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MiniReview

The status of molecular biological research on the plant pathogenic genus *Clavibacter*

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Abstract

The species of the high-GC Gram-positive genus *Clavibacter* can be divided into three groups: (1) The subspecies within *C. michiganensis*; (2) the *Clavibacter* species causing gumming diseases; and (3) the fastidious, xylem-limited subspecies within *C. xyli*. All members of the taxon are plant pathogens. Basic studies have demonstrated the presence of native plasmids in various species and allowed construction of cloning vectors for a few of these species. Infection of plants by *C. michiganensis* and *C. xyli* subspecies can be difficult to detect, since they often give symptomless infections. Therefore, serological and DNA-based detection techniques have been developed to aid in disease control. Efforts to determine what factors may be important in pathogenicity have focused on extracellular polysaccharides in several of the species, but a clear role for these molecules in virulence has not been found. The development of transformation methods for several of the *C. michiganensis* subspecies has allowed elucidation of other pathogenicity factors, e.g., plasmid-encoded extracellular cellulase. For *C. toxicus*, the involvement of a phage in annual ryegrass toxicity has been determined. Of special interest is *C. xyli* ssp. *cynodontis*, which has been genetically engineered for protecting plants from insect damage. Further progress in elucidating the molecular biology of these organisms will require the development of more sophisticated molecular genetic tools.

Keywords: *Clavibacter*; *Corynebacterium*; *Rathayibacter*; Bacterial plant disease; Fastidious bacteria; Molecular plant pathology

1. Introduction

Plant pathogenic bacteria cause serious losses in many different crops. Genetic studies of such phytopathogens, particularly the examination of the genes and gene products involved in disease induction, have focused primarily on the Gram-negative bacteria. This is partially due to the ease of carrying out molecular genetic manipulations with genera such as *Pseudomonas* and *Erwinia* in comparison with most Gram-positive phytopathogens. However, recent advances are encouraging investigators to examine the biochemistry and molecular mechanisms of pathogenesis for Gram-positive plant pathogens.

Most of the plant pathogenic Gram-positive bacteria have a GC-rich genome. Those with coryneform morphology, in which dividing cells are connected at an angle to form a V shape, were originally placed in the genus *Corynebacterium*. Taxonomic refinements have allowed placement of many of these bacteria into other genera: e.g., *Co-*

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Table 1
Clavibacter species and associated diseases

Bacterial name	Host plant	Common name[a]	Symptoms	Refs.[b]
Michiganensis subgroup				
Clavibacter michiganensis ssp. *insidiosus*	Alfalfa	Bacterial wilt	Wilt and stunt	[30]
Clavibacter michiganensis ssp. *michiganensis*	Tomato, pepper	Bacterial canker	Wilt and fruit spot	[30]
Clavibacter michiganensis ssp. *nebraskensis*	Corn	Goss's wilt	Wilt and leaf blight	[30]
Clavibacter michiganensis ssp. *sepedonicus*	Potato	Ring rot	Wilt and tuber rot	[30]
Clavibacter michiganensis ssp. *tessellarius*	Wheat	Leaf spot		
Gumming pathogens of grasses				
Clavibacter iranicus	Wheat		Yellow slime of leaves and inflorescences, leaf spots	[30]
Clavibacter rathayi	Grasses		Yellow slime of leaves and inflorescences	[30]
Clavibacter toxicus	Annual ryegrass (*Lolium rigidum*)	Yellow slime disease	Yellow slime of inflorescences, bacterially infected nematode galls	[4]
Clavibacter tritici	Cereals and grasses	Tundu disease	Yellow slime of leaves and inflorescences	[30]
Xylem limited vascular pathogens				
Clavibacter xyli ssp. *cynodontis*	Bermuda grass		Stunt	[1]
Clavibacter xyli ssp. *xyli*	Sugar cane	Ratoon stunt	Stunt	[1]

[a]Where one exists.
[b]Only one is given due to space limitations.

rynebacterium fascians is now *Rhodococcus fascians*, *Corynebacterium ilicis* is now *Arthrobacter ilicis*, and *Corynebacterium flaccumfaciens* subspecies are all *Curtobacterium* species [1,2]. Currently, phytopathogenic coryneform bacteria whose cell walls contain 2,4-diaminobutyric acid are placed in the genus *Clavibacter*. In this review, we restrict ourselves to examining methods for pathogen detection, molecular methods for genetic manipulations and molecular aspects of pathogenicity in the genus *Clavibacter*. The commercially important species are the most studied, and this is reflected in the relative amount of information available for each.

2. Species and associated diseases

The *Clavibacter* species fall loosely into one of three groups (Table 1): the subspecies within *C. michiganensis* (*C. m.* ssp. *insidiosus*, *C. m.* ssp. *michiganensis*, *C. m.* ssp. *nebraskensis*, *C. m.* ssp. *sepedonicus* and *C. m.* ssp. *tessellarius*); the species causing gumming diseases of grasses (*C. iranicus*, *C. rathayi*, *C. tritici* and *C. toxicus*); and the xylem-limited subspecies within *C. xyli* (*C. x.* ssp. *xyli* and *C. x.* ssp. *cynodontis*).

The *C. michiganensis* group contains subspecies that cause a variety of plant diseases (Table 1), generally involving wilt. While the taxonomy of these subspecies has been debated [3,4], the classification proposed by Davis et al. [1] is the one used in this review. These bacteria have no active insect vectors, are seed transmitted, and some are controlled through the production of certified seed. Development of methods for detection of these pathogens in seed stocks is considered important in improving disease control.

The diseases caused by the gumming bacteria, characterized by yellow bacterial slime on developing seedheads of grasses, and transmitted by a nematode vector, are superficially similar. However, the bacteria causing these diseases belong to separate species

[4]. Separate genus status for this group, excluding *C. toxicus*, under the name *Rathayibacter* has been proposed [5]. *C. toxicus* is of particular interest in Australia, since it produces a toxin in infected plants that is harmful to grazing animals [6].

The two subspecies of the *C. xyli* group are C. *x.* ssp. *xyli*, which causes the economically serious ratoon stunting disease in sugar cane, and *C. x.* ssp. *cynodontis*, which can cause bermuda grass stunt. These are fastidious organisms that seem to be near perfect pathogens, causing few visible symptoms in their host plants in spite of high bacterial titres.

3. Basic molecular biology

Large native plasmids have been reported in *C. m.* ssp. *insidiosus*, *C. m.* ssp. *michiganensis*, *C. m.* ssp. *sepedonicus*, *C. m.* ssp. *nebraskensis*, *C. tritici* and *C. rathayi* [7], and *C. x.* ssp. *cynodontis* [8]. Only those from *C. m.* ssp. *michiganensis*, *C. m.* ssp. *sepedonicus*, and *C. x.* ssp. *cynodontis* have been studied in any detail. The two native plasmids from *C. m.* ssp. *michiganensis* each carry pathogenicity factors (discussed in the following section) and can be cured to give plasmid-free strains. In contrast, *C. m.* ssp. *sepedonicus* almost always carries the native plasmid either in the autonomously replicating form or integrated into the chromosome [9]. This plasmid also contains a sequence that is repeated many times on the chromosome and this may be the site of integration for the plasmid [9]. Similarly, the *C. x.* ssp. *cynodontis* plasmid pCXC100 contains a partial copy of an apparently inactive insertion sequence, IS*1237*, of which there are many copies on the genome [10]; however, there is no evidence that this insertion sequence plays a role in integration of pCXC100.

Transformation methods based on electroporation have been developed for some of these species. Transformation rates are low, ranging between 3×10^3 and 2×10^5 cfu/μg DNA, but are sufficient to allow molecular genetic studies and gene manipulations. Origins of replication from the two native *C. m.* ssp. *michiganensis* plasmids have been isolated and used to construct cloning vectors that transform *C. m.* ssp. *michiganensis* [11] and *C. m.* ssp. *sepedonicus* [12]. One of these vectors, pDM100, has also been reported to transform *C. iranicus*, *C. m.* ssp. *nebraskensis* and *C. m.* ssp. *insidiosus*, but at low rates [11]. *C. x.* ssp. *cynodontis* has been transformed using a cloning vector based on the origin of replication from the native plasmid [8], as well as plasmids of the IncP group from Gram-negative bacteria [13].

Promoter studies have shown that strong promoters from *C. x.* ssp. *cynodontis* appear to be very different from previously determined bacterial promoter consensus sequences [14]. However, a promoter from the pathogenicity gene *pat-1* of *C. m.* ssp. *michiganensis* shows homologies to the consensus sequences for the −10 and −35 regions of *E. coli* σ^{70} promoters [15]. Promoters from many more genes will need to be analyzed in order to determine what sequences are optimal for gene transcription in *Clavibacter*.

4. Detection

Some of the species in this genus cause particular problems because the level of symptom expression in host plants can be minimal. For instance, *C. m.* ssp. *sepedonicus* can cause latent infections in potato, where plants show no symptoms but produce tubers that are infected; hence the importance of disease-free certified seed potato programmes for disease control. Another example is *C. x.* ssp. *xyli*, which causes stunting and reduced yields in sugar cane. Stunting can be caused by a number of biotic and abiotic factors as well, however, and is only seen during conditions of water stress. Hence, the presence of *C. x.* ssp. *xyli* must be confirmed to verify this bacterium as the cause of stunt. Detection of these and other *Clavibacter* species is therefore important for their control, and considerable data have been generated on the use of serological and DNA-based detection and identification methods.

4.1. Serological detection methods

Polyclonal antisera have been used for detection of *C. m.* ssp. *insidiosus*, *C. m.* ssp. *michiganensis*, and *C. m.* ssp. *sepedonicus* in techniques such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and immunofluorescence colony staining.

For *C. x.* ssp. *xyli*, the pathogen can be detected using tissue immunoblotting [16]. However, cross-reactivity of polyclonal antisera often compromises the specificity of identification. Monoclonal antibodies to a cell-wall antigen and extracellular polysaccharide gave much improved specificity, and were highly efficient in indexing asymptomatic potatoes for the presence of *C. m.* ssp. *sepedonicus* [17]. Polyclonal ELISA tests have also been applied to detection of *C. toxicus* in paddocks and in hay for export, without any problems of cross-reactivity [6].

4.2. DNA-based detection methods

Detection of *C. m.* ssp. *sepedonicus* by direct DNA hybridization has focused primarily on plasmid sequences. Probes based on the native plasmid of *C. m.* ssp. *sepedonicus* are fairly specific for the subspecies but some of them weakly cross hybridize with *C. m.* ssp. *insidiosus* and other bacteria (e.g. [18]). DNA probes for *C. m.* ssp. *michiganensis*, based on plasmid-borne genes involved in pathogenicity, were highly specific and in one case even differentiated between virulent and avirulent strains of the bacterium [19]. The sensitivity of using plasmid DNA hybridization probes for detection may be comparable to or less than that of serological assays, depending on bacterial concentration and sample characteristics [20]. For *C. x.* ssp. *xyli*, fragments of genomic DNA served as specific hybridization probes, but weak cross-hybridization occurred with the other subspecies, *C. x.* ssp. *cynodontis* [21].

As with immunofluorescence, in situ hybridization with oligonucleotides carrying a fluorescent label can be used to visualize individual bacterial cells, and this has been successfully applied to *C. m.* ssp. *sepedonicus* [22]. In situ hybridization has also been combined with detection by fluorescent monoclonal antibodies to probe individual *C. m.* ssp. *sepedonicus* cells. This combination of two different detection methods allowed highly accurate identification of bacterial cells even in samples from which the pathogen could not be isolated [23].

4.3. PCR-based detection methods

The superior sensitivity of PCR methods has been applied to detection of several *Clavibacter* species. PCR methods have been developed to detect *C. x.* ssp. *xyli* (S. Brumbley and M. Fegan, personal communication) and *C. m.* ssp. *michiganensis* (R. Eichenlaub, personal communication). For *C. m.* ssp. *sepedonicus*, various primer sets have been defined that specifically amplify native plasmid DNA (e.g. [24]); in one case a single primer is used to amplify the plasmid-borne repetitive element [25]. PCR primers have also been selected from an RNA intergenic spacer [26], and a competitive PCR protocol has been developed for quantitative detection of this subspecies [27]. The various PCR protocols permit specific identification of the target species. However, their practical utility for indexing plant tissue and screening environmental samples for low numbers of the bacteria requires further evaluation.

5. Virulence factors

5.1. Extracellular polysaccharides

It has been proposed that extracellular polysaccharides (EPS) induce wilt symptoms by interfering with water transport [28]. The obvious mucoid nature of many *C. michiganensis* subspecies and strains has spurred investigations of this possibility. *C. m.* ssp. *sepedonicus* was found to produce an extracellular 'glycopeptide', which is actually an EPS with a minor peptide component, that induced wilt in detached tomato leaves [29]. It was suggested that wilt was induced by cellular damage rather than interference with water transport [29], but this idea is no longer considered valid [30]. In a comparison of various strains, non-mucoid variants induced fewer symptoms and reduced tuber yields less than mucoid variants (S. De Boer, unpublished observations), suggesting that EPS might play a role in virulence. However, in eggplant (*Solanum melongena*) assays no correlation between mucoidy and virulence was found [31,32]. A phytotoxic fraction containing polysaccharides with a minor protein component, similar to that from *C. m.* ssp. *sepedonicus*, has also been described from *C. m.* ssp. *michiganensis* [33]. However, the degree of wilting induced by this phytotoxic fraction on tomato cuttings showed no correlation

with the disease resistance or susceptibility of the cultivars from which the cuttings were derived. Additionally, for different bacterial strains there was no correlation between the degree of virulence and the amount of phytotoxin produced in vitro [33]. Recent work further supports this observation since a *C. m.* ssp. *michiganensis* mutant that produced EPS to only 10% of wild-type levels still induced wilting, but a non-virulent EPS positive strain did not, although it retained the ability to colonize tomato plants [34]. However, plants infected with the reduced EPS mutant had a slightly higher biomass, suggesting that EPS has a subtle effect on virulence [34]. For *C. m.* ssp. *insidiosus*, U.V.-induced EPS non-producing strains were able to infect plants, even competing effectively when co-inoculated with wild-type strains [35]. These data collectively suggest that the function of EPS in pathogenicity for *Clavibacter* species is less straightforward than originally believed. A better understanding of the role of EPS may require more sophisticated knowledge of the various processes involved in bacterial colonization, multiplication and induction of disease symptoms than we currently possess.

5.2. Extracellular enzymes

Extracellular enzymes such as cellulase and pectinolytic enzymes that can degrade the plant cell wall are produced by many plant pathogens. For *Clavibacter* species, the involvement of cellulase in virulence for several species has been examined, most thoroughly for *C. m.* ssp. *michiganensis*. In this subspecies, one of the native plasmids carries a cellulase gene. Plasmid cured strains no longer induce wilt, but transformation with a plasmid containing the cellulase gene restores the ability to cause wilt [36], unequivocally demonstrating that the cellulase is necessary for virulence. *C. m.* ssp. *sepedonicus* also produces a cellulase [37], as well as an amylase (M. Laine, unpublished observations). Using chemical mutagenesis, two cellulase and five amylase negative mutants were generated. These strains grew to wild-type levels in culture, but the amylase negative mutants of *C. m.* ssp. *sepedonicus* were somewhat reduced in virulence compared to the parent, and the cellulase non-producers were non-virulent (M. Laine, K. Lehtilä and S. De Boer, unpublished observations). Transformation of the cellulase mutants with the cellulase gene from *C. m.* ssp. *michiganensis* restored expression of cellulase [12] and partially restored virulence (M. Laine, unpublished observations). This suggests that cellulase is also important for virulence in *C. m.* ssp. *sepedonicus*. Additionally, an extracellular amylase seems to play a role in virulence, although it is less important than cellulase. The involvement of amylase in virulence is not surprising given that one of the symptoms of the disease is the decay of starchy tubers. Southern hybridization data show that three other *C. michiganensis* subspecies, *insidiosus*, *nebraskensis* and *tessellarius*, contain sequences hybridizing to the cellulase gene from *C. m.* ssp. *michiganensis*, but *C. iranicus*, *tritici* and *toxicus* do not [19].

5.3. Other virulence factors

In a systematic search for toxic activities, membrane channel forming activity was detected in *C. m.* ssp. *nebraskensis* culture supernatants. A 65 kDa protein was identified as the active moiety, and it produced anion channels in lipid bilayers [38]. This protein may be unique to this subspecies since the activity is not found in culture supernatants from *C. m.* ssp. *michiganensis*. As formation of membrane channels is a common mode of action for toxins, this protein could be a toxin involved in pathogenesis. However, activity of the protein in host plants remains to be evaluated (R. Eichenlaub, personal communication).

One of the two native plasmids from *C. m.* ssp. *michiganensis* encodes a virulence factor called *pat-1*. Like cellulase, this factor is involved in induction of wilt in tomato. Introduction of the *pat-1* gene into a plasmid cured strain restores wilt-inducing ability. According to sequence homologies, the product of this gene may be a serine-type protease, although no protease activity has been detected in *C. m.* ssp. *michiganensis* culture fluids [15].

6. Special issues

6.1. Surrogate vector method

Due to the low virulence nature of *C. x.* ssp. *cy-*

nodontis and the fact that it can be transferred to other hosts artificially, the possibility of using the bacterium as a 'surrogate vector' has been pursued. The principle is to engineer the endophytic bacteria instead of transforming the plant genome. The engineered bacteria producing beneficial proteins in planta could, for example, give protection from plant diseases or pests. Various components necessary for the genetic manipulation of *C. x.* ssp. *cynodontis* have been developed. Plasmid cloning vectors have been developed [8,13], strong promoters to drive expression of foreign genes have been isolated and characterized [14], and integrative expression vectors have been constructed ([39]; M. Haapalainen, unpublished results). The surrogate vector method has been tested in the field, and found to give considerable protection of maize from the European corn borer [40]. However, no increase in yield was observed, possibly due to the additional water stress and nutritional demands of the bacteria. It remains to be seen if this method might be practical for crops other than maize.

6.2. The role of phage in toxigenicity for C. toxicus

C. toxicus, which causes a gumming disease transmitted by nematodes, primarily of the species *Anguina funesta*, causes annual ryegrass toxicity [6]. This disease affects grazing animals feeding on infected, galled seed heads. Corynetoxin, which is responsible for toxicity, is produced by the bacteria only when they are infected with a bacteriophage [41]. Ryegrass can be infected using nematodes from galls as inoculum; however, Koch's postulates have not been fulfilled for this disease, since ryegrass cannot be infected using cultured *C. toxicus*, which have invariably lost the phage (K. Ophel-Keller, personal communication). It has been suggested that phage infection, which makes the bacteria unstable in culture, may facilitate infection of plants, and therefore may play a role in pathogenicity [41]. The phage genome is not big enough to code for the entire synthetic pathway of the corynetoxin, so most of the synthetic apparatus must be coded for by the bacterial chromosome [41]. This is in contrast to *Corynebacterium diptheriae*, where the bacteriophage carries the genes coding for toxin biosynthesis.

7. Concluding comments

Molecular characterization of several *Clavibacter* species is progressing, facilitated by the availability of transformation procedures and plasmid vectors for these species. However, these bacteria remain fairly difficult to work with, some having slow growth characteristics and all giving low rates of transformation. Further progress would be enhanced by development of more advanced techniques, such as transposon mutagenesis. These efforts are ongoing, but are not always straightforward. For instance, although vectors and transposons are available for closely related species, their utility for any particular *Clavibacter* species can not be assumed. This is demonstrated by the instability of *C. x.* ssp. *cynodontis* plasmid vectors when transformed into *C. m.* ssp. *sepedonicus*; conversely, plasmid vectors used with *C. m.* ssp. *sepedonicus* are unstable in *C. x.* ssp. *cynodontis* (M. Haapalainen and M. Laine, unpublished observations). Nonetheless, efforts to develop improved techniques for manipulation of these bacteria are continuing and should allow further molecular characterization of this genus, particularly the factors involved in pathogenicity.

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