ECOLOGICAL AND GENETIC CHARACTERISTICS ASSOCIATED WITH *ALCALIGENES XYLOSOXIDANS DENITRIFICANS*

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ABSTRACT
A bacterium isolated from *Homalodisca coagulata* Say, found also to inhabit xylem of citrus and grape (Lauzon et al. unpubl.), shows potential for use in Symbiotic Control strategies against *Xylella fastidiosa* (*Xf*), the causal organism of Pierce's disease (PD). The biology and "behavior" of the bacterium, identified as *Alcaligenes xylosoxidans denitrificans* (*Axd*), is under study to gather information that can be used to assess its efficacy and risk of use in the field. Real Time-Polymerase Chain Reaction (RT-PCR) was used as a semi-quantitative means of monitoring *Axd* growth in lake water under semi-natural conditions. *Axd* grew better in autoclaved lake water than in lake water that contained indigenous microbial populations. *Axd* growth was also monitored in soil and on leaf surfaces under semi-natural conditions using microbiological and molecular techniques. *Axd* was not retrieved from soils containing indigenous microbial populations unless the soil was autoclaved. *Axd* was retrieved from leaf surfaces from citrus, strawberry, sage, and basil. We are currently examining the effect of introducing *Axd* to citrus leaf microbial communities using denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism. We have also monitored transfer and uptake of two plasmid vectors, DsRed (pIRES-DsRed Express, Invitrogen) and pTZ18r (Amersham Biotech). Transformation attempts included both chemical and electroporation protocols. *E. coli* was used as a control. In both cases, *Axd* resisted transformation while *E. coli* was successfully transformed. In addition, *Axd* was screened for the presence of endogenous plasmids. A strain of *E. coli* containing a single copy plasmid was used as a control. Our data suggest that *Axd* does not contain any endogenous plasmids from 5-150 kb. We are currently examining horizontal gene transfer potential between *Axd* and strains of *E. coli* and *Shigella* sp. that carry fluorescent and antibiotic-marked endogenous plasmids. Horizontal gene transfer is yet one factor used to assess harm and risk. It has been inferred that *Axd* is a potential new human pathogen based in part on its association with *Pseudomonas aeruginosa* (*P. aeruginosa*) infections in Cystic Fibrosis patients. We used RT-PCR to compare the growth of *Axd* and *P. aeruginosa* individually and in co-culture. We found that *Axd* and *P. aeruginosa* significantly affect each others' growth. Our data suggest that *Axd* has the potential to mitigate harm associated with *P. aeruginosa* infections.

INTRODUCTION
Axd is a potential candidate for Symbiotic Control of PD. Symbiotic Control strategies engage beneficial microorganisms to control pathogenic microorganisms. This includes the use of a symbiont to deliver an antimicrobial product (i.e. Beard et al. 2002). The use of *Axd* in the management or control of PD requires that *Axd* remain in ecosystems for limited but effective periods of time and cause minimal and reversible, or no disruption to a host or ecosystem. To begin to assess efficacy and risk associated with the use of *Axd* in the field, we conducted studies aimed to monitor the fate of *Axd* in soil, water, and plant ecosystems under semi-natural conditions. We also examined the potential of *Axd* to engage in horizontal gene transfer. Finally, because *Axd* has been reported as a possible new human pathogen based in part on its association with *P. aeruginosa* infections in Cystic Fibrosis, we examined growth of both bacterial species alone and in co-culture using Real Time PCR.

OBJECTIVES
1. Determine if *Axd* possesses plasmids of high, medium-low, and very low copy number.
2. Determine if *Axd* participates in horizontal gene transfer, namely transformation.
3. Assess the impact of adding GM *Axd* to microbial communities present in various ecosystems.
4. Assess the competitive vigor of *Axd* when grown in co-culture with *P. aeruginosa*.

RESULTS

Objective 1: Determine if *Axd* possesses plasmids

No plasmids were found in *Axd* (Figures 1-3). *E. coli* containing a single copy plasmid/cell was used in all detection preparations as a positive control. High, medium-low and very low copy number procedures were employed including the use of Field Inversion Gel Electrophoresis for improved separation and resolution of DNA (i.e. discerning between a mix of genomic and plasmid DNA). *Axd* does not possess plasmids ranging in size from 5 to 150 kb.

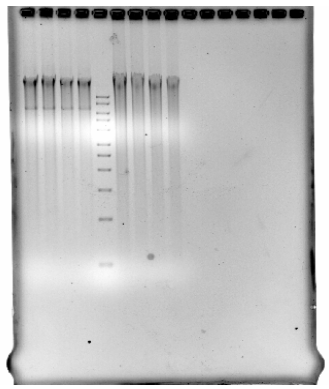


Figure 1: 5 ml and 10 ml culture volumes of *Axd* used for high copy plasmid preparation. A 5 ml– 10 ml culture volume is generally enough to detect a high copy plasmid in bacteria. No DNA bands are present in the lanes 2-5. Lane 1- 10 kb DNA marker. The highest band is 10 kb and the lowest is 1 kb. Lane 2,3 –5 ml culture volume of *Axd* plasmid prep. Lane 4,5 -10 ml culture volume of *Axd* plasmid prep. Note: 5ml and 10 ml refer to the culture volumes that were subjected to plasmid prep and not the amounts that were actually loaded onto the gel.

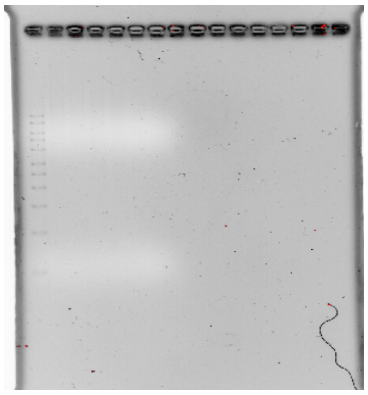


Figure 2: 500 ml culture volume of *E. coli* containing pBeloBac plasmid DNA and *Axd* were used for medium-low plasmid prep. A 500 ml culture volume is generally enough to detect a medium copy plasmid (30-300 copies/cell), low copy (5 – 30 copies/cell) and very low copy (less than 5 copies/cell) of plasmid DNA. Lanes 1-4 –500 ml culture volume of *E. coli* containing pBeloBac plasmid DNA. Lane 5- 10 kb DNA marker with the highest band at 10 kb, and the lowest at 1 kb. The sizes from top to bottom are 10 kb, 8 kb, 6 kb , 5 kb , 4kb, 3 kb, 2.5 kb, 2 kb, 1.5 kb and 1 kb. Lane 6 -9 –500 ml culture volume of *Axd* plasmid prep. No bands were detected below the 10 kb marker.

Figure 3: A 2 liter culture volume of *Axd* used for a very low plasmid prep. A 500 ml culture volume is generally enough to detect a very low copy plasmid such as a single copy plasmid/cell, however, we also performed a 2 liter plasmid prep for *Axd* to be thorough. No bands were observed in lanes 5-8. Lanes 1- 3 – empty. Lane 4- 10 kb DNA marker. The highest band is 10 kb, the lowest is 1 kb. Lanes 5-8 –2 liter culture volume A*xd* plasmid prep. Some bands can be seen above the 10 kb marker size in Lanes 5-7, however, we don't know if this is plasmid DNA or sheared genomic DNA. Therefore, the plasmid prep sample was subjected to FIGE – field inversion gel electrophoresis which helps separate distinct plasmid DNA from smears of genomic DNA much better than separation on an agarose gel. We conclude that *Axd* does not have plasmids of sizes ranging from 5- 150 kb.

Objective 2: Determine if *Axd* participates in horizontal gene transfer, namely transformation

Axd was subjected to chemical ($CaCl_2$) and electroporation techniques in attempts to transform *Axd* with two different plasmids. Plasmids pIRES2-DsRedExpress (Invitrogen) and pTZ18r (Amersham Biotech) were used for all procedures. A strain of *E. coli* amenable to transformation was used as a control. Results show that *Axd* was not easily amenable to transformation. There is a slight possibility that promoters do not work in *Axd* for these plasmids, although this is doubtful. Similar results have been found in the Lampe laboratory (personal communication).

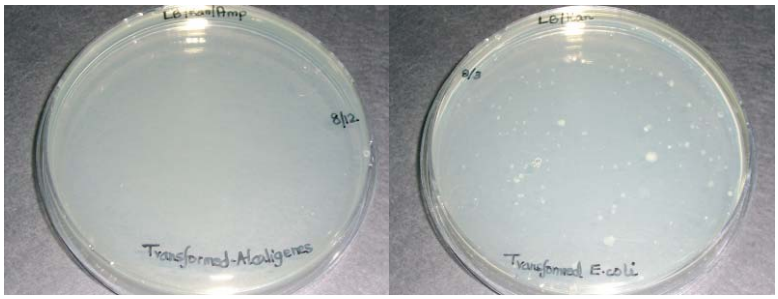


Figure 2. *Axd* plated onto Luria Bertani (left) agar post-transformation protocols. Notice that no growth occurred for *Axd* on a medium that would support the growth of a transformed strain. *E. coli* were transformed (right), grew well, and thus, procedures were conducted properly.

Objective 3: Assess the impact of adding GM *Axd* to microbial communities of various ecosystems
GM *Axd* containing EGFP fluorescent gene was used in all studies. GM *Axd* was applied to leaf surfaces of three different hosts and detected using microbiological and molecular techniques. GM *Axd* was also added to autoclaved and non autoclaved lake water and detected over time using molecular means (Figure 4). Real Time PCR was used to monitor the growth of GM *Axd* over time using primers for EGFP and 16 S rRNA genes. Data reveal poor growth of GM *Axd* in lake water (complete data not shown).

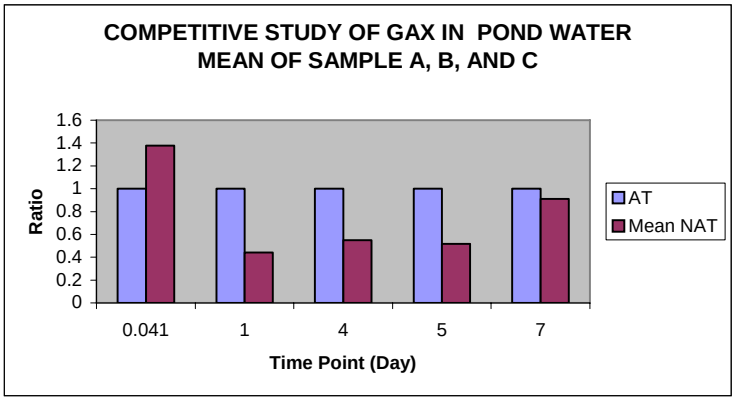
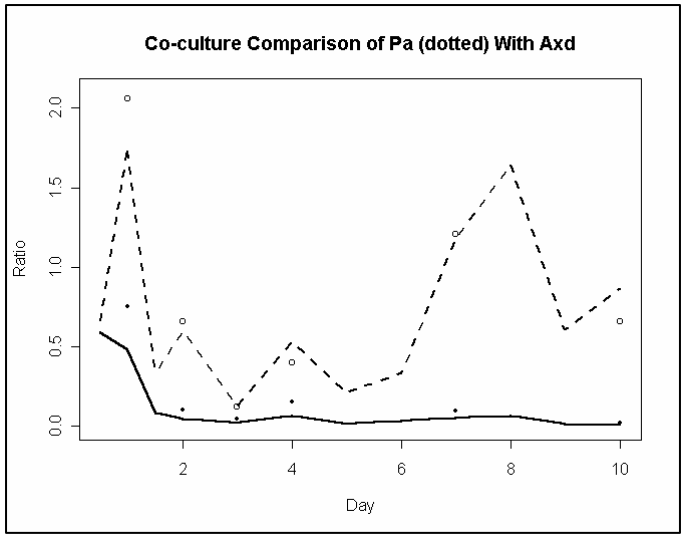


Figure 4.

Studies have recently commenced for determining the impact of inoculating citrus and grape leaf surfaces with *Axd* on community structure. Meyer Lemon leaves were inoculated with both 10 and 100 µL volumes of an 18 h culture of GM *Axd* to confirm detection of *Axd* using both primers for 16S rDNA and primers for EGFP. Detection was successful (data not shown).



Objective 4: Assess the competitive vigor of *Axd* when grown in co-culture with P. aeruginosa
Real Time PCR revealed that when grown together in liquid medium, *P. aeruginosa* and *Axd* significantly affect (decrease) each others' growth. Ratios were generated for mixed samples in relation to individual growth values over time and ranked. Treatments (batches) were replicated and two trials were conducted. No differences were found statistically in batches. ANOVA on ranks were used because residuals in ANOVA on ratios indicated non-normal data. ANOVA ranks showed that *P. aeruginosa* grew better in the presence of *Axd* than did *Axd* in the presence of *P. aeruginosa*; however, *P. aeruginosa* growth was less than that in pure culture. After 24 h, the presence of *Axd* significantly decreases the growth of *P. aeruginosa* but after 7 days of interactions, P. *aeruginosa* cell numbers increase significantly.

CONCLUSIONS
We have found that *Axd* is not easily transformed and does not contain plasmids ranging in size from 5-150 kb. *Axd* also does not establish well in established ecosystems but likely remains in an environment long enough to exert an effect on *Xf* (Bextine et al. 2005). When grown in the presence of the human pathogen, *P. aeruginosa*, *Axd* growth is less than that seen in pure culture. In addition, the presence of *Axd* decreases growth of *P. aeruginosa*. These data question recent inferences that *Axd* is a nosocomial or harmful bacterium to humans. While our findings provide important information regarding risk assessment and use of *Axd* in the field, further studies are necessary, including those that monitor the fate of *Axd* through successive field seasons and changing environmental and plant physiological conditions. To date, no concrete evidence exists that show *Axd* as a harmful bacterium to any ecosystem or host.

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