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# Toxin-antitoxin systems *mqs*R/*ygi*T and *din*J/*rel*E of Xylella fastidiosa

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ABSTRACT

*Xylella fastidiosa* encodes multiple toxin-antitoxin systems, including *dinJ/relE* and *mqsR/ygiT*. Phylogenetic analyses indicate these two toxin-antitoxin systems have distinct evolutionary histories. Comparisons among *X. fastidiosa* subspecies/strains reveal toxin-antitoxin systems are often embedded in prophage sequences, suggesting acquisition via horizontal transfer. Tagged proteins of both toxin-antitoxin systems were over-expressed, purified, and evaluated for activity. Toxins MqsR and RelE are ribonucleases. MqsR cleaved at G▲CU and G▲CC; RelE lacked specificity in vitro. YgiT and DinJ antitoxins inhibit activity of the cognate toxin by direct binding. Single (toxin or antitoxin) and double (toxin and antitoxin) knock-out mutants were constructed in *X. fastidiosa* strain Temecula. Both antitoxin mutants displayed reduced planktonic cell density relative to wild type. The *dinJ*$^{-}$ mutant produced less biofilm, whereas the *ygiT*$^{-}$ mutant produced more biofilm, relative to wild type. Planktonic cell density of both toxin mutants was unaffected, with biofilm reduced slightly. Planktonic cell density and biofilm formation of double mutants were similar to wild type. However, viable cell counts for planktonic cells and biofilm of double mutants were more than one order of magnitude greater than wild type. Collectively, the results indicate that *dinJ/relE* and *mqsR/ygiT* are functional toxin-antitoxin systems and likely play different roles in regulation of *X. fastidiosa* population levels.

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## Introduction

Bacterial toxin-antitoxin (TA) systems typically consist of a stable toxin and a cognate labile antitoxin [1,2]. Most commonly, TA system toxins are ribonucleases targeting mRNA; cognate antitoxins inhibit ribonuclease activity via direct binding [3–10]. Plasmid-encoded TA systems act as addiction systems, conferring stable plasmid inheritance [11–18]. Plasmid maintenance is achieved by post-segregational killing of plasmid-free daughter cells by residual toxin [12]. In contrast, TA systems encoded by bacterial chromosomes mediate growth by inhibiting translation in response to various environmental stresses [1,4,19–23] or during biofilm formation [24–26]. TA system-mediated translational arrest may lead to a state of physiological dormancy [27,28] or result in cell death. Theoretically, either repression or up-regulation of TA systems could lead to translational arrest [19,20]. Upon repression, stable toxin would outlast labile antitoxin, a situation similar to post-segregational killing of plasmid-free daughter cells [12]. Alternatively, up-regulation [21] may lead to a greater number of unbound (active) toxin molecules per cell if the ratio of antitoxin-bound to unbound toxin remains constant.

*Xylella fastidiosa* [29] is a fastidious, xylem-limited bacterium causing Pierce's disease of grapevine and vascular occlusion diseases of numerous horticultural and landscape plants in the Americas [30]. Based on genome comparisons [31,32] and multilocus sequence typing [33,34], four subspecies of *X. fastidiosa* have been characterized. For subspecies *fastidiosa*, the center of diversity is in Central America [35–37]. Strains of subspecies *fastidiosa* from temperate North America exhibit limited polymorphism, leading to the hypothesis that Pierce's disease of grapevine is caused by a subspecies *fastidiosa* lineage introduced from Central America in the late 19th century [37]. Subspecies *multiplex* appears to be native to temperate North America [34,37] where diverse strains cause disease in a wide range of hosts [38–40]. Subspecies *pauca* includes strains infecting coffee and citrus from South America [36,41,42] that may have arisen by recombination between an introduced subspecies *multiplex* lineage and an unidentified native South American lineage [43]. A group of closely related strains infecting oleander [44], likely introduced to the U.S. ca. 1980 [34], comprise subspecies *sandyi*. Mulberry-infecting strains from the U.S. [45]. have a mosaic genome generated by recombination among strains of subspecies *fastidiosa* and subspecies *multiplex* [46].

Although TA systems are generally more common in free-living compared to host-associated prokaryotes [1], examination of

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*X. fastidiosa* genome sequences reveals the presence of multiple TA system homologs [47]. Previously, only the plasmid-borne *X. fastidiosa* homolog of the *pemI/pemK* TA system has been characterized for function. Closely related 25 kbp plasmids, resident in *X. fastidiosa* strains isolated from mulberry [48] and distantly related to Incompatibility Group P-1 plasmids [49], encode the *pemI/pemK* TA system that serves as a classic plasmid addiction system [13]. The *X. fastidiosa* PemK toxin is a ribonuclease with preference for cleavage between the U and A residues of UACU and UACG; PemI is the cognate antitoxin that binds to and blocks ribonuclease activity of PemK [5]. Thus, functional activities of the *X. fastidiosa pemI/pemK* TA system are similar to that of the prototypical *pemI/pemK* TA system from *Escherichia coli* plasmid R100 [10], despite sharing only ~50% amino acid sequence identity [5].

Given the importance of biofilm formation in *X. fastidiosa* virulence and vector transmission [50–54], coupled with different environmental stresses likely experienced in plant xylem versus insect foreguts, examination of TA system homologs encoded by the *X. fastidiosa* chromosome is warranted. Among the numerous TA system homologs [47] present in genome sequences of one or more *X. fastidiosa* subspecies, we selected *mqsR/ygiT* and *dinJ/relE* for analyses. The *mqsR/ygiT X. fastidiosa* homolog was chosen because the prototypical MqsR toxin homolog in *E. coli* is a motility quorum-sensing regulator affecting biofilm formation [24,26] and the *X. fastidiosa* homolog in subspecies *pauca* strain 9a5c is up-regulated in biofilm exposed to antimicrobial compounds [47]. The *dinJ/relE X. fastidiosa* homolog was chosen because the prototypical RelE toxin homolog in *E. coli* causes translational arrest in response to a specific (starvation) stress [21]. Here, as the initial step in characterization of TA system homologs encoded by the *X. fastidiosa* chromosome, we describe (*i*) phylogeny and placement of *mqsR/ygiT* and *dinJ/relE* in the *X. fastidiosa* genome, (*ii*) functional activities of purified proteins encoded by *X. fastidiosa mqsR/ygiT* and *dinJ/relE*, and (*iii*) effects of knock-out mutants of *mqsR/ygiT* and *dinJ/relE* on *X. fastidiosa* planktonic cell density, biofilm formation and cell viability.

## Materials and methods

### *Phylogenetic analyses*

The amino acid sequence of each protein encoded by *mqsR/ygiT* (Gene loci tags PD370 and PD371; protein ID numbers NP_778601 and NP_778602) and *dinJ/relE* (Gene loci tags PD1099 and PD1100; protein ID numbers NP_779305.1 and NP_779306.1) of *X. fastidiosa* strain Temecula (subspecies *fastidiosa*) was used to query the GenBank nonredundant protein database. Subjects returned from BLAST P searches were selected as taxa included in phylogenetic analyses. A neighbor-joining tree (1000 bootstrap replicates) for each protein was constructed based on a multiple alignment of amino acid sequences generated using CLUSTALX. Nodes bearing $\leq$70% bootstrap support were considered unreliable and collapsed to polytomies. The corresponding homolog from *E. coli* strain K12 (Protein ID numbers NP_417494 [MqsR], NP_417493 [YgiT], NP_414761.1 [DinJ], NP_414760.1 [RelE]) was designated as an outgroup for each analysis.

### *Genomic comparisons among X. fastidiosa subspecies and strains*

To determine occurrence and genomic location of *mqsR/ygiT* and *dinJ/relE* in multiple *X. fastidiosa* subspecies/strains, DNA sequences for each TA system and flanking regions were retrieved from GenBank for all available *X. fastidiosa* strains and aligned. Since the two TA systems were (if present) embedded (with one exception) in separate copies of a duplicated prophage region, BLAST searches also were conducted with the flanking prophage genes *W* (gene loci tags PD0367 and PD1098, protein ID numbers NP_778598.1 and NP_779304.1) and *V* (gene loci tags PD0372 and PD1101, protein ID numbers NP_778603.1 and NP_779307.1) from *X. fastidiosa* strain Temecula to determine presence/absence and gene composition of additional copies of the prophage region in each *X. fastidiosa* subspecies/strain.

### *Protein expression and purification*

Open reading frames coding for toxin and cognate antitoxin of the two TA systems were cloned into expression vectors, and over-expressed in *E. coli*. Over-expressed proteins were purified as described for the *X. fastidiosa pemI/pemK* TA system [5]. *E. coli* strain BL21(DE3)pLysS (Stratagene, La Jolla, CA) was used for all protein expression experiments. Toxin and cognate antitoxin were co-expressed using the pETDuet-1 vector, as expression of toxin in the absence of antitoxin inhibits growth of *E. coli* [5]. For each TA cognate pair, one protein was expressed with an S-tag, the other protein was expressed with a $His_{(6)}$-tag. Following induction of expression, toxin-antitoxin native complexes (from dual expression constructs) were purified from soluble protein extracts by affinity chromatography on Ni-nitrotriacetic acid (NTA) columns, which specifically bind $His_{(6)}$-tagged proteins. Toxin was further purified (e.g., antitoxin removed) from toxin-antitoxin native complexes by denaturation, affinity chromatography using S-protein agarose (if S-tagged) or NTA (if $His_{(6)}$-tagged), and renaturation of eluted toxin as described [5]. Each tagged antitoxin also was expressed alone, in the absence of toxin, and purified from soluble protein extracts using the appropriate affinity column. Toxin-antitoxin complexes were reconstituted by mixing and incubating purified toxin and purified cognate antitoxin as described [5]. Proteins in purified samples were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after denaturation by boiling in the presence of SDS. Protein concentration was estimated by measuring absorbance (280 nm) using the ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and an extinction coefficient of 1.0 O.D. for a 1.0 mg/ml solution.

### *Ribonuclease assays*

Ribonuclease assays were conducted essentially as described [5]. Substrate was total RNA (5 μg) extracted from leaves of *Nicotiana tabacum* using TRIzol reagent (Life Technologies, Foster City, CA) as described by the manufacturer. Nuclease assays were conducted by incubation (10 min, room temperature) of RNA substrate with (for each TA system) native toxin-antitoxin complex (0.5 μg), toxin (0.25 μg), antitoxin, (0.25 μg), or reconstituted toxin-antitoxin complex (0.5 μg). Products were evaluated by electrophoresis in 1.4% agarose-formaldehyde gels.

### *Determination of ribonuclease cleavage sites*

*N. tabacum* total RNA substrate (5 μg) was partially digested (3–4 min, room temperature) with purified MqsR or RelE. Digestion products were used as template for 5′-RACE (Rapid Amplification of cDNA Ends) as described [5]. *N. tabacum* 23S rRNA (GenBank accession number AY123764) was selected as the target sequence for amplification in two 5′-RACE reactions (designated RACE1 and RACE2). Reverse transcription was primed using an oligonucleotide annealing to *N. tabacum* 23S rRNA nucleotides 999 to 970 (RACE 1) or nucleotides 1296 to 1276 (RACE 2). Homopolymeric tails (poly A or poly C) were added to the 3′-ends of cDNA products prior to amplification by polymerase chain reaction (PCR). PCR products were ligated into pGEMT-easy (Promega, Madison,