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► **To cite this version:**

Yvan Kraepiel, Marie-Anne Barny. Gram-negative phytopathogenic bacteria, all hemibiotrophs after all?. *Molecular Plant Pathology*, 2016, 17 (3), pp.313-316. 10.1111/mpp.12345 . hal-01501584

HAL Id: hal-01501584

<https://hal.science/hal-01501584>

Submitted on 4 Apr 2017

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Gram-negative phytopathogenic bacteria, all hemibiotrophs after all?

YVAN KRAEPIEL¹ and MARIE-ANNE BARNY^{2*}

¹Sorbonne Universités, UPMC Univ Paris 06, INRA, CNRS, IRD, Diderot Univ Paris 07, UPEC Univ Paris 12, Institut d'Ecologie et des Sciences de l'Environnement de Paris (UMR113) Case 237, 4 place Jussieu, 75252 Paris, France

²UMR1392, INRA, Institut d'Ecologie et des Sciences de l'Environnement, Université Pierre et Marie Curie (UPMC), Bât A7ème Etage Case 237, 7 Quai St.-Bernard, 75252 Paris, France

* barny@upmc.fr, barny@agroparistech.fr

Traditionally, the lifestyles of plant pathogens have been divided into distinct groups predicated on nutrient acquisition and the viability of host tissue. Biotrophs extract nutrients from living cells while necrotrophs feed off killed cells. Necrotrophy is defined as growth and nutrition of the pathogen on dead or dying plant material. Hemibiotrophs share characteristics with both biotrophs and necrotrophs, initially invading cells that are maintained alive prior to a transition to a necrotrophic life style in which nutrients are obtained from killing host cells.

The biotrophic/necrotrophic terminology is not adapted to bacteria

Although this terminology has been developed with pathogenic fungi, it is sometimes used to describe bacterial life styles. However, when applied to bacteria, the picture is not so clear, and we believe that the biotrophic/necrotrophic terminology should not be used for bacteria. A brief survey of the literature shows that *Pseudomonas syringae* is often referred to as biotrophic or hemibiotrophic but is also occasionally described as partly necrotrophic or even necrotrophic. *Xanthomonas* spp. are often referred to as biotrophic. *Erwinia amylovora* is often referred to as necrogenic, which cautiously describes its capacity to induce necrotic symptoms regardless of its life style, but it could also be described as necrotrophic or hemibiotrophic. The soft rot pathogens, *Pectobacterium* spp. and *Dickeya* spp. are mostly described as necrotrophs. Moreover, both biotrophy and necrotrophy have been assigned to *Ralstonia solanacearum* in the literature. The delivered message is obviously rather confusing with bacteria. Why is this?

To understand, let us look back to the initial descriptions of biotrophic and necrotrophic life style. These contrasted lifestyles were initially described following microscopic observation of plant colonisation by pathogenic fungi (Lo Prestiet *al*, 2015). As paradigm of biotrophic fungus, the maize pathogen *Ustilago maydis* initiates colonisation by an

initial intracellular growth, during which its hyphae is encased by the plant plasma membrane, and later switches to predominantly intercellular growth. In addition, the biotrophic tomato pathogen *Cladosporium fulvum* remains exclusively in the extracellular compartment of tomato leaves. During the colonisation processes by these two biotrophic fungi, the plant cells remain alive. These microscopic observations were the first to indicate that plant pathogens may possess tools to avoid plant cell death. However, hyphae of necrotrophic fungi such as *Botrytis cinerea* or *Sclerotinia sclerotiorum*, after a short initial period of intercellular subcuticular growth, kill epidermal cells as soon as they penetrate inside the plant cells. In this mode of infection, the death of host plant cells precedes or accompanies colonization by the pathogen. Hemibiotrophic fungi such as *Colletotrichum* spp. or *Magnaporthe oryzae* initially develop an intracellular bulged hypha encased by the plant plasma membrane which does not kill the plant cell, and later switch to a thin intracellular necrotrophic hypha. When applied to bacteria, these assignments were not driven by close microscopic examination of the disease processes. Indeed, gram negative plant pathogenic bacteria remain extracellular throughout the infection process and it is not possible to classify them as biotrophs or necrotrophs on the basis of microscopic examination, as it has been performed with fungi. This could explain the difficulties to clearly assign a biotrophic or necrotrophic life style to bacteria. One bacterial exception that is not discussed in this opinion letter is *Agrobacterium* which could be considered as a true biotroph because it does not kill its host plant cells to proliferate, but rather induces the development of plant tumours producing metabolites that it can catabolize.

Why has this confusing terminology been applied to bacteria?

The explanation probably lies in the fact that it is important for plant pathologists to be able to distinguish between biotrophic and necrotrophic pathogens, as plant defences against biotrophs and necrotrophs are distinct and antagonistic. Because biotrophic pathogens require a living host, localized controlled cell death triggered through specific recognition of the invading pathogen by specific plant resistance (R) proteins forms part of an effective defence strategy. Necrotrophic pathogens, however, actively kill host tissue and therefore programmed cell death initiated by the plant is intuitively not an effective strategy to limit necrotrophic pathogen growth. It is also widely accepted that the salicylic acid (SA)-dependent defence signalling is effective against biotrophic

pathogens, whereas jasmonic acid (JA) and ethylene (ET)-dependent defence responses are efficient against necrotrophic pathogens. Determining the life style of a given plant pathogen is therefore important. This explains why plant pathologists have attempted to classify bacteria as necrotrophs and biotrophs, as it has been performed with fungi. However, when applied to bacteria, the biotrophic/necrotrophic terminology is not always consistent with experimental results, leading scientists to struggle in endless discussions to fit their data with the dogma. For example, it may be stated in an article dealing with the influence on plant defence of the ERF96 transcription factor belonging to the ethylene responsive factor family «overexpression of ERF96 increased *Arabidopsis* resistance to necrotrophic pathogens such as the fungi *Botrytis cinerea* and *Pectobacterium carotovorum* ssp. *carotovorum* bacteria. However, *Arabidopsis* overexpressing ERF96 was more sensitive to hemibiotrophic bacteria *P. syringae* pv. *Tomato DC3000*» (Catinot et al, 2015) indicating that ERF96 is efficient against necrotrophs. But Brader et al. (2007), while studying the role of the mitogen-activated protein (MAP) kinase kinase MKK2 in SA- and JA-related plant defences, did not qualify the same bacteria as biotrophic or necrotrophic when reporting the similar sensitivity of both bacteria to MKK2-related defence, «MKK2-EE plants were more resistant to infection by *P. syringae* pv. *tomato DC3000* and *Erwinia carotovora* subsp. *carotovora* (now named *Pectobacterium carotovorum* ssp. *carotovorum*), but showed enhanced sensitivity to the fungal necrotroph *Alternaria brassicicola*». In our opinion, the efficiency of a given plant defense mechanism against a given pathogen should not dictate the terminology used to describe the pathogen's life style, this only adds confusion. Indeed, JA-related defence mechanisms, which include the production of most major classes of secondary metabolites and defence-related proteins, trigger efficient defences against many pathogens, whatever their life style (Campos et al, 2014). Furthermore, the dichotomy JA-necrotroph/SA-biotroph has mainly emerged from studies with *Arabidopsis* whereas studies with other species such as Monocots illustrate a much more contrasted reality (De Vleeschauwer et al, 2013). Even on *Arabidopsis*, the picture is not simple for bacterial pathogens. For example, JA-related defences are partly efficient during the symptomatic macerating phase induced by the soft rot pectinolytic bacteria *D. dadantii*, as expected for a "necrotrophic" pathogen. However plant necrosis, which is supposed to benefit to necrotrophic pathogens is also able to block efficiently *D. dadantii* at the

onset of infection (Kraepiel *et al.*, 2011). This clearly indicates that *D. dadantii* can't be classified so easily as biotroph or necrotroph. Similarly both SA and JA/ET pathways are efficient against Pectobacteria and the relative importance of both types of defence depends on the stage of the infection (Davidsson *et al.* 2013).

Biotrophic and hemibiotrophic type III-dependent phyto-bacteria, in addition to defence suppression, also induce cell death

The necrotrophic/ biotrophic classification terminology is often superimposed on the main weapons deployed by different bacterial pathogens. On one hand bacterial pathogens that rely on a functional type III secretion system (T3SS) deploy a battery of injected type III effectors (T3Es) mostly involved in the suppression of plant defences for the benefit of the pathogen. This underscores the importance of the initial biotrophic development of these bacteria and explains why these bacteria are mostly described as biotrophic or hemibiotrophic. On the other hand, soft rot pectinolytic bacteria, which secrete a large set of plant cell wall-degrading enzymes (PCWDEs) through the type II secretion system (T2SS), are particularly effective in macerating the host tissues and in obtaining nutrients from the dead cells, explaining why they have often been referred to as necrotrophic bacteria. However, pathogenicity is not that simple and, as described below, a closer look at the pathogenic weapons deployed by bacterial pathogens rules out this oversimplified terminology.

Let us examine first the pathogenicity of *Pseudomonas*, *Xanthomonas*, *Ralstonia* and *Erwinia* species that rely on a functional T3SS. This T3SS allows the injection of T3Es inside the plant host cells. As injected T3Es can also be detected by plant R proteins, pathogens, during evolution, either lose the recognized effector or acquire new effectors to avoid R protein recognition. This has been conceptualized in the well-known zig-zag model of plant pathogen evolution. As a consequence, biotrophic pathogens often deploy a large set of T3Es, are recognized by many plant R proteins and have a narrow host range. Already, we can see that the pathogenicity of *E. amylovora* and *R. solanacearum* does not fit totally to this scheme. *E. amylovora* has a narrow host range, a characteristic of biotrophs, but there are no known resistance genes against this bacterium, a characteristic mostly shared by necrotrophs (Malnoy *et al.*, 2012). *R. solanacearum* injects a plethora of T3Es involved in the suppression of plant defences, a characteristic of biotrophs, and yet this bacterium has a very large host range similar

necrotrophs (Pouyemiro and Genin, 2009). Although the zig-zag model is extremely powerful in deciphering the mechanisms by which plant-pathogenic bacteria suppress plant defences, this paradigm has also limited our ability to see beyond it. Indeed, biotrophic and hemibiotrophic type III-dependent phyto-bacteria, in addition to defence suppression, also induce cell death. The need for an efficient mode of nutrient acquisition is a possible trigger for the transition to necrotrophy, and cell death is generally the ultimate response to pathogen attack. Type III-dependent phyto-bacteria harbour in their genomes several weapons to achieve this goal. First, some T3Es are involved in cell death induction during disease development. Interestingly, often, these cell death eliciting T3Es are key players for pathogen growth *in planta*, suggesting that induction of cell death is important for nutrients acquisition during infection. For example, PthA, which belongs to the transcription activator-like effectors (TALE) T3E family, is essential for pathogenicity of *X. citri*. PthA is involved in programmed killing of host cells and ectopic expression of *pthA* in citrus cells is sufficient to cause typical disease symptoms: division, enlargement and death of host cells. PthA is required for the production of necrotic cankers on all species of citrus attacked by *X. citri*. TALEs are DNA-binding proteins with a modular DNA-binding domain. The DNA binding domain is predictable, which simplifies elucidation of TALE function *in planta*, and it was shown that PthA activates the expression of the transcription factor CsLOB1, which coordinates pustule formation (Boch et al, 2014). Another T3Es family involved in cell death elicitation is the AvrE-like T3E family. This family is widespread among type III-dependent phyto-bacteria and plays a crucial role for bacterial growth *in planta*. Interestingly, T3Es of this family play a dual role during the disease process; they inhibit SA-mediated plant defences and interfere with vesicular trafficking, but also elicit electrolyte leakage and a slow plant cell death that participates in nutrient release (Degraeve et al, 2015). These dual effects suggest that AvrE-like effectors may be involved in the transition from biotrophy to necrotrophy.

In addition to T3Es, type III-dependent phyto-bacteria harbour other virulence factors devoted to plant cell death. *Pseudomonas*, *Xanthomonas*, and *Ralstonia* species harbour T2SS and PCWDEs. These PCWDEs have often been suggested to participate mainly in the saprophytic life of these pathogens. However, although mutations affecting the T2SS of *P. syringae* have not been reported, *R. solanacearum*, *X. campestris* sp. *campestris*, *X. oryzae* sp. *oryzae* and *X. campestris* sp. *vesicatoria* T2SS mutants are affected in virulence,

indicating that T2SS is also an important virulence factor for these bacteria (Szczesny, 2010).

Other factors involved in plant cell death are the toxins produced by *P. syringae* strains. The coronatine toxin has been shown to act as a mimic leading to the activation of JA-dependent defences and therefore to the suppression of antagonistic SA-dependent defences. Moreover, this toxin is known to injure plant cells and to be involved in disease development and symptoms. For example, the coronatine-defective *P. syringae* pv. *atropurpurea* mutant strain does not cause any symptoms, whereas the coronatine-producing strain induces a severe water-soaking symptoms, on oat (Yao et al, 2002). In addition, other pathovars of *P. syringae* produce other toxins, such as tabtoxin, phaseolotoxin or mangotoxin, which can interfere with the nitrogen metabolism of the host, causing amino acid deficiencies in host cells and concomitant accumulation of nitrogen-containing intermediates that can be metabolized by the pathogen as a nitrogen source. This metabolic imbalance of amino acids leads to chlorosis and even necrosis symptoms in the host plant, and probably aids pathogen growth, because of the release of nutrients (Arrebola et al, 2011).

In *Pectobacterium* and *Dickeya* soft rot necrotrophic pathogens, in addition to brute force killing, the asymptomatic biotrophic phase plays an important role

Now Let us examine the situation of pathogenic soft rot enterobacteria of genera *Pectobacterium* and *Dickeya*. For these bacteria, as described above, pathogenicity relies mostly on a T2SS which allows secretion of a large arsenal of PCWDEs, such as pectinases, cellulases, hemicellulases, and proteases. Once expressed, these large amounts of PCWDEs rapidly disrupt host cell integrity and promote rotting. However soft rot enterobacteria could also be found in latent asymptomatic infections on many host crops. These latent infections, which are highly dependent on environmental conditions, can last for several months with the pathogen reaching high population levels in the absence of visible disease symptoms (Liu et al, 2008). These asymptomatic infections play an important role in the disease dynamics of natural infections. Because these asymptomatic infections are difficult to reproduce in laboratory conditions, the exact nature of this biotrophic phase remains elusive. One of the key elements to explain the switch between this asymptomatic biotrophic phase and the soft-rotting necrotrophic phase is probably the fine-tuning of PCWDEs production. Indeed, the

production of PCWDEs is strictly controlled in a population density-dependent manner through quorum sensing (QS) regulation in *Pectobacterium*, whereas the PecS global regulator prevents the premature expression of *D. dadantii* PCWDEs at the beginning of the infection process before the appearance of symptoms (Mhedbi-Hajri et al, 2011). This fine regulation is probably necessary to prevent premature activation of plant defences, as the action of PCWDEs releases cell wall fragments which trigger defence responses in the host plant (Davidsson et al., 2013).

In addition to PCWDEs regulation, quorum sensing in *Pectobacterium* also regulates other virulence determinants that could be involved in plant defence suppression. Among them, the T3SS and the AvrE-like T3E DspE/A have been found to be important for *P. atrosepticum* virulence. As effectors of this family are involved in the suppression of SA-mediated plant defence, it would be interesting to test whether DspE/A proteins play a similar role in *P. atrosepticum*. Similarly, the *P. atrosepticum* type VI secretion system (T6SS) is also regulated through quorum sensing and T6SS mutants are also affected in virulence. Moreover, *P. wasabiae* harbours two T6SS machineries that have been shown experimentally to have partially overlapping functions during potato infection (Davidsson et al., 2013). The T6SS, like T3SS, allows the injection of type VI effector proteins (T6Es) into eukaryotic cells. The exact nature and role of the T6Es injected by *P. atrosepticum* or *P. wasabiae* are still unknown, but it can be speculated that they may be involved in the suppression of plant defences. Other traits identified through genomic studies could also benefit the bacterium. Among them, a type IV secretion system (T4SS) and a putative polyketide phytotoxin (encoded by the *cfa* cluster) have been shown to contribute to the virulence of *P. atrosepticum*. Once again, thenature and the role of the material translocated through the T4SS of *P. atrosepticum* inside the plant cell remain to be determined.

The diverse life style of phytopathogenic bacteria should be seen as a continuum of hemibiotrophic pathogens.

Irrespective of the bacteria considered, after an initial biotrophic phase, in which the bacteria avoid the elicitation of or suppress plant defences, a switch to necrotrophic stage, in which the bacteria actively kill plant cells, is observed. Both phases are required to induce disease although the length of time of each phase will vary between bacteria. The length of each phase will also vary for the same bacteria between experimental

conditions, as the inoculum concentration, age of the plant and the inoculation procedure will influence the outcome of the interaction. Another difficulty arises because the switch between the initial biotrophic phase and the later necrotrophic phase cannot be visualised through microscopic examination, as it has been performed with fungi. This makes the necrotroph/biotroph classification highly hazardous for phytopathogenic bacteria. Furthermore, all phytopathogenic bacteria possess weapons to suppress or avoid premature elicitation of plants defences, indicating they initially develop on living hosts, and weapons involved in cell death induction, probably resulting in nutrient acquisition. Therefore, phytopathogenic bacteria should always be considered as hemibiotrophs. Although the biotrophic phase has been thoroughly described for type III-dependent phyto-bacteria, it has been overlooked for type II-dependent soft rot pectinolytic bacteria, probably because the asymptomatic biotrophic phase is highly dependent on environmental conditions and is difficult to reproduce in laboratory conditions. Moreover, because the zig-zag model has been so powerful in understanding the biotrophic phase of type III-dependent phyto-bacteria, their necrotrophic phase is often underestimated. To achieve a better comprehension of plant-bacterial interactions, the hemibiotrophic nature of these interactions should be recognized.

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