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80 INQUIRY Volume 4 2003

TRANSCRIPTIONAL CONTROL OF THE *OPP* OPERON IN CLOSTRIDIUM DIFFICILE

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Abstract:

As a serious expense for both the hospital and the patient, nosocomial infections create a burden on the health care industry that is not easily overcome. Among the infections commonly contracted in the hospital environment, those associated with the Clostridium difficile bacterium account for millions of cases each year. Largely due to the nature of C. difficile infection as a response to the disruption of the normal flora of the colon caused by antibiotic activity, no completely effective treatment for this condition has been identified. It is this problem that forms the foundation for research devoted to the development of a control mechanism for the expression of the oligopeptide permease (opp) genes, which are important metabolic structural genes in the C. difficile genome. In the work reported here, a genetic construct with the ability to monitor the activity of the dual promoter known to control the expression of the opp genes was created. When this construct was used in combination with a highly expressed gene for an opp regulatory protein, we found that gene expression associated with the oppDF promoter was enhanced significantly, while oppAB expression was greatly inhibited. This pattern was observed using fructose, glucose, mannitol, and peptides as growth substrates, with the degree of induction and repression varying with the nature of the substrate. Future applications of the pUA442 construct may make it possible to determine the specific conditions that prevent the formation of structures of metabolism in C. difficile on the most basic transcriptional level, and ultimately allow for the regulation of one of the nosocomial infections that so greatly contributes to the problems of the health care industry.

Introduction:

In a recent study, 15% of observed hospital patients developed *Clostridium difficile*-associated nosocomial infections, resulting in 3.6 additional days of hospitalization and a 54% increase in the cost of care. From this and other related data, it was estimated that the United States spends more than 1.1 billion dollars fighting and treating these infections every year (1). As this statistical information shows, *C. difficile*-associated infections pose a serious problem to both the health care industry and its patients. A variety of signs and symptoms and a wide range of severity mark such infections, which may occur without symptoms, as basic diarrhea, or as any of a variety of degrees of colitis (2). However, when minor and largely asymptomatic infections go undiagnosed, serious and even fatal cases of CDAD may occur in the form of toxic megacolon and bowel perforation (3). *C. difficile* infections of all levels of severity are generally the result of antibiotic treatments that alter the normal flora of the colon, ultimately allowing for growth of the Gram-positive, spore-forming bacterium. Following this colonization, *C. difficile* produces toxins A and B, which, along with its adhesive abilities, serve as some of its most important virulence factors (4). These virulence factors prove to be quite successful, as no treatment has been consistent in combating *C. difficile* infections. The strong antimicrobial drugs metronidazole and vancomycin are generally used to treat *C. difficile*, but recurrences of infection following these therapies are common (5).

The very real implications of *C. difficile* infection provide a sufficient motive for studying the bacterium in order to ultimately develop a more reliable treatment, and it is the achievement of this goal through genetic control of the system responsible for *C. difficile* adherence and substrate utilization that forms the foundation of this research. A set of structures thought to provide these abilities has been identified in the oligopeptide permease (Opp) system of the bacterium, which in *C. difficile* is comprised of four subunit types designated Opp A, B, D, and F. In association with each other, these proteins of the Opp system allow for the uptake of peptides of various sizes and sequences, an activity important in *C. difficile* due to the preferential utilization of peptide substrates by the bacterium (6). In addition, the Opp A ligand-binding protein on the surface of the bacterial cell membrane is thought to play a significant role in the adhesive properties of the bacterium, thus acting as a factor of colonization. The implications of this information are far-reaching as control of the creation of Opp system proteins on the genetic level could possibly thwart both the adhesive and substrate utilization abilities of the bacterium, ultimately preventing *C. difficile* infection. The genetic locus of the Opp system has been identified and isolated by the Ivey lab, and detailed analysis has proven its composition to be relatively unique. Interestingly, the *opp* locus is not consistent with typical models of genetic organization in peptide permeases, instead consisting of a dual promoter controlling the *oppA* and *oppB* genes on one side and *oppD* and *oppF* oriented in the opposite direction on the other (Figure 1).

It is the activity of this unique promoter that forms the basis for the presented research, as a more complete understanding of the bacterial *opp* locus can provide important insight into the development of methods of control of the genes that ultimately produce the proteins of the *C. difficile* oligopeptide permease. If activity of the dual promoter is prevented, the *opp* genes it controls cannot be transcribed into messenger RNA, thus preventing translation into respective Opp proteins and the formation of the Opp system necessary for *C. difficile* substrate utilization and adhesion. By this chain of events, the survival mechanisms and infectious abilities of the bacterium would essentially be eliminated, along with associated pathology and disease.

On the most basic level, the achievement of these goals depends entirely on the successful production of a specific dual-reporter plasmid construct according to the methods of recombinant DNA technology and other experimental methods of molecular biology. This construct was designed so that independent genes able to transcribe two slightly different types of luciferase flank the unique *C. difficile* dual *opp* promoter region in order to provide a means of distinguishing the simultaneous expression of the oppositely oriented gene loci (Figure 2). The first of these is a firefly luciferase that emits light in the presence of luciferin (7). A measure of the light produced through the enzymatic action of luciferase can be directly correlated to the level of transcription of the luciferase gene, and thus the activity of the *oppD* and *oppF* promoter of the dual operon system. On the opposite side of the *opp* promoter and oriented in the opposite direction is the *Renilla* luciferase gene, which is able to simultaneously produce a similar but distinct luciferase when exposed to coelenterate-luciferin (8). The activity of the promoter for *oppA* and *oppB*, corresponding to the expression of this *Renilla* gene, can then be compared to that of the *oppD* and *oppF* genes, as indicated by levels of firefly luciferase present, concurrently and under identical conditions. Ultimately, the ability of this proposed construct to monitor promoter activation will make it possible to identify the environmental conditions that initiate and, conversely, prevent gene expression. Furthermore, understanding this bacterial stimulus-response may eventually allow for the development of a chemotherapeutic agent capable of controlling the metabolic and adhesive properties that facilitate the colonization, and thus infection, of *C. difficile*.

Materials and Methods:

Isolation of Plasmid DNA Construct Components

Plasmids that contain the promoter and luciferase genes required for the formation of the desired construct were isolated through the use of the Wizard" Miniprep kit. The pUA319 plasmid was the source for the *opp* promoter and the gene that encodes firefly luciferase, and the pDM543 plasmid provided the *Renilla* luciferase gene (Figure 2). Plasmid isolations from the pUA319 and pDM543 strains were determined to be successful through analysis using agarose gel electrophoresis.

Construct Formation through Ligation-Independent Cloning

The ligation independent cloning process involves the creation of linearized segments with long nucleotide overhangs from a desired vector and insert. The bases of these overhangs are then excised by the 3'—5' exonuclease activity of DNA polymerase until a specific recognition site is reached on each. According to their design, the products of this reaction are complementary to each other, and thus a recombinant plasmid is able to form through a simple annealing reaction and without the use of ligase. The formation and amplification of the desired linear overhang segments of each plasmid was successfully achieved through the use of the polymerase chain reaction (PCR). Agarose gel electrophoresis of each of the PCR products indicated the presence of a band of the desired size just under 5 kb for the pUA319 sample and at 1 kb for the pDM543 plasmid (Figure 3). Purification of these samples through exposure to Dpn1, as well as treatment with chloroform:isoamyl alcohol and further purification using the QIAquick PCR purification kit followed. Treatment with T4 DNA polymerase according to the specific nucleotide overhangs created for each plasmid, as well as digestion with Kpn1 to prevent the formation of background colonies produced from any remaining template plasmid, was used to prepare the linearized samples for the annealing reaction. Annealing was performed at 22°C for 1 h. Annealed products were then used for transformation of E. coli. Subsequent plating on Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml) confirmed the success of ligation-independent cloning. Further confirmation was provided by a rough plasmid isolation using phenol and chloroform, which, through agarose gel electrophoresis, a plasmid of the desired 6kb size. The remaining cultures consistently produced 5kb bands, hinting at background growth from the original pUA319 or pDM543 plasmids. To determine the exact nature of the promising sample, the more precise plasmid purification procedure used previously for the isolation of pUA319 and pDM543 from culture was employed, this time using the QIAprep Spin Miniprep Kit.

Luciferase assays

To measure the response of the pUA442 construct to the repression of its promoters, the Dual-Luciferase, Reporter kit (9) was employed. This system is able to characterize promoter response by preparing cell cultures in such a way that the activity of the firefly and *Renilla* luciferase proteins produced through the transcription and translation of the genes controlled by the dual *opp* promoter may be quantified. Since these proteins are known to emit light as they break down specific types of luciferin, interaction with such compounds then provides a means of measurement of protein presence and activity through light emissions detected by a luminometer. Protein determination

employed the bicinchoninic acid assay, and was performed as described by the manufacturer (Pierce, Rockford, IL).

Results and Discussion:

Analysis of Purified Plasmid

To verify that the plasmid was in fact the desired construct, the purification product was first subjected to the PCR process using primers designed to amplify a portion of the desired construct that contains segments of each of the original pUA319 and pDM543 plasmids. PCR would be predicted to produce an amplified product of about 1 kb in size from the correct plasmid, but no result from any other plasmid (i.e. background). According to the electrophoresis results, gradient PCR process produced strong 1kb bands at all but the highest annealing temperature, thus providing the first proof that the plasmid isolated from the annealing reaction culture was in fact the desired construct (Figure 4A).

This confirmation was further supported through diagnostic tests using restriction endonuclease digestion. Two digests were set up according to knowledge of the location of specific restriction sites on the desired recombinant plasmid. The QIAprep-purified plasmid digested with

Kpn1 was unaffected, as evidenced by the presence of a band just under 6 kb produced through electrophoresis (Figure 4B). This band of the same size as the isolated plasmid indicates that there were no restriction sites specific to the endonuclease, which is consistent with the expected nature of the recombinant plasmid. A double digest with the *Pst1* and *Xba1* endonucleases was also performed, with results analyzed through agarose gel electrophoresis producing bands at 4 and approximately 1.6kb (Figure 4B). The location of unique *Pst1* and *Xba1* restriction sites on the recombinant plasmid determine that digestion with these endonucleases should produce fragments of 4146 and 1681 base pairs in size, thus providing further verification that the plasmid produced through the LIC process and isolated in QIAGEN plasmid purification was in fact the desired recombinant construct. The successfully produced plasmid construct was designated pUA442.

Test of Construct Functional Abilities:

In order to assess the capabilities of the produced construct to measure variability in *C. difficile opp* promoter activity, the effects of repressor activity on the expression of the pUA442 *opp* operon as represented by firefly and *Renilla* luciferase production was tested. A variable, specified as AK_4, and control, known as AK_1, were created for this experimental process through separate co-transformations of the pUA442 construct with different plasmids. The first of these, known as pUA328k and present in the AK_4 experimental variable, is comprised of a kanamycin-resistant vector containing a gene encoding a regulator of the *opp* promoter, while the AK_1 control version of this plasmid known as pWSK129 is simply the kanamycin-resistant vector without the repression gene (Figure 5). Cells separately containing these plasmids plus the pUA442 construct thus provide the experimental system for the test, as the uninhibited activity of the *opp* promoter can be directly compared to that of the same structure under conditions of repression. If the pUA442 construct is functioning as designed, production of more firefly and *Renilla* luciferase proteins and thus higher luminescence measurements would be expected from the AK_1 control sample as compared to the AK_4 repressor variable known to decrease gene expression and protein production.

The results of the reactions set up according to the Dual-Luciferase, protocol (9) can be seen in the data presented as mean light emission values appropriately adjusted for relative protein content (Table 1). The AK_1 and AK_4 samples represented by these results were divided according to incubation time and the presence of the IPTG enhancer known to increase the expression of the repressor genes present in the AK_4 cultures, thus providing even greater levels of repression of the *opp* operon. As the table shows, the first two sets of samples were incubated without the enhancer until reaching mid-log phase at 3.5 hours, while IPTG was added to the next two in the last hour of this incubation. The final four sets of AK_1 and AK_4 measurements were taken from those stationary phase samples incubated for 6.5 hours both without, and then with, the enhancer.

As can be seen by comparing the variable and control in both the mid-log (3.5h) and stationary (6.5h) sample types (Figure 6), light emissions produced from the interaction of the specific luciferin recognized by each of the luciferases were consistently higher in AK_1 than in AK_4, regardless of incubation time and enhancer presence. Furthermore, this same pattern of luciferase gene expression was demonstrated for both the firefly and *Renilla* samples. Although the expression of the firefly genes generally occurred at decidedly lower levels than those of *Renilla* luciferase, the consistency of this deviance suggests that it is the result of the activity of the *opp* promoter. Overall gene expression varied according to the presence of IPTG in the AK_4 samples, as those without the enhancer remained relatively unchanged while those with it showed a slight decrease in protein production over time. However, the general trend of the AK_1 samples of both types showed an increase in protein production, as would be expected of an unrepressed system. It is also interesting to note that a comparison of gene expression in AK_4 samples at the same phase of growth with and without the enhancer shows a decrease in expression in the *Renilla* luciferase genes in the presence of IPTG, but an increase in the expression of the firefly genes. On the whole, these observations prove the functional abilities of the pUA442 construct to monitor the variable expression of the *opp* operon, as it was able to demonstrate the repressor activity known to be a characteristic of the AK_4 variable.

Effect of Growth Substrates on *opp* Promoter Expression:

Following this verification of the functional abilities of the pUA442 plasmid, these capacities were put to work as the created AK_4 construct/repressor system was monitored in the presence of various growth substrates. Preliminary data obtained by analyzing RNA levels of *opp* genes suggests that expression varies in response to peptide availability (A. Richards, personal communication). Thus, cells grown in the presence of peptides or with a variety of carbohydrate substrates can serve as a model system for testing the regulation of expression of the *opp* operon. Due to the abilities of the created construct to act as a dual-reporter of gene expression, a comparison of luciferase production according to type under identical conditions makes it possible to determine the conditions in which both *oppA* and *oppB*, as well as the oppositely oriented *oppD* and *oppF* genes, are preferentially expressed.

In initial experimentation of this type, three carbohydrate growth substrates—fructose, glucose, and mannitol—were selected for testing and used at levels of 0.5% in LB broth media. The AK_4 cultures of each substrate type, as well as an LB control (in which peptides serve as the growth substrate), were further identified according to the presence or absence of the IPTG inducer. To measure levels of *opp* expression in these various growth conditions, cells were assayed for firefly and *Renilla* luciferase activity according to the previously described processes. This experimental procedure was repeated a total of three times, and results averaged to produce the mean values presented here following appropriate adjustments for relative protein content (Table 2).

Examination of these results proves interesting in many respects, the first of which focuses on the effect of the IPTG enhancer on the firefly and *Renilla* expression. As expected, the production and subsequent activity of *Renilla* luciferase is significantly lowered in those samples containing the repressor enhancer, regardless of substrate type (Figure 7A). However, consistent with observations presented in the previous luminescence experimentation, IPTG-induced production of the regulatory protein does not lead to inhibition of the portion of the dual promoter responsible for *oppD* and *oppF* production, here represented as firefly luciferase activity. Light emissions produced through such activity are, in fact, consistently higher in those samples containing the regulatory protein (Figure 7B). From these results it is possible to speculate that while enhancing expression of the portion of the promoter controlling *opp D* and *F* expression, the regulatory protein may block expression of the *opp A* and *B* portion of the dual promoter, thus allowing for the patterns of luciferase expression observed.

A more concrete conclusion made from the luminescence results verifies the hypothesis that specific substrate type has an effect on *opp* promoter activity, as interesting patterns of gene expression according to carbohydrate substrate or the lack thereof were identified in both the *Renilla* and firefly systems. Results of *Renilla* luminescence in enhanced samples show the highest levels of promoter activity in glucose, followed by mannitol, LB alone, and fructose, while those without IPTG were best activated in mannitol, glucose, LB, and then fructose. On the other hand, the highest levels of firefly promoter activity were found in mannitol, LB broth, glucose, and then fructose for samples both with and without the IPTG enhancer. However, due to low levels of expression of the firefly luciferase genes, it is difficult to provide a truly accurate determination of this promoter activity. From these results it can be deduced that the developed plasmid construct is able to successfully monitor variable promoter activity according to growth conditions provided by various substrates.

The successful production of a usable dual-reporter construct and confirmation of its functional and experimental capabilities has important implications for future study and, ultimately, control of the pathogenic activity of the *C. difficile* bacterium. As has been shown, the system can be an important tool in the analysis of the expression of the unique dual operon system of *C. difficile*. Based on its ability to measure transcription of the permeases of the *opp* locus through promoter activity, it is possible to gain a greater understanding of the specific metabolic physical conditions in which *C. difficile opp* genes are expressed. More extensive studies of promoter response to substrate growth conditions may ultimately establish the optimum conditions for the expression of the *C. difficile opp* locus, in turn facilitating the design of specific poisons that utilize the conditions determined to cause chemical deactivation and decrease gene expression. If the genes are not expressed, *C. difficile* will lack the ability to take up and prepare peptide substrates for use, and will be unable to survive. The utilization of this information for the development of a chemotherapeutic agent could make the prevention of the potentially serious diseases associated with *C. difficile* infection a reality.

References:

1. Kyne L, Hamel MB, Polavaram R, Kelly CP. Health care costs and mortality associated with nosocomial diarrhea due to *Clostridium difficile*. *Clin Infect Dis* 34(3):346-53, 2002.

2. Mylonakis E, Ryan ET, Calderwood SB. *Clostridium difficile*-associated diarrhea. *Arch Int Med* 161(4):525, 2001.

3. Fekety R, Shah AB. Diagnosis and treatment of *Clostridium difficile* colitis. *JAMA*. 269(1):71-75, 1993.

4. Poxton IR, McCoubrey J, Blair G. The pathogenicity of *Clostridium difficile*. *Clin Microbiol Infect* 7:421-427, 2001.

5. Pel·ez T, Alcal· L, Alonso R, Rodrìguez-CrÈixems M, Garcìa-Lechuz JM, Bouza E. Reassessment of *Clostridium difficile* susceptibility to Metronidazole and Vancomycin. *Antimicrob Agent Chemo* 46(6):1647-1650, 2002.

6. Seddon SV, Borriello SP. Proteolytic activity of *Clostridium difficile*. *J Med Microbiol*. 36(5):307-311, 1992.

7. Wood, KV. The Chemistry of Bioluminescent Reporter Assays. Promega Notes 65-79, 1998.

8. Sherf BA, Navarro SL, Hannah R, Wood K. Dual Luciferase Reporter Assay: an Advanced Co-Reporter Technology Integrating Firefly and *Renilla* Luciferase Assays. Promega Notes. 57-59, 1996.

9. *Dual-Luciferase, Reporter 1000 Assay System Technical Manual* #046. Promega Corporation. 2001.

Table 1: Luminescence values for construct functional tests
Results of luminescence measurements for the AK_1 control and AK_4 repressor variable in light units. Samples with IPTG are compared to those without the enhancer in both mid-log (3.5h) and stationary (6.5h) phases.

	$AK_1$3.5h		$AK_4$3.5h	AK_1+IPTG3.5h	AK_4+IPTG3.5h		$AK_1$6.5h	$AK_4$6.5h
AK_1+IPTG 6.5h					AK_4+IPTG6.5h			
Firefly	0.790	0.426	1.651	1.094	0.357	0.333	3.702	0.816
Renilla	31.05	28.33	19.76	15.54	64.14	28.05	33.53	13.36

Table 2: Luminescence values for substrate tests
Results of luminescence measurements in light units for AK_4 cultured in various media both with and without the IPTG enhancer.

	Fructose	Fructose+IPTG		Glucose	Glucose+IPTG		Mannitol	
Mannitol+IPTG		LB				LB+IPTG		
Firefly	4.131	9.204	11.71	22.78	26.57	44.20	24.81	30.38
Renilla	185.5	96.15	793.0	327.2	881.4	238.9	482.8	164.9

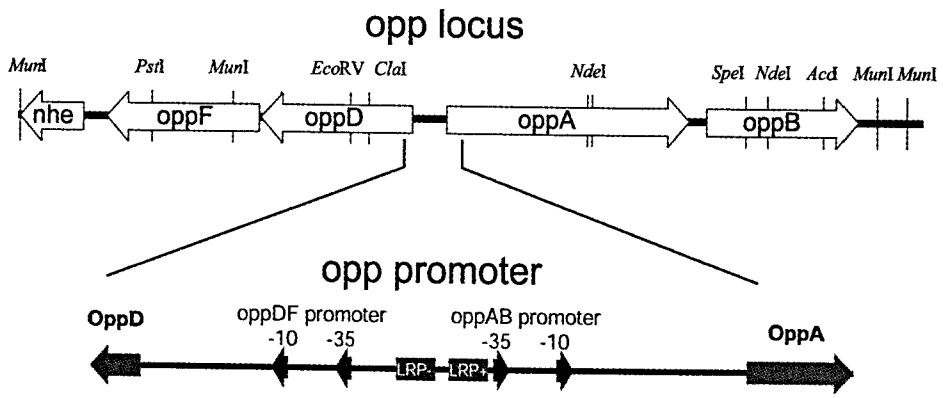
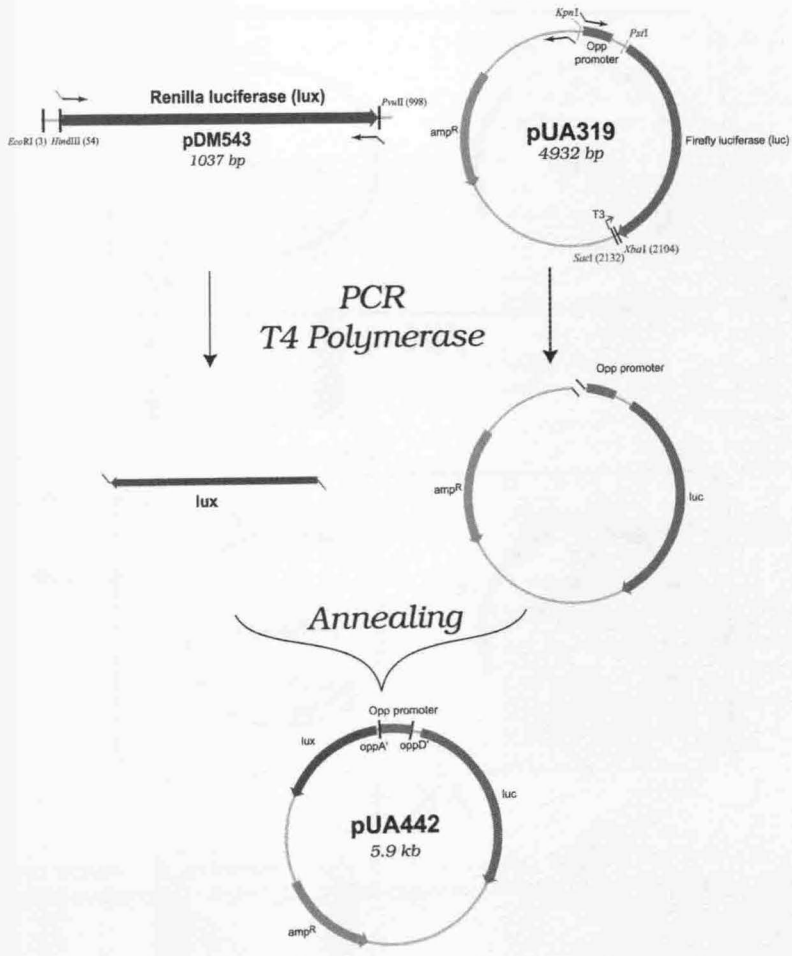


Figure 1: Structural organization of the opp *locus and promoter*
A dual promoter facilitates transcription of oppA *and* oppB *genes oriented in one direction, and* oppD *and* oppF *in the other*



[***Figure 2:*** *Creation of pUA442 construct from pUA319 vector and pDM543 insert. Following PCR amplification of vector and insert with desired nucleotide overhangs, activity of T4 DNA Polymerase works to excise extra bases to a specific recognition site, thus allowing for annealing of complementary ends on the desired pUA319 and pDM543 segments without the use of ligase and completing the process of ligation independent cloning*

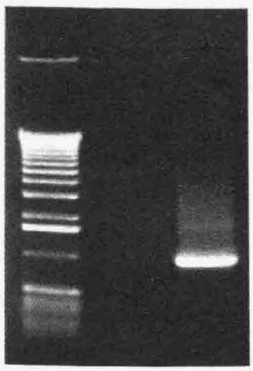


Figure 3: PCR products for ligation-independent cloning. Lane 1, 1kb DNA Ladder; Lane 2, purified pUA319 PCR product for ligation independent cloning at 5kb; Lane 3, purified pDM543 PCR product for ligation independent cloning at 1kb.

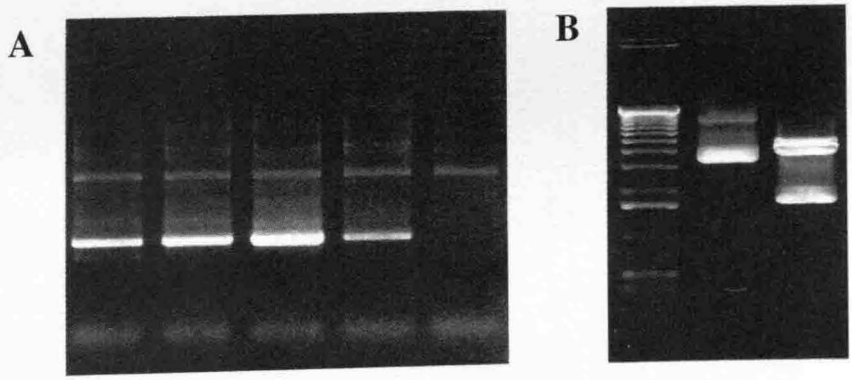


Figure 4: Purified pUA442 PCR products at 1kb. B) Restriction endonuclease verification of pUA442 production- Lane 1: 1kb DNA Ladder; Lane 2, Kpn1*-digested pUA442 plasmid-no signs of restriction endonuclease cuts; Lane 3,* Pst1 *and* Xba1 *double-digested pUA442 plasmid products at 4 and 1.6 kb.*

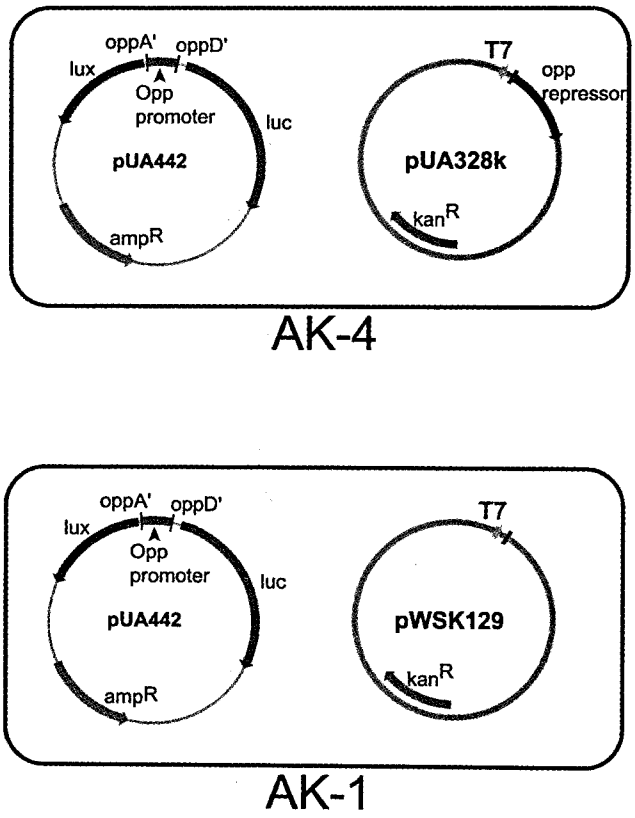
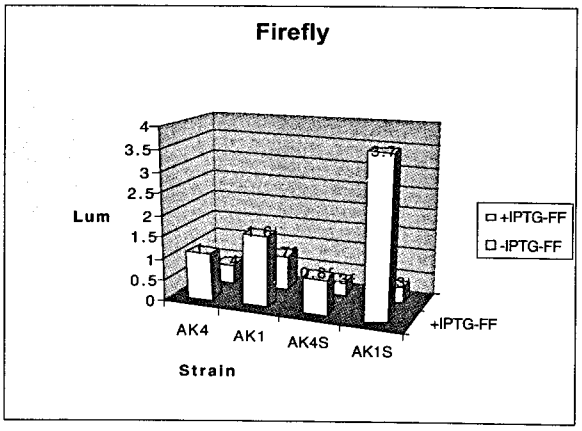


Figure 5: Composition of AK_4 variable and AK_1 control systems. AK_4 contains the ampicillin-resistant pUA442 monitor construct and the kanamycin-resistant pUA328k plasmid with a gene for repression of the opp *promoter, while AK_1 is comprised of pUA442 and the kanamycin resistant pWSK129 plasmid without an* opp *repressor gene.*



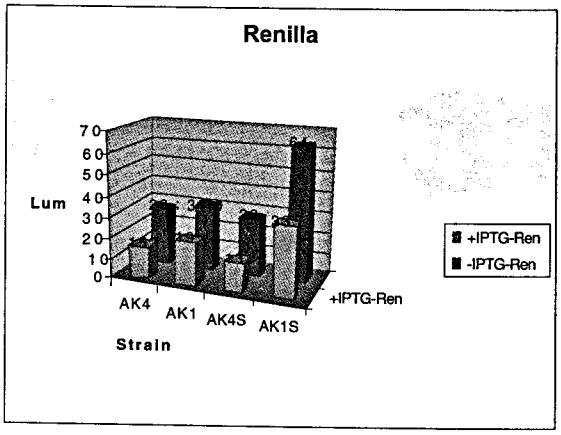


Figure 6: Comparison of luminescence values for construct functional tests
Comparison of firefly and Renilla *luminescence measurements in the AK_1 control and AK_4 repressor variable according to type and presence of the IPTG enhancer.*

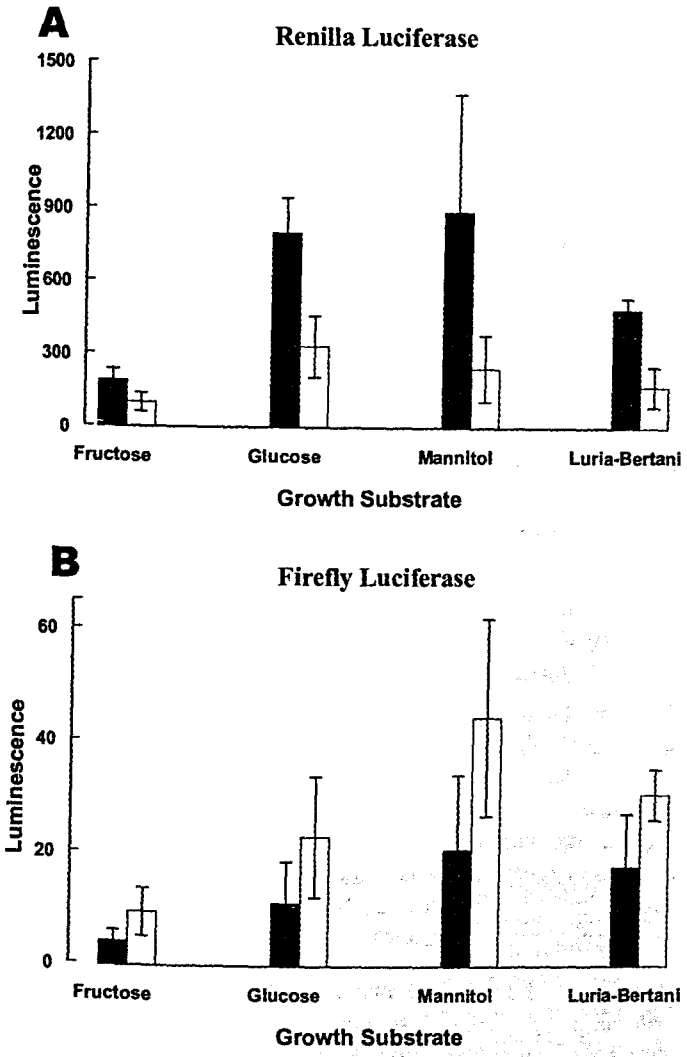


Figure 7: Expression of firefly and Renilla *luciferase in cells grown on various growth substrates. Luciferase measurements were performed as described in Materials and Methods. Measurements (n=3) were taken using fresh cultures on separate days.* Solid bars, *mean values for samples with no IPTG added.* Open bars, *mean values for samples to which IPTG was added. Error bars represent the standard deviation. A) Renilla luciferase activity, and B) firefly luciferase.*

Faculty comment:

D. Mack Ivey, Ms. Davis's mentor, had only the most glowing things to say about her work. He wrote:

> Lindsey Davis is soon to complete her senior year at the University of Arkansas, and will graduate with Honors this spring with B.S. degree in Microbiology and a minor in Spanish. Lindsey performed her thesis research in my laboratory. Lindsey is smart, energetic, curious, independent, and friendly, and has proven to be an exceptional researcher. As a junior, she devoted approximately 20 hours per week to her thesis work, while maintaining a rigorous course load (with a 4.0 GPA) as well as maintaining a busy extracurricular agenda. During the past summer, she worked full time in the emergency room of a Branson, Missouri hospital. This year, her senior year, she has continued her thesis research while taking a full course load. As both a junior and senior, she received departmental awards recognizing the highest academic achievement of any student majoring in Microbiology. She has been awarded a Silo student undergraduate research fellowship, and has received numerous academic awards, including the recent invitation to be a member of Phi Beta Kappa. She has already published an article associated with her Spanish minor.
>
> Lindsey has studied the disease-causing mechanisms of the bacterium Clostridium *difficile*, a human intestinal pathogen that is associated with many cases of hospital-acquired diarrhea and colitis. She has become highly proficient in a variety of recombinant DNA techniques, and is an expert at ligation-independent cloning, which she has used to construct a luciferase dual reporter system to analyze virulence gene regulation. Her analysis of virulence gene expression has lead to the exciting and unexpected discovery of a regulatory protein with both activation and repression functions.
>
> Lindsey is exceptional at lab work. In fact, I strongly suspect that she is exceptional at everything she does. Her experiments are always meticulously planned, and she carries them out with great dexterity. She excels at working independently, but she is not afraid to ask questions when the need arises. She handles the frustrations of the inevitable failed experiment with a very mature equanimity. She is always cheerful and positive. She is the sort of individual who brightens the space around her, wherever she is. She has willingly helped train new lab personnel, and she does more than her share of the general lab maintenance. She holds herself to a high standard, but she is not the least bit critical of others. With some delight, I have observed that her positive attitude and energy have rubbed off on others in my lab. I believe this will happen wherever she goes.
>
> I have been at the University of Arkansas for ten years, and I have supervised an average of four to five undergraduates per year in my lab during this period. Lindsey is one of the two best students I have encountered. Her academic skills are unmatched, and her talent at research is phenomenal.