PI: **Rappleye, Chad A**	Title: Forward genetics-based discovery of Histoplasma virulence genes	
Received: 06/14/2013	FOA: PA11-262	Council: 01/2014
Competition ID: ADOBE-FORMS-B2	FOA Title: NIH SMALL RESEARCH GRANT PROGRAM (PARENT R03)	
1 R03 AI111015-01	Dual:	Accession Number: 3599102
IPF: 6218701	Organization: OHIO STATE UNIVERSITY	
Former Number:	Department: Microbiology	
IRG/SRG: PTHE	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [redacted] Year 2: [redacted]	Animals: N Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Chad Rappleye	The Ohio State University	PD/PI

OMB Number: 4040-0001
Expiration Date: 06/30/2011

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	State Application Identifier

1. * TYPE OF SUBMISSION
☐ Pre-application ☒ Application ☐ Changed/Corrected Application

2. DATE SUBMITTED: 06/14/2013

Applicant Identifier:

4. a. Federal Identifier:

b. Agency Routing Identifier:

5. APPLICANT INFORMATION

*** Organizational DUNS:** [redacted]

* Legal Name: The Ohio State University
Department:
Division:
* Street1: 1960 Kenny Road
Street2:
* City: Columbus
County / Parish: Franklin
* State: OH: Ohio
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 43210-1016

Person to be contacted on matters involving this application
Prefix:
* First Name: Tamara
Middle Name:
* Last Name: Dickey
Suffix:
* Phone Number: [redacted]
Fax Number:
Email: [redacted]

6. * EMPLOYER IDENTIFICATION *(EIN) or (TIN)*: [redacted]

7. * TYPE OF APPLICANT: H: Public/State Controlled 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:
TITLE:

11. * DESCRIPTIVE TITLE OF APPLICANT'S PROJECT:
Forward genetics-based discovery of Histoplasma virulence genes

12. PROPOSED PROJECT:
* Start Date: 04/01/2014
* Ending Date: 03/31/2016

*** 13. CONGRESSIONAL DISTRICT OF APPLICANT**: OH-015

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION
Prefix:
* First Name: Chad
Middle Name: Alan
* Last Name: Rappleye
Suffix:
Position/Title: Associate Professor
* Organization Name: The Ohio State University
Department: Microbiology
Division:
* Street1: 484 W. 12th Ave.
Street2: 540 Biological Sciences Bldg.
* City: Columbus
County / Parish: Franklin
* State: OH: Ohio
Province:
* Country: USA: UNITED STATES
* ZIP / Postal Code: 43210-1292
* Phone Number: [redacted]
Fax Number:
* Email: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested	[redacted]
b. Total Non-Federal Funds	0.00
c. Total Federal & Non-Federal Funds	[redacted]
d. Estimated Program Income	0.00

16. * IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?

a. YES ☐ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ☒ PROGRAM IS NOT COVERED BY E.O. 12372; OR

☐ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious. or fraudulent statements or claims may subject me to criminal, civil, or administrative penalities. (U.S. Code, Title 18, Section 1001)

☒ *** I agree**

**** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.***

18. SFLLL or other Explanatory Documentation

Add Attachment | Delete Attachment | View Attachment

19. Authorized Representative

Prefix: * First Name: Christine Middle Name:

* Last Name: Hamble Suffix:

* Position/Title: Assistant Director

* Organization: The Ohio State University

Department: Division:

* Street1: 1960 Kenny Road

Street2:

* City: Columbus County / Parish: Franklin

* State: OH: Ohio Province:

* Country: USA: UNITED STATES * ZIP / Postal Code: 43210-1016

* Phone Number: [redacted] Fax Number: [redacted]

* Email: [redacted]

*** Signature of Authorized Representative**

Christine Hamble

*** Date Signed**

06/14/2013

20. Pre-application

Add Attachment | Delete Attachment | View Attachment

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

OMB Number: 4040-0010
Expiration Date: 08/31/2011

Project/Performance Site Location(s)

Project/Performance Site Primary Location ☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: The Ohio State University

DUNS Number: [redacted]

* Street1: 1960 Kenny Road

Street2:

* City: Columbus County: Franklin

* State: OH: Ohio

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: 43210-1016 * Project/ Performance Site Congressional District: OH-015

Project/Performance Site Location 1 ☐ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:

DUNS Number:

* Street1:

Street2:

* City: County:

* State:

Province:

* Country: USA: UNITED STATES

* ZIP / Postal Code: * Project/ Performance Site Congressional District:

Additional Location(s) Add Attachment Delete Attachment View Attachment

RESEARCH & RELATED Other Project Information

OMB Number: 4040-0001

1. Are Human Subjects Involved? ☐ Yes ☒ No

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ☐ Yes ☐ No

If yes, check appropriate exemption number. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6

If no, is the IRB review Pending? ☐ Yes ☐ No

IRB Approval Date:

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:

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

4.a. Does this Project Have an Actual or Potential Impact - positive or negative - 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. Is the research performance site designated, or eligible to be designated, as a historic place? ☐ Yes ☒ No

5.a. If yes, please explain:

6. Does this project involve activities outside of the United States or partnerships with international collaborators? ☐ Yes ☒ No

6.a. If yes, identify countries:

6.b. Optional Explanation:

7. Project Summary/Abstract Mutant_Screen___Project_Summary102103 Add Attachment Delete Attachment View Attachment

8. Project Narrative Mutant_Screen___Narrative1021035548.p Add Attachment Delete Attachment View Attachment

9. Bibliography & References Cited Mutant_Screen___Literature_Cited10210 Add Attachment Delete Attachment View Attachment

10. Facilities & Other Resources Mutant_Screen___Facilities_and_Resour Add Attachment Delete Attachment View Attachment

11. Equipment Mutant_Screen___Equipment1021035520.p Add Attachment Delete Attachment View Attachment

12. Other Attachments Add Attachments Delete Attachments View Attachments ☐

Project Summary/Abstract

Forward genetics-based discovery of *Histoplasma* virulence genes

Current understanding of the molecular mechanisms that underly *Histoplasma* pathogenesis remains limited. Unlike opportunistic pathogens, the fungal pathogen *Histoplasma capsulatum* can cause disease even in immunocompetent hosts by parasitizing phagocytes of the host. Only a few virulence factors have been identified and characterized to date. In this proposal, we will use a forward genetics approach to discover the virulence factors that enable *Histoplasma* to subvert the defenses of the macrophage, *Histoplasma's* primary host cell. Random mutants of *Histoplasma* yeasts will be created using insertional mutagenesis. Mutants will be screened for decreased virulence in macrophages using a transgenic macrophage line and a *Histoplasma* strain that has been engineered with fluorescence to provide high-throughput screening capability. Mutants will be classified according to the stage at which *Histoplasma* pathogenesis is blocked by analysis of intramacrophage growth kinetics. The virulence genes represented by each attenuated mutant will be identified by mapping of the mutation. The final collection of virulence-defective mutants will be ranked according to the severity of their impairment, the classification of their pathogenesis defects, and the identity of the virulence gene identities. These rankings will be used to prioritize further characterization of the discovered virulence factors in future studies to define their roles in facilitating *Histoplasma* survival and growth in host macrophages.

Relevance

Histoplasmosis, a respiratory and systemic disease caused by infections with the fungal pathogen *Histoplasma capsulatum*, afflicts thousands each year in the United States regardless of the host's immune status. The mechanisms that enable *Histoplasma* to subvert immune defenses are poorly understood. This proposal will identify new virulence factors through a genetics approach to improve our understanding of *Histoplasma* pathogenesis. Identification of these processes essential to virulence will aid in the development of improved therapeutic options to treat histoplasmosis.

Facilities and Other Resources

Laboratory

The Rappleye lab has 970 ft^2 total laboratory space at Ohio State University located on the 5th floor of the Biological Sciences Building. This includes a 220 ft^2 HEPA-filtered air supply dedicated clean room for BSL-2 work with fungal pathogens.

Computer

The Rappleye lab has 5 desktop PCs (Pentium IV, i5, and i7 dual and quad core processors) and one laptop computer for instrument operation, bioinformatics analyses, data management, and word processing. An HP 1320n network printer is available for trainees. Access to extensive library resources at OSU is also available via the internet.

Office

Dr. Rappleye has 90 ft^2 of office space adjacent to his main laboratory.

Other Resources

Plant-Microbe Genomics Facility (http://pmgf.osu.edu/)

Located immediately downstairs from the Rappleye lab is the Plant-Microbe Genomics Facility that provides a wealth of "-omics" and sequencing services to OSU researchers at a significant discount. Major services and resources relevant to this proposal include:

- DNA sequencing (Sanger and capillary-based): 3730 DNA Analyzer (Applied Biosystems, Inc)
- Quantitative / real-time PCR: CFX96 Real-Time Detection System (BioRad)
- Next-generation / 454 pyrosequencing: GS FLX System (Roche)
- Robotic liquid handling station: Biomek FX 20-deck platform (Beckman)

Scientific Environment

Microbial pathogenesis and microbiology is a significant strength of the research community at Ohio State University. Dr. Rappleye has a dual appointment with the Department of Microbiology and the Department of Microbial Infection and Immunity. In addition, Dr. Rappleye is a member of the Center for Microbial Interface Biology and the Public Health Preparedness for Infectious Diseases initiative. Dr. Rappleye and trainees in his lab have numerous interactions with members of these departments and centers through weekly seminar series and graduate courses, as well as informal meetings.

Department of Microbiology (http://microbiology.osu.edu/)

The Department of Microbiology is comprised of a diverse group of scientists seeking to understand how microbes function and influence their environment. Faculty are experts in genetic, molecular genetic, and genomic approaches including application to organisms with difficult genetics: Dr. Chuck Daniels (genomics of archea bacteria), Tina Henkin (bacterial transcriptional controls), Dr. Mike Ibba (tRNA biology), Dr. Robert Munson (bacterial pathogenesis), Dr. John Reeve (molecular biology of archea), Dr. Natacha Ruiz (genetic dissection of the bacterial cell envelope), Dr. Tom Santangelo (transcriptional regulation in archea), Dr. Dan Wozniak (genetics and pathogenesis of *Pseudomonas*). In addition, Microbiology faculty provide a wealth of

expertise in microbial physiology and biochemistry upon which we can draw for understanding genes potentially discovered that are involved in *Histoplasma* intracellular metabolism: Dr. Birgit Alber (central carbon metabolism), Dr. Joseph Krzycki (biochemistry of archea), and Dr. Robert Tabita (biochemistry and energy pathways in bacteria and archea). The microbiology environment also includes research and expertise with eukaryotic microbes which provides unique eukaryotic perspectives that will benefit our research: Dr. Juan Alfonzo (*Trypanosome* biology) and Dr. Abhay Satoskar (*Leishmania* and immunology of leishmaniasis).

Department of Microbial Infection and Immunity (MII; http://medicine.osu.edu/mii/)

The Department of Microbial Infection and Immunity is part of the College of Medicine at Ohio State University. Laboratories of the MII are located in the Biomedical Research Tower which is adjacent to the building in which Dr. Rappleye's laboratory is located. Core members of the MII department include investigators with expertise in intracellular pathogens, innate immunity, and phagocyte biology including Dr. Larry Schlesinger (*Mycobacterium tuberculosis* pathogenesis and macrophage biology), Dr. John Gunn (*Salmonella* and *Francisella* pathogenesis), Dr. Amal Amer (*Legionella/Burkholderia* pathogenesis and autophagy in macrophages), Dr. Mark Drew (*Plasmodium* pathogenesis), Dr. Stephanie Seveau (*Listeria* pathogenesis and phagocyte cell biology), and Dr. Brian Ahmer (*Salmonella* genetics and pathogenesis), and Dr. Jordi Torrelles (*Mycobacterium* biochemistry). Members of Dr. Rappleye's lab attend bi-weekly work-in-progress meetings with MII faculty and trainees as well as weekly seminars on host-pathogenesis interactions.

Center for Microbial Interface Biology (CMIB; http://cmib.osu.edu/)

Campus-wide, there are over 70 faculty members in the Center for Microbial Interface Biology. Major themes of the CMIB are respiratory infectious diseases, intracellular parasitism, mucosal immunology, biofilms, and therapeutics. The CMIB hosts many seminars on topics central to host-pathogen interactions and infectious diseases. Access to the diverse areas of expertise among members of the CMIB, which ranges from proteomics to animal models of disease, is an invaluable resource for infectious disease work at Ohio State University.

Equipment

Rappleye laboratory

The main laboratory has equipment required for general microbiological, molecular, and biochemical work including microcentrifuges, agarose and polyacrylamide electrophoresis equipment, spectrophotometer, rocking and shaking platforms, bead-beater cell disruptor, and incubators (shaking and static).

Major equipment relevant to this proposal which are available include:

- Synergy 2 microplate reader (UV, visible, and fluorescence (GFP and RFP) capabilities; BioTek)
- epMotion robotic liquid handling station, 4-deck platform (Eppendorf) for setting up and processing 96-well plate-based assays
- Nikon Elcipse Ti deconvolution microscope with: 1.4 megapixel CCD camera (HQ^2, Photometrics), objectives (40X, 100X) for DIC and fluorescence (FITC/GFP, Texas-red/RFP, and DAPI filter sets)
- Nikon E400 microscope with: objectives (10X, 40X, 60X, 100X) configured for phase-contrast and fluorescence microscopy (FITC/GFP, Texas-red/RFP, and DAPI filter sets)
- (2) PCR machines: 96 sample capacity (Applied Biosystems)
- Realplex real-time PCR machine (Eppendorf)
- AlphaImager HP gel documentation and fluorescent imaging system with 1.4 megapixel CCD camera, excitation and emission filters for GFP and RFP (for imaging fluorescent colonies)
- 4°C, -20°C, -80°C storage units
- AirClean 600 nucleic acid workstation

The Rappleye lab BSL-2 room contains equipment and engineering safety for working with infectious agents:

- (2) class II biological safety cabinets
- (2) water-jacketed 37°C CO_2 incubator: 13 cu. ft. total capacity for fungal and tissue culture
- 7.5 cu ft CO_2-supplied shaking incubator for liquid fungal cultures (Multitron, ATR)
- inverted phase microscope (Olympus, 4X, 10X, 20X objectives)
- upright phase microscope (Leica, 10X, 40X, 100X objectives)
- electroporation apparatus (Bio-rad) for transformation of bacteria and fungi

Other University Resources

As a core member of the Center for Microbial Interface Biology, the Rappleye lab also has access to CMIB shared equipment including:

- Biomek robotic liquid handling station: 8-position
- ELISA plate reader (Biotek ELX800)
- Bio-Plex suspension array system (Bio-rad)

OMB Number: 4040-0001
Expiration Date: 06/30/2011

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: | * First Name: Chad | Middle Name: Alan
* Last Name: Rappleye | Suffix:
Position/Title: Associate Professor | Department: Microbiology
Organization Name: The Ohio State University | Division:
* Street1: 484 W. 12th Ave.
Street2: 540 Biological Sciences Bldg.
* City: Columbus | County/ Parish: Franklin
* State: OH: Ohio | Province:
* Country: USA: UNITED STATES | * Zip / Postal Code: 43210-1292
* Phone Number: [redacted] | Fax Number:
* E-Mail: [redacted]

Credential, e.g., agency login: [redacted]

*** Project Role:** PD/PI | **Other Project Role Category:**

Degree Type: PhD
Degree Year: 2002

***Attach Biographical Sketch** CRappleye_Biosketch1021035522 | Add Attachment | Delete Attachment | View Attachment
Attach Current & Pending Support | Add Attachment | Delete Attachment | View Attachment

PROFILE - Senior/Key Person 1

Prefix: | * First Name: | Middle Name:
* Last Name: | Suffix:
Position/Title: | Department:
Organization Name: | Division:
* Street1:
Street2:
* City: | County/ Parish:
* State: | Province:
* Country: USA: UNITED STATES | * Zip / Postal Code:
* Phone Number: | Fax Number:
* E-Mail:

Credential, e.g., agency login:

*** Project Role:** | **Other Project Role Category:**

Degree Type:
Degree Year:

***Attach Biographical Sketch** | Add Attachment | Delete Attachment | View Attachment
Attach Current & Pending Support | Add Attachment | Delete Attachment | View Attachment

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/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
Chad A. Rappleye	Associate Professor of Microbiology
eRA COMMONS USER NAME (credential, e.g., agency login) [redacted]	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Utah, Salt Lake City, UT	B.S.	06/95	Biology
University of California, San Diego, CA	Ph.D.	06/02	Biology
Washington University, St. Louis, MO	postdoc	08/06	Microbial pathogenesis

A. Personal Statement

My research seeks to understand the biology and pathogenic mechanisms of fungal pathogens. Of particular interest are the strategies employed by fungal pathogens to subvert immune defenses and to establish permissive replication niches in the host. The majority of my laboratory's efforts are directed at defining the molecular mechanisms that contribute to the virulence of the primary fungal pathogen *Histoplasma capsulatum*. To investigate the interactions between *Histoplasma* and the host, my laboratory uses molecular genetics, proteomics, and transcriptomics to identify and characterize factors contributing to *Histoplasma*'s ability to infect and grow within host phagocytes. The ability to disrupt gene functions is essential for demonstrating the role of candidate factors. We have pioneered techniques to knock-down and knock-out genes to facilitate these tests in *Histoplasma*, an organism notoriously recalcitrant to homologous recombination and gene replacement strategies.

My laboratory is one of only a handful of labs with the expertise required to molecularly dissect Histoplasma virulence. My work has defined and characterized the role of three of the 5 major virulence factors of *Histoplasma capsulatum* identified to date. These include an α-linked polysaccharide of the yeast cell wall that acts to conceal yeast from the host, a stress response factor that enables intracellular growth, and an efficient extracellular antioxidant system that protects *Histoplasma* from host-derived antimicrobial metabolites. We have identified the constituent proteins comprising the secreted proteome of pathogenic-phase *Histoplasma* cells as a basis for understanding how *Histoplasma* interacts with the host. In addition, we have completed the first transcriptome analysis of two strains of Histoplasma and comparison of the regulons of pathogenic and non-pathogenic phases. These resources will allow us to take advantage of the discoveries made in the current proposal and rapidly move from discovery to mechanism studies. We have expertise in physiologically relevant infection models including isolation and infection of human and murine phagocytes as well as in vivo infections (murine). This allows us to accurately model and investigate histoplasmosis disease.

B. Positions and Honors

Positions and Employment

2002-2006	Postdoctoral Fellow	Department of Molecular Microbiology Washington University, St. Louis, MO
2006-2012	Assistant Professor	Department of Microbiology

		Department of Internal Medicine, Division of Infectious Diseases Ohio State University, Columbus OH
2012-	Associate Professor	Department of Microbiology Department of Microbial Infection and Immunity Ohio State University, Columbus OH

Honors

1997-2002	Howard Hughes Medical Institute Predoctoral Fellowship
2002-2003	NIH/NHLBI Pulmonary Research Training Grant Award, Washington University, St. Louis, MO
2003-2006	Damon Runyon Cancer Research Foundation Postdoctoral Fellowship
2009-2010	Co-chair American Society of Microbiology Conference on Dimorphic Fungal Pathogens
2010-2011	Session convener, American Society of Microbiology General Meeting

C. Selected Peer-reviewed Publications

1. Rappleye CA, Engle JT, and Goldman WE. 2004. RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. Mol Microbiol 53(1):153-165.
2. Marion CL, Rappleye CA, Engle JT, and Goldman WE. 2006. An alpha-(1,4)-amylase is essential for alpha-(1,3)-glucan production and virulence in *Histoplasma capsulatum*. Mol Microbiol 62(4):970-983.
3. Rappleye CA, and Goldman WE. 2006. Defining virulence genes in the dimorphic fungi. Annu Rev Microbiol 60:281-303.
4. Almeida AJ, Carmona JA, Cunha C, Carvalho A, Rappleye CA, Goldman WE, Hooykaas PJ, Leao C, Ludovico P, and Rodrigues F. 2007. Towards a molecular genetic system for the pathogenic fungus *Paracoccidioides brasiliensis*. Fungal Genet Biol 44(12):1387-1398.
5. Krajaejun T, Gauthier GM, Rappleye CA, Sullivan TD, and Klein BS. 2007. Development and application of a green fluorescent protein sentinel system for identification of RNA interference in *Blastomyces dermatitidis* illuminates the role of septin in morphogenesis and sporulation. Eukaryot Cell 6(8):1299-1309. PMC1951135
6. Rappleye CA, Eissenberg LG, and Goldman WE. 2007. *Histoplasma capsulatum* alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor. Proc Natl Acad Sci U S A 104(4):1366-1370. PMC1783108
7. Holbrook ED, and Rappleye CA. 2008. *Histoplasma capsulatum* pathogenesis: making a lifestyle switch. Curr Opin Microbiol 11(4):318-324.
8. Youseff BH, Dougherty JA, and Rappleye CA. 2009. Reverse genetics through random mutagenesis in *Histoplasma capsulatum*. BMC Microbiol 9:236. PMC2781022
9. Edwards JA, Alore EA, and Rappleye CA. 2011. The yeast-phase virulence requirement for alpha-glucan synthase differs among *Histoplasma capsulatum* chemotypes. Eukaryot Cell 10(1):87-97. PMC3019800
10. Holbrook ED, Edwards JA, Youseff BH, and Rappleye CA. 2011. Definition of the Extracellular Proteome of Pathogenic-Phase *Histoplasma capsulatum*. J Proteome Res 10(4):1929-1943. PMC3069693
11. Edwards JA, Zemska O, and Rappleye CA. 2011. Discovery of a Role for Hsp82 in *Histoplasma* Virulence through a Quantitative Screen for Macrophage Lethality. Infect Immun 79(8):3348-3357. PMC3147571
12. Edwards JA, and Rappleye CA. 2011. *Histoplasma* mechanisms of pathogenesis - one portfolio doesn't fit all. FEMS Microbiol Lett 324(1):1-9. PMC3228276
13. Youseff BH, and Rappleye CA. 2012. RNAi-based gene silencing using a GFP sentinel system in *Histoplasma capsulatum*. *In* Brand, A, and MacCallum D (eds.), Host-Fungal Interactions: Manipulation of Fungal Gene Expression, Methods in Molecular Biology, 845:51-66.

14. Zemska O, and Rappleye CA. 2011. *Agrobacterium*-mediated insertional mutagenesis in *Histoplasma capsulatum* *In* Brand, A, and MacCallum D (eds.), Host-Fungal Interactions: Manipulation of Fungal Gene Expression, Methods in Molecular Biology, 845:151-164.

15. Youseff BH, Holbrook ED, Smolnycki KA, Rappleye CA. 2012. Extracellular Superoxide Dismutase Protects Histoplasma Yeast Cells From Host-Derived Oxidative Stress. PLoS Pathogens 8(5):e1002713. PMC3355102

16. Kemski MM, Stevens B, and Rappleye CA. 2013. Spectrum of T-DNA Integrations for Insertional Mutagenesis of *Histoplasma capsulatum*. Fungal Biol 117(1):41-51. PMC 3552300

17. [redacted]

D. Research Support

Ongoing Research Support

R01-AI083335 Rappleye 8/1/2009 – 7/31/2014
NIH / NIAID
Virulence factor discovery in the secreted proteome of *Histoplasma capsulatum*
The major goals of this project are to define the major factors secreted by pathogenic-phase *Histoplasma* cells and to define their potential roles in promoting *Histoplasma* pathogenesis.
Role: PI

CCTS Pilot Grant Award: Rappleye / Tjarks 8/17/2102 - 8/16/2013
NIH / NCRR (UL1RR0025755 PI: Jackson)
Lead candidate antifungal drug development
The goal of this project grant is to focus on integrating the traditional biomedical research process (basic scientific discovery to translation into clinical research to dissemination into the community for improved patient outcomes).
The goal of the pilot grant award, administered through the Center for Clinical and Translational Science (CCTS) at OSU, is to advance a hit compound with potent antifungal activity and to establish basic structure-activity relationships.
Role: PI

Completed Research Support

[redacted]

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:
* First Name: Chad
Middle Name: Alan
* Last Name: Rappleye
Suffix:

2. Human Subjects

Clinical Trial? ☐ No ☐ Yes

* Agency-Defined Phase III Clinical Trial? ☐ No ☐ Yes

3. Applicant Organization Contact

Person to be contacted on matters involving this application

Prefix:
* First Name: Tamara
Middle Name:
* Last Name: Dickey
Suffix:

* Phone Number: [redacted]
Fax Number:
Email: [redacted]

* Title: Sponsored Programs Officer

* Street1: 1960 Kenny Road
Street2:
* City: Columbus
County/Parish: Franklin
* State: OH: Ohio
Province:
* Country: USA: UNITED STATES
* Zip / Postal Code: 43210-1016

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells

* Does the proposed project involve human embryonic stem cells? ☒ No ☐ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/research/registry/. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): ☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Tracking Number:GRANT11428274 Funding Opportunity Number:PA-11-262 Received Date:2013-06-14T11:27:52-04:00

PHS 398 Modular Budget

OMB Number: 0925-0001

Budget Period: 1

Start Date: 04/01/2014 End Date: 03/31/2015

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A	[redacted]
Consortium F&A	
Total Direct Costs	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	On Campus Organized Research	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) DHHS, Ernest Kinneer, [redacted]

Indirect Cost Rate Agreement Date 09/21/2012

Total Indirect Costs [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) [redacted]

Budget Period: 2

Start Date: 04/01/2015 End Date: 03/31/2016

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A	[redacted]
Consortium F&A	
Total Direct Costs	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	On Campus Organized Research	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number) DHHS, Ernest Kinneer, [redacted]

Indirect Cost Rate Agreement Date 09/21/2012

Total Indirect Costs [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($) [redacted]

PHS 398 Modular Budget

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium F&A for Entire Project Period	$	[redacted]
Section A, Total Consortium F&A for Entire Project Period	$	
Section A, Total Direct Costs for Entire Project Period	$	[redacted]
Section B, Total Indirect Costs for Entire Project Period	$	[redacted]
Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period	$	[redacted]

2. Budget Justifications

?	Personnel Justification	Mutant_Screen___Personnel10210	Add Attachment	Delete Attachment	View Attachment
?	Consortium Justification		Add Attachment	Delete Attachment	View Attachment
?	Additional Narrative Justification		Add Attachment	Delete Attachment	View Attachment

Budget Justification

1. Personnel

Salary calculations are based on actual salaries. Fringe benefits, including mandatory contributions to the State of Ohio retirement systems, health insurance, worker's compensation, Medicare tax, and vacation and sick leave costs, as appropriate, are calculated as a percentage of salary and based on employment classification.

Chad A. Rappleye, Ph.D.

Principal Investigator (0 academic months, 0.6 summer months)
Dr. Rappleye will be responsible for administration of the project, experimental design, and analysis and interpretation of results. Dr. Rappleye provides expertise and experience in fungal biology, fungal genetics, and in vitro and in vivo models of infection. Salary support equivalent to 5% annual effort for Dr. Rappleye is requested.

Andrew Garfoot, B.S.

Graduate Research Associate (6 academic months)
Mr. Garfoot will be responsible for mutagenesis and for performance of the genetic screen in Aim 1. The graduate student will also be responsible for recording and analyzing experimental data as well as microbial culture of the fungal strains. Mr. Garfoot will conduct the mapping experiments in Aim 2. Salary and tuition support equivalent to 50% annual effort is requested.

Research assistant (to be appointed)

Research Assistant (3.6 calendar months)
A Research Assistant (bachelor degree-holding) will be hired to assist in the preparation and execution of the genetic screen (Aim 1). In addition, the research assistant will be responsible for maintenance of macrophage lines and set up of virulence assays. Salary support equivalent to 30% annual effort for this individual is requested.

2. Variations in modules requested

There are no variations in budget modules.

OMB Number: 0925-0001

PHS 398 Research Plan

1. Application Type:

From SF 424 (R&R) Cover Page. The response provided on that page, regarding the type of application being submitted, is repeated for your reference, as you attach the appropriate sections of the Research Plan.

*Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

2. Research Plan Attachments:

Please attach applicable sections of the research plan, below.

1. Introduction to Application (for RESUBMISSION or REVISION only)		Add Attachment	Delete Attachment	View Attachment
2. Specific Aims	Mutant_Screen___Specific_Aim	Add Attachment	Delete Attachment	View Attachment
3. *Research Strategy	Mutant_Screen___Proposal102	Add Attachment	Delete Attachment	View Attachment
4. Inclusion Enrollment Report		Add Attachment	Delete Attachment	View Attachment
5. Progress Report Publication List		Add Attachment	Delete Attachment	View Attachment

Human Subjects Sections

6. Protection of Human Subjects		Add Attachment	Delete Attachment	View Attachment
7. Inclusion of Women and Minorities		Add Attachment	Delete Attachment	View Attachment
8. Targeted/Planned Enrollment Table		Add Attachment	Delete Attachment	View Attachment
9. Inclusion of Children		Add Attachment	Delete Attachment	View Attachment

Other Research Plan Sections

10. Vertebrate Animals		Add Attachment	Delete Attachment	View Attachment
11. Select Agent Research		Add Attachment	Delete Attachment	View Attachment
12. Multiple PD/PI Leadership Plan		Add Attachment	Delete Attachment	View Attachment
13. Consortium/Contractual Arrangements		Add Attachment	Delete Attachment	View Attachment
14. Letters of Support		Add Attachment	Delete Attachment	View Attachment
15. Resource Sharing Plan(s)		Add Attachment	Delete Attachment	View Attachment

16. Appendix Add Attachments | Remove Attachments | View Attachments

Specific Aims

The fungal pathogen *Histoplasma capsulatum* causes an estimated 100,000 infections annually in the United States. While most infections are self limiting upon activation of adaptive immunity, thousands each year are hospitalized due to acute respiratory disease and life-threatening disseminated histoplasmosis. Unlike opportunistic fungal pathogens, *Histoplasma* causes disease even in immunocompetent individuals. By itself, the innate immune system is unable to control *Histoplasma* yeasts due to *Histoplasma's* ability to parasitize host phagocytes. The mechanisms that enable *Histoplasma* to survive and replicate with macrophages, ultimately leading to destruction of the phagocyte, are only beginning to be defined.

As the *Histoplasma*-macrophage interaction is key to pathogenesis, our goal is to better understand the factors that enable intracellular growth of *Histoplasma*. Forward genetics is a powerful approach to identify new factors if an efficient mutagen and screen are employed. We have optimized and characterized an insertional mutagen for *Histoplasma* based on *Agrobacterium*-mediated transfer and random integration of T-DNA into fungal chromosomes. In addition, we have developed a high-throughput screen to facilitate identification of mutants unable to persist in the intramacrophage environment. For this, we developed an RFP-fluorescent *Histoplasma* strain and a transgenic *lacZ*-expressing macrophage cell line which permits quantitative monitoring of both intracellular yeast replication and macrophage destruction, respectively. The combination of these mutagenesis and screening advances provides the efficiency necessary for forward genetics-based discovery of new virulence factors that enable *Histoplasma* to overcome innate immune defenses and exploit the macrophage as its host cell.

Aim 1. Screen *Histoplasma* T-DNA insertion mutants for attenuated virulence in macrophages.

Aim 1A. Generate a library of T-DNA insertion mutants in *Histoplasma* yeast.
Agrobacterium-mediated transformation will be used to mutagenize *Histoplasma* yeasts. Individual mutants will be arrayed into 96-well plates to facilitate high-throughput screening and to enable banking of the mutant collection for long term preservation. A library of 40,000 mutants will be generated representing approximately 2.5-fold coverage of the *Histoplasma* genome.

Aim 1B. Identification of mutants deficient in survival and replication within macrophages.
Macrophages will be infected with individual *Histoplasma* mutants and the intramacrophage growth of yeast monitored non-destructively by measurement of yeast-expressed RFP fluorescence. End point macrophage lysis by yeast will be determined by quantifying the remaining macrophage-expressed β-galactosidase activity. *Histoplasma* mutants will be selected that exhibit at least 50% reduction in intramacrophage growth and/or at least 50% decreased ability to lyse macrophages

Aim 2. Determine the identify of genes required for *Histoplasma* virulence in macrophages.

Aim 2A. Map the disrupted loci in attenuated mutants.
Mutants will be tested by PCR to eliminate those with T-DNA disruption of genes known to be required for intramacrophage survival and growth. New virulence genes will be identified by mapping the T-DNA insertions through hemi-specific PCR techniques (e.g., thermal asymmetric interlaced PCR) and sequencing of the amplified regions flanking the T-DNA borders. Disrupted loci will be identified by comparison of sequences flanking the insertion to transcriptome-based gene models (best option) or de novo gene predictions (alternative).

Aim 2B. Classify and prioritize virulence mutants.
Mutants will be classified as: (1) deficient in macrophage entry, (2) impaired survival in macrophages, (3) normal survival but impaired replication in macrophages, and (4) normal replication but deficient ability to cause macrophage lysis. Candidate factors representing each class will be prioritized by the severity of the virulence attenuation, conservation of the factor among intracellular pathogens, and increased expression by pathogenic- compared to non-pathogenic-phase cells.

The virulence genes identified will form the basis of future studies to characterize the factors that promote *Histoplasma* pathogenesis in host macrophages.

1. SIGNIFICANCE

Fungal pathogens. Invasive and systemic fungal infections continue to cause significant morbidity and mortality, causing more deaths in the United States than tuberculosis [1,2]. Infection by most fungal pathogens occurs via the respiratory tract, resulting in varying degrees of respiratory and systemic disease dependent on dose, immunological status of the host, and virulence of the pathogen. In contrast to opportunistic fungi such as *Candida* and *Aspergillus*, certain dimorphic fungal pathogens cause disease even in immunocompetent individuals, which is reflected in the fact they constitute the most common pulmonary fungal infection among otherwise healthy individuals [3,4]. The majority of hospitalizations due to infections by dimorphic fungal infections are caused by *Histoplasma capsulatum* [4] with up to 90% of individuals residing in endemic regions having serological evidence of prior infection with *Histoplasma* [5]. Whereas *Candida* and *Aspergillus* cells are readily controlled and eliminated by innate immune cells of the immunocompetent host (e.g., phagocytes), the innate immune system alone is insufficient to clear infections by *Histoplasma* [6–8].

***Histoplasma* as an intracellular pathogen of macrophages**. Macrophages provide one of the first lines of protection against fungal infections, yet *Histoplasma* yeasts effectively parasitize these innate immune cells. *Histoplasma* yeast enter into and survive within these normally fungicidal cells. This ability results, in part, from expression of virulence factors that effectively conceal *Histoplasma* yeast from pathogen recognition receptors of the host and production of defense strategies that enable *Histoplasma* yeasts to combat antifungal products produced by host immune cells (e.g., reactive oxygen compounds). Accomplishment of these tasks is followed by establishment of an intracellular niche permissive for yeast growth and replication, which includes blocking phagosome acidification and activation of lysosomal hydrolases [9]. Replication of intracellular yeasts ultimately leads to lysis of the macrophage and spread of *Histoplasma* to additional phagocytes. Since the *Histoplasma*-macrophage interaction is central to *Histoplasma* pathogenesis and *Histoplasma's* ability to establish infections in hosts with functional innate immunity, our goal is to better understand the factors that bias the outcome of this interaction in *Histoplasma's* favor.

2. INNOVATION

Current scarcity in defined virulence mechanisms. Although the characteristics of *Histoplasma's* infection of macrophages have been well described, our understanding at a mechanistic level remains quite limited. In contrast to fungi with more facile genetics, techniques for manipulating gene expression (e.g., elimination of gene function, expression of transgenes, etc.) in *Histoplasma* are relatively recent developments. Five central virulence factors have been defined for *Histoplasma*: cell wall α-glucan [10,11], the secreted Cbp1 protein [12], production of siderophores [13,14], the extracellular Yps3 protein [15], and an extracellular ROS-defense system [16,17]. Of these, all were identified through reverse-genetic approaches in which the candidate factor was initially selected based on characteristics suggesting a role in virulence. For example, Cbp1, Yps3, and α-glucan are specifically produced by the pathogenic-form (i.e., yeast) but not the mycelial form of *Histoplasma*. Siderophores and the superoxide dismutase/catalase antioxidant system are secreted factors that were hypothesized to interact with host factors as a consequence of their extracellular localization and putative function. Although a profitable approach, reverse genetics relies heavily on initial guesses regarding the virulence factors. Once candidates are selected, the difficult genetics of *Histoplasma* necessitates large efforts to generate the gene knock-outs or knock-downs required for functional demonstration of their role in virulence.

Forward genetics approach to *Histoplasma* virulence. Application of forward genetics for dissection of *Histoplasma* pathogenesis provides an excellent approach to discovery of virulence factors. Time and again, forward genetics has been instrumental in establishing the molecular components that contribute to a biological process. From identification of regulators controlling the cell cycle to discovery of genes controlling programmed cell death or body patterning, the ability to identify a gene through mutant phenotypes has provided key footholds necessary for developing a mechanistic understanding [18–20]. Often these factors and the mechanisms to which they contribute have been unsuspected gene products that would otherwise have been missed. In addition to the lack of assumptions or hypothesis bias in selecting candidate factors, a forward genetics approach immediately provides the mutant strains necessary for further functional tests.

We propose to discover new virulence factors contributing to *Histoplasma* pathogenesis through a forward genetics approach to identify mutants defective for virulence in macrophages. Towards this goal, we have

optimized insertional mutagenesis methodologies and have developed high-throughput screening techniques necessary for detection of intramacrophage virulence phenotypes. These innovations include the generation of colorimetric and fluorescent reporter lines of macrophages and yeast, respectively, to efficiently quantify intramacrophage yeasts and their ability to lyse host cells. Unlike virulence screens using solid-media plate phenotypes or unnatural hosts, we will use the actual host cell (i.e., the macrophage) in our screens. Some limited success with forward genetics in *Histoplasma* has been achieved in defining factors that control the morphological switch from mycelia to yeast as well as factors that contribute to environmental and temperature stresses [21,22]. However, this proposal represents the first large-scale effort to generate a comprehensive library of *Histoplasma* mutants that define the factors required for infection, intracellular survival and growth, and lysis of host macrophages.

3. APPROACH

3A. PRELIMINARY STUDIES

T-DNA as an insertional mutagen. The use of a mutagen that provides random mutations as well as rapid mapping of the mutations is paramount to the success of a forward genetics screen. *Agrobacterium* has been utilized to transform or mutagenize many different fungal species through transfer and integration of a DNA element known as transfer DNA ("T-DNA"; [23,24]). We and others have shown that *Agrobacterium*-mediated transformation can be used as a mutagen for *Histoplasma* [22,25–29]. Insertional mutagens have the advantage of facilitating rapid mapping of the integration site by providing a known sequence anchor that can be used to isolate unknown sequences flanking the insertion element (i.e, regions adjacent to the T-DNA).

To optimize the mutagenesis efficiency, we examined different T-DNA elements and different *Agrobacterium* strains. Transformation with some T-DNA elements results in a high frequency of carryover of non-T-DNA vector sequences beyond the left border (LB) and right border (RB) ends of the T-DNA element [30–32]. As mapping of the T-DNA relies upon having *Histoplasma* genomic sequence immediately adjacent to known ends of the T-DNA element (see below), transfer of vector backbone or extra sequences frustrates efforts to identify the chromosomal site of T-DNA integration. We determined that the T-DNA element carried on plasmid pBHt2 provides the highest efficiency of transformation (79 ± 20 per $5x10^7$ yeast) and the lowest frequency of vector backbone carryover (8%) [26]. In addition, we determined that transfer of the T-DNA element on pBHt2 preserves the LB and RB T-DNA ends in 87% and 94% of integration events. The T-DNA element on pBHt2 carries the hygromycin phosphotransferase gene (*hph*) for hygromycin resistance-based selection of transformants in which the T-DNA element has stably integrated (Figure 1).

The precision of T-DNA integration enables the known-sequence of the LB and RB ends to be used as anchors for mapping of the T-DNA insertions. We have used thermal asymmetric interlaced PCR ("TAIL-PCR", [33]), a hemi-specific PCR technique, to efficiently map the location of integrated T-DNA in *Histoplasma* [26,34]. A nested series of primers matching the LB and RB ends is combined with random degenerate primers that allow for PCR-based amplification of sequences immediately flanking the T-DNA LB and RB borders (Figure 1). Proximal ends of the recovered sequences are matched to the T-DNA to ensure the PCR products are anchored in the T-DNA ends and the remaining sequence is matched to the *Histoplasma* genome sequence to provide information about the chromosomal context. We have successfully mapped over 100 T-DNA insertions using this procedure [26,34].

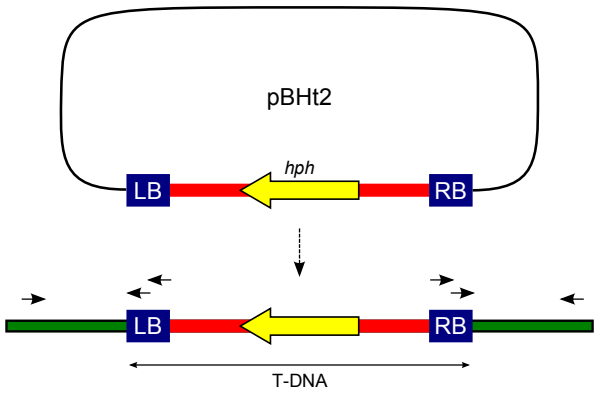

Figure 1. Schematic of the T-DNA element on plasmid pBHt2 and following integration. The T-DNA element is bounded by left- and right border sequences (LB and RB). Short arrows indicate the nested LB- and RB-specific and the degenerate PCR primers used to amplify the *Histoplasma* genome sequence (green) flanking the T-DNA insertion.

Integration of the T-DNA element from pBHt2 into the *Histoplasma* chromosome is random, an essential characteristic for unbiased mutagenesis. We mapped and examined the individual chromosomal location of the T-DNA element in 70 randomly selected *Histoplasma* transformants to determine the integration

characteristics [26]. We found no evidence of insertional hotspots in the *Histoplasma* genome (defined as multiple insertions within 5000 base pairs of each other). While T-DNA integration is random relative to different genetic loci, the site of integration relative to an individual coding sequence often occurs in the promoter region of the gene (36% of integrations; [26]). Nonetheless, we have demonstrated that integrations into regions upstream of genes (promoters), as well as integrations into introns, effectively disrupt gene function thereby expanding the useful target region for mutagenesis beyond the coding sequence itself [22,26,35]. These data validate the use of T-DNA integration as a random insertional mutagen for *Histoplasma* and establish the T-DNA element from pBHt2 as the optimal element with good transformation efficiency and good fidelity of integration (minimal LB and RB truncation and minimal carryover of backbone sequences flanking the LB and RB ends) permitting rapid mapping of T-DNA integrations.

Development of a high-throughput macrophage-based virulence screen. The second requirement of a forward genetics approach is a relevant and efficient screen for the phenotype of interest. The primary limitation of screens for attenuated virulence is the difficulty of screening large numbers of individual mutants directly for decreased virulence. Since infection and residence within macrophages is the central feature of *Histoplasma* pathogenesis, we developed technologies to rapidly screen for mutants defective for intramacrophage growth. The end result of successful macrophage infection and yeast replication is lysis of the macrophage. Thus, yeast virulence in macrophages is inversely proportional to the number of surviving macrophages. This permits identification of yeasts with attenuated virulence based on their inability to reduce macrophage numbers following co-culture of yeasts and macrophages. The following criteria guided our assay design efforts: (1) the assay must measure only macrophage numbers in a mixed population of macrophages and yeast, (2) the assay must be sensitive and quantitative in order to detect partial defects in virulence, and (3) the assay should require minimal manipulations to permit high-throughput screening. To meet these requirements, we engineered macrophages to constitutively express a *lacZ* transgene (P388D1-*lacZ* cells) which can be readily assayed using a one-step lysis and assay buffer in combination with a chromogenic (ONPG; o-nitrophenyl-galactopyranoside; Figure 2) or fluorescent (FDG; fluorescein digalactoside) enzyme substrate [22]. Based on the observed assay variability following infection with wild type *Histoplasma* yeast, we calculated that the sensitivity of the assay is sufficient to discriminate 15% decreased rate of macrophage killing by yeast (i.e., virulence attenuation) with 90% confidence (i.e., 90% statistical power). As proof of principle, we demonstrated that the virulence attenuation of *Histoplasma* yeasts lacking the known Cbp1 virulence factor is accurately recapitulated and quantified with LacZ expressing macrophages [22].

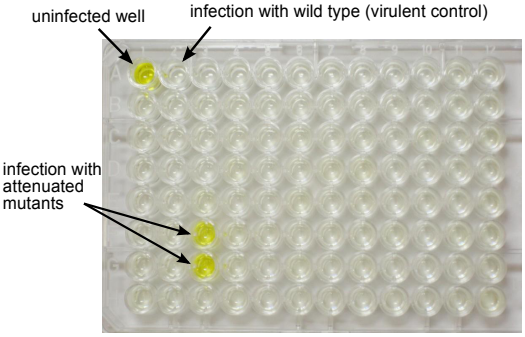

Figure 2. Identification of attenuated mutants using the *lacZ* -macrophage screen . After 6 days of infection with different mutants, remaining macrophages were quantified by LacZ-based conversion of ONPG to its yellow product. Arrows indicate uninfected and wild-type controls as well as two attenuated mutants identified by their inability to reduce the macrophage popuation (resulting in high LacZ activity)

Using the macrophage *lacZ* screen, we have performed pilot screens of T-DNA-mutagenized *Histoplasma* yeasts. Individual yeast transformants were picked into wells of a 96-well plate and grown for 48 hours. Yeasts were diluted and used to infect P388D1-*lacZ* macrophages at a multiplicity of infection (MOI) of 1:1 (yeasts to macrophages) in 96-well plates. We have screened 3478 independent *Histoplasma* mutants and have identified 21 mutants with significantly reduced ability to lyse macrophages (at least 30% attenuation compared to infections by virulent control *Histoplasma* yeasts; Table 1) and whose attenuated phenotype was maintained in repeat assays. Among the mutants isolated to date is an insertion in the promoter of the *CBP1* gene (mutant 12B4) confirming the mutagenesis and screen will identify mutants with attenuated virulence. The virulence mutant isolation frequency is 0.76% ± 0.30% (n=8). Mapping of the integration events in these mutants is ongoing as is work to complement the mutations in order to confirm the phenotype is linked to the mutation. We have already characterized one mutant isolated from the

Table 1: Attenuated mutants

mutant	attenuation	locus
3G3	83%	*VPS41*
4A7	74%	*VAT1*
12B4	71%	*CBP1*
2C10	70%	*PBS2*
8D11	70%	*HCL1*
8G9	66%	*HYP*
7A9	64%	*PEX10*
5D3	60%	*HYP*
5G4	58%	*PCK1*
15C2	57%	*(tbd)*
5C12	56%	*HYP*
5F3	55%	*HYP*
13A6	51%	*VMA2*
16E1	50%	*HSP82*
8B1	44%	*HYP*
2F2	43%	*PEX14*
5E1	43%	*GPR1*
1C8	42%	*(tbd)*
2E10	35%	*GCN2*
4C1	33%	*DDP1*
4A6	31%	*DSC2*

screen with an insertion in the *HSP82* promoter and have demonstrated that the 82 kDa heat shock protein is required for full *Histoplasma* virulence [22]. Importantly, this work also confirms that mutation of genes required for virulence in macrophages translates into attenuated virulence in vivo. Notable, even in this small collection of mutants, are a number hypothetical or genes of unknown function ("*HYP*"; Table 1).

Fluorescent reporter yeast to quantify intramacrophage growth. To facilitate more rapid classification of the virulence defects associated with mutants identified using the macrophage screen, we developed a non-destructive method for quantifying intramacrophage yeasts. Impaired ability of yeasts to destroy macrophages could result from impaired entry, decreased survival in macrophages, defects in intramacrophage replication, or loss of the ability to trigger macrophage lysis. The number of intracellular yeast can be used to distinguish between these classes as low, declining, static, or increasing numbers of intracellular yeasts, respectively. Typically quantification of intracellular yeasts requires plating of macrophage lysates to enumerate yeast colony forming units (CFU). However, this is not practical for high-throughput screens as it is an endpoint assay due to destruction of the macrophages to recover the yeast and it requires substantial manual processing of each sample. To overcome these restrictions, we engineered *Histoplasma* yeast to constitutively express the tandom-dimer red-fluorescent protein (tdTomato-RFP) which permits quantification of intracellular yeasts directly in macrophages based on the total RFP-fluorescence in wells with infected macrophages (Figure 3; [36]). As the fluorescence can be quantified without destruction of the macrophages, the kinetics of intracellular yeast replication can be readily assessed. We have confirmed that RFP fluorescence is proportional to yeast cell numbers and that RFP fluorescence reports intracellular replication (Figure 4). Most importantly, the RFP-fluorescent *Histoplasma* strain has identical entry rate, intracellular survival, and growth within macrophages as wild-type yeasts (Figure 5). The ability to monitor intracellular replication of yeasts and the quantification of macrophage killing by yeasts described above combine to create a powerful screen to identify and classify *Histoplasma* mutants with impaired virulence in macrophages.

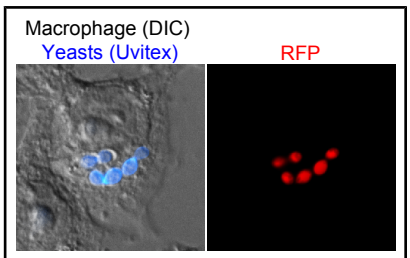

Figure 3. Intramacrophage RFP-fluorescent *Histoplasma* yeasts.

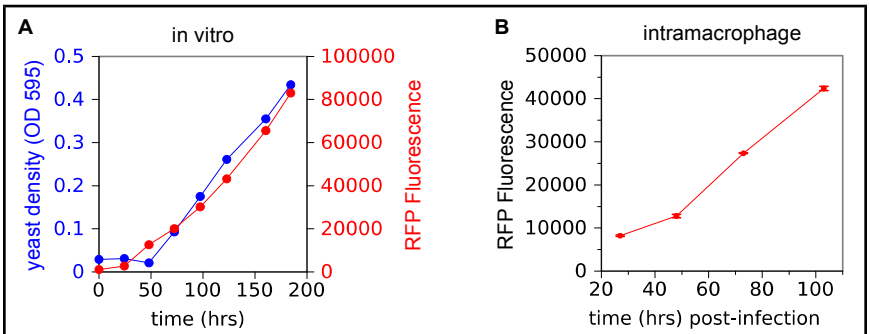

Figure 4. RFP fluorescence can be used as a surrogate means to quantify yeasts in vitro and in macrophages. (A) Liquid cultures of RFP-fluorescent yeasts were measured for yeast density (turbidity at 595 nm) and RFP fluorescence. (B) RFP-fluorescence of intramacrophage yeasts following macrophage infection.

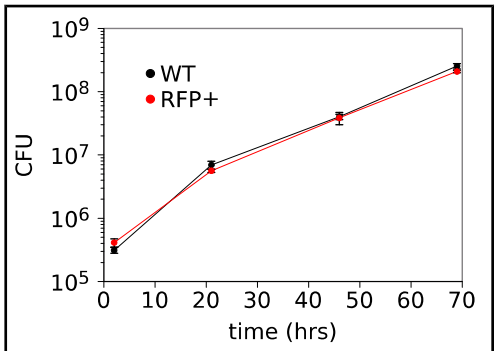

Figure 5. RFP-fluorescent yeasts have equivalent survival and growth in macrophages compared to wild-type yeasts . Data indicates the viable yeast CFUs recovered from macrophages over time.

3B. Experimental Approach

Aim 1. Screen *Histoplasma* T-DNA insertion mutants for attenuated virulence in macrophages.

Rationale. To discover new factors contributing to *Histoplasma* virulence in macrophages, we will perform a large scale genetic screen designed to identify mutants with attenuated virulence. In preliminary studies, we satisfied the two major requirements for our forward genetics approach, namely (1) use of an optimal mutagen (i.e., random insertional mutagenesis by T-DNA integration) and (2) development of a relevant phenotypic screen, which is amenable to high-throughput through the use of macrophage and yeast reporter backgrounds.

Aim 1A. Generate a library of T-DNA insertion mutants in *Histoplasma* yeast. *Agrobacterium*-mediated transformation of *Histoplasma* will be used as the insertional mutagen. The characterized T-DNA element on vector pBHt2 will be used as it provides high transformation efficiency (i.e., mutagenesis) and good fidelity of T-

DNA integration. The RFP-fluorescent *Histoplasma* yeast strain will be used as the genetic background for the mutagenesis as it provides a means of quantifying intramacrophage yeast replication. Yeast cells will be transformed by co-cultivation of *Agrobacterium* and *Histoplasma* yeasts after which yeast transformants will be selected on media with hygromycin. Individual transformants will be picked into *Histoplasma* growth medium and arrayed into 96-well plates. Aliquots of each 96-well plate will be frozen at -80°C for long term preservation of the mutant library and for a resource for other researchers to use in other screens. The arrayed library will also benefit targeted mutation approaches in which the library can be screened by PCR for insertions in specific genes of interest [35].

Table 2: Transcriptome statistics

genome size	41 Mb
total genes	9359
avg CDS length	2041 bp
avg exons / gene	3.1
gene density / 10 kb	2.3

The purpose of the large scale screen is to recover at least one mutation in each gene contributing to virulence. To estimate the number of mutants necessary for 2.5-fold coverage of the encoded genes in the *Histoplasma* genome, we used parameters on the number and size of genes that were determined from our recently completed transcriptome project using multiple strains and growth conditions (Table 2). We estimate the size of the average genetic locus (promoter plus the gene) to be approximately 2600 base pairs. This number encompasses the effective target region for gene disruptions since insertions in promoters and introns as well as the gene proper are also effective in preventing gene functions. The average gene in *Histoplasma* consists of 3 exons and 2041 base pairs of coding sequence (Table 2). To this, 500 base pairs were added as a conservative estimate of a typical promoter based on data from promoter-reporter gene fusions constructed in our laboratory (unpublished data). 80% of introns in Histoplasma range from 54 to 170 base pairs based on our transcriptome analysis (unpublished data), and an additional 50 base pairs for each of two introns were included. Using 2600 base pairs as an effective target region for gene-disrupting T-DNA integrations, a single mutation in each 2600 base pair region of the genome requires 15,769 mutants. 39,423 mutants provides for 2.5-fold coverage. This number is clearly an overestimate of the mutants needed as the number of non-essential genes (and thus recoverable mutations in vitro) is much lower. We plan to generate a library of approximately 40,000 mutants. This will require approximately 400 96-well plates which can be feasibly accomplished in about 1 year by manually arraying 8 to 10 96-well plates of mutants per week.

Aim 1B. Identification of mutants deficient in survival and replication within macrophages. Recovered mutants arrayed into 96-well plates will be used to infect P388D1-*lacZ* macrophages at an MOI of 1:1. After 2 hours, the medium will be replaced to remove non-internalized yeast (2 hours is sufficient for internalization of >90% of yeast). Yeast replication will be monitored daily over 6 days by scanning wells with infected macrophages for *Histoplasma* yeast-expressed RFP fluorescence. After 6 days, the relative number of surviving macrophages will be quantified by measurement of the macrophage-expressed *LacZ* activity. Results will be compared to control wells present on each plate that include (1) uninfected macrophages, (2) macrophages infected with non-mutagenized parental RFP-fluorescent yeast (virulent control), and (3) macrophages infected with a *cbp1* mutant strain (attenuated control). Yeast replication results will be compared to the virulent and attenuated control to identify mutants deficient for intracellular survival and replication and those showing at least 50% reduction in intramacrophage yeast proliferation will be selected for secondary screens. Mutants showing at least a 50% reduction in the ability to destroy macrophages compared to the virulent control will also be selected for follow-up. Presumably, there will be significant overlap in these collections. Selected mutants will be recovered from the inoculum plates and the virulence phenotypes confirmed by infection of macrophages in triplicate and at multiple MOIs.

Aim 2. Determine the identify of genes required for *Histoplasma* virulence in macrophages.

Rationale. Based on the frequency of attenuated mutant isolation observed in preliminary studies (0.76%) and the proposed number of mutants to be generated (40,000), we expect isolation of approximately 300 mutants. These will need to be prioritized for further study and characterization. Prioritization will be based on the identify of the genes disrupted and their putative functions as well as classification and ranking of the severity of the mutant phenotypes.

Aim 2A. Map the disrupted loci in attenuated mutants. The identity of loci required for full virulence in macrophages will be determined by mapping of the T-DNA integrations in attenuated mutants. To rapidly identify mutants representing known virulence factors, mutants will be initially screened by PCR for T-DNA insertions in the *CBP1* (Cbp1 virulence factor), *SOD3* (extracellular superoxide dismutase), *CATB* (extracellular catalase), *SID1* (siderophore biosynthesis), *SRE1* (siderophore biosynthesis), and *YPS3* (secreted factor) genes. Mutations in new genes will be mapped by TAIL-PCR using DNA prepared from each mutant and primers directed outward from the LB and RB ends of the T-DNA. The PCR products generated, representing sequences flanking the T-DNA element, will be matched to the G217B genome sequence [37] using the BLAST algorithm. The identify of the disrupted genes (or integration into their respective promoters) will be determined by mapping the position of T-DNA integration with that of gene models as determined by transcriptome sequencing (unpublished data). This analysis will also enable identification of those mutants representing multiple insertions in a single locus. Putative virulence gene functions will be determined by BLAST of the disrupted locus to existing protein databases to identify homologous genes as well as GO-term annotation of the transcriptome.

Aim 2B. Classify and prioritize virulence mutants. Attenuated mutants will be classified based on their intramacrophage growth phenotype. It is anticipated that attenuated mutants will be deficient to varying degrees in their ability to cause lysis of the macrophage host cell and this will be the primary measure of virulence attenuation. Further classification of these mutants will include: defects in macrophage entry (low RFP fluorescence) defects in intramacrophage *Histoplasma* survival (declining RFP fluorescence during the infection time course), defects in intramacrophage growth (static but not declining RFP fluorescence), and defects in causing macrophage lysis (increasing RFP fluorescence yet lack of significant effects on macrophage survival) (Figure 6). Secondary ranking of mutants will be done according to the severity of the defect in intramacrophage yeast growth kinetics as reflected by RFP fluorescence. For initial rankings, mutants that show impaired growth outside of macrophages (i.e., in liquid culture) will be lowered in priority.

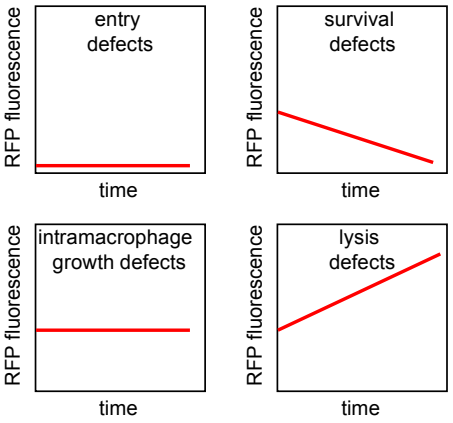

Figure 6. Predicted intramacrophage yeast RFP fluorescence kinetics by mutant class.

The most severely attenuated mutants will be further prioritized according to the candidate gene identities and their potential connections to virulence. For example, secreted factors (as predicted by the Signal P algorithm [38]) will be prioritized since extracellular proteins are strong candidates for factors that directly interact with and potentially modulate the host cell. As the goal of this study is to identify unsuspected factors, novel genes (or genes without a known function) will also be selected. Two criteria will be used to prioritize novel factors: conservation of the factor among intracellular pathogens and preferential expression by pathogenic *Histoplasma* yeast compared to the non-virulent mycelial phase *Histoplasma* cells. Transcriptional profiles will be determined using the *Histoplasma* yeast- and mycelial phase transcriptomes recently assembled by our laboratory (unpublished data). From these criteria we anticipate selecting the top 10% of virulence genes for further study.

4. FUTURE DIRECTIONS.

The virulence genes identified through this forward genetics approach will be further characterized in future studies to describe the mechanisms that facilitate *Histoplasma* survival and growth within host macrophages. For prioritized genes, immediate efforts will focus on complementation of the mutations to directly link loss of virulence to disruption of the candidate locus. In addition, we will confirm that mutants with defects in macrophage virulence are similarly attenuated in vivo using a murine inhalational model of respiratory and disseminated histoplasmosis. We will subsequently define, with finer resolution, the nature of the virulence attenuation through cellular markers of intramacrophage trafficking. Further characterization of the roles of new virulence genes will be guided by gene identities and the relevant defects in stages of macrophage infection. Our experience and expertise in molecular manipulation and cellular analyses of *Histoplasma* and host cells will enable us to readily move beyond virulence factor identification. These follow up studies will provide a mechanistic understanding of how *Histoplasma* yeasts successfully infect, survive, and replicate within macrophages thereby subverting innate immune defenses of the host.

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PHS 398 Checklist

OMB Number: 0925-0001

1. Application Type:

From SF 424 (R&R) Cover Page. The responses provided on the R&R cover page are repeated here for your reference, as you answer the questions that are specific to the PHS398.

* Type of Application:

☒ New ☐ Resubmission ☐ Renewal ☐ Continuation ☐ Revision

Federal Identifier:

2. Change of Investigator / Change of Institution Questions

☐ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:
* First Name:
Middle Name:
* Last Name:
Suffix:

☐ Change of Grantee Institution

* Name of former institution:

3. Inventions and Patents (For renewal applications only)

* Inventions and Patents: Yes ☐ No ☐

If the answer is "Yes" then please answer the following:

* Previously Reported: Yes ☐ No ☐

4. * Program Income

Is program income anticipated during the periods for which the grant support is requested?

☐ Yes ☒ No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

5. * Disclosure Permission Statement

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

☒ Yes ☐ No