PD-A21-385

USAID

U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT

SEP 18 1993

Dr. Walter Sullivan, Vice President for
Sponsored Programs
Morehouse School of Medicine
720 Westview Dr., SW
Atlanta, GA 30310-1495

ENTERED
OCT 15 1993
Section

Subject: Grant No. PCE-5053-G-00-3047-00

Dear Dr. Sullivan:

Pursuant to the authority contained in the Foreign Assistance Act of 1961 and the Federal Grant and Cooperative Agreement Act of 1982, as amended, the Agency for International Development (hereinafter referred to as "A.I.D.") hereby grants to the Morehouse School of Medicine (hereinfter referred to as "Morehouse" or "the Grantee") the sum of $99,942 to provide financial support for the program described in Attachment 2 of this Grant entitled "Program Description."

This Grant is effective and obligation is made as of the date of this letter and shall apply to expenditures made by the Grantee in furtherance of program objectives during the period beginning with the effective date and ending September 30, 1995.

This Grant is made to the Grantee on the condition that the funds will be administered in accordance with the terms and conditions as set forth in Attachment 1 (the Schedule); Attachment 2 (the Program Description); and Attachment 3 (the Standard Provisions); all of which have been agreed to by your organization.

Please sign the original and all enclosed copies of this letter to acknowledge your receipt of this grant and return the original and all but one copy to the undersigned.

If you have any questions, please contact Ms. Karin Kolstrom of my staff at (703) 875-1189.

Sincerely,

Gary V. Kinney

Gary V. Kinney
Grant Officer
Chief, PCE Branch
Division B
Office of Procurement

1

Attachments:

1. Schedule
2. Program Description
~~3. Standard Provisions~~

ACKNOWLEDGED:

Morehouse School of Medicine

BY: Walter W. Sullivan

TYPED NAME: Walter W. Sullivan, Ph.D.

TITLE: Vice President for Health Promotion

DATE: September 30, 1993

97581

FISCAL DATA

A. GENERAL

A.1. Total Estimated A.I.D. Amount: $99,942
A.2. Total Obligated A.I.D. Amount: $99,942
A.3. Cost-Sharing Amount (Non-Federal):$25,977
A.4. Other Contributions (Federal): $0
A.5. Project No.:936-5053
A.6. A.I.D. Project Office: R&D/UC
A.7. Funding Source: A.I.D./W
A.8. Tax I.D. No.: 15-81438873A1
A.9. CEC No.: N/A
A.10. LOC No.: 72-00-1536

B. SPECIFIC

B.1.(a) PIO/T No.: 936-5053-3692944
B.1.(b) Appropriation: 72-1131021.1
B.1.(c) Allotment: 341-36-099-00-20-31
B.1.(d) BPC: DDVA-93-16900-KG11
B.1.(e) Amount: $99,942

ATTACHMENT 1

SCHEDULE

1A. PURPOSE OF GRANT

The purpose of this Grant is to provide financial support for the program described in Attachment 2 of this Grant entitled "Program Description."

1B. PERIOD OF GRANT

The effective date of this Grant is the date of the Cover Letter and the estimated completion date is September 30, 1995. Funds obligated hereunder (see Section 1C.2. below) shall be used to reimburse the Grantee for allowable program expenditures incurred by the Grantee in pursuit of program objectives during such period. Funds obligated hereunder are anticipated to be sufficient for completion by the Grantee of the program described in Attachment 2 of this Grant by the estimated completion date.

1C. AMOUNT OF GRANT AND PAYMENT

1C.1. The total estimated amount of this Grant for its full period, as set forth in Section 1B. above, is $99,942.

1C.2. A.I.D. hereby obligates the amount of $99,942 for the purposes of this Grant during the indicated period set forth in Section 1B. above, thereby fulfilling A.I.D.'s funding requirements. A.I.D. shall not be liable for reimbursing the Grantee for any costs in excess of the obligated amount, except as specified in paragraph (f) of the Standard Provision of this Grant entitled "Revision of Grant Budget."

1C.3. Payment shall be made to the Grantee in accordance with procedures set forth in the Standard Provision of this Grant entitled "Payment - Letter of Credit," as shown in Attachment 3.

1.C.4. The total estimated amount of the program described in Attachment 2 of this Grant is $125,919, of which A.I.D. may provide the amount specified in Section 1C.1. above, and the Grantee will provide $99,942 in accordance with Section 1D below.

1D. GRANT BUDGET

1D.1. The following is the Budget for the total estimated amount of this Grant (see Section 1C.1. above) for its full period (see Section 1B. above). The Grantee may not exceed the

total estimated amount or the obligated amount of this Grant, whichever is less (see Sections 1C.1. and 1C.2., respectively, above). Except as specified in the Standard Provision of this Grant entitled "Revision of Grant Budget," as shown in Attachment 3, the Grantee may adjust line item amounts as may be reasonably necessary for the attainment of program objectives.

1D.2. Budget

Cost Element	A.I.D.	Cost-Sharing Non-Federal	Total
Salaries	$34,820	$4,820	$39,640
Fringe	$8,358	$1,157	$9,515
Travel	$2,000	$0	$2,000
Non-Exp. Equip.	$1,000	$20,000	$21,000
ODC	$24,500	$0	$24,500
Overhead	$29,264	$0	$29,264
Total:	$99,942	$25,977	$125,919

1D.3. Inclusion of any cost in the budget of this Grant does not obviate the requirement for prior approval by the Grant Officer of cost items designated as requiring prior approval by the applicable cost principles (see the Standard Provision of this Grant set forth in Attachment 3 entitled "Allowable Costs") and other terms and conditions of this Grant, unless specifically stated in Section 1I. below.

1E. REPORTING

1E.1. Financial Reporting

1E.1.(a) Financial reporting requirements shall be in accordance with the Standard Provision of this Grant entitled "Payment - Letter of Credit," as shown in Attachment 3. If a Standard Form 269 is required by the aforesaid Standard Provision, the "Long Form" of said form shall be used.

1E.1.(b) All financial reports shall be submitted to A.I.D., Office of Financial Management, FA/FM/CMPD/DCB, Room 700 SA-2, Washington, D.C. 20523-0209. In addition, three copies of all financial reports shall be submitted to the A.I.D. Project Office specified in the Cover Letter of this Grant, concurrently with submission of the Quarterly Technical Reports (See Section 1E.2. below).

1E.1.(c) The frequency of financial reporting and the due dates of reports shall be as specified in the Standard Provision of this Grant referred to in Section 1E.1.(a) above.

1E.2. Program Reporting

1E.2.(a) Annual Workplan

1E.2.(a)(1) The Grantee shall submit an annual workplan for this Grant which shall contain the following:

1E.2.(a)(1)(A) An action-oriented workplan describing planned activities for the next year, delineated by calendar quarter, and linked to the project goals and objectives, which describes the individuals to be involved, the activities to be conducted, and where and when they will be conducted. Planned activities shall be grouped by subject category, and then related to project objectives;

1E.2.(a)(1)(B) A projected budget, utilizing the same budget line items as are set forth in the budget of this Grant, for each calendar quarter, corresponding to the workplan; and

1E.2.(a)(1)(C) Publications, reports, workshops, seminars, and other information dissemination activities planned, by calendar quarter.

1E.2.(a)(2) The Grantee may develop the annual workplan in consultation with the A.I.D. Project Officer for this Grant.

1E.2.(a)(3) Five (5) copies of the annual workplan shall be submitted to the designated A.I.D. Project Officer for this Grant and one copy submitted to the Grant Officer. The annual workplan shall be submitted by the Grantee not later than sixty (60) days from the effective date of this Grant (see Section 1B. above).

1E.2.(b) Quarterly Reports

The Grantee shall submit five (5) copies of brief quarterly program performance reports, which coincide with the financial reporting periods described in Section 1E.1. above, to the A.I.D. Project Office specified in the Cover Letter of this Grant. In addition, two copies shall be submitted to A.I.D., POL/CDIE/DI, Washington, DC 20523-1802. These reports shall be submitted within 30 days following the end of the reporting period, and shall briefly present the following information:

1E.2.(b)(1) A comparison of actual accomplishments with the goals established for the period, the findings of the investigator, or both. If the output of programs can be readily quantified, such quantitative data should be related to cost data for computation of unit costs.

1E.2.(b)(2) Reasons why established goals were not met, if applicable.

1E.2.(b)(3) Other pertinent information including the status of finances and expenditures and, when appropriate, analysis and explanation of cost overruns or high unit costs.

1E.2.(c) Special Reports

1E.2.(c)(1) Within 30 days following the completion of each international trip, the Grantee shall submit 3 copies of a trip report summarizing the accomplishments of the trip to the A.I.D. Project Officer specified in the cover letter of this Grant. If several individuals are travelling together to one site, a single report representing the group will suffice. The report shall include the purpose of the trip, technical observations, suggestions and recommendations, overall impressions of the site situation (if appropriate), and a list of persons visited with their title and organization affiliation.

1E.2.(c)(2) Between the required program performance reporting dates, events may occur that have significant impact upon the program. In such instances, the Grantee shall inform the A.I.D. Project Officer as soon as the following types of conditions become known:

1E.2.(c)(2)(A) Problems, delays, or adverse conditions that will materially affect the ability to attain program objectives, prevent the meeting of time schedules and goals, or preclude the attainment of work units by established time periods. This disclosure shall be accompanied by a statement of the action taken, or contemplated, and any A.I.D. assistance needed to resolve the situation.

1E.2.(c)(2)(B) Favorable developments or events that enable time schedules to be met sooner than anticipated or more work units to be produced than originally projected.

1E.2.(c)(3) If any performance review conducted by the Grantee discloses the need for change in the budget estimates in accordance with the criteria established in the Standard Provision of this Grant entitled "Revision of Grant Budget," the Grantee shall submit a request for budget revision to the Grant Officer and the A.I.D. Project Officer specified in the Cover Letter of this Grant.

1E.2.(d) Environmental Impact

If it appears that the outputs of this project will result in an adverse environmental impact, the Grantee shall notify the A.I.D. Project Officer prior to implementation, in order to allow for orderly preparation of an environmental impact statement. The Grantee shall assure that appropriate U.S. Government and/or host country procedures are followed.

1E.2.(e) Care of Laboratory Animals

If the Standard Provision entitled "Care of Laboratory Animals" applies to this Grant, the Grantee shall include the certificate required by paragraph (c) of said Standard Provision in all of its reports which pertain to the use of laboratory animals.

1E.2.(f) Compliance With Federal Guidelines and Regulatory Procedures Pertaining to Recombinant DNA

The Grantee shall implement any research activities under this Grant which involve recombinant DNA in accordance with: 1) the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules; 2) procedures issued by the U.S. Department of Agriculture (USDA), the Environmental Protection Agency (EPA), or other appropriate Federal agency; 3) A.I.D.'s environmental procedures; and 4) such other Federal guidelines and procedures as may apply during the course of research. The Grantee cannot commence testing in any foreign location until written approval for such testing is obtained from the A.I.D. Project Officer and the government of the country where testing is planned. Testing shall be conducted in accordance with all applicable regulations of that country. In addition, and prior to commencement of any such testing, the Grantee shall make a judgement and communicate same to the A.I.D. Project Officer as to whether the regulations, procedures, or facilities of the country in question are adequate to ensure testing in an environmentally sound manner. In the event such judgement is that they are not, the Grantee and the A.I.D. Project Officer will consult and agree on the conditions to be applied to the testing which will have such environmental effect. Reports submitted to A.I.D. under this Grant will address regulatory issues as noted above related to the activity.

1E.2.(g) Final Report

Within 90 days following the estimated completion date of this Grant (see Section 1B. above), the Grantee shall submit five (5) copies of a final report to the A.I.D. Project Office specified in the cover letter of this Grant. In addition, two copies shall be submitted to A.I.D., POL/CDIE/DI, Washington, DC 20523-1802. It will cover the entire period of the Grant and include all information shown in Sections 1E.2.(a) and 1E.2.(c) above.

1F. SPECIAL PROVISIONS

1F.1. OPTIONAL STANDARD PROVISIONS

The following Optional Standard Provisions for U.S., Nongovernmental Grantees, as listed in Attachment 3 of this Grant, are hereby deleted as follows:

Provision	Page
Payment - Periodic Advance	13
Payment - Cost Reimbursement	15
Local Cost Financing	33
Patent Rights	35
Negotiated Indirect Cost Rates - Provisional	44
Negotiated Indirect Cost Rates - Provisional	46
Participant Training	48
Voluntary Population Planning	49
Protection of the Individual as a Research Subject	56
Title To and Care of Property(U.S. Government Title)	63
Title To and Care of Property(Cooperating Country Title)	67

All other Optional Standard Provisions are hereby incorporated into this Grant and have been checked off on page 9 of Attachment 3.

1F.2. Limitations on Reimbursement of Costs of Compensation for Personal Services and Professional Service Costs

1F.2.(a) Employee Salaries

Except as the Grant Officer may otherwise agree in writing, A.I.D. shall not be liable for reimbursing the Grantee for any costs allocable to the salary portion of direct compensation paid by the Grantee to its employees for personal services which exceed the highest salary level for a Foreign Service Officer, Class 1 (FS-1), as periodically amended.

1F.2.(b) Consultant Fees

Compensation for consultants retained by the Grantee hereunder shall not exceed, without specific approval of the rate by the Grant Officer: either the highest rate of annual compensation received by the consultant during any full year of the immediately preceding three years; or the maximum rate of a Foreign Service Officer, Class 1 (FS-1) (as periodically amended), whichever is less. A daily rate is derived by dividing the annual compensation by 2,087 and multiplying the result by 8.

1F.3. Defense Base Act (DBA) and Medical Evacuation Insurance

Pursuant to Section J.16. of OMB Circular A-21 (for educational institutions) or Section 18 of Attachment B of OMB Circular A-122 (for nonprofit organizations other than educational institutions), the Grantee is authorized to purchase DBA and/or medical evacuation insurance under this Grant. If DBA insurance is purchased, it shall be purchased from the insurance company or agent with which A.I.D. has a contract to provide DBA insurance for A.I.D. contracts. The Grant Officer will provide the name, address, and telephone number of such insurance company or agent upon request.

1G. INDIRECT COST RATE

Pursuant to the Standard Provisions of this Grant entitled "Negotiated Indirect Cost Rates - Predetermined" and "Negotiated Indirect Cost Rates - Provisional (Nonprofits)," a predetermined indirect cost rate or rates shall be established for each of the Grantee's accounting periods which apply to this Grant. Payments on account of allowable indirect costs shall be made on the basis of such predetermined rates. The rate(s) for the initial period and the base(s) to which it is (they are) applied is (are) as follows:

Type	Rate	Base	Period
On-Campus/Home Office	42.0%	1/	7/1/92 to 6/30/95
Off-Campus/Off-Site	26.0%	1/	7/1/92 to 6/30/95

1/ Base of Application: Modified total direct costs consisting of salaries and wages, fringe benefits, materials and supplies, services, travel and subawards up to $25,000 each.

1G.1. Rates for subsequent periods shall be established in accordance with the Standard Provision of this Grant entitled "Negotiated Indirect Cost Rates - Predetermined."

1H. PROPERTY REQUIREMENTS

1H.1. Title to Property

Title to property acquired hereunder shall vest in the Grantee subject to the requirements of the Standard Provision of this grant entitled "Title To and Use of Property (Grantee Title)" regarding use, accountability, and disposition of such property.

1H.2. Equipment Purchases

Equipment purchases under this Grant must be made in accordance with the Standard Provisions entitled "Procurement of Goods and Services" and "AID Eligibility Rules for Goods and Services" included in Attachment 3 of this Grant. Inclusion of costs in the budget of this Grant for the purchase of nonexpendable equipment does not obviate the requirements of Section J.13. of OMB Circular A-21 (for educational institutions) or Section 13 of Attachment B of OMB Circular A-122 (for nonprofit organizations other than educational institutions) for prior approval of such purchases by the Grant Officer, nor any other terms and conditions of this Grant, unless specifically stated in Section 1H.2.(a)(3) below.

1H.2.(a) Requirement for Prior Approval

Pursuant to Sections 1D.3. and 1G.3. above and the Standard Provisions of this Grant entitled "Allowable Costs" and "Revision of Grant Budget," and by extension, Section 13 of Attachment B of OMB Circular A-122, the Grantee must obtain A.I.D. Grant Officer approval for purchases of the following:

1H.2.(a)(1) General Purpose Equipment, which is defined as an article of nonexpendable tangible personal property which is usable for other than research, medical, scientific or technical activities, whether or not special modifications are needed to make them suitable for a particular purpose (e.g., office equipment and furnishings, air conditioning equipment, reproduction and printing equipment, motor vehicles, and automatic data processing equipment), having a useful life of more than two years and an acquisition cost of $500 or more per unit); and

1H.2.(a)(2) Special Purpose Equipment, which is defined as an article of nonexpendable tangible personal property, which is used only for research, medical, scientific, or technical activities (e.g., microscopes, x-ray machines, surgical instruments, and spectrometers), and which has a useful life of more than two years and an acquisition cost of $1,000 or more per unit).

1H.2.(a)(3) Approvals

In furtherance of the foregoing, the Grant Officer does hereby provide approval for the following purchases, which shall not be construed as authorization to exceed the total estimated amount or the obligated amount of this Grant, whichever is less (see Section 1C. above):

- One DNA sequencing gel apparatus

1H.2.(a)(4) Exception for Automation Equipment

Any approval for the purchase of automation equipment which may be provided in Section 1H.2.(a)(3) above or subsequently provided by the Grant Officer is not valid if the total cost of purchases of automation equipment (e.g., computers, word processors, etc.), software, or related services made hereunder will exceed $100,000. The Grantee must, under such circumstances, obtain the approval of the Grant Officer for the total planned system of any automation equipment, software, or related services.

1I. RESOLUTION OF CONFLICTS

Conflicts between any of the Attachments of this Grant shall be resolved by applying the following descending order of precedence:

Attachment 1 - Schedule
Attachment 3 - Standard Provisions
Attachment 2 - Program Description

Proposal to

The Agency for International Development

from

Morehouse School of Medicine
Atlanta, Georgia 30310-1495

Gene Expression during *Entamoeba* Encystation

Vincent C. Bond, Ph.D.
Assistant Professor of Biochemistry
Principal Investigator

16 March 1993

Vincent C. Bond, Ph.D.
Principal Investigator

Walter W. Sullivan, Ph.D.
Vice President for
Sponsored Programs

STATEMENT OF FUNDING:

This proposal was part of an RO1 submitted to NIH. It has since been reviewed and, although recieving a good priority score, will not be funded. Alternative funding thru USAID will allow this project to get off the ground, and allow a stronger RO1 resubmission at some later date using the data generated through the proposed USAID project.

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B. RELEVANCE OF THE PROPOSED WORK TO THE MISSION OF AID.

1. STATEMENT OF THE PROBLEM.

Amebiasis is a tissue invasive disease caused by a number of protozoan parasites including Entamoeba histolytica. An estimated 400 million people, mostly in the less developed tropical countries, are infected with E. histolytica each year (63). Up to 50 million of these suffer up to a week of debilitating symptoms of the disease per episode, translating to economic losses of billions of dollars a year for already impoverished countries. Further, as many as 30,000 directly related deaths can be attributed to this malady. Thus, if health as well as economic levels are to improve for many less developed nations, a means must be found to control amebiasis, which is one of the major tropical parasitic diseases.

A clinical means of disease prevention awaits a clearer understanding of the causative agent (E. histolytica) itself. The long term goal of our research is directed towards development of strategies to prevent transmission of amebiasis by inhibition of Entamoeba histolytica encystation. The research proposed in this project will investigate the regulatory mechanisms of encystation in the amoeboid organism Entamoeba. Entamoeba histolytica, however, cannot be induced into encystation in culture. Consequently, Entamoeba invadens, the reptilian parasite, will be used as a model system as it can be induced to encyst in laboratory cultures.

2. DEVELOPING COUNTRIES PARTICIPATION IN THE PROPOSED RESEARCH.

None.

3. ENVIRONMENTAL EFFECTS.

None.

C. SCIENTIFIC ASPECTS OF THE PROPOSED WORK.

1. THE PROBLEM TO BE ADDRESSED.

1a. The Research Question.

Entamoeba is characterized by two distinct cellular forms, the trophozoite and the cyst. The trophozoite is the motile, feeding stage which has the potential to invade a wide variety of tissue types in the host and is the direct agent of host tissue destruction. Knowledge of this stage has advanced, and a large majority of the molecular studies address the trophozoite and its effects on the host (2,7,8,9,10,20,23,24,26,28,33, 35,36,37,38,39,45,46,47,49,50,51,52,55,59,60,66)

There are no immunological strategies to prevent amebiasis. The incidence of disease in the developed world is limited by sanitation systems that minimize the possibility of fecal-oral transmission of the cysts. Active infections are effectively treated by drugs. Metronidazole, the current drug of choice, is most effective against parasites localized within host tissues (29). However, due to its rapid absorption, this and other nitroimidazoles are least effective against parasites resident in the intestinal lumen, where encystation occurs. Thus parasites often remain resident and continue to encyst in the intestines of "cured" asymptomatic individuals, and these individuals can continue to spread the disease (25).

Consequently, research focusing on the cyst stage could result in alternative therapies that would specifically block encystation. These therapies may not cure the treated host (although an understanding of the cyst stage of this organism could lead to development of remedial strategies). They certainly would result in reducing the spread

of the disease and lowering the general level of infection, in theory breaking the transmission cycle and ultimately eliminating the parasite. However, at present there is little known about the molecular aspects of the encystation process.

1b. Goals of the proposed research.

The long term goal of this research is directed towards development of strategies to prevent transmission of amebiasis by inhibition of Entamoeba histolytica encystation. Thus, the research proposed in this project will investigate the regulatory mechanisms of encystation in the amoeboid organism Entamoeba. Entamoeba histolytica, however, cannot be induced into encystation in culture. Consequently, Entamoeba invadens, the reptilian parasite, will be used as a model system as it can be induced to encyst in laboratory cultures.

The specific objectives of the proposed research are to:

1. Enrich for encystation specific messages.

This will be accomplished through the use of subtractive library techniques.

2. Examine the kinetics of expression of encystation specific clones.

mRNA expression kinetics will be used to identify encystation specific clones, and to place these clones chronologically in this morphological pathway.

3. Characterize encystation specific cDNAs.

Isolated clones will be sequenced and coding regions identified. Nucleic acid sequence, as well as deduced amino acid sequence, will be compared with those in computerized databases for sequence homologies and other structural characteristics that will lead to the identity of function of the gene products.

Successful completion of the proposed studies will provide information on encystation specific genes in Entamoeba, gene products involved in encystation, and the chronological order of expression of these molecules in this morphogenic process. Comparisons with reported gene/protein structures may suggest functions of these molecules in encystation in Entamoeba. Further, this knowledge should allow construction of a logical hypothesis of molecular mechanisms of encystation. Finally, the information gained is certain to reveal fruitful avenues for a more detailed investigation of means to block critical steps in cyst formation.

2. BACKGROUND.

EVIDENCE FOR EXPRESSION CONTROL IN ENCYSTATION

The protozoan parasite, Entamoeba histolytica, is the infective agent of the tissue invasive disease, amebiasis. As many as 50 million people worldwide suffer debilitation and approximately 50,000 die annually from infections with pathogenic strains of E. histolytica (63).

The life cycle of all Entamoeba species is characterized by two distinct cellular forms, the trophozoite and the cyst. The highly motile amoeboid trophozoite is the vegetative form and the direct agent of host tissue destruction. The cyst can survive outside the host for a brief time and is the agent of disease transmission.

15x.

As mentioned above, there are no immunoprotective strategies to combat amebiasis. The disease is prevented in developed countries by sanitation systems that limit fecal-oral transmission of cysts. Symptomatic amebic infections are currently treated with several drugs that kill the trophozoite. The most commonly used are the imidazoles, metronidazole and tinidazole (29). Though effective, extended treatment is often required and side effects can be severe. Further, due to their rapid absorption, they are least effective against organisms resident in the intestinal lumen, where encystation occurs. Thus, parasites often remain resident, and continue to encyst (42) in the intestines of "cured" asymptomatic individuals, and these people will continue to spread the disease (25).

There are a number of species of Entamoeba. All are similar in gross morphology, intracellular structure and life cycle (17,44,67). The most common pathogens are E. histolytica, found in man, and E. invadens, a parasite of reptiles. While conditions that trigger encystation of E. invadens in laboratory cultures have not worked for E. histolytica, the process appears essentially the same, and cysts of these two species share a number of distinguishing structural features. These include a protective chitin wall (1), maturation from a uninucleate to a tetranucleate form (43), and crystalloid aggregates of ribosomes (chromatoid bodies; 48). The similar occurrence of the latter, which form as a consequence of loss of translatable mRNA (30,31), suggests that analogous alterations of macromolecular metabolism also occur during encystation of these species. Thus, while there are certainly numerous subtle differences in the mechanisms of encystation, the overall process and the logic of its regulation is most probably the same in the two species. If Entamoeba encystation is to be investigated at this time, E. invadens is the logical model.

Excystation takes place in the host large intestine, where trophozoites may reside commensurallly (13). A primarily dysenteric disease follows, resulting from invasive destruction of wall of the lower intestine. Bloody diarrhea with accompanying fluid loss and debilitation are the primary symptoms. If the trophozoites penetrate the wall and enter the bloodstream, they may infest extraintestinal tissues, the most common being the liver, where they can cause massive and often fatal abscesses. Cysts are not found associated with the trophozoites in infected tissues, and are normally not passed by patients with active amebiasis (42,50). Encystation occurs more often in the lumen of the large bowel of nonsymptomatic hosts and of clinically recovered patients who carry residual trophozoites within the intestine (34). The environmental conditions that induce encystation in vivo are not known.

Means to effect reproducible encystation of the human pathogen in laboratory cultures have not been discovered. Several ways to induce mass (>50%) encystation[1] of the reptilian parasite, E. invadens in axenic cultures have been found. These include osmotic shock (4,6,41,53), transfer to a medium preconditioned by bacterial growth (61), and removal of glucose from the culture medium (62). While all of these procedures reduce nutrient levels, depletion of nutrients per se has not been shown to serve as the signal to initiate encystation. A common feature of all encystation media is reduced osmolarity compared to the corresponding culture medium. However, the role of osmotic stress in inducing encystation is not known.

A more recent study has measured the appearance of chitin synthase activity in encysting E. invadens (15). Chitin synthase in encysting Entamoeba invadens. Biochem.J. 280: 641-647). This enzyme is responsible for the assembly of GlcNAc residues to form chitin, the major, and a unique component of the cyst wall. Chitin synthase activity increased rapidly and markedly (8-10 fold) following stimulation of encystation.

[1]Traditionally, encystation is recognized microscopically by formation of a hyaline, ionic detergent resistant sphere. Appropriate staining reveals the chitin wall, nuclei and ribosomal aggregates. A 'precyst' form that has been described (3) is not easily identified and has not yet been shown to be an amoeba committed to encystation.

Unfortunately, neither the time course of encystation itself nor the correlation of enzyme specific activity to the appearance of walled cysts were reported. It was clear, however, that expression of this encystation specific protein was initiated by transfer of cells to encystation conditions and well ahead of the appearance of walled cysts. A second report (65) demonstrated an increase in chitinase activity coincident with cyst formation. The role in encystation of this chitin degrading enzyme, which was not detectable in vegetative amoebae, is unclear. A sialoglycoprotein that appears to arise specifically during encystation has also been reported (5).

The time course of encystation and macromolecular metabolism during encystation was studied some years ago in Dr. Gordon Baileys laboratory (57,58). The doubling time for walled cyst formation was 7 times faster than for trophozoites in either growth or encystation cultures. This suggested that events leading to encystation become synchronized following transfer to encystation conditions and that their initiation may not be cell cycle stage dependent.

More than half of the protein and RNA labeled during vegetative growth was lost after transfer of amoebae to encystation conditions, and this loss was most rapid immediately following transfer. Net protein synthesis occurred throughout encystation, reaching a plateau at approximately the time of maximum cyst formation. In contrast, net RNA synthesis reached a maximum prior to the appearance of walled cyst formation whenever added during encystation. However, cyst wall formation proceeded essentially as in control cultures for up to 10 hours after addition of inhibitory levels of actinomycin D. Collectively, these data suggest the following: transcription of trophozoite specific genes ceases, and degradation of trophozoite specific transcript and protein occurs quickly following transfer of amoebae to encystation conditions. At the same time, expression of cyst specific genes is initiated. The synthesis of at least some of the message required for cyst formation occurs well in advance of its translation.

Recently, by digitized image analysis and comparison of the spot patterns on 2-dimensional protein gels of E. invadens trophozoites and cysts, we have been able to detect as many as 72 proteins that may be unique to the cyst (12).

Thus, while only one protein of known function during encystation has been identified, there is considerable preliminary evidence that the overall process depends on a complex series of molecular events including the timed and carefully regulated expression of a number of encystation specific genes. Therefore, the goal of this project is to begin to elucidate the regulatory mechanisms and molecules involved in encystation in Entamoeba by identifying and characterizing genes (and their products) that are expressed exclusively during encystation.

3. EXPERIMENTAL DESIGN

3a. DESCRIPTION OF EXPERIMENTAL DESIGN

We propose to identify, clone and characterize gene(s) that are unique to the encystation process in E.invadens. In AIM I, encystation specific messages will be identified and cloned using subtractive library techniques. cDNA libraries of encystation RNAs will be made from a portion of the RNA harvested from populations of encystation induced cells at various time points during the encystation process. This RNA will be pooled, reverse transcribed, PCR amplified, and directionally inserted into a lambda vector, forming an encystation cDNA library. A trophozoite cDNA library will also be made from a portion of the RNA harvested from vegetatively growing cells. The rest of the RNAs harvested will be used to make the subtraction library. This will be accomplished using biotin labeled cDNA made from the trophozoite RNA as probe. The common cDNAs in the uncloned encystation cDNA will be subtracted using repeated rounds of streptavidin selection enriching for encystation cDNAs unique to that process. These unique cDNAs will then be cloned, plated, and screened using probe made from the encystation and trophozoite

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cDNA libraries described above. This step will allow us to confirm the uniqueness of the subtraction cDNAs to encystation, and to eliminate reiterated clones.

In AIM II, the kinetics of expression of each clone will be examined. Multiple Northern blots will be made using RNA from vegetatively growing cells, and RNA from cells induced to encyst. The encysted RNAs will be from at least 10 post-induction time points, and RNA from each time point will not be pooled but run individually. The unique clones isolated in the first stage will be used individually to screen these Northerns, allowing each clone to be grouped according to its kinetics of expression during encystation.

In AIM III, the unique clones will be characterized, beginning with those representing genes turned on earliest during encystation. This will be accomplished by sequencing the clones, identifying putative coding regions, extrapolating the amino acid sequences, and using these data to screen DNA, RNA, and protein data banks to search for potential sequence homologies.

By using subtractive library techniques in conjunction with PCR amplification of the cDNA pool, the chance of finding encystation specific transcripts is increased. This is due to an amplification of the middle and low abundance cDNA clones relative to the total cDNA pool (from the PCR amplification), and to a substantial enrichment for those unique transcripts (from the subtraction). This should greatly increase the probability of detecting most of the middle abundance unique clones, as well as all of the high abundance unique clones (19,27). However, in spite of these attempts to bias the experiments to maximize the chances of getting these transcripts, it is certain that many of the low abundance encystation specific messages will not be identified . In the future these could potentially be identified using various techniques such as many repeated rounds of PCR and subtraction (32).

This project will identify genes unique to the encystation process. However, there most certainly are trophozoite genes that are shut off by induction of encystation. Additionally, there are genes that will have a quantitative variation in their expression upon encystation induction. Both of these classes of transcripts will not be identified in these experiments. Again, in the future, genes falling into both of these classes will be identified.

3b. METHODS

Diagrams for some of the steps of the Methods (as indicated) are included at the end of this subsection.

AIM I. Enrich for encystation specific messages.

E. invadens strain IP-1, originally obtained from ATCC and maintained in Dr. Gordon Bailey's laboratory for several years, will be grown axenically in TYI-S medium (16) at 25^{0} C. Encystation will be induced by osmotic shock. We find this to be the simplest, most reproducibly successful method. Mid-growth phase trophozoite cultures are centrifuged at 250 xg for 3 minutes to collect the free cells, and 3/4 of the supernatant medium is replaced with sterile deionized water.

1- Isolate RNA from:
   a. Growth phase amoebae.
   b. Amoebae at various points following induction of encystation.

      Will take four postinduction times points and pool the isolated RNAs, which will then be called our induced RNA.

Total RNA will be isolated from each of the above sources by the acid guanidinium thiocyanate-phenol-chloroform method (14). RNA is ethanol precipitated several times to achieve high purity.

Select for polyA$^+$ RNA using a PolyAtract mRNA isolation system III (Promega).

2- Reverse transcribe RNA in each of the two samples, generating cDNA.

A. STRAND-SYNTHESIS (See diagram 2A at end of Methods)

First and second strand cDNA synthesis will be performed according to the procedure of Gubler and Hoffman, 1983 (22). 1-2 μg of methyl mercury (CH_3HgOH) denatured polyadenylated (polyA+) RNA will be used in the first strand synthesis. The reaction volume will be 10-20 μl, and will contain 50mM Tris, Ph 8.3; 75mM KCL; 3mM $MgCl_2$; 10mM DTT; 1.25mM each of dATP, dCTP, dGTP, and dTTP; 100μg/ml oligo-dT; 100μg/ml of a poly T primer; 1500 units/ml RNAsin; and 2000 units/ml MMTV reverse transcriptase. The reaction will be incubated at 37° C for 1 hour. An excess of this reaction mix will be made, and ^{32}P-DCTP will be added to part to monitor cDNA synthesis.

Second strand synthesis will be carried out by diluting the first strand reaction directly into a 100μl final volume of 200mM Tris, pH 7.5; 5mM $MgCl_2$; 100mM KCL; 10mM $(NH_4)2SO_4$; 0.15mM ß-NAD; 50μg/ml BSA; 40μM each of dATP, dCTP, dGTP, and dTTP; 8.5 units/ml RNAseH; 230 units/ml DNA Polymerase I; 10 units/ml E.coli DNA ligase. The reaction will be incubated for 1 hour at 12°C, and then for 1 hour at 22°C. Finally, 5 units of Polymerase K (Klenow fragment) will be added, and the reaction incubated for 30 minutes at 37°C to blunt all ends.

B. CLEAN-UP AND ADAPTER LIGATION (See diagram 2B)

Adapters will be attached to all of the encystation cDNA pool, and part of the trophozoite cDNA pool. The method of Schweinfest et al., 1990 will be used in conjunction with the method of Duguid and Dinauer, 1988 and 1989 (18,19). Two oligonucleotides, dCTCTTGCTTGAATTCGGACTA and dTAGTCCGAATTCAAGCAAGAGCACA, will be synthesized (See Diagram). The two oligo-nucleotides (1mg/ml) will be hybridized in the kinase buffer at 45°C for 10 minutes. This will yield the duplex adapter with a blunt end on one side, a four nucleotide 3'overhang on the other end, and an EcoRI restriction site 7 bases from the blunt end.

The ligation will be accomplished as follows: The ds cDNA mixture will be extracted 2 times with phenol: chloroform (1:1), and layered onto a prespun (400g for 2 minutes) spin column of 2ml bed volume of Sephacryl-200. The spin column will be freshly equilibrated in ligation buffer (66mM Tris, pH 7.5; 1mM spermidine; 15mM DTT; 10mM $MgCl_2$; 200μg/ml BSA). It will be spun at 400g for 2 minutes in a swinging bucket rotor. To the eluate (about 100μl) will be added 1μl of 100mM ATP, 1μl of 10 A260 units/ml adapters, and 5 Weiss units of T4 DNA ligase. Ligation will be done overnight at 14°C (See Diagram). The reaction will be heat inactivated at 65°C for 10 minutes. Subsequently, the ends of the adapters will be phosphorylated by addition of fresh ATP to 10mM; polynucleotide kinase to 100 units/ml, and incubation at 37°C for 2 hours. One phenol:chloroform (1:1) extraction is followed by a repeat of the spin column in a buffer containing 10mM Tris, pH 7.5; 1mM EDTA, 150mM NaCl. The final eluate is about 100μl.

3- PCR amplify the encystation cDNA pool. (See diagram 3)

The cDNA will be precipitated with ethanol, and one-half of the product amplified by PCR through 20 cycles in a 100μl reaction containing 1μg of the 21-mer oligonucleotide above (See Diagram). The following parameters have been calculated and will be used initially: denaturation-30sec, 94°C; annealing-30sec, 50°C; elongation-120sec, 72°C. Vent polymerase (New England Biolabs, NEB) will be used to circumvent the problem of mistakes being introduced through PCR. It is not possible to get a calculation of the T_m for Vent polymerase as most of the ions are not Na^+. However, from the PI's experience with Vent, and information obtained from NEB's technical people, this polymerase in the

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appropriate buffer has an annealing temperature that is either at or a few degrees above that of Taq polymerase. Thus, what we will do is make the T_m calculation for Taq in Taq buffer and use that as a starting point for PCR in Vent.

4- Photobiotin label part of the trophozoite cDNA. (See diagram 4)

The portion of the trophozoite cDNA pool without adapters will be used in the subtraction steps (See Diagram; 11,21,64). After phenol extraction and ethanol precipitation, the DNA samples will be dissolved in 50µl of 5mM HEPES; 1mM EDTA; pH 7.5 (HE), and boiled for 5 minutes. The DNA will be cooled and sonicated to shear the DNA to an average size of 500-1000 bp. This will be done on ice, and in pulses separated by equal cooling periods. The DNA will be ethanol precipitated, redissolved in 50µl HE, and mixed in a 1.5ml tube under darkroom safelights with 50µl of a 1mg/ml solution of photoprobe biotin (Vector Labs; 40). This mixture will be incubated on ice and irradiated with a sunlamp (GE Model RSM, 275 W) for 15 minutes. Following addition of 100µl of 0.1M Tris, pH 9,

the solution will be extracted four times with water saturated 2-butanol. The DNA is precipitated with ethanol and redissolved in water. This process will be repeated.

5- Hybridize biotin labeled trophozoite cDNA to encystation cDNAs.
(See diagram 5)

Aliquots of the biotin labeled trophozoite cDNA (25µg, or 10fold concentration) will be mixed with encystation cDNA (2.5µg, 1x concentration), polyA (2µg), and polyC (2µg), ethanol precipitated, dissolved in 1-5µl of water, and subsequently mixed with hybridization buffer (prewarmed to 65°C) and formamide to give a final volume of 10µl, and a final concentration of 25mM HEPES, pH 7.5; 5mM EDTA; 0.1% SDS; 1.5M NaCl; 11% Dextran Sulfate; and 15% formamide (See Diagram). 200µl of paraffin oil will then be layered on top the aqueous phase. This solution will be denatured at 98-100°C for 2-3 minutes, followed by incubation at a temperature 20-30°C below the melting point (determined by formamide and salt conditions; 64; 40). According to Barr and Emanuel, 1990 (11) these conditions combined with repeated rounds of hybridization should yield the maximum extent of hybridization between the driver (trophozoite) and sample (encystation) DNAs (See Diagram).

6- Streptavidin select out all hybridized molecules. (See diagram 6)

HE will be added to a final aqueous volume of 50µl, most of the paraffin oil removed, and the rest extracted with 50µl chloroform. The aqueous phase will be mixed with 10µl of a 1 mg/ml solution of streptavidin and water, followed by incubation at room temperature for 5 minutes, and extraction with an equal volume of HE-saturated phenol:chloroform (1:1) (See Diagram). The separated organic phase/interface will be back extracted with 50µl of HE, and the two aqueous phases combined. The above will be repeated 3 times.

7- Repeat 5 and 6.

8- PCR amplify the unhybridized encystation cDNA remaining, the adapter ligated trophozoite cDNAs, and the unselected encystation cDNAs. (See diagram 8)

PCR amplification of the unhybridized cDNAs remaining from the subtraction procedure will be amplified as below (See Diagram). This same process will be used to amplify the adapter ligated trophozoite cDNA pool, and the unselected encystation cDNA pool to make libraries of each which will subsequently be used to make a probe for screening the subtraction library.

One of the oligonucleotides used to make the adapters (step 2B) will be used for one oligomer. A second oligomer will be used, "Not I oligomer" TTTTTTTTTTTTTGCGGCCGCATCAGG-CTTAAGd containing a run of 13 T's at the 3' end to give hybridization specificity to the polyA end of the dsDNAs, 13nt from the 5'end of this oligomer corresponding to nt 10-21 from the 5' end of the 21mer oligomer (see step 2), and an internal NotI restriction site (this will allow directional cloning of the resultant dsDNAs). A second Not I oligomer, exactly the same except for having a run of 13 A's at the 3' end, will also be made and used in a separate reaction. The mixture of cDNAs will be amplified through 20 rounds of PCR the products of the two reactions subsequently being pooled. Vent polymerase will be used to reduce the probability of introducing errors. We will start with the parameters described in 3, and adjust the procedure from there.

9- Directional cloning of the subtracted cDNAs, the adapter ligated trophozoite cDNAs, and the unsubtracted encystation cDNAs. (See diagram 9)

Directional cloning of the unhybridized cDNAs remaining from the subtraction procedure will be performed as below. This same process will be used to clone the adapter ligated trophozoite cDNA pool, and the unselected encystation cDNA pool to make libraries of each which will subsequently be used to make a probe for screening the subtraction library.

cDNAs from 8 will be ligated into lambda Zap II/NotI-SpeI, using the Stratagene cloning kit. 1μg of Lambda Zap arms will be mixed with 0.1μg of cDNA insert; 10x ligation buffer (0.5M Tris-HCL, pH 7.5; 70mM $MgCl_2$; 10mM DTT) 10mM ATP; and 2 Weiss units of T4 DNA ligase in up to 5μl final volume. This reaction will be incubated overnight at 4°C. With an insert free of contaminants, and containing a high frequency of ligatable ends, about $2x10^6$ to $1.5x10^7$ recombinant plaques are expected with a high efficiency packaging extract.

The Stratagene Gigapack II Gold packaging extract will be used for packaging the clones. 1μl of the 5μl ligation will be test packaged. Then, 1μl out of the 500μl packaged reaction will be plated with 200μl of $O.D._{600}$=0.5 XL1-Blue cells. The phage and bacteria will be preincubated for 15 minutes at 37°C with gentle shaking, and then 2-3ml of top agar (48°C), 15μl of 0.5M IPTG (in water), and 50μl of 250mg/ml X-gal (in Dimethylformamide) will be added and immediately plated onto the appropriate agar plates. Plaques should be visualized at 6-8 hours of incubation, with background plaques (blue) being less then $1x10^5$ pfu/μg of arms, and positive plaques (white) being 10-100 fold above background. If the test titer gives good results the rest of the ligation will be packaged.

10- Plate out packaged phage at high density for plaques.

The in vivo libraries will be amplified (plate lysates) to make large, stable, and high titer stocks of the libraries. Aliquots of the library will be mixed with 600μl of XL1-Blue at $O.D._{600}$=0.5 cells for almost confluent lysis. The phage and cells will be preincubated at 37°C for 15 minutes. Subsequently, 6.5ml of melted 48°C top agar will be mixed with each aliquot of infected bacteria, and poured evenly onto 150mm bottom agar plates. The plates will be incubated at 37°C for 5-8 hours, not letting the plaques get too large. Then, the plates will be overlayed with 10ml of SM buffer (5.8g NaCl; 2.0g $MgSO_4$; 50ml 1M Tris-HCL, pH 7.5; 5ml 2% gelatin) and left at 4°C overnight with gentle shaking. The high titer phage lysate stocks will be recovered from each plate and pooled. The plates will be rinsed with an additional 2ml of SM and pooled. Chloroform will be added to 5%, the tube inverted to mix, and incubated for 15 minutes at room temperature. Subsequently, the tube will be centrifuged for 5 minutes at 4000g, the supernatant recovered, transferred to sterile polypropylene tube, 0.3% chloroform added, and final solution stored in aliquots at 4°C. The titer will be checked.

11- Make replica filters, 3 filter copies.

To screen the library, about $1x10^6$ plaques need to be examined. This should be accomplished by doing 20 aliquots of 50,000 plaques each which will give defined plaques on a 150mm plate. Thus, recombinant phage from the subtraction library will be plated with host cells in 6.5 ml of top agar on the appropriate 150mm plates at approximately 50,000 plaques/plate. The plates will be incubated at 37°C for 6-8 hours (getting small, defined plaques), and then refrigerated at least 2 hours at 4°C (prevents top agar sticking to filters). The plaques will be transferred, during a 2 minutes exposure, onto pencil-labeled nitrocellulose filters. The orientation of the filters on the respective plates will be marked with waterproof ink in a syringe. Duplicate filters will be made, with the subsequent transfers lasting about 4-6 minutes. The filters will be denatured after lifting by submerging in 1.5M NaCl, 0.5M NaOH for 2 minutes, neutralized for 5 minutes by submerging in 1.5M NaCl, 0.5M Tris-HCL,pH 8.0, and rinsed for 30 seconds in 0.2M Tris-HCL, pH 7.5, 2X SSC. All solutions will be at room temperature. Filters will be blot dried on 3MM filter paper, and DNA crosslinked to the filters with Stratalinker UV Crosslinker for 30 seconds.

12- Screen filters with random primed cDNA from trophozoite or encystation cDNA.

Inserts from the trophozoite cDNA library, and from the unsubtracted encystation cDNA library will be cut out of the vector. They will then be heat denatured in the presence of a hexanucleotide mixture for 10 minutes at 95°C in 2μl, and quickly chilled. Subsequently, a 1μl mixture of dGTP, $dCTP_4$ and dTTP at 5mM each; 1μl of 0.9M HEPES, pH 6.6, 100mM $MgCl_2$; 1μl of 20mM DTT; 3μl [a-^{32}P]dATP (sp. act.>3000Ci/mmol; 10μCi/μl), 1μl H_2O, and 1μl (5 units) Klenow fragment of E.coli DNA Polymerase I will be added, and the reaction brought to 20μl with water. The reaction will be incubated for at least 3 hours at room temperature.

Filters will be prepared for hybridization as described (54; Stratagene). Filters will be hybridized with the ^{32}P-cDNAs at 10μg/ml, one filter screened with trophozoite, the other screened with encystation probe at $5x10^6$ cpm/ml in a volume of 10ml. After a moderate-stringency wash (four changes of 15mM NaCl; 1.5mM trisodium citrate; 0.1% $NaDodSO_4$; pH7.0, for 15 minutes at 50°C), the filters will be autoradiographed.

13- Pick all encyst positive plaques.

Encystation specific clones will be identified by overlaying the autoradiograms obtained with the two probes. The positives will be the plaques that do not hybridize to trophozoite probe, but do hybridize to encystation probe. These plaques, on the original plates (54) will be stabbed with a toothpick, and dropped into a tube with 200μl of $O.D._{600}$=0.5 XL1-Blue cells. After a 15 minute preincubation at 37°C, growth media will be added, and incubation continued at 37°C overnight. Chloroform will be added to lyse any remaining cells.

14- Replate positive clones at low density, and reprobe as above to plaque purify.

Repeat step 11 except:
a) only plate 1000 plaques per 150mm plate
b) will make a set of filters for each plaque picked in 13. Repeat step 12.

15- Pick encyst positive plaques.

Repeat step 13 except this time only one positive plaque will be picked for each set of filters/plates now.

16- Make plate lysates from each positive plaque.

Repeat as in step 10.

17- Purify recombinant DNA, and excise inserts.

XL1-Blue cells will be infected at high multiplicity with each positive phage, incubated, phage purified, stocks made of each clone, and the recombinant DNA extracted and purified as in Sambrook et al., 1989 (54).

Lambda recombinants will be digested with the appropriate restriction enzyme to remove inserts as in Maniatie.

18- Label excised inserts by random priming.

Inserts will be heat denatured in the presence of a hexanucleotide mixture for 10 minutes at 95°C in 2μl, and quickly chilled (54). Subsequently, a 1μl mixture of dGTP, dCTP, and dTTP at 5mM each; 1μl of 0.9M HEPES, pH 6.6, 100mM $MgCl_2$; 1μl of 20mM DTT; 3μl [a-^{32}P]dATP (sp. act.>3000Ci/mmol; 10μCi/μl), 1μl H_2O, and 1μl (5 units) Klenow fragment of E.coli DNA Polymerase I will be added, and the reaction brought to 20μl with water. The reaction will be incubated for at least 3 hours at room temperature.

19- Make multiple dot blots containing all inserts.

Unlabeled inserts will be heat denatured in 1.5M NaCl, 0.5N NaOH, and transferred to nitrocellulose filters in a fixed pattern, using a filtration manifold. Multiple sets of filters will be made containing all the encystation positive recombinant inserts.

20- Screen for reiterated clones using random primed probe.

Using the random primed probe (from step 18), the sets of filters (from step 19) will be screened for reiterated sequences (filter screening will be done essentially as in step 12), put into groups based on this, and the longest sequences in each group kept for further analysis.

Because of the multiple sets of filters, several probes can be used at one time, and the filters can be stripped and reprobed.

AIM II. Examine kinetics of expression of encystation specific clones.

1- Isolate RNA.

Total RNA will be newly isolated from:

a. Vegetatively growing trophozoites.
b. Trophozoites induced to encyst.
   This time divide postinduction into 10 time periods, isolate RNA from each period.

This will be done essentially as in I-1.

2- Make multiple Northern blots using nylon filters with the above RNAs.

RNAs will be electrophoretically separated through formaldehyde gels (54), transferred to nylon membranes, and covalently crosslinked. Multiple filters will be made to allow several clones to be analyzed at one time.

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3- Probe blots with random primed labeled inserts from the unique clones (I-20).

This step will be done as in step I-20. Because multiple filters have been made we will be able to screen with several probes at a time. Also, we can strip and reprobe filters.

AIM III. Characterize these cDNAs.

1- Sequence clones.

The clones will be sequenced beginning with those representing genes turned on early in encystation. This will be done by excising the pBluescript plasmid from the recombinant lambda ZAP II vectors using the in vivo excision protocol provided commercially by Stratagene.

Single-stranded phage DNA will be prepared as in Sambrook et al., 1989 (54). 200μl of $O.D._{600}$= 1.0 XL1-Blue cells will be mixed with 200μl of the appropriate phage stock (see I-), and 1μl of R408 helper phage (>1x10^6 pfu/ml). This prep is incubated for 15 minutes at 37°C, then 5 ml of 2X YT media added and the solution incubated at 37°C for 3 hours with shaking. The solution will be heated for 20 minutes at 70°C to inactivate the E. coli, and spun for 5 minutes at 4000g. The supernatant is carefully decanted. Add 200μl of a solution of 20% polyethylene glycol (PEG 8000) in 2.5M NaCl to each 1.2ml of supernatant. Mix gently and let stand 15 minutes at room temperature. Spin at 12,000g for 5 minutes at 4°C to recover the precipitated bacteriophage particles. The resultant pellet of single-stranded bacteriophage particles will then be organically extracted, and ethanol precipitated to purify the single-stranded DNA.

The unique cDNA inserts will be sequenced using the Sanger dideoxy-mediated chain-termination sequencing method (54). Commercially available universal primers homologous to insert contiguous vector sequences in pBluescript (Stratagene) will be used to do the initial priming off of purified single-stranded DNAs. These data will then be used in generating oligomers to do internal sequencing.

2- Identify coding regions, etc.

The sequence data collected will be loaded onto a Sun workstation. The data will then be processed for coding regions, start and stop codons, etc, using the Intelligenetics suite program package available at this institution.

3- Screen sequence data banks.

To identify any potential homologies, the information gathered in III-2 will then be screened against sequence data banks to identify all significant homologies to other bacterial or eucaryotic sequences.

All data gathered from the successful completion of this project will be used in a screening of developmental data banks, both of bacteria and eucaryotes, looking for homologies between the genes/molecules identified, specifically for well characterized gene products that have known functional roles in sporulation pathways in other organisms. Any potential homologies can subsequently be exploited through the use of gene mutants generated either by classical genetic analytical techniques, and/or by molecular genetic techniques.

The initial subtraction protocol may identify encystation specific genes/gene products that are not turned on early in the encystation pathway. Alternatively, we may find that we can identify no homologies to genes/gene products in other organisms

developmental pathways. To deal with these possible outcomes of the initial project, we plan to: 1) repeat the subtraction technique using dsDNAs generated from mRNAs gathered only early in encystation, and many repeated rounds of PCR and subtraction; 2) use gene mutants generated either by classical genetic analytical techniques, and/or by molecular genetic techniques (the ability to reintroduce manipulated clones back into Entamoeba is currently being developed in the PI's laboratory), and attempt to identify molecules further upstream in the encystation developmental pathway. The knowledge gained from this project will help to open a second, potentially vulnerable developmental process from which amebiasis can be attacked.

25x

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27x

Biographical Sketch.

Education:

BS (Biology) Pennsylvania State University
MS (Genetics) Pennsylvania State University
Ph.D. (Genetics) Pennsylvania State University

Professional Experience:

Laboratory Technician, Pennsylvania State University, Biophysics Department, 1974-1976.

Research Assistant; Teaching Assistant, Pennsylvania State University, Genetics Program, 1976-1979.

Research Assistant, Genetics Program
Teaching Assistant, Molecular and Cell Biology Department, Pennsylvania State University, 1979-1982.

Research Fellow, Division of Biology, California Institute of Technology, 1982-1990.

Assistant Professor of Biochemistry, Morehouse School of Medicine, Present.

Relevant Publications:

DNA Manipulation:

Bond, V.C., and B. Wold. (1987). Mol. Cell. Biol. 7: 2286.

Ngai, J., Bond, V.C., Wold, B., and E.Lazarides. (1987). Mol. Cell. Biol. 7: 3955.

Bond, V.C., and B. Wold. (1993). Mol. Cell. Biol., in press.

Entamoeba:

Bond, V.C., Beanan, M.J., and G.B. Bailey. (1992). Arch. Med. Res. 23(2): 11-13.

E BUDGET INFORMATION AND ESTIMATES

1. ESTIMATED TOTAL FUNDING REQUESTED

Direct Costs:	70677.30
Indirect Costs:	29264.47
Total Requested	99941.77

2. COST SHARING. (None)

3. DETAILED BUDGET.

Salaries:	Effort	Salary	Fringe	Total
Vincent C. Bond, PhD., PI	10%	4820.4	1156.9	5977.30
Research Associate	100%	30000.0	7200.0	37200.00
Personnel Totals:		34820.4	8357.9	43177.30

Travel:	
PI to scientific meetings	2000.00
Equipment:	
DNA Sequencing Gel Apparatus	1000.00
Supplies:	
Membranes/filters	3500.00
Primer preperation	2000.00
Chemicals	1000.00
Disposables	3000.00
DNA kits	3000.00
Radioactive Materials	3000.00
Media	3000.00
Packaging extracts/vectors	1500.00
film/cassettes/screens	2000.00
Other Costs:	
Equipment Maintenance and Repair	750.00
Publication Costs	1000.00
Radioactivity Disposal	750.00

4. IINDIRECT COSTS. (42% of all except equipment) 29264.47

Total: 99941.77

29x

F. BUDGET JUSTIFICATION.

All estimates are based on an anticipated funding time frame of a year and one half.

PERSONNEL: Fringe benefit rate is 24%. The Research Associate is essential for this study. This is a to be filled position. We are looking for a person proficient in molecular biology. If possible he or she will also have some expertise in ameobiasis, although this part is not a prerequisite.

Travel: Travel support is requested for the PI to attend the International Seminar on Amebiasis in Mexico City in 1995. This meeting is integral in allowing the PI to interact with other amebiasis investigators, gather unpublished pieces of information, and convey any data PI will have.

Equipment: A sequencing gel apparatus is requested ($1000, BioRad). While two sequencing systems exist in the Molecular Genetics Core Facility, and a DNA sequenator is on order, the large volume of sequencing that will be required for this project justifies a dedicated apparatus for the PI's laboratory. The PI has the required power supply.

Supplies: Nylon membranes are required at several stages of cDNA library screening. They are very expensive and nitrocellulose will be used for noncritical library screening assays. The original master filters will be made on nylon filters to facilitate repeated rescreening. We have budgeted $3500 for the period.

While we will move to fluoresence based sequencing and hybridizations in the future, we expect to still need radioisotope based procedures. Therefore, we must project expenses of ^{32}P dNTPs cost $150/vial; we expect to use at least 2 vials per month. ^{35}S dATP utilized mainly for DNA sequencing will cost $175/vial, and we exspect to use 1-2 vials per month. Based on these estimates, we have budgeted $3000 for the period.

Library screening and sequencing require X-ray film, cassettes, and intensifier screens. We estimate $2000 for these costs.

Recombinant DNA techniques required in AIMS II and III require bacteria culture media, yeast extract, tryptone, and agar. Amoeba culture requires a variety of similar ingredients plus 15% bavine serum. This latter item is of particular expense. Based on past experience, we request a total of $3000/year for these expenses.

Packaging extracts, cosmid, and cDNA vectors are expected to be

about $1,500.

DNA modifying enzymes used for recombinant DNA manipulations, PCR, probe making, and sequencing are the more expensive items required for this project. General restriction enzymes like EcoRI,

rare cutters like NotI will cost $75-$150/vial. Vent polymerase (NEB) for PCR costs $70/vial; T7 polymerase (Sequenase) for sequencing costs $100/vial. Reverse Transcriptase, Klenow fragment, polynucleotide kinase, DNA ligase, photobiotin labeling kit, polyA mRNA kit -- together, these items will be expected to cost $3000.

A variety of chemicals are required for nucleic acid isolation, hybridization, probe preperation, and gel analysis, ethanol for precipitation, ammonium persulfate for gels, antibiotics for plasmid and phage isolation, CsCl for DNA purifications, organics for nucleic acid purification, beads (like Sephacryl) for column purifications. A total of $1000 is estimated for these.

Disposables, which will be used at all stages of this project include pipets, tips, reaction vials, tubes of various kinds, glassware, bacterial plates, sterilization filters, cell culture vessels, gloves.

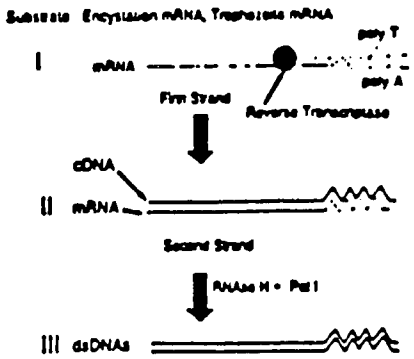
We will have access to an inhouse oligonucleotide synthesizer. Therefore, for primer use in this project we estimate the cost of buying the chemicals to make our own oligonucleotides at about $2000.

<u>Other Direct Costs:</u> Costs for radioactive waste disposal and other safety compliance requirements are estimated at $750. The same amount is requested for equipment repair and maintenance costs. Computer costs include annual institutional network fees for service and maintenance for PI's PC and Sun workstation.

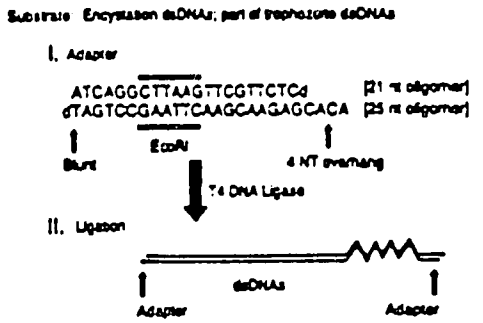
31x

SUBTRACTION LIBRARY CONSTRUCTION

2A. Strand synthesis

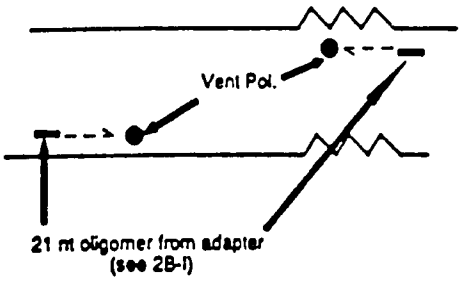


2B. Adapter ligation



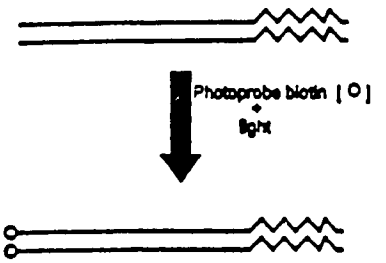
3. PCR Amplification

Substrate: Adapter linked Encystation dsDNA

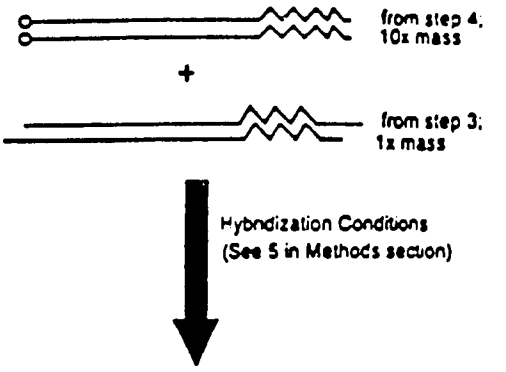


4. Photobiotin labeling

Substrate: Trophozoite dsDNAs without adapters



5. Hybridization of biotin labeled trophozoite DNA with adapter ligated encystation dsDNAs.

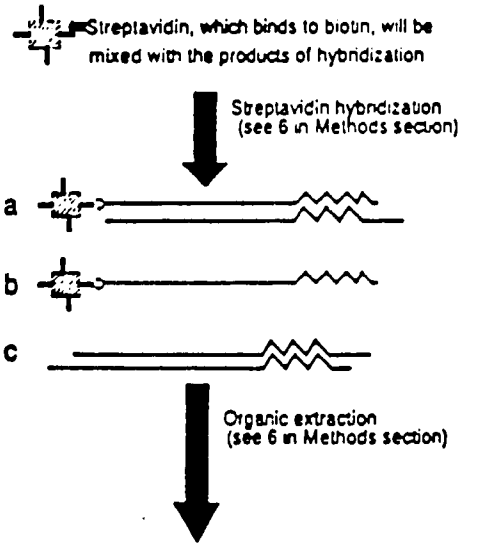


The resultant single stranded biotin labeled trophozoite cDNAs from 4 can:

1) rehybridize to a complementary biotin labeled trophozoite cDNA.
2) stay single stranded.
3) rehybridize to a complementary adapter linked encystation cDNA from 3.

10x fold excess of the biotinylated trophozoite dsDNA will be used to ensure a reaction biased towards (3) enrichment for encystation specific sequences only.

6. Streptavidin subtraction.



During organic extraction, streptavidin and anything bound to it will partition out of the aqueous phase and into the organic phase. Therefore, thru the use of organic extractions, all streptavidin bound molecules (a and b) will be removed, or subtracted. Only those molecules that are not bound to streptavidin (c) will be left in solution. These will be dsDNAs that are unique to encystation.

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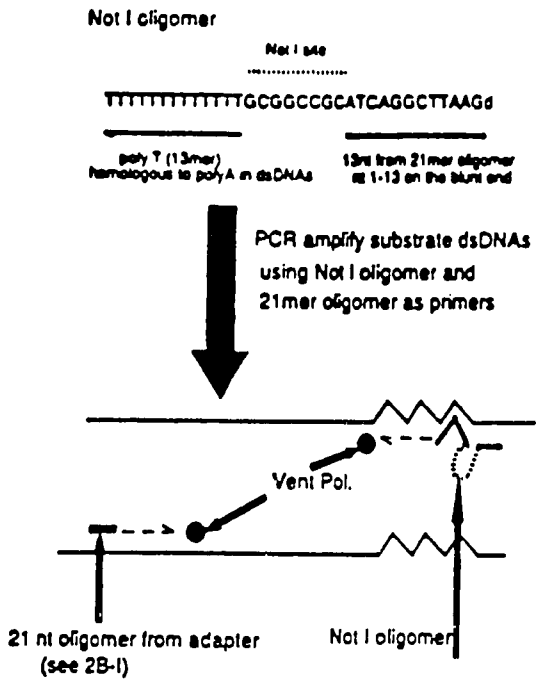
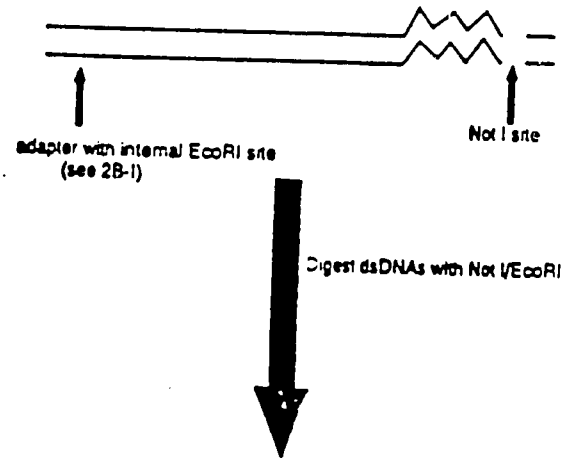
8. PCR Amplify products of subtraction.

Substrate: encystation specific enriched dsDNAs left after steps 6 and 7



9. Digest and directionally clone.

Substrate: dsDNA PCR products of step 8



Directionally clone products of dsDNA digest into lambda vector.

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33x

APPENDIX

Letter of Collaboration and Biographical Sketch

Gordon B. Bailey, Ph.D.

MOREHOUSE
SCHOOL OF MEDICINE

Department of Microbiology & Immunology

Vincent Craig Bond, Ph.D.
Assistant Professor of Biochemistry
Morehouse School of Medicine

16 March 1993

Dear Craig:

I am delighted that you will undertake the study of gene expression during encystation in *Entamoeba*. This could be immensely important to the study of regulation in *Entamoeba*, and in the etiology of amebiasis. To my knowledge, no one else is working in this area, and thus this research will be a valuable contribution. It is exciting to me that you have developed an interest free of any persuasion from me. The ongoing collaboration between our labs on the unrelated USAID *Entamoeba* grant you are finishing has worked to our mutual benefit, and I am sure the present project will as well.

My laboratory will be happy to provide you all the assistance you and your lab staff need to develop your skills in working experimentally with *Entamoeba invadens* and to collaborate in any aspects of the work where my experience can contribute. This will include providing you with axenic cultures, training in culture techniques, and all experimental manipulations with this organism you require. I will also provide you access to all of the bibliographic material I have collected over the years on *Entamoeba invadens* and amebiasis, and will give you whatever advice and ideas I can from my own experience.

Good luck

Sincerely yours,

Gordon B. Bailey

Gordon B. Bailey, Ph.D.
Professor and Chairman of Microbiology

KNOWLEDGE • WISDOM • EXCELLENCE • SERVICE
720 Westview Drive, S.W. Atlanta, Georgia 30310-1495 Telephone (404) 752-1580/1583

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Biographical Sketch.
Gordon B. Bailey, Ph.D.

Education:

BA (Botany) Brown University
MA (Botany/Chemistry) University of Massachusetts
Ph.D. (Biochemistry/Chemistry) School of Medicine,
University of Florida

Appointments:

Professor, Department of Biochemistry, Morehouse School of Medicine, Atlanta, Georgia 1976 - present

Program Director, Research Centers in Minority Institutions (RCMI) Program, Morehouse School of Medicine 1986 - present

Acting Chairman, Department of Biochemistry, Morehouse School of Medicine 1987

Program Director, Biomedical Research Development Program, Morehouse School of Medicine 1978 - 1981

Assistant Professor, Department of Biochemistry, St. Louis University School of Medicine 1973 - 1976

Deputy Director, Anemia and Malnutrition Research Center, Faculty of Medicine, Chiang Mai University (Thailand) 1975 - 1976

Consultant, The World Health Organization 1975 - 1976

Visiting Professor, Department of Biochemistry, Faculty of Medicine, Chiang Mai University 1973 - 1976

Visiting Research Professor, Anemia and Malnutrition Research Center, Faculty of Medicine, Chiang Mai University 1973 - 1976

Field Staff, The Rockefeller Foundation 1966 - 1973

Visiting Professor, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand 1966 - 1973

Publications on *Entamoeba*.

Bailey, G.B., D.B. Day and N.E. McCoomer. *Entamoeba* motility: Dynamics of cytoplasmic streaming, surface capping and organization of the actin cytoskeleton during locomotion by *Entamoeba invadens*. Submitted to J. Protozool.

Bailey, G.B., J.R. Gilmour, N.E. McCoomer. 1990. Roles of target cell membrane carbohydrate and lipid in *Entamoeba histolytica* interaction with mammalian cells. Infect. Immun. 58:2389-2391.

Bailey, G.B., E.D. Nudelman, D.B. Day, J.R. Gilmour and C.F. Harper. 1990. Use of non-cellular models to study the interaction of *Entamoeba histolytica* with mammalian cells. Arch. Invest. Med (Mex.). in press.

Bailey, G.B., E.D. Nudelman, D.B. Day, C.F. Harper and J.R. Gilmour. 1990. The specificity of glycosphingolipid recognition by *Entamoeba histolytica*. Infect. Immun. 58:43-47.

Bailey, G.B. Chemotaxis by *E. histolytica*. in Ravdin J.I. ed., Amebiasis: Human infection by *Entamoeba histolytica*, Wiley and Sons, Inc. New York. 1988, pgs. 339-350.

Bailey, G.B., D.B. Day, C. Nokkaew and C.C. Harper. 1987. Target cell membrane lipid stimulation of *Entamoeba histolytica* actin polymerization and phagocytosis. Infect. Immun. 55:1448-1453.

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Bailey, G.B., D.B. Day and G.J. Leitch. 1985. Chemotaxis by *Entamoeba histolytica*, J. Protozool. 32:341-346.

Leitch, G.J., A.D. Dickey, I.A. Idezulu and G.B. Bailey. 1985. The behavior of *Entamoeba histolytica* trophozoites in the lumen and mucus blanket of the *in vivo* rat colon. Infect. Immun. 47:68-73.

Udezulu, I.A., G.J. Leitch and G.B. Bailey. 1982. Use of indomethacin to demonstrate enterotoxic activity in extracts of *Entamoeba histolytica*. Infect. Immun. 36:795-801.

Bailey, G.B. 1982. Preliminary studies of chemotaxis by *Entamoeba*. Arch. Invest. Med. (Mex.) 13 (Supl. 3):265-271.

Sirijintagarn, P. and G.B. Bailey. 1980. The relationship of DNA synthesis and cell cycle events to encystation by *Entamoeba invadens*. Arch. Invest. Med. (Mex.) 11(Supl. 3):1-6.

Bailey, G.B. and S. Rengpien. 1980. Osmotic stress as a factor controlling encystation by *Entamoeba invadens*. Arch. Invest. Med. (Mex.) 11(Supl. 3):7-10.

Kusamrarn, T., P. Sobhon and G.B. Bailey. 1975. The mechanism of formation of inhibitor induced ribosome helices in *Entamoeba invadens*. J. Cell Biol. 65:529-539.

Kusamrarn, T., K. Vinijchaikul and G.B. Bailey. 1975. Comparison of the structure and function of polysomal and helical ribosomes from *Entamoeba invadens*. J. Cell Biol. 65:540-549.

Rengpien S. and G.B. Bailey. 1975. Differentiation of *Entamoeba*: A new medium and optimal conditions for axenic encystation of *Entamoeba invadens*. J. Parasit. 61: 24-29.

Rengpien S. and G.B. Bailey. 1974. Stimulation of encystation of *Entamoeba invadens* by reduced osmolarity. Proc. 1973 Bangkok Symp. Sci. Res. 2: 248-253.

Boonsong, S. and G.B. Bailey. 1974. Novel mechanisms of energy metabolism in *Entamoeba*. Proc. 1973 Bangkok Symp. Sci. Res. 2: 310-315.

Seng, L. and G.B. Bailey. 1974. The requirement for DNA synthesis and cell division during differentiation of *Entamoeba*. Proc.1973 Bangkok Symposium on Sci.Res. 2:350-357.

Thepsuparangsikul, V., L. Seng and G.B. Bailey. 1971. Differentiation of *Entamoeba*: Encystation of *E. invadens* in monoxenic and axenic cultures. J. Parasit. 57: 1288-1293.

Thepsuparangsikul, V., L. Seng and G.B. Bailey. 1971. Differentiation of *Entamoeba*: Encystation of *E. invadens* in monoxenic and axenic cultures. J. Parasit. 57:1288-1293.

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Recent Abstracts on *Entamoeba*.

Shen, P-S., C. Nokkaew, C. Aloisio and G. B. Bailey. 1990. Liposomes capture membrane proteins from whole cell lysates of *Entamoeba histolytica*. J. Cell Biol. 111:74a.

Shen, P-S. and G. B. Bailey. 1990. Liposome capture: a technique for isolation of plasma membrane proteins from whole cell lysates of *Entamoeba histolytica*. 1st Asian and Pacific Congress on Cell Biology, Shianghai.

McCoomer, N. E. and G. B. Bailey. 1990. Cytofluorescent Study of the Mechanism of Pseudopod Extension by *Entamoeba*. Morehouse College Science Symposium.

Bailey, G. B., D. B. Day, N. E. McCoomer and C. F. Harper. 1989. Mechanisms of *Entamoeba* motility. Abstract, Arch. Invest. Med. (Mex). XIth International Seminar on Amebasis. Mexico City.

Bailey, G.B., E.D. Nudelman, D.B. Day, C.F. Harper and J.R. Gilmour. 1989. Use of non-cellular models to study the interaction of *E. histolytica* with mammalian cells. Abst B-113 pg 49 Amer. Soc. Microbiol.

Bailey, G.B., D.B. Day and C.H. Harper. 1989. Actin Dynamics in *Entamoeba* Locomotion. J. Cell Biol. 107:453a.

Nokkaew, C., C. H. Aloisio and G. B. Bailey. 1988. An *Entamoeba histolytica* Monoclonal Antibody the blocks Target Stimulated Amoeba Actin Polymerization. Amer. Soc. Parsit. Abstr. #84.

Harper, C. C., C. Nokkaew and G. B. Bailey. 1988. Proteins associated with the cytoskeleton of *Entamoeba histolytica*. Fed. Proc. 47:903.