The *pst* operon of enteropathogenic *Escherichia coli* enhances bacterial adherence to epithelial cells

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Enteropathogenic *Escherichia coli* (EPEC) adheres *in vivo* and *in vitro* to epithelial cells. Two main adhesins, the bundle-forming pilus and intimin, encoded by the *bfp* operon and *eae*, respectively, are responsible for the localized and the intimate adherence phenotypes. Deletion of the *pst* operon of EPEC abolishes the transport of inorganic phosphate through the phosphate-specific transport system and causes the constitutive expression of the PHO regulon genes. In the absence of *pst* there is a decrease in the expression of the main EPEC adhesins and a reduction in bacterial adherence to epithelial cells *in vitro*. This effect is not related to PHO constitutivity, because a Δpst *phoB* double mutant that is defective in the transcription of the PHO genes also displayed low levels of adherence and expression of adhesins. Likewise, a PHO-constitutive *phoR* mutation did not affect bacterial adherence. The expression of the *per* operon, which encodes the *bfp* and *ler* regulators PerA and PerC, is also negatively affected by the *pst* deletion. Overall, the data presented here demonstrate that the *pst* operon of EPEC plays a positive role in the bacterial adherence mechanism by increasing the expression of *perA* and *perC* and consequently the transcription of *bfp* and *eae*.

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is one of the most important bacterial causes of infant diarrhoea in developing countries. EPEC is known for its ability to cause attaching-effacing (A/E) lesions, which is histopathologically characterized by an intimate adherence of the bacteria to the brush-border microvilli, and the formation of pedestal-like structures on the epithelial tissue beneath the bacterial adherence sites (Nataro & Kaper, 1998). In the A/E lesions, bacteria adhere intimately through the adhesin intimin that binds to the intimin receptor (Tir) previously exported to the host cell (Kenny *et al.*, 1997). Another EPEC characteristic is the formation of microcolonies on cell monolayers *in vitro*, a pattern known as localized adherence (LA) (Scaletsky *et al.*, 1984).

Most genes implicated in the A/E lesions are situated in a 35 kb pathogenicity island known as the locus of enterocyte effacement (LEE). This region harbours five operons containing 41 genes divided into three functional regions. LEE1, 2 and 3 encode proteins involved in the biogenesis of a type III secretion system (TTSS), which is a multi-protein complex that spans both bacterial membranes and is used to transfer effector proteins into the host

cell. LEE5 contains the *eae* and *tir* loci that encode, respectively, the adhesin intimin and Tir. LEE4 encodes the Esp proteins, which are secreted by a TTSS (Mellies *et al.*, 1999).

Typical EPEC strains carry a large plasmid known as pEAF (plasmid of EPEC adherence factor). At least two operons (*bfp* and *per*) present in pEAF are needed to confer on EPEC the LA phenotype. The *bfp* operon contains 14 genes related to the biogenesis of the bundle-forming pilus (BFP), a type IV fimbria found in typical EPEC strains (Stone *et al.*, 1996). The first gene of the operon, *bfpA*, encodes the main subunit of the fimbria (Donnenberg *et al.*, 1992). PerA, encoded by the *perABC* operon, is responsible for the positive regulation of *bfp* expression (Tobe *et al.*, 1996). PerC activates the transcription of *ler*, a gene present in LEE1 that encodes the main transcriptional activator of LEE (Mellies *et al.*, 1999). In addition, expression of *bfp* and A/E activity is dependent on several environmental factors. Maximum transcription levels of *bfp* and of A/E activity occur at 37 °C and during the exponential growth phase (Puentes *et al.*, 1996; Rosenshine *et al.*, 1996). Expression of *bfp* is also dependent on the presence of calcium ions and is inhibited by ammonium (Puentes *et al.*, 1996).

The PHO regulon of *E. coli* constitutes a group of more than 30 genes and operons whose expression is induced by phosphate limitation and that are related to the transport and assimilation of phosphorylated compounds. Some of

Abbreviations: A/E, attaching-effacing; AP, alkaline phosphatase; BFP, bundle-forming pilus; CAT, chloramphenicol acetyltransferase; EPEC, enteropathogenic *Escherichia coli*; FCS, fetal calf serum; LA, localized adherence; LEE, locus of enterocyte effacement; P_i, inorganic phosphate; TTSS, type III secretion system.

the most well-studied PHO genes are *phoA*, which encodes alkaline phosphatase, the *pst* operon that encodes the high-affinity inorganic phosphate (P_i)-transport system Pst, and the operon *phoB-phoR*, which codes for a two component system that controls the expression of the PHO regulon. When P_i concentration in the medium falls below 4 μ M, the histidine kinase PhoR, located in the inner membrane, auto-phosphorylates and transfers the phosphate group to the regulatory protein PhoB. Once phosphorylated, PhoB-P binds to consensus regions, known as PHO boxes, present in the promoters of all PHO genes thereby activating their transcription (Makino *et al.*, 1988). Under phosphate-excess conditions, PhoR, which also displays phosphatase activity, dephosphorylates PhoB-P and halts the transcription of the PHO regulon genes (Makino *et al.*, 1989).

The *pst* operon is induced under P_i shortage conditions and is composed of five genes, *pstS*, *pstC*, *pstA*, *pstB* and *phoU*, which are transcribed counter-clockwise in that order. Four of these genes encode the Pst system formed by the proteins PstS, PstC, PstA and PstB. PstS is a periplasmic protein that binds P_i with high affinity and carries the P_i molecule to the channel formed by the transmembrane proteins PstC and PstA (Webb *et al.*, 1992). PstB is an ATP-binding protein that energizes Pst transport (Chan & Torriani, 1996). The function of PhoU is still unclear, but it does not participate in the transport of P_i (Steed & Wanner, 1993).

The Pst system also functions as a negative regulator of the PHO regulon, as most mutations in any gene of the *pst* operon lead to the constitutive expression of the PHO regulon (Wanner, 1996). The nature of this regulatory function is still unknown, and some point mutations in *pstC* and *pstA* abolish P_i transport through Pst, without disrupting the Pst regulatory function (Cox *et al.*, 1988, 1989). Mutations in *phoR* that affect the phosphatase activity of the protein also cause the constitutive expression of the PHO regulon (Carmany *et al.*, 2003; Kreuzer *et al.*, 1975). At the protein level, the Pst system of EPEC (strain E2348/69) is 99.2 % identical to that of *E. coli* K-12. At the DNA level, the *pst* ORFs are 97.5 % identical on average. The only significant difference is a 92 bp deletion in EPEC *pst* in the intergenic region between *pstA* and *pstB*.

Several studies have described a positive correlation between some PHO genes and the virulence of *E. coli* and of other bacterial species as well. Mutations in *pstC*, *phoU* and partial deletions of the *pst* operon reduced the virulence of extra-intestinal *E. coli* strains (Buckles *et al.*, 2006; Daigle *et al.*, 1995; Lamarche *et al.*, 2005). Likewise, insertions in the *pstS* gene of a porcine EPEC strain also negatively interfered with bacterial virulence by reducing attachment to the host cells (Batisson *et al.*, 2003) and in *Vibrio cholerae*, a mutation in *phoB* partially impaired the colonization of rabbit intestines (von Krüger *et al.*, 1999).

In the present study, we analysed the influence of the regulatory genes of the PHO regulon, namely the *pst*

operon, *phoB* and *phoR*, on the adherence of EPEC to epithelial cells *in vitro*. We show that *pst* is needed for the full expression of EPEC adhesins and that the lack of *pst* is detrimental to bacterial adherence.

METHODS

Bacterial strains, plasmids and oligonucleotides. These are listed in Table 1.

Media and growth conditions. Medium A is a semi-rich low-phosphate medium (Levinthal *et al.*, 1962) which was called medium A + P_i when supplemented with 1 mM KH_2PO_4 and medium A - P_i when not supplemented. LB medium was used as described by Miller (1992). Dulbecco's Modified Eagle's Medium (DMEM) for epithelial cells (Cultilab-Brazil) was supplemented with 40 μ g proline ml^{-1} when used for the Δ *pst phoB* mutant.

Transduction of PHO mutations. The mutations Δ *pst::Km^R* and *phoB519::Tn5* were transferred to LRT9 by transduction with phage P1 and selection with kanamycin. *phoB23 proC::Tn10* was transduced from strain BS1 into GMF195 (LRT9 Δ *pst::Km^R*) and selected for tetracycline resistance. The *phoR69* mutation was transduced into LRT9 by a two-step procedure. First, *proC::Tn10* from strain BS8 was transduced into LRT9 and selected for tetracycline resistance and proline auxotrophy. Next, a P1 lysate of strain C3T (*phoR69 proC⁺*) was used to transduce LRT9 *proC::Tn10*, and the transductants were selected for growth on minimal medium. The Δ *pst* and *phoR* mutants were tested for alkaline phosphatase (AP) constitutivity and the *phoB* mutants were tested for the lack of AP induction under P_i limitation. All transductions were performed as described by Miller (1992).

Plasmid construction. Plasmid pGM2 was constructed by ligating a *Pst*I- and *Eco*RI-digested PCR product corresponding to the entire *pst* operon of E2348/69 to the low-copy vector pGB2 digested with the same restriction enzymes. The *pst* fragment of E2348/69 was amplified using the oligonucleotides *pst1* and *pst2*, which adds *Pst*I and *Eco*RI restriction sites to the ends of the DNA molecule.

To construct a transcriptional fusion between the *perABC* promoter and *lacZ*, a fragment of 645 bp containing the promoter of *per* was amplified by PCR using plasmid pEAF as a template and the oligonucleotides Per-P1 and Per-P2. The PCR product was first cloned in pGEM T-easy (Promega) and the recombinant plasmid was digested with *Eco*RI. The digested fragment containing the *perABC* promoter region was ligated to the *Eco*RI-digested vector pRKlacZ290 (Gober & Shapiro, 1992) that harbours a promoterless *lacZ* gene, resulting in plasmid pGM17. The orientation of the cloned *per* promoter with respect to *lacZ* was checked by amplifying the region encompassing the fusion *per-lacZ* by PCR with the oligonucleotides Per-P1 and *lacZ*-1391 and sequencing.

Strain GMF247 (LRT9 Δ *pst phoB*) is resistant to tetracycline and could not be transformed with pGM17. A spectinomycin-resistance cassette was therefore inserted into pGM17, resulting in plasmid pGM29. Briefly, pGM17 was partially digested with 0.2 units of *Eco*RV (which digests inside the tetracycline-resistance gene and elsewhere) for 2 h. The spectinomycin-resistance cassette was obtained by digesting pJL74 (LeDeaux & Grossman, 1995) with *Pst*I and *Bam*HI, followed by treatment with Klenow to blunt the fragment ends. The fragments were then ligated and colonies were selected based on their resistance to spectinomycin and sensitivity to tetracycline.

Enzyme assays. AP activity was assayed as described by Spira *et al.* (1995). Bacteria were grown in medium A $\pm P_i$. *p*-Nitrophenyl-

Table 1. Strains, plasmids and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Relevant features or sequence	Reference/source
Strains		
LRT9	EPEC O111 : abH2	L. R. Trabulsi (Instituto Butantan, São Paulo)
GMF195	LRT9 $\Delta(pstSCAB-phoU)$ 560::Km ^R	P1 transduction from BS7 into LRT9
GMF201	pGM2 in GMF195	This study
GMF210	pBS1 in JC1	This study
GMF236	LRT9 <i>phoB23 proC</i> ::Tn10	P1 transduction from BS1 into LRT9
GMF247	LRT9 $\Delta(pstSCAB-phoU)$ 560::Km ^R <i>phoB23 proC</i> ::Tn10	P1 transduction from BS1 into GMF195
GMF257	LRT9 <i>phoR69</i>	P1 transduction from BS8 and C3T into LRT9
GMF264	pBS1 in GMF257	This study
GMF269	pGB2 in GMF195	This study
GMF285	pGM17 in LRT9	This study
GMF286	pGM17 in GMF195	This study
GMF287	pGM17 in GMF201	This study
JC1	LRT9 <i>phoB519</i> ::Tn5	P1 transduction from BW10200 into LRT9
E2348/69	EPEC O127 : H6	Levine <i>et al.</i> (1985)
GMF284	pGM17 in JPN15	This study
JPN15	EPEC E2348/69 cured of pEAF	Levine <i>et al.</i> (1985)
MG1655	Wild-type <i>E. coli</i> K-12	Xiao <i>et al.</i> (1991)
BS1	MG1655 <i>phoB23 proC</i> ::Tn10	P1 transduction from RW3390 into LEP1 and then into MG1655
BS7	MG1655 $\Delta(pstSCAB-phoU)$ 560::Km ^R	Spira <i>et al.</i> (1995)
BS8	MG1655 <i>proC</i> ::Tn10	P1 transduction from RW3390 into MG1655
BW10200	Δlac -169 <i>phoB519</i> ::Tn5 <i>creB510</i> thi	Steed & Wanner (1993)
C3T	Hfr <i>relA1 pit-10 spoT1 tonA22 phoR69</i>	Echols <i>et al.</i> (1961)
LEP1	F ⁻ <i>phoB lac proC thi trp purE tsx</i>	Bracha & Yagil (1973)
RW3390	$\Delta[lacU169]$ <i>proC</i> ::Tn10 <i>leu thi</i> Str ^R	R. Weisberg (NIH, Bethesda, USA)
Plasmids		
pBS1	<i>phoBR</i> from <i>E. coli</i> K-12 cloned in pBR322	Spira & Yagil (1999)
pBS11	<i>pstS</i> promoter cloned in pKK232-8	Taschner <i>et al.</i> (2004)
pGB2	Cloning vector	Churchward <i>et al.</i> (1984)
pGEM T-easy	Cloning vector	Promega
pGM2	<i>pstSCAB-phoU</i> from strain E2348/69 cloned in pGB2	This study
pGM17	<i>perABC</i> promoter cloned in pRKlacZ290	This study
pGM29	<i>perABC</i> promoter cloned in pRKlacZ290 containing a Sp ^R cassette	This study
pKK232-8	Cloning vector carrying a promoterless <i>cat</i> gene	Amersham Biosciences
pRKlacZ290	Low-copy vector carrying a promoterless <i>lacZ</i> gene	Gober & Shapiro (1992)
pJL74	Plasmid bearing a Sp ^R cassette	LeDeaux & Grossman (1995)
Oligonucleotides		
bfp-A	5'-AATGGTGCTTGCCTTGCTGC-3'	
bfp-B	5'-GCCGCTTTATCCAACCTGGT-3'	
eae-A	5'-ACGTTGCAGCATGGGTAAGTC-3'	
eae-B	5'-GATCGGCAACAGTTTCACCTG-3'	
Per-P1	5'-CTTAGCCGCGTGTCCACTAT-3'	
Per-P2	5'-TTCGGTGAATTCTTCTTGTTTC-3'	
lacZ-1391	5'-CCTCTTCGCTATTACGCCAG-3'	
pst1	5'-TCCTGCGAATTCATGTGAC-3'	
pst2	5'-GACCATGCCTGCAGTTATTA-3'	
rpoD1	5'-GCCGAAGCAGTTTGACTACC-3'	
rpoD2	5'-GCCACGGTTGGTGTATTCT-3'	

phosphate (p-NPP) was used as a substrate and Na₂HPO₄ was used to terminate the reaction. AP specific activity was calculated according to the following formula: A⁴¹⁰/time (min)/cell density (OD₅₄₀).

β -Galactosidase was assayed as described by Miller (1992). Briefly, 800 μ l buffer Z (16.1 g Na₂HPO₄ l⁻¹, 5.5 g NaH₂PO₄ l⁻¹, 0.75 g KCl l⁻¹, 0.25 g MgSO₄·7H₂O l⁻¹ and 2.7 ml β -mercaptoethanol l⁻¹) was

added to 200 µl of permeabilized cells and the reaction was started with the addition of 200 µl 4 mg ONPG ml⁻¹. Samples were incubated at 32 °C until a yellow colour developed and 500 µl 1 M Na₂CO₃ was added to terminate the reaction. The reaction product was read at 420 nm and Miller units were calculated.

Chloramphenicol acetyltransferase (CAT) activity of the *pstS-cat* fusion was measured using a modification of the biochemical assay devised by Shaw (1975). Bacteria were grown overnight in LB and resuspended in LB, medium A ± P_i, DMEM supplemented with 0%, 2% or 10% fetal calf serum (FCS), DMEM supplemented with 2% FCS and 1 mM KH₂PO₄ and DMEM medium without FCS. Cells were grown to the mid-exponential phase (OD₅₄₀ 0.4–0.5), washed with 0.1 M Tris/HCl pH 8 and disrupted by incubation in lysis buffer (100 mM potassium phosphate buffer pH 8.0, 2.0 mM EDTA, 1% Triton X-100, 5.0 mg BSA ml⁻¹, 1.0 mM DTT, 5.0 mg lysozyme ml⁻¹) for 15 min at room temperature. The lysate was then centrifuged to remove cell debris and added to a cuvette containing 0.4 mg dithio-bis(2-nitrobenzoic acid) (DTNB) ml⁻¹ and 0.1 mM acetyl-CoA, both diluted in 0.1 M Tris/HCl pH 7.8. The reaction was started by the addition of 0.1 mM chloramphenicol. The enzyme specific activity was calculated using the following formula: $(\Delta A^{412} \times \text{dilution factor}) / (13.6 \times \text{OD}_{540} \times \text{cell volume})$.

Qualitative adherence assay. The adherence of EPEC to Hep-2 cells (larynx carcinoma established culture) was essentially performed as described by Cravioto *et al.* (1979). A suspension containing 10⁵ Hep-2 cells in 1 ml DMEM supplemented with 10% FCS was added to each well of a 24-well tissue plate and grown for 48 h at 37 °C with 5% CO₂. The medium was then removed from the cell monolayer and replaced with 1 ml fresh DMEM supplemented with 2% FCS and 1% mannose. At this point, 5 × 10⁷ bacteria previously grown for 18 h in LB medium at 37 °C were added to each well. After 3 h of incubation, the cell monolayer was washed six times with PBS to remove the non-adherent bacteria. Cells were fixed with methanol and stained with Giemsa and May–Grunwald stains. Adherent bacteria were observed under an optical microscope and photographed.

Quantitative adherence assay. To quantify the level of bacterial adherence, a modification of the method of Minami *et al.* (1987) was employed. This method makes use of the endogenous β-galactosidase produced by the bacteria as a reporter. Hep-2 and bacterial cells were grown as described for the qualitative adherence assay, except that 1 mM IPTG was added to the media to induce expression of the *lacZ* operon. After washing with PBS, the monolayer containing the adhered bacteria was treated with 200 µl lysis buffer (0.1 M Tris/HCl pH 8, 2 mM EDTA, 1% Triton X-100, 1 mM DTT and 5 mg lysozyme ml⁻¹) for 20 min at room temperature; β-galactosidase activity was assayed as described above.

RNA extraction and Northern-blot analysis. Bacteria were grown in DMEM supplemented with 2% FCS without agitation at 37 °C and harvested at an OD₅₄₀ of 0.4–0.6. They were then homogenized with 4 ml acid phenol/guanidine thiocyanate and incubated at 60 °C for 5 min. Following the addition of 1.6 ml chloroform, samples were centrifuged and 1 vol. 2-propanol was added to the supernatant. The precipitated RNA was centrifuged, resuspended in formamide and quantified by spectrophotometry. Twenty micrograms total RNA were electrophoresed in a 1% agarose gel containing 7% formaldehyde and transferred to a nylon membrane by capillarity. Radioactively labelled DNA probes for *bfpA*, *eae* and *rpoD* were synthesized by random primer labelling using [³²P]-dCTP. The DNA templates were amplified by PCR using the oligonucleotide pairs bfp-A/bfp-B, eae-A/eae-B and rpoD+/rpoD-. Membranes were hybridized with the labelled probes at 42 °C in hybridization solution (MRC-HS114F) for at least 16 h, washed and exposed to X-ray films.

To hybridize the RNA with *rpoD*, membranes were stripped of the labelled *bfpA* or *eae* probes and rehybridized with a radioactively labelled *rpoD* DNA probe.

Western-blot analysis. Cells grown overnight in LB were diluted in 10 ml DMEM containing 2% FCS to a final OD₅₄₀ of 0.025 and incubated at 37 °C without agitation. After 4 h, the cultures were centrifuged and resuspended in 1 ml PBS. OD₅₄₀ was measured, and 2 × 10⁹ bacteria from each culture were resuspended in 100 µl loading buffer (0.1 M Tris/HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol) and boiled for 3 min. Ten microlitres of each sample was resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences) by capillarity. The membrane was blocked for 1 h in PBS supplemented with 0.05% Tween-20 (PBS-T) and 5% skim milk, and incubated with the monoclonal antibody anti-intimin (diluted 1:500) and the polyclonal antibody anti-BFP (diluted 1:2000) for 1 h. Peroxidase conjugates of anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G (Promega) were used as secondary antibodies. Detection was performed with the SuperSignal West Pico Chemiluminescent system (Pierce), as recommended by the manufacturer.

RESULTS

To test whether the PHO regulon affects EPEC adherence to epithelial cells, mutations in PHO genes that have regulatory functions were transferred to the wild-type EPEC strain LRT9 by P1 transduction. Unlike many natural *E. coli* isolates, LRT9 is relatively sensitive to P1, which facilitates the transfer of gene markers or mutations from *E. coli* K-12 to EPEC strains, although with a considerably lower efficiency. Mutations in *phoB*, *phoR* and a deletion of the entire *pst* operon were transduced to LRT9. To check the phenotype of the transductants, bacteria were grown overnight in medium A under P_i excess (+P_i) or P_i limitation (−P_i) and tested for AP activity (Table 2). As expected, mutations in *pst* and *phoR* resulted in the constitutive expression of AP. The level of AP under P_i-sufficiency was higher in LRT9 Δ*pst* than in LRT9 *phoR*, confirming that mutations in *phoR* result in less AP production than mutations in *pst* (Kreuzer *et al.*, 1975). Plasmids carrying the respective wild-type genes complemented the constitutive phenotype. Two different null mutations in *phoB* (alleles *phoB23* and *phoB519*) abolished the expression of AP even under P_i-limiting conditions and normal levels of AP expression were restored by complementing *in trans* with a wild-type *phoB* gene. In conclusion, mutations introduced into the PHO regulatory genes of LRT9 caused the expected phenotypes.

Effect of PHO mutations on *in vitro* EPEC adherence

To test the effect of the PHO mutations on EPEC adherence, qualitative and quantitative *in vitro* assays with an established epithelial culture (Hep-2 cells) were performed. As happens with typical wild-type EPEC

Table 2. AP level of overnight cultures

Values represent the mean $\pm$ SE of three independent experiments. +P_i, medium A supplemented with 1 mM KH₂PO₄; -P_i, non-supplemented medium A. See Methods for units.

Strain	Genotype	Plasmid	AP activity	
			+P _i	-P _i
LRT9	Wild-type	–	0.01 $\pm$ 0.01	1.76 $\pm$ 0.47
GMF195	Δpst	–	2.97 $\pm$ 0.73	3.09 $\pm$ 0.39
GMF201	Δpst	pGM2 (<i>ppst</i> ⁺)	0.09 $\pm$ 0.11	1.57 $\pm$ 0.32
JC1	<i>phoB519</i>	–	0	0
GMF210	<i>phoB519</i>	pBS1 (<i>pphoBR</i> ⁺)	0.04 $\pm$ 0.04	1.28 $\pm$ 0.35
GMF257	<i>phoR69</i>	–	1.93 $\pm$ 0.31	1.23 $\pm$ 0.30
GMF264	<i>phoR69</i>	pBS1 (<i>pphoBR</i> ⁺)	0.04 $\pm$ 0.04	1.63 $\pm$ 0.15
GMF247	Δpst <i>phoB23</i>	–	0 $\pm$ 0.02	0.07 $\pm$ 0.11

strains, LRT9 adhered in a localized fashion and formed microcolonies on Hep-2 cell surfaces after 3 h of incubation (Fig. 1a). Deletion of the *pst* operon negatively

Fig. 1. Qualitative *in vitro* adherence assay of PHO mutants. Bacteria (5×10^7 c.f.u. ml⁻¹) were added to a monolayer of Hep-2 cells and incubated for 3 h. Following the incubation period, the cell monolayer was washed and stained. (a) EPEC wild-type strain LRT9; (b) GMF195 (LRT9 Δpst); (c) JC1 (LRT9 *phoB519*); (d) GMF195 transformed with pGM2 (*ppst*⁺); (e) GMF247 (Δpst *phoB23*); (f) GMF257 (LRT9 *phoR69*).

affected the adherence of LRT9, as the microcolonies formed by strain GMF195 (LRT9 Δpst) were more scattered and with fewer bacteria adhered (Fig. 1b). For a quantitative assessment of bacterial adherence, the β -galactosidase activity of the adhered bacteria was used as a reporter (Minami *et al.*, 1987). The adherence level of strain GMF195 was more than twofold lower than that of the wild-type strain (Fig. 2, compare bars B and C). C.f.u. ml⁻¹ counting of adhered LRT9 and of its Δpst mutant confirmed the deleterious effect of Δpst on adherence (not shown). Complementation *in trans* of the Δpst mutation with the low-copy plasmid pGM2 (*ppst*⁺) restored the normal adherence phenotype (Figs 1d and 2, D). pGB2 was used as a negative control and, as expected, did not improve the adherence level of GMF195 (Fig. 2, E). A *phoB* null mutation did not affect the adherence pattern of LRT9 (Fig. 1c), and a 20 % increase in the adherence level of the *phoB* mutant was observed, but it was not statistically significant (Fig. 2, F), indicating that the presence of PhoB is not necessary to obtain normal levels of adherence.

Since mutations in the *pst* operon lead to the constitutive transcription of all PHO genes, we next investigated whether the effect of Pst on EPEC adherence was related to the constitutive expression of the PHO genes. To test this, mutant strains LRT9 *phoR* and LRT9 Δpst *phoB* were obtained. We reasoned that if PHO constitutivity elicited by *pst* deletion is responsible for the inhibition of adherence, inactivation of PhoB would alleviate the deleterious effect of PHO constitutivity on bacterial adherence. Likewise, we reasoned that a *phoR* mutation, which also causes the constitutive expression of the PHO genes, would also inhibit EPEC adherence. The double mutant Δpst *phoB23* displayed an adherence level similar to that shown by the Δpst mutant (Figs 1e and 2, G), indicating that although this strain ceased to express PHO constitutively, it still adhered less than the wild-type or the *phoB* mutant, suggesting that *pst* was epistatic to *phoB* in this case. Interestingly, the opposite occurs in the regulation of PHO, i.e. *phoB* is epistatic to *pst*. The *phoR*

Fig. 2. Quantitative adherence assay of PHO mutants. Bacteria (5×10^7 c.f.u. ml^{-1}) grown in the presence of 1 mM IPTG were added to a Hep-2 cell monolayer. After 3 h, the cells were washed to remove non-adherent bacteria, and the activity of β -galactosidase was measured. A value of 100 % was assigned to the adherence level of the wild-type LRT9 strain. Bars represent the means $\pm$ SE of at least three independent experiments. **, Values significantly different ($P < 0.05$) from the parent strain LRT9 by Student's *t*-test. Bars: A, K-12 strain MG1655; B, LRT9; C, GMF195 (LRT9 Δpst); D, GMF195 transformed with *pGM2* (*ppst*⁺); E, GMF195 transformed with *pGB2* (cloning vector); F, JC1 (LRT9 *phoB519*); G, GMF247 (Δpst *phoB23*); H, GMF257 (LRT9 *phoR69*).

mutation did not significantly affect the adherence of LRT9 (Figs 1f and 2, H). Taken together, these results suggest that the deleterious effect of the Δpst mutation on EPEC adherence is not due to the constitutive expression of PHO.

It might be argued that the negative effect of Δpst on EPEC adherence is caused by an artefact due to a decrease in bacterial growth rate or by an unspecific inhibition of endogenous β -galactosidase expression. To address these concerns, the kinetics of β -galactosidase expression and the growth patterns of strains LRT9, GMF195 (LRT9 Δpst) and GMF195 transformed with *pGM2* (*ppst*⁺) were determined (Fig. 3). Bacteria were suspended in DMEM supplemented with 2 % FCS and 1 mM IPTG at an initial concentration of 5×10^7 cells ml^{-1} and grown without agitation for 9 h at 37 °C. Samples were harvested every hour and assayed for β -galactosidase activity and growth. All three strains grew exponentially with a very similar growth rate for the first 5–6 h (up to $\text{OD}_{540} \sim 0.7$), when they reached the stationary phase (Fig. 3b). It could also be argued that the presence of *pst* would be advantageous under these conditions because a *pst*⁺ strain would take up P_i more efficiently. However, DMEM is rich in P_i (0.9 mM), which enables maximal growth rate and yield even in the absence of the high-affinity P_i transport system Pst. The entry of the cells into the stationary phase after 5 h of growth ($\text{OD}_{540} \sim 0.8$) was probably caused by O_2 depletion and by the presence of 1 mM IPTG, which limited bacterial growth.

The β -galactosidase activity of all three strains reached a plateau at 3 h (Fig. 3a). At this point the strain bearing *pGM2* showed a slightly higher activity that slowly decreased from that point on. Overall, the growth pattern and β -galactosidase activity of all three strains was very similar and it can thus be concluded that the negative effect

of Δpst on adherence is not due to a reduction in growth rate or in β -galactosidase expression.

Fig. 3. Endogenous β -galactosidase activity and growth curve of LRT9 and its *pst* mutant. Bacteria were grown in DMEM supplemented with 2 % FCS and 1 mM IPTG for 9 h. Samples were harvested every hour and assayed for (a) β -galactosidase activity and (b) growth. ■, LRT9; ▲, GMF195 (LRT9 Δpst); ▼, GMF201 (*pGM2* in GMF195). Experiments were repeated twice, and representative results are presented.

The *pst* operon positively regulates the expression of *bfp*, *eae* and *per*

EPEC adherence depends on the expression of at least two different adhesins, BFP and intimin. Deletion of *pst* might affect EPEC adherence by inhibiting the expression of one of these adhesins. To test this hypothesis, Northern- and Western-blot analyses were conducted. Cells were grown in DMEM and harvested at the mid-exponential phase. Total RNA was extracted and hybridized with DNA probes corresponding to the first gene of the *bfp* operon, *bfpA*. The *bfpA* probe hybridized with a 0.6 kb band corresponding to the transcript of *bfpA* alone (Fig. 4a). Interestingly,

Fig. 4. Effect of *pst* on the expression of *bfpA* and BFP. (a) Northern-blot analysis of *bfpA*. RNA was extracted from mid-exponential-phase cultures of strains LRT9, GMF195 (LRT9 Δ pst), GMF201 (pGM2 in GMF195) and GMF247 (Δ pst *phoB*23) grown in DMEM and hybridized with a labelled *bfpA* DNA probe. Densitometric analyses of the *bfpA* band intensities normalized against the mRNA of *rpoD* are shown in the graphs. Each bar corresponds to the mean $\pm$ SE of five independent experiments, except for GMF247, which corresponds to the mean $\pm$ SE of three independent experiments. (b) Western-blot of BFP. Cells were grown as above and total protein extracts were assayed for BFP using a polyclonal anti-BFP serum. The bars represent the mean $\pm$ SE of densitometric analysis of four independent experiments. The pictures of the blots are of typical experiments. **, Significantly different from the parent strain LRT9 by Student's *t*-test ($P < 0.05$).

although the *bfp* operon is composed of 14 genes, bands corresponding to transcripts containing the distal genes of this operon could not be observed under normal conditions. Overexposed autoradiograms, however, revealed the presence of large bands (data not shown). The presence of a stem-loop structure [$\Delta G -16.9$ kcal mol⁻¹ (-70.7 kJ mol⁻¹)] in the intergenic region between *bfpA* and *bfpG*, might be acting as an mRNA stabilizer of the *bfpA* transcript or as a transcription terminator. This would explain why the mRNA of *bfpA* appears to be in excess in relation to the other cognate operon gene transcripts. The blot was rehybridized with a DNA probe corresponding to the housekeeping gene *rpoD* to normalize the RNA present in each sample. The densitometric analysis of the band intensities showed there was, on average, a twofold reduction in *bfpA* mRNA levels in the Δ pst mutant when compared to the wild-type strain, and that this reduced level of *bfpA* transcript was also observed in the Δ pst *phoB* double mutant (Fig. 4a). When the mutant was transformed with the low-copy plasmid pGM2 (*ppst*⁺), the level of *bfpA* mRNA was restored and even slightly increased, probably due to the presence of several copies of *pst*⁺. Similar results were observed when the production of the BFP protein was assessed by Western blotting (Fig. 4b). There was a 1.7-fold decrease in both Δ pst and Δ pst *phoB* relative to the wild-type strain, which was restored by complementation *in trans* with pGM2. The fact that the Δ pst *phoB* double mutant was not able to restore expression of BFP at both transcript and protein level suggests again that the constitutivity of PHO caused by the *pst* deletion is not responsible for the inhibition of *bfp* expression.

Fig. 5(a) shows that a DNA probe corresponding to *eae*, which encodes intimin, hybridized to a 3 kb band, as described by Gómez-Duarte & Kaper (1995), although minor bands of 2.8 and 5.2 kb, corresponding to *cesT-eae* and to the entire *tir-cesT-eae* operon were also observed. The blots were rehybridized with *rpoD* as above and the densitometric analysis of the band intensities showed there was a 2.5-fold reduction in the level of Δ pst *eae* mRNA. pGM2 (*ppst*⁺) restored the level of *eae* mRNA. The Western blot analysis of intimin revealed that the *pst* deletion caused a 1.5-fold decrease in intimin level (Fig. 5b). These results indicate that *pst* is required for the full expression of *bfpA* and *eae*, and consequently for the maximization of synthesis of BFP and intimin.

PerA and PerC are positive regulators of the *bfp* and LEE1 operons, respectively (Mellies *et al.*, 1999; Tobe *et al.*, 1996). LEE1 contains *ler*, which activates *eae*. *perA-perB-perC* form an operon on the pEAF plasmid. Several attempts to measure the effect of Δ pst on *per* expression through Northern-blot analysis failed, because no signal could be observed (data not shown). This suggests that *perA* is transcribed at a very low level, and its mRNA cannot be detected with a moderately sensitive technique, such as Northern hybridization.

Fig. 5. Effect of *pst* on the expression of *eae* and intimin. (a) Northern-blot analysis of *eae*. RNA was extracted from mid-exponential cultures of strains LRT9, GMF195 (LRT9 Δpst) and GMF201 (pGM2 in GMF195) grown in DMEM and hybridized with a labelled *eae* DNA probe. Densitometric analysis of the *eae* band intensities normalized against the mRNA of *rpoD* are shown in the graphs. Each bar corresponds to the mean $\pm$ SE of five independent experiments (b) Western blot of intimin. Cells were grown as above and total protein extracts were assayed for intimin using a monoclonal anti-intimin serum. The bars represent the mean $\pm$ SE of densitometric analysis of four independent experiments. The pictures of the blots are of typical experiments. **, Significantly different from the parent strain LRT9 by Student's *t*-test ($P < 0.05$).

To measure the effect of Δpst on *per* expression, a transcriptional fusion between the promoter of *perA* and *lacZ* was constructed (pGM17) and transformed into strains LRT9, GMF195 (LRT9 Δpst), GMF201 [GMF195 bearing pGM2 (*ppst*⁺)] and JPN15 (EPEC cured of pEAF) as a negative control. Because GMF247 (LRT9 Δpst *phoB*) is resistant to tetracycline, a spectinomycin-resistance cassette was introduced into pGM17, resulting in plasmid pGM29. This plasmid, which behaves very similarly to pGM17 (not shown), was transformed into GMF247. All transformants were grown overnight and resuspended in DMEM supplemented with 2 % FCS and 0.4 % glucose (to repress endogenous β -galactosidase expression) to an

OD₅₄₀ of 0.025, and grown for 9 h without agitation. Growth of all strains was similar; only strain JPN15 (EPEC cured of pEAF and negative control) showed a slightly higher growth rate (Fig. 6b). Samples were taken every hour and assayed for β -galactosidase activity (Fig. 6a). With the exception of JPN15, all strains displayed a gradual increase in β -galactosidase, which reached its peak at the height of the exponential phase (around 6 h), followed by a decrease until a new plateau was reached. At its highest point, the β -galactosidase activity of LRT9 was almost twice as high as that of the Δpst mutant and 2.5-fold above the β -galactosidase level of Δpst *phoB*. This demonstrates that the deleterious effect of Δpst on *per* expression cannot be alleviated by abolishing PHO constitutivity. Introduction of pGM2 (*ppst*⁺) into LRT9 Δpst led to a 70 % increase in the level of β -galactosidase compared to the wild-type strain, suggesting that *pst* has a positive role in the induction of the *per* operon. Since PerA is a positive regulator of *bfp*, *pst* is likely to affect *bfp* expression via *perA*. Analogously, an augmentation in PerC would positively affect the expression of *ler*, which in turn increases the transcription of *eae*. Therefore, the positive effect of *pst* on *eae* expression is likely to be through an increase in *perC* transcription.

Fig. 6. Effect of Δpst on *per* expression. Plasmid pGM17 (*perA-lacZ* fusion) was transformed into LRT9 (■), GMF195 (LRT9 Δpst) (▲), GMF201 (pGM2 in LRT9 Δpst) (▼), and JPN15 (pEAF-cured EPEC) (◆). GMF247 (Δpst *phoB23*) was transformed with plasmid pGM29 (○). Cells were grown in DMEM for 9 h. Samples were taken every hour and assayed for (a) β -galactosidase and (b) growth. Experiments were repeated three times, and typical results are presented.

The *pst* operon is partially induced in DMEM

Genes of the PHO regulon are activated by P_i -shortage. Under P_i -excess conditions, expression of Pst and other PHO genes is only basal (Wanner, 1996). We asked whether a low (basal) expression of Pst would be sufficient to affect the expression of the adhesins, or if under the growth conditions used in the adherence assay (DMEM) the actual level of Pst is increased. To answer this question, the activity of the *pst* promoter was measured under different growth conditions. Plasmid pBS11 (*pstS-cat* transcriptional fusion) was transformed into LRT9 and assayed for CAT and AP activity in bacteria grown exponentially in LB, medium A+ P_i and DMEM alone or supplemented with FCS and 1 mM P_i . Table 3 shows that cells grown under P_i -limiting conditions (medium A- P_i) presented an 11-fold induction in the level of *pst* expression when compared to cells grown in medium A+ P_i or in LB. In contrast, the CAT activity in DMEM+2% FCS (the medium used in the adherence assays) was only fivefold lower than under P_i -starvation conditions (medium A- P_i) and twofold higher than the activity of cells grown in other high- P_i media (medium A+ P_i or LB). Addition of 1 mM P_i to DMEM did not significantly alter the level of *pst* expression, suggesting that the increased *pst* expression in DMEM is not due to a shortage of P_i . Likewise, addition of 10% FCS had no effect on the ability of DMEM to increase *pst* transcription. These results suggest that one or more components of DMEM positively affect *pst* expression. This effect is specific to *pst* because the basal level of another PHO protein, AP, was not affected by DMEM. In conclusion, expression of *pst* under the growth conditions used in the adherence assay was significantly higher for P_i -replete media. The relatively high level of Pst is likely to contribute to its positive effect on the expression of EPEC adhesins.

DISCUSSION

EPEC is characterized by its pattern of localized (primary) and intimate adherence, which are its main virulence

Table 3. CAT and AP activities of strains carrying the *pst-cat* fusion in various growth media

Values represent the mean $\pm$ SE of at least three independent experiments. See Methods for units.

Growth medium	CAT activity	AP activity
LB	2.32 ($\pm$ 0.88)	0.01 ($\pm$ 0.01)
Medium A+ P_i	1.98 ($\pm$ 0.5)	0.02 ($\pm$ 0.01)
Medium A- P_i	22.1 ($\pm$ 9.05)	2.29 ($\pm$ 0.71)
DMEM	8.06 ($\pm$ 3.46)*	0.001 ($\pm$ 0.001)
DMEM+2% FCS	4.3 ($\pm$ 0.3)*	0.01 ($\pm$ 0.01)
DMEM+2% FCS+ P_i	5.58 ($\pm$ 2.08)*	0.001 ($\pm$ 0.001)
DMEM+10% FCS	4.38 ($\pm$ 1.29)*	0.01 ($\pm$ 0.001)

*Values that are statistically significantly different from medium A+ P_i or LB according to Student's *t*-test, with $P < 0.05$.

factors, and by the lack of Shiga-like toxin. EPEC adheres to epithelial cells in a two-step fashion: the first step is dependent on BFP; followed by the intimate adherence which is dependent on intimin. Environmental factors contribute to the regulation of virulence-related genes in EPEC. For instance, a temperature of 37 °C, the presence of calcium ions and low ammonium concentrations are paramount to the development of the LA phenotype and to *bfp* transcription (Puentes *et al.*, 1996). Also, *bfp* expression is growth phase dependent, being transcribed predominantly during the exponential phase. Similarly, the A/E phenotype is also dependent on temperature and growth phase (Rosenshine *et al.*, 1996).

Some PHO regulon genes, particularly the *pst* operon and *phoB*, have been shown to be involved in different aspects of the virulence of *E. coli* and other bacteria. Among pathogenic *E. coli*, point mutations in *phoU*, *pstC* and a *pst* deletion reduced the virulence of different extra-intestinal strains (Buckles *et al.*, 2006; Daigle *et al.*, 1995; Lamarche *et al.*, 2005). Insertions in the *pstS* gene of a porcine EPEC strain resulted in reduced attachment of the bacteria to piglet ileal explants (Batisson *et al.*, 2003). In *Shigella flexneri*, a *pst* mutant displayed a significant reduction in plaque formation, which was suppressed by a mutation in *phoB* and also by the introduction of a non-constitutive *pstA* mutation (Runyen-Janecky *et al.*, 2005). Restoration of normal levels of plaque formation by suppression of the PHO-constitutive phenotype implied that at least in this case, the constitutive expression of the PHO genes was the cause of the inhibition of *S. flexneri* virulence. On the other hand, it was suggested that the lack of a functional Pst in the avian pathogenic *E. coli* O78 reduced its virulence due to changes in cell surface composition (Lamarche *et al.*, 2005). However, in most cases, the exact mechanism through which *pst* enhances pathogenicity remains unclear.

In the present study, we showed that a complete deletion of *pst* caused a reduction in the ability of EPEC to adhere to epithelial cells *in vitro* and to display the LA phenotype. Moreover, the Pst system was required for the full expression of the adhesins BFP and intimin, and of the regulators encoded by the *per* operon. It was also shown that the *pst* operon itself is slightly induced under the conditions of the adherence assay. Unlike in *S. flexneri* (Runyen-Janecky *et al.*, 2005), the constitutivity of the PHO genes caused by Δ *pst* was not responsible for its deleterious effect on EPEC virulence, because introduction of a *phoB* mutation into LRT9 Δ *pst* eliminated PHO constitutivity, but did not restore bacterial adherence to its original levels. Furthermore, a *phoR* mutant, which also constitutively expresses the PHO genes, did not inhibit bacterial adherence. Also, a *pstS* polar mutant of LRT9 inhibited *bfp* expression to the same extent as Δ *pst*, but a non-polar mutation in *pstS* (which is also PHO-constitutive) had no effect on *bfp* (data not shown), confirming the lack of effect of PHO constitutivity on cell adherence.

LRT9 and its Δpst mutant displayed a similar growth rate in DMEM, indicating that the reduction in adherence was not due to a growth disadvantage of the Δpst mutant. Measurement of the intrinsic β -galactosidase activities of the wild-type and Δpst mutant showed that they were very similar. This experiment was important to show that the quantitative method employed to determine bacterial adherence (i.e. endogenous β -galactosidase activity) was not influenced by some unrelated variation in the β -galactosidase expression pattern caused by the pst mutation. Although less common than determining c.f.u. ml⁻¹, the use of β -galactosidase as a reporter of EPEC adherence has already been shown to be reliable and reproducible (Minami *et al.*, 1987). Here, we confirmed the reliability of this methodology using a different EPEC lineage (LRT9) and also by comparing the adherence levels obtained with the β -galactosidase assays with c.f.u. ml⁻¹ calculations of the wild-type and the Δpst mutant, and by microscopic examination of the adhered bacteria.

Northern-blot analysis of *bfp* and *eae*, Western blots of BFP and intimin, and the enzymic assays of the *perA-lacZ* fusion showed that *pst* plays a positive role in the expression of the *bfp*, *tir-cesT-eae* and *per* operons. Since PerA and PerC positively regulate *bfp* and *eae*, respectively (Tobe *et al.*, 1996; Mellies *et al.*, 1999), it seems plausible to suggest that the mechanism by which *pst* enhances transcription of these genes is via induction of *per*, whose products in turn upregulate *bfp* and *eae*.

A possible source of concern was the fact that the adherence assay was performed under conditions of P_i abundance and that the level of *pst* expression under these conditions would be too low to exert any significant effect. However, measurement of *pst* transcription level in DMEM showed that it was twofold higher than that observed for a high-P_i medium such as LB or medium A + P_i. This implies that in DMEM, expression of *pst* is higher than normal and more likely to have a broader effect, for instance as an adherence enhancer, as observed here. The possibility that some component of FCS, which supplements DMEM, is responsible for the increased expression of *pst* was ruled out, because the level of *pst* was even higher in non-supplemented DMEM. The nature of the *pst*-inducing factor in DMEM remains to be investigated.

It has recently been shown that a short RNA sequence in the intergenic region between *pstA* and *pstB* has a regulatory role in σ^S translation (Schurdell *et al.*, 2007). It is not known whether *rpoS* has any effect on EPEC adherence, but in enterohaemorrhagic *E. coli* (EHEC), *rpoS* activates the expression of LEE via DsrA (Laaberki *et al.*, 2006). It is possible that the regulatory region between *pstA* and *pstB* plays a role in *per* transcription via *rpoS* or through another mechanism. The *pstA-pstB* intergenic region of EPEC E2348/69 carries a deletion of 92 bp (from position 3247 to 3338 on the ECOPHOS sequence K01992), but the *rpoS* regulatory sequence (which comprises the 3352–3383 region) is intact in this strain.

At this stage, it is still unclear if the entire *pst* operon or just one of its genes is involved in the regulation of EPEC adherence. A polar *pstS* mutant presented a reduction in the adherence level similar to the one observed for Δpst , while a non-polar mutation in this gene did not affect the level of adherence (not shown). To test the involvement of individual *pst* genes in adherence, single *pst* mutations (polar and non-polar) should be isolated and tested.

In conclusion, this study adds to a number of reports showing that the *pst* operon participates in the mechanism of *E. coli* virulence. This function could not be previously anticipated judging from the main roles of Pst as a P_i transport system and as a negative regulator of PHO. However, it has been recently reported that Pst also plays a role in the regulation of RpoS (Schurdell *et al.*, 2007) and in the phenomenon of bacterial persistence (Li & Zhang, 2007). It is becoming evident that Pst has a broader regulatory impact as a global (though not principal) regulator of pathogenicity in different bacterial species and even as a more general regulator beyond its role in P_i metabolism.

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