XYLEM FLUID CHEMISTRY MEDIATION OF PIERCE'S DISEASE: STIMULATION OF AGGREGATION AND BIOFILM FORMATION

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ABSTRACT
Xylella fastidiosa (Xf) is the causal agent of Pierce's disease in grapevines. The mechanisms of pathogenicity are largely due to occlusion of xylem vessels by aggregation of *Xf* and biofilm formation. Our previous work has documented the effects of xylem constituents on both *Xf* proliferation and biofilm formation. This current research utilizes 1) addition of xylem constituents to defined media *in-vitro*, and 2) exposure of *Xf* to xylem fluids of different *Vitis* germplasms to investigate effects of xylem components on *Xf* growth and biofilm formation. Xylem fluid is typically low in O_2 and our *in-vitro* studies have established the capability of *Xf* to grow under hypoxic conditions. The growth in the defined (minimal) media is often superior or equal under the oxygen-limited conditions as compared to the air-saturated media. These effects were found to be variable with *Xf* strain and media formulation indicating interactive effects between O_2 and specific xylem components. Short term (1 hour) to long term (12 days) exposure of *Xf* to *Vitis* xylem fluids showed highly significant differences in both *Xf* growth rates and biofilm formation dependent on *Vitis* genotype. *Xf* growth in *Vitis* xylem fluid was correlated to the concentration of the organic acid citric acid, many of the amino acids including glutamic acid, glutamine, histidine, valine, methionine, isoleucine and phenylalanine and inorganic ions including copper, magnesium, phosphorous and zinc. Biofilm formation was also correlated to specific xylem constituents. Our next experiments will test the growth and biofilm formation of *Xf* in response to changing the concentration of these constituents noted above in xylem fluid so that the role of each constituent can be assessed.

INTRODUCTION
We have previously established that a functional relationship exists between Pierce's disease (PD) expression and *Xylella fastidiosa (Xf)* colony growth within *Vitis* germplasms. Colony growth results from proliferation of individual *Xf* (cfu), aggregation, and biofilm formation. Both *in-vitro* and *in-vivo* experiments have shown that both *Xf* proliferation and biofilm formation may be impacted by a variety of constituents including specific inorganic ions, O_2, antioxidants, amino and organic acids and sulfhydryl groups. Newly developed defined media result in variable patterns of *Xf* colony growth (i.e. PW+ results in rapid bacterial proliferation with comparatively little biofilm formation whereas our newly defined media CHARDS provides slower bacterial growth but high biofilm formation). CHARDS media is equivalent to CHARD2 (Leite et al. 2004) except that starch at 0.2g/liter is added. These media provide an array of tools to test effects of individual compounds on *Xf* colony growth. Lastly, we can also examine *Xf* colony formation within xylem fluids of varying *Vitis* germplasms, and correlate patterns of growth to composition of the xylem fluid.

OBJECTIVES
1. Utilize defined media to examine the effects of O_2 and other xylem components on *Xf* growth.
2. Quantify the relationship between naturally occurring xylem constituents (inorganic ions, amino acids and organic acids) and *Xf* colony growth utilizing xylem fluid from a variety of *Vitis* germplasms.

Objective 1. The effects of O_2 on *Xf* growth and biofilm
Our initial work focused on the role of O_2 in *Xf* colony growth. The levels of oxygen found in xylem fluid are highly variable between almost atmospheric to anoxic levels (Gansert et al. 2001; Eklund, 2000). There may also be great variation within a plant (Dongen et al. 2003). Levels documented in xylem are generally well below atmospheric levels, and *Xf* has been defined as an obligate aerobe incapable of growth without O_2 (Wells et al. 1987). We subjected *Xf* growing in liquid PW+ media to 5 levels of O_2 ranging from atmospheric O_2 (21%) to anaerobic conditions (0%). Gas treatments were applied for 5 minutes every 24 hours. *Xf* was cultured under these conditions for 15 days at which time *Xf* growth, quantified by optical density (OD), and biofilm formation were measured. OD was measured using a Genosys 8 spectrophotometer at a wavelength of 600nm. The formation of biofilm on the surface of polypropylene tubes was assayed by the crystal violet method (Espinosa-Urgel et al. 2000). The oxygen levels were determined using a LaMotte's Dissolved Oxygen Test Kit (model EDO•code 7414) to insure the accuracy and persistence of treatment conditions.

Our results established a relationship between O_2 concentrations and *Xf* growth. For many strains tested, *Xf* growth rates were highest under atmospheric conditions and declined as O_2 levels declined. Growth comparison of *Xylella fastidiosa* pv. Pierce's disease strain 'Temecula' and *Xanthomonas campestris* under 21% oxygen and 0% oxygen headspaces, indicate that there was a discrepancy among the ability to grow under a hypoxic condition. There was no change in the optical density for *Xanthomonas* but there was continued growth for *Xylella* under the nitrogen gas treatments (0% O_2; Figure 1). This was also

found for other strains of *Xylella*, such as the Pierce's disease strain 'UCLA' and the almond leaf scorch strain 'Tulare' (data not shown). Effects of oxygen on biofilm appear more variable than effects on OD, yet in about half of the experiments conducted biofilm increased significantly under hypoxic conditions. Media formulation had more significant effects on biofilm than did O_2 concentrations.

The absolute rates of *Xf* growth and the subsequent reactions to declining O_2 were strain dependent. Furthermore, the effects of oxygen varied greatly depending on media used during assays (Table 1). When grown in the defined media XDM2 effects of varying oxygen were highly significant, whereas effects were not evident for *Xf* grown in CHARDS. The latter is a defined media based on xylem fluid composition (Leite et al. 2004), whereas the former is based on genomic analysis (Lemos et al. 2003). Variations in effects suggest interactions between oxygen and other xylem constituents that may be important to *Xf* growth.

Preliminary analysis of media composition, CHARDS and XDM2, after sustained *Xf* growth under differing oxygen levels (21% versus 0%) showed both qualitative and quantitative variations in organic acid profiles. These results, along with analysis of terminal oxidase (the high through-put, low affinity cytochrome bo) in the electron transport pathway and the lack of production of hydrogen sulfide, suggest the possibility that the bacterium may be employing an anaerobic energy production pathway. Current research is addressing if such pathways may be fermentative. We are also analyzing these metabolic products to determine if these products are related to pathogenicity.

Objective 2: *Xf* colony growth in xylem fluid from varying *Vitis* germplasms.
Xylem fluid was collected from *Vitis* cultivars ranging in PD susceptibility. These included *Vitis rotundifolia* cvs. Carlos and Noble, *Vitis rupestris* cv. St. George, *Vitis simpsoni* cv. Pixialla, *Vitis champinii* cvs. Dogridge and Ramsey and *Vitis vinifera* cvs. Chardonnay, Chenin blanc and Exotic all collected from cut bleeding spurs (Andersen and Brodbeck 1991) at the NFREC-Quincy Research Center in March 2005 and also from *Vitis vinifera* cv. Chardonnay and *Vitis rotundifolia* cv. Noble in California during March and April 2006. Profiles of inorganic nutrients (phosphorous, potassium, magnesium, zinc, manganese, copper, boron and sodium) amino acids (glutamine, asparagine, aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cysteine, isoleucine, leucine, phenylalanine and lysine), organic acids (oxalic, citric, tartaric, malic, malonic, lactic and succinic acids), electrical conductivity and pH were established for the xylem fluid used in each assay. Cell suspensions of the Temecula strain were pelleted from PW+ medium and re-suspended in xylem fluid from the varying cultivars for periods of one hour, 5 days and 12 days. Bacterial concentration and biofilm formation were quantified for each treatment and correlated to individual chemical constituents in the xylem fluid. Tests were run in March 2006 and again in June 2006 to insure repeatability. We note that the only way to collect sufficient quantities of xylem fluid for this experiment is to use bleeding xylem fluid from cut *Vitis* spurs which is only available in late winter. These fluids from dormant vines may or may not be representative of xylem fluid from specific germplasms in summer when *Xf* is actively proliferating. Thus, this methodology was developed to provide a range of xylem profiles to assess effects of individual xylem components on *Xf* growth rather than to quantify characteristics of *Xf* growth as a function of specific *Vitis* germplasms.

Our results emphasize how dramatically even short term exposure of *Xf* to xylem fluid of varying composition quickly alters *Xf* growth and biofilm formation (Table 2). Significant effects in biofilm ($p<0.0001$) were present after 1 hour and persisted throughout 12 days. Effects on OD were delayed (not apparent after 1 hour) but became highly significant with time ($p<0.0001$ for the 5 and 12 day intervals). Optical density varied greater than 3-fold and biofilm formation greater than 5-fold in the various fluids during the experiments. In all cases both OD and biofilm formation increased with time, but rates of increase varied dramatically between xylem fluid treatments. For example, biofilm formation in Carlos fluid was significantly higher than in the other fluids when measured after one hour, was intermediate in value after five days and was lower than in all other fluids after 12 days. Exposure to specific xylem fluids often had the opposite effect on overall growth rates (OD) and biofilm formation. Optical density for *Xf* incubated in Ramsey fluid was consistently higher than *Xf* in other fluids at each time period, but also consistently lower in biofilm formation.

Analyses of xylem fluids showed that many xylem constituents were highly correlated to optical density and biofilm formation. Optical density (growth) became more highly correlated with these constituents over time with only two significant correlations after 1 hour (Table 3). Glutamine, the predominant amino acid in *Vitis* xylem fluid, was weakly but consistently correlated with OD after 5 and 12 days. Some of the minor amino acids (histidine, valine, methionine, isoleucine and phenylalanine) were much more strongly correlated to *Xf* growth. The organic acid citric acid was also very highly correlated to OD after 5 and 12 days. For the inorganic ions, phosphorous, copper and zinc were well correlated with OD. For all of the constituents mentioned above, results appeared consistent over time as significant relationships apparent after 5 days also persisted through 12 days. We have previously hypothesized the importance of calcium, magnesium, phosphorous and citric acid to *Xf* growth (Andersen 2005). Equations utilizing these as variables yielded higher correlations than regression analyses based on any single chemical constituent ($P<0.0001$; $R^2=0.90$). The strength of these relationships suggest that our original hypothesis merits further investigation.

Biofilm formation was also correlated to xylem constituents Table 4). Both the specific compounds correlated to biofilm formation and the timing of possible effects varied drastically from those found with OD. Six amino acids (glycine, alanine, threonine, arginine, leucine and lysine) and one organic acid (tartaric acid) were related to biofilm formation, but only for a short time (one hour). None of these relationships persisted. The only correlations between xylem constituents and biofilm formation were with xylem pH.

These consistent correlations between xylem constituents and *Xf* growth are important, but further work is needed to suggest a causative relationship between *Xf* growth patterns and individual xylem components. To further investigate potential mechanistic relationships, we are currently repeating these experiments but manipulating xylem chemistry via supplementation of xylem constituents that appear well correlated with *Xf* growth and development. These experiments will allow us to discriminate mechanistic relationships from those that were strictly correlative.

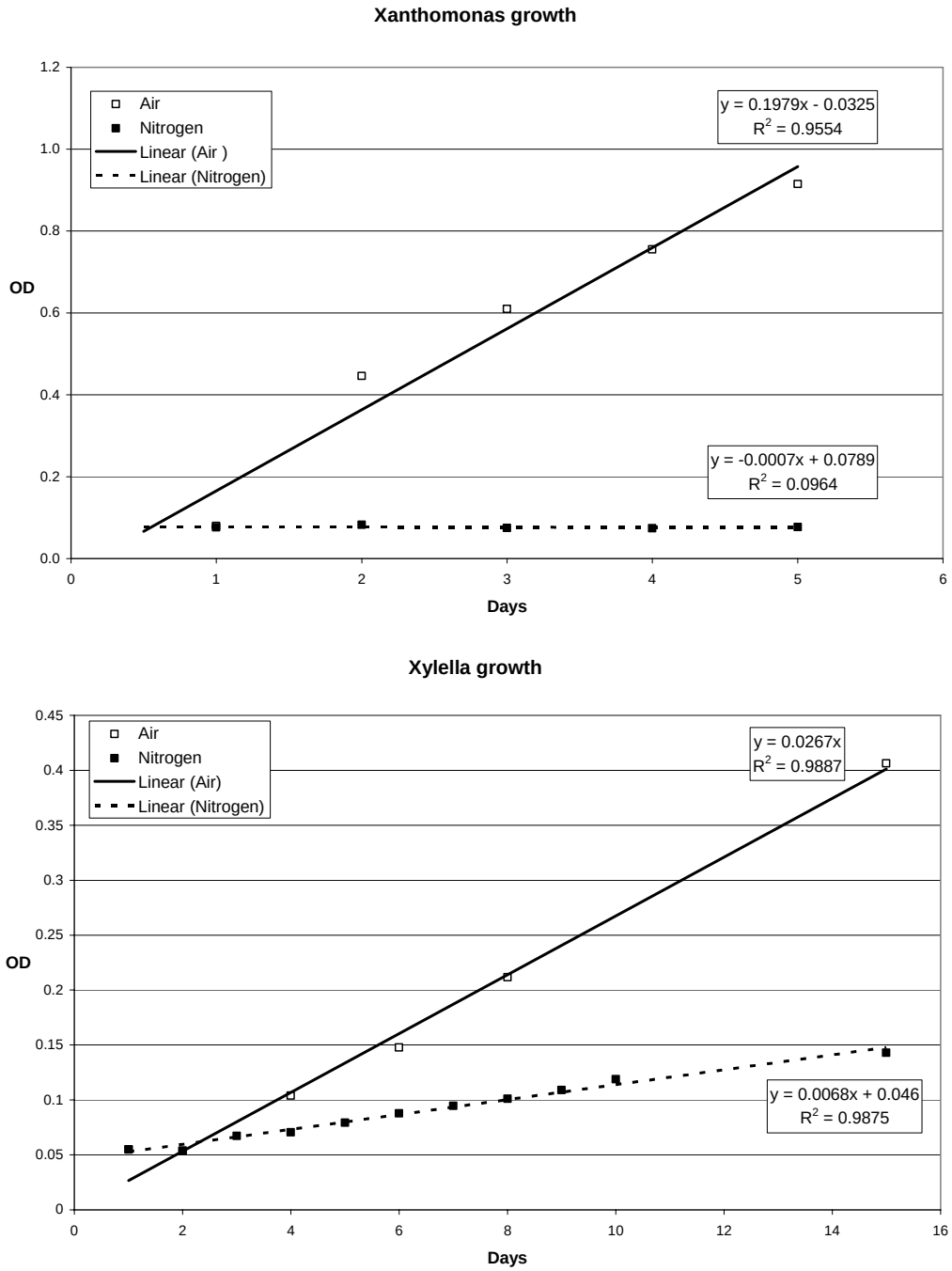

Figure 1. Mean bacterial growth of *Xanthomonas campestris* and *Xf* 'Temecula' in PW+ broth under various oxygen concentrations. Air is 21% oxygen and nitrogen is 0% oxygen. For each treatment level n=3.

Table 1. Optical densities for *Xf* growth under air (21% oxygen) and nitrogen (0% oxygen). On 11/2005 a pooled culture (rather than individual replications) was spun down and resuspended in the appropriate media and then dispensed into the falcon polypropylene test tubes. The experiments in 2006 both had initial optical densities of ~0.1 and the 11/2005 experiment started with OD=0.07.

Media-Gas	**OD** 03/2006	**OD** 04/2006	**OD** 11/2005*
CHARDS - Air	0.165 ±0.005	0.145 ±0.008	0.092 ±0.001
CHARDS - Nitrogen	0.154 ±0.007	0.155 ±.007	0.102 ±0.004
Statistics	P=0.2588	P=0.4123	P=0.0643
XDM2* - Air	0.119 ±0.004	0.107 ±0.005	0.079 ±0.001
XDM2* - Nitrogen	0.168 ±0	0.154 ±0.006	0.092 ±0.003
Statistics	**P=0.0002**	**P=0.0047**	**P=0.0017**

Table 2. Optical density and biofilm formation of *Xf* Temecula suspended in xylem fluid from nine different cultivars of *Vitis* for periods of 1 hour, 5 days and 12 days. Columns with different letters are significantly different for Duncan's MS ($p<0.05$).

	1 hour		5 days		12 days	
	OD	Biofilm	OD	Biofilm	OD	Biofilm
Carlos	0.111	0.139^{a}	0.104^{b}	0.469^{b}	0.106^{b}	0.688^{bcd}
Chardonnay	0.168	0.061^{bc}	0.155^{b}	0.223^{c}	0.137^{b}	0.550^{d}
Chenin Blanc	0.123	0.060^{bc}	0.128^{b}	0.474^{b}	0.140^{b}	0.636^{bcd}
Dogridge	0.129	0.075^{bc}	0.168^{b}	0.656^{ab}	0.160^{b}	0.724^{bcd}
Exotic	0.117	0.071^{bc}	0.124^{b}	0.520^{b}	0.120^{b}	0.847^{b}
Noble	0.131	0.094^{b}	0.112^{b}	0.801^{a}	0.153^{b}	1.200^{a}
Pixialla	0.133	0.065^{bc}	0.136^{b}	0.578^{ab}	0.130^{b}	0.586^{cd}
Ramsey	0.138	0.046^{c}	0.296^{a}	0.134^{c}	0.390^{a}	0.266^{e}
St. George	0.129	0.057^{bc}	0.140^{b}	0.549^{b}	0.166^{b}	0.794^{bc}
Statistics (p<)	NS	0.0001	0.0001	0.0001	0.0001	0.0001

Table 3. The relationship between xylem constituents and optical density of *Xf* Temecula strain suspended in xylem fluid from nine different cultivars of *Vitis* for periods of 1 hour, 5 days and 12 days.

	OD (600 nm)								
	1 hour			5 days			12 days		
	Equation	P<	R^2	Equation	P<	R^2	Equation	P<	R^2
Amino acids									
glu							y = 0.087 + 0.00153x	0.061	0.42
gln				y = 0.111 + 0.0000135x	0.051	0.44	y = 0.105 + 0.0000206x	0.043	0.47
his				y = 0.108 – 0.00144x	0.005	0.70	y = 0.994 + 0.00221x	0.0025	0.75
val				y = 0.100 + 0.000892x	0.024	0.54	y = 0.085 + 0.00143x	0.010	0.63
met				y = 0.111 + 0.0057x	0.047	0.45	y = 0.096 + 0.00276x	0.010	0.63
ile				y = 0.102 + 0.00157x	0.010	0.64	y = 0.0914 + 0.00241x	0.006	0.68
phe				y = 0.103 + 0.00115x	0.005	0.70	y = 0.095 + 0.00172x	0.004	0.71
Organic acids									
cit				y = 0.0745 + 0.000276x	0.0002	0.89	y = 0.0569 + 0.000393x	0.0008	0.82
tar	y = 0.0977 + 0.000327x	0.014	0.60						
Inorganic ions									
P				y = 0.079 + 0.0052x	0.018	0.57	y = 0.0606 + 0.0076x	0.021	0.56
Mg	y = 0.109 + 0.000095x	0.036	0.49						
Zn				y = 0.106 + 0.0793x	0.002	0.77	y = 1.00 + 0.115x	0.003	0.74
Cu				y = 0.084 + 0.846x	0.017	0.58	y = 0.076 + 1.13x	0.04	0.48
(Cit * P) / (Ca * Mg)				y = 0.0939 + 0.0338x	0.002	0.78	y = 0.0776 + 0.0523x	0.0005	0.84
(Cit * P) / (Ca + Mg)				y = 0.101 + 0.00142x	0.0001	0.90	y = 0.0927 + 0.0021x	0.0001	0.90

Table 4. The relationship between xylem constituents and biofilm formation of *Xf* Temecula strain suspended in xylem fluid from nine different cultivars of *Vitis* for periods of 1 hour, 5 days and 12 days.

	Biofilm								
	1 hour			5 days			12 days		
	Equation	P<	R^2	Equation	P<	R^2	Equation	P<	R^2
Amino acids									
gly	y = 0.0529 + 0.00067x	0.032	0.51						
arg	y = 0.0638 + 0.0000839x	0.0015	0.78						
thr	y = 0.0642 + 0.0000695x	0.002	0.77						
ala	y = 0.057 + 0.00115x	0.002	0.76						
leu	y = 0.0611 + 0.00028x	0.0002	0.87						
lys	y = 0.0579 + 0.0033x	0.0008	0.82						
Organic acids									
tar	y = 0.126 – 0.000510x	0.038	0.48						
Inorganic ions									
Cu				y = 0.734 – 3.07x	0.013	0.61			
pH				y = 4.10 – 0.624x	0.0011	0.80	y = 4.51 – 0.659x	0.016	0.59

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THE ROLES THAT DIFFERENT PILI CLASSES IN *XYLELLA FASTIDIOSA* PLAY IN COLONIZATION OF GRAPEVINES AND PIERCE'S DISEASE PATHOGENESIS

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ABSTRACT
Type I and type IV pili of *Xylella fastidiosa* play different roles in twitching motility, biofilm formation, and cell-cell aggregation. Thirty twitching mutants were generated with an EZ::TN transposome system and type IV pilus-associated genes were identified, including *fimT*, *pilX*, *pilY1, pilO*, and *pilR*. Mutations in all resulted in a twitch-minus phenotype except that *pilY1* mutant was twitching-reduced. A *fimA* mutant lacked type I pili and altered biofilm development and twitching. A *fimA/pilO* double mutant lacked both classes of pili, was twitch-minus and produced almost no visible biofilm. The gene for the type IV pilin (*pilA*), was cloned and expressed (predicted 15 KDa protein). The pilin sequence is 38% and 55% identical to that of the type IV pilin from *Pseudomonas aeruginosa* and *P. syringae* pv. tomato, respectively. A monoclonal antibody against the *pilA* gene product (prepilin) is being prepared.

INTRODUCTION
Xylella fastidiosa (*Xf*) has both type I pili and type IV pili located at one pole of the cells, and exhibits twitching motility and biofilm formation (Meng et al, 2005). Twitching functions in host colonization of many gram-negative bacteria. Approximately 40 genes have been identified that are involved in the biogenesis and function of type IV pili in *P. aeruginosa* (Mattick, 2002), including those encoding structural and regulatory proteins. In several bacteria, type IV pili are known to function in attachment and biofilm formation (Hélaine *et al*., 2005, Schilling *et al.,* 2001); known virulence factors. The main structural protein of IV pili, pilin, is encoded by *pilA* and is essential for the twitching motility in *P. aeruginosa* and *P. stutzeri (*Mattick *et al*., 2002; Graupner *et al*., 2000).

Our recent study revealed that type I pili play a central role in cell attachment and biofilm formation, and that type IV pili mediate twitching motility against a flowing current in microfluidic chambers. A *fimA* mutant (no type I pili) was capable of twitching motility. In contrast *pilB* and *pilQ* mutants (no type IV pili) did not twitch and were greatly impaired in their ability to migrate downward in grapevine shoots (Meng *et al.,* 2005). We have identified several previously undescribed genes in *Xf* that are associated with pili development and their associated phenotypes. We have cloned and expressed *pilA* and its product, pilin, the primary structural protein of type IV pili. Antibodies to PilA and other surface proteins will be used for development of diagnostic tests and eventually in the development of novel controls for Pierce's Disease.

OBJECTIVES
1. Characterize the putative type I pili gene cluster and phenotpes associated with genes.
2. Characterize two additional gene clusters that are likely to be involved in regulation of type IV pili and related functions.
3. Development of monoclonal antibodies to *Xf*.

RESULTS
Characterization of gene clusters associated with pil genes. Thirty twitching-defective mutants, representing 12 different open reading frames, were obtained from approximately 3000 Kan^R insertion mutants generated via the EZ::TN <Kan-2> system. Insertions occurred in homologs of pilus-related genes of *P. aeruginosa*, including PD0019 (*fimT*), PD0022 (*pilX*), PD0023 (*pilY1*), PD1693 (*pilO*), and PD1928 (*pilR*) that reside in four different gene clusters (Table 1; Figure 1). Open reading frame PD0062 corresponds to the *fimA* gene of *E. coli* (precursor for type I pili). A second round of mutagenesis in mutant 6E11 (lacks type I pili) was performed with EZ::TN <DHFR-1> system to select mutants that lacked both pilus types. Six non-twitching mutants were obtained having insertions in PD1923 (*pilC* in DM11, DM15), PD1693 *(pilO* in DM12), PD1671 (DM13), PD0609 (DM14) and PD0022 (*pilX* in DM16), respectively. DM12 (*fimA/pilO*) was selected for further study. *pilO* resides in operon *pilMNOPQ* (Van Sluys *et al*., 2003). Homologs in *P. aeruginosa* are required for type IV pilus assembly (Mattick, 2002).