RESEARCH PAPER

Pathogenicity Islands, Virulence Repertoires, and Rational Antivirulence Drug Design Against *Xanthomonas citri* pv. *punicae* in Pomegranate

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Abstract
Bacterial blight (oily spot) of pomegranate (*Punica granatum* L.) is caused by *Xanthomonas citri* pv. *punicae*, a phytopathogen historically classified as *Xanthomonas axonopodis* pv. *punicae*. Comparative genomic investigations reveal that this bacterium utilizes a virulence repertoire encompassing an *hrp*/*hrc*-encoded type III secretion system (T3SS), non-TAL *Xanthomonas* outer protein (Xop) effectors, transcription activator-like effectors (TALEs), pseudo-TALEs, and mobile accessory determinants that drive host colonization and tissue necrosis. The transition to high-contiguity long-read sequencing platforms has resolved repeat-dense genomic neighborhoods, mobile elements, plasmids, and central tandem repeat domains of TALE loci.
Pathogenicity islands (PAIs) represent horizontally acquired genomic segments that accelerate pathotype evolution. In phytopathogens, PAIs are characterized by atypical G+C content, altered dinucleotide frequencies, insertion adjacent to tRNA/tmRNA genes, flanking direct repeats, and mobility-associated elements. However, sequence amelioration and structural rearrangements necessitate multi-algorithmic and functional validation. Furthermore, as conventional bactericides face escalating chemical resistance, the virulence factors encoded within these genomic islands represent candidate targets for antivirulence drug discovery.
This review synthesizes the genomic architecture, effector biology, and mobile genetic landscape of *X. citri* pv. *punicae*, introduces a translational framework for small-molecule and peptide-based inhibitor development targeting secretion machinery and effectors, and outlines an integrated approach to differentiate bona fide PAIs from core virulence clusters.

Keywords: *Xanthomonas citri* pv. *punicae*; *Punica granatum*; pomegranate bacterial blight; pathogenicity islands; type III secretion system; antivirulence therapeutics; drug design and discovery; Xop effectors; comparative pathogenomics.
How to cite this article: Anagha B. Anmod and M. M. V. Baig1*. Pathogenicity Islands, Virulence Repertoires, and Rational Antivirulence Drug Design Against Xanthomonas citri pv. punicae in Pomegranate. Int J Drug Deliv Technol. 2026;16(88s):1853-1858. DOI: 10.25258/ijddt.16.88s.187.

1. Introduction:

Disease Burden, Taxonomic Evolution, and Virulence Architecture

Pomegranate (*Punica granatum* L., family Lythraceae) is an economically significant fruit crop cultivated throughout arid and semi-arid regions worldwide. Commercial orchards face major constraints due to bacterial blight (oily spot or nodal blight), a devastating disease capable of causing 60% to 80% yield losses under warm, humid conditions. The pathogen invades aerial tissues via stomata, hydathodes, lenticels, and physical abrasions. Foliar symptoms initiate as water-soaked lesions that transition into dark necrotic spots with chlorotic halos, frequently resulting in defoliation. On woody twigs and stems, cankers cause shoot dieback, whereas infected fruits develop dark, raised, oily lesions that split into characteristic star-shaped cracks, destroying commercial value.

Initially described as *Xanthomonas punicae* and later grouped as *Xanthomonas campestris* pv. *punicae*, the organism was subsequently designated *Xanthomonas axonopodis* pv. *punicae* following polyphasic revisions. Multilocus sequence analysis (MLSA) and whole-genome average nucleotide identity (ANI) revealed that the pathogen shares ~98.78% ANI with *Xanthomonas citri* pv. *citri*, placing it firmly within the *Xanthomonas citri* species complex as *Xanthomonas citri* pv. *punicae*.

The infection cycle of *X. citri* pv. *punicae* depends on epiphytic attachment, motility via type IV pili and flagella, secretion of extracellular polysaccharides (EPS), and direct translocation of effector proteins into host cells via the type III secretion system (T3SS). Once inside the plant cell, effectors suppress pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), manipulate host transcription, and perturb reactive oxygen species (ROS) homeostasis.

The effector complement comprises non-TAL *Xanthomonas* outer proteins (Xops) and transcription activator-like effectors (TALEs). Non-TAL effectors such as XopN and XopL suppress early immune signaling and programmed cell death (PCD). While individual effectors have been characterized, resolving how these virulence factors are clustered within horizontally acquired genomic islands or maintained on plasmids is essential for understanding pathotype diversification and for designing targeted antimicrobial interventions.

2. Evolutionary Biology and Structural Hallmarks of Bacterial Pathogenicity Islands

Genomic islands (GIs) are horizontally acquired chromosomal segments typically spanning 10 to over 200 kb. A genomic island is classified as a pathogenicity island (PAI) when it encodes factors directly or indirectly driving colonization, immune suppression, or effector translocation.

Horizontal integration imparts diagnostic compositional and structural hallmarks:

1. **Sequence Composition Bias:** Because bacterial taxa exhibit characteristic nucleotide proportions, horizontally transferred segments typically deviate in G+C percentage and codon usage from the core genome. In *Xanthomonas*, where the core chromosome exhibits ~63% to 65% G+C content, acquired virulence islands, effector cassettes, and *hrp*-flanking segments frequently show reduced G+C content (52% to 59%).
2. **Integration at tRNA/tmRNA Loci and Flanking Direct Repeats:** PAIs preferentially integrate into the 3′ end of tRNA or tmRNA (*ssrA*) loci via site-specific recombinases. Recombination between chromosomal *attB* and island *attP* sites generates flanking direct repeats (DRs) that define island boundaries.
3. **Mobility and Recombination Modules:** Canonical islands carry functional or vestigial integrases, transposases, and insertion sequences (IS). In xanthomonads, IS elements (e.g., IS*3*, IS*5*, IS*Xac*) cluster around virulence loci, driving genome rearrangements, pseudogenization, and mobile gene capture.
4. **Mosaicism and Chromosomal Heterogeneity:** PAIs frequently exhibit mosaic structures formed by iterative gene insertions into integration hotspots. Like the tripartite *hrp* cluster in *Pseudomonas syringae*, Xanthomonas *hrp* loci feature a conserved structural core bordered by variable, mobile effector-containing regions.

Over evolutionary timescales, compositional amelioration and deletion of terminal direct repeats can immobilize islands within the chromosome. Consequently, robust identification of PAIs requires integrated computational, structural, and experimental evidence.

3. Genomic Resources and Architecture of *Xanthomonas citri* pv. *punicae*

Pioneering comparative genomics in *Xanthomonas* revealed that while core genomes remain colinear, lineage-specific islands and plasmids encode the specific determinants of host range. The initial Illumina draft assembly of *X. citri* pv. *punicae* strain LMG 859 (4.94 Mb, 64.9% G+C) identified core structural T3SS machinery and non-TAL effectors, but multi-copy IS elements and repetitive *tal* genes were fragmented across short contigs.

Third-generation long-read sequencing technologies, including PacBio SMRT and Oxford Nanopore platforms, have resolved these repetitive loci. PacBio sequencing of strain Bagalkot (5.4 Mb, 63.94% G+C) resolved 11 non-TAL effectors, two full-length TALEs, and four pseudo-TALEs, including two localized on plasmid pUnnamed2. Similarly, long-read assemblies of strain 119 identified seven TALE-encoding genes across chromosomal (four copies) and plasmid-borne (three copies) locations. In silico subtractive genomics against the pomegranate proteome further identified 595 pathogen-specific proteins, including the essential type IV pilus secretin PilQ.

Concurrently, field surveys have documented emerging resistance to agricultural bactericides like streptomycin, demonstrating how accessory genome plasticity influences clinical persistence and control efficacy.

Table 1. Key genomic resources and virulence-associated features of sequenced *Xanthomonas citri* pv. *punicae* strains

Strain / Dataset	Sequencing Platform & Metrics	Resolved Genomic & Virulence Features	Reference
Strain LMG 859	Illumina short-read draft (~4.94 Mb; 64.9% G+C; 219 contigs)	Encodes core *hrp*/*hrc* cluster and non-TAL effectors; repetitive *tal* loci remained collapsed.	Sharma et al. (2012)
Strain Bagalkot	PacBio SMRT long-read assembly (~5.40 Mb; 63.94% G+C; N50 = 5.16 Mb)	Resolves 11 non-TAL effectors, 2 full-length TALEs, and 4 pseudo-TALEs (2 plasmid-borne).	Radhika et al. (2021)
Strain 119	Long-read chromosomal and plasmid assembly (~5.3–5.4 Mb;	Identifies seven TALE genes (four chromosomal, three on	K. et al. (2023)

	~64% G+C)	plasmid), confirming plasmid-borne virulence plasticity.	
Subtractive Dataset	In silico host-pathogen proteome subtraction	Uncovers 595 pathogen-specific proteins; identifies PilQ secretin as a key virulence and motility target.	Dineshkumar & Antony (2022)
Field Isolates	MLSA, rep-PCR, and bactericide assays	Establishes regional lineage diversity; reports emerging resistance to standard streptomycin formulations.	Sharma et al. (2022)

4. Type III Secretion System (T3SS), Non-TAL (Xop) Effectors, and TALEs in Pomegranate Blight

4.1 Organization and Transcriptional Control of the *hrp*/*hrc* Pathogenicity Cluster

The T3SS is encoded within a 23–25 kb chromosomal *hrp* gene cluster composed of over 20 genes organized into polycistronic operons. Nine conserved *hrc* genes form the core export apparatus, inner and outer membrane rings, and the energizing ATPase (HrcN). Flanking *hrp* and *hpa* genes encode the extracellular pilus, the translocon pore protein HrpF, and specific secretion chaperones like HpaB.

Expression is regulated by an apoplast-sensing cascade: the OmpR-type regulator HrpG activates *hrpX*, encoding an AraC-type transcriptional activator. HrpX binds to conserved plant-inducible promoter (PIP) motifs (TTCGB-N15-TTCGB) upstream of *hrp* operons and dispersed effector genes, driving their coordinated induction during host colonization. While the central *hrp* region represents an ancient, stabilized island, flanking loci display lower G+C content and frequent mobile element insertions.

4.2 Non-TAL (Xop) Effectors and Suppression of Host Immunity

X. citri pv. *punicae* utilizes a modular set of non-TAL effectors to disassemble host defenses:

XopN: Encodes an antiparallel α-helical repeat scaffold (HEAT/ARM-like motifs). Upon translocation, it localizes to the host plasma membrane, where it represses PAMP-induced ROS production, prevents callose deposition, and facilitates bacterial multiplication. Gene disruption leads to severe loss of lesion expansion in pomegranate.

XopL: Functions as an E3 ubiquitin ligase via its C-terminal XL-box domain. Knockout mutants ($\Delta xopL$) fail to form typical water-soaked lesions and exhibit attenuated growth in planta. XopL suppresses both the early ROS burst and subsequent programmed cell death, maintaining host tissue viability during colonization.

Conserved and Accessory Effectors: The core effector AvrBs2 possesses a putative glycerophosphoryl diester phosphodiesterase domain essential for intracellular fitness, while accessory effectors (XopC2, XopQ, XopX) target MAPK signaling and stomatal immunity.

4.3 Transcription Activator-Like Effectors (TALEs) and Plasmid Dynamics

Canonical TALEs possess an N-terminal translocation signal, nuclear localization signals, a C-terminal acidic activation domain, and a central repeat domain comprising tandem 34-amino-acid repeats. Residues 12 and 13 within each repeat—the repeat-variable diresidue (RVD)—dictate specific nucleotide recognition within promoter effector-binding elements (EBEs) of host susceptibility (*S*) genes.

In *X. citri* pv. *punicae*, full-length TALEs operate alongside truncated pseudo-TALEs. Truncated TALEs can function as dominant-negative decoys that block host immune receptors or act as recombination substrates. Because TALE and pseudo-TALE loci are flanked by IS elements and reside on both chromosomes and conjugative plasmids, they represent active drivers of genomic divergence and host adaptation.

Table 2. Major virulence effectors and island-associated targets in *Xanthomonas citri* pv. *Punicae*

Virulence Determinant	Structural / Biochemical Domain	Genomic Context & Organization	Biological Function	Primary References
Hrp/Hrc Injectisome	Membrane-spanning nanomachine; HrcN ATPase; HrpF	23–25 kb *hrp* pathogenicity island; bordered by mobile	Translocates virulence effectors into plant cells; control	Buttner (2002); Büttner & Bonas (2010);

	transloco n	eleme nts	led by PIP boxes	Scheib ner et al. (2017)
XopL	LRR domain & C-terminal XL-box E3 ubiquitin ligase	Conser ved chrom osoma l virulen ce island	Suppre sses PAMP-induce d ROS and host cell death to preserv e tissue	Büttne r (2016) ; Soni & Mond al (2018)
XopN	α-Helical repeat scaffold (ARM/H EAT-like motifs)	Chrom osoma l virulen ce island	Targets host plasma membr ane; inhibits early PTI signali ng	White et al. (2009) ; Kuma r et al. (2016)
AvrBs 2	Glycerop hosphory l diester phosphod iesterase domain	Conser ved core chrom osoma l locus	Suppre sses host recogni tion; stabiliz es outer envelo pe integrit y	White et al. (2009) ; Li et al. (2015) ; An et al. (2019)
TALE s / Pseud o-TALE s	Tandem 34-aa repeats (polymor phic RVDs) & AAD	Plasmi ds and chrom osoma l islands ; flanke d by IS eleme nts	Transcr iptional activati on of host suscept ibility genes; decoy suppres sion	Bogda nove et al. (2010) ; Grau et al. (2016) ; Radhi ka et al. (2021)
PilQ	Secretin barrel domain (Type IV pilus)	Chrom osoma l motilit y operon	Drives twitchi ng motilit y, biofilm formati on, and natural transfo rmatio n	Juhas et al. (2009) ; Dines hkuma r & Anton y (2022)

5. Translational Relevance: Antivirulence Targets, In Silico Drug Design, and Inhibitor Discovery

The emergence of antibiotic resistance and chemical phytotoxicity linked to conventional copper compounds and streptomycin formulations necessitates alternative therapeutic strategies. Because bacterial pathogenicity islands encode specialized machinery required for infection rather than basal viability, they provide attractive targets for antivirulence drug design. Antivirulence agents disarm pathogenic fitness without exerting direct bactericidal selection pressure, significantly attenuating the evolutionary drive toward drug resistance.

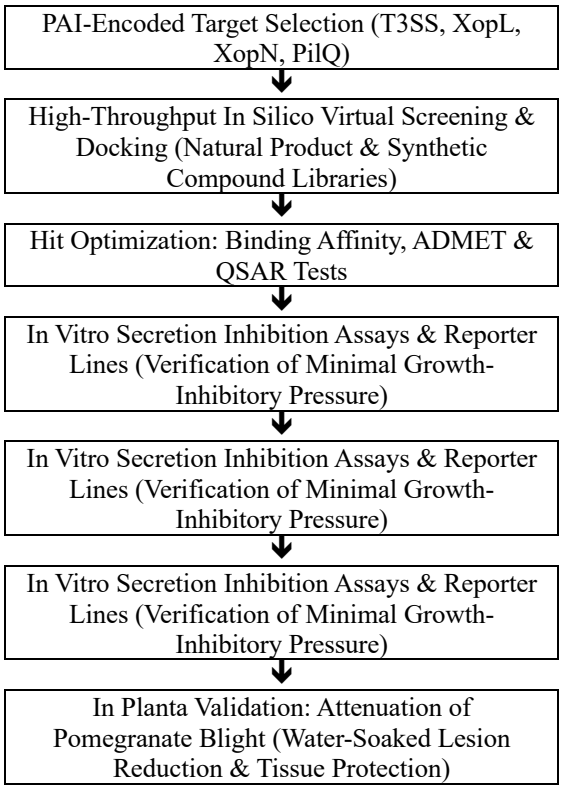


Figure 1. Antivirulence drug discovery workflow for Xanthomonas citri pv. punicae.

5.1 Structure-Guided Targeting of the Type III Secretion Injectisome

The T3SS apparatus represents an established target for antivirulence intervention across Gram-negative phytopathogens. Disrupting the machinery prevents the delivery of all downstream effector proteins (both Xops and TALEs) without killing the bacterium.

HrcN ATPase Inhibition: The cytoplasmic ATPase HrcN powers the unfolding and export of effector proteins through the inner membrane channel. High-throughput virtual screening targeting the conserved ATP-binding pocket enables the identification of competitive small-molecule inhibitors that halt injectisome function, analogous to small-molecule inhibitors validated against *Xanthomonas oryzae*.

Blockade of the HrpF Translocon: HrpF inserts into the host plasma membrane to form the translocation pore. Structure-based design of peptidomimetics or small hydrophobic molecules targeting the transmembrane or oligomerization interfaces of HrpF can prevent pore formation, neutralizing effector entry into pomegranate cells.

Transcriptional Disruption via HrpX and HrpG: Chemical interference with the HrpG-HrpX regulatory cascade prevents the transcriptional activation of the *hrp* regulon. Small molecules that destabilize the DNA-binding domain of the AraC-type activator HrpX prevent binding to PIP-box motifs, silencing the coordinated expression of pathogenicity island cargo genes.

5.2 Direct Inhibition of Effector Enzymes and Surface Secretins

Beyond the structural injectisome, enzymatic effectors and outer-membrane secretins represent viable docking targets:

XopL E3 Ubiquitin Ligase Antagonists: Because XopL possesses a distinct XL-box domain responsible for subverting host ubiquitination and suppressing cell death, molecular docking of focused chemical libraries can identify small molecules that occlude its catalytic cleft. Inhibiting XopL activity restores host ROS accumulation and triggers localized defense responses, restricting bacterial proliferation.

XopN Scaffold Perturbation: Computational modeling of the antiparallel α-helical HEAT/ARM-like repeats of XopN reveals surface grooves responsible for interacting with host plant membrane proteins. Small molecules or engineered cyclic peptides designed to occupy these interfaces prevent the effector from engaging host targets, neutralizing its immunosuppressive activity.

PilQ Secretin Inhibitors: Genome subtraction strategies have prioritized the type IV pilus secretin PilQ as a pathogen-specific, surface-exposed target. Disruption of the PilQ oligomeric ring by membrane-active compounds blocks twitching motility and surface adherence, reducing the initial epiphytic establishment of *X. citri* pv. punicae on pomegranate leaves and fruits.

5.3 Computer-Aided Drug Discovery (CADD) and Screening Pipelines

Modern in silico screening combines structural homology modeling (e.g., AlphaFold-multimer predictions), molecular dynamics (MD) simulations, and receptor-ligand docking across natural product and synthetic compound libraries. Pomegranate tissues are abundant in bioactive polyphenols, including ellagitannins (punicalagin, punicalin) and gallic acid derivatives, which possess documented antimicrobial properties. Molecular docking against Xap virulence proteins provides an avenue to isolate or chemically modify natural plant-derived scaffolds into potent antivirulence formulations. Validated hits can then be evaluated through in vitro T3SS-dependent secretion assays, followed by in planta challenge assays to confirm the reduction of water-soaked oily spots and nodal blight symptoms.

6. Conclusion

Horizontal gene transfer mediated by genomic islands, transposable elements, and conjugative plasmids plays a central role in shaping the virulence repertoire and ecological adaptation of *Xanthomonas citri* pv. punicae. High-contiguity long-read sequencing has resolved a multifaceted pathogenic architecture comprising an *hrp*/*hrc* T3SS core, critical non-TAL effectors such as XopL and XopN, and mobile plasmid-borne TALE repertoires. By moving beyond single-metric compositional predictions, future studies must integrate comparative genomics, AnnoTALE profiling, and targeted mutational assays to delineate authentic pathogenicity islands from core structures. Critically, translating these genomic and structural insights into structure-guided antivirulence drug discovery targeting T3SS components, Xop effectors, and secretins offers a sustainable paradigm to circumvent bactericide resistance and protect commercial pomegranate cultivation.

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