The RNA processing enzyme polynucleotide phosphorylase negatively controls biofilm formation by repressing poly-N-acetylglucosamine (PNAG) production in *Escherichia coli* C

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**Abstract**

**Background:** Transition from planktonic cells to biofilm is mediated by production of adhesion factors, such as extracellular polysaccharides (EPS), and modulated by complex regulatory networks that, in addition to controlling production of adhesion factors, redirect bacterial cell metabolism to the biofilm mode.

**Results:** Deletion of the *pnp* gene, encoding polynucleotide phosphorylase, an RNA processing enzyme and a component of the RNA degradosome, results in increased biofilm formation in *Escherichia coli*. This effect is particularly pronounced in the *E. coli* strain C-1a, in which deletion of the *pnp* gene leads to strong cell aggregation in liquid medium. Cell aggregation is dependent on the EPS poly-N-acetylglucosamine (PNAG), thus suggesting negative regulation of the PNAG biosynthetic operon *pgaABCD* by PNPase. Indeed, *pgaABCD* transcript levels are higher in the *pnp* mutant. Negative control of *pgaABCD* expression by PNPase takes place at mRNA stability level and involves the 5'-untranslated region of the *pgaABCD* transcript, which serves as a cis-element regulating *pgaABCD* transcript stability and translatability.

**Conclusions:** Our results demonstrate that PNPase is necessary to maintain bacterial cells in the planktonic mode through down-regulation of *pgaABCD* expression and PNAG production.

**Keywords:** Biofilm, RNA processing, Degradosome, EPS, Cell adhesion, PNPase

**Background**

Most bacteria can switch between two different lifestyles: single cells (planktonic mode) and biofilms, i.e., sessile microbial communities. Planktonic and biofilm cells differ significantly in their physiology and morphology and in their global gene expression pattern [1-3]. Extensive production of extracellular polysaccharides (EPS) represents a defining feature of bacterial biofilms; EPS are the major constituent of the so-called “biofilm matrix”, which also includes cell surface-associated proteins and nucleic acids [4,5]. In addition to constituting the material embedding biofilm cells and to being a main determinant for surface attachment, the EPS are responsible for cell resistance to environmental stresses such as desiccation [6] and to predation by bacteriophages [7]. In several bacterial species, EPS are also required for swarming motility [8,9].

Expression of genes involved in EPS biosynthesis is controlled by complex regulatory networks responding to a variety of environmental and physiological cues, including stress signals, nutrient availability, temperature, etc. [10-13]. Regulation of EPS production can take place at any level, i.e., transcription initiation, mRNA stability, and protein activity. For instance, the *vps* genes, involved in EPS biosynthesis in *Vibrio cholerae*, are regulated at the transcription level by the CytR protein, in response to intracellular pyrimidine concentrations [14]. The RsmA
### Table 1 Bacterial strains and plasmids

| Strains | Relevant Genotype | Origin or reference |
|---------|-------------------|---------------------|
| C-1a    | E. coli C, prototrophic | [40] |
| C-5691  | Δpnp-751          | [41] |
| C-5928  | ΔbcsA:cat         | by P1 HTF AM72 transduction into C-1a |
| C-5929  | Δpnp-751 ΔbcsA:cat | by P1 HTF AM72 transduction into C-5691 |
| C-5930  | ΔcsgA:cat         | by P1 HTF AM70 transduction into C-1a |
| C-5931  | Δpnp-751 ΔcsgA:cat | by P1 HTF AM70 transduction into C-5691 |
| C-5932  | ΔpgaA:cat         | by P1 HTF AM56 transduction into C-1a |
| C-5933  | Δpnp-751 ΔpgaA:cat | by P1 HTF AM56 transduction into C-5691 |
| C-5934  | ΔwcaD::tet        | by P1 HTF AM105 transduction into C-1a |
| C-5935  | Δpnp-751 ΔwcaD::tet | by P1 HTF AM105 transduction into C-5691 |
| C-5936  | ΔpgaC::kan        | by P1 HTF JW1007 transduction into C-1a |
| C-5937  | Δpnp-751 ΔpgaC::kan | by P1 HTF JW1007 transduction into C-5691 |
| C-5938  | ΔcsrA::kan        | From C-1a by λ Red-mediated recombination; primers: FG2624 and FG2625 |
| C-5940  | ΔcsrB::kan        | From C-1a by λ Red-mediated recombination; primers: FG2524 and FG2525 |
| C-5942  | Δpnp-751 ΔcsrB::kan | From C-5691 by λ Red-mediated recombination; primers: FG2524 and FG2525. |
| C-5944  | ΔcsrC::cat        | From C-1a by λ Red-mediated recombination; primers: FG2585 and FG2586. |
| C-5946  | Δpnp-751 ΔcsrC::cat | From C-5691 by λ Red-mediated recombination; primers: FG2585 and FG2586. |
| C-5948  | ΔcsrB::kan ΔcsrC::cat | From C-5691 by λ Red-mediated recombination; primers: FG2585 and FG2586. |
| C-5950  | Δpnp-751 ΔcsrB::kan ΔcsrC::cat | From C-5691 by λ Red-mediated recombination; primers: FG2585 and FG2586. |
| C-5952  | ΔcsrD::cat        | From C-1a by λ Red-mediated recombination; primers: PL674 and PL675. |
| C-5954  | Δpnp-751 ΔcsrD::cat | From C-5691 by λ Red-mediated recombination; primers: PL674 and PL675. |
| C-5960  | ΔmcaS::kan        | From C-1a by λ Red-mediated recombination; primers: FG2755 and FG2756. |
| C-5962  | Δpnp-751 ΔmcaS::kan | From C-5691 by λ Red-mediated recombination; primers: FG2755 and FG2756. |

| JW1007  | BW25113 ΔpgaC::kan | [68] |
| AM56    | MG1655 ΔpgaA::cat  | [69] |
| AM70    | MG1655 ΔcsgA::cat  | [69] |
| AM72    | MG1655 ΔbcsA::cat  | [69] |
| AM105   | MG1655 ΔwcaD::tet  | From MG1655 by λ Red-mediated recombination with a DNA fragment obtained by PCR of tet10 cassette of EB 1.3 with primers PL372 and PL373. |

| EB 1.3  | MG1655 papaStn10-tet | [33] |

### Plasmids and phage

| Relevant characteristics | Reference |
|--------------------------|-----------|
| pBAD24                   | AmpR, ColE1 | [70] |
| pBAD24ΔΔ1                | pBAD24 derivative with a modified polylinker; carries an unique Ncol site overlapping the araBp transcription start | this work |
| pBADpnp                  | pBAD24 derivative; harbours an EcoRI-HindIII fragment of pEJ01 that carries the pnp gene | this work |
| pBADmb                   | pBAD24 derivative; harbours an HindIII-XbaI fragment of pFCT6.9 that carries the mb gene | this work |
Table 1 Bacterial strains and plasmids (Continued)

| Strain/Plasmid       | Description                                                                 | Source/Comment                  |
|----------------------|-----------------------------------------------------------------------------|---------------------------------|
| pBADΔrnr             | pBAD24Δr derivative; harbours the rnr gene (obtained by PCR on MG1655 DNA with FG2474-FG2475 oligonucleotides) between Ncol-HindIII sites | this work                       |
| pΔLpga               | pJAMA8 derivative, harbours the -116 to +32 region relative to the pgaABCD transcription start site cloned into the SphI/XbaI sites | this work                       |
| pEJ01                | carries a His-tagged pnp allele                                             | [71]                            |
| pFCT69               | carries a His-tagged mb allele                                              | [72]; received from Cecilia Arraiano |
| pGZ119HE             | onV_Cass, Car^r                                                              | [73]                            |
| pJAMA8               | Amp^r, ColE1; luxAB based promoter-probe vector                             | [37]                            |
| pLpga1               | pJAMA8 derivative, harbours the -116 to +234 region relative to the pgaABCD transcription start site cloned into the SphI/XbaI sites | this work                       |
| pLpga2               | pJAMA8 derivative, harbours a translational fusion of pgaA promoter, regulatory region and first 5 codons of pgaA (-116 to +249 relative to transcription start site) with luxA ORF (Open Reading Frame) | this work                       |
| pTLUX                | pJAMA8 derivative, harbours ptac promoter of pGZ119HE cloned into the SphI/XbaI sites | this work                       |
| P1 HTF               | High transduction frequency phage P1 derivative                             | [74]; received from Richard Calendar |

Protein negatively regulates EPS production in *Pseudomonas aeruginosa* by repressing translation of the *psl* transcript [15]. Finally, cellulose production in *Glucanacetobacter xylinum* and in various enterobacteria requires enzymatic activation of the cellulose biosynthetic machinery by the signal molecule cyclic-di-GMP (c-di-GMP) [16,17], a signal molecule which plays a pivotal role as a molecular switch to biofilm formation in Gram negative bacteria [18]. The great variety of regulatory mechanisms presiding to EPS biosynthesis, and the role of c-di-GMP as signal molecule mainly devoted to its control, underline the critical importance of timely EPS production for bacterial cells.

Polynucleotide phosphorylase (PNPase) plays an important role in RNA processing and turnover, being implicated in RNA degradation and in polymerization of heteropolymeric tails at the 3′-end of miRNA [19,20]. PNPase is an homotrimeric enzyme that, together with the endonuclease RNase E, the DEAD-box RNA helicase RhlB, and enolase, constitute the RNA degradosome, a multiprotein machine devoted to RNA degradation [21,22]. Despite the crucial role played by PNPase in RNA processing, the *pnp* gene is not essential; however, *pnp* inactivation has pleiotropic effects, which include reduced proficiency in homologous recombination and repair [23,24], inability to grow at low temperatures [25] and inhibition of lysogenization by bacteriophage P4 [26]. Moreover, lack of PNPase affects stability of several small RNAs, thus impacting their ability to regulate their targets [27].

In this work, we show that deletion of the *pnp* gene results in strong cell aggregation and biofilm formation, due to overproduction of the EPS poly-N-acetylglucosamine. Increased biofilm formation was observed both in *E. coli* MG1655 and C-1a strains, being more pronounced in the latter. We demonstrate that PNPase negatively controls expression of the PNAG biosynthetic operon *pgaABCD* at post-transcriptional level, thus acting as a negative determinant for biofilm formation. Our observation that PNPase acts as an inhibitor of biofilm formation is consistent with previous findings highlighting the importance of regulation of EPS production and biofilm formation at mRNA stability level [28].

**Methods**

**Bacteria and growth media**

Bacterial strains and plasmids are listed in Table 1. *E. coli* C-1a is a standard laboratory strain [29], whose known differences with *E. coli* MG1655 reside in its restriction/modification systems [30] and in the presence of a functional *rph* gene, encoding ribonuclease PH, which, in contrast, is inactivated by a frameshift mutation in *E. coli* MG1655 [31]. For strain construction by λ Red-mediated recombination [32], if not otherwise indicated, the parental strains were transformed with DNA fragments obtained by PCR using either pKD3 (for amplification of DNA fragments carrying chloramphenicol-resistance cassettes) or pKD13 (for DNA fragments carrying kanamycin-resistance cassettes) as template. The sequences of oligonucleotides utilized in this work are reported in Additional file 1: Table S1. Bacterial cultures were grown in the following media: LD (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl); M9 (82 mM Na2HPO4, 24 mM KH2PO4, 85 mM NaCl, 19 mM NH4Cl, 1 mM MgSO4, 0.1 mM CaCl2, 0.1 μg/ml thiamine); M9/sup (M9 supplemented with 0.25 g/l tryptone, 0.125 g/l yeast extract, 0.125 g/l NaCl). Unless otherwise stated, 0.4% glucose was added to give either M9Glu or M9Glu/sup.
media. When needed, media were supplemented with 100 μg/ml ampicillin.

**Cell aggregation and adhesion assays**

Cell aggregation was assessed as follows: overnight cultures grown in LD at 37°C on a rotatory device were diluted 50-fold in 50 ml of M9Glu/sup in a 250 ml flask. The cultures were then incubated at 37°C with shaking at 100 rpm. Cell adhesion to the flask walls was assessed in overnight cultures grown in M9Glu/sup medium at 37°C. Liquid cultures were removed and cell aggregates attached to the flask glass walls were stained with crystal violet for 5 minutes to allow for better visualization. Quantitative determination of surface attachment to

![Graph](image)

**Figure 1** Cell aggregation and adhesion by *E. coli* C PNPase-defective strain. A. Growth curves of *E. coli* C-1a (pnp+; solid symbols) and *E. coli* C-5691 (Δpnp-751; open symbols) in different media (M9Glu/sup, diamonds; M9Glu, triangles) (left panel). Cell clumping by the C-5691 (Δpnp) strain led to deposition of ring-like aggregates on the flask walls (indicated by the arrow; right panel). The picture was taken in the late exponential phase (OD<sub>600</sub> = 5–6). B. Cultures of strains carrying pBAD24 derivatives grown up to OD<sub>600</sub> = 0.6–0.8 in M9Glu/sup at 37°C with aeration were harvested by centrifugation, resuspended in 0.04 vol M9 and diluted 25 fold in pre-warmed M9/sup with either 0.4% glucose (solid symbols) or 1% arabinose (empty symbols). Incubation at 37°C was resumed and growth monitored spectrophotometrically. Left panel: PNPase complementation. Right panel: suppression by RNase II.
polystyrene microtiter wells was carried out using crystal violet staining as previously described [33]. Binding to Congo red (CR) was assessed in CR agar medium (1% casamino acid, 0.15% yeast extract, 0.005% MgSO4, 2% agar; after autoclaving, 0.004% Congo red and 0.002% Coomassie blue). Overnight cultures in microtiter wells were replica plated on CR agar plates, grown for 24 h at 30°C, and further incubated 24 h at 4°C for better detection of staining.

Gene expression determination
RNA extraction, Northern blot analysis and synthesis of radiolabelled riboprobes by \textit{in vitro} transcription with T7 RNA polymerase were previously described [34,35]. The DNA template for PGA riboprobe synthesis was amplified by PCR on C-1a genomic DNA with oligonucleotides FG2491/39 and FG2492/22. Autoradiographic images of Northern blots were obtained by phosphorimaging using ImageQuant software (Molecular Dynamics). Quantitative (real time) reverse transcriptase PCR (quantitative RT-PCR) was performed as described [33]. Oligonucleotides PL101/21 and PL102/19 were used for 16S rRNA reverse transcription and PCR amplification. mRNA half-lives were estimated as described [36] by regression analysis of mRNA remaining (estimated by real time PCR) versus time after rifampicin addition.

Figure 2 Identification of the factor responsible for C-5691 (Δpnp) aggregative phenotype. A. Cell aggregation in C-1a (pnp+), C-5691 (Δpnp) and C-5691 derivatives carrying mutations in genes encoding for adhesion determinants (ΔpgaC, C-5937; ΔbcsA, C-5929; ΔcsgA, C-5931; ΔwcaD, C-5935). Cell aggregates were stained with crystal violet for better visualization. B. Surface adhesion of the same set of strains to polystyrene microtiter plates. The adhesion unit values, assessed as previously described [33], are the average of three independent experiments and standard deviation is shown. The overall \( p \)-value obtained by ANOVA was \( p = 5.11 \times 10^{-12} \). Letters provide the representation for posthoc comparisons. According to posthoc analysis (Tukey’s HSD, \( p < 0.05 \)), means sharing the same letter are not significantly different from each other. C. Phenotype on Congo red-supplemented agar plates. D. Phase contrast micrographs (1,000 x magnification) of pnp+ (C-1a), Δpnp (C-5691), ΔpgaC (C-5936), and Δpnp ΔpgaC (C-5937) strains grown overnight in M9Glu/sup medium at 37°C. The images were acquired with a digital CCD Leica DFC camera.
Luciferase assays were performed as in [37]. Oligonucleotides utilized for Northern blot, real time PCR, and construction of reporter plasmids are listed in Additional file 1: Table S1.

**PNAG detection**

PNAG production was determined as described [38]. Bacteria were grown overnight in 3 ml of M9 Glu/ sup medium at 37°C. Cells were collected by centrifugation and diluted in Tris-buffered saline [20 mM Tris–HCl, 150 mM NaCl (pH 7.4)] to an OD_{600} = 1.5. 1ml of suspension was centrifuged at 10,500 x g, resuspended in 300 μl of 0.5 M EDTA (pH 8.0), and incubated for 5 min at 100°C. Cells were removed by centrifugation at 10,500 x g for 6 min and 100 μl of the supernatant was incubated with 200 μg of protease K for 60 min at 60°C. Protease K was heat-inactivated at 80°C for 30 min. The solution was diluted 1:3 in Tris-buffered saline and 10 μl was spotted onto a nitrocellulose filter using a Dot-blot apparatus (Bio-Rad). The filter was saturated for about 2 hours in 0.1 M Tris–HCl (pH 7.5), 0.3 M NaCl, 0.1% Triton (Sigma Aldrich) and 5% milk and then incubated overnight at 4°C with a 1:1,000 dilution of purified PNAG antibodies (a kind gift from G.B. Pier [39]). PNAG antibodies were detected using a secondary anti-goat antibody (dilution 1:5,000) conjugated with horseradish peroxidase. Immunoreactive spots were revealed using ECL Western blotting reagent (Amersham Pharmacia Biotech).

**Statistical analysis**

When applicable, statistically significant differences among samples were determined using a t-test of analysis of variance (ANOVA) via a software run in MATLAB environment (Version 7.0, The MathWorks Inc.). Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine significance of the data. Statistically significant results were depicted by p-values <0.05.

**Results**

**Lack of PNPase induces cell aggregation in E. coli C**

The *E. coli* C *pnp* deletion mutant C-5691 (a derivative of *E. coli* C-1a [40,41]) showed an apparent growth arrest when grown at 37°C in M9 minimal medium with glucose as sole carbon source (M9Glu, Figure 1A, left panel). The growth defect was overcome by supplementing M9Glu with 0.25 g/l tryptone, 0.125 g/l yeast extract, 0.125 g/l NaCl (M9Glu/sup medium); however, in such conditions, C-5691 optical density drastically decreased at the onset of stationary phase. Such drop was due to cell flocculation, leading to formation of macroscopic cell clumps sedimenting onto the flask glass wall (Figure 1A, right panel). Cell flocculation also occurred when either arabinose or glycerol were added to M9/sup media instead of glucose (data not shown).

The aggregative phenotype of the C-5691 (Δ*pnp*) strain was complemented by basal expression from a multicopy plasmid of the *pnp* gene under araBp promoter, indicating that low PNPase expression is sufficient to restore planktonic growth. Conversely, arabinose addition did not completely restore a wild type phenotype (Figure 1B, left panel), suggesting that PNPase overexpression may also cause aggregation. Ectopic expression of RNase II suppressed the aggregative phenotype of the *pnp* mutant (Figure 1B, right panel), thus suggesting that such a phenotype is controlled by the RNA degrading activity of PNPase. In contrast, however, RNase R overexpression did not compensate for lack of PNPase, indicating that different ribonucleases are not fully interchangeable in this process.

**Inactivation of the pnp gene induces poly-N-acetylglucosamine (PNAG) production**

In addition to macroscopic cell aggregation (Figures 1 and 2A), deletion of *pnp* stimulated adhesion to poly-styrene microtiter plates in a standard biofilm formation assay [33] (Figure 2B) and resulted in red phenotype on solid medium supplemented with Congo red, a dye binding to polymeric extracellular structures such as amyloid fibers and polysaccharides (Figure 2C). Cell aggregation was also observed by phase contrast microscopy (Figure 2D). Altogether, these observations strongly suggest that inactivation of *pnp* triggers the expression of one or more extracellular factors implicated in cell aggregation and adhesion to solid surfaces. In order to identify such factor(s), we searched for deletion mutants in genes encoding known adhesion factors and biofilm determinants that could suppress the aggregative phenotype of the C-5691 (Δ*pnp*) mutant strain. The following adhesion factors were targeted by appropriate mutations (Table 1): curli fibers (ΔcsgA), which strongly promote attachment to abiotic surfaces and constitute the main determinant for Congo red binding [42,43]; cellulose (ΔbcsA) and PNAG (ΔpgaA and ΔpgaC), two extracellular polysaccharides able to promote surface adhesion and to affect Congo red binding to the bacterial cell [44,45]; and the capsular polysaccharide colanic acid (ΔwcaD), which promotes biofilm maturation acting synergistically with other adhesion factors such as curli fibers or conjugal pili [46,47].

The aggregative phenotype of the C-5691 (Δ*pnp*) mutant, as determined by cell aggregation, surface adhesion, and Congo red binding experiments, was totally abolished by deletion of *pgaC* (Figure 2), which encodes the polysaccharide polymerase needed for biosynthesis of PNAG from UDP-N-acetylglucosamine [48]. Deletion of
pgaA, also part of the PNAG biosynthetic operon pgaABCD, produced identical effects as pgaC (data not shown). In contrast, no significant effects on either Congo red binding or cell aggregation and adhesion were detected in any Δpnp derivative unable to produce curli or colanic acid (Figure 2). Finally, deletion of the bcsA gene, which encodes cellulose synthase, led to a significant increase in cell adhesion to the flask glass walls (Figure 2A); this result is consistent with previous observations suggesting that, although cellulose can promote bacterial adhesion, it can also act as a negative determinant for cell aggregation, particularly in curli-producing E. coli strains [49,50]. In the C-1a strain, carrying a wild type pnp allele, inactivation of genes involved in biosynthesis of curli, PNAG, cellulose and colanic acid did not result in any notable effects on cell aggregation (Additional file 2: Figure S1).

To establish whether induction of PNAG-dependent cell aggregation in the absence of PNPase is unique to E. coli C-1a or it is conserved in other E. coli strains, we performed adhesion assays comparing the standard laboratory strain MG1655 to its Δpnp derivative KG206. Similar to what observed for the E. coli C strains, deletion of the pnp gene in the MG1655 background resulted in a significant increase in adhesion to solid surfaces, which was totally abolished by pgaA deletion (Additional file 3: Figure S2). However, cell aggregation was not observed in KG206 liquid cultures (data not shown), suggesting that the effect of pnp deletion is less pronounced in the MG1655 background.

Our results clearly indicate that PNAG is required for the aggregative phenotype of pnp mutant strains, suggesting that PNPase may act as a negative regulator of PNAG production. We thus determined by western blotting PNAG relative amounts in both C-1a (WT) and C-5691 (Δpnp) strains using anti-PNAG antibodies. As shown in Figure 3, the Δpnp mutants (both with the single Δpnp mutation and in association with either ΔcsgA or ΔwcaD) exhibited higher PNAG levels relative to the pnp+ strains. As expected, no PNAG could be detected in pgaC mutants, whereas bcsA inactivation, which abolishes cellulose production, led to stimulation of PNAG biosynthesis. Despite increased PNAG production, the pnp+ ΔbcsA strain did not show any detectable cell aggregation (Additional file 2: Figure S1). Discrepancies between PNAG levels and aggregative phenotype in some mutants might be explained by presence of additional adhesion factors, or different timing in PNAG production.

**PNPase downregulates pgaABCD operon expression at post-transcriptional level**

In E. coli, the functions responsible for PNAG biogenesis are clustered in the pgaABCD operon [48]. By northern blot analysis we found that the pgaABCD transcript was much more abundant in the Δpnp strain than in pnp+ (Figure 4A), suggestive of negative control of pgaABCD transcript stability by PNPase. Increased transcription of the pgaABCD operon was also detected in the E. coli MG1655 Δpnp derivative KG206 (data not shown), in agreement with biofilm formation experiments (Additional file 3: Figure S2). We investigated the mechanism of pgaABCD regulation by PNPase and its possible connections with known regulatory networks controlling pgaABCD expression. pgaABCD expression is positively regulated at the transcription initiation level by NhaR, while pgaABCD mRNA stability and translation are negatively regulated by the CsrA protein [51,52]. The 234-nucleotide long pgaABCD 5′-UTR carries multiple binding sites for the translation repressor CsrA [51]. Two small RNAs, CsrB and CsrC, positively regulate pgaABCD by binding CsrA and antagonizing its activity [53]. Stability of the two small RNAs is controlled by CsrD, which triggers RNase E-dependent degradation by
a still unknown mechanism [54]. Recently, a third sRNA, McaS, has been involved in this regulatory system as a positive regulator of pgaABCD expression [55].

Enhanced stability of pgaABCD mRNA may account for (or at least contribute to) the increase in pgaABCD expression. Indeed, RNA degradation kinetics experiments performed by quantitative RT-PCR showed a small, but reproducible 2.5-fold half-life increase of pgaA mRNA in the Δpnp mutant (from 0.6 min in C-1a to 1.5 min in the pnp mutant; Additional file 4: Figure S3). A comparable effect was elicited by deletion of the csrA gene (estimated mRNA half-life, 1.5 min; Additional file 4: Figure S3), known to regulate pgaABCD mRNA stability in E. coli K12 [38,51].

Post-transcriptional regulation of the pgaABCD operon by the CsrA protein targets its 234 nucleotide-long 5’-UTR. Therefore, we tested whether this determinant was also involved in pgaABCD control by PNPase. To this aim, we constructed several plasmids (see Table 1) harboring both transcriptional and translational fusions between different elements of the pgaABCD regulatory region and the luxAB operon, which encodes the catalytic subunits of Vibrio harveyi luciferase, as a reporter [37]. Luciferase expression in both pnp+ and Δpnp strains was tested using the transcriptional fusion plasmids pLpga and pLpga1, which harbor the pgaABCD promoter region (pgaAp) alone (−116 to +32 relative to the transcript start site) and a region encompassing pgaAp and the entire pgaA leader (without its ATG start codon), respectively. In these constructs, translation of the luxAB transcript depends on the vector translation initiation region (TIR). Conversely, pLpga2 carries a translational fusion of the whole 5’-UTR and the first 5 codons of pgaA with luxA. A plasmid expressing luxAB from Ptac promoter (pTLUX) and the vector TIR was also tested as a control of PNPase effects on luciferase mRNA expression. The results of a typical experiment of luciferase activity determination are reported on the right.

### Table 1

| Plasmid | pnp | Δpnp-751 | R.A. |
|---------|-----|----------|------|
| pTLUX   | 85.5| 104.4    | 1.2±0.2 |
| pΔLpga  | 258.4| 346.7    | 1.3±0.1 |
| pLpga1  | 8.1 | 32.9     | 4.3±0.9 |
| pLpga2  | 6.1 | 77.9     | 12.8±0.4 |

**Figure 4 Analysis of pgaABCD regulation by PNPase.** A. Northern blot analysis of pgaABCD operon transcription. 15 μg of total RNA extracted from E. coli C-1a (pnp+) and E. coli C-5691 (Δpnp-751) cultures grown up to OD600 = 0.8 in M9Glu/sup at 37°C were hybridized with the radiolabelled PAGA riboprobe (specific for pgaA). B. Identification of in cis determinants of pgaABCD regulation by PNPase. Map of pJAMA8 luciferase fusion derivatives and luciferase activity expressed by each plasmid. Details about plasmid construction and coordinates of the cloned regions are reported in Methods and in Table 1. Construct elements are reported on an arbitrary scale. For relative luciferase activity (R.A.) in E. coli C-5691 (Δpnp-751) vs. E. coli C-1a (pnp+) strains, average and standard deviation of at least two independent determinations are reported. Although the absolute values of luciferase activity could vary from experiment to experiment, the relative ratio of luciferase activity exhibited by strains carrying different fusions was reproducible.
affect *pgaABCD* expression (namely, *csrB*, *csrC* and *mcaS*), or *csrD*, whose gene product favors CsrB and CsrC degradation [54]. We also readily obtained the Δ*csrA::kan* mutation in C-1a (*pnp*+), indicating that, unlike in K-12 strains [58], *csrA* is not essential in *E. coli* C. Conversely, in spite of several attempts performed both by λ Red mediated recombination [32] and by P1 reciprocal transductions, we could not obtain a Δ*pnp* Δ*csrA* double mutant, suggesting that the combination of the two mutations might be lethal.

Each mutant was assayed for the expression of *pgaA* by quantitative RT-PCR and for PNAG production by western blotting. The results of these analyses showed that, both in the C-1a (*pnp*+) and in the C-5691 (Δ*pnp*) backgrounds, each tested mutation increased both *pgaA* mRNA expression (Figure 5A) and PNAG production (Figure 5B). This result was unexpected for mutants lacking CsrB, CsrC or McaS that, according to the current model of *pgaABCD* regulation, should act as positive regulators of such operon [51]. Thus, while our results support the role of CsrA as a major regulator of *pgaABCD* expression, they also suggest that the current model for *pgaABCD* post-transcriptional regulation, which is based on data obtained in *E. coli* K-12, may not readily apply to *E. coli* C. The additive effect observed upon combining Δ*pnp*-751 with deletions targeting different sRNAs suggest that PNPase and the sRNAs may act independently on *pgaABCD* regulation.

### Discussion

In this report, we have shown that PNPase negatively regulates the production of the adhesion factor PNAG, thus maintaining the bacterial cells in a planktonic state (Figures 1-3) when grown at 37°C in supplemented minimal medium. Our results are in line with previous works by other groups connecting PNPase to regulation of outer membrane proteins in *E. coli* [59] and curli production in Salmonella [60]. Thus, PNPase seems to play a pivotal role in regulating the composition of cell envelope and the production of adhesion surface determinants. PNPase-dependent regulation of PNAG production requires its ribonuclease activity, as suggested by the observation that overexpression of RNase II can compensate for lack of PNPase (Figure 1B). Cell aggregation in the absence of PNPase is suppressed by RNase II, but not by RNase R. This reminds what previously showed for cold sensitivity in *pnp* mutants, which is also solely suppressed by RNase II [61] and reinforces the notion that, albeit partially redundant, RNA degradation pathways possess a certain degree of specificity and are not fully interchangeable [62].
The precise mechanistic role played by PNPass in regulation of pgaABCD expression, as well as the physiological signals to which it responds, remain elusive. PNPass activity is modulated (at least in vitro) by cyclic-di-GMP [63], a signal molecule implicated in biofilm formation [18]. However, deletion of the dos gene, encoding a c-di-GMP phosphodiesterase which co-purifies with the RNA degradosome [63], did not affect pgaABCD expression (data not shown). Key molecules in energy metabolism and carbon flux, such as ATP and citrate also influence PNPass activity [64,65]. Thus, it can be speculated that environmental or physiological signals might regulate pgaABCD expression by controlling the level of specific metabolites that could directly modulate PNPass activity.

Our data clearly indicate that PNPass controls PNAG production by negatively regulating the pgaABCD operon at post-transcriptional level and that it targets the 5′-UTR of the pgaABCD transcript, thus similar to the translational repressor CsrA (Figures 4–5 and Additional file 4: Figure S3). This would suggest that the two proteins might belong to the same regulatory network. However, probing this hypothesis is complicated by the observation that in E. coli C, the mechanisms of CsrA-dependent gene expression regulation and its modulation by small RNAs might be more complex than in E. coli K-12, where the current model for CsrA regulation has been developed. This notion is somehow suggested by the fact that, while deletion of the csrA gene is lethal for E. coli K-12 when grown on glucose-based media [55], this is not the case for E. coli C. Moreover, to our surprise, the lack of putative positive regulators such as CsrB, CsrC and McsA resulted in an increase of pgaABCD expression levels both in the Δpnp and in its parental strain C-1a, which would suggest a negative role of these sRNAs in pgaABCD control (Figure 5). Genes encoding cell surface-associated structures seem to constitute a “hotspot” for post-transcriptional regulation involving small non coding RNAs. For instance, multiple control of gene expression by sRNAs has already been demonstrated for csgD, which encodes the master regulator for the biosynthesis of thin aggregative fimbriae (curli), one of the major adhesion factors in E. coli [28,55,66,67]. It is thus possible that, in E. coli C, increased pgaABCD expression in mutant strains carrying deletions of sRNA-encoding genes might be due to feedback induction of yet unidentified factors which might play a role in CsrA-dependent regulation. This possibility is supported by the observation that CsrB, CsrC and McsA mutually control their transcript level both in E. coli K and C [53] (T. Carzaniga and F. Briani, unpublished data). pgaABCD operon regulation appears to be an intriguing model system for the study of post-transcriptional modulation of gene expression in bacteria.

Conclusions
In this work, we have unravelled a novel role for PNPass as a negative regulator of pgaABCD expression and PNAG biosynthesis. Thus, PNPass activity contributes to keeping E. coli cells in the planktonic state. Our findings underline the importance of post-transcriptional regulation for genes encoding cell surface-associated structures and factors involved in biofilm formation and suggest the existence of strain-specific variability in these regulatory mechanisms. Indeed, small RNA-dependent post-transcriptional regulation of pgaABCD expression in E. coli C is more complex than the model proposed for E. coli K-12, possibly connected to a central role played by PNAG as a determinant for biofilm formation in the former strain.

Additional files

Additional file 1: Table S1. Primers used in this work.

Additional file 2: Figure S1. Effects of inactivation of genes encoding adhesion factors and biofilm determinants in the C-1a strain. C-1a (pnp+), KG206 (Δpnp), and KG206 derivatives carrying mutations in genes encoding for adhesion determinants (ΔpnpC, impaired in PNAG production; ΔcsgA, impaired in cellulose production; ΔpgaA, impaired in curli production; ΔmcaS, impaired in colanic acid production) were grown over night in M9Gluc at 37°C in glass flasks. Cell aggregates were stained with crystal violet.

Additional file 3: Figure S2. Surface adhesion of pnp deletion mutant derivative of E. coli MG1655 and identification of the adhesion factor involved. Surface adhesion to polystyrene microtitre plates by MG1655 (pnp+), KG206 (Δpnp), and KG206 derivatives carrying mutations in genes coding for adhesion determinants (ΔpnpA, AM56; ΔcsgA, AM72; ΔcsgB, AM70; ΔmcaS, AM102) was assessed at 37°C in M9Gluc at a multiplicity of infection (MOI) of 0.8. Adhesion unit values, assessed as previously described [33], are the average of three independent experiments and standard deviation is shown. The overall p-value obtained by ANOVA is indicated in the graph. Letters provide the representation for posthoc comparisons. According to posthoc analysis (Turkey’s HSD, p < 0.05), means sharing the same letter are not significantly different from each other.

Additional file 4: Figure S3. pgaA mRNA decay analysis. Bacterial cultures of C-1a (pnp+), C-5691 (Δpnp) and C-5938 (ΔcsgA) were grown up to OD550 = 0.8 in M9Gluc sup, rifampicin (final concentration of 0.4 mg/ml) was added, and samples for RNA extraction were taken at different time points immediately before (t = 0) and after antibiotic addition. pgaA mRNA degradation kinetics was estimated by quantitative RT-PCR with oligonucleotides PL99 and PL100, as detailed in Methods.

Authors’ contributions
FB, GD and PL conceived the project and designed the experiments. FB and PL wrote the manuscript. TC and DA designed and performed the experiments. All authors read and approved the final manuscript.

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