The bacterial AAA + enhancer-binding proteins (EBPs) HrpR and Hrps (HrpRs) of Pseudomonas syringae (Ps) activate σ54-dependent transcription at the hrpL promoter; triggering type-three secretion system-mediated pathogenicity. In contrast with singly acting EBPs, the evolution of the strictly co-operative HrpRs pair raises questions of potential benefits and mechanistic differences this transcription control system offers. Here, we show distinct properties of HrpR and Hrps variants, indicating functional specialization of these non-redundant, tandemly arranged paralogues. Activities of HrpR, Hrps and their control proteins HrpV and HrpG from Ps pv. tomato DC3000 in vitro establish that HrpRs forms a transcriptionally active heterohexamer, that there is a direct negative regulatory role for HrpV through specific binding to Hrps and that HrpG suppresses HrpV. The distinct HrpR and Hrps functionalities suggest how partial paralogue degeneration has potentially led to a novel control mechanism for EBPs and indicate subunit-specific roles for EBPs in σ54-RNA polymerase activation.
M any bacterial pathogens use a three-protein secretion system (T3SS) to deliver proteins into host cells (reviewed in ref. 1). hrp and hrc genes establish the regulatory and structural functions associated with the T3SS from the group I-type hrp/hrc cluster of Pseudomonas, Erwinia and former Erwinia phytopathogens (for example, Pectobacterium and Dickeya spp.2,3). hop genes encode secreted pathogenicity effectors and the majority of hop genes and hrp/hrc cluster genes are regulated by the extracytoplasmic function (σE)-factor HrpL, which is regulated by the σE-dependent hrpL promoter4,5. Regulation of σE-RNA polymerase (σE-RNAP) activity is achieved by the action of specific enhancer-binding proteins (EBPs)6,7. In phytopathogenic fluorescent Pseudomonas spp., other than Pseudomonas syringae (Ps), and in the phytopathogenic species in the Enterobacteriaceae, a single EBPs-HrpS, activates transcription from the hrpL promoter8. In Ps, hspR expression is activated by a co-dependent pair of EBPs; HrpR and HrpS.

EBPs are molecular machines belonging to the AAA+ (ATPases associated with various cellular activities) superfamily9 of P-loop ATPases. They remodel σE-RNAP at its promoter sites and, via a carboxy (C)-terminal HTH domain, bind promoter upstream activator sites (UASs). The ATPase activity of EBPs relies on the formation of hexameric ring-like assemblies6. This feature is commonly exploited by cis-acting regulatory domains to control the activity of EBPs by either impairing (as in DctD and NtrC1) or promoting (as in NtrC) hexamer assembly6,9. The EBPs HrpR and HrpS, similar to the well-studied EBPs PspF, lack cis-acting regulatory domains and comprise only the AAA+ and HTH domains. PspF activity is directly negatively regulated by an additional protein, PspA10. HrpV is thought to fulfill a similar regulatory role in the HrpRS and probably HrpS systems11. Repression of HrpV is proposed to be released through the action of HrpG12.

The vast majority of bacterial AAA+ proteins studied so far form homo-hexameric AAA+ rings, whereas their eukaryotic isoenzymes often function as heteromeric assemblies. For instance, the mini-chromosomal maintenance (MCM) complex, a processive replicative helicase, functions as a typical AAA+ homo-hexameric ring in archaea13, but the eukaryotic isoenzyme (MCM: Mcm2-7p) is heteromeric14. The hrpRS genes are transcribed as an operon, proposed to form a functional heteromeric HrpRS AAA+ complex of unknown quaternary structure15. The advantage that such a dual EBP system confers on T3SS-mediated pathogenicity in Ps is unclear.

Multiple sensory systems as well as abiotic and biotic inputs are used in regulating expression from group I hrp regions. Among these sensory systems are Lon, GacA, HrpXY, rsaA/B, HrpRS, HrpV, HrpG and quorum-sensing components16,17,18,19. Here, we study the HrpRS, HrpV and HrpG components of Ps DC3000 and report: direct evidence for a single mixed composition functional hexameric HrpRS complex; that HrpR and HrpS each contact σE; that HrpR and HrpS make unequal contributions to the ATP-dependent and HrpV-controlled functioning of the hetero-hexamer and; specific binding of HrpV to HrpS is used in a novel inhibition and regulation mechanism that is sensitive to HrpG action. These findings provide detailed insights into the molecular evolution of an EBP system that distinguishes Ps from non-syringae plant pathogens.

Results Gene duplication leading to HrpRS is distinctive for Ps. In Ps (including DC3000), hprR and hprS are arranged in tandem (hrpRS), transcribed as a single operon4 and exhibit high sequence similarity, suggesting that they have evolved from a single ancestral gene duplication event. A search for the highest similar sequences for group I-type pathogenicity islands (PAIs) identified ten non-redundant hrpRS tandem arrangements (in Ps pathovars) and ten single-hrpS sequences (in Erwinia-type pathovars as well as the hrpS homologue rspr from P. fluorescens). All 20 inspected PAIs also harbour the regulatory genes hrvV and hrvG, including P. mendocina (a P. aeruginosa-related human pathogen20), suggesting that the appearance of hrpRS is more recent than that of the presumed hrvS regulatory genes (hrvV and hrvG). Although regulatory roles for HrvV and HrvG remain to be established for non-syringae strains they have been clearly annotated as hrvV and hrvG across genera and the synteny of the intra-operon loci are strictly conserved. A phylogenetic tree based on protein sequences derived from the 30 EBPs obtained is shown in Supplementary Figure S1. Where present HrpR and HrpS have 55–65% sequence identity and 70–79% sequence similarity to each other (Supplementary Table S1—compared with other well-studied bacterial EBPs, such as ZraR, PspF, NtrC1, which have 38–41% identity; Supplementary Table S2). Strikingly, hrpRS was found in all Ps pathovars, making the dual hrpRS EBPs system a distinguishing feature in the hrp/hrc gene cluster of group I PAI in Ps pathovars. Further, no polymorphism (hrpRS or hrvS) has been reported for Ps. We did not find polymorphism among identified PAI (including among redundant sequences); strongly suggesting that hrpRS in Ps provides a selective advantage compared with singly acting hrvS. The exact branch pattern symmetry between the HrpSps, and HrpR groups (Supplementary Fig. S1) suggests that following gene duplication, hrvR and hrvS may not have diverged freely as is commonly assumed in gene duplication due to functional redundancy11,12. Inspection of the HrpR and HrpS sequences of Ps DC3000 and their predicted structures indicate no obvious basis of their co-dependency. They show clear congruence with other EBPs (Supplementary Fig. S2), in that the seven conserved regions identified for all EBPs and the conserved AAA+ motifs are present (Fig. 1a).

Specialization of HrpR and HrpS mechanism and regulation. Initially, we expressed wild-type (WT) HrpR, HrpS, HrvV and HrvG in Escherichia coli, and assayed activities using a P. syr.1::P_hrvL::lacZ reporter integrated into the chromosome (see Methods and Supplementary Table S3). As shown in Figure 1b, co-expressed HrpR and HrpS (expressed simultaneously from different plasmid constructs) or HrpR (co-expressed as a single operon), but not HrpS or HrvG alone (expressed singly), resulted in elevated β-galactosidase activity—in line with the strict requirement for both HrpR and HrvG to activate hrvL transcription. Western blotting confirmed that both HrpR and HrvG were stably expressed, excluding gross protein instability at the level of proteolysis as a basis of their co-dependence in vivo (Supplementary Fig. S3), although HrpR–HrpS assembly defects cannot be excluded. Co-expression of HrpRS and HrvG resulted in reduced transcription activation (Fig. 1b, HrpR + HrvG), demonstrating that HrvG negatively controls the functionality of HrpR in the absence of other Ps-specific factors in vivo. Notably, expression of HrvG partly released the negative effect of HrvP on HrvR activity.

Biochemical studies with AAA+ proteins, including EBPs, suggest that non-synchronized ATP hydrolysis occurs between individual subunits of homomeric complexes11,23. To address this notion more directly, we introduced equivalent single amino-acid substitutions into EBPs functionally important motifs in either HrpR or HrpS (Fig. 1a) to delineate the specific roles of conserved EBPs sequences within the context of heteromeric HrpRS6. We assayed the effect of amino-acid substitutions, on in vivo P_hrvL transcription activation (Fig. 1c,d). As shown in Figure 1c,d, the β-galactosidase activities of HrpR and HrpS variant forms demonstrated that the integrity of the Walker A (P-loop) motif was required for in vivo promoter activation. Substitutions in the HrpS Walker B motif (E108, Fig. 1d), expected to be essential for ATP hydrolysis, and the sensor II residue R226, retain significant residual activity (51 Miller Units (MU) and 32 MU, respectively). In contrast (Fig. 1c) the corresponding substitutions in HrpR showed no detectable activity (E110 and R228; 2 MU and 1 MU, respectively; where the background level is 2 MU and 100% activity corresponds to 350 MU).
Given that Walker B residues and the sensor II ‘R’ residue are critical for ATP hydrolysis and EBP functionality (in terms of the ability to stimulate open complex (OC) formation)\cite{23,24}, these data suggest mechanistic subunit specialization in the ATP hydrolysis-associated functionalities of HrpR and HrpS.

EBPs contact $\sigma^{04}$ via the consensus GAFTGA motif\cite{25}. Such interactions depend on the nucleotide-bound state\cite{26,27,28} and rely on the integrity of the ‘F’ and ‘T’ residues\cite{29,30}. In HrpR, this sequence is predominantly GAFTGV and in HrpS is always GAYTGA (including in singly acting HrpS; Supplementary Fig. S2). Deviation from the consensus GAFTGA motif in HrpR and HrpS may reflect subunit specialization in contacting $\sigma^{04}$. Substituting Y for F in HrpS, resulted in a 50% increase in activity compared with WT HrpRS (Fig. 1e), indicating that HrpRs can interact with $\sigma^{04}$, yet appears to limit the activity of HrpRS. As substituting Y for F in HrpS did not overcome the requirement for HrpR, we infer that the non-consensus residue Y85 is not the basis of HrpRS co-dependency. Substitution of the near invariant T residue (present in >97% of identified EBPs; a key $\sigma^{04}$-contacting residue, yet does not affect the self-assembly or ATPase activities of PspF\cite{31}) for A in either HrpR or HrpS led to a
loss of transcription activation; demonstrating that both the HrpR and HrpS σ^54-contacting GAFTGA sequences are strictly required.

Clearly HrpR and HrpS have each evolved mechanistic specialization, providing direct evidence for subunit-specific roles of this novel EBP system in activating σ^54-RNAP.

**Interactions between the Hrp proteins.** We analysed the protein–protein interactions that occur within the HrpRS, HrpV and HrpG regulatory complexes in vivo using a bacterial two-hybrid system, BACTH. We verified that all two-hybrid fusion proteins were similarly expressed (Supplementary Fig. S3)—thereby establishing that failure to detect an interaction was not simply due to unstable Hrp-fusion proteins. Consistent with earlier findings, HrpS self-associates and interacts with both HrpR and HrpV, but not HrpG (Fig. 1f). HrpV also interacts with HrpG. HrpR did not detectably interact with itself, HrpV or HrpG (Fig. 1f and refs 8, 13). Glutaraldehyde crosslinking with co-purified HrpRS and purified HrpV, and followed by western blotting using antibodies specific to HrpR, HrpS (His-tag) and HrpV established direct binding of HrpV to HrpS can occur independently of additional factors (Supplementary Fig. 3). To distinguish which HrpS domain contributed to the different binding interactions, we created a C-terminal HTH truncation variant, HrpS_1−275, (the HrpS AAA + domain), and analysed interactions with full-length (FL) HrpS, HrpR, HrpV and HrpG. In contrast to HrpR, HrpS_1−275 showed no detectable interactions with itself or HrpR—suggesting oligomerization determinants in HrpS reside in the HTH domain, consistent with dimerization functionalities in NtrC. Importantly, interaction activities are retained between HrpS_1−275 and HrpV, suggesting that HrpV acts directly by binding the HrpS AAA + domain.

Regulation of EBPs is commonly achieved by altering higher-order oligomer formation (and hence ATPase activity). We therefore tested whether HrpV affected subunit-binding interactions within the HrpRS complex by co-expressing native HrpV together with the BACTH protein fusions to form a three-component system (Fig. 1g). Our results demonstrate that expression of native HrpV greatly increased HrpS σ^54-containing and moderately increased HrpR–HrpS interactions (Fig. 1h), suggesting that HrpV binding to HrpS somewhat alters subunit–subunit interactions within the self-assembled HrpRS complex. Western blotting confirmed that the levels of HrpR and HrpS production were similar in the presence of the extra copy of HrpV (Supplementary Fig. S3). Given the absence of detectable interactions between HrpS and HrpG (even in the presence of HrpV), we infer that HrpV may not bind HrpS and HrpG simultaneously and that HrpG relieves HrpV inhibition by precluding HrpV–HrpS interactions.

**HrpV and HrpG act antagonistically to control HrpRS activity.** The ways in which heteromeric EBPs may differ from singly acting EBPs are not known. We therefore investigated features in the HrpRS system that are common to EBPs: the presence of and cooperative binding to UAS DNA; a facilitating role of integration host factor (IHF) to increase productive interactions between the EBP and σ^54-RNAP closed complex; a strict nucleotide-dependency during transcription activation, and the regulation of EBPs by modulating the oligomeric state and/or DNA-binding activity.

To gain insights into the HrpRS system, we purified FL HrpR, HrpS, HrpRS (co-expressed), IHF, HrpV and HrpG. In vitro studies of many other EBPs have been performed using isolated EBP domains, because of the difficulties associated with limited solubility of FL EBPs. HrpR and HrpS when expressed and purified separately resulted in substantially lower protein yields than co-expressed HrpRS, suggesting that HrpR/HrpS mutually stabilize a soluble heteromeric conformation (in line with oligomerization determinants residing within the AAA+ and HTH domains; Fig. 1c,d,f). Further, attempts to co-purify HrpRS complexes harboring Walker B alanine substitutions in either HrpR or HrpS resulted in very low protein yields, probably due to a failure to form stable HrpRS complexes as observed with the equivalent substitutions in PspF. The activity of the purified HrpRS complex was measured using an in vitro single-round transcription assay from the supercoiled hrpL promoter. As shown in Figure 2a, FL transcript formation required σ^54-RNAP, DATP and HrpRS. Purified IHF greatly stimulated the amount of FL transcript obtained in vitro. Notably, the absence of FL transcripts with HrpR alone, HrpS alone or separately purified and mixed in vitro HrpR and HrpS (HrpR + HrpS), suggests that in vivo self-assembly of the HrpRS complex is important for forming the active HrpRS complex (Fig. 2b). HrpRS activated transcription when RNAP was reconstituted with either Klebsiella pneumoniae (KP) or Ps σ^54 (Fig. 2c), indicating that HrpRS can function independently of Ps-specific σ^54.

Purified HrpV inhibited HrpRS-dependent activation of σ^54-RNAP transcription (Fig. 2d) at a 1:1 ratio (HrpV:HrpRS), independent of the constant concentrations of DNA, σ^54-RNAP and IHF. We infer that HrpV directly acts to diminish productive interactions HrpRS makes with the closed promoter complex. To establish that HrpV specifically inhibits the action of the HrpRS complex, we tested whether HrpV could inhibit transcription activation by an alternate EBP (the AAA + domain of PspF), using the σ^54-dependent Sinorhizobium meliloti nifH test promoter, which lacks the HrpRS UASs present in the hrpL promoter. As shown in Figure 2e, although PspF and HrpRS are both able to activate σ^54-dependent transcription at the nifH promoter, only the activity of HrpRS is inhibited by HrpV. Clearly, the action of HrpV is specific to the HrpS subunit of the HrpRS complex, not previously observed in vitro (Ps σ^54)-RNAP complexes, suggesting HrpV acts before OC formation (Fig. 2g: compare OC to OC + HrpRS–V and OC + HrpV). HrpV did not inhibit the ATPase activity of HrpRS (where the ATPase turnover rate per min for 1 μM HrpRS was: 0.180 ± 0.02 (minus HrpV), 0.192 ± 0.02 (plus 1 μM HrpV) and 0.183 ± 0.02 (plus 10 μM HrpV) and the ATPase buffer did not prevent HrpS from interacting with HrpV, Fig. 2h). Overall, these data establish that HrpV acts via a mechanism specific to the HrpS subunit of the HrpRS complex, not previously described for other EBPs.

Further, HrpG, when added to the HrpRS–V regulatory complex (Fig. 2e), relieved some of the inhibitory action of HrpV (as observed in vivo) but had no stimulatory effect on transcription activation by PspF. These results establish that HrpG functions as a specific suppressor for HrpV activity and that HrgP and HrpV can function independently of any Ps-specific factors.

**HrpRS co-dependency is hrpL promoter independent.** We established that the hrpL promoter (P_{hrpL}) contains a functional UAS between positions –147 to –36 relative to the transcription start-site (Fig. 3a,b), to which purified HrpR, HrpS and HrpRS bind in electrophoretic mobility shift assays (Fig. 3c,d). Consistent with the in vitro observations, in which addition of IHF greatly stimulated transcription (Fig. 2a), hrpL-dependent in vivo transcription in an IHF deletion strain was marginal (Fig. 3b). The specific IHF-binding site is present in the promoter probe (Fig. 3e), probably promoting productive interactions between HrpRS and σ^54-RNAP, as evidenced by the increased transcription activity in vivo (Fig. 3b) and in vitro (Fig. 2a). Compared with either HrpR alone or HrpS alone or when HrpR and HrpS were mixed together (denoted as HrpR + HrpS, Fig. 3c), co-purified HrpRS bound the UAS DNA with greater affinity (Fig. 3d), indicating co-operative binding to the P_{hrpL}. Lack of increased UAS binding in the presence of pre-mixed, separately purified HrpR and HrpS, probably reflects incompetent binding.
Figure 2 | Regulation of the HrpRS complex in vitro. (a) In vitro full-length (FL) transcription assays (~470 nucleotides) using the supercoiled hrpL promoter in the presence (+) or absence (−) of σ54-RNAP (100 nM), dATP (4 mM), co-purified HrpRS (0.8 μM) and integration host factor (IHF) (20 nM). (b) HrpR alone (0.8 μM), HrpS alone (0.8 μM) and pre-mixed HrpR and HrpS (0.8 μM) (HrpR + HrpS) failed to activate transcription. (c) As in (a), including dATP (4 mM), IHF (20 nM) with reconstituted σ54-RNAP using either purified σ54 from Ps (σ54-Ps) or from σ54 K. Pneumoniae (σ54-Kp). (d) HrpRS-dependent activation from the hrpL promoter as in (a), in the presence of increasing HrpV concentrations. (e) Transcription from a test promoter (S. meliloti nifH), with the EBP AAA + domain PspF1-275, or HrpRS, and in the presence (+) or absence (−) of HrpV (2 μM) and HrpG (2 μM). Gel images of transcripts for Figure 2a–e are shown in Supplementary Figure 4. (f) A bar graph showing electrophoretic mobility shifted hrpL DNA promoter probe comprising UAS sequences (in percentage of total hrpL DNA promoter signal) in the presence of HrpRS (2.5 μM) or HrpRS (2.5 μM and HrpV (4.2 μM)). (g) A bar graph depicting the percentage of DNA bound in the closed promoter complex (CC) and transcriptionally proficient open promoter complex (OC; formed by PspF activation) in the presence of HrpRS–V or HrpV. (h) A bar graph depicting the relative amount of glutaraldehyde crosslinked HrpRS–HrpV species in either ATPase buffer (used to measure the ATPase activity of HrpRS + / − HrpV) or HGned buffer. The relative amount of crosslinked HrpS–V complex was determined by fluorescence scanning. In f–h, estimated errors of measurements are shown as ±10%.

HrpR and HrpS are not equivalent for nucleotide binding. Identical subunits in EBPs and other AAA + proteins make delineation of mechanistic roles of individual subunits experimentally challenging. To directly probe any non-equivalence of HrpR and HrpS subunits (as suggested by the mutagenesis studies; Fig. 1c, d), in the context of the purified functional HrpRS complex, we tested nucleotide binding with non-equilibrium covalent ultraviolet crosslinking of [α-32P]ATP using PspF as a control. Recall that the highly conserved Walker A motif (GXXXXXG[K/T/S]), especially, the ‘K’ residue, is strictly required for nucleotide binding in AAA+ proteins, including EBPs. As shown in Figure 4, HrpR and HrpS were separated by SDS-PAGE and the relative protein concentrations evaluated by SYPRO ruby protein stain fluorescence (Sy). The relative amount of [α-32P]ATP crosslinked protein species was determined by PhosphorImager analysis. Side-by-side comparison of the relative [α-32P]ATP fluorescence intensities (Fig. 4a) of the HrpR and HrpS bands indicate that [α-32P]ATP preferentially crosslinks to HrpR and at a level comparable to that of the structurally and functionally characterized EBP PspF19 (Fig. 4b). Specific binding of [α-32P]ATP to HrpR is further demonstrated by the competitive binding of non-radiolabelled ADP, ATP and ATPγS (Fig. 4a). Differential nucleotide binding to particular subunits of the strictly co-dependent HrpRS system provides direct evidence for non-equivalent nucleotide-dependent roles for individual subunits in a functional EBP complex.

The HrpRS hetero-hexamer is the most active assembly. Gel filtration of co-purified HrpRS (Fig. 5a) resolved at least two distinct species with molecular weights (MWs) corresponding to 212 kDa (apparent 6.1 mer) and 52 kDa (apparent 1.4 mer)—where the MW of HrpR is 34.9 kDa and HrpS65 is 34.5 kDa. The lower MW peak does not permit assignment of subunit composition(s) and may comprise a mixture of monomers and dimers. SDS–PAGE analysis (Fig. 5b) of the fractions demonstrates that HrpR and HrpS are present in both peaks. Interestingly, we note that the relative intensities of HrpR and HrpS differ significantly between the hexameric (hex) species (where HrpS predominates over HrpR) and monomer/dimer species (where HrpR predominates over HrpS). The relative HrpR/HrpS fluorescence intensities are consistently scored as ~0.5 in fractions 5–8 suggesting a fixed HrpR/HrpS subunit assembly (in line with the in vitro transcription assays; Fig. 2b). These features of the hrpL promoter in good agreement with other EBP interactions at σ44-dependent promoters in terms of co-operative binding to the UAS and exclude promoter architecture as the major basis of HrpRS co-dependency41.
the hexameric fraction was too low to detect transcription in vitro (Fig. 5e). However, the gel-filtration purified HrpRS monomer/dimer fraction (fraction 10) was unable to detectably activate S^4-RNAP, whereas the original loading sample (diluted to the same concentration as the gel-filtered HrpRS monomer/dimer fraction) supported transcription (Fig. 5e). We infer that the hexameric HrpRS assembly is the active form. These results suggest that a defined subunit composition of a HrpRS hexamer confers the highest transcription and ATPase activities.

**Discussion**

Gene duplication events followed by recombination and/or diversification are thought to be the major driving force for evolutionary innovation\(^{46}\), but knowledge of the molecular mechanisms that underpin selection of duplicated genes is fragmented\(^{49}\). Despite considerable \(hrp/hrc\) gene similarities, the evolution of \(hrpRS\) through duplication is the most distinguishing feature of \(P s\) PAIs (pathovars include tomato, tagetes (marigold), phaseolica (bean) and oryzae (rice)). We now provide insights into the mechanistic diversification of HrpR and HrpS and their regulation by HrpV and HrpG. Uncommon for gene duplication events, duplication of an EBP ancestor has not resulted in functional independence, but has locked the diverging HrpR and HrpS subunits into strict co-dependence, possibly explaining their high sequence conservation. HrpR appears to have become specialized in nucleotide-dependent functionalities, whereas HrpS retained a HrpV-dependent regulatory function. Our results suggest no obvious mechanistic advantage of the HrpRS system in activating S^4-dependent transcription when compared with singly acting EBPs, rather there may be regulatory advantages conferred by having only HrpS targeted by HrpV. In line with this, deletion of Lon protease correlates with HrpR (and not HrpS) accumulation in vivo—although it is unclear whether Lon degrades HrpR directly\(^{16,17,19}\). The divergent roles of HrpR and HrpS described here provide one example of regulatory specialization of one subunit of a strictly co-dependent system, thus illustrating how partial sub-functionalization (for example, reduced ATP binding by HrpS, inability of HrpR to interact with HrpV) could occur in combination with neo-functionalization (for example, acquisition of a novel interaction between HrpR and Lon)\(^{19}\).

Purified HrpRS is competent to activate transcription, is negatively regulated in trans by HrpV and this repression is released by
HrpG, establishing that the HrpRSVG system is sufficient for regulated expression at the hrpL promoter in vivo. Activity of HrpRS in E. coli, with K. pneumoniae σ41 and S. mellioti promoter sequences indicated that the HrpRS system does not require Ps-specific factors to function. Amino-acid residues essential for nucleotide binding and hydrolysis are important for HrpRS activity in vivo, particularly in the case of HrpR. However, as the ATPase activity and oligomeric states have not been tested in vivo, we cannot exclude that assembly defects account for lack of activity. Significantly, mutations in catalytic residues in HrpS (D107, E108 and R226), thought essential for activity in EBPs, did not abolish transcription activity in HrpRS. The requirement for both HrpR and HrpS σ54-binding motifs suggests an active, and not merely structural role, for each protein in the HrpRS complex. Binding of HrpV to HrpS and not HrpR implies a subunit-specific negative control mechanism not previously described for other EBPs. HrpV can completely repress HrpRS activity, but does not directly inhibit each subunit of the functional hexamer; potentially acting allosterically and/or sterically blocking access to the RNA polymerase closed complex. Allostery could be achieved through topological effects on the HrpRS hexameric ring in line with HrpV-mediated changes in the interactions between HrpR and HrpS (suggested in the BACTH assays; Fig. 1h). The action of HrpV is distinct in that it does not alter the ATPase or DNA-binding activities of HrpRS. In contrast, NifH diminishes the ATPase and DNA-binding properties of the EBP NifA, and PsdB inhibits the ATPase activity of PsdF.

Heteromeric AAA+ proteins have been reported as stacked homo-hexameric rings (for example, the bacterial proteases ClpA/ClpX3 and human ATPases Tip48/Tip49 (ref. 53)), or as a single hetero-hexameric ring (in the case of eukaryotic MCM; Mcm2-7p), or as homo-multimers (as in prokaryotic and archeal MCM)p55. Notably, the six distinct subunits of Mcm2-7p demonstrate clear mechanistic specialization in binding and hydrolyzing ATPp5. We now show that HrpRS forms a hetero-hexameric structure. The distinct ATP-binding activities and functional roles of HrpR and HrpS provide direct evidence that individual subunits in EBPs may not be functionally equivalent, and that asymmetric functioning may underpin their action.

**Methods**

**Bacterial strains and plasmids.** FL hrpR, hrpS, hrpRS (as a single operon), hrpV and hrpC genes were amplified by PCR from P. DC3000 genomic DNA (see Supplementary Table S3 for all strains and plasmids used in this study). The bacterial two-hybrid (BACTH) fusion proteins were amplified with flanking 5′-XbaI and 3′-KpnI restriction sites and cloned into the corresponding pUT18C and pKT25 vectors. To construct the pUT18C-hrpS/hrpV plasmid (co-expressing T18-HrpS and WT HrpV) a DNA fragment encompassing the Shine–Dalgarno sequence and hrpV was PCR amplified to include flanking 5′-KpnI and 3′-EcoRI restriction sites and cloned into pUT18C-hrpS (see Fig. 1g). Mutations in hrpR or hrpS were introduced using the QuikChange method (Agilent technologies) as instructed. All recombinant plasmids were verified by sequencing. The Pαv, translational lacZ reporter fusion (hrpL–lacZ) was constructed by amplifying 0.6kb of the hrpL promoter region as an EcoRI-BamHI fragment from P. DC3000 genomic DNA and subcloning into pBluescript (ref. 54)—creating pAVM415. The single-copy chromosomal fusion of hrpL–lacZ was constructed as describedp56. Briefly, phage λCD551 (mmr) was grown in a strain carrying pAVM415. The resulting λ phages were used to generate lysogens in SA1943 and screened for a Gal– phenotype on MacConkey galactose plates supplemented with ampicillin (100 µg ml−1). P1 phage was grown on the lysogens and the resultant lysates used to transduce the recipient strain DY226, which was screened for ampicillin resistant transductants at 42°C. The constructed strain carrying the Pαv-lacZ fusion was then screened for the Bio+ phenotype and the presence of promoter sensitivity to β-galactosidase. Finally, the Pαv-lacZ fusion was moved by P1 transduction (by selecting for ampicillin resistance) into strain MC4100, generating strain MJ2806.

**In vivo β-galactosidase assays.** β-Galactosidase assaysp47 and BACTH assays were performed as describedp56. Briefly, proteins were fused to either the T18 or T25 fragments of adenylate cyclase (plasmids pT18C and pT25, respectively) and co-transformed into BTH101 cells. Interaction efficiencies were quantified by measuring the β-galactosidase activity of the cultures following induction (for 2 h at 25°C) with 0.5 mM isopropyl-β-D-thiogalactoside at mid-log phasep56.
protease inhibitor cocktail (Roche), disrupted by sonication and the resulting soluble fraction purified by metal affinity chromatography using a linear gradient of 0–1 M imidazole (in buffer A). The desired protein fractions were dialysed overnight against storage buffer (20 mM Tris pH 8.0, 50 mM NaCl, 0.1 mM EDTA and 5% glycerol). HrpG was purified from the soluble cell fraction by urea-detergentation (which involved resuspending the cell pellet in buffer A containing 8 M urea. The rest of the Ni-affinity purification protocol remained the same. IIFH was purified exactly as described37.

**FL transcription assays.** FL transcription assays were performed in either TTH (10 mM Tris–HCl, pH 7.5, 70 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP, 12.5% glycerol and 0.1% w/v Triton X-100) or STA (25 mM Tris-acetate, pH 8.0, 80 mM Mg-acetate, 10 mM KCl and 3.5% w/v PEG 6000) buffer (for the hrp or nif promoters respectively) in a 10 µL volume containing 100 µM α32P-NAP (reconstituted at a 1:5 ratio RNA:Phospho-32P) and 20 µM supercoiled promoter DNA and where stated 4 mM ATP, 20 mM IIFH and equimolar (to [HrpRS]) concentrations of HrpV and/or HrpG (unless stated). OC formation was initiated by addition of 0.8 µM HrpR, HrpS or HrpRS (for the hrp promoter, unless otherwise stated) or 2 µM HrpRS or Phospho-P (for the nif promoter, unless otherwise stated) and incubated at room temperature (RT) for 1 h. Concentration stated refers to the monomer concentration for HrpR, HrpS or Phospho-P and the dimer concentration for HrpRS. Transcription was initiated with a mix containing 100 µg/mL 1 µM ATP, CTP, GTP and 0.05% (v/v) glycerol, incubated with 30 µCi [α32P]UTP and incubated for 10–20 min at RT (hrp) or 37°C (nif). Reactions were quenched with loading dye and analysed on 6% denaturing gels. Gels were dried and transcribed visualized and quantified using a Fuji FLA-5000 PhosphorImager.

**In vitro guluronic acid crosslinking.** Assays were conducted in a total reaction volume of 10 µL, in HGNED buffer (25 mM HEPES, pH 8.0, 1 mM dithiothreitol, 100 mM NaCl, 0.2 mM EDTA, 0.05% (v/v) NP-40 and 10% (v/v) glycerol)) containing 2.5 µM of the specified proteins. Samples were treated with guluronic acid (at a final concentration 0.1%) and incubated at RT for 10 min. Reactions were stopped by addition of 2 µl stop solution (200 mM Tris–HCl pH 7.5, 200 mM glycerine). Crosslinked protein complexes were separated by 10% SDS–PAGE and analysed by western blotting (see below).

**Western blotting.** Crosslinked complexes were separated by SDS–PAGE and transferred onto polyvinylidene difluoride membrane using a semidyaltransfer blotting system (Bio-Rad). Western blotting was performed as described38 using antibodies specific to either Ps HrpR (α-HrpR; 1:10,000), Ps HrpS (α-HrpS; 1:10,000), Ps HrpV (α-HrpV; 1:5,000), E. coli Phospho-P (α-Phospho-P; 1:4,000), His-tag (to detect HrpR; α-His; 1:1,000; Promega) or the BACTH fusion construct pK2 (α-pK2; 1:500). Proteins were detected using the ECL plus Western Blotting Detection Kit (GE Healthcare) as instructed.

**ATP binding and hydrolysis assays.** ATP binding was carried out in 20 µL total reaction volume containing 10 µM of purified protein in reaction buffer (20 mM Tris pH 8.0, 50 mM NaCl, 0.1 mM EDTA and 5% glycerol)) supplemented with 40 µCi [α32P]ATP (3000 Ci mmol−1) (NEN). Sample drops were spotted onto a 4°C cooled glass plate and exposed to ultraviolet light, illuminating (254 nm, UVG-54, UV-5) for 15 min on ice. Reactions were stopped by adding 10 µl NaOH and proteins were resolved by 10% SDS–PAGE analysis. Gels were stained by Sypry-rub fluorescent protein stain (Invitrogen) and protein band intensities visualized and quantified on Fuji FLA-5000 PhosphorImager (Fuji).
26. Rappas, M., Schumacher, J., Niwa, H., Buck, M. & Zhang, X. Structural basis of the nucleotide driven conformational changes in the AAA+ domain of transcription activator PapE. *J. Mol. Biol.* 357, 481–492 (2006).

27. Bordes, P. et al. The ATP hydrolyzing transcription activator phase shock protein F of Escherichia coli identifies a surface that binds sigma 54. *Proc. Natl Acad. Sci. USA* 100, 2278–2283 (2003).

28. Chaney, M. et al. Binding of transcriptional activators to sigma 54 in the presence of the transition state analog ADP-Aluminum fluoride: insights into activator mechaconochemical action. *Genes Dev.* 15, 2282–2294 (2001).

29. Chen, B., Sysoeva, T. A., Chowdhury, S. & Nixon, B. T. Regulation and action of bacterial enhancer-binding proteins. *Science* 307, 1972–1975 (2005).

30. Zhang, N. et al. The role of the conserved phenylalanine in the sigma54-interacting GAFTGTA motif of bacterial enhancer binding proteins. *Nucleic Acids Res.* 37, 5981–5992 (2009).

31. Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl Acad. Sci. USA* 95, 3752–3756 (1998).

32. Wang, Y. K., Park, S., Nixon, B. T. & Hoover, T. R. Nucleotide-dependent transcriptional activation by bacterial PspF AAA+ protein. *Genes Dev.* 17, 2042–2052 (1995).

33. Rombel, I. et al. The bacterial enhancer-binding protein NIFA as a molecular machine: ATP hydrolysis is coupled to transcriptional activation. *Nature Commun.* 519–533 (2009).

34. Lee, S. Y. et al. Structural insights into the activity of enhancer-binding proteins. *Science* 307, 1972–1975 (2005).

35. Inoue, H. & Kondrashov, F. The evolution of gene duplications: classifying and distinguishing between models. *Nat. Rev. Genet.* 11, 97–108 (2010).

36. Puri, T., Wendler, P., Sigala, B., Saibl, H. & Tsaneva, I. R. Dodecameric structure and ATPase activity of the human TIP48/TIP49 complex. *J. Mol. Biol.* 366, 179–192 (2007).

37. Burrows, P. C. et al. Functional roles of the pre-sensor I insertion sequence in an AAA+ bacterial enhancer binding protein. *Mol. Microbiol.* 73, 519–533 (2009).

Acknowledgments

We thank M. Abellan and B. Vignoto for help with preparing some of the hpr constructs; D. Court for ARBDC531, DY226 and SA1943 strains; H. Nash for K574b; John Pinney and John Mansfield for advice and comments on evolutionary aspects and *Pseudomonas syringae* biology, respectively; N. Joly and S.R. Wigneshwararaj for technical suggestions. We thank the above and members of the MB lab, especially G. Jovanovic for critical reading of the manuscript. This work was supported by the BBSRC, Leverhulme Trust and Wellcome Trust grants to MB. F.G.M.R. was the recipient of a CNPq-funded fellowship.

Authors contributions

M.I. prepared most constructs, designed, carried out and analysed all *in vitro* work (transcription, BACTH, UAS mapping, IFH); performed to *in vitro* transcription with hrpL, EMSA, including IFH purification and contributed to writing of Materials and Methods. E.H.I. worked on the evolution, purification of HrpRS, HrpV and HrpG; HrpG work and analysis; contributed to editing manuscript and contributed to writing of Materials and Methods. P.C.B. designed and established the heterologous *in vitro* transcription assay, including abortive conditions and linear DNA probes, collated and prepared figures for publication and contributed significantly to editing manuscript. F.G.M.R. purified HrpRS and carried out DNA binding and guanidinoacetate crosslinking experiments and also purified *Ps* Δ*hrp* for *in vitro* transcription experiments and contributed to writing of Materials and Methods. M.B. conceived the project design and experimental approaches, oversaw the study, interpretations of data and writing of the manuscript. I.S. designed, carried out and analysed *in vitro* work (ATPase, gel filtration, ultraviolet crosslinking), conceived the evolution work, contributed to the project design, oversaw the study, interpreted data and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Jovanovic, M. et al. Regulation of the co-evolved HrpR and HrpS AAA+ proteins required for *Pseudomonas syringae* pathogenicity. *Nat. Commun.* 2:177 doi: 10.1038/ncomms1177 (2011).

License: This work is licensed under a Creative Commons Attribution-NonCommercial-Share Alike 3.0 United States License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/