# Distribution and inheritance of a gene cluster encoding a sulfated tyrosine peptide in *Xanthomonas* spp.

Running title: *raxX-raxSTAB* gene cluster in *Xanthomonas* spp.

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## ABSTRACT

Tyrosine sulfation is a post-translational modification that influences interaction specificity between certain receptors and their protein ligands in diverse biological processes. For example, rice XA21 receptor-mediated recognition of the sulfated bacterial protein RaxX activates an immune response and triggers resistance to the phytopathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). A five kb *raxX-raxSTAB* gene cluster of *Xoo* encodes RaxX, the RaxST tyrosylprotein sulfotransferasea and the RaxA and RaxB components of a predicted proteolytic maturation and ATP-dependent peptide secretion complex. The complete *raxX-raxSTAB* gene cluster was found only in *Xanthomonas* species, and its distribution is consistent with multiple gain and loss events during *Xanthomonas* speciation. Homologs of the *raxST* gene are present in genome sequences of diverse bacterial species. Together, these results establish a foundation for investigating biological roles for tyrosine sulfation in bacteria.

ABSTRACT = 132 words (maximum 200)

## INTRODUCTION

Host receptors activate innate immunity pathways upon pathogen recognition (Ronald and Beutler 2010). The gene encoding the rice XA21 receptor kinase (Song *et al.,* 1995) confers broad spectrum resistance against the gamma-proteobacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Wang *et al.,* 1996). This well-studied XA21-*Xoo* model provides a basis from which to understand molecular and evolutionary mechanisms of host-microbe interactions.

Several *Xoo rax* genes are **r**equired for **a**ctivation of **X**A21-mediated immunity (**Fig. 1a**). The *raxX-raxSTAB* gene cluster encodes the 60-residue RaxX predicted precursor protein that undergoes sulfation by the RaxST tyrosylprotein sulfotransferase at residue Tyr-41 (Pruitt *et al.,* 2015). We hypothesize that the RaxABC proteolytic maturation and ATP-dependent peptide secretion complex (da Silva *et al.,* 2004) further processes the sulfated RaxX precursor by removing its double-glycine leader peptide prior to secretion (Holland *et al.,* 2016). Located outside the *raxX-raxSTAB* gene cluster, the *raxC* gene, an ortholog of the *tolC* gene, encodes the predicted outer membrane channel for this complex (da Silva *et al.,* 2004). Finally, the *raxPQ* genes encode enzymes to assimilate sulfate into 3'-phosphoadenosine 5'-phosphosulfate (Shen *et al.,* 2002), the sulfodonor for the RaxST tyrosylprotein sulfotransferase (Han *et al.,* 2012).

Tyrosylprotein sulfotransferase is confined to the Golgi complex in both plants and animals (Moore 2009). Thus, this post-translational modification is targeted to a subset of cell surface and secreted proteins that influence a variety of eukaryotic physiological processes (Matsubayashi 2014; Stone *et al.,* 2009). For example, tyrosine sulfation of the chemokine receptors CCR5 and CXCR4 is necessary for high-affinity binding not only to chemokines, but also to the HIV-1 surface glycoprotein (Farzan *et al.,* 1999;

Kleist *et al.,* 2016). In plants, sulfated tyrosine peptides influence xylem development, root growth, and/or plant immune signaling (Matsubayashi 2014; Zhou *et al.,* 2017).

RaxST sulfation of RaxX residue Tyr-41 is the only example of tyrosine sulfation reported in bacteria (da Silva *et al.,* 2004; Pruitt *et al.,* 2015). Strikingly, RaxX residues 40-52 share sequence similarity with mature active **p**lant peptide containing **s**ulfated t**y**rosine (PSY) hormones (Amano *et al.,* 2007; Pruitt *et al.,* 2015; Pruitt *et al.,* 2017). Indeed, RaxX, like PSY1, can enhance root growth in diverse plant species (Pruitt *et al.,* 2017). Moreover, RaxX also contributes to *Xoo* virulence in the absence of the XA21 immune receptor (Pruitt *et al.,* 2017). This apparent hormone mimickry by RaxX therefore may serve broad functions in *Xoo* pathogenesis. To further elucidate the biological role of bacterial tyrosine sulfation, we sought to identify the species distribution and possible origin of genes in the *raxX-raxSTAB* gene cluster.

Here we show that the *raxX-raxSTAB* gene cluster is confined to a subset of *Xanthomonas* species. In all cases examined, the *raxX-raxSTAB* gene cluster lies between two core (housekeeping) genes, *gcvP* encoding a subunit of glycine dehydrogenase, and a gene encoding a major facilitator subfamily transporter (*"mfsX"*). Examination of nucleotide sequence conservation across the *raxX-raxSTAB* gene cluster, and at its boundaries with the *gcvP* and *"mfsX"* genes, suggests that the *raxX-raxSTAB* gene cluster was acquired through lateral transfer by *X. translucens*, a pathogen of diverse cereal species (Langlois *et al.,* 2017), and separately by *X. maliensis*, associated with but nonpathogenic for rice (Triplett *et al.,* 2015). Finally, genes homologous to *raxST* are present in bacterial genomes from a wide range of species, raising the possibility that RaxST-catalyzed tyrosine sulfation may occur in other genomic and biological contexts in addition to RaxX.

## RESULTS

### The *raxX-raxSTAB* gene cluster is present in a subset of *Xanthomonas* spp.

We searched databases at the National Center for Biotechnology Information to identify bacterial genomes with the *raxX-raxSTAB* gene cluster. We found the *raxX-raxSTAB* gene cluster exclusively in *Xanthomonas* spp., and ultimately detected it in more than 200 unique genome sequences among 413 accessed through the RefSeq database (O'Leary *et al.,* 2016).

*Xanthomonas* taxonomy has undergone substantial changes over the years (Vauterin *et al.,* 2000; Young 2008); see (Midha and Patil 2014) for a representative example). At one point, many strains were denoted as pathovars of either *X. campestris* or *X. axonopodis*, but today over 20 species are distinguished, many with multiple pathovars (Rademaker *et al.,* 2005; Vauterin *et al.,* 1995). Because many of the genome sequences we examined are from closely-related strains, in some cases associated with different species designations, we constructed a phylogenetic tree in order to organize these sequences by relatedness (**Fig. S1**).

The phylogenetic relationships among *Xanthomonas* spp. was assessed using the entire genome assembly with Andi v0.10 (Haubold *et al.,* 2015; Klotzl and Haubold 2016). We compared our topology with several other *Xanthomonas* phylogenetic trees published previously (Ferreira-Tonin *et al.,* 2012; Gardiner *et al.,* 2014; Hauben *et al.,* 1997; Midha and Patil 2014; Parkinson *et al.,* 2007; Parkinson *et al.,* 2009; Rademaker *et al.,* 2005; Triplett *et al.,* 2015; Young *et al.,* 2008). Most share broad similarity with each other and with the whole-genome tree presented here in defining relationships

between well-sampled species. To facilitate discussion, we represent our phylogenetic tree as a cladogram (**Fig. 2**).

We detected the *raxX-raxSTAB* gene cluster in six lineages that consistently are identified as being distinct from one another (Rademaker *et al.,* 2005; Vauterin *et al.,* 1995) (**Fig. 2**). One lineage includes the two *X. oryzae* pathovars, *oryzae* and *oryzicola*, pathogenic on rice (Niño-Liu *et al.,* 2006). A second lineage includes *X. vasicola*, strains of which are pathogenic on sugarcane, sorghum or maize, together with strains denoted as *X. campestris* pv. *musacearum*, pathogenic on banana (Aritua *et al.,* 2008). The third lineage includes *X. euvesicatoria* and *X. perforans*, pathogenic on pepper and tomato (Potnis *et al.,* 2015), together with strains denoted as *X. alfalfa* subsp. *citrumelonis* (pathogenic on citrus) and *X. dieffenbachiae* (anthuriums) (Rademaker group 9.2; (Barak *et al.,* 2016; Rademaker *et al.,* 2005). The fourth lineage includes strains denoted as *X. axonopodis* pathovars *manihotis* (pathogenic on cassava) and *phaseoli* (bean) (Rademaker group 9.4; (Mhedbi-Hajri *et al.,* 2013; Rademaker *et al.,* 2005).

The fifth lineage includes *X. translucens*, different strains of which are pathogenic on one or more cereal crops such as wheat and barley, and/or non-cereal forage and turfgrass species (Langlois *et al.,* 2017). *X. translucens* is within the distinct cluster of "early-branching" species whose divergence from the remainder apparently occurred relatively early during *Xanthomonas* speciation (Parkinson *et al.,* 2007). The sixth lineage comprises *X. maliensis*, associated with but nonpathogenic on rice (Triplett *et al.,* 2011); phylogenetic analyses place this species between the "early-branching" species and the remainder (Triplett *et al.,* 2015).

Notably, the *raxX-raxSTAB* gene cluster is absent from the *X. citri* pathovar group, pathogenic on a range of dicots including citrus. This group, which includes certain strains denoted as *X. axonopodis* or *X. campestris* (Bansal *et al.,* 2017), clusters phylogenetically among the *X. oryzae*, *X. euvesicatoria* and *X. axonopodis* pv. *manihotis* groups (Midha and Patil 2014; Rademaker *et al.,* 2005; Vauterin *et al.,* 1995) (**Fig. 2**).

Together, these observations suggest that the *raxX-raxSTAB* gene cluster experienced multiple gains and/or losses during *Xanthomonas* speciation.

**Sequence conservation of the *raxX-raxSTAB* gene cluster suggests lateral transfer between *Xanthomonas* spp.**

From the initial analysis described above, we selected 15 species, representing the phylogenetic range of *Xanthomonas*, for more detailed analyses of *rax* gene cluster composition, organization, and inheritance (**Fig. 2; Table 1**). The corresponding genome sequences are accompanied by published descriptions (**Table 1**). The close relative *Stenotrophomonas maltophilia*, which does not contain the *raxX-raxSTAB* gene cluster, serves as a reference (Moore *et al.,* 1997).

Both the organization and size of the *raxX-raxSTAB* gene cluster are conserved across all six lineages in which it resides. To address hypotheses for patterns of *raxX-raxSTAB* gene cluster inheritance, we compared individual phylogenetic trees for each of the four *rax* genes to the overall *Xanthomonas* phylogenetic tree (**Fig. 3**) (Kuo and Ochman 2009). For all four genes, sequences in *X. translucens*, in the early-branching group, cluster separately from sequences in the other lineages. This finding is congruent with the hypothesis, that *X. translucens* acquired the *raxX-raxSTAB* gene

cluster relatively early during *Xanthomonas* speciation. For *X. maliensis*, the *raxX-raxSTAB* genes assort among those from *X. euvesicatoria* and the *X. axonopodis* pathovars *manihotis* and *phaseoli* (**Fig. 3**), even though the *X. maliensis* genome sequence itself is more distantly related (**Fig. 2**). This finding suggests that *X. maliensis* acquired the *raxX-raxSTAB* gene cluster relatively late during *Xanthomonas* speciation.

The *raxX-raxSTAB* gene cluster lies between two core (housekeeping) genes (**Fig. 1a**). One, *gcvP*, encodes the pyridoxal-phosphate subunit of glycine dehydrogenase. An approximately 170 nt riboswitch (*gcvR* in **Fig. 1a**) controls GcvP protein synthesis in response to glycine (Mandal *et al.,* 2004). The other, "*mfsX*", encodes a **m**ajor **f**acilitator **s**ubfamily (MFS) transporter related to Bcr and CflA efflux proteins (da Silva *et al.,* 2004). Here, "*mfsX*" is only a provisional designation absent functional characterization.

We further examined phylogenetic relationships by comparing nucleotide sequence identity across the *gcvP, raxX, raxST, raxA, raxB* and *"mfsX"* coding regions, each from the initiation through termination codon (**Table 2**). For comparison, values are presented also for **g**enome-wide **a**verage **n**ucleotide **i**dentity (gANI) as well as the **a**lignment **f**raction (AF), which estimates the fraction of orthologous genes (Varghese *et al.,* 2015). For context, a widely-used criterion assigns 95% **a**verage **n**ucleotide **i**dentity (ANI) as the cut-off point for species delineation (Goris *et al.,* 2007). Sequence from *X. euvesicatoria* is the reference.

The *raxST*, *raxA* and *raxB* coding sequences from *X. axonopodis* pv. *manihotis* and *X. maliensis* display the highest identity to those from *X. euvesicatoria*, at least 95% in each case. Sequences from *Xoo* and *X. vasicola* (also known as *X. campestris* pv. *malvacearum*) are about 90% identical, and those from *X. translucens* roughly 75%

identical (**Table 2**). The *raxX* coding sequences are more divergent, with identity to the *X. euvesicatoria* sequence ranging from almost 90% for *X. maliensis* and *Xoo* to only 63% for *X. translucens* (**Table 2**).

## Boundaries flanking the *raxX-raxSTAB* gene cluster and adjacent genes suggest lateral transfer through general recombination

Comparison of the *gcvP* - *[raxX-raxSTAB]* - "*mfsX*" region from all 16 reference species reveals sharp boundaries flanking the position of the *raxX-raxSTAB* gene cluster. On the left flank, substantial nucleotide identity spans the *gcvP* gene, the *gcvR* riboswitch, and a presumptive promoter –10 element (Mitchell *et al.,* 2003) (**Fig. 1b**). On the right flank, identity begins shortly after the "*mfsX*" initiation codon. Accordingly, upstream signals for "*mfsX*" gene transcription (Mitchell *et al.,* 2003) and translation (Ma *et al.,* 2002) are conserved within, but not between, *raxX-raxSTAB*-positive and -negative sequences (**Fig. 1b**).

Between these boundaries in *raxX-raxSTAB* gene cluster-negative species, the compact (≤ 200 nt) *gcvP*-"*mfsX*" intergenic sequence is modestly conserved in most genomes (about 60-80% overall identity) (**Fig. 1b**). Much of this identity comes from the "*mfsX*" potential transcription and translation initiation sequences described above. The overall intergenic sequence is less conserved in the early-branching species (*X. albilineans*, *X. hyacinthi* and *X. sacchari*), displaying about 50-65% overall identity.

In *raxX-raxSTAB* gene cluster-positive genomes, sequence flanking these boundaries appears unrelated to the *gcvP*-"*mfsX*" intergenic sequence from *raxX-raxSTAB* gene cluster-negative genomes (**Fig. 1b**). Rather, it is well-conserved even in the early-

branching species, *X. translucens*. These results suggest that anomolous *raxX-raxSTAB* gene cluster phylogenetic distribution results from lateral gene transfer.

Sequences of the adjacent *gcvP* gene display length polymorphisms (**Fig. 4**) that do not align with overall *Xanthomonas* species relationships (**Fig. 2**). Polymorphisms of this type are unusual, and indicate recombination (Nelson *et al.,* 1997). Their occurrence in a gene adjacent to the *raxX-raxSTAB* gene cluster independently supports the model, that this genomic region evolves through lateral gene transfer.

### *raxST* homologs are present in genomes of diverse bacterial species

As we searched genome sequences available through GenBank for evidence of the *raxX-raxSTAB* gene cluster outside of *Xanthomonas* spp., we identified sequences encoding proteins with about 40% identity to, and approximately the same length as, the *Xoo* RaxST protein. Sequence identity is high in residues that form the binding pocket for the cofactor, 3'-phosphoadenosine 5'-phosphosulfate (da Silva *et al.,* 2004; Kakuta *et al.,* 1998), consistent with assignment of these encoded proteins as sulfotransferases. It is not known if these genes encoding tyrosylprotein sulfotransferases, as there are no defined sequence features that distinguish such enzymes from other sulfotransferases that have non-protein substrates (Dong *et al.,* 2012; Teramoto *et al.,* 2013).

These *raxST* homologs are in a range of bacterial phyla including Proteobacteria and Cyanobacteria (**Fig. 5**). Nevertheless, for most species represented by multiple genome sequences, the *raxST* homolog was detected in a minority of individuals, so it is not part of the core genome in these strains. Moreover, relationships between species in a *raxST* gene phylogenetic tree bear no resemblance to those in the overall

tree of bacterial species. For example, in the *raxST* gene tree, sequences from Cyanobacteria are flanked on both sides by sequences from Proteobacteria (**Fig. 5**). Together, these findings provide evidence for lateral transfer of *raxST* homologs transfer (Kuo and Ochman 2009).

## DISCUSSION

We hypothesize that the *raxX-raxSTAB* gene cluster originated in an ancestor to the lineage containing *X. oryzae*, *X. euvesicatoria*, and related species, with further gains or loss through lateral transfer as described below (**Fig. 2**). Analysis suggests that relatively few events were necessary to form the *raxX-raxSTAB* gene cluster. The *raxAB* genes are homologous to those encoding proteolytic maturation and ATP-dependent peptide secretion complexes (da Silva *et al.,* 2004; Lin *et al.,* 2015), related to type 1 secretion systems but specialized for secreting small peptides such as bacteriocins and peptide pheromones (Holland *et al.,* 2016). Frequently, the gene encoding the secreted substrate is adjacent to genes encoding components of the secretion complex (Dirix *et al.,* 2004). The *raxX* gene therefore might have evolved from the gene for the seccreted peptide substrate of the RaxAB ancestor. Finally, as we show here, homologs for the *raxST* gene are distributed broadly (**Fig. 5**).

The *raxX-raxSTAB* gene cluster does not exhibit features, such as a gene for a site-specific recombinase, characteristic of self-mobile genomic islands (Hacker *et al.,* 1997). Moreover, variant-length alleles of the adjacent *gcvP* gene (**Fig. 4**) provide evidence for general recombination in the vicinity (Nelson *et al.,* 1997). Thus, the simplest model for *raxX-STAB* gene cluster lateral transfer is that it occurred through general recombination between genes flanking each side of the *raxX-STAB* gene cluster (**Fig. 1b**).

Three examples provide further evidence for lateral transfer. First, the *raxX-raxSTAB* gene cluster from the early branching species *X. translucens* has essentially the same size, composition and structure as the others. However, the *X. translucens raxX-raxSTAB* sequences are more divergent (**Table 2**). This predicts that the *raxX-raxSTAB*

gene cluster has been part of the *X. translucens* genome sufficiently long for sequence alterations to accumulate (Kuo and Ochman 2009).

In the second example of evidence for lateral transfer, the *X. maliensis raxSTAB* sequences share strong similarity to those of *X. euvesicatoria* and the *X. axonopodis* pathovars *manihotis* and *phaseoli*, whereas their genome sequences are more divergent (**Table 2**). This suggests that *X. maliensis* acquired the *raxX-raxSTAB* gene cluster relatively recently (Kuo and Ochman 2009).

The final example of evidence for lateral transfer considers apparent loss of the *raxX-raxSTAB* gene cluster during differentiation of *X. citri* from the large group including *Xoo, X. euvesicatoria* and related species (Midha and Patil 2014; Rademaker *et al.,* 2005; Vauterin *et al.,* 1995) (**Fig. 2**). Sequence in the *gcvP*-"*mfsX*" intergenic region is conserved among *raxX-raxSTAB* gene cluster-negative species, including *X. citri* (**Fig. 1b**). This is consistent with loss from the *X. citri* lineage mediated by recombination, with the *gcvP*-"*mfsX*" region from a *raxX-raxSTAB* gene cluster-negative species. The result of this recombination would be replacement of the *raxX-raxSTAB* gene cluster with a conserved *gcvP*-"*mfsX*" region.

In an alternative scenario, where the *X. citri raxX-raxSTAB* gene cluster was lost through deletion, the remaining sequence in the intergenic region would more closely resemble the *raxX-raxSTAB* gene cluster-positive boundary sequence. This conclusion is not supported by the intergenic region found in *X. citri*. A second alternative scenario, that the *raxX-raxSTAB* gene cluster formed after *X. citri* speciation, is not supported by the analysis of *X. translucens* sequences described above.

Broad phylogenetic distribution of the *raxX-raxSTAB* gene cluster implies that its associated phenotypes can contribute to host interactions with diverse *Xanthomonas* spp. The *raxX-raxSTAB* gene cluster was identified in the context of the rice XA21-mediated immune response (da Silva *et al.,* 2004; Pruitt *et al.,* 2015), but the sequence and functional similarities between the bacterial RaxX and the plant PSY sulfopeptides suggests that RaxX may mimic PSY phytohormone activies to facilitate infection (Pruitt *et al.,* 2017). Indeed, *Xoo* strains that cannot synthesize sulfated RaxX exhibit reduced virulence (Pruitt *et al.,* 2017).

Evidence for lateral transfer to *X. translucens* and *X. maliensis* suggests that *raxX-raxSTAB* gene cluster acquisition may contribute to emergence of new species or pathovars. The potentially useful phenotype of PSY hormone mimickry conceivably could introduce a particular strain to previously inaccessible hosts or niches. On the other hand, loss of the *raxX-raxSTAB* gene cluster apparently occurred during formation of the *X. citri* lineage (**Fig. 2**), perhaps indicating that the *raxX-raxSTAB* gene cluster did not enhance fitness in this case.

Pathovar phenotypes that differentiate bacterium-plant interactions, characterized extensively in members of the genus *Xanthomonas* (Jacques *et al.,* 2016), are not predicted by the presence or absence of the *raxX-raxSTAB* gene cluster. Some species that infect monocots exclusively contain the *raxX-raxSTAB* gene cluster (e.g., *X. oryzae*, *X. translucens*), whereas others do not (e.g., *X. arboricola*, *X. hyacinthi*). Likewise, some species that infect dicots exclusively contain the *raxX-raxSTAB* gene cluster (e.g., *X. euvesicatoria*), whereas others do not (e.g., *X. campestris* pv. *campestris*; *X. citri*). Similarly, there is no association with tissue specificity; for example, a single species contains both vascular (*X. oryzae* pv. *oryzae*) and nonvascular (*X. oryzae* pv. *oryzicola*) pathogens.

## MATERIALS AND METHODS

### Survey of *raxX-STAB* gene clusters in *Xanthomonas* spp.

All available *Xanthomonas* genomes were downloaded from the NCBI ftp server on January 29, 2016 (413 genome accessions). The genome fasta files were used to build a local blast database using BLASTv2.27+ (Camacho *et al.,* 2009). For all genes in and surrounding the *raxSTAB* operon blastn (evalue cutoff of 1e-3) was used to identify homologs in the local blast database. Due to the small size of RaxX, tblastn was required to identify homologs (evalue cutoff of 1e-3). Fasta files for each blast hit were generated using a custom python script (available upon request). Alignments of all genes were performed with Muscle v3.5 (Edgar 2004) implemented in the desktop tool Geneious v9.1.8 (Kearse *et al.,* 2012). Alignment ends were trimmed so that each sequence was equal in length and in the first coding frame. Maximum likelihood trees were built with RaxML v8.2.4 (Stamatakis 2014) with the following settings: (-m GTRGAMMA F -f a -x 3298589 -N 10000 -p 23). Trees shown in all figures are the highest scoring ML tree and numbers shown on branches are the resampled bootstrap values from 1000 replicates. Trees were drawn in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Whole genome phylogenies were generated using the entire genome assembly with the program Andi v0.10 (Haubold *et al.,* 2015; Klotzl and Haubold 2016). These distance matrices were plotted as neighbor-joining tree using Phylip v3.695 (Felsenstein 1981). Numbers on the branches represent the proportion (0-100) that the branch appeared in the “bootstrapped” distance matrices using Andi.

**Sequence analyses**

Nucleotide and deduced amino acid sequences were edited and analyzed with the programs EditSeq™ (version 14.1.0), MegAlign™ (version 14.1.0) and SeqBuilder™ (version 14.1.0), DNASTAR, Madison, WI. The Integrated Microbial Genomes interface (Chen *et al.,* 2017) was used to compare genome segments from different species, and also to extract values for genome-wide Average Nucleotide Identity and genome Alignment Fraction (Varghese *et al.,* 2015).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**Figure 1.** The *raxX-raxSTAB* gene cluster. A. The *rax* genetic region, drawn to scale. B. Boundary sequences. Sequences conserved within a group but different from other groups are colored green, brown, or yellow. Black sequence is conserved in all lineages, and blue sequence represents matches to consensi for transcription and translation initiation sequences. An "*mfsX*" +1 frameshift in *Xoo* sequences is indicated by the vertical red line. Abbreviations: *S. maltophilia, Sm; X. albilineans, Xa; X. arboricola pv. juglandis, Xaj; X. axonopodis pv. manihotis, Xam; X. campestris pv. campestris, Xcc; X. campestris pv. musacearum, Xcm; X. cannabis, Xc; X. citri subsp. citri, Xac; X. euvesicatoria, Xe; X. fragariae, Xf; X. hyacinthi, Xh; X. maliensis, Xm; X. oryzae pv. oryzae,Xoo; X. sacchari,; Xs X. translucens, Xt; X. vesicatoria, Xv.*

**Figure 2.** Model for r*axX-raxSTAB* inheritance during *Xanthomonas* speciation. The *Xanthomonas* spp. cladogram is based on published phylogenetic trees; see text for references. Gray lines depict lineages for strains that lack the *raxX-raxSTAB* gene cluster, whereas black lines depict those that carry the cluster. Numbers indicate *gcvP* length polymorphism in each species (see **Fig. 4**). Hypothetical events are: A, formation of the *raxX-raxSTAB* gene cluster; B, lateral transfer to *X. translucens*; C, lateral transfer to *X. maliensis*; D, loss from *X. citri*.

**Figure 3.** Phylogenetic trees for *rax* gene nucleotide sequences. The best scoring maximum likelihood trees for (A) *raxA*, (B) *raxB*, (C) *raxX* and (D) *raxST* in *Xanthomona*s spp. Numbers shown on branches represent the proportion of branches supported by 10,000 bootstrap replicates (0-100). Bootstraps are not shown for branches with less than 50% support, nor for branches too short to easily distinguish.

**Figure 4.** GcvP length polymorphisms. The relevant portion of the GcvP amino acid sequence is shown for each of the reference strains. Species in red lack the *raxX-raxSTAB* gene cluster, whereas those in blue lines carry the cluster. Numbers denote different allelic types. The positions of residues Gly-733 and Val-738 (numbering for allelic type 1) are indicated. Abbreviations are as in Fig. 1b.

**Figure 5.** Phylogenetic tree for *raxST* homologs in diverse bacterial genera. Distribution of *raxST* homolgs across bacterial genera. The tree shown was constructed by neighbor-joining with 1000 bootstrap replicates; branches with < 50% bootstrap support are not drawn. The *raxST* sequence from *Xoo* strain PXO99$^{A}$ was used as query for tBLASTn.

**Figure S1.** Whole genome-based *Xanthomonas* phylogenetic tree. Phylogenetic tree constructed from comparison of whole genome sequences; see text for details. See **Fig. 2** for the corresponding cladogram. Red lines depict lineages for strains that lack the *raxX-raxSTAB* gene cluster, whereas blue lines depict those that carry the cluster.

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**Figure S1**.

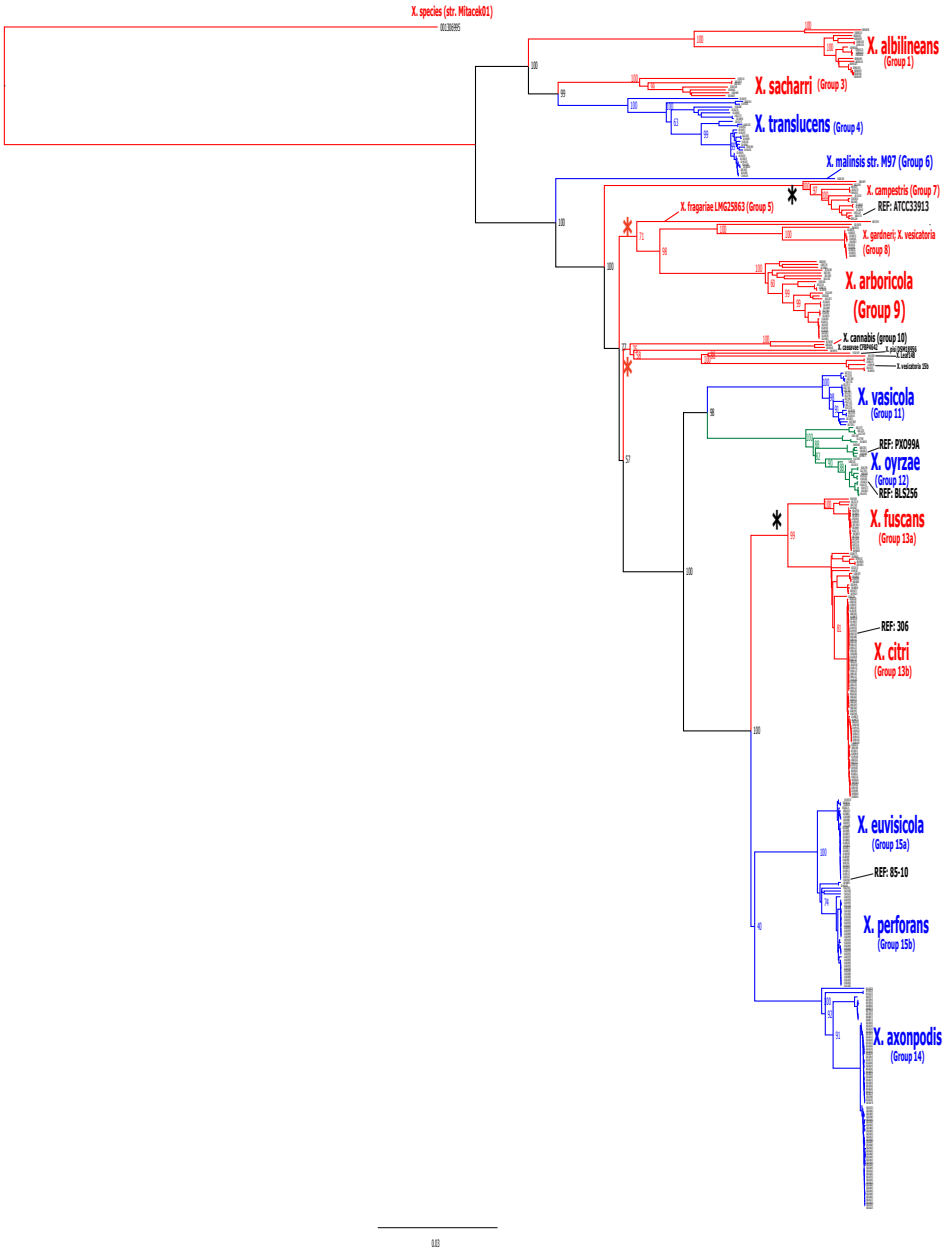


**Table 1.** Reference strains for sequence comparisons.

| Species | Strain | *raxX-raxSTAB* | Accession | Reference |
|---|---|---|---|---|
| *S. maltophilia* | K279a | – | NC_010943.1 | (Crossman *et al.,* 2008) |
| *X. albilineans* | GPE PC73 | – | NC_013722.1 | (Pieretti *et al.,* 2015) |
| *X. arboricola* pv. *juglandis* | Xaj 417 | – | NZ_CP012251.1 | (Pereira *et al.,* 2015) |
| *X. axonopodis* pv. *manihotis* | UA536 | + | NZ_AKEQ00000000 | (Bart *et al.,* 2012) |
| *X. campestris* pv. *campestris* | ATCC 33913 | – | NC_003902.1 | (da Silva *et al.,* 2002) |
| *X. campestris* pv. *musacearum* | NCPPB 4392 | + | NZ_AKBI00000000.1 | (Wasukira *et al.,* 2012) |
| *X. cannabis* | NCPPB 2877 | – | NZ_JSZE00000000.1 | (Jacobs *et al.,* 2015) |
| *X. citri* subsp. *citri* | 306 | – | NC_003919.1 | (da Silva *et al.,* 2002) |
| *X. euvesicatoria* | 85-10 | + | NZ_CP017190.1 | (Thieme *et al.,* 2005) |
| *X. fragariae* | LMG 25863 | – | NZ_AJRZ00000000.1 | (Vandroemme *et al.,* 2013) |
| *X. hyacinthi* | DSM 19077 | – | JPLD00000000.1 | (Naushad *et al.,* 2015) |
| *X. maliensis* | M97 | + | NZ_AQPR00000000.1 | (Triplett *et al.,* 2015) |
| *X. oryzae* pv. *oryzae* | PXO99$^{A}$ | + | NC_010717.2 | (Salzberg *et al.,* 2008) |
| *X. sacchari* | R1 | – | NZ_CP010409.1 | (Studholme *et al.,* 2011) |
| *X. translucens* | DAR61454 | + | GCA_000334075.1 | (Gardiner *et al.,* 2014) |
| *X. vesicatoria* | 15b | – | NZ_JSXZ00000000.1 | (Vancheva *et al.,* 2015) |

**Table 2.** Nucleotide sequence identity between *rax* genes.

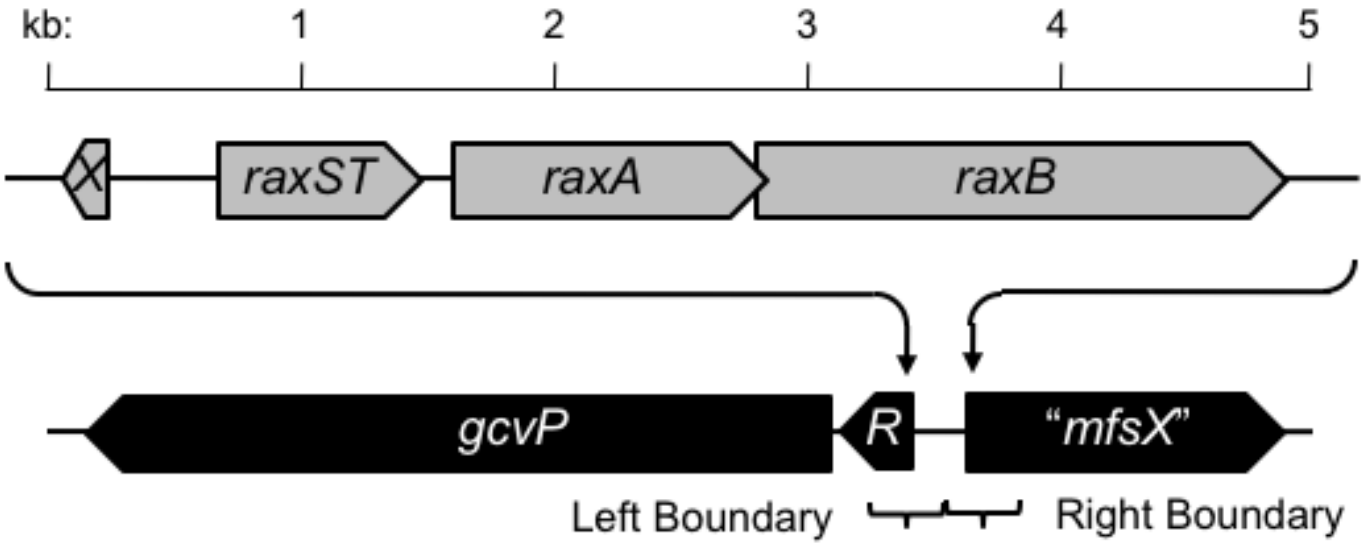
| | Comparison to *X. euvesicatoria* sequence | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | % Nucleotide identity | | | | | | Genome | |
| Species | *gcvP* | *raxX* | *raxST* | *raxA* | *raxB* | *"mfsX"* | gANI [a] | AF [b] |
| *X. citri* subsp. *citri* | 94.8 | — [c] | — | — | — | 97.3 | 94.9 | 0.80 |
| *X. axonopodis* pv. *manihotis* | 94.1 | 99.0 | 96.1 | 97.6 | 95.8 | 97.3 | 95.0 | 0.79 |
| *X. maliensis* | 93.5 | 89.5 | 96.4 | 95.0 | 96.4 | **85.0** | **83.5** | **0.66** |
| *X. campestris* pv. *malvacearum* | 91.3 | 82.2 | 91.4 | 90.9 | 93.2 | 93.6 | 91.2 | 0.72 |
| *X. oryzae* pv. *oryzae* | 92.8 | 88.5 | 91.4 | 87.6 | 89.5 | 91.6 | 91.2 | 0.61 |
| *X. translucens* | 88.7 | 63.1 | 80.7 | 71.0 | 77.3 | 80.4 | 80.4 | 0.54 |

[a] genome-wide Average Nucleotide Identity (Varghese *et al.,* 2015).

[b] genome Alignment Fraction, from *X. euvesicatoria* to subject species (Varghese *et al.,* 2015).

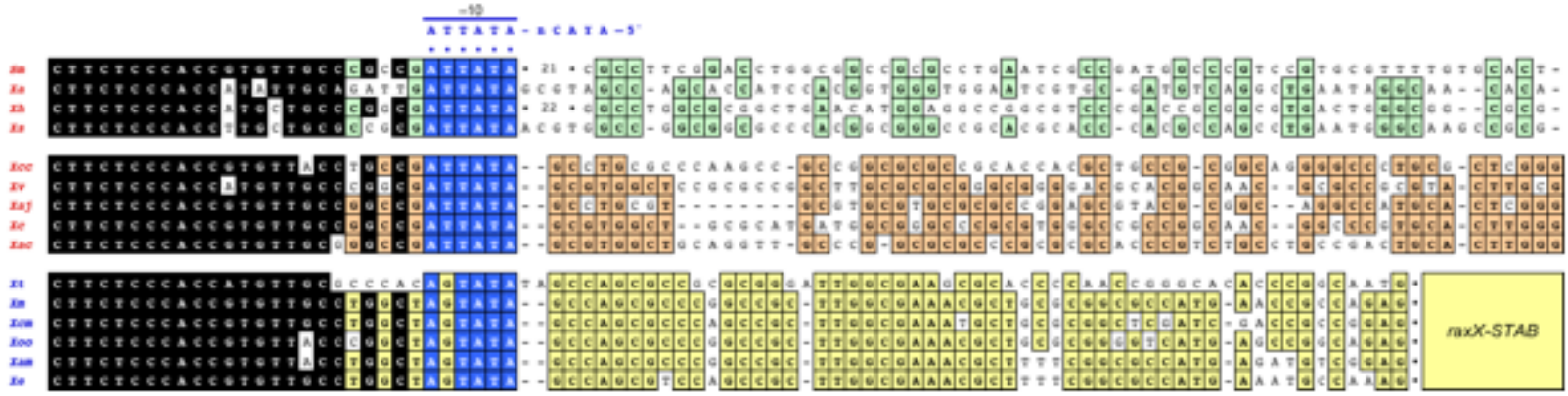
[c] —, gene not present

**A**




**B**

## Left Boundary:




## Right Boundary: