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Environmental phosphate differentially affects virulence phenotypes of uropathogenic *Escherichia coli* isolates causative of prostatitis

M Grillo-Puertas¹, MG Martínez-Zamora¹, MR Rintoul¹, SM Soto², and VA Rapisarda^{1,*}

¹Instituto Superior de Investigaciones Biológicas, INSIBIO (CONICET-UNT) and Instituto de Química Biológica “Dr Bernabé Bloj”, Facultad de Bioquímica, Química y Farmacia (UNT); Tucumán, Argentina; ²Barcelona Center for International Health Research (CRESIB; Hospital Clinic-University of Barcelona); Barcelona, Spain

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K-12 *Escherichia coli* cells grown in static media containing a critical phosphate (Pi) concentration ≥ 25 mM maintained a high polyphosphate (polyP) level in stationary phase, impairing biofilm formation, a phenomenon that is triggered by polyP degradation. Pi concentration in human urine fluctuates according to health state. Here, the influence of environmental Pi concentration on the occurrence of virulence traits in uropathogenic *E. coli* (UPEC) isolated from acute prostatitis patients was evaluated. After a first screening, 3 isolates were selected according to differential biofilm formation profiles depending on media Pi concentration. For each isolate, biofilm positive and negative conditions were established. Regardless of the isolate, biofilm formation capacity was accompanied with curli and cellulose production and expression of some key virulence factors associated with adhesion. When the selected isolates were grown in their non-biofilm-forming condition, low concentrations of nalidixic acid and ciprofloxacin induced biofilm formation. Interestingly, similar to laboratory strains, polyP degradation induced biofilm formation in the selected isolates. Data demonstrated the complexity of UPEC responses to environmental Pi and the importance of polyP metabolism in the virulence of clinical isolates.

INTRODUCTION

Pathogenic *Escherichia coli* comprise a diverse group of strains associated with both intestinal and extraintestinal infections.¹ Uropathogenic *E. coli* (UPEC) is the predominant causative agent of urinary tract infections (UTIs), representing up to 85% of community acquired UTIs.² In comparison to commensal strains, UPEC has several virulence factors that allow it to colonize host mucosal surfaces, being important for establishing infection; these include adhesins, toxins, iron acquisition systems, and capsular antigens.³ Biofilm formation may be considered another pathogenic determinant, which allows these strains to persist a long time in the genito-urinary tract and interfere with bacterial eradication by host defense mechanisms and antibiotics.^{4,5}

Curli fimbriae and/or cellulose are produced in *E. coli*, *Salmonella* spp. and other Enterobacteriaceae influencing the adherence properties of several biofilm-forming microorganisms.^{6–10} As a virulence factor associated to biofilm formation, flagella, allows fixation of bacteria to epithelial cells.¹¹ In pathogenic bacteria such as *Salmonella* spp, *E. coli*, *Bordetella bronchiseptica* y *Bordetella pertussis*, it has been demonstrated that motility plays an essential role, mainly in initial phase of infection.^{12,13}

Inorganic polyphosphate (polyP) was shown to be critical for attributes such as motility, quorum sensing, biofilm

formation, resistance to oxidative, osmotic, heat, nutritional and alkaline stress, and stationary-phase survival in several microorganisms.^{14–23} PolyP is a linear chain of tens to many hundreds of phosphate residues linked by high-energy phosphoanhydride bonds.^{14, 24} PolyP is usually accumulated during exponential phase of growth, and degraded at the beginning of stationary phase.¹⁵ Previous reports from our laboratory shown that *E. coli* cells grown in static media containing a critical phosphate (Pi) concentration ≥ 25 mM maintained a high polyP level in stationary phase.²⁵ This characteristic was related to the impairment in biofilm formation, since polyP degradation is a cellular mechanism necessary to trigger this phenomenon.²⁵

Human urine Pi concentration ranges are between 0.02–2 mM, although values higher than 2 mM could be found depending on eating habits, age, muscle mass, renal function, and other factors.²⁶ Hyperphosphaturia, an excess of phosphate in urine, was described under several health disorders, such as cases of multiple myeloma, kidney diseases or excess of vitamin D in the body, among others.^{27–33} Depending on the clinical case, Pi concentrations in urine from 20 to 50 mM were found.

In the present study, the capacity to form biofilm of clinical UPEC isolates from acute prostatitis patients was analyzed in response to the media Pi concentration. Also, the relationship

*Correspondence to: Viviana A Rapisarda; Email: vrapisarda@fbqf.unt.edu.ar

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between *in vitro* biofilm formation and expression/production of some virulence traits was determined.

RESULTS

Biofilm formation of uropathogenic *E. coli* isolates

A total of 55 UPEC strains causing acute prostatitis were isolated from 44 patients and analyzed for biofilm formation in media containing different Pi concentrations (2 to 60 mM). A total of 38 isolates were positive for *in vitro* biofilm formation. Out of these isolates, 16 isolates formed biofilm independently of media Pi concentration. However, 11 isolates increased biofilm formation with increasing Pi concentrations, 7 presented maximum biofilm formation in media containing 20 mM Pi, and 4 decreased biofilm formation with increasing Pi concentrations.

Considering previously reported data about Pi influence on biofilm formation capacity in laboratory strains, the present study was focused in those isolates in which biofilm formation capacity varied with Pi concentration. Thus, 3 isolates, named MGP16, MGP12 and MGP45, were selected according to differential biofilm formation profiles depending on media Pi concentration (Fig. 1). For MGP16, a decrease in biofilm formation was observed when Pi concentration increased, conversely to the behavior of MGP45 isolate. For MGP12, biofilm formation reached a maximum at 20 mM Pi decreasing at higher concentrations. For further studies, two Pi concentrations corresponding to biofilm positive (BF+) and biofilm negative (BF-) conditions were established for each of the 3 selected isolates (see Table 1), according to results in Figure 1.

Biofilm structure and related phenotypes

To evaluate biofilm structures formed by each isolate, confocal laser scanning microscopy (CLSM) and scanning electronic microscopy (SEM) microscopies were carried out. CLSM analysis allowed the reconstruction of 3D-images of biofilms from

Table 1. Pi concentrations established to each biofilm formation conditions

Isolate	Pi concentration (mM)	BF condition
MGP16	2	+
	60	-
MGP12	20	+
	60	-
MGP45	2	-
	60	+

MGP45 and MGP12 strains (Fig. 2A). In BF+ condition, MGP45 produced a biofilm thicker than the one from MGP12. For both strains, projections could be distinguished on the biofilm surfaces, giving them the appearance of a “Christmas tree forest.” Figure 2B (left panels) shows SEM images of the 3 isolates in BF+ conditions. In general, cells had sizes ranging between 1.2 and 2 μm in length. MGP16 and MGP12 showed similar biofilm phenotypes with abundant extracellular material, presenting the latter higher number of cells attached to the glass surface. At higher magnification, cells exhibited smooth or slightly rough surfaces, a great amount of extracellular vesicular material, and potential conjugating structures (arrows in inserts i and ii of Fig. 2B). In MGP45 biofilm, formation of protrusions that rise to the surface was observed (arrows). Using higher magnification, the presence of extracellular matrix was observed (arrow in insert iii). In addition, a peculiarity identified in this isolate was the presence of chains of bacilli as larger as 7 to 8 μm forming a nest in which most of the cells presented no septum (insert iv of Fig. 2B). Using CLSM and SEM, no structure was visualized in BF- conditions (right panels in Fig. 2A and B).

For further characterization, curli, cellulose and motility were analyzed as aspect related to the biofilm formation in the selected *E. coli* isolates. On Congo red plates (Fig. 3A), the 3 isolates presented violet colonies in BF+ conditions, indicating *rdar* morpho-type which is characterized by curli fiber and cellulose production. In BF- conditions, isolates grew as pink colonies, indicative of *pdar* morphotype, characteristic of cells expressing only cellulose. Indeed, production of cellulose, cellulose-like, or extracellular material was observed in both BF+ and BF- conditions, given by a strong Calcofluor White fluorescence in biofilms and unattached single cells, respectively (Fig. 3B). All isolates presented swimming motility in BF+ conditions (Fig. 3C). It is noteworthy that MGP12 and MGP45 presented an exacerbated motility in BF- conditions and, conversely, no motility was observed for MGP16 in BF- (Fig. 3C).

Another isolate of each group was selected to determine curli and motility phenotypes (supplementary Fig. S1 A and B, top panels). The newly analyzed 3 isolates were MGP42 forming biofilm similarly to MGP16, MGP18 to

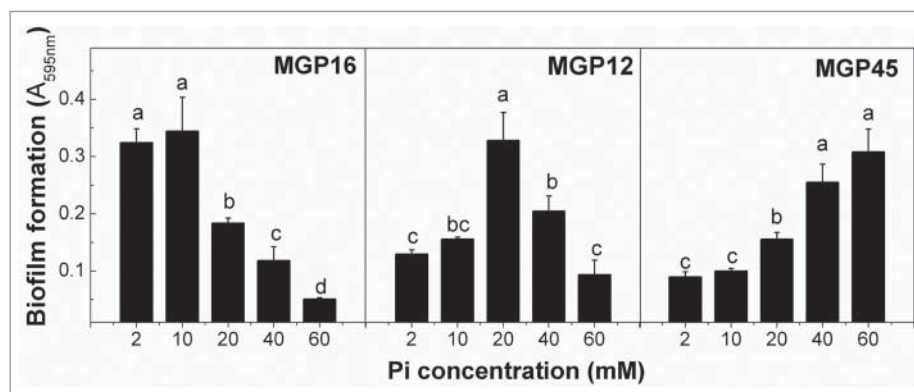


Figure 1. Biofilm formation in different Pi concentration media. Selected isolates (MGP16, MGP12, and MGP45) were grown in static conditions for 48 h in M63 medium modified with the indicated Pi concentrations. Biofilm formation was quantified by cristal violet technique. Data represent the mean \pm SD of at least 3 independent experiments. Different letters indicate significant differences according to Tukey's test with a p -value \geq 0.05.

MGP12 and MGP04 to MGP45. MGP42, MGP18 and MGP04 presents similar curli phenotypes than isolates from the corresponding group described above.

Virulence factor expression

To assess whether selected known virulence genes (including adhesin-related encoding genes (*fimB*, *fimA*, *papA*, *agn-43*), siderophore-related genes (*iroN*, *iutA*), and toxin-encoding genes (*cnf-1*, *hlyA*) were present in the 3 isolates, PCR was used. *fimB*, *agn-43*, *iroN*, *iutA*, and *hlyA*, were detected in MGP16, MGP45 and MGP12, while *fimA*, *papA*, and *cnf-1* were not found in any of the selected strains. Differential expression of genes related to each virulence trait, e.g. *fimB*, *iroN*, and *hlyA*, were determined by q-PCR in BF+ conditions relative to BF-conditions. In all isolates, *fimB* and *iroN* were induced, while *hlyA* was repressed (Fig. 4).

Antibiotic susceptibility

Nalidixic acid and ciprofloxacin susceptibility of the selected isolates was determined as MIC values. MGP12 and MGP16 isolates were found to be resistant up to 200 $\mu\text{g ml}^{-1}$. For MGP45, MICs for ciprofloxacin and nalidixic acid were 1 and 30 $\mu\text{g ml}^{-1}$, respectively. Additionally, the effect on biofilm formation of nalidixic acid added at zero time was tested in MGP12 and MGP45 isolates (Fig. 5). Biofilm formation was inhibited in BF+ conditions by high antibiotic concentration for both isolates, mainly in MGP45. In BF- conditions, low concentrations of antibiotic strongly induced biofilm formation.

Modulation of polyP levels related to biofilm formation

To analyze whether intracellular polyP was implicated in the biofilm formation phenotype of the selected isolates, polyP levels were measured in cells grown in BF+ and BF- conditions at 30°C during 48 h. Regarding the isolates grown in the BF+ conditions, polyP was synthesized and subsequently degraded in all cases (Fig. 6 A, left panels). Peaks of polyP levels were achieved between 6–10 h of growth for MGP16 isolate and in about

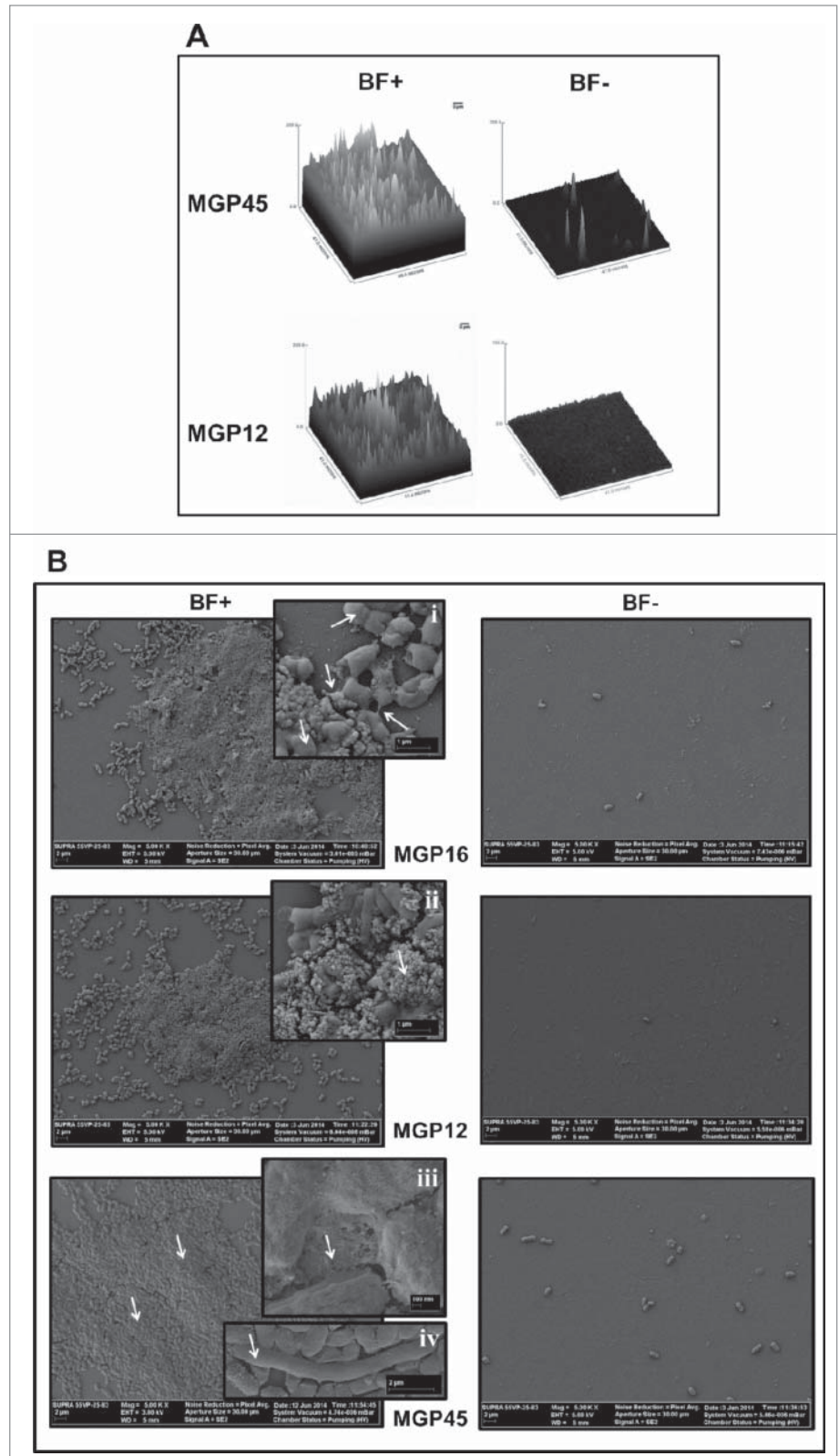


Figure 2. Microscopy analysis of *E. coli* isolates. Biofilm formation of the indicated *E. coli* isolates grown in static conditions for 48 h in M63 medium in BF+ and BF- conditions was analyzed (A) by CLSM and represented by 3-dimensional reconstruction of biofilm image or (B) by SEM microscopy, magnification: 5,000x. Inserts i and ii, 30,000x; insert iii, 80,000x; insert iv, 20,000x. Data are representative from 2 independent experiments.

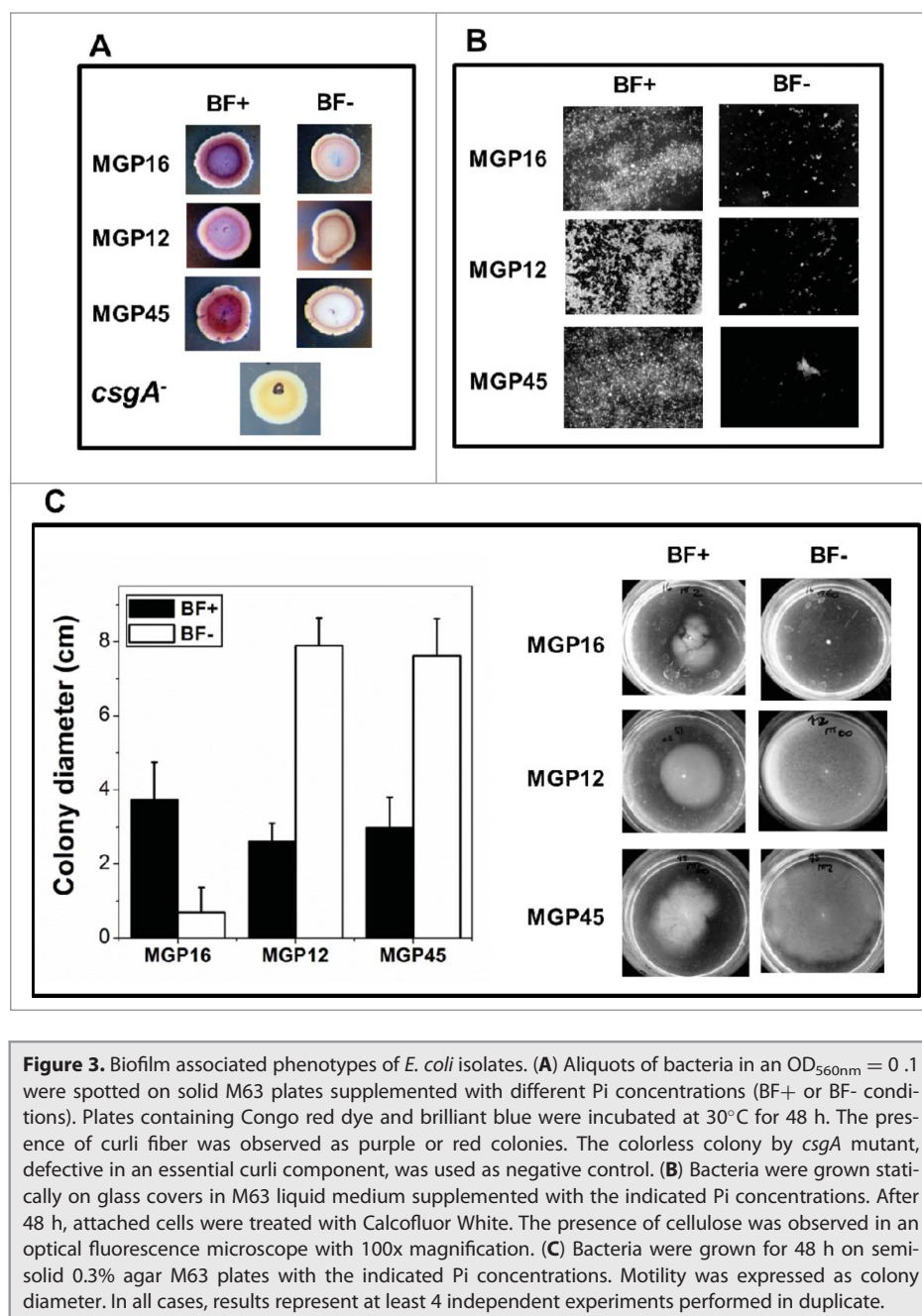


Figure 3. Biofilm associated phenotypes of *E. coli* isolates. (A) Aliquots of bacteria in an $OD_{560nm} = 0.1$ were spotted on solid M63 plates supplemented with different Pi concentrations (BF+ or BF- conditions). Plates containing Congo red dye and brilliant blue were incubated at 30°C for 48 h. The presence of curli fiber was observed as purple or red colonies. The colorless colony by *csgA* mutant, defective in an essential curli component, was used as negative control. (B) Bacteria were grown statically on glass covers in M63 liquid medium supplemented with the indicated Pi concentrations. After 48 h, attached cells were treated with Calcofluor White. The presence of cellulose was observed in an optical fluorescence microscope with 100x magnification. (C) Bacteria were grown for 48 h on semi-solid 0.3% agar M63 plates with the indicated Pi concentrations. Motility was expressed as colony diameter. In all cases, results represent at least 4 independent experiments performed in duplicate.

2 h for MGP12 and MGP45. It should be noted that in BF- conditions, isolates MGP12 and MGP45 were deficient in polyP synthesis during the entire growth curve and MGP16 was unable to degrade the polymer in stationary phase (Fig. 6 A, left panels). To further analyze the involvement of polyP metabolism in biofilm formation, polyP levels (Fig 6A, middle and right panels) and biofilm formation (Fig. 6 B) were assayed in double mutants MGP12*ppkppx* and MGP45*ppkppx* and in the corresponding complemented strains (*ppk*⁺). Regarding the polymer levels, both *ppkppx* mutants were unable to reach the peak of polyP characteristic of parental strains in BF+ conditions (Fig 6A, middle panels). However, complemented strains were unable to

degrade the polymer, showing elevated polyP levels in the entire growth curve in all tested conditions (Fig 6A, right panels). Note that all mutant strains did not show biofilm formation as the parental isolates (Fig 6B). As expected, polyP degradation was observed in MGP42, MGP18 and MGP04 isolates in biofilm forming conditions (Fig. S1C).

DISCUSSION

Here, a high variability among the capacities to form biofilms *in vitro* of UPEC isolated from prostatitis patients was observed, in agreement with previous studies with other pathogenic *E. coli* strains.^{34–37} Remarkably, the ability of UPEC isolates to form biofilms was shown to be different according to medium Pi concentration.

Considering differential biofilm formation phenotype related to media Pi concentration, characterization of virulence aspects were carried out. Selected isolates were found to form prominent biofilms, presenting extracellular matrix or vesicular material. High quantities of extracellular material such as outer membrane vesicles are common among clinical isolates due to the necessity to secrete virulence effectors to attack the host.³⁸ In BF+ conditions, selected isolates presented *rdar* (red, dry and rough) morphotype, as reported by Römling et al.³⁹ This phenotype includes adhesion to abiotic surfaces (biofilm formation) and expression of curli fimbriae and cellulose as extracellular matrix components.³⁵ Thus, the capacity of these strains to form biofilms at solid-liquid interfaces would be

a consequence of curli and cellulose production. Similarly, it was previously reported that expression of curli fimbriae and cellulose (*rdar* morphotypes) determine the medium or high biofilm-forming capacity of UTI isolates under at least one of the growth conditions tested.³⁵ On the other hand, the cellulose production found in BF- conditions is in agreement with the fact that cellulose is associated with both single and biofilm forming cells.^{40, 41} Additionally, motility seems to be required for biofilm formation in the selected isolates. It is worth to note that MGP12 and MGP45 showed an exacerbated motility in BF- condition, which may lead to a reversible attachment of bacteria to the surface, as previously described by Dunne.⁴² However, variability in respect

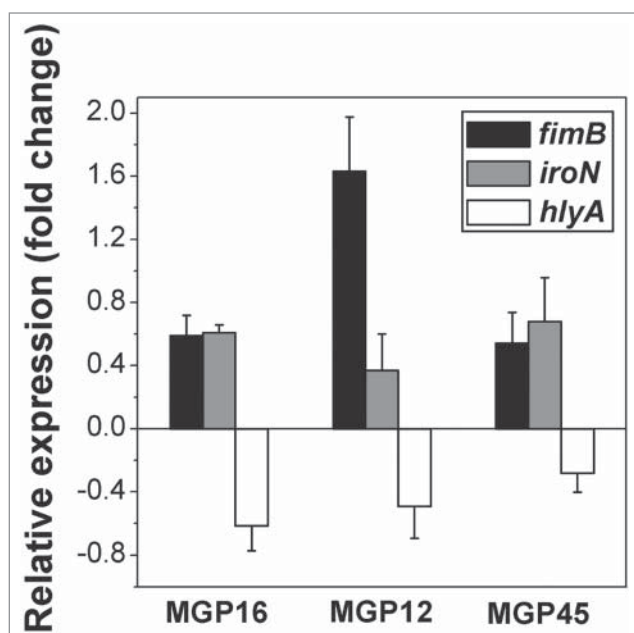


Figure 4. Relative expression of virulence factors related to biofilm formation. Expression of *fimB*, *iroN* and *hlyA* genes of the selected isolates was determined by q-PCR. Values are depicted as x-fold of up- or down-regulation of BF+ conditions compared to BF- conditions. Data represent the mean \pm SD of at least 2 independent experiments performed in triplicate.

to motility results within the isolates and their mutants among conditions (Fig. S1 B) suggest that this feature is not directly related to biofilm phenotype.

In *E. coli*, initiation, attachment and subsequent maturation of biofilm requires the expression of a set of genes, encoding a variety of virulence factors such as haemolysin, fimbriae, secreted proteins, capsules, and iron-acquisition systems, among others.^{43,44} Consistently, *iutA* (not shown), *fimB* and *iroN* genes were induced in BF+ conditions in the selected isolates.

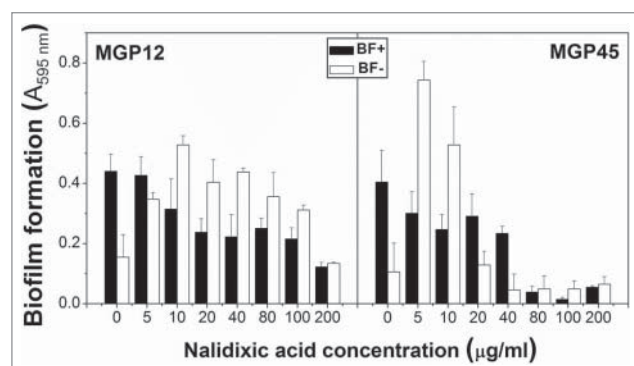


Figure 5. Antibiotic resistance in static conditions. ON cultures of the selected isolates were diluted to an $OD_{560nm}=0.1$ and incubated in multiwell plates in M63 medium containing different Pi concentrations and the indicated nalidixic acid concentrations. Plates were incubated at 30°C for 48 h and biofilm formation was assessed by crystal violet staining. Results represent the mean \pm SD of 3 different experiments.

Haemolysin was also expressed in the selected isolates but it was repressed in BF+ conditions. These results may be explained by the fact that, although associated to higher potential to form biofilm,^{4,45} haemolysin is a trait involved in invasiveness and could be secreted in the planktonic lifestyle in order to injure host tissue and then colonize it by forming the biofilm. Unexpectedly, even when *fimB* was found in the genetic pool of the 3 isolates, *fimA* gene was not detected as the other virulence factors; this may be due to the loss of genes or pathogenicity islands in UPEC associated with different environmental conditions, according to Middelendorf et al.⁴⁶ Induction of siderophore related genes in BF+ conditions indicate that UPEC may use a broad repertoire of systems to acquire iron in order to survive within the iron-limited urinary tract.⁴⁷ Interestingly, UPEC strains causative of asymptomatic bacteriuria, while lacking classical virulence factors such as fimbriae, express the full complement of iron acquisition systems, providing further evidence for the requirement of iron uptake during successful urinary tract colonization.⁴⁸

Fluoroquinolones have been the antimicrobial treatment of choice in febrile and acute UTIs.⁴⁹⁻⁵² Thus, susceptibility to nalidixic acid and ciprofloxacin was tested. From the 55 isolates tested, 83% and 36% were sensitive to ciprofloxacin and nalidixic acid, respectively. Biofilm producing strains were significantly more resistant than those unable to form biofilm (not shown). However, Soto et al.⁴⁹ described that strains able to form biofilms were significantly less resistant to nalidixic acid than non-biofilm producers. This discrepancy could be due to genome plasticity in pathogen isolates, which is responsible for the phenotypic diversity.⁵³ Interestingly, when antibiotics were added at low concentration in a BF- conditions, biofilm formation induction was achieved. Similar result were previously observed in *Staphylococcus epidermidis* isolates using macrolides⁵⁴ and in *Salmonella enteric* serovar Typhimurium in which biofilm and EPS production was improved after treatment with sub-inhibitory concentrations of cefotaxime.⁵⁵ It is worth to note that, when antibiotic was added at 24 h, the above mentioned induction of biofilm formation was not observed in the BF- conditions (not shown).

Pathogenic strains associated with human diseases are remarkably diverse, reacting differently to environmental conditions.⁵⁶ *E. coli* has a collection of more than 30 genes, which respond to variation in media Pi concentrations, known as Pho regulon. This regulon is involved in transport and assimilation of phosphorylated compounds and its transcription is activated by Pi deficiency in the external environment.^{57,58} *E. coli* possesses two Pi transport systems, a high-affinity phosphate transport and low affinity Pi transporter called Pst and Pit system, respectively.⁵⁹ Inactivation of the Pst system attenuate virulence of both extraintestinal pathogenic *E. coli* (ExPEC) and enteropathogenic *E. coli* (EPEC) strains⁶⁰⁻⁶⁴ (7, 15, 19, 24, 42). In *pst* operon UPEC mutants, Pho regulon is activated even under phosphate-replete conditions, reducing colonization of the murine urinary tract.^{65, 66}

A very important aspect reported here is that, regardless of the media Pi concentration, biofilm formation in all of the isolates requires the presence and the degradation of polyP. These data

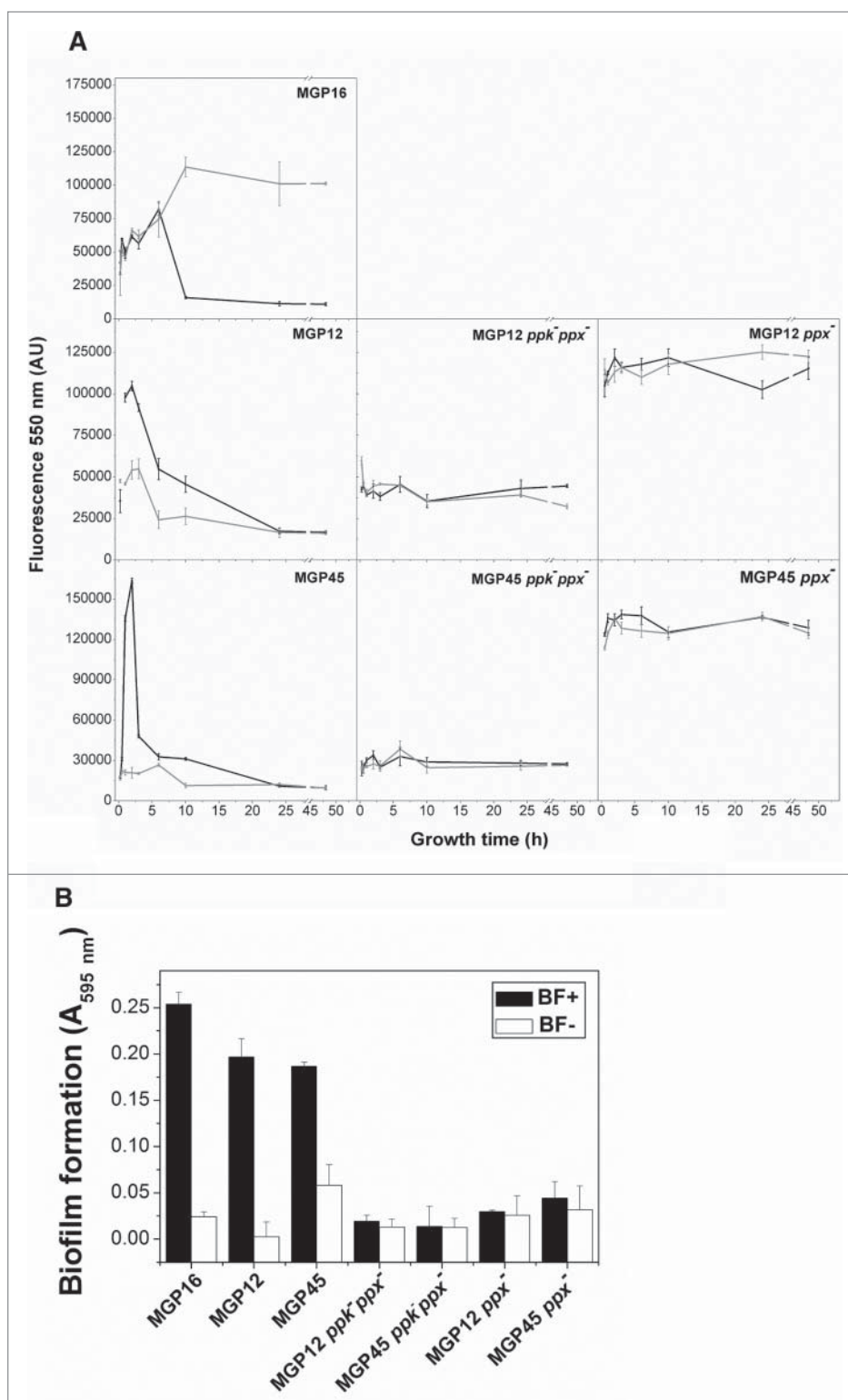


Figure 6. Intracellular polyP levels and biofilm formation in parental and mutant strains. **(A)** Cells of the indicated strains were grown for 48 h in static M63 medium modified with the Pi concentrations corresponding to BF+ (black line) and BF- (gray line) conditions. At the indicated time of growth, polyP was quantified using DAPI fluorescence, as described in Methods. DAPI emission was undetectable in cell free controls. **(B)** Indicated strains were grown in static conditions for 48 h in M63 medium modified with the indicated Pi concentrations corresponding to BF+ conditions (black columns) and to BF- conditions (white columns). Biofilm formation was quantified by crystal violet technique. Data are expressed as average \pm SD of 4 independent experiments.

genes to modify synthesis or degradation of the polymer. Although the complexity of isolates genetic backgrounds, our study highlights the implication of Pi as a possible physiological signal to regulate biofilm phenotype in *E. coli* species.

METHODS

Bacterial strains and growth media

E. coli strains collected from acute prostatitis patients at the Hospital Clinic of Barcelona were analyzed. Patients with prostatitis had a mean age of 59.6 \pm 16 years-old and remained hospitalized for at least 24 h. All urinary tract infection episodes were community acquired and uncomplicated, which means that no patient had an underlying comorbidity, apparent urological abnormality or an urethral catheter in place. MGP12 *ppk*⁻ *ppx*⁻ and MGP45 *ppk*⁻ *ppx*⁻ were constructed by P1 phage transduction.⁶⁷ Complemented strains MGP12 *ppk*⁻ *ppx*⁻ (*ppk*⁺) and MGP45 *ppk*⁻ *ppx*⁻ (*ppk*⁺) were obtained by transformation with pSPK1 plasmid,⁶⁸ containing *ppk* gene. Cells were grown using M63 minimal medium supplemented with 0.4% glucose. M63 medium contained: 100 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 1.7 μ M FeSO₄·7H₂O and 1 mM MgSO₄.⁶⁹ When indicated, medium was prepared with Pi concentrations other than 100 mM.

were corroborated with mutant strains and are in agreement with previous results from our laboratory.²⁵ Further studies would be necessary to elucidate the regulatory mechanism involved in this process. Additionally, it would be interesting to consider the development of alternative therapies with *ppk* and/or *ppx* as target

Quantification of biofilm formation

Biofilm formation was assayed on the basis of the ability of cells to adhere and grow on polystyrene microtiter plates and stained by crystal violet.⁷⁰ Overnight (ON) cultures in Luria Broth (LB) (L3022, Sigma) were diluted to an $OD_{560} = 0.1$ (corresponding to around 6×10^7 CFU ml^{-1}) with fresh M63 glucose medium with different Pi concentrations. Cells were grown in microtiter plates under static conditions at 30°C for 48 h. Then, unattached cells were removed by washing the plates with deionized water. Two hundred microliters of 0.1% crystal violet solution was added to each well and plates were incubated at room temperature for 5 min. Then, wells were rinsed 3 times with water. Finally, the absorbed crystal violet was extracted with 200 μ l of 95% ethanol and absorbance at 595nm was measured (Spectra MaxPlus384 Absorbance Microplate Reader, US). Six replicates were performed for each experimental condition.

PolyP level measurement

Intracellular polyP was measured by a fluorescence approach using 4',6-diamidino-2-phenylindole (DAPI) in cell suspensions growing in static conditions.⁷¹ Briefly, cells were washed and resuspended in T buffer (100 mM Tris-HCl, pH 8). Seventeen μ M DAPI (D9542, Sigma) was added to cuvettes containing cell suspensions ($OD_{560} = 0.02$) in T buffer, with 0.00075% SDS and chloroform for cell permeabilization.²⁵ After incubation for 5 min at 37°C with agitation, the DAPI fluorescence spectra (excitation, 415 nm; emission, 445–650 nm) were recorded using an ISS PCI spectrofluorometer (ISS Inc., Champaign, IL). Fluorescence of the DAPI-polyP complex at 550 nm was used as a measurement of intracellular polyP, since emissions from free DAPI and DAPI-DNA are minimal at this wavelength.⁷¹

Microscopic analysis

Microscopic images of biofilms were obtained by CLSM or by SEM. *E. coli* strains were grown under the same conditions used in the biofilm formation assay in a 6-well polystyrene plate containing M63 medium with different Pi concentration with a glass coverslip inside. After incubation for 48 h, unattached cells were removed by pipetting and cells on coverslips were then rinsed with water and dried at 37°C for 10 min. For CLSM the adhered cells were stained with 15 μ l Calcofluor White (18909 Sigma) for 10 min in the dark. The coverslips were then rinsed with sterile water and removed from the wells. Images were captured using a Leica Confocal Microscope TCS-SP5 (Unitat de Microscopia confocal, UB, Barcelona, Spain). For SEM, cells on coverslips were fixed with 2.5% glutaraldehyde and 2.5% paraformaldehyde, acetone and ethanol dehydrated, and gold coated with anion sputter JFC-1100 (JEOL). Coverslips containing the biofilms were then attached to aluminum holders and analyzed using a Carl Zeiss SUPRA-55 scanning electron microscope from CIME (UNT-CONICET) with a resolution of 1.0 nm at 15 kV and 1.7 nm at 1 kV in high-vacuum (HV) mode and 2 nm at 30 kV in variable-pressure mode (VP).

Curli and cellulose production assays

Curli production was examined by the Congo red-binding assay according to Da Re and Ghigo.⁷² Briefly, 5 microliters of each ON culture grown at 37°C in LB medium were spotted onto M63 agar plates, supplemented with 40 μ g ml^{-1} Congo red and 20 μ g ml^{-1} brilliant blue containing different Pi concentrations and incubated for 48 h. Colony morphologies on Congo red plates were scored according to the basic morphotypes previously detected in *S. Typhimurium*: *rdar* (violet colony, expresses curli fimbriae and cellulose), *pdar* (pink colony, expresses cellulose), *bdar* (brown colony, expresses curli fimbriae) and *saw* (colorless colony, no expression of curli fimbriae nor cellulose).³⁹

Cellulose production was followed using the Calcofluor White qualitative assay with modification.⁷³ Strains were grown onto a coverslip immersed in M63 medium containing different Pi concentrations at 30°C during 48 h. After incubation attached cells were washed twice with distilled water and stained with 15 μ l of Calcofluor White for 10 min. Cellulose were visualized under a fluorescence microscope Olympus BX51TF equipped with an Olympus QColor5 digital camera (Q-imaging, Surrey, BC, Canada) at excitation and emission wavelengths of 395 and 440 nm, respectively.

Motility assays

Motility was evaluated according to Ulett et al. with modifications.⁷⁴ Briefly, ON LB cultures were washed and resuspended with fresh M63 medium to an $OD_{560nm} = 0.1$. Cells were spotted onto the center of 0.3% agar M63 plates supplemented with different Pi concentrations, using a sterile toothpick for inoculation. Plates were incubated at 30°C for 48 h and swimming motility was determined by measuring diameters of growth. For irregular halos, colony diameters were calculated as an average of several diameters around the colony. Data are expressed as the mean diameter (cm) of movement for 3 independent experiments.

Antibiotic susceptibility assays

Susceptibility to nalidixic acid or ciprofloxacin was determined by a disc-plate diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI). Antibiotic minimal inhibitory concentrations (MICs) were determined by E-test strips (AB Biodisk, Solna, Sweden), as the intersection of the ellipse of inhibition with the strip. To test the antibiotic concentration able to inhibit biofilm formation or to disassemble a pre-formed biofilm, increasing antibiotic concentrations (0–200 μ g ml^{-1}) were added from the beginning or after 24 h of growth, respectively. Plates were incubated at 30°C and biofilm was quantified at 48 h.

Detection and expression of virulence factors genes

The presence of the virulence factor genes, haemolysin (*hlyA* gene), cytotoxic necrotizing factor (*cnfI*), type I fimbriae regulator (*fimB* gene), type I fimbriae (*fimA* gene), P-fimbriae (*papA* gene), siderophores (*iroN* and *iutA* genes), was analyzed by PCR with specific primers (Table 2), using as template genomic DNA

Table 2 Primers used for detection and expression of virulence factor genes

Primers	Sequence 5'-3'	Target	Reference
FimA-f	TGCGGGTAGCGCAACAA	<i>fimA</i>	This work
FimA-r	ACGCAGTCCTGTTTTATCCA	<i>fimA</i>	This work
FimB-f	TCTCGACTCCGGTGGTAATG	<i>fimB</i>	This work
FimB-r	GCGTAACATGTGCGGATGAA	<i>fimB</i>	This work
PapA-f	TTTTTCGGGTGCCAAGTG	<i>papA</i>	This work
PapA-r	TGTTGACCCGACGGTCTGT	<i>papA</i>	This work
Agn-43-f	ACAGGCTGGTATGGAATCACATG	<i>agn-43</i>	This work
Agn-43-r	GTGCGCGGGCCAGTT	<i>agn-43</i>	This work
Cnf-1-f	CCAGGAGGTAAACAGTAATCA	<i>cnf1</i>	This work
Cnf-1-r	TTCGGGCAACAATTGTCGTA	<i>cnf1</i>	This work
HlyA-f	GGCAGCGGCTACTAAACAG	<i>hlyA</i>	This work
HlyA-r	CGTTCGGTGAGGCCAATG	<i>hlyA</i>	This work
IutA-f	AGCCGAGCAATCGAAT	<i>iutA</i>	This work
IutA-r	CATTTTCGATAACCCAGGTGGTT	<i>iutA</i>	This work
IroN-f	CAGTCTGAATCTTTCGAGGAA	<i>iroN</i>	This work
IroN-r	GGGCAACCCCTGCTTTG	<i>iroN</i>	This work
16S-f	CCCCAACGCAGGTTCTG	16S	This work
16S-r	CGGCGAACATGCGTTTC	16S	This work

Primers were desing by Primer Express[®] Software v3.0.1 (Life Technologies)

extracted from each strain with Wizard[®] Genomic DNA Purification Kit (A1120, Promega).

RNA was extracted from each strain culture grown in M63 medium supplemented with different Pi concentrations at 30°C for 48 h, using the SV Total RNA Isolation System (Z3100, Promega), according to the manufacturer's recommendations. Reverse transcription-PCR (RT-PCR) was carried out using M-MLV Reverse Transcriptase (M1701, Promega) with random nonamer primers (Sigma) and Quantitative PCR (q-PCR) was performed using IQ Sybr Green Super Mix (1708880, Bio-Rad)

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with gene-specific primers in an Applied Biosystems 7500 Real-Time PCR System. Specific primers used for q-PCR analysis are listed in Table 2. Each q-PCR reaction was done in triplicate and the calculated threshold cycle (CT) was normalized to the CT of the 16S gene (used as a reference internal gene) amplified from the corresponding sample. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Genes with a fold change above or below the defined threshold of 2 were considered differentially expressed.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) followed by Tukey's test with Statitix 9.0 Analytical Software 2008 for Windows (USA). Differences at p -value ≥ 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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