PhoB activation in non-limiting phosphate condition by the maintenance of high polyphosphate levels in the stationary phase inhibits biofilm formation in *Escherichia coli*

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Polyphosphate (polyP) degradation in *Escherichia coli* stationary phase triggers biofilm formation via the LuxS quorum sensing system. In media containing excess of phosphate (Pi), high polyP levels are maintained in the stationary phase with the consequent inhibition of biofilm formation. The transcriptional-response regulator PhoB, which is activated under Pi limitation, is involved in the inhibition of biofilm formation in several bacterial species. In the current study, we report, for the first time, we believe, that *E. coli* PhoB can be activated in non-limiting Pi conditions, leading to inhibition of biofilm formation. In fact, PhoB was activated when high polyP levels were maintained in the stationary phase, whereas it remained inactive when the polymer was degraded or absent. PhoB activation was mediated by acetyl phosphate with the consequent repression of biofilm formation owing to the downregulation of c-di-GMP synthesis and the inhibition of autoinducer-2 production. These results allowed us to propose a model showing that PhoB is a component in the signal cascade regulating biofilm formation triggered by fluctuations of polyP levels in *E. coli* cells during stationary phase.

INTRODUCTION

Phosphate (Pi) is an essential nutrient, used in the assembly of ATP, lipopolysaccharides, nucleic acids and other cell components (Wanner, 1996). Pho regulon is a global regulatory mechanism involved in bacterial Pi management (Wanner & Chang, 1987). The most common genes of the Pho regulon encode: extracellular enzymes capable of obtaining Pi from organic phosphates, Pi-specific transporters and enzymes involved in the storing and saving of nutrients (Rao & Torriani, 1990; Hsieh & Wanner, 2010; Santos-Beneit *et al.*, 2008). The Pho regulon not only is a regulatory circuit of Pi homeostasis but also plays an important adaptive role in bacterial stress and virulence (Chekabab *et al.*, 2014). Several new members of the Pho regulon have emerged in the last few years in several bacteria; however, there are still many unknown questions regarding the activation and function of the whole system (Santos-Beneit, 2015). In *Escherichia coli*, the Pho regulon responds to low extracellular Pi concentrations via the two-component system PhoBR. During Pi limitation, the histidine kinase sensor, PhoR, phosphorylates the response regulator PhoB, which binds to a consensus Pho box sequence with the promoters of Pho regulon genes activating or repressing their transcription (Makino *et al.*, 1993; Wanner, 1996; Rao & Torriani, 1990; Hsieh & Wanner, 2010). Under Pi-sufficient conditions, PhoR acts as a net phospho-PhoB phosphatase, deactivating PhoB (Wanner, 1996).

Inorganic polyphosphate (polyP), found in bacteria, archaea, fungi, protozoa, plants and animals, is a linear chain of tens or many hundreds of Pi residues linked by phosphoanhydride bonds (Kulaev, 1979; Kornberg *et al.*, 1999). Some attributes of polyP are substitution of ATP in kinase reactions, Pi storage, chelation of divalent metals and regulatory roles in growth, development, stress resistance and nutrient deprivation (Kulaev, 1979; Kornberg *et al.*, 1999). In a variety of microorganisms, such as *Pseudomonas aeruginosa, E. coli, Salmonella enterica* serovar Dublin, *Vibrio cholerae, Bacillus cereus* and *Porphyromonas gingivalis*, the presence of polyP is critical to certain features, such as motility, quorum sensing, biofilm formation and survival in stationary phase (Kulaev, 1979; Rao & Kornberg, 1996; Rashid & Kornberg, 2000; Rashid *et al.*, 2000a, b; Jahid *et al.*, 2006; Kim *et al.*,

Table 1. *E. coli* strains and plasmids used in this work

Strains and plasmids	Relevant genotype or description	Reference
MC4100	*araD lac rpsL flbB deoC ptsF rbsR relA1*	Casadaban & Cohen (1979)
LSB022	MC4100 (*ppkppx*:: Km)	Schurig-Briccio *et al.* (2009a)
LSB022/pBC29	(*ppkppx*:: Km/*ppk*⁺, Ap)	Grillo-Puertas *et al.* (2012)
BW25113	K-12 derivative, Δ(*araD-araB*)567 Δ*lacZ4787*(:: *rrnB-3*) λ⁻ *rph-1* Δ(*rhaD-rhaB*)568 *hsdR514*	CGSC
JW0389-1	F⁻ Δ(*araD-araB*)567 Δ*lacZ4787*(:: *rrnB-3*) Δ*phoB763*:: kan λ⁻ *rph-1*, Δ(*rhaD-rhaB*)568 *hsdR514*	CGSC
JW0390-2	F⁻ Δ(*araD-araB*)567 Δ*lacZ4787*(:: *rrnB-3*) Δ*phoR764*:: kan λ⁻ *rph-1* Δ(*rhaD-rhaB*)568 *hsdR514*	CGSC
BW29134	BW25113 (*phoB⁻phoR*)	CGSC
MGP006	BW29134 (*ppkppx*:: Km, *phoB⁻phoR⁻*)	This work
MGP006/pBC29	(*ppkppx*:: Km, *phoB⁻phoR⁻/ppk*⁺, Ap)	This work
MG1655	F⁻ lambda⁻ *ilvG⁻rfb⁻50 rph⁻1*	CGSC
pta ackA	MG1655 (*pta ackA*:: Km)	Mizrahi *et al.* (2006)
KJ295	MG1655 (*ydeH*:: Cm)	Jonas *et al.* (2009)
MG1655 *ydaM⁻*	MG1655 (*ydaM*:: Cm)	Weber *et al.* (2006)
MGP008	BW29134 (*ydeH*:: Cm, *phoB⁻phoR⁻*)	This work
JW2662-1	BW25113 (*luxS*:: Km)	CGSC
MGP009	BW29134 (*luxS*:: Km, *phoB⁻phoR⁻*)	This work
pBC29	(*ppk*⁺, Ap)	Yanofsky *et al.* (1991)

2002; Ogawa *et al.*, 2000; Price-Carter *et al.*, 2005; Tan *et al.*, 2005; Scrurig-Briccio *et al.*, 2009b). In *E. coli*, the main enzymes associated with polyP metabolism are the polyphosphate kinases (PPK, encoded by the *ppk* gene), responsible for the synthesis of polyP from ATP, and the exopolyphosphatase (PPX, encoded by the *ppx* gene), responsible for the hydrolysis of polyP (Rao & Kornberg, 1996; Ahn & Kornberg, 1990; Akiyama *et al.*, 1993).

PolyP levels in *E. coli* fluctuate during the growth curve according to the concentration of Pi in the medium. In Pi limitation or sufficiency, polyP is accumulated during the exponential phase of growth and degraded at the beginning of the stationary phase (Ohtake *et al.*, 1998; Ault-Riché *et al.*, 1998; Nesmeyanova, 2000; Schurig-Briccio *et al.*, 2009b). In contrast, it has been demonstrated in our laboratory that, in media containing high Pi concentration (>25–37 mM), *E. coli* cells maintained the polyP pool during the stationary phase (Scrurig-Briccio *et al.*, 2009b; Grillo-Puertas *et al.*, 2012). This maintenance of polyP levels has been related to the maintenance of the expression of aerobic respiratory chain genes, protection against oxidative stress and inhibition of biofilm formation (Schurig-Briccio *et al.*, 2008, 2009a; Grillo-Puertas *et al.*, 2012, 2015). Actually, to trigger biofilm formation, not only the presence of polyP but also its degradation is required (Grillo-Puertas *et al.*, 2012, 2015). When polyP is degraded in the stationary phase (in sufficient Pi concentration media), the production of the quorum sensing signal autoinducer-2 (AI-2) induces biofilm formation (Grillo-Puertas *et al.*, 2012).

The intracellular signals generated by polyP fluctuations and their relation with AI-2 production and biofilm formation capacity remain unknown. It has been reported that PhoB activation inhibits biofilm formation in *Pseudomonas fluorescens, Pseudomonas aureofaciens* and *V. cholerae* (Monds *et al.*, 2001, 2007; Sultan *et al.*, 2010; Pratt *et al.*, 2010). Thus, considering that PhoB could be a component in the signal cascade of polyP-dependent biofilm formation, the aim of this work was to investigate whether PhoB activity is modulated by polyP levels in the stationary phase of *E. coli* and whether it is involved in biofilm formation capacity in such conditions.

METHODS

Bacterial strains and media. Bacterial strains used in this study are listed in Table 1. Cells were grown in the saline minimal media MT (2 mM phosphate) (Simon & Tessman, 1963), MT+P (defined as MT prepared without phosphate and supplemented with 40 mM phosphate) (Schurig-Briccio *et al.*, 2008), or MT−P (defined as MT prepared without phosphate and supplemented with 0.003 mM phosphate). MT minimal medium contains 0.272 g KH₂PO₄ (corresponding to 2 mM), 5.8 g NaCl, 3.7 g KCl, 0.15 g CaCl₂.2H₂O, 1.1 g NH₄Cl, 0.142 g Na₂SO₄, 12.1 g Tris [tris(hydroxymethyl) aminomethane], 0.27 mg FeCl₃ and 0.2 g MgSO₄.7H₂O per litre of distilled water. Sodium phosphate (Na₂HPO₄/NaH₂PO₄, Sigma) buffer pH 7 was used to supplement minimal media. In all experiments, the media were supplemented with 0.4 % glucose and 0.1 % tryptone. When required, antibiotics were used – 100 µg ml⁻¹ of ampicillin, 50 µg ml⁻¹ of kanamycin or 30 µg ml⁻¹ of chloramphenicol. Conditioned media (CM) are 24 h spent media obtained from bacterial cultures by two sequential centrifugations at 15 000 r.p.m. and filtration with 0.2 µm pore size discs.

Alkaline phosphatase (AP) activity determination. Alkaline phosphatase (AP) is an enzyme encoded by *phoA*, a Pho regulon member. Since *phoA* gene transcription is positively regulated only by PhoB, AP activity has been used largely as a reporter of PhoB activation (Wanner

& Latterell, 1980; Lamarche *et al.*, 2005, 2008). AP activity was determined using the chromogenic substrate *p*-nitrophenylphosphate (pNPP) according to a previously described method (Lamarche *et al.*, 2005) with modifications. Briefly, cells were grown statically at 30 °C in the indicated media and aliquots were extracted at different times of growth. For permeabilization, cells were resuspended at an $OD_{560 \, nm}$ of 0.5 in 1 M Tris-HCl buffer pH= 8 to a final volume of 1 ml with the addition of 30 µl of 0.1 % SDS and 30 µl of chloroform and incubated for 30 min at room temperature. Permeabilized cells were incubated with 2 mM pNPP (Sigma) at 37 °C for 20 min or up to colour development. Absorbances at 405 and 550 nm were determined. AP activity was calculated with the following equation, where *T* is the reaction time in minutes:

Measurements of polyP levels. Intracellular polyP was measured in cell suspensions using a DAPI (4′,6-diamidino-2-phenylindole) based fluorescence approach (Aschar-Sobbi *et al.*, 2008). Cells growing at 30 °C in static conditions were washed and resuspended in buffer T (100 mM Tris HCl, pH 7.5). DAPI (17 µM Sigma) was added to cuvettes containing cell suspensions at an $OD_{560 \, nm}$ of 0.02, with 15 µl of 0.1 % SDS and 15 µl chloroform for cell permeabilization. After 5 min of stirring at 37 °C, the DAPI fluorescence spectra (excitation, 415 nm; emission, 445 to 650 nm) were recorded using an ISS PCI spectrofluorometer (Champaign, IL). Fluorescence (expressed as arbitrary units) of the DAPI-polyP complex at 550 nm was used as a measure of the intracellular polyP, since fluorescence emissions from free DAPI and DAPI-DNA are minimal at this wavelength (Aschar-Sobbi *et al.*, 2008).

Quantification of biofilm formation. Biofilm formation was assayed on the basis of the ability of cells to adhere and grow on 96-well polystyrene microtitre plates (O'Toole & Kolter, 1998). Overnight stationary phase cultures in MT, MT+P or MT−P were diluted to $OD_{560 \, nm}$=0.1 (corresponding to c.f.u. ml^{-1} from 5 to 6×10^7) with fresh medium and incubated in static conditions at 30 °C in microtitre plates for 48 h. After removing the unattached cells and rinsing the plates three times with deionized water, quantification of attached cells was performed as follows. Two hundred microlitres of 0.1 % crystal violet solution was added to each well and the plates were incubated at room temperature for 30 min in darkness. Then, the wells were rinsed again three times with water. The absorbed crystal violet was extracted with 200 µl of 95 % ethanol and measured at 595 nm (SpectraMax Plus 384 absorbance microplate reader). Six replicates for each condition were performed in each experiment.

RESULTS

Fluctuations in polyP levels modulate PhoB activity in stationary phase

To determine if PhoB activity is modulated by fluctuations of polyP, AP activity and polyP levels were measured in the cells grown in MT−P (deficient phosphate medium with 0.003 mM Pi), MT (sufficient phosphate medium with 2 mM Pi) and MT+P (high phosphate medium with 40 mM Pi). AP activity of MC4100 wild-type (WT) cells grown in MT was low at all growth times tested (Fig. 1). In the limiting Pi condition (MT−P), AP activity was high during the exponential phase and extremely low in the stationary phase. In contrast, in cells grown in MT+P, the activity increased progressively from the exponential to the stationary phase. Similar AP activity results were obtained with BW25113 and MG1655 WT strains (not shown). *phoB*
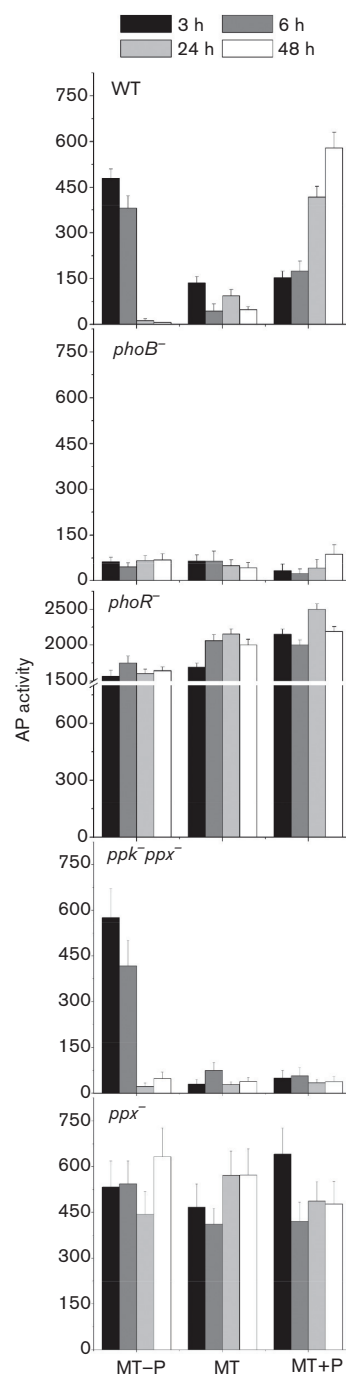

Fig. 1. AP activity in different Pi concentration media. Cells of the indicated strains were grown at 30 °C in MT−P, MT and MT+P media. At 3, 6, 24 and 48 h, aliquots were removed and AP activity was determined according to the Methods. Results represent the mean ± SD of four independent experiments.

mutant was used as negative control, while *phoR* mutant was used as positive control. Considering that PhoR is the only phosphatase known to dephosphorylate PhoB, its deficiency produces a constitutive activation of the response
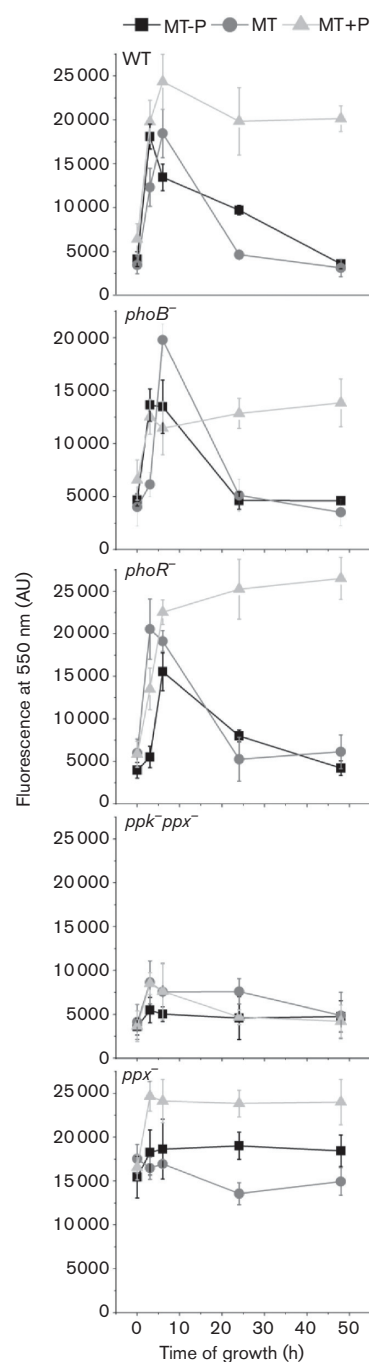
Fig. 2. PolyP levels during growth in different Pi concentration media. Cells of the indicated strains were grown at 30 °C for 48 h in static MT−P (black line), MT (grey line) or MT+P (light grey line) medium. At 3, 6, 24 and 48 h, polyP was quantified using DAPI fluorescence, as described in Methods. DAPI emission was undetectable in cell free controls. Data are expressed in fluorescence arbitrary units (AU) as the average ± SD of three independent experiments.

regulator (phosphorylated state). In all tested conditions, *phoB* single mutant showed low AP activity values, whereas *phoR* single mutant presented extremely high AP activity (Fig. 1).

Considering the unusually high AP activity in the stationary phase of WT cells grown in high Pi concentration medium MT+P, we hypothesized that the maintenance of polyP levels results in the activation of PhoB. To test this hypothesis, AP activity was also determined in strains deficient in polyP synthesis (*ppx⁻ppk*) or degradation (*ppx⁻*) (Fig. 1). The mentioned unusual AP activity was not observed in *ppx⁻ppk⁻* cells. However, the *ppx⁻* strain showed high AP activity at all the assayed growth times and phosphate concentrations. *phoBR⁻*, *phoBR⁻ppk⁻ppx⁻* and *phoBR⁻ppx⁻* strains showed low AP activity in all the conditions tested (data not shown).

Fig. 2 shows the polyP levels in the tested strains at different times of growth. MC4100 WT, *phoB⁻*, and *phoR⁻* cells, grown in MT−P or MT, synthesized polyP in the exponential phase and degraded it in the stationary phase, whereas cells grown in MT+P maintained polyP levels even in the stationary phase. As expected, in all tested conditions, *ppkppx* and *ppx* mutant strains presented low and high polyP levels, respectively. PolyP profiles in the BW25113 WT strain were similar to those of MC4100 (not shown). *phoBR⁻*, *phoBR⁻ppk⁻ppx⁻* and *phoBR⁻ppx⁻* strains showed similar polyP profiles as against the WT, *ppk⁻ppx⁻* and *ppx⁻* strains, respectively (data not shown).

It has been previously reported that cells grown for 24 h are able to adapt to changes between media with Pi concentration (Schurig-Briccio *et al.*, 2009b; Grillo-Puertas *et al.*, 2012). Indeed, when WT cells grown for 24 h in MT+P were shifted to fresh MT medium (MT+P→24MT), polyP was degraded. On the contrary, the addition of 40 mM phosphate buffer to WT cells grown for 24 h in MT medium (MT+24P) induced the re-synthesis and the maintenance of polyP. Considering that the PhoB activation/deactivation responds to polyP levels in the stationary phase, the reversibility of this phenotype was evaluated using the approach mentioned earlier. As shown in Fig. 3, AP activity in cells grown in MT for 24 h increased after the addition of 40 mM Pi (see 48 h in MT+24P condition), consistent with polyP re-synthesis and maintenance. In contrast, AP activity in cells grown in MT+P decreased when polyP was degraded by the switch to MT (see 48 h in MT+P→24MT condition).

PhoB activation by the maintenance of polyP levels in the stationary phase inhibits biofilm formation

The results here support the hypothesis that polyP maintenance during the stationary phase activates PhoB. Because the inability of the cells to degrade polyP in the stationary phase inhibits biofilm formation, we wondered whether PhoB could participate in the inhibition of biofilm formation under such conditions. To answer this issue, the biofilm formation by WT, Pho and polyP related-mutants cultivated in different Pi concentration media was

analysed. MC4100 WT cells grown in MT and MT−P formed biofilms; in contrast, cells grown in MT+P did not (Fig. 4). Similar results were obtained with BW25113 and MG1655 WT strains (data not shown). *phoB* and *phoBR* mutants formed biofilms independent of Pi concentration, while the *phoR⁻* strain did not form biofilms in any of the conditions tested (Fig. 4). Additionally, the *ppx* mutant did not form biofilm but the *phoBR ppx* triple mutant caused the suppression of this biofilm negative phenotype in all media. Together, the results indicate that PhoB activation, linked to the maintenance of polyP levels in MT +P stationary phase cells, inhibits biofilm formation. However, polyP seems to have other roles that trigger biofilm formation in our experimental conditions, since *phoBR* mutation in a *ppkppx* background did not suppress the biofilm negative phenotype. Note that all strains were able to grow under the tested conditions.

Acetyl phosphate is involved in the PhoB activation in the stationary phase

Acetyl phosphate (AcP), a physiologically relevant small molecule, can serve as a phosphoryl donor to a subset of the two-component response regulators that regulate diverse cellular processes (Wolfe, 2010). On the basis of this, we wondered if AcP is involved in the PhoB phosphorylation (activation) that occurred in the stationary phase cells grown in MT+P. Thus, we carried out AP activity and biofilm formation experiments using the *pta⁻ackA⁻* strain (deficient in genes encoding phosphotransacetylase and acetate kinase), which is unable to synthetise AcP. The results indicate that the absence of AcP suppressed the PhoB activation in the stationary phase (Fig. 5a) and stimulated biofilm formation in the cells grown in MT+P (Fig. 5b).

c-di-GMP induces biofilm formation when PhoB is inactive

c-di-GMP has been frequently reported as a signal that triggers bacterial biofilm formation (Wolfe & Visick, 2010; Simm *et al.*, 2004; Romling & Amikam, 2006; Hengge, 2009). As a preliminary approach to study if this nucleotide is involved in thebiofilm formation capacity of the cells grown in our experimental conditions, biofilm formation assay was performed with *ydeH* and *ydaM* diguanylate cyclase mutants (strains deficient in synthesis of c-di-GMP). The results in Fig. 4 show that the *ydeH* and *ydaM* mutants were unable to form biofilms in MT−P and MT, suppressing the WT phenotype. Moreover, *ydeH* mutation in a *phoBR* background (*ydeHphoBR* mutant) suppressed the biofilm formation capacity of the *phoBR⁻* strain in all media tested. Thus, c-di-GMP is a signal involved in the induction of biofilm formation in our experimental conditions, when PhoB is inactive.

PhoB activation is linked to the inhibition of AI-2 production

In *E. coli*, AI-2 is synthesized by LuxS, encoded by the *luxS* gene (Xavier & Bassler, 2003). We have previously described that AI-2 production triggers biofilm formation when polyP is degraded in the stationary phase (Grillo-Puertas *et al.*, 2012). Therefore, could polyP-dependent PhoB activation inhibit AI-2 production? To answer this question, biofilm formation assay using a *luxS* deficient strain exposed to conditioned media (CM) was carried out (Fig. 6). Briefly, *luxS⁻* cells grown for 24 h in MT medium were shifted to CM and incubated for a further 24 h before biofilm quantification. CM from MC4100 WT cells grown
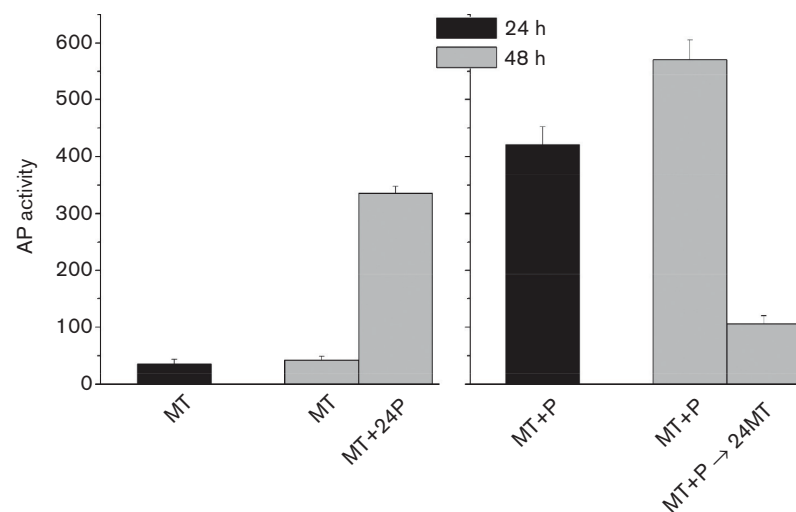

Fig. 3. AP activity after changes in medium phosphate concentration during the stationary phase. AP activity by MC4100 was measured at indicated times of growth in MT and in MT with the addition of 40 mM phosphate buffer pH 7 at 24 h (MT+24P) or in MT+P and in MT+P culture switched to fresh MT at 24 h (MT+P→24MT). Results represent the mean ± SD of at least three independent experiments performed in triplicate.
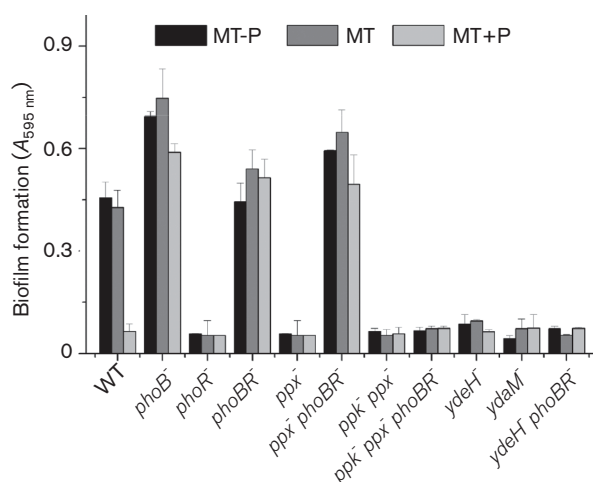
Fig. 4. Biofilm formation in PhoBR and polyP related mutants. The biofilm amount was determined at 48 h in the indicated strains grown at 30 °C in static conditions in MT−P, MT or MT+P medium. Data are expressed as average ± SD of five independent experiments.

Fig. 6. Biofilm formation by *luxS* mutant in conditioned media. *luxS⁻* cells growing in MT medium for 24 h were re-inoculated in CM from the indicated strains grown for 24 h in MT or MT+P. Biofilm formation was determined 24 h after the shift to CM. 48 h MT culture of the *luxS* mutant was used as control (dashed line). Data are expressed as average ± SD of four independent experiments.

in MT or from *phoB⁻* and *phoBR⁻* cells grown in MT and MT+P were able to induce biofilm formation in the *luxS* mutant. This effect was not observed using CM from *phoR* or *phoBRluxS* mutants. These data indicate that AI-2 production is inhibited by PhoB activation in the stationary phase.

DISCUSSION

Here, we demonstrated that PhoB can be activated during the stationary phase in *E. coli* cells grown in a medium that contains high Pi concentration, a condition in which cells maintain high polyP levels. This finding is supported by the absence of AP activity in *ppkppx* mutant grown in Pi
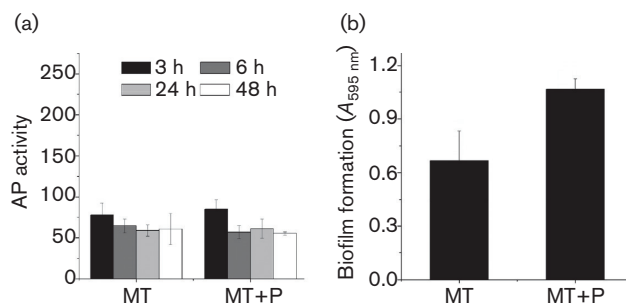

Fig. 5. AP activity and biofilm formation in *pta ackA* mutant. *pta⁻ackA⁻* cells were grown statically at 30 °C in MT and MT+P medium. (a) At 3, 6, 24 and 48 h, aliquots were taken and AP activity was determined as described in Methods. (b) Biofilm formation was determined at 48 h (b). The results represent the mean ± SD of three independent experiments.
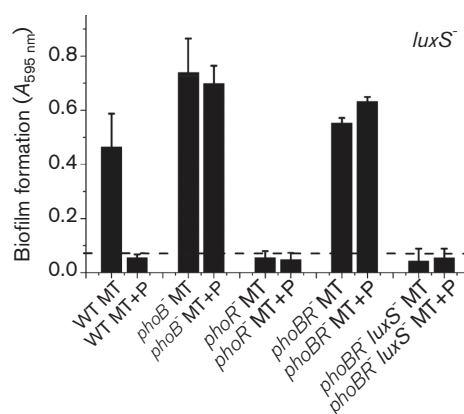
abundance and by the elevated AP activity observed in the *ppx⁻* strain in all tested conditions. The PhoB activation related to the maintenance of high polyP levels in the stationary phase is due to phosphorylation via AcP. In our experimental conditions, the activated regulator inhibits the synthesis of c-di-GMP and the production of AI-2, negatively regulating biofilm formation.

We consider that the polyP level fluctuations control the intracellular Pi concentration. Although we have not identified the mechanism controlling the balance between intracellular Pi and polyP, a Pi deficiency could be generated when polyP was maintained or not degraded during the stationary phase, with consequent PhoB activation. In fact, Nesmeyanova (2000) and Motomura *et al.* (2011) suggested that polyP can serve as a Pi reservoir, participating in the maintenance of the intracellular Pi concentration.

The exponential phase cells grown in MT and MT+P did not activate PhoB even though polyP levels were as high as those of the stationary phase cells grown in MT+P. Indeed, the dynamics of polyP synthesis/degradation that occurs in the exponential phase may allow Pi release from polyP, keeping PhoB inactive. It was previously reported that polyP accumulation in the exponential phase is a dynamic process in which PPX is highly active (Nesmeyanova, 2000).

The unusual PhoB phosphorylation during the stationary phase in non-limiting Pi concentration can occur through direct interaction with the polyP or indirectly by other cellular components. Eisenbach (1996) described that polyP may be a substitute for ATP in protein phosphorylation in chemotaxis systems or that phospho-PPK could directly transfer the Pi group to proteins (cross-phosphorylation). In addition, several PhoR-independent pathways for PhoB phosphorylation in *E. coli* have been previously described.
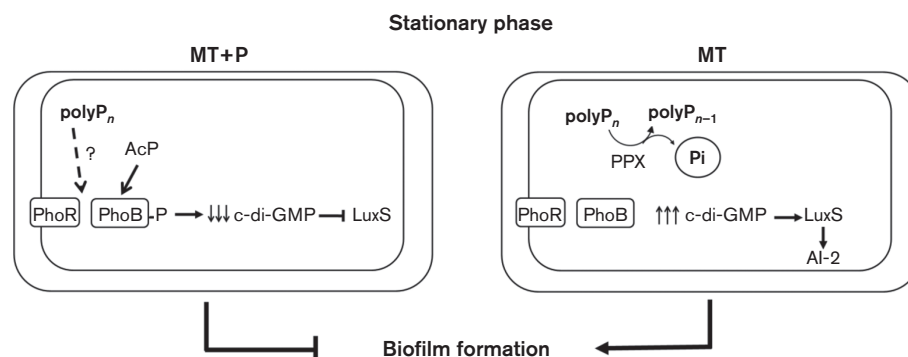
Fig 7. Simplified model of the mechanism proposed for biofilm formation in *E. coli*. In high Pi medium (MT+P), inhibition of biofilm formation is a consequence of the maintenance of high polyP levels in the stationary phase, activation of PhoB via AcP and downregulation of c-diGMP synthesis. In sufficient Pi medium (MT), polyP degradation during the stationary phase may generate available Pi in the cytoplasm, inactivation of PhoB and activation of the signal cascade for biofilm formation mediated by AI-2. polyP: Polyphosphate; PPX: exopolyphosphatase; Pi: phosphate; PhoR: Pi sensor response protein; PhoB-P: phosphorylated regulatory response protein, active; PhoB: regulatory response protein, inactive; AcP: acetyl phosphate; LuxS: protein synthesizing AI-2; AI-2: autoinducer-2, quorum sensing factor.

PhoB activity can be induced by CreC (Wanner *et al.*, 1988), five other non-partner histidine kinases (ArcB, KdpD, QseC, BaeS and VanS) (Haldimann *et al.*, 1996; Fisher *et al.*, 1995), or AcP (Kim *et al.*, 1996; Wanner & Wilmes-Riesenberg, 1992; Wolfe, 2010). As a first insight into this issue, our results using the *pta ackA* mutant strain indicate that AcP acts as the phosphoryl donor for PhoB activation in MT+P in the stationary phase.

In our experimental conditions, biofilm formation required PhoB inactivation or absence. However, the inability to form a biofilm of the $ppk^- ppx^-$ strain is not suppressed by a *phoBR* mutation. Thus, other roles can be attributed to the polymer in the biofilm formation, besides the modulation of PhoB activity. In agreement with this, the pleiotropic effects of polyP in bacterial physiology have been widely reported (Kornberg, 1999; Rashid & Kornberg, 2000; Rashid *et al.*, 2000a, b; Gray & Jakob, 2015). It is worth noting that *E. coli* cells grown under high Pi concentrations present an enhanced stationary phase fitness and resistance to oxidants (Schurig-Briccio *et al.*, 2009a; Grillo-Puertas *et al.*, 2012; 2014). In addition, it has been reported that both polyP and PhoBR system are required for growth and to overcome environmental stress in the stationary phase in *V. cholerae* (Lery *et al.*, 2013; Jahid *et al.*, 2006).

Although the present study is not focused on second messengers associated with the regulation of biofilm formation, the results from strains that are unable to synthesize c-di-GMP indicate the participation of this nucleotide in the signal cascade that induces biofilm formation in MT. In *Pse. fluorescens*, it has been reported that inhibition of biofilm formation by PhoB was in turn mediated by the activation of a c-di-GMP phosphodiesterase, which catalysed the degradation of c-di-GMP (Monds *et al.*, 2007). In addition, connections between quorum sensing, c-di-GMP and biofilm formation have been reported in *Pse aeruginosa* and *V. cholerae* (Ueda & Wood, 2009; Waters *et al.*, 2008; Srivastava & Waters, 2012).

In conclusion, in MT+P medium, the maintenance of high polyP levels in the stationary phase induces the activation of PhoB via AcP, inhibiting the c-di-GMP synthesis and the AI-2 production with the consequent impairment of biofilm formation capacity (see model in Fig. 7). In contrast, in MT medium, the polymer degradation in the stationary phase keeps PhoB inactive, allowing the production of c-di-GMP and AI-2 to trigger biofilm formation (Fig. 7). This is the first time that a relationship between PhoB regulation, polyP fluctuations and biofilm formation has been reported.

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