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PrfA regulation offsets the cost of Listeria virulence outside the host

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Summary

Virulence traits are essential for pathogen fitness, but whether they affect microbial performance in the environment, where they are not needed, remains experimentally unconfirmed. We investigated this question with the facultative pathogen Listeria monocytogenes and its PrfA virulence regulon. PrfA-regulated genes are activated intracellularly (PrfA 'ON') but shut down outside the host (PrfA 'OFF'). Using a mutant PrfA regulator locked ON (PrfA*) and thus causing PrfA-controlled genes to be constitutively activated, we show that virulence gene expression significantly impairs the listerial growth rate ($\mu$) and maximum growth (A) in rich medium. Deletion analysis of the PrfA regulon and complementation of a L. monocytogenes mutant lacking all PrfA-regulated genes with PrfA* indicated that the growth reduction was specifically due to the unneeded virulence determinants and not to pleiotropic regulatory effects of PrfA ON. No PrfA*-associated fitness disadvantage was observed in infected eukaryotic cells, where PrfA-regulated virulence gene expression is critical for survival. Microcosm experiments demonstrated that the constitutively virulent state strongly impaired L. monocytogenes performance in soil, the natural habitat of these bacteria. Our findings provide empirical proof that virulence carries a significant cost to the pathogen. They also experimentally substantiate the assumed, although not proven, key role of virulence gene regulation systems in suppressing the cost of bacterial virulence outside the host.

Introduction

The ability of a microbe to infect and cause harm (virulence) correlates with its multiplication rate within the host, itself a direct determinant of between-host transmission success (Read, 1994; Lipsitch and Moxon, 1997). High virulence, however, may immobilize or cause the death of the host, impairing transmission to new hosts and hence pathogen fitness. Virulence has thus been theorized to hinge on a trade-off balance with transmissibility and to be potentially costly to the pathogen (Anderson and May, 1981; Antia et al., 1994; Bull, 1994; Alizon et al., 2009). This relationship is easily intuited for micro-parasites depending on a live host for transmission (i.e. obligate pathogens) and is at the core of virulence theory (Bull and Lauring, 2014). However, whether microbial virulence also affects the performance of indirectly transmitted pathogens in the environment remains to be clarified and is largely neglected by evolutionary models.

Virulence determinants have specifically evolved to confer an advantage within the host, and the gratuitous expression of microbial traits in a situation in which they are not required is known to carry fitness penalties (Nguyen et al., 1989; Eames and Kortemme, 2012). Despite the obvious potential significance for pathogen evolution, experimental information about the costs associated with unneeded virulence traits in a non-host system is essentially lacking. A number of studies with phytopathogens have examined the fitness costs of ‘avirulence’ gene mutations to virulence in susceptible plant populations without the matching resistance (R) gene (where the pathogen’s avirulence/virulence gene is irrelevant) (Leach et al., 2001; Bahri et al., 2009; Huang et al., 2010; Montarry et al., 2010). These studies have generally measured the cost of virulence via the effects on within-host fitness attributes (e.g. in planta multiplication, amount of disease symptoms or pathogen released from leaves) but not on saprophytic growth and survival (Sacristan and Garcia-Arenal, 2008). In animal
pathogens, a recent report on Salmonella addressed the cost of virulence factors in in vitro culture (Sturm et al., 2011). In this study, Sturm and colleagues showed that expression of the type III secretion system (TTSS)-1 was associated with significant growth retardation. Gene deletion analysis suggested that the growth defect was at least in part attributable to TTSS-1 virulence factor expression, although the possibility that it was also due to global, pleiotropic regulatory effects was not excluded (Sturm et al., 2011).

Listeria monocytogenes is a prototypic facultative pathogen that can live both as a soil saprotroph or an intracellular parasite of animals and people (Vazquez-Boland et al., 2001b; Freitag et al., 2009). Listerial virulence is conferred by a set of proteins that promote host cell invasion (internalins InlA and InlB), phagocytic vacuole escape (pore-forming toxin Hly, phospholipases PlcA and PlcB, metalloprotease Mpl), cytosolic replication (sugar phosphate transporter Hpt) and actin-based cell-to-cell spread (surface protein ActA, internalin InlC) (Cossart, 2011). The genes encoding these nine virulence factors are coordinately regulated by the transcriptional activator PrfA (Mengaud et al., 1991; Chakraborty et al., 1992) (Fig. 1). PrfA-regulated genes are normally very weakly expressed outside the host but strongly induced during intracellular infection (Moors et al., 1999; Shetron-Rama et al., 2002; Chatterjee et al., 2006; Joseph et al., 2006; Toledo-Arana et al., 2009). This activation is thought to require PrfA to allosterically switch from its native, weakly active (‘OFF’) conformation to a highly active (‘ON’) state (Scotti et al., 2007; de las Heras et al., 2011) and is essential for Listeria virulence (Deshayes et al., 2012). Single amino acid substitutions that lock PrfA in an ‘always-ON’ (PrfA*) state have been identified (Ripio et al., 1997; Vega et al., 2004; Wong and Freitag, 2004). Listeria monocytogenes mutants carrying one such PrfA* substitution, G145S, constitutively express the PrfA-regulated genes in vitro to levels similar to the wild type during intracellular infection (Ripio et al., 1997; Vega et al., 2004; Deshayes et al., 2012). prfA*G145S mutants therefore provide a unique tool to investigate the cost of virulence traits in non-host conditions.

Taking advantage of the properties conferred by the prfA* allele, we show that virulence gene activation imposes a significant burden on L. monocytogenes outside the host. We also show that this burden limits the survival and competitive ability of L. monocytogenes in soil. Our data provide the first formal demonstration that the virulence traits that make a microbe pathogenic entail a significant fitness cost. We also experimentally substantiate that a primary key role of virulence gene regulation systems in facultative pathogens is to neutralize the cost of virulence outside the host, thereby maximizing between-host pathogen fitness in the environmental reservoir.

**Results**

When first identified in our laboratory (Ripio et al., 1996; 1997), we observed that prfA* mutants exhibited impaired growth in broth medium, suggesting a fitness defect (unpubl. data). The prfA*-associated growth reduction was also noted by others, although the effect was relatively minor compared with wild-type prfA (prfAWT) and was not statistically confirmed (Marr et al., 2006). More recently, L. monocytogenes bacteria carrying prfA* alleles were found to have increased sensitivity to stress and a competitive disadvantage upon repeated passage in broth culture (Bruno and Freitag, 2010), although no growth defect in rich medium was directly observed in monoculture (Port and Freitag, 2007; Bruno and Freitag, 2010). The interpretation of these reports was complicated by possible regulatory interference of PrfA ON with listerial carbon nutrition/metabolism (Marr et al., 2006; Bruno and Freitag, 2010). Moreover, effects on fitness could have been obscured in these studies by the use of strains trans-complemented with the prfA gene on a multicopy plasmid (Marr et al., 2006), or carrying enzymatic and antibiotic resistance cassettes under the control of a PrfA-dependent promoter (Port and Freitag, 2007; Bruno and Freitag, 2010).

**Cost of PrfA activation in vitro**

To avoid possible confounding effects due to the potential burden introduced by multicopy plasmids or reporter genes, we investigated the fitness consequences of PrfA regulon activation using a naturally occurring prfAWTG1448S strain (P14A) (Ripio et al., 1997) and an isogenic, unmarked prfAWT allelic exchange revertant thereof.

![Fig. 1. Schematic of L. monocytogenes PrfA virulence regulon and ON–OFF PrfA switching. Dotted lines indicate relevant transcriptional units.](image-url)
(P14Rev). The latter was obtained by double homologous recombination using fosfomycin to counterselect the original prfA* genotype (see Experimental procedures). This selection strategy is based on the ability of the listerial PrfA-dependent sugar phosphate permease Hpt to confer susceptibility to fosfomycin when the PrfA system is activated (Scortti et al., 2006). Bacterial fitness was measured by determining the exponential growth rate (μ) and maximum growth yield (A) in brain–heart infusion (BHI) broth, a rich culture medium in which Listeria growth is optimal and wild-type PrfA-dependent gene expression is maximally downregulated at 37°C (Ripio et al., 1996; 1997; Shetron-Rama et al., 2003). As controls, an isogenic in-frame prfA deletant (ΔprfA) and the parent prfA WT strain of P14A (isolate P14) were also tested.

P14A exhibited a clear growth defect in BHI, as evidenced by its significantly lower μ and A values (F3,10 = 8.07 P = .005 and 54.98 P < .0001 respectively) (Fig. 2). Replacement of P14A’s prfA* allele by prfA WT (P14Rev) restored growth to wild-type (P14) levels. On the other hand, the growth dynamics of P14 and P14Rev, both expressing a PrfAWT protein, was identical to that of the ΔprfA strain lacking PrfA (Fig. 2). These data indicate (i) that the constitutively active PrfA*G145S protein, driving high (‘in vivo’ equivalent) levels of PrfA-dependent gene expression in in vitro conditions (Ripio et al., 1997; Deshayes et al., 2012), significantly impairs L. monocytogenes fitness in rich medium; and (ii) that PrfA WT, associated with negligible levels of PrfA-dependent gene expression in vitro (Ripio et al., 1997; Deshayes et al., 2012), has a neutral effect on L. monocytogenes performance.

PrfA* does not impair L. monocytogenes fitness in infected host cells

Since PrfA-regulated virulence determinants are unlikely to be necessary for extracellular growth in vitro, the fitness disadvantage observed with the prfA* allele in BHI could reflect the burden typically associated with expressing dispensable gene products (Dong et al., 1995; Stoebel et al., 2008; Shachrai et al., 2010). If this explanation is correct, then no significant growth impairment is expected to occur in an infection setting, where bacterial fitness depends on the expression of virulence genes. To confirm this, we compared the behaviour of the prfA* and prfA WT bacteria in intracellular proliferation assays in eukaryotic cell monolayers.

P14A did not differ from P14Rev (and P14) in intracellular growth in HeLa cells (F2,3 = 0.04 P = .9575) (Fig. 3). This result is in agreement with previous data showing that prfA* and prfA WT L. monocytogenes have similar or comparable virulence in vivo in mice and in infected cells (Ripio et al., 1996; Shetron-Rama et al., 2003; Bruno and Freitag, 2010; Deshayes et al., 2012). Thus, despite the significant growth defect observed in vitro in rich medium, the PrfA* protein did not seem to impair L. monocytogenes fitness in vivo in a host system. This is consistent with the notion that PrfA* is locked in the ON state presumably adopted by PrfA WT in vivo during infection, resulting in similar levels of virulence gene expression for both prfA* and prfA WT bacteria within host cells (de las Heras et al., 2011; Deshayes et al., 2012).

The fitness cost is due to PrfA regulon components

The growth reduction associated with the prfA* allele in nutrient-rich BHI could be due to the cost of expressing unneeded virulence products, or alternatively to PrfA ON

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Complementation of ΔprfA with the prfA\textsuperscript{*} allele, but not prfA\textsuperscript{WT} or empty vector, caused growth inhibition, with significant reduction in both μ and A (F\textsubscript{2,8} = 8.17 P = .0117 and 34.04 P < .0001 respectively) (Fig. 5A). This mirrored the previous data with the isogenic strains carrying the prfA gene in its native chromosomal location, confirming that the growth reduction was solely due to the activity of PrfA\textsuperscript{*}. In contrast, no significant differences were observed between the complemented ΔREG strains (μ P = .1397, A P = .9142) (Fig. 5B), or between these and ΔprfA complemented with prfA\textsuperscript{WT} or empty vector (μ P = .4104, A P = .1719). These data show that the growth reduction caused by PrfA ON requires the presence of the PrfA-dependent virulence genes on the listerial chromosome.

Partial PrfA regulon mutants in P14A were analysed to determine the contribution of specific PrfA-regulated loci to the fitness loss. Deletion of the internalin genes inlAB and inlC or the hpt monocistron did not relieve the growth defect caused by PrfA\textsuperscript{*} (Fig. S1). In contrast, deletion of

Interfering with some listerial housekeeping function important for listerial growth, as previously suggested (Marr et al., 2006). To address this question, we constructed a P14A mutant lacking the entire PrfA regulon (ΔREG), i.e. Listeria pathogenicity island 1 encompassing the prfA, plcA, hly, mpl, actA and plcB genes (LIPI-1), the internalin loci inlAB and inlC, and the organophosphate transporter gene hpt (also known as uhpT) (Fig. 1). ΔREG was complemented with either prfA\textsuperscript{WT} (from P14) or prfA\textsuperscript{G145S} (from P14A) inserted in monocopy in a permissive site of the listerial chromosome using an integrative vector (pPL2) (Lauer et al., 2002; Deshayes et al., 2012).

P14A ΔprfA, which possesses the entire PrfA regulon except the deleted prfA gene, was also complemented with the same prfA constructs as a control. Western blot analyses confirmed that the PrfA protein was correctly expressed in prfA-complemented ΔREG and ΔprfA (Fig. 4A). They also confirmed that the prfA\textsuperscript{*} and prfA\textsuperscript{WT} constructs induced, respectively, the expected high and low/undetectable expression levels of PrfA-regulated products in BHI (Fig. 4B).

Fig. 3. Intracellular proliferation of L. monocytogenes prfA\textsuperscript{*} (strain P14A) and prfA\textsuperscript{WT} (P14A isogenic wild-type allele-replacement revertant P14\textsuperscript{Rev} and parent strain P14) in human HeLa cells. Upper panel, intracellular colony forming units (cfu); lower panel, data expressed as normalized intracellular growth coefficient (IGC, see Experimental procedures). Mean ± SEM of three experiments.

Fig. 4. Western immunoblot analysis. A. Detection of PrfA in cell extracts of ΔprfA and ΔREG bacteria complemented with prfA\textsuperscript{WT} or prfA\textsuperscript{*} alleles. Protein loaded: 10 μg.
B. Detection of selected PrfA-dependent virulence factors in the cell extracts or culture supernatants of ΔprfA complemented with prfA\textsuperscript{WT} or prfA\textsuperscript{*} alleles. The two arrows in PlcB indicate the unprocessed and mature form of the enzyme. Protein loaded per lane: 20 μg, 5 μg for Hly.
LIPI-1 rescued the growth defect in the presence of prfA* (Fig. 6). Some recovery of the wild-type phenotype was observed for single hly or actA deletion mutants within LIPI-1, although the effect was not statistically significant (Figs S2 and S3). Thus, the PrfA*-associated growth impairment is mainly attributable to LIPI-1 and depends on the expression of several PrfA-regulated genes. Together, our results are consistent with the growth reduction caused by PrfA ON being due to the burden associated with the expression of PrfA regulon virulence determinants.

**PrfA switch-off is required for optimal fitness in soil**

We next sought to investigate the effect of PrfA activation on fitness in a non-host model more closely approximating the conditions encountered by *L. monocytogenes* in nature. Soil rich in decaying plant matter is considered to be the main *Listeria* environmental reservoir (Weis and Seeliger, 1975; Vazquez-Boland *et al*., 2001b; Freitag *et al*., 2009; Vivant *et al*., 2013) and was chosen for these experiments. Sterile topsoil of neutral pH was used to ensure optimal *L. monocytogenes* growth/survival (Botzler *et al*., 1974; McLaughlin *et al*., 2011; Locatelli *et al*., 2013; Vivant *et al*., 2013). P14A (prfA*) and its isogenic P14Rev (prfAWT) and ΔprfA derivatives were inoculated in axenic microcosms at a dose of ≈6 × 10^6 cfu g⁻¹, and viable bacterial numbers in soil were regularly monitored for 17 days by plate counting. Although the pPL2 vector had previously demonstrated stable chromosomal integration in a variety of conditions (*in vitro* in culture media or *in vivo* in infected cells and mice) (Lauer *et al*., 2002; Deshayes *et al*., 2012; this study), the prfAWT and prfA* pPL2 constructs (and control empty vector) were rapidly lost in soil by the complemented ΔprfA strain (within the first 48 h) and could not be used. P14A again showed significantly different behaviour (genotype × time points F₂,₇₄ = 5.02 P < .0001; two-way analysis of variance (ANOVA) with Tukey’s post-hoc multiple comparisons): after an initial population increase for the three strains, P14A counts steadily dropped from day 3, while P14WT and ΔprfA continued to grow until day 5, followed by stabilization until declining after day 11 (Fig. 7). Thus, consistent with our observations in rich...
medium, prfA* bacteria also exhibited diminished fitness in soil compared with prfA*WT and ΔprfA bacteria.

**Competition experiments**

To internally control for possible inter-sample variation in growth due to physicochemical/nutritional micro-environment heterogeneity in soil (Vivant et al., 2013), the strains were tested in mixed culture in the same soil microcosms. This approach also permits direct determination of the competitive ability and an estimate of the strength of selection acting against the less fit genotype (Lenski, 1992). Either prfA* or prfA*WT bacteria were co-inoculated in a 1:1 ratio with ΔprfA used as a common reference. This allowed confirmation of the relative frequencies of the competing genotypes by polymerase chain reaction (PCR) screening of the specific deletion in ΔprfA (see Experimental procedures).

prfA* bacteria were clearly outcompeted by ΔprfA after the first 24 h (competitive index (CI) < 1) until their total disappearance by day 9 (Fig. 8A). In contrast, no differences in the relative fitness of prfA*WT and ΔprfA genotypes (CI not significantly different from 1) were observed throughout the experiment (Fig. 8B). These data indicate that (i) the burden imposed by the activation of the PrfA virulence regulon compromises *L. monocytogenes* survival in soil, and (ii) the virulence-associated fitness cost in soil is effectively compensated by the ON–OFF switchable PrfA regulator.

**Discussion**

Microbial growth is a correlate of the fitness status of the prokaryotic cell and responds to the principle of cost–benefit optimality. To ensure maximal fitness, microbial cells need to optimize the allocation of limited resources to competing traits (Dekel and Alon, 2005; Molenaar et al., 2009; Berkhout et al., 2013). This is often achieved by coupling gene expression to beneficial processes under specific conditions, as classically illustrated by studies with the *lac* operon or antibiotic resistance determinants (Koch, 1983; Nguyen et al., 1989; Dekel and Alon, 2005; Stoebel et al., 2008; Eames and Kortemme, 2012). Here we analysed the fitness consequences of expressing virulence traits in conditions in which they are not directly beneficial, i.e. during saprophytic growth outside the host. Notwithstanding its undeniable potential significance in pathogen evolution and transmission dynamics, this question had been insufficiently investigated. Using *L. monocytogenes* and a mutant form of its

![Fig. 6. Growth in BHI of ΔLIPI-1 complemented with prfA*WT, prfA* or empty vector. ΔprfA bacteria complemented with prfA*WT, prfA* or empty vector were used as a control. A. Growth curves. B. Corresponding μ (growth rate) and A (maximum growth) values expressed in OD600 units. Mean ± SEM of three experiments. ΔprfA complemented with prfA* used as reference in post-hoc multiple comparison. Numbers indicate P values; ns, not significant.](image)

![Fig. 7. Monoculture experiments in soil. Microcosms were seeded with 6 × 10^6 cfu g⁻¹ of *L. monocytogenes* prfA* (P14A), prfA*WT (P14WT) or ΔprfA, and the bacterial population dynamics for each strain regularly monitored in soil by plate counting during static incubation at room temperature. See Experimental procedures for details. Results expressed as mean cfu g⁻¹ ± SEM of three replicates. The prfA* and prfA*WT alleles remained stable throughout the experiments (see Fig. S5).](image)
master virulence regulator, PrfA*G145S (Ripio et al., 1997), which causes virulence genes to be constitutively expressed in vitro to the same high levels seen in vivo during infection (de las Heras et al., 2011; Deshayes et al., 2012), we demonstrate that virulence traits impose a significant burden on bacterial fitness. The fitness disadvantage was evident in extracellular conditions but not in infected cells where the virulence products are indispensable, reflecting that, during infection, the burden associated with virulence factor synthesis is compensated by the beneficial effects on within-host fitness. Using a soil model, we further show, for the first time, that the virulence-associated fitness cost translates into significantly impaired bacterial survival in an environmental milieu relevant for pathogen transmission.

PrfA* had no effect on growth in the absence of the target PrfA regulon genes, indicating that the impaired performance was clearly linked to the expression of the virulence factors and not due to PrfA ON disturbing an unrelated housekeeping or metabolic pathway(s). A possible explanation is that some PrfA regulon product(s) might exert a direct inhibitory effect on L. monocytogenes via unknown mechanisms. Alternatively, and more plausibly, the PrfA*-associated growth deficiency may be the consequence of the gratuitous expression of unneeded PrfA regulon products. Indeed, growth reduction is the typical penalty response observed when wasteful proteins are expressed by bacterial cells, aka protein cost (Dong et al., 1995; Dekel and Alon, 2005; Stoebel et al., 2008; Shachrai et al., 2010). The growth deficiency was readily apparent in monoculture in resource-replete conditions, indicating that the impact of PrfA regulon activation on Listeria fitness is substantial. LIPI-1, which contains six of the nine PrfA-regulated genes (Fig. 1), appeared to

Fig. 8. Competition experiments in soil. (A) prfA* (P14A) versus ΔprfA. (B) prfAWT (P14™) versus ΔprfA. Microcosms were inoculated with ≈10^7 cfu g^-1 of 1:1 mixes of the indicated L. monocytogenes strains. Left panels, bar charts: bar height indicates log total cfu g^-1; black and grey areas within bars indicate the proportion of competing bacteria. Right panels, competitive index (CI). P values for statistically significant differences with the reference value 1 are indicated (see Experimental procedures). Mean ±SEM of three replicates.
account for the entire burden. Growth rate ($\mu$) and growth yield ($A$) were both impaired, as would be expected if rate limiting bacterial biosynthetic resources are diverted for virulence factor expression until a critical nutrient(s) is exhausted from the medium.

Protein cost is a major driving force in the shaping of regulatory systems (Dekel and Alon, 2005; Babu and Aravind, 2006; Kalisky et al., 2007; Stoebel et al., 2008; Gao and Stock, 2013). The rapid elimination of the prfA* genotype in the competition experiments in soil equates to a selection coefficient of about −0.33 d$^{-1}$ (roughly a 33% difference in fitness measured over a day) (Lenski, 1992), indicating very strong selection against constitutive virulence gene expression in this environment. This selection is expected to be even greater in non-sterile soil, where the presence of competing microbiota has been shown to significantly impair L. monocytogenes growth/survival (McLaughlin et al., 2011; Locatelli et al., 2013; Vivant et al., 2013). Whether expressing PrfA$^{WT}$ or lacking the PrfA regulator, no significant differences in L. monocytogenes fitness were observed in either rich medium or soil. The cost neutrality of PrfA$^{WT}$ in the tested extracellular conditions therefore indicates that the acquisition of an ON-OFF switchable PrfA regulator has been critical in the evolution of L. monocytogenes as a facultative parasite.

The instability in soil (but not BHI or other conditions) of the chromosomally integrated pPL2 constructs indicates that PrfA$^{WT}$, and indeed the empty complementation vector itself, imposed a burden. This implies that soil is a strongly selective environment for L. monocytogenes in which, despite PrfA-dependent genes being down-regulated (Piveteau et al., 2011), any leaky expression due to the basal activity of PrfA$^{WT}$ in the OFF state (Deshayes et al., 2012) may be disadvantageous. Indeed, although not apparent in BHI, ΔprfA bacteria also exhibit some fitness advantage over prfA$^{WT}$ bacteria in certain circumstances (e.g. chemically defined medium; our unpublished observations). Listeria monocytogenes possesses other mechanisms in addition to ON-OFF PrfA switching to ensure that the PrfA regulon is effectively silenced outside the host. For example, an RNA thermoswitch prevents efficient prfA gene translation at environmental temperatures (≤30°C) (Johansson et al., 2002). Growth on cellobiose and other plant-derived β-glucosides, presumably abundant in the decaying vegetation-rich soil habitat, also strongly represses PrfA regulated genes (Brehm et al., 1999). The existence of these redundant PrfA-downregulating mechanisms is consistent with preventing any virulence-related fitness loss being critically important for L. monocytogenes outside the host.

Since dispensable genes tend to be readily eliminated from bacterial genomes (Cooper et al., 2001; Mira et al., 2001), L. monocytogenes is expected to lose the ability to express the PrfA regulon – and indeed the PrfA regulon altogether – during its existence as a free-living organism. This appears to have occurred during evolution and is the presumed mechanism that gave rise to the obligate saprophytic species of the genus, typified by Listeria innocua (Vazquez-Boland et al., 2001a; Schmid et al., 2005; Hain et al., 2006). Some strains of Listeria seeligeri, another non-pathogenic species, still possess a partially conserved PrfA regulon undergoing gene decay processes. (Vazquez-Boland et al., 2001a; den Bakker et al., 2010). Similarly, spontaneous prfA disabling mutations are not uncommon among L. monocytogenes food isolates (Roche et al., 2005). This predicts a scenario of rapid decline and even extinction of the pathogenic L. monocytogenes, which is clearly not supported by this species’ known widespread distribution and epidemiology (Vazquez-Boland et al., 2001b; Freitag et al., 2009). Arguably, therefore, virulence must somehow confer an evolutionary advantage to L. monocytogenes. The maintenance of the PrfA regulon may be positively selected in the environmental habitat for a number of reasons. For example, PrfA-regulated virulence factors may promote survival by helping Listeria to evade predation by soil bacterivorous protozoa (Greub and Raoult, 2004). The PrfA regulon may also facilitate the subclinical colonization of the intestinal tract of animal hosts and subsequent fecal-oral enrichment of virulent L. monocytogenes bacteria in the environment (Vazquez-Boland et al., 2001b).

While essential for within-host microbial proliferation, virulence, if excessive, may also reduce the time the infected host remains viable and producing pathogen offspring for transmission to new hosts. Based on this tenet, evolutionary theory posits that pathogen fitness is optimized through a trade-off between virulence and transmission (Anderson and May, 1981; Antia et al., 1994; Bull, 1994; Bull and Lauring, 2014). This assumption, however, is host-centric and based on direct host-to-host transmission models, neglecting that pathogens are also indirectly transmitted from environmental sources (Anderson and May, 1981; Roche et al., 2011; Mikonranta et al., 2012). Moreover, many pathogens, like L. monocytogenes, not only ‘sit-and-wait’ in the environment for new hosts (Walther and Ewald, 2004) but reproduce as free-living organisms (Menkanto et al., 2012). Here, we provide with the facultative pathogen L. monocytogenes the first formal demonstration that virulence traits are intrinsically costly to the microbe, impairing pathogen proliferation outside the host. A significant implication is that, contrary to current belief (Bonhoeffer et al., 1996; Gandon, 1998; Walther and Ewald, 2004; Roche et al., 2011), the evolutionary dynamics of facultative pathogens that do not depend directly on a host for
transmission is also constrained by a virulence-transmission trade-off. We suggest that this trade-off has been a key determinant in the evolution of virulence regulation systems in facultative pathogens, as exemplified here with the Listeria PrfA switch. A deeper insight into how microbes control the costs of virulence both within and outside the host, and incorporating this knowledge into virulence theory, will be key to improve our understanding of pathogen ecology and the evolution of virulence.

### Experimental procedures

**Bacteria, plasmids, media and reagents**

The strains and plasmids used are listed in Table 1. *Listeria monocytogenes* bacteria were all derived from the serovar 4b human isolate P14 (Ripio et al., 1996; 1997). *Listeria* and *Escherichia coli* were grown at 37°C in BHI (Difco-BD) and Luria–Bertani (Sigma) media, respectively, supplemented with 1.5% agar (w/v) and/or antibiotics as appropriate. Chemicals and oligonucleotides were purchased from Sigma-Aldrich unless stated otherwise.

**General DNA techniques**

Chromosomal *Listeria* DNA was extracted and purified as previously described (Ripio et al., 1997). Plasmid DNA was extracted from *E. coli* using the Spin Miniprep kit from Qiagen and introduced into *L. monocytogenes* by electroporation (Ripio et al., 1997) using a Gene Pulser Xcell apparatus (Bio-Rad). Polymerase chain reaction was carried out with Taq DNA polymerase (Biotools, Spain) for detection/mapping purposes or high-fidelity ProofStart DNA polymerase (Qiagen) for mutant construction or gene complementation.

### Table 1. Bacterial strains and plasmids used in this study.

| Strain/plasmid | Genotype/description | Source (reference) | Internal strain collection no. |
|----------------|----------------------|-------------------|------------------------------|
| **L. monocytogenes** | | | |
| P14 | prfA<sup>WT</sup>, wild-type strain of serovar 4b, human clinical isolate | Our laboratory (Ripio et al., 1996; 1997) | PAM 14 |
| P14<sup>ΔprfA</sup> | prfA<sup>G145S</sup> isogenic derivative of P14 | Our laboratory (Ripio et al., 1996; 1997) | PAM 50 |
| P14<sup>ΔactA</sup> | ΔactA, allele exchange wild-type revertant of P14A | This study | PAM 3757 |
| ΔprfA<sup>ΔactA</sup> | In frame prfA deletion mutant of P14A | Our laboratory (Deshayes et al., 2012) | PAM 373 |
| ΔprfA (vector) | ΔprfA, PAM 373 complemented with pPL2 empty vector | Our laboratory (Deshayes et al., 2012) | PAM 3293 |
| ΔprfA (prfA<sup>WT</sup>) | prfA<sup>WT</sup>, PAM 373 complemented with pPL2 prfAbc<sup>WT</sup> | This study | PAM 3319 |
| ΔprfA (prfA<sup>*</sup>) | prfA<sup>G145S</sup>, PAM 373 complemented with pPL2prfAbc<sup>*</sup> | This study | PAM 3320 |
| ΔREG | ΔLIP1-1, ΔinlAB-ΔinlC, Δhpt, PrfA regulon deletion mutant of P14A | | PAM 3691 |
| ΔREG (vector) | PAM 3691 complemented with pPL2 empty vector | This study | PAM 3734 |
| ΔREG (prfA<sup>*</sup>) | PAM 3691 complemented with pPL2 prfAbc<sup>*</sup> | This study | PAM 3694 |
| ΔLIP1-1 | ΔprfA plcA hly mpl actA plcB, LIP1-1 deletion mutant of P14A | This study | PAM 3695 |
| ΔLIP1-1 (vector) | PAM 3732 complemented with pPL2 empty vector | This study | PAM 3732 |
| ΔLIP1-1 (prfA<sup>WT</sup>) | PAM 3732 complemented with pPL2 prfAbc<sup>WT</sup> | This study | PAM 3750 |
| ΔLIP1-1 (prfA<sup>*</sup>) | PAM 3732 complemented with pPL2 prfAbc<sup>*</sup> | This study | PAM 3751 |
| ΔinlABC | ΔinlAB-ΔinlC in frame deletion mutant of P14A | Our laboratory (unpublished) | PAM 3657 |
| Δhpt | Δhpt in frame deletion mutant of P14A | Our laboratory (Scortti et al., 2006) | PAM 377 |
| Δhly | Δhly in frame deletion mutant of P14A | Our laboratory (Deshayes et al., 2012) | PAM 3730 |
| ΔactA | ΔactA in frame deletion mutant of P14A | Our laboratory (Suarez et al., 2001) | PAM 185 |
| **E. coli** | | | |
| DH5α | Cloning host strain | Our laboratory | |
| **Plasmids** | | | |
| pPL2 | Integrative vector for single-copy gene complementation in *L. monocytogenes* | M. Loesnser (Lauer et al., 2002) | |
| pMAD | Thermosensitive shuttle vector for allelic exchange in Gram-positives | M. Debarbouille (Amaud et al., 2004) | |
| pLSV1 | Thermosensitive shuttle vector for allelic exchange in Gram-positives | J. Kreft (Wuenscher et al., 1991) | |
| pPL2 prfAbc<sup>WT</sup> | pPL2 inserted with PrfA-autoregulated ΔplcA-prfAbc<sup>WT</sup> bicistronic construct | This study | |
| pPL2 prfAbc<sup>*</sup> | pPL2 inserted with PrfA-autoregulated ΔplcA-prfAbc<sup>*</sup> bicistronic construct | This study | |
| pLS5’prfAb<sup>WT</sup> | pLSV1 inserted with a 5’-truncated prfAbc<sup>WT</sup> used in P14<sup>ΔprfA</sup> construction | This study | |
| pMAD LIP1 | pMAD inserted with recombinogenic construct for deletion of LIP1-1 | This study | |
| pLSV hpt | pLSV1 inserted with recombinogenic construct for deletion of hpt | Our laboratory | |
The PCR products were purified with the PCR purification kit from Qiagen and analysed by standard gel electrophoresis in 1.0% agarose (Biotools). DNA sequences were determined on both strands by Sanger sequencing. Restriction enzymes were used according to the manufacturer’s instructions (New England Biolabs).

**prfA** WT revertant from prfA* 

P14 WT was constructed by replacing the prfA G145S allele of strain P14A with prfA WT following a procedure described in detail elsewhere (J. Monzó i Gil, PhD thesis, University of Bristol, UK, 2007). Briefly, primers PrfAalleI and PrfAalleII-long (Table S1), the latter with a Sall site, were used to amplify the prfA gene from wild-type *L. monocytogenes* P14 (Table 1). The PCR product was digested with Sall and EcoRI (naturally occurring internal site 25 bp downstream from the prfA start codon), and the resulting 5′-end-truncated prfA fragment (which includes codon 145) was inserted into the thermosensitive shuttle vector pLSV1 (Wuenscher et al., 1991), giving rise to the allele replacement plasmid pLS5′ (Table 1). The PCR products were purified with the PCR purification kit previously described (Suarez et al., 2001). 

For prfA complementation, prfA WT and prfA G145S from P14 and P14A, respectively, with all native promoters including the PrfA-dependent plcA promoter that positively auto-regulates prfA expression (Mengaud et al., 1991; Scortti et al., 2007) (see Fig. 1), were inserted in monocopy in the *L. monocytogenes* chromosome using the integrative vector pPL2 (Lauer et al., 2002) as previously described (Deshayes et al., 2012). prfA constructs were generated by in-frame deleting the plcA gene from the plcA-prfA bicistron from either P14 or P14A by splicing overlap extension PCR using suitable primer combinations (Table S1). After electroporation into ΔprfA or ΔREG, pPL2 integrants were selected in BHI plates containing 7.5 μg ml−1 chloramphenicol. All gene deletions were confirmed by PCR and DNA sequencing.

**Western immunoblotting**

*Listeria* were grown in 10 ml BHI until OD600 ≈ 1.0–1.2 and the cultures (1 ml) were centrifuged at ~ 7000 × g for 5 min at 4°C to separate the supernatant and the bacterial cells. The cell-free supernatant was precipitated with 16% trichloroacetic acid overnight at 4°C. After centrifugation at 18 000 × g for 10 min at 4°C, the protein pellet was washed with acetone, dried, then re-suspended in 2% SDS 6 M urea Tris-HCl buffer and stored at −80°C. For cell-associated proteins, the bacterial pellet was re-suspended in cold lysis solution (50 mM NaH2PO4, 300 mM NaCl, pH 7.4) with protease inhibitor cocktail (Roche), transferred to Lysis Matrix B tubes containing 0.1 mm silica beads (Q-Biogene) and homogenized in a FastPrep instrument (Q-Biogene) (three cycles of 30 s at speed set to 6). Cell debris was removed by centrifugation at 12 000 × g for 20 min at 4°C and the supernatant stored at −80°C. After determining total protein concentration (colorimetric DC protein assay, Bio-Rad), protein samples were separated by SDS-PAGE using 4–12% NuPAGE Bis–Tris mini gels (Novex Life Technologies) and electro-transferred to polyvinylidene difluoride membranes using a Mini-Protein II cuvette. Membranes were blocked for 2 h with 0.05% Tween 20 5% skim milk (w/v) phosphate-buffered saline pH 7.2 (PBS) and incubated (1 h or overnight at room temperature) with appropriate primary (see below) and secondary (1:5000-diluted anti-rabbit and 1:2000-diluted anti-mouse, horseradish peroxidase-conjugated) antibodies in the same solution. After washing, immunoreactive proteins were detected using Amersham’s ECL chemiluminescent detection reagents (GE Healthcare). The following primary antibodies were used: PrfA rabbit polyclonal (Vega et al., 1998); PlcA and PlcB mouse monoclonals (J. Wehland, Braunschweig, Germany); Hly mouse monoclonal (T. Chakraborty, Giessen, Germany); InIA and InIB mouse monoclonals (P. Cossart, Paris, France); and InIC rabbit polyclonal (raised against an InIC-specific peptide).

**Deletion mutants and prfA complementation**

Unmarked gene deletion mutants were constructed in *L. monocytogenes* P14A (Table 1) by allelic exchange using a thermosensitive shuttle vector. The in-frame deletion mutants ΔprfA, Δhly, ΔactA, Δhpt and ΔinlABC were previously available in our laboratory (Table 1). For deleting LIPI-1, DNA fragments of 893 bp and 684 bp corresponding to the chromosomal regions encompassing the prfA and plcB genes at both side of the pathogenicity island (see Fig. 1) were PCR-amplified using primer pairs PrsF1/PrsR2 and PrsF3/PrsR4 (Table S1), then fused together by splicing overlap extension PCR (Pogulis et al., 1996) using the complementary 3′ sequence tails carried by PrsR2 and PrsF3 and a second PCR reaction with PrsF1 and PrsR4. The EcoRI and BamHI sites carried by the latter primers (Table S1) were used to insert the resulting 1577 bp PCR product into the pMAD vector (Arnaud et al., 2004), giving rise to the plasmid pmΔLIPI-1 (Table 1). The ΔREG mutant was constructed by deleting LIPI-1 and hpt from P14A ΔinlABC (Table 1). The hpt gene was in frame deleted using the pLSV1-based pLSA_hpt allele replacement plasmid (Table 1). After electroporation, the first and second recombinants were selected and checked by PCR mapping as previously described (Suarez et al., 2001).

Growth curves

Overnight BHI cultures were diluted 1:100 into fresh BHI and grown at 37°C with rotary shaking (200 r.p.m.) until OD600 ≈ 1.0. OD600. Bacteria were collected by centrifugation, washed twice in PBS and suspended in pre-warmed BHI to give an
OD_{600} = 0.05. Triplicate 200 μl aliquots of the bacterial suspensions were transferred to different positions of flat-bottom 96-well microplates (Costar). Plates were incubated at 37°C with shaking (200 r.p.m.) and bacterial growth monitored by measuring the OD_{600} every 30 min in an automated plate reader (FluoStar Optima or Omega machines, BMG Labtech). Cultures were monitored by phase-contrast microscopy to exclude bacterial clumping as a potential source of variation. The maximum growth rate during exponential growth (μ) and maximum bacterial cell density reached during the growth curve (A) were estimated from spline-fits of OD_{600} values using the GROFIT package in R (Kahm et al., 2010).

**Intracellular infection assay**

*Listeria monocytogenes* intracellular proliferation was tested in human epithelial HeLa cell monolayers using a gentamicin protection assay as previously described (Deshayes et al., 2012). Due to the constitutive activation of their PrfA-regulated cell invasion determinants, prfA* bacteria are more invasive than (broth-grown) prfA^WT bacteria (see Fig. 3, upper panel). Intracellular proliferation data were therefore normalized to the number of internalized *L. monocytogenes* bacteria using an intracellular growth coefficient calculated with the formula: 

\[ \text{IGC} = \left( \frac{\text{IC} - \text{IB}_0}{\text{IB}_0} \right) \]

where IB_0 and IC are the intracellular bacterial numbers at any specific time point (t = n) and t = 0, respectively (Deshayes et al., 2012).

**Soil experiments**

For each experiment, subsurface topsoil samples were collected within a depth of ≈ 10 cm from several locations of a residential garden in Edinburgh (UK). Soil was carefully mixed, sieved through 6 mm mesh to remove coarse particles and autoclaved (121°C-15 min). The soil used had a pH of 7.23 (range 7.2-7.3) and average moisture content of 25.3% (range 24.1 and 26.5). The pH was measured in the liquid phase of a soil suspension prepared by vigorously stirring 25 g of soil in 50 ml distilled water. The water content was determined in 10 g samples by the oven-dry method. Prior to the experiments, the soil was tested for the presence of antimicrobial or inhibitory activity against *L. monocytogenes* (P14A, P14Rev and ΔprfA). For this, a soluble extract was prepared by suspending 50 g of soil in 50 ml distilled water. After mixing vigorously, the suspension was allowed to settle for 5 min, and the supernatant was decimally diluted and plated for viable count determination. The maximum growth rate during exponential growth (μ) and the supernatant was left to sediment for 20 min at room temperature.

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