RELATIVE TOXICITY OF DEMETER PEPTIDES ON *ERWINIA AMYLOVORA* isolate 581

Series of experiments were carried out with four lytic peptides supplied by Demeter Biotechnologies Inc. to select one peptide which can totally inhibit *E. amylovora* at a low concentration. The summary of the results achieved is presented below

CULTURE OF ORGANISM: The *E. amylovora* 581 was streaked on NYDA (25 g Sigma nutrient agar, 10 g dextrose and 5 g yeast extract) plates and grown overnight in 26 °C incubator. This overnight-grown culture plate was used to inoculate 150 ml NYDB (8 g Sigma nutrient broth, 10 g dextrose, and 5 g yeast extract) medium in 250 ml flask. Two flasks of broth cultures were incubated overnight in a shaker (150 rpm) at 26 °C. After pelleting the bacteria by centrifugation at 7000 rpm at 4 °C, the cells were suspended in 0.025% or 0.1% sodium chloride solution. The bacterial population in the suspension was adjusted to 10^8 colony forming units (CFU)/ml using a Klett photoelectric colorimeter (140 Klett reading equals approx. 10^8 CFU/ ml and absorbency of 0.387 at 420 nm in a spectrophotometer). Further dilution with salt solution was done to get 10^7 or 10^6 CFU/ ml.

PEPTIDE STOCK SOLUTION: One mM stock solutions of all four peptides (**D5C1a, D2A21, D5CT,** and **D4E1**) were made in autoclaved nanopure water and aliquots (50-100 ul) of stock solutions in microfuge tubes were stored in frost-free -20 freezer. The frozen aliquots were thawed only once. In other words, aliquots once thawed were not used again for the test.

	Mol Wt.	Conc.per mg supplied	Purity	Quantity used for 1mM aqueous solution (stock)
D5C1a	5228.20	0.143 µmol	74.75%	6.99 mg/1ml
D5CT	4001.16	0.180 µmol	72.02%	5.56 mg/1ml
D2A21	3364.20	0.240 µmol	80.74%	4.16 mg/1ml
D4E1	2611.87	0.343 µmol	89.5%	2.91 mg/1ml

Demeter supplied 10 mg (lyophilized) of each peptide. **Dr. Jaynes faxed that the peptides are stable in water for several months.**

- **FINAL CONCENTRATION OF PEPTIDE IN THE TEST CULTURES**

The following concentrations of each peptide were used in our test with ***E. amylovora* isolate 581**

0, 1, 2, 4, 5, 10, 20, 30 µM

PEPTIDE TOXICITY TEST PROCEDURE: One ml of bacterial suspension in a 1.5 ml microfuge tube served as experimental unit for all experiments. Various concentrations of peptides were added to the bacterial suspension (10^6 or 10^7 CFU/ ml) and the cultures were incubated for an hour at 26 °C in a shaker (100 rpm). After one hour of incubation, cultures were diluted (50x for peptide-treated and 500 to 1000x for control) with salt solution, and plated with Autoplater (Spiralbiotech Autoplate model 3000) and the plates were incubated at 26 °C. Three replications were used for each treatment, and two plates were plated for each replication. Therefore there were 6 plates for each treatment including control. Autoplater use 50 µl per plating. In addition, undiluted cultures (50 or 100 µl) were also plated manually. Bacterial colonies appeared 24-36 hours in control plates. Peptide-treated-cultures took 36-48 hours or longer to show colonies, if any. The colonies were counted using with Laser Bacterial Enumerator interfaced with software BEN (Spiralbiotech).

RESULTS AND CONCLUSIONS:

Initial experiments showed that all four peptides killed (total inhibition of growth on plate) ***E. amylovora*** **isolate 581** (10^5 CFU/ ml) in one hour at higher concentrations (5-30 µM) when added to the cultures suspended in 0.1% sodium chloride solutions. High concentrations (5-20 µM) of one peptide (D2A21) was tested in cultures suspended in NYDB medium. This peptides did not completely kill the bacteria(10^6CFU/ml) suspended in NYDB medium even at high concentrations. This may be due to the binding of the peptides by the constituents of NYDB medium or by inactivation of the peptides by this medium. Therefore, cultures were routinely suspended in 0.025% or 0.1% sodium chloride solution and peptides were tested at 1, 2 and 4 µM concentrations to select a peptide which can completely kill a higher bacterial population at a lower concentration. The results are presented in the following table:

	Colony Forming Units per ml (mean of 6 plates)				
Bacterial Population used	**10^6**			**10^7**	
PEPTIDES	1µM	2 µM	4 µM	1 µM	2 µM
D5C1a	0	0	0	2.56×10^5	0
D2A21	0	0	0	Too many to count	3.43×10^5
D4E1	0	37	465	Not tested	
D5CT	5695	1073	330	Not tested	
Control (Population on plate)	8.5×10^5			2.16×10^7	

- The peptides **D5C1a**, **D4E1** and **D2A21** killed 10^6 CFU/ ml even at 1 µM concentration.
- Only **D5C1a** completely killed even a higher population (10^7 CFU/ ml) of this bacterium at 2 µM concentration (No bacterial colony was observed even after one week of incubation).
- Cecropin B was used for comparison in one test. It killed 10^6 CFU/ ml at 2 µM concentration.

Based on the results, the gene for the peptide **D5C1a** (which inhibits a high population (10^7 CFU/ ml) of ***E. amylovora*** isolate **581** at a low concentration) can be used for transforming pear explants to produce fire blight-resistant transgenic pear.