CARACTÉRISATION GÉNOTYPIQUE ET PHÉNOTYPIQUE D'ISOLATS DE *XANTHOMONAS HORTORUM* PV. *VITIANS* CAUSANT LA TACHE BACTÉRIENNE DE LA LAITUE AU CANADA

par

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Au Canada, la culture de la laitue (*Lactica sativa* L.) demeure depuis des années un marché très lucratif parmi les autres types de cultures. Étant un légume feuille, tous facteurs biotiques et abiotiques affectant l'aspect, la quantité et la taille des feuilles d'une laitue peuvent compromettre sa valeur marchande. Parmi ces facteurs, les maladies d'origine bactérienne demeurent un enjeu important autant pour les producteurs que pour la communauté scientifique, car la laitue est très sensible à plusieurs produits phytosanitaires. Les pesticides à base de cuivre causent généralement des dommages phytotoxiques aux plants, rendant les traitements contre les agents pathogènes bactériens très difficiles. C'est en effet le cas pour la maladie de la tache bactérienne de la laitue, causée par *Xanthomonas hortorum* pv. *vitians*. En 2017, un premier pesticide fut homologué contre cet agent pathogène. Toutefois, l'usage continu du même pesticide peut mener à des cas de résistance, et les traitements chimiques sur la partie consommable du légume demeurent une préoccupation pour le consommateur. Afin de réduire l'incidence de cette maladie, l'alternative la plus prometteuse reste le développement de cultivars tolérants. Par contre, afin de permettre de bien tester la tolérance face à la maladie des nouveaux cultivars développés, une bonne connaissance de la diversité de l'agent pathogène est primordiale.

Ainsi, l'objectif de ce projet de recherche était de procéder à une caractérisation génotypique et phénotypique de plusieurs isolats de *X. hortorum* pv. *vitians* recueillis dans la principale région productrice de laitue au Canada, d'identifier et de distinguer les pathotypes présents et enfin, de fournir des outils diagnostiques permettant la détection de ces pathotypes, à partir de cultures pures, en utilisant des techniques de qPCR.

La réception hebdomadaire d'échantillons de laitues symptomatiques durant les saisons estivales de 2014 à 2017 a supporté la création d'une importante collection d'isolats de *X. hortorum* pv. *vitians* purifiés à partir de laitues provenant d'une dizaine de producteurs de la région de la Montérégie. L'extraction de tissus végétaux infectés, la macération dans une solution saline, une sonication et l'étalement sur milieu de culture solide a permis de purifier

694 isolats qui ont tous pu être associés au pathovar *vitians* par amplification PCR et séquençage Sanger de quatre gènes constitutifs. L'inoculation par aspersion de trois cultivars de laitue, classés sensible (Paris Island Cos), intermédiaire (Romora) ou tolérant (Little Gem) à la tache bactérienne, suivie d'une évaluation rigoureuse des symptômes ont permis l'identification de six pathotypes de *X. hortorum* pv. *vitians* présents dans la zone de production de laitue de la Montérégie. Ces pathotypes montrent des profils différents de pouvoir pathogène sur les cultivars testés, dont la majorité ne cause pas de symptômes sur le cultivar tolérant. De plus, deux traits phénotypiques ont été étudiés *in vitro* afin de distinguer les pathotypes. La sécrétion d'un exopolysaccharide, le xanthane, et la production d'un pigment jaune, la xanthomonadine, ont été caractérisées pour plusieurs isolats appartenant aux différents pathotypes. Bien que des taux de sécrétion variables entre pathotypes aient été observés pour chacun des traits phénotypiques étudiés, les résultats semblent indiquer que la caractérisation des productions *in vitro* de xanthane et de xanthomonadine ne constituerait pas un moyen adéquat pour distinguer ces pathotypes. Afin de caractériser plus en profondeur les pathotypes, des techniques de séquençage de nouvelle génération ont été employés afin de séquencer plusieurs isolats appartenant aux divers pathotypes. Ce séquençage a permis d'identifier plusieurs éléments, tels que du polymorphisme nucléotidique, des délétions ou encore des régions ayant un nombre de copies variable au sein des génomes, pouvant être associés significativement au pouvoir pathogène observée sur le cultivar tolérant. Enfin, à l'aide de techniques de qPCR, ces éléments ont servi au développement d'outils permettant la détection, à partir de cultures pures, d'isolats de *X. hortorum* pv. *vitians* susceptibles de causer la maladie sur le cultivar tolérant. Les travaux effectués dans le cadre de ce projet ont permis d'identifier plusieurs pathotypes de *X. hortorum* pv. *vitians*, ainsi que le développement d'outils moléculaires permettant la détection de *X. hortorum* pv. *vitians* susceptible de causer des symptômes sur le cultivar tolérant à la maladie de la tache bactérienne de la laitue.

Mots clés : *Xanthomonas hortorum* pv. *vitians*, tache bactérienne, laitue, pouvoir pathogène, pathotype, séquençage de nouvelle génération, diagnostic.

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Unité de mesure

$	Dollar canadien (CAD)
°C	Degré Celsius
µl	Microlitre
µM	Micromole
bp	« base pair »
CFU	« Colony forming unit »
cm	Centimètre
m	Mètre
ml	Millilitre
mm	Millimètre
g	Gramme
h	Heure
L	Litre
min	Minute
ng	Nanogramme
nm	Nanomètre
rpm	« Rotation per minute »
sec	Seconde
xg	« Gravitational force »

Milieu de culture

YDC « Yeast Dextrose Carbonate »

Autre

ADN Acide désoxyribonucléique
am *Ante meridiem*
Blvd Boulevard
DNA « Deoxyribonucleic acid »
PCR « Polymerase chain reaction »
pv Pathovar
pm *Post meridiem*
qPCR « Quantitative polymerase chain reaction »
SNP « Single-nucleotide polymorphism »

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INTRODUCTION GÉNÉRALE

1.1 La culture de la laitue

La production de laitue et chicorée a connu un essor depuis les années 80. En effet, au niveau mondial, la production de laitue et chicorée s'est chiffrée à 26 088 462 tonnes en 2015 comparé à 8 449 156 tonnes en 1980, représentant une augmentation de plus de 208 %. La superficie récoltée a également presque triplé au cours de ces 35 ans, passant de 451 545 à 1 205 266 hectares. L'Asie demeure le plus grand producteur pour l'année 2015, totalisant 68,1 % de la production mondiale, suivi par les Amériques (18,3 %), l'Europe (11,1 %), l'Afrique (1,8 %) et l'Océanie (0,7 %). Le Canada se retrouve au 16^{e} rang des plus grands producteurs mondiaux en 2015, totalisant 109 228 tonnes de laitue et chicorée produites, dominé par la Chine (14 639 119 tonnes), les États-Unis (3 795 260 tonnes) et l'Inde (1 085 373 tonnes) (FAOSTAT, 2018; Agriculture et agroalimentaire Canada, 2016). Au Canada, sur les 109 228 tonnes de laitues produites en 2015, la province de Québec en avait produit 99 167, totalisant 90,7 % de la production au pays (MAPAQ, 2016). La production de laitue au Québec se fait majoritairement dans la région des Jardins de Napierville (Statistique Canada, 2018), où près de 50 % des laitues cultivées sont de type pommée, et près de 35 % sont de type romaine (Consortium PRISME, 2016). Pour l'année 2015, la production de laitue a rapporté des recettes de plus de 95 millions de dollars au Canada, dont plus de 83 millions avaient été générés par les producteurs du Québec.

La culture conventionnelle de la laitue consiste à faire croître les laitues en champs, à l'air libre. Cette méthode permet l'usage de machinerie afin d'augmenter l'efficacité des nombreuses étapes au cours de la culture. Selon la méthode utilisée, les semis peuvent être effectués plus tôt, pour ensuite être transplantés au champ, ou le semi peu se faire directement au champ en temps opportun. Le semi est effectué à une profondeur d'environ 0,6 cm à l'aide d'un semoir de précision (Crops and Commitee, 1988). Une fois les deux premières feuilles développées, les

plants sont éclaircis afin de favoriser la formation des cœurs. Tout au long de la croissance, les laitues sont irriguées à l'aide de gicleurs et fertilisées avec de l'azote et du phosphore. Selon la variété cultivée, la récolte se fait environ 65 jours après le semi, et peut aller jusqu'à 100 jours (Kerns *et al.*, 1999).

Depuis quelques années, la production de laitues en serre, principalement la culture hydroponique, gagne en popularité. Cette culture consiste à faire croître des plants sans sol, en utilisant des nutriments dissous dans l'eau (Santos *et al.*, 2012). Certaines méthodes permettent la récolte en aussi peu que 35 jours (Brechner et Both) et la tenue de 8 à 10 cycles de production par année (Agriculture et agroalimentaire Canada, 2006). Cette méthode culturale a ses avantages, mais aussi ses inconvénients. Une récente étude a permis de comparer les demandes en énergie, en eau et en superficie pour les cultures hydroponiques conventionnelles de la laitue (Barbosa *et al.*, 2015). La culture hydroponique permettrait d'obtenir des rendements d'environ 11 fois plus élevés qu'en cultures conventionnelles. Elle permettrait aussi de réduire la consommation en eau d'environ 13 fois, mais augmente la demande en énergie d'environ 82 fois par rapport aux méthodes conventionnelles. De plus, la culture hydroponique permettrait d'éliminer les risques liés aux agents pathogènes d'origine tellurique, mais augmenterait les chances de propagation de maladies, si celles-ci se développent (Paulitz, 1997; Paulitz et Bélanger, 2001).

1.2 Les agents pathogènes et les maladies de la laitue

Plusieurs facteurs biotiques et abiotiques peuvent affecter la culture de la laitue. Parmi les facteurs biotiques, les champignons demeurent les agents pathogènes les plus redoutables sur la laitue, particulièrement le champignon *Bremia lactucae* Regel, agent causal du mildiou (Nordskog *et al.*, 2007). Cette maladie se manifeste généralement suite à des périodes prolongées de temps frais et d'humidité (Blancard, 2013). Du côté des virus, le virus de la mosaïque de la laitue, du genre *Potyvirus*, reste l'agent pathogène d'origine virale le plus dangereux pour la laitue (Revers *et al.*, 1997). Ce virus a été répertorié dans le monde entier et peut être transmis aussi bien par les semences que par des insectes (Tomlinson, 1962). Bien qu'à

une échelle un peu moins importante, les bactéries constituent des agents pathogènes dévastateurs dans la culture de la laitue. *Erwinia*, *Pseudomonas*, *Rhizomonas* et *Xanthomonas* sont les quatre genres bactériens les plus dommageables (Blancard *et al.*, 2003).

Le genre *Erwinia* comporte plusieurs espèces, dont la majeure partie est associée aux plantes (Kado, 2006). Parmi ces espèces, *E. carotovora* subsp. *carotovora* (Jones) Bergey *et al*., aussi connu sous le nom de *Pectobacteirum carotovorum* (Jones) Waldee, proposé par Waldee (1942), est l'agent responsable de la pourriture humide et noire du pivot de la pomme de laitue (bacterial soft rot). Cette bactérie a été rapportée à travers le monde, et cause la maladie autant au champ que durant le transport, ou lors de l'entreposage des légumes et des fruits (Bhat *et al.*, 2010). La présence d'*E. aroideae* et d'*E. chrysanthemi* a également été rapportée sur la laitue (Blancard *et al.*, 2003).

Pseudomonas est un genre bactérien hétérogène qui contient plus de 120 espèces validées (Pascual *et al.*, 2012). Parmi ces espèces, deux ont été répertoriées comme étant capables de causer des maladies sur la laitue : *P. cichorii* (Swingle) Stapp. et *P. marginalis* pv. *marginalis* (Brown) Stevens, agents responsables de la maladie des taches et des nervures noires et de la maladie des taches et nécroses foliaires marginales (marginal leaf spot), respectivement. La maladie des taches et des nervures noires regroupe deux types de maladies, soit la maladie des taches noires (varnish spot) et la maladie des nervures noires (midrib rot). *P. cichorii* va causer la maladie des taches noires sur les laitues de type iceberg alors qu'elle va causer la maladie des nervures noire sur des laitues de type beurre cultivées en serre (Pauwelyn *et al.*, 2011; Cottyn *et al.*, 2009). Bien que les symptômes causés par *P. marginalis* pv. *marginalis* soient peu sévères, la maladie des taches et nécroses foliaires marginales a été répertoriée dans plusieurs champs à travers le monde (Paine et Branfoot, 1924; Berger, 1967).

Rhizomonas suberifaciens van Bruggen, Jochimsen and Brown, plus récemment connue sous le nom proposé de *Sphingomonas suberifaciens* (van Bruggen, Jochimsen and Brown 1990) Comb. Nov. (Yabuuchi *et al.*, 1999), est l'agent responsable de la maladie du gros pivot infectieux, puisque l'on observe l'élargissement du diamètre de la racine centrale ainsi que son

durcissement d'où son autre appellation de la maladie des racines liégeuses (corky root), qui affecte surtout la racine principale du plant. L'isolation et l'identification de cette bactérie pathogène sont difficiles (van Bruggen *et al.*, 1988), mais des symptômes ont été rapportés dans plusieurs pays, dont le Canada (Busch et Barron, 1963; Guzman, 1981).

Le genre *Xanthomonas* contient plus d'une trentaine d'espèces capables de causer des maladies sur au moins 124 plantes monocotylédones et au moins 268 plantes dicotylédones (Leyns *et al.*, 1984). Parmi ces espèces, *X. campestris* pv. *vitians* (Brown) Dye, aussi nommée *X. hortorum* pv. *vitians* (Vauterin *et al.*, 1995), est l'agent pathogène responsable de la maladie de la tache bactérienne de la laitue (bacterial leaf spot). Cette maladie a été rapportée sur tous les continents (Wallis et Joubert, 1972 ; Davis *et al*., 1997 ; Daboin et Tortolero, 1993; Toussaint *et al.*, 1998), et des pertes économiques substantielles pour les producteurs de laitue ont suivi les épidémies observées durant les années passées (Robinson *et al.*, 2006; Toussaint, 1999).

Xanthomonas hortorum pv. *vitians*, tout comme la majorité des autres membres du genre *Xanthomonas*, est une bactérie Gram négative capable de sécréter un exopolysaccharide et un pigment jaune, nommés xanthane et xanthomonadine, respectivement. Plusieurs études ont démontré que ces deux composés seraient importants lors du processus d'infection et joueraient un rôle dans le maintien de l'aptitude phénotypique (« fitness ») (Denny, 1995; He *et al.*, 2011). Ces deux composés sont responsables de l'allure jaune et mucoïde des colonies sur milieux solides. Cette bactérie aérobique, en forme de bacille, est non sporulante et possède un flagelle polaire (Toussaint, 1999).

1.3 Xanthane et xanthomonadine

Le xanthane a été découvert dans les années 1950 par Allene Rosalind Jeanes à partir de la bactérie *X. campestris* (Born *et al.*, 2005). Semblable à la cellulose, le xanthane est en fait une chaine de glucoses liés par des liens glycosidiques β-1,4 avec des chaines latérales composées de β-D-mannose, d'acide β-1,4-D-glucuronique, d'α-1,2-D-mannose et d'unités d'acide pyruvique (Rosalam et England, 2006). La biosynthèse de ce polysaccharide, dirigée par les

gènes du groupe *gum*, se fait en premier lieu dans le cytoplasme. Suite à une translocation, la synthèse se poursuit dans le périplasme, puis le xanthane est exporté à l'extérieur de la cellule (Vorhölter *et al.*, 2008). Quelques études on permis de mettre en évidence le rôle du xanthane au sein du pouvoir pathogène. Sutton et Williams (1970) ont démontré que l'exopolysaccharide bloquerait les vaisseaux, ce qui provoquerait des maladies vasculaires alors que d'autres ont démontré que le xanthane supprimerait la déposition de callose, un moyen de défense de la plante (Yun *et al.*, 2006). Outre ses propriétés bénéfiques pour la bactérie, le xanthane est largement utilisé dans l'industrie sous forme de gomme de xanthane. Commercialisé en 1964, le xanthane est utilisé depuis dans plusieurs aliments, boissons et autres produits non comestibles comme épaississant, émulsifiant, gélifiant, stabilisateur et autres (Sutherland, 2001).

Les xanthomonadines, des pigments jaunes originalement identifiés comme étant des caroténoïdes, sont en fait des esters aryl-polyènes halogénés ayant comme structure des acides 17-(4-bromo-3-methoxyphenyl)-17-bromo-heptadeca-2,4,6,8,10,12,14,16-octanoïque (Andrewes *et al.*, 1976). Ces pigments, dont la production est gérée par sept unités transcriptionnelles nommées *pigA* à *pigG* (Poplawsky *et al.*, 1993; Poplawsky et Chun, 1997), sont associés exclusivement à la membrane externe de la bactérie (Stephens et Starr, 1963). Les xanthomonadines protégeraient la bactérie des dommages photo-oxydatif (Jenkins et Starr, 1982) et aideraient à la survie en tant qu'épiphyte (Chun *et al.*, 1997). Par contre, une étude a démontré que ces pigments ne seraient pas nécessaires à l'agent pathogène suite à l'infection (Poplawsky et Chun, 1997). Étant uniques aux xanthomonades, les xanthomonadines, dont 15 groupes ont été identifié, ont servi au développement de marqueurs chimiotaxonomiques (Starr *et al.*, 1977). Aucun autre usage commercial n'a été rapporté dans la littérature pour les xanthomonadines.

1.4 La tache bactérienne de la laitue

La maladie de la tache bactérienne de la laitue a été rapportée pour la première fois en 1918, aux États-Unis (Brown, 1918). Les symptômes typiques consistent, en premier lieu, à des taches

foncées sur les feuilles qui s'apparentent à des cellules gorgées d'eau. Ces taches, que l'on nomme taches huileuses, évoluent rapidement en taches noires et brunes correspondant à du tissu nécrosé. Les taches peuvent se regrouper pour ainsi former de grandes lésions sur les feuilles et ce, rapidement (Figure 1.1). Bien que la température de croissance optimale *in vitro* de *X. hortorum* pv. *vitians* soit de 28 ºC (Toussaint, 1999), les conditions favorables au développement de la maladie de la tache bactérienne de la laitue ne sont pas clairement établies (Robinson *et al.*, 2006). Toutefois, une humidité élevée est requise. La principale source d'inoculum proviendrait des semences (Umesh *et al.*, 1996), mais jusqu'à présent, aucune méthode n'a réussi à détecter la bactérie pathogène dans des lots commerciaux de semences de laitue. De plus, il a été démontré que *X. hortorum* pv. *vitians* a la capacité de survire quelque temps sur les débris végétaux dans le sol (Barak *et al.*, 2001), lui conférant l'habilité de causer des épidémies lors de subséquentes cultures de laitue au même endroit. *X. hortorum* pv. *vitians* a aussi la capacité de causer des symptômes sur le poivron et la tomate (Robinson *et al.*, 2006) et peut survivre en tant qu'épiphyte sur plusieurs espèces végétales faisant parties de la famille des *Asteracea*, des *Chenopodiaceae*, des *Malvaceae*, des *Polygonaceae*, des *Portulacaceae* et des *Solanaceae* (Toussaint *et al.*, 2012), multipliant ainsi les sources possibles d'inoculum.

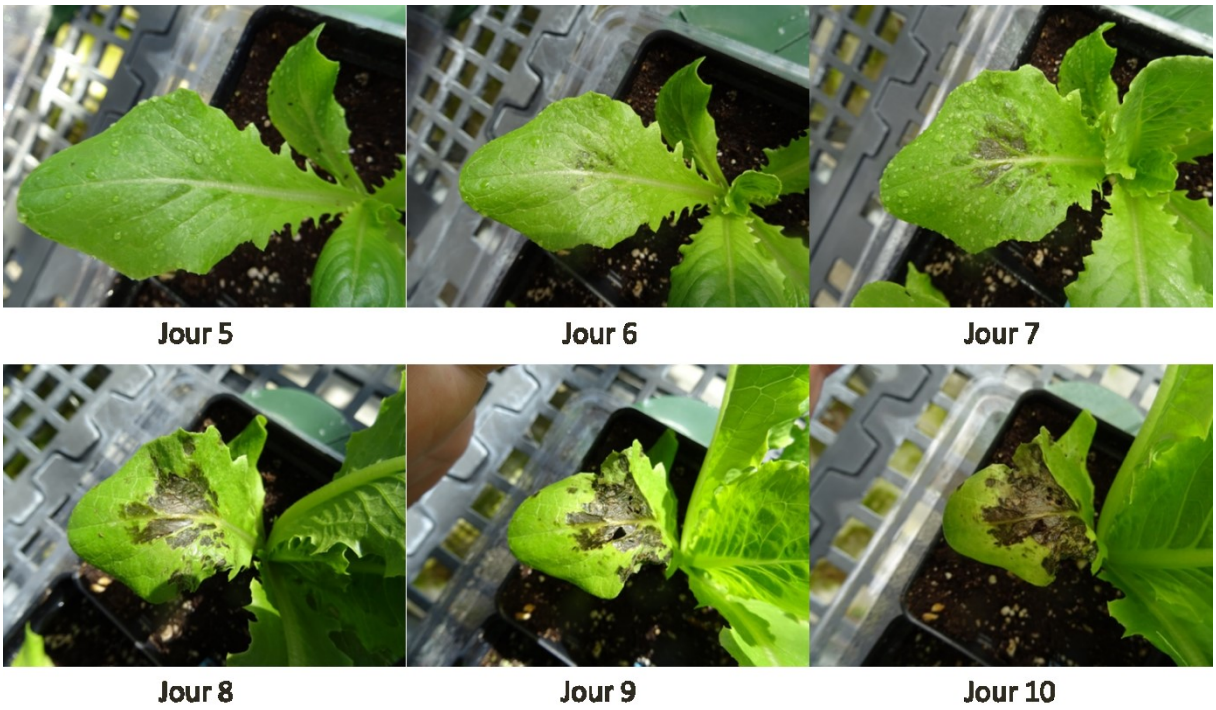

Figure 1.1. Évolution des symptômes de la tache bactérienne de la laitue, causée par la bactérie *Xanthomonas hortorum* pv. *vitians*, sur le cultivar sensible Paris Island Cos.

Avant 2017, aucun pesticide n'avait été homologué pour réduire l'incidence de la maladie dans les champs. De plus, les produits couramment utilisés dans les autres cultures afin de traiter les bactérioses contiennent du cuivre, ce qui est peu souhaitable pour la laitue, car le cuivre cause des effets phytotoxiques sur la laitue (Carisse *et al.*, 2000). Le seul produit homologué contre la tache bactérienne au Canada, à ce jour, est le Confine™ Extra (Winfield Solutions LLC, Shoreview, MN, États-Unis), un fongicide à base de sels de potassium de l'acide phosphoreux homologué contre le mildiou également. Par contre, il n'est homologué que sur la laitue feuille, en ce qui trait à la tache bactérienne (Réseau d'avertissements phytosanitaires, 2017). Comme quelques cas de résistance aux phosphonates ont déjà été recensés (Brown *et al.*, 2004; Khilare *et al.*, 2003), l'usage répété de ce fongicide pourrait mener à d'autres cas de résistance. Le développement de méthodes moins risquées a donc été entrepris, comme l'usage de cultivars tolérants, qui pour l'instant constitue le moyen le plus désirable de lutte contre la maladie.

Diverses études ont permis de classer plusieurs cultivars de laitue en fonction de leur tolérance face à la maladie de la tache bactérienne (Bull *et al.*, 2007; Carisse *et al.*, 2000; Sahin et Miller, 1997; Wang *et al.*, 2015), ce qui a permis d'identifier au moins un gène de résistance, *Xar1* (Hayes *et al.*, 2014). Ce gène a été retrouvé dans les cultivars La Brillante, Pavane et Little Gem, trois cultivars identifiés comme tolérant à la maladie de la tache bactérienne de la laitue (Bull *et al.*, 2007; Hayes *et al.*, 2013; Hayes *et al.*, 2014). Le cultivar La Brillante étant de type Batavia, et les cultivars Pavane et Little Gem étant de type Latin, le gène de résistance *Xar1* n'a pas été identifié dans des laitues de type Romaine ou Iceberg, qui sont très cultivés en Amérique du Nord.

1.5 Diversité de *Xanthomonas hortorum* pv. *vitians*

La diversité, autant génotypique que phénotypique, de *X. hortorum* pv. *vitians* a été démontrée au cours de plusieurs études. Sahin *et al.* (2003) ont identifié deux groupes démontrant un pouvoir pathogène différent, basés sur les symptômes causés sur quelques espèces végétales. Le premier groupe cause des taches nécrotiques et des symptômes de maladie systémique, alors que le second groupe cause seulement des taches nécrotiques. Ces deux groupes ont aussi été identifiés au niveau sérologique, en se basant sur le profil des antigènes présents à la surface de la paroi cellulaire. Ils ont également noté quelques divergences entre ces groupes à ce qui trait à l'utilisation de sources de carbone. Des différences pour l'utilisation de l'acide α-hydroxybutyrique, de l'acide α-cetobutyrique ainsi que de l'acide aminé L-Sérine ont été observées. Barak et Gilbertson (2003) ont démontré que certains isolats de *X. hortorum* pv. *vitians* différaient quant à leur profil de plasmides. Deux plasmides de tailles distinctes ont été observés, et certains isolats possédaient les deux. Des patrons de digestions enzymatiques différents ont également été observés chez les isolats ayant un seul plasmide, mais aussi chez les isolats ayant les deux. Une analyse de séquence multilocus a également permis de mettre en lumière la diversité génétique au sein d'isolats de *X. hortorum* pv. *vitians* provenant de la laitue et d'identifier trois groupes distincts, selon les huit gènes utilisés, soit *fusA*, *gap-1*, *gltA*, *gyrB*, *lacF*, *lepA*, *JF1* et *hrpB* (Fayette *et al.*, 2016). Le niveau diversité des isolats de *X. hortorum* pv. *vitians* semble être affectée par la provenance des isolats, en ce sens où le niveau de diversité

observée avec les isolats provenant de la Californie (Barak et Gilbertson, 2003) ne concorde pas nécessairement avec le niveau de diversité observé avec ceux provenant de l'Ohio (Sahin *et al.*, 2003) ou de la Floride (Fayette *et al.*, 2016).

1.6 Enjeu du projet de recherche

Ce présent projet de recherche s'insère donc dans la problématique du développement de cultivars de laitue tolérants à la maladie de la tache bactérienne. Afin de réduire l'usage de pesticides et ainsi réduire le risque de développement de résistance chez *X. hortorum* pv. *vitians*, les cultivars tolérants développés doivent être le plus efficace possible. Afin de favoriser cette efficacité, une bonne connaissance de la diversité de l'agent pathogène est essentielle. C'est pourquoi le but de ce projet de recherche est de caractériser, au niveau génotypique et phénotypique, plusieurs isolats de *X. hortorum* pv. *vitians* récoltés dans les zones de production de laitue au Québec. La diversité au sein des isolats de cet agent pathogène a été mise en lumière à plusieurs reprises aux États-Unis, mais ces études n'incluaient pas d'isolats en provenance du Canada. De plus, aucune étude comparable à celle-ci n'a été effectuée en sol canadien. Le développement local de cultivars tolérants manque donc d'information afin de bien tester les nouvelles lignées potentielles face à la tache bactérienne. De bons indicateurs de la diversité de cet agent pathogène en sol québécois ont été les tests de pouvoir pathogène effectué au Centre de Recherche et de Développement de Saint-Jean-sur-Richelieu au cours des dernières années. Alors que plusieurs isolats de *X. hortorum* pv. *vitians* avaient été inoculés par aspersion sur différents cultivars de laitue, une uniformité des symptômes causés au sein d'un même cultivar n'était pas observée. En d'autres mots, pour un cultivar donné, certains isolats étaient plus agressifs que d'autres, variant de peu ou pas de symptômes à beaucoup de symptômes. Ces tests ont été répétés et les mêmes résultats ont été observés à chaque fois, ce qui laissait croire en la présence de plus d'un pathotypes de *X. hortorum* pv. *vitians* sévissant dans les cultures de laitue au Québec. Or, la présence de pathotypes de l'agent causal de la maladie de la tache bactérienne de la laitue au Québec représenterait une information clé pour les améliorateurs génétiques de cultivars de laitue, surtout si certains de ces pathotypes ont la capacité de causer la maladie sur les cultivars actuellement connus comme tolérants. Ainsi, le but étant de caractériser la diversité

génétique et pnénotypique de la population de *X. hortorum* pv. *vitians* au Canada, le projet de recherche est composé de quatre objectifs spécifiques. Le premier objectif spécifique correspond à poursuivre l'analyse de séquence multilocus avec les isolats recueillis en 2016 et 2017, afin de vérifier l'évolution de la diversité des isolats par rapport à ceux recueillis en 2014 et 2015. Le deuxième objectif spécifique consiste en une caractérisation de traits phénotypiques d'isolats de *X. hortorum* pv. *vitians* recueillis de 2014 à 2016. Cette caractérisation se fait en premier lieu en serre, avec des tests de pouvoir pathogène, ce qui permettra de déterminer les potentiels pathotypes. Ensuite, la production de xanthane et de xanthomonadine sera investiguée au laboratoire. Le troisième objectif spécifique est la caractérisation génétique de ces mêmes isolats, plus particulièrement par le séquençage du génome entier à l'aide d'un séquenceur MiSeq, utilisant des technologies de séquençage de nouvelle génération. Enfin, le dernier objectif spécifique consiste au développement d'outils diagnostic permettant la détection des pathotypes de *X. hortorum* pv. *vitians* identifiés précédemment.

CHAPITRE 2
ÉTUDE GÉNOTYPIQUE ET PHÉNOTYPIQUE D'ISOLATS DE LA BACTÉRIE PATHOGÈNE DE LA LAITUE XANTHOMONAS HORTORUM PV. VITIANS PURIFIÉS AU QUÉBEC, CANADA

2.1 Justification de l'article

La maladie de la tache bactérienne de la laitue est provoquée par la bactérie *X. hortorum* pv. *vitians*. Débutant avec des taches d'apparence huileuses, les symptômes évoluent rapidement en lésions nécrotiques sur la surface des feuilles, causant beaucoup de pertes pour les producteurs. Un seul pesticide est homologué contre cette maladie au Canada, mais comme l'usage répété du même composé peut mener à des cas de résistance, le développement de cultivars de laitue tolérant envers la tache bactérienne reste le moyen de lutte le plus prometteur. Peu, sinon aucune étude n'a été effectuée au niveau de la diversité de *X. hortorum* pv. *vitians* au Québec ou au Canada, laissant les améliorateurs génétiques de cultivars de laitue avec de l'information obsolète concernant cette bactérie pathogène qu'ils essaient de déjouer. Ce manque d'informations fiables a donc mis en lumière le besoin d'évaluer la diversité de cette bactérie présent dans les champs de laitue canadiens.

Les travaux effectués au cours de cette étude ont permis d'identifier plusieurs pathotypes de l'agent causal de la maladie de la tache bactérienne de la laitue pour ainsi mettre en évidence la diversité de cette bactérie au Québec. Les résultats acquis ont également contribué à l'avancement des connaissances sur l'épidémiologie de cet agent pathogène et ont permis de fournir à l'industrie de la laitue et à la communauté scientifique un outil de diagnostic qui pourrait permettre la détection des isolats les plus susceptibles de causer la maladie à la source de l'inoculum, soit la semence.

Les travaux accomplis, de même que les résultats recueillis lors de cette étude sont exposés sous la forme d'un article scientifique nommé « Genotypic and phenotypic study of isolates of lettuce

bacterial pathogen *Xanthomonas hortorum* pv. *vitians* collected in Québec, Canada ». Les auteurs sont Pierre-Olivier Hébert, Martin Laforest, Dong Xu, Marie Ciotola, Mélanie Cadieux, Carole Beaulieu et Vicky Toussaint. Pierre-Olivier Hébert a accompli la majorité des expériences ainsi qu'effectué la rédaction du manuscrit. Martin Laforest a contribué en participant à l'analyse bio-informatique des génomes séquencés ainsi qu'au développement des outils diagnostiques. Dong Xu a contribué au développement des techniques d'analyse de séquences Sanger. Marie Ciotola et Mélanie Cadieux ont contribué au développement des techniques d'inoculation de laitues en serre, et ont participé aux tests de pouvoir pathogène. Carole Beaulieu et Vicky Toussaint ont supervisé l'ensemble du projet. Martin Laforest, Dong Xu, Carole Beaulieu et Vicky Toussaint ont révisé le manuscrit.

Genotypic and phenotypic characterization of lettuce bacterial pathogen *Xanthomonas hortorum* pv. *vitians* isolates collected in Quebec, Canada

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Key words : *Xanthomonas hortorum*, bacterial leaf spot, lettuce, pathogenicity, genome-wide association study

2.2 Abstract

Bacterial leaf spot of lettuce, caused by *Xanthomonas hortorum* pv. *vitians*, is an economically important worldwide disease that caused millions of dollars in losses in only a few months in winter of 1992 in Florida. While only one pesticide is registered to control this disease in

Canada, development of lettuce cultivars tolerant to bacterial leaf spot remains the most promising approach, in a host-specific resistance context, to reduce its incidence and severity in lettuce fields. However, the lack of information about the diversity of the pathogen impairs breeding programs mainly when disease resistance is tested on newly developed germplasm lines. To evaluate the diversity of *X. hortorum* pv. *vitians*, a multilocus sequence analysis was performed on 694 isolates collected in Quebec through summers of 2014 to 2017, and one collected in 2007. All isolates tested were clustered into five phylogroups. Six pathotypes were identified following pathogenicity tests conducted in greenhouses, but when phylogroups were compared with pathotypes, no correlation could be drawn. *In vitro* production of xanthan and xanthomonadins was also investigated and higher production rates of xanthomonadins have been correlated to the disability of causing disease on cultivar Little Gem. Whole-genome sequencing was undertaken with 95 isolates belonging to pathotypes II to VI, and *de novo* assembly made with reads unmapped to the reference strain's genome resulted in 694 contigs ranging from 128 to 120,795 bp. Variant calling was performed prior to genome-wide association studies computed with single-nucleotide polymorphisms (SNPs), copy-number variants and gaps. Significant p-values were only found on tolerant cultivar Little Gem. Our results allowed identification by molecular assays of isolates likely to cause bacterial leaf spot of lettuce, using two SNPs identified through genome-wide association study.

2.3 Introduction

Plant pathogens use a wide array of pathogenesis mechanisms to infect their hosts. Many fungi and Oomycetes invade plants via appressoria or enzymatic activity (Widmer *et al.*, 1998) and viruses use vectors to reach host plants and enter them through damaged cells (Mandadi & Scholthof, 2013) while bacteria often use a cell-communication system known as quorum sensing to coordinate colonization of hosts (von Bodman *et al.*, 2003) and inject effector proteins in plant cells using diverse secretion systems (Büttner & He, 2009) (for a more complete review, see Giraldo & Valent, 2013). Members of the bacterial genus *Xanthomonas*, which possess type I to VI secretion systems (Büttner & Bonas, 2010), are divided in 27 species known to cause disease on many mono- and di-cotyledons plants. Those species are also subdivided

into pathovars, to differentiate strains according to their pathogenicity on different host species. Pathovars can also include pathotypes, to differentiate strains within pathovars according to their pathogenicity on varieties of specific host species.

Xanthomonas campestris pv. *vitians* (Brown) Dye, or also known as *Xanthomonas hortorum* pv. *vitians*, the causative agent of bacterial leaf spot (BLS) of lettuce, is a rod-shaped, aerobic Gram negative bacteria. It is non-sporulating and possesses one polar flagellum. The recently sequenced *X. hortorum* pv. *vitians* strain B07-007 has a chromosome and a plasmid of 5,175,249 bp and 75,655 bp, respectively. This bacterium, like many other xanthomonads, secretes an exopolysaccharide called xanthan and produces brominated aryl-polyene pigments called xanthomonadins. Those two products play important roles in maintaining the fitness and in the infection process (He *et al.*, 2011; Denny, 1995) and are responsible of the yellow and mucoid aspect of colonies on solid media.

Since its first report in South Carolina in 1918 (Brown, 1918), BLS has been reported in many countries such as Australia (Davis *et al*., 1997), Brazil (Bradbury, 1986), France (Allex & Rat, 1990), Germany (Davis *et al*., 1997), India (Wallis & Joubert, 1972), Italy (Zoina & Volpe, 1994), Japan (Bradbury, 1986), South Africa (Wallis & Joubert, 1972), Turkey (Sahin, 2000), United States (Pernezny *et al*., 1995) and Venezuela (Daboin & Tortolero, 1993). In Canada, the first report went back to 1994 (Toussaint *et al.*, 1998) and since, the disease was observed in many lettuce fields over the years.

Conflicting reports have been written on required environmental conditions for disease development (Robinson *et al.*, 2006). The optimum temperature reported for outbreaks to occur varies from cold to warm. Although conditions for BLS to occur remain unclear, symptoms are easily distinguishable. They consist of water-soaked lesions scattered on leaves that later become necrotic and papery. V-shaped lesions near veins due to systemic infection can also be observed (Sahin & Miller, 1997).

According to the Food and Agriculture Organisation of the United Nations, lettuce production almost doubled in the last 20 years, going from 14 million tonnes in 1994 to 26 million tonnes in 2016. With a farm gate value of more than $78 million in 2017, lettuce was Canada's fourth most grown vegetables according to Statistics Canada. In the United States of America, the value of production was established by the U. S. Department of Agriculture to be over $3 billion in 2017.

Prior to July 2017, no chemicals were registered in Canada against BLS. Currently, the only registered pesticide uses mono- and di-potassium salt of phosphorous acid. However, continuous use of the same pesticide could lead to resistance from the targeted pathogen (Cooksey, 1990; Ritchie & Dittapongpitch, 1991). Therefore, alternative ways such as development of tolerant cultivars of lettuce towards BLS tend to be amongst the most promising option for controlling the disease (Lu *et al.*, 2014). Consequently, knowledge of *Xanthomonas hortorum* pv. *vitians* diversity present in lettuce fields in Canada is crucial to lettuce breeders in order to develop efficient tolerant cultivars.

The goals of this study were to characterize the pathotypes of *Xanthomonas hortorum* pv. *vitians* present in lettuce fields in Quebec through genotypic and phenotypic analyses and to develop a detection tool using molecular markers identified through next-generation sequencing that could be used for diagnostic purposes.

2.4 Materials and methods

2.4.1 Purification of bacterial isolates

Bacterial isolates were purified from symptomatic lettuces harvested by the company Phytodata Inc. (Sherrington, Quebec, Canada) in fields located in the Monteregie region, in Quebec, during the summers of 2014 to 2017. Upon reception of the samples, symptomatic leaves were washed with water, and five discs were cut with a 1-cm diameter punch while ensuring that both healthy and diseased tissues were sampled for disc. Discs were then washed with a 70 % ethanol solution

for one minute, and then washed for another minute with a 0.05 % bleach solution. After being rinsed three times with sterile water, discs were cut in half and transferred in 10 ml of saline solution (NaCl 0.85% w/v in distilled water), and then sonicated in a FS20H ultrasonic bath (Thermo Fisher Scientific, Waltham, MA, USA) during 5 minutes. These bacterial suspensions were plated on Yeast Dextrose Carbonate (YDC) media (Wilson *et al*., 1967) amended with cycloheximide (50 mg/L) (Sigma-Aldrich, St-Louis, MO, USA) and incubated at 28 °C until isolated yellow and mucoid colonies developed. Purified bacterial isolates were stored at -80 °C in a preservation broth (Tryptic soy broth 30 g/L, glycerol 100 ml/L) until usage.

2.4.2 DNA extraction and multi-locus sequence analyses

Five ml of nutrient broth (Thermo Fisher Scientific, Waltham, MA, USA) was inoculated with a single colony and incubated overnight at 150 rpm at room temperature. A volume of 2 ml of bacterial culture was centrifuged (5000xg, 1 min) and DNA was extracted from pellets using Norgen Bacterial Genomic DNA Purification kit (Norgen Biotek, Thorold, ON, Canada). Following manufacturer's instructions, 10 to 100 ng of DNA was used in a 50-µl PCR reaction using One*Taq*® polymerase (New England Biolabs, Ipswich, MA, USA) and previously described primers (Young *et al.*, 2008) for partial housekeeping genes *gyrB*, *rpoD* and *dnaK* and primers XanfyuA 4F 5′-AYTCSTACGTGCAGAGCCTG-3′ and XanfyuA3R 5′-CGTRTAGCCSGGCATCTTCA-3′ for partial housekeeping gene *fyuA*. Conditions for PCR reaction were the following: 95 ºC for 2 min followed with 30 cycles of 94 ºC for 20 sec, 54 ºC for 30 sec, 68 ºC for 1 min and a final elongation at 68 ºC for 10 min. PCR was performed in a SureCycler 8800 (Agilent Technologies, Santa Clara, CA, USA) and PCR products were migrated on 0.7 % agarose (BioShop Canada Inc., Burlington, ON, Canada) gel in 1X Tris-acetate-EDTA (TAE) buffer (Invitrogen, Burlington, ON, Canada) with SYBR Safe dye (Invitrogen, Burlington, ON, Canada) before being visualized with a G:BOX (Synoptics Ltd, Cambridge, UK). Amplicons were then sent to the McGill University and Génome Québec Innovation Centre (Montreal, QC, Canada) for Sanger sequencing. Sequences were verified using SeqMan Pro (DNASTAR, Madison, WI, USA) and a neighbor-joining phylogenetic tree, with a bootstrap value of 1000, was generated with the concatenated sequences using MEGA

5.1 (Pennsylvania State University, PA, USA). Reference strains *X. axonopodis* (CFBP 4924), *X. axonopodis* pv. *alfalfae* (CFBP 3836), *X. axonopodis* pv. *allii* (CFBP 6107), *X. axonopodis* pv. *begonia* (CFBP 2524), *X. axonopodis* pv. *glycines* (CFBP 2526), *X. axonopodis* pv. *maculifoliigardeniae* (CFBP 1155), *X. axonopodis* pv. *malvacearum* (CFBP 7153 and CFBP 2530), *X. axonopodis* pv. *phaseoli* (CFBP 2534), *X. axonopodis* pv *poinsettiicola* (CFBP 7277), *X. axonopodis* pv. *vasculorum* (CFBP 5823), *X. axonopodis* pv. *vignicola* (CFBP 7112), *X. axonopodis* pv. *vitians* (CFBP 2538), *X. campestris* pv. *aberrans* (CFBP 6865), *X. campestris* pv. *barbareae* (CFBP 5825), *X. campestris* pv. *betae* (CFBP 5852), *X. campestris* pv. *bilvae* (CFBP 3136), *X. campestris* pv. *campestris* (CFBP 5241), *X. campestris* pv. *esculenti* (CFBP 5857), *X. campestris* pv. *incanae* (CFBP 2527), *X. campestris* pv. *mangiferaeindicae* (CFBP 1716), *X. campestris* pv. *paulliniae* (CFBP 5862), *X. campestris* pv. *raphani* (CFBP 5827), *X. campestris* pv. *viegasii* (CFBP 5866 and CFBP 4477), *X. hortorum* pv. *carotae* (GenBank CM002307.1), *X. hortorum* pv. *hederae* (CFBP 4925), *X. hortorum* pv. *perlargonii* (CFBP 2533), *X. hortorum* pv. *taraxaci* (CFBP 410) and *X. hortorum* pv. *vitians* (NCPPB 2248) were also characterized for comparison with our isolates. All reference strains used in this study came from CIRM-CFBP: International Center for Microbial Resources (https://www6.inra.fr/cirm_eng/) except *X. hortorum* pv. *vitians* (NCPPB 2248) which came from the National Collection of Plant Pathogenic Bacteria and sequences from *X. hortorum* pv. *carotae* which were retrieve from GenBank. The nomenclature of Vauterin, Hoste, Kerster and Swings was used (Vauterin *et al.*, 1995).

2.4.3 Pathogenicity assay

One hundred and seventy *X. hortorum* pv. *vitians* isolates were spray-inoculated on three lettuces cultivars (Romora, Little Gem and Paris Island Cos). Lettuces seeds were sown in 128 cells plates containing ProMix (ProMix BX Mycorrhizae, Premier Tech Biotechnologies, Rivière-du-Loup, QC, Canada) and grown in an Adaptis germinator (Conviron, Winnipeg, MB, Canada) during 14 days at 18 °C, at 75 % humidity and a 16 h photoperiod at a light intensity of 400 µmol/m^2/sec. Plants were then transplanted in 10 cm plastic pots, fertilized with 10-52-10 fertilizer (Master Plant-Prod Inc., Brampton, ON, Canada) and incubated in the same

conditions, but at 20 °C instead, for three days. Lettuces were spray-inoculated on both abaxial and adaxial leaf surfaces until run-off using a Phantom 100 air-brush (Createx colors, CT, USA) with bacterial suspensions adjusted to $1x10^8$ CFU/ml prepared with saline solution (0.85 % w/v). *X. hortorum* pv. *vitians* strain B07-007 (Xu *et al.*, 2017, unpublished) was used as positive control while saline solution was used as negative control. Bacterial suspensions were also used to verify pathogenicity on tobacco leaves (*Nicotiana tabacum* L. cv. Xanthi) by injecting suspension into abaxial side of leaves with 1 ml needleless syringes (Terumo, Tokyo, Japan) until the solution infiltrated an area of approximately 2 cm of diameter. Inoculated plants were kept for two hours at room temperature and then incubated in a greenhouse at 24 °C during daytime and 19 °C during nighttime, at 70 % humidity. The first two days after inoculation, lights were turned off, shade houses were deployed and misting was triggered every 30 minutes, during 20 seconds, from 8 am to 8 pm. From the third day, 16 hours of photoperiod without shade house were daily applied and misting was triggered at every hour. During the whole study, plants were fertilized continuously with 6-11-31 fertilizer (Master Plant-Prod Inc., Brampton, ON, Canada) and 15.5-0-0 fertilizer (Yara International, Oslo, Norway). Lettuces disease symptoms were evaluated 8 to 10 days after inoculation using a disease scale taking into account the size of lesions (Table 2.1). Scores were converted to parametric values to simplify analyses. All statistical analyses were conducted with the statistical analysis add-in software XLSTAT 2010 (Addinsoft, Paris, France).

Table 2.1 Disease rating scale used for evaluation of symptoms on lettuce.

Diameter of lesions	Score	Occurrence of lesions	Score
< 2 mm	1	On one inoculated leaf	.1
2 mm ≤ lesions ≤ 5 mm	2	On less than half of inoculated leaves but on more than one	.2
5 mm ≤ lesions ≤ 9 mm	3	On more than half of inoculated leaves	.3
9 mm ≤ lesions ≤ 20 mm	4		
20 mm ≤ lesions ≤ 41 mm	5		

2.4.4 Purification of xanthan

Purification of xanthan was carried out with 8, 16, 8 and 16 isolates from previously identified pathotypes III, IV, V and VI respectively, using a described protocol (Steffens *et al*., 2016). Briefly, 90 ml of broth (glucose 25 g/L, yeast extract 3 g/L, KH_2PO_4 2 g/L and $MgSO_4$ 1 g/L) were inoculated with 10 ml of bacterial suspension adjusted to $1x10^{-8}$ CFU/ml in saline solution (0.85 %) before being incubated at 150 rpm during 96 h at 28 °C. After incubation, 10 ml of culture was centrifuged for 1 h at 12 500 xg. Both the pellet and the supernatant were kept separately. For the supernatant, isopropanol (30 ml) was added and mixed, followed by incubation on ice for 30 minutes prior to centrifugation for 1 h 30 at 9000 xg. The supernatant was discarded and both pellets were dried at 60 °C for 2 h and then dried in a vacuum bell containing Drierite desiccant (W A Hammond Drierite Co. LTD., Xenia, OH, USA) for four days at room temperature. Xanthan production was expressed in grams of xanthan produced per gram of biomass.

2.4.5 Purification of xanthomonadins

The protocol previously described by He *et al.* (2011) was used to purify xanthomonadins for the same isolates used for xanthan purification. Briefly, 50 ml of no. 1 nutrient broth (Sigma-Aldrich, St-Louis, MO, USA) were inoculated with a single colony and incubated at 150 rpm, 28 °C, until the stationary phase (approximately 32 h). Cultures were adjusted to an optical density of 1.0 at 600 nm. One hundred µl was sampled and used to do serial dilutions with sterile water. Dilutions from 10^{-4} to 10^{-7} were plated on Pseudomonas Agar F medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Five ml from adjusted cultures were also centrifuged (5 min, 4000 xg) and pellets were kept. One ml of methanol was added to the pellet and the solution was mixed and heated to 100 °C (Starr & Stephens, 1964) for 10 minutes. Optical density of methanol extracts was measured at 441 nm (Poplawsky & Chun, 1997) on a Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) after centrifugation (5 min, 5000 xg). Wet weights of pellets were also weighed.

2.4.6 Bacterial genomes sequencing & *de novo* assembly

Genomic DNA of 96 *Xanthomonas hortorum* pv. *vitians* isolates purified from lettuces between 2014 and 2016 were sequenced using MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA). Libraries were prepared following the instruction of the Nextera XT library preparation kit (Illumina, San Diego, CA, USA), but instead of normalizing the library with magnetic beads, we quantified the libraries using the NEBNext Library Quant Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Reads were filtered for a quality score of 15 with Trimmomatic (Bolger *et al.*, 2014). After a first mapping with BWA 0.7.12 (Li & Durbin, 2009) against the reference strain *Xanthomonas hortorum* pv. *vitians* B07-007 (GenBank accession no. CP016878.1 (genome) and CP016879.1 (plasmid)), unmapped reads were used to do a *de novo* assembly with SPAdes 3.12 (Bankevich *et al.*, 2012) to investigate possible insertions in isolate's genomes.

2.4.7 Genome-wide association study (GWAS)

Reads were mapped once again to the reference strain *Xanthomonas hortorum* pv. *vitians* strain B07-007 and to *de novo* contigs generated previously (Supplementary file 2.2) using BWA. Variant calling was made with Samtools 1.4.1 (Li *et al.,* 2009; Li, 2011) following a workflow available online (http://www.htslib.org/workflow/) and associations between the genotype and observed phenotypes were completed using PLINK 1.07 software (Purcell *et al.*, 2007). PLINK is available online (http://pngu.mgh.harvard.edu/purcell/plink/) as a free open-source software. PLINK was run with three datasets corresponding to single-nucleotide polymorphisms (SNP) identified with Samtools, nucleotide with a coverage of zero present on sequenced genomes (Perl scripts), which are basically gaps, and copy number variant (CNV) obtained with CNV-Seq (Xie & Tammi, 2009). CNVs were investigated to identify regions or genes present in multiple numbers of copies that would be missed with the gap analysis alone.

2.4.8 Diagnostic tools

On the 170 isolates tested on lettuce, the 75 unsequenced isolates were used for qPCR amplification assays with rhAmp® probes (IDT, Coralville, IA, USA). Two rhAmp® assays were developed with the rhAmp® Genotyping Design Tool based on bi-allelic SNPs identified with PLINK. The first assay is using a SNP located at the position 590,650 of the reference strain *X. hortorum* pv. *vitians* B07-007's chromosome (GenBank accession no. CP016878.1), with a p-value of 2.84×10^{-13}, and the second assay uses a SNP located at the position 549 of *de novo* contig 245, with a p-value of 5.62×10^{-11}. Quantitative PCR amplifications were performed on an Mx3000P qPCR system with MxPro qPCR software (Agilent Technologies, Santa Clara, CA, USA) following the instructions of the rhAmp® SNP Genotyping kit. Yakima Yellow dye was detected with the HEX dye channel while FAM dye was detected with the FAM channel.

2.5 Results

2.5.1 Multi locus sequence analyses

Housekeeping genes *fyuA*, *gyrB*, *rpoD* and *dnaK* were partially amplified and sequenced for 694 *Xanthomonas hortorum* pv. *vitians* isolates purified during the summers of 2014 to 2017. Strain B07-007 was collected in 2007. The neighbor-joining tree generated with the concatenated sequences of purified isolates, along with the reference strains, allowed us to position all the isolates into five distinct phylogroups, named A to E. Isolates' sequences of phylogroup A are identical to the sequence of the reference strain *X. hortorum* pv. *vitians* B07-007 while other phylogroups are all genetically close, but different, to the reference strain (Figure 2.1), based on those sequences. Phylogroups A, B, C, D and E represent 427, 2, 21, 25 and 220 isolates respectively. Although all phylogroups were present in 2014, only phylogroups A and E were observed in the following three years.

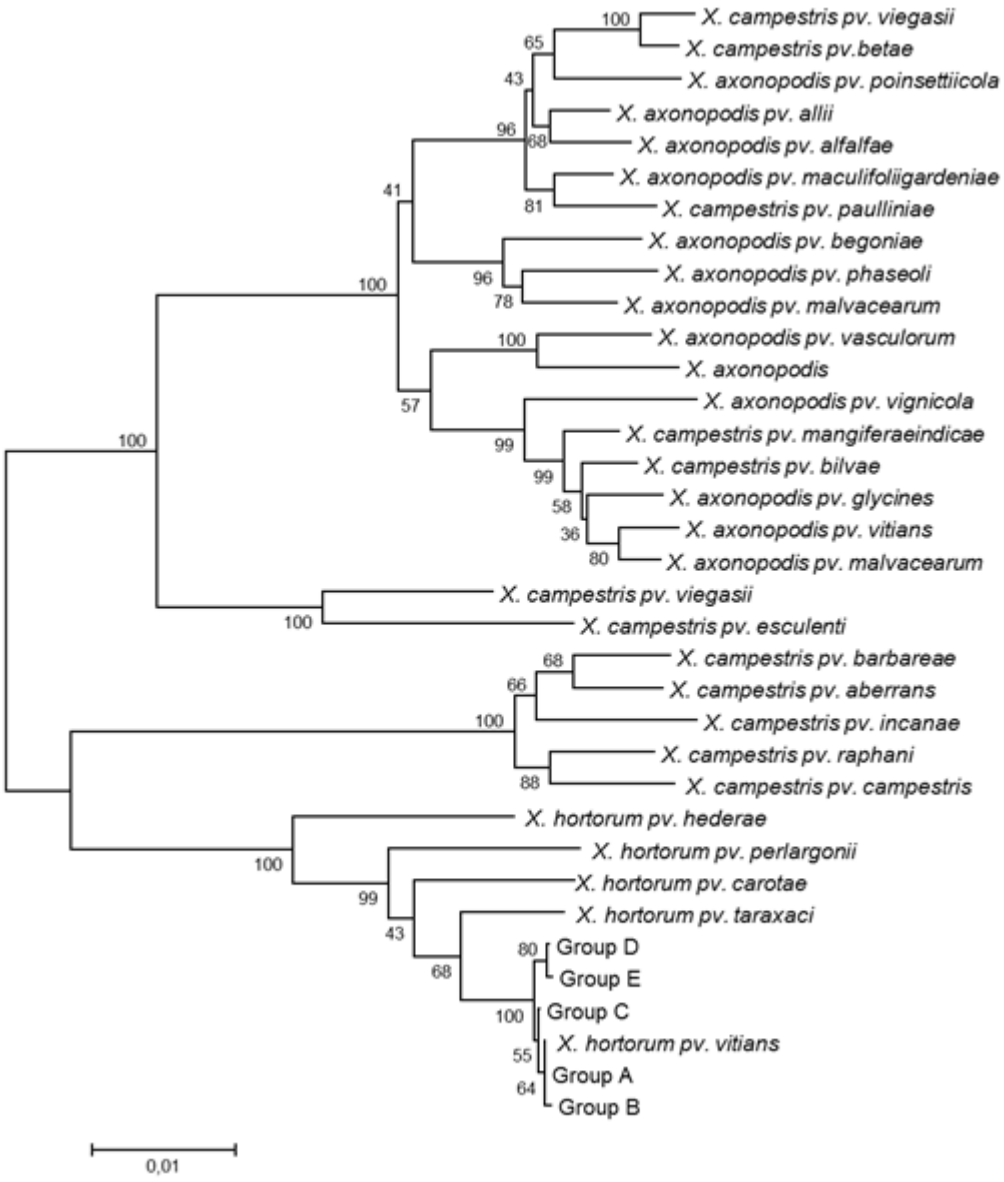

Figure 2.1. Neighbor-joining tree of *Xanthomonas hortorum* pv. *vitians* isolates purified from 2014 to 2017 in Quebec, Canada, and 30 xanthomonads reference strains, based on multi-locus sequence analyses. Bootstrap support values are shown at nodes and bar lengths are in number of substitutions per site. Phylogroups A, B, C, D and E are composed of 427, 2, 21, 25 and 220 isolates of *X. hortorum* pv. *vitians* respectively.

2.5.2 Pathogenicity assay

Disease severity caused by 170 *Xanthomonas hortorum* pv. *vitians* isolates purified in 2014, 2015 and 2016 were evaluated on three lettuce cultivars classified as susceptible (Paris Island Cos), intermediate (Romora) and tolerant (Little Gem) (Carisse *et al.*, 2000; Bull *et al.*, 2007; D. Rekika, pers. comm.). The disease severity observed in the greenhouse during the pathogenicity assays matched the disease tolerance of each cultivar. Overall, we observed most severe symptoms on Paris Island Cos, and least on Little Gem, while symptoms on Romora were intermediate. Eight to ten days after inoculation, symptoms developed at least on one lettuce cultivar for 169 isolates. The remaining isolate, belonging to group B, caused only an hypersensitive reaction (HR) on the three cultivars tested, indicating that these isolates were not possibly *X. hortorum* pv. *vitians*. Also, all isolates caused HR on tobacco leaves, indicating that our isolates were plant pathogenic bacteria (Schaad *et al.*, 2001). The average disease severity was determined for all isolates tested and groups were compared to each other. All groups are generally more aggressive on Paris island Cos than on the two other cultivars. While, on average, groups A and C were more aggressive on cultivar Romora than on Little Gem, groups B, D and E were more aggressive on cultivar Little Gem than on Romora, but when we compared isolates to each other, we observed that within a group, isolates did not cause the same disease severity (Figure 2.2). Since there is only a weak to very weak correlation between the aggressiveness of the isolates on the different cultivars, it is impossible to form groups on that criterion (Figure 2.3). Therefore, a new classification was developed based on the ability of each isolates to cause symptoms or not, for each lettuce cultivar, independently and five pathotypes of *X. hortorum* pv. *vitians*, named I to V, were determined. Pathotypes I, II and III did not cause symptoms on Little Gem, pathotype I did not cause symptoms on Paris Island Cos and pathotypes II and IV did not cause symptoms on Romora (Figure 2.4). Pathotypes I, II, III, IV and V include 4, 17, 67, 21 and 60 isolates respectively (Table 2.2).

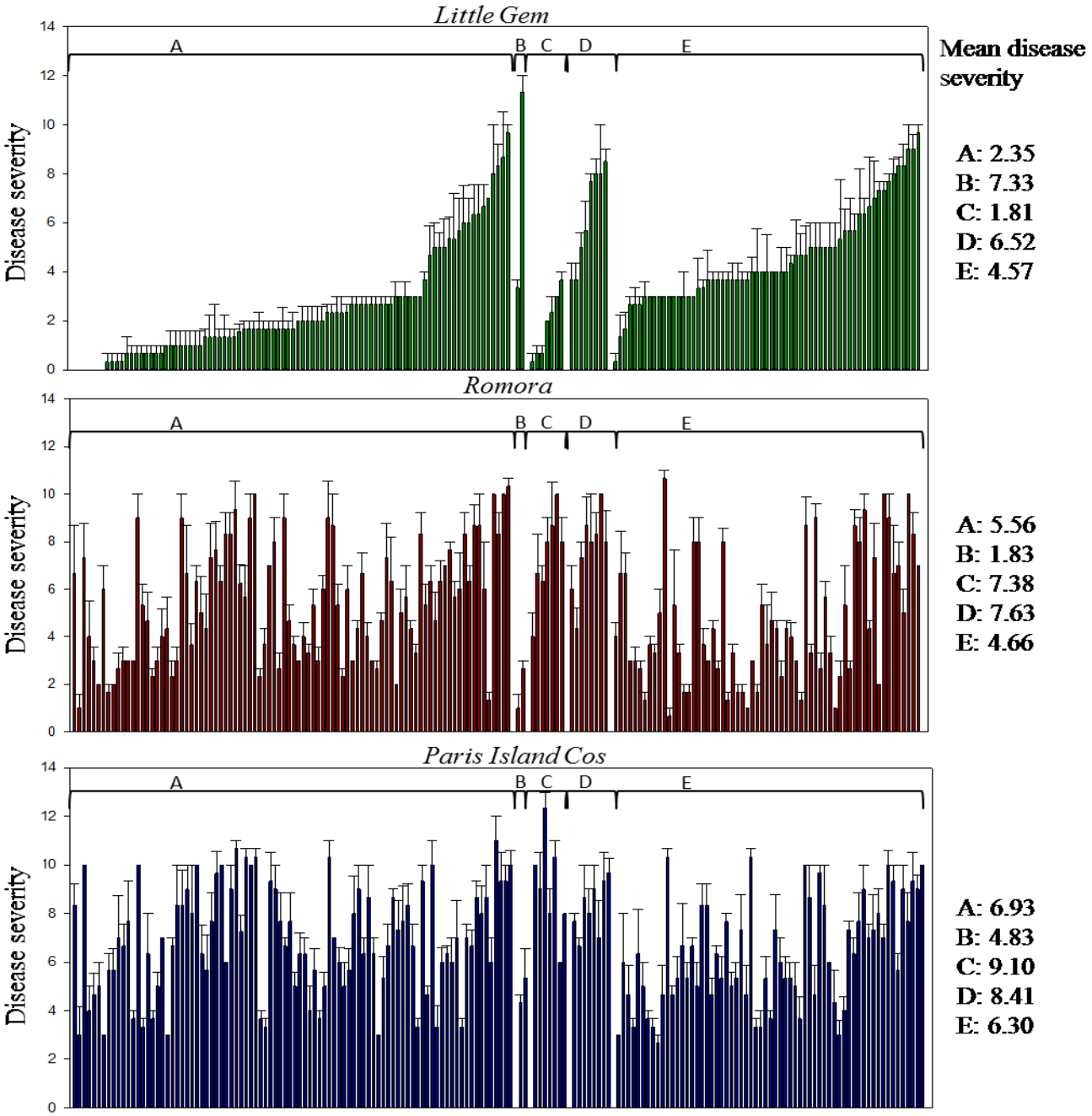

Figure 2.2. Disease severities of *X. hortorum* pv. *vitians* isolates tested on different lettuce cultivars. Little Gem (top panel), Romora (panel in the middle) and Paris Island Cos (bottom panel). Each group, based on multilocus sequence analyses, are shown (A to E). Isolates are sorted in ascending order of their symptoms severity on cultivar *Little Gem* and the same order was kept for the other lettuce cultivars.

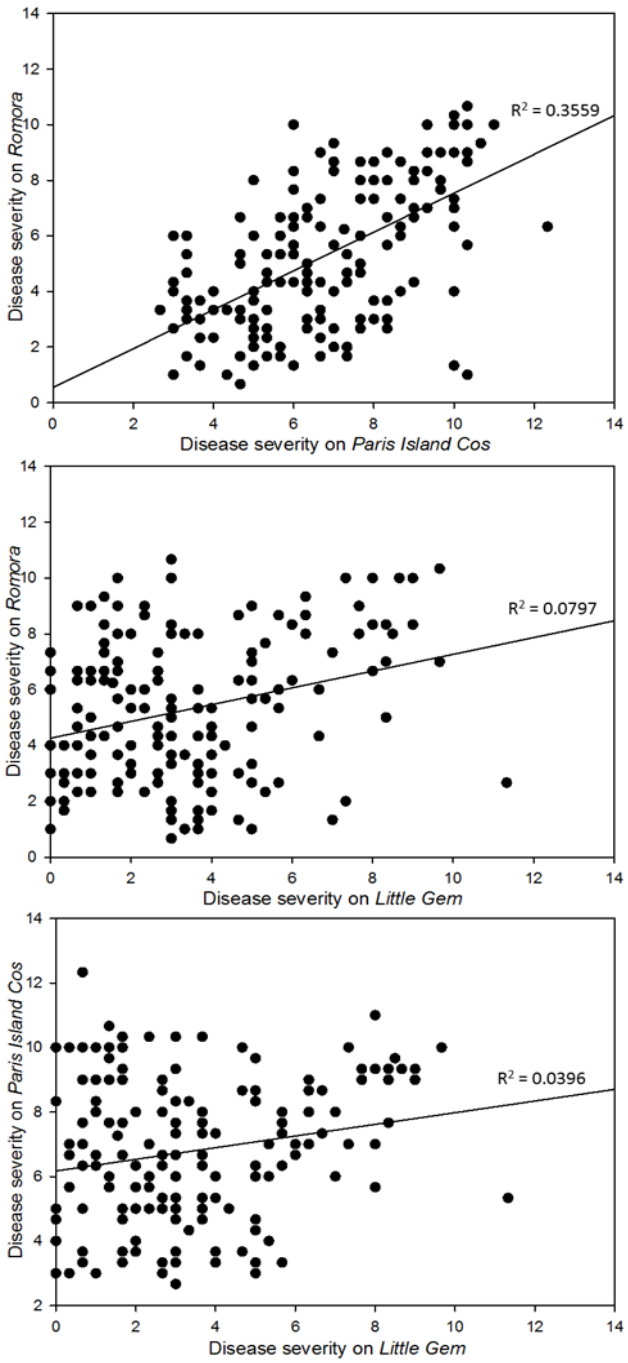

Figure 2.3. Correlation of disease severity caused by *X. hortorum* pv. *vitians* isolates on three lettuce cultivars. The absence of correlation is indicated by the low R^2 values.

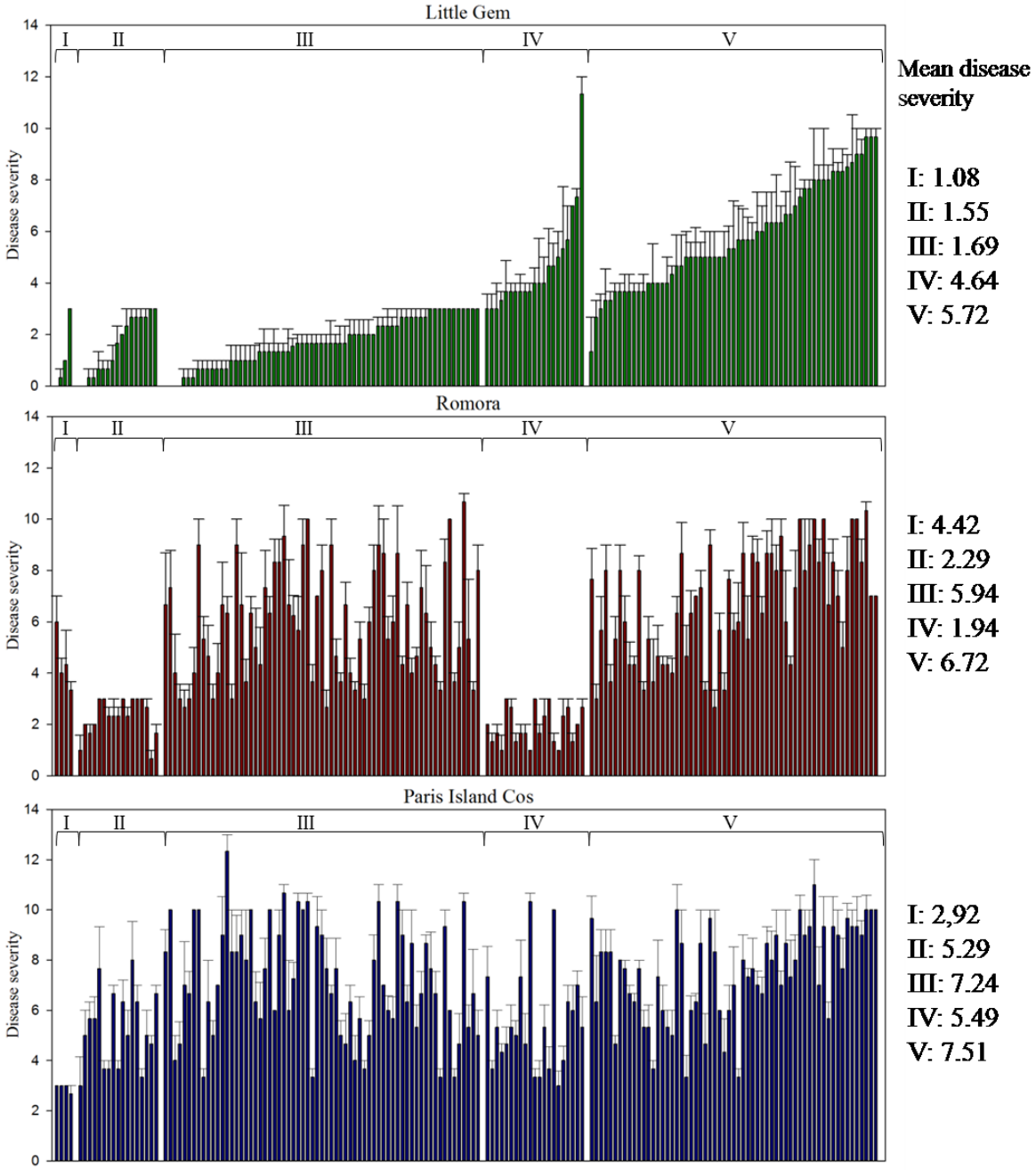

Figure 2.4. Disease severities of *X. hortorum* pv. *vitians* isolates reclassified in pathotypes by their ability to cause symptoms on each lettuce cultivars inoculated. Isolates in each pathotypes are sorted in ascending order of their symptoms severity on cultivar *Little Gem* and the same order was kept for the other lettuce cultivars.

Table 2.2 Pathotype classification of *X. hortorum* pv. *vitians* isolates based on their pathogenicity on the three lettuce cultivars tested.

Pathotypes	Number of isolates	Symptoms observed		
		Little Gem	Paris Island Cos	Romora
I	4	-	-	+
II	17	-	+	-
III	67	-	+	+
IV	21	+	+	-
V	60	+	+	+

(+) ability to cause symptoms; (-) ability to cause HR or absence of symptoms.

2.5.3 Purification of xanthan

Xanthan was purified from forty-eight isolates belonging to pathotypes II to V. Because pathotype I contains only a few isolates, it was not included in this test. To the isolates level, production rate ranged from 0.514 g to 2.882 g of xanthan per gram of biomass. When pathotypes were compared, pathotype II produced a significantly lower amount of xanthan per gram of biomass with an average production rate of 1.704 g compared to 2.229 g, 2.247 g and 2.254 g for pathotypes III, IV and V respectively (Figure 2.5).

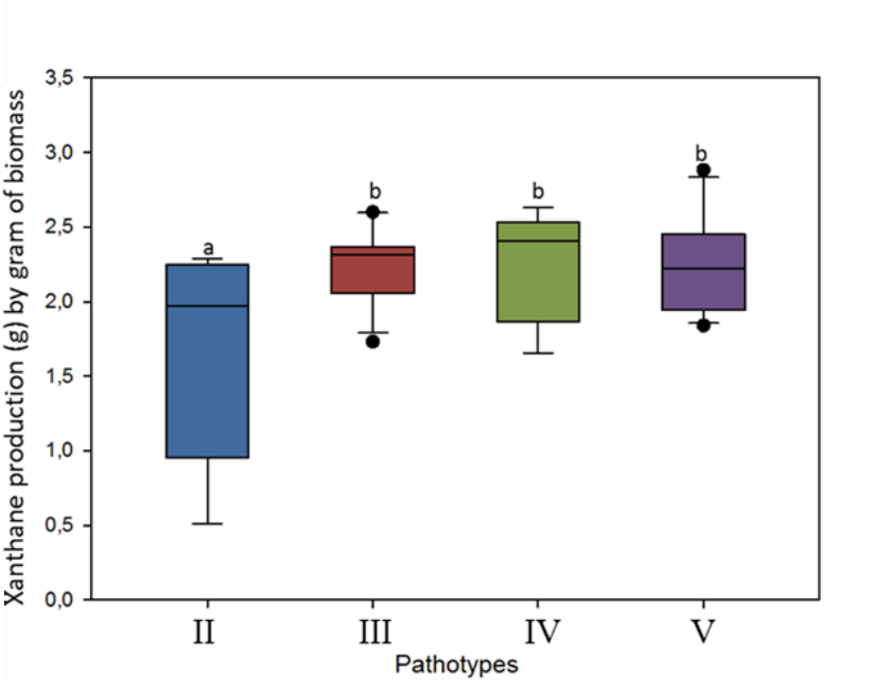

Figure 2.5. Xanthan production of *X. hortorum* pv. *vitians* isolates expressed in grams of xanthan produced by gram of biomass. Fisher's F-test reveals significant difference in xanthan production (P = 0.013), as shown by the letters a and b on top of bars. Outlier values are shown as black dots.

2.5.4 Purification of xanthomonadins

The production of xanthomondins was quantified as the absorbance at 441 nm of the methanol extracts. The lowest 441 nm absorbance was measured at 0.057 while the highest 441 nm absorbance was measured at 0.160. When we compared pathotypes with each other, pathotypes IV and V produced significantly fewer amount of pigments with average absorbance of 0.092 and 0.089 respectively (Figure 2.6). Pathotypes II and III produced equal amount of pigments, expressed by an average absorbance at 441 nm of 0.110.

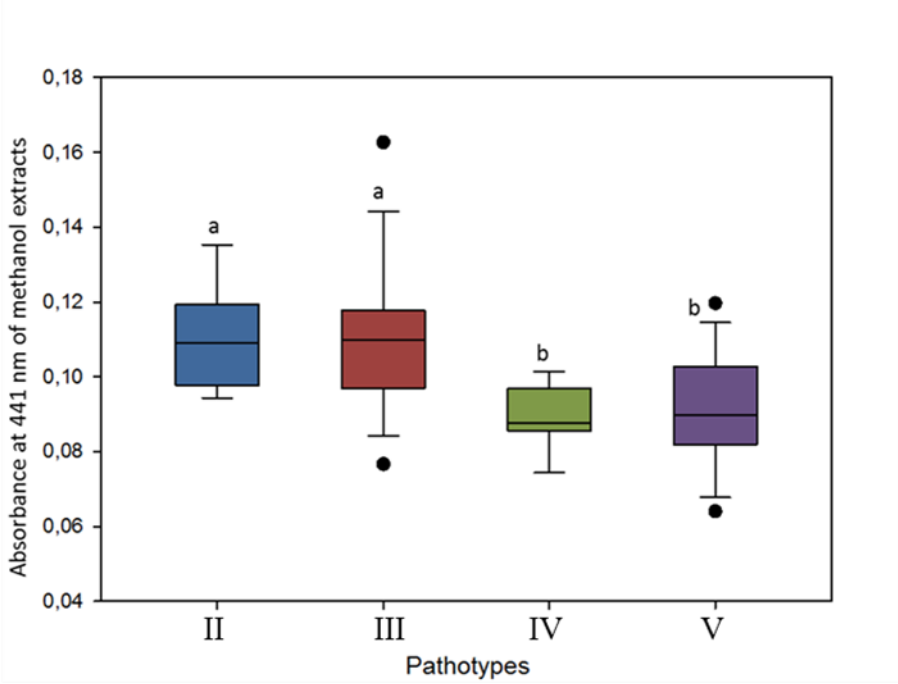

Figure 2.6. Xanthomonadins production of *X. hortorum* pv. *vitians* isolates expressed by the absorbance at 441 nm of methanol extracts. Fisher's F-test reveals significant difference in xanthomonadins production ($P = 0.002$), as shown by the letters a and b on top of bars Outlier values are shown as black dots.

2.5.5 Bacterial genomes sequencing & *De novo* assembly

Over 18 million reads were provided by the 95 isolates successfully sequenced on the MiSeq. No reads were obtained from isolate ID060814K. Reads were mapped for a first time against the reference strain *X. hortorum* pv. *vitians* strain B07-007 to obtain unmapped reads and these were used for *de novo* assembly to investigate sequences of *X. hortorum* pv. *vitians* pan genome absent from the reference sequence. All forward and reverse unmapped reads from all isolates served as input for a single assembly. Using SPAdes, 694 contigs were generated with these unmapped reads. Contigs ranged from 128 bp to 120,795 bp and were later used as reference sequences, along with B07-007 data, for a second round of read mapping. After the second round of read mapping, we observed that the entire chromosome of the reference B07-007 was not fully covered with reads by our sequenced isolates. Only one isolate had the whole 5,175,249

bp chromosomal sequence of B07-007 covered, while the others ranged from 4,594,047 bp to 5,174,085 bp covered.

Further analyzes of mapped reads revealed that the sequenced isolates had different portions of the B07-007 covered with reads. While the 75,648 bp plasmid sequence of B07-007 was fully covered for some isolates, we observed two other major populations of isolates with about 47,500 bp and about 56,100 bp of the reference sequence covered. The isolate with the least coverage had about 31,300 bp of the 75,648 bp reference sequence. In addition, for each isolates successfully sequenced, coverage of B07-007 plasmid sequence and disease severity were compared. On lettuce cultivar Little Gem, isolates which has the reference's plasmid sequence entirely covered clustered together (Figure 2.7). All these isolates caused less severe symptoms when artificially inoculated on lettuce leaves. For cultivar Romora and Paris Island Cos, wide range of disease severity was observed for isolates of each major populations of reference's plasmid sequence coverage. When pathotypes were compared, we observed that isolates from pathotypes IV and V had average plasmid coverage significantly lower than the other pathotypes and that isolates from pathotype III tended to have the entire B07-007 plasmid covered (data not shown).

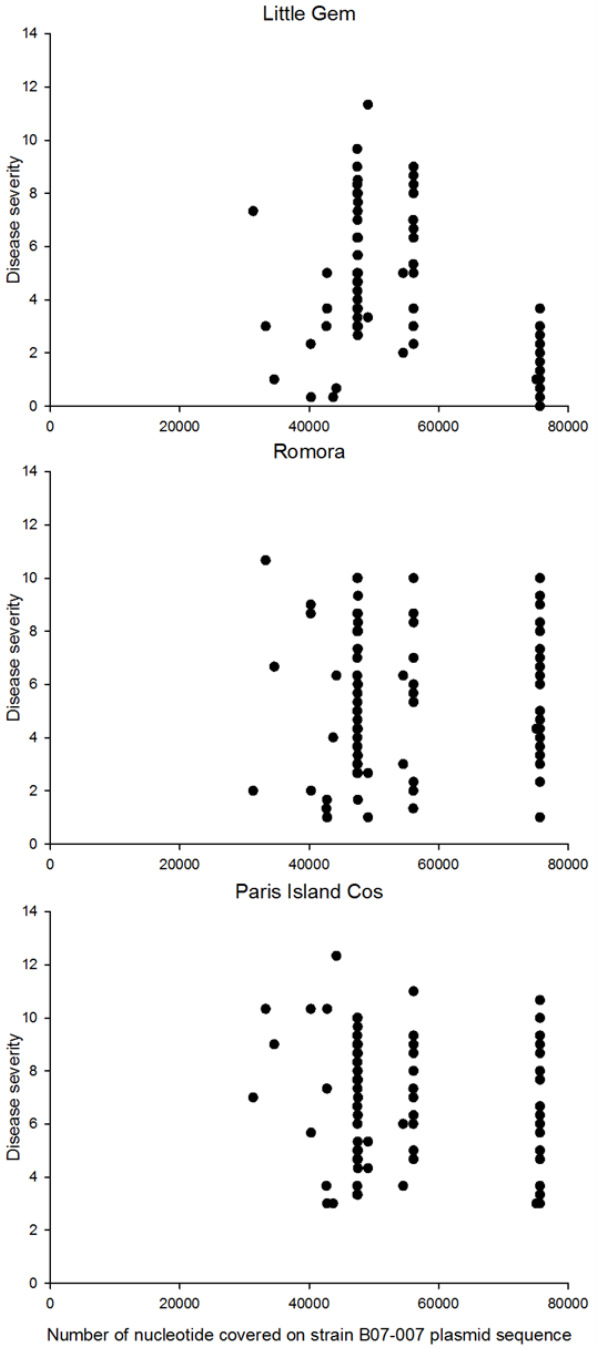

Figure 2.7. Disease severity on different lettuce cultivar based on approximate number of nucleotide covered on reference B07-007 strain plasmid sequence. Little Gem (top panel), Romora (middle panel) and Paris Island Cos (bottom panel). Far right values are equivalent to the 75,648 bp B07-007 strain plasmid sequence. Each dot represents a single isolate sequenced and inoculated on lettuce leaves.

2.5.6 GWAS

After the second mapping and variant calling with Samtools, genomes were analyzed in different ways with PLINK software. Firstly, 23,119 SNPs were identified on both genomic and plasmidic sequences, as well as on *de novo* contigs after variant calling. From these SNPs, we identified with PLINK 4,301 SNPs significantly associated with disease severity on cultivar Little Gem, from which 135 are tri-allelic (where three different nucleotides can be observed at the same position). No SNPs were associated with the phenotypes for the two other cultivars. Significant SNPs are located mostly on the chromosome, but were also located on the plasmid and on *de novo* contigs (Figure 2.8).

Secondly, 2,707,953 nucleotides with coverage of zero, which are corresponding to gaps on genomic and plasmidic sequences and on *de novo* contigs, were retrieved with Perl scripts. Of these nucleotides identified, 172,842 were significantly associated to disease severity on cultivar Little Gem. Most of them were located on the chromosome. Many zero-coverage nucleotides were identified for the 95 *X. hortorum* pv. *vitians* isolates, but no gaps were clearly identified because of the high variability observed within the isolates sequenced.

Lastly, CNV regions were identified with CNV-Seq. On all sequences, 86,111 CNVs of 100 bp of length were identified from which 863 were significantly associated to disease severity on cultivar Little Gem. The majority of CNVs were located on *de novo* contigs. None were identified on the plasmid (Figure 2.8). For PLINK analyses, the significance levels were adjusted with the Bonferroni correction to a significance level of 2.16×10^{-6}, 1.84×10^{-8} and 5.8×10^{-7} for SNPs, nucleotides with coverage of zero and CNVs respectively.

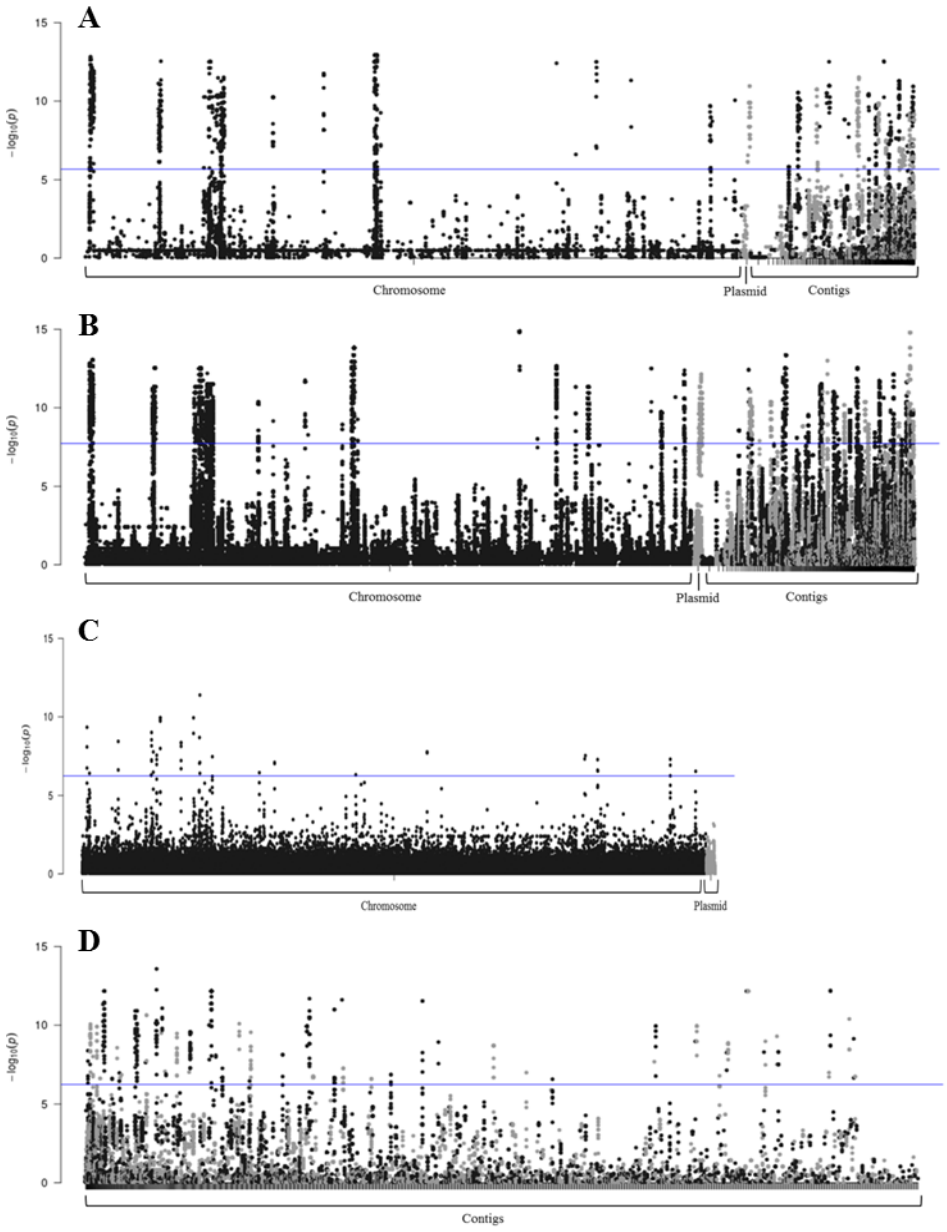

Figure 2.8. Manhattan Plot of divers elements significantly associated to disease severity on tolerant lettuce cultivar Little Gem. SNPs (A), gaps (zero-coverage nucleotides) (B) and CNVs identified during GWAS on chromosome and plasmid (C) or on *de novo* contigs (D). The line represents the significance level adjusted with the Bonferroni correction.

2.5.7 Detection method

We have developed two rhAmp® genotyping assays to identify isolates based on their capacity to induce bacterial leaf spot on the tolerant lettuce cultivar Little Gem. *X. hortorum* pv. *vitians* isolates tested for their pathogenicity on lettuce, but not sequenced on MiSeq were used for these genotyping assays. Isolate ID060814K was not successfully sequenced on MiSeq, so it was included as well. All isolates which have a high disease severity on cultivar Little Gem were positive with the first genotyping assay using a SNP located on *de novo* contig 245, showing Ct values of 24.84 or less. Isolates with a Ct value of 31.48 and more were considered negative. Reciprocally, all isolates with low disease severity were positive, with Ct values of 32.49 or less with the second genotyping assay using a SNP located at position 590,650 of reference strain's chromosome. A Ct value of 37.36 or more was considered negative (Figure 2.9). Phenotyped, but non-sequenced isolates were used to validate the rhAmp® assays. With few exceptions, *X. hortorum* pv. *vitians* isolates likely to cause severe bacterial leaf spot on the tolerant lettuce cultivar Little Gem were detected with rhAmp® genotyping assays we developed.

Isolates	Contig 245	Genome 590,650	Average disease severity	Isolates	Contig 245	Genome 590,650	Average disease severity
	Ct	Ct	Little Gem		Ct	Ct	Little Gem
190716A	21.04	29.92	6.0	240914AN	31.48	26.84	0.7
270716U	21.23	40	3.0	170914I	31.8	28.42	3.3
100816Q	21.33	40	5.0	140916A	31.85	37.36	0.7
190716C	21.4	30.53	5.3	060814K	32.1	29.65	0.7
100816U	21.45	40	9.7	030914AG	32.12	32.18	2.7
030914AC	21.6	40	5.0	170914Y	32.46	29.39	0.0
190716I	21.69	40	4.7	240914F	32.47	27.96	1.3
170914S	21.77	40	4.0	170914AJ	32.58	31.22	2.0
070815F	21.83	40	3.7	060814AA	32.8	30.78	2.7
100816C	21.9	40	4.0	240914P	32.89	28.03	2.3
140916O	21.96	40	6.0	100914A	33.09	29.5	2.0
030914A	22.04	40	4.0	060814Z	33.16	30.49	1.3
240914A	22.05	40	8.0	030914K	33.37	29.8	0.0
060814V	22.05	40	3.3	030914T	33.47	30.76	0.7
260814AC	22.07	40	3.0	030914O	33.49	29.83	1.3
170914AK	22.12	40	3.0	040816B	33.69	31.6	1.7
030914W	22.13	39.97	5.3	100914L	33.72	31.68	1.7
260814AF	22.23	40	5.7	100914F	33.94	29.03	1.7
100914X	22.23	40	0.7	170914A	34.13	29.96	2.7
030914E	22.26	40	4.0	170914T	34.36	29.46	1.7
030914AD	22.27	40	5.0	200814I	34.38	28.49	2.3
030914J	22.28	40	3.7	170914Q	34.67	30.34	0.7
240914AK	22.31	40	3.0	030915I	36.02	32.49	1.7
240914BC	22.41	40	3.0	260814E	36.79	29.24	3.0
200814N	22.42	40	4.0	190716K	36.9	28.24	1.0
110815K	22.46	40	7.7	030915A	37	39.09	1.7
160615A	22.46	40	1.7	260814V	37.02	29.35	0.0
100914Q	22.47	31.21	2.7	260814AR	37.14	28.55	0.3
110815S	22.49	40	3.7	260814P	38.01	28.54	2.7
240914Q	22.54	40	4.0	070815H	38.15	29.61	2.7
100914AG	22.54	40	0.3	070815D	38.33	31.73	2.7
100914V	22.55	40	3.0	110815AH	38.89	29.74	0.7
100816F	22.59	40	3.0	110815AF	39.04	30.97	1.3
070815B	22.63	31.18	2.0	110815Z	40	32.19	0.0
240914AT	22.66	40	4.0	160615C	40	27.7	1.0
110815D	22.87	40	8.3	290715K	40	38.74	2.7
110815AB	23.67	40	6.7				
270716A	24.58	29.41	0.7				
030914Y	24.84	40	2.7				

Figure 2.9. Ct values of *X. hortorum* pv. *vitians* isolates tested with the rhAmp® genotyping assays developed. Isolates on the left are considered positive for the rhAmp® Contig 245 assay while isolates on the right are considered negative. False-positive (left) and false-negative (right) are shown in bold.

2.6 Discussion

Knowledge of the diversity of the pathogen is important in order to develop effective lettuce cultivars that could be tolerant to most of the strains encountered. Here, we identified five pathotypes of *X. hortorum* pv. *vitians* present in lettuce fields in the province of Quebec, Canada, based on their host ranges on three cultivars, Paris Island Cos, Romora and Little Gem, the latter being tolerant. It has been previously demonstrated (Hayes *et al.*, 2014) that resistance to bacterial leaf spot in lettuce cultivars is conferred by a resistance gene, *Xar1*. Bull *et al*. (2007) and Wang *et al*. (2015) observed moderate to high resistance to bacterial leaf spot when cultivar Little Gem was artificially inoculated with *X. hortorum* pv. *vitians* isolates collected in California and Florida, respectively. Nevertheless, despite the presence of *Xar1* in cultivar Little Gem, our results showed that many isolates recovered in lettuce fields located in Monteregie (Quebec), the most important lettuce production area in Canada, were able to induce considerable amount of symptoms on cultivar Little Gem. Because the pathogen is thought to be seed-borne (Umesh *et al.*, 1996; Sahin & Miller, 1997), and that the majority of Monteregie's lettuce growers buy their seeds from California, it suggests that isolates able to cause disease on tolerant cultivar Little Gem are also present in lettuce fields in California.

Genetic diversity of *X. hortorum* pv. *vitians* isolates recovered in lettuce fields from 2014 to 2017 was firstly evaluated with the Young *et al*. (2008) method. Young et al. (2008) conducted a MLSA to differentiate species of *Xanthomonas* by sequencing the *fyuA*, *gyrB*, *rpoD* and *dnaK* housekeeping genes. All 694 isolates collected in Quebec were subdivided into five phylogroups, and reference strains included did differentiate well, indicating that this MLSA provided enough depth to be able to distinguish our isolates at the pathovar level. We tried to correlate phylogroups, based on the MLSA conducted, and pathotypes obtained through pathogenicity assays, to see if these pathotypes could be identified directly by MLSA, but no correlation was observed because each phylogroup, excepting phylogroup B which contain only two isolates, are scattered in different pathotypes, thus indicating that genes tonB-dependent receptor (*fyuA*), DNA gyrase subunit B (*gyrB*), RNA polymerase sigma factor (*rpoD*) and chaperone protein dnaK (*dnaK*) are not linked to pathogenicity in *X. hortorum* pv. *vitians*.

Because MLSA was an inadequate method to identify pathotypes in this study, we investigated the production of xanthan and xanthomonadins to characterize the isolates and possibly find a faster way to assign them to a specific pathotype. Considering both compounds are involved in the infection process, we hypothesized that differential production levels of these products could be correlated to the pathotypes described in this study. Although xanthan play an important role in inducing plant susceptibility (Yun *et al.*, 2006), we were unable to establish any correlation between *in vitro* production of the polysaccharide and symptoms observed on the three lettuce cultivars inoculated. The xanthan production was characterized by measuring the amount of xanthan produced by grams of biomass, which allowed us to compare the xanthane production between isolates. Even if pathotype II produced significantly lower amount of xanthan than pathotypes III to V, it could not be correlated to the absence of symptoms on either cultivars Little Gem or Romora because it was also observed for pathotypes III and IV respectively, which also have low amount of xanthan but are less aggressive on these cultivars. When production of xanthomonadins was characterized, we made sure that each isolates tested had the same theoretical number of cells by counting CFU after dilution plating and by weighing cell pellets, making comparison between isolates possible. Isolates from pathotypes II and III, which have produced on average greater amounts of xanthomonadins, were unable to cause symptoms on cultivar Little Gem. Moreover, isolates from pathotypes IV and V, which have non-significantly different average production rate, do not have the same pathogenicity on cultivar Romora. Xanthomonadins are thought to protect xanthomonads from photo oxidative stress (He *et al.*, 2011), thus helping survival of bacteria on plants and increasing chances of infection leading to disease. He and colleagues (2011) also demonstrated that when xanthomonadin biosynthesis is impaired, a reduction of the virulence can be observed. However, in our study we did not observed a reduction of aggressiveness for the pathotypes that produced significantly less xanthomonadins. Taken together, these observations indicates that *in vitro* production of either xanthan or xanthomonadins might not be the best method to characterize and identify pathotypes of *X. hortorum* pv. *vitians*. *In planta* secretion rates could be investigated instead.

We proceeded to a second evaluation of the genetic diversity by sequencing whole genomes of 95 *X. hortorum* pv. *vitians* isolates belonging to pathotypes I to V on a MiSeq. Isolates from pathotype I were not tested for their xanthan and xanthomonadins *in vitro* production, but were included in whole genome sequencing to evaluate the greatest diversity possible. Variant calling performed after the second round of read mapping allowed us to identify many SNPs, zero-coverage nucleotides and CNVs located on the chromosome, the plasmid or on contigs. For many isolates, we observed that when some gaps were located on the chromosome, some contigs were covered by reads, as if some contigs were only covered, in a complementary way, if some gaps were on the chromosome. It suggests that some loci are polymorphic and that they could be associated with the aggressiveness of *X. hortorum* pv. *vitians* towards the tolerant cultivar Little Gem. The genetic diversity observed within our isolates might play an important role in the infection process.

The non-uniform coverage of reference strain B07-007 plasmid sequence observed after read mapping is intriguing because many pathogenicity-related genes are not covered for isolates of populations of about 47,500 bp or about 56,100 bp of the reference sequence covered. While many isolates from these two populations caused severe symptoms on all three lettuce cultivars inoculated, *virB3*, *virB8* and *virB10* genes from type IV secretion system are not covered, along with a type III and a type VI secretion system gene. It has been previously demonstrated that the capacity to kill Gram-negative bacteria was conferred to *Xanthomonas citri* by its type IV secretion system (Souza *et al.*, 2015), thus conferring a growth advantage over other bacterial communities present on leaf surface. The fact that some isolates are able to cause BLS on the three lettuce cultivars despite the lack of pathogenicity- and competition-related genes may be because these genes located on the plasmid are facultative, as they are also located on the chromosome.

As mentioned before, contigs obtained with SPAdes are not covered by all isolates sequenced. Interestingly, only three isolates (ID060814AJ, ID100916B and ID260814AH) have the entire 120,795-bp contig sequenced, corresponding to plasmidic DNA when compared to the NCBI NT database using BLAST (Altschul *et al.*, 1990). Canteros *et al.* (1995) had already observed

various sizes of plasmids for *X. campestris* pv. *vesicatoria*. The non-uniform coverage of strain B07-007's plasmid sequence added to the 120,795-bp plasmidic sequence obtained by *de novo* assembly represent major clues that various sizes of plasmids exist for *X. hortorum* pv. *vitians* as well.

The reference strain B07-007 used for mapping is unable to cause bacterial leaf spot on tolerant lettuce cultivar Little Gem. Therefore, a variant calling step right after the first round of read mapping could have limited the analyses because genes related to pathogenicity on tolerant cultivars could be missed due to their absence on non-pathogenic isolates towards tolerant cultivar Little Gem. Consequently, instead of doing the variant calling step right away, we decided to retrieve unmapped reads to compute a *de novo* assembly with them and then run a second round of read mapping with *de novo* contigs added to the reference sequences. This way, sequences of *X. hortorum* pv. *vitians* pan genome absent from the reference sequence are not lost and are included in the analyses. As it is not a flawless method, it would be interesting to use an isolate known to be able to cause the disease on the tolerant cultivar as reference for read mapping. Perhaps different SNPs could be identified and associated with the phenotypes observed.

GWAS may be arduous to adapt to microbial variants (see Power *et al.*, 2017 for review). Lineage effects in population structure and linkage disequilibrium (LD) may affect microbial GWAS results (Earle *et al.*, 2016). In this study, we demonstrated that we had enough diversity to achieve bacterial GWAS. As we can see on figure 2.8, significant SNPs are grouped in distinct regions that could correspond to linkage blocks in our population. These linkage blocks indicate that in our study, LD might not be as strong as Earle *et al.* (2016) suggest, because many regions showed very low association between SNPs and phenotype. To our knowledge, this is the first GWAS conducted on xanthomonads.

The SNP selected at position 590,650 of chromosome is located in an intergenic region, and the SNP at position 549 of contig 245 is located in a hypothetical protein, based on a BLAST on NCBI NT database. These mutations are probably not responsible for disease severity on

tolerant cultivar Little Gem, but because of the genomic structure observed, they might be adjacent to the ones that are, thus decreasing the number of potentially involved genes. As Power *et al.* (2017) also pointed out; the effect of SNPs alone may not always be possible to focus on. This is what happened with the two bi-allelic SNPs chosen for molecular identification. Both SNPs are located in genomic portions that are not covered by all isolates sequenced, associated to a high or low severity of symptom, for rhAmp® genotyping assays contig 245 and Genome 590,650 respectively. Thereby, for the molecular identification assays developed, the detection of any of the alleles is considered positive, and the absence of the allele is considered negative. SNPs being located on genomic portions not covered by all isolates, we would have expected significant CNVs for these regions as well. The GWAS conducted with gaps identified both regions containing the SNPs used for molecular detection as significantly associated with disease severity on the tolerant cultivar. However, only nucleotides 247 to 1,074 of contig 245 were identified as CNVs by CNV-seq. The lack of sensitivity of CNV-seq pinpointed by Brynildsrud *et al.* (2015) may be the reason why no CNVs were detected at position 590,650 of sequenced isolates' chromosome.

No association was found between polymorphism and phenotypes for the two lettuce cultivars Paris Island Cos and Romora. This could be for several reasons. Firstly, more than one gene or loci could be involved in the pathogenicity process, and the size of the population studied is not large enough to consider gene interactions. Secondly, the number of members of a gene family could be different from one isolate to another. Whole genome assembly, with PacBio reads for example, might be needed to resolve gene duplication. Lastly, differences in gene expression, which have not been investigated in our study, could interfere with the amount of effectors required to induce disease.

We tried to develop rhAmp® genotyping assays to detect every pathotype identified in this study, but SNPs, gaps and CNVs were only significantly associated to cultivar Little Gem, thus making it impossible to discriminate each pathotypes. Instead, the genotyping assays were developed to detect isolates able to cause disease on tolerant cultivar Little Gem. As seen on figure 2.9, perfect detection of isolates likely to cause bacterial leaf spot on the tolerant cultivar

was not achieved. As the evaluation of symptoms is based on an observable scale, misjudgment of symptoms can occurs. Re-evaluation of symptoms induced under the same conditions for problematic isolates could be done to confirm their aggressiveness on the three lettuce cultivars assayed. It is also possible that other factors, like genes involved in the successful pathogenic interaction were missed due to the relatively small size of our sample set. As the phenotype measured is quantitative, it is likely that many genes are involved in the host-pathogen interaction.

The results in this study indicate that there are actually at least five pathotypes of *Xanthomonas hortorum* pv. *vitians* in lettuce fields in the province of Quebec, Canada, based on pathogenicity tests conducted in greenhouse on lettuce cultivars Little Gem, Paris Island Cos and Romora. Of these pathotypes, three were able to cause bacterial leaf spot on tolerant cultivar Little Gem, pointing out the importance of including these pathotypes in lettuce breeding programs for resistance assays. Bacterial GWAS was achieved with 95 isolates belonging to the five pathotypes and over four thousand SNPs were significantly associated with disease severity on the tolerant cultivar Little Gem, allowing us to develop two genotyping assays to detect isolates of *X. hortorum* pv. *vitians* able to cause bacterial leaf spot on this cultivar. These genotyping assays could eventually be used to detect the pathogen in seed lots to prevent disease development in lettuce fields. It would also be interesting to see in which genes involved in pathogenicity these SNPs are located. Knowledge about their function may help to understand the host-pathogen interaction and, in turn, develop new phytosanitary products based on this information.

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2.8 Supplementary content

Supplementary file 2.1. List of SNPs, zero-coverage nucleotide and CNVs with their positions and p-values identified with GWAS.

Supplementary file 2.2. List of contigs obtained after *de novo* assembly.

CHAPITRE 3
DISCUSSION ET CONCLUSION GÉNÉRALE

Les résultats obtenus au cours de ce projet de recherche ont permis d'évaluer la diversité dans la population de *Xanthomonas hortorum* pv. *vitians*, agent responsable de la maladie de la tache bactérienne de la laitue. Ces recherches ont également permis le développement d'un outil permettant la détection de cette bactérie pathogène de la laitue.

3.1 Détermination des pathotypes de *X. hortorum* pv. *vitians*

Cinq pathotypes de *X. hortorum* pv. *vitians* ont été identifiés suite à des tests de pouvoir pathogène sur trois cultivars de laitue; Little Gem, Paris Island Cos et Romora. Le choix des cultivars à tester s'est arrêté sur ceux-ci pour deux raisons majeures : leur niveau de tolérance était préalablement connu, et ces trois cultivars constituaient l'éventail complet des niveaux de tolérance, soit sensible, intermédiaire et tolérant. Comme les 170 isolats utilisés pour ces tests ont été récoltés dans plusieurs fermes de la région des Jardins de Napierville, en Montérégie, et que les trois cultivars inoculés ne font pas partie de ceux cultivés par ces producteurs, il serait intéressant d'ajouter les cultivars tels que Estival, Prestige, Salanova, ou encore Sunbelt afin de déterminer le profil des pathotypes identifiés sur ces cultivars et ainsi dresser un portrait plus actuel pour les producteurs locaux. Toutefois, nos résultats ont permis de mettre en évidence deux pathotypes (IV et V) capables de causer des symptômes sur un cultivar tolérant à la maladie de la tache bactérienne de la laitue. Cette information demeure très pertinente autant pour la communauté scientifique que pour les améliorateurs génétiques de la laitue. Plusieurs études ont été effectuées auparavant, testant la pouvoir pathogène d'isolats de *X. hortorum* pv. *vitians* sur plusieurs cultivars de laitue, où le cultivar Little Gem démontrait une tolérance élevée (Bull *et al.*, 2007; Bull *et al.*, 2015) ou modérée (Wang *et al.*, 2015) à la maladie. Nous avons observé, dans nos essais, une tolérance modérée pour le cultivar Little Gem, mais certains isolats ont causé beaucoup de symptômes, ce qui n'avait pas été observé dans les études mentionnées précédemment. Ces isolats sont d'une grande importance pour les programmes d'amélioration

génétique de la laitue, puisque ceux-ci pourraient être ajoutés aux bactéries utilisées pour tester la tolérance des nouvelles lignées de laitue développées.

Certains isolats non catégorisés lors de nos essais ont été utilisés lors de tests de pouvoir pathogène sur le cultivar Paris Island Cos, mais aussi sur différents hôtes végétaux afin de vérifier leur pouvoir pathogène sur ceux-ci (Annexe 2). Tous les isolats ont causé des symptômes sur laitue, rendant la classification difficile car ils pourraient faire partie des pathotypes II à V. Par contre, ils n'ont pas tous causé des symptômes sur la carotte. L'ajout d'hôtes supplémentaires pourrait donc constituer de l'information additionnelle intéressante aux pathotypes identifiés lors de ce projet de recherche, permettant de mieux différencier ceux-ci.

3.2 Étude d'association à l'échelle du génome (Genome-wide association study)

La performance de notre étude d'association à l'échelle du génome a permis de faire progresser les connaissances dans le domaine d'études d'associations bactériennes. Cette technique a été, et est encore grandement utilisée pour le génome humain. Depuis la première publication concernant une étude d'association à l'échelle du génome avec des bactéries en 2013 (Sheppard *et al.*, 2013), plusieurs études ont paru, effectuant des associations avec des bactéries d'intérêt clinique. Le domaine agricole étant aussi d'importance pour notre économie, nos recherches ouvrent donc la voie à d'autres études du même genre dans des cultures de plus grandes envergures comme le maïs, affecté par *Erwinia stewartii* ou encore le riz, aux prises avec *Xanthomonas oryzae* pv. *oryzae*, par exemple. Tout comme dans le domaine clinique, les résultats permettraient de trouver de nouvelles cibles moléculaires pour le développement d'agents de contrôle chimiques ou biologiques.

Nous avons choisi la souche B07-007 de *X. hortorum* pv. *vitians* comme référence pour les analyses, car nous connaissons bien son agressivité sur les trois cultivars de laitue testés. Nous avons récolté cette souche en 2007, et son chromosome ainsi que son plasmide ont été séquencés et annotés récemment (Annexe 1), permettant de localiser facilement les gènes sur lesquels les mutations sont présentes. La souche B07-007 étant incapable de causer des symptômes sur le

cultivar tolérant Little Gem, il serait intéressant de réaliser de nouveau cette analyse, mais avec une souche ayant la capacité de causer des symptômes sur ce même cultivar tolérant, afin de vérifier si de nouvelles cibles moléculaires pourraient être identifiées, multipliant ainsi les possibilités de développer des agents de contrôle contre cette bactérie pathogène.

3.3 Outils de détection de *X. hortorum* pv. *vitians*

La réussite de l'étude d'association à l'échelle du génome nous a également permis d'identifier plusieurs cibles potentielles pour des outils de détection. Nos deux outils se sont avérés efficaces d'une part pour la détection d'isolats susceptible de causer la maladie et d'une autre pour les isolats ne causant que peu ou pas de maladie. Les cibles que nous avons choisies se retrouvent dans une région intergénique et dans une protéine hypothétique, ce qui porte à croire que ces mutations ne sont probablement pas responsables du phénotype associé. Grâce au déséquilibre de liaison observé durant les analyses, il y a de bonnes chances que les mutations choisies soient près des mutations causatives. Il serait donc intéressant de conduire des analyses ainsi que des expériences plus approfondies afin de cibler les mutations causatives. De cette manière, de nouveaux gènes reliés au pouvoir pathogène pourraient être identifiés chez *X. hortorum* pv. *vitians*.

Le but principal de ces outils diagnostic est de permettre la détection de la bactérie pathogène à partir de la semence, source soupçonnée de l'inoculum primaire (Umesh *et al.*, 1996). Nous avons tenté de détecter *X. hortorum* pv. *vitians* dans quelques lots de semences provenant de producteurs de la région, mais comme les échantillons dataient de très longtemps et que les conditions de conservations n'ont pas été optimales, aucune colonie s'apparentant aux colonies typiques de *X. hortorum* pv. *vitians* n'a été obtenu suite aux multiples essais avec diverses méthodes d'extraction. Nous n'avons donc pas été en mesure de tester notre outil diagnostique avec des semences comme prévu. Il serait donc très profitable de trouver des lots de semences, qui ont été semés en champs, et dans lesquels la maladie a été observée. Ainsi, nous pourrions tenter d'extraire la bactérie des semences suspectées d'être contaminées, et si contamination il y a, nous pourrions tester notre outil à partir de celles-ci.

3.4 Conclusion

Les résultats recueillis au cours de ce projet de recherche ont collaboré à faire progresser les connaissances en rapport avec l'agent pathogène responsable de la maladie de la tache bactérienne de la laitue, *Xanthomonas hortorum* pv. *vitians*. Les caractérisations génotypiques et phénotypiques effectuées ont permis de confirmer notre hypothèse de départ, soit qu'il y a vraisemblablement présence de différents pathotypes de cette bactérie sévissant dans les champs de laitue au Québec. Ces résultats ont également permis de soulever plusieurs autres questions par rapport au pouvoir pathogène de cette bactérie, ouvrant la voie à d'autres projets de recherche afin d'approfondir les connaissances envers cet agent pathogène. Il serait intéressant d'approfondir sur les régions sur le chromosome ou le plasmide de *X. hortorum* pv. *vitians* où plusieurs sites présentant du polymorphisme nucléotidique ont été identifiés lors de ce projet de recherche afin d'identifier de nouveaux gènes potentiellement reliés au pouvoir pathogène de cette bactérie, ou encore de se pencher un peu plus sur les xanthomonadines, afin de savoir si les différents pathotypes identifiés au cours de ce projet produisent tous les même groupes de xanthomonadines, ou si *X. hortorum* pv. *vitians* produit un nouveau groupe de xanthomonadines. De plus, ces informations ont permis de développer un outil diagnostic, qui pourrait éventuellement permettre aux producteurs de s'assurer que leurs semences sont exemptes de l'agent pathogène, réduisant grandement l'incidence de la maladie sur leurs terres.

ANNEXE 1

L'article suivant traite du séquençage et de l'assemblage du génome et du plasmide d'un isolat de *Xanthomonas hortorum* pv. *vitians*, purifié en 2007 à partir d'un échantillon de laitue symptomatique. L'isolat en question, nommé B07-007, a été utilisé dans les travaux présentés aux chapitres précédents comme témoin positif lors des tests de pouvoir pathogène ainsi que comme souche de référence lors de l'assemblage des séquences. Ce manuscrit a été ajouté en annexe à ce mémoire, car l'annotation du génome et du plasmide de la souche B07-007 a grandement facilité les analyses lors de l'appel de variants effectué suite au séquençage des 95 isolats de *X. hortorum* pv. *vitians*, puisque nous étions en mesure de déterminer la position exacte, et sur quels gènes les mutations étaient identifiées, en plus de pouvoir faire le parallèle entre les mutations observées, et le pouvoir pathogène sur les trois cultivars de laitue inoculés. Ma contribution à cet article est la suivante : J'ai collaboré au typage par séquençage multilocus en effectuant plusieurs réactions par PCR et en procédant aux analyses bio-informatiques de ce typage. J'ai également collaboré à la révision du manuscrit. Ce manuscrit est en cours de soumission. Pour ce mémoire, des modifications ont été apportées au manuscrit et n'engagent que la responsabilité de l'étudiant.

Comparative genomic analysis of the lettuce bacterial leaf spot pathogen *Xanthomonas hortorum* strain B07-007 and other xanthomonads: insights into relationships and pathogenicity

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Key words: *Xanthomonas hortorum*, genomic analysis, type III effectors, pathogenicity, phylogenetic relationship, lettuce bacterial leaf spot

4.1 Abstract

Xanthomonas hortorum strains, causal agents of bacterial leaf spot of lettuce in Quebec, Canada, were studied. We performed PacBio third generation sequencing and Illumina HiSeq 2000 sequencing to determine the genome for the strain B07-007. PacBio sequencing reads data was used to assemble draft genome which was then used as reference for assembling a high-quality genome sequence with HiSeq short reads. Data mining allowed us to gain an insight into the specific characteristics of the bacterium. B07-007 is an entity of *Xanthomonas hortorum* based on phylogenomic and housekeeping gene (*dnaK*, *fyuA*, *gyrB* and *rpoD*) analysis. The strain has a genome of 5.18 Mb and a plasmid of 75.7 kb with GC contents of 63.7% and 59.6% respectively. It possesses complete type II, III and IV secretion systems. The T3SS pathogenicity island (PAI) was highly conserved but rearrangements by transposable elements IS3 and IS30-like were observed. An IS5-like element split the *virB4* gene of T4SS. Twenty-seven genes coding for 23 different effectors and nine additional effector candidates were identified by BLASTp and T3 promoter (PIP boxes) search. T3 effector repertoire comparison showed that B07-007 was distinct from any other species and strains and suggested its specific evolutionary itinerary.

4.2 Introduction

The Gram-negative, rod-shaped, mostly yellow-pigment producer, *Xanthomonas* species often cause bacterial cankers, necrosis, spots and blights on leaves, stems and fruits of a variety of plants covering hundreds of monocotyledonous and dicotyledonous species (Hayward, 1993). The *Xanthomonas* genus includes 27 species and numerous pathovars (Bull et al., 2010).

Parkinson et al. (2007, 2009) drew the phylogeny of *Xanthomonas* genus using the housekeeping gene *gyrB*, discriminated diversities among different species, and suggested a phylogenetic structure of the genus. *X. campestris* was a heterogenetic species. Vauterin et al. (1995) separated strains of this species and revised or renamed them into 15 different species using DNA-DNA hybridization. *X. hortorum* was a renamed species from this restructure of the old *X. campestris* species and these authors also proposed a new pathovar name, *X. hortorum* pv. *vitians*, for the strain LMG 938 from *X. campestris* pv. *vitians*. However, its systematic position is not consistent. Currently, *X. hortorum* contains four valid pathovars (pvs. *carotae*, *hederae*, *pelargonii* and *taraxaci*). The lettuce pathogens were separated into two distinct genomic types: Type A and Type B. The Type A pathogen was accepted as *X. axonopodis* pv. *vitians*. However the proposition for the Type B strain, LMG 938, was considered invalid for the reason of not conforming to the standard for definition of pathovar (Young et al. 1996). The strain is thus included in the *X. hortorum* species without further pathotype determination (Saddler and Bradbury, 2005). Even though the strain LMG 938 and its highly related entities had been classified in the *X. hortorum* species, the name *X. campestris* pv. *vitians* was still wrongly used for LMG 938 and related entities pathogenic to lettuce (Sahin et al. 2003; Fayette et al. 2016). The continued use of the name *X. campestris* pv. *vitians* is not based on their phylogenetic relations but rooted from some confusion in pathovar determination. So, clarification of this undetermined pathotype position becomes necessary for this group of pathogens. *X. hortorum* members are pathogens of several economic crops. Their hosts include lettuce, tomato, pepper, carrot, geranium, ivy plants, and Russian dandelion etc. Symptoms on lettuce by *X. hortorum* included dark brown to black water-soaked lesions along margins of lower leaves and small black spots on the leaf surface (Sahin and Miller, 1997). *X. hortorum* is a relatively small group of plant pathogens. It forms a cluster together with *X. gardneri* and *X. cynarae* (Young et al. 2008). The genome of *X. hortorum* pv. *carotae* (*Xhc*) strain M081 was sequenced and assembled with gaps (Kimbrel et al. 2011). These authors identified several clusters of virulence-related genes carrying 21 type III effector candidates and proved two genes, *avrBs2* and *xopQ*, responsible for HR (hypersensitive response) in heterologous plants.

The next-generation sequencing (NGS) technologies have impacted the ways of biological studies and pushed rapid advances in bio-information. Data mining of the ever expanding biological information, including DNA, RNA and proteins, has led into deep insights for inter-organism relationship, genetics, species evolution, metabolism mechanisms, etc. Numerous prokaryotic genome sequences are now publicly available. For xanthomonads, several hundreds of Bio-projects have been registered in GenBank, and since the first two *Xanthomonas* genomes were assembled in 2002 (da Silva et al.), more than 50 genomes of the genus have been successfully completed. Mining such data helped revealing the composition of different pathogenicity-related secretion systems, quorum sensing systems, flagellum constituents and synthesis, lipopolysaccharide (LPS) synthesis, and significant enrichment of the repertoire of type III effector candidates (Potnis et al. 2011; Moreira et al. 2010; Bart et al. 2012; Zhai et al. 2013; Wichmann et al. 2013; Arrieta-Ortiz et al. 2013; Vandroemme et al. 2013). Genomic analysis has also allowed the detection of inter-kingdom gene transfer (Gardiner et al. 2014).

Xanthomonads, causal agents of many plant diseases, principally possess the type III secretion system (T3SS) for target detection and protein secretion, and a set of specific proteins called effectors, which are released through the secretion system into the host plant cells. These effectors suppress the plant defense system and orientate the physiological processes toward favoring the pathogen development. A set of pathogenicity-related genes are arranged within a region of more than 20 kb in length, i.e. the pathogenicity island (PAI). The PAI is composed of subsets of *hrp* (hypersensitive response and pathogenicity), *hrc* (*hrp* and conserved) and *hpa* (hrp-associated) genes (Kim et al. 2003). These pathogenicity genes are regulated by at least seven plant-inducible promoters (PIP) (Kim et al. 2003). The PIP boxes contain consensus sequences of TTCGB-N8,15-16,21-TTCGB (Kim et al. 2003; Koebnik et al. 2006; Huguet and Bonas, 1997; Guo et al., 2012) composing two TTCG perfect direct repeat sequences. The distances between the two TTCGB boxes vary, depending on particular pathogenicity genes. Sequence modification experiments suggested that a downstream hexamer box at position of the -10 region also plays an important role in the activation of the *hrp* genes in *Ralstonia solanacearum* (Cunnac et al. 2004). Such hexamer box (YRNNNT) was also found at the -10 region in pathogenic xanthomonads (Koenik et al. 2006; Furutani et al. 2006). HrpX was

reported as a transcriptional activator of the T3SS and the effectors, and binds to the PIP box to activate the *hrp-hrc-hpa* operons (Koebnik et al. 2006). A previous model suggests that HrpG regulates hrpX (Tang et al. 2006). However, Liu et al. (2014) thought that HrpG may switch on the expression of HrcT via the T3 promoter. The expression of hrpX is regulated by HrcT, a structural component of T3SS (Liu et al. 2014). It is interesting that RsmA, a repressor of secondary metabolism, participates in hrpG regulation (Andrade et al. 2014). RsmA stabilizes hrpG mRNA by binding to its 5' untranslated region.

Elucidation of xanthomonad pathogenicity mechanism would be much helpful in developing molecular and genetic strategies to control *Xanthomonas*-induced plant diseases. To gain insights about the mechanisms involved in the pathogenicity of xanthomonads, we used NGS and bioinformatics to assemble a high-quality genome of the pathogen *X. hortorum* strain B07-007. We then compared B07-007 to different *Xanthomonas* genomes and revealed phylogenetic relations with other species and strains. Interestingly, this strain is distinct from any other published *Xanthomonas* species, pathovars or strains. We also compared the composition of different secretion systems, including the T3SS pathogenicity island and type III effectors, between B07-007 and other *Xanthomonas* species and strains. Mobile elements and their roles were discussed.

4.3 Materials et methods

4.3.1 Bacterial strains

Bacterial strains used in this study are listed in Table 4.1. The causal bacterial pathogens for lettuce were isolated from diseased leaves (*Lactuca sativa L.*) in the monteregie area, Quebec, Canada. The pathogenicity of the isolates was verified by the Koch's postulation methods. *Xanthomonas* reference strains were obtained from CFBP Collection Centre. Both field isolates and reference strains were grown on Yeast dextrose carbonate medium at 28 °C for 48 hours.

Table 4.1. Bacterial strains used in this study.

Species/pathovars	Strains‡	Sources
X. hortorum		
	B07-007	Wild type†
	VT106	Wild type†
	B14-313	Wild type†
	B14-370	Wild type†
	B14-531	Wild type†
[pv. *vitians*]§	LMG 938	DNA sample, courtesy gift from C. Bull
pv. *hederae*	CFBP4925^{pT}	International culture Collections, FCBP
pv. *perlargonii*	CFBP2533^{pT}	International culture Collections, FCBP
pv. *taraxaci*	CFBP410^{pT}	International culture Collections, FCBP
X. campestris		
pv. *incanae*	CFBP2527^{pT}	International culture Collections, FCBP
pv. *aberrans*	CFBP6865^{pT}	International culture Collections, FCBP
pv. *barbareae*	CFBP5825^{pT}	International culture Collections, FCBP
pv. *betae*	CFBP5852^{pT}	International culture Collections, FCBP
pv. *bilvae*	CFBP3136^{pT}	International culture Collections, FCBP
pv. *campestris*	CFBP5241^{pT}	International culture Collections, FCBP
pv. *esculenti*	CFBP5857^{pT}	International culture Collections, FCBP
pv. *mangiferaeindicae*	CFBP1716^{pT}	International culture Collections, FCBP
pv. *paulliniae*	CFBP5862^{pT}	International culture Collections, FCBP
pv. *raphani*	CFBP5827^{pT}	International culture Collections, FCBP
pv. *viegasii*	CFBP5866^{pT}	International culture Collections, FCBP
pv. *zinniae*	CFBP4477^{pT}	International culture Collections, FCBP
X. axonopodis		
pv. *axonopodis*	CFBP4924^{T}	International culture Collections, FCBP

pv. *alfalfa*	CFBP3836^{pT}	International culture Collections, FCBP
pv. *allii*	CFBP6107^{pT}	International culture Collections, FCBP
pv. *begonia*	CFBP2524^{pT}	International culture Collections, FCBP
pv. *glycines*	CFBP2526^{pT}	International culture Collections, FCBP
pv. *maculifoliigardeniae*	CFBP1155^{pT}	International culture Collections, FCBP
	CFBP2530^{pT}	International culture Collections, FCBP
pv. *malvacearum*	CFBP7153^{pT}	International culture Collections, FCBP
pv. *malvacearum*	CFBP2534^{pT}	International culture Collections, FCBP
pv. *phaseoli*	CFBP7277^{pT}	International culture Collections, FCBP
pv. *poinsettiicola*	CFBP5823^{pT}	International culture Collections, FCBP
pv. *vasculorum*	CFBP7112^{pT}	International culture Collections, FCBP
pv. *vignicola*	CFBP2538^{pT}	International culture Collections, FCBP
pv. *vitians*		

Note: §, pathotype nomination removed from Names of plant pathogenic bacteria 1864-1995 (Young et al., 1996). ‡, T, type strain of the species; pT, pathotype strain. † Isolated from lettuce bacterial leaf spot, Monteregie, Quebec, Canada.

4.3.2 Molecular techniques

Genomic DNA for NGS was isolated from pure bacterial culture. Bacterial cells were collected from fresh bacterial lawn on King's B agar plates and suspended in sterile TE buffer (Tris-base at 10 mM, EDTA at 1 mM, pH 8.0). Wizard Genome DNA purification Kit (Promega, Madison, USA) was used and manufacturer's instruction followed. Final DNA samples were suspended in sterile deionized water. The third generation DNA sequencing developed by Pacific Biosciences (Menlo Park, CA, USA) was applied to sequence the B07-007 genome. About 10 µg of genomic DNA was used for DNA shearing and subsequent sequencing library preparation. Two SMRT cells were used and sequenced in a PacBio RSII sequencer (Pacific Biosciences) by McGill University and Genome Quebec Innovation Centre (MUGQIC), Montreal, QC,

Canada. For Illumina HiSeq 2000 sequencing, DNA library was prepared according to the manufacturer's instructions for TruSeq DNA Preparation (Illumina, San Diego, CA, USA). Paired-end sequencing of 100mers was performed at the IRIC's Genomics Core Facility (IRIC: Institute for Research in Immunology and Cancer, Montreal University, Montreal, QC, Canada).

To identify our field isolates, we amplified internal sequences of four housekeeping genes. Sequences of three pairs of DNA primers were used according to Young et al. (2008). These were XdnaK1F (GGTGGAAGACCTGGTCAAGA) and XdnaK1R (TCCTTGACYTCGGTGAACTC) for *dnaK*, XgyrB1F (ACGAGTACAACCCGGACAA) and XgyrB1R (CCCATCARGGTGCTGAAGAT) for *gyrB*, and XrpoD1F (TGGAACAGGGCTATCTGACC) and XrpoD1R (CATTCYAGGTTGGTCTGRTT) for *rpoD*. The fourth pair, XanfyuA-F4 (AYTCSTACGTGCAGAGCCTG) and XanfyuA-R3 (CGTRTAGCCSGGCATCTTCA) for *fyuA* was designed. PCRs were performed in 50 µl of reaction cocktail containing 1 X TopTaq PCR buffer with 15 mM MgCl2, 200 µM of each dNTP, 0.25 µM of primers and 1.25 units of TopTaq DNA polymerase (Qiagen, Mississauga, ON, Canada). DNA amplification reaction was run under the following conditions: 95 °C for 2 min, 30 cycles at 94 °C for 20 s, 54 °C for 30 s, and 72 °C for 1 min, with an additional extension at 72 °C for 10 min after the final cycle. Amplification was visualized on 0.7% agarose gel and DNA was Sanger-sequenced at MUGQIC.

4.3.3 Genome assembly and annotation

The DNA subreads generated with PacBio sequencing were assembled using the Hierarchical Genome Assembly Process (HGAP) workflow (Chin et al. 2013). A preassembly (BLASR) was carried out (Chaisson and Tesler, 2012), which consists of aligning short subreads on long subreads to correct errors. The long corrected reads were used as seeds into Celera assembly, which generated contigs. The contigs were aligned again with raw reads (BLASR) and processed through Quiver algorithm to generate high quality consensus sequences. These consensus sequences were used as reference sequences to assemble Illumina short read sequences with CLC Genomics Workbench v7.0.4. Illumina raw sequences were trimmed using

quality score of 0.001. Paired-end sequences were merged with mismatch cost score of 3, maximum unaligned end mismatch score of 0, and minimum score of 11. The trimmed merged, unmerged, and orphan sequences were aligned with mismatch cost of 3, insertion cost of 3, deletion cost of 3, length fraction of 0.5 and similarity fraction of 0.8. Uncertain positions were re-aligned locally and when necessary, PCR primers were generated, corresponding regions amplified and Sanger-sequenced. The newly obtained consensus sequences were used as new reference sequences and CLC assembly was repeated to verify the accuracy. The final assembly resulted in a complete genome and was annotated via the Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) at the NCBI (Tatusova et al. 2016). Genes encoded for type III effectors and other pathogenicity-related proteins were annotated manually.

4.3.4 Multilocus sequence typing (MLST), phylogenetic and phylogenomic analyses

The four *Xanthomonas* housekeeping genes *dnaK*, *fyuA*, *gyrB* and *rpoD* from isolates in this study and CFBP reference strains and sequences retrieved from GenBank were respectively aligned, trimmed and linked together to make concatenated DNA sequences. The concatenated sequences were aligned with Clustal W (Larkin at al. 2007) and neighbor joining trees were constructed and bootstrapped for 1000 random samplings of sites from the alignments using MEGA version 6 (Tamura et al. 2013).

Whole genome alignments were performed with genome sequences of B07-007 and 20 other *Xanthomonas* strains. Tree file was built using the online pipeline Realphy v1.10 (Bertels et al. 2014) (http://realphy.unibas.ch/fcgi/realphy) and visualized with TreeViewX v0.5.0 (Page 1996).

4.3.5 Genome alignment

The MAUVE program v2.3.1 (Darling et al. 2004) was run for whole genome alignment between B07-007 and five different genomes from GenBank using default values.

4.3.6 Type III effector identification

Effector candidates were identified based on the known type III effectors and suggested candidates by White et al. (2009) (http://www.xanthomonas.org/t3e.html) updated until August 8, 2016, as model type III effectors in the study. The model effector amino acid sequences and amino acid sequences of the CDSs of 58 completed genomes (Figure 4.8) were retrieved from GenBank and converted into local BLAST databases. BLASTp 2.0 integrated in BioEdit v7.2.5 was used and the threshold E-value was set to 1e-10. No filter for low complexity regions was activated. Putative Type III effectors were also identified using DNAMAX v3.0, searching for PIP-box plus -10 box-like motifs (TTCGB-N15-TTCGB-N30-32-YRNNNT, in which B is referred to any bases except adenine, R referred to A or G, and Y referred to C or T) (Kim et al. 2003; Koebnik et al. 2006; Jacobs et al. 2015; Guo et al., 2012; Furutani et al. 2006).

4.4 Results and discussion

4.4.1 Lettuce bacterial leaf spot pathogen – *Xanthomonas hortorum* strain B07-007

Bacterial leaf spot of lettuce was first observed in 1994 in the South shore regions (Monteregie) of Montreal, Quebec, Canada (Toussaint, 1999). The pathogen infected Romaine and Crisphead cultivars and caused water-soaked lesions at leaf margin and midrib or dark-brown coloration on the leaves. No systemic infection was observed. Since 2007, we purified three hundred seventy-six field isolates from several local farms. Preliminary identification was performed by colony morphology and BIOLOG system. Subsequent molecular identification was followed using four pairs of primers from *Xanthomonas* housekeeping genes (*dnaK*, *gyrB*, *rpoD* and *fyuA*). These isolates were finally identified as clones of five different strains of *X. hortorum*, i.e. B07-007, VT106, B14-346, B14-357 and B14-531 (data not shown).

4.4.2 Genome sequencing and global assembly

We chose to sequence the B07-007 strain for a profound study because it represents the dominant strain in the region. Hybrid assembly using both Illumina and PacBio reads was used as a strategy to recover the full genome sequence of B07-007. PacBio sequencing generated about 0.18 million reads with mean read length of 5.9 kb, of which more than 10 thousand reads were over 15 kb long, representing about 1 billion base pairs. The PacBio de novo assembly generated two contigs, one chromosome and one plasmid. Long read coverage was about 140X for both contigs. The Illumina HiSeq sequencing for B07-007 generated about 260M 100bp paired-end reads, which were used to polish the PacBio de novo assembly. The assembly showed a continuous sequence with no zero coverage regions. The average coverage with unique Illumina data is 2681X. However, we noticed that the efficiency of Illumina HiSeq sequencing varied much with the GC content – coverage was negatively correlated to the GC percentage (Figure 4.1). As a result, a much lower coverage was observed in some GC-rich regions of the genome. A circular chromosome of 5,175,249 bp and a plasmid of 75,655 bp were obtained with GC content of 63.7% and 59.6% respectively. The plasmid was named pB07007. Plasmid profile and size were confirmed on agarose gel (Figure 4.2).

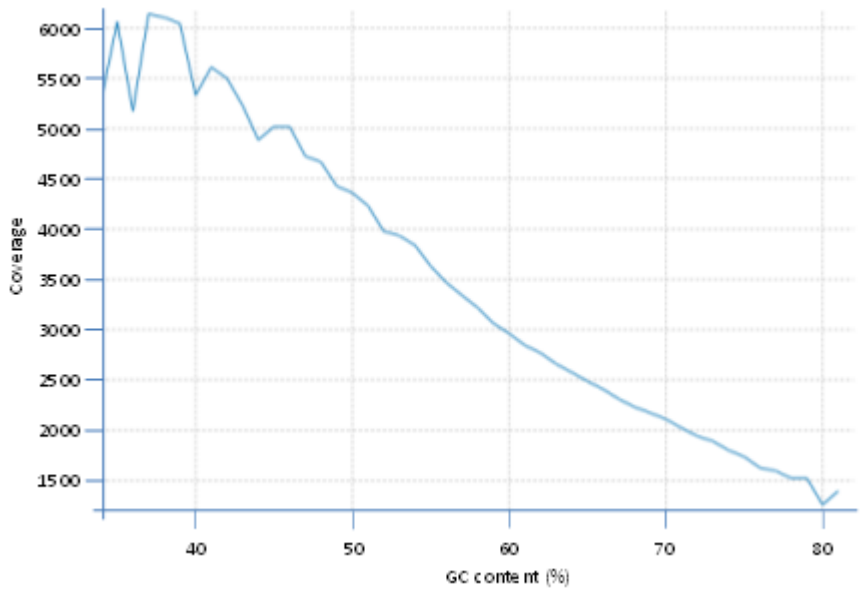

Figure 4.1. Relation of GC content and assembly coverage of the HiSeq sequence data. The plot displays, for each GC content level (0-100%), the mean read coverage of 100 bp reference segments with that GC content.

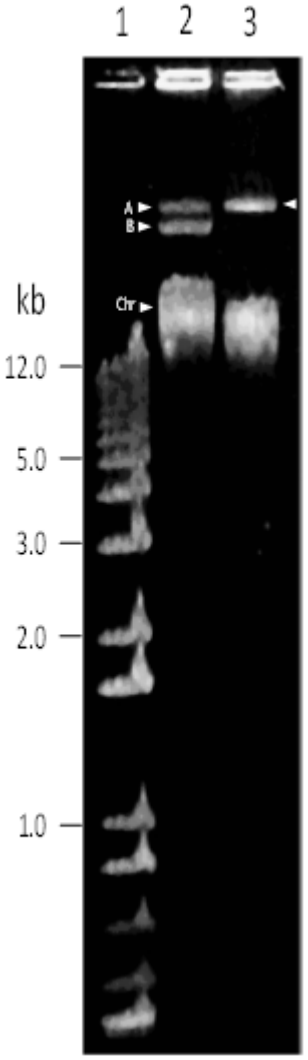

Figure 4.2. Plasmid profile of strain B07-007. A plasmid isolation protocol (Crosa & Falkow 1981) was applied to purify bacterial megaplasmids. Lane 1, linear DNA molecular weight marker in kb (Sigma-Aldrich Canada Co., Ontario, Canada). Lane 2, Pseudomonas syringae pv. tomato DC3000 as reference, arrow A, pDC3000A (73.7 Kb), and arrow B, pDC3000B (67.5 kb). Lane 3, B07-007, an arrow indicates the sole 75.6-kb plasmid. Chr, chromosomal DNA.

4.4.3 B07-007 genome analysis

The B07-007 genome sequences were annotated with NCBI PGAAP. The genome (chromosome and plasmid) contains a total of 4489 genes including two rRNA operons (5S, 16S and 23S), 53 tRNAs, 40 ncRNAs and 4260 CDSs (Figure 4.3). The genome possesses Types II, III and IV secretion systems. It contains a total of 127 transposase sequences related to different insertion sequence elements (Supplementary Table S4.1).

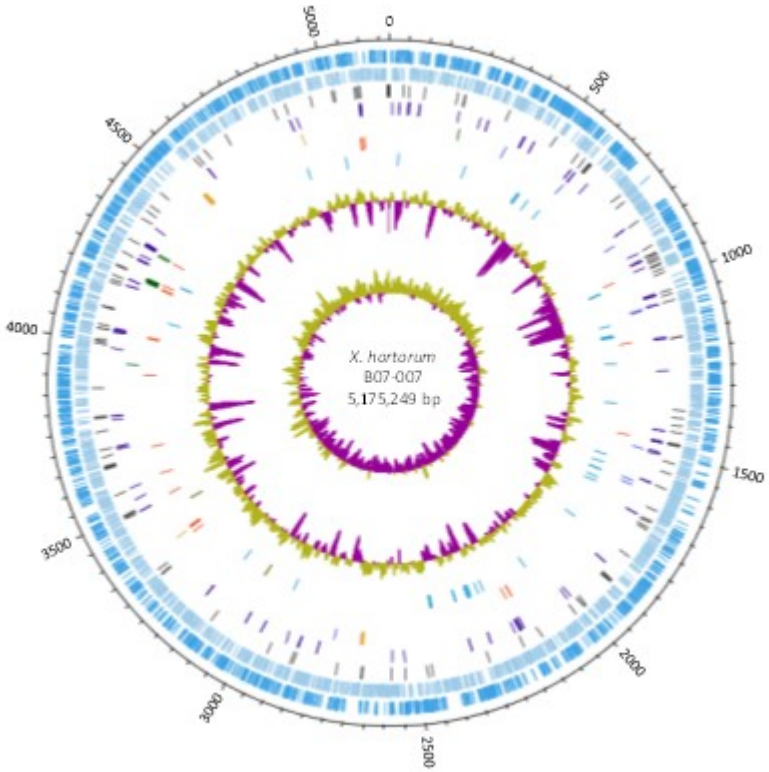

Figure 4.3. Genome of *X. hortorum* strain B07-007. The genome ring illustrates, from outside to inside, genome coordinates in kb, protein-coding genes in forward and reverse strands, pseudo-genes, transposases, secretion system proteins (Types II (orange), III (green) and IV (royalblue)), Type III effectors, tRNA (light blue) and rRNA (olive) genes, percent G+C content, and GC skew. GC skews shows (G-C)/G+C) in 10 kb windows.

4.4.4 Phylogenomic relationships

Kimbrel et al. (2011) inferred a relative position of *X. hortorum* among several different *Xanthomonas* strains by phylogenomic analysis. To establish the phylogenetic position of B07-007, its chromosome sequence was compared to 21 other *Xanthomonas* chromosome sequences (Figure 4.4). Five distinct groups were inferred. Group I includes *X. citri*, *X. axonopodis*, *X. fuscans* and *X. campestris* strains, which are pathogens infecting citrus, bean, soybean, pepper and tomato plants. *X. oryzae* members were quite conserved and clustered in Group II. Strain B07-007 and the Xhc M081 grouped together in Group III. The four *X. campestris* strains analysed, all pathogens of Arabidopsis and cruciferous vegetables, clustered together into Group IV. Finally, *X. translucens* strains clustered together as Group V. Exceptionally, *X. fragariae*, *X. sacchari*, and *X. albilineans* were singly separated from others.

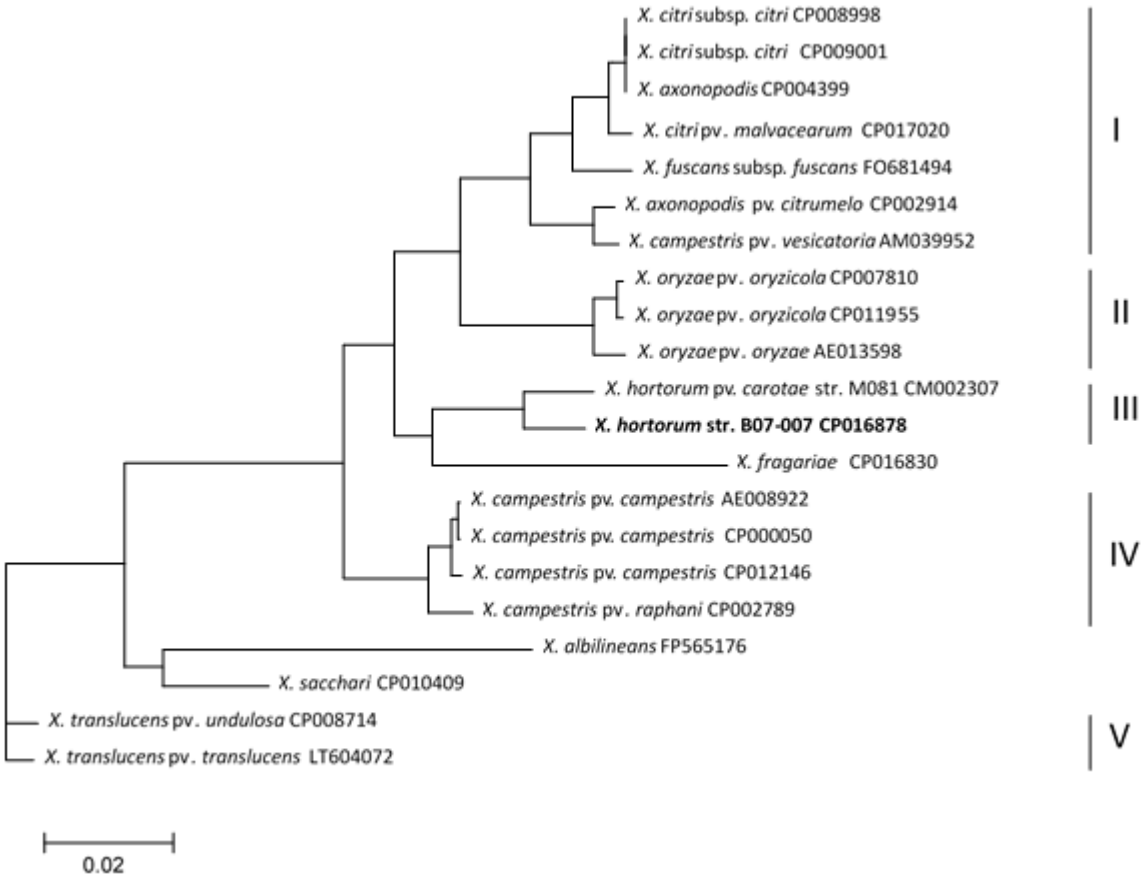

Figure 4.4. Phylogenomic relationships. Phylogenetic tree generated from whole genome sequences of *X. hortorum* strain B07-007 and other *Xanthomonas* species and strains. GenBank accession numbers are indicated following each of the corresponding bacterial names. A scale bar below the tree refers to number of nucleotide substitutions per site.

4.4.5 Relationship of B07-007 and its sister strains

A set of concatenated DNA sequences of four housekeeping genes of the five laboratory strains and 30 reference strains was generated and their phylogenetic tree was constructed. The dendrogram in Figure 4.5 shows four major clusters: Cluster I, composed of two close sub-groups, Ia (members of *X. axonopodis*) and Ib (members of *X. citri*); Cluster II included two strains, *X.* sp. pv. *esculenti* and *X.* sp. pv. *zinnia*; Cluster III, *X. campestris*, and Cluster IV, the *X. hortorum* members. B07-007 and four other lab strains clustered with the group of *X.*

hortorum. It is interesting that the five lab strains were closely related to the strain LMG 938. The five lab strains in this study are phylogenetically distinct from the four known pathovars of *X. hortorum* and now remain in the specific level without pathovar proposition. Their pathotype determination is considered in a future study.

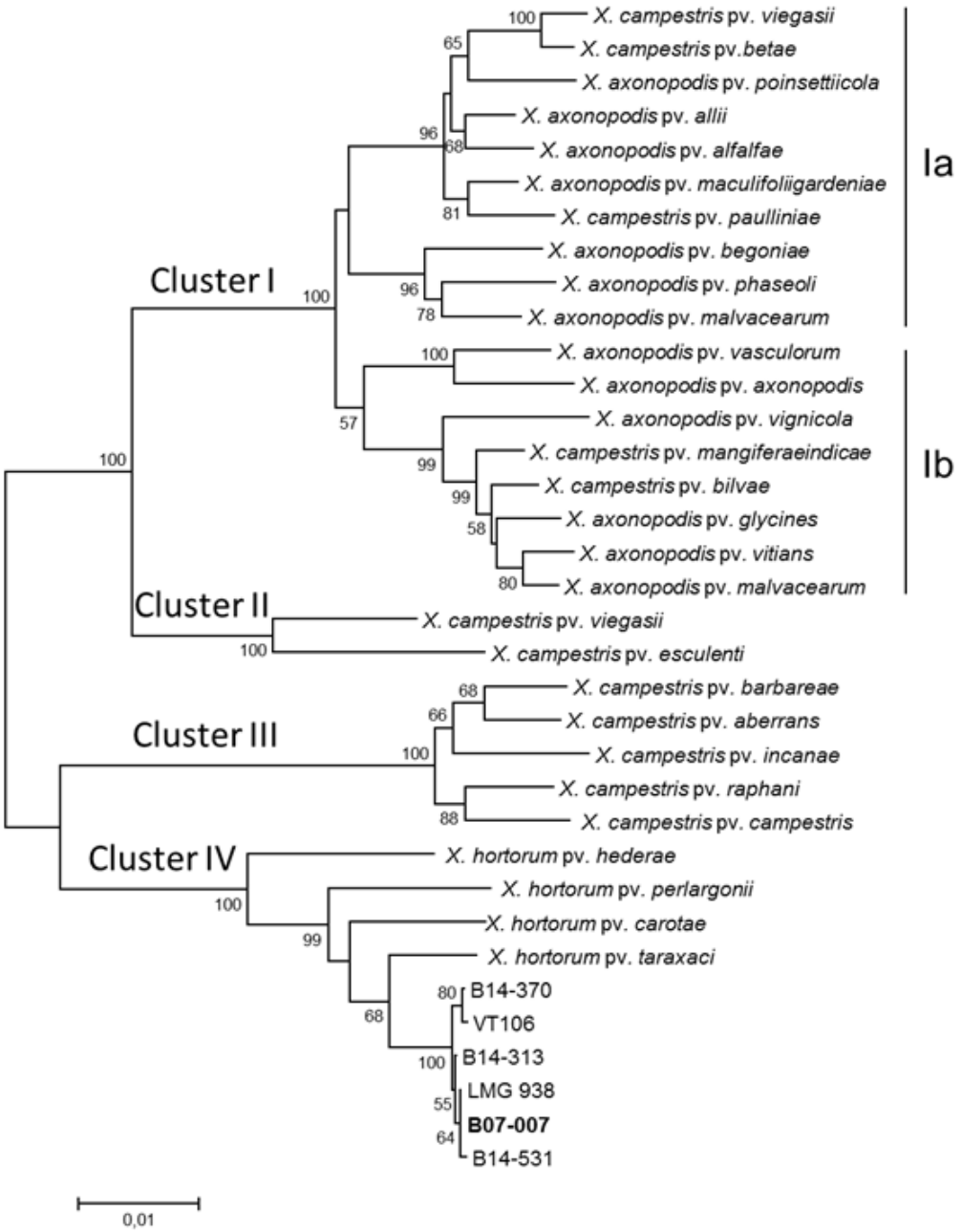

Figure 4.5. Phylogenetic determination of the systematic position of the lettuce strains isolated from Monteregie, QC, Canada. Neighbor-joining tree constructed with concatenated nucleotide sequences of internal regions of the genes *dnaK*, *fyuA*, *gyrB* and *rpoD* from 30 reference strains, B07-007 and four other lettuce strains is illustrated. The number at the nodes indicates the levels of bootstrap 1000 random samplings with replacement. Values greater than 50% are given. Scale bar represents the number of nucleotide substitutions per site.

4.4.6 Genome comparison

Phylogenomic analysis suggests that the B07-007 genome is highly homologous to *Xhc* M081 (Figure 4.4). To estimate synteny between B07-007 and other xanthomonads, genome alignment was run with Mauve program between B07-007 and five *Xanthomonas* species/pathovars. Figure 4.6 shows blocks of conservation between B07-007 and *Xhc* M081 and four other *Xanthomonas* strains from Groups I, II and IV (Figure 4.4). Although the phylogenomic analysis suggests that B07-007 and M081 are highly homologous, we noticed two main large blocks of inversion-translocation incidents over the genomes. The first block occurred between 780000 and 1780000 nt and the second between 3,300,000 and 4,430,000 nt on the B07-007 genome (positions adjusted in the alignment) compared to *Xhc* M081. We then carefully checked the alignment of our genome assembly with special attention in these two regions and did not find uncertain positions possibly causing wrong assemblies of such large blocks. These two blocks are not duplicates. The whole genome comparison to *Xhc* M081 and other *Xanthomonas* species revealed a lot of sequence rearrangements: translocations, inversions and deletions. This phenomenon may reflect their respective evolution from a common ancestor.

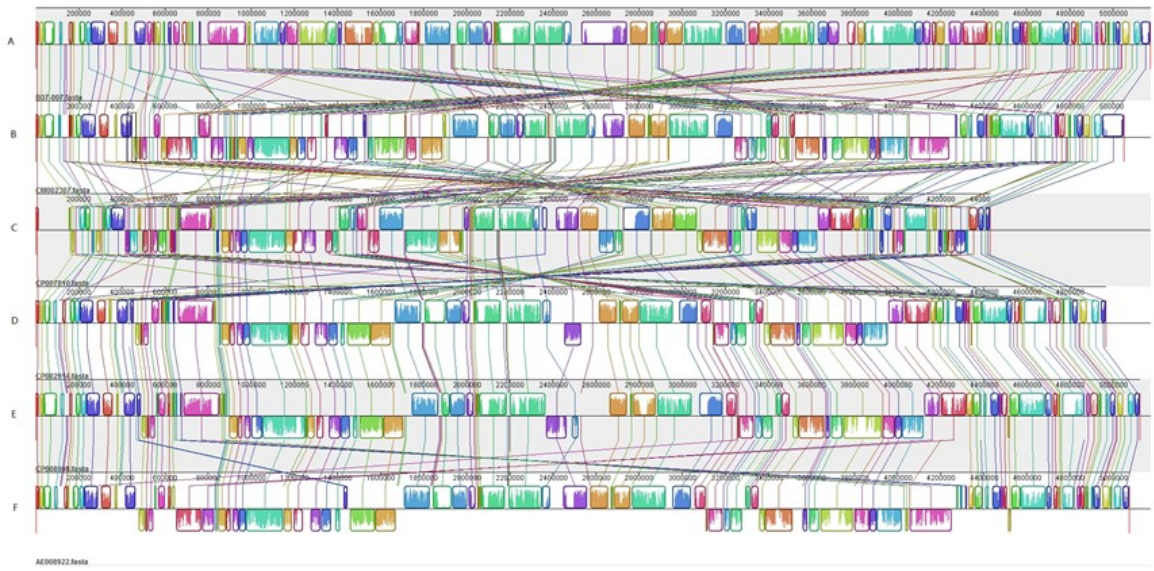

Figure 4.6. Genome alignments of *X. hortorum* strain B07-007 to different *Xanthomonas* species. Whole genome alignment of the high-quality assembled genome sequence of B07-007 (CP016878) (A) with *X. hortorum* pv. *carotae* (CM002307) (B), *X. oryzae* pv. *oryzicola* (CP007810) (C), *X. axonopodis* pv. *citrumelo* (CP002914) (D), *X. citri* subsp. *citri* (CP008998) (E), and *X. campestris* pv. *campestris* (AE008922) (F). Genome sequences were adjusted to the same start with *X. hortorum* pv. *carotae* (CM002307). The start point of B07-007 corresponds to position 3584645 of the originally deposited sequence in GenBank for the alignment. The line control blocks (LCB, rounded rectangles) represent similarities between aligned genome sequences within which are shown the similarity plots (bundles of vertical lines). Connecting lines link the homologous blocks between genomes. Spaces between LCBs are regions without significant homologies. There is a base line for each genome. LCBs above the base line are forward sequences and under the line, reverse-complement sequences. Genome boundary lines crossing the base line are drawn at the start and the end of each genome. Sequence length scale is shown above each genome.

Genetic behaviors such as DNA sequence inversion, insertion, deletions, duplication and translocations often link to transposons and insertion sequences in bacteria. B07-007 genome contains many segments of DNA related to transposable elements. These elements (complete or fragmented) are derivatives belonging to nine distinct groups (IS3, IS4, IS5, IS30, IS110, IS1595, ISxcc1, ISxac1, ISxac3 and IS200 families) and some unclassified. They were present in multiple copies except for IS30, IS110 and IS1595. IS3, IS4, and IS5 were frequent in *Xanthomonas* species (Bogdanove et al. 2011) and also in B07-007, in multiple copies. Among them, members of IS5 family were most dominant (Supplementary Figure S4.1 and Table S4.1). Transposase 00020 in a single copy was grouped with IS110 and neighbored to IS4 family, while transposase XJ27_20990, also in single copy and clustered to IS1595, was clearly distinct from any other known IS families in the strain. XJ27_18140 is 546 bp long and highly similar to the moiety of N-terminus of IS30. IS200-like IS elements include two members, XJ27_09335 and XJ27_18885. These two are highly similar to a transposase from *X. gardneri* which belongs to the IS200/IS605 family. IS200 was first found in *Salmonella typhimurium* (Lam and Roth 1983) and since then related elements were observed in a variety of bacterial species including both Gram-negative and Gram-positive (Mahillon and Chandler 1998), suggesting its wide range of horizontal transfer.

4.4.7 Secretion systems

Secretion is an essential function for bacteria to transport proteins, DNA and certain secondary metabolites into the environment, target eukaryotic cells or other bacteria. Bacterial secretion systems, based on their functions, are classified in seven different systems (I to VII) and several specific pathways (Green and Mecsas 2016). Gram-negatives share T1SS to T6SS. *X. hortorum* B07-007 possesses T2SS-, T3SS- and T4SS-related proteins (Table 4.2).

Table 4.2. Secretion systems, effectors and pathogenicity-related genes of *Xanthomonas hortorum* strain B07-007.

Location[a]	Gene identifier	Secretion system	Protein name/function	Distance from PIP box, -10 box	PIP box, -10 box[f]
Secretion systems:					
chr	XJ27_11760	II	GspD		
chr	XJ27_11795	II	GspH		
chr	XJ27_11800	II	GspG		
chr	XJ27_11805	II	GspF		
chr	XJ27_11810	II	GspE		
chr	XJ27_14765	II	GspE		
chr	XJ27_19580	II	GspC		
chr	XJ27_19585	II	GspD		
chr	XJ27_19590	II	GspE		
chr	XJ27_19595	II	GspF		
chr	XJ27_19600	II	GspG		
chr	XJ27_19605	II	GspH		
chr	XJ27_19610	II	GspI		
chr	XJ27_19615	II	GspJ		
chr	XJ27_19620	II	GspK		
chr	XJ27_19625	II	GspL		
chr	XJ27_19630	II	GspM		
chr	XJ27_19635	II	GspN		
chr	XJ27_20975	II	GspO		
chr	XJ27_20980	II	GspF		
chr	XJ27_18135	III	HrpF		
chr	XJ27_18170	III	HpaB		
chr	XJ27_18175	III	HrpE		
chr	XJ27_18180	III	HrpD6		
chr	XJ27_18185	III	HrpD5		
chr	XJ27_18190	III	HpaA		
chr	XJ27_18195	III	EscS/YscS/HrcS		
chr	XJ27_18200	III	EscR/YscR/HrcR		
chr	XJ27_18205	III	HrcQ	174	T3E-15N-31N
chr	XJ27_18210	III	HpaP/HpaC		
chr	XJ27_18215	III	EscV/YscV/HrcV		
chr	XJ27_18220	III	EscU/YscU/HrcU	33	T3E-15N-32N
chr	XJ27_18225	III	HrpB1	45	T3E-15N-30N
chr	XJ27_18230	III	HrpB2		
chr	XJ27_18235	III	EscJ/YscJ/HrcJ		
chr	XJ27_18240	III	HrpB4		
chr	XJ27_18245	III	HrpB5		

chr	XJ27_18250	III	EscN/YscN/HrcN		
chr	XJ27_18255	III	HrpB7		
chr	XJ27_18260	III	EscT/YscT/HrcT		
chr	XJ27_18265	III	EscC/YscC/HrcC		
chr	XJ27_01615	IV	PilT		
chr	XJ27_02520	IV	VirD4		
chr	XJ27_02555	IV	Rhs		
chr	XJ27_03525	IV	PilW		
chr	XJ27_04565	IV	virB6		
chr	XJ27_06500	IV	Rhs		
chr	XJ27_06920	IV	VirB9		
chr	XJ27_06935	IV	VirD4		
chr	XJ27_06985	IV	PilV		
chr	XJ27_12125	IV	PilT		
pla	XJ27_22310	IV	VirB11		
pla	XJ27_22315	IV	VirB10		
pla	XJ27_22320	IV	VirB9		
pla	XJ27_22325	IV	VirB8		
pla	XJ27_22330	IV	VirB6		
pla	XJ27_22335	IV	VirB5		
pla	XJ27_22340	IV	VirB4_C[c]		
pla	XJ27_22350	IV	VirB4_N[c]		
pla	XJ27_22355	IV	VirB3		
pla	XJ27_22360	IV	VirB2		
Type III effectors and potential virulence-related factors:					
chr	XJ27_04030	III	XopE2	29	T3E-15N-30N
chr	XJ27_04990	III	XopD[d]		
chr	XJ27_06365	III	XopAF		
chr	XJ27_09245	III	XopAM	459	T3E-15N-30N
chr	XJ27_09330	III	XopQ	39	T3E-15N-31N
chr	XJ27_14735	III	XopZ[d]		
chr	XJ27_14770	III	XopAD		
chr	XJ27_15275	III	XopL[d]	285	T3E-15N-31N
chr	XJ27_15550	III	AvrXccA1		
chr	XJ27_15920	III	AvrBs2		
chr	XJ27_16520	III	HopX1		
chr	XJ27_16940	III	XopN		
chr	XJ27_17065	III	XopR	716	T3E-15N-30N
chr	XJ27_17450	III	XopB		
chr	XJ27_17465	III	XopC1		
chr	XJ27_18160	III	XopZ[d]		
chr	XJ27_18165	III	HrpW		
chr	XJ27_18270	III	XopA	100	T3E-15N-31N
chr	XJ27_18625	III	XopX[d]		

chr	XJ27_18630	III	XopX[d]		
chr	XJ27_21665	III	XopL[d]		
chr	XJ27_21690	III	XopAU		
chr	XJ27_21695	III	XopK	223	T3E-15N-30N
chr	XJ27_21745	III	XopD[d]		
pla	XJ27_22075	III	AvrBs3		
pla	XJ27_22115	III	XopJ5		
pla	XJ27_22130	III	AvrBs1		
pla	XJ27_22445	III	XopE2		
chr	XJ27_05305	N/A[b]	putative T3 effector	263	T3E-15N-31N
chr	XJ27_05435	N/A	secreted protein	373	T3E-15N-31N
chr	XJ27_05455	N/A	polygalacturonase	205	T3E-15N-31N
chr	XJ27_11675	N/A	hypothetical protein	406	T3E-15N-32N
chr	XJ27_17070	N/A	hypothetical protein	339	T3E-15N-30N
chr	XJ27_17385	N/A	glycosyl hydrolase family 3	455	T3E-15N-31N
chr	XJ27_18275	N/A	lytic transglycosylase	244	T3E-15N-31N
chr	XJ27_18415	N/A	lipase	248	T3E-15N-31N
chr	XJ27_20805	N/A	aminopeptidase	27	T3E-15N-31N
Other pathogenicity-related[e]:					
chr	XJ27_08950	N/A	pathogenicity-related protein		
chr	XJ27_14745	N/A	pathogenicity-related protein		
chr	XJ27_15550	N/A	avirulence		

Note: *a*, abbreviation, chr, chromosome, pla, plasmid; *b*, N/A, not analysed; c, truncated protein, _C refers to C-terminal part and _N, N-terminal part; d, there exist two homologous but not identical copies; e, gene descriptions according to PGAAP annotations; f, T3E-15N-30N, T3E-15N-31N and T3E-15N-32N refer to TTCGB $-N_{15}$-TTCGB-$N_{30\text{-}32}$-YRNNNT.

Thirteen T2SS-related proteins are present in B07-007 genome. The T2SS mainly form two gene blocks (*gspEFGH* and *gspCDEFGGIJKLMN*), which each are arranged in tandem. Another gene, *gspO*, is located separately from these two gene blocks. A functional T2SS is usually composed of T2S GspCDEFGHIJKLMN and GspO proteins. T2SS is responsible of secreting enzymatic proteins to digest carbohydrates, polysaccharides, pectin, and various kinds of proteins including some toxins.

B07-007 genome also has genes coding for T4SS, which has a variety of functions including a role in conjugation. The T4SS is also involved in protein translocation into host cells. Roles of

T4SS have widely been studied in human diseases as well as in the *Agrobacterium* spp. plant pathogens (Voth et al. 2012), but its roles in other pathogens have received less attention. The T4SS includes genes involved in pilus formation. A functional T4SS requires at least the presence of the *virB* operon for the synthesis of pili and *virD4* as ATPase and coupling protein. In B07-007, a block of genes, *virB2* to *virB11*, were present on its plasmid. Between *virB3* and *virB5*, two CDSs (XJ27_22350 and XJ27_22340) were found to respectively belong to the N-terminal and C-terminal domains of *virB4* (here named *virB4_N* and *virB4_C*). *virB4_N* (XJ27_22350) codes for a 266 aa protein and is highly homologous to the N-terminal domain of *X. euvesicatoria VirB4* (WP_042841380) from aa 1 to 266. *virB4_C* (XJ27_22340) codes for a 607 aa long protein, corresponding to the C-terminal domain of this *X. euvesicatoria* VirB4 protein from aa 267 to 873. Between *virB4_N* and *virB4_C*, an IS5 transposable element (XJ27_22345) is found, but it is thought to be disarmed because it has no inverted repeats or direct repeats in its adjacent regions. This incomplete IS5 spatially separates the original *virB4* into two parts without losing any coding regions of the latter. Whether the two separated VirB4 domains retain their functionality and whether this IS5 blocks the expression of the downstream genes from *virB4_C* to *virB11* remains to clarify. Finally, ORF XJ27_22360, upstream of the *virB2*, might be a variant or a degenerated form of *virB1*.

T3SS, also called the "injectisome", is often related to plant diseases and plays a pivotal role in many plant disease pathogens. T3SS is the most studied and the most important secretion system in relation to plant pathogenicity. In *Xanthomonas* species, this system is composed of many genes locating in a region just over 20 kb long called the T3SS pathogenicity island (PAI). Figure 4.7 shows the T3SS PAI region of six different strains from five different *Xanthomonas* species. The T3SS PAI for the key genes responsible for pathogenicity from *hpaB* to *hrcC* are much conserved among the different species analysed. Their gene sizes and orientations remain conserved. A set of the genes from *hrpB1* to *hrcC* is forwardly arranged in tandem, while the set from *hrcU* to *hpaB* is in the reverse orientation. Both sets have operon structures. A similarity plot of this region between strains B07-007 and M081 suggests their high conservation with only some more variation in *hrpE*, *hrpD6*, *hrpD5* and *hrpA* (SP in Figure 4.7). An exception is the putative translocon *hrpF* (Büttner et al. 2002). Its position and orientation on the T3SS PAI

are variable among the different strains analysed. *X. campestris* pv. *campestris* ATCC 33913, the first *Xanthomonas* complete genome published, has two effector genes *xopF* and *hrpW* in a short segment between *hrpF* and *hpaB*. Within the same region, *X. citri* subsp. *citri* MN12 has a truncated *xopF1* and *hpa1* genes, and *X. axonopodis* pv. *citrumelo* F1 contains *xopF1* and *hpa3* while *X. oryzae* pv. *oryzicola* B8-12 possesses *xopF1* and a long noncoding region. Strains B07-007 and M081 both contain *hrpW* and *xopZ* downstream the *hpaB* gene while M081 also has *xopF1* downstream of *xopZ*, which is absent in B07-007. Although a *hrpF* is present in both strains, the one in M081 is located nearly 1 Mb away from the *xopZ*. It is interesting to note that B07-007 has an IS3 and a possible IS30 derivative in the *hrpF-xopZ* region. The IS3 is located just downstream of *xopZ*. The IS3 alone, or cooperatively with the possible IS30 derivative, may have conducted rearrangement in the region and induced the loss of *xopF1* in B07-007. Instead, an epoxide hydrolase (EPHX) and a prolonged non-coding region were moved in between the two IS elements.

The three secretion systems T2SS, T3SS and T4SS are related to pathogenicity. Each system plays specific roles in pathogenesis. Considering the presence of these three together in B07-007, it is interesting to probe in depth their interrelationship in how effectively invading host tissues and developing symptoms. Cooperative actions between different secretion systems exist in bacteria owning multiple systems of secretion (Cianciotto 2005).

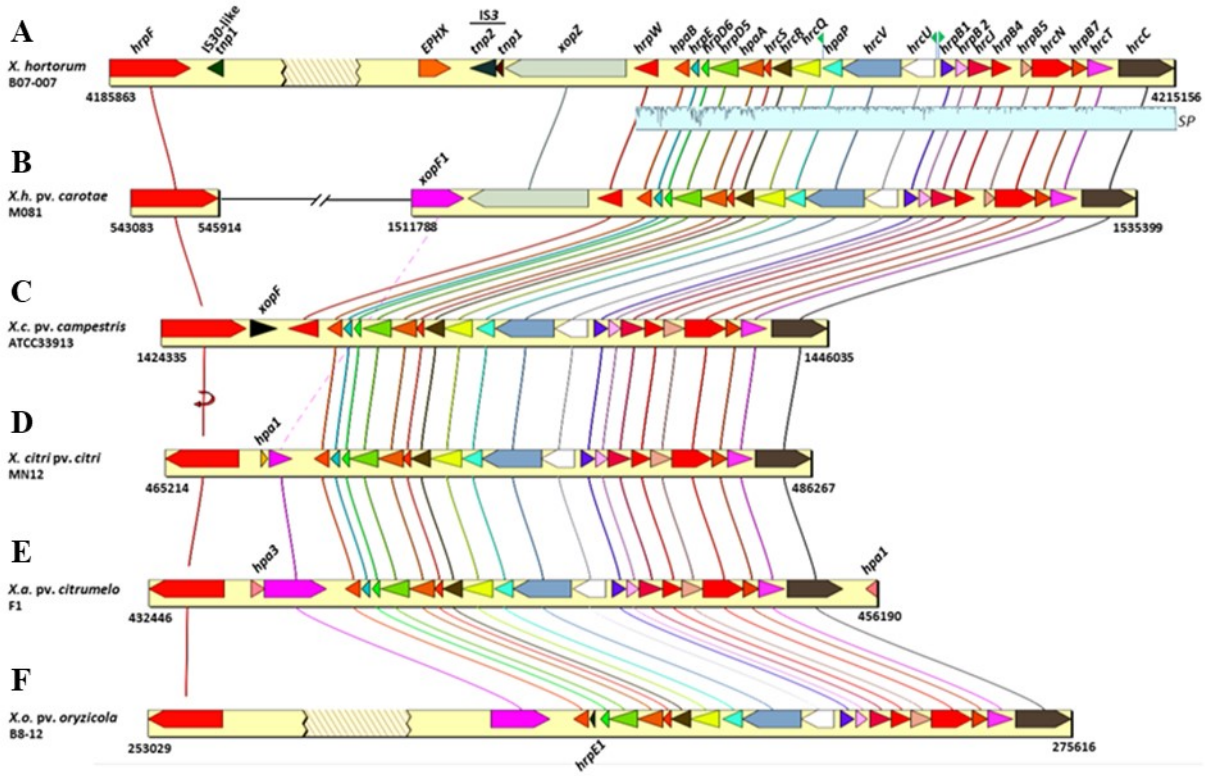

Figure 4.7. Syntenic comparison of the T3SS pathogenicity island (PAI). The T3SS pathogenicity-related gene regions between hrpF and hrcC are shown from genomes of six *Xanthomonas* strains. (A) *X. hortorum* B07-007; (B) *X. hortorum* pv. *carotae* M081; (C) *X. campestris* pv. *campestris* ATCC 33913; (D) *X. citri* subsp. *citri* MN12; (E) *X. axonopodis* pv. *citrumelo* F1 and (F) *X. oryzae* pv. *oryzicola* B8-12. Orthologous genes share single color across species and linked by curved lines. Gene names are labelled above the B07-007 PAI region. Names of specific genes or gene variants are denoted accordingly. The positions of both extremities of each region/segment are numbered. Putative promoters were indicated with green triangle flags pointing the corresponding transcription directions. Collapsed regions with diagonal lines are prolonged non-coding sequences. The diagram was generated using SimpleSynteny on-line program (Veltri et al. 2016). A similarity plot (SP) generated with MAUVE for the region from hrpW to hrcC between both *X. hortorum* B07-007 and M081 strains is shown below the B07-007 PAI. Curved arrow represents shift of gene direction.

4.4.8 Type III effectors

Predicted proteins of B07-007 and all the 58 completed *Xanthomonas* genomes available in GenBank (August 2016) were mined for type III effectors. Effector aa sequences from White et al. (2009) served as model effectors. Each model effector was composed of a set of homologous aa sequences which may be from *Xanthomonas*, *Pseudomonas* and *Ralstonia* genera. A total of fifty-nine genomes were investigated for 57 known type III effectors (Figure 4.8). Ten *Xanthomonas* species including 13 pathovars have type III effectors. Among the 57 effectors studied, 16 were frequent, i.e. present in over 40 genomes, and 26 were rare, i.e. appeared in fewer than ten genomes. Exceptionally, XopJ4 was not present in any of the investigated genomes according to our screening method. Most strains analysed contained multiple type III effector genes with some exceptions. *X. albilineans* (FP565176) had no type III effector at all, while *X. sacchari* (CP010409) had only one and *X. campestris* (CP011256), two. *X. albilineans* (FP565176) has a complete set of type III secretion system, but had conducted reductive genome evolution (Pieretti et al. 2009), during which the type III effectors may have been lost. *X. sacchari* (CP010409) also had an obviously smaller genome than other *Xanthonmonas* strains and may have also conducted a reductive evolution leading to the loss of some type III effectors. The other 56 strains each contained more than ten different type III effectors. Homologous strains showed similar repertoires of type III effectors.

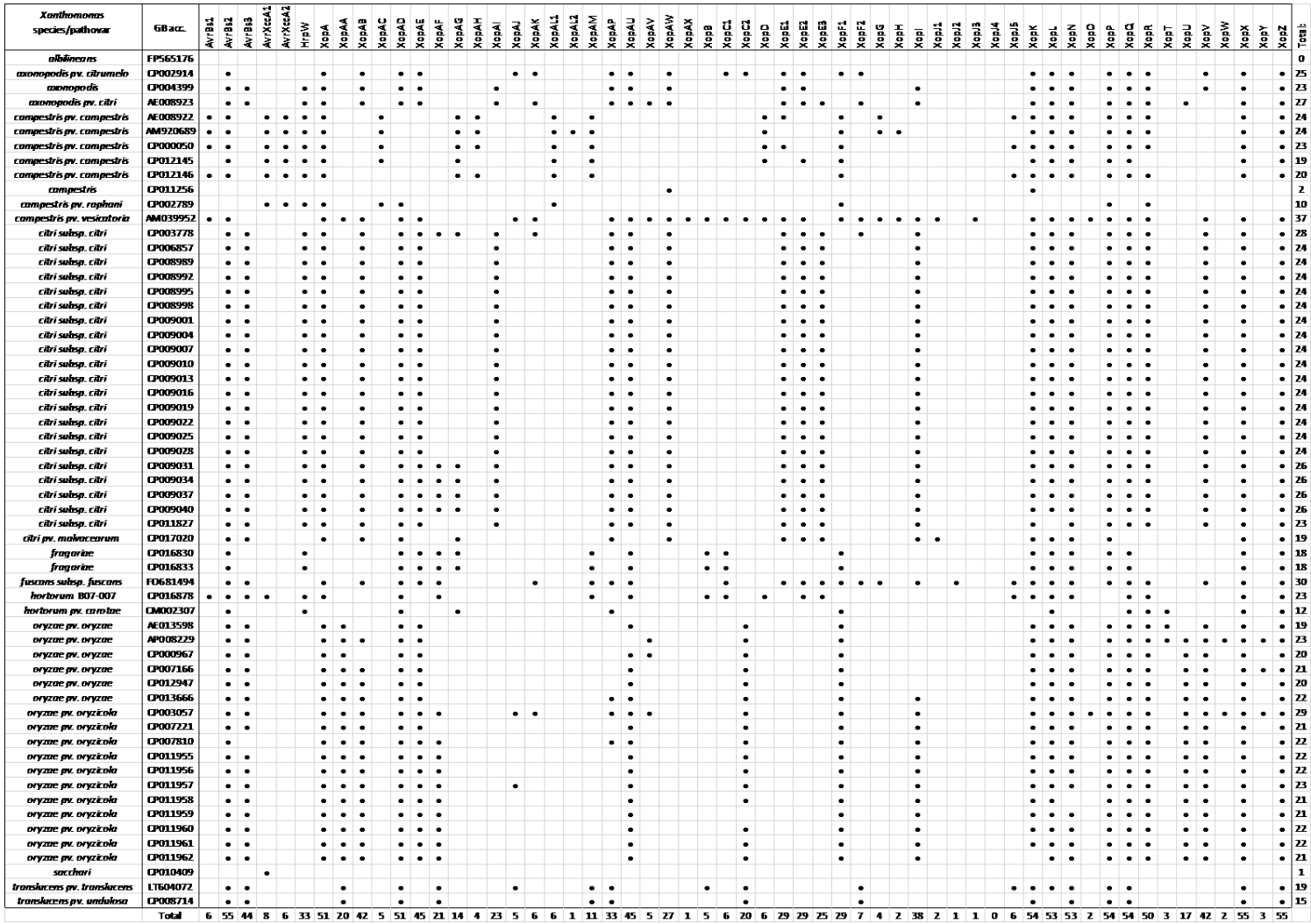

Figure 4.8. Presence/absence of T3 effectors in *Xanthomonas* strains. Analysis with all complete *Xanthomonas* genomes available in GenBank and effector data from http://www.xanthomonas.org/t3e.html (up to August 2016).

A neighbor-joining analysis for the presence of the different effectors clusters these strains in five major groups: Citri, Oryzae, Fragariae, Campestris and Translucens (Figure 4.9). The Citri group included all strains of *X. citri* subsp. *citri*, and pv. *malvacearum*. Some *X. citri* strains shared identical repertoires of effectors while others showed distinct differences. Oryzae group included all *X. oryzae* pv. *oryzae* and pv. *oryzicola* strains. Both *X. fragariae* strains sharing identical repertoire were together for Fragariae group. Campestris group clustered several *X. campestris* strains and finally, the two *X. translucens* pathovars were placed to Translucens group. These groupings are in accordance with genetic similarity of members of *Xanthomonas* (Rodriguez-R et al. 2012). Both *X. hortorum* strains B07-007 and M081 were clustered in neither group nor are they together. They each had a specific set of type III effectors. B07-007 contained 27 candidates belonging to 23 type III effectors. Of these 23 effectors, twelve (AvrBs2, AvrBs3, XopA, XopAD, XopAU, XopK, XopL, XopN, XopQ, XopR, XopX and XopZ) were most popular (≥40 strains studied) in different species/strains, five (AvrBs1, AvrXccA1, XopD and XopJ5) were frequent (>10 and <40) and six (AvrBs1, AvrXccA1, XopB, XopC1, XopD and XopJ5) were rare (≤10). Four effectors (XopD, XopL, XopX and XopZ) were present in two different copies. Phylogenetic analysis was respectively performed for each of the four effectors with corresponding model effector aa sequences. The two XopD copies were variants possibly stemming from the same origin (Supplementary figure S4.2 A). But both counterparts respective to XopL, XopX and XopZ clustered in different and even rather distant branches, suggesting that the two different copies for each effector may have been acquired from different origins (Supplementary Figure S4.2 B to D). The XopE paralogs (XopE2 and XopE3) in B07-007 were determined by phylogenetic grouping (Supplementary Figure S4.2 E). XopE2 in B07-007 has two isoforms. One (XJ27_04030) is located in the chromosome and the other (XJ27_22445) is plasmid-borne. XJ27_16520 clustered in the XopE3 group.

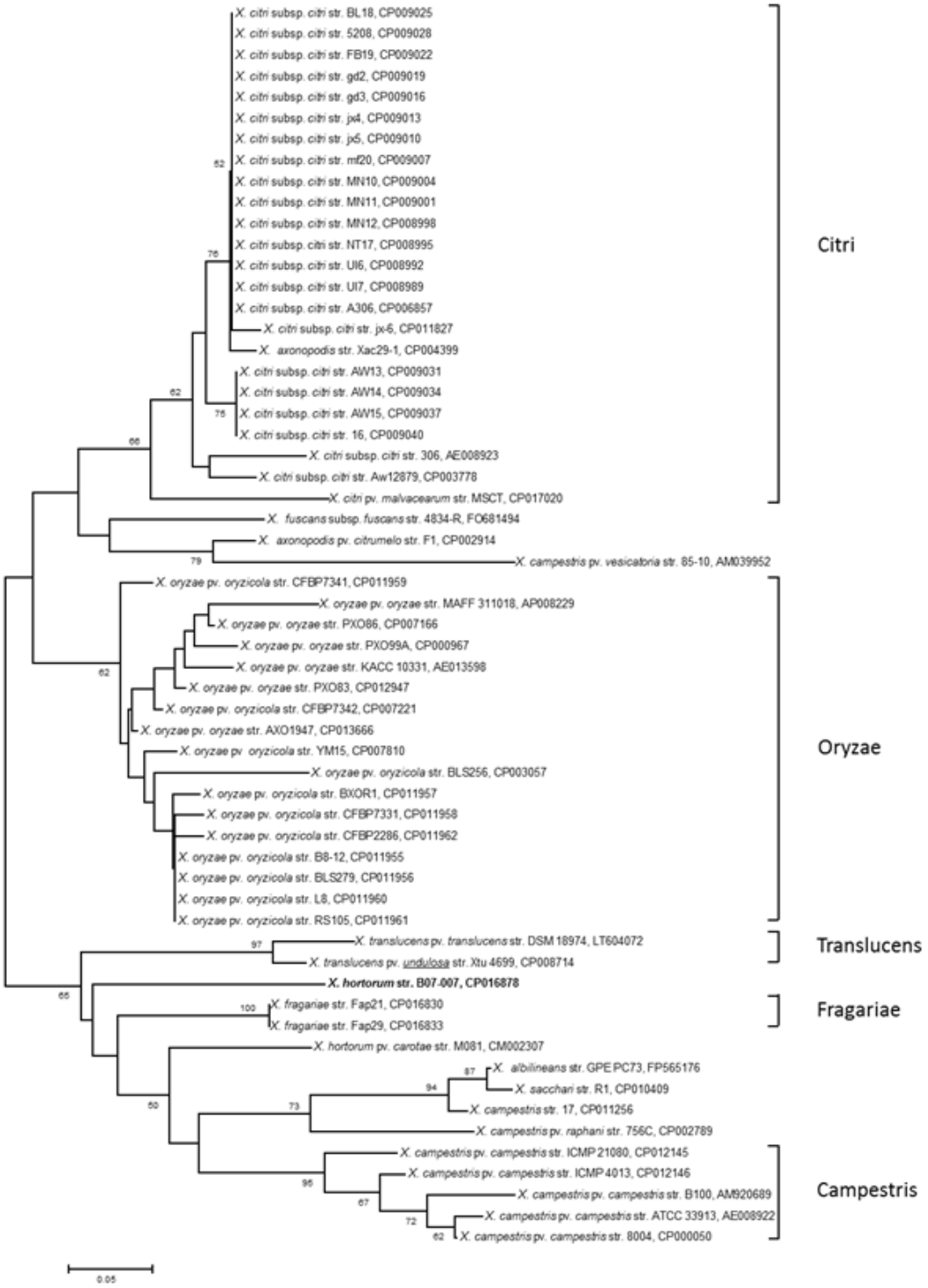

Figure 4.9. Dendrogram of type III effectors in *Xanthomonas* species. A Neighbor-Joining tree based on type III effector data in Figure 4.8 was constructed with MEGA6. Bootstrap values (r=1000) higher than 50% are indicated.

The two *X. hortorum* strains, B07-007 and Xhc M081, did not share similar repertoires of the type III effectors. M081 had 12 different type III effectors based on the effector search standard we used. We did not find the effector AvrXccA1 as reported for M081 (Kimbrel et al. 2011) and the gene XHC_4368 product showed too low similarity with the reference XopAE amino acid sequences. Our results suggest that it is more likely a XopL, a leucine rich protein. We found a XopAP (XHC_2406) in M081 which was not reported previously. Among these 12 effectors, M081 shares eight in common with B07-007. Effectors XopAG, XopAP, XopF1 and XopT present in M081 are absent in B07-007. Although whole genome phylogeny clustered the two strains together (Figure 4.4) and their T3SS PAIs are highly homologous (Figure 4.7), their type III effector genes may have been acquired quite differently or both strains may have respectively followed different evolutionary itineraries to adapt to their respective host specificity. The fact that both *X. hortorum* strains carry different sets of type III effectors infers their difference in host plant preference.

We conducted a predicted plant-induced promoter (PIP) motif search for pathogenicity-related genes over the B07-007 genome. PIP-box is often present upstream of HrpX-regulated genes (Furutani et al. 2006). The 'TTCGB –N15-TTCGB' PIP-box combined with putative –10 promoter region (YRNNNT) was used for the search. Thirty-eight PIP-boxes plus -10 boxes were identified in total, of which 21 were within 800 bp upstream of a CDS (Table 4.2). Of these 21 CDSs, three were in the T3SS PAI region. The first PIP-box, located before *hrpB1*, is thought to be responsible for the control of the expression from *hrpB1* to *hrcC*. The second PIP-box, upstream of *hrcU*, may regulate the genes from *hrcU* to *hpaB*. The third PIP-box present before *hrcQ* may play a role of enhancing the downstream expression (Figure 4.7). We did not find any promoter region for the translocon gene *hrpF* as described in some other studies (Kimbrel et al. 2011; Kim et al. 2003). We then enlarged our search scope by using 'TTCGB –N8-21-TTCGB-N30-32-YRNNNT' for new screening. Seven PIP-box candidates were found upstream of known type III effector, but none were before *hrpF*. Those effector genes with upstream PIP-boxes were *xopE2*, *xopAM*, *xopQ*, one of the two *xopL* alleles, *xopR*, *xopA* and *xopK* and were scattered all over the genome. We found nine other candidates (XJ27_05305, XJ27_05435,

XJ27_05455, XJ27_11675, XJ27_17070, XJ27_17385, XJ27_18275, XJ27_18415 and XJ27_20805) harboring a PIP-box potentially involved in pathogenesis. The amino acid sequence of XJ27_05305 showed a high homology to XHC_2558 of Xhc M081, a putative T3 effector, but its exact role is not known. Product of XJ27_05435 is a Serine/cysteine peptidase protein likely secreted. Cysteine peptidases are often key proteolytic virulence factors (Hotson et al. 2003). XJ27_05455 codes for a polygalacturonase which is capable of hydrolyzing polygalacturonan, a carbohydrate component of pectin in plant cell walls. The virulence factor Peh, a polygalacturonase, is an effector presumably secreted by T2SS (Zhao et al. 2005). Thus, XJ27_05455 may be a T2 effector and potentially regulated by HrpX for T3SS. XJ27_17385 belongs to glycosyl hydrolase family 3, members of which were reported to be virulence factors (Herron et al. 2000). XJ27_18275 encodes a lytic transglycosylase. This enzyme may be implicated in the T3SS and T4SS (Oh et al. 2007). XJ27_18415, coded for a lipase, may be secreted by T2SS (Salmond 1994). XJ27_20805 is also considered as a possible pathogenicity-related candidate. Its product - an aminopeptidase, may relate to virulence because this kind of enzyme(s) was found in an Xac secretome (Ferreira et al. 2016).

4.5 Conclusion

By comparing the whole genome sequence of B07-007 with other *Xanthomonas* species, we were able to gain insights about xanthomonads. Phylogenetic analysis showed that B07-007 clusters with four other lettuce strains to *X. hortorum* while distinct divergence appears between these lettuce strains and other pathovars. The presence of three key secretion systems (II, III and IV) and a specific set of type III effectors support its pathogenicity and its host specificity. Although the T3SS PAI is quite conserved between B07-007 and *Xhc* M081, their respective constitution of type III effectors is quite different. The type III effector repertoire of B07-007 seems to be unique among the xanthomonads. B07-007 genome also contains many IS mobile elements which caused rearrangements in the T3SS PAI and other secretion systems. Activities of mobile elements over the genome and environmental selection from various mutations may have shaped the strain for some of its specific characteristics. IS mobile element intervention in

secretion systems may have fine-tuned the pathogenic capacity and the host specificity of the strain.

4.6 Acknowledgement

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Table S4.1. Transposases found in B07-007 genome.

Identifier #	Association	Copy or derivative number
XJ27_00245	IS3	6
XJ27_02530	IS3	
XJ27_03835	IS3	
XJ27_04985	IS3	
XJ27_09370	IS3	
XJ27_18150	IS3	
XJ27_00360	IS4	8
XJ27_01775	IS4	
XJ27_05445	IS4	
XJ27_08705	IS4	
XJ27_09350	IS4	
XJ27_09355	IS4	
XJ27_18465	IS4	
XJ27_21730	IS4	
XJ27_00260	IS5	15
XJ27_00355	IS5	
XJ27_01145	IS5	
XJ27_04005	IS5	
XJ27_04415	IS5	
XJ27_04420	IS5	
XJ27_04995	IS5	
XJ27_09345	IS5	
XJ27_11340	IS5	
XJ27_12795	IS5	
XJ27_15535	IS5	
XJ27_16020	IS5	
XJ27_18475	IS5	

XJ27_18665	IS5	
XJ27_21735	IS5	
XJ27_00020	IS110	1
XJ27_18885	IS200	1
XJ27_00855	IS1595	12
XJ27_03710	IS1595	
XJ27_04405	IS1595	
XJ27_05005	IS1595	
XJ27_06505	IS1595	
XJ27_08640	IS1595	
XJ27_12610	IS1595	
XJ27_15395	IS1595	
XJ27_17260	IS1595	
XJ27_17480	IS1595	
XJ27_18480	IS1595	
XJ27_20990	IS1595	
XJ27_16355	ISxac1	3
XJ27_21710	ISxac1	
XJ27_21740	ISxac1	
XJ27_04430	ISxac3	7
XJ27_04530	ISxac3	
XJ27_06495	ISxac3	
XJ27_07325	ISxac3	
XJ27_09795	ISxac3	
XJ27_17500	ISxac3	
XJ27_18660	ISxac3	
XJ27_02560	ISxcc1	7
XJ27_06335	ISxcc1	

XJ27_06860	ISxcc1
XJ27_16030	ISxcc1
XJ27_16410	ISxcc1
XJ27_17455	ISxcc1
XJ27_20885	ISxcc1
XJ27_00115	not determined
XJ27_00120	not determined
XJ27_00250	not determined
XJ27_01240	not determined
XJ27_01245	not determined
XJ27_01760	not determined
XJ27_01765	not determined
XJ27_01925	not determined
XJ27_01930	not determined
XJ27_02525	not determined
XJ27_02565	not determined
XJ27_02725	not determined
XJ27_03405	not determined
XJ27_03410	not determined
XJ27_03840	not determined
XJ27_03855	not determined
XJ27_04425	not determined
XJ27_04535	not determined
XJ27_06220	not determined
XJ27_06225	not determined
XJ27_06340	not determined
XJ27_06350	not determined
XJ27_06355	not determined

XJ27_06360	not determined
XJ27_06490	not determined
XJ27_06855	not determined
XJ27_07320	not determined
XJ27_08010	not determined
XJ27_08015	not determined
XJ27_09295	not determined
XJ27_09300	not determined
XJ27_09335	not determined
XJ27_09365	not determined
XJ27_09440	not determined
XJ27_09790	not determined
XJ27_10160	not determined
XJ27_10165	not determined
XJ27_11205	not determined
XJ27_12300	not determined
XJ27_12305	not determined
XJ27_12600	not determined
XJ27_12605	not determined
XJ27_13150	not determined
XJ27_13155	not determined
XJ27_13375	not determined
XJ27_13380	not determined
XJ27_14040	not determined
XJ27_14045	not determined
XJ27_15225	not determined
XJ27_15230	not determined
XJ27_16035	not determined

XJ27_16405	not determined
XJ27_17460	not determined
XJ27_17505	not determined
XJ27_18065	not determined
XJ27_18070	not determined
XJ27_18155	not determined
XJ27_18530	not determined
XJ27_18640	not determined
XJ27_18645	not determined
XJ27_18655	not determined
XJ27_18680	not determined
XJ27_18685	not determined
XJ27_19815	not determined
XJ27_19820	not determined
XJ27_20890	not determined
XJ27_21720	not determined

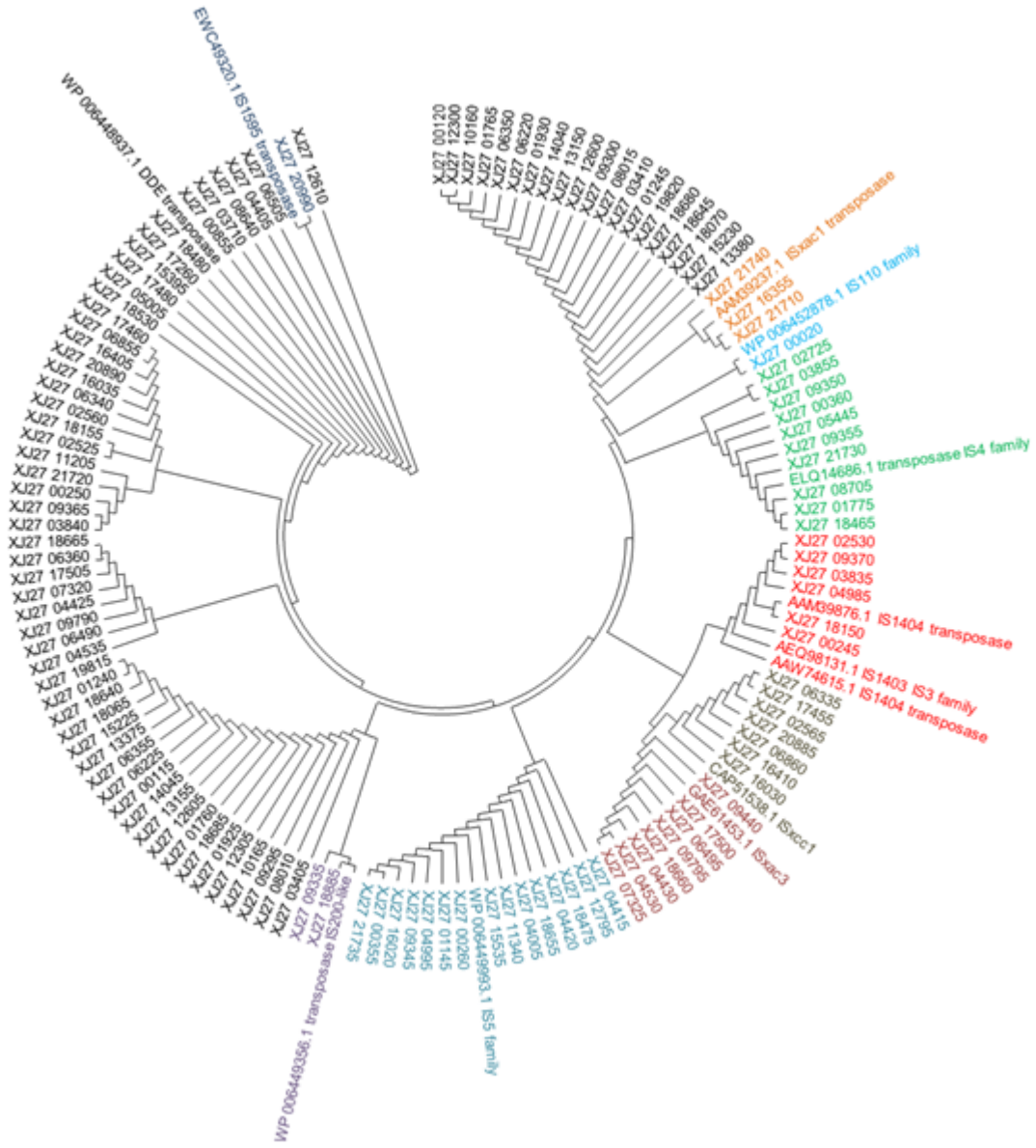

Figure S4.1. The evolutionary history of the different transposases whose genes were present in B07-007 genome was inferred by the Maximum Likelihood method. Reference transposases retrieved from GenBank were included. Identifier codes by locus tags were used for the B07-007 transposases, and GenBank accession numbers and IS names or families for the reference transposases are indicated. The analysis was conducted in MEGA6 using default parameters.

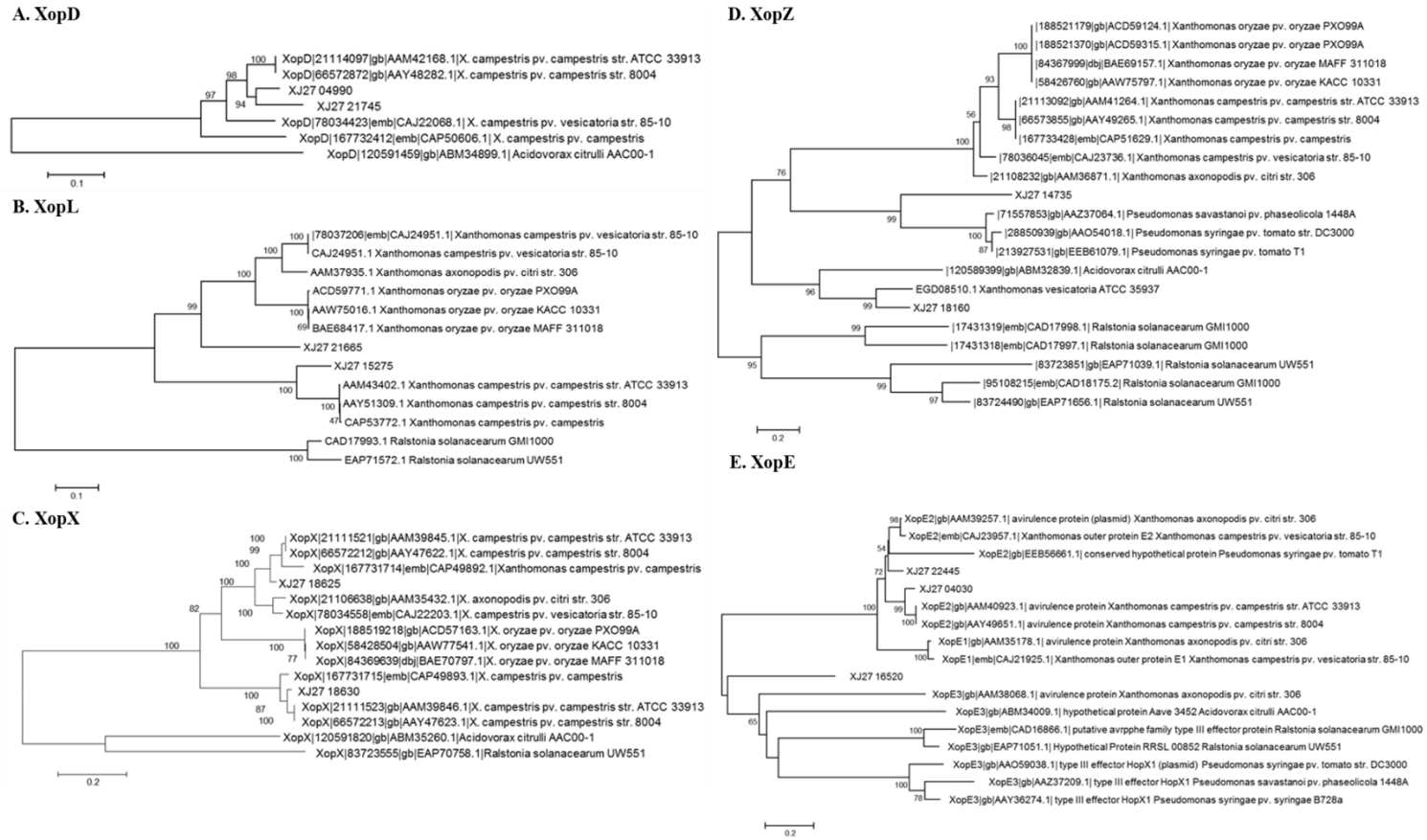

Figure S4.2. Phylogenetic relation of isoform effectors for XopD, XopL, XopX, XopZ and XopE in B07-007 strain. Amino acid sequences for each reference type III effector were from the updated effector list (http://www.xanthomonas.org/t3e.html) by White et al. (2009). Neighbor-joining method was used and MEGA6 was run for the analyses. Default values were applied. (A) was drawn for XJ27_04990 and XJ27_21745 (XopD); (B) for XJ27_15275 and XJ27_21665 (XopL); (C) for XJ27_18625 and XJ27_18630 (XopX); (D) for XJ27_14735 and XJ27_18160 (XopZ); and (E) for XJ27_04030, XJ27_16520 and XJ27_22445 (XopE).

ANNEXE 2

L'article suivant traite de la reconsidération de l'espèce *Xanthomonas hortorum* pv. *vitians* (Vauterin *et al.,* 1995) comme nom valide dans la nomenclature de cet agent pathogène de la laitue. Le groupe de Vauterin (1995) a proposé le nom de *Xanthomonas hortorum* pv. *vitians* suite à des tests moléculaires fait avec plusieurs isolats du genre *Xanthomonas* montrant un séparation des isolats causant la maladie de la tache bactérienne de la laitue des autres isolats faisant parti de l'espèce *Xanthomonas campestris*. Cet ouvrage avait donc pour but de valider la proposition du nom *Xanthomonas hortorum* pv. *vitians* en déterminant la gamme d'hôtes de cet agent pathogène de la laitue. Ce manuscrit a été ajouté en annexe puisque l'étude qui en découle a permis de soutenir les résultats obtenus lors du typage par séquençage multilocus effectué dans ce projet de maîtrise. De plus, l'inoculation de plusieurs isolats de *X. hortorum* pv. *vitians* sur plusieurs espèces végétales, incluant le cultivar de laitue Paris Island Cos, a permit de valider les résulats obtenus lors des essais de pouvoir pathogène en serres au cours de ce projet de maîtrise. Ma contribution à cet article est la suivante : J'ai participé à la préparation des solutions bactériennes à inoculer, j'ai aider à l'inoculation des différents plants lors des essais en serres, j'ai contribué à l'évaluation des symptômes sur ces plants et j'ai également collaboré à la révision du manuscrit. Ce manuscrit a été soumis à la revue Phytopathology. Pour ce mémoire, des modifications ont été apportées au manuscrit et n'engagent que la responsabilité de l'étudiant.

Reconsideration of the pathovar *Xanthomonas hortorum* pv. *vitians* (Vauterin et al. 1995) for lettuce pathogens and combination of *X. gardneri* species into the new pathovar *X. hortorum* pv. *Gardneri*

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GenBank/EMBL/DDBJ accession numbers for the genome sequences of *Xanthomonas hortorum* strains NCPPB 2248, VT106, B14-346 , B14-357 , B14-531 , B17-001 , CFBP 2533 , CFBP 4925 , CFBP 410 and CFBP 7705 are SRZ189793-802; of *Xanthomonas axonopodis* NCPPB 976 is SRZ189803; of Xanthomonas gardneri NCPPB 881 is SRZ189804; of *Xanthomonas cynarae* NCPPB 4356 is SRZ189805.

5.1 Abstract

Xanthomonas hortorum pv. *vitians* has been considered an invalid pathovar for a group of lettuce pathogens. This issue has remained unsolved in the taxonomy of this plant pathogen for more than 20 years. We tested here the host range for seven lettuce strains, four *X. hortorum* pathotype strains, the *X. gardneri* and *X. cynarae* type strains and the *X. axonopodis* pv. *vitians* pathotype strain. Their genomes were sequenced and genome-based phylogenetic analysis and digital DNA-DNA hybridization (dDDH) was performed. The lettuce strains were the only pathogens infecting lettuces. These lettuce strains closely clustered together forming a phylogenetically distinct group with inferred dDDH values above 96% between the strains by GGDC method. *X. gardneri* NCPPB 881T (=ATCC 19865T) was phylogenetically grouped in *X. hortorum* and close to the lettuce strains (dDDH of 83%) and showed a specific host range to tomato, pepper, carrot, ivy and Russian dandelion. From its original wet-lab DDH data reported previously, *X. gardneri* NCPPB 881T (=ATCC 19865T) showed DDH value above the species delineation threshold of 70% with *X. hortorum* pv. *taraxaci* ATCC 19318pT. Here, we

propose restoring the pathovar name *X. hortorum* pv. *vitians* Vauterin et al. 1995 for the lettuce pathogens with NCPPB 2248 (= LMG 938) as pathotype strain, and combining *X. hortorum* and *X. gardneri* into one species, retaining the species name of *X. hortorum* Vauterin et al. 1995 and assigning a new pathovar, pv. *gardneri* with NCPPB 881 as pathotype strain.

5.2 Introduction

Lettuce *Xanthomonas* pathogens often caused severe lettuce production loss. The bacterial leaf spot disease of lettuce (*Lactuca sativa L.*) can be an important factor affecting the yield and quality of the vegetable worldwide. Many plant pathologists have been studying this group of pathogens, but these small bugs are still in undetermined status with regards to their subspecific classification. Species *Xanthomonas hortorum* was proposed along with 19 other species reclassification or creation by Vauterin et al. (1995), based on a comprehensive DNA-DNA hybridization study. Under the species *X. hortorum*, five pathovars were ever proposed: pv. *hederae*, pv. *carotae*, pv. *pelargonii*, pv. *taraxaci* and [pv. *vitians*]. Two strains pathogenic to lettuces, LMG 937 and LMG 938, were distinctly separated into genomic Type A and Type B, respectively. This Type A strain was assigned to *X. axonopodis* pv. *vitians* and the Type B strain, to *X. hortorum* [pv. *vitians*]. In 1996, the pathovar assignations were reviewed and the pathovar *X. axonopodis* pv. *vitians* was retained with doubt while *X. hortorum* [pv. *vitians*] was not accepted for the reason of not conforming to the standard for definition of pathovar (http://www.isppweb.org/about_tppb_naming.asp) (Young et al. 1996). Strains which originally belonged to *X. campestris* pv. *vitians*, except for LMG 937, have since been referred to *X. hortorum* (Bull et al. 2010).

Xanthomonas hortorum forms a small group of pathogens which currently includes only the above four pathovars and a group of lettuce pathogens. These pathogens are conducive to diseases on carrots (*Daucus carota ¬L.*), some ornamental plants like geraniums and ivy plants, Russian dandelion (*Taraxacum kok-saghyz* L. E. Rodin) and lettuce. The lettuce pathogens are the only group which pathotype has not been determined. As different researchers still use *X.*

campestris pv. *vitians* for the taxonomic name when reporting the pathogens of the lettuce bacterial leaf spot disease, it results in confusion of their taxonomic position.

In the other hand, some studies revealed that strains of *X. gardneri* and *X. cynarae* were phylogenetically close to *X. hortorum* (Young et al. 2008; Parkinson et al. 2007). The study of Hamza et al. (2012) further indicated that *X. gardneri* and *X. cynarae* were hardly distinguishable based on MLSA with four housekeeping genes. These studies suggested high genetic relatedness of the three species. *X. gardneri* represents a group of pathogens on cultivated tomato and pepper genetically distinct from *X. perforans*, *X. vesicatoria* and *X. euvesicatoria* (Potnis et al. 2011). *X. cynarae* was reportedly pathogen on artichoke and a new species was created accordingly (Trébaol et al. 2000). Because of the phylogenetic closeness of *X. gardneri* and *X. cynarae* to *X. hortorum*, they were included in the present study.

To determine the taxonomic attribution of the lettuce pathogens, we tested the host range of our field strains and other related strains obtained from two culture collections (CFBP and NCPPB) and sequenced their entire genomes for phylogenomic analysis and digital DNA-DNA hybridization estimation. Based on our experimental results and subsequent analysis, we propose the restoration of the pathovar name *X. hortorum* pv. *vitians* Vauterin et al. 1995 to distinguish the pathogens causing lettuce bacterial leaf spot disease and the combination *of X. gardneri* into *X. hortorum* pv. *gardneri*.

5.3 Materials and methods

5.3.1 Bacterial strains

Strains B07-007, VT106, B14-346, B14-357 and B14-531 were isolated from diseased lettuce leaves in the Monteregie area, QC, Canada. Strain B17-001 was received from the Laboratoire d'expertise et de diagnostic en phytoprotection, Quebec, QC, Canada. Reference strains *X. hortorum* pv. *pelargonii* CFBP 2533pT, *X. hortorum* pv. *hederae* CFBP 4925T,pT, *X. hortorum* pv. *taraxaci* CFBP 410pT, *X. hortorum* pv. *carotae* CFBP 7705 were obtained from CIRM-

CFBP (International Center for Microbial Resources - French Collection for Plant-associated Bacteria, France), and *X. hortorum* [pv. *vitians*] NCPPB 2248pT, *X. axonopodis* pv. *vitians* NCPPB 976pT, *X. gardneri* NCPPB 881T, *X. cynarae* NCPPB 4356T, from National Collection of Plant Pathogenic Bacteria, UK. The bacterial strains were grown on YDC agar (g/L: Yeast Extract, 5; Glucose, 10; CaCO3, 30; Agar, 15) plates at 28 °C for 48 hours. Fresh bacterial suspension of 108 CFU/ml was prepared in sterile Millipore Super-Q filtered water for bioassays.

5.3.2 Pathogenicity test

Seeds of Romaine lettuce "Paris Island" (*Lactuca sativa L*. var. *longifolia*), Mega Fantastic tomato (*Solanum lycopersicum L*.) and Giant Marconi pepper (*Capsicum annuum L*.) were purchased from Norseco Inc. (Laval, QC, Canada). Carrot seeds (*Daucus carota* ¬*L*. subsp. *carota*, PI 390887) were provided by USDA-ARS-GRIN Plant Collection. Geranium (*Pelargonium peltatum L*.) and ivy (*Hedera helix L*.) plants were obtained from Botanix Centre de Jardinage St-Jean, St-Jean-sur-Richelieu, QC, Canada. Russian dandelion (*Taraxacum kok-saghyz* L. E. Rodin) seeds were kindly provided by the Ontario Ministry of Agriculture, ON, Canada. Young plants at the four-leaf stage were used for the pathogenicity tests. Three inoculation methods were used depending on the kinds of plants to be tested. Inoculum spraying on the whole plant was used for lettuce, tomato, pepper, carrot and geranium at 25 psi, local infiltration with 1-ml syringe without needle (Terumo Medical Co., Somerset, NJ, USA) was performed on leaves of ivy and Russian dandelion while artichoke bracts were scarified with a sterile scalpel, inoculated by depositing 1 ml of bacterial suspension on the bracts (Trébaol et al. 2000). Inoculated lettuces, geranium and ivy plants were cultivated at the 12/12 light/dark cycles with temperature accordingly set to 24/17 °C while the inoculated tomato, pepper, carrot and Russian dandelion were maintained at the 12/12 light/dark cycles and accordingly to 28/25 °C. A water mist was applied daily for 20 sec every hour following the light cycle. The inoculated artichoke bracts were placed in sterile Petri dishes and incubated at 28 °C for four days. Symptom developments were observed daily and final evaluations were performed after 10 days for lettuce, 14 days for tomato, sweet pepper, geranium and Russian dandelion, 25 days

for carrot and 4 days for artichoke. Inoculated ivy leaves were evaluated at 4th, 7th and 11th days. Three replicates were performed for the controls and treatments.

5.3.3 Genome sequencing

Fresh bacterial cells were harvested from bacterial lawn on agar plates and suspended in sterile TE buffer (Tris-base at 10 mM, EDTA at 1 mM, pH 8.0). DNA was isolated with Wizard Genome DNA purification Kit (Promega, Madison, USA) following manufacturer's instructions. Two hundred ng of genomic DNA was sheared in microTUBE-15 AFA Beads Screw-Cap by Covaris M220 Focused Ultrasonicator (D-Mark Biosciences Inc., Toronto, ON, Canada) set at 20 °C for 23 sec with peak incident power to 30 Watts, duty factor at 20%, and 50 cycles per burst. Genomic library was prepared with TruSeq Nano DNA Library Prep (Illumina, CA, USA) following manufacturer's instructions. The DNA library was paired-end sequenced with MiSeq Reagent Kit v3 on an Illumina MiSeq system.

5.3.4 Phylogenomic analysis

Illumina raw sequences were trimmed and paired-end sequences were merged with CLC Genomics Workbench v7.0.4 (Qiagen, Redwood, CA, USA). The trimmed merged, unmerged and orphan sequences were assembled *de novo* to construct contig fragments. Contigs longer than 50 nt were retained and used in subsequent analysis. The B07-007 genome (CP016878-9) in addition to the newly assembled genome of 13 strains and 11 other *Xanthomonas* genomes retrieved from GenBank were included for phylogenetic analysis. The names and GenBank accession numbers of the retrieved genome sequences are *X. hortorum* pv. *carotae* M081 (CM002307), *X. campestris* pv. *campestris* ATCC 33913T (AE008922), *X. campestris* pv. *vesicatoria* str. 85-10 (AM039948-52), *X. vesicatoria* ATCC35937 T (AEQV00000000), *X. citri* str. 306 (AE008923-5), *X. axonopodis* pv. *citrumelo* F1 (CP002914), *X. fragariae* LMG 25863 (AJRZ00000000), *X. oryzae* pv. *oryzae* KACC 10331 (AE013598), *X. gardneri* ATCC 19865T (AEQX00000000), *X. gardneri* ICMP7383 (CP018731-4) and *X. cynarae* CFBP4188T (MDFM00000000). The sequence data files of the strains were submitted to the online pipeline

Realphy 1.12 (Bertels et al. 2014) (http://realphy.unibas.ch/fcgi/realphy) for phylogenetic analysis. Genome sequences of *X. hortorum* B07-007, *X. vesicatoria* ATCC35937 T, *X. citri* str. 306 and *X. campestris* pv. *campestris* ATCC33913T were assigned as reference genomes for the phylogenetic tree construction. A tree file from merged alignments was constructed and visualized with MEGA v7.0.14.

Digital DNA-DNA hybridization analysis. DNA contigs and genomes were analysed for digital DNA-DNA hybridization with GGDC 2.0 at http://ggdc.dsmz.de (Meier-Kolthoff et al. 2013).

5.4 Results and discussion

5.4.1 Pathogenicity

Fourteen *Xanthomonas* strains were tested on eight different plant species (Table 5.1). Each of these species is a reported host for at least one of the reference strains. Among the tested strains, only the six field strains (B07-007, VT106, B14-357, B14-346, B14-531 and B17-001) and NCPPB 2248pT caused symptoms on lettuce. These strains (called lettuce strains hereafter) induced grey-black to black-brown necrotic lesion spots with chlorotic yellowish halos on lettuce leaves, sometimes spots merged together and developed larger water-soaked lesions. However, no systemic symptom development was observed. These symptoms correspond to the Group B of Sahin et al. (2003). The lettuce strains also caused dark brown necrotic leaf spots on tomato, sweet pepper, and carrot by spray inoculation. Symptoms on tomato were much milder for all the lettuce strains compared to the known tomato pathogen *X. gardneri* NCPPB 881T. The lettuce strains caused round or irregular brown spots on the middle and at the edges of carrot leaves with yellow halos. Sometimes, the centre of the brown spot was whitened. If infection appeared at the tips of leaves, it showed blight symptoms. The severity of the symptoms on carrots varied between the different strains. Strains NCPPB 2248pT, B07-007, B14-346 and B17-001 showed higher severity while VT107 and B14-531 caused milder symptoms and B14-357 did not induce any symptoms. Lettuce strains did not infect geranium plant. On ivy plant, these strains showed symptoms somewhat atypical as compared to the

known pathogen *X. hortorum* pv. *hederae* CFBP 4925T,pT. In the first 72h after inoculation, dark brown spot began to appear on the injection site and, in some cases, the spot could cover more than 50% of the injected area, while the positive control *X. hortorum* pv. *hederae* CFBP 4925T,pT did not show any symptoms for a week. This early appearance of symptoms was similar to hypersensitive response (HR) induced by *X. hortorum* pv. *pelargonii* CFBP 2533pT which induced necrosis of the whole inoculated region within 72h. The lettuce strains developed over time brown spots over the entire injected area accompanied by obvious yellowish halos, sometimes the diseased areas were expanded outside the injected area after seven days of inoculation while CFBP 4925T,pT did not show these symptoms until the eleventh day. On Russian dandelion leaves, the lettuce strains caused black necrosis in the injected area. On occasions, the symptoms developed to the main vein and their adjacent regions and could cause whole leaf loss.

Table 5.1 Pathogenicity of *Xanthomonas* species on different host plants.

Species/pv./strain	Lettuce[a]	Tomato	Sweet pepper	Carrot	Geranium	Ivy	Russian dandelion	Artichoke
X. hortorum pv. *pelargonii* CFBP 2533^{pT}	-	+(w)	+	+	+	HR	+	-
X. hortorum pv. *hederae* CFBP4925$^{T, pT}$	-	+(w)	-	+	-	+	HR	-
X. hortorum pv. *taraxaci* CFBP410 ^{pT}	-	-	-	+	-	+	+	-
X. hortorum pv. *carotae* CFBP 7705	-	-	-	+	-	NA	HR	-
X. hortorum pv. *vitians* NCPPB 2248^{pT}, B07-007, VT106, B14-346, B14-357, B14-531, B17-001	+	+(w)	+	+	-	+(at)	+	-
X. axonopodis pv. *vitians* NCPPB 976 ^{pT}	-	+(w)	-	+	-	+	HR	-
X. gardneri NCPPB 881^{T}	-	+	+	+(w)	-	+	+	-
X. cynarae NCPPB 4356^{T}	-	+	+	+	-	+(at)	+	-

Note: a, - = no symptom; + = with symptom; (w), weak; HR = hypersensitive response; (at), atypical symptom; NA, not analysed.

X. gardneri NCPPB 881T and *X. cynarae* NCPPB 4356T are respectively classified in two different species. However, both strains shared the same host range. They were capable of infecting tomato, sweet pepper, carrot, ivy and Russian dandelion but not lettuce, geranium and artichoke. NCPPB 881T was highly aggressive to tomato and sweet pepper plants while NCPPB 4356T was capable of infecting both plants but did not cause the same degree of damage as the former. Both strains induced grey-brown necrotic spots in round to irregular forms on tomato leaves and veins. Some spots showed a pale centre with thin halo zone. Halo zones were not obvious on some spots. Some infected leaves were curled. On carrots, both strains caused brown leaf spots with clear halo zones. NCPPB 881T showed milder symptoms than NCPPB 4356T. Both strains developed dark brown spots on the injected area of ivy leaves. NCPPB 881T showed a symptom development similar to the positive control strain CFBP 4925T,pT while NCPPB 4356T acted like the lettuce strains, with atypical symptom development. On injected areas of Russian dandelion leaves, NCPPB 881T and NCPPB 4356T caused black necrosis and the symptoms developed to adjacent regions and the central vein. It is noteworthy that the NCPPB 4356T, which was reported to be an artichoke pathogen, was not able to infect artichoke bracts in our tests. We repeatedly tested this strain and others but no symptoms were observed. In fact, no strains used in this study were able to induce symptoms on bracts of commercially obtained artichoke flowers with the methods described by Trébaol et al. (2000). So, host range test did not support the separation of both NCPPB 881T and NCPPB 4356T into two distinct species.

The strain NCPPB 976pT received a taxonomic name of *X. axonopodis* pv. *vitians* (Vauterin et al. 1995). Host range tests in this study did not show its pathogenicity on lettuce, in agreement with other studies (Sahin et al. 2003; Barak and Gilbertson 2003). However, NCPPB 976pT showed weak symptoms on tomato and no infection on sweet pepper. It could also induce necrotic spot symptoms on carrot and ivy leaves, and HR on Russian dandelion.

5.4.2 Phylogenetic relationship

Contig or genome sequences of the field and reference strains were analysed with the online pipeline RealPhy program. The merged tree was generated from multiple sequence alignments reconstructed by combining sequences pre-aligned to the four individual reference genomes respectively in order to increase the quality of the inferred phylogeny (Figure 5.1). The *X. hortorum* strains were clustered together. All the lettuce strains were very closely grouped. Considering their identical host range, these lettuce strains may share the same origin. It is interesting to note that strains of NCPPB 881T and NCPPB 4356T were grouped together with the *X. hortorum* members and very close to the lettuce strains. To verify the exactness of both strains used in the study, we retrieved the genomic sequences of *X. gardneri* ATCC 19865T (synonym of NCPPB 881T) and *X. cynarae* CFBP 4188T (synonym of NCPPB 4356T) from GenBank and included them in the phylogeny study. As illustrated in Figure 5.1, there is no difference between NCPPB 881T and ATCC 19865T, and likewise, between NCPPB 4356T and CFBP 4188T. A branch formed by *X. gardneri* NCPPB 881T and *X. cynarae* NCPPB 4356T is close to the lettuce strains within a genetic distance of ~1%.

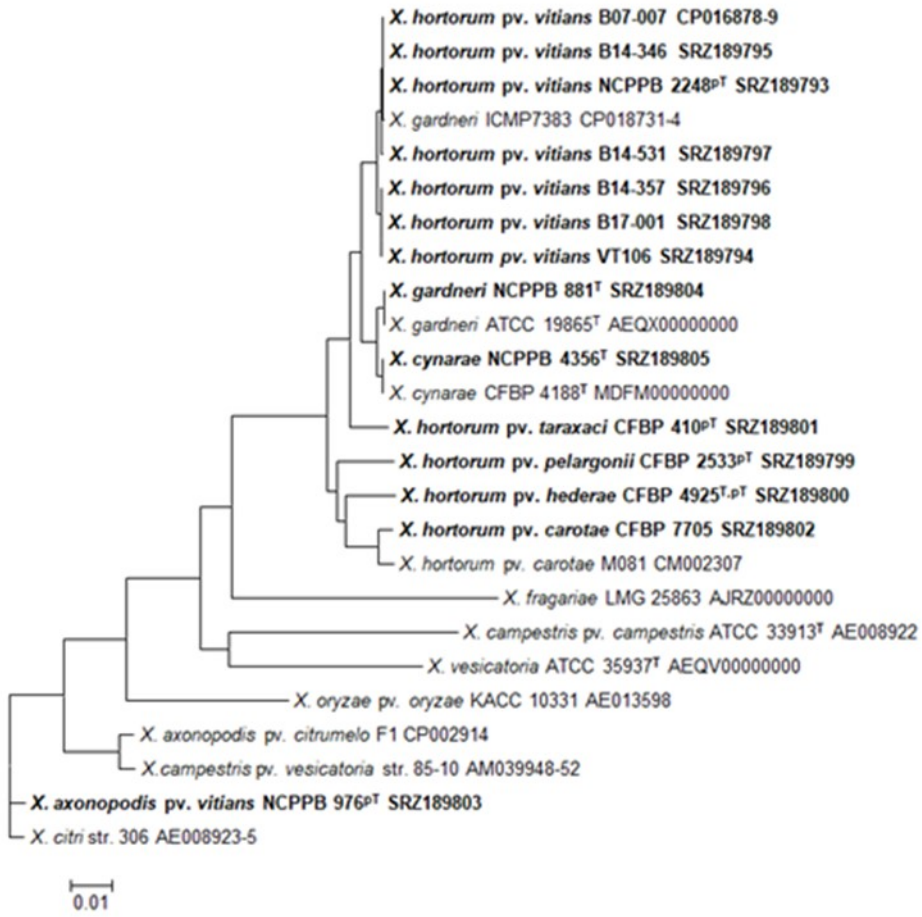

Figure 5.1. Phylogeny and relationship of *Xanthomonas hortorum* strains. The unrooted phylogenetic tree was reconstructed with combined multiple sequence alignments of the whole genomes to the assigned reference genomes by REALPHY v1.09. The genomes sequenced in this work are highlighted in bold. The bar length represents one substitution over 100 nucleotides.

5.4.3 Digital DNA-DNA hybridization

The dDDH technique was used as it was shown to mimic the wet-lab DDH values (Meier-Kolthoff et al. 2013). We applied the recommended formula 2 of the GGDC 2.0 program to infer the wet-lab DDH results for the concerned strains. We conducted genome comparisons between the lettuce strains and obtained dDDH estimates for NCPPB 2248pT as reference to

B07-007 for 99.6%, to B14-346 for 99.6%, to B14-357 for 96.4%, to B14-531 for 98.1%, to B17-001 for 96.8% and to VT106 for 96.8% respectively. These values were all much higher than the 70% threshold for species delineation. To further subclassify genetically highly close strains, Meier-Kolthoff et al. (2013) suggested a threshold of 79% dDDH for delineating subspecies. Again, the dDDH values among lettuce strains were in fact much higher than 79% which further affirmed their affinity at the subspecific level. We also ran the comparison between the *X. gardneri* and *X. cynarae* type strains. The dDDH value between NCPPB 881T and NCPPB 4356T was 93.1%, indicating their high similarity. When both NCPPB 881T and NCPPB 4356T compared to NCPPB 2248pT, they showed 83.4% and 84.7% respectively, suggesting that NCPPB 881T and NCPPB 4356T and NCPPB 2248pT were close enough even at the subspecific level. Genome-based phylogeny strongly supports this inference (Figure 5.1). However, there is a clear difference in their host spectra. NCPPB 881T and NCPPB 4356T did not infect lettuce although they shared the same hosts with the lettuce strains for the rest of the tested plants. On the other hand, comparison between two *X. gardneri* strains NCPPB 881T and ICMP 7383 showed only 83.4%, in contrast to 98.9% between NCPPB 2248pT and ICMP 7383. This inference reflected directly to the phylogenetic tree (Figure 5.1) that both NCPPB 2248pT and ICMP 7383 were undistinguishable. The strain ICMP 7383 was originally isolated from tomato (Richard et al. 2017), it is highly similar to the lettuce strains and somewhat distant from NCPPB 881T. According to our results, it would be genetically classified in the lettuce group. A host range test is needed to know if ICMP 7383 is able to infect lettuces.

For the strain NCPPB 4356T, although our genome sequence comparisons suggested that the strain was very close to NCPPB 881T, and to a lesser extent to NCPPB 2248pT, we carefully verified the information described in the original paper by Trébaol et al. (2000). Reported ratio of DNA-DNA hybridization of this strain with *X. hortorum* pv. *pelargonii* CFBP 2533pT was low (49%). By comparing the previous BIOLOG GN test results, there were 26 differences in the utilisation of 95 carbon compounds between the described *X. cynarae* strains (Trébaol et al. 2000) and *X. hortorum strains* (Vauterin et al. 1995) suggesting metabolic difference between both species. Finally, the strain NCPPB 4356T we used was not pathogenic to artichoke. All this information suggests that the strain NCPPB 4356T deposited in NCPPB and in CIRM-

CFBP as CFBP 4188T may not be the same strain described by Trébaol et al. (2000). Because of this conflicting information, we terminated its further study.

From the above analyses, both *X. gardneri* and *X. hortorum* are sufficiently related and should be combined into one species as they showed a very high level of similarity. Jones et al. (2004) proposed the new species *X. gardneri* based on DDH results, phenotype, and metabolic profiles. The authors included the *X. hortorum* pv. *taraxaci* pathotype strain ATCC 19318pT in their DDH experiment and obtained DDH values of 71% and 75% between ATCC 19865T and ATCC 19318pT (Table 2 in Jones et al. 2004), above the 70% threshold as recommended for defining a genospecies by Wayne et al. (1987). We simulated dDDH between ATCC 19318pT and ATCC 19865T and obtained 76%, very close to the real web-lab data. So, information obtained in this and previous studies warrants the ATCC 19865 sharing the same species with *X. hortorum*.

5.5 Conclusion

In conclusion, our results indicate the lettuce strains are genetically close one to another and phylogenetically distinct from the current valid *X. hortorum* pathovars, grouping in a specific pathological group. These characteristics indicate the lettuce strains are a distinct pathovar. In 1995, Vauterin et al. (1995) proposed the pathovar with the name pv. *vitians*. Here, the data presented support the proposal of this pathovar. We also propose the combination of *X. gardneri* into *X. hortorum* species. NCPPB 881 shows a specific host range and forms a short yet distinct phylogenetic branch within *X. hortorum*. Requirements for a separate pathovar are met. We thus propose the creation of a new pathovar for this specific group of tomato-pepper strains.

Pathovar description of *Xanthomonas hortorum* pv. *gardneri*: The original species of *X. gardneri* is combined with *X. hortorum* and the species name *Xanthomonas hortorum* Vauterin et al. 1995 is retained. The description of the pathovar is the same as that of the species. The strains are usually isolated from necrotic lesion spots of tomato or pepper leaves. They can also

infect carrot, ivy and Russian dandelion under warm and highly humid conditions. The pathotype strain is NCPPB 881 (= ATCC 19865).

Metabolic distinction is as described by Jones et al. (2004).

Pathovar description of *Xanthomonas hortorum* pv. *vitians*, Vauterin et al. 1995: The description of the pathovar is the same as that of the species. The strains are usually isolated from necrotic lesion spots of lettuce leaves. They can also infect tomato, sweet pepper, carrot, ivy and Russian dandelion under warm and highly humid conditions. The pathotype strain is NCPPB 2248 (= LMG 938).

Metabolic distinction is as described by Vauterin et al. (1995).

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