A novel transcriptional autoregulatory loop enhances expression of the \textit{Pantoea stewartii} subsp. \textit{stewartii} Hrp type III secretion system

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\textbf{Abstract}

The \textit{hrp} type III secretion regulon of \textit{Pantoea stewartii} is regulated by a cascade involving the HrpX/HrpY two-component system, the HrpS enhancer-binding protein and the HrpL alternate sigma factor. \textit{hrpXY} is both constitutive and autoregulated; HrpY controls \textit{hrpS}; and HrpS activates \textit{hrpL}. These regulatory genes are arranged in the order \textit{hrpL}, \textit{hrpXY} and \textit{hrpS} and constitute three operons. This study describes a novel autoregulatory loop involving HrpS. Genetic experiments using a chromosomal \textit{hrpS}-\textit{lacZ} fusion demonstrated that ectopic expression of HrpS increases \textit{hrpS} transcription and that this effect is blocked by polar mutations in \textit{hrpXY} and \textit{hrpL} and by a nonpolar mutation in \textit{hrpY}. RT-PCR and Northern blot analysis revealed a \textit{hrpL}-\textit{hrpXY} polycistronic mRNA. These results suggest that HrpS-mediated autoregulation is due to activation of \textit{hrpS} by increased levels of HrpY resulting from read-through transcription of \textit{hrpXY} from the \textit{hrpL} promoter. This novel autoregulatory loop may serve to rapidly induce \textit{hrp} genes during infection and to compensate for negative regulatory mechanisms that keep the regulon off in the insect vector.

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\section*{1. Introduction}

\textit{Pantoea stewartii} subsp. \textit{stewartii} (synon. \textit{Erwinia stewartii}) is a bacterial pathogen of sweet corn and maize that causes wilting and leaf blight, preceded by formation of water-soaked lesions on young leaves [1]. The disease is known as Stewart’s bacterial wilt and leaf blight and it is transmitted by corn flea beetles. The bacteria need a Hrp type III secretion system to grow in the host to high cell density and for symptom development [2–5]. Once bacteria are introduced into the vascular system by an insect vector, bacterial cells and extracellular polysaccharide slime cause blockage of xylem vessels resulting in severe wilting and death of seedlings [6,7].

Type III secretion systems (TTSS) are used by both animal and plant pathogenic bacteria to inject pathogenicity proteins directly into host cells. In plant pathogens, TTSSs are encoded by \textit{hrp} (for hypersensitive response and pathogenicity) genes. Two major groups of \textit{hrp} gene clusters are known, which differ significantly in their organization and mode of regulation. Group I \textit{hrp} clusters are found in \textit{Pseudomonas}, \textit{Erwinia} and \textit{Pantoea} spp. and group II clusters occur in \textit{Xanthomonas} and \textit{Ralstonia} spp. Group I \textit{hrp} genes are activated by complex regulatory pathways ending in HrpS and HrpL [8–10].
HrpS is an NtrC-like enhancer-binding factor that activates hrpL and HrpL is an alternate sigma factor that is needed for expression of the secretion and effector genes in the Hrp regulon. Additional upstream regulation in Erwinia and Pantoaea spp. involves the HrpX/HrpY two-component regulatory system. In Pantoaea spp., HrpY regulates both hrpS and its own production. For P. stewartii, each of these steps has been confirmed by reconstruction experiments in Escherichia coli and HrpX has been shown to sense internal signals resulting from growth in nitrogen deficient minimal media [11]. In spite of the high amino acid similarity of the HrpX, HrpY, HrpS and HrpL proteins among the erwinias and pantoeas and the conserved map order of the genes encoding them, their 5' regulatory and intergenic regions are quite different. This sequence divergence is reflected in differing responses of the hrp regulon to environmental stimuli, in different basal levels of expression of hrpS, in the occurrence of alternate autoregulatory loops, and possibly in the target gene(s) regulated by HrpY. In P. stewartii and Pantoaea agglomerans pv. gypsophilae, phosphorylated HrpY activates transcription of hrpS [11,12; Merighi and Coplin, manuscript in preparation]. In addition, the hrpXY operon is autoregulated by HrpY [11,12]. In Erwinia amylovora, Wei et al. [13] also found that hrpXY and hrpS control hrpL expression. In addition, they reported that hrpS is environmentally regulated, but it does not appear to be autoregulated. Rho-independent transcriptional terminators are not apparent downstream of hrpL and hrpY in P. stewartii and P. agglomerans pv. gypsophilae, but they are in E. amylovora [11–13].

The hrpS promoter appears to be a key point in the regulatory cascade, where various specific and global regulators converge to directly or indirectly affect hrp gene expression. In preliminary epistasis experiments to delineate the regulatory cascade in P. stewartii, we observed that overexpression of hrpS resulted in increased expression of a chromosomal hrpS-lacZ reporter gene fusion. The purpose of this study was to elucidate the mechanism of this autoregulation. We initially studied the effect of ectopic expression of hrpS in chromosomal mutants with a polar insertion in one regulatory gene and a reporter gene fusion in another and found that autoregulation was blocked by upstream polar mutations, so that it could not be due to the effect of HrpS on its own promoter. We then determined that P_{hrpL}, but not P_{hrpXY}, along with activated HrpY were required for this effect. Finally, we analyzed hrpL-hrpXY and hrpXY-hrpS interoperon transcription using RT-PCR and Northern blot analysis. We propose a model whereby HrpS activates hrpL-hrpXY interoperon transcription from P_{hrpL}, and the increased level of HrpY further increases hrpS expression. The possible roles of this novel autoregulatory loop are discussed.

2. Materials and methods

2.1. Bacterial strains, culture media and nucleotide sequences

The bacterial strains and plasmids used in this study are given in Table 1 and Fig. 1. Bacteria were either grown in Luria–Bertani (LB) broth and agar [14] for recombinant DNA procedures or in IM minimal liquid medium at pH 5.5 (IM5.5) [10] or casamino acids–peptone–glucose broth (CPG) [9] for induction of hrp gene expression [11]. For analysis of hrp gene expression, cultures were grown overnight in LB with shaking, diluted 1:20 in fresh LB and grown to exponential phase. Then the cells were centrifuged, resuspended in IM5.5 at OD$_{600}$ = 0.1, and again grown 4–16 h (typically until OD$_{600}$ reached 1.0–1.1). The nucleotide sequences of the P. stewartii hrpL, hrpXY, and hrpS genes and their coordinates are found in Genbank Accession No. AF282857.

2.2. Molecular genetics and cloning techniques

Plasmids were conjugally transferred between strains by biparental or triparental matings using pRK2013::Tn7 as a helper plasmid [15] or they were introduced by electroporation. Double hrp mutants were constructed by allele exchange of mutations cloned in the suicide vector pLD55 with positive selection for tetracycline sensitive (Tcr) double recombinants in fusaric acid medium as described by Metcalfe et al. [16] and modified by Merighi et al. [11]. Preparation of cell lysates, DNA purification, molecular cloning, Southern blot hybridization and PCR were performed following standard procedures [14]. Total RNA was isolated from bacterial cultures grown in IM5.5 [10] as described by Piper et al. [17].

2.3. Strain construction

To construct a polar mutation in hrpL to use for allelic exchange in P. stewartii, the Ω spectinomycin resistance (Sp') interposon was purified as a 2-kb Smal fragment from plasmid pHP45:Ω and ligated into the Nvi I site of plasmid pDM2533, which contains hrpL in a 1.3-kb PCR fragment. The resulting plasmid, pDM2862, was digested with Kpn I and Spe I and the 3.3-kb hrpL::Ω fragment was subcloned into suicide plasmid pLD55 to create pDM2868. E. coli strain BW20339(pDM2868) was crossed with strains MEX1 hrpS lacZ and MEX116 hrpX lacZ by triparental mating and transconjugants were selected for tetracycline resistance (Tc'). Single cross-over mutants were streaked on fusaric acid medium to select for the second recombination event. Putative Tc' Sp' double-recombinants of MEX1 and MEX116 were identified by PCR and
designated DM2824 and DM2830, respectively. Interposon-target gene mutations were confirmed by Southern blot analysis. Strain DM2858 hrpS-lacZ hrpY [D57N] was constructed in a similar manner by introduction of the hrp Y [D57N] mutation from pMM246 [11] into strain MEX1.

2.4. Measurement of β-glucuronidase and β-galactosidase activities

β-Glucuronidase and β-galactosidase activities in bacterial cultures were measured using the fluorogenic substrates 4-methyl-umbelliferyl-glucuronide (MUG) and 4-methyl-umbelliferyl-galactoside (MUGal), respectively [11]. One unit of enzyme activity (1 U) was equal to 1 pmol 4-methyl-umbelliferone (MU) min⁻¹ OD₆₀₀ ml⁻¹ of culture.

2.5. RT-PCR analysis

Analysis of interposon transcripts was performed using the Titan-One-Tube RT-PCR protocol according to the supplier’s instructions (Roche, Nutley, NJ, USA) in an MJ Research (Watertown, MA, USA) PTC-100 thermal cycler. For the synthesis and amplification of cDNAs from internal portions of hrpS (fragment A) and hrpL (fragment D), the primer pairs RTSR (⁴⁶⁵⁰GTAAGCTAAGTTGTTGAC⁴⁶³³) +...
were used to analyze the various transcripts, were synthesized by PCR using plasmid pMM58 as a template and primers LFB (662CGCG-GATCCACATTAAGCCAAACGGCAAAT[14]) and LRE (125CGGAATTCTGGTTTACCCCCGTTCAGTT1237) to amplify hrpL and primers YFH (2855CCGAGCTTTC-GATAACATGATAGATAAC2877) and YRH (3521CCGAGCTTATGAGCATCTAAGA2350) to amplify hrpY[11]. PCR products were labelled by random priming using [α-32P]dATP and the Klenow fragment of DNA polymerase I [14]. About 2 × 10^6 cpm (Cerenkov) of probe were used for each hybridization experiment. Hybridization and post-hybridization washes were performed at high stringency. The signals from the blots were detected by exposure to Biomax MS X-ray film (Kodak, Rochester, NY, USA) at −80 °C for 24 to 48 h.

3. Results

3.1. Ectopic expression of HrpS indirectly upregulated hrpS expression by acting on the hrpL promoter

In culture, P. stewartii hrpS was optionally induced by transferring cells from a rich medium (LB) to a semi-defined hrp-inducing medium (IM5.5), which was lower in pH, salts and nitrogen and contained sucrose and a small amount of casamino acids. Under these conditions, the hrpS lacZ fusion in strain MEX1 was typically induced from a starting level of <50 U (i.e. the level of hrpS expression in LB) up to >2000 after 4–6 h, when hrp expression reaches a maximum (Fig. 2(a)). When the same strain carried low-copy plasmid pRF205 P lac-hrpS, overexpression of HrpS increased hrpS lacZ expression by 6-fold (Fig. 2(a)). In three experiments, pRF205 increased expression of hrpS by 2 to 6-fold following a shift from LB to IM5.5 or when cells were continuously grown to late-log/early stationary phase in CPG broth. However, pRF205 did not increase hrpS-lacZ expression under hrp-repressing conditions in LB broth (data not shown), so that phosphorylation of HrpY by HrpX might be needed. In all cases, it was important that the cells were in log or late log phase before shifting them to IM5.5 medium.

The apparent autoregulation of hrpS did not appear to be due to the ability of HrpS to directly enhance transcription initiation at P hrpS. This was indicated by the following observations: (1) There is no consensus binding site for σ54 factors in the hrpS promoter region [11]; (2) expression of the P hrpS-uidA fusion in pMM50 was not affected by a mutation in hrpS [11], so HrpS is not required for its own synthesis; (3) hrpS-lacZ expression in strain DM2830 hrpL::Ω was not increased by pRF205 (Fig. 2(a)); and (4) a similar upstream, polar

### 2.6. Northern hybridizations

For each strain, ca. 10 μg of total bacterial RNA was electrophoresed in a 0.9% agarose gel in 1× MOPS/formamide buffer [14]. RNA gels were blotted overnight onto a Nytran (S&S, Keene, NH, USA) nylon filter in 5× SSC buffer and fixed to the filter by UV cross-linking following standard protocols [14]. The hrpL and hrpY probes, which

Fig. 1. Physical map of the hrp regulatory region, mutants and plasmids. Grey boxes represent open reading frames and the solid box represents a remnant of an IS element. The solid circle indicates a Tn5 insertion, the open circle indicates an Ω interposon insertion, and arrows indicate Tn5HoHol-lacZYA insertions and the orientation of the lacZYA reporter in the mutant alleles that were used to construct the strains described in Table 1. The inserts of the plasmids used in this study are shown beneath the map.

RTSF (1497ACTCATTGACCACACATGC4414) and RTLX (931GGGCAATACCAGATACCC914) + RTLF (700AACCCTGCACCCTACTGAG683) were used to analyze the various transcripts, were synthesized by PCR using plasmid pMM58 as a template and primers LFB (662CGCG-GATCCACATTAAGCCAAACGGCAAAT[14]) and LRE (125CGGAATTCTGGTTTACCCCCGTTCAGTT1237) to amplify hrpL and primers YFH (2855CCGAGCTTTC-GATAACATGATAGATAAC2877) and YRH (3521CCGAGCTTATGAGCATCTAAGA2350) to amplify hrpY[11]. PCR products were labelled by random priming using [α-32P]dATP and the Klenow fragment of DNA polymerase I [14]. About 2 × 10^6 cpm (Cerenkov) of probe were used for each hybridization experiment. Hybridization and post-hybridization washes were performed at high stringency. The signals from the blots were detected by exposure to Biomax MS X-ray film (Kodak, Rochester, NY, USA) at −80 °C for 24 to 48 h.

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hrpY::Tn5 mutation in strain DM733 likewise abolished the autoregulatory effect of pRF205 (Fig. 2(a)). On this basis, we hypothesized an autoregulatory loop, in which HrpS enhances transcription of hrpL and downstream operons by read-through transcription, rather than by activating its own promoter.

This model predicts that hrpXY would also be stimulated by overexpression of HrpS. To test this, we first determined the basal level of the HrpXY promoter and then showed that it was increased by ectopic expression of hrpS. When the hrpX promoter region was expressed from a plasmid borne promoter probe vector (pMM25), it showed high basal expression in both IM5.5 (the average GUS activity ± SD in an early stationary phase culture was 252 ± 11 U; n = 3) and LB (335 ± 91 U). We then assayed hrpX lacZ expression in the presence and absence of pRF205 using strain MEX116 as the genetic background and found that hrpXY expression was up-regulated ca. 4-fold by ectopic expression of HrpS (Fig. 2(a)). Furthermore, this effect was blocked in strain DM2824, a MEX116 derivative carrying a polar hrpL::Ω mutation (Fig. 2(a)).

3.2. HrpS-induced autoregulation required functional HrpY protein

We postulated that, once HrpS stimulates transcription from P_{hrpL} into hrpXY, increased expression of hrpS could occur by increased levels of HrpY stimulating P_{hrpS}, by read-through transcription proceeding into hrpS, or by both mechanisms. To test the extent of these possibilities genetically, we introduced a nonpolar hrpY [D57N] null mutation into a hrpS::lacZ background, thereby inactivating the phosphorylation site and the transcriptional activator function of HrpY [11]. In this strain, DM2858, ectopic expression of HrpS did not upregulate hrpS transcription, even though transcription should not have been blocked (Fig. 2(a)). This finding suggests that the HrpS-mediated autoregulatory effect is primarily dependent on activation of P_{hrpS} by HrpY. To further test our hypothesis that the autoregulatory effect involves trans-activation of hrpS by HrpY, we repeated this experiment using a plasmid-borne hrpS-uidA fusion (Fig. 2(b)). In wild-type strain DC283 carrying pMM50, ectopic expression of hrpS^+ from high-copy plasmid pRF8 increased GUS expression ca. 3-fold after 16 h growth in IM. This stimulation was abolished by either a polar or a nonpolar mutation in hrpY.

Fig. 2. Effects of the ectopic expression of HrpS on chromosomal hrpS-lacZ and hrpX-lacZ fusions and a plasmid borne hrpS-uidA fusion in various genetic backgrounds. (a) P. stewartii strains carrying chromosomal fusions were grown in IM5.5 to OD_{600} 0.7–1.0 and β-galactosidase assays were performed as described in Section 2. Strain MEX1 is hrpS-lacZ, strain DM733 is hrpY::Tn5 hrpS-lacZ, strain DM2824 is hrpX::Ω hrpS-lacZ, strain DM2858 is hrpY[Δ57N] hrpS-lacZ, strain MEX116 is hrpX-lacZ, and strain DM2830 is hrpX::Ω hrpX-lacZ. (b) P. stewartii strains carrying plasmid-borne uidA fusions were grown overnight in IM5.5 after pregrowth in LB to exponential phase and β-glucuronidase assays were performed. Strain DC283 is wild-type, DM064 is hrpY::Tn5, MM254 contains a nonpolar hrpY[Δ54N] mutation, pMM50 carries a plasmid borne hrpS-uidA fusion, and pRF8 is a hrpS^+ expression plasmid. One unit of enzyme activity was equal to 1 pmol 4-methyl-umbelliferone (MU) min^{-1} OD_{600} ml^{-1} of culture. Data are averages from a representative experiment with two replicates and two technical repeats per treatment. Both experiments were reproduced three times and in all cases the relative standard deviation for a given treatment was <15% of the measured activity.
3.3. RT-PCR revealed the absence of transcription termination downstream of hrpL and hrpXY

To further demonstrate that read-through transcription of hrpXY from P_{hrpL} was the underlying molecular mechanism for hrpS autoregulation, we used RT-PCR to detect transcripts spanning the various regulatory operons. Oligonucleotide primers were designed to amplify cDNA transcripts spanning the hrpL-hrpXY and hrpY-hrpS intergenic regions and internal portions of the hrpL and hrpS genes using a “one-tube” RT-PCR reaction (fragments C, B, D and A, respectively; Fig. 3(a)). Total RNA was purified from P. stewartii DC283 grown in IM5.5 to early stationary phase. The 254- and 232-bp control products from within the hrpL regulatory region located upstream of the hrpS promoter (271 bp, fragment B; Fig. 3(b)). Based on the RT-PCR results, transcription downstream of hrpY is not terminated immediately and can proceed almost to the hrpS promoter -35 box (Fig. 3; product B), which is located 196 bp downstream of hrpY (Merighi and Coplin, manuscript in preparation). Attempts to obtain PCR products with primers RTYSF and RTSR, which amplify the entire 5’hrpS regulatory region, repeatedly failed, indicating that either transcription terminates between -40 and +911 bp from the transcription start site or this mRNA is too unstable to detect. No product was amplified from the control lacking the reverse transcriptase enzyme, which was run using primers for the internal portion of hrpS and DNA-free total RNA as template (Fig. 3(b), lane 7). The identity of the PCR products was confirmed by direct sequencing of the fragments. These results indicate that transcription from P_{hrpL} may terminate downstream of hrpY, but before the start of hrpS.

3.4. Northern blot analysis revealed the presence of a long transcript spanning hrpL and hrpXY

To confirm the results of the RT-PCR analysis, we analyzed the transcripts of the hrp regulatory regions by Northern blotting using hrpL and hrpY gene probes (Fig. 4). DNA-free total RNA was prepared from strains DC283 (wild-type) and MEX105 hrpL::Tn3HoHoI grown in IM5.5 to early stationary phase. In DC283, the hrpL probe detected a strong 2.9-kb band with a very long leading edge, which may be degradation products. The size of this band is consistent with a transcript starting at the hrpL promoter and spanning hrpL, hrpXY and part of the hrpS 5’ regulatory region (Fig. 4). The hrpY probe also revealed this transcript in DC283, as well as a 2.2-kb transcript that corresponds in size to the hrpXY operon. The two bands were about equal in intensity, suggesting that both the hrpL and hrpX promoters contribute significantly to hrpXY expression. Using the hrpL probe, only a 0.5-kb transcript could be detected in MEX105. This transcript corresponds in size to the distance from the hrpL σ^{34} promoter to the site of the Tn3HoHoI insertion in this mutant. We did not find a 4.5-kb transcript that would include the entire hrpLXYXS region.

4. Discussion

The hrp regulon in P. stewartii is regulated by a cascade consisting of HrpX, HrpY, HrpS and HrpL (Fig. 5) [10,11]. The basal expression of the hrpXY operon is constitutive, but the expression of all the regulatory genes is affected either transcriptionally or post-translationally by environmental signals, redox, quorum sensing and specific feedback mechanisms. We previously showed that the hrpXY operon is directly autoregulated [11] and the experiments described in this study demonstrate that hrpS is also autoregulated, but by a novel, indirect mechanism. This did not appear to be direct autoregulation of hrpS by its own product because: (1) there is not a consensus sequence for σ^{34} factors upstream of hrpS, making direct binding of HrpS to this
We verified that the activation of the last gene, hrpS, by the regulatory region in P. stewartii was autoregulated [11], and therefore of hrpXY. This loop augments the direct hrpXY expression, whereas it was blocked by polar mutation of P_{hrpL} and hrpXY. Similarly, hrpXY expression was also turned up by overexpression of HrpS and this effect was also blocked by a polar mutation in hrpL. The results obtained from the RT-PCR and Northern blot analyses were consistent with the absence of transcriptional terminators downstream of hrpL, but not hrpY. Our Northern blots revealed a 2.9-kb mRNA, which could include all of hrpL and hrpXY, that was absent in a polar hrpL mutant. However, we did not find the predicted 4.5-kb hrpLXYS transcript. Consequently, it appears that transcription from P_{hrpL} proceeds only through hrpXY and then HrpY activates P_{hrpS} (Fig. 5). We then demonstrated that ectopic expression of hrpS can also stimulate P_{hrpS} in trans, in a HrpY-dependent manner. This second model would further require that HrpY be activated by the HrpX sensor-kinase. This was shown to be the case by introducing a non-phosphorylatable hrpY [D57N] allele into a hrpS-lacZ reporter strain. The D57 residue is the only phosphorylation site in HrpY (Merighi and Coplin, manuscript in preparation). This nonpolar mutation abolished the HrpS autoregulatory effect, indicating that it requires phosphorylated HrpY. Furthermore, this model is consistent with our observation that HrpS-mediated autoregulation only occurs in minimal, Hrp-inducing media.

Fig. 5. Model of the hrp regulatory cascade including the autoregulatory loop triggered by HrpS. The activation of hrpL by HrpS is not only part of the core cascade that activates the Hrp regulon, but also triggers a positive autoregulatory loop that increases the transcription levels of hrpXY and therefore of hrpS. This loop augments the direct autoregulation of hrpXY, thereby stabilizing the “on” state of the regulatory circuit.
appears to be a pure feedback mechanism, at least in first approximation.

Pantoea and Erwinia spp. control the expression of their hrp genes via similar regulatory cascades that amplify plant and environmental signals at each step. Like many biotrophic plant pathogenic bacteria, the erwinias employ a “stealth” strategy following entry into a host and do not immediately deploy their pathogenicity mechanisms, such as Hrp effector proteins and degradative enzymes. Instead, they wait until they reach a critical population that insures a successful infection. Thus, the lack of read-through transcription into hrpS from the constitutive hrpX promoter in P. stewartii makes sense if hrp genes need to be turned off at certain times during infection or when the pathogen is in the insect vector. Later, as nutrients become available from damaged tissues, it may again turn off expression of the hrp genes to save energy. The autoregulation provided by HrpY and HrpS could provide a mechanism that very rapidly increases hrp gene expression once the decision to attack the host has been made. In addition, these autoregulatory mechanisms may be needed in order to compensate for high turnover rates of various regulatory molecules, such as the possible degradation of HrpS by Lon protease [25]. Since terminators are also absent between the hrp regulatory genes in P. agglomerans pv. gypsophilae, but not E. amylovora, HrpS-mediated transcriptional autoregulation may be an adaptation of Pantoea spp., which are more recently evolved pathogens.

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