The Yersinia enterocolitica Phospholipase Gene yplA Is Part of the Flagellar Regulon

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Yersinia enterocolitica yplA encodes a phospholipase required for virulence. Virulence genes are often regulated in response to environmental signals; therefore, yplA expression was examined using a yplA:lacZY transcriptional fusion. Maximal yplA expression occurred between pH 6.5 and pH 7.5 and was induced in the mid-logarithmic growth phase. Potential Fnr, cyclic AMP (cAMP)-cAMP receptor protein (Crp), and σ⁷ regulatory sites were identified in the nucleotide sequence. Reduction of yplA expression by aeration, addition of glucose and sucrose, and application of high temperature and salt is consistent with Fnr-, cAMP-Crp-, and σ⁷-mediated regulation, respectively. Expression of yplA was reduced in flhDC and flhA null strains, indicating that yplA is part of the flagellar regulon.

Yersinia enterocolitica is a human pathogen and a causative agent of gastroenteritis which is transmitted by the oral-fecal route (8). As is frequently true of gastrointestinal parasites, Y. enterocolitica is capable of prolonged survival outside a human host, in soil and water at temperatures as low as 4°C (9). Consequently, it has adapted to grow in two environments, in a generally nutrient-rich host at 37°C. The adaptation to a nutrient-limited natural environs at ambient temperature and Consequence, it has adapted to grow in two environments, in a generally nutrient-rich host at 37°C. The adaptation to a nutrient-limited natural environs at ambient temperature and

The ability of Y. enterocolitica strain 8081v (39) and its derivatives to colonize tissues and to induce a more vigorous inflammatory response (41). To better understand the role of this phospholipase in pathogenesis, we characterized the pattern of yplA expression in Y. enterocolitica strains derived from 8081v (Table 1) in response to environmental conditions, especially those conditions which affect the expression of other known virulence factors. In addition, we examined the possibility that yplA is regulated as part of the flagellar regulon because it has a potential flagellar σ⁷ promoter and it has been demonstrated that the phospholipase is secreted through the flagellar type III secretion system (49).

Sequence analysis of the yplAB promoter region. The nucleotide sequence was scanned for potential regulatory sequences that might influence yplA expression; this required additional sequence information upstream of yplA (GenBank accession no. AF0678496). The template pDH520 (41) was purified by the alkaline lysis method (31), sequenced using the ABI Prism Bigdye Terminator Cycle Sequencing System (Perkin-Elmer), and read on an ABI Prism 377 DNA Sequencer (Nucleic Acid Chemistry Laboratory, Washington University). Sequence analysis using the Wisconsin Sequence Analysis Package (GCG, Madison, Wis.) indicated that the arrangement of genes and their predicted amino acid sequences are very similar to those in the phiAB locus of Serratia liquefaciens (41). S. liquefaciens phiAB encodes a phospholipase A₂ and its putative accessory protein, respectively (18, 19). Located 221 bp upstream of yplAB is another open reading frame, yplX, transcribed from the same strand as yplAB. The predicted amino acid sequence of Y. enterocolitica yplX is 90% identical (over 111 residues) to that of similarly placed orfX found in S. liquefaciens (17), as well as 85 and 83% identical to hypothetical proteins encoded in E. coli and Haemophilus influenzae (6, 15). All of these small proteins share significant identity (80% identity over 61 residues) with the acetyltransferase domain of E. coli pyruvate formate lyase (formate acetyltransferase) (48). Further examination of the Y. enterocolitica nucleotide se-
sequence indicated that divergently transcribed genes flank the hypothetical acetyltransferase yplX and yplAB, suggesting that the maximal possible transcriptional unit is restricted to yplXAB.

Examination of the upstream regions for potential regulatory sequences such as promoters and regulatory protein binding sites has identified several regions of interest. Preceding yplX by 113 bp is a sequence with similarity to the binding site consensus for Fnr (46). Fnr is a global activator of genes expressed under anaerobic conditions that is itself oxygen sensitive (4). Examination of the intergenic sequence between yplX and yplA had confirmed a potential binding site for flagellin (21, 41) and Crp (7). The potential Fnr (46) and Crp binding sites were found similarly positioned in the S. liquefaciens regulatory sequences such as promoters and regulatory protein binding sites.

Plasmids

<table>
<thead>
<tr>
<th>Source or reference</th>
<th>Genotype or relevant characteristics</th>
<th>Y. enterocolitica strains</th>
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<tr>
<td>pDHS20</td>
<td>6.0-kb HindIII fragment harboring yplAB in pUC19; Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>39</td>
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<tr>
<td>peP185.2</td>
<td>mob&lt;sup&gt;+&lt;/sup&gt; of pACYC184 with R6K origin; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>37</td>
</tr>
<tr>
<td>pFUSE</td>
<td>lacZYA&lt;sup&gt;+&lt;/sup&gt; derivative of PEPEP185.2; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td>pDHS45</td>
<td>2.2-kb NhelI/XbaI fragment harboring region 5' to yplA in pFUSE to give yplXA&lt;sup&gt;+&lt;/sup&gt;:lacZY; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pTM100</td>
<td>mob&lt;sup&gt;+&lt;/sup&gt; derivative of pACYC184; Cm&lt;sup&gt;r&lt;/sup&gt; Tet&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>2.2-kb EcoRV fragment harboring flaA in pTM100; Cm&lt;sup&gt;r&lt;/sup&gt; Tet&lt;sup&gt;r&lt;/sup&gt;</td>
<td>2</td>
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<sup>a</sup> ypl<sup>+</sup> strains carry the virulence plasmid of the O:8 serovar; pYVO8<sup>+</sup> strains do not. PLA<sup>-</sup> indicates that the strain produces phospholipase activity on plates; PLA<sup>-</sup> indicates that the strain does not.

Environmental signals affecting yplAB expression. The Y. enterocolitica reporter strain YEDS16 was generated with yplX::lacZY located in the appropriate chromosomal environment in single copy to reflect expression of yplA (Fig. 1). YEDS16 is a merodiploid with a fully intact yplX yplAB (yplXAB) region with another copy of yplX:: transcriptionally fused to lacZY. To generate a transcriptional fusion in the Yersinia chromosome, pDHS45 (yplA::lacZY) was mated into Y. enterocolitica strain JB809v (26) as previously described (42). pDHS45 replicates from an R6K origin; thus, it can only be stably maintained if the plasmid integrates into the chromosome by homologous recombination (27, 42). Y. enterocolitica transconjugants were selected on Luria broth (LB) supplemented with nalidixic acid at 20 μg/ml and chloramphenicol at 25 μg/ml. This strain, YEDS16, was found to be β-galactosidase and phospholipase positive on plates (1% tryptone agar or MacConkey agar base) incubated at 26°C supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside at 20 μg/ml or 0.2% egg yolk lecithin and 1 mM CaCl₂, respectively (data not shown); under the same conditions, 8081v is also positive on lecithin plates (41). This is consistent with the functional phospholipase promoter being duplicated and driving lacZY and yplA expression in YEDS16.

An initial observation indicated that levels of phospholipase activity produced by Y. enterocolitica decreased when salt was added to the medium (when assayed on lecithin plates). Consistent with this observation, comparison of the β-galactosidase activity produced by overnight cultures of the reporter strain, YEDS16, grown in either 1% tryptone broth, 1% tryptone with 200 mM NaCl, or LB (which contains 171 mM NaCl) determined that yplA expression was greatest in the 1% tryptone broth cultures (data not shown), β-Galactosidase assays were performed and values were calculated as previously described (35). This finding suggests that the inhibitory effect of salt on YplA activity is exerted at the transcriptional level. Thereafter, all experiments used Y. enterocolitica cultures grown in 5 ml of 1% tryptone broth based media under standard conditions, i.e., incubation in a culture tube on a wheel at 26°C for 8 h, unless otherwise indicated. Cultures for determination of yplA expression were inoculated from overnight LB cultures, conditions...
known to produce very low levels of yplA expression. Thus, residual β-galactosidase activity from the overnight cultures would not be detected in the samples assayed. The inhibitory effect of NaCl was further examined to distinguish among several possible environmental cues which might be causing the effect: NaCl concentration, ionic strength, and osmolarity (Fig. 2A). The effects of ionic strength versus osmolarity were tested in 1% tryptone broth supplemented with increasing salt (0 to 300 mM KCl or NaCl) or rhamnose (0 to 600 mM) concentrations, as indicated. The level of β-galactosidase activity (yplA expression) decreased similarly with increasing concentrations of NaCl and KCl, but only the highest concentration of rhamnose (600 mM) caused significant repression. Thus, the environmental cue triggered by NaCl seems to be either chloride concentration or ionic strength generally rather than osmolarity, although high osmotic strength is also apparently inhibitory.

The level of expression was monitored at various times during the growth of YEDS16 to determine at which phase of growth yplA is most highly expressed (Fig. 2B). β-Galactosidase activity peaked during mid-to-late logarithmic phase, but significant activity was detected late into stationary phase. Induction of expression at the transition from logarithmic growth to stationary phase has been demonstrated for other virulence genes, including inv, yst, ail, and myfA (22, 34, 37, 38). Although the initial increase of yplA transcription is initiated in late log phase, significant transcriptional activity continues well into stationary phase.

Experiments examining phospholipase activity produced by *Y. enterocolitica* on lecithin plates established that phospholipase activity was produced at 26°C but was not detected at 37°C. There are several possible explanations for this temperature effect: yplA may not be efficiently transcribed, YplA may not be efficiently secreted, or YplA is not active at 37°C. Active phospholipase had been produced from a high-copy plasmid in *E. coli* grown at 37°C, suggesting that YplA is active at 37°C, which is consistent with the role of YplA as a virulence factor (data not shown). To determine whether the temperature effect was due to transcriptional regulation, YEDS16 was grown at 37°C and assayed for β-galactosidase activity at various times during growth (Fig. 2B). In vitro, there was no significant induction of expression in 1% tryptone at 37°C at any phase of growth. Therefore, the lack of phospholipase activity displayed by *Y. enterocolitica* in vitro at 37°C is due to temperature regulation at the level of transcription.

Superficially, this pattern of temperature regulation is contradictory to the role of YplA as a virulence factor. However, inv (invasin) and yst (heat-stable enterotoxin) are also virulence genes expressed at low temperature rather than at 37°C in vitro (13). Yet, at slightly acidic pH or high osmolarity, respectively, these virulence genes are expressed at 37°C in vitro (34, 37). Moreover, invasin has been detected in *yersiniae* recovered from mouse tissues 45 h after peroral inoculation in amounts similar to those produced by the same number of bacteria cultured at 26°C in vitro (37). Therefore, high-temperature repression can be superseded by other conditions that stimulate expression in the host. Alternatively, low-temperature induction could reflect the need for the virulence factors early in the infectious process, as has been suggested for invasin (37) and the *Y. enterocolitica* urease (12). Invasin is thought...
to promote initial entry through the intestinal epithelium, and urease is thought to promote survival in the acidic environment of the host stomach. However, a secreted virulence factor would likely be diluted or degraded and exert little effect unless produced and secreted within the host. Thus, it seems probable that some cue induces expression of \( yplA \) in vivo. Future experiments will attempt to demonstrate the presence of YplA or expression of \( yplA \) in yersiniae infecting mouse tissues, but that is beyond the scope of this study.

A number of important \textit{Yersinia} virulence genes, the \textit{yop} genes and \textit{yadA}, are expressed under low-calcium conditions at 37°C in vitro (39). Experiments with YEDS16 demonstrated that removal of calcium by addition of 20 mM NaC\(_2\)O\(_4\) and 20 mM MgCl\(_2\) at 26°C slightly repressed expression of \( yplA \), but addition of extra Ca\(^{2+}\) (2.5 mM CaCl\(_2\)) had no significant effect (data not shown). There was no apparent effect of calcium limitation at 37°C, although \textit{Y. enterocolitica} does not grow well at elevated temperature without calcium. Virulence factors from many pathogenic bacterial species are regulated in response to Fe\(^{2+}\). Experiments in which either additional 150 \( \mu \)M FeCl\(_3\) was added or available Fe\(^{2+}\) was removed (chelated using 100 \( \mu \)M 2,2′-dipyridyl) demonstrated there was no significant effect on transcription of \( yplA \) in response to Fe\(^{2+}\) concentration (data not shown).

\textit{Y. enterocolitica} survives over a large pH range (pHs 4.0 to 10.0), which is certainly beneficial as it traverses the acidic stomach and is carried into the alkaline small intestine (5). Therefore, \( \beta \)-galactosidase activity was assayed in cultures grown in a series of buffered broth media (1% tryptone) inoculated in parallel. The effect of pH was examined using 1% tryptone broth containing a 50 mM concentration of the following buffers equilibrated at the indicated pH values: citric acid for pHs 5.0 and 6.0; HEPES for pHs 7.0 and 7.5, and TAPS [\( N \)-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid] for pHs 8.5 and 9.5. After 8 h of incubation, bacteria were harvested for \( \beta \)-galactosidase assays and the pH of the culture media was tested. In no case had the pH significantly changed (\( \pm 0.2 \) pH U) from the original pH of the buffered media. In the pH range tested, pHs 5.0 to 9.5, the cultures reached similar cell densities (optical density at 600 nm [OD\(_{600}\)] 1.0 to 1.2), except for the most alkaline medium (pH 9.5; OD\(_{600}\) 0.5 to 0.7). These results suggest that \( yplA \) was efficiently transcribed at pHs 6 to 7.5 and expression peaked at pH 7.0 (Fig. 2C).
Given the identification of potential regulatory binding sites 5' to the yplAB locus, environmental cues which affect the regulatory proteins were tested. First, the potential Fnr binding site upstream of yplX suggests that transcription of yplX, and perhaps yplA, might be regulated in response to oxygen tension. Few investigators have examined the response of Y. enterocolitica virulence gene expression at different oxygen levels: expression of both inv and ail is reduced under anaerobic conditions in vitro (inv is only significantly affected in rich medium at 26°C and not at 37°C or in minimal media) (36, 37). Therefore, the effect of aeration was determined using 5-ml cultures grown under the following conditions of increasing aeration, from least to greatest: in a culture tube on a wheel, in a slanted culture tube shaking at 250 rpm, and in a 125-ml flask shaking at 250 rpm. Static cultures were not included for comparison because these cultures grew slowly to a low cell density in 1% tryptone, which would not allow meaningful comparison of promoter activities. Vigorously shaken cultures produced a fifth of the β-galactosidase activity of cultures grown in tubes on a wheel (Fig. 3A). As maximum expression occurs when cell density is high and free oxygen is scavenged quickly, the oxygen tension in these culture is probably low. These results are consistent with Fnr-dependent regulation of yplA expression. Similar conditions of low oxygen tension occur in the gut lumen, so this condition may have relevance in the host (43).
β-galactosidase activity to a quarter and approximately a third of that observed in the 1% tryptone broth, respectively (Fig. 3B). Addition of other utilisable carbon sources at 20 mM, including maltose, galactose, arabinose, glycerol, and lactose, did not have a significant effect on the levels of yplA expression (Fig. 3B and data not shown). While lactose is a disaccharide composed of glucose and galactose, it had no significant effect on levels of β-galactosidase activity. This result is consistent with the fact that Y. enterocolitica is unable to utilize lactose as a carbon source. Furthermore, all of these results are consistent with the regulation of yplA by catabolite repression.

To confirm that the patterns of transcriptional regulation determined using the reporter fusion reflected the production of YplA protein, Y. enterocolitica grown under various conditions was examined by Western analysis using antisem generated against YplA. Polyclonal antisem was raised in a rabbit using MalE-YplA produced from pDHS28 in E. coli and purified as previously described (49). Confounding specificity for common bacterial antigens was removed by adsorption to acetone powders prepared from Y. enterocolitica YEDS10 (phospholipase null mutant) and E. coli containing pMal-p2 (20). In all cases, the amount of YplA detected correlated with the levels of expression determined using the yplA::lacZY fusion (data not shown).

yplA expression requires the flagellar regulators (FlhDC and FliA). The identification of a potential flagellar sigma factor-dependent promoter, export of YplA by the flagellar type III apparatus, and regulation of yplA and flagellar genes in response to temperature and ionic strength suggest that yplA is part of the flagellar regulon. This hypothesis was tested by introducing yplA::lacZY into Y. enterocolitica strains YEDS10 and JB400v, which have mutations in the flagellar regulatory genes flhDC (50) and fliA (2), respectively. The correct insertion of the reporter plasmid construct pDHS45 into ΔflhDC or ‘fliA mutant strains was confirmed by Southern analysis (Fig. 1; data not shown). When bacteria were grown under standard conditions, β-galactosidase activity was reduced to ~20% of wild-type levels in either YEDS29 (ΔflhDC) or YEDS18 (fliA) compared to YEDS16 (Fig. 3C). Parent strains JB400v and YEDS16 do not produce detectable phospholipase activity on plates. To confirm that the ΔflhDC or ‘fliA mutation was responsible for the decreased β-galactosidase production, each mutation was complemented with the functional gene(s) carried on a plasmid, pGY10 (50) or pJB222 (2), respectively. For comparison, the plasmid vector pTM100 was introduced into the strains as well. The ‘fliA mutation was complemented to wild-type levels of β-galactosidase activity by pJB222. Similarly, the ΔflhDC mutation was complemented by pGY10 yet it induced even greater β-galactosidase activity than that of the wild type, YEDS16 (Fig. 3C). In neither case did the pTM100 vector alone complement the mutation. To confirm that the patterns of transcriptional regulation determined using the reporter fusion reflected the production of YplA protein, Y. enterocolitica mutant strains with and without complementing plasmids were examined by Western analysis using antisem generated against YplA. For all strains, the amount of YplA correlated with levels of lacZ expression (Fig. 3D). Thus, the results demonstrate that yplA is part of the flagellar regulon and are consistent with direct promotion of yplA transcription by FliA (σF).

FliA-dependent regulation would provide a mechanism for both inhibition of yplA expression by ionic strength and temperature regulation of yplA. The flagellin genes flcABC are similarly repressed at 37°C and expressed at 26°C, and their expression is dependent on FliA (σF) (23). In vitro, fliA expression is immediately arrested if the temperature is increased to 37°C, which explains the loss of motility and reduced expression of flagellin genes and yplA (24). Repression of yplA expression by ionic strength may also reflect its regulation as part of the flagellar regulon. Motility and flagellin production are also repressed by ionic strength, although the mechanism has not been elucidated (50). Thus far, characterization of the Y. enterocolitica flagellar regulatory cascade has determined that it is very similar to the E. coli and Salmonella paradigm (21, 30). The master regulator flhDC is required for expression of fliA, and FliA (σF), in turn, promotes expression of the class III flagellar genes which complete the flagellum. Therefore, FlhDC is needed for expression of both class II and III genes but FliA is only necessary for expression of class III genes. Consequently, the effects of mutations in flhDC and fliA on expression are consistent with direct promotion of yplA transcription by σF, possibly by binding to the identified σF consensus site. With respect to the patterns of regulation by oxygen tension (Fnr), catabolite repression (cAMP-Crp), and a flagellar regulator (FliA), the behavior of yplA is identical to that of phlA of S. liquefaciens (16, 17). Indeed, the organization of the Y. enterocolitica yplXAB and S. liquefaciens orxphlAB loci and the proteins encoded therein are very similar. Since both Serratia and Yersinia spp. are members of the family Enterobacteriaceae, it is certainly possible that other species have phospholipase genes at similar loci. The potential role of the PhlA phospholipase in pathogenesis has not been addressed, although S. liquefaciens is considered an opportunistic pathogen.

Based on the E. coli and Salmonella paradigm, flhDC is predicted to be under catabolite repression control; cAMP-Crp is required for flhDC transcription (21, 30). Interestingly, for Y. enterocolitica, the response to the addition of glucose differs for flagellin (and motility); with or without the addition of glucose, Y. enterocolitica is motile and similar levels of flagelin are produced (50). However, the phospholipase production appears to be catabolite repressed. This is consistent with the presence of a CAMP-Crp site upstream of yplA and suggests one mechanism to explain why yplA regulation does not always parallel the flagellar regulon. These data suggest that other regulatory mechanisms are superimposed on yplA expression, in addition to regulation by FliA and FlhDC.

Another consideration for the phospholipase is its secretion into the extracellular milieu, which is undoubtedly necessary for its role as a virulence factor. Indeed, a number of bacterial phospholipases have been identified as virulence factors, and without exception, they are all secreted proteins (reviewed in references 44 and 47). This Y. enterocolitica phospholipase has been shown to utilize the flagellar type III secretion apparatus (49). The expression of class II flagellar genes encoding this apparatus is dependent on FlhDC but does not require FliA or expression of the class III genes. Interestingly, the class II flagellar genes flhBAE of Y. enterocolitica are not transcriptionally regulated by temperature (14). As FliA and FlhB are thought to be part of the secretion apparatus (1, 30), a functional flagellar type III secretion apparatus may be produced at 37°C although the flagellum is not complete and the yersiniae are not motile. Production of a functional flagellar secretion apparatus without a functioning flagellum might appear wasteful, unless the flagellar secretion apparatus serves another purpose, such as secretion of nonflagellar proteins.

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