Characterization of Mutations in *hpmB* of the *Proteus mirabilis* Hemolysin System that Result in Altered Activation and Secretion Functions of the Hemolysin (HpmA)

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ABSTRACT

The hemolysin system of *Proteus mirabilis* consists of two proteins, HpmA and HpmB. HpmB is found in the outer membrane and functions in the activation and extracellular secretion of the hemolysin HpmA. Activated HpmA once released into the external environment lyses a variety of human cells including red blood cells by forming pores in the cell membrane. The activation and secretion functions of HpmB to date have not been separated. The goal of this project is to separate the activation and secretion functions by characterizing mutants of this hemolysin system. Random mutagenesis of *hpm*B generated clones with different classes of altered phenotypes. The two HpmB mutant phenotypes examined here are enhanced activation (superhemolytic), and activation+/secretion-. Generation of these two phenotypes demonstrate that the activation and secretion functions of HpmB are separable events. Characterization of the superhemolytic mutant clones was carried out in a tightly regulated *Escherichia coli* expression system. *hpm*B was cloned into pUC 19 and is under the control of the *lac* promoter, while *hpm*A was cloned into pET 29A and transcription is under the control of the T7 promoter. The activation+/secretion- mutant clones were characterized in a constitutive expression system. In preparation for liquid hemolysis assays, the mutant strains were grown in broth cultures and three different cell fractions were collected: whole cell, supernatant, and sonicate. Liquid hemolysis assays and Western blots were performed on all cell fractions and the results confirm the mutant phenotypes.

INTRODUCTION

Proteus mirabilis is an opportunistic pathogen that causes a variety of human diseases but primarily urinary tract infections (UTIs) (5). One of its most potent virulence factors is a calcium independent hemolysin system consisting of two proteins HpmA and HpmB. HpmB is found in the outer membrane of the bacteria, and functions in the activation and extracellular secretion of the hemolysin HpmA. Activated HpmA once released into the external environment forms pores in and lyses a variety of human cells including red blood cells (10).

In previous research, *hpm*B was cloned on a high copy plasmid (8). This plasmid was mutated with hydroxylamine and transformed into *Escherichia coli* cells expressing the wild

type HpmA. Without the Hpm proteins, these *E. coli* cells do not otherwise possess a hemolysin system or express a hemolytic phenotype (9). This was done to ensure that any hemolytic activity observed by the cells was due to the HpmA and HpmB from the *P. mirabilis*. The mutants generated above were grown on blood agar plates. Increased, reduced, and nonhemolytic mutants were selected and further characterized based on liquid hemolysis assay results. The two categories of mutants that we examined were:

1) Superhemolytic: These mutants show an increase in hemolytic activity compared to wild type. Melissa Boeldt studied 2 mutants expressing this phenotype.
2) Activation+/ Secretion-: These mutants are capable of activating HpmA but are unable to secrete it extracellularly. Amy Green studied 2 mutants that display this phenotype. There are other mutant phenotypes that are beyond the scope of this project.

MATERIALS AND METHODS

TABLE 1. Bacterial Strains used in this Study

Mutant Name	*E. coli* Strain	Phenotype	Plasmids	Antibiotic Resistance
RAU 181	DH1	Wild Type Hemolytic	WT *hpm*A in pACYC 184, WT *hpm*B in pUC19	Amp, Cm
RAU 95	DH1	HpmB def. Non . Hem	WT *hpm*A in pACYC 184 pUC 19	Amp, Cm
RAU 157	DH1	Superhemolytic	WT *hpm*A in pACYC184, MUT *hpm*B in pUC 19	Amp, Cm
RAU 159	DH1	Superhemolytic	WT *hpm*A in pACYC184, MUT *hpm*B in pUC 19	Amp, Cm
RAU 161 [77221b]	DH1	Act+/ Sec-	WT *hpm*A in pACYC184, MUT *hpm*B in pUC 19	Amp, Cm
RAU 163 [92417a]	DH1	Act+/ Sec-	WT *hpm*A in pACYC184, MUT *hpm*B in pUC 19	Amp, Cm
RAU 188	BL21 DE3	Wild Type Hemolytic	WT *hpm*A in pET29 WT *hpm*B in pUC19	Amp, CM, Kan
RAU 187	BL21 DE3	HpmB def. Non . Hem	WT *hpm*A in pET29 pUC19	Amp, CM, Kan
RAU 197	BL21 DE3	Superhemolytic	WT *hpm*A in pET29 MUT *hpm*B in pUC19	Amp, CM, Kan
RAU199	BL21 DE3	Superhemolytic	WT *hpm*A in pET29 MUT *hpm*B in pUC19	Amp, CM, Kan
RAU 201	BL21 DE3	Act+/ Sec-	WT *hpm*A in pET29 MUT *hpm*B in pUC19	Amp, CM, Kan
RAU 203	BL21 DE3	Act+/ Sec-	WT *hpm*A in pET29 MUT *hpm*B in pUC19	Amp, CM, Kan

Note: All BL21 DE3 strains also contain pLysE

Bacterial strains and Plasmids: Bacterial strains used in this study are listed in Table 1. *E. coli* Bl21 and pET vectors were obtained from Novagen Corp. [Madison, WI].

Uninduced liquid hemolysis assays: Inoculate 5ml of LB broth containing 100µg/ml ampicillin and 25µg/ml chloramphenicol to an OD_{600} of 0.05 of desired *E. coli* strain. Bacteria were incubated at 37ºC with constant aeration. Harvest the bacteria at an OD_{600} of 0.9 and pipette 1.5ml into 2 microcentrifuge tubes and centrifuge for 5 min. at 14,000 x g. Transfer 0.75ml of the supernatant from each centrifuge tube and place in a new microcentrifuge tube labeled supernatant. Discard the remaining supernatant and resuspend the pellet from one tube in 1.5ml phosphate buffered saline (PBS) [label this as washed whole cell] and resuspend the other pellet in 0.75ml of PBS [label this as washed sonicate]. Place all fractions on ice. Sonicate this fraction at 50%, 1 second cycles with the output control set at 4 for 15 seconds. Repeat this cycle twice [Heat Systems W-385, Plainview, NY]. Incubate 500µl of each sample with 50µl of 20% blood and 450µl of PBS for 1 hr in a 37°C water bath. Centrifuge the samples after incubation for 5 min. at 14,000 x g. Hemolytic activity is expressed as the OD_{540} of this supernatant.

Construction of the pET expression system: (Figure 1) The mutants were grown on Tryptic Soy Agar (TSA) plates containing 100mg/ml and 25mg/ml chloramphenicol and plasmid DNA was purified using a standard alkaline mini-prep protocol (9). These mini-preps were digested with *Cla* I overnight at 37°C. An *E. coli* strain containing *hpm*A on the pET 29a+ vector and chromosomal DNA containing the DE 3 lysogen was made competent and transformed with the restriction digestion. The resulting strain was made competent and transformed with pLYSE (9). These transformants were then renamed as RAU strains.

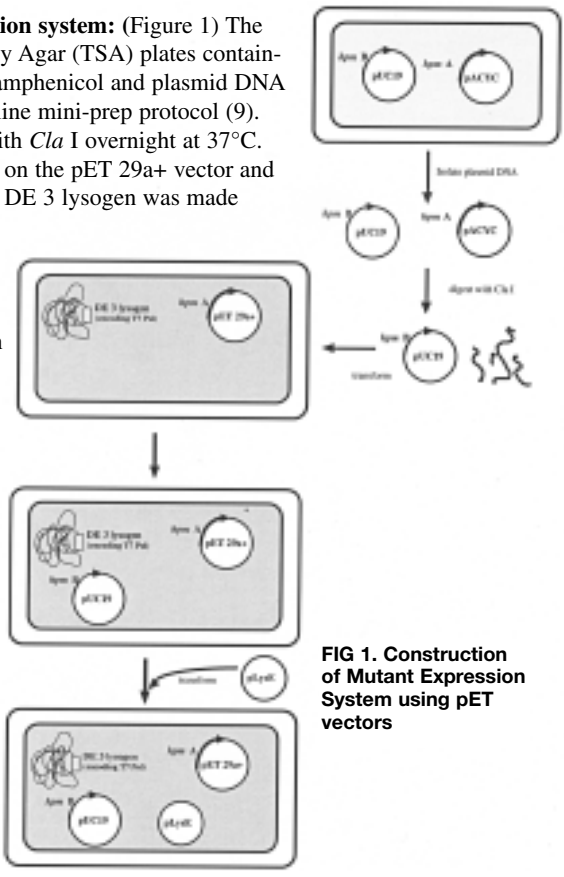

FIG 1. Construction of Mutant Expression System using pET vectors

Liquid hemolysis assays with induction: Inoculate 5ml of LB broth containing 100µg/ml ampicillin and 25µg/ml chloramphenicol and 10µg/ml kanamycin to an OD_{600} of 0.05 of desired *E. coli* strain. Bacteria were incubated at 37ºC with constant aeration. Induce the culture with 50µl of 0.1M IPTG at an OD_{600} of 0.25. Harvest the bacteria at an OD_{600} of 0.9 and pipette 1.5ml into 2 microcentrifuge tubes and centrifuge for 5 min. at 14,000 x g. Transfer 0.75mls

of the supernatant from each centrifuge tube and place in a new microcentrifuge tube labeled supernatant. Discard the remaining supernatant and resuspend the pellet from one tube in 1.5ml phosphate buffered saline (PBS) [label this as washed whole cell] and resuspend the other pellet in 0.75ml of PBS [label this as washed sonicate]. Place all fractions on ice. Sonication was performed as described above. For the Act+/Sec- mutants, incubate 500µl of the sample with 50µl of 20% blood and 450µl of PBS for 1 hr in a 37°C water bath. For the Superhemolytic mutants, 10µl of the cell and sonicate fraction and 200µl of the supernatant was incubated with 50µl of 20% blood and 940µl of PBS for the cell and sonicate and 750µl of PBS for the supernatant for 1 hr in a 37°C water bath. Centrifuge the samples after incubation for 5 min. at 14,000 x g. Hemolytic activity is expressed as the OD_{540} of this supernatant.

Western Blotting: Whole cell and supernatant samples were prepared according to Sambrook et al. (9). For mutants and control strains, 50µl of the whole cell and 200 µl of the supernatant of the total culture were loaded on an 8% SDS-PAGE gel and stacked at 110 V. The voltage was increased to 220V for resolution. The gel was then transferred to nitrocellulose for one hour in 0.5X Tris-glycine (pH 8.3) buffer containing 10% methanol and 0.03% SDS. Nonspecific binding sites were blocked with a solution containing 5% casein for at least two hr at room temperature. The membrane was washed 3 times for 5 min each in wash solution and incubated in the primary antibody (polyclonal rabbit anti-HpmA) at 1:10,000 dilution containing 5% BSA overnight at 4°C. The membrane was washed as before and incubated with secondary antibody (monoclonal goat anti-rabbit conjugated with horse radish peroxidase) at a 1:3000 dilution prepared in blocking solution for 1 hr and washed as before. Secondary antibody was detected by the Lumi-Light Western Blotting Substrate according to manufacturer's protocol. The membrane was exposed to x-ray film and developed after a series of incubation times ranging from 1-5min.

RESULTS

Characterize the Hemolytic Phenotype of HpmB Mutants

Inconsistent preliminary hemolysis assay results suggested that constitutive expression of HpmA can result in an unstable hemolytic phenotype (TABLE 2). This instability was most apparent in activation+/secretion- mutants, which produced discrete differences in colony

TABLE 2. Hemolytic activity as a percentage of wild type in original expression system

Date	Mutant	Phenotype	Supernatant	Washed Whole Cell	Washed Sonicate
10/28/99	7221b[a]	Act+/Sec-	0	0	149
	91417a[b]	Act+/Sec-	0	9	142
11/12/99	7221b[a]	Act+/Sec-	0	0	5.3
	91417a[b]	Act+/Sec-	0	0	2.8
11/6/99	RAU 157	Super Hemolytic	12.3	36.8	139
	RAU 159	Super Hemolytic	17.7	21.8	58.8
11/7/99	RAU 157	Super Hemolytic	69.3	98.2	120.2
	RAU 159	Super Hemolytic	14.5	28.7	58.6

[a] contains *hpmB* from RAU 162 [b] contains *hpmB* from RAU 163

sizes and hemolytic zones on blood agar plates. These differences were not apparent on TSA plates without blood.

Control Expression of HpmA to Stabilize Mutant Phenotypes

Due to the negative effects of constitutive HpmA expression, controlling the expression of HpmA became crucial to maintaining the viability of our mutants. This was accomplished through the use of the T7 polymerase (pET) expression system (FIG. 1).

Liquid Hemolysis Assays of Stable *E. coli* strains with Controlled HpmA Expression

Hemolysis assays were then performed on the mutants with the pET expression system (TABLE 3). With expression of the hemolysin controlled, hemolysis data is consistent and confirms the superhemolytic phenotypic characterization. On the other hand, controlling HpmA expression did not stabilize the activation+/secretion- phenotype, therefore, the constitutive expression system was reexamined. Upon observation of mutant growth on blood agar plates, two discrete colony morphologies were present. When the smaller colony type was selected and analyzed, the activation+/ secretion- phenotype was consistently observed (TABLE 4).

Western Blotting of Liquid Hemolysis Assay Fractions

The distribution of HpmA in the whole cell and culture supernatant fractions can be visual-

TABLE 3. Hemolytic activity as a percentage of wild type in the pET expression system

Mutant	Date	Phenotype	Supernatant	Washed Whole Cell	Washed Sonicate
RAU197[a]	3/31/00	Superhemolytic	296	144	158
	4/20/00	Superhemolytic	401	103	55.2
	4/28/00	Superhemolytic	56.6	111	199
		Mean	**251**	**120**	**137**
RAU199[b]	3/31/00	Superhemolytic	242	157	197
	4/20/00	Superhemolytic	452	97	116
	5/6/00	Superhemolytic	165	96.5	58.2
		Mean	**286**	**117**	**124**
RAU201[c]	3/3/00	Act+/Sec-	22	2.1	6.1
	4/2/00	Act+/Sec-	0	2	1.5
		Mean	**11**	**2.1**	**3.8**
RAU203[d]	3/3/00	Act+/Sec-	0	1	.4
	4/2/00	Act+/Sec-	0	1	1
		Mean	**0**	**1**	**.7**

[a] *hpmB* from RAU 157 expressed in pET system
[b] *hpmB* from RAU 159 expressed in pET system
[c] *hpmB* from RAU161 expressed in pET system, contains *hpmB* from 7221b
[d] *hpmB* from RAU163 expressed in pET system, contains *hpmB* from 91417a

TABLE 4. Re-examination of Act+/Sec- mutants in the constitutive expression system

Mutant	Date	Phenotype	Supernatant	Washed Whole Cell	Washed Sonicate
7221b[a]	5/10/00	Act+/Sec-	2.3	10.9	97.1
	5/18/00	Act+/Sec-	ND[c]	0.2	69.3
	Mean		**2.3**	**0.2**	**83.2**
91417a[b]	5/10/00	Act+/Sec-	7.5	4.1	119
	5/18/00	Act+/Sec-	0	0	77.1
	Mean		3.75	2.1	98.1

[a] contains *hpmB* from RAU 161 [b] contains *hpmB* from RAU 163 [c] Not Determined

ized for the superhemolytic phenotype (FIG. 2) and the activation+/secretion- phenotype (FIG. 3). Both strains of superhemolytic mutants produce about the same amount of HpmA and localize it in the same pattern as the wild type. Mutant 7221b is not strictly activation+/ secretion- because it is also capable of secreting an inactive form of HpmA. The amount of HpmA secreted is equivalent to the HpmA found in the wild type supernatant. Mutant 91417a only localizes the Hpm A intracellularly. Both activation+/secretion- mutants appear to have reduced HpmA expression when compared to wild type and the negative control which expresses no HpmB.

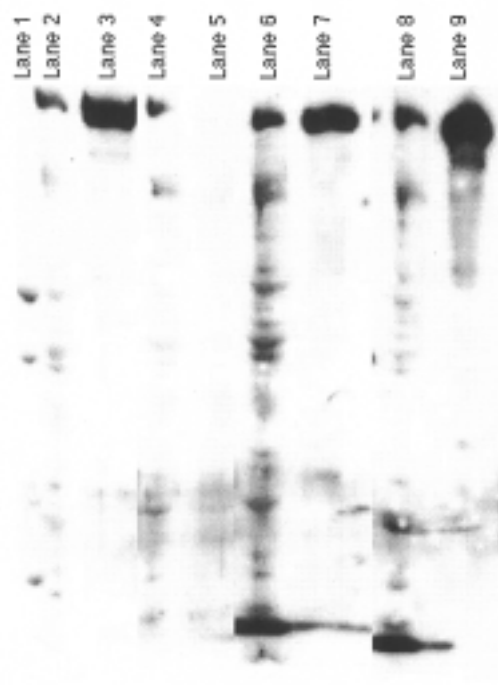

FIG. 2. Western blot of superhemolytic mutants in the pET expression system from E. coli BL21 cells. Lane 1, Protein marker; Lane 2, RAU 187 [WT HpmA&B] Cell; Lane 3, RAU 187 Supernatant; Lane 4, RAU 188[WT HpmA & no HpmB] Cell; Lane 5, RAU 188 Supernatant; Lane 6, RAU 197 [WT HpmA & mutant B] Cell; Lane 7, RAU 197 Supernatant; Lane 8, RAU 199 [WT HpmA & mutant B] Cell; Lane 9, RAU 199 Supernatant.

DISCUSSION

Superhemolytic Mutants: With the creation of the new expression system, we have alleviated the phenotypic instabilities (TABLE 3) due to constitutive Hpm A expression, among the superhemolytic mutants. The superhemolytic phenotype does not demonstrate a separation of activation and secretion functions, however the phenotype is inherently interesting. These mutant phenotypes are the products of hydroxylamine mutagene-

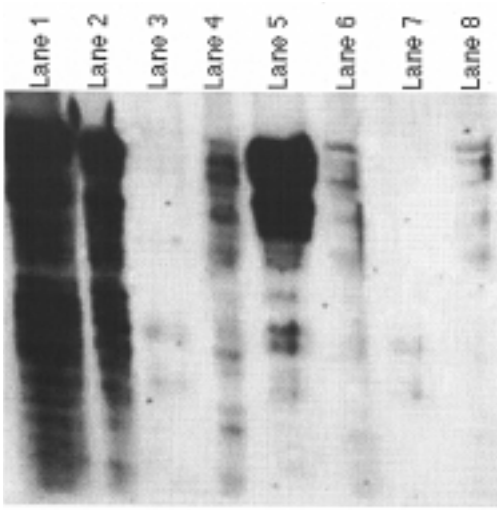

FIG. 3. Western blot of activation+/secretion- mutants in the constitutive expression system, E. coli DH1. Lane 1, RAU181 [WT HpmA&B] Supernatant; Lane 2, RAU 181 Cell; Lane 3, RAU 95 [WT HpmA & no HpmB] Supernatant; Lane 4, RAU 95 Cell; Lane 5 , 7221b [WT HpmA & mutant B] Supernatant; Lane 6, 7221b Cell; Lane 7, 91417a [WT HpmA & mutant B] Supernatant; Lane 8, 91417a Cell.

sis of *hpmB*. Generally mutagenesis will reduce or completely eliminate the protein function, however in this case the mutations enhanced the function of HpmB (increase in hemolytic activity). This increase in hemolytic activity could be a result of HpmB activating and secreting more HpmA or "superactivating" a smaller amount of HpmA. The Western blot of the superhemolytic hemolysis assay fractions suggests the latter. FIG. 2 shows a steady state expression of HpmA in the mutant strain comparable to that expressed in the presence of WT HpmB being produced, even though the mutant displays greater hemolytic activity than the wild type.

Activation+/Secretion- Mutants: Control of HpmA expression in the activation+/secretion- mutants generated results inconsistent with the previously observed phenotype (TABLES 2&3]. The low hemolytic activity found in the sonicate fraction in TABLE 3 may be due to an over expression of HpmA by the wild type strain. The efficiency of wild type HpmB is exploited in this system [TABLE 3] such that it is able to activate and secrete 10-20 times more HpmA than in the constitutive system [TABLE 2] because more HpmA is expressed during induction. This elevated HpmA expression/activity seen with WT HpmB significantly lowers the percentage of hemolysis demonstrated by the mutant, presumably because the mutant HpmB cannot interact with HpmA as efficiently. In an effort to alleviate this artifact of HpmA expression, hemolysis assays were performed after the wild type and mutant strains were induced with 10% and 20% of the normal concentration of isopropylthiogalactoside (IPTG) (data not shown). Results indicated that lower levels of induction do not significantly reduce the amount of hemolysis produced by the wild type. Time constraints did not allow further investigation of this phenomena. These results, and the fact that the desired mutant phenotype has previously been seen in the constitutive expression system prompted the re-examination of that system. Re-examination of these mutants using strict selection criteria resulted in confirmation of the original phenotype (TABLE 4).

Western blot results for the activation+/ secretion- were surprising because, it shows two mutants with similar hemolytic phenotypes have different localization patterns of HpmA

(FIG. 3). Mutant 7221b is capable of activating an intracellular form of HpmA, but also capable of secreting an inactive HpmA. The secretion capabilities of 7221b was not previously observed since the inactive hemolysin is not detectable in hemolysis assays. Mutant 91417a only localizes HpmA intracellularly. In both mutants the intracellular expression of HpmA is less than that of the wild type and the negative control, although the hemolytic activity of the intracellular (sonicate) sample approaches that of wild type which suggests that a greater percentage of the intracellular HpmA in these mutants is hemolytically active.

No one to date has identified the mechanism by which HpmB secretes and activates HpmA. Phenotypic characterizations of the mutants performed here suggest that activation and secretion of HpmA are separable events and the mutations in *hpmB* can enhance this the hemolytic of wild type HpmA. Several other pathogenic bacteria contain protein systems consisting of an accessory B protein that activates and secretes the larger A protein. These Hpm experiments will serve as a model to study the related protein systems in *Serratia marcescens* (7), *Edwardsiella tarda* (4), *Haemophilus ducreyi* (6), *Bordetella pertussis* (3), *Haemophilus influenza* (1) and *Erwinia chrysanthemi* (2).

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