Celebrating the 20th Anniversary of the First *Xanthomonas* Genome Sequences – How Genomics Revolutionized Taxonomy, Provided Insight into the Emergence of Pathogenic Bacteria, Enabled New Fundamental Discoveries and Helped Developing Novel Control Measures – A Perspective from the French Network on Xanthomonads

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Nothing in Biology Makes Sense Except in the Light of Evolution

(Theodosius Dobzhansky, 1973)

— 50 years ago —

PROLOGUE

Since 2008, the French Network on Xanthomonads (FNX) unites those French research teams working on phytopathogenic bacteria of the *Xanthomonadaceae* family, now designated as *Lysobacteraceae* [Naushad et al. 2015], including three genera (*Xanthomonas*, *Xylella* and *Stenotrophomonas*) responsible for diseases on most major crops (rice, wheat and other cereals, banana, citrus, cabbage, common beans, pepper, tomato, sugarcane, coffee, grape, olive, etc.). This academic network integrates a large range of expertise (incl. diagnostics, epidemiology, phylogenetics, molecular biology, bacterial and plant genetics, comparative genomics, transcriptomics, proteomics, metabolomics) and coordinates research on xanthomonads in interaction with their biotic and abiotic environment.

FNX activities involve hosting of students in the partner laboratories, co-supervision of PhD students, organization of workshops, training courses and international meetings, coordination of collaborative R&D projects, and organization of annual scientific meetings. In the last years, the network has extended internationally thanks to the coordination of the EuroXanth COST Action (2017-2021) and to the southern hemisphere through the International Scientific Coordination Network ‘NSSN-X’ (2019-2023, Burkina Faso, Ivory Coast, Mali, Colombia and Ecuador).

The FNX consortium has been established to perpetuate and strengthen these national and international partnerships, which contribute to the development of solutions for the sustainable management and anticipation of emerging diseases caused by members of the *Lysobacteraceae*. Here, select players in the consortium provide their

personal view on what they consider as breakthrough discoveries in our field since bacterial genomics had revolutionized molecular plant pathology 20 years ago.

Nicolas W. G. Chen (Lecturer in phytopathology at L'Institut Agro; Molecular plant pathologist and genomicist specialized on legume-infecting xanthomonads): Two-decade history of *Xanthomonas* genomes

The information contained in genomes is a gold mine for us as plant pathologists, allowing us to improve diagnostics and search for traits involved in epidemiology and plant-microbes interactions, as well as the evolutionary processes behind them. The year 2022 marked the 20th anniversary of the first two *Xanthomonas* whole-genome sequences published in Nature [da Silva et al. 2002]. I joined the *Xanthomonas* community ten years later to work on host adaptation, and this publication was one of the very first *Xanthomonas* papers I read. A central aspect of this work was the comparison of two *Xanthomonas* pathovars, *Xanthomonas citri* pv. *citri* and *Xanthomonas campestris* pv. *campestris*, pathogenic on citrus and Brassicaceae, respectively. This approach allowed the authors to identify strain-specific genes and to propose mechanisms that may explain the distinct host specificities and pathogenic processes, two burning issues in our community [Harris et al. 2020; Jacques et al. 2016]. This comparative genomics analysis was pioneering in many ways and it took more than three years for the next *Xanthomonas* genomes to be published. A few years later, whole genome sequencing was 'democratized' with the advent of ever faster and cheaper sequencing technologies [Zhao and Grant 2011], soon leading to the release of several dozens, then several hundreds of *Xanthomonas* genome sequences per year (Fig. 1).

The accumulation of genomic data has led to an increasingly precise search for genes that may be involved in contrasting pathogenic processes. In 2011, comparative analysis of ten *Xanthomonas* genomes allowed to define clusters of genes that might be linked to monocotyledonous versus dicotyledonous host plants, and/or to vascular vs. non-vascular lifestyles [Bogdanove et al. 2011]. Later on, a remarkable combination of genomics and functional analyses highlighted the cellobiohydrolase CbsA as a key factor of tissue specificity [Gluck-Thaler et al. 2020]. Strikingly, heterologous expression of *cbsA* was sufficient to confer vascular pathogenesis to the non-vascular pathogen

Xanthomonas transluscens pv. *undulosa*. Analysis of presence/absence and horizontal gene transfers led the authors to hypothesize that repeated gain and loss of *cbsA* modulated the evolution of vascular versus non-vascular lifestyles. Remarkably, da Silva et al. had also mentioned cellobiohydrolases as possible factors for the symptom differences observed between citrus and Brassicaceae diseases [da Silva et al. 2002]. This fact further emphasises that these plant cell wall-degrading enzymes play a key role in the interaction between *Xanthomonas* and plants, and once again demonstrates the power of comparative genomics in uncovering important genes in the interaction between plants and pathogens.

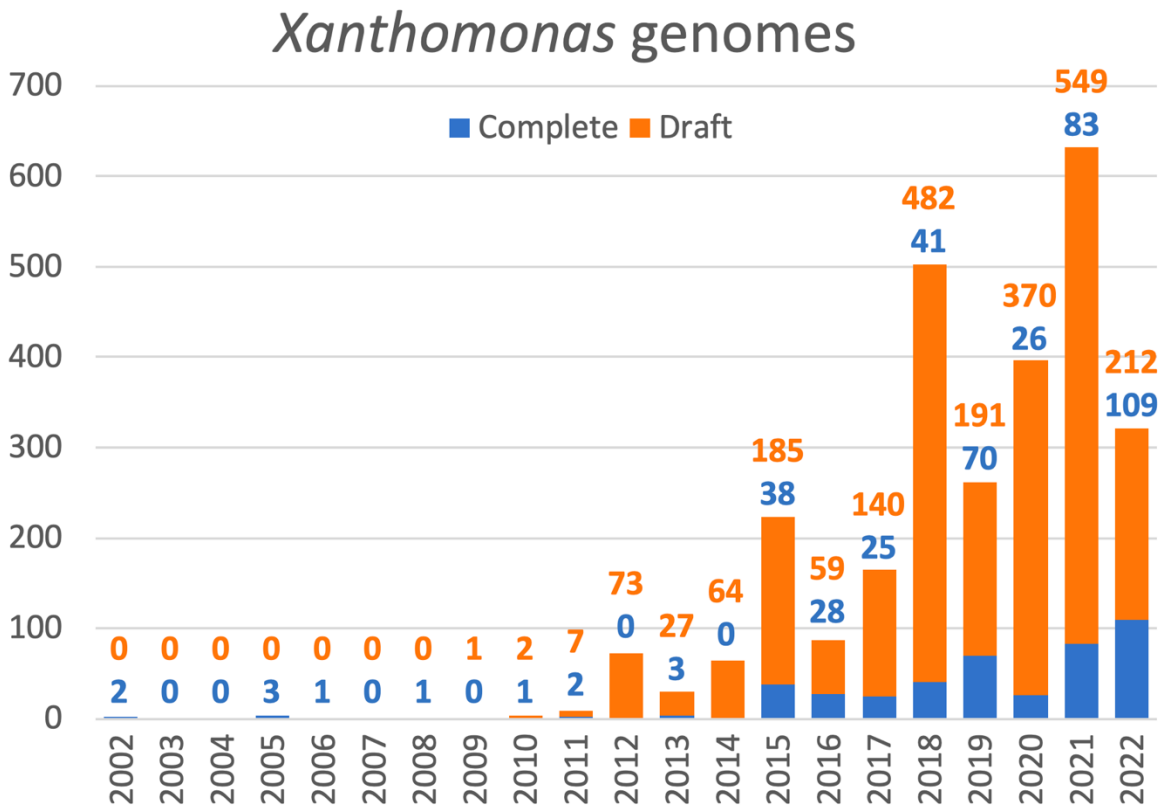

Fig. 1. NCBI *Xanthomonas* genome statistics (as of 13 July 2023). *Xanthomonas* genome assembly metadata were extracted from NCBI GenBank at https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=338. GenBank assembly levels 'Contig', 'Scaffold' and 'Chromosome' were considered together as Draft level. The complete list of genomes and relevant metadata are available in Supplementary Table S1.

I've always been fascinated how the study of DNA sequences allows us to go back in time, explore events that happened in the past and gain insight into what could happen

next. Over the past decade, the field of ancient genomics has yielded considerable contributions to the study of emergence and evolutionary history of various pathogens, including those affecting crops. In this context, herbarium collections have been an important source of dated, identified and preserved DNA samples, whose use in comparative genomics and phylogeography may shed light into the emergence and evolutionary history of plant pathogens. Recently, thirteen genome sequences of *X. citri* pv. *citri* were reconstructed from infected historical *Citrus* specimens dating back to 1845 [Campos et al. 2023]. Following authentication based on ancient DNA damage patterns, the authors compared them with a large set of modern genomes. Their analyses indicated that historical genomes had similar characteristics to contemporary *X. citri* pv. *citri* strains, including a complete type 3 secretion system. Remarkably, integration of the ancient genomes brought temporal signals to perform phylogenetic tip-dating inferences. Their findings revealed that *X. citri* pv. *citri* originated in Southern Asia around 11,500 years ago and diversified during the beginning of the 13th century, after *Citrus* diversification and before spreading to the rest of the world. This evolutionary scenario connects the *X. citri* pv. *citri* specialization to the Neolithic climatic change and revolution of agriculture, and links *X. citri* pv. *citri* diversification with the human-driven expansion of citriculture through the early East-West trade and later colonization. More generally, this work has demonstrated that ancient genomes can be considered as important sources of evolutionary information that can help us better understand past, present and future *Xanthomonas* epidemics.

The rise of the genomic era has led to many more discoveries and insights, some of which will be addressed by my colleagues below.

Laurent D. Noël (Researcher at CNRS Toulouse; Molecular geneticist of *Xanthomonas* pathogenicity and plant immunity, *Xanthomonas campestris*/Brassicaceae pathosystem): What do xanthomonads have to teach us about host specificity?

Nearly thirty years after my first encounter with *Xanthomonas*, I am still amazed by how little we know and learned about host specificity in the plant pathogen community despite the broad scientific and economic relevance of this question in terms of understanding and predicting host jumps and epidemics [Wernham 1948]. The

Xanthomonas genus could appear as a perfect system because, while *Xanthomonas* are collectively able to infect nearly every living vascular plant, each species or subspecies has usually a narrow host range: *Xanthomonas oryzae* infects only rice, *X. campestris* only brassicas, *X. citri* pv. *citri* only citrus, etc. The FNX community wrote a review on this topic which, rather than highlighting the extent of our knowledge, evidenced the lack of genetic or molecular data supporting the determinism of host specificity [Jacques et al. 2016].

A paper that has been particularly puzzling me is a simple comparative study of type 3 effector gene content in xanthomonads [Hajri et al. 2009]. The authors concluded that phylogenetically distinct species of *Xanthomonas* infecting common beans share a similar effector gene repertoire in what was coined as 'repertoire-for-repertoire' hypothesis, reminiscent of Flor's 'gene-for-gene' hypothesis [Flor 1955]. This correlative evidence suggested that host plants would impose a strong selective pressure on the composition of the effector gene repertoire, thus defining which *Xanthomonas* can be pathogenic on which plant(s). This observation goes much beyond the role of some effectors simply restricting host range such as *xopQ* recognition in *Nicotiana benthamiana* [Adlung et al. 2016]. Type 3 effector repertoires are highly variable, even at the intra-specific level, and their functional characterization is essentially restricted to one-gene-at-a-time analyses [Arroyo-Velez et al. 2020]. Their systematic and comprehensive investigation has only now become easier thanks to advanced genome editing protocols [Li et al. 2023]. In parallel, synthetic biology will enable the construction of mini-effector repertoires and provide new perspectives to explore how effector repertoires function and determine, at least in part, host specificity [Cunnac et al. 2011; Ruiz-Bedoya et al. 2023].

Marion Fischer-Le Saux (Researcher at INRAE in Angers with a research focus on emergence, systematics and ecology of plant-associated bacteria): Systematics of bacterial plant pathogens and appreciating non-pathogenic strains

I came to plant pathology via microbial systematics. When I was asked to select key papers that have shaped my thinking, I thought of a handful of papers that represent milestones in the development of this discipline and in our understanding of the diversity and phylogeny of the *Xanthomonas* genus.

When I started to work on *Xanthomonas*, my reference paper was Vauterin *et al*. (1995), a comprehensive taxonomic study based on DNA-DNA hybridizations that contributed to classifying the overwhelming number of pathovars [Vauterin et al. 1995]. One should bear in mind that in the late 20th century and still in the early 2000's it was almost a 'mission: impossible' to determine the taxonomic position of a strain without performing the cumbersome DNA-DNA hybridizations. This method I routinely used during my PhD required huge quantities of high-quality DNA and cautious handling of tritium, a long-lived radionuclide. For phylogenetic studies and molecular identification, 16S rRNA gene sequencing was the gold standard, even though, within *Xanthomonas*, it does not include information at the species level [Hauben et al. 1997].

One of the first well-resolved phylogenetic trees of the *Xanthomonas* genus was based on the partial sequencing of four housekeeping genes (Multilocus Sequence Analysis, MLSA) [Young et al. 2008]. The authors demonstrated that strains from the same species (as determined by DNA-DNA hybridizations) formed tight clusters, well-separated from other species. Thus, MLSA represented a powerful tool to classify *Xanthomonas* strains and to determine their phylogenetic relationships. One year later it was shown that partial sequencing of the *gyrB* gene alone made it possible to classify almost all pathovars of *Xanthomonas* into species-level clades [Parkinson et al. 2009]. It was so amazing to realize that such a small part of the genome (530 bp) contained the same level of information, from a taxonomic perspective, as the whole genome DNA relatedness determined with the tedious DNA-DNA hybridizations! This work revealed that many pathovars needed to be reclassified and inspired a series of taxonomic papers to improve the delimitation of *Xanthomonas* species.

Even though Young and coworkers had demonstrated the usefulness of MLSA for species delineation within the *Xanthomonas* genus and described a new species, *Xanthomonas dyei*, based on this method [Young et al. 2010], the new gold standard that has ultimately replaced DNA-DNA hybridizations is Average Nucleotide Identity (ANI) [Konstantinidis and Tiedje 2005]. The first *Xanthomonas* species described with ANI was *Xanthomonas maliensis* [Triplett et al. 2015]. This article focused on non-pathogenic strains isolated from rice, which was a new direction in the field at that time. Indeed, in previous decades, little attention had been paid to non-pathogenic strains, precluding our

ability to embrace the real diversity of the genus. Since then, the growing interest in non-pathogenic strains has enabled the description of several new species [Mafakheri et al. 2022; Martins et al. 2020; Vicente et al. 2017] and provided novel insight in *Xanthomonas* evolution and pathogen adaptation.

Population genetics tools applied to a collection of pathogenic and non-pathogenic strains allowed to unveil an epidemic population structure in *Xanthomonas arboricola* [Merda et al. 2016], similar to what was found in some human pathogens [Maynard-Smith et al. 2000]. Moreover, robust phylogenies and comparative genomics gave insight in the gain and loss of pathogenicity genes and enabled proposing molecular scenarios of patho-adaptation [Jacobs et al. 2015; Merda et al. 2017], thus highlighting the relevance of *Xanthomonas* as a model to study pathogen emergence.

Perrine Portier (Director of CIRM-CFBP, the French Collection for Plant-associated Bacteria, at INRAE Angers. https://cirm-cfbp.fr): Strain classification in the genomics era

As the curator of CIRM-CFBP (https://cirm-cfbp.fr), the French Collection for Plant-associated Bacteria, which preserves strategic resources for plant health, my interest lies in the diversity and phylogeny of plant-pathogenic bacteria. Managing these resources was my entry point into the world of *Xanthomonas*. *Xanthomonas* indeed makes up a large part of the resources we have in our collection. As the contents of the collection reflect over time the research interests of the plant-pathogenic bacteria research community, this illustrates the importance of *Xanthomonas* for managing plant health.

Accurate identification of a pathogen can provide a lot of information about the ecology of this organism, the threat it represents to crops and inform us on how to mitigate its impact through appropriate management techniques. Indeed, the first question the users of the collection ask is 'what is this strain?'. This question seems trivial at first. All of us hope that scientists who deposited these strains in a collection and curators took great care of the identification of the resources. In recent decades, access to DNA sequences has completely reshaped the taxonomy of bacteria, including *Xanthomonas*.

Since the first genus-scale study of *Xanthomonas* phylogeny using DNA-DNA hybridization [Vauterin et al. 1995], many authors refined the taxonomy of the genus. One of the most prominent papers in the field addressed the diversity of strains that cause

anthurium bacterial blight, assumed to belong to *Xanthomonas axonopodis* pv. *dieffenbachiae*. [Constantin et al. 2016]. At that time, members of the species *X. axonopodis* were already well known to be very diverse and thought to belong to several genetic groups that may even represent several species [Ferreira-Tonin et al. 2012; Parkinson et al. 2009; Rademaker et al. 2005]. Constantin and co-workers used a polyphasic approach, combining the partial sequences of seven house-keeping genes, DNA-DNA hybridization, ANI calculation and phenotypic characteristics which demonstrated the important diversity within the *X. axonopodis* species. Starting with anthurium-pathogenic strains, this article revealed the need to redescribe the entire *X. axonopodis* species complex. The former *X. axonopodis* species was divided into four species: *Xanthomonas euvesicatoria*, *Xanthomonas phaseoli*, *X. citri* and *X. axonopodis*. It's interesting to note that these newly described or redefined species corresponded relatively nicely to the groups defined by Rademaker and co-workers in 2005, *X. euvesicatoria* corresponding to Rademaker group 9.2, *X. axonopodis* to group 9.3, *X. phaseoli* to group 9.4 and *X. citri* to groups 9.5 and 9.6.

Following this work, similar taxonomic refinements were performed, leading for instance to the re-definitions of the species *Xanthomonas hortorum* and *X. arboricola* [Morinère et al. 2020; Zarei et al. 2022]. In parallel, further new *Xanthomonas* species were discovered and described. The genus *Xanthomonas* now consists of 32 valid species, 8 of which have been described since 2015 (Fig. 2). Interestingly, some of these new species concern strains associated with plants but not necessarily being pathogenic to them (*X. maliensis*, *Xanthomonas nasturtii*, *Xanthomonas floridensis*, *Xanthomonas euroxanthea*) showing a shift in the plant-pathology community, taking more into account the ecology of the plant and its accompanying microorganisms in its environment [Martins et al. 2020; Triplett et al. 2015; Vicente et al. 2017].

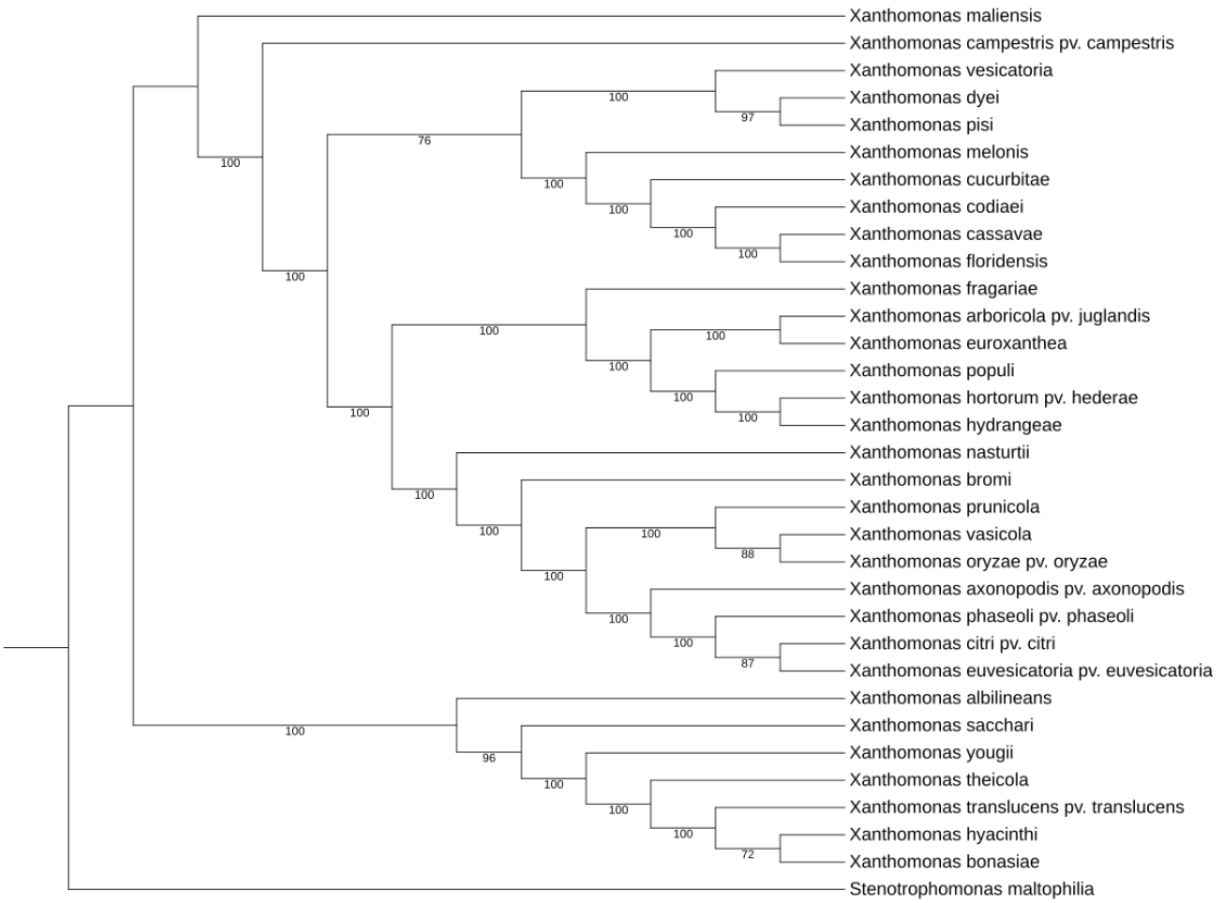

Fig. 2. Phylogenetic tree of the 32 valid species of *Xanthomonas* provided after TYGS analysis [Meier-Kolthoff et al. 2022]. Tree inferred with FastME 2.1.6.1 [Lefort et al. 2015] from GBDP distances calculated from genome sequences retrieved from Genbank. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers on branches are GBDP pseudo-bootstrap support values > 70% from 100 replications, with an average branch support of 97.2% [Farris 1972]. The Newick file was edited in iTOL https://itol.embl.de/ and rooted on the outgroup *Stenotrophomonas maltophilia*. The complete list of genomes and GenBank Assembly accession numbers are available in Supplementary Table S2.

The work published by Constantin and co-workers opened the way to a systematic taxonomic (re)classification of strains within the genus *Xanthomonas* [Constantin et al. 2016]. However, the strains belonging to the Rademaker group 9.1 were not considered by Constantin et al. One of the reasons may be because only a few strains of this group are available in the collections worldwide. Though, the pathovars of this group (including pv. *begoniae* and pv. *spondiae*) still have an unclear taxonomic status. As the other Rademaker's groups, this group corresponds probably to a separate species but more

work is necessary to clarify that. More strains available in the culture collections are also necessary to better represent this part of the *Xanthomonas* diversity. Moreover, if Constantin's work reclassified some pathovars from the former *X. axonopodis* species complex, a lot of pathovars are still not formally classified. Recently, Bansal and collaborators (2021) published the reclassification of twenty pathovars within the species *X. citri* and Harrison et al. (2023) did the same for twenty pathovars reclassified within the species *X. euvesicatoria.* These investigations further contribute to the complete and current classification of strains from various origins belonging to the genus *Xanthomonas* [Bansal et al. 2022; Harrison et al. 2023]. The easy access to the complete genome sequences of bacteria speared a profound taxonomic revolution within *Xanthomonas*, which is still underway, and shed light on the work still needed to be done to have a clear understanding of the diversity of this genus.

Marie-Agnès Jacques (Researcher at INRAE Angers; Bacteriologist interested in seed health and epidemiology of plant pathogenic bacteria), Olivier Pruvost (Researcher at CIRAD La Réunion; Bacteriologist interested in population biology of xanthomonads), Adrien Rieux (Researcher at CIRAD La Réunion; Population genomicist): Emergence of *Xylella fastidiosa* in Europe

As European plant pathologists involved in eco-evolutionary analyses of emerging *Xanthomonas* populations, we have been deeply interested in the emergence of *Xylella fastidiosa* in centuries-old olive groves in southern Italy a decade ago, and subsequently in other crops and EU territories [EFSA 2013, 2019]. Despite *Xylella* being phylogenetically related to *Xanthomonas* [Naushad et al. 2015], the two genera differ strikingly in their biological characteristics. *X. fastidiosa* is a fastidious, insect-transmitted bacterium and is an obligate colonizer of its host plants and insect vectors [Rapicavoli et al. 2018; Sicard et al. 2018]. In contrast to strains of *Xanthomonas*, which exhibit a high degree of host specialization [Jacques et al. 2016], *X. fastidiosa* is a more generalist pathogen, with some lineages able to infect several distinct plant families [Landa et al. 2022]. The wide range of *Xanthomonas* niches linked to the colonization of plant organ surfaces, parenchyma, and xylem contrasts as well with the narrow niches of *Xylella*, inhabiting only the plant xylem and the insect foregut.

The first genome of a phytopathogenic bacterium to be sequenced was that of a strain of *X. fastidiosa* in 2000, which has a relatively small genome of 2.8 Mbp in size, roughly half the size of a typical *Xanthomonas* genome [Simpson et al. 2000]. This first genomic resource opened the way for developing reliable molecular detection and typing tools that are key for the epidemio-surveillance of this pathogen. Since then, the numerous genomic resources available for this phytopathogenic species have been used to identify possible mechanisms involved in the expansion of its host range, its biological peculiarities, as well as to date and understand the scenarios of introduction in Europe [Donegan et al. 2023; Dupas et al. 2023; Gerlin et al. 2020; Landa et al. 2022].

The work conducted by colleagues from the Balearic Islands, Spain, is particularly remarkable and comprehensive as it has made it possible to trace and date back the introduction of several strains of *X. fastidiosa* into Majorca from California [Moralejo et al. 2019, 2020]. These strains are responsible for the decline of almond trees (*X. fastidiosa* subsp. *fastidiosa* ST1, *X. fastidiosa* subsp. *multiplex* ST7 and ST81), the symptoms of which were observed from 2003 onwards, and Pierce's disease of grapevine (*X. fastidiosa* subsp. *fastidiosa* ST1). The interest of their work is the complementarity of the numerous analyses that were used from (i) epidemiology with large inventories of *X. fastidiosa* genotypes and their distribution, measurements of the incidence and the severity of the disease in numerous orchards and vineyards, epidemic dynamics by the analysis of Google street-view panoramic images, and the numerous pathogenicity and transmission tests, (ii) dendrochronology (dating of growth rings, detection by qPCR in the rings of trees being uprooted and appropriate statistical analysis), (iii) climatic data, (iv) phylogenomics (ML and Bayesian trees and tip-dating), up to (v) technical literature analysis to establish possible links between the Majorcan and Californian almond industries. Finally, the most probable scenario was that almond scions infected or co-infected with ST1 and ST81 strains were introduced in 1993 in Majorca from California. In Majorca, they were grafted onto local rootstocks. The pathogens spread on the island thanks to the spittlebug *Philaenus spumarius*, which also led to the infection of many other species, including wild olive trees (*X. fastidiosa* subsp. *multiplex*) and grapevines (*X. fastidiosa* subsp. *fastidiosa*). The favorable dynamics of the grapevine production and concomitantly unfavorable conditions of almond production in Majorca, linked to many socio-economic

reasons, periods of drought and high incidence of fungal pathogens have led to very different management of vineyards and almond orchards that currently present low and very high incidences of diseases due to *X. fastidiosa*, respectively. This type of study clearly justifies the modification of the control measures that shifted from eradication to containment as a consequence of the long-overlooked presence and dissemination of *X. fastidiosa* in the Balearic Islands.

Understanding the mechanisms leading to pathogen adaptation, either to a new crop or environmental condition is one of our top research priorities since it is a first step towards the development and implementation of effective management and control strategies. Such research appears particularly pertinent for emerging pathogens such as *X. fastidiosa* with a capacity to adapt to multiple crops as well as having expanding geographic and host ranges. In this context, current knowledge suggests that subsp. *fastidiosa* was introduced once from Central America to the United States ~150 years ago, where the bacteria would have acquired the ability to infect grapevines and led to the emergence of Pierce's disease of grapevines [Vanhove et al. 2019]. To better understand how *X. fastidiosa* evolved with the emergence of a novel plant disease and diversified in allopatry in different regions of the United States, Castillo and co-workers sampled, sequenced and analyzed the entire genome of 175 isolates using various population genomic inference methods [Castillo et al. 2021]. Their findings first highlighted that following their establishment in the US, Pierce's disease-causing strains split into two populations on the East and West Coasts, from which subsequent introductions in Taiwan and Spain occurred, respectively. Interestingly, their results also demonstrated that *X. fastidiosa* populations diversified via multiple co-occurring evolutionary forces acting at both intra- and inter-population level via changes in gene content (gain/loss) and variations in nucleotide sequence (mutation and recombination). Compared to natural environments, the reduced diversity of monoculture agricultural landscapes can help bacterial plant pathogens to quickly adapt to local biological and ecological conditions, ultimately leading to pathogen specialization.

It has been known for some time that bacteria in the *X. fastidiosa* species are naturally competent [Kung and Almeida 2011]. Homologous recombination, first detected from genotyping data such as MLST (MultiLocus Sequence Typing), was suggested as a

significant but yet not paramount driver of evolution as compared to some other freely recombining bacteria [Scally et al. 2005]. An important contribution came from Potnis and co-workers, who investigated recombination patterns in outbreak strains and in strains evolving in lab experiments (*i.e.*, as the result of natural transformation) [Potnis et al. 2019]. The authors analyzed the ecological significance from whole genome sequence data in relation to the infraspecific genetic structure of *X. fastidiosa*. Genomic Integration of 'foreign' DNA though natural transformation consisted of fragments 2–31 kb in size. Evidence of extensive, but yet sublineage-dependent, intra- and inter-subspecific recombination that occurred in the course of *X. fastidiosa* evolution was found. In some cases, *e.g.*, mulberry (*Morus alba*) or oleander (*Nerium oleander*) isolates, genomes appeared chimeric between subsp. *fastidiosa* and subsp. *multiplex.* Comparing experimentally evolved strains and strains from outbreaks or interceptions indicated genomic regions recurrently showing recombination, suggesting several recombination hotspots in *X. fastidiosa* genomes. In these recombinant regions, genes involved in regulatory and signaling cascades, such as genes encoding for production and sensing of the quorum sensing diffusible signal factor, or genes shown to be important for virulence or nutrient acquisition were detected at high frequencies. Globalization as a whole facilitates a sympatric occurrence (*i.e.*, co-infections) of distinct lineages in the same ecological niche, favoring DNA exchange among strains and the possible emergence of some recombinogenic strains for which the rearrangement(s) provide(s) a selective advantage.

Given the disastrous impact *X. fastidiosa* has had in Europe since its first outdoor detection in 2013, numerous prevention and mitigation measures are being applied [EFSA 2023]. The early detection of *X. fastidiosa* in crops by canine olfactory detection as achieved for Candidatus *Liberibacter asiaticus* [Gottwald et al. 2020] and/or the use of spectral imaging [Zarco-Tejada et al. 2018], further confirmed by in-field molecular detection by LAMP or RPA-based assays [Cesbron et al. 2023], are, in our view, the most promising in terms of application potential. The use of modelling to identify the most favourable areas for the establishment of the bacterium [Martinetti and Soubeyrand 2019], based on currently infected areas per subspecies and the known host range will be a considerable aid to the effectiveness of surveillance of the territory, further allowing (i) the

precise knowledge of strains associated with these new outbreaks through evolutionary genomics studies, and (ii) the implementation of control methods (use of resistant varieties, for example) in the areas most at risk for its spread to mitigate its impact.

Ralf Koebnik (Researcher at IRD Montpellier; Molecular microbiologist and bacterial geneticist with special interest in protein secretion and comparative genomics): Type 4 protein secretion – another mechanism to kill bacteria

Ever since I first immersed myself in scientific research in 1979, when I was cleaning disposable pipette tips and was developing Maxam-Gilbert DNA sequencing X-ray films at what is now the Max Delbrück Center for Molecular Medicine in Berlin (Germany), I have been fascinated by protein secretion and other phenomena on lipid bilayer membranes. It is therefore no surprise that I consider the discovery of type 4 protein secretion as a weapon in bacterial competition in *Xanthomonas* as one of the outstanding discoveries in recent years.

The story began about two decades ago, when Chuck Farak and collaborators were looking for protein-protein interactions in type 3 and type 4 secretion systems (T3SS, T4SS) once the genome sequence of *X. citri* was available. Using yeast two-hybrid assays they found a set of previously uncharacterized *Xanthomonas* proteins to interact with VirD4, whose gene is adjacent to the chromosomal type 4 secretion *virB* locus [Alegria et al. 2005]. Remarkably, all these uncharacterized proteins were found to possess a conserved 120-amino-acid domain in their C termini, which led the authors to speculate that they may represent a family of cofactors or substrates, *i.e.* effectors, of the *Xanthomonas* T4SS.

In a follow up study published in Nature Communications in 2015, the same team found that the *X. citri* T4SS provides to these cells the capacity to kill other Gram-negative bacterial cells in a contact-dependent manner [Souza et al. 2015]. Moreover, it was also shown that the secretion of one type 4 effector protein requires the conserved C-terminal domain that had been identified ten years before. This was the first demonstration of the involvement of a T4SS in bacterial killing, similar to the T6SS-mediated interbacterial interactions [Sgro et al. 2019]. Until that time, examples of T4SS-mediated macromolecule transfer from one bacterium to another was restricted to protein-DNA complexes during bacterial conjugation.

The Brazilian colleagues then investigated the atomic details of the type 4 secretion process and how its activity is regulated. Solution NMR spectroscopy and isothermal titration calorimetry revealed how the C-terminal effector domain, characterized by multiple conserved motifs and a glutamine-rich tail, interacts with the VirD4 protein that recruits the effectors for secretion [Oka et al. 2022]. However, since the production of this multi-subunit molecular machine involves significant metabolic costs, one can imagine that its production should be restricted unless there are prey bacteria in the vicinity of the bacterium. Indeed, Farah and co-workers found that the chromosomal *virB* operon, which encodes the structural genes of this T4SS in *X. citri*, is regulated by the conserved global regulator CsrA [Cenens et al. 2020]. Bacteria subjected to a wide range of growth conditions were found to maintain a constant density of T4SSs in the cell envelope and concomitant interbacterial competitiveness. Hence, CsrA provides a constant, albeit partial, repression on the *virB* operon, regardless of the growth conditions, and in this way limits T4SS-related metabolic costs while maintaining the aggressive nature of *X. citri* towards competitors.

We now better understand how type 4 and type 6 secretion systems provide an advantage to the bacteria that produce them, both by eliminating potential competitors and perhaps by using the cell contents of the lysed bacteria as nutrients. It will be interesting to monitor type 4 and type 6 secretion activity in situ and to analyze how these secretion systems shape the microbiota of plant and human pathogens, but also soil-borne bacteria in the *Xanthomonadales* order. Remember that bacteria of the genera *Lysobacter* and *Stenotrophomonas* are known as biocontrol organisms [Hayward et al. 2010]. As these bacteria belong to the same family as *Xanthomonas*, it was not so surprising that strains of both *Lysobacter* and *Stenotrophomonas* were found to encode and utilise the secretion system to kill other bacteria [Bayer-Santos et al. 2019; Nas et al. 2021; Shen et al. 2021]. This antibacterial activity can be detrimental to the rational development of biocontrol communities, as potentially beneficial bacteria may kill each other. Indeed, it has been shown that only inactivation of T4SS-mediated antibacterial activities enabled the development of a biocontrol alliance of two inherently incompatible *Lysobacter* species [Wu et al. 2021]. Therefore, a detailed characterisation of contact-dependent killing mediated by T4SS and T6SS will facilitate the engineering of beneficial

synthetic communities. Moreover, exciting insights into how the various effectors exert their toxic effects are expected in the coming years, which could lead to new biotechnological applications.

Boris Szurek (Researcher at IRD Montpellier; Molecular plant pathologist with a strong interest in TAL effectors, their function and evolution in rice- and cassava pathogens): Cracking the DNA-binding code of TAL effectors and its biotechnological applications

1989 is the year of the fall of the Berlin wall. It is also the year of the discovery of *avrBs3*, the founding member of the family of *avrBs3*/*pthA* effectors (which have now been renamed TAL for Transcription Activator-Like) by Ulla Bonas, as she was initiating her research on the *Xanthomonas*/pepper pathosystem [Bonas et al. 1989]. If the first event immediately and dramatically changed the face of the world, the second kept since 1989 and up to now an entire community of scientists in suspense, with discoveries each more surprising and fascinating than the previous one. Having spent over 25 years studying these fascinating molecules, the joint discovery by the Bonas and the Bogdanove labs of the TAL code (which governs the specificity of interaction between the effector protein and its target DNA sequence) is for me one of the most intellectually stimulating discoveries since the isolation of *avrBs3*, not mentioning all the implications of this finding for TAL effector (TALE) biology research and biotech applications [Boch et al. 2009; Moscou and Bogdanove 2009].

It was known for long that the repeat identity and their ordering in the central region of the protein determined TALE specificity [Herbers et al. 1992]. Later on, it was shown that the repeat central region was able to bind DNA, and, interestingly, that the AvrBs3 binding sequence was of the same size in nucleotides as the number of repeats in AvrBs3 [Kay et al. 2009; Yang et al. 2000]. For both teams and as simple as it sounds, the revelation came from the alignment between the most variable residues of the repeats, also called repeat variable residues (RVDs), and the few TALE target DNA sequences known at the time, hinting to a one-TALE-repeat per one DNA-base-pair recognition [Schornack and Boch 2010]. While the Bonas lab cracked the code by testing through GUS reporter gene assays the association between TALEs and predicted effector-binding elements (EBEs), as well as mutated versions of them in order to investigate the

nucleotide specificity of repeat types, the Bogdanove lab took a bioinformatic approach and scanned for RVD-nucleotide alignments with minimal entropy for each of ten known TALE target gene promoter pairs, resulting in the finding of one matching box for each effector.

The consequences of the discovery of the TAL code were huge. On the one hand, the code allows to predict the host genes induced by xanthomonads in the course of infection, leading to the identification of a wealth of new susceptibility (*S*) genes and the unique chance to decrypt the host molecular mechanisms underlying disease development. The modular nature of TALEs, which can be considered as remote controls for gene induction [Scholze and Boch 2011], allows the design of synthetic TALEs, also known as dTALEs or ArtTALs [Morbitzer et al. 2010; Streubel et al. 2013]. This approach has become the state-of-the-art in confirming predicted *S* genes, but has also been pioneered in other heterologous systems, including human cells [Geissler et al. 2011; Miller et al. 2011; Zhang et al. 2011].

The programmable DNA-binding specificity of the TALE repeat region has been used to create designer restriction enzymes that cleave double-stranded DNA at any desired position [Christian et al. 2010; Li et al. 2011; Mahfouz et al. 2011; Miller et al. 2011]. Relying on the cell's DNA repair machinery, these enzymes allow to introduce small deletions in the genome of virtually all eukaryotic organisms. One of the first applications was the use of TALENs to edit the EBEs of *S* genes, thus disarming xanthomonads that rely on the activity of the corresponding TALEs [Li et al. 2012]. On the other hand, it is also possible to place the promoter sequences of the induced plant *S* genes in front of certain resistance genes, called executor genes, and in this way to prevent the propagation of xanthomonads [Römer et al. 2009]. Both aspects open up new perspectives for engineering disease-resistant plants. In addition to the FokI DNA cleavage domain, as used in most TALENs, it is possible to direct other protein domains by fusion to customized DNA-binding modules at any desired location of a DNA molecule - an aspect that has opened up a wide range of biotechnological and medical applications [Becker and Boch 2021].

Without doubt the most spectacular application was the first medicinal use of TALENs in a last-ditch attempt to cure a child with otherwise untreatable leukemia [Begley

2015]. Scientists at the Ormond Street Hospital in London, UK, edited genes in immune cells to make them hunt down and destroy the malignant blood cells that threatened the year-old girl's life [Qasim et al. 2017]. Since then, CRISPR-Cas technology has superseded the early success of TALENs in gene therapy but also in agricultural and veterinary applications [Dhakate et al. 2022; Negi et al. 2022; Khlidj 2023; Sahu et al. 2023]. However, TALE technologies have once again come into play as a tool for epigenomic reprogramming, but are again in competition with CRISPR-Cas technology [Nomura 2018; Qi et al. 2023].

Perhaps the most interesting area for TALE technologies lies in the modification of organelle genomes. Here, base editing within organelle DNA using CRISPR-Cas technology has thus far been hindered by challenges associated with the delivery of guide RNA into the mitochondria [Mok et al. 2020]. By fusing a bacterial cytidine deaminase, derived from a T6SS effector toxin, to a programmable TALE repeat arrays and a uracil glycosylase inhibitor site-specific base conversions were achieved in human mitochondrial DNA with high target specificity [Mok et al. 2020]. Mitochondrial diseases, which affect an estimated 1 in 5,000 people, can cause a vast array of health concerns, including fatigue, weakness, strokes, seizures, cardiomyopathy, developmental or cognitive disabilities, diabetes mellitus, impairment of hearing, vision, growth, liver, gastrointestinal, or kidney function [Cohen 2019]. Therefore, mitochondrial genome engineering holds great promise [Barrera-Paez and Moraes 2022].

Much progress has also been made in using TALE-based tools for site-specific engineering of plastid and mitochondrial genomes in flowering plants [Maliga 2022]. The successful knockout of a conserved plant mitochondrial gene paved the way to systematic reverse genetic studies in plant mitochondrial genomes and also suggests a new strategy for building synthetic cytoplasmic male sterility (CMS) systems in crops [Forner et al. 2023]. CMS systems represent a valuable tool in the production of hybrid seed in self-pollinating crop species, including maize, rice, cotton, and a number of vegetable crops, and improving hybrid technology can contribute both to supplying the world's growing population with food and to conserving land [Eckardt 2006].

Mathilde Hutin (Researcher at IRD Montpellier; Molecular plant pathologist specialized on rice-infecting *Xanthomonas*): TALE-dependent resistance and susceptibility genes

The elucidation of the TALE code was definitely a turning point in the understanding of the molecular dialogue established between most of the pathogenic *Xanthomonas* species and their hosts. This discovery has made it possible to use major TALEs as probes to easily identify new resistance and susceptibility genes, by combining transcriptomic and bioinformatic prediction. For me, one of the best examples is the identification of the *Bs4c* resistance gene in pepper plants, which is induced by the TALE AvrBs4 [Strauß et al. 2012]. Having joined the *Xanthomonas* community at this exciting time, I tend to forget that there was a before. Indeed, although more than 18 functional targets of TALE have been characterized, including susceptibility and resistance genes, four of them had been identified before the TALE code was deciphered.

The fact that certain TALEs are major virulence or avirulence factors, leading to a strong phenotype when mutated, has facilitated their characterization and the identification of their unique function in *Xanthomonas* pathogenicity. The AvrBs3 and PthA proteins, identified in *X. euvesicatoria* pv. *euvesicatoria* (formerly known as *X. campestris* pv. *vesicatoria*) and *X. citri* pv. *citri* (formerly known as *X. axonopodis* pv. *citri*), respectively, were the first members of the TALE family to be identified [Bonas et al. 1989; Swarup et al. 1991]. However, the first TALE-dependent *R* gene to be cloned was *Xa27* from rice, which determines the specific recognition of the *X. oryzae* pv. *oryzae* strains expressing the TALE AvrXa27 [Gu et al. 2005]. The authors demonstrated that the central repeats are responsible for the specificity of the interaction and that the nuclear localization signals (NLS) and the acidic activation domain of AvrXa27 are required for disease resistance. The authors further showed that *Xa27* was induced only in the resistant rice variety in the presence of AvrXa27 and they found that the coding sequence of *Xa27* was identical between susceptible and resistant rice varieties. They were the first to suggest that polymorphisms in the promoter region of a TALE-targeted plant gene could explain its differential transcriptional activation. *Xa27* encodes a protein of 119 amino acids with no similarity to any other resistance protein known at that time. *Xa27* was the

first member of a class of genes, called executor (*E*) genes, *i.e.* genes whose induction is necessary and sufficient to kill cells and prevent the development of the disease.

Two years after the cloning of *Xa27*, two functional targets of AvrBs3 were cloned. The *E* gene *Bs3* was cloned from BAC clones of a pepper cultivar that is resistant to *X. euvesicatoria* bacteria expressing AvrBs3 [Römer et al. 2007]. The authors showed that AvrBs3 was able to specifically bind to the *Bs3* promoter and to induce the expression of the *Bs3* gene. In the same year, the ability of AvrBs3 to directly induce *upa20*, a cell size regulator involved in cell enlargement and bacterial dispersion during the infection, was shown [Kay et al. 2007]. As with *upa20* in pepper, the induction of *Os8N3* (now *OsSWEET11*) in rice is required for disease development upon infection by *X. oryzae* strains that rely on the TALE PthXo1 [Yang et al. 2006]. *OsSWEET11* was the first cloned *S* gene and belongs to a widely conserved family of sugar transporters, called SWEETs for Sugar Will Eventually be Exported Transporters [Chen et al. 2010].

Later on, a second *S* gene belonging to the *SWEET* family, *OsSWEET14*, was identified [Antony et al. 2010]. The authors showed that the recessive resistant allele *xa13,* which contains a deletion in the promoter of *OsSWEET11*, is defeated by the presence of AvrXa7 and PthXo3, two other TALEs from different *X. oryzae* strains that induce *OsSWEET14* [Antony et al. 2010]. *xa13* was the first recessive resistance allele shown to act by loss of susceptibility. This discovery opened the way to provide new sources of TALE-dependent resistances by exploiting polymorphisms in the promoters of *S* genes that already exist or have been generated by genome editing, ironically using TALENs [Hutin et al. 2015; Li et al. 2012]!

Upa20 and *OsSWEET11* are emblematic not only because they were the first virulence TALE targets to be identified but also because they paved the way to the discovery of more and more TALE targets. This allowed a better understanding of the whole pathosystem, in particular what *Xanthomonas* requires to cause disease, and consequently new ways to control *Xanthomonas*-mediated diseases.

I find this evolutionary convergence within a species, but also between different species, by targeting different members of the sucrose transporter family particularly impressive. Even more so because, despite the number of *SWEET* genes identified to date, we still do not understand how inducing *SWEET* gene expression favors disease

development. In *Xanthomonas*, the number of TALE genes varies between species, but also between pathovars of the same species and even among strains within the same pathovar. *X. oryzae* strains generally contain a large number of TALE genes (between 8 and 30 copies per genome), but only a few of them appear to play a major role in virulence. What about the others? Do they act as a genetic reservoir? Are they required for interaction with weed species that could serve as alternative hosts? Do they play a role in other stages of the infection cycle? To me, there is no doubt that we still have a lot to understand and that we will continue to be surprised by this fascinating effector protein family.

Alvaro Perez-Quintero (Researcher at IRD Montpellier; Bioinformatician studying coevolution of *Xanthomonas oryzae* TAL effectors and rice): Interfering with TALE-mediated *R* gene recognition

One of the reasons I am so fascinated by *Xanthomonas* and its mechanisms to cause disease is that this organism does not stop surprising us. After the TALE code was deciphered one would think few mysteries remained: we knew how to predict their targets and we could focus on finding more and more susceptibility genes. We also knew plants were able to thwart TALE activity through loss-of-susceptibility or executor genes, or by direct recognition of TALEs. As postulated by the 'zig-zag' model for plant-pathogen arms-races [Jones and Dangl 2006], we expected bacteria to also have counter-defense mechanisms to suppress resistance, but it was still remarkable to discover that one such mechanism would involve the 'pseudogenization' of TALEs themselves.

The modular structure of TALEs is well conserved: all TALEs with known virulence role have a secretion signal, a central repeat domain consisting of nearly identical 33-34 aa repeats, several NLSs and a C-terminal eukaryotic transcription activation domain. Yet, some deviations were identified, such as TALEs that contain longer, 'aberrant', repeats that introduce flexibility in binding and overcome 'loss-of-susceptibility' alleles [Richter et al. 2014]. A curious case was a group of 'pseudogenes' found in *X. oryzae* strains, which were TALE sequences with multiple in-frame deletions resulting in a lack of an activation domain (they also contained only one NLS, and often short 28-aa repeats). Since the product of these 'pseudogenes' likely lacked transcriptional activation activity, they were thought to be non-functional. However, their sequences were highly conserved

even across the two genetically distinct *X. oryzae* pathovars *oryzae* and *oryzicola*. This fact suggested that these genes are not just pseudogenes. Indeed, in 2015, two teams demonstrated that these genes, termed interfering (iTALEs) or truncated TALEs (truncTALEs), are in fact functional and can interfere with the function of dominant rice *R* genes [Ji et al. 2016; Read et al. 2016]. *Xa1* and *Xo1* are broad spectrum *R* genes that can directly recognize the canonical TALE structure, and the peculiar structure of iTALEs/truncTALEs disrupts in some way *Xa1*/*Xo1* activity (likely through competitive binding) and leads to disease [Ji et al. 2016; Read et al. 2016].

What excites me about this system is the evolutionary implication, showing that *X. oryzae* and rice are in an ever-escalating arms race and that the action of TALEs is likely a major player in the co-evolution of these organisms.

Sophie Cesbron (Engineer at INRAE Angers; interested in epidemiology, emergence and control of plant pathogenic bacteria): Phage-nanoparticle conjugates – a new strategy for controlling bacterial plant diseases?

Bacterial plant diseases are difficult to control. In the absence of durable resistance to the disease in the host plant, few methods exist to control a bacterial disease. Some antibiotics and heavy metals can be used in plant cultivation but their use is increasingly restricted or even banned, depending on the country, because of the appearance of resistance over the last twenty years and the risk that they pose to the environment and human health [Larsson and Flach 2022; Richard et al. 2017]. Hence, there is a renewed interest in bacteriophages, discovered more than a century ago, to combat pathogenic bacteria. Many articles describe them as a new, promising method to control phytopathogenic bacteria such as *Lysobacteraceae*, because of their specificity. Even if there is still a lot of work to characterize bacteriophages in addition to their bacterial hosts, this research topic has been revolutionized by the advances in DNA sequencing and metagenomics [Strathdee et al. 2023].

At the same time, nanotechnologies are attracting increasing interest in agriculture, for example for pathogen detection or as nanopesticides able to fight plant diseases and to improve the efficiency of chemicals at lower doses. Gold nanoparticles in particular have optical properties that make them powerful colorimetric biosensors [Khanna et al. 2022]. These particles coupled with antibodies have been used a few years ago for the

detection of bacteria in complex systems (plant extracts, food and body fluids). But these particles also have other properties, such as the release of heat under infra-red radiation, sufficient to kill bacteria.

It is therefore not surprising that scientists are seeking to couple bacteriophages to nanoparticles to obtain new means of detection or new means of rapid control, being more efficient, more sensitive and highly specific. This is the case with the work of Irene Chen and collaborators who combined chimeric phages with gold nanoparticles, first for the detection, and then for the destruction of pathogenic bacteria such as *X. campestris* pv. *campestris* and *X. euvesicatoria* pv. *euvesicatoria* [Peng and Chen 2018; Peng et al. 2020]. In these studies, the backbone of the M13 phage was engineered to express a foreign receptor-binding protein to specifically target the desired bacterial species and allowed the detection of as little as 100 cells. This chimeric phage was then thiolated to promote interaction with gold and brought into contact with the targeted bacteria. The added gold nanoparticles will finally aggregate on the thiolated phages and change color. The same team then used the properties of gold to convert infra-red radiation into heat. This heat generation at a submicrometer to micrometer distance effectively killed >99% of the targeted bacterial cells within ten minutes, as well as the chimeric phage, thus reducing the potential risks associated with phage therapy.

This nice proof of concept opens new perspectives for the control of other phytopathogenic bacteria. However, the mode of application, the distribution and the *in planta* effects of these molecules still need to be tested before a future development in precision agriculture.

EPILOGUE

With this personal essay, we wanted to share our fascination about some key moments in *Xanthomonas* research with the readership. We hope that this fascination will inspire the following generations of phytopathologists. Sound epidemiological models, profound risk assessment, deep molecular understanding of virulence and resistance mechanisms, insights into the interplay of all organisms and their interaction with environmental factors

- all this will help to ensure sustainable agriculture and conservation of our environment in the future.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

AUTHOR CONTRIBUTIONS

All authors were involved in conceptualization, writing of the original draft, reviewing and final editing the manuscript. RK, NWGC and PP contributed to data curation, formal analyses, and visualization. The project was administered by NWCG, LDN, OP and RK, and the whole study was supervised by RK.

DATA, SCRIPTS, CODE, AND SUPPLEMENTARY INFORMATION AVAILABILITY

The data in Supplementary Tables S1 and S2 are available from Zenodo under DOI 10.5281/zenodo.10683038.

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