Implications of toxins in the ecology and evolution of plant pathogenic microorganisms: Bacteria

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Abstract. This review attempts to rationalise what is known about bacterial phytotoxins and associate it with the ecology and possible evolution of the producing organisms. Study of non-toxin producing variants gives insight into the ecological role of the toxin. Elucidation of chemical structures of phytotoxins has shown that many exist as families of analogous compounds. Studies on the variation of chemical structures and how they are distributed across species and genera can lead to development of hypotheses on evolutionary relationships. Knowledge on biosynthetic pathways to toxins allows recognition of specific enzymatic steps involved in developing the characteristic features of the structures. Phytotoxins often have a potent biochemical activity, and in some cases the producing organism has associated mechanisms to prevent action of the toxin upon itself; in such cases toxigenesis is clearly not a chance event. The various aspects of bacterial toxigenesis indicate that bacterial phytotoxins are special secondary metabolic products that play beneficial roles to the producing organisms in their various ecological niches.

Key words. Phytotoxins; ecology; phylogeny; evolution; biosynthesis; coronatine; phaseolotoxin; rhizobitoxine; syringomycin; syringotoxin; syringostatin; tabtoxin; tagetitoxin; tropolone; fireblight toxin; thaxtomin; 3-methylthiopropionic acid; carboxylic acids; *Pseudomonas*; *Xanthomonas*; *Streptomyces*; *Erwinia*; *Bradyrhizobium*.

Introduction

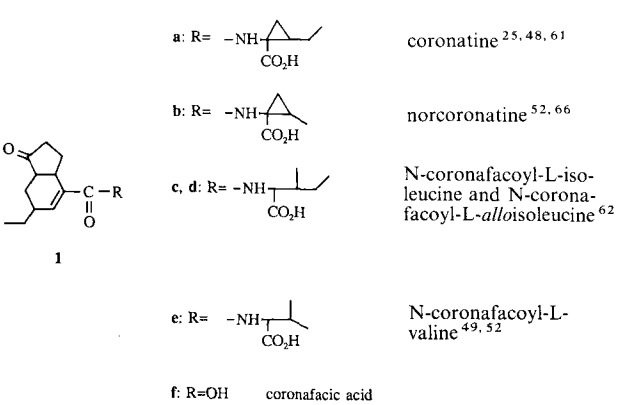
Phytotoxic metabolites of plant pathogenic bacteria have been studied for about 25 years. The principal driving force has been the curiosity generated over the biological activity of these materials. What is their significance? What roles do phytotoxins play in the interactions between plant and bacteria? Why and how have these organisms acquired these traits?

There is increasing evidence that phytotoxins are an important ecological factor contributing significantly to the pathogenic life-style of many bacteria and this review focusses on this theme. In contrast the place of toxins in the evolution and phylogeny of these organisms is at the present time tentative and speculative.

There are two perspectives in this review on phytotoxins. The first is that of the individual toxins and their importance to the organisms that produce it. The second examines the distribution of toxins between the genera and species of bacteria (as they are currently known) and speculates on its evolutionary significance. The examination of individual toxins covers many aspects. Only toxins that are chemically defined are considered. The nature of structural variations within and between species may reflect divergence in genetic information or a lack of specificity at specific steps in their biosynthesis. Knowledge of toxin biosynthetic pathways allows both the recognition of the special features utilised by the organism, some of which might be important in the regulation or limitation of toxin production, and also the recognition of key biosynthetic intermediates as indicators of a potential for toxigenicity in non-toxigenic organisms. Finally, the importance of physiological aspects associated with toxin production is assessed. The mode of action of phytotoxins in the plant is an important topic overlapping with the present treatment that is the subject of a separate review by R. D. Durbin (see pp. 776 of this issue).

Coronatine

Natural occurrence, chemistry. Coronatine, structure **1a**[26], is the parent phytotoxin produced by many pathovars of *Pseudomonas syringae*; pv. *atropurpurea*, pv. *glycinea*, pv. *morsprunorum* and pv. *tomato*[50]. An important structural feature of coronatine is the bicyclic component, coronafacic acid (**1f**). A family (termed coronatines) of amides between coronafacic acid and various amino acids exists in nature, indicating that synthesis of coronafacic acid itself is subject to rigorous enzymatic control, whereas the coupled amino acid is less specific (structures **1a** → **1e**, from various *P. syringae* pathovars). However, coronatine, which contains the uncommon cyclopropane part-structure, is the most abundant component in liquid cultures of *P. syringae* pathovars and **1e**



usually the second most abundant, while the others are minor products that have been characterised and identified from *P. syringae* pv. *glycinea*. The pattern of carboxylic acid products in the GLC analysis of some *P. syringae* pv. *tomato* strains indicates the presence of a

similar distribution of these minor components also[6]. Coronatine has been suggested to occur in cultures of *Xanthomonas campestris* pv. *phormiicola*, a pathogen of New Zealand flax[97]. However, analysis of the products of *X. campestris* pv. *phormiicola* by GLC and GC-mass spectrometry has shown that two components were present and these were tentatively identified as the coronatines **1c** and **1e** (R. E. Mitchell, unpublished observations). Neither coronatine per se nor cyclopropane compounds were present and this shows up a significant difference between this xanthomonad and pseudomonads where the cyclopropane product coronatine is always present, and in most cases the most abundant component.

Biosynthesis and phylogeny. The two chemical components of coronatine are biosynthesized from different pathways. The coronafacic acid moiety is a polyketide, derived from 5 acetate units and the C-2 and C-3 of pyruvate[75]. It appears to be biosynthesized from two polyketide chains, with the pyruvate serving as a starter unit for one of these (fig. 1). The mechanisms by which C-2 and C-3 of pyruvate, and the two polypeptide chains are coupled together to give an intermediate bicyclic structure are not known.

The cyclopropyl moiety of coronatine is derived from isoleucine. Radioactivity from L-[U-^{14}C]isoleucine is incorporated specifically into the cyclopropyl moiety[51], and the ^{13}C-1 of DL-isoleucine was incorporated specifically into the carboxylic acid C[75].

Final assembly of the coronatine molecule requires the formation of an amide bond between a product of the coronafacic acid pathway and an amino acid component. The tacit assumption that the coupled unit is coronafacic acid itself is supported by the observation that coronafacic acid is secreted by many, but not all, coronatine-producing strains (R. E. Mitchell, unpublished observations). Neither the mechanism of cyclisation to the cyclopropane ring nor the point at which this occurs is known. The synthesis of 1-amino-1-carboxy-2-ethylcyclopropane could be completed prior to it being coupled in the final step with coronafacic acid (fig. 2). However, the alternative route coupling coronafacic acid with an amino acid of primary metabolism, followed by cyclization of the coronafacoyl-amino acid to form the 3-membered ring is more attractive as this would explain in a logical way the natural occurrence of coronafacoylvaline **1e** and coronafacoylisoleucines **1c** and **1d** which would be biosynthetic intermediates of norcoronatine and coronatine respectively.

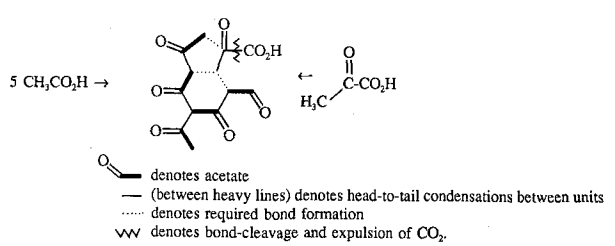


Figure 1. Biosynthetic origin of the carbon skeleton of coronafacic acid[75].

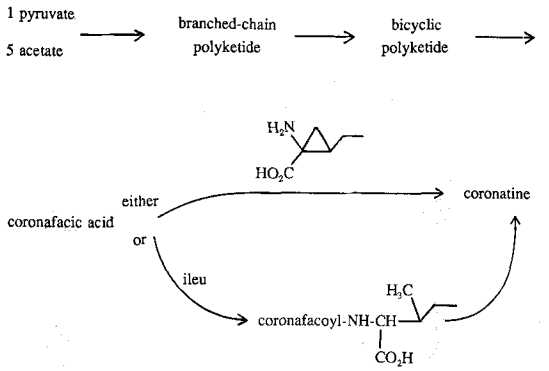


Figure 2. Possible biosynthetic routes to coronatine[62, 75].

In liquid cultures supplied with various aliphathic L-amino acids a coupling reaction occurs which results in the formation of the coronafacoyl derivative of the amino acid supplied[57]. The yield of this product increased with increasing length of the aliphatic chain of amino acid[57], i.e. for $H_2N-CH(R)-CO_2H$ the yield of coupled product when

$$R=-CH_3 < R=-CH_2CH_3 < R=-CH_2CH_2CH_3.$$

This is interpreted to be an increasing coupling efficiency as the 3-carbon chain length, corresponding to the chain length in isoleucine, is approached.

Because all coronafacoyl amides have phytotoxic activity[94] it can be suggested that the cyclopropyl derivative may be evolutionarily more recent. An attractive surmise is that the biosynthesis of the coronatines involves the coupling of coronafacic acid and aliphatic amino acids, in particular valine and isoleucine, and that the cyclization step forming the cyclopropane of the coronatine molecule occurs via coronafacoylisoleucine and is a more recently acquired trait. If this hypothesis is substantiated it may provide an insight into the phylogeny of species distributed between different genera such as *Pseudomonas* and *Xanthomonas*.

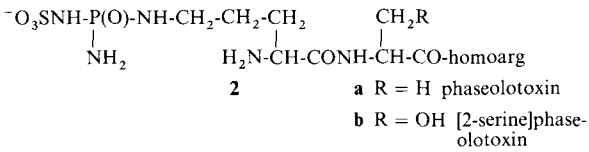
Ecology. As it is now apparent that production of coronatines is widespread in nature it can be argued that this phytotoxin contributes to the biological fitness of the organisms that can produce it. In order to understand more about the relative roles of coronatine and its noncyclopropyl analogues, more research is necessary into the biological significance of these compounds.

Coronatine itself is the cause of a number of biological effects on plants, which have been documented[50], but in contrast it is not known to have significant antibiotic activity against other microorganisms. In a recent study[7], nontoxigenic Tn5 mutants (cor$^-$) of *P. syringae* pv. *tomato* were compared with their parent wild-type (cor$^+$) strain. The cor$^-$ mutants colonized tomato leaves

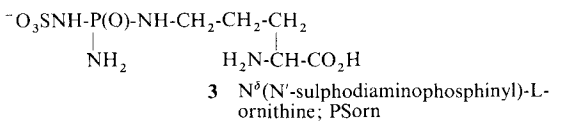
and produced the same number of lesions as the wild-type, but the lesions were smaller (0.2 mm diam., compared with 0.6 mm diam.), ceased to expand several days sooner than those of the wild-type, and had a lower bacterial population. The bacterial population in the lesions of the cor$^-$ mutant also declined earlier than those of the wild-type. Thus the capacity to produce coronatine enabled the organism to achieve higher population numbers and to persist longer, both being features likely to contribute to its success in nature.

Phaseolotoxin

Natural occurrence, chemistry. Phaseolotoxin is a tripeptide phytotoxin known to be produced by only the one bacterial species, *Pseudomonas syringae* pv. *phaseolicola*[65]. The chemistry and biochemistry of phaseolotoxin have been reviewed[47,50]. The chemical structure of phaseolotoxin is **2a**[45,67] and by analogy, the serine analogue of phaseolotoxin, which is a minor constituent (ca. 5%) of the total *P. phaseolicola* toxin[46,60], is **2b**. The chirality at the phosphorus has not to date been determined[54,67]. The most potent biochemical form of the toxin[98] is PSorn[55], structure **3**[67], a compound



2 **a** R = H phaseolotoxin
b R = OH [2-serine]phaseolotoxin



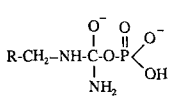
3 N^{δ}(N'-sulphodiaminophosphinyl)-L-ornithine; PSorn

that is unlikely to be a product of cellular biosynthesis[50]. Rather, PSorn is formed extracellularly in planta, but not in liquid culture, by peptidases[33,45,55,67].

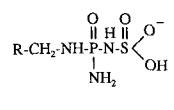
Biosynthesis. A speculative discussion on the biosynthesis has been presented by Chatterjee and Vidaver[9]. *P. syringae* pv. *phaseolicola* readily incorporates radioactivity from $^{35}SO_4^{2-}$ and $^{32}PO_4^{2-}$ into the S and P moieties respectively[45], and ^{15}N from $^{15}NH_4^+$ into all three nitrogens bonded to P[67]. Furthermore, the organism utilises [U-^{14}C]ornithine which appeared specifically in the ornithyl residue of phaseolotoxin (R. E. Mitchell, unpublished observations). Other studies showed that ^{3}H-labelled lysine gave rise to the homoarginyl moiety (R. E. Mitchell, unpublished observations). A key enzyme in phaseolotoxin biosynthesis may be an amidino transferase[84], which catalyzes the transfer of the amidino moiety of arginine to the side chain N of lysine, with concomitant release of ornithine. Correlations have been reported between activity of this enzyme and toxin production in different strains of the organism, and at different growth temperatures between 18 and 30 °C[84]. A considerable challenge for future studies is to determine the mechanism of assembly of the inorganic moiety and its attachment to the δ-N of ornithine, or of the ornithyl residue of a peptide. The tripeptide may be derived from the controlled assembly of activated amino acids[30]. The genes encoding biosynthesis of phaseolotoxin have been found to be clustered in a portion of chromosomal DNA[78,83].

Ecology. An important ecological topic is the resistance of *P. syringae* pv. *phaseolicola* to phaseolotoxin. A complex mechanism prevents this potential problem. The gene cluster encoding phaseolotoxin biosynthesis also encodes for an ornithine transcarbamoylase (OTCase) resistant to phaseolotoxin[77] which is produced only under conditions permissible for phaseolotoxin biosynthesis. In contrast, tox$^-$ *P. syringae* pv. *phaseolicola* strains produce only a toxin-sensitive OTCase that is typical of other organisms and also tox$^+$ *P. syringae* pv. *phaseolicola* strains[100]. The biochemistry of the two enzymes have been compared, and the insensitivity of the toxin-resistant one to inhibition by phaseolotoxin and PSorn is accounted for by a lower affinity for carbamoyl phosphate and a slower binding of ornithine. This enzyme will still efficiently catalyse the synthesis of citrulline (and hence arginine) given a sufficiently high concentration of ornithine[100]. Significantly, an elevated ornithine concentration occurs in planta in the presence of PSorn, by inactivation of the toxin-sensitive plant OTCase[98].

The chemical structure of phaseolotoxin is relatively simple yet elegantly refined with many features relating to the organism's ecology. The tripeptide structure is a common feature of many bacterial bio-active products such as antibiotics, probably because it is associated with export of such compounds from the cell. Often the two 'carrier' amino acid residues of such tripeptides form a dialanyl unit. In comparison phaseolotoxin bears an alanylhomoarginine unit, which implies a significance to the homoarginyl residue. This could be the increased (over alanyl) chain length, or the strongly basic nature of the guanidino group. The presence of the homoarginyl residue quite certainly 'masks' the strongly anionic character of the $-NHSO_3^-$ moiety and may thus play a key role in mobilisation of phaseolotoxin across membranes to the exterior of the cell. This chemical difference is clearly illustrated in the different electrophoretic mobilities of PSorn and phaseolotoxin[45] where phaseolotoxin migrates as a cation at pH 2 and PSorn migrates as an anion. PSorn, the chemical form of toxin that results in planta exterior to the bacterial cell[55,57], approaches the perfect design based on the biochemical data of Temple-



Transition-state structure



PSorn

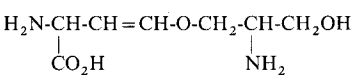
R-CH_2-NH- = δ-ornithyl

ton et al.[98 - 100]. Structurally PSorn is a mimic of the transition-state in the reaction between ornithine and carbamoyl phosphate.

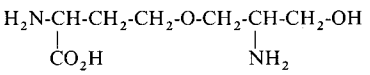
Do phaseolotoxin or PSorn play a precise role in the ecology of *P. syringae* pv. *phaseolicola*? Their presence may offer a territorial advantage, since phaseolotoxin is inhibitory to the growth of other microorganisms[96]. The effects on the biochemical function of bean plants as a result of OTCase inhibition have been found to be a block of arginine synthesis leading to a halt in accumulation of protein and reduced chlorophyll synthesis[105]. The consequences in plants infected with toxigenic *P. syringae* pv. *phaseolicola* is a growth reduction and stunting. Other reports suggest that phaseolotoxin suppresses phytoalexin synthesis in bean[19] and is a factor in the systemic invasion of the host plant[76], while toxin production in general is considered to enhance virulence[50]. The information collectively suggests that phaseolotoxin is an important factor in the ecology of *P. syringae* pv. *phaseolicola*.

Rhizobitoxine

Natural occurrence, chemistry. Rhizobitoxine is the 2-amino-4-alkoxy-but-3-enoic acid **4**[74], and therefore a 4-alkoxy derivative of vinylglycine.



4 rhizobitoxine



5 dihydrorhizobitoxine

It is commonly produced by *Bradyrhizobium japonicum*, and other species of *Bradyrhizobium* from diverse geographical areas (USA, Africa, Central & South America) and from various hosts[14, 34, 41], and its presence in root nodules is often accompanied by dihydrorhizobitoxine, **5**[43], which is not known to be biologically active. Rhizobitoxine is also produced by a high proportion of *Pseudomonas andropognis* strains[58, 64], and its involvement in bacterial stripe disease of corn[109] is apparent[58]. Given the extremely diverse range of plants (representatives from 13 families) that can be host to *P. andropogonis*[24], rhizobitoxine most certainly has a widespread involvement in plant diseases. Additionally, the presence of dihydrorhizobitoxine in cultures of *P. andropogonis* has recently been established (R. E. Mitchell, unpublished observations).

Two further 4-alkoxy derivatives of 2-amino-3-butenoic acid have been reported from other bacteria, the 4-methoxy compound from *Pseudomonas aeruginosa*[90] and the 2'-aminoethoxy compound from a *Streptomyces*[82]. It is likely that the genetic material necessary for the production of this class of compound is widely spread in nature.

Biosynthesis. Many strains of *P. andropogonis* secrete 2-amino-3,4-dihydroxybutanoic acid (hydroxythreonine)[58], while the 3-carbon compound serinol

$(HOCH_2-CH(NH_2)-CH_2OH)$,

has been reported[44] to accumulate in root nodules colonized by *B. japonicum*. On the basis of carbon-chain homologies and functional group interrelationships, hydroxythreonine and serinol are the probable immediate biosynthetic precursors of rhizobitoxine. Thus, coupling between C-4 of hydroxythreonine and serinol to form an ether linkage, followed by dehydration across the C-3/C-4 bond of hydroxythreonine could result in rhizobitoxine. Whereas the involvement of serinol is speculative, that of hydroxythreonine was established when *P. andropogonis* was found to incorporate up to 40% of radioactivity from ^{14}C-hydroxythreonine into rhizobitoxine[53]. The biosynthesis of hydroxythreonine is via aspartic acid → threonine pathway with a branch point at, or soon after, homoserine, since *P. andropogonis* incorporated radioactivity into rhizobitoxine from ^{14}C-aspartate (2%) and ^{14}C-homoserine (3.2%), but not from ^{14}C-threonine. Also, exogenous threonine substantially reduces the incorporation of ^{14}C-aspartate into hydroxythreonine and rhizobitoxine, presumably by reducing the flux through the aspartate → homoserine pathway by feedback inhibition (R. E. Mitchell, unpublished observations). Aspartic acid is a specific precursor, since 4-^{13}C-aspartic acid, where C-4 is scrambled to C-1 by way of fumarase, gives rise to ^{13}C-specifically at C-4 of hydroxythreonine, and C-4, C-1 of rhizobitoxine (R. E. Mitchell, Phytochemistry, in press). Possible biosynthetic routes to rhizobitoxine are detailed in figure 3.

Dihydrorhizobitoxine usually co-occurs with rhizobitoxine. In *P. andropogonis* it is generally the minor constituent (R. E. Mitchell, unpublished observations), while in *B. japonicum* it is generally the major constituent (fourfold or higher) in comparison to rhizobitoxine[41]. One possible origin of dihydrorhizobitoxine is by the direct coupling of homoserine and serinol, or alternatively, it may not deviate from the rhizobitoxine pathway so early.

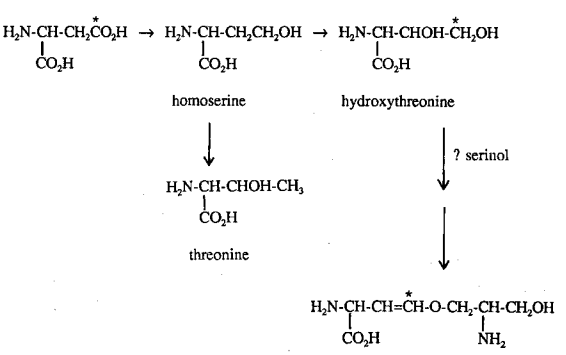


Figure 3. Possible biosynthetic route to rhizobitoxine. Carbons marked with an asterisk were established to be ^{13}C-enriched.

Ecology. The phytotoxicity of rhizobitoxine may arise from inactivation of plant β-cystathionase-pyridoxal phosphate[18]. In a similar way the structural analogue aminoethoxyvinylglycine inhibits reactions mediated by pyridoxal phosphate[39]. Rhizobitoxine is inhibitory to the growth of microorganisms such as *Bacillus subtilis*[34], and *Salmonella typhimurium* by inhibition of β-cystathionase[72]. Rhizobitoxine production may in this manner provide potential competitive or territorial advantage, and could also be important for saprophytic survival prior to infection. The toxin is able to inhibit the growth of the fungus *Macrophomina phaseolina* and has been suggested to play a role in protecting soybean roots nodulated with rhizobitoxine-producing *B. japonicum* from infection by *M. phaseolina*[8].

A potent effect of rhizobitoxine is the inhibition of ethylene biosynthesis[73]. This may be an important factor in the ecology of *Bradyrhizobia* species as ethylene is known to substantially reduce nodule formation. In the presence of $1 \rightarrow 10$ μm aminoethoxyvinyl glycine, a well known ethylene inhibitor, the ethylene biosynthesis rate of whole alfalfa plants was reduced to $< 50\%$ and nodule formation was significantly increased[81]. This effect, when induced by rhizobitoxine production at the root-site may be even more extensive. Many effects of ethylene antagonise processes associated with nodule formation, and the presence of rhizobitoxine may therefore enhance nodulation by reducing the antagonism.

Infections of plants by rhizobia or pseudomonads may induce ethylene biosynthesis by the host plant which in turn signals the activation of defense mechanisms. A key ecological role of rhizobitoxine therefore may be to suppress this signal mechanism by inhibition of ethylene biosynthesis in the host plant, and thus allow more effective colonisation of the plant.

There are marked differences between *P. andropogonis* and *B. japonicum* in the proportions of rhizobitoxine to dihydrorhizobitoxine. In the former case dihydrorhizobitoxine is a minor component compared to rhizobitoxine in liquid culture (R. E. Mitchell, unpublished observations), while with *B. japonicum* the levels of dihydrorhizobitoxine in planta relative to rhizobitoxine are several-fold higher[41,43]. Assuming this is species-related, rather than from arising from a difference between the conditions of 'in planta' and 'in culture', it is compelling to ask what is the role of dihydrorhizobitoxine production? Is its abundance (in *Bradyrhizobium*) a result of a mechanism to limit the quantity of free rhizobitoxine, or does its presence limit the biological activity of rhizobitoxine? Indeed, it would seem that for the symbiotic relationship developed by species of *Bradyrhizobium* excesses of rhizobitoxine would not be beneficial whereas in a pathogenic interaction involving a pseudomonad a higher level of rhizobitoxine may be an advantage.

The possibility of an ecological role of hydroxythreonine itself should not be overlooked since this compound functions as an antimetabolite (inhibition of *Escherichia coli* reversed by serine or threonine) that has been reported as a product of a *Streptomyces* sp. isolated from soil[111].

Phylogeny. All of the known structural variants of rhizobitoxine have hydroxythreonine as their common precursor, with variation in the 4-alkoxy moiety. Thus an important factor in the production of rhizobitoxine is the capability to synthesize hydroxythreonine. Since this is only a minor deviation from a primary biosynthetic pathway, it follows there may be as few as even one specific gene product necessary to mediate the conversion of homoserine to hydroxythreonine. The enzyme system that couples either hydroxythreonine or homoserine with a hydroxyl-containing compound (to form rhizobitoxine or dihydrorhizobitoxine respectively) may not even be a gene product that is specific to rhizobitoxine synthesis. It may be closely related to, or even the same as that which forms O-alkyl homoserine in a wide range of bacteria[69,70]. The enzymology of the coupling reaction and of hydroxythreonine synthesis are aspects that could contribute to information on the phylogeny of rhizobitoxine biosynthesis.

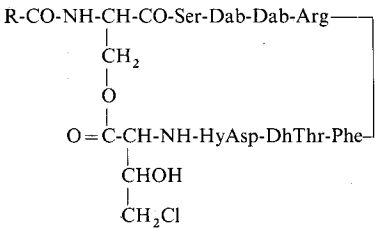
Two symbiotic genotypes, I and II, of *B. japonicum*[95] apparently represent two highly divergent evolutionary lines, and phenotypic correlations consistent with these genotype groups have been recorded[41,42]. Only genotype II rhizobia produce rhizobitoxine and these may have acquired this capability at an early stage of evolution. The suggestion has been made that this evolutionary line of *Bradyrhizobium* may be closely related to species of *Pseudomonas*[41]; an example could be *P. andropogonis*.

Syringomycin, syringotoxin, syringostatin

Natural occurrence. Syringomycin (SR), syringotoxin (ST) and syringostatin (SS) represent a family of interrelated phytotoxins produced by *Pseudomonas syringae* pv. *syringae*, a bacterial pathogen of numerous monocot and dicot plants. This class of compound is recognised by a wide biocidal activity often observed against the fungus *Geotrichum candidum*[24]. The nature of compounds produced is correlated to the host of origin of the bacterial strain. Thus, SR is produced by strains pathogenic on stone fruit, pears and grass hosts[23], ST is produced by citrus isolates[20], and SS has recently been reported from a strain of *P. syringae* pv. *syringae* causing blight of lilac (*Syringa vulgaris*)[27,28]. While SR and ST differ in both biological activity and chemical characteristics[20,24], the results of recent studies (discussed below under syringostatin) indicate that SS is an amino acid analogue of ST. Gross and DeVay[23] reported that syringomycin-like activity was restricted to *P. syringae* pv. *syringae*, since 14 other pseudomonads (*P. fluorescens*, *P. cepacia*, *P. avenae*, *P. viridiflava*, and 10 pathovars of *P. syringae*) were non-producers. In a more comprehensive survey of *P. syringae*, 27 pathovars, including at least 6 strains in

23 cases, were negative for syringomycin-like activity (J. M. Young, personal communication of unpublished data); however, the 4 pathovars *P. syringae* pv. *aptata* (6 strains), *P. syringae* pv. *atrofaciens* (6 strains), *P. syringae* pv. *japonica* (9 strains) and *P. syringae* pv. *syringae* (79 strains positive, 8 strains negative) did produce syringomycin-like activity.

Chemistry. Syringomycin has been reported to exist as a mixture of several distinct analogues[3-5,93] with the main components designated SR-B, SR-E, and SR-G (in order of elution from reverse-phase HPLC), with SR-E largely exceeding the others. The structures of three compounds, SR-A_1, SR-E and SR-G, have been determined to be homologous lipodepsinonapeptides[92], where SR-A_1 is a minor component. Recent data require that the structures previously reported[92] for these compounds be revised to **6**, **7** and **8** (A. Ballio, personal communication of unpublished data). The 3-dimensional conformations that these macrocyclic molecules adopt may be important for biological activity. For example, there is likely to be an intramolecular ionic interaction between the free carboxyl group and the basic moieties contained in Dab and Arg. In SR-E the chiral C-3 of 4-Cl-threonine and 3-OH-aspartate were determined by NMR to have the *threo* configuration, and the 2,3-dehydrothreonine residue was the Z isomer. The configurations of some of the amino acid residues has been reported[3], and work is in progress to assign the stereochemistry of the remaining chiral carbons.

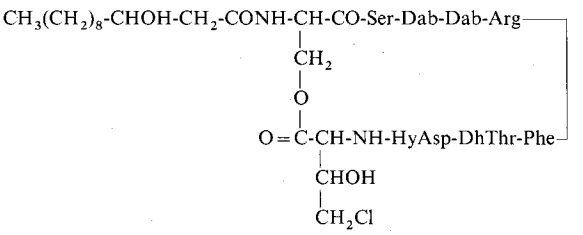


Dab = diaminobutyric acid
HyAsp = 3-hydroxyaspartic acid; α-peptide linked
DhThr = 2,3-dehydrothreonine

6 SR-E: R = -CH_2-CH(OH)-$(CH_2)_8CH_3$
7 SR-A_1: R = -CH_2-CH(OH)-$(CH_2)_6CH_3$
8 SR-G: R = -CH_2-CH(OH)-$(CH_2)_{10}CH_3$

A total of six further (mostly minor) components in the syringomycin complex have been characterised by HPLC and FAB-MS data[4]. These apparently do not contain a chlorine substituent as they have a normal isotopic pattern of MH^+, and four of them, together with SR-A_1, display slight or no antifungal activity. Out of the total mixture, SR-E and SR-G are the two major components and also the most active in the antifungal assay[4]. Since there is a marked variation in the biological activity of some of the analogues[5], the precise chemical definition of the minor analogues may lead to valuable information on structure-activity relationships.

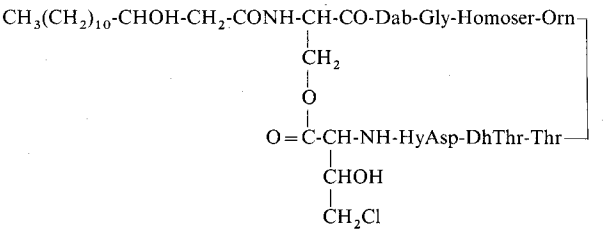
A strain of *P. syringae* pv. *syringae* isolated from sugar cane in Japan has been reported to produce syringomycins, and the structure of the major component has been determined as **9** by FAB-MS and NMR data[17]. This compound is identical to SR-E, structure **6**, and the two independent studies on syringomycin therefore make a strong case for the structural assignments made.



Dab = diaminobutyric acid
HyAsp = 3-hydroxyaspartic acid; α-peptide linked
DhThr = 2,3-dehydrothreonine

9

Syringotoxin is another lipodepsinonapeptide (A. Ballio, personal communication of unpublished data). 3-Hydroxytetradecanoic acid acylates an N-terminal serine (as in SR-G) on which the macrocyclic lactone is closed. The other amino acid units are equimolar amounts of 4-chlorothreonine, 3-hydroxyaspartic acid, 2,3-dehydrothreonine and 2,4-diaminobutyric acid (as found in SR) and also glycine, homoserine, ornithine and threonine (differing from SR). The amino acid sequence of ST has been determined, and the structure established as **10** (A. Ballio, personal communication of unpublished data).

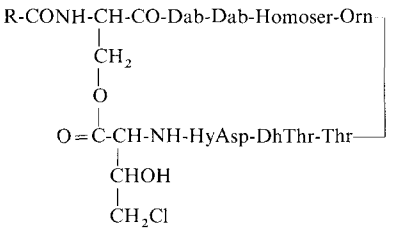


HyAsp = 3-hydroxyaspartic acid; α-peptide linked
DhThr = 2,3-dehydrothreonine

10 syringotoxin

Syringostatins, like SR, exist as a family of lipodepsinonapeptides[27]. The various components of the SS mixture were resolved by HPLC. The two most abundant, SS-A and SS-B, also had strong antifungal activity and the structures of these have been elucidated[28] as **11** and **12**. Compared with syringomycins the syringostatins have differing amino acid compositions, and differing amino acid sequences where some residues occupy equivalent positions (N-acylser, dab, 2,3-dehydrothreonine, 3-hydroxyaspartate and 4-chlorothreonine residues). In contrast the structure of SS differs from that of ST in only one amino acid residue (Dab replacing Gly residue). Thus SS and ST are structural analogues and consideration needs to be given to adopting one generic name for these compounds. If the amino acid residues are numbered clockwise from N-acylserine, then SS should be-

come 3-(dab) syringotoxin. Preference given to ST over SS is historically based.



Dab = diaminobutyric acid
HyAsp = 3-hydroxyaspartic acid; α-peptide linked
DhThr = 2,3-dehydrothreonine

11 SR-A: R = -CH_2-CHOH-CH_2-$(Ch_2)_9$-CH_3
12 SS-B: R = -CH_2-CHOH-CHOH-$(CH_2)_9CH_3$

The apparent ease of hydrolysis of SS-A and SS-B to linear N-acylated peptides without chlorine[28] indicates the possibility that minor components of the SR complex and SS complex are extracellular products and not direct cellular biosynthetic products.

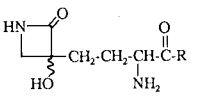
Biosynthesis. Little is known about syringomycin biosynthesis. Large proteins have been characterised that are suggested to function as peptide synthetases in the biosynthesis of SR[114] and ST[68].

Ecology. The production of syringomycin by *P. syringae* pv. *syringae* may be an important factor in the ecology of the organism, since it was found in 55 out of 75 strains from various hosts[23]. Production of SR is dependent upon iron, and under conditions of low iron siderophore production may be closely associated with maintenance of SR production[21,22]. SR acts in an as yet unknown way on the plasma membrane of the host causing a cascade of physiological/biochemical changes which have individually been characterised by the work of Takemoto and colleagues (refer to R. D. Durbin, pp. 776 in this issue). Some indication of the importance of SR to the ecology of the organism has come from the study of tox^- mutant strains of *P. syringae* pv. *syringae* obtained by Tn5 transposon mutagenesis[113]. SR was not required for pathogenicity but contributed significantly to virulence – lesions of tox^- strains were approximately one third the size of those from tox^+ strains. However, this result must be treated with caution as it was based on the presumption that a non-quantitative assessment of tox^- strain by *G. candidum* bioassay corresponded to non-toxigenicity in planta. It is known that the production of SR is sensitive to the growth conditions[21], and also that different strains respond differently to variations in conditions. A quantitative assessment, such as by the method of Ballio et al.[4], of SR components produced in liquid cultures of mutant strains could add stronger weight to the conclusions. Although the tox^- mutants of Xu and Gross[113] grew to similar population levels in planta as the parental strain, data was not given on comparative population levels over a longer period of time than 3 days. It is possible that SR contributes to the longer term survival of *P. syringae* pv. *syringae* in planta, as is the case with tox^+ strains of other *P. syringae* pathovars. In contrast, another group of tox^- mutant strains were non pathogenic and produced population levels much lower (>200-fold) than the wild type. Again, data for populations beyond 3 days were not given, but the implication in this case is that without production of SR these tox^- strains are considerably disadvantaged.

There are many unanswered questions concerning this group of toxins. How do the structural variations affect biological activities? Are there variations in modes of action with structural variation? How do citrus isolates and lilac isolates differ genetically from other *P. syringae* pv. *syringae* isolates? Is there phylogenetic significance in the production of the different toxins SR, ST and SS? Does the presence of different toxins reflect the adaptation of the organism within different ecological niches?

Tabtoxin

Natural occurrence, chemistry. The chemistry of the tabtoxin family, comprising the three compounds **13**, **14**, and **15** has been reviewed[47,50]. Various combinations of these compounds are produced by *P. syringae* pv. *tabaci*, pv. *coronafaciens*, and pv. *garcae*, depending upon the



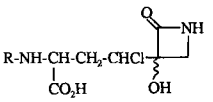
13 R = OH; tabtoxinine-β-lactam (TβL)
14 R = -NH-CH-CO_2H; tabtoxin
CH(OH)-CH_3
15 R = -NH-CH-CO_2H; (2-serine)tabtoxin
CH_2OH

growth environment or culture conditions and on the strain. Two pathovars associated with this group are *P. syringae* pv. *angulata* and pv. *striafaciens* which are considered to be non-toxigenic forms of *P. syringae* pv. *tabaci* and pv. *coronafaciens* respectively[9,112]. Tabtoxin and TβL are the predominantly significant phytotoxins while (2-serine)tabtoxin is only a minor component. Experimental evidence suggests that tabtoxin is the primary intracellular metabolic product of *P. syringae* pathovars[12]. The mechanism of occurrence of TβL, both in culture and in planta, is by amide bond cleavage of tabtoxin by aminopeptidase, both within the cell and extracellularly[38,108]. The majority of *P. syringae* pv. *tabaci* strains (14 out of 17 tested) produce TβL both in the intercellular fluid of plants infected by the strains, and in liquid culture supplemented with Zn^{2+}[12,13]. In contrast, a small proportion of *P. syringae* pv. *tabaci* strains (3 out of 17 tested) produced only tabtoxin both in culture and in planta[12]. The occurrence of TβL has been ascribed to the presence of a Zn-dependent dipeptide-specific aminopeptidase present in the periplasm of

P. syringae pv. *tobaci*[38], and a correlation was found between the production of TβL in liquid cultures containing 25 μM Zn^{2+} and the occurrence of aminopeptidase activity in a periplasmic extract of cells. The three strains of *P. syringae* pv. *tabaci* that produced only tabtoxin in culture and in planta lacked this activity. Non-toxigenic strains of *P. syringae* pv. *tabaci* were found to retain the aminopeptidase activity, and therefore may have derived at an earlier time from the toxigenic form.

It is apparent that ecologically the most common and the most significant extracellular product of *P. syringae* pv. *tabaci* and related strains is TβL, and that the primary intracellular biosynthetic product of the organism is the dipeptide tabtoxin[11].

The report of a dipeptide **16** resembling tabtoxin from a *Streptomyces* sp.[89] indicates these compounds may be more widespread in nature than presently known. However, it is not known whether **16** is phytotoxic, but since the TβL moiety is the N-terminal residue rather than C-terminal as in tabtoxin, different ecological factors might be involved.



16 R= Ala

Biosynthesis. A detailed analysis of the distribution of ^{13}C label in TβL biosynthesized in the presence of D-[1-^{13}C]glucose, D-[2-^{13}C]glucose and D-[U-^{13}C]glucose[110] enabled the origin of the carbons of TβL to be identified (fig. 4). The four carbons of aspartic acid were carried through to give the same labelling pattern in TβL, showing that aspartic acid is a key biosynthetic precursor. The structural homology between the carbon skeleton of TβL and lysine indicates that the intact aspartate carbon skeleton may enter into at least the early stages of the lysine biosynthetic pathway. This involves the condensation of aspartate semialdehyde with pyruvate, and the decarboxylative loss at some stage of C that was the C-1 of pyruvate. Lysine was not incorporated into TβL[110] and therefore if any part of the lysine biosynthetic pathway is utilised, it is an early portion of it.

Now that the nature of the 'building blocks' of tabtoxin have been exposed, the details of the pathway can be explored. Many steps, each controlled by different gene products, are likely to be specific to this pathway. As pointed out[110], knowledge of the biosynthetic pathway is an essential component to the overall broad understanding of the organism and its ecology.

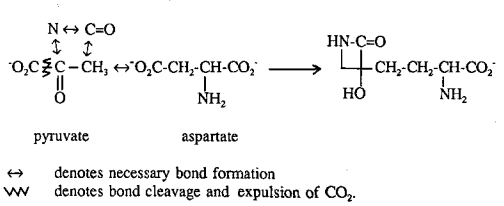


Figure 4. Biosynthetic origin of tabtoxinine-β-lactam[110].

Ecology. A powerful function of TβL in planta is the disruption of normal metabolic function by the inactivation of glutamine synthetase[35, 36, 106]. In contrast, tabtoxin itself does not have this inhibitory function. Therefore, the mechanism developed by a high proportion of *P. syringae* pv. *tabaci* to ensure TβL, rather than tabtoxin, is the extracellular product would appear to be ecologically advantageous. The few strains reported that do not have this capacity and produce only tabtoxin as the extracellular product are an interesting case. Do these represent a more ancient form of *P. syringae* pv. *tabaci*? Conversely, is the capacity to produce TβL a relatively recent evolutionary development? An important corollary to this is whether tabtoxin has an alternative target site in planta; however, this is not absolutely a necessary requirement since plant enzymes themselves do have the capacity to hydrolyse tabtoxin to TβL[108], and studies to date[104] have found no evidence for another site of action.

The self protection of *P. syringae* pv. *tabaci* from TβL is an important ecological topic. Unlike the OTCases of *P. syringae* pv. *phaseolicola*, the glutamine synthetase of *P. syringae* pv. *tabaci* has only one form and this is susceptible to inactivation by TβL[101]. One possible protection mechanism could result if the liberation of TβL from tabtoxin occurred at a location that normally prevented contact with the glutamine synthetase, although however, other protection mechanisms have been established. One of these is an adenylation of glutamine synthetase, rendering it less susceptible to inactivation by TβL[31]. Adenylation is activated at the onset of TβL formation, and has been suggested to be initiated by threonine released from tabtoxin during TβL formation. Another mechanism of protection involves a β-lactamase[32] which hydrolyses the β-lactam ring of TβL to tabtoxinine, the open chain product. This activity appeared to be located either in the inner membrane or within its envelope, and represents a mechanism to inactivate any TβL that threatens to make contact with intracellular glutamine synthetase.

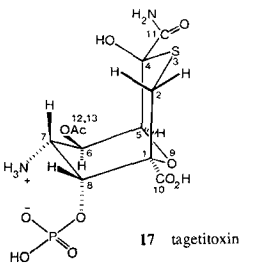
Ecological benefits to an organism producing TβL are very likely. Because glutamine synthetase is of universal importance with an apparent conserved active-site, TβL can be regarded to have general biocidal activity that may be significant to the general adaptability of the organism to different environmental circumstances, or hosts. The toxin enhances the virulence of the organism[107] and is important for the survival of the organism, since toxigenic strains of *P. syringae* pv. *tabaci* supported a 10-fold higher population density than a non-toxigenic strain 14 days after inoculation, in spite of similar growth rates during the first 3 days[107]. Turner suggested[106]

that the higher population levels achieved by virtue of toxigenesis is the result of a less hostile environment as a consequence of an alteration of the properties of toxin-affected tissue.

Tagetitoxin

Natural occurrence, chemistry. Tagetitoxin is produced by only one bacterial species, *Pseudomonas syringae* pv. *tagetis*, which is a pathogen of several species of *Compositae*[85]. A significant disease symptom in each case is a chlorosis of newly developing leaves at the plant apex. In spite of this, most strains of *P. syringae* pv. *tagetis* growing in liquid culture impart very little, or no biologically active component to the culture fluid[10]. However, a limited number of isolates are capable of producing a relatively high titre of biological activity in vitro, and in this instance tagetitoxin was the single chemical component obtained that was responsible for the phytotoxic (apical chlorosis) activity[56]. The bicyclic structure of tagetitoxin **17**[63], was recently revised from that previously proposed[59] on the basis of FAB mass spectrometry and more extensive NMR data. Small quantities (10–20 ng) of the pure toxin cause apical chlorosis symptoms in plants identical to those caused by infections with *P. syringae* pv. *tagetis*. Nothing is currently known however about specific structural features of this molecule which might be important for its biological activity. Biosynthetic studies have not been undertaken.

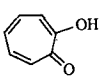
Ecology. Tagetitoxin is known to inhibit RNA formation in the chloroplast[10] but it is not known how this might be ecologically advantageous to the organism. Tagetitoxin is not known to have antibiotic properties.



17 tagetitoxin

Tropolone

Natural occurrence, chemistry, phytotoxic effects. Tropolone, structure **18**, was first reported as a product from



18 tropolone

an unidentified species of *Pseudomonas*[40]. Subsequently, tropolone has been reported from an unrelated pseudomonad that was pathogenic on rice seedlings[2]. The pure toxin (tropolone) inhibited growth of rice seedlings, and inhibited root elongation, effects that correlated with symptoms on rice seedlings infected with the pseudomonad[2]. This *Pseudomonas* sp. has since been described as a new species, *Pseudomonas plantarii*[1].

Ecology. Tropolone is bacteriostatic for both Gram-positive and Gram-negative species, and at concentrations higher than 0.02 M it is bacteriocidal against both[40, 102]. Lindberg et al.[40] noted that possibly there was ecological significance in the isolation of their tropolone-producing pseudomonad among colonies of *Helminthosporium cynodontis* isolated from Bermuda grass (*Cynodon dactylon*). Clearly *P. plantarii* has a resistance mechanism to counter the potential adverse effects of tropolone, and one can speculate that the production of the compound offers the organism an ecological competitive advantage. A comparison of the data of Trust[102] on the bacteriocidal activity of tropolone with that of Azegami et al.[2] on the biological effects of tropolone on rice suggests a minimum bacteriostatic tropolone concentration of ca. 0.7 μg/ml in the former case, and a minimum concentration of ca 10 μg/ml for growth effects in rice. This may suggest that the antibacterial properties of tropolone are ecologically more significant for *P. plantarii*.

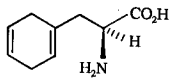
The tropolone ring structure occurs naturally within the family Cupressaceae, such as in 4-isopropyltropolone (thujaplicin). Such compounds also have bacteriostatic and antifungal properties[103] and it is possible that these tropolone derivatives contribute significantly to the extreme durability of wood from genera of this family[102]. Whether there are any evolutionary links between the genes encoding the biosynthesis of tropolone in bacteria and higher plants is a question open to speculation.

Other toxins

The material discussed in this review has been restricted to chemically defined phytotoxins. In the many other reports of phytotoxic effects in various bacterial plant pathogenic interactions, in some cases further work is necessary to characterise a particular phytotoxin and to establish its biological role, and in other cases, proof of the functioning of a specific chemically-definable phytotoxin has not been achieved. This latter situation can arise for many reasons, including difficulties with a chemically labile or interactive chemical component, or difficulties with biological variability such as organisms toxigenic in planta not producing sufficient quantity (if any) in culture. There are also many well established cases of bacteria that produce plant hormones such as auxins and cytokinins, which have not been discussed here (for examples, see Gross and Cody[22]).

The remaining examples of toxins have recently been chemically defined, but little information is yet available on how they function, or on their biological significance. It is hoped that mentioning them will encourage further studies on biological aspects to be undertaken.

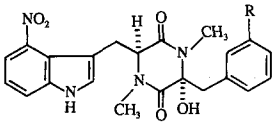
Fireblight toxin. Erwinia amylovora, which causes fireblight disease on rosaceous species, produces in a vigorously aerated minimal medium an active component that causes growth inhibition and a grey discoloration of cultured pear cells[15,16]. This has been purified and shown to be L-(−)-2,5-dihydrophenylalanine **19** (DHP). There seems to be good evidence to suggest that this chemical



19 DHP

is responsible for the widespread necrosis symptoms that are characteristic of fireblight disease. DHP is a reducing agent, and readily dehydrogenates to form phenylalanine, a reaction that has hampered isolation and characterisation of DHP. The compound interferes with normal metabolic activity – it is a known enzyme inhibitor and inhibits the growth of microorganisms[16] – and on this basis there are many possible ways in which DHP may contribute to the ecological success of *E. amylovora*.

Thaxtomin. Streptomyces scabies causes a disease of potato tubers that is characterised by scab-like symptoms. When *S. scabies* was cultured on aseptically cultured minitubers two chemical components were produced that induced scab-like lesions on potato tubers. These have been isolated and characterised[29,37] and their chemical structures, **20** and **21**, were elucidated from FAB-MS and NMR data[29]. The two compounds, thaxtomin A and B, have also been isolated from the scabby tissue of infected field grown potato tubers.

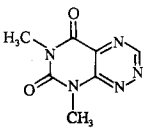


20 thaxtomin A R = OH
21 thaxtomin B R = H

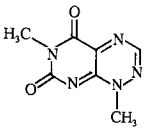
The two compounds can be envisaged to be derived biosynthetically from the combination (diketopiperazine ring formation) of two amino acids: 4-nitrotryptophan with α-hydroxy-m-tyrosine for thaxtomin A and 4-nitrotryptophan with α-hydroxyphenylalanine for thaxtomin B, and at some point methylation of both N functions. The 4-nitro substituent of the indole ring of tryptophan is an unusual feature which may be a characteristic of this type of compound.

In an independent earlier report[87] extracellular products that were inhibitory to the root-growth of rice seedlings were found in liquid cultures of *Streptomyces* sp. associated with potato scab. The chemical components responsible for this activity are probably different from the thaxtomins, as it is implied by King et al.[29] that the thaxtomins were not produced in the medium of Sakaii et al.[87]. A number of biologically-active products of *Streptomyces* therefore may be present in the infection of potato tubers.

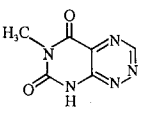
P. glumae toxins. Pseudomonas glumae, which causes grain rot of rice, produces several related compounds that induce chlorosis on leaves of rice seedlings at concentrations as low as 10 μg/ml, and that cause growth reduction in the leaves and roots[88]. These are fervenulin **22**, toxoflavin **23**, and reumycin **24**.



22 fervenulin



23 toxoflavin



24 reumycin

3-Methylthiopropionic acid, and other low MW carboxylic acids. A product of *Xanthomonas campestris* pv. *manihotis* in liquid culture responsible for producing blight symptoms in cassava leaves, similar to those induced in bacterial infections, has been identified as 3-methylthiopropionic acid, **25**[80]. This compound has also been detected in extracts of cassava leaves infected with *X. campestris* pv. *manihotis*[79] and a correspondence was

CH_3-S-CH_2CH_2-CO_2H **25** CH_3-S-CH=CH-CO_2H **26**

noted between the appearance of blight symptoms, the approach of the maximum bacterial population, and an increase in 3-methylthiopropionic acid.

Other workers have reported low molecular weight carboxylic acids, including **25** and **26**, produced by *Xanthomonas campestris* pv. *oryzae*[71] and that these were toxic to rice seedlings. Robeson and Cook[86] reported the production of **25** and **26** in liquid cultures of *Xanthomonas campestris* pv. *campestris*, pv. *amoraciae*, pv. *raphani* and pv. *carotae*. The two compounds arose from the methionine used in the culture medium, and did not occur in the absence of methionine. However, the result of Perreaux et al.[79] from *X. campestris* pv. *manihotis* indicates that in the plant the biosynthetic mechanism is operative, and the necessary precursors are available to the organism for the production of 3-methylthiopropionic acid. The two compounds **25** and **26** inhibited the growth of cabbage seedlings[86], but the magnitude of the inhibition was inversely related to the pH of the solution applied and was also not specific for **25** and **26** since compounds such as benzoic acid and isovaleric acid behaved similarly. It was therefore suggested that such low molecular weight carboxylic acids are unlikely to play a major role during pathogenesis. However, they may contribute non-specifically to the ecology of the organisms. Certainly my own observations show that most bacteria produce varying amounts of carboxylic acids in liquid culture[48] that are assumed to be generally of low molecular weight. Unknown factors are the capacity and rate of strains to produce such compounds in planta. The available data suggests that there will be biological effects at least above a certain concentration.

Distribution of bacterial phytotoxins across genera

Toxins*	*Xanthomonas*	*Pseudomonas*	*Streptomyces*	*Bradyrhizobium*	*Erwinia*
3-MTPA	●				
Coronatines	●	●			
Phaseolotoxin		●			
Tagetitoxin		●			
Lipodepsinonapeptides**		●			
Tropolone		●			
Tabtoxins		●	●		
Rhizobitoxines		●	●	●	
Thaxtomin			●		
Dihydrophenylalanine					●

3-MTPA = 3-methylthiopropionic acid; * the plural case = the family of toxins; ** these include syringomycins, syringotoxin, syringostatins.

Overview

Bacterial phytotoxins considered here are secondary metabolites of low molecular weight and diverse chemical structure. As seen in this review, the chemical oddities that occur, and the overall chemical variety of phytotoxins, demonstrates that there are many specific biosynthetic steps, and associated specific genes, involved.

Our current knowledge on the distribution of various phytotoxins in nature is sparse. There are good indications however that the genetic material encoding for the biosynthesis of some toxins is apparently widespread in nature (table). This is seen with rhizobitoxines, found in the genera *Bradyrhizobium*, *Pseudomonas*, and *Streptomyces*, and with coronatines, found in *Pseudomonas* and *Xanthomonas*. The evolutionary relationships need to be explored using recent advances in molecular genetics. Variation in the nature of the final products is observed, showing that there is some divergence in the biosynthetic pathways between the different organisms. Some variation may be a consequence of the different cellular environment of the toxin-producing genes or may reflect the differences in particular ecological niches of the organisms concerned.

Some toxins, such as phaseolotoxin and tagetitoxin, are unique in their restriction to a single species or pathovar. This may merely reflect an inadequacy in the scientific search, but if this is the case in reality then one must ask how and why the genetic material has *not* been passed on? Does this again tell us something about the ecology and evolution of such organisms?

There is considerable progress being made in the studies of bacterial phytotoxins. Interwoven interdisciplinary activities are a significant factor towards the progress and it is important that this continues to develop. Many research areas are worthy of more activity, such as the biochemistry of the many specific steps involved in phytotoxin synthesis, mutant analyses and ecological studies by microbiologists to define ecological roles of phytotoxins, and studies of genes encoding toxin synthesis to develop a phylogeny of toxigenesis in phytopathogenic bacteria.

Acknowledgments. I am very grateful to R. E. Beever and M. D. Templeton for their critical evaluations of the manuscript, and to Julie Bradley for the preparation of the manuscript.

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