THREE NEW PATHOGENICITY EFFECTORS OF PIERCE'S DISEASE NOT FOUND IN BIOCONTROL STRAIN EB92-1

Principal Investigator:
Dean Gabriel
Dept. of Plant Pathology
University of Florida
Gainesville, FL 32611
dgabr@ufl.edu

Researcher:
Shujian Zhang
Dept. of Plant Pathology
University of Florida
Gainesville, FL 32611
sjzhang@ufl.edu

Researcher:
Pranjib Chakrabarty
Dept. of Plant Pathology
University of Florida
Gainesville, FL 32611
pranjib@ufl.edu

Cooperator:
Donald Hopkins
Mid-Florida REC
University of Florida
Apopka, FL 32703
dhop@ufl.edu

Reporting Period: The results reported here are from work conducted November 2010 to October 2011.

ABSTRACT

Xylella fastidiosa (*Xf*) infects a wide range of plant hosts and causes economically serious diseases, including Pierce's disease (PD) of grapevines. *Xf* biocontrol strain EB92-1 is infectious to grapevines but does not cause symptoms. The draft genome of EB92-1 revealed: 1) that it was nearly identical in gene order and sequence to Temecula; 2) no unique or additional genes were found in EB92-1 that were not previously identified in Temecula, and 3) EB92-1 appeared to be missing genes encoding 10 potential pathogenicity effectors found in Temecula (Zhang et al 2011). The latter included a type II secreted lipase (LipA; PD1703), two identical genes from a duplicated prophage region encoding proteins similar to zonula occludens toxin (Zot; PD0915 and PD0928) and all six predicted hemagglutinin-like proteins (PD0986, PD1792, PD2108, PD2110, PD2116 and PD2118). PCR analyses and subsequent sequencing of the PCR products confirmed that all 10 genes were missing, at least from their expected locations, in EB92-1. Leaves of tobacco and citrus inoculated with crude protein extracts of the Temecula PD1703 gene over-expressed in *Escherichia coli* exhibited hypersensitive cell collapse in less than 24 hrs. PD1703, driven by its native promoter, was cloned in shuttle vector pBBR1-MCS5 downstream from the *lacZ* promoter; this construct conferred strong secreted lipase activity to *Xanthomonas citri*, *E. coli*, and EB92-1 in plate assays. Although pathogenicity of the *X. citri* exconjugant expressing PD1703 was not evidently increased on citrus, pathogenicity of the EB92-1 exconjugant with PD1703 showed significantly increased symptoms on grapes as compared with an EB92-1 exconjugant carrying the empty vector. Similarly, Temecula PD0928 (Zot) and PD0986 (hemagglutinin) were also cloned in shuttle vector pBBR1-MCS5 and downstream from the *lacZ* promoter; when moved into EB92-1, both exconjugants also showed significantly increased symptoms on grape in comparison to EB92-1 with the empty vector. These results suggest that the Temecula PD1703 lipase, the two redundant PD0915 and PD0928 Zot proteins and at least one hemagglutinin all contribute to the pathogenic symptoms elicited by *Xf* on grapes.

LAYPERSON SUMMARY

Xylella fastidiosa strain EB92-1 is infectious to grapevines but causes no symptoms and has been used for biological control of Pierce's disease (PD). We determined the genomic DNA sequence of EB92-1 to 98% completion, allowing comparisons of this strain to strain Temecula, which causes PD. Most of the EB92-1 genes were nearly identical in gene order and protein sequence with those found in Temecula. No unique or additional genes were found in EB92-1 that were not previously identified in Temecula. However, 11 genes found in Temecula were not found in EB92-1; 10 of these encoded predicted secreted pathogenicity effectors that had not previously been associated with PD. Four of these missing genes were functionally tested to determine if they actually contributed to disease or not, and all four were found to enhance PD symptoms. This data identifies new molecular targets with potential to suppress disease symptoms.

INTRODUCTION

Symptoms of Pierce's disease (PD) include concentric rings of leaf scorch, almost reminiscent of a toxin. Symptoms of citrus variegated chlorosis (CVC) include brown, gummy lesions with necrotic centers. How these symptoms are elicited has been an open question for years. Hopkins (2005) discovered an effective PD biocontrol strain, *Xylella fastidiosa (Xf)* EB92-1, which infects grapevine and survives for many years, yet without causing any symptoms of PD. EB92-1 can be inoculated in a single location in a *Vitis vinifera* grapevine and the entire plant is protected from PD for years (Hopkins, 2005). How does this strain infect grape, and yet not cause disease? What factors are different? We reasoned that comparisons of the genomic sequence of EB92-1 with Temecula might reveal genetic differences enabling discovery of important PD pathogenicity factors and/or factors present in EB92-1 that may be triggers of host defense.

OBJECTIVES

The objectives of the two-year proposal are listed below.

1. Obtain nearly the complete EB92-1 genome DNA sequence. This objective was completed and is now published (Zhang et al., 2011);
2. Compare EB92-1 with Temecula and identify all unique open reading frames (ORFs) and differences, ranking the top 40 candidate ORFs for evaluation as elicitors. This objective was completed and is now published (Zhang et al., 2011).

3, 4, & 5. Evaluate two defense response assays designed to test the hypothesis that EB92-1 produces an elicitor that Temecula does not, to identify such elicitors and perform defense response assay screens. These objectives were not pursued because it became apparent from the results of sequence comparisons in Objective 2 that Temecula produced

pathogenicity effectors that EB92-1 did not. No genes were identified in EB92-1 that were not already present in Temecula. EB92-1 appeared to be a more highly evolved PD strain than Temecula, having lost specific pathogenicity factors---some redundant---that cause disease. It is not yet clear if EB92-1 is more likely to survive than Temecula, as some of the pathogenicity factors may enhance parasitic survival.

RESULTS AND DISCUSSION

The *Xf* biocontrol strain EB92-1 genome is very similar and syntenic to Pierce's disease (PD) strains. By far the majority of the primary BLAST hits were to Temecula; 92% of predicted EB92-1 proteins had more than 99% identity with Temecula proteins. Genome comparisons of EB92-1 revealed a high level of synteny with Temecula1. A plasmid sequence similar to the Temecula plasmid (pXFPD1.3) was also found in EB92-1. No unique or additional genes were found in EB92-1 that were not previously identified in Temecula1. However, 11 genes found in Temecula were not found in EB92-1; 10 of these encoded predicted secreted pathogenicity effectors (Zhang et al., 2011). Two predicted Temecula type II secreted enzymes, a serine protease (PD0956) and a lipase (PD1703), appeared to be missing from EB92-1. Two of three predicted zonula occludens toxin (Zot) genes, PD0928 and PD0915, also appeared to be missing in EB92-1. Zot is an important secreted virulence factor for *Vibrio cholerae* (Uzzau et al., 1999) and has been suggested as a potential virulence factor in CVC strain 9a5c (da Silva et al., 2007). Surprisingly, all six predicted hemagglutinin-like proteins (PD0986, PD1792, PD2108, PD2110, PD2116, and PD2118) appeared to be missing in EB92-1. Two of these (PD1792 and PD2118) carry a type V two-partner secretion (TPS) domain (Clantin et al., 2004); the remaining four have no TPS domain. Independent knockout mutations of PD1792 and PD2118 in Temecula1 caused an increase in virulence (Guilhabert et al., 2005). In contrast, a knockout of the only functional hemagglutinin-like protein with a TPS domain in *Xanthomonas axonopodis* pv. *citri* caused a loss of virulence (Gottig et al., 2009). Reported below are the results of experiments designed to functionally test the roles of four representatives of these 10 potential secreted pathogenicity factors.

A predicted type II secreted esterase, LipA, (PD1703), was entirely missing from EB92-1, evidently as a result of a deletion. This conclusion was confirmed by PCR analysis (**Figure 1**) and subsequent sequencing. PD1703 is an apparent *lipA* ortholog of *Xanthomonas oryzae*; *lipA* is known to directly contribute to pathogenic symptoms of *X. oryzae* by degradation of host cell walls, eliciting programmed cell death (Rajeshwari et al, 2005; Jha et al, 2007; Aparna et al 2009).

PD1703 exhibited strong esterase activity in *Xanthomonas* citri, *E.coli,* and EB92-1. PD1703 was cloned with its native promoter (690bp) and predicted secretion leader peptide in pBBR1MCS-5 (downstream from the *lacZ* promoter), creating pSZ26. An *in vitro* lipase assay was conducted using Tween 20 as the substrate and 0.01% Victoria Blue B as indicator, exactly as described by Samad et al. (1989).

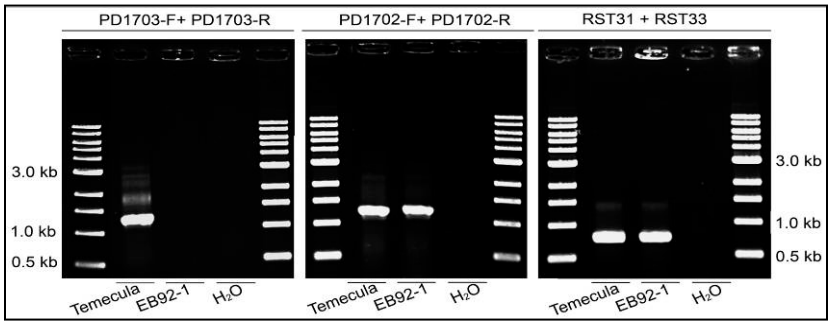

Figure 1. PCR confirmation of PD1703 in Temecula and EB92-1.

Agar plates containing the substrate and indicator were poured and wells created by removal of agar with a cork borer. Culture supernatants from centrifuged cells grown to late mid-log phase (ca. OD = 0.7) were added to the wells as indicated in **Figure 2**. The third row in **Figures 2A and 2B** shows the reaction of the indicator to supernatants from *X. citri* B21.2 and *E. coli* Mach1-T1 exconjugants, respectively, carrying cloned PD1703 (pSZ26), respectively, and the fourth row in **Figure 2C**, shows the reaction to the supernatant from the EB92-1 exconjugant carrying cloned PD1703 (pSZ26), demonstrating relatively strong amounts of secreted lipase in these culture supernatants (the crude supernatants were not concentrated or purified). These levels of lipase activity were not present in the supernatants of wild type Temecula or EB92-1, nor in *X. citri* B21.2, *E. coli* Mach1-T1, *X. citri* B21.2 and *E. coli* Mach1-T1 transconjugant with another lipase (PD1702, with its native promoter) cloned from Temecula1, or these same strains carrying the empty vector pBBR1MCS-5.

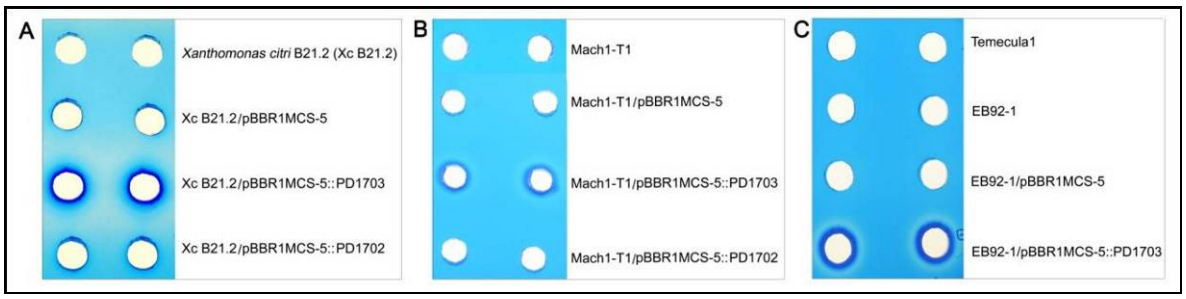

Figure 2. *In vitro* secreted esterase assay of culture supernatants from *X. citri* B21.2, *E. coli*, and *Xf* strains Temecula and EB92-1.

Crude protein extracts of PD1703 overexpressed in BL21 (DE3)/pET-27b induced hypersensitive cell collapse in tobacco and citrus. Crude protein was extracted from *E. coli* BL2 (DE3) carrying pET27b and expressing PD1703 from *lacZ* using a Qiagen protein extraction kit. Protein was resuspended in 200 ul of supplied buffer; some protein remained insoluble but was applied in a suspension and labeled "total protein" in **Figure 3** below. Total protein was centrifuged at high speed to pellet the insoluble protein, and the remaining soluble protein is labeled "supernatant" in **Figure 3** below. A total of 50 ul of total and 50 ul of soluble protein was added to the wells created in the agar plate shown in **Figure 3A**, below. Approximately 10 ul of crude protein suspension ("total protein") was injected into the tobacco and citrus leaves for each inoculation zone circled in **Figure 3B**.

An amount of crude protein suspension that measured 15-18 mg/ml elicited a rapid cell collapse that was visible starting 14 hrs post inoculation in tobacco. The reaction became stronger by two days. Photos of reacting tobacco and citrus leaves at 48 hrs post inoculation are shown in **Figure 3B**. By comparison, 4-5 ug/ml of purified *X. oryzae* LipA were required to elicit browning of rice in infiltrated zones by degradation of cell walls (Jha et al. 2007).

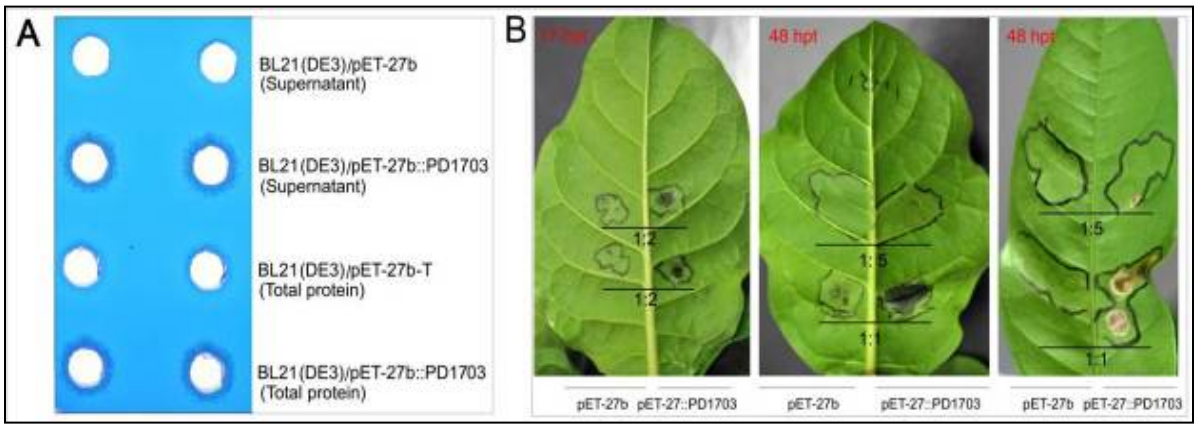

Figure 3 PD1703 crude protein exhibited esterase activity in agar plate assays (A), and induced hypersensitive cell collapse in SR1 tobacco (B, left and center) and citrus (B, right). Equivalent amounts of crude protein from empty vector (pET-27b) or expressing PD1703 (pET-27b::PD1703) were inoculated. A dilution range of 1:1, 1:2, and 1:5 was applied to these leaves; the 1:2 dilution represents 15 – 18 mg/ml of suspended crude ("total protein").

Temecula PD1703 lipase (in pSZ26), PD0928 Zot (in pSZ41), and PD0986 (in pPC3.1) hemagglutinin all contribute to the pathogenic symptoms elicited by *Xf* on grapes. All *Xf* strains were grown in PD3 medium for 36-48 hours at 28° C. As mentioned above, PD1703 was cloned with its native promoter (690bp) and predicted secretion leader peptide in pBBR1MCS-5 (downstream from the *lacZ* promoter), creating pSZ26. For pathogenicity assays, 4-6 week old *V. vinifera* cv. Carignane were inoculated with 10 ul cultures of each strain (OD_{600} = 0.245). Bacterial cultures were diluted in SCP buffer and were inoculated by stem puncture at two points in each of the three internodes, starting with the second internode. Each experiment was repeated at least three times with 3-4 replications in each independent experiment. Plants were maintained under green house and carefully observed for the appearance of symptoms. Observations were recorded from the time the first visible symptoms appeared (6-7 weeks post inoculation). Inferences were drawn from at least three independent experiments.

Pathogenic symptoms elicited by Temecula began to appear by the 5th week after inoculation, and continued to develop up to 12 weeks. Most of the inoculated plants were killed after 12 weeks. Plants inoculated with EB92-1/pSZ26 (Lipase;

Figure 4) and EB92-1/pSZ41 (ZOT; **Figure 5**) showed slightly delayed pathogenesis with visible symptoms becoming evident by the end of six weeks. The infection progressed slowly and remained restricted to 9-10 internodes in case of strains pSZ26 and pSZ41. At the end of three months, plants inoculated with EB92/pSZ26 reached a total of 30% infection and EB92/pSZ41 reached a total of 22 %. EB92-1/ pBBRMCS5 (empty vector) never reached higher than 12% during the period (**Table 1**). Plants inoculated with EB92-1/pPC3.1 (hemagglutinin; **Figure 6**) exhibited symptoms almost as rapidly as the wild type (by the 5th week after inoculation) but with reduced severity (40% infection for EB92-1/pPC3.1 vs. 62% for Temecula by 48 days post inoculation).

Plants inoculated with EB92-1/pSZ26 produced necrosis delimited with pinkish areas. As the infection progressed the entire lamina underwent necrosis leading to defoliation. However the infection progressed slowly compared to wild type strains and remained restricted to 9-10 internodes above the lowest point of inoculation. By contrast, wild type Temecula infections advanced to the tip of the vine (refer **Figure 4**).

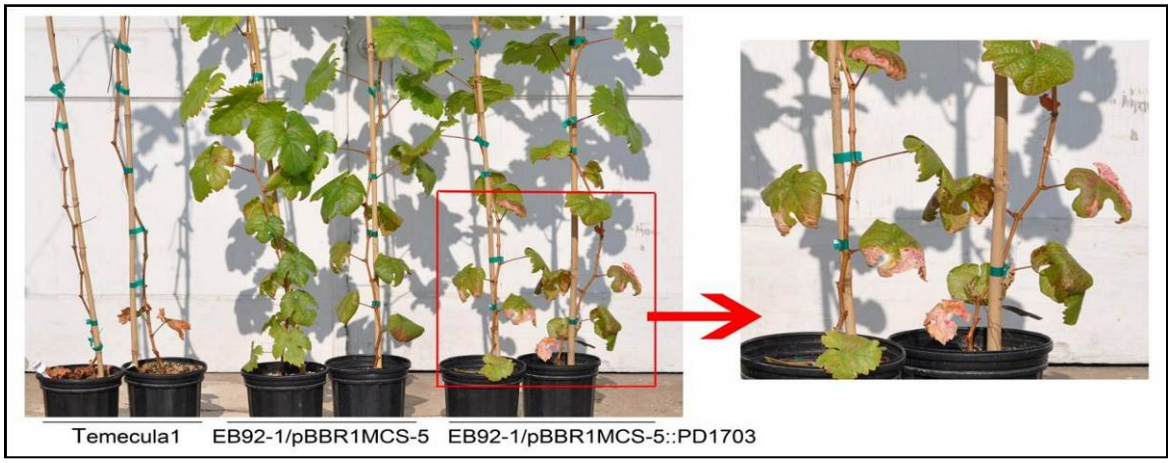

Figure 4. Pathogenic symptoms elicited by Temecula, EB92-1/ pBBRMCS5 (empty vector), and pSZ26 (PD1703 lipase), respectively. (Enlargement shows boxed area on right). Photos taken 90 days post-inoculation.

The infection of EB92-1/pSZ41 (ZOT) was also comparatively slow with visible symptoms becoming evident only by the end of six weeks. The inoculated plants showed prominent anthocyanosis with green veinal areas. As the infection advanced the infected leaves underwent necrosis resulting in defoliation (**Figure 5**).

Figure 5. Pathogenic symptoms elicited by pSZ41 (ZOT; PD0928). These lower leaves dropped off 10 days later. Photo taken 90 days post-inoculation.

The infection of EB92-1/pPC3.1 (hemagglutinin; PD0986) was surprisingly fast by comparison with the other two transconjugants, with visible symptoms elicited by the transconjugants becoming evident by the beginning of the 5th week in

two independent experiments (15 grape plants inoculated with transconjugants total). All plants inoculated with transconjugants showed prominent necrotic areas very similar to those observed with the wild type (**Figure 6**).

Figure 6 Pathogenic symptoms elicited by EB92-1/pPC3.1 (hemagglutinin; PD0986). Photo taken 48 days post-inoculation.

REFERENCES CITED

Aparna, G., et al. 2009. A cell wall-degrading esterase of *Xanthomonas oryzae* requires a unique substrate recognition module for pathogenesis on rice. Plant Cell 21:1860-1873.

Clantin, B., et al. 2004. The crystal structure of filamentous hemagglutinin secretion domain and its implications for the two-partner secretion pathway. Proc. Natl. Acad. Sci. U. S. A. 101:6194-6199.

da Silva, V. S., et al. 2007. Comparative genomic characterization of citrus-associated *Xylella fastidiosa* strains. BMC Genomics 8:474-489.

Gottig, N., et al. 2009. A filamentous hemagglutinin-like protein of *Xanthomonas axonopodis* pv. citri, the phytopathogen responsible for citrus canker, is involved in bacterial virulence. PLoS ONE 44(2): e4358. doi:10.1371/journal.pone.0004358.

Guilhabert, M. R. et al. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute to *X. fastidiosa* biofilm maturation and colonization and attenuate virulence. Molec. Plant- Microbe Interact. 18:856-868.

Hopkins, D. L. 2005. Biological control of Pierce's disease in the vineyard with strains of *Xylella fastidiosa* benign to grapevine. Plant Dis. 89:1348–1352.

Jha, G., et al. 2007. Functional interplay between two *Xanthomonas oryzae* pv. *oryzae* secretion systems in modulating virulence on rice. Molec. Plant-Microbe Interact. 20:31-40.

Rajeshwari, R., et al. 2005. Role of an *in planta*-expressed xylanase of *Xanthomonas oryzae* pv. *oryzae* in promoting virulence on rice. Molec. Plant-Microbe Interact. 18:830-837.

Samad et al., 1989. A plate assay for primary screening of lipase activity. J. Microbiol. Meth. 9:51-56

Uzzau, S., et al. 1999. Expression of a *Vibrio cholerae* zonula occludens toxin and analysis of its subcellular localization. Microb. Pathogenesis 27:377-385.

Zhang, S., et al. 2011. The genome of *Xylella fastidiosa* biocontrol strain EB92-1 is very similar to Pierce's Disease strains. J Bacteriol. 193: 5576–5577.

FUNDING AGENCIES

Funding for this project was provided by the USDA-funded University of California Pierce's Disease Research Grants Program.

Table 1. Results of 3 independent experiments comparing pathogenicity of Temecula & EB92-1 exconjugants carrying pBBRMCS5 (empty vector), pSZ41(PD0928; Zot) or pSZ26 (PD1703; lipase).

	# Total leaves	# Asympto-matic leaves	# Sympto-matic leaves	# Bare petioles	# Bare nodes	# Total leaf production	# Total diseased leaves	Diseased leaves/ total	Avg. diseased leaves/ total
Temecula (WT)	9	1	8	3	23	35	34	0.97	0.70
	11	0	11	4	23	38	38	1.00	
	7	0	7	4	20	31	31	1.00	
	4	0	4	6	23	33	33	1.00	
	38	17	21	3	4	45	28	0.62	
	39	28	10	0	0	38	10	0.26	
	53	42	11	1	10	64	22	0.34	
	61	38	23	0	2	63	25	0.40	
	33	17	16	0	2	35	18	0.51	
	25	13	12	0	8	33	20	0.61	
	0	0	0	0	20	20	20	1.00	
	23	12	11	0	20	43	31	0.72	
EB92/ pBBRMCS5	31	30	1	0	0	31	1	0.03	0.12
	40	35	5	0	2	42	7	0.17	
	40	37	3	0	2	42	5	0.12	
	40	39	1	0	1	41	2	0.05	
	38	34	4	0	3	41	7	0.17	
	30	29	1	0	4	34	5	0.15	
	24	23	1	0	4	28	5	0.18	
	37	36	1	0	0	37	1	0.03	
	76	71	5	0	0	76	5	0.07	
	46	41	5	0	3	49	8	0.16	
	43	40	3	0	3	46	6	0.13	
	40	37	3	0	4	44	7	0.16	
	43	39	4	0	4	47	8	0.17	
EB92/ pSZ41 (Zot)	41	35	6	0	0	41	6	0.15	0.22
	33	29	4	0	3	36	7	0.19	
	30	27	3	0	2	32	5	0.16	
	32	30	2	0	4	36	6	0.17	
	41	36	5	0	1	42	6	0.14	
	33	28	5	1	0	34	6	0.18	
	29	23	6	0	0	29	6	0.21	
	13	10	3	0	5	18	8	0.44	
	41	34	7	0	0	41	7	0.17	
	57	47	10	0	6	63	16	0.25	
	18	12	6	1	3	22	10	0.45	
EB92/ pSZ26 (lipase)	34	28	6	0	3	37	9	0.24	
	38	36	2	0	3	41	5	0.12	
	30	22	8	0	5	35	13	0.37	
	41	37	4	0	3	44	7	0.16	
	32	23	9	0	1	33	10	0.30	
	34	28	6	0	7	41	13	0.32	0.30
	23	18	5	1	4	28	10	0.36	
	36	28	8	0	0	36	8	0.22	
	33	20	13	0	0	33	13	0.39	
	7	4	3	1	2	10	6	0.60	
	35	24	11	0	0	35	11	0.31	