Development of Potent Type III Secretion System Inhibitors

(OTT ID 1112 and 1200)

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New Antimicrobial Drugs: Problems and Solutions

Problem:

- Inappropriate antibiotic use in human medicine and intensive agricultural production has led to increases in microbial resistance
- Antibiotics target cellular processes essential for bacterial survival leading to selection for mutations that confer drug resistance
- Major pharmaceutical companies have sharply decreased efforts for research and development for new antibiotics and antimicrobials
- Plant disease in major cash crops leads to billions of dollars worth of direct and indirect losses each year

Solution:

- Target bacterial virulence, rather than bacterial survival
- Reduce selection pressure on bacterial pathogens to develop drug-resistant mutations
- Drs. Yang and Chen have developed compounds that target components of the Type III secretion system of multiple Gram-negative pathogens
- These compounds should not effect the normal non-pathogenic microbial flora

Potent T3SS Inhibitors: Market Potential

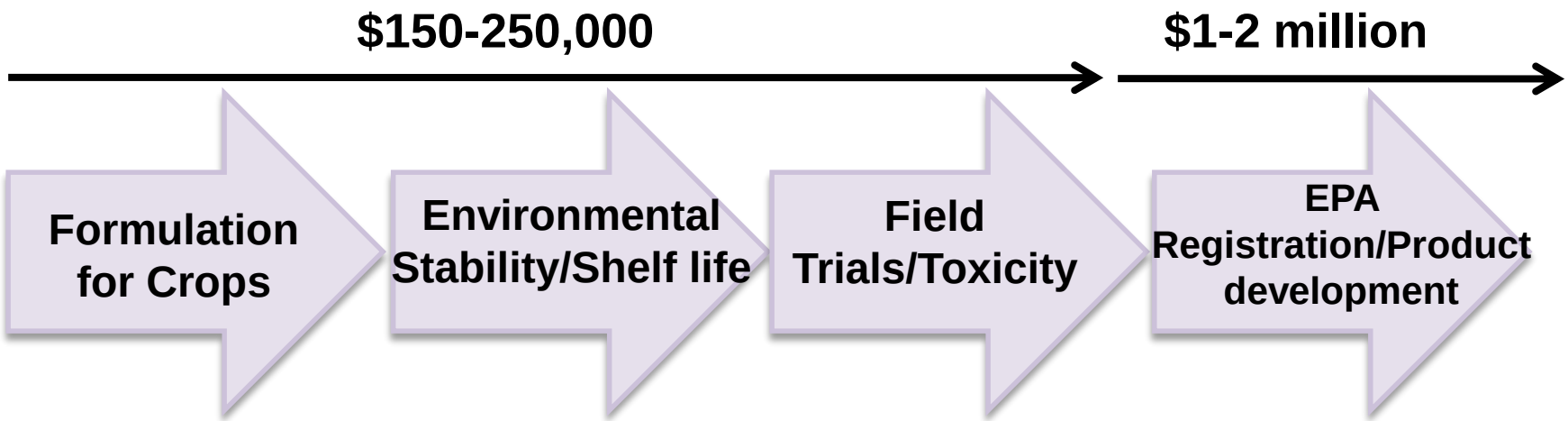
Applications for T3SS Inhibitors:

- Plant pathogens; agricultural cash crops
- Animal/human pathogens; veterinary medicine
- Fish pathogens in aquaculture
- Food safety:
  - Reduce risk of co-contamination by human pathogens
  - Prevention of post-harvest infection in storage crops
- Household antimicrobials
- Estimated $250,000 needed for next development steps of toxicity testing and plant field trial assays ($250K per pathogen tested)

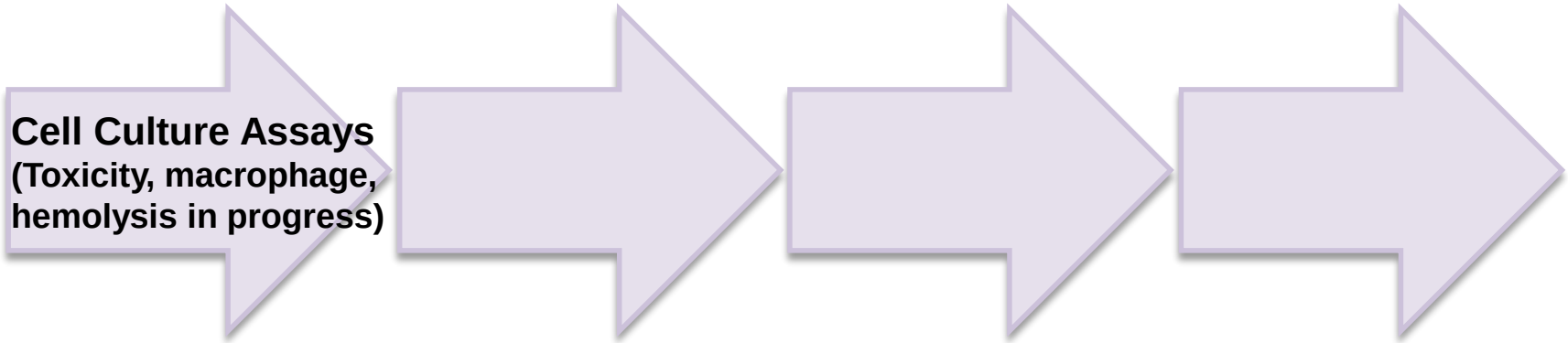
Market

- Plant diseases lead to billions of dollars worth of direct and indirect losses every year
- The worldwide pesticide industry was $52 billion in 2008 and the market is expected to grow annually at a rate of 7% between 2008-2013 (SBI reports)
- The antibacterial market is predicted to grow to over $45 billion by 2012 (Arrowhead Publishers)
- In the U.S. alone hospital bacterial infections result in $4.5 billion in excess healthcare costs

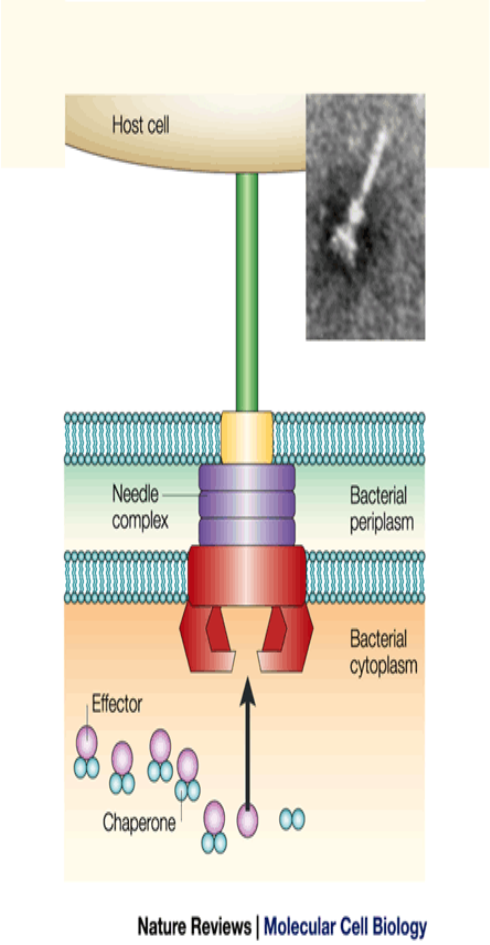
Road to Commercialization: Next Steps



Human/Animal (*P. aeruginosa*)



Type III Secretion System (T3SS)



- **T3SS is a membrane spanning system that bacteria utilize to inject virulence proteins directly into host cells**
- **T3SS mutants are significantly attenuated for virulence**
- **Most enterobacterial pathogens, such as *D. dadantii*, *E. coli*, *Pseudomonas* and *Yersinia* spp, encode T3SS**
- **T3S systems are present in many plant, animal, and human pathogens, but are not present in their non-pathogenic counterparts**
- **Several T3SS components are conserved among different pathogens**

Diseases caused by bacteria using a T3SS

Dickeya dadantii 3937

•A necrotic phytopathogen that causes soft rot, wilts, and blight diseases on a wide range of plant species

•Major pathogenicity factors are pectinolytic enzymes and T3SS

Pseudomonas syringae

• Infects a wide variety of fruits, vegetables, and ornamental plants and responsible for a number of economically important diseases in the Pacific Northwest

•Bacterial speck of tomato caused by *Pseudomonas syringae* pv. *tomato* DC3000

Erwinia amylovora

•Causes fire blight in pear, apple and other Rosaceous plants.

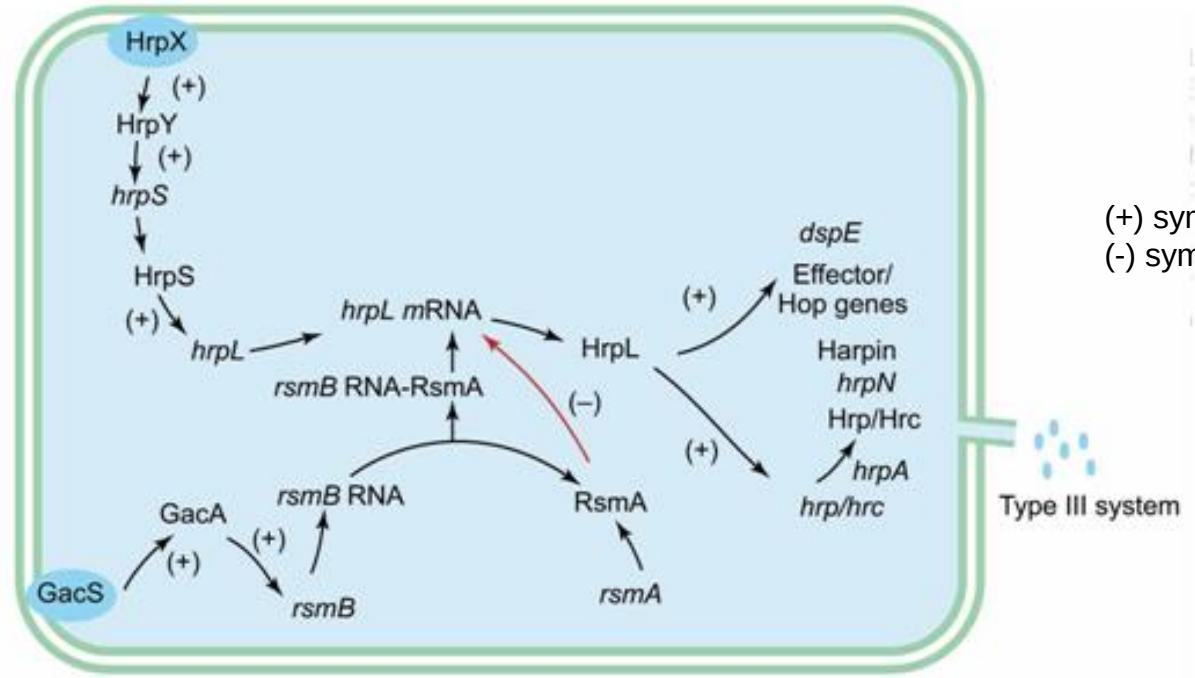
Pseudomonas aeruginosa

•A cause of nosocomial infections in immunocompromised individuals (e.g., HIV and cancer)

•Serious problems in patients hospitalized with cystic fibrosis, pneumonia, urinary tract infections, and burns

•Mortality rate approaching 50%

Regulatory Network Controlling *D. dadantii 3937* T3SS



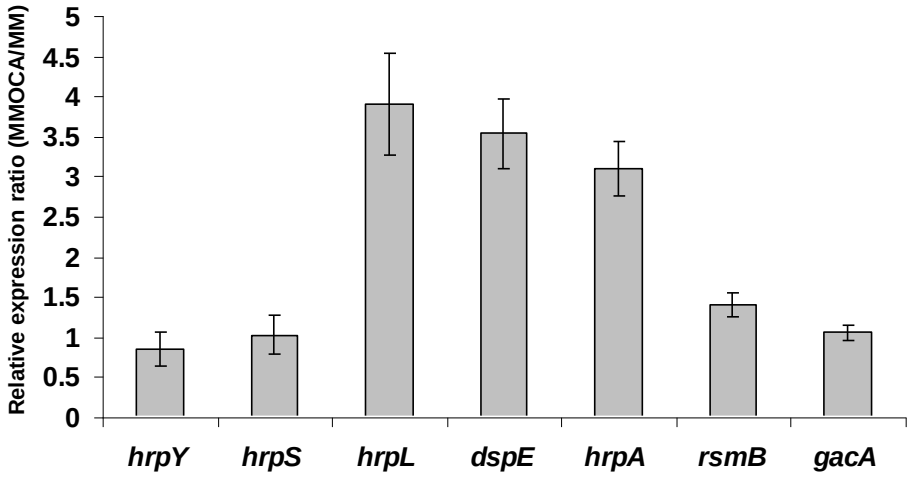
(+) symbol designates positive regulation
(-) symbol designates negative regulation

- The *D. dadantii* T3SS is regulated by the HrpX/HrpY-HrpS-HrpL and the GacS/GacA-RsmA-*rsmB*-HrpL regulatory pathways.
- The two-component system **HrpX/HrpY** activates *hrpS*, which encodes a σ^{54}-enhancer. **HrpS** is required for expression of the alternative sigma factor, *hrpL*.
- **HrpL** activates expression of genes encoding the T3SS apparatus and its secreted substrates.
- **RsmA** is a small RNA-binding protein that acts by lowering the half-life of *hrpL* mRNA.
- **GacS/GacA** upregulates the expression of ***rsmB*,** which increases the mRNA level of *hrpL* by sequestering RsmA.

Identification of T3SS Inducers and Inhibitors

- Phenolic compounds constitute an important class of organic substances produced by plants
- Dr. Yang's group recently discovered that two phenolic compounds , *meta*-coumaric acid (MCA, Yang 005) and *ortho*-coumaric acid (OCA, Yang 006), induce expression of the 3937 hrpA T3SS gene encoding the pilus required for protein translocation into plant cells
- Screened MCA and OCA analogs and isomers for effects on 3937 *hrpA* expression levels
- Identified an isomer PCA (*p*-coumaric acid, Yang 004) which repressed the expression of T3SS genes in 3937
- Then screened ~60 analogs of PCA, OCA, and MCA (30 newly synthesized at Duke University) for effects on *hrpA* expression in 3937
- *Para*-coumarohydroxamic acid (Yang 103) had greater inhibitory effects than PCA
- The compounds are now also being tested against *P. syringae* DC3000
- 2 analogs show inhibition in T3SS *hrpA* of DC3000 but did not inhibit *hrpA* in 3937 indicating these analogs might be very target-selective and could thus support development of pathogen-specific antimicrobials in many different bacterial species with T3SS

Induction of T3SS genes in *D. dadantii* 3937 using OCA

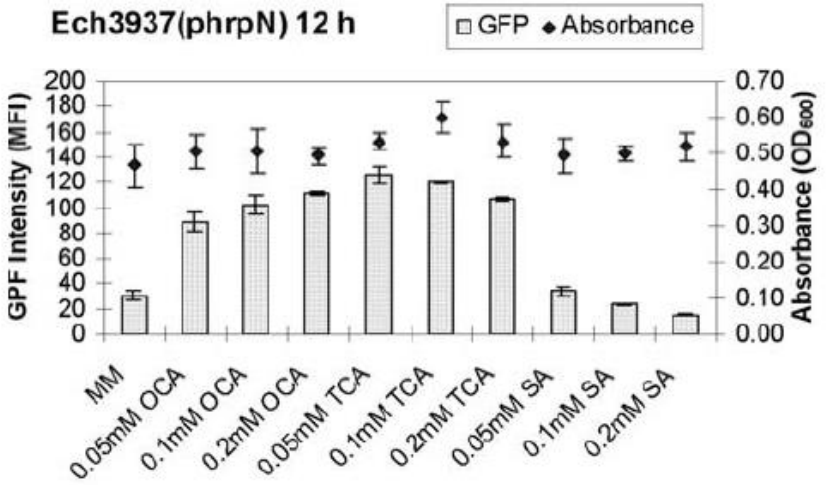


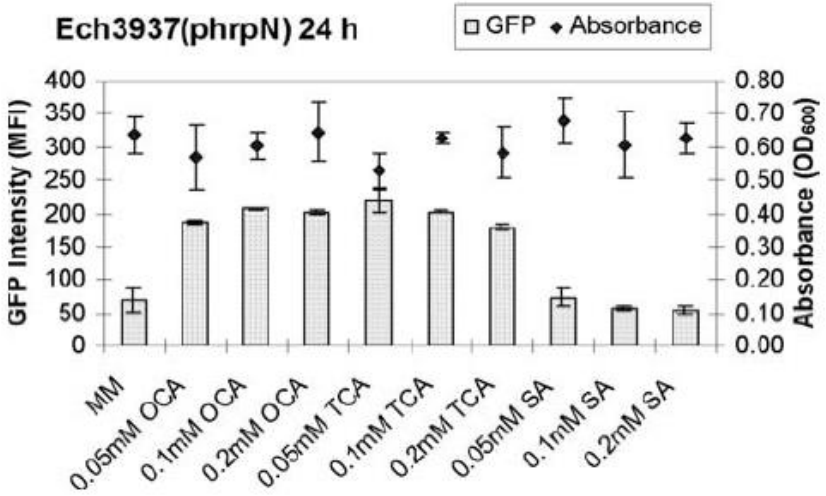
3937 grown in minimal medium (MM) with or without 0.1mM OCA

*The phenolic compounds OCA and *t*-Cinnamic acid induce T3SS genes while salicylic acid (SA) does not affect gene expression

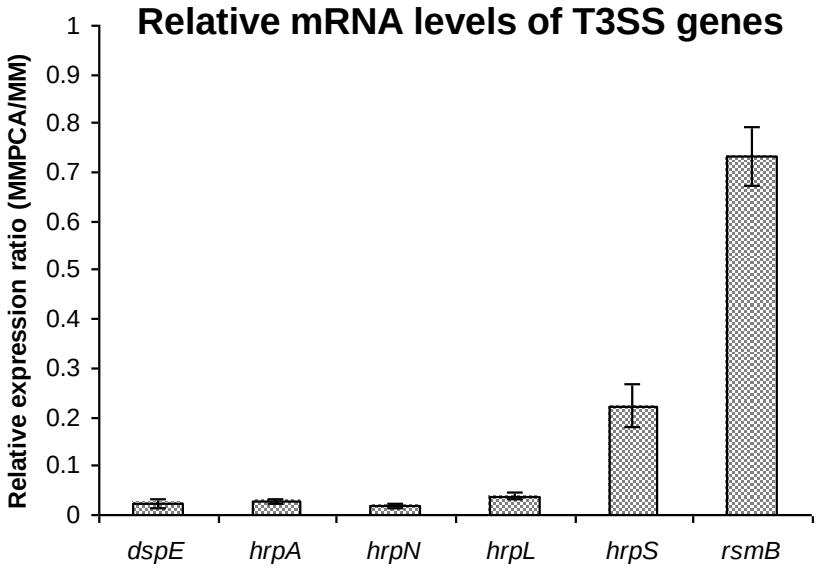
Yang et al. 2008. PLoS ONE. 3(8). e2973

GFP Promoter fusion expression for *hrpN* and growth curve during OCA, TCA or SA treatment



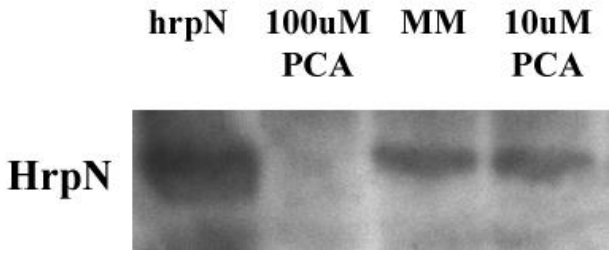


Repression of T3SS genes in *D. dadantii* 3927 using PCA

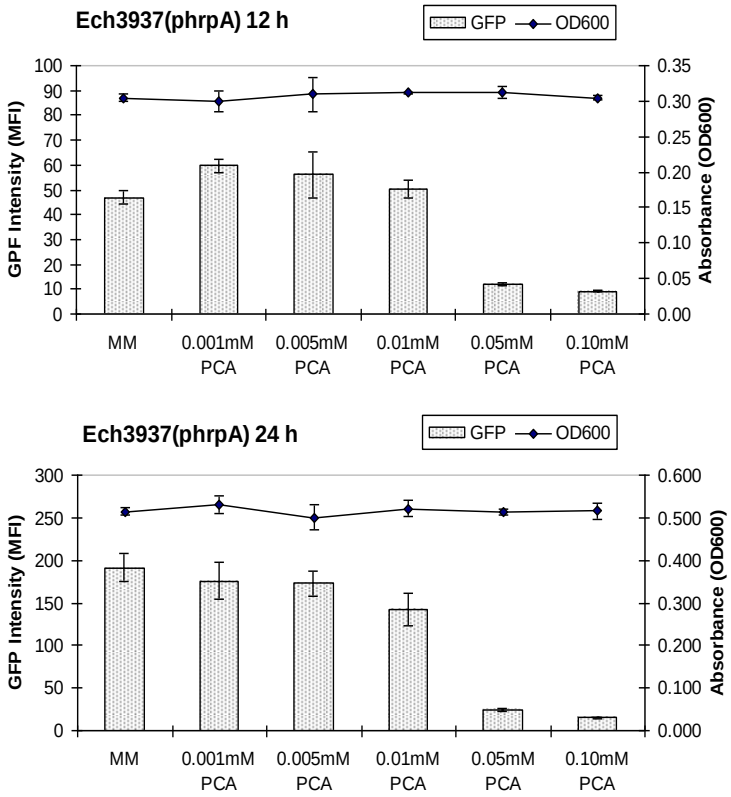


3937 grown in minimal medium (MM) with or without 0.1mM PCA

Protein expression levels of HrpN



GFP Promoter fusion expression for *hrpA* and growth curve during PCA treatment



***PCA inhibits RNA and protein expression of T3SS genes but does not kill the bacteria (OD600)**

New Analogs and Inhibition Screening for pathogenic bacteria

- Structure-activity relationship analogs were designed based on PCA
- 60 analogs of PCA-related phenolic compounds were screened (30 newly synthesized) for inhibition of *hrpA* from *D. dadantii*
- 21 showed an inhibitory effect against *hrpA* expression of *D. dadantii*
- 42 showed an inhibitory effect against *hrpA* expression of *P. syringae* pv. *tomato*
- 52 compounds have been screened thus far for *Erwinia amylovora ,* 19 of which exhibited an inhibitory effect against *hrpA* expression
- 101 compounds have been screened thus far for *Pseudomonas aeruginosa*, a human pathogen, and 2 show promising inhibitory effects; further compounds will be synthesized based on a SAR approach

Inhibitory Effect on T3SS *hrpA* expression

Phenolic compound[a]	12 h[b]	24 h
MM	35.4±0.8	90.6±11.7
Yang-100	45.3±3.4*	119.1±22.1
Yang-101	17.5±0.7*	19.9±0.5*
Yang-102	21.1±1.2*	60.8±10.6
Yang-103	8.7±0.3*	11.9±0.8*
Yang-104	3.0±0.1*	12.9±1.8*
Yang-105	22.9±2.6*	82.4±5.8
Yang-106	10.5±0.8*	49.4±4.1*
Yang-107	45.9±2.7*	122.1±17.9
Yang-108	31.6±2.5	109.0±19.8
Yang-109	35.7±6.3	81.5±3.8
Yang-110	63.4±8.0*	183.8±19.2*
Yang-111	23.9±5.2	54.7±7.2
Yang-112	23.8±4.3	60.7±6.4
Yang-113	24.4±0.9*	68.5±5.7
MM-pAT	2.0±0.0*	3.4±0.3*
Phenolic compound[a]	**12 h[b]**	**24 h**
MM	51.4±6.7	77.1±9.1
Yang-114	55.4±1.0	76.5±5.6
Yang-115	57.9±1.6	79.6±3.3
Yang-116	24.3±1.5*	39.3±3.5*
Yang-117	49.8±1.6	73.1±2.0
Yang-118	59.0±0.4	83.8±1.7
Yang-119	47.8±3.2	68.2±10.4
Yang-120	49.9±2.6	68.6±6.0
Yang-121	57.1±10.0	72.2±10.6
Yang-122	38.6±1.8	60.9±0.8
Yang-123	52.3±2.0	77.7±6.8
Yang-124	44.9±3.5	78.1±3.4
Yang-125	17.4±0.9*	25.6±2.0*
Yang-126	7.8±0.2*	13.4±0.3*
Yang-127	21.0±3.0*	35.1±3.0*

[a]MM and MM supplemented with 100 µM of different compounds. These compounds were assayed two different times with MM as the control treatment for each set of experiments. MM-pAT is vector control.

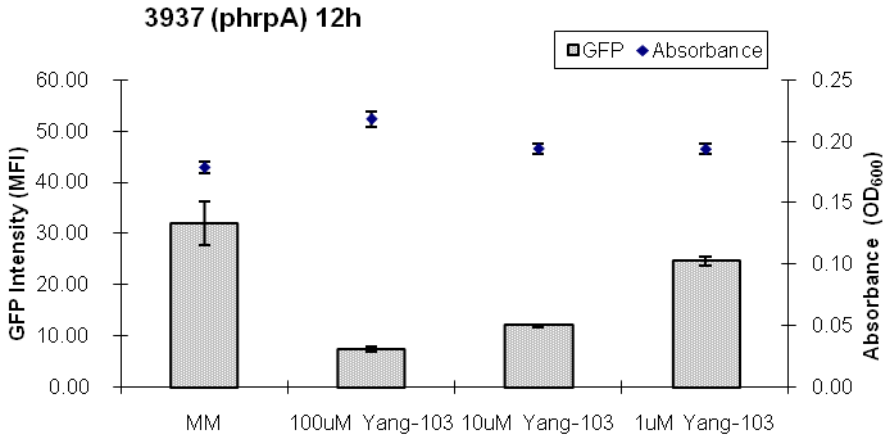
[b]*D. dadantii* 3937 cells carrying GFP reporter phrpA . Promoter activities compared at 12 and 24 h of bacterial growth. GFP intensity was determined on gated populations of bacterial cells by flow cytometry. N=2 with 3 replicates per experiment. The value is presented as the average of three replicates with standard deviation (SD).

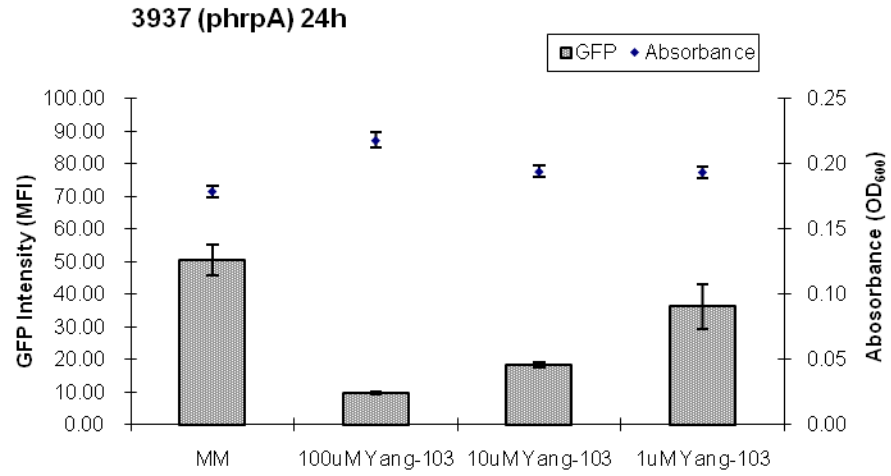
*Statistically significant difference in GFP intensity between bacterial cells grown in MM and MM supplemented with different compounds ($P < 0.01$, Student's *t*-test).

***11 compounds were inhibitory at 12 and 24 hrs**

The Expression level of T3SS gene *hrpA* is repressed by Yang-103 in *D. dadantii* 3937

GFP Promoter fusion expression for *hrpA* and growth curve during Yang-103 treatment





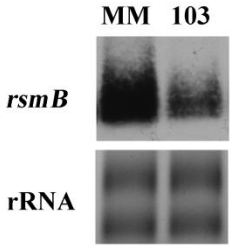
- Yang-103 is more potent than PCA in inhibiting *hrpA* expression
- Bacterial growth is not changed during the application of Yang-103
- This demonstrates that the repression is not due to toxicity of the compounds or nutritional status
- Targeting virulence rather than survival will reduce selection pressure for bacterial genetic mutations

Novel Compound Yang-103 Inhibits Expression of Multiple T3SS genes and the *rsmB* regulatory RNA

	12 h		24 h	
Gene Promoter	**MM**	**MM103**	**MM**	**MM103**
3937 (phrpA)	58.7±6.1	8.9±0.7*	66.5±5.4	8.9±0.2*
3937 (phrpN)	46.8±2.9	5.8±0.7*	49.9±2.1	5.4±0.3*
3937 (phrpS)	73.2±0.6	27.7±1.5*	90.9±1.2	26.4±0.6*
3937 (phrpL)	20.2±1.8	7.6±0.2*	21.5±0.5	7.7±0.2*
3937 (pmrp)	70.6±0.2	81.2±0.7*	80.9±0.9	67.7±2.5*
3937 (pPROBE-AT)	4.2±0.5	3.0±0.6	5.7±1.8	4.3±0.6

- Experimental conditions same as previous table slide 10
- GFP intensity determined by flow cytometry

* Indicates statistical significance

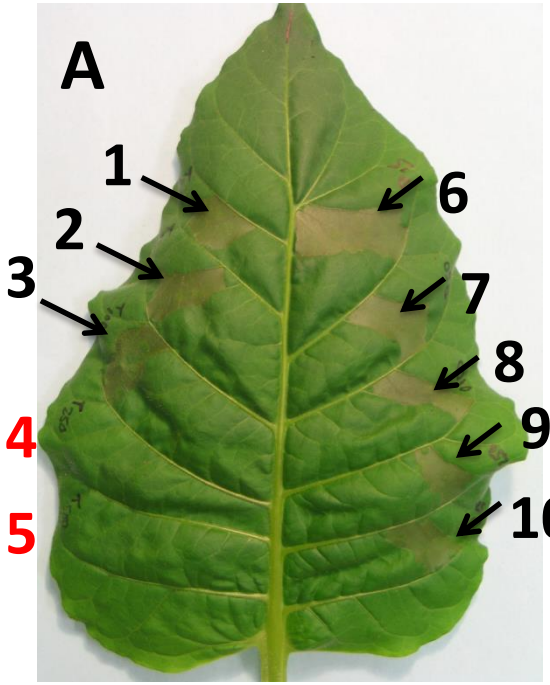


MM: minimal media
103: MM supplemented with 100 mM Yang103
rRNA: control

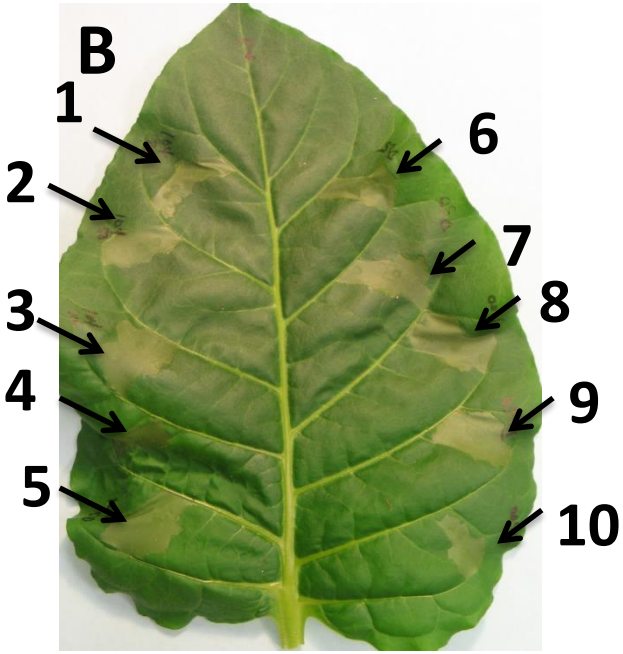
- Quantification of *rsmB* RNA of *D. dadantii* 3937 using Northern blot analysis
- Addition of Yang-103 led to reduced levels of regulatory *rsmB* RNA
- Suggests that Yang-103 inhibits T3SS through the *rsmB*-HrpL pathway
- This indicates the first known incidence of an inhibitor of a regulatory RNA
- *rsmB* is involved in the regulation of T3SS and other virulence factors such as biofilm formation, quorum sensing signals, cellulases, pectate lyases, proteases, and toxins in many bacteria

Yang-001 suppresses the Hypersensitive Response (HR) to *Pseudomonas syringae* pv. *tomato* DC3000 infection in tobacco plant

Yang-001



Yang-104



Tobacco leaves were infiltrated with DC3000 (OD_{600}= 0.1) or DC3000 (OD_{600}= 0.1) supplemented with different concentrations of T3SS inhibitor Yang-001 or Yang-104. **1.** DC3000 supplemented with 25 µM of compound. **2.** DC3000 supplemented with 50 µM of compound. **3.** DC3000 supplemented with 100 µM of compound. **4.** DC3000 supplemented with 250 µM of compound. **5.** DC3000 supplemented with 500 µM of compound. **6.** to **10. :** DC3000 alone was infiltrated into the tobacco leaf.

- Yang-001 (A) suppressed the HR in tobacco (250 and 500uM in red) while analog Yang-104 (B) had no effect

- Yang-001 also suppressed *P. syringae* DC3000 *hrpA* expression in a GFP FACS assay

Treatment with Phenolic Compound Yang-117 Protects Tomato Plants from DC3000

DC3000 with Yang-117

DC3000 alone

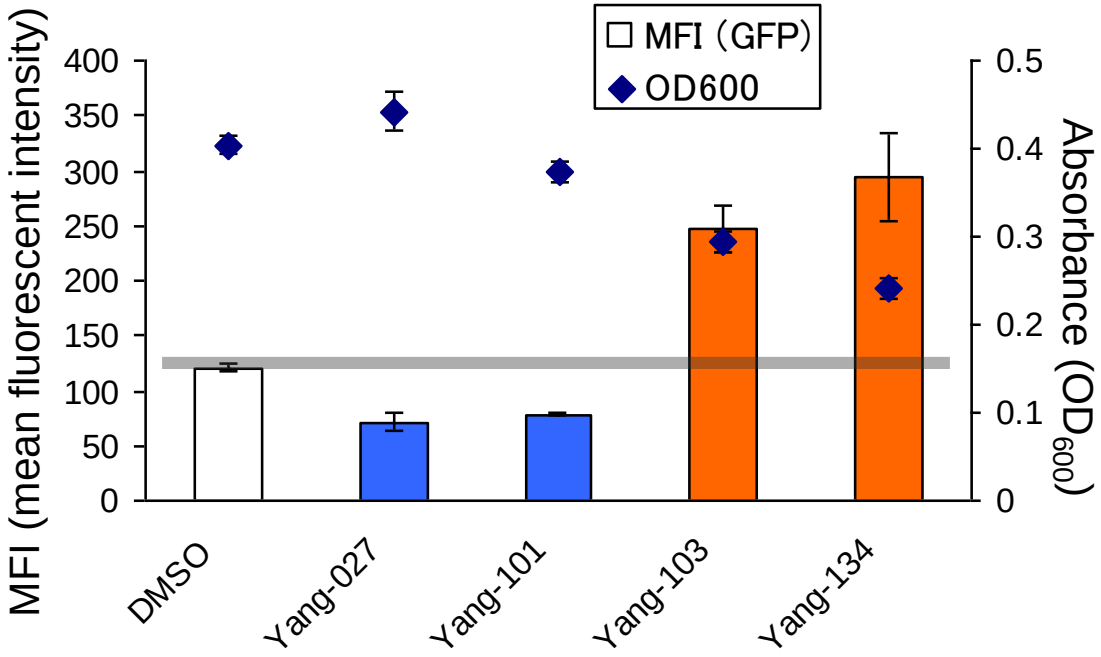
- **Bacterial suspension of DC3000 was diluted to 4 X10^6 CFU/ml in 10 mM $MgCl_2$ and 0.02% Silwet L-77 for dip-inoculation.**

(A) DC3000 supplemented with 250 µM of Yang-117.

(B) DC3000 alone.

- **Reduced lesions of bacterial speck were observed in tomato plants treated with Yang-117**

Inhibition of T3SS genes in *P. aeruginosa* using an *exoS* GFP reporter assay



Compound 250μM

Gray Line = GFP control level for DMSO alone treatment

•Cells treated with compound for 6hrs

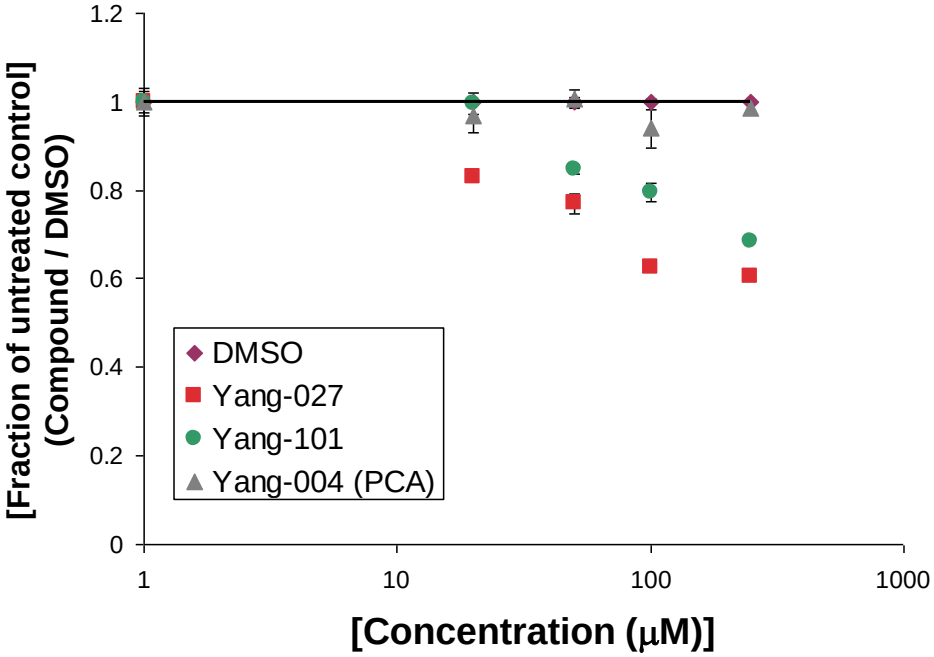
•Yang-027 and Yang-101 were the strongest inhibitors of the T3SS effector (blue bars)

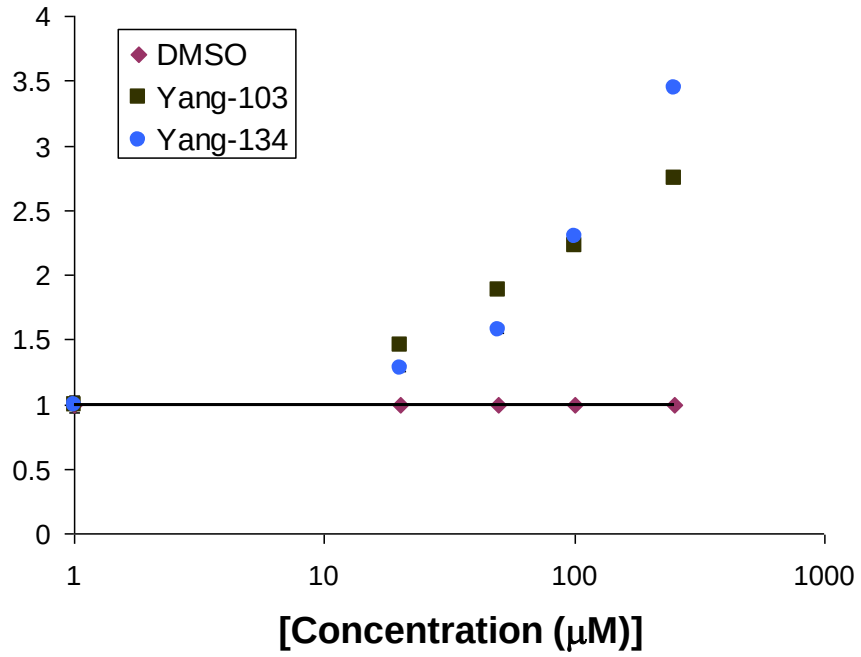
•Yang-103 and Yang-134 were the strongest inducers (orange bar)

•Growth was measured after 12hr by optical density and compared to the DMSO only control (blue circles)

•Bacterial growth is unaffected after treatment with either Yang-027 or Yang-101 compared to untreated control

Dose Response Results for Strongest Yang Inhibitors and Inducers in *P. aeruginosa exoS* Reporter Assay

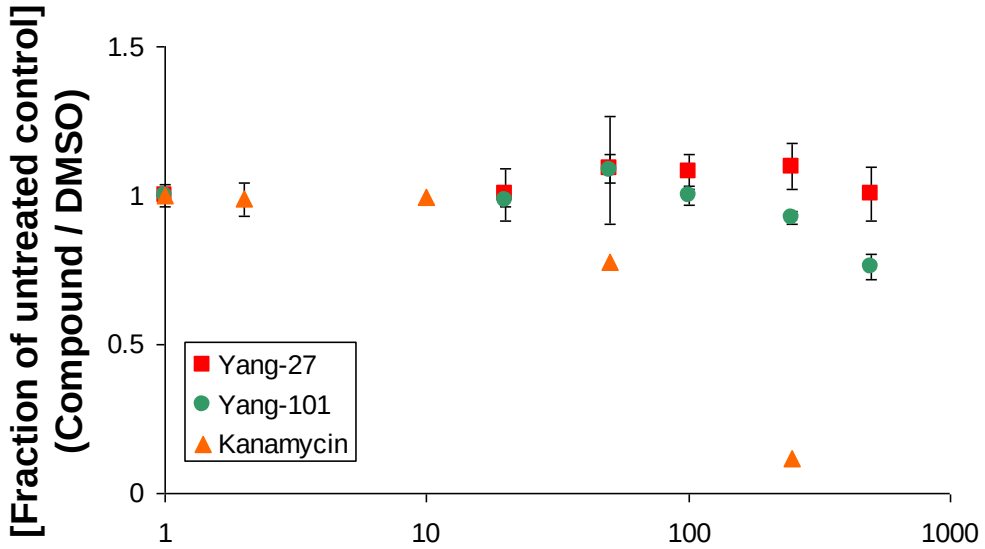


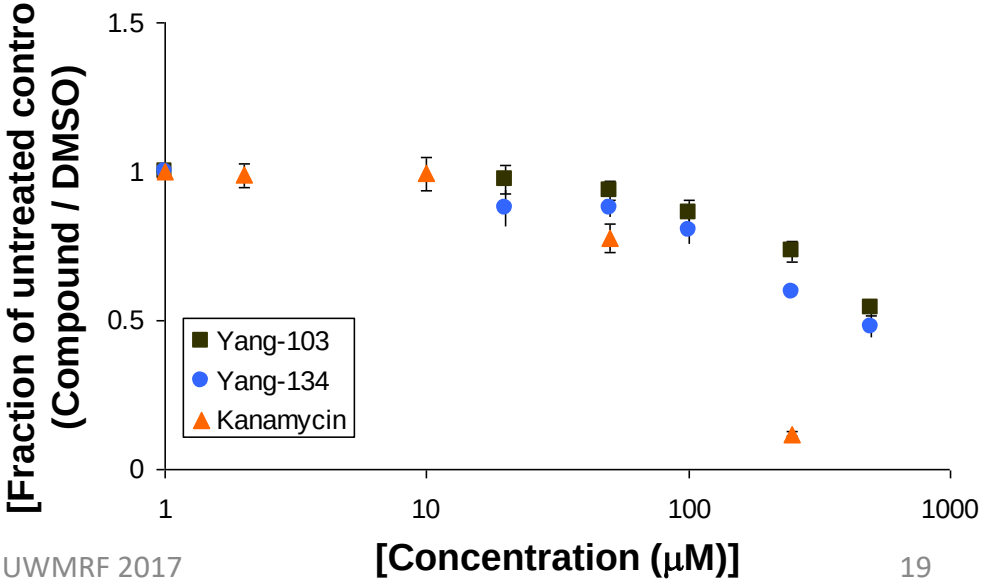


PCA (Yang-004) = negative control; phenolic compound
Black line = Control level for DMSO only treatment
Fraction of untreated control = Ratio of *exoS* expression levels: compound/DMSO
•Cells treated with compound for 6hr followed by flow cytometry to determine mean fluorescence intensity

- Yang-027 and Yang-101 show a dose-dependent inhibitory effect on *exoS*
- Yang-103 and Yang-134 show a dose-dependent induction effect on *exoS*

Evaluation of T3SS inhibitors and inducers on *P. aeruginosa* bacterial growth





- No significant effect on bacterial growth for inhibitory compounds Yang-027 and Yang-101 up to 250 uM (500 uM for Yang-027) compared to kanamycin control

- T3SS inducing compounds Yang-103 and Yang-134 show relatively strong inhibition of bacterial growth compared to kanamycin control

- **Growth was measured using OD = 600nm after 12 hr of treatment with compounds**

In Summary

- Numerous phenolic compounds have been screened and tested for inhibition of T3SS genes in plant pathogens *D. dadantii* 3937, *P. syringae* pv. *tomato* DC3000, and *Erwinia amylovora,* and the human pathogen *P. aeruginosa*
- Several of the Yang compounds are effective against one or more of the above plant pathogens and the human pathogen *P. aeruginosa*
- Yang compounds have been shown to suppress the hypersensitive response *in planta;* this is the first report of HR suppression using T3SS inhibitory compounds.
- Several of the compounds are also able to prevent bacterial speck lesion in a tomato host caused by DC3000.
- Many of the Yang compounds repress virulence genes but do not affect the growth of the bacteria unlike most currently used antimicrobials and antibiotics which kill bacteria
- Targeting of virulence rather than growth and survival is a novel approach that reduces the chance for bacterial mutation and resistance to the compounds
- There is urgent need for novel approaches in developing new antimicrobial drugs due to the continued increase in antibiotic resistance among bacterial pathogens during the last decade

Work in Progress

- Synthesis and testing of novel phenolic compound analogs for inhibition of T3SS in *P. syringae* pv. *tomato* DC3000
- Further screening for inhibition of T3SS using the compound library against the pathogen *Erwinia amylovora* Ea273
- Further *in planta* assays in African violet, pear, and tomato plants
- Plan for formulation studies for use of compounds in plant field trials
- Cell culture efficacy assays for *P. aeruginosa*
- Synthesis of more compounds for *P. aeruginosa* testing based on SAR data; new compounds will be designed based on the inhibitory and inducing effects of Yang-27, -101, -103, and -134
- Evaluation of the selectivity of the inhibitors on T3SS pathogens by measuring the effect on indigenous non-pathogenic microflora

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