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., 1999). Some 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., 2000; Rashid et al., 2000b; Rashid et al., 2000c; Rashid et al., 2000d).
exopolyphosphatase (PPX, encoded by the gene), responsible for the synthesis of polyP from ATP, and the degradation is required (Grillo-Puertas et al., 2012, 2015). When polyP is degraded in the stationary phase (Scrurig-Briccio et al., 2008, 2009a; Grillo-Puertas et al., 2005; Scrurig-Briccio et al., 2006). 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; Akijama 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., 2000; Price-Carter et al., 2005; Scrurig-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 maintain 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 aeruginosa 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 & Tesman, 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 (tri(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 et al., 2002; Ogawa et al., 2000; Price-Carter et al., 2005; Tan et al., 2005; Scrurig-Briccio et al., 2009b).

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant genotype or description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>araD lac rpd, fliB deoC pscF rheR relA1</td>
<td>Casadaban &amp; Cohen (1979)</td>
</tr>
<tr>
<td>LSBO22</td>
<td>ppp phx:: Km</td>
<td>Schurig-Briccio et al. (2009a)</td>
</tr>
<tr>
<td>BW25113</td>
<td>K-12 derivative, Δ araD-araB 567 Δ lacZ 4787 (:: rpsL-3) λ− rph-1 Δ (rhaD-rhaB) 568 hsdR514</td>
<td>CGSC</td>
</tr>
<tr>
<td>JW0389-1</td>
<td>F− Δ araD-araB 567 Δ lacZ 4787 (:: rpsL-3) Δ phoB 763:: kan λ− rph-1 Δ (rhaD-rhaB) 568 hsdR514</td>
<td>CGSC</td>
</tr>
<tr>
<td>JW0390-2</td>
<td>F− Δ araD-araB 567 Δ lacZ 4787 (:: rpsL-3) Δ phoR 764:: kan λ− rph-1 Δ (rhaD-rhaB) 568 hsdR514</td>
<td>CGSC</td>
</tr>
<tr>
<td>BW29134</td>
<td>BW25113 (phoB− phoR−)</td>
<td>CGSC</td>
</tr>
<tr>
<td>MG009</td>
<td>BW29134 (ppk ppk:: Km phoB− phoR−)</td>
<td>This work</td>
</tr>
<tr>
<td>MG006/ppBC29</td>
<td>(ppk ppk:: Km phoB− phoR−/ppk, Ap)</td>
<td>This work</td>
</tr>
<tr>
<td>MG1655</td>
<td>F− λ− ivG− rpl− 50 rph-1</td>
<td>CGSC</td>
</tr>
<tr>
<td>pta aikA</td>
<td>MG1655 (pta aikA:: Km)</td>
<td>Mizrahi et al. (2006)</td>
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<td>K295</td>
<td>MG1655 (ydeH:: Cm)</td>
<td>Jonas et al. (2009)</td>
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<td>MG1655 ydaM−</td>
<td>MG1655 (ydaM:: Cm)</td>
<td>Weber et al. (2006)</td>
</tr>
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<td>MG008</td>
<td>BW29134 (ydeH:: Cm phoB− phoR−)</td>
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<tr>
<td>JW2662-1</td>
<td>BW25113 (luxS:: Km)</td>
<td>CGSC</td>
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<tr>
<td>MG009</td>
<td>BW29134 (luxS:: Km phoB− phoR−)</td>
<td>This work</td>
</tr>
</tbody>
</table>

http://mic.microbiologyresearch.org
& 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_{2005} 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:

$$AP\ activity = \frac{A_{500nm}}{T} \times A_{530nm}$$

**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 spectrophuorometer. 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_{600 nm}=0.1 (corresponding to c.f.u. ml$^{-1}$ from 5 to 6 x 10$^3$) 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.

**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.

Similar AP activity results were obtained with BW25113 and MG1655 WT strains (not shown). phoB mutant was used as negative control, while phoR mutant was used as positive control. Considering that PhoR is the only phosphatase...
PhoB activation by high intracellular polyP levels

known to dephosphorylate PhoB, its deficiency produces a constitutive activation of the response 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−/ppk+) (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, phoR−, and phoR−/ppx− 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, ppk−/ppx− and ppk−/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 ppk−/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 concentrations (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 ppx ppx 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 synthesise 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 the biofilm 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 (ydeH phoBR 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 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 phoBR luxS 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 *ppk ppx* mutant grown in Pi 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-diGMP 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 can serve as a Pi reservoir, participating in the maintenance of high polyP levels in the stationary phase.

**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. 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.
In addition, several PhoR-independent pathways for PhoB phosphorylation in *E. coli* have been previously described. 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 *phoBR* strain is not suppressed by 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 *P. 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 *P. aeruginosa* and *V. cholerae* (Ueda & Wood, 2009; Waters et al., 2008; Srivas-tava & 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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**REFERENCES**


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