Deciphering the Acylation Pattern of Yersinia enterocolitica Lipid A

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Abstract

Pathogenic bacteria may modify their surface to evade the host innate immune response. Yersinia enterocolitica modulates its lipopolysaccharide (LPS) lipid A structure, and the key regulatory signal is temperature. At 21°C, lipid A is hexa-acylated and may be modified with aminooarabinose or palmitate. At 37°C, Y. enterocolitica expresses a tetra-acylated lipid A consistent with the 3′-O-deacylation of the molecule. In this work, by combining genetic and mass spectrometric analysis, we establish that Y. enterocolitica encodes a lipid A deacylase, LpxR, responsible for the lipid A structure observed at 37°C. Western blot analyses indicate that LpxR exhibits latency at 21°C, deacylation of lipid A is not observed despite the expression of LpxR in the membrane. Aminooarabinose-modified lipid A is involved in the latency. 3-D modelling, docking and site-directed mutagenesis experiments showed that LpxR D31 reduces the active site cavity volume so that aminooarabinose containing Kdo2-lipid A cannot be accommodated and, therefore, not deacylated. Our data revealed that the expression of LpxR is negatively controlled by RovA and PhoPQ which are necessary for the lipid A modification with aminooarabinose. Next, we investigated the role of lipid A structural plasticity conferred by LpxR on the expression/function of Y. enterocolitica virulence factors. We present evidence that motility and invasion of eukaryotic cells were reduced in the lpxR mutant grown at 21°C. Mechanistically, our data revealed that the expressions of fhbDC and rovA, regulators controlling the flagellar regulon and invasin respectively, were down-regulated in the mutant. In contrast, the levels of the virulence plasmid (pYV)-encoded virulence factors Yops and YadA were not affected in the lpxR mutant. Finally, we establish that the low inflammatory response associated to Y. enterocolitica infections is the sum of the anti-inflammatory action exerted by pYV-encoded YopP and the reduced activation of the LPS receptor by a LpxR-dependent deacylated LPS.

Introduction

Lipopolysaccharide (LPS) is one of the major surface components of Gram-negative bacteria. The molecular structure of LPS is rather unique: an amphiphile with a hydrophobic region, the so-called lipid A, adjacent to a densely negatively charged polysaccharide. In Escherichia coli K-12, the lipid A is a (1′-6)-linked disaccharide of glucosamine phosphorylated at the 1 and 4′ positions with positions 2, 3, 2′, and 3′acylated with R-3-hydroxymyristoyl groups, the so-called lipid IV A. The 2′ and 3′R-3-hydroxymyristoyl groups are further acylated with laureate (C12) and myristate (C14), respectively, by the action of the so-called late acyltransferases LpxL (HtrB) and LpxM (MsbB), respectively [1]. When E. coli is grown at 12°C, LpxP, the cold-temperature-specific late acyltransferase, acts instead of LpxL, adding palmitoleate (C16:1) [1]. Although the enzymes required to synthesize the lipid A are conserved throughout all Gram-negative bacteria there is heterogeneity on lipid A structure among Gram-negative bacteria compared to the E. coli K-12. This is due to differences in the type and length of fatty acids, in the presence of decorations such as aminooarabinose or phosphoethanolamine and even in the removal of groups such as phosphates or fatty acids from lipid A [2].

LPS plays a crucial role during recognition of microbial infection by the host immune system. In fact, the lipid A moiety is a ligand of the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 complex [3]. The stimulation of this receptor complex triggers the activation of signalling cascades resulting in the induction of antimicrobial genes and release of cytokines, thereby initiating inflammatory and immune defence responses. Perusal of the literature demonstrates that changes in the number of acyl chains and in the phosphorylation status of the headgroup greatly affect the biological activity of lipid A. It is not surprising that some pathogens modulate their lipid A structure to alter their detection by the host; being these regulated changes important virulence traits (for a review see [4]). Furthermore, given the importance of the LPS structure to the homeostasis of the outer membrane, it is possible that the aforementioned changes may also affect the physiology of the outer membrane as was recently demonstrated for Salmonella [5].
Author Summary

Lipopolysaccharide (LPS) is one of the major surface components of Gram-negative bacteria. The LPS contains a molecular pattern recognized by the innate immune system. Not surprisingly, the modification of the LPS pattern is a virulence strategy of several pathogens to evade the innate immune system. Yersinia enterocolitica causes food-borne infections in animals and humans (yersiniosis). Temperature regulates most, if not all, virulence factors of yersiniae including the structure of the LPS lipid A. At 21°C, lipid A is mainly hexa-acylated and may be modified with aminoorabinose or palmitate. In contrast, at 37°C, Y. enterocolitica expresses a unique tetra-acylated lipid A. In this work, we establish that Y. enterocolitica encodes a lipid A deacylase, LpxR, responsible for the lipid A structure expressed by the pathogen at 37°C, the host temperature. Our findings also revealed that the low inflammatory response associated to Y. enterocolitica infections is the sum of the anti-inflammatory action exerted by a Yersinia protein translocated into the cytosol of macrophages and the reduced activation of the LPS receptor complex due to the expression of a LpxR-dependent deacylated LPS.

The genus Yersinia includes three human pathogens: Y. pestis, Y. pseudotuberculosis and Y. enterocolitica. The latter can cause food-borne infections in animals and humans (yersiniosis), with symptoms such as enteritis and mesenteric lymphadenitis [6]. Y. enterocolitica is endowed with a repertoire of virulence factors that help bacteria to colonize the intestinal tract and to resist host defence mechanisms [7,8]. Temperature regulates most, if not all, virulence factors of yersiniae [7,8]. Recent studies have shown that temperature also regulates the structure of yersiniae lipid A [9–14]. Thus the number and type of the lipid A fatty acids and the substitutions of the 1- and 4'-positions in the glucosamine disaccharide can vary. Rebeil and co-workers [12] elegantly demonstrated that a shift in temperature induces a change in the number and type of acyl groups on the lipid A of the three Yersinia species. At 21°C, lipid As are mainly hexa-acylated whereas at 37°C they are tetra-acylated [12]. The temperature-dependent regulation of the lipid A acyltransferases underlines the shift in lipid A acylation both in Y. pestis and in Y. enterocolitica [12,14,15]. Pathogenic yersiniae also express hepta-acylated lipid A due to the addition of C10, in Y. pestis and Y. pseudotuberculosis, or C16 (palmitate), in Y. enterocolitica [12,14,15]. PagP is the acyltransferase responsible for the addition of palmitate to the lipid A in Y. enterocolitica [15]. Other lipid A species are consistent with the substitution of the phosphate at the 4' end of the glucosamine disaccharide with aminoorabinose [15]. The aminoorabinose content is temperature-regulated in Y. pestis and in Y. enterocolitica [12,15,16]; being higher in bacteria grown at 21°C than at 37°C. Similar to other Gram-negative bacteria, the products of ugd and pmrHJFKL (ambC) [hereafter pmrF operon] are required for the synthesis and addition of aminoorabinose to lipid A in Y. enterocolitica [15]. Finally, we and others [9–14,17] have reported a unique tetra-acyl lipid A species (m/z 1388) found only in Y. enterocolitica grown at 37°C. Evidence support the notion that this species lacks the ester-linked R3-hydroxyximyristoyl group further acylated with laurate [C12] [12,14,17]. Indeed, mass spectrometry analysis did confirm that the nonreducing glucosamine of the lipid A is substituted with only one (amide-linked) R3-hydroxyximyristoyl group further acylated with myristate [C14] [17]. Altogether, these findings strongly suggest that the tetra-acyl lipid A species (m/z 1388) may be caused by a deacylase removing the 3'-acyloxyacyl residue of the lipid A. The work described in this article gives experimental support to this hypothesis and explores the impact of the lipid A structure on Y. enterocolitica virulence traits.

Results

Identification of the Y. enterocolitica 3’-O-deacylase of lipid A

Further confirming previous findings [14,15], lipid A isolated from Y. enterocolitica 8001 serotype O:8 (hereafter YeO8; Table 1) grown at 37°C appeared to be identical to those reported by Rebeil et al. and Oertelt et al. [12,17]. The main species were a 3’-O-deacylated form (m/z 1389) containing two glucosamines, two phosphates, three 3-OH-C14, and one C14; and a hexa-acylated form (m/z 1797) (Figure 1A). In bacteria grown at 21°C, a minor species (m/z 1414) was detected and may represent a 3’-O-deacylated form containing three 3-OH-C14 and one C16:1 [12]. S. enterica serovar typhimurium and Helicobacter pylori also express 3’-O-deacylated lipid A species [18,19]. A membrane located hydrolase, named LpxR, removes the 3’-acyloxyacyl residue of lipid A in both organisms [18,19]. In silico analysis of the YeO8 genome (accession number AM286415) [20] revealed that this pathogen may encode an LpxR orthologue (locus tag YE3039). The predicted YeO8 LpxR (YeLpxR) has 73% and 20% amino acid identities to S. enterica and H. pylori LpxR proteins, respectively. Furthermore, YeLpxR has 100% amino acid identity to Y. enterocolitica Y11 serotype O:3 (locus tag Y11_05741; accession number FR729477) and Y105 serotype O:9 (locus tag YE105_2442; accession number CP000224) LpxR homologs. Analysis of the available Y. pestis and Y. pseudotuberculosis genomes revealed that they do not encode any gene similar to lpxR.

YeO8 lpxR was mutated to determine whether this gene is indeed responsible for removing the 3’-acyloxyacyl residue of lipid A. MALDI-TOF mass spectrometry studies showed that, at 37°C, the lpxR mutant (YeO8-ΔlpxR) produced a lipid A which lacked the unique tetra-acyl lipid A species (m/z 1388) found in YeO8 and only contained the hexa-acylated species (m/z 1797; four 3-OH-C14, one C12 and one C14) (Figure 1B). At 21°C, lipid A isolated from YeO8-ΔlpxR was similar to that of YeO8 although without the minor species m/z 1414 (Figure 1B). Complementation of the mutant with pTM1LpxR restored the presence of the tetra-acyl species (Figure 1C).

In summary, our results confirmed the predicted function of Y. enterocolitica O:8 lpxR homolog as the lipid A 3’-O-deacylase.

Expression of lpxR

The LpxR-dependent lipid A deacylation was more evident on bacteria grown at 37°C than at 21°C, hence suggesting that the expression and/or function of the deacylase might be temperature-regulated, being higher at 37°C than at 21°C. To monitor transcription of lpxR quantitatively, a transcriptional fusion was constructed in which a promoterless lucFF gene was placed under the control of the lpxR promoter region (see Material and Methods); thereafter lpxR::lucFF was introduced into YeO8 and the luciferase activity was determined. The expression of the fusion was higher at 21°C than at 37°C (Figure 2A). Real time (RT) quantitative PCR (RT-qPCR) experiments showed that lpxR mRNA levels were also higher at 21°C than at 37°C (Figure 2B).

To assess LpxR levels, the C-terminus of the protein was tagged with a FLAG epitope and the construct was cloned into the medium-copy plasmid pTM100 to obtain pTM1LpxRFLAG (see Materials and Methods). This plasmid restored the presence of the tetra-acyl species (m/z 1414 and m/z 1388) in the lipid A of YeO8-

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## Table 1. Strains and plasmids used in this study.

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ΔlpxRKm (data not shown). Western blot analysis of purified membranes from YeO8-ΔlpxRKm containing pTMLpxRFLAG showed that LpxR levels were higher in membranes from bacteria grown at 21°C than at 37°C (Figure 2C). Altogether, it can be concluded that the expression of lpxR is indeed temperature-regulated, but, in contrast to our initial hypothesis, its expression is higher at 21°C than at 37°C.

The apparent contradiction between the mass spectrometry analysis, more deacylation at 37°C, and the Western blot data, higher levels of LpxR at 21°C than at 37°C, led us to explore whether low temperature may affect the function of the enzyme. Since E. coli has been used as surrogate host to characterize Salmonella LpxR (StLpxR) function [18], we mobilized pTMLpxR into E. coli MG1655 to analyze lipid A species by mass spectrometry in bacteria grown at 21°C and 37°C. Results shown in Figure 3 demonstrate that LpxR did deacylate the E. coli lipid A from bacteria grown either at 21 or 37°C as detected by the presence of species m/z 1360 (Figure 3C-D). This species was found previously in E. coli expressing StLpxR [18]. Of note, the species m/z 1414, which is consistent with the deacylation of the species m/z 1850 containing palmitoleate (C16:1) instead of laurate (C12:0), was observed only in E. coli grown at 21°C. LpxP is the cold-temperature-specific late acyltransferase responsible for the addition of palmitoleate [1]. Altogether, our results indicate that the reduced LpxR-dependent deacylation found in YeO8 grown at 21°C cannot be attributed to a general lack of function of the enzyme at this temperature.

Lipid A modification with aminoarabinose affects LpxR-dependent deacylation

We sought to determine why LpxR activity was not observed in YeO8 grown at 21°C despite the detection of the enzyme in the membrane. Among other possibilities, we speculated that specific features of YeO8 lipid A found only at 21°C might be responsible for the reduced LpxR activity. Furthermore, these features should be absent in E. coli grown at 21°C since LpxR-dependent activity was observed here. A conspicuous difference between YeO8 and E. coli lipid A is the presence of aminoarabinose and palmitate (m/z 1954 and 2063, respectively) only in the former [14,15]. Therefore, we explored whether any of these modifications could account for the reduced LpxR activity. In YeO8, similarly to other Gram-negative pathogens, the products of the pmrF operon are required for the synthesis and addition of aminoarabinose to lipid A whereas the acyltransferase PagP is required for the addition of palmitate to lipid A [15]. The lipid A from the pagP mutant, YeO8-ΔpagPGB, grown at 21°C resembled that of the wild-type strain, except that the species containing palmitate (m/z 2063) was not detected (Figure 4A). In contrast, the tetra-acylated species (m/z 1414) was clearly observed in the lipid A from YeO8-ΔpmrF grown at 21°C (Figure 4C). This was dependent on LpxR activity since the peak was absent in the double mutant YeO8-ΔpmrF-ΔlpxRKm (Figure 4E). LpxR-dependent deacylation of lipid A (m/z 1388) observed in bacteria grown at 37°C was not affected in either pmrF or pagP single mutants (Figure 4B, D). Control experiments revealed that lpxR expression was not affected in YeO8-ΔpmrF since the expression of the lpxR::lucFF fusion was not significantly different between YeO8 and the pmrF mutant either grown at 21°C or at 37°C (Figure 4G).

On the whole, these results are consistent with the notion that the reduced LpxR activity observed in YeO8 at 21°C is associated with the lipid A modification with aminoarabinose.

LpxR 3-D modelling

Our findings might suggest that aminoarabinose-containing LPS may directly inactivate the lipid A deacylase activity of YeLpxR. Alternatively, modification of lipid A with aminoarabinose could inhibit the physical interaction of LPS with YeLpxR. To explore this, the 3-D structure of YeLpxR was modeled (Figure 5A). The amino acids 1–296 (following the putative signal sequence) could be modeled based on the crystal structure of StLpxR (PDB code 3FID; [21]) and the sequence alignment between StLpxR and YeLpxR (Figure S1). The fold of the resulting model is likely to be of good quality, since YeLpxR has such a high sequence identity to StLpxR (75%). Additionally, the important StLpxR amino acids identified by Rutten and co-workers [21] are conserved in YeLpxR. Six amino acids differ between the YeLpxR and the StLpxR active sites (Figure S1). Major differences are D31 and Q35 in YeLpxR, of which D31 is closer to the active site (Figure 5B). The corresponding amino acids are much smaller in StLpxR, glycine and an alanine, respectively, which cause StLpxR to have a bigger cavity. StLpxR has a protruding cavity close to K67, which cannot be found in YeLpxR (Figure 6A). The difference is induced by D31 in YeLpxR, which occupies more space than G31 in StLpxR. As a consequence, the conserved K67 adopts a different conformation in the YeLpxR model. Due to D31, the cavity in YeLpxR is divided into two parts with a narrow connection, and this amino acid also prevents YeLpxR from forming an inward protruding cavity similar to the one found near G31 in StLpxR (Figure 6A).

Docking of a modified Kdo2-lipid A molecule (see Materials and methods) to the model of YeLpxR showed that the phosphate group, which attaches aminoarabinose to Kdo2-lipid A, binds into the cavity in the vicinity of K67 and D31 (Figure 6B). Docking of the same molecule to the crystal structure of StLpxR yielded a result where the phosphate group was located in the protruding cavity close to K67 (Figure 6C). As expected, docking of the modified Kdo2-lipid A molecule with aminoarabinose to the
YeLpxR model did not give any valuable result. On the other hand, when the same molecule was docked to the StLpxR crystal structure, aminoarabinose was bound close to G31. It occupies the space corresponding to the narrow connection of the two larger cavities in YeLpxR (Figure 6D).

As a result from the modeling and docking studies, we suggest that Kdo2-lipid A with aminoarabinose cannot fit into the active site of YeLpxR due to D31, hence leading to the inability of YeLpxR to deacylate Kdo2-lipid A with aminoarabinose.

To confirm our predictions, we constructed LpxR mutants by site-directed mutagenesis (see Material and Methods). In addition to the amino acids corresponding to the active site amino acids in StLpxR, we wanted to study the effect of the D31G mutation for YeLpxR as the modelling and docking studies suggested that D31 has an important role in the YeLpxR specificity for the Kdo2-lipid A species. The constructs were introduced into E. coli MG1655 and the lipid A from the transformants grown at 37°C was

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**Figure 1. Lipid A analysis from Y. enterocolitica lpxR mutant.** (A) Negative ion MALDI-TOF mass spectrometry spectra of lipid A isolated from YeO8 grown at 21°C and 37°C. (B) Negative ion MALDI-TOF mass spectrometry spectra of lipid A isolated from YeO8-ΔlpxR (ΔlpxR) grown at 21°C and 37°C. (C) Negative ion MALDI-TOF mass spectrometry spectra of lipid A isolated from YeO8-ΔlpxR carrying pTMLpxR grown at 21°C and 37°C. The results in all panels are representative of three independent lipid A extractions.

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analyzed by MALDI-TOF mass spectrometry. Most of the constructs containing LpxR mutants did trigger the deacylation of E. coli lipid A, detected by the presence of species m/z 1360, (Table 2). In contrast, constructs containing LpxR mutants, LpxR(N9A), LpxR(D10A), LpxR(S34A), and LpxR(H122A) did not deacylate E. coli lipid A. These results were expected since Ruten and co-workers have reported that these residues are located in the StLpxR active site and all of them are conserved in LpxR homologues [21]. Next, only those constructs triggering deacylation of E. coli lipid A were introduced into YeO8. When the YeO8 strains were grown at 37°C, all LpxR mutants restored the presence of the tetra-acyl species (m/z 1388) in the lipid A of YeO8-ΔlpxR (Table 2). Additionally, the mass spectrometry analysis revealed that LpxR(D31G) mutant did trigger the deacylation of lipid A in bacteria grown at 21°C as it was detected the presence of lipid A species m/z 1414 and m/z 1545 (Figure 7B). The latter is consistent with the deacylation of the lipid A species modified with aminoarabinose (m/z 1554).

In summary, our results further confirmed the amino acids important for the catalytic activity of YeLpxR. Moreover, our results confirmed the molecular modelling predictions, thereby demonstrating that the presence of D31 in the active site pocket of YeLpxR causes steric hindrance for the binding and deacylation of lipid A species modified with aminoarabinose.

Regulation of lpxR expression

In YeO8 the expression of the loci responsible for the lipid A modification with aminoarabinose, ugd and pmrF operon, is temperature regulated, being higher at 21°C than at 37°C [15]. Mechanistically, this is so because the expression of the positive regulators phoPQ and pmrAB, which control the expression of ugd and the pmrF operon, is also higher at 21°C than at 37°C [15]. In turn, the temperature-dependent regulation of phoPQ and pmrAB is explained by H-NS-dependent negative regulation alleviated by RovA, another major regulator of Yersinia [22,23], at 21°C [15]. Moreover, there is cross-talk between the regulators in such way that PhoPQ and PmrAB regulate positively the expression of rovera and the effect of PhoPQ is more important [15].

The inverse correlation between the substitution of the lipid A with aminoarabinose and lipid A deacylation, prompted us to evaluate whether phoPQ and pmrAB might negatively regulate lpxR. Results shown in figure 8 revealed that the expression of lpxR::lucFF was significantly up-regulated in the phoPQ and pmrAB mutants at 21°C and 37°C (Figure S2). However, the expression of lpxR reached wild-type levels in the double phoPQ::pmrAB mutant regardless the bacteria growth temperature (Figure 8A). RT-qPCR experiments showed that the levels of lpxR mRNA were higher in the phoPQ and pmrAB mutants than in the wild type and double phoPQ::pmrAB mutants, which were not significantly different (Figure S2).

Recently, we have shown that rovera expression is downregulated in the phoPQ and pmrAB single mutants, being the lowest in the phoPQ mutant, whereas in the phoPQ::pmrAB double mutant rovera expression is not significantly different to that in the wild type [15]. Therefore, the fact that lpxR expression follows the opposite trend in these mutants led us to analyze whether rovera negatively regulates the expression of lpxR. Indeed, luciferase activity was higher in the rovera mutant than in the wild type and the levels were not significantly different that those observed in the phoPQ mutant when bacteria were grown either at 21°C or 37°C (Figure 8A). Similar results were obtained when the lpxR mRNA levels were analyzed by RT-qPCR (Figure S2). The increased lpxR expression observed in rovera and phoPQ single mutants at 21°C was no longer found in the double mutant rovera-phoPQ (Figure 8A and Figure S2).
When bacteria were grown at 37°C, lpxR expression in the rovA-phoPQ mutant was significantly lower than those observed in the rovA and phoPQ single mutants (p<0.05 for each comparison versus rovA-phoPQ mutant) although still higher than that in the wild type (Figure 8A and Figure S2). Of note, the expression of lpxR was no longer temperature regulated in the rovA-phoPQ mutant (Figure 8B).

The fact that the expression of lpxR::lucFF in the triple mutant rovA-phoPQ-pmrAB at 21°C was less than in the wild-type strain may support the notion that, in the absence of the negative regulator RovA, PmrAB and/or a PmrAB-modulated regulator positively regulates lpxR. At 37°C, lpxR expression in the triple mutant was not significantly different than those found in the double mutant phoPQ-pmrAB and the wild type (Figure 8A and Figure S2).

Collectively, our data revealed that the expression of lpxR is negatively controlled by the same regulators that activate the loci necessary for the substitution of the phosphate at the 4′ end of the glucosamine disaccharide with aminoarabinose.

Flagellar regulon and lipid A acylation

In a previous study, we observed the down regulation of YeO8 virulence factors in mutants lacking the lipid A late acyltransferases LpxM, LpxL or LpxP [14]. These results raised the possibility that lipid A acylation may act as a regulatory signal by acting on a transduction pathway(s) [14]. In this context, we sought to determine the impact of LpxR to the expression/function of YeO8 virulence factors.

Virulence genes can be regulated as part of the flagellar regulon, indicating that this regulon contributes to L. enterococitica pathogenesis [24]. YeO8 is motile when grown at 21°C but not at 37°C [25] and previously we showed that LpxM and LpxP mutants are less motile than the wild type [14]. We examined the influence of LpxR on the flagellar regulon. We quantified the migration of the wild type and YeO8-DlpxR Km in motility medium (1% tryptone-0.3% agar plates). Figure 9 shows that YeO8-DlpxR Km was less motile than the wild type.

Yersinia motility is related to the levels of flagellins which, in turn, are regulated by the expression of flhDC, the flagellum master regulatory operon [25,26]. We hypothesized that the expression of flhDC could be lower in the lpxR mutant than in the wild type. To address this, the flhDC::lucFF transcriptional fusion [26] was introduced into the chromosome of the strains and the luciferase activity was determined. At 21°C, luminescence was lower in the lpxR mutant than in the wild type (Figure 9B). Complementation of the lpxR mutant with pTMYeLpxR restored flhDC::lucFF expression to wild-type levels (Figure 9B). Notably, the catalytic inactive LpxR mutants LpxR(N9A) and LpxR(S34A), encoded by pTMLpxR(N9A) and pTMLpxR(S34A) respectively, also complemented the lpxR mutant (Figure 9B). Western blot analysis of purified membranes from YeO8-DlpxR Km containing pTMLpxR(N9A)/FLAG or pTMLpxR(S34A)/FLAG showed that
Deacylation of \textit{Y. enterocolitica} Lipid A

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Deacylation of \textit{Y. enterocolitica} Lipid A}
\end{figure}
the mutant proteins were expressed (Figure S3). When the strains were grown at 37°C, YeO8 and YeO8-DlpxR Km produced the same luminescence (Figure 9B).

One virulence gene that is regulated as part of the flagellar regulon is yplA and hence its expression is regulated by flhDC [24,27,28]. Considering that flhDC expression was downregulated in the lpxR mutant, we speculated that yplA expression could be affected in this mutant. The transcriptional fusion yplA::lacZYA [29] was introduced into the chromosome of the wild type and the lpxR mutant and their β-galactosidase activities were measured. Indeed, the β-galactosidase activity was lower in YeO8-DlpxR Km than in the wild type (Figure 9C). Plasmids pTMYeLpxR, pTMLpxR(N9A) and pTMLpxR(S34A) complemented the phenotype (Figure 9C).

In summary, these results indicate that the flagellar regulon is downregulated in the lpxR mutant with a concomitant decrease in motility and downregulation of yplA expression.

Invasin and lipid A acylation

Inv is an outer membrane protein of Y. enterocolitica responsible for invasion of the host [30,31]. Since YeO8 lipid A mutations affect inv expression [14], we asked whether inv expression is altered in the lpxR mutant. An inv::phoA translational fusion [32] was introduced into the genome of YeO8 and YeO8-DlpxR Km and inv expression was monitored as alkaline phosphatase (AP) activity (Figure 10A). AP activity was significantly lower in the lpxR mutant than in the wild type. Plasmids pTMYeLpxR, pTMLpxR(N9A) and pTMLpxR(S34A) restored AP activity to wild-type levels. These differences in inv expression prompted us to study the ability of YeO8-DlpxR Km to invade HeLa cells by using a gentamicin protection assay. The amount of intracellular bacteria was 55% lower when cells were infected with the lpxR mutant than with the wild type (Figure 10B).

RovA is required for inv expression in Y. enterocolitica [33]. Therefore, among other possibilities, the low inv expression found in the lpxR mutant could be caused by downregulation of rovA expression. To address this, the rovA::lacFF transcriptional fusion [14] was introduced into the genome of the wild type and the lpxR mutant and the luminescence was determined. Results shown in figure 10C demonstrate that rovA expression was downregulated in YeO8-DlpxR Km. This phenotype was complemented with plasmids pTMYeLpxR, pTMLpxR(N9A) and pTMLpxR(S34A).

Together, our data show that the down-regulation of inv expression found in the lpxR mutant is most likely caused by downregulation of rovA expression, the positive transcriptional regulator of inv.

Impact of lipid A acylation on pYV-encoded virulence factors

Y. enterocolitica harbours a plasmid (pYV)-encoded type III secretion system which is required for virulence. A set of virulence factors, called Yops, are secreted by this system and enable Y. enterocolitica to multiply extracellularly in lymphoid tissues [34–36]. In several pathogens, LPS polysaccharide status affects the expression of the type III secretion systems [37–39]. Therefore, we asked whether the production of the Yersinia pYV-encoded type III secretion system is altered in the lpxR mutant. At 37°C and under low calcium concentrations, this system secretes the Yops to

Figure 4. Lipid A analysis from Y. enterocolitica lipid A mutants. Negative ion MALDI-TOF mass spectrometry spectra of lipid A isolated from the indicated Y. enterocolitica strains grown at 21°C (A,C,E) and 37°C (B,D,F). The results in all panels are representative of three independent lipid A extractions. (G) Analysis of the expression of lpxR by measuring luciferase activity of YeO8 (white bars) and YeO8-DpmrF (gray bars) carrying lpxR::lacFF transcriptional fusion, which were grown at 21°C or 37°C. Data are presented as mean ± SD (n = 3).

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Figure 5. Modelling of Y. enterocolitica O:8 LpxR. (A) The model of YeLpxR based on the StLpxR crystal structure. The β-barrel is colored as rainbow, the helices are green and loops are wheat. (B) Close-up view of the active site. The amino acids mutated in this study are shown as sticks in yellow and pink.

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the culture supernatant [40]. Analysis of Yop secretion revealed that the wild type and the \( lpxR \) mutant secreted similar levels of Yops (Figure 11A). We sought to determine whether the translocation of Yops to the cytosol of eukaryotic cells is affected in the \( lpxR \) mutant. Detection of cytoskeleton disturbances upon infection of epithelial cells is one of the most sensitive assays to establish Yop translocation [41]. The injection of YopE into the cytosol of A549 cells by wild-type bacteria induced disruption and condensation of the actin microfilament structure of the cells whereas this was not the case when cells were infected with YeO8-\( \Delta lpxR \)mut (Figure 11B). YopE translocation to A549 cells was not affected in the \( lpxR \) mutant background (Figure 11C). As expected, A549 cells infected with YeO8-\( \Delta lpxR \)Km displayed similar cytoskeleton disturbances than those cells infected with the wild type (Figure 11B).

\( yadA \) is another pYV-encoded virulence gene whose expression is only induced at 37\(^\circ\)C [42]. YadA is an outer membrane protein mediating bacterial adhesion, bacterial binding to proteins of the extracellular matrix and complement resistance (for a review see [43]). Analysis of YadA expression by SDS-PAGE demonstrated that YeO8-\( \Delta lpxR \)Km and YeO8 produced the same amount of the protein (Figure 11D). To assess YadA functionality, we asked whether the YadA-dependent binding to collagen is altered in the \( lpxR \) mutant. To this end, we analyzed the binding of YadA-

Figure 6. Docking of Kdo\(_2\)-lipid A to LpxR. (A) The differences in surface cavities between YeLpxR (pink) and StLpxR (green) with K67 as sticks with pink carbon atoms for YeLpxR and green carbon atoms for StLpxR and YeLpxR D31 as sticks with pink carbon atoms. G31 in StLpxR is hidden behind D31 in YeLpxR. (B) The YeLpxR model with Kdo\(_2\)-lipid A (sticks with yellow carbon atoms) in the active site. The surface cavity of YeLpxR is shown in pink and K67 and D31 as sticks with pink carbon atoms. (C) The StLpxR model with Kdo\(_2\)-lipid A (yellow sticks) in the active site. The surface cavity of StLpxR is shown in green and K67 and G31 as sticks with green carbon atoms. (D) The StLpxR model with Kdo\(_2\)-lipid A including aminoareshinose in the active site. The surface cavity of StLpxR is shown in green along with K67 and G31 (green sticks).

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expressing whole bacteria to collagen type I by immunofluorescence (see Material and Methods). In contrast to the negative control, a pYV-cured strain (YeO8c), YeO8 and YeO8-D

pxR Km bound to collagen without differences between them (Figure 11E–F).

Taken together, these results suggest that the production and function of the pYV-encoded virulence factors Yops and YadA are not altered in the \( \text{lpXR} \) mutant.

Lipid A acylation and innate immunity

Cationic antimicrobial peptides (CAMPs) belong to the arsenal of weapons of the innate immune system against infections. In the case of Gram-negative bacteria, CAMPs interact with the lipid A moiety of the LPS [44–47] and lipid A modification is one of the strategies employed by Gram-negative bacteria to counteract the action of CAMPs. We and others have used polymyxin B as a model CAMP since it also binds to lipid A. Furthermore, resistance to this peptide reflects well the resistance to other mammalian peptides and correlates with virulence [48–51]. Therefore we evaluated the resistance of the \( \text{lpXR} \) mutant to polymyxin B. Results shown in figure 12A demonstrate that the mutant was as resistant as the wild type to the peptide when grown either at 21°C or at 37°C. Of note both strains were more susceptible to polymyxin B when grown at 37°C than at 21°C (Figure 12A).

Table 2. Effect of \( \text{lpXR} \) mutations on lipid A deacylation.

| Lipid A deacylation |  
|---|---|
| Mutations | \( E. \text{coli} \) (37°C) | \( Y. \text{enterocolitica} \) (21°C) |
| | \( m/z \) 1360 | \( m/z \) 1414 |
| N9A | No | No |
| D10A | No | n.a. |
| S34A | No | n.a. |
| H122A | No | n.a. |
| Q118A | No | No |
| Q57A | Yes | No |
| Y130A | Yes | No |
| G36A | Yes | No |
| F79A | Yes | No |
| P62A | Yes | No |
| W133A | Yes | No |
| D31G | Yes | Yes |

n.a.; Not analyzed.

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Figure 7. Presence of D31 in the active site pocket of \( Y. \text{enterocolitica} \) O:8 LpxR affects the deacylation activity of the enzyme.

Negative ion MALDI-TOF mass spectrometry spectra of lipid A isolated from: (A) \( E. \text{coli} \) MG1655 (\( E. \text{coli} \)) carrying pTMLpxR(D31G) grown at 37°C. (B) YeO8-D\( \text{lpXR} \)Km (\( \Delta \text{lpXR} \)) carrying pTMLpxR(D31G) grown at 21°C. (C) YeO8-D\( \text{lpXR} \)Km (\( \Delta \text{lpXR} \)) carrying pTMLpxR(D31G) grown at 37°C. The results in all panels are representative of three independent lipid A extractions.

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Figure 8. Y. enterocolitica PhoPQ, PmrAB two-component systems and RovA control the expression of lpxR. (A) Analysis of the expression of lpxR by YeO8 (white bar), and mutants (grays bars) YeO8-DphoPQ (DphoPQ), YeO8-DpmrAB (DpmrAB) and YeO8-DphoPQ-DpmrAB (DphoPQ-pmrAB), Yvm927 (DrovA-Yvm927-DphoPQ-DpmrAB) carrying the transcriptional fusion lpxR::lucFF grown at 21°C or 37°C. Data are presented as mean ± SD (n = 3). *, results are significantly different (p < 0.05; two-tailed t test) from the results for YeO8 grown at the same temperature. (B) This panel displays the same results shown in panel A for YeO8 and the double mutant Yvm927-DphoPQ (DrovA-DphoPQ) and it is included for the sake of clarity.

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Figure 9. Flagellar regulon is downregulated in the Y. enterocolitica O:8 lpxR mutant. (A) Motility assays were performed with YeO8, and YeO8-DlpxRKm (DlpxR) in a semisolid agar plate (3% agar and 1% tryptone). Plates were incubated at 22°C for 24 h. (B) Analysis of flhDC expression by YeO8, YeO8-DlpxRKm (DlpxR), and YeO8-DlpxRKm with the plasmids pTMYE-lpxR (DlpxR/pTMYE-lpxR); pTMYE-lpxRS34A (DlpxR/pTMYE-lpxRS34A), and pTMYE-lpxR(N9A) carrying the transcriptional fusion flhDC::lucFF grown at 21°C and 37°C. (C) β-galactosidase activity production by yplA’::lacZYA present in YeO8, YeO8-DlpxRKm (DlpxR), and YeO8-DlpxRKm with the plasmids pTMYE-lpxR (DlpxR/pTMYE-lpxR); pTMYE-lpxR(N9A) (DlpxR/pTMYE-lpxR(N9A)), and pTMYE-lpxRS34A (DlpxR/pTMYE-lpxRS34A) [β-galactosidase values given in Miller units, mean ± SD (n = 3)]. *, results are significantly different (p < 0.05; two-tailed t test) from the results for YeO8.

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The mammalian immune system recognizes and responds to E. coli LPS via the TLR4 complex, resulting in the synthesis and secretion of pro-inflammatory cytokines that recruit immune cells to the site of infection. The ability of LPSs to evoke inflammatory responses and the potency of them are directly related to the structure of the molecule. It has been reported that underacylated LPSs are less inflammatory than hexa-acylated ones, being the E. coli lipid A (LPS) the prototype of hexa-acylated LPSs [52]. Therefore, the dramatic changes in lipid A acylation displayed by the $\Delta lpxR$ mutant at 37°C led us to evaluate the immunostimulatory properties of YeO8 and YeO8-$\Delta lpxR$. As cellular read-out, we determined TNF$\alpha$ levels secreted by macrophages infected either with the wild type or the $\Delta lpxR$ mutant grown at 21°C and 37°C. YeO8 and YeO8-$\Delta lpxR$ induced similar levels of TNF$\alpha$ although the levels induced by bacteria grown at 37°C were significantly lower than those triggered by bacteria grown at 21°C (p<0.05 for comparison of TNF$\alpha$ levels between temperatures for a given strain) (Figure 12B). This was dependent on the well known anti-inflammatory action of the pYV-encoded YopP [53,54], since a yopP mutant grown at 37°C induced similar levels of TNF$\alpha$ than those induced by wild-type bacteria grown at 21°C (Figure 12B). Therefore we sought to determine whether YopP could be counteracting the immunostimulatory response induced by YeO8-$\Delta lpxR$. Indeed, YeO8-$\Delta yopP$-$\Delta lpxR$ induced the highest levels of TNF$\alpha$ (Figure 12B). Further sustaining this notion, the TNF$\alpha$ levels induced by the yopP mutant cured of the pYV virulence plasmid grown at 37°C were significantly higher than those induced by the virulence plasmid negative wild-type strain but not different than the YeO8-$\Delta yopP$-$\Delta lpxR$Km-triggered TNF$\alpha$ levels (Figure 12B). Of note, the TNF$\alpha$ levels induced by the virulence plasmid negative wild-type strain grown at 37°C were significantly lower than those triggered by bacteria grown at 21°C hence further highlighting the importance of lipid A acylation on the immunostimulatory properties of YeO8.

Discussion

Pathogenic yersiniae show a temperature-dependent variation in lipid A acylation [9–14]. At 21°C, Y. enterocolitica synthesizes hexa-acylated lipid A containing four 3-OH-C$_{14}$, one C$_{12}$ and either one C$_{16:1}$ or one C$_{14}$. At 37°C, Y. enterocolitica lipid A presents a tetra-acylated species (m/z 1388) and a hexa-acylated one containing four 3-OH-C$_{14}$, one C$_{12}$ and C$_{14}$. In a previous work, we identified and characterized the acyltransfarses, lpxM, lpxL and lpxP, responsible for the addition of C$_{12}$, C$_{14}$ and C$_{16:1}$, respectively, to lipid A [14]. Moreover, we demonstrated that the expressions of these enzymes are temperature regulated [14]. However, the unique tetra-acyl lipid A found in the wild type grown at 37°C (m/z 1388) remained to be explained at the molecular level. We and others have established that this species is consistent with 3'-O-deacylation of lipid A [12,14,17]. In this work by combining biochemistry, genetics and molecular modelling we present evidence that LpxR is the lipid A 3'-O-deacylase of Y. enterocolitica.

YeLpxR is one of the closest homologues to StLpxR. Despite the presence of StLpxR in the Salmonella outer membrane, the bacterium does not produce 3'-O-deacylated lipid A species under any growth conditions tested to date [18]. This has been termed as enzyme latency and similar findings have been reported for the Salmonella lipid A 3'-O-deacylase PagL and E. coli PagP [55,56]. Our data revealed that YeLpxR is also latent in the membrane of YeO8 grown at 21°C. However, this is not a general feature of lipid A deacylases since H. pylori LpxR is constitutively active [19]. Several explanations could underlie YeLpxR latency at 21°C. Firstly, we explored whether low temperature may affect the function of the enzyme. The fact that YeLpxR did deacylate E. coli lipid A when grown at 21°C does not support that low temperatures grossly inhibit the enzyme activity. Nevertheless, we do not by any means completely rule out that temperature may affect YeLpxR activity, and thorough biochemical analyses are warranted to rigorously define the functional parameters of YeLpxR activity. This will be the subject of future studies. We next hypothesized that specific features of YeO8 lipid A, which do not exist in the E. coli lipid A, may be responsible for YeLpxR latency. The first conspicuous difference is the type of secondary fatty attached to the lipid IV$_A$. In E. coli the late acyltransferases LpxL and LpxM add laureate (C$_{12}$) and myristate (C$_{14}$) respectively [1] whereas in YeO8 these enzymes transfer myristate (C$_{14}$) and laureate (C$_{12}$) respectively [14]. However, this cannot

Figure 10. inv expression is altered in Y. enterocolitica O8 $\Delta lpxR$ mutant. (A) Alkaline phosphatase (AP) activities exhibited by inv::phoA translational fusion present in YeO8, YeO8-$\Delta lpxR$Km ($\Delta lpxR$), and YeO8-$\Delta lpxR$ with the plasmids pTMYeLpxR ($\Delta lpxR$/pTMYeLpxR), pTMYeLpxR(N9A) ($\Delta lpxR$/pTMYeLpxR(N9A)), and pTMYeLpxR(S34A) ($\Delta lpxR$/pTMYeLpxR(S34A)) [AP is expressed in enzyme units per OD$_{600}$ unit; mean ± SD (n = 3)]. (B) Invasion of HeLa cells by YeO8, and YeO8-$\Delta lpxR$Km ($\Delta lpxR$). Invasion assays were done in triplicate without centrifugation (n = 3). (C) Analysis of rovA expression by YeO8, YeO8-$\Delta lpxR$Km ($\Delta lpxR$), and YeO8-$\Delta lpxR$ with the plasmids pTMYeLpxR ($\Delta lpxR$/pTMYeLpxR); pTMYeLpxR(N9A) ($\Delta lpxR$/pTMYeLpxR(N9A)), and pTMYeLpxR(S34A) ($\Delta lpxR$/pTMYeLpxR(S34A)) carrying the transcriptional fusion rovA::lucFF. *, results are significantly different (p<0.05; one-tailed t test) from the results for YeO8.

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account for the reduced LpxR activity since the enzyme did deacylate E. coli lipid A. The presence of palmitoleate in YeO8 lipid A at 21°C but not at 37°C cannot be the reason since YeLpxR deacylated E. coli lipid A containing palmitoleate, found in E. coli grown at 21°C. Instead, our results revealed that the lipid A substitution with aminoarabinose is associated with YeLpxR latency since LpxR-dependent lipid A deacylation was clearly observed in the pmrF mutant grown at 21°C. Notably, the lack of aminoarabinose also releases Salmonella PagL from latency [56], hence suggesting a key role for the lipid A modification with aminoarabinose in LPS remodelling.

The molecular modelling and docking experiments further highlighted the importance of lipid A substitution with aminoarabinose for YeLpxR function. D31 in YeLpxR forces the conserved K67 to adopt a different conformation compared to StLpxR. According to the docking results, the resulting loss of cavity space in the vicinity of K67 in YeLpxR, causes the phosphate at the 4’ end of Kdo2-lipidA to bind somewhat differently to YeLpxR than to StLpxR. In the latter, the phosphate binds in the cavity near K67, while in YeLpxR it is forced to bind more outwards from the enzyme. The docking of Kdo2-lipidA with aminoarabinose to StLpxR showed that aminoarabinose occupies the cavity space, which corresponds to a narrow connection between two larger cavities in YeLpxR. The large reduction in cavity volume at this particular site causes this space to be too small for the accommodation of aminoarabinose. Hence, D31 seems to cause steric hindrance for the binding of aminoarabinose-containing Kdo2-lipidA to YeLpxR. Therefore, we predicted that D31 could have an important role for the YeLpxR substrate specificity. Indeed, the site-directed mutagenesis experiments validated that the presence of D31 in the active site pocket of YeLpxR causes a steric hindrance for the binding and
decacylation of lipid A species modified with aminoarabinose. Nevertheless, at present we do not rule out that other residues of YeLpxR also contribute to its latency. In this regard, Salmonella PagL is released from latency when specific amino acid residues located at extracellular loops of the enzyme are mutated and it has been postulated that these residues are involved in the recognition of aminoarabinose-modified lipid A [56–58]. Studies are going to explore whether residues located at extracellular loops of LpxR also contribute to enzyme latency.

The inverse correlation between the aminoarabinose content in the LPS and the LpxR-dependent lipid A deacylation prompted us to evaluate whether the same regulatory network governing the expression of the pmrF operon and ugd could regulate lpxR. Recently, we have shown that the global regulators RovA, PhoPQ, and PmrAB positively control the expression of the loci necessary for aminoarabinose biosynthesis at 21°C [15]. Furthermore, there is a cross-talk between these regulators since the expressions of phoPQ and pmrAB are downregulated in the rogA mutant whereas rogA expression is downregulated in phoPQ and pmrAB single mutants [15]. Our findings support the notion that RovA and PhoPQ are negative regulators of lpxR since its expression was higher in phoPQ and rogA single mutant backgrounds than in the wild type. In turn, the two-component system PmrAB and/or a PmrAB-regulated system may act as a positive regulator because lpxR expression was similar in the wild-type and rogA-phoPQ backgrounds.

One striking finding of our study is that motility and invasion of eukaryotic cells were reduced in the lpxR mutant grown at 21°C. Mechanistically, our data revealed that the expressions of flhDC and rncA, the key regulators controlling the flagellar regulon and invasin respectively [22,25,33], were down-regulated in the lpxR mutant. Although we have reported that lipid A acylation status affects motility and invasion [14], the phenotypes were found in mutants lacking the late-acyltransferases and hence displaying major changes in the lipid A structure at 21°C [14]. This is in contrast to the lpxR mutant grown at 21°C, where the LpxR-dependent deacylation was hardly observed. The fact that YeLpxR is in latent stage at this growth temperature may suggest that, in the lpxR mutant background, the absence of the enzyme in the outer membrane, not the lipid A deacylation, acts as the regulatory signal underlying the reduced expressions of flhDC and rncA. Given experimental support to this hypothesis, the catalytically inactive proteins, such as LpxR, may launch transmembrane signal transduction pathways upon sensing outer membrane perturbations [59], in our case, the absence of the protein itself. Therefore, it can be speculated that those systems sensing extracytoplasmic stresses could underlie the regulatory connection between the absence of LpxR and the expression of Y. enterocolitica virulence factors. Giving indirect support to our speculation, it has been reported that lipid A deacylation induces σ52-dependent responses in E. coli [60], the Cpx system senses changes in LPS O-polysaccharide [61]. Experiments are underway to test whether the activation status of the Cpx and/or σE systems is altered in the lpxR mutant background and whether any of these systems is responsible for the reduced expression of flhDC and rncA found in the mutant.

Figure 12. Impact of lpxR on Y. enterocolitica O:8 interplay with the innate immune system. (A) YeO8 (black circle) or YeO8-ΔlpxR Km (white circle) grown at 21°C or 37°C were exposed to different concentrations of polymyxin B. Each point represents the mean and standard deviation of eight samples from four independently grown batches of bacteria. (B) TNFα secretion by infected macrophages with YeO8 (WT), YeO8-ΔlpxR Km (ΔlpxR), YeO8-ΔyopF (ΔyopF), YeO8- ΔyopP-ΔlpxR Km (ΔyopP-ΔlpxR),”c” denotes bacteria without the virulence plasmid. Strains were grown at 21°C (denoted as 21) and 37°C (denoted as 37). The data are means and s.e.m. *, p<0.05 (for the indicated comparisons). doi:10.1371/journal.ppat.1002978.g012
The LPS contains a molecular pattern recognized by the innate immune system thereby arousing several host defence responses. On one hand, CAMPs target this LPS pattern to bind to the bacterial surface, which is necessary for their microbicidal action. On the other hand, recognition of the LPS by the LPS receptor complex triggers the activation of host defence responses, chiefly the production of inflammatory markers. Not surprisingly, the complex triggers the activation of host defence responses, chiefly

On the other hand, recognition of the LPS by the LPS receptor bacterial surface, which is necessary for their microbicidal action. To our initial purified LPS and the LPS expressed in the complex lipid environment of the bacterial outer membrane. To our initial studies highlight the critical role of pYV-encoded Yops, chiefly YopP, to prevent the activation of inflammatory responses in a variety of cells, including macrophages. Nevertheless, Rebeil and co-workers [12] conclusively demonstrated that purified LPS from Y. enterocolitica grown at 37°C is less inflammatory than that purified from bacteria grown at 21°C. This is in agreement with the concept that underacylated LPSs are less inflammatory than hexaacylated ones [52]. Therefore, it was plausible to speculate that the LpxR-dependent decylation of LPS at 37°C was responsible for the reduced stimulatory potential of the LPS described by Rebeil and co-workers. To confirm this speculation we chose to challenge macrophages with alive bacteria instead of using purified LPS since there might be differences between the cellular recognition of purified LPS and the LPS expressed in the complex lipid environment of the bacterial outer membrane. To our initial surprise, we observed that the lpxR mutant elicited similar inflammatory response than the wild type when both strains were grown at 37°C. The fact that these responses were significantly lower than those elicited by bacteria grown at 21°C suggested that pYV-encoded factors were attenuating the inflammatory response. Therefore, we hypothesized that the arsenal of Yops injected to the cell were efficiently counteracting the activation of inflammatory responses exerted by the lpxR mutant LPS. In fact, previous data demonstrated that the production and function of the pYV-encoded virulence factors were not affected in the lpxR mutant. Giving support to our hypothesis, the inflammatory response elicited by the lpxR mutant cured of the pYV virulence plasmid grown at 37°C was significantly higher than that induced by the virulence plasmid negative wild-type strain. Moreover, our findings suggest that, among all Yops, YopP plays a major role in counteracting the inflammation elicited by the lpxR mutant since the TNFα levels induced by the lpxR mutant cured of the pYV virulence plasmid grown at 37°C were not different than those triggered by YeO8-ΔyopPΔlpxR. On the whole, our results and those reported by Rebeil and co-workers [12] are consistent with a model in which the characteristic low inflammatory response associated to Y. enterocolitica infections might be the sum of the anti-inflammatory action exerted by YopP and the reduced activation of the LPS receptor complex due to the expression of a LpxR-dependent deacylated LPS. In this scenario, the latency of LpxR may facilitate a quick bacterial response upon entering the host to reduce the initial recognition of the pathogen by the LPS receptor complex. This will allow the pathogen to activate other host countermeasures, among others the pYV-encoded type III secretion system, which is a time consuming process.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, Yersinia strains were grown in lysogeny broth (LB) medium at either 21°C or 37°C. When appropriate, antibiotics were added to the growth medium at the following concentrations: ampicillin (Amp), 100 μg/ml for Y. enterocolitica and 50 μg/ml for E. coli; kanamycin (Km), 100 μg/ml in agar plates for Y. enterocolitica, 50 μg/ml in agar plates for E. coli, and 20 μg/ml in broth; chloramphenicol (Cm), 20 μg/ml; trimethoprim (Tp), 100 μg/ml; tetracycline (Tet) 12.5 μg/ml; and streptomycin (Str), 100 μg/ml.

Y. enterocolitica mutant construction

In silico analysis led to the identification of Y. enterocolitica 8081 homologue of lpxR (YE3093), yopP (YE0008) and yopE (YE0005) [accession number AM286415; [20]). To obtain the lpxR, yopP, and yopE mutants two sets of primers (Table S1) were used for each gene to amplify two different fragments from each gene, LpxRUP and LpxRDOWN, YopPUP and YopPDOWN, YopEUP and YopEDOWN, respectively. Both fragments were BamHI-digested, purified, ligated, amplified as a single PCR fragment using a mixture of GoTag Flex polymerase (2.5 units/reaction; Promega) and Vent polymerase (2.5 units/reaction; New England Biolabs), gel purified and cloned into pGEM-T-Easy (Promega) to obtain pGEMTAΔlpxR, pGEMTAΔyopP, and pGEMTAΔyopE respectively. A kanamycin resistance cassette flanked by FRT recombination sites was obtained as a BamHI fragment from pGEMTFRKm and it was cloned into BamHI-digested pGEMTAΔlpxR and pGEMTAΔyopP to generate pGEMTAΔlpxR·Km and pGEMTAΔyopP·Km respectively. ΔlpxR·Km, and ΔyopP·Km allelics were amplified using Vent polymerase (New England Biolabs) and cloned into Smal-digested pKNG101 to obtain pKNGAΔlpxR·Km and pKNGAΔyopP·Km, respectively. ΔyopE allele was obtained by PvuII-digestion of pGEMTAΔyopE, gel purified and cloned into Smal-digested pKNG101 to obtain pKNGAΔyopE·Km. YeO8-ΔyopP was obtained by Flp-mediated recombination [63] using pFLP2Tp. Plasmids were introduced into E. coli CC118-Δpir from which they were mobilized into Y. enterocolitica 8081 by triparental conjugation using the helper strain E. coli HB101/pRK2013. Bacteria were diluted and aliquots spread on Yersinia selective agar medium plates (Oxoid) supplemented with Stre. Bacteria from 5 individual colonies were pooled and allowed to grow in LB without any antibiotic overnight at RT. Bacterial cultures were serially diluted and aliquots spread in LB without NaCl containing 10% sucrose and plates were incubated at RT. The recombinants that survived 10% sucrose were checked for their antibiotic resistance. The appropriate replacement of the wild-type alleles by the mutant ones was confirmed by PCR and Southern blot (data not shown). In the case of YeO8-ΔlpxR·Km and YeO8-ΔyopP·Km mutants, the kanamycin cassette was excised by Flp-mediated recombination [63] using plasmid pFLP2Tp. This plasmid is a derivative from pFLP2 constructed by cloning a trimethoprim resistance cassette, obtained by Smal digestion of p34S-Tp [64], into Scal-digested pFLP2. The generated mutants were named YeO8-ΔlpxR and YeO8-ΔyopP, respectively. YeO8-ΔyopP·ΔlpxR·Km and YeO8-ΔyopE·ΔlpxR·Km double mutants were obtained mobilizing the pKNGAΔlpxR plasmid into YeO8-ΔyopP and YeO8-ΔyopE, respectively. The replacement of
the wild-type alleles by the mutant ones was done as described above and confirmed by PCR (data not shown).

To cure the pTY plasmid from YeO9-ΔlpxRMKm, bacteria were grown at 37°C in Congo Red Magnesium oxalate agar plates [65]. Colony size and lack of uptake of Congo Red were used to detect loss of the virulence plasmid. This was further confirmed by testing the YadA-dependent autoagglutination ability [66].

Construction of lpxR::lucFF reporter fusion

A 443 bp DNA fragment containing the promoter region of lpxR was amplified by PCR using Vent polymerase (see Table S1 for primers used), EcoRI digested, gel purified and cloned into EcoRI-Smal digested pGPl01Tp suicide vector [15]. This vector contains a promoterless firefly luciferase gene (lucFF) and a R6K origin of replication. A plasmid in which lucFF was under the control of the lpxR promoter was identified by restriction digestion analysis and named pGPl01TpYlplxR. This plasmid was introduced into E. coli DH5α-lpxRM from which it was mobilized into Y. enterocolitica by triparental conjugation using the helper strain E. coli HB101/pRK2013. Strains in which the suicide vectors were integrated into the genome by homologous recombination were selected. This was confirmed by PCR (data not shown).

Complementation of lpxR mutant

To complement the lpxR mutant, a DNA fragment of 1.5 kb was PCR-amplified using TaKaRa polymerase (see Table S1 for primers used) gel purified, and cloned into pGEMT-Easy (Promega) to obtain pGEMTComplpxR. A fragment, containing the putative promoter and coding region of the deacylase, was obtained by PvuII digestion of pGEMTComplpxR, gel purified and cloned into the ScaI site of the medium copy plasmid pT1M100 [40] to obtain pT1MplxR. For the construction of plasmid pTMLpxRFLAG, the lpxR coding region with its own promoter and a FLAG epitope sequence right before the stop codon was PCR amplified using Vent polymerase, and primers LpxRtagging and LpxrFLAG (Table S1) and genomic DNA as template. The fragment was phosphorylated, gel purified and cloned into ScaI-digested pT1M100 [40]. Plasmids were introduced into E. coli DH5α-lpxRM and then mobilized into Y. enterocolitica strains by triparental conjugation using the helper strain E. coli HB101/pRK2013.

Lipid A analysis and structural modeling of LpxR

Lipid A was extracted using an ammonium hydroxide/isobutyric acid method and subjected to negative ion matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis [14, 67]. Analyses were performed on a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Incorporated) in negative reflective mode with delayed extraction. Each spectrum was an average of 300 shots. The ion-accelerating voltage was set at 20 kV. Dihydroxybenzoic acid (Sigma Chemical Co., St. Louis, MO) was used as a matrix. Further calibration for lipid A analysis was performed externally using lipid A extracted from E. coli strain MG1655 grown in LB at 37°C. Interpretation of the negative-ion spectra is based on earlier studies showing that ions with masses higher than 1000 gave signals proportional to the corresponding lipid A species present in the preparation [9, 12, 17, 68]. Important theoretical masses for the interpretation of peaks found in this study are: lipid IVα, 1405; C12, 182, C14a, 210; C16:0, 296;2; aminoarabinose ( AraNH2), 131.1; C16:0 239.

Site-directed mutagenesis

Site-directed mutagenesis of the lpxR gene was performed by PCR [69]. Plasmid pTMLpxR, obtained with a minipreparation kit (Macherey-Nagel), was used as template and the desired mutations were introduced by the primer pairs described in Table S1. Amplifications were carried out in 50 μl reaction mixture using Vent DNA polymerase (New England BioLabs.). The PCR was started with initial 70 sec incubation at 95°C and then steps (95°C 50 sec, 60°C 75 sec and 72°C 6 min) were repeated 20 times followed by a 10 min extension time at 72°C. The obtained PCR products were gel purified, phosphorylated with T4 polynucleotide kinase, ligated, and digested with DpnI to break down any remaining template plasmid. The ligated PCR-product was transformed into E. coli C600. Plasmid DNA was isolated from transformants and the lpxR gene was completely sequenced to confirm the generated mutations and to ensure that no other changes were introduced. The name of each mutant construct includes the wild-type residue (single-letter amino acid designation) followed by the codon number and mutant residue (typically alanine).

For the construction of plasmids pTMLpxR(N9A)FLAG and pTMLpxR(S34A)FLAG, the lpxR alleles encoded into pTMLpxR(N9A) and pTMLpxR(S34A) were PCR amplified using Vent polymerase, and primers LpxRtagging and LpxrFLAG (Table S1). The fragments were phosphorylated, gel purified and cloned into Scal-digested pT1M100 [40]. Plasmids were introduced into E. coli DH5α-lpxRM and then mobilized into Y. enterocolitica strains by triparental conjugation using the helper strain E. coli HB101/pRK2013.

Purification of membrane proteins and Western blot analysis of LpxR FLAG tagged proteins

Overnight 5-ml cultures of Y. enterocolitica strains were diluted 1:20 into 100 ml of LB in a 250-ml flask. Cultures were incubated with aeration at 21°C or 37°C until OD600 0.8. Bacteria were recovered by centrifugation (6500×g, 10 min, RT) and they were resuspended in 2 ml of 10 mM Tris/HCl (pH 7.4) + 5 mM MgSO4 containing 2% Triton X-100 (v/v). Cells were broken by sonication (Branson digital sonifier; microtip 1/8” diameter, amplitude 10%) for 15 × 1 min cycles, each cycle comprised 1 min sonication step separated by 1 min intervals. Unbroken cells were eliminated by centrifugation (2000×g, 20 min), and cell envelopes were recovered by ultracentrifugation (Beckman 70.1 Ti rotor; 45 000×g, 1 h, 4°C). The cell envelopes were resuspended in 500 μl of distilled water. The protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific).

50 μg of proteins were separated on 4–12% SDS-PAGE, and semi-dry electrotransferred onto a nitrocellulose membrane using as transfer buffer SDS-PAGE-urea lysis buffer [a freshly prepared 1:1 mix of 1× SDS running buffer (12 mM Tris, 96 mM glycine, 0.1% SDS) and urea lysis buffer (10 mM Na2HPO4, 1% β-mercaptoethanol, 1% SDS, 6 M urea) [70]. Membrane was blocked with 4% skim milk in PBS. Membranes were stained using anti-Flag antibody (Macherey-Nagel) and visualized using the Basic Local Alignment Search Tool (BLAST) at NCBI (http://blast.ncbi.nlm.nih.gov/). A pairwise sequence alignment was made using the program MALIGN [71] in the BODIL modeling environment [72], and a picture of the alignment was created using ESPript [73]. The essential water molecule and the zinc ion in the StLpxR crystal structure were also included in the YeLpxR model. A set of ten models was created with the program MODELLER [74], from which the model with the lowest value of the MODELLER objective function was analyzed and compared to the crystal structure of StLpxR by superimposing with the

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program VERTAA (Johnson & Lehtonen, 2004) in BODIL. Different rotamers for D10 and D31 were searched with the program Jackal [http://wiki.c2b2.columbia.edu/honiglab_public/index.php/Software/Jackal]. D10 was changed to the same rotamer as in the crystal structure of StLpxR, while the rotamer used for D31 was the one with the lowest energy according to Jackal. SURFNET [75] was used to detect surface cavities, while PyMOL (Version 1.4, Schrodinger, LLC) was used for preparing pictures. For the SURFNET calculations, the minimum radius for gap spheres was set to 1.5 Å and the maximum radius was 4.0 Å.

For the docking studies, a Kdo₂-lipid A, both with and without aminoarabinose, was modified from the coordinates for the LPS molecule in the crystal structure of BhuA [76]. The fatty acyl chains were removed from the Kdo₂-lipid A molecule in order to reduce the number of rotatable bonds and make the docking more reliable. Aminoarabinose was added to the modified Kdo₂-lipid A molecule with SYBYL (Version 8.0, Tripos Associates, Inc., St Louis, MO, USA), and the structure was minimized with the molecule with SYBYL (Version 8.0, Tripos Associates, Inc., St Louis, MO, USA), and the structure was minimized with the program using 10% polyacrylamide gels and proteins visualized by Coomassie brilliant blue staining.

Luciferase activity

The reporter strains were grown at 21°C or at 37°C on an orbital incubator shaker (180 r.p.m.) until OD₅₄₀ 1.6. The cultures were harvested (2500 × g, 20 min, 4°C) and resuspended to an OD₅₄₀ of 1.0 in PBS. A 100 μl aliquot of the bacterial suspension was mixed with 100 μl of luciferase assay reagent (1 mM D-luciferin [Synchem] in 100 mM citrate buffer pH 5). Luminescence was immediately measured with a Luminometer LB9507 (Berthold) and expressed as relative light units (RLU). All measurements were carried out in quintuplicate on at least three separate occasions.

Analysis of motility and flhDC expression

Phenotypic assays for swimming motility were initiated by stabbing 2 μl of an overnight culture at the centre of agar plates containing 0.3% agar and 1% tryptone [25,26]. Plates were analyzed after 24 h of incubation at RT and the diameters of the halos migrated by the strain from the inoculation point were compared. Experiments were run in quadruplicate in three independent occasions.

To measure flhDC expression, plasmid pRSFflhDC08 [26] encoding the transcriptional fusion flhDC::lacFF was integrated into the genomes of the strains by homologous recombination. This was confirmed by Southern blot (data not shown). Luminescence was determined as previously described.

β-galactosidase and alkaline phosphatase activities

β-galactosidase activity was determined as previously described with bacteria grown in 1% tryptone at RT [77]. Alkaline phosphatase activity was determined in permeabilized cells and the results are expressed in enzyme units per OD₅₄₀ as previously described [78]. Experiments were run in duplicate in three independent occasions.

Real-time quantitative PCR (RT-qPCR)

Bacteria were grown at 21°C or at 37°C in 5 ml of LB medium on an orbital incubator shaker (180 r.p.m.) until an OD₅₄₀ of 0.3. 0.5 ml of ice-cold solution EtOH/phenol [19:1 v/v [pH 4.3]] were added to the culture and the mixture was incubated on ice for 30 min to prevent RNA degradation. Total RNA was extracted using a commercial NucleoSpin RNA II kit as recommended by the manufacturer (Macherey-Nagel).

cDNA was obtained by retrotranscription of 2 μg of total RNA using a commercial M-MLV Reverse Transcriptase (Sigma) and random primers mixture (SABiosciences, Qiagen), 50 ng of cDNA were used as a template in a 25-μl reaction. RT-PCR analyses were performed with a Smart Cycler real-time PCR instrument (Cepheid, Sunnyvale, CA) and using a KapaSYBR Fast qPCR Kit as recommended by the manufacturer (Cultek). The thermocycling protocol was as follows; 95°C for 3 min for hot-start polymerase activation, followed by 45 cycles of 95°C for 15 s, and 60°C for 30 s. SYBR green dye fluorescence was measured at 521 nm.

cDNAs were obtained from three independent extractions of mRNA and each one amplified by RT-qPCR in two independent occasions. Relative quantities of lpxR mRNAs were obtained using the comparative threshold cycle (ΔΔCT) method by normalizing to rpoB and tonB genes (Table S1).

Analysis of Yop secretion

Overnight cultures of Y. enterocolitica strains were diluted 1:50 into 25 ml of TSB supplemented with 20 mM MgCl₂ and 20 mM sodium oxalate in a 100-ml flask. Cultures were incubated with aeration at 21°C for 2.5 h, and then transferred at 37°C for 3 h. The optical density at 540 nm of the culture was measured and the bacterial cells were collected by centrifugation at 1500 × g for 30 min. Ammonium sulphate (final concentration 47.5% w/v) was used to precipitate proteins from 20 ml of the supernatant. After overnight incubation at 4°C, proteins were collected by centrifugation (3000 × g, 30 min, 4°C) and washed twice with 1.5 ml of water. Dried protein pellets were resuspended in 50 to 80 μl of sample buffer and normalized according to the cell count. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels and proteins visualized by Coomassie brilliant blue staining.

Control experiments revealed that the secretion of Yops was not affected in yopE and yopP mutants except that each mutant did not produce either YopE or YopP, respectively (data not shown).

Analysis of YadA production

Bacteria were grown overnight in 2 ml RPMI 1640 medium lacking phenol red at 37°C without shaking. The OD₅₄₀ of the culture was measured and CFUs were determined by plating serial dilutions. Bacteria from 1-ml aliquot were recovered by centrifugation (16 000 × g, 10 min, 4°C) and resuspended in 200 μl of SDS-sample buffer. Samples were incubated for 4 h at 37°C and kept frozen at −20°C. Samples were analyzed by SDS-PAGE using 10% polyacrylamide gels and proteins visualized by Coomassie brilliant blue staining. Samples were normalized according to the cell count and they were not boiled before loading the gel.

Binding assay to collagen-coated slides

Overnight cultures of Y. enterocolitica strains grown at 37°C were diluted 1:10 into 5 ml of LB and grown with aeration at 37°C for 2.5 h. Bacteria were pelleted, washed once with PBS and resuspended to an OD₅₄₀ of 0.3 in PBS.

12 mm circular coverslips in 24-well tissue culture plates were coated overnight at 4°C with 10 μg/ml human collagen type I (Sigma) in PBS (final volume 100 μl). Coverslips were washed three times with TBS and later they were blocked for 1 h at 4°C with 2% BSA in TBS. Finally, coverslips were washed three times
and were incubated at 37°C with 100 μl of the bacterial suspension. After 1 h incubation, the coverslips were washed three times with PBS and then bacteria fixed with 3.7% paraformaldehyde (PFA) in PBS pH 7.4 for 20 min at room temperature. PFA fixed cells were incubated with PBS containing 0.1% saponin, 10% horse serum and Hoechst 33342 (1:2500) for 30 min in a wet dark chamber. Finally, coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H2O, mounted on Aqua Poly/Mount (Polysciences) and analysed with a Leica CTR6000 fluorescence microscope. Bacteria were counted in images from three randomly selected fields of view obtained at a magnification of ×100 taken with a Leica DFC350FX camera. Wild-type adhesion was set to 100%.

Actin disruption by Yersinia infection

Carcinomic human alveolar basal epithelial cells (A549, ATCC CCL-185) were maintained in RPMI 1640 tissue culture medium supplemented with 1% HEPES, 10% heat inactivated foetal calf serum (FCS) and antibiotics (penicillin and streptomycin) in 25 cm² tissue culture flasks at 37°C in a humidified 5% CO2 atmosphere as previously described [79]. For infections, A549 cells were seeded on 12 mm circular coverslips in 24-well tissue culture plates to 70% confluence. Cells were serum starved overnight. Overnight cultures of Y. enterocolitica strains grown at 21°C were diluted 1:10 into 5 ml of LB and grown with aeration at 21°C for 1.5 h and then 1 h at 37°C. Bacteria were pelleted, washed once with PBS and resuspended to an OD600 = 1 (approximately 10⁵ CFU/ml) in PBS. Cells were infected with this suspension to get a multiplicity of infection of 25:1. After 1 h incubation, the coverslips were washed three times with PBS and then cells fixed with 3.7% PFA in PBS pH 7.4 for 20 min at room temperature. PFA fixed cells were incubated with PBS containing 0.1% saponin, 10% horse serum, Hoechst 33342 (1:2500), and OregonGreen 514-phalloidin (1:100) (Invitrogen) for 30 min in a wet dark chamber. Finally, coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H2O, mounted on Aqua Poly/Mount (Polysciences) and analysed with a Leica CTR6000 fluorescence microscope. Images were taken with a Leica DFC350FX camera.

YopE translocation

YopE translocation into A549 cells was done as previously described [38]. Briefly, A549 cells were seeded in 12-well tissue culture plates to 80% confluence. Cells were serum starved 16 h before infection. Overnight cultures of Y. enterocolitica strains grown at 21°C were diluted 1:10 into 5 ml of LB and grown with aeration at 21°C for 1.5 h and then 1 h at 37°C. Bacteria were pelleted, washed once with PBS and resuspended to an OD600 = 1 (approximately 10⁵ CFU/ml) in PBS. Cells were infected with this suspension to get a multiplicity of infection of 25:1. After 1 h incubation, the coverslips were washed three times with PBS and then cells fixed with 3.7% PFA in PBS pH 7.4 for 20 min at room temperature. PFA fixed cells were incubated with PBS containing 0.1% saponin, 10% horse serum, Hoechst 33342 (1:2500), and OregonGreen 514-phalloidin (1:100) (Invitrogen) for 30 min in a wet dark chamber. Finally, coverslips were washed twice in 0.1% saponin in PBS, once in PBS and once in H2O, mounted on Aqua Poly/Mount (Polysciences) and analysed with a Leica CTR6000 fluorescence microscope. Images were taken with a Leica DFC350FX camera.

Western blotting

Lipid A of Y. enterocolitica and Y. pseudotuberculosis was extracted as described [80]. Bacteria were washed once with PBS and resuspended to an OD600 = 1 (approximately 10⁵ CFU/ml) in PBS. Cells were infected with this suspension to get a multiplicity of infection of 25:1. To synchronize infection, plates were centrifuged at 200 x g during 5 min. After a 30 min infection, cells were washed twice with PBS and then incubated for an additional 180 min in medium containing gentamicin (100 μg/ml). Supernatants were removed from the wells, cell debris removed by centrifugation, and samples were frozen at −80°C. TNFα present in supernatants of culture cells was determined by ELISA (Bender MedSystems) with a sensitivity <4 pg/ml.

YopE translocation

Cells were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting using rabbit polyclonal antisera raised against YopE (1:2000 dilution).

Invasion to HeLa cells

Strains were grown aerobically for 16 h at RT, pelleted and resuspended to an OD600 of 0.3 in PBS. Bacteria suspensions were added to subconfluent HeLa cells at a multiplicity of infection of ~25:1. After a 30 min infection, monolayers were washed twice with PBS and then incubated for an additional 90 min in medium containing gentamicin (100 μg/ml) to kill extracellular bacteria. This treatment was long enough to kill all extracellular bacteria. After this period, cells were washed three times with PBS and lysed with 0.5% saponin in PBS and bacteria were plated. Experiments were carried out in triplicate on three independent occasions. Invasion is expressed as CFUs per monolayer.

Polymerin B susceptibility assay

Bacteria were grown either at 21°C or 37°C in 5 ml LB in a 15-ml Falcon tube with shaking (180 rpm), and harvested (2500 x g, 20 min, 24°C) in the exponential growth phase (OD600 0.8). Bacteria were washed once with PBS and a suspension containing approximately 1x10⁸ CFU/ml was prepared in 10 mM PBS (pH 6.5, 1% Tryptone Soy Broth (TSB, Oxoid), and 100 mM NaCl. Aliquots (5 μl) of this suspension were mixed in 1.5 ml microcentrifuge tubes with various concentrations of polymerin B (Sigma). In all cases the final volume was 30 μl. After 1 h incubation at the bacterial growth temperature, the contents of the tubes were plated on LB agar. Colony counts were determined and results were expressed as percentages of the colony count of bacteria not exposed to antibacterial agents. All experiments were done with duplicate samples on at least four independent occasions.

Macrophage culture and TNFα ELISA

Murine macrophages RAW264.7 (ATCC, TIB71) were grown on DMEM tissue culture medium supplemented with 10% heat-inactivated foetal calf serum (FCS) and Heps 10 mM at 37°C in an humidified 5% CO2 atmosphere. For bacterial infection, cells were seeded in 24-well tissue culture plates 15 h before the experiment at a density of 7x10⁵ cells per well. Overnight cultures of Y. enterocolitica strains grown at 21°C were diluted 1:10 into 5 ml of LB and grown with aeration at 37°C or 21°C for 3 h. Bacteria were pelleted, washed once with PBS and resuspended to an OD600 = 1 (approximately 10⁹ CFU/ml) in PBS. Cells were infected with this suspension to get a multiplicity of infection of 25:1. To synchronize infection, plates were centrifuged at 200 x g during 5 min. After a 30 min infection, cells were washed twice with PBS and then incubated for an additional 180 min in medium containing gentamicin (100 μg/ml). Supernatants were removed from the wells, cell debris removed by centrifugation, and samples were frozen at −80°C. TNFα present in supernatants of culture cells was determined by ELISA (Bender MedSystems) with a sensitivity <4 pg/ml.

Statistical analysis

The results were analyzed by the one-sample t test using GraphPad Prism software (GraphPad Software Inc.). Results are given as means ± SD. A P value of <0.05 was considered to be statistically significant.

Supporting Information

Figure S1  Sequence alignment of StLpxR and YeLpxR. Conserved residues are shown with black background. The amino acid sequences were aligned using the Needleman-Wunsch algorithm and the aligned sequences were displayed using the MUSCLE algorithm. The alignment was visualized using the JalView application.
acids that were mutated in this study are highlighted with grey background, with the two biggest differences, G/D31 and A/Q35, indicated by grey stars. The secondary structure for StLpxR is shown on top of the alignment.

(Fig)

Figure S2 Analysis of the expression of Y. enterocolitica lpxR. Analysis of lpxR mRNA levels assessed by RT-qPCR. Total RNA was extracted from YeO8 (white bar), and mutants (grays bars) YeO8-ΔphoPQ (ΔphoPQ, YeO8-ΔpmrAB (ΔpmrAB) and YeO8-ΔphoPQΔpmrAB (ΔphoPQ ΔpmrAB). Ymv927 (Δσ54), Ymv927-ΔphoPQΔpmrAB (Δσ54ΔphoPQ ΔpmrAB). (A) Bacteria were grown at 21°C. Wild-type bacteria (YeO8) expression levels were set to 1. (B) Bacteria were grown at 37°C. Wild-type bacteria (YeO8) expression levels were set to 1.

(Fig)

Figure S3 Analysis of LpxR levels. Cell envelopes were purified from YeO8-ΔphoRkm mutant (YeO8-ΔphoR) carrying plasmids pTM1pxRFLAG, pTM1lpxR/N9AFLAG or pTM1lpxR/S34AFLAG. Strains were grown at 21°C. 80 μg of proteins were run in SDS-12% polyacrylamide gel, electrotransferred onto a nitrocellulose membrane, and used for anti-Flag antibodies.

(TIF)

Table S1 Primers used in this study.

(DOC)

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Author Contributions

Conceived and designed the experiments: TAS JAB. Performed the experiments: MR EL KMD CPG CML NT. Analyzed the data: MR EL KMD CPG CML NT TAS JAB. Wrote the paper: MR EL KMD TAS JAB.

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