Haverford College

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

Haverford College received $18,238 in formula funds for the grant award period January 1, 2013 through June 30, 2015. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Pathoadaptivity of Cell Detaching Escherichia coli – We have accumulated preliminary evidence pointing to cell detaching *Escherichia coli* (CDEC) as diarrheal pathogens. We hypothesize that *E. coli* strains that fit this description evolved greater virulence through loss of a common gene encoding the lactose repressor. We will determine whether CDEC represent a distinct diarrheagenic *E. coli* lineage and will identify potential virulence targets that could be repressed by *lacI.*

Anticipated Duration of Project

1/1/2013 – 6/30/15

Project Overview

Cell-detaching *E. coli* (CDEC) detach cultured epithelial cells from solid supports. In 1993 CDEC were identified as unusual fecal isolates associated with infantile diarrhea. Cell detachment was later attributed to specific bacterial toxins, which with the adhesins also elaborated by CDEC, are produced by E. coli that cause urinary tract infections. Some researchers suspect that these organisms are merely potential uropathogens in stool. A handful of other studies however support the idea that CDEC may be diarrheal pathogens. CDEC isolates are predominantly unable to utilize lactose and the question as to whether or not they can cause diarrhea remains unanswered. The lactose repressor (LacI) is an *E. coli* protein that ensures that lactose-utilization genes (*lacAYZ*) are only expressed when that sugar is available. LacI is a recently uncovered 'antivirulence' factor in *Salmonella* because it blocks virulence gene expression. The current evolutionary model proposes that negative selection led to loss of *lacI* in *Salmonella* and then created selective pressure for subsequent loss of *lacAYZ*, accounting for lactose-negativity. Noting that pathoadaptive evolutionary paradigms have played out in multiple pathogens, we screened CDEC isolates from an earlier epidemiological study for *lacI* and found that CDEC as a whole were not significantly associated with disease, but *lacI*– negative CDEC were ($p<0.05$). A CDEC strain expressing LacI is less virulent than the isogenic wildtype strain in a nematode model. We hypothesize that CDEC are a diarrheagenic lineage of

E. coli that lost the *lacI* gene, and subsequently other *lac* genes, as they evolved virulence because *lacI* represses one or more CDEC virulence factors. We will test this hypothesis through the following specific aims: (1) Do CDEC represent a distinct lineage of diarrhea-causing *E. coli*? We will use multilocus sequence typing to determine whether CDEC strains from different individuals belong to the same or different *E. coli* lineages. (2) Is loss of *lacI* by CDEC pathoadaptive? We will determine whether LacI binds to virulence genes and/or their promoters and thereby represses the transcription of genes that are important for virulence.

Principal Investigator

Iruka N. Okeke, PhD
Associate Professor of Biology
Haverford College
370 Lancaster Avenue
Haverford, PA 19041

Other Participating Researchers

Erin Remaly BSc, MSc – employed by Haverford College
Ann Wolski HC'13 – student at Haverford College

Expected Research Outcomes and Benefits

The principal outcome of our study will be to determine whether CDEC are indeed pathogens, and therefore merit further study. This project will also contribute to the understanding of pathodaptivity through loss of house-keeping genes, as well as to the current knowledge on gene regulation by the *E. coli* lactose repressor. Strains that fit the description of CDEC have only been found in studies where under-nutrition was observed. It is therefore possible that CDEC are only virulent in a subset of individuals (for example the very young, the very old or the undernourished) that are at high risk of persistent diarrhea of unknown etiology and that epidemiologic studies that are not appropriately stratified will miss them. In the event that we are able to identify CDEC lineages and/or elucidate virulence mechanisms, these preliminary data will form the basis for collaborative research to define the epidemiology and risk groups for CDEC diarrhea as well as the potential for interventions available or in development for uropathogenic *E. coli* to ameliorate CDEC disease. Should our data support CDEC diarrheagenicity and the pathoadaptivity of LacI in these bacteria, we will institute collaborations to investigate their role in diarrheal disease.

The experiments described in this project will be performed largely by mentored undergraduate students working in the laboratory of the PI. The students will receive training in molecular microbiology research and opportunities to present their work to a scientific audience. Findings from the study will be disseminated through publication in the peer-reviewed scientific literature and presentation and a national microbiology conference.

Summary of Research Completed

Specific aim 1: To determine whether Cell Detaching *Escherichia coli* (CDEC) represent a distinct lineage of diarrhea-causing *E. coli*.

CDEC strains from children with diarrhea belong to very few sequence types

If cell-detaching *Escherichia coli* (CDEC) represent a true pathotype, they will be defined by a core gene set and will therefore belong to a small number of lineages and/or share conserved mobile elements. We aimed to determine whether CDEC strains belong to one or a few lineages by multi-locus sequence typing (MLST) using the Wirth et al scheme [Wirth et al. Mol Microbiol 60: 1136-1151, 2006]. According to that scheme, we sequenced seven housekeeping genes, *adk, fumB, gyrB, icd, mdh, purA* and *recA*, from fourteen CDEC strains originally obtained from children with diarrhea. Thirteen of the strains were isolated from children with diarrhea in Nigeria and strain A70.1 was from an aborigine child with diarrhea in Australia. We entered the data into the *E. coli* MLST database at www.mlst.net in order generate allele profiles and determine sequence types (STs).

As shown in Table 1, the results reveal that six of the strains, including isolate A70.1, belonged to the ST12 complex (four of these to ST12) and two each to ST1808 and ST505. The data in Table 1 demonstrate that all but four of the tested CDEC strains place into one of these three MLST-defined groups, strongly suggesting that, although there is unlikely to be a single CDEC lineage, pathogenic CDEC belong to a small number of specific lineages. The STs and ST complexes seen with CDEC have also been recorded for uropathogenic *E. coli* and other invasive *E. coli* which share hemolysin, adhesin and cytotoxic necrotizong factor genes with CDEC. However, only a few of the dozens of uropathogenic sequence types were seen with CDEC and only three lineages have so far been documented more than once.

Figure 1 shows a phylogeny for the *E. coli* sequence-type complex 12 produced using eBURST [version 3 at http://eburst.mlst.net/ ; Feil et al. J Bacteriol 186: 1518-1530]. Strains belonging to the founder ST (ST12), and all other STs in ST 12 Complex, have been recovered from invasive human infections, most notably urinary tract infections and sepsis. The CDEC strains belong to STs 12, 438 and 493 only, which, as illustrated in Figure 2 represent a single lineage within the ST12 Complex. Altogether, the data collected to date suggest that pathogenic CDEC may belong to very few lineages. As there is likely to be more than one CDEC lineage but CDEC strains share virulence loci and lactose negativity, it is possible that CDEC lineages evolved through distinct, if similar paths. On-going research is testing other CDEC from diarrhea, as well as eight strains from the Nigeria collection that were isolated from healthy children.

Specific aim 2: To determine whether loss of *lacI* by CDEC is pathoadaptive.

Integrating lacI *on the chromosome of CDEC strain C06b alters in vitro phenotypes associated with virulence*

Using our earlier prepared construct containing *lacI* (the lac repressor gene) and a kanamycin/neomycin resistance gene integrated at *att*Tn*7* (which lies between the genes *pstS* and

glmS) on the chromosome and a similar isogenic construct containing the neomycin/kanamycin resistance cassette without *lacI* at the same site, we studied *in vitro* phenotypes associated with virulence. The two phenotypes we investigated were biofilm formation and HEp-2 cell detachment. We built biofilms of CDEC and control strains in DMEM over 48 hours in 24-well plates. The biofilms were washed and fixed at defined time points, after which we used crystal violet staining and elution to quantify biofilm mass as performed by O'Toole and Kolter [Mol Microbiol 28: 449-461, 1998]. Strain 17-2, a cell-detaching enteroaggregative *E. coli* strain was used as positive control because enteroaggregative *E. coli* are exceptional biofilm formers. C600, a laboratory *E. coli* strain capable of forming biofilms was also tested. To quantify cell-detachment, the defining phenotype for CDEC, we counted HEp-2 cells remaining attached after incubation with a CDEC or control strain. Counting was done manually, after Giemsa staining of the treated monolayer and using a light microscope. Prototypical CDEC strain A70.1 from Australia was the positive control and laboratory *E. coli* strain C600 was used as negative control for this experiment.

The results, shown in Figure 2 demonstrate that inserting *lacI* and a kanamycin/neomycin resistance cassette into CDEC strain C06b produced a detectable but modest reduction in biofilm formation and cell-detaching activity that was not seen when the kanamycin resistance cassette was inserted alone. Based on these findings, we conclude that expressing *lacI* from attTn7 in strain C06b may affect the expression of one or more targets involved in virulence. Experiments in progress will quantify expression of LacI and candidate targets in these strains. Because the results seen were subtle, it is plausible that there are determinants of virulence in CDEC lineages that are independent of LacI regulation. For this reason, and because the best way to identify such determinants would be through a genome-wide screen, we are preparing to use Illumina technology to sequence the genome of CDEC strain C06b. Genome sequencing will also make it possible to find a genetic definition for CDEC.

Table 1: Multilocus sequence typing data from fourteen cell-detaching *E. coli* strains. Data from strains belonging to ST12 complex is shaded in blue and the ST1808 and ST505 strain data in grey and brown respectively. Data from strains belonging to other STs is unshaded.

CDEC strain	MLST alleles adk	fumC	gyrB	icd	mdh	purA	recA	ST	ST COMPLEX
C06b	37	38	229	37	17	8	26	1808	95
D03b	37	38	229	37	17	8	26	1808	95
E01b	13	13	9	13	16	10	9	12	12
E26	13	13	9	13	16	10	9	12	12
G30a	40	13	9	13	16	75	9	438	12
G76	6	11	4	10	7	8	6	93	168
G75	ND	35	61	52	5	5	4	ND	
C11a	40	13	9	13	16	10	9	493	12
G40a	13	13	9	13	16	10	9	12	12
E05a	24	11	4	ND	8	ND	2	ND	
E08a	6	11	5	1	20	ND	9	ND	
E29a	53	40	104	13	26	28	29	505	None
G02a	53	40	104	13	26	28	29	505	None
A70.1	13	13	9	13	16	10	9	12	12

ND= Not yet determined: pending data

Figure 1: The ST12 sequence type complex of *E. coli*. The size of each node is proportional to the number of database strains used to compile the data and the founder ST, ST12, is marked in blue. All STs within the complex have been recovered from invasive human infections including urinary tract infections and sepsis. In this study, CDEC strains have only been detected within the sequence types boxed in red.

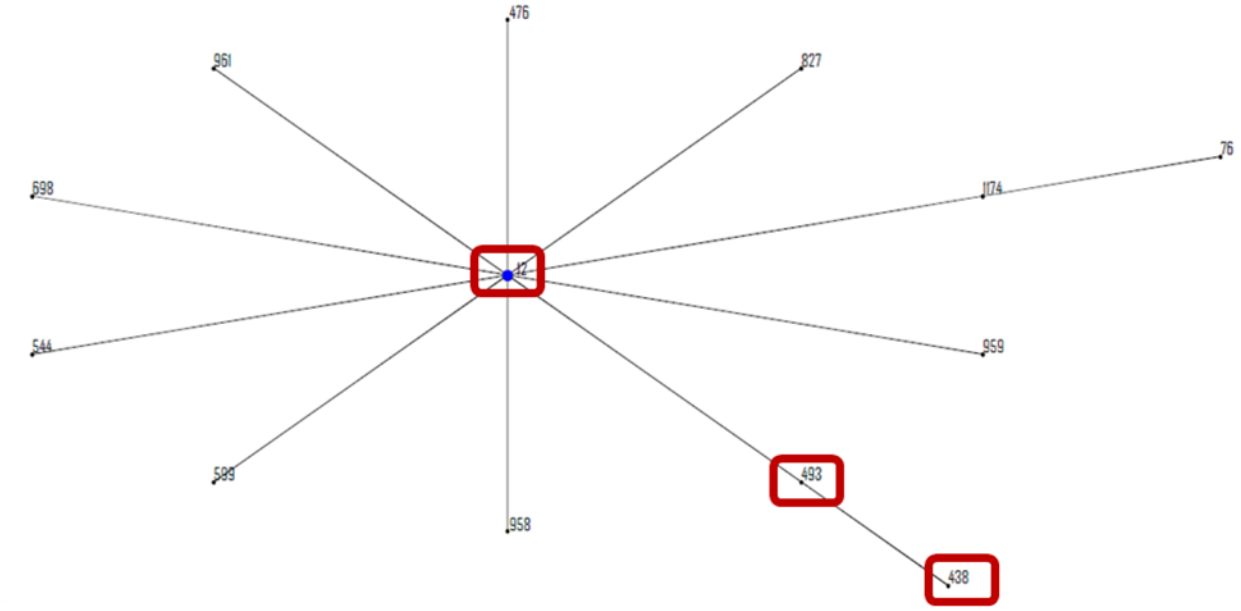
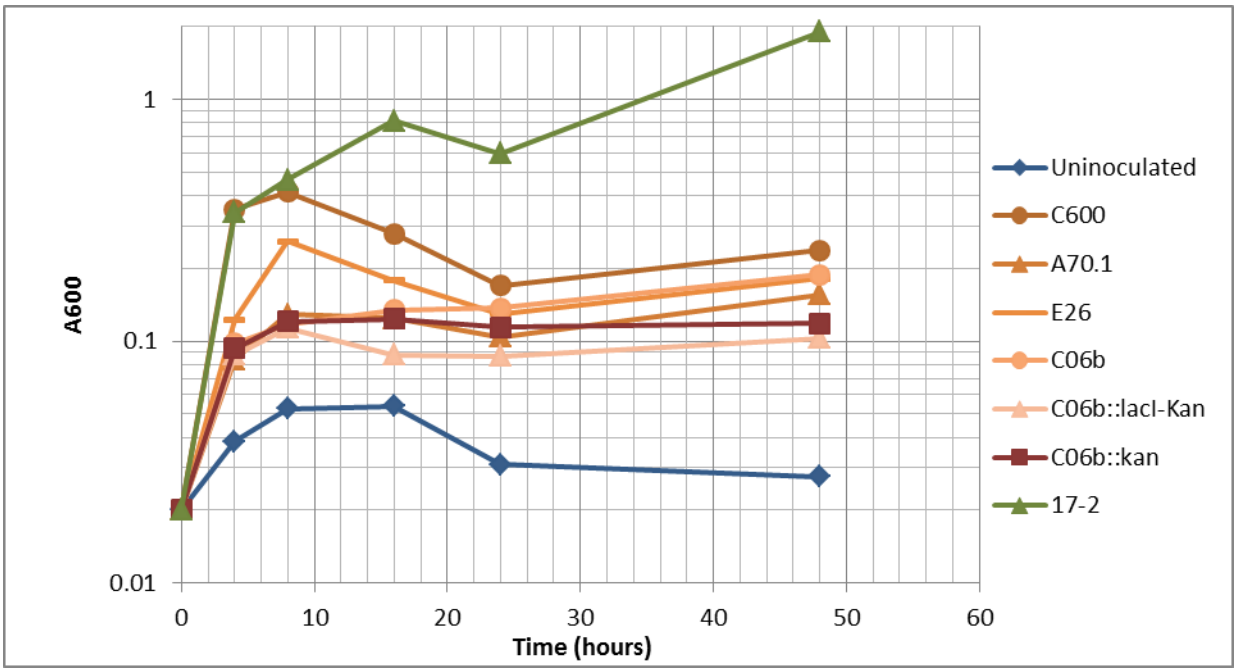
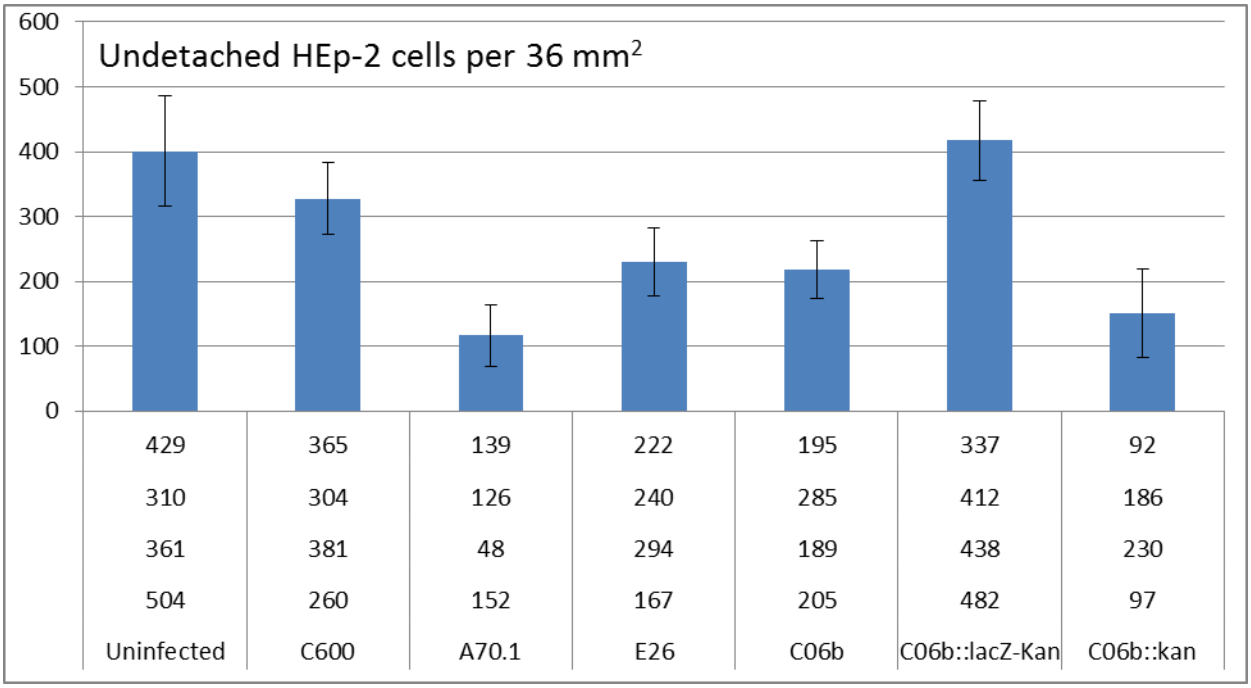


Figure 2: Inserting *lacI* onto the chromosome of CDEC strain C06b produces modest reductions in biofilm formation and HEp-2 cell detachment. (a) biofilm formation quantified by crystal violet staining and elution. CDEC strains C06b, A70.1 and E26 are poor biofilm formers and insertion of *lacI* into C06b led to a modest reduction in biofilm formation that was not seen when only a neomycin/ kanamycin-resistance cassette was introduced into the strain. (b) Cell detachment is the defining phenotype of CDEC. The number of undetached cells per 36mm^2 surface was increased when *lacI* was added to C06b. 17-2 and C600 are control strains.

2A



2B