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The bacterial *hrp* genes which induce the hypersensitive response in plant.

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Introduction

In nature plants are resistant to the majority of pathogens, and many bacterial live in close contact with the plant without causing any harm. Most commonly, this bacteriaum is found growing asymptomatically on plant surfaces. The ability of a strain to cause disease thus is the exception rather than the rule. To be a successful pathogen the invading bacterium has to overcome the plant's defense. During evolution plant pathogenic bacteria have acquired multiple functions that enable them to colonize and multiply in living tissue. Among 1600 different species known in the bacterial kingdom only a small number (about 80) are plant pathogenic and in most cases highly specialized with respect to the plant that can be attacked. .

Many strains of *Pseudomonas syringae* have the capacity to cause disease in several economically important plant species (Agrios, 1988). Disease occurs when bacteria are introduced into tissue of a susceptible plant species and begin to multiply in the intercellular spaces. Tissue necroses, called leaf blights, commonly develop following colonization of tissue, but some strains have the capacity to induce tissue hyperplasias. Extracellular polysaccharides, peptide toxins, and phytohormones appear to be the major virulence factors for this bacterium (Long and Stasckwaic, 1993)

Individual straisn of *P. syringae* usually only cause disease in one or a few plant species, and in some cases, are limited to specific variants (cultivars or ecotypes) of a plant species (Keen, 1992). In most other plants, a *P. syringae* strain elicits a set of inducible cellular defense responses which restricts further multiplication of the bacteria and invasion of tissue. The plant's defense mechanisms include the production of active oxygen species, secondary metabolites with antimicrobial activity and hydrolytic enzymes with lysozymal activity as well as alteration of plant cell wall structure (Keen, 1992). The initiation of these defense mechanisms is the hypersensitivie response (HR). The HR was first observed as a rapid localized collapse of

tobacco leaf tissue after infiltration of high numbers of bacterial pathogens that are host specific for other plant species (Klement, 1963; Klement et al., 1964). Low levels of inoculum are sufficient for *P. syringae* initiate pathogenesis., but high levels (>5x106 cells/ml) are required for *P. syringae* to cause the macroscopic collapse. An incompatible pathogen at lower concentrations cuases the HR only in scattered, individual plant cells, with one bacterium eliciting the death of one cell. The macroscopic HR is a laboratory manifestation of a cellular hypersensitivity that can occur under natural conditions. The HR can be elicited in nonhosts or resistant hosts by most plant pathogenic bacteria, including *Xanthomonas* spp., *Pseudomonas* spp., *Erwinia* spp., and *Ralstonia solanacearum*. However, it is important to emphasize that the HR is not elicited by nonpathogen species like *P. fluorescens*. This implies an underlying relationship between the ability to be a plant pathogen and to elicit the HR. Because the ability to elicit the HR is a unique attribute of the necrogenic pathogens and these bacteria can avoid or suppress its elicitation in their hosts, the HR phenomenon appears central to bacterial pathogenicity and host specificity (Klement, 1982; Goodman and Novacky, 1994; Dangle et al., 1996).

Two classes of genes have been identified which affect the ability of *P. syringae* strains to elicit the HR and thus affect the host range. The *hrp* genes control the basic pathogenicity of a *P. syringae* strain in susceptible plant hosts and are also essential for the elicitation of the HR in nonhost plant species (Bonas, 1994; Willis et al., 1991). Most *P. syringae hrp*::Tn mutants lose both pathogenicity in the susceptible plant species as well as the ability to elicit the HR in other nonhost plant species (Huang et al., 1991; Lindgren et al., 1986). A *hrp* cluster cloned from *P. syringae* pv. *syringae* Pss61 enables nonpathogenic bacteria, like *E. coli* and *P. fluorescens*, to elicit the HR in tobacco (Huang et al., 1988). In addition to the *hrp* genes, many *P. syringae* strains also carry avirulence (*avr*) genes which fucntion to restrict the host range of the strain to those variants of the susceptible plant species which lack a corresponding resistance (R) gene product (Dangle, 1994; Staskawicz et al., 1995).

Isolation, organization and function of hrp genes

hrp genes have been isolated from all major gram-negative plant pathogenic bacteria excep *Agrobacterium*. The *hrp* genes were originally described for the bean pathogen *Pseudomonas syringae* pv. *phaseolicola* by identifying Tn5 transposon mutants that grew normally in minimal media but failed to elicit the HR in nonhost tobacco or cause disease or multiply in host bean (Lindgren et al., 1986). Since then *hrp* gene clusters have been cloned from a number of different bacteria. In all of the cases, the *hrp* genes are organized in clusters of 22-40 kb. The *hrp* clusters of *P. s. syringae* Pss61 and *E. amylovora* Ea321, carried on recombinent cosmids pHIR11 and pCPP430, respectively, enable nonpathogenic bacteria such as *P. fluorescens* and *E.*

coli to elicit the HR in tobacco and many other plants.

Mutational analyses have shown that most *hrp* genes cluster in a 25 kb region of the chromosome and are conserved among *P. syringae* strains (Lindgren et al., 1988). The complete mucleotide sequence of the *hrp* gene cluster isolated from a strain of *P. syringae* pv. *syringae* (Pss61) has recently been assembled through the efforts of several laboratories (He et al., 1993; Heu and Hutcheson, 1993; Huang et al., 1992; 1995; Lidell and Hutcheson, 1994; Xiao et al., 1994). The deduced organization of the cluster indicates the presence of 26 genes organized as at least 8 transcriptional units. Striking similarities have found between the *hrp* genes of pathogens belonging to different genera. (Figure 1).

DNA sequence analysis of the *hrp* genes has revealed some important clues to their possible biochemical functions. Extensive similarities exist between the deduced gene products of the *hrp* cluster and virulence determinants of enteric bacteria pathogenic to mammals, such as the *ysc* genes of *Yersinia* spp., the *spa/mxi* genes of *Salmonella* and *Shigella* and the *sep* genes of enteropathogenic *E. coli* strains, that are required for the secretion of virulence proteins (Figure 2) (Huang et al., 1995). Similarities are also observed with the *fli* genes of *Bacillus* and *Salmonella* that function in flagellar biosynthesis (Huang et al., 1995; Hutcheson et al., 1994; Lidell and Hutcheson, 1994). Most of the genes exhibiting similarities to the *P. syringae hrp* gene products appear to function in the recently described Type III protein secretion pathway, thereby establishing the existence of the conserved type III secretion system in Gran-negative bacteria (Salmond and Reeves, 1993; Vangijsegem et al., 1994). This has led to nomenclatural changes and refinement of the *hrp* gene (Bogdanove et al., 1996). The nine *hrp* genes that are broadly conserved in plant and animal pathogens have been redesignated as *hrc* (hypersensitive response and conserved). The *hrp* genes, and particularly the hrc subset, are now considered tobe fundamentally involved in type III protein secretion in phytopathogenic bacteria. Proteins secreted by this mechanism lack an obvious N-terminal signal sequence and do not appear to be processed during the secretion process. In *Yersinis* strains, each secreted Yop protein also has a dedicated chaperone to facilitate the secretion process (Cornelis, 1994). The mechanism for protein secretion by the Hrp pathway has not been fully elucidated. An indication of the organization of the *hrp* gene products in the membrane may be indicated by the similarities with the gene products of many of the late genes invloved in flagellar biosynthesis. These genes form the secretion system for flagellin and the physical relationships of several gene products established (MacNab, 1992; Shapiro, 1995). FliF is thought to form the mounting plate for the secretion system. Attached to the mounting plate is a switch complex which includes FliG and FliN. Built upon this complex is the export apparatus formed from FlhA and FliI together with other proteins(Possibly FliJ, FliP, FliQ, FliR and FlhB). FlhB has been postulated to function as a gatekeeper for the secretion apparatus (Shapiro, 1995). Since homologs to these proteins are present in the *P. syringae hrp* cluster, it tempting to speculate that the Hrp gene products may

form a similar structure in the inner membrane. Consistent with this hypothesis, HrpJ2, HrpU4, HrpU5, HrpU6, HrpU7 (also known as HrpI, HrpW, HrpO, HrpX, HrpY, respectively) contain regions of hydrophobic residues typical of transmembrane domains (Huang et al., 1992; Huang et al., 1995) and suggestive of an inner membrane location. HrpH2 (also known as HrpH) has properties of an outer membrane protein (Huang et al., 1992). HrpJ4 appears to be an ATPase and is predicted to be associated with the cytoplasmic face of the inner membrane (Lidell and Hutcheson, 1994).

At least one protein, termed $Harpin_{pss}$, has been shown to be secreted by the Hrp secretion system and to function as an elicitor of the HR in some plants, such as tobacco (He et al., 1993). $Harpin_{Pss}$ is the product of the second gene of the *hrpZ* operon (*hrpZ2*) (Huang et al., 1995; Xiao and Hutcheson, 1994). It is a 35 kD protein which is relatively glycine-rich and heat-stable. Deletion derivatives of *hrpZ2* carrying either the N or C portion of the protein retain at least partial elicitor activity, suggesting that at least two domains in the protein carry HR eliciting activity (Collmer et al., 1994). Specific deleiton of the *hrpZ2* gene in several strains of *P. syringae*, however, had little or no effct on the pathogenicity of the mutant or its ability to elicit the HR. Since inactivation of the genes encoding components of the secretion system causes a loss of pathogenicity and ability to elicit the HR, this observation suggests other proteins necessary for pathogenesis are secreted by the hrp secretion system. Recently, several other proteins have been identified which may also be secreted by the *hrp* secretion system in *P. syringae* pv. *tomato* DC3000(collmer et al., 1994). It has not yet been established whether nay of these secreted proteins function as an elicitir of the HR or are required for pathogenicity. The specific biochemical fucntion of these proteins has not been elucidated. The earliest effects of harpin detected in plant cells responding hypersensitively include production of active oxygen and leakage of K+ from plant cells (Baker et al., 1993)..

Regulation of *hrp* genes

The expression of *hrp* genes is affected by growth conditions, including amino source, carbon source, osmoticum, and pH (Huynh et al., 1989; Rahme et al., 1992; Xiao et al., 1992). Thus far several positive acting transcription factors have been identified in the *hrp* cluster which mediate the environmental regulation of *hrp* genes. HrpR and HrpS share sequence similarity with members of a large protein family that usually function in two component regulatory systems and activate σ 54-dependent promoters (Felley et al., 1991; Grimm et al., 1995; Grimm and Panopolos, 1989; Xiao et al., 1994). While the domains deduced to interact with σ 54-RNA polymerase holoenzyme and a helix-turn-helix involved in DNA binding (Morett and Segovia 1993) are detected in both HrpR and HrpS, the N-terminal domains thought to

function in the modulation of the regulatory activity are absent in both proteins. The *hrp*R gene appears to be expressed from σ 70-dependent promoter located about 100 bp upstream of the initiation codon. The Pss61 *hrp*R and *hrp*S ORF's are separated by a 50 bp intergenic region which lacks an obvious terminator or sequences similar to known promoter consensus sequences, suggesting these genes may be part of a single transcriptional unit.

By using plasmid-borne constructs to express the Pss61 *hrp*R and/or *hrp*S in an *E. coli* strain singly or in combination, HrpR and HrpS were shown to be positive activators of the hrpL promoter (Xiao et al., 1994). Both HrpR and HrpS were required to activate the *hrpL* promoter in E. coli. A dissection of the *hrpL* promoter revealed the presence of a strong candidate σ 54 promoter as predicted from the deduced structural features of HrpR and HrpS (Hutcheson et al., 1994; Zhang and Jones, 1995). The transcriptional start site was consistent with the functionality of the σ 54 promoter consensus sequence and the HrpR/S-dependent activity of the *hrpL* promoter was lost in a *rpoN* mutant of *E. coli* (Heu and Hutcheson, 1997). Proteins similar to HrpR and HrpS frequently bind to enhancer-like elements located 100-500 bp upstream of a σ 54 promoter (Morett and Segovia, 1993). Approximately 100 bp upstream of the σ 54 promoter consensus sequence in the *hrpL* promoter is a region of dyad symmetry similar to an enhancer-like element (Hutcheson et al., 1994). Deletion analysis showed this region to be essential to *hrpRS*-dependent activation of the *hrpL* promoter (Heu and Hutcheson, 1997).

The HrpL gene product has properties which suggest that it functions as an alternative sigma factor controlling transcription of *hrp* and *avr* genes (Xiao et al., 1994). It is a required positive acting transcriptional factor for the *hrpK, hrpJ, hrpU, hrpH,* and *hrpZ* operons as well as for several alternative sigma factors such as AlgU and CnrA (Xiao et al., 1994). A conserved bipartite sequence motif, originally identified in the promoter active regions of several *avr* genes (GGAACCNAN14CCACNNA) (known as a hrp box or avr box) (Innes et al., 1993; Salmeron and Staskawicz, 1993; Shen and Keen, 1993), has been shown to function as a HrpL-dependent promoter (Xiao and Hutcheson, 1994). This putative promoter motif shares in common with the promoters recognized by related sigma factors a conserved -35 region but only weak similarity is observed in the -10 region (Lonetto et al., 1993; Salmeron and Staskawicz, 1993; Shen and Keen, 1993).

These results indicate that the expression of the hrp cluster is controlled by a regulatory cascade involved σ 70 (*hrpR* promoter), σ 54 (*hrpL* promoter), and σ L (various HrpL-dependent promoters). An alternative model for the action of HrpR and HrpS has been proposed which suggests HrpS alone functions to regulate expression of the *hrp* genes in *P. syringae* strains (Grimm et al., 1995). An overview of the apparent *hrp* regulatory network is shown in Figure 3.

The genetics of *hrp* regulation are surprisingly different in X. *c. vesicatoria* and *R. solanacearum*. There is no hrp box sequence in *Xanthomonas hrp* gene promoters. Another

sequence motif that occurs in the promoter region of *hrp* loci in *X. c. vesicatoria* was recently identified. This PIP (plant inducible promoter) box has the sequence TTCGC-N15-TTCGC and occurs upstream of the -35 consensus sequence in four out of six *hrp* promoters (Fenselau et al., 1996). *R. solanacearum hrp* expression is dependent on HrpB, a member of the AraC family of positive activators, and the homologous HrpX appears to have the same function in *Xanthomonas* spp (Genin et al., 1992; Wengelnkik and Bonas, 1996).

Interaction of *hrp* and *avr* genes

A long standing quandary in plant-microbe interactions has been the function of *avr* genes. *avr* genes have been cloned from a number of *P. syringae* strains and demonstrated to control race-spcific interactions with the susceptible plant species (Dangle, 1994; Keen, 1990; Staskawicz, et al., 1995). *P. syringae* strains carrying an avr gene elicit the HR in those cultivars of the susceptible plant species which express the corresponding R gene. Current models to describe these interactions invoke race specific elicitors produced directly or indirectly by *avr* genes which are recognized by receptors in the plant cell that are encoded by R-genes to initiate the HR (Keen, 1992). With the exception of *avrD* (Midlands et al., 1993), however, the identity of the postulated race specific elicitor has been elusive. Cell-free fractions of bacteria carrying constitutively expressed *avr* genes (other than *avrD*) have failed to exihibit elicitor activity. The deduced gene products thus far lack features indicative of a biochemical activity. Indications that Hrp and Avr gene products might inteact to control the host range of *P. syringae* strains comes from a obsevation that in one strain of *P. syringae* hrp mutations suppressed the phenotype of an *avr* gene (Huynh et al., 1989) and *avr* genes are regulated by the same mechanism as the *hrp* operons in *P. syringae* strains.

Exprimental evidence demonstrating that the *hrp* gene cluster mediates the phenotypic expression of *avr* genes came from studies employing the heterologous expression of *hrp* genes in *E. coli* (Pirhonen et al., 1996). *E. coli* is not a plant pathogen and thus fails to elicits a response when inoculated into tobacco or any other plant. Both the *hrp*-linked secretion system and the *hrp* regulatory system have been shown to be active in *E. coli* (Heu and Hutcheson, 1993; Lidell and Hutcheson, 1994; Xiao et al., 1994). *E. coli* strains carrying the Pss61 *hrp* cluster are capable of eliciting the HR in tobacco but a relatively high inoculum level is required, even when expression of the cluster is increased by the presence of a constitutively expressed *hrpL* construct. In other plants, such as soybean cultuvars or ecotypes of *Arabidopsis thaliana*, a null response is consistently obseved. In *E. coli* transformants carrying a constitutively expressed *hrpL* construct, the cloned *P. syirgnae* Pss61 *hrp* cluster and *avrB* expressed from vector *lacUV5* promoters elicit the HR specifically in those soybean cultivars which carry the reactive

Rpg1 resistance gene (Pirhonen et al., 1996). Similar experiments demonstrated that phenotypic expression of *avrA*, *avrC*, *avrPto*, *avrRpt2*, *avrRpm1*, and *avrPph3* in *E. coli* also requires an activity of the *hrp* cluster. *E. coli* transformants carrying each of these *avr* genes and the induced Pss61 *hrp* cluster elicited the HR in those soybean cultivars which carry a reactive R-gene. Therefore at least 7 *avr* genes isolated from *P. syringae* strains, phenotypic expression in an *E. coli* strain is dependent directly upon the *hrp* genes.

Subsequent experiments showed that all three activities associated with the *P. syringae hrp* cluster are required for phenotypic expression of *avr* genes. As discussed above, transcription of *avr* genes is controlled by the HrpL-dependent regulatory system. Mutational analyses indicated that both the *hrp*-linked secretion system and harpin production appear also to be necessary for phenotypic expression of *avr* genes. These observations provides an indication of a mechanism by which the Avr phenotype is generated. One possibility could be that the complete *hrp* lcuster is needed for the presentation of the Avr gene product to the plant cell. Alternatively, harpin and the Avr gene product could serve as a co-elicitors in a dual stimulus model or the *avr* gene products could catalyze a posttranslational modification of harpin to product the postulated race specific elicitor.

Concluding remarks

It is now apparent that the *hrp* genes play a central role in the pathogenicity of *P. syringae* strains, controlling both virulence and host range. Thus in phytopathogenic bacteria and in several enteric bacteria pathogenic to mammals, a conserved type III protein secretion system is a necessary component for virulence. The similarity in organization and components of the *hrp*-related secretion systems found in the *P. syringae* strains and the enteric bacteria, such as *Yersinia* strains, suggest a common origin. Because *P. syringae* strains are common epiphytes of many plants and are capable of conjugal transfer of DNA to heterologous bacteria, lateral transfer of a functional secretion system between bacteria could have occured. Alternatively, the similarity with the late genes of flagellar biosynthesis could suggest these secretion systems evolved from a duplication and adaptation of these genes. Further analysis will be necessary to determine if either of these models are correct.

In the mammalian pathogens, the virulence factors secreted by this secretion system facilitate attachment, and in some cases, invasion of target cells. Pathogenesis of *P. syringae* strains differs from that of most mammalian pathogens: strains remain external to the cell wall of living plant cells in colonized tissue. Pathogenesis by both types of pathogens, however, share a common requirement for contact with the host cell before a response is initiated. In view of the similarity with the flagella biosynthesis systems, it may be that TypeIII protein secretion systems

from structures that inject secreted proteins into the parasitized cell (Rosqvist et al., 1994). Since resistance gene products are apparently cytoplasmic (Dangle, 1995), the function of *hrp* genes in *P. syringae* strains might be to introduce an *avr*-linked elicitor or the *avr* gene products directly into plant cells.

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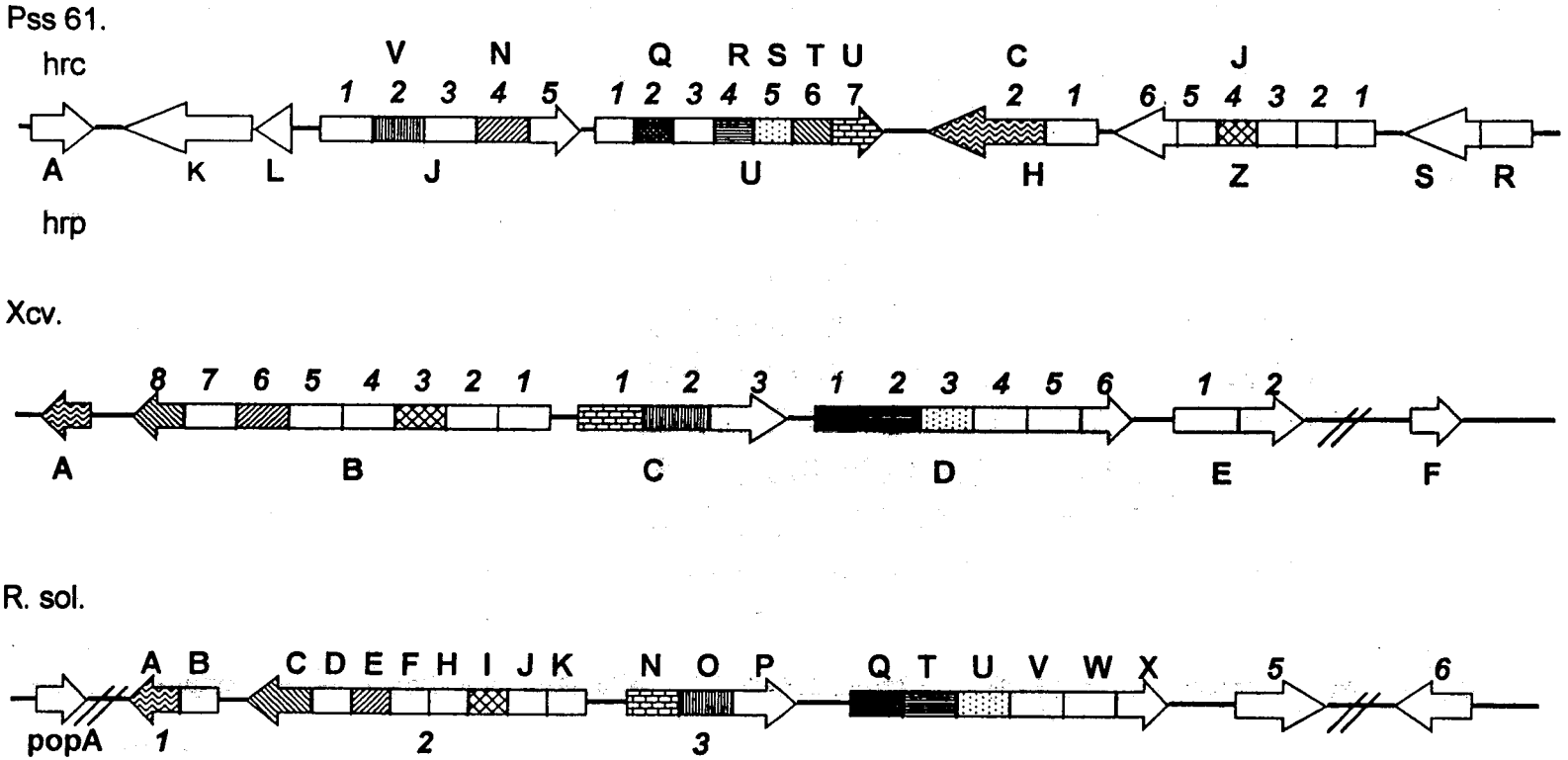

Figure 1. Genetic and translational organization of the hrp gene cluster of different plant pathogenic bacteria. Pss61; *Pseudomonas syringae* pv. *syringae* Pss61; Xcv: *Xanthomonas campestris* pv. *vesicatoria*; R. sol.: *Ralstonia solanacearum* (*Pseudomonas solanacearum*). *hrc* (hypersensitive response and conserved) is a new nomenclature for the conserved *hrp* (hypersensitive response and pathogenicity) genes as mentioned in the text. Arrows represent transcription units as determined by genetic analysis. Boxes correspond to sequences of open reading frames (ORFs) that have been published. In case of sequence similarities between ORFs in different clusters the boxes are filled with the same pattern.

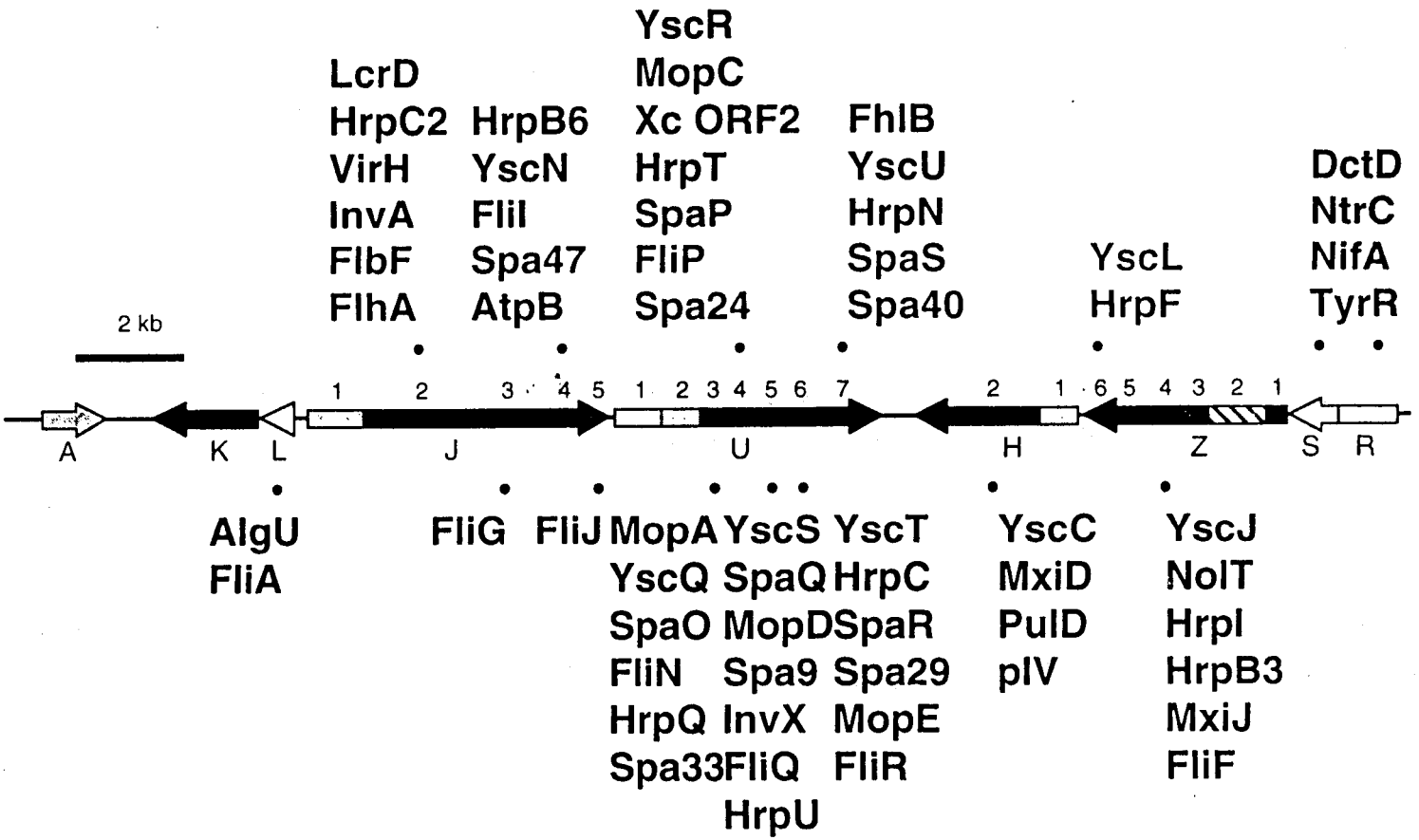

Figure 2. Organization of the *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* cluster and sequence similarities with related proteins. Genes identified within the *P. syringae* Pss61 *hrp* are shown. Direction of transcription is indicated by the arrowheads. Operons designated by the capital letters and genes by numbers. Gray filled boxes indicate *hrp* gene products functioning in protein translocation. The light shaded boxes indicate gene products with regulatory activity. The hatched box indicateds the gene producing $harpin_{pss}$. Gene products exhibiting sequence similarities are indicated. Source organisms: *ysc*, *Yersinia* sp.; *spa & mxi*, *Salmonella & Shigella* sp.; *sep*, EPEC *Escherichia coli*; *mop*, *Erwinia carotovora*; *fli & flh*, *Salmonella & Bacillus* sp.; *Xc*, *Xanthomonas campestris*; *hrp*, *Xanthomonas campestris* or *Ralstonia solanacearum*; *alg*, *P. aeruginosa*.

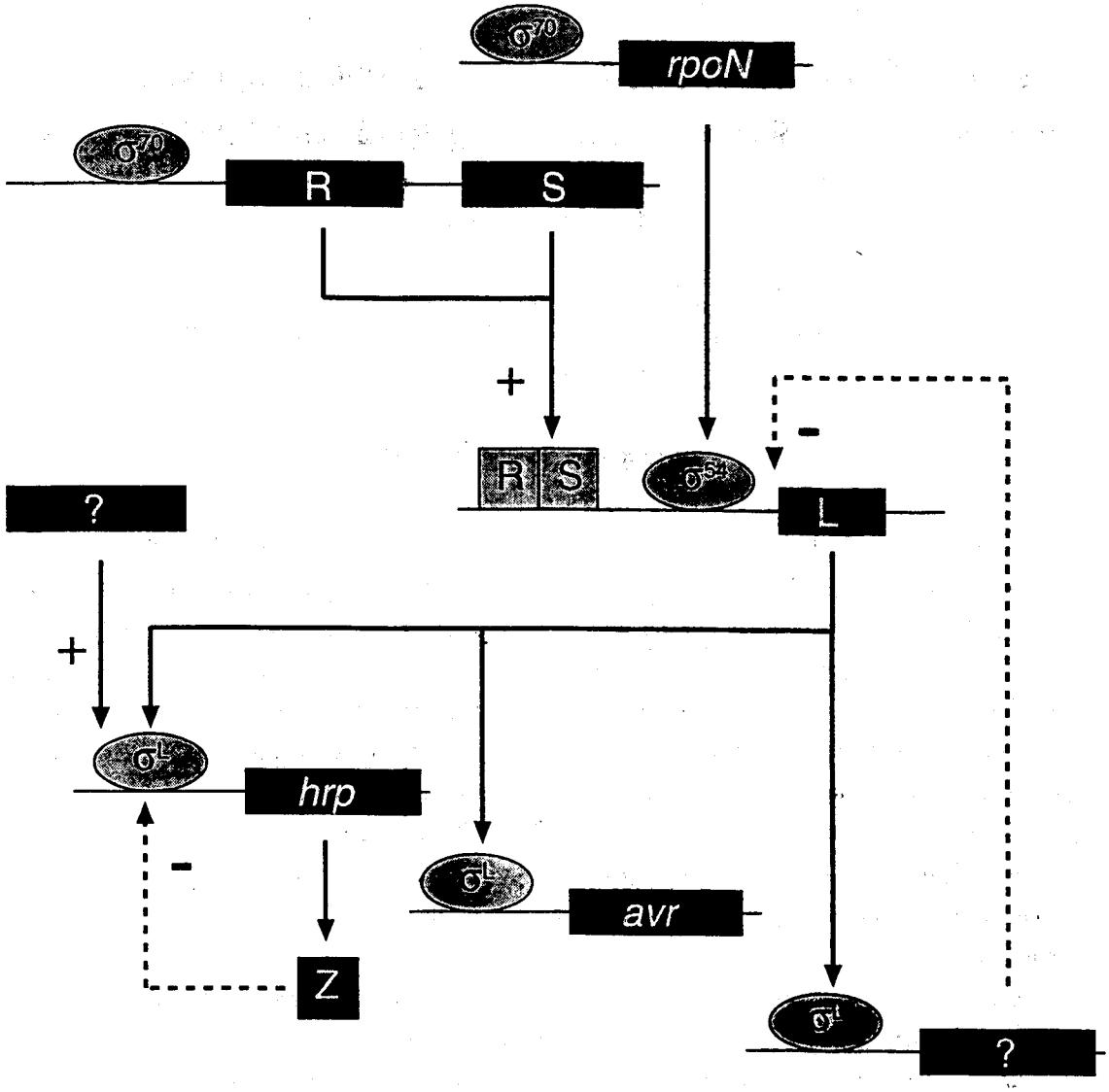

Figure 3. Transcriptional factors controlling expression of *hrp* and *avr* genes in *P. syringae* Pss61. Sigma factors are indicated by the ovals. Promoters are represented by lines and genes by dark shaded recangles. Regulatory components shown as boxes. Arrows indicate target promoters for regulatory factors. Dashed lines indicated negative-acting regulatory components. The *hrpR* promoter appears tobe constitutively expressed whereas promoters below are conditional. Current analyses suggest that a presently unidentified positive activator and a negative-acting component linked to HrpZ2 mediates the activity of HrpL (Rowley, and Hutcheson, unpublished results).