliction of humans (HIV) is accepted to have adapted from primates. The frequent emergence of new strains of influenza (e.g. the avian influenza strain H5N1 and the recent case of H1N1) is another example. In these cases the effects of social and environmental factors are quite apparent (e.g., Fauci, 2006). The following text is mostly focused on host-bacterial interactions as examples, due to the direct relevance to the proposed goals. Even in the case of a natural setting like the human microbiome, at different locations of the body there are a different mix of microbes (Dethlefsen et. al., 2007). The same microbes while well adapted and controlled and which may even perform essential functions through the delicate balance of a large number of features of host and different microbes in the mix, like in mammalian gut, can cause disease accompanied by host damage at another niche in the same host. The other common example is the often encountered food poisoning by *E. coli* and *Salmonella* where the environment temporarily provides an existence niche for these microbes. A similar situation is not difficult to envisage in environment-induced emergence of new microbial diseases or maladaptations. A prominent example is the emergence and persistence of nosocomial infections where microbes (like *Candida* sp. and *P. aeruginosa*) adapt to form and survive in biofilms in catheters in hospital settings and the availability of susceptible/vulnerable hosts in the form of immune compromised patients (e.g., Lynch and Robertson, 2008). The adaptation of microbes to new hosts, the failure to eradicate them, and continued availability of an appropriate host niche, could lead to permanent fixation. Such emergence of opportunistic pathogens pose a serious threat and can have a significant heath care and economic impact (e.g., methicillin resistant *Staphylococcus aureus*, MRSA).

Host-microbe recognition and coevolution

Typical studies on host-pathogen interactions can be broadly classified into three broad categories: (i) the study of mode of action of specific virulence factors and toxins, (ii) studies of innate immunity using PAMPs (pathogen associate molecular patterns) and MAMPs (microbe associated molecular patterns to elicit host responses, and (iii) use of whole microbes/pathogens while adding to complexity can highlight mechanisms occurring at the organismal levels of host and microbe. These approaches led to the development of PRRs - pattern recognition receptors (Janeway, 1989; Janeway and Medzitov, 2002). Some well studied PRRs include toll like receptors (TLRs) and NOD like receptors (NLRs) both of which are characterized by the presence of leucine rich repeats (LRRs) and use different class of adaptors (CARD domain containing adaptors and TIR domain containing adaptors, respectively – (Akira et al. 2006; Fritz et al. 2006; Meylan et al. 2006; Beutler et al. 2007). These receptors recognize a variety of pathogen components including proteinaceous molecules, lipopolysaccharides, toxins of bacteria, viral proteins and CpG DNA of invading bacteria and single and dsRNA of viruses. The recognition by PRRs is usually accompanied by one or more of the following events: engagement of a variety of adaptor molecules, kinase cascades, followed by convergence of many different pathways onto one or few transcriptional regulators (e.g., NF-KB, IRFs) that trigger a variety of immune related functions. The third approach ranges from the study of microbial communities in hosts, niche effects on microbiome and ecological and evolutionary principles of host-microbe interactions that focus on the second stage of adaptation - genetic fixation, e.g., genome wide association studies (Backhead et. al., 2005; Dethlefsen et. al., 2007; Quintana-Murci et. al., 2007; Pamer 2007) to study of specific aspects of interactions of single host and microbe. The study of classically defined adaptive immunity in mammals (often involving memory based antibody mediated immunity) is not discussed here.

On the other hand the ability of microbes to suppress or subvert host innate immunity has been recognized for a long time (Schwab, 1975). A major example is the conservation of type III secretion system in many gram-negative microbes that play a key role in pathogenesis of a variety of hosts in different kingdom (Galan and Collmer, 1999). Number of proteins directly delivered into the host by this secretion system target key signaling and other host processes. Many pathogens have evolved a suite of effectors that affect many aspect of host immunity and homeostasis [e.g., (Bhavsar et al. 2007; Roy and Mocarski 2007) for some recent reviews on this topic]. There have been references to environment-induced adaptations, changes in signals and outputs and less often the impact of the environment on metabolic status as it pertains to modifying the outcome of a host-microbe interaction. This study posits that early stages of host-microbe adaptations that can potentially convert into maladaptation is brought about by rewiring and altered regulation using existing cellular machinery (without need for hard-wired genetic change at that juncture). This has remained one of the least studied aspects of host-microbe interactions, in part due to lack of realization and good systems for study.

Prominent role for model systems in the study of host-microbe interactions

The study of host-microbe interactions and their various intricate machineries has been aided by numerous models including cell culture systems, vertebrate models like mouse, emerging models like zebra fish (*Danio rerio*) and a number of invertebrate models and even single cell organisms like yeast. Cell culture based systems offer advantages of directly derived from the host of interest often human and other mammals, very high throughput when a good phenotypic or reporter readout for a phenomena or a pathway are available. An aspect of direct relevance to this proposal is the successful use of invertebrate models to discern microbial virulence factors and mechanisms and host immune response pathways. This has often yielded remarkable success in highlighting pathways, mode of action, and components or modular structures conserved across different kingdoms. These whole organism models offer advantages of study of non-cell autonomous processes, together with number of tools to rapidly test hypotheses and gene functions.

Indeed, the toll gene was cloned in Drosophila as a gene required for dorso-ventral patterning, and subsequently shown to play a role immunity (Hashimoto et al. 1988; Lemaitre et al. 1996). Some background in plants and *C. elegans* relevant to this proposal is outlined below. Since their first cloning from *Arabidopsis* (Mindrinos et. al., 1993; Bent et. al., 1993) it has been realized that plants have a large family of LRR containing proteins identified as genetically recognizing specific and general components of a variety of pathogens, e.g., parts of bacterial flagellin and elongation factor Tu (Gomez-Gomez and Boller 2000; Zipfel et

al. 2006). For example the LRR containing receptor recognizing flagellin (FLS2) is structurally and functionally similar to recognition and response to a different region of flagella by TLR5 in mammals. Consistent with the intracellular presence of many of the LRR containing receptors recognizing specific bacterial components, it has been shown that the corresponding bacterial effector proteins are also functional inside the plant cell - as initially demonstrated for the effector protein AvrB – (Gopalan et al. 1996), delivered into the host by the type III secretion apparatus that is present in most gram-negative pathogens (reviewed in Galan and Collmer 1999). Other major classes of pathogen recognition molecules cloned in plants include kinases (Martin et al. 1993) and enzymes that detoxify toxins of pathogens (Johal and Briggs 1992; Scott-Craig et al. 1992). In both plants and *C. elegans* MAP kinase cascades (Ligterink et al. 1997; Zhang and Klessig 2001; Asai et al. 2002; Kim et al. 2002), cell death (Klement 1964; Aballay and Ausubel 2001) and transcription factors that trigger immunity (in case of plants, a protein NPR1 that shares many features of NFKB – (Cao et al. 1997; Ryals et al. 1997), and in the case of *C. elegans* a transcription factor DAF-16 that is downstream of, and is negatively regulated by the insulin-like receptor DAF2 (Garsin et al. 2003) that also controls longevity have been identified. It is likely that both these models will highlight different components and conceptually similar modules that are conserved across different kingdom, including mammals.

Like in other kingdoms the type III secretion system and effectors target key host processes in model plants (recently reviewed in Boller and He, 2009). In addition microbial pathogens of plants also makes hormones and host hormone mimicks thus modulating host processes that alter the outcome of host-microbe interactions (recently reviewed in Grant and Jones, 2009). The structural relatedness of components and mechanisms raise the question if these conceptually similar arms in different kingdoms arose independently or through convergent evolution (Ausubel 2005).

Translating knowledge from model organisms to other higher organisms of direct relevance

As mentioned the advances in genome sequencing and other technologies for measuring system-wide levels (and changes) in cellular components have brought about a large shift in the way biological systems are studied. This shift, in principle, enables studying cellular signaling and metabolic processes and their regulation and alterations under specific conditions in a unified and often unbiased manner, often enabled by powerful reverse genetics tools (e.g., RNAi, gene knockouts). Together with the use of this information (e.g., information of predicted orthologs – e.g., COGS, Inparanoid (Sayers et. al., 2009; Berglund et. al., 2008), prediction of coregulated modules (Eisen et. al., 1998) and *de novo* predicted and curated upstream elements e.g., TRANSFAC (Matys et. al., 2006) and technologies (transcriptome, proteome, interactome, metabolome etc.) a number of computational and bioinformatic tools have evolved, including curated databases of molecular, phenotypic and metabolic functions of cellular components (e.g., GO, KEGG, BioCyc/Metacyc – Ashburner et. al., 2000; Kanehisa, 2008; Caspi et. al., 2008). This systems approach is further aided by tools to integrate network models, visual representations (e.g., Cytoscape – Cline et. al., 2007) enable (i) inferences of cross-talk and rewiring between previously thought to be linear chain of pathways (canonical pathways), (ii) identification of new modules, (iii) new functions for genes and components previously known for different functions in the context where they were first identified, and (iv) annotation of functions of biological components of previously unknown functions. In addition ortholog information enable the prediction and extension of inference obtained from a simpler organism to more complex organism or conversely to test the inference from a more complex organism using simpler models with more tools for that purpose.

A model metasystem to study environment induced and emerging host-microbe adaptations

Environment-induced host-microbe adaptations and prediction of emergence of microbe related diseases through the study of system status during such interactions is a key goal of this proposal. The above background sets the stage to indicate the feasibility based on accomplished biological studies of this nature and the constantly emerging technological, computational and visualization tools. As mentioned earlier detection of such environments and study of emergence in natural settings and testing of any developed hypotheses are extremely difficult. Thus a metasystem comprised of model organisms (the host *Arabidopsis thaliana*) and well studied microbes representing different classes of microbes and different modes of interactions has been developed to aid rapid study of such phenomena. In the conditions of this metasystem of interaction well studied microbes including human opportunistic pathogens, agricultural pathogens and

commonly considered innocuous microbes (*E. coli* and *B. subtilis*) cause characteristic host damage in each case. The preliminary study outlines a number of lines of evidence indicating that these combinations of hosts and microbes represent different modes of interaction and involve active contribution of host, microbe and the interaction environment. Earlier it had been shown that in certain assays *E. coli* and *B. subtilis* can elicit characteristic immune gene expression responses in Drosophila (Lemaitre et. al., 1997) and involvement of active defense for survival of infection by commonly non-pathogenic *E. coli* (Naitza et. al., 2002). *B. subtilis* is also highly pathogenic to Drosophila (Tzou et. al., 2002). While those studies highlight that these commonly considered non-pathogenic microbes can also infect other hosts also under appropriate conditions, a significant advantage of the system developed here is amenability to high-throughput automation assisted screens for components that perturb these interactions (e.g., genetic and chemical compound screens).

Thus the study of this model metasystem during the progression of different modes of host microbe interactions should contribute to build a knowledge framework of system status adaptations, and signaling and metabolic pathway alterations. This knowledge framework should aid prediction of emergence of new adaptations in nature and environments favoring them. In addition, it might also be possible to predict the extent of adaptations from this knowledge base.

4. PRELIMINARY RESULTS

As highlighted in the previous section, the evolution of new host-microbe adaptations in natural settings are difficult to identify and difficult to study. Further, when environments, cellular components or pathways are identified as potentially causal, it will be difficult to study and prove a causal relationship. Thus a model system that comprises different modes of interaction would allow us to predict system status during initial stages of adaptation that could potentially lead to maldaptation (a microbe related disease). Here a metasystem of framework model organisms has been set up using seedlings of the model plant Arabidopsis as host interacting with a number of microbes including the human opportunistic pathogen (and a multi-host pahogen) *Pseudomonas aeruginosa*, a plant pathogen *Pseudomonas syringae* pv. *tomato*, and two commonly innocuous microbes that are laboratory work horses, *Escherichia coli* and *Bacillus subtilis*. Importantly, under the conditions of these interactions even the commonly considered innocuous microbes cause host damage. The setting up of the metasystem, data demonstrating that they represent different modes of interaction, and other intriguing properties that should enable use of this system to infer broad principles of environment induced host-microbe adaptations are described below. Some results are also included concerning Arabidopsis seedlings interacting with the *Xanthomonas campestris* pv. *campestris* (Xcc) or *Xanthomonas campestris* pv. *raphani* (Xcr), which represent susceptible and resistant reactions, respectively (Parker et. al., 1993). Different species of *Xanthomonas* affect a variety of plants including many vegetables, fruits, rice and cotton. Finally, also described are results obtained using the laboratory model strain NCTC8325 of the human opportunistic pathogen *Staphylococcus aureus*. Much of the published information (Gopalan and Ausubel, 2008) has been reproduced as such.

A variety of microbes can infect Arabidopsis seedlings under appropriate conditions (host damage and seedling growth inhibition as readouts)

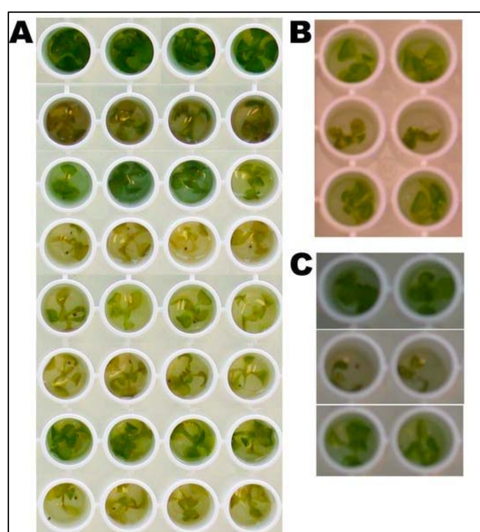


Fig. 1 Visual phenotype of seedlings infected with different microbes. Seedlings grown in 96 well plates (one per well) were infected with indicated microbes and symptoms recorded (**A**) 5dpi, (**B**) 3dpi and (**C**) 4dpi. (**A**) the different rows display seedlings 1. untreated, or treated with 2. *P. aeruginosa* -PA14, 3. PA14::*lasR*, 4. *B. subtilis*, 5. *E. coli* (Dh5a), 6. DC3000, 7. DC3000::*hrcC*, 8. DC3000(pAvrB). (**B**) rows: top - untreated control, middle - *E.coli*, 3. bottom - *E.coli* expressing GFP in a plasmid. (**C**) rows: top – untreated control, middle - *B. subtilis*, bottom - *Staphylococcus aureus*.

When 10-day old Arabidopsis seedlings growing submerged in liquid plant growth medium in 96 well microtiter plates are infected with the plant pathogen *Pseudomonas syringae* pv. *tomato* (DC3000) or the multi-host opportunistic human pathogen *Pseudomonas aeruginosa* (PA14) the seedlings developed chlorotic disease symptoms and exhibited growth arrest (Fig. 1A). Surprisingly, the gram-negative and gram-positive laboratory microbes *E. coli* and *B. subtilis*, respectively, also inflicted damage and caused growth arrest of Arabidopsis seedlings under these conditions (Fig. 1A, Fig. 1B and Fig. 1C). The pathogenic behavior of DC3000 and PA14 was not surprising - PA14 had earlier been shown to infect a variety of invertebrate hosts,

Table 1 Growth of different microbes in the plant growth medium and in the presence of seedlings.

	day 0	day 3		
microbe		medium	conditioned medium	whole well
PA14	5.38	6.9 SD 0.27	7.7 SD 0.39	> 9.5
PA14::*lasR*	5.30	ND	ND	> 9.5
B.subtilis	4.00	4.4 SD 0.5	5.6 SD 0.16	6.6 SD 0.18
E. coli	5.62	6.3 SD 0.15	5.35 SD 0.13	7.7 SD 0.3
DC3000	4.84	6.9 SD 0.02	5.04 SD 0.35	> 9.5
DC3000/AvrB	4.70	ND	ND	> 9.5
DC3000::*hrcC*	4.84	ND	ND	> 9.5

Indicated are the growth (log_{10}CFU) of the different microbes in the plant growth medium (MS), in the conditioned medium (i.e., seedlings were removed at the time of addition of microbes), or in the presence of seedlings, 3 dpi.

including plants, in addition to mammals (Rahme et. al., 1995; Tan et. al., 1999; Mahajan-Miklos et. al., 2000). But the fact that laboratory microbes generally considered to be nonpathogenic can affect

seedlings similarly to known pathogens was not expected and provided an opportunity to study host-microbe maladaptations in a controlled environment. In particular, *B. subtilis* was especially potent in inflicting damage, such that in three days the seedlings were quite bleached and there was a significant loss of tissue integrity (Fig.7B and Fig. 1A).

Importantly, under the conditions of these assays, the microbes do not grow significantly in the plant growth medium, but grow well in the presence of seedlings, indicating that active host-microbe interaction contributes to microbial growth and host damage (Table 1 and Songnuan et. al., 2008). In contrast, seedlings treated with the extensively studied laboratory model strain NCTC8325 of the human opportunistic gram-positive pathogen *Staphylococcus aureus* (Iandolo et. al., 2002), elicited very minor disease symptoms, as evidenced by visibly robust seedlings with no sign of reduction in size or degreening (Fig. 1C).

In contrast to submerged seedlings, 10 day old seedlings growing on soil were not susceptible when sprayed with *B. subtilis* or *E. coli*, whereas DC3000 caused potent chlorosis and stunting (Fig. 2 and data not shown). ***This property was used in a screen to identify Arabidopsis mutants impaired in susceptibility and several have been identified. The characterization of these mutants are not part of this proposal.*** Unlike seedlings, leaves of adult Arabidopsis plants infiltrated with *B. subtilis* did show tissue damage limited to the infiltrated area, indicating effective restriction of this microbe to the infiltrated area. This damage was characteristically different, confined (Fig. 3) and slower compared to the typical spreading chlorotic symptoms of DC3000 at similar time points (data not shown). No damage was apparent when adult leaves were infiltrated with *E. coli*. ***These data indicate that the characteristics of the environment of interaction in these assay conditions alter the host and/or microbial system status to enable the observed host damage and microbial growth of even commonly considered innocuous microbes.***

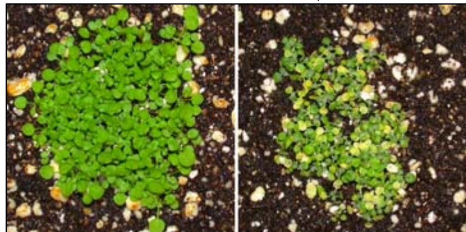


Fig. 2 Symptom development after treatment of soil grown Arabidopsis seedlings treated with *P. syringae* pv. *tomato* strain DC3000. The seedlings were sprayed with water (left panel) or DC3000 (right panel) at 10 and 12 days and symptom recorded 5 days after second spray. Seedlings treated similarly with *P. aeruginosa* PA14, *B. subtilis* or *E. coli* did not show any symptom.

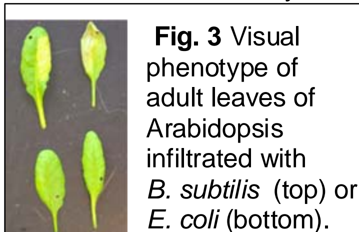


Fig. 3 Visual phenotype of adult leaves of Arabidopsis infiltrated with *B. subtilis* (top) or *E. coli* (bottom). Leaves of 4 week old soil grown plants were infiltrated with bacteria at ABS_{600nm} of 0.02 and symptoms recorded 4 dpi.

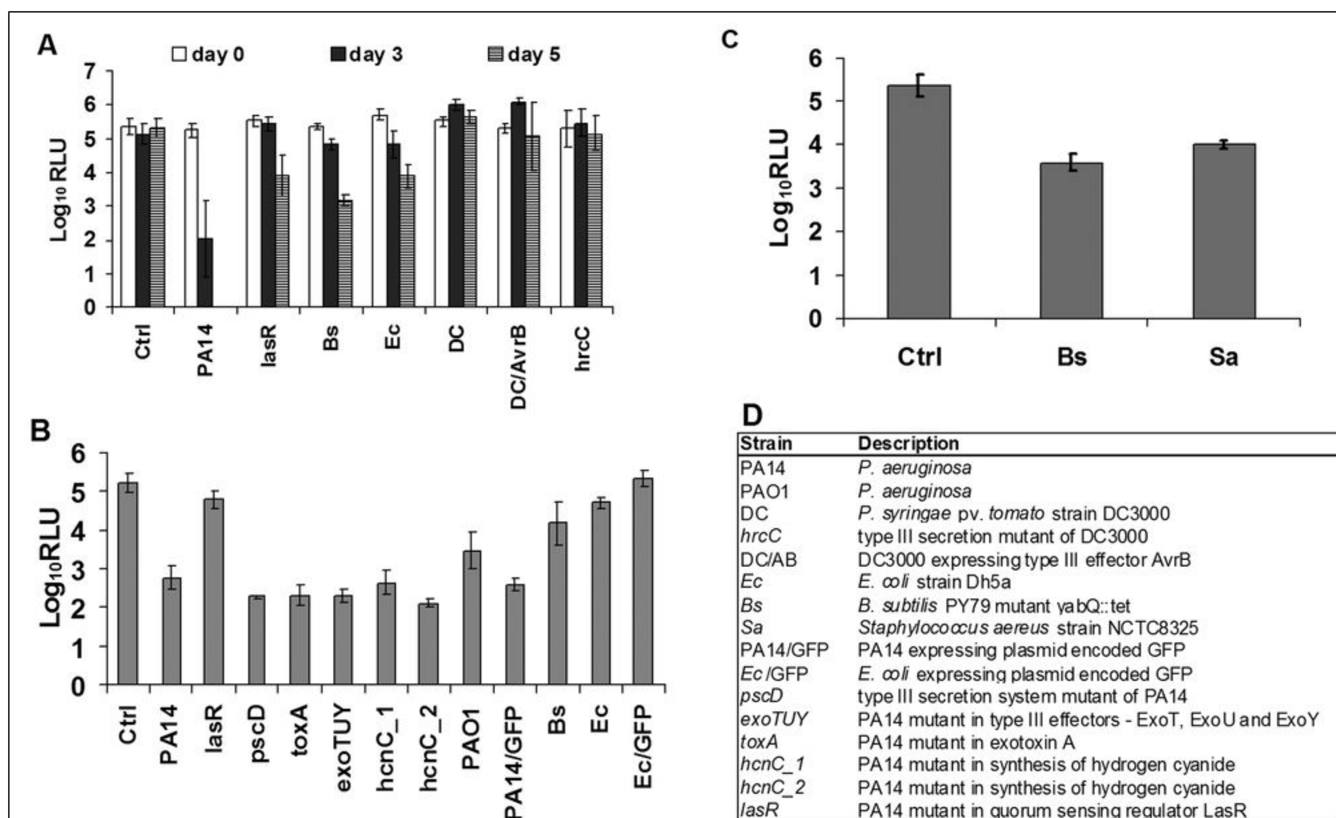
Supporting the conclusion that the interactions between Arabidopsis seedlings and the two known plant pathogens in the seedling assay emulate well-studied aspects of host-pathogen interactions are the observations that the bacterial mutants PA14::*lasR* and DC3000::*hrcC*, which are impaired in the synthesis or delivery of a subset of virulence factors, respectively, were attenuated in their ability to inflict host damage in the submerged seedling assay. LasR is a key regulatory factor controlling the quorum sensing response in *P. aeruginosa* (Gray et. al., 1994), which in turn affects the production of a variety of virulence factors and has been shown to affect the virulence of PA14 in adult plants (Rahme et. al., 1995), and other hosts (Tan et. al., 1999). *P. syringae* HrcC is a key structural component of the type III secretion system conserved in a wide range of gram negative pathogens, that delivers a suite of virulence effectors directly into host cells (Galan and Collmer, 1999). These data, the data in Table 1 on microbial load during the different interactions tested, and other data presented below also support the notion that the damage is not simply correlated to microbial load. Even in the case of *E. coli* mediated host damage and seedling stunting this seemed to be the case, as an *E. coli* strain Dh5α harboring a plasmid expressing GFP was much less effective in inflicting host damage (Fig. 1B). In contrast, when the GFP expressing plasmid was transformed into a different DH5α background, the reconstructed GFP expressing strain was just as virulent as wild-type DH5α. This result implies that the original GFP-expressing strain had likely accumulated a genetic change further supporting the conclusion that the ***host damage caused by Dh5α in this system involves an active host-microbe interaction***. In addition, PA14 with GFP expressed from a plasmid, elicited equivalent visual symptoms as PA14 (not shown).

The assays described here have been conducted under two different conditions, either high fluence 75 uE (typical) or low fluence 40 uE (Fig. 1A and Fig. 4A), with the rate of appearance of nearly all described phenotypes more rapid under high fluence conditions. ***In addition assays done with a 10 fold lower initial***

microbial inoculum or a 100 fold higher inoculum did not make a substantive difference in most of the outcome reported here, thus highlighting the robustness of the assays.

A quantitative high-throughput assay for host damage based on the constitutively expressed luciferase activity reveals interaction specific features

In order to have a high-throughput quantitative measure of host damage, we used the activity of a constitutively expressed transgenic luciferase gene (Subramanian et. al. 2006) – under the control of a 35S promoter- as a surrogate of plant health. As can be seen in Fig. 4A, PA14 effectively shuts down the luciferase activity and the activity was completely abrogated by 5 dpi, whereas a PA14::*lasR* mutant only had a modest effect on luciferase activity during this time period. In contrast to PA14, and despite the disease symptoms shown in Fig. 1A, there was no reduction in luciferase activity in seedlings infected with *P. syringae* DC3000. Similar results were obtained with DC3000::*hrcC*, or DC3000 expressing the avirulence gene product AvrB (a type III effector that elicits a potent and rapid cell death response when recognized inside infected cells accompanied by strong resistance, when recognized by a cognate resistance (R) gene product of the host (Dangl and Jones, 2001; Gopalan et. al., 2006) – Fig. 4A. ***The fact that the highly adapted plant pathogen DC3000 does not cause a reduction in luciferase activity seems counterintuitive, but is consistent with the fact that it is generally considered to be a biotrophic pathogen that depends on its host for nutrition.*** Biotrophs like DC3000 cause slow host damage to maximize the amount of host-derived nutrition. *B. subtilis* was more effective than E. coli in the ability to reduce the luciferase activity, and both were intermediate between PA14 and DC3000. But the fact that DC3000 expressing AvrB, that is expected to elicit an HR and kill the seedlings did not abrogate the luciferase activity in the seedlings and did not seem to reduce the bacterial load significantly (Table 1), is unexpected and highlights unique aspects of this system (discussed later).



Strain	Description
PA14	*P. aeruginosa*
PAO1	*P. aeruginosa*
DC	*P. syringae* pv. *tomato* strain DC3000
hrcC	type III secretion mutant of DC3000
DC/AB	DC3000 expressing type III effector AvrB
Ec	*E. coli* strain Dh5a
Bs	*B. subtilis* PY79 mutant yabQ::tet
Sa	*Staphylococcus aereus* strain NCTC8325
PA14/GFP	PA14 expressing plasmid encoded GFP
Ec /GFP	*E. coli* expressing plasmid encoded GFP
pscD	type III secretion system mutant of PA14
exoTUY	PA14 mutant in type III effectors - ExoT, ExoU and ExoY
toxA	PA14 mutant in exotoxin A
hcnC_1	PA14 mutant in synthesis of hydrogen cyanide
hcnC_2	PA14 mutant in synthesis of hydrogen cyanide
lasR	PA14 mutant in quorum sensing regulator LasR

Fig. 4 Activity of seedlings engineered to constitutively expressed luciferase after infection with different microbes.
(**A**) The seedlings were infected with microbes as in Fig. 1 and the luciferase activity were measured on the same seedlings before and after addition of microbes (Panel **D**) at indicated time points. (**B**) Seedlings were infected with different microbes or different mutants of *P. aeruginosa* strain PA14 (Panel **D**) and luciferase activity measured 2 dpi. (**C**) Seedlings were infected with *B. subtilis* or *S. aureus* and luciferase activity measured 3 dpi. Ctrl is control uninfected seedlings. Shown are log10 relative luminescence units. (**D**) List of strains used in the luciferase assays.

As was observed earlier, the *E. coli* strain expressing GFP in a plasmid (a possible mutant) also had reduced effect on reduction of luciferase activity (Fig. 4B). PA14 expressing GFP treated seedlings had similar luciferase activity compared to PA14. In contrast, though *S. aureus* did not cause any significant visual phenotype it did cause reduction in luciferase activity, though not as dramatic as PA14.

These data indicate that certain aspects of host-microbe interactions as evidenced by host damage can be studied using the luciferase activity assay in a highly automation assisted and extremely high-throughput manner. This should enable genetic and compound perturbation screens for host-microbe interactions and other studies of complex signaling interactions, which are not part of proposed goals of this application. The exceptions and their implications are discussed in other contexts below.

Evaluation of PA14 mutants in several known virulence factors or virulence regulators using the luciferase assay system reveals additional factors and regulators that can be revealed by this system

The same assay for abrogation of constitutive luciferase activity was used to test *P. aeruginosa* strain PAO1 (Holloway, 1955) and a number of mutants of PA14 affected in known *P. aeruginosa* virulence factors. PAO1 was less potent, than strain PA14. As shown in Fig. 4B, and in contrast to the *lasR* mutant, none of the other tested PA14 mutants were significantly affected in their ability to abrogate the luciferase activity of the seedlings compared to PA14 wild type. Similarly, none of these mutants elicited diminished visual symptoms (not shown). Among the mutants tested were a type III secretion mutant (*pscD*), mutants in three different type III virulence effectors (ExoT, ExoU, and ExoY), an exoTUY triple mutant, a mutant in a secreted exotoxin (*toxA*), and two independent mutants in a gene involved in the production of toxic hydrogen cyanide (*hcnC*). The type III secretion system and the three effectors tested were previously shown to be required for full virulence in insects and mammals (Miyata et. al., 2003), but not in *C. elegans* or adult Arabidopsis plants. *hcnC* is a key mediator of toxin-mediated killing in *C. elegans* (Gallagher and Manoil, 2001). ToxA, despite having a minor role in virulence in adult Arabidopsis plants and in a burned mouse model (Rahme et. al., 1995), does not have any effect in this system.

GacA is considered a master regulator upstream of the quorum sensing system (including LasR) and has been shown to regulate a number of virulence factors in other conditions (Heurlier et. al., 2004; Goodman et. al., 2009). Surprisingly, a *gacA* mutant that is impaired to some extent in adult plant assays (Rahme et. al., 1995) and almost completely impaired in the *C. elegans* slow killing system (Tan et.al., 1999) did not show any significant difference from PA14 by visual phenotype or host luciferase activity assay (data not shown).

These data together indicate that the seedling infection system while preserving some well-established aspects of host-pathogen interactions observed in other pathosystems, is unique in some aspects. Thus, particular features of this infection system fundamentally modifies host and pathogen status such that maladaptive interactions of some microbes that would not normally be initiated are favored. This also highlights the potential to identify novel virulence factors, virulence mechanisms, and virulence regulatory mechanisms that can be highlighted by features characteristic to this system.

Histopathogical analysis of interaction of seedlings with different microbes further indicate different modes of host-microbe interactions

To test if the four different model microbes (*P. aeruginosa, P. syringae, E. coli, B.subitilis*) cause similar histopathological damage in Arabidopsis seedlings, a cell impermeable nucleic acid dye Sytox Green (SG) was used to probe the different interactions. SG enters cells with compromised membrane integrity and fluoresces when interacting with double stranded nucleic acids. Fig. 5 shows green fluorescence due to SG staining of permeabilized cells of Arabidopsis seedlings during interaction with the four different bacterial strains. The control seedlings are bigger and do not exhibit SG staining, whereas the infected seedlings show staining to varying extents. Of particular interest is the fact that seedlings interacting with *B. subtilis* are transparent, indicative of extensive damage and loss of tissue integrity. A slightly higher magnification using wide angle microscopy (Fig. 6) showed a characteristic circular staining pattern on the leaves of seedlings interacting with PA14, more pervasive staining during interaction with PA14::*lasR*, uniform staining of seedlings interacting with DC3000, a large punctuate staining pattern (what appears to be clusters of membrane permeable cells) in

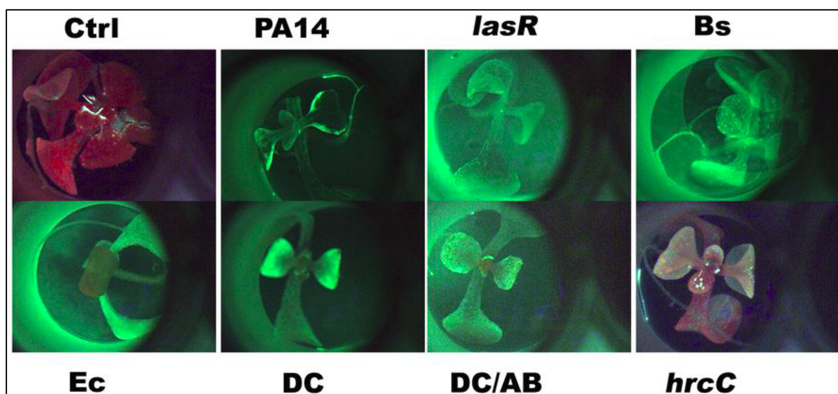


Fig. 5 Wide angle micrographs of membrane permeabilization in seedlings infected with different microbes.
Wide angle microscopy of seedlings sytox green staining in green fluorescence channel. Seedlings were stained 3 dpi with membrane impermeable dye Sytox green (SG) and imaged in transparent bottom wells of 96 well plates.

seedlings treated with DC3000 expressing AvrB, extremely bright fluorescence in seedlings interacting with *B. subtilis* (indicating extensive damage), and bright fluorescence in patches in seedlings interacting with *E. coli* (Fig. 6). A higher magnification of leaves from seedlings interacting with DC3000 and DC3000 expressing AvrB and PA14 is shown in Fig. 7A. These results indicate that AvrB likely causes permeabilization (probably cell death) in clusters of cells compared to DC3000, despite the fact that in both cases the seedlings do not seem to lose significant luciferase activity. This indicates that while the characteristic cell death response expected of an Avr-R gene interaction is still active, the resistance response that normally accompanies the cell death is not effective. Previous studies have shown that submerged seedlings do express genes strongly associated with disease resistance (e.g., PR1) in response to treatment with host and microbe derived defense elicitors (oligogalacturonides and flagellin, respectively) (Denoux et. al., 2008). These data again emphasize the fact that characteristic interaction patterns of known host-pathogen interactions are active in the system (e.g., behavior of *lasR* and *hrcC* mutants, characteristic staining patterns of seedlings treated with different microbes) but the environment-mediated alterations of the host and microbe in the submerged seedling assay favors continued interaction which might otherwise be terminated or severely diminished (e.g., DC3000 expressing AvrB, *E. coli* and *B. subtilis*).

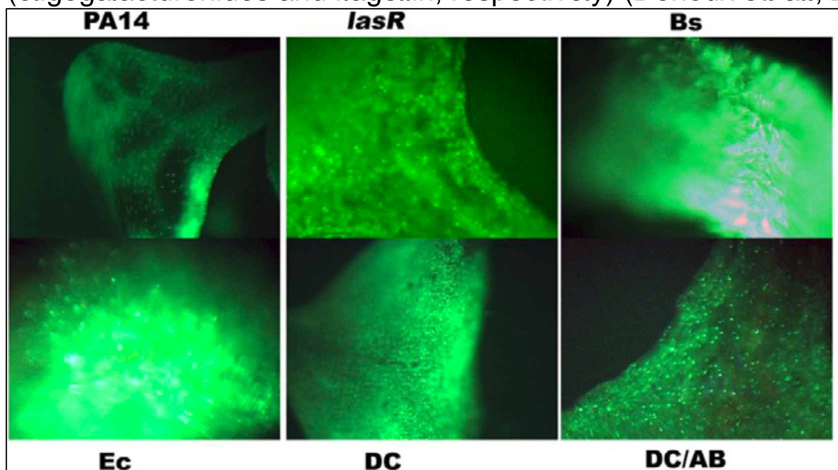


Fig. 6 Higher magnification wide angle micrographs of membrane permeabilization in seedlings infected with different microbes.
Wide angle microscopy of seedlings sytox green staining in green fluorescence channel. Seedlings were stained 3 dpi with membrane impermeable dye Sytox green (SG) and imaged in transparent bottom wells of 96 well plates.

The unusual staining patterns of leaves from seedlings infected with PA14 or *B. subtilis* were further examined at higher magnification (Fig. 5, Fig. 6, Fig. 7). In the case of seedlings interacting with *B. subtilis*, loss of tissue integrity was often seen as big gaps between cells in some locations, corroborating the visually severe damage and strong staining and transparent leaves observed at lower magnifications (Fig.5). Many other locations also showed an amorphous mass of cells indicative of loss of tissue integrity. The latter fact is highlighted in micrographs of a leaf from a seedling infected with *B. subtilis* stained with Syto 59, a dye that stains both intact and membrane permeabilized cells (Fig. 7B).

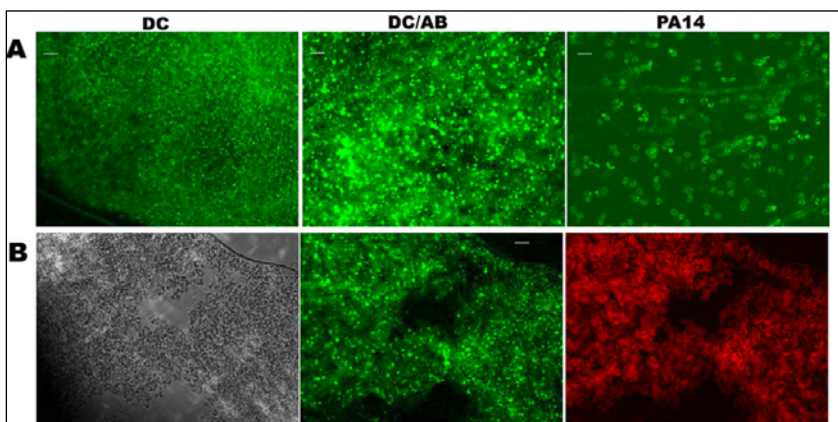


Fig. 7 Characteristic cellular damage in seedlings infected with different microbes.
(**A**) Seedlings were stained 3 dpi with membrane impermeable dye Sytox green after treatment with *P. syringae* pv. *tomato* DC3000 (DC), DC3000 expressing the avirulence protein AvrB (DC/AB), and *P. aeruginosa* PA14. (**B**) Seedlings were stained 3 dpi with membrane impermeable dye Sytox green or the membrane permeable dye Syto 59 after infection with *B. subtilis*, left to right: bright field, Sytox green staining, and Syto 59 staining. Scale bars indicate 50 µm.

Leaves of seedlings interacting with PA14 showed a very unique pattern of staining of many but not all stomata (Fig. 7A, Fig. 8

and Fig. 9B and consistent with Fig. 6). Interestingly, this staining was observed throughout the stomatal (guard) cells as opposed to punctuate nuclear staining or diffuse background expected of this class of stain. In order to rule out the possibility that permeant stomatal guard cells were uniformly staining because they had a change in cellular status (e.g., physiological status like redox) specific to SG that increased green fluorescence in the absence of nucleic acid binding, the seedlings were costained with another nucleic acid staining membrane impermeable dye, propidium iodide – PI

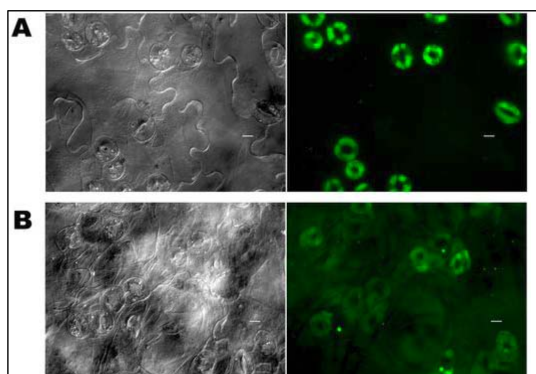


Fig. 8 Characteristic changes of seedlings infected with *P. aeruginosa* PA14 or a *lasR* mutant. Seedlings were stained 3 dpi with membrane impermeable dye Sytox green (SG) after infection with (**A**) *P. aeruginosa* (PA14), (**B**) PA14::*lasR*. Bright field image on left, sytox green fluorescence on the right. Scale bar indicates 10 μm.

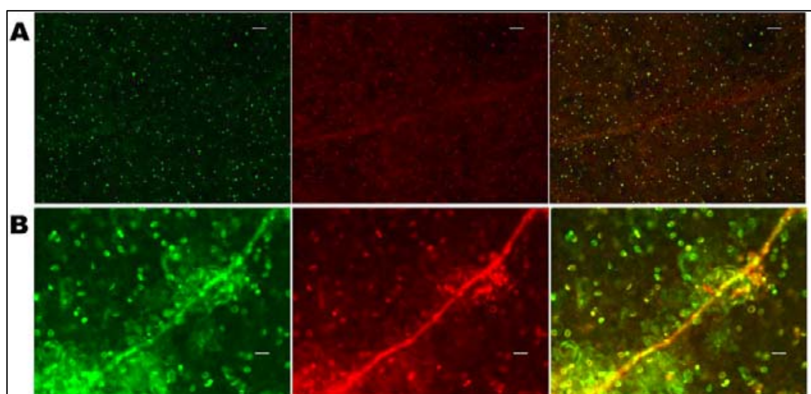


Fig. 9 Multiple dyes reveal the same characteristic changes of seedlings infected with. *P.aeruginosa* or *P. syringae* pv. *tomato*. Seedlings were stained 3 dpi with membrane impermeable dye Sytox green (SG) in combination with propidium iodide (PI) – another cell impermeant nucleic acid stain after infection with (**A**) *P. syringae* pv. *tomato* DC3000 (PA14), or (**B**) *P. aeruginosa*. Left: SG staining, middle: PI staining, and right: merge of both. Scale bar indicates 50 μm.

As shown in Fig.9A seedlings interacting with DC3000 showed staining consistent with the expected pattern of nuclear staining, and the fluorescence from the SG channel (green) and PI channel (red) overlapped. In the case of seedlings interacting with PA14 the staining was uniform over the stomatal (guard) cells and the staining in the SG and PI channels overlapped perfectly (Fig. 9B). These results indicate that the whole cell staining observed in many guard cells is not specific to SG. This raises the intriguing possibility that PA14 invades the stomatal guard cells, as both these dyes can stain permeabilized bacterial cells as well. Typically, bacterial pathogens of plants are intercellular. In the case of PA14 it has earlier been shown by scanning electron microscopy that some bacterial cells seem to punch holes through some plant cell types, and the plant cell walls become ruffled (Plotnikova et. al., 2000). However, the profile of bacterial cells (unstained and alive i.e., not stained by SG – as in Fig. 8A) as well as observations with GFP expressing PA14 provided no evidence for the presence of live or intact bacteria in guard cells. Similarly, there is no obvious evidence for intact bacteria inside the guard cells in electron micrographs (Fig. 10). In addition culture filtrates from PA14 grown in the presence of seedlings – in this system (3 dpi) – do not cause this stomatal guard cell staining or visual phenotype observed with seedlings infected with PA14 (data not shown). Such analysis was not carried out in the case of *B. subtilis*.

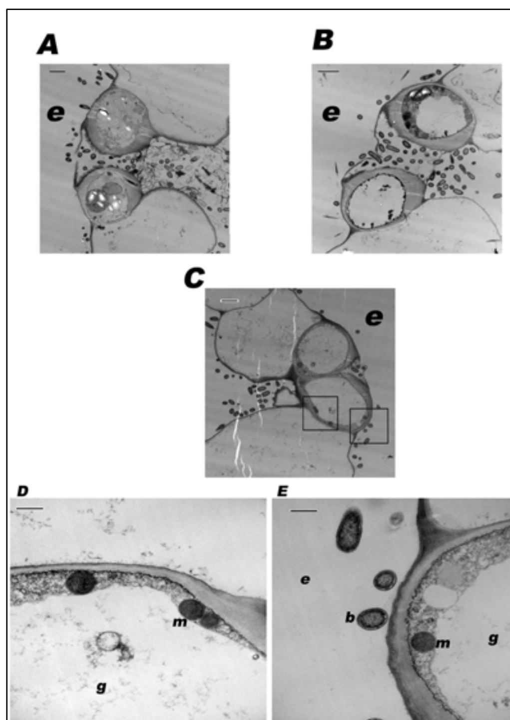


Fig. 10 Electron micrographs from liquid grown seedlings treated with *P. aeruginosa* PA14 do not show intact bacterial cells inside the guard cells.
Leaves of liquid grown seedlings treated with PA14 (on day 10) were subjected to transmission electron microscopy 3 dpi. Three pairs of guard cells from PA14 treated seedlings are shown (panels **A – C**), because many and not all guard cells show the characteristic staining phenotype upon infection with PA14. (**D**) and (**E**) indicate higher magnification of the regions indicated by squares on the left and right in panel (**C**), respectively. Representative mitochondria indicated by the letter **m** beside them, bacteria indicated by letter **b** beside it, regions outside the plant leaf surface are indicated by letter **e**, and guard cells indicated by letter **g** inside. Scale bars represent 2 μm in panels **A – C**, and 500 nm in panels **D** and **E**.

In the case of seedlings interacting with the PA14::*lasR* mutant, the staining was pervasive and diffuse in many cell types, but was less intense in the stomatal guard cells (Fig. 8B). This indicates that LasR plays a major role in the stomatal staining phenotype. It can also be inferred that virulence factors produced by PA14 in the presence of functional LasR incites rapid

and potent damage to the host. The *lasR* mutant is still able to elicit damage (despite attenuated) and causes stunting of seedlings that differs from wild type PA14 (Fig. 1A, Fig. 4, and Fig. 8B), indicating the presence of additional LasR independent factors that by themselves cause this partially impaired and phenotypically different damage (e.g., pervasive staining of many cell types compared to stomatal staining of PA14).

As indicated previously in terms of visual host damage and effect on host luciferase marker activity, the staining pattern characteristic of PA14 (and different from the PA14::*lasR* mutant) was maintained in the PA14::*gacA* mutant (data not shown). In addition a single point transcriptome done at 24 h post infection in this system (three independent replicates) reveals nearly identical expression pattern in PA14 and PA14::*gacA* mutant treated seedlings (data not shown). ***This reinforces the fact that in this system there is indeed a previously unrecognized GacA independent (but still maintaining LasR dependent phenotypes tested) regulatory circuit that can be elucidated using this system.***

Cell patterning defects in seedlings indicate hormonal and other intercellular signaling defect in this system

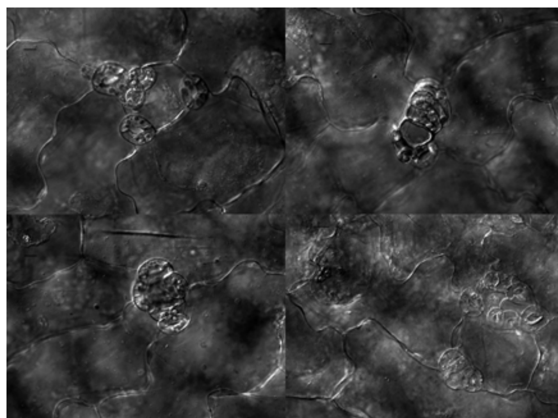


Fig. 11 Impaired guard cell patterning on leaves of liquid grown seedlings. Shown are three examples (DIC micrographs) of leaves from a 13 day old control untreated seedlings and a PA14 treated seedling, 3dpi infected on day 10, (bottom right) to highlight impairment in guard cell patterning and specification. All frames were taken at same magnification. Scale bar (upper left) represent 10 μm.

Support for the conclusion that the environment of the submerged seedling assay also alters the host in fundamental ways is the fact that often two and sometimes more guard cells could be observed in contact with each other (Fig. 5, Fig. 6A, Fig. 6B and Fig. 7B). This is the case in control (Fig. 11) as well as seedlings infected with various microbes, and violates fundamental rules of normal cell patterning in leaves. Normally, guard cells prevent their neighbors from attaining the same cell fate (Nadeau and Slack, 2002). A set of LRR containing receptor like kinases, a probable secreted protein ligand, members of a map kinase cascade and myb family transcription factors controlling this patterning and cell fate decision have been identified (Bergmann et. al., 2004; Hara et. al., 2007; Lai et. al., 2005; Shpak et. al., 2005). It should be noted that the signaling cascade module is remarkably similar in architecture to known signaling cascades in defense signaling. ***Thus, the patterning defect observed in this submerged seedling system indicates that while the seedlings look normal, overall hormone and other soluble signals or local concentrations that operate in cell-cell communication are impaired in this environment.*** Such signals that alter host-microbe interactions and defense pathways are likely responsible in part for the differences in responses observed in this system and probably operative in nature.

Additionally, this defect was not confined to the constitutive luciferase constructs used in above studies and is also observed in assays done in another batch of Col seedling.

The above data also clearly indicate the need for multiple read-outs in these analyses, as opposed to luciferase as a sole indicator used in many studies or the loss of chlorophyll used as a marker in one study *(Schreiber et. al., 2008)*.

A Jasmonate induced defense reporter suggest dysregulation of hormone signaling

Sytox green staining (Fig. 7A) and visual response (data not shown) do indicate more rapid cell death in the presence of the hypersensitive programmed cell death (HR) eliciting DC3000(AvrB) and in the case of the incompatible pathogen Xcr, that is also expected to elicit the HR. It is also known that the HR cell death response are operative as early as the cotyledon leaves that emerges out of the seeds, and probably even more potent in true leaves. This fact can be inferred from earlier experiments with transgenic expression of AvrB in Arabidopsis with appropriate recognition component R gene (Gopalan et. al., 1996). As mentioned

earlier it has been shown that PR1 and some other defense genes are induced in this assay system by pathogen and host derived defense elicitors (Denoux et. al., 2008). In that case, Flg22 - a peptide fragment derived from the flagella of bacteria was used as the bacterial MAMP (microbe associated molecular pattern). Two different conserved regions of flagella are recognized by LRR containing receptor kinases FLS2 (Zipfel et. al., 2004) and TLR5, in Arabidopsis and mammalian systems, respectively to elicit innate immune responses leading to certain basal level of host defense against pathogens. OG (oligogalacturonide) a plant cell wall derived elicitor thought to be generated by degradative enzymes of fungal pathogens was the other elicitor in that study. These data clearly indicate the capability of the system to elicit a defense response, but a change in the system status prevents execution of effective defense.

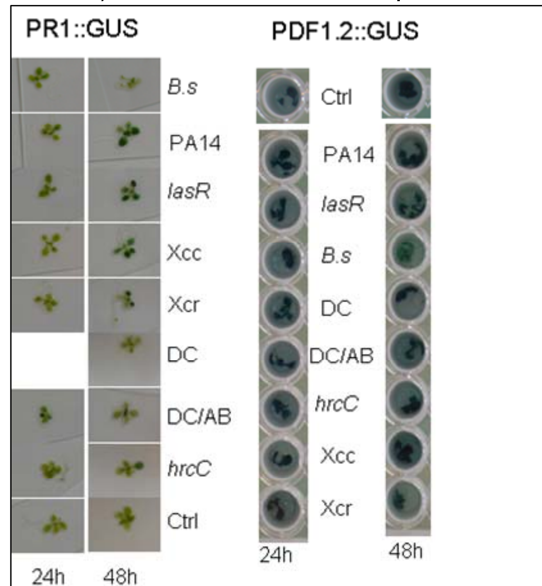


Fig. 12 Expression of PR1::GUS (top left); PDF1.2::GUS (top right); and PR1 and PDF1.2 transcripts (bottom) - Affymetrix chips, Signal Intensity. 24 h time point unless noted).

One example of environment induced change (cell patterning defect, was highlighted in the earlier section). A further example along these lines can be inferred from the behavior of a defense gene reporter considered a hallmark of signaling by the developmental and defense associated hormone jasmonate, viz., *PDF1.2*, detailed below. Two of the best studied arms of defense responses in plant are mediated by salicylic acid (SA) and jasmonic acid (JA). The SA and JA mediated arms of defense primarily plays a role in different types of infection, biotrophic (pathogens that damage, but not kill the host cells quickly to obtain nutrients) and necrotrophic (pathogens kill host cells) pathogens, respectively (Glazebrook, 2005). JA activates pathogen defense responses with another hormone, ethylene (e.g., Penninckx, 1998). In addition, a major theme that has been recognized as a result of these studies is the fact that these two pathways of defense are often antagonistic to each other (e.g., Spoel and Dong, 2008). There is also an extensive interaction between the different hormone pathways affecting defense (reviewed in Pieterse et. al., 2009). Typically, transcriptional (and some times checked at the protein level) induction of *PR1* and *PDF1.2* are considered hallmarks of SA and JA mediated arms of defense, respectively. A single point transcriptome at 24 h post infection (three replicates) treated with PA14, PA14::*lasR*, DC3000, *B. subtilis*, *E. coli*, DC3000(AvrB) and a few representative experiments from 48 h post infection treated with PA14, *B. subtilis* and DC3000 (AvrB) done in this study reveals that in this environment there could be significant exceptions to the antagonistic phenomena observed in other systems (Fig. 12). Further, the *PDF1.2* promoter GUS fusions used as reporter here indicate a constitutive high level of GUS activity even in untreated seedlings. That does not seem to correlate with the transcript levels observed. The transcriptional data compared to the qualitative GUS assays, in addition to several aspects observed with the luciferase reporter indicate there is a mismatch between the transcriptome and proteome. Some contributing factors that could be envisaged are the host damage and manipulation of host machinery by microbes that affect protein synthesis and/or stability. For example, extensive manipulation and utilization of the ubiquitin conjugation machinery by plant and mammalian pathogens is very well documented (e.g., Sweet et. al., 2007; Rosebrock et. al., 2007 – opposite functions). ***These data argue for care and highlight serious limitations in interpreting data based on transcriptome data alone without associated data on the proteome, in the study of host-microbe interactions.***

Non-trivial regulation of the potent *B. subtilis* induced host damage

All of the above experiments involving *B. subtilis* were done with the sporulation deficient mutant *yabQ*::tet (Fawcett, 2000). When a wild-type strain PY79 was used, the damage was as potent and preliminary observation under the microscope at 3 dpi did not reveal any obvious sporulation. In addition, a number of mutants (mostly in strain 168, obtained from the Bacillus Genetic Stock Center, BGSC) altered (decrease or

increase) in regulation of degradative enzymes reveal the characteristic and potent host damage accompanied by loss of tissue integrity is not simply a function of production of enzymes one would predict to cause plant tissue damage (data not shown). Thus a complete evaluation of a library of knockouts in all the genes of *B. subtilis* is expected to reveal previously unrecognized factors controlling its ability to cause potent host damage highlighted by this system and its environment. In addition the potent loss of integrity during the interaction suggest that under appropriate conditions the same factors could potentially cause damage to other hosts (e.g., mammals) and would be worth an exploration. For example, some gram positive and gram-negative microbes are known to cause soft tissue damage especially in immune compromised conditions as is the case with necrotizing fasciitis (Kotrappa et. al., 1996).

Some salient features highlighted by preliminary analysis of the transcriptome data

As mentioned earlier a single time point transcriptome data set has been generated and is being evaluated and prepared for publication. Thus detailed analysis is not included in this proposal at this juncture. I have experience with analyses of such data and functional inference in combination with biological data and curated gene function information with bacterial, mammalian and plant transcriptome (Deziel et. al., 2005; Padfiled et. al., 2005; Astrakas et. al., 2005; Padfield et. al., 2006; Denoux et. al., 2008). Thus, besides the extracted data on the expression of PR1 and PDF1.2 and the concordance of transcriptome of seedlings treated with PA14 and PA14::*gacA* mutant one aspect is highlighted below.

In another line of study, an *ad hoc* algorithm (unpublished) was used to represent putative causal expression correlation (up or down regulation – represented by red or blue edges, respectively) in a network format and correlated transcripts were separated based on connectivity, in an older version of Cytoscape (Cline et. al., 2007). A central and the largest correlational network is shown in Fig.13. When the presence of known upstream elements (promoters) represented in the PLACE (A Database of Plant Cis-acting Regulatory DNA Elements; Higo et. al., 1999) database (searched for using a simple *ad hoc* Perl script upstream 500 bp of each gene represented– location and number were not used at this stage) were integrated with that sub-network, one could see two genes that appeared to be commonly involved in downregulating one module and upregulating another module. The upstream elements used here represent multiple overlapping motifs. These two genes happen to be transcription factors from two different gene families. The effect (downregulation or upregulation) correlates with the presence of either two classes or single class of upstream elements. A double mutant is being constructed to validate this inference using qPCR for gene expression phenotype and to test the effect of these modules on host-microbe interactions. ***The bulk of these genes are present in this modular format in all the treatments tested here as well as in publicly available datasets from adult plants treated with different bacteria.*** Since the modularity and functions are being tested the figure does not show the names of the genes or the upstream elements. The above example is shown simply to demonstrate the knowhow and *ad hoc* algorithm development process that I have been involved with (besides systematic use of different computational tools and data organizing schemes that have been successful and being developed). The other salient example that would highlight this strength is the discovery of a novel property of datasets, the concept (termed ReSurfX) that has been developed into a data analyses and technology design algorithm with a recently issued US patent (Gopalan, 2009).

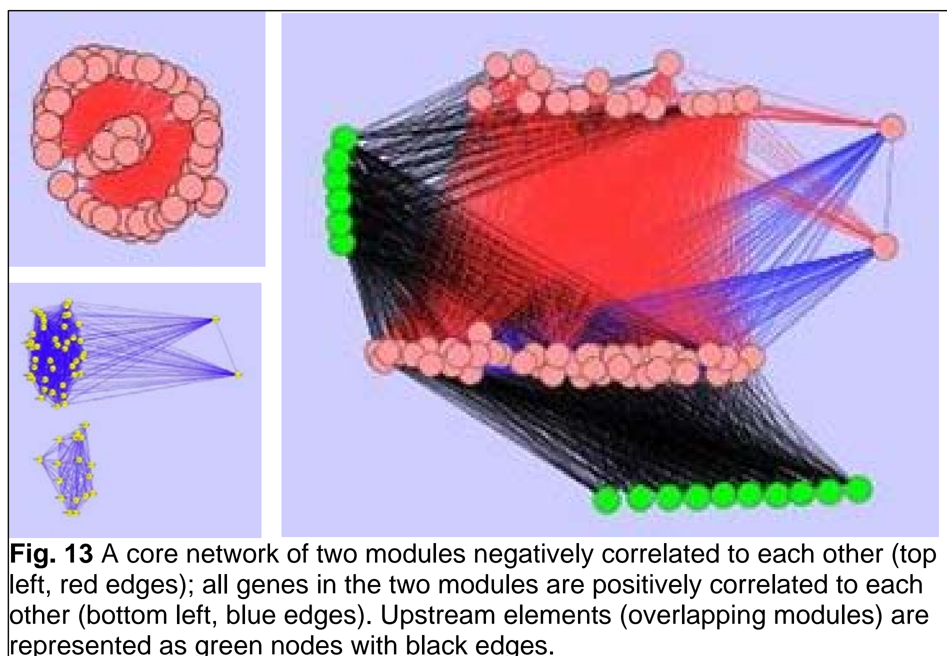


Fig. 13 A core network of two modules negatively correlated to each other (top left, red edges); all genes in the two modules are positively correlated to each other (bottom left, blue edges). Upstream elements (overlapping modules) are represented as green nodes with black edges.

5. RESEARCH DESIGN AND METHODS

The main goals of the proposal are to try and gain a comprehensive understanding of different modes of interactions between host and microbes using a model metasystem that has been developed recently (Gopalan and Ausubel, 2008). The metasystem consists of a host (seedlings of model plant *Arabidopsis thaliana*) and microbes representing different classes of interactions (ranging from a well adapted plant pathogen, a broad host opportunistic pathogen that cause infections in immune compromised humans, and commonly considered innocuous microbes), all causing host damage in characteristic ways under the conditions of these experiments. The rationale being that early stages of adaptation of host and microbe (to avoid elimination by host innate immunity) occurs by adaptive system status change (biochemical and signaling state changes of preexisting machinery) in nature, under certain environments. This model system represents a time compressed paradigm of a similar scenario occurring in nature. The use of extensively studied host and microbes with many tools and a large knowledge base and comparisons between different modes of interaction will aid understanding the underlying mechanisms that lead to this kind of adaptation. It is reasonably assumed that this knowledge framework can be extrapolated to understand early stages of real life infections that evolve by this mode. In all cases the extensive knowledge base of these organisms will be comprehensively evaluated with data generated in this proposal to form hypotheses and identify components and mechanisms relevant to the goals of the project. When some aspect of the project is to be carried out using core facilities or there is prior publication/preliminary data indicating my familiarity of the techniques or analyses, basic details of experiments are not provided.

The metasystem has been set up in a manner amenable to high throughput automation assisted perturbation screens (genetic and compounds) and with the aim of developing a broad platform to study other aspects of signaling interactions. These goals are not part of this proposal.

Aim 1: Gain additional biological insight via the study of progression of host damage and changes (cellular and physiological) in the host and microbes in this model metasystem

The data on the characteristic host damage shown in “Preliminary Data” clearly indicate that these interactions represent different characteristic progressions/end points, in addition to evidence that many well known aspects of pathogen host interactions (in the case of *P. aeruginosa* and *P. syringae*) are conserved in this system. Further, the data also indicate that the observations on the reporters and some unusual responses elicited by these host-microbe combinations would serve as a powerful handle to understand the host and microbial biology during these interactions that would be difficult to study using many conventional models. It also serves to highlight novel modes of regulation of virulence (e.g., in the case of *gacA* and *lasR* mutants of *P. aeruginosa*) and defense responses (e.g., behavior of *PDF1.2*::GUS reporter) that needs to be understood.

Aim 1A. Microscopy of host integrity and structural changes during the progression of host-microbe interaction

The ability to detect cellular structural components, their integrity and cellular metabolic activity has been advanced by the availability of improved dyes together with fluorescent microscopy and/or uv imaging (in case of cell wall visualization). Another tool is the ability to tag structural components (e.g., actin) and in some cases specific subcellular compartments with fluorescent proteins and commercially available fluorescent tagged antibodies. Many of the preliminary results described above obtained using the membrane impermeable stain Sytox Green® (Molecular Probes, CA) under low to medium magnification were very helpful in discerning the differences in the mode of interaction between the different microbes and the host. Of particular significance are the facts that *P. aeruginosa* (in this case tested with strain PA14) displays a characteristic staining pattern of host stomatal guard cells (different than expected for a nucleic acid dye in a membrane permeabilized cell). In contrast, *P. syringae* pv. *tomato* (DC3000) caused uniform permeabilization of most host cells with a staining pattern reminiscent of a nucleic acid stain (prominent spots presumably at the nucleus), whereas DC3000 expressing the avirulence protein AvrB displayed a staining pattern suggestive of clusters of dead host cells. AvrB when delivered into the plant cell by the type III secretion apparatus is expected to elicit a programmed cell death in these seedlings as they harbor the corresponding resistance (R) gene product.

In this aim, a more comprehensive time course analysis to determine these changes in terms of cell permeability as well as other structural and metabolic components, will be carried out, which should provide new valuable information. Where available, seeds of transgenic plants with markers for the structural and subcellular components will be used in the experiment. For example, actin, tubulin and microtubule reporter transgenics have been used earlier for other purposes. While initial studies (as in 'Preliminary Results' section) will be carried out in fluorescent mode without optical sectioning, subsequent studies will be done using a confocal microscope or when possible simply on a microscope with apotome function. This careful study is expected to highlight the nature of interaction from an outcome based perspective (i.e., phenotypic progression of host damage). In addition to the microbes studied earlier by microscopy in this system (*P. aerugiosa*, *P. syringae* pv. *tomato* with and without an avirulence gene product, *B. subtilis* and *E. coli*), *S. aureus*, Xcc, and Xcr that have been included in subsequent studies in preliminary results will be included for these studies.

This aim primarily focuses on characterizing the different modes of interactions that could help uncover novel regulatory aspects in pathogenesis and defense. When possible, tools that have already built in the community will be utilized. Use of plant and bacterial mutants (previously known, and to be discovered as part of this proposal) should be very informative.

Aim 1B. Elucidate the nature of unique staining patterns observed during interaction with *P. aeruginosa*

The unique guard cell staining pattern observed during interaction with *P. aeruginosa* but not with any of the other microbes studied here raises some important questions and forms a good assay to decipher the mode of pathogenesis. Two key questions that arise are (i) is this staining pattern a unique artifact of staining due to some status change of a physical or chemical property of the cell (e.g., redox, pH etc.), or (ii) have *P. aeruginosa* invaded the guard cells extensively? These two possibilities are not mutually exclusive.

1. Thus these experiments need to be done more carefully with other nucleic acid dyes like DAPI to see if staining of the guard cells reflects disintegrated bacteria or host cell nucleus. It should be noted that in some cases punctuate staining in the guard cells in addition to the whole cell staining is observed.
2. To test the possibility of bacteria invading the guard cells and getting degraded, *in situ* hybridization with *P. aeruginosa* fluorescently labeled specific 16R rRNA probes (Jansen et. al., 2000) will be used.
3. Electron microscopy to check for integrity of the host cells along the course of infection will be carried out. This will be done at the EM core of Center for Membrane Biology and Center for Systems Biology, MGH.
4. Screen for PA14 transposon mutants that alter this staining pattern (part of Aim 1F below).

*Another important observation is that unlike the seedlings treated with other microbes, there is no staining of epidermal cells (*other cell types to be examined - guard cells and vascular cells treated wit PA14 typically seem to stain completely with Sytox Green). This includes the lack of punctuate staining reminiscent of nuclear staining. This indicates that either those cells have either internally disintegrated or that they are killed in a different way (as supported by loss of luciferase activity in these seedlings). *Experiments 1 and 3 above should address this aspect directly and experiment 4 should help identify bacterial factors contributing to these phenotypes.*

Aim 1C. Novel GacA independent regulatory circuitry in PA14 controlling virulence under these conditions

Based on existing knowledge the master regulator GacA (a two-component regulator) of *P. aeruginosa* plays a key upstream regulatory role in controlling quorum sensing (both Las and Rhl dependent systems) and virulence related functions in many tested systems (Heurlier et. al., 2004; Goodman et. al., 2009). GacA has also been shown to play key role in virulence in some other bacteria (e.g., *P. syringae* and *Erwinia* sp. Chatterjee et. al., 2003; Cui et. al., 2001). The same has been shown with model systems as well. For example in the case of slow killing of *C. elegans* by *P. aeruginosa gacA* mutants are extremely attenuated to the extent that they behave more like their well adapted food source *E. coli* (Tan et. al., 1999). Even in adult plant assays with PA14 it has been shown that *gacA* mutants are attenuated in virulence (Rahme et. al., 1995). Surprisingly, in the seedling assay system used here a *gacA* mutant maintained all characteristics tested similar to wild type PA14. But a *lasR* mutant is partially impaired in the visual host damage phenotype, ability to abrogate the

constitutive luciferase activity as the wild type, and has a much weaker stomatal guard cell staining pattern, as well as a pervasive staining pattern very different than wild type or a *gacA* mutant. These data clearly indicate that quorum sensing does play a role in virulence of PA14 in this system, like in most other systems tested. Further, these data also indicate that LasR dependent quorum sensing only affects part of the virulence and in the absence of those, PA14 switches to a different mode of interaction.

This goal is to elucidate how the lack of GacA is circumvented in this system to confer virulence and host damage apparently similar to wild type. In other words, it is hypothesized that there exits a previously unidentified regulation independent of GacA that acts upstream of LasR in parallel to GacA. It can be reasonably assumed that such mode of action is operative in nature in medically significant conditions and has not been discerned due to limitations of phenotypes assayed. The following specific experiments will be used to test these observations and hypotheses:

1. Test the expression of *lasR* and known *lasR* dependent genes in *gacA* mutant in this system.

2. Conduct a microarray (transcriptome) analysis of *gacA* and *lasR* mutants and compare to wild type PA14 in this system at two time points (early time points of relevance will be determined from Aim 1A). Identify potential key modulators of virulence and test their role by mutagenesis and constitutive expression in PA14 and *gacA* mutant. Reporters for LasR and LasR dependent genes will also be used.

3. Identify genomic regions bound by GacA in PA14 and test for occupancy of those sites in a *gacA* mutant in this system. To test the sites occupied by GacA, a 6X His or FLAG tagged GacA in PA14 by precise replacement will be used. Subsequently DNA fragments bound to GacA will be immunoprecipited using antibodies to the tags and sequenced using standard protocols. To test the occupancy of the sites in a *gacA* mutant by another factor, one or more DNA fragments (selected based on most enriched subset) will be used as probes in the recently developed PICh approach to identify factors binding to selected regions (Dejardin & Kingston, 2009). Alternatively it is theoretically possible that GacA bound regions are not responsible for this circumvention of its requirement. In that case it is predicted that candidate genes/pathway can be inferred from the transcriptome data. Mutants and constitutive expression of these candidates genes in a *gacA* mutant background, and if needed PA14, should help to elucidate the alternate regulatory mechanism, with abrogation of seedling luciferase activity assay and the guard cell staining as read outs.

Aim 1D. An animal model to emulate potent *B. subtilis* induced damage in this metasystem

The data presented here indicate that *B. subtilis* surprisingly is a very potent inducer of host damage in this system. In comparison to all the other microbes used in this system, it has a very unique feature in the sense that there is near complete loss of tissue integrity, in addition to rapid loss of chlorophyll. It also abrogates the constitutive luciferase activity expressed in the seedlings, albeit slower than in the case of *P. aeruginosa*. These data indicate that *B. subtilis* has unique features compared to all the other microbes tested. The fact *B. subtilis* is commonly innocuous, and even beneficial in certain conditions (Bais et. al., 2004), provides a powerful model to dissect this unique mode of virulence and to understand how under this condition these microbes evade, disarm or circumvent host immunity.

The unique feature that *B. subtilis* seems to rapidly affect the structural integrity of tissue structure is reminiscent of necrotizing fasciitis. Necrotizing fasciitis is a condition caused by certain bacterial pathogens, e.g., *Streptococcus. pyogenes* and *Staphylococcus aureus*, typically in trauma patients and causes severe tissue damage (giving them the common term “flesh eating bacteria”). Thus, based on the kind of damage observed, it would be interesting to study if *B. subtilis* can cause similar damage in animal hosts under appropriate conditions.

1. Study infection (and nature of infection and damage) in the burn mouse model and another murine model for soft tissue infections under different conditions that alter the interaction environment.

This will be done in collaboration with the Wessels and Rahme laboratories (HMS and MGH). The burn mouse model has been successfully adapted for the study of *P. aeruginosa* pathogenesis (Rahme et. al., 1995) and is being used in the laboratory of Laurence Rahme at MGH. The murine soft tissue infection model for necrotizing

fasciitis (Bricker et. al., 2005), is being used by the laboratory of Michael Wessels, HMS. (***Letters of support/collaboration from Michael Wessels and Laurence Rahme are enclosed***).

Expected results: This goal is based purely on the observed mode of tissue integrity loss and extrapolation to other hosts. It is predicted in none of the two mouse models the infection will be readily apparent, thus a few conditions that enable the mode of environment induced adaptation observed here will be emulated to test this hypothesis. The success of setting up such a model will be extremely fruitful for further studies and will be a clean demonstration of the hypothesis that is proposed and the goals built on in this application. Success will also be followed up in collaboration with these laboratories with some immunological and histopathological studies to make it a publishable unit. These will include study of cytokines and infiltration of certain cell types (e.g., neutrophils) that normally shed light on some aspect of the immune regulation.

Aim 1E. Screen for bacterial genes conferring potent *B. subtilis* induced damage in this metasystem

B. subtilis is an extensively studied organism in terms of basic metabolism, genetics, and extensively studied with respect to sporulation. More recently, studies have also revealed other novel features previously not realized before including population behavior (Suel et. al., 2007) and survival (Gonzalez-Pastor et. al., 2003), biofilm formation (Lopez et. al., 2009) etc. Thus many mutations in sporulation and stationary phase events, production of degradative and other secretory enzymes and in their regulatory circuitry have been identified and are available in the public domain (e.g., The Bacillus Genetic Stock Center - BGSC, run by Prof. Daniel Zeigler). Preliminary data indicate the damage observed cannot be explained by the trivial presence of these enzymes. But, an ordered insertion library like the one available for PA14 mentioned in Aim 1F (Liberati et. al., 2006) is not available. The latter is being proposed by a team of *B. subtilis* laboratories including David Rudner (HMS), Richard Losick (Harvard) and Carol Gross (UCSF) together with the Broad Institute of Harvard and MIT as an economic stimulus package initiative GO grant. Thus specific goals of this Aim are:

1. Test a complete, to be constructed, library of *B. subtilis* insertion mutants to identify factors affecting *B. subtilis*-host interactions in this system (***loss of chlorophyll, seedling stunting, and effect of constitutively expressed luciferase activity in the host seedlings***).

B. subtilis being one of the most extensively worked on organisms is being promoted to have additional tools to harness as a model for systems and synthetic biology. The complete knock out library expected to be developed over the first year will be used in this system to identify mutants affecting the readouts. ***Letter from Dr. David Rudner enclosed.*** An adult plant leaf infiltration screen will also be carried out for factors affecting localized virulence and host damage as shown in Fig. 3. In case their grant is not funded and that initiative not undertaken, the following targeted approach will be undertaken.

2. Study potential factors will be through targeted deletion of conserved genes and proteins with domains shared with other pathogenic bacteria.

Collection of information on the large number of microbial genomes like in COGS (Sayers et. al., 2009 – part of NCBI) together with directed search for known and new factors affecting virulence will be used for this purpose. *Targeted insertions of candidate genes in B. subtilis will be generated when not available from the Bacillus Genetic Stock Center.* The genes found to play a role will be integrated with other data from this proposal and with the large knowledge base, datasets and metabolic pathways information available for this organism.

Expected results: As mentioned in the "Preliminary Results" section the host damage inflicted by *B. subtilis* cannot be explained by a trivial effect involving some known secreted enzymes that could affect plant tissue. Thus it is expected that this approach will uncover potentially novel and/or combinatorial effects that contribute to such potent damage during interaction with host under these conditions. In some cases effect of single factors and positive and negative regulatory effects can be confirmed using over expression constructs. Transformation of laboratory strains of *B. subtilis* is trivial, so combination mutants of several factors identified to have an effect can be easily constructed. Some of the genes identified should directly shed light on the metabolic and signaling pathways affected and/or rewired during these interactions. Specific components can also be studied using promoter YFP fusions being proposed to be built by Rudner et. al. In addition, the transcriptome analysis during interaction with the metabolic pathway information should highlight fundamental physiological changes potentially contributing to the interaction that can be tested.

Aim 1F. Screen for virulence factors and regulators of *P. aeruginosa* affecting different host phenotypes

As has been mentioned earlier *P. aeruginosa* (in this case strain PA14) inflicts potent host damage, seedling stunting and killing, complete abrogation of a constitutively expressed luciferase activity, and a unique staining pattern on stomatal guard cells by the membrane impermeable dye Sytox green with no apparent staining of epidermal cells, and loss of chlorophyll fluorescence. In addition a *lasR* mutant (a quorum sensing regulator that has been shown to have a role in attenuating virulence in model invertebrates to mammals and humans) is attenuated in and altered in several of these responses. For example a *lasR* mutant does not abrogate the constitutively expressed luciferase activity, stains the stomatal guard cells rarely and weakly with Sytox Green, and shows a pervasive staining of other cell types. Strain PA14 is extensively studied in the laboratory of Fred Ausubel (where I am currently housed) and has been shown to infect a variety of invertebrate and mammalian hosts with several common virulence effectors and regulators. To this effect, a screen is being conducted using the *C. elegans* model and the non-redundant PA14 insertion library collection to look for other virulence factors, among other approaches. As has been shown above, the metasystem described and used in this proposal has unique phenotypic read-outs, and is probably capable of highlighting novel virulence mechanisms or modes of action. Indeed, a blind screen of collection of two 96 well plates of PA14 transposon insertions (identified in a different screen for a different phenotype in the Ausubel laboratory) retested for alteration of abrogation of seedling luciferase activity picked up a *lasI* mutant as the third most affected mutant (data not shown). ***This also proves the robustness and screen readiness of this assay.***

The set up for this metasystem has been accomplished in a high-throughput automation amenable manner in 96 well plates. Thus using a high-throughput luminescence reader and a screening microscope the PA14 insertion library of ~5,500 clones (Liberati et. al., 2006) will be screened for altered phenotypes mentioned above. Since PA14 virulence determinants are of central interest of the Ausubel lab, the outcome of this screen will be published as a collaborative effort. Optical zoom using and a wide angle field lens can detect the circular staining pattern characteristic of PA14 treated seedlings stained with Sytox Green (data not shown).

Expected results: The results expected and follow up will be similar to what is written above in Aim 1E. Any multiple mutants or reporter constructs needed will be constructed and used. In addition the results will be combined with other screens carried out by the Ausubel lab and close collaborators for virulence and associated functions (e.g., in *C. elegans*, *Drosophila melanogaster* etc.). This is expected to highlight common and system/environment specific adaptations of PA14 in host interactions. Novel aspects identified will also tested in the burn mouse model in collaboration with Laurence Rahme, and other models as appropriate. ***In Aims 1C, 1E and 1F and 1H (below) the primary interest will be to integrate hits into the knowledge framework to be assembled - Aim 2C. Only predicted key nodes (upstream) in signaling, metabolic and de novo pathways and key downstream (or direct regulators) are to be analyzed in this proposal – Aim 3.*** In-depth characterization of other hits will be part of a different proposal.

Aim 1G. Elucidate the biological basis of the mismatch between host damage and the constitutively expressed luciferase marker gene expression

As has been highlighted before, though the different microbes used here seem to cause different mode of host damage as extensively documented by Sytox Green staining, the abrogation of constitutively expressed luciferase activity does not always seem to have a correlation with the extent of host damage. The glaring example is the complete lack of reduction in luciferase activity of seedlings treated with DC3000, the *hrcC* mutant of DC3000, or the DC3000 expressing AvrB which is expected to cause a rapid programmed cell death. One explanation for this observation with DC3000 and derivatives is possibly the mode of interaction, that DC3000 being a biotrophic pathogen, would prefer not to kill the host cell rapidly so as to obtain nutrients. Then one would have to further predict that this feature of DC3000 is dominant over the cell death response when expressing AvrB (at least in this system). There is also significant lack of correlation between host damage and luciferase activity in interactions involving PA14, *B. subtilis* and *E. coli*.

This could be due to several reasons including the way the host cells are damaged, effect on physiological properties altered in the host cells that could have an effect on luciferase as an enzyme, or other properties like

protein degradation, or gene or protein expression. Some aspects of this phenomenon may be beyond the scope of this proposal (though luciferase as a marker is used for a wide range of assays). Aspects that can reveal the mechanistic basis and nature of interactions between the host and the microbe will be of interest. For example, microbes could harbor factors that enhance or decrease protein degradation or synthesis or alter host cell physico-chemical status in some cases, and in other cases be directly proportional to host damage. This study will be initially carried out using the following three experiments:

1. Quantitative RT PCR of luciferase transcripts after different treatments and at different time points.
2. Quantitative protein levels of luciferase using the monoclonal antibody available for this protein from Perkin Elmer.
3. Lyse plant cells treated with different bacteria representing different behavior and add lysates from untreated plants (with known amount of luciferase activity, or purified luciferase) and assay for physico-chemical condition changes affecting the activity after different treatments.
4. In conditions representing different behaviors, look for protein modifications by immunoprecipitation followed by mass spectrometry.

Another effective way to address this would be to screen a transposon insertion library of DC3000 to identify factors contributing to this mode of interaction (especially the lack of abrogation of luciferase activity by DC3000 induced damage, even when it harbors the avirulence protein that seem to maintain the expected programmed cell death response). A recent conversation with Prof. Alan Collmer (Cornell University) who spearheaded the sequencing of this strain indicated there is no complete annotated and ordered transposon library at this time.

Aim 1H. Elucidate the molecular mechanism of impaired regulation of Jasmonate mediated immune response as revealed by the expression of *PDF1.2*::GUS reporter in this system

The fact that bacteria typically considered to be innocuous can initiate active host-microbe interactions under the conditions of this system and some unusual features highlighted by the different assays used here is consistent with the hypothesis that environment induced adaptation of host and microbe can significantly alter the outcome.

Two prominent cases of host alterations in this system highlighted by these studies are, i) the cell patterning defect observed in seedlings that violates the single cell spacing rule that a guard cell's neighbor is actively prevented from attaining the same fate, and ii) the surprising constitutive high level expression of GUS expression (and activity) of a *PDF1.2* promoter reporter, considered a hallmark of Jasmonate (JA) based defense arm. It should be reiterated at this stage that numerous studies indicate that the two arms of defense induced by SA and JA are antagonistic. JA is a close analog of prostaglandins in mammals. Thus following this observation to gain mechanistic insight and eventually integrate with signaling networks altered and operative in this system together with objectives of Aim 2 should be extremely informative towards the broad goals of this proposal. In addition, it can shed light on some aspects of additional similarities and mechanistic aspects of SA and JA (and broadly lipid signaling) in progression of host-microbe adaptations.

To address the question a combined genetic and metabolomic approach will be used as follows:

1. **Genetic approach:** Many genetic components of JA biosysnthesis and signaling are well known at this stage. Mutants are available in many components of this pathway outlined in the next few lines. The biosynthetic enzyme allene oxide synthase (AOS) a cytochrome P450 enzyme that commits the precursor 13-hydroperoxylinolenic acid to synthesis of jasmonates (e.g., Park et. al., 2002). JAR1 that conjugates JA to JA-Ile (Staswick et. al., 1992) which is the predominantly active form in defense related functions, the F-box protein coronatine insensitive 1 (COI1; Xie et. al., 1998) that upon binding to JA-Ile and JAZ proteins executes its ubiqutin dependent degradation and activate the jasmonate signaling cascade (Thines et. al., 2007; Chini et. al., 2007). JIN1 a MYC2 bHLH transcription factor that represses a set of defense genes (e.g., *PDF1.2*) and these latter genes are positively regulated by the ethylene responsive factor (ERF1) – Lorenzo et. al., 2004. JIN1 also upregulates a subset of JA induced genes, that are repressed ethylene. The induction of JA responsive defense genes is antagonized by SA mediated signals via NPR1 (a central regulator of many SA dependent defense functions). These data

are summarized in Farmer et. al., in Science STKE; and Pieterse et. al., 2009. Thus, the *PDF1.2*::GUS reporter line will be crossed with these mutants and the resultant seeds will be used in this assay for constitutive and microbe induced expression of the reporter. In some cases like *coi1*, the seeds can only be obtained as heterozygotes, in those cases the reporter expression phenotypes will be correlated with genotyping to check if they are homozygous or heterozygous for those mutations. In addition a GUS antibody will be used for gross changes by western blotting indicative of modifications leading to alterations in turn over. Additionally, as with the case of experiments with luciferase in Aim1G, the GUS protein will be immunoprecipitated and subjected to mass spectroscopy.

2. **Metabolomic approach:** The above experiments should place the cause for constitutive expression on the canonical JA signaling cascade (or indicate the existence of an alternative mode of regulation of the JA mediated defense gene regulation). In addition it will be important to know the levels of SA (given the major role these two hormone pathways play in regulating each other) under these conditions. The methods for extracting the plant material and analyzing multiple hormones by LC-MS established recently (Pan et. al., 2008; Forcat et. al., 2008) will be used. If the data suggest impairment of the biosynthetic or feedback pathways at specific points, then the analyses will be extended to known and predicted branch points and derivatives of the components that are now not channeled properly.
3. **Live imaging of interactions and cross regulation between SA and JA mediated defense pathways:** The availability of key marker genes for the two known major pathways of defense response involving SA and JA provide powerful tools for live imaging based study of their signaling during the different kind of interactions. This can also be compared with soil grown plants where their antagonistic interactions are well demonstrated. Some conceived similarities of these two defense mediators and their signaling interaction in mammals provides additional incentive to study their potential rewiring under these conditions. Thus fluorescence resonance energy transfer (FRET) and Bioluminescence resonance energy transfer (BRET) based assays using reporters of defense response genes (based on promoters of SA and JA mediated genes, *PR1* and *PDF1.2*) will be constructed for this purpose. Both FRET and BRET have been shown to work in plants (e.g., Hink et. al., 2002; Xu et. al., 2007). Both FRET and BRET can be used in non-destructive mode and can form powerful systems for monitoring live course of events. Since only two components are to be used here, a split intein based constructs (e.g., Yang et. al., 2006) will be constructed under the regulation of these promoters. FRET/BRET will be followed during the course of infection of the different microbes. ***If found necessary, and possible to conduct, a plate based assay on seedlings germinated on agar and then submerged in liquid medium, a mutagenized collection of either the double reporter or PDF1.2::GUS reporter can be screened using non-destructive GUS assay or BRET based assay for mutants affected in the impaired response.***

Expected results and contingency plan: This goal will likely give a key clue as to how the host system status is altered that prevents effective execution of defense response it is capable of eliciting. The genetic characterization using known components of JA, ethylene and SA pathway (biosynthesis and signaling) would likely localize at the point the defect originates. There could be also an unusual problem because the transcript level does not seem to correlate with the reporter activity. This does not seem to be a generalized defect in impaired protein turnover, as *PR1*::GUS also having some basal expression of transcript does not show high levels of reporter activity. If there is an effect on transcript stability the genetic experiments may not answer, then experiments will be initiated to test this possibility. These two genes used as examples here are end point markers for defense related responses by the two most prominent defense pathways mediated by hormonal responses. An alternative possibility is that there is an unusual regulation of the *PDF1.2* promoter by non-jasmonate dependent factors. ***Another jasmonate responsive gene, a VSP1::GUS reporter is also being tested. The results will indicate if the ethylene downregulated arm of JA induced genes (VSP1 belongs to that class) is affected in this system.*** Additionally, a *PDF1.2*::GUS from an independently constructed source will also be tested. Jasmonate synthesis or misregulation of responses may be only one factor contributing to inability of these seedlings to effect the immune response as evidenced by the cell patterning defect in stomatal guard cells that uses similar perception and signaling architecture compared to perception of pathogen elicitors and induction of defense response. This would likely become evident or have to be carefully elucidated from the combined studies of phenotypes, hormone levels either based on the response and transcriptome based analyses to infer a bottleneck or rechanneling of other metabolic pathways. In the latter case targeted metabolite analysis will be carried out. For this purpose a collaborator with appropriate strength in metabolite analyses and complementary interest will be sought. ***The FRET and BRET assays will pave***

way for many other analyses that are foreseen from the output of these studies and will also serve to standardize the assay as a platform technology for studying signaling interactions in general.

Aim 2. Generation and integration of system wide data and other data available for these framework model organisms to build a knowledge base to aid prediction and better study of host-microbe adaptations

The primary hypotheses of this proposal is that intermingling of new combinations of host and microbe and host interactions with even commonly considered innocuous or beneficial microbes can turn detrimental when interacting under appropriate conditions. Further, it is hypothesized that this change in adaptation is brought about by environment induced changes in system status (defined as biochemical and signaling alterations brought about primarily by rewiring of pre-existing cellular components). In addition to large databases populated with information on cellular components and their interactions (as components and pathways), the framework organisms used in the metasystem developed and used here has a long standing tradition of being some of the most well studied, understood and have tools to test any new hypotheses generated under new conditions. This aim capitalizes on the latest developments (and poised to contribute to further development of knowledge) and the knowledge developed over many decades and proposes to identify system status changes that occur during the transition and progression of host damage and evolution of maladaptations. Goals of Aim 2 consist of two broad parts: (i) generating system-wide data on three cellular components (transcriptome, proteome and phosphoproteome) during the course of the diverse host-microbe interactions represented by this system, and (ii) organizing this knowledge in a framework suitable for inference of system status changes and integrating them with the knowledge base of interactions of components that have been studied to confer other know phenotypes to infer changes that likely bring about these interactions.

Aim 2A-1. Host and microbial transcriptome during the progression of these interactions

Transcriptome analysis is one of the most mature technologies and the most widely used (thus large volume of data available from different organisms at different conditions). In many cases known knowledge have been recapitulated and new insights and regulatory themes have been recognized using transcriptome data. Thus, the transcriptome of the host (Arabidopsis seedlings) and the microbes during the process of these host-microbe interactions will be determined. In all these cases the GeneChip technology (Affymetrix, CA) will be utilized, that are available for all these organisms. Since one of the objectives is to determine if the knowledge base created from this proposal could also be used to predict the extent of progression of adaptation, time course data will be collected. For this purpose 4, 8, 12, 24, 36 and 48 hrs post infection are targeted for the host. In the case of microbes data from 8, 12, 24, 30, 36 and 48 hrs post infection will be collected. The time course data generation will be staggered to provide room for changes and maximize the use of data generated at specific time points. ***The data for the 24 hr time point has already been collected and some observation (in-depth analyses are being done and will be published shortly) has been presented earlier***. I have extensive experience with Affymetrix technology design and analyses, evidenced by publications (listed earlier) and a patent that has special advantages in analyzing data and technology designs involving multiple independent measure of each parameter (Gopalan, 2009). The data will be analyzed using ANOVA with time and treatment as factors, with appropriate precautions in controlling ratio of true and false positives identified.

Expected results and contingency plan: A major problem with many data sets from Arabidopsis transcriptome studies is that very large number of genes are differentially regulated by each treatment. Thus many standard list-based analyses need to be evaluated in light of other phenotypic data. A second approach will be to validate some of them for any causal role through qPCR using appropriate mutants. There are certain problems associated with determining the host transcriptome in later time points in these kind of experiments involving two organisms. One way of circumventing it would be to use quantitated polyA RNA as the starting material. Similar safeguards will be undertaken to identify any systematic misleading aspects with the microbial transcriptome before using the transcripts identified as differentially regulated for downstream decision making. One way would be to do a preliminary data collection for one microbe and one time point and validate as above or using a reporter gene construct in plant tissue associated bacteria and free bacteria in the medium. This latter aspect will be undertaken earlier in the data generation pipeline, and if needed alternative

sequencing based strategies for quantitative determination of the transcriptome will be explored. The Department has a Solexa sequencer (Illumina Corp.).

Aim 2A-2. Determining the proteome of the host and microbes during the different interactions represented by the metasystem

There is a significant degree of mismatch between the transcriptome and the protein reporter activities in this metasystem. This probably represents the nature of the system and the interactions explored here. Thus it becomes imperative to determine the proteome in addition to transcriptome to make confident interpretations of the data and to build the knowledge base. Thus a combined host-microbe trancriptome will be determined initially at two time points without replication. Shotgun/MudPit proteomics followed by analysis of results using 6 frame translation of the host and microbe genome will be utilized (Yates et. al., 2009; Swanson et. al., 2009). These type of analyses are becoming standard and are offered by core facilities. An additional time point or replication will be determined based on the outcome and nature of the results. The Partners Core facility that caters to system-wide proteomic needs of researchers at Harvard and the Hospital system is being rearranged. Thus the best approach to make a balance between absolute and relative quantitation ideally using post extraction tagging or the emerging use of spiked in tagged proteins and peptides will be used. Some existing and emerging methods and their comparisons are covered in a number of reviews (e.g., Bantscheff, 2007; Ong and Mann, 2005).

Expected results and contingency plan: It is assumed that it is reasonably safe to try to do the host and microbe proteome together and deconvolute using 6 frame translations of both genomes as database. One problem that could complicate this type is sample preparation. But it is predicted at this stage that the use of the harsher of the conditions required between the host and microbe will solve this problem. A general proteome (as opposed to specific modifications with higher regulatory potential – see below) is targeted at this stage due to the potential to identify previously unidentified components. Thus a whole proteome, in addition to resolving the transcript-protein mismatch would also enable more confident prediction of signaling and metabolic pathways operative at different stages.

Aim 2A- 3. Determine the phosphoproteome to aid regulatory node identification

The main goal of the proposal is to organize the phenotypic information, data from bacterial mutant screens, some metabolic information and the system-wide transcriptome and proteome data collected above and integrate with the large knowledge base available for these organisms (see Aim 2B). Thus it might become a necessary step to include one other data set from this system that has high level of control point information. Two such technologies that are maturing are small RNA sequencing to identify microRNA based controls and phosphoproteome. Both these have the potential to identify key modulatory steps and the responsible regulators. It is predicted that involvement of regulatory involvement of miRNAs can be predicted by computational analysis of datasets continuing to be generated.

Thus a phosphoproteome analysis will be carried out at 12 h or 18 h (the time point considered about midway between infection and irreversible damage sets in). The technology for separation of phosphorylated proteins (e.g., reviewed in Nita-Lazar et. al., 2008) followed by shotgun sequencing of this subfraction is being extensively used in literature. Again the decision to choose between a comparative proteome in combinations of two vs. single treatment will be made based on the state of the advances at that stage and in consultation with the technical expert at the proteomics core.

Expected results and contingency plan: Determining the phosphoproteome is not expected to be a problem. But the time point estimated here may need change based on the transcriptome and other careful time course phenotypic analysis (e.g., the microscopy based analysis proposed in Aim 1A). The phosphorylation differences identified may not represent components in modules identified predicted as causal or characteristic of a particular class of host-microbe interaction. Thus additional information in terms of other known relationships like interactome etc. may need to be utilized to find the key links and placement in the network.

Aim 2B. Infer system status and causal components using the data generated and the knowledge of nature of the host-microbe interaction using integrative biological and computational tools

A main hypothesis of the proposal is that initial stages of host-microbe adaptations occur by changes in system status (defined here as primarily adaptive changes in signaling networks and metabolic output) of the host and microbe, and such changes under appropriate conditions. This hypothesis further predicts that this precedes long term adaptations through Mendelian changes and this kind of system status adaptation can be predicted by careful integrative study of systems representing different host-microbe interactions. The systems and the constituent microbes represented in the metasystem studied here have been extensively studied using classical genetics and biochemical tools for many decades. These studies, sequencing and application of newer technologies have generated a very large knowledge framework including causal genetic and regulatory modules (canonical) and metabolic pathways affecting them. Two overarching themes emerged from these of studies: (i) system-wide approaches redefine or expand known roles for many known biological components, and (ii) integrating a compendium of knowledge from different approaches can shed light into new and rewired components of previously known processes potentially leading to a different outcome. In addition a well chosen model with a large number of tools to test inferred or hypothesized components and modules can lead to rapid progress of an otherwise difficult to test hypothesis or difficult to study natural phenomenon.

Dynamics of host-microbe interactions posits that components and modules in both organisms need to be functioning in a particular way to lead to the outcome. Much of the analysis in this goal will depend on the following advances/organizational principles:

1) Extensive metabolic pathway organization for each of the component organisms (typically constructed in a canonical framework),
2) Large compendium of data sets (transcriptome, proteome, interactome, metabolome etc.) under different conditions and perturbations,
3) Excellent visualization tools in terms of static pathways and networks – thus the ability to detect/predict changes in component structural organization (i.e., flux through networks and metabolic pathways),
4) Development and availability of user friendly network decomposition tools,
5) Availability of large literature curated relationship networks with tools to organize a given set of components in the context of known relationships as well as in terms of canonical organization,
6) Extensive framework of organization of functional information (e.g., gene ontology consortium) to infer significant changes in components associated with well studied and annotated processes,
7) Organized genome and proteomic structural features (e.g., promoters, conserved sequence and structural features of proteins), and tools to predict such elements *de novo* from a collection of genes or proteins,
8) Availability of large curated/putative orthologs of the host with higher plant and mammalian organisms the recognition and prediction of evolution of diseases is the goal. For example, over 30% of annotated Arabidopsis proteins have predicted orthologs in humans, and 66 % in rice (from Inparanoid database)
9) The above point also highlights the use of data collected from different disease, development and data on infection and immune related scenarios can be used to form the integrative framework.

As has been shown by numerous publications and an example shown using an *ad hoc* algorithm in Fig. 13, appropriate utilization and integration of knowledge base can illuminate *de novo* modular networks. Broadly, two approaches will be used. One, an approach, as in above example, either collected individually and reassembled into networks with more information and decomposed again. The kind and relevancy of information combined is going to determine the outcome in these networks. The other approach, that is complementary and can be combined with the first approach, is using different canonical modules of signaling and metabolic networks and assembled newly collected information on top of them and extract relevant parts and make a new connectivity network. The commonality and differences to some extent can be discerned using approaches somewhat similar in principle to the one used in a recent study of complex traits in Drosophila (Ayroles et. al., 2009). Using an approach related in principle and a hierarchical network organization I discerned the involvement of lipid signaling as a direct upstream components of the expression of an Arabidopsis homolog related to the defense induced gene *hin1* (Gopalan, 2005 – conference abstract; Gopalan et. al., 1996). In case of analyses of time course data to discern causal (hierarchical) connection between components/modules, the approach used would have to include coefficients representing relationship between adjacent time points. The finally arranged networks can now be used in flux analyses between different pathways and compared to canonical arrangement to discern changes that are occurring during these interactions.

Multiple modes of dimensionality reduction in terms of phenotypic association and one or more of the above listed features might be a necessary step for achieving some of the goals. It is also envisaged that when different features are used for dimensionality reduction, different aspects of system status change will be highlighted. It should also be emphasized that the whole library screens of Aim 1E and Aim 1F in addition to screening targeted mutants of the different microbes and of host components will provide key informative nodes of inference.

The phosphoproteome proposed in Aim 2A-3 should also serve to highlight key regulatory nodes. Yet another regulatory component that could be easily identified when they have a role are miRNAs. Small RNAs and microRNAs have been shown to play diverse role in a variety of processes including mammalian and plant immune pathways (e.g., Xiao and Rajewsky, 2009; Navarro et. al., 2006). Since a large body of work is being carried out and organized in databases like miRBase (Griffiths-Jones et. al., 2008) MPSS (Lu et. al. 2005) and Arabidopsis small RNA project (Backman et. al., 2008). The data will be analyzed for evidence of significant regulation of key modules predicted to be affected and relevant results incorporated into the networks.

Expected results and contingency plan: This aim will build networks of signaling and metabolic interactions based on results from this work and existing knowledge base. A major unmet need that is envisaged for this goal of building large number of networks operative under these conditions is a complementary need to have the easy to visualize information in different formats and packages in one machine readable form to redraw and visualize in different context. Some aspects of required improvement in commonly used software packages for biological inference (not discusses here) will be attempted as part of this proposal and implemented as an add-on or stand-alone module. In some cases, specific hierarchy within modules may not be predictable without additional experimentation, even when modules can be organized in a hierarchy. ***Much of the refinement and additional development of tools and models are expected to continue beyond the term of this proposal. This would probably entail a proposal involving a multi-disciplinary team.***

Aim 3. Prove the validity of the hypotheses generation work flow by removal and addition of specific component(s)/pathways to the host and/or microbe based on the hypotheses generated.

The main advantages of using a model metasystem is the ability to test hypotheses generated rapidly either using direct modulation of components/modules or in combination with mutants in canonical pathways affected or rearranged by these analyses. On the first stage, modules affecting specific host-microbes individually in the host or microbe will be tested by use of mutants, RNAi, overexpression, dominant mutant competitors, or when available specific (or reasonably specific) inhibitors of certain processes. In addition, live imaging of interaction of two or more cannonical or newly identified modules will be setup using FRET and BRET, as was described for a simpler case in Aim 1H. Ideal proof would be modulation of modules in a matched manner in the host and microbe that together are predicted to bring about the phenotype. For example, this could represent (i) one or more related virulence modules of the microbe affecting a specific host process, (ii) metabolic processes affecting specific preferred substrate utilization in the microbe from the host and equivalent processes affected by that metabolite in the host, or (iii) prevention of accumulation of an antimicrobial metabolite normally produced by the host, due to rechanneling of metabolic flux through other pathways (or opposite, i.e., increased accumulation), either through host and or microbial processes involved in the interaction.

The main goal is to be able to predict broader themes of emergence of host-microbe maladaptations. Thus other model organisms, single cell models (like mammalian cell culture – when there is no specific evidence for non-cell autonomous processes), and other animal models for representing an equivalent kind of interaction discerned from the phenotypic and other system-wide data generated here will be used to test the hypotheses generated. An example of the last type of tests will be like the prediction that the potent damage induced by *B. subtils* in this system seems to be reminiscent of necrotizing fasciitis. So the knowledge generated will be constantly compared to known and emerging diseases to discern such similarities. In some cases, the evaluation of these modules and mode of action against equivalent modules (using orthologs) being assembled based on literature curated and *de novo* generated networks for different disease conditions will be used to predict environments or host niches that could lead to new maladaptations. ***This aim will serve to highlight the proof of principle and test key hypotheses (the low hanging fruits) and much of the other***

hits from screens and modules and nodes identified from the networks (and system-wide data) will have to be tested as part other proposals.

SUMMARY: The metasystem of model organisms assembled here to study different well adapted and commonly considered innocuous microbes interacting and eliciting host damage through active contributions from the host and the microbe is being explored to understand the progression of different modes of host-microbe interactions. This time-compressed model for some natural environment and niche induced adaptations emerging in nature should highlight valuable information on common themes and modules involved in the evolution of new host-microbe maladaptations in different kingdoms. ***The main goal is to be able to predict broader themes of emergence of host-microbe maladaptations. Thus, in-depth analysis of a single combination of host and microbe or a specific phenotype is not the target of this proposal.*** Instead, a mix of cell biology, genetics of host and microbes (genome wide screens of two microbes for multiple phenotypes), and novel features highlighting the potential changes that could be affecting the changes in this metasystem will be studied. In addition, an elaborate attempt to assemble the above data, system-wide data to be collected during progression of the different modes of interactions, large knowledge base available for the well studied organisms comprising the model, and information from orthologs are to be integrated to form a knowledge framework for easy prediction of natural evolution of new host-microbe maladaptations. The hypotheses generated will be tested in the model metasystem used here and in other models to prove generality. The knowledge framework and data generated should prove to be valuable resource for the scientific community for a variety of purposes. ***The grand scheme will grow along with the developments in the field, identify advantages and limitations, and set the stage for future design of such studies and train personnel in this emerging approach of study.***

LETTERS OF SUPPORT from Prof. Fred Ausubel (MGH and HMS), Prof. George Church (HMS, Harvard and MIT), Prof. Michael Wessels (Children's Hospital and HMS), Dr. Laurence Rahme (MGH), and Dr. David Rudner (HMS) are enclosed.

Animals

One of the Aims proposes testing the hypotheses that *B. subtilis* can be infective in a burn mouse model or soft tissue infection model of mouse under appropriate conditions. These experiments are currently slated to be done in collaboration with Prof. Michael Wessels and Dr. Laurence Rahme, whose laboratories are equipped for and have the IACUC approval. Changes in protocols will be communicated accordingly to see if any modifications are needed in the current approval of those laboratories, and approval obtained if changes are suggested.

Select Agents List

Not Applicable

LITERATURE CITED

1. Aballay A and Ausubel FM (2001). Programmed cell death mediated by ced-3 and ced-4 protects *Caenorhabditis elegans* from *Salmonella typhimurium*-mediated killing. Proc. Nat. Acad. Sci. USA 98(5): 2735-2739.

2. Akira S, Uematsu S and Takeuchi O (2006). Pathogen recognition and innate immunity. Cell 124(4): 783-801.

3. Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, et al. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415(6875): 977-983.

4. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25(1): 25-29.

5. Astrakas LG, Goljer I, Yasuhara S, Padfield KE, Zhang Q, et al. (2005). Proton NMR spectroscopy shows lipids accumulate in skeletal muscle in response to burn trauma-induced apoptosis. FASEB J. 19(11): 1431-1440.

6. Ausubel FM (2005). Are innate immune signaling pathways in plants and animals conserved? Nat. Immunol. 6(10): 973-979.

7. Backhed F, Ley RE, Sonnenburg JL, Peterson DA and Gordon JI (2005). Host-Bacterial mutualism in the human intestine. Science 307(5717): 1915-1920.

8. Backman TWH, Sullivan CM, Cumbie JS, Miller ZA, Chapman EJ, et al. (2008). Update of ASRP: the Arabidopsis Small RNA Project database. Nucl. Acids Res. 36(suppl_1): D982-985.

9. Bais HP, Fall R and Vivanco JM (2004). Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol. 134(1): 307-319.

10. Bantscheff M, Schirle M, Sweetman G, Rick J and Kuster B (2007). Quantitative mass spectrometry in proteomics: a critical review. Anal. Bioanal. Chem. 389(4): 1017-1031.

11. Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, et al. (1994). RPS2 of Arabidopsis thaliana: a leucine-rich repeat class of plant disease resistance genes. Science 265(5180): 1856-1860.

12. Berglund A-C, Sjolund E, Ostlund G and Sonnhammer ELL (2008). InParanoid 6: eukaryotic ortholog clusters with Inparalogs. Nucl. Acids Res. 36(suppl_1): D263-266.

13. Bergmann DC, Lukowitz W and Somerville CR (2004). Stomatal development and pattern controlled by a MAPKK kinase. Science 304(5676): 1494-1497.

14. Beutler B, Eidenschenk C, Crozat K, Imler JL, Takeuchi O, et al. (2007). Genetic analysis of resistance to viral infection. Nat. Rev. Immunol. 7(10): 753-766.

15. Bhavsar AP, Guttman JA and Finlay BB (2007). Manipulation of host-cell pathways by bacterial pathogens. Nature 449(7164): 827-834.

16. Boller T and He SY (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science 324(5928): 742-744.

17. Bricker AL, Carey VJ and Wessels MR (2005). Role of NADase in virulence in experimental invasive Group A Streptococcal infection. Infect. Immun. 73(10): 6562-6566.

18. Cao H, Glazebrook J, Clarke JD, Volko S and Dong X (1997). The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88(1): 57-63.

19. Caspi R, Foerster H, Fulcher CA, Kaipa P, Krummenacker M, et al. (2008). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome databases. Nucleic Acids Res 36(Database issue): D623-631.

20. Chatterjee A, Cui Y, Yang H, Collmer A, Alfano JR, et al. (2003). GacA, the response regulator of a two-component system, acts as a master regulator in Pseudomonas syringae pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. Mol. Plant Microbe Interact. 16(12): 1106-1117.

21. Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature 448(7154): 666-671.

22. Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, et al. (2007). Integration of biological networks and gene expression data using Cytoscape. Nat. Protoc. 2(10): 2366-2382.

23. Cui Y, Chatterjee A and Chatterjee AK (2001). Effects of the two-component system comprising GacA and GacS of *Erwinia carotovora* subsp. *carotovora* on the production of global regulatory *rsmB* RNA, extracellular enzymes, and $harpin_{Ecc}$. Mol. Plant Microbe Interact. 14(4): 516-526.

24. Dangl JL and Jones JD (2001). Plant pathogens and integrated defence responses to infection. Nature 411(6839): 826-833.

25. Dejardin J and Kingston RE (2009). Purification of proteins associated with specific genomic Loci. Cell 136(1): 175-186.

26. Denoux C, Galletti R, Mammarella N, Gopalan S, Werck D, et al. (2008). Activation of Defense Response Pathways by OGs and Flg22 Elicitors in Arabidopsis Seedlings. Mol Plant: ssn019.

27. Dethlefsen L, McFall-Ngai M and Relman DA (2007). An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449(7164): 811-818.

28. Deziel E, Gopalan S, Tampakaki AP, Lepine F, Padfield KE, et al. (2005). The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI*, *rhlRI* or the production of N-acyl-L-homoserine lactones. Mol. Microbiol. 55(4): 998-1014.

29. Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, et al. (2008). Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J. Exp. Med. 205(2): 323-329.

30. Eisen MB, Spellman PT, Brown PO and Botstein D (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Nat. Acad. Sci. USA 95(25): 14863-14868.

31. Farmer EE, Liechti R, Gfeller A (2009) Arabidopsis Jasmonate Signaling Pathway. *Sci. Signal.* (Connections Map in the Database of Cell Signaling, as seen 22 May 2009), http://stke.sciencemag.org/cgi/cm/stkecm;CMP_13931.

32. Farmer EE, Dubugnon L, Liechti R (2009) Jasmonate Biochemical Pathway. *Sci. Signal.* (Connections Map in the Database of Cell Signaling, as seen 25 May 2009), http://stke.sciencemag.org/cgi/cm/stkecm;CMP_7361.

33. Fauci AS (2006). Emerging and re-emerging infectious diseases: influenza as a prototype of the host-pathogen balancing act. Cell 124(4): 665-670.

34. Fawcett P, Eichenberger P, Losick R and Youngman P (2000). The transcriptional profile of early to middle sporulation in *Bacillus subtilis*. Proc. Nat. Acad. Sci. USA 97(14): 8063-8068.

35. Finlay BB and Falkow S (1997). Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61(2): 136-169.

36. Forcat S, Bennett MH, Mansfield JW and Grant MR (2008). A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress. Plant Methods 4: 16.

37. Fritz JH, Ferrero RL, Philpott DJ and Girardin SE (2006). Nod-like proteins in immunity, inflammation and disease. Nat. Immunol. 7(12): 1250-1257.

38. Galan JE and Collmer A (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284(5418): 1322-1328.

39. Gallagher LA and Manoil C (2001). *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. J. Bacteriol. 183(21): 6207-6214.

40. Garsin DA, Villanueva JM, Begun J, Kim DH, Sifri CD, et al. (2003). Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens. Science 300(5627): 1921.

41. Glazebrook J (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Ann. Rev. Phytopathol. 43: 205-227.

42. Gomez-Gomez L and Boller T (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol. Cell. 5(6): 1003-1011.

43. Goodman AL, Merighi M, Hyodo M, Ventre I, Filloux A, et al. (2009). Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. Genes Dev. 23(2): 249-259.

44. Gopalan S (2005). Inference of comprehensive network neighborhoods associated with phenotypes: a case study using hin1/ndr1 gene family. 16th International Conference on Arabidopsis Research, Wisconsin, USA, http://www.arabidopsis.org/servlets/TairObject?type=publication&id=501717113

45. Gopalan S (2009). Methods and systems for high confidence utilization of datasets. US Patent 7,480,593.

46. Gopalan S and Ausubel FM (2008). A metasystem of framework model organisms to study emergence of new host-microbe adaptations. PLoS ONE 3(12): e3891.

47. Gopalan S, Bauer DW, Alfano JR, Loniello AO, He SY, et al. (1996). Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. Plant Cell 8(7): 1095-1105.

48. Gopalan S, Wei W and He SY (1996). *hrp* gene-dependent induction of *hin1*: a plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. Plant J 10(4): 591-600.

49. Grant MR and Jones JD (2009). Hormone (dis)harmony moulds plant health and disease. Science 324(5928): 750-752.

50. Gray KM, Passador L, Iglewski BH and Greenberg EP (1994). Interchangeability and specificity of components from the quorum-sensing regulatory systems of *Vibrio fischeri* and *Pseudomonas aeruginosa*. J. Bacteriol. 176(10): 3076-3080.

51. Griffiths-Jones S, Saini HK, van Dongen S and Enright AJ (2008). miRBase: tools for microRNA genomics. Nucl. Acids Res. 36(suppl_1): D154-158.

52. Hashimoto C, Hudson KL and Anderson KV (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52(2): 269-279.

53. Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, et al. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 186(10): 2936-2945.

54. Hink MA, Bisselin T and Visser AJ (2002). Imaging protein-protein interactions in living cells. Plant Mol. Biol. 50(6): 871-883.

55. Holloway BW (1955). Genetic recombination in *Pseudomonas aeruginosa*. J. Gen. Microbiol. 13(3): 572-581.

56. Iandolo JJ, Worrell V, Groicher KH, Qian Y, Tian R, et al. (2002). Comparative analysis of the genomes of the temperate bacteriophages phi 11, phi 12 and phi 13 of *Staphylococcus aureus* 8325. Gene 289(1-2): 109-118.

57. Janeway CA, Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 54 Pt 1: 1-13.

58. Janeway CA, Jr. and Medzhitov R (2002). Innate immune recognition. Annu. Rev. Immunol. 20: 197-216.

59. Johal GS and Briggs SP (1992). Reductase activity encoded by the HM1 disease resistance gene in maize. Science 258(5084): 985-987.

60. Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, et al. (2008). KEGG for linking genomes to life and the environment. Nucleic Acids Res 36(Database issue): D480-484.

61. Kim DH, Feinbaum R, Alloing G, Emerson FE, Garsin DA, et al. (2002). A conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity. Science 297(5581): 623-626.

62. Klement Z, Farkas, GL, Lovrekovich, L (1964). Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54: 474-477.

63. Kotrappa KS, Bansal RS and Amin NM (1996). Necrotizing fasciitis. Am. Fam. Physician 53(5): 1691-1697.

64. Lai LB, Nadeau JA, Lucas J, Lee E-K, Nakagawa T, et al. (2005). The Arabidopsis R2R3 MYB proteins FOUR LIPS and MYB88 restrict divisions late in the stomatal cell lineage. Plant Cell 17(10): 2754-2767.

65. Lemaitre B, Nicolas E, Michaut L, Reichhart JM and Hoffmann JA (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86(6): 973-983.

66. Lemaitre B, Reichhart J-M and Hoffmann JA (1997). Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Nat. Acad. Sci. USA 94(26): 14614-14619.

67. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, et al. (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc. Nat. Acad. Sci. USA 103(8): 2833-2838.

68. Ligterink W, Kroj T, zur Nieden U, Hirt H and Scheel D (1997). Receptor-mediated activation of a MAP kinase in pathogen defense of plants. Science 276(5321): 2054-2057.

69. Lopez D, Fischbach MA, Chu F, Losick R and Kolter R (2009). Structurally diverse natural products that cause potassium leakage trigger multicellularity in *Bacillus subtilis*. Proc. Nat. Acad. Sci. USA 106(1): 280-285.

70. Lorenzo O, Chico JM, Sanchez-Serrano JJ and Solano R (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16(7): 1938-1950.

71. Lu C, Tej SS, Luo S, Haudenschild CD, Meyers BC, et al. (2005). Elucidation of the small RNA component of the transcriptome. Science 309(5740): 1567-1569.

72. Lynch AS and Robertson GT (2008). Bacterial and fungal biofilm infections. Ann. Rev. Med. 59(1): 415-428.

73. Mahajan-Miklos S, Rahme LG and Ausubel FM (2000). Elucidating the molecular mechanisms of bacterial virulence using non-mammalian hosts. Mol. Microbiol. 37(5): 981-988.

74. Martin GB, Brommonschenkel SH, Chunwongse J, Frary A, Ganal MW, et al. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262(5138): 1432-1436.

75. Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, et al. (2006). TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic Acids Res 34(Database issue): D108-110.

76. Meylan E, Tschopp J and Karin M (2006). Intracellular pattern recognition receptors in the host response. Nature 442(7098): 39-44.

77. Mindrinos M, Katagiri F, Yu GL and Ausubel FM (1994). The *A. thaliana* disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell 78(6): 1089-1099.

78. Miyata S, Casey M, Frank DW, Ausubel FM and Drenkard E (2003). Use of the *Galleria mellonella* caterpillar as a model host to study the role of the Type III secretion system in *Pseudomonas aeruginosa* pathogenesis. Infect. Immun. 71(5): 2404-2413.

79. Nadeau JA and Sack FD (2002). Control of stomatal distribution on the Arabidopsis leaf surface. Science 296(5573): 1697-1700.

80. Naitza S, Rosse C, Kappler C, Georgel P, Belvin M, et al. (2002). The Drosophila immune defense against gram-negative infection requires the death protein dFADD. Immunity 17(5): 575-581.

81. Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, et al. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312(5772): 436-439.

82. Ong SE and Mann M (2005). Mass spectrometry-based proteomics turns quantitative. Nat. Chem. Biol. 1(5): 252-262.

83. Padfield KE, Astrakas LG, Zhang Q, Gopalan S, Dai G, et al. (2005). Burn injury causes mitochondrial dysfunction in skeletal muscle. Proc. Nat. Acad. Sci. USA 102(15): 5368-5373.

84. Padfield KE, Zhang Q, Gopalan S, Tzika AA, Mindrinos MN, et al. (2006). Local and distant burn injury alter immuno-inflammatory gene expression in skeletal muscle. J. Trauma 61(2): 280-292.

85. Pamer EG (2007). Immune responses to commensal and environmental microbes. Nat. Immunol. 8(11): 1173-1178.

86. Pan X, Welti R and Wang X (2008). Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry. Phytochemistry 69(8): 1773-1781.

87. Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, et al. (2002). A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J 31(1): 1-12.

88. Penninckx IA, Thomma BP, Buchala A, Metraux JP and Broekaert WF (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell 10(12): 2103-2113.

89. Pieterse CMJ, Leon-Reyes A, Van der Ent S and Van Wees SCM (2009). Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5(5): 308-316.

90. Pirofski LA and Casadevall A (2008). The damage-response framework of microbial pathogenesis and infectious diseases. Adv. Exp. Med. Biol. 635: 135-146.

91. Plotnikova JM, Rahme LG and Ausubel FM (2000). Pathogenesis of the human opportunistic pathogen *Pseudomonas aeruginosa* PA14 in Arabidopsis. Plant Physiol. 124(4): 1766-1774.

92. Quintana-Murci L, Alcais A, Abel L and Casanova J-L (2007). Immunology in natura: clinical, epidemiological and evolutionary genetics of infectious diseases. Nat. Immunol. 8(11): 1165-1171.

93. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, et al. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. Science 268(5219): 1899-1902.

94. Rosebrock TR, Zeng L, Brady JJ, Abramovitch RB, Xiao F, et al. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature 448(7151): 370-374.

95. Roy CR and Mocarski ES (2007). Pathogen subversion of cell-intrinsic innate immunity. Nat. Immunol. 8(11): 1179-1187.

96. Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, et al. (1997). The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. Plant Cell 9(3): 425-439.

97. Sayers EW, Barrett T, Benson DA, Bryant SH, Canese K, et al. (2009). Database resources of the National Center for Biotechnology Information. Nucl. Acids Res. 37(suppl_1): D5-15.

98. Schneider DS and Ayres JS (2008). Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. Nat. Rev. Immunol. 8(11): 889-895.

99. Schreiber K, Ckurshumova W, Peek J and Desveaux D (2008). A high-throughput chemical screen for resistance to Pseudomonas syringae in Arabidopsis. Plant J 54(3): 522-531.

100. Schwab JH (1975). Suppression of the immune response by microorganisms. Microbiol. Mol. Biol. Rev. 39(2): 121-143.

101. Scott-Craig JS, Panaccione DG, Pocard JA and Walton JD (1992). The cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7-kilobase open reading frame. J. Biol. Chem. 267(36): 26044-26049.

102. Shpak ED, McAbee JM, Pillitteri LJ and Torii KU (2005). Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science 309(5732): 290-293.

103. Spoel SH and Dong X (2008). Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe 3(6): 348-351.

104. Staswick PE, Su W and Howell SH (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Nat. Acad. Sci. USA 89(15): 6837-6840.

105. Swanson SK, Florens L and Washburn MP (2009). Generation and analysis of multidimensional protein identification technology datasets. Methods Mol. Biol. 492: 1-20.

106. Sweet CR, Conlon J, Golenbock DT, Goguen J and Silverman N (2007). YopJ targets TRAF proteins to inhibit TLR-mediated NF-kappaB, MAPK and IRF3 signal transduction. Cell Microbiol. 9(11): 2700-2715.

107. Swertz MA and Jansen RC (2007). Beyond standardization: dynamic software infrastructures for systems biology. Nat. Rev. Genet. 8(3): 235-243.

108. Tan M-W, Mahajan-Miklos S and Ausubel FM (1999). Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc. Nat. Acad. Sci. USA 96(2): 715-720.

109. Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448(7154): 661-665.

110. Tzou P, Reichhart J-M and Lemaitre B (2002). Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proc. Nat. Acad. Sci. USA 99(4): 2152-2157.

111. Xiao C and Rajewsky K (2009). MicroRNA control in the immune system: Basic principles. 136(1): 26-36.

112. Xie DX, Feys BF, James S, Nieto-Rostro M and Turner JG (1998). COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science 280(5366): 1091-1094.

113. Xu X, Soutto M, Xie Q, Servick S, Subramanian C, et al. (2007). Imaging protein interactions with bioluminescence resonance energy transfer (BRET) in plant and mammalian cells and tissues. Proc. Nat. Acad. Sci. USA 104(24): 10264-10269.

114. Yang J, Fox GC, Jr. and Henry-Smith TV (2003). Intein-mediated assembly of a functional beta - glucuronidase in transgenic plants. Proc. Nat. Acad. Sci. USA 100(6): 3513-3518.

115. Yates J, Ruse CI and Nakorchevsky A (2009). Proteomics by mass spectrometry: Approaches, Advances, and Applications. Annu Rev Biomed Eng.

116. Zhang S and Klessig DF (2001). MAPK cascades in plant defense signaling. Trends Plant Sci. 6(11): 520-527.

117. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, et al. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428(6984): 764-767.

MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

Department of Molecular Biology
Richard B. Simches Research Center
185 Cambridge Street, CPZN-7250
Boston, Massachusetts 02114
Tel: 617-726-5969, Fax: 617-726-5949
email: ausubel@molbio.mgh.harvard.edu

Frederick M. Ausubel, Ph.D.
Professor of Genetics
Harvard Medical School

May 27, 2009

Dr. Suresh Gopalan
Department of Molecular Biology
Richard B. Simches Research Center
Massachusetts General Hospital
Boston, MA 02114

Dear Suresh,

I am very pleased to fully support your R01 grant application entitled “Metasystem of model organisms to study emergence of host-microbe maladaptations”, in which you are proposing to use the model host-microbe metasystem you have developed to study the mechanistic basis of how new host-pathogen relationships develop. This is an ideal model in which to explore fundamental questions about the emergence of new infectious diseases.

In carrying out this project, you will be able to utilize space in the Department of Molecular Biology allocated to my laboratory until you have secured independent laboratory space of your own. You will also have unlimited access to the equipment and infrastructure that is available to all researchers in the Department of Molecular Biology. This is possible because of the manner in which supplies and equipment are shared between laboratories in our Department. The Department of Molecular Biology is fully equipped to carry out all aspects of modern molecular biology research and you will have unfettered access to this infrastructure. Finally, the Department of Molecular Biology is fully equipped to carry out plant-related research.

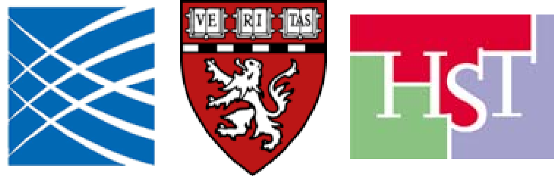
As you know, within the Department of Molecular Biology, my laboratory and Jen Sheen’s laboratory work with Arabidopsis as a model host to study bacterial pathogenesis. There is not only a great deal of know how among our laboratories, but also a variety of reagents that will be available to you, including a *Pseudomonas aeruginosa* PA14 non-redundant transposon mutation library. Finally, the work in my laboratory and the Sheen laboratory is complementary, but not overlapping to what you have proposed in your R01 application. In addition, my laboratory is studying the pathogenesis of *P. aeruginosa* PA14 in relation to its ability to cause a lethal infection in the intestine of the model nematode *Caenorhabditis elegans*. I will be pleased to make available to you the full resources of my laboratory and I am looking forward to working with you as appropriate on this exciting and important project.

Best wishes,

Frederick M. Ausubel

A Teaching Affiliate of Harvard Medical School

Harvard Medical School
Department of Genetics
77 Avenue Louis Pasteur
Boston, MA, USA 02115
http://arep.med.harvard.edu



May 24, 2009
To: Suresh Gopalan, Dept. Molecular Biology, MGH

Dear Suresh,

I am writing in support of your grant application on the use of the metasystem of model organisms to study the emergence of new host-microbe adaptations. Your data are very convincing that the host damage induced in this metasystem is environmentally induced. Based on this, and the role of known virulence determinants, I am convinced that this is a good model system to study the emergence of host-microbe adaptations. The advances in technologies for system-wide measurement of biological components and advances in the development of tools to integrate them, makes your timely effort to use this model to build a knowledge framework feasible. It should be very productive.

As we discussed, your project will need a good deal of computational effort to integrate the data and make inferences of broader consequence. You have been working on these aspects as evidenced by your recently issued, and well deserved, patent on "Methods and Systems for High Confidence Utilization of Datasets", and other examples of network decomposition and integration you have discussed with me. I am confident you will rise up to the challenges, use appropriate tools and develop solutions of broader utility as needed.

As you know, we have been using a variety of systems biology technologies, as well as developing new ones. We have also been developing computational tools for over a decade now. Thus, we would be glad to assist, advise and collaborate with you on your endeavor as needs arise. In addition, we are generating data on human derived cell lines (from individual participants) for potential therapeutic purposes, as part of the Personal Genome Project (PGP). If you need to rapidly test any new algorithms you develop, you could also use these systems and data derived from them.

Best wishes for getting your grant application funded.

Sincerely,

George M. Church, Professor of Genetics & Biophysics
Director, NIH-CEGS, PGP, & DOE Centers
Wyss Inst. of Biologically Inspired Engineering, Broad Inst. of Harvard & MIT

Children's Hospital Boston
Department of Medicine
Division of Infectious Diseases

HARVARD MEDICAL SCHOOL
Department of Pediatrics
Department of Medicine

Michael R. Wessels, MD
Chief, Division of Infectious Diseases
John F. Enders Professor of Pediatrics and Professor of Medicine
Children's Hospital Boston
300 Longwood Avenue
Boston, Massachusetts 02115
main phone 617-919-2900 | *fax* 617-730-0254
michael.wessels@childrens.harvard.edu
www.childrenshospital.org

May 21, 2009

Suresh Gopalan, PhD
Department of Molecular Biology, Massachusetts General Hospital &
Department of Genetics, Harvard Medical School
Richard B. Simches Research Building
185 Cambridge Street, CPZN7250
Boston, MA 02114-2790

Dear Suresh,

I am writing to affirm my support of your R01 grant application on the use of a model organism based metasystem for understanding host-microbe adaptations.

It is extremely interesting that in your experimental system a variety of microbes can cause host damage. It is well known that environmental factors can change the outcome of host-microbe interactions. For example, group A *Streptococus* often causes soft tissue infection in patients with minor trauma or other defects in the epithelial barrier. Thus a facile system to study such adaptations and its use for natural infections is very attractive.

Particularly intriguing is your observation of extensive loss of tissue integrity caused by *B. subtilis*, and your hypothesis that it seems to resemble necrotizing fasciitis. As you know, we have used a murine model of invasive soft tissue infection for our studies of group A streptococcal pathogenesis. I would be glad to assist you and your colleagues and collaborate as needed to test if under appropriate conditions *B. subtilis* can cause infection similar to necrotizing fasciitis in the murine model we have used. We can also collaborate to compare immune responses when your experimental system is set up.

Best wishes for success in your project,

Michael R. Wessels, MD

MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

Department of Surgery
Director, Molecular Surgical Laboratory
Thier Research Building, Room 340A
50 Blossom Street
Boston, MA 02114
Tel: 617 724-5003
Fax: 617 724-8558
Email: rahme@molbio.mgh.harvard.edu

Laurence Rahme, Ph.D
Associate Professor of Surgery
(Microbiology and Molecular Genetics)

Suresh Gopalan, PhD
Department of Molecular Biology
Massachusetts General Hospital &
Department of Genetics, Harvard Medical School
Richard B. Simches Research Building
185 Cambridge Street, CPZN7250
Boston, MA 02114-2790

May 24, 2009

Dear Suresh,

I am writing in support of your R01 grant application to use the model organism based metasystem you have published recently for understanding host-microbe adaptations. Our discussion the other day on the system you developed was very useful in helping me understand that your system has the potential to uncover novel modes of virulence regulation, and to assess host damage effects by a variety of pathogens and innocuous microbes.

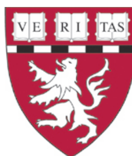
You have access to several tools to test new molecules and hypotheses you generate using your model, as several laboratories including Dr. Fred Ausubel's and mine have developed. The usefulness of your system is further validated by the requirement for known virulence factors of PA14 that we have shown to be important in other invertebrate and mammalian models (e.g., LasR). Based on the potent effect of *B. subtilis* in your model, you are proposing to test the virulence potential of *B. subtilis* in the burned mouse model that we have adapted for the identification and study of PA14 virulence factors. We will be glad to assist you with the use of the burn mouse model to test your hypothesis, and collaborate on other aspects of the project as needed. You could conduct the initial experiments in my laboratory using our approved animal protocol.

Best wishes,

Laurence G. Rahme, Ph.D.

HARVARD MEDICAL SCHOOL
Department of Microbiology
and Molecular Genetics

DAVID RUDNER, PH.D.
Assistant Professor
Harvard Medical School



Harvard Medical School
200 Longwood Avenue
Boston, MA 02115
Ph: (617) 432-4455
Fax: (617) 738-7664
email: rudner@hms.harvard.edu

M
May 24, 2009

Dear Suresh

I am writing to express my enthusiastic support for your R01 application on the use of a metasystem where different microbes interact with Arabidopsis seedlings and cause host damage. The dependence on known virulence factors makes it an attractive model to study environment induced emergence of host-microbe adaptations that you propose to study.

Particularly attractive to me is the fact that *B. subtilis* causes potent host damage in your Arabidopsis seedling-based system. The observation that this cannot be accounted for by trivial enzymatic digestion based on your preliminary studies makes it very attractive to identify novel virulence effectors and their mechanisms of action. Your interest in screening a library of null mutants makes perfect sense. The fact that your infection and readouts are done in 96-well plates will certainly expedite this screen. As you know, there is a wealth of information about *B. subtilis* on various aspects of growth, physiology, metabolism and regulatory mechanisms. This large body of data collected over many decades should aid you with your goal of extending your studies to understand environment induced emergence of microbe related diseases. Unfortunately, there is no publicly available ordered library of B. subtilis mutants to facilitate your proposed screens. However, Richard Losick, and I in conjunction with the Broad Institute, and a consortium of other investigators have submitted a GO grant to generate a variety of *B. subtilis* tools.

We have proposed to construct a library of clean null mutants with yeast barcodes in laboratory and "wild" strains of *B. subtilis* (168, PY79, and 3610). The library of knock-out strains will be available in forty 96-well plates and should greatly faciliate your screens. In addition, we have also proposed to construct an ordered over-expression library and promoter fusions to yfp (for 300 genes). We are happy to give you access to all of these tools as soon as they are constructed. This toolkit will be available to the entire community of researchers.

We are very excited about the possibility of constructing these genome-scale resources, and hope they will shed light into the host-microbe interaction processes that you are studying.

your sincerely,

David Rudner

Resource Sharing

All 'model organisms' generated by this project will be distributed freely or deposited into a repository/stock center (e.g., Arabidopsis Biological Resource Center, Bacillus Geneic Stock Center etc.) making them available to the broader research community, either before or immediately after publication. If we assume responsibility for distributing the newly generated model organisms, we will fill requests in a timely fashion. In addition, we will provide relevant protocols and published genetic and phenotypic data upon request. Other resources generated (e.g., organismal scale gene expression data and high-throughput sequencing data) will be deposited in community repositories in accordance with community standards. New resources and knowledge framework generated that fall outside the scope of these community repositories will be disseminated through dedicated web sites and databases to be generated in the last two years of the proposal. Material transfers will be made with Simple Letter Agreement (SLA) that conform to the intent of Uniform Biological Materials Transfer Agreement (UBMTA). Should any intellectual property arise which requires a patent, we will ensure that the technology (materials and data) remains widely available to the research community in accordance with the NIH Principles and Guidelines document and that of Massachusetts General Hospital and Harvard University.