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Research article

Exploring multitasking proteins in *Xanthomonas* secretomes: Insights into mechanisms of plant-pathogen interactions

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ABSTRACT

Recent advances in large-scale functional genomic analysis have significantly increased interest in multitask proteins. The role of these proteins in *Xanthomonas* phytopathogens, a model for plant-pathogen interaction studies, remains largely underexplored. In this study, we introduce an innovative systematic comparative analysis of secretomes from 18 different Xanthomonas species, integrating data from multiple proteomic studies to identify potential multitasking proteins. This approach led to the identification of 93 proteins primarily involved in central metabolism that are secreted under various physiological conditions, including 16 previously characterized moonlighting proteins. Promiscuity analysis of five selected enzymes revealed that three (asparaginase, chorismate mutase, and phosphoenolpyruvate synthase) exhibit high potential for catalyzing reactions with non-canonical substrates, suggesting additional functional roles beyond their primary enzymatic activities. Additionally, we re-annotated previously hypothetical secreted proteins, assigning functions related to central metabolism and indicating a high potential for promiscuous activity. This comprehensive compilation of potential moonlighting and promiscuous proteins in Xanthomonas provides new insights into the molecular mechanisms driving plant-pathogen interactions and establishes a foundation for future experimental validations of these multifunctional proteins.

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1. Introduction

Xanthomonas are Gram-negative gamma-proteobacteria cause a wide range ofpathologies in both monocotyledonous and dicotyledonous plants. These bacteria are generally associated with canker, necrosis, or parenchymal and vascular disease affecting numerous socioeconomically important crops [1,2]. They are responsible for numerous plant diseases, such as bacterial spots on pepper and tomato plants (*X. campestris* pv. *vesicatoria*), black rot of crucifers (*X. campestris* pv. *campestris*), bacterial leaf blight of rice (*X. oryzae* pv. *oryzae*), bacterial leaf streak of rice (*X. oryzae* pv. *oryzicola*), and citrus canker (*X. axonopodis* pv. *citri*) [2].

During the invasion of plant tissue, *Xanthomonas* species transition from epiphytic or saprophytic organisms (capable of surviving outside the plant) to phytobacteria upon in contact with internal tissues of compatible hosts [3–5]. Consequently, *Xanthomonas* must adapt to the stressful conditions imposed by plants during early stages of infection [6]. This leads to the bacteria expressing genes associated with pathogenicity and virulence, including type III secretion system effectors (T3SSe), cell wall-degrading enzymes, and genes involved in biofilm formation and quorum sensing, among others [7]. Thus, if the microorganism is susceptible and unable to adapt within the plant, damage to multiple microbial molecules can inhibit replication or induce cell lysis, potentially resulting to cell death. However, some microorganisms can metabolize or inactivate reactive oxygen species (ROS), facilitating plant tissue colonization and disease induction [8]. This sophisticated repertoire of proteins, coupled with the ability to cause damage while evading the plant's immune system, enables phytopathogens to induce various diseases and harm agricultural products, highlighting their economic significance.

As structural and functional genomics continue to evolve, the identification and characterization of proteins responsible for tissue damage and the attenuation of plant defense responses have become increasingly efficient [9–13]. This detailed characterization of the effectome of these pathogens has significantly enhanced our understanding of interactions with both compatible and incompatible hosts [14–16]. However, beyond proteins traditionally associated with pathogenicity and virulence, multifunctional proteins (those that perform multiple physiological roles) have begun to garner significant attention [17,18]. These proteins can be classified into two categories: moonlighting proteins and promiscuous proteins [19]. Moonlighting refers to individual proteins capable of executing more than one function without resulting from gene fusions, splice variants, or post-translational modifications [20]. They are proteins capable of refer to those that can utilize multiple substrates or enzymes catalyzing multiple steps within the same metabolic pathway [21]. One of the first examples is the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primarily involved in glycolysis but also performs various additional functions such as transcription factor, signal transduction, cell death, and abiotic stress. These diverse roles exemplify its moonlighting capabilities [22–24]. A particularly intriguing secondary role of this protein is its ability to facilitate membrane fusion [25]. In contrast, promiscuous proteins exhibit a secondary activity within the active site responsible for the primary function. These enzymes have the capability to recognize multiple substrates and/or generate multiple products at a single active site [26].

Indeed, most moonlighting proteins are conserved metabolic enzymes, receptors, transcription factors, adhesins, or molecular chaperones with additional biological functions related to bacterial virulence or adaptation [27]. Some moonlighting proteins can perform both primary and secondary functions simultaneously, while others, change functions in response to environmental changes [27]. For example, enolase, a key enzyme in glycolysis responsible for converting 2-phosphoglycerate to phosphoenolpyruvate, is also secreted and associates with the cell surface where it functions as a plasminogen receptor, facilitating bacterial adherence and invasion of host tissues [38].

Moreover, moonlighting proteins are notable for exhibiting additional functions beyond their canonical roles, often associated with central carbon metabolism pathways, including glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway [30]. For instance, the flagellin (a structural protein of the flagellum [31,32]), elongation factor Tu (associated with the translation machinery [33,34]), chaperones (involved in protein refolding [35,36]), and even enzymes linked to energy metabolism [37,38], have been implicated in a broad spectrum of actions, including immunomodulation and the induction of host defense or virulence responses [28,39]. Surprisingly, not all homologous proteins exhibit moonlighting functions, making the study of these roles across different model organisms even more challenging [40].

In contrast, promiscuous enzymes exhibit a remarkable ability to adapt to changing genetic or environmental conditions by acquiring novel functions [41]. When subjected to genetic mutation or environmental alterations, these enzymes can be co-opted to catalyze reactions crucial for enhanced fitness or even survival [42]. This adaptive capacity underscores the dynamic nature of enzyme functionality and highlights its pivotal role in evolutionary processes [43]. Furthermore, the versatility of promiscuous enzymes provides a fascinating glimpse into the molecular mechanisms underlying biological adaptation and diversification. In phytopathogens, promiscuous enzymes can catalyze multiple reactions, enabling them to produce a broader range of metabolites that can be advantageous in the arms race against host plants [44]. Due to their physiological roles, these proteins become attractive targets for control through inhibitory substrates. For example, the necrosis- and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), which are known to induce necrosis and play a role in the virulence of various plant pathogens by triggering plant defense responses [45]. In this context, although proteins with promiscuous functions are less frequently reported than moonlighting proteins, both categories contribute significantly to the elusive 'dark matter' of biological systems.

The discovery of new moonlighting and promiscuous proteins often occurs serendipitously. To address this, several studies have focused on developing alternative search methodologies [46–60], and establishing specialized repositories for storing information associated with these proteins [61–64]. However, none of these approaches have specifically focused on pathosystems involving phytopathogens. In this study, we strive to advance systematic search methodologies for moonlighting proteins using the phytopathogen *Xanthomonas* as a model.

The primary objective of this study is to conduct a systematic comparative analysis of the available *Xanthomonas* secretomes studies to identify and characterize potential multitasking proteins, including moonlighting and promiscuous enzymes, with the goal of advancing our understanding of their roles in plant-pathogen interactions. Our approach is based on the premise that analyzing secretomes provides an effective strategy for uncovering proteins that are actively secreted during host-pathogen interactions, providing insights into their roles in virulence, adaptation, and interaction mechanisms with plant hosts. In this investigation, we selected 18 previously published secretome studies, each with a different *Xanthomonas* species, to compare protein categories exhibiting high expression under infection conditions. Our innovative systematic approach identified 93 proteins primarily related to *Xanthomonas* central metabolism that could potentially serve as novel moonlighting or promiscuous proteins within the context of plant-pathogen interactions.

2. Results

2.1. Identification of multitasking proteins in Xanthomonas secretomes

Upon conducting an initial search for publications involving proteomic analysis, we found that between 2005 and 2021, a total of 194 articles were published. Among these, 18 specifically focused on *Xanthomonas* secretome studies, encompassing various species and pathovars (Fig. 1A, Table 1). These studies employed diverse experimental conditions and methodologies, providing a broad overview of the secreted protein repertoire within the genus. By consolidating data from these studies, we aimed to identify proteins consistently secreted across multiple conditions and species, thereby highlighting potential multitasking proteins with fundamental roles in Xanthomonas biology.

2.2. Functional categorization and comparative analysis

In comparing and selecting potential moonlighting proteins across the 18 *Xanthomonas* secretome studies, we observed that the total number of secreted proteins identified in each study ranged from four to 424. These studies assessed proteins under diverse physiological conditions, including *in vitro* versus *in vivo* evaluations and mutant versus wild-type strains. Our comparative analysis categorized the proteins from these 18 studies into four functional categories (Fig. 1B and Table 2): cellular processes and metabolism (CP/M), virulence and adaptation (V/A), membrane-associated and transport (MA/T), and hypothetical proteins (HP). Across all secretome studies analyzed, the majority of identified proteins fell into the cellular processes and metabolism category.

Interestingly, these proteins, not typically expected to be secreted and lacking a signal peptide, are characteristic of moonlighting proteins. Of the 93 proteins identified in at least two studies, 62 (67 %) were classified in the CP/M category, 15 (16 %) in the V/A

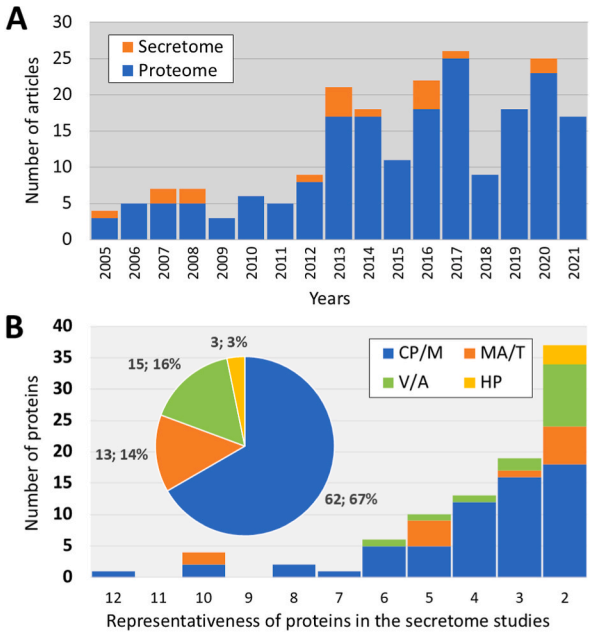


Fig. 1. **(A)** Total articles published involving *Xanthomonas* proteomics and secretome data between 2005 and 2021. (**B)** Representativeness of the proteins identified in the 18 secretomes based on the functional classification. The pie chart highlights the total number and the respective percentage of potential moonlight in each of the functional categories (total and percentage of representation). The histogram highlights how many times the proteins were identified in the secretomes evaluated, following the functional classification. CP/M: Cellular processes and metabolism; HP: Hypothetical protein; V/A: Virulence and adaptation; MA/T: Membrane-associated and transport proteins.

Table 1
General features and identification of the 18 selected secretomes.

#	Author	Strain	Experiment	Medium	Type	Approach	Proteins	Categories (%)			
								CP/M	HP	V/A	MA/T
A	Gonzáles 2012	Xoo XKK.12	*In planta* x control	PY	*In planta* secreted proteins (xylem sap)	LC-MALDI-MS/MS	64	42,19	26,56	12,50	18,75
B	Wang 2013	Xoo K3	*In planta* x *in vitro*	PSA x PS	*In planta* secreted proteins	2DE MALDI-TOF-MS	88	60,23	15,91	6,82	17,04
C	Xu 2013	Xoo ZJ173	*In vitro*	NB	Proteome database for Xoo	2DE MALDI-TOF/TOF-MS	19	78,95	5,26	0	15,79
			In vitro x *in planta*	Resistant (NA + PCA)	PCA-resistance proteins		54	74,07	7,41	9,26	9,26
D	Robin 2014	Xoo BAI3	*In vitro* (*hrpX** x wt)	PSA/NB	HrpX-regulated proteins	2D-DIGE LC-MS/MS	6	50	16,67	33,33	0
E	Chen 2016	Xoo PXO124	*In vivo* (rice embryo cell) x *in vitro*	PS	Xoo-secreted proteins	2D-DIGE MALDI-TOF/TOF-MS	4	0	75	25	0
F	Qian 2013	Xoc Rs105	*In vitro* (*rpfF* x wt)	NB	DSF-regulated extracellular proteins	2DE MALDI-TOF-MS	33	60,61	18,18	9,09	12,12
G	Watt 2005	Xcc B100	*In vitro*	M9	Cytosolic proteome	2DE MALDI-TOF-MS	28	78,57	14,29	3,57	3,57
					Extracellular proteome		65	56,92	13,85	9,23	20,00
					Periplasmic proteome		49	75,55	18,37	2,04	2,04
H	Chung 2007	Xcc 17 x Xcc 11A	*In vitro*	TYG	Virulent x avirulent strain	2DE MALDI-TOF-MS	22	77,27	13,64	9,09	0
I	Sidhu 2008	Xcc B100	*In vitro*	M9 x XVM2	OMV-associated protein	MALDI-TOF-MS and nano-LC-ESI-MS/MS	31	0	0	67,74	32,26
							22	0	0	59,09	40,91
J	Andrade 2008	Xcc ATCC 33913	*In planta* x control x *in vitro*	NYG	*In vivo* expression	2DE MALDI-TOF/TOF	14	100	0	0	0
K	Musa 2013	Xcc B100	*In vitro*	M9	*Xanthomonas* phosphoproteome	2D-PAGE MALDI-TOF/TOF-MS and LC-ESI-MS	6	100	0	0	0
L	Ferreira 2016	Xac 306	*hrpB4* x wt	NB and XAM1	Xac-secreted proteins	nLC-ESI-UPLC-MS/MS	55	41,82	23,64	20	14,54
M	Artier 2016	Xac 306	*In vitro*	NB x XAM-M	Periplasmic-enriched proteome	2DE LC-MS/MS	18	77,78	11,11	5,55	5,55
							33	63,64	15,15	12,12	9,09
N	Carnielli 2016	Xac 306	*In planta* x *in vitro*	NB	Surface proteins	2D-DIGE and LC-ESI-MS/MS	36	47,22	13,89	2,78	36,11
O	Mi 2007	*Xanthomonas*	*In vitro* (wt x *hrp* mutants)	*hrp*-inducing XVM2	Interaction proteome of Hrp proteins	1-DE and MALDI-TOF-MS	13	30,77	0	69,23	0
P	Wang 2017	Xoo K3	*In planta* x *in vitro*	PSA	Xoo secreted proteins	SDS-PAGE and MudPIT	424	46,69	30,2	8,49	14,62
Q	Zandonadi 2020	XAC XauB	*In vitro (*XAC x XauB*)*	XAM-M	Periplasmic-enriched proteome	2DE LC-MS/MS	67	71,66	10,44	2,98	14,92
R	Wang 2020#	Xoo K3	*In vitro (wt x* PXO_0317*)*	NB	Glycosylated Outer Membrane Proteins	SDS-PAGE and nLC-MS	13	0	0	23,08	76,92

#Paper identification; *Ectopically expression; **Host x non-host interaction; PY – Peptone Yeast; PSA – Peptone Sucrose Agar; TYG – Tryptone Yeast Glucose; NYG – Nutrient Yeast Glycerol; CP/M – Cellular processes and metabolism; HP – Hypothetical protein; V/A – Virulence and adaptation; MA/T – Membrane-associated and transport proteins. Each protein was only considered once even though it may occur at several positions on the gel. # Differential proteins had their expression altered by glycosylation.

category, 13 (14 %) in the MA/T category, and three (3 %) in the HP category (Fig. 1B). As anticipated, most proteins exhibited low representation across the secretomes investigated due to variability in global expression profiles and physiological conditions. However, at least eight proteins were identified in six or more secretomes, indicating a low representation variability across the different secretomic profiles. Examples include chaperonins (DnaK, GroEL, GroES), elongation factor Tu (TufA and TufB), ATP synthase subunits, ribosomal proteins, malate dehydrogenase, aminopeptidase (PepA and PepN), superoxide dismutase (SodM), phosphoglucomutase/phosphomannomutase (XanA), and xylose isomerase (XylA) in the CP/M category; outer membrane proteins and TonB-dependent receptors in the MA/T category; and cellulase (CelS, EngXCA, Egl) in the V/A category.

These findings suggest that proteins involved in cellular processes and metabolism are not only central to the basic physiology of Xanthomonas but also possess additional roles that may contribute to pathogenicity and adaptation. The identification of these proteins across multiple secretomes underscores their potential as multitasking proteins with conserved functions essential for host interaction and survival under varying environmental conditions.

2.3. Revisiting characterized moonlighting proteins in Xanthomonas

Similar to other models, certain proteins exhibiting moonlighting function have already been identified in *Xanthomonas* (Table 3). For example, proteins associated with energy metabolism and tryptophan metabolism have been linked, either directly or indirectly, to pathogenicity and virulence. Furthermore, the majority of these proteins have some degree of association with host-plant interactions based on their non-canonical function. This indicates that multifunctional proteins play significant roles in the adaptability and virulence of Xanthomonas, highlighting their importance in plant-pathogen interactions and presenting potential targets for controlling bacterial diseases in crops.

2.4. Structural and promiscuity analysis of selected proteins

Five proteins previously identified as potential moonlighting proteins were selected for promiscuity analysis of their active sites: three enzymatic (asparaginase, chorismate mutase, and phosphoenolpyruvate synthase) and two non-enzymatic proteins (elongation factor Tu, Ef-Tu, and chaperonin 60). As expected, Ef-Tu and chaperonin 60 were inadequate for promiscuity site analysis due to the absence of evident catalytic sites, contrasting with the enzymatic proteins. Despite the absence of evident catalytic sites, Ef-Tu and chaperonin 60 were included in the promiscuity analysis due to their recognized multifunctional roles in bacterial physiology and pathogenicity [65,66]. This inclusion aimed to explore potential non-catalytic multitasking functions that may not involve traditional enzymatic promiscuity.

The asparaginase (XAC3092 GenBank accession AAM37937.1) from *Xanthomonas* presents the highest identity (61 %, and similarity 74 %) with solved protein structures from *Elizabethkingia* (PDB 1P4K) [75] (Fig. 2A). The active site of this enzyme comprises Trp11/Asn151/Thr203 [67], where the Asp151Asn substitution completely abolishes catalysis [68]. In the *Xanthomonas* asparaginase, CLASP (CataLytic Active Site Prediction) software was utilized to identify the corresponding triad - Trp17/Asn171/Thr223 [69]. In addition to the canonical asparaginase catalytic triad, CLASP identified other potential active site residues based on structural similarities to active sites from other enzyme types. Fig. 2B shows the top 10 protein matches using three and four residues as queries. The *Escherichia coli* uracil DNA glycosylase (PDBid:1EUG) shows close structural similarity of the active site residues (Fig. 2C). Both are hydrolases, suggesting some similarity in their catalytic activities. Interestingly, in some of investigated proteins (Fig. 2B), the same potential catalytic residues associated with promiscuous functions in Xanthomonas were identified, indicating that these residues may facilitate the binding or catalysis of multiple substrates, thereby suggesting the enzyme's ability to perform diverse biochemical reactions. Potential catalytic residues with promiscuous function in *Xanthomonas* were identified, such as phosphatidylcholine and NS3 protease (three residues query), and muconate cycloisomerase, chloromuconate cycloisomerase and mandelate racemase/muconate lactonizing (four residues query) (Fig. 2D). Thus, the identification of additional potential catalytic residues (associated with other enzymatic functions) located within the same pocket as the canonical triad in *Xanthomonas asparaginase* strongly suggests the possible functional promiscuity of this enzyme.

Based on these findings, we attempted to inactivate this gene to understand its involvement in *in vitro* growth and potential involvement with virulence reduction in *Xanthomonas citri* pv. *citri* using *Citrus sinensis* plants. Despite successful gene deletion (Fig. 2E), no change in the growth profile (Fig. 2F), or significant reduction in the virulence phenotype *in vivo* was observed (Fig. 2G and Supplementary Fig. S1). This indicates that while the asparaginase has the potential for promiscuous activity based on active site analysis, its role in virulence and growth under the tested conditions may be redundant or compensated by other proteins. This redundancy suggests that multiple proteins may collaborate or compensate for each other's functions, ensuring the robustness of Xanthomonas pathogenic mechanisms. Future studies could explore double or multiple gene knockouts to assess potential compensatory effects and further elucidate the functional significance of asparaginase in Xanthomonas biology.

For chorismate mutase, the closest PDB structure to the *Xanthomonas* is from *Burkholderia phymatum* (PDB:5TS9, 47 % identity, 57 % similarity), followed by *Mycobacterium tuberculosis* (PDB:2AO2, 28 % identity, 46 % similarity) (Fig. 3A). Despite significant sequence divergence, the structures are highly conserved (Fig. 3B). The reported active site in *Mycobacterium tuberculosis* consists of Arg49/Lys60/Glu106, while in *Burkholderia*, it consists of Arg34/Lys45/Gln94. The corresponding triad (Arg44/Lys55/Gln104) using CLASP shows almost exact spatial superimposition in *Xanthomonas* (Fig. 3C). Fig. 3D shows the top 10 protein matches using three and four residues as queries. These structural similarities suggest potential convergent evolution of active site configurations, which may underline promiscuous activities, although experimental validation is necessary to confirm such functions. This convergence highlights how different organisms can evolve similar catalytic mechanisms independently, potentially enabling similar multifunctional

Table 2
Categorization of proteins from the 18 secretome studies.

CAT	PROTEINS	ARTICLES																	Rep	MP	TOTAL	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R			Sec.	Prot.
CP/M	*Chaperonin (DnaK, GroEL, GroES)	X	X	X				X	X		X		X	X	X	X	X	X		12	Y	12	23
CP/M	Elongation factor Tu (TufA and tufB)	X	X	X				X	X		X		X	X	X			X		10	Y	10	15
CP/M	*ATP synthase alpha/beta chains(AtpA and AtpD)	X	X	X			X	X			X			X	X		X	X		10	N	10	13
CP/M	*Peptidyl-prolyl cis-trans isomerase (SurA and FkpA)	X	X	X				X	X				X	X			X			8	Y	9	17
CP/M	*50S ribosomal proteins	X	X					X	X				X		X		X	X		8	Y	8	14
CP/M	*30S ribosomal proteins	X	X	X				X					X		X		X			7	Y	7	12
CP/M	Malate dehydrogenase (Mdh)		X	X			X	X	X						X					6	Y	6	6
CP/M	Aminopeptidase (PepA and PepN)			X				X	X				X	X			X			6	Y	6	7
CP/M	Superoxidase dismutase (SodM)	X	X				X	X						X			X			6	N	5	5
CP/M	Phosphoglucomutase/phosphomannomutase (XanA)			X				X				X		X			X	X		6	Y	4	6
CP/M	Xylose isomerase (XylA)	X	X	X				X									X	X		6	N	4	6
CP/M	Elongation factor Ts and G (Tsf)		X					X			X			X	X					5	N	5	5
CP/M	Aspartate semialdehyde dehydrogenase (Asd)		X					X	X		X						X			5	N	4	5
CP/M	ATP-dependent Clp protease proteolytic subunit		X					X						X	X		X			5	N	4	5
CP/M	Glyceraldehyde-3-phosphate dehydrogenase (GapA)		X	X				X									X	X		5	Y	4	5
CP/M	Cysteine protease	X					X	X					X				X			5	Y	4	5
CP/M	Alanyl dipeptidyl peptidase	X		X				X						X						4	N	4	4
CP/M	Enolase (Eno)		X					X			X	X								4	Y	4	4
CP/M	Flagellar L-ring protein (FlgG, FlgH and FliD)	X					X	X					X							4	N	4	4
CP/M	Flagellin (FliC)	X	X				X						X							4	N	4	4
CP/M	1,4-beta-glucosidase		X	X				X									X			4	N	3	4
CP/M	ABC transporter sulfate binding protein (Sbp)						X	X						X			X			4	N	4	4
CP/M	Adenylate kinase (Adk)				X			X							X			X		4	N	4	4
CP/M	Fructose-bisphosphate aldolase (Fba)							X				X			X		X			4	N	4	4
CP/M	Phosphom./GDP-mannosep. (XanB)							X			X				X			X		4	N	4	4
CP/M	Transketolase 1 (TktA)			X				X						X			X			4	N	4	4
CP/M	*DNA-binding related protein	X	X														X	X		4	N	4	4
CP/M	Pyruvate kinase type II		X											X			X	X		4	Y	4	4
CP/M	Dihydrolipoamide succinyltransferase (SucB)	X	X	X																3	N	3	3
CP/M	Glutamine synthetase (GlnA)	X						X							X					3	Y	3	3
CP/M	Inorganic pyrophosphatase (Ppa)							X	X					X						3	N	3	3
CP/M	*Oxidoreductase			X				X	X											3	Y	3	4
CP/M	Phosphoglycerate kinase (Pgk)			X				X			X									3	Y	3	3
CP/M	Ribokinase (RbsK)						X	X						X						3	N	3	3
CP/M	RNA polymerase alpha chain (RpoR)		X					X							X					3	N	3	3
CP/M	Two-comp. regulatory protein (PhoA, PhoP)		X	X				X												3	N	3	4
CP/M	3-isopropylmalate dehydrogenase (LeuB)		X					X									X			3	N	3	3
CP/M	Alkaline phosphatase		X				X										X			3	N	3	3
CP/M	Alkyl hydroperoxide reductase subunit C (AhpC)			X				X									X			3	N	3	3
CP/M	Citrate synthase (GltA)							X			X						X			3	Y	3	3
CP/M	Fimbrial assembly protein (PilQ)	X		X													X			3	N	3	3
CP/M	*Serine protease						X						X				X			3	Y	3	5
CP/M	Succinyl-CoA synthetase subunit alpha			X										X				X		3	Y	4	4
CP/M	Transaldolase (Tal, TalB)							X				X					X			3	N	3	3
CP/M	2-h-2,4-d-1,7-d.5-carbox.-2-oxo-hex-3-ene-1,7-dioat.		X					X												2	N	2	2
CP/M	3-isopropylmalate dehydratase (LeuD)		X					X												2	N	2	2
CP/M	Adenylosuccinate synthetase (PurA)						X	X												2	N	2	2

(*continued on next page*)

Table 2 (*continued*)

CAT	PROTEINS	ARTICLES																	Rep	MP	TOTAL	
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R			Sec.	Prot.
CP/M	DNA-directed RNA polymerase alpha chain		X								X									2	N	2	2
CP/M	Electron transfer flavoprotein alpha subunit (EftA)		X					X												2	N	2	2
CP/M	Enolase-phosphatase		X											X						2	N	2	2
CP/M	Fumarylacetoacetate hydrolase (UptA)						X		X											2	Y	2	2
CP/M	Glutaredoxin-like protein		X					X												2	N	2	2
CP/M	Organic hydroperoxide resistance protein (Ohr)		X						X											2	N	2	2
CP/M	Phosphoenolpyruvate synthase (PpsA)							X				X								2	N	2	2
CP/M	Phosphoglycerate mutase (Gpm)		X					X												2	Y	2	2
CP/M	Polynucleotide phosphorylase/polyadenylase		X	X																2	N	2	2
CP/M	Polyvinyl alcohol dehydrogenase							X					X							2	N	2	2
CP/M	Septum site-determining protein (MinD)			X				X												2	N	2	2
CP/M	Thioredoxin (Trx)							X			X									2	Y	2	2
CP/M	Type II citrate synthase			X			X													2	Y	2	2
CP/M	Type IV pilus assembly protein (PilY1)		X										X							2	N	2	2
CP/M	UTP-glucose-1-phosphate uridylyltransferase (GalU)		X					X												2	N	2	2
HP	Conserved hypothetical protein							X	X											2	Y	2	2
HP	Hypothetical protein												X	X						2	N	2	2
HP	Hypothetical protein (HrcC)	X	X																	2	N	2	2
MA/T	*Outer membrane proteins		X	X			X	X		X			X	X	X		X		X	10	Y	10	26
MA/T	*TonB-dependent receptors	X	X	X			X	X		X					X		X	X	X	10	N	9	17
MA/T	*Ferric enterobactin receptor (BfeA)		X	X				X							X		X			5	N	4	5
MA/T	*Oar protein		X					X		X					X					5	N	4	6
MA/T	*OmpA-related protein	X	X					X							X				X	5	N-	4	6
MA/T	Polyphosphate-selective porin O (OprO)	X		X			X						X					X		5	N	4	5
MA/T	Peptidoglycan-associated outer memb. lipop. (Pcp)	X											X						X	3	N	3	3
MA/T	*OmpA family protein	X											X							2	–	2	2
MA/T	*Outer membrane antigen		X												X					2	N	2	2
MA/T	*Outer membrane hemin receptor (PhuR)							X						X						2	N	2	2
MA/T	Outer membrane protein (XadA)	X	X																	2	N	2	3
MA/T	TonB-like protein	X		X																2	N	2	2
MA/T	Translocation protein (TolB)			X				X												2	N	2	2
V/A	*Cellulase (CelS, EngXCA, Egl)			X				X	X	X			X				X			6	N	6	10
V/A	Periplasmic protease (MucD)	X						X						X	X		X			5	N	4	5
V/A	VirK protein (VirK)	X	X										X				X			4	N	4	4
V/A	Lipase esterase (EstA, LipA)	X				X		X												3	N	3	3
V/A	Lytic murein transglycosylase (Mlt, MltB)												X	X				X		3	N	3	3
V/A	Avirulence protein									X						X				2	N	3	7
V/A	*Extracellular protease						X						X							2	N	2	2
V/A	Hypersensitive response secretion protein (HrpA1)		X													X				2	N	3	3
V/A	Lipoprotein (VacJ, RlpA, RlpB)									X			X							2	N	2	3
V/A	Polygalacturonase			X			X													2	N	2	2
V/A	Regulator of pathogenicity factors (RpfN)		X										X							2	N	2	2
V/A	TldD protein (TldD)							X						X						2	N	2	2
V/A	Toluene tolerance protein (yrbC)				X								X							2	N	2	2
V/A	Two-component system regulatory protein (ColR)	X		X																2	Y	2	2
V/A	Xanthomonas adhesion (XadA1)		X							X										2	N	2	2

CAT – Categories; CP/M – Cellular processes and metabolism; HP – Hypothetical protein; V/A – Virulence and adaptation; MA/T – Membrane-associated and transport proteins; MP – Previously described as moonlight protein; Sec – Number of secretomes they are found; Prot – Number of proteins. * Proteins that have pairs of orthologous paralogs.

Table 3
Proteins described as moonlighting in Xanthomonas. Ref: PubMed IDs from the NCBI database.

Protein	Product	Canonical function	Moonlight function	Specie	Ref	Secretomes (n = 18)
Pgk	Phosphoglycerate kinase	Glycolysis enzyme	Required for pathogenicity	Xoo Xoo*	18943766 17981946	3
GapA	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis enzyme	Required for EPS production and full pathogenicity	Xcc Xoo*	19372163 17981946	5
DsbD	C-type cytochrome biogenesis protein	Cytochrome biogenesis	Required for pathogenesis	Xcc	28655353	NI
FbaB	Fructose-bisphosphate aldolase	Glycolysis enzyme	Play critical roles in coordinating *hrp* gene expression	Xoo* Xoo*	22384086 17981946	4
Gk	Glucose kinase	Glycolysis enzyme	Required for extracellular polysaccharide production	Xcc	18048941	NI
PpsA	Phosphoenolpyruvate synthase	Gluconeogenesis enzyme	Required for virulence	Xcc Xoo*	16109965 17981946	2
DsbB	Disulfide bond formation protein B	Disulfide bond formation in some periplasmic proteins	Required for pathogenicity	Xcc	18616400	NI
AroQ	Chorismate mutase	Tryptophan metabolism	Required for virulence	Xoo Xoo*	20128700 17981946	NI
AroE	Shikimate dehydrogenase	Tryptophan metabolism	Required for pigment production and virulence	Xoo	11133452	NI
Idh	Isocitrate dehydrogenases	TCA enzyme	Required for virulence	Xcc	27282849	NI
Hfq	RNA chaperone	Protein folding	Required for virulence, motility and stress tolerance	Xcc	29901272	NI
FliC	Flagellin	Synthesis of flagella	Required for host–pathogen interactions	Xoo	25187853	4
EftA	Elongation factor Tu	Protein biosynthesis	Required for enhancement of plant innate immune system	Xoo	25358295	10
Pgi	Phosphoglucose Isomerase	Glycolysis enzyme	Required for pathogenicity	Xoo	18943766	NI
PurH	Bifunctional purine biosynthesis protein	Purine metabolism	Required for virulence	Xoo	16175206	NI
Hlp	HU-like protein	Global transcriptional regulators	Required for virulence	Xcc	34424610	4

NI – Not identified; Xoo – *Xanthomonas oryzae* pv. *oryzae*; Xoo* – *Xanthomonas oryzae* pv. *oryzicola*; Xcc – *Xanthomonas campestris* pv. *campestris*.

capabilities in their respective proteins.

Finally, analysis of the phosphoenolpyruvate synthase (PpsA) structure revealed a high degree of superposition with the rifampin phosphotransferase protein from *Listeria monocytogenes* (PDB:5HV6) (Fig. 4A). When evaluated for the conservation of its catalytic sites, PpsA from *Xanthomonas* showed a high degree of conservation with pyrophosphate-dependent phosphofructokinase from *Borrelia burgdorferi* (PDB:1KZH). Despite no sequence or structural similarity, this high degree of conservation suggests a potential scenario of convergent evolution (Fig. 4B). This observation indicates that PpsA may possess promiscuous catalytic capabilities, allowing it to interact with non-canonical substrates, although further experimental studies are required to substantiate this hypothesis. The identification of such convergent features may inform future studies aimed at understanding enzyme versatility and potential cross-reactivity in biochemical pathways.

2.5. Reannotation and structural characterization of hypothetical proteins

Three hypothetical proteins were identified in at least two of the secretomes investigated (XOO0094, XCC4094, and XAC0868). Reannotation of these genes identified XOO0094 as the *hrcQ* gene, which encodes a protein essential for the type III secretion system [70]. XCC4094 is a hypothetical conserved protein widely distributed among several genera, with a signal peptide (1–31, SEC/SPI – score 0.9991) and a SYLF domain (DUF500 between residues 37 and 232), potentially related to lipid metabolism (Fig. 5A). Structural analysis revealed that the C-terminal portion, which lacks any known domain, presented high error rates during conformational prediction, complicating reliable partial folding (Fig. 5B). Superposition analysis of this protein with a reference lipoprotein structure from *Burkholderia pseudomallei* (PDB 7OFN), showed reliable overlap indices (P-value = 1.82e-04, raw FATCAT score = 171.37, and RMSD = 3.17 Å without conformational twists) (Fig. 5C). Although XCC4094 is structurally similar to a lipoprotein, the signal peptide predictions by SignalP of XCC4094 orthologs in other *Xanthomonas* were not classified as a lipoprotein signal. This suggests that XCC4094 may also act as a membrane lipoprotein, supporting its identification in associated secretomes. Experimental studies will be necessary to determine the protein's true localization and function.

Reannotation of XAC0868 revealed the presence of a signal peptide/lipoprotein signal peptide sequence (1–21, SEC/SPI – score 0.5219 and SEC/SPII – score 0.477), along with two BULB-Lectin domains (29–155 and 160–269) (Fig. 5D), both with low conformational error estimates (Fig. 5E). When evaluated for overlap with a reference *Pseudomonas* LLPA bacteriocin structure (PDB 3M7H), the overlap indices were highly reliable (P-value = 6.20e-10, raw FATCAT score = 414.71, and an RMSD of 2.67 Å with 1 twist) (Fig. 5F). Interestingly, unlike XCC4094, this protein is primarily found in *Xanthomonas* species that infect citrus, with lower representation in nine other species, making it relatively restricted to certain *Xanthomonas* genomes (Fig. 5G). This restricted distribution suggests a specialized role in specific host-pathogen interactions, potentially linked to its promiscuous functionality in lipid

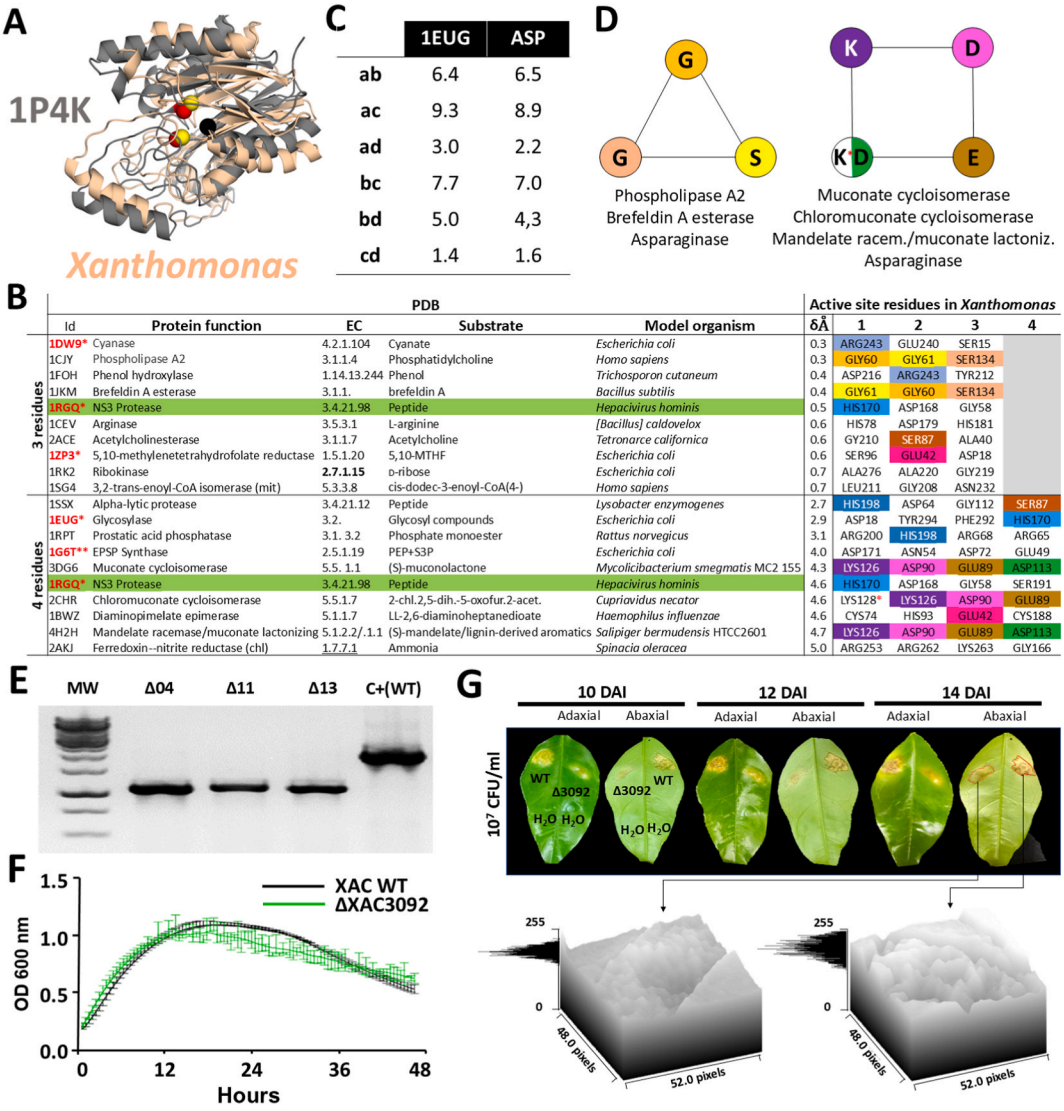
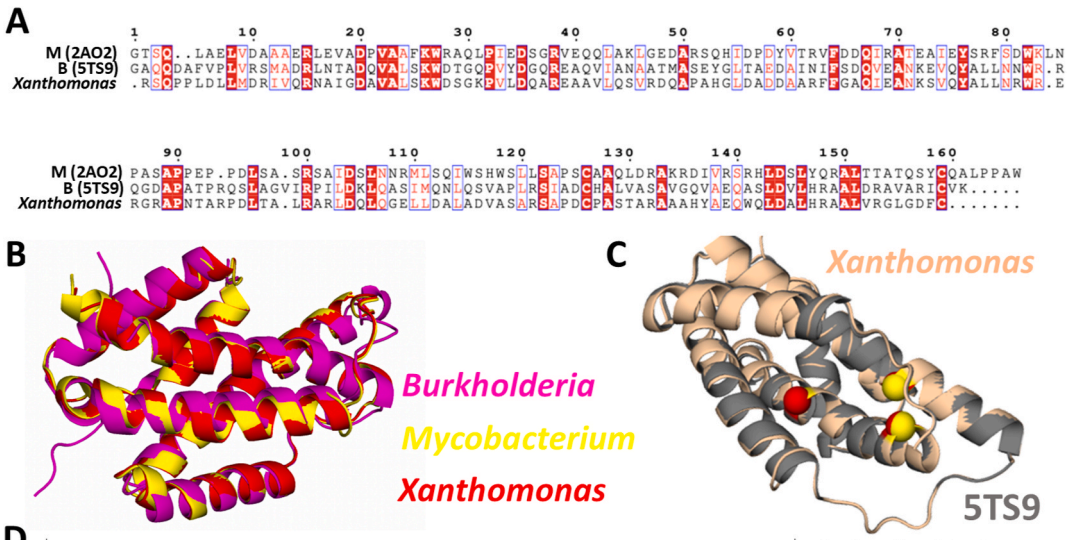


Fig. 2. Analysis of the glycosylase promiscuity potential of Asparaginase. **(A)** Superimposition of asparagine proteins based on active sites of asparaginase from *Elizabethkingia meningoseptica* (PDB 1P4K). Black sphere (Trp11), upper spheres (Asn151), lower spheres (Thr203). **(B)** Predicted active site residues in asparaginase for promiscuous activities using the Catalytic Site Atlas (CSA) database: *Xanthomonas* asparaginase catalytic residues are Trp11/Asn151/Thr203. Those with similar characteristics are marked with *, while those having at least one exact match residue are marked with **. gLines highlighted in green identify the same reference proteins in the analyses for 3 or four residues. The background colors in the cells of the catalytic residues identify the same residues obtained for different reference proteins. **(C)** The active site residues given in CSA for 1EUG are Asp64/Tyr66/Phe77/His187 (a/b/c/d). CLASP detected homologous residues are Asp18/Tyr294/Phe292/His170 (a/b/c/d). The pairwise distances (Å) in these protein structures demonstrate structural homology. **(D)** Conservation profile of 3 or 4 amino acid residues of *Xanthomonas* catalytic site for different reference proteins. The letters inside the circles correspond to single-letter abbreviations referring to the amino acid identified in B. **(E)** Electrophoresis in a 0.8 % agarose gel confirms deletion of the *aspG* gene (XAC3092). MW: molecular weight (1 Kb ladder), Δ4-11-13 (three mutant clones with deletion of the XAC3092 gene), C+: positive control (intact XAC3092). **(F)** *In vitro* growth curves of wild-type and XAC3092 mutant. The lines denote the mean of the experiments performed in triplicate, while the vertical bars indicate the standard deviation for each data point. **(G)** Analysis of the *in vivo* virulence profile in *Citrus sinensis* plants. DAI: days after inoculation; CFU: colony forming unit. At 14 DAI the injured areas were established and the surface structural profile was characterized using imageJ [158].

metabolism or bacteriocin activity. The association with citrus-infecting species may indicate an adaptation to specific host environments, highlighting the evolutionary diversification of multitasking proteins in response to host-specific pressures.

2.6. *Investigating protein moonlighting in various biological models*

As anticipated, the functional complexity of proteins with moonlighting function was made explicit in the literature search for



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	PDB Id	Protein function	EC	Substrate	Model organism	Active site residues in *Xanthomonas* δÅ	1	2	3	4
3 residues	1J49	D-Lactate-dehydrogenase	1.1.1.28	D-lactate	*Lactobacillus delbrueckii*	0.8	ARG44	ASP39	GLU138	
	12AS	Asparagine synthase	6.3.5.4	L-aspartate + L-glutamine	*Escherichia coli*	1.6	ASP57	ARG112	GLN104	
	1LTQ	Polynucleotide kinase	2.7.1.78	5'-dephospho-DNA	Tequatrovirus T4	1.6	LYS55	ASP64	ARG129	
	1EZF	Farnesyl-diphosphate farnesyltransferase	2.5.1.21	t-Farnesyl diphosphate	*Homo sapiens*	1.7	TYR105	ARG44	ARG182	
	1P4N	UDP-MurNAc-pentapeptide	2.7.8.13	UDP-Mur2Ac+undecaprenyl P	*Staphylococcus aureus*	1.7	LYS101	ASP57	ARG44	
	1MYR	Myrosinase	3.2.1.147	glucosinolates	*Sinapis alba*	1.8	ARG116	GLN104	SER102	
	1MRQ	Aldo-keto reductase family 1 member C1	1.1.1.300	Carbonyl-containing compounds	*Homo sapiens*	2.0	ASP49	TYR105	LYS55	
	1EC9	Glucarate dehydratase	4.2.1.40	D-glucarate	*Escherichia coli*	2.3	LYS60	LYS55	ASP57	
	1ZIO	Adenylate kynase	2.7.4.3	ATP + AM	*Geobacillus stearothermophilus*	2.3	LYS101	ARG44	ARG110	
	1PIX	Glutaconyl-CoA decarboxylase A subunit	7.2.4.5	(2E)-4-carboxybut-2-enoyl-CoA	*Acidaminococcus fermentans*	2.4	ASP64	LYS60	LYS55	
4 residues	1G99	Acetate kinase	2.7.2.1	Acetate	*Methanosarcina thermophila*	4.5	SER102	ARG44	ASP39	GLY185
	12AS	Asparagine synthase	6.3.5.4	L-aspartate + L-glutamine	*Escherichia coli*	5.0	ASP57	ARG112	GLN104	ASP64
	1MYR	Myrosinase	3.2.1.147	glucosinolates	*Sinapis alba*	5.2	ARG116	GLN104	SER102	ASP09
	3KB9	Epi-isozizaene synthase	4.2.3.37	(2E,6E)-farnesyl diphosphate	*Streptomyces coelicolor*	5.5	PHE92	PHE93	PHE187	ARG44
	1GTK	Porphobilinogen deaminase	2.5.1.61	4 porphobilinogen	*Escherichia coli*	6.0	LYS55	ASP57	ARG112	ARG110
	1B6T	Phosphopantetheine adenylyltransferase	2.7.7.3	4'-Phosphopantetheine	*Escherichia coli*	6.1	HIS176	LYS55	ARG182	SER102
	1JCH	Colicin E3	NI	Polypeptide toxins	*Escherichia coli*	6.5	ASP141	HIS82	GLU138	ARG44
	1RBA	RUBISCO	4.1.1.39	3-phospho-D-glycerate	*Rhodospirillum rubrum*	8.2	LYS55	LYS101	ASN100	HIS176
	1URO	Uroporphyrinogen decarboxyase	4.1.1.37	uroporphyrinogen III	*Homo sapiens*	8.3	ARG44	ARG131	ARG129	ASP49
	1GSA	Glutathione synthetase	6.3.2.3	L-glutamyl-L-cysteine + glutamine	*Escherichia coli*	8.5	LYS101	LYS55	ARG44	ARG131

Fig. 3. Promiscuity potential analysis in chorismate mutase (CM). **(A)** Multiple sequence alignments of CM from *Mycobacterium* (M), *Burkholderia* (B) and *Xanthomonas*. The active site in *Mycobacterium tuberculosis* consists of different residues (PDB 2AO2: Arg49/Lys60/Glu106) than that reported in *Burkholderia phymatum* (PDB 5TS9: Arg34/Lys45/Gln94). **(B)** Superposition of *Burkholderia*, *Mycobacterium* and *Xanthomonas* structures showing high structural conservation in spite of sequence divergence. **(C)** Superimposition of predicted *Xanthomonas* chorismate mutase structure with the CM from *Burkholderia phymatum* (PDB:5TS9) based on active sites (Active2 results) in the corresponding triad (Arg44/Lys55/Gln104) using CLASP, with almost exact spatial superimposition in *Xanthomonas*. Superposition of the active sites aligns the complete proteins as well. **(D)** Predicted active site residues in CM for promiscuous and moonlighting activities using the Catalytic Site Atlas database: These are all selected to have at least one residue from the active site (Arg44/Lys55/Gln104). D-lactate dehydrogenase from *Lactobacillus bulgaricus* (PDB:1J49) is a good match, suggesting a similar function for CM.

empirically identified roles of such proteins. For instance, chaperonins, besides their classic role in protein folding, were shown to serve as secreted effectors, membrane receptors, modulators of cytotoxicity, and participate in cell adhesion. A summary of these functions for other moonlighting proteins is provided in Table 4. Notably, most of these proteins, in addition to their canonical function, have the capability to act as Pathogen-Associated Molecular Patterns (PAMP), thereby triggering virulence or avirulence responses in compatible hosts. This multifaceted functionality underscores the versatility of moonlighting proteins in modulating host-pathogen interactions and highlights their potential as targets for therapeutic interventions aimed at disrupting these critical processes.

3. Discussion

Beyond the empirical characterization of moonlighting functions - often identified serendipitously - numerous in silico research endeavors have aimed to identify other multitasking proteins [46–60]. These efforts have systematically enhanced our structural understanding of these proteins. However, no methodology to date has proposed to identify these proteins through a systematic evaluation of secreted protein repertoires derived from a comparative secretome analysis. This positions our proposal as a pioneering approach that can potentially be implemented across other pathosystems. We selected *Xanthomonas* as a model in this study because it represents one of the bacterial genera causing the most widespread damage to a variety of agricultural crops [71–73] and has been extensively studied genetically and functionally [74,75] (Fig. 6A).

A comprehensive examination of secretomes from various bacteria can yield pivotal biological insights that guide future experimental decisions. As proposed by Jeffery, the ability of a single protein to fulfill multiple functions and participate in different multi-

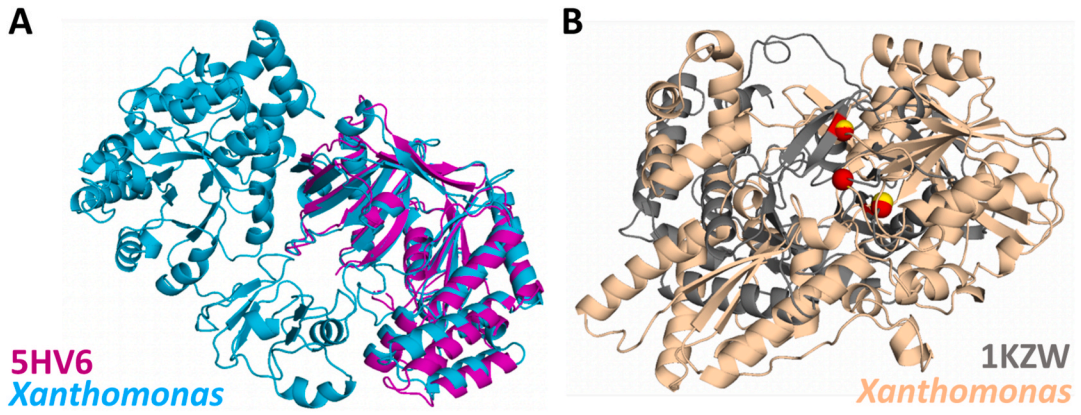


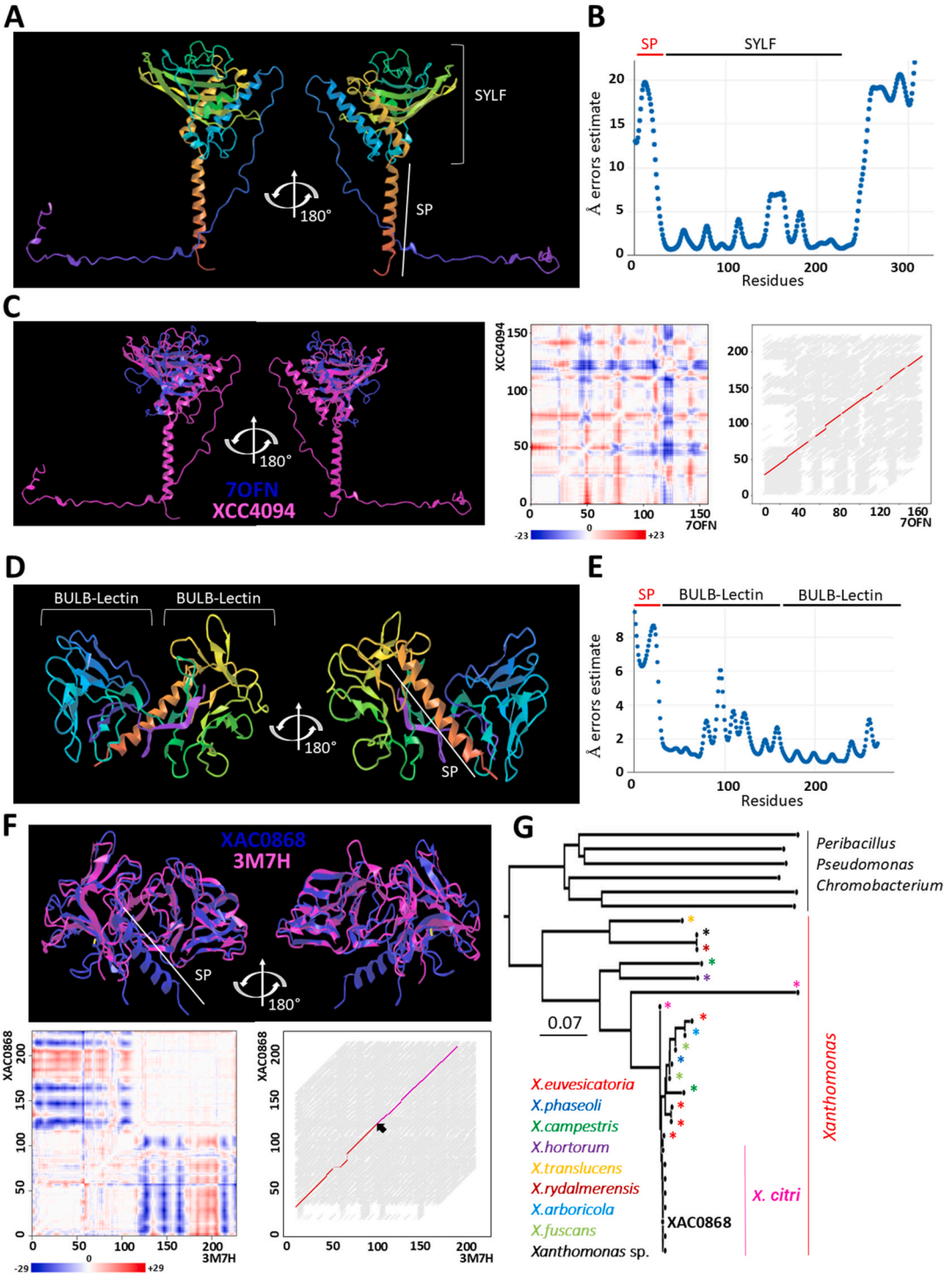
Fig. 4. Superposition of the predicted *Xanthomonas* phosphoenolpyruvate synthase (PpsA) structure and rifampin phosphotransferase from *Listeria monocytogenes* (PDB:5HV6). This result suggests that the *Xanthomonas* PEPs N-terminal has rifampin deactivating properties. **(B)** Superimposition of the predicted *Xanthomonas* PpsA structure and pyrophosphate-dependent phosphofructokinase from *Borrelia burgdorferi* (PDB:1KZH).

protein complexes or biochemical pathways can illuminate unanticipated results from proteomics projects [27] (Fig. 6B). In the context of systems biology, moonlight proteins can play key roles in the complex yet organized network of cellular biochemical pathways. They have the potential to connect metabolic pathways or cellular locations that were previously unimaginable, thus imposing a form of fuzzy logic on cellular metabolism [29]. In our approach, we conducted a systematic analysis of 18 previously published studies (Table 1), identifying 93 potential moonlight proteins. Most of these proteins had not been previously attributed to this function in the *Xanthomonas* genus. We categorized these proteins into four main groups, providing an integrated perspective of their canonical roles.

Interestingly, of the total proteins identified, 62 (66.7 %) were categorized into cellular processes and metabolism (CP/M). As shown in Table 1, CP/M was the most represented category in all the analyzed secretomes even though it is the category that, in theory, should present the lowest number of identified proteins, assuming that metabolic proteins should not be found in studies that evaluate secreted proteins supposedly related to virulence induction. This group includes most of the proteins previously described as potential moonlighting in different biological models, with emphasis on chaperones, proteins associated with flagellar synthesis, elongation factors, ATP synthase subunits, ribosomal proteins, enzymes associated with energy metabolism (such as phosphoglucomutase, glyceraldehyde-3-phosphate dehydrogenase, enolase, fructose-bisphosphate aldolase, among others), or involved in redox processes (such as superoxide dismutase and glutaredoxin-like protein).

Chaperones, elongation factors, especially Ef-Tu, and flagellin, in addition to their canonical functions, have long been known as immunomodulators of response in animal and plant hosts [33,76–78], being categorized as important PAMP proteins and, therefore, PTI response inducers [79]. In *Xanthomonas*, functions associated with immunoregulation and virulence induction have also been reported for these proteins [80–83] (Fig. 6B). Likewise, proteins involved in energy metabolism (Embden-Meyerhof-Parnas glycolytic and Szent-Györgyi-Krebs cycle pathways) have already been described as moonlighting in different biological models, either acting as regulatory factors of gene expression [84,85] or as modulators of other cellular responses [86,87]. In *Xanthomonas*, such enzymes have also been related as fundamental to pathogenicity and virulence processes, some of which induce hypersensitivity responses when mutated, thus demonstrating their importance in the molecular processes of plant-pathogen interaction [88–93].

In addition to proteins associated with central metabolism, more recently it has been shown that oxidative stress proteins are directly related to other biological functions, such as lysine biosynthesis, DNA integrity, and chronological life survival and cell-adhesion [94,95]. In our investigations, superoxide dismutase (SodM) was identified in six of the evaluated secretomes, alkyl hydroperoxide reductase (AhpC) [96] in three, and glutaredoxin-like (Grx) and organic hydroperoxide resistance protein (Ohr) in only two. In *M. tuberculosis*, SodM is a moonlighting protein that functions as an adhesin, which facilitates penetration of host cells and binds to several host moonlighting proteins, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aldolase [95]. Interestingly, several attempts to obtain a *sodM* deletion mutant were unsuccessful, suggesting the encoded protein to be essential for bacterial survival [97] (a characteristic observed for most multitasking proteins). Indeed, there are consistent reports that this protein can multitask, since it is already described as having an important role in disease induction [98–100]. As for Grx and Ohr, Sagawa and collaborators demonstrated that they are overexpressed under infection conditions, which justifies their potential in the detoxification of reactive species produced as the first line of plant defense [101]. Especially for Ohr, as it is associated with sensing, metabolism, and physiological adaptive response to lipid hydroperoxide [102], and it is produced by plant lipoxigenases during pathogen invasion, it may trigger an important role in the defense responses against phytopathogens [103]. Finally, although AhpC has so far been reported in *Xanthomonas* to be important in redox stress processes [96,104], in other pathosystems it is also associated with virulence [105]. Therefore, as they have been identified externally to the pathogen, in addition to the ROS detoxification function, they may be involved with other signaling pathways in the host cell, thus shedding light on the investigation of molecular interaction with plants. It is important to highlight that, similar to what is observed for catalase (KatE) [106] and thioredoxin (Trx) [107], although they were not shown as potential moonlight as they were identified in only one of the analyzed secretomes, they are strong candidates to act as



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moonlighting proteins in *Xanthomonas* together with all the most representative proteins involved in redox stress, similar to what is observed in other pathosystems [17,40].

Unlike the proteins classified in the CP/M category, whose cellular location is mainly cytoplasmic, highlighting them as potential moonlighting proteins when identified externally to the cell, for the proteins grouped in the (MA/T) category this possibility is not so evident [108]. Considering that gram-negative bacteria are surrounded by an outer membrane containing lipopolysaccharide, the

Fig. 5. Structural analysis of two of the hypothetical proteins identified in the secretomes analyzed. **(A)** 3D structure prediction of XCC4094 using ROBETTA, in two planes of rotation. The sequence corresponding to the signal peptide is identified with SP. SYLF corresponds to the motif identified in the protein. **(B)** Error estimation graph in Angstroms (Å) for each amino acid residue of the XCC4094 protein. **(C)** Superposition of the 3D structures of XCC4094 and lipoprotein from *Burkholderia pseudomallei* (PDB: 7OFN), in two planes of rotation, using FATCAT. The graphs on the side represent the Differential Distance Matrix (whose legend establishes the distance difference in Angstroms) and FATCAT chaining result, respectively. **(D)** Prediction of the 3D structure of XAC0868 using ROBETTA, in two planes of rotation. The sequence corresponding to the signal peptide is identified with SP. BULB-Lectin corresponds to the motifs of the same name identified in the protein. **(E)** Error estimation graph in Angstroms for each amino acid residue of the XAC0868 protein. **(F)** Superposition of the 3D structures of XAC0868 and bacteriocin LLPA from *Pseudomonas* sp (PDB: 3M7H), in two planes of rotation, using FATCAT. The graphs below, respectively, represent the Differential Distance Matrix and FATCAT chaining results (whose black arrow identifies a twist region between the respective BULB-Lectin domains). **(G)** Phylogeny established from the multiple alignment of amino acid sequences of proteins homologous to XACO868 previously identified by Blast. The colors of the asterisks correspond to the respective species identified by the same color in the legend.

proteins associated with this membrane (outer membrane proteins – OMP) are of fundamental importance for the interaction with the environment since they have already been reported to have many diverse roles, including acting as adhesion factors in virulence, channels for the uptake of nutrients, siderophore receptors and enzymes such as proteases and lipases [109].

The presence of intracellular enzymes within secretomes can be partially attributed to the release of outer membrane vesicles (OMVs) by Gram-negative bacteria like *Xanthomonas*. OMVs facilitate the transport of various proteins, including those not traditionally secreted, thereby complicating the identification of multitasking proteins. Understanding the role of OMVs in protein secretion is essential for accurately interpreting secretomic data and discerning true multifunctionality from vesicle-mediated transport. OMVs are linked to several stages of the life cycle of the soil-dwelling myxobacterium *Myxococcus* xanthus, which is a predator of a variety of bacteria and fungi [110]. Interestingly, GAPDH, an enzyme with multiple moonlight functions, may enhance the antimicrobial properties of *M. xanthus* OMVs in natural environments by facilitating their fusion with prey cell membranes [111]. This study illustrates how seemingly unrelated virulence factors from different pathogens can work together to improve bacterial colonization and infection, highlighting the complex interactions that contribute to pathogenicity.

Among all *Xanthomonas* secretomes analyzed, 13 OMP families were identified in at least two of the 18 secretomes evaluated, highlighting the TonB-dependent receptors (TBDR), typically related to the binding and transport of ferric chelates called siderophores, vitamin B12, and nickel complexes [112]. Their transport requires energy in the form of proton-motive force in complex with TonB-ExbB-ExbD proteins [112]. However, it was described recently in *Xanthomonas* that these proteins are also related to plant carbohydrate scavenging, which may contribute to the adaptation of *Xanthomonas* in their host plants [113,114], and were also associated with the transport of aromatic compounds derived from lignin in *Sphingomonas* [115]. Vorhölter and collaborators described that TBDR are involved in bacterial trans-envelope signaling in the context of a pathogenic interaction with a plant [116], influencing host specificity and bacterial pathogenicity, these findings are corroborated by other authors [116,117]. Considering that in 10 of the 18 investigated secretomes, these proteins were identified, they may have other functions associated with molecular interaction with the host. Due to a relatively high copy number of these genes in the genomes of these pathogens, empirical investigations of other possible non-canonical functions are essential.

It is important to point out two other outer membrane proteins, polyphosphate-selective porin O (OprO) and outer membrane protein XadA, respectively identified in five and three of the evaluated secretomes. Interestingly, OprO had already been described in *Xanthomonas* as a porin possibly regulated under conditions that simulate cell tissue, or even during the infectious process [118,119]. XadA is a non-fimbrial adhesion required for optimal virulence in *Xanthomonas* [120]. In both cases, there is no evidence associated with an established non-canonical function for these proteins, nor even in other pathosystems, making them potential targets in the search for other associated molecular functions.

Difficulties in inferring a moonlighting function for proteins in the MA/T category are also evident for proteins classified in the V/A category. In this case, the fact that all of them have already been reported as fundamental to the virulence and adaptation process since most are naturally secreted, any moonlighting function that is not linked to the interaction with the host and the ability to induce the classic phenotypes ceases to be investigated, to the detriment of the main function. Even so, it is worth mentioning the enzymes that degrade the structural polymers of plants, in special cellulases (CelS/Egl/Eng) identified in six secretomes, periplasmic protease (MucD) identified in five secretomes, and virulence-associated proteins like VirK and LipA identified in three and five secretomes, respectively.

Although there is no report of the non-canonical function of cellulases in *Xanthomonas*, in other pathosystems this has already been demonstrated. In the fungus *Rhizoctonia solani*, the causal agent for Rhizoctonia foliar blight, cellulase can act as an elicitor of the plant immune system [121]. A similar response was also observed for cellulases from *Trichoderma harzianum* that trigger induced systemic resistance against *Curvularia* leaf spot in maize and elicit defense [122]. In contrast, Mlt has been reported with multiple functions in *Xanthomonas*, either acting as a protein associated with the type III secretory system involved with effector translocation [123,124], cell separation [125], or acting as an epimerase [126].

Furthermore, two of the most interesting proteins in the category of virulence and adaptation are LipA and VirK. In a recent study, Assis et al., using comparative genomics, identified seven effector protein families with different adaptive and evolutionary histories in plant-associated members of the Xanthomonadaceae, including LipA and VirK [12]. LipA mutants in *Xanthomonas* showed a clear reduction in virulence potential [12,127], a similar phenotype to that found for the mutation in the homologous gene LesA in *Xylella fastidiosa* [128]. Interestingly, in addition to being directly associated with virulence, in *Xanthomonas* it has been shown that LipA is capable of inducing innate immune responses in rice plants [129], which highlights it as a new potential moonlighting for this

Table 4
Proteins identified on *Xanthomonas* secretomes and described as moonlighting in other species.

Protein	Canonical functions	Moonlight functions (non-canonical)	Biological model	PMID PMCID/DOI
GroEL	Chaperone	Secretion/effectors	*Mycoplasma*	4990256
GroES			*Clavibacter*	23821058
DnaK		Membrane receptor	*Haemophilus*	16880000
		Cytotoxicity	*Actinobacillus*	9784537
		Cell-adhesion	*Chlamydia*	18310329
			Clostridium	11160803
			Lactobacillus	16368998
			Plesiomonas	17172512
			Mammalian	14742538
			Helicobacter	9872496
			Histoplasma	12496435
			Legionella	9746556
			Mycobacterium	19470749
			Salmonella	1639475
			Haemophilus	9488422
TufA/TufB	Elongation factor	Secretion/effectors	*Leishmania*	12384497
			Staphylococcus	20847047
		Cell-adhesion	*Mycoplasma*	12421310
			Pseudomonas	17709513
			Cryptosporidium	3837153
			Streptococcus	25046156
			Leptospira	24312361
			Francisella	2551611
			Mycobacterium	17849409
			Mycoplasma	16054780
			Lactobacillus	15039339
			Acinetobacter	3362023
			Candida	12622818
		Post-translational modification	*Mycoplasma*	17182197
		MAMP/PAMP	*Ralstonia*	16713565
			Mycoplasma	4990256
			Burkholderia	21179405
			Mycoplasma	17182197
Eno	Glycolysis enzyme	Cell-adhesion	*Aeromonas*	19270100
			Bacillus	18456007
			Bifidobacteria	19574304
			Borrelia	22087329
			Candida	12867553
			Lactobacillus	17892475
			Leishmania	17653767
			Onchocerca	12818429
			Paracoccidioides	19429745
			Schistosoma	20609522
			Staphylococcus	15158195
			Streptococcus	17964283
Fba	Glycolysis enzyme	MAMP/PAMP	*Streptococcus*	15498039
			Streptococcus	17492599
		Stress tolerance	*Listeria*	27489951
			Arabidopsis	10.5010/JPB.2012.39.2.106
		Cell-adhesion	*Echinococcus*	22750316
			Mycobacterium	21949126
			Neisseria	20199602
GapA	Glycolysis enzyme	Membrane receptor	*Escherichia*	19784925
		Cell-adhesion	*Candida*	9573088
			Lactobacillus	17379720
			Mycoplasma	12787366
			Neisseria	21062461
			Paracoccidioides	16368993
			Bacillus	20727989
			Trichomonas	19380472
		Post-translational modification	*Streptococcus*	10024547
GlnA	Glutamine metabolism	Cell-adhesion	*Lactobacillus*	22389474
Pgk	Glycolysis enzyme	Cell-adhesion	*Streptococcus*	21729749
			Pneumococcus	24196407
SodM	Redox enzyme	Cell-adhesion	*Mycobacterium*	14687559
		Chronological life survival	*Candida*	25479837
AtpD/AtpP	Synthesis of ATP	Morphogenesis	*Arabidopsis*	22328157

(*continued on next page*)

Table 4 (*continued*)

Protein	Canonical functions	Moonlight functions (non-canonical)	Biological model	PMID PMCID/DOI
		Regulation of intracellular pH	*Mammalian*	20626349
FliC	Flagellum biosynthesis	MAMP/PAMP	*Pseudomonas*	2752798
			Stenotrophomonas	21415208
			Escherichia	3434589
			Salmonella	16648855
		Cell-adhesion	*Campylobacter*	2065653
			Stenotrophomonas	21415208
			Pseudomonas	11880301
Mdh	TCA enzyme	Chloroplast Development	*Arabidopsis*	29934433
		Regulation of intracellular pH	*Streptococcus*	25583521
		Cell-adhesion	*Brucella*	24609497
		MAMP/PAMP	*Brucella*	28629726
			Toxoplasma	26514423

pathosystem. VirK, on the other hand, remains enigmatic. Identified in *Xanthomonas* proteomics studies in infective condition [101, 130], it has been described in *E. coli* as a periplasmic protein essential for efficient secretion of plasmid toxins [131]. VirK can be a potential moonlight protein as it was identified in most sequenced *Xanthomonas*, has a signal peptide that favors its secretion, and has two domains with distinct functions, transferase, and immunoglobulin [12].

Only three hypothetical proteins (XOO0094 - BAE66849; XCC4094 - AAM43315; and XAC0868 – AAM35756) were identified in two of the 18 secretomes investigated. Initially described as a hypothetical gene inserted in the cluster of genes encoding the type III secretion apparatus, reannotation of XOO0094 in parallel with a search for its empirical function in other publications revealed that it actually corresponds to the gene encoding the HrcQ protein (HrpD) whose promoter region has a consensus region of PIP-BOX (plant-inducible promoter) [132], which might play a role in transcription activation of the *hrpB* operon [133].

In contrast, XCC4094 corresponds to a hypothetical conserved protein widely distributed beyond the genus *Xanthomonas*. It has a coding sequence for a signal peptide, which corroborates the fact that it was identified as a secreted protein. The presence of the SYLF domain categorizes it as a potential homologous lipoprotein from *Burkholderia pseudomallei* [134].

For the hypothetical protein XAC0868, a signal peptide sequence was also identified, in addition to two BULB-Lectin domains [135], in tandem. Comparative structural analysis revealed that this protein in *Xanthomonas* has a structure very similar to a bacteriocin from *Pseudomonas* LLPA [136]. This bacteriocin has high potential for biocontrol, and its expression under diverse environmental stress conditions interferes with the regulation and synthesis of this protein [137].

Finally, the search for promiscuous catalytic residues in three enzymes previously classified as potential moonlighting proved to be very interesting. We first analyzed the enzyme phosphoenolpyruvate synthase (PpsA) which converts pyruvate into phosphoenolpyruvate, an important step to utilize pyruvate or lactate as a carbon source or to use phosphoenolpyruvate itself directly as an energy source for the phosphotransferase system of sugar uptake [138]. Disruption of *ppsA* leads to reduced growth on specific carbon sources like pyruvate and dicarboxylates, alongside a significant decrease in the secretion of virulence factors essential for plant infection, indicating a link between carbon metabolism and pathogenicity regulated by *ppsA* [139,140]. Comparative structural analysis showed that the N-terminal portion of PpsA from *Xanthomonas* presents a high degree of overlap with the enzyme rifampin phosphotransferase from *Listeria monocytogenes*, responsible for conferring resistance to this antibiotic. Our in silico analysis indicates a high degree of structural similarity to characterized PpsA enzymes from other organisms, such as the rifampin phosphotransferase from Listeria monocytogenes (PDB:5HV6), suggesting a possible convergent evolution scenario. Future experimental studies are necessary to confirm the functional similarities and potential promiscuous activities of *Xanthomonas* PpsA. Another interesting fact is that Rifampin is one of the most potent and broad-spectrum bactericidal antibiotics against pathogens, and the possible resistance of some strains of *Xanthomonas* to this antibiotic could be due to the activity of this non-canonical function of PpsA [141]. Additionally, the superposition of the structure of PpsA from *Xanthomonas* showed high similarity of the active site with another unrelated protein, indicating this could be an instance of convergent evolution.

The enzyme chorismate mutase (CM) was also selected for promiscuity analysis. First, however, it is important to highlight that *Xanthomonas* has two copies of CM. The first has around 400 residues and is bifunctional and cytoplasmic (chorismate mutase/prephenate dehydratase - PheA). The second, with around 160 residues (AroQ), with the presence of a signal peptide and speculated to be an important factor in controlling plant metabolism during the infectious process, modulating the shikimate pathway [12], thereby inducing an increase in the synthesis of tyrosine and phenyalalanine to the detriment of the synthesis of salicylic acid. The copy used in these investigations refers to this smaller, secreted copy, whose relationship with virulence has also been described for other models [142], and are drug targets being absent in mammals [143].

This enzyme presents a fascinating example of how active sites can be tinkered with to generate different functions. In *Burkholderia/Xanthomonas*, Active1 has conserved Arg49 and Lys60, with Glu106 mutated to Lys, a differently charged amino acid. Correspondingly, Active2 in *Mycobacterium* has conserved Arg34 and Lys55, with Gln94 mutated to Glu, also differently charged. The best CLASP match using three-residue motifs is a D-lactate dehydrogenase from *Lactobacillus bulgaricus* (PDB:1J49), corroborating the known dehydrogenase activity of CM.

The last enzyme analyzed, asparaginase is conserved and catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia and is used in treating acute lymphoblastic leukemia and lymphoblastic lymphoma in humans [144]. In *Xanthomonas*, it was identified

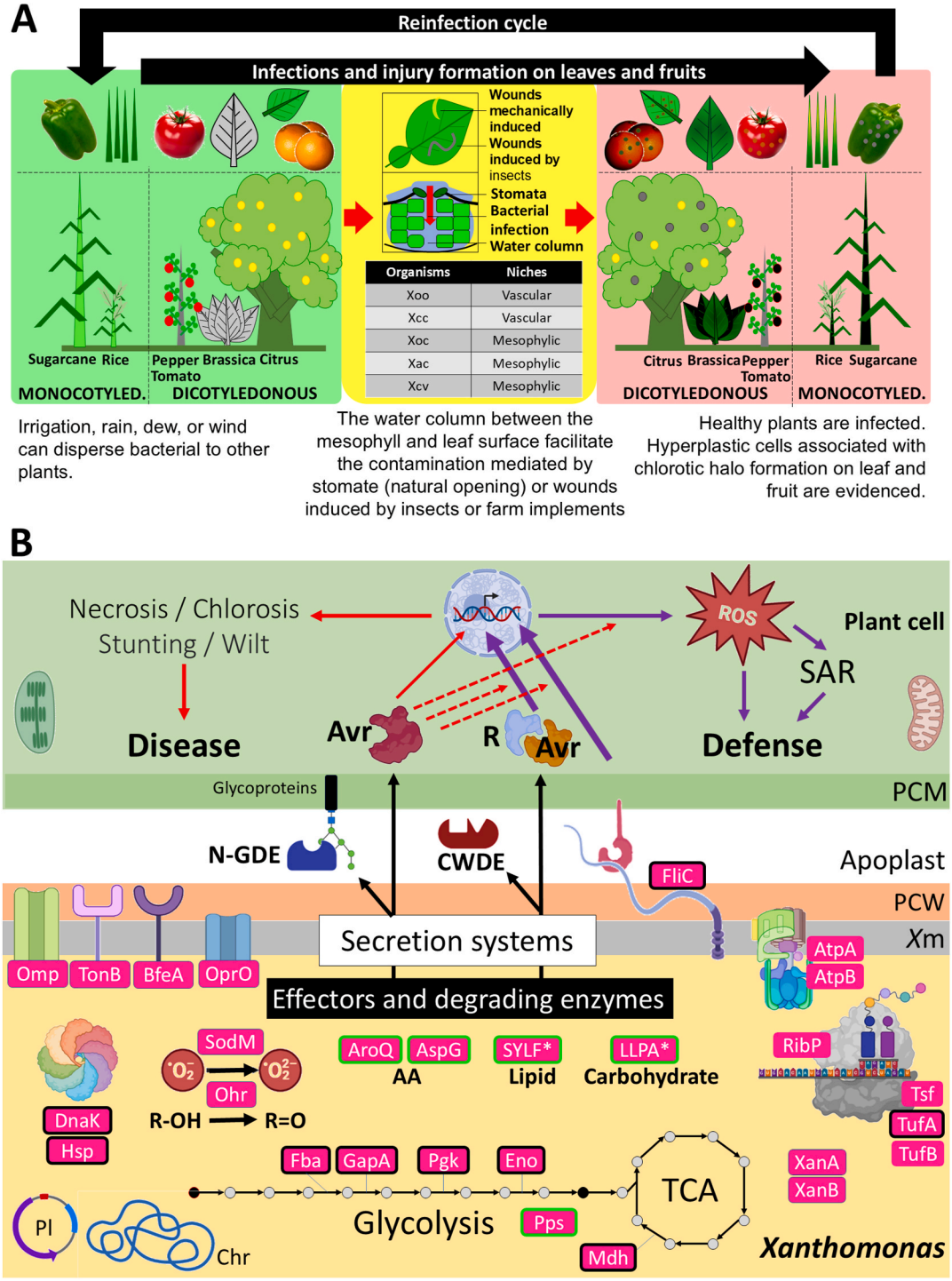

Fig. 6. **(A)** Schematic representation of *Xanthomonas* life cycle. Xoo – *Xanthomonas oryzae* pv. *oryzae*, Xcc – *Xanthomonas campestris* pv. *campestris*, Xoc – *Xanthomonas oryzae* pv. *oryzicola*, Xac – *Xanthomonas citri* pv. *citri*, Xcv – *Xanthomonas campestris* pv. *vesicatoria*. **(B)** Schematic representation of *Xanthomonas* moonlight and promiscuous proteins localization and function. Proteins are identified in pink. Proteins previously described as having a moonlight function in *Xanthomonas* are highlighted with a black outline. Proteins that have a putative promiscuous function in *Xanthomonas* are highlighted with a green outline. Xm – *Xanthomonas* membrane; PWC – Plant cell wall; PCM – Plant cell membrane; CWDE – Cell wall degrading enzymes; N-GDE – N-glycans degrading enzymes; Avr – Avirulence proteins; R – Plant resistance proteins; SAR – Systemic acquired resistance; TCA – Tricarboxylic acid cycle; AA – Amino acid metabolism; Pl – plasmid; Chr – Chromosome.

with the highest similarity to solved protein structures from *Elizabethkingia* [68], and its active site composition was determined using CLASP, revealing potential promiscuity. Despite attempts to inactivate the gene in *Xanthomonas citri* pv. *citri*, no significant reduction in growth or virulence was observed, indicating its non-canonical function may not play a major role in this pathosystem.

Overall, our comparative analysis of 18 different Xanthomonas secretomes successfully identified conserved multitasking proteins across multiple species and conditions, highlighting those fundamental to plant-pathogen interactions. This integrative approach leverages existing data to uncover fundamental proteins that may play pivotal roles in plant-pathogen interactions, providing a valuable resource for future functional studies and therapeutic target identification. However, this broad approach may overlook species-specific multitasking proteins essential for adaptation to specific hosts or niches. In contrast, detailed analyses of individual secretomes can provide in-depth insights into unique virulence factors pertinent to each strain. Integrating both comparative and single secretome analyses would offer a more comprehensive understanding of multitasking proteins in Xanthomonas. Moreover, this study acknowledges the lack of experimental validation for the predicted functions of the identified multitasking proteins. While our in silico analyses provide foundational insights, the functional roles of these proteins in plant-microbe interactions require empirical confirmation. Additionally, our enzyme promiscuity analysis, based solely on the conservation of active site residues, serves as a predictive tool rather than definitive evidence of multifunctionality. Enzyme promiscuity involves the ability to act on multiple substrates or catalyze different reactions, influenced by active site topology and catalytic mechanisms. Thus, experimental validation is essential to confirm these predictions. Future research should prioritize functional assays, such as gene knockout studies and protein-protein interaction analyses, to validate the predicted roles of these multitasking proteins and explore their contributions to the virulence and adaptability of Xanthomonas species.

4. Methods

Fig. 7 illustrates the methodological pipeline employed in this study, outlining the sequential steps taken to identify multitask proteins within Xanthomonas secretomes. This includes secretome selection, protein identification and categorization, analysis of moonlighting and promiscuous functions, and structural evaluations.

4.1. Secretome studies selection

A systematic search for scientific articles published between 2005 and 2021 involving proteomic analysis was conducted via PubMed. Searches were restricted to the *Xanthomonas* pathosystem model, selecting investigations focusing on secretomes regardless of the protein identification techniques used.

4.2. Identification and comparison among secreted proteins

The list of secreted proteins and their respective locus tags in the genomes were acquired from reference articles. We then compared these proteins across the 18 studies using two methods: (a) KEGG's [145] orthologs tool from the input locus tag, or (b) a Blastp [146] identity analysis against the NCBI non-redundant (nr) database, restricting the search to *Xanthomonas* species (both with default parameters). Data were compiled into a table and sorted by the frequency of protein identification.

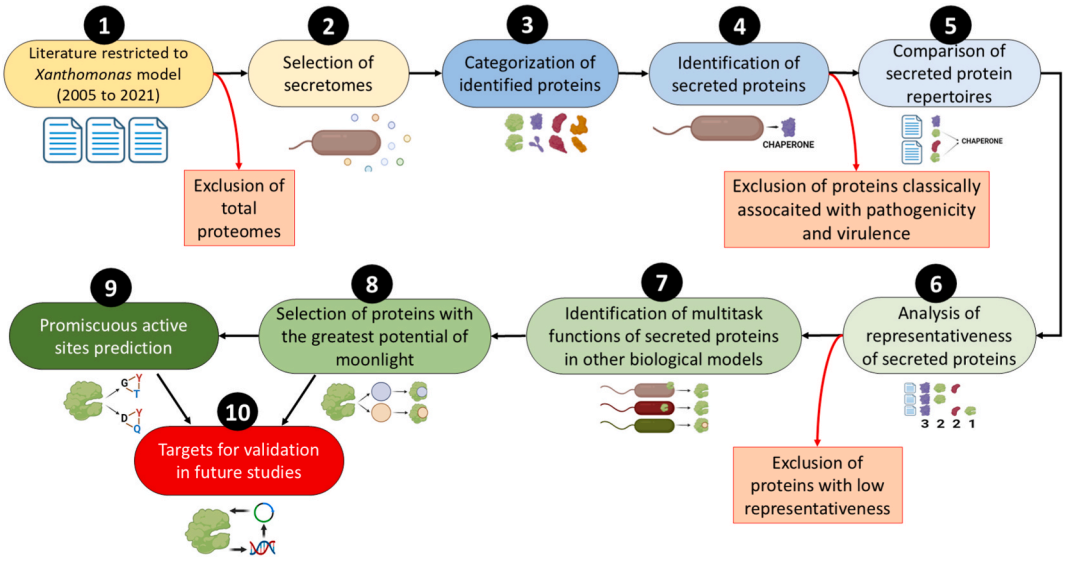

Fig. 7. Flowchart describing the methods and steps employed to search for multitask proteins in *Xanthomonas*.

4.3. Categorization of identified proteins

Proteins identified in the secretomes were classified into four functional categories: CP/M (Cellular processes and metabolism); V/A (Virulence and adaptation); MA/T (Membrane-associated and transport); and HP (Hypothetical protein). This classification aimed to separate proteins related to pathogenesis from other metabolic or structural/transport proteins, based on GeneOntology classification [147].

4.4. Identification of moonlight and promiscuous orthologs in other model organisms

We initiated a targeted search centered on the pre-classified moonlighting roles already identified for orthologous proteins in other models using PubMed, and the MoonProt 3.0[62] and MultitaskProtDB [61] databases, in all cases using keywords.

4.5. Presence of signal peptide and domains

Potential moonlighting proteins were evaluated for the presence of a signal peptide, using the SignalP 6.0 [148] tool and domain with InterPro [149], (both with default parameters).

4.6. Cellular localization of putative moonlighting proteins

Proteins were analyzed for cellular localization based on literature and data repositories like KEGG [145] and Uniprot [150], using different model organisms as a reference.

4.7. Moonlighting functions in Xanthomonas secretomes

We examined the classical and moonlighting functions of the most representative proteins in *Xanthomonas* secretomes, validated in other model organisms using PubMed, MoonProt 3.0[62] and MultitaskProtDB [61], in all cases using keywords.

4.8. Literature search for moonlighting functions

A refined literature search targeted articles highlighting presumptive moonlighting functions identified in other bacterial species.

4.9. Definition of catalytic residues in putative promiscuous proteins

Five proteins (asparaginase, chorismate mutase, phosphoenolpyruvate-synthase, elongation factor Tu, and chaperonin 60) were selected for promiscuity analysis of their active sites using CLASP (CataLytic Active Site Prediction) [69], using default parameters. This selection was based on variations in secretome representation and the diversity of related cellular processes. For each of the five selected proteins, a list of ten proteins with similar catalytic sites was identified. Using the default parameters, the dataset from the Catalytic Site Atlas (CSA) [151] was utilized to identify proteins with more than three or four active site residues for querying the selected moonlighting proteins from *Xanthomonas*. CAS [151] provides an original hand-annotated set containing information extracted from primary literature, using defined criteria to assign catalytic residues, and an additional homologous set, containing annotations inferred by PSI-BLAST and sequence alignment to one of the original sets. This dataset was used to extract proteins with more than three (N = 519) and four (N = 401) active site residues. These were used to query the ten selected moonlighting proteins from *Xanthomonas*.

4.10. Phylogenetic analysis of putative hypothetical proteins

Using reference Fasta sequences for each hypothetical proteins (XCC4094 and XAC0868) a blast analysis (BlastP against nr) was conducted. Homologous Fasta sequences were acquired and multiple alignment analysis was performed using Clustal Omega [152], using default parameters. The NW file was exported to FigTree (http://tree.bio.ed.ac.uk/software/figtree/) for structural topology creation, using midpoint rooting.

4.11. Prediction and comparison of 3D structure of putative hypothetical proteins

Selected proteins were reannotated using Blast [146]. For those without a predicted function, 3D structure was investigated with ROBETTA [153]. Superposition analysis of 3D structures was performed using g FATCAT [154]. Additionally, promiscuity analysis of active sites was also performed using CLASP.

4.12. Construction and analysis of XAC3092 (asparaginase – AspG) mutant

Deletion mutagenesis by overlap extension PCR [155] and homologous recombination was performed by site-directed mutagenesis using two pairs of non-chimeric and chimeric primers (A, B, C and D) designed to amplify the flanking regions of the sequence to be

deleted and ligate the two products (Supplementary Table S1). The PCR product of 1119 bp was cloned into the suicide vector pNPTS138 [156] using *Nhe*I and *Hin*dIII restriction enzymes. An internal fragment of 1065 bp was deleted. The recombinant vector was transformed into *Xanthomonas axonopodis* pv. *citri* 306 (Xac) by electroporation for homologous recombination. The colonies showing resistance to kanamycin and susceptibility to sucrose (provided by the suicide vector in NA medium) were selected and replicated. Mutant confirmation was performed with PCR followed by DNA sequencing to confirm *aspG* gene deletion.

4.13. In planta pathogenicity test

Mutant and wild-type (WT) Xac strains were cultivated overnight in NB at 28C and then centrifuged at 3000×*g* for 12 min at room temperature. The supernatant was discarded and the pellets resuspended in autoclaved tap water to an OD 600 nm of 0.3 ABS, equivalent to 10^8 CFU/mL. A bacterial suspension was injected directly into young leaves at two points on the abaxial side of three young leaves (technical replicates) in three different "Pera Rio" orange (*C. sinensis* L. Osbeck) plants (biological replicates) using needleless hypodermic syringes [157]. Xac306 WT was infiltrated on each leaf on the left-hand side of the central vein, while *aspG* mutant was infiltrated on the right-hand side, so that symptom progression could be compared side by side. One leaf in each plant was infiltrated with sterile distilled water as negative control.

CRediT authorship contribution statement

Renata de Almeida Barbosa Assis: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Rafael Marini Ferreira:** Writing – review & editing, Writing – original draft, Investigation. **Amanda Carolina Paulino de Oliveira:** Writing – review & editing, Writing – original draft, Investigation. **Flávia Maria Souza Carvalho:** Writing – review & editing, Writing – original draft, Investigation. **Jesus Aparecido Ferro:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Robson Francisco de Souza:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Elena Graciela Orellano:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Nalvo Franco Almeida:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Camila Carrião Machado Garcia:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Abhaya M. Dandekar:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Sandeep Chakraborty:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Alessandro M. Varani:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Leandro Marcio Moreira:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study did not involve human participants or animals.

Declaration of the use of generative artificial intelligence

In this scientific work, generative artificial intelligence (AI) has not been used.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:LEANDRO MARCIO MOREIRA reports financial support was provided by Federal University of Ouro Preto. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42979.

References

[1] J. Swings, L. Vauterin, K. Kersters, The bacterium Xanthomonas, in: Xanthomonas, Chapman and Hall, London, 1993, pp. 121–156, *chapter 2*.
[2] F. Leyns, M.D. Cleene, J.-G. Swings, J. De Ley, The host range of the genus Xanthomonas, Bot. Rev. 50 (1984) 308–356.
[3] M. Goto, K. Ohta, N. Okabe, Studies on saprophytic survival of Xanthomonas citri (Hasse) Dowson. 2. longevity and survival density of the bacterium on artificially infested weeds, plant residues and soils, 日本植物病理学会報 41 (1975) 141–147.
[4] J.H. Graham, Survival of Xanthomonas campestris pv. citri in citrus plant debris and soil in Florida and Argentina, Plant Dis. 71 (1987) 1094.
[5] J.H. Graham, T.R. Gottwald, J. Cubero, D.S. Achor, Xanthomonas axonopodis pv. citri: factors affecting successful eradication of citrus canker, Mol. Plant Pathol. 5 (2004) 1–15.
[6] B.C. Freeman, G.A. Beattie, An overview of plant defenses against pathogens and herbivores. https://dr.lib.iastate.edu/entities/publication/27a4e983-bebc-4150-acac-d322ca26a53b, 2008.
[7] R.P. Ryan, et al., Pathogenomics of Xanthomonas : understanding bacterium–plant interactions, Nat. Rev. Microbiol. 9 (2011) 344–355.
[8] Q. Yang, et al., Broad-spectrum chemicals block ROS detoxification to prevent plant fungal invasion, Curr. Biol. 32 (2022) 3886–3897.e6.
[9] R.A.B. Assis, et al., A comparative genomic analysis of Xanthomonas arboricola pv. juglandis strains reveal hallmarks of mobile genetic elements in the adaptation and accelerated evolution of virulence, Genomics 113 (2021) 2513–2525.
[10] N.P. Fonseca, et al., Analyses of seven new genomes of Xanthomonas citri pv. aurantifolii strains, causative agents of citrus canker B and C, show a reduced repertoire of pathogenicity-related genes, Front. Microbiol. 10 (2019).
[11] R. Bart, et al., High-throughput genomic sequencing of cassava bacterial blight strains identifies conserved effectors to target for durable resistance, Proc. Natl. Acad. Sci. U. S. A 109 (2012) E1972–E1979.
[12] R. de A.B. Assis, et al., Identification and analysis of seven effector protein families with different adaptive and evolutionary histories in plant-associated members of the Xanthomonadaceae, Sci. Rep. 7 (2017) 16133.
[13] J.S.L. Patané, et al., Origin and diversification of Xanthomonas citri subsp. citri pathotypes revealed by inclusive phylogenomic, dating, and biogeographic analyses, BMC Genom. 20 (2019) 700.
[14] D. Blüher, et al., A 1-phytase type III effector interferes with plant hormone signaling, Nat. Commun. 8 (2017) 2159.
[15] J. Boch, U. Bonas, T. Lahaye, TAL effectors–pathogen strategies and plant resistance engineering, New Phytol. 204 (2014) 823–832.
[16] D. Teper, N. Wang, Consequences of adaptation of TAL effectors on host susceptibility to Xanthomonas, PLoS Genet. 17 (2021) e1009310.
[17] D.H.E.W. Huberts, I.J. van der Klei, Moonlighting proteins: an intriguing mode of multitasking, Biochim. Biophys. Acta BBA - Mol. Cell Res. 1803 (2010) 520–525.
[18] N. Singh, N. Bhalla, Moonlighting proteins, Annu. Rev. Genet. 54 (2020) 265–285.
[19] B. Jia, G.-W. Cheong, S. Zhang, Multifunctional enzymes in archaea: promiscuity and moonlight, Extremophiles 17 (2013) 193–203.
[20] C.J. Jeffery, Moonlighting proteins, Trends Biochem. Sci. 24 (1999) 8–11.
[21] C.J. Jeffery, Multifunctional proteins: examples of gene sharing, Ann. Med. 35 (2003) 28–35.
[22] C. Tristan, N. Shahani, T.W. Sedlak, A. Sawa, The diverse functions of GAPDH: views from different subcellular compartments, Cell. Signal. 23 (2010) 317.
[23] C. Nicholls, H. Li, J.-P. Liu, GAPDH: a common enzyme with uncommon functions, Clin. Exp. Pharmacol. Physiol. 39 (2012) 674–679.
[24] H. Zhang, Y. Zhao, D.-X. Zhou, Rice NAD+-dependent histone deacetylase OsSRT1 represses glycolysis and regulates the moonlighting function of GAPDH as a transcriptional activator of glycolytic genes, Nucleic Acids Res. 45 (2017) 12241.
[25] P.E. Glaser, R.W. Gross, Rapid plasmenylethanolamine-selective fusion of membrane bilayers catalyzed by an isoform of glyceraldehyde-3-phosphate dehydrogenase: discrimination between glycolytic and fusogenic roles of individual isoforms, Biochemistry 34 (1995) 12193–12203.
[26] S.D. Copley, Enzymes with extra talents: moonlighting functions and catalytic promiscuity, Curr. Opin. Chem. Biol. 7 (2003) 265–272.
[27] C.J. Jeffery, Moonlighting proteins: complications and implications for proteomics research, Drug Discov. Today Targets 3 (2004) 71–78.
[28] B. Henderson, A. Martin, Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease, Infect. Immun. 79 (2011) 3476–3491.
[29] H. Liu, C.J. Jeffery, Moonlighting proteins in the fuzzy logic of cellular metabolism, Molecules 25 (2020) 3440.
[30] C. Pan, B. Li, M.C. Simon, Moonlighting functions of metabolic enzymes and metabolites in cancer, Mol. Cell 81 (2021) 3760–3774.
[31] R. González-Stegmaier, A. Aguirre, C. Cárcamo, P. Aguila-Torres, F. Villarroel-Espíndola, Recombinant domain of flagellin promotes in vitro a chemotactic inflammatory profile in human immune cells independently of a dendritic cell phenotype, Molecules 28 (2023) 2394.
[32] G. Sobol, et al., Tomato receptor-like cytoplasmic kinase Fir1 is involved in flagellin signaling and preinvasion immunity, Plant Physiol. 192 (2023) 565–581.
[33] C. Zipfel, et al., Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation, Cell 125 (2006) 749–760.
[34] M. Widjaja, et al., Elongation factor Tu is a multifunctional and processed moonlighting protein, Sci. Rep. 7 (2017) 11227.
[35] L.-X. Chai, et al., A putative nuclear copper chaperone promotes plant immunity in Arabidopsis, J. Exp. Bot. 71 (2020) 6684–6696.
[36] C.-J. Park, Y.-S. Seo, Heat shock proteins: a review of the molecular chaperones for plant immunity, Plant Pathol. J. 31 (2015) 323–333.
[37] B. González, P. Vera, Folate metabolism interferes with plant immunity through 1C methionine synthase-directed genome-wide DNA methylation enhancement, Mol. Plant 12 (2019) 1227–1242.
[38] L. Yang, et al., Reduction of the canonical function of a glycolytic enzyme enolase triggers immune responses that further affect metabolism and growth in Arabidopsis, Plant Cell 34 (2022) 1745–1767.
[39] T. Nakano, et al., A novel moonlight function of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for immunomodulation, Biofactors 44 (2018) 597–608.
[40] C.J. Jeffery, Moonlighting proteins–an update, Mol. Biosyst. 5 (2009) 345–350.
[41] S.D. Copley, An evolutionary biochemist's perspective on promiscuity, Trends Biochem. Sci. 40 (2015) 72–78.
[42] M. López-Iglesias, V. Gotor-Fernández, Recent advances in biocatalytic promiscuity: hydrolase-catalyzed reactions for nonconventional transformations, Chem. Rec. N. Y. N 15 (2015) 743–759.
[43] O. Khersonsky, D.S. Tawfik, Enzyme promiscuity: a mechanistic and evolutionary perspective, Annu. Rev. Biochem. 79 (2010) 471–505.
[44] G.A. Castillo Villamizar, et al., Characteristics of the first protein tyrosine phosphatase with phytase activity from a soil metagenome, Genes 10 (2019) 101.
[45] K. Pirc, et al., Nep1-like proteins as a target for plant pathogen control, PLoS Pathog. 17 (2021) e1009477.
[46] Y. Chen, S. Li, J. Guo, A method for identifying moonlighting proteins based on linear discriminant analysis and bagging-SVM, Front. Genet. 13 (2022) 963349.
[47] S. Hernández, et al., Bioinformatics and moonlighting proteins, Front. Bioeng. Biotechnol. 3 (2015).
[48] I.K. Khan, D. Kihara, Computational characterization of moonlighting proteins, Biochem. Soc. Trans. 42 (2014) 1780–1785.
[49] Y. Li, et al., De novo prediction of moonlighting proteins using multimodal deep ensemble learning, Front. Genet. 12 (2021).
[50] C.E. Chapple, et al., Extreme multifunctional proteins identified from a human protein interaction network, Nat. Commun. 6 (2015) 7412.
[51] I. Khan, et al., Genome-scale identification and characterization of moonlighting proteins, Biol. Direct 9 (2014) 30.
[52] Y. Pritykin, D. Ghersi, M. Singh, Genome-wide detection and analysis of multifunctional genes, PLoS Comput. Biol. 11 (2015) e1004467.

[53] L. Cheng, K.-S. Leung, Identification and characterization of moonlighting long non-coding RNAs based on RNA and protein interactome, Bioinformatics 34 (2018) 3519–3528.
[54] A. Jain, H. Gali, D. Kihara, Identification of moonlighting proteins in genomes using text mining techniques, Proteomics 18 (2018) e1800083.
[55] X. Liu, et al., IdentPMP: identification of moonlighting proteins in plants using sequence-based learning models, PeerJ 9 (2021) e11900.
[56] C.J. Jeffery, Mass spectrometry and the search for moonlighting proteins, Mass Spectrom. Rev. 24 (2005) 772–782.
[57] F. Shirafkan, S. Gharaghani, K. Rahimian, R.H. Sajedi, J. Zahiri, Moonlighting protein prediction using physico-chemical and evolutional properties via machine learning methods, BMC Bioinf. 22 (2021) 261.
[58] M. Krantz, E. Klipp, Moonlighting proteins - an approach to systematize the concept, Silico Biol. 13 (2020) 71–83.
[59] I. Khan, J. McGraw, D. Kihara, MPFit: computational tool for predicting moonlighting proteins, in: D. Kihara (Ed.), Protein Function Prediction: Methods and Protocols, Springer, New York, NY, 2017, pp. 45–57, https://doi.org/10.1007/978-1-4939-7015-5_5.
[60] Q. Wei, I.K. Khan, Z. Ding, S. Yerneni, D. Kihara, NaviGO: interactive tool for visualization and functional similarity and coherence analysis with gene ontology, BMC Bioinf. 18 (2017) 177.
[61] S. Hernández, et al., MultitaskProtDB: a database of multitasking proteins, Nucleic Acids Res. 42 (2014) D517–D520.
[62] C. Chen, et al., MoonProt 3.0: an update of the moonlighting proteins database, Nucleic Acids Res. 49 (2021) D368–D372.
[63] D.M. Ribeiro, G. Briere, B. Bely, L. Spinelli, C. Brun, MoonDB 2.0: an updated database of extreme multifunctional and moonlighting proteins, Nucleic Acids Res. 47 (2019) D398–D402.
[64] B. Su, Z. Qian, T. Li, Y. Zhou, A. Wong, PlantMP: a database for moonlighting plant proteins, Database 2019 (2019) baz050.
[65] K.L. Harvey, V.M. Jarocki, I.G. Charles, S.P. Djordjevic, The diverse functional roles of elongation factor Tu (EF-Tu) in microbial pathogenesis, Front. Microbiol. 10 (2019) 2351.
[66] M. Maguire, A.R.M. Coates, B. Henderson, Chaperonin 60 unfolds its secrets of cellular communication, Cell Stress Chaperones 7 (2002) 317.
[67] L. Sui, D. Lakshminarasimhan, S. Pande, H.-C. Guo, Structural basis of a point mutation that causes the genetic disease aspartylglucosaminuria, Struct. Lond. Engl. 1993 22 (2014) 1855–1861.
[68] X. Qian, C. Guan, H.-C. Guo, A dual role for an aspartic acid in glycosylasparaginase autoproteolysis, Struct. Lond. Engl. 1993 11 (2003) 997–1003.
[69] S. Chakraborty, R. Minda, L. Salaye, S.K. Bhattacharjee, B.J. Rao, Active site detection by spatial conformity and electrostatic analysis—unravelling a proteolytic function in shrimp alkaline phosphatase, PLoS One 6 (2011) e28470.
[70] C. Lorenz, J. Hausner, D. Büttner, HrcQ provides a docking site for early and late type III secretion substrates from Xanthomonas, PLoS One 7 (2012) e51063.
[71] S. Sapkota, M. Mergoum, Z. Liu, The translucens group of Xanthomonas translucens: complicated and important pathogens causing bacterial leaf streak on cereals, Mol. Plant Pathol. 21 (2020) 291–302.
[72] W.W. Shane, J.S. Baumer, P.S. Teng, Crop losses caused by Xanthomonas streak on spring wheat and barley, Plant Dis. (1987) 927–930, https://doi.org/10.1094/PD-71-0927.
[73] P.L.H. Health, P. on P. E, et al., Pest categorisation of Xanthomonas oryzae pathovars oryzae and oryzicola, EFSA J. 16 (2018) e05109.
[74] S. Timilsina, et al., Xanthomonas diversity, virulence and plant–pathogen interactions, Nat. Rev. Microbiol. 18 (2020) 415–427.
[75] S.-Q. An, et al., Mechanistic insights into host adaptation, virulence and epidemiology of the phytopathogen Xanthomonas, FEMS Microbiol. Rev. 44 (2020) 1–32.
[76] B. Henderson, et al., Caught with their PAMPs down? The extracellular signalling actions of molecular chaperones are not due to microbial contaminants, Cell Stress Chaperones 15 (2010) 123–141.
[77] F. Lu, et al., Enhancement of innate immune system in monocot rice by transferring the dicotyledonous elongation factor Tu receptor EFR, J. Integr. Plant Biol. 57 (2015) 641–652.
[78] M. Zhong, H. Yan, Y. Li, Flagellin: a unique microbe-associated molecular pattern and a multi-faceted immunomodulator, Cell. Mol. Immunol. 14 (2017) 862–864.
[79] J. Zhang, J.-M. Zhou, Plant immunity triggered by microbial molecular signatures, Mol. Plant 3 (2010) 783–793.
[80] C. Yu, H. Chen, F. Tian, J.E. Leach, C. He, Differentially-expressed genes in rice infected by Xanthomonas oryzae pv. oryzae relative to a flagellin-deficient mutant reveal potential functions of flagellin in host–pathogen interactions, Rice 7 (2014) 20.
[81] M.L. Malvino, A.J. Bott, C.E. Green, T. Majumdar, S.R. Hind, Influence of flagellin polymorphisms, gene regulation, and responsive memory on the motility of Xanthomonas species that cause bacterial spot disease of solanaceous plants, Mol. Plant-Microbe Interactions® 35 (2022) 157–169.
[82] Y.-L. Gan, et al., The C-terminal domain of the type III secretion chaperone HpaB contributes to dissociation of chaperone-effector complex in Xanthomonas campestris pv. campestris, PLoS One 16 (2021) e0246033.
[83] J.-L. Lai, et al., The RNA chaperone Hfq is important for the virulence, motility and stress tolerance in the phytopathogen Xanthomonas campestris, Environ. Microbiol. Rep. 10 (2018) 542–554.
[84] A.E. Boukouris, S.D. Zervopoulos, E.D. Michelakis, Metabolic enzymes moonlighting in the nucleus: metabolic regulation of gene transcription, Trends Biochem. Sci. 41 (2016) 712–730.
[85] Y. Zhang, et al., A moonlighting role for enzymes of glycolysis in the co-localization of mitochondria and chloroplasts, Nat. Commun. 11 (2020) 4509.
[86] Z. Lu, T. Hunter, Metabolic kinases moonlighting as protein kinases, Trends Biochem. Sci. 43 (2018) 301–310.
[87] E. Henry, N. Fung, J. Liu, G. Drakakaki, G. Coaker, Beyond glycolysis: GAPDHs are multi-functional enzymes involved in regulation of ROS, autophagy, and plant immune responses, PLoS Genet. 11 (2015) e1005199.
[88] W. Guo, et al., Fructose-bisphophate aldolase exhibits functional roles between carbon metabolism and the hrp system in rice pathogen Xanthomonas oryzae pv. oryzicola, PLoS One 7 (2012) e31855.
[89] G.-T. Lu, et al., Glyceraldehyde-3-phosphate dehydrogenase of Xanthomonas campestris pv. campestris is required for extracellular polysaccharide production and full virulence, Microbiol. Read. Engl 155 (2009) 1602–1612.
[90] S. Tsuge, et al., Involvement of phosphoglucose isomerase in pathogenicity of Xanthomonas oryzae pv. oryzae, Phytopathology 94 (2004) 478–483.
[91] L. Wang, S. Makino, A. Subedee, A.J. Bogdanove, Novel candidate virulence factors in rice pathogen Xanthomonas oryzae pv. oryzicola as revealed by mutational analysis, Appl. Environ. Microbiol. 73 (2007) 8023–8027.
[92] G.-T. Lu, et al., The role of glucose kinase in carbohydrate utilization and extracellular polysaccharide production in Xanthomonas campestris pathovar campestris, Microbiol. Read. Engl. 153 (2007) 4284–4294.
[93] C. Lv, et al., Two isocitrate dehydrogenases from a plant pathogen Xanthomonas campestris pv. campestris 8004. Bioinformatic analysis, enzymatic characterization, and implication in virulence, J. Basic Microbiol. 56 (2016) 975–985.
[94] M. Briones-Martin-Del-Campo, et al., The superoxide dismutases of Candida glabrata protect against oxidative damage and are required for lysine biosynthesis, DNA integrity and chronological life survival, Microbiol. Read. Engl. 161 (2015) 300–310.
[95] V.M. Reddy, F.G. Suleman, Mycobacterium avium-superoxide dismutase binds to epithelial cell aldolase, glyceraldehyde-3-phosphate dehydrogenase and cyclophilin A, Microb. Pathog. 36 (2004) 67–74.
[96] S. Mongkolsuk, W. Whangsuk, P. Vattanaviboon, S. Loprasert, M. Fuangthong, A Xanthomonas alkyl hydroperoxide reductase subunit C (ahpC) mutant showed an altered peroxide stress response and complex regulation of the compensatory response of peroxide detoxification enzymes, J. Bacteriol. 182 (2000) 6845–6849.
[97] D.A.L. Cabrejos, et al., Structural characterization of a pathogenicity-related superoxide dismutase codified by a probably essential gene in Xanthomonas citri subsp. citri, PLoS One 14 (2019) e0209988.
[98] S.G. Smith, T.J. Wilson, J.M. Dow, M.J. Daniels, A gene for superoxide dismutase from Xanthomonas campestris pv. campestris and its expression during bacterial-plant interactions, Mol. Plant-Microbe Interact. MPMI 9 (1996) 584–593.
[99] J. Artier, et al., Comparative proteomic analysis of Xanthomonas citri ssp. citri periplasmic proteins reveals changes in cellular envelope metabolism during in vitro pathogenicity induction, Mol. Plant Pathol. 19 (2018) 143–157.

[100] S.A. Watt, V. Tellström, T. Patschkowski, K. Niehaus, Identification of the bacterial superoxide dismutase (SodM) as plant-inducible elicitor of an oxidative burst reaction in tobacco cell suspension cultures, J. Biotechnol. 126 (2006) 78–86.
[101] C. H. D. Sagawa, et al., Proteome analysis of walnut bacterial blight disease, Int. J. Mol. Sci. 21 (2020) 7453.
[102] C. Klomsiri, W. Panmanee, S. Dharmsthiti, P. Vattanaviboon, S. Mongkolsuk, Novel roles of ohrR-ohr in Xanthomonas sensing, metabolism, and physiological adaptive response to lipid hydroperoxide, J. Bacteriol. 187 (2005) 3277–3281.
[103] M.V. Kolomiets, H. Chen, R.J. Gladon, E.J. Braun, D.J. Hannapel, A leaf lipoxygenase of potato induced specifically by pathogen infection, Plant Physiol. 124 (2000) 1121–1130.
[104] X. Li, et al., Mutation of alkyl hydroperoxide reductase gene *ahpC* of *Xanthomonas oryzae* pv. *oryzae* affects hydrogen peroxide accumulation during the rice–pathogen interaction, Res. Microbiol. 165 (2014) 605–611.
[105] S. La Carbona, et al., Comparative study of the physiological roles of three peroxidases (NADH peroxidase, Alkyl hydroperoxide reductase and Thiol peroxidase) in oxidative stress response, survival inside macrophages and virulence of Enterococcus faecalis, Mol. Microbiol. 66 (2007) 1148–1163.
[106] M.L. Tondo, S. Petrocelli, J. Ottado, E.G. Orellano, The monofunctional catalase KatE of Xanthomonas axonopodis pv. citri is required for full virulence in citrus plants, PLoS One 5 (2010) e10803.
[107] S.Y. Yi, D. Choi, C.-M. Ryu, Implication of a pepper h-type thioredoxin in type I- and II-nonhost resistance to Xanthomonas axonopodis, Plant Biotechnol. Rep. 1 (2007) 117–123.
[108] V. Amblee, C.J. Jeffery, Physical features of intracellular proteins that moonlight on the cell surface, PLoS One 10 (2015) e0130575.
[109] S.E. Rollauer, M.A. Sooreshjani, N. Noinaj, S.K. Buchanan, Outer membrane protein biogenesis in Gram-negative bacteria, Philos. Trans. R. Soc. B Biol. Sci. 370 (2015) 20150023.
[110] D.E. Whitworth, Myxobacterial vesicles death at a distance? Adv. Appl. Microbiol. 75 (2011) 1–31.
[111] D.E. Whitworth, B.H. Morgan, Synergism between bacterial GAPDH and OMVs: disparate mechanisms but Co-operative action, Front. Microbiol. 6 (2015) 1231.
[112] N. Noinaj, M. Guillier, T.J. Barnard, S.K. Buchanan, TonB-dependent transporters: regulation, structure, and function, Annu. Rev. Microbiol. 64 (2010) 43–60.
[113] S. Blanvillain, et al., Plant carbohydrate scavenging through tonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria, PLoS One 2 (2007) e224.
[114] S. Dupoiron, et al., The N-Glycan Cluster from Xanthomonas campestris pv. campestris, J. Biol. Chem. 290 (2015) 6022–6036.
[115] M. Fujita, et al., A TonB-dependent receptor constitutes the outer membrane transport system for a lignin-derived aromatic compound, Commun. Biol. 2 (2019) 1–10.
[116] F.-J. Vorhölter, et al., Involvement of bacterial TonB-dependent signaling in the generation of an oligogalacturonide damage-associated molecular pattern from plant cell walls exposed to Xanthomonas campestris pv. campestris pectate lyases, BMC Microbiol. 12 (2012) 239.
[117] E.A. Newberry, et al., Independent evolution with the gene flux originating from multiple Xanthomonas species explains genomic heterogeneity in Xanthomonas perforans, Appl. Environ. Microbiol. 85 (2019) e00885, 19.
[118] R.M. Ferreira, et al., Unravelling potential virulence factor candidates in Xanthomonas citri. subsp. citri by secretome analysis, PeerJ 4 (2016) e1734.
[119] S. Kim, et al., Time-resolved pathogenic gene expression analysis of the plant pathogen Xanthomonas oryzae pv. oryzae, BMC Genom. 17 (2016) 345.
[120] S.K. Ray, R. Rajeshwari, Y. Sharma, R.V. Sonti, A high-molecular-weight outer membrane protein of Xanthomonas oryzae pv. oryzae exhibits similarity to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence, Mol. Microbiol. 46 (2002) 637–647.
[121] Y. Ma, et al., Fungal cellulase is an elicitor but its enzymatic activity is not required for its elicitor activity, Mol. Plant Pathol. 16 (2015) 14–26.
[122] K. Saravanakumar, et al., Cellulase from Trichoderma harzianum interacts with roots and triggers induced systemic resistance to foliar disease in maize, Sci. Rep. 6 (2016) 35543.
[123] J. Hausner, N. Hartmann, M. Jordan, D. Büttner, The predicted lytic transglycosylase HpaH from Xanthomonas campestris pv. vesicatoria associates with the type III secretion system and promotes effector protein translocation, Infect. Immun. 85 (2017) e00788, 16.
[124] A.C.P. Oliveira, et al., Transposons and pathogenicity in Xanthomonas: acquisition of murein lytic transglycosylases by TnXax1 enhances Xanthomonas citri subsp. citri 306 virulence and fitness, PeerJ 6 (2018).
[125] L. Wang, et al., Two lytic transglycosylases of Xanthomonas campestris pv. campestris associated with cell separation and type III secretion system, respectively, FEMS Microbiol. Lett. 366 (2019) fnz073.
[126] A.C.P. de Oliveira, et al., XAC4296 is a multifunctional and exclusive Xanthomonadaceae gene containing a fusion of lytic transglycosylase and epimerase domains, Microorganisms 10 (2022) 1008.
[127] G. Aparna, A. Chatterjee, R.V. Sonti, R. Sankaranarayanan, A cell wall–degrading esterase of Xanthomonas oryzae requires a unique substrate recognition module for pathogenesis on rice, Plant Cell 21 (2009) 1860–1873.
[128] R. Nascimento, et al., The type II secreted lipase/esterase LesA is a key virulence factor required for Xylella fastidiosa pathogenesis in grapevines, Sci. Rep. 6 (2016) 18598.
[129] D. Sinha, M.K. Gupta, H.K. Patel, A. Ranjan, R.V. Sonti, Cell wall degrading enzyme induced rice innate immune responses are suppressed by the type 3 secretion system effectors XopN, XopQ, XopX and XopZ of Xanthomonas oryzae pv. oryzae, PLoS One 8 (2013) e75867.
[130] G. Astua-Monge, et al., Expression profiling of virulence and pathogenicity genes of Xanthomonas axonopodis pv. citri, J. Bacteriol. 187 (2005) 1201–1205.
[131] G. Tapia-Pastrana, L. Chavez-Dueñas, H. Lanz-Mendoza, K. Teter, F. Navarro-García, VirK is a periplasmic protein required for efficient secretion of plasmid-encoded toxin from enteroaggregative Escherichia coli, Infect. Immun. 80 (2012) 2276–2285.
[132] A. Furutani, et al., Identification of novel HrpXo regulons preceded by two cis-acting elements, a plant-inducible promoter box and a −10 box-like sequence, from the genome database of Xanthomonas oryzae pv. oryzae, FEMS Microbiol. Lett. 259 (2006) 133–141.
[133] S. Fenselau, U. Bonas, Sequence and expression analysis of the hrpB pathogenicity operon of Xanthomonas campestris pv. vesicatoria which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems, Mol. Plant-Microbe Interact. MPMI 8 (1995) 845–854.
[134] G. Quilici, et al., Solution structure of the BPSL1445 protein of Burkholderia pseudomallei reveals the SYLF domain three-dimensional fold, ACS Chem. Biol. 17 (2022) 230–239.
[135] G. Hester, H. Kaku, I.J. Goldstein, C.S. Wright, Structure of mannose-specific snowdrop (Galanthus nivalis) lectin is representative of a new plant lectin family, Nat. Struct. Biol. 2 (1995) 472–479.
[136] M.G.K. Ghequire, et al., Structural determinants for activity and specificity of the bacterial toxin LlpA, PLoS Pathog. 9 (2013) e1003199.
[137] P.E. De Los Santos, A.H.A. Parret, R. De Mot, Stress-related Pseudomonas genes involved in production of bacteriocin LlpA, FEMS Microbiol. Lett. 244 (2005) 243–250.
[138] P.W. Postma, J.W. Lengeler, G.R. Jacobson, Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria, Microbiol. Rev. 57 (1993) 543–594.
[139] S. Kasem, S. Prathuangwong, S. Tsuyumu, Evidence that ppsA in Xanthomonas Axonopodis Pv . Glycines Affects Carbon Utilization and Secretion of Virulence Factors, 2008.
[140] D.-J. Tang, et al., Xanthomonas campestris pv. campestris possesses a single gluconeogenic pathway that is required for virulence, J. Bacteriol. 187 (2005) 6231.
[141] D.M. Weller, A.W. Saettler, Rifampin-resistant Xanthomonas phaseoli var. fuscans and Xanthomonas phaseoli: tools for field study of bean blight bacteria, Phytopathology 68 (1978) 778–781.
[142] R. Qamra, P. Prakash, B. Aruna, S.E. Hasnain, S.C. Mande, The 2.15 A crystal structure of Mycobacterium tuberculosis chorismate mutase reveals an unexpected gene duplication and suggests a role in host-pathogen interactions, Biochemistry 45 (2006) 6997–7005.
[143] O.A. Asojo, et al., Crystal structure of chorismate mutase from Burkholderia phymatum, Acta Crystallogr. Sect. F Struct. Biol. Commun. 74 (2018) 187–192.
[144] S.A. Alrumman, et al., Production and anticancer activity of an L-asparaginase from Bacillus licheniformis isolated from the red sea, Saudi Arabia, Sci. Rep. 9 (2019) 3756.
[145] M. Kanehisa, S. Goto, KEGG: Kyoto encyclopedia of genes and genomes, Nucleic Acids Res. 28 (2000) 27–30.

[146] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.
[147] M. Ashburner, et al., Gene ontology: tool for the unification of biology. The Gene Ontology Consortium, Nat. Genet. 25 (2000) 25–29.
[148] F. Teufel, et al., SignalP 6.0 predicts all five types of signal peptides using protein language models, Nat. Biotechnol. 40 (2022) 1023–1025.
[149] T. Paysan-Lafosse, et al., InterPro in 2022, Nucleic Acids Res. 51 (2023) D418–D427.
[150] The UniProt Consortium, UniProt: the universal protein knowledgebase in 2023, Nucleic Acids Res. 51 (2023) D523–D531.
[151] C.T. Porter, G.J. Bartlett, J.M. Thornton, The Catalytic Site Atlas: a resource of catalytic sites and residues identified in enzymes using structural data, Nucleic Acids Res. 32 (2004) D129–D133.
[152] F. Sievers, D.G. Higgins, Clustal Omega for making accurate alignments of many protein sequences, Protein Sci. 27 (2018) 135–145.
[153] D.E. Kim, D. Chivian, D. Baker, Protein structure prediction and analysis using the Robetta server, Nucleic Acids Res. 32 (2004) W526–W531.
[154] Y. Ye, A. Godzik, FATCAT: a web server for flexible structure comparison and structure similarity searching, Nucleic Acids Res. 32 (2004) W582–W585.
[155] J. Lee, H.-J. Lee, M.-K. Shin, W.-S. Ryu, Versatile PCR-mediated insertion or deletion mutagenesis, Biotechniques 36 (2004) 398–400.
[156] K. Kaniga, I. Delor, G.R. Cornelis, A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*, Gene 109 (1991) 137–141.
[157] M.L. Laia, et al., New genes of Xanthomonas citri subsp. citri involved in pathogenesis and adaptation revealed by a transposon-based mutant library, BMC Microbiol. 9 (2009) 12.
[158] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675.