I. INTERIM PROGRESS REPORT FOR CDFA AGREEMENT NUMBER 13-0096-SA

II. TITLE OF PROJECT. Development of a biological control for Pierce's disease

III. PRINCIPAL INVESTIGATOR, CO-INVESTIGATORS, AND COOPERATORS.

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IV. TIME PERIOD COVERED BY THE REPORT. October 2014-February 2015

V. INTRODUCTION.

X. fastidiosa (*Xf*) is a Gram-negative, xylem-limited bacterium that causes Pierce's disease (PD) of grapevines (Chatterjee et al. 2008). *Xf* is transmitted to plants by insect vectors and once in the xylem, *Xf* is postulated to migrate, aggregate, and form biofilm that clogs the vessels leading to PD. We, and others, have studied *Xf* proteins and regulators involved in these steps (Guilhabert and Kirkpatrick 2005, Meng et al. 2005, Feil et al. 2007, Li et al. 2007, Shi et al. 2007, da Silva Neto et al. 2008, Cursino et al. 2009, Cursino et al. 2011) with the goal of better understanding PD in order to develop prevention strategies.

We deleted the *Xf* PD1311 gene (ΔPD1311), a putative acyl-CoA synthetase (ACS). ACSs catalyze long-chain fatty acyl-CoAs (Black et al. 1992) and are involved in numerous processes including pathogenicity (Barber et al. 1997). We have found that PD1311 is a functional enzyme (data not shown), and that the ΔPD1311 strain grows in sap (**Fig. 1A**). ΔPD1311 also has reduced motility (**Fig. 1B**), reduced aggregation (**Fig. 1C**), and less biofilm production (**Fig. 1D**) in comparison to wild-type cells. Interestingly, ΔPD1311 is altered in expression of key genes involved in virulence (**Table 1**). Consistent with our phenotype findings, the preliminary data suggests ΔPD1311 is downregulated in the PD1926 gene, which is involved in type IV pili and therefore motility. Additionally, ΔPD1311 is downregulated in the *fimA* (associated with type I pili) and *hxfB* genes, which are both involved in adhesion (Feil et al. 2007, Li et al. 2007).

Unexpectedly, the adhesion gene *xadA* is upregulated in ΔPD1311. Other than the *xadA* finding, all of the phenotypes for the ΔPD1311 strain would suggest that it would be less virulent in plants as similar phenotypes have been showed to have reduced, but not eliminated, PD (Cursino et al. 2009, Cursino et al. 2011). When tested in plants in 2013 we found that ΔPD1311 was avirulent (**Fig. 2**). In addition, we found that ΔPD1311 reduced wild-type *Xf* biofilm formation *in vitro* (**Fig. 1E**).

Given our findings, we proposed that ΔPD1311 has potential as a biocontrol for PD. The weakly virulent *Xf* elderberry strain EB92-1 has been studied as a potential PD biological control (Hopkins 2005, Hopkins 2012). Other approaches towards controlling PD include resistant rootstocks (Cousins & Goolsby 2011) and transgenic vines (Dandekar 2014, Gilchrist et al. 2014, Gilchrist & Lincoln 2014, Kirkpatrick 2014, Lindow 2014, Powell & Labavitch 2014). Continued research of PD controls is warranted. Given the avirulent phenotype of ΔPD1311 and its ability to limit wild-type induced PD, this strain provides new potential for a commercialized biological control.

fimA	PD1926	*hxfB*	*xadA*
0.23± 0.11	0.03± 0.03	0.08± 0.02	15.15± 3.77

Table 1. Expression of key virulence-related genes in ΔPD1311 strain. Wild-type or ΔPD1311 mutant cells were grown in PD2 broth for 3 days for RNA extraction. *petC* and *nuoA* were used as reference genes. Data is gene expression in mutant cells relative to wild-type cells. Experiment performed once with three biological samples per strain. Genes encode adhesion type I pili (*fimA*), motility type IV pili (PD1926), adhesion proteins HxfB and XadA (Feil et al. 2007, Li et al. 2007).

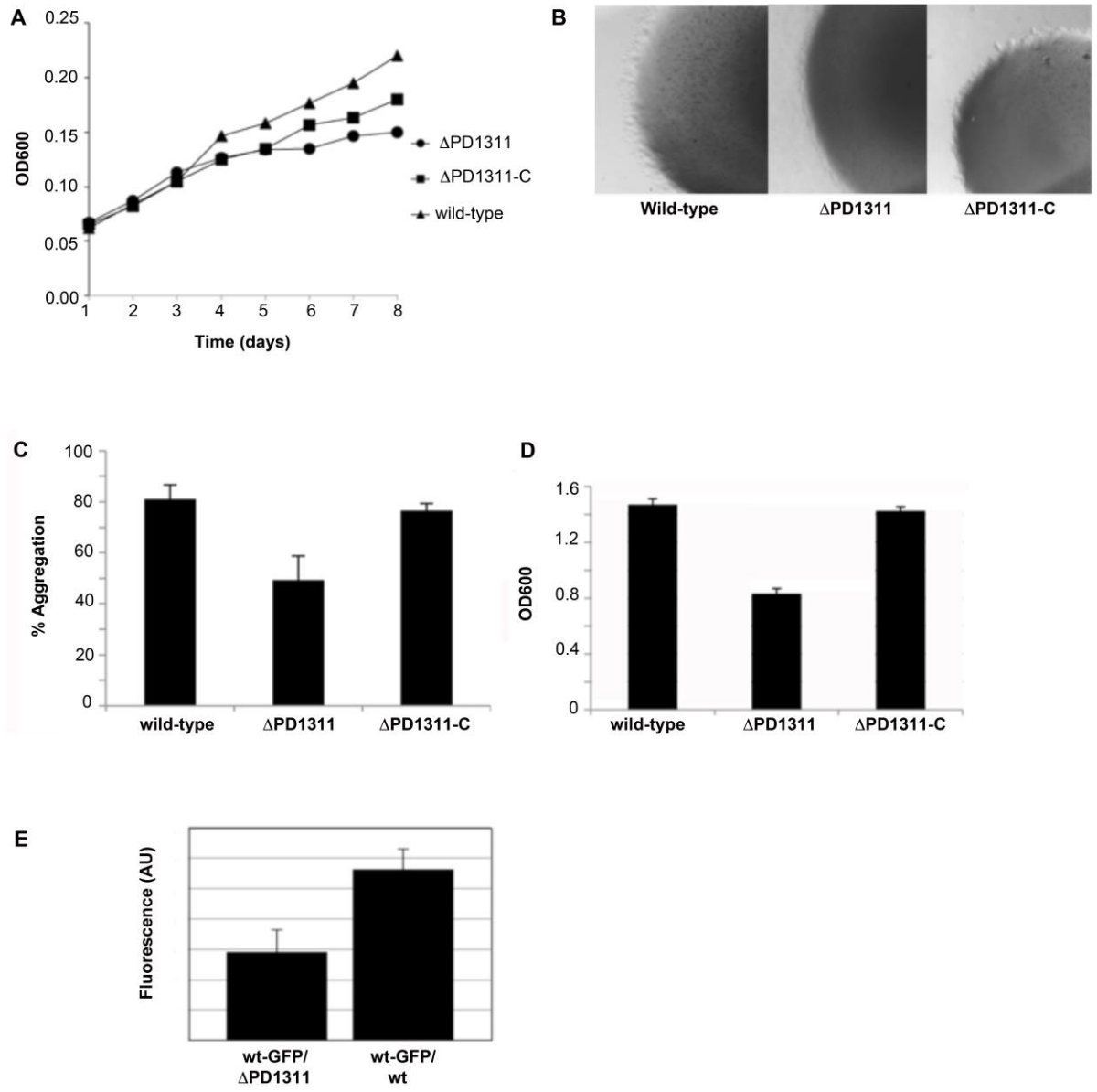

Fig. 1. Previous phenotypic findings with the ΔPD1311 strain. A) ΔPD1311 strain growth. Wild-type *Xf* (triangle), mutant (ΔPD1311 - circle), and complemented mutant (ΔPD1311-C – square) strains were grown for eight days in 100% *Vitis vinifera* cv. Chardonnay xylem sap and growth was determined by OD_{600} readings. **B) Motility of ΔPD1311 mutant strain**. Colony fringes of strains were assayed on PW agar overlaid with cellophane. Colonies were assessed after five days of growth (Meng et al. 2005, Li et al. 2007). Colonies photographed at 90X magnification. Experiment was repeated three times. **C) Aggregation of ΔPD1311 strain.** Aggregation of wild-type, mutant and complemented mutant strains grown in test tubes for five days in 3 ml of PD2 (Burdman et al. 2000, Davis et al. 1980, Shi et al. 2007). The experiment was repeated three times. **D) Biofilm of ΔPD1311 strain.** Quantification of biofilm formation in 96 well plates (Zaini et al. 2009). Experiment was repeated three times with 24 replicates each. **E) ΔPD1311 strain impacts biofilm formation by wild-type cells.** Quantification of biofilm in 96 well plates with agitation with equal amounts of wild-type *Xf* constitutively expressing green fluorescent protein (WT-GFP) and either wild-type *Xf* (wt) or the ΔPD1311 strain. Experiment was performed with 24 replicates. Fluorescence in arbitrary units (AU).

VI. LIST OF OBJECTIVES.

The overall goal is to test if the ΔPD1311 *Xf* strain functions as a biocontrol for PD and to understand how PD1311 affects virulence. To examine these questions, we proposed the following:

Objective 1. Determine the biocontrol potential of the ΔPD1311 strain.

Objective 2. Examine motility and how the PD1311 protein impacts virulence.

VII. DESCRIPTION OF ACTIVIES.

Objective 1. Determine the biocontrol potential of the ΔPD1311 strain.

We previously performed one round of *in planta* experiments and found that the ΔPD1311 strain is avirulent. We have now completed two additional rounds, which confirm this finding (**Fig. 2**).

Given our findings that the *Xf* ΔPD1311 Temecula strain does not cause PD and impacts wild-type biofilm production, we began pilot greenhouse studies to determine if the ΔPD1311 can be a viable biocontrol. We inoculated *V. vinifera* cv. Cabernet franc vines per standard procedures (Cursino et al. 2011) and recorded disease development of PD using the five-scale assessment (Guilhabert & Kirkpatrick 2005). We created three different inoculation conditions: i) wild-type *Xf* after a two week pre-treatment with the ΔPD1311 strain [following procedures used in *Xf* elderberry EB92.1 strain biocontrol studies (Hopkins 2005)], ii) wild-type and ΔPD1311 strain co-inoculated, and iii) ΔPD1311 strain after a two week pre-treatment with the wild-type strain. Our controls included vines inoculated with wild-type Temecula, the ΔPD1311 strain, or buffer control (Hopkins 1984). We found that the inoculation with ΔPD1311 strain inhibits PD; inoculation with ΔPD1311 before

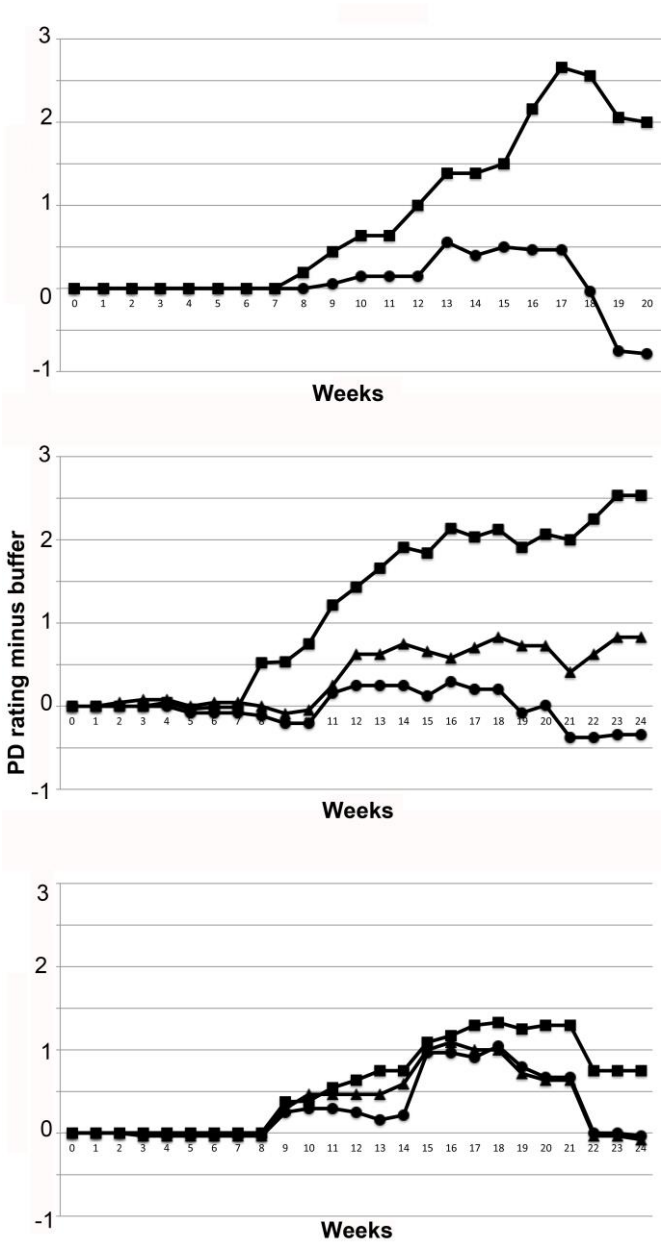

Fig. 2. Development of Pierce's disease. Grapevines were inoculated with wild-type *Xf* (square), ΔPD1311 strain (circle), ΔPD1311 complement (triangle), and buffer. Symptoms were monitored and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005, Cursino et al. 2009). Trial one from 2013 (top graph) did not include the complement strain.

wild-type *Xf* was equal or superior to co- or post-treatment (**Fig. 3**).

Objective 2. Examine motility and how the PD1311 protein impacts virulence.

We have preliminary results showing that the ΔPD1311 strain impacts wild-type cells *in vitro* (**Figure 1E**), survives in plants (**Table 2**), and limits wild-type induced disease (**Fig. 3**). Therefore we would like to know how the two strains spread through the plant when both are inoculated. We will soon begin organizing the experiments in order to address objective 2b.

	Plant 1		Plant 2		Plant 3		Plant 4	
	up	down	up	down	up	down	up	down
WT	-	-	-	-	-	+	-	-
ΔPD1311	-	+	-	-	+	+	-	-

Table 2. ΔPD1311 strain detected *in planta*. Five microliters of 10^9 CFU/mL of wild-type (WT) or mutant (ΔPD1311) *Xf* were inoculated into young grapevines in the 6-7th node counting from the top. The petioles directly above (up) and below (down) the inoculation point were sampled 10 days post-inoculation for PCR detection using *Xf* specific primers. + or – represents the presence or absence of the characteristic band.

VIII. SUMMARY OF ACCOMPLISHMENTS AND RESULTS FOR EACH OBJECTIVE.

Xf motility, aggregation, and biofilm formation are key steps in PD development (Chatterjee et al. 2008). Concerning objective 1, we confirmed that the ΔPD1311 strain is avirulent, and we found that it can significantly reduce PD development by wild-type *Xf*. For objective 2, our preliminary results show that the mutant can survive *in planta*, but need to perform further experiments to confirm these results. Overall, this work will help further understanding of disease development and prevention.

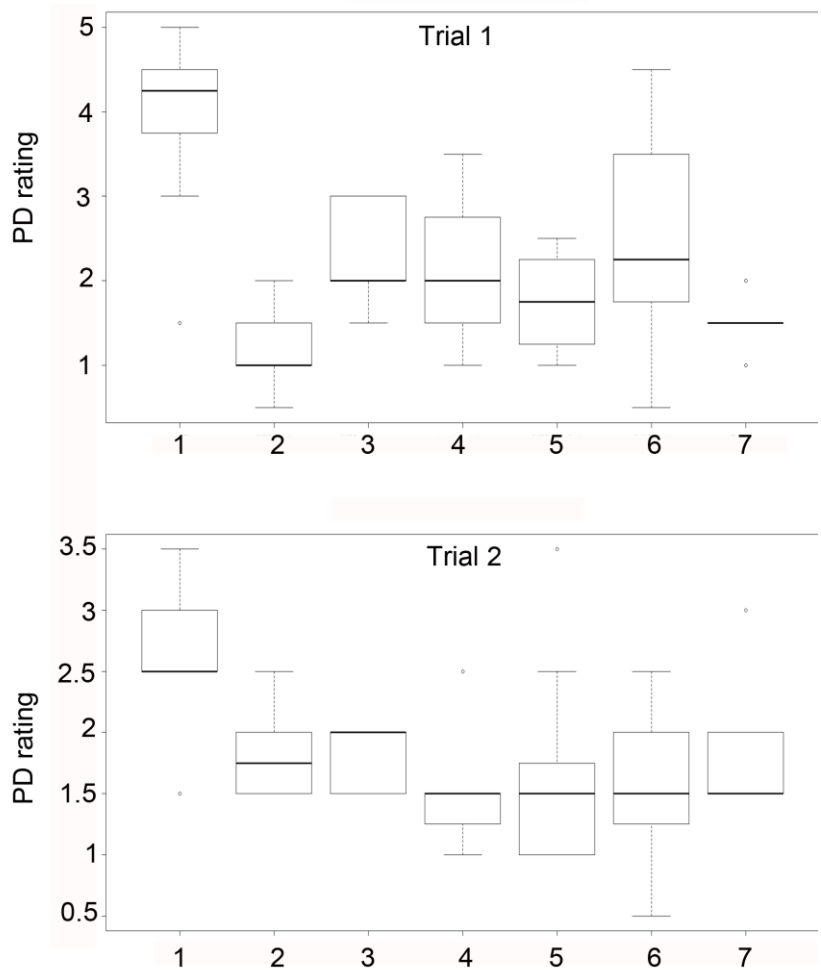

Fig. 3. ΔPD1311 strain reduces PD. Grapevines were inoculated with wild-type *Xf* (1), ΔPD1311strain (2), ΔPD1311 complement strain (3), co-inoculation ΔPD1311 and wild-type *Xf* (4), pre-treat with ΔPD1311 strain two weeks before wild-type cells (5), post-treat with ΔPD1311 strain two weeks after wild-type cells (6), and buffer (7). Bold lines represent the median values and circles representing outliers of each data group. Symptoms have been monitored on 12 plants for each treatment for 24 weeks and rated on a scale of 0-5 (Guilhabert and Kirkpatrick 2005).

IX. PUBLICATIONS PRODUCED AND PENDING, AND PRESENTATIONS MADE THAT RELATE TO THE FUNDED PROJECT.

Publications (Peer reviewed and Proceedings).

Cursino, L., Athinuwat, D., Patel, K.R., Galvani, C.D., Zaini, P.A., Li, Y., De La Fuente, L., Hoch, H.C., Burr, T.J., and Mowery, P. Characterization of the *Xylella fastidiosa* PD1671 gene encoding degenerate c-di-GMP GGDEF/EAL domains, and its role in the development of Pierce's disease. *PlosOne*. In press.

Burr, T.J., Mowery, P., Cursino, L., and Hao, L. Identification of a new virulence factor required for Pierce's disease and its utility in development of a biological control. Proceedings of the Pierce's Disease Research Symposium 2014, pp. 42-49. Proceedings.

Burr, T.J., Mowery, P., Cursino, L., and K. Johnson. Identification of a new virulence factor required for Pierce's disease and its utility in development of a biological control. Proceedings of the Pierce's Disease Research Symposium 2013, pp. 41-47. Proceedings.

Mowery, P., T.J., Burr, Hoch, H.C., Cursino, L., Johnson, K., Galvani, C., Athiuwat, D., and Shi, X. Exploiting a chemosensory signal transduction system that controls twitching motility and virulence in *Xylella fastidiosa*. Proceedings of the Pierce's Disease Research Symposium 2012, pp. 59-64. Proceedings.

Cursino, L., Galvani, C.D., Athinuwat, D., Zaini, P.A., Li, Y., De La Fuente, L., Hoch, H.C., Burr, T.J., and P. Mowery. 2011. Identification of an Operon, Pil-Chp, that Controls Twitching Motility and Virulence in *Xylella fastidiosa*. *Mol. Plant Microbe Interact.* 24:1198-1206.

Mowery, P., T.J., Burr, Hoch, H.C., Cursino, L., Athiuwat, D., and Galvani, C. Exploiting a chemosensory signal transduction system that controls twitching motility and virulence in *Xylella fastidiosa*. Proceedings of the Pierce's Disease Research Symposium 2011, pp. 71-75. Proceedings.

Pending Publications.

Johnson, K.L., Cursino, L., Burr, T.J., and Mowery, P. Potential complications when developing gene deletion clones in *Xylella fastidiosa*. Accepted to *BMC Res. Notes* pending revisions.

Presentations and Posters.

Burr, T.J. PD1311, a virulence factor required for Pierce's disease and its utility in development of a biological control. Pierce's Disease Research Symposium, Sacramento, CA, 2014. Presentation.

Burr, T.J. How *Xylella fastidiosa* is able to move in plants. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Presentation.

Johnson, K, Mowery, P., and Burr, T.J. Impact of aggregation on development of *Xylella fastidiosa* mutant clones. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Poster.

Mowery, P., Johnson, K.L., Cursino, L., and Burr, T.J. Identification of a new virulence factor required for Pierce's disease and its utility in development of a biological control. Pierce's Disease Research Symposium, Sacramento, CA, 2013. Poster.

Johnson, K. Role of a thioredoxin family protein in *Xylella fastidiosa* virulence. APS-MSA, Austin, TX, 2013. Presentation.

Mowery, P., Johnson, K.L., Cursino, L., and Burr, T.J. *Xylella fastidiosa* virulence factor mutant strain as a potential biocontrol for Pierce's disease. APS-MSA, Austin, TX, 2013. Poster.

Mowery, P. "How does your vineyard grow? Understanding the grapevine pathogen, *Xylella fastidiosa*." Department of Biology. Ithaca College. Ithaca, NY, 2013. Presentation.

X. RESEARCH RELEVANCE STATEMENT.

Xylella fastidiosa is an important phytopathogen that infects a number of important crops including citrus, almonds, and coffee. The *X. fastidiosa* Temecula strain infects grapevines and induces Pierce's disease. We recently deleted the *X. fastidiosa* PD1311 gene and found that the strain was no longer pathogenic. Based on sequence analysis, PD1311 appears to encode an acyl-CoA synthetase, which is a class of enzymes involved in many different processes including secondary metabolite production. We have characterized the ΔPD1311 strain and found phenotypes consistent with reduced virulence. We have found the ΔPD1311 strain to be avirulent and to reduce the virulence of wild-type *X. fastidiosa*. Therefore, we propose that the ΔPD1311 acts as a potential biocontrol for Pierce's disease.

XI. LAY SUMMARY OF PROJECT ACCOMPLISHMENTS.

We discovered that deleting the *X. fastidiosa* Temecula gene, PD1311, results in a strain that does not cause Pierce's disease. This project will examine how PD1311 plays such a central role in disease. In addition to not causing disease, we have found that the PD1311 mutant functions as a biocontrol; pre-treatment of grapevines with the PD1311 mutant significantly reduced Pierce's disease. Options for managing Pierce's disease are limited, which makes investigation of possible biocontrols critically important. Although the PD1311 mutant is a genetically modified organism, other such organisms are registered for use in agriculture in the U.S. Together the results from these aims will expand our understanding of Pierce's disease and provide information in relation to preventing disease.

XII. STATUS OF FUNDS.

$18,707 of the funds are left of which $18,189 are to cover salary and fringe benefits of the post-doctoral fellow performing the work.

XIII. SUMMARY AND STATUS OF INTELLECTUAL PROPERTY ASSOCIATED WITH THE PROJECT.

No intellectual property has resulted from research done under this grant.

XIV. LITERATURE CITED.

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